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Anti-inflammatory and anticancer compounds isolated from *Ventilago madraspatana* Gaertn., *Rubia cordifolia* Linn. and *Lantana camara* Linn.

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Abstract

Objectives The aim was to search for anti-inflammatory and anticancer compounds from three medicinal plants, viz. *Ventilago madraspatana* Gaertn., *Rubia cordifolia* Linn. and *Lantana camara* Linn.

Methods The NO• scavenging potential of selected plant extracts was determined on LPS/IFN-γ activated murine peritoneal macrophage cultures, and iNOS and COX-2 expression was evaluated by Western blot analysis. Bio-assay guided fractionation yielded four compounds: physcion and emodin from *V. madraspatana*, 1-hydroxytectoquinone from *R. cordifolia*, and oleanonic acid from *L. camara*. The anti-inflammatory activity of these compounds was tested through the carrageenan-induced rat-paw oedema model. They were then tested against a murine tumour (Ehrlich ascites carcinoma), and three human cancer cell lines, namely A375 (malignant skin melanoma), Hep2 (epidermoid laryngeal carcinoma) and U937 (lymphoma).

Key findings All four compounds dose dependently inhibited NO• through suppression of iNOS protein without affecting macrophage viability. Physcion and emodin caused 65–68% reduction of oedema volume at 40 mg/kg, which validated their in-vivo anti-inflammatory effect. 1-hydroxytectoquinone and oleanonic acid exhibited promising cytotoxicity against A375 cells.

Conclusions Ethnomedical reports on these traditional medicinal plants have been rationalised through an insight into the anti-inflammatory as well as anticancer potential of four constituents, characterised to be prospective candidates for designing novel therapeutic agents.

Keywords anticancer activity; anti-inflammatory activity; inducible nitric oxide synthase; *Lantana camara*; *Rubia cordifolia*; *Ventilago madraspatana*

Introduction

Inflammatory diseases are a longstanding medical problem and a major cause of morbidity worldwide. To get symptomatic relief in inflammatory conditions such as arthritis and rheumatism, treatment mostly involves the application of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), which are in great demand these days. However, prolonged use of NSAIDs often leads to renal problems, gastrointestinal irritation and other side effects, while some of these specific COX-2 inhibitors, such as rofecoxib and celecoxib, have recently been implicated in a high possibility of myocardial infarction/strokes, and have been marketed with a 'black-box' warning.[1]

The inflammatory pathway is characterised by several mediators, one of which is the nitric oxide radical (NO•). It is generated from the terminal guanido-nitrogen of L-arginine through a five-electron oxidation process, aided by the NADPH-dependent enzyme known as nitric oxide synthase (NOS; EC 1.14.13.39).[2] During inflammatory reactions in mammals, the Ca^{2+}-independent inducible form of nitric oxide synthase (iNOS) is stimulated by either bacterial lipopolysaccharide (LPS, a bacterial endotoxin), or pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β), and expressed within macrophages, hepatocytes, and endothelial or smooth muscle cells.[2] This leads to the generation of NO•, which plays an essential role in the defence system of the host. However, in the event of

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overproduction of NO• through iNOS, this reactive radical induces damages to normal tissues, causing a variety of pathophysiological processes, such as chronic inflammation, septic shock, atherosclerosis and autoimmune diseases, often leading to cancer.[3–6] Hence, the use of specific modulators to downregulate iNOS may be an effective strategy against these maladies.[3,5]

Historically, medicinal plants have been considered to be prospective sources of 'novel' anti-inflammatory agents, following the discovery of aspirin from willow bark. Recently, the search for plant-derived COX, LOX or iNOS modulators led to the recognition of potential iNOS inhibitors, such as curcumin, limonene, gingerol, yakuchinone, garcinol, etc., which were also found to exhibit antiproliferative effects against a number of cancer cells.[7] In fact, excessive and prolonged iNOS-mediated NO• generation may be linked to inflammation as well as tumourigenesis.[6,8] In the present study therefore, three plant samples were chosen, namely *Ventilago madraspatana* Gaertn. (Rhamnaceae; Red Creeper) stem-bark, *Rubia cordifolia* Linn. (Rubiaceae; Indian Madder) root and *Lantana camara* Linn. (Verbenaceae; Wild Sage) root bark. This selection was based on the plants' traditional usage in Ayurveda and other indigenous systems of medicine, where they are used in the treatment of rheumatism, asthma and other inflammatory conditions.[9,10]

In fact, *R. cordifolia* and *L. camara* are popular medicinal herbs used in many countries of the world, and have been previously investigated for the possible isolation of their phytochemical constituents.[11,12] However, a literature search apparently revealed the lack of any systematic study to link these compounds to their respective pharmacological activity. Most of the available reports are on the crude extracts of these two plants.[13–22]

In the case of *V. madraspatana*, although its chemical constituents have been well-documented,[23,24] the biological validation of the traditional usage of this species of *Ventilago* was found to be inadequate.[25]

Previously, we found that the crude extracts of the selected plant samples could inhibit NO• radical generation in the LPS-stimulated murine macrophages through suppression of iNOS protein.[26] In the present study we identified four pure compounds, isolated from the three plants through bioassay-guided fractionation, and validated their anti-inflammatory activity in an animal model for the first time. Moreover, these compounds were screened for their antiproliferative potential against three human cancer cell lines, A375 (malignant skin melanoma), Hep2 (epidermoid laryngeal carcinoma), U937 (lymphoma), and a murine tumour model (EAC; Ehrlich ascites carcinoma). This has not previously been reported to the best of our knowledge.

Materials and Methods

Chemical and reagents

RPMI-1640, HEPES, L-glutamine, streptomycin, penicillin G, LPS from *Escherichia coli* 0127: B8, IFN-γ, N^G-monomethyl-L-arginine (L-NMMA), *N*-(1-naphthyl) ethylenediamine dihydrochloride, SDS, glycerol, β-mercaptoethanol, nitrocellulose membrane (0.25 μm), camptothecin, doxorubicin hydrochoride and carrageenan were products of Sigma Chemicals Co, St Louis, MO, USA. Fetal bovine serum (FBS), anti-rabbit iNOS polyclonal antibody and alkaline phosphatase-labelled goat anti-rabbit antibody were purchased from HyClone, USA, BD Transduction Laboratories, USA, and Bangalore Genei, India, respectively. Anti-rabbit COX-2, and β-actin polyclonal antibody were procured from Santa Cruz Biotechnology, USA. Gentamicin was purchased from HiMedia Laboratories Private Limited, Mumbai, India. Starch, sodium nitroprusside, sulfanilamide, orthophosphoric acid, 3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tris, EDTA, bromophenol blue, nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), DMSO, and organic solvents of analytical grade were procured from Sisco Research Laboratories, Mumbai, India.

Experimental animals

Female colony-bred Swiss A mice (6–8 weeks old; 18–20 g), obtained from Chittaranjan National Cancer Institute, Kolkata, were used for isolation of murine peritoneal macrophage cells and routine maintenance of EAC tumour. Colony-bred female albino Wistar rats (130–150 g) were used for the paw oedema experiments. The experiments with animals, and their maintenance, were conducted under the Institutional Animal Ethics Committee guidelines.

Plant materials and preparation of crude extracts

V. madraspatana stem-bark [JU/BH/002] was collected from Bolangir district, Orissa; *R. cordifolia* [JU/BH/010] root was obtained from a traditional medicine practitioner, and *L. camara* [JU/BH/011] root-bark was collected locally. The plant materials were authenticated by the Botanical Survey of India, Kolkata, and voucher specimens were preserved in our laboratory. Plant samples (500 g) were shade-dried, pulverised, and soaked in water/methanol/chloroform (2.5 l) at room temperature for 1 month. The respective extracts were obtained by removing the solvents *in vacuo*, followed by activity-guided fractional separation through preparative thin layer chromatography (PTLC) on silica gel-G, or column chromatography using silica gel (60–120 mesh). Thereafter, the active compounds were purified by crystallisation, followed by characterisation through routine spectroscopic analysis. DMSO was used to dissolve the compounds for biological assessments, where its final concentration did not exceed 0.1% (v/v) in cell culture media.

Bioassay guided fractionation of extracts and characterisation of active principles

An *ex-vivo* macrophage model was employed to evaluate suppression of iNOS protein in order to carry out bioassay-guided fractionation of the selected plant extracts.

Chloroform extract of V. madraspatana

The chloroform extract of *V. madraspatana* gave the active fractions by elution with petroleum ether (bp 60–80°C) and chloroform mixtures (60 : 40, and 50 : 50, v/v, respectively) through column chromatography. The first fraction was obtained through column chromatography and was then subjected to PTLC using a mixture of petroleum ether and

chloroform (80 : 20 v/v), yielding physcion (**1**; 0.22% w/w), which was crystallised from methanol (yellow plates; mp 201°C). Similarly, another fraction yielded emodin (**2**; 0.31% w/w), which was obtained by PTLC using a mixture of chloroform and ethyl acetate (80 : 20 v/v), and was crystallised from methanol into orange needles (mp 255°C).

Methanol extract of R. cordifolia

Four major fractions[F1 (R_f : 0.92), F2 (R_f : 0.78), F3 (R_f: 0.40), F4 (R_f : 0.11)] were isolated from the methanol extract of *R. cordifolia*, when subjected to activity-guided PTLC using the solvent system $CHCl_3$: EtOAc : MeOH in ratios of 3 : 2 : 1 (v/v/v). Fraction F1 was found to be the most active. Column chromatography was therefore performed on *R. cordifolia* root to obtain more F1, which was further purified by PTLC. Elution with a mixture of petroleum ether and ethyl acetate (3 : 1 v/v; 0.004% w/w) yielded 1-hydroxytectoquinone (**3**), which was crystallised from a CH_2Cl_2-ether mixture (orange needles; mp 182°C).

Methanol extract of L. camara

Column chromatography with petroleum ether-chloroform (1 : 9 v/v) afforded a white residue (400 mg), which was further fractionated by PTLC using a mixture of chloroform-ethyl acetate-petroleum ether (3 : 2 : 1 v/v/v). The major active constituent was isolated as a white solid (1.5% w/w), which was crystallised from a mixture of dichloromethane and petroleum ether to furnish oleanonic acid (**4**; mp 180–182°C).

Evaluation of iNOS-inhibition and anti-inflammatory properties

Isolation of peritoneal macrophages

Swiss A mice were injected intraperitoneally (i.p.) with 1.0 ml of starch solution (4% w/v). After 48 h, peritoneal exudate cells were obtained by lavage with ice-cold RPMI-1640. Cells were washed, resuspended in HEPES-buffered RPMI-1640 medium (supplemented with 10% heat-inactivated FBS, and antibiotics: 100 μg/ml of streptomycin, 100 U/ml of penicillin G, and 100 μg/ml of gentamicin). These were seeded (1×10^6 cells/ml) in sterile disposable culture plates (35 mm; Tarson, India). The cells were maintained at 37°C for 4 h in a humidified atmosphere containing 5% CO_2. The non-adherent cells were removed by washing with RPMI-1640 and fresh culture medium was added to the adherent cells to grow at 80–90% confluency for further experiments.

Nitric oxide production in LPS and IFN-γ-stimulated macrophages

The adherent murine peritoneal exudate cells (1×10^6 cells/ml) were stimulated with either LPS (10 μg/ml), or IFN-γ (10 U/ml), in the presence or absence of the active compounds (**1–4**; 10, 20 and 40 μM). After incubation in a humidified 5% CO_2 incubator at 37°C for 24 h with IFN-γ, or 48 h with LPS, the culture supernatant accumulating the resultant NO_2^- was mixed with an equal volume of Griess reagent (1% sulfanilamide dissolved in 5% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride in water),[27] and the absorbance was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech) at a wavelength of 550 nm. Nitrite concentration was determined based on a standard curve for sodium nitrite solutions prepared with RPMI-1640. L-NMMA was used as the positive control. Suitable experiments were carried out to ensure that the samples did not interfere with the detection of nitrite by Griess reagent.

Cell viability

In the previous experiment, the adherent macrophages remaining after removal of the supernatant were treated with fresh culture medium containing 0.4 mg/ml of MTT, and further incubated for 4 h under similar conditions. The viable cells reduced the MTT into violet crystals of formazan, which were dissolved in DMSO. The extent of the reduction was estimated spectrophotometrically at 570 nm to determine viability of the cells treated with the compounds **1–4** in comparison to untreated controls.[28]

Western blot analysis

Murine peritoneal macrophage cells (2×10^6 cells/ml) treated with LPS (10 μg/ml), in the presence or absence of different doses (10, 20 and 40 μM) of compounds (**1–4**) were incubated at 37°C for 48 h in humidified 5% CO_2. Adherent cells were scraped out from the culture plates, washed in cold phosphate buffered saline (PBS), and boiled with the lysis buffer. The lysate from each cell sample (25 μg of protein) was separated electrophoretically on a 10% SDS-polyacrylamide gel, and the proteins were electrically transferred onto a nitrocellulose membrane. After blocking with 3% non-fat dry milk, the membranes were incubated with either anti-rabbit iNO S (dilution 1 : 10,000) or anti-COX-2 (1 : 2000 dilution), and anti-β-actin polyclonal primary antibody for 2 h. Then the membranes were washed and incubated with the corresponding alkaline phosphatase-labelled goat anti-rabbit secondary antibody (dilution 1 : 3000) for 1 h. The protein bands were visualised using NBT and BCIP as the substrate for phosphatase.

Carrageenan-induced rat paw oedema

Wistar rats (15) were divided into five groups for the in-vivo anti-inflammatory study. Three groups were treated,with 10, 20 and 40 mg/kg i.p. of the test compound, respectively, while the other two groups were injected either with indomethacin (10 mg/kg; standard drug) or DMSO (vehicle control). After 30 min, a subcutaneous injection of carrageenan suspension (1% in normal saline; 0.1 ml)[29] was administered to the subplantar region of the right hind paw of each experimental rat to induce acute inflammation. The paw volume was measured plethysmographically (using water) prior to administration of this phlogistic agent, and thereafter at hourly intervals up to 6 h. The results represented the mean of three independent experiments and the percentage of inhibition was calculated as follows:$[(E_c\text{-}E_t)/E_c] \times 100$, where E_c is the oedema volume of the control, and E_t is the oedema volume of the treated group.

Antiproliferative activity against murine and human cancer cell lines

Maintenance of murine and human cancer cell lines

A murine tumour model, EAC, was serially maintained by routine intraperitoneal transplantation (1×10^6 cells/mouse) in colony-bred Swiss A mice. EAC cells were collected on days 12–14 post-transplantation, suspended in PBS (pH 7.4), centrifuged and washed with cold PBS, taking care to remove adhering red blood cells, if any.[30] The cells were counted using a haemocytometer, and the viability (>95%) was checked by Trypan blue exclusion assay. The cells were resuspended in the HEPES-buffered RPMI-1640 medium without phenol red, supplemented with 10% heat-inactivated FBS and antibiotics: penicillin (100 U/ml), streptomycin (150 μg/ml) and gentamicin (150 μg/ml). They were then incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

Human cancer cell lines, U937 (human lymphoma) cells, were obtained from the Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The cells were grown in HEPES-buffered RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics as above. The cells were routinely subcultured in a humidified 5% CO_2 incubator at 37°C.

The other two cell lines, A375 (malignant skin melanoma) and Hep2 (epidermoid laryngeal carcinoma), were obtained from the National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FBS containing a 5% mixture of penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (3 μg/ml) in the presence of 5% CO_2 in air at 37°C. They were routinely subcultured using a 0.25% trypsin/0.02% EDTA solution.

Isolation of human peripheral blood mononuclear cells

Fresh heparinised whole blood was collected from a normal human volunteer with informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation.[31] The blood (5 ml) was layered carefully over the hypaque (3 ml, Sigma Diagnostics, USA), and centrifuged at room temperature at 1000 rpm for 45 min. The buffy coat layer containing PBMC at the interface was carefully taken out, washed twice with PBS and centrifuged at 1500–2000 rpm for 10 min. The cells were suspended in the HEPES-buffered RPMI-1640 medium without phenol red, supplemented with 20% heat-inactivated FBS and antibiotics: penicillin (100 μg/ml), streptomycin (150 μg/ml) and gentamicin (150 μg/ml). It was then incubated in the presence of 5% CO_2 in air at 37°C.

Cytotoxicity assay

The antiproliferative effect of the four compounds on the selected cancer cell lines and normal human lymphocytes (PBMC) was assessed *in vitro* by MTT reduction assay.[32] Briefly, cells (2×10^5/ml) were seeded in 96-well flat-bottomed microplates (Nunc, Roskilide, Denmark), and treated with the test compounds (1–100 μM) in triplicate. After 24 h of incubation in a humidified atmosphere of 5% CO_2 in air at 37°C, the culture medium was replaced with MTT solution (100 μl; 1 mg/ml in sterile PBS) for a further period of 24 h (4 h in the case of EAC cells). The precipitated crystals of formazan blue were solubilised by adding DMSO (200 μl) to each well. The optical density was measured with a microplate reader (Bio-Rad, Model 680) at a wavelength of 570 nm. Doxorubicin and camptothecin were used as positive controls in this experiment. The results represented the mean of three independent experiments and were expressed as IC50, i.e. the concentration at which the optical density of the treated cells was reduced by 50% with respect to the untreated control.

Morphological study on human cancer cell lines

Human cancer cell(s) (2×10^5/ml; 200 μl each) were seeded onto cover slips, and were cultured with the active compounds, one at a time. After 24 h of incubation at 37°C in a 5% CO_2 atmosphere, the supernatant was removed and the adhering cells were fixed with methanol, followed by staining with Giemsa solution for microscopic observation (Olympus, Japan).

Statistical analysis

All the results were expressed as mean ± SD. Student's *t*-test was used to determine the statistical significance for differences between the two means. Statistical comparisons between experimental groups were performed by the Kruskal–Wallis test followed by Dunn's post-hoc test using GraphPad Prism software (Version 4.0, GraphPad Software, Inc., La Jolla, CA, USA). Values were considered statistically significant when $P < 0.05$.

Results

Bioassay-guided fractionation of the chloroform extract of *V. madraspatana* stem-bark led to the isolation and spectroscopic characterisation of two structurally related anthraquinonoids, namely physcion (1,8-dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone; **1**) and emodin (1,3,8-trihydroxy-6-methyl-9,10-anthraquinone; **2**).[33]

Compound (**3**) was isolated from *R. cordifolia* root and was determined to be 1-hydroxytectoquinone (1-hydroxy-2-methyl-9,10-anthraquinone) through routine spectroscopic analysis, this structure being corroborated by an earlier report.[34] Furthermore, we confirmed its structure through two-dimensional ^{13}C–^{1}H NMR correlations (HMQC and HMBC). From the ^{1}H-NMR spectrum, the proton appearing as a singlet at δ 12.97 is assignable to peri-1-OH (chelated with one of the quinone carbonyls). The signals displayed at δ 7.80 (2H, ddd) and 8.31 (2H, dd) indicate the presence of four contiguous protons in one benzene ring. The ^{1}H-NMR spectrum also shows the presence of two ortho-coupled aromatic protons at δ 7.55 (1H, d, J = 7.5 Hz) and 7.76 (1H, d, J = 7.5 Hz), respectively. In the HMBC spectrum, the protons at δ 7.76 and 8.31 are correlated to the carbonyl carbon resonating at δ 182.4, which is assignable to C-10. The signal for an aromatic proton at δ 7.55 and the methyl protons at δ 2.39 correlate with a carbon resonance displayed at δ 161.1, assignable to C-1, which unequivocally settles the location of methyl group at the 2-position of the anthraquinone ring.

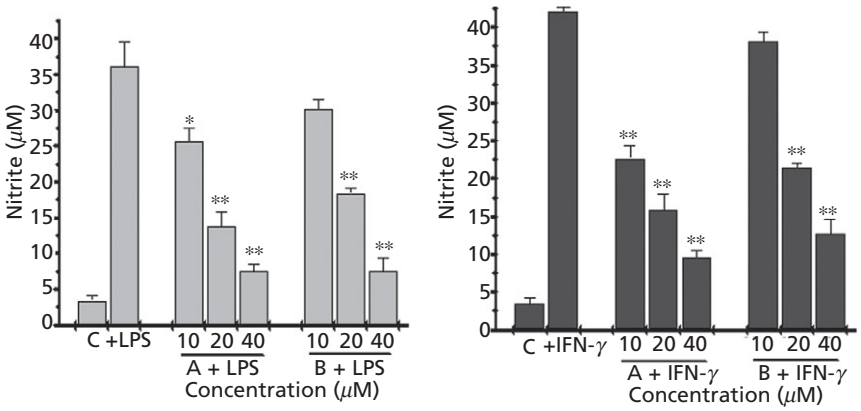

Figure 1 Effect of 1-hydroxytectoquinone and oleanonic acid on NO• production. Murine peritoneal macrophages (1×10^6cells/ml) were treated with lipopolysaccharide (10 μg/ml) or interferon-γ (10 U/ml) and incubated at 37°C in a humidified 5% CO_2 incubator in the presence or absence of the test compounds (10–40 μM). After 24 h (for IFN-γ) or 48 h (for LPS), supernatants were collected and the nitrite accumulation was measured by Griess reaction. Concentrations of nitrite were determined with sodium nitrite as standard. L-NMMA, a non-selective inhibitor of NOS, was used as a control (IC50: 41 μM). A, 1-hydroxytectoquinone; B, oleanonic acid; LPS, lipopolysaccharide; IFN-γ, interferon-γ. *$P < 0.05$, **$P < 0.01$ indicate significant differences from the LPS/IFN-γ-treated control groups. Data were obtained from three independent experiments and expressed as means ± SD.

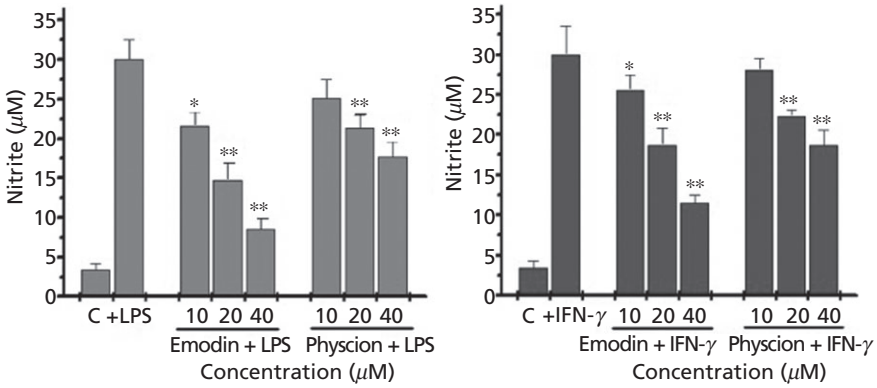

Figure 2 Effect of emodin and physcion on NO• production. Murine peritoneal macrophages (1×10^6cells/ml) were treated with lipopolysaccharide (10 μg/ml) or interferon-γ (10 U/ml) and incubated at 37°C in a humidified 5% CO_2 incubator in the presence or absence of the test compounds (10–40 μM). After 24 h (for IFN-γ) or 48 h (for LPS), supernatants were collected and the nitrite accumulation was measured by Griess reaction. Concentrations of nitrite were determined with sodium nitrite as standard. L-NMMA, a non-selective inhibitor of NOS, was used as a control (IC50: 41 μM). LPS, lipopolysaccharide; IFN-γ, interferon-γ. Data were obtained from three independent experiments and expressed as means ± SD. *$P < 0.05$; **$P < 0.01$ indicate significant differences from the LPS/IFN-γ-treated control groups.

The major bioactive fraction from the methanol extract of *L. camara* was identified as oleanonic acid through spectroscopic analysis and corroboration with literature data.[35–37]

From the bioassay experiments described above it was found that the tested compounds dose-dependently (10, 20, and 40 μM) inhibited the production of NO• in the murine peritoneal macrophage culture stimulated with LPS/IFN-γ (Figures 1 and 2). At the lowest tested concentration (10 μM) of 1-hydroxytectoquinone (**3**) and emodin (**2**), a significant inhibition of NO• occurred (~25 μM nitrite; $P < 0.05$) in comparison to the control sets treated with LPS/IFN-γ (36–42 μMs nitrite). However, in the case of oleanonic acid (**4**) and physcion (**1**), significant reduction of nitrite accumulation (~18–22 μM nitrite; $P < 0.05$) was noted at a slightly higher dose of 20 μM. It was noted that in the presence of 40 μM of **2**, **3** and **4**, the generation of nitrite from the activated macrophages was reduced nearly to the level of the untreated control (7–11 μM nitrite; $P < 0.01$). The tested compounds in fact exhibited stronger activity than L-NMMA, which was used as a positive control in the experiment (Figures 1 and 2).

Next, a dose-dependent (10, 20 and 40 μM) immunoblot analysis was carried out in order to determine the effect of **1**–**4** on the expression of iNOS protein in murine macrophages. Furthermore, the modulating property of the isolated constituents on the level of COX-2 protein in the LPS-activated murine macrophages was similarly assessed using anti-COX-2 specific antiserum. In fact, Salvemini has strongly suggested that NO• generated through catalytic activity of iNOS could play a critical role in the activation of COX-2

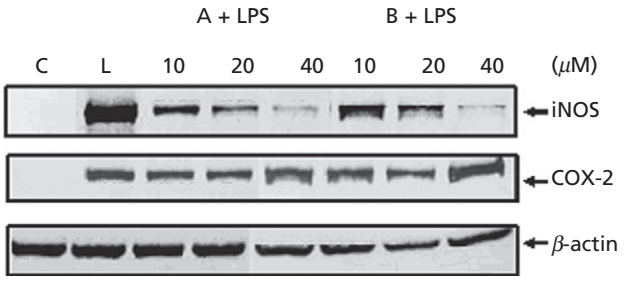

Figure 3 Effect of 1-hydroxytectoquinone and oleanonic acid on iNOS, COX-2, and β-actin protein expression in lipopolysaccharide-induced cultured murine peritoneal macrophages. Cells (2×10^6 cells/ml) were stimulated with lipopolysaccharide (10 μg/ml) in the presence or absence of different concentrations (10–40 μM) of the pure compounds. After 48 h, cells were harvested and lysed. Lysates (25 μg of protein/lane) were subjected to SDS-PAGE, followed by Western blotting with iNOS, COX-2 and β-actin (internal control) antiserum. A, 1-hydroxytectoquinone; B, oleanonic acid; C, untreated control; L, lipopolysaccharide-treated.

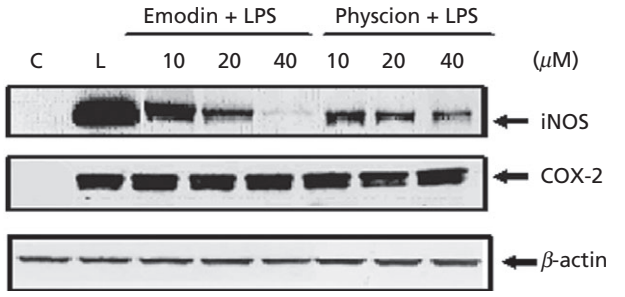

Figure 4 Effect of emodin and physcion on iNOS, COX-2, and β-actin protein expression in lipopolysaccharide-induced cultured murine peritoneal macrophages. Cells (2×10^6 cells/ml) were stimulated with lipopolysaccharide (10 μg/ml) in the presence or absence of different concentrations (10–40 μM) of the pure compounds. After 48 h, cells were harvested and lysed. Lysates (25 μg of protein/lane) were subjected to SDS-PAGE, followed by Western blotting with iNOS, COX-2 and β-actin (internal control) antiserum. C, untreated control; L, LPS-treated.

enzyme in cultured macrophages following treatment with pro-inflammatory stimuli, such as LPS.[38]

It was observed that all four compounds could specifically suppress the expression of iNOS protein in a concentration-dependent manner, as indicated by the diminishing intensity of the protein bands, which became almost undetectable at the highest tested concentrations (40 μM) of emodin (**2**; Figure 4), 1-hydroxytectoquinone (**3**; Figure 3) and oleanonic acid (**4**; Figure 3). Physcion (**1**), meanwhile, exhibited only comparatively moderate activity (Figure 4). This observation may confirm that the inhibition of NO• in the activated cultured macrophages did occur through the reduction of iNOS protein levels. Furthermore, none of the tested compounds showed any modulating effect on COX-2 protein expression. In addition, the presence of equally distinct bands of β-actin protein was observed for all compounds, which implies that the inhibition of NO• through the suppression of iNOS is not associated with general cellular toxicity, at least in the tested range of concentrations (10–40 μMs; Figures 3 and 4).

Thereafter, these iNOS inhibitory constituents were assessed through carrageenan-induced rat-paw oedema, an experimental arthritis model, in order to validate their anti-inflammatory activity *in vivo*. Earlier studies have indicated that carrageenan-induced oedema formation in mammals may cause site-specific expression of iNOS enzyme,[39–41] which is reportedly suppressed by the prophylactic administration of iNOS inhibitors, namely, L-NAME (N^G-nitro-L-arginine methyl ester), 7-NI (7-nitroindazole) and L-NIL (*N*-iminoethyl-L-lysine).[39,40] In our study, the results (Table 1) clearly show that the tested compounds can reduce oedema volume in a dose- and time-dependent (10, 20 and 40 mg/kg body weight) manner without any noticeable toxicity on the experimental rats. Incidentally, the in-vivo test could not be performed with physcion (**1**), owing to the small quantities of this product that we were able to prepare. In the case of emodin (**2**) and 1-hydroxytectoquinone (**3**), marked activity was found after 6 h of administration of carrageenan: reductions in the size of the oedema of ~68% ($P < 0.01$) and ~65% ($P < 0.05$), respectively, were observed at the highest tested concentration (40 mg/kg), as compared with the vehicle control. However, under similar conditions, oleanonic acid (**4**) could reduce only ~45% of the inflammation, while indomethacin, a standard positive control, showed ~82% ($P < 0.001$) inhibition at 10 mg/kg.

Next, compounds **1**–**4** and two standard anticancer agents (doxorubicin and camptothecin) were evaluated *in vitro* against one murine carcinoma (EAC), and three human cancer cell lines, namely A375 (malignant skin melanoma), Hep2 (epidermoid laryngeal carcinoma) and U937 (lymphoma). Results, as obtained from the MTT-reduction assay, showed that all the compounds could inhibit 50% of EAC cell proliferation at less than 10 μMs concentration (Table 2). Marked inhibition was observed when A375 cells were incubated for 24 h with 1-hydroxytectoquinone (**3**), as well as oleanonic acid (**4**) (IC50 values: 3.2 and ~11 μM, respectively). However, these compounds were relatively less toxic against Hep2 cells (IC50 >50 μM). Only a moderate cytotoxicity of the tested samples was observed against U937 cell line, with the IC50 ranging between 19 and 28 μM. Furthermore, none of the tested compounds were found to be cytotoxic against normal PBMC up to 100 μM concentration.

Finally, the antiproliferative potential of **3** and **4** was further demonstrated through microscopic observation of A375 cells treated with 10 μM of the compounds for 24 h (Figure 5). When treated with 1-hydroxytectoquinone (**3**), the cultured melanoma cells were observed to have irregular plasma membrane and condensed nuclei (Figure 5B), whereas, in the case of oleanonic acid (**4**), these cells were found to be in a more condensed form as compared to the untreated control cells, showing visible intracellular granules and shattered cytoplasm (Figure 5C).

Discussion

In this study, emodin and physcion isolated from *V. madraspatana* exhibited prospective iNOS inhibitory activity, in corroboration of some earlier reports on these

Table 1 Effects of tested compounds on carrageenan-induced rat paw oedema

Tested compounds	Dose (mg/kg body weight)	Oedema volume (ml) (% inhibition)			
		1 h	2 h	4 h	6 h
Vehicle control	–	0.70 ± 0.1	0.91 ± 0.1	1.24 ± 1.1	1.70 ± 0.1
Emodin (2)	10	0.73 ± 0.1	0.87 ± 0.1	1.20 ± 1.1	1.62 ± 1.2
		(NI)	(4.4 ± 1.7%)	(3.2 ± 1.0%)	(4.7 ± 1.5%)
	20	0.71 ± 0.2	0.77 ± 0.1*	0.90 ± 0.2	1.13 ± 0.3
		(NI)	(15.4 ± 1.1%)	(27.4 ± 1.2%)	(33.5 ± 1.6%)
	40	0.70 ± 0.2	0.69 ± 0.2**	0.55 ± 0.1*	0.54 ± 0.2**
		(NI)	(24.1 ± 1.9%)	(55.6 ± 1.4%)	(68.2 ± 1.3%)
1-Hydroxytectoquinone (3)	10	0.72 ± 0.3	0.89 ± 0.2	1.21 ± 1.3	1.64 ± 0.1
		(NI)	(2.2 ± 1.3%)	(2.4 ± 1.4%)	(3.5 ± 1.5%)
	20	0.73 ± 0.1	0.84 ± 0.2	0.98 ± 0.3	1.26 ± 0.2
		(NI)	(7.7 ± 1.8%)	(21.0 ± 1.5%)	(25.9 ± 1.8%)
	40	0.71 ± 0.2	0.80 ± 0.1	0.63 ± 0.1	0.60 ± 0.1*
		(NI)	(12.0 ± 1.2%)	(49.2 ± 1.7%)	(64.7 ± 1.2%)
Oleanonic acid (4)	10	0.73 ± 0.1	0.92 ± 0.3	1.20 ± 0.3	1.61 ± 0.3
		(NI)	(NI)	(3.2 ± 1.8%)	(5.3 ± 1.1%)
	20	0.70 ± 0.1	0.93 ± 0.1	1.10 ± 0.1	1.43 ± 0.1
		(NI)	(NI)	(11.3 ± 1.3%)	(15.9 ± 1.8%)
	40	0.72 ± 0.2	0.91 ± 0.2	0.89 ± 0.2	0.94 ± 0.1
		(NI)	(NI)	(28.2 ± 1.7%)	(44.7 ± 1.2%)
Indomethacin	10	0.38 ± 0.1	0.28 ± 0.1***	0.32 ± 0.1**	0.3 ± 0.1***
		(45.6 ± 1.4%)	(69.3 ± 1.5%)	(74.2 ± 1.2%)	(82.4 ± 1.1%)

Values are mean ± SD of three experiments, $n = 5$ in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared with vehicle control (Kruskal–Wallis test followed by Dunn's post-hoc test). NI, no inhibition.

Table 2 Cytotoxicity of tested compounds against human cancer cell lines and peripheral blood mononuclear cells

Compound (no.)	IC50 (μM)				
	EAC	A375	Hep2	U937	PBMC
Physcion (**1**)	8.4 ± 1.5	>100	>100	27.9 ± 1.7	>100
Emodin (**2**)	6.5 ± 1.2	74.8 ± 1.5	>100	18.8 ± 1.1	>100
1-Hydroxytectoquinone (**3**)	5.7 ± 1.8	3.2 ± 1.2	51.2 ± 1.5	23.4 ± 1.9	>100
Oleanonic acid (**4**)	7.1 ± 1.3	10.9 ± 1.5	59.3 ± 1.1	16.5 ± 1.3	>100
Doxorubicin[a]	>10	0.007 ± 0.001	0.42 ± 0.04	0.003 ± 0.001	15.51 ± 1.74
Camptothecin[a]	ND	0.003 ± 0.001	20.13 ± 2.42	0.100 ± 0.001	32.28 ± 2.04

Data represent mean values ± SD for three independent determinations. ND, not determined; [a]clinical anticancer drugs, i.e. doxorubicin and camptothecin taken as positive controls.

anthraquinonoids, which are also found in other medicinal plants.[42] Another analogous compound, 1-hydroxytectoquinone from *R. cordifolia*, showed similar activity, which is reported here for the first time although this activity of the *R. cordifolia* root itself has been reported previously.[43] Oleanonic acid, a pentacyclic triterpenoid, was also found to be an effective inhibitor of iNOS,[44] which further confirmed our earlier observations on the crude extract of *L. camara* root.[26] Three of these iNOS inhibitors were applied to carrageenan-induced rat paw oedema to validate their anti-inflammatory property in this in-vivo model. This is the first time such a test has been performed on these compounds.

Other studies on this model showed that inflammation of rat hind-paw induced by intraplantar injection of carrageenan comprised a relatively rapid early phase (up to 2 h), followed by an extended late phase (2–6 h).[39,40] The early phase of inflammation is known to be triggered by the concerted release of histamine, bradykinin and 5-hydroxytryptamine at the inflamed site, while the late-phase response is primarily due to the formation of pro-inflammatory prostanoids, coupled with persistent production of NO• through the iNOS pathway.[39] In fact, iNOS protein expression was found to attain its highest level at 10 h, and was not even detectable for 6 h. According to Handy and Moore, as well as Salvemini *et al*.,[39,40] some of the selective iNOS inhibitors, L-NIL (*N*-iminoethyl-L-lysine) or AG (aminoguanidine), will not inhibit paw oedema before 4 h of carrageenan administration, but will sustain the effect for 10 h.

Since our results indicated that the tested compounds could reduce the paw volume only after the sixth hour of carrageenan administration, this corroborates the supposition that these compounds also act through the suppression of the iNOS protein. Hence, it is reasonable to speculate that inhibition of NO• production through the modulation of iNOS

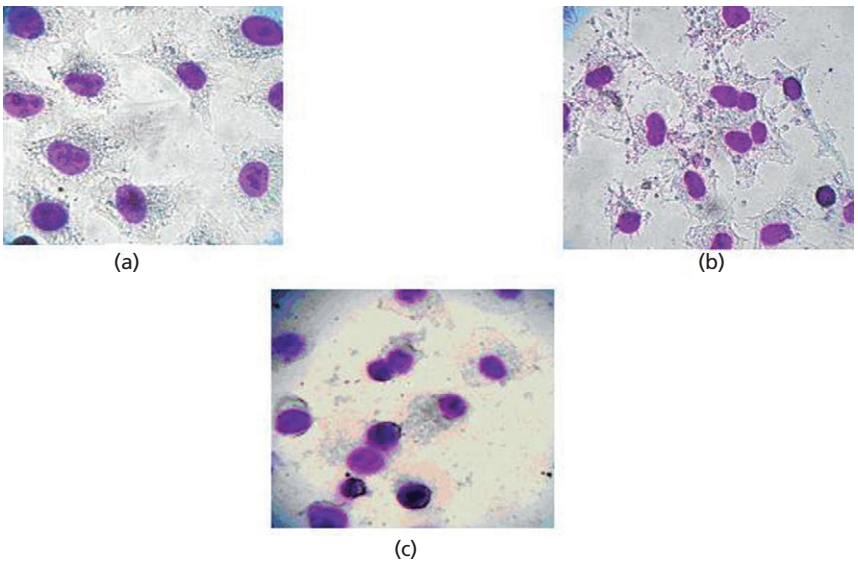

Figure 5 Morphological observation of the cultured A375 cells under light microscope. (a) untreated control cells; (b) treated with 1-hydroxytectoquinone (10 μM); (c) treated with oleanonic acid (10 μM); all for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Magnification × 1000.

may account, at least in part, for the anti-inflammatory properties of the selected plant samples or their customary herbal formulations. Further study of the detailed mechanistic pathway is merited in order to explain the contribution of these compounds at a molecular level.

The antiproliferative potential of these compounds was also explored, in view of the recent observation that iNOS expression may have a functional role in promoting the growth and progression of solid tumours, such as human gynaecological cancer, breast cancer and tumours of the nervous system.[7] In fact, iNOS inhibitors such as *N*-(3-(aminomethyl)benzyl) acetamidine (1400W), *S,S*-1,4-phenylene-bis(1,2-ethanediyl) bis-isothiourea, and L-*N*6-1-iminoethyl)lysine tetrazole-amide (SC-51) represent a novel approach for cancer chemotherapy.[8] Here, reasonably strong cytotoxicity of 1-hydroxytectoquinone and oleanonic acid against human malignant melanoma (A375) was observed for the first time, although some other constituents of *R. cordifolia*, particularly the cyclic hexapeptides, have been reported to be active against other cancer cells.[45,46] Similarly, the cytotoxicity of oleanonic acid obtained from different plant sources has been reported against other types of cancer.[47,48] Taken together, the present study reveals the anti-inflammatory potential of compounds **1**–**4**. Furthermore, the antiproliferative potential of 1-hydroxytectoquinone and oleanonic acid against human melanoma cells is presumably mediated through the down-regulation of iNOS protein, which would merit further mechanistic investigation of these compounds as potential 'leads' for the development of novel therapeutic agents.

Conclusions

The present paper explores three traditional medicinal plants in the light of their ability to suppress the expression of iNOS protein. Here, for the first time, we identify the active compounds and validate their iNOS inhibitory properties through the carrageenan-induced rat-paw oedema model *in vivo*. Through this combined approach it can be speculated that the inhibition of NO• through the modulation of iNOS (as established by Western blot analysis), partly accounts for the anti-inflammatory properties of the selected medicinal plants (as confirmed through an in-vivo animal study). Moreover, in compliance with the recent observation that iNOS inhibition may have a functional role in reducing cancer progression, the isolated compounds were tested for the first time for cytotoxicity against certain cancer cell lines. There remains ample scope to study the signalling pathway in detail, with a view to generating leads for designing novel chemotherapeutic agents.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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