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Cardamonin, inhibits pro-inflammatory mediators in activated RAW 264.7 cells and whole blood

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Abstract

Some chalcones, such as hydroxychalcones have been reported previously to inhibit major pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and reactive oxygen species production by suppressing inducible enzyme expression via inhibition of the mitogen-activated protein kinase (MAPK) pathway and nuclear translocation of critical transcription factors. In this report, the effects of cardamonin (2′,4′-dihydroxy-6′-methoxychalcone), a chalcone that we have previously isolated from *Alpinia rafflesiana*, was evaluated upon two cellular systems that are repeatedly used in the analysis of anti-inflammatory bioactive compounds namely RAW 264.7 cells and whole blood. Cardamonin inhibited NO and PGE_2 production from lipopolysaccharide- and interferon-γ-induced RAW cells and whole blood with IC_{50} values of 11.4 μM and 26.8 μM, respectively. Analysis of thromboxane B_2 (TxB_2) secretion from whole blood either stimulated via the COX-1 or COX-2 pathway revealed that cardamonin inhibits the generation of TxB_2 via both pathways with IC_{50} values of 2.9 and 1.1 μM, respectively. Analysis of IC_{50} ratios determined that cardamonin was more COX-2 selective in its inhibition of TxB_2 with a ratio of 0.39. Cardamonin also inhibited the generation of intracellular reactive oxygen species and secretion of TNF-α from RAW 264.7 cells in a dose responsive manner with IC_{50} values of 12.8 μM and 4.6 μM, respectively. However, cardamonin was a moderate inhibitor of lipoxygenase activity when tested in an enzymatic assay system, in which not a single concentration tested was able to cause an inhibition of more than 50%. Our results suggest that cardamonin acts upon major pro-inflammatory mediators in a similar fashion as described by previous work on other closely related synthetic hydroxychalcones and strengthens the conclusion of the importance of the methoxyl moiety substitution on the 4′ or 6′ locations of the A benzene ring.

Keywords: Cardamonin; *Alpinia rafflesiana*; RAW 264.7; Whole blood; Inflammatory mediator

1. Introduction

Many flavonoids are known to possess antioxidant (Bors et al., 1990), free radical scavenging, anti-inflammatory (Chen et al., 1998), and anti-tumour activity (Anto et al., 1995). A number of chalcones, a subclass of flavonoids, and their derivatives have also been found to inhibit the synthesis of nitric oxide (NO) (Cheng et al., 2001; Huang et al., 2001) and prostaglandin (PG) (Herencia et al., 1999, 1998; Huang et al., 2001; Kim et al., 2001), products of the nitric oxide synthase (NOS) and cyclooxygenase (COX) pathways, respectively. These bioactive chalcones are both naturally occurring and synthetic in nature. Recently DCDC (2′,5′,-dihydroxy-4-chloro-dihydrochalcone) was shown to inhibit NO synthesis in the lipopolysaccharide (LPS)-stimulated murine macrophage

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cell line RAW 264.7 through inhibition of inducible NOS (iNOS) expression (Huang et al., 2001). This activity was shown to be due to the inhibition of inhibitor of kappa B-α (IκB-α) degradation, a factor responsible for preventing nuclear translocation of the transcription factor nuclear factor-kappa B (NF-κB). In another study by Zhao et al. (2003), five chalcones were isolated from *Humulus lupulus* L. namely xanthohumol, xanthohumol D, dihydroxanthohumol, xanthohumol B and a new chalcone which was an oxidation product of xanthohumol, all demonstrated NO inhibitory activity via suppression of iNOS expression. It was concluded that the double bond between the α and β positions may be important for NO inhibitory activity, however the occurrence of a prenyl chain was not a prerequisite. In yet another study, a naturally occurring chalcone, broussochalcone A, isolated from *Broussonetia papyrifera* was shown to inhibit NO synthesis (Cheng et al., 2001). Apart from NO, chalcones have also been shown to inhibit the synthesis of prostaglandins. A recent study has shown that synthetic methoxychalcone derivatives inhibit the biosynthesis of prostaglandin E_2 (PGE_2) and this activity is related to the occurrence of 2′- and 4′-methoxyl groups (Kim et al., 2001). The seeds of *Alpinia blepharocalyx* have yielded diarylheptanoids that bear chalcone or flavanone moieties and strongly inhibited NO production form murine macrophages (Prasain et al., 1997). It is clear through structure activity relationship studies that not all chalcones possess anti-inflammatory activity. An example here involves chloroquinolinyl chalcones, whereby substituents (especially halogen) in the B ring, or the presence of pyridine at B, favour anti-inflammatory effects (Herencia et al., 1998). It is evident that chalcones are becoming an interesting class of compounds that may be used in the management of diseases that are primarily manifested by inflammatory processes.

Cardamonin (2′,4′-dihydroxy-6′-methoxychalcone; [$C_{16}H_{14}O_4$]; molecular mass 270.28) is a naturally occurring chalcone (Fig. 1). This compound was initially isolated from the seeds of *Amomum subulatum* (Bheemasankara et al., 1976) and subsequently from other zingiberous plant species. To date, very few biological effects have been ascribed to this compound. Cardamonin that was isolated from *Boesenbergia pandurata* demonstrated significant anti-mutagenic effects upon activation of heterocyclic amines (Trakoontivakorn et al., 2001). In another study, cardamonin isolated from *Alpinia henryi* caused vasorelaxation of rat mesenteric artery via NO-mediated processes (Wang et al., 2001). We have recently isolated cardamonin from the fruits of *Alpinia rafflesiana* and the anti-inflammatory activity of this compound in cellular models of inflammation is the subject of this report.

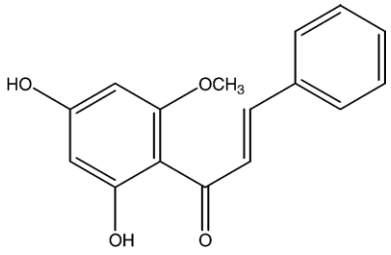

Fig. 1. Chemical structure of cardamonin (2′,4′-dihydroxy-6′-methoxychalcone).

2. Materials and methods

2.1. Reagents

Foetal calf serum was purchased from Mycoplex™ (PAA Lab. GmbH, Austria). Antibiotics (5000 U/ml penicillin and 5000 μg/ml streptomycin) and Dulbecco's Modified Eagle's Media (DMEM) were purchased from Flowlab™, Australia. Recombinant mouse IFN-γ was purchased from BD Pharmingen, USA. Absolute ethanol was purchased from Hayman Limited, UK. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Fluka, BioChemika, Buchs, Switzerland. Hank's Balanced Salt Solution (HBSS) was purchased from GIBCO BRL, Life Technologies™, Scotland. Dimethyl sulfoxide (DMSO) and toluene were purchased from BDH Laboratory Supplies, England. Radiolabeled [^{3}H]PGE_2 and [^{3}H]TxB_2 were purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. Tris–HCl was purchased from GIBCO BRL, USA and Tris-base was purchased from Pharmacia Biotech, Sweden. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Compound

Cardamonin or 2′,4′-dihydroxy-6′-methoxychalcone ($C_{16}H_{14}O_4$, MW 270.28) was isolated from fruits of *A. rafflesiana* was 99.99% pure and the structure was elucidated by spectroscopic methods (Mohamad et al., 2004). The compound was dissolved in 100% dimethyl sulphoxide (DMSO) as a stock at 50 mM and diluted to appropriate concentrations for assay as described in Materials and methods and Results. The final concentration of DMSO was kept constant at 0.1%, preliminary experiments determined that this concentration did not affect the formation of any of the mediators measured. This DMSO concentration allows solubilization of pure compounds in aqueous solution without toxic effects upon cells.

2.3. Cell culture and stimulation

RAW 264.7 cells were purchased from the European Collection of Cell Cultures (CAMR, UK) and maintained in DMEM supplemented with 10% foetal calf serum, 4.5 g/L glucose, sodium pyruvate (1 mM), L-glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml). Cells at a confluency of 80–90% were scraped out and centrifuged at 110×*g*/4 °C for 10 min. The concentration was then adjusted to 1×10^6 cells/ml and cell viability was always >95% as determined by trypan blue dye exclusion. Fifty microliters of cell suspension was dispensed into wells of a tissue culture grade 96-well plate (5×10^4 cells/well) except for blanks and incubated for 2 h at 37 °C, 5% CO_2 to attached the cells. After 2 h, unattached cells were gently discarded. Attached cells were then induced with both 200 U/ml of recombinant mouse interferon gamma (IFN-γ) and 10 μg/ml of *E. coli* LPS (strain 055:B5) in

the presence or absence of cardamonin at a final volume of 100 μl/well. The cardamonin stock was serially diluted to decreasing concentrations ranging from 50 to 0.78 μM (final concentration in all assays except TxB_2). Untreated and drug controls were stimulated with both LPS/IFN-γ and also had the same amount of DMSO in culture media. Cells were then incubated at 37 °C, 5% CO_2 for 17–20 h.

2.4. Nitrite determination

Supernatants of spent cell culture media were assayed for nitrite (NO_2^-) by the Griess reaction (Dirsch et al., 1998). Briefly, an equal volume of Griess reagent (1% sulfanilamide/0.1% naphtylethyenediamine dihydrochloride in 2.5% H_3PO_4) was mixed with cell culture supernatants and colour development was assessed at λ 550 nm with a microplate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA). Fresh culture medium was used as the blank in all the experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve (0–100 μM) freshly prepared in deionized water. Percent inhibition was calculated against control cells that were not treated but induced with LPS/IFN-γ and contained 0.1% DMSO.

2.5. Cell viability

Cell viability was assessed following removal of spent media. One hundred microliters of DMEM containing 5% FBS was added to each well followed by 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml). After 3 h, the formazan crystals were dissolved with 100 μl/well of 100% DMSO. The absorbance was measured at λ 570 nm with a microplate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA). Cell viability was determined as percentage of untreated stimulated cells.

2.6. Intracellular oxidative stress assay

Quantification of the inhibitory effect of cardamonin upon cellular oxidative stress was conducted by using the method of Wang and Joseph (1999) with some modifications. Black flat-bottomed tissue culture grade plates were used in place of transparent plates. Briefly, RAW 264.7 cells were induced into an inflammatory state as described previously. Following 17–20-h incubation at 37 °C, 5% CO_2, cell culture supernatants were discarded and replaced with 100 μl/well of 2′,7′-dichlorofluorescin diacetate (DCFH-DA; 100 μM, dissolved in RPMI-1640 containing 1% FBS) and incubated (5% CO_2, 37 °C) for 30 min. Following incubation, cells were gently washed with 200 μl/well of sterile warm (37 °C) HBSS to remove the unbound DCFH-DA, this was repeated twice. One hundred microliters of HBSS was then added to each well and the level of intracellular oxidation of DCFH to highly fluorescent dichlorofluorescein (DCF) by reactive oxygen species generated in the cells was measured using a SpectraMax GeminiXS multiwell fluorescence plate reader (Molecular Devices, Inc., USA). The excitation was set at 485 nm and emission at 530 nm, fluorescence was recorded every 5 min over a 30-min interval. The percentage increase of fluorescence was calculated using the following formula: $[Ft_{30} - Ft_0/Ft_0 \times 100]$ where, Ft_{30} is the fluorescence at 30 min and Ft_0 is the fluorescence at 0 min. Differences in the percentage increase of fluorescence of treated cells were compared to untreated controls and quercetin was used as a positive control.

2.7. Whole blood PGE_2 assay

Blood was drawn from healthy volunteers into heparin-coated vacutainer tubes. Two hundred microliters of heparinized blood was added to 800 μl RPMI 1640 (supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin) that contained 100 μg/ml heparin and 40 μg/ml acetylsalicylic acid. Monocytes were stimulated to produce PGE_2 by the addition of 10 μg/ml of *E. coli* serotype 0111:B4 LPS. Following a 24-h incubation at 37 °C in 5% CO_2, blood cells were sedimented by centrifugation and plasma was frozen at −80 °C. A radioimmunoassay was used to quantify the concentration of plasma PGE_2. Briefly, plasma (100 μl) was added to polystyrene tubes followed by addition of 100 μl of anti-PGE_2 antiserum (serum was diluted 1:8 with Tris buffer containing 1 g/L gelatine, 0.008 M Tris-base and 0.042 M Tris–HCl, pH 7.4) and 100 μl of $[H^3]PGE_2$ (stock diluted 1:150 with Tris buffer) and incubated overnight at 4 °C. Following overnight incubation, 200 μl of charcoal/dextran buffer (20 g/L activated charcoal and 4 g/L of dextran diluted in Tris buffer) was mixed into the tubes and incubated on ice for 5 min. Tubes were then centrifuged at 1000 × *g* at 4 °C for 10 min and the supernatant of each sample was gently poured into scintillation vials followed by the addition of 4 ml of scintillation cocktail (0.18 mM POPOP, 12 mM PPO, dissolved in 1 L of toluene and diluted with Triton X-100 at 2:1 ratio) and vortexed. Radioactivity was then counted over 5-min intervals in a liquid scintillation counter (Beckman Instruments, LS Analyser, Inc. USA). A standard curve was generated for the estimation of PGE_2 concentrations and both negative and positive controls were included in each run. A four-parameter logistic curve-fitting program was used to estimate PGE_2 concentrations.

2.8. Whole blood TxB_2 assay

TxB_2 concentrations of plasma were quantified following synthesis via both COX-1 and COX-2 pathways. Blood was drawn from healthy volunteers into heparin-coated vacutainer tubes and processed as described for PGE_2 estimation. For COX-1-derived TxB_2 quantification blood platelets were induced with 2 μl of calcium ionophore, A23187 (30 μM) and for COX-2-derived TxB_2 blood monocytes were induced with *E. coli* serotype 055:B5 LPS (10 μg/ml). Concentration of TxB_2 in samples was quantified by radioimmunoassay. The RIA protocol is similar to that used for PGE_2 quantification however the concentration of TxB_2 antiserum was used at 1:10 dilution and $[H^3]TxB_2$ was diluted 1:30. All other steps were the same as described for PGE_2 quantification.

2.9. Tumour necrosis factor-α (TNF-α) immunoassay

TNF-α secretion was measured using an OPTEIA™ Mouse TNF-α Set kit, Pharmingen according to the protocol of the manufacturer. The spent media was used without dilution.

2.10. Lipoxygenase (LOX) kinetic assay

One hundred and sixty microliters of 100 mM sodium phosphate, pH 8.0 was added into each well of UV transparent microplates (Greiner, Sigma, USA) followed by 10 μl of test compound in triplicate. The compound was assayed at seven 2-fold dilutions starting at 50 μM. The plate was pre-read at 234 nm in a microplate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA). Then 20 μl of enzyme (Soybean Lipoxygenase Type I-B, Sigma, USA) at a final concentration of 80 U/well was dispensed into each well. The plates were then incubated at room temperature for 10 min before adding 10 μl/well of 0.3 mM linoleic acid (substrate) and absorbance at 234 nm was measured every min over a 6-min interval. The percent inhibition of enzyme activity was calculated by following formula: $[1-(\text{slope}_{\text{sample}}-\text{slope}_{\text{control}})]\times 100$.

2.11. Statistical analyses

The IC_{50} values were calculated using one parameter model $[y=100/(1+a/x)]$ using GraphPad Prism software. Differences between groups were determined by one-way analysis of variance (ANOVA) followed by post hoc comparisons using least significant difference (LSD) method. Statistical significance of differences between groups was accepted at $p<0.05$.

3. Results

3.1. Effect of cardamonin on NO_2^- production and cell viability

The induction of RAW 264.7 cells into an inflammatory state by treatment with LPS/IFN-γ caused synthesis and secretion of NO. The breakdown product of secreted NO namely NO_2^- was detected in media at a mean concentration of 16.14±2.04 μM (Fig. 2). Cells that were not induced released trace amounts of NO. Cardamonin showed a dose-related inhibition of NO production in which significant inhibition was still evident at 6.25 μM. The IC_{50} was calculated at 11.39±1.53 μM. L-NAME, a standard NOS inhibitor, was used as a positive control and caused a significant inhibition (76.67±3.81%) of NO at 250 μM. Cardamonin did not affect cell viability at all concentrations used as assessed by mitochondrial reduction of MTT following a 17–20-h treatment, viability was always >95% (data not shown).

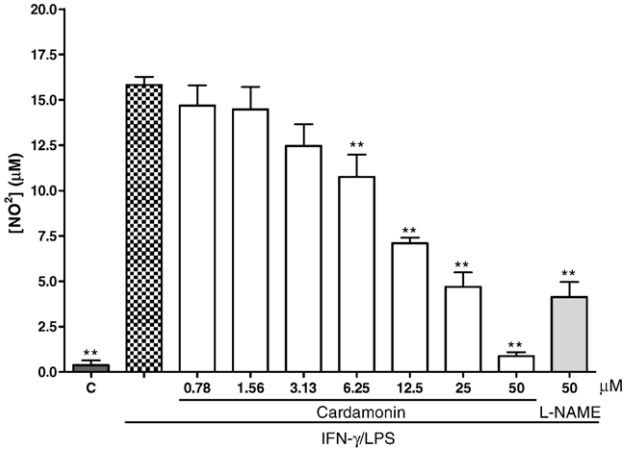

Fig. 2. Effect of cardamonin on nitric oxide production from RAW 264.7 cells. Cells were stimulated for 17–20 h with 200 U/ml recombinant murine IFN-γ and 10 μg/ml *E. coli* LPS and treated with increasing concentrations of cardamonin. Concentrations of NO_2^- in the media were determined by the Griess assay. The IC_{50} was calculated at 11.39±1.53 μM. All values are the mean±S.E.M. of three independent experiments. $^{**}P<0.01$, significantly different from the LPS/IFN-γ-treated control group.

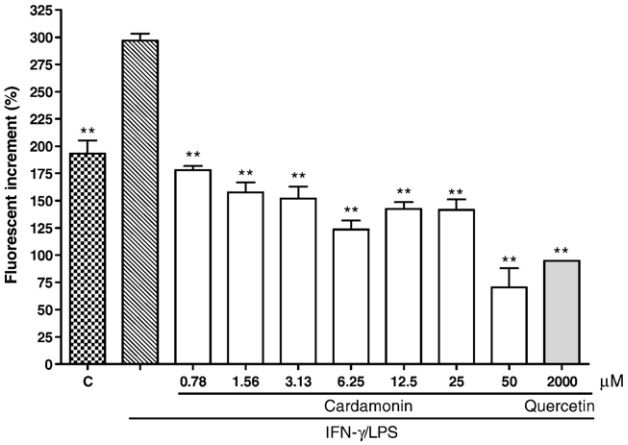

Fig. 3. Effect of cardamonin on reactive oxygen species production from RAW 264.7 cells. Cells were stimulated for 17–20 h with 200 U/ml recombinant murine IFN-γ and 10 μg/ml *E. coli* LPS and treated with increasing concentrations of cardamonin. Concentrations of reactive oxygen species in the media were determined by intracellular fluorometric assay. The IC_{50} was calculated at 12.79±7.07 μM. All values are the mean±S.E.M. of three independent experiments $^{**}P<0.01$, significantly different from the LPS/IFN-γ-treated control group.

3.2. Effect cardamonin on reactive oxygen species production

Treatment of induced RAW 264.7 cells with cardamonin showed dose-dependent inhibition of intracellular reactive

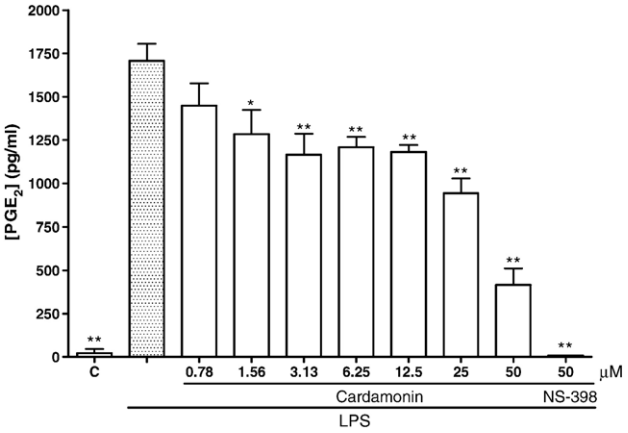

Fig. 4. Effect of cardamonin on prostaglandin E_2 (PGE_2) production from whole blood. Cells were stimulated for 24 h with 10 μg/ml *E. coli* LPS and treated with increasing concentrations of cardamonin. Concentrations of PGE_2 in the plasma were determined by radioimmunoassay. The IC_{50} was calculated at 26.79±4.88 μM. All values are the mean±S.E.M. of three independent experiments. $^{**}P<0.05$, $^{**}P<0.01$, significantly different from LPS-treated blood.

oxygen species generation (Fig. 3). The compound was significantly active at all concentrations tested with an IC_{50} value of 12.79±7.07 μM. Quercetin is known for its inhibitory effects upon RAW cell intracellular reactive oxygen species generation and was used as a positive control which afforded 62.6±5.61% inhibition at 2 mM. Cardamonin was equivalent in its inhibitory effects albeit at a much lower concentration.

3.3. Effect of cardamonin on PGE_2 production

As shown in Fig. 4, cardamonin inhibited PGE_2 production in a dose-dependent manner. Significant inhibition was attainable with concentrations of cardamonin as low as 1.56 μM. The IC_{50} value was 26.79±4.88 μM. NS-398, a COX-2 inhibitor, was used as a drug control and significantly inhibited the production of PGE_2 at 97.27±0.08% at a concentration of 50 μM.

3.4. Effect of cardamonin on TxB_2 production

Fig. 5 shows the effect of cardamonin upon production of TxB_2 from leucocytes following stimulation of both COX-1 and COX-2 pathways. Stimulation of TxB_2 production with calcium ionophore (A 23435) released maximal amounts of TxB_2 (647.48 pg/ml±33.65). This production was dose-dependently decreased following treatment with cardamonin and inhibition was significant at doses as low as 0.24 μM. The IC_{50} was 2.88±0.22 μM. When blood cells were exposed to LPS for 3 h mean plasma concentrations of TxB_2 increased to 37.44±1.55 pg/ml. This was due to activation of the COX-2 pathway of eicosanoid synthesis. Similarly cardamonin strongly inhibited TxB_2 production via the COX-2 pathway in a dose-dependent manner with an IC_{50} of 1.07±0.11 μM.

Ketoprofen was used as a drug control in both assays for TxB_2 and demonstrated dose-dependent inhibition of production via both COX pathways. However, the inhibitory effect was more pronounced upon TxB_2 production via the COX-1 pathway, this was to be expected since ketoprofen is more preferential to COX-1 inhibition (Cryer and Feldman, 1998). The IC_{50} ratio for cardamonin was calculated to be 0.39, which shows that this compound has a preference for inhibiting the COX-2 pathway of TxB_2 synthesis.

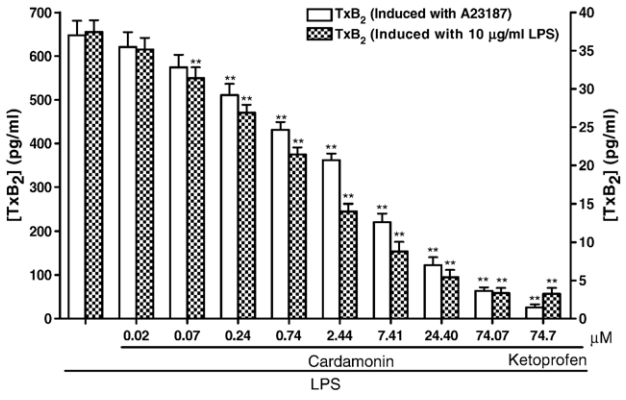

Fig. 5. Effect of cardamonin on thromboxane B_2 (TxB_2) production from whole blood. Cells were either stimulated with 10 μg/ml *E. coli* LPS or 30 μM A23187 calcium ionophore for 24 h and treated with increasing concentrations of cardamonin. Concentrations of TxB_2 in the plasma were determined by radioimmunoassay. The IC_{50} was calculated at 1.07±0.11 μM (LPS activation of COX-2) and 2.88±0.22 (A23187 activation of COX-1). All values are the mean±S.E.M. of six independent experiments. $^{**}P<0.01$, significantly different from LPS/A23187-treated blood.

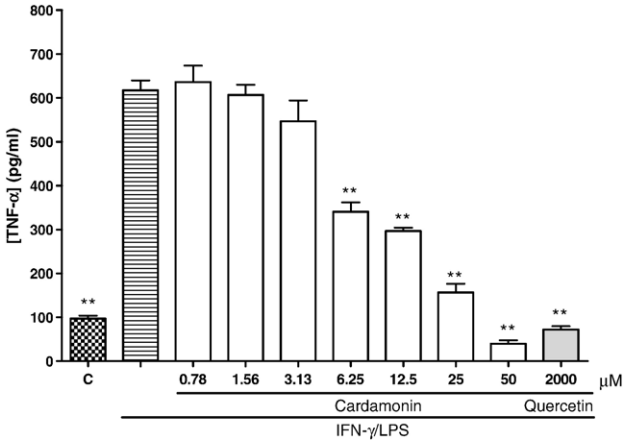

Fig. 6. Effect of cardamonin on tumour necrosis factor-α (TNF-α) production from RAW 264.7 cells. Cells were stimulated for 17–20 h with 200 U/ml recombinant murine IFN-γ and 10 μg/ml *E. coli* LPS and treated with increasing concentrations of cardamonin. Concentrations of TNF-α in the media were determined by ELISA. The IC_{50} was calculated at 4.57±0.25 μM. All values are the mean±S.E.M. of six independent experiments. $^{**}P<0.01$, significantly different from LPS/IFN-γ-treated control group.

3.5. Effect of cardamonin on TNF-α production

Fig 6 shows the effect of the various treatments upon TNF-α production from RAW cells. The basal medium concentration of TNF-α in the absence of IFN-γ/LPS was 96.83±18.26 pg/ml. Following exposure of RAW 264.7 cells to LPS/IFN-γ, a significant amount of TNF-α was released into the media

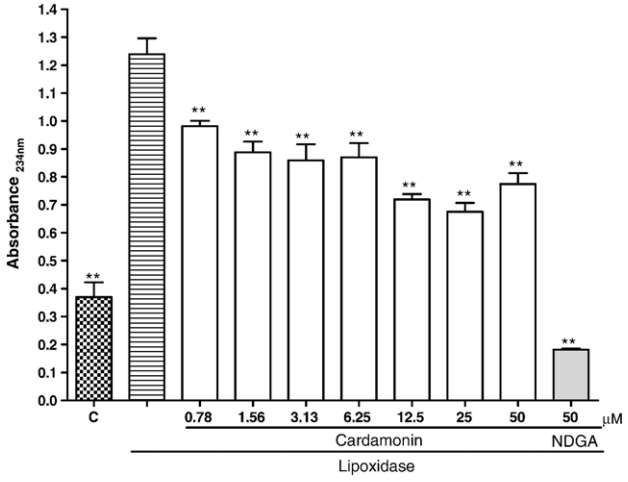

Fig. 7. Effect of cardamonin on lipoxygenase activity. Cells were either stimulated with 10 μg/ml *E. coli* LPS or 30 μM A23187 calcium ionophore for 24 h and treated with increasing concentrations of cardamonin. The IC_{50} was calculated at 1.07±0.11 μM (LPS activation of COX-2) and 2.88±0.22 (A23187 activation of COX-1). All values are the mean±S.E.M. of three independent experiments. $^{**}P<0.01$, significantly different from control.

(616.83±65.2 pg/ml). Cardamonin demonstrated a dose-dependent inhibitory effect upon TNF-α production in which doses as low as 6.25 μM were significantly suppressive. The IC_{50} was calculated at 4.57±0.25 μM. Quercetin was used as a drug control at the published effective concentration and strongly inhibited the production of TNF-α.

3.6. Effect of cardamonin on lipoxygenase enzyme activity

Fig. 7 shows the effect of the various treatments upon LOX activity. Following reaction of LOX with linoleic acid a significant increase in absorbance was observed. All doses of cardamonin demonstrated a dose-dependent inhibitory effect upon LOX activity. Despite significant inhibition of enzyme activity cardamonin was not strongly inhibitory and none of the doses used could induce an inhibitory percentage of more than 50%, therefore the IC_{50} was not calculated. Nordyhydroguaretic acid (NDGA) was used as a drug control at the published effective concentration and strongly inhibited the activity of LOX (90.00±2.4%).

4. Discussion

Chalcones are abundantly present in the plant kingdom and have various biological activities which include anti-inflammatory, anti-allergy, antioxidant and antimicrobial effects (Busse et al., 1984; Middleton and Drzewiecki, 1984; Lopez et al., 2001). Several recent reports have shown that 2′-hydroxychalcone derivatives possess potent anti-inflammatory activity (Kim et al., 2001; Ban et al., 2004; Won et al., 2005; Ko et al., 2003). *Alpinia* species have been used traditionally for their anti-inflammatory activity (Burkill, 1966), however the principle component responsible for this activity has not been described to date. Preliminary screening for iNOS inhibition of compounds isolated from this species in our laboratory has led us to believe that cardamonin is possibly the compound responsible for anti-inflammatory activity. Our experiments have concluded that cardamonin has significant inhibitory effects upon pro-inflammatory mediator release in the cellular systems employed. It is therefore highly possible that cardamonin is the active principle when used in folkloric medicine.

Our results strengthen the findings of several reports regarding the anti-inflammatory activity of chalcones and flavonoids. Although a direct comparison cannot be made with other compounds reported in the literature due to differences in experimental procedures, it is evident that cardamonin shows similar effects as described by Kim et al. (2001) in their SAR study of chalcones in which they showed that among 2′-hydroxychalcone derivatives a methoxyl group at 4′ and 6′ is essential for the expression of PGE_2 inhibitory activity. They also demonstrated that substitution of the B ring was not essential for activity. On a similar note, we have also shown that cardamonin is active despite the absence of functional group substitution on the B ring, cardamonin in fact has a methoxyl substitution on the 6′ of the A ring. Similarly, Ban et al. (2004) had shown several 2′-hydroxychalcone derivatives were potent inhibitors of NO and TNF-α via suppression of nuclear translocation of NF-κB.

It is interesting to note that the activation of COX and iNOS genes is mediated by the excess production of reactive oxygen species (Morel and Barouki, 1999). Nuclear translocation of the transcription factor NF-κB is essential for the activation of COX and iNOS genes and redox regulation is involved in NF-κB activation (Piette et al., 1997; Sen and Packer, 1996). Recently, Ban et al. (2004) demonstrated that several synthetic 2′-hydroxychalcones exhibit inhibitory effects upon NF-κB translocation. Our findings have demonstrated anti-oxidative effects of cardamonin and it is therefore possible that cardamonin may inhibit the products of COX and iNOS, namely PGE_2 and NO, due to the scavenging properties of this compound that leads to repression of gene transcription. Furthermore, some chalcones have also been demonstrated to scavenge NO (Herencia et al., 2002) and therefore it is also possible that cardamonin may possess this activity.

The effect of cardamonin upon prostanoid generation was very significant in that both the production PGE_2 and TxB_2 were significantly reduced in a dose-dependent fashion. The inhibition of prostanoid synthesis is the major desirable effect of NSAID's which leads to reduction of pain and swelling (Bley et al., 1998). As discussed earlier, these steroids are the product of the COX enzyme system and therefore cardamonin may have inhibited their production either through redox regulation of NF-κB translocation or via scavenging of reactive oxygen species that activate COX genes or via direct inhibition of COX enzyme activity. These possibilities are being evaluated in our laboratory.

Cardamonin also demonstrated moderate inhibitory activity against enzymatic activity of LOX, another key enzyme involved in the inflammatory response which is involved in the synthesis of leukotrienes. Although cardamonin significantly reduced enzymatic activity, the doses used failed to inhibit activity more than 50% and thus the IC_{50} value was not attainable. Nevertheless, it is interesting to note that cardamonin has dual LOX/COX inhibitory activity albeit at moderate levels. Modification of the compound may lead to enhanced activity and selectivity. Nakadate et al. (1997) showed that some chalcone derivatives inhibit both LOX and COX in mouse epidermis. Hydroxy groups on the A ring markedly influenced the inhibitory action of chalcones. The presence of hydroxy groups on the benzene ring B was not essential for the inhibitory action on LOX/COX but slightly modified the potencies of their inhibitory action for both enzymes.

TNF-α is a significant proinflammatory mediator which is involved in the pathogenesis of several inflammatory diseases via induction of secretion of IL-1, IL-6 and IL-10 and activation of T cells (Marriot et al., 1997) and therefore suppression of TNF-α would be beneficial in the management of inflammatory-related diseases. The significant inhibition of TNF-α production by cardamonin may be due to the fact that inhibition of NF-κB nuclear translocation causes reduced expression of TNF-α genes (Yao et al., 1997). Due to the close similarity of cardamonin's structure to that of several 2′-hydroxychalcone derivatives evaluated by Ban et al. (2004), it is highly possibly that cardamonin inhibits NF-κB activation leading to reduced TNF-α synthesis.

In conclusion, it is evident that cardamonin isolated from the fruits of *A. rafflesiana* possesses anti-inflammatory properties in cellular models and we speculate the most possible mechanism involved is the inhibition of activation of the NF-κB pathway. Our current research involves evaluating the effect of cardamonin upon pro-inflammatory signaling pathways in order to determine exactly its target molecule.

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