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In vitro and *in vivo* anti-inflammatory activities of columbin through the inhibition of cycloxygenase-2 and nitric oxide but not the suppression of NF-κB translocation

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ABSTRACT

Columbin, a diterpenoid furanolactone, was isolated purely for the first time from the plant species Tinspora bakis. The anti-inflammatory effects of columbin were studied *in vitro*, *in silico* and *in vivo*. The effect of columbin on nitric oxide was examined on lipopolysaccharide–interferon-gamma (LPS/IFN) induced RAW264.7 macrophages. *In vitro* and *in silico* cyclooxygenase-1 and cyclooxygenase-2 inhibitory activities of columbin using biochemical kit and molecular docking, respectively, were investigated. Mechanism of columbin in suppressing NF-kappaB-translocation was tested using Cellomics®NF-κB activation assay and ArrayScan Reader in LPS-stimulated RAW264.7 cells. Moreover, effects of columbin *in vivo* that were done on carrageenan-induced mice paw-oedema were tested. Lastly, the *in vitro* and *in vivo* toxicities of columbin were examined on human liver cells and mice, respectively. Treatment with columbin or N^{ω}-nitro-L-arginine methyl ester (L-NAME) inhibited LPS/IFN-γ-induced NO production without affecting the viability of RAW264.7. Pre-treatment of stimulated cells with columbin did not inhibit the translocation of NF-κB to the nucleus in LPS-stimulated cells. COX-1 and COX-2 inhibitory activities of columbin were 63.7 ± 6.4% and 18.8 ± 1.5% inhibition at 100 μM, respectively. Molecular docking study further helped in supporting the observed COX-2 selectivity. Whereby, the interaction of columbin with Tyr385 and Arg120 signifies its higher activity in COX-2, as Tyr385 was reported to be involved in the abstraction of hydrogen from C-13 of arachidonate, and Arg120 is critical for high affinity arachidonate binding. Additionally, columbin inhibited oedema formation in mice paw. Lastly, the compound was observed to be safe *in vitro* and *in vivo*. This study presents columbin as a potential anti-inflammatory drug.

1. Introduction

Recent advances in fundamental biomedical research have established an essential role for inflammation in mediating some human afflictions. This list of inflammatory diseases would run to over 100 each of which causes the deterioration of tissue in one or more parts of the body (Kharitonov, 2004; Nathan and Ding, 2010). Therefore, the inflammatory response must be actively ended when no longer needed to prevent unnecessary harmful biological processes. Mechanisms which serve to terminate inflammation include various cellular and immunological responses and could be initiated using potential anti-inflammatory compounds, which work specifically by inhibiting inflammatory components or activating transcription factors (Tak and Firestein, 2001).

Current anti-inflammatory drugs can inhibit inflammation as curative agents. These conventional drugs have not been successful to cure chronic inflammatory disorders. Natural products play a significant role in human health in relation to the prevention and treatment of inflammatory conditions. Therefore, there is a need for new and safe anti-inflammatory agents and one of the on-going research candidates are plant constituents used in herbal and traditional medicine (Nam, 2006; Read, 1995). *Tinospora bakis* Miers (Menisoermaceae), locally known in Sudan as "Erg-elhagar", is used traditionally to treat headache and rheumatism (Broun and Massy, 1929; El Ghazali et al., 2003). Recently, Zafinindra et al. (2003) indicated that the aqueous extract of *T. bakis* roots has shown *in vivo* antipyretic effects. Alkaloidal extract of this plant have revealed also *in vitro* antimalarial activity against *Plasmodium falciparum* chloroquine-resistant strain

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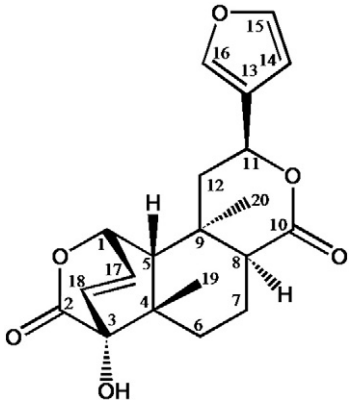

Fig. 1. Chemical structure of columbin.

W2 (Ouattara et al., 2006). Recently our laboratory confirmed that the plant ethanolic crude extract has potent immunosuppressive and antioxidant activities.

Biologically guided phytochemical study in our laboratory led to the isolation of columbin (Fig. 1), a diterpenoid furanolactone, in a bulk amount for the first time from the root of *T. bakis* (Koko et al., 2008, 2009). This is thus the fourth report of its occurrence in this family. Originally isolated from colombo root (*Jateorhiza palmata* Miers, Memspermaceae), columbin has more recently been found in seeds of *Sphenocentrum Jollyanum* (Memspermaceae). It has, however, been found once in an unrelated family, in *Melothria maderaspatana* Cogn. (Cucurbitaceae), so that it cannot be regarded as being entirely specific to the Menispermaceae family. Nevertheless, the occurrence of several oxides of columbin in another member of the family, *Fdxzurru chloroleucu*, suggests that this type of diterpenoid bitter principle may be fairly widespread in this plant group (Cava and Soboczenski, 1956; Cava et al., 1959; Chen et al., 1973; Gilbert et al., 1967; Hanuman et al., 1986; Ramstad et al., 1975). The crude drugs *Jateorhiza columba*, *T. capillipes*, *T. sagittata*, *Dzoscoreophyllum cummznsiz*, *Sphenocentrum Jollyanum* and *M. maderaspatana* contain columbin, and were used for traditional healing of various diseases including inflammatory ones (Cava and Soboczenski, 1956; Cava et al., 1959; Chen et al., 1973; Gilbert et al., 1967; Hanuman et al., 1986; Ramstad et al., 1975). However there were few reports on the biological activities of this bitter substance. Therefore, the current study was designed to investigate the mechanistic anti-inflammatory action of columbin *in vitro*, *in silico* and *in vivo*. We have also tested toxicity of the compound on normal mice and human normal liver cells (WRL-68).

2. Materials and methods

2.1. Cell lines and reagents

RAW264.7 cell line was obtained from American Type Culture Collection (ATCC), USA. Dulbecco's Modified Eagle Medium (DMEM) both with and without phenol red, phosphate buffered saline and Hanks' balanced salt solution (HBSS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phosphate buffered saline (PBS) and Griess reagent were from Invitrogen (Carlsbad, USA). Foetal bovine serum (FBS), LPS from *E. coli* serotype 0111:B4, Indomethacin, L-NAME [L-NG-nitroarginine methyl ester (hydrochloride)], dimethylsulfoxide (DMSO), and sodium nitrite were obtained from Sigma (St Louis, USA). Interferon gamma (IFNγ) was from BD Biosciences (New Jersey, USA). NF-κB translocation kit was from Cellomics (Pittsburg, USA). All other chemicals and reagents used were of HPLC grade.

2.2. Plant material and compound isolation

Tinospora bakis Miers was collected in April 2009 from its natural habitat in the Central Sudan. The voucher specimen was identified by Dr. Wai'l S. Abdalla, a Senior Botanist at the Herbarium of Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan, where the specimen was also deposited and coded with GG6-07. Columbin (Purity 98.8%) was isolated from the whole plant of *T. bakis* according to the method described earlier (Koko et al., 2009).

2.2.1. Identification of columbin

It is a diterpenoid furanolactone isolated from neutral chloroform fraction of *T. bakis* in a very bulk amount (23 g; 0.3%) through 40 mm diameter size column chromatography with 250 g of column silica gel eluted with 50% acetone/hexane system. The compound was purified by washing with methanol (less soluble). Physical status: colourless crystal. R_f: 0.55 (3% chloroform/methanol). Melting point: 191–193 °C. $[\alpha]_D^{20}$: 52.7° (c = 10, pyridine). UV ($CHCl_3$) λ_{max} nm (log ε): 215 (furan ring). IR ($CHCl_3$) $\nu_{max}cm^{-1}$: 3460, 3485 (OH); 1740, 1720 (lactone) 1500, 890 (furan); 1610 (C = C). ^{1}HNMR ($CDCl_3$, 300 MHz,): 7.60 (1H, m, H-16), 7.45 (1H, m, H-15), 6.67 (1H, m, H-14), 6.40 (1H, dd, J = 5.0, 8.0 Hz, H-17), 6.12 (1H, dd, J = 8.5, 1.5 Hz, H-18), 5.47 (1H, dd, J = 11, 4.5 Hz, H-11), 5.21 (1H, dd J = 5.0, 1.5 Hz, H-1), 2.50 (1H, m, H-5), 2.40 (1H, m, H-8), 2.33 (2H, d, J = 4.7 Hz, H-6), 2.21 (2H, m, H-12), 2.01 (2H, d, J = 11.0 Hz, H-7), 1.10 (3H, s, Me-19), 0.85 (3H, s, Me-20). The UV, IR and ^{1}HNMR are identical with literature values for columbin (Gilbert et al., 1967; Moody et al., 2006). ^{13}CNMR ($CDCl_3$, 100 MHz): 175.1 (C-2), 173.2 (C-10), 144.7 (C-15), 140.2 (C-16), 135.8 (C-17), 132.8 (C-18), 130.3 (C-13), 110.4 (C-14), 77.1 (C-3), 76.6 (C-1), 72.4 (C-11), 51.9 (C-8), 43.5 (C-12), 42.2 (C-9), 39.5 (C-5), 32.4 (C-4),31.6 (C-6), 22.7 (C-7), 20.1 (C-19), 17.6 (C-20). HREI MS: m/z 358.142 (calcd 358.1416 for $C_{20}H_{22}O_6$). EI MS (rel.int. %): m/z 358 (13), 314 (8), 252 (32), 210 (46), 193 (25), 142 (28), 121 (31), 107 (100). The HREI MS gave the formula $C_{20}H_{22}O_6$ (calcd 182.1416) with loss of (O − C═O) at the first peak (314) *i.e.* decarboxylated. UV showed the absorption furan, while IR spectrum showed the presence of OH, lactone as well as furan. The ^{1}HNMR and ^{13}CNMR spectra together with DEPT experiment indicated the presence of lactone and ester carbonyls (δ 175.1 and 173.2) a furan ring (δ 7.6 m, 7.5 m, 6.7 m, 144.7, 140.2, 130.3 and 110.4). The presence of hydroxyl is indicated by the presence of downfield chemical shift of C-3 (δ 77.1). The singlet coupling at proton 19 and 20 δ 1.1 and 0.9 indicates the presence of two methyl groups at δ 20.1 and 17.6 respectively. The chemical shift of methyl group at C-9 was observed at high field due the influence of furan ring on the same side as the C-9 methyl group. The structure and stereochemistry of the compound were compared with previously authenticated one (Atta-urRahman and Ahmad, 1988; Gilbert et al., 1967; Moody et al., 2006).

2.3. Effect of columbin on nitric oxide and PGE_2 production

2.3.1. Cell culture and stimulation

The murine monocytic macrophage cell line (RAW 264.7) was maintained in DMEM supplemented with 10% FBS, 4.5 g/l glucose, sodium pyruvate (1 mM), L-glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml) at 37 °C and 5% CO_2. Cells at confluency of 80–90% were centrifuged at 120 × g at 4 °C for 10 min and cell concentration was adjusted to (2×10^6) cells/ml, whereby the cell viability always more than 90%, as determined by trypan blue exclusion. A total of 50 μL of cell suspension was seeded into a tissue culture grade 96-well plate (4×10^5 cells/well) and incubate for 2 h at 37 °C, 5% CO_2 for cells attachment. Then, the cells were stimulated by using 100 U/ml of IFN-γ and 5 μg/ml of LPS with or without the presence of columbin tested at the final volume of 100 μL/well. DMSO was used as vehicle,

where the final concentration of DMSO was maintained at 0.1% of all cultures. Cells were further incubated at 37 °C, 5% CO_2 for 20 h. The culture supernatant was subjected to Griess assay for nitrite determination and the cells remaining in the well were tested for cell viability assay by using MTT reagent.

2.3.2. Griess assay

To evaluate the inhibitory activity of columbin on nitric oxide (NO) production, culture media was assayed using Griess reaction (Granger et al., 1996). Briefly, an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-(ι-naphthyl)-ethylene diamine dihydrochloride, dissolved in 2.5% H_3PO_4) was mixed with culture supernatant and colour development was measured at 550 nm using a microplate reader (SpectraMax Plus, Molecular Devices Inc., Sunnyvale, CA, USA). The amount of nitrite in the culture supernatant was calculated from a standard curve (0–100 μM) of sodium nitrite freshly prepared in deionized water. Percentage of the NO inhibition was calculated by using nitrate level of IFN-γ/LPS-induced group as the control.

$$NO\ inhibitory(\%) = \frac{[NO_2^-]_{control} - \left[NO_2^-\right]_{sample}}{[NO_2^-]_{control}} \times 100$$

2.3.3. PGE_2 assays

PGE_2 level in macrophage culture medium was quantified by EIA kits according to the manufacturer's instructions. NS-398 was used as a positive control in the assay.

2.3.4. Cell viability of RAW 264.7 macrophage

Potential anti-NO agents are preferred to be safe. Therefore, the cytotoxicity of columbin on cultured cells was determined by assaying the reduction of MTT reagents to formazan salts (Mossman, 1983). After removing of supernatant, the MTT reagents (0.05 mg/ml dissolved in sterile PBS, pH7.0) were added into each well. The cells remaining were incubated at 37 °C for 4 h and the formazan salts formed were dissolved by adding 100 μl of 100% DMSO in each well. The absorbance was then measured at 570 nm using SpectraMax Plus microplate reader (Molecular Devices, USA). The percentage of cell viability was calculated by using the cell viability of IFN-γ/LPS-induced group as the control.

$$Cell\ Viability(\%) = \frac{OD_{control} - OD_{sample}}{O_{control}} \times 100$$

2.4. Effect of NF-κB translocation pathway

RAW264.7 was seeded overnight at 1.2 or 2.5×10^5 cells/ml, in a 96-well plate. The cells were either pre-treated for 1 h with different concentrations of columbin or were left untreated. RAW264.7 cells were then stimulated with 10 ng/ml of LPS for 30 min. The medium was discarded and cells were fixed and stained using Cellomics®NF-κB activation kit from Thermo Scientific according to the manufacturer's instructions. The assay plate was evaluated on ArrayScan HCS Reader. The Cytoplasm to Nucleus Translocation BioApplication software was used to calculate the ratio of cytoplasmic and nuclear NF-κB intensity (Ding et al., 1998). The average intensity of 200 objects (cells) per well was quantified. The ratios were then compared among stimulated, treated, and untreated cells.

2.5. In vitro evaluation of COX-1 and COX-2 inhibitory activity of columbin

The inhibition of the enzymes COX-1 and COX-2 are considered as one of the mechanisms of anti-inflammatory actions; therefore, columbin was tested for COX-1 and COX-2 inhibitory activity using a COX-inhibitor screening kit (Catalog No.560101, Cayman Chemical, USA) according to the manufacturer's instructions. The EIA kit to determine the COX-1 and COX-2 inhibitory activity has been used earlier (Herrera-Salgado et al., 2005). COX is involved in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins. COX catalyses the conversion of arachidonic acid to PGH_2. This assay measures the production of PGF2α generated by $SnCl_2$, in the presence of PGH_2. The initial reactions take place in test tubes heated at 37 °C. In the background tubes reaction buffer and heme are mixed. In the 100% activity tubes reaction buffer, heme, the enzyme in question and solvent are added. In the sample tubes reaction buffer, heme, inhibitor at different concentrations and enzyme are added. The tubes are incubated for 15 min at 37 °C. Then arachidonic acid is added and the tubes were incubated for 2 min. HCl 1 M is used to stop the reaction and stannous chloride traps the reaction product and reduces it to a more stable form. The tubes are incubated a final time for 5 min at room temperature. The tubes were then diluted while the backgrounds and left as they are. A 96-well plate coated with mouse anti-rabbit IgG is provided. In the wells of the plate nonspecific binding, maximum binding, standards, and the inhibitor dilutions are added with tracer and antiserum. The plate is incubated at room temperature for 18 h, washed 5 times with wash buffer, developed with Ellman's Reagent and read on a microplate reader at 410 nm. The stock solution of the compound was dissolved in DMSO with final concentration of 0–100 μM. The percentage of inhibition for the respective COX enzyme was graphically determined from three-point curves. Indomethacin was used as reference standard. EC50 was calculated and the selectivity of columbin for COX enzymes was calculated based on the method mentioned previously (Gierse et al., 2008).

2.6. Paw oedema induced by carrageenan

The carrageenan-induced mice paw oedema test was used as an experimental model for screening the anti-inflammatory activity according to the modified method of Dordevic et al. (2007). Male Balb/c mice (5–6 weeks of age) were obtained from Animal House, University of Malaya, Kuala Lumpur, Malaysia, left for 7 days to acclimatize and were only used once throughout the experiments. All the experiments were conducted in accordance with the ethical guidelines on animal experimentation approved by the Animal Care Unit Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Animals were fasted 12 h prior to experiment. Mice ($N = 60$) were randomly divided into six groups, and thus each group consisted of 10 animals. Columbin was intra-peritoneally administered to mice at the dose of 30, 100, 300 and 700 mg/kg. Aspirin, an anti-inflammatory drug, was used as a positive control. To induce acute phase inflammation in paw, rats were injected subcutaneously into the right hind paw with a 1% solution of carrageenan dissolved in saline 30 min after vehicle or columbin treatment. The paw volumes were measured up to 5 h after the injection at intervals of 1 h. Paw volume was measured with a plethysmometer (Ugo Basile, Italy) immediately prior to the injection of carrageenan and thereafter at an interval of 1 h for a period of 5 h. Oedema inhibitory activity was calculated according to the following formula:

$$Percentage\ inhibition = \frac{[(C_t - C_0)_{control} - (C_t - C_0)_{treated}]}{[(C_t - C_0)_{control}]} \times 100$$

where, C_t = mean paw volume for each group at time t, and C_0 = mean paw volume for each group before carrageenan injection.

2.7. Molecular docking

The aim of this docking is to investigate the interactions involved in the binding of columbin to active sites of COX-1 and COX-2 via *in*

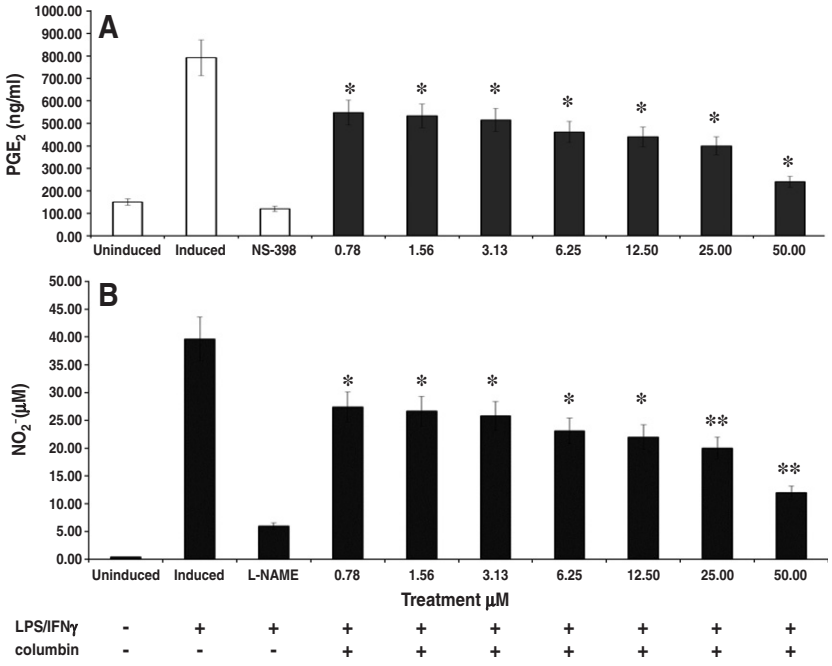

Fig. 2. The effects of columbin on NO and PGE_2 production: murine macrophage cells were left untreated or pretreated with the indicated concentrations of columbin. The cells were then either left in medium or were pretreated with LPS/IFN-gamma. The data are average of 3 independent experiments. *Significant at 0.05 and ** at 0.01 as compared to induced cells.

silico computational method. The three-dimensional structures of COX-1 and COX-2 were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb; accession codes 1EQG and 1PXX, respectively) (Berman et al., 2000). The structure of columbin was built using ChemBioDraw Ultra software, and optimized using HyperChem Professional software (Hypercube Inc.) with PM3 parameters using the steepest descent and conjugate gradient (Polak-Ribiere) algorithms (convergence criteria were set to maximum of 500 cycles or 0.1 kcal/Å mol RMS gradient). Docking files were prepared using AutoDock Tools v.1.5.4 software (http://www.scripps.edu/~sanner/python/adt) (Coon et al., 2001). For the protein molecules, all heteroatoms including the drugs, water molecules and molecules originating from the crystallization buffers, were removed. Polar hydrogen atoms were then added and non-polar hydrogen atoms were merged, Kollman charges and solvation parameters were assigned by default. For the ligand, Gasteiger charges were added, non-polar hydrogen atoms were merged, and all bonds were made non-rotatable (for flexible-ligand docking). Docking jobs were performed on an HP Z600 workstation, running on Kernel Linux 2.6.35-28-generic operating system, Intel® Xenon® processor 2.40 GHz and 3.4 GB RAM. All docking calculations were done with the AutoDock 4.2 software package (Morris et al., 1998) using the Lamarckian genetic algorithm (LGA). A population size of 150 and 2,500,000 energy evaluations were used for 100 search runs. The grid box, with grid spacing of 0.375 Å was centred on the active sites of the macromolecule. RMSD tolerance used was 0.5 Å. The active sites on COX-1 and COX-2 were determined based on the study by Gautam et al. (2011). The conformations from the docking experiments were analysed using the Discovery Studio 3.0 (http://www.accelrys.com) visualization tool, which also identified the H-bonds and van der Waals interactions between the active sites of COX-1 and COX-2 with the ligand. The LIGPLOT program (Wallace et al., 1995) was used to map out hydrophobic interactions.

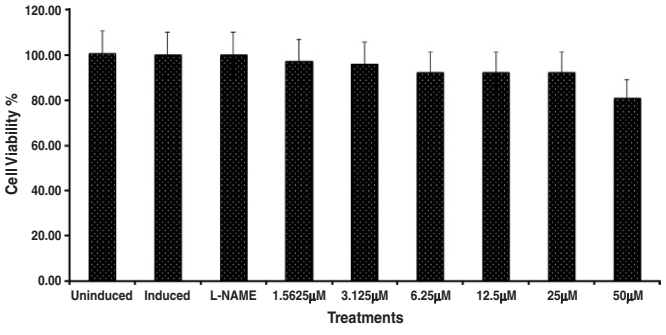

Fig. 3. The effects of columbin on RAW264.7 cells' viability: cells were pretreated with the indicated concentrations of columbin or were left untreated. Data are the average of three independent experiments (±SD), and were analysed using one way ANOVA.

2.8. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay on human normal hepatic cells (WRL-68)

This colorimetric assay, is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells (Mosman, 1986), was used to determine any potential cytotoxicity. Human normal hepatic cells (WRL-68) were obtained from American Type Cell Collection (ATCC), maintained in a 37 °C incubator with 5% CO_2 saturation and maintained in Dulbecco's modified Eagle's medium (DMEM). Medium were supplemented with 10% foetus calf serum (FCS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For measurement of cell viability, cells were seeded at a density of 1×10^5 cells/ml in a 96-well plate and incubated for 24 h at 37 °C and 5% CO_2. Cells were treated with columbin and incubated for 24 h. After 24 h, MTT solution at 2 mg/ml was added for 1 h. Absorbance was measured at 570 nm. Results were expressed as a percentage of control giving percentage cell viability after 24 h exposure to test agent. The potency of cell growth inhibition for columbin was expressed as an IC_{50} value, defined as the concentration that caused a 50% loss of cell growth. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

2.9. Gastric tolerability test

When animals were sacrificed, their stomach was removed and opened along the greater curvature. Lesions were examined under

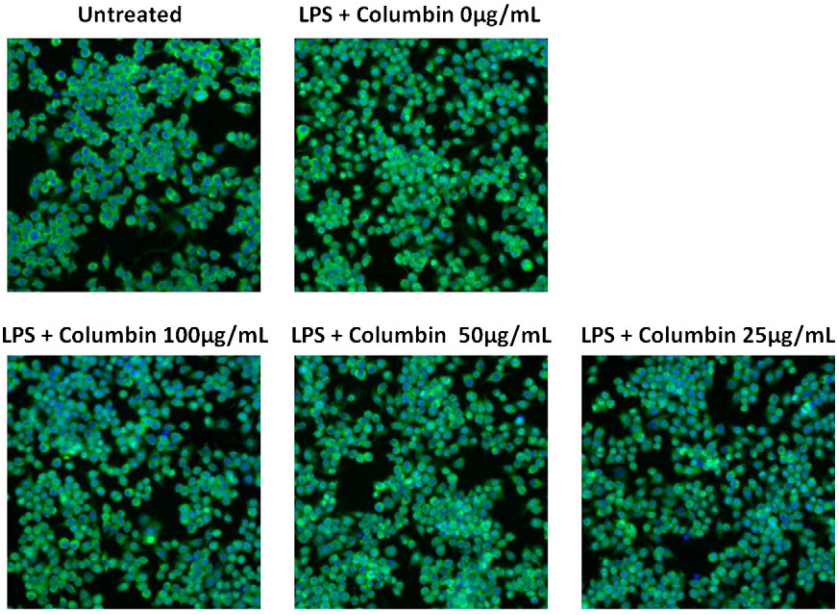

Fig. 4. NF-κB (DyLight™ 488, light green), was sequestered in the cytoplasm in cells treated with DMEM alone (untreated), and the nuclei of the cells appear blue (Hoescht). However, NF-κB translocates into the nucleus upon LPS stimulation. Pretreatment with columbin did not prevent NF-κB translocation in the presence of LPS.

an illuminated magnifier (1.8×). The intensity of gastric lesions was assessed according to a modified scoring system of Adami et al. (1964): (0: no lesions; 0.5: slight hyperaemia or ≤5 petechiae; 1: ≤5 erosions ≤5 mm in length; 1.5: ≤5 erosions ≤5 mm in length and many petechiae; 2: 6–10 erosions ≤5 mm in length; 2.5: 1–5 erosions >5 mm in length; 3: 5–10 erosions >5 mm in length; 3.5: >10 erosions >5 mm in length; 4: 1–3 erosions ≤5 mm in length and 0.5–1 mm in width; 4.5: 4–5 erosions ≤5 mm in length and 0.5–1 mm in width; 5: 1–3 erosions >5 mm in length and 0.5–1 mm in width; 6: 4 or 5 grade 5 lesions; 7: ≥6 grade 5 lesions; 8: complete lesion of the mucosa with haemorrhage).

2.10. Preliminary acute toxicity assessment

Animal experiments were performed in accordance with the guidelines for animal experimentation issued by the Animal Care and Use Committee at the University of Malaya (Ethics Number: FAR/11/03/2009/MA(R)). The method described by Lorke was employed [16]. In brief, mice were separated into four groups of 6 mice each. They were fasted overnight and then were orally administered with the columbin at the doses of 30, 500, and 1000 mg/kg, while the control group only received the vehicle. The mice were observed for any abnormal behaviour such as sedation, respiratory distress, motor impairment, and hyperexcitability for 3 h. Furthermore, the incidence of mortality for each group was recorded up to 24 h after administration. Food and water were provided *ad libitum*.

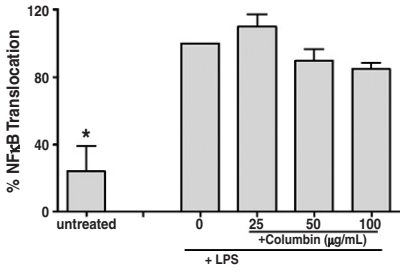

Fig. 5. The effects of columbin on NF-kB translocation: murine macrophage cells were left untreated or pretreated with the indicated concentrations of columbin. The cells were then either left in medium or were pretreated with LPS for 30 min. Percentage NFkB translocation to the nucleus was then compared between the untreated cells and the different concentrations of columbin to cells treated with LPS alone (0 µg/ml) using ANOVA test. The data are average of 2 independent experiments. (* $P<0.05$, *** $P<0.001$).

2.11. Statistical analysis

The data obtained was statistically analysed using one-way ANOVA. This was followed by Dunnett's or Tukey's post hoc tests

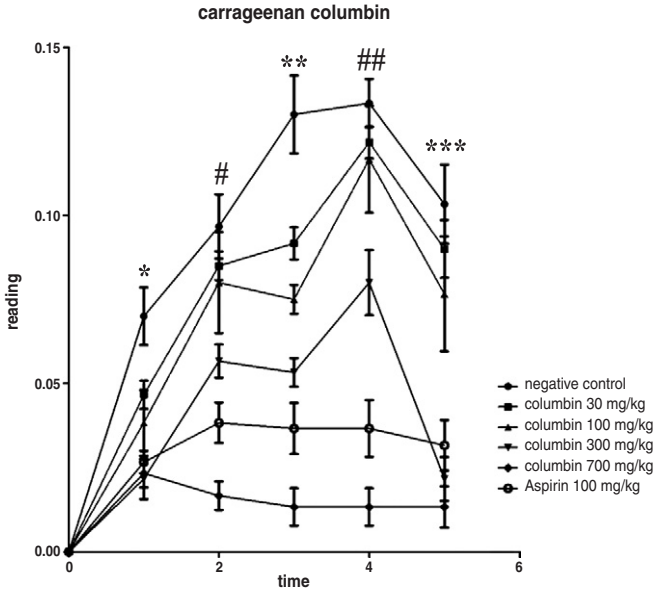

Fig. 6. Effects of columbin and aspirin on paw oedema induced by carrageenan in rats. The animals (10 per group) were injected intra-peritoneally with columbin (30, 100, 300, and 700 mg/kg), aspirin (100 mg/kg), or vehicle (distilled water). Thirty minutes after receiving these drugs, each animal was injected with carrageenan in the right hind paw. The oedema was measured immediately prior to the carrageenan injection and 1, 2, 3, 4, and 5 h later. Its volume determined with a plethysmometer as the difference between the final and initial volumes. Data are reported as means ± S.E.M. Symbols indicate the statistical significant difference between the control and treatment groups.

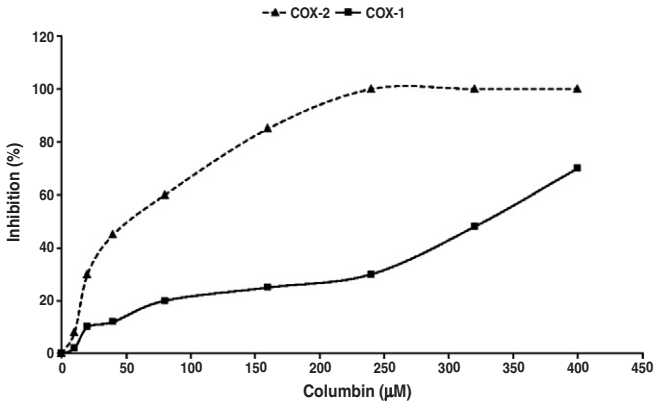

Fig. 7. Inhibition of COX enzymes. Columbin was evaluated in a COX catalysed prostaglandin biosynthesis assay. Data represent mean ± S.E.M.

when the ANOVA produced significant results. All data were expressed as the mean ± S.E.M. The tests were performed using GraphPad Software ver 5.01 (GraphPad Software Inc., San Diego, CA). Differences are considered significant when $P<0.05$.

3. Results

3.1. Effect of columbin on NO_2^- and PGE_2 production and RAW cells viability

The induction of RAW 264.7 cells into an inflammatory state by treatment with LPS/IFN-γ caused significant increase in NO and PGE_2 as shown in Fig. 2. As shown in Fig. 2A, columbin also inhibited the production of PGE_2 in a dose-dependent manner. The breakdown product of secreted NO namely NO_2^- was detected in media at a mean concentration of 39.60 ± 3.32 μM. Cells that were not induced released trace amounts of NO. Columbin showed a dose-related inhibition of NO production in which significant inhibition was still evident at 0.78 ± 0.12 μM. The IC_{50} was calculated at 36.1 ± 4.12 μM. L-NAME, a standard NO inhibitor, was used as a positive control and caused a significant inhibition (84.54 ± 5.77%) of NO at 250 μM (Fig. 2B). Fortunately, columbin did not affect cell viability at 0–50 μM as assessed by mitochondrial reduction of MTT following a 17–20-h treatment; viability was always >80% at 50 μM (Fig. 3).

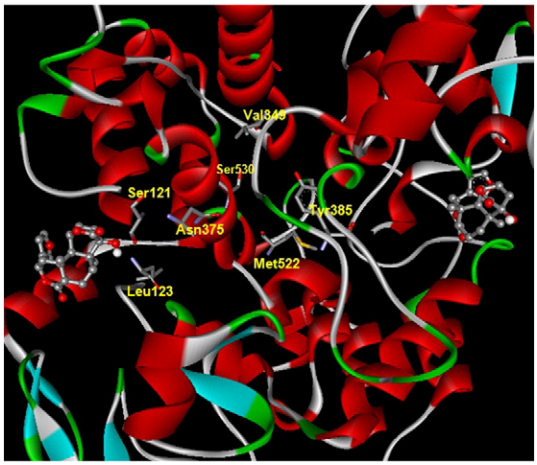

Fig. 8. Binding of columbin to COX-1 (protein secondary structures are represented by ribbons format). Columbin is shown as ball and stick. Residues of the active site are shown as stick and labelled.

3.2. Effects of columbin on NF-κB translocation

The ability of columbin to inhibit the transcription factor NF-κB was investigated. Treatment of RAW264.7 cells with 10 ng/ml LPS for 30 min lead to a significant increase ($P<0.05$) in NF-κB translocation as compared to untreated cells (Fig. 4). Pretreatment of stimulated cells with columbin did not inhibit the translocation of NF-κB to the nucleus in LPS-stimulated cells (Fig. 5).

3.3. Carrageenan-induced rat paw oedema

The anti-inflammatory activity of columbin was measured at the doses of 30, 100, 300 and 700 mg/kg b.w. against acute paw oedema induced by carrageenan (Fig. 6). Columbin has shown to inhibit inflammation at the 3rd hour for all doses used. At doses of 300 mg/kg and 700 mg/kg, columbin inhibited inflammation from 0 to 5 h and the results were comparable to that of aspirin as a standard anti-inflammatory drug. Based on the present results it can be suggested that the inhibitory effect of columbin on carrageenan-induced paw oedema in mice may be due to the suppression of the release of mediators responsible for inflammation including prostaglandin.

3.4. Effects of columbin on cycloxygenase-1 and 2

Columbin was evaluated for *in vitro* COX-1 and COX-2 inhibitory activity in a COX catalysed prostaglandin biosynthesis assay. As shown in Fig. 7, columbin (100 μM) selectively inhibits COX-2 (63.7 ± 6.4%), with minor inhibition on COX-1 (18.8 ± 1.5). The EC_{50} for columbin for COX-2 and COX-1 is 53.1 ± 1.4 and 327 ± 8.3, respectively with selectivity ratio of 6.5. Indomethacin as non-selective COX-1 and COX-2 inhibitor shows inhibition of 81.37 ± 5.5 and 92 ± 6.5, respectively.

3.5. Molecular docking

Experimental results show that columbin is more active in inhibiting COX-2 compared to COX-1. Docking results show that columbin does not bind to the active site of COX-1, but possibly bind to two different binding sites in COX-1 (Fig. 8). Figs. 9 and 10 illustrate the binding conformations of columbin at the two different binding sites of COX-1. From Fig. 9C, it is shown that columbin forms hydrogen bonds (H-bonds) with three residues, namely Ile124, Gln372 and Lys532. Bond distances 22-OH of columbin and C═O of Ile124 is 2.05 Å (H···O); O20 of columbin and OH of Gln372 is 2.09 Å (O···H); O24 of columbin and OH of Gln372 is 2.22 Å (O···H); O24 of columbin and NHZ1 of Gln372 is 1.97 Å (O···H); O24 of columbin and NHZ2 of Gln372 is 2.10 Å (O···H). Hydrophic and van der Waals interactions were also observed between columbin and the surrounding residues in the binding site. From Fig. 10C, it is shown that when columbin binds to the other binding site, no H-bonds were formed with the surrounding residues in the binding site. Only hydrophobic and van der Waals interactions were observed.

Docking of columbin to COX-2 exhibits binding of the ligand to the active site (Fig. 11). This could explain the higher bioactivity of columbin towards COX-2 than COX-1. From Fig. 11C, it is shown that columbin forms H-bonds Tyr385, with bonding distance between O25 of columbin and OH of Tyr385 is 2.30 Å (O···H). In addition, pi–pi interaction was observed between columbin and Tyr355 with centroid–centroid separation, R_{cen}, is 5.17 Å, and π–cation interaction between columbin and Arg120 with distance of 4.80 Å. The fact that columbin interacts with Tyr385 and Arg120 may also signify the higher activity in COX-2, as Tyr385 was reported to be involved in the abstraction of hydrogen from C-13 of arachidonate, and Arg120 is critical for high affinity arachidonate binding (Thuresson et al., 2001). Hydrophic and van der Waals interactions were also observed between columbin and the surrounding residues in the active site.

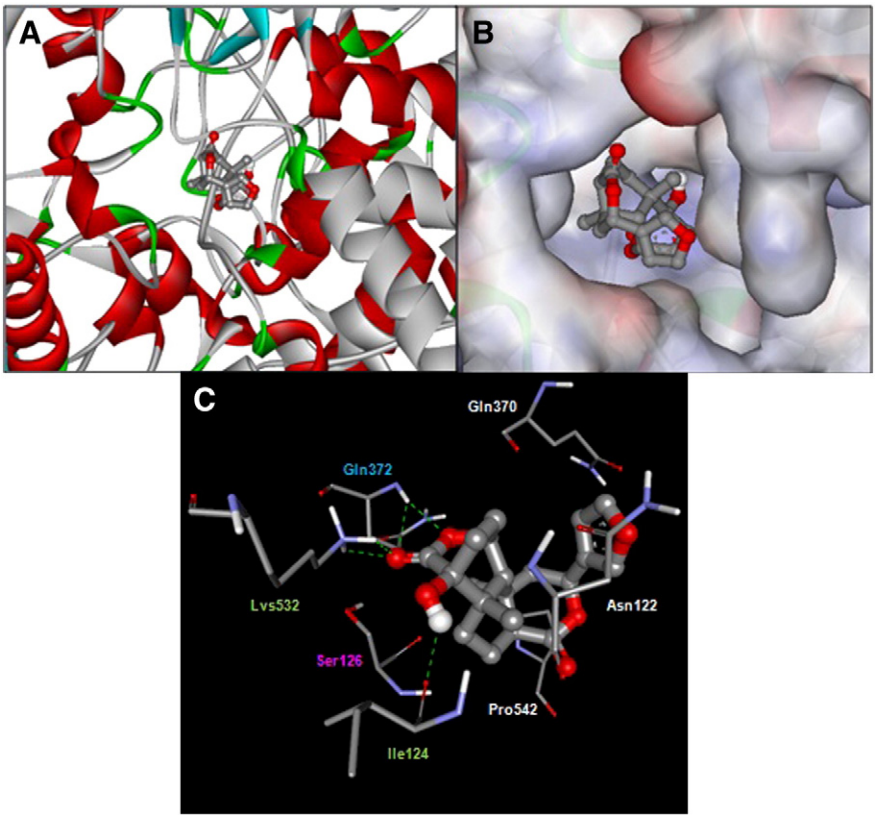

Fig. 9. (A) View of columbin at the binding site 1 of COX-1 (protein secondary structures are represented by ribbons format). Columbin is shown as ball and stick. (B) Transparent connolly surface representation of columbin in the binding site. (C) Simplified view of columbin interacting with surrounding residues. Green dashed lines indicate H-bonds. Residues interacting with the ligand are shown as sticks. Residues labelled in green interact with the ligand via H-bonds, labelled in white exhibit hydrophobic interactions, labelled in blue interacts via van der Waals and H-bonds, and labelled in pink interacts via van der Waals and hydrophobic interactions.

3.6. Gastric tolerability

Columbin did not produce any significant gastric lesions. The changes observed were in range of 0–1 according to the Adami's scoring scale. Namely, only few petechiae were registered in rat stomach regardless of given dose.

3.7. Preliminary acute toxicity assessment and MTT assay

Administration of columbin (30, 500, and 1000 mg/kg, p.o.) did not produce any noticeable effect on behaviour or mortality in treated animals during observation period. The IC_{50} of columbin on WRL-68 was observed to be very high with the value of 160.2 ± 4.67 μg/ml which indicates the safety of this natural compound.

4. Discussion

The current study was designed to investigate the *in vitro*, *in vivo* and *in silico* anti-inflammatory activities of columbin. Although this compound was isolated previously (Atta-urRahman and Ahmad, 1988), to the best of our knowledge this is the first time it's isolation from the species *T. barkis* is reported. Moreover, this is the first time the compound was shown to inhibit COX-2 and NO.

Nitric oxide plays an important role in various inflammatory conditions where it is produced by the inducible form of nitric oxide synthase (iNOS) from the amino acid L-arginine (Kojda and Harrison, 1999). NO in tissues is susceptible to manipulation by proinflammatory cytokines (Bonavida et al., 2010; Wimalawansa, 2008). NO has important immune, cardiovascular and neurological second messenger functions that are implicated in sepsis, cancer and inflammation. LPS and IFN-γ were shown to induce the expression of this enzyme, resulting in the production of abundant amounts of NO. The obtained results suggest that columbin has dose-dependent anti-inflammatory activities related with its inhibition of NO and PGE_2 production in macrophages without affecting the viability of these cells. Our results are in line with previous findings which showed that compounds structurally similar to columbin are able to inhibit the production of NO (Chiou et al., 2000).

Cyclooxygenases are inducible enzymes that catalyse the production of prostaglandins, which contribute to the inflammatory process and tissue damage. It is reported that COX-2 can also be activated by high concentrations of nitric oxide, contributing towards more intense inflammatory responses as seen in many chronic inflammatory disorders. In the current study, it was observed that columbin was able to inhibit COX-2 when tested using EIA kit (Fig. 7). Several natural products of plant origin have been shown to transmit their anti-inflammatory activities through suppression of COX-2, however, for that suppression of nitric oxide production is critical (Tian et al., 2008).

Having demonstrated that columbin possesses anti-inflammatory activity *in vitro*, next we tested it in an *in vivo* model of inflammation of carrageenan. The injection of carrageenan in mice produces a typical biphasic oedema associated with the production of several inflammatory mediators, such as COX enzymes, prostaglandins, nitric oxide and cytokines. The carrageenan test is highly sensitive to non-steroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies (Bucci et al., 2005). The current findings showed that the degree of swelling of the carrageenan injected paws was maximal the 3rd hour after injection as depicted in Fig. 6. Statistical analysis revealed that columbin significantly inhibited the development of oedema at the fourth hour after treatment ($P<0.05$; Fig. 6). It is well known that the third phase

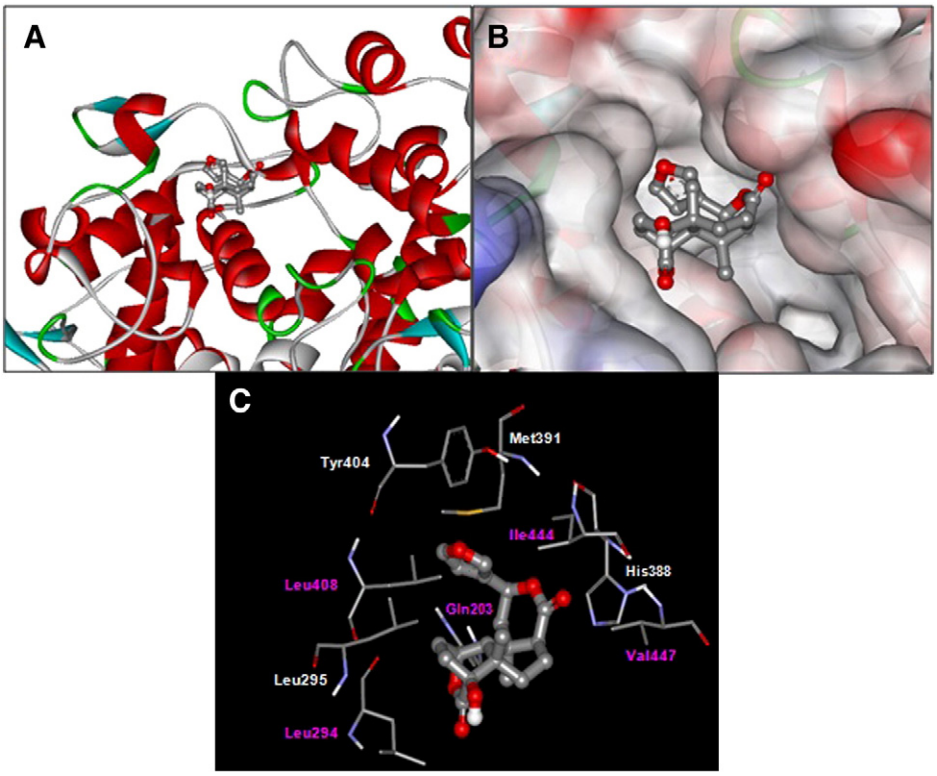

Fig 10. (A) View of columbin at the binding site 2 of COX-1 (protein secondary structures are represented by ribbons format). Columbin is shown as ball and stick. (B) Transparent connolly surface representation of columbin in the binding site. (C) Simplified view of columbin interacting with surrounding residues. Residues interacting with the ligand are shown as sticks. Residues labelled in white exhibit hydrophobic interactions, and labelled in pink interact via van der Waals and hydrophobic interactions.

of the oedema induced by carrageenan, in which the oedema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction (Bastos et al., 2001; Neto et al., 2005).

COX-1 was proposed to regulate physiological functions while COX-2 to mediate pathophysiological reactions such as inflammation. In particular, it was suggested that maintenance of gastric mucosal integrity relies exclusively on COX-1. Recently, it was shown that a selective COX-1 inhibitor does not damage the mucosa in the healthy rat stomach, although mucosal prostaglandin formation is near-maximally suppressed. However, concurrent treatment with a COX-1 and a COX-2 inhibitor induces severe gastric damage (Forones et al., 2008). This indicates that in normal mucosa both COX-1 and COX-2 have to be inhibited to evoke ulcerogenic effects. The current study showed that columbin did not produce any significant gastric lesions. This suggests that the selectivity of this natural diterpenoid furanolactone to COX-2 enzyme may play a major role in the absence of any gastric lesions in the experimental rats. Selectivity for COX-2 reduces the risk of peptic ulceration, and is the main feature of celecoxib, rofecoxib and other members of these NSAIDs (Hawkey and Langman, 2003).

To the best of literature survey, this is the first report of the molecular modelling studies of this molecule with the COX enzymes. The interaction with amino acid Ser530 is important for enzyme inhibitory activity. Tyr385 is responsible for the abstraction of 13-pro-S-hydrogen from arachidonic acid. In case of complex of columbin with COX-1, the hydrogen bonding interaction with Ser530 was found to be absent, which could be a probable reason for the observed selectivity of columbin towards COX-2 inhibition (Gautam et al., 2011). Further, the scoring function of columbin complex with COX-2 suggests them as the preferred ligand for COX-2 than COX-1 and provides rationale for selectivity of enzyme inhibitory activity. The selectivity issue was further supported by our experimental analysis showing better inhibitory activity with COX-2. COX2 has larger sized molecule seems to be more selective towards COX-2 due to its increased active site volume.

It was shown previously that columbin from *Calumbae Radix* inhibits azoxymethane-induced rat colon carcinogenesis (Kohno et al., 2002). The inactivation of COX-2 enzyme activity in this particular model is known to inhibit colon carcinogenesis (Rao et al., 2009). Since columbin was shown to inhibit COX-2, the anti-inflammatory effects of this compound may be attributed, to a certain extent, to the inhibition of this transcription factor.

Inducible NF-kB is accountable for the ruling of various inflammatory pathways. Due to the fact that the production of both iNOS and COX enzymes is regulated, at least in part, by the transcription factor NF-κB (Wang et al., 2009), we investigated the role columbin plays on inhibiting this transcription factor's translocation. LPS was shown to cause NF-κB activation in RAW264.7 (Shin et al., 2009). Columbin was not able to inhibit the translocation of NF-κB in LPS-treated RAW264.7 cells. This suggests that the *in vitro* and *in vivo* anti-inflammatory activity of columbin is through the inhibition of cycloxygenase-2 and nitric oxide but not the suppression of NF-KB translocation. In order to confirm the results of NF-κB involvement in columbin effect, the use of other techniques such as EMSA is highly recommended.

In conclusion, columbin inhibited LPS/IFN-gamma-induced NO production, EIA assayed cyclooxygenases and carrageenan-induced rat paw oedema. Molecular docking study further helped in supporting the observed COX-2 selectivity. This natural diterpenoid furanolactone was not toxic to human normal liver cells, murine RAW 264.7

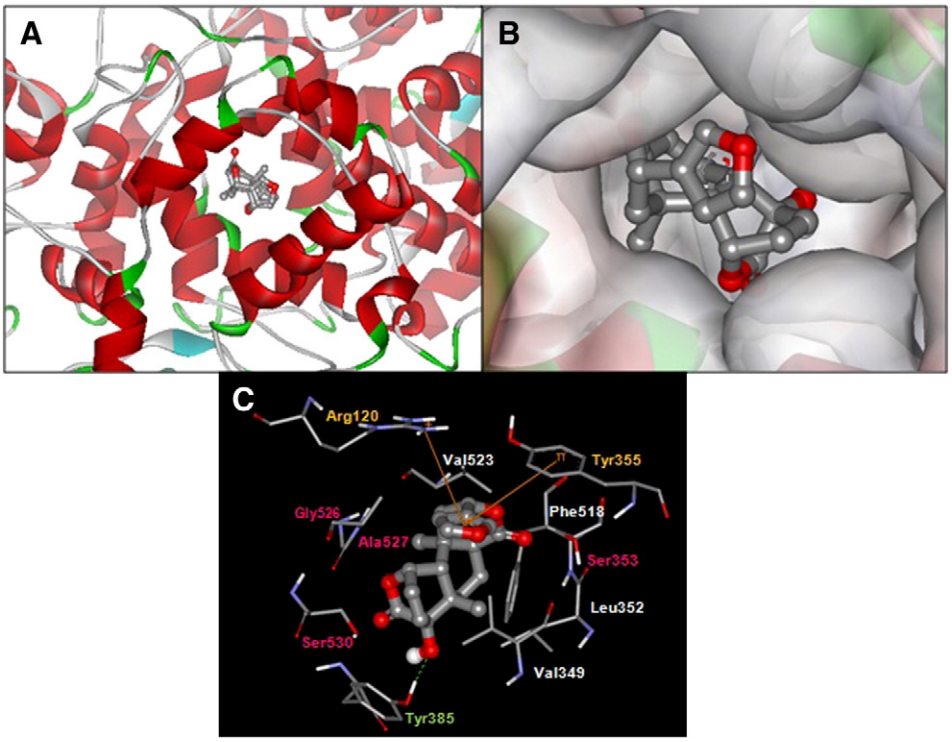

Fig. 11. (A) View of columbin at the active site of COX-2 (protein secondary structures are represented by ribbons format). Columbin is shown as ball and stick. (B) Transparent connolly surface representation of columbin in the binding site. (C) Simplified view of columbin interacting with surrounding residues. Residues interacting with the ligand are shown as sticks. Green dashed line indicates H-bond. Residues labelled in green interact with the ligand via H-bond, labelled in white exhibit hydrophobic interactions, and labelled in pink interact via van der Waals and hydrophobic interactions. Residues in orange have pi–pi/pi–cation interactions with the ligand.

macrophages and mice. The findings of the study inferred that the dual functioning of columbin as NO and COX inhibitor render it as a lead molecule for further development of new anti-inflammatory agent(s).

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