RESEARCH ARTICLE

Inhibition of Human Neutrophil Functional Responsiveness by *Cryptolepis buchanani* Extract

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Abstract

Cryptolepis buchanani Roem. & Schult. (Asclepiadaceae), a climbing tree and commonly known as "Thao En On" in Thailand, is widely used in traditional folk medicine in Southeast Asia. In Thailand, the stem of this plant is traditionally used for the treatment of inflammation, including arthritis and muscle and joint pain. Alcoholic extract from stem of *C. buchanani* was found to possess anti-inflammatory activities in animal models. This study aimed to investigate the *in vitro* effects of *C. buchanani* extract on human neutrophil functional responsiveness in order to elucidate the underlying cellular mechanisms of its acute anti-inflammatory effects. Human neutrophil functional responsiveness was determined by measuring *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis, superoxide anion generation (SAG), and release of myeloperoxidase (MPO) and elastase. Apoptosis was assessed morphologically and by flow cytometry. Neutrophil viability was assessed by trypan blue exclusion and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide (XTT) cytotoxic assay. Although *C. buchanani* extract did not affect neutrophil viability and apoptosis, treatment of neutrophils with the herbal extract concentration-dependently inhibited fMLP-induced chemotaxis, SAG, and MPO release. In addition, neutrophil apoptosis was slightly stimulated by the herbal extract. In conclusion, these findings suggest that the anti-inflammatory property of *C. buchanani* extract is mediated, in part, by inhibition of neutrophil functional responsiveness. The results lend support to the effectiveness of *C. buchanani* in combating inflammation. However, further investigations are required to fully identify the biologically active compound(s) of *C. buchanani* and to define the underlying molecular and cellular mechanisms of action involved.

Keywords: *Cryptolepis buchanani*, anti-inflammatory activity, antioxidant activity, neutrophil functional responsiveness, neutrophil apoptosis

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การยับยั้งการทำงานตอบสนองของเม็ดเลือดขาวนิวโทรฟิลของสารสกัดเถาเอ็นอ่อน

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บทคัดย่อ

เถาเอ็นอ่อน (*Cryptolepis buchanani* Roem. & Schult.) เป็นพันธุ์ไม้เลื้อยในตระกูล Asclepiadaceae ที่นำมาใช้ทางการแพทย์พื้นบ้านและแผนโบราณอย่างแพร่หลายในแถบภูมิภาคเอเชียตะวันออกเฉียงใต้ ในประเทศไทย ลำต้นของเถาเอ็นอ่อนนำมาใช้รักษาการอักเสบ รวมทั้งการอักเสบของข้อและอาการปวดกล้ามเนื้อและข้อ สารสกัดอัลกอฮอล์จากส่วนลำต้นของเถาเอ็นอ่อนพบว่ามีฤทธิ์ต้านอักเสบในสัตว์ทดลอง การศึกษาครั้งนี้มีวัตถุประสงค์ในการศึกษาผลของสารสกัดจากส่วนลำต้นเถาเอ็นอ่อนต่อการทำงานตอบสนองของนิวโทรฟิล เพื่ออธิบายถึงกลไกระดับเซลล์ของฤทธิ์ต้านอักเสบเฉียบพลันของสารสกัดเถาเอ็นอ่อน การทำงานตอบสนองของนิวโทรฟิลทำได้โดยการวัดการเคลื่อนที่ของนิวโทรฟิลที่มีตัวกำหนดทิศทางซึ่งเหนี่ยวนำโดย *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) การสร้างซุปเปอร์ออกไซด์แอนไอออน และการปล่อยเอนไซม์ไมอีโลเพอร์ออกซิเดสและอีลาสเตส การตายแบบอะพอพโทซีสของนิวโทรฟิลประเมินจากรูปร่างของเซลล์และโดยใช้โฟลไซโตเมทรี ความอยู่รอดของนิวโทรฟิลตรวจวัดโดย trypan blue exclusion และการทดสอบความเป็นพิษต่อเซลล์โดยใช้ 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)- *2H*-tetrazolium-5-carboxanilide (XTT) แม้ว่าสารสกัดเถาเอ็นอ่อนไม่มีผลต่อความอยู่รอดและการตายแบบอะพอพโทซีสของนิวโทรฟิล แต่พบว่ามีฤทธิ์ยับยั้งการเคลื่อนที่ของนิวโทรฟิลแบบมีทิศทางซึ่งเหนี่ยวนำโดย fMLP การสร้างซุปเปอร์ออกไซด์แอนไอออนและการปล่อยเอนไซม์ไมอีโลเพอร์ออกซิเดสได้โดยขึ้นกับความเข้มข้นของสารสกัด นอกจากนี้ยังพบว่าสารสกัดเถาเอ็นอ่อนมีผลกระตุ้นการตายแบบอะพอพโทซีสของนิวโทรฟิลเล็กน้อย โดยสรุปกลไกการออกฤทธิ์ต้านอักเสบของเถาเอ็นอ่อนบางส่วนเป็นผลจากการยับยั้งการทำงานตอบสนองของนิวโทรฟิล ผลการศึกษานี้สนับสนุนประสิทธิภาพของเถาเอ็นอ่อนในการต้านการอักเสบ อย่างไรก็ตามควรมีการศึกษาเพื่อระบุสารออกฤทธิ์สำคัญและกลไกการออกฤทธิ์ที่เกี่ยวข้องทั้งในระดับโมเลกุลและเซลล์ต่อไป

คำสำคัญ: เถาเอ็นอ่อน, ฤทธิ์ต้านการอักเสบ, ฤทธิ์ต้านอนุมูลอิสระ, การทำงานตอบสนองของนิวโทรฟิล, การตายของนิวโทรฟิลแบบอะพอพโทซีส

Introduction

Cryptolepis buchanani Roem. & Schult. (Asclepiadaceae), commonly known as "Thao En On" in Thailand, is a climbing tree found in evergreen forest in India, China, Nepal and Indo-china. The principal constituents of C. *buchanani* are sarverogenin and isosarverogenin glycosides, cardenolides such as cryptosin, buchanin, a novel pyridine alkaloid buchananine and a serine protease cryptolepain.[1] This plant is used in Indian folkloric medicine (Ayurveda) as antidiarrheal, anti-ulcerative, anti-inflammatory, blood purifier, antibacterial, anti-cough, demulcent, diaphoretic and diuretic and for the treatment of childhood rickets[1] and bone fractures.[2] It is also widely used in folk medicine in Southeast Asia. In Thailand, the alcoholic extract from stem of *C. buchanani* has been commonly used for the treatment of inflammatory conditions such as arthritis and muscle and joint pain.[3-5] Alcoholic extract from stem of *C. buchanani* possesses analgesic, chondoprotective[6], and anti-inflammatory properties.[4-6] In addition, *C. buchanani* aqueous leaf extract showed broad spectrum antimicrobial activity against food-borne pathogen bacteria, nosocomial infection bacteria and some normal flora bacteria.[7] Marked *in vitro* antifungal activity was also demonstrated by its methanolic and aqueous extracts.[8] Recently, *C. buchanani* oil formulation demonstrated a clinical efficacy comparable to 1% indomethacin solution in topical therapy for patients with mild to moderate osteoarthritis (OA) of the knee with lower rates of adverse events than indomethacin solution.[9]

Polymorphonuclear neutrophils (PMNs) play an important role in host defense. Exposure to bacteria or bacterial products activates these cells, as part of the inflammatory response, resulting in the clearance of pathogens. However, inappropriate or excessive neutrophil activation can cause severe tissue damage, contributing to the pathology of a range of inflammatory diseases. Neutrophils have significant roles in acute and chronic inflammatory and autoimmune diseases, infectious and non-infectious conditions.[10] Dysregulated recruitment, activation or clearance of neutrophils leads to the liberation of granule products, release of toxic oxygen metabolites e.g. superoxide anion (O_2^-) and production of various inflammatory mediators, which can contribute to tissue damage associated with inflammatory disorders (e.g. ischaemia/reperfusion injury, myocardial infarction, atherosclerosis, and rheumatoid arthritis).[11] Aged neutrophils undergo spontaneous apoptosis (programmed cell death), a process that can be modulated by cytokines or other pro-inflammatory agents. During apoptosis the neutrophil retains its granule contents and loses the ability to secrete them in response to secretagogues. Apoptotic neutrophils are ingested by inflammatory macrophages employing novel phagocytic recognition mechanisms that fail to provoke a macrophage proinflammatory response.[12,13] If apoptotic neutrophils are not rapidly cleared, they undergo secondary or post-apoptotic necrosis, resulting in the release of potentially toxic intracellular contents with induction of tissue injury and inflammatory responses. Moreover, the inflammatory responses can be prolonged.[14] Thus apoptosis is considered as a crucial process for the control and resolution of inflammation. In acute inflammation, large numbers of neutrophils infiltrate the inflamed tissues. Activated neutrophils then trigger the release of toxic reactive oxygen species (ROS), antimicrobial peptides and proteolytic enzymes, all of which cause tissue damages. Suppression of neutrophil function

can control the inflammatory responses. This effect has been implicated in the mechanisms of action of some non-steroidal anti-inflammatory agents, since inhibition of constitutive and inducible cyclooxygenase does not seem to account for all of their anti-inflammatory effects.[15] Arthritis is an inflammatory joint disorder, of which osteo-arthritis, gout and rheumatoid arthritis are the most common types. Neutrophils play significant role in the initiation and progression of rheumatoid arthritis[16-19], whereas their role in osteoarthritis is in early stage of pathogenesis.[20] Thus the modulation of neutrophil functions could be useful for the treatment of all forms of arthritis.[21,22]

Although, *C. buchanani* has been widely used in folk medicine as an anti-inflammatory agent, the cellular mechanisms underlying its anti-inflammatory activity remain elusive. Therefore, the purpose of the present study was to investigate the *in vitro* effects of *C. Buchanani* extracts (CBE) on antioxidant activity and human neutrophil functional responsiveness and apoptosis. The research protocol was reviewed and approved by the Committee on Human Rights Related to Researches Involving Human Subjects, based on the Declaration of Helsinki, Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

Materials and Methods

Chemicals and reagents

Limulus amebocyte lysate (LAL) (Pyrogent® 5000) was obtained from Lonza Walkersville (Walkersville, MD, USA). Polymorphprep® was purchased from Axis-Shield (Oslo, Norway). Diff-Quik® was purchased from Gamidor Ltd (Abingdon, UK). Dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-pycrylhydrazyl (DPPH), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), trypan blue, cytochrome c, cytochalasin B, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), phenazine methosulfate (PMS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 3,3′,5,5′-tetramethylbenzidine (TMB), dexamethasone, and indomethacin were purchases from Sigma-Aldrich (St. Louis, MO, USA). Citric acid monohydrate, potassium phosphate monobasic anhydrous ($KHPO_4$), potassium chloride (KCl), calcium chloride ($CaCl_2$), sodium chloride (NaCl), sodium phosphate monobasic ($NaHPO_4$), sodium phosphate dibasic (Na_2HPO_4), sodium bicarbonate ($NaHCO_3$,) and N-*tert*-butoxy-carbonyl-L-alanine-*p*-nitrophenyl ester (Boc-ala-ONp) were purchased from ICN Biomedicals (Irvine, CA, USA). Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine (without phenol red), Iscove's modified Dulbecco's medium (MDM) (without phenol red) and penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Annexin V-FITC was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Plant extract

The stems of *C. buchanani* were collected in Chiang Mai province and a voucher specimen BKF no. 137513 was deposited at the Forest Herbarium, Royal Forestry Department, Bangkok, Thailand. Dried and finely powdered stems (3.85 kg) was percolated at room temperature with 4 × 64 L of methanol. The filtrate was evaporated under a reduced pressure and freeze dried to give 452 g of the crude extract. The stock solution of CBE (100 mg/mL) was freshly prepared in DMSO and serial diluted using phosphate buffered saline (PBS) or RPMI 1640 or Iscove's MEM as required.

Assessment of Lipopolysaccharide (LPS) contamination

CBE was checked for bacterial LPS contamination using a commercial test kit of limulus amebocyte lysate (LAL).

Determination of DPPH scavenging activity

The antioxidant activity of CBE, based on the scavenging of stable free radical, was determined using DPPH scavenging assay with some modifications.[23] When a solution of DPPH, a stable free radical, is mixed with a substrate that can act as a hydrogen atom donor, a stable non-radical form of DPPH is obtained. In brief, DPPH in methanol (1.8 mL; 0.15 mM**)** was added to 200 µL of CBE at different concentrations (1-200 µg/mL). After incubation for 10 min, the absorbance was determined spectrophotometrically at a wavelength of 517 nm. The scavenging activity was calculated as a percentage of radical reduction. Trolox, a known radical scavenger, at the concentration range of 3.125-200 µg/mL was used as a reference compound.

Preparation of human neutrophils

Human neutrophils were isolated by density gradient centrifugation using density gradient media Polymorphprep®. Venous blood samples were taken from healthy donors at Department of Pharmacology, Faculty of Science, Mahidol University. Briefly, venous blood, using heparin as anticoagulant, was mixed with an equal volume of Polymorphprep® and the mixture was centrifuged at 400×g for 35 min at room temperature. After centrifugation, PMNs were harvested and washed with PBS. Any contaminating red cells were removed by hypotonic lysis. Most cells were viable (greater than 99%) as determined by trypan blue exclusion assay and were re-suspended as required.

Cytotoxicity assay

The cytotoxicity of CBE was tested in cultured human neutrophils prior to the investigation of its effects on human neutrophil responsiveness and apoptosis. XTT is a new tetrazolium salt used as a substrate in the colorimetric assay for mammalian cell survival.[24] It can be metabolically reduced by mitochondrial dehydrogenase enzymes of living cells to yield a soluble, colored formazan product. Human neutrophils (3×10^6 cells/mL) in RPMI 1640 with 10% autologous serum were preincubated with CBE (1-1000 µg/mL) in 96-well plates at 37°C for 45 min. Then 50 µL of a mixture of XTT (1 mg/mL) and 1% PMS was added and incubated at 37°C for 3 h in a moist, 5% CO_2 atmosphere. The optical density of XTT formazan product was measured spectrophotometrically at a wavelength of 450 nm with a reference wavelength of 650 nm using a microplate reader (Packard SpectraCount, Packard BioScience, Meriden, CT, USA). All experiments were done at least 5 times with each treatment performed in triplicate. The results expressed in absolute absorbance units, which were relative to the number of viable cells. The percentage of cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = 100 - \frac{(OD_{\text{CBE test}} - OD_{\text{CBE control}}) \times 100}{(OD_{\text{cell control}} - OD_{\text{medium control}})}$$

The cytotoxicity of CBE was expressed as 50% cytotoxic concentration (CC_{50}), i.e., the concentration of the extract required for the reduction of cell viability by 50%.

Assessment of neutrophil functional responsiveness

Chemotaxis assay An *in vitro* assay for chemotaxis of human neutrophils was performed using 96-well chemotaxis chambers (Neuro Probe, Cabin John, MD, USA) as previously described.[25] In brief, the bottom wells of the chamber were filled with chemoattractant (1 μM fMLP). The top plate of chemotaxis chamber with the installed polyvinylpyrrolidone-free polycarbonate filter (3 μm) was placed onto the filled bottom plate containing neutrophils (3×10^6 cells/mL in RPMI 1640) that were pretreated with CBE (0.1-100 μg/mL) or indomethacin (0.1-100 μg/mL) for 10 min. After incubation at 37°C for 45 min in a humidified, 5% CO_2 atmosphere, the filter was removed, washed, fixed and stained with Diff-Quik®. Chemotaxis was quantified spectrophotometrically by measuring absorbance at a wavelength of 550 nm. The magnitude of the absorbance taken was directly proportional to the number of cells that migrated and were trapped in the filter. Basal absorbance was taken as cells without fMLP. Each treatment was carried out in triplicate.

Superoxide anion generation (SAG) Human neutrophil SAG was determined by spectrophotometric evaluation of the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c to ferrocytochrome c as previously described.[26] Briefly, aliquots of neutrophils in PBS (1.5×10^6 cells/mL) were preincubated with CBE (0.1-100 μg/mL) or indomethacin (0.1-100 μg/mL or the vehicle (PBS 5 μL) for 10 min at 37°C, followed by addition of fMLP (100 nM) and incubated at 37°C for 10 min. SAG was measured at a wavelength of 550 nm. Basal absorbance was taken as cells without fMLP. Each treatment was carried out in triplicate.

Myeloperoxidase (MPO) production Human neutrophil MPO production was determined spectrophotometrically using TMB as a substrate. The assay is based on the oxidation of TMB by neutrophil MPO in the presence of H_2O_2 with some modification.[27] Briefly, cells in PBS (2.5×10^6 cells/mL) were preincubated with CBE (0.1-100 μg/mL) or indomethacin (0.1-100 μg/mL) or vehicle at 37°C for 10 min and then stimulated with cytochalasin B (5 μg/mL) and fMLP (100 nM) at 37°C for 10 min. After centrifugation at 320×g, at 4°C for 10 min, supernatants were incubated with the reaction mixture (1 mg/mL TMB in 0.05M citrate phosphate buffer (pH 5.0) supplemented with 0.012% H_2O_2) at 37°C for 10 min. Oxidized TMB formed a soluble chromophore. The reaction was terminated after 5 min by the addition of 4M H_2SO_4 and the absorbance was measured spectrophotometrically at a wavelength of 450 nm. Each treatment was carried out in triplicate.

Elastase release by human neutrophils Human neutrophil elastase activity was determined using t-Boc as a substrate.[28] Briefly, cells in PBS (2.5×10^6 cells/mL) were preincubated with CBE (0.1-100 μg/mL) or indomethacin (0. 1-100 μg/mL) or vehicle at 37°C for 10 min and then stimulated with cytochalasin B (5 μg/mL) and fMLP (100 nM) at 37°C for 10 min. After centrifugation at 2000×g, at 4°C for 10 min, supernatants were incubated with t-Boc (200 μM) at 37°C for 20 min. Then the extent of *p*-nitrophenol release was measured spectrophotometrically at a wavelength of 414 nm in a microplate reader.

The effects of CBE and indomethacin on fMLP-induced human neutrophil functional responsiveness were expressed as the percentage of the inhibition of the

responses produced by a submaximal effective concentration of fMLP, which were calculated from the difference between those of the CBE or drug-treated group and the control group. The IC_{50} values or the concentrations of CBE or indomethacin that inhibited 50% of the maximal effects produced by fMLP were calculated by non-linear curve fitting from at least five concentrations of CBE or indomethacin using Microsoft Excel.

Assessment of human neutrophil apoptosis

Cell culture Apoptosis of human neutrophils was assessed *in vitro* as previously described.[29] Briefly, human neutrophils (5×10^6 cells/mL) were re-suspended in Iscove's MEM containing 100 IU/mL penicillin and 100 IU/mL streptomycin and supplemented with 10% autologous serum. Neutrophils were cultured in flat-bottomed 96-well polypropylene plates (at 37°C, 5% CO_2) for 20 h in the presence of either Iscove's MEM (control) or CBE at various concentrations (10-500 μg/mL). Dexamethasone (1 μM) was used as a reference drug. All experiments were done at least 4 times and each treatment was performed in triplicate.

Morphological assessment of neutrophil apoptosis After 20 h of culture either in the presence or absence of CBE at various concentrations (10-500 μg/mL) or dexamethasone (1 μM), neutrophils were re-suspended. One hundred microliter of cell suspension harvested from each well was cytocentrifuged using Cytospin II (Shandon, Chesire, UK). Centrifuged slides were then air-dried, fixed in methanol and stained with Diff-Quik®. Cell morphology was examined using oil immersion light microscopy (×100 objective) (Olympus BX60, Olympus, Southend-on-Sea, UK) to determine the proportion of apoptotic neutrophils.[30] For each treatment, slides were prepared in duplicate, a total of at least 500 neutrophils per slide were counted over a minimum of five high power fields. Trypan blue exclusion assay was performed in parallel to determine cell viability.

Annexin V apoptosis assay The assessment of neutrophil apoptosis was performed by flow cytometry using fluorescein isothiocyanate (FITC)-labelled recombinant human annexin V (Bender MedSystems, Vienna, Austria). Since Annexin V has a strong affinity for phosphatidylserine that are exposed on the outside membrane surface of apoptotic cells, it can be used as a probe for detecting apoptosis.[31] Stock FITC-Annexin V (180 μL), diluted to 1:2000 in binding buffer (HBSS plus 5 mM $CaCl_2$), was added to 20 μL of neutrophils (5×10^6 cells/mL) pre-incubated for 20 h either in the presence or absence of CBE or dexamethasone (used as a reference drug). Following a 10-min incubation at 4°C, the samples were analysed for apoptotic neutrophils using a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with EXPO®32 data analysis software. In brief, gates and region were place around populations of apoptotic neutrophils with marker expression (phophatidylserine) that have been stained with antibodies conjugated to fluorophore (FITC-annexinV) to investigate and quantify apoptotic neutrophil population. Data was expressed in a histogram which displayed relative fluorescence or light scatter intensity on the X-axis and the number of events (cell count) on the Y-axis. Cells with marker expression were called the positive dataset. There were two peaks which can be interpreted as the positive and negative datasets. Using an analytical software, measurement and statistics can be obtained for many parameters in addition to the number of cells and percentage of cells within the gate.

Statistical analysis

Results were expressed as mean±standard error of mean (SEM). Statistical analysis of data was performed by one-way analysis of variance (ANOVA) using Microsoft Excel, followed by the Dunnett's test using the GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA) for comparison between control and CBE or drug-treated groups. Differences were considered statistically significant when $p<0.05$.

Results

LPS contamination in the extract

The LPS concentration in the CBE determined by the LAL test was 2.9×10^{-4} EU/μg.

Antioxidant effect of CBE

The methanolic extract of *C. Buchanani* exhibited marked antioxidant activity by scavenging DPPH in a concentration-dependent manner with EC_{50} of 55.8±4.5 μg/mL, compared to trolox, which showed very high radical scavenging activity with EC_{50} of 6.1±2.5 μg/mL (Figure 1).

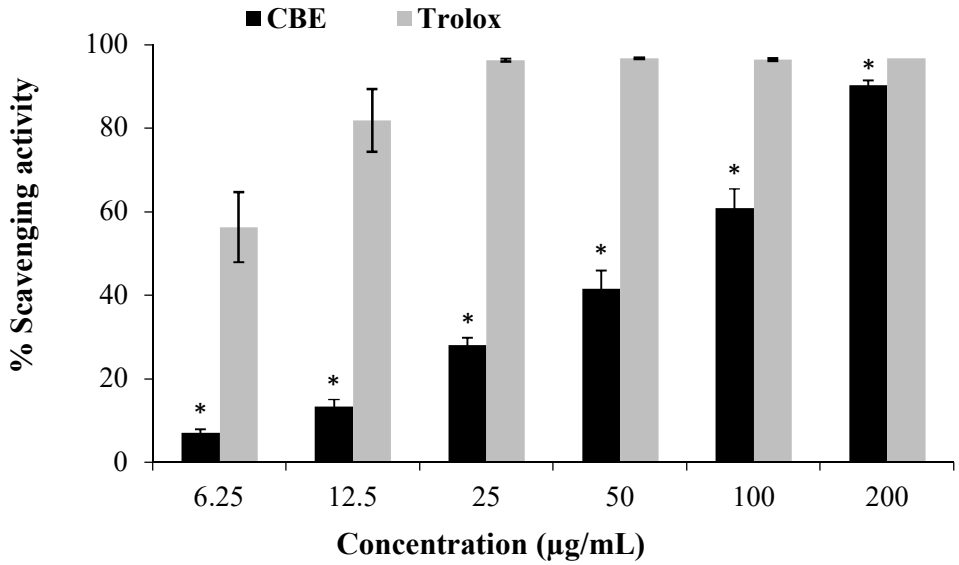

Figure 1. DPPH radical scavenging activities of CBE and trolox. Results are presented as mean±SEM of six experiments. $^{*}p<0.05$ indicates a statistically significant difference from trolox groups.

Cytotoxicity of CBE in human neutrophils

CBE at the concentrations up to 100 μg/mL did not significantly affected neutrophil viability during 45 min incubation period when compared to the control group, but its cytotoxic effect was observed on neutrophils at the concentration of 1000 μg/mL (CC_{50} >1000 μg/mL) (Figure 2).

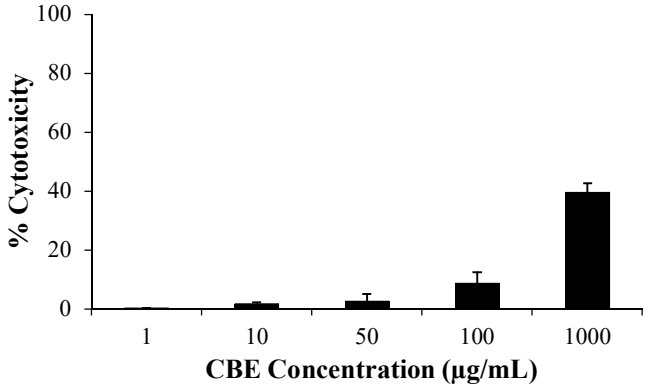

Figure 2. Cytotoxic effect of CBE (1-1000 µg/mL) on human neutrophils after 45 min incubation. Results are presented as mean±SEM of six experiments using the cells collected from different donors.

Effect of CBE on neutrophil chemotaxis

fMLP at a concentration of 1 µM induced a submaximal migration of human neutrophils. When the cells were preincubated with CBE at the concentration range of 0.1-100 µg/mL at 37˚C for 10 min, neutrophil chemotaxis induced by fMLP (1 µM) was significantly suppressed in a concentration-dependent manner compared to control (p<0.05, ANOVA), with IC_{50} of 140.4±18.2 µg/mL (Figure 3). Indomethacin (0.1-100 µg/mL) exerted stronger inhibitory effects on fMLP-induced neutrophil chemotaxis than those of CBE, giving IC_{50} of 17.7±2.5 µg/mL (p<0.05, ANOVA).

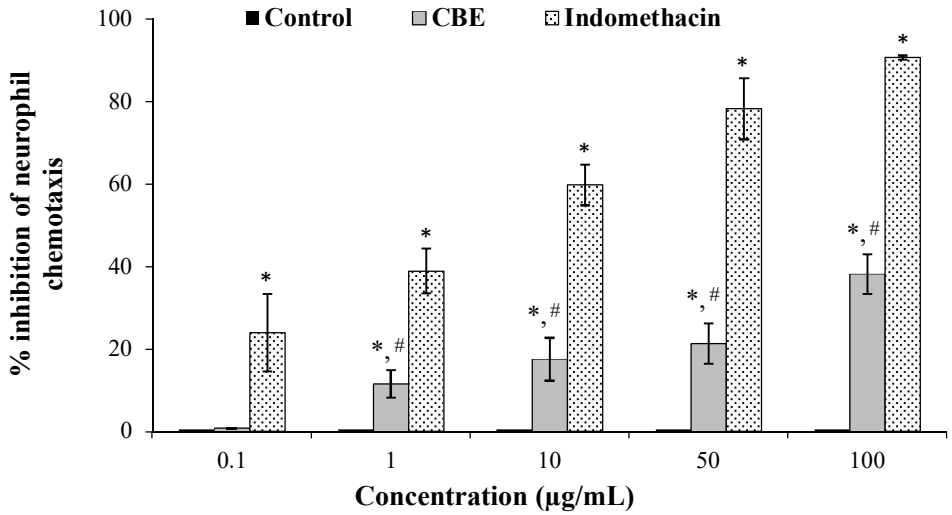

Figure 3. Inhibitory effects of CBE and indomethacin (0.1-100 µg/mL) on fMLP-induced human neutrophil chemotaxis. Results are presented as mean±SEM of six experiments using the cells collected from different donors. $^{*}p$<0.05 indicates a statistically significant difference from the control group. $^{\#}p$<0.05 indicates a statistically significant difference from the indomethacin treated group.

Effect of CBE on generation of O_2^- by neutrophils

Neutrophils released O_2^- into the extracellular medium upon stimulation with fMLP (100 nM), as determined by SOD-inhibitable cytochrome c reduction. Preincubation with CBE at the concentration range of 0.1-100 μg/mL at 37°C for 10 min significantly suppressed fMLP-induced SAG by human neutrophils in a concentration-dependent manner (p<0.05, ANOVA), with IC_{50} of 42.2±3.5 μg/mL (Figure 4). Indomethacin (0.1-100 μg/mL) also demonstrated strong inhibition of neutrophil SAG induced by fMLP (IC_{50} of 31.0±3.2 μg/mL; p<0.05, ANOVA). The results indicated that inhibitory effects on neutrophil SAG of CBE and indomethacin at 10-100 μg/mL were comparable.

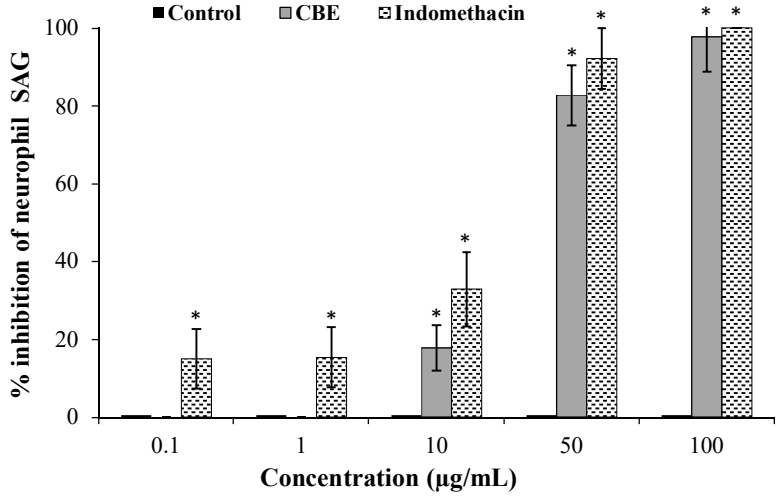

Figure 4. Inhibitory effects of CBE and indomethacin (0.1-100 μg/mL) on fMLP-induced SAG in human neutrophils. Results are presented as mean±SEM of seven experiments using cells collected from different donors. $^*p$<0.05 indicates a statistically significant difference from the control group.

Effect of CBE on neutrophil MPO production

The methanolic extract of *C. buchanani* at the concentration range of 0.1-100 μg/mL significantly inhibited the fMLP-induced MPO production in human neutrophils in a concentration-dependent manner (p<0.05, ANOVA) with IC_{50} of 108.3±8.9 μg/mL (Figure 5). Indomethacin exerted strong inhibitory effect on fMLP-induced human neutrophil MPO production with IC_{50} of 43.7±3.6 μg/mL.

Effect of CBE on neutrophil elastase release

Preincubation of isolated human neutrophils with CBE (0.1-100 μg/mL) elicited a very weak inhibition of human neutrophil elastase release induced by fMLP (100 nM) with IC_{50}>7000 μg/mL, in contrast to indomethacin (1-100 μg/mL) which caused a significantly inhibition with IC_{50} of 52.3±6.5 μg/mL (p<0.05, ANOVA) (Figure 6).

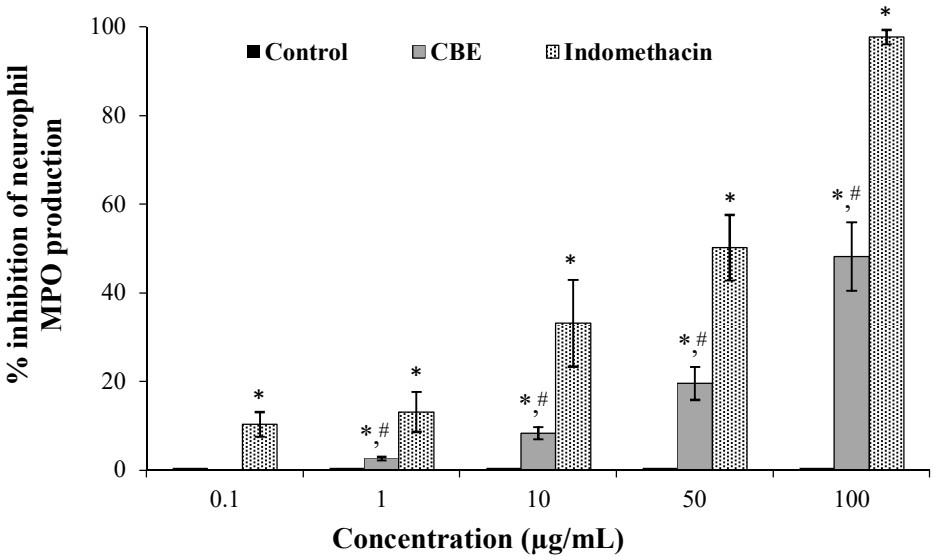

Figure 5. Inhibitory effects of CBE and indomethacin (0.1-100 µg/mL) on fMLP-induced MPO production in human neutrophils. Results are presented as mean±SEM of seven experiments using cells collected from different donors. $^{*}p<0.05$ indicates a statistically significant difference from the control group. $^{\#}p<0.05$ indicates a statistically significant difference from the indomethacin treated group.

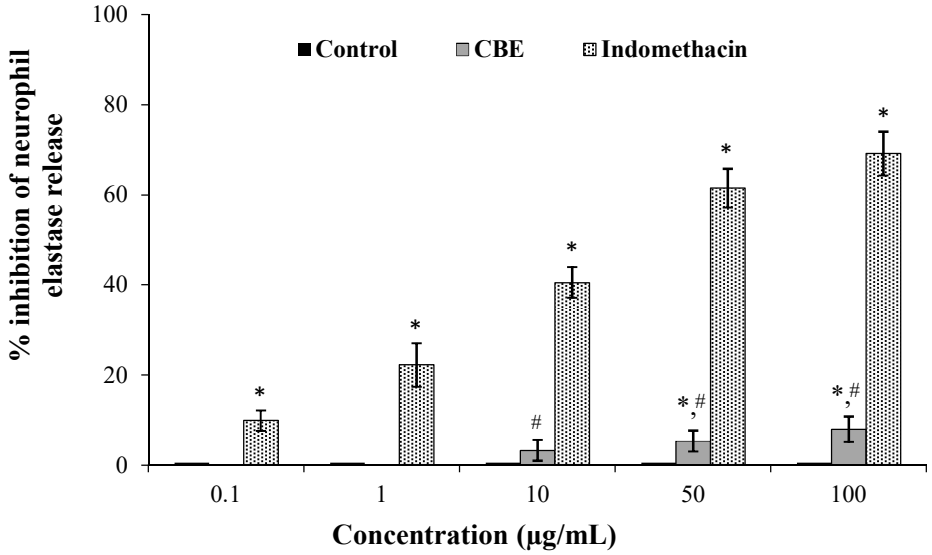

Figure 6. Inhibitory effects of CBE and indomethacin (0.1-100 µg/mL) on fMLP-induced elastase release in human neutrophils. Results are presented as mean±SEM of six experiments using cells collected from different donors. $^{*}p<0.05$ indicates a statistically significant difference from the control group. $^{\#}p<0.05$ indicates a statistically significant difference from the indomethacin treated group.

Effect of CBE on neutrophil apoptosis

Neutrophil necrosis at 20 h culture was negligible with >98% cell viability, assessed by trypan blue exclusion. Neutrophil apoptosis is characterized by dramatic morphological changes where apoptotic neutrophils exhibit hyperchromatic pyknotic nuclei. After 20 h culture, in the absence of either CBE or dexamethasone, about 60% of neutrophils underwent apoptosis as assessed by morphology (60.2%, Figure 7A) and flow cytometry using FITC-labelled recombinant human annexin V (61.9%, Figure 8A). CBE at 100 μg/mL showed slightly stimulatory effect on neutrophil apoptosis as assessed by morphology (62.7%, Figure 7B) and flow cytometry (64.0%, Figure 8D). In addition, CBE at concentrations of 1 and 10 μg/mL also slightly induced neutrophil apoptosis (62.6% and 63.3%, Figure 8B and 8C) compare with the control group. Interestingly, CBE at 500 μg/mL demonstrated a significant inhibition on neutrophil apoptosis (55.1% apoptosis) (Figure 8E). Dexamethasone (1 μM) significantly inhibited neutrophil apoptosis with 51.2 % and 52.1% being apoptotic as assessed by morphology (Figure 7C) and annexin V binding (Figure 8F), respectively.

Discussion

Previous studies have demonstrated the anti-acute inflammatory activity of CBE in ethyl phenylpropiolate (EPP)-induced rat ear edema and carrageenan-induce rat paw edema models.[5,6] The herbal extract also showed potential for its anti-chronic inflammatory activity in cotton thread-induced granuloma model.[5] Moreover, CBE was shown to reduce eicosanoid production in stimulated rat peritoneal leukocytes and tumor necrosis factor (TNF)-α release from activated human monocytic cell lines (THP). Both eicosanoids and TNF-α are known to be distinctive inflammatory mediators involved in acute and chronic inflammation, respectively.[5] As neutrophils play pivotal roles in the acute inflammatory process, the *in vitro* effects of CBE on human neutrophil function responsiveness were evaluated in the present study. Prior to the investigation of the effects of CBE on human neutrophil responsiveness and apoptosis, the herbal extract (1-1000 μg/mL) was tested for its cytotoxicity on cultured human neutrophils. CBE at concentrations of up to 100 μg/mL did not affect the viability of neutrophils during a 45-min incubation period.

A number of factors including cytokines, chemical mediators and bacterial products such as LPS, have been shown to induce neutrophil functional responsiveness and to prolong the functional lifespan of neutrophils.[12,31] Neutrophils, under LPS stimulation, generate oxygen radicals, nitric oxide and other components of the inflammatory processes. Human neutrophils constitutively undergo apoptosis and this process is critical for the resolution of inflammation. Neutrophil apoptosis can be modulated by a wide variety of agents including granulocyte-macrophage colony-stimulating factor (GM-CSF), LPS and TNF-α. Sabroe et al.[32] demonstrated that LPS could enable prolonged neutrophil survival at early (4 h) but not late (22 h) time points by inhibiting spontaneous apoptosis of neutrophils via a nuclear factor kappa B (NF-κB)-mediated survival pathway, leading to inflammation. At extremely low concentrations (0.01 ng/mL), LPS almost completely prevented early neutrophil apoptosis via Toll-like receptor (TLR)-4 stimulation. It is possible that the herbal extract may be contaminated with small amount of LPS. To ascertain that the effects of CBE on neutrophil functions and apoptosis are not related to the action of

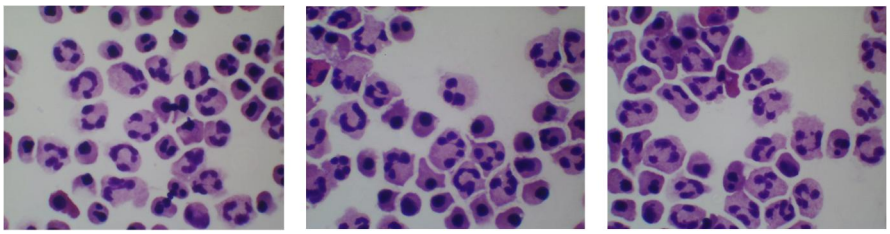

Figure 7. Changes in morphology of neutrophils treated with (A) vehicle control, (B) CBE (100 μg/mL) and (C) dexamethasone ((1μM) at 20 h culture.

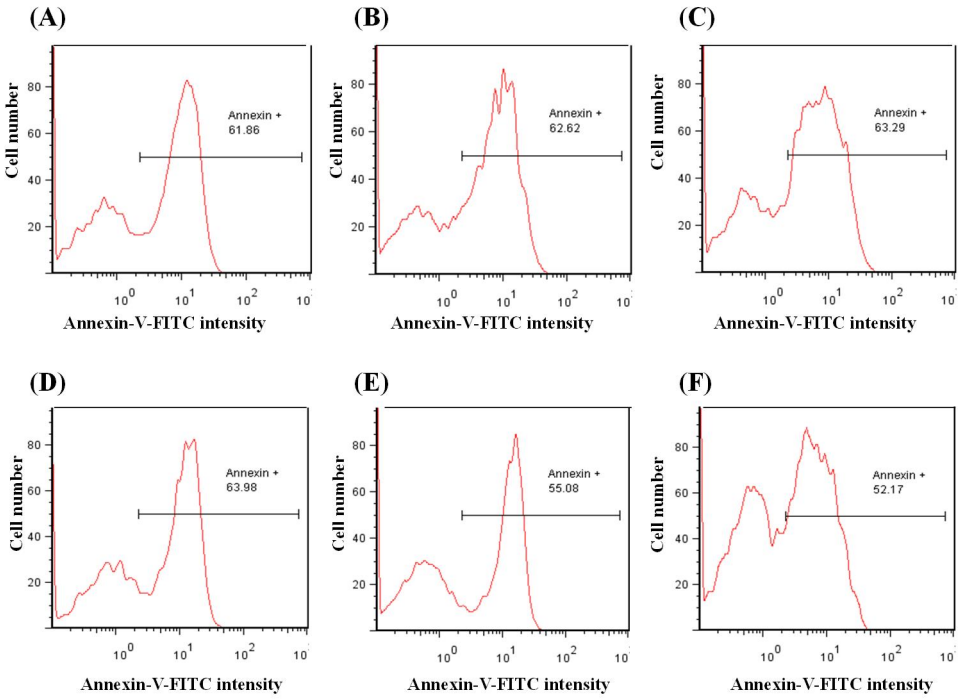

Figure 8. Effects of (A) vehicle control, (B) CBE (1 μg/mL), (C) CBE (10 μg/mL), (D) CBE (100 μg/mL), (E) CBE (500 μg/mL) and (F) dexamethasone (1 μM) on phosphatidylserene expression at 20 h culture, assessed by flow cytometry using FITC-labelled recombinant human annexin V. Mean fluorescence values are shown for a minimum of 5,000 cells for each condition.

contaminating LPS, the amount of LPS in the herb extract was determined before conducting further experiments. The results showed that CBE contained negligible quantities of LPS. Furthermore, since the extract did not induce neutrophil response *per se* but did inhibit neutrophil function and slightly stimulated neutrophil apoptosis, it can rule out the possibility that LPS is a biologically significant contaminant.

During inflammation neutrophils migrate to sites of trauma and release O_2^- and tissue destructive enzymes such as MPO and elastase. This process can exacerbate the inflammatory response and lead to tissue destruction. Formulated peptides such

as fMLP are liberated from bacteria and are powerful neutrophil agonists. The effect of CBE on neutrophil functional responsiveness was compared to that of indomethacin, a prototype of anti-inflammatory drug used in this study. Pretreatment of neutrophils with CBE (0.1-100µg/mL) significantly inhibited fMLP-induced neutrophil chemotaxis in a concentration-dependent manner. Activation of neutrophils also results in both the extracellular release of O_2^- and the intracellular production of radical, which may be attributed to distinct pools of NADPH oxidase.[33] In this study, fMLP concentration-dependently triggered neutrophil SAG; an effect significantly inhibited by CBE. In addition, the herbal extract exhibited marked antioxidant activity in a concentration-dependent manner as determined by DPPH scavenging activity assay. These results are consistent with previous findings.[34] Neutrophil activation also results in degranulation with release of several enzymes such as MPO and elastase. MPO, an abundant chloride peroxidase found in neutrophil azurophilic granules, is involved in microbial killing and inflammatory tissue damage. Anti-inflammatory activities of certain drugs have been attributed to the inhibition of leukocyte MPO activity.[35] Our study showed that CBE inhibited fMLP-induced MPO production in a concentration-dependent manner. In response to divergent stimuli, recruited neutrophils at the site of infection or inflammation also released elastase from gelatinase granules. Neutrophil elastase promotes inflammation and inhibits the healing process in chronic inflammatory process. The effect of CBE on fMLP-induced elastase release in neutrophils was assessed and it was found that CBE showed slight inhibition of fMLP-induced elastase release.

Resolution of inflammation involves the clearance of excess or effete inflammatory cells by a process of physiological programmed cell death (apoptosis) and the subsequent recognition and removal of apoptotic cells by phagocytes. Human neutrophils constitutively undergo apoptosis. Apoptosis, a process which controls the functional longevity of granulocytes *in situ*, provides a granulocyte clearance mechanism that tends to limit tissue injury and promotes resolution of inflammation.[12] Failure to remove neutrophils and their toxic products from tissues likely results in chronic persistent inflammation. The two pathways of apoptosis, mitochondrial pathway and death receptor pathway, differ in their induction and regulation, and both culminate in the activation of "executioner" caspases. A wide variety of agents can modulate neutrophil apoptosis through multiple and complex receptor-signalling pathways. Granulocyte apoptosis is also regulated by an inducible form of the transcription factor NF-κB. Inhibition of this transcription factor may be exploited for therapeutic benefit in inflammatory conditions where granulocytes play a prominent role. Determination of the CBE effect on constitutive neutrophil apoptosis was carried out in this study. CBE at the concentrations of 10-100 µg/mL was found to slightly induce neutrophil apoptosis. Thus the early increased apoptosis of neutrophils by CBE is likely to promote resolution of inflammation. On the contrary, CBE at a concentration 500 µg/mL caused inhibition of neutrophil apoptosis, leading to enhancing the longevity of these cells, and therefore delaying inflammatory resolution. The opposite effects of CBE on neutrophil apoptosis observed at different concentrations might be caused by different mechanisms. The glucocorticoid dexamethasone which was used as a reference drug showed an inhibitory effect on neutrophil apoptosis.[36]

Conclusion

We have demonstrated that the methanolic extract from *C. buchanani* exerted *in vitro* inhibitory effects on human neutrophil functional responsiveness, i.e., chemotaxis, SAG, and MPO production, without having a significant cytotoxic effect. In addition, this herbal extract caused slightly stimulatory effects on neutrophil apoptosis. These suggested that the effects of CBE may, in part, be attributable to its anti-inflammatory activity. Thus these results lend support to the benefits of using CBE in Thai traditional medicine for the treatment of inflammatory conditions in which neutrophils play the major roles. However, further investigation is required to fully identify the biologically active ingredients with their activities and to define the underlying molecular mechanisms of the inhibitory effects of CBE on neutrophil functional responsiveness and CBE stimulatory effect on neutrophil apoptosis.

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References

1. Sharma D, Sahu AN, Mujeeb M, Bharti A, Sharma A, Tripathi RK et al. Development of pharmacognostical profile of *Cryptolepis buchanani* Roem. & Schult. Int J Pharm Pharm Sci. 2012;4(2):615-8.
2. Tayung K, Saikia N. *Cryptolepis buchanani* - A less-known medicinal plant used in bone fracture. Indian J Tradit Know. 2003;2(4):371-4.
3. Panthong A, Kanjanapothi D, Talor WC. Ethnobotanical review of medicinal plants from Thai traditionnal books, Part I: Plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. J Ethnopharmacol. 1986 Dec;18(3): 213-28.
4. Laupattarakasem P, Houghton PJ, Hoult JR, Itharat A. An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. J Ethnopharmacol. 2003 Apr;85(2-3):207-15.
5. Laupattarakasem P, Wangsrimongkol T, Surarit R, Hahnvajanawong. *In vitro* and *in vivo* anti-inflammatory potential of *Cryptolepis buchanani*. J Ethnopharmacol. 2006 Dec 6;108(3):349-54.
6. Hanprasertpong N, Teekachunhatean S, Chaiwongsa R, Ongchai S, Kunanusorn P, Sangdee C, et al. Analgesic, anti-inflammatory, and chondroprotective activities of *Cryptolepis buchanani* extract: *in vitro* and *in vivo* studies. Biomed Res Int. 2014;2014:978582.
7. Sittiwet C, Puangpronpitag D. Anti-bacterial activity of *Cryptolepis buchanani* aqueous extract. Int J Biol Chem. 2009;3(2):90-4.
8. Viniyaka KS, Prashith Ketkuda TR, Mallikarjun N, Sateesh VN. Anti-dermatophyte of *Cryptolepis buchanani* Roem. & Schult. Pharmacogn J. 2010 Mar;2(7):170-2.

9. Hanprasertpong N, Panthong A, Sangdee C, Kunanusorn P, Kasitanon N, Lhieochaiphant S, et al. *Cryptolepis buchanani* oil formulation versus indomethacin solution in topical therapy for osteoarthritis of the knee: A randomized controlled trial. J Herb Med. 2017 Mar;7:18-26.
10. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013 Mar;13(3):159-75.
11. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischaemia/reperfusion injury. Pharmacol Rev. 2001 Mar;53(1):135-59.
12. Walker A, Ward C, Taylor EL, Dransfield I, Hart SP, Haslett C, et al. Regulation of neutrophil apoptosis and removal of apoptotic cells. Curr Drug Targets Inflamm Allergy. 2005 Aug;4(4):447-54.
13. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med. 1999 Nov;160(5 Pt 2):S5-11.
14. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov. 2004 May;3(5)::401-16.
15. Kankaanranta H, Moilanen E, Vapatalo H. Effects of non-steroidal anti-inflammatory drugs on polymorphonuclear leukocyte functions *in vitro*: focus on fenamates. Naunyn Schmiedebergs Arch Pharmacol. 1994 Dec;350(6):685-91.
16. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol. 2001 Aug 1; 167(3):1601-8.
17. Bombini G, Canetti C, Rocha FA, Cunba FQ. Tumour necrosis factor-alpha mediates neutrophil migration to the knee synovial cavity during immune inflammation. Eur J Pharmacol. 2004 Aug 2;496(1-3:197-204.
18. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. Nat Rev Rheumatol. 2014 Oct;10(10):593-601.
19. Chen W, Wang Q, Ke Y, Lin J. Neutrophil function in an inflammatory milieu of rheumatoid arthritis. J Immunol Res. 2018 Dec 3;2018:8549329.
20. Kandahari A, Yang X, Dighe A, Pan D, Cui Q. Recognition of immune response for the early diagnosis and treatment of osteoarthritis. J Immunol Res. 2015; 2015;2015:192415.
21. Kraan MC, Koster BM, Elferink JG, Post WJ, Breedveld FC, Tak PP. Inhibition of neutrophil migration soon after initiation of treatment with leflunomide or methotrexate in patients with rheumatoid arthritis: Finding in a prospective, randomized, double-blind clinical trial in fifteen patients. Arthritis Rheum. 2000 Jul;43(7):1488-95.
22. den Broeder AA, Wanten GJ, Oyen WJ, Naber T, van Riel PL, Barrera P. Neutrophil migration and production of reactive oxygen species during treatment with a fully human anti-tumor necrosis factor-α monoclonal antibody in patients with rheumatoid arthritis. J Rheumatol. 2003 Feb;30(2):232-7.
23. Cavin A, Hostettmann K, Dyatmyko W, Potterat O. Antioxidant and lipophilic constituents of *Tinospora crispa*. Planta Med. 1998 Jun;64(5):393-6.

24. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods. 1991 Sep 13;142(2):257-65.
25. Wanikiat P, Woodward DF, Armstrong RA. Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils. Br J Pharmacol. 1997 Nov;122(6):1135-45.
26. Talpain E, Armstrong RA, Coleman RA, Vardey CJ. Characterisation of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. Br J Pharmacol. 1995 Apr;114(7):1459-65.
27. Suzuki K, Ota H, Sasagawa S, Sakatani T, fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal Biochem. 1983 Jul 15;132(2):345-52.
28. Barrett AJ. Leukocyte elastase. Method Enzymol. 1981;80 Pt C:581-8.
29. Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, et al. NF-kappaB activation is a critical regulator of human granulocyte apoptosis *in vitro*. J Biol Chem. 1999 Feb 12;274(7):4309-18.
30. Ward C, Dransfield I, Chilvers ER, Haslett C, Rossi AG. Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. Trends Pharmacol Sci. 1999 Dec;20(12):503-9.
31. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, Van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994 Sep 1;84(5):1415-20.
32. Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN, et al. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol. 2003 May 15;170(10)::5268-75.
33. Karlsson A, Nixon JB, McPhail LC. Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidyl-inositol 3-kinase. J Leukoc Biol. 2000 Mar;67(3): 396-404.
34. Ashwini SK, Kiran R, Soumyn KV, Sudharshan SJ, Prashith Kekuda TR, Vinayaka KS, et al. Insecticidal and *in vitro* antioxidant potency of extracts of *Cryptolepis buchanani* Roem. & Schult. Int J Ph Sci. 2010;2(1);418-25.
35. Ramos CL, Pou S, Rosen GM. Effects of anti-inflammatory drugs on myeloperoxidase-dependent hydroxyl radical generation by human neutrophils. Biochem Pharmacol. 1995 Apr 18;49(8):1079-84.
36. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophlic granulocytes. J Immunol. 1996 Jun 1;156(11):4422-8.