In vitro and *in vivo* anti-inflammatory potential of *Cryptolepis buchanani*

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Abstract

Cryptolepis buchanani Roem. & Schult. (Asclepiadaceae), a climbing tree, is used as 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. In the current study, the potential anti-inflammatory activity of a 50% ethanol extract of this plant was evaluated in a number of experimental models. For anti-acute inflammatory activity, results showed that the extract caused reduction of carrageenan-induced rat paw edema in addition to significant reduction of eicosanoid production from calcium ionophore A23187-stimulated rat peritoneal leukocytes. In a test for anti-chronic inflammatory potential utilizing the cotton thread-induced granuloma, the extract caused significant lowering of granulation tissue formation. The reduction of tumor necrosis factor-α (TNF-α) release from LPS-stimulated human monocytic cell line (THP-1) was also demonstrated in cells that were pre-incubated with the extract. An additional important feature of *Cryptolepis buchanani* is its low toxicity, especially by oral treatment, which significantly encourages clinical trials of this extract in the human. In conclusion, the results give scientific support to the traditional use of this plant for combating inflammation. Further investigations are required to identify the active constituents responsible for the anti-inflammatory activity of *Cryptolepis buchanani*. Subacute and chronic toxicological studies in animals are also needed before clinical trials.

Keywords: *Cryptolepis buchanani*; Asclepiadaceae; Anti-inflammation; Acute toxicity; Traditional medicine

1. Introduction

Cryptolepis buchanani Roem. & Schult (Asclepiadaceae), a climbing tree, is widely used in folk medicine in southeast Asia. In Thailand, the alcoholic extract of stem of this plant is commonly used for the treatment of inflammatory conditions such as arthritis, and muscle and joint pain (Panthong et al., 1986; Laupattarakasem et al., 2003). A weak inhibition of eicosanoid generation from rat leukocytes was previously reported but this was not dose-dependent (Laupattarakasem et al., 2003). Therefore, we designed experiments to re-evaluate the anti-inflammatory potential of this plant in both acute and chronic inflammation using several *in vitro* and *in vivo* experimental models. An acute toxicity test was included in this preliminary toxicity study.

Inflammation is a process involving multiple factors acting in a complex network. The ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions (Colditz, 1985; Kasama et al., 1995). Neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively (Kasama et al., 1993). At the inflamed site, the recruited cells are activated to release many inflammatory mediators which elicit the initiation and maintenance of an inflammatory response, causing a change from the acute phase to the chronic phase of inflammation. Therefore, inhibition of the cellular reactions is one of the targets that are generally used as an *in vitro* model for

Abbreviations: 5-LO, 5-lipooxygenase; AA 861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone; ASA, acetylsalicylic acid; COX, cyclooxygenase; DMSO, dimethylsulfoxide; EIA, enzymeimmunoassay; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IDM, indomethacin; LD_{50}, lethal dose$_{50}$; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; PMA, phorbol-12-myristate-13-acetate; PMNs, polymorphonuclear; THP-1, human leukemia monocytic cell line; TNF-α, tumor necrosis factor-α; TXB_2, thromboxane B_2

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anti-inflammatory testing. For *in vivo* tests, inflammation can be induced in animals by many substances. Rat paw edema is the most commonly used model for acute inflammation while subcutaneous implantation of biomaterial is usually used for the chronic inflammatory model. In the present study, evaluation of the anti-inflammatory potential of 50% ethanol extract of *Cryptolepis buchanani* was performed using both *in vitro* and *in vivo* tests. Inhibition of eicosanoid generation from rat neutrophils and pro-inflammatory cytokine (e.g., tumor necrosis factor-α, TNF-α) release from macrophages are used as markers for *in vitro* tests for acute and chronic inflammation, respectively. Carrageenan-induced rat paw edema and cotton thread-induced granulation in rats were used as the model for acute and chronic inflammation.

2. Materials and methods

2.1. Chemicals

Calcium ionophore (A23187), oyster glycogen (Type II), indomethacin (IDM), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone (AA 861), acetylsalicylic acid (ASA), ethyl acetate, dimethylsulfoxide (DMSO), carrageenan, phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS) from *E.coli* 026:B_6 were obtained from Sigma–Aldrich (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin, human endothelial SFM medium, medium 199, trypsin-EDTA solution were purchased from Gibco-BRL (Karlsruhe, Germany). Thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) enzymeimmunoassay kits were purchased from Amersham Biosciences (New Jersey, USA). TNF-α enzyme-linked immunosorbant assay kit was purchased from BD Biosciences (New Jersey, USA).

2.2. Plant material and extract preparation

Cryptolepis buchanani was identified by Dr. Arunporn Itharat and collected from Songkla Province in southern Thailand. Voucher specimens of this plant (SKP A 0180101) were deposited in the Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Science, Prince of Songkla University, Hat Yai, Thailand. After the plant material was dried, it was refluxed twice with 50% ethanol. The extract was then filtered and centrifuged at $800 \times g$ for 10 min. The ethanol was removed under reduced pressure by rotary evaporation and the water residue was removed by lyophilisation. The percent yield was calculated on the basis of the dried plant material weight. For testing, the extract was dissolved in a vehicle and diluted to the desired concentrations.

2.3. Animals

Sprague-Dawley rats (200 ± 50 g) and Swiss albino mice (25 ± 5 g) were obtained from the Faculty of Medicine, Khon Kaen University. They were kept in standard environmental conditions and maintained on a standard rodent diet with water given *ad libitum*. All procedures involving the use of animals were undertaken in accordance with the guidelines of the Animal Ethics Committee, Khon Kaen University.

2.4. Eicosanoid generation by ionophore-stimulated rat peritoneal leukocytes

Rat peritoneal leukocytes were prepared according to a standard method (Moroney et al., 1988). Briefly, the accumulation of leukocytes was stimulated by intraperitoneal injection of 6% oyster glycogen in saline. After 16–20 h, rats were sacrificed and the peritoneal leukocytes were immediately lavaged with ice-cold modified Hank's balanced salt solution (HBSS) free of Ca^{2+} and Mg^{2+}. After centrifuging at $400 \times g$ for 10 min at 4 °C, contaminating erythrocytes in the pellet were lysed with Tris-buffer NH_4Cl. With further centrifugation, the cell pellet was re-suspended in complete HBSS at 2.5×10^6 cells/ml containing Ca^{2+} and Mg^{2+}. Cell viability, based on trypan blue exclusion, was greater than 95%. By this method, mixed peritoneal leukocytes comprised approximately 75% polymorphonuclear neutrophils leukocytes (PMNs).

Triplicate aliquots of 0.5 ml PMNs were pre-incubated for 10 min, at 37 °C with the extract, vehicle, or reference drugs: indomethacin (5 μM) for cyclooxygenase inhibition, or AA 861 (1 μM) for 5-lipooxygenase inhibition, and then stimulated for another 10 min by addition of calcium ionophore A23187 (1 μM). The cells were then pelleted by centrifugation at $400 \times g$ for 10 min at 4 °C and the supernatants were then taken and frozen at −20 °C. Aliquots (2–25 μl) of the thawed samples were subjected to enzymeimmunoassay (EIA) for thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) (Amersham, USA). The assay was based on the competition between TXB_2 and LTB_4 for a limited number of binding sites on a TXB_2/LTB_4-specific antibody. With fixed amounts of antibody and peroxidase labeled TXB_2/LTB_4, the amount of peroxidase labeled ligand bound by the antibody would be inversely proportional to the concentration of added unlabeled ligand. The concentration of unlabeled TXB_2/LTB_4 in the sample can then be determined by interpolation from a standard curve.

2.5. TNF-α production from LPS-stimulated THP-1

Human leukemia monocytic THP-1 cells were cultured in 75 cm^2 plastic flasks bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL, Germany). The cell culture was maintained at 37 °C in a humidified 5% CO_2 atmosphere. According to a method described by Singh et al. (2005), cells at the exponential growth phase were trypsinized and the cell suspension was prepared in complete medium at 5×10^5 cells/ml. About 500 μl of cell suspension was pre-activated with PMA (100 nM) in a 24-well plate for 48 h to undergo transformation into macrophages before pre-incubation with 100 μl of various concentrations (1, 5, 50, 100, 200 μg/ml) of *Cryptolepis buchanani* extract. Cells with dexamethasone (1 μM) and vehicle (0.5% DMSO) were used as a reference drug and control, respectively. After 30 min pre-treatment with test compounds, cells were further incubated with 1 μg/ml LPS (Sigma, USA) for 24 h. Cell-free supernatants were collected

and TNF-α level was determined by EIA (BD Biosciences, USA).

2.6. Carrageenan-induced hind-paw edema

The anti-acute inflammatory activity of *Cryptolepis buchanani* was investigated using the model of carrageenan-induced rat paw edema as described by Winter et al. (1962). Rats weighing 200 ± 50 g were used. The animals were pre-treated with the extract at doses of 100, 250, and 500 mg/kg by intraperitoneal injection or 1000 mg/kg per oral to each group of six rats for 60 min before sub-plantar injection of 0.1 ml of 1% carrageenan solution. Aspirin (300 mg/kg, orally) was used as a positive control. The edema volume was determined at 1, 2 and 3 h after carrageenan injection by a water displacement method (Plethysmometer, model 7150, Ugo Basile). Edema volumes were compared between the treatment and control groups.

2.7. Cotton thread-induced granulation tissue in rats

Tissue granulation was induced by cotton thread implantation in male Sprague-Dawley rats, according to a published procedure (Ghosh et al., 2002) with slight modifications. Cotton thread was washed with ethyl acetate overnight, dried at room temperature, cut into 2 cm in length (7 ± 0.5 mg each) and sterilized by dry heat at 160 °C for 2 h. The ethanol extract of *Cryptolepis buchanani* was administered at concentrations of 150 and 300 mg/kg by intraperitoneal injection, or 1000 mg/kg orally to each group of six rats 60 min before subcutaneous implantation of the cotton thread on each rat's back under anesthesia. About 1 mg/kg of dexamethasone was administered orally as reference drug while the control rats received the same volume of the vehicle. Each group of rats was further treated with the extract or control substance once daily for six consecutive days. On the 8th day, the rats were sacrificed and the threads covered with granulation tissue were dissected out and dried in a hot air oven at 60 °C until a constant weight was obtained. The granulation weight was compared between the treatment and control groups.

2.8. Acute toxicity test

The acute toxicity of *Cryptolepis buchanani* was evaluated with the lethal dose test, involving single administrations of the plant extract to a group of male and female mice at increasing doses in order to determine the dose that would kill 50% of mice (LD_{50}) within a set time-frame. The mice (weight, 20–25 g) were divided into five groups ($n = 6$). Administration of the extract was performed orally at a dose of 1000 mg/kg, or by intraperitoneal injection at 200, 400, 800 and 1000 mg/kg. Observations for toxic symptoms and mortality were recorded at 1, 2, 3 and 24 h, then once daily for 7 days and the LD_{50} of the extract was estimated.

3. Statistical analysis

The statistical significance of the results was analyzed by Student's *t*-test for unpaired observations. The results were expressed as the mean ± standard error of mean (S.E.M.), the values of statistical significance being set at $^{*}P \leq 0.05$ and $^{**}P \leq 0.001$ levels.

4. Results

4.1. Effects of plant extract on eicosanoid generation

Addition of 1 μM calcium ionophore A23187 to the suspension of rat mixed leukocytes (PMN neutrophils and mononuclear cells) caused the generation of eicosanoids via cyclooxygenase (COX) and 5-lipooxygenase (5-LO) pathways, resulting in the release of TXB_2 and LTB_4, respectively (Fig. 1). Two compounds, known to be potent and specific inhibitors, were used as reference drugs. Pre-treatment of cells with indomethacin (COX

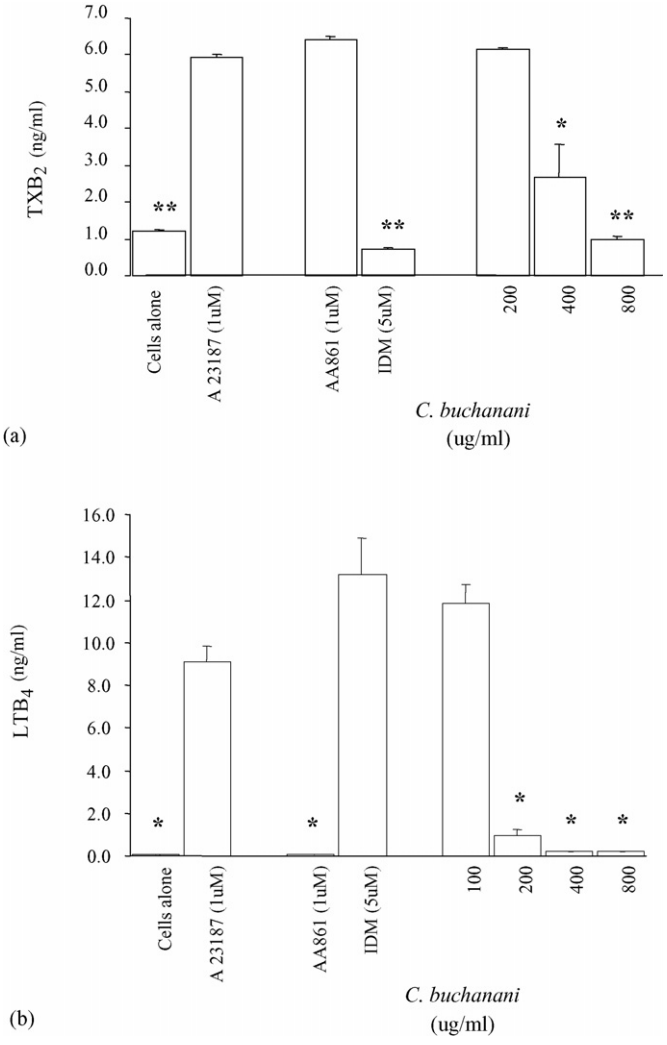

Fig. 1. Inhibition of TXB_2 (a) and LTB_4 (b) generation in rat peritoneal leukocytes treated with 1 μM calcium ionophore A23187 by 50% ethanol extract of *Cryptolepis buchanani* and the reference standards: cyclooxygenase inhibitor (indomethacin, IDM) and 5-lipooxygenase inhibitor (AA 861). Columns show mean ± S.E.M. The symbols (*,**) indicate statistically significant inhibition with respect to A23187 by unpaired Student's *t*-test, $P \leq 0.05$ and $P \leq 0.001$, respectively ($n = 3$).

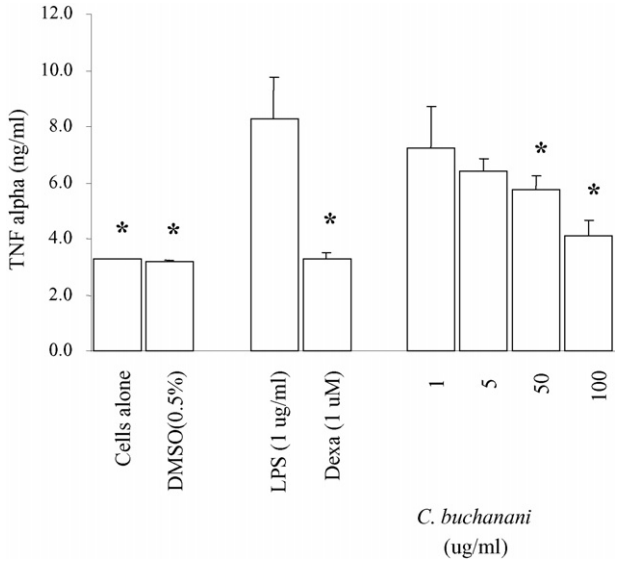

Fig. 2. The inhibitory effect of *Cryptolepis buchanani* on the production of TNF-α from monocyte cell line (THP-1 cells). THP-1 cells were primed with PMA (100 nM) for 48 h before culture in the presence or absence of test compounds for 30 min prior to LPS (1 μg/ml) stimulation for 24 h. Supernatants were assayed in triplicate for TNF-α by ELISA. Values are expressed as mean ± S.E.M. $^{*}P \leq 0.05$ statistical significance compared with control group by unpaired Student's *t*-test ($n = 3$). Dexa, dexamethasone (1 μM).

inhibitor, 5 μM) caused suppression of TXB_2 level while LTB_4 suppression was caused by AA 861 (5-LO inhibitor, 1 μM). Cells pre-treated with the 50% ethanol extract of *Cryptolepis buchanani* showed similar inhibitory effects on both COX and 5-LO pathways, resulting in TXB_2 (Fig. 1a) and LTB_4 (Fig. 1b) inhibition in a dose-dependent manner. Notably, at the highest dose (800 μg/ml), the inhibitory effect of the extract was similar to indomethacin and AA 861 for COX and 5-LO products, respectively.

4.2. Effects of plant extract on TNF-α production

The results of treatment of LPS-stimulated monocytic cell line (THP-1 cells) with various concentrations of the 50% ethanol extract of *Cryptolepis buchanani* in the dose range 1–100 μg/ml is shown in Fig. 2. The plant extract significantly inhibited TNF-α production in a dose-dependent manner. The inhibitory effects of *Cryptolepis buchanani* (100 μg/ml) and dexamethasone (1 μM) were 52.19 and 60.17%, respectively.

4.3. Effects of plant extract on the rat hind-paw edema

In the carrageenan-induced paw edema, there was a gradual increase in the edema paw volume in the control group during the whole experiment (3 h). A significant dose-related reduction in edema was shown in the animals that were pre-treated with the intraperitoneal injection of the extract, whilst no inhibitory effect was seen with the oral route (Table 1).

Table 1
Effects of 50% ethanol extract of *Cryptolepis buchanani* on carrageenan-induced rat paw edema

Experiments	Edema volume (ml), mean ± S.E.M.		
	1 h	2 h	3 h
Control	0.29 ± 0.04	0.53 ± 0.05	0.65 ± 0.04
ASA (300 mg/kg, p.o.)	0.06 ± 0.01**	0.14 ± 0.03**	0.29 ± 0.05**
Cryptolepis buchanani			
1000 (mg/kg, p.o.)	0.32 ± 0.03	0.63 ± 0.04	0.70 ± 0.04
100 (mg/kg, i.p.)	0.23 ± 0.03	0.42 ± 0.049	0.53 ± 0.05*
250 (mg/kg, i.p.)	0.17 ± 0.02*	0.39 ± 0.01**	0.57 ± 0.02*
500 (mg/kg, i.p.)	0.13 ± 0.02**	0.30 ± 0.04**	0.47 ± 0.06**

Drugs were administered for 60 min before subplantar carrageenan injection. The change of footpad volume was determined at 1, 2, and 3 h after irritant injection. Each value represents the mean ± S.E.M. of six rats per group. Statistically significant difference with respect to the control is expressed as $^{*}P \leq 0.05$, $^{**}P \leq 0.001$ (unpaired Student's *t*-test).

4.4. Effect of plant extract on the cotton thread-induced granulation tissue in rats

In a cotton thread-induced inflammation model, the 50% ethanol extract of *Cryptolepis buchanani* tested at the dose of 300 mg/kg i.p. and 1000 mg/kg p.o. showed anti-inflammatory activity (Fig. 3). After 7 days, the mean dry weight of granulomatous tissue surrounding the threads was significantly lower for the group treated with extract as compared to the control group. The significant anti-inflammatory activity was also demonstrated with dexamethasone at a dose of 1 mg/kg p.o. used as the reference control.

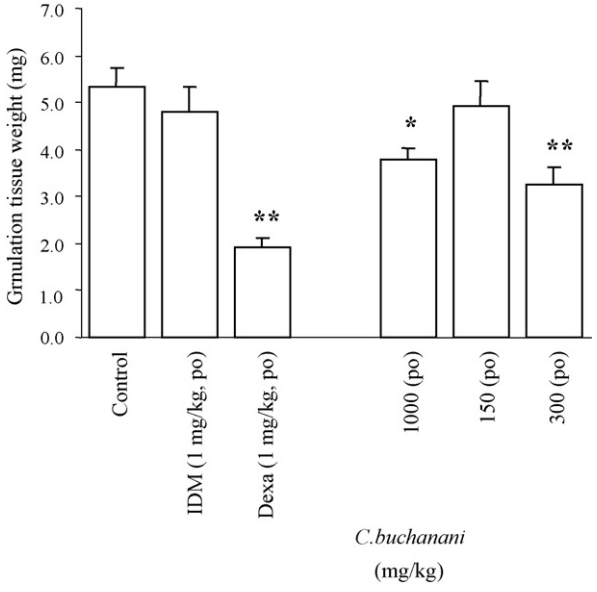

Fig. 3. Effects of 50% ethanol extract of *Cryptolepis buchanani* on the cotton thread-induced tissue granulation. The animals were pre-treated with various concentrations of the extract and reference drugs 60 min before subcutaneous implantation of the cotton thread (2 cm, 7 mg) in the dorsum of rats and once a day for the following six consecutive days. The rats were sacrificed 7 days after cotton implantation and the granulation tissue weight was determined as described in Section 2. Values are the mean ± S.E.M. of six rats. Statistical significance is represented by $^{*}P \leq 0.05$ and $^{**}P \leq 0.001$, respectively (unpaired Student's *t*-test).

Table 2
The mortality in mice was recorded during the experimental period of 7 days after a single dose of *Cryptolepis buchanani* was administered ($n = 6$)

Substance	Route	Dose (mg/kg)	Number of death/six mice
Cryptolepis buchanani	p.o.	1000	–/6
	i.p.	200	–/6
	i.p.	400	–/6
	i.p.	800	2/6
	i.p.	1000	6/6

4.5. Acute toxicity

Oral treatment of mice with the 50% ethanol extract up to 1000 mg/kg (approximately equivalent to 12 g/kg of dried material powder) showed no obvious behavioral changes after administration up to 7 days (Table 2). No lethal dose or LD_{50} could be determined since the highest dose tested caused no mortality. In contrast, hypersensitivity effects (tremor and fur) were observed in all animals within 2 h of intraperitoneal administration. A dose of 800 mg/kg i.p. caused death in one-third (33%) of the animals within 2 h, whilst 100% death could be observed when given with 1000 mg/kg i.p. From this data, the estimated LD_{50} was 900 mg/kg which was equivalent to 12 g/kg of the dried powder. By comparing with the toxicity-rating chart (Gosselin et al., 1984), the extract was classified as a non-toxic substance (oral administration) or slightly toxic substance (intraperitoneal injection). Additionally, macroscopic examination for gross lesions in the organs at autopsy did not reveal any change due to the extract administration.

5. Discussion and conclusion

Our results provide evidence that a 50% ethanol extract of *Cryptolepis buchanani* stems possesses anti-inflammatory activity. The extract was tested with the standard models for both acute and chronic inflammation. For the acute inflammatory model, the *in vitro* test was performed using the calcium ionophore A23187-stimulated rat leukocyte model. It was found that the extract inhibited both TXB_2 and LTB_4, the products of arachidonic acid mediated by COX and 5-LO pathways, respectively. The COX pathway leads to the generation of prostaglandins and thromboxanes, which mediate the pain and edema associated with inflammation, while the 5-LO pathway produces leukotrienes, including the leukocyte chemo-attractant LTB_4. This result suggests that the extract inhibited both the COX and 5-LO pathways, which differs from standard non-steroidal anti-inflammatory drugs, and probably accounted for its anti-inflammatory effect. The inhibitory effect of the extract on arachidonate metabolism was supported by the rat paw edema model. The present study shows that the 50% ethanol extract from the stem of *Cryptolepis buchanani*, especially at the high dose (500 mg/kg i.p.), has an inhibitory effect on edema formation in both early and late phases of the carrageenan-induced rat paw model. Aspirin, a COX inhibitor, at 300 mg/kg p.o., markedly reduced the paw edema. The paw edema induced by the subplantar injection of carrageenan in rats is biphasic; the early phase involves the release of serotonin, histamine, and kinins while the late phase is mediated by prostaglandins (Vinegar et al., 1969). This result tends to suggest that the inhibitory effect of the extract on edema formation is probably due to the inhibition of the synthesis and/or release of these mediators, especially the cyclooxygenase products.

For chronic inflammation, LPS-stimulated monocytes and cotton thread-induced granulation were used as *in vitro* and *in vivo* tests, respectively. Activated monocytes can release a series of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α). Therefore, the release of TNF-α after stimulation of monocytes with LPS is a valid model system to test novel compounds for potential anti-inflammatory effects (Singh et al., 2005). In the present work, the inhibitory effect of the extract on TNF-α release from LPS-stimulated human monocytic cell line (THP-1) was demonstrated in a dose-dependent manner. TNF-α plays a critical role in both acute and chronic inflammation (Holtmann and Neurath, 2004). TNF-α facilitates inflammatory cell infiltration by promoting the adhesion of neutrophils and lymphocytes to endothelial cells (Dore and Sirois, 1996). Additionally, TNF-α stimulates neutrophils to transcribe and release cytokines, and chemokines biosynthesis (Marucha et al., 1990; Fernandez et al., 1996). Interaction between these mediators thus enhances further inflammatory reactions (Gouwy et al., 2005) and inhibition of TNF-α release can reduce the severity of inflammation. The anti-chronic inflammatory activity of the extract was also demonstrated in cotton thread-induced granuloma in rats. Tissue granulation, one of the distinctive features of chronic inflammation, which is composed of marked infiltration macrophages and neovascularization, was induced by subcutaneous implantation of biomaterials. The implanted material induces a host inflammatory response and modulates the release of inflammatory mediators which finally leads to tissue proliferation and granular formation (Remes and Williams, 1992; Tang and Eaton, 1995; Hu et al., 2001). Cellular accumulations and pro-inflammatory cytokines, TNF-α and IL-1β, were demonstrated within the first 2 weeks (Cardona et al., 1992; Tang et al., 1998a,b; Dalu et al., 2000). Kato et al. (2002) also reported that TNF-α can accelerate both angiogenesis and matrix degrading by induction of vascular endothelial growth factor and various matrix metalloproteinases. Therefore, the inhibition of tissue granuloma by the extract, at least in part, may be through interference with TNF-α release.

In vivo toxicological studies of 50% ethanol extract of *Cryptolepis buchanani* have given contrasting results, depending on the route of administration. The present study showed that oral treatment of mice with the extract did not induce mortality or significant clinical symptoms of toxicity, while intraperitoneal injection of extremely high dose (800 and 1000 mg/kg) induced toxic effects causing the death of 33 and 100% of the animals, respectively. Although the compound responsible for this effect has not been identified, the effect might be caused by cardenolides, the potent positive inotropic agent that is found in *Cryptolepis buchanani* (Rao and Banning, 1990a,b).

In conclusion, a 50% ethanol extract from the stems of *Cryptolepis buchanani* exerts anti-inflammatory activity in several models. At this stage of investigation, the mechanism of

action is not clear, but it might be attributed to the inhibitory effect of the extract on leukocyte activations, resulting in the reduction of the release of inflammatory mediators and pro-inflammatory cytokines, as well as amelioration of the inflammation in both acute and chronic inflammatory animal models. These results lend support to the effectiveness of *Cryptolepis buchanani* in combating inflammation via multilevel regulation of inflammatory reactions. An additional important feature of *Cryptolepis buchanani* is its low toxicity, especially by oral treatment, that encourages clinical trials of this extract in humans. However, subchronic and chronic toxicological studies in animals are needed before these trials. Further investigations are needed to identify the active constituents responsible for the anti-inflammatory activity of *Cryptolepis buchanani*.

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