Original Article

In vivo analgesic and anti-inflammatory activities of a standardized *Rhinacanthus nasutus* leaf extract in comparison with its major active constituent rhinacanthin-C

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Abstract

Rhinacanthus nasutus (*R. nasutus*) leaf extract was prepared and standardized to obtain rhinacanthins rich extract that contained total rhinacanthin-C (Rn-C) of not less than 70% w/w. Rn-C was also isolated from the standardized *R. nasutus* leaf extract (SRLE). SRLE was investigated on pain and inflammatory models in parallel with its main naphthoquinone constituent, Rn-C in order to compare their efficacy in experimental animals. The analgesic activities of SRLE and Rn-C were evaluated by the acetic acid-induced writhing test, a hot-plate test and formalin test at doses of 20, 40, and 80 mg/kg. The anti-inflammatory activities were investigated by carrageenan induced paw edema and the cotton pellet induced granuloma in rats at doses of 80, 160, and 320 mg/kg. SRLE and Rn-C inhibited the acetic acid induced writhing in a dose dependent manner; inhibited the early phase of the formalin test at 80 mg/kg and the late phase at 40 and 80 mg/kg. However, none of the tested doses were effective in protecting against the hot plate test. In the animal models of inflammation, SRLE and Rn-C dose dependently inhibited edema formation in the carrageenan induced paw edema and suppressed granuloma formation in the cotton pellet induced granuloma in rats. The effects of SRLE in these tests were similar to those of the Rn-C. This study confirms the analgesic and anti-inflammatory activities of SRLE and Rn-C in animal models as well as demonstrating that they have a similar efficacy.

Keywords: rhinacanthins rich extract, naphthoquinone, inflammation, pain

1. Introduction

Rhinacanthus nasutus (Linn.) Kurz (Acanthaceae) is a small shrub widely distributed and cultivated in India, China, Taiwan, and in Southeast Asia including Thailand (Siripong *et al.*, 2006). Different parts of this plant have long been used in traditional medicine for the treatment of a variety of ailments. In Thailand, the leaves are used as an antipyretic, anti-inflammatory, antihypertensive, antisnake venom and as a detoxicant (Farnsworth and Bunyapraphatsara, 1992). In India, the leaves and roots of the plant are widely used for the treatment of rheumatism (Cheruvathur *et al.*, 2012).

Although, several groups of compounds like flavonoids, anthraquinones, triterpenes and steroids have been identified in extracts from the plant, naphthoquinones are reported to be the most biologically active constituent (Sendl *et al.*, 1996). Among several naphthoquinone analogues identified, Rn-C is found to be the major naphthoquinone in the leaf extract (Panichayupakaranant *et al.*, 2009) and has shown several pharmacological activities. Rn-C has shown potent antiviral activity (Sendl *et al.*, 1996), cytotoxicity (Wu *et al.*, 1998), antiproliferative (Siripong *et al.*, 2006), anti-

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inflammatory (Tewtrakul *et al.*, 2009a) and antiallergic (Tewtrakul et al., 2009b) activities. Recently, a rhinacanthins-rich *R. nasutus* leaf extract that contained a total amount of rhinacanthins of not less than 70% showed antifungal (Panichayupakaranant *et al.*, 2009) as well as antibacterial (Puttarak *et al.*, 2010) activities with a potency similar to that of Rn-C.

Despite the evidence from the reports of the various aforementioned biological activities, analgesic and anti-inflammatory activities of the standardized *R. nasutus* leaf extract (SRLE), standardized to contain total rhinacanthin-C of not less than 70% w/w, and its main active compound, Rn-C, have not been explored in any animal model. Therefore, this study was designed to evaluate the analgesic and anti-inflammatory activities of SRLE in parallel with Rn-C, the major active constituent, in order to compare their efficacy in experimental animals.

2. Materials and Methods

2.1 Chemicals and reagents

Indomethacin was obtained from Fluka BioChemika, Japan; morphine sulphate and carrageenan lambda were from Sigma (St. Louis, U.S.A.); acetic acid was from J.T. Baker Inc. U.S.A.; propylene glycol and formalin were from Vidhyasom Co., Ltd., Thailand; tween-80 was from Srichand United Dispensary Co., Ltd., Thailand. All other chemicals and solvents used in this study for extraction of plant material and quantitative analysis were of reagent or HPLC grade. Test compounds and indomethacin were dissolved in a cosolvent consisted of propylene glycol, tween 80 and distilled water (4:1:4) with the exception of morphine sulfate that was dissolved in normal saline. All were prepared just before commencing the experiment.

2.2 Plant material

Fresh leaves of *R. nasutus* were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, in June 2011. A voucher specimen (No. 0011814) was identified by Dr. P. Panichayupakaranant (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand), and deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The collected leaves were dried in a hot air oven at 50°C for 24 hrs and grounded into a fine powder.

2.3 Preparation of crude extract

The powdered material (2.0 kg) was macerated with 8 L of 95% ethyl acetate at room temperature for three days with frequent stirring. After three days, the macerate was filtered and evaporated to obtain a syrupy mass. The marc was remacerated again with ethyl acetate four times, filtered, and evaporated until it became a thick viscous mass (160 g).

2.4 Preparation of standardized *R. nasutus* leaf extract

Rhinacanthins rich extract was prepared from the crude extract according to the method previously described (Panichayupakaranant *et al.*, 2009). The crude ethyl acetate extract (135 g) was dissolved in methanol, filtered and loaded to a column packed with Amberlite® IRA-67 (Sigma, St. Louis, U.S.A.). The solution was allowed to drain from the lower valve of column at a flow rate of 1.5 mL/min. The column was then washed with methanol to remove chlorophyll. The rhinacanthins that remained in the column was then eluted with 10% acetic acid in methanol. The collected solution was evaporated under reduced pressure to give the rhinacanthins rich extract (48 g).

Quantitative analysis of the Rn-C content in the rhinacanthins rich extract was then performed by the reverse-phase high performance liquid chromatographic method using a TSK-gel ODS-80Ts column (Tosoh Bioscience, Tokyo, Japan). The mobile phase consisted of methanol and 5% aqueous acetic acid (80:20, v/v) pumped at a flow rate of 1 mL/min. The injection volume was 20 iL. The quantification wavelength was set at 254 nm. The SRLE thus obtained with an Rn-C content of not less than 70% w/w was used for the isolation of pure Rn-C as well as for further animal experiments.

2.5 Isolation of rhinacanthin-C

The SRLE obtained was chromatographed over silica gel using hexane and ethyl acetate (99:1) as the solvent system to obtain pure Rn-C. The first fraction obtained from the silica gel column was again chromatographed through a silica gel column using the same solvent system to remove any remaining interfering compounds and further purify Rn-C. The pure Rn-C thus obtained was elucidated by ^{1}H NMR spectral data. The compound was verified by comparing the ^{1}H NMR spectral data obtained with those previously reported (Sendl *et al.*, 1996).

2.6 Animals

Animals used in this study were male ICR mice weighing 25-35 g and male Wistar rats weighing 150-200 g. They were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Thailand. The animals were housed in standard cages for at least one week in the laboratory animal room prior to testing. Food and water were given *ad libitum* unless otherwise specified. All experimental protocols were approved by the Animal Ethics Committee, Prince of Songkla University (MOE 0521.11/533).

2.7 Analgesic activities

2.7.1 Writhing test

An acetic acid-induced writhing test was carried out according to the method described previously (Koster *et al.*, 1959). Male ICR mice were divided into eight groups with ten animals each. Different groups were orally administered with cosolvent (10 mL/kg), indomethacin (5 mg/kg), SRLE and Rn-C (20, 40, and 80 mg/kg), respectively. After 30 min, 0.6% acetic acid solution (10 mL/kg) was injected into the peritoneal cavity and the number of writhing episodes was counted for 20 min. A reduction in the writhing number compared to the control group was evaluated for analgesia which was expressed as the percentage inhibition of writhings.

2.7.2 Hot plate test

The hot plate test was carried out according to the method previously described (Woolfe and Macdonald, 1944). Male ICR mice were divided into nine groups with ten animals in each group. Different groups were administered with cosolvent (10 mL/kg), indomethacin (5 mg/kg), morphine sulfate (5 mg/kg, s.c.), SRLE and Rn-C (20, 40, and 80 mg/kg), respectively. Thirty minutes after oral administration of the test agents, or 15 min after subcutaneous injection of morphine sulphate, the nociceptive response was measured every 15 min over a 60 min period by individually placing each mouse on a hot plate (Harvard Apparatus Ltd., UK) maintained at a constant temperature of $55°C \pm 1°C$. Latency of the nociceptive response such as licking of a hind limb or jumping was noted. The cut-off time was fixed at 45 sec to prevent tissue damage. Only the mice that showed a nociceptive response within 15 sec were used in the experiments.

2.7.3 Formalin test

The formalin test was performed as previously reported (Hunskaar *et al.*, 1985). Male ICR mice were divided into nine groups with ten animals in each group. Different groups were administered with cosolvent (10 mL/kg), indomethacin (5 mg/ kg), morphine sulfate (5 mg/kg, s.c.), SRLE and Rn-C (20, 40, and 80 mg/kg), respectively. Thirty minutes after oral administration of the test agents, or 15 minutes after subcutaneous injection of morphine sulfate, 20 μL of 2.5% formalin in normal saline solution was injected subcutaneously to a right hind paw of each mouse. The time spent by each mouse on licking the injected paw was recorded. The data were expressed as the total licking time in the early phase (0-5 min) and the late phase (15-30 min) after formalin injection.

2.8 Anti-inflammatory activities

2.8.1 Carrageenan-induced rat paw edema

The acute anti-inflammatory activity was evaluated by carrageenan-induced rat paw edema model as previously described (Winter *et al.*, 1962). Male Wistar rats were divided into eight groups with six animals in each group. The initial volume of right hind paw of each rat was measured using a plethysmometer (Ugobasile, Milan, Italy). Different groups of rats were orally administered with cosolvent (5 mL/kg), indomethacin (5 mg/kg), SRLE and Rn-C (80, 160, and 320 mg/kg), respectively. After 30 minutes, each rat of every group was subcutaneously injected with 0.1 mL of 1% (w/v) carrageenan in normal saline into the subplantar region of the right hind paw. The volume of right hind paw was measured at 1, 3 and 5 h after carrageenan injection, and the edema volume was determined.

2.8.2 Cotton pellet-induced granuloma in rats

The cotton pellet-induced granuloma was performed according to the technique previously described (Swingle and Shideman, 1972). Male Wistar rats were divided into eight groups with six animals in each group. The rats of each group were orally administered with cosolvent (5 mL/kg), indomethacin (5 mg/kg), SRLE and Rn-C (80, 160, and 320 mg/kg), respectively. After 20 min, the rats were anesthetized and sterile cotton pellets weighing 20±1mg each were aseptically implanted in both sides of the groin regions by making small subcutaneous incisions. The incisions were sutured. The respective doses were administered once daily for seven consecutive days. On the 8th day, the animals were sacrificed and the pellets were removed carefully and dried in a hot air oven at 60°C until the weight became constant. The mean weight of the granuloma tissue formed around each pellet was recorded. The weight of the pellets removed from the test groups were compared with those removed from the control group. Inhibition of granuloma was evaluated for the anti-inflammatory activity that was expressed as a percentage inhibition of granuloma.

2.9 Statistical analysis

Data obtained are expressed as a mean±S.E.M. and were evaluated by one-way ANOVA followed by Dunnett's test. A significant difference is considered at $p<0.05$.

3. Results

3.1 Effects of SRLE and Rn-C on nociceptive responses in mice

3.1.1 Writhing test

Oral administration of SRLE and Rn-C significantly decreased the number of writhings produced by intraperitoneal administration of 0.6% acetic acid in a dose-dependent manner as shown in Figure 1. Both the SRLE and Rn-C at the dose of 80 mg/kg produced an inhibitory effect with a maximal inhibition of 62.30%, and 70.49%, respectively that
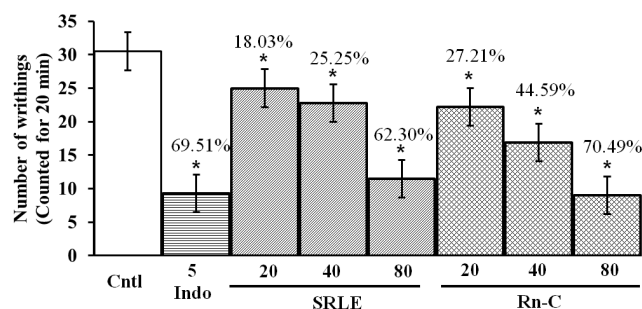
Figure 1. Effect of SRLE, Rn-C and indomethacin on 0.6% acetic acid-induced writhing in mice. Doses are expressed as mg/kg. Data are presented as a mean ± S.E.M. (n=10). The number above each column denotes the inhibition percentage. *p<0.05, significantly different compared with the control (Dunnett's test). Cntl = Control, Indo = Indomethacin.

were comparable to 69.51% inhibition produced by the standard drug indomethacin (5 mg/kg).

3.1.2 Hot plate test

In the hot plate test, none of the test substances at any of the tested doses produced significant effect, but the opioid analgesic morphine sulphate (5 mg/kg) significantly increased the latency of nociceptive response (Table 1).

3.1.3 Formalin test

As shown in Figure 2, oral administration of SRLE and Rn-C at the doses of 40 and 80 mg/kg significantly (p<0.05) decreased the licking time in the late phase but only the higher dose of 80 mg/kg of both the SRLE and Rn-C significantly decreased the licking time in the early phase. The percentage inhibition of SRLE at doses of 40 and 80 mg/kg

in the late phase was 22.81 and 67.57% and that of Rn-C (40 and 80 mg/kg) was 28.62 and 74.37%, respectively. In the early phase, the SRLE and Rn-C at dose of 80 mg/kg produced 37.18 and 46.86% inhibition. The standard drug, indomethacin, exhibited a significant effect only on the late
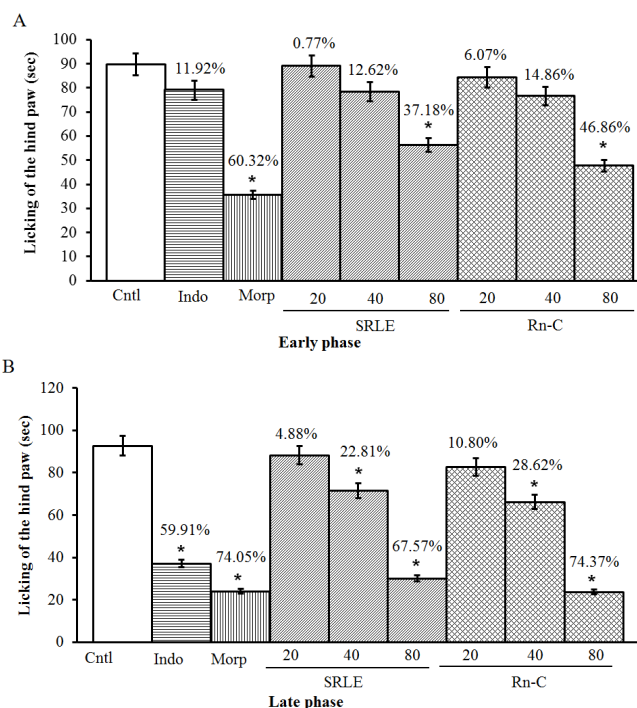

Figure 2. Effect of SRLE, Rn-C, indomethacin and morphine on the early phase (A) and the late phase (B) of formalin-induced paw licking in mice. Doses are expressed as mg/kg. Data are presented as a mean ± S.E.M. (n=10). The number above each column denotes the inhibition percentage. * p<0.05, significantly different compared with the control (Dunnett's test). Cntl = Control, Indo = Indomethacin, Morp = Morphine.

Table 1. Effect of SRLE, Rn-C, indomethacin and morphine on heat-induced pain in mice.

Treatment	Dose (mg/kg)	Latency of nociceptive response (sec)				
		30 min	45 min	60 min	75 min	90 min
Control	-	11.30 ± 0.56	11.67 ± 0.58	11.37 ± 0.44	10.71 ± 0.37	9.60 ± 0.63
Indomethacin	5	12.28 ± 0.82	12.75 ± 0.97	11.87 ± 0.56	12.28 ± 1.02	10.44 ± 0.92
Morphine	5	18.03 ± 0.96*	19.48 ± 1.11*	22.44 ± 1.67*	22.55 ± 1.09*	21.12 ± 1.26*
SRLE	20	10.73 ± 0.59	12.27 ± 0.80	11.76 ± 0.84	12.24 ± 0.82	11.16 ± 0.80
	40	11.93 ± 0.72	12.26 ± 0.76	11.99 ± 0.89	11.20 ± 0.78	12.48 ± 0.90
	80	12.55 ± 0.77	12.34 ± 0.65	12.48 ± 0.71	12.65 ± 0.66	10.81 ± 1.08
Rn-C	20	10.19 ± 0.96	10.52 ± 1.01	12.07 ± 1.35	11.72 ± 0.89	10.78 ± 1.07
	40	10.68 ± 0.62	10.79 ± 0.50	11.38 ± 0.85	10.61 ± 0.93	9.66 ± 1.05
	80	10.73 ± 0.59	11.56 ± 0.71	11.18 ± 0.73	11.47 ± 1.33	9.06 ± 0.74

* p<0.05, significantly different compared with the control (Dunnett's test).
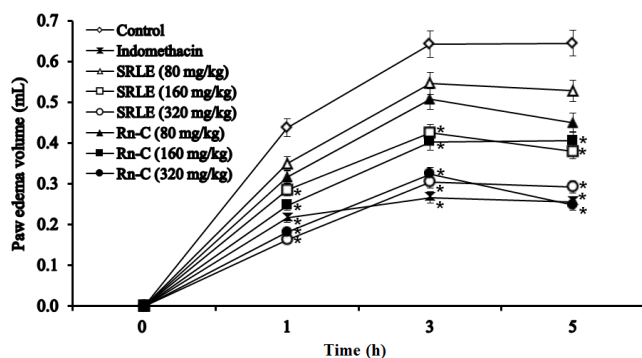Values are presented as a mean ± S.E.M (n=10).

Figure 3. Effect of SRLE, Rn-C and indomethacin on carrageenan-induced paw edema in rats. Data are presented as a mean ± S.E.M. (n=6). * p<0.05, significantly different compared with the control (Dunnett's test).

phase (59.91% inhibition) whereas morphine sulfate decreased the licking time both in the early and the late phases (60.32 and 74.05% inhibition). The result showed that the effects of the SRLE and Rn-C at dose of 80 mg/kg, indomethacin (5 mg/kg) and morphine sulphate (5 mg/kg) were comparable to each other in the late phase of formalin test.

3.2 Effects of SRLE and Rn-C on inflammations in rats

3.2.1 Carrageenan-induced paw edema

Figure 3 shows the results of the carrageenan-induced rat paw edema. The volume of the carrageenan injected paw increased progressively and reached a maximum after three hours in the control group, whereas in the SRLE and Rn-C treated groups, both exhibited a significant inhibition of paw edema induced by carrageenan in a dose dependent manner. SRLE and Rn-C at the dose of 320 mg/kg produced 54.69 and

60.94% inhibition of edema that was comparable to 59.38% inhibition produced by the standard drug indomethacin (5 mg/kg) at 5 hrs of carrageenan injection.

3.2.2 Cotton pellet-induced granuloma

Both, the SRLE and Rn-C at doses of 80, 160, and 320 mg/kg significantly decreased the granuloma weight (Table 2) although their activity was a little lower than that of the standard drug indomethacin (5 mg/kg). SRLE at the dose of 80 mg/kg produced 18.22% inhibition of granuloma, Rn-C at the same dose produced 17.81% and the standard drug indomethacin, produced 24.29% inhibition of granuloma.

4. Discussion

In the present study, both SRLE and Rn-C showed analgesic as well as acute and chronic anti-inflammatory activities in the animal models with a similar efficacy. The acetic acid-induced writhing test is the most common method for screening potential analgesic activity. Intraperitoneal administration of acetic acid produces a nociceptive response consisting of a wave of constriction and elongation that passes caudally along the abdominal wall followed by extension of the hind limbs (Collier *et al.*, 1968). Nociception is produced by the direct activation of peritoneal nociceptors (Julius and Basbaum, 2001) as well as by the release of prostaglandins (Deraedt *et al.*, 1980) which play an important role in inflammatory pain. In the present study, both SRLE and Rn-C at the dose of 80 mg/kg exhibited the suppression of acetic acid-induced writhing with the magnitude comparable to the standard drug indomethacin indicating their antinociceptive activity.

Further investigation of analgesic activity was done by a hot plate test as this can distinguish between the centrally acting analgesic drugs from the peripherally acting

Table 2. Effect of SRLE, Rn-C and indomethacin on cotton pellet-induced granuloma formation in rats.

Treatment	Dose (mg/kg)	Granuloma weight (mg)	Granuloma weight (mg/mg cotton)	GI (%)
Control	-	49.31 ± 0.44	2.47 ± 0.02	-
Indomethacin	5	37.48 ± 1.32*	1.87 ± 0.07*	24.29
SRLE	80	40.95 ± 0.59*	2.05 ± 0.03*	17.00
	160	40.83 ± 0.66*	2.04 ± 0.03*	17.41
	320	40.37 ± 0.50*	2.02 ± 0.02*	18.22
Rn-C	80	41.90 ± 1.05*	2.10 ± 0.05*	14.98
	160	41.43 ± 0.32*	2.07 ± 0.02*	16.19
	320	40.61 ± 0.91*	2.03 ± 0.05*	17.81

*p<0.05 significantly different from the control (ANOVA followed by Dunnett's test).
GI: Granuloma inhibition. Values are a mean±S.E.M. (n=6).

analgesic drugs. Pain induced by the heat can be alleviated by a centrally acting narcotic analgesics (Janicki and Libich, 1979) whereas peripherally acting drugs will be ineffective (Vogel, 2002). In our experiment, all the tested doses of both SRLE and Rn-C showed no significant effect in the hot plate test so this precluded the involvement of opioid receptors in their analgesic activity. Morphine sulfate (opioid analgesic) on the other hand significantly delayed the nociceptive response in the test whereas indomethacin was ineffective in inhibiting the nociceptive response.

The formalin test was performed in order to further understand the mechanism of analgesic effect. The formalin test has two phases, (1) an early phase (0-5 min) of short-lasting nociception (neurogenic pain) is caused by direct stimulation of nociceptors with the chemical irritant, while (2) the late phase (15-30 min) of tonic pain (inflammatory pain) is due to the release of inflammatory mediators and changes in the central processing (Tjølsen *et al.*, 1992). Substance P and bradykinin are responsible for the early phase while inflammatory mediators like histamine, serotonin, prostaglandins and bradykinin are involved in the late phase (Shibata *et al.*, 1989). Centrally acting drugs can inhibit both phases however, peripherally acting drugs like most of the NSAIDs inhibit only the second phase (Hunskaar and Hole, 1987). In this study, as SRLE and Rn-C at their higher tested dose (80 mg/kg) significantly inhibited the nociceptive response in the early phase as well as late phase while the moderate dose (40 mg/kg) inhibited only the late phase. This indicated the involvement of a central effect depending on the dose although both were more effective in the inflammatory pain. On the other hand, indomethacin was effective only in the late phase.

In the present study, carrageenan induced rat paw edema was selected as an acute model of inflammation. It is a suitable animal model for evaluation of an anti-inflammatory activity of a new compound (Vinegar *et al.*, 1969). The response induced by injection of carrageenan involves three phases each is attributed to different chemical mediators. The initial phase of 1.5 hrs is caused by the release of histamine and serotonin followed by the second phase from 1.5 to 2.5 hrs that is mediated by kinins, and the third phase occurs from 2.5 to 6 hrs and is presumably mediated by prostaglandins (Di Rosa, 1972). In this test, both SRLE and Rn-C produced a significant inhibitory effect on edema formation in a dose dependant manner. This effect was significant from the first measurement and was maintained throughout the study period of five hours. This indicated that the anti-inflammatory mechanism of action of the tested compounds may be through the inhibition of early mediators like histamine and serotonin as well as the products of the prostaglandin biosynthesis pathway. These results also corroborated the previous report that Rn-C inhibits the prostaglandin E_2 and the nitric oxide release by inhibiting the gene expression of COX-2 and the inducible nitric oxide synthase (Tewtrakul *et al.*, 2009a).

SRLE and Rn-C also significantly inhibited the granuloma formation in the cotton pellet-induced granuloma test. This is an established model for studying the efficacy of compounds in the chronic inflammation. Proliferation of macrophages, neutrophils and fibroblasts are the basic sources of granuloma formation (Li *et al.*, 2008). Therefore, inhibition of granuloma formation by SRLE and Rn-C indicates the suppression of the proliferative phase of the inflammatory response. The efficacy of SRLE and Rn-C against both the carrageenan induced edema and the cotton pellet induced granuloma indicated that they are active against both the exudative and the proliferative phases of the inflammatory response.

From the findings of our study, it is evident that Rn-C, the major active constituent, is responsible for the analgesic and anti-inflammatory activities of SRLE. However, as the activities of SRLE in all experimental models were comparable to Rn-C, it might be possible that its activity is amplified by the synergistic activity with other rhinacanthins such as rhinacanthin-D and -N (Puttarak *et al.*, 2010) presented in the SRLE, which have also shown anti-inflammatory activities in an *in vitro* experiment (Tewtrakul *et al.*, 2009a). Therefore, SRLE is superior over Rn-C in terms of cost effectiveness and time.

From these investigations, it was concluded that SRLE and Rn-C both have analgesic and anti-inflammatory activities. The analgesic activity of these substances may be mediated through central and peripheral mechanism depending on the dose. Their anti-inflammatory activity can be attributed at least to the inhibition of prostaglandin synthesis. Moreover, the most important finding of these investigations is that the efficacy of both SRLE and Rn-C were similar in analgesic and anti-inflammatory activities. This reinforces SRLE as a potential phytomedicine for pain and inflammatory conditions.

Acknowledgements

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