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In vivo Antinociceptive Activity of Leaf Extract of *Crinum asiaticum* and Phytochemical Analysis of the Bioactive Fractions

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Abstract: *Crinum asiaticum* is a perennial bulbous herb native to tropical South-eastern Asia. The plant is useful in Asian and African traditional medicine as an analgesic agent. This report aimed to investigate the antinociceptive effects of leaf extracts of *Crinum asiaticum* on rats and to highlight the major chemical groups of the bioactive fractions. Powdered leaf of *Crinum asiaticum* was sequentially extracted using petroleum ether, chloroform and methanol. The extracts were investigated for their antinociceptive activity on rats using the carrageenan-induced paw hyperalgesia method. Pain threshold response was measured by applying pressure on the rat's paw using analgesy-meter. Oedema was induced by injection of 0.1 mL 1% (w/v) carrageenan dissolved in normal saline into the footpad of the right hind paw of the rat and allowed to develop. The test samples were administered orally in a dose of 1000, 500 and 250 mg kg^{-1} (10 mL kg^{-1}). Rats administered orally with vehicle were used as negative group. Indomethacin (1 mg kg^{-1}) was used as positive control. After 4 h, the pain threshold of each rat was measured. The best activity was shown by the chloroform extracts at the dose of 250 mg kg^{-1} (n = 4-5 in each group; $p<0.05$), followed by the methanol extracts. No activity was observed with the petroleum ether extract (1000 mg kg^{-1}). Indomethacin was used as positive control (1 mg kg^{-1}). Phytochemical screening of the active fractions indicated the presence of alkaloids, coumarins, glycosides, triterpenes and flavonoids. The results obtained support the reported uses of the plant as traditional analgesic agent.

Key words: Amaryllidaceae, *Crinum asiaticum*, nociception, pain threshold, analgesic agents, traditional medicine

INTRODUCTION

Crinum asiaticum Linn. (Amaryllidaceae) known in Peninsular Malaysia as *bunga tembaga suasa* is a perennial bulbous herb native to tropical South-eastern Asia. It is also found in subtropical and warm temperate regions in Africa, Australia and the Americas. The plant has a wide range of application in Asian (including Malaysia, Indonesia and Philippines) and African traditional medicine. It is useful in the treatment of inflamed joints, sprains, vomiting, haemorrhoids, contusions, fractures, earache and other inflammations (Singh *et al*., 2010). It is also reported to be used as antidote for wounds from poisoned arrows (Samud *et al*., 1999). Roasted bulbs of *Crinum* species are used by the Zulu in South Africa for the treatment of aching joints, rheumatism, varicose veins and backache (Hutchings *et al*., 1996).

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Nociception is the mechanism by which pain information is passed to the central nervous system (Frizelle, 2006). Drugs used to relieve the pain are varied depending on the severity, duration and the nature of the painful stimulus. The currently available analgesic drugs including opioids and non Steroidal Anti-Inflammatory Drugs (NSAIDs) provide in many cases only 50% pain relief to about 30% of patients (Hewitt *et al*., 2009). The complex nature of the chain of central and peripheral mechanisms that underline pain sensation and the fact that no simple test is good enough to predict the efficiency of a test agent in humans makes the use of various experimental models imperative when screening crude extract or compounds for pharmacological activity (Okunrobol *et al*., 2009). Evidences also showed that NSAIDs frequently cause gastrointestinal disorders while opiates lead to tolerance, physical dependency and addiction (Dogruer *et al*., 1998). The search therefore for other alternatives is urgently needed.

The genus *Crinum* is a true representative of Amaryllidaceae as it exhibits all the major chemical traits of the family such as alkaloids, flavonoids, coumarins and terpenoids (Fennell and van Staden, 2001). These

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chemical groups are known to be responsible for many biological activities including antinociceptive effects (Chan *et al*., 1998; Leal *et al*., 2000).

Previous investigation carried out in our laboratory confirmed the presence of active anti-inflammatory agents in the methanol extract of *Crinum asiaticum* (Samud *et al*., 1999). Some researchers reported that plant extracts with anti-inflammatory effects were also found to be antinociceptive. This may indicate a possible correlation between the two biological activities (Yim *et al*., 2009). This report aimed to investigate the antinociceptive effects of leaf extracts of *Crinum asiaticum* on rats and to highlight the major chemical groups of the bioactive fractions.

MATERIALS AND METHODS

Collection of plant materials and preparation: Samples of fresh green leaves of *C. asiaticum* were collected from Penang Island, Malaysia in June 2008. A voucher specimen has been deposited at the herbarium of the School of Biological Sciences, Universiti Sains Malaysia (Specimen No. 10106). The leaves were washed, dried in an oven at 40°C, grounded and powdered. The coarse powder (3.5 kg) was sequentially extracted by soxhlet using petroleum ether, chloform and methanol respectively. The extract was filtered, dried under reduced pressure, further freeze dried at -40°C and kept in glass bottles prior to pharmacological screening. Yield percentages were 3.5, 1.3 and 13.4% for petroleum ether, chloroform and methanol extracts, respectively.

Experimental animals: Experiments were performed on male albino rats (Sprague Dawley) with a body weight of 150-200 g. The animals were bred and housed in the animal house of the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the Universiti Sains Malaysia, Health campus guideline (protocol number: PPSG/07 (A) 044 dated 18 th May 2003).

Evaluation of antinociceptive activity: The antinociceptive activity of the extracts was determined following the method described by El-Mahdi *et al*. (1990) with slight modifications. Animals were fasted one night before starting the experiment. Pain threshold response of the right hind paw was measured by applying pressure on the rat's paw using analgesy-meter. Oedema was induced by injection of 0.1 mL 1% (w/v) carrageenan dissolved in normal saline into the footpad of the right hind paw of the rat and allowed to develop for 2 h. And then (after 2 h) the test samples were administered orally in a dose of 1000, 500 and 250 mg kg^{-1} (10 mL kg^{-1}). Rats administered orally with vehicle were used as negative group. Indomethacin (1 mg kg^{-1}) was used as positive control. After 4 h the pain threshold of each rat was measured. The pressure needed to evoke a reaction to pain which was either in the form of withdrawal of the squeezed foot or sharp squeak, was considered as the end point.

Fractionation and phytochemical analysis of the bioactive factions: The chloroform extract (15 g) was further fractionated based on activity guided process using a dry column chromatography. Steps of the fractionation were shown in Fig. 3. Solvent used were: petroleum ether: chloroform (1:9 and 1:1), 100% chloroform and methanol: chloroform (1:1). Fractions with the same Rf values were combined and tested for antinociceptive effects. The most active fractions were again combined and further fractionated using solvent chloroform: methanol (7:3). Twenty eight fractions were obtained. Aliquots of these fractions were spotted on TLC plates and developed using solvent chloroform: methanol (7:3). Detection of the chemical groups was carried out using the method of Wagner and Bladt (1996) for the detection of alkaloids, coumarins, flavonoids glycosides and triterpenes.

Statistical analysis: Data are expressed as Mean±SEM. Statistical analysis was performed using one way ANOVA followed by Dunnett multiple comparison test. The p-value less than 0.05 was considered as significant.

RESULTS

The present study indicated that chloroform and methanol leaf extracts of *C. asiaticum* had analgesic activity when tested at doses ranged between 250 and 1000 mg kg^{-1} while petroleum ether showed no significant effects at the highest dose used (1000 mg kg^{-1}). The concentration of the chloroform extract administered was reduced (from 1000 to 250 mg kg^{-1}) because its activity at high dose of 1000 mg kg^{-1} was associated with side effect of hyperactivity which led to the death of some of the test animals (data not shown). Results showing the antinociceptive effects of the test extracts at the dose of 250 mg kg^{-1} are presented in Fig. 1.

The chloroform extract was further fractionated using dry flash column chromatography. Five fractions f1-f5 were obtained. Fraction f4 and f5 showed strong antinociceptive effects when compared to the control ($p<0.01$ and $p<0.05$, respectively) but no activity was observed with fraction f1, f2 and f3 (Fig. 2).

Fraction f4-f5 were combined and subjected to phytochemical analysis. Results obtained indicated the presence of alkaloids, heterocyclic and nitrogen compounds, coumarins, glycosides, triterpenes and flavonoids (Fig. 3). Alkaloids, heterocyclic and nitrogen

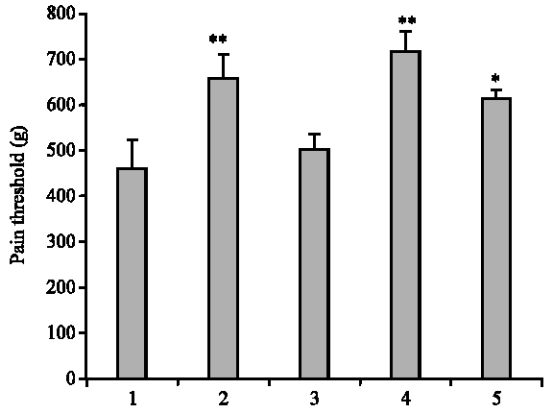

Fig. 1: Antinociceptive effect of *C. asiaticum* extracts (250 mg kg^{-1}) on rats (n = 4-5 in each group). Results are expressed as means of pain threshold (g)±SEM. *p<0.05 and **p<0.01 indicate significant differences from the control. 1: Negative control; 2: Indomethacin (positive control); 3: Petroleum ether extract; 4: Chloroform extract and 5: Methanol extract

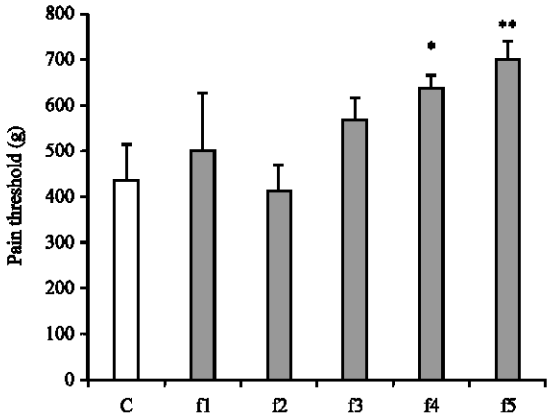

Fig. 2: Antinociceptive effect of fractions f1, f2, f3, f4 and f5 obtained from the chloroform extract of *C. asiaticum* on rats (n = 7-8 in each group). Dose used was 250 mg kg^{-1}. Results are expressed as means of pain threshold (g)±SEM. *p<0.05 and **p<0.01 indicate significant differences from the control. C: Control

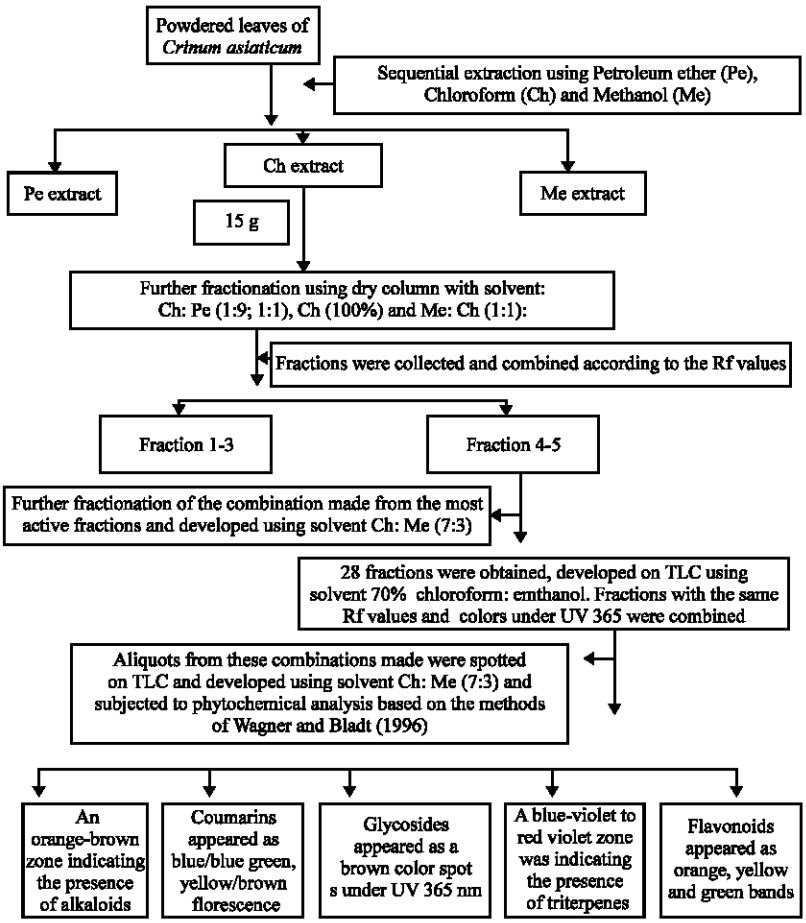

Fig. 3: Extraction of the plant material and phytochemical analysis of the chloroform leaf extract of *Crinum asiaticum*

compounds were indicated as orange-brown zones after spraying with Dragendorff's reagent whereas coumarins (sprayed with 10 mL KOH solution) appeared yellow/brown/blue/blue-green fluorescence. Glycosides were observed as brown spots after spraying with 15 mL^{-1} of antimony trichloride reagent and heated for 6 min at 100°C. Triterpenes were appeared as blue-violet to red violet zones when sprayed with anisaldehyde-sulphuric acid reagent and heated for 5 min at 100°C. Flavonoids were appeared as orange, yellow and green bands. This was obtained using two reagents: 10 mL of methanolic diphenylboric acid-β-ethyl amino ester followed by 8 mL of ethanolic polyethylene glycol. The plate was then examined under UV 365 nm.

DISCUSSION

Pain is a subjective symptom that is affected by psychological factors. A wide variety of plants-derived chemicals such as alkaloid, triterpenes and flavonoids were used to alleviate or kill pain (Huo *et al*., 2007; Okunrobo *et al*., 2009). Ratnasooriya *et al*. (2005) reported that leaf extracts of *Crinum bulbispermum* showed remarkable antinociceptive activity when evaluated in the formalin test. These findings are in agreement with the obtained results in the present study suggesting possible occurrence of similar bioactive agents in the two investigated *Crinum* species.

Phytochemical screening of the active chloroform fraction indicated the presence of alkaloids, coumarins, flavonoid, triterpenes, glycosides and heterocyclic nitrogen compounds (Fig. 3). A number of naturally occurring alkaloids was reported presence in plants of the family Amaryllidaceae including *Crinum* species. Some of these alkaloids such as pyrrolo[de]phenanthridine (e.g., galanthine), lycorenine, pretazettine, dibenzofuran (e.g., galanthamine) and 5,10 b-ethanophenanthridine group are known to possess antinociceptive effects *in vivo* (Ghosal *et al*., 1985; Calixto *et al*., 2000; Fennel and van Staden, 2001).

Terpenoid and steroid compounds are widely distributed in the plant and exhibit distinctive pharmacological properties. Naturally occurring terpenoids were known to possess anti-inflammatory and antinociceptive properties, inhibit platelet aggregation and interfere at the intracellular level with several steps of signal transduction mechanisms (Calixto *et al*., 2000).

Antinociceptive effects of flavonoids were also reported (Meotti *et al*., 2006). Preliminary studies have demonstrated that various flavonoids, including rutin, quercetin, luteolin and gossypin possessed significant antinociception *in vivo* (Calixto *et al*., 2000). During a clinical study of analgesic effects, a plant extract contains flavonglycosids had improved the nerve function and pain associated with autonomic neurophathy in ten patients (Koltringer *et al*., 1989). Coumarins and amino acids were also proved to be involved in pain transmission (Grubb, 1998; Millan, 1999). All these chemical groups were confirmed presence in the chloroform leaf extract of *Crinum asiaticum* investigated in this study (Fig. 3). The antinociceptive activity observed by the chloroform extract and the fractions in this study may be due to the effects of these chemical agents.

CONCLUSION

Our findings in this study support the traditional uses of *Crinum asiaticum* as traditional crude analgesic agent.

The relatively higher activity observed with the chloroform extract when compared to the methanol extract suggests the presence of promising non-polar compound(s) that need to be further investigated.

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