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Effects of opioid and non-opioid antagonists, pH and enzymes on *Corchorus olitorius* antinociception in mice

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SUMMARY

The present study was carried out to determine the involvement of opioid and non-opioid receptor and the effect of pH and enzymes on the recently reported antinociceptive activity of aqueous extract of *Corchorus olitorius* (AECO) leaves using the abdominal constriction test. The extract was prepared by soaking the dried powdered leaves of *Corchorus (C.) olitorius* in distilled water overnight, and the supernatant obtained was considered as a stock solution with 100% concentration/strength. The extract, administered subcutaneously in the concentrations/strength of 10, 50 and 100%, was found to show a significant concentration-independent antinociception. The 50% concentration AECO were further used to study on the above mentioned parameters. The extract exhibited: significant ($P < 0.05$) decreased in activity when pre-treated (*s.c.*) against 10 mg/kg naloxonazine, bicuculine (10 mg/kg), phenoxybenzamine (10 mg/kg), 10 mg/kg pindolol, and 5 mg/kg mecamylamine, but not 10 mg/kg naltrindole, 10 mg/kg atropine, respectively; significant ($P < 0.05$) decreased in activity after pre-treatment against 10% a-amylase, but not 1% protease or 10% lipase and; significant ($P < 0.05$) decreased in activity after exposure to alkaline condition (pH between 9 and 13) while maintaining the activity at acidic condition, respectively. The *C. olitorius* leaves antinociception, which involved, at least in part, activation of μ-opioid, α- and β-adrenergic, and nicotinic receptors, was found to decrease under alkaline condition and in the presence of α-amylase.

Key words: *Corchorus olitorius*; Antinociception; Abdominal constriction test; Opioid; Non-opioid; Enzymes; pH

INTRODUCTION

Corchorus (C.) olitorius L., known to the Malaysian's Sabahan people as '*Senaung betina*', is an annual herb belonging to the family Tiliaceae. Its leaves and roots are used as herbal medicine and eaten as vegetable by local people in various part of the world, including Malaysia (Zegichi *et al.*, 2003). Traditionally its leaves are used as demulcent, diuretic, febrifuge and tonic, and also in the treatment of fever, pain and tumours, to name a few (Abu-Hadid *et al.*, 1994; Zegichi *et al.*, 2003). According to Kirtikar and Basu (1975), the cold infusion is said to restore appetite and strength.

Earlier study has demonstrated that the seeds of *C. olitorius* contain oil (Watt and Breyer-Brandwijk, 1962), which was later proven by Sharaf *et al.* (1979)

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to possess estrogenic activity. In addition, Negm *et al.* (1980) have reported on the presence of high content of hydrogen cyanide and several cardiac glycosides, for examples Corchoroside A and Corchoroside B, in the said seeds. On the other hand, Yen *et al.* (2001) have demonstrated that *C. olitorius* extracts possessed moderate and weaker inhibitory effect towards the mutagenicity of 2-amino-3-methyl-imidazo[4,5-f]quinoline or benzo [a]pyrene in S. typhimurium TA98 and TA100, respectively. Recently, the methanolic extract of *C. olitorius* seed was also reported to possess significant anticonvulsive property, which is suggested to be due to its ability to alter the level of catecholamines and brain amino acids in mice (Gupta *et al.*, 2003). In addition, Innami *et al.* (2005) reported on the *C. olitorius* leaves ability to suppress elevation of postprandial blood glucose levels in rats and humans and that this activity is attributed to its viscous soluble dietary fiber ability to delay absorption of glucose from the intestinal membrane in the upper digestive tract. Furthermore, Ohtani *et al.* (1995) have isolated an acidic polysaccharide from the water-soluble mucilage extracted from dried leaves of *C. olitorius*, which contain high amount of uronic acid, and consisted of rhamnose, glucose, galacturonic acid, glucuronic acid as well as a methyl group. The isolated polysaccharide was also found to show proliferative activity toward the murine splenocyte.

Our recent study using the abdominal constriction test and hot plate test has demonstrated that the aqueous extract of *C. olitorius* leaves possessed significant peripheral and central antinociceptive activities (Zakaria *et al.*, 2005). The said activities were also suggested to be mediated, at least in part, via the opioid receptor based on our observation on the ability of naloxone, a non-specific opioid receptor antagonist, to block/reverse *C. olitorius* antinociceptive activity observed in both types of tests (Zakaria *et al.*, 2005). Furthermore, the activity was also found to resist the effect of temperature up to the boiling point of 100°C.

Based on our literature's search, we were unable to find any scientific reports reporting on *C. olitorius* antinociceptive and other pharmacological activities, other than those mentioned above. This seems to indicate lack of exploration on the pharmacological activities of this plant despite the traditional claimed described earlier. Based on that fact and on our recent findings described earlier, we decided to take this opportunity to study, at this moment, on its potential peripheral antinociceptive activity and factors that might influence the activity. Our aims are to elucidate the involvement of opioid and non-opioid receptors in *C. olitorius* antinociception and also to determine the effects of pH and enzymes on the antinociceptive activity of *C. olitorius*.

MATERIALS AND METHODS

Plant material

The leaves of *C. olitorius* were collected from the area of Shah Alam (Selangor, Malaysia) between January and February 2004 and identified by Mr. Shamsul Khamis from Institute of Bioscience, Universiti Putra Malaysia, Malaysia. A voucher specimen (SK 963/04) was deposited at the Herbarium of Institute of Bioscience, Universiti Putra Malaysia, Malaysia.

Preparation of *C. olitorius* leaves aqueous extract

The leaves of *C. olitorius* were washed and rinsed with water to remove dirt and unwanted particles and then oven-dried at a temperature of 50°C. The dried leaves were then ground into small particles, weighed (200 g) and added with distilled water (dH_2O) in the ratio of 1 : 25 (w/v). This ratio was found to be the best ratio for full soaking of the leaves with dH_2O. This mixture was then shaken for 24 h under room temperature (27 ± 2°C) and the supernatant was collected and filtered using Whatman No.1 filter paper while the remaining plant residue was kept in an oven for future uses, which include further extraction using organic solvents such as methanol and chloroform and

determination of the respective extracts anti-inflammatory, anti-pyretic, antibacterial activities etc. The supernatant obtained, labeled as aqueous extract of *C. olitorius* (AECO) and regarded as the stock solution with 100% concentration/strength, was then subjected to a freeze-drying process to yield a dried semi-solid crude extract weighing approximately 27.5 g. The stock solution was then diluted with dH_2O to the concentrations/strengths of 10% and 50%, and used together to build the antinociceptive profile for AECO. Further studies will be carried out using the lowest dose that showed significant antinociceptive activity.

Preparation of drugs

Acetylsalicylic acid (Bayer, Singapore) and morphine (Sigma, Germany), prepared in their respective dose of 100 mg/kg (Sulaiman *et al.*, 2004) and 0.8 mg/kg (Mat Jais *et al.*, 1997) by dissolving them in dH_2O, were used for the purpose of comparison (positive control group). Except for 10 mg/kg bicuculline (Sigma), a gamma aminobutyric acid (GABA) receptor antagonist, which is prepared by dissolving it in slightly heated dH_2O, the respective antagonists of opioid (10 mg/kg naloxonazine or naltrindole; Sigma), α- and β-adrenergic (10 mg/kg phenoxybenzamine and pindolol; Sigma), muscarinic (5 mg/kg atropine; Sigma), and nicotinic (5 mg/kg mecamylamine; Sigma) receptors, were prepared to the required doses by dissolving them in dH_2O. The different doses of the antagonists used in this study were selected according to previous works (Bentley and Starr, 1986; Verma and Kulkarni, 1991; Ono and Satoh 1992; Baratti *et al.*, 1993; Clojnacka-Wojcik *et al.*, 1994; Santos *et al.*, 1995; Pieretti *et al.*, 1999). The enzymes, α-amylase (Sigma) and lipase (Sigma), in the concentration of 10%, while protease (Sigma), in the concentration of 1%, were prepared according to our report in Zakaria *et al.* (2004).

Experimental animals

Male ICR mice (25 - 30 g; 5 - 7 weeks old), used in this study, were obtained from the Veterinary Animal Unit (Universiti Putra Malaysia (UPM), Malaysia) and kept under room temperature (27 ± 2°C; 70 - 80% humidity; 12 h light/darkness cycle) in the Animal Holding Unit (UPM) for at least 48 h before use. Food and water were supplied *ad libitum* up to the beginning of the experiments. At all times the mice were handled in accordance with current UPM guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). All experiments were conducted between 09:30 and 12:30 h (Mat Jais *et al.*, 1997) to minimize the effects of environmental changes.

Antinociceptive studies involving AECO

In the first study, the AECO, in the concentration/strength of 10%, 50%, and 100%, was administered subcutaneously (*s.c.*) in mice 30 min prior to subjection to the antinociceptive assay. The rest of the studies were carried out using the lowest concentration of AECO that showed significant antinociceptive activity. In the second study, the mice were pre-treated (*s.c.*) for 10 min against different types of receptor antagonists (naloxonazine, b-funaltrexamine, naltrindole, phenoxybenzamine, pindolol, bicuculline, atropine, or mecamylamine) followed by the respective extract administration (*s.c.*). In the third study, the respective extract was pre-treated against an acidic (pH 3 or 5) or alkaline (pH 9, 11 or 13) conditions for 2 h and then neutralized before administered (*s.c.*) into mice. In the fourth study, the respective extract was pre-treated against various enzymes (a-amylase, protease, or lipase) for 2 h before administered (*s.c.*) into mice. 30 min after the respective extract administration, the mice were subjected to the antinociceptive assay. All solutions and drugs were administered in the volume of 10 ml/kg of mice.

ANTINOCICEPTIVE ASSAY

Abdominal constriction test

The abdominal constriction test (Dambisya and

Lee, 1995) was used to establish the antinociceptive profile, and to determine the involvement of opioid and non-opioid receptors as well as the effects of pH and enzymes on the antinociceptive activity of AECO. The animals (n = 10) were injected (*s.c.*) with dH_2O, acetylsalicylic acid, morphine, or AECO (10%, 50% or 100%) followed by intraperitoneal (*i.p.*) administration of 0.6% acetic acid (J.T. Baker, U.S.A.) 30 min later. The mice were placed individually into glass beakers and 5 min were allowed to elapse. The number of abdominal constrictions produced in these animals was counted for 25 min, commencing 5 min after the acetic acid administration. The abdominal constriction resulting from the injection of acetic acid consisting of a contraction of the abdominal together with a stretching of at least one of the hind limbs (Correa *et al.*, 1996). Antinociceptive activity was indicated by the reduction in the mean of the number of abdominal constrictions in the test groups compared to the control group.

Statistical analysis

The results are presented as Mean ± Standard Error of Mean (S.E.M.). The one-way Analysis of Variance (ANOVA) test with Duncan post-test was used to analyse and compare the data, with $P < 0.05$ as the limit of significance.

RESULTS

The antinociceptive profile of AECO

The antinociceptive profile of AECO, at concentrations of 10, 50, and 100%, was compared with acetylsalicylic acid (100 mg/kg) and morphine (0.8 mg/kg) as shown in Fig. 1. From the data obtained, AECO exhibited significant ($P < 0.05$) antinociceptive activity in a concentration independent manner, with only the 50% concentration extract show significant activity. Increasing the concentration of the extract to 100% was found to cause a total loss of antinociception. Interestingly, the 50% concentration extract produced an activity that is equipotent to that of 0.8 mg/kg morphine.

The involvement of opioid and non-opioid receptor in AECO antinociceptive activity

The effect of various types of receptor antagonists on the extract (50% concentration) antinociceptive activity was shown in Fig. 2. Pre-treatment with naloxonazine, bicuculine, phenoxybenzamine, pindolol, or mecamylamine was found to significantly

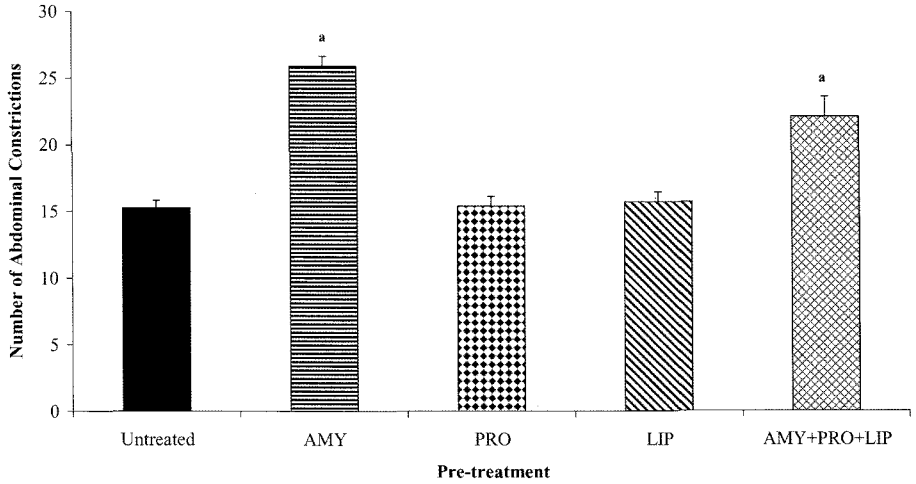

Fig. 1. The peripheral antinociceptive profile of *Corchorus olitorius* aqueous extract in mice assessed by abdominal constriction test. [a,b]Data with different superscript differ significantly ($P < 0.05$) when compared against the control group (dH_2O-treated).

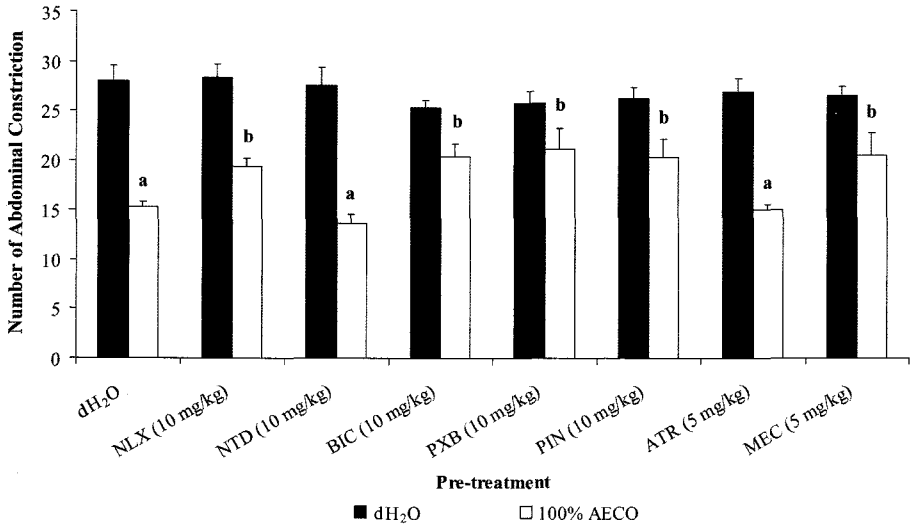

Fig. 2. Effect of various receptor antagonists on *Corchorus olitorius* aqueous extract peripheral antinociceptive activity. [a,b]Data with different superscript differ significantly ($P < 0.05$) when compared against their respective control group (dH_2O-treated).

($P < 0.05$) reversed, but not blocked, the AECO observed activity. This seems to indicate the involvement of m-opioid, GABA, α- and β-adrenergic, and nicotinic receptors in the AECO antinociception. The failure of naltrindole and atropine to block/revers the antinociceptive activity of AECO seems to suggest that the δ-opioid and muscarinic receptors did not involved in the AECO antinociception.

Effect of pH on AECO antinociceptive activity

The effect of pH (pH 3, 5, 9, 11, or 13) on the respective AECO (50% concentration; pH 6.5) antinociceptive activity was shown in Fig. 3. The activity was found to decrease significantly ($P < 0.05$) after exposure of the extract to alkaline condition (between pH 9 to 13) but maintained under acidic condition.

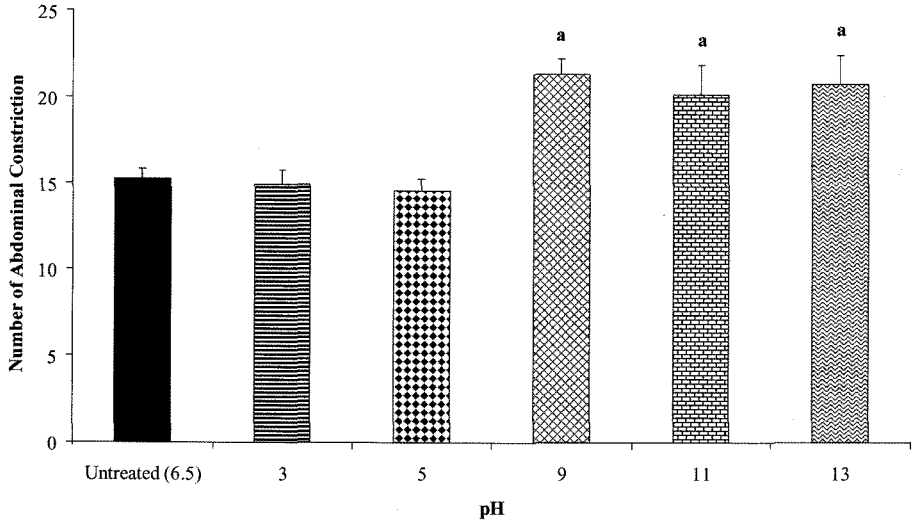

Fig. 3. Effect of pH on the *Corchorus olitorius* aqueous extract peripheral antinociceptive activity. [a]Data with superscript differ significantly ($P < 0.05$) when compared against the unadjusted group (normal extract). Untreated group = Control group.

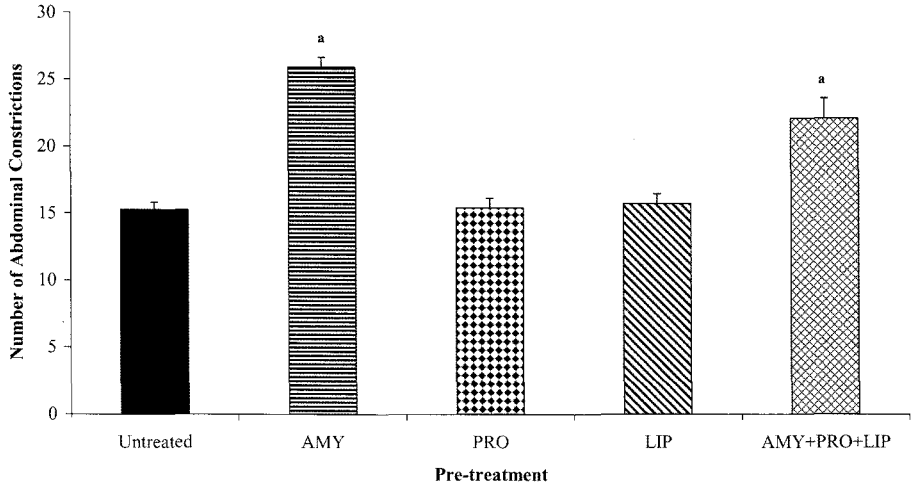

Fig. 4. Effect of enzymes (α-amylase, protease, lipase and their combination (A + P + L)) on the *Corchorus olitorius* peripheral antinociceptive activity. [a]Data with superscript differ significantly ($P < 0.05$) when compared against the untreated group (normal extract). Untreated group = Control group.

Effect of various enzymes on AECO antinociceptive activity

The effects of various enzymes, namely α-amylase, protease, lipase or their combination (A + P + L), on the respective AECO (50% concentration) antinociceptive activity was illustrated in Fig. 4. The antinociceptive activity of AECO decreased significantly after pre-treatment with α-amylase or (A + P + L) indicating the influence of α-amylase in the AECO observed activity.

DISCUSSION

In the present study, the antinociceptive activity of AECO were investigated and the involvements of opioid and non-opioid receptors in the antinociceptive activity as well as the effects of pH and various enzymes on the observed activity was elucidated using the abdominal constriction test. The abdominal constriction test can detect analgesia of compounds/dose levels that may be inactive in other tests, like hot plate test or tail flick test, due to its high sensitivity (Katzung, 1995). The analgesic activity observed with this test is though to involve, at least in part, the activation of local peritoneal receptors, such as several different types of opioid receptors, found at the surface of the cells lining the peritoneal cavity (Bentley *et al.*, 1981) and, thus, characterized as peripheral analgesia. In contrast, the hot plate test, which is categorized as central mechanism, is thought to involve the spinal reflex. Drugs acting centrally, such as morphine, produced antinociception in both types of assays (Sulaiman *et al.*, 2004) while drugs acting peripherally, such as aspirin and indomethacin, produced the antinociceptive effect only in the abdominal constriction test (Seigmund *et al.*, 1957; Hendershot and Forsaith, 1959). Our previous study has demonstrated that the AECO possessed both the peripheral and central activities (Zakaria *et al.*, 2005). In addition, the peripheral, but not central, antinociceptive activity was observed in a concentration-independent manner (Zakaria *et al.*, 2005). Our recent study has also demonstrated the same pattern of activity and, thus, confirming the previous finding. The previous and recent findings are in line with traditional claim by peoples in various places (Zegichi *et al.*, 2003) that the leaves are used for treatment of various ailments such as to provide relief from gastric ulcers and to reduce swelling of the prostate gland (Kirtikar and

Basu, 1975). Although this study was carried out using the abdominal constriction test, which is generally accepted as the assay for elucidating peripheral antinociception(Mat Jais *et al.*, 1997) the fact that AECO also exhibited central antinociception when administered *S.C.* (Zakaria *et al.*, 2005) should also be considered as part of the effects observed.

Interestingly, the highest concentration of AECO used (100%) was found to produce total loss of antinociceptive activity. Speculatively, the lack of dose dependent relationship is most probably accounted for the fact that only a small percentage of AECO is needed to interrupt the acetic acid-induced synthesis of prostaglandins. This kind of dose-response relationship is in line with report by Tripathi (1994) that the present of high concentrations of its active principle can sometimes lead to reduction of drugs effectiveness. This type of relationship also indicates that the concentrations used in this test have to be within the therapeutic window of AECO in which certain drugs exert their maximum curative effect Tripathi (1994). According to Katzung (1995), the presence of high concentration of the respective extract bioactive compound can also result in deactivation of the antinociceptive-inducing receptors within the peritoneal cavity, which lead to the lost in antinociceptive activity of the AECO at the highest concentration used.

The present study has also demonstrated the involvement of at least five types of receptors, namely m-opioid, GABA, α- and β-adrenergic, and nicotinic receptors, in the peripheral antinociceptive activity of AECO. This finding was in line with our previous report on the involvement of opioid receptor in the extract antinociceptive activity (Zakaria *et al.*, 2005) while the involvement of opioid and adrenergic receptors were expected based on reports made by (Bentley *et al.*, 1981, 1983) on the presence of both types of receptors in the peritoneal cavity. In addition, the involvement of GABA and muscarinic receptors in antinociception have also been reported by Costa *et al.* (1982) and Barocelli *et al.* (2001). The opioid receptor is known to mediate morphine's beneficial as well as adverse side effects (Katzung, 1995). Our finding in the present and previous studies on the involvement of opioid receptor in the peripheral as well as central antinociceptive activities of the AECO have proven that *C. olitorius* could be a promising substitute for morphine and other synthetic opioids with adverse side effects in the near future. This is based on our finding that the AECO, at the concentrations or dosages used, did not produce any toxic or lethal effects.

This finding is expected since we are using the crude extract, which is generally known to contain various types of bioactive compounds such as flavonoids, tannins, essential oil and steroids that are found abundantly in the leaves of plants (Kaneda *et al.*, 1991; Peres *et al.*, 1998; Calixto *et al.*, 2000; Su *et al.*, 2003). Furthermore, Calixto *et al.* (2000) and Peres *et al.* (1998) have also reported on the ability of these compounds to exhibit antinociceptive activity. Although isolation and purification of different fraction from the aqueous extract of *C. oitorius* as well as assaying for antinociceptive activity of each fraction were not the objective of this study, nevertheless, based on the results of the study and supported by previous reports, it is plausible to suggest that the antinociceptive activity of the extract may be attributed to inhibition of the above mentioned receptors or prostaglandin release, or blocking of the enzyme, cyclo-oxygenase, that is responsible for prostaglandin production, and similar mediators involved in nociceptive process (Spector, 1962; di Rosa *et al.*, 1971). Yoshikawa *et al.* (1997) have also reported on the isolation of various types of compounds, such as three new ionone glucosides named corchoionosides A, B, and C, as well as seven known compounds namely an ionone glucoside (6S, 9R)-roseoside, a monoterpene glucoside betulalbuside A, two flavonol glucosides astragalin and isoquercitrin, two coumarin glucosides scopolin and cichoriine, and chlorogenic acid. Of these compounds, Corchoionosides A and B and

(6S, 9R)- roseoside were found to inhibit the histamine release from rat peritoneal exudate cells induced by antigen-antibody reaction. The ability to inhibit histamine release reflex the compounds, as well as the AECO, ability to block inflammation and nociception since histamine is known as a mediators of inflammation and nociception processes. This finding is also in line with our recent report on the extract ability to exhibit anti-inflammatory and antipyretic effects (Zakaria *et al.*, 2005b).

The ability of AECO to withstand the effects of acidic, but not alkaline, condition indicated its stability under the former condition. This finding was against report made by Dambisya *et al.* (1999) on the ability of mucus extract of freshwater fish, *Channa striatus*, to withstand the effects of both conditions. Furthermore, the loss of activity of the AECO seen under alkaline condition was against our recent finding on the *Muntingia calabura* extract activity. The *M. calabura* antinociceptive activity was found to improve significantly under alkaline condition and maintained under acidic condition (*unpublished data*). Furthermore, our finding is also in line with previous report made by Ohtani *et al.* (1995) on the presence of acidic polysaccharide that was rich in uronic acid (65%), and consisted of rhamnose, glucose, galacturonic acid, and glucuronic acid and exhibited proliferative activity toward the murine splenocyte.

Further study carried out using various types of common enzymes have demonstrated that the AECO was unstable in the presence of α-amylase as can be seen from the total loss of activity of the AECO after pre-treatment against the enzyme or its combination (A + P + L), but not protease or lipase. This finding seems to indicate the presence of carbohydrate, such as polysaccharide, as part of the major constituents in the bioactive compounds that are responsible for the antinociceptive activity observed in the AECO. It is generally known that the α-amylase is responsible for denaturation or breakdown of carbohydrate, including polysaccharide (Bowman and Rand, 1980; Ginsburg and Robbins, 1981; Zakaria *et al.*, 2004). The suggestion on the presence of carbohydrate, in the form of polysaccharide, is plausible since *C. olitorius* leaves is known to release sticky and mucilaginous mucus after soaking in water. Furthermore, Ohtani *et al.* (1995) have also reported on the presence of acidic polysaccharide as mentioned earlier.

The AECO was prepared in concentration/ strength, namely 10%, 50% and 100%, which was later converted to dosage (mg/kg), based on our previous studies (Mat Jais *et al.*, 1997; Zakaria *et al.*, 2005c). Since this is a preliminary study carried out to develop the antinociceptive profiles of the extract, the AECO was used directly by diluting the stock solution in dH_2O to a required concentrations rather than using it in doses form.

In conclusion, *C. olitorius* possesses a peripherally mediated antinociceptive activity, which is thought to involve, at least in part, activation of μ-opioid, GABA, α- and β-adrenergic, and nicotinic receptors. The bioactive compounds responsible for the antinociceptive activity are unstable under alkaline condition and in the presence of α-amylase.

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