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Effects of Various Receptor Antagonists, pH and Enzymes on *Muntingia calabura* Antinociception in Mice

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Abstract: The present study was carried out to determine the involvement of various receptor antagonists in and the effect of pH and enzymes on the recently reported antinociceptive activity of aqueous extract of *Muntingia calabura* leaves (MCAE) using the abdominal constriction test. The extract was prepared by soaking the dried powdered leaves of *M. calabura* in distilled water (dH_2O) overnight and the supernatant obtained was considered as a stock solution with 100% concentration/strength. The MCAE, administered s.c. at the concentrations of 5, 50 and 100%, were found to show significant antinociceptive activity in a concentration-dependent manner. The 50% concentration MCAE was 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^{-1} naloxonazine, 10 mg kg^{-1} pindolol and 5 mg kg^{-1} atropine, but not 10 mg kg^{-1} β-funaltreaxamine, 10 mg kg^{-1} naltrindole, 10 mg kg^{-1} phenoxybenzamine, 10 mg kg^{-1} bicuculine or 5 mg kg^{-1} mecamylamine, respectively. The extract exhibited significant ($p<0.05$) increased in activity after pre-treatment at alkaline pH (pH 9 and 11) while maintaining the activity at the extreme acidic and alkaline conditions (pH 2 and pH 13), respectively. The extract activity was not changed after pre-treatment against α-amylase, protease, lipase or their combination, when compared to the dH_2O-pre-treated group, respectively. Based on the results, we conclude that the *M. calabura* leaves peripheral antinociception involved, at least in part, activation of μ-opioid, β-adrenergic and muscarinic receptors and resist the effect of extreme acidic and alkaline conditions as well as various enzymes.

Key words: *Muntingia calabura*, antinociception, abdominal constriction test, opioid, non-opioid, pH, enzymes

INTRODUCTION

Muntingia calabura L., locally known to the Malays as '*Kerukup siam*', belongs to the family Elaeocarpaceae (Morton, 1987). Although, it is not native to the Southeast Asia region, it is wildly cultivated and has become one of the most common roadside trees in the Southeast Asia region, including Malaysia (Jensen, 1999). The fruits are widely eaten out-of-hand by children and adults and are often cooked in tarts or made into jam by the Mexican while the leaf infusion is drunk as a tea-like beverage (Morton, 1987).

M. calabura flowers are traditionally belief to possess antiseptic properties and its infusion is valued as an antispasmodic. It is taken to relieve headache and the first symptoms of a cold (Jensen, 1999; Verheij *et al*., 1992). The leaves of *M. calabura* are used in Peru folklore medicine, either by boiling or steeping in water, to treat gastric ulcers or to reduce swelling of the prostate gland, respectively (Morton, 1987). In addition, the strips of its bark are boiled and used as a wash to reduce swelling in the lower extremities.

Scientifically, the plant has been reported to possess anti-cancer compounds. For example, Kaneda *et al.* (1991) have reported on the isolation of 12 new flavonoids from the roots of *M. calabura* with most of them demonstrated cytotoxic activity when tested against cultured P-388 cells. The authors also claimed that the flavans are more active than the flavones in the above-mentioned test. Furthermore, certain of these structurally related flavonoids exhibited somewhat selective activities when evaluated with a number of human cancer cell lines. Su *et al.* (2003) reported on the isolation of a flavanone with an unsubstituted B-ring, (2R,3R)-7-methoxy-3,5,8-trihydroxyflavanone, as well as 24 known compounds, which were mainly flavanones and flavones, during the

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activity-guided fractionation of an EtOAc-soluble extract of the leaves of *M. calabura* using an *in vitro* quinone reductase induction assay with cultured Hepa 1c1c7 (mouse hepatoma) cells. According to the authors, of the isolates obtained, in addition to (2R,3R)-7-methoxy-3,5,8-trihydroxyflavanone, (2S)-5-hydroxy-7-methoxyflavanone, 2',4'-dihydroxychalcone, 4,2',4'-trihydroxychalcone, 7-hydroxyisoflavone and 7,3',4'-trimethoxyisoflavone were found to induce quinone reductase activity.

In addition, our recent study has demonstrated that the *M. calabura* leaves aqueous extract possessed peripheral and central antinociceptive activity when assessed using the abdominal constriction test and hot plate test (Zakaria *et al*., 2004). Further study has also demonstrated that the extract antinociceptive activity involved, at least in part, activation of peripheral and central opioid receptors and that it is resistance against the effect of temperature (Zakaria *et al*., 2004).

Various studies have utilized the pharmacological tools such as various types of receptor antagonists to identify the basic mechanism of action responsible of antinociceptive agents (Bentley *et al*., 1981; Bentley and Starr, 1986; Ellis *et al*., 1999; Ono and Satoh, 1992; Pieretti *et al*., 1999) and one study, for example, has also utilized the effects of temperature and pH to establish the physical properties of the bioactive compound (s) responsible for the said activity of *C. striatus* (Dambisya *et al*., 1999). Based on the facts that *M. calabura* are wildly cultivated in Malaysia and that no serious study have been carried out to explore the pharmaceutical benefits of this plants, together with our finding on the potential antinociceptive activity exhibited by the aqueous extract of *M. calabura* leaves, we take the opportunity to elucidate the involvement of opioid and non-opioid receptors in and determine the effect of pH and various basic enzymes on, the extract observed activity, by focusing, at this moment, on its peripherally mediated mechanism.

MATERIALS AND METHODS

Experimental animals: Male ICR mice (25-30 g; 5-7 weeks old) were used in this study and obtained from the Veterinary Animal Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), Malaysia. The animals were 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 used. 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 with n = 10 were conducted between 09.30 and 18.30 h to minimize the effects of environmental changes.

Preparation of drugs: A 100 mg kg^{-1} acetylsalicylic acid (ASA) (Bayer, Singapore) and 0.8 mg kg^{-1} morphine (Sigma, Germany) were used for the purposed of comparison. Naloxonazine, naltrindole, pindolol, phenoxybenzamine and bicuculine, in the dose of 10 mg kg^{-1} and atropine and mecamylamine, in the dose of 5 mg kg^{-1}, were used to study the involvement of opioid and non-opioid receptors in the said activity of MCAE. α-Amylase and lipase, in the concentration of 10% and protease, in the concentration of 1%, were used to determine the effect of various enzymes on MCAE antinociception. All drugs and chemicals were prepared by dissolving them in dH_2O.

Plant material: The leaves of *M. calabura* were collected in January-February 2004 from its natural habitat in Shah Alam, Selangor, Malaysia and identified by Mr. Shamsul Khamis from the Institute of Bioscience, UPM, Malaysia. A voucher specimen (SK 964/04) was deposited at the Herbarium of the Institute of Bioscience, UPM, Malaysia.

Preparation of *Muntingia calabura* Aqurous Extract (MCAE): The leaves of *M. calabura* were rinsed with water to remove all the dirt and unwanted particles and then oven-dried at the temperature of 40°C. The dried leaves were then grinded into small particles, weighed and added with distilled water (dH_2O) in the ratio of 1:25 (w/v). The mixture was soaked for 24 h and then the supernatant was collected and filtered using Whatman No. 1 filter paper while, the remaining plant residue was kept in an oven for future used. The supernatant obtained, label as MCAE and considered as stock solution with 100% concentration/strength, was diluted with dH_2O to the concentration/strength of 5 and 50% and used together in the antinociceptive study. Thirty milliliter of the obtained supernatant was also subjected to freeze-drying process to determine the amount of crude dried MCAE present in every 10 mL of the supernatant.

Antinociceptive studies involving MCAE

Determination of the peripheral antinociceptive activity of MCAE: In the first study to establish the antinociceptive profile of the MCAE, the room-temperature (27±2°C) prepared extracts, in the concentration of 5, 50 and 100%, were administered subcutaneously (s.c.) in mice (n = 10) and left for 30 min prior to subjection to the antinocicepive assay.

Determination of the effects of opioid and non-opioid antagonists on the peripheral antinociceptive activity of MCAE.

The second study was carried out using only the 50% concentration extract that showed significant antinociceptive activity. All antagonists were administered (s.c.) followed 10 min later by the s.c. administration of extracts, respectively. Thirty minutes after the extract administration, the mice (n = 10) were subjected to the antinociceptive assay.

Determination of the effects of pH on the peripheral antinociceptive activity of MCAE: In the third study, 50% concentration extracts with pH 5.1 were pre-treated against a series of different pH (3, 5, 7, 9, 11 or 13) for 2 h and then neutralized back to pH 5.1 followed by *s.c.* administration into mice (n = 10), respectively. Thirty minutes later, the mice were subjected to the antinociceptive assay.

Effects of various enzymes on peripheral antinociceptive activity of MCAE: In the fourth study, the 50% concentration extracts were pre-treated with 10% concentrations α-amylase or lipase, or 1% concentration protease for 2 h in a water bath at 40°C. Each extract were then cool down to room temperature before administered s.c. into mice (n = 10). Thirty minutes after the respective extract administration, the mice were subjected to the antinociceptive assay.

Antinociceptive assay: The abdominal constriction test was used as described by Dambisya and Lee (1995). Briefly, the animals were injected (s.c.) with dH_2O, ASA, morphine or MCAEs (5, 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 the number of abdominal constrictions produced in these animals was counted for 25 min, commencing 5 min after the acetic aid 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 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. The percentage of analgesia (%) was calculated based on the formula described by Mat Jais *et al*. (1997): [(Saline control group mean-test group mean)/Saline control mean]×100.

Statistical analysis: The results are presented as Mean±Standard Error of Mean (SEM). The One-way Analysis of Variance (ANOVA) test followed by the Dunnett post hoc test was used to analyse and compare the data, with $p<0.05$ as the limit of significance.

RESULTS

Determination of crude dried MCAE: Thirty milliliter of MCAE supernatant, which were divided into 3 plastic test tubes of 10 mL each and subjected to freeze-drying process, was found to yield approximately 0.27 g of crude dried extract.

The peripheral antinociceptive profile of MCAE: From the data obtained, the MCAE was found to demonstrate a significant ($p<0.05$) antinociceptive activity in a concentration-dependent manner (Fig. 1). The activity was observed at the concentration as low as 5% and together with the 50% concentration MCAE producing an activity that was equipotent to that of morphine (0.8 mg kg^{-1}) or ASA (100 mg kg^{-1}), respectively. The highest concentration (100%) of MCAE was found to cause highly significant ($p<0.05$) decreased in the number of abdominal constrictions (9.40 ± 0.91) when compared to the control group (30.14±1.68), with the percentage of analgesia of 68.81%.

Effects of opioid and non-opioid antagonists on peripheral antinociceptive activity of MCAE: The results obtained after pre-treatment of mice with various types of antagonists followed by the administration of 50% concentration MCAEs were shown in Fig. 2. The extract antinociceptive activity was significantly ($p<0.05$) reversed by 10 mg kg^{-1} naloxonazine, 10 mg kg^{-1} pindolol and 5 mg kg^{-1} atropine, respectively. Pre-treatment with 10 mg kg^{-1} naltrindole, 10 mg kg^{-1} phenoxybenzamine, 10 mg kg^{-1} bicuculine or 5 mg kg^{-1} mecamylamine failed to cause significant change in the extract antinociceptive activity.

Effects of pH on peripheral antinociceptive activity of MCAE: Pre-treatment of the respective 50% concentration MCAE against a series of different pH conditions, namely pH 3, 5, 7, 9, 11 and 13, was found to affect the extract antinociceptive activity as shown in Fig. 3. Although, the acidic condition, with pH ranging from 3-5, did not cause any significant change in the MCAE antinociceptive activity, it does reflect on the extract/bioactive compound resistance against the effect of acidic condition. Interestingly, under the alkaline condition with pH ranging between 9 and 11, the MCAE activity was found to significantly ($p<0.05$) enhance while at the extreme alkaline condition (pH 13), the activity was still retain as seen in the acidic condition.

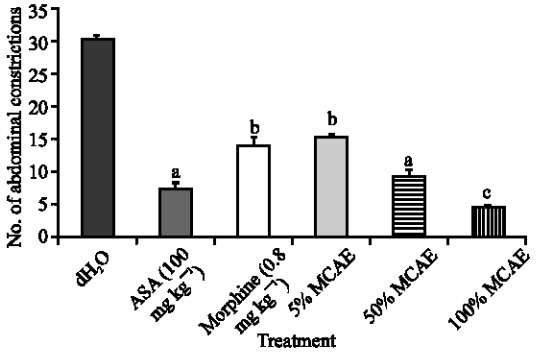

Fig. 1: The antinociceptive profile of *Muntingia calabura* aqueous extract in mice assessed by abdominal constriction test. [a,b]Data with different superscript differ significantly ($p<0.05$) when compared against the control group (dH_2O-treated)

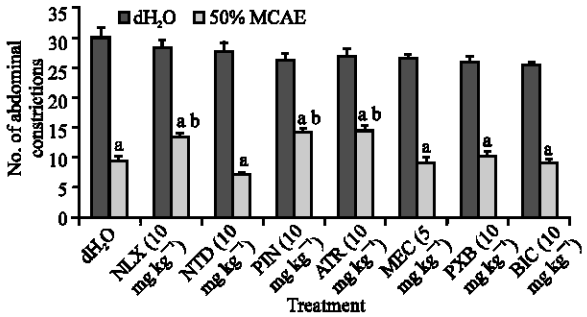

Fig. 2: Effect of various receptor antagonists on *Muntingia calabura* aqueous extract antinociceptive activity. [a]Data differ significantly ($p<0.05$) when compared against their respective control group (dH_2O-treated), [b]Data differ significantly ($p<0.05$) when compared against the positive control group (100% MCAE + DH_2O)

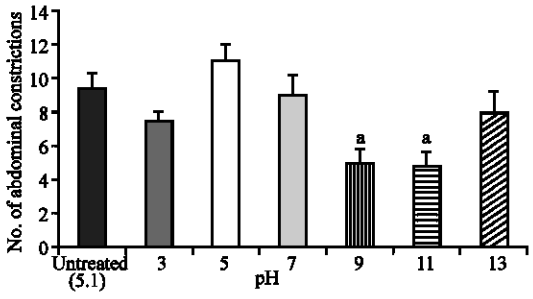

Fig. 3: Effect of pH on the *Muntingia calabura* aqueous extract antinociceptive activity. [a]Data with superscript differ significantly ($p<0.05$) when compared against the untreated group (normal extract), Untreated group = Control group = 50% concentration MCAE

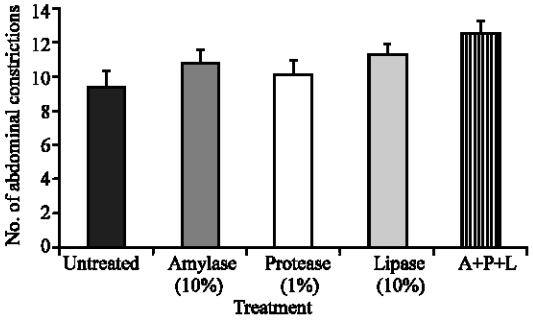

Fig. 4: Effect of enzymes (α-amylase, protease, lipase and their combination (A+P+L)) on the *Muntingia calabura* antinociceptive activity. Untreated group = Control group = 50% concentration MCAE

Effects of enzymes on peripheral antinociceptive activity of MCAE: The results obtained for pre-treatment of the respective 50% concentration MCAE against common enzymes, namely α-amylase, protease, lipase or their combination (A+P+L), was shown in Fig. 4. As can be seen from the Fig. 4, pre-treatment of the MCAE against the three enzymes or their combination did not cause any significant change in its antinociceptive activity and, thus, indicating the extract resistance against the chemical reaction of the respective enzyme.

DISCUSSION

In the present study, the peripheral antinociceptive activity of MCAE was evaluated and the involvement of opioid and non-opioid receptors in the said activity as well as the effect of pH and enzymes on the extract properties were determined. The abdominal constriction test was used to evaluate the peripheral antinociceptive activity of the extract.

The abdominal constriction test is a very sensitive test that can detect analgesia of compounds/dose levels that may be inactive in other test (Bentley *et al*., 1981; Bentley *et al*., 1986). The analgesic activity observed using this assay involved, at least in part, the local peritoneal receptors, such as opioid and adrenergic receptors, found at the surface of the cells lining the peritoneal cavity. According to Deraedt *et al*. (1980), the acetic acid, used as the inducer of nociception in the said test, causes irritation to the peritoneal cavity, which then leads to an increase in peritoneal fluid levels of prostaglandins (PGE_2 and $PGE_{2\alpha}$). This will then lead to enhance inflammatory pain due to increase capillary permeability induced by the prostaglandins (Vogel and Vogel, 1997). Our previous and recent study has demonstrated the MCAE ability to induced concentration-

dependent antinociception and was in line with claimed made on its ability to treat various types of ailments (Jensen, 1999; Verheij *et al*., 1992). However, the significant antinociceptive activity observed with the writhing test was not specific since the test did not indicate whether the activity was due to central and/or peripheral activity (Chan *et al*., 1995). On going study using the formalin test has demonstrated the extract ability to inhibit the early phase (neurogenic pain) and late phase (inflammation pain) (data not published), which is in line with our recent observation on the extract peripheral and central antinociception (Zakaria *et al*., 2004). Centrally acting drugs, like morphine, have been reported to produce antinociceptive effect in both types of assays (Sulaiman *et al*., 2004), while peripherally acting drugs, like aspirin and indomethacin, produced their antinociceptive effect only in the abdominal constriction test (Siegmund *et al*., 1957; Hendershot and Forsaith, 1959; Amanlou *et al*., 2005). In addition, the 5 and 50% concentration extracts were found to produce an antinociceptive activity that are equipotent to 100 mg kg^{-1} ASA and 0.8 mg kg^{-1} morphine, respectively.

Pre-treatment with various types of receptor antagonists were found to demonstrated the involvement of μ-opioid, β-adrenergic and muscarinic receptors in the MCAE antinociception. This finding was in line with our previous report on the involvement of opioid receptor in the extract antinociceptive activity (Zakaria *et al*., 2004; Bentley *et al*., 1981), while the involvement of β-adrenergic and muscarinic receptors were expected based on reports made by Bentley *et al*. (1981) and Bentley and Starr (1986), on the presence of both types of receptors in the peritoneal cavity. Recent study by Zakaria *et al*. (2006a), has also demonstrated the involvement of L-arginine/nitric oxide/cyclic guanosine monophosphate (L-arginine/ NO/cGMP) pathway in the MCAE antinociceptive activity. The fact that acetylcholine and cholinomimetic agents with predominant muscarinic action are known to increase the concentration of cGMP by activation of nitric oxide signaling pathway in the nociceptive conditions (Patil *et al*., 2004), seems to support our findings on the involvement of muscarinic receptors in the L-arginine/NO/cGMP pathway mediated MCAE antinociception. Although, there was no report to associate the adrenergic receptors activation to the involvement of L-arginine/NO/cGMP pathway in antinociceptive mechanism, at least one study has linked the lack of adrenergic-induced vasoconstriction in the skeletal muscle with the reduced expression of nNOS within the said muscle (Thomas *et al*., 1998).

The involvement of the above-mentioned receptors in MCAE antinociception were expected since the extract used are of a crude type, which is known to contain various types of compounds, such as flavonoids, steroids, essential oil and tannins that are found abundantly in the leaves of plants (Kaneda *et al*., 1991; Calixto *et al*., 2000; Peres *et al*., 1998). Although, isolation and purification of different fraction from the aqueous extract of *M. calabura* and assaying for antinociceptive activity of each fraction were not the objective of this study, nevertheless, based on the results of the study, we can suggest that the antinociceptive activity of the extract may be attributed to inhibition of the above mentioned receptors, prostaglandin release or blocking of the enzyme, cyclo-oxygenase, that is responsible for prostaglandin production and similar mediators involved in nociceptive process (Di Rosa *et al*., 1971; Spector, 1962). Inhibition of prostaglandin or cyclo-oxygenase were suggested based on our on going studies with the MCAE that was shown to show positive effects in the formalin, the carrageenan-induced paw edema and brewer's yeast-induced pyrexia tests (data not published).

The additional studies, carried out to observe the effects of pH and enzymes, were actually aimed at getting some additional information on the physical properties of the bioactive compound (s) responsible for the MCAE antinociception. The ability to withstand the effects of pH and enzymes, for examples, are very important during the pharmacodynamic and pharmacokinetic studies of the bioactive compound (s) in the future. The ability of MCAE to withstand the effects of extreme acidic and alkaline condition, as well as the denaturation effect of enzymes were expected to be due to the present of stable short chain macromolecules or bioactive compounds (Dambisya *et al*., 1999). Interestingly, our results also show that the extract activity was significantly enhanced at alkaline condition, but maintained at acidic condition. The presence of activity even after pre-treatment under extreme acidic and alkaline conditions indicate the bioactive compounds resistance to the pH's denaturing effect. Furthermore, our previous study has also demonstrated that the extract resist the effect of temperature up to 100°C (Zakaria *et al*., 2004), which is also believed to be due to the presence of short chain and stable macromolecules. It is known that short chain macromolecule possessed high stability and resist the effect of temperature as well as pH (Dambisya *et al*., 1999).

The ability of abdominal constriction test to determine the antinociceptive activity of opioid agonists and non-steroidal anti-inflammatory agents (Vogel and Vogel, 1997) has lead to the chosen of morphine and ASA as our reference drugs. In any case, the consistent of the results of this study with ASA or morphine activities can take into account to confirm the folklore use of *M. calabura* as an antinociceptive agent and to treat various ailments.

CONCLUSION

Based on the results obtained, it is conclude that the observed *M. calabura* leaves peripheral antinociception might be attributed to the presence of bioactive compounds that were resistance against the denaturation effects of pH and enzymes and act via opioid, β-adrenergic and muscarinic receptors. Further study is being carried out in our lab to identify the role of the above parameters on the extracts central antinociceptive activity.

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