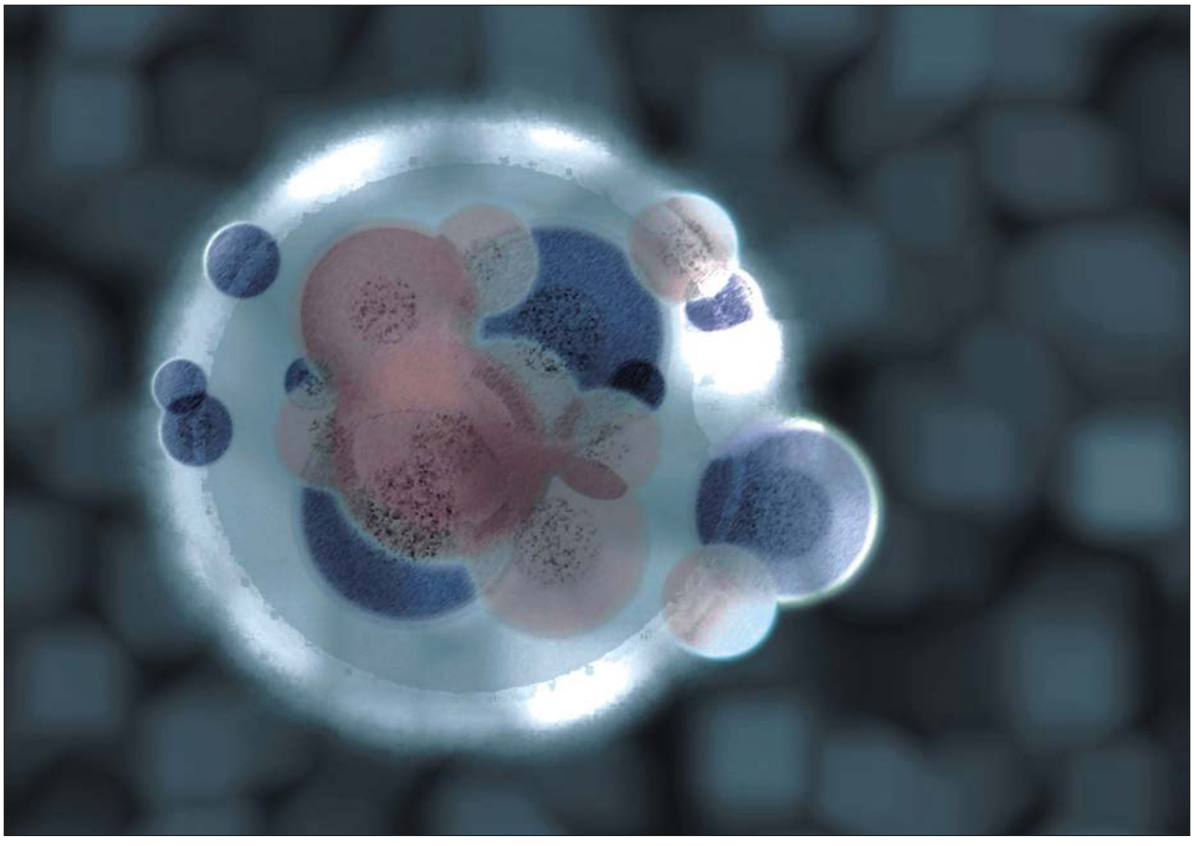

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Report on Some of the Physical Properties of Bioactive Compounds Responsible for the *Channa striatus* Fillet Extract Antinociceptive Activity

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Abstract: The present study was carried out to elucidate some of the basic physical properties of the bioactive compounds responsible for *Channa striatus* antinociception. The aqueous extract of *C. striatus* was obtained after soaking the fish fresh fillet in the ratio of 1:2 (w/v) in chloroform: methanol (2:1; v:v) overnight. The extract, in the concentrations of 10, 50 and 100%, was subjected to the centrifugation-filtration process using the Millipore Ultrafree-CL low binding cellulose filters with different pore size (5,000, 10,000 and 30,000 dalton) prior to the abdominal constriction test in mice, respectively. Subsequently, the 100% concentration extract was also subjected to the analytical high performance liquid chromatography (using a Gilson 715 HPLC equipped with two slave 306 pumps and analytical reverse-phase Vydac C-18 column (10 μm, 0.46×25 cm) set at 40°C, with the flow rate of 0.5 mL min^{-1} absorbance at the wavelength of 220 nm was monitored by a wavelength UV detector; the eluant A was 100% methanol and eluant B was 80% methanol in dH_2O) to establish its chromatogram profiles and the first fraction obtained was collected and subjected to the same antinocceptive assay. The non-filtered extract was found to exhibit a concentration-dependent antinociception ($p<0.05$). Except for the 30,000 dalton extract, the 5,000 and 10,000 dalton extracts antinociception were significantly different ($p<0.05$) from the non-filtered extract. Furthermore, the chromatogram profiles of *C. striatus* aqueous extract revealed the presence of four major fractions while the first fraction, prepared in the concentrations of 0.005, 0.05, 0.5 and 5 mg kg^{-1}, was found to exhibit significantly ($p<0.05$) concentration-dependent antinociception. Based on the present data obtained, it is plausible to suggest that at least four types of bioactive compounds with molecular weight ranging from lower than 5,000 dalton as well as between 10,000 to 30,000 dalton presences in the aqueous extract of *C. striatus*.

Key words: Channa striatus, aqueous extract, antinociception, molecular weight, high performance liquid chromatography, abdominal constriction test

INTRODUCTION

Channa striatus, a freshwater and carnivorous snakehead fish, is known to the Malays as 'Haruan' and used traditionally to promote wound healing and also to alleviate post-operative pain and discomfort (Mat Jais *et al*., 1994, 1997). The fish is indigenous to many Southeast Asian countries, including Malaysia (Wee, 1982) and consumed entirely as a dietary medicine by dry-frying, boiling in porridge or grilling. Earlier study has demonstrated that its fillet and mucus extracts possessed peripherally, but not centrally, mediated antinociception (Mat Jais *et al*., 1997), with the latter was later demonstrated to involve activation of a non-opioid mechanism Dambisya *et al*. (1999). The mucus extract of *C. striatus* antinociception was also reported to resist the effect pH and temperature (Dambisya *et al*., 1999) and diminished after pre-treatment with lipase, but not amylase and protease (Zakaria *et al*., 2004). Recent studies have demonstrated that the reported non-opioid mechanism of *C. striatus* involved activation of at least, four different types of receptor systems namely serotonergic, muscarinic, α-adrenergic and $GABA_A$-ergic (Zakaria *et al*., 2005a) as well as activation of L-arginine/nitric oxide/cyclic guanosine monophosphate pathway (Zakaria *et al*., 2005b).

Although not yet proven, Dambisya *et al*. (1999) have suggested the presence of stable and short chain

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bioactive compound while Zakaria *et al.* (2004) have suggested the presence of lipid-based compound as part of the major constituent of the bioactive compound. Previous finding by Huang *et al.* (2001) that one types of lipoamino acid, namely arachidonylglycine, possessed antinociceptive activity when assessed using a formalin test seems to support the above suggestions since the earlier and recent studies have also demonstrated the presence of arachidonic acid and glycine (Mat Jais *et al.*, 1994; 1998a, b; Zuraini *et al.*, 2005), both being the precursor for arachidonylglycine formation, in *C. striatus* fillet as well as roe extracts. Other than that, the fish also contain certain fatty acids that have been reported to produce anti-inflammatory activity, such as stearic acid and oleic acid, which might also help to explain its ability to exhibit anti-inflammatory activity (Somchit *et al.*, 2004) since fatty acids, including the two mentioned above, have been reported to attenuate polymorphonuclear leukocytes (PMN) activity and thus, suppressed inflammatory processes (Crocker *et al.*, 2001).

In the present study, we undertook an investigation to determine the molecular weight range of the said bioactive compounds and also to establish the chromatographic profiles for the aqueous extract of *C. striatus*.

MATERIALS AND METHODS

Preparation of fresh haruan fillet: Six month old (150-400 g) haruan fish, cultured in Pontian, Johor, Malaysia and transferred to Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia, were acclimatized for at least 3 days prior to experiments and were used throughout the study. Preparation of fresh haruan fillet was carried out using the method described by Mat Jais *et al.* (1997). Precleaned live *C. striatus* were weighed and slaughtered and the fillets were obtained by carefully cutting the fish lengthwise along the backbone to gain maximum amount of flesh without any backbone.

Preparation of channa striatus aqueous extract: The aqueous extract of *C. striatus* were prepared using 2:1 (v:v) chloroform:methanol (CM) system as described previously (Zakaria *et al.*, 2005a, b). The fresh fillet obtained was soaked in CM, in the ratio of 1:2 (w:v), overnight and then filtered. The supernatant obtained was left for 30 min to settle down into two layers. The upper layer, which is an aqueous extract of *C. striatus* (AECS), was collected and evaporated to remove any methanol residues present while the lower layer, which is the lipid-based extract of *C. striatus* was discarded. The AECS obtained was considered as stock solution with 100% concentration/strength and used throughout the study.

Centrifugation and filtration of aqueous extract of *C. striatus*: The stock solution was divided four parts in which the first part was not centrifuged and filtered at all, prepared in three different concentrations (25 and 100%) and used directly in the antinociception evaluation. The rest parts of AECS were subjected to the centrifugation-filtration processes using different types of filters with different size of pores. Initially, 2 mL of the second, third and fourth parts of AECS were loaded into the filter cup (upper part of the filters) of each filters and centrifuged (Heraeus Sepatech, Germany) at 5,000 rpm for 10 min by using the said Ultrafree-CL filters (Millipore, USA), which filtered compounds with molecular weight of 5,000, 10,000 or 30,000 dalton, respectively. After the centrifugation process, the supernatant obtained in the filtrate collection tubes (bottom part of the filters) were collected and considered as the respective stock solution (100% concentration/strength). Except for stock solutions filtered using the 5,000 and 10,000 daltons pore size filters that was prepared in the concentration of 5, 10 and 25%, the stock solution filtered using the 30,000 dalton pore size filter was diluted to the concentrations of 25 and 50% by using distilled water (dH_2O). The reason for this discrepancy in the concentration of extracts prepared was attributed to the fact that the volume of filtrate obtained in the smaller pore size filters (5,000 and 10,000 dalton) was very little.

Chromatographic profilings of aqueous extract of *C. striatus*: Subsequently, the AECS, lyophilised overnight (24 h), was subjected to the high performance liquid chromatography according to the method described by Hornby *et al.* (2001) but with slightly modification. We have tried several procedures by manipulating the volume of sample injection, the flow rate of sample or the absorbance wavelength, and the conditions given below was found to be the best for separation of peptides from AECS. Briefly, a 5 μL sample of ASH was injected into a Gilson 715 HPLC equipped with two slave 306 pumps and analytical reverse-phase Vydac C-18 column (10 μm, 0.46×25 cm) set at 40°C, with the flow rate of 0.5 mL min^{-1}. The absorbance at the wavelength of 220 nm was monitored by a wavelength UV detector (Applied Biosystem 759A). The eluant A was 100% methanol and eluant B was 80% methanol in dH_2O.

Antinociceptive profile of the first fraction of AECS obtained after HPLC procedures: The first fraction collected after subjection of the AECS, filtered using the

30,000 dalton pore size filters, to HPLC procedures was dried under vacuum, reconstituted in dH_2O to the concentrations of 0.005, 0.05, 0.5 and 5 mg kg^{-1} and then used in the antinociceptive assay.

Preparation of drug: 100 mg kg^{-1} acetylsalicylic acid (ASA) (Bayer, Singapore), used for the purposed of comparison, was prepared by dissolving them in dH_2O.

Experimental animals: Male Balb-C mice (25-30 g; 5-7 weeks old) were used in this study. The animals were obtained from the Animal Source Unit, Faculty of Veterinary Medicine, Universiti Putra (UPM), Malaysia and kept under room temperature (27±2°C; 70-80% humidity; 12 h light/darkness cycle) in the Animal Holding Unit, Faculty of Medical and Health Sciences, 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 cared for in accordance with current UPM principles and guidelines for the care of laboratory animals and the UPM ethical guidelines for investigations of experimental pain in conscious animals as adopted from Zimmermann (1983).

All mice were equally divided into several groups of 10 mice each (n = 10). The first six groups were subjected to the administration of 10, 50 and 100% concentrations AECS, either non-filtered or filtered using the filters with pore size of 30,000 dalton, respectively. The other groups of mice were subjected to the administration of 5, 10 and 25% concentrations of AECS, non-filtered and filtered using the filters with pore size of 5,000 or 10,000 daltons, respectively. Other groups of mice were also administered with the 0.005, 0.05, 0.5 and 5 mg kg^{-1} of the AECS first fraction collected after the HPLC procedures. dH_2O and ASA were used as negative and positive groups. Thirty minutes after the test solutions administration the respective mice were subjected to the antinociceptive assay. All of the test solutions were administered subcutaneously (s.c.) in the volume of 10 mL kg^{-1} body weight (Mat Jais *et al*., 1997).

ANTINOCICEPTIVE ASSAY

Abdominal constriction test: The abdominal constriction test used is based on the report made by Dambisya and Lee (1995). The acetic acid (J.T. Baker, USA) prepared as 0.6% (v:v) solution in dH_2O and used to induce pain in mice peritoneal cavity, was administered intraperitoneally (i.p.) in a volume of 10 mL kg^{-1} of mice, 30 min after the s.c. administration of respective dH_2O, aspirin or ASH. The abdominal constriction resulting from the injection of acetic acid consisting of a contraction of the abdominal region together with a stretching of the hind limbs (Correa *et al*., 1996). The number of abdominal constrictions was counted cumulatively over the period of 25 min, commencing 5 min following the acetic acid administration. 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) as given below:

$$\frac{\text{Control group mean - Test group mean}}{\text{Control group mean}} \times 100\%$$

Statistical analysis: The results are presented as Mean±Standard Error of Mean (SEM). The one-way ANOVA test with Dunnet post-hoc test was used to analyze and compare the data, with $p<0.05$ as the limit of significance.

RESULTS

Table 1 shows the antinociceptive profiles of AECS, non-filtered an filtered using the 30,000 dalton pore size filters and compared their effectiveness in relieving nociception assessed by acetic acid-induced abdominal constriction test. Both types of extracts induced equipotent antinociception indicated by the insignificant ($p<0.05$) data obtained for the respective concentration of AECS. The said activity occurred in a concentration-dependent manner with the highest concentration (100% concentration) almost completely inhibited the abdominal constriction.

Table 2 shows the antinociceptive profiles of AECS, non-filtered or filtered using the 5,000 or 10,000 daltons pore size filters, at the concentrations of 5, 10 and 25%, respectively. It was observed that decreased in the size of the filters pore caused significant ($p<0.05$) decreased in the pain relieving properties of the AECS. However, the decreased was still within the antinociceptive range.

Figure 1 shows the chromatographic profile of AECS, filtered using the 30,000 dalton pore size filters, after subjection to HPLC procedures. Four major peaks at the retention times of 8.919, 9.841, 10.263 and 10.744 can be seen, which seems to indicate the presence of at least four major fractions (Fig. 1). The first fraction, collected from the HPLC procedures and evaporated to dryness before being reconstituted in dH_2O, was also found to exhibit a concentration-dependent antinociception (Table 3). Interestingly, even the lowest concentration of AECS used (0.005 mg kg^{-1}) produced approximately 30%

Table 1: The antinociceptive profile of aqueous extract of *Channa striatus*, non-filtered or filtered using the 30,000 NMWL pore size filters, assessed using the abdominal constriction test

Treatment	Dose (mg kg^{-1}) or concentration (%)	No. of mice	No. of abdominal constrictions±SEM	Percentage of pain inhibition (%)
dH_2O	-	9	34.40±0.87	-
Morphine	5 mg kg^{-1}	9	13.82±1.42*	59.83
ASA	100 mg kg^{-1}	9	7.4±0.85*	78.49
	25%	9	12.86±0.99*	63.73
Non-filtered AECS	50%	9	7.14±0.86*	79.89
	100%	9	4.38±0.57*	87.66
	25%	9	10.78±0.83*	69.63
Filtered AECS	50%	9	6.56±0.56*	81.52
	100%	9	2.33±0.50*	93.44

Data with superscript differ significantly ($p<0.05$) when compared to the control group (dH_2O-treated)

Table 2: The antinociceptive profile of the respective 5%, 10% and 25% concentrations aqueous extract of *Channa striatus*, non-filtered or filtered using the 5,000, 15,000 or 30,000 NMWL pore size filters, assessed using the abdominal constriction test

Treatment		Dose (mg kg^{-1}) or Concentration (%)	No. of mice (n)	No. of abdominal constrictions±SEM	Percentage of pain inhibition (%)
dH_2O		-	9	34.40±0.87	-
Morphine		5 mg kg^{-1}	9	13.82±1.42*	59.83
ASA		100 mg kg^{-1}	9	7.4±0.85*	78.49
		5%	9	24.63±1.30*	28.48
Non-filtered AECS		10%	9	23.14±0.55*	32.81
		25%	9	12.86±0.99*	62.66
		5%	9	26.29±1.02*	23.66
	30,000 NMWL	10%	9	27.33±0.87*	17.74
		25%	9	10.78±0.83*	68.70
		5%	9	26.38±1.02*	23.40
Filtered AECS	15,000 NMWL	10%	9	23.00±0.46*	33.21
		25%	9	16.43±1.19*	52.29
		5%	9	25.89±1.05*	24.83
	5,000 NMWL	10%	9	23.89±0.96*	30.63
		25%	9	21.22±0.91*	38.39

Data with superscript differ significantly ($p<0.05$) when compared to the control group (dH_2O-treated)

Table 3: The antinociceptive profile of the first fraction of aqueous extract of *Channa striatus* obtained after HPLC procedures assessed using the abdominal constriction test.

Treatment	Dose (mg kg^{-1}) (n)	No. of mice constrictions±SEM	No. of abdominal inhibition (%)	Percentage of pain
dH_2O	-	9	34.40±0.87	-
	0.005	9	24.43±1.41*	29.07
AECS	0.05	9	23.56±1.22*	31.59
Fraction	0.5	9	21.50±1.31*	37.57
	5	9	18.00±1.63*	47.74

* Data with superscript differ significantly ($p<0.05$) when compared to the control group (dH_2O-treated)

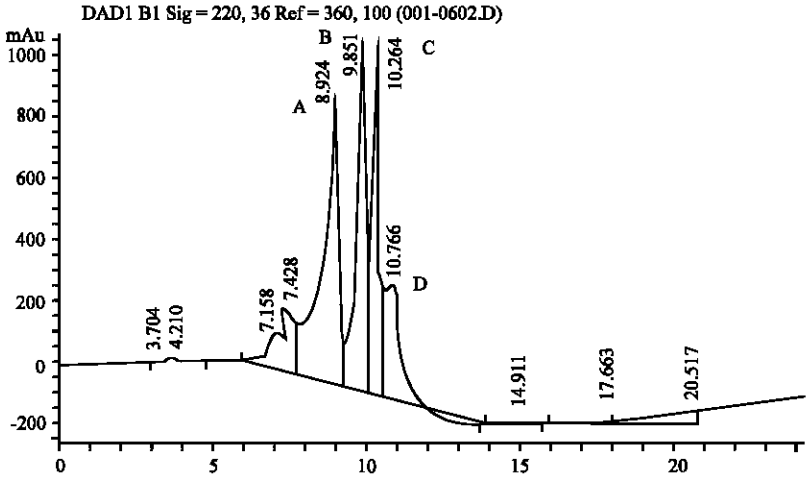

Fig. 1: The chromatographic profile of aqueous extract of *Channa striatus* after subjection to HPLC procedures. The chromatogram demonstrated the presence of four major fractions labeled as A, B, C and D

percentage of analgesia while the highest concentration used (5 mg kg^{-1}) exhibited approximately 50% percentage of analgesia.

DISCUSSION

C. striatus has been used by the Malays for a long time to hasten the healing of wound (Wee, 1982). The said activity has been proven scientifically by several researchers (Mat Jais *et al*., 1998; Baie and Sheikh, 2000a, b) and is attributed to the high concentration of fatty acids, particularly arachidonic acid and amino acids, particularly glycine, in its fillet (Mat Jais *et al*., 1994; Zuraini *et al*., 2005) which are necessary for the growth of wound healing tissue (Mat Jais *et al*., 1994). The fillet extract of *C. striatus* has also been reported to possess peripheral, but not central, antinociception (Mat Jais *et al*., 1997; Zakaria *et al*., 2005a) that was mediated via the activation of muscarinic, $GABA_A$, α-adrenergic and serotonergic receptor systems (Zakaria *et al*., 2005a) and L-arginine/nitric oxide/cyclic guanosine monophosphate pathway (Zakaria *et al*., 2005b). The AECS has also been found to possess high concentration of glycine but low concentration of arachidonic acid (data not published). The presence of arachidonic acid and glycine in the AECS has been associated with report made by Huang *et al*. (2001) on the presence of arachidonylglycine, a conjugated lipoamino acid, with antinociceptive activity. This suggestion is strengthen by our findings that the AECS also produce antinociceptive activity when assessed by the formalin test (data not published) as reported on arachidonylglycine (Huang *et al*., 2001).

The present study has showed that the AECS, either non-filtered or filtered using the 30,000 daltons pore size filters, possessed a concentration-dependent antinociception as previously reported (Mat Jais *et al*., 1997). Furthermore, the 25% concentration extract, either non-filtered or filtered using the 5,000, 10,000 or 30,000 daltons pore sizes filters, exhibited significant decreased in activity as the pore size of the filters decreased. In addition, decreased in the concentrations of filtered and non-filtered AECS to 5 and 10% was found to produce significantly lower antinociception, which did not depend on the concentration of extracts used. Based on the present study, two classes of bioactive compounds responsible for the AECS antinociception could be suggested. The first class of compounds is believed to possess molecular weight less than 5,000 dalton based on the finding that the antinociceptive activity is still observed in the AECS filtered using the 5,000 dalton pore size filters. Another group of compound is suggested to possess the molecular weight between 15,000 and 30,000 daltons based on the observation that the AECS filtered using the 15,000 and 30,000 daltons pore size filters were also found to exhibit the said activity.

Further studies have revealed the present of at least four major fractions in the AECS, which are believed to be polypeptides or conjugated compounds with hydrophilic/polar characteristic. Several reasons could be used to support the above suggestions. Firstly, several reports have demonstrated the presence of various types of amino acids and fatty acids in the *C. striatus* fillet (Mat Jais *et al*., 1998, Zuraini *et al*., 2005). Furthermore, we have also recently demonstrated the presence of fatty acids and amino acids in the AECS (data not published). The presence of various types of amino acids are believed to directly contribute to the observed antinociception (Aimone and Gebhart, 1986; Jacquet, 1988) or to be the building blocks of various types of polypeptides, which have been reported to possess the antinociceptive activity in other species (Montecucchi *et al*., 1981; Newcomb *et al*., 1995; Livett *et al*., 2004). On the other hand, the fatty acids themselves are believed to be precursors for the synthesis of several compounds with antinociceptive activity, such as cyclo-pentanone prostaglandins (Hosoi *et al*., 1999). Prostaglandins with antinociceptive activity like PGE_1 and PGE_2 have been shown to derive from the breakdown of fatty acids, including ARA, which is found abundantly in *C. striatus* fillet (Zeldin, 2001). Other than that, the amino acids and fatty acids present in *C. striatus* could also formed several types of conjugated compounds with antinociceptive activity, like *N*-arachidonylglycine (Huang *et al*., 2001) or palmitoylethanolamide (Griffin *et al*., 2000). The presence of four major fractions in AECS seems to support earlier study on the presence of two classes of bioactive compounds with different molecular weight. This finding also seems to support our earlier report on the involvement of at least four different types of non-opioid receptor systems, namely muscarinic, $GABA_A$, α-adrenergic and serotonergic receptors (Zakaria *et al*., 2005a).

Several suggestions could be proposed to explain the mechanism involved in the observed activities of *C. striatus*. According to Hiramatsu *et al*. (2001) the mechanism of non-opioid antinociceptive activity of antinociceptive agents could be due to several factors, which may include (1) direct agonist activity of any of the major compounds in AECS on NMDA receptors causing neuronal excitability; (2) the release of excitatory amino acids, which may lead to central sensitization and (3) other mechanisms mediating some neuronal and/or endocrine systems. In addition, the observed antinociception may

be attributed to the blocking of prostaglandin release or inhibition of the enzyme, cyclo-oxygenase, that is responsible for prostaglandin production, and similar mediators involved in the processes (Griffin *et al*., 2000). Taken into account the fact that *C. striatus* contain high amount of arachidonic acid, a precursor for prostaglandin synthesis (Mat Jais *et al*., 1997; Zuraini *et al*., 2005), second suggestion that the mechanism of antinociception observed in AECS is different from the one produced by ASA was proposed. This might include attenuation of other types of nociception mediators, such as chemokines and cytokines (Ghosh and Karin, 2002) or in part ARA-independent mechanisms (Calder *et al*., 2002). The fact that we have identified the involvement of, at least, muscarinic, $GABA_A$, α-adrenergic and serotonergic, in the AECS antinociception (Zakaria *et al*., 2005a) seems to support the second suggestion.

Finally, it is concluded that there are at least two classes of bioactive compounds responsible for the *C. striatus* fillet extract antinociception with molecular weight of one class is lesser than 5,000 NMWL and the other is between 15,000 to 30,000 NMWL. The chromatographic profile of the extract also demonstrated the presence of at least four major fractions of bioactive compounds that needs further thorough investigation.

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