Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
22(3): 630-641, May/Jun. 2012

Antinociceptive and anti-ulcerogenic activities of the ethanolic extract of *Annona muricata* leaf

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Article

Received 23 Dec 2010
Accepted 23 Oct 2011
Available online 6 Jan 2012

Keywords:
abdominal writhing
Annonaceae
Annona muricata
antinociceptive
anti-ulcerogenic
formalin test

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2012005000001

Abstract: Ethanolic extract of *Annona muricata* L., Annonaceae, leaf (AML) was used to investigate its antinociceptive and anti-ulcerogenic activities and the involvement of the mechanism of ethanolic leaves extract of AML in various animal models. Antinociceptive activity of AML extract was done using acetic acid-induced abdominal writhing in mice, formalin test in rats and hot plate test in mice. Furthermore, the anti-ulcerogenic effect of AML extract was studied in ethanol-induced ulcer model in rats, ethanol-induced gastric lesions in L-NAME-pre-treated rats as well as ethanol-induced gastric lesions in NEM-pre-treated rats test model to determine its mechanism. AML exhibited significant and dose-dependent antinociceptive activity. It also significantly decreased the ulcerative lesion produced by ethanol in rats in a dose-dependent manner. Pre-treatment with *N*-ethymaleimide, a thiol blocker, including mucosal nonprotein sulfhydryl groups, reduced the anti-ulcerogenic effect of AML extract in the same ulcer model, suggesting that AML extract may have active substances such as tannins, flavanoids and triterpenes that increase the mucosal nonprotein sulfhydryl group content.

Introduction

Annona muricata L., Annonaceae, commonly known as soursop, is an edible tropical fruit which is common, readily available and cheap in the producing country (Morton, 1987). The bark, leaves and roots are claimed to be sedative, antispasmodic, hypoglycemic, hypotensive, smooth muscle relaxant and a tea is made for various disorders for those purposes (Holdsworth, 1990). Several studies by different researches over the years have demonstrated that the leaf, bark, root, stem, and fruit seed extracts of *Annona muricata* are antibacterial *in vitro* against numerous pathogen (Misas et al, 1979; Sundarrao, 1993), and the bark has antifungal properties (Heinrich, 1992). In 1990 to 1993, there were two studies indicated that *A. muricata* has the properties against malaria (Arkcoll, 1990; Antoun et al., 1993). In addition, *A. muricata* leaf extract possesses antioxidant (Baskar et al.,2007) and molluscicidal properties (Santos & Sant`Ana, 2001; Luna et al., 2006). Recent studies also showed anti-inflammatory and analgesic properties in the leaves of the plant (Roslida et al., 2010; De Sousa et al., 2010) Among the chemical constituents found in the leaf of *A. muricata* are alkaloids (Le Bouef et al., 1981; 1982), essential oils (Pélissler et al., 1994; Kossouh et al., 2007) and acetogenins (Wu et al., 1995a; 1995b; 1995c; 1995d; Zeng et al., 1996; Kim et al., 1998; Chang et al.,2003).

In the present study, we investigated the antinociceptive effect of *Annona muricata* leaf extract in various animal models as well as anti-ulcer effect in absolute ethanol induced ulcer model. Possible participation of either nitric oxide or/and sulfhydryl group in the extract was also determined in its anti-ulcerogenic activity.

Material and methods

Plant material

Leaves of *Annona muricata* L., Annonaceae, were collected from Raub, Pahang, Malaysia in December 2007. Samples were authenticated by Dr Richard Chung (Forest Research Institute Malaysia) and a voucher specimen (number FRI 57966) has been deposited in the herbarium of Forest Research Institute Malaysia (FRIM), Kepong, Selangor, Malaysia. The leaves were cut into small pieces and dried at 40-42 ºC for three days. The dried leaves were then grounded and macerated in aqueous ethanol (80%) for few days. The solvent extract was then filtered and concentrated under reduced pressure using the rotary evaporator until the solvent was totally removed. The yield of the ethanol extract of AML

was obtained by dry weight method and at time of use, the extract was dissolved in 1% Tween 80 at desired dose concentration (10, 30, 100 and 300 mg/kg) administered orally 1 h prior to the inducers.

Animals

Healthy *Sprague dawley* rats weighing between 200-300 g and adult ICR mice (25-40 g) were obtained from Animal Unit of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia with ethics approval from the Animal Ethics Committee of Universiti Putra Malaysia (UPM/FPSK/PADS/BRUUH/00266). The animals were fed on standard laboratory diet and allowed free access to water. Animals for anti-ulcerogenic activity were fasted 24 h prior to the experiment.

Drugs

Acetic acid, acetylsalicylic acid, piroxicam, carbenoxolone, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) and *N*-ethylmaleimide (NEM) were purchased from Sigma (USA). Naloxone was obtained from Fluka (Netherland), absolute ethanol was purchased from JT Baker (Malaysia), formalin was purchased from Systerm (Malaysia) and morphine was obtained from Lipomed (Switzerland).

Acetic acid-induced abdominal writhings

The acetic acid-induced abdominal writhing test was performed as described previously by Koster et al. (1959). ICR mice were selected for this study where 36 mice weighing 30-40 g were used in which six mice will be allotted for each group. Mice were administered orally (*p.o*.) with negative control (1% Tween 80, 10 mL/kg), AML (10, 30, 100 and 300 mg/kg) and positive control with piroxicam (100 mg/kg) prior to intraperitoneal (*i.p*.) injection of 0.6% acetic acid (10 mL/kg). The number of writhing was recorded for 60 min, starting 5 min after the injection of acetic acid. The evaluation of antinociceptive activity was expressed as the percentage reduction or inhibition of the number of total abdominal writhes using the following ratio: (control mean-treated mean)×100/ control mean (Dambisya & Lee., 1994).

Formalin test

The formalin test was assessed according to procedures described by Dubuisson & Dennis (1977), and Wheeler-Aceto et al., (1990). Briefly, rats received either AML (10, 30,100 and 300 mg/kg, *p.o*.) or acetylsalicylic acid (100 mg/kg, *i.p*.) or 1% Tween 80 1 h prior to the subcutaneous injection of 50 µL of 5% formalin (v/v in distilled water) into the dorsal surface of the left hind paw of the rats. The spontaneous responses after the injection including biting, licking and scratching of the injected paw were observed and the time spent was recorded up to 1 h. Two phases of the behavioral responses, the first 5 min was considered as early phase (neurogenic phase) and the time of 15-60 min as the late phase (inflammatory phase) of the nociceptive response. The effect of pretreatment with naloxone (5 mg/kg, *i.p*.) on the analgesia produced by AML (300 mg/kg) was determined in a separate group of animals. Morphine (5 mg/kg, *i.p*.), in the absence and presence of naloxone treatment, was used as a reference.

Hot-plate test

The test was performed using the method previously described by Abdel Zaher et al., (2006). Before the test was carried out, the mice were subjected to the selection process in which the untreated mice were placed on the hot plate (Ugo Basile 7280, Italy) maintained at 53±0.5 ºC. Those mice with response latency times between 7 and 10 s were selected for this study. The selected mice were equally divided into six groups (n=6). The mice were treated with 1% Tween 80, morphine (5 mg/kg) or AML (10, 30, 100 or 300 mg/kg) 1 h prior to the test. The latency time of response for nociceptive behavior, *e.g*. licking the paw or jumping was recorded. Mice were removed from the hot plate immediately after the response shown. Response latencies were measured at half hourly interval for the duration of 4 h after the substance administration. The animals were remained not more than 20 s on the hot plate to avoid excessive tissue damage. The prolongation of the latency times was an indicator of analgesic activity and was compared with the values of control animals for statistical comparison. The effect of pretreatment with naloxone (5 mg/kg, *i.p*.) on the analgesia produced by the AML extract (300 mg/kg) was determined in a separate group of animals. Morphine (5 mg/kg, *i.p*.), in the absence and presence of naloxone treatment, was used as a reference.

Anti-ulcerogenic activity

Ethanol-induced ulcer

The experiment was performed as described by Morimoto et al. (1991). In this model, rats were distributed into six groups of six animals each that had been fasted 24 h with free access of water prior to receiving an oral dose of 1 mL of vehicle (1% Tween 80 aqueous solution), and the second group was treated with lansoprazole (30 mg/ kg). The remaining four groups received 10, 30, 100 and 300 mg/kg of AML extract, respectively. One hour after the treatment, all rats received 1 mL of 99.5% ethanol induced gastric ulcer. One hour later, the animals were

sacrificed under deeply ether anesthesia. The stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with normal saline to remove the gastric contents and blood clots. The stomach was dissected for examination of the total surface area of lesions. The photographs were taken and the lesion areas were traced with a transparent graph paper to count the total surface area of lesions in mm^2.

Ethanol-induced gastric mucosal lesion in L-NAME pre-treated rats

The experiment was performed according to the method described by Arrieta et al. (2003). The rats were fasted 24 h with water *ad libitum* and randomly placed in six cages of six rats per cage. The rats were treated with 70 mg/kg (*i.p.*) of L-NAME (an inhibitor of nitric oxide synthase) or saline (*i.p.*) 30 min before administration of 1% Tween 80 (10 mL/kg), carbenoxolone (100 mg/kg) or AML (300 mg/kg). After 1 h, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction (Matsuda et al., 1999). One hour after ethanol administration, animals were killed under deeply ether anesthesia and the stomachs excised and gastric damage was determined as described previously.

Ethanol-induced gastric mucosal lesion in NEM pre-treated rats

This test was assessed using the method described by Matsuda et al. (1999). Rats were divided into six groups with six animals that fasted for 24 h with free access of water. The animals were previously treated subcutaneously with NEM (*N*-ethylmaleimide, 10 mg/kg) or saline. Thirty minutes later, the different groups received an oral dose of the vehicle (10 mL/kg), carbenoxolone (100 mg/kg) and AML (300 mg/kg). After 60 min, all groups were orally administrated with 1 mL of absolute ethanol for gastric-ulcer induction. Animals were sacrificed 1h after the administration of ethanol by deeply ether anesthesia and the stomachs excised and gastric damage was determined as described previously.

Histological evaluation

Histological evaluation of stomach was done using the method modified by Al-Bekairi et al (1992). Histological changes were divided into four categories and grades were given based on the characteristic of cell tissue stomach. First category is edema, second category is congestion, third category is hemorrhage and fourth category is necrosis. All this changes categories are measured by using graded on scale (1=normal, 2=title effect, 3=appreciable effect, 4=severe effect and 5=intensively severe effect).

Statistical analysis

The values of statistical analysis were expressed as mean values±SEM. The results of the experiment were performed as changes of percentage from control value. Data was analyzed by ANOVA followed by Dunnett's multiple comparison tests. $p<0.05$ was considered to be statistical significance.

Results

Antinociceptive activity

Acetic acid-induced writhing test

The antinociceptive effect of the AML extract on abdominal writhes of mice induced by 0.6% acetic acid is shown in Figure 1. The oral administration of AML crude extract at all dosages *i.e.* 10, 30, 100 and 300 mg/kg elicited significant inhibition and dose-dependent reduction in the number of abdominal writhes by 5.52, 34.25, 53.04 and 95.30% of inhibition, respectively, as compared with respective control value. At 100 mg/kg, AML is as potent as piroxicam (Figure 1).

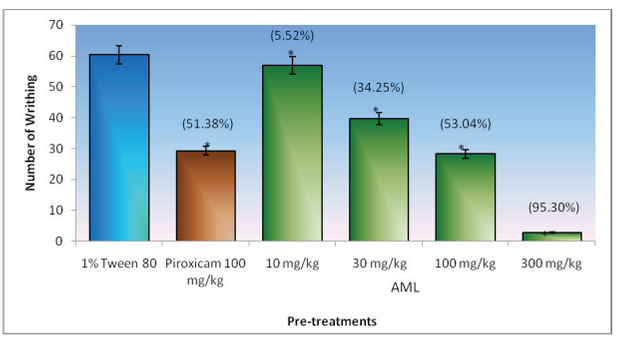

Figure 1. Percentage inhibition of acetic acid-induced writhing response in mice on various doses of leaves extract of AML and piroxicam. Data is expressed as mean±SEM of six mice. *$p<0.05$ indicates significant difference when compared with control by using ANOVA followed by Dunnett's Test.

Formalin test

The antinociceptive effects of AML on formalin test are summarized in Figure 2. This formalin test promoted a biphasic characteristic response. The time spent licking for the control group in the first phase (0-5 min) was 71.76±2.97 s and in the second phase (15-60 min) was 275.5±6.18 s. Sixty minutes after the treatment, administration of the extract demonstrated significant dose dependent inhibition in both early (13.11, 18.97, 44.01 and 82.54%) and late phase (14.09, 21.66, 47.36 and 66.21%)

at all dosage administered. The effect of morphine (5 mg/kg, *i.p.*) produced significant suppression of both early phase (51.73%) and late phase (76.61%). On the other hand, ASA only produced significant suppression in the late phase.

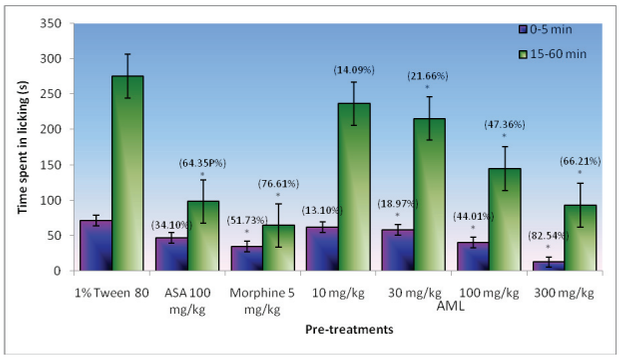

Figure 2. Effects of the ethanol extract from AML leaves on formalin-induced pain in rats. Data are expressed as mean±SEM of six mice. **p*<0.05 indicates significant difference when compared with control by using ANOVA followed by Dunnett's Test.

Hot-plate test

Antinociceptive effect of AML was evaluated by recording the reaction time of mice (licking the paw or jumping) exposed to the hot plate for every 30 min for the duration of 4 h. As shown in Table 1, the AML ethanol extract prolonged the reaction time of mice exposed to the hot plate when compared with control group. At 100 mg/kg, AML significantly increased the reaction time at 90 min (53.26%) and 120 min (53.92%). At 60 and 90 min, AML given orally at dose 300 mg/kg significantly prolonged the latency time of discomfort reaction with 50.23 and 96.17%, respectively. On the other hand, morphine started to exhibit its significant effect at 60 min and extended the latency time of discomfort reaction time until 240 min when compared to control group. On the contrary, morphine pre-treated naloxone has shown prominent attenuation of latency time of discomfort reaction prolongation when compared to morphine treated group in the hot plate test. Whilst, AML at 300 mg/kg pre-treated naloxone showed significant reduction in the prolonged latency time of reaction when compared to AML 300 mg/kg treated group. Naloxone, known as an opioid antagonist that blocked the morphine action and antagonized the antinociceptive effect of the AML extract as well.

Anti-ulcerogenic activity

Absolute ethanol induced ulcer

Significant reduction of the total area of gastric lesions in the absolute ethanol-induced ulcerogenesis in comparison to the control group was not observed in the lowest dose of the AML extract (10 mg/kg). However, at higher dosage, *i.e.* 30, 100 and 300 mg/kg, AML significantly reduced the total area of gastric lesion from 273.3±11.1 to 131.8±8.1, 67.2±6.8 and 19.7±2.9, respectively. AML exhibited its gastro protective effect

Table 1. The effect of oral administration of different doses of AML on the hot plate reaction time (s).

Treatment	Dose (mg/kg)	Latency time (min) of discomfort reaction, s, mean±SEM (n=6) (% inhibition)					
		0	60	90	120	180	240
Control AML	1% Tween 80	8.54±0.38	8.82±0.47	7.83±0.19	8.68±0.40	9.17±0.32	8.86±0.48
	10	8.82±0.49	11.13±0.67	10.17±0.96	10.83±0.64	10.90±0.92	10.57±0.49
		(3.28)	(26.19)	(29.89)	(24.77)	(18.870	(19.30)
	30	8.91±0.45	9.17±0.25	9.09±0.28	10.5±0.46	9.87±1.25	10.00±0.72
		(4.33)	(3.97)	(16.09)	(20.97)	(7.63)	(12.87)
	100	7.69±0.67	11.15±2.00	12.00±1.50**	13.36±1.03**	12.63±1.11	11.76±0.48**
		(0.00)	(26.42)	(53.26)	(53.92)	(37.73)	(32.73)
	300	8.50±0.65	13.25±0.52**	15.36±0.79***	15.28±1.30***	15.59±1.94**	12.62±0.85***
		(0.00)	(50.23)	(96.17)	(76.04)	(70.01)	(42.44)
Morphine	5	7.80±0.51	13.63±0.36**	17.90±0.43***	18.48±0.47***	14.3±0.36**	11.45±0.39**
		(0.00)	(54.54)	(128.61)	(112.90)	(55.94)	(29.23)
Morphine +	5+5	7.14±0.29	7.87±0.36***	8.30±0.34***	10.03±0.30***	8.14±0.24***	7.55±0.21***
Naloxone		(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
AML +	300+5	7.83±0.65	9.66±0.31***	10.15±0.34***	10.00±0.36**	9.01±0.38**	8.41±0.43***
Naloxone		(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)

Without naloxone, data was compared with control (Tween 80 1% 10 mL/kg). With naloxone (5 mg/kg *i.p.*), was administered 15 min prior to extract or morphine. All the results were compared with their respective test substances in the absence of naloxone. Values are reported as mean±SEM for group of six animals. The data were analyzed by ANOVA followed by Dunnett's test. Asterisks indicated statistically significant values from control. **p*<0.05, ***p*<0.01, ****p*<0.001. The independent t-test was used for comparison between two groups.

in a dose dependent manner (Figure 3). This designated that AML may possessed antioxidant properties, which was able to reduce the effect of free radicals appearance produced by ethanol and the release of free radicals can generate toxic effects and reduce the proliferation of cells.

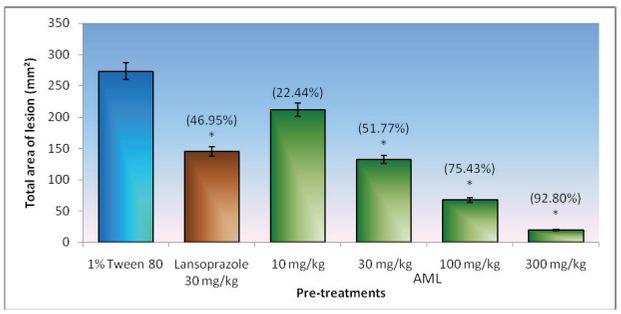

Figure 3. Effects of oral administration of *Annona muricata* leaf extract on acute gastric ulcer induced by absolute ethanol in rat. Data are expressed as mean±SEM of six mice. *p<0.05, indicate significant difference when compared with control by using ANOVA followed by Dunnett's Test.

Ethanol-induced gastric mucosal lesion in L-NAME pre-treated rats

The AML extract maintained its antiulcerogenic activity when tested on ethanol-induced ulcer in L-NAME (a NO-synthase inhibitor) pretreated rats when compared to the saline pre-treated group. At 300 mg/kg, AML reduced the ulcerative lesion from 378.7±31.22 mm² to 23.2±4.4 mm² with the inhibition of 93.88% (Figure 4a). This result is only slightly higher than the protective activity observed by pre-treatment of AML with saline as compared with the control (86.83%) (Figure 4a). This show that pre-treatment of L-NAME did not alter AML-induced protection, thus excluding role of NOs in mediating protective effect of AML. On the other hand, carbenoloxone exhibited significant difference in inhibiting the gastric ulceration area in both saline pre-treated group and L-NAME pre-treated group.

Ethanol-induced gastric mucosal lesion in NEM pre-treated rats

The results showed increased gastric lesions in control groups of pre-treatment with NEM (379.67±15.47 mm²) compared to control of saline pre-treated group (186.33±18 mm²) (Figure 4b). On the contrary, AML treatment with saline showed significantly better inhibition (82.02%) than with NEM (58.21%). Thus, this significant attenuation (p<0.001) indicated that there is a strong participation of endogenous SH in the gastro protective effects of AML. Besides that, result showed that carbenoxolone pre-treated saline group exhibited slightly greater inhibition (69.05%) than NEM pre-treated group (57.37%). Hence, this may also suggest a possible participation of -SH group in the mechanism of anti-ulcer effect of carbenoxolone.

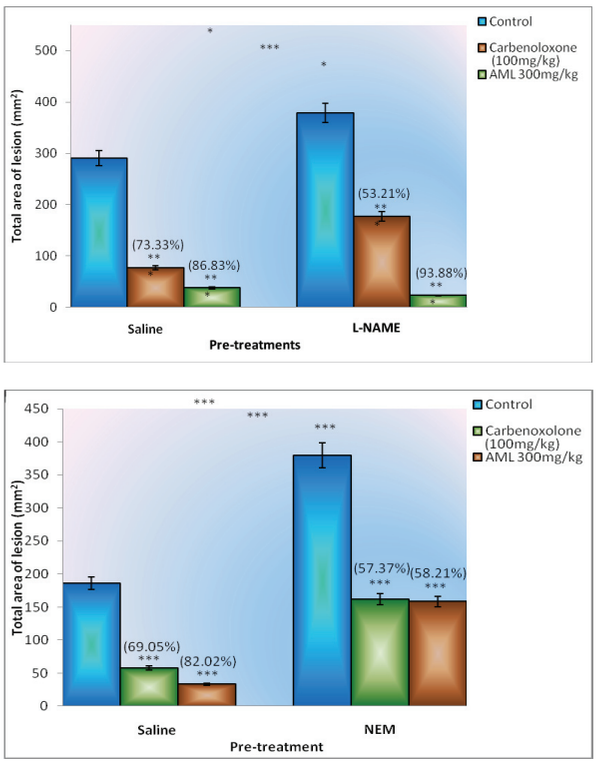

Figure 4. A. Effects of AML ethanolic extract on gastric lesions induced by ethanol in rats pre-treated with L-NAME (an inhibitor of NO synthase). Results are expressed as mean±SEM. *p<0.05, ***p<0.001 indicates significant difference from the control group by using ANOVA followed by Dunnett's test. n=6 animals; B. Effects of AML ethanolic extract on gastric lesions induced by ethanol in rats pre-treated with NEM (a blocker of SH). Results are expressed as mean±SEM. ***p<0.001 indicated significant difference from the control group by using ANOVA followed by Dunnett's test.

Histological study

Ethanol-induced ulcer

The light microscopic analysis showed a severity reduction by AML treatment in oedema, blood congestion, hemorrhage and necrosis features in a dose-dependent manner when compared to control group (Table 2). From the current study, control group showed prominent features on blood congestion, hemorrhage and necrosis. However when treated with AML 100 mg/kg, there were only slight effects of oedema, blood congestion, hemorrhage and necrosis observed from the slides compared to the control group. At the highest dose *i.e.* 300 mg/kg,

AML exhibited significant differences in mean score of blood congestion, hemorrhage and necrosis. Whilst, the positive control, lansoprazole only presented significant reduction on hemorrhage and necrosis effects. The result showed by AML at 100 mg/kg was slightly greater than lansoprazole.

Table 2. Effect of *Annona muricata* crude extract in histology study induced by absolute ethanol in rats.

Group	Oedema	Blood congestion	Hemorrhage	Necrosis
Control (1% Tween 80)	0.67±0.21	2.00±0.26	3.67±0.21	3.17±0.31
Lansoprazole (30 mg/kg)	0.50±0.22	1.00±0.37	1.33±0.21***	1.50±0.22**
AML 10 mg/kg	0.83±0.31	1.50±0.22	2.17±0.31**	2.00±0.45
AML 30 mg/kg	0.50±0.22	1.67±0.33	1.83±0.48***	1.67±0.42**
AML 100 mg/kg	0.33±0.21	0.83±0.31*	1.17±0.31***	0.83±0.17***
AML 300 mg/kg	0.33±0.21	0.83±0.31*	0.50±0.22***	0.33±0.21***

Results are presented as mean±SEM. *p<0.05, **p<0.01, p<0.005 indicates significant difference from control using ANOVA followed by Dunnett's Test.

Ethanol-induced gastric mucosal lesion in L-NAME pre-treated rats

From the histological studies, control group induced with absolute ethanol in pre-treated saline and L-NAME animals showed pronounced effects on the mean score of oedema, blood congestion, hemorrhage and necrosis. The results shown by carbenoxolone in pre-treated L-NAME group were slightly greater than carbenoxolone in pre-treated saline group. Carbenoxolone showed significant increase in the mean score of blood congestion (2.17±0.17), hemorrhage (2.00±0.26) and necrosis (1.33±0.21) in pre-treated L-NAME group compared to blood congestion (1.33±0.21), hemorrhage (1.00±0.26) and necrosis (0.67±0.21) in pre-treated normal saline group, respectively (Table 3). Nevertheless, there is no significant difference between AML at 300 mg/kg pre-treated with L-NAME and AML at 300 mg/kg pre-treated with normal saline. Thus, it may show that there is no involvement of L-NAME in the ethanol-inducer ulceration which supported the findings shown previously in the macroscopic observation.

Ethanol-induced gastric mucosal lesion in NEM pre-treated rats

Based on the microscopic observation for control group in pre-treated saline and pre-treated NEM animals, there was no significant difference shown on severity of the features in oedema, blood congestion, hemorrhage and necrosis between both group of animals. On the contrary, AML at 300 mg/kg pre-treated with NEM showed significant difference in blood congestion (1.50±0.22) and necrosis (2.00±0.26) scoring when compared to AML at 300 mg/kg pre-treated with normal saline, blood congestion (0.67±0.21) and necrosis (0.50±0.22), respectively (Table 4). Thus, this significant attenuation (p<0.05) in inhibition showed an evidence of a possible participation of SH in gastroprotection effect of AML in ulcerogenic activity.

Discussion

The antinociceptive activity of orally administered ethanol extract of AML was demonstrated in mice and rats in this study by two different chemical nociceptive test models and another model of thermal nociceptive. Acetic acid-induced abdominal writhing and formalin test represents the chemical nociceptive test model, whilst the hot plate test represents the thermal nociceptive model. Results showed that oral administrations of AML exhibited pronounced antinociceptive activity when evaluated in all the nociceptive models.

Acetic acid that injected by intraperitoneal route can lead to the peritoneal inflammation (acute peritonitis) which illustrated by contraction of the abdominal muscle in conjunction with an extension of the forelimbs and elongation of the body (Koster et al., 1959). This model was permitted to evaluate the antinociceptive activity caused by both neurogenic and/or inflammatory pain. Besides that, acetic acid writhing test is also used to evaluate the compounds for peripheral antinociceptive activities in general. Therefore, the writhing test is useful for distinguishing between central and peripheral nociception (Le Bars et al., 2001).

Acetic acid injected into peritoneal cavity is believed to be able lead to an increase of cyclooxygenase (COX) and lipooxygenase (LOX) products in peritoneal

Table 3. Effects of *Annona muricata* L. crude extract in histology study induced by absolute ethanol in rats pre-treated with L-NAME.

Group	Oedema	Blood congestion	Hemorrhage	Necrosis
Normal saline control (*i.p.*)	0.83±0.31	1.50±0.22	2.17±0.17	2.17±0.40
Normal saline (*i.p.*) + carbenoxolone	0.50±0.22	1.33±0.21	1.00±0.26	0.67±0.21
Normal saline (*i.*p.) + 300 mg/kg AML	0.67±0.21	0.83±0.17	0.83±0.17	0.50±0.22
L-NAME control	1.17±0.31	1.67±0.42	1.67±0.33	2.00±0.37
L-NAME + carbenoxolone	0.67±0.21	2.17±0.17*	2.00±0.26*	1.33±0.21*
L-NAME + 300 mg/kg AML	0.50±0.22	1.33±0.21	0.50±0.22	0.83±0.31

Results are presented as mean±SEM. *p<0.05 indicates significant difference from control using T-test.

Table 4. Effects of *Annona muricata* crude extract in histopathology study induced by absolute ethanol in rats pre-treated with NEM.

Group	Oedema	Blood congestion	Hemorrhage	Necrosis
Normal saline control (*s.c.*)	1.33±0.21	1.67±0.21	2.50±0.22	2.00±0.26
Normal saline (*s.c.*) + carbenoxolone	0.67±0.21	1.17±0.31	1.33±0.21	0.83±0.31
Normal saline (*s.c*) + 300 mg/kg AML	0.50±0.22	0.67±0.21	1.33±0.21	0.50±0.22
NEM control	1.00±0.26	1.67±0.33	2.00±0.26	2.67±0.21
NEM + carbenoxolone	0.83±0.31	1.67±0.33	1.33±0.21	1.17±0.31
NEM + 300 mg/kg AML	1.00±0.26	1.50±0.22*	1.67±0.21	2.00±0.26**

Results are presented as mean±SEM. *p<0.05 indicates significant difference from control using T-test.

fluids as well as promoting the release of other inflammatory mediators such as bradykinin, substance P, TNF-α, IL-1β, IL-8, which finally stimulate the primary afferent nociceptors entering dorsal horn of the central nervous system (Ikeda et al., 2001). The results reported herein demonstrated that the administration of the AML significantly reduced the number of abdominal writhing induced by acetic acid in a dose-dependent manner. Thus, the present result may also suggest that the mechanism of AML may be partly mediated by the inhibition of COX and/or LOX and other inflammatory mediators in peripheral tissues. Also, the antinociceptive activity of AML could also be suggested by the interruption of signal transduction in primary afferent nociceptors (Ong et al., 2011).

The formalin test is not only a very well-described method in assessing antinociceptive drugs but also useful in the elucidation of the action mechanism. The advantage of using the formalin model of nociception is that it can distinguish between central and peripheral pain components (Tjolsen et al., 1992). This test is a model of nociceptive response which can be characterized by two distinct phases involving different mechanisms. The first phase or the neurogenic pain is an intensely painful stage caused by the direct formalin action through the activation of nociceptive neurons in the periphery and the second phase correspond to the inflammatory pain occurs at the spinal cord level through the activation of the ventral horn neurons (Shibata et al., 1989).

In the present study, AML has shown to produce antinociception against both neurogenic and inflammatory phases of formalin test. The time spent in licking the injured paw was significantly reduced by oral administration of the AML in both phases. This has been supported by the administration of the morphine in this study, which acts as positive control. Morphine, the centrally acting drugs, has inhibited both phases equally in this test which is in accordance with Shibata et al (1989), whilst ASA, peripherally acting drugs, only significantly inhibited the second phase of the formalin (Figure 2). On the other hand, AML administered at 300 mg/kg, *p.o*. possessed stronger antinociceptive effect than ASA (100 mg/kg, *p.o*.) but less than morphine (5 mg/kg, *i.p.*) in the formalin. Due to the inhibitory possessions by AML on both neurogenic and inflammatory phase of the formalin-induced paw licking test, therefore it is suggested that AML may contain active ingredients which may act both centrally and peripherally.

The central antinociceptive action of the AML was supported by the result obtained in the hot plate test as it is a sensitive and specific method used to demonstrate the involvement of central mechanism (Nemirovsky et al., 2001; Sulaiman et al., 2009). In the present study, oral administration of AML has shown a significant dose-related prolongation in the latency time when induced by the thermal nociceptive stimulus. At the dose of 300 mg/kg, AML provoked the longest latency when compared with control, hence confirming the central activity of this extract (Table 1).

Therefore, the results obtained in the antinociceptive tests appear to suggest that AML possesses significant centrally and peripherally mediated antinociceptive properties. The central nociception effect may be mediated via inhibition of central pain receptors, whilst the peripheral nociception activity may be mediated through inhibition of cyclooxygenase and/or lipoxygenase (and other inflammatory mediators) (Humberto et al., 2010). These results are in regard with the reports produced by Eddy & Leimbach (1953), Williamson et al. (1996) and Koster et al. (1959) whom suggested that acetic acid writhing and hot plate test models are useful techniques for the evaluation of centrally and peripherally acting antinociceptive drugs, respectively.

Besides that, it is well described that endogenous opioid system is largely involved in the central regulation of pain, as well as in the action of opioid-derived analgesic drugs (). The present results exerted that the antinociception elicited by AML seems to be dependent of the activation of opioid system. This is postulated based on the result on a pre-treatment with a non-selective opioid receptor antagonist, naloxone. Naloxone, an opioid antagonist has shown to significantly antagonize the antinociceptive effect of AML but significantly reversed the antinociceptive effect of morphine in the hot plate test (Table 1). These findings clearly suggested that AML possesses its antinociceptive activity which was mediated through opioid mechanism. The antinociceptive effect may due to the activation of

opioid or central receptors or the modulation of the effect of endogenous opioid peptides which may participate in the antinociceptive activity at both peripheral and central levels (Ong et al., 2011).

In this study, we also utililized another model for gastric lesion induced by absolute ethanol. Gastric ulcer normally occurs in people who take non-steroidal anti-inflammatory drugs (NSAID), which is known to possess antinociceptive and anti-inflammatory effects depending on the particular drug at different doses. All of the NSAID appear to share a common mechanism, namely inhibition of cyclo-oxygenase (COX) enzyme(s), which leads to a decrease in the synthesis of various prostaglandins and thromboxanes.

It has been previously demonstrated that AML possesses an anti-inflammatory effect (Roslida et al., 2010; De Sousa et al., 2010). A search for new compounds or plant extracts with anti-inflammatory activity could represent a promising and practical approach for the management of inflammatory diseases, especially if these compounds do not harm the gastrointestinal tract after long term consumption.

Absolute ethanol can lead to severe damage of the gastric mucosa and it provokes multiple hemorrhagic red bands (patches) of different sizes along the long axis of the glandular stomach (Mincis et al., 1995). Even though the pathogenesis of ethanol-induced gastric mucosal damage is still unknown, but it may be due to stasis in gastric blood flow, which contributes to the development of the hemorrhage and necrotic aspects of tissue injury (Guth et al., 1984).

Gastric mucus acts as an important protective factor for the gastric mucosa. It is not only capable of acting as an antioxidant agent but also reducing mucosal damage mediated by oxygen free radicals. However, the protective properties of the mucus barrier depend on the gel structure and also on the amount or thickness of the layer covering the mucosal surface (Penisi & Piezzi, 1999). Therefore, antiulcer agents should provoke mucosa-strengthening effect and cicatrisation action with low occurrence of side effects. This effect is known as cytoprotection. In addition, according to the previous report, AML have antioxidant effects in different *in vitro* models (Baskar et al., 2007). Thus, these data may suggest that antioxidative effect of AML could be one of the contributory factors in this experimental model in producing its anti-ulcerogenic effect.

In the present study, the control group treated orally with ethanol clearly produced the expected characteristic zone of necrotizing mucosal lesions (Figure 3). On the other hand, oral administration of AML significantly decreased the total lesion area and the percentage of lesion. The best inhibitory effect on the ulcerative area was observed with oral treatment of AML which showed a significant ulcer reduction by 51.77% at 30 mg/kg, 75.43% at 100 mg/kg and 92.80% at 300 mg/kg, respectively, when compared to the control group. These results indicate that AML extract displays a dose dependable anti-ulcerogenic effect which relates with cytoprotective activity, since it significantly reduced the absolute ethanol-induced ulcer. In addition, AML is as potent as lansoprazole at dose of 30 mg/kg (Figure 3). The preliminary phytochemical screening showed that the presence of tannins, flavonoids and triterpenes in AML may exert its effect in preventing gastric damage development on gastric mucosal (John & Onabanjo, 1990; Gracioso et al., 2002; Gonzalez & Di Stasi, 2002; Lewis & Hanson, 1991).

Studies designate the involvement of NO, an endogen substance that enhances the mechanisms of cytoprotection of mucosa observed experimentally by promoting blood vessel dilatation and reducing lipoperoxidation while acting similarly to an anti-inflammatory drug (Ancha et al., 2003). It is also well recognized that NO was involved in the modulation of acid and alkaline secretion, mucus secretion and gastric mucosal blood flow (Chandranath et al., 2002). Besides that, nitric oxide also modulates the secretion of some cellular growth factors, primarily the epidermal growth factor (Elliot et al., 1995).

In order to investigate the role of endogenous NO in cytoprotection, we used the NOS inhibitor, L-NAME to investigate the protective effect of AML on ethanol-induced gastric haemorrhagic lesions. Pre-treatment with L-NAME did not alter the cytoprotection induced by AML. Oral administration of AML (300 mg/kg) to L-NAME (70 mg/kg) - pre-treated animals produced a reduction (23.2±4.4 mm2) in the gastric haemorrhagic lesions when compared with the L-NAME-pre-treated control value of 378.7±31.2 mm² (Figure 4b). This gastro protective effect of AML observed by pre-treatment with L-NAME was very similar to the protective activity observed by pre-treatment with saline (86.83%). This result showed that the previous administration of L-NAME, a NO-synthase inhibitor did not alter the AML-induced cytoprotection of ethanol-induced gastric lesions, thus it is possible to affirm the exclusion role of endogenous NO in mediating cytoprotective effect of AML.

On the other hand, carbenoxolone has shown the opposite result when it significantly altered the anti-ulcer effect in L-NAME pretreated groups compared to its antiulcer effect in saline pretreated group (Figure 4b). This may postulate a possible involvement of NO groups in its mechanism of action as a cytoprotective agent.

Besides NO, the role of endogenous SH compounds in mucosal protection has been demonstrated in ethanol-induced gastric injury where the development of damage was accompanied by a lowering in production of mucosal SH compounds (Avila et al., 1996). The

synthesis of mucus which strengthens the mucosa barrier against harmful agents also plays its vital role in gastric production. The continuous adherent mucus layer is also a barrier to luminal pepsin, thus protecting the underlying mucosa from proteolytic digestion (Allen & Flemstrom, 2005).

Therefore, we investigated the possible involvement of endogenous SH in the gastroprotective effect of AML extract by pre-treating animals with NEM (a SH-blocker) in gastric lesion induced by ethanol. Pre-treatment with NEM alters the cytoprotection induced by AML, yet, pre-treatment of animals with NEM noticeably increased the gastric lesions when compared to control groups. The animals treated with AML (300 mg/kg) reduced the gastric lesion almost by one third (82.02% with saline versus 58.21% with NEM). This significant (p<0.001) attenuation of gastric lesions indicated the strong involvement of endogenous SHs in the gastroprotection effects of AML. This might be due to the anti-oxidative compounds in the extract (Baskar et al., 2007).

SH compounds may be involved in scavenging oxygen derived free radicals and controlling the production and nature of mucus (Allen et al., 1984, Salim, 1993). The SH compounds bind to the free radicals that are formed following tissue injury by noxious agents. These agents may also protect mucus, since mucus subunits are joined by disulfide bridges, if reduced, render mucus water-soluble. Therefore, the anti-oxidative compounds present in AML can prevent the loss of gastric mucus and non-protein SH, since they are able to remove free radicals formed due to ethanol-induced mucosal ulcer (Avila et al., 1996).

In the histology study, histological changes of ethanol-induced gastric lesion were focused on four features in cell which are oedema, congestion, hemorrhage and necrosis. Administration of ethanol can cause congestion of blood vessel, intense damage of gastric mucosa and it induces multiple different sizes hemorrhagic red bands (patches) and finally leads to necrosis.

In ethanol-induced ulcer model, a significant difference in the hemorrhagic is exhibited in AML treated group at all doses (*i.e*. 10, 30, 100 and 300 mg/kg) when compared with control group (Table 2). AML at higher dosage (*i.e*. 100 and 300 mg/kg) demonstrated significant reduction of severity in blood congestion effect when compared to the control group (Table 2). Furthermore, when treated with AML at 30, 100 and 300 mg/kg, it significantly reduced the effect of necrosis by the inhibition of 47.32, 73.82 and 89.59%, respectively (Table 2).

Treatment of AML in NEM pretreated animals significantly showed the reduction of the gastric mucosal protection against ethanol-induced depletion of SH contents (Figure 4b) but there was no significant reduction observed between L-NAME and saline pretreated animals (Table 3). In NEM pretreated animals treated with AML, there were a significant increased in the mean score of blood congestion and necrosis when compared with saline pretreated group treated with AML. This is in agreement with a few studies which have been reported previously that ethanol-induced gastric lesions will lead to decrease of sulfhydryl compounds in the gastric mucosa (Szabo *et al.*, 1981; Miller et al., 1985 ; Tariq & Aqeel, 1990

Therefore, from the current histology result in ethanol-induced gastric mucosal lesion treated with AML in NEM-pretreated rats, there was significant increment of certain inflammatory features, indicating the worsening of the ulcer when compared with the group treated with AML in saline pretreated group. Indeed, this indicates a strong involvement of SHs pathway in gastroprotection of AML (Figure 4a) which also in agreement with the result of macroscopic done previously.

In conclusion, AML ethanolic extract possesses significant antinociceptive effect on both peripheral and central mechanisms as well as the involvement of opioid mechanism. AML showed its significant inhibitory effect on both acetic acid-induced writhing and formalin test. Besides that, AML also exerted antinociceptive effect in the hot plate test which is a specific central antinociceptive test and this effect was antagonized by naloxone, an opioid antagonist. Therefore, we may suggest that it is possible that AML also perform its antinociceptive effect via central opioid receptors or promoted release of endogenous opiopeptides (Hosseinzadeh et al., 2000). In addition, AML has been observed to exhibit its antiulcerogenic effect in dose-dependent manner which relates to cytoprotective and antioxidant properties. We postulated that AML produced significant inhibition in ethanol-induced ulcer model by decreasing the formation of free radicals. Furthermore, from the data obtained, the gastroprotective effect of AML in ethanol-induced ulcer model was found to be mediated not by the NO pathway, but the involvement of endogenous sulfhydryl that increase the defence mechanism of the gastric mucosa against aggressive factor in the stomach. This might be due to the similar anti-oxidative compounds reported in the plant previously (Baskar et al., 2007). In this experiment, the exact nature of the active compound has yet to be determined. Thus, it is worthwhile to further investigate the pharmacological properties of AML, especially, in identifying the composition of the active compound in order to help in building a profile of its bioactive constituents.

Acknowledgment

The authors wish to thank the Faculty of Medicine & Health Sciences, Universiti Putra Malaysia for the facilities and funding (Project no : 04/01/07/1019RU) provided for the study.

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