INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 9, No. 1, p. 255-267, 2016

RESEARCH PAPER OPEN ACCESS

Effects of *Annona squamosa* leaf extract on *Mus musculus* exposed to mercury acetate

Joycelyn C. Jumawan[1,2*], Edna A. Amparado[2]

[1]*Biology Department, Caraga State University, Butuan City, Agusan Del Norte, Philippines*

[2]*Institute of Biology, University of the Philippines-Diliman Quezon City, Philippines*

Key words: *Annona squamosa*, Oxidative stress, Mercuric acetate.

http://dx.doi.org/10.12692/ijb/9.1.255-267 Article published on July 30, 2016

Abstract

The effects of solo and combinatorial exposure to ethanolic extract of *Annona squamosa* Linn. leaves and mercuric acetate (MA) were studied in gestating ICR mice and their fetuses *in-utero*. Results show that mercuric acetate significantly increased maternal mortality and fetal resorption index while inducing decreased fetal size. Exposure of mercuric acetate and leaf extract doses significantly enhanced this effect. Solo exposure of ethanolic extract at 25 mg/kg body weight induced abortion and resorption of embryos to a lesser extent than combinatorial exposure. Lipid peroxidation assay reveal lower MDA values in liver and brain of maternal mice exposed to solo and combinatorial exposure but not with their fetal counterpart. Maternal mice and fetuses exposed to leaf extract of *A. squamosa* with MA show marked hepatic and neurologic histological injury similar to positive control. The results suggest that *A. squamosa* leaves have the potential to protect against mercury-induced oxidative stress in maternal mice but may not be applied during gestation

* **Corresponding Author:** Joycelyn C. Jumawan ✉ joycejumawan@gmail.com.

Introduction

Antioxidants provide protection to living organism from damage caused by the uncontrolled production of ROS-concomitant lipid peroxidation, protein damage, and DNA strand breaking (Quig, 1998; Ercal *et al.,* 2001). These interfering effects of anti-oxidants on the damaging effects of sulfhydryl reactive metals have significant consequences on the developing embryo. It has been proposed that plants with potent antioxidant properties may also have the potential to interfere and mediate the effects of heavy metal toxicity (Patrick, 2002; Flora *et al.,* 2008; Woo *et al.,* 2009).

Annona squamosa Linn. locally known as sugar apple is widely popular for its edible fruit and for its use as powerful pesticide (Chang *et al.,* 1998; Hopp *et al.*, 1998; Kempraj and Bhat, 2011). The ethanolic extracts of its leaf has been reported to have hypoglycaemic and anti-diabetic effects (Gupta *et al.,* 2005; Gupta *et al.,* 2008; Shirwaikar *et al.,* 2004). *In vitro* studies utilizing ethanolic and flavonoid positive fractions have shown that its leaves and seeds have antioxidant, chemo-preventive and chemo-therapeutic activities (Punzalan-Brady, 2008) with flavonoids exhibiting the highest inhibitory activity when assayed for the ability to scavenge diphenylpicrylhydrazyl (DPPH) free radical (Punzalan *et al.,* 2006). The water extract of *A. squamosa* leaves possessed antioxidant activity as shown by increased activities of scavenging enzymes, catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione reductase (GR) and glutathione-transferase (GST) and decreased malondialdehyde levels in various organs of Winstar rats (Gupta *et al.*, 2008). The roots and seeds of the plant are traditionally used to induce abortion (Goswami *et al.,* 2011; Kumar *et al.,* 2012).

The possible maternal-fetal protective attenuation of *A. squamosa ethanolic* leaf extract against mercury-induced oxidative stress during gestation has not been explored so far. This study aims to explore possible effects of the extract to minimize congenital hepatic and neurotoxic effects of mercuric acetate on fetus exposed *in utero*. Findings of this study could offer new insights on its protective function on embryonic development.

Materials and methods

Preparation of leaf extract

Mature leaves of authenticated *A. squamosa* were collected from the forests of Morong, Bataan and later processed at the Institute of Biology, University of the Philippines. Leaves were deveined, rinsed and shade-dried until crispy. Forty-five (45) grams of the dry, powdered material was immersed in 95% analytical grade ethanol for 48 hours. The mixture was filtered and evaporated to dryness using a rotary evaporator. The resulting product was lyophilized and was reconstituted in deionized water to make a stock solution of 25 mg/ml and 75 mg/ml (Gupta *et al.,* 2008).

Test agents

Deionized water was used as negative control treatment. 4 mg/kg of mercuric acetate (MA) (Fisher Scientific Company) was used as positive control. Twenty-five mg/kg of ethanolic leaf extract from *A. squamosa* was used to test the independent effect of the extract. Two other extract doses (25 mg/kg and 75 mg/kg) of *A. squamosa* were used in combinatorial treatment with 4 mg/kg mercuric acetate (MA) as test treatments.

Animal Stock

Fifteen (15) adult breeder males (>6 months old; 28-33 g) and thirty-six (36) female (8 weeks old; 28-31 g) ICR strain white mice *Mus musculus* purchased from Bureau of Food and Drugs, Animal Research Division were used in this study. Mice were fed with standard high protein pellets and water *ad libitum* and maintained under laboratory conditions (temperature 26±2°C, 12h natural light/dark cycle).

Breeding and treatment set-up

Adult females were initially induced to estrus 72 hours prior to exposure to males. Females undergoing estrus were then randomly paired overnight with sexually experienced males in 2 female: 1 male ratio

per cage. Successful mating was confirmed by the presence of vaginal plugs, the observance of which was considered as gestational day 0 (GD 0) of pregnancy. Subjects were randomly partitioned into 6 groups with 6 vaginal plug-positive females as replicates and treated as follows: Group 1: distilled water *ad libitum* (negative control 1; -C1); Group 2: subcutaneous injection of distilled deionized water at 10 ml/kg BW (negative control 2; -C2); Group 3: 4 mg/kg BW mercuric acetate (MA) in distilled deionized water medium; Group 4: ethanol leaf extract of *A. squamosa* (ELEAS) at 25 mg/kg BW; Group 5: 25 mg/kg ethanolic leaf extract of *A. squamosa* and 4 mg/kg BW mercuric acetate and Group 6 : 75 mg/kg ethanolic leaf extract of *A. squamosa* and 4 mg/kg mercuric acetate. The extracts and vehicles were given subcutaneously for three consecutive days starting at GD 14 as gestation is already prominent, and passage of mercuric acetate in the mice placenta is enhanced at this stage. Females were sacrificed on GD 19, and the numbers of viable and resorbed fetus and resorption sites were recorded.

Gross anatomical examination, morphometry and isolation of vital organs

Pregnant mice were weighed daily to monitor weight changes. On GD 19, isolated fetuses were examined for morphologic abnormalities such as hydrocephalus, oedema, cleft lip and palate. The number of fetuses in the uterus/dam were recorded, and their corresponding weights taken. Percent embryonic loss after implantation was calculated as (number of implantations- number of fetuses in development/ number of implantations) x 100 and used as a measure of toxicity effect (Khan *et al*., 2004). The mortality rate of pregnant mice during treatment per group was calculated using the formula: Mortality rate: (Number of deaths/Treated females) x100. Morphometry of individual fetuses was measured using a digital caliper. The following indices were measured—crown-rump length (CRL) as the measurement from the skull vertex (crown) to the midpoint between the apices of the buttocks (rump); head-lip length (HdLL) as the measurement from the back of the head to the tip of the recognizable lip, forelimb length (FL) as the measurement between the top and the tip of the forelimbs and hind-limb length (HL) as the measurement between the top and tip of the recognizable hind limb.

Thiobarbituric acid reactive substances (TBARS) assay for lipid peroxidation

Lipid peroxidation assay (Ohkawa *et al.,* 1979) of the liver and brain of the maternal mice and their fetuses were done. Liver and brain samples were isolated and processed rapidly after sacrifice at GD 19. Samples were weighed and homogenized in a glass tissue grinder using 0.05M phosphate buffer solution to yield a concentration of 100 mg net tissue weight per mL of the homogenizing medium. 0.5 mL of the homogenate was added to 2.5 mL trichloroacetic acid (TCA) and one mL of thiobarbituric acid (TBA) and the resulting mixture vortexed. Test tubes containing the mixture were placed in boiling water (100°C) for 30 minutes, cooled to room temperature and added with four mL n-butanol. The mixture was vortexed, and the n-butanol layer was centrifuged at 3300 rpm and 25°C for 10 minutes. The absorbance of the organic layer was measured spectrophotometrically at 535 nm.

Histological preparation and microscopic observation

Brain and liver from the maternal and fetal samples were fixed in 10% buffered formalin and subjected to routine histological preparation.

Three sections from three maternal and fetal samples for each treatment group were examined using a light microscope. Histology of cross sections from the cerebral cortex, cerebellum and hippocampus were done to assess neurotoxicity of the brain. % of the occurrence of any form of pathology or lesion was noted for each treatment group. Liver hyperplasia was scored manually by counting the number of nuclei in a liver area of 25,500 μm2 (mean of 5 counts/ section). Frequencies of occurrence of necrotic, hypertrophic cells or steatosis per replicate section were noted.

Statistical analysis

Parameters in percentages (%) - Mortality rate, and % resorption were subjected to nonparametric Kruskal-Wallis test to determine dose or treatment response. Analysis of variance (ANOVA) was utilized to determine differences between treatment groups for values such as maternal weight gain, fetal size, fetal weights, morphometric measurements and MDA (nmol protein^{-1}) values. When the ANOVA revealed significant differences among treatment groups, Duncan's multiple range test (DMRT) was used to particular treatment differences. Differences were considered statistically significant if < 0.05.

Results

Uteri of the dams treated with MA were seen with resorbed embryos or resorbed sites and swellings that marked previous implantations (Figure 1) even prior to GD 19. Some representative samples treated with MA and combinations with ELEAS had 100% resorption of embryos. Maternal mortality in mice treated with solo ELEAS is significantly lower than those treated with MA or in the combination of the two doses. Nonetheless, this relatively "improved" response is still significantly lower compared to the negative controls across all indices ($p<0.05$) (Table 1).

Table 1. Reproductive indices/outcomes in the maternal and fetal *Mus musculus* exposed to varying concentrations of ethanolic leaf extract from A. squamosa (ELEAS) and mercuric acetate (MA) and their combinations.

Indices	Maternal Wt.gain (g)	Fetal size (n)	Fetal Wt. (g)	Resorption Index (%)	% death	CRL (mm)	HdLL (mm)	FL (mm)	HL (mm)
-Control 1	15.50±1.65^{a}	11.83±1.64^{a}	2.05±0.14^{a}	0	0	20.9± 0.70^{a}	10.27±0.87^{a}	7.43±0.40^{a}	8.10±0.70^{a}
Control 2	15.83±2.13^{a}	10.00±1.24^{a}	1.97±0.06^{a}	4.74	0	19.52±1.55^{a}	10.88±0.53^{a}	8.06±0.12^{a}	7.40±0.05^{a}
4mg/kg MA	4.67±1.98^{c}	4.17±1.71^{c}	1.00±0.014^{b}	50	50	14.80±1.27^{b}	8.93± 1.29^{b}	6.53±0.53^{b}	6.00±0.47^{b}
25mg/kg ELEAS	13.33±2.26^{a}	8.00±2.11^{b}	1.38±0.12^{b}	40	17	13.90±0.92^{b}	7.73± 0.41^{b}	5.96±0.44^{b}	5.90±0.25^{b}
MA+25mg/kg ELEAS	6.33±1.94^{b}	5.83±1.75^{c}	1.15±0.12^{b}	53	33	10.97±0.42^{c}	6.46± 0.16^{b}	4.36±0.89^{b}	2.23±0.17^{c}
MA+75mg/kg ELEAS	6.17±2.94^{b}	3.83±1.83^{c}	1.00±0.05^{b}	56	50	12.09±0.19^{c}	6.92±0.35^{b}	5.18±0.35^{b}	4.69±0.42^{c}

Means with different letters (columns) are significantly different from each other ($p<0.05$).

Comparison of morphometric variables reveals that fetuses treated with MA and the two doses of ELEAS were significantly smaller than the negative control (Table 1; Figure 2). However, no abnormal phenotypes such as cleft palate or cleft lip, or deformed digits were observed in fetuses gestational exposed to solo ELEAS. Abnormal swellings/outgrowth in the neck portion of embryos in the simultaneous exposure of MA+75 mg/kg ELEAS were observed.

Comparison of oxidative stress response showed that all treatments have significantly higher MDA (nmol mg protein^{-1}) levels compared to the negative controls (Table 2; Figure 3).

Table 2. MDA (nmol protein $^{-1}$) levels through lipid peroxidation assay in maternal and fetal brain and liver.

TREATMENT	BRAIN		LIVER	
	Maternal	Foetal	Maternal	Foetal
-Control 1	0.22±0.16^{c}	0.06±0.40^{d}	0.26±0.21^{d}	0.18±0.46^{c}
- Control 2	0.17±0.01^{c}	0.18±1.36^{d}	0.23±0.66^{d}	0.22±0.15^{c}
4mg/kg MA	1.10±0.014^{a}	0.54±1.90^{b}	1.13±1.29^{a}	0.70±0.76^{a}
25mg/kg ELEAS	0.42±0.18^{b}	0.49±0.72^{c}	0.34±0.55^{c}	0.15±0.84^{c}
MA+25mg/kg ELEAS	0.47±0.12^{b}	0.31±0.82^{c}	0.72±0.21^{b}	0.75±0.76^{a}
MA+75mg/kg ELEAS	0.56±0.08^{b}	0.73±0.21^{a}	0.86±0.39^{b}	0.54±0.67^{b}

Means with different letters (columns) are significantly different from each other ($p<0.05$).

Comparison however between maternal-fetal brain and liver MDA levels show that maternal mice treated with ELEAS have significantly higher MDA values ($p<0.05$) compared to their fetuses were MDA levels were not significantly different from the positive control ($p>0.05$). MDA levels of the fetal brain and liver in the solo treatment of 25 mg/kg ELEAS were significantly smaller compared to those gestational exposed to MA. MDA values of the latter two treatments were significantly lower than MA ($p<0.05$).

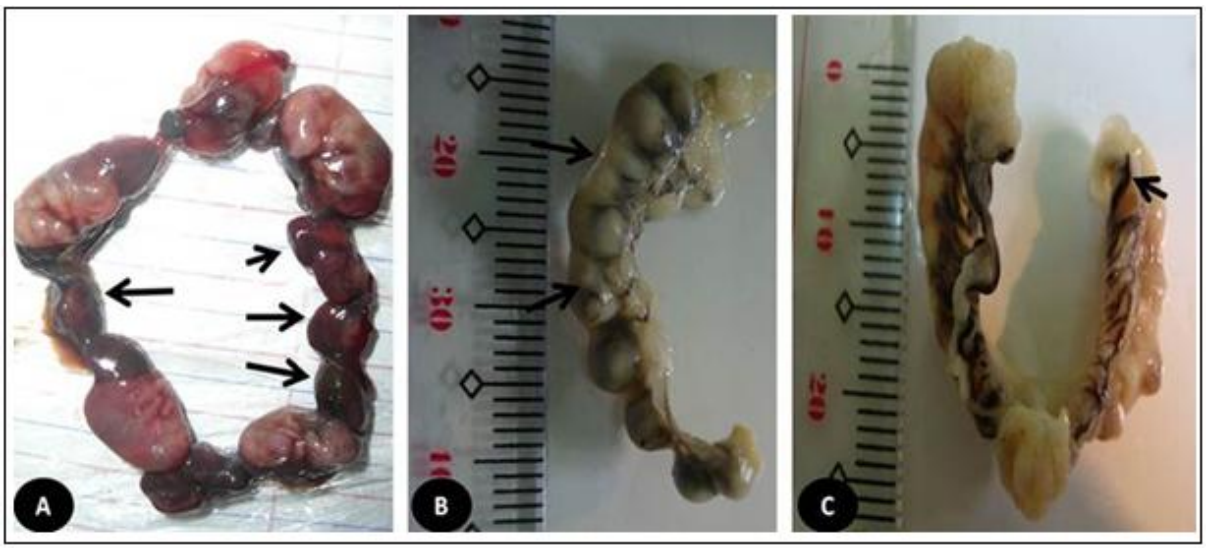

Fig. 1. A. Resorbed embryos (arrows) in a female treated with 4 mg/kg MA (GD 17). B. 100% fetal resorption in pregnant mice treated with MA + ELEAS (GD 17) C. 100% resorption in a dam treated with MA +75mg/kg ELEAS.

Maternal liver exposed to single and combinatorial treatments of MA and ELEAS show varying histologic injury compared to the negative controls (Figure 4). Maternal liver exposed to MA showed consistent dissociation of necrotic hepatocytes (Figure 4 C, D). This pathological observation seemed to be enhanced by the subsequent treatment of 25 and 75 mg/kg ELEAS where vacoulations and massive steatosis were observed (Figure 4. E, F). Solo treatment of 25 mg/kg ELEAS also caused the minimal incidence of pathologies in the liver (30%). Combinatorial treatment of MA and ELEAS resulted in mixed lympho-monocytic infiltrations as well as the formation of cell aggregates (Figure 4 G, H).

Observations of histologic injury in the fetal liver were higher (65%) than in the maternal counterpart (32%). Hepatocellular necrosis and abundance of neutrophils near terminal hepatic venules in the fetal liver exposed *in utero* to MA are of frequent occurrence in many samples (Figure 5 D, F). Steatosis, vacuolations and invasion of megakaryocytes and neutrophils especially near venules and extravasation of red blood cells were consistently abundant in combinatorial treatments of MA with the two doses of ELEAS (Figure 5 C, D).

Maternal mice exposed to MA exhibited decreased density of neurons in the hippocampus (Figure 6). Observations of cerebellar sections in the maternal brain exposed to MA reveal consistent hemorrhagic lesion in the white matter which was also consistently observed in the MA+75mg/kg ELEAS combination (Figure 6 B, C). Distinct vacuolations, irregularly dispersed Purkinje cells and occurrence of neoplastic aggregation of cellular mass in the combinatorial exposure were also observed in the maternal brain (Figure 6 F).

In-utero exposure of the fetus to MA and combinations of low and high doses of ELEAS destroyed the cellular integrity in the fetal brain

(Figure 7). Extensive loss and degeneration of neurons exposed to MA (Figure 7 B) and pronounced disintegration and pyknosis in combination treatments of MA and ELEAS at 25 mg/kg and 75 mg/kg were consistently observed (Figure 7 C). Frequent observations of abnormal lesions along with mild and severe vacuolations of cells in fetal brain exposed *in utero* to low and high combinations of ELEAS with MA were also observed (Figure 7 H, I). The occurrence of pathology in the fetal brain is more frequent (76%) compared to pathologies in the maternal brain (49%).

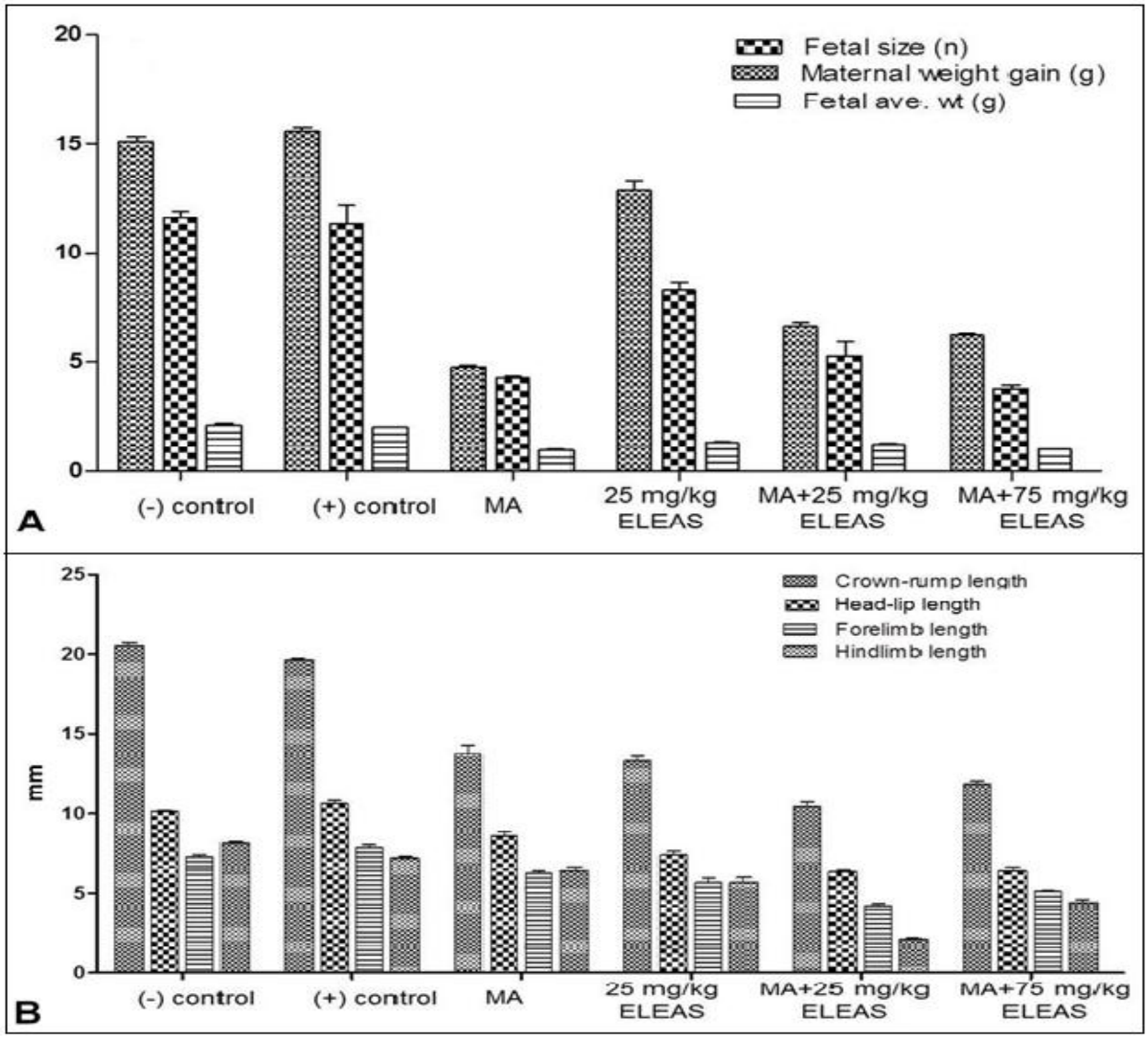

Fig. 2. Morphometric (A) and reproductive (B) indices of maternal and fetal *Mus musculus* exposed to ethanolic leaf extract from *A. squamosa* (ELEAS).

Discussions

The present study evaluated the effects of ethanolic leaf extract from *A. squamosa* in mercuric acetate-induced oxidative stress and the possible attenuation of the antioxidant properties of the extract in the reproductive response and maternally-relayed effects to the developing liver and brain of the fetus. The adapted dosage of *A. squamosa* (25 mg/kg and 75 mg/kg) from anti-diabetic studies (Gupta *et al.*, 2008) was shown to cause toxicity and some incidence of fetal resorption in this study. Although the researchers are yet to explore the effects of single exposure of 75 mg/kg ELEAS in the future, observations on incidence of fetal resorption on the much lower dose (25 mg/kg ELEAS) indicate that this dosage may not be appropriate during gestation and that *A. squamosa* may have a potential abortifacient effect if treated at GD 14 onwards in mice. The effects

of the lower and higher doses of this extract should be investigated in subsequent studies. The seed extract of *A. squamosa* at high concentrations (300–600 mg/kg BW) did not interfere with the reproductive performance of pregnant rats exposed at GD 10 (Damasceno *et al.*, 2002). Further, the active lipid-soluble annonin in the seeds might be the active component responsible for the abortive and anti-implantation activities when given to pregnant rats (Gale, 1974).

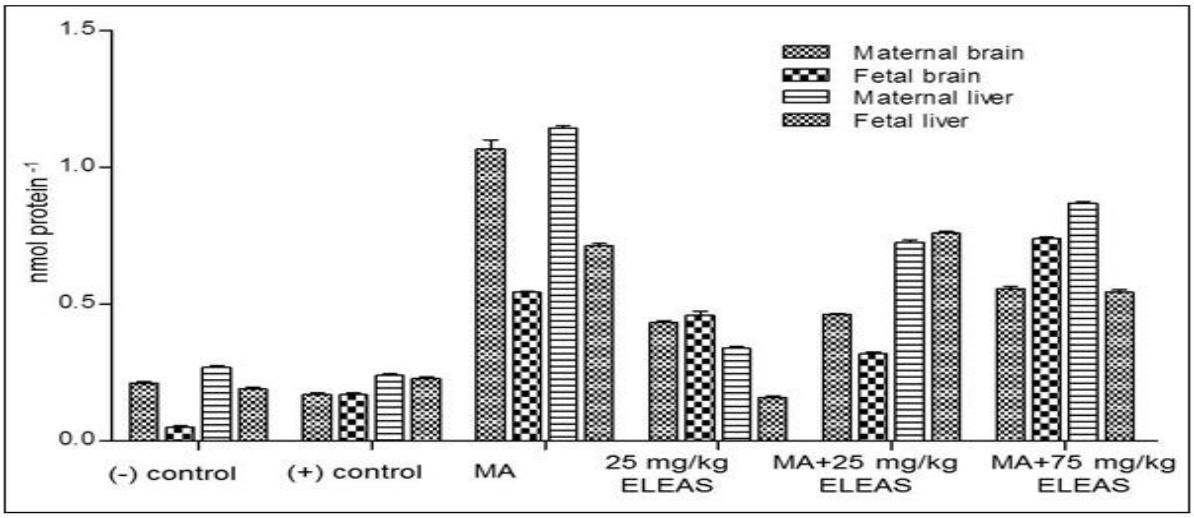

Fig. 3. Malondialdehyde (MDA) levels (nmol protein^{-1}) through lipid peroxidation assay in the maternal and fetal brain and liver.

It was likely that the malformations seen in the combinatorial exposure of MA+ELEAS might have been caused by MA since no distinct form of teratogenesis was observed with exposure to the plain leaf extract of *A. squamosal*. Oral exposure of mercuric chloride at a lower dose (0.25-1.0 mg/kg) produced adverse effects on the reproductive performance of mice in the absence of overt mercury toxicity (Khan *et al.,* 2004). Pregnant hamsters gavaged with a single 22 mg/ kg of mercuric acetate at GD 8 resulted in increased incidence of resorption of embryos (Gale, 1974) while dose-related reduction of mean litter size resulted from treatment of male rats with 1, 2.5, or 5 mg of mercury/kg/day of methyl mercuric chloride prior to mating (Khera, 1974).

Mercury has long been identified to cause several effects in early mammalian development. Methylmercury (MeHg) is a potent neurotoxin that affects the fetus at even low and medium prenatal and postnatal exposure to MeHg results in locomotor, motor coordination and learning deficits (Glover *et al.,* 2009). Apart from leaking through the placental barrier, mercury can also be concentrated in the brain of the developing fetus resulting in teratogenesis in affected embryos (Yoshida *et al.,* 2002). Mercury can also affect the detoxifying activity of the liver resulting in various forms of hepatoxicity (Strubelt, 1996; Quig, 1998; Sausen de frietas *et al.*, 2009).

Oxidative stress has been pointed out as an important molecular mechanism in methyl mercury (MeHg) intoxication (Ali *et al.,* 1992; Ercal *et al.,* 2001). The hazardous effects of MeHg are well known and seem to be related to thiol depletion that, in turn, can lead to an increase in intracellular oxidative stress (Husain *et al.,* 1987).

Combinatorial treatments of MA and *A. squamosa* that showed significantly reduced MDAs reflect that it was able to counteract the oxidative stress induced by MA exposure. This result affirms the antioxidant activity of the plant as reported (Gupta *et al.,* 2008) and the hepatoprotective effect of the alcoholic extract on experimentally-induced liver injury in rodents (Raj *et al.,* 2009; Saleem *et al.,* 2008; Saleem *et al.*, 2011).

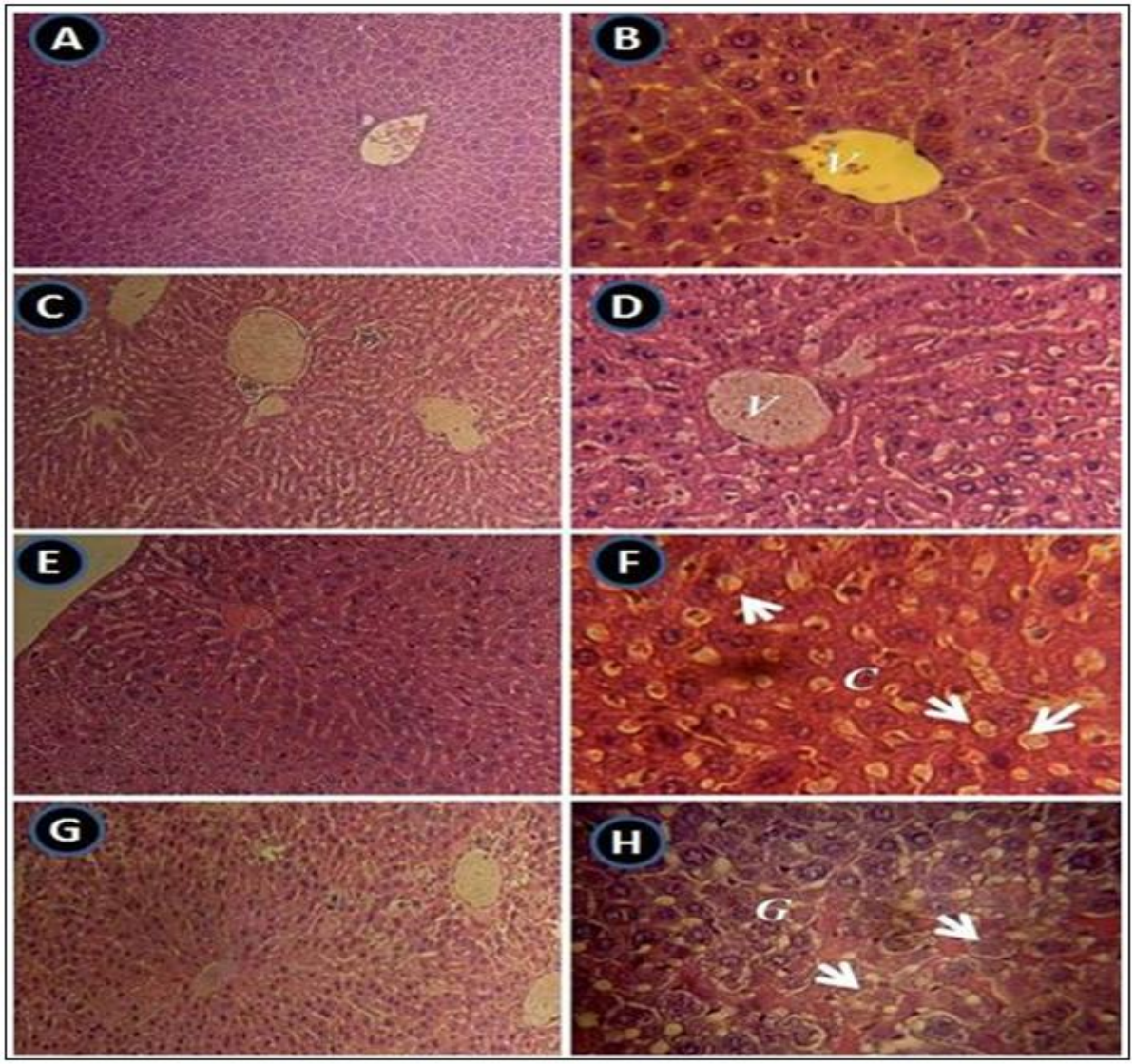

Fig. 4. Maternal liver exposed to solo and combinatorial treatments of MA and ELEAS. A-B. Liver from negative control; C-D. Maternal liver exposed to MA; E-F. Maternal liver treated with 25 mg/kg ELEAS; G-H. Maternal liver exposed to combinatorial treatment of MA and ELEAS. C, white arrows in F- fatty exchange. G,white arrows in H-aggregates. (H&E stain; A,C,D,G=100X; B,D,F,H=400X).

While it is noted that the placenta can enhance the passage of mercury through this barrier system (Ercal *et al.,* 2011; Goyer, 1990; Yoshida, 2002), the metal-binding protein metallothionein may also play a significant part in this response. The probable unregulated passage of mercury in the fetal brain may have profound adverse effects later in development. Prenatal exposure to MeHg disrupts the postnatal development of the glutathione antioxidant system in the mouse brain, pointing to an additional molecular mechanism by which MeHg induces pro-oxidative damage in the developing CNS and the liver (Stringari *et al*., 2008).

The results of the present study reinforce that mercuric acetate-mediated oxidative stress plays a crucial role in metal-induced neurotoxicity. The hippocampal pathology observed in this study upon exposure is a common manifestation of mercury-induced toxicity (Flora *et al*., 2008). Cognitive studies of prenatally exposed mice to MA were shown to have spatial and cognitive defects that may persist throughout life (Stringari *et al.,* 2008). Chronic intrauterine exposure to low-dose MeHg induces a decrease in neuron population in the limbic system, and the offspring may have impaired higher brain function (Kakita *et al*., 2000).

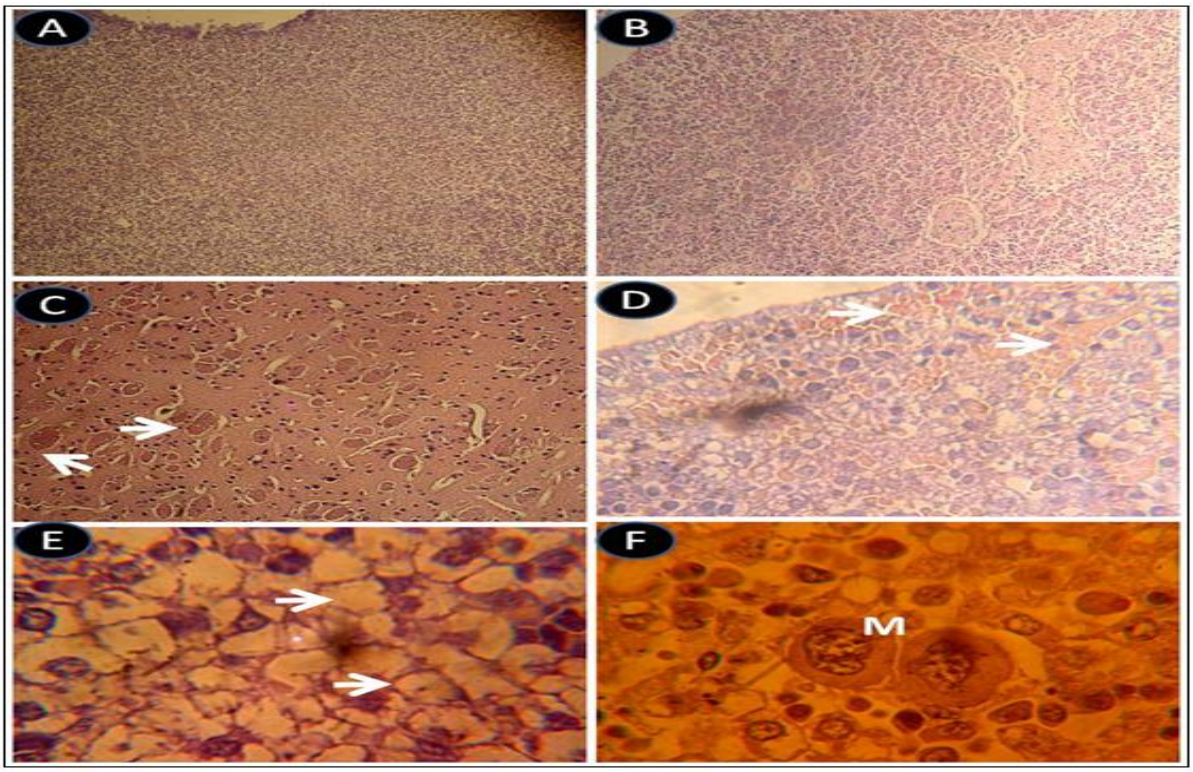

Fig. 5. Fetal liver exposed in utero to single and combinatorial treatments of MA and ELEAS. A. Liver from negative control; B. Fetal liver gestationally exposed to 25 mg/kg ELEAS; C-D. Combinatorial exposure of MA +25mg/kg ELEAS. C. Clumping of cellular mass in some hepatic lobules (white arrows). D. Extravasation of red blood cells (white arrows). E-F. Combinatorial exposure of MA +75mg/kg ELEAS. E. Steatosis and vacuolations (white arrows). F. Invasion of megakaryocytes (M). (H&E stain; A-B=100X; C-D, F= 400X; E= 1000X).

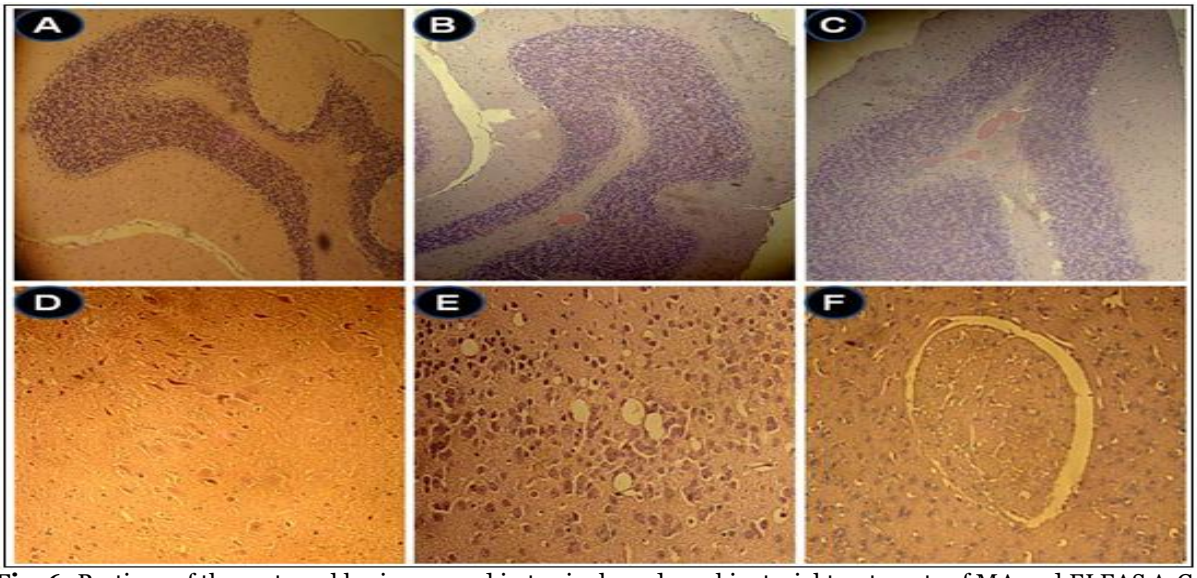

Fig. 6. Portions of the maternal brain exposed in to single and combinatorial treatments of MA and ELEAS.A-C. Representative section at the region of the rostral cerebellum. A. Normal distinctive architecture between the granular and molecular layers; B-C.Hemorrhagic lesion in the white matter (white arrow) in a cerebellar section exposed to MA, consistently observed in the MA + 75mg/kg ELEAS; D. Cerebrum showing normal histology. E. Defined vacoulations and irregularly dispersed purkinje in the same region of a brain exposed to MA; F.Frequent observations of neoplastic aggregation of cellular mass (white arrows) in the combinatorial treatments of MA+ ELEAS.(H&E stain; A-C= 100X; D-F= 400X).

Results of the present study suggest that *A. squamosa* may have the potential to rescue the maternal liver and the brain from oxidative stress, however, possible attenuation in combinatorial treatments should not be applied in gestating females as it may not be able to reduce oxidative stress and it can in fact cause premature farrowing, abortion and resorption at a certain degree therefore compromising the development of the fetus at the expense of protecting against maternal metal-induced toxicity. The noteworthy facets of the extract—the protective capacity of the extract against metal-induced oxidative stress as well as its potential to be abortifacient in gestating mice requires further studies to elucidate if these two facets are separate mechanisms and the active agents in this plant responsible for such developmental outcomes.

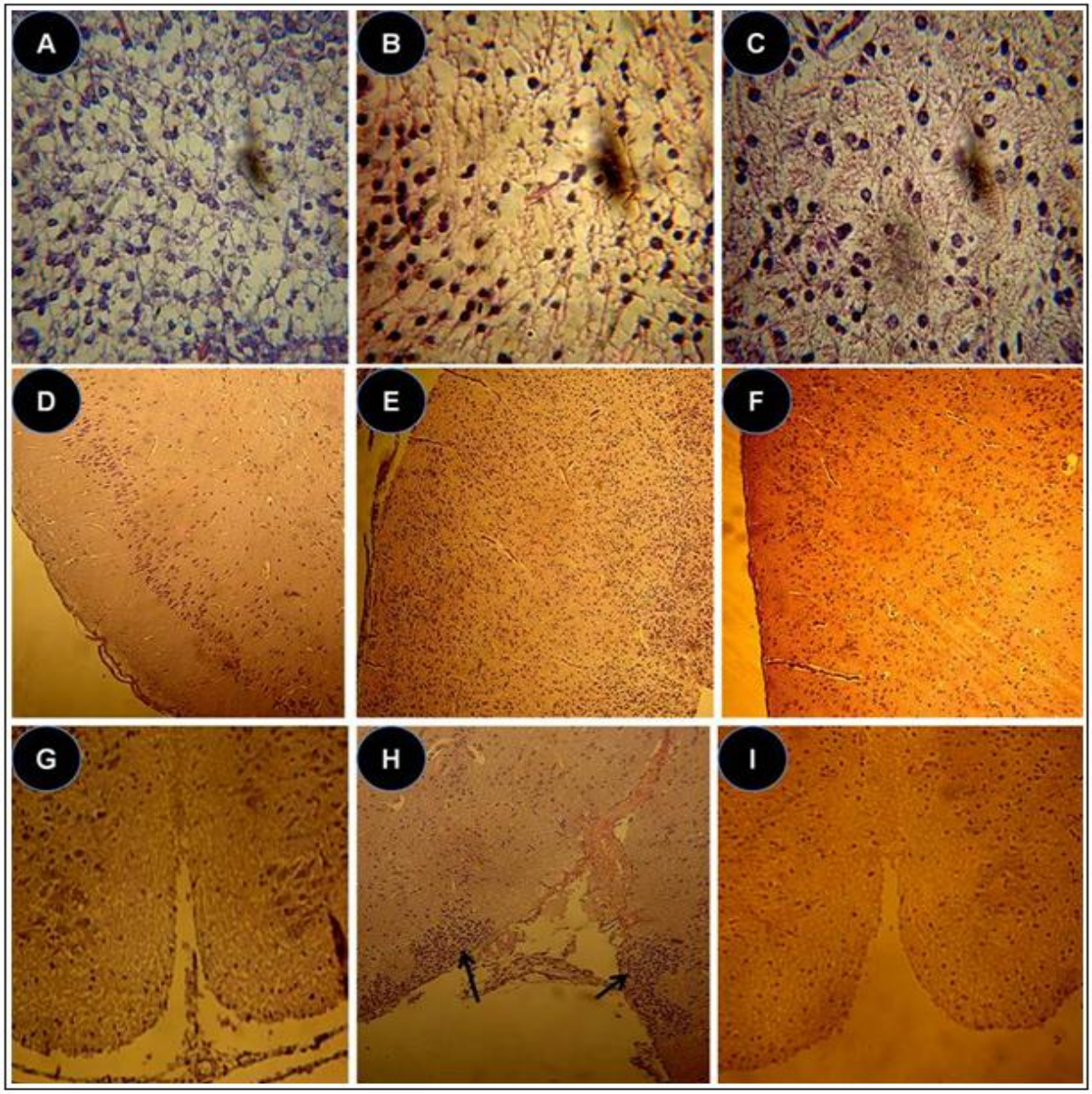

Fig. 7. Portions of the fetal brain gestationally exposed to single and combinatorial treatments of MA and ELEAS.S. A-C. Cross section of the cerebellum; A. Intact and proportioned layers in the negative control; B. section from brain exposed to MA+ ELEAS; E-F. Irregular aggregation of neurons in the pia matter exposed to MA and combinatorial treatments of MA+ 25 mg/kg bw (F) compared to the negative control (D); G-I. Sections along the pia and arachnoid layers of the brain meninges. All treatments caused reduction and disintegration of neurons near the posterior region (H-I). (H&E stain; A-C=400X; D-I= 100X).

Acknowledgements

The authors thank the Philippine Kidney Dialysis Foundation (PKDF) Histopathology Laboratory for histological processing of samples. The study was funded by the Commission of Higher Education-Science and Engineering Graduate Scholarships (CHED-SEGS) for JC Jumawan.

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