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EMBRYO-TOXIC AND TERATOGENIC ACTIVITIES OF LYOPHILIZED WATER EXTRACTS OF *Persea americana* MILL. AND *Syzygium cumini* (L.) SKEELS LEAVES IN *Danio rerio*

RICH MILTON R. DULAY[1*] AND MA. ELLENITA G. DE CASTRO[2]

1:Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines

2:Department of Biology, College of Science, De La Salle University, Taft Avenue, Manila, Philippines

***Corresponding Author, Email:** richmiltondulay@clsu.edu.ph

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ABSTRACT

Teratogen could be a potential anticancer agent. In this paper, the toxic and teratogenic effects of lyophilized water extracts of *Persea americana* and *Syzygium cumini* leaves in *Danio rerio* embryo model were investigated. Results of the assay revealed that the two plant leaves extracts affect the survivability of embryos in concentration and time of exposure manner. A 100% mortality of embryos was found at 1000 µg/ml and higher concentrations of *P. americana* at 5000 µg/ml and higher concentrations of *S. cumini* after 48 hours of exposure. No mortality was noted in embryos at 50 µg/ml of both extracts. All embryos exposed to 50 µg/ml of *P. americana* extract, 100 µg/ml and lower concentration of *S. cumini* extract, and control embryos did not show delayed development. A 100 µg/ml of both extracts significantly registered lower percentage malformations having 33.33% in *P. americana* and 16.67% in *S. cumini*. The *P. americana* leaves extract showed hook-like tail larva at 500 µg/ml and C-shaped tail larva at 100 µg/ml while *S. cumini* extract had bent tail larvae at both 500 µg/ml and 100 µg/ml. These significant effects of the two plant leaves extracts strongly indicate their promising potential as source of anticancer agents.

Keywords: *Persea americana*, *Syzygium cumini*, zebrafish, teratogen, anticancer

INTRODUCTION

Plants are important to humankind. They are rich source of bioactive phytochemicals and nutrients that play an important role in the physiology of human body systems. Plants are considered in alternative medicines due to their minor side effects and less toxicity but very strong bioactivities. Some of these plants include avocado and duhat, which are known to have antibacterial and antioxidant properties **[1, 2]**.

Persea americana Mill. (Lauraceae) is popularly known as avocado. It is a terrestrial, erect, deciduous, evergreen tree reaching up to 15 to 20 m high. This tropical tree possesses medicinal properties as it is being utilized as therapeutic alternative for ulcer, dysentery, dyspepsia, bacterial infection, and skin diseases. Its fruit is delicious that is rich in fatty acids such as linoleic, oleic, palmitic, stearic, linolenic, capric, and myristic acids. In several studies, the seed of avocado have been reported to exhibit antioxidant, antihypertensive, larvicidal, fungicidal, hypolipidemic, amoebicidal, and giardicidal activities **[3-6]**.

Syzygium cumini(L.) Skeels (Myrtaceae) is commonly called duhat. This large tropical tree is native to the Philippines and is reported to contain flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoid, cardiac glycosides, and tannins and showed inhibitory activity against both gram negative and positive bacteria**[7]**. The leaves are also used to treat fever, gastropathy, strangury, dermopathy, leucorrhoea, and stomachalgia **[8]** while the seed extract is used for diabetes and has antioxidant property **[9]**.

Inspite of the various biological activities, it is still necessary to assess the toxic and teratogenic effects, if any, of *P. americana* and *S. cumini* leaves extracts. Teratogenicity can be a desirable property because many anticancer drugs are teratogenic, and teratogens can be used as anticancer drugs **[10]**. To observe the teratogenic effect, zebrafish (*Danio rerio*) is used an ideal animal model due to remarkable similarity to human physiological response. The effect of varying concentrations of lyophilized water extracts of *P. americana* and *S. cumini* is reported in this paper with the intention of establishing their potential as sources of teratogens and/or anticancer agents.

MATERIALS AND METHODS

Source of Plant Samples

The leaves of *P. americana* and *S. cumini* were collected from Bambanaba, Cuyapo, Nueva Ecija, Philippines, and separately placed in a plastic bag and properly labeled.

Leaves were washed and air-dried for 10 days. These were milled and prepared for water extraction and lyophilization.

Extraction and Treatment Preparation

The active components of the milled leaves were obtained following the hot water extraction using the protocol of Eguchi et al. **[11]**. Ten grams each leaves was extracted individually in 300 ml hot water at 80 - 90ºC in a water bath for 2 hours. Extracts were filtered using Whatman filter paper No. 2 and the filtrates were freeze-dried for 24 hours. The different treatment concentrations of the extract were prepared by diluting to embryo water medium **[12]**. Ten ml of each treatment concentration of the extracts was prepared (50, 100, 500, 1000, 5000, and 10000 µg/ml) and control (embryo water) and placed into each well of the 12-well ELISA plate.

Spawning of Zebrafish

The protocol adopted in this study was based from Nagel **[13]**. A non-treated stock of tap water in a glass aquarium with oxygen saturation was used for spawning of zebrafish where mature females and males were present at 1:2 ratio. The condition was 26 ± 1°C at a 12 hour day/night light regime. The fish were fed with dry flakes twice a day. To ensure optimum water quality excess food was removed daily. In order for the zebrafish to spawn, the aquarium was covered with black plastic for 12 hours. Adult zebrafish were localized in a plastic mesh to prevent the released eggs from cannibalism. After incubation in the dark, eggs were exposed to lighted condition for another 12 hours. Fertilization occurs within 30 minutes after light was turned on. Twelve hour after fertilization, the adult fish localized in the plastic mesh were transferred into another aquarium, and the embryos were siphoned out of the aquarium using a hose. They were placed in a watch glass and observed under the dissecting microscope to examine uniformity and normal condition of embryos.

Evaluation of Toxicity and Teratogenicity

The protocol of Dulay et al. **[14]** on the toxicity and teratogenicity using zebrafish embryos was followed in the present work. Four embryos at segmentation phase were transferred into each well containing the different treatments. The plate was incubated at 26°C ± 1°C. Teratogenic activity was examined using a dissecting microscope after 12, 24, 36, and 48hours of incubation. Morphological endpoint evaluation of zebrafish was based on the parameters established by Nagel **[13]**: Lethal (coagulation, tail not detached, no somites, and no heart-beat), Teratogenic (malformation of head, tail and heart,

scoliosis, deformity of yolk, and growth retardation), and Normal. Percentage hatchability, delayed growth, malformations, and mortality were determined. A test was classified as valid, if 100% of the embryos in the control (embryo water) show normal conditions. Data were analyzed using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) was used to compare the means at 5% level of significance.

RESULTS AND DISCUSSION

Toxic Effects of *P. americana* and *S. cumini* Extracts

Some plants are less toxic and some are considered highly toxic. The toxic effects of plants include cardiotoxicity, neurotoxicity, embryotoxicity, nephrotoxicity, hepatoxicity, and could cause allergic reactions. Thus, it is imperative to assess the toxic effects of plants especially the edible ones. In this study, the embryotoxic effects of *P. americana* and *S. cumini* leaves were carried out in *D. rerio*. Embryotoxicity is defined by the coagulated embryos and no visual heartbeat. The percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations of lyophilized water extracts of *P. americana* and *S. cumini* leaves is shown in Table 1. Apparently, the toxic effects of the two leaves extracts were dose and time of exposure dependent. After 12 hours of exposure, embryos at 10000 µg/ml of both plant extracts recorded 100% mortality whereas embryos at 100 µg/ml and lower concentrations of both extracts and control embryos registered 0% mortality. However, after 48 hours of extract exposure of embryos, 100% mortality of embryos was found at 1000 µg/ml and higher concentrations of *P. americana* and at 5000 µg/ml and higher concentrations of *S. cumini*. Lower percentage mortality was observed in embryos at lower concentrations. Embryos at 500 and 100 µg/ml of *P. americana* significantly recorded lower percentage mortality having means of 41.67% and 8.33%, respectively. On the other hand, *S. cumini* extract at 500 µg/ml had 25% morality of embryos while 100 µg/ml registered 8.33% mortality, which is comparable with the control embryos. The results of the study strongly suggest that higher certain concentrations of both plant extracts are more toxic as it directly affects the survival of zebrafish embryos. Comparing the two plant extracts, *P. americana* leaf extracts showed more embryo-toxic than *S. cumini* leaves against *D. rerio* embryos.

Table 1. Mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure to varying concentrations of *P. americana* and *S. cumini* lyophilized water extracts

Extract	Concentration (µg/ml)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
P. americana	10000	100.00[a]	100.00[a]	100.00[a]	100.00[a]
	5000	91.67[a]	100.00[a]	100.00[a]	100.00[a]
	1000	66.67[b]	66.67[b]	91.67[a]	100.00[a]
	500	25.00[c]	33.33[c]	33.33[b]	41.67[b]
	100	0.00[d]	0.00[d]	8.33[c]	8.33[c]
	50	0.00[d]	0.00[d]	0.00[c]	0.00[c]
	0	0.00[d]	0.00[d]	0.00[c]	0.00[c]
S. cumini	10000	100.00[a]	100.00[a]	100.00[a]	100.00[a]
	5000	66.67[b]	91.67[a]	100.00[a]	100.00[a]
	1000	25.00[c]	33.33[b]	41.67[b]	66.67[b]
	500	8.33[d]	16.67[b]	16.67[c]	25.00[c]
	100	0.00[d]	0.00[c]	0.00[d]	8.33[d]
	50	0.00[d]	0.00[c]	0.00[d]	0.00[d]
	0.00	0.00[d]	0.00[c]	0.00[d]	0.00[d]

Treatment means of each plant extract having the same letter of superscript are not significantly different from each other at 5% level of significance using LSD.

These embryotoxic effects of the two leaves extracts could possibly be accounted to their bioactive chemical constituents. *P. americana* leaves contain persin [15; (2R,12Z,15Z)-2-hydroxy-4-oxoheneicosa-12,15-dienyl acetate], which showed toxic activity in acinar epithelium of lactating mammary gland and the myocardium of livestock **[15]**. Aside from persin, flavonoid such as quercetin showed virustatic effects against HIV syncytium formation and viral p24 antigen formation **[16]**. Avocado leaves extract also showed cardiotoxic effects in mammals and birds **[17-20]**. In addition, the seeds of *P. americana* consist of phytosterols, triterpenes, fatty acids, and abscisic acid **[21]**.On the other hand, *S. cumini* is rich in compounds such as anthocyanins, glucoside, ellagic acid, isoquercetin, kaemferol and myrecetin **[22]**.The bioactive compounds present in this plant showed inhibitory activity against bacteria, fungi and virus and responsible to the wide range of pharmacological properties such as anti-inflammatory, anti-ulcerogenic, cardioprotective, anti-allergic, anticancer, chemopreventive, radioprotective, free radical scavenging, antioxidant, hepatoprotective, anti-diarrheal, hypoglycemic and anti-diabetic effects **[23]**.

Teratogenicity of *P. americana* and *S. cumini* Extracts

Teratogenic effects are characterized by the delayed development and malformation on the developing embryos of zebrafish. The bioactive components of the test plants may

act as teratogen, which are used as anti-cancer and anti-epileptic drug **[24]**. In this study, the teratogenic effects of lyophilized water extracts of *P. americana* and *S. cumini* leaves in zebrafish was also investigated. Table 2 presents the percentage delayed development and percentage malformation of *D. rerio* exposed to varying concentrations of the two plant extracts.

In terms of delayed development, 1000 µg/ml and higher concentrations of *P. americana* extract and 5000 µg/ml and higher concentrations of *S. cumini* extract showed 100%. The 500 µg/ml of the two extracts significantly recorded lower percentage delayed development having 25.0% for *P. americana* and 33.33% for *S. cumini*. All embryos exposed to 50 µg/ml of *P. americana* extract, 100 µg/ml and lower concentration of *S. cumini* extract, and control embryos did not show delayed development. However, in terms of percentage malformation, the highest was evident in embryos exposed at 500 µg/ml of both leaves extracts having 58.33% in *P. americana* and 41.67% in *S. cumini*. No malformation was noted at higher concentrations due the early arrested growth as manifested by the coagulation of embryos. A 100 µg/ml of both extracts significantly registered lower percentage malformations having 33.33% in *P. americana* and 16.67% in *S. cumini*. Control embryos showed no malformation.

Table 2. Percentage delayed growth after 48 hours and percentage malformation after 72 hours of *D. rerio* exposed to varying concentrations of *P. americana* and *S. cumini* lyophilized water extracts

Extract	Concentration (µg/ml)	Delayed development (%)	Malformation (%)
P. americana	**10000**	**100.00^{a}**	**Coagulated***
	5000	**100.00^{a}**	**Coagulated***
	1000	**100.00^{a}**	**Coagulated***
	500	**25.00^{b}**	**58.33^{a}**
	100	**8.33^{c}**	**33.33^{b}**
	50	**0.00^{c}**	**8.33^{c}**
	0	**0.00^{c}**	**0.00^{c}**
S. cumini	**10000**	**100.00^{a}**	**Coagulated***
	5000	**100.00^{a}**	**Coagulated***
	1000	**91.67^{a}**	**33.33^{a}**
	500	**33.33^{b}**	**41.67^{a}**
	100	**0.00^{c}**	**16.67^{b}**
	50	**0.00^{c}**	**0.00^{c}**
	0	**0.00^{c}**	**0.00^{c}**

Treatment means of each plant extract having the same letter of superscript are not significantly different from each other at 5% level of significance using LSD. *No malformation was observed due to coagulation of embryos.

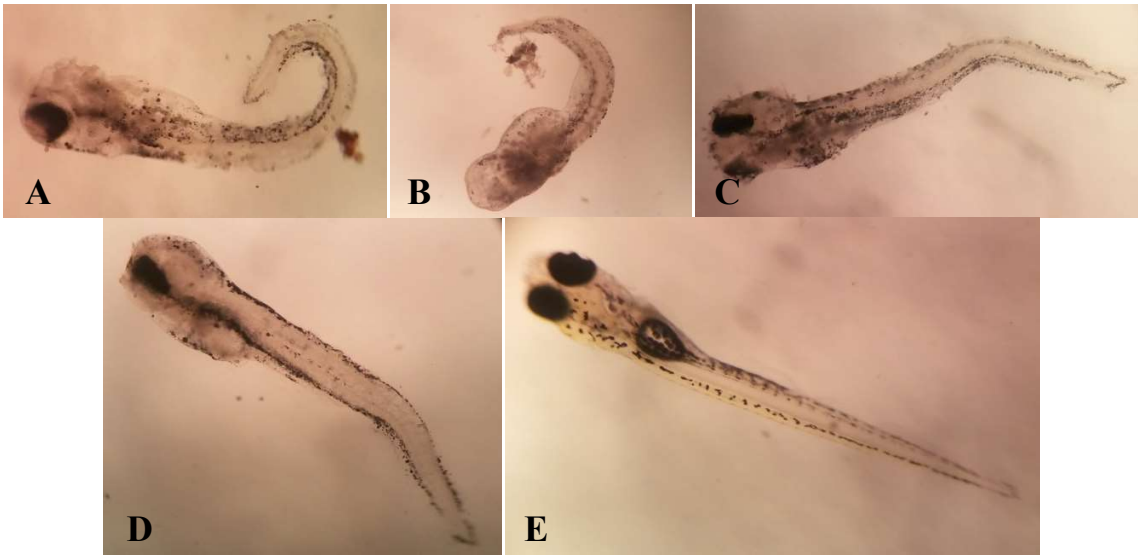

Figure 1: Morphological abnormalities of embryos exposed to the extracts of *P. americana*: A) hook-like taillarva at 500 µg/ml, B) C-shaped tail larva at 100 µg/ml, and of *S. cumini*: C) bent tail larva at 500 µg/ml, D) bent tail larva at 100 µg/ml, and E) normal larva at embryo water after 72 hours.

The different morphological abnormalities of *D. rerio* were observed after 72 hours of treatment exposure and the results are shown in Figure 1. It can be seen that the most marked abnormalities were tail malformations. The *P. americana* leaves extract showed hook-like tail larva at 500 µg/ml and C-shaped tail larva at 100 µg/ml. On the other hand, bent tail larvae were observed at both 500 µg/ml and 100 µg/ml of *S. cumini* leaves extract. These malformations were also observed as teratogenic effects of other plants that we previously assayed.

Many plants used as food or in traditional medicine also contain toxic and teratogenic effects. For instance, *Plectranthus barbatus* extract at high doses treated before the period of embryo implantation in rat caused a decrease in the number of implantation sites, fetal growth retardation, increased incidence of skeletal abnormalities and reduced number of ossification centers of the fetuses **[25]**. In addition, ingestion of high doses of *R. graveolens* extract during pre-implantation in mice induced changes in the blastocyst formation, reduced the number and delayed the development of embryos **[26]**. As such, even if a plant is considered edible and medicinal, it may also have detrimental effect as it contain xenobiotic agents, substances foreign to the human body, which are potentially toxic. Thus, assessment of the toxic and teratogenic effects of various medicinal plants must be continuously conducted.

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