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Phytochemical Constituents and Teratogenic Effect of Lyophilized Extracts of *Bixa orellana* L. (Achuete) and *Piper betle* L. (Ikmo) Leaves in *Danio rerio* Embryos

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ABSTRACT

This work highlighted the preliminary chemical screening and toxic and teratogenic effects of lyophilized extracts of leaves of Bixa orellana and Piper betle in developing zebrafish, Danio rerio, embryos. Chemical analysis revealed that both B. orellana and P. betle contained flavonoids, cardiac glycosides, and saponins in varying amount while the former has an addition of terpenoids and the latter has tannins as additional compounds present. In toxicity assay, a 100% mortality of embryos was observed at 10000 µg/ml concentration after 36 hours and at 5000 µg/ml concentration after 48 hours of exposure in both plants. Plant extracts affect the hatchability of embryos, which can be accounted to the observed delayed development. Coagulated embryo was the most distinct toxic effect while delayed development and tail malformation were the most observed teratogenic effects of both plant extracts.

Keywords: *Bixa orellano, Piper betle*, teratogens, zebrafish, phytochemicals.

INTRODUCTION

Plant is one of the most diverse groups of organism. Many plants are considered medicinal which can be source of plant-derived substances with versatile applications. Due to the presence of several bioactivities and functionalities of plants, considerable attention was given to same plant groups. They can be valuable resource of natural drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [1]. Medicinal plants are potentially toxic that could lead to a serious health risks and sometimes fatal [2]. Some of the medicinal plants used in the Philippines are achuete (*Bixa orellana*) and ikmo (*Piper betle*).

Bixa orellana L. (Bixaceae), commonly known as *achuete*, is a profusely fruiting tall shrub to small evergreen tree, reaching 6-10 meter high at maturity. The fruits are in clusters with prickly heart shaped pods contain small reddish-orange seeds. It is a folk herbal medicine; the leaf is used to treat headache and hematoma and its decoction is used to cure skin diseases and burns and as a purgative and in the treatment of dysentery. Ayurveda practitioners in India use it as an astringent and mild purgative and are considered as a good remedy for treating dysentery and kidney diseases. The root bark is anti-parasitic and antipyretic.

Piper betle L. (Piperaceae), also known as *ikmo*, is a perennial dioceious, semi woody climber, nodes swollen, papillose when young, glabrous at maturity, leaves alternate, simple, bright green in color, berries are rarely produced, cocresent into a fleshy spadix. The leaves have a strong pungent aromatic flavor and are widely used as *nganga* by the native communities in the Philippines. Its fresh crushed leaves are used as antisepsis of wounds and

cuts. Pharmacologically, this plant is effective antimicrobial, antidiabetic, gastroprotective, immunomodulatory, platelet inhibitory, antioxidant, antifertility, hepato-protective, and anticancer [3].

Zebrafish (*Danio rerio*), a vertebrate animal, is currently one of the leading models for studying development and diseases. The embryos and larvae are transparent, develop rapidly, and similar to mammalian development. As such, this animal model is used to assess toxic and teratogenic effects of the substances tested and is used in the preliminary screening of new toxic compounds with potential anticancer properties [4]. Teratogenecity is characterized by the malformation or abnormal development of the shapes and forms on the body of the developing embryo.

Several findings have been reported regarding the pharmacological and folkloric uses of these two medicinal plants. However, their teratogenic and embryotoxic effects have not been studied yet. Thus, this study highlighted the effects of lyophilized extracts of *B. orellana* and *P. betle* in the developing embryos of zebrafish as animal model. The important phytochemical attributes responsible for the different bioactivities were likewise elucidated.

MATERIALS AND METHODS

Source of Plant Samples

Leaves of *B. orellana* were collected from Brgy. Tanaytay while leaves of *P. betle* were acquired from Brgy. Lecsab Poblacion, Alaminos City, Pangasinan, Philippines. Samples were washed three times and air-dried for 7 days. These were pulverized and processed for phytochemical analysis and extraction for teratogenic assay.

Phytochemical Analyses

The chemical screening of the aqueous extracts of the plants were carried out following the procedures described by Sofowora [5]. Among the phytochemicals considered include alkaloids, cardiac glycoside, flavonoids, saponins, tannins, and terpenoid. Distilled water was used a control and was used as a gauge in the changes of color/intensity of the reaction. Three replicates were laid out for each test parameter.

Extraction of Functional Components for Teratogenic Assay

The functional components of the air-dried plant samples were obtained following the hot water extraction following the protocol of Eguchi et al. [6]. The pulverized plant sample (10 g each part) 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 extract filtrates were lyophilized in a freeze-dryer for 2 days. The lyophilized extracts were used for the different treatment concentrations for toxicity assay by diluting the extract to embryo water medium [7].

Spawning of Zebrafish

The protocol of this study was based on that of Nagel [8]. 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 on the toxicity and teratogenicity using zebrafish embryos established by Dulay et al. [9] was followed. Ten ml of each treatment concentration of the extracts was prepared using embryo water as diluent (50 µg/ml, 100 µg/ml, 500 µg/ml, 1000 µg/ml, 5000 µg/ml, and 10000µg/ml) and control (embryo water) and placed into each well of the 12-well ELISA plate. 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 48h of incubation. Morphological endpoint evaluation of zebra fish was based on the parameters established by Nagel [8]: 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, heartbeat rate, 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

Phytochemical Compositions

Plants are valuable source of new chemical entity. In order to elucidate these active chemicals, the present study screened the phytochemical compositions of *B. orellana* and *P. betle* leaves. The results of the phytochemical screening are presented in Table 1. Among the six phytochemicals, five were found present in *B. orellana* while *P. betle* had four phytochemicals. *B. orellana* contained flavonoids, terpenoids, cardiac glycosides, saponins, and tannins. On the other hand, *P. betle* had flavonoids, cardiac glycosides, saponins, and tannins. Alkaloids was not detected in both plants while terpenoids was absent in *P. betle*. The results clearly indicate that the two plants hold essential biochemicals with effective functional activities and provide evidences supporting the various ethnobotanical uses.

Table 1. Phytochemical compositions of *B. orellana* and *P. betle*

Phytochemicals	*B. orellana*	*P. betle*
Flavonoids	+	+
Terpenoids	+	**0**
Cardiac Glycosides	+	+
Saponins	+	+
Alkaloids	**0**	**0**
Tannins	+	+

+ positive, 0 negative.

The two plants contain appreciable amounts flavonoids, cardiac glycosides, saponins, and tannins. Flavonoids are one of the principal physiologically active constituents of folkloric medicinal plants. They exhibit broad spectrum of biological activities including cardio-protection, antioxidant, anticancer, anti-inflammatory and antimicrobial [10]. Flavonoid from the extracts of seeds and callus tissues of *Gossypium* species showed antibacterial activity against *B. cereus*, *P. aeruginosa*, *S. typhimurium* and *S. aureus* [11]. Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products. There are several biological effects that have been ascribed to saponins including immunostimulant, hypocholesterolaemic, anticarcinogenic, antioxidant, antifungal and antiviral properties [12]. On the other hand, in the review conducted by Serrano et al. [13], tannins are a unique group of phenolic metabolites, which are widely distributed in almost all plant foods and beverages. Highly polymerised tannins exhibit low bioaccessibility in the small intestine and low fermentability by colonic microflora. They also reported that the biological properties of tannins are antioxidant, antimicrobial, antiviral, and antidiabetic effects. Cardiac glycosides are class of steroid-like compounds, which are effective in the treatment of congestive heart failure and as anti-arrhythmic agents. It was also revealed that these compounds are involved in complex cell-signal transduction mechanisms, resulting in selective control of human tumor but not normal cellular proliferation [14]. Therefore, these plants studied can be seen as a potential source of useful drugs.

Toxic Effects of the Plant Extracts

Mortality is the measure of toxicity. The toxicity assay confirmed the toxic effects of the two plant extracts on zebrafish embryos. The results of the study strongly suggest that at higher certain concentrations of both plant extracts are toxic as it affects the survival of zebrafish embryos and these effects are dependent on dose and time of exposure. The percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations of lyophilized water extract of *B. orellana* and *P. betle* is shown in Table 2. Apparently, in both plant extracts, mortality was only observed at the highest concentration after 12 hours of exposure and at 5000 µg/ml concentration after 24 hours of exposure. However, at these periods, significant difference on percentage mortality was only noted between *B. orellana*-exposed embryos and control embryos. A 100% mortality of embryos was observed at 10000 µg/ml concentration after 36 hours of exposure and at 5000 µg/ml concentration after 48 hours of exposure in both plants. At 48 hours post treatment application, no mortality was observed in embryos exposed at 100 µg/ml or lower concentrations of *B. orellana* extract and in embryos at 500 µg/ml or lower concentrations of *P. betle* extract. Thus, *B. orellana* extract is more toxic to embryos than *P. betle* extract.

The toxic effects of both plants could be attributed to their strong bioactive compounds. The active ingredients of *P. betle* leaves are primary a class of allyl benzene compounds, chavibetol (betlephenol; 3-hydroxy-4-methoxyallylbenzene), chavicol (p- allyl-phenol; 4-allylphenol), estragole (p-allylanisole; 4-methoxy-allylbenzene), eugenol (allylguaiacol; 4-hydroxy-3-methoxyallylbenzene; 2-methoxy-4-allyl-phenol), methyl eugenol (eugenol methyl ether; 3,-dimethoxy-allylbenzene) and hydroxycatechol (2,4-dihydroxy-allylbenzene)[15]. Among these compounds, hydroxychavicol and eugenol are the most important phytochemicals which are reported to contribute to various bioactivities such as antimutagenic, anticarcinogenic, antidiabetic, anti-inflammatory and antibacterial activities [16]. However, an aqueous extract of leaves of *P. betle* showed potent cytotoxicity with a mean CTC_{50} value of 96.25 µg/ml against Hep-2 cell line suggesting a potential anticancer property [17]. On the other hand, the

seed of *B. orellana* has four major active components including (Z,E)-farnesyl acetate (11.6%), occidentalol acetate (9.7%), spathulenol (9.6%), and ishwarane (9.1%) [18] whereas its leaves have ishwarane, phytol, polyprenol, stigmasterol, and sitosterol [19]. The ethanolic extract of *B. orellana* exhibits cytotoxic effect human lung carcinoma cell at concentrations below 100 µg/ml [20].

Table 2. Mortality of *D. rerio* embryos after 12, 24, 36 and 48 hours of exposure to varying concentrations of *B. orellana* and *P. betle* lyophilized water extracts

Extract	Concentration (µg/ml)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
B. orellana	10000	16.67^{a}	66.67^{a}	100.00^{a}	100.00^{a}
	5000	0.00^{b}	16.67^{b}	66.67^{b}	100.00^{a}
	1000	0.00^{b}	0.00^{c}	25.00^{c}	58.33^{b}
	500	0.00^{b}	0.00^{c}	0.00^{d}	16.67^{c}
	100	0.00^{b}	0.00^{c}	0.00^{d}	0.00^{c}
	50	0.00^{b}	0.00^{c}	0.00^{d}	0.00^{c}
Control	0	0.00^{b}	0.00^{c}	0.00^{d}	0.00^{c}
P. betle	10000	8.33^{a}	41.67^{a}	100.00^{a}	100.00^{a}
	5000	0.00^{a}	16.67^{ab}	83.33^{a}	100.00^{a}
	1000	0.00^{a}	0.00^{b}	33.33^{b}	50.00^{b}
	500	0.00^{a}	0.00^{b}	0.00^{c}	0.00^{c}
	100	0.00^{a}	0.00^{b}	0.00^{c}	0.00^{c}
	50	0.00^{a}	0.00^{b}	0.00^{c}	0.00^{c}
Control	0.00	0.00^{a}	0.00^{b}	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.

Effect of Plant Extracts on the Hatchability of Embryos

During normal hatching process, the chorion is digested by the hatching enzyme, chorionase secreted from the hatching gland cells of the embryo which in turn accumulated in the perivitelline space, reaches the chorion and induces its breakdown releasing the free-living larva. The effect of the two plant extracts on the hatchability of exposed embryos was determined and the results are also presented in Table 3. Apparently, the varying concentrations of plant extracts affected the hatchability of embryos: as the extract concentration increased the percent hatchability decreased. Control embryos and embryos treated with 50 µg/ml of both plant extracts showed 100% hatchability. Significantly lower percentage hatchability was noted in embryos at 100 µg/ml concentration of *B. orellana* extract having 66.67%. Although lower, the 91.67% hatchability of embryos at 100 µg/ml concentration of *P. betle* was found statistically comparable with the control embryos. However, no hatched was observed in embryos exposed at 500 µg/ml concentration of both plant extracts. The results strongly suggest that the two extracts interrupt the normal hatching process of the zebrafish. This could be due to the delayed development and morphological abnormalities of the embryos.

Table 3. Hatchability and heartbeat rate of *D. rerio* after 48 hours of exposure to varying concentrations of *B. orellana* and *P. betle* lyophilized water extracts

Extract	Concentration (µg/ml)	Hatchability (%)	Delayed development (%)
B. orellana	10000	0.00^{c}	100.00^{a}
	5000	0.00^{c}	100.00^{a}
	1000	0.00^{c}	100.00^{a}
	500	0.00^{c}	100.00^{a}
	100	66.67^{b}	33.33^{b}
	50	100.00^{a}	0.00^{c}
Control	0	100.00^{a}	0.00^{c}
P. betle	10000	0.00^{b}	100.00^{a}
	5000	0.00^{b}	100.00^{a}
	1000	0.00^{b}	100.00^{a}
	500	0.00^{b}	100.00^{a}
	100	91.67^{a}	8.33^{b}
	50	100.00^{a}	0.00^{b}
Control	0	100.00^{a}	0.00^{b}

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.

Delayed Development of Embryos Treated with the Plant Extracts

Delayed growth is one of the most remarkable teratogenic effects of the two plant extracts (Table 3). Embryos at 500 µg/ml or higher concentrations in both plant extracts significantly recorded the 100% delayed development. However, at 100 µg/ml concentration, 33.33% of embryos showed delayed development in *B. orellana* extract while 8.33% of embryos were delayed in *P. betle* extract. No delayed development was noted in embryos at 50 µg/ml concentration of both plant extracts and control embryos. These results clearly indicate that the development of

embryos is greatly affected by the varying concentrations of the plant extracts. This delayed growth could be accounted to the active components of the extracts that possibly interrupt the embryonic physiological processes by inhibiting substances or enzymes responsible for the growth and development of the embryos. This observed delayed development could induce the different morphological abnormalities.

Teratogenic Effects of the Plant Extracts

Teratogenic effect is considered sub-lethal effect of the compound or substance being tested. In the present study, the different morphological abnormalities of embryos treated with the varying concentrations of the two plant extracts were observed after 72 hours of extract exposure. The different morphological endpoints are shown in Figure 1. It can be seen that most marked abnormalities are tail malformations. The *P. betle* extract showed bent tail tip embryo at 500 µg/ml and curved tail embryos at both 500 µg/ml and 100 µg/ml. On the other hand, bent tail embryos at 500 µg/ml and 100 µg/ml and L-shaped tail embryo at 500 µg/ml were observed in *B. orellana* extract. Comparing the severity of abnormalities, embryos exposed to *B. orellana* extract showed a more severe malformation than *P. betle* extract. Moreover, teratogenic effects appeared at the lower concentrations due to the early arrested development of embryos at higher concentrations of both extracts. Therefore, these two medicinal plants could exhibit teratogenic effects in the developing embryos of zebrafish.

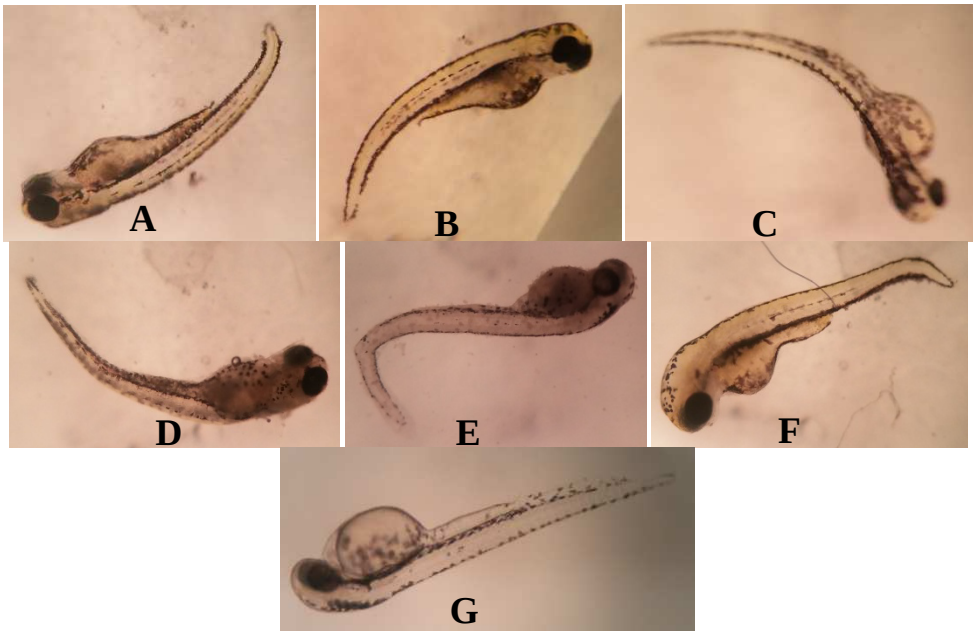

Figure 1. Teratogenic effects of varying concentrations lyophilized extracts of P. betle: A) bent tail tip embryo at 500 µg/ml, B) curved tail embryo at 500 µg/ml, C) curved tail embryo at 100 µg/ml, and of B. orellana: D) bent tail embryo at 500 µg/ml, E) L-shaped tail embryo at 500 µg/ml, F) bent tail tip embryo at 100 µg/ml, and G) normal hatched at embryo water

In the present study, the phytochemical components and the toxic and teratogenic activities of *B. orellana* and *P. betle* leaves extracts against zebrafish are highlighted. The results showed the pharmacological importance of these two plants, which can provide a direction in discovering bioactive compounds with great functional and biological activities.

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