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Phytochemical Screening and Teratogenic Effect of Lyophilized Water Extracts from *Ocimum sanctum* L. (Holy Basil) and *Tamarindus indica* L. (Tamarind) Leaves in *Danio rerio* Embryos

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ABSTRACT

The toxic and teratogenic effects of lyophilized water extracts of Ocimum sanctum and Tamarindus indica in Danio rerio embryos and their active phytochemicals were assessed and analyzed in this study. Analysis revealed that O. sanctum contained flavonoids, terpenoids, saponins, and tannins while T. indica had flavonoids, terpenoids, saponins, and alkaloids. In D. rerio assay, a 100% mortality was observed in embryos exposed to 5000 ppm and 10000 ppm of O. sanctum and to 10000 ppm of T. indica after 12 hours of exposure. At 48 hours of exposure, O. sanctum extract-treated embryos showed 25.00% and 58.33% mortality while T. indica had 8.33% and 33.33% mortality at 100 and 500 ppm concentrations of each extract, respectively. Embryos at 100 ppm or higher concentrations significantly recorded lower heartbeat rate while no heartbeat was observed in embryos at 1000 ppm or higher concentrations of O. sanctum and at 5000 ppm or higher concentrations of T. indica. Embryos exposed to 100 ppm and 500 ppm significantly recorded lower hatchabilities (66.67% and 16.67% for O. sanctum and 91.67% and 58.33% for T. indica, respectively) when compared to the control embryos. No hatched was noted in 1000 ppm of both plant extracts. Growth retardation, yolk deformity, and tail malformation were the most observed teratogenic effects of both plant extracts.

Keywords: *Ocimum sanctum, Tamarindus indica, Danio rerio,* teratogens.

INTRODUCTION

Medicinal plants are important source of medicines which are used to cure a wide range of diseases due to their powerful bioactive secondary metabolites and phytochemicals. Plants contain alkaloids, flavonoids, tannins, saponins, glycosides, phenols, sterols, quercetin, anthocyanidins, lignans, and among others. In the Philippines, a number of medicinal plants have been identified and utilized as natural remedy for jaundice, skin diseases, anemia, dyspepsia, fever, bacterial infection, inflammation, urinary diseases and other health problems. Some of these medicinal plants include holy basil and tamarind.

Ocimum sanctum L. (Lamiaceae), also known as holy basil, is an erect shrub of 35-70 cm tall with simple opposite green and purple leaves and with strong scent and hairy stem. Its different parts have been traditionally used for the treatment of diarrhea, arthritis, eye diseases, dysentery, skin disease, bronchitis, malaria, insect bites and exhibits antimicrobial, cardioprotective, analgesic, antispasmodic, anti-fertility, anticancer, antidiabetic, antifungal, and adaptogenic actions [1]. On the other hand, *Tamaridus indica* L. (Caesalpinaceae) or tamarind, as it commonly called, is a medium-sized tree with pinnately compound yellow green to dark green leaves and sour taste fruit. All

parts of this plant have medicinal properties. The leaves are the most valuable part which has been reported to exhibit hepatoprotective and antibacterial properties [2, 3].

Danio rerio (zebrafish) assay is a sensitive test used to assess the toxic and teratogenic effects of certain compounds or substances. Zebrafish embryo is considered as a suitable and reliable animal model for evaluation of the teratogenicity due to their small size, transparency, rapid development, high fecundity, and developmental similarity to human development [4, 5]. This is also used in various toxicological researches such as safety assessment of new pharmaceutical drugs and in the preliminary screening of new toxic compounds with potential anticancer properties [6].

Considering the remarkable biological activities of *O. sanctum* and *T. indica*, their teratogenic effects were not yet investigated, hence, this study. The toxic and teratogenic effects of the two medicinal plants were assessed in the developing embryos of zebrafish. The phytochemical constituents were also investigated.

MATERIALS AND METHODS

Source of Plant Samples

Plant leaves were collected from Barangay Bolaney, Alaminos City, Pangasinan, Philippines. Samples were washed three times and air-dried in a shaded condition 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 [7]. The different mycochemicals namely; alkaloids, cardiac glycoside, flavonoids, saponins, tannins, and terpenoids were analyzed. Results were compared with distilled water as control and determined based on the 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. [8]. 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 prepared for the different treatment concentrations for toxicity assay by diluting the extract to embryo water medium [9].

Spawning of Zebrafish

The protocol of this study was based on that of Nagel [10]. 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. [11] was followed. Ten ml of each treatment concentration of the extracts was prepared using embryo water as diluent (50 ppm, 100 ppm, 500 ppm, 1000 ppm, 5000 ppm, and 10000 ppm) 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 [12]: 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 Constituents

Medicinal plants are rich in chemical substances with potential therapeutic benefits. These chemical substances are also known as phytochemicals. In the present study, the phytochemical compositions of *O. sanctum* and *T. indica* leaves were screened and the results are presented in Table 1. Out of the six phytochemicals screened, each plant sample contained four phytochemicals. *O. sanctum* contained flavonoids, terpenoids, saponins, and tannins while *T. indica* had flavonoids, terpenoids, saponins, and alkaloids. These results indicate that these two plants hold chemical substances that are associated in the treatment of many diseases.

Table 1. Phytochemical composition of *O. sanctum* and *T. indica*

Phytochemicals	*O. sanctum*	*T. indica*
Flavonoids	+	+
Terpenoids	+	+
Cardiac Glycosides	**0**	**0**
Saponins	+	+
Alkaloids	**0**	+
Tannins	+	**0**

+ positive, 0 negative.

Both plants have flavonoids, terpenoids, and saponins. Flavonoids are polyphenolic compounds that have been reported to exhibit antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant [12]. Terpenoids, on the other hand, are good expectorants which also act as antioxidant, modify hormones, cholesterol absorption blocker, and protect cellular differentiation [13]. Saponins are compounds that possess anti-inflammatory, antibiotic, antioxidant, anticholesterolemic, immune boosters, and prevent cancers and heart diseases [14, 15]. Alkaloids are detected in *T. indica* but not in *O. sanctum* while tannins are present in *O. sanctum* but absent in *T. indica*. Alkaloids are powerful chemicals known to treat some types of cancer, reduce spasms, and relieve pains and inflammation [16]. Important alkaloids of plant origin include caffeine, nicotine, cocaine, and the synthetic O,O-acetylated morphine derivative heroin and those currently in clinical use that include the analgesics morphine and codeine; the anticancer agents vinblastine and taxol; the gout suppressant colchicine; the muscle relaxant (+)-tubocurarine; the antiarrythmic ajmaline; the antibiotic sanguinarine, and the sedative scopolamine [17]. Tannins possess anti-inflammatory, antifungal, antioxidant and healing properties [18] and act on arachidonic acid metabolism in leucocytes with important roles in reversing inflammations [19] and they are used in treatments fostering wound healing.

Table 2. Mortality of *D. rerio* embryos after 12, 24, 36 and 48 hours of exposure to varying concentrations of *O. sanctum* and *T. indica* lyophilized water extracts

Extract	Concentration (ppm)	Mortality (%)			
		12 hours	24 hours	36 hours	48 hours
O. sanctum	10000	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	5000	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	1000	66.67^{b}	91.67^{a}	100.00^{a}	100.00^{a}
	500	33.33^{b}	33.33^{b}	50.00^{b}	58.33^{b}
	100	8.33^{c}	8.33^{c}	16.67^{b}	25.00^{c}
	50	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{d}
Control	0	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{d}
T. indica	10000	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
	5000	66.67^{b}	100.00^{a}	100.00^{a}	100.00^{a}
	1000	41.67^{b}	41.67^{b}	58.33^{b}	83.33^{a}
	500	8.33^{c}	25.00^{b}	25.00^{c}	33.33^{b}
	100	0.00^{c}	0.00^{c}	0.00^{d}	8.33^{c}
	50	0.00^{c}	0.00^{c}	0.00^{d}	0.00^{c}
Control	0.00	0.00^{c}	0.00^{c}	0.00^{d}	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.

Toxic Effects in Extract Treated D. rerio Embryos

Coagulated and no heartbeat embryos were the basis of determining the toxic effect of the extracts. The percentage mortality of *D. rerio* embryos after 12, 24, 36, and 48 hours of exposure in varying concentrations of lyophilized water extract of *O. sanctum* and *T. indica* is shown in Table 2. The embryo-toxic effects of both extracts were found dependent on dose and time of exposure. After 12 hours of exposure, mortality was observed in embryos exposed to 100 ppm or higher concentrations of *O. sanctum* extract and to 500 ppm or higher concentrations of *T. indica* extract. A 100% mortality was observed in embryos exposed to 5000 ppm and 10000 ppm of *O. sanctum* and to 10000 ppm of *T. indica*. After 24 and 36 hours, percentage mortality of embryos was continually increased at the

different concentrations that showed mortality after 12 hours of treatment application. *O. sanctum* extract-treated embryos showed 25.00% and 58.33% mortality while *T. indica* had 8.33% and 33.33% mortality at 100 and 500 ppm concentrations of each extract after 48 hours of exposure, respectively. However, no mortality was noted in embryos exposed at 50 ppm concentrations of both extract in all observation periods. The results of the present study indicate that both plant leaves extracts possess toxic effects against *D. rerio* at specific level or concentrations. These strong embryo-toxic activities significantly dictate the anticancer, antitumor and apoptotic properties of these two important medicinal plants.

The toxic effect of both plant extracts could be due to their various biologically active compounds. The *O. sanctum* leaves contain eugenol (1-hydroxy-2-methoxy-4-allylbenzene), euginal, urosolic acid (2,3,4,5,6,6a, 7,8,8a,,10,11,12, 13,14b-tetradecahydro-1H-picene-4a-carboxylic acid), carvacrol (5-isopropyl-2-methylphenol), linalool (3,7-dimethylocta-1,6-dien-3-ol), limatrol, caryophyllene (4,11,11-trimethyl-8-methylene-bicyclo[7.2.0] undec-4-ene, methyl carvicol (also called Estragol: 1-allyl-4-methoxybenzene) while the seed volatile oil have fatty acids and sitosterol [1, 20, 21]. These compounds are responsible to the toxic effect *O. sanctum* against other cellular model and organisms. The aqueous and ethanolic extracts of *O. sanctum* significantly reduced the incidence of papillomas and squamous cell carcinomas in 7,12-dimethylbenzaanthracene (DMBA) induced (0.5%) hamster buccal pouch [22]. The eugenol derived from *O. sanctum* exhibited putative anthelmintic activity with an ED50 value of 62.1 µg/ml against *Caenorhabditis elegance* [23]. Moreover, aqueous extract of *O. sanctum* at 60 mg/kg inhibited the growth of *Klebsiella, Escherichia coli, Proteus, Staphylococcus aureus* and *Candida albicans* while its alcoholic extract showed inhibitory activity against *Vibrio cholera* [24]. *T. indica*, on the other hand, has phenol and flavonoids as active compounds with inhibitory effect. The α and β-pinene, linalool and nerol have proven activity against *E. coli* and other bacteria [25]. In addition, the combination of root extracts of *T. indica* and *Carica papaya* and the pure root extract of *T. indica* significantly increased the larvae mortality of *Meloidogyne incognita* [26].

Effect of Extracts on the Heartbeat Rate of Embryos

The heart is the first functional organ developed in zebrafish and other vertebrates. In zebrafish embryo assay, heartbeat is an important endpoint in assessing embryo toxicity and considered as one of the parameters established by Nagel [10]. This endpoint was therefore determined in the present study. The heartbeat rates of extract treated embryos are shown in Table 3. Apparently, no heartbeat was observed in embryos at 1000 ppm or higher concentrations of *O. sanctum* and at 5000 ppm or higher concentrations of *T. indica*. This is due to the early arrested development of embryos as toxic effect of the two extracts. Among the different concentrations of both extracts, the heartbeat rate of embryos at 50 ppm was found statistically comparable to the control embryos. However, embryos at 100 ppm or higher concentrations significantly recorded lower heartbeat rate, which strongly indicate cardio-toxicity. The abnormal function of the heart could be originated from the lesion of the yolk sac, which may block the supply of nutrients, thus, heart function is greatly affected due to the limited energy source [27]. Heart function impairment such as low heartbeat rate could lead to the failure of organogenesis. In the present study, since heartbeat rates were significantly decreased by the two plant extracts, exposed embryos were anticipated to show different morphological abnormalities.

Table 3. Hatchability and heartbeat rate of *D. rerio* after 48 hours of exposure to varying concentrations of *O. sanctum* and *T. indica* lyophilized water extracts

Extract	Concentration (ppm)	Hatchability (%)	Heartbeat rate (/min)
O. sanctum	10000	0.00^{d}	0.00^{c}
	5000	0.00^{d}	0.00^{c}
	1000	0.00^{d}	0.00^{c}
	500	16.67^{c}	119.00^{b}
	100	66.67^{b}	121.33^{b}
	50	100.00^{a}	142.33^{a}
Control	0	100.00^{a}	145.33^{a}
T. indica	10000	0.00^{c}	0.00^{d}
	5000	0.00^{c}	0.00^{d}
	1000	0.00^{c}	105.33^{c}
	500	58.33^{b}	130.00^{b}
	100	91.67^{a}	137.67^{b}
	50	100.00^{a}	141.67^{a}
Control	0	100.00^{a}	145.67^{a}

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 Extracts on the Hatchability of Embryos

Hatching process is another sensitive endpoint of *D. rerio* assay because extract may influence the hatching. The effect of the two plant extracts on the hatchability of exposed embryos was determined and the results are also

presented in Table 3. In both extract, embryos at 50 ppm of extract showed 100% hatchability. Embryos exposed to 100 ppm and 500 ppm significantly recorded lower hatchabilities (66.67% and 16.67% for *O. sanctum* and 91.67% and 58.33% for *T. indica*, respectively) when compared to the control embryos. No hatched was noted in 1000 ppm of both plant extracts. These results clearly indicate that the two extracts affect the hatching process of the embryos. The low hatchability or no hatched could be due to the delayed growth and very limited movement which possibly attributed to the abnormalities of extract treated embryos. Hatching process is also prevented by embryos because chorion is served as an important defense shield of not having directly exposed to extract. Chorion acts as a barrier for exposure to chemicals, thus, it can reduce toxic effects.

Teratogenic Effect in Extract Treated D. rerio Embryos

The different morphological endpoints of embryos exposed to the varying concentrations of the two plant extracts were observed after 48 to 72 hours of extract exposure. Figure 1 shows the different morphological endpoints which are considered as the toxic and teratogenic effects of the extracts tested. It can be noticed that teratogenic effects appeared at the lower concentrations due to the early arrested development of embryos at higher concentrations of both extracts. Embryos exposed at 100, 500, and 1000 ppm concentrations of *O. sanctum* extract showed loop-like tail, bent wavy tail tip, yolk deformity, growth retardation or underdeverloped as morphological endpoints. On the other hand, in *T. indica* extract, hook-like tail, bump back, and growth retardation of embryos were the observed morphological endpoints. Moreover, coagulated embryos, the most marked toxic effect, were apparent at 1000 ppm or higher concentrations of both extracts. Therefore, these two medicinal plants could exhibit teratogenic effects in the developing embryos of zebrafish.

In conclusion, the presence of bioactive phytochemicals and functional activities such as embryo-toxicity and teratogenicity in medicinal plant samples strongly signify their anticancer, antitumor and apoptotic properties. In this work, it was found that *O. santum* and *T. indica* contain valuable phytochemicals which are related or associated in the treatment of several diseases. Bothe plant extracts exhibit lethal and teratogenic effects against zebrafish embryos. Coagulated embryos was the most marked lethal effect while growth retardation, yolk deformity, and tail malformation were the most observed teratogenic effects of both medicinal plant extracts.

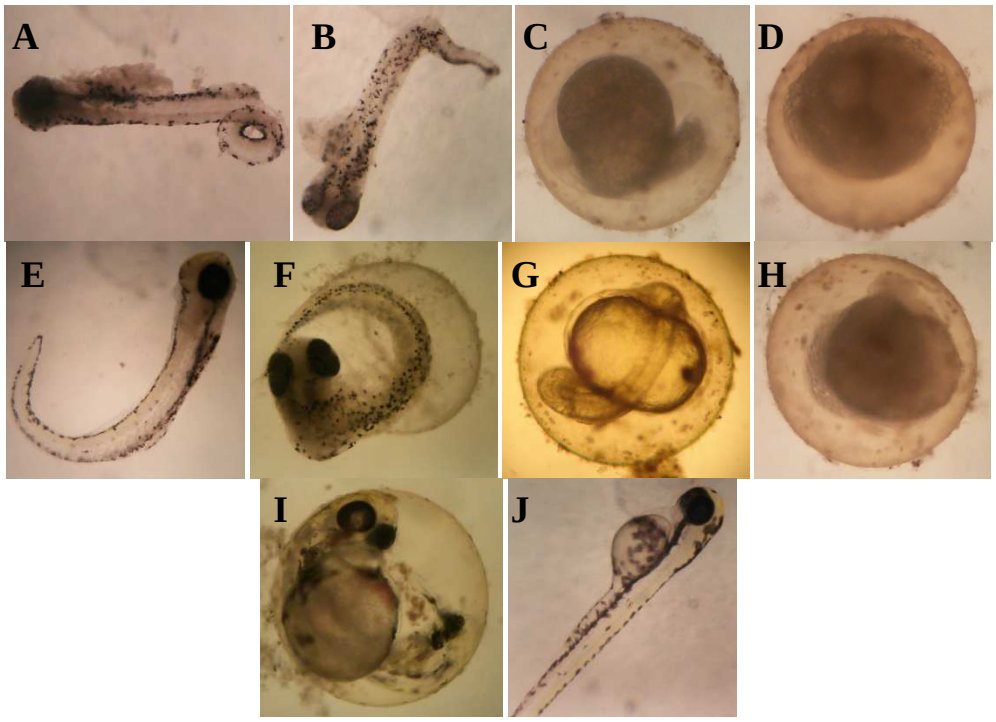

Figure 1. Toxic and teratogenic effects of varying concentrations lyophilized water extracts of O. sanctum: A) loop-like tail and yolk deformity at 500 ppm, B) bent wavy tail tip and yolk deformity at 100 ppm, C) dead underdeveloped embryo at 1000 ppm, D) coagulated embryo at 5000 ppm, and T. indica: E) hook-like tail at 500 ppm, F) bump back at 100 ppm, G) growth retardation at 500 ppm, H) coagulated embryo at 5000 ppm, I) non-hatched normal embryo at 100 ppm, J) normal hatched at embryo water

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