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Research Article

PHYTOCHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF *DENDROBIUM PHALAENOPSIS* FITZG. FLOWERS

Ma. Francheska C. Echavaria, Mary Jhane G. Valentino*, Eden S. David

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

**Corresponding Author:*
E-mail: maryjhane.valentino@clsu2.edu.ph

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ABSTRACT. The phytochemical constituents and biological activities such as anti-oxidant activity, antimicrobial and teratogenicity of *Dendrobium phalaenopsis* flowers ethanolic extract were evaluated in the present study. Using Thin Layer Chromatography method, essential oils, phenols, fatty acids, anthraquinones, anthrones, tannins, flavonoids, and alkaloids were detected in the ethanolic extract of *D. phalaenopsis* flowers. In addition, the ethanolic extract has a total phenolic content of 374.472 mg Gallic Acid Equivalent/g and an anti-oxidant activity of 613.1 µg/ml. The extract inhibited *Staphylococcus aureus*, but showed no inhibition against *Escherichia coli*. Meanwhile, findings in protectant test suggests that the ethanolic extract can be a potential protectant agent against *E. coli* and *S. aureus*. Moreover, no antifungal activity was observed in the ethanolic extract against *A. flavus*. Lastly, for the teratogenicity using *Danio rerio* as model, *D. phalaenopsis* flowers ethanolic extract caused lethal effect such as embryo coagulation and lack of heartbeat while its teratogenic effect were severe growth retardation, little pigmentation, and yolk sac edema with an estimated LC_{50} of $2.15x10^3$ ppm. Thus, the findings suggest the medicinal and anti-cancer potential of *D. phalaenopsis* flowers ethanolic extracts.

Keywords: *Antibacterial, antifungal, Dendrobium phalaenopsis, phytochemical constituents, teratogenicity.*

INTRODUCTION

Dendrobium phalaenopsis is an epiphytic orchid native to Larat Island in eastern Indonesia, where it grows on the branches of tiny trees in scrubby areas and was designated as Queensland's floral emblem in November 1959 [1, 2]. It has a very beautiful flower with purple color and it is highly prized for its decorative and medicinal properties [2].

Traditionally, *Dendrobium* species have been used in Asia, Europe, and Australia [3, 4] for their medicinal properties, including tonic, astringent, analgesic, antipyretic, and anti-inflammatory properties [5]. Furthermore, *Dendrobium* flowers are consumed in Asian countries as snacks, cake decorations, and pickles [6].

Despite the applications and consumption in many parts of the world, there are limited studies and articles on the probable effects of *Dendrobium* species, especially *D. phalaenopsis* flower intake. Thus, phytochemical analysis of *D. phalaenopsis* flowers can

provide important information about the consumption and medicinal properties of this plant. Moreover, investigating the biological activities can be important in identifying potential sources of new antibiotics, antioxidants, and other pharmaceuticals.

This study aimed to detect the phytochemical constituents of *D. phalaenopsis* flowers ethanolic extracts and examine their biological activities including the antibacterial activity, antifungal activity, and teratogenic activity.

MATERIALS AND METHODS

Preparation of Plant Extract

Powdered flowers of *D. phalaenopsis* was extracted using ethanol by maceration method [7]. Fifty grams was submerged in 250 mL of 95% ethanol for 72 hours and stirred every 12 hours. The mixture was filtered and subjected to a rotary evaporator (50℃, 150 rpm) to remove the solvent. It was stored in an amber bottle, labeled and refrigerated until use.

Detection of Phytochemical Composition and Radical Scavenging Activity

Phytochemical screening was performed on the ethanolic extract of *D. phalaenopsis* flowers to determine the presence of secondary metabolites such as essential oils, triterpenes, sterols, phenols, fatty acids, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, steroids, alkaloids, and amino acids. The protocol was adapted from the study of Guevara et al. [8] with some modifications. Each plant extract was marked, labeled, and subjected to TLC (Thin Layer Chromatography). The 7x4 cm plate was developed in a 16mm acetate-methanol (7:3) solution. The spots for certain metabolites were visualized on TLC plates and subjected to UV light and a hot plate to examine the separation of different chemicals. A vanillin-sulfuric acid reagent was used for typical secondary metabolite visualization. This solution can detect the presence of phenols, sterols, triterpenes, and essential oils. Anthraquinones and anthrones were tested using methanolic potassium hydroxide, while phenolic compounds and tannins was detected using the potassium ferricyanide-ferric chloride reagent. Alkaloids was detected using Dragendorff's reagent, and flavonoids was detected using antimony (III) chloride. Total phenolic content was done using Folin-Ciocalteu technique [9] and DPPH RSA (2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Activity) as described by Baliyan et al. [9] for the detection anti-oxidant activity was carried out.

Antibacterial Assay

Escherichia coli and *Staphylococcus aureus* were used for the antibacterial assay with streptomycin sulfate as positive control and distilled water as negative control following the protocol by Austria et al. [10]. Both eradicant and protectant tests were done. Zone of bacterial colonization was measured for protectant test and zone of inhibition for eradicant test at 12 and 24hrs of incubation.

Antifungal Assay

Aspergillus flavus was inoculated in Potato Dextrose Broth (PDB) and incubated for 72 hours at 37ºC. The antifungal assay was done as described by Valentino et al. [11] with some modifications. The paper discs were soaked in the fungal suspension (1.5 x

10^6 spores/mL) and were seeded equidistantly in the plates flooded with ethanolic extracts. Zone of mycelial colonization was measured after the incubation period.

Teratogenicity Assay

Teratogenicity test was done following the protocol by Lindain et al. [12]. The mortality, hatchability, and morphological abnormalities were investigated, and assessed using a compound microscope at 12 hours, 24 hours, 36 hours, and 48 hpta (hours post treatment application).

RESULTS AND DISCUSSION

Phytochemical Composition

The phytochemicals detected are presented in Table 1. Accordingly, *Dendrobium* spp. were found to contain a variety of phytochemical substances. The flowers of *D. nobile* contains flavonoids, organic acids, phenols, nucleotides, carbohydrates, lipids, terpenoids, and alkaloids [13]. Moreover, *D. phalaenopsis* and *D. lineale* also contains alkaloid, flavonoid, and terpenoid [14]. In addition**,** a total phenolic content of 374.472 mg GAE/g was recorded (Table 2). The detected phytochemicals are known to have valuable importance in the field of medicines. These are the major secondary metabolites with anti-cancer, anti-diabetic, anti-microbial and anti-oxidant properties [13]. Similarly, phenolic chemicals with wide range of biological actions have been previously discovered in flowers of *Dendrobium* species including *'Sonia Jo Daeng'*, Shavin white, Snow Rabbit, and *D. sabin* [15-17]. Furthermore, phenolic compounds can be found in plants, particularly herbs, spices, fruit peels, and vegetables which exhibit biological actions to alleviate several chronic diseases [18-20]. Accordingly, plants with various secondary metabolites such as phenols, alkaloids, tannins, essential oils and steroids can be served as natural materials for drug discovery [21-23].

Table 1. *Phytochemical constituents of D. phalaenopsis flowers ethanolic extract*

PHYTOCHEMICAL CONSTITUENTS	PRESENCE/ABSENCE
Essential oils	+
Triterpenes	-
Sterols	-
Phenols	+
Fatty acids	+
Sugars	-
Anthraquinones	+
Coumarins	-
Anthrones	+
Tannins	+
Flavonoids	+
Steroids	-
Alkaloids	+
Amino acids	-

** "+" indicates presence, "-"indicates absence*

Anti-oxidant Activity

Antioxidants protect the body from free radicals by either eliminating or preventing other oxidation reactions by oxidizing themselves and it lowers the chain reactions that can cause cell damage leading to several life-threatening diseases [24, 25]. The ethanolic extract of *D. phalaenopsis* flowers exhibited an anti-oxidant activity of 613.1 µg/ml (Table 2). This is higher as compared to anti-oxidant activity of *D. lindleyi* with 111.79 µg/ml as reported by Rahman et al. [26]. In addition, anti-oxidant activity of vitamins can be ranged from 10-900 µg/ml, thus, the anti-oxidant activity of *D. phalaenopsis* is within the acceptable range. The presence of bioactive compounds, including saponins, tannins, flavonoids, terpenoids, coumarins, cardiac glycosides, favonoids, proteins, phenols, quinines, resins, steroids, and lignins contribute to anti-oxidant activities of plants [27].

This finding is congruent with previous researches that *D. catenatum, D. crepidatum, D. loddigesii, D. nobile,* and *D. officinale* are utilized as traditional medicines. According to Zhao et al. [28], flowers of *D. devonianum* and *D. officinale* showed strong antioxidant activity with dose-dependent capabilities. Furthermore, flowers of *'Sonia Jo Daeng'*, Sonia, Sonia Pink, Snow Rabbit, Shavin White, and *D. sabin*, were reported to exhibit notable anti-oxidant properties after undergoing a DPPH scavenging assay and total phenolic content assessment [29-31].

Table 2. *Total phenolic content and radical scavenging activity of D. phalaenopsis flowers ethanolic extract*

SAMPLE DESCRIPTION	TOTAL PHENOLIC CONTENT (mg GAE/g)	ANTI OXIDANT ACTIVITY (µg/ml)
D. phalaenopsis flowers extract	374.472	613.1%
Catechin (control)	-	799.3%

**Abs DPPH = 0.548;*Wavelength 517 nm BK UV 1000 Spectrophotometer*

Antibacterial Activity

Eradicants prevent illnesses caused by harmful bacteria [10]. Table 3 demonstrated the ability of the ethanolic extracts of *D. phalaenopsis* flower to suppress the growth of *S. aureus* with a zone of inhibition (ZOI) of 7.12 mm and 6.87 mm at 12 and 24 hours, respectively. In contrast, no ZOI was detected against *E. coli*. As protectant, the smaller the zone of bacterial colonization formed the more efficient the extract is, given that the action of protectant is to inhibit or hinder the bacterial growth [10]. Thus, if the bacterial growth were stopped in the first place, there will be no need for eradicating agent and in turn, antibacterial resistance could be prevented [28]. As shown in table 4, zone of colonization of *E. coli* on the ethanolic extract were 9.38 mm at 12 hours of incubation and grew to 15.62 mm after 24 hours. Meanwhile, zone of colonization of *S. aureus* were 8.13 mm and 8.97 mm after 12 hours and 24 hours of incubation, respectively. Statistical analysis indicates that its effect is not comparable with the commercial antibiotic. Findings suggested that *D. phalenopsis* flowers ethanolic extract can be a potential protectant agent against *E. coli* and *S. aureus* but may need to enhance its efficacy by increasing its concentration and combining with other agents.

Similarly, Sufaati et al. [7] found that the ethanol extracts of *D. antennatum* and *D. violaceoflavens* stems had weak antibacterial activity against *S. aureus*. The extracts were even less effective against *E. coli*. Furthermore, Agustini et al. [33] reported that ethanol extract of *D. lasianthera* stem and leaves inhibited *S. aureus* with ZOI of 8.42 mm and 6.77 mm, respectively, but had no inhibition against *E. coli*.

Results also showed that *E. coli* appeared to be less sensitive to the ethanolic extract since gram negative bacteria have a thin peptidoglycan layer covered by multiple thin membrane layers that eject toxins, whereas gram positive bacteria have a thick peptidoglycan layer that absorbs surrounding materials [29]. In addition, lack of outer membrane may expose them to secondary metabolites [30-32].

Table 3. *Mean diameter of zone of inhibition (mm) affected by the treatments against E. coli and S. aureus after 12 and 24 hours of incubation (Eradicant test).*

TREATMENT	**DIAMETER OF ZONE OF INHIBITION**			
	E. coli		***S. aureus***	
ERADICANT TEST	**12 HRS**	**24 HRS**	**12 HRS**	**24 HRS**
Ethanolic extract	0.00^{a}±0.00	0.00^{a} ±0.00	7.12^{b}±0.28	6.87^{b}±0.28
Streptomycin sulfate (+ control)	27.10^{b}±1.26	26.92^{b}±1.12	30.73^{c}±2.33	36.67^{c}±1.29
Distilled water (- control)	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00
PROTECTANT TEST	**DIAMETER OF ZONE OF COLONIZATION**			
Ehanolic extract	9.38^{b}±0.53	15.62^{b}±1.30	8.13^{b}±0.23	8.97^{b}±0.69
Streptomycin sulfate (- control)	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00	0.00^{a}±0.00
Distilled water (+ control)	13.13^{c}±0.76	22.43^{c}±0.15	9.28^{c}±0.65	11.98^{b}±1.98

Antifungal Activity

Table 5 shows that zone of mycelial colonization of *Aspergillus flavus* in plates treated with ethanloic extract *of D. phalaenopsis* measured 17.15 mm and 29.93 mm after 24 and 48 hours of incubation. Statistical analysis showed that at all incubation period, the *D. phalaenopsis* flowers ethanolic extract are not significantly different to negative control which suggest its lack of antifungal activity.

Table 5. *Mean diameter of zone of mycelial colonization (mm) of A. flavus against D. phalaenopsis flower ethanolic extract after 24 and 48 hours of incubation.*

TREATMENT	**DIAMETER OF ZONE OF COLONIZATION**	
	A. flavus	
	24 HRS	**48 HRS**
Ethanolic extract	17.15^{b}±1.21	29.93^{b}±2.26
Nystatin (positive control)	0.00^{a}±0.00	0.00^{a}±0.00
Distilled water (negative control)	17.26^{b}±0.31	29.97^{b}±1.33

Similarly, Setyati et al. [34] reported that antifungal activity of leaf extract of *D. linearifolium* was low against *A. flavus* while according to Marasini & Joshi [35], *D.*

nobile and *D. amoeneum* showed very weak and no antifungal activity against fungal pathogens, respectively. Moreover, coumarins which are naturally occurring bioactive chemicals have a wide range of pharmacological activity, including antifungal, antibacterial, antiviral, anticancer, anti-inflammatory, and anticoagulant properties [36]. However, coumarins are not detected in the phytochemical screening of the present study.

Teratogenicity

Percentage Mortality

In all treatments, no mortality was recorded at 12 hours post-treatment application (hpta). However, longer exposure of embryos to various doses of *D. phalaenopsis* flowers ethanolic extract increased the mean percentage mortality, which was apparent at 10000 and 5000 ppm. Furthermore, a mean percentage of 100% mortality at 10000 ppm concentration was observed at 24 hpta. Whereas, 5000 ppm concentration recorded a mean percentage of 55.56 % mortality at 36 hpta and which further increased to 100% at 48 hpta (Table 6).

Table 6. *Mean percentage mortality of Danio rerio embryos after 24, 36, and 48 hours of exposure to different concentrations of ethanolic extract of D. phalaenopsis flowers.*

CONCENTRATION (PPM)	TREATMENT EXPOSURE		
	24 HRS	36 HRS	48 HRS
0.00	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$
500	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$
1000	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$	$0.00^{a}\pm0.00$
5000	$0.00^{a}\pm0.00$	$55.56^{b}\pm19.25$	$100.00^{b}\pm0.00$
10000	$100.00^{b}\pm0.00$	$100.00^{c}\pm0.00$	$100.00^{b}\pm0.00$

Using probit analysis, the LC_{50} of ethanolic extract of *D. phalaenopsis* flowers in *Danio rerio* embryo were revealed to be 2152.84 ppm. The LC_{50} (lethal concentration) suggest a 50% mortality at a lower concentration which is usually used in preparation of safe concentrations of drugs [26]. Analysis of variance revealed that mean percentage mortality at 24 hpta for 10000 ppm concentration is significantly different to the concentrations ≤5000 ppm. Meanwhile, at 36 hpta and 48 hpta, 5000 ppm and 10000 ppm concentrations are significantly different from the control. These implies that the harmful effects of *D. phalaenopsis* flowers extract was found to be concentration and duration dependent, as evidenced by increasing embryo mortality at greater concentrations and longer exposure to various treatments. The adverse effects can be associated to the phytochemicals present in *D. phalaenopsis* ethanolic extract which could have significant effects on embryos, resulting in mortality as early as 24 hours and further exposure to the extract. Elsayed et al. [38] found out that essential oils from *Moringa oleifera* and *Moringa peregrina* were toxic to *Danio rerio* embryos at various concentrations. While Clemen-Pascual et al. [39] reported that negative impacts could be due to alkaloids found in most extracts of 53 Philippine medicinal plants.

Percentage Hatchability

Hatching is an important indicator of successful embryonic development [40]. As shown in table 7, *Danio rerio* embryos treated with 500 ppm and 1000 ppm of ethanolic extracts had a mean hatchability of 100% at 48 hours while otherwise at 5000 ppm and 10000 ppm ethanolic extracts. Many factors may influence the rate of hatchability including early coagulation and significant delay in the embryonic growth. Furthermore, the ethanolic extract of *D. phalaenopsis* flowers may delay the hatching process through the inhibition of the release of chorionase, an enzyme needed for hatching [41].

Table 7. *Hatchability of Danio rerio embryos treated with different concentrations of ethanolic extract of D. phalaenopsis flowers.*

CONCENTRATION (PPM)	48 HRS
0.00	100.00^{b}±0.00
500	100.00^{b}±0.00
1000	100.00^{b}±0.00
5000	0.00^{a}±0.00
10000	0.00^{a}±0.00

Heartbeat Rate

Normal embryonic heartbeat rate of *Danio rerio* embryos is considerably closer to humans, at 120-180 beats per minute [29]. Table 9 shows that embryos exposed to 0 ppm of *D. phalaenopsis* flowers ethanolic extract had the highest mean heartbeat rate with 168 beats per minute which is significantly higher compared to those treated with 1000 ppm with 159 beats per minute. For 5000 and 10000 ppm, no detectable heartbeat was observed. Lack of detectable heartbeat during the observation period implies delay in growth. Based on the findings, as the extract concentration increased, the heartbeat rates were also reduced significantly. Lack of detectable heartbeat, blood circulation, movement for 20 seconds, and coagulation are signs of mortality in *Danio rerio* [29].

Table 8. *Heartbeat of Danio rerio embryos after 36 hours of exposure to different concentrations of ethanolic extract of D. phalaenopsis flowers.*

CONCENTRATION (PPM)	HEARTBEAT
0.00	168.89^{c}±5.05
500	163.56^{bc}±4.07
1000	159.11^{b}±3.36
5000	0.00^{a}±0.00
10000	0.00^{a}±0.00

Morphological Endpoints of Treated Zebra Fish Embryos

Presented in Table 9 are the lethal and teratogenic effects observed in *Danio rerio* embryos that were exposed to various concentrations of *D. phalaenopsis* flowers. Figure 1 shows that 1000 ppm concentration exhibit teratogenic effects such as yolk sac edema and little pigmentation. Meanwhile, 5000 ppm and higher concentrations cause lethal effects such as coagulation and teratogenic effects including growth retardation. The

delayed growth of embryos treated with 5000 ppm and 10000 ppm concentrations of the extract could be due to the phytochemical constituents present, such as alkaloids, flavonoids, and phenolic compounds, which have been linked to cytotoxic properties. The most noticeable teratogenic effect caused by *D. phalaenopsis* flowers ethanolic extract was growth retardation at 5000 ppm and 10000 ppm concentrations. Other teratogenic effects observed were yolk sac edema and little pigmentation on *Danio rerio* embryos treated with 1000 ppm concentration at 48 hpta. Meanwhile, coagulation (lethal effect) was observed in embryos treated with 10000 ppm concentrations at 24 hpta and in 5000 ppm at 48 hpta. This notable teratogenic activity is important for assessing the anti-cancer, anti-tumor, and apoptotic effects of secondary metabolites [41].

Secondary metabolites that act as anti-oxidants can also exhibit antiviral, antitumor and anticancer [42]. As such, saponins, terpenoids and alkaloids prevents different types of cancers [17-19]. Vinblastine, vincristine and taxol are the known alkaloids anti-cancer agents that were first isolated in plants [42]. Meanwhile for lycopene and tocopherol for terpenoids, resveratrol and curcumin for polyphenols, kaempferol and apigenin are bioactive compounds with anti-cancer properties. Their mechanisms include the inhibition of cancer cell growth and inducing cell apoptosis. Metabolic regulation and activation of signaling pathways through interactions to other compounds [43]. Independently or synergistically with other compounds through regulation of metabolic and signaling pathways alters the physiology of cancer cells [44].

Table 9. *Lethal and teratogenic effects of various concentrations of D. phalaenopsis flowers ethanolic extract at 12, 24, 36, and 48 hours of exposure.*

TOXICOLOGICAL ENDPOINTS	TIME OF TREATMENT EXPOSURE (H)	CONCENTRATION (PPM) 10000	5000	1000	500	0
Lethal						
Coagulation	12	-	-	-	-	-
	24	+	-	-	-	-
	36	+	-	-	-	-
	48	+	+	-	-	-
No heartbeat	12	-	-	-	-	-
	24	-	-	-	-	-
	36	+	+	-	-	-
	48	+	+	-	-	-
Teratogenic						
Growth	12	+	+	-	-	-
Retardation	24	+	+	-	-	-
	36	+	+	-	-	-
	48	+	+	-	-	-
Limited	12	-	-	-	-	-
pigmentation	24	-	-	-	-	-
	36	-	-	+	-	-
	48	-	-	+	-	-
Yolk sac edema	12	-	-	-	-	-
	24	-	-	-	-	-
	36	-	-	-	-	-

48	-	-	+	-	-

**presence (+) or absence (-) of lethal or teratogenic effects; parameters not observed in all concentrations at various observation hours were removed from the table*

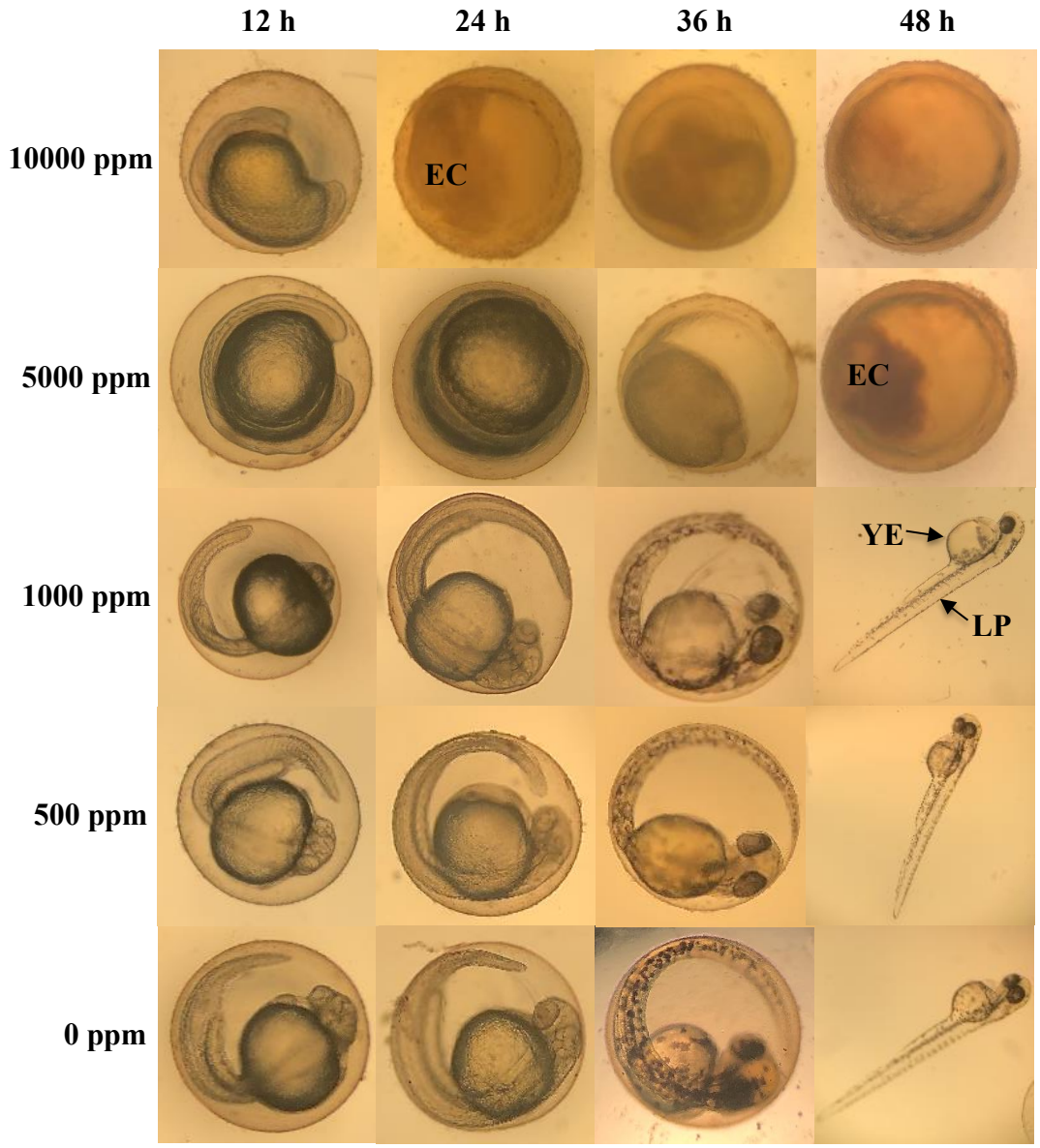


Fig 1. *Morphological development of embryos exposed to different concentrations of D. phalaenopsis flowers ethanolic extract. Lethal effects: embryo coagulation (EC) 24 hpta in 10000 ppm; embryo coagulation (EC) 48 hpta in 5000 ppm; teratogenic effects: yolk sac edema (YE) and little pigmentation (LP) at 48 hpta in 1000 ppm.*

CONCLUSION

D. phalaenopsis flowers ethanolic extract contain several phytochemicals including essential oils, phenols, fatty acids, anthraquinones, anthrones, tannins, flavonoids, and alkaloids. Moreover, the extract exhibited anti-oxidant activity. Zone of inhibition was observed against *S. aureus*, but none against *E. coli*. Meanwhile, findings in protectant

test suggests that the extract can be a potential protectant against *E. coli* and *S. aureus*. Moreover, no antifungal activity was observed against *A. flavus*. Furthermore, the can be considered toxic with LC_{50} of 2152.84 ppm. Lethal effects to *Danio rerio* embryos were observed at ≥5000 ppm and teratogenic effects at 1000 ppm concentration

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