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PHYTOCHEMICAL COMPOSITIONS AND BIOLOGICAL ACTIVITIES OF LEAF EXTRACT OF OREGANO (*Origanum vulgare*)

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ABSTRACT

This paper reported the chemical compositions and biological activities of leaves of *Origanum vulgare*. The phytochemical constituents, antioxidant properties, cytotoxic, embryo-toxic and teratogenic effects were investigated. *O. vulgare* contained alkaloids, tritepenes, phenols, essential oils, fatty acids, and anthraquinones while flavonoids, coumarins, tannins, and cardiac glycosides were absent. The extract showed scavenging activity of 43.22% against DPPH free radicals. Cytotoxic assay revealed that water extract of *O. vulgare* leaves is non-toxic. However, 10000 µg/ml of extract was found to be embryo-toxic while 1000 µg/ml and 100 µg/ml of extract showed delayed growth and morphological abnormalities including yolk deformity and stunted and bent tail as teratogenic effects, respectively. Therefore, *O. vulgare* leaves could be promising source of phytochemicals with significant functionalities.

Keywords: *Origanum vulgare, Danio rerio*, bioactivities, DPPH free radical

INTRODUCTION

Plants play a vital role when it comes to medicinal purposes. Scientific investigations are needed to properly identify and recognize the status of medicinal plants in the field of pharmacology. Oregano (*Origanum vulgare*) is one of the medicinal plants which are being utilized in folkloric medicine. Its leaves are used as natural remedy for severe bronchitis, asthma, diarrhea, epilepsy, cough, and fever. It is also reported that oregano has antibacterial and antioxidant properties, and rich source of vitamin K that promotes bone growth, maintain bone density, and produce blood clotting protein **[1]**. Moreover, it also possesses potent cytotoxic and antibacterial activities which are attributed mostly to the presence of the isomeric phenolic constituents **[2]**. The major compounds found in oregano were quinic, rosmarinic, 3-(3,4-dihydroxyphenyl)-2- hydroxypropanoic acids, luteolin-7-0- and glucurunide **[3]**.

Zebrafish (*Danio rerio*) is an efficient and well-suited animal model for teratogenicity and embryo-toxicity evaluation due to their small size, transparency, rapid development, high fecundity, and developmental similarity to human development **[4]**. On the other hand, brine shrimp (*Artemia salina*) is being used to screen the cytotoxic effects of certain compounds or extracts. These assays are considered preliminary tests in order to determine the biological and functional activities such as anticancer and antitumor. Antioxidants are compounds that scavenge free radicals and discovering the natural sources of these agents is of high interest nowadays. Some of these natural antioxidants are phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, and ascorbic acid **[5, 6]**.

Herein, we reported the phytochemical compositions, radical scavenging activity, cytotoxic and teratogenic effects of *O. vulgare* leaf extract in order to establish its status as safe and effective natural agents in pharmaceutical industries.

MATERIALS AND METHODS

Source of Plant Sample

Leaves of *O. vulgare* were collected from the Department of Biological Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. Samples were washed three-times, cut into pieces, air-dried at room temperature for 3 days, and pulverized using a blender.

Extraction

The 20 g of powdered air-dried leaves of *O. vulgare* was soaked in 600 ml of 95% ethanol for 48 hours. After soaking, this was filtered using Whatman filter paper No. 2 and the filtrate was concentrated in rotary evaporator. The extract yield was determined and this was used in the phytochemical screening and radical scavenging activity assay. On the other hand, hot water extraction was also done for the cytotoxicity and teratogenicity assays. Powdered leaves (10 g) was placed in 1000 ml-capacity Erlenmeyer flask containing 300 ml of double distilled water and then subjected to waterbath at 80-90°C for 2 hours. This was filtered and prepared for the different concentrations.

Phytochemical Screening

Phytochemical screening protocol of Guevarra et al. **[7]** was carried out to detect the secondary metabolites present in *O. vulgare*. Extract was spotted on mark and labeled TLC (thin layer chromatography) 7 x 4 cm, and was developed in the acetate-methanol (7:3) mixture in the developing chamber. The spots for a certain metabolite were visualized on the TLC plates and were exposed under UV light and hot plate to check the separation of the different compound. For typical visualization of secondary metabolites, vannilin-sulfuric acid reagents were utilized. This solution can determine the presence of phenols, sterols, triterpenes and essential oils. Methanolic potassium hydroxide was used to test antraquinones, coumarins, and anthrones while phenolics compound and tannins were detected through the use of potassium ferricyanide-ferric chloride reagent. Dragendorff's reagent was used to spot alkaloids and antimony (III) chloride will be used to detect the presence of flavonoids.

Radical scavenging activity assay

The concentrated extract was used to make a stock solution and aliquot was taken to make 1000 ppm dilution and 1000 µg/ml of catechin as control (1mg/mL). One mL of prepared stock solution was mixed with 4 mL of 0.1 mM DPPH solution in separate plastic cuvette. Reactions were done in triplicate. The prepared mixture was incubated in the dark at 37˚C for 30 minutes. The absorbance readings were monitored at 517 nm using a UV VIS spectrophotometer. A lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical scavenging activities were compared to the activity of the control catechin. The ability to scavenge the DPPH radical was calculated using the formula: % Radical Scavenging Effect = [($A_{control}$– A_{sample}) / $A_{control}$] x 100, where $A_{control}$ is the absorbance of the control which is the DPPH without the test sample, A_{sample} is the absorbance of the test sample containing the mixture of the DPPH and the sample. This procedure was followed after Kolak et al. **[8]**.

Brine shrimp lethality assay

The extract was diluted to saline solution following the different concentrations namely; 0, 1, 10, 100, 1000, 10000 μg/ml and then dispensed into vials. Triplicates were done per concentration. Twenty nauplii were transferred into each replicate. The number of dead nauplii was determined after 24 hours. The percentage mortality was calculated by dividing the number of dead nauplii to the total number, multiplied by 100%. Using probit analysis, the 50% lethality concentration (LC_{50}) was determined.

D. rerio **embryo-toxicity and teratogenicity sssay**

An aquarium comprising of untreated and clean water with continuous aeration at 26±1°C was maintained for adult female and male zebrafish at a ratio1:2, respectively. Fish were then acclimatized for 1 week before using for spawning and fertilization. They were nourished daily with a high protein diet flakes. Zebrafish were confined in a plastic mesh to prevent cannibalism. After which, the zebrafish were subjected in dark condition by wrapping the aquarium with black plastic bag to allow spawning. After 12 hours in the dark condition, the aquarium was exposed to lighted condition for another 12 hours. Fertilization occurs 30 min after the light was turned on. Twelve-hour post fertilized embryos were siphoned out from the aquarium using a hose and transferred in a beaker. Embryos were rinsed three times with distilled water and placed in a Petri plate to check the uniformity and normal conditions of embryos using a compound microscope. Unfertilized or coagulated eggs were discarded.

The different treatment concentrations (0, 1, 10, 100, 1000, and 10000 μg/ml) were prepared by diluting the extract to embryo water. Two ml of each treatment concentration of extract was dispensed 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 under 40X magnification using a compound microscope after 12, 24, 36 and 48 hours of incubation. Morphological endpoint evaluation of treated zebrafish was based on the parameters established by Nagel **[9]**: lethal (coagulation, tail not detached, no somites, and no heart-beat), teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement), and normal. Hatchability, malformation and mortality rates were recorded. A test was classified as valid, if 100% of the embryos in the control (embryo water) showed normal conditions. Data were analyzed using Analysis of Variance

(ANOVA) and compared using Duncan Multiple Range Test (DMRT) at 5% level of significance.

RESULTS AND DISCUSSION

Phytochemical Constituents of *O. vulgare*

Phytochemicals are important component of plants that play a major role in the biological activities of plants. In this study, the phytochemical constituents of *O. vulgare* were elucidated and the results are presented in Table 1. Apparently, the leaf of *O. vulgare* has alkaloids, tritepenes, phenols, essential oils, fatty acids, and anthraquinones while flavonoids, coumarins, tannins, and cardiac glycosides were absent. Alkaloid has been widely studied for their potential use in the elimination and reduction of human cancer cell lines **[10]**. Some triterpenes can be important remedies for curing diabetes mellitus and they can be used for the development of new multitarget bioactive drugs. They also have a strong antioxidant capacity and it inhibit the formation of advanced glycation end products, implicated in the pathogenesis of diabetic nephropathy, neuropathy or impaired wound healing and embryopathy **[11]**. Phenolic compounds are known for its preventive activity against infectious and degenerative diseases, inflammation and allergies via antioxidant, antimicrobial and enzymes / protein modulation / neutralization mechanisms. The roles of phenolic components in food industry are antioxidant, anti-mutagenic, and scavenging activity of free radicals and prevention of pathologies such as cardiovascular heart diseases and cancer **[12]**. Essential oils are widely used for bactericidal, fungicidal, virucidal, antiparasitical, medicinal, insectidal and cosmetic applications, mostly in pharmaceutical, agricultural, sanitary and food industries. Based from its cytotoxic capacity, essential oils can also be used for antiseptic and antimicrobial agents for personal use. It also shows a potential for antimutagenic activity and anticarcinogenic activity **[13]**. Fatty acids play an important role in the body. It serves as fuel, serves structural functions as part of cell membranes, transports fat-soluble vitamins and it involves in cell regulation and signalling. For clinical applications, it is used in managing diseases, giving them a unique role as a nutraceutical, which is a nutrient that has properties of a drug **[14]**. Anthraquinones, however, has therapeutic potential in autoimmune diabetes and it also shows an anti-inflammatory activity **[15]**.

Table 1: Phytochemical compositions of *O. vulgare*	
Bioactive Components	**Results**
Alkaloids	Present
Flavonoids	Not Detected
Fatty acids	Present
Anthraquinones	Present
Cardiac Glycosides	Not Detected
Triterpenes	Present
Coumarins	Not Detected
Tannins	Not Detected
Phenols	Present
Essential Oils	Present

Radical Scavenging Activity of *O. vulgare*

DPPH radical scavenging assay is widely used to determine the ability of compounds as antioxidants or free radical scavengers. In this study, the free radical scavenging activity of *O. vulgare* extract was evaluated. The assay revealed that the extract showed scavenging activity with a mean of 43.22%. Although lower than the radical scavenging effect of control catechin (71.68%), this value dictates the promising potential of *O. vulgare* leaves as source of natural antioxidant. Kyoji et al. **[16]** also reported the antioxidant and anti-inflammatory activities of oregano in mice.

Cytotoxic Effect and LC_{50} of *O. vulgare* Extract

Cytotoxic activity of certain compounds or extracts is determined using brine shrimp nuplii in order to preliminary establish their bioactivities and toxic effects. In this study, the cytotoxic effect of water extract of *O. vulgare* was evaluated. The percentage mortality of the nuplii was recorded after 24 hours of exposure to the different concentrations of the extract. The 10000 μg/ml had 58.33% mortality whereas the 1000 μg/ml showed 35.00% mortality. The median lethal concentration (LC_{50}) value of *O. vulgare* extract is higher than 5000 ug/ml. This LC_{50} value indicates that *O. vulgare* water extract is non-toxic based on the toxicity rating established by Clarkson et al. **[17]**.

Embryo-toxic and Teratogenic Effects of *O. vulgare*

Zebrafish embryo is the most common model in assessing embryo-toxic and teratogenic effects of extracts or compounds. In this study, the embryo-toxic and teratogenic effects of water extract of *O. vulgare* in developing embryos of zebrafish were investigated. Percentage mortality was determined every after 12 hours of extract exposure (data not shown) and this was defined as coagulated and no visual of heartbeat. No mortality was observed in all concentrations after 12 hours of exposure.

However, 100% mortality was started to observe after 24 hours to the embryos exposed at the highest concentration used. These results indicate that *O. vulgare* water extract is not toxic to zebrafish embryo and this conforms to the non-toxic effect of the extract in brine shrimp toxicity assay.

The heart is the known as the first organ to form and function during vertebrate embryo development. Heart development of the zebrafish can be considered as a miniature version of the process that occurs in mammals which is an ideal mode to study the mechanism of cardiac development and other related congenital cardiac defects in human **[18]**. The heartbeat rate of exposed embryos was also observed and data are presented in Table 2. Apparently, control embryos showed the highest heartbeat rate of 146.67 per min, which is not statistically different to those exposed at 1 µg/ml (132.00 per min). However, embryos at 1000 µg/ml recorded the lowest rate of 116.33 per min. It can be noticed that the rate of heartbeat had decreased in increasing concentration of the *O. vulgare* water extract. No heartbeat was observed in embryos at the highest concentration due to the observed coagulation after 24 hours of exposure.

Hatching process was also monitored after 48 hours of treatment exposure. It can be seen that all extract-treated embryos showed lower percentage hatchability except those at 1 ug/ml, which is statistically comparable with the control. No hatching was noted in embryos at 1000 and 10000 µg/ml. The no to low percent hatching indicate delayed growth among embryos. Delayed growth is one of the most distinct teratogenic effects of *O. vulgare* extract and this was obvious to embryos exposed to 1000 and 10000 µg/ml of extract (Figure 1). Aside from delayed growth, there were two morphological abnormalities observed including yolk deformity and stunted and bent tail hatched (Figure 2). Embryo with yolk deformity was observed in 100 µg/ml after 12 hours exposure while the stunted and bent tail hatched was seen to those exposed for 48 hours in 100 µg/ml. Thus, 100 µg/ml of *O. vulgare* is teratogenic to zebrafish.

Table 2: Hatchability and heart beat rate of zebrafish after 48 hours

Extract (µg/ml)	Hatchability (%)	Heart beat (per min)
0	91.67^{a}	146.67^{a}
1	75.00^{ab}	132.00^{ab}
10	50.00^{b}	126.33^{bc}
100	66.67^{b}	120.00^{bc}
1000	0.00^{c}	116.33^{c}
10000	0.00^{c}	NHB*

In each column, treatment means having the same letter of superscript are not significantly different from each other at 5% level of significance using LSD. *NHB; no heart beat

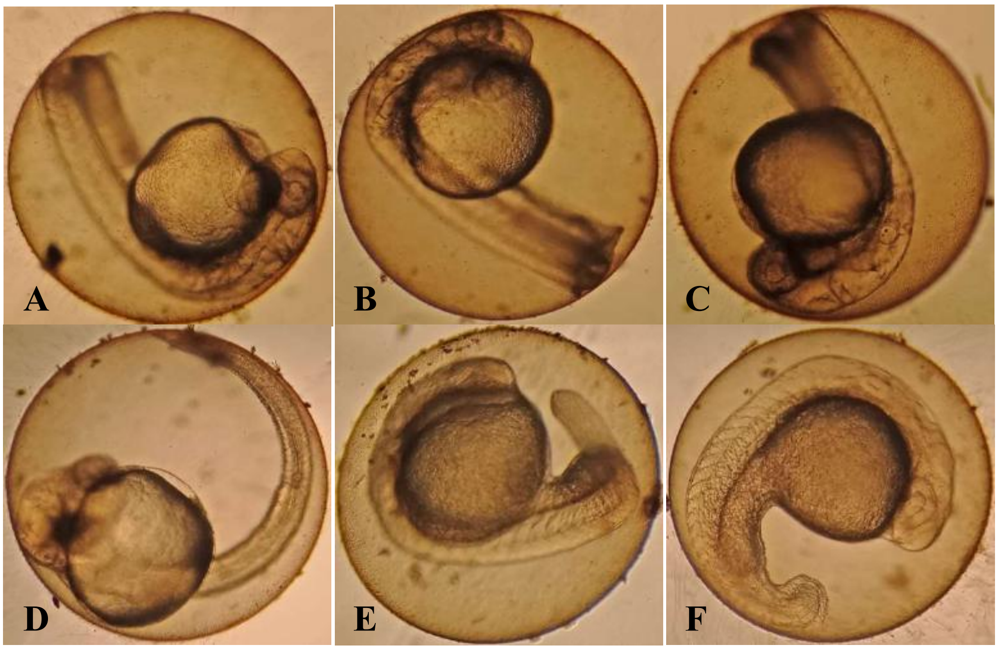

Figure 1: Zebrafish embryos after 12 hours exposure to varying concentration of extract of *O. vulgare*: (A) 0, (B) 1, (C) 10, (D) 100, (E) 1000, and (F) 10000 µg/ml, Delayed growth was obvious to embryos exposed to 1000 and 10000 µg/ml of extract

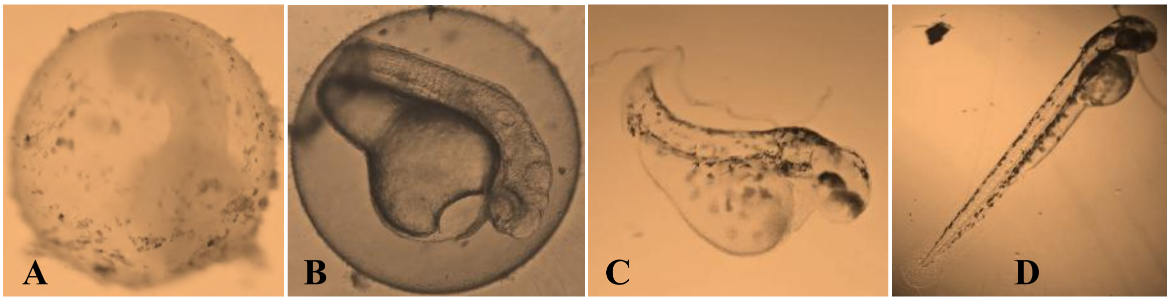

Figure 2: Toxic effect and morphological abnormalities of embryos exposed to varying concentrations of the extract of *O. vulgare*: (A) Coagulated embryo exposed at 10000 µg/ml after 24 hours; (B) Embryo with yolk deformity at 100 µg/ml after 12 hours; (C) Stunted and bent tail hatched exposed at 100 µg/ml after 48 hours; and (D) control normal hatched zebrafish after 48 hours

CONCLUSION

In conclusion, *O. vulgare* leaves contain phytochemicals that exhibit biological activities. Its ethanol extract showed scavenging activity against DPPH free radicals while its water extract is non-toxic to brine shrimp nuplii but showed toxic and teratogenic effects in developing embryos of zebrafish.

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