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Research Article

Identification and assessment of biological activities of *Gymnanthemum amygdalinum* (Delile) Sch.Bip. ex Walp. collected from Bongabon, Nueva Ecija

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Abstract: The medicinal potential of plants encompasses a diverse array of compounds with therapeutic applications. These compounds have the potential to contribute to the development of innovative pharmaceuticals that enhance overall health. This study highlights the molecular identification, phytochemical analysis, teratogenic and cytotoxic effects of *Gymnanthemum amygdalinum* collected from Bongabon, Nueva Ecija. Morphological and molecular identification confirmed the identity of *G. amygdalinum* having 100 % similarity to their corresponding sequences. Also, *G. amygdalinum* exhibited secondary metabolites such as essential oils, phenols, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, steroids, and alkaloids. The plant extract has teratogenic effects as mortality rate was observed at 1000 and 10,000 ppm, correlated with low hatchability rate at the same concentrations. These findings demonstrated the potential for anticancer, leading to further evaluation of cytotoxicity employing *Artemia salina* and hepatocellular carcinoma cell lines (HepG2). As a result, *G. amygdalinum* was found to be moderately toxic in brine shrimp lethality assay with a mortality rate of 10 ppm and higher. Similarly, it is moderately toxic in HepG2 at a median concentration of 1000 ppm and highly toxic at 4000 ppm. Collectively, *G. amygdalinum* extract exhibits teratogenic and cytotoxic effects and is suitable for further studies at the same or higher concentrations. Accordingly, it is recommended to proceed to the next phase of study for anticancer and antiproliferative. This study provides a scientific foundation for future research, supporting the researchers in uncovering the medicinal potentials of not only *G. amygdalinum* but other medicinal plants as well.

1. INTRODUCTION

The use of medicinal plants is an important aspect of traditional medicine in the Philippines, particularly among those who live in distant mountainous areas remote from cities. Its origins are found in the traditions among various ethnic Filipino communities. To date, herbal medicines have been recognized by researchers to be one of the foundations for discovering the potential medication of plants.

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Phytochemicals from plants are being studied for direct medicinal applications as well as prototype lead compounds for the development of newly manufactured synthetic or semisynthetic medicines (Chattopadhyay & Maurya, 2015). Locals in Bongabon, Nueva Ecija, commonly use medicinal plants instead of commercially produced medicines since these are more readily available and it is a traditional practice. When residents become ill or infected with a disease, they simply boil the leaves of those well-known medicinal plants found in their vicinity or from the nearby mountains. Furthermore, there is a particular plant in their area that is highly utilized for its medicinal value even though this plant has no local name and is still unidentified. In this regard, it is vital to identify this plant using a molecular approach to provide the local people residing in the area with accurate information regarding the identity of the plant they utilized. DNA barcoding is being used in an increasing number of studies to identify unknown organisms and determine their taxonomy. This plant molecular identification method involves obtaining one or more DNA sequences of one or more candidate genes and comparing them to a repository of genes associated with plant taxa (Simpson, 2019).

Locals, on the other hand, believe that due to its high efficacy as a traditional medicine in treating diseases, this plant could potentially be used to treat cancer. However, extensive research and several clinical trials are required before one can claim such potential of this plant. In connection to this, several bioassays, teratogenicity, and cytotoxicity were conducted to come up with the initial data on the anti-cancer potential of this plant. Teratogens are chemical, physical, or biological agents that can cause developmental defects. Some teratogens cause death, while others cause cell apoptosis. Teratogens are also capable of causing developmental abnormalities by altering gene expression patterns, inhibiting cell interactions, or preventing the morphogenetic movement of cells (Fenderson, 2009). The zebrafish is a good animal model that can be used to assess the teratogenic effects of some medicines because of its characteristics and traits that are similar to those of other vertebrate animals particularly humans (Dulay *et al*., 2012). Teratogens and teratogenic agents may be developed as anticancer drugs, therefore, anticancer and teratogenic effects are two closely related concepts (Blagosklonny, 2005). It has been proven through experimentation that anticancer plants have cytotoxic effects (Ghorani *et al*., 2018). Cytotoxicity test is one method of determining toxicity, which is an *in vitro* test that is mainly performed to screen potentially toxic compounds that affect basic cellular functions (Fotakis & Timbrell, 2006). Therefore, this study aims to molecularly identify the medicinal plant collected from Bongabon, Nueva Ecija, to determine its secondary metabolites and anti-cancer potential by evaluating its teratogenic and cytotoxic effect on zebrafish, brineshrimp nauplii, and hepatocellular carcinoma cell lines. In addition, this research can significantly contribute to the body of knowledge, mainly in the pharmaceutical and biomedical fields.

2. MATERIALS and METHODS

2.1. Sample Collection

The plant material was collected in Brgy. Calaanan, Bongabon, Nueva Ecija. Plant leaves were collected and used in the study since this is the part of the plant that was utilized by the local people. To prevent deterioration of plant components, leaves were immediately air dried after collection. Fresh leaves, on the other hand, were collected for molecular identification and placed in a 2 mL microtube containing Cetyl trimethylammonium bromide (CTAB) buffer.

2.2. Morphological Identification

The collected plants were photographed in their natural habitat. The main external plant structures and shapes were observed. A taxonomist from the Department of Biological Sciences, College of Science, Central Luzon State University confirmed and authenticated the identification of the plant specimen.

2.3. Nucleotide Sequencing

The genomic DNA of the plant samples was extracted from the dried leaf sample using the (CTAB) method of Murray and Thompson (1980) with minor modifications. About 100 mg of plant sample was grounded using mortar and pestle. Six hundred (600) µL of 2X CTAB buffer and 70 µL of 20 % Sodium dodecyl sulfate (SDS) were added to the ground sample and then thoroughly homogenized and incubated for 45 min in a dry bath (Labnet AccublockTM). Chloroform iso-amyl (19:1 v/v) was added and spun (Beckman Coulter) at 12,000 rpm for 30 min. After transferring the aqueous phase, 500 µL of ice-cold isopropanol was added, and incubated overnight. Following incubation, it was spun for 10 minutes at 10,000 rpm with 70 % ethanol.

The pellet DNA was dried for 3 hours of incubation at room temperature, and 100 µL 1X TE Buffer was added to completely dissolve the DNA pellet. To check the DNA quality, 2 µL of the DNA sample and 2 µL of loading dye were loaded in each well of a 1 % agarose gel and run in gel electrophoresis (Accuris My GelTM) for 30 min at 100V. The gel was then visualized in a UV trans-illuminator (UVitec Cambridge). DNA samples were purified, then aliquoted into 1:50 µL of nuclease-free water and used for PCR amplification.

The nucleotide ribosomal DNA region of the samples was amplified using the *rbcL* gene marker and the following components of GoTaq® Green Mastermix with 1 µL of 10 µM forward primer *rbcL* ATG TCA CCA CAA ACA GAG ACT AAA GC and 1 µL of 10 µM reverse primer *rbcL*724 GTA AAA TCA AGT CCA CCR CG. The PCR was performed using a thermal cycler (BioRad T100™) and the parameter was set to 95°C for 3 minutes (initial denaturation), followed by 35 cycles of 95°C for 30 seconds (denaturation), 51.1°C for 30 seconds (annealing), 72°C for 1 minute (extension), 72°C for 10 minutes (final extension). The PCR product was checked using gel electrophoresis (Accuris My Gel™) for 30 minutes at 100V; 2 µL from the PCR product was loaded in each well. The gel was visualized in the UV trans-illuminator Gel Documentation System (Uvitec Cambridge Gel Documentation System). Once amplified, DNA was quantified using the Spectrophometer (Multiskan™ Go™ ThermoFisher). When the expected size of amplified DNA fragments was confirmed, PCR amplicons were stored in the microtubes and sent to Apical Scientific Sdn Bhd in Malaysia for PCR purification and double pass DNA sequencing procedure using the forward and reverse primers. The BioEdit software was used for the visual presentation of chromatograms to check the sequence quality. The sequences of the samples were queried using BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) to compare with similar nucleotide sequences stored in the Genbank for proper identification.

2.4. Phytochemical Analysis

2.4.1. Preparation of extract

Ethanol extraction was used in the study. The air-dried leaves were ground using a mill grinder and sieved to remove large portions of the leaves. Ten grams of powdered leaves were soaked in ninety percent (90 %) ethanol and kept in a well-sealed flask for three days with constant agitation. After 3 days, the sample was filtered using filter paper (Whattman No. 2), then the filtrate was concentrated to dryness using a rotary evaporator (DLabTM).

2.4.2. Phytochemical screening

The screening was performed to detect the secondary metabolites of the crude extract. The extract was spotted on a 7 x 4 cm labeled TLC (thin layer chromatography). This was made in the developing chamber using an acetate-methanol (7:3) mixture. To test the separation of the different substances, the spots for specific metabolites were seen using TLC plates that were subjected to UV light and a hot plate. Vanillin-sulfuric acid reagents were used to detect the presence of phenols, steroids, triterpenes, and essential oils. Secondary metabolites such as

anthraquinones, coumarins, and anthrones were detected using methanolic potassium hydroxide. The potassium ferricyanide-ferric chloride reagent was used to identify the phenolic compounds and tannins. Finally, the presence of flavonoids was determined using a Dragendorff's reagent.

2.5. Teratogenicity Assessment

The teratogenic assessment used in this study was based on the published article by De Leon *et al*. (2020). The treatment concentrations were calculated using the standard dilution method, $C_1V_1 = C_2V_2$, with the extract being diluted with embryo water (Hank's solution). Each vial contained three milliliters of treatment concentration, along with four embryos in the segmentation phase and incubated at 26±°C. After 12, 24, 36, and 48 hours of incubation, teratogenic activity was observed under a compound microscope (Bell Photonics) at 40x magnification. Teratogenic (head and tail malformations, scoliosis, growth retardation, stunted tail, and limited movement) and lethal (coagulation, tail not detached, no somites, and no heartbeat) morphological endpoints were evaluated. The hatchability and mortality rate of the eggs were also assessed.

2.6. Brine Shrimp Lethality Assay

Brine shrimp lethality assay based on McLaughlin *et al*. (1998) as cited by De Leon *et al*. (2020) was used to evaluate the cytotoxicity of the *G. amygdalinum* leaf extract. The LC_{50} was evaluated according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of <249 µg/mL is highly toxic, LC_{50} of 250-499 µg/mL is moderately toxic and LC_{50} of 500-1000 µg/mL is mildly toxic. Moreover, values above 1000 µg/mL are non-toxic according to the rating of (McLaughlin *et al*., 1998). Under laboratory conditions, all the treatments were laid out in a completely randomized design (CRD), at a 5 % level of significance, and one-way analysis of variance (ANOVA) was used to determine the least significant differences (LSD) between treatments.

2.7. Cytotoxicity Assay using Hepatocellular Carcinoma Cell lines

A total of 10 mg of leaf extract was weighed in 2 mL capacity microtubes, added with 250 µL of Dimethylsulfoxide (DMSO- ATCC® 4-X™), and mixed for 40 minutes, acquiring 40,000 parts per million (ppm) concentration. Each extract was diluted to 4000 ppm concentration from 40,000 ppm concentration using the Eagle's Minimum Essential Medium (EMEM) (ATCC® 302003™). Extracts were serially diluted in two-folds from 4000 ppm concentration, (highest concentration) until it reached 125 ppm (lowest concentration) used for the treatment. The positive control: 5-fluorouracil (Sigma-Aldrich, Germany) was also prepared with a similar concentration and diluted with EMEM.

Human hepatocellular carcinoma [HepG2] (ATCC® HB-8065™) cells were cultured in T25 flasks using Eagle's Minimum Essential Medium (ATCC® 302003™), supplemented with 10% Fetal Bovine Serum (FBS) (HyClone™ Sera SH30071.03) and 1% Penicillin-Streptomycin Solution (ATCC® 302300™). Cells were grown and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells at 80 % confluence were used for cell seeding.

The cytotoxicity activity of the extract was tested against [HepG2] (ATCC® HB-8065™) using Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH). A 5000 cells/100 µL media were seeded in 96 well plates, it was then incubated for 4 hours in a CO_2 incubator allowing cells to attach. Once attached, cells were treated in triplicates with the prepared extract of leaves and the controls. After 16-18 hours of incubation, controls were treated with lysis solution, following the manufacturer's protocol with minor modifications, and incubated for 45 mins. The incubated plates were spun down using a refrigerated centrifuge (Centurion Scientific Limited, United Kingdom) at 500 rcf, 25°C for 5 mins. A total of 30 µL of supernatant from the plates was transferred into a new plate and 30 µL of Promega CytoTox 96® Non-

Radioactive Cytotoxicity Assay (LDH) reagent was added and incubated again for 30 minutes. The plates were then read using a Multiskan Go™ (Thermo Fisher Scientific, USA) microplate reader with 20-second low shaking settings at 490 nm absorbance. Following a logarithmic model of *in vitro* cytotoxicity versus compound concentration, as established by multiple standard industry assays, compounds with percent cytotoxicity near 50 % were immediately flagged indicating that the least active concentration would be toxic to the patient. The value of 10 % cytotoxicity is arbitrarily chosen as the threshold for flagging compounds for testing discretion. This level of cytotoxicity implies that a subsequent increase in compound concentration to increase bioactivity may potentially lead to cytotoxicity of 50 %. Samples were tested in duplicates in two (2) independent trials. Samples with a highly cytotoxic profile were given an extended margin of 5 % only if the other trial exhibited a moderately cytotoxic, mildly cytotoxic, or non-cytotoxic profile (Table 1). The decision to pursue (Discontinue or Proceed) downstream experiments and orthogonal assays is based on the concurrence of both hepatic and nephric cytotoxic classifications of the samples.

$$\% \text{ Cytotoxicity} = \frac{(Experimental - Blank\ 3) - (Negative\ control - Blank\ 1)}{(Positive\ control\ 1 - Blank\ 2) - (Negative\ Control - Blank\ 1)}$$

The experimental data were all analyzed with GraphPad Prism Version 8.0.2 and the mean and standard deviation were presented. Nonlinear Regression was used to examine the LC_{50} for Dose Response Inhibition. For statistical analysis, an unpaired t-test was used, and a P-value of 0.05 was considered statistically significant.

Table 1. Criteria for classification of cytotoxicity.

Classification*	Cytotoxicity (%)
Non-cytotoxic	< 0 %
Mildly cytotoxic	0-1 %
Moderately cytotoxic	1-10 %
Highly cytotoxic	>10 %

*Based on NIMBB-DMBEL, UPD (Bataclan *et al.*, 2019).

3. RESULTS

3.1. Morphological Identification

Plant morphology plays a vital role in plant identification as it provides a visual and structural basis for differentiating between different plant species. As identified, it is a large shrub 2-3 meters long. The leaf is characterized as elliptical with entire margins, a reticulate venation pattern, and an alternate phyllotaxy arrangement. Alara and Abdurahman (2021) stated that this plant can reach a height of 1-6 m above sea level and has elliptical-shaped petiolate leaves that are 6 mm in diameter and 20 cm long. More so, an expert plant taxonomist from the Department of Biological Sciences, College of Science, Central Luzon State University has confirmed and authenticated the identity of the collected plant as *Gymnanthemum amygdalinum*.

3.2. Nucleotide Sequencing

To further confirm the identity of the collected medicinal plant from Bongabon, Nueva Ecija, molecular identification was conducted using the *rbcL* gene marker. By providing the DNA sequences of the collected medicinal plant, the National Center for Biotechnology Information-BLAST analysis confirms the identity of *G. amygdalinum*, with 100 % similarity to their respective GenBank sequences (Table 2). The identity was determined by the maximum percent similarity of plant samples obtained from GenBank as well as the comparison of Genbank sequences to the actual photograph and morphological characteristics of the plant. Considering

the 100% similarity and 100% query cover, which indicate sequence diversity between the collected plant and the *G. amygdalinum* species in GenBank, the *rbcL* gene used for identifying *G. amygdalinum* proves to be a potent molecular marker.

Table 2. Molecular Identification of *G. amygdalinum.*

Plant	BLAST Identity	% Identity	Query Cover	Accession Number
	*Gymnanthemum amygdalinum*	100 %	100 %	MN627973.1

3.3. Phytochemical Analysis

Detecting phytochemicals in plants can reveal their potential health benefits. Different phytochemicals present in *G. amygdalinum* extract were analyzed to understand its nutritional and medicinal properties and how it can be used for various purposes. The extract was evaluated for the presence of 14 secondary metabolites (Table 3), out of these 10 secondary metabolites was found present in *G. amygdalinum* extract which includes alkaloids, anthraquinones, anthrones, coumarins, essential oils, flavonoids, phenols, steroids, sugars, and tannins.

Table 3. Phytochemical composition of *G. amygdalinum.*

Plant constituents	Results
Alkaloids	+
Amino acids	-
Anthraquinones	+
Anthrones	+
Coumarins	+
Essential oils	+
Fatty acids	-
Flavonoids	+
Phenols	+
Steroids	+
Sterols	-
Sugars	+
Tannins	+
Triterpenes	-

*present (+), absent (-)

3.4. Teratogenicity Assessment

The lethal effect of various concentrations of *G. amygdalinum* ethanol extract on zebrafish embryos was assessed, and the mean percentage mortality of the embryos after 12, 24, 36, and 48 hours of exposure is presented in Table 4. At 12 hours post-treatment application, mortality was observed as 33.33 % in the 10,000 ppm and 22.20 % in the 1000 ppm which increased to 55.60 % and 33 %, respectively, after 24 hours. Moreover, at 48-hour exposure, the mortality increased at 77 % at 10000 ppm and 44.40 % at 1000 ppm. No mortality was recorded at lower concentrations of 100 ppm, 10 ppm, 1 ppm, and control throughout the observation period. As a result, the mortality of zebrafish embryos was proportionate to the concentrations of plant

extract and to the time the embryos were exposed. The percentage of mortality increased along with an increase in concentration and exposure length.

This finding indicates that *G. amygdalinum* ethanol extract has teratogenic effects on embryos which correlates to the percent hatchability at lower concentrations after 48 hours of exposure. The percent hatchability of zebrafish embryos after 48 hours of exposure to various concentrations is shown in Table 5. Hatchability is a major indicator of a successful development process. As a result, a disrupted hatching process could imply distinctive developmental problems. All embryos from concentrations ranging from 0 ppm to 100 ppm were fully formed and had hatched. However, at higher concentrations, a delay in embryo hatching was observed having a 55.60 % hatchability at 1000 ppm and 22.20 % at 10000 ppm which are the same concentrations that have exhibited mortality rates. Furthermore, the lethal effect of no heartbeat was observed at concentrations 1000 and 10,000 ppm (Table 6), while other toxicological endpoints such as coagulation and not detached tail were not observed at all concentrations. Thus, the teratogenicity assay confirmed the lethal effect of *G. amygdalinum* on zebrafish embryos. Figure 1 shows the morphological development of embryos exposed to various concentrations of *G. amygdalinum* extract. At higher concentrations (1000 ppm and 10,000 ppm), stunted growth of the embryos was observed compared to normal development at lower conce The lethal effect of various concentrations of *G. amygdalinum* ethanol extract on zebrafish embryos was assessed, and the mean percentage mortality of the embryos after 12, 24, 36, and 48 hours of exposure is presented in Table 4. At 12 hours post-treatment application, mortality was observed as 33.33 % in the 10,000 ppm and 22.20 % in the 1000 ppm which increased to 55.60 % and 33 %, respectively, after 24 hours. Moreover, at 48-hour exposure, the mortality increased to 77 % at 10000 ppm and 44.40 % at 1000 ppm. No mortality was recorded at lower concentrations of 100 ppm, 10 ppm, 1 ppm, and control throughout the observation period. As a result, the mortality of zebrafish embryos was proportionate to the concentrations of plant extract and to the time the embryos were exposed. The percentage of mortality increased along with an increase in concentration and exposure length.

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3.5. Cytotoxicity Assay using *Brine shrimp*

Given that *G. amygdalinum* extract was revealed to be teratogenic to the zebrafish embryo in the teratogenicity assay, this is a positive indicator that the plant extract might also be cytotoxic. To examine the cytotoxicity, an *in vivo* cytotoxicity assay was performed using brine shrimp (*Artemia salina*). The brine shrimp lethality assay is an efficient approach for preliminary toxicity research and testing which employs brine shrimps instead of mice and rats as *in vivo*

animal models. Table 7 shows the mortality rate of brine shrimp nauplii after 24 hours of exposure to various concentrations of *G. amygdalinum* extract. The highest mortality rate was observed at 10,000 ppm with 100 % while the lowest was observed at 10 ppm with 6.67 % mortality. The analysis of variance showed that, after 24 hours of extract exposure, there was no significant difference in all treatment concentrations at a 5 % level of significance. Based on the data, the median lethal concentration (LC_{50}) of the extract was obtained using probit regression analysis. LC_{50} value is the most important measure in determining the toxicity of a plant extract. The computed LC_{50} value is 263.03 which is considered to be moderately toxic according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of <249 µg/mL is highly toxic, LC_{50} of 250-499 µg/mL is moderately toxic, and LC_{50} of 500-1000 µg/mL is mildly toxic.

Table 4. Mortality rate at 12, 24, 48 hpta of embryo at different concentrations.

Concentration (ppm)	% Mortality		
	12 Hours	24 Hours	48 Hours
0	0.00^{b}	0.00^{c}	0.00^{c}
1	0.00^{b}	0.00^{c}	0.00^{c}
10	0.00^{b}	0.00^{c}	0.00^{c}
100	0.00^{b}	0.00^{c}	0.00^{c}
1000	22.20^{a}	33.00^{b}	44.40^{c}
10000	33.33^{a}	55.6^{a}	77.80^{c}

*Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

Table 5. Percent hatchability after 48 hpta exposure at different concentrations

Concentration (ppm)	% Hatchability
	48 Hours
0	100.00 ^{a}
1	100.00 ^{a}
10	100.00 ^{a}
100	100.00 ^{a}
1000	55.60 ^{b}
10000	22.20^{c}

*Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

Table 6. Lethal effects of various concentrations at 12, 24, and 48 hours of exposure.

Toxicological Endpoints	Time of exposure	Concentration (ppm)					
		0	1	10	100	1000	10000
Coagulation	12	–	–	–	–	–	–
	24	–	–	–	–	–	–
	48	–	–	–	–	–	–
No Heartbeat	12	–	–	–	–	+	+
	24	–	–	–	–	+	+
	28	–	–	–	–	+	+
Tail not detached	12	–	–	–	–	–	–
	24	–	–	–	–	–	–
	28	–	–	–	–	–	–

*observed (+), not observed (-)

Table 7. Mortality rate of Brine shrimp nauplii after 24 hours of exposure to different concentrations.

Concentration (ppm)	% Mortality
	24 Hours
0	0.00[d]
1	0.00[d]
10	6.67 [d]
100	23.33[c]
1000	63.33[b]
10000	100.00[a]

∗Means with the same letter superscript are not significantly different from each other at a 5% level of significance.

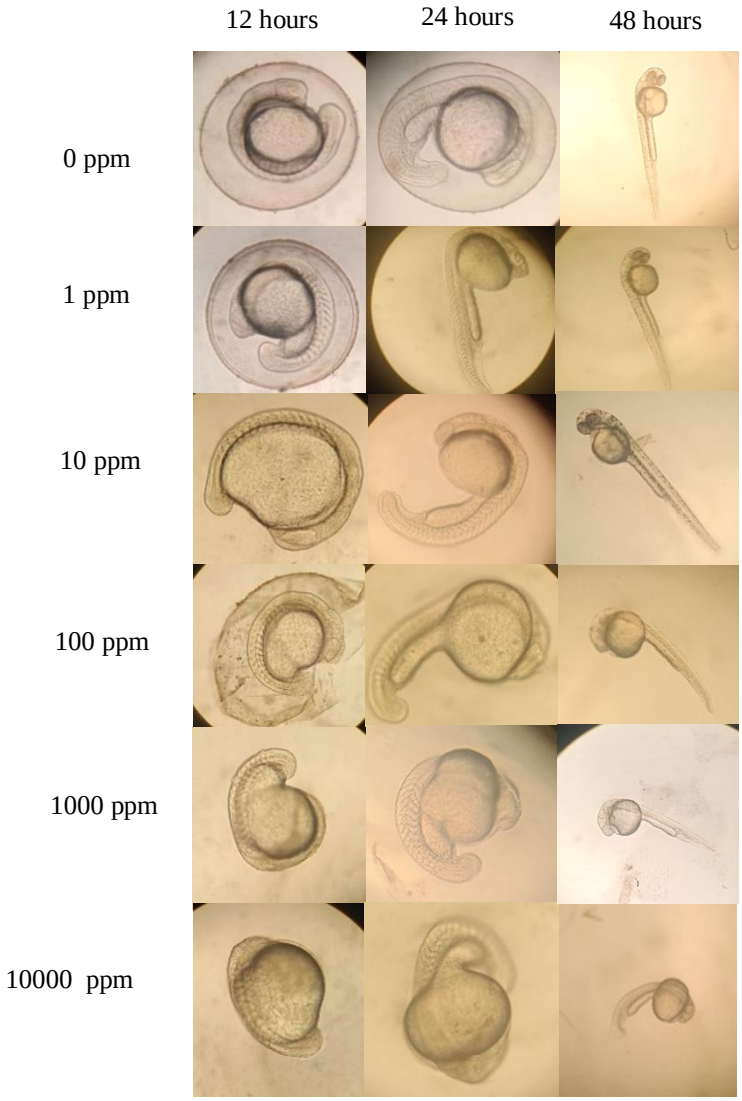

Figure 1. Morphological development of embryos exposed to different concentrations.

3.6. Cytotoxicity Assay Using Hepatocarcinoma Cell Lines

With regards to the findings that *G. amygdalinum* extract exhibits cytotoxic effects in an *in vivo* assay using brine shrimp, an *in vitro* assay was carried out using human hepatocarcinoma cell lines. These cell lines are efficient in *in vitro*, and they also maintain the genomic and transcriptome landscapes of primary HepG2. The cell-based cytotoxicity of *G. amygdalinum*

extracts was analyzed using five concentrations, with 1000 ppm being used as the median concentration, since teratogenic and *in vivo* cytotoxic effects were observed at this concentration. Starting at 4000 ppm and decreasing by half until reaching 250 ppm was found to be the lowest concentration.

Table 8 shows that after two separate trials, four (4) concentrations were found to be cytotoxic. The percent cytotoxicity was 1.82 for 500 ppm, 8.11 % for 1000 ppm, 17.21 % for 2000 ppm, and 21.26 % for 4000 ppm. Cytotoxicity levels of 500 ppm and 1000 ppm were categorized as moderately toxic, while concentrations of 2000 ppm and 4000 ppm were categorized as highly toxic. Figure 2 illustrates a dose-dependent trend in which the cytotoxicity increases as the concentration increases. The results indicate that a lower concentration of 250 ppm was not acceptable for further analysis as its toxicity level was found non-cytotoxic. However, 500 ppm, 1000 ppm, 2000 ppm, and 4000 ppm can be used in the next phase for anti-cancer, anti-proliferative, and apoptotic studies since they have the potential to suppress various cancer cell actions and are advantageous for thorough studies. Also, different cell lines could well be employed for further research using the same concentrations.

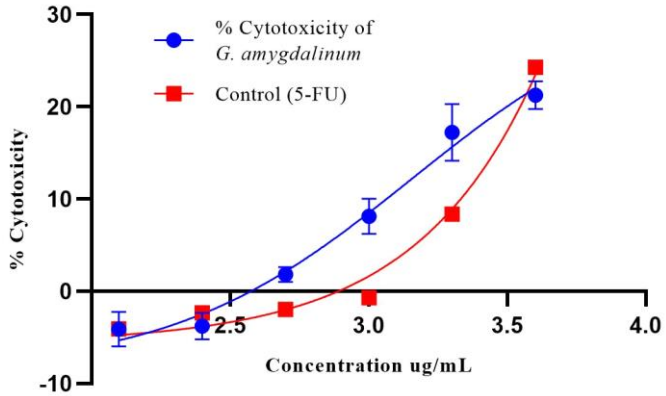

Figure 2. Cytotoxicity index plots for HepG2 cell lines (1 ppm =1 µg/mL).

Table 8. Calculated percent cytotoxicity in HepG2 cell lines.

Concentration (ppm)	% Cytotoxicity of the plant	Toxicity Level
4000	21.2595	Highly cytotoxic
2000	17.2105	Highly cytotoxic
1000	8.1175	Moderate cytotoxic
500	1.824	Moderate cytotoxic
250	-3.7765	Non-cytotoxic

4. DISCUSSION

All over the world, medicinal plants are used as alternative or complementary medications. For centuries, medicinal plants have been used in medicine and folk medicines to prevent and cure diseases by utilizing various indigenous biological systems (Elnour *et al*. 2023). In Bongabon, Nueva Ecija, the local people implement their accumulated traditional knowledge, thus they are very fascinated with utilizing medicinal plants rather than pharmaceutical products as it provides a less expensive option for treating various diseases. Studies on these medicinal plants include accurate identification, pharmacological, and toxicological evaluations are of the utmost importance for the development of new drugs.

Proper identification of plant species is therefore important to further studies into the various advantages they provide. In the present study, a medicinal plant that was widely utilized by the

locals in Bongabon, Nueva Ecija was morphologically and molecularly identified as *Gymnanthemum amygdalinum* with 100 % similarity to their corresponding GenBank sequences. The *rbcL* gene marker, which was utilized to identify *G. amygdalinum*, is a powerful molecular marker as Tnah *et al*. (2019) and Eraga *et al*. (2020) have both successfully employed this molecular marker to identify *G. amygdalinum*. In addition to this, there have been reported plants that belong to the same family of Asteraceae that confirmed its identity by also using *rbcL* gene in molecular identification such as *Cirsium arvense* (Cao *et al*., 2022a), *Argyranthemum frutescens* (Zhao *et al*., 2022), *Mikania glomerata* and *Mikania laevigata* (Bastos *et al*., 2011).

The naturally occurring bioactive compounds known as phytochemicals are derived from various plant parts and are primarily responsible for all plants' biological activities. It is used to describe plant chemicals that are not nutrients but may have health benefits by lowering the risk of developing chronic diseases (Cao *et al*., 2022b). Table 3 shows the presence of essential oils, phenols, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, steroids, and alkaloids in *G. amygdalinum* extract. *G. amygdalinum* belongs to the family of Asteraceae, according to Soković *et al*., (2019), certain plants belonging to the family Asteraceae are rich in phytochemicals. The majority of phytochemicals proved to have useful attributes, including anti-arthritic, antibacterial, antimalarial, antidiabetic, and others. It can strengthen the immune system, reduce the rate at which cancer cells grow, and protect against DNA damage, which can result in cancer and other illnesses (Kumar *et al*., 2023). In this study, the presence of important phytochemicals such as flavonoids was found. Flavonoids have a broad range of anticancer properties, making them suitable candidates for further research into the development of novel cancer chemopreventive medicines, as flavonoid-rich foods could induce advantageous changes in the gut microbiota, lowering the risk of developing cancer and normalizing vital cellular functions (Kopustinskiene *et al*., 2020).

Furthermore, renowned for having a bitter flavor, the *G. amygdalinum* is commonly named a bitter leaf. The bitterness of the *G. amygdalinum* prevents the locals of Bongabon from eating the leaves as vegetables. However, according to Engel (2007), bitterness generally denotes toxicity, it is possible that bitterness in plants could be a useful indicator of therapeutic potential. In teratogenicity assay, to determine direct-acting teratogens and conduct preliminary evaluations of embryotoxic substances (Weigt *et al*., 2011), zebrafish was used as it has increasingly been recognized as an efficient animal model in determining teratogenic effects. The embryos were considered dead if no visible heartbeat or observed as coagulated during the experiment. The number of deaths caused by a specific condition is known as mortality. In this study, mortality was observed at greater concentrations of 1000 ppm and 10,000 ppm (Table 4), which is correlated with the low hatchability rate at the same concentrations (Table 5). On the other hand, the lethal effects of no heartbeat were also observed at higher concentrations.

Teratogenicity assay confirmed the lethal effect of *G. amygdalinum* on zebrafish embryos. Additionally, there were no abnormalities in the morphological development of embryos exposed to various concentrations of Gamygdalinum extract, however, there was stunted growth of embryos at higher concentrations (1000 ppm and 10,000 ppm) compared to the normal development at lower concentrations. Thus, *G. amygdalinum* extract is teratogenic to the embryos. The mortality of zebrafish embryos was found to be related to the concentrations of *G. amygdalinum* extract used and the duration that the embryos were exposed. As the concentration and duration of exposure increased, the percent mortality correspondingly increased. This finding indicates that *G. amygdalinum* extract has teratogenic effects on embryos. In the study of teratogenicity of a rhizome plant using zebra fish, the toxicity effects were also discovered to be dose-dependent at dosage above 62.50 µg/mL, while, at 125.0 µg/mL, mortality of embryos was observed (Alafiatayo *et al*., 2019) Similar results were seen in the *Carica papaya* extract (De Castro *et al*., 2015) and *Moringa oleifera* (David *et al*., 2016)

as embryo-toxic in which teratogenicity and mortality were both concentration-dependent. Likewise, in the study of Jose *et al.* (2016), the toxic effects of *Garcinia mangostana* extract on developing zebrafish embryos were discovered to depend on the period of exposure, concentrations, and parts of the plant. The lyophilized water extract from the plant leaves was more toxic than the extract from stem bark.

Considering that *G. amygdalinum* extract was revealed to be teratogenic to the embryo in the teratogenicity assay. Determination of whether the plant extract possesses an impact on cell proliferation or exhibits direct cytotoxic effects, is of utmost importance. Drug screening typically uses cell cytotoxicity and proliferation assays (Adan, *et al*., 2016), thus, an *in vivo* cytotoxicity assay was performed using brine shrimp (*Artemia salina*). The highest mortality rate of 100 % was observed at 10,000 ppm while the lowest was observed at 10 ppm with 6.67 % mortality (Table 7). Results showed that, after 24 hours of extract exposure, there was no significant difference in all treatment concentrations at a 5 % level of significance. The computed LC_{50} value is 263.03 which is considered to be moderately toxic according to the rating of Aldahi *et al*. (2015) stating that LC_{50} of 250-499 µg/mL is moderately toxic. The assay results show that as the extract concentrations increased, the mortality of brine shrimp nauplii correspondingly increased, indicating that the mortality rate is considered high. Correspondingly, other reported studies have confirmed the moderate toxicity of *V. amygdalina* (Dosumu *et al*., 2017; Ijeh & Onyechi, 2010; Omede *et al*., 2018) while other species of *Vernonia* genus like *V. anthelmintica* shows mild cytotoxic activity (Patnaik & Bhatnagar, 2015).

To support the findings that *G. amygdalinum* extract shows cytotoxic effects in an *in vivo* assay employing brine shrimp, an *in vitro* assay was performed using human hepatocarcinoma cell lines (HepG2). Results show that the toxicity level was non-cytotoxic at lower concentrations (250 ppm) while the toxicity level was found to be moderate to highly cytotoxic at 500 ppm, 1000 ppm, 2000 ppm, and 4000 ppm. Therefore, it has the potential to be used in the next stage of cancer research. Congruent to the results, endemic plants from the same family of *G. amygdalinum*, such as *S. musilii* whole and *A. monosperma* leaves, demonstrated a capable anticancer effect when assessed with HepG2 (Khan *et al*., 2022). On the other hand, the study of Wong *et al*. (2013) on *G. amygdalinum* inhibits cancer growth in MCF-7 and MDA-MB-231 cells. The effect was mediated by the inhibition of breast cancer cell proliferation. When combined with doxorubicin, it showed synergism, implying that it can supplement current chemotherapeutic treatment.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Central Luzon State University, 2023-511.

Authorship Contribution Statement

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