International Journal of Agricultural Technology 2024 Vol. 20(2):551-564
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

DNA profiling and evaluation of bioactivities of *Sapindus saponaria*: Medicinal plant from Bongabon, Nueva Ecija Philippines

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De Leon, D. T. C., Fermin, S. M. C., Undan, J. R., Dulay, R. M. R. and De Leon, A. M. (2024). DNA profiling and evaluation of bioactivities of *Sapindus saponaria*: Medicinal plant from Bongabon, Nueva Ecija Philippines. International Journal of Agricultural Technology 20(2):551-564.

Abstract Findings revealed mild toxicity to zebrafish embryos and discovered teratogenic effects at the highest concentration (10,000ppm) used. To support these results for its potential, additional assessment employed a brine shrimp bioassay, unveiling an LC_{50} of 280.85µg/ml which found to be moderately toxic. For this observation, there is needed for a more specific bioassay, prompting further exploration into the plant's effects on HepG2 cells revealed a substantial and concentration dependent cytotoxic effect against hepatocellular carcinoma at 24.28% cytotoxicity making it highly toxic. Collectively, the study revealed the toxicity profile of *S. saponaria* extracts through assessments using zebrafish embryos, brine shrimp nauplii, and HepG2 cells as experimental models. There is limited to understand the plant's constituent components. Prospective research directions should involve thorough investigations into its properties and impacts on various cell lines.

Keywords: Cytotoxicity, Herbal treatment, Hepatocellular carcinoma, Teratogenicity

Introduction

Natural medicine is becoming increasingly popular around the world. Herbal medicine practices, unlike traditional licensed pharmaceuticals, are rarely supported by effectiveness, efficacy, or safety research, raising concerns about teratogenicity. Medicinal herbs and herbal treatments can contain toxins that are harmful to both human body and fetus. Embryotoxicity, teratogenicity, and abortifacient effects are all possible side effects of inappropriate medicinal herb usage (Bernstein *et al*., 2020).

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In the Philippines, medicines and services from physicians and other healthcare professionals are particularly difficult to obtain in geographically distant and underprivileged locations where people are physically or economically constrained. Traditional Filipino medicine, which includes herbal therapy, has been practiced for generations and is widely recognized in rural regions (Lazarte, 2020). One plant used by the locals in the Barangay Calaanan, Bongabon, Nueva Ecija, Philippines is the *Sapindus saponaria*. Locals just refer to this plant as *"Ahas"* and are unaware of its exact identity. Therefore, it is essential to identify plants using their molecular identity. This plant is used by the locals for treating snake bites by covering the layer of the affected bite with the leaves, used for various skin diseases and they also used this as a remedy for common colds by drinking the decocted leaves.

Few species of the Sapindaceae family were already known to have medicinal benefits. Some known uses of this family were diuretic, stimulant, expectorant, natural surfactant, sedative, vermifuge and against stomachache and dermatitis in many parts of the world (Tsuzuki *et al.,* 2007). There are over 1300 species of soapberry in the world, many of which are toxic. A well-studied and close species to *S. saponaria is Sapindus mukorossi*, the fruit is valued for the saponins present in the pericarp and constitutes up to 56.5% of the drupe known for inhibiting tumor cell growth (Tanaka *et al.,* 1976; Upadhyay and Singh, 2012). Soapnuts are renowned for their detergent and insecticidal properties due to the presence of saponins. These fruits also hold significant medicinal value, being employed in the treatment of various conditions such as excessive salivation, pimples, epilepsy, chlorosis, migraines, eczema, and psoriasis (Kirtikar and Basu, 1991; Upadhyay and Singh, 2012). The leaves are employed in baths for alleviating joint pain, while the roots find application in treating gout and rheumatism (Singh *et al.,* 2010; Upadhyay and Singh, 2012). Therefore, there is a need for a comprehensive study to elucidate the phytochemical components, toxicity, teratogenicity, of *S. saponaria*. This is particularly important as previous research has revealed diverse uses of plants within the Sapindaceae family. The aim was to determine the molecular identity using the *rbc*L gene marker and to assess the phytochemical, teratogenic, and cytotoxic effects of the "Ahas" plant.

Materials and methods

Molecular identification

DNA Extraction

The fieldwork was commenced by engaging in consultations with members of the local community. The purpose of these discussions was to

delineate the study's scope and to gather insights into the diverse herbal remedies employed for treating various ailments. The plant leaves were obtained in February 2022, 2022 from Barangay Calaanan in Bongabon, Philippines. The extraction of DNA was followed by the study of De Leon *et al.,* 2018.

PCR amplification

PCR amplification of the nucleotide rDNA was carried out using primer pair rbcL forward (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and rbcL reverse (5'-GTAAAATCAAGTCCACCRCG-3') (CBOL Plant Working Group *et al.,* 2009). The gene rbcL has the strongest characterization among plastid gene. It is now simple to retrieve across land plants owing to advances in primer design, and it is ideal for recovering high-quality bidirectional sequences (Fazekas *et al.,* 2008; CBOL Plant Working Group *et al.,* 2009). The PCR reaction is composed of 25 µL mixture containing the following: 1 µL of genomic DNA; 1 µL of each rbcL primer; 12.5 µL of 1X GoTaq® Green Master Mix (Promega Corporation, USA); and 9.5 µL of nuclease-free water. Samples were run into Veriflex™ 96-Well Thermal Cycler (Applied Biosystems, California, USA) conditions and programmed as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 51.2°C for 30 sec, extension at 72°C for 60 sec, and final cycle of extension at 72°C for 5 min. The quality of PCR amplicons were checked following the same method described in the preceding section. The expected amplicon size was approximately 550 bp. The unpurified PCR product was quantified using Qubit 4 Fluorometer (Invitrogen Thermo Fisher Scientific, USA) before sequencing.

DNA sequencing and sequence alignment

Unpurified PCR products were sent to Apical Scientific SdnBhd in Malaysia for Unpurified PCR Product Clean up and DNA sequencing using rbcL reverse and forward primers. The sequence data generated using the forward primer and reverse primer were edited by cutting the first 20 sequences at the start of each sequences, aligned using pairwise alignment tool, and created a consensus sequences were conducted using the BioEdit 7.2 (Hall, 1999) application. The nucleotide sequence comparisons were performed using the standard nucleotide Basic Local Alignment Search Tool (BLASTN) against the National Center for Biotechnology Information (NCBI) GenBank database.

Teratogenicity assessment

Teratogenic Assessment of De Leon *et al.* (2020) was the basis of the procedure of this study.

Zebrafish (Danio rerio) teratogenicity and toxicity assay

Three milliliters of each treatment concentration were placed in each vial with four embryos at segmentation phase. The vials were incubated at 26ºC. Teratogenic activity was observed under a compound microscope with 40x magnification after 12, 24, 36, and 48 hours of incubation. Morphological endpoint evaluation such as teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement), lethal (coagulation, tail not detached, no somites, and no heartbeat) was examined. Hatchability and mortality rate were also recorded. Pictures were taken out and death was recognized as coagulated embryos.

Brine shrimp lethality assay

Artemia salina lethality assay of De Leon *et al*. (2020) was used to assess the cytotoxicity of *S. saponaria* leaves extracts. The LC50 was evaluated according to the rating of Aldahi *et al.* (2015) stating that LC50 of <249 µg/mL is highly toxic, LC50 of 250-499 µg/mL is moderately toxic and LC50 of 500-1000 µg/mL is mildly toxic. Moreover, values above 1000 µg/mL are non-toxic according to the rating of McLaughlin & Rogers (1998).

Cytotoxicity of leaves extract using human hepatocellular carcinoma (Hep G2) cells

Preparation of extracts

Leaves were air dried for and grounded into powder using a homogenizer. The dried leaves (g) were weighed and placed into flask and added distilled water in 1:20 ratio. The flask containing the extract was heated using water bath for 2 hours at 80˚C. Extract were then filtered using filter paper and were placed in a centrifuge concentrator (Eppendorf Concentrator Plus™, Germany) at 45˚C to remove the excess water.

Dilution of leaves extract and control

A total of 10 mg of leaves extract were weighed in a 2 mL capacity microtubes, added with 250 uL of DMSO (ATCC® 4-X™) and vortexed for 40 minutes, this is now the 40,000 parts per million (ppm) concentration. Each extract was diluted to 4000 ppm concentration using the Eagle's Minimum Essential Medium (EMEM) (ATCC® 302003™). From the 4000-ppm concentration being the highest concentration, extracts were serially diluted two-folds until it reaches 250 ppm being the lowest concentration used for the treatment. The positive control; 5-fluorouracil (Sigma-Aldrich, Germany) were also prepared with the similar concentration and diluted also with EMEM.

Cell culture maintenance

Human hepatocellular carcinoma [Hep G2] (ATCC® HB-8065™) were cultured in T25 flask 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^{o}C in a humidified atmosphere containing 5% CO^2 while cells at 80% confluence were used for cell seeding.

Cytotoxicity assay

The cytotoxic activity of the leaves extract was tested against Hepatocellular carcinoma [Hep G2] (ATCC® HB-8065™) using Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH) assay. A 5000 cells/100 ul media was seeded in a 96 well plates. It was then incubated for 4 hours at CO^2 incubator allowing cells to attach. Once attached, cells were treated in triplicates with the prepared extract of leaves and the controls. Following an incubation period of 16-18 hours, the control cells underwent treatment with a lysis solution. With minor adjustments, this step was carried out in accordance with the manufacturer's protocol and the cells were then incubated for 45 minutes. The plates were then read using Multiskan Go™ (Thermo Fisher Scientific, USA) microplate reader with 20 seconds low shaking setting at 490 nm absorbance.

Data analysis

For the Teratogenicity Assessment: Analysis of Variance (Anova) and Tukey's significant test at 5% level of significance in the Minitab Statistical Software was used to study and compare the data. For Brine Shrimp Lethality Assay: The median lethal concentration LC50 was analyzed using probit analysis. For Hepatocellular Carcinoma Cytotoxicity: Compounds that resulted in cytotoxicity greater than 10% are tagged as highly cytotoxic; compounds that resulted in cytotoxicity within the range of 1–10% are tagged as moderately cytotoxic; and compounds that resulted in cytotoxicity within the range of 0–1% are tagged as mildly cytotoxic. Compounds with negative values of percent cytotoxicity are tagged as non-cytotoxic (Bataclan *et al.,* 2019). The sample were tested in duplicates in two (2) independent trials.

Formula used:

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

The experimental data were all processed using GraphPad Prism Version 8.0.2 and presented the mean and standard deviation. Unpaired t-test was

performed for statistical analyses and a P-value 0.05 was considered to be statistically significant.

Results

Molecular identification

In the present study, the plant commonly referred to the local people of Bongabon Nueva Ecija, Philippines as "*Ahas*" (Figure 1) was molecularly identified (Table 2). PCR amplification of rbcL forward and reverse primer pair were used to establish and produce a single band of approximately 570 base pairs.

Figure 1. Collected leaves of *Sapindus saponaria*

A few regional and international plant species were successfully identified and verified using universal primer sequences created by CBOL. Results showed that plant has 99.64% identity and 98% query cover (Table 2) which indicated the sequence divergence between the collected plant and Sapindus species in the Genbank repository of NCBI.

Table 2. BLAST Analysis results of chloroplast DNA of AHA plant using rbcL marker inferred from GenBank

BLASTn Identity	Accession Code from GenBank	Query Cover (%)	Identity (%)
Sapindus saponaria	AY724366.1	98	99.64

Toxic effects of Sapindus saponaria extract

The varying concentrations of *S. saponaria* extracts toxic effect to zebrafish were assessed. The percentage mortality of embryos exposure to the various treatments in their observation time points is shown on Table 3. It was

found out that there was no significant difference from the control and with the concentrations ranging from 1-1000 ppm. Since the embryos were developed (Figure 1), concentrations from the lowest to the highest were examined in 48 hrs for the malformation that might occur. The mild toxic property of the plant extracts fully manifested at 48 hrs at 10,000 ppm concentration which exhibited a 22.2% mortality rate.

Table 3. Mortality of zebrafish embryos after 12, 24 and 48 hours of exposure at different concentrations of *Sapindus saponaria*

	Mortality (hours)		
Concentration (ppm)	**12hrs**	**24hrs**	**48hrs**
10000	22.2[a]	22.2[a]	22.2[a]
1000	0.00[b]	0.00[b]	0.00[b]
100	0.00[b]	0.00[b]	0.00[b]
10	0.00[b]	0.00[b]	0.00[b]
1	0.00[b]	0.00[b]	0.00[b]
0 (control)	0.00[b]	0.00[b]	0.00[b]

*Means that do not share a letter are significantly different

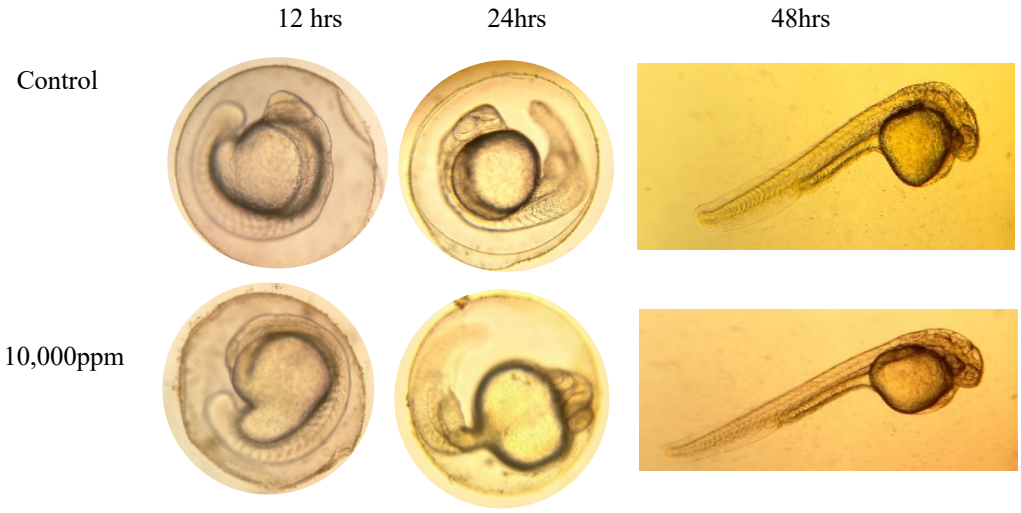

Figure 1. Morphological development of embryos exposed with control and 10,000 ppm (highest concentration)

Teratogenic effects of Sapindus saponaria extract

The morphological abnormalities including tail malformation, head deformation, and delayed development were observed within 12, 24, and 48 hours post treatment application. Only two abnormalities were observed and this were shown in Figure 2. The tail malformations were observed in the highest

concentration at 48 hrs. These tail malformations were having a kinks or bending of the tails in two trials. In addition to the tail malformation, there was also an observed yolk deformity, which has been incorporated into the toxicological analysis as a potential teratogenic effect.

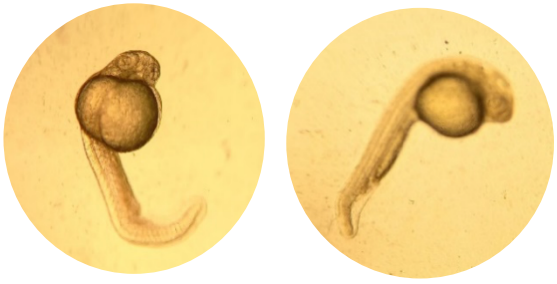

Figure 2. Morphological abnormalities (bent tail and delayed development) of embryos exposed with 10,000 ppm (highest concentration)

Brine shrimp lethality assay

The brine shrimp lethality assay (BSLA) was a basic and low-cost bioassay that was used to evaluate the potency of phytochemicals found in plant extracts. The results of the present study showed that the lethality's degree was directly related to extract concentration. The cytotoxic activity of *S. saponaria* extract was determined by performing brine shrimp lethality assay and analyzed using probit analysis. The mean percentage mortality of *A. salina* 48-hour old nauplii treated to varied concentrations of the extract during the assay is shown in Table 4.

Table 4. *A. salina* treated with varied concentrations of *S. saponaria* leaves extract

Concentration (ppm)	Mortality (%)
10000	76.67^{a}
1000	66.67^{ab}
100	53.33^{bc}
10	50^{c}
1	50^{c}
0	0^{d}

*Means that do not share a letter are significantly different

Mortality was observed in 48-hour-old *A. salina* nauplii treated with various concentrations including the lowest concentration. It was noted that the mortality rate is seen immediately at the lowest concentration used. At 1ppm, 50% of the mortality was recorded, this is in comparable with the next two succeeding concentration. Furthermore, it reached about 76.67% for the highest

concentration. In this regard, the computed LC_{50} value for *S. saponaria* extract was analysed (Figure 4). Results showed that at 280.85 µg/ml this was classified as moderately cytotoxic test.BSLA is inadequate in determining the action of the bioactive substance in plant but this is useful by providing preliminary screening that can be supported by other specific assay. Therefore, additional studies are necessary to corroborate the findings obtained through BSLA.

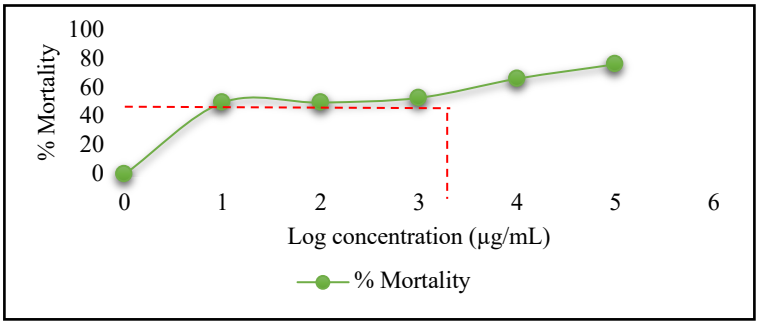

Figure 4. Point estimate of LC50 value of *S. saponaria* extract after 48 hours of exposure of *A. salina*

Cytotoxicity of leaves extract using human hepatocellular carcinoma (Hep G2) cells

The cytotoxic potential of extracts from "AHA" (*S. saponaria*) was evaluated through a series of six concentrations. The concentrations ranged from 4000 ppm as the highest to 125 ppm as the lowest, with a twofold decrease between each concentration. Same instruction was followed for the positive control (5-FU). The treated AHA extracts and control were incubated for a period of 16-18 hours. Following the incubation, Cytotox 96® NonRadioactive Cytotoxicity Assay was employed in accordance with the manufacturer's instructions. The outcomes showed the computed percentage of cytotoxicity for the extract of *S. saponaria* leaves (Table 5).

Table 5. Calculated percent cytotoxicity of *S. saponaria* leaves extract in Hep G2 cell lines

Concentration (ppm)	% Cytoxicity of Plant	Toxicity Level
4000	22.39	Highly Cytotoxic
2000	15.03	Highly Cytotoxic
1000	11.13	Highly Cytotoxic
500	2.26	Moderately Cytotoxic
250	-2.45	Non-Cytotoxic

Following two separate trials, three concentrations exhibited significant cytotoxicity: ranging from 11.13% at 1000 ppm to 22.39% at 4000 ppm. Conversely, concentrations of 500 ppm displayed moderate toxicity at 2.26%. However, concentrations of 250 ppm at -2.45% were determined to be non-cytotoxic. It is advisable to employ cytotoxic concentrations for future investigations such as studies involving anti-proliferative effects, induced cell apoptosis, and assessments of cell viability. These concentrations had the potential to induce or inhibit specific cancer cell responses, making them valuable for in-depth analysis. *S. saponaria* extracts exhibited a substantial and concentration dependent cytotoxic effect against HepG2 cells (Figure 5). There was no significant difference between the *S. saponaria* and the cancer cell damage effects of control (5-FU) having 24.28% cytotoxicity. The extract and its 2-fold dilution increased the cytotoxicity the same with the control.

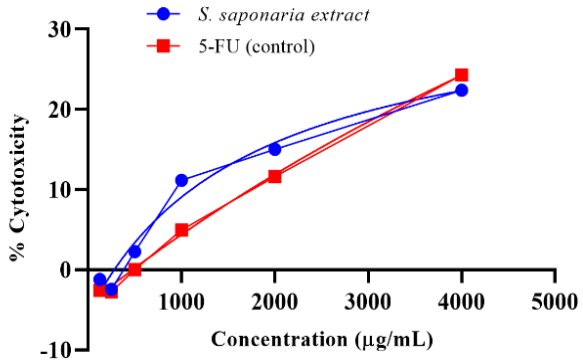

Figure 5. Cytotoxicity index plots of the extracts of *S. saponaria* and their respective serial dilutions (2-fold dilutions) against human hepatocellular carcinoma (HepG2) cell lines

Discussion

Herbal medicines are gaining significant attention in the global health sectors due to their potential health benefits and growing commercial importance (Thakur *et al.,* 2019). The unique composition of the genetic material for each species makes DNA-based identification a useful tool for accurate species identification (Dapar *et al*., 2020). For quality control and safety evaluation of herbal medicines and neutraceuticals, DNA-based authentication of medicinal plants would be helpful. This would be greatly increased the medical potential and commercial profitability of herbal products in the future (Ganie *et al.,* 2015). In the study, only the rbcL primer pair was used as this marker possesses robust discriminatory power for DNA species identification and authentication purposes.

After its identification, *S. saponaria* were subjected to different bioactivity assay to assess its potential. For its teratogenic activity, results showed the mild toxic property of the plant at the highest concentration used and it was observed in 48 hours. While medicinal plants are generally regarded as safe, they are not completely devoid of potential side effects or toxicity (Mounanga *et al*., 2015; Anywar *et al*., 2021). The level of toxicity exhibited by medicinal plants depends on the specific chemical makeup of each plant. Conversely, it was also observed that the plant has teratogenic activity to the developing embryo of zebrafish showed tail malformation and yolk deformity at the highest concentration within 48 hrs. The potential for toxicity in traditional herbal remedies can emerge from both short-term and long-term exposures, even when dealing with extracts of minimal toxicity. Moreover, the plant extract might possess mutagenic or carcinogenic properties (Ferreira-Machado *et al*., 2004). As of now, no reports have indicated teratogenicity in *S. saponaria*. This has sparked significant interest in exploring the pharmaceutical effects associated with this plant.

In addition, the extracting solvent should be also considered. The choice of ethanol as the extraction solvent could be considered as this provides many advantages over other organic solvents, which is relatively safer (less toxic) (Synder, 1974; Abarca-Vargas *et al*., 2016). Also an alcohol yields more extraction than water as most of the phytochemicals are more soluble in alcohol than water.

Considering the reported toxicity of this plant's family and having a mild toxic property in zebra fish, additional test for toxicity was conducted. A basic, high throughput cytotoxicity test for bioactive compounds was the brine shrimp lethality bioassay. It is based on research into the potential of test substances to kill brine shrimp (*Artemia salina*) (Harwig and Scott, 1971; Wu, 2014). The assay revealed that the *S. saponaria* extract's LC_{50} value classified it as moderately cytotoxic. Signifying a mild level of toxicity in zebrafish, this again supports the results of the initial findings, While the specific mechanism of action causing plant toxicity remains uncertain, it is often correlated with more targeted bioactivity testing. (Gadir, 2012; Karchesy *et al*. 2016). The study of utilizing *S. saponaria* plant has very limited study. There were still no reports regarding its toxicity however few studies conveyed that the plant has antifungal activity (Tsuzuki *et al*., 2007), antiulcer activity (Albiero *et al.,* 2002), and supported that plant has anti-snake venom activity (da Silva *et al*., 2012).

As the outcomes from the initial sub-studies exhibited a good correlation, indicating both with mild cytotoxicity to zebrafish and moderately cytotoxic to brine shrimp, an additional investigation was conducted using Hepatocellular carcinoma (HepG2) cell line to validate the plant's toxicity. The liver, due to its specific role and position within the body, is a central site vulnerable to systemic

harm caused by chemicals. The toxicity instigated by chemicals in the liver typically stems from a combination of non-selective cell-damaging effects and actions that particularly affect liver tissue (Tabernilla *et al*., 2021). Hepatocellular carcinoma cell lines have several advantages so this was used in the study. Making them ideal models for such toxicity research, this includes an infinite lifespan, and a consistent phenotypic, (Donato *et al*., 2013). In the present study, it was found out that the plant extract exhibited high cytotoxicity within the concentration range from 1000 to 4000 ppm to HepG2. In the research conducted by Liu *et al.* (2018), they investigated the effect of aqueous extracts from both leaves and stems of *Sapindus mukorossi*. These species share a close relation to *S. saponaria*. The study demonstrated significant effectiveness in reducing the invasiveness of lung cancer cell lines. Importantly, these extracts did not induce any adverse effects such as organ damage, immunotoxicity, or off-target inflammation. These findings suggest that *S. saponaria* holds promise as a source of innovative and valuable leads for addressing a range of diseases.

Cumulatively, it is elucidated the toxicity profile of extracts from *Sapindus saponaria* leaves, employing human hepatocellular carcinoma cells, zebrafish and brine shrimp nauplii as test models. The study revealed toxicity at higher doses. While these observations yielded a relatively low mortality rate and few malformations in zebrafish embryos, caution is advised among the local population regarding the use of *S. saponaria*, primarily due to the lack of reported information concerning its phytochemical constituents and other uses. Based on these results, researchers may consider conducting a more comprehensive investigation focusing on antiproliferative properties, apoptosis, cell migration, invasion, and adhesion assays. Additionally, they could explore the effects of the plant on various human cancer cell lines as well as normal cell lines. This study is of utmost importance, given that the local population in Bongabon, Nueva Ecija, Philippines relies on the use of this plant for various purposes. To date, there has been no documented information regarding the phytochemical composition, teratogenic potential, or toxicity of this plant.

Acknowledgements

The authors would like to express their sincerest gratitude to Mr. Emmanuel Gandalera, Mr. Paul Henric Gojo Cruz, Ms. Minette Trinidad, Ms. Lariza Mae Pajarillaga, Ms. Janice Aguilar, Project staffs of Ethnobotanical and Folkloric Studies of Plants Utilized by The Ilocano and Zambal of Nueva Ecija and Zambales, to the Department of Science and Technology-Regional Office III Provincial Science and Technology Center – Nueva Ecija RGIA project and CLHRDC, and to the residents of Barangay Calaanan, Bongabon Nueva Ecija for helping in the conduct of this study. The main author would also like to thank the DOST-SEI ASTHRDP Scholarship for the thesis grant funding.

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(Received: 28 September 2023, Revised: 8 January 2024, Accepted: 14 February 2023)