Assessment of Cytotoxic Activity of Five Common Philippine Medicinal Plants Using Brine Shrimp Lethality Assay

Lloyd O. Balinado[1*] and Merab A. Chan[2]
[1]College of Arts and Sciences
Cavite State University
Indang, Cavite 4122 Philippines
lloydbalinado@gmail.com

[2]School of Science and Engineering
Ateneo de Manila University
Quezon City, 1108 Philippines

Abstract

A wide variety of plants in the Philippines are used to address several medical ailments; however, many of which have not yet undergone thorough pharmacological studies. The problem on the possible side effects caused by these medicinal preparations has, therefore, increased the interest in validating their safety for human use. The present study was conducted to determine the cytotoxicity of five common local medicinal plants. Annona muricata L., Cymbopogon citratus DC., Graptophyllum pictum (L.) Griff, Jatropha curcas L., and Piper betle L. were selected and subjected to crude aqueous leaf extraction. Extracts were then investigated for cytotoxicity potential using brine shrimp lethality assay. The experiment resulted in median lethal time (LT_{50}) values ranging between 21.23 to 24.06 hours. In addition, all these extracts had median lethal concentrations (LC_{50}) lower than 1.00 mg/mL, suggesting the presence of cytotoxic constituents that could further be investigated both for safety and for anticancer potential.

1. Introduction

The Philippines has a wide variation of medicinal plant use. This may be thought to be in association with its existing plant diversity pattern that is a product of its archipelagic nature, geographical history, and climatic and topographic features (Framework for Philippine Plant Conservation Strategy and Action Plan, 2009). This high quantity of medicinal plants then entails a

wider scope for healing (Hawkins, 2008) – a knowledge predating even the local introduction of antibiotics and other recent drugs (Manuel *et al.*, 2012).

Since there are countless extracts that can be obtained from local medicinal plants (Penecilla and Magno, 2011), Philippine ethnomedicine could then essentially be a key to discover new antibiotic compounds and anti-viral and anticancer drugs (Roberson, 2008; Oladele *et al.*, 2011), and other treatments against a number of existing, emerging, and re-emerging diseases (Gutierrez *et al.*, 2013). However, only 10 plants are recommended by the Department of Health (DOH) and the Philippine Institute for Traditional and Alternative Health Care (PITAHC) for medicinal use since there is a lack of pharmacological studies concerning other local plants (Principe and Jose, 2002).

Five medicinal plants commonly used in the country are *Annona muricata* L. (vernacular name: guyabano), *Cymbopogon citratus* DC. (tanglad, salay), *Graptophyllum pictum* (L.) Griff. (morado), *Jatropha curcas* L. (tuba-tuba) and *Piper betle* L. (litlit, ikmo) These plants are usually prepared as decocted leaves and more commonly being administered to patients via internal routes (Balinado and Chan, 2017). This then demands higher attention to test for its safety since it may directly target delicate organs (Asiimwe *et al.*, 2014). With this assumption, this study was carried out generally to assess, using brine shrimp lethality assay, the cytotoxic activities of the crude aqueous leaf extracts of these plants.

2. Methodology

2.1 Collection of Leaf Samples

The leaves of *A. muricata* L., *C. citratus* DC., *G. pictum* (L.) Griff., *J. curcas* L. and *P. betle* L. from Mendez, Cavite (14.1312° N, 120.9016° E) were collected into labeled Ziploc bags and were temporarily stored in an ice cooler before transporting to the drying site (Biswas *et al.*, 2013). This was done from October to December 2015. Representative fresh samples of these plants were submitted to the National Museum of the Philippines, and taxonomic identity was verified by John Rey C. Callado, Museum Researcher I of the Botany Division.

The remaining leaf samples were then first washed with running water to eliminate unwanted particles and were subsequently air-dried under shade (average temperature = 26.59 °C; relative humidity = 81.52%). Resulting dried leaves were finely ground and pulverized using an electric blender.

2.2 Crude Aqueous Leaf Extraction

Water is the most common solvent used in medicinal plant preparation in Cavite (Balinado and Chan, 2017); hence, aqueous extraction was followed. For every 50 g of powdered leaves, 250 mL distilled water was added and mechanically shaken for 48 h (Gakunga *et al.*, 2014). It was then heated and allowed to boil for 20 minutes before subjecting to filtration using Whatman filter paper No. 1 (Penaduka *et al.*, 2011). Filtrates were then transferred into clear, wide-mouthed glass vials and were oven-dried at 50 °C until dried crude extract was left (Igbokwe *et al.*, 2010). Amounts of 10% dimethyl sulfoxide (DMSO) were then added into each vial for dissolution or reconstitution of the crude extract to give a final crude extract concentration of 10 mg/mL (Bhargava *et al.*, 2015). The crude extracts were finally sterilized by autoclaving (Hashemi *et al.*, 2008) and were kept at 4 °C prior to experimental use (Selvamohan *et al.*, 2012).

2.3 Brine Shrimp Lethality Assay (BSLA)

With slight modifications, BSLA was done following the methods of Peteros and Uy (2010), Khan *et al.* (2013), Olowa and Nuñeza (2013), and Kurian *et al.* (2018). Cysts of brine shrimp were brought in tanks containing artificial seawater (ASW) which was prepared by adding 2.8 g of commercially available sea salt to 100 mL of sterile distilled water. Tanks were provided with constant stirring of the solution for 2 h (Ahmed *et al.*, 2014), a constant supply of oxygen, and proper illumination. Eggs were then permitted to hatch and reach the nauplii stage for two days (48 hours [h]).

Separate glass vial containers were first filled with 500, 250, 125, 50 and 25 μL of the previously prepared crude extracts before adding approximately 4 μL of ASW. Using a Pasteur pipette, a suspension containing inexact number of nauplii was next transferred into each of these vials, where total count of nauplii in every vial was determined at the end of the experiment by immobilizing the remaining live brine shrimps with 100-μL methanol and finally counting them (Pisutthanan *et al.*, 2004; Hamidi *et al.*, 2014). The volume in each vial was then adjusted by adding appropriate amounts of ASW to achieve a 5-ml final volume. This respectively resulted to the following

concentrations: 1.00, 0.50, 0.25, 0.10 and 0.05 mg/mL. Vials with potassium dichromate and DMSO were also prepared to serve as positive and negative control setups, respectively. The assay was performed in triplicates and was kept open under illumination.

2.4 Determination of Median Lethal Time (LT_{50}) and Lethal Concentration (LC_{50})

Brine shrimp mortality was determined by counting the total number of dead or immobile brine shrimp nauplii at the bottom of each vial using a magnifying lens (Peteros and Uy, 2010; Khan *et al.*, 2013). The observation was performed every 2 h for a total of 24 h. Percentage mortality per vial was calculated using the formula, % mortality = (count of dead nauplii/initial count of live nauplii) × 100.

Gathered data were then arcsine transformed before subjecting to one-way analysis of variance (ANOVA) using statistical package for social sciences (SPSS) 16.0. This determined the level of significant difference among percentage mortalities in (a) different time of observation per extract concentration, and (b) different extract concentrations at the 24th hour of observation. Results were interpreted as significant when $p < 0.05$ and not significant when $p > 0.05$. Specific comparisons using Duncan's multiple range test (DMRT) was then employed using the same statistical software.

In determining LT_{50} or the time that resulted to a 50% decline in the brine shrimp population upon exposure to each test concentration, the two-hourly data on mortality were utilized and were subjected to probit analysis. Only those concentrations that were reported to kill or immobilize at least 50% of the brine shrimp nauplii after 24 h were analyzed. Resulting values were then compared with the R values obtained from regression analysis.

For LC_{50} or the concentration that resulted to a 50% decrease in the brine shrimp population, the 24th-hour data on mortality were utilized. Similar to LT_{50} determination, data were subjected to probit analysis. These values were compared to LC_{50} obtained from the regression analysis. Resulting values that fell below 1.00 mg/mL were interpreted as active or potent concentrations, while values that were above 1.00 mg/mL indicated non-toxicity (Meyer *et al.*, 1982). Furthermore, Clarkson's toxicity criterion was also used to assess the calculated LC_{50} values. This classified the extract concentrations as (a) non-toxic if it was above 1.00 mg/mL, (b) lowly toxic if between 0.50 and 1.00 mg/mL, (c) moderately toxic if between 0.10 and 0.50, and (d) highly toxic if below 0.10 mg/mL (Clarkson *et al*., 2004).

3. Results and Discussion

3.1 Effect of Exposure Time on Brine Shrimp Mortality

Mortality of nauplii exposed to varying concentrations of plant extracts was initially recorded after 16 to 18 h of incubation and at highest concentrations used, in general. Maximum sensitivity or the highest mortality percentage of brine shrimp nauplii was achieved after 24 h.

Generally, significant differences among % mortalities $(p < 0.05)$ recorded based on exposure time in each plant extract concentration were noted. In addition, a directly proportional relationship was recorded between the length of exposure time and mortality rates (Figures 1 to 6). This is shown by the fact, for instance, that at the highest concentration, *A. muricata* extracts resulted to 8, 14, 26 and 61% of brine shrimp mortalities as exposure time increased from 18 to 24 h recorded at a 2-h interval (i.e., 18, 20, 22, 24 h) (Figure 1).
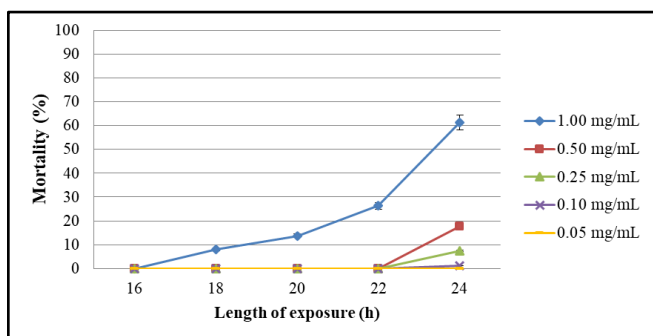

Figure 1. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to *A. muricata* crude aqueous leaf extract

Additionally, 10, 44 and 97%; and 6, 7, and 42% mortality rates were recorded for 1.00- and 0.50-mg/mL concentrations of *C. citratus*, correspondingly, at 20-, 22- and 24-h incubation time (Figure 2). Likewise, in 1.00-, 0.50- and 0.25-mg/mL concentrations, *G. pictum* extracts showed increasing mortality rates of 8, 16, 30 and 90%; 4, 7, 20 and 54%; and 5, 7, 12 and 18%, respectively (Figure 3). *J. curcas* also revealed the same relationship after 6, 19, 46 and 70%; 3, 18, 35 and 67%; and 3, 8, 26 and 44% of mortality rates were observed in 1.00-, 0.50- and 0.25-mg/mL concentrations, after 18, 20, 22

and 24 h of exposure to extracts (Figure 4). In addition, for *P. betle*, mortality rates were increasingly recorded as 8, 20, 35 and 72% in 0.50-g/mL concentration during the same exposure time, while 2, 11, 27, 49 and 95% in 1.0-mg/mL concentration from 16 to 24 h of incubation (Figure 5). Lastly, the same time-dependent increase in mortality in every test concentration was also observed in brine shrimp nauplii exposed to different concentrations of the positive control, potassium dichromate (Figure 6).
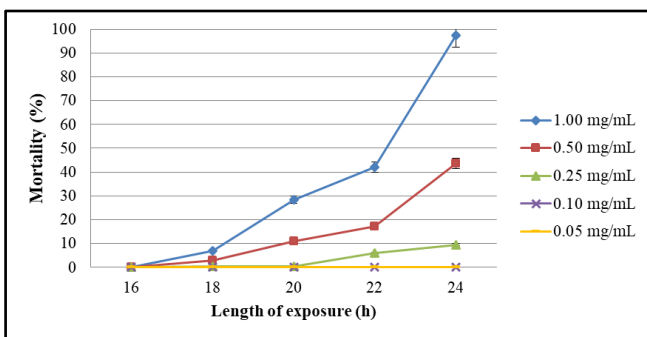

Figure 2. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to *C. citratus* crude aqueous leaf extract
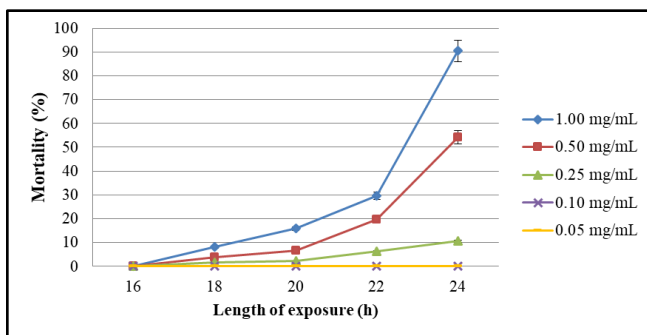

Figure 3. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to *G. pictum* crude aqueous leaf extract
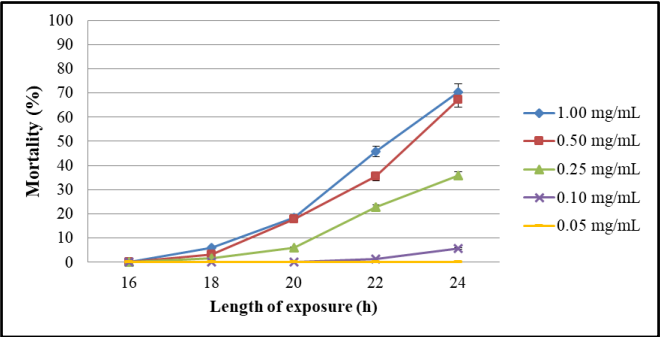
Figure 4. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to *J. curcas* crude aqueous leaf extract
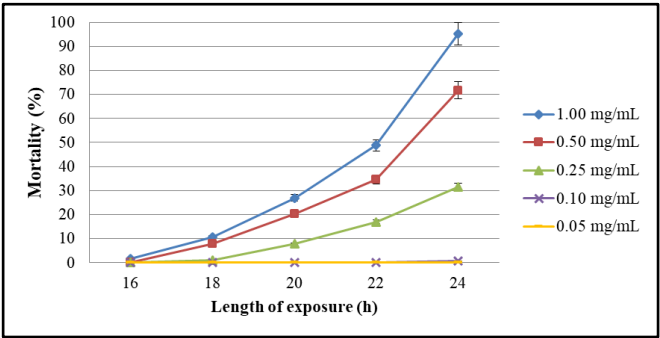

Figure 5. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to *P. betle* crude aqueous leaf extract
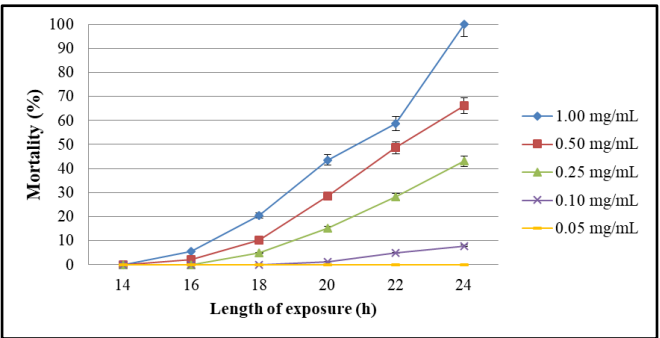

Figure 6. Mortality rates of brine shrimp nauplii in response to varying concentrations and exposure time to potassium dichromate (positive control)

For concentrations that had mortality rates of 50% and higher within 24 h (i.e., *A. muricata*, 1.00 mg/mL; *C. citratus*, 1.00 mg/mL; *G. pictum*, 0.50 and 1.00 mg/mL; *J. curcas*, 0.50 and 1.00 mg/mL; *P. betle*, 0.50 and 1.00 mg/mL), LT_{50} was calculated using probit analysis available in SPSS 16.0. These values were comparable to the results of regression analyses from where R values were taken. LT_{50} values for all plant extract concentrations were found to lie between 21.23 and 24.06 h (Table 1). This means that at these given exposure times, the population of brine shrimp nauplii is expected to decline by half. Determining the effect of time on mortality rates at several concentrations is significant especially when the speed of causing death is of prime interest since mortality rate also changes with time (Thorne *et al.*, 1995; Otieno *et al*., 2013; Umaru *et al.*, 2018).

Table 1. Median lethal time (LT_{50}) values (in h) and confidence limits of crude aqueous leaf extracts

Plant Name	Concentration (in mg/mL)	LT_{50} (h)	Lower Toxic Time (h)	Upper Toxic Time (h)	R-Value
A. muricata	1.00	23.46	22.84	24.33	0.98
C. citratus	1.00	21.40	20.87	21.99	0.99
G. pictum	1.00	22.10	21.67	22.60	0.98
	0.50	24.06	23.43	24.97	0.99
J. curcas	1.00	22.36	22.01	22.76	0.99
	0.50	22.78	22.32	23.33	0.99
P. betle	1.00	21.23	20.77	21.74	0.99
	0.50	22.57	21.97	23.37	0.99
Positive control	1.00	20.28	19.89	20.69	0.99
	0.50	22.21	21.83	22.65	0.98

3.3 Effect of Varying Concentrations on Brine Shrimp Mortality

As what is also shown in previous tables and figures, all plant extracts were lethal at the highest concentration. In general, mortality rates were observed to be significantly declining ($p < 0.05$) down to 0% compared to positive control as concentrations also decreased to 0.05 mg/mL. This directly proportional concentration-lethality relationship was also reported in literature (Krishnaraju *et al.*, 2006; Dosmu *et al.*, 2010; Peteros and Uy, 2010; Olowa and Nuñeza 2013; Otang *et al.*, 2013; Urmi *et al.*, 2013; Del Socorro *et al.*, 2014; Hamidi *et al.*, 2014; Sreeshma and Nair, 2014; Vieira *et al.*, 2014; Parvez *et al.*, 2015; Uddin *et al.*, 2015), where highest mortality rates were recorded at the highest concentrations. On the other hand, the highest concentration at which no mortality was observed could be considered the highest tolerable concentration level (Parvez *et al.*, 2015). This study revealed

that the highest tolerable level was 0.05 mg/mL for *A. muricata*, *J. curcas*, and *P. betle*, and 0.10 mg/mL for *C. citratus* and *G. pictum*.

Shown in Figure 7 are the recorded mortality rates of brine shrimp nauplii after a 24-h exposure to all plant extract concentrations.
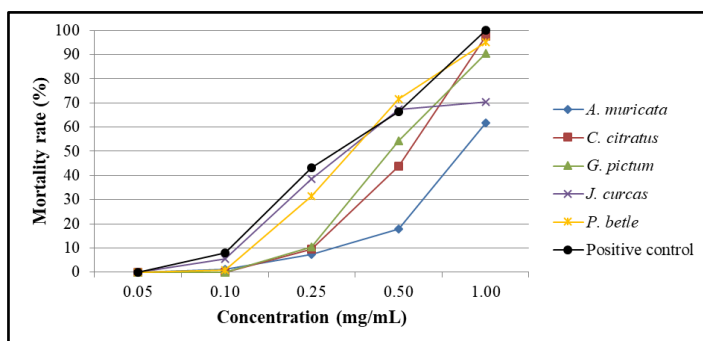

Figure 7. Mortality rates of brine shrimp nauplii after 24 h of exposure to varying concentrations of five crude aqueous leaf extracts

LC_{50} was calculated by probit analysis using these values and results are presented in Table 2. These values were comparable to the results of regression analyses from where R values were taken. Based on the results, crude aqueous leaf extracts of *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas*, and *P. betle* had LC_{50} values of 0.87, 0.49, 0.48, 0.43 and 0.35 mg/mL, respectively. On the other hand, potassium dichromate, which served as positive control, was recorded to have an LC_{50} value of 0.30 mg/mL, a value similarly obtained by Syahmi *et al.* (2010) and Parvez *et al.* (2015). No death was found in the negative control. This was due to the insensitivity of nauplii to this solvent as reported in other BSLA studies (Musa, 2012; Ahmed *et al.*, 2014; Hamidi *et al.*, 2014). All these values are lower than 1.00 mg/mL, thus suggesting the toxicity of extracts (Meyer, 1982). Furthermore, following Clarkson's toxicity criterion (Clarkson 2004), crude aqueous leaf extracts of *A. muricata* was lowly toxic (LC_{50} = 0.50-1.00 mg/mL), while extracts of the other four plants were moderately toxic (LC_{50} = 0.10-0.50 mg/mL).

Table 2. Median lethal concentration (LC_{50}) values (in mg/mL) and confidence limits of crude aqueous leaf extracts, and classification of toxicity based on Meyer's (1982) and Clarkson's (2004) toxicity criteria

Plant Name	LC_{50} (mg/ mL)	Lower Toxic Concentration	Upper Toxic Concentration	Toxicity according to		R-Value
				Meyer	Clarkson	
A. muricata	0.87	0.71	1.17	Toxic	Low	0.98
C. citratus	0.49	0.45	0.54	Toxic	Moderate	0.99
G. pictum	0.48	0.44	0.53	Toxic	Moderate	0.99
J. curcas	0.42	0.33	0.55	Toxic	Moderate	0.96
P. betle	0.35	0.31	0.39	Toxic	Moderate	0.98
Positive control	0.30	0.28	0.32	Toxic	Moderate	0.99

It is considered that extracts having LC_{50} values higher than 1.00 mg/mL are not toxic, thus may further be pharmacologically investigated for drug development. On the contrary, LC_{50} values that are lower than 1.00 mg/mL, like in the case of all the five plant extracts used, could indicate the presence of active toxic compounds playing a role in showing significant bioactivity (Meyer *et al.*, 1982; Otang *et al.*, 2013).

As reported by Agu and Okolie (2017), *A. muricata* leaf has potent antioxidant capacity that could be due to its phytochemical constituents like phenols, alkaloids, and flavonoids. In addition, Najinah *et al.* (2016) confirmed the presence of acetogenins in these leaves which are of valuable potential to cure a wide range of diseases including cancer. In terms of the cytotoxic activity recorded for *C. citratus*, the same was observed by Halabi and Sheikh (2014) as they suggested the antiproliferative potential of the plant against human cancer cell lines. It was further revealed by Anes *et al.* (2017) that *C. citratus* leaf extracts contain tannins, flavonoids, and alkaloids that could contribute to its medicinal effect. Meanwhile, flavonoids, tannins, steroids, saponins, and phenolics were found to be present in *G. pictum*. These were suggested by Jiangseubchatveera *et al.* (2017) to be of antioxidant significance; however, were non-cytotoxic to Vero cell culture. On the other hand, *J. curcas* leaves were reported by Tomar *et al.* (2015) to possess high contents of tannins, phenols and phytic acids. Similarly, the cytotoxic activity of this plant, and its antitumor potential, was also presented by Nayak *et al.* (2016).

Among the five plant extracts, *P. betle* was recorded as the most active and was similarly reported in other studies (Krishnaraju *et al.*, 2006; Del Socorro *et al.*, 2014). This cytotoxic activity of *P. betle* also reflects its anticancer effect including that of against the cancer-causing agents present in tobacco

(*Nicotiana tabacum* L.) and areca nut (*Areca catechu* L.). *P. betle*, *N. tabacum* and *A. catechu* are known components of betel quid (locally known as nganga). Betel quid is regularly chewed by some individuals and is mainly associated with oral cancer (Rai *et al.*, 2011).

Given the findings of this study, all crude plant extracts exhibited significant activities against brine shrimp nauplii. This means that these extracts could contain cytotoxic compounds. This then opens questions regarding the safety of these plants for human use, especially that four of these plants, namely *A. muricata*, *C. citratus*, *J. curcas*, and *P. betle*, were reported to be administered internally. These compounds could directly target and affect delicate organs; thus, further studies must be conducted. However, this finding does not necessarily indicate their total toxicity since it may suggest anticancer or antitumor potential of these plants as well similar to findings presented by Rai *et al.* (2011), Khan *et al.* (2013), Del Socorro *et al.* (2014), Halabi and Sheikh (2014), Najinah *et al.* (2016), and Nayak *et al.* (2016). Thus, further investigations on this aspect may also be carried out.

4. Conclusion

Cytotoxic activities of *A. muricata*, *C. citratus*, *G. pictum*, *J. curcas*, and *P. betle* revealed that their crude aqueous leaf extracts were lethal to 50% of brine shrimp nauplii population (LT_{50}) at 21.23 to 24.06 h of exposure. In addition, these extracts had LC_{50} values that were lower than 1.00 mg/mL. These extracts were classified as moderately toxic except for *A. muricata* that was lowly toxic. This then implies the presence of cytotoxic constituents in these extracts that could further be investigated for the safety of use.

5. Acknowledgement

6. References

Agu, K., & Okolie, P. (2017). Proximate composition, phytochemical analysis, and in vitro antioxidant potentials of extracts of *Annona muricata* (Soursop). Food Science & Nutrition, 5(5), 1029-1036.

Ahmed, W., Ahmad, M., Rauf, A., Feroz, S., Khan, Z., & Zia-Ul-Haq. (2014). Phytochemistry and cytotoxicity of *Proposis spicigera* using brine shrimp lethality assay. Topclass Journal of Herbal Medicine, 3(3), 14-16.

Anes, U., Malgwi, T., Dibal, M., Otalu, O., & Aliyu, N. (2017). Preliminary phytochemical screening and antimicrobial activity of *Cymbopogon citratus* (DC.) Stapf. (Poaceae) leaf ethanol extract against selected microbes. American Journal of Microbiology and Biotechnology, 4(5), 61-66.

Asiimwe, S., Namutebi, A., Karisson, B., Mugisha, M., & Origa, H. (2014). Documentation and consensus of indigenous knowledge on medicinal plants used by the local communities of western Uganda. Journal of Natural Product and Plant Resources, 4(1), 34-42.

Balinado, L., & Chan, M. (2017). An ethnomedicinal study of plants and local health care practices in District 7, Cavite, Philippines, Proceedings on of the 2017 International Conference on Chemical, Agricultural, Biological and Medical Sciences, Manila, Philippines, 131-143.

Bhargava, L., Kumar, T., Aggarwal, S., Zinzarde, S., Sanap, A., & Patil, P. (2015). Comparative evaluation of the antimicrobial efficacy of neem, green tea, triphala and sodium hypochlorite: an in vitro study. Journal of Dental Research and Review, 2(1), 13-16.

Biswas, B., Rogers, K., McLaughlin, F., Daniels, D., & Yadav, A. (2013). Antimicrobial activities of leaf extracts of guava (*Psidium guajava* L.) on two gram-negative and gram-positive bacteria. International Journal of Microbiology, 2013, 1-7.

Clarkson, C., Maharaj, V., Crouch, N., Grace, O., Pillay, P., Matsabisa, M., Bhagwandin, N., Smith, P., & Folb, P. (2004). In vitro antiplasmodial activity of medicinal plants native to or naturalized in South Africa. Journal of Ethnopharmacology, 92, 177-191.

Del Socorro, M., Bendoy, C., & Dacayana, C. (2014). Cytotoxic effects of betel vine, Piper betle Linn. leaf extracts using *Artemia salina* Leach (brine shrimp lethality assay). Journal of Multidisciplinary Studies, 3(1), 100-111. doi:10.7828/jmds.v3i1.629.

Gakunga, N., Mugisha, K., Owiny, D., & Waako, P. (2014). Effects of crude aqueous leaf extracts of *Citropsis articulata* and *Mystroxylon aethiopicum* on sex hormone levels in male albino rats. International Journal of Pharmaceutical Science Invention, 3(1), 5-17.

Gutierrez, R., Baculi, R., Pastor, N., Puma-ar, T., & Balangcod, T. (2013). Antibacterial potential of some medicinal plants of the Cordillera Region, Philippines. Indian Journal of Traditional Knowledge, 12(4), 630-637.

Framework for Philippine Plant Conservation Strategy and Action Plan. (2009). Framework for Philippine plant conservation strategy and action plan. Retrieved from http://www.bmb.gov.ph/index.php?option=com_docman&task=doc_download&gid=211&Itemid=234

Halabi, M., & Sheikh, B. (2014). Anti-proliferative effect and phytochemical analysis of *Cymbopogon citratus* extract. BioMed Research International, 2014(1-8).

Hamidi, M., Jovanova, B., & Panovska, T. (2014). Toxicological evaluation of the plant products using brine shrimp (*Artemia salina* L.) model. Macedonian Pharmaceutical Bulletin, 60(1), 9-18.

Hashemi, S., Zulkifli, I., Zunita, Z., & Somchit, M. (2008). The effect of selected sterilization methods on antibacterial activity of aqueous extract of herbal plants. Journal of Biological Sciences, 8, 1072-1076. doi:10.3923/jbs.2008.1072.1076.

Hawkins, B. (2008). Plants for life: medicinal plant conservation and botanic gardens. United Kingdom: Botanic Gardens Conservation International, 49.

Igbokwe, N., Igbokwe, I., & Sandabe, U. (2010). Effect of prolonged oral administration of aqueous *Ficus sycomorus* stem-bark extract on testicular size of growing albino rat. International Journal of Morphology, 28(4), 1315-1322.

Jiangseubchatveera, N., Liaewruangrath, S., Teerawutgulrag, A., Santiarworn, D., Pyne, S., & Liawruangrath, B. (2017). Phytochemical screening, phenolic and flavonoid contents, antioxidant and cytotoxic activities of *Graptophyllum pictum* (L.) Griff. Chiang Mai Journal of Science, 44(1), 193-202.

Khan, M., Asad, S., Zanan, G., Rehman, H., Rehman, S., Iqbal, A., Ullah, A., Ullah, I., Shahnoor & Ali, S. (2013). Antioxidant and cytotoxic activities of crude methanolic extract of *Medicago polymorpha*. IOSR Journal of Pharmacy, 3(8), 32-37.

Krishnaraju, A., Rao, T., Sundararaju, D., Vanisree, M., Tsay, H. & Subbaraju, G. (2006). Biological screening of medicinal plants collected from Eastern Ghats of India using *Artemia salina* (brine shrimp test). International Journal of Applied Sciences and Engineering, 4(2), 115-125.

Kurian, S., Joseph, L., & Josekumar, V. (2018). Phytochemical evaluation, cytotoxicity screening, and in vitro free radical scavenging activity of the leaf petiole of *Artocarpus heterophyllus* Lam. (Family: Moraceae). Journal of Pharmacy Research, 12(4), 455-460.

Manuel, J., Balangcod, T., Patacsil, M., Laruan, L., & Cortez, S. (2012) Suppression of growth of some medically important pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*) with plant extracts of selected indigenous semi-temperature medicinal plants in the Philippines. Tangkoyob Research Journal, 6(1), 1-12.

Meyer, B., Ferrigni, N., Putnam, J., Jacobsen, L., Nichols, D., & McLaughlin, J. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. Planta Medica, 45, 31-34.

Musa, A. (2012). Cytotoxicity activity and phytochemical screening of *Cochlospermum tinctorium* Perr Ex A. Rich rhizome. Journal Applied Pharmaceutical Science, 2(7), 155-159.

Najinah, N., Daud, N., Ya'akob, H., & Rosdi, M. (2016). Acetogenins of *Annona muricata* leaves: characterization and potential anticancer study. Integrative Cancer Science and Therapeutics, 3(4), 543-551.

Nayak, A., Satapathi, K., & Sahoo, S. (2016). Comparative studies on the phytochemistry, antimicrobial and antioxidant activities of *Jatropha* species (*J. curcas L.* and J. *gossypifolia* L.) of Odisha. International Journal of Pharmacognosy and Phytochemical Research, 8(10), 1614-1624.

Oladele, A., Alade, G., & Omobuwajo, O. (2011). Medicinal plants conservation and cultivation by traditional medicine practitioners (TMPs) in Aiyedaade Local Government Area of Osun State, Nigeria. Agriculture and Biology Journal of North America, 2(3), 476-487. doi:10.5251/abjna.2011.2.3.476.487.

Olowa, L., & Nuñeza, O. (2013). Brine shrimp lethality assay of the ethanolic extracts of three selected species of medicinal plants from Iligan City, Philippines. International Research Journal of Biological Sciences, 2(11), 74-77.

Otang, W., Grieson, D., & Ndip, R. (2013). Assessment of potential toxicity of three South African medicinal plants using the brine shrimp (*Artemia salina*) assay. African Journal Pharmacy and Pharmacology, 7(20), 1272-1279.

Otieno, G., Waititu, G. & Salifu, D. (2013). Generalized estimating equations for repeated measures logistic regression in mosquito dose-response. Open Journal of Statistics, 3, 293-298.

Parvez, M., Hussain, F., Ahmad, B., Al, J., & Hassan, S. (2015). Cytotoxic activity evaluation of *Euphorbia granulate* Forssk. American-Eurasian Journal of Toxicological Sciences, 7(1), 39-42. doi:10.5829/idosi.aejts.2015.7.1.92177.

Penaduka, D., Okoh, O., & Okoh, A. (2011). In-vitro antagonistic characteristics of crude aqueous and methanolic extracts of *Garcinia kola* (Heckel) seeds against some *Vibrio* bacteria. Molecules, 16, 2754-2765. doi:10.3390/molecules16042754.

Penecilla, G., & Magno, C. (2011). Antibacterial activity of extracts of twelve common medicinal plants from the Philippines. Journal of Medicinal Plants Research, 5(16), 3975-3981.

Peteros, N., & Uy, M. (2010). Antioxidant and cytotoxic activities and phytochemical screening of four medicinal plants. Journal of Medicinal Plants Research, 4(5), 407-414.

Pisutthanan, S., Plianbangchang, P., Pisutthanan, N., Ruanruay, S., & Muanrit, O. (2004). Brine shrimp lethality activity of Thai medicinal plants in the family Meliaceae. Naresuan University Journal, 12(2), 13-18.

Principe, E., & Jose, A. (2002). Propagation management of herbal and medicinal plants. Research Information Series on Ecosystems, 14(2), 12.

Rai, M., Thilakchand, K., Palatty, P., Rao, P., Rao, S., Bhat, H., & Baliga, M. (2011). *Piper betle* L. (betel vine), the maligned Southeast Asian medicinal plant possesses cancer preventive effects: time to reconsider the wronged opinion. Asian Pacific Journal of Cancer Prevention, 12, 2149-2156.

Roberson, E. (2008). Medicinal plants at risk: nature's pharmacy, our treasure chest. Tucson, AZ: Center for Biological Diversity.

Selvamohan, T., Ramadas, V., & Shibila Selva Kishore, S. (2012). Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. Advances in Applied Science Research, 3(5), 3374-3381

Sreeshma, L. & Nair, B. (2014). Brine shrimp lethality assay in two species of *Biophytum* DC. (Oxalidaceae). International Journal of Pharmacy and Pharmaceutical Sciences, 6(4), 582-586.

Syahmi, A., Vijaratna, S., Sasidharan, S., Latha, L., Kwan, Y., Lau, Y., Shin, L., & Chen, Y. (2010). Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* Jacq. (oil palm leaf) methanol extract. Molecules, 15, 8111-8121. doi: 10.3390/molecules15118111.

Thorne, J., Weaver, D., Chew & Baker, J. (1995). Probit analysis for correlated data: multiple observations over time at one concentration. Journal of Economic Entomology, 88(5), 1510-1512.

Tomar, N., Sharma, M., & Agarwal, R. (2015). Phytochemical analysis *of Jatropha curcas* L. during different seasons and developmental stages and seedling growth of wheat (*Triticum aestivum* L) as affected by extracts/leachates of *Jatropha curcas* L. Physiology and Molecular Biology of Plants, 21(1), 83-92.

Uddin, F., Uddin, S., Hossain, D., & Manchur, M. (2015). Antioxidant, cytotoxic and phytochemical properties of the ethanol extract of *Piper betle* leaf. International Journal of Pharmacy Sciences and Research, 6(10), 4252-4258.

Umaru, I., Badruddin, F., & Umaru, H. (2018). Cytotoxicity brine shrimp activity of *Leptadenia hastata* (PER) Decne leaves, stem-bark and root extract. International Journal of Biochemistry and Physiology, 3(2), 1-8.

Urmi, K., Mostafa, S., Begum, G., & Hamid, K. (2013). Comparative brine shrimp lethality bioassay of different plant parts of *Bauhinia purpurea* L. Journal of Pharmaceutical Sciences and Research, 5(10), 190-192.

Vieira, G., Mourao, J., Angelo, A., Costa, R., & Vieira, R. (2010). Antibacterial effect (in vitro) of *Moringa oleifera* and *Annona muricata* against Gram positive and Gram negative bacteria. Revista do Instituto de Medicina Tropical de São Paulo, 52(3), 129-132. doi:10.1590/S0036-46652010000300003.