



## Comparative toxicity, phytochemistry, and use of 53 Philippine medicinal plants

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### ABSTRACT

The study compares the toxicity of 53 selected medicinal plants commonly used in the Philippines to treat various diseases. It uses as a benchmark *Vitex negundo* L., which was approved by the Philippine Food and Drug Administration as an herbal drug for cough and asthma after passing clinical trials for safety and efficacy. The methods were chosen for their simplicity and accessibility even for resource-limited laboratories. Extracts (95 % ethanol) of the medicinal parts of the plants were (1) chemically profiled using qualitative phytochemical tests that detect the presence of key classes of bioactive compounds; and (2) evaluated for toxicity using the brine shrimp (*Artemia* sp.) lethality assay (BSLA). General phytochemical screening revealed the presence of tannins in 50 plant extracts, alkaloids in 43, glycosides in 33, flavonoids in 31, steroids in 21, triterpenoids in 20, anthraquinones in 10, and saponins in 8. Extracts from eight plants had LC<sub>50</sub> values lower than the potassium dichromate control (approximately 12 µg/mL) and were considered highly toxic; extracts from 21 plants had LC<sub>50</sub> values between 12 µg/mL and 100 µg/mL and were considered moderately toxic; extracts from 19 plant extracts, including *Vitex negundo* and some common vegetables, had LC<sub>50</sub> values between 100 µg/mL and 500 µg/mL, and were considered mildly toxic and likely to have reasonable safety margins; five plant extracts, including common vegetables, had LC<sub>50</sub> values above 500 µg/mL and were considered essentially nontoxic. No apparent correlation could be found between toxicity and chemical diversity or a specific class of phytochemicals present. Our findings may serve as a guide for herbal drug and nutraceutical development, especially in prioritizing plants for more detailed safety studies.

The use of medicinal plants in treating various diseases is gaining increasing attention worldwide. The World Health Organization estimated that up to 80 % of the total population rely on herbal medicines for their primary health care [1,2]. In the Philippines, the use of herbal medicines has been a practice for centuries, especially in rural areas. Among Filipinos, the folkloric history of herbal medicines was inherited from their ancestors through oral tradition, developed by trial-and-error method, and handed on from generation to generation until today [3,4].

The Philippines recorded about 13,500 plant species, according to the Department of Environment and Natural Resources [5]. About 3500 of them are endemic [6], and 1500 were found to have medicinal and therapeutic value [7]. A number of medicinal plants are under different stages of development as herbal drugs [8].

Starting in the 1970s and 1980s, a major national research program

sought to develop herbal medicines for the mainstream, focusing on the following criteria: safety, efficacy, quality, availability of raw material, and availability of propagation studies of the raw herbs, in order to allow scaling up for commercialization [9]. Studies initially focused on *Blumea balsamifera* Linn., family Compositae, locally known as sambong; *Senna alata* L., family Fabaceae, locally known as akapulko; and *Vitex negundo* Linn., family Verbenaceae, locally known as lagundi. More were later added to make the initial ten priority medicinal plants and the expanded list of priority medicinal plants (Expanded herbal plants from DOH) [10].

*Vitex negundo* became the pioneer commercial herbal medicine. Through technology transfer and partnership with the private sector, *Vitex negundo* leaf became the second most popular commercial cough medicine in the country by 2011 [9]. Ten years later, a number of brands

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and formulations of *Vitex negundo* leaf, from tablets and capsules to cough syrups, are registered as herbal medicines for cough and asthma by the Philippine Food and Drug Administration or FDA Philippines. [11]. At present, the Philippine Department of Science and Technology is studying its possible use as therapy for mild cases of COVID-19 [12].

To be registered as herbal medicine with therapeutic claims by FDA Philippines, the product must pass plant authenticity and chemical profile standards, comply with quality assurance and good manufacturing practice, be non-mutagenic as shown by assays such as the Ames and micronucleus tests, and show safety and pharmacologic effect for the therapeutic claim in pre-clinical animal studies and in clinical trials [13].

One of the key concerns modern medical practitioners have about medicinal plants is their safety. Researchers have used different laboratory-based assays to measure safety in the use of herbal plants and herbal plants derived products, but challenges remain [14]. For example, although there are many reports on the potential of medicinal plants, there are few studies of the toxicogenic effects when consumed in large amounts [15]. Also, it is not straightforward to compare toxicity data for different plants, given differences in experimental conditions, as well as plant sources.

To address the question of acute toxicity of medicinal plants, we studied a collection of 53 plants sourced from selected areas of North Cotabato, in the island of Mindanao, based on their availability and their traditional use. These plants are used for a variety of health conditions, and have a diverse set of bioactivities, as shown in Table 1. While some have been studied in detail for bioactivity and chemical composition, some are less well-described. By studying them in parallel, their toxicity and therapeutic potential can be compared.

To make our comparisons of Philippine plants potentially useful to a broader set of researchers, we focused on three key points. (1) We chose methods that use only small amounts of material, and are simple and readily available even in laboratories with limited resources. (2) The range of plants we studied includes those that are used in other medicinal plant traditions, such as traditional Chinese medicine or Ayurvedic medicine. This would allow comparison with other studies, even in cases where the specific results, such as LC<sub>50</sub> values, may vary due to differences in plant growth or experimental conditions. (3) We used potassium dichromate as a positive control in the toxicity assay, instead of specific cytotoxic drugs which may not be readily available in laboratory stockrooms. A large number of studies use this as a toxicity control, and comparisons can be made across studies, again despite variations in specific LC<sub>50</sub> values due to differences in experimental conditions. We note though that potassium dichromate may be a regulated chemical, and local authorities often have guidelines and reporting requirements for proper procurement, use, storage, and disposal [16,17].

To profile the chemical constituents of the 53 plants in parallel, standard phytochemical screening was conducted to determine the presence of alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids.

To assess the toxicity of the plants in parallel, we used the brine shrimp lethality assay (BSLA), which is a simple, reliable, inexpensive micro-scale bioassay technique that can detect a range of bioactivity in ethanolic extracts as well as isolated purified bioactive natural products [18]. This assay is based on the ability of the extract to kill laboratory-cultured brine shrimp nauplii, from the genus *Artemia* [19–21]. Brine shrimp lethality assay only requires small amounts of sample. Unlike vertebrate and especially mammalian species, *Artemia* species are not typically subject to strict animal research regulations.

Toxicity testing using BSLA is often done for two different purposes. These two lines of research using BSLA offer different perspectives on how the results are interpreted and used. First, it is often used as a first-pass tool to screen libraries of extracts or compounds for possible bioactivity, prior to more detailed, more complex bioactivity assays of selected candidates [22]. Second, BSLA may also be used as an indicator for toxicity in various contexts, such as ecotoxicology [23,24].

Drawing from these two lines of research, our study aimed to investigate the comparative toxicity of the ethanolic extracts of these selected medicinal plants using the brine shrimp lethality assay and thus, contribute towards estimating the safety of the plant extracts. Extrapolating from *Artemia* species to human toxicity is not straightforward. For example, efforts have been made to model acute toxicity of chemicals in humans from a number of parameters, including toxicity in *Artemia*, with promising but limited results [25,26]. To partially address this, the study includes comparison or benchmark plants where some level of human toxicity information is available from practice: the over-the-counter medicine *Vitex negundo* leaf, as discussed previously, and some common vegetables, such as *Allium cepa* (onion) leaf, and *Ipomoea batata* (sweet potato) leaf, which are part of the diet and may be considered as safe.

## 1. Materials and methods

### 1.1. Plant materials

Plant materials were collected from private farms in the province of North Cotabato, Philippines. The plant parts were chosen based on known ethnobotanical information as summarized in Table 1. The vouchered specimens were identified by Prof. James V. LaFrankie of the University of the Philippines and deposited at the University of the Philippines Herbarium.

### 1.2. Chemicals

Potassium dichromate, ethanol, dimethyl sulfoxide (DMSO), and other chemicals used were reagent grade, unless otherwise noted.

### 1.3. Sample preparation and extraction

The plant samples were washed with running water to remove dirt and other contaminants and final washing was done with distilled water. After washing, the plant materials were dried and transferred into small plastic net bags for further drying. Then, these were air dried until the samples were brown and brittle. Samples that needed further drying were dried in an oven at 40 °C for 24 h. The dried samples were ground using a blender. The ground samples were kept in clean, dry bottles and stored at 4 °C. Each powdered plant part was soaked with 95 % ethanol for 72 h. The collected extracts were filtered and concentrated *in vacuo* at 30–35 °C. The resulting extracts were kept refrigerated at 4 °C and used for phytochemical screening and cytotoxicity tests.

### 1.4. General phytochemical screening

The phytochemical constituents were qualitatively determined following a standard method described by Trease and Evans; Harborne, and Kokate [100–102]. Phytochemical analysis was conducted to determine the presence of alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids.

### 1.5. Brine shrimp lethality assay

Brine shrimp lethality assay (BSLA) was carried out following the filter disc procedure previously described by Meyer et al. [22], McLaughlin et al. [103], Krishnaraju et al. [104] and Peteros and Uy [105].

#### 1.5.1. Sample preparation

Plant samples for the assay were prepared by initially dissolving 50 mg of extract with 5 drops of dimethyl sulfoxide (DMSO) in a test tube and further diluted with 5 mL 95 % ethanol to make a 10 mg/mL stock solution. Appropriate amounts of this stock solution (5, 50, and 500 µL) were used to give the final concentrations of 10, 100, and 1000 µg/mL in

**Table 1**

Ethnobotanical information of the Philippine medicinal plants in this study, arranged in alphabetical order.

| Plant No. | Scientific Name                                 | Family Name    | Common name  | Plant parts | Medicinal uses and properties   | References <sup>a</sup>           |
|-----------|---|----------------|--|-------------|---|-----------------------------------|
| 1         | <i>Abelmoschus esculentus</i> (L.) Moench       | Malvaceae      | Okra (English, Filipino)   | Fruit       | For inflammation, gastric ulcer, diarrhea, urinary problems   | [27,28]                           |
| 2         | <i>Adonidia merrillii</i> (Becc.) Becc.         | Arecaceae      | Manila palm (English), bunga de Jolo (Filipino)                      | Young fruit | For diarrhea; seeds stimulate mental alertness  | [28]                              |
| 3         | <i>Aglaia odorata</i> Lour.                     | Meliaceae      | Chinese perfume plant (English), kutsaritas (Filipino)               | Leaves      | As post-partum bath, for fractures and injuries from falls  | Traditional use, [28]             |
| 4         | <i>Allium cepa</i> L.                           | Amaryllidaceae | Onion (English), sibuyas dahunan (Filipino)                          | Leaves      | For abdominal pain; antimicrobial, antioxidant  | Traditional use, [29,30,31]       |
| 5         | <i>Annona reticulata</i> L.                     | Annonaceae     | Custard apple (English), anonas (Filipino)                           | Leaves      | For abdominal pain, diarrhea, boils, over-fatigue, fever, inflammation; antiparasitic, insecticidal, anticancer | Traditional use, [32,33,34]       |
| 6         | <i>Arcangelisia flava</i> (L.) Merr.            | Menispermaceae | Albutra (Filipino)   | Leaves      | For malaria, itching, and topical ulcers  | Traditional use, [31]             |
| 7         | <i>Artemisia vulgaris</i> L.                    | Compositae     | Mugwort (English), damong-Maria (Filipino)                           | Leaves      | For cough and diarrhea  | Traditional use, [35,36]          |
| 8         | <i>Artocarpus heterophyllus</i> Lam.            | Moraceae       | Jackfruit (English), langka (Filipino)                               | Leaves      | For diabetes, wounds, and abdominal pain  | Traditional use, [37]             |
| 9         | <i>Bauhinia integrifolia</i> Roxb.              | Fabaceae       | Agpoi (Filipino)   | Stem        | For relapse; antibacterial and antifungal   | Traditional use, [38]             |
| 10        | <i>Bauhinia purpurea</i> L.                     | Fabaceae       | Butterfly tree (English), alibangbang (Filipino)                     | Leaves      | For wounds and constipation   | [39,40]                           |
| 11        | <i>Bidens pilosa</i> L.                         | Compositae     | Spanish needle (English), tuway-tuway (Filipino)                     | Whole plant | For diabetes, fatigue, inflammation, malaria, dysentery   | Traditional use, [28]             |
| 12        | <i>Bixa orellana</i> L.                         | Bixaceae       | Annatto (English), atchuete (Filipino)                               | Leaves      | For headache, burns, and wounds; antimicrobial, antifungal, antileishmanial, antihistamine                      | Traditional use, [41,42]          |
| 13        | <i>Cananga odorata</i> (Lam.) Hook.f. & Thomson | Annonaceae     | Ylang-ylang (English, Filipino)                                      | Leaves      | For urinary tract and other microbial infections  | Traditional use, [43,44]          |
| 14        | <i>Carmona retusa</i> (Vahl) Masam              | Boraginaceae   | Scorpionbush (English), tsaang gubat (Filipino)                      | Leaves      | For cough, colic, diarrhea, dysentery, and stomachache; antioxidant   | Traditional use, [36,45], [28,46] |
| 15        | <i>Ceiba pentandra</i> (L.) Gaertn.             | Malvaceae      | Kapok (English, Filipino)  | Leaves      | For diabetes  | [28,46]                           |
| 16        | <i>Citrus maxima</i> (Burm.) Merr.              | Rutaceae       | Pomelo (English), Suha (Filipino)                                    | Leaves      | As postpartum bath; anticancer  | [47,48]                           |
| 17        | <i>Coleus blumei</i> Benth.                     | Lamiaceae      | Coleus (English), mayana (Filipino)                                  | Leaves      | For bruises and sprains; antibacterial, antioxidant   | Traditional use, [49,50]          |
| 18        | <i>Colocasia esculenta</i> (L.) Schott          | Araceae        | Taro (English), gabi (Filipino)                                      | Leaves      | For hypertension and itching; diuretic and antioxidant  | Traditional use, [51,52]          |
| 19        | <i>Corchorus aestuans</i> L.                    | Malvaceae      | Jute (English), saluyot (Filipino)                                   | Leaves      | For sprain, inflammation, fever, and constipation   | Traditional use, [53]             |
| 20        | <i>Crescentia cujete</i> L.                     | Bignoniaceae   | Calabash tree (English), Cujete (Filipino)                           | Leaves      | For diabetes and fever, as diuretic; fruit is antibacterial   | Traditional use, [54]             |
| 21        | <i>Croton tiglium</i> L.                        | Euphorbiaceae  | Purging croton (English), tuba (Filipino)                            | Stem        | For snakebite, skin diseases, sprains and bone pains  | Traditional use, [28,55]          |
| 22        | <i>Cyperus kyllingia</i> Endl.                  | Cyperaceae     | White-top sedge (English), anuwang (Filipino)                        | Whole plant | For fever, snakebite, acne and excessive sweating; antibacterial  | Traditional use, [56,57,58]       |
| 23        | <i>Diospyros blancoi</i> A.DC.                  | Ebenaceae      | Velvet apple (English), mabolo (Filipino)                            | Leaves      | For colds, diarrhea, hypertension, heart disease, diabetes, stomachache, eczema, and spider bites               | Traditional use, [28,59]          |
| 24        | <i>Durio zibethinus</i> L.                      | Malvaceae      | Durian (English, Filipino)   | Roots       | For dengue; antibacterial and antioxidant   | Traditional use, [60,61]          |
| 25        | <i>Ficus minahassae</i> (Teijsm. & Vriese) Miq. | Moraceae       | Cluster fig tree (English), hagimit (Filipino)                       | Roots       | For bone fracture and rheumatism; to promote lactation; astringent and antibacterial                            | Traditional use, [28,62]          |
| 26        | <i>Ficus septica</i> Burm.f.                    | Moraceae       | Hauli fig tree (English), leng guo rong (Chinese), hawili (Filipino) | Leaves      | For rheumatism; diuretic; anticancer  | [62]                              |
| 27        | <i>Garcinia mangostana</i> L.                   | Clusiaceae     | Mangosteen (English, Filipino)                                       | Leaves      | For cough; neuroprotective and antioxidant  | Traditional use, [36,63]          |
| 28        | <i>Heliconia rostrata</i> Ruiz & Pav.           | Heliconiaceae  | Lobster claw, false bird of paradise (English)                       | Leaves      | Antimicrobial   | [64]                              |
| 29        | <i>Hibiscus rosa-sinensis</i> L.                | Malvaceae      | Hibiscus (English), gumamela (Filipino)                              | Leaves      | For diabetes and inflammation   | [65]                              |
| 30        | <i>Hydrocotyle asiatica</i> L.                  | Apiaceae       | Centella (English), takip-kohol (Filipino)                           | Whole plant | For anxiety, depression, and mental or neurological conditions, inflammation, infection, diabetes               | [66,67]                           |
| 31        | <i>Ipomoea batatas</i> (L.) Lam.                | Convolvulaceae | Sweet potato (English), kamote (Filipino)                            | Leaves      | Antioxidant   | [68]                              |
| 32        | <i>Jasminum elongatum</i> (P.J. Bergius) Willd. | Oleaceae       | Sampaguita-gubat (Filipino)  | Leaves      | For fever, headache and vertigo; antibacterial  | Traditional use, [28,69]          |
| 33        | <i>Kalanchoe pinnata</i> (Lam.) Pers.           | Crassulaceae   | Goethe plant (English), katakataka (Filipino)                        | Leaves      | For cold with fever, whooping cough, malaria, snakebites, bone fracture, wound healing                          | Traditional use, [70]             |
| 34        | <i>Lansium domesticum</i> Corrêa                | Meliaceae      |  | Leaves      |   | [71,72]                           |

(continued on next page)

Table 1 (continued)

| Plant No. | Scientific Name  | Family Name    | Common name  | Plant parts  | Medicinal uses and properties   | References <sup>a</sup>        |
|-----------|--|----------------|--|--------------|---|--------------------------------|
| 35        | <i>Manihot esculenta</i> Crantz  | Euphorbiaceae  | Langsat (English), lansones (Filipino)<br>Cassava (English), kamoteng kahoy (Filipino) | Leaves       | For dysentery, microbial infection, and aging<br>For urinary tract infection, abdominal pain; antioxidant     | Traditional use, [28,73,74]    |
| 36        | <i>Mentha arvensis</i> L.  | Lamiaceae      | Wild mint (English), yerba buena (Filipino)  | Leaves       | For insect bites, fevers, toothaches, headaches   | [28]                           |
| 37        | <i>Momordica charantia</i> L.  | Cucurbitaceae  | Bitter melon (English), ampalaya (Filipino)  | Leaves       | For diabetes, wounds, skin diseases, burns and scalds   | [28,36]                        |
| 38        | <i>Muntingia calabura</i> L.   | Muntingiaceae  | Jamaica cherry (English), aratiles (Filipino)  | Leaves       | For loose bowel movement and bacterial infection  | Traditional use, [28,75]       |
| 39        | <i>Nephelium lappaceum</i> L.  | Sapindaceae    | Rambutan (English, Filipino)   | Leaves       | Antioxidant and antibacterial   | [76]                           |
| 40        | <i>Pandanus amaryllifolius</i> Roxb.                                     | Pandanaceae    | Pandan (English, Filipino)   | Leaves       | For fever; antibacterial and anticancer   | [36,77],<br>Traditional use    |
| 41        | <i>Peperomia pellucida</i> (L.) Kunth                                    | Piperaceae     | Pepper-elder (English), pansit-pansitan (Filipino)                                     | Whole plant  | For arthritis, rheumatism, abscesses, boils, inflammation; antioxidant  | Traditional use, [28,36,78,79] |
| 42        | <i>Persea americana</i> Mill   | Lauraceae      | Avocado (English, Filipino)  | Leaves       | For menstruation, wounds, diabetes, loose bowel movement; antiviral   | [80,81,82,83,84,85]            |
| 43        | <i>Piper betle</i> L.  | Piperaceae     | Betel (English), ikmo (Filipino)   | Leaves       | For sprains, cough, bronchitis, rheumatism and bone and joint pain; antibacterial and antifungal              | [28,31,86,87]                  |
| 44        | <i>Polyscias guilfoylei</i> (W. Bull.) L. H. Bailey                      | Araliaceae     | Geranium aralia (English), San Francisco (Filipino)                                    | Leaves       | As postpartum bath; antimicrobial, anticancer, antioxidant  | Traditional use, [28,88]       |
| 45        | <i>Pseudelephantopus spicatus</i> (B. Juss. ex Aubl.) Rohr ex C.F. Baker | Compositae     | Dog's tongue (English); dilangaso (Filipino)   | Stems/leaves | For eczema, wounds, sprains   | [28,49,80]                     |
| 46        | <i>Psidium guajava</i> L.  | Myrtaceae      | Guava (English), bayabas (Filipino)  | Bark         | For diarrhea and prevention of liver damage   | Traditional use, [28,36,89,90] |
| 47        | <i>Pterocarpus indicus</i> Willd.  | Fabaceae       | Rosewood (English), Narra (Filipino)   | Leaves       | As post-partum bath; antimicrobial, anti-angiogenic   | Traditional use, [28,91,92]    |
| 48        | <i>Senna alata</i> (L.) Roxb.  | Fabaceae       | Emperor's candlesticks (English), akapulco (Filipino)                                  | Leaves       | For constipation, skin diseases, asthma; anticancer   | [36,93,94]                     |
| 49        | <i>Stachytarpheta jamaicensis</i> (L.) Vahl                              | Verbenaceae    | Blue porterweed (English), kandikandilana (Filipino)                                   | Leaves       | For sprains, bruises, inflammation, cough, asthma, malaria, worms, parasites                                  | Traditional use, [28]          |
| 50        | <i>Theobroma cacao</i> L.  | Malvaceae      | Cacao (English, Filipino)  | Leaves       | Anticancer, antioxidant   | [95,96]                        |
| 51        | <i>Tinospora rumphii</i> Boerl.  | Menispermaceae | Guduchi (English), makabuhay (Filipino)  | Stem         | For wounds, skin diseases, fertility regulation, rheumatism, malaria, fever, diarrhea, dysentery, stomachache | [28,36]                        |
| 52        | <i>Typhonium trilobatum</i> (L.) Schott                                  | Araceae        | Bengal arum (English), gabingnuno (Filipino)   | Rhizomes     | For skin eruption, nausea, diarrhea, gastric ulcer, asthma, cough, headache, rheumatism                       | [28,97]                        |
| 53        | <i>Vitex negundo</i> L.  | Lamiaceae      | Five-leaved chaste tree (English), lagundi (Filipino)                                  | Leaves       | For cough, asthma, colds, flu, fever, malaria; antimicrobial, anticancer                                      | [36,98,99]                     |

<sup>a</sup> Traditional use – uses of medicinal plants as practiced by the local people in the community.

the final 5-mL assay solution. The stock solutions were dispensed to 1.25-cm discs of filter paper (Whatman filter paper no. 42, Cat No. 1442-125) in a glass vial, air dried, and then dried further *in vacuo* for one hour. Drying by nitrogen gas was performed when necessary to completely remove the solvent. Additional dilutions were processed in a similar way to prepare samples with final concentrations of 0.10, 0.25, 0.50, 0.75, 1.00, and 10.00 µg/mL for selected plant extracts, as noted in Table 2. Control vials were prepared using artificial sea water, potassium dichromate, DMSO and ethanol. These were done in triplicates for each concentration.

### 1.5.2. Hatching of brine shrimp

Brine shrimp eggs (*Artemia* sp.) were obtained from the Institute of Fisheries Research and Development – Mindanao State University, Naawan Campus, Philippines. The eggs were hatched in artificial sea water prepared by dissolving 38 g of rock salt or sea salt in 1 L of distilled water and then filtered to remove the particulates. The eggs were incubated in a specially designed two-compartment glass tank, illuminated, and fully aerated. After 48 h incubation at 32 °C, the nauplii (larvae) were attracted to one side of the tank with a light source and collected with a glass pipette.

### 1.5.3. Bioassay

Four mL of artificial sea water was added to each of the previously prepared sample vials containing the loaded filter paper discs. Ten

actively swimming nauplii (estimated at second-third instar stage, based on hours from hatching) were transferred using a clear glass pipette to each sample vial. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. More artificial sea water was added to make up the 5 mL final assay volume. A drop of dry yeast suspension (Red Star) (3 mg in 5 mL artificial sea water) was added as food to each vial. The vials were maintained under illumination at 32 °C. After 24 h, survivors were counted and the percentage mortality in each vial and the controls was determined using the equation:

$$\% \text{ mortality} = (\text{no. of dead nauplii} / \text{initial no. of nauplii}) \times 100$$

The resulting data were analyzed using the Statistical Package for Social Sciences (SPSS) and R with R Studio. The LC<sub>50</sub> values were calculated using logarithmic or linear regression as appropriate for the concentration ranges used. LC<sub>50</sub> data are expressed as means. Correlations between each phytochemical class or phytochemical diversity and LC<sub>50</sub> were tested by one-way ANOVA. The limit of significance was set at p < 0.05.

## 2. Results

The results of the phytochemical screening and BSLA on the ethanolic extracts of the Philippine medicinal plants are shown in Table 2. The general phytochemical assessment showed that of the 53 plant extracts profiled, 43 tested positive for alkaloids, 10 for anthraquinones,

Table 2

Results of phytochemical screening on ethanolic extracts of fifty-three Philippine medicinal plants, listed in alphabetical order.

| Plant no. | Scientific Name  | Family Name        | Plant parts   | Alkaloids | Anthraquinones | Flavonoids | Glycosides | Saponins | Steroids | Tannins | Triterpenoids | LC <sub>50</sub> (in µg/mL)* |
|-----------|--|--------------------|---------------|-----------|----------------|------------|------------|----------|----------|---------|---------------|------------------------------|
| 1         | <i>Abelmoschus esculentus</i> (L.) Moench                                | Malvaceae          | fruit         | -         | -              | -          | -          | -        | +        | +       | -             | 465                          |
| 2         | <i>Adonia merrillii</i> (Becc.) Becc.                                    | Arecaceae          | young fruit   | +         | -              | +          | -          | -        | +        | +       | +             | 51.3                         |
| 3         | <i>Aglai odorata</i> Lour.   | Meliaceae          | leaves        | +         | -              | +          | +          | -        | +        | +       | -             | 424                          |
| 4         | <i>Allium cepa</i> L.  | Amaryllidaceae     | leaves        | -         | -              | -          | -          | +        | -        | +       | -             | 503                          |
| 5         | <i>Annona reticulata</i> L.  | Annonaceae         | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 1.83**                       |
| 6         | <i>Arcangelisia flava</i> (L.) Merr.                                     | Menispermaceae     | leaves        | +         | -              | +          | -          | -        | -        | +       | -             | 2.32**                       |
| 7         | <i>Artemisia vulgaris</i> L.   | Compositae         | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 84                           |
| 8         | <i>Artocarpus heterophyllus</i> Lam.                                     | Moraceae           | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 454                          |
| 9         | <i>Bauhinia integrifolia</i> Roxb.                                       | Fabaceae           | stem          | +         | +              | -          | -          | -        | +        | -       | +             | 409                          |
| 10        | <i>Bauhinia purpurea</i> L.  | Fabaceae           | leaves        | +         | -              | -          | -          | -        | -        | +       | +             | 584                          |
| 11        | <i>Bidens pilosa</i> L.  | Compositae         | whole plant   | +         | +              | +          | +          | -        | +        | +       | +             | 137                          |
| 12        | <i>Bixa orellana</i> L.  | Bixaceae           | leaves        | +         | -              | +          | +          | -        | +        | +       | +             | 54.8                         |
| 13        | <i>Cananga odorata</i> (Lam.) Hook.f. & Thomson                          | Annonaceae         | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 370                          |
| 14        | <i>Carmona retusa</i> (Vahl) Masam                                       | Boraginaceae       | leaves        | +         | -              | +          | -          | -        | -        | +       | -             | 15.9                         |
| 15        | <i>Ceiba pentandra</i> (L.) Gaertn.                                      | Malvaceae          | leaves        | +         | -              | +          | -          | +        | +        | +       | +             | 409                          |
| 16        | <i>Citrus maxima</i> (Burm.) Merr.                                       | Rutaceae           | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 485                          |
| 17        | <i>Coleus blumei</i> Benth.  | Lamiaceae          | leaves        | -         | +              | -          | +          | +        | -        | +       | +             | 454                          |
| 18        | <i>Colocasia esculenta</i> (L.) Schott                                   | Araceae            | leaves        | -         | -              | +          | +          | -        | -        | +       | -             | 60                           |
| 19        | <i>Corchorus aestuans</i> L.   | Malvaceae          | stem & leaves | +         | -              | -          | +          | -        | -        | +       | -             | 43                           |
| 20        | <i>Crescentia cujete</i> L.  | Bignoniaceae       | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 25.6                         |
| 21        | <i>Croton tiglium</i> L.   | Euphorbiaceae      | stem          | +         | +              | -          | -          | -        | +        | -       | +             | 1.32**                       |
| 22        | <i>Cyperus kyllingia</i> Endl.   | Cyperaceae         | whole plant   | +         | -              | +          | +          | -        | -        | +       | -             | 2.22**                       |
| 23        | <i>Diospyros blancoi</i> A.DC.   | Ebenaceae          | leaves        | -         | +              | -          | +          | +        | +        | +       | +             | 90                           |
| 24        | <i>Durio zibethinus</i> L.   | Malvaceae          | roots         | -         | -              | +          | -          | -        | +        | +       | +             | 31.1                         |
| 25        | <i>Ficus minahassae</i> (Teijsm. & Vriese) Miq.                          | Moraceae           | roots         | +         | +              | -          | -          | -        | +        | -       | +             | 61.2                         |
| 26        | <i>Ficus septica</i> Burm.f.   | Moraceae           | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 37.9                         |
| 27        | <i>Garcinia mangostana</i> L.  | Clusiaceae         | leaves        | +         | +              | +          | -          | -        | +        | +       | -             | 70.1                         |
| 28        | <i>Heliconia rostrata</i> Ruiz & Pav.                                    | Heliconiaceae      | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 64.5                         |
| 29        | <i>Hibiscus rosa-sinensis</i> L.   | Malvaceae          | leaves        | +         | -              | -          | +          | +        | -        | +       | -             | 465                          |
| 30        | <i>Hydrocotyle asiatica</i> L.   | Apiaceae           | whole plant   | +         | -              | -          | +          | -        | -        | +       | +             | 41.1                         |
| 31        | <i>Ipomoea batatas</i> (L.) Lam.   | Convolvulaceae     | leaves        | +         | -              | +          | -          | -        | -        | +       | -             | 511                          |
| 32        | <i>Jasminum elongatum</i> (P.J. Bergius) Willd.                          | Oleaceae           | leaves        | +         | +              | -          | -          | -        | -        | +       | -             | 113                          |
| 33        | <i>Kalanchoe pinnata</i> (Lam.) Pers.                                    | Crassulaceae       | leaves        | +         | -              | +          | +          | +        | +        | +       | +             | 283                          |
| 34        | <i>Lansium domesticum</i> Corrêa   | Meliaceae          | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 0.51                         |
| 35        | <i>Manihot esculenta</i> Crantz  | Euphorbiaceae      | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 409                          |
| 36        | <i>Mentha arvensis</i> L.  | Lamiaceae          | leaves        | +         | +              | +          | +          | -        | -        | +       | -             | 421                          |
| 37        | <i>Momordica charantia</i> L.  | Cucurbitaceae      | leaves        | -         | -              | -          | -          | -        | -        | +       | -             | 111                          |
| 38        | <i>Muntingia calabura</i> L.   | Muntingiaceae      | leaves        | +         | -              | +          | -          | -        | +        | +       | +             | 23.1                         |
| 39        | <i>Nephelium lappaceum</i> L.  | Sapindaceae        | leaves        | +         | -              | -          | +          | -        | -        | +       | -             | 2.78                         |
| 40        | <i>Pandanus amaryllifolius</i> Roxb.                                     | Pandanaceae        | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 17.8                         |
| 41        | <i>Peperomia pellucida</i> (L.) Kunth                                    | Piperaceae         | whole plant   | -         | -              | -          | +          | +        | -        | +       | -             | 12.9                         |
| 42        | <i>Persea americana</i> Mill   | Lauraceae          | leaves        | +         | -              | +          | +          | +        | +        | +       | -             | 1.92**                       |
| 43        | <i>Piper betle</i> L.  | Piperaceae         | leaves        | +         | -              | +          | +          | -        | +        | +       | +             | 106                          |
| 44        | <i>Polyscias guilfoylei</i> (W. Bull) L. H. Bailey                       | Araliaceae         | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 13.3                         |
| 45        | <i>Pseudelephantopus spicatus</i> (B. Juss. ex Aubl.) Rohr ex C.F. Baker | Compositae         | whole plant   | -         | -              | -          | +          | -        | -        | +       | -             | 503                          |
| 46        | <i>Psidium guajava</i> L.  | Myrtaceae          | bark          | +         | -              | +          | -          | -        | +        | +       | +             | 12.5                         |
| 47        | <i>Pterocarpus indicus</i> Willd.  | Fabaceae           | leaves        | -         | -              | -          | -          | -        | -        | +       | -             | 46.4                         |
| 48        | <i>Senna alata</i> (L.) Roxb   | Fabaceae           | leaves        | +         | +              | +          | +          | -        | +        | +       | +             | 206                          |
| 49        | <i>Stachytarpheta jamaicensis</i> (L.) Vahl                              | Verbenaceae        | whole plant   | +         | -              | +          | +          | -        | +        | +       | +             | 9.43                         |
| 50        | <i>Theobroma cacao</i> L.  | Malvaceae          | leaves        | +         | -              | +          | +          | -        | -        | +       | -             | 754                          |
| 51        | <i>Tinospora rumphii</i> Boerl.  | Menispermaceae     | stem          | +         | -              | -          | -          | -        | +        | +       | +             | 199                          |
| 52        | <i>Typhonium trilobatum</i> (L.) Schott                                  | Araceae            | rhizomes      | +         | -              | +          | +          | -        | -        | +       | -             | 29.3                         |
| 53        | <i>Vitex negundo</i> L.  | Lamiaceae          | leaves        | +         | -              | +          | -          | -        | +        | +       | +             | 465                          |
|           | Potassium dichromate (K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> )    | (positive control) |               |           |                |            |            |          |          |         |               | 12.24                        |

\*\*\*

\*Mean of three replicates.

\*\*Confirmed using 6 different concentrations (0.10, 0.25, 0.50, 0.75, 1.00, 10.00 µg/mL).

\*\*\*Solvent controls showed 100 % survival of the brine shrimp nauplii.

31 for flavonoids, 33 for glycosides, eight for saponins, 21 for steroids, 50 for tannins and 20 for triterpenoids. Most extracts were chemically diverse: on average, the plants tested positive for four different classes of secondary metabolites. *Momordica charantia* and *Pterocarpus indicus* showed the least diversity by testing positive only for tannins, and *Senna alata* and *Bidens pilosa* showed the greatest diversity by testing positive for all classes except saponins.

Table 2 also shows the LC<sub>50</sub> in brine shrimp after 24 h of exposure to the ethanolic extracts. The toxicity values ranged from less than 1 µg/mL to over 700 µg/mL. The potassium dichromate control had an LC<sub>50</sub> of 12.24 µg/mL. All brine shrimp nauplii survived in the solvent controls, showing no apparent toxicity from solvent. This is not surprising since the filter disc method involves evaporating the solvent prior to reconstituting the assay solution using artificial sea water.

Based on the results, toxicity values were classified according to Table 3. The most toxic category has a cut-off based on the potassium dichromate control's LC<sub>50</sub>, rounded off to 12 µg/mL. The rationale for the full classification is detailed in the discussion. No clear correlations were found between the classes of phytochemicals present and the toxicity classification, as shown in Fig. 1. However, alkaloids were found in all the highly toxic extracts. No apparent correlation was seen between the phytochemical diversity, as measured by the number of phytochemical classes present, and the LC<sub>50</sub> values.

Among the least toxic extracts were those from common vegetables, such as *Ipomoea batatas* (sweet potato) leaves, with LC<sub>50</sub> = 511 µg/mL; and *Allium cepa* (onion) leaves, with LC<sub>50</sub> = 503 µg/mL. Another common vegetable, *Abelmoschus esculentus* (okra) fruit, shared the same LC<sub>50</sub> of 465 µg/mL with the FDA-approved benchmark plant *Vitex negundo*. Some other vegetables showed greater toxicity: the leaves of *Momordica charantia* (bitter melon, with LC<sub>50</sub> = 111 µg/mL), the leaves of *Colocasia esculenta* (taro, with LC<sub>50</sub> = 60 µg/mL), and the leaves of *Corchorus aestuans* (locally known as *saluyot*, a type of jute, with LC<sub>50</sub> = 43 µg/mL). The ethanolic extract of the leaves of *Pandanus amaryllifolius* (pandan), which is used for flavor and aroma across Southeast Asia, exhibited an LC<sub>50</sub> of 17.8 µg/mL, which would classify it as moderately toxic, at least to brine shrimp.

### 3. Discussion

#### 3.1. Phytochemical screening

The pharmacologic effects of medicinal plants are due to the presence of bioactive constituents. For example, a number of flavonoid compounds—casticin, chrysoptanol D, luteolin, isoorientin and luteolin-

**Table 3**  
Proposed brine shrimp lethality assay (BSLA) toxicity classification scheme based on LC<sub>50</sub> values.

| LC <sub>50</sub> Value (µg/mL) | Toxicity              | Rationale  |
|--------------------------------|-----------------------|--|
| LC <sub>50</sub> ≤ 12          | highly toxic          | Similar to or higher toxicity than potassium dichromate control (LC <sub>50</sub> = 12.24 µg/mL in this study, rounded off to 12 µg/mL for simplicity) Parallels classification in other schemes   |
| 12 < LC <sub>50</sub> ≤ 100    | moderately toxic      |  |
| 100 < LC <sub>50</sub> ≤ 500   | mildly toxic          | Non-toxic in some schemes [199,200], moderately toxic in others [197,198]; includes some vegetables and <i>Vitex negundo</i> leaf, which has preparations approved by the Philippine FDA as over-the-counter cough and asthma medication based on clinical studies for safety and efficacy     |
| 500 < LC <sub>50</sub>         | essentially non-toxic | Includes some common vegetables; in preliminary screens for drug discovery purposes, where toxicity is used as a surrogate marker for other bioactivities, this category is often considered as sufficiently toxic for further study as a drug candidate if LC <sub>50</sub> < 1000 µg/mL [22] |

7–O-glucosides—were isolated from the leaves of *Vitex negundo* L., three of which showed antihistamine and bronchial-relaxing activity [98]. In addition, four iridoids were isolated from the pharmacologically active fraction, and shown to have anti-inflammatory activity [106].

Phytochemical screening of the plant extracts provides a quick profile of their chemical constituents. Such phytochemicals exert biological properties through different mechanisms. While the results of this type of screening are preliminary, they give an estimate of the chemical diversity of the plant extract, and may provide us the basis for developing the leads for new compounds with potential bioactivities.

The overall picture shows that the extracts tend to be chemically diverse. This highlights the complex chemistry of medicinal plants. This makes their possible pharmacological effects complex as well, whether on brine shrimp, cultured cells, model animals, or humans [107].

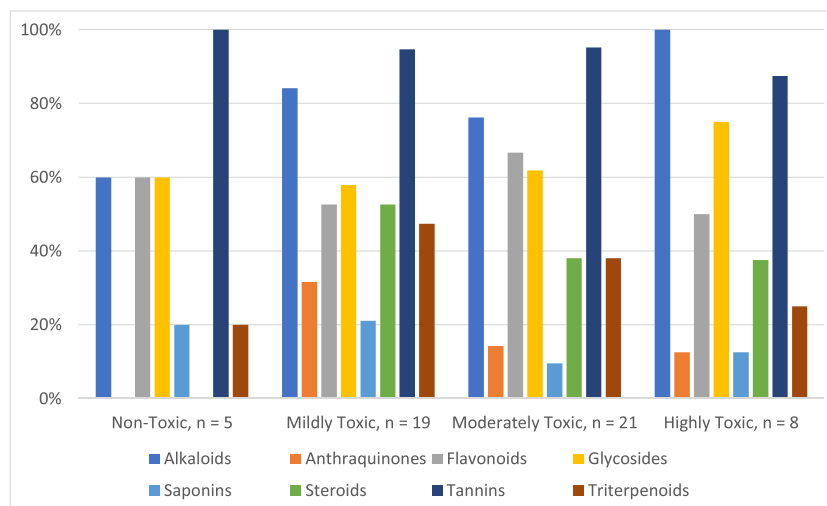
Alkaloids, which were present in most of the extracts examined, have been shown to be generally bioactive. Studies of alkaloids show they possess cytotoxic, antioxidant, anti-inflammatory, anti-asthmatic, and anti-anaphylactic, vasorelaxant, and antiplatelet activating effects [108–110]. From the results, both Annonaceae species, *Annona reticula* and *Cananga odorata*, tested positive for alkaloids. These results are consistent with the literature describing the presence of isoquinoline alkaloids in Annonaceae family [111]. Alkaloidal compounds such as ornithine, euplauridine, anomontane have also been isolated from *Ostenyhopetalum amazonicum*, *Cananga odorata* and *Annona montana*, which are members of the Annonaceae family [112]. *Cyperus kallingia*, from the Cyperaceae family, showed the presence of alkaloids, flavonoids, glycosides and tannins, which is consistent with the compilation of Stuart [28]. The presence of alkaloids in *Croton tiglium* is also consistent with the result of Koche et al. [113].

Anthraquinones were detected in ten plant extracts studied. Natural anthraquinones are distinguished by structural variety, a wide range of biological activity, and low toxicity. They possess strong anti-plasmodial activity *in vitro* [114]. They are also known for astringent, purgative, anti-inflammatory, and bactericide effects; they participate in the processes of metabolism, respiration, division of cells, oxidative phosphorylation, complexation with DNA as shown by anti-tumor activity, and in other physiological processes of vital importance [115]; and they are components of many medicines of plant origin [114]. They are also known for their laxative and antibacterial properties [116,117].

In this study, the results revealed the presence of anthraquinones in the extracts from *Bauhinia integrifolia* and *Senna alata*. The genus *Senna* is known for anthraquinone-containing plants, and our results are consistent with the phytochemical analysis of *Senna alata* conducted by Sule et al. [118]. *Senna alata* is known for its laxative activity, a property it shares with *Cassia fistula*, another member of the family Fabaceae [116]. Similarly, the plant extract of *Bidens pilosa* also revealed the presence of anthraquinones, which is consistent with the phytochemical screening conducted by Moabe and co-workers [119].

Flavonoids, which were present in 31 plant extracts studied, are naturally occurring polyphenolic substances with variable chemical structures, including anthocyanins, flavones, flavonols, isoflavones, flavonones and chalcones. They possess various biological and pharmacological activities and are found in fruits, vegetables, grains, tree barks, roots, stems, and flowers, as well as in tea and wine [105,120,121]. Current evidence strongly supports a contribution of polyphenols to the prevention of cardiovascular diseases, cancers, and osteoporosis, and suggests a role in the prevention of neurodegenerative diseases and diabetes mellitus [122]. Because of their antioxidant and anti-inflammatory properties, flavonoids may also beneficially influence other chronic diseases involving oxidative stress or inflammation, such as rheumatoid arthritis and chronic obstructive pulmonary disease (COPD) [123].

The presence of flavonoids in most of the plant extracts is in agreement with the results of previous studies conducted on the same families. For example, in family Araceae, anthocyanins, flavones C-glycosides and proanthocyanidins were the major flavonoids in the



**Fig. 1.** Phytochemical profiles of extracts for each toxicity category, expressed in percent of the number of plant extracts in that category that test positive for each class of phytochemicals.

species [124]. Specifically, flavonoids have been shown to be present in *Colocasia esculenta* [125] and *Typhonium flagelliforme* [126]. The results in *Artemisia vulgaris*, *Bidens pilosa*, and *Pseudelephantopus spicatus* are consistent with the positive result for flavonoids reported in the literature [127–129]. *Diospyros blancoi* yielded the same result with the phytochemical screening conducted by Howlader et al. [130]. *Manihot esculenta* also showed the presence of flavonoids, which is consistent with the result of Ebuehi et al. [131]. Our results from *Senna alata* are also consistent with the findings of El-Mahmood and Doughari [132]. The flavonoids in the family Bixaceae, Bombaceae, Boraginaceae are in accordance with similar findings in the literature [133–135]. Family Clusiaceae, Convolvaceae, Crassulaceae and Cyperaceae also exhibited positive results in flavonoids, which are consistent with similar studies conducted on the same plants [135–138]. Family Heliconiaceae, Myrtaceae, Aracaceae, Pandanaceae, Piperaceae, Rutaceae, and Verbanaceae showed positive results for flavonoids, which are in agreement with previous reports [139,140].

Glycosides were present in 33 plant extracts belonging to family Annonaceae, Araceae, Asteraceae, Lamiaceae, Meliaceae, and Piparaceae. Some glycosides are known to treat heart conditions. Cardiac glycosides help improve symptoms of congestive heart failure and cardiac arrhythmia by inhibiting the  $\text{Na}^+/\text{K}^+$  ATPase pump and promote  $\text{Na}^+/\text{Ca}^{2+}$  exchange within the myocardial cells resulting to improved cardiac output and reduced distention of heart [119,141]. Glycosides have been reported to exhibit antidiabetic characteristics [119], which is consistent with our result for *Persea americana*, which tested positive for glycosides and is used traditionally to treat diabetes. In addition, some glycosides also exhibit a hepatoprotective property [126,127], consistent with our result for *Psidium guajava*, which tested positive for glycosides [89]. These findings support the traditional medicinal uses of the plant extracts being studied.

Glycosides have been isolated and characterized from some of the 33 plants that tested positive for them. Two novel flavonol monoglycosides, isolated from the antiviral fractions of *Persea americana* leaf infusion, strongly inhibited herpes simplex virus type 1 (HSV-1), and Aujeszky's disease virus (ADV) in cell culture [81]. Another novel bioactive polyacetylenic glucoside, cytopiloyne, isolated from *Bidens pilosa*, modulated T cell differentiation and prevented the development of non-obese diabetes (NOD) in NOD mice. This lends support to the ethnopharmacological observation of anti-diabetic activity of *Bidens pilosa* extract [142]. *Annona squamosa* leaf extracts contain quercetin-3-O-glucoside, which has antidiabetic and antioxidative effects [143]. Isovitexin, isolated from the leaf of *Colocasia esculenta*, showed scavenging activity for reactive oxygen species when tested

[125].

Saponins were detected in eight plant extracts. The positive results of *Allium cepa*, *Ceiba pentandra*, and *Peperomia pellucida* are consistent with preliminary phytochemical screening results in the literature [144–147]. Similarly, *Diospyros blancoi* is also positive for saponins, in agreement with phytochemical analysis of different *Diospyros* species [148–150]. *Persea americana* tested positive for saponins, consistent with the report that saponins are present in the leaves, fruit and seeds of the plant [151]. *Kalanchoe pinnata*, which is used to treat whooping cough, also tested positive for saponins. This is in agreement with the phytochemical result of the roots, leaves and stem of the plant [152, 153].

Steroidal compounds were present in 21 plant species belonging to eighteen families. Plants from family Verbenaceae tested positive for steroids, consistent with literature. For example, steroidal glucosides have been isolated from *Stachytarpheta jamaicensis* [154]. These have been found to possess anti-inflammatory, antioxidant, anti-asthmatic, bronchodilator, anti-spasmodic and liver detoxifying activities. *Vitex negundo*, belonging to the same family, also tested positive for steroids. Sharma and co-workers [155] showed that the steroidal extract of *Vitex negundo* exhibited antimicrobial activity, and was nontoxic to fresh human erythrocytes. In addition, *Bidens pilosa* and *Bixa orellana* also tested positive for steroidal compounds, in agreement with previous reports [156,157].

In the family Fabaceae, *Senna alata* and *Bauhinia integrifolia* tested positive for steroids, which is consistent with previously reported phytochemical analyses [39,158]. The presence of steroids in *Ficus minhassae*, family Moraceae; *Persea americana*, family Lauraceae; and *Tinospora rumphii*, family Menispermaceae, are also consistent with the literature [159,160].

Triterpenoids, a large class of unique and potentially usable biological compounds, are biosynthesized in plants by the cyclization of squalene [161]. They are studied for anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotoxic, sedative and tonic effects [162,163]. They also possess antioxidant, antimicrobial, antiviral, anti-allergic, antipruritic, anti-angiogenic and spasmolytic activities [164,165]. Triterpenoids were also reported to have anti-HIV-1, anti-leishmanial and anti-cancer activities [166,167].

Twenty plants tested positive for triterpenoids. Together with tannins, terpenoids are attributed for analgesic and anti-inflammatory activities [154]. For some plants, previous studies have identified terpenoids of different bioactivities. Two new triterpenoids, methyl 27-O-trans-caffeoylcylicodiscate and methyl 27-O-cis-caffeoylcylicodiscate were isolated from the wood bark extract from *Durio zibethinus*

[168]. Another study on the ethyl acetate extract of the air-dried leaves of *Diospyros blancoi* identified bioactive triterpenes that exhibited antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus* and *Trichophyton mentagrophytes*, and were found inactive against *Bacillus subtilis*, *Aspergillus niger*. The study also reported significant analgesic and anti-inflammatory activities [169]. Moreover, two new diterpenes were obtained from the leaves of *Tinospora rumphii*, along with the known compounds tinotufolin D and vitexilactone [170]. Dolabellane diterpenoids from *Aglaia odorata* showed weak cytotoxicity against the human cancer cell lines [171].

Tannins, which were present in most of the ethanolic plant extracts, are secondary metabolites that have pharmacological importance due to their astringent action. They promote rapid healing and formation of new tissues on wounds and inflamed mucosa [172,173]. They exhibit antimicrobial, antidiabetic, anti-inflammatory, antibacterial, and anti-tumor activities and reportedly inhibit HIV replication [119, 174–176]. Tannins are also studied for their anti-diarrheal, hemostatic and antihemorrhoidal compounds [177].

The positive results in *Artocarpus heterophyllus*, and *Persea americana*, which are known for their antidiabetic properties, are consistent with the literature. Similarly, *Anona reticulata*, *Carmona retusa*, *Diospyros blancoi*, and *Typhonium trilobatum*, showed positive for tannins, which may support the anti-diarrheal activity of the plants. The presence of tannins in *Bauhinia purpurea*, *Bixa orellana*, *Momordica charantia*, and *Persea americana* also lend support to the potential wound healing capacity of these plants.

### 3.2. Brine shrimp lethality assay

The use of *Artemia* as a test organism for bioassays was proposed in 1956 [178]. Many toxicity assay methods using the genus *Artemia* are available [23,179]. The recently published genome of *Artemia franciscana* Kellogg provides some insights to its ability to survive in extreme conditions [180].

Toxicity testing is often used for two different purposes, and the toxicity classification cut-offs depend on these goals. (1) It is used as a preliminary test for potential bioactivity in screening studies, to identify candidates for more intensive subsequent testing. In these cases, the researcher has to balance where to set the cut-off for moving a candidate to the next round of study: a cut off value that is too stringent, such as a low concentration for LC<sub>50</sub>, could mean missing many promising candidates. On the other hand, setting a cut-off that is too permissive would not narrow down the field sufficiently so that the number of candidates can be efficiently handled in the next round of assays, which are usually more expensive and time consuming.

In most cases, the results of the brine shrimp lethality assay correlate reasonably well with cytotoxicity and anti-tumor properties of terrestrial plant extracts [103,181]. For example, McLaughlin et al. in 1998 noted that the results from BSLA correlate well with cytotoxicity results in human solid tumor cell lines they tried, with ED<sub>50</sub> values in cell lines about one-tenth of the LC<sub>50</sub> values in brine shrimp [103]. BSLA results can also potentially indicate a wide range of pharmacological activities such as antiviral, antimicrobial, insecticidal, pesticidal, and other activities [18].

(2) Toxicity testing using *Artemia* is also a way for evaluating potential environmental toxins, whether via BSLA or assays using other endpoints [23,24,182]. Nunes and coworkers reviewed some of the key factors in using the genus *Artemia* for ecotoxicity testing [24]. Although there is an ISO standard method for toxicity assessment of nanomaterials using brine shrimp (ISO/TS 20787:2018) [183], there is no single standard for general brine shrimp toxicity assessment. But a number of efforts have been done to develop standards for reliability and reproducibility of brine shrimp assays for ecotoxicity over the decades, with good results, as shown by intercalibration studies [23,182,184–186].

While there are correlations between BSLA results and acute human

toxicity, the relationship is not straightforward. Efforts have been made to model acute toxicity of chemicals in humans based on *Artemia* toxicity tests, often in combination with other assays and compound physicochemical parameters, but these are not yet well-established [26,27, 179–182,189]. Nevertheless, the simplicity and accessibility of BSLA make it a useful preliminary screen for estimating general toxicity.

Ideas from these two different purposes for using BSLA—medicinal plant screening for bioactivity, and ecotoxicological testing—were used in this study to provide a rough assessment of possible toxic effects of medicinal plants. Methods for medicinal plant research vary, but are often adapted from the work of Meyer, McLaughlin, and coworkers. These methods look at mortality, rather than detailed physiology or behavioral parameters, as the endpoint [18,22]. The method uses a 48-h hatching and incubation period of *Artemia*, followed by 24 h of exposure to the test substance or extract. This is in agreement with other studies, mainly from ecotoxicological research, which point to a greater sensitivity of the nauplii to toxins at 48 h [23,179]. For example, a detailed study with *Artemia franciscana* using potassium dichromate as the standard toxicant showed an LC<sub>50</sub> of 21 µg/mL at Day 1 after hatching, 9.12 µg/mL at Day 2, and 15 µg/mL at Day 3 [190]. Greatest sensitivity was at Day 2, when the larvae are expected to be a mixture of instar II and III. This time frame for testing is recommended for brine shrimp assays [24,182,187,188]. This is consistent with our method, in which eggs were incubated and allowed to hatch over 48 h, and exposed to the test substance (control or extract) for another 24 h.

In this study, the results of the BSLA are expressed in LC<sub>50</sub> values of the plant extracts. It can be observed that the medicinal plant extracts have a wide range of toxicity values (Table 2). We expect this variability due to the range of bioactivities and ethnomedical uses associated with our plants (Table 1), and thus, their possible modes of toxicity to brine shrimp. In addition, the multicomponent nature of medicinal plant extracts can lead to the additional complexity of additive, synergistic or antagonistic effects [107]. Future studies may shed light on the possible mechanisms of toxicity of these medicinal plants.

The toxicity control, potassium dichromate, showed an LC<sub>50</sub> of 12.24 µg/mL. This is similar to values found by several other groups using *Artemia*, and within the range of an intercalibration exercise involving 59 laboratories by Persoone and coworkers [184,191–193]. Given the results of our potassium dichromate control, we estimate that the *Artemia* nauplii under our experimental conditions are close to or at the recommended stage of high sensitivity to possible toxicants [184].

No toxicity was observed for the solvent controls. Although some solvents may be somewhat toxic to *Artemia* [194], the filter disc method adapted from Meyer and coworkers minimizes solvent interference by drying the solvents and reconstituting the assay solution in artificial sea water.

Despite the growing popularity of the BSLA in studies of plant toxicity [195], no single toxicity classification scale is used by workers in the field. In our study, fifty-three Philippine medicinal plants were examined, eight of which appear to be highly toxic against brine shrimp at concentrations which are lower than the control, potassium dichromate, as shown in Table 2. We rounded this to 12 µg/mL and propose a classification scheme, shown in Table 3, which categorizes extracts with LC<sub>50</sub> ≤ 12 µg/mL as highly toxic. This cut-off need not be fixed at a particular value, but set at the LC<sub>50</sub> of a highly toxic control, such as potassium dichromate. Above the LC<sub>50</sub> of potassium dichromate, we propose a classification for moderately toxic, mildly toxic, and essentially non-toxic medicinal plant extracts, similar to classification schemes from the work of other researchers, but also informed by the results from our benchmark plants.

For those using BSLA as a bioactivity screening tool, LC<sub>50</sub> < 100 µg/mL is often taken as indicative of the presence of potential cytotoxic or insecticidal compounds [22,196], while extracts with LC<sub>50</sub> > 1000 µg/mL are often considered nontoxic, in part because this is usually the upper limit for sample concentrations used in the experiments. Extracts considered nontoxic were usually not pursued for further study as



potential anticancer compounds [22].

The range of LC<sub>50</sub> values between 100 µg/mL and 1000 µg/mL have been classified in different ways by different researchers. For example, Nguta and coworkers refined the scale while doing a preliminary screen using the BSLA of 45 aqueous extracts of 31 different Kenyan plant species for possible antimalarial activity [197]. They classified those with LC<sub>50</sub> values between 100 µg/mL and 500 µg/mL as moderately toxic, and those with LC<sub>50</sub> values between 500 µg/mL and 1000 µg/mL as mildly toxic. Karchesy et al. used a similar scale to screen plants from the American Pacific Northwest, choosing those with strong toxicity for further study for insecticidal and antimicrobial candidates [198]. Moshi and coworkers studied Tanzanian medicinal plants to draw some inferences on their safety, as well as to explore their bioactivity [199]. They categorized extracts with LC<sub>50</sub> values above 100 µg/mL as nontoxic, and refined their broad classification from previous studies to several categories: highly toxic (LC<sub>50</sub> < 1.0 µg/mL), toxic (1.0 µg/mL < LC<sub>50</sub> < 10.0 µg/mL), moderately toxic (10.0 µg/mL < LC<sub>50</sub> < 30.0 µg/mL), and mildly toxic (30 µg/mL < LC<sub>50</sub> < 100 µg/mL) in comparison to cyclophosphamide (LC<sub>50</sub> = 16.3 µg/mL). On the other hand, Mousseux screened for bioactive compounds in extracts from marine sources and classified their toxicity using 4 levels: highly toxic for LC<sub>50</sub> < 10 µg/mL, moderately toxic for 10 µg/mL ≤ LC<sub>50</sub> < 50 µg/mL, mildly toxic for 50 µg/mL ≤ LC<sub>50</sub> < 100 µg/mL, and nontoxic for LC<sub>50</sub> ≥ 100 µg/mL [200].

These illustrate that researchers have used different toxicity scales for BSLA, depending on the purpose of the assay. For drug discovery, when toxicity is the desired endpoint, whether for cancer cells, insects, or plasmodium species, focusing on the highly toxic candidates is usually desirable. As the researchers proceed to do more detailed studies on their candidate extracts or compounds, they then have to consider the therapeutic window to avoid toxic effects on humans.

The BSLA results for the highly toxic plant extracts correlate with results from other toxicity studies. The toxicity result of *Croton tiglium* (Euphorbiaceae), whose LC<sub>50</sub> = 1.32 µg/mL, is consistent with the anticancer activity exhibited by 12-O-tetradecanoylphorbol-13-acetate (TPA), a major active constituent of croton oil [201], although it is lower than the 30 µg/mL reported by Meyer et al. in one of the pioneer studies using BSLA for active plant constituents [20]. The BSLA result of *Arcangelisia flava*, belonging to family Menispermaceae, is similar to the findings of Soonthornchareonnon and co-workers [202]. The LC<sub>50</sub> of *Annona reticulata* and *Arcangelisia flava* are in the highly toxic range due to the presence of phytochemical compounds such as alkaloids, glycosides and tannins that are thought to be responsible for its anticancer, insecticidal property and germicidal property, respectively [28].

The high toxicity of *Lansium domesticum* leaf extract may be due to the compounds thought to be responsible for its anti-skin cancer activity [28]. The LC<sub>50</sub> of *Nephelium lappaceum* also revealed a high toxicity in BSLA, and is likely to be due to bioactive compounds responsible for its antibacterial and other activities [28]. No plant parts commonly consumed as vegetables were found in the highly toxic category.

Similar to classification schemes mentioned above [197,199,200] we used a range of 12–100 µg/mL for the next level of toxicity. Twenty-one plant extracts fall in this category, which we classified as moderately toxic. Extracts from some of these plants have also been shown to exhibit cytotoxicity. For example, the toxicity result of *Colocasia esculenta* (taro) leaves agrees with the previously described dose-dependent toxicity on anticancer effects *in vitro* [203,204]. However, taro leaves, as well as roots, when properly prepared and cooked, are also considered vegetables. *Ficus* species, specifically *Ficus minahassae* and *Ficus septica*, showed potential bioactivities. The result of *Ficus septica* is consistent with the studies previously conducted by Nugroho et al. [205]. They reported that the n-hexane insoluble fraction of *Ficus septica* leaves enhanced the action of doxorubicin, increasing growth inhibition and apoptotic induction over doxorubicin alone in breast cancer T47D cell lines.

The brine shrimp toxicity of *Psidium guajava* leaves is consistent with

the BSLA result found by Rana et al. [206] and is consistent with the cytotoxicity results on HT-29 human colon cancer cells [207]. The result of the ethanolic extract of *Pandanus amaryllifolius* (pandan) is consistent with the cytotoxic activity of this plant in inducing apoptosis on the hormone independent breast cancer cell line MDA-MB-231 [77]. Pandan leaves are used across Southeast Asia to add flavor and aroma to during cooking, but are not vegetables consumed in large quantities. *Peperomia pellucida* leaf extract demonstrated a LC<sub>50</sub> = 12.92 µg/mL, which is consistent with the BSLA result conducted by Khan and co-workers [208]. This is also consistent with the anticancer activity against human breast adenocarcinoma (MCF-7) cell line [209]. The ethanolic leaf extract of *Carmona retusa* showed an LC<sub>50</sub> = 15.9 µg/mL, which would classify it as moderately toxic in the BSLA. But sufficient safety and efficacy of *Carmona retusa* leaf preparations have allowed product registration as over-the-counter herbal medicine for diarrhea in the Philippines [210].

As with several classification schemes mentioned above [197,198], we used a range of 100–500 µg/mL for the next level of toxicity, which we classified as mildly toxic. We note though that for some researchers, extracts with LC<sub>50</sub> in this range would already be considered non-toxic [199,200]. Nineteen plant extracts had LC<sub>50</sub> values in this range. This group includes the ethanolic extract of *Vitex negundo* leaves. Khan et al. [211] reported that the n-hexane and chloroform extracts from the bark were toxic to brine shrimp. Another study on leaf extracts using different solvents showed toxicity, with the methanolic extract showing a value of 12.5 µg/mL [212]. Nevertheless, our results place *Vitex negundo* leaf ethanolic extracts in the category of mildly toxic. Clinical experience agrees with this classification. *Vitex negundo* leaf is safe enough to be an over-the-counter herbal medicine in the Philippines [9,11].

The result in *Cananga odorata* (LC<sub>50</sub> = 370.00 µg/mL) can be related to the bioactivity value of the acetone extract (LC<sub>50</sub> ≤ 150.00 µg/mL) reported by Caloprisco and co-workers [213]. Our results are also consistent with studies conducted by Zakaria et al. [214] on *Manihot esculenta*, and Krishnaraju et al. [104] on *Hibiscus rosa sinensis*.

Results for *Artocarpus heterophyllus* leaves show that it is mildly toxic, with LC<sub>50</sub> of 454 µg/mL. Burci and coworkers studied seed extracts for various types of toxicity and the ethanolic extract showed similar results to ours in the BSLA, with a value of 389 µg/mL, with only the hexane extract showing moderate toxicity [215]. They did not observe any hemolytic activity. When tested against a number of cell lines, some of the extracts showed activity against cancer cell lines but no measurable toxicity against a mouse fibroblast cell line. While their results are from the seed rather than the leaves, these agree with the low toxicity we observe for this plant. The mildly toxic category also includes the ethanolic extracts from common vegetables: *Abelmoschus esculentus* (okra) fruit or pod, LC<sub>50</sub> of 465 µg/mL, and *Momordica charantia* (bitter melon) leaves, LC<sub>50</sub> of 111 µg/mL. Both these vegetables are generally cooked before being consumed.

At the least toxic end of the spectrum, five ethanolic plant extracts showed LC<sub>50</sub> values over 500 µg/mL. Traditional use and other studies outlined in Table 1 support the potential bioactivity of these extracts. For purposes of assessing medicinal plant safety, we classified this group as essentially non-toxic, since this group includes *Allium cepa* L. leaves and *Ipomoea batatas* L. leaves, which are common vegetables. This group are also shows LC<sub>50</sub> values far larger and less toxic than *Vitex negundo*. The low measured toxicity suggests the potential of this set of plants as nutraceuticals or food supplements.

Using the vegetables as a benchmark, the results suggest that plant extracts with low to moderate toxicity may be sufficiently safe for as possible agents for various diseases and health conditions. However, the results also indicate the need for further study. Our method, based on standard practice in medicinal plant research, uses ethanolic extracts of dried plant parts. Since the vegetables are usually cooked rather than eaten raw, our findings may not sufficiently reflect toxicity or safety of the plant material as used in actual ethnomedical practice. For example, traditional healers may use fresh or dried plant material; a single plant

may be used on their own or combined with other medicinal plants or other substances; heating may or may not be part of the process; the plant material itself may be used, or extracted juices, infusions and decoctions in water, or alcoholic tinctures; the material or extract may be applied topically or administered orally [28,31,36]. A more detailed assessment of toxicity of particular medicinal plants should take these preparation methods into account.

Taken together with the result from *Vitex negundo*, plant extracts that exhibit low to even moderate toxicity using BSLA may be good candidates for development as herbal drugs, especially where modulation of cellular processes, rather than cell or pathogen death, is the desired bioactivity. In addition, it is possible that plant extracts with low toxicity in BSLA may have safety profiles amenable even for long term use for the treatment of chronic diseases. However, long term safety will have to be explored using assays designed to study toxic effects during prolonged exposures. These assays, even using brine shrimp, are more challenging to implement than assays for acute exposure [23].

By comparing a range of medicinal plants for toxicity using BSLA, our results provide a guide to prioritizing which medicinal plants need to be studied in greater detail for assessment for safety. Extensive pre-clinical and clinical studies, similar to those done for *Vitex negundo*, will be needed to establish these plants as mainstream herbal medicines. Even if particular plants are shown to be effective and safe through such studies, food and drug regulatory agencies and the WHO require rigorous assessment of processing conditions, to ensure that herbal products on the market are standardized, effective and safe [216–218].

#### 4. Conclusion

Our study investigated the comparative toxicity of the ethanolic extracts of 53 selected medicinal plants used in the Philippines, with the aim to contribute towards estimating the safety of the plant extracts. To make the results more broadly useful, we used simple methods that are accessible even in relatively resource-limited contexts: chemical profiles and chemical diversity were estimated using standard simple qualitative phytochemical screening tests, and toxicity was estimated using the brine shrimp lethality assay. The collection also includes plants used in other ethnomedical traditions, to allow comparisons across studies even if the precise quantitative findings may differ due to experimental conditions.

Since extrapolating from brine shrimp to humans is not straightforward, we used a benchmarking approach using plants with some level of safety information. A key benchmark in the collection is a plant that passed clinical trials for safety and efficacy: *Vitex negundo*, whose leaf preparations have been approved by the Philippine FDA as over-the-counter medications for cough and asthma. In addition, the collection includes plants that are commonly used vegetables, and regarded as safe to ingest: for example, *Allium cepa* (onion) leaves and *Ipomoea batatas* (sweet potato) leaves.

The results obtained from this work revealed that most plant extracts were chemically diverse, despite the low resolution of simple qualitative tests. On average, the plants tested positive for four different classes of secondary metabolites. Of the 53 plant extracts profiled, 43 tested positive for alkaloids, 10 for anthraquinones, 31 for flavonoids, 33 for glycosides, eight for saponins, 21 for steroids, 50 for tannins and 20 for triterpenoids.

A broad range of toxicity against brine shrimp was also observed. Based on our findings from our benchmark plants, and informed by classification schemes by a number of other researchers, we propose a modified scheme for classifying toxicity levels. Five plant extracts had LC<sub>50</sub> values over 500 µg/mL, and these included vegetables that are part of the diet and commonly considered as safe. These findings support the classification of this group as essentially non-toxic to humans despite observable LC<sub>50</sub> values in brine shrimp.

Another 19 plant extracts had LC<sub>50</sub> values between 100 µg/mL and 500 µg/mL, including the clinically-validated benchmark plant *Vitex*

*negundo*, as well as other common vegetables. This supports the classification of this category as mildly toxic. Our results suggest that plants in this category are likely to be safe, although clinical experience is needed to validate this, as was done for *Vitex negundo*.

Twenty-one plant extracts showed moderate toxicity, with LC<sub>50</sub> values between 12 µg/mL and 100 µg/mL. Finally, eight plant extracts had LC<sub>50</sub> values lower than potassium dichromate (less than 12.24 µg/mL, rounded to 12 µg/mL), suggesting potent cytotoxicity, and consistent with anticancer activity observed in other studies of some of the plants. While these plants may have possible pharmacological effects, care must be taken to study the therapeutic index, whether in ethnomedical practice or drug discovery efforts.

No clear trend was found between which classes of chemical compounds correlate with higher levels of toxicity. Nor was chemical diversity correlated with toxicity. The classes of phytochemicals present in the plants are merely starting points for more detailed chemical analysis of the plant extracts. Further studies are needed to confirm the specific bioactivities of the plant components.

Furthermore, other toxicity test methods using vertebrate models, mammalian models or human cell lines are needed to better relate the toxicity information observed in brine shrimp to possible effects on humans. Nevertheless, our findings offer suggestions for which plants to prioritize for safety studies in order to better understand the therapeutic index of these plant extracts, the potential toxic effects in pre-clinical or clinical research, and the possible side effects in ethnomedical practice. In the future, the results of these kinds of detailed studies can help in weighing the risks and benefits of medicinal plants.

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#### CRediT authorship contribution statement

**Lydia M. Clemen-Pascual:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Funding acquisition. **Rene Angelo S. Macahig:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision. **Nina Rosario L. Rojas:** Conceptualization, Methodology, Formal analysis, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

#### Declaration of Competing Interest

The authors declare no conflict of interest.

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