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General bioactivity screening of *Homalocladium platycladum* (Tapeworm plant) via brine shrimp lethality assay

Javen Domugho, Janus B. Pansacala*

Chemistry Department, College of Science and Mathematics, Western Mindanao State University, Normal Road, Baliwasan, Zamboanga City, Philippines

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Abstract

This study aimed to evaluate the presence of bioactive compounds in *Homalocladium platycladum* (Tapeworm plant) through bioactivity screening and toxicity assessment. The powdered mature stems of the plant were extracted using different solvents, namely n-hexane (HEX), dichloromethane (DCM), ethyl acetate (ETA), n-butanol (BUT), and methanol-water (WAT). The resulting fractions were prepared and tested at various concentrations using the brine shrimp lethality assay. The results indicated that the HEX, DCM, and BUT fractions exhibited immediate and acute toxicity, suggesting the presence of bioactive compounds. Furthermore, all fractions demonstrated toxicity at longer exposure times, indicating the potential for chronic toxicity. The median lethal concentration (LC_{50}) analysis using the Miller-Tainter method revealed moderate toxicity for the HEX and DCM fractions, with LC_{50} values of 199.9 ppm and 457.3 ppm, respectively. Moreover, all fractions, including HEX, DCM, ETA, BUT, and WAT, exhibited bioactivity potential at longer exposure times, with calculated LC_{50} values of 19.79 ppm, 38.82 ppm, 14.77 ppm, 5.565 ppm, and 131.3 ppm, respectively. Notably, all fractions, except for the methanol-water fraction, displayed high toxicity after 24-hour exposure. These findings suggest that the Tapeworm plant contains bioactive compounds with pharmacological potential. Therefore, further investigation of these compounds is warranted for the development of novel pharmaceutical agents.

* **Corresponding Author:** Janus B. Pansacala ✉ janus.pansacala@wmsu.edu.ph

Introduction

Medicinal plants have been extensively utilized for centuries, particularly in rural areas, as remedies for various health conditions. These plants have been found to contain phytochemicals, which are vital components in combating specific illnesses. The pharmaceutical industry has recognized the potential of natural products derived from plants, whether in the form of pure compounds or standardized extracts, for their pharmacological properties. Hence, these natural products have gained considerable interest and are being incorporated into pharmaceutical research and development.

Bioactive compounds are chemical substances synthesized by plants that possess medicinal or toxicological effects on humans and animals. The production of bioactive compounds is a common trait among plants, although the types and quantities may vary depending on the species, as well as the propagation and maturity of the plant (Bernhoft, 2010). To harness the pharmacological potential of these compounds, they must first be extracted from the plant material.

Solvent-solvent extraction, also known as liquid-liquid extraction, is a commonly used method for extracting bioactive compounds. This technique relies on the solubility (polarity) of the compounds in different solvents. One well-established partitioning protocol for liquid-liquid extraction is the Kupchan method. It is particularly effective in extracting even minute amounts of target substances and has led to the discovery of numerous new compounds that were previously unattainable using traditional extraction approaches (Kupchan *et al.*, 1972, 1973). The method involves separating and extracting compounds based on their solubility in two solvents with contrasting polarities.

In the study of medicinal plants, extensive extraction and analysis have been carried out over many years to determine their pharmacological potential by identifying the presence of bioactive compounds (Abubakar & Haque, 2020). The choice of solvent in the extraction process is crucial, as different types of bioactive compounds exhibit varying affinities for solvents based on their polarity. Consequently, polar or non-polar solvents can selectively extract specific types of bioactive compounds depending on the chemical composition of the molecules involved.

The Brine Shrimp Lethality Assay (BSLA) is a well-known and versatile bioassay that serves multiple purposes, including general toxicity testing, detection of pharmaceutical compounds, and evaluation of bioactive compounds derived from plant extracts (Hamidi *et al.*, 2014; Krishnaraju *et al.*, 2005). BSLA is highly valued for its practicality, as it can be easily conducted using commercially available brine shrimp eggs (artemia cysts). The assay is cost-effective, as it requires simple and inexpensive materials, and does not necessitate sophisticated equipment. This accessibility and simplicity contribute to the widespread utility of the BSLA in various research and screening applications.

Homalocladium platycladum (*H. platycladum*), commonly known as centipede plant, ribbon bush, or tapeworm plant, is a plant in the Philippines with significant potential. It belongs to the *Polygonaceae* family and is a perennial shrub with semi-herbaceous characteristics. Originally from Papua New Guinea and the Solomon Islands, *H. platycladum* is used as a medicinal plant in Chinese and traditional Malay medicine. It is also recognized for its folkloric medicinal value in Brazil, India, and Indonesia, (Manfron *et al.*, 2007; Nuria, 2010) particularly in the treatment of snake and centipede bites. Despite its recognized traditional uses, there is limited scientific data and research supporting the medicinal or pharmaceutical effects of *H. platycladum*. Therefore, investigating the general cytotoxicity of the plant is crucial in determining the presence or absence of bioactive compounds in its crude extract. The process will involve the solvent-solvent partitioning, specifically the liquid-liquid extraction using the Kupchan Method, along with the brine shrimp lethality assay (BSLA). By employing the techniques, the study aims to provide further evidence regarding the bioactivity of *H. platycladum* and its promising potential for pharmacological applications.

Materials and methods

Collection and Preparation of Sample and Materials

Fresh leaves of *Homalocladium platycladum* were purposively sampled from Menzi and Cabunbata in Isabela City, Basilan Province. The mature leaves were randomly collected from each plant and stored in a clean Styrofoam box.

Leaves collected from different sampling sites were combined in a single container and thoroughly mixed before undergoing the air-drying process. Brine shrimp eggs for the Brine Shrimp Lethality Assay (BSLA) were obtained from a local pet shop and used as received, with the eggs placed in a transparent plastic container. For the extraction and partitioning process, hexane, dichloromethane, ethyl acetate, n-butanol, and methanol were selected as solvents. These solvents were procured and utilized without further modification.

Preparation of H. platycladum Crude Alcoholic Extract

The freshly collected leaves of *H. platycladum* were thoroughly washed under running tap water to remove any dust or impurities. Subsequently, the washed leaves were air-dried by placing them on a net and suspending them in a shaded area for a period of 30 days. Once fully air-dried, the *H. platycladum* leaves were cut into smaller pieces and pulverized using a kitchen grinder. Approximately 180 g of the resulting dried powder was carefully transferred into a clean, airtight, flat-bottomed amber glass container with a capacity of 4.0 L.

To extract the bioactive compounds, 2.4 L of methanol was added to the powdered sample, and the mixture was allowed to soak for 15 days at room temperature, with regular shaking each day to ensure proper mixing. Following the 15-day soaking period, the liquid extract was separated from the solid residue by filtration using Whatman filter paper no. 1. The obtained filtrate was then transferred to a pre-weighed evaporation flask, and the solvent was removed using rotary evaporation. The remaining dried extract was weighed to determine the amount of crude extract obtained, measured in grams.

Liquid-liquid Partitioning of Crude Extract via Modified Kupchan's Method

To perform the solvent-solvent extraction of the crude extract, a modified version of Kupchan's method was employed (Otsuka, 2008). Approximately 7.6078g of the crude extract was dissolved in 135mL of methanol, resulting in a concentration of approximately 90% methanol.

The solution was then transferred to a 500mL separatory funnel, and 15mL of distilled water was added. The crude extract was subjected to three successive extractions with 150mL of hexane. After each extraction, the hexane layer was collected in a pre-weighed evaporating flask and subjected to rotary evaporation to obtain the hexane-soluble fraction (HEX). The remaining water-methanol layer was returned to the separatory funnel for dichloromethane extraction. The aqueous methanol layer was extracted three times with 150mL of dichloromethane, and the resulting dichloromethane-soluble fraction (DCM) was collected and rotavaped in a pre-weighed evaporating flask. For the third extraction, the aqueous solution was partitioned against 150mL of ethyl acetate. The obtained ethyl acetate fraction (ETA) was collected and rotavaped. The residual aqueous layer was then subjected to three extractions with n-butanol, resulting in the n-butanol soluble fraction (BUT). Additionally, the remaining methanol-water soluble fraction (WAT) was obtained and rotavaped. The dried fractions, including HEX, DCM, ETA, BUT, and WAT, were weighed and reported as grams of fraction obtained. Furthermore, these dried fractions were carefully stored in sealed containers for subsequent analysis.

Brine Shrimp Lethality Assay (BSLA) of H. platycladum Fractions

Brine shrimp lethality assay (BSLA) was used to evaluate the potential bioactivity property of each fraction by means of its toxicological behavior. Each dried fractionate extracts of *H. platycladum* underwent to toxicity screening. The process involved seawater preparation for hatching the (*Artemia)* brine shrimp eggs, formulation of appropriate dosages, bioassay testing, LC_{50} computation.

Preparation of Sterile Natural Seawater

The collection of natural seawater for experimental purposes involved careful consideration of the collection site, ensuring its distance from any nearby village to minimize potential contamination. Once collected, the natural seawater underwent a series of essential procedures to ensure its suitability for the experiment. Firstly, the seawater was subjected to filtration to remove any impurities or debris present. Subsequently, sterilization of the filtered seawater was achieved through autoclaving, employing standard protocols to eliminate any potential microbial contaminants. The resulting filtered and sterilized seawater served as the basis for the preparation of fractionate solutions utilized in the brine shrimp lethality assay (BSLA). Various concentrations of 10 ppm, 100 ppm, 500 ppm, and 1000 ppm were prepared using the sterilized seawater, enabling the assessment of bioactivity through the BSLA. These carefully prepared fractionate solutions served as crucial components in the subsequent evaluation of the experimental outcomes.

Preparation of Dosages for BSLA

The brine shrimp lethality assay (BSLA) required the preparation of various dosages of fractions, as well as a positive control and a blank. Stock solutions with a concentration of 50,000 ppm were prepared for each fraction (HEX, DCM, ETA, BUT, and WAT). To create the 50,000-ppm stock solution for HEX, 829.5 mg of n-hexane dried extract was dissolved in 16.59mL of sterilized seawater, along with a tween 80 solution consisting of 15.5mL of seawater and 1.09mL of tween 80. Similarly, the 50,000-ppm stock solution for DCM involved dissolving 723.3 mg of dichloromethane dried extract in 14.40mL of sterilized seawater, along with a tween 80 solution of 13.0mL of seawater and 1.40mL of tween 80. For ETA, the dried ethyl acetate extract weighing 2242.6 mg was dissolved in 43.85mL of sterilized seawater, with the addition of 1.00mL of tween 80, resulting in a 50,000-ppm solution. The BUT fraction required dissolving 1199.8 mg of butanol extract in 23.0mL of sterilized seawater, along with 1.00mL of tween 80, to obtain the 50,000-ppm solution. Finally, the WAT stock solution was created by dissolving 1958.2 mg of the extract in 39.16mL of sterilized seawater, resulting in a concentration of 50,000 ppm. All the prepared 50,000 ppm solutions were then used to prepare dosages at four different concentrations: 10 ppm, 100 ppm, 500 ppm, and 1000 ppm. For the positive control, a 50,000-ppm stock solution was prepared by dissolving 500.0 mg of thymol in 10.0mL of sterilized seawater. The blank consisted solely of sterilized seawater.

Hatching of Artemia cysts

A modified container was utilized to facilitate the preparation and collection of brine shrimp eggs (*Artemia* cysts) and their larvae after 24 hours of hatching. The setup involved using a rectangular fish aquarium measuring 22 x 32 cm, along with a colored plastic cup equipped with five small holes in the middle body and a prototype air passage. The container was filled with natural seawater, and the *Artemia* cysts were placed inside the colored plastic cup, which was then covered. After an incubation period of 24 hours at room temperature (22-29 °C), the active artemia nauplii (larvae) that had hatched and were free from eggshells could be observed in the glass aquarium. The light source outside the colored plastic cups attracted the active larvae, making them visible in the aquarium. These active larvae were carefully collected and transferred to a clean 250mL beaker for easy handling and transference, using a Pasteur pipette.

Bioassay Testing

The *Artemia* nauplii (larvae) were enumerated within the stem of a Pasteur pipette against a background with appropriate lighting. Each sample was transferred with ten (10) larvae to four test tubes of each fraction, containing 3.0mL of sterilized seawater and varying volumes of the 50,000-ppm solution (1 µL, 10 µL, 50 µL, and 100 µL, respectively). The test tubes were labeled according to their concentrations: 10 ppm, 100 ppm, 500 ppm, and 1000 ppm. Additional sterilized seawater was added to achieve a total volume of 5.0mL, and the test tubes were exposed to constant illumination throughout the entire process. The assay involved monitoring the number of alive and dead larvae over a specific period. After a 6-hour exposure period, the remaining

alive and dead brine shrimp larvae were counted, and the same procedure was repeated after 24 hours of exposure. By determining the number of surviving and deceased brine shrimp larvae, the percentage (%) mortality was calculated and used to evaluate the median lethal concentration (LC_{50}).

Computation of the Median Lethal Concentration (LC_{50})

The brine shrimp larvae, both alive and deceased, were carefully examined and tallied within each dosage of the various fractions after a 6-hour and 24-hour exposure period to detect any signs of toxicity. The median lethal concentrations (LC_{50}) for all the fractions were calculated using the probit analysis method, employing a conversion table that links the percentage of mortality to corresponding probit values as outlined by Finney (Finney & Stevens, 1948). Additionally, the determination of the probit values at zero- and one-hundred percent mortality for each fraction at different dosages was accomplished using the Miller-Tainter method, incorporating corrected probit equivalents (Arambasic & Randhawa, 2014; Randhawa, 2009).

Results and discussion

Extraction of H. platycladum leaves

The dried crude extract of *H. platycladum* exhibited a notable dark green color and contained brownish crystals upon drying.

These visual characteristics could be attributed to the presence of concentrated phytochemicals in the absence of a solvent. The dried extract weighed 7.6078g, which provided a sufficient amount for further fractionation using the modified Kupchan's method. This method involved employing solvents with increasing polarity, namely hexane, dichloromethane, ethyl acetate, and n-butanol, to extract specific fractions from the original crude methanolic extract.

As a result, five distinct fractions were obtained, namely the hexane fraction (HEX), dichloromethane fraction (DCM), ethyl acetate fraction (ETA), n-butanol fraction (BUT), and the methanol-water fraction (WAT). Table 1 presents the weights (yields) of the methanolic crude extract of *H. platycladum* as well as the subsequent yields of the obtained fractions.

Table 1. Weight of *Homalocladium platycladum* crude extract and fractions.

Weight crude methanolic extract	Weight of dried fractions					Total yield of fractions
	HEX	DCM	ETA	BUT	WAT	
7.6078 g	1.0497 g	0.7233 g	2.2455 g	1.1998 g	1.9582 g	7.1765 g
% Yield	13.8%	9.51%	29.5%	15.8%	25.7%	94.3%

The results of the solvent-solvent extraction process revealed that solvents with intermediate to high polarity yielded higher quantities of dried fractions, whereas solvents with lower polarity resulted in smaller amounts of fractions. It is worth noting that during the rotary evaporation process, all solvents used in the extraction were effectively removed from the dried fractions. This was confirmed through visual inspection, which ensured that no residual solvents were present in the final fractions. The removal of solvents was further facilitated by subjecting the samples to an airflow in a fume hood for several days, promoting the complete drying of the fractions. Overall, these findings demonstrate the successful extraction and drying of the different fractions obtained from the crude extract of *H. platycladum*.

Bioactivity screening of H. platycladum fractions with brine shrimp lethality assay (BSLA)

The brine shrimp lethality assay (BSLA) was conducted to evaluate the potential bioactivity of the different fractions (HEX, DCM, ETA, BUT, and WAT) obtained from H. platycladum. Stock solutions of each fraction were prepared at a concentration of 50,000ppm.

The assay involved exposing the brine shrimp larvae to different dosages of each fraction for a 6-hour and 24-hour period, representing acute and chronic toxicity levels, respectively. The number of dead larvae was recorded at each time point, and the percentage of mortality was calculated by comparing the number of dead larvae to the total number of larvae.

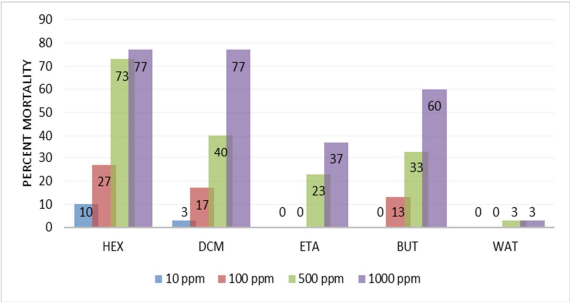

Fig. 1. Toxicity testing of the five fractions at 6-hour exposure.

Fig. 1 illustrates the number of dead brine shrimp larvae and the corresponding percentage of mortality after 6 hours of exposure to the five fractions. HEX exhibited the highest number of dead larvae at the 6-hour period, followed by DCM, BUT, ETA, and WAT in descending order. Consequently, when converting the total number of dead larvae into percentage mortality, HEX also demonstrated the highest values (10%, 27%, 73%, and 77%) among the fractions tested. DCM exhibited percentages of mortality at 3%, 17%, 40%, and 77%, while BUT displayed 0%, 13%, 33%, and 60% mortality for the respective dosages of 10 ppm, 100 ppm, 500 ppm, and 1000 ppm. ETA exhibited percentages of mortality at 0%, 0%, 23%, and 37%, while WAT had the lowest percentages of mortality at 0%, 0%, 3%, and 3% for the same dosages.

These results align with expectations, as there is a clear positive correlation between the concentration of each fraction and the percentage of mortality observed. Higher concentrations of each fraction led to higher percentages of mortality, particularly evident in the 1000 ppm dosages. The findings from the BSLA provide initial evidence of potential bioactivity within the fractions of *H. platycladum*, warranting further investigation to identify and isolate the bioactive compounds responsible for the observed effects.

The results obtained from the brine shrimp lethality assay (BSLA) provide insights into the acute and long-term toxicity of the fractions obtained from *H. platycladum*. In terms of acute toxicity, the HEX, DCM, and BUT fractions exhibited notable bioactivity, with percentages of mortality reaching 77%, 77%, and 60%, respectively, at a dosage of 1000 ppm. Conversely, the ETA and WAT fractions showed minimal acute toxicity, with percentages of mortality at 37% and 3%, respectively, indicating a lack of significant bioactivity. To further investigate the long-term effects, a 24-hour exposure period was conducted for the fractions, and the results are presented in Fig. 2.

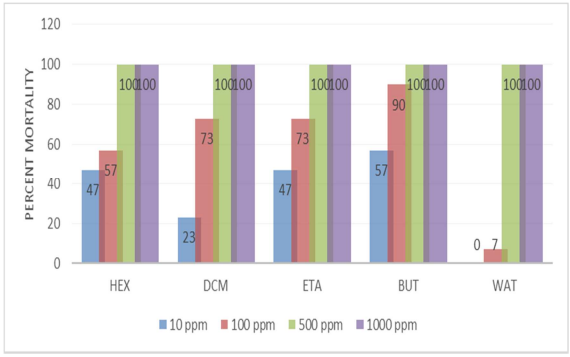

Fig. 2. Toxicity testing of the five fractions at 24-hour exposure.

Fig. 2 illustrates that extended exposure times led to increased mortality of the brine shrimp larvae, correlating with higher dosages of each fraction. Remarkably, the BUT fraction exhibited the highest number of dead larvae, followed by ETA, HEX, DCM, and WAT in descending order of decreasing mortality values. Specifically, the BUT fraction displayed the highest percentage of mortality among the five fractions, with values of 50%, 90%, 100%, and 100% for dosages of 10 ppm, 100 ppm, 500 ppm, and 1000 ppm, respectively. It was followed by ETA (47%, 73%, 97%, and 97%), HEX (47%, 57%, 100%, and 100%), DCM (23%, 73%, 100%, and 100%), and WAT (0%, 7%, 100%, and 100%).

The findings from the 24-hour exposure period indicate that all fractions exhibited toxicity, suggesting the presence of bioactive constituents in the leaves of *H. platycladum*. The effects of these constituents become more pronounced with longer exposure times. These results highlight the potential pharmacological significance of the plant and warrant further investigation to identify and isolate the specific bioactive compounds responsible for the observed toxic effects. Understanding the bioactivity

and toxicity profiles of these fractions contributes to the overall knowledge of *H. platycladum* and its potential applications in various fields, including pharmacology and natural product discovery.

Calculation of median lethal concentrations (LC_{50}) of H. platycladum fractions via Miller-Tainter Method.

The Miller-Tainter method, which incorporates a modified probit analysis, was employed to calculate the LC_{50} values for each fraction of *H. platycladum*. The method involves transforming the percent (%) mortality values into probit equivalents using Finney's table. These probit values, which show a linear relationship with the log concentration of each fraction, were utilized to determine the LC_{50} values through linear regression. The probit value of 5.00 corresponds to 50% mortality, and the log ppm concentration of each fraction represents the median lethal concentration. Notably, the Miller-Tainter method required special considerations for the transformation of 0% and 100% mortality values, for which existing probit equivalents were not available. However, with the use of a specific formula, equivalent transformations for both 0% and 100% mortality were obtained. Table 2 presents a summary of the computed LC_{50} values for each fraction during the 6-hour and 24-hour exposure periods.

Table 2. Computed LC_{50} values for 6-hour and 24-hour period of exposure.

Fractions	Period: 6-hour		Period: 24-hour	
	LC_{50}	Clarkson's Scale	LC_{50}	Clarkson's Scale
HEX	199.9 ppm	Moderately toxic	19.79 ppm	Highly toxic
DCM	457.3 ppm	Moderately toxic	32.82 ppm	Highly toxic
ETA	4601 ppm	Non-toxic	14.77 ppm	Highly toxic
BUT	910.3 ppm	Low toxic	5.565 ppm	Highly toxic
WAT	1.909×10^{44} ppm	Non-toxic	131.3 ppm	Moderately toxic

The results indicate that the presence of bioactive compounds in the fractions influences their exhibited toxicities and LC_{50} values. Lower LC_{50} values suggest a higher likelihood of a fraction containing phytochemicals with potential bioactivity. Moreover, it was observed that longer exposure times resulted in enhanced toxicological effects. The acute and chronic toxicity estimates provided by the different exposure periods offer insights into the potency levels and assertiveness of the compounds present in each fraction.

The HEX and DCM fractions displayed immediate biological activity at shorter exposure periods, indicating their potential pharmacological application as antifungal, antimicrobial, and anticancer agents. The literature suggests that these fractions may contain alkaloids, glycosides, steroids, flavonoids, saponins, tannins, sterols, emodols, coumarins, carotenoids, and terpenoids (Azmir *et al.*, 2013; Dhawan & Gupta, 2016; Kemboi *et al.*, 2016; Tiwari *et al.*, 2011). On the other hand, the gradual increase in bioactivity over time observed in the BUT, ETA, and WAT fractions suggests that the compounds they contain may find use as anti-helminthic and anti-hypertensive drugs, reflecting their potential pharmacological properties.

The findings highlight the diverse bioactive compounds present in *H. platycladum* and their potential applications in various therapeutic areas. Further investigation, including isolation and identification of the specific bioactive compounds, is warranted to unlock the full pharmacological potential of this plant. These results contribute to the expanding knowledge of *H. platycladum* and its importance in natural product research and drug discovery.

Conclusion

In conclusion, this study focused on the extraction, partitioning, and bioactivity screening of *Homalocladium platycladum* (Tapeworm plant). The crude methanolic extract was successfully obtained and further fractionated using modified Kupchan's method. The bioactivity screening through the brine shrimp lethality assay (BSLA) revealed acute toxicity for the hexane (HEX), dichloromethane (DCM), and n-

butanol (BUT) fractions, indicating the presence of bioactive compounds. However, the ethyl acetate (ETA) and methanol-water (WAT) fractions did not exhibit significant bioactivity at shorter exposure times.

Additionally, the 24-hour exposure period demonstrated that all five fractions (HEX, DCM, ETA, BUT, and WAT) displayed toxicity, indicating the presence of compounds with potential bioactivity over longer exposure times. The calculation of median lethal concentration (LC_{50}) using the Miller-Tainter method further supported these findings.

The study provides valuable insights into the toxicity and bioactivity profiles of *H. platycladum* and highlights its potential as a source of pharmacologically active compounds. Further investigation is necessary to isolate and identify the specific bioactive constituents responsible for the observed effects. Understanding the bioactivity and toxicity of these fractions contributes to our knowledge of *H. platycladum* and its potential applications in pharmacology and natural product discovery.

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