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Phytochemical Analysis and Cytotoxicity Potential of *Pittosporum pentandrum* (Mamalis) Crude Leaf Extract Using Brine Shrimp Lethality Assay

Stephanie Ocal, Ricky Boy Ferrer, Karla Namoc, Sharlene Cherry S. De Guzman
BS Biology, Natural Science Department, Pangasinan State University-Lingayen Campus
Lingayen, Pangasinan, Philippines

Abstract *- In the Philippines,* Pittosporum pentandrum*, locally known as* Mamalis*, is intently utilized by traditional herbalists to treat patients with various cases. Although Mamalis have been medicinally useful internationally and locally, only few studies have provided scientific data to confirm its cytotoxic activities. In this present study, crude extract of* Pittosporum pentandrum *or* Mamalis *leaves was tested in vivo for its cytotoxic effects against brine shrimp nauplii and relate its toxicity results with its known phytochemical and ethnobotanical activities. In summary,* Mamalis *crude extract contain phytochemicals such as alkaloids, triterpenoids, flavonoids and tannin that may be attributed to its ability to be used as a herbal medicine against pathogenic microorganisms or cancerous cells. Using BSLA, the cytotoxic capability of* Mamalis *crude extract is evident at 0.01% (100 (100 µg/ml) to 0.09% (900 µg/ml) with a* LC_{50} *at 0.05% (500 µg/ml). The toxicity criteria of the said* LC_{50} *is 'toxic' and 'moderately toxic' according to Meyer's and Clarkson's toxicity index, respectively. These results also proved that* Mamalis *crude extract can be further examined as an antimicrobial or antitumor agent.*

Keywords – cancer, herbal medicine, pathogenic microorganism

INTRODUCTION

The exploration of new medicine that comes from plants is now the current trend in conducting research regarding the use of alternative ways to combat the menace of pathogenic microorganisms, tumor and cancer agents that are resistant to commercial drugs [1]. Plants can be used using their crude or advanced form to become medicine in their pure state [2]. This is also due to the fact that plants are rich in secondary metabolites that can be utilized in different biological and pharmacological activities, thus enabling them as starting point in the development of modern medicine [3]. Although many plants have valuable properties, some of them are known to carry toxicological properties as well [1]. The toxicity of plant may originate from the substances present in them such as alkaloids, essential oils, tannins, resins, and many others [4].

Several species of *Pittosporum* plant have been studied and proven effective in fighting antimicrobial and antifungal activities. In Egypt, *Pittosporum tobira* was found out to be active against *Rastonia solanacearum*, a fungal pathogen that causes soft rot and brown rot diseases in potato, tomato, tabaco, banana and eggplant and some ornamental plants [5]. In Brazil, *Pittosporum undalatum* was studied to inhibit the production of aflatoxins produced by a fungal pathogen, *Aspergillus flavos* [6]. In India, *Pittosporum pentandrum,* is used for various treatment such as external cleansing of wounds, applied as alternative antibiotic against *Mycobacterium aureus* which causes acute low back pain, and is also used as an antidote to snake poison [7].

In the Philippines, *Pittosporum pentandrum,* local known as *Mamalis*, is intently utilized by traditional herbalists to treat patients with various cases. Some of its ethnomedicinal uses include using its extracts to cleanse wounds. An aromatic decoction from its boiled leaves is also used by women as bath after giving birth

while the bark of the *Mamalis* is worn as a necklace by the *Negritos* which, they believe, are a good way to treat cough and headache [8]. In other places, small doses of powdered bark is diluted into a juice and drank to reduce fever [9]. Although *Mamalis* have been medicinally useful internationally and locally, only few studies have provided scientific data to confirm its activities. An analysis of the phytochemical properties and cytotoxic capabilities of Mamalis using biological assay will serve as additional evidences in its antimicrobial and antitumor effects. Experimental bioassays such as brine shrimp lethality assay is practiced as a starting point in the development of new drugs.

Brine Shrimp Lethality Assay (BSLA) is a technique used to screen the toxicity of plant extracts [10]. The dormant eggs (nauplii) of brine shrimp are available commercially and can be used as experimental cells with which plant extracts are tested to identify its toxicity activities [11]. Since its introduction, this *in vivo* test has been successively employed for active cytotoxic and antitumor agents [12]. BSLA is now considered an effective method in generating the toxicological data of ethnomedicinal plants since it can explore the general toxicity of herbal remedies simply by using plant crude extracts [11].

In this present study, crude extract of *Pittosporum pentandrum* or Mamalis was tested *in vivo* for its cytotoxic effects against brine shrimp nauplii and relate its toxicity results with its known phytochemical and ethnobotanical activities. Specifically, this study determined the lethal concentration at 50% (LC_{50}) of *Mamalis* extract to identify the toxicity criteria it belongs using Meyer's and Clarkson's toxicity indexes. The findings of this study can be used as a basis for the development of new tools of great medicinal importance.

OBJECTIVES OF THE STUDY

This study investigated the different active phytochemicals present in *Mamalis* leaves crude extract. It also explored the percent mortality rate of brine shrimp in the following concentrations (1) 99% of Artificial saltwater and 1% DMSO (2) 0.2% Doxorubicin (3) 0.01% – 0.09% *Mamalis* extract and (4) 100% of Distilled water. After conducting the experiment, the LC_{50} of *Mamalis* leaves extract was also identified and the toxicity criteria was determined using Meyer's and Clarkson's index. Statistical analysis was conducted to determine the significant difference in the percent mortality rate of the different *Mamalis* extract concentrations and the control solutions.

MATERIALS AND METHOD

Research Design

This research used experimental method to test the cytotoxicity of Mamalis leaves extract using Brine Shrimp Lethality Assy.

Plant Collection

The identified area for the collection of *Mamalis* leaves was in Sitio Aliling, Barangay Laoag, Aguilar, Pangasinan where the said plant was naturally grown and the population is not endangered. A total of (1) kg of young fresh *Mamalis* leaves was brought to the laboratory for phytochemical testing and crude extraction.

Phytochemical Analysis and Plant Extraction

Mamalis leaves was prepared and brought to the Pharmacological Laboratory of Virgen Milagrosa University Foundation, San Carlos City on March 1, 2018. Certification was issued true and results were considered valid [13]. The extraction was done in the same laboratory.

Preliminary Testing of *Mamalis* Crude Extract Formulations

The first set of formulation was done by diluting the the pure Mamalis crude extract into four increasing concentrations – i.e., (µg/ml)25%, 50%, 75% and 100%. BSLA was then employed and determined its LC_{50}. However, the said formulations did not reveal the LC_{50} and so another set of formulations was prepared. A second set of nine formulations was diluted from pure Mamalis crude extract – 0.1% (1000µg/ml), 0.2% (2000µg/ml), 0.3%(3000µg/ml), 0.4%

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(4000µg/ml), 0.5% (5000µg/ml), 0.6% (6000µg/ml), 0.7% (7000µg/ml), 0.8% (8000µg/ml), and 0.9%(9000µg/ml). BSLA was then employed and observed for $LC_{50.}$ None of the said concentration levels resulted to a $LC_{50.}$ A third set of formulations was established – i.e., 0.01%(100µg/ml), 0.02% (200µg/ml), 0.03% (300µg/ml), 0.04% (400µg/ml), 0.05% (500µg/ml), 0.06% (600µg/ml), 0.07% (700µg/ml), 0.08% (800µg/ml), and 0.09% (900µg/ml). BSLA was conducted and the set up was observed for the LC_{50}. One of the said formulations was detected was LC_{50} and thus, the final set of formulations was used in the conduct of this experiment.

Treatment set up

A total of twelve treatments (Table 1) was established. Each treatment has three replications. For the different formulations, a mixture of Mamalis crude extract, Dimethyl sulfoxide (DMSO) and artificial saltwater (ASW) was made in different concentrations with a total volume of two (2) ml per formulation in each replicate vial. The positive control was a 2000µg/ml (2%) concentration of Doxorubicin, an anti-tumor agent. The negative control was simply using artificial saltwater only and distilled water only. The set up (Figure 1) was incubated under a study lamp for 24 hours before measurement of mortality rate.

Table 1. Treatment setup

Treatment Code	Amount of DMSO (0.02%)	Amount of Mamalis Crude Extract	Concentration	Control
T1			2ml	Artificial Salt Water
T2			2ml	Doxorubicin
T3	1.9 ml	0.01ml	0.01% (100 µg/ml)	
T4	1.8ml	0.02ml	0.02% (200µg/ml)	
T5	1.6mL	0.03mL	0.03% (300µg/ml)	
T6	1.4mL	0.04mL	0.04% (400µg/ml)	
T7	1.2mL	0.05Ml	0.05% (500µg/ml)	
T8	1.0mL	0.06mL	0.06% (600µg/ml)	
T9	0.9mL	0.07mL	0.07% (700µg/ml)	
T10	0.8mL	0.08mL	0.08% (800µg/ml)	
T11	0.9mL	0.06Ml	0.09% (900µg/ml)	
T12			2ml	Distilled Water

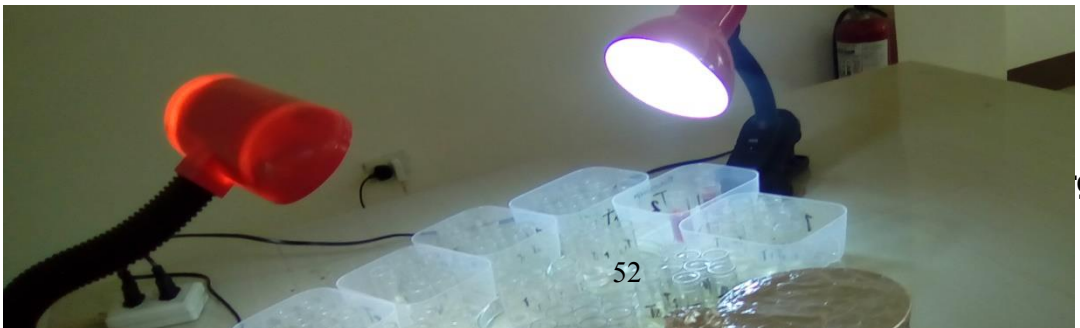

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Figure 2. set up and the stock solution for the experiment

Hatching Eggs of Brine Shrimp

Dormant eggs were purchased at a local pet store and stored in the refrigerator to maintain its freshness. A solution of artificial saltwater was then prepared by dissolving 20 g of rock salt in 540 ml of distilled water. A $^1/_8$ teaspoon of the unhatched eggs was then added to the ASW solution and incubated with an aerator to create oxygen under a study lamp to attract the live nauplii to the surface once hatched. This is done for easy collection of live nauplii samples as they are easily attracted to light.

Brine Shrimp Lethality Assay

Using a vial with 10ml capacity, each formulation of Mamalis crude extract together with the control solutions was distributed across a total of 108 vials – with a sample distribution of 2 ml in formulation in each vial and addition of ten (10) live nauplii in each vial. The vials were then incubated for 24 hours and exposed to light before observation of dead nauplii. The percent mortality rate of nauplii in each concentration was measure using the following formula:
Percent mortality = (# of dead nauplii/total nauplii counted) x 100

The data was then used to construct a graph that shows the linear regression of the mortality rate and the level of concentration at LC_{50}.

Treatment of Data

The LC_{50} value was determined through linear regression analysis. Toxicity criteria was defined using Meyers Toxicity Index and Clarksons Toxicity Index (Table 2). One way Analysis of Variance followed by Multiple Comparison Test using Scheffe were then employed to determine any significant differences in the various concentrations of *Mamalis* crude extract and the control solution.

RESULTS AND DISCUSSION

Phytochemical analysis of *Mamalis* Crude Extract

Phytochemical analysis of active constituents presents in *Mamalis* leaves showed that this plant contains secondary metabolites such as alkaloids, unsaturated sterols and triterpens, flavonoids and also tannin and phenolic compounds (Table 3)

Table 2. Meyer's* and Clarkson's** toxicity index

MEYER'S* INDEX		CLARKSON'S** INDEX	
LC_{50}	**Description**	**LC_{50}**	**Description**
< 1000 µg/ml	Toxic	> 1001 µg/ml	Non-toxic
> 1000 µg/ml	Non-toxic	501 – 1000 µg/ml	Low toxic
		101 – 500 µg/ml	Moderately toxic
		0 – 100 µg/ml	Highly toxic

*Meyers et al., 1982
** Clarkson et al., 2004

Table 3. Phytochemicals Analysis of *Mamalis* leaves

Test Performed	Positive Result	Actual Result	Remark
Alkaloids			
1. Mayer's Reagent	Production of Precipitate	Presence of Precipitate	+
2. Wagner's Reagent	Production of Precipitate	Presence of Precipitate	+
3. Bouchardat's Reagent	Production of Precipitate	Presence of Precipitate	+
4. Valser's Reagent	Production of Precipitate	Presence of Precipitate	+
Unsaturated Sterols and Triterpenes			
1. Lieberman's Burchard Test	Blue or Green Colour Sol'n	Green Colour Sol'n	+
Flavonoids			
1. Cyanidin Test	Green or Red Colour	Green Colour Sol'n	+
Tannin and Phenolic Compounds			
1. Gelatin Test	Production of Precipitate	Presence of Precipitate	+
2. Gelatin Block Test	Production of Precipitate	Presence of Precipitate	+
3. Ferric Chloride Test	Greenish Blue/Greenish Black Colour	Greenish Black Sol'n	+

Legend: + = Indicates presence of active constituents, - = Indicates absence of active constituents

These secondary metabolites such as alkaloids that were naturally occurring in plants stated that their mechanisms considered from its anti-oxidant, anti-cancer, analgesic, relieving action of ephedrine for asthma, and anti-inflammatory [13], [14]. The Unsaturated sterols and triterpenes, sterols are studied from its hypoglycaemic, antioxidant, anti-inflammatory and analgesic activities [15] and triterpenoids are studied

for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and free radical scavengers [16], [17]. According to Peteros and Uy [16], flavonoids possess numerous pharmacological properties such as antiviral, anti-fungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticancer, hepatoprotective, and cytotoxic activities of flavonoids have generated interest in studies of plants. In the study of [18] flavonoids were inhibit cell toxicity in cancer cells. Tannins considered as antimicrobials, antioxidant, antifungal, antiviral effect antioxidant, and anti-inflammatory [19], [16].

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Mortality Rate of Nauplii using BSLA

Figure 3 shows the mortality rate of nauplii after treating with the different formulations of *Mamalis* crude extract. Using the formula stated in the methodology, mortality rate was accounted for in all formulations. As seen in the table, the highest mortality rate (64.4%) was achieved at 0.09% (900 µg/ml) and lowest (35.6%) at 0.01% (100 µg/ml) of *Mamalis* crude extract. The positive control, Doxorubicin showed 100% mortality rate while the negative control 5.6% and 0% for distilled water and ASW, respectively

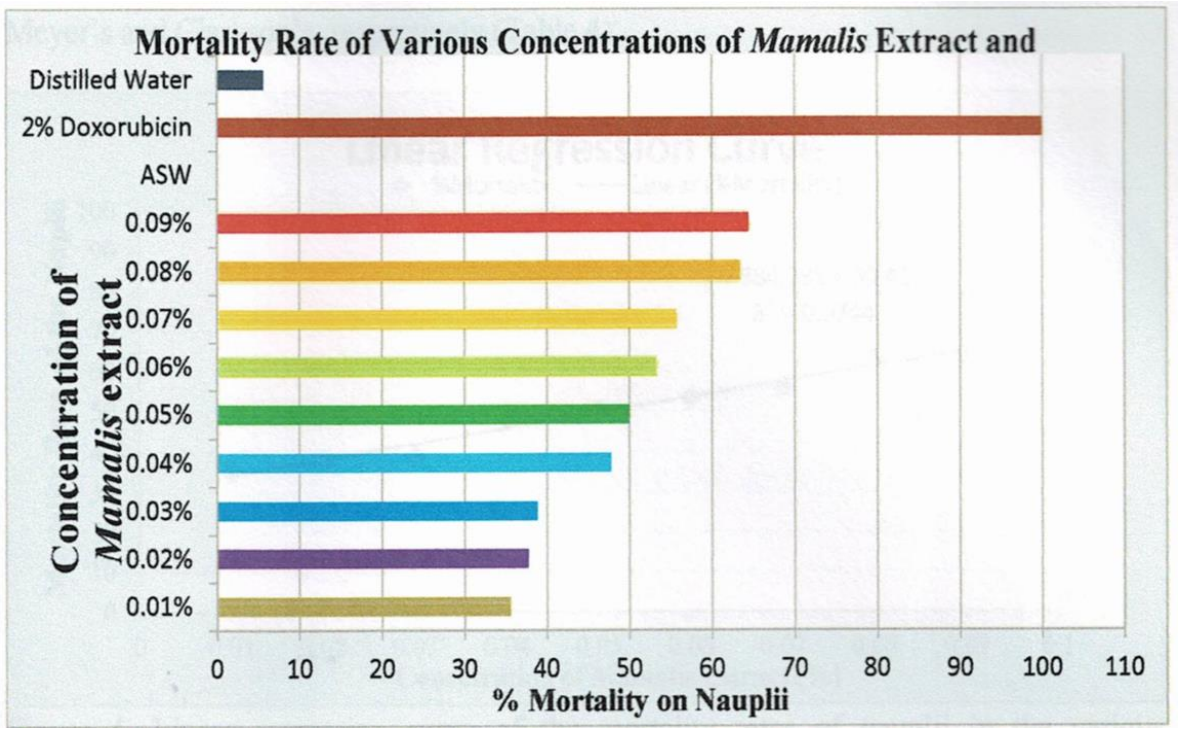

Figure 3. Mortality rate of various concentration of *Mamalis* leaves crude extract and controls

According to previous studies on the different species *Pittosporum* sp, mortality rate of brine shrimp was also observed at concentrations ranging from less than 1000µg/ml. One study of P. viridiflorum showed maximum mortalities occurred at the highest extract concentrations of 2µg/ml and Pittosporum lanatum mortality rate against brine shrimp was at 27.4µg/ml [12].

Determination of LC_{50} and Toxicity Criteria

Figure 4 indicates the linear regression curve of the mortality rate across the nine formulations of *Mamalis* crude extract. It can be seen from this figure that LC_{50} was achieved at the concentration of

500 µg/ml. Similar results were observed in the three replications of the said concentration. Based on the toxicity index in Table 2, at 500 µg/ml $LC_{50,}$ the Mamalis crude extract is identified as toxic and moderately toxic for Meyer's and Clarkson's, respectively (Table 4).

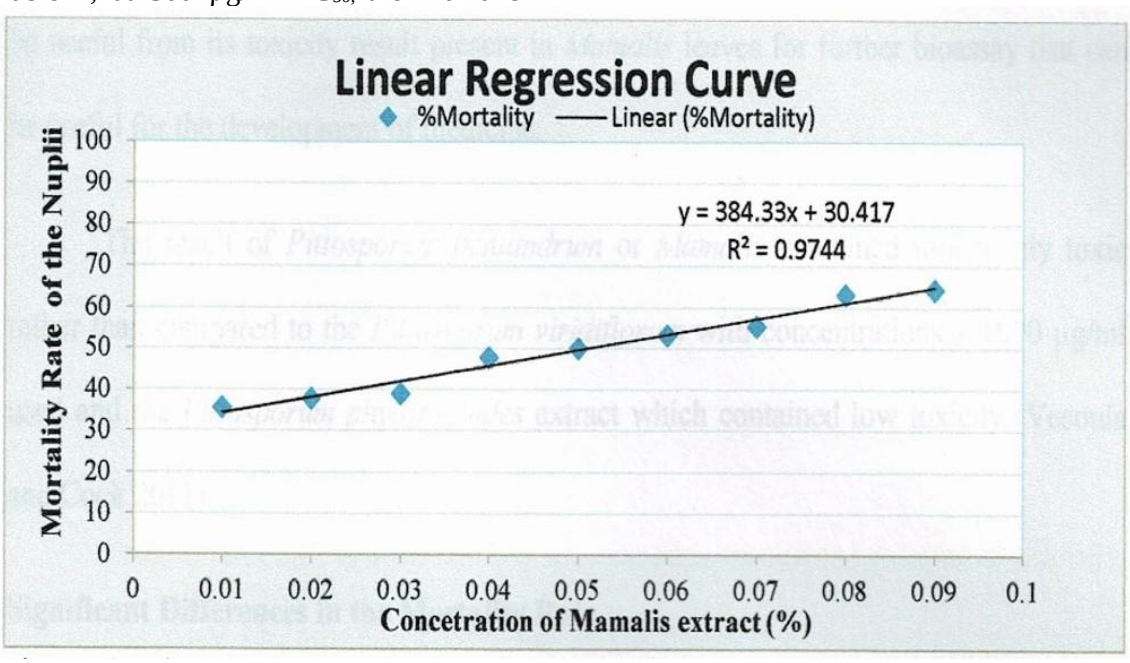

Figure 4. Linear regression curve of the mortality rates of nauplii in the various formulations of *Mamalis* crude extract.

According to previous studies on the different species *Pittosporum sp,* the LC_{50} of *Pittosporum petandrum* was observed at concentration of 0.05% (500µg/ml) compared to other *Pittosporum sp* One study of P. viridiflorum showed that this species had a LC_{50} at 1.13µg/ml (Vesouka and Cocka, 2011) and study on the *P. phylliraeoides* shows against brine shrimp with an LC_{50} at 2.52µg/ml [12].

Table 4. Toxicity criteria of the LC_{50} of Mamalis crude extract

Toxicity Index	LC_{50} of Mamalis (500 µg/ml)	Descriptive rating
Meyer's	LC_{50}> 1000 µg/ml	**Toxic**
Clarkson's	LC_{50} of 500-1000 µg/ml	**Moderately Toxic**

Table 4 shows the toxicity of the Mamalis leaves crude extracts expressed as LC_{50} values in of Meyer's or to Clarkson's toxicity index. The Table 4 show the LC_{50}<1000 µg/ml that indicated to be toxic using Meyer's toxicity index, where the Clarkson's toxicity index indicated the concentration of different concentration shows LC_{50} of 500 µg/ml represent as amoderately toxic present in *Mamalis* leaves.

From this study, it can be useful from its toxicity result present in *Mamalis* leaves for futher bioassay that can be useful for the development of medicine.

The result of *Pittosporum pentandrum* or *Mamalis* contained moderately toxic rather than compared to the *Pittosporum viridiflorum* with concentration ≤ 1000 µg/ml used and the *Pittosporum phylliraeoides* extract which contained low toxicity [20].

Significant Differences in the Mortality Rate

Table 5 shows the ANOVA of the different treatments indicating that the differences in the mortality across treatments is highly significant at α=0.05. Table 6 highlights the comparison on the mortality rate of the different formulations of *Mamalis* crude extract and the control solutions. It can be seen from the table that Scheffe test resulted to a significant difference between the mortality rates of 0.01% (100 µg/ml), 0.02% (200 µg/ml) and 0.03% (300 µg/ml) as compared to 0.09% (900 µg/ml) of *Mamalis* crude extracts with a p-values of 0.010, 0.030 and 0.048, respectively. When compared to Doxorubicin, the Scheffe test showed high significant differences as compared with the mortality rates of *Mamalis* crude extract.

Table 5. ANOVA of the mortality rate across treatments

Source	F	Prob > F
Across treatments **Across replications PLS** **SHOW DATA HERE**	44.84	0.0000
Level of significance at α=0.05		

Table 6. Multiple comparison on the mortality rates using Scheffe

Treatments	p-values	Descriptive rating
100 µg/ml and 900 µg/ml	0.010	Significant
200 µg and 900 µg/ml	0.030	Significant
300 µg and 900 µg	0.048	Significant
100 µg/ml and Doxorubicin	0.000	Significant
200 µg and Doxorubicin	0.000	Significant
300 µg and Doxorubicin	0.000	Significant
400 µg and Doxorubicin	0.000	Significant
500 µg and Doxorubicin	0.000	Significant
600 µg and Doxorubicin	0.000	Significant
700 µg and Doxorubicin	0.000	Significant
800 µg and Doxorubicin	0.000	Significant
900µg and Doxorubicin	0.000	Significant
Level of significance is at α=0.05		

Table 6 shows results on the multiple comparisons test the number of mortalit rate of the brine shrimp upon application of the different concentrations of *Mamalis* leaves crude extract together with the Doxorubicin (positive control). The different concentrations of *Mamalis* with the doxorubicin indicated statistically significant showed in the number of the mortality rate of

brine shrimp treated of *Mamalis* leaves crude extract between 100µg/ml and 900µg/ml, 200µg/ml and 900µg/ml, 300µg/ml and 900 µg/ml. Although the different formulation show significantly difference, the 500µg/ml, 600µg/ml, 700µg/ml, 800µg/ml, and 900µg/ml show LC_{50} that considerably showed toxicity activity. The toxicity effect of the various concentration of the *Mamalis* leaves crude extract may be because the present active phytochemical.

Multiple comparison among the mortality rates of the nine concentration of *Mamalis* crude extract implies that there is a significant difference in the cytotoxic capability of 900µg/ml compared to 100-300µg/ml. It is also implies that 900µg/ml of *Mamalis* leaves crude extract does not provides the same level of toxicity level of 2000µg/ml doxocubicin because the comparison result is significantly different. The doxocubicin or the positive controlindicated 100% mortality to brine shrimp and considered to be highly toxic which considerably significant to the *Mamalis* extracts. The toxicity of plants was determined by comparing their LC_{50} values with highly toxic chemicals to be used as positive controls for this BSLA such as: potassium dichromate, thymol, cyclophosphamide, pure DMSO, Doxocubirin [10], [1].

CONCLUSION AND RECOMMENDATION

The present investigation revealed that *Mamalis* crude extract has potential cytotoxic capabilities due to its present active phytochemicals. This proves that *Mamalis* crude extract is a promising source in the development of new antimicrobial or antitumor agents.

The researchers recommend the further elucidation on the active components responsible for the cytotoxic properties of *Mamalis***.** *Mamalis* extracts and isolates stored in phytochemical laboratories have yet to be tested in more specific biological assays using laboratory rats or cancer cell lines to further confirm the results of this study.

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