Brine Shrimp Lethality Bioassay and Phytochemical Screening of Primates-Consumed Plants from Pangandaran Beach Conservation Area

Ferry Ferdiansyah Sofian[1*], Ami Tjitraresmi[1], Dudi Runadi[1], Yulien Ratu Kania[2], Anas Subarnas[3]

[1]Natural Product Pharmacy Laboratory, Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Indonesia - 45363

[2]Apothecary Profession Student, Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Indonesia - 45363

[3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Indonesia - 45363

Email *: ferry.ferdiansyah@unpad.ac.id

ABSTRACT

Aim:

In this study, we investigated the toxicity of ethanol extracts of primates-consumed plants, including *Dysoxylum caulostachyum, Eugenia aquea*, *Garcinia celebica*, and *Psychotria valetonii* collected from Pangandaran beach conservation area using brine shrimp lethality bioassay. The toxicity of the fractions from the most active extract were also investigated. Furthermore, the phytochemical screening test of ethanol extracts was executed to detect the presence of groups of secondary metabolite compounds contained in the extracts.

Methods:

The brine shrimp lethality bioassay was conducted for investigating the toxicity of extracts and fractions. The bioassay was evaluated using the statistical method of PROBIT analysis with the aid of the SPSS 23 computation package in order to determine the value of LC_{50}. The phytochemical screening was executed by using established method.

Results:

As results, in study of brine shrimp test, ethanol extracts of *D. caulostachyum*, *G. celebica* and *P. valetonii* leaves showed the toxicity with the LC_{50} values of 34.14, 705.09 and 586.50 µg/mL, respectively. The ethanol extract of *E. aquea* leaf was not performed the toxicity which was indicated by the LC_{50} values > 1000 µg/mL. Furthermore, the fractions of n-hexane, ethyl acetate and water from *D. caulostachyum* extract had the toxicity with the LC_{50} values of 12.46, 83.59 and 478.18 µg/mL, respectively. Based on the results of the phytochemical screening test, secondary metabolite compounds contained in each extract were flavonoids, quinones, phenols, monoterpenoids and sesquiterpenoids.

Conclusions:

As a conclusion, the ethanol extract of *D. caulostachyum* leaf revealed more toxic properties than the ethanol extracts of *G. celebica* and *P. valetonii* leaves. Moreover, the extract of *E. aquea* leaf was not performed the toxicity. The toxicity revealed by the fractions from *D. caulostachyum* extract clearly indicated the toxic property. This study should be continued to search for potentially active compounds of the plant.

Keywords:

Brine shrimp lethality bioassay, phytochemical screening, primates-consumed plants, toxicity

INTRODUCTION

Interest in traditional systems of medicine and, in particular, herbal medicines, have increased substantially in both developed and developing countries over the past two decades [1]. Plants and plant-derived sources not only provide us foodstuff, shelter but also, they provide remedies for many years. Different chemical constituents contained in plant exhibit different activities for alleviating abnormal health of human or animals. Therefore, traditional medicine practitioners appreciate to use different parts of plant having several chemical constituents [2].

The area of nature reserves in Indonesia, especially in Pangandaran, is an area that has geological elements where local people are invited to participate in protecting and improving the function of natural heritage, including the archaeological, ecological and cultural values in it. In this area, there is a natural potential that can be used as a source of medical treatments [3].

In previous study, Subarnas *et al.* (2012) have tested 42 primates-consumed plants from Pangandaran beach conservation area for their antiproliferative activity against cell lines of human breast adenocarcinoma (MCF-7). The results showed that four extracts of *Dysoxylum caulostachyum*, *Eugenia aquea*, *Garcinia celebica*, and *Psychotria valetonii* leaves strongly inhibited the MCF-7 cell proliferation with IC_{50} values of 12, 58, 87, and 87 µg/mL, respectively [4]. In the series of the investigation, Sofian *et al.* (2018) had recently evaluated two species of Indonesian primate-consumed plants for their in vitro antiplasmodial activity against *Plasmodium falciparum* 3D7 in the search for a new natural antimalarial agent. The plants used for the study were *D. caulostachyum* and *G. celebica*. The results showed that the extracts of *D. caulostachyum* and *G. celebica* leaves exhibited moderate to high antiplasmodial activity (1.1-10 µg/mL) against *P. falciparum* [3].

In this study, we investigated the toxicity of ethanol extracts of primates-consumed plants, including *D. caulostachyum*, *E. aquea*, *G. celebica*, and *P. valetonii* collected from Pangandaran beach conservation area using brine shrimp lethality bioassay. Then, the toxicity of the fractions from the most active extract were also investigated. Furthermore, the phytochemical screening test of ethanol extracts was executed to detect the presence of groups of secondary metabolite compounds contained in the extracts.

MATERIALS AND METHODS

Plant Materials

The plant materials used in this study were *D. caulostachyum*, *E. aquea*, *G. celebica*, and *P. valetonii* leaves collected from Pangandaran beach conservation area, Ciamis, West Java, Indonesia. Dry leaves were roughly chopped in order to obtained smaller pieces of sample and ready to be extracted. All the plants were authenticated and determined (number 426-429/HB/08/2017) in herbarium by a senior taxonomist scientist at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematic and Natural Science, Universitas Padjadjaran, Indonesia.

Preparation and Extraction of Plant Materials

All the leaves were air dried in the laboratory at room temperature (30 ± 2°C) for 10 days and roughly chopped in order to obtain smaller pieces of sample. The extraction was executed by maceration method. Each of sample (250 g) was put into the macerator and soaked in 2.0 L of ethanol (70%). The extraction process is repeated with 1.0 L of ethanol (70%) for 24 hours and then 0.75 L for another 24 hours. The each of liquid extracts obtained was filtered using Whatman paper No.1 and then evaporated using IKA® RV 10 rotary evaporator at a temperature of 40-50°C with velocity of 65 rpm and evaporated using Memmert® water bath at a temperature of 50°C to produce viscous extract with constant weight.

Phytochemical Screening of Extracts

The extracts were quantitatively tested for presence of the group of secondary metabolite compounds, including groups of alkaloids, flavonoids, quinones, phenols, saponins, tannins, steroids and triterpenoids, monoterpenoids and sesquiterpenoids. This screening was executed according to the Farnsworth method [5], namely:

Test of Alkaloids

The 0.5 g of each extracts was rinsed with 10 mL ammonia (10%), then added with 5 mL of chloroform, and crushed strongly. The chloroform layer was pipetted in while filtered, then into it was added 2 N of hydrochloric acid. The mixture was shaken firmly until there were formed two layers. The acid layer was pipetted, then it was divided into three parts. The first part was added by Mayer's reagent. The occurrence of sediment or turbidity was observed. If there was turbidity or white precipitate, it was possible that extract was contained alkaloids. The second part was added by Dragendorff's reagent. The occurrence of sediment or turbidity was observed. If there was turbidity or yellow orange-colored precipitate, it means that the extract may contain alkaloids. The third section was used as a comparative blank.

Test of Flavonoids

The 0.5 g of each extracts was mixed with 50 mL of hot water, little amount of magnesium powder and 5 mL of hydrochloric acid (2N) in the test tube. The mixture was heated over a water bath, then it was filtered. Amyl alcohol was added to the filtrate in the test tube, then it was shaken vigorously. The presence of flavonoids was characterized by the formation of yellow to red that could be drawn by amyl alcohol.

Test of Quinones

The 0.5 g of each extracts was mixed with 50 mL of water in a test tube and heated over a water bath, then filtered (Filtrate A). Then, 5% of potassium hydroxide reagent was added to the Filtrate A in the test tube. The presence of quinone compounds was characterized by the occurrence of a yellow solution.

Test of Phenol

The 1% of Iron (III) chloride reagent was added to the Filtrate A in the test tube. The presence of phenolic compounds was characterized by the occurrence of green-blue color to black solution.

Test of Saponins

After cooling, 10 mL of Filtrate A in the test tube was shaken tightly for approximately 30 seconds. The formation of a foam that was at least 1 cm high and persistent for approximately 10 minutes and was not lost on the addition of 1 drop of dilute hydrochloric acid indicated that the extract contained saponins.

Test of Tannins

The 1% of gelatin was added to the Filtrate A in the test tube. The presence of tannin compounds was characterized by the occurrence of white colored precipitate.

Test of Triterpenoids and Steroids

The 0.5 g of each extracts was mixed with 5 mL of ether, then pipetted while filtered (Filtrate B). The filtrate was placed in a vapor plate, then allowed to evaporate until it was drying. Liebermann Burchard reagent was added to the dried filtrate. The occurrence of purple color indicated the presence of triterpenoid compounds, whereas the presence of blue-green color indicated the presence of steroid compounds.

Test of Monoterpenoids and Sesquiterpenoids

The Filtrate B was placed in a vapor plate, then allowed to evaporate until it was drying. The 10% of vanillin solution in concentrated sulfuric acid was added to the dried filtrate. The occurrence of colors indicated the presence of monoterpenoid and sesquiterpenoid compounds.

Fractionation of Extract

Fractionation was conducted by using liquid-liquid extraction. About 30 g of viscous extract was weighed and dissolved with 500 mL of water. The extract solution was then filled into the separating funnel. N-hexane solvent was added in the same volume as water (1:1) into that funnel. The mixture was shaken and the formed gas was occasionally removed by opening a cap of that funnel. Then, the

mixture in that funnel was slowly formed into two separate layers, that were the water phase and the n-hexane phase. The n-hexane phase was collected and then another n-hexane was added into the water phase with the same volume. This treatment was repeated until a clear of n-hexane fraction was obtained in that funnel. All the collected n-hexane fraction was evaporated using IKA® RV 10 rotary evaporator at a temperature of 40-50°C with velocity of 65 rpm to obtain a viscous fraction of n-hexane phase.

After extracted by n-hexane, the water phase filled in the separating funnel was mixed with ethyl acetate in the same volume (1:1). The mixture was shaken and the formed gas was also occasionally removed by opening a cap of that funnel. Then, the mixture in that funnel was slowly formed into two separate layers, that were the water phase and the ethyl acetate phase. The ethyl acetate phase was collected and then another ethyl acetate was added into the water phase with the same volume. This treatment was repeated until a clear ethyl acetate fraction was obtained in that funnel. The resulting ethyl acetate fraction was evaporated solvent using a rotary evaporator and a water bath to obtain a viscous fraction. All the collected ethyl acetate fraction was evaporated using IKA® RV 10 rotary evaporator at a temperature of 40-50°C with velocity of 65 rpm to obtain a viscous fraction of ethyl acetate phase.

The water phase obtained from the liquid-liquid extraction was evaporated using Memmert® water bath at a temperature of 50°C to produce a viscous fraction of water phase.

Brine Shrimp Lethality Bioassay

The sample toxicity test method was executed by using Meyer Method (1982) with some modifications using *Artemia salina* Leach larvae [6,7,8,9] with the following steps:

Sample Preparation

Samples were prepared by dissolving 4 mg of extract or fraction in 20 µL of dimethyl sulfoxide (DMSO) and then filled the artificial seawater until the total volume was 1 mL to obtain the solution concentration of 4,000 µg/mL (Solution A). Serial dilutions were conducted by diluting the Solution A with the artificial seawater to obtain different concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.90625 µg/mL).

Hatching the Shrimp

Brine shrimp eggs were hatched for 48 hours in a conical flask containing 300 mL of seawater. The flasks were well aerated with the aid of an air pump and kept in a water bath at 29–30°C. A bright light source was left on and the nauplii hatched within 48 hours [8].

Bioassay

An aliquot of each concentration (1 mL) was transferred, in triplicate, into clean sterile universal vials with pipette, and aerated seawater (9 mL) was added. Ten shrimp nauplii were transferred to each vial (10 shrimps per concentration). The 1% DMSO in seawater were used as negative controls, respectively. After 24 hours, the numbers of survivors were counted and percentage of death calculated. The concentration that killed 50% of the nauplii (LC_{50} in µg/mL and Confidence Intervals 95%) was determined using the statistical method of PROBIT analysis with the aid of the SPSS 23 computation package (SPSS Inc.) [8]. Criterion of the toxicity for extracts and fractions was established according to Déciga-campos *et al*. [8,9,10]: LC_{50} values > 1000 µg/mL (non-toxic), ≥ 500 ≤ 1000 µg/mL (weak toxicity) and < 500 µg/mL (toxic).

RESULTS

Phytochemical Screening of Extracts

Data of phytochemical screening of some primate-consumed plant extracts are shown in Table 1. The results showed that the ethanol extract of *D. caulostachyum* leaf indicated the presence of flavonoids, quinones, phenols, monoterpenoids and sesquiterpenoids. The ethanol extract of E. *aquea* and *G. celebica* leaves indicated the presence of alkaloids, flavonoids, quinones, phenols, saponins, tannins, monoterpenoids and sesquiterpenoids. Moreover, all the secondary metabolites tested, including alkaloids, flavonoids, quinones, phenols, saponins, tannins, steroids and triterpenoids, monoterpenoids and sesquiterpenoids were identified in the ethanol extract of *P. valetonii* leaf. For comparison,

secondary metabolite compounds contained in each extract were flavonoids, quinones, phenols, monoterpenoids and sesquiterpenoids.

Table 1: Phytochemical analysis of some primate-consumed plant extracts

Secondary metabolite	*Ethanol extracts*			
	D. caulostachyum	***E. aquea***	***G. celebica***	***P. valetonii***
Alkaloids	-	+	+	+
Flavonoids	+	+	+	+
Quinones	+	+	+	+
Phenols	+	+	+	+
Saponins	-	+	+	+
Tannins	-	+	+	+
Steroids and triterpenoids	-	-	-	+
Monoterpenoids and sesquiterpenoids	+	+	+	+

Note: (+) Present, (-) Absent

Brine Shrimp Lethality Bioassay

The results of brine shrimp test for some primate-consumed plant extracts were showed in Table 2. In study of brine shrimp test, three extracts showed the toxicity with the LC_{50} values ≤ 1000 µg/mL. There were extracts of *D. caulostachyum*, *G. celebica* and *P. valetonii* leaves with the LC_{50} values of 34.14, 705.09 and 586.50 µg/mL, respectively. It can be assessed that three of them potentially had toxic properties with LC_{50} values < 1000 µg/mL. *D. caulostachyum* extract had more toxic properties (LC_{50} values < 500 µg/mL) than that *G. celebica* and *P. valetonii* (LC_{50} values ≥ 500 ≤ 1000 µg/mL). It can be indicated *G. celebica* and *P. valetonii* extracts had weak toxicity. Another case with extract of *E. aquea* leaf, the result showed that the extract was not performed the toxicity which was indicated by the LC_{50} values > 1000 µg/mL (non-toxic) [8,10].

Furthermore, the toxicity assay was continued against the fractions from *D. caulostachyum* extract which had the highest toxicity (LC_{50} value: 34.14 µg/mL). The results showed that the fractions of n-hexane, ethyl acetate and water from *D. caulostachyum* extract had the toxicity with the LC_{50} values of 12.46, 83.59 and 478.18 µg/mL, respectively (Table 3). All the LC_{50} values indicated the toxic properties (LC_{50} values < 500 µg/mL). Other studies explained that LC_{50} values which were less than 250 µg/mL indicated presence of bioactive compounds in the extracts [11,12].

Table 2: Brine shrimp lethality bioassay results of some primate-consumed plant extracts

Extract	LC_{50} value (µg/mL)	Regression equation	R^2
1. *Dysoxylum caulostachyum* Miq.	34.14	y = 1.8357x + 2.2301	0.964
2. *Eugenia aquea* Burm.F.	> 1000	y = 0.3866x + 3.7554	0.965
3. *Garcinia celebica* L.	705.09	y = 0.8189x + 2.6320	0.952
4. *Psychotria valetonii* Hochr.	586.50	y = 0.7408x + 2.9848	0.784

Table 3: Brine shrimp lethality bioassay results of fractions from *D. caulostachyum* extract

Fraction	LC_{50} value (µg/mL)	Regression equation	R^2
1. n-Hexane	12.46	y = 0.4153x + 4.5458	0.750
2. Ethyl acetate	83.59	y = 0.3873x + 4.2502	0.778
3. Water	478.18	y = 0.3392x + 4.0776	0.831

DISCUSSION

The toxicity revealed by the fractions from *D. caulostachyum* extract clearly indicated the presence of potent bioactive compounds. Preliminary the toxicity of ethanolic leaf extract of *D. caulostachyum* was screened previously and potential brine shrimp lethality was reported (LC_{50} values < 500 µg/mL) [10,12].

Meanwhile, this study should be continued to search for potentially active compounds of the plant, particularly from the fractions of n-hexane and ethyl acetate. Furthermore, this study should be extended to more specific activity testing, such as of anticancer, antitumor, antibacterial, pesticidal, and antimalarial activities.

The phytochemical screening technique can be a valuable aid in the selection of biologically or medically beneficial plants, either on the basis of one or more approaches defined under the phytopharmacological approach, or through some other ways. The goal is to isolate one or more constituents which is responsible for a particular activity [5].

CONCLUSION

As conclusion, the ethanol extract of *D. caulostachyum* leaf revealed toxic properties (LC_{50} value < 500 µg/mL) with an LC_{50} value of 34.14 µg/mL, while the ethanol extracts of *G. celebica* and *P. valetonii* leaves had weak toxicity (LC_{50} value ≥ 500 ≤ 1000 µg/mL) with LC_{50} values of 705.09 and 586.50 µg/mL, respectively. Another case with extract of *E. aquea* leaf, the result showed that the extract was not performed the toxicity which was indicated by the LC_{50} values > 1000 µg/mL. The toxicity revealed by the fractions from *D. caulostachyum* extract clearly indicated the toxic property. Meanwhile, this study should be continued to search for potentially active compounds of the plant, particularly from the fractions of n-hexane and ethyl form the ethanol extract of *D. caulostachyum* leaf.

ACKNOWLEDGEMENTS

The authors acknowledgement the Universitas Padjadjaran for the financial support, encouragement, and providing facility to conduct the research through Fundamental Research Programme of Universitas Padjadjaran.

REFERENCES

[1] World Health Organization, *WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants*, WHO, Geneva 2003, pp 1.

[2] Kabir, M.S.H., Hossain, M.M., Kabir, M.I., Rahman, M.M., Hasanat, A., Emran, T.B., Rahman, M.A., Phytochemical screening, antioxidant, thrombolytic, α-amylase inhibition and cytotoxic activities of ethanol extract of *Steudnera colocasiifolia* K. Koch leaves, *J Young Pharm.* 2016, 8(4), 391-397.

[3] Sofian, F.F., Tjitraresmi, A., Runadi, D., Tanti, G.A., Hamida, A., Halimah, E., Subarnas, A., Asih, P.B.S., In vitro antiplasmodial activity of *Dysoxylum caulostachyum* (Miq) and *Garcinia*

celebica (L) leaf extracts against *Plasmodium falciparum, J Pharm Sci & Res.* 2018, 10(2), 391-393.

[4] Subarnas A, Diantini A, Abdulah R, Zuhrotun A, Yamazaki C, Nakazawa M, Koyama, H., Antiproliferative activity of primates-consumed plants against MCF-7 human breast cancer cell lines, *E3 J Med Res.* 2012, 1(4), 38-43.

[5] Farnsworth, N.R., Review biological and phytochemical screening of plants, *J Pharm Sci.* 1966, 55(3), 225-276.

[6] Meyer, B.N., Ferrigni, N.A., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L., Brine shrimp: a convenient general bioassay for active plant constituents, *J Med Plant Res.* 1982, 45, 31-4.

[7] Zuhrotun, A., Suganda, A.G., Wirasutisna, K.R., Wibowo, M.S., Toxicity of selected Apocynaceae, Magnoliaceae and Simaroubaceae of Indonesian plants using brine shrimp lethality bioassay, *Res J Pharm Bio & Chem Sci*. 2017, 8(18), 10-15.

[8] Bastos, M.L.A., Lima, M.R.F., Conserva, L.M., Andrade, V.S., Rocha, E.M.M., Lemos, R.P.L., Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae) extracts and their main constituents, *Ann Clin Micro & Antimicro*. 2009, 8(16), 1-6.

[9] Solis, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P., Phillipson, J.D., A microwell cytotoxicity using *Artemia salina* (Brine shrimp), *Planta Med.* 1993, 59, 250-252.

[10] Déciga-Campos, M., Rivero-Cruz, I., Arriaga-Alba, M., Castañeda-Corral, G., Angeles-López, G.E., Navarrete, A., Mata, R., Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine, *J Ethnopharmacol.* 2007, 110, 334-342.

[11] Kabir, S.M.T., Sabrin, F., Hasan, M.N., Islam, K.D., Billah, M.M., Glenway, L., Investigation of preliminary cytotoxic activity of ethanolic stem and leaf extracts of *Amoora cucullata*, *J Innov Dev Strategy*. 2012, 6(2), 24-28.

[12] Pervin, R., Afrin, S., Sabrin, F., Zohora, U.S., Rahman, M.S., Islam, K.D., Billah, M.M., Antioxidant, Antibacterial and Brine Shrimp Lethality Bioassay of *Amoora cucullata*, a Mangrove Plant, *J Young Pharm.* 2016, 8(1), 33-38.