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Research Article

ANTIMUTAGENIC ACTIVITY OF WATER FRACTION AND ITS SEPARATING FRACTION FROM THE PUSPA PLANT (*SCHIMA WALLICHII* KORTH) BY THE MICRONUCLEUS METHOD

Danni Ramdhani [1]*, Anas Subarnas [2], Ahmad Muhtadi [2], Cucu Hadiansyah [3]
[1]Pharmaceutical Analysis and Medicinal Chemistry Departement, Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Sumedang, West Java, Indonesia
[2]Pharmacology and Clinical Pharmacy Departement, Faculty of Pharmacy, Universitas Padjadjaran, Jatinangor, Sumedang, West Java, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor, Sumedang, West Java, Indonesia
Corresponding author email: ramdhani07@gmail.com

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ABSTRACT

Objective: An investigation on mutagenic activity of two fraction separated from the water fractions of *Schima wallichi* Korth has been carried out on Swiss Webster male mice using the micronucleus method. Methods: The simplicia of puspa leaves was extracted by maceration method with methanol solvent for 2 days. The crude extract obtained was fractionated based on the principle of polarity, using n-hexane, ethyl acetate, n-butanol and water solvents. Each fraction was administered orally at the same doses of 350 and 700 mg/ kg of body weight. Results: Antimutagenic activity was slowly decreasing frequency of micronucleus in every 1000 erythrocytes of bone marrow preparation for each test animal. The result of the investigation indicated that the two fractions, at a dose of 700 mg/kg of body weight decreased the frequency of micronucleus significantly as compared with the control group, with the inhibitory of 34.25 % and 44.29 % respectively potency. Phytochemical screening of the fractions indicated that the first fraction contained two main compounds, quinone and polyphenol, and the second one contained saponin in addition to quinone and polyphenol. Conclusion: The antimutagenic activity of the puspa (*Schima wallichii* Korth) plant from the water fraction and its separation showed significant results. The biggest percentage of inhibition of mutation is indicated by fraction 3 which is 44.29 %. There is an alleged correlation between the presence of saponin compounds in fraction 3 with the ability to inhibit the occurrence of mutations.

Keywords: Antimutagenic, *Schima wallichii* Korth, micronucleus assay, cyclophosphamide, polyphenol, saponin.

INTRODUCTION

Mutations are the cause of congenital metabolic disorders cellular system that can trigger morbidity and mortality in living organisms[1]. Mutagen compounds are involved in the process of initiation and promotion of several human diseases especially cancer. The ability of new bioactive compounds phytocompounds in counteracting promutagenic and carcinogenic effects get some satisfying results[2]. Antimutagenic and anticarcinogenic properties of a wide variety of dietary constituents and plant secondary metabolites have been reported. The antimutagenic effect is closely related to many classes of phytocompounds, especially flavonoids, phenolic, and saponin compounds present in plants[3].

Puspa (*Schima wallichi* Korth) plant is an Indonesian tropical plant. Puspa plants are found to grow in groups in tropical forests on Java and Sumatra. Puspa plants belonging to the tribe of Theacea, are widely used empirically in traditional medicinal herbs by the Javanese tribe[4].

The study of genotoxicity through an *in vivo* micronucleus (MN) test on mouse bone marrow is an important part of the tests used to identify the mutagen hazards[5]. Schmid and Heddle have the initiative that a simpler approach to measuring chromosome damage *in vivo* is to measure micronucleus[6]. Micronuclei frequencies have been considered to be a reliable index for detecting chromosome breakages and loss[7]. MN is cell nucleus that contains a chromosome fragment that is damaged in part or all of its chromosomes. In the process of cell division, micronucleus is not inserted into the nucleus. MN can be induced by defects incell repair machines and accumulated DNA damage and chromosomal aberrations. Genotoxic agents can cause the formation of MN which causes cell death, genomics instability, or development of cancer[8].

Previous research using the micronucleus method using mice animals showed that methanol extract from the puspa plant had antimutagenic activity[9].

MATERIALS AND METHODS

Plant Material and Sample Preparations

Plant material used in this study is the leaf part of the puspa plant (*Schima wallichii* Korth) obtained from the Ciwidey forest, in West Java Province, Indonesia. Puspa leaves obtained are then dried and crushed with a mechanical grinder. The resulting powder material is then extracted by maceration with methanol

for 3 days. Every day methanol solvents are replaced with fresh ones. After 2 days the methanol solvent is then evaporated at 80ºC to get a semisolid mass. Semisolid methanol extract is fractionated sequentially with solvents, n-hexane, ethyl acetate, n-butanol, and water. The water fraction obtained was then separated by column chromatography with the stationary phase of silica gel, the mobile phase of the combination of $CHCl_3$ and Methanol solvents and obtained Fractions 1, 2 and 3. The plant extract was mixed with distilled water and 350 mg/kg and 700 mg/kg the mouse body weight.

Column Chromatography

The column employed for this purpose is a borosilicate column, measuring the length of about 50 cm, with an internal diameter of 3 cm. The Silica gel with 230–400 μm particle size was used for this study.

Phytochemical screening

All the chemicals used were of analytical grade and were purchased from Merck and Sigma-Aldrich. The methods of Harbone[10] and Trease and Evans[11] were used to identify the following phytochemicals in the extracts: alkaloids, saponins, tannins, anthraquinones, flavonoids, terpenoids and cardiac glycosides. Dried samples of the extracts were used in each of the specific methods for testing for the presence of the afore-mentioned phytochemicals as described by Harbone[10] and Trease and Evans[11].

Cyclophosphamide (Endoxan-Asta)

Endoxan-Asta is the trade name of Cyclophosphamide (CPA). It is a whitecrystalline powder with molecular weight 261. It has been widely used as a positive control in genetic toxicology. Giemsa Stain (NICE Chem Pvt Ltd Cohine) Geimsa stain (Sigma Chemical Company, New York USA), Methanol, n-butanol, etil asetat (Brataco Chemical), were used in the study.

Animals

A total of 24 male Swiss albino mice obtained from the animal facility, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. Animals were maintained under standard conditions in the animal house approved by Committee. All mice were certified for good health at the time of receipt. Age of the animals at the start of the treatment was approximately 8- 12 weeks, and weighing between 22-25 g. The animals were housed in Poly propylene cages and maintained at 24 ± 2ºC under 12 h light/ dark cycle and were fed *ad libitum* with standard pellet diet and had free access to water. During the acclimatization period, the mice were observed for animal ethical clearance signs. Ethical Clearance number: 06/UN6.KEP/EC/2018

Experimental design

The fraction resulting from the separation of the water fraction was tested using 2 doses of 350 mg / kg body weight and 700 mg/kg body weight. Each fraction is dissolved with distilled water. In the normal group and negative controls pulvis gummi arabicum (PGA) was added to increase solubility. Giving the test substance is given by single oral gavage in a volume of 20 ml/kg to the test animal group for the 24, 8 and 72 harvest time point. Cyclophosphamide dissolved in sterile deionized water served as positive control and was administered in a dose of 25 mg/kg, i.p. to groups for marrow harvest at the 24, 48 and 72 h time point[12].

Bone marrow extraction

The animal was killed by cervical dislocation or made unconscious using anesthetic ether. Femur and tibia were cutopen. Upper end of femur was cut open. Small opening was visible. Needle is inserted through aperture and 0.5 ml of suspending medium was flushed through the lower epiphyseal end in a clean cavity block. Flushing was performed four times to collect about 2 ml of bone marrow in a suspending medium. It was then centrifuged at 1000 rpm for 8 min[12].

Fixing and staining

Narrow film was prepared by smearing of the supernatant and dried. Smear fixing is performed in absolute methanol for 10 min. Slide was then is kept in coupling jar containing May-Grunwald's stain in phosphate buffer (1:1, pH 6.8). The slides were transferred to Geimsa stain freshly diluted to it phosphate buffer (1:6) and kept for 30 min. Slide were washed (3-4 times) with distilled water. Slide is air-dried[12].

Micronucleus Scoring

Coded slides were analysed for micronuclei and the incidence of micronuclei in polychromatic erythrocytes (PCE) was recorded in 1000 PCE in each slide. Four animals were analysed for each group. Besides, the ratio of polychromatic to normochromatic erythrocytes (NCE) was also estimated by counting the 1000 erythrocytes in each slide.

Statistical analysis

A compound is considered mutagenic in this test system if at any of the preparation intervals, a statistically significant increase in the number of micronucleated PCEs is found in comparison to the vehicle controls. Data were analyzed using ANOVA followed by Dunnet comparison test to observe any significant differences amongst the dosage sets and harvest periods to asses the genotoxicity effects at $p < 0.05$ level of significance.

RESULTS AND DISCUSSIONS

In the test group added a test fraction with a dose of 350 mg/kg, and 700 mg/Kg, it was seen that there was a significant decrease in the number of micronucleus when compared with negative controls. The results of the *in vivo* micronucleus tests are presented in Table 1.

Table 1: Mutation Test Results with the Micronucleus Method

Treatments	Dose (mg/Kg)	Eritrosit	Micronucleus (MNCE)	Number MNCE (mean ± SD)
Normal Control	-	4000	7	1.75 ± 0.83
Negative Control	-	4000	648	162 ± 7.44
Test Group (Fraction 2)	350	4000	531	132.7 ± 3.03
	700	4000	426	106.5 ± 2.29
Test Group (Fraction 3)	350	4000	419	119.7 ± 3.11
	700	4000	361	90.25 ± 5.49

Significant decrease in the number of micronucleus can be seen clearly in Figure 1. In the administration of fraction 3 at a dose of 700 mg/Kg there was a clear decrease in the number of micronucleus, which means that at this dose fraction 3 gave the best percentage of mutation inhibition.

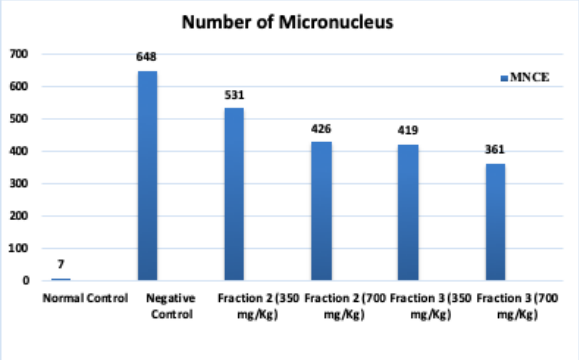

Figure 1: Number of micronucleus in testing the fraction of leaf separation of Puspa (*Schima wallichii* Korth)

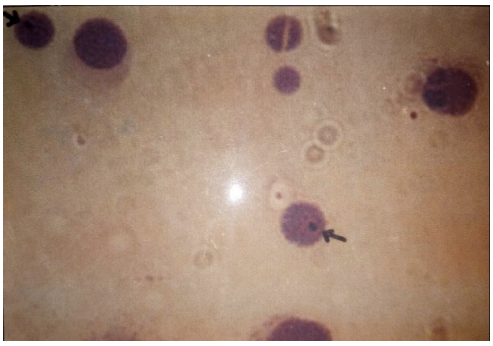

Figure 2: Micronucleus in Erythrocyte Cells

The chromosome fragments or whole chromosomes may lag behind during cell division and form micronucleus as seen in positive control Cyclophosphamide – treated mice. In the normal control treatment group, the animal not added to the test substance was only added to the PGA suspension. Whereas the negative control of the test animal was added to cyclophosphamide as a mutation inducing agent[13]. There is an increase in the number of micronucleus per 1000 erythrocyte cells for each test animal significantly with the administration of cyclophosphamide dose 50 mg / kg BB compared to normal control. Cyclophosphamide (CPA) is a commonly used chemotherapeutic agent that is an alkylating agent and induces DNA double strand breaks[14]. Micro nuclei separated from in addition to the main nucleus a cell are the results of a centric chromosomes or lagging chromosomes that fail to incorporate into either of daughter nuclei during the telophase of the miotic cells. Briefly the animals are treated with fraction of puspa prior to the administration of known carcinogen and bone marrow cells were examined for reduction in the MNPCE[15].

Extraction and Fractionation

The extraction process of puspa leaves simplicia was 450 grams by using methanol by maceration for 2 x 24 hours, producing a thick extract weighing 92 grams.Crude extract (70 g) was subjected to column chromatography on silica gel Silica gel with 230–400 µm particle size (Merck) packed and eluted with mixture of n-Hexane, chloroform, ethyl acetate, methanol, n-butanol and water of increasing polarity to obtain fractions. Crude extract (70 grams) is used to fractionate with n-hexane solvent: water with a ratio of 3: 1 produces a n-hexane fraction of 1.25 g. Then the fraction of water was fractionated again with ethyl acetate solvents, and ethyl acetate fractions of 3.8 grams were obtained. Then the last step, the fraction of water is fractionated with n-butanol solvent, and the n-butanol fraction of 17.6 grams is obtained, and the water fraction is 15.5 g.

Figure 3: Puspa Plants (*Schima wallichii* Korth)

Phytochemical screening shows similar results with previous studies, where in the water fraction there are groups of quinone compounds, polyphenols, and saponins[9]. The novel results were obtained in phytochemical screening in the fraction resulting from the separation of water fractions, where in the fractions 1 and 2 there were no groups of saponin compounds detected. This is different from the results in fraction 3, where saponin group compounds were detected. The presence of saponin in fraction 3 is thought to affect the activity of the antimutagenic effect, where fraction 3 shows the highest percentage of mutation inhibition, which is 44.29 %. Saponin compounds have been known to have potential adipocyte differentiation, and also antimutagenic activity[16-18].

Column Chromatography

The water fraction used for column chromatography separation process is 10 grams. The column chromatography process was carried out using the silica gel stationary phase, and the mobile phase combination between chloroform and methanol solvents. The first column chromatography process used an eluent ratio of chloroform: methanol (1: 1) as much as 500 ml, and produced an effect of 1 as much as 0.03 g. The second column chromatography process uses an eluent with a ratio of (1: 2) as much as 500 ml resulting in a fraction 2 of 2.27 g. The third column chromatography process using methanol eluent as much as 500 mL resulted in a fraction 3 of 0.07 g.

Phytochemical screening

Phytochemical screening has been carried out in previous studies, showing that the methanol extract and water fraction contain groups of quinone compounds, polyphenols, and saponins. In this study phytochemical screening was carried out on the water fraction, and the fractions resulting from the separation. Phytochemical screening results can be seen in Table 2 below:

Table 2: Phytochemical Screening Results from Water Fractions and Separation Fractions

Group of Compounds	Water Fraction	Fraction 1	Fraction 2	Fraction 3
Quinone	+	+	+	+
Polyphenol	+	+	+	+
Saponin	+	-	-	+

Notes: + : identified, - : not identified

CONCLUSION

The antimutagenic activity of the puspa (*Schima wallichii* Korth) plant from the water fraction and its separation showed significant results. The biggest percentage of inhibition of mutation is indicated by fraction 3 which is 44.29 %. There is an alleged correlation between the presence of saponin compounds in fraction 3 with the ability to inhibit the occurrence of mutations.

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