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Antimutagenic potential and phytochemical analysis of selected Philippine plants

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ABSTRACT

Chemopreventive agents can act to prevent or stop genetic mutation that can lead to cancer. Several promising chemopreventive agents include vitamins, minerals and phytochemicals, all of which are obtained from plants. Natural products derived from plants continue to be a fertile source of cure for cancer. In this study, selected Philippine plants were tested for their ability to inhibit chromosomal damage induced by tetracycline in the *in vivo* micronucleus test (MT). The methanolic extract of *Canarium ovatum* Engl., locally recognized as *pili*, showed the most promising activity by reducing the number of micronucleated polychromatic erythrocytes by 54.41% in the preliminary MT screening. Bioactivity-guided fractionation of the crude methanol extract of *C. ovatum* leaves have identified the most active portion in the hexane fraction. Further isolation and purification will be done to determine the constituent/s responsible for the observed antimutagenic activity in *C. ovatum.* Identification and characterization of the bioactive compound/s in this plant could be a starting point in the search for other chemopreventive substances, and for the possible synthesis and development of more efficacious agents.

Keywords: *Canarium ovatum,* chromosomal damage*, Crateva religiosa,* micronucleus test, phytochemicals, *Pouteria campechiana, Premna odorata.*

INTRODUCTION

Worldwide, cancer causes more deaths than AIDS, tuberculosis, and malaria combined (1). Although several anticancer drugs are already commercially available, a number of adverse effects sometimes occur during chemotherapy (2). To reduce this unwanted effects many chemotherapeutic agents in the clinic are derived from natural products or designed on the basis of original compounds found in natural products (3). In the search for new cancer chemopreventive agents from natural sources, this research is directed towards the screening of plants with antimutagenic activity in the micronucleus test (MT) based on the method of Schmid (4). MT is a highly sensitive *in vivo* test designed to detect carcinogenic effects (5). In addition, this study is also focused on the isolation and characterization of the antimutagenic expressions of the plant identified with the highest activity using a bioassay directed scheme.

As a part of a systematic investigation of identifying bioactive compounds from Philippine plant extracts, a library of Philippine plant extracts was subjected to different investigator-initiated screens at the Institute of Chemistry and Cell Biology (ICCB) Screening Facility at Harvard Medical School. The selected plants that were subjected to the preliminary screening for bioactivity in MT include *Premna odorata* Blanco*, Pouteria campechiana* Baehni*, Crataeva religiosa* Forst and *Canarium ovatum* Engl.

These plants were chosen since they were among the few plant extracts that gave positive results in various assays from the Philippine plant extract libraries deposited at the ICCB (6). Literature search revealed that *C. ovatum* has not been studied for any bioactivity. *P. campechiana* has exhibited antimitotic activity against HeLa cells (7). Iridoid glycosides were isolated from *P. odorata* (8). Cytotoxic triterpenes were identified from the seeds of *C. religiosa* (9)

MATERIALS AND METHODS

Chemicals and reagents

Methanol (MeOH), hexane, and ethyl acetate (EtOAc) used for extraction and fractionation were technical grade purchased from Malcor Chemicals (Sampaloc, Manila), and were single-distilled before use. Dimethylsulfoxide (DMSO) was purchased from Merck (Hohenbrunn, Germany). Tetracycline was obtained from Sigma Chemicals (St. Louis, MO). Fetal Bovine Serum (FBS) was acquired from GIBCO (Grand Island, NY). May-Grunwald and Giemsa Stain were purchased from Himedia Laboratory (Mumbai, India). Silica gel 60 G purchased from Merck (Hohenbrunn, Germany) was used in the Normal Phase Liquid Chromatography (NP-VLC) of the sample extract.

Plant material

P. odorata Blanco, *P. campechiana* Baehni, *C. religiosa* Forst, and *C. ovatum* Engl. leaves were all collected in UP Diliman campus and submitted to the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines, Diliman.

Plant extraction and fractionation

MeOH extract of each plant was prepared from dried leaves, filtered and concentrated *in vacuo* at 40°C using a rotary evaporator (Heidolph). MeOH extract of plant with the highest bioactivity was partitioned between hexane and deionized water (2:1). The alcoholic portion was further extracted with EtOAc (2:1). The hexane and EtOAc extracts were concentrated *in vacuo*.

NP-VLC was done on the portion with the highest activity using hexane, 10% gradient ratios of EtOAc in hexane, and MeOH. Column fractions were pooled based on their Thin Layer Chromatography (TLC) profile resulting into 8 fractions. The most active hexane fraction was further subjected to NP-VLC using gradient elution of EtOAc in hexane.

Antimutagenicity bioassay

ICR mice, 15–25 grams and 4–5 weeks old, were purchased from the Bureau of Food and Drugs (BFAD, Alabang, Muntinlupa, Philippines), and acclimatized for one week prior to the assay. All the animals used in the experiments were treated in accordance with the rules and regulations of Republic Act 8485, Philippine Animal Welfare Act of 1998. The protocol followed for the micronucleus test was reviewed and approved by the College of Science Animal Care and Use Committee (CSACUC) of the College of Science, University of the Philippines, Diliman, Quezon City.

Micronucleus Test. Five mice were used for each test sample, and also for the positive, solvent, and spontaneous controls. The required weights of plant extracts were dissolved in 20% DMSO with a dosage of 5.0 mg/20 g mouse. Tetracyline (TET) was dissolved in an appropriate volume of distilled water to give a dosage of 55 mg TET per kg mouse. TET was administered by intraperitoneal injection and the test samples, orally by gavage. Two administrations of the test samples were made, 30 hours and 6 hours before the mice were sacrificed by cervical dislocation. The experimental procedures of the micronucleus test were essentially based on those described by Schmid (4). Briefly, both femurs were removed from each treated mouse and the proximal ends were cut to expose the bone marrow. The bone marrow was aspirated with fetal bovine serum into a centrifuge tube. The cells were collected by centrifugation at 1000 rpm for 5 minutes, and the slides were prepared. The slides were prepared by smearing the cells on a glass slide and stained with undiluted May-Grunwald stain for 3 minutes, 2 minutes in 50% May-Grunwald stain solution diluted with distilled water, and 10 minutes in 15% aqueous Giemsa stain solution. Three slides per mouse were prepared and coded before microscopical analysis.

The slides were scored for the number of micronucleated polychromatic erythrocytes (MN-PCE) per 1000 polychromatic erythrocytes (PCE). The anti-mutagenic potential of the plant extracts were determined by computing the % decrease in MN-PCE using the following equation:

$$\frac{no.ofMN-PCE(TET+20\%DMSO)-no.ofMN-PCE(TET+testsample)}{no.ofMN-PCE(TET+20\%DMSO)}x100\%$$

Data gathered were assessed statistically using the one-way analysis of variance (ANOVA) with post-hoc test. *P* values less than 0.05 were considered to indicate statistical significance.

Phytochemical analysis

Phytochemical screening of the crude methanol extracts of *C. ovatum*, *C. religiosa*, *P. campechiana* and *P. odorata* was

based on several procedures with slight modification (10–13)

Test for tannins: About 2 mg of sample was dissolved in 5 mL distilled water followed by dropwise addition of 15% $FeCl_3$ solution. Formation of a blue-black precipitate indicates the presence of hydrolysable tannins while brownish-green precipitate indicates condensed tannins.

Test for saponins: Approximately 5 mg of sample was boiled in 5 mL distilled water, allowed to cool, and was shaken vigorously to observe for frothing which suggests the presence of saponins. This was further verified by layering the solution with corn oil followed by vigorous shaking, then observing for the presence of emulsion at the froth-water interface.

Test for terpenoids: A chromatographic test utilizing Silica Gel 60 F_{254} was done to test for the presence of terpenoids. A TLC chromatogram of the samples was developed in $CHCl_3$, and sprayed with vanillin-H_2SO_4 solution. The formation of red to purple spots upon heating of the TLC plate signifies a positive result for terpenoids. The result of this test was confirmed by the Salkowski test. This was performed by treating 2mg of sample with 2 mL $CHCl_3$, followed by layering with H_2SO_4, and then observing for the formation of a reddish brown coloration of the interface.

Test for flavonoids: About 2 mg of sample was dissolved in 2 mL 1M NaOH followed by addition of a few drops of 0.6 M HCl. A yellow to orange solution with NaOH that turns colorless upon addition of HCl denotes the presence of flavonoids. A chromatographic test was also conducted to confirm the presence of flavonoids. Samples were dissolved in 70% EtOH, and spotted on a Silica Gel 60 F_{254} TLC plate. The chromatogram was developed in Forestal solution (30:3:10 glacial acetic acid: concentrated HCl: water), dried, viewed under UV light, and then fumed with NH_4OH. Observed spots in the resulting chromatogram were noted for fluorescence or change in color.

Test for Cardiac Glycoside (Killiani-Keller test): One drop of 15% $FeCl_3$ was added to 2 mg of sample dissolved in 2 mL distilled water. This was followed by layering of the solution with 1 mL concentrated H_2SO_4. Formation of a brown ring at the interface indicates the presence of cardiac glycosides.

Test for Phenolic compounds: Two mg of sample dissolved in 2 mL distilled water was added with few drops of 1% $FeCl_3$. Formation of a green, purple, blue, or black solution implies the presence of phenolic compounds.

Test for steroids: Two mL of diluted H_2SO_4 was added to 2 mg of sample dissolved in 2 mL acetic anhydride. Formation of a blue or green solution is indicative of the presence of steroids.

Test for Alkaloids: Five mg of sample was dissolved in 2 mL distilled water and added with 3 drops of Wagner's reagent (2 g iodine and 6 g potassium iodide in 100 mL water). Formation of a blue black precipitate confirms the presence of alkaloids.

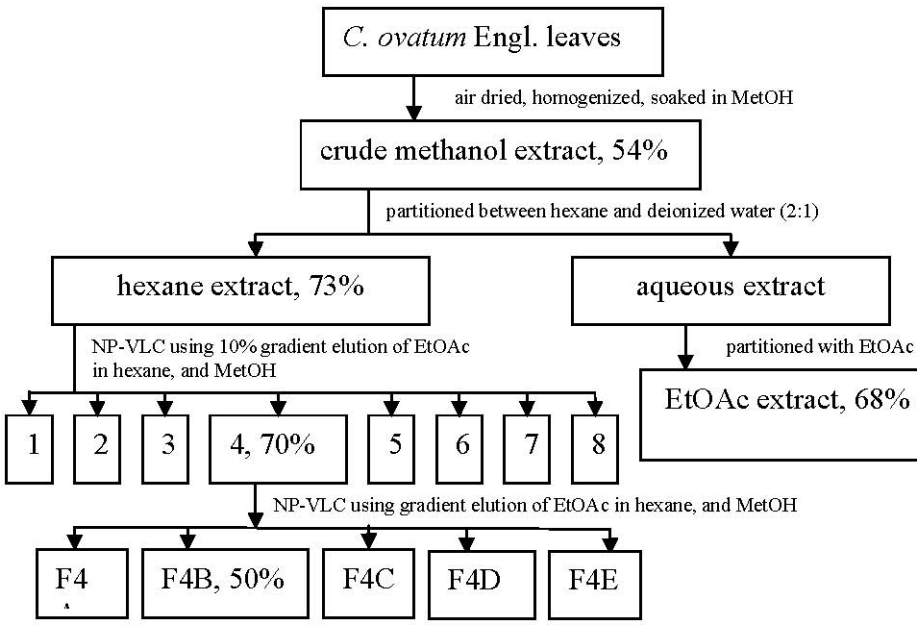

Figure 1: MT-directed isolation scheme for *C. ovatum*.

RESULT AND DISCUSSION

Four Philippine plants that are being used in folkloric medicine and as food were evaluated for their antimutagenic activities. A portion of this work has been presented at the 24th Philippine Chemistry Congress and the Asia-Pacific Conference on Chemistry Education. The phytochemical profiles of these plants were also obtained. Table 1 shows the preliminary screening result using MT on the crude methanol extracts and has revealed the most promising bioactivity for *C. ovatum*. At a concentration of 5 mg per 20 g mouse of *C. ovatum*, the number of MN-PCE induced by TET at a concentration of 55 mg per kg mouse, was lowered by 54.41%. The crude methanol extract of *C. ovatum* was then subjected to a bioassay-guided directed scheme and this is diagrammed in Figure 1.

Bioassay on hexane and EtOAc extracts of *C. ovatum* indicated that both extracts are active in MT at a concentration of 5 mg per 20 g mouse. The hexane extract lowered the number of MN-PCE induced by TET which was administered at a concentration of 55 mg per kg mouse, by 73.13%. EtOAC on the other hand reduced it by 68.66% using the same dosage of plant extract and TET (Table 2). These extracts are considerably more active than the methanol extract. The hexane extract was further purified and the resulting MT on fractions are summarized in Table 3. Fraction 4 has shown the highest significant activity by a reduction of 70.26% of MN-PCE induced by TET using the same concentration. Further fractionation followed by bioactivity testing consequently identified F4B to be the most active at 50% antimutagenicity (Table 4). The decrease in the bioactivity

Table 3: Antimutagenicity of hexane fractions of *C. ovatum Engl* in MT.

Test compound	No. of MN-PCE expressed as mean ± S.E.M (n=15)	% antimutagenicity
TET + 20% DMSO	4.27 ± 0.330	–
TET + F1	1.60 ± 0.190*	62.53
TET + F2	1.83 ± 0.271*	57.14
TET + F3	1.73 ± 0.248*	59.48
TET + F4	1.27 ± 0.345*	70.26
TET + F5	1.47 ± 0.291*	65.57
TET + F6	1.67 ± 0.333*	60.89
TET + F7	1.47 ± 0.256*	65.57
TET + F8	1.86 ± 0.294*	56.44
spontaneous	1.00 ± 0.239	–
TET	4.07 ± 0.330	–

*bioactivity is significant at $p<0.05$

Table 2: Antimutagenicity of hexane and EtOAc fractions of *C. ovatum Engl* in MT.

Test compound	No. of MN-PCE expressed as mean ± S.E.M (n=15)	% Antimutagenicity
20% DMSO + TET	4.47 ± 0.50	–
C. ovatum Engl. HEX + TET	1.20 ± 0.24*	73.13
C. ovatum Engl. EtOAc + TET	1.40 ± 0.32*	68.66
TET	4.80 ± 0.45	–
spontaneous	0.67 ± 0.29	–

*bioactivity is significant at $p<0.05$

Table 1: Antimutagenicity of crude methanol extracts in MT.

Test compound	No. of MN-PCE expressed as mean ± S.E.M (n=15)	% Antimutagenicity
20% DMSO + TET	4.53 ± 0.31	–
C. ovatum Engl. + TET	2.07 ± 0.278*	54.41
P. campechiana Baehni + TET	2.73 ± 0.18*	39.70
C. Religiosa Forst + TET	2.53 ± 0.19*	44.11
P. odorata Blco. "alagaw" + TET	2.71 ± 0.24*	40.12
TET	7.07 ± 0.36	–
spontaneous	0.20 ± 0.11	–

*bioactivity is significant at $p<0.05$

Table 4: Antimutagenicity of hexane sub-fractions of *C. ovatum Engl* in MT.

Test compound	No. of MN-PCE expressed as mean ± S.E.M (n=15)	% Antimutagenicity
TET + 20% DMSO	4.00 ± 0.37	–
TET	3.20 ± 0.37	–
TET + F4A	2.25 ± 0.22*	43.75
TET + F4B	2.00 ± 0.28*	50
TET + F4C	2.47 ± 0.27*	38.33
TET + F4D	2.07 ± 0.27*	48.33
TET + F4E	2.53 ± 0.17*	36.67
normal	1.93 ± 0.23	–

*bioactivity is significant at $p<0.05$

Table 5: Phytochemical screening results of the tested plants

Plant	Tannin	Saponin	Terpenoid	Flavonoid	Cardiac Glycoside	Phenolic Compounds	Steroid	Alkaloid
C. ovatum	+ bg ppt	+	+	+	+	+	–	–
C. religiosa	–	+	+	–	+	–	+	–
P. campechiana	+ bg ppt	+	–	+	+	+	+	–
P. odorata	–	+	+	+	+	+	+	–

of the fractions could mean that components of Fraction 4 are showing synergistic effect on their antimutagenicity and warrants further investigation.

Phytochemical screening of the various plant extracts as shown in Table 5 revealed that the plants contain several secondary metabolites. The crude methanol extracts from the four different plants were all negative for the presence of alkaloids. *C. ovatum* was positive for the presence of condensed tannins, saponins, terpenoids, flavonoids, cardiac glycosides and phenolic compounds. Many studies have shown that compounds belonging to these classes exhibit antimutagenic activities. For example, various saponins isolated from different plant sources have shown antimutagenic activities (14–16). Some terpenes have likewise exhibited considerable antimutagenicity using bacterial reverse mutation assay (17). Flavonoids from *Aspalathus linearis* (18), *Rhus verniciflua* (19), *Spinacia olaracea* (20), *Chrysanthemum morifolium* (21) and *Pogostemon cablin* (22) have been previously studied. The antimutagenicity of phenolic compounds from maple products (23) and *Phaseolus vulgaris* against aflatoxin have been established (24). Antimutagenic steroids from the thorns of *Gleditsia sinensis* have also been identified (25). Therefore, it is most likely that compounds belonging to these classes are responsible for the observed bioactivity.

CONCLUSION

This study has established the antimutagenic activity of *C. ovatum, C. religiosa, P. campechiana* and *P. odorata*. This research has identified the most active sub-fraction, F4B, in the hexane portion of *C. ovatum* via MT-guided isolation. This indicates that F4B might be valuable in cancer chemoprevention. Further isolation and purification of this sub-fraction is now in progress to determine the compound/s responsible for the observed bioactivity. Further purification on the ethyl acetate portion of *C. ovatum* is currently being done. These plants may be potential sources of lead chemopreventive agents.

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