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Phytochemistry, Genotoxicity and Anti- Genotoxicity Screening of *Calamus spinifolius, Becc.* Ethanol Leaf Extract

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Abstract

The study evaluated the bio-active components of ethanol leaf extract of *Calamus spinifolius* Becc., an endemic rattan plant species of Pampanga, Philippines and its genotoxic and anti-genotoxic potentials using the Laboratory Test tube Method and Thin Layer Chromatography and Micronucleus Test for genotoxicity and anti-genotoxicity studies, respectively. The study revealed that ethanol leaf extract of *C. spinifolius* had primary alkaloids, 2-deoxysugars (steroids), anthraquinone, leucoanthocyanin (flavonoids), saponin and condensed tannins. Micronucleus Test showed that the ethanol leaf extract did not produce considerable number of micronucleated polychromatic erythrocytes (MPCEs) on the bone marrow cells of *Mus musculus* to be considered genotoxic; however, it was able to reduce the number of MPCEs when given in combination with tetracycline, hence, anti-genotoxic. Data were statistically validated using the Analysis of Variance and the Tukey's Multiple Range Test. Result of the study can help different agencies in the discovery of potential medicinal values of different plants, particularly the endemic plants in the Philippines.

Keywords: Anti-genotoxicity; Genotoxicity; Micronucleated; Polychromatic Erythrocytes; Phytochemistry.

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1. Introduction

Traditionally, when we look at the forest we only see the timber and the completed revenue it can be significantly add to the country's coffers. We do not give much attention to the other possible commercially valuable components of the forest especially the so-called non-wood or minor forest products, which abound in the forest with some species that can be literally yield foreign exchange from the world market. These non-wood forest products are minimally utilized. Their importance are overshadowed by the forest's primary products. These non-wood forest products were ignored, destroyed and left to rot during logging operations or gathered only for local or domestic consumption. However, these plants are also the most prolific laboratories in the world. They produce a great number of compounds of various chemical structures. Of these are the secondary metabolites, often unique to a particular species, which include the alkaloids, the steroids, the flavonoids and others [1]. They provide an abundant supply of biological materials that can be screened for medicinal activities.

Among the non-wood species, rattan of the family Palmae ranks foremost to timber and this distinction has put a severe stress on the natural rattan stands. Natural rattan stands are only known as main sources of the new materials for the furniture industry thus, to the scientists and researchers, rattan opened doors to other wide variety of research areas - from production, utilization and to conservation. Another promising area to be considered is on the determination of its bioactive components and anti-genotoxic effects. Of the species of rattan, *Calamus spinifolius* Becc . is one species yet to be explored. It belongs to the 32 of the 45 Philippine species which are endemic in Luzon particularly in Mt. Arayat, and Zambales. It is also found in Panay and Maguindanao and in Southeast Asian tropical rain forests [2].

Figure 1: The experimental plant: *Calamus spinifolius* Becc.

So far, no studies have been undertaken with regards to the phytochemical and medicinal potentials of this plant species, thus, the purpose of this study is to discover its biological components, genotoxic and antigenotoxic potentials, with an end goal of providing cheaper nature-based alternative medicine produced by pharmaceutical companies. The study, however, will not be isolating the identified bio-active components. The study will only

determine the genotoxic and antigenotoxic potentials of the ethanol leaf extract of *Calamus spinifolius*.

Genotoxicity is defined as a destructive effect on a cell's genetic material affecting its integrity [3]. It refers to interaction between various agents and DNA or the cellular machinery that regulates fidelity of the genome [4]. A substance that has the property of genotoxicity is known as mutagen/genotoxin. It can alter the structure of DNA of a living cell. Genotoxicity to somatic cells , when not repaired, can induce cancer in the presence of promoters while genotoxicity to germ cells can lead to genetic disorders that can be transmitted from generation to the next [5]. Thus, it is imperative to determine ways and means to reduce genotoxic effects of proven genotoxins/mutagens.

Genotoxicity testing is an important part of the hazard assessment of any chemicals or substances for regulatory purposes [6]. In vivo genotoxicity assay provides detailed information of biological and physiological significance [7]. One method that has been developed for genotoxicity /mutagenicity detection testing of chemicals that induce the formation of a small membrane bound DNA fragments in cells, known as micronucleus is the Micronucleus Test developed by Schmid.

However, there are antigenotoxins that can reduce the frequency of alterations of DNA. They may act by preventing the alkylation of DNA, preventing intercalations of DNA, inhibiting error-free repair mechanisms [8].

2. Materials and Methods

2.1. Plant Collection

The experimental plant was collected in the northwestern part of Mt. Arayat, Pampanga, Philippines. The collected leaves of the plant were washed thoroughly with distilled water to remove impurities.

2.2. Sample Preparation

For ethanol leaf extract, one kilo of air-dried leaves of *C. spinifolius* were chopped and ground into fine pieces using a blender and placed in colored bottles with 500 ml of 80% ethyl alcohol to submerge the material. The leaves were soaked for 48 hours, filtered through a clean and fine cloth and extract was concentrated with a rotary evaporator.

The extract was stored in tightly stoppered containers in the refrigerator at about 0-5°C until used for the phytochemistry, genotoxicity and anti- genotoxicity screening.

2.3. Phytochemical Screening

The plant extract was subjected to series of phytochemical screenings: the test for alkaloids, test for steroids, test for anthraquinone, test for flavonoids, test for saponin, and test for tannins using the Test-tube Method and Thin Layer Chromatography procedures.

2.4. Genotoxicity and Anti-Genotoxicity Screening

The Micronucleus Test/ was utilized for the genotoxicity and anti- genotoxicity screening of the plant extract. It is a cytogenetic analysis for the breakage of bone marrow chromosomes and malfunction of the spindle apparatus [9].

The following treatments were used with three replications, each with one mice per replicate. Three slides were prepared for each mice, with one thousand cells per slide were counted and scored for the presence of micronuclei, a basis in determining the genotoxicity and anti- genotoxicity potentials of the leaf extract.

To- Negative control (Distilled water)

T1- Ethanol leaf extract of *C. spinifoloius*

T2- Ethanol leaf extract of *C. spinifoloius* + Tetracycline

T+- Positive Control (Tetracycline)

Two doses, each with 0.5 ml per 20 g body weight of mice of the *C. spinifolius* leaf extract, including the positive and negative control were introduced to the mice through the gavage method. For anti-genotoxocity test, 0.5 ml of tetracycline per 20 g body weight was introduced first, immediately followed by the leaf extract. The first treatment was administered twenty-four (24) hours before the second treatment. Six hours after the last treatment, the mice was sacrificed and excised through cervical dislocation.

Immediately after sacrificing the mice, both femora were removed by cutting through the pelvis and tibia. The bone was freed from the muscle with the use of the gauze and fingers. The proximal end of the femora was carefully shortened with scissors until a small opening in the bone marrow become visible.

Then, 0.2 ml fetal calf serum was sucked into a 1 mL syringe with a needle, which was inserted into the proximal part of the bone. Fetal calf serum helped preserved the perfect morphology of erythrocytes as well as the nucleated cells. Femur was submerged in the serum and was squeezed against the wall to prevent the slipping of the needle. By gentle flushing, the marrow was forced out through the opening around the needle. Suspension was centrifuged at 1000 rev/min for five minutes to sediment the cells and supernatant was removed. Cells in the sediment were gently mixed with a Pasteur pipette. A small drop of the viscous suspension was placed at one end of a microscope slide and was smeared according to the conventional hematological method. Three slides per mouse were prepared and were air dried for at least 24 hours.

Staining of slides was done a day after the preparation of the slides to have good results. Slides were stained with undiluted May-Gruenwald solution for three minutes, then transferred in a diluted 50% May-Gruenwald solution for two minutes. Slides were washed with distilled water before and after they were stained for 10 minutes with 15 % Aqueous Giemsa stain solution and blotted dry with a tissue paper before drying in the air. Slides were screened at a medium magnification for a region of a suitable technical quality. The stain of mature

erythrocytes must be red in color and strong bluish tint in the young polychromatic erythrocytes. Using a hand tally counter, one thousand cells per slide were counted giving a total of three thousand cells per mice. Polychromatic cells inhibiting micronucleus was scored. The number of Micronucleus Polychromatic Erythrocytes (MPCEs) in the three slides per mouse were averaged and resulting figures were recorded as the score of the animal.

As a rule, to be considered genotoxic, the number of MPCEs, produced by the test sample must be twice or more than twice the negative control (distilled water). To be considered anti- genotoxic, the number of MPCEs produced by the test sample in combination with tetracycline must be lowered when compared with tetracycline alone

2.5. Statistical Treatment

Data were analyzed using the Analysis of Variance (ANOVA) and Tukey's Multiple Range Test.

3. Results and Discussion

3.1 Bio-active components of the ethanol leaf extract of Calamus spinifolius, Becc

Calamus spinifolius ethanol leaf extract contained the following bio-active components: alkaloids, steroid, anthraquinone, flavonoid, saponin and tannin (Table 1).

Table 1: Bio-active components of the ethanol leaf extract of *Calamus spinifolius*, Becc.

Bio-active components	Remarks
Alkaloids	Primary alkaloids
Steroid	2-deoxysugars
Anthraquinone	present
Flavonoids	Leucoanthocyanin
Saponin	present
Tannin	Condensed tannin

Alkaloids are usually derivatives of amino acids, and many have a bitter taste. They are found as secondary metabolites in plants, animals, and fungi. Various alkaloids have pharmacological effects on humans and animals. Many are poisonous, but some are used medicinally as analgesics (pain relievers) or anesthetics, particularly morphine and codeine. Some, such as vinblastine, are used to treat certain types of cancer [10]. Alkaloids have strong anti-bacterial and anti-cancer biological activities and they are widely used as a compound of drug and herbal therapy formulation [11].

Aside from alkaloids, it also contained steroids, in the form of 2-deoxysugars. Steroids are a large group of

naturally occurring and synthetic lipids, or fat-soluble chemicals, with a great diversity of physiological activity which include the development and control of the reproductive tract in humans as well as contributed to a wide range of therapeutic application. Some of its therapeutic uses are cardiotonics (digitoxine), vitamin D precursors (orgeosterols), and anabolic agents (androgens). Deoxy-sugars are monosaccharide derivatives formed by the deoxidation of a hydroxyl group in an aldose or ketose. Often one or more of the carbons of a carbohydrate will lack an oxygen subsitituent [12].

Further, it also contained anthraquinone, which is a chemical in making dyes. It is a yellow crystalline chemical that is used in the manufacture of dyes having the formula of $C_{14}H_8O_2$ [13].

Flavonoids in the form of leucoanthocyanin which are phenolic plant pigments generally containing the γ-benzopyrone nucleus were also noted. Leucoanthocyanins occupy an important position among the water soluble organic compounds present in the tissues of plant. They have been implicated as being responsible for the astringent taste of unripe fruits [14]. Most flavonoids have anti-inflammatory properties, anti-viral, anti-fungal, cytotoxic activities and they inhibit platelet function through binding to the thromboxane A2 receptor [15].

Saponin is also present in ethanol leaf extract of *C. spinifolius*. Saponin is a group of naturally occurring oily glycosides that foam freely when shaken with water and they occur in a wide variety of plants. Plant saponins have interesting biological activities such as spermicidal and molluscicidal activities [16]. It has pharmacological relationship with cortisone, an anti-inflammatory agent used in the synthesis of hormone, vitamin D and cardiac glycosides. Saponin also acts as expectorant through the stimulation of a reflex of the upper digestive tract [17].

Finally, tannins are also present in the leaves of *C. spinifolius*, particularly condensed tannins. Tannin is any of a group of pale-yellow to light-brown amorphous substances in the form of powder, flakes, or a spongy mass, widely distributed in plants and used chiefly in tanning leather, dyeing fabric, making ink, and in various medical applications. Tannin solutions are acid and have an astringent taste. Tannin is responsible for the astringency, color, and some of the flavor in tea. Tannins occur normally in the roots, wood, bark, leaves, and fruit of many plants [18]. Recent reports show that tannins may have potential value as cytotoxic and/or antineoplastic agents. Condensed tannins are polymers of phenolic compounds related to flavonoid pigments, frequent constituents of woody plants. Studies showed that condensed tannin when administered by subcutaneous injection in rats or mice produced both local sarcomas and liver tumors [19].

3.2. Chromosome breaking effects of the ethanol leaf extract of C. spinofolius, Becc. using the Micronucleus Test

Micronucleus test was used in the study for assessing chromosome damage because it enables both chromosome loss and chromosome breakage to be measured reliably. The assay detects damage to the chromosome or the mitotic apparatus of immature red blood cells found in the bone marrow. During cell division, undamaged chromosomes give rise to normal daughter nuclei, if chromosomes are broken or the

mitotic apparatus of the cell is damaged, chromosomes or chromosome fragments may be incorporated in a second nucleus, instead of the main nuclei called micronucleus [20].

Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division, appearing in the cytoplasm of interphase cells as small additional nuclei. The detection of micronuelei provides a readily measurable index of chromosome breakage and loss [21]. Decrease in the percentage of polychromatic erythrocytes (PCEs) and increase in percentage of micronucleated polychromatic erythrocytes (MPCEs) are indicators of the chromosomal aberrations and these damages implicate as the major cause for the appearance of nuclei at the last stage of mitosis and as an indicator of genotoxic insult to the nuclei [22].

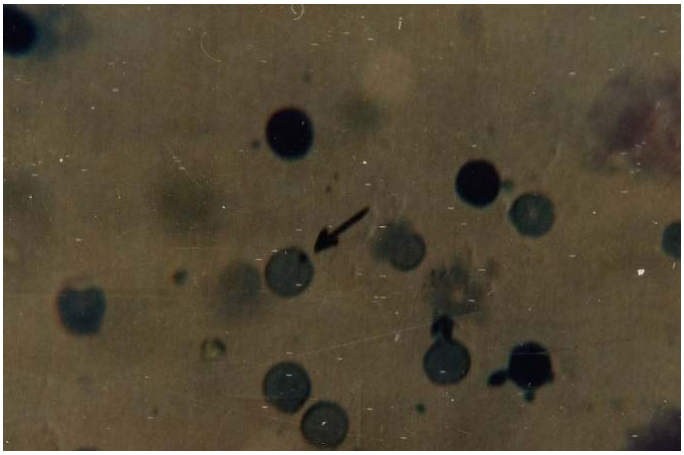

Figure 1: Micronucleated polychromatic erythrocytes (MPCEs) in the

bone marrow cells of *Mus musculus* as shown by the arrow

To be considered genotoxic, the MPCEs (Figure 1) produced by the test samples must be twice or more than twice the negative control. To be considered anti-genotoxic, the MPCEs produced by the test samples in combination with a proven mutagen must be lower when compared with the mutagen alone [23].

Statistical analysis revealed that results of the study were significant at 5% level. Analysis of the means revealed no significant difference in the mean number of MPCEs in the bone marrow cells of *Mus musculus* produced by the ethanol leaf extract of *C. spinifolius* (T1) with the negative control (T0-distilled water). Tetracycline (T+), however, showed significant difference when compared with T0 and T1. It was also found out that tetracycline's value was twice or more than twice the negative control (Table 2). The result showing no significant difference on the MPCES produced between the ethanol leaf extract of *C. spinifolius* and the negative control (distilled water) implies that no chromosome breaking effects were exhibited by the ethanol leaf extract of *C. spinifolius.*

Even if it had produced considerable number of MPCEs, the value obtained is below the standard that is twice or more than twice the negative control. This shows that it did not alter the chromosome of the bone marrow cells of *M. musculus*, hence, non-genotoxic. Breakage of chromosomes will induce formation of appreciable amounts of micronucleated polychromatic erythrocytes.

Table 2: Chromosome breaking effects of the ethanol leaf extract of *C. spinifolius*, Becc. using the Micronucleus Test

	No. of Micronucleated Polychromatic Erythrocytes per thousand bone marrow cells				
Treatments	Replications			Total	Mean
	I	II	III		
To (distilled water)	1.79	1.47	1.33	4.59	1.53[b]
T1 (ethanol leaf extract of *C. spinifolius*)	1.78	1.68	1.87	5.33	1.78[b]
T+ (tetracycline)	5.67	6.00	6.33	18.00	6.00[a]

Legend: Means of the same letter are not significantly different at 5% level

Genotoxin, such as tetracycline on the other hand, induced appreciable formation of MPCEs. Tetracyline is a group of antibiotic drugs which shares common basic activity. Sylianco has proven that tetracycline can induce formation of micronuclei in mammalian somatic cells. It acts by blocking the attachment of amino acyl transfer RNA to RNA complex, thereby interfering with protein synthesis and by alkylating the bases of DNA stabilized by hydrogen bonding. Both intercalation and hydrogen bonding can destabilize DNA helix leading to fragmentation. Fragmentation results in the formation of MPCEs in the bone marrow cells of *M. musculus*, indicating the chromosome breaking capacity of tetracycline [24].

3.3 Antigenotoxic activity of the ethanol leaf extract of C. spinifolius against Tetracycline

As shown in Table 2, the chromosome-breaking effect of tetracycline was reduced by the ethanol leaf extract of *C. spinifolius*. Statistical analysis revealed that results were significant at 5% level. Tukey's Multiple Range Test revealed no significant difference in the mean number of MPCEs produced by T2 (ethanol leaf extract of *C. spinifolius* co-administered with tetracycline) and with that of the positive control, T+ (tetracycline).

Results showed that the combination of tetracycline and the ethanol leaf extracts of the plant sample produced a lesser number of MPCEs when compared to tetracycline alone. The reduction is significant as shown by the statistical evaluation using ANOVA. The reduction of formation of micronucleated polychromatic erythrocytes by the ethanol leaf extract of *C. spinofolius* indicates that the fragmentation of the chromatin material by tetracycline is reduced. Since tetracycline is metabolized to alkylating agent of DNA, there is a possibility that the ethanol leaf extract of *C. spinifolius* inhibits its metabolism to species reactive with DNA, thus, reducing the chromosome-breaking effects of tetracycline, hence, anti-genotoxic. This could be attributed to some constituents of the leaf extract from *C. spinifolius* such as flavonoids, steroids, and saponin. Flavonoid is an anti-oxidant, saponin is a good source of vitamins, which are already proven anti-mutagenic and steroids are precursors of vitamin D [25]. In addition, fibers, which are present in the family Palmae, may also trap the tetracycline. Protein fibers crisscross the marrow, forming a meshwork that supports the developing blood cells clustered in the space between the fibers. It interspersed with the developing blood cells preventing the

formation of MPCEs [26]. It is also possible that substances of undetermined structures are responsible for the reduction of genotoxic effects.

Table 3: Antigenotoxic activity of the ethanol leaf extract of *C. spinifolius*, Becc. Against Tetracycline

	No. of Micronucleated Polychromatic Erythrocytes per thousand bone marrow cells				
Treatments	Replications			Total	Mean
	I	II	III		
To (distilled water)	1.79	1.47	1.33	4.59	1.53[c]
T1 (ethanol leaf extract of *C. spinifolius*)	1.78	1.68	1.87	5.33	1.78[c]
T2 (ethanol leaf extract of C. spinifolius + tetracycline	3.14	3.08	2.97	9.19	3.06[b]
T+ (tetracycline)	5.67	6.00	6.33	18.00	6.00[a]

Legend: Means of the same letter are not significantly different at 5% level

4. Conclusion

Ethanol leaf extract of *C. spinifolius* has been found to contain the following: primary alkaloids, 2-deoxysugars (steroids), anthraquinone, leucoanthocyanin (flavonoids), saponin and condensed tannins. It did not produce considerable number of micronucleated polychromatic erythrocytes (MPCEs) on the bone marrow cells of *Mus musculus* to be considered genotoxic, hence non-genotoxic; however, it was able to reduce the number of MPCEs when given in combination with tetracycline, hence, anti-genotoxic.

5. Recommendation

Isolation and characterization of the bio-active components of the ethanol leaf of *Calamus spinifolius* is recommended and identify their specific actions.

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