GENOTOXIC, ANTIGENOTOXIC AND PROTECTIVE EVALUATION OF *TINOSPORA CORDIFOLIA* STEM BY PERIPHERAL BLOOD MICRONUCLEUS ASSAY

Kevin Ace Pañares[1], Fema Abamo[1*], and Merell Billacura[2]
[1]Biology Department, College of Natural Sciences and Mathematics (CNSM), Mindanao State University (MSU), Main Campus, Marawi City 9700, Philippines
[2]Chemistry Department, CNSM, MSU, Main Campus Marawi City, Philippines
[*]For correspondence; Tel. + (63)9177015099, E-mail: yadfem@yahoo.com

ABSTRACT: *The search for new drugs from ethnobotanicals is growing tremendously in the Philippines. In traditional medicine, Tinospora cordifolia popularly known as Guduchi, is a famous climbing shrub for curing variety of diseases from common colds, allergies and infections to complicated jaundice and diabetes. However, no study has been carried out to evaluate the genotoxic, antigenotoxic and protective properties of the methanol extract of the plant's stem against the erythrocytes of albino mice. This study therefore aimed to evaluate the short term genotoxicity testing of T. cordifolia stem's methanol extract against the mice erythrocytes using peripheral blood micronucleus assay and to assess the antigenotoxic and protective properties of the extract against methyl methanesulfonate (MMS)-induced genotoxicity in mice utilizing the same assay. Results revealed that the extract was not genotoxic to the erythrocytes of mice even at higher dose of 500 ppm. Significantly the extract demonstrated both the protective and the antigenotoxic properties against MMS-induced genotoxicity in mice. The protective potential of the extract worked in dose-dependent manner but not its antigenotoxic property, i.e. lower dose of 250 ppm did not differ significantly from 500 ppm in reducing the micronuclei count induced by MMS. Further studies should be conducted to exploit the potential of T. cordifolia as alternative source of natural compounds to cure genotoxic-related problems.*

Keywords: Genotoxic and antigenotoxic tests, protective test, micronucleus assay, *Tinospora cordifolia*, methyl methanesulfonate

1. INTRODUCTION

The search for new cure is a relentless activity of man to survive diseases and abnormalities. Researches in ethnomedicinal plants as possible source of bioactive compounds with pharmaceutical values are growing enormously. *Tinospora cordifolia* popularly known as Guduchi but locally called as Panyawan, is one of the commonly used medicinal plants not only in Philippines but also in other countries such as India for curing various diseases including common colds, dental infections, rheumatism, jaundice, diabetes and hypertension [1]. This plant is also reported to possess remarkable medicinal properties against stress, allergies, arthritis, spasm, inflammation, malaria, leprosy, tumor including immunomodulatory, anti-periodic and hepatoprotective activities [2]. *T. cordifolia* is a climbing shrub that belongs to the family Menispermaceae**.** It is glabrous with aerial roots that are fleshy, long and filiform and with rather succulent stem. Its leaves are cordate and membranous with bark spiraling left appearing gray or creamy. Diverse classes of chemical compounds found in *T. cordifolia* include phenolics, alkaloids, steroids, diterpenoid lactones, sesquiterpenoid, glycosides, polysaccharides, and aliphatic compounds. Secondary metabolites were reported to include anthraquinones, terpenoids, and saponins [3].

Studies have been conducted on its medicinal efficacy on antimicrobial, antioxidant, and anticancer activities. For the plant stem extract, it has been characterized to contain alkaloids, carbohydrates, glycosides, protein, amino acids, fixed oils and fats, tannins, saponins, steroids, flavonoids, and phenols [4]. However, no scientific study has been carried out to evaluate the genotoxic, antigenotoxic and protective activities specifically on its stem's methanol extract. This extract maybe potentially useful but chemical mixtures might be harmful and genotoxic that may cause damage to the genetic material of the living cells leading to mutations causing serious problems to the host including cancer and birth defects, hence genotoxicity testing is necessary [5]. To assess genotoxic activities of natural compounds against living systems, peripheral blood micronucleus assay has been utilized [6,7]. For a decade now, micronuclei present in cells have been used as biomarker of genotoxity. These micronuclei appear as small satellite nucleus surrounding the cell nucleus; these are actually chromosomes or fragments of chromosomes which are left behind after cell division [8]. Moreover, micronuclei formation in mice erythrocytes can be induced by using a mutagen methyl methanesulfonate (MMS) [9]. This study was undertaken therefore to evaluate the genotoxicity of the methanol extract of *T. cordifolia* stem against the erythrocytes of albino mice by peripheral blood micronucleus assay. Using the same assay, the study further aimed to determine the antigenotoxic and protective potentials of the stem's extract against MMS-induced genotoxicity in mice.

2. MATERIALS AND METHODS

T. cordifolia (Figure 1) was identified with the aid of the book "A Flora of Manila" [10]. Fresh stems of the plants were cleaned, air-dried, crushed and ground. Exactly 250g powder stem were soaked up to 1000 mL of absolute methanol for 72 hours in a 1000 mL; the filtrate was obtained using #1 Whatman filter paper and the solvent methanol was removed using a rotary evaporator under 115 rpm at 40^{o}C. A dark brown residue obtained was dissolved in Dimethyl Sulfoxide (DMSO) and diluted with distilled water to prepare two concentrations at 250 ppm and 500 ppm.

A total of twenty-four (24) albino mice (*Mus musculus*), 7-12 weeks old of either sex, were used in the tests. The experimental design for the evaluation of the genotoxic, antigenotoxic and protective activities of the methanol extract of *T. cordifolia* stem is summarized in Table 1; it has eight treatments replicated thrice with one mouse per replicate. Negative control treatments were distilled water (T1) and DMSO (T2) while positive control (T3) was the mutagen MMS (Sigma Aldrich) at 50mg/kg mouse. For genotoxic evaluation, a higher dose of the extract at 500 ppm (T4) was administered to the mice for five days. For antigenotoxic group, the mice were pre-treated with MMS for two days prior to extract treatment for three days at two different dosages, 250 ppm (T5) and 500 ppm (T6). For the protective evaluation, two extract dosages also at 250 ppm (T7) and 500 ppm (T8) were administered for three days ahead of MMS treatment for two days. All treatments were given to mice by oral gavage once daily at 24-hour interval at 0.2mL/20g mouse body weight.

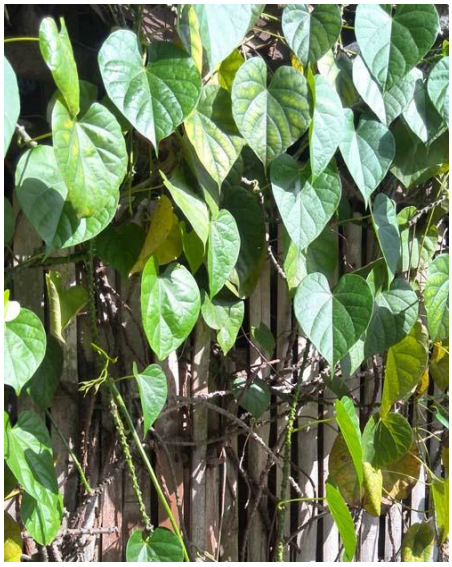

Figure (1) *Tinospora cordifolia*

Table (1) Treatment groups

Treatments	Administration/Duration
(T1) Treatment 1 (Negative control)	Distilled water for 5 days
(T2) Treatment 2 (DMSO)	DMSO for 5 days
(T3) Treatment 3 (Positive control)	Distilled water for 3 days + MMS for 2 days
(T4) Treatment 4 (Genotoxic test)	Extract at 500 ppm for 5 days
(T5) Treatment 5 (Antigenotoxic test)	MMS for 2 days + 250 ppm extract for 3 days
(T6) Treatment 6 (Antigenotoxic test)	MMS for 2 days + 500 ppm extract for 3 days
(T7) Treatment 7 (Protective test)	250 ppm Extract for 3 days + MMS for 2 days
(T8) Treatment 8 (Protective test)	500 ppm extract for 3 days + MMS for 2 days

The method outlined in the CGSMT [6,7] was followed for the peripheral blood micronucleus assay with some modifications. Briefly, for all the treatment groups, 30 hours after final treatment, the tails of mice were cut and blood was smeared into glass slide; the smeared blood were air-dried, fixed by dipping once in 95% methanol for two seconds, and air-dried again prior to staining with Acridine Orange (Sigma Aldrich) at 0.1g/100mL dilution by distilled water; and the stained blood samples were air-dried overnight and finally covered with cover slip ready for micronuclei scoring. Five blood smears (slides) were prepared per treatment replicate and 1000 erythrocytes per slide were scored blindly for micronuclei using a Ken-α-vision light microscope at 1000x magnification. The counted micronuclei from all treatments were statistically analyzed using Kruskal Wallis and Tamhane tests at 5% and 1% levels of significance.

3. RESULTS AND DISCUSSION

Micronuclei observed in the mice erythrocytes from all treatments were counted and the means were tabulated in Table 2. The graphic representation of the data is shown in Figure 2. Results showed that the mutagen MMS as the positive control (T3) had the highest incidence of micronuclei formation in the experiment while the genotoxic treatment (T4) at 500 ppm including the negative control treatments (T1 and T2) had the lowest micronuclei counts. Interestingly, the antimutagenic (T5 and T6) and protective (T7 and T8) tests revealed lower counts of micronuclei than the positive control. Statistical analyses of the mean variations are also given in Table 2. Kruskal Wallis test confirmed that the differences of micronuclei counts from each treatment were highly significant at 1%.

The positive control using MMS as mutagen was expected to yield higher counts of micronuclei as it was used to induce mutations. Tamhane test confirmed that T3 having the highest count was significantly higher than the rest of the treatments illustrating that MMS is a potent mutagen that effectively generated micronuclei in the mice erythrocytes (Figure 3). MMS, an alkylating agent, is known for its ability to interact with DNA *in vitro* and *in vivo* producing genotoxic damage. Figure 4 shows the metabolic activation of MMS and the formation of the alkylated DNA [11]. When DNA is alkylated for instance in guanine, apurinic sites could be created which could induce mispairing of nucleotide bases. This may eventually cause destabilization of the DNA structure which may lead to chromosome breakage leaving some DNA fragments behind during cell division hence generating micronuclei formation.

Furthermore, the data illustrated that the genotoxic test (T4) had the lowest micronuclei score but the count was not significantly different from the negative controls (T1 and T2) by Tamhane test (Table 2). Importantly, it was statistically lower than the positive control and even from the rest of the treatments by the same Tamhane test. This result implied that the stem extract even at higher dose of 500 ppm did not exhibit any significant genotoxic activity against the mice erythrocytes. The minimal counts of micronuclei observed in

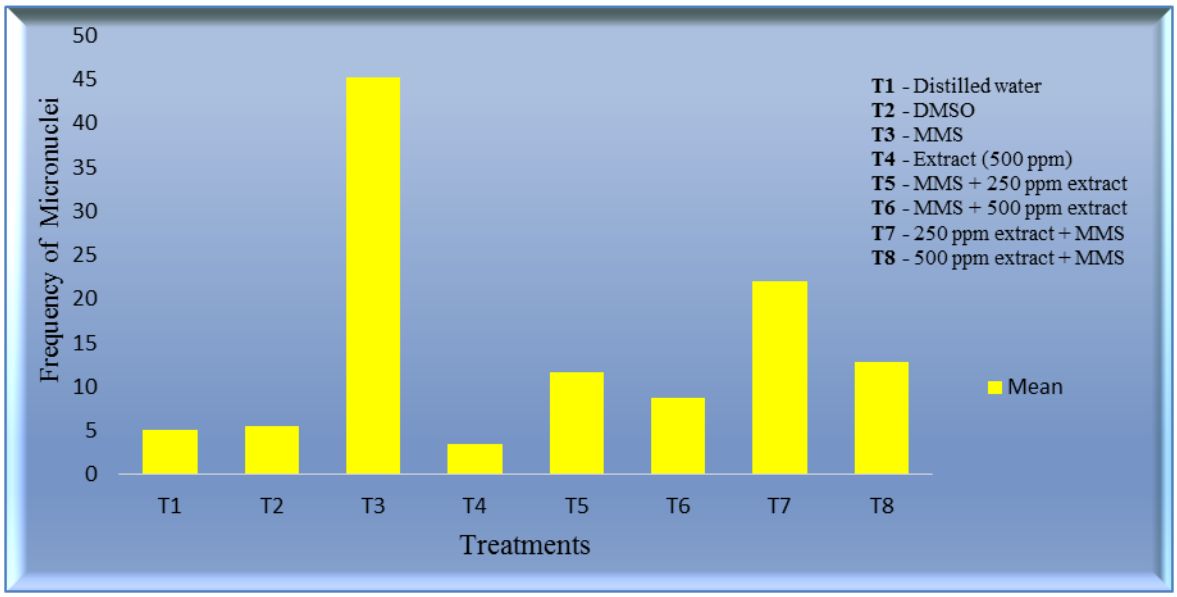

Figure (2) Frequency distribution of micronuclei from eight treatments

Table (2) Frequency distribution of micronuclei in all treatments and the statistical Kruskal Wallis and Tamhane tests

Treatments	R1*	R2*	R3*	Mean**	Kruskal Wallis Test
T1 – Distilled Water	5.4	4.6	5.4	5.13 A	
T2 – DMSO	5.6	5.2	5.8	5.53 A	
T3 – MMS	44.6	46	45	45.2 E	Chi-square = 22.513
T4 – Genotoxic test (Extract only at 500 ppm)	3.4	3.2	3.6	3.4 A	
T5 – Antigenotoxic test (MMS + 250 ppm extract)	11.2	11.8	11.8	11.6 B	P value = 0.002***
T6 – Antigenotoxic test (MMS + 500 ppm extract)	8	7.6	10.8	8.8 B	
T7 – Protective test (250 ppm extract + MMS)	19	23.2	23.8	22.0 D	
T8 – Protective test (500 ppm extract + MMS)	13.2	12.6	12.6	12.8 C	

*values of the replicates are means of the five blood smears
**Tamhane test: means having the same letters are not significantly different at $\alpha \leq 0.05$
***highly significance at p value ≤ 0.01

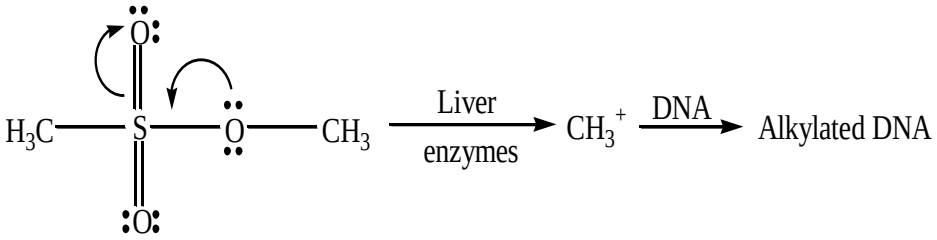

Figure (3) Metabolic activation of methyl methanesulfonate [11]

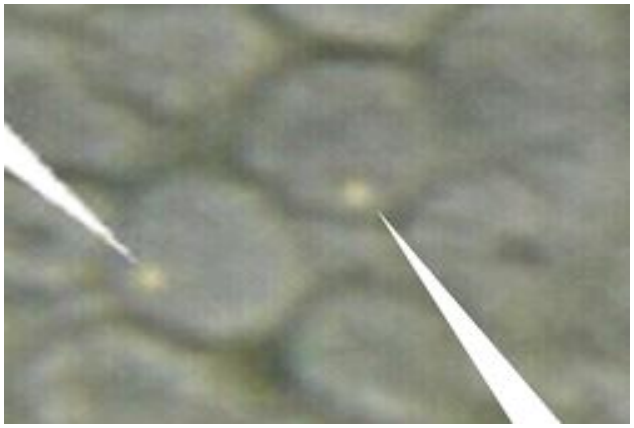

Figure (4) Photomicrograph of mice erythrocytes stained with acridine orange at 1000x showing MMS-induced micronuclei (white arrows)

the control treatments were probably due to some spontaneous chromosomal aberrations naturally happening in the cell [7].

The antigenotoxic evaluation of the extract was carried out by pre-treating the mice with MMS mutagen prior to extract treatment; should there be reduced counts of micronuclei after the extract administration, then the natural compounds in the plant extract must have repaired the DNA damage induced by MMS thus antigenotoxic [6]. Indeed T5 and T6 yielded remarkably lower frequencies of micronuclei (Figure 3), significantly lower than the positive control (Table 2). Somehow, the micronuclei generated by MMS in the mice erythrocytes were reduced, although Tamhane test confirmed that two different extract dosages were not significantly different in reducing micronuclei frequencies. Thus, 250 ppm methanol extract from the plant's stem would suffice in reducing the micronuclei counts induced by MMS. There are many mechanisms, by which natural compounds can bring about antigenotoxic, mutagenic effects, i.e. inhibiting tumor progression, modulating replication, DNA metabolism and repair, promoting apoptosis and detoxifying carcinogenic agents or controlling of gene expression [1]. *T. cordifolia* is known to contain flavonoids [4] and the study of Kuno *et al.* [12] suggested that natural compounds such as flavonoids may work through induction of apoptosis in the damage cells. This apoptotic action probably accounts for the reduction of micronuclei counts in treated mice erythrocytes. The affected erythrocytes with micronuclei were probably induced to undergo the apoptotic process by the natural compounds in the extracts hence explaining the reduction of micronuclei counts in these antigenotoxic tests.

In the protective group, the mice were pre-treated with the stem extract prior to mutagen treatment. In this set up, the extract should inhibit micronuclei formation in mice erythrocytes upon treatment with MMS, hence demonstrating protective action [6]. Certainly the results corroborated the hypothesis; T7 and T8 had lower counts of micronuclei and significantly lower than the positive control by Tamhane test (Table 2) and the reduction worked in a dose-dependent manner since a corresponding decrease of micronuclei count was observed as dosage increased. The protective compounds from the stem extract probably work by changing the activation and detoxification of the mutagen MMS by modulating the metabolism of the xenobiotics through absorption; or by inhibiting the functions of superactive oxygen species. The active compounds in the extract may directly interact with MMS without affecting the DNA molecules. The bioactive compounds may inactivate the MMS by reacting directly with the latter's methyl cation. Another mode of action is possibly through the interaction of the DNA's nucleophilic sites with the bioactive compounds present in the extract hence the mutagen could no longer bind to these sites [13].

4. CONCLUSION

The present study significantly demonstrated that methanol extract of *T. cordifolia* stem had no genotoxic activity against albino mice erythrocytes at 500 ppm by peripheral blood micronucleus assay. On the other hand, the extract displayed both the protective and antigenotoxic properties against the MMS-induced mutagenicity in mice by the same assay. The protective potential of the stem extract worked in dose-dependent manner, i.e. the higher the dosage the lower the micronuclei induction by MMS. For antigenotoxic property, the low dosage at 250 ppm extract would suffice in reducing the micronuclei incidence induced by MMS. Further studies should be conducted to exploit the potential of *T. cordifolia* as alternative source of natural compounds to cure genotoxic-related problems.

5. REFERENCES

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