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The Genotoxic Potential of Alugbati Leaf Extracts on MCF-7 Cells

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Abstract

To determine the genotoxicity of alugbati (*Basella alba* Linn. var. rubra) leaf extracts on breast adenocarcinoma, the Comet assay was employed on MCF-7 cells incubated with the following: lyophilized alugbati juice extracts reconstituted with 2 % DMSO (AJ) and in aqueous media (AJ2), and lyophilized alugbati hydrolysate (exogenous myrosinase (E.C. 3.2.3.1) assisted) re- dissolved with 2 % DMSO in culture media (AH). Untreated MCF-7 cells in 2 % DMSO served as the negative control. MANOVA and Post hoc Tukey's HSD were employed to determine statistically significant differences among the samples. First, mutant cells in AJ and AH formed pronounced comet tails indicating that DNA damage had occurred significantly compared to that of the control. Post hoc comparisons between AJ and AH indicated that both samples exhibited comparable effects to MCF-7 cells. Due to the similarity of AJ to AH, it was presumed that hydrolysis occurred during the mechanical process of juice extraction. Second, AJ2 exhibited analogous results with the control; whereas, AJ was found to be statistically different. Aberrant cells incubated with the control and AJ2 trials exhibited relatively minimal genotoxicity as evidenced by intact nuclei. Overall, multiple comparisons illustrated that the most prominent DNA damage was observed by extracts AJ and AH in all parameters. The results of this study suggested that alugbati leaves subjected to enzyme-assisted hydrolysis or juice extractions prepared in DMSO caused considerable DNA damage in MCF-7 cells.

Keywords: Alugbati, Glucosinolates, Myrosinase, Genotoxicity, Comet Assay, MCF-7, *Basella alba* Linn. var. rubra

1. Introduction

As of 2014, statistics showed that Asia accounts for approximately 50 % of the global incidence of cancer, and is projected to increase from 6.1 million in 2008 to 10.6 million cases in 2030 (Sankaranarayanan *et al*., 2014). Among the fifteen Asian countries assessed by the Pfizer Medical Division, the Philippines was found to be ranked third with the highest cancer prevalence (McDonald *et al*., 2008). In fact, the occurrence of malignant neoplasm continued to increase in the ten leading causes of mortality in the Philippines from 9th to 3rd in occurrence in only a span of two decades in the Philippines (Tayag *et al.*, 2011). In this regard, different dietary practices and nutrition have become a focus of several researches which are associated with the prevention of noncommunicable diseases such as cancer (Vainio and Weiderpass, 2006); (Key, 2011). Several epidemiological studies show that the consumption of fruits and vegetables was strongly linked to risk reduction of prevailing forms of cancer and that a diet rich in this food was suggested as a primary preventive factor (Tayag *et al.*, 2011; Rick *et al.*, 2013). Riboli and Norat (2003) proposed an intake of approximately of 350 grams of fruits and vegetables per day as proportional to the preventable level of cancer types such as colorectal, esophageal and gastric. The high intake of fruits and vegetables were also correlated to the risk reduction of breast cancer by the hormone steroid receptor status (Emaus *et al*., 2015). In addition, a large cohort of case studies recommended that the intake of cruciferous vegetables, such as broccoli, reduces the risk of stomach, colon and lung cancer. In general, dietary components such as flavonoids, phenolic compounds, and glucosinolates are the potential factors contributing to such health benefits (Johnson, 2002).

Figure 1. *Basella alba* Linn. var. rubra (alugbati).

B. alba, a relatively understudied but widely consumed vegetable grown in Asia is believed to exhibit a wide range of biological functions. This plant is very popular in the

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central and southern Philippines, and is often a popular component of a concoction vegetable dish called "laswa" with its characteristic jelly-like consistency (Figure 1). Alugbati is recognized as a medicinal plant known for its antioxidant, antibacterial and anticancer potentials (Sushila *et al.*, 2010). The leaves and stem of alugbati have been used in Indian Ayurvedic treatments for curing diseases such as melanoma, leukemia and oral cancer. The traditional use of alugbati has been attributed and correlated to the presence of bioactive phenolics and flavonoids in the plant (Adhikari *et al.*, 2012). Moreover, preliminary screening in the laboratory showed that the extracts of alugbati species contain significant levels of glucosinolates (GSLs) (Malabed and Sandoval, De La Salle University – Manila, unpublished data). GSLs are phytochemicals and secondary metabolites known for the anticancer properties of their hydrolysis products. They are present along with myrosinase which is responsible for their enzymatic breakdown into various hydrolytic products such as isothiocyanates (ITCs) which are known to possess a number of marked biological activities (Calmes *et al.*, 2015). For instance, isothiocyanates were established to block metabolic activation and enhance the detoxification of chemically-induced carcinogens. Other breakdown products such as allyl-ITCs and benzyl-ITCs exhibited high bioavailabity of up to 90 % in orally administered trials in bladder cancer cells, and decreased the growth of pancreatic tumor cells (Zhang, 2010; Boreddy *et al.*, 2011). Several studies have led to the proposal of some mechanisms and actions of GSL hydrolysis products on cancer. The induction of Phase II enzymes such as glucoronosyl transferase (GT) and glutathione S-transferase (GST), modification of steroid hormone metabolism, and protection against oxidative damages are the general ways by which these compounds exhibit their anticancer properties (Das *et al*., 2000).

This research is aimed at determining the genotoxic capabilities of the juice and semi-purified enzymatically-hydrolyzed preparations of *B. alba* on breast adenocarcinoma immortalized cell line. To the best of the researchers' knowledge, this is the first reported study using this methodology of genotoxicity analyses on the extracts of *B. alba* against the aforementioned MCF-7 cells.

2. Materials and Methods

2.1. Preparation of Alugbati Juice Samples

Fresh alugbati leaves were chopped and blended with a small amount of water. The mixture was filtered to separate the juice from the pulp. The alugbati juice was frozen and lyophilized using a Labconco Freeze Dry System/Frozone®. Prior to the assay, 0.034 g lyophilized alugbati juice was reconstituted in 10 mL DMSO (AJ), and 0.095 g of the powdered sample was reconstituted in 10 mL distilled water (AJ2).

2.2. Preparation of Alugbati Hydrolysate

Fresh alugbati leaves were cut into small pieces, and were frozen and lyophilized. About 2.0 g of ground freeze-dried samples were mixed with 30.0 mL distilled water, and 100.0 µL myrosinase (E.C. 3.2.3.1) (3 units/mL). The hydrolysis mixture was homogenized and incubated for one hour at room temperature. 30.0 mL of the solvent DCM was added to the hydrolysate. The mixture was centrifuged for three minutes at 4000 rpm, and the organic layer was separated from the aqueous layer. The organic layer was dried over anhydrous Na_2SO_4 and was subsequently concentrated in a rotary evaorator (Malabed and Noel, 2012). The extract weighing 0.017g was dissolved in 10 mL DMSO (AH).

2.3. Comet Assay (Single cell gel electrophoresis)

Human breast adenocarcinoma cell line (MCF-7) was treated with extracts AJ, AJ2 and AH. Untreated MCF-7 cells in DMSO served as the negative control. MCF-7 cells, in complete growth medium, composed of Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific Gibco®, USA) containing 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, Gibco®, USA), and 1x antibiotic- antimycotic (Thermo Fisher Scientific, Gibco®, USA), and kept at 37°C with 5 % CO_2 in a 98 % humidified incubator, were placed in separate flat-sided culture test tubes and were incubated with 15 µL extract AJ, 10 µL of extract AJ2 and 25 µL of hydrolysate AH resulting in 0.75 % (v/v) DMSO, 0.53 % (v/v) water and 1.25 % (v/v) DMSO in solution, respectively. After twenty-four hours, the cells were harvested and incubated with 2 mL trypsin-EDTA and 5 % CO_2 at 37^{o}C for five minutes. Subsequently, 4 mL 1 x PBS was added, and the mixture was centrifuged for ten minutes at 22^{o}C and 1,200 rpm. The pellet was re-dissolved in PBS, transferred to an Eppendorf tube, and subjected to comet assay.

The bioassay was performed using Trevigen's reagent kit for Comet Assay® and the assay protocol specified by Trevigen Inc. (Gaithersburg, USA) was followed. About 50 µL of the cell culture mixture (250 µL low melting agarose and 25 µL of the MCF-7 cells) was layered onto the wells of the Comet slide and placed in the dark at 4^{o}C until the mixture solidified. After that, the slides were immersed in a pre-chilled lysis solution for one hour at 4^{o}C. Consequently, the slides were incubated in the dark with 50 mL alkaline unwinding solution (pH>13 (200 mM NaOH, 1 mM EDTA)) for twenty minutes at room temperature. Electrophoresis was performed as described in the Trevigen's Alkaline Comet Assay protocol. Following electrophoresis, the air-dried slides were stained with 50 µL of SYBR Green, and after subsequent drying, the images were captured using fluorescence microscopy.

Statistical evaluations of the different preparations of *B. alba* leaves (AJ, AJ2, and AH) were done on all the accumulated data gathered from the Comet assay. DNA damage was evaluated by measuring three parameters: tail length (*TL*) of the resulting fragmented DNA, the percentage of DNA in tail (*%DNA*), and the tail moment (*TM*). Cells with extensive DNA single and double stranded fragmentations were characterized according to the degree of nucleic dispersion and migration visualized as pronounced tails. The resulting images of cells or comets were scrutinized using OpenComet and ImageJ software.

2.4. Statistical Analyses

Multivariate analysis of variance (MANOVA) test was performed using SAS 9.3 Software and graphical representations were plotted using GraphPad Prism 7.04. Pillai's Trace was used with statistical significance level

(α) of p≤0.05. Post-hoc Tukey's honest significant difference (Tukey's HSD) was performed in the three dependent variables, and values of the least square means were also compared. Treatments having the same letter were considered not significantly different at 95 % confidence level.

3. Results

3.1. Comparison of the Alugbati Juice Extract and Hydrolysates in DMSO

Lyophilized alugbati juice (AJ) and alugbati hydrolysate (AH), both in DMSO, were assessed for possible genotoxic effects on MCF-7 cell lines. The mean scores and corresponding standard deviations (SD) in the three parameters (*TL*, *%DNA*, and *TM*) for the control, AJ and AH were presented in Table 1 and Figure 2. The control exhibited minimal DNA damage for *TL* (36.98 ± 10.56), *%DNA* (15.55 ± 8.66) and *TM* (40.95 ± 6.37).

Table 1. Post hoc comparison: Tukey's HSD on *B. alba* trials.

Extract	Parameter		
	TL	*%DNA*	*TM*
control	36.99±10.56[b]	40.95±8.66[b]	15.55±6.37[b]
AJ	119.74±71.61[a]	72.27±18.65[a]	81.40±51.30[a]
AH	118.18±114.11[a]	78.64±16.69[a]	84.22±83.75[a]
AJ2	61.40±53.47[b]	40.96±27.89[b]	32.44±40.12[b]

* Means with the same letter are not significantly different.

MCF-7 cells incubated with AJ and AH extracts showed loss of DNA integrity and substantial migration represented in numerous comets with extensive tails compared to the control with intact nuclei (*Figure 3*). Moreover, comets parameters of AJ (TL = 119.74 ± 71.61; % DNA= 72.27 ± 18.65; TM = 81.40 ± 51.30) and AH (TL = 118.18 ± 114.11; % DNA = 78.64 ± 16.69; TM = 84.22 ± 83.75) (Table 1 and Figure 2). Multivariate testing indicated that there was a statistically significant difference between the parameters of the control with both AJ and AH (F (6, 292) = 31.30, p = 0.0001; Pillai's trace = 0.78).

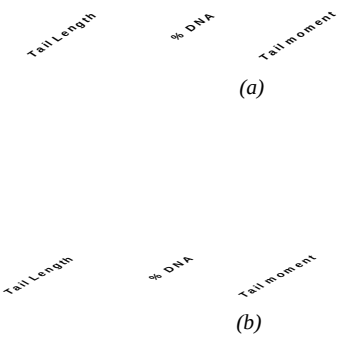

Figure 2. Comparison of the comet assay parameters incubated with the *(a)* **AJ** and **AH** and with *(b)* **AJ** and **AJ2.**

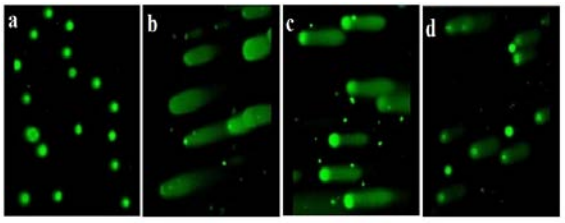

Figure 3. Images of MCF-7 cells subjected to Comet assay: *(a)* control *(b)* **AJ** *(c)* **AH** *(d)* **AJ2.**

3.2. Comparison of Alugbati Juice Extracts in Water and DMSO

The effect of changes in the matrix (lyophilized alugbati juice in DMSO (AJ) and in water (AJ2)) was observed to verify if the solubility of the constituents played a role in the genotoxicity of *B. alba* extracts. In addition, untreated MCF-7 cells in 2 % DMSO served as the negative control, and the results of the assay divulged that the solvent did not exhibit significant genotoxicity to the untreated cells. As seen in Table 1, data gathered for AJ were considerably different compared to the control (TL = 36.98 ± 10.56; %DNA = 40.95 ± 8.66; TM = 15.55 ± 6.37) and AJ2 (TL = 61.40 ± 53.47; %DNA = 40.96 ± 27.89; TM = 32.44 ± 40.12) in all the generated data. AJ (TL = 119.74 ± 71.61, %DNA = 72.27 ± 18.65 and TM = 81.40 ± 51.30) showed the most substantial DNA damage. However, the resulting images for AJ2 (Figure 3) exhibited fewer moderate to extensive diffused nuclei. In comparison with AJ, comets from AJ2 exhibited relatively shorter comet tails and the fluorescence of the comets were faint and less prominent. The findings dictate that greater DNA damage occurred in MCF-7 incubated with AJ compared to AJ2.

The outcome of the Pillai's trace statistics, F (6, 292) = 16.85, p = 0.0001; Pillai's trace = 0.51, signified that there were statistical differences between the control, AJ, and AJ2. Post hoc analyses showed that AJ was significantly different from the control and AJ2. This implied that the alugbati juice in DMSO exhibited significant DNA damage and genotoxicity to MCF-7 cell lines. On the other hand, the control and AJ2 were statistically equivalent indicating that the two exhibited the same effect.

4. Discussion

The Comet assay is one of the most sensitive techniques that can observe genotoxic effects in cancer cells (Florent *et al.*, 1999). Several factors could initiate DNA damage such as ionizing radicals, topoisomerases inhibitors, alkylating agents, sulfonates, *etc.* (Cann and Hicks, 2007). Bioactive constituents have been found to initiate DNA double strand breaks through the alkylation of DNA bases; consequently, DNA helices are disrupted and DNA breaks are induced. Elevation of reactive oxygen species levels trigger oxidative stress which also leads to unstable genomic processes of cells and double strand breaks (George and Rupasinghe, 2017). The appearance and formation of comets with pronounced tails in the assay were indicative of DNA strand breaks caused by pretreatments of the cells. The present investigation was performed to determine whether the extracts from *B. alba*

leaves could cause substantial DNA damage on MCF-7 cells and possess chemopreventive properties.

Alugbati juice's pharmacognosy has been reported to be linked to constituents such as flavonoids, phenolics and intact GSLs (Tongco *et al.*, 2015); Myrosinase assisted hydrolysis of GSLs primarily produce degradation products specifically ITCs (Angelino *et al.,* 2015). The evaluation of the collected images of comets from **AJ** and **AH** trials suggested that the presence of bioactive compounds in the juice extract which could consist of hydrolysis products can cause genotoxicity against breast adenocarcinoma. During the preparation of extracts **AJ** and **AJ2**, it was observed that powdered alugbati juice was soluble in DMSO, but partially dissolved in water. The results of the Comet assay exhibited that the extracts reconstituted with DMSO had greater efficacy due to the polar-aprotic solvent which had the capability to dissolve both polar and small nonpolar compounds (Capriotti and Capriotti, 2012). Even though the results of the water extract did cause minimal nucleic fragmentation, the bioactive constituents may not have been completely soluble, and therefore did not contribute to substantial genotoxicity on the MCF-7 cell line. Since the two extracts, **AJ** and **AJ2**, were prepared using the same freeze-dried alugbati juice, it may be inferred that the difference in their genotoxicity could be attributed to the solvent used.

Herbal extracts of *B. alba* were shown to exhibit immense anti-inflammatory, antioxidant, antibacterial, antifungal, and anti-ulcer activities (Sushila *et al.,* 2010). Such observations were correlated to the considerable concentrations of flavonoids, phenolics and betalains present in the plant (Kumar *et al.,* 2015). Cytotoxicity studies proposed that alugbati extracts exhibit chemotherapeutic actions through the following: modification of the permeability of mutagens through membranes, and prevention of mutagen transfer into cytosols by binding or inserting phenolics into the transporters of the outer cell membrane (Adhikari *et al.,* 2012). In another research, MTT assay showed that the red-stem variety of alugbati showed strong cytotoxicity against cervical carcinoma cells after a twenty-four-hour treatment with 50 mg/mL *B. alba* extract. Morphological changes, such as shrinkage and blebbing of the cancer cells, and the significant decrease of the live cancer cell count were indicative of the genotoxic activity of the alugbati extract (Kumar *et al.,* 2015).

It was found from preliminary studies in the laboratory that alugbati contained significant levels of GSLs which could be a possible cause of its genotoxic activities against MCF-7 cells. Fifteen local vegetables were analyzed for their total GSL content, and it was found that alugbati contains the highest levels of GSLs (85.30 μmol/g) (Malabed and Sandoval, De La Salle University – Manila, unpublished data). Moreover, initial identification of GSLs in *B. alba* indicated that extracts could also contain breakdown products such as isothiocyanates. Tan, *et al*. (2017) proposed pathways by which ITCs which could direct cell death. Inhibition of Phase I enzyme generates electrophiles, and introduces polar groups to xenobiotics. Consequently, activation of Phase II enzymes via antioxidant response factors (ARE) detoxifies and aids in the excretion of these carcinogens. It was observed that ITCs participate in mercapturic acid pathway in certain cancer cells. In this pathway, depletion of GSH level result in the activation of caspase dependent pathways, and the increase of S-thiocarbamoyl derivatives activates the expression of tumor suppressor genes (p53, Ink 4A, BRCA 1 & 2, ATF 2) both of which leads to cell death. Cell toxicity of hydrolysis products such as ITCs and indole-3-carbinol had been primarily studied on human lung, breast and prostate cancer cell lines in order to determine their anti-tumor activities. ITCs are known to cause pathogen and fungal death through the induction of cellular oxidative stress and redox homeostasis which causes glutathione depletion. It was found that such mechanisms were similar to its cellular targets in the said mammalian cancer cells. This observation corroborates the effects of ITCs in human lung, breast and prostate cancer cell lines (Calmes *et al*., 2015).

5. Conclusion

The analyses of the Comet assay on MCF-7 cells exhibited that lyophilized alugbati juice (**AJ**) and hydrolysate (**AH**) trials, both in 2 % DMSO, displayed notable DNA damage to the mutant cells observed by the appearance of pronounced tails. No significant differences were found between the genotoxic effects of the **AJ** and **AH** extracts which were attributed to the possibility of hydrolysis also taking place during the juice extraction. Moreover, determining the effects of change in the matrix revealed that alugbati juice in 2 % DMSO (**AJ**) exhibited greater DNA damage compared to the alugbati juice extract reconstituted in water (**AJ2**). Evidence was found to indicate that the bioactive constituents which possibly induced nucleic fragmentation were more soluble in DMSO than in water. Generally, extracts **AJ** (*TL* = 119.74 ± 71.61; *PD* = 72.27 ± 18.65; *TM* = 81.40 ± 51.30) and **AH** (*TL* = 118.18 ± 114.11; *PD* = 78.64 ± 16.69; *TM* = 84.22 ± 83.75) resulted in the most significant damage to MCF-7 cells. Overall, the current research shows that the extracts from *B. alba* caused significant DNA damage to MCF-7 cells. It supports preliminary studies on the biochemical constituents of alugbati as well as possible GSLs affirming significant chemotherapeutic properties of the plant extracts. To the researchers' knowledge, this is the first research that reports the genotoxic activity of alugbati extracts against breast cancer cells. A thorough and extensive investigation is recommended to further characterize the bioactive compounds and glucosinolates present in alugbati, and it will now be of interest to establish specific mechanisms by which alugbati constituents trigger cell cycle arrest on MCF-7 cells.

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