

## Free Radical Scavenging and *In Vitro* Cytotoxic Activity of Bugnay (*Antidesma bunius*) Leaves Extract Against A549 Human Lung Adenocarcinoma and HCT-116 Human Colorectal Cancer Cell Lines

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### Abstract

Cancer is one of the significant causes of mortality worldwide. Studies on antineoplastic drugs focused on natural products have revealed several mechanisms to inhibit cancer cells. Bugnay (*Antidesma bunius*) leaves showed potentials due to its activity observed against brine shrimp and breast cancer cells. However, there is still limited knowledge about its activity against other human cancer cells. This study focused on determining the phytochemical compounds in *A. bunius* leaves extract, the free radical scavenging activity of the extract using the Diphenylpicrylhydrazyl (DPPH) method, and *in vitro* cytotoxic activity against two cancer cell lines, namely HCT-116 human colorectal and A549 human lung adenocarcinoma cancer cell lines by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The phytochemicals identified were unsaturated lactones, flavonoids, phenolics, diterpenes, saponins, tannins, carbohydrates, and reducing sugars. The extract showed significant free radical scavenging activity and a direct correlation of activity with concentration levels. It also exhibited cytotoxic activity against HCT-116 human colorectal and A549 human lung adenocarcinoma. Hence, *A. bunius* leaves have the potential to be a source of antioxidant and antineoplastic compounds. This warrant further isolation of the compounds for chemotherapeutic purposes.

**Keywords:** *Antidesma bunius*, Bugnay, Cancer, Cytotoxicity, Radical

### INTRODUCTION

Cancer has been identified as the second leading cause of mortality worldwide; it exhibited public health concerns and burdens to the socioeconomics (Siegel, *et al.*, 2019). Among the different kinds of cancer, lung and breast cancers are the malignancies with the highest incidences followed by colorectal globally. Due to the rise in

early detection and treatment, cancer patients' life expectancy became higher (Bray, *et al.*, 2018). However, even now, cancer deaths continuously impose a significant problem. The main culprit

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has been identified, and it is the ability of tumors to become resistant to therapy through epigenetic (Zhou, *et al.*, 2013) or genetic mechanisms (Calin, *et al.*, 2005).

In the interest of combating cancer, recent developments have led to a rise in medicinal plants use for screening and identification of pharmacologically active and natural compounds against cancer. Some of these plant isolates, namely Vincristine, Vinblastine, and Paclitaxel, are now marketed for cancer treatments (Zulkipli, *et al.*, 2015). These discoveries significantly contributed to developing cancer drugs and extensive studies of promising medicinal plants for cancer, which may provide less toxicity and chemoprotective potentials (Mondal, *et al.*, 2012). Despite the various benefits acquired from natural resources, the emphasis for its utilization is still undervalued though their considerable contributions to modern pharmaceuticals and in traditional medicine are evident (Reid, *et al.*, 2018).

*Antidesma bunius* is a fruit tree that is native to Southeast Asia and Northern Australia. It is common in Northern Luzon, Philippines, particularly in the Cordillera region and in Mindanao with its local Philippine name Bignay, Bugnay, or Bignai. *A. bunius* plant has been featured in several types of research evaluating its vast potential (Butkhup and Samappito, 2008; Kassem, *et al.*, 2008; Mohan, *et al.*, 2013). Notably, a study revealed its antiangiogenic property (Juan, *et al.*, 2014), and cytotoxicity of leaves extract against brine shrimp (Micor, *et al.*, 2005) and MCF-7 breast cancer cell line (Subarnas, *et al.*, 2012). There are still few studies regarding the cytotoxic activity of the plant against human cancer cell lines. Hence, a more focused study in this plant's potential against other cancer cell lines is highly suggestible.

This study intended to determine the phytochemical substituents present in *A. bunius* leaves, to determine the free radical scavenging activity of the extract on Diphenylpicrylhydrazyl (DPPH) and to determine if there is a significant difference between the *in vitro* cytotoxic activity of

*A. bunius* leaves extract and Doxorubicin against HCT-116 human colorectal and A549 human lung adenocarcinoma cancer cells.

## MATERIALS AND METHODS

### General Experimental Procedures

The study utilized an experimental approach. The assessment of *A. bunius* was constricted to qualitative phytochemical screening, quantitative free radical inhibition analysis using DPPH method, and cytotoxicity assay under completely randomized design (CRD) with three trials per treatment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All reagents used were of analytical grade.

### Collection and Authentication of Plant

The plant was obtained from Alfonso Lista, Ifugao, Philippines. The parts of the plant sample (branch, leaves, flowers, and fruits) were sent for proper botanical identification and verification at the School of Natural Sciences, Saint Louis University (Baguio City, Philippines).

### Preparation of Extracts

*A. bunius* leaves were washed with tap water followed by distilled water then chopped into fine, thin pieces. The chopped leaves were dried using the oven (Olympia, USA) at 70°C for 12 h to remove moisture. The dried leaves were crushed and pulverized using an electric blender (Kyowa 1000). Crushed leaf samples were macerated in 80% ethanol (Merck, Darmstadt, Germany) in a closed container for three days with constant agitation.

Ethanol and methanol are efficient solvents in extracting antioxidant phenolic compounds from different natural resources. However, since ethanol has a lower toxicity than methanol, it is selected as the best solvent to extract antioxidant compounds (Do, *et al.*, 2014; Ballesteros, *et al.*, 2014).

The obtained sample was filtered using a Whatman No. 2 filter paper and the ethanol component of the sample was allowed to evaporate.

The crude extract was subjected to phytochemical and antioxidant determination, while the portion obtained via rotary evaporation (Laborota 4011, Heidolph) was submitted for *in vitro* cytotoxicity testing.

### Qualitative Phytochemical Analysis

The extract's phytochemical screening was conducted at Saint Louis University (Baguio City, Philippines) by the Natural Sciences Research Unit (NSRU). The extract was analyzed for the presence of phytochemical compounds such as flavonoids, phenolic compounds, alkaloid, saponins, glycosides, phytosterols, carbohydrates, and proteins using the method of Guevara, *et al.* (2005).

### Free Radical Scavenging Analysis

The analysis was conducted according to the standard method by Clarke, *et al.* (2013). Four concentrations of the *A. bunius* leaves extract (625, 1250, 2500, and 5000 µg/mL) were prepared via serial dilution with Dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, USA) as the solvent. Two hundred microliters of each concentration of the extract were added to 1.8 mL of DPPH (Sigma Aldrich) in ethanol (0.1 mM) (Merck) using separate test tubes. After leaving the test tubes in the dark for 30 minutes, the absorbance was measured at 518 nm using a VIS spectrophotometer (Labomed Inc., Los Angeles, California, USA). The same procedure was done using the different concentrations of L-Ascorbic acid (62.5, 125, 250, 500 µg/mL) (Sigma Aldrich, Singapore) as a control. The procedure was performed in triplicates per concentration with repeated measurements of absorbance per replicate. The DPPH radical scavenging activity was determined using the formula where Abs neg control is the absorbance of DPPH and ethanol (Clarke, *et al.*, 2013; Barcelo, 2015):

$$\% \text{Radical Scavenging Activity} = \left[ \frac{(\text{Abs neg control} - \text{Abs extract})}{(\text{Abs neg control})} \right] \times 100$$

### Cell Culture

The human lung adenocarcinoma (A549) and human colorectal (HCT-116) cancer cell lines were acquired from the American Type Culture Collection (ATCC, Manassas, USA). The cell lines were cultured in Dulbecco's Modified Eagle Medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), 0.14% sodium bicarbonate (Gibco), and 0.1 mM sodium pyruvate (Gibco). The cell lines were maintained in a CO<sub>2</sub> incubator (PHCbi, Illinois, USA) with a 5% CO<sub>2</sub> atmosphere and 95% humidity at 37°C.

### *In vitro* Cytotoxicity Assay

In determining cell survival and toxicity, MTT assay was performed following the method of Mosmann (1983). In greater detail, the cells of HCT-116 human colorectal and A549 human lung adenocarcinoma cancer cell lines were seeded separately. It was seeded at 4×10<sup>4</sup> cells/mL/well in sterile 96-well microtiter plates (Corning, NY, USA) and incubated overnight at 37°C (5% CO<sub>2</sub> at 98% humidity) wherein the log phase of their growth curve was attained.

In a master dilution plate (MDP), 4 mg/mL of extracts were diluted successively to the concentration of 1000 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL. Ten microliters were retrieved after the MDP and were distributed to the plated cells to get the final concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL. Eight two-fold dilutions of the sample were used as treatments starting from 100 µg/mL down to 0.78125 µg/mL. Concentrations were determined in line with the study of Dapat, *et al.* (2013) and Tantengco, *et al.* (2015).

The positive control was eight three-fold dilutions of Doxorubicin (Sigma, St. Louis, USA), an anthracycline antibiotic with antineoplastic activity used for cancer chemotherapy. A solvent for the extracts known as DMSO (Sigma) serves

as the negative control. Following incubation, cells were treated with each extract dilution. The treated cells were again incubated for 72 h at 37°C and 5% CO<sub>2</sub>.

Following the incubation period, the media was removed and 20 µL of MTT dye (Sigma Aldrich) at 5 mg/mL phosphate-buffered saline (Roche, Germany) was dispensed to all the wells. There was another incubation period for the cells for 4 h at 37°C (5% CO<sub>2</sub>). After incubation, 150 µL of DMSO was added to every well. A Ledetect reader was used at 570 nm to read the absorbance. A linear regression of the absorbance graph against concentration was used to compute the half-maximal inhibitory concentration (IC<sub>50</sub>), which is the concentration needed to kill 50% of the cell population.

Assay optimization was done by subtracting the MTT absorbance of cancer cells treated with *A. bunius* extract and the plant extract's absorbance to eliminate possible color interference at 570 nm.

### Statistical Analysis

In determining the phytochemical substituents of *A. bunius* leaves crude extract, the results were presented as + (positive) and – (negative), depending on which substituent was present in the extract.

For DPPH assay, results were presented as frequencies and mean±SD of triplicates from three independent experiments. GraphPad Prism 8 was then used to calculate for the IC<sub>50</sub> of DPPH and MTT cytotoxicity assay. The IC<sub>50</sub> value was calculated to determine the sample concentration required to inhibit 50% of the free radicals. The computed two IC<sub>50</sub> per assay were compared using independent samples t-test. Specifically, t-test was used to determine if there is a significant difference in the IC<sub>50</sub> of (1) *A. bunius* leaves extract and L- Ascorbic acid, (2) *A. bunius* leaves extract and Doxorubicin on HCT-116 human colorectal and, (3) *A. bunius* leaves extract and Doxorubicin on A549 human lung adenocarcinoma cancer cell line. Other data needed was assessed in the software SPSS 23.0, and differences with *p*<0.05 was considered significant.

## RESULTS

### Phytochemical Screening

Table 1 below showed the different phytochemical compounds present in *A. bunius* leaves extract. The result of analyses revealed reducing sugars, carbohydrates, unsaturated lactones, saponins, diterpenes, phenolics, tannins, and flavonoids.

Table 1. Qualitative Phytochemical Analysis of *A. bunius* Leaves Extract.

Test for Constituents	Constituents Detected	Results	Test for Constituents	Constituents Detected	Results
Detection of Alkaloids			Detection of Phytosterols		
• Mayer's test	Alkaloids	-	• Cupric acetate test	Diterpenes	+
• Dragendorff's test	Alkaloids	-	• Salkowski's test	Triterpenes	-
• Hager's test	Alkaloids	-	• Liberman Burchard test	Phytosterols	-
• Wagner's test	Alkaloids	-			
Detection of Carbohydrates			Detection of Phenolic Compounds		
• Molisch's test	Carbohydrates	+	• Ferric Chloride test	Phenolics	+
• Fehling's test	Reducing sugars	+	• Gelatin test	Tannins	+
Detection of Glycosides			Detection of Flavonoids		
• Modified Borntrager's test	Anthraquinone glycosides	-	• Alkaline Reagent test	Flavonoids	+
• Kedde test	Unsaturated lactones	+	• Lead Acetate test	Flavonoids	+
Detection of Saponins			Detection of Proteins		
• Froth Test	Saponins	+	• Xanthoproteic test	Proteins	-
• Foam test	Saponins	+			

### Diphenylpicrylhydrazyl (DPPH) Assay

Figure 1 indicated that the  $IC_{50}$  of the *A. bunius* leaves extract was increased in a concentration-dependent manner. Among the four concentrations of *A. bunius* crude extract, 5000  $\mu\text{g/mL}$  showed the highest free radical scavenging activity ( $67.29 \pm 0.56$ ).

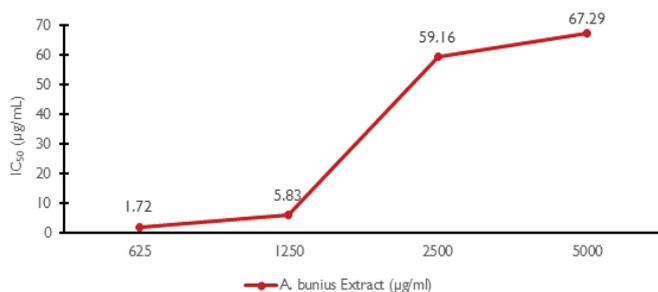


Figure 1. Free radical scavenging activity of the four concentrations of *A. bunius* leaves extract on DPPH Assay.

Figure 2, on the other hand, showed the comparison between the free radical scavenging activity of L-Ascorbic acid and *A. bunius* leaves extract. Independent samples t-test revealed that *A. bunius* leaves extract ( $M=3.27$ ,  $SD=0.00208$ ) was significantly different ( $p<0.001$ ) from L-ascorbic acid ( $M=2.43$ ,  $SD=0.00493$ ). This indicated that *A. bunius* leaves extract antioxidant activity was active but lower than L-ascorbic acid.

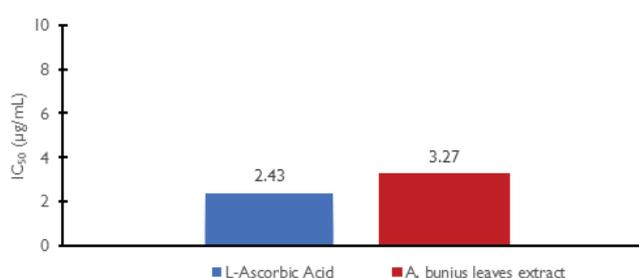


Figure 2. Log  $IC_{50}$  values of L-Ascorbic acid and *A. bunius* leaves extract on DPPH Assay.

significantly different ( $p<0.05$ ) from Doxorubicin ( $M=0.0567$ ,  $SD=0.015$ ). This result indicated that *A. bunius* leaves extract has cytotoxic activity against HCT-116 human colorectal cancer cells but lower than Doxorubicin.

Figure 4 showed the difference between the  $IC_{50}$  of *A. bunius* leaves extract and Doxorubicin against the A549 lung adenocarcinoma cell line. Independent samples t-test showed that *A. bunius* leaves extract ( $M=1.75$ ,  $SD=1.12$ ) was not

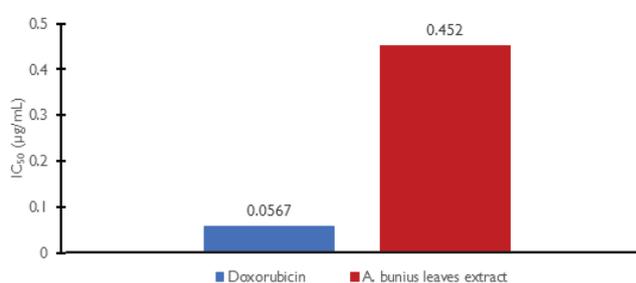


Figure 3.  $IC_{50}$  values of Doxorubicin and *A. bunius* leaves extract against HCT-116 human colorectal cancer cell line.

significantly different ( $p>0.05$ ) from Doxorubicin ( $M=0.14$ ,  $SD=0.05$ ). This result indicated that *A. bunius* leaves extract has a similar dose-dependent cytotoxic activity of Doxorubicin against A549 human lung cancer cells.

### DISCUSSION

Qualitative phytochemical analysis of *A. bunius* leaves revealed the presence of unsaturated lactones, flavonoids, phenolics, diterpenes, saponins, and tannins. These exhibited both

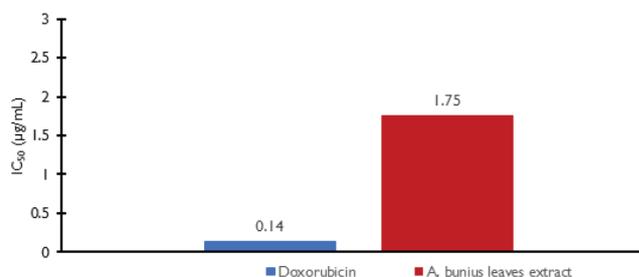


Figure 4.  $IC_{50}$  values of Doxorubicin and *A. bunius* leaves extract against A549 lung adenocarcinoma cell line.

antioxidant and anticancer properties reported in several studies. In a study by Tantawy, *et al.* (2015), on the hypoglycemic property evaluation of *A. bunius* leaves, phytochemicals identified were the same as the present study. Another study by Jorjong, *et al.* (2015), using the fruit sample of *A. bunius*, has demonstrated flavonoids and phenolics determined to be present in *A. bunius* leaves.

The antioxidant activity of the extract was demonstrated through the DPPH assay. This test was used because of its characteristics and availability. According to Škrovánková, *et al.* (2012), the DPPH test is commonly used because of the short time of analysis, the free radical's stability, and measurable reaction. DPPH has been widely adopted in measuring the free radical scavenging activity of antioxidants. This assay is solely based on the reduction of an alcoholic DPPH solution using a hydrogen-donating antioxidant (Fatiha and Abdelkader, 2019).

The IC<sub>50</sub> value indicated that *A. bunius* leaves extract has less potent radical scavenging activity than L-ascorbic acid; therefore, to yield similar effects with L-ascorbic acid, a higher dose is needed. Free radical scavenging activity either prevents the generation of reactive oxygen species or eliminates them before they damage the cell's vital components (Riaz, *et al.*, 2012). According to a study from the US National Cancer Institute, antioxidants prevent damage to cells by donating electrons to stabilize the free radicals, heal damaged cells, and delay or prevent cancer (Olsen and Ware, 2018).

In a study by Islam, *et al.* (2018), methanolic leaves extract of *A. bunius* possessed excellent antioxidant activity similar to that of the ethanolic leaves extract of *A. bunius* used in this study. Several studies revealed phytochemical compounds that exhibit free radical scavenging activity, which was present in *A. bunius* leaves extract. Such compounds include unsaturated lactones, saponins, diterpenes, phenolics, and tannins (Yang, *et al.*, 2016; Chen, *et al.*, 2014; Perez and Ahuatzí, 2015; Daglia, 2012).

Literature has revealed the antioxidant property of unsaturated lactones and flavonoids through inhibiting nitric oxide (Yang, *et al.*, 2016). Phenolics are widely known to have protective effects as antioxidants by lowering free radicals present in the body. In recent years, evidence has shown that small amounts of phenolics have modulatory effects on selective intracellular signaling pathways critical for cellular functions such as proliferation and apoptosis (Ozcan, *et al.*, 2014). Diterpenes, on the other hand, are found to be effective antioxidant by inhibiting lipid peroxidation and lipoxygenase (Perez and Ahuatzí, 2015).

The MTT assay of *A. bunius* leaves extract revealed activity against HCT-116 human colorectal and A549 lung adenocarcinoma cancer cell lines. The cytotoxic activity of *A. bunius* is also supported by the previous studies against brine shrimp (Zaman, *et al.*, 2018) and MCF-7 breast cancer cell line (Subarnas, *et al.*, 2012).

An extract that exhibits in vitro cytotoxicity against cancer cells can be used as a possible drug source if it reveals an IC<sub>50</sub> value not greater than 30 µg/mL (Suffness and Pezzuto, 2009). In this study, the IC<sub>50</sub> value of the *A. bunius* leaves extract is lower than 30 µg/mL in both cancer cell lines. Therefore, these findings support the notion that *A. bunius* extract can be a potential source of antineoplastic compounds.

The cytotoxic activity may be attributed to the phytochemical compounds, namely, saponins, diterpenes, tannic acid, polyphenols, and flavonoids. Flavonoids found in different plant sources showed activity against lung cancer cells (Al-Taweel, *et al.*, 2015). Antitumor properties of flavonoids include decreasing mitochondrial potential, overexpressing Fas and FADD ligands, and promoting DNA fragmentation (Abotaleb, *et al.*, 2019). A study done by Gong, *et al.* (2014) revealed a positive inhibition of nicotine-induced lung cancer cells by flavonoid compounds through interference with genes (MMPs) that regulate the initiation of metastasis. Reduction in the risk of

tumor formation is associated with flavonoids' dietary intake (Donato and Romagnolo, 2012).

Based on this study's phytochemical determination, *A. bunius* contains saponins that were found to possess antitumor effects on many cancer cells (Shuli Man, 2010). As studied by He, *et al.* (2014), it effectively inhibits the invasion and metastases of the A549 lung cancer cell line by mechanisms similar to flavonoids inhibiting matrix metalloproteinase (MMP) genes. Studies showed that diterpenes exhibit antitumor property and have been proven to possess moderate or strong antiproliferative effects on various human cancer cell lines, including colon carcinomas (Vasas, *et al.*, 2014). Polyphenols are thoroughly studied, and it has been recognized as a chemopreventive agent against cancer such as colorectal cancer (Araújo, *et al.*, 2011). Tannic acid is reported to be effective against colon carcinogenesis by ameliorating inflammatory damage and oxidative stress (Oday and Hamiza, 2012).

A breakthrough in pharmacologic medicine is the discovery of a complex diterpene marketed as Paclitaxel. Paclitaxel is an anticancer agent that inhibits the metaphase-anaphase transition in cancer cells by suppressing the spindle microtubule dynamics leading to mitosis inhibition inducing apoptosis (Khanna, *et al.*, 2015). Saponins can induce apoptosis and inhibit cancer cell growth through G1 cell cycle arrest (Yu, *et al.*, 2015). Lastly, tannins are found to cause cell cycle arrest in S phase and promotion of apoptosis (intrinsic pathway) through BCL-XL downregulation accompanied by the mitochondrial release of cytochrome C into the cytoplasm leading to activation of caspase 9 (initiator) and caspase 3 (effector) (Yildirim and Kutlu, 2015).

The study was limited to the qualitative determination of the phytochemicals, colorimetric antioxidant assay, and *in vitro* cytotoxic assay. Further studies to quantitatively evaluate and isolate *A. bunius* leaves active components, determination of the total antioxidant capacity, evaluation of the

mechanism of cytotoxicity, and tumor selectivity determination are recommended.

To our knowledge, this is the pioneer study to report about the biologic activity of the *A. bunius* against the mentioned carcinoma cells. The findings of this study were geared towards its contribution to drug discovery and development, responding to the challenge of drug innovations on cancer chemotherapy and chemoprevention. This study supports promoting and exploring indigenous plants, particularly in the Philippines, on unraveling the potentials found within the said plants.

## CONCLUSION

*A. bunius* leaves extract is a rich source of a variety of phytochemicals. The extract showed significant free radical inhibition activity. Also, it exhibited cytotoxic activity against lung and colorectal cancer. Hence, *A. bunius* leaves have the potential to be a source of natural antioxidant and antineoplastic compounds. This study recommends further isolation and evaluation of the active compounds for chemotherapeutic purposes.

## CONFLICT OF INTEREST

All authors declare no conflict of interest.

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