

Bioactive Compounds and Cytotoxicity of Ethyl Acetate Extract From *Broussonetia luzonica* (Moraceae) Blanco Leaves against Hepatocellular Carcinoma (Hepg2) Cell Lines

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ABSTRACT

Introduction: *Broussonetia luzonica* (Moraceae) Blanco is an edible and endemic plant in the Philippines. Other species of the plant are used traditionally in Chinese medicine to treat impotency and eye disorders and was proven to have anticancer potential. To date, there are no published scientific evidences yet to prove the cytotoxicity against hepatocellular carcinoma cell lines (HepG2) of *B. luzonica*. Furthermore, the bioactive compounds of the ethyl acetate leaf extract were determined. **Methods:** Bioactive compounds were determined using Gas Chromatography-Mass Spectrometry (GC-MS). To determine the IC₅₀, the percentage HepG2 Cell inhibition of the extract at 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL And 12.5 µg/mL concentrations against (HepG2) was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. **Results:** GC-MS revealed the top three major bioactive compounds of ethyl acetate leaf extract based on quantity (%). These are 1,2,3-propanetriol, monoacetate (21.21%), phytol (20.28%) and squalene (6.85%). MTT assay showed that ethyl acetate extract at different concentrations exhibited marked inhibition of the HepG2. The concentration of the extracts that will inhibit 50% of the

cancer cell lines (IC₅₀) was also determined. The assay revealed that compared to positive control (doxorubicin) with IC₅₀ 5.068 µg/mL, Ethyl Acetate Extract statistically exhibited greater cytotoxic effect against HepG2 Cell Lines With IC₅₀ 1.118 µg/mL ($P=0.001$). **Conclusion:** The presence of several bioactive compounds in ethyl acetate extract from the leaves of *B. luzonica* confirms the importance of the plant in treatment of diseases. Furthermore, the extract manifested more potent cytotoxic activity than the positive control, indicating promising chemotherapeutic potential of the plant.

Key words: Endemic, Liver Cancer, GC-MS, MTT Assay.

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INTRODUCTION

Cancer is the leading cause of mortality and morbidity worldwide, representing a major public health burden in all parts of the world. World cancer report 2014 states that there were estimated 14.1 million new cancer cases, 18.2 million cancer mortality, and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide.¹ Of these, more cases were in men (7.4 million) than in women (6.7 million). The number of reported cases is expected to rise to 24 million by 2035.² In addition, cancer causes one in every four deaths in the United States.³

A very malignant and most common type of cancer, hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide.⁴ Since HCC is seldom detected early, high percentage of patients visiting health service facilities with symptoms of HCC are confirmed on malignant stage.⁵ Therefore, there is a need to address this alarming health threat to mankind.

Even though there are several approved chemotherapeutic agents available for preventing and treating cancer, the scientific community is still challenged to reduce the increasing incidence of this chronic disease. At present, there is a continuous and growing interest in search for safe and effective compounds that could prevent, reverse, and block cancer development in order to address the alarming health problem.⁶⁻⁸ These researches focus more on the use of available natural resources and further investigate different mechanisms leading to cancer which could be the possible targets in suppressing its emergence.⁹⁻¹¹ Plants play a significant role in the prevention and treatment of diseases and can even prevent and minimize the adverse effects of conventional treatments.¹² They can be source of chemical compounds of biological and pharmacological

importance. History reveals that plants are sources of successful drugs, and will continuously be an important pool for screening lead compounds.¹³ Among the successful chemotherapeutic agents derived from plants are vincristine and paclitaxel¹⁴ which lead researchers to evaluate cancer therapeutic potential of other plants.¹⁵⁻¹⁸

B. luzonica commonly known as himbabao or alukon is endemic in the Philippines. Its leaves and flowers are locally eaten by Filipinos as part of vegetable recipes. Edible plants are not only source of nutrition but of medicinal value.¹⁹⁻²¹ Currently, there are no published studies on biological properties of the edible and endemic *B. luzonica*.²² However, several investigations on the other species of *Broussonetia* which is *B. papyrifera* revealed various biologic activities. *B. papyrifera* exhibited marked cytotoxicity against breast, lung and liver cancer cell lines,²³ showed antioxidant property²⁴ and has the ability to inhibit certain tumor necrosis factor- α (TNF- α) and interleukin 1b (IL1b).²⁵ According to Sousa (2012), plants under the same genera could also exhibit the same biologic activities.²⁶ Therefore, chemical components of *B. luzonica* leaves and the bioactivity were investigated. This is the first time to report the bioactive compounds and the cytotoxicity of the ethyl acetate extract from the leaves of *B. luzonica* against HepG2 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MATERIALS AND METHODS

Plant Sample

The plant specimen of *B. luzonica*, collected by the researcher from Solano, Nueva Vizcaya, Philippines on June, 2015. It was verified and authenticated at the UST Herbarium, Research Center for Natural and Applied

Sciences, University of Santo Tomas and was assigned an accession number of 012978.

Extraction of Ethyl Acetate Extract

The leaves of the plant were cleaned and air dried at room temperature for about 3 days. The dried leaves were ground using wiley mill model no. 2 (Arthur h. Thomas co.). About 3 kg of dried and powdered samples underwent 24 hrs percolation using 3 liters of ethyl acetate as solvent for extraction. Extract was collected and percolation was repeated on the same plant sample.²⁷⁻²⁸ The solvent present in the pool of the extract was evaporated to dryness using Heidolphrotavapor (Germany).

The Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

Analysis of bioactive compounds from ethyl acetate extract obtained from the leaves of *B. luzonica* by GC-MS was done using Agilent Technologies GC systems (Gc-7890a/MS-5975c Model, Santa Clara, CA, USA) equipped with Hp-5ms column (30 M Length X 250 µm Diameter X 0.25 Mm Thickness of Film). Spectroscopic detection by GC-MS which is an electron ionization system had utilized high energy electrons of 70 ev. pure helium gas (99.995%) was the carrier gas at a flow rate of 1 ml/min. The initial temperature is set at 50°C to 150°C with ramp rate of 3°C/min and holding time of about 10 mins. Finally, the temperature is increased to 300°C at 10°C/min. one microliter (1 µl) of the prepared 1% sample of the extracts diluted with respective solvents was injected in a splitless mode. Relative quantity of the chemical compounds present in the extract of *B. luzonica* was expressed as percentage based on peak area produced in the chromatogram. GC-MS analysis was done in Nanocast Laboratory, Department of Physical Sciences, Mahidol University, Thailand.

Identification of Chemical Constituents

Bioactive compounds extracted from different extracts of *B. luzonica* were identified based on GC retention time on HP-5MS column and matching of the spectra with standards on computer software data (repliband mainlab data of GC-MS systems).

MTT Assay

MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) Assay is based on the method of Mossmann with some modifications.²⁹ Hepg2 (Human Caucasian Hepatocyte Carcinoma) Cell Lines were seeded into 96-well plates with 5 X 10⁵ cells/mL and incubated at 37°C humidified incubator with 5% CO₂ for 24 hrs. Five concentrations of sample were used (200, 100, 50, 25, 12.5 µg/mL) in treating the cells and Doxorubicin HCl as positive control. Cells were treated with the above mentioned sample concentrations and control with 100 µl volume for each well and incubated for 24 hrs. At 37°C humidified incubator with 5% CO₂. After incubation, media from each well was removed and 20 µl(MTT) with 5 mg/mL concentration dissolved in phosphate buffer saline pH 7.2 was added into each well containing the untreated and treated cells. The 96-well plate containing HepG2 cells was incubated for 4 hrs at 37°C humidified incubator with 5% CO₂. After incubation, 200 µl of DMSO was added to each well and the absorbance was read at 570 nm using Corona Sh-1000lab Microplate Reader. The percentage growth inhibition was calculated using the following formula.³⁰

$$\%Cell\ Inhibition = 100 - \left\{ \frac{(As - Ab)}{(Ac - Ab)} \right\} \times 100$$

Where,

As = Absorbance value of Sample Compound

Ab = Absorbance value of Blank

Ac = Absorbance value of Control

From the percentage inhibition, concentration that could inhibit or kill 50% of cancer cell population or IC₅₀ was calculated statistically using graphpad prism software.

Statistical Analysis

The results were expressed as mean ± sd of three trials. IC₅₀ estimates for Hepg2 cell lines were computed based on % inhibition values using four parameter logistic model, performed at graphpad prism version 6.0 (graphpad soft ware, la jolla california usa, www.graphpad.com). To determine, if there is significant difference between the IC₅₀ of the control and the extracts, results were treated with analysis of variance followed by tukey's *post-hoc* analysis³¹ to determine which pair of the extracts significantly differ from one another using spss version 20-32 bit.p values of <0.05 were considered statistically different.

RESULTS

Since the discovery of successful chemotherapeutic agents obtained from plants, vast number of studies on plants increases.^{32,33} Isolated compounds from plants having cancer healing potential were also investigated for possible new source of chemotherapeutic agents.³⁴ This investigation was conducted to determine the chemical compounds present in all the extracts. Furthermore, to study the cytotoxicity of the different crude extracts from the leaves of *B. luzonica* against hepatocellular carcinoma (HepG2) cell lines as compared to a standard drug, doxorubicin. GC-MS confirmed that the ethyl acetate crude extract has several bioactive components. Based on abundance, the top three major compounds in the extract are phytol (20.288%), 1,2,3-propanetriol, monoacetate (21.211%) and squalene (6.8%). Table 1 shows the identified bioactive compounds present in ethyl acetate extract. Their identification and characterization are based on their elution order or retention time on a HP-5MS column. The retention time (min), molecular formula and the amount in % of these compounds are also presented. Figure 1 represents the GC-MS chromatogram of the ethyl acetate extract.

The extract and positive control exhibited marked Hepg2 cell inhibitory property at various concentrations as shown in Figure 2.

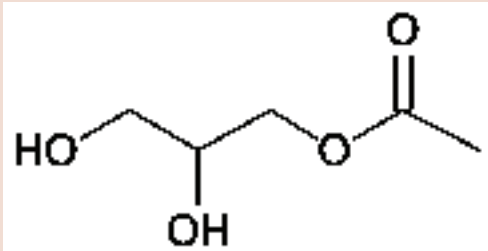
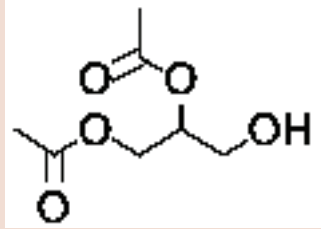
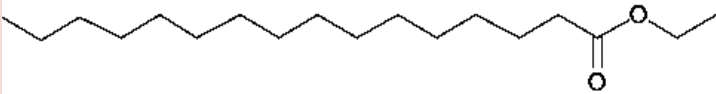
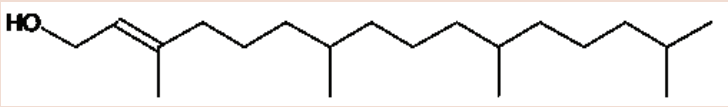
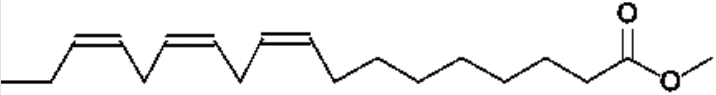
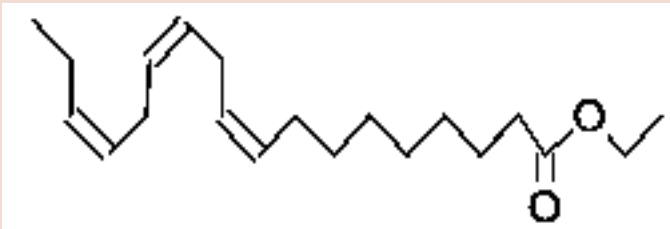
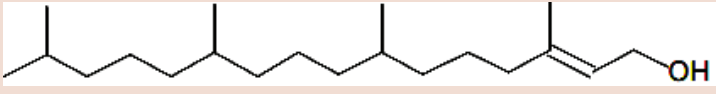
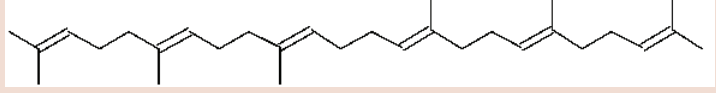
Figure 2a shows the inhibitory effect of Doxorubicin against Hepg2 Cells. At 20 µg/ML, it can inhibit 80.80% of the cancer cells while at 1.25 µg/ML, It can inhibit 17.10 % of the cancer cells. Figure 2b shows that *B. luzonica* ethyl acetate leaf extract inhibited 82.62% and 65.16% of Hepg2 Cells at 200 µg/ML and 12.5 µg/ML concentrations, respectively. Figure 3 shows the IC₅₀ of the extract compared to the standard (Doxorubicin). Ethyl Acetate extract showed the more potent cytotoxicity having IC₅₀ of 1.12 µg/ML than Doxorubicin having IC₅₀ of 5.06 µg/ML.

DISCUSSION

Based on studies, some of the constituents revealed by GC-MS are biologically active compounds. They were proven to possess pharmacologic activities which may contribute to the cytotoxic activity of the plant under study. Phytol, precursor of synthetic vitamin e and vitamin k was found to be cytotoxic against breast cancer cell lines (mcf7).^{35,36} Propanetriol-monoacetate was confirmed to be present in other plants revealing antimicrobial, anti-inflammatory, diuretic and anticancer effects.³⁷ Same compound was proven to be a precursor of tricetin (antifungal) but may also serve as a prodrug and vehicle for anticancer agents.³⁸ Squalene has antioxidant, chemopreventive, antitumor and hypocholesterolemic activities.³⁹⁻⁴⁰ These compounds are present in ethyl acetate extract from *B. luzonica* leaves which supports the cancer therapeutic potential of the plant against hepg2 cell lines.

Based on the criteria of United States National Cancer Institute, a compound is classified as cytotoxic if it has an ic₅₀ of <20 µg/ml.⁴¹ Statistical analysis revealed that there is significant difference between the IC₅₀ of

Table 1: Bioactive Compounds of Ethyl Acetate Extract from *B. luzonica* leaves

Retention Time (Min)	Name of Compounds (Molecular Formula)	Chemical Structure	%
20.08	1,2,3-Propanetriol, monoacetate (C ₅ H ₁₀ O ₄)		21.211
24.6	1,2,3-Propanetriol, diacetate (C ₇ H ₁₂ O ₅)		4.351
50.11	Hexadecanoic acid, ethyl ester (C ₁₈ H ₃₆ O ₂)		2.386
51.7	Phytol (C ₂₀ H ₄₀ O)		20.288
51.56	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (C ₁₉ H ₃₂ O ₂)		2.465
52.3	9,12,15-Octatrienoic acid, ethyl ester, (Z,Z,Z)- (C ₂₀ H ₃₄ O ₂)		2.753
52.9	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (C ₂₀ H ₄₀ O)		6.765
58.0	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-,(all-E)- or Squalene (C ₃₀ H ₅₀)		6.853

B. luzonica ethyl acetate leaf extract compared to the standard with $p = 0.001$ indicating that the extract is more potent than that of the standard since the concentration of ethyl acetate extract that inhibit 50% of the hepg2 cell lines is lower (1.118 $\mu\text{g/ml}$) significantly than that of the standard (5.08 $\mu\text{g/ml}$). This means that ethyl acetate extract has greater cytotoxic activity than doxorubicin. This study is the first one to report the cytotoxicity of the crude ethyl acetate extract obtained from *B. luzonica* leaves.

CONCLUSION

Based on the results, this endemic and edible plant from the Philippines contains bioactive compounds demonstrated marked cytotoxicity against hepg2 can be a source of promising chemotherapeutic agent.

ACKNOWLEDGEMENT

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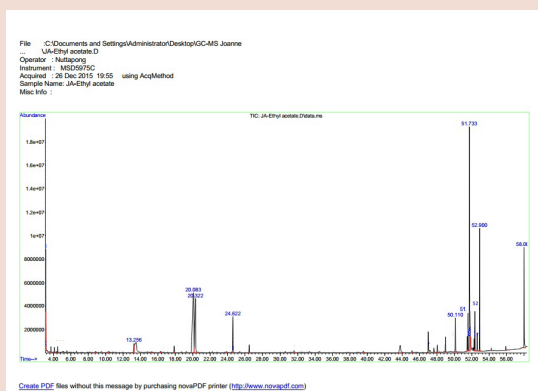


Figure 1: GC-MS Chromatogram of the Ethyl Acetate Extract from *B. luzonica* Leaves.

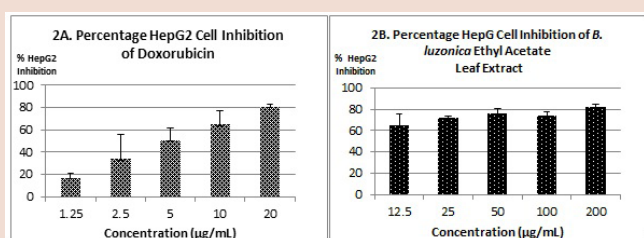


Figure 2: Percentage HepG2 Cell Inhibition of Doxorubicin (2A) and Ethyl Acetate Extract (2B).

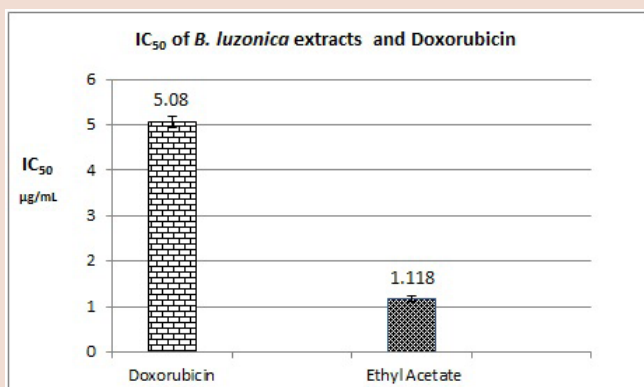


Figure 3: Comparison of the IC₅₀ of Doxorubicin and Ethyl Acetate Extract.

tion, Mammalian Tissue Culture Laboratory (MTCL), Research Center for Natural and Applied Sciences (RCNAS), University of Santo Tomas (UST) Manila, Philippines Department of Science and Technology (DOST) and Commission on Higher Education (CHED), Philippines. Acknowledgement is extended to Asst. Prof. Ophelia Laurente, botanist-curator of UST Herbarium, RCNAS and Nuttapon of Nanocast Laboratory, Physical Sciences Mahidol University, Thailand.

HepG2 cell lines were kindly donated by Librado Santiago, Ph.D., to MTCL, RCNAS, UST, Manila, Philippines, which were purchased from European Collection of Authenticated Cell Cultures (ECACC).

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ABBREVIATIONS USED

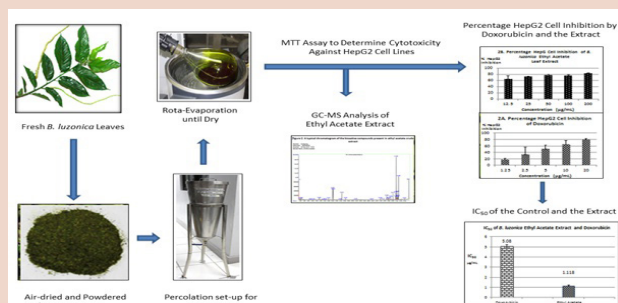
HepG2: Hepatocellular Carcinoma Cell Lines; **MTT:** 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide; **TNF- α :** Tumor Necrosis Factor Alpha; **IC₅₀:** Concentration in $\mu\text{g/mL}$ that will inhibit 50% of cell population; **GC-MS:** Gas Chromatography-Mass Spectrometry.

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PICTORIAL ABSTRACT



SUMMARY

- The main components of ethyl acetate extract of *B. luzonica* leaves are 1,2,3-propanetriol, monoacetate, phytol and squalene.
- At different concentrations, the ethyl acetate extract showed marked inhibition of HepG2 cell lines.
- The ethyl acetate extract ($IC_{50} = 1.118 \mu\text{g/mL}$) showed more potent cytotoxicity than doxorubicin ($IC_{50} = 5.068 \mu\text{g/mL}$) against HepG2 cell lines.

ABBREVIATIONS USED

HepG2: Hepatocellular Carcinoma Cell Lines; **MTT:** 3-(4,5-Dimethylthiazol-2yl)-2,5-Diphenyltetrazolium Bromide; **TNF- α :** Tumor Necrosis Factor Alpha; **IC₅₀:** Concentration in $\mu\text{g/mL}$ that will inhibit 50% of cell population; **GC-MS:** Gas Chromatography-Mass Spectrometry.

ABOUT AUTHORS



Franelyne P. Casuga: Is a doctoral student at the University of Santo Tomas taking Ph.D. in Pharmacy degree. She graduated with Bachelor of Science in Pharmacy and Master of Science in Pharmacy in Centro Escolar University. Her study focused on *In vitro* Cytotoxicity and *In vivo* Anti-inflammatory and Hepatoprotective Properties of Plants.



Mary Jho-Anne T. Corpuz: Obtained her Ph.D. in Pharmacy Degree from the Graduate School, University of Santo Tomas. She is currently affiliated with the Faculty of Pharmacy in the same university. Her study focused on the Antioxidant, Chemopreventive property and Cytotoxicity of plants. Furthermore included in her specialties are preformulation and formulation studies.



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