CYTOTOXICITY, ANTIOXIDANT ACTIVITY AND CHEMICAL CONSTITUENTS OF *Piper sarmentosum* ETHANOLIC EXTRACT

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ABSTRACT

Plants hold a high value for mankind due to its richness in properties. Plants secondary metabolites are of particular interest as they possess medicinal properties that can be utilised by human. Among local plants of interest is *Piper sarmentosum* which is locally known as Kaduk. It is a plant from *Piper* species with numerous medicinal properties. This study evaluated the cytotoxicity property, antioxidant activity, and chemical composition for *P. sarmentosum* of ethanolic extract. The leaves of *P. sarmentosum* were extracted through Soxhlet method using absolute ethanol. The cytotoxic study was performed through MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay using HT-29 human colon adenocarcinoma cell line, while the antioxidant activity was assessed through 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay. The crude was also evaluated for its chemical constituents by gas chromatography coupled with mass spectrometer (GC-MS). Results demonstrated that the cytotoxicity effect of *P. sarmentosum* on HT-29 was exhibited with an IC_{50} of 25 µg/mL. The same extract was also found to exhibit antioxidant activity by DPPH scavenging assay with an IC_{50} of 126.39 µg/mL. Meanwhile, the GC-MS analysis showed that myristicin is the highest chemical constituent in *P. sarmentosum* ethanolic extract followed by β-caryophyllene, asarone, naphthalene, copaene, and lastly elemicine. These findings suggested that the ethanolic extract of *P. sarmentosum* was relatively active and may be considered as potent agent for future healthcare and pharmaceutical industries.

Keywords: *Piper sarmentosum*, cytotoxicity, MTT, HT-29, antioxidant, DPPH, IC_{50}.

INTRODUCTION

Medicinal plants are highly sought after for their bioactive compounds as they can be used to treat various health problems. Men depended on herbal plants since ancient and this perpetual continuity is attributed to their ready accessibility and socio-cultural reasons. One

of the notable local medicinal plants is from the *Piper* genus. *Piper* species are widely studied in various fields (Jarmillo et al., 2008; Greig, 2004). One of the valuable *Piper* species is the *Piper sarmentosum*. *P. sarmentosum* was discovered earlier from the Northeast India to South China and Malaysia. It is a 30 cm tall monoecious plant which is normally small shrubs but sometimes may grow as climber (Chaveerach et al., 2008). Traditionally, *P. sarmentosum* is used to treat various diseases in many countries. They can be found growing in Malaysia, Thailand, Indonesia, Laos, Vietnam, Cambodia, India, China, Burma, and the Phillipines. In Malaysia, the leaves water decoction is consumed to treat cough, headache, arthritis, and waist pain, the root water decoction is useful in improving urination and curing menstrual pain (Aida Azlina et al., 2009; Wiart, 2006).

Among the latest researches showed that *P. sarmentosum* has insecticidal activity (Hematpoor et al., 2017), antimicrobial activity (Chanprapai and Chavasiri, 2017; Fernandez et al., 2012), antibacterial property (Sharifah Farhana et al., 2016), antihypertensive activity (Mohd Zainudin et al., 2015), antidiabetic properties (Thent et al., 2012), osteoporotic fracture healing properties (Estai et al., 2011), and anti-cholesterolemic properties (Amran et al., 2010). The ethanolic extract of *P. sarmentosum* also possesses anti-inflammatory, antipyretic, and anti-nociceptive activities. *P. sarmentosum* ingestion had been tested in rats and the results showed that there is no toxicity effect in the liver, kidney and haematological profile (Mohd Zainuddin et al., 2013).

Colon cancer occurs when there is an abnormal growth of cells in the colon where food digestion continues and nutrients assimilated. Colorectal cancer begins as a non-cancerous growth known as polyp which develops on the colon inner lining. The cells can spread through the blood vessels to other organs and tissues such as the lungs and liver. Colorectal cancer has no early symptoms. The signs such as bleeding only occur at the late stage of the disease (American Cancer Society, 2017). The International Agency for Research on Cancer estimated colorectal cancer (CRC) as the third leading cause of cancer death in the world for both sexes combined (Bray et al., 2018). Meanwhile in Malaysia, the Malaysian National Cancer Registry Report 2007-2011 recorded colon cancer as the top and the second most common cancers among males and females respectively. Colorectal cancer incidence was also reported to be increasing with age and highest among the Chinese for both males and females, followed by Malays and Indians (Azizah et al., 2016).

Anticancer and antioxidant properties of a plant have attracted the interest of many researchers. Anticancer is the property of a substance to inhibit cancer. Anticancer agent aims to damage the DNA of the cancer cells besides inhibiting the synthesis of new DNA strands to halt the progression of the cancer (Ophardt, 2017). An antioxidant can be defined as a substance that can prevent cellular components, damage arising from the chemical reactions involving free radicals (Young and Woodside, 2001). Free radicals are highly reactive species that can cause oxidative stress, a process that can trigger cell damage (National Centre for Complementary and Integrative Health, 2013). In this present study, *P. sarmentosum* ethanolic extract was tested on colon cancer cell line, HT-29 to study its anticancer properties. The antioxidant and chemical constituents of *P. sarmentosum* were also evaluated to analyse the plants potential activities.

MATERIALS AND METHODOLOGY

Collection of Plant Materials

Piper sarmentosum leaves were harvested from the Glasshouse and Nursery Complex of the International Islamic University Malaysia (IIUM) Kuantan, Pahang. The taxonomic identification was performed by Dr. Shamsul Khamis, a botanist from the Faculty of Science and Technology, The National University of Malaysia (UKM). A voucher specimen (No:

PIIUM 0239-3) was deposited in the Herbarium at the Kulliyyah of Pharmacy, IIUM. The leaves were washed thoroughly with tap water for three times to remove impurities. They were then immediately cut into small pieces using a knife before left to dry in the dryer at a temperature of 60 ^{o}C.

Soxhlet Extraction

The dried leaves sample was pulverised to powder form using a blender. About 70 grams of the leaf's powders were weighed and filled in porous cellulose thimbles with cotton wool loaded tightly on top of those powders within the thimbles. Soxhlet extraction was preceded by loading the thimbles filled with crushed plant materials into the Soxhlet extractor. Following this, absolute ethanol was added to the round bottomed flask. The extraction process continued until the solvent became colourless. The extract was then concentrated using rotary evaporator to obtain crude extract. The percentage yield of the extract was calculated using formula proposed by Mushore & Matuvhunye (2013).

GC-MS Analysis

The characterization of chemical compositions in *P. sarmentosum* leave sample through gas chromatography mass spectrometry (GC-MS) analysis was performed based on the modified version of Syed Ab Rahman et al. (2014). The crude extract of *P. sarmentosum* was diluted in ethanol at the range of 1:100. The solution was then filtered with a syringe and 0.45 µm filter to remove any impurities. 1000 µL of the solution was pipetted into the GC-MS auto sampler glass vial to be analysed. The crude extract was run using the instrument upon admission through autosampler. GC-MS analysis of the ethanolic extract was performed using a Perkin-Elmer GC Clarus 680 system. Helium gas was used as carrier gas at a constant flow rate of 1 mL/min. The analysis was performed for one hour. The interpretation on mass spectrum GC-MS was constructed using the database of National Institute Standard and Technology (NIST) by comparing the spectrum of unknown molecules with the spectrum of known molecules stored in NIST library. The peak areas and retention times were measured by electronic integration. The relative amount of individual components was expressed as a percentage by area. The name, retention time, structure, area, and percentage of the components of the test materials were tabulated.

***In vitro* Cytotoxicity Activity (MTT Assay)**

Human colorectal adenocarcinoma cell line (HT-29) was provided by Dr Mohd Hamzah Mohd Nasir of the Central Research & Animal Facility (CREAM) Institute, IIUM Kuantan.

Cells with Extract Treatment

100 mg of *P. sarmentosum* crude extract was weighed and diluted in 1 mL DMEM 5% FBS into concentration 100 mg/mL. The 100 mg/mL stock solution was diluted to concentration of 200 µg/mL. The solution was filtered with syringe of 0.45 µm filter to strain out insoluble crude extract. 400 µL of 200 µg/mL was pipetted into microcentrifuge tubes for serial dilution. The 96-well plate 24 hours seeded with 10^6 of HT-29 cells concentration was taken out from incubator and the old media was discarded. 200 µL of extract of nine different concentrations (100 – 1.5625 µg/mL) was added into each well. For the negative control well, DMEM 5% FBS was added while for positive control, 1 mM hydrogen peroxide (H_2O_2) of concentration was added. The plate was incubated for 72 hours at 37^{o}C in 5% CO_2. For every 24 hours, the cells were observed under light microscope (Leica, Germany).

MTT Assay

MTT powder was prepared into stock solution of 5 mg/mL. After 72 hours, the old media was discarded from the cells. 100 µL of DMEM with 5% FBS and 20 µL of MTT reagent were

added in each well in the dark. The plate was shaken gently to ensure MTT reagent mixed well within each well. The plate was incubated for 3 hours 37°C in 5% CO_2 in the dark. After the formazan crystal formed, the media was removed from all cells before adding 100 µL of DMSO and shaken the plate to dissolve the formazan crystal. The absorbance was measured in a microplate reader at 570 nm. All the data were analysed using GraphPad Prism software and expressed as percentage cell viable ± (standard error of mean) SEM. A graph was plotted with log concentration over mean percentage of triplicate data. Based on the non-linear regression analysis of the graph, IC_{50} value was determined.

DPPH Free Radical Scavenging Activity Assay

Stock solution of crude extracts was prepared at 10 mg/mL in absolute ethanol and diluted to concentrations of 3.125 µg/mL to 200 µg/mL. Similar concentrations were applied for ascorbic acid as positive control. Meanwhile for negative control, ethanol was used. 100 µL of each concentration was added to the well. Following this, 5 µL of DPPH radical solution was added to each well. The plate was shaken gently before incubated in the dark for 30 minutes. The absorbance was measured at 517 nm using microplate reader. Then, the percentage of DPPH activity was plotted against samples concentration to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (IC_{50}). The assay was performed in triplicate and the data were expressed as mean ± SEM. A one-way ANOVA was performed between *P. sarmentosum* and ascorbic acid for $p<0.05$.

RESULTS AND DISCUSSION

ETHANOLIC EXTRACT OF *Piper sarmentosum*

Soxhlet extraction was conducted to prepare the crude extract of *P. sarmentosum* from the leave samples for *in vitro* cytotoxicity assay. The percentage yield of the extract was 20.52%. The crude extract was used for GC-MS analysis, antioxidant study by DPPH assay and cytotoxicity study against colorectal cancer lines, HT-29.

GAS CHROMATOGRAPHY-MASS SPECTROMETER (GC-MS) ANALYSIS

The chemical constituents of *P. sarmentosum* was analysed with GC-MS instrument. GC-MS is an instrument that coupled gas chromatography (GC) with mass spectrometry (MS). This instrument is used to determine the constituents and their respective amount present in the mixture. Gas chromatograph separates the components present, while mass spectrometer detects them through mass-to-charge ratio. The mass spectrum provides information on the molecular weight and elemental composition of the mixture (Stashenko and Martinez, 2014). From the GC-MS analysis of the crude ethanolic extract of *P. sarmentosum*, 6 chemical compounds were detected. The data showed that myristicin was the main active compound in the crude extract of *P. sarmentosum* (Figure 1 & Table 1).

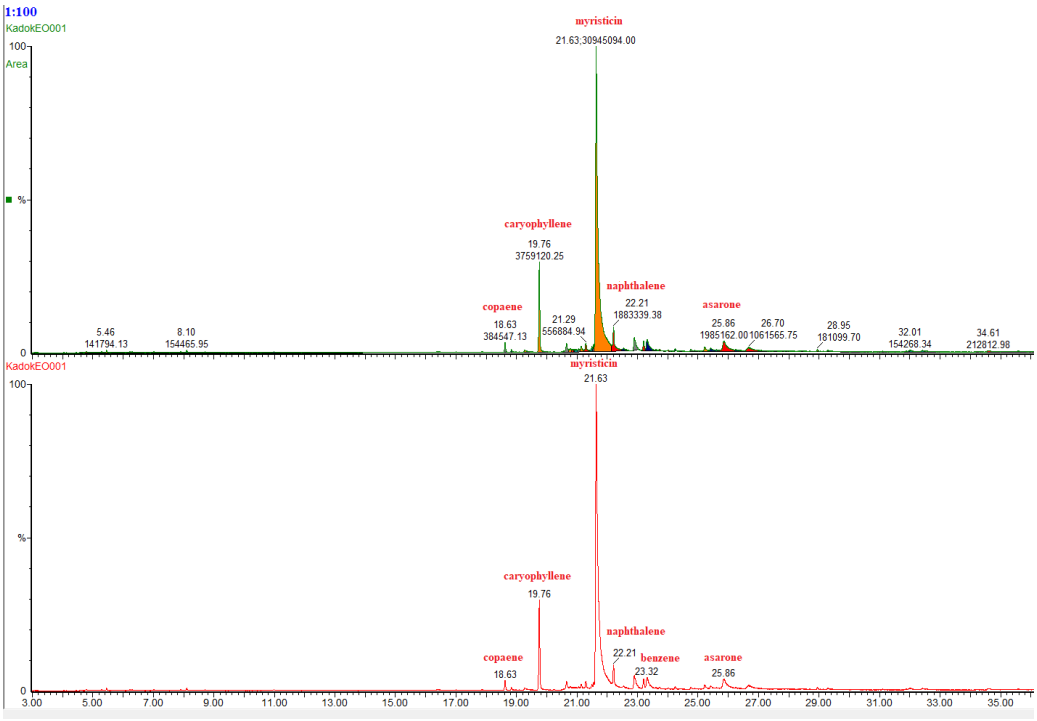

Figure 1 GC-MS analysis of *P. sarmentosum*

Table 1
GC-MS Analysis

Name	Retention time	Area	Percentage
Copaene, $C_{15}H_{24}$	18.63	384 547.13	0.99
Caryophyllene, $C_{15}H_{24}$	19.76	3 759 120.25	9.65
Myristicin, $C_{11}H_{12}O_3$	21.63	30 945 094.0	79.43
Naphthalene, $C_{10}H_8$	22.21	1 883 339.38	4.83
Elemicine, $C_{12}H_{16}O_3$	23.32	-	-
Asarone, $C_{12}H_{16}O_3$	25.86	1 985 162.0	5.10

The results showed that myristicin is the highest in *P. sarmentosum* ethanolic extract followed by β-caryophyllene, asarone, naphthalene, copaene, and lastly elemicine. This is in accordance with the findings by Qin et al. (2010) that found myristicin and caryophyllene as the major components of the species. Myristicin at 79.43% was the compound with the highest percentage. Myristicin or myristin, 1-allyl-3,4-methylenedioxy-5-methoxybenzene, is a naturally occurring alkenylbenzene compound found in various herbs and vegetables such as nutmeg, parsley, and carrot. Myristicin is a polyphenolic compound, and polyphenols are suggested to possess apoptosis inducing properties in its anticancer activity. Plant polyphenols are also able to interfere with proteins that available in cancer cells (Greenwell and Rahman, 2015). Lee et al. (2005) found that myristicin has the ability to induce cytotoxic and apoptotic effects on human neuroblastoma SK-N-SH cells. The second highest compound was β-caryophyllene, a bicyclic sesquiterpene used as cosmetics and food additives (Fidyt et al., 2016). Dahham et al. (2015) reported β–caryophyllene to possess the ability to suppress metastasis of colon cancer, induce apoptosis, antimicrobial properties, and anti-inflammatory activity.

CYTOTOXICITY OF *P. sarmentosum* ETHANOLIC EXTRACT

P. sarmentosum ethanolic extract with concentrations of 1.5625 µg/mL, 3.125 µg/mL, 6.125 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, and 100 µg/mL were tested on HT-29 cells by MTT assay. From the absorbance readings, a non-linear graph as shown in Figure 2 was constructed for the IC_{50} value.

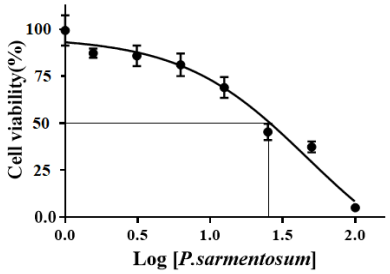

Figure 2 Log [*P. sarmentosum*] to Percentage of HT-29 Cell Viability
Note: the straight line depicted IC_{50} value

The graph depicted IC_{50} at 25 µg/mL from the non-linear regression analysis. Based on the American National Cancer Institute (NCI), the criteria of cytotoxicity activity established that the IC_{50} values of extract must be lower than 30 µg/mL (Yiaile et al., 2018). Therefore, the extracted plant is considered as active against the tested cancer cells due to its low of IC_{50} value. Positive control used in this assay was 1 mM hydrogen peroxide (H_2O_2). H_2O_2 is a broadly effective apoptosis inducer, but the dose ranges from 0.05 to 10 mM that differs by cell type (Xiang et al., 2016). The concentration in this assay was set at 1 mM and was supported by Park et al. (2006) that found 1 mM H_2O_2 was able to induce apoptosis in HT-29. According to the study, a low non-toxic concentration specifically at 10 µM increased cell proliferation than control, but a high toxic concentration at 1 mM increased nuclei cleavage or apoptosis of the cells.

ANTIOXIDANT ACTIVITY OF *P. Sarmentosum* ETHANOLIC EXTRACT

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method is considered a valid, easy and economic method to evaluate radical scavenging activity of antioxidants as the radical compounds are stable and need not be generated (Kedare and Singh, 2011; Huang et al., 2005). *P. sarmentosum* ethanolic extract was tested for its antioxidant activity and the result was interpreted as graph in Figure 3.

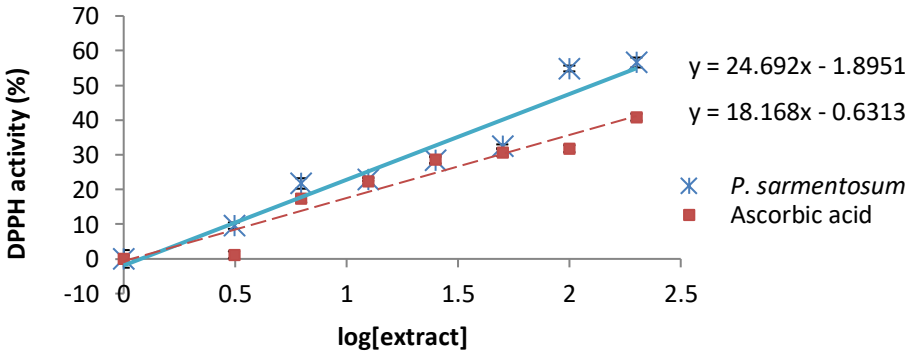

Figure 3 DPPH Activity to log[sample] of *P. sarmentosum* and Ascorbic Acid (Positive Control)

From the linear equations, the IC_{50} of *P. sarmentosum* was 126.39 µg/mL while ascorbic acid was at 612.124 µg/mL. The IC_{50} value of *P. sarmentosum* was lower than ascorbic acid demonstrating *P. sarmentosum* to have a higher antioxidant activity than the positive control. Through a one-way ANOVA analysis conducted, all the data were $p<0.05$, demonstrating that *P. sarmentosum* data were significantly different from that of ascorbic acid.

CONCLUSION

Piper sarmentosum was discovered to be a good anticancer and antioxidant agent. Anticancer properties of *P. sarmentosum* was positive on HT-29 with an IC_{50} value of 25 µg/mL that is in accordance with the criteria established by American National Cancer Institute (NCI) of IC_{50} value lower than 30 µg/mL. The antioxidant effect of the extract was found to be higher than the positive control. The statistical data showed there is significant difference between *P. sarmentosum* and positive control (ascorbic acid). Through GC-MS, the chemical constituent of *P. sarmentosum* was evaluated with myristicin as the highest percentage in the extract. Thus, it can be concluded that the ethanolic crude extract of *P. sarmentosum* was relatively active by its anticancer and antioxidant properties.

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