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Anti-Cancer Potential of Pseuduvaria Macrophylla in Human Cancer Cell Lines

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Abstract – *This is a short report on the recent investigation on the anticancer and antioxidant potential of Pseuduvaria macrophylla stem bark and leaf extract on human breast cancer cell (MCF-7), human prostate cancer cell (PC-3) and human colon cancer cell (HT29). LCMS experiment was done to identify the chemical composition in the methanolic extracts while GCMS analysis was done to determine the chemical composition in the hexanolic extracts. For antioxidant activity, the most active extract was leaf methanolic with 69.22 % of DPPH inhibition. Additionally, the leaf methanolic extract inhibited the better proliferation especially on human breast cancer cell (MCF-7) compared to hexane crude extracts due to more than 90% of cancer cell death and lowest IC50 value at range of 80.70 µg/mL ± 0.08 – 106.70 µg/mL ± 0.07. The major detected compounds were α-Cadinol, neophytadiene, palmitic acid, linoleic acid, oleic acid and isopolycerasoidal and isopolycerasoidal methyl ester. The findings of this study support the anti-cancer claims of Pseuduvaria macrophylla leaf.*

Keywords: annonaceae, antioxidant, anti-cancer, oxidative stress, MTT assay

1.0 INTRODUCTION

In a provincial disease registry, cancer is classified by the type of cell that is initially affected. The World Health Organization (WHO) estimated that 7.6 million of deaths in 2005 were caused by cancer with the majority of cases from lung, stomach and breast cancer [1]. Based on the latest Health Facts 2013 released by Ministry of Health (MoH) Malaysia, cancer is one of the top five causes of death in Malaysia [2]. In Malaysia, the incidence of cancer increased from 32,000 new cases in 2008 to about 37,000 in 2012 [3]. The prevalent type of cancer on the list is breast cancer [4]. Chinese women has listed as the most notable with almost 60 every 100,000, followed by Indians with 55.8 every 100,000 and Malays with 33.9 every 100,000 [5].

The next most common cancers are prostate cancer and colorectal cancers. Same as breast cancer, Chinese had the outstanding reported occurrence of colorectal tumor compare to other races. Prostate cancer is positioned sixth among male diseases and expected to increase each year [6]. In general, various synthetic anti-cancer drugs have been given to cancer patient as a pain relievers. Nevertheless, in long term usage, those drugs are more likely to produce side effects. Hence, an alternative approach from natural plant products is essentially needed for the treatment of cancer. Unfortunately, ethno botanical and traditional use of the medicinal plants has rarely been studied and the effort of establishing the documentation is still on-going [7] Pseuduvaria macrophylla is a member of the Annonaceae family traditionally known as Cagau Biasa which comprises of 119 plant genera and about 1,750-1780 species [8]. Majority plant from this family are reported to contain secondary metabolites including acetogenins, terpenoids, phenolics and alkaloid compounds with various biological activities [9,10]. This species has been studied for various biological activities but little is known on its anticancer effect. Therefore, a short report on the recent study [21] was done to provide additionally finding from the previous study [10].

2.0 MATERIALS AND METHODS

2.1 Chemical Reagents and Solvents

Chemicals that have been used were Fluorescence sodium sail solution, DPPH, analytical grade Fisher Scientific Methanol, AAPH solution analytical grade Fisher Scientific Hexane Polyvinyl pyrrolidone (PVP), Trolox standard,Copper, 0.1% Formic Acid, Ascorbic Acid, gallic acid (Sigma – Aldrich, German) and Folin–Ciocalteu's phenol reagent.

2.2 Plant Source

Pseuduvaria macrophylla was collected from Kenong Forest Park, Malaysia. Voucher specimen (HIR 0009) was deposited at the herbarium, Chemistry Department of the University of Malaya.

2.3 Extraction

Methanol and hexane were used to extract *P. macrophylla* leaves and bark. About 2 L of solvents was poured into a flask containing 500 g of the sample. The solution was left for three days before supernatant was removed. This procedure was repeated atleast twice before filtered it to obtain the solvent extract. Solvents have been removed by means of rotary evaporator, yielding the extracted compounds.

2.4 DPPH Assay

An amount of 25 µL of extracts was added to 175 µL of methanol in a 96-well microplate. The DPPH assay was performed according to the method reported by Orhan *et al*., (2009) and Brem *et al*, (2004) with modifications. Briefly, 50 µl of 0.02% stable DPPH free radical in 100 mL methanol has been added to 20 µL of standard/sample/control and 130 µL of methanol (total assay volume 200 µL) in a 96-well plate. As positive control, Ascorbic acid was used and blank solvent acted as negative control. The SUNRISE Microplate Absorbance Reader was used at absorbance of 517 nm.

2.5 Half Maximal Inhibitory Concentration (IC_{50})

IC_{50} represents the concentration of a substances (inhibitor) that is required to inhibition 50 % of particular biological process. IC_{50} was calculated in DPPH assay to measure the effectiveness of each concentration of sample to inhibit 50% free radical activity. A serial dilutions of samples and standards were prepared before being subjected to DPPH assay.

2.6 The oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed according to Huang *et al*. (2002), in a 96-well black microplate with 25 µL of samples/standard/positive control and 150 µL of fluorescence sodium salt solution, followed by 25 µL of AAPH solution after 45 min incubation at 37°C (200 µL total well volume). Serial dilutions for the Trolox standards were prepared accordingly. The positive control was ascorbic acid and the negative control was blank solvent/PBS. Finally the fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm using Plate Chameleon ™ V Multilabel Counter (Hidex, Turku, Finland). The results were expressed as µM of Trolox Equivalents (TE) per 100 µg/mL of sample.

2.7 MTT cell proliferation assay

MCF-7, PC-3 and HT29 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies, Inc, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine, 1% penicillin and streptomycin. An amount of 1×10^4 human cancer cells per well were seeded into 96-well plate overnight. Cells were treated with various concentrations of compound or extract (dissolved in dimethyl sulfoxide, DMSO) for 24 hours. As negative control, cells will be treated with vehicle (DMSO) only. Next, cells were incubated with 50 µl of MTT (2 mg/ml) at 37°C for 2 hours. After dissolving the formazan crystals in DMSO, plates were read in Chameleon™ multitechnology microplate reader (Hidex, Turku, Finland) at 570 nm against 620 nm. This experiment was performed in triplicates. Mean values ± SD for each concentration will be determined. Cell viability (in percentages, %) has been shown as ratio of absorbance ($A_{570\ nm}$) in treated cells relative to absorbance in control cells (DMSO) ($A_{570\ nm}$). The IC_{50} was defined as the concentration of sample needed to reduce 50% of absorbance relative to the vehicle (DMSO)-treated control [22].

2.8 GCMS Identification and Chemical Analysis of Crude Extract

GCMS was used for the analysis of non-polar compound. The plant extract was performed on JEOL JMS 600H Aglest 68, equipped with 30m x 0.32 mm HP-5 column, stationary phase coating 0.50 um. The temperature of the column should be started at 250°C for 2 min and the temperature rate was constantly increased at the rate of 5°C per min up to 250° C. Helium gas act as a carrier gas with flow rate at 1.8 ml/min. The temperature was used for injector also same as column temperature. The standard split ratio used was 1: 35. To prepare sample, 1 mg of hexane extract was needed and reconstitute with 1mL solvent [21].

2.9 Liquid Chromatography Mass Spectrophotometry/Mass spectrophotometry (LCMS/MS- IT-TOF)

Polar compound is best measured by LCMS/MS-IT-TOF. One mg of plant extract was dissolved with 1 mL of methanol which give concentration of 1mg/mL. A mass spectrum was

recorded on LCMS/MS Shimadzu IT-TOF. The column used for this analysis was Waters X-Bridge RP C-18 column (2.1 mm id x 50 mm, particle size 2.5 um). For solvent A, deionized water in 0.1% Formic Acid was used whereas for solvent B HPLC grade acetonitrile in 0.1% Formic Acid was used [21].

3.0 STATISTICAL ANALYSIS

Each experiment was performed in triplicates. Results were expressed as the means values ± standard error of the means (SEM). The data were analyzed for significance by one-way ANOVA. The test value of $p<0.05$ was considered significant. The graphing was performed using MS Excel 2007 to calculate the percentage of DPPH inhibition, AUC, IC_{50}, TPC and Graphpad Prism 4 Software to obtain the samples' values.

4.0 RESULTS AND DISCUSSION

Human require oxygen to breathe through respiration process, therefore trigger the body constantly to react with oxygen to produce more energy. As the consequence of this activity, free radicals and oxidative stress occurs within the cells and exposed human to many critical health problem such as cancer. Literature survey documented various pharmaceutical potential of the other member of Annonaceae family thus supporting the additional findings of this particular species in the present study [10-13]. The antioxidant capacities of methanol and hexane extracts of *P.macrophylla* in DPPH assay are shown in Fig. 1.

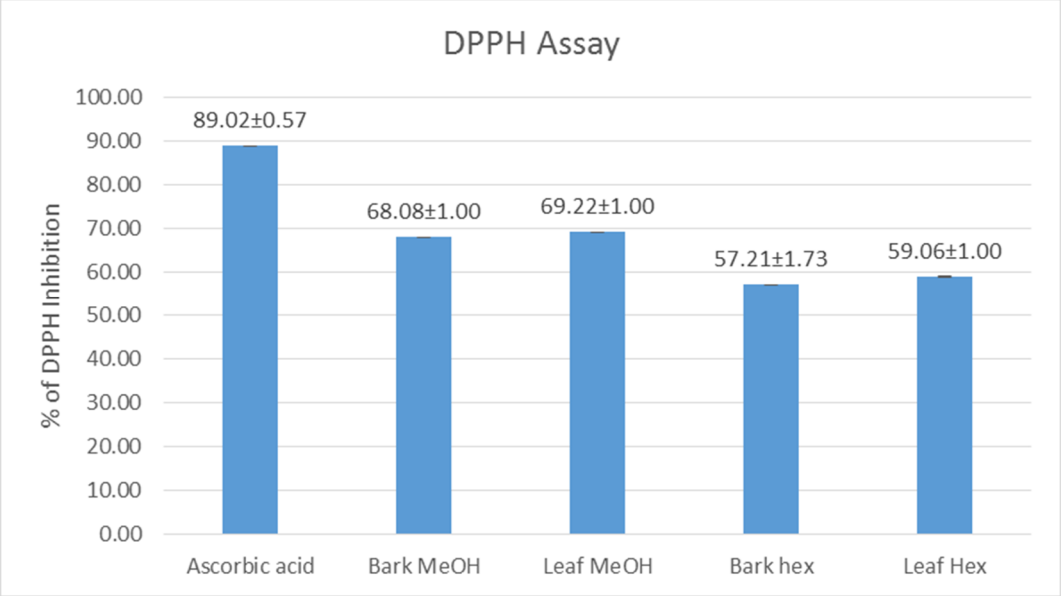

Figure 1: The percentage (%) of DPPH Inhibition of plant extracts. (Each value is the average of three measurements ± SEM)

The results show that the percentage inhibition of 25 µg/ml of leaf methanolic extracts is 69.22%, which is lower than the standard/ascorbic acid (89.02%). Methanol leaf extract has the highest level of protection against free radicals followed by leaf hexane extract. The percentage of DPPH inhibition is lowest in the bark hexanolic leaves extract.

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In DPPH assay, the half maximal inhibitory concentration (IC_{50}) of all extracts were measured to determine the concentration of plant extracts needed to inhibit free radical activity by half. The IC_{50} values of *P.macrophylla* extracts were only calculated in DPPH assay since this assay only measured the percentage of DPPH inhibition without developing a standard curve as in ORAC assay.

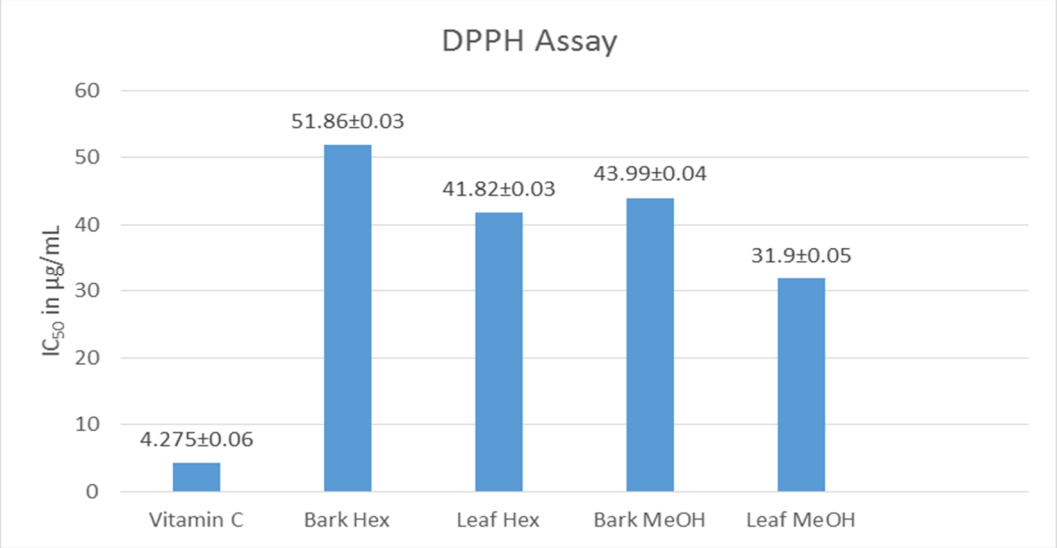

Figure 2: The IC_{50} value of antioxidant activity. (Data are mean ± SEM)

The crude methanolic extract of the bark and leaf of *P. macrophylla* conveyed better antioxidant activity with lower IC_{50} values compared to hexanolic extracts. Test of homogeneity of variances using one-way ANOVA displayed that methanolic extracts have significant value ($p<0.05$) compare to hexanolic extracts in DPPH assay.

An optimum IC_{50} value in other Annonaceae species provided by previous study is within 32-128 µg/mL (Kiatfuengfoo *et al*., 2011). Leaves extracts of *P. macrophylla* convey higher antioxidant activity against DPPH free radical compare to hexanolic crude extracts with IC_{50} values of 31.9µg/ml±0.05, 41.82µg/ml±0.03, 43.99µg/ml±0.04 and 51.86 µg/ml±0.03 respectively (Fig. 2).

Since ORAC assay is a kinetic assay, the Area under curve (AUC) value of Trolox standards were determined by calculating the Relative fluorescence unit in each extracts within 30 minutes. Trolox is a water soluble vitamin E and also an antioxidant. AUC refers to the quantity of fluorescent molecule (fluorescein) protected by potential antioxidant from samples. All extracts and positive control were prepared at a concentration of 20µg/mL and the results have been reported as Trolox Equivalents (TE) in µM (Table 1). As a positive control, Vitamin C was used meanwhile methanol blank acted as a negative control.

The antioxidant capacity of the plant extracts evaluated by the ORAC method (Table 1) illustrated that the TE values for bark and leaf methanolic extracts reflect similar results as DPPH assay ($p<0.05$) by which methanolic crude extracts shows greater activity compared to the hexanolic crude extracts. In addition, test of homogeneity of variances using one-way ANOVA showed that leaves extracts exhibited significant activity ($p<0.05$) compare to bark extracts in both assays.

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Table 1: Antioxidant capacity of plant extracts evaluated by the ORAC method.

Extract	ORAC (μM TE)
Leaf Methanol	19.61±0.18
Bark Methanol	18.92±0.06
Bark Hexane	4.65±0.02
Leaf Hexane	13.15±0.49
Ascorbic Acid	49.22±0.05

Each value is the average of three measurements ± SEM.

Among all of the extracts, leaf methanolic extract of *P.macrophylla* demonstrated highest radical scavenging property against free radical in DPPH assay with smallest concentration of IC_{50} value to inhibit free radical activity by half. In ORAC assay, the obtained results reflect similar finding as DPPH assay which shows that methanolic crude extracts especially leaf has greater antioxidant activity compared to the hexane crude extracts. High level of antioxidant of methanolic extracts in both assays has been supported by the findings from other study which showed that high antioxidant activity in methanol extract of *Pseuduvaria macrophylla* was responsible for the elevation of blood glucose levels and improved serum insulin and C-peptide levels by reducing the level of glutathione (GSH) and lipid peroxidation (LPO) which led to their anti-diabetic potential. Additionally, this plant was also able to protect the pancreas of diabetic rats against peroxidation damage by downregulating oxidative stress and elevated hyperglycaemia based on histopathology analysis [10].

All the tested methanolic and hexanolic extracts exhibited very different cytotoxicity against the three selected human cancer cell lines. One-way ANOVA using test of homogeneity of variances has been used to calculate cytotoxicity activity for each extract. As shown in Fig. 3 and Table 2, leaf methanolic extract strongly inhibited the proliferation of all tested cancer cells especially the breast cancer (MCF-7) compare to other extracts due to more than 90% of cancer cell death and lowest IC_{50} value at range of 80.70 μg/mL ± 0.08 – 106.70 μg/mL ± 0.07. Meanwhile, leaf hexane extract showed inhibition of the proliferation mostly in breast cancer cell followed by colon cancer cells and prostate cancer cells.

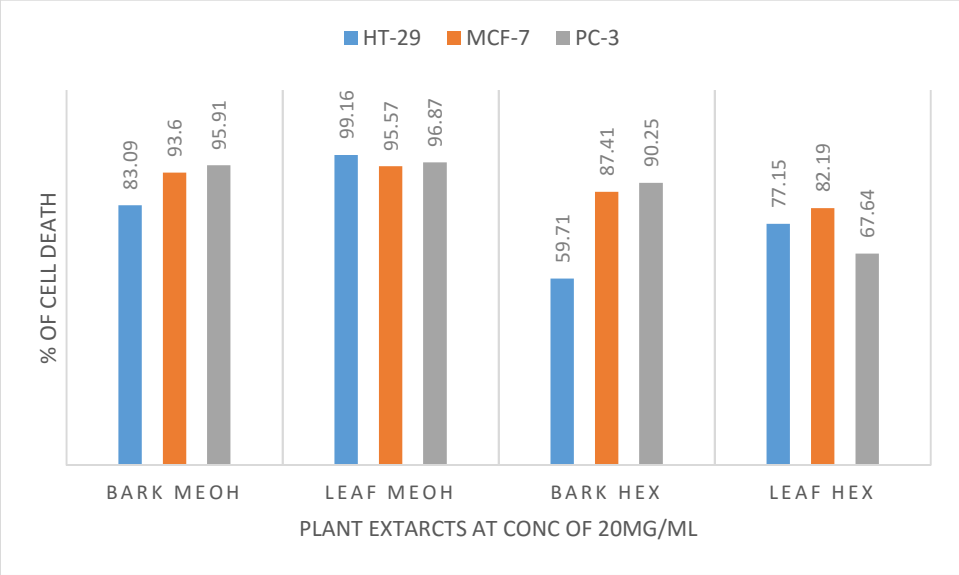

Figure 3: The percentage of cell death of crude extracts at concentration of 20 *ug/mL*.

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Table 4: Effect of P. macrophylla extracts on cells expressed as IC_{50} values in 24 hours MTT assay.

	Bark MeOH	Leaf MeOH	Bark Hex	Leaf Hex
Cell lines				
HT-29	100.13 µg/ml±6.99	94.36 µg/ml±0.33	264.14 µg/ml±3.04	99.83 µg/ml±0.80
MCF-7	85.74 µg/ml±1.44	80.70 µg/ml±0.08	252.33 µg/ml±0.59	95.67 µg/ml±0.40
PC-3	94.96 µg/ml±3.57	106.70 µg/ml±0.70	261.59 µg/ml±5.64	108.65 µg/ml±0.10

* IC_{50} which represent the concentration of plant extracts causing a 50% cytotoxic effect. Mean±SEM

In this context, it has been proved that, few extracts have potential anti-cancer properties, which responsible for the high cytotoxicity towards tested cancer cells [15]. Our study revealed that the leaf methanolic extract showed significant and potent cytotoxicity towards the human breast cancer cells followed by prostate and colon cancer cells. Other experiment on the leaves of *Pseuduvaria* species has composed of tremendous compounds such as aporphine alkaloid; 8-hydroxy-1,4,5-trimethoxy-7-oxoaporphine or 8 hydroxyartabonatine C [11], 1,2,3-trimethoxy-5-oxonoraporphine and ouregidione-induced which reported to potentially have anti-tumor activity [14]. In addition, *Pseuduvaria* species may able to induce cell death via apoptosis with an increase in phosphotidylserine exposure demonstrated by flow cytometry [15].

The chemical constituent analysed by GCMS reported detected 6 major compounds which are α-Cadinol, Neophytadiene, Palmitic acid, Linoleic acid and Oleic Acid (Table 5 & Table 6). Meanwhile, polar chemical compounds analysed by LCMS reported 2 compound which are Isopolycerasoidol & Isopolycerasoidol methyl ester (Fig. 5 and Fig. 6).

Table 5: Compounds tentatively identified in *Pseuduvaria macrophylla* leaf hexane crude.

Peak No	RT[a]	Percentage of the peak[b]	Molecular weight	Molecular formula	Similarity index	Compound
1	14.965	14.8934	222	C15 H26 O	90	α-Cadinol
2	17.170	19.7944	278	C20 H38	94	Neophytadiene
3	18.519	23.7536	256	C16 H32 O2	93	Palmitic acid
4	20.360	41.5685	283	C18 H32 O2	93	Linoleic acid
Total		**100**				

Table 6: Compound tentatively identified in Pseuduvaria macrophylla bark hexane crude extract.

Peak No	RT[a]	Percentage of the peak [b]	Molecular weight	Molecular formula	Similarity index	Compound
1	18.107	24.7798	270	C17 H34 O2	96	Methyl ester
2	20.420	75.2202	282	C18H34O2	85	Oleic Acid
Total		**100**				

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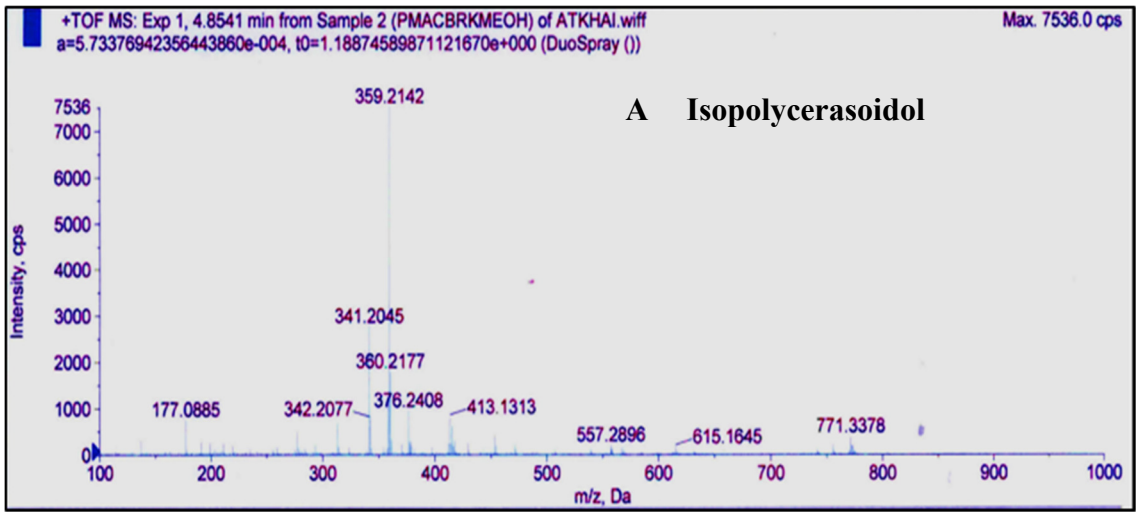

Figure 5: Mass fragmentation of isopolycerasoidol in bark and leaf methanol extract.

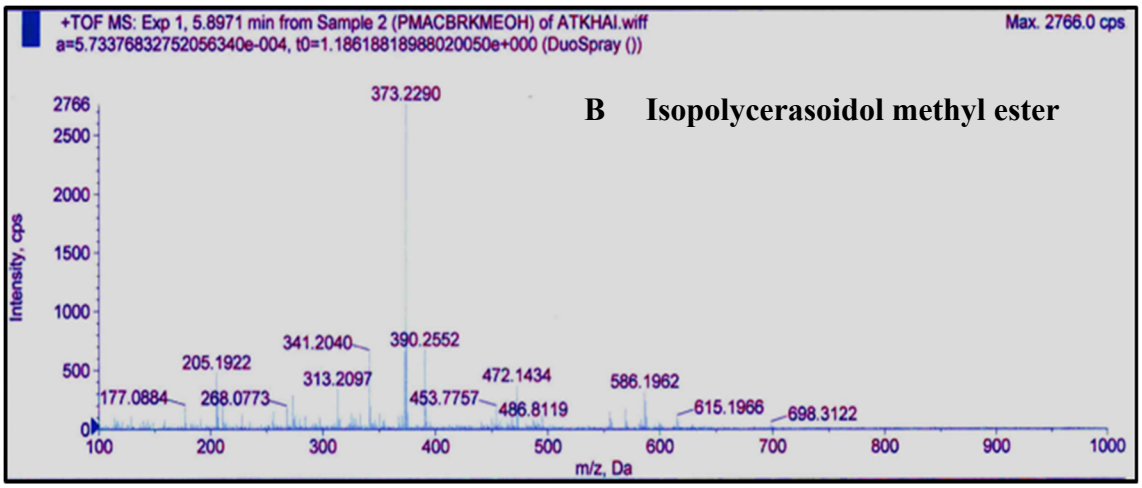

Figure 6: Mass fragmentation of isopolycerasoidol methyl ester in bark and leaf methanol extract.

Phytochemical analysis of bark and leaf methanolic and hexanolic extracts of P.macrophylla revealed the presence of seven major constituents; alkaloids; α-Cadinol 1, Neophytadiene 2, fatty acids; Palmitic acid 3, Linoleic acid 4, and Oleic Acid 5 based on the peak intensity. Meanwhile, polar chemical compounds reported 2 outstanding compound which are alkaloid; Isopolycerasoidol 6 & Isopolycerasoidol methyl ester 7 based on the structure of the same mass fragmentation found in the previous study [15] which could be responsible for the anticancer activity. One of the major compound in leaf hexane extracts, α-Cadinol was proposed as a possible remedy for drug-resistant tuberculosis [16]. In addition, it has been reported that α-cadinol shows selective toxicity especially against human colon adenocarcinoma cell line HT-29 [17]. On the other hand, 2 major compound detected in methanolic extracts, Isopolycerasoidol & Isopolycerasoidol methyl ester may induce mitochondrial-mediated apoptosis in human breast cancer cells by elevating ROS production causing reduction of mitochondrial membrane potential and increasing plasma membrane permeability in breast cancer cells. This finding has become the first pharmacological evidence on *P.macrophylla* for their future development as anticancer agents [Taha et al., 2015]. Additionally, previous

experiment on medicinal plants proved that alkaloids and phenols does contribute to the anti-cancer activity of the plants [18].

The presence of Neophytadiene compound in medical plant contributed to the significant level of antioxidant activity [19] and this may lead to the elevation cytotoxic effect of *P.macrophylla* against tested cancer cells. The chemical constituent in leaf crude extract analysed by GCMS demonstrated the presence of common fatty acids which are Linoleic acid (polyunsaturated). And Palmitic acid (saturated). These compound was believed to be active against human cancer cell lines by which Palmitic acid induces apoptosis in the human leukemic cell line MOLT-4 at 50 micrograms/ml whereas Linoleic acid potentially obstruct the development and spread of malignant tumors, primarily by influencing cell replication and systems of carcinogenesis [20].

5.0 CONCLUSION

Altogether, the study results conclude that *P.macrophylla* leaf methanolic extract has a strong potential as a treatment for cancer as it may upregulating the antioxidant defense system and inducing cell death via apoptosis.

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