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Screening and identification of anti-cancer agents in a selected indigenous plant

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GRAPHICAL ABSTRACT

The selected indigenous plant used for ultra-pure water extraction and decoctions method.

ABSTRACT

A selected indigenous plant (species name not revealed to protect from illegal harvesting) from Borneo is used to treat breast cancer by the local tribe in Sarawak traditionally. However, due to insufficient scientific data, this traditional practice is not globally accepted as potential cure of cancer. To recognize the indigenous plant as potential cure, screening and identification of the potential anti-cancer agents were done using UHPLC-Q-TOF/MS and UHPLC-QQQ/MS. Extractions using ultra-pure water and decoctions in presence of ethanol were used. There were total of eighty two recognized compounds identified from the UHPLC-MS/MS analyses where eight of them were known anti-cancer agents while three of them were potential anti-cancer agents. The classes compound of known and potential anti-cancer agents identified were phenylpropanoids, pyrimidine nucleosides, terpenoids, c-Jun NH2-terminal kinase (JNK) inhibitors and sphingolipids. This study successfully served as first scientific proof that the selected indigenous plant has anti-cancer active compounds and can be further studied as potential cure for cancer.

Keywords: Anti-cancer, UHPLC-MS/MS, indigenous plant, ultra-pure water extractions, decoctions, phytochemicals.

1. INTRODUCTION

Anti-cancer agent is the term used for compounds that have anti-tumor properties. They generally help inhibited the cancer cells growth with their properties. Plant has a complete natural formula which is excipient to each other. Each compounds form plants has its own constituents which helps reduced cancer activity towards the cells. Currently, some of the group anti-cancer agents discovered from phytochemicals are terpenes, carotenoids and phenolics, alkaloids, nitrogen containing compounds, organosulphur compunds and even sphingolipids [1]. Plants are well-known as the major source of phytochemicals and largest source of anti-cancer compounds found naturally in natures.

The selected indigenous plant is used by local tribe in Sarawak to treat cancer traditionally. However, this traditional practice is not globally accepted as potential cure due to insufficient scientific data of the plant. Hence, in order to recognize it as potential cure, screening and identification of anti-cancer agent in the selected indigenous plant served as the first step in publishing scientific data of this plant. Figure 1.1 showed the selected indigenous plant.

Figure 1.1 The selected indigenous plant

The screening and identification were done using ultra-pure water extractions and decoctions method. It is then analysed using UHPLC-MS/MS. UHPLC-MS/MS were used due to its suitability for analysis of complex mixture compounds which commonly found in biological matrices such as plant tissues. Advantage of UHPLC-MS/MS is that separation and structural elucidation of compounds can be achieved in a continuous manner without the need for purification or derivatization [2]. The use of tandem MS is very beneficial because the capacity to perform multi reaction monitoring (MRM) has the advantage of rapid and sensitive detection of several compounds even if they show similar retention times during liquid chromatography separations.

This research emphasized on identifying anti-cancer agents and its class compounds through literature reviews, database searches and additional reading materials. Successfully, the total identified known anti-cancer agents in this research were 8 while identified potential anti-cancer agents were 3.

2. EXPERIMENTAL

2.1. Dry weight analysis

Three plant samples (~0.5 g) were dried using oven (model UN 110, Memmert, Germany) at ~95°C for 5 days until constant weight was achieved. Weight initial, final, average weight losses were recorded.

2.2. Ultra-pure water extractions

Three plant samples (~0.5 g) were ground using a clean pestle and mortar. Ground samples were mixed with 5 mL ultra-pure water, sonicated for 68Hz in 120 minutes (model Branson 3800 Ultrasonic Cleaner - $1\frac{1}{2}$ gallon, Branson Ultrasonics, USA) and were left overnight. The sonicated samples were centrifuged for 30 min at 5000 rpm (model EBA 20, Hettich Zentrifugen, Germany) and the supernatants were removed. The supernatants (2.5 mL) were diluted with 7 mL of 99.7% ethanol. The diluted samples were filtered using 0.45 µm and consequently, 0.22 µm PTFE filters. The filtrates (1 mL) were diluted again 4 times with 99.7% ethanol. The diluted solutions (2 mL) were sent for analysis using Agilent 6520 Accurate-Mass Q-TOF LC/MS and Agilent 6410 Triple Quad LC/MS (AgilentCorp., SantaClara,CA, USA).

2.3. Decoctions

Four plant samples (~0.5 g) were ground using a clean pestle and mortar. Ground samples were boiled with ultra-pure water for 30 min using a hot plate at 100°C until 50 mL final volume remaining. Boiled samples were centrifuged for 30 min at 5000 rpm (model EBA 20, Hettich Zentrifugen, Germany) and the supernatants were removed. Supernatants extract were filtered using 0.45 µm and consequently, 0.22 µm PTFE filter. The filtrates (1 mL) were diluted 4 times with 10% (v/v) ethanol. The diluted solutions (2 mL) were sent for analysis using Agilent 6520 Accurate-Mass Q-TOF LC/MS and Agilent 6410 Triple Quad LC/MS (AgilentCorp., SantaClara, CA, USA)

2.4. UHPLC-MS/MS parameters

UHPLC-Q-TOF/MS analysis was using Agilent 6520 Accurate Mass Q-TOF LC/MS coupled with Agilent 1290 Infinity Series UHPLC consists of binary pump, an online vacuum degasser, an auto sampler, and a column oven, through an electrospray ionization (ESI) source (AgilentCorp., SantaClara, CA, USA). The compounds were separated using an Agilent eclipse XDB-C_{18} column (150 mm × 4.6 mm, 5.0 µm, Agilent, USA) and the column temperature was set at 35˚C. The mobile phase was acetonitrile (solvent A) and water with 0.1% acetic acid (solvent B) at gradient elution of 0–5 min, 30–100% A; 5–10 min, 100% A; 10-11 min, 100–30% A; 11–30 min, 30% A. The flow rate was 0.5 mL/min with 1 µL injection volume. The ESI source with positive ion polarity mode was used. The drying gas was liquid nitrogen at flow rate of 8.0 L/min, temperature of 350˚C, 45 psig nebulizer, 4000 V capillary voltage and 125 V fragmentor voltage. The acquisition mode used was MS followed by auto MS/MS. The MS/MS analysis was conducted on multiple reactions monitoring (MRM) mode. Data acquisition was performed using MassHunter software, version B.06.01.

UHPLC-QQQ/MS analysis was using Agilent 6410 triple Quad LC/MS coupled with Agilent 1290 Infinity Series UHPLC (AgilentCorp., SantaClara,CA, USA). The separation of UHPLC, ESI source of parameter and drying gas parameters used were same setting as UHPLC-Q-TOF/MS analysis. The acquisition used was MS/MS with MRM mode. Data acquisition was performed using MassHunter software, version B.06.01.

3. RESULTS AND DISCUSSIONS

3.1. Dry weight analysis

From the analysis, percentage average weight remained was 54.8205% ± 2.7410 which means 45.1795% ± 2.2590 water molecules were loss from the samples. Weight remained was used to calculate concentrations dry weight basis of the sample.

3.2. Screened and identified anti-cancer agents

The analyses results obtained presented the relative name of compounds identified and its chemical formula from the instrument's data base. The recognized compounds identified from analyses were investigated by cross referenced with databases available such as ChemSpider, PubChem and Kegg for their anti-cancer properties. In addition, the reliability and evidence of each compound as anti-cancer agents were further investigated by literature reviews of journals available. The

compounds were then categorized into known anti-cancer agents or potential anti-cancer agents. Based on the analyses, the total compounds recognized were 82 where 8 were known anti-cancer agents and 3 were potential anti-cancer agents. Some of the known and potential anti-cancer agents were from group of phytochemicals such as terpenoids and phenylpropanoids.

3.3. Identification of known and potential anti-cancer agents

The known anti-cancer agents were identified based on review of in vivo and/or in vitro of human or animal cancer cell lines preclinical studies, clinical studies, cytotoxicity studies of cancer cell lines and other possible test that proved the compounds had anti-cancer activities. The known anti-cancer agents were also selected based on the test that used the compound alone, or the compound alone with its derivatives. Basically, the known anti-cancer compounds were selected if it had anti-cancer activities when tested directly using itself in any published and recognized tests.

The potential anti-cancer agents were identified based on review of in vivo and/or in vitro of human or animal cancer cell lines preclinical studies, clinical studies, cytotoxicity studies of cancer cell lines and other possible test that proved the compounds had anti-cancer activities. However, for potential anti-cancer compounds, most of them are yet to be thoroughly tested with those tests. This means the test used the compound indirectly which includes combination of the compound with others or tests that involved its derivatives only. Basically, the potential anti-cancer compounds were selected only if it had anti-cancer activities when tested with combination of other compounds or by only its derivatives in any published and recognized tests. There are three potential anti-cancer agents identified in this study

There were 8 identified known anti-cancer agents that were from phenylpropanoids, pyrimidine nucleosides, sesquiterpenoids, JNK inhibitors and sphingolipids class compounds while 3 of the potential anti-cancer agents comes from sphingolipids and terpenoids. The classes compound that exhibited anti-cancer activities would be discussed in details in section below.

3.3.1. Phenylpropanoids

Phenylpropanoids are parent molecules for biosynthesis of numerous structurally and functionally diverse plant polyphenols that play multiple essential roles in plant physiology [3]. Lignin is a constituent of cell walls of almost all dry plants and is a subclass of phenylpropanoids. There are three major classifications of lignins depending on the plant species which are lignins of softwoods (gymnosperms), lignins of hardwoods (angiosperms), and lignins of grasses (non-woody or herbaceous plant) [4].

Lignin is considered as the third most abundance polymer in nature, topping 300 billion metric tons on earth. In plant tissues, lignin is bonded to polymers like cellulose and hemicellulose. Lignin and cellulose work synergistically in providing structural function of plant. The lignin or epoxy resin provide stiffness and rigidity while the cellulose and fibrous components provide the primary load-bearing element matrix. Lignin may have different possibility pattern of bonding between its individual units making the stuructures to be complex. Lignins and its derivatives complexes have anti-oxidant, anti-hypertensive and anti-cancer properties.

Burseran or 3-(3,4-methylenedioxybenzyl)-4-(3,4,5-trimethoxybenzyl)tetrahydrofuran is a lignin isolated from *Bursera microphylla* (Burseraceae) A. Gray. This plant is used in traditional medicine to make a tincture for gum sores, cold sores, and abscessed teeth.

The very first paper that reported burseran cytotoxicity was from Cole et al., (1969). In their paper, burseran was reported to show activity in the cell culture test system of the Cancer Chemotherapy National Service Center (CCNSC) [5]. Since the discovery, this paper has been used to investigate further properties of Buserace species and their anti-cancer activities towards cancer cell lines.

In another study, burseran's anti-proliferative properties were investigated against murine cancel cell lines (RAW246.7). The cell viability assay was evaluated using modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] calorimetric method. At half maximal inhibition concentration (IC_{50}) of 0.4 μM burseran, it showed active anti-proliferative activity against RAW246.7. This results proven that burseran has anti-cancer properties [6]. Figure 3.1 show chemical structure of burseran.

3.3.2. Pyrimidine nucleosides

Pyrimidine nucleoside is one of the primary analogues of nucleosides and nucleobase family which includes anti-viral agents, immunosuppressive molecules and cytotoxic compounds. The pyrimidine nucleosides behave as anti-metabolites while competing with physiological nucleosides and interacted with many intracellular targets to induce cytotoxicity. Recently, progress in identification and characterisation of nucleoside transporters and the enzymes of nucleoside metabolism has been made and greater understanding of molecular mechanisms of the anti-cancer activity made it possible to assess their anti-cancer effects.

Cytarabine is a deoxycytidine derivative of pyrimidine nucleoside analogue is commonly used in the treatment of acute leukaemia without activity in solid tumors. In the treatment of acute leukaemia, 30% cases reduction were observed at conventional doses of 100–200 mg/m^2 that administered intravenously each day from days 1–7. In addition, 65-75% complete reduction rate was achieved in previously untreated patients and 30–50% in patients with reoccurrence of acute myeloid leukaemia when cytarabine was administered with combination of anthracycline. At high doses (2–3 g/m^2 infused intravenously over 2–3 hours at maximum of 12 doses), reduction cases rate of 65-75% were achieved [7].

In an initial dose-findings study, effective concentration of cytarabine at 100-200 mg/kg and its new analogue, P-4055 (Elaidic Acid-Cytarabine) at 20-30 mg/kg successfully prolong survival rates in nude mice. The Raji Burkitt's lymphoma leptomeningeal carcinomatosis model investigation showed that nude mice and rats treated with P-4055 had longer survival days (>70 days) compared to cytarabine-saline treated with mean of 13.2 days. Whereas, Raji Leukemia model in nude mice showed that 80% survival for more than 80 days compared to none of the cytarabine treated mice with mean survival 34.2 days.

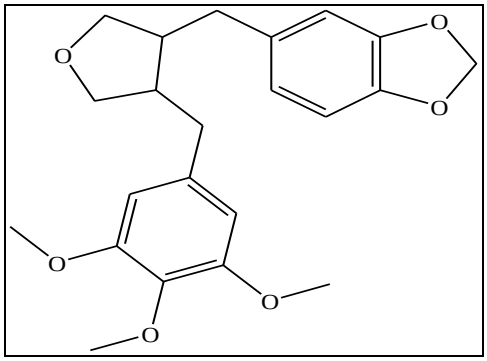

Figure 3.1 Chemical Structure of burseran

Whereas, in its xenograft models, P-4055 suppressed partially or complete cancer degeneration of lung carcinoma, as well as all three malignant melanomas in vivo study in tumor models of nude mice and rats for human cancer. P-4055 showed more potential and efficiency in treatment of cancer compared to cytarabine [8].

Recently, a fluorinated derivative of cytarabine called gemcitabine has activity in various solid tumours and some hematological malignant diseases. It also showed significant anti-cancer activity in pancreatic, breast, bladder, and non-small-cell lung cancer [9]. Currently there were two forms of cytarabine which firstly is conventional cytarabine for acute lymphocytic leukaemia, acute myeloid leukaemia and meningeal leukaemia. Secondly, liposomal cytarabine for intrathecal treatment of lymphomatous meningitis [10]. Figure 3.2 show chemical structure of cytarabine.

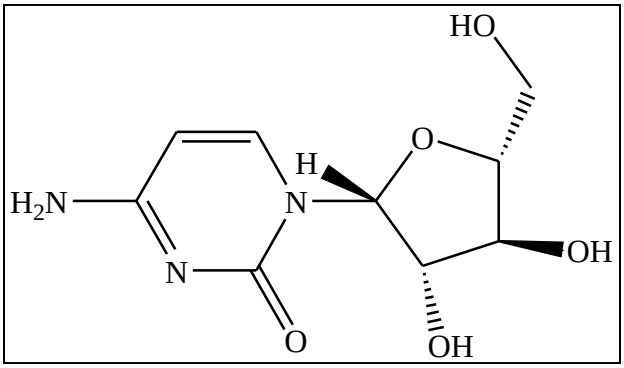

Figure 3.2 Chemical Structure of cytarabine

3.3.3. Terpenoids and Sesquiterpenoids

Terpenoids are a class of natural product which its basic structures contains of five carbon isoprene units. The terms terpenoid derived from terpenes but it may has oxygen related functionality in its structure. Terpenes building block is the

isoprene unit with formula $(C_5H_8)_n$. Terpenes are known as compound inducing anti-cancer, anti-microbial, anti-viral, anti-hyperglycemic, anti-parasitic and anti-inflammatory activities [11]. A natural monocyclic monoterpenes, D-limonene is in pre-clinical studies due to ascertain chemopreventive and chemotherapeutic activities and low toxicity. This monoterpene may act during initiation phase of carcinogenesis, preventing interaction of DNA with carcinogens or during promotion phase, inhibited cancer cell growth and migration [12].

Terpenoids are composed of subclasses based on their structures which are hemiterpenoids, monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids. Sesquiterpenoids and also specifically sesquiterpenoid lactones has many biological activities such as anti-cancer, anti-phlogistic and anti-plasmodoial activities. Some sesquiterpenoid lactones also dispalyed anti-microbial activities, disrupting the cell wall of fungi and invasive bacteria, whereas others protected the plant from environmental stresses that would otherwise cause oxidative damage.The functional groups from the sesquiterpenoids such as α,β-unsaturated carbonyl functions, conjugated esters, epoxides or additional alkylating groups may contributed to enhance the cytotoxic activities towards cancer cell lines.

An example of sesquiterpenoids is an encelin isolated from *Saussurea Parviflora*. It showed strong anticancer activity in survival studies of human hepatocytes (L02), human hepatoma cell (SMMC-7721) and human ovarian neoplasm cell (HO-8910) at half maximal inhibition concentrations (IC_{50}) of 1.47±0.01 μg/ml, 0.57±0.26 μg/ml and 0.85±0.06 μg/ml respectively. The survival study of the cancer cell lines were determined by applying the MTT calorimetric assay method [13]. Figure 3.3 below showed chemical structure of encelin.

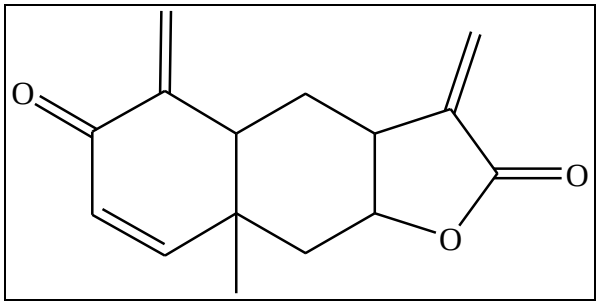

Figure 3.3 Chemical Structure of encelin

3.3.4. c-Jun NH_2-terminal kinase (JNK) Inhibitors

c-Jun NH_2-terminal kinase (JNK) is a stress-activated protein kinase that phosphorylates c-Jun on two sites in the NH_2-terminal activation domain. JNK played an important role in the signal transduction pathways. Notably, in response to stress, cyotokines and many anti-cancer drugs, JNK expression is increased. JNK is also required for some forms of stress-induced apoptosis. On the other hand, JNK also is considered to promote cell survival or growth. Several cancer lines have been reported to possess active JNK. The activation of JNK lead to the phosphorylation of a number of transcription factors named AP-1, and cancer genes such that cancer suppressor named p53 and proto-oncogene named Bcl-2 [15].

The most widely studied JNK inhibitor is SP 600125. In a study by Xia et al., (2006), three gastric cancer cell lines, AGS, BCG-823 and MKN-45, and three colorectal cancer cell lines, SW1116, COLO205 and HT-29, were treated with 20 μM SP-600125. SP-600125 inhibited cell proliferation by 10–80% for the cell lines and increased apoptosis by 1.5–4.5 folds for COLO205, BCG823, MKN-45 and AGS cells. It also caused G2/M cell cycle arrest and elevation of cyclin B1 and Cyclin-dependent kinase inhibitor 1B ($p27^{kip}$). This indicated SP-600125 inhibited proliferation, induced apoptosis and causes cell cycle arrest in gastrointestinal cancer cells. The method used for cell viability assay was MTT calorimetric assay [15].

In another study by Mili et al., (2016), treatment of 20 μM SP-600125 with various concentrations of human cervical cancer cells (HeLa) induced significant SP600125 induce mitosis arrest in G2/M, endoreduplication, mitotic spindle aberrations and apoptosis. The cell viability assay used was an immunofluorescence and apoptosis was characterized using Western Blotting method [14].

In an in vitro study of SP 600125 towards thyroid cancer cell lines, it was found out that at concentration 10 μM - 30 μM, SP 600125 was able to induce cell death through mitotic catastrophe, inhibited cancer cell migrations and replications [16]. Figure 3.4 below showed chemical structure of SP 600125.

3.3.5. Sphingolipids

Sphingolipids is one of the main classes in lipids that present in variety of organisms including eukaryotes and bacteria. It is an essential component of endomembrane system in plants [17]. In plants, the major sphingolipids can be divided into four classes which were ceramides, glycosylceramides (GlcCers), glycosylinositolphosphoceramides (GIPCs), and free long-chain bases (LCBs) [18]. Sphingolipids structure consists of a polar head group bounded to ceramides [17]. Ceramide is composed of sphingosine which is an amide linked to fatty acyl with varying length of 14-26 carbon atoms [19]. Figure 3.5 show chemical structure of complex sphingolipids consist of a polar head group bounded to a hydrophobic ceramides.

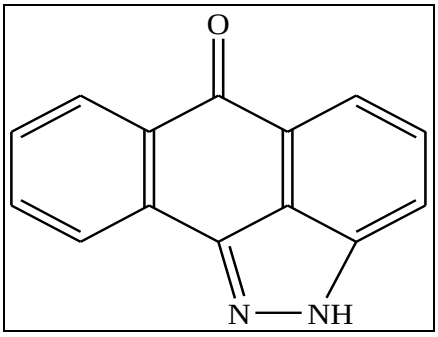

Figure 3.4 Chemical Structure of SP 600125

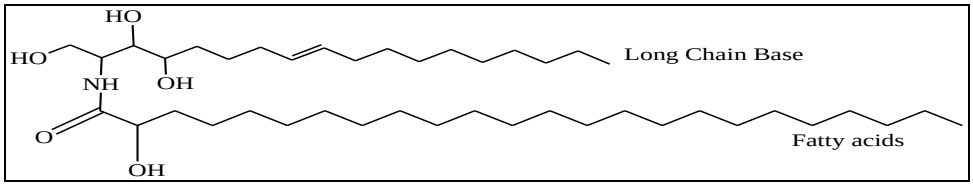

Figure 3.5 Chemical Structures of complex sphingolipids consist of a polar head group bounded to a hydrophobic ceramides

Plant sphingolipids functions as anti-cancer by controlling various aspects of cell growth and proliferation. Ceramides have a very important role as anti-cancer agents as it generally mediates the anti-proliferative responses such as inhibiton of cell growth and induction of apoptosis. A sphingolipid analogue, enigmol has potential anti-cancer activity in a NCI cell line screen and confirmed to be more cytotoxic and persistent than naturally occurring sphingoid bases using HT29 cells, which is colon cancer cell line.

A sphingolipids base, phytosphingosine had been reported as potentially induced-apoptosis compound in human cancer lines via caspase activation and caspase-independent cytochrome c release. Capsase is a family of protease enzymes playing essential roles in programmed cell death. Enzymatic activity measurements of caspases revealed that caspase-3 and caspase-9 were activated in phytosphingosine induced apoptosis, but there was little activation of caspase-8 suggesting that phytosphingosine influences mitochondrial functions.

In dose and time dependant viability analysis of mitochondria in human T-cell lymphoma Jurkat cells, 8 μM of phytosphingosine reduced the cells viability via DNA fragmentation and G2/M cell-cycle arrest. Phytosphingosine induced apoptosis in HL-60RG cells, with protein Kinase B (Akt) activity being attenuated in the steady state. This finding suggested that direct perturbation of mitochondria that enhanced by the Akt dephosphorylation may be the major apoptotic pathway of phytosphingosine-induced apoptosis. Overall, the results of the study indicated that phytosphingosine perturbs mitochondria both directly and indirectly to induce apoptosis.

3.4. Screening and identification ability of UHPLC-Q-TOF/MS and UHPLC-QQQ/MS

From all the data obtained, total of 82 compounds were recognized. UHPLC-Q-TOF/MS screened total of 174 compounds recognized while UHPLC-QQQ/MS only screened a total of 29 compounds with redundant compounds included (data not shown). This numbers proved that triple quad MS is more selective and precise in molecular mass fragmentation screening rather than Q-TOF MS.

In triple quadrupole mass spectrometer, there are three quadrupole analysers. Quadrupole analyser consists of a set of four parallel metal rods. After ionization, the analyte entered the first quadrupole which allow only ions with targeted mass to charge ratio (m/z) to pass through and produced precursor ions. The second quadrupole is called the collision cell. The collision cell contains inert gas such as nitrogen or argon. In this collision cell, the analyte undergoes further fragmentation by colliding with the inert gas particles – this process is called collision induced dissociation. The product ions from this reaction are called the fragments ions. The second quadrupole served as the mass filter of the fragment ions produced from the collision cell which can be set to a specific m/z ratio to pull specific product ions. This setting allowed the targeted ions with specific m/z to be quantitatively analyzed. The analytes are sent to the detectors which qualitatively and quantitatively producing signals of the analyte.

In Q-TOF MS, both mass analyzers, quadrupole and time of flight are combined. A combination of constant and varying voltages allow the transmission of a narrow band of m/z ratio values along the axis of the roads. Time of flight (TOF) is one of the mass analyzers in mass spectrometry. In TOF analysis, all the accelerating gas-phase analyte ions to the detector are given the same amount of energy. However due to difference of mass from each of the ions, the time it took for each ions to arrive at the detector varies from each other. The smaller ions would reach the detector first due to their high velocity and vice versa. The mass is determined from the ions's time of flight. The arrival time at the detector is dependent upon the mass, charge, and kinetic energy (KE) of the ion. Hence, its m/z ratios and the analyte molecules can be qualitatively identified. Because all ion masses are measured for each transient, Q-TOF mass spectrometers are well-suited for the analysis of both targeted and non-targeted analytes and unknown samples.

The higher number of filtration masses produced using triple quad MS produced more specific fragmentation of m/z ratio of analytes compared to only a quadrupole and mass fragmentation from time of flight each analyte in Q-TOF MS. This specificity of fragmentation produced, gave triple quad MS more selective screening and quantitation of compounds than Q-TOF MS. However, Q-TOF MS proved to be very good in terms of qualitative analysis of unknown samples compared to triple quad MS.

4. CONCLUSION

Data obtained from UHPLC-MS, UHPLC-Q-TOF/MS and UHPLC-QQQ/MS successfully showed there were 82 recognized compounds identified from the selected indigenous plant where 8 of them were known anti-cancer agent while 3 of them were potential anti-cancer agents. The classes compound found were from phenylpropanoids, pyrimidine nucleosides, terpenoids, JNK inhibitors and sphingolipids. The effectiveness of the analysis using triple quad proved more selective screening and quantitation of compounds than Q-TOF MS. However, Q-TOF MS proved to be very good in terms of qualitative analysis of the identification of unknown samples compared to triple quad MS. This research successfully served as the first scientific proof that the selected indigenous plant used by local tribe has anti-cancer active compounds that can be further studied as potential cure for cancer. There were several others identified plants used for other remedies that need to be studied as well.

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