CHAPTER ONE
INTRODUCTION

1.1 General

Plants have variety of chemical constituents called ‘phytochemicals’ with protective and disease preventive properties. The term ‘phytochemicals’ are chemical substances that produced naturally in plants that demonstrate various biological activities that include antioxidant, antibacterial, antifungal, anti-inflammatory, anti malarial and anticancer activities [1].

These phytochemicals include isoflavones, flavanoids and lycopene which are clinically proven to be of valuable therapeutic importance which may help in the treatment of many diseases such as cancer, cardiovascular disease, degenerative diseases and HIV infection [2-3]. Vegetables, fruits, herbs and seeds are some of the plants that contain phytochemicals that are rich in phenolic compounds such as flavanoids, phenolic acids, anthraquinones and coumarins [4].

Research in natural products seemed to be unbounded and limitless, and recently the interest in this area of study has revived and increased considerably especially with the latest development of technology in separation methods, spectroscopic techniques and advanced bioassays. Over the years, there have been significant findings and evidence that naturally-occurring compounds derived from higher plants have the potential to be developed as modern therapeutic drugs.

The search for bioactive compounds from plant-based medicines or organisms for the development of conventional drugs are now reviving and becoming more
commercialized in the modern medicine throughout the world. Approximately, about 25% of the drugs used in the world today originate from higher plants and among them were taxol, morphine, quinine caffeine, atropine, and reserpin [5].

It is undeniable that products from natural origin still continue to be valuable sources for new therapeutic agents especially with the profound understanding of the biological significance and mechanism of actions of the active compounds. Until now, about 1% of tropical species have been studied and screened for their pharmaceutical potential against various diseases including cancer, diabetes and cardiovascular disease [6].

Plants and their extracts and derivatives still serve as a major contribution in pharmacy and medicine because of the increasing demand for medicines and foods (nutraceuticals) from the ageing population especially in Europe, Australasia and North America [7-8].

As new diseases emerge and the trend for drug resistance and side-effects increases, there is the need to discover new medicinal drugs from natural origin with specific targets and the least side-effects. On the other hand, synthetic drugs tend to show rapid onset of action with more side-effects in comparison to naturally occurring drugs [9].

1.2 History of Natural Products

The use of plants with medicinal properties has a long story in human history and folklore medicine since the beginning of mankind where plants and herbs were used to treat various symptom of diseases by the indigenous people and traditional healers [10]. The documentation of medicinal uses of plants such as Glycyrrhiza glabra (licorice) and Papaver somniferum (poppy juice), were first recorded on clay tablets from Mesopotamia in about 2600 BC [11].
Ayuverda is one of the ancient medicines originated from India which is based on the balance of the nature. Ayuverdic plants include *Azadirachta indica* (neem) and *Elettaria cardamomum* (cardamomum). *Vinca rosea* and *Taxus spp* were among the medicinal plants derived from folk medicine which were traditionally used for the treatment of diabetes [12].

1.3 Natural Products

Natural products, also referred as secondary metabolites, are bioactive compounds synthesized by natural resources such as plants, marine organisms and microorganisms primarily as a defense mechanism against herbivores and predators in order to survive in their surroundings. Other than that, secondary metabolites may also serve as metal transporting agents, as agents of symbiosis, as sexual hormones and as differentiation effectors [13-14].

Secondary metabolites are naturally occurring products of secondary metabolism from primary products such as the carbohydrates, fatty acids and lipids. They possess complex carbon skeleton structures and biological properties that are unique to the plant [15]. Secondary metabolites are biosynthesized in certain plant species or in specific part of the plants. They are not ubiquitous and their occurrences are limited. They also do not participate in the biosynthesis of primary metabolites.

On the other hand, primary metabolites (carbohydrates, amino acids, enzymes, nucleotides, etc.) occur naturally in plants and they are essential in the normal growth and development of the plants. Secondary metabolites are not required in the vital growth of the plant and due to that they are not biosynthesized in a higher concentration and usually their presence in plants are affected or induced in response to the surrounding
factors, conditions and stress such as the climate, the soil condition, herbivores, etc. [16-17].

Secondary metabolites are grouped according to their structures, biosynthetic pathways and the plants which produce them. Alkaloids, terpenoids and phenolic compounds are the three major groups of secondary metabolites where alkaloid is the largest class of secondary metabolites with over 6,500 known compounds [18]. Alkaloids are nitrogenous organic molecules biosynthesized primarily from amino acids such as tryptophan, phenylalanine, lysine and arginine. Most alkaloids discovered were found to exhibit pharmacological properties such as antimitotic, antibiotic and anti tubercular agents [19-21].

Terpenoids are important in plants as a defense system against herbivores because of their toxicities. The triterpenoid azadirachtin found abundantly in neem plant (Azadirachta indica A. Juss) is a well known natural insecticidal agent [22-23]. Some secondary metabolite families are involved in cell pigmentation in flower and seed which contribute to plant reproduction. For example, Anthocyanin, a polyphenol antioxidant, is a brightly colored pigment in blue and red flowers that attract insects and seed feeders to facilitate pollination [24]. Phenolic compounds from plants with antioxidant activity play an important role in the prevention of oxidative degradation of lipids by neutralizing the free radicals [25].

Recently, there is an increasing interest in secondary metabolites that exhibit active biological properties. Many bioactive secondary metabolites show a variety of pharmacological effects like cytotoxic, antibiotic, antifungal, antiviral, antimalarial and many others. Some of the well known examples of secondary metabolites with pharmacological activities that have been developed as drugs were paclitaxel (anticancer
agent) isolated from Taxus brevifolia and salicin (analgesic agent) isolated from willow [26].

1.4 Drugs from Natural Products

Plants still and will continue to be the primary source for the discovery of new therapeutic agents with pharmaceutical and medicinal properties. Over than 80% of natural products derived from plant origin but only a small percentage from several hundred thousand plant species has been investigated phytochemically and pharmacologically [27].

A substantial number of effective drugs have been developed from natural products through the discovery of bioactive compounds that were originally isolated from the plants or their secondary metabolites. They include paclitaxel, camptothecin, artemisinin, mevastatin and etoposide.

Morphine, an analgesic agent, isolated from *Papaver somnifrum* (opium poppy) was the first alkaloid synthesized in the laboratory in 1816 and this discovery has laid the foundation for the beginning of phytochemistry which is the study of chemistry of plants [28].

The history of aspirin went as far back as 500 AD where the Greek physician Hippocrates documented a concoction made up from the willow bark that was used to treat pain and fever. The Native American Cherokees also used the willow bark to treat symptoms of fever. It was later discovered in the 1800s that the analgesic and antipyretic properties of the willow bark were actually produced by a compound called salicin. This
was then followed by the synthesis of acetylsalicylic acid and marketed under the trade name, aspirin.

Today, aspirin is not only used to treat pain, fever and inflammation but also help to reduce the risk of heart attack and stroke by preventing platelet aggregation. In the United States, over 50 million adults take aspirin regularly for long term prevention of cardiovascular disease [29].

Quinine which was isolated from the bark of the cinchona tree was another breakthrough in drug discovery from plants. Traditionally, it was made a concoction by the native Indians in Peru to treat fevers. It was later discovered that it can also treat malaria [30-31].

According to a survey on the origin of drugs developed between 1981 and 2002, about 63% of the drugs sold on the market were either produced directly or synthetically from lead compounds of natural products such as plants, microorganisms and marine organisms [32-33].

In the United States alone, 13 clinical drugs approved between 2005 and 2007 were derived from natural products with five of them belongs to the first members of new classes [34-35].

Nitisinone, a derivative from Callistemon citrinus used for the treatment of hereditary tyrosinaemia type 1 (HT-1) and galantamine hydrobromide, an Amaryllidacea alkaloid from Galanthus nivalis were among the approved drugs in the last 5 years that were developed from natural origin [36-37]. Doxorubin and paclitaxel (antitumor agents), cyclosporine A and tacrolimus (immunosuppressive agent) and lovastatin (cholesterol lowering agent) are among well known and widely used drugs from natural products [38-39].
This clearly suggests that natural products are important sources for new therapeutic agents and they have significantly contributed as lead compounds in drug development project. Other than plants, chemical compounds from other biological resources such as bacteria, fungi and marine organisms also contribute towards the development of new drugs [40-41].

The increasing demand for medicines and health supplements (nutraceuticals) from the ageing population has prompted the researchers to discover new bioactive compounds from natural products.

In a report by World Health Organization (WHO), more than 80% of the population of the developing countries still depends on traditional medicines from plants for the treatment of common symptoms and diseases. To this date, a major part of this world still uses traditional medicinal plants in their primary health care. [42-43].

The selection of plant materials for bioactive natural products is based on various approaches including random screening, ethnobotanical method, chemotaxonomical knowledge and ethnopharmacological approach [44-46].

In the process of drug discovery, random screenings of various compounds in natural products are tested initially in vitro or in vivo where subsequently only compounds that show significant biological activities and selectivity will proceed to the next level of evaluation and clinical trials [47-48].

The search for pharmaceutical agents through random screening has led to the discovery of many beneficial clinical drugs. Vincristine and vinblastine were discovered from Catharantus through random screening during the search for potential oral hypoglycemic agent [49-50].
In the bioassay-guided fractionation approach, the crude extracts of the plants are screened first and this is followed by bioassay–guided isolation where every stage of fractionation and isolation is guided by bioassays [51-52]. The active chemical constituents are then isolated from the bioactive fractions and the structures of the active compounds are further determined and identified by structural elucidation using chromatographic and spectroscopic methods such as NMR, 2D NMR, UV, IR and MS.

Another method is through chemotaxonomical approach that utilizes the previous informations of a certain group of plants with specific chemical compounds to establish the compounds in another plant in the same taxonomic group [53].

Dereplication is a process where recurrence of similar or known compounds is avoided by differentiating between known and unknown compounds. This is done through the use of high throughput screening (HTS) by applying hyphenated techniques such as the liquid chromatography–electrospray ionization–mass spectrophotometry (LC/ESI/MS), liquid chromatography-photoiodide array detection (LC/PDA) and liquid-chromatography-nuclear magnetic resonance (LC/NMR) chromatography where unknown compounds are identified in a shorter time without the need for extensive purifications compared to the traditional method [54-55].

Recently, there was an effort to establish a database for the dereplication of natural products mixture to facilitate fast identification of known compounds and to provide some insight to unknown compounds [56].

It is quite a major effort to isolate bioactive pure compounds from plants and organisms due to their complex molecules especially in the case of secondary metabolites which are usually not found in a larger amount. Most of the time, the isolated pure compounds were yielded in a very small quantities (in nanograms) which are not enough for further
structural elucidations and bioactivity tests. In general, at least 3mg of sample is required for NMR analysis.

It would be ridiculous to harvest a massive volume of raw plant materials as this may result in the extinction of the species and causing environmental disturbances. Furthermore, the process of extracting and isolating each pure compound are often time-consuming and require expensive chemical solvents, reagents and tools. Not to mention the capacity, efficiency and sensitivity of the instruments used for structural elucidation that also should be considered.

Thus, this problem is usually overcome by synthesizing the compounds in the laboratories in a larger amount where lead compounds from natural products are chemically synthesized by molecular modification of the functional groups to generate structural analogue [57-58].

Another alternative is to use plant tissue culture technology to specifically increase the production of secondary metabolites at a larger scale [59]. For example, several studies have demonstrated that paclitaxel can be produced and purified from plant cell cultures to yield a larger quantity and higher purity of the compounds [60-61].

To this date, with a keen interest in natural products, scientists and researchers from all backgrounds have continuously screening plants and other organisms to discover the lead compounds for antioxidant, anticancer and antimicrobial agents including other significant pharmaceutical agents.

The search for pure compounds with biological activities from plant-based medicines or organisms for the development of potential drug candidate are now expanding in the field of modern medicine throughout the world especially with more plants and organisms being screened.
In fact, with the latest development, plant cell cultures can be genetically developed to produce the selective bioactive metabolites which can be produced in larger volume and cost effective [62-63].

1.5 Natural Antioxidants

Plants contain several compounds that possess antioxidant activity. Natural antioxidant compounds in plants play an important role in scavenging free radicals. Free radicals include hydrogen peroxide, hydroxyl radicals and superoxide anions. They are the by-products of biological redox reaction in the cellular system that are formed from environmental factors such as environmental pollutions and contamination, UV light, cigarette smoke, chemicals and toxic waste [64-65].

The free radicals are highly reactive chemicals with an odd or unpaired electron that can attack the cells to stabilize the odd electron. This reaction will result in cell damage and tissue injury that will eventually cause a number of diseases and disorders such as heart disease, cancer, chronic lung diseases (CKD), stress, aging and neurovascular diseases [66-67].

However, this occurrence can be prevented with diets rich in antioxidant compounds. It was shown that foods like fruits, vegetables, tea and red wine that contain high amount of antioxidants such as tocopherol and carotene were able to reduce the risk of heart disease and cancer [68]. These days, antioxidants are now considered as important nutraceuticals in the human diet in disease prevention [69-70].
Scientific evidence has demonstrated that diets rich in vitamin C, vitamin E and phenolic compounds play an important role in controlling oxidative stress, cardiovascular disease, muscle atrophy and inflammation [71-72].

Other studies also found that antioxidants contribute greatly in preventing the accumulation of reactive oxygen species that can cause cellular damage to the DNA, proteins and nucleic acids [73]. There were also reports that natural antioxidants can delay or prevent degenerative diseases such as Alzheimer’s and Parkinsons’ and increase the human lifespan [74-76].

Primary sources of natural antioxidants such as phenolic compounds and flavanoids can be found mostly in fruits, vegetables, nuts and grain. Vitamin C, vitamin E, carotenes and phenolic acids are among plant sourced antioxidants [77]. Phenolic and polyphenolic compounds are the main class of natural antioxidants that are found mostly in fruits, vegetables, legumes and grains. They exist as substituted benzoic and cinnamic acid compounds [78]. Even though there are synthetic antioxidant compounds on the market such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are commonly used in the food industry, there were some evidence that these chemicals can produce side effects with long term consumption [79]. Therefore, more attention is given now towards the discovery of new natural antioxidants from natural products.

1.6 Cancer

Cancer is a disorder characterized by rapid multiplication of normal cells which develop into uncontrolled growth that eventually led to death. There are many types of cancer and its symptoms depend on the categories of malignancy and how advanced is the process of metastasis. In developed countries, cancer is one of the dominant causes of death with thousands of patients succumbing to death each year from different types of cancer.
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 [80]. In the United States, cancer is the second leading cause of death where one in four deaths is due to cancer that include children and adolescents [81]. It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer around the world [82]. According to the World Cancer Report in 2003, the rate of cancer cases was expected to increase by 50% to 15 million new cases in the year 2020 [83].

Back in 1960, the United States National Cancer Institute (NCI) has initiated an extensive plant collection program leading to the discovery of numerous novel cytotoxic agents, including the taxanes and camptothecins.

To date, the National Cancer Institute has accumulated about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity [84]. Taxol, vinblastine, vincristine, topotecan and irinotecan, camptothecin derivatives and etoposide are among the anticancer agents discovered from plant origin [85].

The search for anti-cancer agents has evolved and expanded to a higher level ever since the discovery of vinblastine and vincristine (natural alkaloids). Vincristine and vinblastine were two vinca alkaloids discovered from the Madagascar periwinkle, *Catharantus roseus* from family Apocynaceae. These were two famous anticancer agents which have advanced successfully through clinical trials and at present they are clinically used for the treatment of lymphoma and leukemia [86].

Another amazing discovery of anticancer agent from natural products was Taxol, which was isolated from the bark of *Taxus brevifolia* from family Taxaceae. Camptothecin, a natural alkaloid isolated from *Camptotheca acuminata* is used as chemotherapeutic agent
in the treatment of stomach, colon and bladder cancers [87]. Aside from plants, more studies are now looking at marine organisms as potential source for anticancer drugs. Some of the new anticancer compounds from marine sources are cryptophycin, aplidine and Halicondrin B [88-90].

Approximately about 74% of the anticancer drugs on the market are either natural products or of a natural origin [91]. Currently, there are nine plant-derived anticancer drugs that have been approved for cancer treatment which are vinblastine, vincristine, etoposide, teniposide, taxol, navelbine, taxotere, topotecan and irinotecan [92]. Up until now, many higher plants have been screened and evaluated to identify new and effective drug for the cancer chemoprevention, as well as to elucidate the mechanisms of cancer prevention and apoptosis.

Quite a number of studies have also reported that extracts from natural products such as fruits, vegetables and medicinal herbs have positive effects against cancer. For example, some of the flavanoids such as kaempferol and genistein showed antiproliferative activity [93]. Quercitin, a flavanoid found mostly in fruits and vegetables demonstrated anti-tumor effect including increasing the effectiveness of some chemotherapeutic agents through synergistic effects [94].

Recent studies of plant extracts that have been studied and have shown cytotoxic effects on cancer cell lines include those of Solanum lyratum which were tested on human colon adenocarcinoma cell line (colo 205) [95], Annona glabra on human leukemia cell lines (CEM/VLB) [96] and Artocarpus altillis on human breast cells (T47D) [97].

There is an upward trend of interest from the pharmaceutical and commercial industries in the continuing discovery of new anticancer agents from plants especially for natural compounds that can overcome the problem of multi drug resistance (MDR) in cancer
chemotherapy [98]. The search for an effective anticancer drug based on natural products has been actively carried out all over the world including Malaysia.

1.7 Natural Products in Malaysia

Malaysia ranks the 12th largest in the world in term of biodiversity [99]. More than 60% of the woods in the Malaysian tropical forest are processed and commercially exported to other countries. Apart being the main source for timber industry, Malaysian tropical forest too is rich with all kinds of plants and flora with medicinal properties which have long been utilized by the local people and traditional practitioners.

Among the popular traditional medicinal plants were *Eurycoma longifolia* (Tongkat Ali) which is scientifically known to increase male potency and *Labisia pumila* (Kacip Fatimah) which is commonly used as postpartum medication for women. In term of commercial purposes, these two herbs are commonly added in coffee mixtures as health supplements, and widely consumed by the consumers. Indigenous people in Malaysia, Orang Asli, used the bark of Tongkat Ali (*Eurycoma longifolia*) to treat small pox and malaria [100]. Other plants used traditionally include Kacip Fatimah (*Labisia* sp.), mempisang (*Gonothialamus* sp) and bunga pakma (*rafflesia hasselti*) [101].

In Sabah, about 30% of traded non-timber forest products sold in the local markets (tamu) was sourced from medicinal plants as part of the socio-economic activity. These include akar mengkudu (*Morinda citrifolia*), tongkat ali (*Eurycoma longifolia*) etc.

It would be a great loss if we do not take this opportunity to screen our natural products for active chemical compounds especially when the technology is made available to us. On top of that, the Government of Malaysia has given more priorities in the scientific
research field by providing more funds and incentives. Therefore, it is imperative that we evaluate our natural products and compile the data for future reference and scientific research as part of our contribution to the world of knowledge.

1.8 Selection of Plant for the Study

1.8.1 Previous work

One study was previously done on the chemical constituents of *Pseuduvaria rugosa* (KLA4709) by Rosmahaida Jamaluddin back in 1999 which has described the isolation and identification of nine compounds with most of them were alkaloids [102].

1.8.2 Justification of present study

*Pseuduvaria rugosa* was selected for the study because literature survey revealed that there was only one thesis documentation on the chemical constituents from the stem bark of *Pseuduvaria rugosa* (KL 4709) which was not published in any journal and so far to the best of the author’s knowledge, there has not been any report on the biological activities of any chemical compounds found from *Pseuduvaria rugosa*. From the literature review, the number of *Pseuduvaria* species that have been chemically investigated is still very small and only a few biological activities of some compounds were reported.

In other *Pseuduvaria* species, alkaloids from *Pseuduvaria setosa* was reported to possess strong anticancer activity against epidermoid carcinoma (KB) and breast cancer cell (BC) cell lines. Interestingly, in the taxanomic approach, *Pseuduvaria rugosa* shared the same class of phylogeny and similar DNA characteristics as *Pseuduvaria setosa*. Previously, most of our research and studies have concentrated on identifying the chemical
compounds in the higher plants, but now studies are more oriented on investigating the biological activities of the phytochemicals as well.

In ethnobotanical context, the plant is not traditionally known to possess medicinal properties among the locals though it belongs to the family Annonaceae which is known to exhibit pharmacological properties. Furthermore, little was known about the medicinal value of this plant though there were claims that this plant may possess anticancer agents which were earlier reported from *Pseuduvaria setosa*. Hence, this study can provide the data and information on the chemical constituents and biological activities in comparison with that of *Pseuduvaria setosa* and other *Pseuduvaria* species.

In this study, the sample of *Pseuduvaria rugosa* was collected from a different location and given a different voucher number which was not the same in the previous study. Again, this study can provide more data and information on the chemical compounds and profile of *Pseuduvaria rugosa* that was collected from a different location.

This study was also not intended to focus on the alkaloid compounds of the plant only but also on other class of compounds. In addition to that, GC/MS analysis was also carried out to investigate the compounds present in the hexane extract of *Pseuduvaria rugosa*. 
1.9 Objectives of the study

In consideration of the above facts, the main purpose of this study is to investigate the chemical constituents of *Pseuduvaria Rugosa* (Annonaceae) and evaluate the biological activities of the crude extracts and the isolated compounds:

a) To investigate and isolate the chemical constituents from the bark of *Pseuduvaria rugosa* using chromatographic methods

b) To identify the isolated compounds by structural elucidations using UV, IR, H-NMR, $^{13}$CNMR, 2D NMR and mass spectrometers.

c) To evaluate the antioxidant and anticancer activities of the crude extracts and identify the active compounds.

d) To report and publish the data and information on the chemical constituents and biological activities of *Pseuduvaria rugosa*.
2.1 Classification and taxanomy of *Pseuduvaria* genus [103]

Kingdom : Plantae  
Division : Magnoliophyta  
Class : Magnoliopsida  
Order : Magnoliales  
Family : Annonaceae  
Genus : *Pseuduvaria*

2.2 The Annonaceae Family

The family Annonaceae is one of the largest family of the flowering plants from magnolia order (Magnoliales) which consists of approximately 128 genera and over 2,220 species [104].

There are about 51 genera and 950 species of the Annonaceous plants in Asia and Australia, 40 genera and 450 species in Africa, and 38 genera and 740 species in the American continent [105].

In Malaysia, there are 38 genera, 198 native and 5 cultivated species including 17 varieties of Annonaceae plants and they are abundantly found in the lowland forests mostly below 2,000 feet [106].
The members of the Annonaceae family are made up of small trees, shrubs and woody climbers that be found mainly in the tropical and subtropical regions [107]. The plants from Annonaceae family have been known to be rich in alkaloids especially the isoquinoline alkaloids where they are found mainly in most parts of the plants [108-109].

Most of the species from Annonaceae were used in folk medicine and many of the compounds isolated have exhibited potent biological activities such as cytotoxic, antitumor, antimalarial, antibacterial, antifungal, antiplatelet aggregation and immunosuppressive activities [110-113].

Traditionally, the plants of Annonacea (mempisang) have been used by the local healers to treat symptoms of fever and stomachache. And recently, they were claims that the plants can be also used in the treatment of cancer.

### 2.2.1 Vernacular names [114]

1. Akau (Sarawak)
2. Antoi, jangkang (Peninsular Malaysia)
3. Pisang-pisang, mempisang (Peninsular Malaysia and Sabah)
4. Mempisang (Brunei)

### 2.2.2 Economic value

In Malaysia, the Annonaceae family is locally identified by the foresters and traditional healers as Mempisang. Another local name is akar larak that describes the climbing plants which is known among the traditional practitioners for mystic purposes. Apart as
a source of firewood, the Annonaceous woods are used in the furniture making, packing cases and crates, while the heavier species are used for parquets and strip flooring [115].

### 2.2.3 Pharmacological value

The members of Annonaceae family are reported to contain secondary metabolites including acetogenins, terpenoids, phenolics and alkaloid compounds with various biological activities [116-117]. Recently, there has been a growing interest in the species of the Annonacea family in the pharmaceutical research and ethnobotanical investigation [118]. In a literature review, 319 secondary metabolites have been reported from 150 species of the Annonaceae that include alkaloids, non-alkaloid compounds and essential oils [119].

These secondary metabolites with interesting biological activities can be potential pharmaceutical agents for the treatment of cancer, microbial infection, hypertension and brain dysfunctions. For example, goniotriocin, an annonaceous acetogenin isolated from *Goniothalamus giganteus*, showed potent and selective cytotoxicity against six human tumour cell lines [120]. Benzylisoquinoline alkaloids isolated from *Annona salzmanii* DC have antibacterial and antifungal activities [121].

Annonaceous plants have been used by traditional healers for medicinal purposes such as stomachache, asthma, cough, fever and wounds [122-123].

In the tropical region, about 50 species of the Annonaceae are commonly used for the treatment of fever, stomach ailment and skin problems. In a recent documentation of traditional medicinal plants in Sabah, Annonaceae plant or *Mempisang* was listed as one of the local medicinal plants regularly used by the local healers to treat clinical symptoms [124].
2.3 Genus *Pseuduvaria*

*Pseuduvaria* is a rainforest plant species that is incorporated in the family Annonaceae. Plants in this genus are in the major group of flowering plants (Angiosperms) that are made up of shrubs and trees usually found in the rainforest population [125]. It was reported that the *Pseuduvaria* species originated from Sundaland in the late Miocene and later migrated to the other part of the region [126].

The *Pseuduvaria* species are commonly found in Malaysia, Thailand, Burma, and Indonesia and in the northeastern part of Queensland, Australia (Figure 2.1). Currently, there are about 52 *Pseuduvaria* species that were classified and documented (Figure 2.2) but only a few have been investigated chemically and biologically.

The *Pseuduvaria* genus was formerly known as *Orophea rugosa*, *Mitrephora rugosa* and *Uvaria rugosa* due to their close resemblances and similar morphological characteristics [127]. They are known to contain both alkaloid and non-alkaloid compounds, including essential oils. Some of the *Pseuduvaria* species are traditionally used in treating cough, fever and stomach ailments.

Previous researches have revealed the presence of alkaloids in most *Pseuduvaria* species that were studied. Some of the alkaloids found in the *Pseuduvaria* species include liriodenine, anonaine, normuciferine, etc. Some studies have also demonstrated that crude extracts and alkaloids isolated from *Pseuduvaria species* exhibit biological and cytotoxic activities [128].
Figure 2.1: Distribution of *Pseuduvaria* species in South East Asia

(indicated in pink color) [126].
Figure 2.2: Chronogram of *Pseuduvaria* species [127]
2.4 *Pseuduvaria rugosa*

2.4.1 Morphological description

The tree is 5-7 m high with slender and dark colored young twigs. The leaves are simple and alternate, membraneous and papery with lanceolate shape (spear shaped). The fruits are small which look like a bunch of berries consist of a group of fleshy carpels that is attached on a torus. (Figure 2.3). The bark is fibrous and aromatic, smooth and grey to brown in color (Figure 2.4).

The flowers are bisexual and inflorescences which are arranged in axillary fascicles either solitary, paired or clustered and they are characterized by a cyclic perianth of three trimerous whorls, an androecium of several stamens and a gynoecium of free carpels on a flat or conical receptacle [129].

In *Pseuduvaria* species, the flower is mostly solitary (outer petals 1–1.5 cm, shorter than inner petals). The pollen of 42 species of *Pseuduvaria* is described as inaperturate, isopolar and radically symmetrical [129]. Figure 2.5 illustrates some of the flowers and fruits from *Pseuduvaria* species.

2.4.2 Distribution

*Pseuduvaria rugosa* is distributed in Malaysia, Thailand, Indonesia and Borneo. In Malaysia, *Pseuduvaria rugosa* can be found in Selangor, Malacca, Perak, Perlis, Pulau Pinang and Kelantan [130].
Figure 2.3: Fruits and flower of *Pseuduvaria rugosa*
Figure 2.4: The stem bark of *Pseuduvaria rugosa*

Figure 2.5: Some of the fruits and flowers of *Pseuduvaria species*
2.5 Chemical aspects of *Pseuduvaria* species

In the past years, a number of phytochemical constituents including alkaloids, terpenoids and essential oils have been isolated and characterized from several *Pseuduvaria* species. Literature reviews indicated that only several species in the genus *Pseuduvaria* have been investigated for its chemical components. And recently, researchers are also interested in looking into their biological activities.

Previous researches have revealed the presence of alkaloids in most *Pseuduvaria* species that were studied. Some of the alkaloids commonly found in the *Pseuduvaria* species include liriodenine, ouregidione, anonaine, nornuciferine, O-methylmoschatoline etc.

Previous investigation on the stem bark of *Pseuduvaria rugosa* (KL 4709) by Rosmahaida (1999) have described the isolation and characterization of nine compounds, of which seven of them were alkaloids [102]. The isolated alkaloid compounds were liriodenine 1, atherospemidine 2, oxoputerine 3, 3-methoxycepharadione-B 4, ouregidione 5 and norcepharadione B 6. Rugosanine 7 was a new alkaloid with an aldehyde group at C-7. In addition to that, two chromans namely rugosin-A 8 and rugosin-B 9 were isolated from the petroleum-ether extract. However, there were no biological activities reported.
Figure 7: Structural formula of compound 7.

Figure 8: Structural formula of the trans-form of compound 8.

Figure 9: Structural formula of the cis-form of compound 9.
In another investigation of *Pseuduvaria macrophylla*, two aporphinoid alkaloids were isolated from the stem bark of *Pseuduvaria macrophylla* that were collected in the primary forest close to Taman Negara, Pahang, Malaysia [131] and no biological activity was reported.

The two compounds were are O-methylmoschatoline 10 and N-Methylouregidione (1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydraporphine) 11. Medicinally, the roots of *Pseuduvaria macrophylla* was used as one the essential concoction for the treatment of stomach ailment [132].

![Chemical structures of O-methylmoschatoline 10 and N-Methylouregidione 11](image)

*Pseuduvaria setosa* (King) J. Sinclair is another species of *Pseuduvaria* genus that has been investigated. In Malaysia, the fruits of *Pseuduvaria setosa* are normally consumed by fruit bats as one of their main diets during the fruiting season [133].

In Thailand, *Pseuduvaria setosa* is found in the southern part of the country. There were two dioxoaporphine alkaloids isolated, N-methylouregidione 11 and ouregidione 5 and
two oxoaporphine alkaloids, liriodenine 1 and oxostephanine 12 from the aerial part of

*Pseuduvaria setosa* (King) J. Sinclair [134].

![Chemical structure of oxostephanine 12](image)

Phytochemical investigation on the stem bark of *Pseuduvaria indochenesis* [135] has led to the isolation and characterization of a novel protoberberine alkaloid, dehydroscoulerine 13, and scoulerine 14 along with three other known oxoaporphine alkaloids, liriodenine 1, atherospermidine 2 and oxoanolobine 15.

![Chemical structure of dehydroscoulerine 13](image)
Three diterpenes were isolated from the stem bark of *Pseuduvaria indochinensis* and identified on the basis of spectroscopic data and x-ray diffraction resulted as ent-8α-hydroxypimar-15-en-18-oic acid 16, ent-pimaric acid 17, and ent-16α,17-dihydroxykauran-19-oic acid 18. Both 16 and 17 appeared to be novel [136].
The alkaloids found from *Pseuduvaria cf. grandifolia* were liriodenine 1, anonaine 19, and nornuciferine 20 and from *Pseuduvaria cf. dolichonema*, the alkaloids found were glaucine 21, 2-hydroxy-1,9,10-trimethoxynoraporphine 22 and norglaucine 23 [137].
In Australia, a study was done on the essential oils of the leaf of Australian species of *Pseuduvaria*. The species investigated were *Pseuduvaria mulgraveana* var. *mulgraveana*, *Pseuduvaria mulgraveana* var. *glabrescens*, *Pseuduvaria hylandii*, *Pseuduvaria villosa*, and *Pseuduvaria froggattii*. [138].

These species are mainly found in the wet tropics of northeastern Queensland [139]. Some of the compounds present in the leaf oils of the *Pseuduvaria* species include β-caryophyllene 24, α-curcumene 25, α-copene 26, elimicin 27, methyl eugenol 28, spathulenol 29, caryophyllene oxide 30 and froggart ether (a fourt dihydroagarofuran isomer) 31. β-caryophyllene 32, a sequisterpene found in essential oils has been reported to potentiate the anticancer activity when combined with paclitaxel [140-141].
2.6 Biological Activities of *Pseuduvaria* Genus

Several studies have demonstrated that crude extracts and alkaloids isolated from *Pseuduvaria* species exhibit biological and cytotoxic activities.

M. Othman et al (2009) studied the antimicrobial and antioxidant activities of the crude extracts (hexane, ethyl acetate and ethanol) of *Pseuduvaria macrophylla*. The antimicrobial activity was evaluated by agar-based pour plate disc diffusion (PPDD) and broth-based turbidometric (TB) assay [142].

The investigation revealed that the crude extracts of *Pseuduvaria macrophylla* showed antimicrobial activity against two gram negative strains, (Escherichia coli and *Citrobacter freundii*) and four gram positive strains (*Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus cereus* and *Bacillus subtilis*) in a dose depending manner.

In the antioxidant test, all crude extracts showed high antioxidant activities in the β-carotene bleaching (BCB) assay test comparable to trolox and quercetin, but in Ferric Reducing Antioxidant Properties (FRAP) test, only the ethanol extract was active than the hexane and ethyl acetate extracts. However, the chemical compounds responsible for the bioactivities, have yet to be isolated.

In Thailand, the root of *Pseuduvaria setosa* has been traditionally used to treat cough and fever [143]. Biological activities of the four alkaloids, N-methyloureigdione 11, liriodenine 1 and oxostephanine 12, isolated from the aerial part of *Pseuduvaria setosa* showed *in vitro* antituberculosis activity against *Mycobacterium tuberculosis* with MIC values of 100, 12.5 and 25 g/ml, respectively [134].
These results have demonstrated that aporphine alkaloids can be developed as potential anti-tubercular agents in chemotherapy [144]. In the same study, liriodenine 1 isolated from *Pseuduvaria setosa* displayed antimalarial activity against *Plasmodium falciparum* (K1, multidrug-resistant) with 50% inhibitory concentration (IC$_{50}$) of 2.8 µg/ml.

In the cytotoxic study of *Pseduvaria setosa*, liriodenine 1 and oxostephanine 12 showed strong cytotoxic activity against epidermoid carcinoma (KB) and breast cancer (BC) cell lines, whereas ouregidione 5 was moderately active against BC cells. Both N-methylouregidione 11 and ouregidione 5 were active against small cell lung cancer (NCI-H187) cell line and were able to stimulate lymphocyte proliferation with stimulation indices (SI) of more than 1.

In another study on bioactivity, ethanol crude extracts from *Pseuduvaria setosa* was found to stimulate lymphocyte proliferation in *in vitro* immunostimulating activity test. The isolated pure compounds, i.e., kaurenoic acid 33, β-sitosterol 34, quebrachitol 35, and ouregidione 5 were capable to enhanced IL-12 secretion from J774A.

Diterpenes, ent-8α-hydroxypimar-15-en-18-oic 16, ent-pimaric acid 17, and ent-16α,17-dihydrokauran-19-oic acid 18 isolated from *Pseuduvaria indochenesis* was found to inhibit DNA topoisomerase activity [136].
3.1 Phytochemical study of *Pseuduvaria rugosa* (Annonaceae)

The stem bark of *Pseuduvaria rugosa* (KL 5186) has been studied for its chemical constituents and biological activities. This species belongs to the family Annonaceae. Phytochemical investigation on the stem bark of *Pseuduvaria rugosa* was carried out through the extraction, separation, structural elucidation and biological activity test. The extraction of the plant sample has been conducted using hexane, followed by dichloromethane and acid-base extraction to yield crude alkaloid and finally with methanol.

The dried and grounded stem bark of *Pseuduvaria rugosa* was first defatted with hexane to remove chlorophyll and lipid matters using Soxhlet extractor. The plant residue was moistened with ammonia and extracted with dichloromethane. Dichloromethane extract was then concentrated to about half of its original volume and then extracted with 5% hydrochloric acid.

The hydrochloric acid extract was basified with ammonia followed by extraction with dichloromethane. Then the crude dichloromethane extract was evaporated to dryness to give crude alkaloid. The plant residue was re-extracted with methanol and evaporated to dryness to give crude methanol extract. All the crude extracts were subjected to TLC, column chromatography and HPLC analysis. The hexane extract was subjected to GC/MS analysis.

The structures of the pure phytochemical compounds were established through UV, IR,
1D and 2D NMR and MS analysis, and also by comparing data and mass spectral from previous published literature.

This work has led to the isolation of seven compounds from the alkaloid and methanol crude extracts.

The six compounds were aporphine alkaloids of which three of them are known compounds, Liriodenine 1, Ouregidione 5 and N-methylouregidione 11 and the other three are new aporphine alkaloids, *Pseuduvarine A* 36, *Pseuduvarine B* 37 and *Pseuduvarine C* 38. One new non-alkaloid compound, *Pseuduvarin* 39 which belongs to the benzo [7] annulene skeleton has also been isolated from this plant.

GCMS analysis of the hexane extract revealed 5 major compounds which are elemicin 24, isoelimicin 40, 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41, 3- Hydroxy-1-propenyl-2-methoxyphenol 42, stigmasterol 43 and γ-sitosterol 44. Other minor compounds were spathulenol 29, caryophyllene oxide 30, selina-6-en-4-ol, asarone, palmitic acid and ethyl 9-octadecenoate.
3.2 PR1: Liriodenine 1

PR1 was crystallized from hexane-EtOAc as yellow needles with melting point at 270-271°C (Lit. 279-281°C) [145]. It showed bright yellow fluorescence under the UV light and gave a positive reaction to Dragendorff’s test with the appearance of orange spot. An oxoaporphinic nature was deduced based on the formation of deep red coloration when dissolved in trifluoroacetic acid.

The oxoaporphine skeleton was deduced by its UV spectrum that exhibited absorption bands at 255, 276 (shoulder), 327 and 401 nm indicating a highly unsaturated chromophoric system of aporphine alkaloids.

Its mass spectrum showed a pseudomolecular ion peak [M+H]^+ at m/z 276.0448 corresponding to the molecular formula of C_{17}H_{9}NO_{3} (Figure 3.1). The loss of
formaldehyde [M-CHO]⁺ can be observed at m/z 247 followed by the loss of CO at m/z 219 [M-CO]⁺ which indicated the presence of methylenedioxy group in the compound (Figure 3.2). These fragmentations provide important information to confirm the structures of aporphine alkaloids.

The infrared spectrum (IR) showed the absorption peak at 1651 cm⁻¹ due to the presence of carbonyl group and the peak at 1309 and 1261 cm⁻¹ was observed which is a characteristic of methylenedioxy group (Figure 3.3).

The ¹H NMR spectrum (CDCl₃ 400 MHz) (Figure 3.4) showed the characteristic of the AB system for H-4 and H-5 peaks. The H-4 and H-5 peaks were observed at δ 7.77 and δ 8.89 as doublet (J = 5.0 Hz), respectively. A singlet with one proton appeared at δ 7.19 was assigned to proton at C-3. Another singlet with two protons was also observed at δ 6.36 which belongs to a methylenedioxy group attached at C-1 and C-2.

A peak appeared as a doublet was observed at δ 8.58 (J = 7.8 Hz) corresponding to proton at C-11. The H-8 appeared at δ 8.64 as a doublet with J=8.3 Hz and it is more deshielded compared to H-11 due to the adjacent carbonyl group at C-7. Two set of triplet signals were observed at δ 7.57 with J=7.5 Hz and δ 7.76 with J=8.2 Hz which were attributable to H-9 and H-10, respectively.

The ¹³C NMR spectrum (Figure 3.5) revealed a total of 17 carbon signals that consisted of one sp³ carbon and 16 sp² carbons. The sp³ carbon belongs to the methylenedioxy group which was observed at δ 102.56.

The C-3 signal appeared at δ 103.36 while the methine carbons C-4, C-9, C-11, C-8, C10 and C-5 signals were observed at δ 124.3, 127.5, 128.7, 128.9, 134.1 and 145.0, respectively. The carbonyl carbon appeared at very low field which is at δ 182.5. The
detailed analysis of quaternary carbons is tabulated in Table 3.1.

Finally, comparison of this spectroscopic data with those reported in the literature confirmed that PR1 was a known compound identified as liriodenine 1 which was also discovered in *Pseuduvaria rugosa* (KL 4709), *Pseuduvaria setosa* and *Pseuduvaria indochedesis*. 
Table 3.1: $^1$H (400 MHz) and $^{13}$C NMR (100 MHz) Spectral data of PR1 in CDCl$_3$

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</thead>
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<td>124.3</td>
</tr>
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<td>145.0</td>
</tr>
<tr>
<td>6a</td>
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<td>-</td>
<td>182.5</td>
</tr>
<tr>
<td>7a</td>
<td>-</td>
<td>131.5</td>
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<td>127.5</td>
</tr>
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<td>10</td>
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<td>134.1</td>
</tr>
<tr>
<td>11</td>
<td>8.58, □, 7.8</td>
<td>128.7</td>
</tr>
<tr>
<td>11a</td>
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</tr>
<tr>
<td>-O-CH$_2$-O-</td>
<td>6.36, s</td>
<td>102.6</td>
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</table>
Figure 3.1: A) Mass spectra and B) UV spectra of PR1
Figure 3.2: MS/MS of PR1
Figure 3.3: IR Spectrum of PR1
Figure 3.4: $^1$H NMR spectrum of PR1 in CDCl$_3$
Figure 3.5: $^{13}$C NMR spectrum of PR1 in CDCl$_3$
3.3 PR2: Ouregidione 5

![Chemical Structure of PR2]

Compound **PR2** was afforded as orange amorphous solid and decomposed at 262°C. The UV spectrum (Figure 3.6) exhibits maximum absorption at 213, 229, and 488 nm indicating a highly conjugated system. An absorption maximum or shoulder between 252 and 265 nm is a diagnostic for dehydroaporphine.

Its mass spectrum (Figure 3.6) established its pseudomolecular ion peak at m/z 338.077 [M+H]^+ corresponding to the molecular formula of C_{19}H_{15}NO_{5}, thus implying highly unsaturated. The MS/MS spectrum displayed the fragmentation ions at m/z 322, 321 and 309.

The peak at m/z 322 is consistent with the loss of methyl group [M-CH_{3}]^+ from one of the aromatic methoxyl substituents. The following ion fragmentation showed the elimination of a carbonyl group [M-CO]^+ at m/z 309 (Figure 3.7).

The IR spectrum showed a conjugated ketone or six member ring lactam at 1733, 1667 and 1455 cm\(^{-1}\). The presence of N-H group is evidenced from the absorption peak at
The $^1$H NMR (400 MHz) (Figure 3.9) of PR2 measured in deuterated trifluoroacetic acid (TFA) as solvent showed the presence of three methoxyl signals appearing as three singlets with the intensity of three protons each at $\delta$ 4.73, 4.52 and 4.49, and positioned at C-3, C-2, and C1, respectively.

Among the aromatic protons, the signal for H-11 is shifted to a lower field, which is at $\delta$ 9.84 as a doublet with $J =$ 8.5 Hz. This circumstance is typical since C-1 is substituted with a methoxyl group in ring A. The peak at $\delta$ 8.73 appeared as one proton singlet belongs to proton at C-7.

Another peak appeared as one proton doublet at $\delta$ 8.42 with $J =$ 8.5 Hz was assigned for H-8. The peak at $\delta$ 8.09 appeared as a one proton triplet belongs to H-9 and the proton signal at $\delta$ 8.19 appeared as a triplet was assigned for H-10 (Figure 3.10). The details of $^1$H NMR data of PR2 are summarized in Table 3.2.

Based on the spectral data of PR2 and comparison with the literature value [134], it is confirmed that PR2 is a known alkaloid identified as ouregidione 5 which was previously isolated from Pseuduvaria rugosa (KL 4709) and Pseuduvaria setosa.
Table 3.2: $^1$H NMR Data of PR2 (400 MHz) in CDCl$_3$

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H, δ, (J, Hz)</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6a</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>8.73, s, 1H</td>
</tr>
<tr>
<td>7a</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>8.42, d, 1H, 8.5</td>
</tr>
<tr>
<td>9</td>
<td>8.19, t, 1H, 8.5</td>
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<td>8.09, t, 1H, 8.5</td>
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<td>C1-OCH$_3$</td>
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<td>C2-OCH$_3$</td>
<td>4.52, s</td>
</tr>
<tr>
<td>C3-OCH$_3$</td>
<td>4.73, s</td>
</tr>
</tbody>
</table>
Figure 3.6: A) Mass spectra and B) UV spectra of PR2
Figure 3.7: MS/MS of PR2
Figure 3.8: IR Spectrum of PR2
Figure 3.9: $^1$HNMR spectrum of PR2 in CF$_3$COOD
Fig 3.10: Expansion of the proton signals of PR2 in the aromatic region
3.4 PR3: N-methylouregidione 11

PR3 was obtained as fluorescence bright yellow amorphous solid decomposed at 199°C. PR3 showed an orange spot when sprayed with Dragendorff’s reagent meaning that it is an alkaloid. The characteristic of an oxoaporphine is showed by the deep red color when mixed with acid medium.

The UV spectrum of PR3 showed absorption bands at \( \lambda_{\text{max}} \) 212, 242, 275, 306, and 416 nm indicating a highly conjugated unsaturated oxoaporphine structure. The mass spectrum exhibited a molecular ion peak at m/z 352.0985 \([\text{M+H}]^+\) corresponding to the molecular formula of C\(_{20}\)H\(_{17}\)NO\(_5\). (Figure 3.11)

The peak at m/z 337 \([\text{M-CH}_3]^+\) corresponded to the loss of methyl group attached to the nitrogen atom at position 7. This confirmed the existence of \(N\)-methyl group in compound PR3. (Figure 3.11)
Its IR spectrum revealed a broad absorption peak at 1712 cm\(^{-1}\) which corresponds to the presence of two carbonyl functional groups in ring B (Figure 3.12).

The \(^1\)H-NMR (400 MHz) spectrum in CDCl\(_3\) (Figure 3.13) displayed three methoxyl signals at \(\delta\) 4.18, 4.09 and 4.01 which correspond to methoxyl groups at C-3, C-2 and C-1, respectively. An N-methyl group resonates at \(\delta\) 3.45 (Figure 3.14).

The presence of five aromatic protons found at \(\delta\) 7.62 (\(J = 8.8\) Hz) as a triplet which belongs to H-9. The signal at \(\delta\) 7.56 (\(J = 6.8\) Hz) as a triplet assigned for H-10 whereas the peak at \(\delta\)7.96 appeared as a doublet with \(J = 8.0\) Hz belongs to H-8. The H-11 signal appeared at very lower field which is at \(\delta\) 9.40 as a doublet with \(J = 8.8\) Hz. This gave an indication that ring D is unsubstituted. The proton signal at C-7 appeared at \(\delta\) 7.88 as one proton singlet. The proton assignments of \textbf{PR3} are summarized in Table 3.3.

The spectroscopic data obtained were in agreement with that of the previous literature [131] and \textbf{PR3} was established as \textit{N}-methylouregidione which was isolated from \textit{Pseu duvaria setosa} and \textit{Pseu duvaria macrophylla}. 


Table 3.3: $^1$H NMR data of PR3 (400 MHz) in CDCl$_3$

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H, δ, (J, Hz)</th>
</tr>
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<tbody>
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<td>1</td>
<td>-</td>
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<tr>
<td>1a</td>
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<td>1b</td>
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<td>2</td>
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<td>-</td>
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<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6a</td>
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</tr>
<tr>
<td>7</td>
<td>7.88, s, 1H</td>
</tr>
<tr>
<td>7a</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>7.96, d, 8.0, 1H</td>
</tr>
<tr>
<td>9</td>
<td>7.62, t, 8.8, 1H</td>
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<tr>
<td>N-CH$_3$</td>
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Figure 3.11: A) Mass spectra and B) UV spectra of PR3
Figure 3.12: IR Spectrum of PR3
Figure 3.13: $^1$H NMR spectrum of PR3
Figure 3.14: Expansion of $^1$H NMR in the aromatic region (PR3)
3.5 PR4 : Pseuduvarine A 36

PR4 was isolated as an orange amorphous solid and showed a positive reaction to Dragendorff’s test meaning that it is an alkaloid which contains nitrogen atom. It did not dissolve well in deuterated chloroform but it was highly soluble in methanol.

An aporphine skeleton was suggested by its UV spectrum showing the $\lambda_{\text{max}}$ at 216, 261, 323 and 425 nm indicating a highly conjugated system.

The mass spectrum of PR4 showed a pseudomolecular ion peak at m/z 323.0764 [M+H]$^+$ suggesting a molecular formula C$_{18}$H$_{14}$N$_2$O$_4$ (Figure 3.15). The IR absorptions showed the characteristic for carbonyl function group at 1687 and 1614 cm$^{-1}$ and NH$_2$ group at 3434 cm$^{-1}$ (Figure 3.16).

The $^1$H-NMR (700 MHz) spectrum measured in deuterated dimethylsulphoxide (DMSO d$_6$) (Figure 3.17) showed signals at $\delta$ 3.98 and $\delta$ 4.07 corresponding to two methoxyl groups at position C-1 and C-2. The proton H-11 appeared as a doublet at lower field which is at $\delta$ 9.29 ( $J$ = 8.0 Hz) and the proton H-8 resonated as doublet at $\delta$ 7.96 ($J$=8.0Hz).
The proton resonated at $\delta$ 7.83 belongs to H-7 and appeared as a singlet. The signal appeared as a multiplet between $\delta$ 7.53 to $\delta$ 7.56 belonging to the protons positioned at C-9 and C-10 (Figure 3.18). This means that the ring D in the aporphine skeleton is unsubstituted.

The peak appeared at $\delta$ 9.35 as a broad singlet was assigned to N-H$_b$ group and the signals resonated at $\delta$ 11.90 belongs to N-H$_a$ group and the broad singlet peak at $\delta$ 12.60 was assigned to N6-H group. N-H$_a$ and N6-H were deshielded by the adjacent carbonyl group and the signals appeared at very lower field. From the $^1$H NMR data, compound PR4 was presumed to be 1,2,3-trisubstituted-4, 5-dioxoaporphine.

The $^{13}$C NMR data (Figure 3.19) supported the 4, 5-dioxoaporphine skeleton by carbonyl signals at $\delta$ 177.6 and $\delta$ 156.3 which were attributable to C-4 and C-5, respectively. Sixteen other carbon signals were observed which consist of two methoxyl groups, five aromatic C-H, and nine sp$^2$ quaternary carbons.

The two methoxyl carbon signals appeared at $\delta$ 59.8 and 60.9 which are attached to C-2 and C-1, respectively. The signal resonated at $\delta$ 114.2 belongs to C-7 whereas C-8, C-9, C-10 and C-11 appeared at $\delta$ 128.3, 125.2, 126.3 and 125.6, respectively. The C-3 bearing NH$_2$ group resonated at $\delta$ 151.00. The sp$^2$ carbons C-1a, C-1b, C-3a, C-6a, 7a and 11a resonated at $\delta$ 115.1, 116.0, 105.4, 128.4, 130.8 and 127.0, respectively (Figure 3.20). The complete assignments of all protons and carbons of compound PR4 are illustrated in Table 3.4.

In COSY spectrum, there are cross-peaks between H-11 and H-10, H-10 and H-9, H-9 and H-8, and also N-Ha and N-Hb. The structure for PR4 was further established and confirmed by the analysis of the 2D-NMR correlation [$^1$H-$^1$H COSY, HSQC, HMBC, and $^1$H-$^{15}$N HMBC] as shown in Figure 3.21, 3.22, 3.23, 3.24 and 3.25).
The presence of NH and one NH$_2$ functional moieties was concluded from the observed split cross-peaks in the $^1$H-$^{15}$N HMBC spectrum. The position of the NH moiety was deduced from the HMBC correlations of H-7 to N-6 and NH to C-1b and C-4. Finally, the substitution pattern for ring A was deduced from the HMBC correlations of NH$_2$ to C-3a ($\delta$ 105.4) (Figure 3.26 and 3.27).

Therefore, PR4 was established as a new aporphine alkaloid and it was elucidated as 3-amino-1, 2-dimethoxy-4, 5-dioxoaporphine or Pseuduvarine A 36. This is the first isolation of a dioxoaporphine alkaloid with an amino group at C3 in ring A.
Table 3.4: $^1$H(700 MHz) and $^{13}$C(175 MHz) NMR data of PR4 at 300K in DMSO d$_6$

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<td>128.3</td>
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<td>9</td>
<td>7.53 (1H, t, 8.0)</td>
<td>125.2</td>
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<td>10</td>
<td>7.56 (1H, t, 8.0)</td>
<td>126.3</td>
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<td>9.29 (1H, d, 8.0)</td>
<td>125.6</td>
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<tr>
<td>11a</td>
<td>-</td>
<td>127.0</td>
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<td>C1-OMe</td>
<td>4.07 (3 H, s)</td>
<td>60.9</td>
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<td>C2-OMe</td>
<td>3.98 (3H, s)</td>
<td>59.8</td>
</tr>
<tr>
<td>N6-H</td>
<td>12.60 (1H, s)</td>
<td>-237.8$^a$</td>
</tr>
<tr>
<td>N-H$_b$</td>
<td>9.35 (1H, s)</td>
<td>-273.3$^a$</td>
</tr>
<tr>
<td>N-H$_a$</td>
<td>11.90 (1H, s)</td>
<td>-273.3$^a$</td>
</tr>
</tbody>
</table>

$^a$ δ$_N$ in ppm was estimated from the cross peaks in $^1$H-$^{15}$HMBC spectra.
Figure 3.15: A) Mass spectra and B) UV spectra of PR4
Figure 3.16: IR Spectrum of PR4
Figure 3.17: $^1$HNMR spectrum of PR4 at 700 MHz in DMSO d$_6$
Figure 3.18: Expanded $^1$H NMR spectrum of PR4 in DMSO $d_6$
Figure 3.19: $^{13}$C NMR spectrum of PR4 in DMSO $D_6$
Figure 3.20: Expanded $^{13}$C-NMR spectrum of PR4 in DMSO $d_6$
Figure 3.21: COSY spectrum of PR 4
Figure 3.22: HSQC spectrum of PR4 in DMSO d$_6$
Figure 3.23: HMBC spectrum of PR4 in DMSO d$_6$
Figure 3.24: $^1$H-$^{15}$N HMBC spectrum of PR 4 in C$_5$D$_5$N
Figure 3.25: Projection of $^1$H-$^{15}$N HMBC spectrum of PR 4
Figure 3.26: Selected 2D-NMR correlation for PR4.

Figure 3.27: $^1$H-$^15$N HMBC correlation of PR4.
3.6 PR5: Pseuduvarine B

PR5 was obtained as orange amorphous solids that gave a positive reaction (orange spot) with Dragendorff’s reagent. The UV and IR spectrum of PR5 were similar to those of PR4. The UV spectrum exhibited absorption maxima at 214, 261, 323 and 421 nm indicating a highly conjugated system and the mass spectrum showed the [M+ H]^+ ion at m/z 337.0812 suggesting a pseudomolecular formula C_{19}H_{16}N_{2}O_{4} (Figure 3.28). Its IR spectrum showed absorption bands of two carbonyl groups at 1684 and 1608 cm\(^{-1}\) (Figure 3.29).

The \(^1\)H NMR (700 MHz) spectrum in deuterated DMSO (Figure 3.30) showed the presence of two methoxyl groups at \(\delta\) 3.98 and \(\delta\) 4.06 and most probably positioned at C-1 and C-2, respectively. The proton H-11 appeared at lower field as a doublet at \(\delta\) 9.28 \((J = 8.0\) Hz\) and the proton H-8 resonated at \(\delta\) 8.09 \((J = 8.0\) Hz\) as a doublet (Figure 3.31).

There is one proton singlet resonated at \(\delta\) 8.17 and it probably belongs to H-7. There is no other singlet peak in the spectrum and it means that C-3 was substituted with NH\(_2\) group. The
two peaks appeared as triplet resonated at δ 7.56 and δ7.60 belongs to H-9 and H-10, respectively in ring D. The N-methyl group appeared at δ 3.90 as a singlet. The N-Hₐ and N-Hₐ groups resonated at δ 12.04 and 9.38, respectively. See Table 3.5 for ¹H NMR and ¹³C NMR spectral data.

The ¹³C NMR spectrum (Figure 3.32 and 3.33) supported the 4,5-dioxoaporphine skeleton by the carbon signals showing at δ 177.4 and δ 156.2 which is a characteristic of two carbonyl groups at C-4 and C-5, respectively. The N-methyl carbon signal resonated at δ 30.8 and the other signals resembled PR4 with a slight different chemical shift.

The structures of PR5 were further established with 2D NMR experiments (COSY, HSQC and HMBC) which are shown in Figure 3.34, 3.35, 3.36 and 3.37. In the COSY spectrum of PR5, the proton on C11 is clearly coupled to the proton on C-10, H10 coupled to H9; H9 coupled to H8, and N-Hₐ proton coupled to N-Hₐ proton.

The existence of one N-Hₐ and one N-Hₐ functional moieties was concluded from the observed split cross-peaks in the ¹H-¹⁵N HMBC spectrum (Figure 3.38). Further analysis of the 2D NMR data confirmed the methyl position at N-6 from the HMBC correlations of N6-CH₃ to C-6a and C-5 (Figure 3.39 and 3.40).

Based on these spectral data, it is concluded that PR5 is a new aporphine alkaloid and identified as N-methyl-3-amino-1,2-dimethoxy-4,5-dioxoaporphine or Pseuduvarine B 37.
Table 3.5: $\textsuperscript{1}H$(700 MHz) and $\textsuperscript{13}C$(175 MHz) NMR data of PR5 at 300K in DMSO $d_6$

<table>
<thead>
<tr>
<th>Position</th>
<th>$\textsuperscript{1}H$, $\delta$, (ppm, Hz)</th>
<th>$\textsuperscript{13}C$, $\delta$</th>
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<td>1</td>
<td>-</td>
<td>158.7</td>
</tr>
<tr>
<td>1a</td>
<td>-</td>
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<td>1b</td>
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<td>116.6</td>
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<td>3</td>
<td>-</td>
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<tr>
<td>3a</td>
<td>-</td>
<td>104.6</td>
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<td>4</td>
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<td>177.4</td>
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<tr>
<td>5</td>
<td>-</td>
<td>156.2</td>
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<tr>
<td>6a</td>
<td>-</td>
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<td>114.8</td>
</tr>
<tr>
<td>7a</td>
<td></td>
<td>130.9</td>
</tr>
<tr>
<td>8</td>
<td>8.09, d, 8.0, 1H</td>
<td>129.0</td>
</tr>
<tr>
<td>9</td>
<td>7.56, t, 8.0, 1H</td>
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<td>10</td>
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<td>126.7</td>
</tr>
<tr>
<td>11</td>
<td>9.28, d, 8.0, 1H</td>
<td>125.5</td>
</tr>
<tr>
<td>11a</td>
<td>-</td>
<td>126.9</td>
</tr>
<tr>
<td>C1-OMe</td>
<td>3.98, s, 3H</td>
<td>60.9</td>
</tr>
<tr>
<td>C2-OMe</td>
<td>4.06, s, 3H</td>
<td>59.8</td>
</tr>
<tr>
<td>N-H$_a$</td>
<td>12.04, s, 1H</td>
<td>-242.0$^a$</td>
</tr>
<tr>
<td>N-H$_b$</td>
<td>9.38, s, 1H</td>
<td>-272.0$^a$</td>
</tr>
<tr>
<td>N6-Me</td>
<td>3.90, s, 3H</td>
<td>30.8</td>
</tr>
</tbody>
</table>

$^a$ $\delta_N$ in ppm was estimated from the cross peaks in $\textsuperscript{1}H$-$\textsuperscript{15}N$ HMBC spectra.
Figure 3.28: A) Mass spectra and B) UV spectra of PR 5
Figure 3.29 : IR Spectrum of PR5
Figure 3.30: $^1$H NMR spectrum of PR5 in DMSO $d_6$
Figure 3.31: Expanded $^1$H NMR spectrum of PR5 in DMSO d$_6$
Figure 3.32: $^{13}$C-NMR spectrum of PR5 in DMSO $d_6$
Figure 3.33: Expanded $^{13}$C-NMR spectrum of PR5 in DMSO d$_6$
Figure 3.34: COSY spectrum of PR5 in DMSO d₆
Figure 3.35: HSQC spectrum of PR 5 in DMSO $d_6$
Figure 3.36: HMBC spectrum of PR6 in DMSO $d_6$
Figure 3.37: Expanded HMBC spectrum of PR6 in DMSO d$_6$
Figure 3.38: $^1$H- $^{15}$N HMBC spectrum of PR 5 in DMSO $d_6$
Figure 3.39: Projection of $^1$H-$^{15}$N HMBC spectrum of PR 5

Figure 3.40: $^1$H-$^{15}$N HMBC correlation of PR5
3.7 PR6 : Pseudevarine C 38

PR6 was obtained as amorphous solid. The UV of PR6 showed the maximum absorptions at $\lambda_{\text{max}}$ 214, 251 323 and 424 nm indicating a highly conjugated molecule. The mass spectrum of PR6 (Figure 3.41) revealed the [M+H]$^+$ peak at m/z 324.0679 corresponding to the molecular formula of C$_{18}$H$_{13}$NO$_5$. The fragment ion peaks at m/z 214 and m/z 196 showed the lost of one hydroxyl (OH).

Its IR spectrum showed the absorption peak at 3366 cm$^{-1}$ assigned for OH functional group and NH group at 3583 cm$^{-1}$. The absorption peak at 1662 cm$^{-1}$ was due to the carbonyl groups in the molecule (Figure 3.42).

The $^1$H-NMR in CDCl$_3$ (400 MHz) (Figure 3.43) of PR6 showed the presence of two methoxy singlets at $\delta$ 4.02 and $\delta$ 4.13 which positioned at C-1 and C-2, respectively. There are five CH aromatic peaks in the spectrum indicating that the ring D was unsubstituted. The H-7 proton appeared at $\delta$ 7.70 as a singlet.
The peak at $\delta$ 9.36 was assigned for H-11 and appeared as a doublet with $J = 9.2$ Hz whereas the H-8 peaks was observed at $\delta$ 7.86 as a doublet with $J = 7.8$Hz. The H-9 and H-10 were resonated at $\delta$ 7.48 and 7.55, respectively and appeared as a triplet with $J = 6.8$ Hz. The OH and NH peaks were observed at $\delta$ 5.26 and $\delta$ 11.70, respectively as broad peak.

The $^{13}$C-NMR (Figure 3.44) spectrum showed eighteen carbon signals consist of two methoxyl groups, five $sp^2$ methine, nine $sp^2$ quaternary carbons and two carbonyl groups. The two methoxyl peaks appeared at $\delta$ 60.60 and $\delta$ 61.1 which attached to C-1 and C-2 respectively. The C-7 peak resonated at $\delta$ 115.4 whereas C-10, C-9, C-11 and C-8 appeared at $\delta$ 125.6, 126.4, 126.9 and 128.33, respectively.

The two carbonyl groups resonated at $\delta$ 160.1 and $\delta$ 170.2 belong to C-5 and C-4, respectively whereas the C-3 bearing OH group resonated at 178.5. The C-1 and C-2 bearing the methoxyl group appeared at $\delta$ 145.0 and $\delta$ 157.5, respectively. The rest of the quaternary carbons peaks are tabulated in Table 3.4.

In COSY spectrum of PR6, there are correlation between H-8 with H-9; H-9 with H-10 and H-8; H-10 with H-9 and H-11; and H-11 with H-10 (Figure 3.45). The other 2D NMR spectrum could not be carried out due to insufficient amount of PR6.

Based on the spectral data, it is concluded that compound PR6 is a new aporphine alkaloid identified as 3-hydroxy, 1, 2-dimethoxy, 4,5-dioxoaporphine or Pseudo uvarine C 38.
Table 3.6: $^1$H and $^{13}$C NMR Spectral data of PR6 in CDCl$_3$ (400 and 100 MHz)

<table>
<thead>
<tr>
<th>POSITION</th>
<th>$^1$H, δ , (J, Hz)</th>
<th>$^{13}$C, δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
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<td>115.4</td>
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<tr>
<td>7a</td>
<td>-</td>
<td>132.1</td>
</tr>
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<td>8</td>
<td>7.86, d, 7.8</td>
<td>128.3</td>
</tr>
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<td>9</td>
<td>7.48, t, 6.8</td>
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<td>125.6</td>
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<td>9.36, d, 9.2</td>
<td>126.9</td>
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<tr>
<td>11a</td>
<td>-</td>
<td>133.0</td>
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<td>OH</td>
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<td>NH</td>
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<td>C1-OCH$_3$</td>
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<tr>
<td>C2-OCH$_3$</td>
<td>4.13</td>
<td>61.1</td>
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</tbody>
</table>
Figure 3.41: A) Mass spectra and B) UV spectra of PR6
Figure 3.42: IR Spectrum of PR6
Figure 3.43: $^1$H NMR spectrum of PR6
Figure 3.44: $^{13}$C NMR spectrum of PR6
Figure 3.45 : COSY spectrum of PR6
3.8 PR7: Pseuvarin 39

PR7 was isolated as orange amorphous in very little amount that overlapped with PR4. The retention time of PR7 was so close to that of PR4, 5.390 min and 5.490 min, respectively (Figure 3.46).

The UV spectrum of PR7 showed absorption bands at 216, 264 and 424 nm indicating a highly conjugated bond (Figure 3.47). Its IR spectrum displayed broad hydroxyl absorption at 3366.94 cm$^{-1}$ and a carbonyl band at 1667.56 cm$^{-1}$ (Figure 3.48).

The structural elucidation of PR7 was solely based on the mass spectroscopic data. From the mass spectroscopic chromatogram, PR7 had nearly the same retention time as PR4. It was first detected using the LCMS/MS in a negative ion mode. At first, it was thought that the unknown compound was an alkaloid, but further test with Dragendorff’s reagent gave a negative result.

The mass spectrum of PR7 showed a pseudomolecular ion peak [M+ H]$^+$ at m/z 323.0807 suggesting a possible molecular formula of C$_{15}$H$_{14}$O$_8$. The base peak appeared
at m/z 307 indicating the loss of one methyl group which was followed by another fragment ion at m/z 292 indicating another loss of methyl group. The subsequent product ions at m/z 264, 236, 208 and 180 indicated the lost of four carbonyl groups (Figure 3.49). The fragmentation pattern of PR7 is shown as below:
Based on the fragmentation pattern of PR7, we deduce that PR7 is 1-Hydroxy-2,3,9-trimethoxy-9-methyl-5H-benz[7] annulene-5,6,7,8(9H)-tetraone or Pseuduvarin 39. This is a new compound which has never been isolated from any plant species.

Figure 3.46: HPLC chromatograms of PR4 and PR7 in MeOH extract.
Figure 3.47: A) Mass spectrum and B) UV spectrum of PR7
Figure 3.48: IR spectrum of PR7
Figure 3.49: Total ion mass spectrum of PR7 in negative ion mode
3.9 GCMS Analysis of the Hexane Extract

The hexane extract of *Pseuduvaria rugosa* was analysed by GC/MS analysis. The structures of the compounds were identified based on the percentage similarity values and the fragmentation pattern of the mass spectra in comparison with those of reference standard compounds in the computer database using NIST standard library reference (NIST – National Institute of Standards and Technology-Chemistry) and also by matching data from those reported in the previous literature. The relative content of each component was determined by peak area normalization. About 50 peaks were detected and 41 compounds with more than 80% similarities were observed.

The five major components identified in the hexane extract of *Pseuduvaria rugosa* were elimicin 24, isoelimicin 40, 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41, 3-Hydroxyl -1-propenyl-2 methoxyphenol 42, stigmasterol 43 and gamma sitosterol 44.

A representative chromatogram of the hexane extract is shown in Figure 3.50.

The largest peak, which eluted at retention time 24.78 minute, was identified as isoelimicin 40 with a molecular weight of 208. The second larger peak eluted at retention time 22.57 minute was identified as elimicin 24.
The following third prominent peak at retention time 23.49 minute with molecular weight of 238 was assigned for 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41.

![Chemical Structure of 1,2,3,4-Tetramethoxy-5-(2-propenyl)benzene](image)

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The fourth peak at retention time 26.733 was matched to a phenolic compound, identified as 3-Hydroxy-1-propenyl-2 methoxyphenol 42 with a molecular weight of 180.

![Chemical Structure of 3-Hydroxy-1-propenyl-2 Methoxyphenol](image)

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The last two major peaks at longer retention time, 65.74 and 70.82 minute with molecular weights of 412 and 414, respectively, belong to stigmasterol 43 and gamma-sitosterol 44.
Other minor components were some monoterpenes, sesquiterpenes, diterpenes and ether that include spathulenol 29, caryophyllene oxide 30, selina-6-en-4-ol, asarone, palmitic acid and ethyl 9-octadecenoate.

The mass fragment ions of target compounds compared to that of the standard compounds in the NIST library with more than 80% similarities are shown in Table 3.7.
Table 3.7: GCMS spectral data of the compounds in the hexane extract of *P. rugosa*

<table>
<thead>
<tr>
<th>Peaks</th>
<th>RT(min)</th>
<th>Area %</th>
<th>SI</th>
<th>MW</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Elemicin 24</td>
<td>22.558</td>
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<td>C_{12}H_{16}O_{3}</td>
</tr>
<tr>
<td>2. 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41</td>
<td>23.498</td>
<td>4.66</td>
<td>87</td>
<td>238</td>
<td>C_{13}H_{18}O_{4}</td>
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<td>4. Selina-6-en-4-ol</td>
<td>23.917</td>
<td>1.26</td>
<td>83</td>
<td>222</td>
<td>C_{13}H_{26}O</td>
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<tr>
<td>5. Spathulenol 29</td>
<td>24.258</td>
<td>1.92</td>
<td>85</td>
<td>220</td>
<td>C_{13}H_{24}O</td>
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<td>6. Isoelemicin 40</td>
<td>24.783</td>
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<td>92</td>
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<td>7. Caryophyllene oxide30</td>
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<td>81</td>
<td>220</td>
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<td>8. Asarone</td>
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<td>89</td>
<td>208</td>
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<tr>
<td>9. 3-Hydroxy-1-propenyl-2methoxyphenol 42</td>
<td>26.733</td>
<td>3.16</td>
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<td>180</td>
<td>C_{10}H_{12}O_{3}</td>
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<td>10. Palmitic acid</td>
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<td>93</td>
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<td>11. Ethyl 9-octadecenoate</td>
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<td>85</td>
<td>310</td>
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<td>12. Stigmasterol 43</td>
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<td>412</td>
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<td>13. Gamma -Sitosterol 44</td>
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<td>6.25</td>
<td>95</td>
<td>414</td>
<td>C_{25}H_{50}O</td>
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</table>
Figure 3.50: GCMS Chromatogram of hexane extract
Elimicin 24

1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41

Selina-6-en-4-ol
Spathulenol 29

Isoelimicin 40

Caryophyllene oxide 30
Asarone

3-Hydroxyl -1- propenyl-2 methoxyphenol 42

Palmitic acid
Ethyl 9-octadecenoate

Stigmasterol 43

Gamma-Sitosterol 44
3.10 BIOACTIVITY EVALUATION

The hexane, dichloromethane and methanol extracts of *Pseuduvaria rugosa* and isolated compounds PR 36 and PR 37 were tested for their *in vitro* antioxidant and cytotoxic activity. Other isolated compounds were not enough for antioxidant and cytotoxic activity tests. The total phenolic content of the crude extracts were also determined with gallic acid as the standard.

3.10.1 Antioxidant Activity by DPPH method

The antioxidant activity of the crude extracts of hexane, dichloromethane and methanol of *Pseuduvaria rugosa* were preliminary screened *in vitro* using DPPH free radical scavenging assay at 100 µg/ml. The results are shown in Table 3.8.

Results showed that the hexane extract exhibited very poor scavenging activity with 3.86% of inhibition when compared with standard ascorbic acid. The dichloromethane extract showed lower scavenging activity (19.32% inhibition) and the methanol extract showed moderate scavenging activity (34.78 % of inhibition). In general, more than 50% inhibition is considered active.

Overall, all the crude extracts did not show any significant radical scavenging activity compared to ascorbic acid as a standard. There was no correlation between antioxidant activity and total phenolic content of the crude extracts. The total phenolic content revealed that the crude extracts did not contain phenolic compounds as the major component.

Since the results in the preliminary screening did not show any significant activity, the analysis on the antioxidant activity of the isolated compounds was not carried out due to insufficient samples. From the preliminary screening of antioxidant activity, it can be
concluded that the crude extracts from the stem bark of *Pseuduvaria rugosa* may not be a suitable potential sources for natural antioxidant.

### Table 3.8. Antioxidant activity and total phenolic content of crude extracts.

<table>
<thead>
<tr>
<th>Crude extract</th>
<th>% inhibition*</th>
<th>Total phenolic content (ugGAE/1mg )</th>
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<tr>
<td>Hexane</td>
<td>3.86</td>
<td>119.21</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>19.32</td>
<td>195.66</td>
</tr>
<tr>
<td>Methanol</td>
<td>34.78</td>
<td>114.10</td>
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<tr>
<td>Ascorbic acid</td>
<td>84.22</td>
<td>–</td>
</tr>
<tr>
<td>(standard)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*percentage of DPPH inhibition of extract at 100 µg/mL

#### 3.10.2 *In vitro* Cytotoxicity test

The crude extracts were initially evaluated for their cytotoxic activities on seven cancer cell lines. The results of the cytotoxic activity of hexane, dichloromethane and methanol crude extracts are summarized in Table 3. The results showed that the hexane, dichloromethane and methanol extracts of *Pseuduvaria rugosa* exhibited significant activity against all the seven cancer lines at 100 µg/ml. The hexane and dichloromethane extract exhibited potent cytotoxicity against breast (MCF7), colon (HT-29) and pancreas cell lines (PxPC3) with more than 90% of cell death.
Table 3.9: Cytotoxic activity of crude extracts of *Pseuduvaria rugosa*

<table>
<thead>
<tr>
<th>Cancer cell lines</th>
<th>Hexane</th>
<th>Dichloromethane</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast (MCF7)</td>
<td>100</td>
<td>97.7</td>
<td>91.5</td>
</tr>
<tr>
<td>Breast (MDA-MB-231)</td>
<td>68.6</td>
<td>87.5</td>
<td>63.6</td>
</tr>
<tr>
<td>Colon (HT-29)</td>
<td>93.7</td>
<td>96.0</td>
<td>57.3</td>
</tr>
<tr>
<td>Lung (A549)</td>
<td>54.2</td>
<td>76.7</td>
<td>9.30</td>
</tr>
<tr>
<td>Pancreas (PxC PC-3)</td>
<td>93.2</td>
<td>94.6</td>
<td>94.8</td>
</tr>
<tr>
<td>Prostate (DU145)</td>
<td>60.3</td>
<td>79.2</td>
<td>45.2</td>
</tr>
<tr>
<td>Liver (HepG-2)</td>
<td>82.7</td>
<td>87.7</td>
<td>13.3</td>
</tr>
</tbody>
</table>

Note: MCF7- Estrogen receptor positive, MD-MB-231- estrogen receptor negative

The cytotoxicity of the new two new alkaloid compounds, **PR36** and **PR37** were tested against breast cells (MCF7), liver cells (HepG2) and human lung cells (HL-60) using the MTT assay.

Results showed that **PR36** and **PR37** showed significant and potent cytotoxicity and selectivity towards the tested cells, with Pseuduvarine A **36** being the most toxic against breast cells (MC7) at IC$_{50}$ value of 0.9 µM and Pseuduvarine **37** being the most toxic and selective on human promyelocytic leukemia (HL-60) cell lines.
Table 3.10: Cytotoxic activity of PR36 and PR37 at IC$_{50}$

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>MCF7</th>
<th>HEPG2</th>
<th>HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*IC$_{50}$ (uM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound PR36</td>
<td>0.90</td>
<td>21.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Compound PR37</td>
<td>&gt;50</td>
<td>15.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>

*IC$_{50}$ represents the concentration causing a 50% cytotoxic effect.
CHAPTER 4
CONCLUSION

The phytochemical investigation of the stem bark of *Pseuduvaria rugosa* have led to the isolation of seven compounds, six of them were aporphone alkaloids of which three were known compounds: Liriodenine 1, ouregidione 5 and *N*-methylouregidione 11 while the other three compounds were new aporphone alkaloids, pseuduvarine A 36, pseuduvarine B 37 and pseuduvarine C 38. The seventh compound, pseuduvarin 39 is believed to be a new compound having benzo[7]annulene skeleton which is not fully identified that has not been isolated before from any plant species.

The GC/MS analysis of the hexane extract revealed some fatty acids and terpenoids, of which the major compounds were isoeliminic 40, 1,2,3,4-tetramethoxy-5-(2-propenyl)benzene 41 (phenolic ether) and 3-Hydroxy-1-propenyl-2 methoxyphenol 42.

The other compounds are elimicin 24, spathulenol 29 and caryophyllene oxide 30 which were previously identified from leaf essential oils of *Pseuduvaria froggattii* and *Pseuduvaria mulgraveana*. The sterol compounds found in *Pseuduvarisa rugosa* are stigmasterol 43 and gamma-sitosterol 44.

In this study, the antioxidant and cytotoxic activities from the bark of *Pseuduvaria rugosa* were evaluated. The results of the study revealed that the crude extracts of *Pseuduvaria rugosa* demonstrated strong cytotoxic activity but moderate antioxidant activity.

Preliminary screening of the crude extracts of *Pseuduvaria rugosa* on the antioxidant activity using DPPH method revealed low to moderate radical scavenging activities in
all hexane, dichloromethane and methanol crude extracts, thus, it can be concluded that the plant is not a potential source for natural antioxidant.

In the preliminary screening to determine the cytotoxicity of the crude extracts against seven cancer cell lines, all the crude extracts (hexane, dichloromethane and methanol) showed significant cytotoxic activity against the seven cancer cell lines with more than 80% of cell death.

The hexane and dichloromethane extracts of *Pseudevaria rugosa* showed potent cytotoxic activity against breast, lung and pancreas cell lines. Further test with the isolated compounds, ouregidione 5, *N*-methylouregidione 11, pseuduvarine C 38 and pseuduvarine 39 showed moderate activity or no activity at all in the cytotoxic bioassay test. It could be the synergetic effects from other compounds in the crude extracts that demonstrated the significant cytotoxic activities.

However, the two new alkaloids with the amino group at C-3, pseuduvarine A 36 and pseuduvarine B 37 isolated from the methanol extract showed significant activity and selectivity in the cytotoxic test against three cancer cell lines namely human breast adenocarcinoma (MCF7), human liver carcinoma cells (HepG2) and human promyelocytic leukemia cells (HL-60). This justified the claims that this plant may possess anticancer properties.

Some of the limitations in this study include insufficient amount of the isolated compounds which were yielded in very small quantities that were not enough for further structure elucidations and further bioactivity tests. In the case of pseuduvarin C 38 and pseuduvarin 39, the yield for the pure compound was so little to be fully analysed by NMR and 2D NMR. Most of the time, most of the compounds overlapped with one another.
The information and data from this study will enable other scientists and researchers to compare data from other *Pseuduvaria* species which were quite limited at this time and hopefully with further investigations, the new compounds PR36 and PR37 can be developed to be a potential candidate for anticancer agent.

It is proposed that further study to be carried out to isolate pseuduvarin C 38 and pseuduvarin 39 in a larger amount in order to confirm the structure of the compounds by means of NMR and 2D NMR.
CHAPTER FIVE

EXPERIMENTAL

5.1 General Experimental Procedures

5.1.1 Solvents

All solvents used such as hexane, dichloromethane and methanol for soxhlet extraction, column separation and fractionation are of analytical grade (AR). Solvents such as acetonitrile (ACN) and methanol were of HPLC grade for HPLC analysis.

5.2 Instrumentation and Spectroscopic Methods

5.2.1 Optical rotation

Optical rotation was measured on a JASCO DIP-1000 polarimeter with methanol and chloroform as solvent.

5.2.2 UV Spectra

Ultraviolet (UV) spectra were recorded on LCMS- IT-TOF equipped with a PDA detector set at the range of 200-400nm.

5.2.3 IR Spectra

The infrared (IR) spectra were recorded on Perkin Elmer FT-IR Spectrometer Spectrum RX1 using chloroform as the solvent.
5.2.4 NMR Spectra

NMR spectra and data analysis were obtained using JEOL FT NMR, JEOL ECA400 FT NMR and 700 MHz with deuterated chloroform (CDCl₃), deuterated methanol (CD₃OD) and deuterated DMSO used as solvents. Chemical shifts were reported in ppm and coupling constants were measured in Hertz (Hz).

5.2.5 Mass Spectra

Mass spectra were recorded on LCMS/MS Shimadzu IT-TOF with Waters X- Bridge RP C-18 column (2.1 mm id x 50 mm, particle size 2.5 um) using HPLC grade acetonitrile in 0.1% Formic Acid (Solvent B) and deionized water in 0.1% Formic Acid (Solvent A).

5.2.6 GCMS

The analysis of the hexane 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 column temperature was kept at 250°C for 2 min with increased at the rate of 5°C per min up to 250°C. Injector temperature 250°C, split ratio 1:35, the carrier gas (Helium) flow rate 1.8 ml/min.

5.3 Chromatography Separation

5.3.1 Thin layer Chromatography (TLC)

TLC was carried out using aluminium supported silica gel 60 F₂₅₄ TLC sheets to detect the separation of the compounds. The spots were visualized under UV light at wavelength 254 nm and 366 nm using the CAMAG TLC UV Visualizer.
5.3.2 Preparative Thin Layer Chromatography (PTLC)

PTLC silica gel 60 F$_{254}$ (Merck) glass plates (20x20cm) were used for the compound separations.

5.3.3 Column Chromatography (CC)

Separation and fractionation of the crude extracts were conducted using chromatographic columns packed with Silica Gel 60F (230-400 mesh) with ratio 30:1 for the crude samples.

5.3.4 High Performance Liquid Chromatography (HPLC)

HPLC analysis, fractionation and purification were performed by preparative reverse phase HPLC using Waters Nova-Pak C18 column (25 x 100 mm, particle size 6 um) using acetonitrile and water with 0.1% formic acid as the eluants. HPLC chromatograms were monitored at 250-450 nm. HPLC analysis of the crude fractions previously partitioned using column chromatography was conducted on 150 x 3.9 mm i.d Nova Pak C18 RP (Waters) column, using solvent A (Acetonitrile in 0.1% FA) and solvent B (Water in 0.1% FA). A program of isocratic elution with 100% A from 0 to 15 min, a linear gradient to 100% B from 15 to 45 min, at a flow rate of 0.8 ml/min was used.

5.4 Detector Reagent

Mayer’s and Dragendorff’s reagents were used to detect the presence of alkaloids and heterocyclic nitrogen compounds.

a. Mayer’s reagent (Potassium mercury iodide)

b. Dragendorff’s reagent (purchased from Sigma)
5.5 Plant Material

The stem bark of *Pseuduvaria Rugosa* (KL 5186) was collected at Hutan Simpan Hijau, Lenggong, Perak and identified by L.E. Teo & J.C. Tetu (Herbarium group of Phytolab) from the Department of Chemistry, University of Malaya. It was tagged and kept in the herbarium until analysis.

5.5.1 Sample Extraction, Fractionation and Isolation

The extraction of crude extracts was carried out by Soxhlet extraction using the Soxhlet extractor. The advantage of this method is that large amount of compounds can be extracted with smaller amount of solvents, and also the solubility of the active principle and gradient concentration and polarity increases with the increasing of temperature that will result an enhanced mass transfer of active principle from solid material to the solvent [146]. By using this method, the sample is repeatedly extracted by every cycle of fresh solvent.

About 500g of dried ground bark of *Pseuduvaria Rugosa* was subjected to Soxhlet extraction for 16 hours using 2.5 L of hexane to remove fatty acids and lipid matters (Scheme 5.1). The defatted plant sample was filtered and dried, and moistened with 15% ammonia (NH$_4$OH) and left to soak overnight. Ammonia helps to free the alkaloid compounds from their salt forms [147]. Then the sample was exhaustively extracted with dichloromethane solvent by Soxhlet extractor for about 16 hours at a controlled temperature. (Overheating of the sample can decompose the compounds)

The crude dichloromethane extract (10.49g) obtained as dark green extract was subjected to acid-base extraction to yield crude alkaloid. The dichloromethane extract was concentrated to 500 ml using the rotary evaporator. The concentrated sample was further re-extracted with 5% HCL and partitioned using liquid-liquid extraction until
Mayer’s test is negative. The aqueous base was basified to pH 11 with ammonia and re-extracted with dichoromethane.

The organic layer was collected, washed with distilled H₂O and sodium chloride solution, and excess water was removed by sodium sulfate anhydrous, and filtered. Finally, the dichloromethane extract was evaporated to yield crude alkaloids (1.58 g) (0.32%).

The hexane extract (27.45g) was subjected to GCMS analysis to identify the chemical constituents. The methanol extract (21.95g) was further fractionated with preparative reverse-phase HPLC. A gradient of 30% to 100% (v/v) acetonitrile in water was applied with a flow rate of 12 ml/min over 60 minutes. From the methanol extract, 2 peaks were detected and the fractions (27-30) and (42-45) from the 2 peaks were later isolated and evaporated.

Purified compounds PR4; Pseuduvarine A 36 (3.1 mg) was obtained from fraction 29, compound PR5; Pseuduvarine B 37 (2.9 mg) was obtained from fraction 44 , PR6 Pseuduvarine C 38 was afforded from fraction 28, and PR 6; Pseuduvarin 39 was afforded from fraction 30 of the methanol extract using the HPLC (Figure 5.1).

Purified compounds PR1; Liriodenine 1, PR2; Ouregidione 5 and PR3; N-methyl ouregidione 11 were obtained from fraction, 17-18, 28-39 and 40-46 of the alkaloid extract, respectively, using the column chromatography. All purified compounds are summarized in Table 5.8
5.5.2 Isolation of the crude alkaloids

The crude alkaloid extract (1.5g) was then subjected to chromatographic separation over a column of silica gel in ethyl acetate and eluted with increasing amount of methanol. Each fraction of about 100ml was collected in a clean tube.

The homogeneity of the fractions and the Rf values were then monitored by TLC and visualized under the UV light at wavelength 254 nm and 366 nm (Figure 5.2). Fractions with similar TLC profiles and Rf values were then combined for further chromatographic analysis. The spots on TLC sheets were sprayed with Drangendorff’s reagent to detect the presence of alkaloids.

A total of 85 fractions were collected. Fraction 1-18 (10.8 mg), fraction 19-27 (9.8 mg), fraction 28-39 (33.6 mg), fraction 40-46 (14.4 mg), fraction 47-55 (79.9 mg), fraction 56-61 (94 mg ), fraction 62-66 (42.4 mg), fraction 67-70 (18.9 mg), fraction 71-79 (17.9 mg) and fraction 80-85 ( 17.7 mg).

5.6 Total Phenolic Contents

Total phenolic content of the bark of Pseuduvaria rugosa was determined according to the Folin-Ciocalteau method by using gallic acid as standard C [148]. 150 ul of sample was added to 750 ul of Folin-Ciocalteau reagent and 600 ul of sodium carbonate in a tube. Then the tubes were incubated at 50°C for 10 min. The absorbance is read was read at 760 nm against a blank methanol. The results were expressed as ug of total phenolics in mg of extract as GAE.
5.7 Antioxidant and Cytotoxic Activity Test

5.7.1 DPPH method

DPPH is a simple and widely used method to preliminary evaluate the antioxidant activity of a compound or plant extracts [149]. This method measure radical scavenging activity of the antioxidants against free radicals (DPPH) 1, 1-diphenyl-2-picrylhydrazyl radical.

The principle of the method: DPPH is a stable free radical that was originally purple in color (when absorbed at 517 nm). When the radical is scavenged by the process of hydrogen radical or electron donation, DPPH change to yellow color due to the formation of diphenylpicrylhydrazine [150].

In this study, the determination of DPPH radical scavenging assay was carried out by method Blois (1958) with some modifications [151]. DPPH, ascorbic acid and methanol were obtained from Sigma Chemical, Malaysia.

In a 96 well plates, 20 uL of sample from sample concentration (200ug/ml) was added to 50 uL of DPPH solution (0.02%) and 130 uL of methanol to reach the total volume of 200 uL. For the control, 20 uL of Ascorbic acid was added to 50 uL of DPPH solution (0.02%) and 130 uL of methanol. Ascorbic acid is used as the reference standard and blank methanol was used as the negative control. The mixtures were shaken and the 96 well plate was incubated in the dark for 30 minute at room temperature. Then the absorbance was measured at 517 nm using UV Spectrophotometer. The percentage inhibition of the sample was calculated following the formula:

\[
% \text{ of inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100
\]
5.7.2 In vitro cytotoxicity test

5.7.2.1 Materials

Crude extracts of hexane, dichloromethane and methanol (200ug/mL) were tested against seven cancer cell lines obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) to investigate the cytotoxic activity.

Dulbecco’s modified Eagle’s medium (DMEM), 100 mM non-essential amino acids, phosphate buffer solution (pH 7.2), 50 µg/mL gentamycin and 2.5 µg/mL amphotericin B were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

200 mM L-glutamine, foetal bovine serum, 0.25% trypsin-EDTA, dimethyl sulphoxide (DMSO), cisplatin and vinblastine sulphate were purchased from Sigma–Aldrich (St. Louis, MO, USA). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] assay kit (CellTiter 96® AQueous One Solution) was obtained from Promega (Madison, WI, USA).

5.7.2.2 Method

(MTS Assay)

The cytotoxicity of the compounds was evaluated against breast cancer cell line (MCF-7). Cell lines were cultured in DMEM media supplemented with 2 mM L-glutamine, 10% foetal bovine serum, 50 µg/mL gentamycin and 2.5 µg/mL amphotericin B, maintained in a 37 °C humid atmosphere of 5% CO₂ cell incubator. Samples and drug standards (cisplatin, vinblastine sulphate and doxorubicin HCl) were dissolved in DMSO and immediately diluted with DMEM media to yield a final DMSO
concentration of less than 0.5% v/v. Cells were plated into 96-well microplates at 10,000 cells per well and maintained in the cell incubator for 24 hour. Then, 100 µL of samples were introduced in triplicates to a final concentration of 15–150 µM for pure compounds and 15–150 µg/mL for extract.

Culture media were carefully refreshed with 100 µL of DMEM media, followed by 20 µL per well of MTS reagent. Microplates were returned to the incubator for 1-2 hours and absorbance of the formazan product was read on a microplate reader at 490nm with 690nm as the background wavelength (Infinite 200, Tecan, Männedorf, Switzerland). IC\textsubscript{50} of samples and drug standards were determined using dose-response curves in Prism 5.02 software (GraphPad Software Inc., La Jolla, CA, USA).

Cell viability and death were calculated as percentage from the absorbance readings following the equations:

Cell viability (%) = \( \frac{A\text{ (sample)}}{A\text{ (control)}} \times 100\% \)

Cell death (%) = 100% - cell viability (%)
HL-60, human promyelocytic leukemia cells were maintained in RPMI-1640 medium; MCF7, human breast adenocarcinoma; and HepG2, human liver carcinoma cells were maintained in Dulbecco’s modified eagle medium (DMEM) medium. Both growth medium were supplemented with 10% fetal calf serum and 1% Penicillin-Streptomycin.

The cells (5 x 10^3 cells/well) were cultured in Nunc disposable 96-well plates containing 90 µL of growth medium per well and were incubated at 37°C in a humidified incubator of 5% CO₂. 10 µL of samples were added to the cultures at 24 hours of incubation. After 48 hours of incubation with the samples, 15 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/ml) were added to each of the wells.

The cultures were incubated for another 3 h before the cells supernatant are removed. After the removal of the cells supernatant, 50 µL of dimethyl sulfoxide (DMSO) was added to each well. The formed formazan crystal was dissolved by re-suspension by pipette.

The optical density was measured using a microplate reader (Bio-Rad, USA) at 550 nm with reference wavelength at 700 nm. In all experiment, three replicates were used. Cisplatin was used as positive control (IC₅₀: 0.87 µM for HL-60, 27.7 µM for MCF7, and 12.3 µM for HepG2).
Figure 5.1: Diagram of extraction and isolation of pure compounds from *Pseuduvaria rugosa.*
Figure 5.2: TLC fractions of crude alkaloid extracts under UV light at 254 nm (top) and 366 nm (bottom)
Figure 5.3: HPLC Chromatogram of MeOH extract
Table 5.1: Yield of isolated compounds from the stem bark of *Pseuduvaria rugosa*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Yield (mg)</th>
<th>Percentage yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liriodenine 1</td>
<td>3.5</td>
<td>0.00070</td>
</tr>
<tr>
<td>Ouregidione 5</td>
<td>3.0</td>
<td>0.00060</td>
</tr>
<tr>
<td>N-methylouregidione 11</td>
<td>2.8</td>
<td>0.00056</td>
</tr>
<tr>
<td>Pseudoarine A 36</td>
<td>3.1</td>
<td>0.00062</td>
</tr>
<tr>
<td>Pseudoarine B 37</td>
<td>2.9</td>
<td>0.00058</td>
</tr>
<tr>
<td>Pseudoarine C 38</td>
<td>2.5</td>
<td>0.00050</td>
</tr>
<tr>
<td>Pseudoavin 39</td>
<td>0.8</td>
<td>0.00016</td>
</tr>
</tbody>
</table>
5.8 Physical and Spectral Data of the Isolated Compounds

**Liriodenine 1**
- Molecular formula: \(C_{17}H_9NO_3\)
- UV \(\lambda_{\text{max}}\) (MeOH): 255, 276, 327, 401 nm
- IR max cm\(^{-1}\): 965, 1660
- Mass spectrum \(m/z\): 276.0448, 247, 219
- \(^1\)H NMR (CDCl\(_3\)) ppm: Refer Table 3.1
- \(^{13}\)C NMR (CDCl\(_3\)) ppm: Refer Table 3.1

**Ouregidione 5**
- Molecular formula: \(C_{19}H_{15}NO_5\)
- UV \(\lambda_{\text{max}}\) (MeOH): 213, 229, 488
- IR max cm\(^{-1}\): 1733, 1667, 1455, 3436
- Mass spectrum \(m/z\): 338, 323, 321, 309
- \(^1\)H NMR (CDCl\(_3\)) ppm: Refer Table 3.2
- \(^{13}\)C NMR (CDCl\(_3\)) ppm: Not available

**N-methyl Ouregidione 11**
- Molecular formula: \(C_{20}H_{17}NO_5\)
- UV \(\lambda_{\text{max}}\) (MeOH): 212, 242, 275, 306, 416
- IR max cm\(^{-1}\): 1712, 1601, 1274
- Mass spectrum \(m/z\): 352, 338, 323, 292, 276
- \(^1\)H NMR (CDCl\(_3\)) ppm: Refer Table 3.3
- \(^{13}\)C NMR (CDCl\(_3\)) ppm: Not available
**Pseuduvarine A 36**

- Molecular formula: $C_{18}H_{14}N_2O_4$
- UV $\lambda$ max (MeOH): 216, 261, 323, 425
- IR max cm$^{-1}$: 1687, 1614
- Mass spectrum m/z: 323, 295, 276, 214
- $^1$H NMR (CDCl$_3$) ppm: Refer Table 3.3
- $^{13}$C NMR (CDCl$_3$) ppm: Refer Table 3.3

**Pseuduvarine B 37**

- Molecular formula: $C_{19}H_{16}N_2O_4$
- UV $\lambda$ max (MeOH): 214, 261, 323, 421
- IR max cm$^{-1}$: 1684, 1608
- Mass spectrum m/z: 337, 308, 275, 214
- $^1$H NMR (CDCl$_3$) ppm: Refer Table 3.4
- $^{13}$C NMR (CDCl$_3$) ppm: Refer Table 3.4

**Pseuduvarine C 38**

- Molecular formula: $C_{18}H_{13}NO_5$
- UV $\lambda$ max (MeOH): 214, 251, 323, 424
- IR max cm$^{-1}$: 3366, 3583
- Mass spectrum m/z: 324, 276, 214
- $^1$H NMR (CDCl$_3$) ppm: Refer Table 3.6
- $^{13}$C NMR (CDCl$_3$) ppm: Refer Table 3.6
<table>
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<th><strong>Pseuduvarine 39</strong></th>
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<td>UV ( \lambda ) max (MeOH)</td>
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<tr>
<td>IR max cm(^{-1})</td>
<td>: 1667, 3366</td>
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<tr>
<td>Mass spectrum m/z</td>
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<td>(^{13}) C NMR (CDCl\textsubscript{3}) ppm</td>
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REFERENCES


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