METABOLIC FINGERPRINTING OF SABAH *RUELLIA TUBEROSA* PLANT EXTRACTS FOR THE IDENTIFICATION OF POTENTIAL ANTICANCER COMPOUNDS

JAN RENEE STEPHANIE JIORRY

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF MASTER OF SCIENCE (MOLECULAR BIOLOGY)

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2014

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Jan Renee Stephanie Jiorry

MZ1212001T

CERTIFICATION

NAME	:	**JAN RENEE STEPHANIE JIORRY**
MATRIC NO	:	**MZ1212001T**
TITLE	:	**METABOLIC FINGERPRINTING OF SABAH *RUELLIA TUBEROSA* PLANT EXTRACTS FOR THE IDENTIFICATION OF POTENTIAL ANTICANCER COMPOUNDS**
DEGREE	:	**MASTER OF SCIENCE (Molecular Biology)**
VIVA DATE	:	**26 MAY 2014**

DECLARED BY

SUPERVISOR **SIGNATURE**

MISS CHEONG BO ENG

ACKNOWLEDGEMENT

First and foremost, I would like to thank Almighty God, for His wonderful blessing that I was able to accomplish this dissertation as a partial fulfilment of the requirements for the degree of master science at Universiti Malaysia Sabah.

I would like express my deepest gratitude and appreciation to my supervisor, Miss Cheong Bo Eng for all her advices, guidance and support throughout this research work that lead to the completion of this dissertation. I would also thank her for her supervision and critical reading of this dissertation.

I wish to thank all my friends in Natural Product Chemistry Laboratory for their kind assistance, helpful advices and encouragement. Apart from that, my sincere thank goes to the lab assistants for their guidance throughout my research.

Lastly, I would like to thank my parents for their support, encouragement and contribution that have helped me in the completion of this dissertation. Without all of you, I would not have done it.

ABSTRACT

Ruellia tuberosa is a Minnie root medicinal plant which is also known as Cracker plant. This plant has been used traditionally by the local people to treat several diseases such as diuretic, antidiabetic, antipyretic and many more. A recent study has shown that this plant exhibits excellent anticancer properties especially on the breast cancer (MCF-7) cells. However, the full complement of bioactive compounds in *R. tuberosa* especially on the anticancer compounds has yet to be elucidated. Hence, the aim of this study was to identify the potential anticancer compounds of the Sabah *R. tuberosa* through metabolic fingerprinting approach. In this study, gas chromatography-mass spectrometry (GC-MS) was used to obtain the metabolic fingerprints of *R. tuberosa* plant extracts. The GC-MS analysis of *R. tuberosa* leaf extract revealed the presence of 15 compounds such as squalene, vitamin E, campesterol, stigmasterol, gamma-tocopherol, gamma-sitosterol, 9, 12, 15-octadecatrienoic acid (Z, Z, Z), alpha-amyrin, hexadecanoic acid trimethylsilyl ester, cholesterol, alpha-linolenic acid trimethylsilyl ester, sucrose, octakis (trimethylsilyl) ether, alpha-tocopherol trimethylsilyl ether, silane, [[(3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl- and beta-sitosterol trimethylsilyl ether. Meanwhile, 10 compounds were detected in the stem extract such as vitamin E, squalene, stigmasterol, campesterol, gamma-sitosterol, lupeol, sucrose, octakis (trimethylsilyl) ether, silane, [[3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl-, beta-sitosterol trimethylsilyl ether and alpha-tocopherol trimethylsilyl ether. Similarly, 10 compounds were detected in root extract such as vitamin E, stigmasterol, campesterol, squalene, gamma-sitosterol, lupeol, alpha-tocopherol trimethylsilyl ether, hexadecanoic acid trimethylsilyl ester, silane, [[3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl- and beta-sitosterol trimethylsilyl ether. A chemical database was constructed using PCDL software. By analysing the chemical database and the MTT assay result of *R. tuberosa* against breast cancer (MCF-7) cell line, the potential anticancer compounds were speculated. Hence, we highly speculated that squalene, stigmasterol, campesterol, vitamin E and lupeol were the compounds responsible for the anticancer activity of *R. tuberosa*. Therefore, further study should be conducted to isolate these compounds and study their proliferative activity against MCF-7 cell line.

ABSTRAK

Cap Jari Metabolik bagi Ekstrak *Ruellia Tuberosa* Dalam Mengenal Pasti Sebatian yang Mempunyai Potensi Sebagai Anti-Kanser

Ruellia tuberosa merupakan suatu tumbuhan perubatan *Minnie root* yang juga dikenali sebagai tumbuhan Cracker. Tumbuhan ini telah digunakan secara tradisional oleh penduduk tempatan untuk merawat beberapa penyakit seperti diuretik, antidiabetic , antipiretik dan banyak lagi. Beberapa kajian yang dilakukan baru-baru ini telah menunjukkan bahawa tumbuhan ini mempunyai ciri-ciri anti-kanser yang sangat baik terutamanya terhadap kanser payudara (MCF -7). Walau bagaimanapun, belum ada penjelasan lengkap mengenai komponen aktif di dalam tumbuhan ini. Oleh yang demikian, kajian in bertujuan untuk mengenal pasti sebatian-sebatian di dalam *R. tuberosa* daripada Sabah yang mempunyai potensi sebagai anti-kanser melalui pendekatan cap jari metabolik. Dalam kajian ini, teknik gas kromatografi – berjisim spektrometer (GC -MS) telah digunakan untuk mendapatkan cap jari metabolik daripada ekstrak-ekstrak *R. tuberosa*. Analisis GC -MS daripada ekstrak daun *R. tuberosa* menunjukkan kehadiran 15 sebatian seperti squalene, vitamin E, kampesterol, stigmasterol, gamma-tokoferol, gamma-sitosterol, 9, 12, 15 asid-octadecatrienoic (Z, Z, Z), alfa-amyrin, asid heksadekanoik trimethylsilyl ester, kolesterol, alfa-linolenik asid trimethylsilyl ether, sukrosa, octakis (trimethylsilyl) ether, silane, [[3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl-, alfa-tokoferol trimethylsilyl ether dan beta-sitosterol trimethylsilyl ether. Selain daripada itu, 10 sebatian telah dikesan dalam ekstrak batang seperti vitamin E, squalene, stigmasterol, kampesterol, gamma-sitosterol, lupeol, sukrosa, octakis (trimethylsilyl) ether, silane, [[3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl-, beta-sitosterol trimethylsilyl ether dan alfa-tokoferol trimethylsilyl ether. Selain itu, 10 sebatian juga telah dikesan dalam ekstrak akar seperti vitamin E, stigmasterol, kampesterol, squalene, gamma-sitosterol, lupeol, alpha-tokoferol trimethylsilyl ether, asid heksadekanoik trimethylsilyl ester, silane, [[3β, 24R)-ergost-5-en-3-yl]oxy]trimethyl- dan beta-sitosterol trimethylsilyl ether. Satu pangkalan data telah dibina menggunakan perisian PCDL. Sebatian yang mempunyai potensi sebagai anti-kanser telah dikenalpasti berdasarkan pangkalan data tersebut dan keputusan asai MTT yang telah dilakukan keatas *R. tuberosa* terhadap kanser payudara (MCF-7). Kami membuat spekulasi bahawa squalene, stigmasterol, kampesterol, vitamin E dan lupeol merupakan sebatian yang bertanggungjawab untuk aktiviti anti-kanser bagi *R. tuberosa*. Oleh yang demikian, kajian lanjut harus dilakukan untuk mengisolasi sebatian-sebatian tersebut dan mengkaji aktiviti anti-proliferatif sebatian tersebut keatas kanser payudara (MCF-7).

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LIST OF ABBREVIATIONS

EAC	-	Ehrlich Ascites Carcinoma
EAE	-	Ethyl Acetate Extract
EaF	-	Ethyl Acetate Fraction
EAFME	-	Ethyl Acetate Fraction of Methanol Extract
CfF	-	Chloroform Fraction
FT-IR	-	Fourier Transform Infrared
GC-MS	-	Gas Chromatography-Mass Spectrometry
HxF	-	Hexane Fraction
LC-MS	-	Liquid Chromatography-Tandem Mass Spectrometry
ME	-	Methanol Extract
mg/kg	-	Milligram per kilogram
MW	-	Molecular Weight
NMR	-	Nuclear Magnetic Resonance
PCDL	-	Personal Compound Database and Library
PPM	-	Part per million
RT	-	Retention Time
TLC	-	Thin Layer Chromatography
UV	-	Ultraviolet
WE	-	Water Extract
WHO	-	World Health Organization
WtF	-	Water Fraction
µg/ml	-	Microgram per mililitre

CHAPTER 1

INTRODUCTION

Cancer refers to the uncontrolled growth of abnormal cells in the body and is considered as one of the leading causes of death worldwide. As many as 95 % of all types of cancers are usually caused by human lifestyle and may take as long as 20-30 years to develop (Bhanot *et al.*, 2011). The conventional cancer therapies such as chemotherapy and radiation therapy may cause mild to serious side effects to patients and at best, only extend the patient's lifespan by a few years (Amin *et al.*, 2009). Due to this reason, alternative approaches were considered and one of them involves the use of plants.

Plants and their extracts have been traditionally used for medicinal purposes. These plants are an expensive gift from nature due to the fact that they could serve as the source of important therapeutic aids for relieving human ailments (Geetha *et al.*, 2013). Plants also constitute an important source of active natural products that differ extensively in structures, biological properties and mechanism of actions (Sangeetha and Vijayalakshmi, 2011). Since ancient time, people have been using plants to treat certain diseases ranging from minor diseases such as headache, stomach ache to major ones like cancers. The World Health Organization (WHO) reported that more than 80% of the world's population relies on the traditional medicine for their primary healthcare needs (Duraipandiyan *et al.*, 2006). The importance of medicinal plants lies not only in their therapeutic values but also in their potential as sources of novel drug discovery (Vital and Rivera, 2011). Today, plants become a vital source of drugs (Geetha *et al.*, 2013) and a lot of extensive researches have been done on medicinal plants that led to the discovery of novel drugs to be used in treatment and prevention of diseases.

Furthermore, plants have played great role as a source of effective anticancer agents and have been used traditionally in the treatment of cancer (Bhanot *et al.*, 2011). The natural compounds from the plants are being used as

cancer therapies as well as chemopreventive compounds (Amin *et al.*, 2009). According to Bhanot *et al.* (2011), medicinal plants were recognized as the source of anticancer agents through the discovery and development of vinca alkaloids (vinblastine and vincristine) and isolation of the cytotoxic podohyllotoxins. It is also reported that the natural products derived from plants possess biological activities from antioxidant to anticancer (Sangeetha and Vijayalakshmi, 2011). As of today, various numbers of plants are studied for their anticancer activity against various experimental models (Reddy *et al.*, 2013). Apart from that, masses attention have been given to plant-derived natural products such as flavonoids, steroids, alkaloids and terpenoids to identify their pharmacological activities (Reddy *et al.*, 2013).

Ruellia tuberosa Linn. is a Minnie root medicinal plant; a tropical perennial plant that is widely distributed in Southeast Asia, including Thailand and Laos (Chen *et al*, 2006; Arirudran *et al.*, 2011b). It belongs to family Acanthaceae and is a native of Central America. *R. tuberosa* is medicinally used in West Indies, Central America, Guiana and Peru (Chothani and Mishra, 2012) as an antihelmintic, against joint pains and strained muscles (Arirudran *et al.*, 2011a). These authors also reported its traditional uses as diuretic, antidiabetic, antipyretic, analgesic, anti-hypertensive, thirst-quenching and antidotal agent. Apart from that, *R. tuberosa* has recently being incorporated as one of the components in an herbal drink in Taiwan (Chen *et al.*, 2006). Moreover, various experiments have been conducted and it was proven that *R. tuberosa* possess antioxidant (Chen *et al.*, 2006; Chothani and Mishra, 2012; Geetha *et al.*, 2013), antimicrobial (Arirudran *et al.*, 2011a), anticancer (Reddy *et al.*, 2013) antihyperlipidemic (Krishna *et al.*, 2012), gastroprotective activity (Arambewela *et al.*, 2003), antidiabetic and hepatoprotective activity (Rajan *et al.*, 2012). Recently, Cheong *et al.* (2013) have studied about the *R. tuberosa* from Sabah and found that the plants possess excellent anticancer activity especially against the breast cancer (MCF-7) cell line.

Despite its potential medicinal value, there is relatively little information on chemical constituents of *R. tuberosa* and their pharmacological activities (Arirudran *et al.*, 2011a; Chen *et al.*, 2006). Plants consist of many constituents

and therefore are varied (Chothani *et al.*, 2011). The knowledge of the chemical constituents of plants is necessary for the discovery of potential therapeutic agent as well as disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances (Sermakkani and Thangapandian, 2012). This is followed by the isolation, characterization and determination of bioactivity of the identified compounds for its pharmaceutical exploitation (Patra *et al.*, 2012). To study on the chemical constituents of plants, metabolomics approach was used. Metabolomics has become a well-known technique in studying all types of organisms and complements the data obtained by the other 'omics' such as genomics, transcriptomics and proteomics (Schripsema, 2010). This author defined metabolomics as the identification and quantification of all metabolites in a biological system. Hence, metabolomics analysis aims to identify and quantify all metabolites in a given biological system (Weckwerth and Fiehn, 2002).

There are several approaches in metabolomics analysis including targeted analysis, metabolic profiling, metabolic footprinting and metabolic fingerprinting. In this study, a comprehensive metabolic fingerprinting that represents the pharmacologically active compounds from *R. tuberosa* is carried out to be used for the identification of potential medicinal values. Metabolic fingerprinting appears to be the easiest approach in metabolomics whereby it utilizes all detector readings for numerical analysis to unambigouosly identifies specific constituents present in plants (Mahdi *et al.*, 2010). Schripsema (2010) defined metabolic fingerprinting as the unbiased, global screening approach to classify samples based on the metabolites patterns or "fingerprints". It helps us to gain better insight into the biochemical composition of the plant in a semi-automated and essentially, untargeted manner (Schripsema, 2010).

In this study, we carried out the gas chromatography-mass spectrometry (GC-MS) based metabolic fingerprinting to investigate the chemical constituents in the leaf, stem and root of Sabah *R. tuberosa.* GC-MS is a technique that involves the combination of gas chromatography and mass spectrometry. It is one of the most commonly used technique in metabolic fingerprinting in which it has been reported that more than 300 compounds were able to be distinguished with GC-MS (Jonsson *et al.*, 2004). A preliminary phytochemical studies conducted by

Arirudran *et al.* (2011c) reported that this plant contains steroids, triterpenoids, phenol, flavonoids, tannin and sugars. Meanwhile, Lin *et al.* (2006) reported that they have successfully isolated five flavonoids namely cirsimaritin, cirsimarin, cirsiliol 4'-glucoside, sorbifolin and pedalitin along with betulin, vanilic acid and indole-3-carboxaldehyde from the ethyl acetate extract of *R. tuberosa* which showed cytotoxicity against KB cell line and HepG2 cell line. Therefore, the data obtained in this study will give us further insight on the chemical constituents of Sabah *R. tuberosa* which may then lead to the discovery of anticancer properties of this plant.

Hence, the objectives of this study were:

1. To obtain the metabolic fingerprints of Sabah *Ruellia tuberosa* plant extracts.
2. To construct chemical database based on the metabolite profiles for each of the Sabah *Ruellia tuberosa* plant extracts.
3. To identify the potential anticancer compounds from Sabah *Ruellia tuberosa*.

CHAPTER 2

LITERATURE REVIEW

2.1 *Ruellia tuberosa*

Ruellia is a large and variable genus of mostly tropical perennial herb and shrub with the sizes that range from few centimetres to large, bushy plants (Long, 1976). Being the second largest genus in the family of Acanthaceae, this genus has approximately 300 species (Tripp and Monas, 2008) of geographically widespread and morphologically diverse (Tripp, 2007).

Ruellia tuberosa Linn. is a native of Central America which has been introduced into Indian garden as ornament (Chothani *et al.*, 2011). It is widely distributed in Southeast Asia (Ananthakrishnan and Doss, 2012; Arirudran *et al.*, 2011a; Chen *et al.*, 2006). It is regarded as environmental weed despite its striking blue-violet colour. *R. tuberosa* is also known as Cracker plant in English and Pattaskai in Tamil (Arirudran *et al.*, 2011c) which describe the explosive phenomenon of its seed capsule when it comes in contact with water releasing the seeds it contain. The people in Cayman, Florida called this plant heartbush which refers to the roots being used to treat heart disease. Other names for this plant include Minnie root, popping pod, Cracker plant, snapdragon root, sheep potato, bluebell, Duppy gun, Daniel's great gun and large-bell flower (Chothani *et al.*, 2010).

Kingdom: Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Scrophulasiales
Family : Acanthaceae
Genus : Ruellia
Species : *Ruellia tuberosa*

Chothani *et al.* (2010) also stated the synonyms of *Ruellia tuberosa* as *Ruellia picta* and *Ruellia clandestine*.

2.1.1 Morphology of *Ruellia tuberosa*

Ruellia tuberosa is an erect, sub erect of diffuse perennial herb (Chothani *et al.*, 2011) growing up to 60-70 cm with a hairy stem (Arirudran *et al.*, 2011b). The simple leaves of this plant are opposite, elliptic or ovate with a wavy edge. A review on *R. tuberosa* by Chothani *et al.* (2010) stated that the plants have leaves with up to 2 cm long petiole, 5-9 x 2-4 cm in length, shining, basally cuneate to attenuate, entire to undulate, obtuse to somewhat acute. Arirudran *et al.* (2011c) also reported that this plant only flowers after the start of rainy season. The flowers of *R. tuberosa* are blue-violet in colour, attractive enough to be used as an ornament plant in Indian garden. They are eye-catching, with funnel shape, five-lobed corolla up to 5 cm in size. On the other hand, the roots are slender, elongated and have a tuberous finger like structure. Meanwhile, the pods of *R. tuberosa* consist of seven to eight seeds which burst open when they come in contact with water causing the seeds to be hurdled away (Chothani *et al.*, 2010). The leaves, flowers, roots, stems and pods of *R. tuberosa* were as illustrated in Figure 2.1.

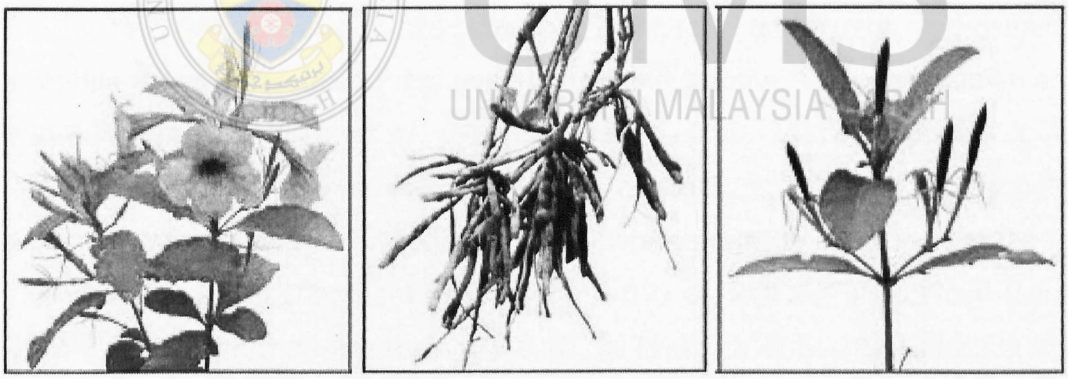

Figure 2.1: Images of *R. tuberosa* showing its leaves, tuberous roots, stems and the pods (source: Philippine Medicinal Plants, 2013).

2.1.2 Ethnomedicinal Uses of *Ruellia tuberosa*

Since ancient time, plants have been a rich source of effective and safe medicine (Meena and Rao, 2010). The authors also stated that these indigenous remedies are popular among the people of both urban and rural areas because of their safe to be use, effective and inexpensive nature. Apart from that, *R. tuberosa* has been

used medicinally by local people in West Indies, Central America, Guiana and Peru (Chothani *et al.*, 2011).

The dried and ground root of this plant were used in abortion which causes bleeding and the expelling out the foetus (Kamble *et al.*, 2010). The leaf juice is orally administered to relieve asthma (Rahmatullah *et al.*, 2010). Additionally, the local in Rajasthan used the tubers to relieve abdominal pain after delivery (Meena and Rao, 2010) as well as to relieve stomach ache (Swarnkar and Katewa, 2008). Some local in Bangladesh used *R. tuberosa* to treat sexually transmitted diseases (STD) including syphilis and gonorrhoea (Mollik *et al.*, 2010). In addition to its potential in treating bladder stone, *R. tuberosa* also possess emetic activity and can act as the substitute of ipecac (Chothani and Mishra, 2012). Moreover, the leaf juice of this plant is used to for the scorpion bite (Ushakumari *et al.*, 2012). In Suriname's traditional medicine system, *R. tuberosa* is used as anthelminthic and also in soothing joint pain and strained muscle (Chothani *et al.*, 2010; Reddy *et al.*, 2013).

As in folk medicine, it has been used as diuretic, anti-pyretic, anti-diabetic, antidotal agent, thirst-quenching, analgesic, antinociceptive, anti-hypertensive and anti-inflammatory (Chen *et al.*, 2006; Arirudran *et al.*, 2011a; Shahwar *et al.*, 2011). The root infusion is used for kidney diseases; in the form of syrup for whooping cough; infusion or decoction for a diabetes remedy and tubers in tea for cleansing the blood (Chothani and Mishra, 2012) as well as for 'cooling' (Lans, 2006). Apart from that, the roots are used for livestock as oestrus induction and anthelminthic (Lans and Brown, 1998). In the other hand, the fruits are crushed and applied on blisters (Sharma *et al.*, 2012). The paste of leaves can also be applied externally on the wound (Rudrapal *et al.*, 2012). Additionally, *R. tuberosa* has been used externally in Thai traditional medicine as an anti-inflammatory, antiseptic and as antidote for detoxification of poison (Phakeovilay *et al.*, 2013).

2.1.3 Phytochemistry of *Ruellia tuberosa*

The preliminary phytochemical studies conducted by Arirudran *et al.* (2011c) revealed the presence of tannin, flavonoid, steroid, triterpenoids and phenol in different extracts of *R. tuberosa* whole plant. They found that the n-hexane

used medicinally by local people in West Indies, Central America, Guiana and Peru (Chothani *et al.*, 2011).

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extract contains steroid and triterpenoids while the chloroform extract contains steroid, triterpenoids and phenols. On the other hand, both ethyl acetate and aqueous extracts contain steroid, triterpenoids, phenols, flavonoids, tannin and sugars. Chothani *et al.* (2012) reported that the methanolic extract of roots of *R. tuberosa* contain carbohydrates, saponins, flavonoid, phenolic compound and sterols. They also found that the water extracts contain phenolic compound, saponins and carbohydrate. There is also the presence of sitosterols which was reported to have anti-diabetic effect (Shahwar *et al.*, 2011). Apart from that, the leaves were reported to contain a trace of apigenin and luteolin whereas malvidin -3, 5- diglucoside were found in the flowers (Chothani *et al.*, 2010). It is also found that the leaves of *R. tuberosa* possess significant amount of vitamin (C, K), carotenoids and phenols (Manikandan and Doss, 2010). Figure 2.2 showed some of the chemical constituents that have been found in *R. tuberosa*.

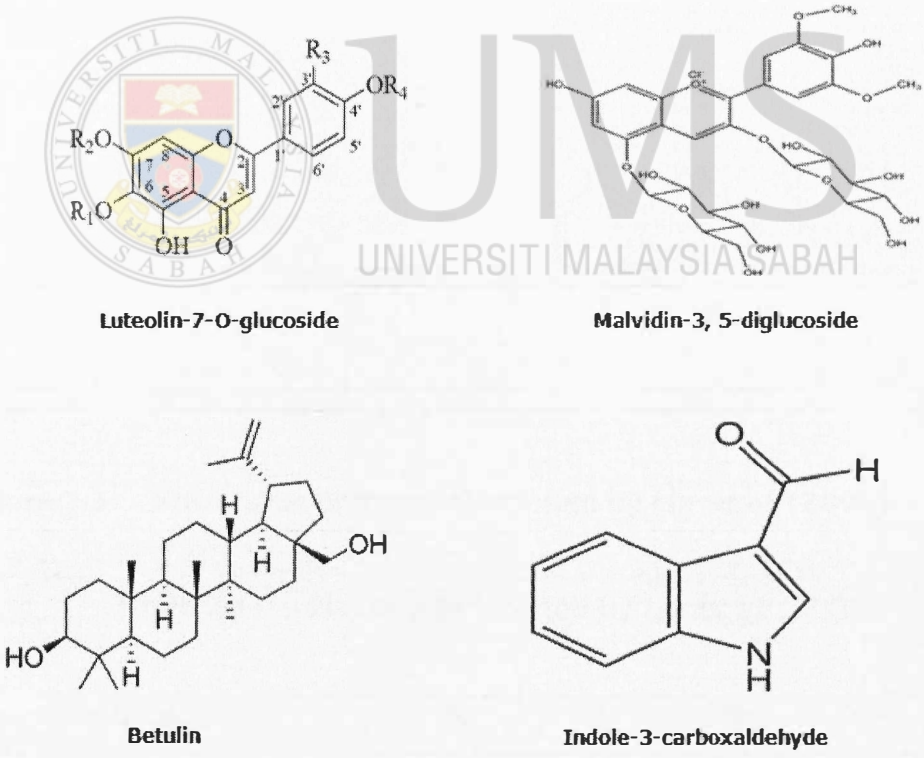

Figure 2.2: Some of the chemical constituents of *R. tuberosa* (source: Chothani *et al.*, 2010).