A PHYTOCHEMICAL AND BIOMEDICAL INVESTIGATION OF *CISSUS REPENS* LAM. (WA-ROUND-CHIN) AND *BOESENBERGIA ROTUNDA* (L.) MANSF. (SEIK-PHOO-CHIN)

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ABSTRACT

The present research focused on the evaluation of bioactive principles from the locally cultivated two medicinal plants: *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin) including the phytochemical analyses, isolation and structural elucidation of organic compounds and biological activity screening such as antimicrobial activity, antioxidant activity, cytotoxicity test, antitumor activity and antiproliferative activity. The whole plants of *C.repens* and aerial parts and rhizomes of *B.rotunda* were collected from Kyauk Tan Village, Bago Region and their respective scientific name has been identified at Botany Department, University of Yangon. The preliminary phytochemical tests revealed the presence of alkaloids, carbohydrates, flavonoids, glycoside, organic acid, phenolic compounds, saponins, steroids, tannins and terpenoids in the whole plant of *C.repens* (WRC), aerial parts and rhizomes of *B.rotunda* (SPCA and SPCR).

Determination of nutritional valves of WRC, SPCA and SPCR have also been carried out by AOAC method, resulting 3.11%, 3.28% and 4.49% of moisture, 6.80%, 14.75% and 7.00% of ash, 12.18%, 9.23% and 6.82% of protein, 13.73%, 28.54% and 12.24% of fiber, 7.90%, 5.81 % and 3.62% of fat, 56.28%, 38.39% and 65.83% of carbohydrate respectively based on the dried samples.

In addition, some soluble matter contents of the samples were also analysed using PE, EtOAC, CH_3COCH_3, EtOH and water as solvents. On silica gel column chromatographic separation, eight compounds were isolated from PE extract of WRC, EtOAc and $CHCl_3$ extracts of SPCR, characterized by some physical and chemical properties and structurally identified by the combination of UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectroscopic methods and also by comparing with the reported data. The compounds isolated from PE extract of WRC were identified to be friedelin (**A**, 0.014 %, m.pt. 258-259 °C) and friedelinol (**B**, 0.008 %, m.pt. 278-279 °C). Two compounds isolated from EtOAc extract of SPCR were identified to be β-sitosterol (**C**, 0.26 %, m.pt.138-140 °C) and β-sitosterol-β-D-glucoside (**D**, 0.9 %, m.pt-272-274 °C) whereas the compounds obtained from $CHCl_3$ extract of SPCR were pinostrobin (**E**, 0.26 %, m.pt. 96-98 °C), 4′, 7-dimethylkaempferol (**F**, 0.25 %,

m.pt 179-182 °C), galanal A (**G**, 0.43 %, m.pt. 165-167 °C) and galanal B (**H**, 0.83 %, m.pt, 134-134.5 °C).

The antimicrobial activity of PE, EtOAc, EtOH and MeOH extracts from WRC, SPCA and SPCR was screened on six microorganism such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* by agar well diffusion method. All of these crude extracts have pronounced antimicrobial activity with inhibition zone diameters ranged between 13mm ~ 55mm in WRC, 14mm ~ 55mm in SPCA and 13mm ~ 35 mm in SPCR respectively. The MIC values of EtOAc extracts (24.4141 μg/mL, 2500 μg/mL) of WRC, (25000 μg/mL, 25000 mg/mL) of SPCA, (100000 μg/mL, 100000 μg/mL) of SPCR and some isolated compounds **A-D** [friedelin (12.5 μg/mL, 25 μg/mL), friedelinol (100 μg/mL, 100 μg/mL), β-sitosterol (25 μg/mL, 25 μg/mL), β-sitosterol-β-D-glucoside (100 μg/mL, 100 μg/mL)] of WRC, SPCA and SPCR were also determined against two species of organisms such as *Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method.

The antioxidant activity of ethanol and watery crude extracts of WRC, SPCA and SPCR and some isolated compounds (friedelin, friedelinol, β-sitosterol-β-D-glucoside) was determined by DPPH radical scavenging assay by using UV spectrophotometric technique. The order of radical scavenging activity was observed as SPCA > SPCR > WRC for ethanol extracts whereas the order of radical scavenging activity was SPCA > WRC > SPCR for watery extracts.

The antioxidant potential of isolated compounds were found to be in the order of β-sitosterol-β-D-glucoside > friedelin. Friedelinol did not show % inhibition in the range of concentration 28.71-47.69 μg/mL.

Ethanol crude extract of SPCR possessed mild cytotoxicity on brine shrimp. Its LD_{50} was determined to be 370.50 μg/mL after 24 hours while the other crude extracts did not exhibit their cytotoxic effect up to the optimum dose of 1000 μg/mL.

Antitumor activity of ethyl acetate, ethanol and methanol extracts of WRC, SPCA and SPCR and some isolated compounds **A-D** (friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside) were also tested on tumor produced bacteria using PCG (Potato crown Gall) test. From this experiment, all of the test extracts from

all samples were significantly found to inhibit the formation of tumor in the dose of 0.2 g/disc. Among test compounds, friedelin, β-sitosterol and β-sitosterol-β-D-glucoside showed to prevent the tumor formation with the dose of 0.1 μg/disc, however tumor formation was not prevented by isolated compound friedelinol and β-sitosterol-β-D-glucoside with the dose up to 0.1 μg/disc.

Moreover, antiproliferative activity of MeOH extract of WRC, SPCA and SPCR, $CHCl_3$ extract of SPCR and four isolated compounds (pinostrobin, 4′, 7-dimethylkaempferol, galanal A and galanal B) was determined by using ten human cancer cell lines.

The antiproliferative activity of four extracts were found to be in order of SPCA (MeOH extract) > SPCR ($CHCl_3$ extract) > SPCR (MeOH extract) > WRC (MeOH extract) against lung (LK-2), lung (A549), stomach, breast human cancer cell lines and normal human fibroblast. The order of antiproliferative activity of three extracts were observed as SPCA (MeOH extract > SPCR (MeOH extract) > WRC (MeOH extract) against colon, liver, cervix, leukemia and prostate human cancer cell lines. Among the isolated compounds, galanal A and galanal B were found to be more potent than pinostrobin and 4′,7-dimethylkaempferol in antiproliferative activity against lung (LK-2), lung (A549), stomach, breast human cancer cell lines and normal human fibroblast.

Keywords : *Cissus repens*, *Boesenbergia rotunda*, antioxidant activity, antitumor activity, antiproliferative activity

LIST OF ABBREVIATIONS

%	:	percent
°C	:	degree Celsius
μg	:	microgram
^{13}C NMR	:	Carbon 13 Nuclear Magnetic Resonance
^{1}H NMR	:	Proton Nuclear Magnetic Resonance
^{1}H-^{1}H COSY	:	^{1}H, ^{1}H-Correlated Spectroscopy
aq	:	aqueous
ca	:	circa, about
CC	:	Column Chromatography
CCK-8	:	Cell counting kit-8
cm^{-1}	:	per centimeter
cm^{3}	:	cubic centimeter
d	:	doublet
dd	:	doublet of doublet
DMSO	:	Dimethyl sulfoxide
DPPH	:	1,1-Diphenyl-2-picryl hydrazyl
e.g. ,	:	for example
EI MS	:	Electron Impact Mass Spectrometry
ESI MS	:	Electron Spray Ionization Mass Spectrometry
et al.	:	et allii, and others
etc	:	et cetera, and other rings
F	:	Fraction
f	:	fraction
FT IR	:	Fourier Transform Infrared Spectroscopy
g	:	gram
HMBC	:	Heteronuclear Multiple Bond Correlation
HSQC	:	Heteronuclear Single Quantum Correlation
i.e	:	id ext, that is
IC_{50}	:	50% Inhibitory Concentration
J	:	coupling constant

kg	:	kilogram
m/z	:	mass/charge
mg	:	milligram
MHz	:	Mega Hertz
mL	:	millilitre
mp	:	melting point
nm	:	nanometer
NOESY	:	Nuclear Overhauser Effect Spectroscopy
oop	:	out-of-plane
PBS	:	Phosphate Buffered Saline
PE	:	Petroleum Ether
ppm	:	part per million
ppt	:	precipitate
R_f	:	Retension factor
s	:	singlet
SD	:	Standard Deviation
SE	:	Standard Error
SPCA	:	Aerial parts of *B. rotunda*
SPCR	:	Rhizomes of *B. rotunda*
t	:	triplet
TLC	:	Thin Layer Chromatography
UV	:	Ultraviolet
V	:	volume
v/v	:	volume by volume
viz	:	videlicet, namely
W	:	weight
WRC	:	The whole plant of *C. repens*
δ	:	Bending Vibration, Chemical shift
λ_{max}	:	Wavelength of Maximum Absorption
υ	:	Stretching vibration

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CHAPTER I

1. INTRODUCTION

Medicinal plants constituent a very important natural resource used by indigenous medicinal system for the last 300 years. Plant-based drugs have been used against various diseases since long time. The nature has provided abundant plant wealth for all living creatures, which possess medicinal virtues.

In developing countries, herbal medicine is still the mainstay of health care, using local traditional and beliefs. A medicinal plant is any plant which, in one or more of its organs, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. Medicinal plants are one of important natural wealth of a country. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other countries. In this way, indigenous medicinal plants play significant role of an economy of a country.

Of the 250, 000 higher plant species on earth, more than 80,000 species are reported to have at least some medicinal value and around 5,000 species have specific therapeutic value (antimalarial, anticancer, antiulcer, anti-diabetic, anti-cholesterol, anti-inflammatory, antiviral, antibacterial, antifungal, antiprotozoal, anti-diarrhea, hypotensive, tranquilizing, anaesthetic, spamolytic, anti-allergic, hepatoprotective). Herb plants produce and contain a variety of chemical substance that act upon the body. The drugs are derived either from the whole plant or from different parts of the plant like root, stem, bark, heartwood, leaf, flower, fruit and seed etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. These medicinal principles are separated by different processes: of which the most common is extraction. Extraction is the separation of the required constituents from plant materials using a solvent.

The Myanmar Traditional Medicine is one with profound medical treatises, a variety of potent and effective medicines and a diversity of therapies. The government is giving impetus to developing Traditional Medicine systematically reach

international standards and to manufacturing potent and efficacious Traditional Medicine based on scientific evidences and practices. With the aim to extend the scope of health care services for both rural and urban areas, health care by Myanmar Traditional Medicine services is provided through Myanmar Traditional Medicine hospitals and clinics in all states and divisions of Myanmar.

Development of the use of traditional and herbal medicine is also one of the sectors in the national health policy in our country. In the present research work, Wa-round-chin and Seik-phoo-chin were selected for the investigation of some bioactivities such as antimicrobial activity, antioxidant activity, cytotoxicity, antitumor activity, antiproliferative activity and their organic constituents were studied. There was no scientific information about this plants having antitumor activity, antiproliferative activity in Myanmar. This study intended to illustrate the scientific proof of Myanmar medicinal plant used as good remedies in the treatment of tumor and cancer.

1.1 Selected Myanmar Traditional Medicinal Plants

1.1.1 Botanical aspects of *Cissus repens* Lam. (Wa-round-chin, WRC)

Botanical name	:	*Cissus repens* Lam.
Genus	:	*Cissus*
Species	:	*repens*
Family	:	Vitaceae
Myanmar name	:	Wa-round-chin
English name	:	Som Toum, *Cissus*, Veour Sanda, Grape and Variegate
Part used	:	Stem and leaves

(a) Distribution

Cissus repens Lam. belongs to the family Vitaceae, is a species of tropical rainforest vine in the grap-family (Figure 1.1). It is distributed in India to Southern, China, Philippines, Malaysia, Cambodia, Vietnam, Myanmar, Taiwan and Thailand (Fernandeds and Banu, 2012).

Figure 1.1 Photographs of *Cissus repens* Lam. (Wa-round-chin, WRC)

(b) Description

It is a slender glabrous climber ; branches glaucous, tendril stout, forked. Leaves up to 15 x 9 cm; broadly ovate, cordate at base, acuminate at apex; dentate-crenate, glabrous petioles up to 7 cm long. Flowers in leaf-opposed compound umbellate cymes, small, greenish yellow, peduncles solitary or fascicled, pedicels slender, reddish. Calyx truncate, Berries ellipsoid-pyriform, 5 mm across, 1-seeded, black when ripe. Flowering occurs mainly between August and October (Yeo *et al.,* 2012).

(c) Chemical constituents in *C. repens*

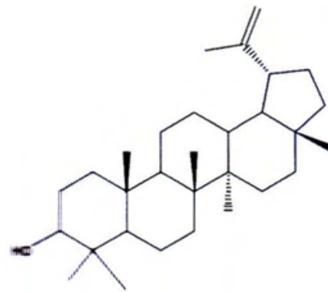
Investigation on the whole plant reported the presence of flavonoids, terpenoids, stilbene, alkaloids. The isolation of ursolic acid, asiatic acid, lupeol, friedelin, and epifriedelanol was from the whole plant (Chang *et al.,* 2012).

3-keto steroids, onocer-7-en-3a, 21b-diol and onecer-7-en-3a, 21a-diol have been isolated from the stems of *C. repens*. (Riviere *et al.,* 2012).

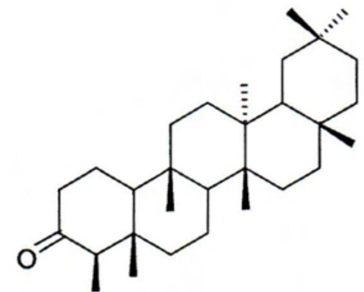
Rahim *et al.* (2012) isolated four stilbene C-glucosides namely trans-3-O-methyl-resveratrol-2-C-beta-glucoside,cis-3-O-methyl-resveratrol-2-C-beta-glucoside, trans-3-O-methyl-resveratrol-2-(2-p-coumaric)-C-beta-glucoside (cissuside A) and trans-3-O-methyl-resveratrol-2-(3-p-coumaric)-C-beta-glucoside (cissuside B) from the aerial parts of *C. repens*.

Bergenin, isolariciresinol and 1-[(3-methyl-butyl) phloroglucinol]-β-D-glucopyranoside, 4-O-methyl gallate, protocatechuic, gallic acid, 3-O-galloyl bergenin, 2α, 3β, 23-trihydoxy-olean-12-en-28-oic-acid and pallidol were isolated from the aerial parts of *C. repens* reported by Rahim *et al.,* (2012).

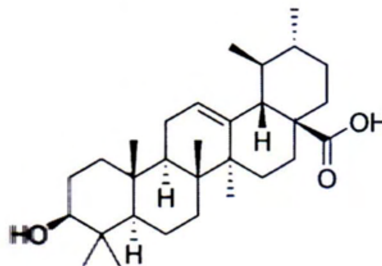
The structures of some chemical constituents present in *C. repens* are illustrated in Figure 1.2.



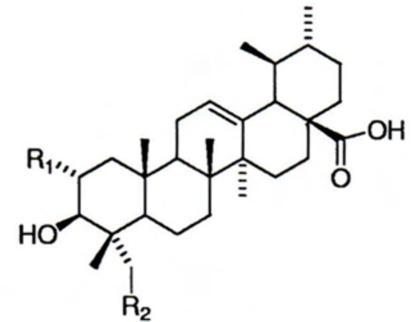
Lupeol



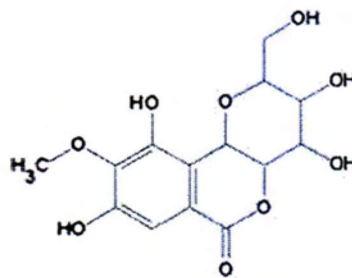
Friedelin



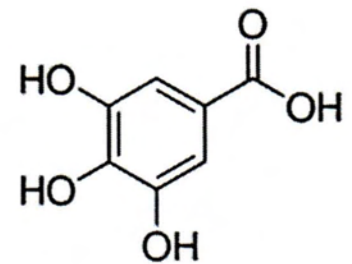
Ursolic acid



Asiatic acid



Bergenin



Gallic acid

Figure 1.2 Structures of some chemical constituents isolated from *Cissus repens* Lam.

(d) Medicinal properties and uses

The roots and stems of *Cissus repens* Lam. are used for snake bites, rheumatic pain and the stems are also applied to the treatment of nephritis, long-term coughs, and diarrhea. The rhizome is used for the treatment of sore, carbuncles, ulcers, hepatitis and jaundice, peptic uler, tumors and hypertension in Myanmar traditional medicine. The young shoots are succulent pleasantly acidic and edible, often it is substituted for sorrel. The leaves are also eaten raw or in soup. Fruit is acid but eaten. The stem can be made into ropes. The paste of the root and also of the leaf is applied as a suppurate. Leaves are warmed and rubbed on the skin for skin diseases and itch. The fruit is antiscorbutic, dyspepsia, heart diseases, thirst and ulcers. It overcomes loss of appetite, indigestion, flatulence, liver and spleen diseases, cough and other respiratory disorders. The literature shows that this genus has anti-inflammatory analgesic, antagonist of endothelin and venom, inhabited gastric ulcer, anti-osteoporosis, anti-oxidant, anti-bacterial, anti-allergic, anti-cancer, cure diabetes and other active (Chang *et al.,* 2012).

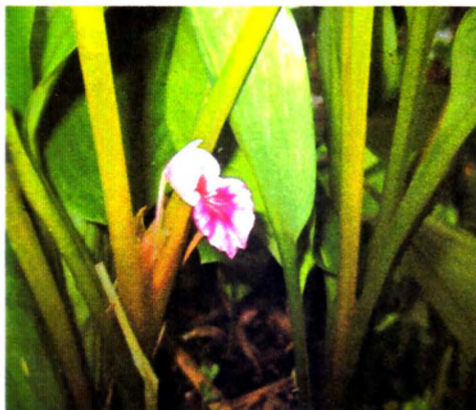
1.1.2 Botanical aspects of *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin, SPC)

Botanical name	:	*Boesenbergia rotunda* (L.) Mansf.
Genus	:	*Boesenbergia*
Species	:	*rotunda*
Family	:	Zingiberaceae
Myanmar name	:	Seik-phoo-chin (Figure 1.3)
English name	:	Finger root, Chinese ginger, Chinese key
Parts used	:	Leaves, stem and rhizomes

(a) Distribution

Boesenbergia rotunda is native from Southern Yunnan Province, China, to west Malaysia. It grows in dense forest and is common in its natural range. It is widely cultivated throughout South-East Asia, in small-scale subsistence farming systems, and has become naturalized in some countries. Species in the ginger family

The whole plant



Flower

rhizomes

Figure 1.3 Photographs of *B.rotunda* (Seik-phoo-chin, SPC)

(Zingiberaceae) usually grow in damp shaded lowland areas or on hill slopes, as scattered plants or thickets (Isa *et al.,* 2012).

(b) Description

Boesenbergia rotunda, a herb in the ginger family (Zingiberaceae) is a medicinal and culinary herb. Its rhizomes are often shaped like a bunch of fingers, hence its common English name Fingerroot.

Fingerroot is a small, erect herb, up to 50 cm tall. The rhizome are bright yellow and strongly aromatic, and they resemble fingers growing from a central point. There are usually 3-4 leaves upto 12 cm wide and 50 cm long, which are undivided, ovate-oblong in shape. The flowers are tubular, pink and aromatic and produced in terminal inflorescene (Isa *et al.,* 2012).

(c) Chemical constituents of *Boesenbergia rotunda* (L.) Mansf.

Boesenbergia rotunda contains 1 to 3% of an essential oil. The essential oil of *B.rotunda* consists of largely amount of oxygenated and non-oxygenated monoterpenes. The major compounds of essential oils isolated by various methods and solvents are γ-terpinene, geraniol, camphor, β-ocimene, 1,8-cineole, myrcene, borneol, camphene, methyl cinnamate, terpineol, geranial and neral (Sukari *et al.,* 2008).

B.rotunda contains a number of important bioactive compounds such as boesenbergin, cardamonin, pinostrobin, pinocembrin, panduratin A and 4-hydroxypanduratin A.

Several new prenylated chalcones, namely (+)-krachaizin A, (-)-krachaizin A, (+)-krachaizin B, (-)-krachaizin B, (+)-panduratin A, (-)-panduratin A, (+)-4-hydroxypanduratin A, (-)-4-hydroxypanduratin A, (+)-isopanduratin A, (-)-isopanduratin A can be also found in *B. rotunda* (Morikawa *et al.,* 2008). Figure 1.4 shows the structures of some compounds in *B. rotunda.*

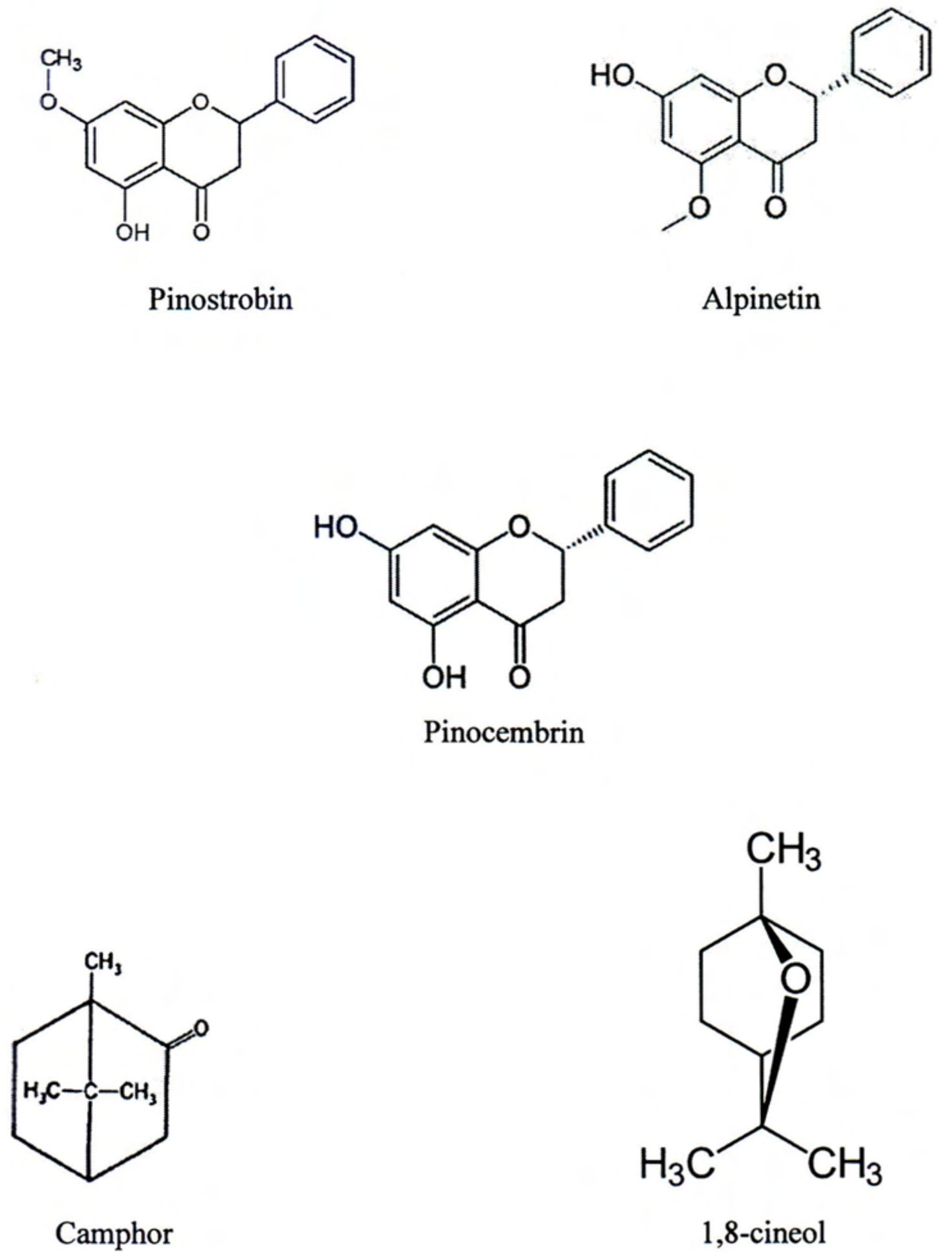


Figure 1.4 **Structures of some chemical constituents contained in *Boesenbergia rotunda* (L.) Mansf.**

(d) Medicinal properties and use

Rhizomes, it is commonly used as a folk medicine in Southeast Asia for treating several diseases including aphthous, dry mouth, stomach discomfort, leucorrhea and dysentery. Scientific investigations in the past have reported that the extracts isolated from the *Boesenbergia rotunda* plant using various solutions (such as methanol, hexane or chloroform) have neuroprotective, antibacterial, anticancer, antifeedant and antiviral effects. The methanol-based extract was shown to contain chemical are known to play critical role in cascades or processes. When the hexane or chloroform is used in the isolation process, the resulting extract contains other important antioxidants; three flavonones (pinostrobin, pinocembrin and alpinetin) and two chalcones (Salama *et al.,* 2012).

In tradition medicine, rhizomes and roots are used in post-partum tonic mixtures (such as the popular Indonesian tonic, 'jamu'), as a stomachic (improve appetite and digestion) and carminative (to aid digestion and reduce gas) and as a remedy for coughs and mouth ulcers. Crushed rhizomes and roots are also applied externally to treat rheumatism. Scientific research is underway to investigate their possible antioxidant, anti-inflammatory, antibacterial and anticancer properties. Fingerroot is used as a flavouring and eaten as a vegetable, as well as having a variety of medicinal use. It is cultivated for its rhizomes and roots in Indonesia, Malaysia, Indochina and India where they are used as a spicy flavouring in food and pickles. The rhizomes are also edible and leaves are used together with those from the teak tree (*Tectona gradis*) to wrap fermented soya bean cake ('tempeh') a traditional Indonesian food.

1.2 Antimicrobial Activity

Medicinal plants are known to produce certain bioactive molecules which reacts with other organisms in the environment, inhibiting bacterial and fungal growth (Chopra *et al*., 1992)

1.2.1 Microorganisms

Microorganisms are small living forms of life, which we cannot see with the eyes. Bacteria, yeasts (fungi) and the molds are three types of microorganisms.

Bacteria can be seen when studied with a powerful microscope that enlarges 1000 times. Yeast must be viewed through a microscope that minifies several hundred times. Molds can be seen with only slight magnification and use of an ordinary magnifying glass. Bacteria, yeasts and molds can be found everywhere. They are present on animals, people and even in the air we breathe. Microorganisms have a direct impact on our daily lives. Some are helpful. They aid our bodily processes by helping break down complex foods into simplex substances. Some, called germs, are harmful to use by the role they play in causing diseases.

1.2.2 Classification of microorganisms

The structure and the shape of bacteria are prokaryotic cell structure, spherical or oval (cocci), rod shaped (bacilli), comma-shaped (vibrios), non-flexuous, spiral (spirilla), fexuous, spiral (spirochetes) and filamentous and branching (actinomycetes). The types of microorganisms can be classified as mentioned in Table 1.1 (Cruckshank, 1960). In the present work, six types of microorganisms shown in this table were applied for determination of antimicrobial activity.

Table 1.1 Types of Microorganisms and Their Effects

Organisms	Gram	Shape	Types	Effect
Bacillus subtilis	+	Rod	Bacteria	causes conjunctivities, food poisoning
Staphylococcus aureus	+	Spherical	Bacteria	causes pneumonia, abscesses, wound sepsis, burns, food poison, carbuncle, soft tissue infection, bone and joint infections, gastrointestinal, septicemia,
Pseudomonas aeruginosa	-	Rod	Bacteria	chronic lung, ear infections, burn infections, surgical wounds, ocular infection
Bacillus pumilus	+	Rod	Bacteria	eye infection, soft tissue infection, food poison
Candida albicans	+	Capsules	Fungus	bowel disorders, sinus irritation, intense itching, sores, ringworm
Escherichia coli	-	Spherical	Bacteria	urinary tract infection, bloody diarrhea, enteric diseases

1.2.3 Methods of detection of antimicrobial activity

The general standard methods for detecting an in *vitro* antimicrobial include plate diffusion test, serial dilution test and streak test.

In the plate diffusion test, the surface of an agar nutrient contained in a petri dish is uniformly inoculated with a measure of the test bacterial culture. The test solutions are added to such a plate by pipetting them either into circular hole cut into the agar or into previously applied glass or metal cylinders or these are absorbed onto the filter paper discs which are put on the surface of the agar. The test substance diffuse into the agar with decreasing concentration towards the periphery. In the case of positive reaction, an inhibitory zone can be observed after incubation (at 37°C) for several hours. The diameter of the inhibitory zone is proportional to the logarithm of the concentration of the antibiotic under constant experimental conditions. When comparing different antibiotics at known concentrations, the inhibitory zone diameter is taken as a measure of the antimicrobial activity. This method was applied for examination of antimicrobial activity in the present work.

In the serial dilution test, the growth of the test organism is investigated in a liquid culture medium with decreasing concentrations of an antibiotic. After incubation (at 37°C), the last still inhibitory concentration of the antibiotic the so-called minimum inhibitory concentration (MIC), it is necessary to expose the bacteria to a number of different concentrations of the antibiotic and after a shorter or longer period of time improve them to an antibiotic free nutrient medium (subculture) to test if they again grow or in other word if they are still capable of reproduction or not.

The streak permits the determination of antibiotic effect of a test compound on several microorganisms simultaneously and is hence suitable for the determination of the spectrum of activity. A filter paper disc impregnated with 10 μg/cm of the antibiotic to be examined is placed in the middle of a petri dish containing nutrient agar. The suspension of the test organisms is radially streak on the plate with a platinum loop. After incubation, it is established that some of the test organism have been inhibited in the growth in the diffusion area to different extent (Finegold *et al.*, 1978).

1.3 Role of Free Radicals and Antioxidants

1.3.1 Free radicals

A free radicals is an atom or group of atoms that have one or more unpaired electrons. In chemistry, free radicals are atoms, molecules or ions with unpaired electrons on an otherwise open shell configuration. Free radicals play an important role in combustion, atmospheric chemistry, polymerization, plasma chemistry, biochemistry and many other chemical processes, including human physiology. The human body is composed of many different types of molecules. Molecules consist of one or more atoms of one or more elements joined by chemical bonds. Normally, bonds do not split in a way that leaves a molecule with an odd, unpaired electron. But when weak bonds split or oxygen interacts with certain molecules, free radicals are formed.

Free radicals are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability. When a free radical is born, it goes around the body looking for another compound to steal and electron form. When the "attacked" molecules loses its electron, it becomes a free radicals itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, RNA, protein, enzymes or the cell membrane. Cells may function poorly or die if this occurs (Diaz *et al.,* 1997).

Many forms of cancer are thought to be the results of reactions between free radicals and DNA, resulting in mutations that can adversely affect the cell cycle and potentially lead to malignancy.

1.3.2 Oxidative Stress

Oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants. Oxidation, or the loss of an electron, can sometime produce reactive substances known as free radicals that can cause oxidative stress or damage to the cells. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. In addition, oxidative stress occurs when the

quantity of free radicals the body has to cope with exceeds the availability of antioxidants .

Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. DNA damage induced by ionizing radiation is similar to oxidative stress, and these lesions have been implicated in aging and cancer. Consumption of antioxidants is thought to provide protection against oxidative damage and contribute positive health benefits (Diaz *et al.,* 1997).

1.3.3 Antioxidants

Antioxidants means "against oxidation". Antioxidants, also known as "free radical scavengers" are compounds that either reduce formation of free radicals or react with an neutralize them.

Antioxidants work many functions. They help alleviate the symptoms and side effects of many diseases and help to neutralized the production of free radicals which are chemically complexes that cause harm to our cell and play a major role in the disease process. Antioxidants fight cancer by neutralizing DNA damaging free radicals and prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain and help lower risk of cardiovascular disease and dimentia, including Alzheimer's disease.

There are three potential sources of anitoxidants, those produced by the body, those derived from certain food substances and those taken as nutritional supplements.

The primary antioxidants are phytochemicals; they come from our diet by eating fruits and vegetable. The primary antioxidants are carotenoids (such as lycopene, lutein, beta-carotene), vitamin C, vitamin E, selenium and anthocyanins. They can be found in colourful fruits and vegetables including spinach, blue berries, garlic, carrots, red bell pepper, tomatoes and green tea (Goedrow *et al.,* 2006).

Antioxidants are also thought to have a role in slowing the aging process and preventing heart disease and strokes, but the data is still inconclusive. Perhaps the best advice, which comes from several authorities in cancer prevention, is to eat five servings of fruit or vegetables per day (Halliwell, 1999).

1.3.4 Measurements of radical scavenging activity

Commonly used for measurements of radical scavenger activity are as follows.

(a) Conjugated diene assay

This method allows dynamic quantification of conjugated dienes (CD) formed as a result of initial PUFA (Poly-Unsaturated Fatty Acid) oxidation by meaning UV absorbance at 234 nm. The principle of this assay is that, during linoleic acid oxidation the double bonds are converted into conjugated double bonds which are characterized by strong UV absorption at 234 nm.

(b) Lipid peroxide assay

While linoleic acid is oxidized, oxidation starts at its allylic position in a neospecific reaction to form an unstable mixture of lipid peroxides. The total amount of lipid peroxide can be detected iodometrically by PD assay. The amount of lipid peroxides accumulated first reaches a maximum, there after declining while forming aldehydes.

(c) Thiobarbituric acid assay

During Lipid peroxidation, lipid peroxides are formed with a subsequent formation of peroxyl radicals, followed by a decomposition phase to yield the aldehydes such as hexanal, malondialdehyde (MDA) and 4-hydroxynomenal. This assay is based on the detection of a stable product which is formed between aldehydes and thiobarbituric acid (TBA) in aqueous phase.

(d) The Carotene-linoleic acid system

The modification of the above assay is based on the determination of the coupled oxidation of carotene and linolenic acid. This assay is simple, reproducible and time-efficient for a rapid evaluation of antioxidant properties.

(e) 1,1 Diphenyl-2- Picryl-Hydrazyl (DPPH) Assay

This assay measures the free radical scavenging capacity of a compound. DPPH is a molecule containing a stable free radical that tends to capture hydrogen

from the antioxidant. Due to its free radical, the DPPH solution is violet colour, being neutralized by the test sample. The colour changes upon neutralization of the free radical from violet to pale yellow. The decolouration of the initial colour is proportional to the test samples anti-radicalizing power. In the present research, antioxidant activity was investigated by using this assay due to its simplicity.

(f) Cyclic volumetry (V)

In this technique, the antioxidant activity of tissue or plasma was measured on the bases of the reducing properties of the tested sample, by measuring their oxidation potential (Soares *et al.,* 1997).

1.4 Toxicity Test

1.4.1 Brine shrimp

Brine shrimp (Figure 1.5) is a small fairy shrimp that live in vine pool and is used as food for aquarium fish. The scientific classification of brine shrimp are as follows :

Scientific name	:	*Artemia salina*
Family name	:	Artemiidae
Genus	:	*Artemia*
Marketing name	:	Sea-Monkeys
Species	:	*salina*
Common name	:	Brine shrimp

Figure 1.5 Image of brine shrimp (*Artemia salina*)

Brine shrimp are small members of the fairy shrimp order Anostraca, generally reaching only 0.6 inches (15 millimeters) in length. The brine shrimp's body is distinctly separated into head, thorax and abdomen. The head consists of two sections. The first one with the antennae and eyes, and the second one bearing the jaws (mandibles and maxillae). The first pair of antennae, known as antennules, are usually pair of considerable length, but not segmented. The second pair is more muscular, especially in males. The males use their antennae to grasp females while mating. In some males these antennae are equipped with numerous outgrowths and are quite unusual in shape. At the sides of the head is a pair of stalked compound eyes and an unpaired naupliar eye at the top of the head.

Color of brine shrimp varies from nearly hyaline (glass like, colorless, or transparent) to a bright red. Brine shrimp are filter feeders. Brine shrimp are found in inland saltwaters worldwide, in such places as salt lakes and salt marshes. They can inhabit water both many times the salinity of seawater, and also salinities much less concentrated than seawater.

Artemia, as *Artemia salina*, were first discovered in lymington, England, in 1755, although this particular population is now extinct (Lee *et al.,* 1999).

1.4.2 Brine shrimp toxicity test

In the present work, brine shrimp lethality assay was applied for toxicity test since it is considered as a useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities in plant extracts. The assay is considered a useful tool for preliminary assessment of toxicity and it has been useful for the detection of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides and cytotoxicity testing of dental materials.

Although most researchers have made use of the hatched nauplii, other assays based on the inhibition of hatching of the cyst have also been used. For the past 30 years, the Artemia nauplii have been used to detect general toxicity, in teratology screens and in ecotoxicology. From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extracts (Lee *et al.,* 1999).

1.5 Tumor

A tumor is an abnormal growth of body tissue and actually refers to any type of lump or swelling in the body. Also known as a neoplasm (meaning "new formation"), a tumor can be either benign (non-cancerous or not serious) or malignant (cancerous or deadly). In general, tumors appear to occur when there is a problem with the dividing of cells in the body. Cells are the building blocks of living things. Typically, the division of cells in the body is strictly controlled. New cells are created to replace older ones or to perform new functions. Cells that are damaged or no longer needed die to make room for healthy replacements. If the balance of cell division and death is disturbed, a tumor may form.

Tumor can develop in almost any organ or tissue, such as the lung, colon, breast, skin, brain, liver, ovary, kidney, uterus, bones, or nerve tissue. There are multiple causes of tumors. Tobacco causes more deaths from cancer than any other environmental substance. Other causes include obesity, inactivity (sedentary lifestyle), excessive alcohol consumption, radiation, genetic problems, excessive sunlight exposure, benzene, a number of other chemicals and certain poisonous mushrooms and aflatoxins (a poison produced by organisms that can grow on peanut plants). Certain viruses can play a role in the development of tumors, such as cervical

cancer and hepatocellular carcinoma. However, the cause of many tumors remains unknown. The most common cause of cancer-related death is lung cancer (Galvis *et al.,* 1999).

1.5.1 Antitumor activity test

(a) Potato crown gall (PCG) test

The crown gall tumor assay is one of several bench top bioassays recommended for the rapid screening of plants with antitumor activity. Crown gall is a neoplastic plant disease induced by the gram negative bacterium *A. tumefaciens.* Infected plants, exhibit tumorgenic growth symptoms in stem collars and other parts of the plant. Crown Gall is a common disease of dicot plants including many woody shrubs and various herbaceous plants. During infection of the plant material with the bacterium, a large tumor-inducing (Ti) plasmid, found in the DNA, is incorporated into the plant's chromosomal DNA. The phenols released when the plant is wounded activate the Ti plasmid of the bacterium, which induces cell proliferation without the cells going through apoptosis. Thus, transforming normal wounded cells into autonomous tumors cells. Since the induced tumor is similar in nuclei acid content and histology to human and animal cancers, the test system has been used to evaluate and pre-screen the antitumor, cytotoxic properties of natural products.

In 1980, Galsky *et al.,* demonstrated that inhibition of Crown Gall tumor initiation on potato disc showed appearance agreement with compounds and plant extracts known to be active in the 3 PS (in *vivo,* mouse leukemia) antitumor assay. In 1981 these workers expanded their study to show that inhibition of the growth of the tumors, in addition to the inhibition of tumor initiation, agreed well with 3PS activity. The results suggest that the potato disc assay is safe, simple, rapid, inexpensive and statistically reliable prescreen for 3PS antitumor activity.

The present, investigation was initiated to modify Galsky's potato disc assay for the routine assay of plant fractionation extracts and to test the effectiveness of the modified assay as an antitumor prescreen for crude plant extracts. Tumors were initiated on potato disc, and two modifications were made :

(1) The use of dimethylsulfoxide (DMSO) as a universal solvent for the plant extracts; and

(2) The use of iodine/potassium iodide solution to stain the background, starch-containing, nontumorous (normal) cells to facilitate tumor counts. DMSO did not affect bacteria viability, but the DMSO did not inhibit tumor initiation. However, inoculation of control disc with identical final concentrations (12.5 % is used routinely) of DMSO compensated for this inhibition. The assay also gives indication of tumor-promoting or carcinogenic properties of the test samples (Ferrigni *et al.,* 1982).

(b) Tumor producing bacteria

Agrobacterium tumefaciens is the causal agent of Crown Gall disease (the formation of tumors) in over 140 species of dicot. It is cosmopolitan in distribution, causes Crown Gall disease in dicotyledonous plants. Tomato, peach, apple, grape, shade and nut tree and vines plants are common test plants. *Agrobacterium* possess both linear and circular DNA chromosomes in addition to a plant-tumor-including (Ti) plasmid. The *A. tumefaciens* infects the plant through its Ti plasmid. The Ti plasmid integrates a segment of its DNA, known as T-DNA, into the chromosomal DNA of its host plant cells. The plasmid T-DNA is incorporated into the genome of the host cell by homologus recombination, and the virulence (vir) genes on the T-DNA are expressed, causing the formation of a gall.

A. tumetaciens is an alpha-proteobacterium of the family Rhizobiaceae, which includes the nitrogen fixing legume symbionts. Unlike the nitrogen fixing symbionts, tumor producing *Agrobacterium* are parasitic and do not benefit the plant. The wide varieties of plants affected by *Agrobacterium* made it of great concern to the agriculture industry (Moore *et al.,* 1997).

A. tumefaciens is a rod shaped, grow aerobically and Gram negative soil bacterium. The cells are non-sporing and motile, 0.6-1.0 mm by 1.5-3.0 mm and may exist singly or mixed in culture. It has rapid growth on meat extract or yeast extract peptone media and also growth on carbohydrate-containing media usually accompanied by polysaccharide slime. Colonies are non-pigmented and voluminous, slimy appearance (Collin, 2001).

1.6 Antiproliferative Activity

Natural products, especially plants, have been used for the treatment of various diseases for thousands of years. Terrestrial plants have been used as medicine in Egypt, China, Myanmar, India and Greece from ancient time and an impressive number of modern drugs have developed from them. The first writer records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Alkaidians. The World health Organization estimates that approximately 80% of the world's inhabitants rely on traditional medicine for their primary health care (Farnsworth *et al.,* 1985).

From the earliest times, herbs have been prized for their pain-relieving and healing abilities and today we still rely largely on the curative properties of plants. According to World health Organization, 80% of the people living in rural areas depend on medicinal herbs as primary healthcare system. Cancer is a general term applied of series of malignant disease that may affect different parts of a body. Cancer is a major public health burden in both developed and developing countries. 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 in 2002 (Parkin *et al.,* 2002).

The extra cells can form a mass called a tumor. Tumors can be benign or malignant. Benign tumors aren't cancer while malignant ones are. Cells from malignant tumors can invade nearby tissues. They can also break away and spread to other parts of the body. Cancer is not just one disease but many diseases. There are more than 100 different types of cancers. Most cancers are named for where they start.

Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. Antiproliferative activity were studied *in vitro* using human cancer cell lines.

Fruits and vegetables are natural medicines and have been used in our daily diet. Phytochemcials present in the dietary fruits and vegetables have antiproliferative properties. Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales (Buttet, 2004). So far, pharmaceutical companies have screened more than 25, 000 plants for anti-cancer drugs.

1.6.1 Cancer cell line

Cancer cell line is the cancer cells that keep dividing and growing over time, under certain conditions in a laboratory. Cancer cell lines are used in research to study the biology of cancer and to test cancer treatment.

The first human cell line was established in a Baltimore laboratory over 50 year ago by George Gey. This cell line was hela-named after henrietta lacks, the lady from whom the cell line was derived, who had cervical carcinoma. Gey's vision paved the way of cell culture as we know it today, allowing its widespread development into an important experimental tool in cancer research. One of the major benefits of using cultured cell lines in cancer research is that they offer an infinite supply of a relatively homogeneous cell population that is capable of self-replication in standard cell culture medium (Teerasripreecha *et al.,* 2012).

1.7 Scope of the Present Research

Medicinal plants constituent a very important natural resource used by indigenous medicinal system for the last 300 years. Plant-based drugs have been used against various diseases since long time. The nature has provided abundant plant wealth for all living creatures, which possess medicinal virtues. The Myanmar Traditional Medicine is one with profound medical treatises, a variety of potent and effective medicines and a diversity of therapies. Traditional Medicine based on scientific evidences and practices.

In the present research work, Wa-round-chin and Seik-phoo-chin were selected for the investigation of some bioactivities such as antimicrobial activity, antioxidant activity, cytotoxicity, antitumor activity, antiproliferative activity and their organic constituents were studied. There was no scientific information about this plants having antitumor activity and antiproliferative activity in Myanmar. This study intended to illustrate the scientific proof of Myanmar medicinal plant used as good remedies in the treatment of tumor and cancer.

1.8 Aim and Objectives of the Present Work

The aim of the present study was to investigate the phytochemical constituents and biological activities such as antimicrobial, antioxidant, cytotoxicity, antitumor

and antiproliferative activities of *Cissus repens* Lam. (Wa-round-chin) and of *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin).

To achieve this aim, the research was carried out according to the following objectives.

(1) Collecting and identifying the whole plant of *Cissus repens* (Wa-round-chin) and aerial parts and rhizomes of *Boesenbergia rotunda* (Seik-phoo-chin).

(2) Performing the preliminary phytochemical tests of the collected samples.

(3) Determining the extractable matter of both plants by WHO method.

(4) Determining the nutritional values of the sample such as moisture, ash, protein, fat, fiber and carbohydrate by AOAC method.

(5) Preparing crude extracts of the whole plant of *Cissus repens*, aerial parts and rhizomes of *Boesenbergia rotunda* using some solvents.

(6) Isolating some organic compounds from the crude extracts of the whole plants of *C. repens* and rhizome of *Boesenbergia rotunda* by thin layer and column chromatographic methods.

(7) Identifying the isolated compounds by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI-MS.

(8) Screening the antimicrobial activity of various crude extracts by agar well diffusion method.

(9) Evaluating the antioxidant activity of crude extracts and the isolated compounds by DPPH assay.

(10) Investigating the cytotoxicity of water and ethanol extracts of the whole plant of *C. repens*, aerial parts and rhizomes of *B. rotunda*.

(11) Screening the antitumor activity of crude extracts and the isolated compounds by Potato Crown Gall assay (PCG).

(12) Screening the antiproliferative activities of crude extracts and the isolated compounds by using ten cell lines.

CHAPTER II

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Samples

The selected plants used in this study were two Myanmar medicinal plants: the whole plant of *Cissus repens* Lam. (Wa-round-chin, WRC) and the aerial parts and rhizomes of *Boesenbergia rotunda* L. (Mansf.). (Seik-phoo-chin, SPCA, SPCR). They were purchased from Kyauk Tan Village, Bago Region on September 2012. After collection, the scientific name of *C. repens* and *B. rotunda* were identified by authorized botanists at Botany Department, Yangon University. The collected samples were washed with water and dried in an over at 50 °C. The dried pieces were made into powder by using grinding machine. The powdered sample was separately stored in air-tight container to prevent moisture changes and other contaminations. The dried powdered samples were used for chemical and biological investigations.

2.2 Preliminary Phytochemical Investigation of *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* L. (Mansf.) (Seik-phoo-chin)

The prepared air-dried powdered were subjected to preliminary phytochemical tests in order to find out the types of phytoorganic constituents such as alkaloids, α-amino acid, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, steroids, tannins and terpenoids present in the samples.

2.2.1 Chemicals required

Acetic acid, acetic anhydride, chloroform, ethanol, ethyl acetate, ferric chloride, 1% gelatin, hydrochloric acid, iodine, $K_3Fe(CN)_6$, lead acetate, magnesium ribbon, α-naphthol, ninhydrin, potassium hydroxides, potassium iodide, sodium chloride, sodium hydroxide and sulphuric acid.

2.2.2 Procedures

(1) Test for alkaloids

Dried powdered sample (3 g) was boiled with 50 mL of 1% hydrochloric acid for about 10 minutes and allowed to cool and then filtered. The fittrate was divided into three portions and tested separately with Mayer's reagent, Dragendroff's reagent and Wagner's reagent. Observation was made to see the coloured precipitates, indicating the presence of alkaloids (Trease, 1980).

(2) Test for α-amino acid

Dried powdered sample (3 g) was boiled with 50 mL of distilled water for 10 minutes and filtered. A few drops of filtrate was spotted on a filter paper using a capillary tube, allowed to dry and sprayed with ninhydrin reagent. The filter paper was dried at room temperature and then kept in an over at 110 °C for a few minutes to see if pink or violet coloured spot appeared indicating the presence of α-amino acids (Marini-Bettolo, 1981).

(3) Test for carbohydrates (Molish's Test)

Dried powdered sample (3 g) was boiled with distilled water for about 20 minutes and filtered 5 mL of filtrate was placed into a test tube and a few drops of 10 % α-naphthol was added and shaken. The test tube was inclined at an angle of 45° and about 1 mL of concentrated sulphuric acid was slowly introduced along the inner side of the test tube to see a red ring formed between the two layers that indicates the presence of carbohydrates (Shriner, 1980).

(4) Test for cyanogenic glycosides

Dried powdered sample (1 g) was mixed with distilled water in boiling tube. Then about 5 drops of concentrated sulphuric acid was added and sodium picrate paper was trapped in the neck of the test tube by means of a cork. The resulting mixture was heated by using a sprit burner. Observation was made to see if the paper turned brick red which indicated the presence of cyanogenic glycosides (Trease, 1980).

(5) Test for flavonoids (Cyanidin Reaction)

The dried powdered sample (3 g) was socked in 50 mL of ethanol for about 6 hours and filtered. A piece of magnesium turning and a few drops of concentrated hydrochloric acid were added into 5 mL of ethanol extract to see if pink colour appeared, indicating the presence of flavonoids (Robinson, 1983).

(6) Test for glycosides

About 3g of powdered sample was soaked in 50 mL of ethanol for 6 hours and filtered 5 mL of filterate were taken and treated with a few drops of 10% lead acetate solution. If white precipitate were formed, it was noticed as the presence of glycosides (Marini-Bettolo, 1981).

(7) Test for organic acids

Dried powdered sample (3 g) was boiled with distilled water for about 10 minutes and filtered. 5 mL of filtrate were taken and treated with a few drops of bromocresol green indicator to see appearing if yellow colouration which indicated the presence of organic acids (Robinson, 1983).

(8) Test for phenolic compounds

Dried powdered sample (3 g) was socked in 50 mL of ethanol for 6 hours and filtered. 5 mL of filtrate were taken and treated with a few drops of freshly prepared 1:1 mixture of 1 % potassium ferricyanide and 1% ferric chloride solution. The change of colour of solution indicated the presence of phenolic compounds (Marini-Bettolo, 1981).

(9) Test for reducing sugars

About 3 g of dried powdered samples were boiled with 25 mL of distilled water for about 10 minutes and filtered. The resultant solution was boiled with Benedict's reagent for two minutes to see the formation of brick red precipitates, on cooling down the solution, indicating the presence of reducing sugars (Finar, 1969).

(10) Test for saponins

Dried powdered sample (3 g) was put into the conical flask followed by the addition of distilled water and the mixture was vigorously shaken for a few minutes. Observation was made to see producing of permanent frothing (Shriner, 1980).

(11) Test for steroids (Liebermann Burchard Test)

The dried powdered sample (3 g) was socked in 50 mL of petroleum ether (60-80 °C) for about 6 hours and filtered. 3 drops of acetic anhydride and 1 drop of concentrated sulphuric acid were added to 5 mL of petroleum ether extract and recorded the observed colour. If the colour changed to blue or greenish blue or green, the steroids were present (Tin Wa, 1970).

(12) Test for tannins

Dried powdered sample (3 g) was boiled with 50 mL of distilled water for about 10 minutes and filtered. 5 mL of water extract were taken and treated with a few drops of gelatin and 1%. $FeCl_3$. Observation was made to see precipitate were formed; then the presence of tannins (M-Tin Wa, 1970).

(13) Test for terpenoids

The dried powdered sample (3 g) was soaked in 50 mL of chloroform for about 6 hours and filtered. 3 drops of acetic anhydride and one drop of concentrated sulphuric acid were added to 5 mL of chloroform extract and recorded the observed colour. Red or pink colouration indicated the presence of terpenoids (M-Tin Wa, 1970).

2.3 Determination of Some Physico-chemical Properties of the Samples

In the present study, some nutritional values such as moisture, fat, ash, fibre, protein and carbohydrate and energy values of the whole plant of *C. repens* (Wa- round-chin, WRC) and aerial parts and rhizome of *B. rotunda* (Seik-phoo-chin, SPCA and SPCR) were determined by AOAC methods (AOAC, 2000).

2.3.1 Determination of moisture content

The moisture content of WRC, SPCA and SPCR were determined by oven drying method. The moisture content was the weight loss due to the evaporation of water at the drying temperature.

(i) Sample

Dried power of the whole plant of *C. repens* (Wa- round-chin, WRC) and aerial parts and rhizomes of *B. rotunda* (Seik-phoo-chin, SPCA and SPCR).

(ii) Apparatus requirement

Porcelain crucibles, electronic balance, oven and air-tight desiccators.

(iii) Procedure

Sample (2 g) was placed in the porcelain crucibles, which had previously been dried and cooled in air-tight desiccator, and accurately weighed. The porcelain crucibles with the samples were placed in an oven and dried for 30 minutes at 105 °C. Then, they were removed from the oven and cooled in the air-tight desiccator at room temperature and weighed. The procedure was repeated until the loss in weight had not been changed. The moisture content can be calculated by the following formula.

$$\text{Moisture (\%)} = \frac{\text{Loss in weight (g)}}{\text{Weight of sample (g)}} \times 100\ \%$$

2.3.2 Determination of fat content

Fat content was determined by Soxhlet extraction method.

(i) Samples

Dried powder of WRC, SPCA and SPCR.

(ii) Chemical requirement

Petroleum ether (b. pt 60-80 °C)

(iii) Apparatus requirement

Soxhlet extractor, condenser, water bath, round-bottomed flask (250 mL), oven and cloth bag.

(iv) Procedure

Sample (10 g) was weighed, placed in a cloth bag and the bag was then placed in a Soxhlet extractor. Petroleum ether (250 mL) was poured into the extractor until some of it overflowed into the flask. The flask was heated on a water bath. The extraction was assumed to be complete when a small amount of extract placed on a watch glass did not leave any residue on evaporation of solvent.

A duration of about 8 hours was required for complete extraction. The petroleum ether was removed by simple distillation until the volume of the petroleum ether was remained to about 10 mL. The last trace of the solvent was then removed by placing the content in an oven at about 100 °C until the constant weight was obtained. The fat content of sample was calculated by the following equation.

$$\text{Fat (\%)} = \frac{\text{Weight of fat (g)}}{\text{Weight of sample (g)}} \times 100\ \%$$

2.3.3 Determination of ash content

The ash content is the inorganic residue remaining after the organic matter has been burnt away.

(i) Sample

Dried powder of WRC, SPCA and SPCR.

(ii) Apparatus requirement

Porcelain crucible, burner, Muttle furnace, air-tight desicators and electronic balance.

(iii) Procedure

Sample (2 g) was introduced into a predried and cooled porcelain crucible, and accurately weighed. Then, it was heated gently on an electronic stone until the sample was thoroughly charred. The crucible and content were then transferred to the Muffle furnace at 600 °C for 2 hours until the residue was free from carbon. Then, the crucible containing residue was cooled in a desiccator and weighed. Heating, cooling and weighing were repeated until constant weight was obtained.

The ash content of the sample was calculated using the following equation.

$$\text{Ash (\%)} = \frac{\text{Loss of residue (g)}}{\text{Weight of sample (g)}} \times 100\ \%$$

2.3.4 Determination of fibre content

Fibre content of samples was determined by acid-base digestion method.

(i) Sample

Dried powder of WRC, SPCA and SPCR.

(ii) Chemicals requirement

Sulphuric acid solution (1.25 % v/v) and sodium hydroxide solution (1.25 % w/v).

(iii) Apparatus requirement

Round-bottomed flask (500 mL), condenser, filter paper, glass rod, Buchner funnel, water suction pump, beaker, oven, measuring cylinder and conical flask.

(iv) Preparation of solutions

(a) 1.25 % (v/v) sulphuric acid solution

Sulphuric acid (1.25 mL) was dissolved in distilled water and the volume was made up to 100 mL with distilled water.

(b) 1.25 % (w/v) sodium hydroxide solution

Sodium hydroxide pellets (1.25 g) was dissolved in (100 mL) distilled water and it was kept overnight.

(v) Procedure

Sample (2 g) was accurately weighed and introduced into 500 mL round-bottomed flask. Then, 200 mL of 1.25 % sulphuric acid was poured into the flask. The flask was connected with reflux condenser and digested for about 1 hour. The flask was rotated with hand every few minutes in order to mix the contents and to remove particles from the sides. The contents in the flask were filtered through a filter paper supported in a Buchner funnel with water suction pump and washed with boiling distilled water to be free from acid. The residue was then washed down into the flask with 1.25 % sodium hydroxide (200 mL) and boiled for about 1 hour, rotating the flask in 5 minutes intervals. After boiling, the flask was removed and filtered through the same filter paper used in acid hydrolysis. The residue was washed thoroughly with boiling distilled water until free from alkali. The contents of the filter paper was cooled and weight.

The crude fibre content of sample was calculated by the following equation.

$$\text{Fibre (\%)} = \frac{\text{Weight of fibre (g)}}{\text{Weight of sample (g)}} \times 100\ \%$$

2.3.5 Determination of protein content

The crude protein content was determined by Micro-Kjeldahl's method.

(i) Sample

Dried powder of WRC, SPCA and SPCR.

(ii) Chemicals requirement

Potassium sulphate, copper (II) sulphate pentahydrate concentrated sulphuric acid, 40 % sodium hydroxide, 2 % boric acid, 0.1 M hydrochloric acid, distilled water and methyl red indicator.

(iii) Apparatus requirement

Kjeldahl's digestion flask and Micro-Kjeldah distillation apparatus, digestion rack, conical flask (50 mL), round bottom flask and micro-burette.

(iv) Preparation of solutions

(a) 40 % sodium hydroxide solution

Sodium hydroxide pellets (40 g) was dissolved in (100 mL) distilled water and it was kept overnight.

(b) 2 % Boric acid solution

Boric acid (2 g) was dissolved in distilled water and the volume was made up to 100 mL with distilled water.

(c) 0.1 M hydrochloric acid solution

Hydrochloric acid (8.9 mL) was dissolved in distilled water and the volume made up to 100 mL to obtain a 1 M HCl solution.

This 1 M HCl solution (100 mL) was diluted with distilled water and the volume was made up to 1 L in a volumetric flask.

(d) Methyl red indicator solution

Methyl red (0.1 g) was dissolved in 95 % ethanol (60 mL) and the volume was made up to the mark in a 100 mL volumetric flask with distilled water.

(v) Procedure

Accurately weighed sample (0.5 g) was introduced in the dry Kjeldahl's digestion flask. Potassium sulphate (0.2 g) and copper II sulphate (0.05 g) were added to the flask. Concentrated sulphuric acid (10 mL) was then poured into the flask in such a way as to wash down any solid adhering to the neck and the contents were shaken until well mixed. The flask was placed on the digestion rack and a small funnel was placed in the neck of the flask. The contents were digested and heated over a small flame so that the liquid boiled gently. Digestion was continued until the mixture become clear and almost pale green colour. Then, the flask was allowed to

cool at room temperature and about 20 mL of distilled water was added carefully into the flask with frequent shaking 40 % NaOH (25 mL) was poured into the flask. The solution and rinsing was transferred completely to the steam distillation apparatus which had previously been cleaned by passing steam through the apparatus for about 30 minutes. The tip of the condenser was dipped beneath the surface of 2 % boric acid solution and 2 drops of methyl red solution in a conical flask (receiver flask). The steam liberated from boiling water in the flask was passed through the mixture in apparatus. When ammonia evolved was carried by steam and condensed in condenser, the distillation was collected in the receiver flask. The distillation was assumed to be completed 15 minutes after boiling of the solution. Then, the receiver flask was removed and titrated with 0.1 M hydrochloric acid solution. The percentage of protein content can be calculated by using the following equation.

$$\text{Nitrogen (\%)} = \frac{1.4007 \times V \times M}{W}$$

Where, 1.4007	=	milliequivalent weight of N × 10
V	=	mL of HCl titrant used for test portion
M	=	Molarity of HCl solution
W	=	test portion weight, g
Protein (%)	=	Nirogen (%) × 6.25
Where, 6.25	=	value of primary nitrogen in protein

2.3.6 Determination of carbohydrate content

Carbohydrate content was determined by subtraction method. The total carbohydrate content of samples can be obtained as the difference between 100 and the sum of the percentage of ash, fat, fibre, moisture and protein contents by the following equation.

Carbohydrate (%) = 100 % – (ash + fat + fibre + moisture + protein)

2.3.7 Determination of energy values

The energy value of samples was calculated by the following equation.

$$\text{Energy value (kcal/100 g)} = (4 \times \text{protein}) + (4 \times \text{carbohydrate}) + (9 \times \text{fat})$$

2.4 Determination of the Soluble Matter Contents

This method determines the amount of active constituent extracted with solvents from a given amount of medicinal plant material. The extractive values provide an indication of the extract of polar, moderately polar and non-polar components present in the medicinal plant material.

(i) Sample

Dried powder of *C. repens* (Wa- round-chin, WRC) and aerial part and rhizomes of *B. rotunda* (Seik-phoo-chin, SPCA and SPCR).

(ii) Chemical requirement

Pet-ether (60-80 °C), ethyl acetate, acetone, 95 % ethanol and distilled water.

(iii) Apparatus requirement

Conical flasks, water bath, measuring cylinders, funnels, filter paper, shaker and porcelain crucibles.

(iv) Procedure

The accurately weighted coarsely powdered air-dried material (4 g) was placed in a conical flask. The solvent specified (100 mL) was macerated for the plant material concerned for 6 hours, shaking frequently, then allowed to stand for 18 hours. The extract was filtered rapidly taking care not to lose any solvent, and transferred to a preweighed porcelain basin and evaporated to dryness on water-bath. The dried filtrate was then placed in oven, maintained till constant weight, at 105 °C.

For ethanol soluble extractable matter, the concentration of solvent specified was used in the test procedure for the plant material concerned. For water soluble extractable matter, water was used a solvent. Other solvents were used as specified in

the test procedure (WHO, 1998). The contents of extractable matter calculated according to the following relationship.

2.5 Preparation of Crude Extracts by Successive Solvent Extraction Method

(i) Samples

Dried powdered samples of WRC and SPCR.

(ii) Chemicals

95 % ethanol, petroleum ether (60-80 °C), ethyl acetate and distilled water.

(iii) Apparatus

Beakers, conical flask, measuring cylinders, funnels, vaccum rotary evaporator and water bath.

(iv) Procedure

The dried powdered sample (500 g) was percolated with 95 % ethanol (2 L) for one week and filtered. This procedure was repeated for three times. The combined ethanol extracts were concentrated under reduced pressure by means of a rotary evaporatory. 95 % ethanol extract was obtained.

Then, 95 % ethanol extract was partitioned with petroleum ether (60-80 °C). The combined petroleum ether layers were concentrated under reduced pressure by means of a rotary evaporator. Consequently, pet-ether soluble extract was obtained.

The defatted residue was further successively partitioned between ethyl acetate and water. The combined ethyl acetate layers were concentrated by means of a rotary evaporator. After that ethyl acetate soluble extract was obtained.

In this way, 95 % ethanol, pet-ether and ethyl acetate soluble extracts of both plants samples were prepared. The general procedure for the preparation of crude extracts form *C. repens* and rhizomes of *B. rotunda* is described in Figure 2.1.

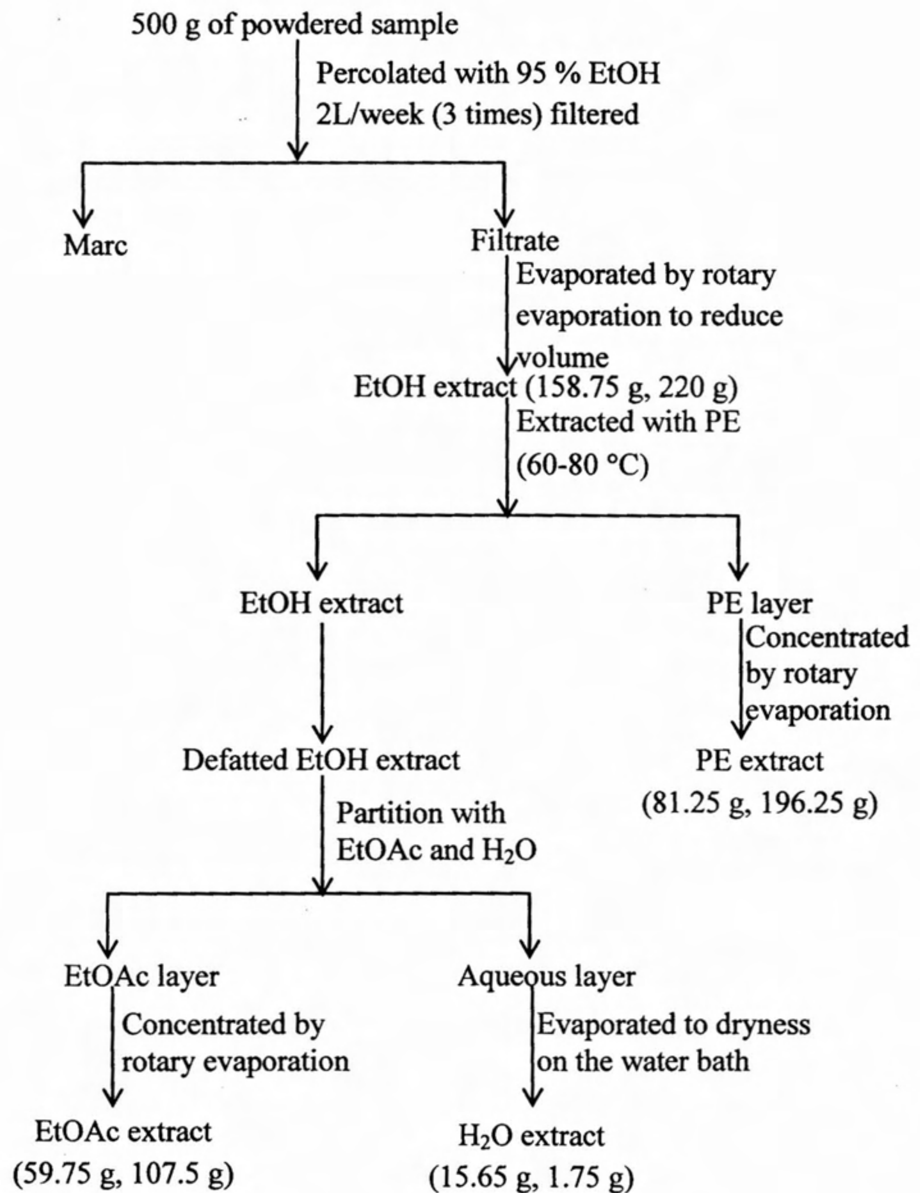


Figure 2.1 Procedure for preparation of crude extracts of the whole plant of *C. repens* (WRC) and rhizomes of *B. rotunda* (SPCR)

2.6 Isolation of Phytoconstituents from Selected Plant Samples

2.6.1 Separation and isolation of some organic constituents from pet-ether crude extract of the whole plant of *C. repens*

(i) Sample

Pet-ether crude extract of *C. repens* (WRC) obtained from Section 2.5.

(ii) Chemicals

Pet-ether (60-80 °C), ethyl acetate, methanol, pre-coated TLC plate (GF_{254} Aluminium plates, Merck), silica gel (40-60 mm, Wakogel), 5 % H_2SO_4, vanillin and ansisaldehyde.

(iii) Apparatus

UV lamp (365-254 nm), heater, glass, chromatographic column (60 × 2.5 cm), chromatographic tank, beaker, capillary tubes, measuring cylinder, stand, glass funnel and pasture pipette.

(iv) Procedure

A glass chromatographic column (60 × 2.5 cm) with a tap attached was clamped so that it was perfectly vertical. The column was packed with silica gel, using solvent system (PE: EtOAc, 100 % v/v). Firstly, the column was plugged by pushing a small piece of cotton wool through the solvent with a glass rod. Care was taken so that no air bubbles were trapped in the cotton wool. 100 g of silica gel were measured and placed in a beaker and made into slurry by adding PE : EtOAc (100: 1 v/v) and suspension was poured into the column and at the same time the tap was opened so that the solvent flowed at slow but constant rate. As the column material slowly settled to the bottom, the column was lightly taped with a rubber tubing around the outside wall so as to achieve an air bubble free, uniform packing. Column materials sticking to the upper walls of the column were washed down with the solvent. When the level of solvent had fallen to reach a few millimeters above the top of the silica gel, the tap was closed.

5 g of pet-ether extracts were mixed with 5 g of silica gel. The mixture was allowed to evaporate with continuous agitation so that free flows dry silica gel of

which the sample was uniformly adsorbed. The resulting powdered mixture was added to the column using a small long necked funnel. The top of the layer was net with solvent that had previously been allowed to remain above the gel by opening the top. Some absorbed gel sticking on the inner wall was washed down with the solvent. A piece of cotton wool was placed between the solvent and the column gel. The tap was opened and the fractions were collected at the rate of one drop per four seconds. Gradient elution was performed successively with PE: EtOAc solvent systems in the ratios of 100 : 1, 60: 1, 40: 1, 30: 1, 20 : 1, 15: 1 v/v and a total of 165 fractions were collected. The fractions were monitored by TLC. The fractions gave similar appearance on TLC were combined and finally six main fractions (FI to F VI) were collected. After removal of the solvents, fractions F III and F IV provided solid substances. All of these solid materials were purified by washing with ethyl-acetate followed by crystallization from methanol.

70 mg (0.014 %) of compound **A,** (white needle) was isolated from fraction F III (f_{38}-f_{59}). From fraction F IV (f_{60}-f_{78}), 38 mg (0.008 %) of compound **B** were also isolated as colourless crystals.

2.6.2 Separation and isolation of some organic constituents from ethyl acetate crude extract of rhizomes of *B. rotunda* (SPCR)

(i) Sample

Ethyl acetate crude extract of *B. rotunda* (SPCR) obtained from Section 2.5.

(ii) Chemicals

The chemicals used in this section were similar to these mentioned in Section 2.6.1 (ii).

(iii) Apparatus

The apparatus required for this section was the same as those mentioned in Section 2.6.1 (iii).

(iv) Procedure

6 g of ethyl acetate crude extract from the rhizomes of *B. rotunda* were subjected to column chromatographic separation technique using silica gel (40-60 μm). The detailed procedure for this section was similar to that mentioned in Section 2.6.1 (iv). Gradient elution was performed successively with PE : EtOAc (15:1, 9:1, 5:1, 3:1, 1:3, 1:5, 1:9, 1:15 v/v) and 210 fraction were combined on the basic of their behaviors on TLC to give eight main fractions. From fraction F III and F VII, compound **C** and **D** were isolated and F I, F II, F IV, F V, F VI and F VIII occurred as mixtures. The solid material obtained from fraction F III (f_{30}-f_{50}) was washed with ethanol and purified by crystallization from pet ether to give 63 mg (0.26 %) of compound **C** as a colourless crystal.

From fraction F VII (45 mg, 0.9 %) of compound **D** as a white powder was provided, after purification by washing with acetone followed by crystallization from methanol.

2.7 Extraction, Separation and Isolation of Organic Compounds from the Rhizomes of *B. rotunda*

(i) Sample

The dried powder of SPCR.

(ii) Chemicals

Chloroform, ethyl acetate, n-hexane, methanol, dichloromethane, benzene, distilled water, precoated TLC plate (GF_{254} and RP-18F_{254} plates, Merck, 0.25 or 0.50 mm thickness), silica gel (silica gel 60 N, Spherical, neutral, 40-50 μm, Karto Chemical Co., Inc, Japan) and cosmosil 75C18 – OPN (Nacalai Tesque Inc., Kyoto, Japan).

(iii) Apparatus

Beakers, conical flask, measuring cylinders, funnels, vacuum rotary evaporator, water bath, UV lamp (365-254 nm), heater, glass chromatographic columns, chromatographic tank, capillary tubes, stand, pasture pipette.

(iv) Procedure

The dried powdered sample (200 g) were extracted with chloroform (1000 mL) for 6 hours at 30 °C and filtered. This procedure was repeated for three times. The combined extracts were evaporated under reduced pressure by means of a rotatory evaporation. Consequently chloroform extract (6 g) was obtained. The chloroform crude extract was separated by column chromatographic separation technique using silica gel (40-60 μm). The detail procedure for this section was similar to that mentioned in Section 2.6.1 (iv). Gradient elution was performed successively using EtOAc: n-hexane in the ratios of 10:90, 15:85, 20:80, 25:75, 30:70, 35:65 and 50:50 v/v. Successive fractions obtained were combined on the basis of their behaviour on TLC. Finally, seven main fractions F-I to F-VII was obtained. After the solvents have been evaporated, fraction F-I (f_{1-15}), FIII (f_{51-70}), F-V (f_{87-107}) and F-VII ($f_{159-179}$) were obtained as a mixture.

Fraction F-II (f_{16-50}) (950 mg) was rechromtographedon silica gel with *n*-hexane: CH_2Cl_2:EtOAc solvent system to give seven subfraction. Subfraction – II was subjected to normal-phase preparative TLC with C_6H_6:CH_2Cl_2 (2:1 v/v) to give compound **E** (15.8 mg, 0.26 %) as colorless crystal.

Fraction F-IV (f_{71-80}) (730 mg) was rechromatographedon cosmosil 75C18-OPN with MeOH: H_2O (5:1 v/v) to give two subfractions (4-1 and 4-2). Subfraction 4-1 was purified by Sephadex LH20 with methanol to give compound **F** (15 mg, 0.25 %).

Fraction F-VI ($f_{108-158}$) (197 mg) was rechromatographedon cosmosil 75C18-OPN with MeOH: H_2O (5:1 v/v) to give three subfractions (6-1, 6-2 and 6-3). Subfraction 6-1 was purified by Sephadex LH20 with methanol followed by normal-phase preparative TLC with *n*-hexane: CH_2Cl_2: EtOAc (2:2:1 v/v) to give compound **G** (25.7 mg, 0.43 %). Similarly, subfraction 6-2 was purified by the same procedure as a subfraction 6-2 to give compound **H** (50 mg, 0.83 %).

2.8 Physicochemical Characterization of Isolated Compounds (Compound A to H)

The isolated compounds (**A** to **H**) were characterized by determination of some physical properties such as melting points, R_f values and solubility and some chemical properties by some colour tests.

2.8.1 Determination of melting point

A small amount of isolated compound was introduced into a capillary tube and its melting point was measured on Gallenkamp melting point apparatus. Each experiment was repeated three times and the average was taken as its melting point. And the observed melting points of all isolated compounds were then recorded.

2.8.2 Determination of R_f values

The isolated compound was subjected to TLC analysis and its R_f value was determined. In this experiment, GF_{254} silica gel precoated aluminium plate (Merck) was employed the chromatogram was developed in the appropriate solvent system for each isolated compound. After the plate was dried, the R_f values of isolated compounds were measured localization of spot with visualizing agents. The R_f values observed for isolated compounds were then recorded.

2.8.3 Determination of solubility of isolated compounds

The solubility of isolated compounds in some organic solvents such as pet-ether, ethyl acetate, chloroform, ethanol and methanol was determined.

2.8.4 Determination of chemical properties of isolated compounds

The isolated compounds were treated with some coloured reagents such as 2, 4-DNP, 10 % $KMnO_4$, 10 % $FeCl_3$, 10 % KOH, 10 % ammonia solution, Mg and concentrated HCl, 10 % lead acetate, bromocresol blue solution, Liebermann-Burchard reagent (conc. HCl and acetic anhydride), anisaldehyde, vanillin and 5 % H_2SO_4 to study their chemical characteristics.

2.9 Identification of Isolated Compounds

The isolated compounds were structurally identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, HMQC and ESI-MS spectroscopy.

2.9.1 Study on UV spectroscopy

For the identification of isolated compounds, UV spectra of the isolated compounds (**E, F, G, H**) were measured on a Shimadzu UV-160A spectrophotometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.9.2 Study on FT IR spectroscopy

The isolated compounds (**A, B, C, D**) were measured as 1 % KBr pellet form and measured by FT IR (8400) spectrophotometer (Shimadzu, Japan) at Department of Chemistry, University of Yangon and recorded by FT IR (Perkin Elmer, Spectrum two) spectrophotometer in order to identify the functional group of pure compound. Infrared spectra of the isolated compounds (**E, F, G, H**) were recorded as KBr pellets on a JASCO FT/IR-460 Plus spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.9.3 Study on ^{1}H NMR spectroscopy

^{1}H NMR spectra of the isolated compound **A** was recorded using $CDCl_3$ as solvent by 125 MHz NMR spectrometer at Department of Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany and that of the isolated compounds **B** and **D** were recorded in $CDCl_3$, by 400 MHz NMR spectrometer at Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

The ^{1}H NMR spectra of isolated compounds (**E, F, G, H**) were recorded in $CDCl_3$ by Joel-500 spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan to examine the type and number of hydrogen present.

2.9.4 Study on ^{13}C NMR spectroscopy

^{13}C NMR spectra of the isolated compound **A** was recorded using $CDCl_3$ as solvent by 125 MHz NMR spectrometer at Department of Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany and that of the isolated compounds **B** and **D** were recorded in $CDCl_3$, by 100 MHz NMR spectrometer at Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

The ^{13}C NMR spectra of isolated compounds (**E, F, G, H**) were recorded in $CDCl_3$ by Joel-500 spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan to examine the type and number of carbon present.

2.9.5 Study on COSY spectroscopy

COSY spectra gives the proton-proton correlation in pure compound. COSY spectra of the isolated compounds (**B** and **D**) were recorded by 400 MHz NMR spectrometer at Graduate School of Bioagricultural Sciences, Nagoya University, Japan and those for the isolated compounds (**G** and **H**) were recorded by 500 MHz NMR spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.9.6 Study on HSQC spectroscopy

Heteronuclear Single Quantum Coherence spectra give direct correlation between the proton and carbon. HSQC spectra of the isolated compounds (**B** and **D**) were recorded by 400 MHz NMR spectrometer at Graduate School of Bioagricultural Sciences, Nagoya University, Japan.

2.9.7 Study on HMBC spectroscopy

Heteronucler Multiple Bond Coherence spectra give the long range correlation between proton and carbon. HMBC spectra of the isolated compounds (**B** and **D**) were recorded by 400 MHz spectrometer Graduate School of Bioagricultural Sciences, Nagoya University, Japan and that of the isolated compounds (**E, F, G, H**) were recorded by 500 MHz spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.9.8 Study on HMQC spectroscopy

Heteronuclear Single Quantum Correlation spectra give direct correlation between the proton and carbon. HMQC spectrum of the isolated compound **(E, F, G, H)** were recorded by 500 MHz spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.9.9 Study on NOESY spectroscopy

NOESY spectra give the proton-proton correlation in pure compound. NOESY spectra of the isolated compounds (**B** and **D**) were recorded by 400 MHz spectrometer at Graduate School of Bioagricultural Sciences, Nagoya University, Japan and that of the isolated compounds (**G** and **H**) were recorded by 500 MHz spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan

2.9.10 Study on ESI MS spectrometry

For the identification of isolated compounds, the mass spectra were recorded to examine the molecular weight and fragmentation patterns. The mass spectra of the isolated compound **A** was recorded by ESI MS spectrometer at the Department of Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany and that of the isolated compounds (**E, F, G, H**) were recorded by Shimadzu LCMS spectrometer at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

2.10 Screening of Some Bioactivities of the Whole Plant of *Cissus repens* Lam (Wa-round-chin) and the Aerial Parts and Rhizomes of *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin)

This section included five parts. The first part concerns with antimicrobial activity test, the second part with determination of antioxidant activity, the third part with the investigation of cytotoxicity test, the fourth part with antitumor activity and the final part with antiproliferative activity on the whole plant of *C. repens* (WRC) and aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR).

2.10.1 Preparation of crude extracts for bioactivity test

(i) Samples

Dried powdered samples of WRC, SPCA and SPCR.

(ii) Chemical

Pet-ether, ethyl acetate, 95 % ethanol and methanol.

(iii) Apparatus

Conical flasks, beakers, measuring cylinders, glass funnel, round-bottomed flask, rotary evaporator, filter paper and water bath.

(iv) Preparation of pet-ether, ethyl acetate, 95 % ethanol and methanol extracts

Pet-ether, ethyl acetate, 95 % ethanol and methanol extracts of three samples were prepared by percolation method. The dried powdered samples (300 g) was separately percolated with 1000 mL of respective solvents for one week and filtered. After filtration, the solvent was removed by mean of rotary evaporator. Percolation, filtration and evaporation were repeated three times. The combined extract of each extraction was stored in refrigeration for further biological tests.

2.10.2 Screening of antimicrobial activity

The antimicrobial activity of four crude extract such as pet-ether, ethyl acetate, 95 % ethanol and methanol from on the whole plant of *C. repens* and aerial parts and rhizomes of *B. rotunda* were determined against six strains of microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* by employing agar well diffusion method. The test were screened at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon, Myanmar.

2.10.2.1 Preparation of medium

(i) Chemicals

Trypticase soy broth from Difco U.S.A, trypticase soy agar from Becton, U.S.A, Mueller-Hinton agar (Hi-Media) and triple sugar iron agar from Becton, U.S.A, Pet-ether, ethyl acetate and ethanol.

(ii) Apparatus

Distilled water, sterile conical flasks, aluminum foil, a stirrer, an autoclave (Torr Seiko Co., Ltd, Tokyo, Japan), a constant temperature both (Yamato Scientific Co., Ltd, Japan), sterile petri-dish, spirit burner, polyethylene, plastic bag, a refrigerator and the incubator.

(iii) Procedure

(a) Preparation of agar medium

Trypticase soy agar 40 g was suspended in 100 mL of distilled water in a sterile conical flask and covered with aluminium foil. Then, suspension was mixed thoroughly and heated suspension was mixed thoroughly and heated to completely dissolve the powder on a hot plate stirrer. The trypticase soy agar solution was sterilized in an autoclave at 121 °C for 15 minutes. The temperature of agar solution was reduced to 50 °C on a constant temperature bath. Trypticase soy agar was then poured into the sterile petri dish near the flame spirit burner. The agar medium was allowed to solidify and sealed tightly in a polyethylene plastic bag. The medium was stored in a refrigerator until it was used. The solidified agar medium was dried in an incubator at 42 °C before use (Finegold, 1978).

(b) Preparation of agar slant medium

Triple sugar iron agars 65 g was suspended in 1000 mL of distilled water in a sterile conical flask, cover the aluminium foil an mixed thoroughly and heated to completely dissolve the powder on a hot plate stirrer. The triple sugar iron agar solution was transferred into the tubes (4 mL for each) and sterilized by autoclaving at 121 °C for 15 minutes. After sterilization, the test tube were placed in a slant position and allowed to solidify (Finegold, 1978).

(c) Preparation of broth medium

Trypticase soy broth (30 g) was suspended in 1000 mL of distilled water in a conical flask, covered with aluminum foil, mixed thoroughly and heated to completely dissolve on a hot plate stirrer. The broth solution was transferred into the test tube (3 mL in each tube) and sterilized by autoclaving for 15 minutes at 121 °C (Finegold, 1978).

2.10.2.2 Culture of bacteria

A few colonies of the organism to be tested were inoculated into the triple sugar iron agar and incubated at 37 °C for 24 hours in an incubator. A few colonies of the organism from the triple sugar iron agar were introduced into the trypticase soy broth and incubated for 3 hours at 37 °C to obtain the suspension of moderate cloudiness. This contained approximately 10^6 to 10^7 organisms per mL.

2.10.2.3 Antimicrobial activity screening by agar well diffusion method

(i) Samples

Pet-ether, ethyl acetae, 95 % ethanol and methanol extracts of WRC, SPCA and SPCR.

(ii) Chemical

Pet-ether, ethyl acetate, 95 % ethanol, methanol and 0.1 M sodium hydroxide solution.

(iii) Preparation of standard 0.1 M sodium hydroxide solution

Sodium hydroxide (4 g) was dissolved in distilled water and the volume made up to 1000 mL with distilled water.

(iv) Preparation of samples

The extract (1 g each) were introduced into sterile petri dishes and dissolved in 1 mL of their respective solvents: pet-ether, ethyl acetate, 95 % ethanol and methanol.

(v) Procedure

Meat extract (0.5 g), peptone (0.5 g) and sodium chloride (0.25 g) were mixed with distilled water and the solution made up to 100 mL with the distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was added. The nutrient agar medium was put into sterile conical flask and plugged with cotton wool and then autoclaved at 121 °C for 15 minutes. After cool down to 40 °C, one drop of suspended strain was inoculated to the nutrient agar medium with the help of a sterile disposable pipette near the burner. About 20 mL of medium was poured into the sterile petri dish and left 10-15 minutes in order to set the agar. After that the agar wells were made with a 7 mm sterile cork borer and the wells were filled with 0.1 mL of extract samples to be tested. And the plates were incubated at 27 °C for 24 hours. After incubation, the diameters of inhibition zones including 10 mm wells were measured.

The microorganisms of *Bacillus subtilis, Staphylococcus aureus* (ATCC-12277), *Pseudomones aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* (ACCT-25922) were tested.

2.10.2.4 Screening of minimum inhibitory concentration (MIC) by agar well diffusion method

(i) Samples

Ethyl acetate extracts of WRC, SPCA and SPCR and some isolated compounds **A, B, C** and **D** (friedelin, friedelinol, β-sitosterol and β-sitosterol-β-D-glucoside)

(ii) Chemicals

Pet-ether, Ethyl acetate, 95 % ethanol, methanol

(iii) Preparation of test sample solutions

Each sample (100 mg) and 1 mL of ethyl acetate were thoroughly mixed by shaker; the mixture solution was filtered and the stock solution was obtained. The

sample solution (10^5, 5×10^4, 2.5×10^4, 1.2×10^4 and 6.2×10^3 μg/ mL etc) was prepared from this stock solution by dilution with appropriate amount of ethyl acetate.

0.1 mg of compound was dissolved in 1 mL of their respective soluble solvents : PE, EtOAc and MeOH were thoroughly mixed by vortex mixer. The mixture solution was filtered and the filtrated was used as a stock solution. Desired concentrations (100, 50, 25, 12.5 and 6.3 μg/ mL etc) of sample solutions were prepared from this stock solution by dilution with appropriate amount of their respective soluble solvents.

(iv) Procedure

Minimum inhibitory concentration (MIC) values of ethyl acetate extract and all isolated compounds were determined by agar well diffusion method. The detailed procedure for this section was similar to that mentioned in Section 2.10.2.3 (v). 0.1 mL of test extract solution from 100 mg per 1 mL mixture was introduced into the agar well of first plate to obtain the concentration of 10^5 μg/mL. And then, 0.1 mL of test sample solution from 50 mg per 1 mL mixture was put into the agar well of second petri dish to obtain the concentration of 5×10^4 μg/mL. By this way, 0.1 mL of each test sample solution was introduced into the agar well of different plate to obtain the concentrations of 10^5 μg/mL, 5×10^4 μg/mL, 2.5×10^4 μg/mL, 1.2×10^4 μg/mL, 6.2×10^3 μg/mL, 3.1×10^3 μg/mL, 1.5×10^3 μg/mL, 7.8×10^2 μg/mL, 3.9×10^2 μg/mL, 1.9×10^2 μg/mL, 97.7 μg/mL, 48.8 μg/mL, 24.4 μg/mL, 12.2 μg/mL, 6.1 μg/mL, 3.1 μg/mL, 1.5 μg/mL, 0.7 μg/mL, 0.4 μg/mL, 0.2 μg/mL.

In this way, 0.1 mL of test compound solution from 0.1 mg per 1 mL mixture was introduced into the agar well of first plate to obtain the concentration of 100 μg/mL. Similarly, 0.1 mL of each test sample solution (compound) was put into the agar well of different plate to obtain the concentrations of 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.3 μg/mL, 3.1 μg/mL, 1.6 μg/mL, 0.8 μg/mL, 0.4 μg/mL, 0.2 μg/mL, 0.1 μg/mL, 0.04 μg/mL, 0.02 μg/mL, 0.01 μg/mL, 0.006 μg/mL, 0.003 μg/mL, 0.02 μg/mL, 0.01 μg/mL, 0.0004 μg/mL, 0.0002 μg/mL. All the plates were incubated to 27 °C for 24 hours. After incubation the diameters of inhibition zones

including, 10 mm wells were measured. The amount of samples that showed the least inhibition zone diameter was determined as the corresponding MIC value.

The microorganisms of *Staphylococcus aureus* and *Escherichia coli* were tested.

2.10.3 Investigation of antioxidant activity by DPPH free radical scavenging assay

DPPH (2, 2-diphenyl-1-picryl hydrazyl) free radical scavenging assay was chosen to assess the antioxidant activity of plant materials. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenols in food system (Leea *et al.,* 2002).

(i) Sample

Ethanol extract and watery extract of the whole plant of *C.repens* (WRC) and aerial parts and rhizomes of *B. rotunda* (SPCA and SPCR) and some isolated compounds **A**, **B**, and **D** (friedelin, friedelinol and β-sitosterol-β-D-glucoside)

(ii) Chemical requirement

Ethanol, 2, 2-diphenyl-1-picryl hydrazyl (DPPH), gallic acid, vitamin C and distilled water.

(iii) Apparatus requirement

UV-visible spectrophotometer (UV-7504, KWF, China), electric balance, syringe (5 mL) (3 mL).

(iv) Preparation of solutions

(a) Preparation of 0.002 % (w/v) DPPH solution

DPPH (2 mg) was thoroughly dissolved in ethanol (100 mL). This solution was freshly prepared in the brown coloured bottle and must be stored in the refrigerator for no longer than 24 hours.

(b) Preparation of test sample solutions

The stock solution (400 μg/mL) of the crude extract was prepared by dissolving 4 mg of respective crude extract in 10 mL of ethanol. This stock solution was two-fold diluted serially with ethanol to get the sample solutions with the concentration of 400, 200, 100, 50, 25 and 12.5 μg/mL.

(c) Preparation of blank solution

Blank solution was prepared by mixing the sample solution (1.5 mL) with ethanol (1.5 mL).

(v) Procedure

DPPH free radical scavenging activity was determined by UV-visible spectrophotometric method according to the procedure described by Marinova and Batchvarov (2011). The control solution was prepared by mixing 1.5 mL of 0.002 %. DPPH solution and 1.5 mL of ethanol in the brown bottle. The sample solution was also prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of test sample solution. These bottles were incubated at room temperature and were shaken on shaker for 30 minutes. After 30 minutes, the absorbance of these solutions was measured at 517 nm by using UV-visible spectrophotometer. The percent radical scavenging activity was calculated by the following equation.

$$\% \text{ RSA} = \frac{\left(A_{\text{DPPH}} - A_{\text{Sample}}\right) - A_{\text{blank}}}{A_{\text{DPPH}}} \times 100$$

Where, % RSA = % radical scavenging activity

A_{DPPH} = absorbance of DPPH in EtOH solution

A_{Sample} = absorbance of sample + DPPH solution

A_{Blank} = absorbance of sample + EtOH solution

The antioxidant powder (IC_{50}) is expressed as the test substances concentration (μg/mL) that result in a 50 % reduction of initial absorbance of DPPH solution and that allow to determine the concentration. IC_{50} (50 % inhibitory concentration) value were calculated by linear regressive excel program. The standard deviation was also calculated by the following equation.

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x}-x_1)+(\bar{x}-x_2)^2+...(\bar{x}-x_n)^n}{(n-1)}}$$

2.10.4 Investigation of cytotoxicity by brine shrimp bioassay

Cytotoxicity of the whole plant of *C. repens* (Wa-round-chin, WRC), aerial parts and rhizomes of *B. rotunda* (Seik-phoo-chin, SPCA and SPCR) was investigated by brine shrimp bioassay according to the procedure described by Dockery and Tomkins, (2000).

(i) Samples

Water extract and ethanol extract of WRC, SPCA and SPCR.

(ii) Chemicals requirement

Sodium chloride, potassium dichromate, caffeine and distilled water.

(iii) Apparatus requirement

Syringes (3 mL) (5 mL), beakers, chambers, pusteur pipette, lamp and water bottle (1.5 L).

(iv) Preparation of solutions

(a) Preparation of sample solution

The sample solution was prepared by dissolving 5 mg of respective crude extract in 5 mL of distilled water. The stock solution was tenfold diluted serially with distilled water to get the sample solutions with the concentration of 1000, 100, 10 and 1 μg/mL.

(b) Preparation of standard solution (potassium dichromate and caffeine)

The stock solutions of standard potassium dichromate and caffeine were prepared by dissolving 5 mg each of potassium dichromate and caffeine in 5 mL of distilled water. The stock solution was tenfold diluted serially with distilled water to get the standard solution with the concentrations of 1000, 100, 10 and 1 μg/mL.

(c) Preparation of artificial sea water

Sodium chloride (38 g) was dissolved in distilled water (1000 mL).

(v) Hatching of brine shrimp

The brine shrimp (*Artemia salina*) was used in this study for cytotoxicity bioassay (Ali *et al.,* 2013). They were purchased as brine shrimp cysts from pet shop, Baho Road, Hlaing Township, Yangon Region.

Brine shrimp cysts (0.5 g) were added to 1 L of artificial sea water. The bottle was placed near a lamp and supplied O_2 for 24 hours. After 27 hours incubation, hatching of brine shrimp cysts was occurred and the alive brine shrimp (napulli) were ready for cytotoxicity test.

(vi) Procedure

Test solution (1 mL) was mixed with 9 mL of artificial sea water and placed in the chamber of ice cup. Alive brine shrimp (10 napulli) was taken with pusteur pipette and placed into each chamber which was kept at room temperature for about 24 hours. After 24 hours incubation, the number of survival brine shrimp was counted and 50 % lethality dose (LD_{50}) was calculated (Dockery and Tomkins, 2000). The control solution was prepared as the above procedure by using distilled water instead of sample solution. The cytotoxicity of different doses of tested samples are described in Table 3.38.

2.10.5 Screening of antitumor activity

In this section, antitumor activity screening of 95 % ethanol, ethyl acetate, methanol extracts and some isolated compounds of the whole plant of *C. repens* (Wa-round-chin, WRC), aerial parts and rhizomes of *B. rotunda* (Seik-phoo-chin, SPCA and SPCR) was carried by Potato Crown Gall (PCG) test (or) Potato Disc Assay (PDA) method (Ferrigni *et al.,* 1982) at Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon.

(a) Preparation of YEP agar medium

Meat extract (0.5 g), yeast extract (0.1 g), peptone (0.1 g) and sucrose (0.5 g) were mixed with 100 mL of distilled water. The pH of this solution was adjusted at 7.2 and 1.5 g of agar powder was added. The YEP agar medium was put into sterile conical flask and plugged with cotton wool and then autoclaved at 121 °C for 15 minutes.

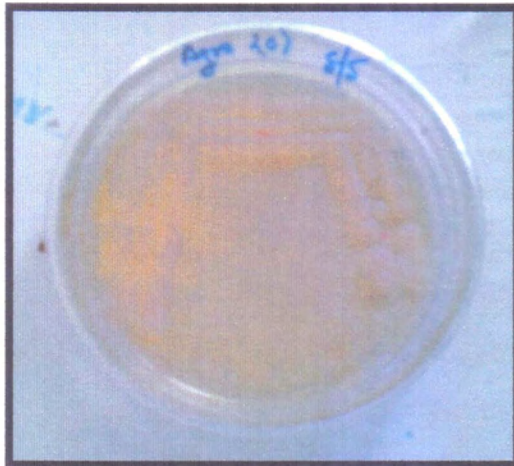
(b) Preparation of YEP agar slant medium

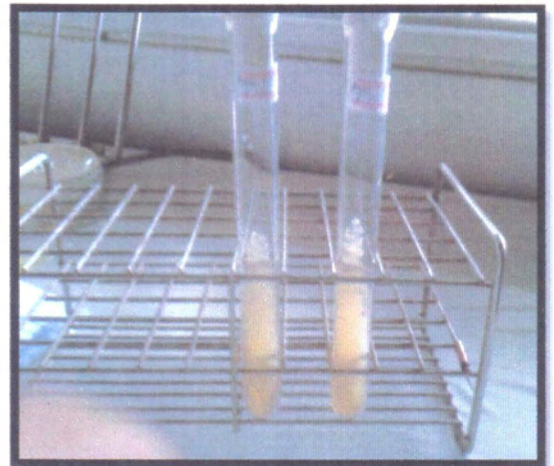
Meat extract (0.5 g), yeast extract (0.1 g), peptone (0.1 g) and sucrose (0.5 g) were mixed with 100 mL of distilled water. The pH of this solution was adjusted at 7.2 and 1.5 g agar powder were mixed thoroughly and heated to completely dissolve the agar powder on a hot plate stirrer. The YEP agar medium was transferred into sterile test tube (about 5 mL for each) and plugged with cotton wool and then autoclaved at 121 °C for 15 minutes. After autoclaving, the test tubes were stopped at about 15° from the horizontal position and allowed to solidify.

2.10.5.1 Isolation of tumor producing bacteria of serial dilution method

The bacteria from the infected leaf of *Sandorium koetjape* Merr. (Thitto) were extracted with Tween 80 or polyoxyethylene (20) sorbitan monooleatal. 1 mL of the extract solution was made up to 100 mL with distilled water to obtain 10^2 dilutions. Then 1 mL of this 10^2 dilution solution was made up to 100 mL with distilled water and 10^3 dilution solutions were obtained. By this way, 10^4, 10^5 and 10^6 dilution was made as serial dilution. And then 1 mL of each dilution solution was transferred to the petri dish with the help of a sterile disposable pipette near the burner. About 20 mL of YEP agar medium (after autoclaving cool down to 40 °C) was poured into the petri dish containing 1 mL of each dilution solution and incubated at 27 °C. After 24 hours, the bacterium were grown on YEP agar medium as colonies. Among the colonies, creamy coloured colonies were transferred into YEP agar slant with the help of inoculation loop near the flame of a spirit burner and incubated again at 27 °C for 24 hours to get pure culture. The photographs of the isolation of pure culture are described in Figure 2.2. The room must be sterilized and it is important not to talk during the cultivation process.

(a)





(b) **(c)**

Figure 2.2 Photographs showing the steps in the isolation of tumor producing bacteria

(a) Tumor on the leaf of *Sandorium koetjap* Merr. (Thitto)

(b) Bacteria culture on YEP agar medium

(c) The bacteria sub-culture transferred on the YEP slants

2.10.5.2 Identification of tumor producing bacteria

The isolated bacteria was identified by its Morphology, Gram staining, spore staining, some biochemical tests and compared with the reference.

(1) Morphology

The morphology of isolated bacteria was examined under Microscope Nikon, Japan (Cruickshank, 1960).

(2) Gram staining method

To perform the Gram staining method, the following reagents were prepared.

(i) Ammonium oxalate crystal violet (Hucker's solution)

Solution A was prepared by dissolving 2 g of crystal violet (90 % dye content) in 20 mL of 95 % ethanol.

Solution B was prepared by dissolving 0.8 g of ammonium oxalate in 80 mL of distilled water. And then solution A and B were mixed.

(ii) Gram's modification of Lugol's solution

1 g of iodine and 2 g of potassium iodide were dissolved in distilled water and the volume made up to 300 mL with distilled water.

(iii) Counter stain (Saffranin) solution

10 mL of Saffranin (25 % solution in 95 % ethanol) was added to 100 mL of distilled water.

(iv) Procedure

One drop of distilled water was mount on the glass slide. A small amount of bacteria was transferred to the slide by the inoculation loop and fixed and the strain by passing onto the flame about 3-4 times. After the fixation of strain, ammonium oxalate crystal violet solution was applied on the strain and rest for 1 minute. And it was washed with running water for not more than 10 seconds. Then iodine solution was stained for 1 minute and washed with running water and washed with 95 %

ethanol until violet colour was disappeared. And then, it was soaked with blotting paper. After that, the slide was counter stained with Saffranin solution for 10 seconds and washed with running water and dried again with blotting paper. Finally, the strain on the slide was observed under a microscope. Red colour showed for the species of Gram negative and blue colour for Gram positive species (Collin, 1964).

(3) Some biochemical tests

(a) Motility test

0.5 g of sodium chloride, 0.5 g of peptone and 0.2 g of yeast extract were mixed with 100 mL of distilled water. Then the medium was adjusted to pH 7.2 with 0.1 M sodium hydroxide solution and 0.4 g of agar powder was mixed thoroughly and heated the contents for complete dissolution. The medium was transferred into sterile test tubes (about 5 mL for each) and plugged with cotton wool and then autoclaved at 121 °C for 15 minutes. After autoclaving, the test tubes were allowed to semi-solid agar medium in the vertical position.

The isolated bacterium was inoculated into the semi-solid agar medium straight down the centre of the test tube to about half the depth of the medium and then incubated for 24 hours at 27 °C. After incubation, the medium in the test tubes were checked to observe if the bacteria grow along the margin or not (Cowan, 1979).

(b) Gelatin liquefaction test

(i) Preparation of mercuric chloride solution

Mercuric chloride (12.0 g) was dissolved in concentrated hydrochloric acid (16 mL) and the solution made up to 100 mL with distilled water in a volumetric flask (Cowan, 1974).

(ii) Preparation of nutrient gelatin agar medium

Meat extract (0.5 g), peptone (0.5 g) and NaCl (0.25 g) were mixed with distilled water and the solution made up to 100 mL with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and agar powder (1.5 g) was added. After that, gelatin (0.4 g) was dissolved in distilled water

(5 mL) and added to the nutrient agar medium. The nutrient gelatin agar medium was put into sterile conical flask and plugged with cotton wool and then autoclaved at 121 °C for 15 minutes. After autoclaving, the medium was poured into the sterile petri dish (about 25 mL for each) and allowed to cool the agar plate.

(iii) Procedure

A loopful of the isolated bacteria was inoculated into gelatin agar medium and incubated at 27 °C for 3 days. After this, the surface was flooded with 5 mL of mercuric chloride solution; clear zone indicated areas of gelatin hydrolysis (Harrigen, 1966). The formation of clear zones in this medium indicated a positive test.

(c) Nitrate reduction tests

(i) Preparation of 5 M acetic acid solution

Glacial acetic acid (28.6 mL) was made up to 100 mL with distilled water in a volumetric flask.

(ii) Preparation of 0.8 % sulphanilic acid solution

Sulphanilic acid (0.8 g) was dissolved in 5 M acetic acid (100 mL) to obtain solution A (Cowan, 1979).

(iii) Preparation of 0.5 % naphthylamine solution

α-Naphthylamine (0.5 g) was dissolved in 5 M acetic acid (100 mL) to obtain solution B (Cowan, 1979).

(iv) Procedure

A mixture of potassium nitrate (0.02 g) and peptone (0.5 g) was placed in a 250 mL conical flask. To this mixture was added sterilized distilled water (100 mL) and the resulting solution sterilized at 121 °C for 15 minutes (Harrigen & McCance, 1966). After sterilization, 5 mL of this solution were distributed into sterile test tubes in a vertical position near the flame of a spirit burner. A loopful of the isolated bacteria was inoculated into the nitrate agar medium and incubated at 27 °C for

96 hours. Immediately before use, equal volumes of solution A and solution B were mixed to give the test reagents. After incubation, one drop each of the reagent was added to the test culture. A red colour developing within a few minute was indicative of a positive nitrate reduction test (Cowan, 1979). If the original purple colour is retained the test is negative.

(d) Indole test

(i) Preparation of Kovac's reagent

p-Dimenthylamino benzaldehyde (5 g) was dissolved in isoamyl alcohol (75 mL). Concentrated hydrochloric acid (25 mL) was added slowly and the reagent stored at 27 °C (Cowan, 1979).

(ii) Procedure

Peptone (2.0 g) and sodium chloride (0.5 g) were placed in a 250 mL conical flask. Sterilized distilled water (100 mL) was added to obtain peptone-water medium (Cowan, 1974). The pH of the resulting solution was adjusted to 7.2 with 0.1 M sodium hydroxide solution (Cruickshank, 1995). Then the medium was sterilized by autoclaved at 121 °C for 15 minutes. After this, 5 mL each of the medium was cooled sand dispensed near the flame of a spirit burner into sterile test tubes.

A loopful of the isolated bacteria was inoculated into peptone-water broth and medium incubated at 27 °C for 48 hours. After this period for the occurrence of indole reaction, 0.5 mL of Kovac's reagent was added to the test culture and was shaken gently. The yellow colour developed within a few seconds in the alcohol layer so that the indole test was taken as a negative one. A positive test is marked by the appearance of red colour.

(e) Urease test

(i) Preparation of approximately 0.1 M sodium hydroxide solution

Sodium hydroxide (0.6 g) was weighed and dissolved in sufficient quantity of distilled water and the volume was made up to 100 mL.

(ii) Preparation of approximately 0.1 M hydrochloric acid solution

Concentrated hydrochloric acid (0.86 mL) was added to sterilized distilled water and the volume made up to 100 mL in a volumetric flask (Cruickshank, 1995).

(iii) Preparation of approximately 10 % sterilized glucose solution

Glucose (1.0 g) was dissolved in sterilized distilled water (10 mL) in a sterile test tube and sterilized in an autoclave at 121 °C for 15 minutes each for three successive days (Cruickshank, 1960).

(iv) Preparation approximately 20 % sterilized urea solution

Urea (2.0 g) was dissolved in sterilized distilled water (10 mL) in a test tube and the solution was sterilized by autoclaving at 121 °C for 15 minutes (Vogel, 1968).

(v) Preparation approximately 0.2 % phenol red solution

Phenol red (0.2 g) was dissolved in a mixture of 0.1 M sodium hydroxide solution (10 mL) and sterilized distilled water (20 mL). It was heated on a hot plate, 0.1 M HCl solution (10 mL) was added, and the volume was made up to 100 mL (Vogel, 1968).

(vi) Procedure

A mixture of peptone (1 g), sodium chloride (0.5 g), dipotassium hydrogen phosphate (0.2 g), phenol red solution (0.6 mL), agar powder (0.2 g) and telephone agar powder (0.67 g) was placed in a 250 mL conical flask. To this mixture was added sterilized distilled water (100 mL) and resulting mixture was heated to dissolve the contents. Then, the pH of the resulting solution was adjusted to 7.2 with 0.1 M sodium hydroxide solution. It was sterilized in the autoclave at 121 °C for 15 minutes. After autoclaving, the medium was cooled to 27 °C, and 10 % sterilized glucose solution (1 mL) and 20 % urea solution (10 mL) were added. Then, 5 mL of each media was dispensed into 20 sterile test tubes near the flame of a spirit burner and finally the test tubes were inclined at about 15 ° from the horizontal position and left to solidity.

A loopful of inoculum was inoculated into the surface of area slant agar medium evenly spread and incubated at 27 °C for 4 days. After this, the appearance of a red colour on the urea slant agar medium indicated a positive urease test (Cruickshank, 1960).

(f) Voges-proskauer test

(i) Preparation of approximately 10 % sterilized glucose solution

Glucose (1.0 g) was dissolved in sterilized distilled water (10 mL) in a sterile test tube and sterilized in an autoclave at 121 °C for 15 minutes each for three successive days (Cruickshank, 1960).

(ii) Preparation of approximately 40 % potassium hydroxide solution

Potassium hydroxide (pellets) (40 g) was dissolved in distilled water and the volume made up to 100 mL in a volumetric flask (Cruickshank, 1960).

(iii) Preparation of approximately 5 % α-naphthol solution

α-Naphthol (5.0 g) was dissolved in absolute ethanol (100 mL) (Cruickshank, 1960).

(iv) Procedure

Peptone (0.5 g) and dipotassium hydrogen phosphate (0.5 g) were placed in a 100 mL conical flask. Sterilized distilled water (100 mL) was added and the pH of the solution adjusted to 7.2 with 0.1 M sodium hydroxide solution and then sterilized in an autoclave at 121 °C for 15 minutes (Cruickshank, 1960). This solution was cooled and 5 mL of sterilized glucose solution added quickly to it near the flame of a spirit burner to get glucose phosphate peptone-water medium. The medium was distribute to each of the sterile test tubes in 5 mL amounts.

A loopful of the isolated bacteria was inoculated into the glucose phosphate agar medium and incubated at 24 °C for 48 hours. After incubation, 40 % potassium hydroxide solution (1 mL) and 5 % α-naphthol (3 mL) in absolute ethanol solution were added to the test culture and shaken quickly. The development of a pink colour

within 2-5 minutes was indicative of a positive VP test (Cruickshank, 1960). A yellow colour indicated a negative test.

2.10.5.3 Antitumor activity screening by potato crown gall test or potato disc assay method

(i) Microorganism

Isolated *Agrobacterium tumefacien* has been maintained as solid slants under refrigeration. For inoculation into the potato discs, 48 hours Broth cultures containing $5 \times 10^7 - 5 \times 10^9$ cell/ mL were used.

(ii) Test samples

Ethyl acetate extract, 95 % ethanol extract and methanol extract of WRC, SPCA and SPCR and some isolated compounds **A**, **B**, **C** and **D** (friedelin, friedelinol, β-sitosterol and β-sitosterol-β-D-glucoside.

(iii) Chemicals

Ethyl acetate, 95 % ethanol, methanol, sodium hypochlorite (Clorox), dimethyl sulphoxide (DMSO), agar powder, I_2 and KI.

(iv) Preparation of sample for testing

Ethyl acetate, 95 % ethanol and methanol extracts of the whole plant of *C. repens* and aerial parts and rhizomes of *B. rotunda* were obtained from Section 2.10.1 and isolated compounds from Sections 2.6.1 and 2.6.2.

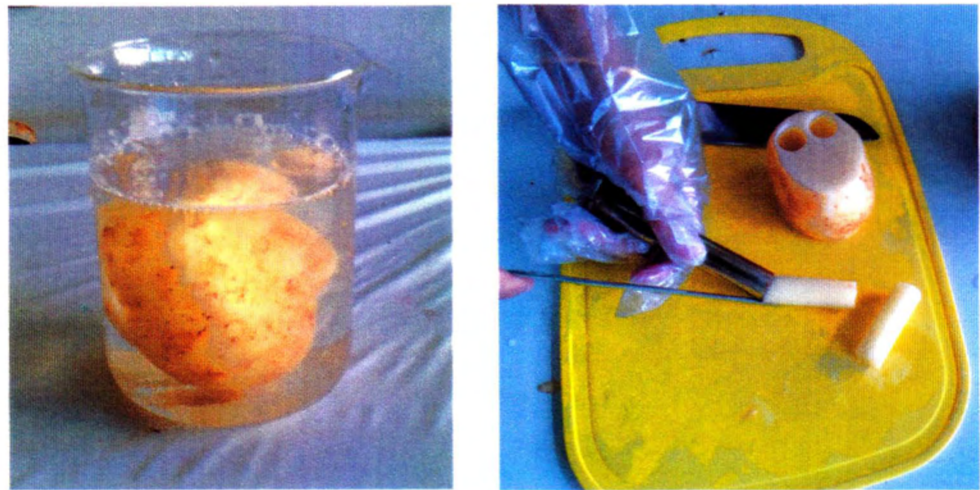
(v) Preparation of bacterial culture

Agrobacterium tumefaciens strain was cultured on Luria-Bertani (LB) agar medium which was prepared by dissolving a mixture of yeast extract (1 g), peptone (1 g), NaCl (0.5 g) and agar (2 g) in distilled water (100 mL) according to Devi *et al.*, 2001. Single colony was transferred into LB broth and incubated at 30 °C for 48 hours in the shake. Six to seven loops of bacterial suspensions (1.0×10^9 cfu) were transferred into sterilized phosphate buffer saline (PBS) and this was used during antitumor assay inoculums.

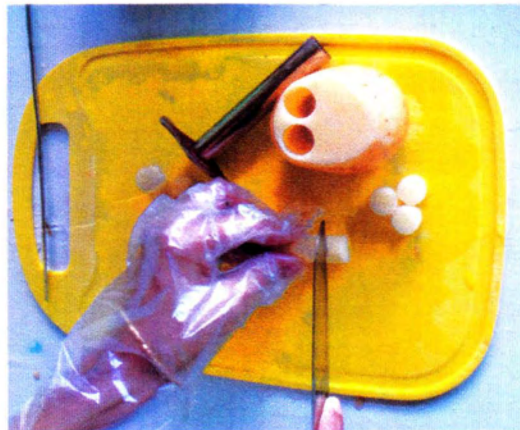
(vi) Procedure

Fresh, disease-free potatoes were purchased from a local market. Tubers of moderate size were surface sterilized by immersion in 0.1 % sodium hypochlorite for 20 minutes. Ends were removed and the potatoes were soaked an additional 10 minutes. A core of the tissue was extracted from each tuber with a surface-sterilized 1.0 cm cork borer. Pieces of 2 cm were removed from each end and discarded. The remainder of the cylinder was cut 0.5 cm thick disc with a surface sterilized sealpel. The discs were then transferred to agar plates (1.5 g of agar dissolved in 100 mL distilled water, autoclaved for 20 minutes at 121 °C, 20 mL poured into each Petri dish. Each plate contained four potato discs and 4 plates, were used for each sample dilution.

Sample (0.2, 0.4 g) were respectively dissolved in DMSO (2 mL) and filtered through millipole filters (0.22 µm) into sterile tube. This solution (0.5 mL) was added to sterilized distilled water (1.5 mL), and broth culture of *A. tumetaciens* in PBS (2 mL) was added. Controls were made in this way; DMSO (0.5 mL) and sterilized distilled water (1.5 mL) were added to the tube containing 2 mL of broth culture of *A. tumefaciens.* Using a sterile disposable pipette, 1 drop (0.05 mL) from these tubes was used to inoculate each potato disc, spreading it over the disc surface. The plate were sealed with tape to minimize moisture loss and incubated at room temperature for three weeks at 27.30 °C. Tumors were observed on potato discs after 21 days under stereo-microscope followed by staining with Lugol's iodine (10 % KI and 5 % I_2) after 30 minutes and compared with control. The antitumor activity was examined by observation of tumor produced or not. Figure 2.3 illustrates the procedure of preparation steps for antitumor activity screening by using Potato Crown Gall test.



(a) (b)



(c)

Figure 2.3 **Photographs for screening of antitumor activity by Potato Crown Gall (PCG) test**

(a) **Surface sterilization of potato in Clorox**

(b) **Extraction of potato core with cork borer**

(c) **Cutting 0.5 cm potato disc**

2.10.5.4 Antitumor activity screening by agar well diffusion method

Antitumor activity of various crude extract such as ethyl acetate, 95 % ethanol, methanol extracts of WRC, SPCA and SPCR and the isolated compounds **A, B, C** and **D** (friedelin, friedelinol, β-sitosterol and β-sitosterol-β-D-glucoside) were studied by agar well diffusion method at the Fermentation Laboratory, Pharmaceutical Research Department, Ministry of Industry, Yangon.

(ii) Preparation of nutrient agar medium

3 g of agar powder, 1 g of sodium chloride, 1 g of peptone, 0.4 g of yeast extract and 200 mL of distilled water were mixed in a 500 mL sterile conical flask and the contents were heated on a hot plate for 30 minutes. The mouth of the flask was covered with a piece of aluminum foil. This medium was adjusted to pH 7.4 with 0.1 M sodium hydroxide solution and was sterilized in an autoclave at 121 °C for 30 minutes.

After autoclaving, half of this medium (100 mL) was dispensed, 5 mL each, into sterile test tubes are inclined at about 15 ° from the horizontal position for the subculture of bacteria and the remaining half of this medium (100 mL) was used for the culture of bacteria.

(ii) Preparation of Inoculums

The microorganism used in the investigation of antitumor activity is *A. tumefaciens* from the leaf of *Sandorium koetjape* Merr. (Thitto)

A few colonies of the organism to be tested were incubated at 37 °C for 24 hours in an incubator. These organisms were introduced into the trypticase soy broth and incubated for 3 hours at 37 °C to obtain the bacterial suspension of moderated cloudiness.

(iii) Screening by agar well diffusion method

The agar well plate diffusion method was used to test the antitumor action of the extracts on 4 hours broth culture of the organisms used.

The extracts of ethyl acetate, 95 % ethanol, methanol and the isolated compounds (**A, B, C, D**) were dissolved in their respective solvent. 1 mL each of the bacterial suspension of 24 hours of nutrient agar was streaked evenly onto the surface of trypticase soy agar plates with sterile cotton swab. Immediately after hardening of the agar well were made with a 10 mm sterile cork borer from each seeded agar. After removing the agar, the wells were filled with the drug extract to be tested. The plates were incubated at 37 °C for 24 hours. The diameters of the inhibition zone were measured and recorded in mm.

2.10.6 Antiproliferative activity

Antiproliferative activity of WRC, SPCA, SPCR and some isolated compounds were studied *in vitro* using cancer cell lines at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

(i) Cancer Cell Lines

The cell lines used were LK-2, A 549 (human lung cancer), ECC 4 (human stomach cancer), COLO 205 (human colon cancer), HuH7 (human liver cancer), Hela (human cervix cancer), K 562 (human leukemia cancer), DU 145 (human prostate cancer), MCF 7 (human breast cancer), and WI-38 (normal human fibroblast). Roswell Park Memorial Institute 1640 medium (RPMI 1640, Wako) was used for LK-2, COLO 205, ECC 4, DU 145, HuH7 and K 562. α-Minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) was used for A 549, Hela, MCF 7 and WI-38. All media were supplemented with 10 % fetal bovine serum (FBS, sigma) and 1 % antiobiotic antimycotic solution (Sigma). For MCF 7 cell, 1 % 0.1 M non-essential amino acid (NEAA, Gibco) and 1 % 1 mM sodium pyruvate (Gibco) were also supplemented.

(ii) Sample

Methanol extracts of WRC, SPCA, SPCR, chloroform extract of SPCR and some isolated compounds **E, F, G** and **H** (pinostrobin, 4',7-dimethykaempferol, galanal A and galanal B).

(iii) Procedure

The *in vitro* antiproliferative activity of the crude extracts and some isolated compounds was determined by the procedure described (Win *et al.,* 2015). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 hours. After the cells were washed with PBS (Nissui Pharmaceuticals), serial dilutions of the tested samples were added. After 72 hours incubation, the cells were washed with PBS and 100 µL of medium containing 10 % WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 hours incubation, the absorbance at 450 nm was measured. The concentrations of the serial dilutions of the tested samples were 100, 10, 1 µg/ mL for crude extract, 100, 10, 1, µM for isolated compounds and 10, 1, 0.1 mM for positive control. Cell viability was calculated from the mean values of the data from three wells using the equation below and antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value. 5-fluorouracil was used as positive control.

$$(\%)\ \text{Cell viability} = 100 \times \frac{\{\text{Abs}_{(\text{test samples})} - \text{Abs}_{(\text{blank})}\}}{\{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}\}}$$

CHAPTER III

3. RESULTS AND DISCUSSION

For this research, the whole plant of *C. repens* (WRC) and the aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR) collected from Kyauk Tan Village, Bago Region in September, 2012 and identified at Botany Department, University of Yangon, Myanmar were selected. 3.49 kg of the cleaned and dry powder of whole plant of *C. repens* and 2.54 kg of the aerial parts and 5.76 kg of rhizomes of *B. rotunda* were prepared and stored in separate air-tight containers to prevent contaminations and kept for isolation of organic compounds and bioactivity tests. In this section, the results obtained from the phytochemical analyses, isolation and structural elucidation of organic compounds from the selected samples and screening of biological activities such as antimicrobial activity, antioxidant activity, cytotoxicity test, antitumor activity and antiproliferative activity will be discussed.

3.1 Phytochemicals Present in the Whole Plant of *C. repens* (WRC) and the Aerial Parts and Rhizomes of *B. rotunda* (SPCA, SPCR)

Phytochemicals mean chemicals obtained from plants. The phytochemicals present in the plant samples were tested by test tube method according to the procedure as mentioned in Section 2.2.

The phytochemical tests revealed that alkaloids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, saponins, steroids, tannins and terpenoids were found to be present but α-amino acids, cyanogenic glycosides and reducing sugars were absent in all selected samples. The results are shown in Table 3.1.

Table 3.1 Results of Preliminary Phytochemical Tests on the Selected Samples

No	Test	Extract	Test Reagent	Observation if present	Remark		
					I	II	III
1.	Alkaloids	1% HCl	Mayer's reagent	White ppt	+	+	+
			Dragendorff's reagent	Orange ppt	+	+	+
			Wagner's reagent	Reddish brown	+	+	+
2.	α-amino acids	H_2O	Ninhydrin	No pink spot	-	-	-
3.	Carbohydrates	H_2O	10 % α-Naphthol and Conc. H_2SO_4	Red ring	+	+	+
4.	Flavonoids	EtOH	Conc. HCl and Mg turnings	Pink colour	+	+	+
5.	Cyanogenic glycosides	H_2O	Sodium picrate	No brick red colur	-	-	-
6.	Glycosides	EtOH	10 % lead acetate	White ppt	+	+	+
7.	Organic acids	H_2O	Bromocresol green indicator	Yellow colour	+	+	+
8.	Phenolic compounds	H_2O	1 % Potassium ferricyanide and 1 % ferric chloride	Green colour	+	+	+
9.	Reducing sugars	H_2O	Fehling's A and Fehling's B	No brick red	-	-	-
10.	Saponins	H_2O	Distilled water	Frothing	+	+	+
11.	Steroids	PE	Acetic anhydride and Conc H_2SO_4	Greenish blue	+	+	+
12.	Tannins	EtOH	1 % Gelatin	Green colour	+	+	+
13.	Terpenoids	$CHCl_3$	Acetic anhydride and Conc. H_2SO_4	Pink or red colour	+	+	+

(+) = Present, (-) = Absent

I = the whole plant of *C.repens* (WRC)

II = aerial parts of *B. rotunda* (SPCA)

III = rhizomes of *B. rotunda* (SPCR)

3.2 Nutritional Values of the Whole Plant of *C. repens* (WRC) and Aerial Parts and Rhizomes of *B. rotunda* (SPCA, SPCR)

The nutritional values such as moisture, ash, protein, fiber, fat and carbohydrate of WRC, SPCA and SPCR were determined according to the procedure as mentioned in Section 2.3.

The moisture content is a major factor since it determines the actual weight of the powdered samples, impacts shelf life and sets the stage for the product stability or degradation either via hydrolytic and oxidative avenues or the potentiation of mold and microbiological growth. The moisture content of WRC, SPCA and SPCR was found to be 3.11%, 3.28 % and 4.49 %, which suggested that the dried samples of WRC, SPCA and SPCR could be kept for further investigations and discourage spoilage.

The total ash is particularly important in the evaluation of purity of samples. It was obtained by burning off the organic matter and measuring the residue of ash. The remaining ashes indicated the total mineral content for each samples. The ash content of WRC, SPCA and SPCR was found to be 6.80%, 14.75 % and 7.00 %.

Protein content of WRC, SPCA and SPCR were measured by Micro Kjeldah's method and found to be 12.18 %, 9.23 % and 6.82 %. The main work of protein is to build the body and to repair the warm out tissues, but any protein eaten in excess of the amount required by the body can be used to repair the warm out the amount required by the body can be used to provide energy.

The fat content of WRC, SPCA and SPCR (7.90%, 5.81 % and 3.62 %) were obtained using Soxhlet extraction of a known weight of sample with petroleum ether. Among the selected samples, fat content of WRC was relatively higher than SPCA and SPCR. It can be assumed that fat was present in low amount in rhizomes. Dietary fat function is the increase of food by absorbing and retaining flavours. A diet providing 1-2% of its caloric of energy as fat is said to be sufficient to human beings as excess fat consumption is implicated in certain cardiovascular disorders such as atherosclerosis, cancer and antiaging.

Fiber content of WRC, SPCA and SPCR were determined by acid-base digestion and found to be 13.73 %, 28.54 % and 12.24 %. Dietary fiber is a physiological and nutritional concept relating to those carbohydrate components of

foods that are digested in the small intestine.

Carbohydrate content of WRC, SPCA and SPCR were 56.28%, 38.39 %, 65.83 %. Total carbohydrate content was determined by subtracting the total crude protein, fiber, ash and moisture from the total weight of sample. The energy value estimation was done by summing the multiplied values for crude protein, fat and carbohydrate by the respective at factors (4, 9, 4).

In this study, the moisture and carbohydrate contents of SPCR are higher than that of WRC and SPCA. The protein and fat contents of WRC are higher than that of SPCA and SPCR. The ash and fiber contents of SPCA are higher than that of SPCR and WRC. The results are shown in Table 3.2.

Table 3.2 Nutritional Values of the Selected Samples

No.	Parameters	Contents%		
		WRC	**SPCA**	**SPCR**
1	Moisture	3.11	3.28	4.49
2	Ash	6.8	14.75	7
3	Protein	12.18	9.23	6.82
4	Fiber	13.73	28.54	12.24
5	Fat	7.9	5.81	3.62
6	Carbohydrate	56.28	38.39	65.83
7	Energy Value (kcal/100 g)	344.94	242.77	323.18

WRC = the whole plant of *C.repens*

SPCA = aerial parts of *B. rotunda*

SPCR = rhizomes of *B. rotunda*

3.3 Soluble Matter Content of the Whole Plant of *C. repens* (WRC) and Aerial Parts and Rhizomes *B. rotunda* (SPCA and SPCR)

The extractable matter contents in WRC, SPCA and SPCR in petroleum ether, ethyl acetate, acetone, ethanol and water were determined by WHO standard method (WHO, 1998).

The results are shown in Table 3.3. According to the results, water soluble matters were found to be the highest in all samples. Therefore, the resulted data suggested that the soluble matter contents increase with increasing polarity of the solvents. It also indicated that all selected samples contain polar compounds as major constituents.

Table 3.3 Results of Extractable Matter of the Selected Samples

No.	Solvents used	Extractable matter (mg/g)		
		WRC	SPCA	SPCR
1.	Petroleum ether	65.1	39.4	1.4
2.	Ethyl acetate	88.0	17.9	47.8
3.	Acetone	77.0	43.8	21.7
4.	Ethanol	86.2	9.0	12.5
5.	Water	157.9	171.0	127.5

WRC = the whole plant of *C.repens*

SPCA = aerial parts of *B. rotunda*

SPCR = rhizomes of *B. rotunda*

3.4 Separation, Isolation and Purification of Some Organic Compounds from the Whole Plant of *C. repens* (WRC) and Rhizomes of *B. rotunda* (SPCR)

To separate and isolate some organic constituents presents in two selected medicinal plant samples, PE, EtOAc, 95% EtOH and H_2O extracts were prepared according to the general procedure mentioned in Figure 2.1 (Section 2.5). In these experiments, all the extracts were kept for separation and isolation of chemical constituents and for some bioactivity tests.

The samples (500 g) were firstly percolated with 95% ethanol at room temperature. The 95% ethanol extracts were successively partitioned with pet-ether (60-80°C) between EtOAc and water. The extracted values of both samples are listed in Table 3.4.

It was observed that the amount of non-polar constituents present in the WRC is higher than that present in SPCR. By contrast, the polar components occurred in the later with the larger amounts than the former.

Pet-ether crude extract from WRC and ethyl acetate crude extract from SPCR were subjected to silica gel column chromatographic separation for isolation of some organic constituents.

5 g of pet-ether crude extract of WRC was separated by column eluents, pet-ether and ethyl acetate (100:1, 60:1, 40:1, 30: 1, 20:1, 15:1 v/v) (Section 2.6.1). The general procedure is described in Figure 3.1. In this experiment, compound **A** and compound **B** were isolated and purified by washing with ethyl acetate followed by crystallization from methanol. After purification and crystallization, 70 mg (0.014 %) of compound **A** (white needles), 38 mg (0.008 %) of compound **B** (colourless crystal) were obtained based on pet-ether extract.

Table 3.4 Contents of Various Crude Extracts from Two Selected Medicinal Plants

Sample	Contents of various crude extracts (%)			
	95% EtOH	PE	EtOAc	H_2O
WRC	31.75	16.25	11.95	3.13
SPCR	44	39.25	21.5	0.35

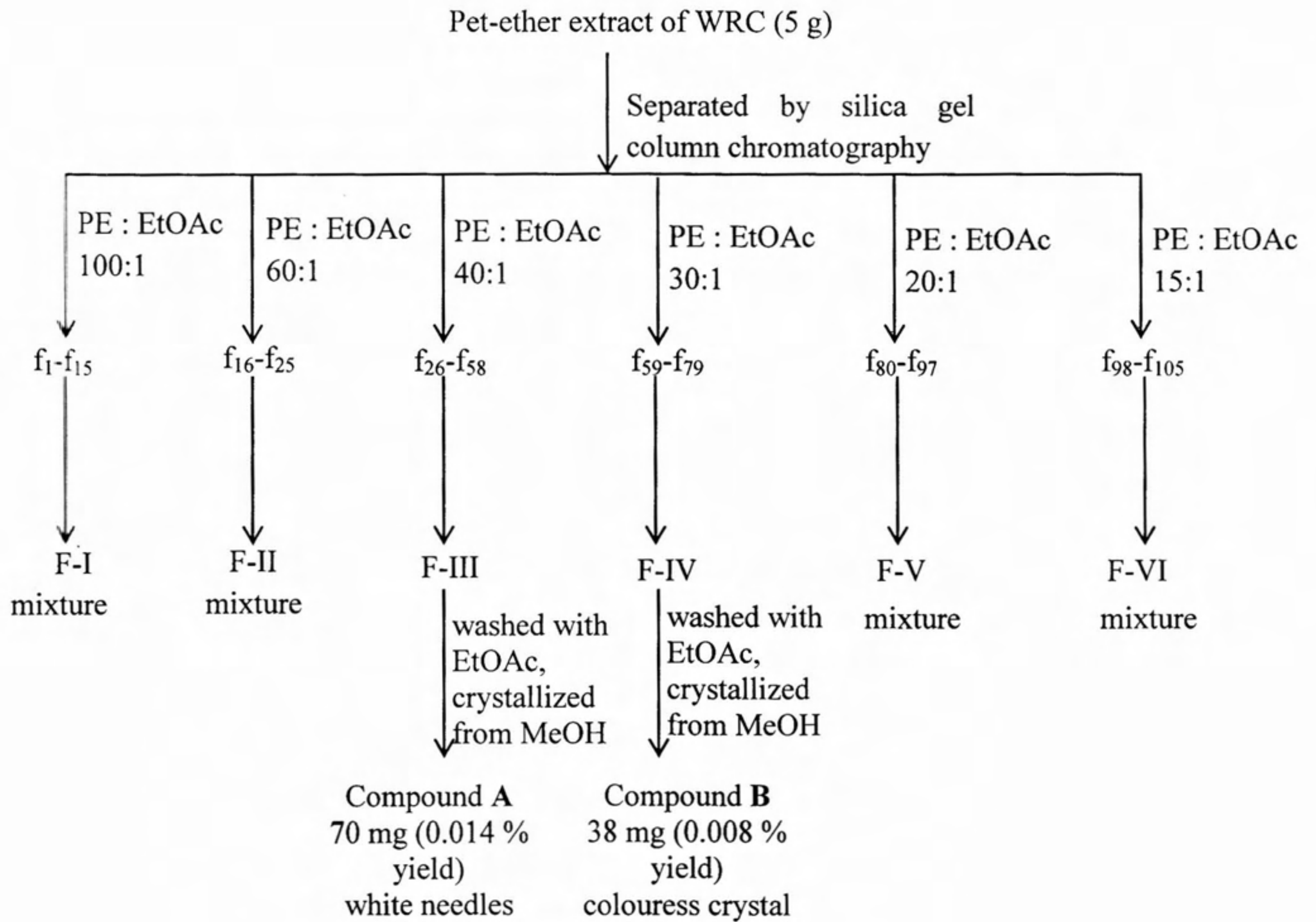


Figure 3.1 Flow diagram for the separation of pet-ether crude extract of WRC by column chromatography

When 6 g of ethyl acetate crude extract of SPCR were separated by column chromatographic method using silica gel GF_{254} and eluting with PE : EtOAc (15:1, 9:1, 5:1, 3:1, 1:3, 1:5, 1:9, 1:15 v/v) as solvent systems (Section 2.6.2), compounds **C** and **D** were isolated.

The separation and isolation procedure is generally illustrated in Figure 3.2. From this separation, eight main fractions (F-I to F-VIII) were obtained. 63 mg (0.26 %) yield of compound **C** was isolated from fraction F-III as a colourless needles, after purification by washing with ethanol followed by crystallization from pet-ether. In addition, 49 mg (0.9 %) of compound **D** was obtained from fraction F-VII as a white powder. It was purified by washing with acetone crystallized from methanol.

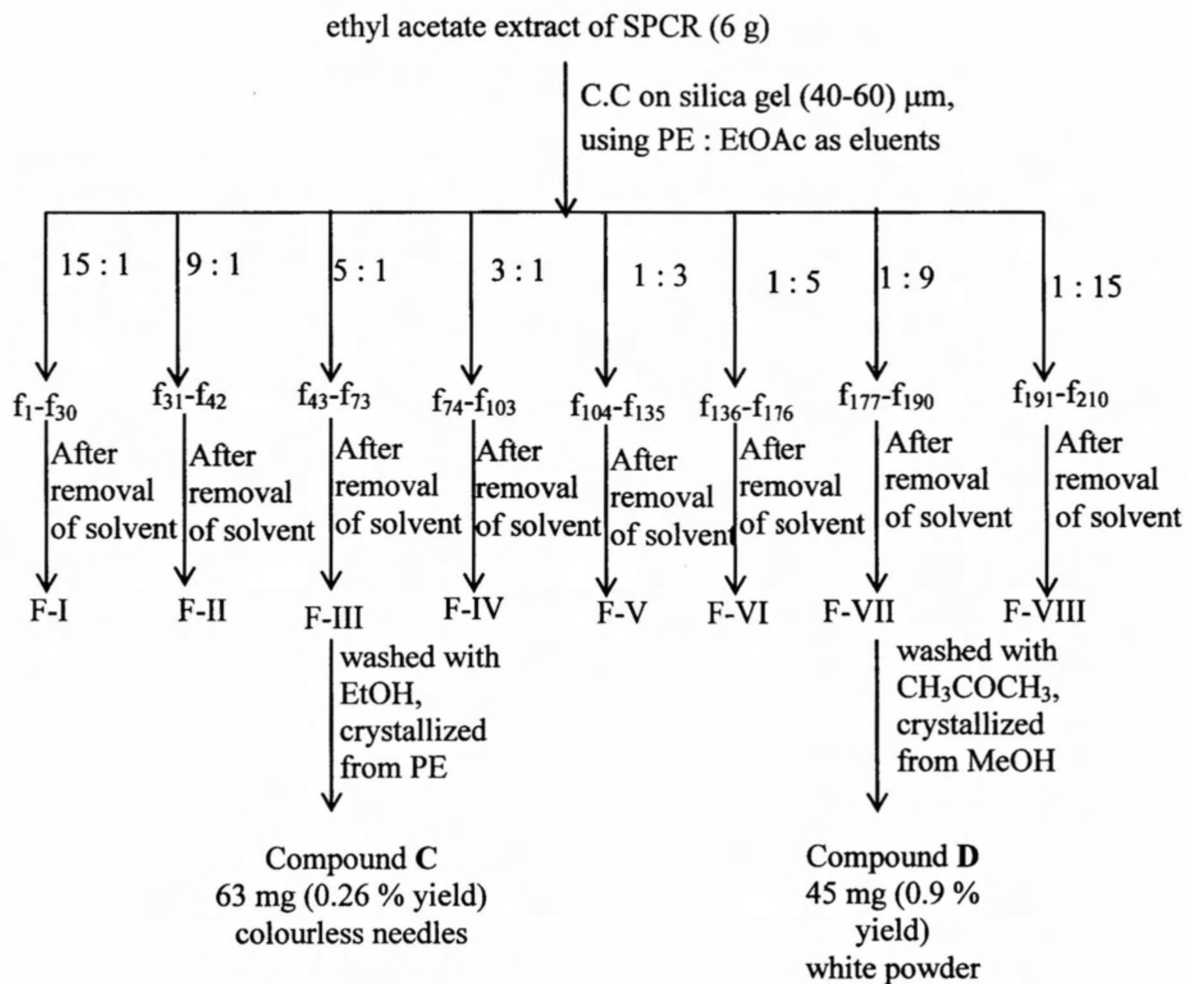


Figure 3.2 Flow diagram for the separation of ethyl acetate crude extract of SPCR by column chromatography

The chloroform crude extract of SPCR were prepared according to the procedure as mentioned in Section 2.7 and compound **E** (pale yellow amorphous powder, 15.8 mg, 0.26 %), compound **F** (yellow needles, 15 mg, 0.25 %), compound **G** (amorphous powder, 25.7 mg, 0.43 %) and compound **H** (amorphous powder, 50 mg, 0.83 %) were isolated from 6 g of chloroform extract of SPCR by column chromatography using silica gel (40-60 μm) as adsorbent, and EtOAc: n-hexane (10:90, 15:85, 20:80, 25:75, 30:70, 35:65 and 50:50 v/v) solvents as eluents (Section 2.7). The procedure for extraction and isolation of chloroform extract of SPCR is illustrated as a flow diagram shown in Figure 3.3.

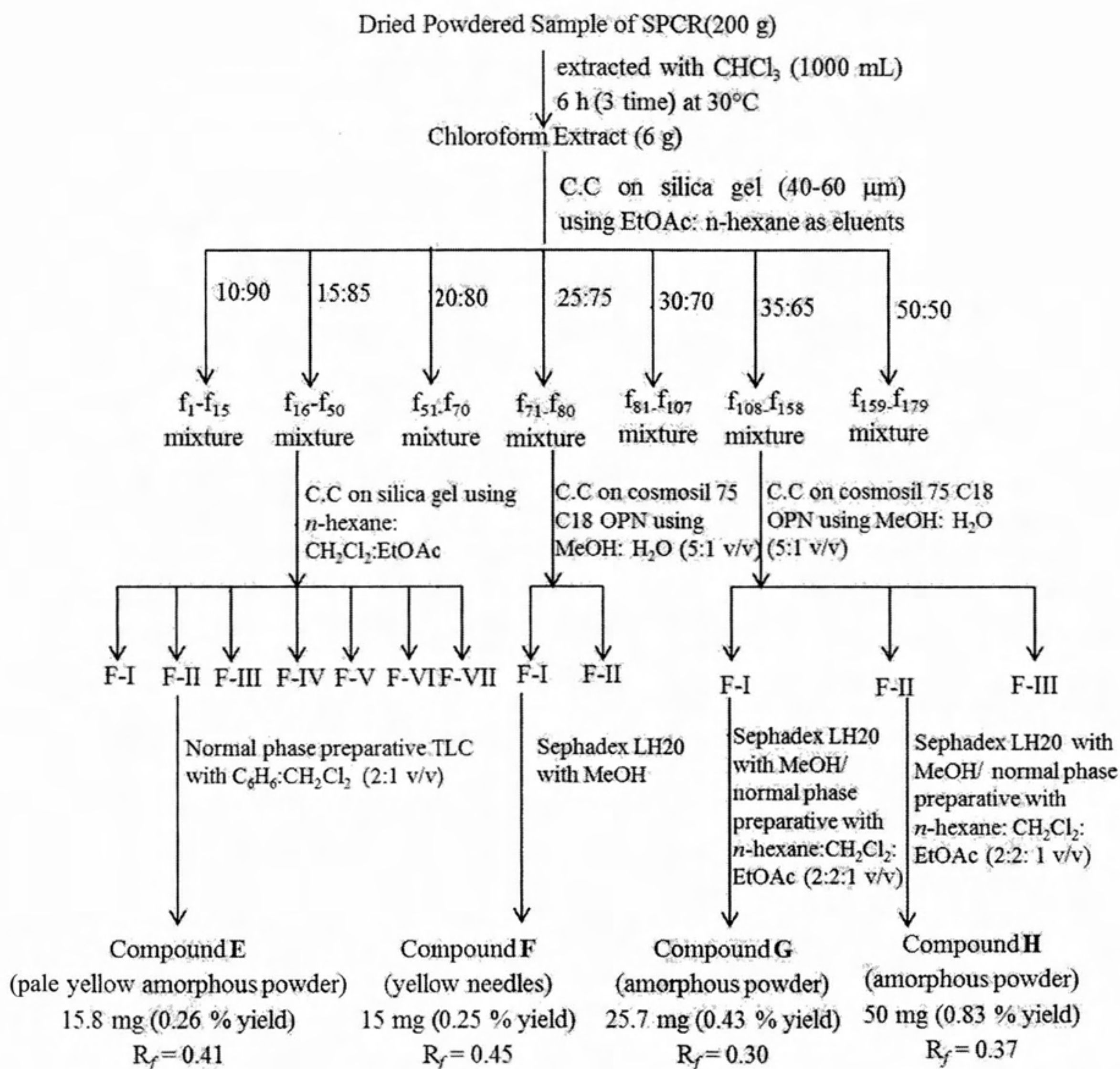
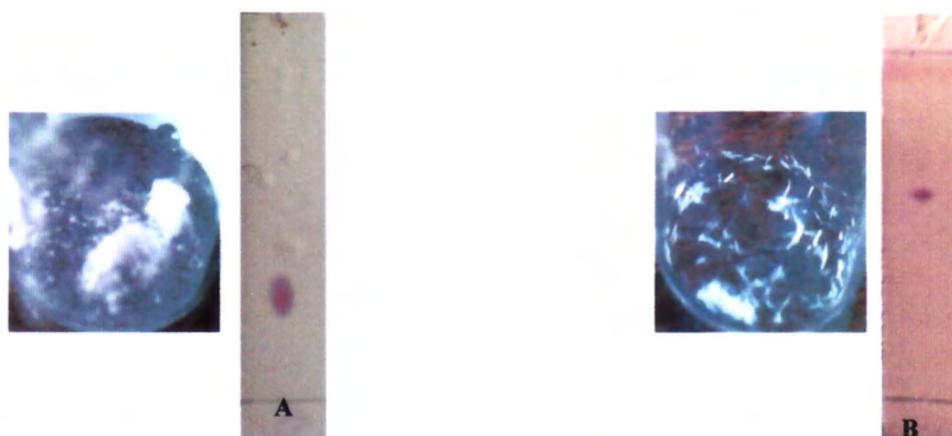


Figure 3.3 Flow diagram for the separation of chloroform crude extract of SPCR by column chromatography

3.5 Characterization and Identification of Isolated Compounds

The isolated compounds **A** to **H** obtained from pet-ether extract of WRC, ethyl acetate extract and chloroform extract of SPCR were firstly characterized by their physical properties such as melting points, R_f values, solubilities and by some chemical properties especially colour reactions. Photographs of the TLC chromatograms of these isolated compounds are shown in Figures 3.4, 3.5 and 3.6. All of these observed physical and chemical properties of eight isolated compounds are summarized in Table 3.5. The isolated compounds **A** to **H** were classified by using reported phytochemical tests and they were identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectrometry.



Compound A

Solvent = PE: EtOAc, 30:1

Reagent = 5 % H_2SO_4, Δ

R_f = 0.36

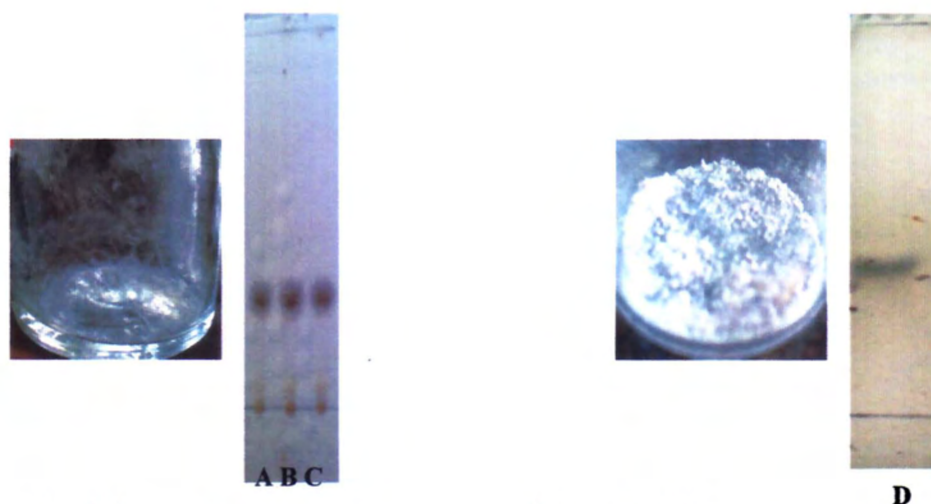
Compound B

Solvent = PE: EtOAc, 5:1

Reagent = 5 % H_2SO_4, Δ

R_f = 0.53

Figure 3.4 Thin layer chromatograms of isolated compounds A and B from PE extract of WRC



Compound C

A = Compound C

B = mixture of β-sitosterol & C

C = authentic β-sitosterol

Solvent = PE : EtOAc, 5:1

Reagent = 5 % H_2SO_4, Δ

R_f = 0.55

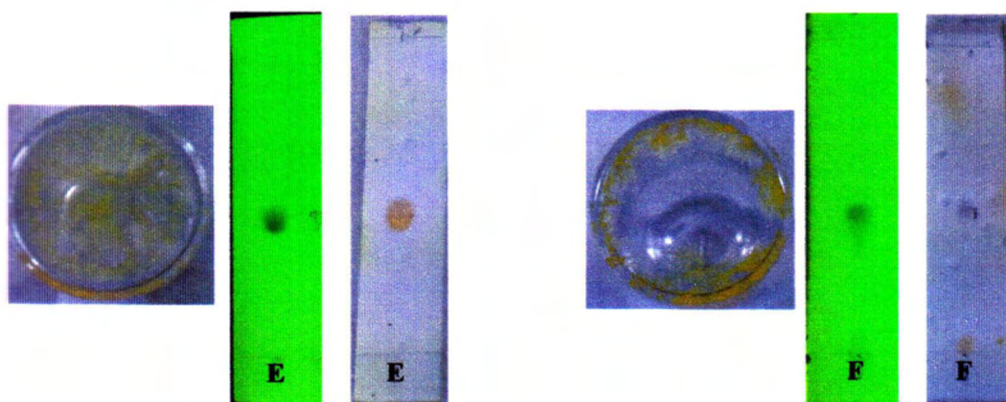
Compound D

Solvent = $CHCl_3$: MeOH, 9:1

Reagent = 5 % H_2SO_4, Δ

R_f = 0.23

Figure 3.5 Thin layer chromatograms of isolated compounds C and D from EtOAc extract of SPCR



Compound E

Solvent = *n*-hexane: CH_2Cl_2, 3:1

Reagent = 1 % Ce $(SO_4)_2$/10 % H_2SO_4, Δ

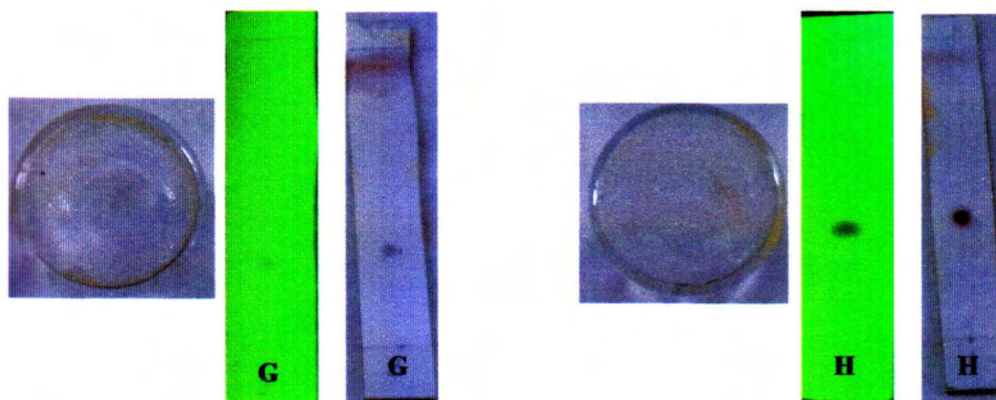
R_f = 0.41

Compound F

Solvent = *n*-hexane: EtOAc, 4:1

Reagent = 1 % $Ce(SO_4)_2$/10 % H_2SO_4, Δ

R_f = 0.45



Compound G

Solvent = *n*-hexane: EtOAc, 2:1

Reagent = 1 % Ce $(SO_4)_2$/10 % H_2SO_4, Δ

R_f = 0.30

Compound H

Solvent = *n*-hexane: EtOAc, 2:1

Reagent = 1 % $Ce(SO_4)_2$/10 % H_2SO_4, Δ

R_f = 0.37

Figure 3.6 Thin layer chromatograms of isolated compounds E, F, G and H from $CHCl_3$ extract of SPCR

Table 3.5 Some Physical Properties of Isolated Compounds (A-H) from the Whole Plant of *C.repens* and Rhizomes of *B. rotunda*

Isolated compounds	Physical State	Melting point (°C)	Solubility						
			PE	$CHCl_3$	EtOAc	CH_3COCH_3	MeOH	EtOH	H_2O
A	White needle	258-259	+	+	-	+	+	+	+
B	Colourless crystal	278-279	+	+	-	+	+	+	+
C	Colourless needle	138-140	+	+	+	+	-	-	-
D	White powder	272-274	+	+	+	-	+	+	+
E	Pale yellow amorphous powder	96-98	-	+	+	+	+	+	+
F	Yellow needles	179-182	-	+	-	-	+	Δ,+	+
G	Amorphous powder	165-167	+	+	+	-	+	-	+
H	Amorphous powder	134-134.5	+	+	+	-	+	-	+

Table 3.6 Some Chemical Properties and Classification of Isolated Compounds

Isolated Compounds	Observation after treating with various reagents										Types of compounds
	I	II	III	IV	V	VI	VII	VIII	IX	X	
A	Pink	Purple	Purple	Pink	No color changed	ND	Decolourized	Yellow ppt	ND	No ppt	Terpene
B	Pink	Purple	Purple	Pink	No color changed	ND	Decolourized	Yellow ppt	ND	No ppt	Terpene
C	Cherry red to blue	Blue	Purple	Greenish blue	No color changed	ND	Decolourized	No yellow ppt	No ppt	White ppt	Steroid
D	Pink	Blue	Blue	Green	No color changed	ND	Decolourized	No ppt	ND	White ppt	Steroidal glycoside
E	Yellow	Blue	Blue	ND	Pink	Brown	Decolourized	Yellow ppt	ND	No ppt	Flavonoid
F	Yellow	Blue	Blue	ND	Pink	Brown	Decolourized	Yellow ppt	ND	No ppt	Flavonoid
G	Pink	Purple	Purple	Pink	No color changed	ND	Decolourized	Yellow ppt	ND	No ppt	Terpene
H	Pink	Purple	Purple	Pink	No color changed	ND	Decolourized	Yellow ppt	ND	No ppt	Terpene

I = 5 % H_2SO_4, Δ IV = Liebermann Burchard VII = 10 % $KMnO_4$ X = 10 % lead acetate

II = Vinillin, Δ V = Conc: HCl and Mg turnings VIII = 2, 4-DNP ND = Non-detected

III = Anisaldehyde, Δ VI = 10 % $FeCl_3$ IX = 5 % KOH

3.6 Identification of Isolated Compounds

In this section, structural educidation of isolated compounds (**A-H**) by using joint application of physicochemical properties and modern spectroscopic data (such as UV, FT IR, 1D NMR, 2D NMR and ESI MS) will be discussed.

For the identification of isolated compounds, FT IR spectra of the isolated compounds (**A, B, C, D**) were measured at Department of Chemistry, University of Yangon.

^{1}H NMR, ^{13}C NMR and ESI MS spectra of the isolated compound **A** were measured at Department of Organic and Biomolecular Chemistry, Georg-August University, Goettingen, Germany.

^{1}H NMR, ^{13}C NMR, COSY, NOESY, HSQC and HMBC spectra of the isolated compounds **B** and **D** were measured at Bioactive Natural Products Chemistry Laboratory, Nagoya University, Nagoya, Japan.

UV, FT IR, ^{1}H NMR, ^{13}C NMR, HMBC, HMQC and ESI MS spectra of the isolated compounds (**E, F, G, H**) and COSY and NOESY spectra of the isolated compounds **G** and **H** were measured at Division of Natural Product Chemistry, Institute of Natural Medicine, University of Toyama, Japan.

3.6.1 Structural elucidation of compound A

Compound **A** (0.014 % yield) was isolated as a white needle from pet-ether extract of the whole plant of *C. repens* (WRC) and its melting point was found to be 258-259 °C. It is soluble in pet-ether, chloroform, acetone, methanol, ethanol and water but insoluble in ethyl acetate. It is UV inactive and R_f value was found to be 0.36 in PE: EtOAc (20: 1 v/v). Compound **A** was classified as terpenoid compound since the reaction with Liebermann Burchard reagent gave pink colouration. The observed yellow precipitate in 2,4-DNP test confirmed the presence of carbonyl group. It gave a purple spot on TLC chromatogram by spraying with vanillin, and a pink colour spot with 5 % H_2SO_4 reagent after heating. Some physico-chemical properties of compound **A** are described in Table 3.7.

Table 3.7 Some Physico-chemical Properties of Isolated Compound A

Experiment	Observation	Remark
UV	Inactive	No conjugated double bond
Liebermann Burchard reagent	Pink	Terpenoid compound
2,4-DNP solution	Yellow ppt	C=O present
Vanillin reagent	Purple spot	on TLC
5 % H_2SO_4, Δ	Pink colour spot	on TLC
R_f value	0.36 (PE:EtOAc, 20:1 v/v)	Non-polar organic compound

The structure of compound **A** was also studied by FT IR, ^{1}H NMR, ^{13}C NMR and ESI MS spectral data. Compound **A** was UV inactive suggesting the absence of conjugated double bond system.

The functional groups present in compound **A** were also studied by FT IR spectroscopy. FT IR spectrum of isolated compound **A** is illustrated in Figure 3.7. In FT IR spectrum, an intense peak was observed at 1714 cm^{-1} indicating the presence of a carbonyl group. In addition, the presence of sp^3 C-H stretching and C-H bending were revealed by absorption bands at 2926 and 1456 cm^{-1}, respectively. The spectral data of compound **A** are listed in Table 3.8.

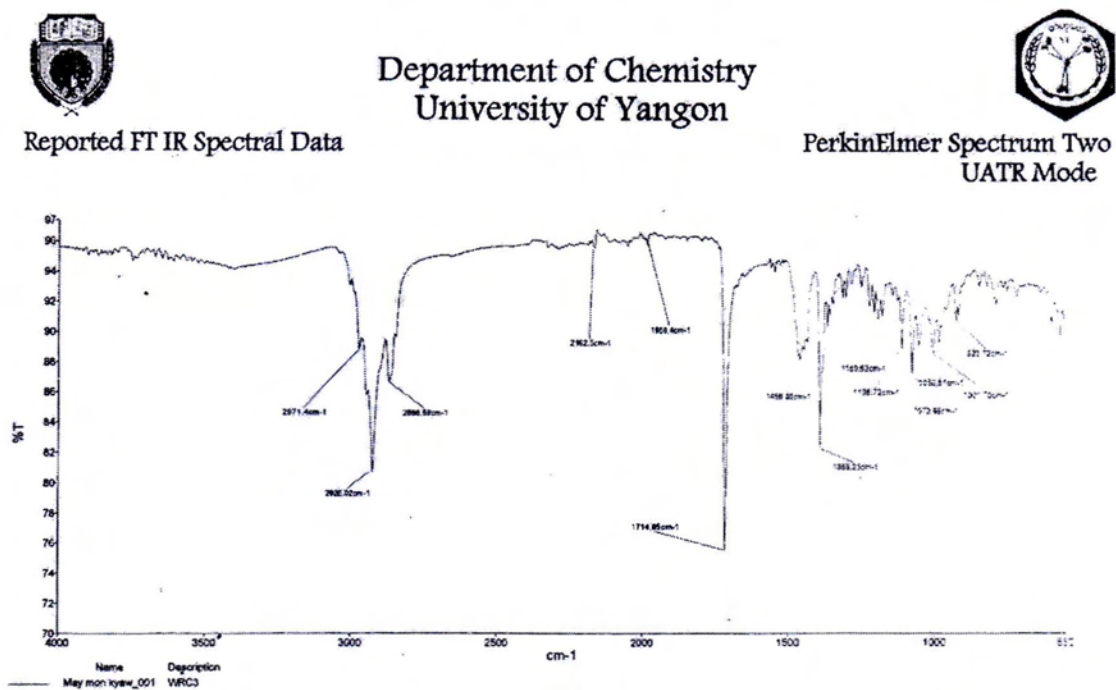


Figure 3.7 FT IR spectrum of isolated compound A

Table 3. 8 FT IR Spectral Data of Isolated Compound A and Reported Friedelin

Wave number (cm^{-1})		Band Assignment
Compound A	**Friedelin***	
2926, 2868	2930, 2870	Asymmetric and symmetric C-H stretching vibration
1714	1716	C = O stretching vibration
1456, 1389	1460, 1388	C-H bending vibration of $-CH_2$ and $-CH_3$ groups
1072	1074	C-H in plane bending vibration

* Susidarti *et al.,* 2009

There were a total of seven methyl singlets observed at δ 0.65 (H-24), 0.80 (H-25), 0.94 (H-26), 0.97 (H-27), 0.65 (H-28) and 0.93 (H-29 and H-30) in the ^{1}H NMR spectrum (Figure 3.8). Besides, a doublet signal of methyl was found at 0.81 (J = 6.1 Hz, H-23), a multiplet signal of methine proton was found at δ 2.18 (H-4) and two groups of methylene protons gave signals at δ 1.90 and 1.65 (m, H-1_a and H-1_b) and 2.32 (dd, J = 11.2 Hz and 5.2 Hz, H-2_a) and 2.22 (m, H-2_b). There was no vinylic proton signal observed and the remaining 17 protons signals were observed in the range of δ 1.2-2.00, which in a highly shielded region.

In the ^{13}C NMR (Figure 3.9), a total of 30 carbons were observed. Among the 30 carbons, a keto carbon signal was observed at δ 212.1 (C-3) which has highly deshielded. There were a total of eight methyl groups as revealed by carbon signals at δ 6.74 (C-23), 14.60 (C-24), 17.80 (C-25), 20.23 (C-26), 18.62 (C-27), 32.0 (C-28) and 31.81 (C-29) and 33.4 (C-30). Meanwhile, six quaternary carbon signals were observed at δ 42.12 (C-5), 37.4 (C-9), 28.14 (C-20), 20.23 (C-26), 18.62 (C-27) and 32.0 (C-28). The rest of the carbon signals were found at δ 22.21 (C-1), 41.40 (C-2), 58.20 (C-4), 41.12 (C-6), 17.9 (C-7), 53.02 (C-8), 59.53 (C-10), 35.81 (C-11), 30.38 (C-12), 39.60 (C-13) 38.21 (C-14), 32.30 (C-15), 36.0 (C-16), 30.01 (C-17), 42.87 (C-18), 35.01 (C-19), 32.80 (C-21) and 39.24 (C-22). Both ^{1}H NMR and ^{13}C NMR data of compound **A** were found to match closely with those of literature values reported for Friedelin (Susidarti *et al.,* 2009). Table 3.9 shows ^{1}H NMR and ^{13}C NMR spectral data for compound **A** in comparison with the literature values of Friedelin.

From ESI MS spectrum (Figure 3.10), the spectral data of isolated compound **A** is indicated the presence of molecular ion peak $[M+H]^+$ at m/z 427 which corresponded to the molecular formula $C_{30}H_{50}O$ (Susidarti *et al.,* 2009). All of the above mentioned ^{1}H NMR, ^{13}C NMR, ESI MS spectral data were consistent with those of reported friedelin and the structure is illustrated in Figure 3.11.

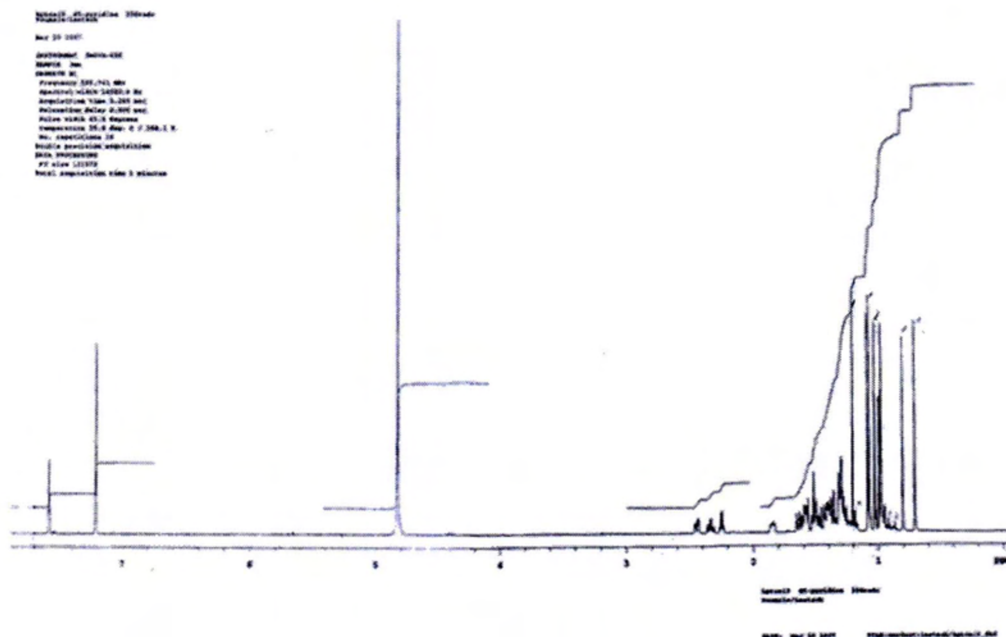


Figure 3.8 ^{1}H NMR (600 MHz, $CDCl_3$) spectrum of isolated compound A

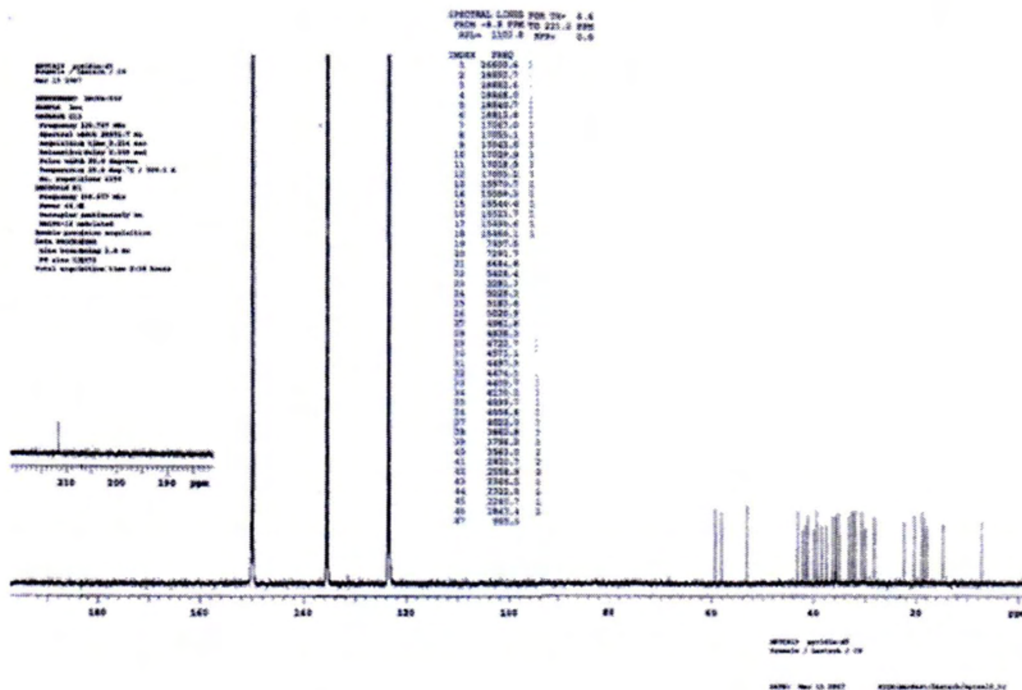


Figure 3.9 ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of isolated compound A

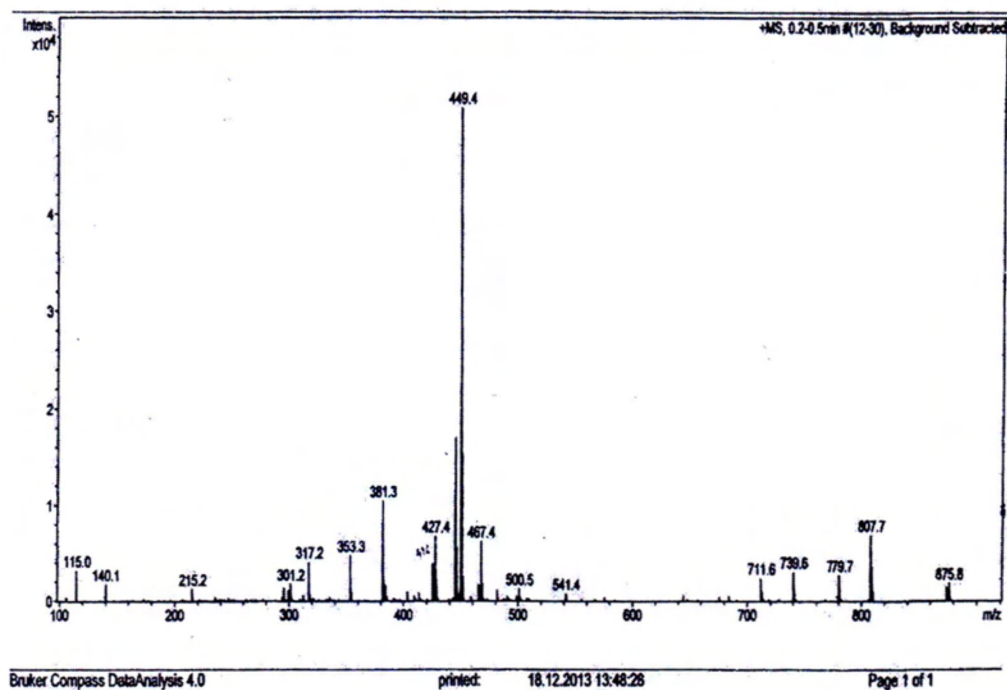


Figure 3.10 ESI MS spectrum of isolated compound A

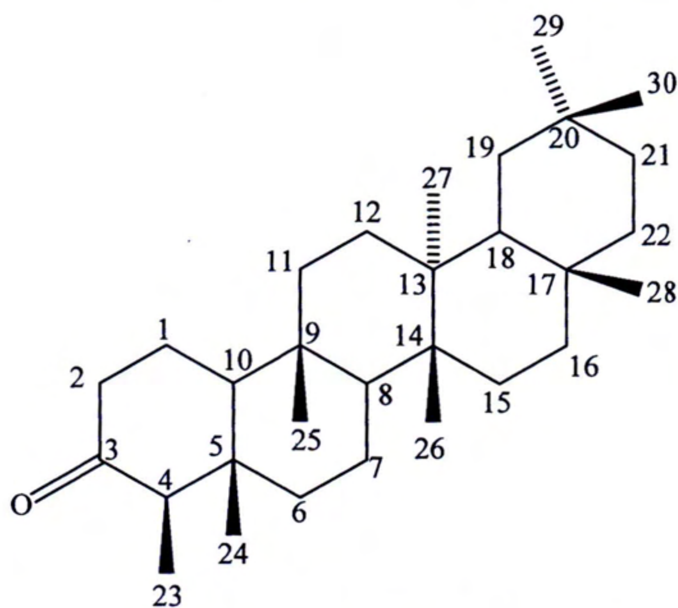


Figure 3.11 Chemical structure of friedelin ($C_{30}H_{50}O$)

Table 3.9 1D NMR Spectral Data of Isolated Compound A and Reported Friedelin

Position	Compound A		Friedelin*	
	δ_H	δ_C	δ_H	δ_C
1	1.90 (m, 1H)	22.21	1.90 (m, 1H)	22.2
	1.65 (m, 1H)		1.65 (m, 1H)	
2	2.32 (dd, 1H, J=11.2 Hz)	41.40	2.32 (dd, 1H, J = 11.3 Hz)	1.4
	2.22 (m, 1H, J= 5.2 Hz)		2.22 (m, 1H, J = 5.4 Hz)	
3		212.1		213.1
4	2.18 (q, 1H)	58.20	2.18 (q, 1H)	58.2
5		42.12		42.1
6	1.66 (m, 1H)	41.12	1.66 (m, 1H)	41.1
	1.21 (m, 1H)		1.21 (m, 1H)	
7	1.66 (m, 1H)	17.9	1.66 (m, 1H)	18.2
	1.21 (m, 1H)		1.21 (m, 1H)	
8	1.35 (m, 1H)	53.02	1.35 (m, 1H)	53.0
9		37.4		37.4
10	1.48 (m, 1H)	59.53	1.48 (m, 1H)	59.5
11	1.38 (m, 1H)	35.81	1.38 (m, 1H)	35.8
	1.19 (m, 1H)		1.19 (m, 1H)	
12	1.31 (m, 1H)	30.38	1.31 (m, 1H)	30.4
	1.24 (m, 1H)		1.24 (m, 1H)	
13		39.60		39.6
14		38.21		38.2

Table 3.9 Continued

Position	Compound A		Friedelin*	
	δ_H	δ_C	δ_H	δ_C
15	1.49 (m, 1H)	32.30	1.49 (m, 1H)	32.3
16	1.50 (m, 1H)	36.0	1.50 (m, 1H)	36.0
	1.27 (m, 1H)		1.27 (m, 1H)	
17		30.01		30.0
18	1.51 (m, 1H)	42.87	1.51 (m, 1H)	42.9
19	1.31 (dd, 1H)	35.01	1.31 (dd, 1H)	35.0
	1.14 (m, 1H)		1.14 (m, 1H)	
20		28.14		28.1
21	1.42 (m, 1H)	32.80	1.42 (m, 1H)	32.8
	1.37 (m, 1H)		1.37 (m, 1H)	
22	1.41 (m, 1H)	39.24	1.41 (m, 1H)	39.2
	0.90 (m, 1H)		0.90	
23	0.81 (d, 3H, J = 6.1 Hz)	6.74	0.81 (d, 3H, J = 6.1 Hz)	6.7
24	0.65 (s, 3H)	14.60	0.65 (s, 3H)	14.6
25	0.80 (s, 3H)	17.80	0.80 (s, 3H)	17.8
26	0.94 (s, 3H)	20.23	0.94 (s, 3H)	20.2
27	0.97 (s, 3H)	18.62	0.97 (s, 3H)	18.6
28	0.65 (s, 3H)	32.0	0.65 (s, 3H)	32.0
29	0.93 (s, 3H)	31.81	0.93 (s, 3H)	31.8
30	0.87 (s, 3H)	33.41	0.87 (s, 3H)	33.4

* Susidarti *et al.*, 2009

3.6.2 Structural elucidation of compound B

Compound **B** was isolated as a colourless crystal from pet-ether crude extract of WRC by column chromatographic separation using silica gel eluting with PE: EtOAc (30 : 1 v/v) according to the procedure shown in Figure 3.1. Its R_f value was found to be 0.53 in PE: EtOAc (5 : 1 v/v) solvent system. It was purified by crystallizing from EtOAc and has the melting point of 278-279 °C. It gave a purple spot on TLC chromatogram by spraying with vanillin, and a pink colour spot with 5 % H_2SO_4 reagent after heating. It also gave pink colouration with Liebermann Burchard reagent indicating the characteristics of a terpenoid. Compound **B** was UV inactive suggesting the absence of conjugated double bond system. Some physico-chemical properties of compound **B** are described in Table 3.10.

Its structure was also studied by FT IR, ^1H NMR, ^{13}C NMR and 2D NMR spectral data. The FT IR spectrum of compound **B** is shown in Figure 3.12 and the interpreted spectral data are illustrated in Table 3.11. The IR spectrum showed an intense and broad peak of O-H stretch at 3469 cm^{-1} and a C-O stretch at 1019 cm^{-1}. These two bands further confirmed the presence of the O-H functional group in friedelinol. CH stretches at 2924 and 2869 cm^{-1} and a CH_3 bending at 1455 cm^{-1} were also observed in the spectrum.

Table 3.10 Some Physico-chemical Properties of Isolated Compound B

Experiment	Observation	Remark
UV	Inactive	No conjugated double bond
Liebermann Burchard reagent	Pink	Terpenoid compound
2,4-DNP solution	Yellow ppt	C=O present
Vanillin reagent	Purple spot	on TLC
5 % H_2SO_4, Δ	Pink colour spot	on TLC
R_f value	0.53 (PE: EtOAc, 5:1 v/v)	Non-polar organic compound

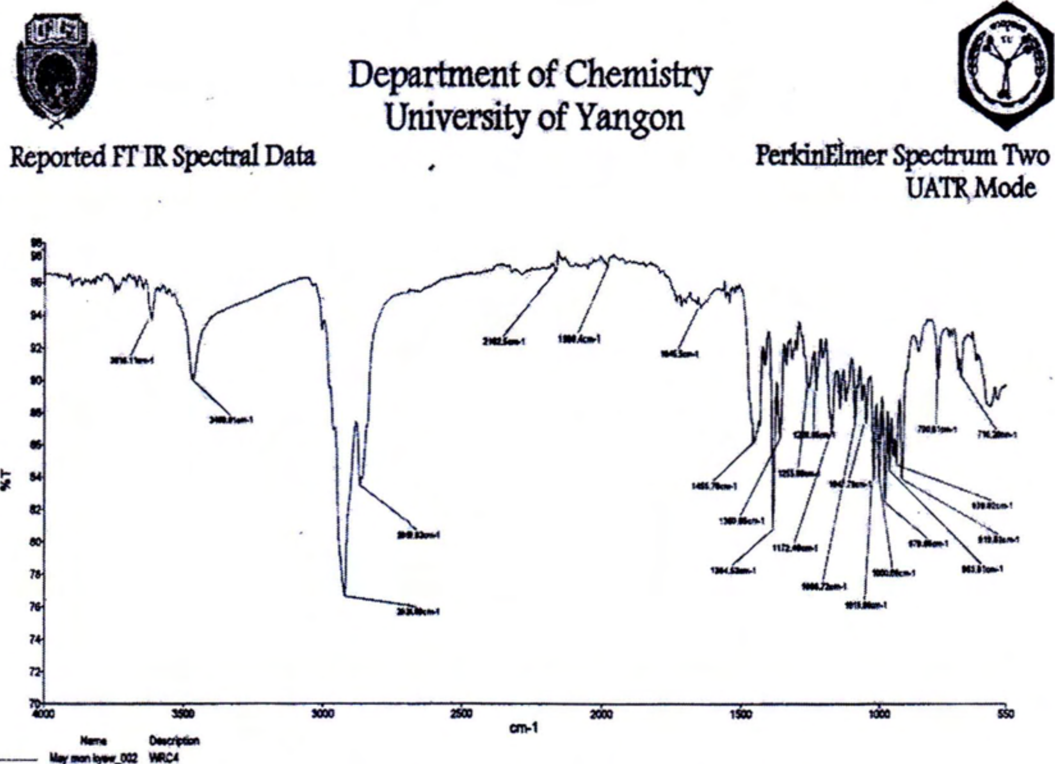


Figure 3.12 FT IR spectrum of isolated compound B

Table 3.11 FT IR Spectral Data of Isolated Compound B and Reported Friendelinol

Wave number (cm^{-1})		Band Assignment
Compound B	**Friedelinol***	
3469	3429	O-H stetching vibration of alcoholic O-H group
2924, 2869	2924, 2869	Asymmetric and symmetric C-H stretching vibration of $-CH_2$ and $-CH_3$ groups
1455	1466	C-H bending vibration of $-CH_3$ groups
1019	1009	C-O stretching vibration of alcohol group
979, 963	978, 962	C-H out of plane bending vibration of aromatic ring

* Ying, 2014

The ^1H NMR spectrum of compound **B** (Figure 3.13) exhibited a total of eight methyl proton signals at δ 0.94 (H-23), 0.91 (H-24), 0.85 (H-25), 0.99 (H-26), 0.99 (H-27) and 1.16 (H-28), 1.00 (H-29) and 0.94 (H-30). The deshielded proton signal at 3.73 ppm was assigned to oxymethine proton, H-3 which was directly attached to a hydroxyl group. There were a doublet of triple signals observed at δ 1.90 and 1.56 (dt, J = 8 Hz, 2 Hz) assigned to proton H-2 and a multiplet signal at 1.72 ppm assigned to proton H-6. The remaining proton signals were assigned to methylene protons, found in the range of δ 0.88-1.55 (Ying, 2014). ^1H ^1H COSY spectrum of isolated compound **B** is show in Figure 3.15. The peak correlating signals at δ 3.73, 1.56 and 1.9 were observed in ^1H ^1H COSY spectrum. In addition, δ 1.72 (H-6) was coupled with the other protons δ 0.92 (H-24). Absolute confirmed to be S based on the coupling constants of the methylene protons $H_{2\alpha, 2\beta}$ (J_{ax} = 8Hz and J_{eq} = 2 Hz).

^{13}C NMR spectrum (Figure 3.14) of compound **B** indicated a total of 30 carbon signals revealing the presence of 30 carbons in the assigned compound. From these 30 carbons, there were eight methyl carbons which gave signals at δ 11.6 (C-23), 16.3 (C-24), 18.2 (C-25), 18.6 (C-26), 20.1 (C-27), 32.3 (C-28), 35.2 (C-29) and 31.7 (C-30). In addition, oxymethine carbon, C-3 gave a relatively deshielded signal at 72.7 ppm. The rest of the carbon signals were observed at δ 15.8 (C-1), 35.5 (C-2), 49.2 (C-4), 37.1 (C-5), 41.7 (C-6), 17.5 (C-7), 53.2 (C-8), 37.8 (C-9), 61.3 (C-10), 35.3 (C-11), 30.6 (C-12), 38.3 (C-13), 39.2 (C-14), 39.6 (C-15), 36.1 (C-16), 30.0 (C-17), 42.8 (C-18), 35.5 (C-19), 28.1 (C-20), 32.3 (C-21) and 39.6 (C-22). In the HSQC spectrum (Figure 3.17), each proton signal correlated with corresponding carbon. The attachment of hydroxyl group at C-3 was confirmed from HMBC correlation (Figure 3.18) of H-3 (δ 3.73). ^1H NMR ^{13}C NMR, ^1H ^1H COSY, HSQC and HMBC spectral data are described in Table 3.12. The assigned data were found to be consistent with those of literature values (Ying, 2014). Therefore compound **B** was identified as friedelinol (molecular formula, $C_{30}H_{52}O$).

WRC IV/CDCl3

Figure 3.13 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of isolated compound B

WRC IV/CDCl3, 13C

Figure 3.14 ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of isolated compound B

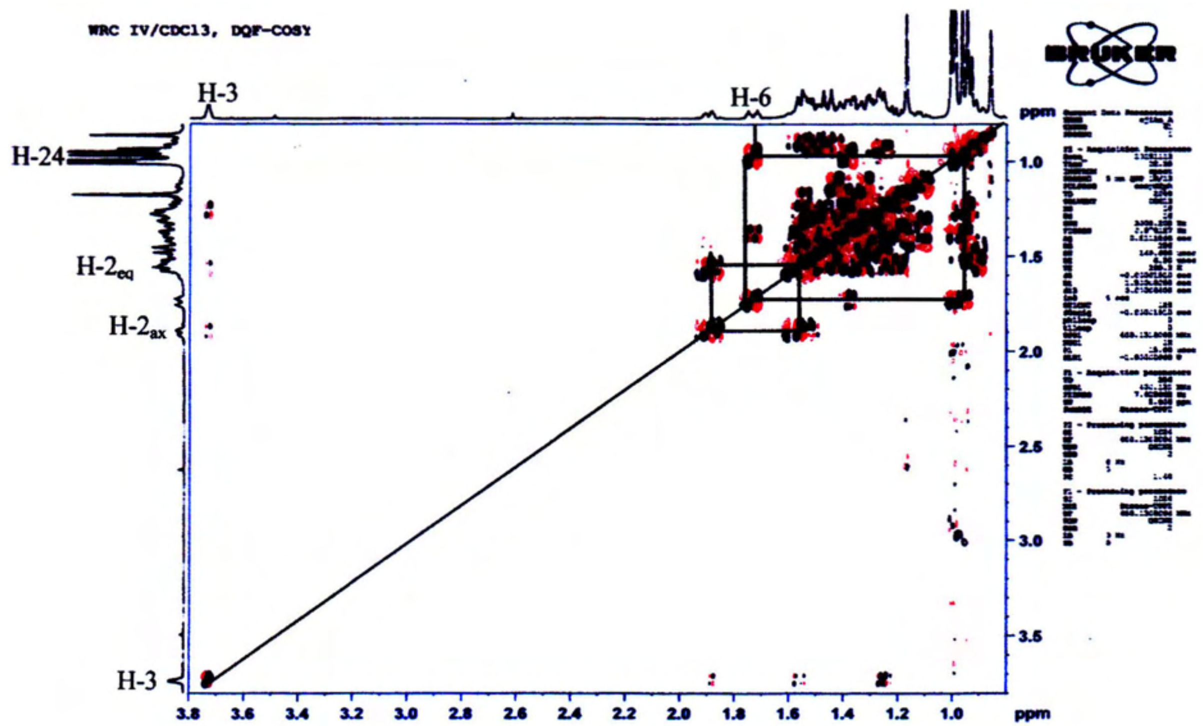


Figure 3.15 ^{1}H ^{1}H **COSY (400 MHz, $CDCl_3$) spectrum of isolated compound B**

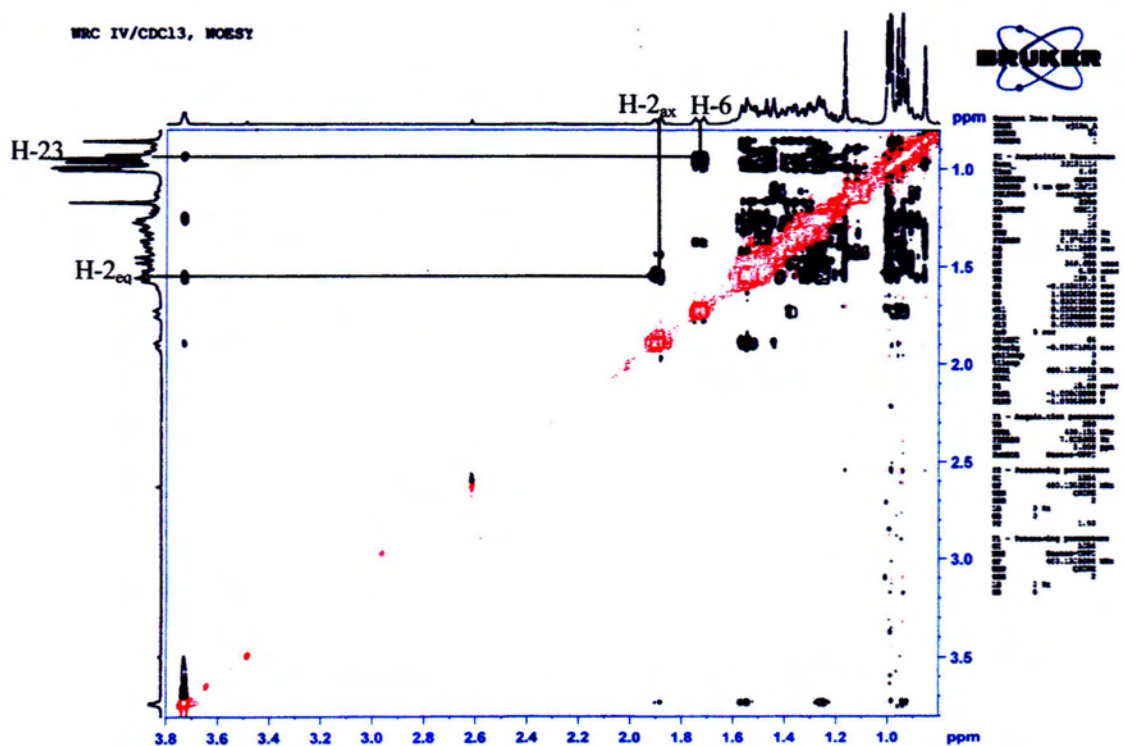


Figure 3.16 **NOESY (400 MHz, $CDCl_3$) spectrum of isolated compound B**

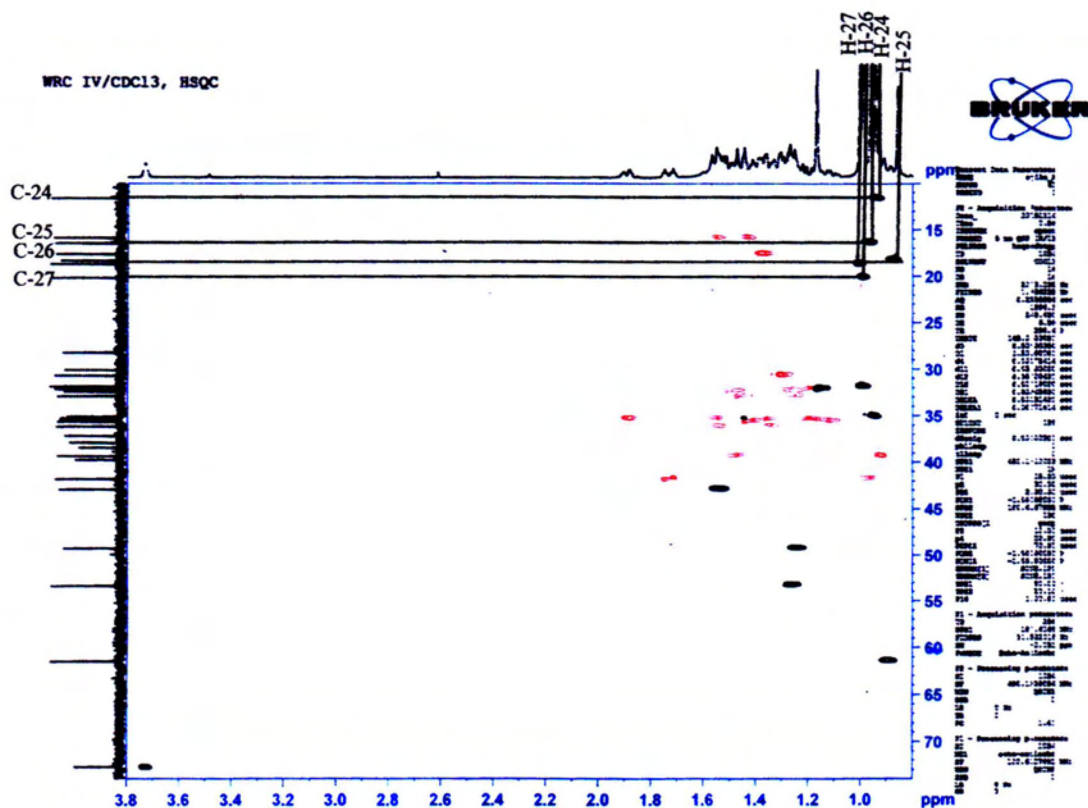


Figure 3.17 HSQC (400 MHz, $CDCl_3$) spectrum of isolated compound B

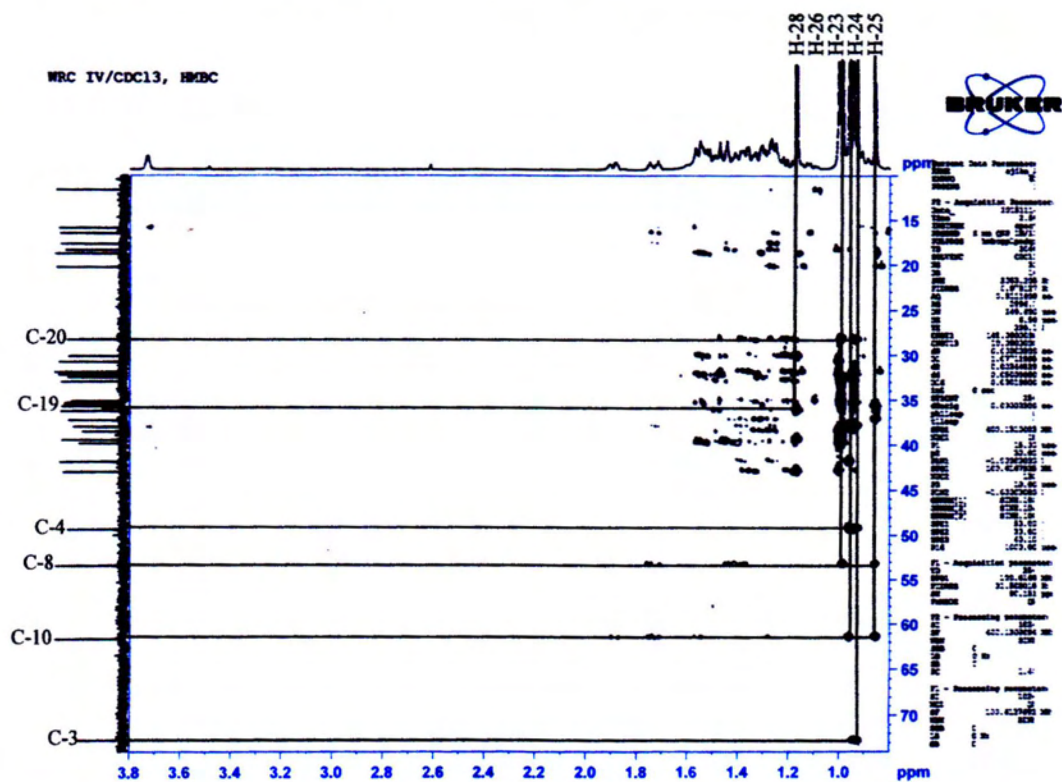
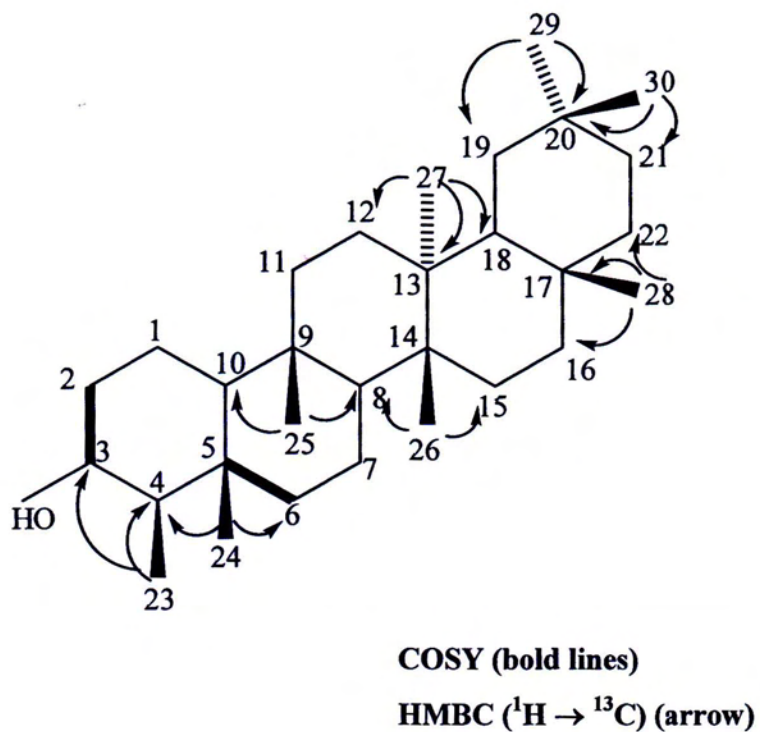


Figure 3.18 HMBC (400 MHz, $CDCl_3$) spectrum of isolated compound B



COSY (bold lines)

HMBC ($^{1}H \rightarrow {}^{13}C$) (arrow)

Figure 3.19 Chemical structure of friedelinol ($C_{30}H_{52}O$)

Table 3.12 1D and 2D NMR Spectral Data of Isolated Compound B and Reported Friedelinol

Position	Compound B				Friedelinol*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_C
1		15.8				35.8
2	1.9 (dt, *J*= 8 Hz, 2 Hz)	35.35			1.9 (dt, *J* = 9.2, 2.1)	35.4
	1.56					
3	3.73	72.7	H_2		3.73	72.8
4		49.2				49.2
5		37.8				37.2
6	1.72	41.7	H_{24}		1.73	41.8
7		17.5				17.6
8		53.2				53.2
9		37.1				37.9
10		61.3				61.4
11		35.21				35.3
12		30.6				30.7
13		38.3				38.4
14		39.2				39.4
15		32.8				32.9

Table 3.12 Continued

Position	Compound B				Friedelinol*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_C
16		36.1				36.2
17		30.0				30.1
18		42.8				42.8
19		35.58				35.6
20		28.1				28.3
21		32.3				32.2
22		39.6				39.8
23	0.94 (d, *J*= 5.2 Hz)	11.6		C_3, C_4	0.94 (d. *J*=6.2)	11.7
24	0.92	16.3		C_4, C_6	0.91	16.5
25	0.85	18.2		C_8, C_{10}	0.85	18.3
26	0.99	18.6		C_8, C_{15}	0.99	18.7
27	0.99	20.1		C_{12}, C_{13}, C_{18}	0.99	20.3
28	1.16	32.3		C_{19}, C_{20}	1.16	32.4
29		35.2		C_{20}, C_{21}	1.00	35.1
30		31.7		C_{20}, C_{21}	0.95	31.9

* Ying, 2014

3.6.3 Structural elucidation of compound C

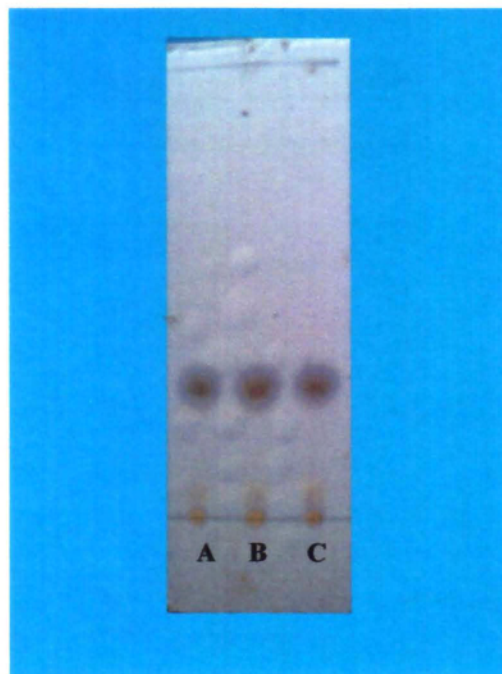
Compound **C** isolated from ethyl acetate extract of rhizomes of *B. rotunda* (SPCR) was obtained as colourless needle in 63 mg, 0.26 % yield and its melting point was found to be 138-140 °C. It was soluble in pet-ether, chloroform, ethyl acetate and acetone but insoluble in methanol, ethanol and water. Its R_f value was 0.55 in PE:EtOAc (5:1 v/v) and it was UV inactive. It gave yellow spot with iodine vapour on TLC chromatogram. According to the results obtained from 2,4-DNP test (Table 3.13), carbonyl group was absent in compound **C**. Decolourization of 10 % $KMnO_4$ solution with compound **C** indicated the presence of C=C bond in it. Since it gave green colour when treated with acetic anhydride and concentrated sulphuric acid, compound **C** was classified as a steroid compound. The R_f value of compound **C** was found to be identical with that of β-sitosterol in any solvent system and they also gave the same behaviours on Co-TLC chromatogram as described in Figure 3.20.

The FT IR spectrum of isolated compound **C** (Figure 3.21) showed that a broad strong band absorbed at 3440 cm^{-1} due to O-H stretching vibration band. The asymmetric and symmetric aliphatic C-H stretching bands appeared at 2939 and 2877 cm^{-1}. A weak band at 1643 cm^{-1} that corresponded to C=C stretching revealed the presence of olefinic band in this compound. In addition, C-H bending bands of CH_3 and CH_2 groups appeared at 1458 cm^{-1}. Absorption band at 1373 cm^{-1} indicated that the compound **C** possessed the CH $(CH_3)_2$ isopropyl skeleton. The band at 1049 cm^{-1} can be attributed to C-O stretching vibration band. Weak intensity band at 956 cm^{-1} was attributed to C-H bending vibration of olefinic bond. These absorption frequencies resembled the absorption frequencies observed for a sterol. The observed FT IR spectral data of compound **C** were also studied by comparing with those of reported β-sitosterol and tabulated in Table 3.13. All of the results such as melting point, R_f value, chemical properties and FT IR spectral data of compound **C** were found to be similar to those of reported β-sitosterol. So the compound **C** was assigned as β-sitosterol. Consequently, compound **C** was identified as β-sitosterol and its chemical structure is shown in Figure 3.21.

Table 3.13 Some Physico-chemical Properties of Isolated Compound C

Experiment	Observation	Remark
Physical state	Colourless needle	Colourless needle (Reported β-sitosterol)
Melting point (°C)	138-140	138-140, β-sitosterol*
UV (254 nm & 365 nm)	Inactive	Inactive
I_2 vapour	Yellow	C=C present compound
Acetic anhydride & Conc: H_2SO_4	Green	Steroid compound
2,4- DNP solution	No Yellow ppt	C=O absent
10% $KMnO_4$ solution	Decolourization	C=C present
5% H_2SO_4, Δ	Cherry red	On TLC
Vanillin, Δ	Blue	On TLC
Anisaldehyde, Δ	Purple	On TLC
R_f value	0.55 (PE:EtOAc- 5:1 v/v)	0.55 (PE:EtOAc- 5:1 v/v)

* Merck Index, 2001.



Compound C

A	=	Compound **C**
B	=	mixture of β-sitosterol & C
C	=	authentic β-sitosterol
Solvent	=	PE : EtOAc, 5:1
Reagent	=	5 % H_2SO_4, Δ
R_f	=	0.55

Figure 3.20 Co-TLC chromatogram of isolated compound C and reported β- sitosterol

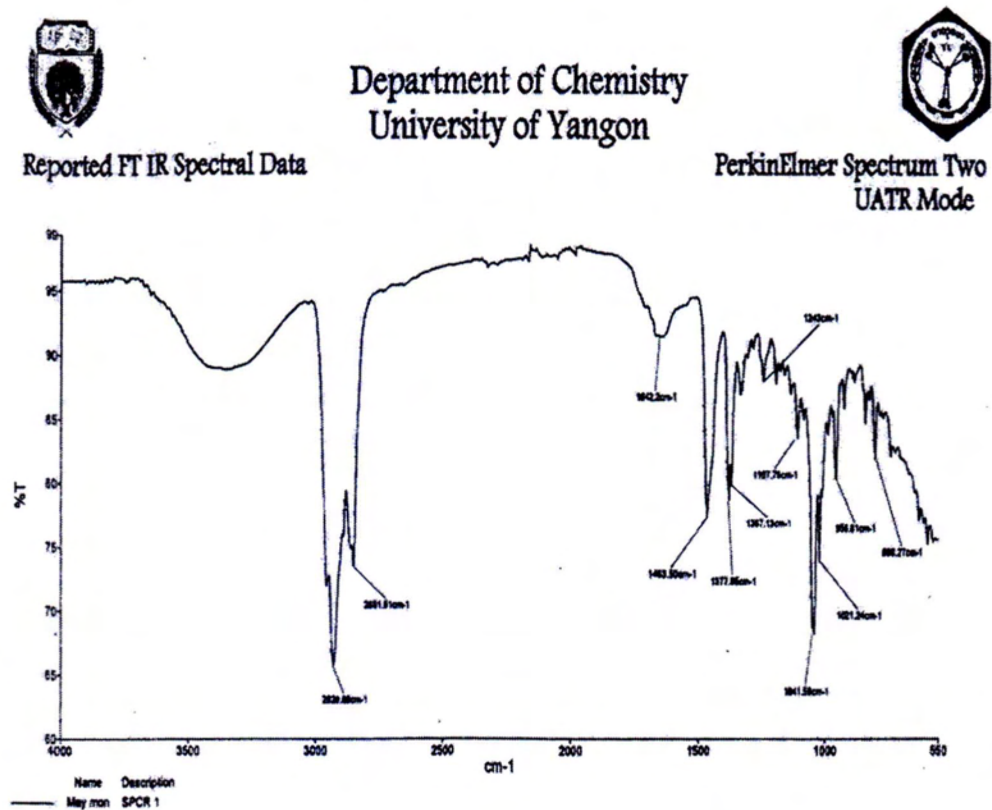


Figure 3.21 FT IR spectrum of isolated compound C

Table 3.14 FT IR Spectral Data of Isolated Compound C and Reported β-Sitosterol

Wave number (cm^{-1})		Band Assignment
Compound C	Reported β-Sitosterol	
3440	3474	ν_{O-H} of alcoholic O-H group
2939,2877	2935,2867	ν_{C-H} of asym and sym CH_3 and CH_2 groups
1643	1637	$\nu_{C=C}$ of olefinic group
1458	1465	δ_{C-H} vibration of CH_2 and CH_3 groups
1373	1377	CH_3 deformation of isopropyl group
1049	1063	ν_{C-O} of cyclic alcohol
956	958	δ_{OOP} (C-H) deformation in benzene

Arjun *et al.*, 2010.

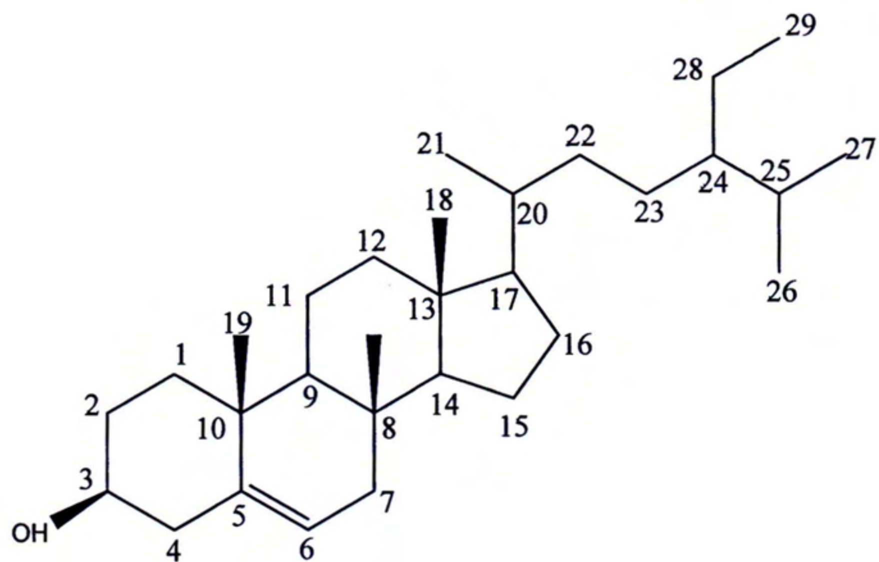


Figure 3.22 Chemical structure of β-sitosterol ($C_{29}H_{50}O$)

3.6.4 Structural elucidation of compound D

Compound **D** was obtained from EtOAc crude extract of rhizomes of *B.rotunda* (SPCR) as white powder. According to the physico-chemical properties as shown in Table 3.15, compound **D** was classified as steroidal glycoside. The melting point of compound **D** was found 272-274°C. Compound **D** was structurally identified by FT IR, ^{1}H NMR ^{13}C NMR, ^{1}H ^{1}H COSY, NOESY, HSQC and HMBC spectral data compared with the reported data.

The structure of compound **D** was also studied by FT IR, ^{1}H NMR, ^{13}C NMR and 2D NMR spectral data. The functional groups present in compound **D** were also studied by FT IR spectroscopy. FT IR spectrum of isolated compound **D** is illustrated in Figure 3.23.

According to FT IR spectrum, compound **D** must contain the functional groups such as hydroxyl group and olefinic double bond due to their respective stretching vibration frequencies of 3392cm^{-1} and 1655cm^{-1}. The band at 1071cm^{-1} appeared due to C-O-C stretching vibrational, indicating the presence of cyclic C-O-C group. Asymmetric and symmetric stretching vibration of C-H provided the bands at 2932cm^{-1}, indicating the presence of $-CH_2$ and $-CH_3$ groups.

Table 3.15 Some Physico-chemical Properties of Isolated Compound D

Experiment	Observation	Remark
UV	Inactive	No conjugated double bond
10% lead acetate	White ppt	Steroidal glycoside
Liebermann Burchard test	Green	
2,4- DNP test	No yellow ppt	C=O absent
10% $KMnO_4$	Decolourized	C=C present
5% H_2SO_4,Δ	Purple	On TLC
R_f value	0.23 ($CHCl_3$: MeOH-9:1 v/v)	Non-polar organic compound

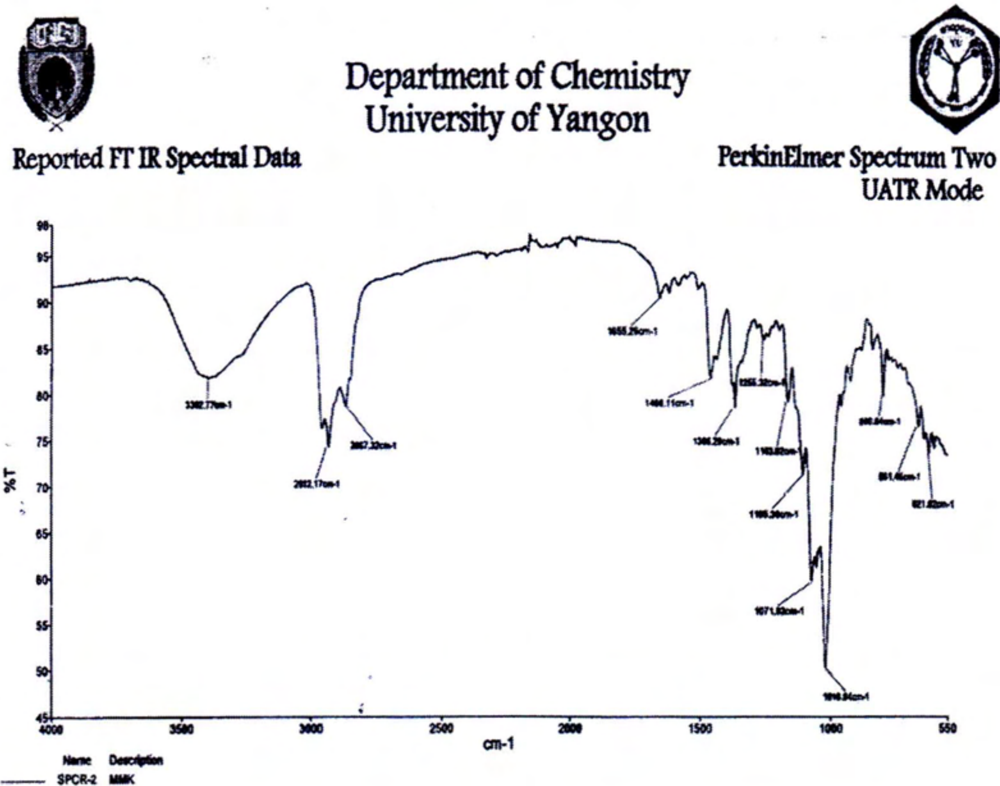


Figure 3.23 FT IR spectrum of isolated compound D

Table 3.16 FT IR Spectral Data of Isolated Compound D and Reported β-Sitosterol-β-D-glucoside

Wave number (cm^{-1})		Band Assignment
Compound D	β-Sitosterol-β-D-glucoside	
3392	3400	O-H stretching vibration of alcoholic O-H group
2932, 2867	2920, 2850	Asymmetric and symmetric –CH stretching vibration of $-CH_2$ and $-CH_3$ group
1655	1620	C=C stretching vibration of olefinic group
1460	1445	C-H bending vibration of $-CH_2$ and $-CH_3$ groups
1071, 1105	1020, 1105	C-O-C stretching vibration of ether group
1018	1028	C-O stretching vibration of alcohol group
800	795	C-H out of plane bending vibration

* Khatun *et al.*, 2012.

The ^1H NMR spectrum of compound **D** is shown in Figure 3.24. The ^1H NMR spectrum of compound **D** showed six spectra at δ. 0.68, 0.82, 0.84, 0.86, 0.93 and 1.10 ppm for methyl hydrogen ($-CH_3$) at H-18, H-29, H-27, H-26, H-21 and H-19 respectively. One proton at H-3 appeared as multiplet at 3.44 ppm and a doublet of 5.36 ppm was the characteristics of double bond in the ring between quaternary carbon and methine carbon H-5 and H-6. The presence of glycoside moiety was confirmed by the resonance between 2.41 ppm and 4.40 ppm. The doublet signal at δ 4.40 ppm with *J*=8 Hz was related to the C-1′ proton which appeared at downfield, indicating that the glucose unit must be attacked by ether linkage (C-O-C) between C-1′ and the aglycone moiety. It is meant that compound **D** may be steroidal-O-glycoside. The signals at the resonance of δ 3.27, 3.24, 3.28 and 2.41 ppm were appeared due to the carbinol methine proton (-CHOH-) of glucose unit. ^1H ^1H COSY spectrum and ^1H ^1H NOESY spectrum of compound **D** are shown in Figures 3.26 and 3.27.

In the ^1H ^1H COSY spectrum, an oxygenated methine protons at δ_H 4.40 (H-1′, *J*=8Hz) correlated with another oxygenated methine proton at δ 3.38 ppm (H-3). Similarly, an oxygenated methine proton at δ 3.58 ppm (H-5′) correlated with oxygenated methylene proton δ 2.41 ppm (H-6′). These ^1H ^1H COSY correlations inferred the position of glucose unit.

The ^{13}C NMR spectrum of isolated compound **D** is shown in Figure 3.25. Methyl corbons are C-18, C-19, C-21, C-26, C-27, C-29 and appeared at 11.79, 18.61, 19.15, 18.61, 19.61, 11.68 ppm. Methylene carbons are C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23, C-28 and appeared at 37.12, 29.50, 42.20, 39.63, 20.93, 39.63, 25.97, 28.09, 38.60, 29.05, 22.94 ppm. Methine carbons are C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24, C-25 and appeared at 79.08, 122.06, 31.75, 49.46, 55.94, 56.62, 36.59, 49.24, 25.97 ppm and quaternary carbons are C-5, C-10, C-13 and appeared at 139.97, 36.00, 45.74 ppm respectively. The glucose unit contained six carbons of which oxygenated carbon C-1′ appeared at 100.96 ppm and methylene carbon C-6′ appeared at 70.06 ppm. The other four carbons of the glucose molecule were appeared at 75.54, 77.00, 73.43, 76.68 ppm. On the basics of HSQC spectrum (Figure 3.28), the corresponding signals were assigned as shown in Table 3.17.

Analysis of HMBC spectrum indicated the methyl group (δ_H 0.93 ppm) to be at C-20 (δ_C 36.59 ppm) and the methyl group (δ_H 0.82 ppm) to be at C-28 (δ_C 22.94 ppm) respectively. On the basis of 1D and 2D NMR spectral data, compound **D** was found to be consistent with those of β-sitosterol-β-D-glucoside are shown in Figure 3.30 (Khatun *et al.*, 2012).

Since compound **D** was found to possess 35 carbons, 60 protons and 2 oxygen atoms, its molecular formula may be assigned as $C_{35}H_{60}O_6$.

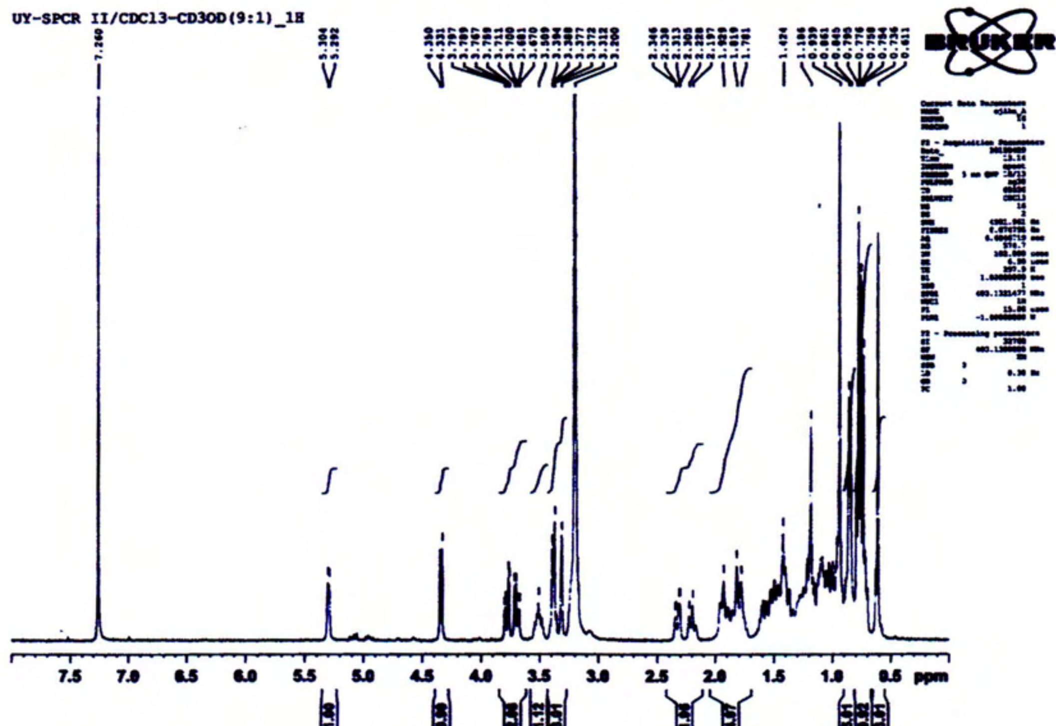


Figure 3.24 ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of isolated compound D

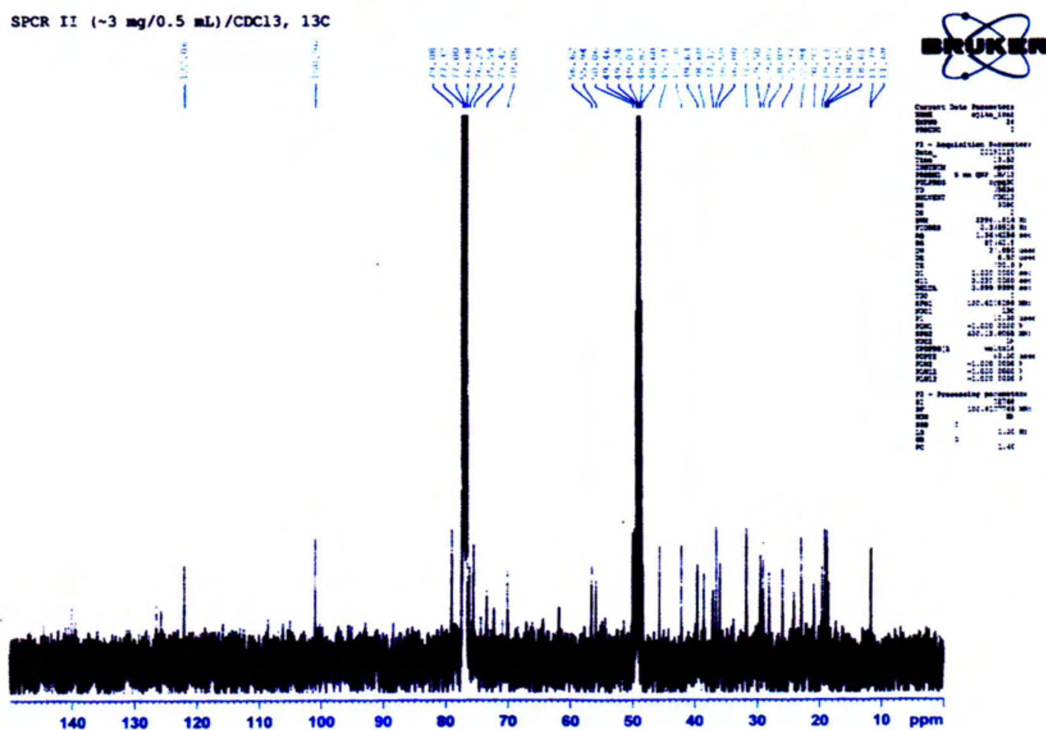


Figure 3.25 ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of isolated compound D

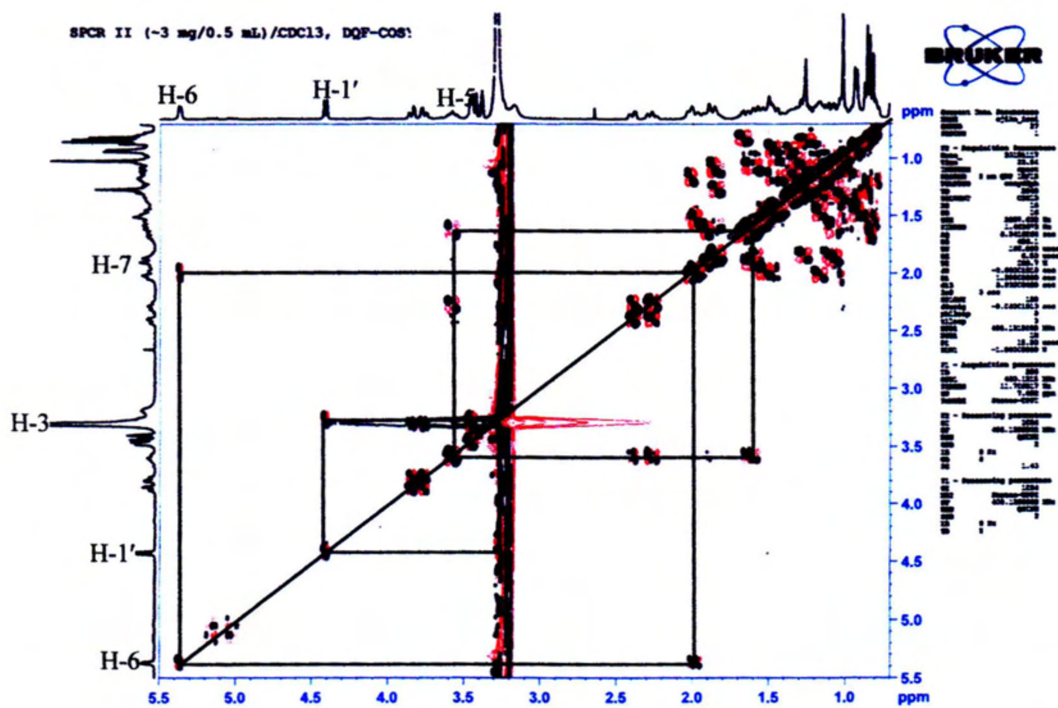


Figure 3.26 COSY (400 MHz, $CDCl_3$) spectrum of isolated compound D

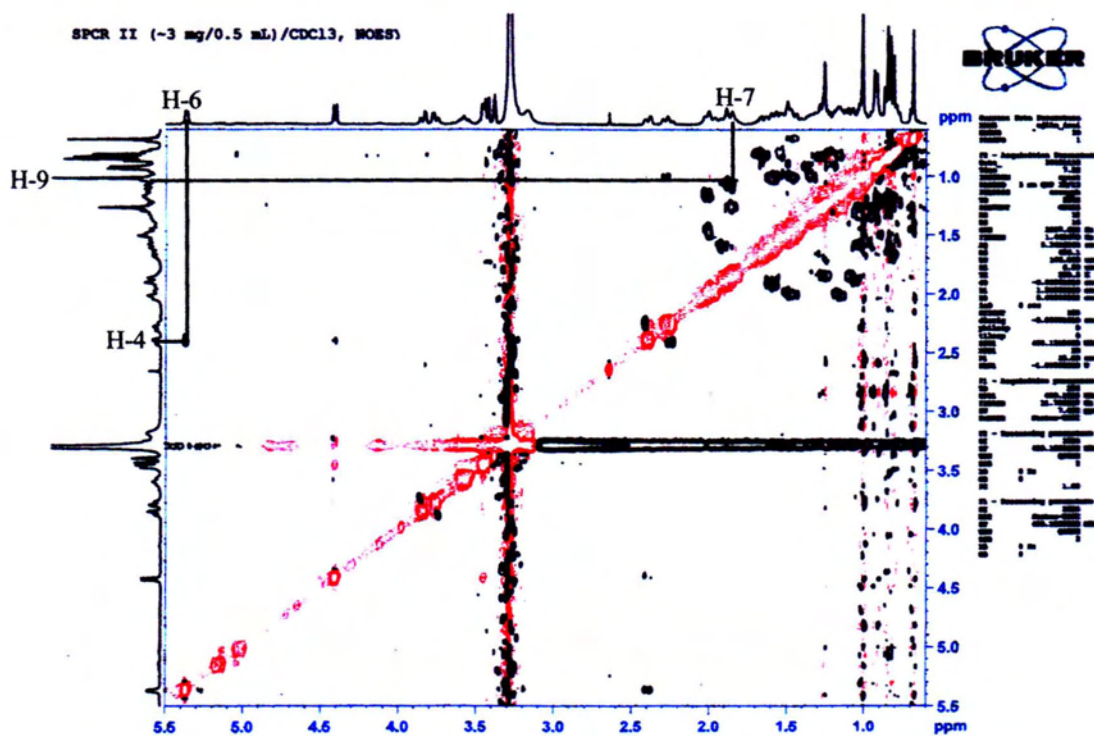


Figure 3.27 NOESY (400 MHz, $CDCl_3$) spectrum of isolated compound D

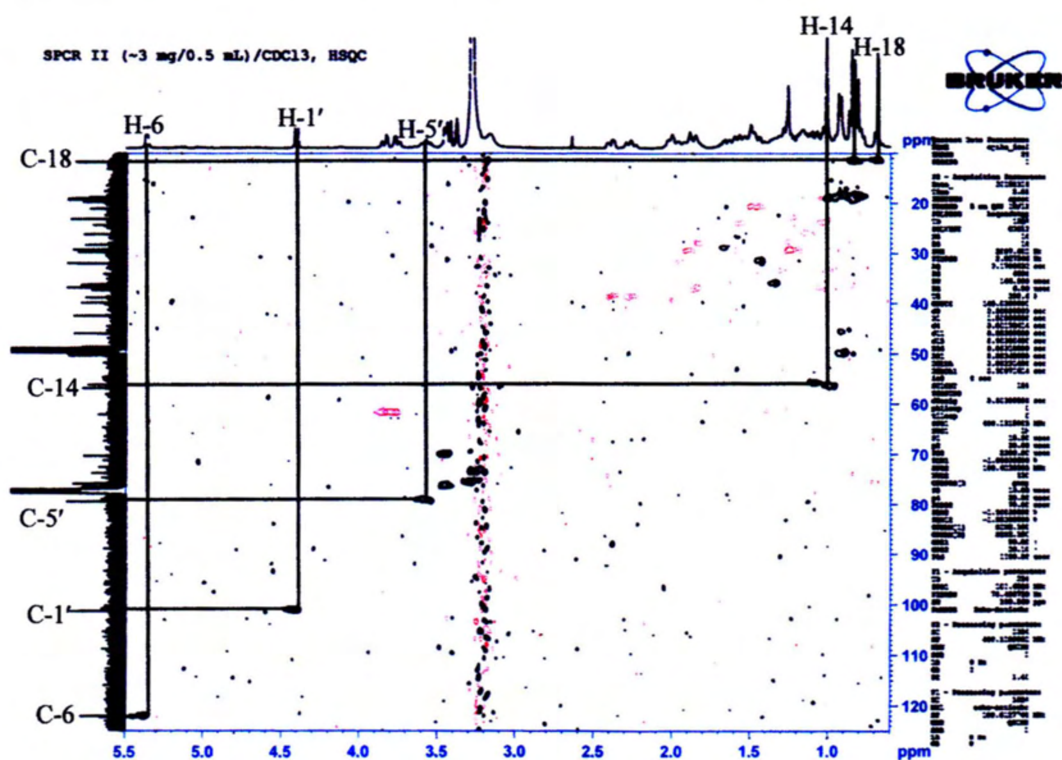


Figure 3.28 HSQC (400 MHz, $CDCl_3$) spectrum of isolated compound D

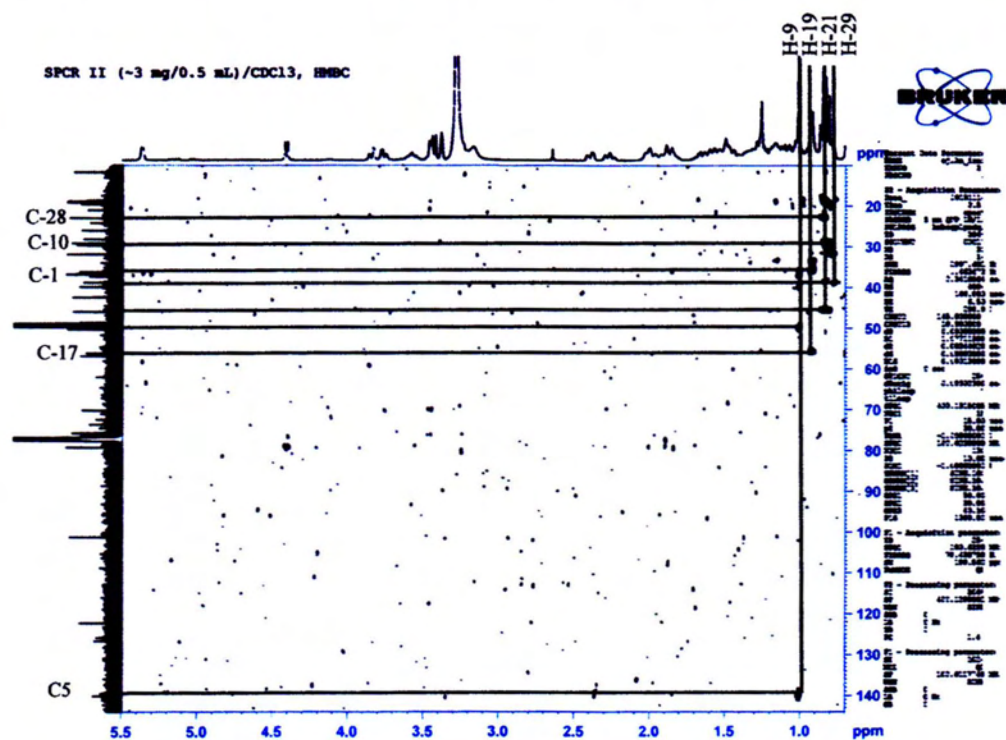


Figure 3.29 HMBC (400 MHz, $CDCl_3$) spectrum of isolated compound D

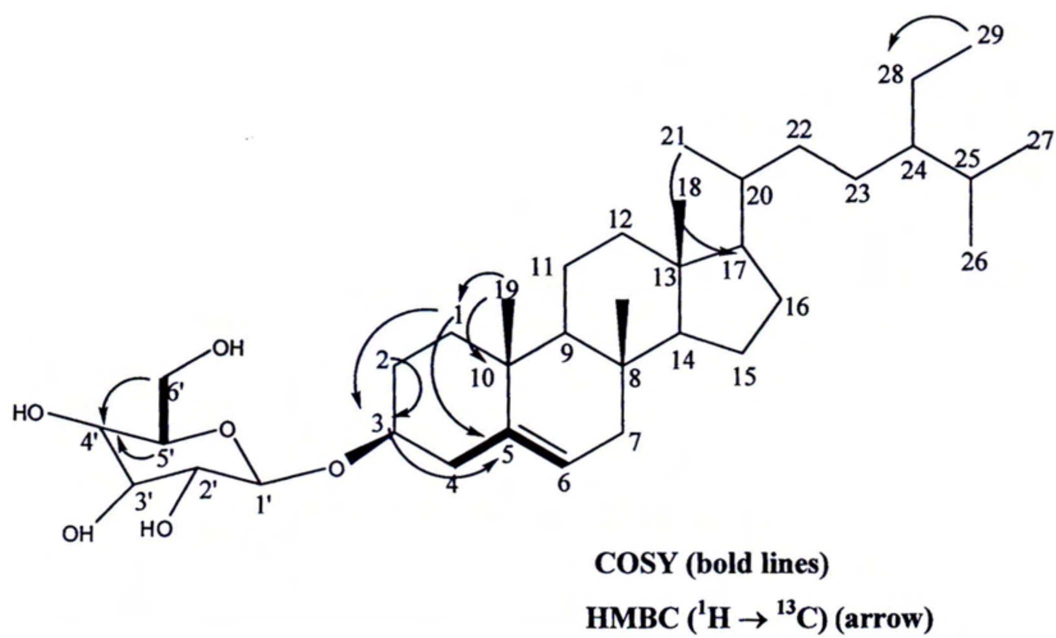


Figure 3.30 Chemical structure of β-sitosterol-β-D-glucoside ($C_{35}H_{60}O_6$)

Table 3.17 1D and 2D NMR Spectral Data of Isolated Compound D and Reported β-sitosterol-β-D-glucoside

Position	Compound D				β-sitosterol-β-D-glucoside*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_C
1	1.25 (m, 2H)	37.12		C-3, C-5	1.25 (m, 2H)	37.5
2	1.85 (m, 2H)	29.50		C-3	1.33 (m, 2H)	29.44
3	3.38 (m, 2H)	79.08		C-5	3.13 (m, 1H)	78.96
4	2.37 (m, 2H)	42.20			2.14 (m, 2H)	42.13
5		139.97				139.98
6	5.36 (s, 1H)	122.06	H-4		5.09 (bs, 1H)	122.1
7	1.88 (m, 2H)	39.63	H-8		1.73 (m, 2H)	39.20
8	1.01 (m, 1H)	31.75			1.22 (m, 1H)	31.70
9	1.01 (m, 1H)	49.46			1.22 (m, 1H)	50.01
10		36.00				35.93
11	1.85 (m, 2H)	20.93			1.33 (m, 2H)	20.85
12	1.85 (m, 2H)	39.63			1.33 (m, 2H)	39.56
13		45.74				45.68
14	1.01 (m, 1H)	55.94			1.22 (m, 1H)	55.87
15	1.88 (m, 2H)	25.97			1.73 (m, 2H)	25.9
16	1.88 (m, 2H)	28.09			1.73 (m, 2H)	28.00
17	1.88 (m, 2H)	56.62			1.73 (m, 2H)	56.56
18	0.68 (s, 3H)	11.79			0.62 (s, 3H)	11.66
19	0.93 (s, 3H)	18.61		C1-C10	0.94 (s, 3H)	18.73

Table 3.17 Continued

Position	Compound D				β-sitosterol-β-D-glucoside*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_C
20	1.25 (m, 1H)	36.59			1.32 (m, 1H)	30.91
21	0.91 (d, 3H, J=6.4Hz)	19.15		C-17	0.84 (d, 3H, J=6.3,Hz)	19.03
22	1.88 (m, 2H)	38.60			1.73 (m, 2H)	38.49
23	1.88 (m, 2H)	29.05			1.73 (m, 2H)	28.99
24	1.01 (m, 1H)	49.24			1.12 (m, 1H)	49.30
25	2.36 (m, 1H)	25.97			2.14 (m, 1H)	24.05
26	0.86 (d, 3H, J=8Hz)	18.61			0.75 (d, 3H, J=7.7Hz)	18.50
27	0.84 (d, 3H, J=1.6Hz)	19.61			0.73 (d, 3H, J=1.6Hz)	19.48
28	1.85 (m, 2H)	22.94			1.33 (m, 2H)	22.86
29	0.82 (t, 3H, J=6.8Hz)	11.68		C-28	0.77 (t, 3H, J=7.8Hz)	11.58
1'	4.40 (d, 1H, J=8Hz)	100.96	H-3		4.11 (d, 1H, J=7.8Hz)	100.92
2'	3.37 (m, 1H)	73.4			3.14 (m, 1H)	75.60
3'	3.42 (m, 1H)	74.7			3.14 (m, 1H)	77.03
4'	3.45 (m, 1H)	70.1			3.14 (m, 1H)	70.0
5'	3.44 (m, 1H)	75.5			3.35 (m, 1H)	76.70
6'	3.77 (m, 2H)	62.1	H-5'	C-4'	2.94 (m, 1H)	61.66

* Khatun *et al.*, 2012

3.6.5 Structural elucidation of compound E

Compound **E** was isolated as a pale yellow amorphous powder in 0.26 % yield from chloroform extract of rhizomes of *B. rotunda* (SPCR) and it has the melting point of 96-98°C. Its R_f value was found to be 0.41 with n-hexane: CH_2Cl_2 (3:1 v/v) solvent system and it was UV active compound. The optical activity of compound **E** is $[\alpha]_D^{25} -31$ (*C* 0.1, MeOH). Compound **E** may be classified as flavonoid due to appearance of pink colouration when the compound was dissolved in ethanol and then treated with concentrated HCl and Mg ribbon. It was observed that carbonyl group was present due to positive 2,4-DNP test. It gave a brown spot on TLC chromatogram while spraying with 10% $FeCl_3$, a yellow spot with iodine vapour and an orange spot with 1 % $Ce(SO_4)_2$/10 % H_2SO_4 followed by heating. Some physico-chemical properties of compound **E** are described in Table 3.18. Compound **E** was calssified as a flavonoid.

Its structure was also studied by UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectral data.

The UV spectrum (Figure 3.31) of compound **E** showed the two strong absorptions bands at 288 and 328 nm in MeOH, indicating the presence of conjugated double bond. The corresponding spectral data are shown in Table 3.19. These absorption wavelengths were observed to be identical with those of pinostrobin (Ching *et al.,* 2007).

The FT IR spectrum of compound **E** is shown in Figure 3.32 and the interpreted spectral data are illustrated in Table 3.20. The absorption bands appeared at 3060 cm^{-1} and 3033 cm^{-1} were due to O-H stretching of alcoholic and phenolic O-H groups which O-H bending was appeared at 1303cm^{-1}. The absorption band at 1159 cm^{-1} was due to C-O stretching of alcoholic and phenolic OH groups. The bands at 2849 cm^{-1} showed asymmetric and symmetric C-H stretching vibration of CH_2 group and their C-H bending vibration occurred at 1443cm^{-1}. The band at 1647 cm^{-1} showed stretching vibration of α, β-unsaturated carbonyl group. The band at 1580 cm^{-1}, 1523 cm^{-1} and 1443 cm^{-1} suggested the stretching vibration of C=C of aromatic groups. The absorption bands appeared at 1259 cm^{-1} and 1092 cm^{-1} was due to the stretching vibration of C-O-C in Ar-O group. A broad absorption band ranging between 888-572 cm^{-1} showed the out of plane bending deformation of C-H in benzene.

Table 3.18 Some Physico-chemical Properties of Isolated Compound E

Experiment	Observation	Remark
UV (254 nm)	Active	Presence of conjugated double bond
I_2 vapour	Yellow	C=C present compound
Mg/HCl in EtOH	Pink	Flavonoid compound
10% $FeCl_3$ solution	Brown	Phenolic-OH present
2, 4-DNP solution	Yellow ppt	C=O present
1% Ce $(SO_4)_2$/10%H_2SO_4,Δ	Orange	On TLC
R_f value	0.41 (*n*-hexane:CH_2Cl_2-3:1v/v)	Moderately polar organic compound

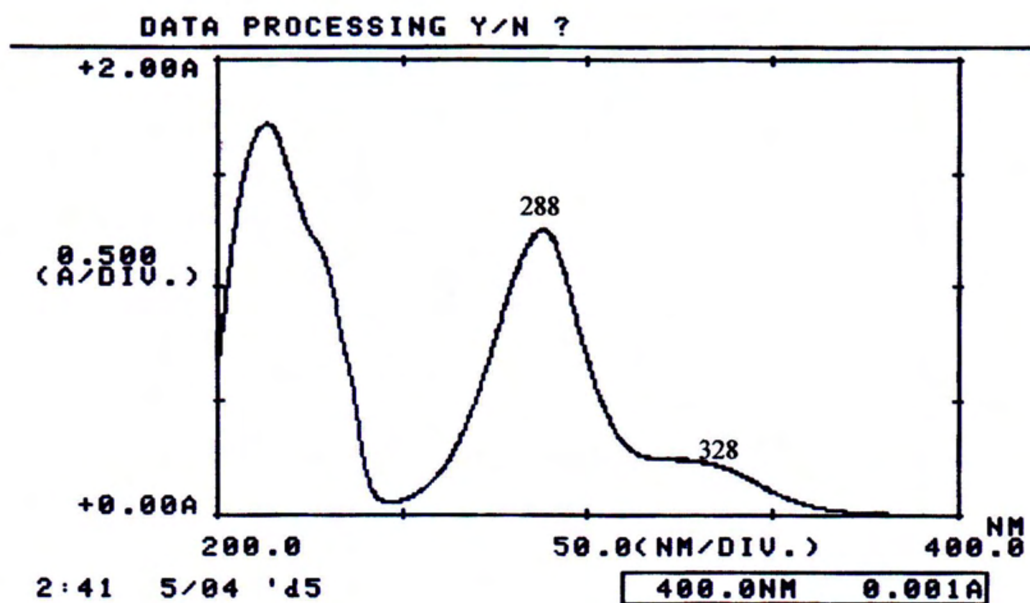


Figure 3.31 UV spectrum of isolated compound E in MeOH

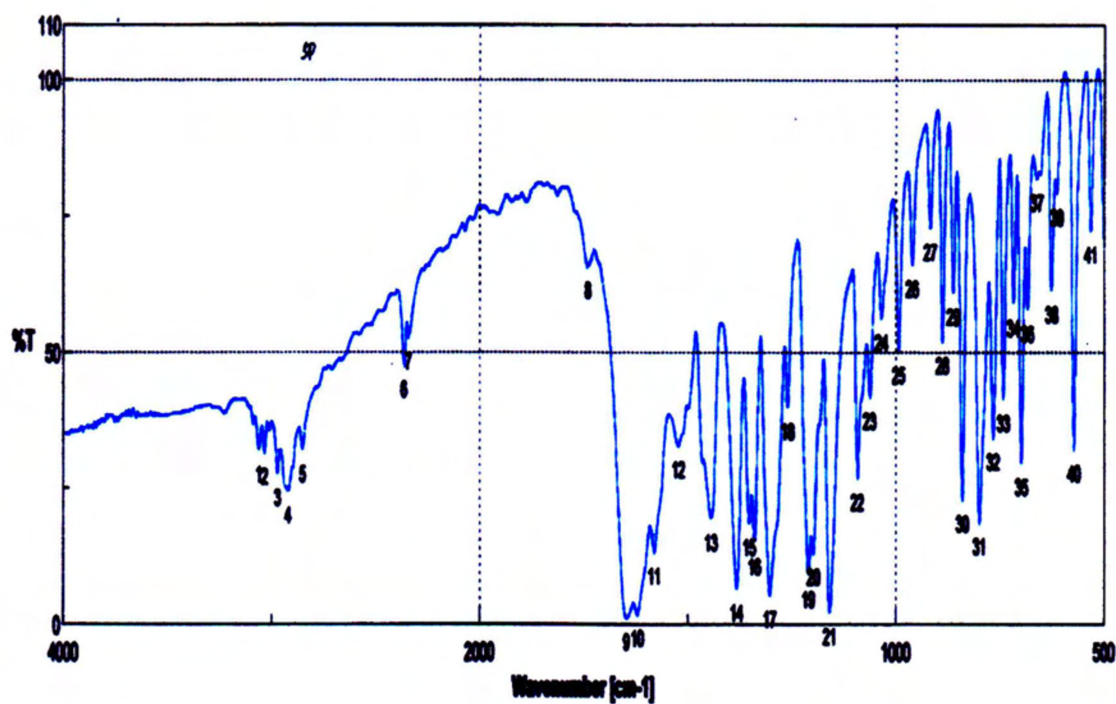


Figure 3.32 FT IR spectrum of isolated compound E

Table 3.19 UV Spectral Data Assignment of Isolated Compound E and Reported Pinostrobin

Solvent	λ_{max} (nm)		Remark
	Compound E	Pinostrobin*	
MeOH	288	289	Band II (275-295 nm) for ring A
	328	327	Band I (300-300 nm) for ring B & C

* Ching *et al.,* 2007

Table 3.20 FT IR Spectral Data of Isolated Compound E

Wave number (cm^{-1})	Band Assignment
3060, 3033	O-H stretching vibration of alcoholic O-H and phenolic O-H group
2971, 2919	C-H stretching vibration of asymmetric and symmetric CH_3 and OCH_3 group
2849	C-H stretching vibration of asymmetric and symmetric CH_2 group
1647	C=O stretching vibration of carbonyl group
1580, 1523, 1443	C=C stretching vibration of aromatic group
1382	C-H bending vibration of $-CH_2$ group
1339	O-H bending vibration in plane of hydroxyl group
1259, 1092	C-O-C stretching vibration in aromatic –O– group
1159	C-O stretching vibration in C-OH group
840, 741	C-H out of plane bending vibration deformation in benzene

The ^{1}H NMR spectrum of compound **E** is shown in Figure 3.33. A one-proton singlet at δ_H 12 ppm sited the free hydroxyl at position 5 on ring A. A five-proton multiplet at δ_H 7.44 ppm confirmed the non-substitution of the B-ring. Two doublets at δ_H 6.07 (J= 2.3 Hz) and 6.09 (J=2.3 Hz) attributable to meta H-6 and H-8 of ring A, placed the methoxyl group on C-7. In the ^{1}H NMR spectrum, a methoxy group was seen as a singlet at δ_H 3.82 ppm. The ^{1}H NMR spectrum shows the typical characteristics of flavonone according to three double-doublet signals of H-3 protons and H-2 proton with ABX coupling system.

The X part appeared as a doublet-doublet for a proton centred. δ_H 5.43 ppm (J=13, 3 Hz) attributable to H-2 and the AB portion, as two doublet-doublet of one proton each at δ_H 2.83 (J= 17, 3Hz) and 3.10 (J=17, 13 Hz) attributable to H-3 (cis) and H-3 (trans) respectively.

The ^{13}C NMR spectrum shows 16 carbons signals including one methyl, one methylene, 8 methine and 6 quaternary carbon atoms. Among these signals the carbon signal of methoxy group is clearly assigned due to the presence of chemical shift 55.6. Assigned and confirmed by using HMBC technique, 6 carbon atoms of mono-substituted aromatic ring (ring B) are located at δ_C 138.3 (C-1′), 126.1 (C-2′, C-6′) and 128.8 (C-3′, C-4′ and C-5′). The spectrum showed 6 singals of carbon in Ring A, located at δ_C 103.1(C-4a), 162.7(C-5), 95.1 (C-6), 167.9 (C-7), 94.2 (C-8) and 164.1 (C-8a). At ring C, the chemical shift of a carbonyl carbon was assigned at δ_C 195.7 (C-4) and two carbon signal (C-2) and C-3) were located at δ_C 792 and 43.3 respectively. On the basic of HMBC spectrum, each proton signal correlated with corresponding carbon. The ^{1}H NMR, ^{13}C NMR and HMBC spectral data are described in Table 3.21.

From ESI MS spectrum, the spectral data of isolated compound **E** is indicated the presence of a molecular ion peak $[M+H]^+$ at m/z 271 which corresponded to the molecular formula $C_{16}H_{14}O_4$. All of the above mentioned ^{1}H NMR, ^{13}C NMR, HMBC and ESI MS spectral data were consistent with those of reported pinostrobin which is one of the constituent present in SPCR (Ching *et al.*, 2007) and the structure is illustrated in Figure 3.38.

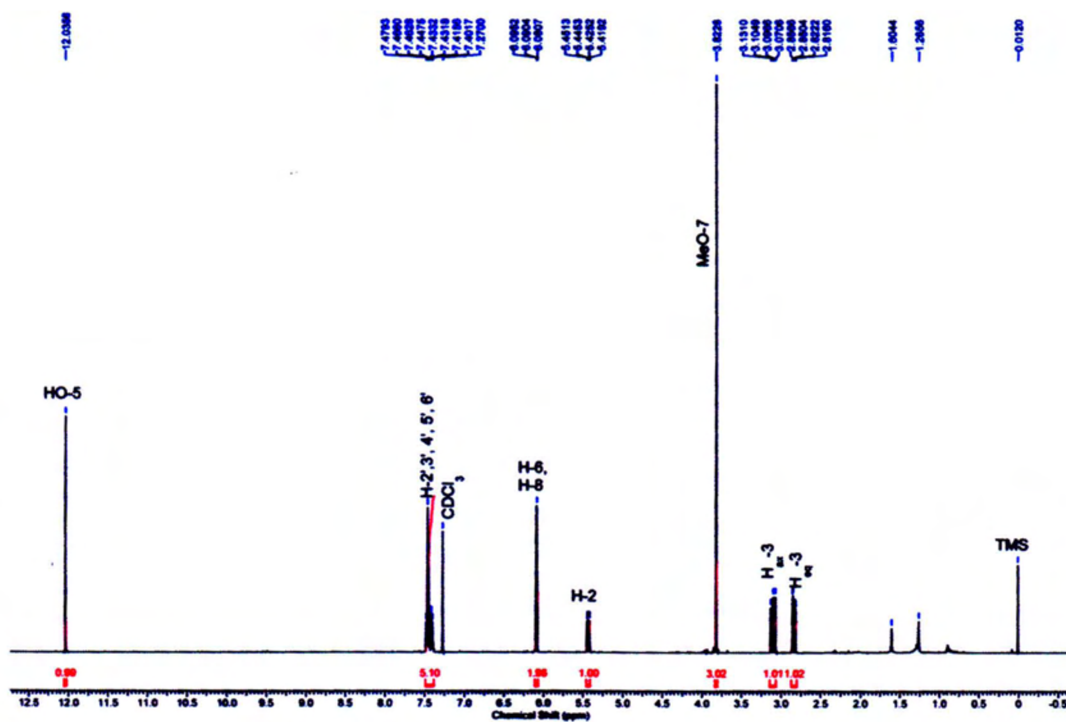


Figure 3.33 ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of isolated compound E

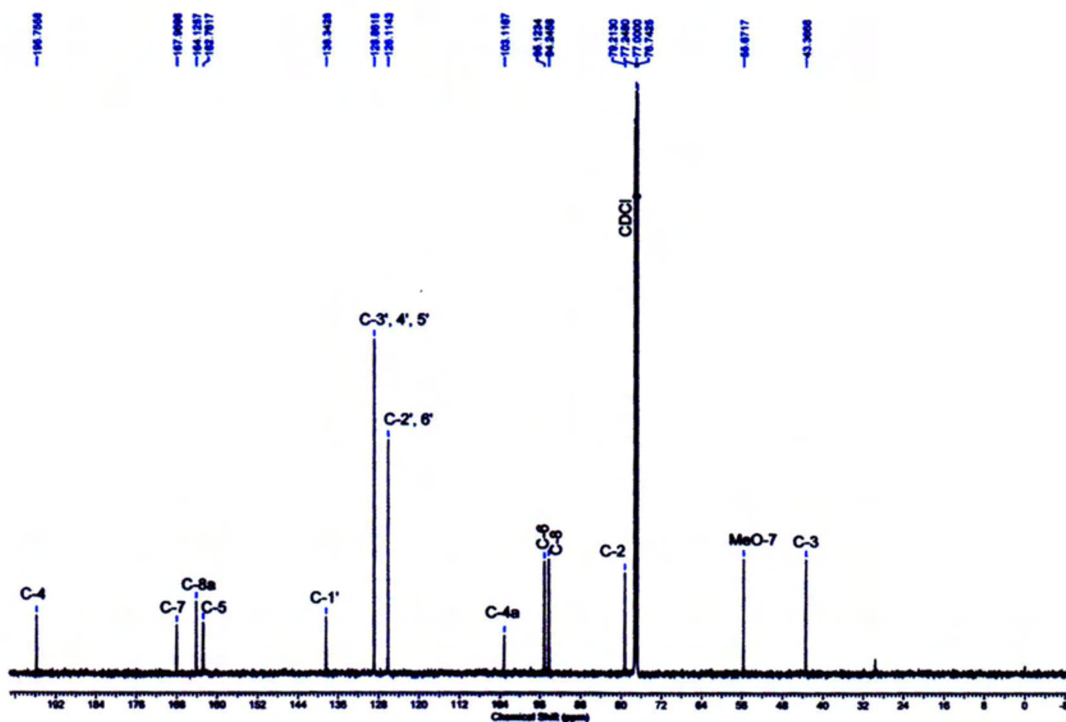


Figure 3.34 ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of isolated compound E

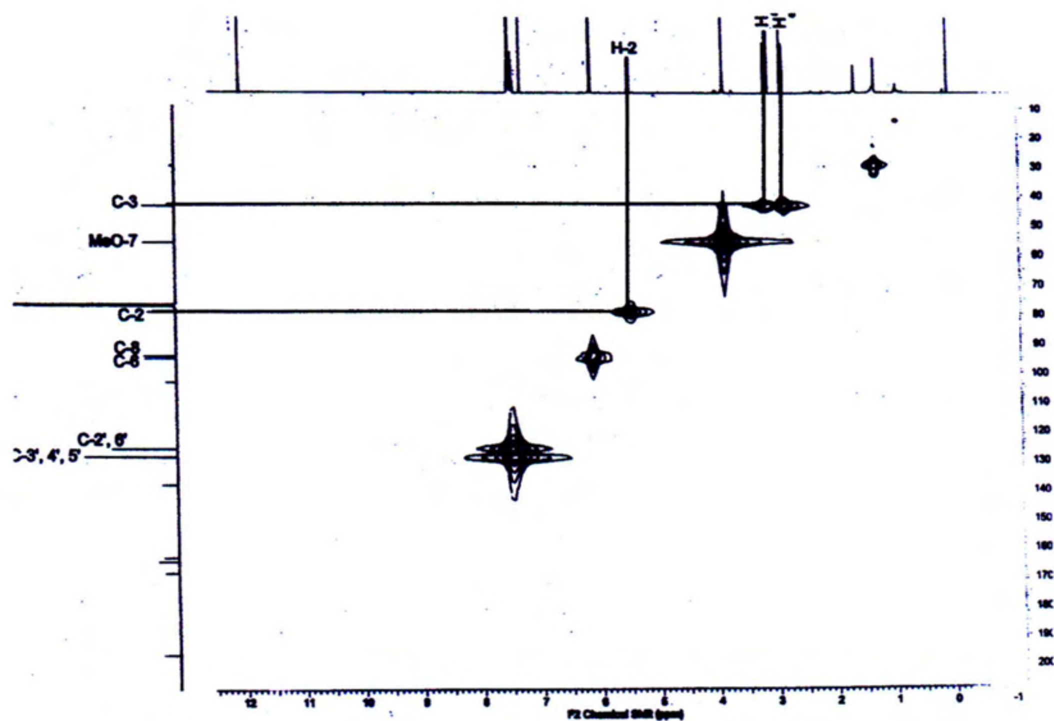


Figure 3.35 HMQC (500 MHz, $CDCl_3$) spectrum of isolated compound E

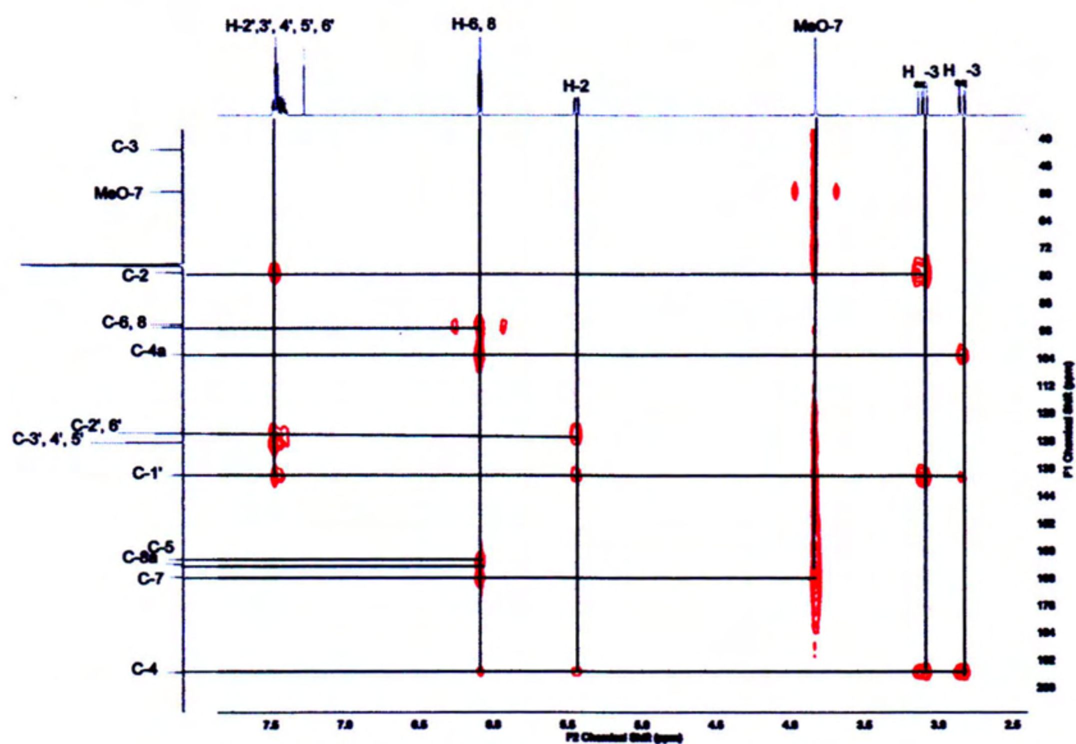


Figure 3.36 HMBC (500 MHz, $CDCl_3$) spectrum of isolated compound E

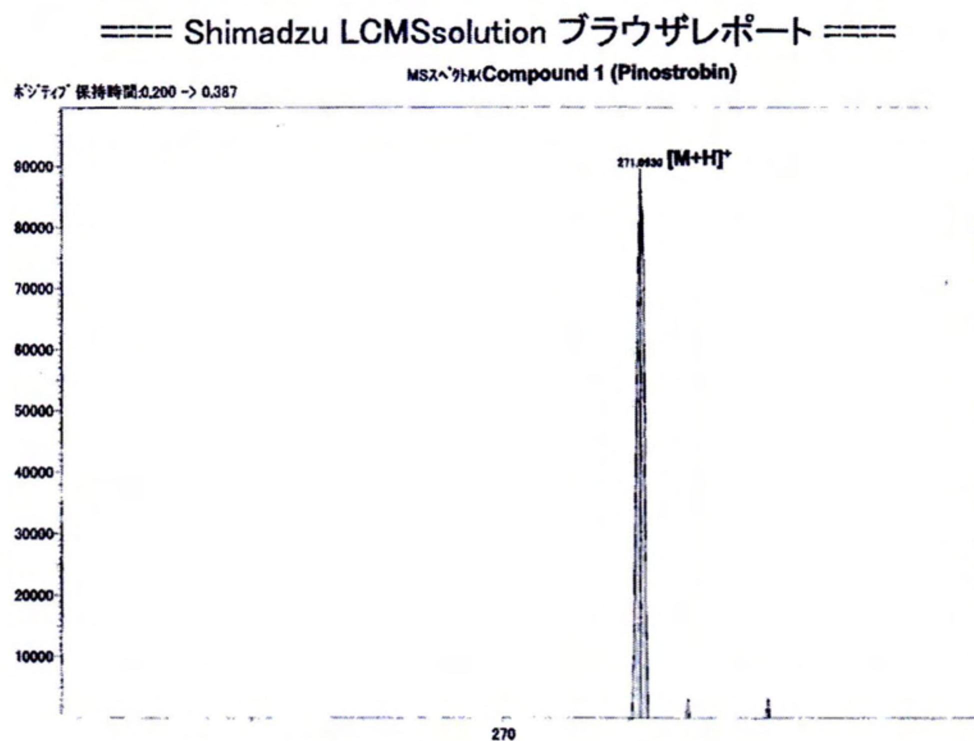
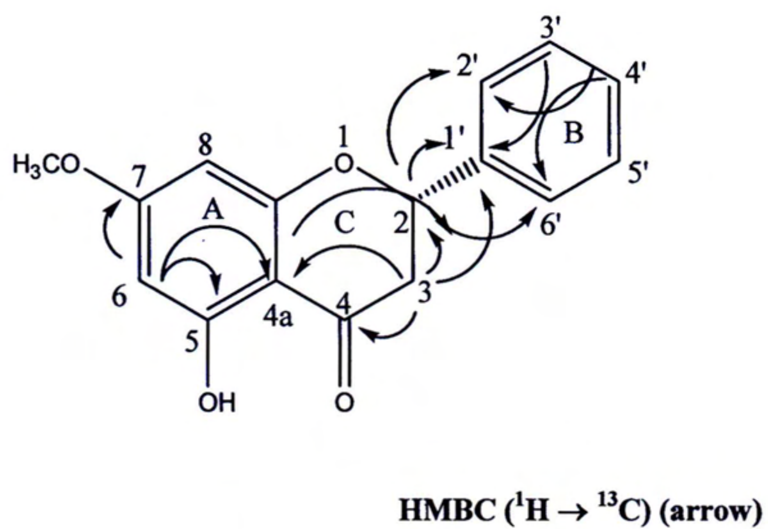


Figure 3.37 ESI MS spectrum of isolated compound E



HMBC ($^{1}H \rightarrow {}^{13}C$) (arrow)

Figure 3.38 Chemical structure of pinostrobin ($C_{16}H_{14}O_4$)

Table 3.21 **1D and 2D NMR Spectral Data of Isolated Compound E and Reported Pinostrobin**

Position	Compound E			Pinostrobin *	
	δ_H	δ_C	HMBC	δ_H	δ_C
2	5.43 (1H, dd, 13.0, 3.0)	79.2	C-2′,6′,1′	5.39 (1H, dd, 12.84, 2.76)	79.0
3_{ax}	3.10 (1H, dd, 17.0, 13.0)	43.3	C-2′,1′,4	3.06 (1H, dd, 15.14, 12.84)	43.2
3_{eq}	2.83 (1H, dd, 17.0, 3.0)		C-4a	2.79 (1H, dd, 14.68, 2.76)	
4		195.7			195.7
4a		103.1			103.0
5		162.7			182.7
6	6.07 (1H, d, 2.3)	95.1	C-4a, 5, 7	6.05 (1H, d, 2.72)	95.0
7		167.9			167.8
8	6.09 (1H, d, 2.3)	94.2	C-4a, 5, 7	6.05 (1H, d, 2.72)	94.1
8a		164.1			164.0
1′		138.3			138.3
2′	7.44 (1H, m)	126.1		7.41 (1H, m)	126.0
3′	7.44 (1H, m)	128.8	C-1′	7.41 (1H, m)	126.0
4′	7.44 (1H, m)	128.8	C-2′, 6′	7.41 (1H, m)	128.8
5′	7.44 (1H, m)	128.8		7.41 (1H, m)	126.0
6′	7.44 (1H, m)	126.1	C-2	7.41 (1H, m)	126.0
HO-5	12.03 (1H, s)			12.0 (1H, s)	
CH_3O-7	3.82 (3H, s)	55.06	C-7	3.79 (3H, s)	55.6

* Ching *et al.*, 2007

3.6.6 Structural elucidation of compound F

Compound **F** was isolated as a yellow needles in 0.25 % yield from $CHCl_3$ extract of rhizomes of *B.rotunda* (SPCR) and it has the melting point of 179-182°C. It was soluble in chloroform, ethyl acetate, acetone, methanol, ethanol and water. Its R_f value was found to be 0.45 with n-hexane: EtOAc (4:1 v/v) solvent system and it was UV active compound. The optical activity of compound **F** is $[\alpha]_D^{25} +34$ (*C* 0.1, MeOH). Compound **F** may be classified as flavonoid due to appearance of pink colouration when the compound was treated with concentrated HCl and Mg ribbons. It was observed that carbonyl group was present due to positive 2,4-DNP test. It gave a brown spot on TLC chromatogram while spraying with 10% $FeCl_3$ indicating the presence of phenolic group. In addition, it also gave a yellow spot with iodine vapour and an orange spot with 1 % Ce $(SO_4)_2$/ 10 % H_2SO_4, followed by heating the TLC chromatogram. Some physico-chemical properties of compound **F** are described in Table 3.22.

The structure of compound **F** was studied by UV-visible and FT IR, NMR and Mass spectral data. The UV spectrum (Figure 3.39) was recorded in MeOH. The maximum absorption (λ_{max}) in methanol of compound **F** were found to be 255 nm and 364 nm due to π-π* (K-band) and n $\rightarrow$ π* (R-band) transitions indicating the characteristic of double bond conjugation in flavonol skeleton.

The functional groups present in compound **F** were also studied by FT IR spectroscopy. The FT IR spectrum is shown in Figure 3.40 and the interpreted spectral data are shown in Table 3.24. The FT IR spectrum of compound **F** showed the absorption bands at 3651 cm^{-1} and 3311 cm^{-1} due to –OH stretching vibration of alcoholic and phenolic O-H group. The band at 3020 cm^{-1} showed CH stretching vibration of =C-H of aromatic ring. 2924 cm^{-1} is due to the presence of CH_3, CH groups. The absorption band at 1662 cm^{-1} indicated the C=O stretching for pyrrone ring. The bands at 1619 cm^{-1} and 1507 cm^{-1} suggested that the stretching vibration of C=C of aromatic groups. The absorption bands at 1260 cm^{-1} appeared due to the stretching vibration of C-OH and asymmetric and symmetric –C–O bending at 1117 cm^{-1} and 1037 cm^{-1}. The broad band at 879-728 cm^{-1} suggested the out-of-plane bending of aromatic –OH group. A broad absorption band at 701-525 cm^{-1} showed the out-of-plane bending detormation of –CH in benzene.

Table 3.22 Some Physico-chemical Properties of Isolated Compound F

Experiment	Observation	Remark
UV	Active	Presence of conjugated double bond
I_2 vapour	Yellow	C=C present
Mg/HCl in EtOH	Pink	Flavonoid compound
10% $FeCl_3$ solution	Brown	Phenolic-OH present
2,4-DNP solution	Yellow spot	C=O present
1% Ce $(SO_4)_2$/10%H_2SO_4,Δ	Orange	On TLC
R_f value	0.45 (n-hexane: EtOAc-4:1 v/v)	Polar organic compound

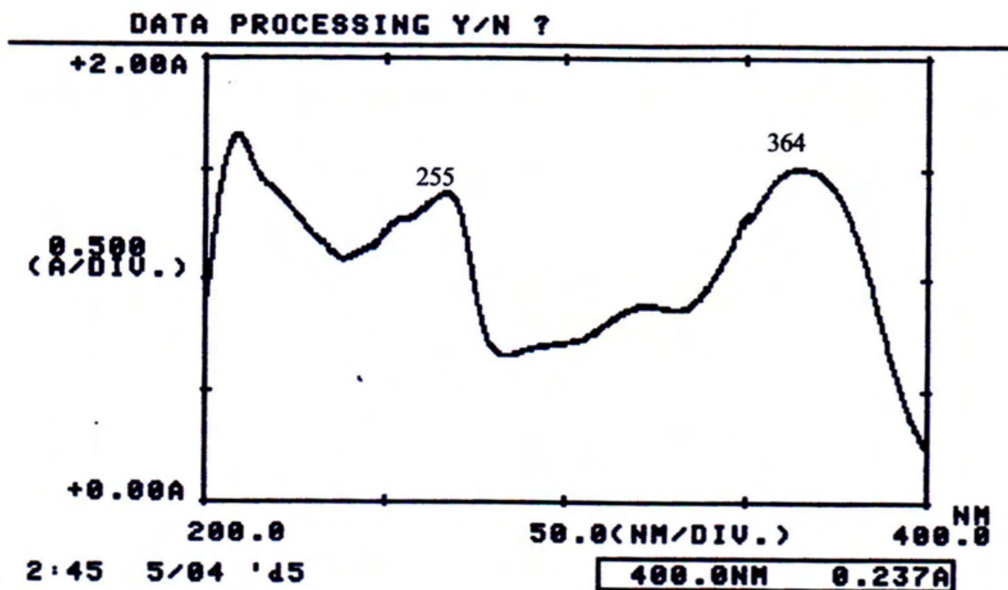


Figure 3.39 UV spectrum of isolated compound F (MeOH)

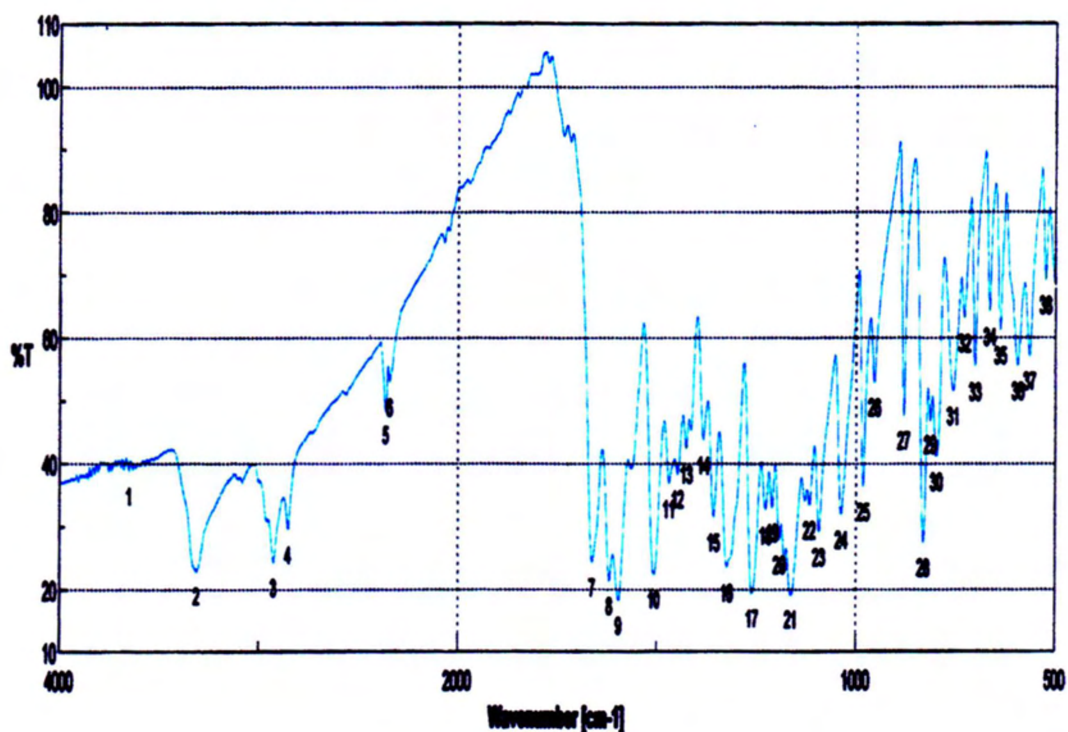


Figure 3.40 FT IR spectrum of isolated compound F

Table 3.23 UV Spectral Data Assignment of Isolated Compound F and Reported 4′,7-Dimethylkaempferol

Solvent	λ_{max} (nm)		Remark
	Compound F	4′,7-dimethyl kaempferol*	
MeOH	255	265	$\pi \rightarrow \pi^*$ (K band)
	364.2	365	$\pi \rightarrow \pi^*$ (R band)

* Oliverira *et al.,* 2012

Table 3.24 FT IR Spectral Data of Isolated Compound F

Wave number (cm^{-1})	Band Assignment
3651, 3311	O-H stretching vibration of alcoholic O-H and phenolic O-H group
3020	=CH stetching vibration of aromatic ring
2924	C-H stretching vibration of CH_3 and CH groups
1662	C=O stretching vibration for pyrrone ring
1619, 1507	C=C stretching vibration of aromatic ring
1383	O-H bending vibration of aromatic ring
1260	Stretching vibration of C-OH
1117	Asymmetric-C–O bending vibration of aromatic –CO group
1037	Symmetric –C–O bending vibration of aromatic –CO group
879-728	symmetric –C–O bending vibration of aromatic –CO group
701-525	Out of plane C-H bending deformation in benzene

The ^1H NMR spectrum of compound **F** is shown in Figure 3.41. The ^1H NMR spectrum showed signals for flavonol substituted at ring A at H-6 and H-8 [δ_H 6.38 and 6.50 (d, J=2.2 each) respectively] and ring B at H-3′, H-5′ and H-2′, H -6′ [δ_H 7.05 and 8.98 (d, J=9.1 each) respectively]. In addition, a signal for two methoxyl groups at (δ_H 3.90) and a hydroxyl group in hydrogen bond at δ_H 11.7 were observed.

The ^{13}C NMR spectrum of compound **F** is shown in Figure 3.42. The ^{13}C NMR spectral data displayed signals for fifteen carbon atoms, which chemical shifts are compatible with the presence of a flavonol at δ_C 135.7 (C, C-3) substituted at ring A δ_C 97.9 and 92.2 (CH each, C-6 and C-8, respectively) and ring B at δ_C 129.4 and 114.1 (CH each, C-3′, C-5′ and C-2′, C-6′, respectively), as well as signals for one carbonyl (δ_C 175.2) and two methoxyl (δ_C 55.5 and 55.9) groups. On the basis of HMQC and HMBC spectrum (Figures 3.43 and 3.44), each proton signal correlated with corresponding carbon. The ^1H NMR, ^{13}C NMR, HMBC spectral data of compound **F** are shown in Table 3.25.

From ESI MS spectrum (Figure 3.45), the spectral data of isolated compound **F** is indicated the presence of a molecular ion peak $[M + H]^+$ at m/z 315 which corresponding to the molecular formula $C_{17}H_{14}O_6$.

All of the above mentioned ^1H NMR, ^{13}C NMR, HSQC, HMBC and ESI MS spectral data were consistent with those of reported 4′, 7- dimethylkaempferol (Oliverira *et al.*, 2012) and its chemical structure is shown in Figure 3.46.

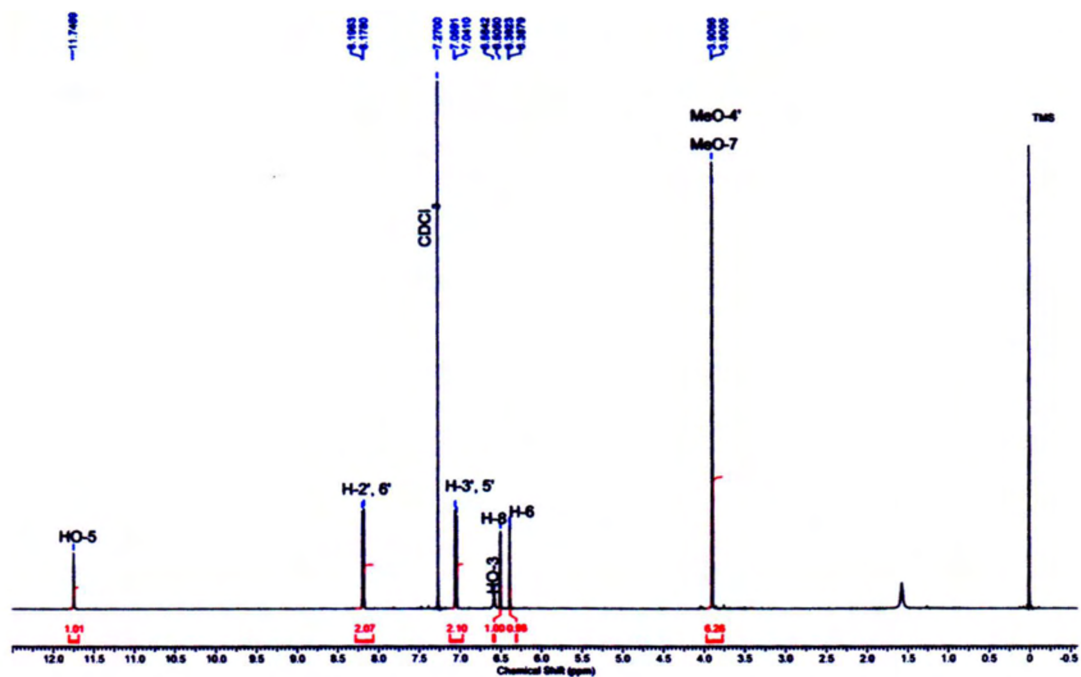


Figure 3.41 ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of isolated compound F

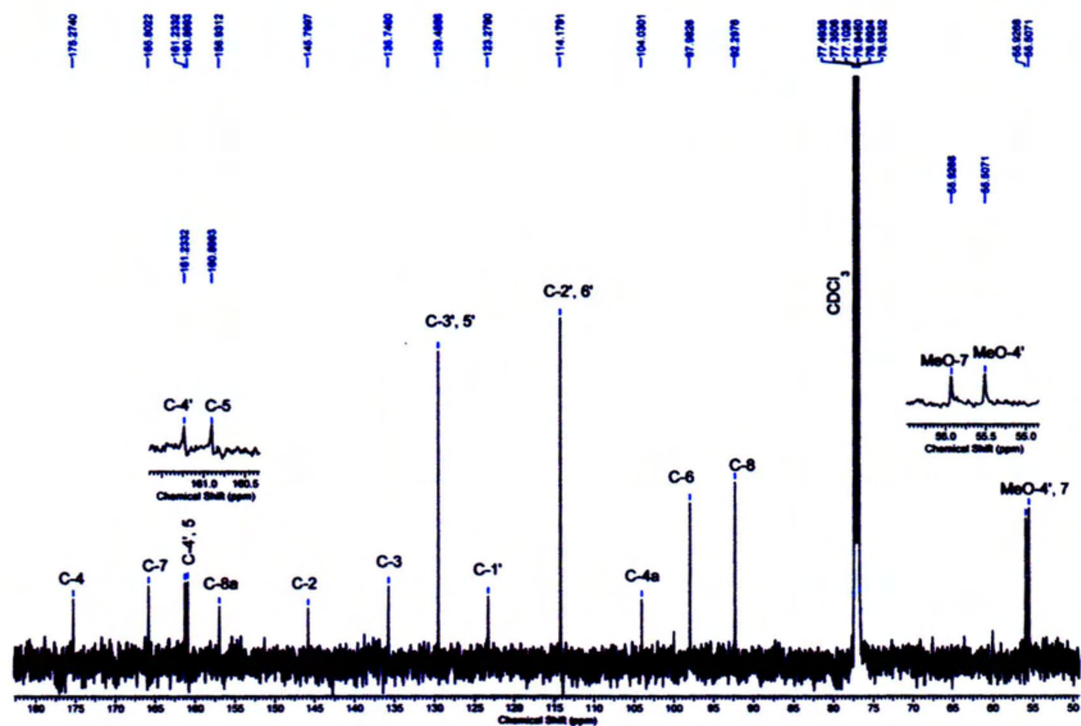


Figure 3.42 ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of isolated compound F

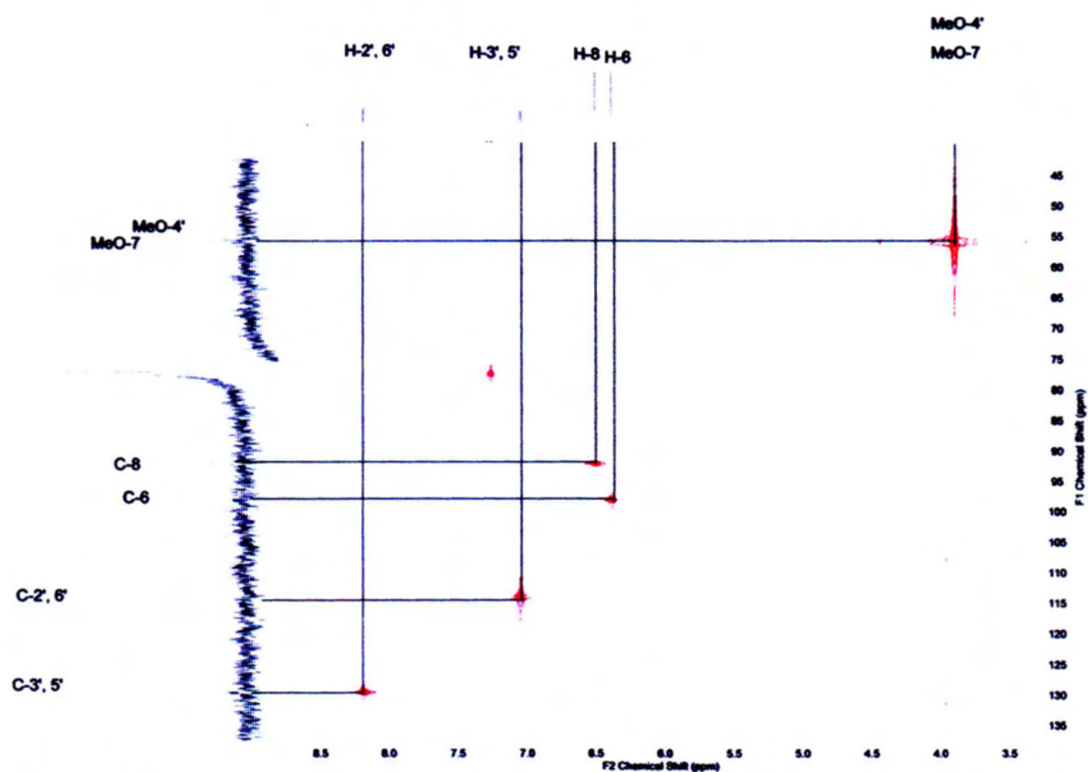


Figure 3.43 HMQC (500 MHz, $CDCl_3$) spectrum of isolated compound F

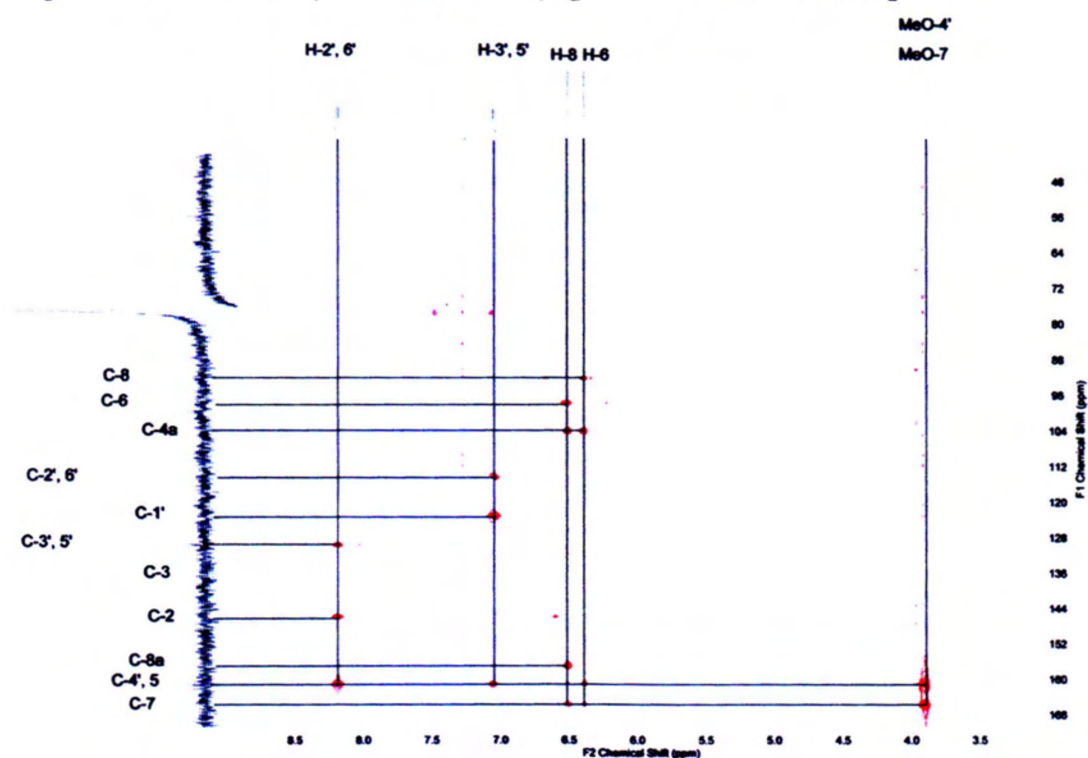


Figure 3.44 HMBC (500 MHz, $CDCl_3$) spectrum of isolated compound F

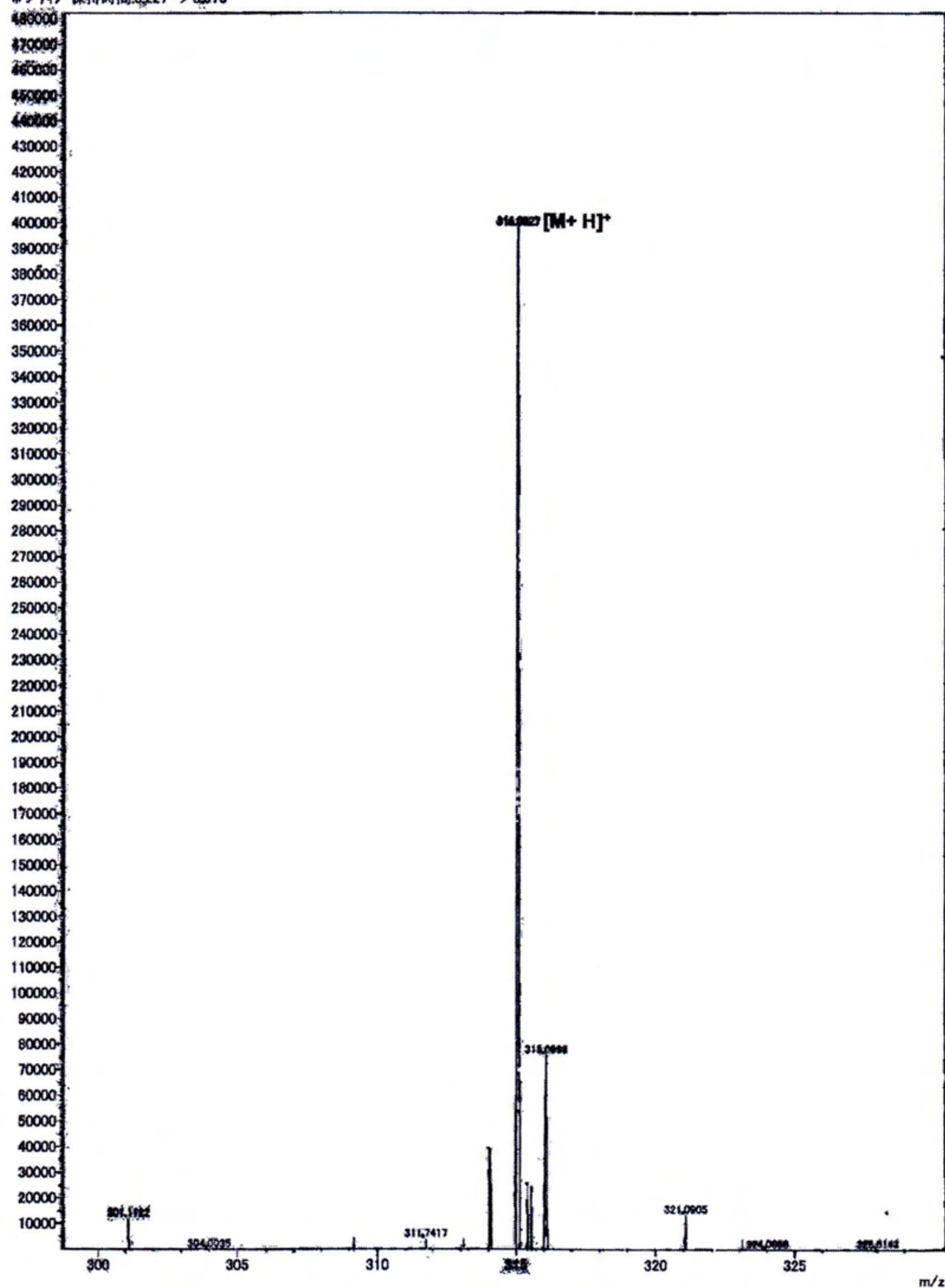


Figure 3.45 ESI MS spectrum of isolated compound F

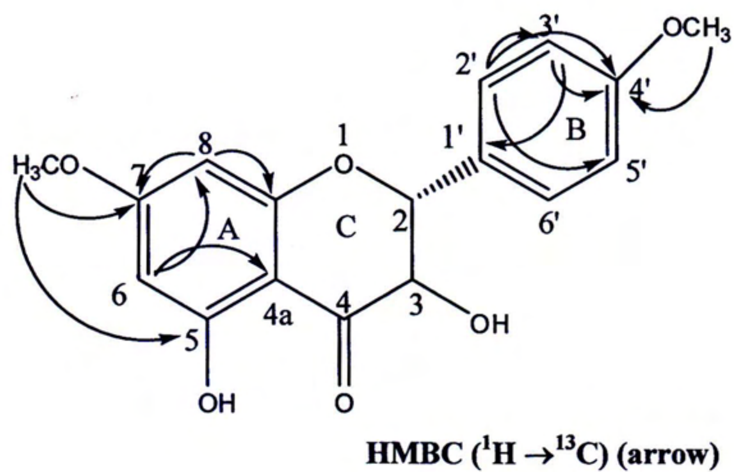


Figure 3.46 Chemical structure of 4′,7-dimethyl kaempferol ($C_{17}H_{14}O_6$)

Table 3.25 1D and 2D NMR Spectral Data of Isolated Compound F and Reported 4′,7-Dimethylkaempferol

Position	Compound F			4′,7-Dimethylkaempferol	
	δ_H	δ_C	HMBC	δ_H	δ_C
2		145.7			
3		135.7			135.67
4		175.2			175.70
4a		104.0			
5		160.8			
6	6.38, d (2.2)	97.9	C-8, C-4a	6.31, d (2.0)	92.21
7		165.8			
8	6.50, d (2.2)	92.2	C-6, C-4a, C-8a, C-7	6.42, d (2.0)	97.90
8a		156.9			
1′		123.2			
2′	8.98, d (9.1)	114.1	C-2, C-3′, C-5′, C-4′	8.09, d (8.8)	114.09
3′	7.05, d (9.1)	129.4	C-2′, C-6′, C-1′, C-4′	6.98, d (8.8)	129.40
4′		161.2			
5′	7.05, d (9.1)	129.4	C-1′, C-2′, C-4′, C-6′	6.98, d (8.8)	129.40
6′	8.98, d (9.1)	114.1	C-2′, C-3′, C-5′, C-4′	8.09, d (8.8)	114.09
HO-3	6.58, s				
CH_3O-4′	3.90, s	55.5	C-4′	3.82, s	55.43
HO-5	11.7, s			11.67, s	
CH_3O-7	3.90,s	55.9	C-7′, C-5′	3.83, s	55.85

* Oliveira *et al.*, 2007

3.6.7 Structural elucidation of compound G

Compound **G** isolated as amorphous powder in 0.43 % yield from $CHCl_3$ extract of the rhizomes of *B.rotunda* and it has the melting point of 165-167 °C. It was soluble in chloroform, ethyl acetate, methanol, ethanol, pet-ether and water. Its R_f value was found to be 0.30 in *n*-hexane: EtOAc (2:1 v/v) solvent system and it was UV active compound. The optical activity of compound **G** is $[\alpha]_D^{25} -10$ $(C\,0.1, MeOH)$. Compound **G** was assumed to be terpenoids since they gave pink colours when tested with Liebermann Burchard reagent. It was observed that aldehyde group was present due to positive 2,4-DNP test. It gave a yellow spot on TLC chromatogram while spraying with iodine vapour and a pink spot with 1 % Ce $(SO_4)_2$/ 10 % H_2SO_4 followed by heating. Some physico-chemical properties of compound **G** are described in Table 3.26.

Its structure was also studied by UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectral data.

In UV-Vis spectrum in MeOH (Figure 3.47, Table 3.27), compound **G** showed an absorption maximum at 236.6 nm ($\pi \rightarrow \pi^*$ transition). It was found to be consistent with that of galanal A (232 nm) (Morita *et al.,* 1986).

The FT IR spectrum of isolated compound **G** was also presented in Figure 3.48 and Table 3.28. The band at 3448 cm^{-1} was due to OH stretching vibration of alcoholic O-H group. The stretching bands at 2925 cm^{-1} showed asymmetric and symmetric C-H stretching vibration of CH_2 group and their C-H bending vibration occurred at 1461 cm^{-1}. The band at 1718 cm^{-1} showed normal aldehydic C=O stretching vibration. The C-H bending for –CH, $-CH_2$ and $-CH_3$ groups displayed at 1461 cm^{-1}. The peak due to C=C stretching vibration of cycloalkane occurred at 1637 cm^{-1} and 1543 cm^{-1}. The absorption band at 1051 cm^{-1} appeared due to alcoholic C-O stretching vibration of secondary alcohol. The band at 909 cm^{-1} showed out of plane C-H bending vibration. The bands at 821 and 752 cm^{-1} were appeared by out of plane aromatic C-H bending vibration. The absorption band at 660 cm^{-1} was attributed to out of plane O-H bending vibration.

Table 3.26 Some Physico-chemical Properties of Isolated Compound G

Experiment	Observation	Remark
UV_{254}	Active	Presence of double bond
I_2 vapour	Yellow	C=C present compound
Liebermann Burchard reagent,Δ	Pink	Terpenoid compound
2, 4-DNP solution	Orange ppt	-CHO present
1 % $Ce(SO_4)_2$/10 % H_2SO_4, Δ	Pink	On TLC
R_f value	0.30 (*n*-hexane: EtOAc-2:1 v/v)	Non-polar organic compound

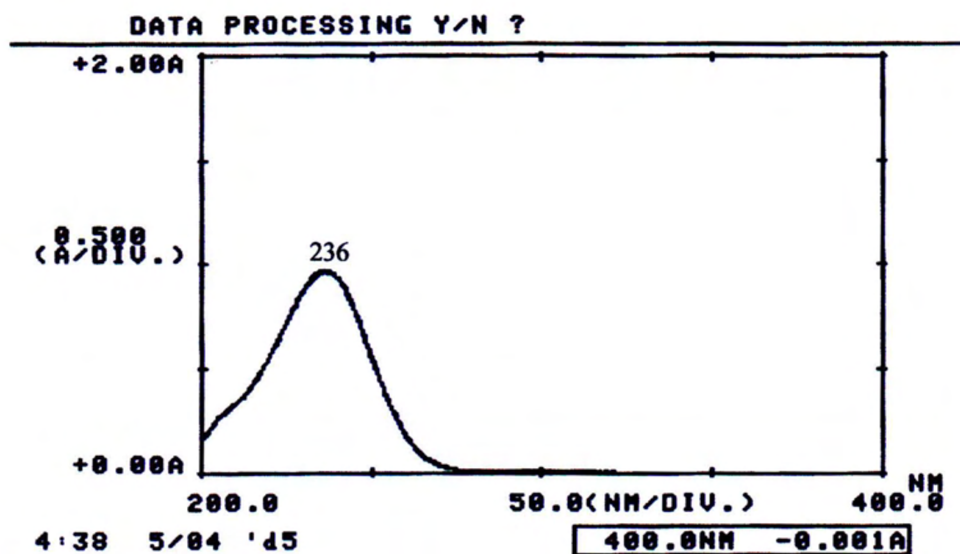


Figure 3.47 UV spectrum of isolated compound G in MeOH

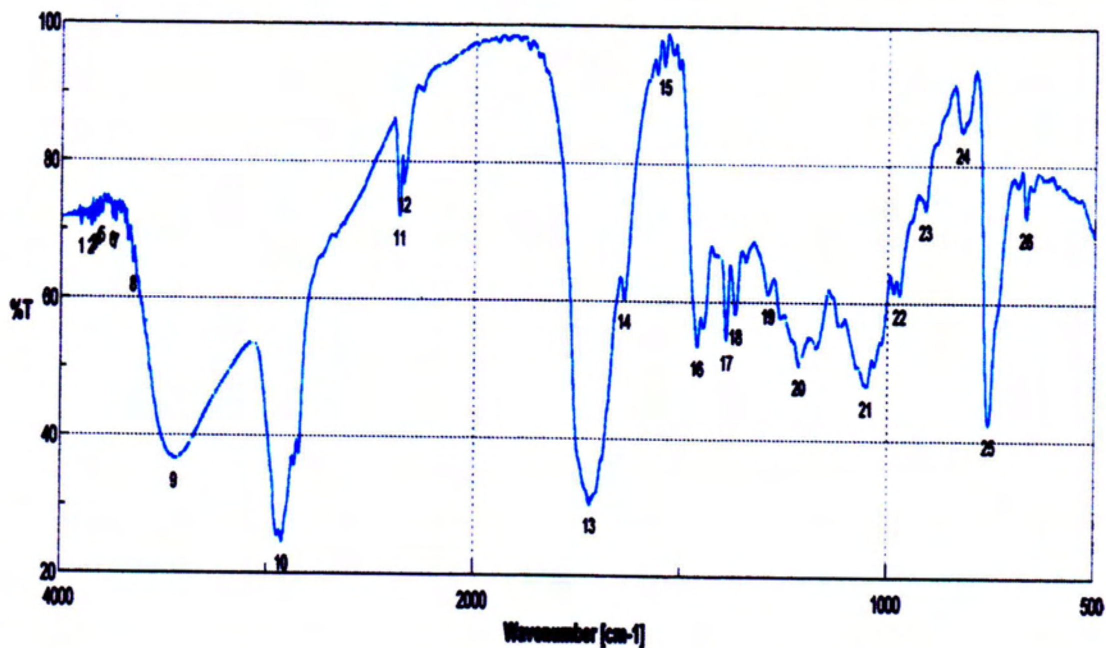


Figure 3.48 FT IR spectrum of isolated Compound G

Table 3.27 UV Spectral Data Assignment of Isolated Compound G and Reported Galanal A

Solvent	λ_{max} (nm)		Remark
	Compound G	Galanal A*	
MeOH	236.6	232	$\pi \rightarrow \pi^*$ transition

* Morita *et al.,* 1986

Table 3.28 FT IR Spectral Data of Isolated Compound G

Wave number (cm^{-1})	Band Assignment
3448	O-H stretching vibration of alcoholic O-H group
2925	C-H stretching vibration of asymmetric and symmetric CH_2 group
1718	C=O stretching vibration of normal aldehyde group
1637,1543	C=C stretching vibration of cycloalkane
1461	C-H bending vibration of $-CH_3$ and $-CH_2$ group
1389	C-H bending vibration of aldehyde group
1214	C-O-C stretching vibration
1051	Alcoholic C-O stretching vibration of secondary alcohol
971,909	C-H out of plane bending vibration
821, 755	C-H out of plane bending vibration of aromatic ring

^{1}H NMR spectrum of compound **G** is shown in Figure 3.49. It was found that there are 3 singlet signals appeared at δ_H 0.77, 0.89 and 0.93 ppm, each for 3 protons and it was indicated the presence of $3CH_3$ groups. The two singlet signals occurred at δ_H 9.38 and 10.11 ppm confirmed and presence of 2CHO groups.

The double doublet signal occurred at δ_H 4.07 ppm confirmed the presence of a secondary carbinol proton (-CHOH-) adjacent to a pair of diastereotopic methylene protons. This observation indicated that there is a $-CH_2$-CHOH-C- moiety in compound **G**. The double doublet signal at downfield, δ_H 6.89 ppm was assigned due to an olefinic proton of –C=CH-group, indicating that compound **G** possesses only an olefinic group. The remaining signals were assigned as presented in Table 3.29.

From the ^{1}H NMR spectral data assignment, compound **G** may be assumed to possess about 30 protons. ^{1}H ^{1}H COSY spectrum of isolated compound **G** is shown in Figure 3.51. The peak correlating signals at δ 2.50, 2.79, 2.65, 4.07, 6.89 ppm were observed in ^{1}H ^{1}H COSY spectrum. Aromatic methane proton δ 6.89 [dd (8.3, 1.6), H-12] was coupled with the methylene protons δ 2.50 [dd (18.5, 8.3)] and 2.79 ppm (H-11 and H_{ax}-11). Oxygenated methane proton δ 4.07 [d (8.9, 3.1) H-15] was correlated with methylene proton δ 2.65 (H-14). One methane proton δ 2.79 ppm (H-11_{ax}) was coupled with δ 2.50 ppm [dd (18.5, 8.3, H-11] and δ 1.61 ppm (H-9). Similarly, One methane proton δ 0.93 ppm (H-5) was coupled with δ 1.78 ppm (H_{ax}-6). On the basics of ^{1}H NMR and ^{1}H ^{1}H COSY spectral data, compound **G** was assumed as galanal A.

The types and number of carbons present in compound **G** were also studied by ^{13}C NMR spectrum (125 MHz, $CDCl_3$) (Figure 3.50) and the corresponding spectral data assignment is described in Table 3.29. It was observed that there are about 20 signals and therefore it was assumed to have 20 carbon atoms including two aldehyde carbon due to the signals at δ_C 193.3 and 206.5 ppm, two olefinic carbons due to the signals at δ_C 142.2 and 156.1 ppm and one carbinol carbon due to the peak appeared at δ_C 71.2 ppm. The remaining 15 carbon atoms were assigned as the sp^3 methyl, methylene, methine and quaternary carbon.

On the basis of HMQC and HMBC spectrum, (Figures 3.53 and 3.54), each proton signal correlated with corresponding carbon. The ^{1}H NMR, ^{13}C NMR, ^{1}H ^{1}H COSY, NOESY, HMBC spectral data are described in Table 3.29.

From ESI MS spectrum (Figure 3.55), the spectral data of isolated compound **G** is indicated the presence of a molecular ion peak $[M+H]^+$ at m/z 319 which corresponded to the molarcular formula $C_{20}H_{30}O_3$. All of the above mentioned UV, ^{1}H NMR, ^{13}C NMR, ^{1}H ^{1}H COSY, NOESY, HSQC, HMBC and ESI MS spectral data were consistent with those of reported galanal A (Abe *et al.*, 2002) and the structure is illustrated in Figure 3.56.

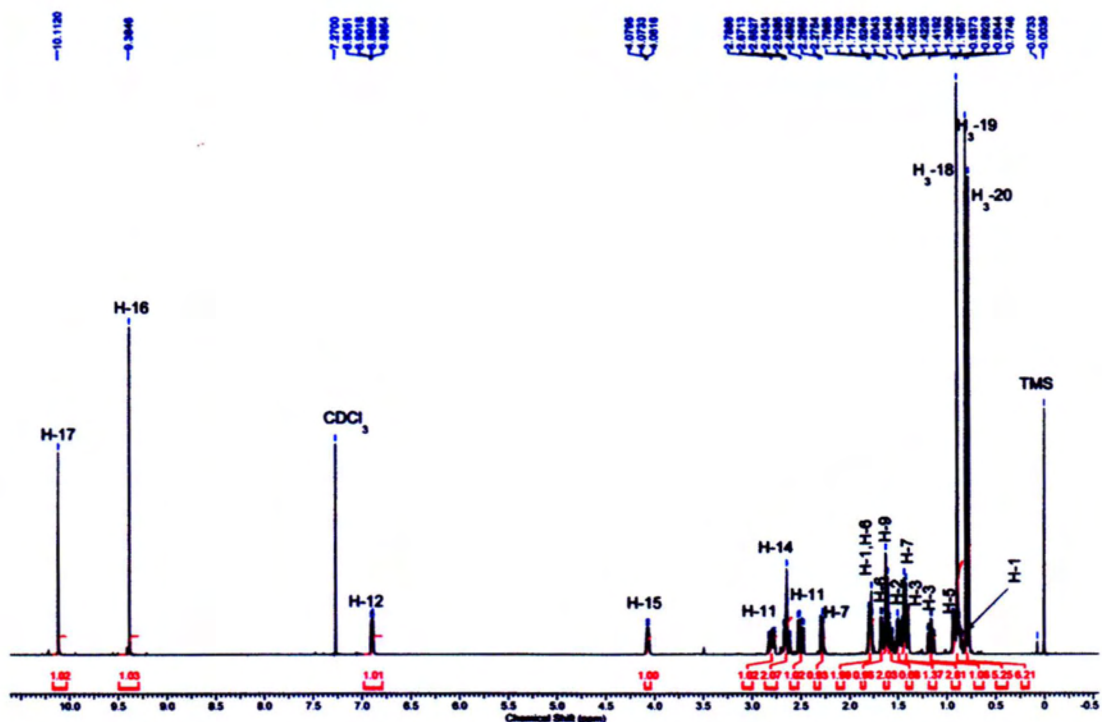


Figure 3.49 ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of isolated compound G

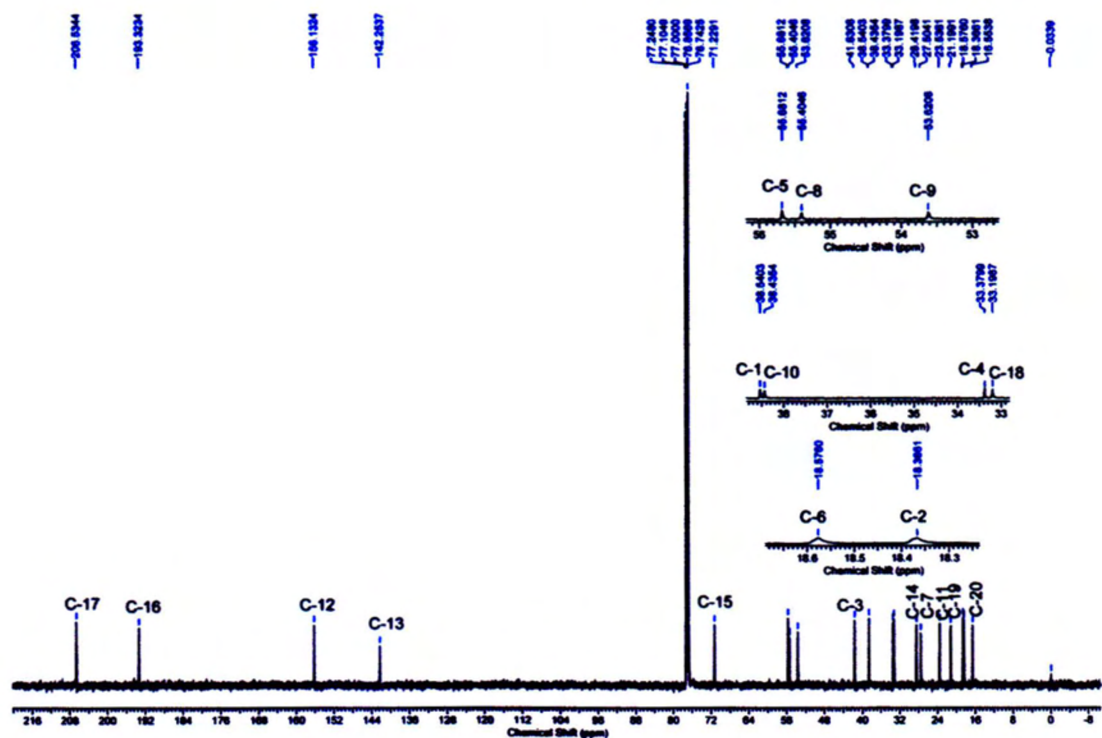


Figure 3.50 ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of isolated compound G

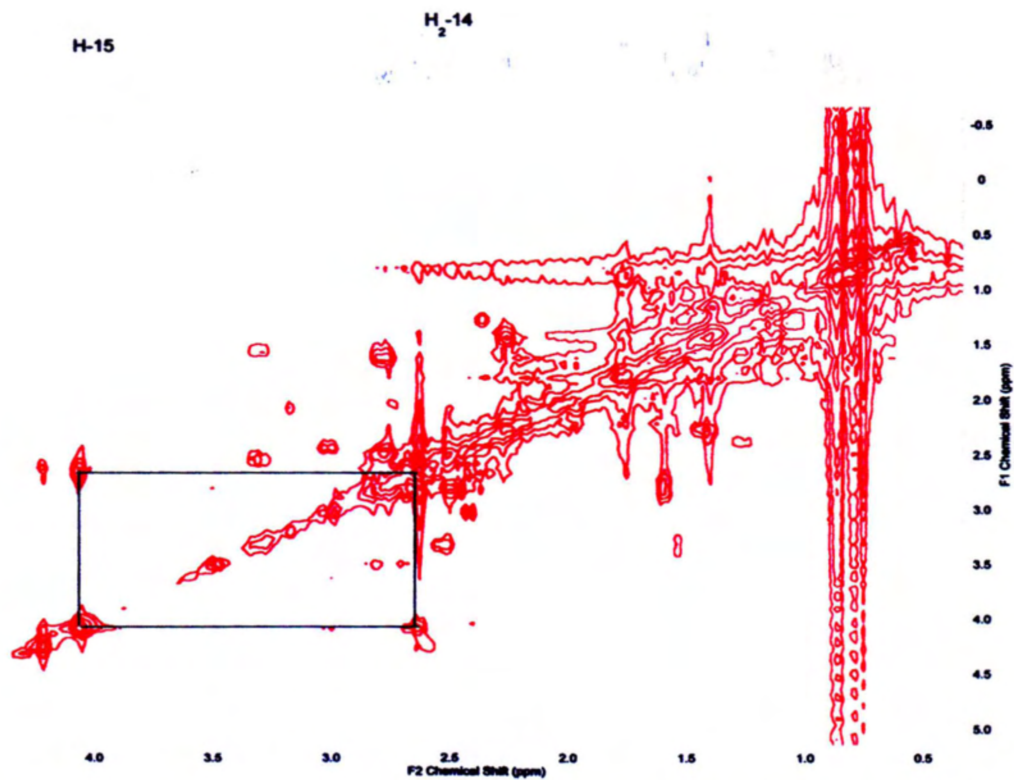


Figure 3.51 ^{1}H ^{1}H COSY (500 MHz, $CDCl_3$) spectrum of isolated compound G

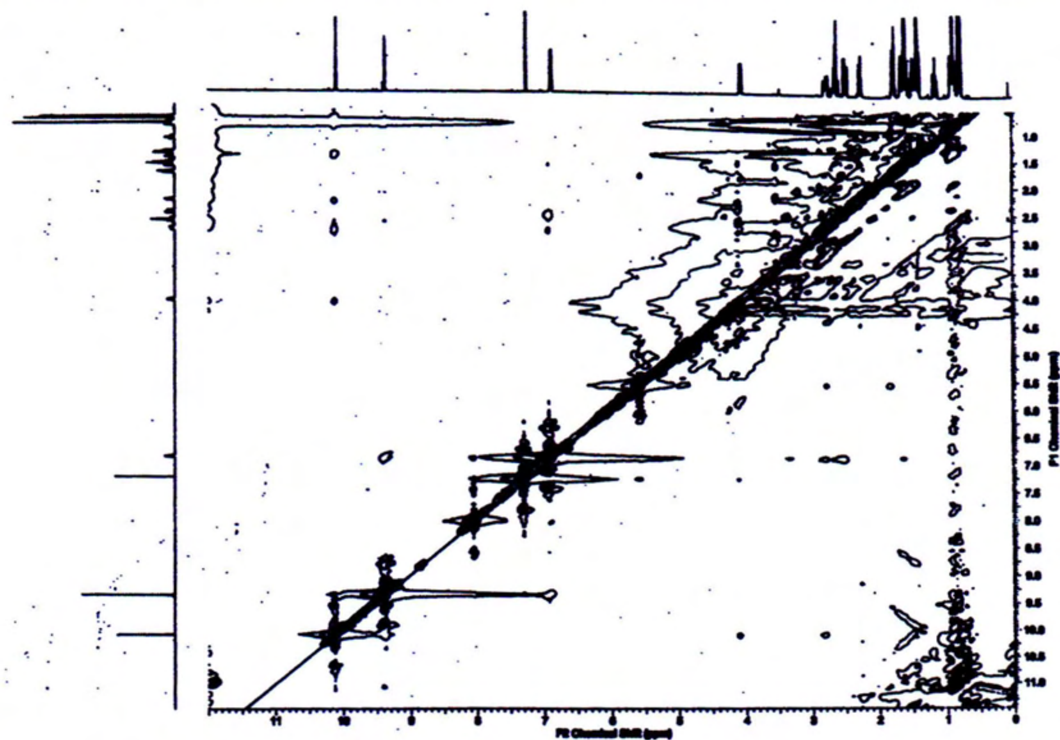


Figure 3.52 NOESY (500 MHz, $CDCl_3$) spectrum of isolated compound G

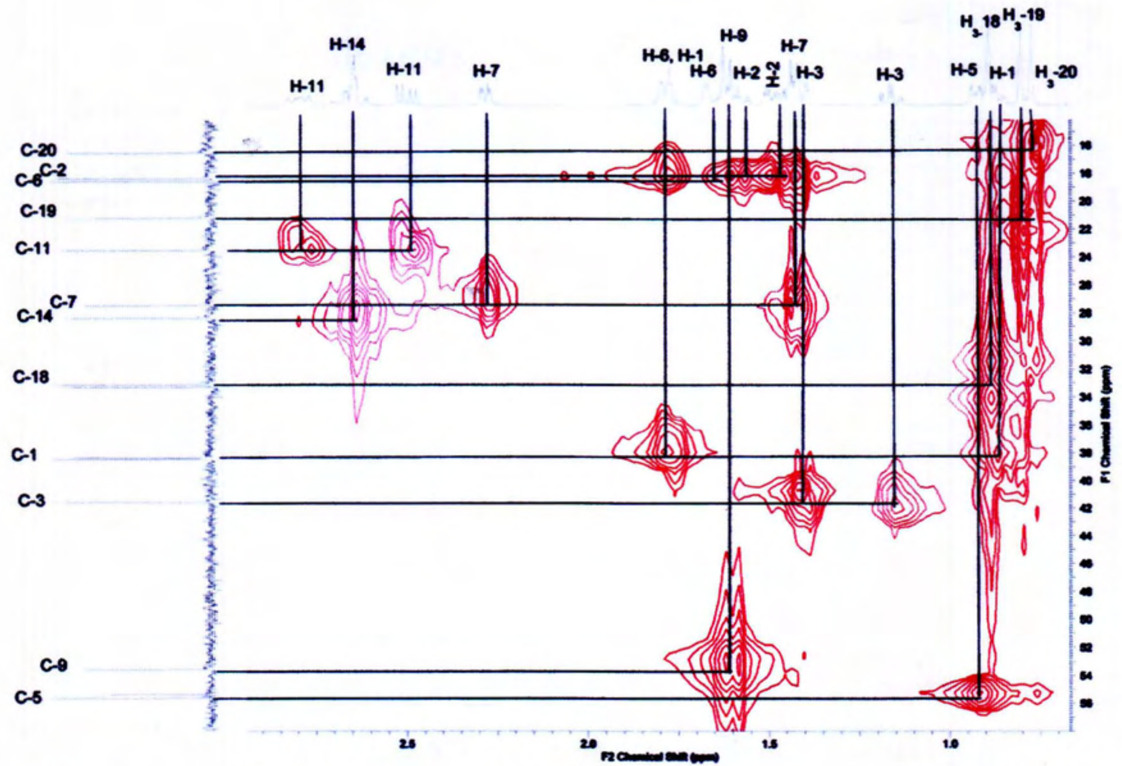


Figure 3.53 HMQC (500 MHz, $CDCl_3$) spectrum of isolated compound G

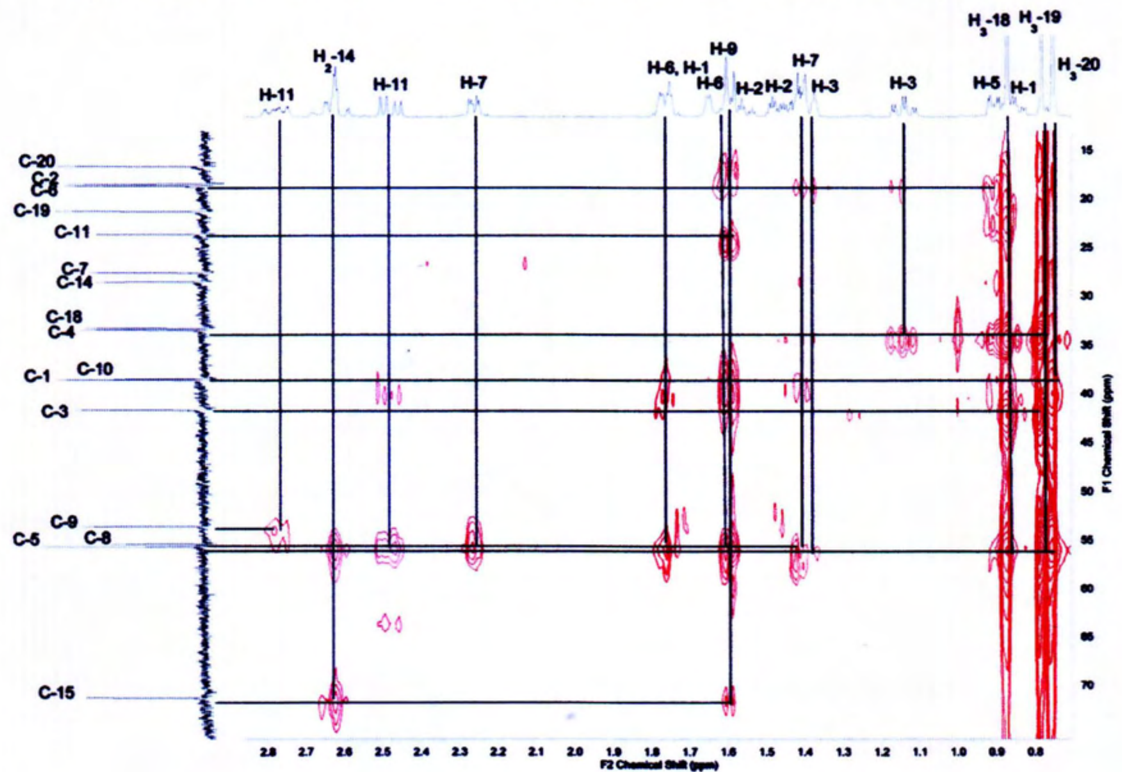


Figure 3.54 HMBC (500 MHz, $CDCl_3$) spectrum of isolated compound G

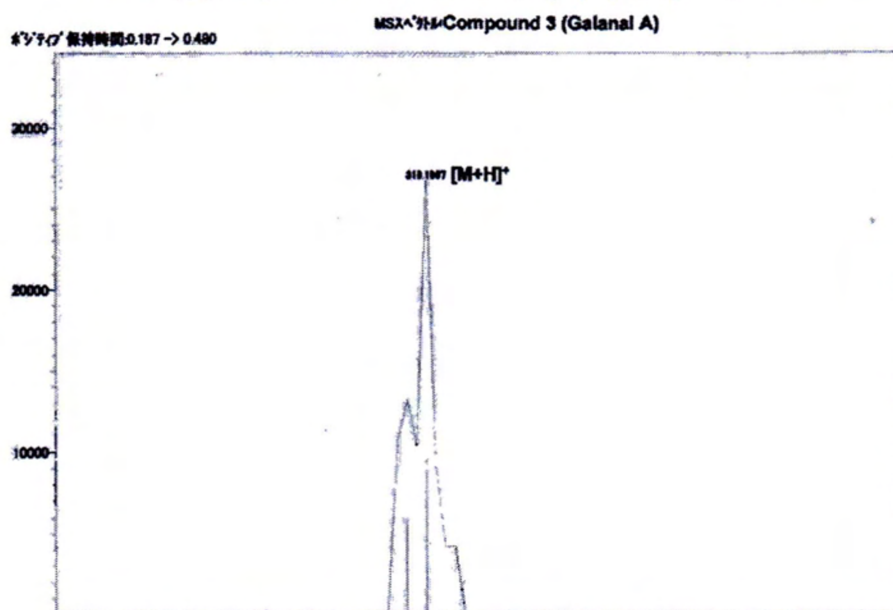
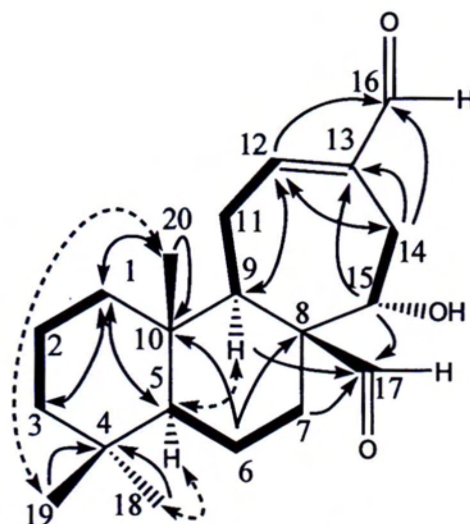


Figure 3.55 ESI MS spectrum of isolated compound G



COSY (bold lines)

NOESY (dashed arrows)

HMBC ($^{1}H \rightarrow {}^{13}C$) (arrow)

Figure 3.56 Chemical structure of galanal A ($C_{20}H_{30}O_3$)

Table 3.29 1D and 2D NMR Spectral Data of Isolated Compound G and Reported Galanal A

Position	Compound G				Galanal A*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_c
1	0.87, m	38.5				38.7
	1.78, m					
2	1.45, m	18.3				18.5
	1.56, m					
3	1.16, dd (13.4, 4.1)	41.6				41.7
	1.40, m					
4		33.3				33.4
5	0.93, m	55.6	H_{ax}-6			53.6
	1.67, m					
6	1.78, m	18.5				18.7
	1.43 m					
7	2.28 m	27.5		C-17		28.3
8		55.4				55.5
9	1.61, m	53.6	H_{ax}-11	C-12, C-17	1.62	55.8
10		38.4				38.5

Table 3.29 Continued

Position	Compound G				Galanal A*	
	δ_H	δ_C	COSY	HMBC	δ_H	δ_c
11	2.50, dd (18.5, 8.3)	23.5		C-12, C-13	2.51-dd (8.0, 17.0)	23.6
	2.79, m				2.78, m	
12	6.89, dd (8.3, 1.6)	156.1	H-11, H_{ax}-11	C-16, C-14	6.88, dd (8.1, 2.0)	156.0
13		142.2				142.2
14	2.65, m	28.4		C-12, C-13	2.70, m	27.7
15	4.07, dd (8.9, 3.1)	71.2	H-14	C-13	4.06, dd (9.0, 3.0)	71.3
16	9.38, s	193.3		C-12, C-13	9.38, s	193.3
17	10.11, s	206.5		C-8	10.11, s	206.5
18	0.93	33.1			0.89, s	33.3
19	0.89	21.1			0.80, s	21.3
20	0.77	16.5			0.78, s	16.6

*Abe *et al., 2002*

3.6.8 Structural elucidation of compound H

Compound **H** is isolated from $CHCl_3$ crude extract of the rhizomes of *B.rotunda* (SPCR) was obtained as amorphous powder and has the melting point of 134-134.5°C. The optical activity of compound **H** is $[a]_D^{25}$ + 0.7 (*C* 0.1, MeOH). It was soluble in chloroform, ethyl acetate, methanol, ethanol, pet-ether and water. Its R_f value was found at 0.37 (*n*-hexane: EtOAc, 2:1 v/v) and it was UV active. According to chemical test, aldehyde group was present due to 2,4-DNP test. Decolourization of 10% $KMnO_4$ solution indicated the presence of olefinic group in compound **H**. It gave pink colouration when treated with Liebermann Burchard reagent. Thus, compound **H** was assigned as a terpenoid compound. Some physico-chemical properties of compound **H** are described in Table 3.30.

The structure of compound **H** was also studied by UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectral data. The UV spectrum (Figure 3.57) of compound **H** showed the maximum absorptions wavelength λ_{max} at 226 nm in MeOH indicating the presence of double bond $\pi \rightarrow \pi^*$ transition. The corresponding spectral data are shown in Table 3.31 and it was identical with that of galanal B (Abe *et al.*, 2002).

The functional groups present in compound **H** were also studied by FT IR spectroscopy. FT IR spectrum of isolated compound **H** is illustrated in Figure 3.58 and the corresponding spectral data are listed in Table 3.32. The absorption band which occurs at 3447 cm^{-1} was due to the O-H stretching vibration of alcoholic OH groups. The absorption band due to O-H in plane bending vibration appeared at 1339 cm^{-1}. The absorption band at 1389 cm^{-1} showed to aldehydic C-H bending vibration. The band at 1733 cm^{-1} indicated the presence of normal aldehydic C=O stretching vibration. The absorption band at 1543 cm^{-1} appeared due to C=C stretching vibration of cycloakene. The band at 1049 cm^{-1} indicated the presence of alcoholic C-O stretching vibration.

The peak due to C-H out of plane bending vibration of aromatic ring occurred at 871 cm^{-1} and 732 cm^{-1}.

Table 3.30 Some Physico-chemical Properties of Isolated Compound H

Experiment	Observation	Remark
U_{254}	Active	Presence of double bond
I_2 vapour	Yellow	C=C present compound
Liebermann Burchard reagent	Pink	Terpenoid compound
2,4-DNP solution	Orange ppt	-CHO present
1% $Ce(SO_4)_2$/ 10% H_2SO_4, Δ	Pink	On TLC
R_f value	0.37 (*n*-hexane: EtOAc, 2:1 v/v)	Non-polar organic compound

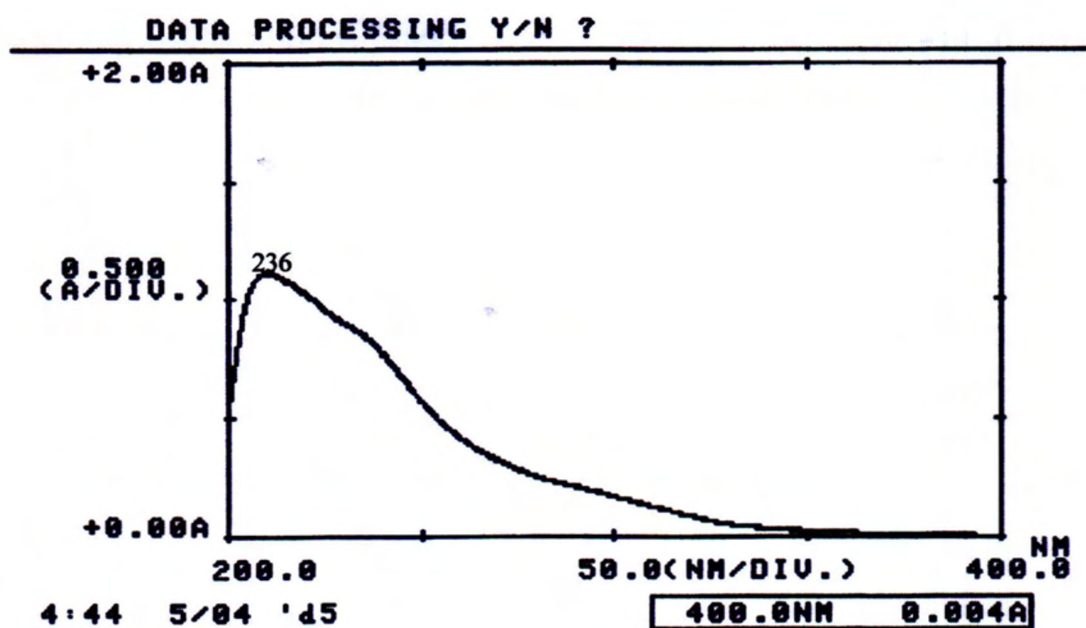


Figure 3.57 UV spectrum of isolated compound H in MeOH

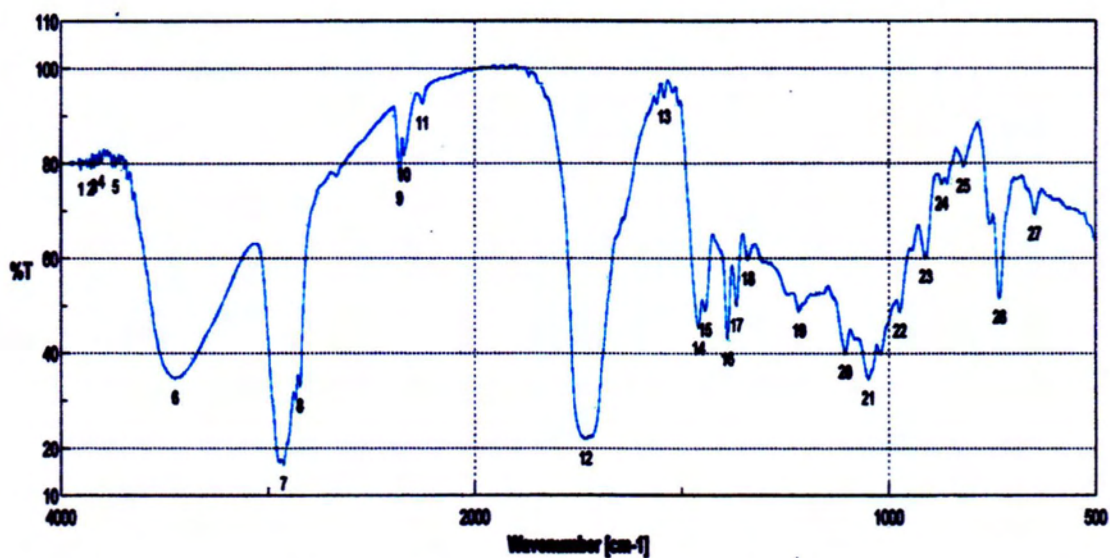


Figure 3.58 FT IR spectrum of isolated compound H

Table 3.31 UV Spectral Data Assignment of Isolated Compound H and Reported Galanal B

Solvent	λ_{max} (nm)		Remark
	Compound H	Galanal B*	
MeOH	236	232	$\pi \rightarrow \pi^*$ transition

* Abe *et al.*, 2002.

Table 3.32 FT IR Spectral Data of Isolated Compound H

Wave number (cm^{-1})		Assignment
Compound H	Galanal B*	
3736,3447	3620	O-H stretching vibration of alcoholic OH group
2925,2845		C-H stretching vibration of asymmetric and symmetric $-CH_3$ and $-CH_2$ group
1733	1710	C=O stretching vibration of normal aldehyde group
1543	1637	C=C stretching vibration of cycloalkane
1460		C-H bending vibration of $-CH_2$ group
1398		C-H bending vibration of aldehyde group
1339		O-H in plane bending vibration
1216		C-O-C stretching vibration
1049		Alcoholic C-O stretching vibration
973,910		C-H out of plane bending vibration
819,732		C-H out of plane bending vibration of aromatic ring

*Abe *et al.*, 2002

In ^{1}H NMR spectrum in $CDCl_3$ (Figure 3.59), the proton signal at δ 10.19 ppm as singlet indicated that the presence of CH-OH proton. The proton signals at δ 89.38 ppm and δ 10.19 ppm as singlet indicated that the presence of CH proton. The three quaternary methyl signals as singlets at δ 0.76 (3H), δ 0.99 (3H) and 0.85 (3H) were coincident with those of reported galanal B (Table 3.33). In the ^{1}H ^{1}H COSY spectrum (Figure 3.61) of compound **H**, methine proton at δ 1.48 correlated with another methine at δ 3.12. Similarly, methine proton at δ 2.53 correlated with another methane at δ 1.48. NOESY spectrum (Figure 3.62) was observed between the protons at H_{α}-9 and H_{α}-15 in compound **H**, indicating the relative configuration of a hydroxyl group was to be β.

The ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of isolated compound **H** is shown in Figure 3.60. It can be seen that twenty ^{13}C peaks were observed. The ^{13}C NMR spectrum of these carbons were confirmed from HMQC and HMBC spectra (Figure 3.63 and 3.64). The most upfield one methyl group and the most downfield one carbonyl signal were observed at δ 15.7(C-20) and δ 208.2(C-17) respectively. In the HMQC spectrum, each proton signal correlated with the corresponding carbon. The HMBC correlation of methine proton (δ_H 1.48) to C-17 (δ_C 208.2) and (δ_C 140.5) confirmed the position of this proton to be at C-15 and that of hydroxyl group at C-15. The ^{1}H NMR, ^{13}C NMR, 2D NMR spectral data are described in Table 3.33.

From ESI MS spectrum (Figure 3.65), the spectral data of isolated compound **H** is indicated a molecular ion peak $[M+H]^+$ at m/z 319 which corresponded to the molecular formula $C_{20}H_{30}O_3$. Therefore, UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectral data of isolated compound **H** were found to be consistent with those of literature (Abe *et al.*, 2002, Morita *et al.*, 1986) and the structure is illustrated in Figure 3.66.

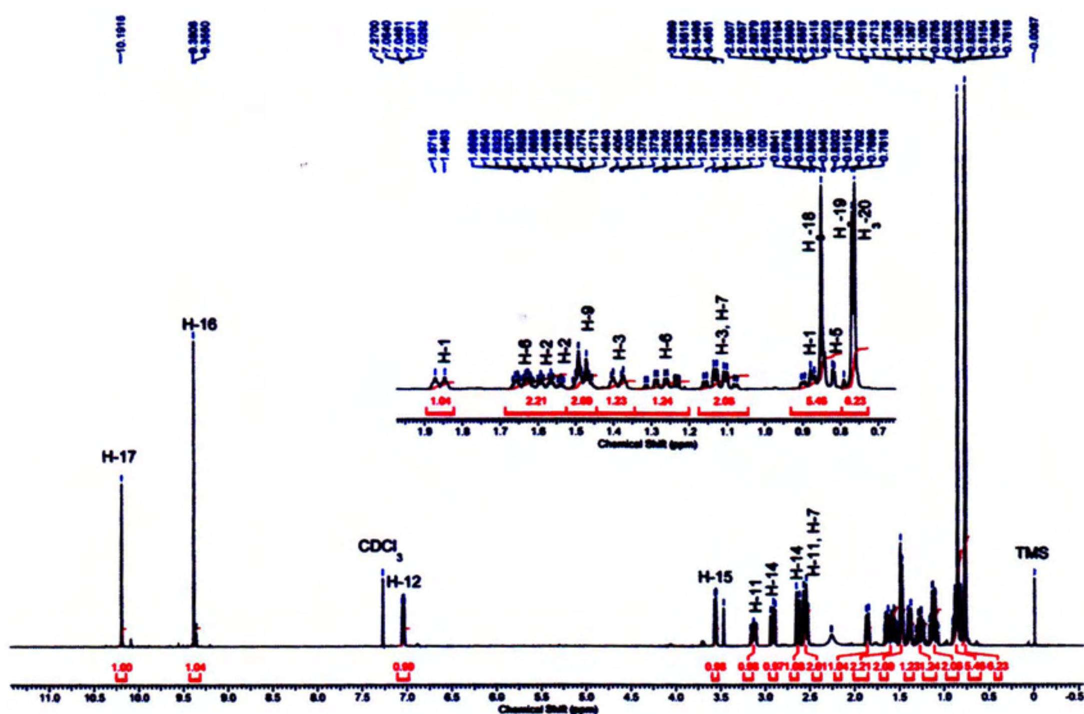


Figure 3.59 ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of isolated compound H

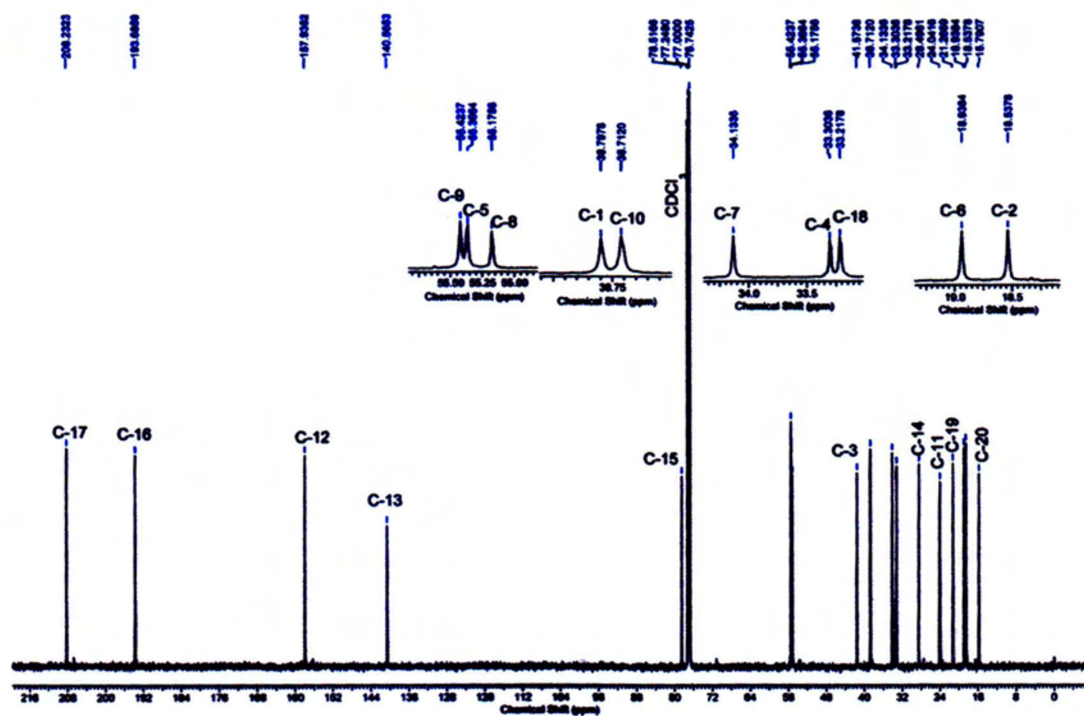


Figure 3.60 ^{13}C NMR (125 MHz, $CDCl_3$) spectrum of isolated compound H

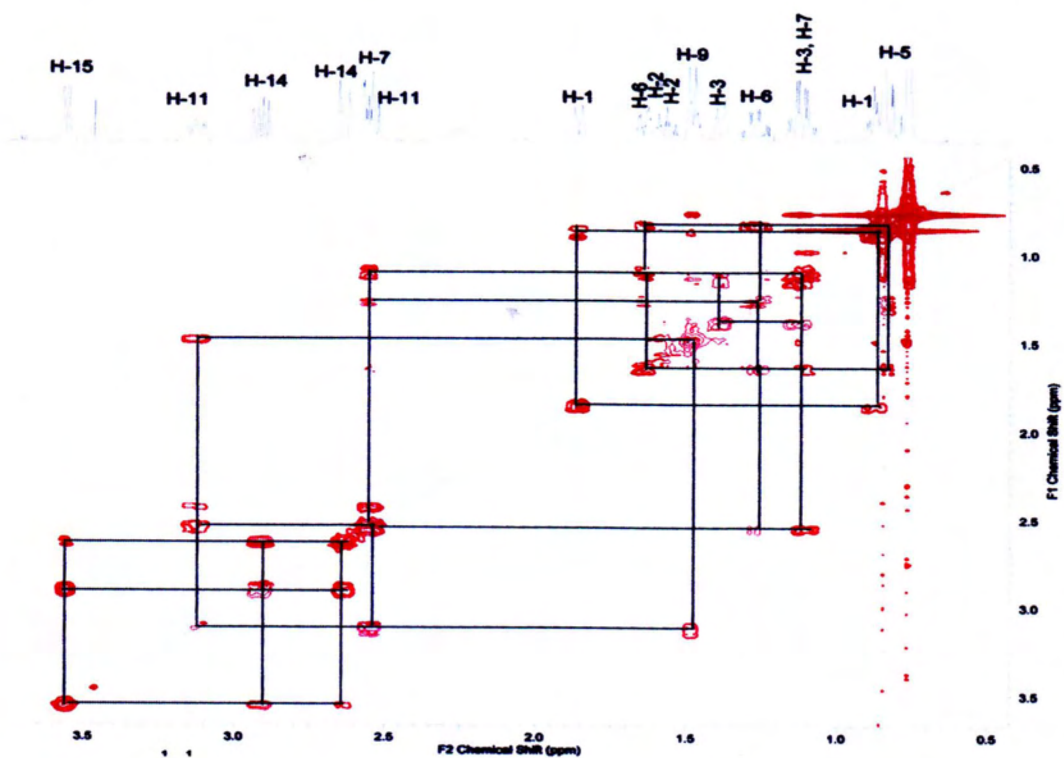


Figure 3.61 ^{1}H ^{1}H COSY (500 MHz, $CDCl_3$) spectrum of isolated compound H

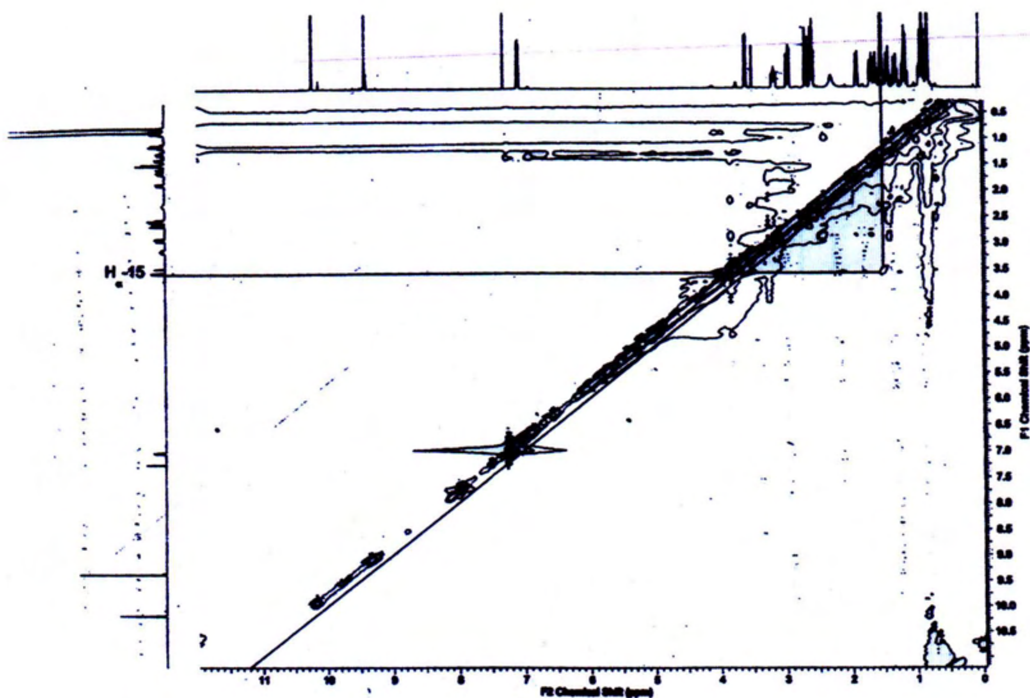


Figure 3.62 NOESY (500 MHz, $CDCl_3$) spectrum of isolated compound H

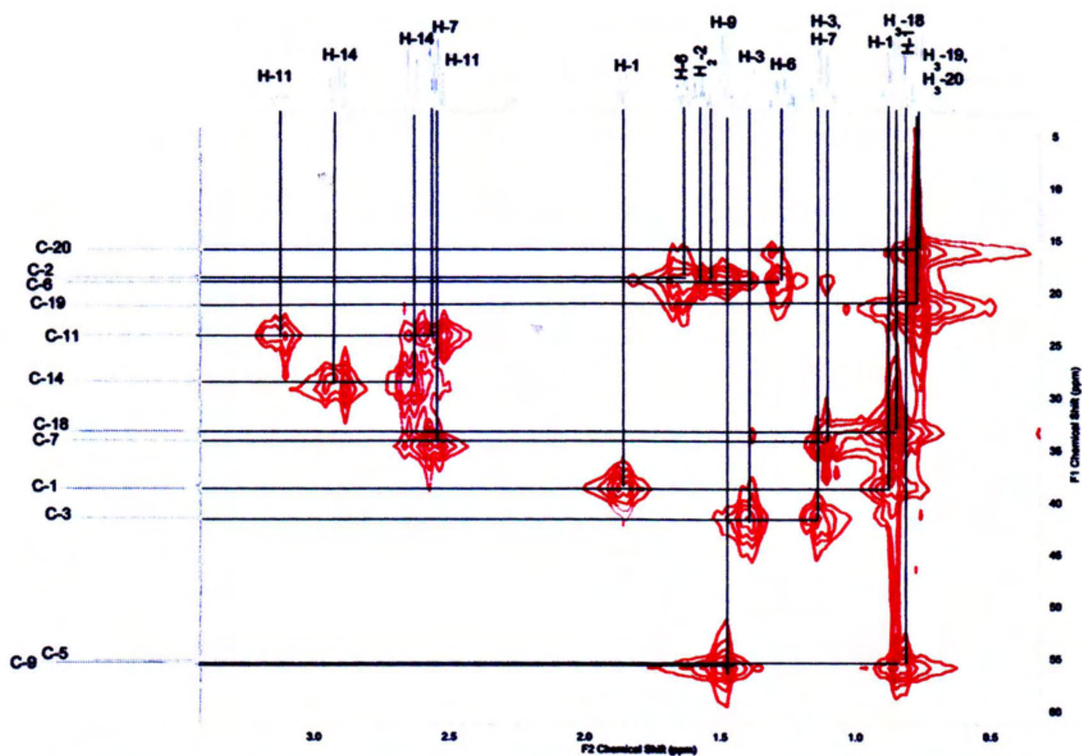


Figure 3.63 HMQC (500 MHz, $CDCl_3$) spectrum of isolated compound H

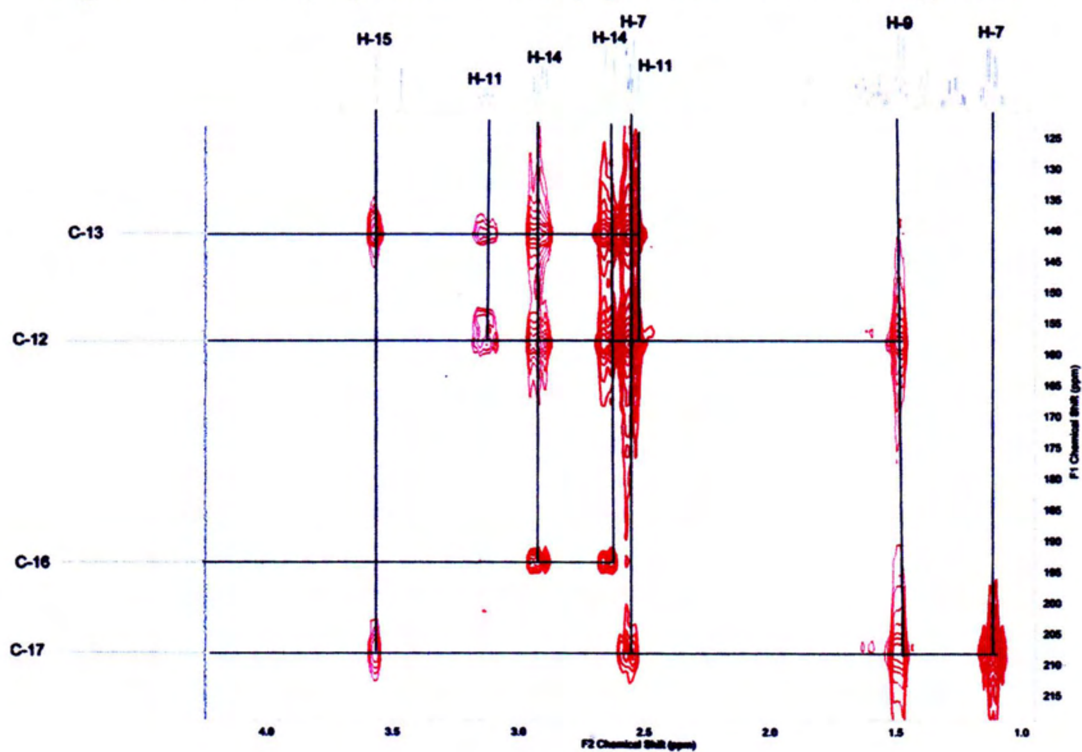


Figure 3.64 HMBC (500 MHz, $CDCl_3$) spectrum of isolated compound H

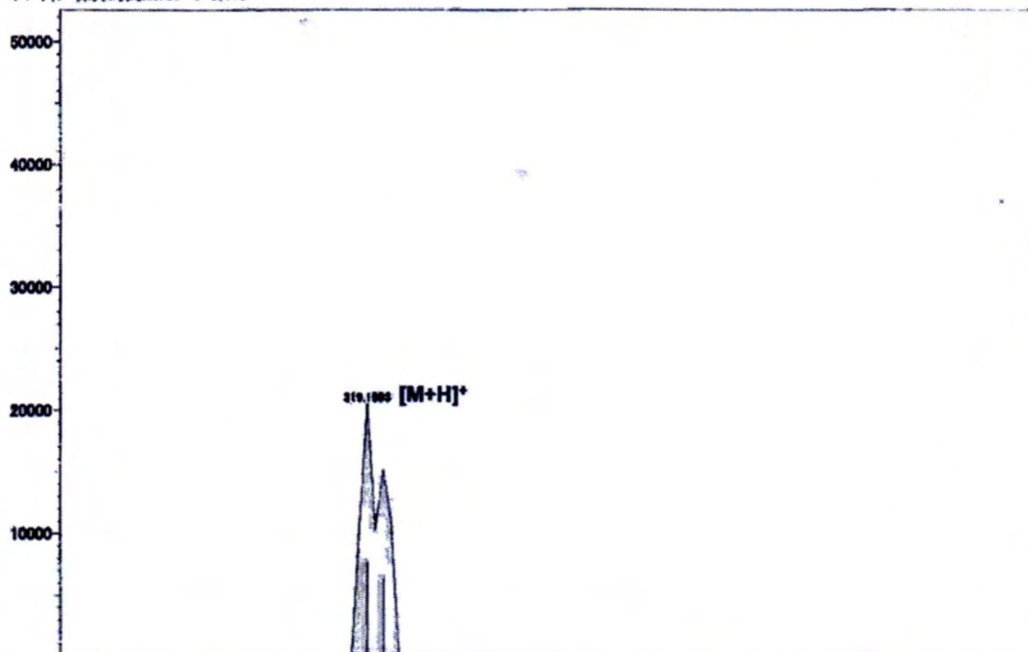
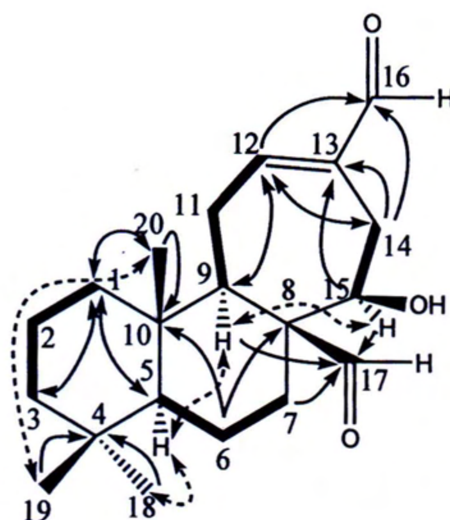


Figure 3.65 ESI MS spectrum of isolated compound H



COSY (bold lines)

NOESY (dashed arrows)

HMBC ($^{1}H \rightarrow ^{13}C$) (arrow)

Figure 3.66 Chemical structure of galanal B ($C_{20}H_{30}O_3$)

Table 3.33 1D and 2D NMR Spectral Data of Isolated Compound H and Reported Galanal B

Position	Compound H					Galanal B*	
	δ_H	δ_C	COSY	NOESY	HMBC	δ_H	δ_c
1	0.82, m 1.85, dd(J=12.6Hz, 2.5Hz)	38.7					38.9
2	1.54, m 1.57, m	18.5					18.7
3	1.10, m 1.38, m	41.5					41.7
4		33.3					33.4
5	0.82, m	55.3					55.5
6	1.26, m 1.64, m	18.9			C-8, C-10		19.1
7	1.10, m 2.56, m	34.1	H-6		C-17		34.5
8		55.1					55.4
9	1.48, m	55.4		H-15	C-17	1.47	55.6
10		38.7					39.0

Table 3.33 Continued

Position	Compound H					Galanal B*	
	δ_H	δ_C	COSY	NOESY	HMBC	δ_H	δ_c
11	2.53, dd 3.12, m	24.0	H-9			2.55, dd 3.12	24.1
12	7.03, dd (*J*=8.4Hz, 3.9Hz)	157.9	H-11			7.04, dd (*J*=8.5Hz, 4.5Hz)	157.7
13		140.5					140.8
14	2.91, dd (*J*=16.4Hz, 8.8Hz) 2.63, dd (*J*=16.4Hz, 1Hz)	28.4	H-15		C-13, C-16	2.92, dd (*J*=16.4Hz, 9.0Hz) 2.67,dd (*J*=16.4Hz, 1.Hz)	28.7
15	3.55, dd (*J*=8.8Hz, 1Hz)	78.5	H-14		C-13, C-17	3.54, dd (*J*=9.0Hz, 1.8Hz)	78.7
16	9.38, s	193.6			C-12, C-14	9.40, s	193.5
17	10.19, s	208.2			C-7	10.20, s	208.1
18	0.85, s	33.2			C-4	0.77, s	33.3
19	0.768, s	21.2			C-4	0.86, s	21.4
20	0.761, s	15.7			C-10	0.78, s	15.9

*Abe *et al.*, 2002

3.7 Some Biological Activties of the Whole Plant of *C. repens* (WRC), Aerial Parts and Rhizomes of *B. rotunda* (SPCA, SPCR)

The results of screening of some bioactivities such as antimicrobial, antioxidant, cytotoxicity, antitumor activity and antiproliferative activity of WRC, SPCA, SPCR and some isolated compounds as described in Section 2.10 will be discussed in this section.

3.7.1 Antimicrobial activity of crude extracts by agar well diffusion method

Screening of antimicrobial activity of various crude extracts such as PE, EtOAc, EtOH and MeOH extracts of WRC, SPCA and SPCR were done by employing agar well diffusion method (Section 2.10.2.3). In this study, the samples were tested on six pathogenic microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* species. The inhibition zones of all crude extracts against six microorganisms tested are shown in Figures 3.67, 3.69 and 3.71 and the corresponding inhibition zone diameters are summarized in Tables 3.34, 3.35 and 3.36.

From these results, it was found that PE extract of WRC did not exhibit any antimicrobial activity against all tested microorgansims whereas EtOAc, EtOH and MeOH extracts from WRC exhibited inhibition zone diameters ranged in 17 ~ 55, 14 ~ 15, 13 ~ 15 mm respectively against all microorganisms tested. The WRC EtOH extract showed less activity and EtOAc extract was observed most effective in antimicrobial activity.

In the case of SPCA, PE, EtOAc, EtOH and MeOH extracts showed inhibition zone diameters ranged in 14, 20 ~ 55, 14 ~ 16 and 14 mm respectively against all microorganism tested. PE and MeOH extracts of SPCA was exhibited less microbial activity and EtOAc extract was the most active.

PE, EtOAc, EtOH and MeOH extracts of SPCR exhibited inhibition zone diameter ranged in 14, 14 ~ 35, 13 ~ 30 and 17 mm respectively against five microorganisms except *Bacillus pumilus.* Therefore, PE extract of SPCR exhibited less microbial activity and EtOAc extract had the highest effectiveness.

Therefore all the crude extracts of three sample; except PE extract of WRC, exhibited antimicrobial activity against all microorganisms tested. Among the crude extracts tested, EtOAc extracts of three samples showed the most pronounced antimicrobial activity against all microorganisms tested. Thus, WRC, SPCA and SPCR might be effective in the formulation of medicine for the treatment of diseases infected by the microorganisms, such as diarrhea, dysentery, eye-infection, urinary tract infections, skin diseases and wound infections.

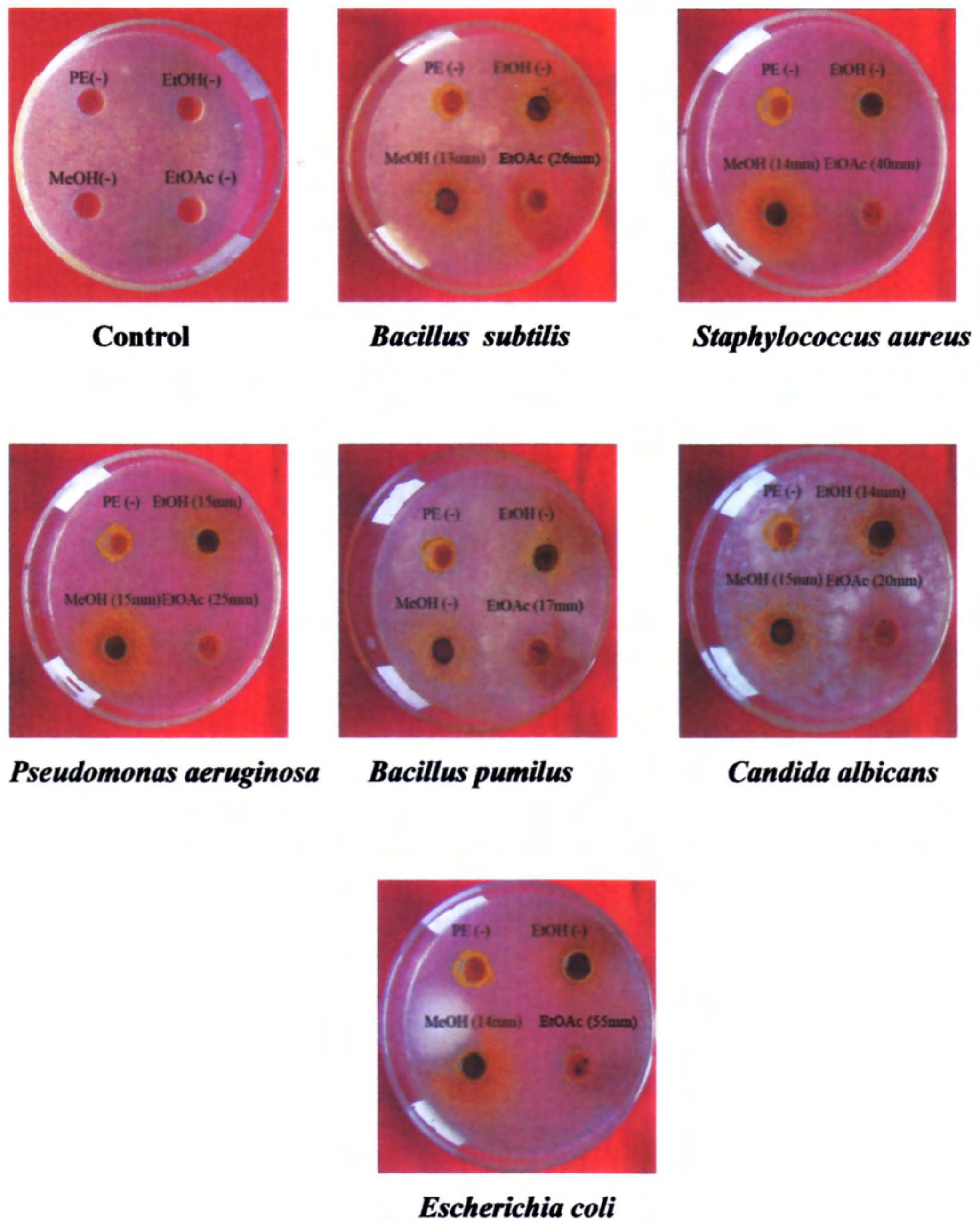


Figure 3.67 Antimicrobial activity screening of different crude extracts from the whole plant of *C. repens* by agar well diffusion method

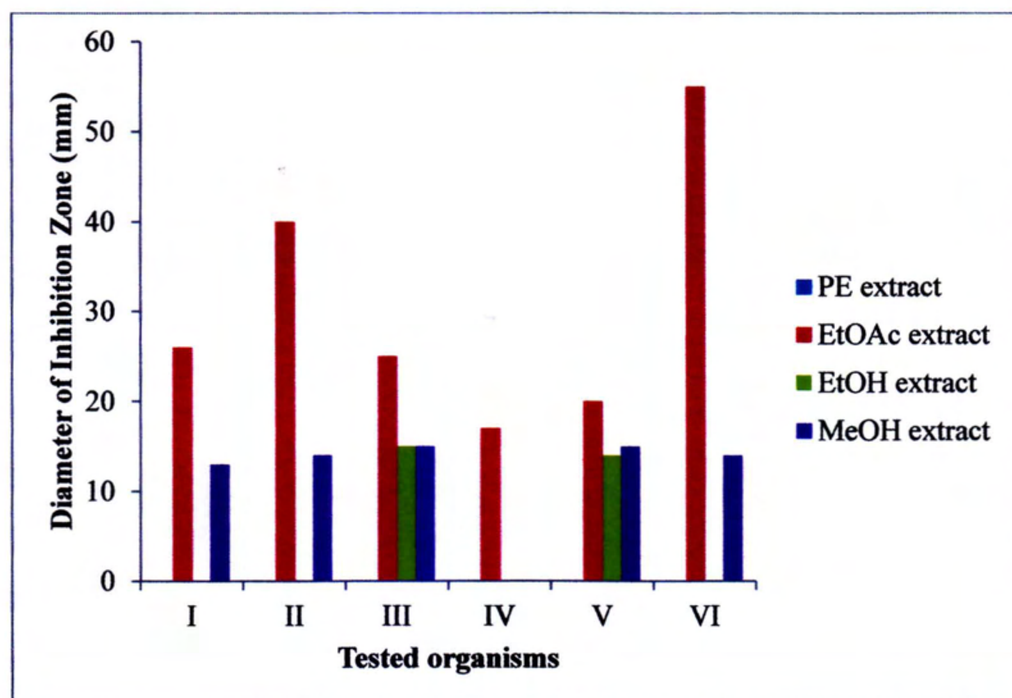
Table 3.34 Inhibition Zone Diameters (mm) Provided by Different Crude Extracts of the Whole Plant of *C. repens*

Test Extracts	Diameter of Inhibition Zone (mm) in different microorganisms					
	I	**II**	**III**	**IV**	**V**	**VI**
PE	-	-	-	-	-	-
EtOAc	26	40	25	17	20	55
EtOH	-	-	15	-	14	-
MeOH	13	14	15	-	15	14

Agar well - 10 mm

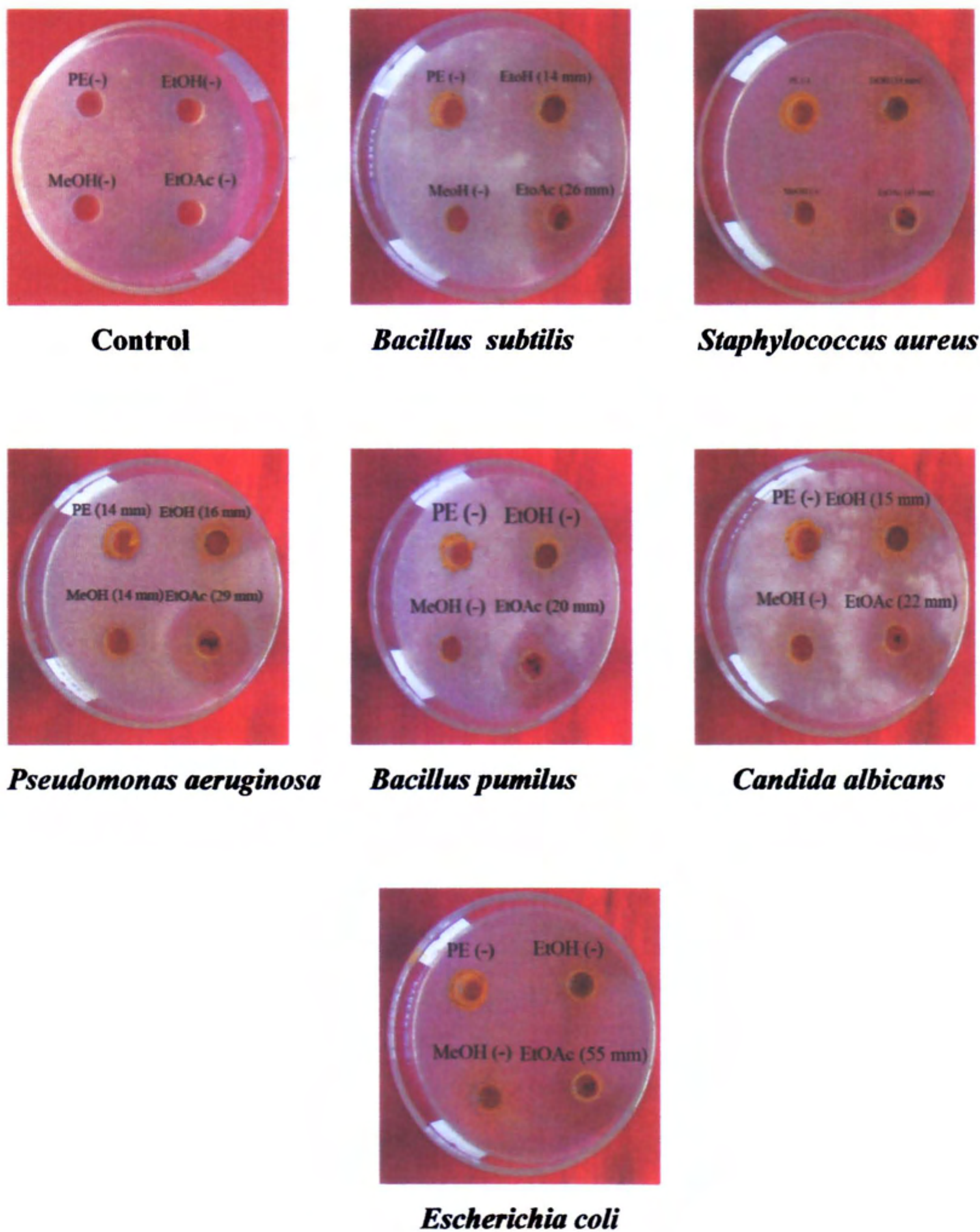
10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm above (+++)

Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus;* V. *C. albicans*; VI. *E. coli*



Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus* V. *C. albicans*; VI. *E. coli*

Figure 3.68 Histogram showing antimicrobial activity of different extracts of the whole plants of *C. repens*



Control **_Bacillus subtilis_** **_Staphylococcus aureus_**

Pseudomonas aeruginosa **_Bacillus pumilus_** **_Candida albicans_**

Escherichia coli

Figure 3.69 Antimicrobial activity screening of different crude extracts from aerial parts of _B. rotunda_ by agar well diffusion method

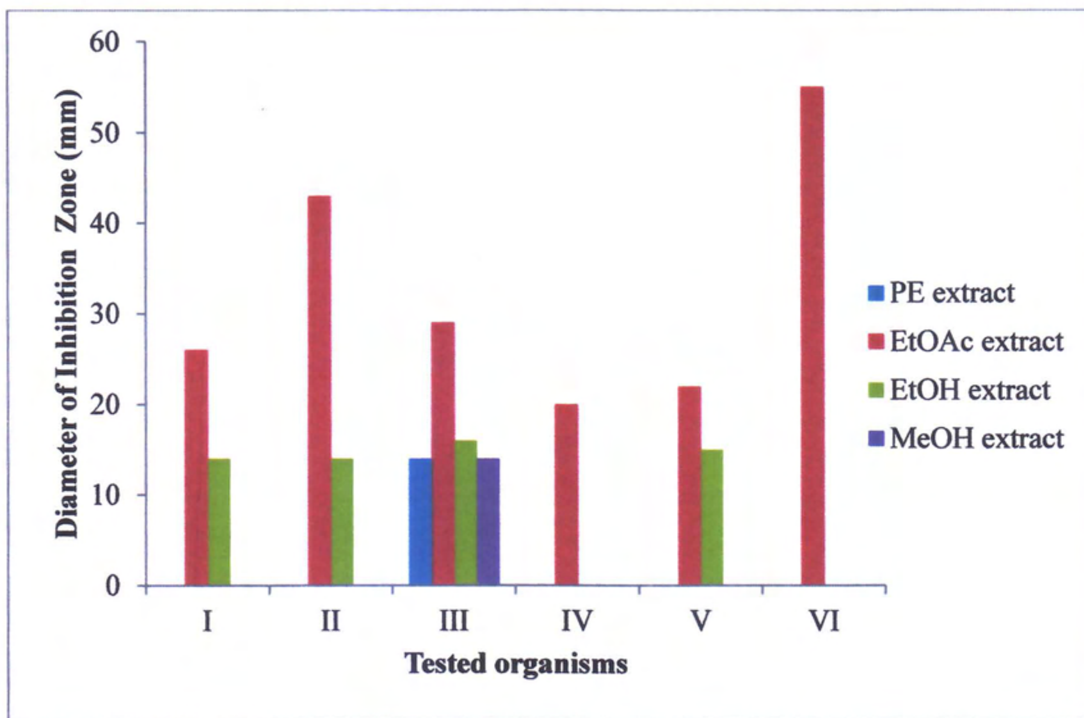
Table 3.35 Inhibition Zone Diameter (mm) Provided by Different Crude Extracts of Aerial Parts of *B. rotunda*

Test Extracts	Diameter of Inhibition Zone (mm) in different microorganisms					
	I	II	III	IV	V	VI
PE	-	-	14	-	-	-
EtOAc	26	43	29	20	22	55
EtOH	14	14	16	-	15	-
MeOH	-	-	14	-	-	-

Agar well - 10 mm

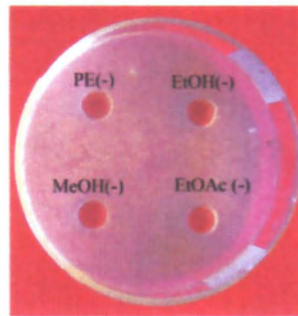
10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm above (+++)

Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus;* V. *C. albicans*; VI. *E. coli*

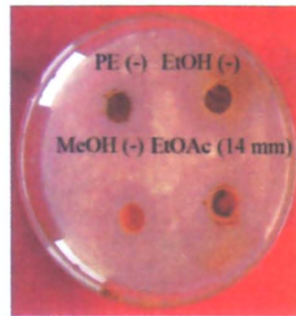


Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus* V. *C. albicans*; VI. *E. coli*

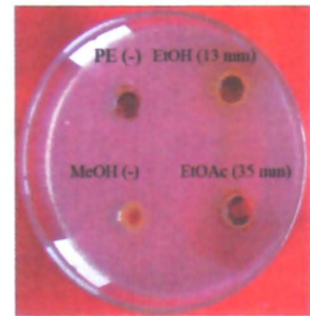
Figure 3.70 Histogram showing antimicrobial activity of different extracts of aerial parts of *B. rotunda*



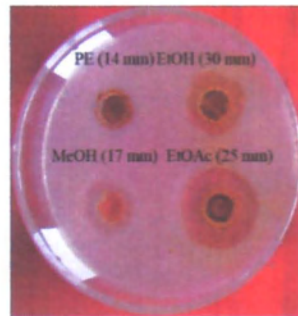
Control



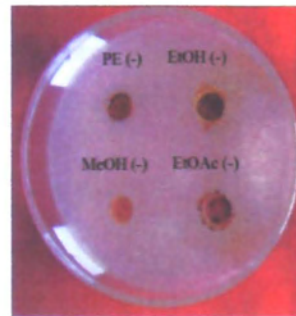
Bacillus subtilis



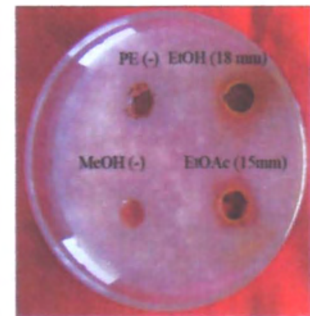
Staphylococcus aureus



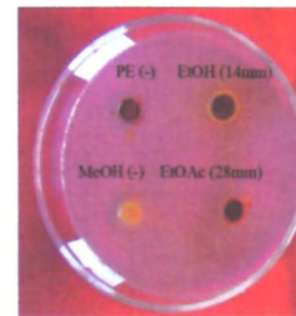
Pseudomonas aeruginosa



Bacillus pumilus



Candida albicans



Escherichia coli

Figure 3.71 Antimicrobial activity screening of different crude extracts from rhizomes of *B. rotunda* by agar well diffusion method

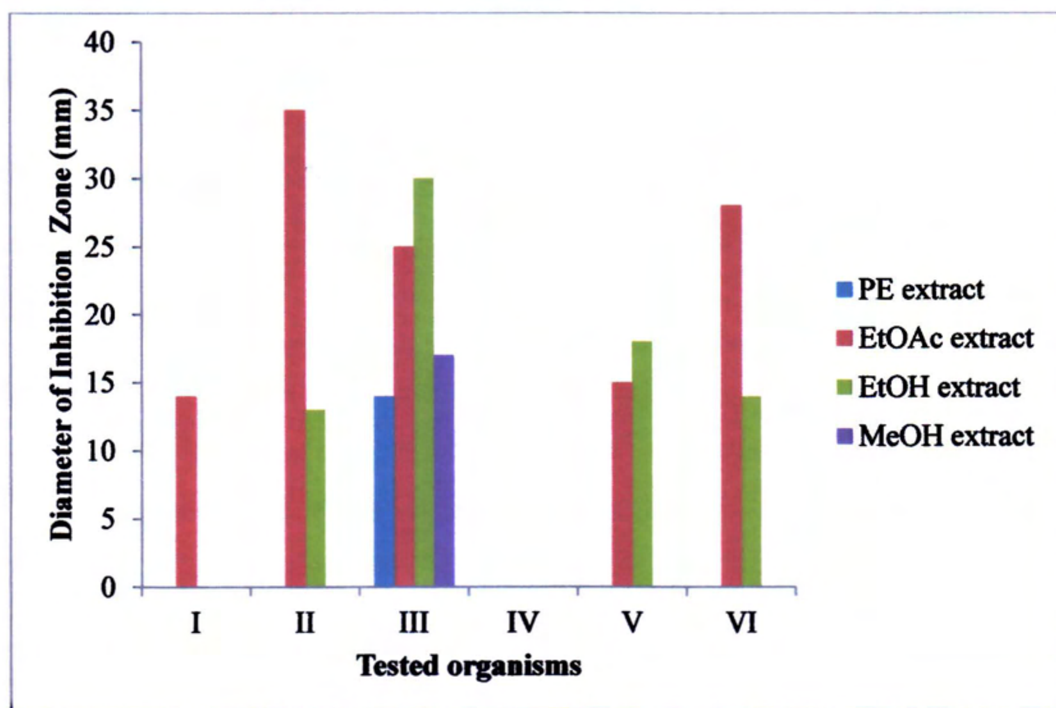
Table 3.36 Inhibition Zone Diameter (mm) Provided by Different Crude Extracts of Rhizomes of *B. rotunda*

Test Extracts	Diameter of Inhibition Zone (mm) in different microorganisms					
	I	II	III	IV	V	VI
PE	-	-	14	-	-	-
EtOAc	14	35	25	-	15	28
EtOH	-	13	30	-	18	14
MeOH	-	-	17	-	-	-

Agar well - 10 mm

10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm above (+++)

Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus;* V. *C. albicans*; VI. *E. coli*



Tested organisms: I. *B. Subtilis*; II. *S. aureus*; III. *P. aeruginosa*; IV. *B. pumilus* V. *C. albicans*; VI. *E. coli*

Figure 3.72 Histogram showing antimicrobial activity of different extracts of rhizomes of *B. rotunda*

3.7.1.1 Minimum inhibitory concentration of isolated compounds (A-D) by agar well diffusion method

The MIC values of ethyl acetate extracts and some isolated compounds **A** to **D** (friedelin, friedelinol, β-sitosterol and β-sitosterol-β-D-glucoside) from WRC, SPCA and SPCR were determined by agar well diffusion method (Section 2.10.2.4). In this study, they were tested on two species of microorganisms; *Staphylococcus aureus* and *Escherichia coli* with different concentrations. Since the crude extracts showed the most pronounced activity against these two microorganisms.

The MIC values of ethyl acetate extracts were ranged from 10^5 μg/ mL to 0.2 μg/ mL concentration. The lowest MIC values for ethyl acetate extracts of WRC, SPCA and SPCR were found to be 24.4 μg/mL, 2.5×10^4 μg/ mL and 10^5 μg/ mL respectively against *Staphylococcus aureus.* In addition, ethyl acetate extracts of WRC, SPCA and SPCR exhibited the lowest MIC values of 2.5×10^4 μg/mL, 2.5×10^4 μg/ mL and 10^5 μg/ mL against *Escherichia coli.* The MIC values of some isolated compound friedelin, friedelinol, β-sitosterol and β-sitosterol-β-D-glucoside were ranged from 100 μg/ mL to 0.002 μg/ mL concentration. The lowest MIC values for the isolated compounds **A, B, C, D** exhibited 12.5 μg/mL, 100 μg/mL, 25 μg/mL and 100 μg/mL respectively against *Staphylococcus aureus.* The lowest MIC values for the isolated compounds **A, B, C, D** exhibited 25 μg/mL, 100 μg/mL, 25 μg/ mL and 100 μg/ mL respectively against *Escherichia coli.* These results are reported in Table 3.37.

From these results, it can be concluded that ethyl acetate extract of WRC and the isolated compound **A** (Friedelin) have most pronounced antimicrobial activity than other extracts and compounds. Friedelin was isolated from WRC it can be inferred that WRC possessing the high effectiveness of antimicrobial activity than SPCA and SPCR.

Table 3.37 Minimum Inhibition Concentration of Ethyl Acetate Extracts and Some Isolated Compounds from WRC, SPCA and SPCR

No.	Organisms	MIC (μg/mL)						
		I	II	III	IV	V	VI	VII
1.	*Staphylococcus aureus*	24.4	2.5×10^4	10^5	12.5	100	25	100
2.	*Escherichia coli*	2.5×10^4	2.5×10^4	10^5	25	100	25	100

I = WRC (EtOAc-extract)

II = SPCA (EtOAc-extract)

III = SPCR (EtOAc-extract)

IV = Friedelin

V = Friedelinol

VI = β-sitosterol

VII = β-sitosterol-β-D-glucoside

3.7.2 Antioxidant activity of ethanol and water crude extracts of the whole plant of *C. repens* (WRC) and aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR) and some isolated compounds by DPPH radical scavenging assay

Antioxidant compounds in plant play an important role as a health-protect factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids and phytoestrogens have been recognized having the potential to reduce disease risk.

The antioxidant activity of WRC, SPCA, SPCR and some isolated compounds **A, B, D** (Friedelin, Friedelinol, β-sitosterol-β-D glucoside) was evaluated by DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging assay (Marinova and Batchvarov, 2011). The radical scavenging effects were determined for ethanol and water extracts of two selected plants. The extracts and some isolated compounds or their constituents decolourized DPPH due to hydrogen donating ability. The radical scavenging activity of the sample were expressed in terms of % RSA or % oxidative inhibition and IC_{50} (50% inhibitory concentration). These results are shown in Tables 3.38, 3.39, 3.40, and Figures 3.73, 3.74, 3.75, 3.76.

According to Figure 3.8, if the concentration of samples increased the % RSA or % inhibition was also increased. It can be suggested that one required to scavenge effectively the radicals the more concentrated samples were needed.

From the experimental results, SPCA was found to have the highest antioxidant activity in both ethanol IC_{50}= 63.65 μg/ mL and water extracts IC_{50} 131.24 μg/mL respectively. The antioxidant activity (IC_{50}) of ethanol and water extracts of WRC and SPCR were observed to be 84.82 μg/mL (WRC-EE), 281.03 μg/mL (WRC-WE), 80.49 μg/mL (SPCR-EE) and > 400 μg/mL (SPCR-WE). On the other hand, among the tested isolated compounds, β-sitosterol-β-D-glucoside (IC_{50} = 21.63 μg/mL) was found to be more potent than friedelin (IC_{50} = 191.11 μg/mL) and friedelinol (IC_{50} > 200 μg/mL) in antioxidant activity.

Although their antioxidant potency were concluded to be very weak if

compared with the potency of standard vitamin C (IC_{50}=0.53 μg/mL) and gallic acid (IC_{50} = 0.91 μg/mL), it can be generally inferred that SPCR extracts possessed the higher antioxidant potency than WRC and SPCR, due to the presence of antixoidants such as β-sitosterol-β-D-glucoside.

Table 3.38 Radical Scavenging Activity (% RSA) of Crude Extract of the Whole Plant of *C. repens* (WRC), Aerial Parts and Rhizomes of *B. rotunda* (SPCA and SPCR)

Sample	% RSA± SD at Different Concentrations (µg/mL)					
	12.5	25	50	100	200	400
WRC-EE	7.67±10.76	16.06±1.26	40.35±2.30	54.28±0.51	84.28±0.23	94.10±1.26
WRC-WE	9.36±11.42	18.56±7.33	19.06±2.84	31.77±6.62	42.14±0.47	61.53±0.95
SPCA-EE	16.10±1.59	28.65±4.50	43.07±1.06	67.79±1.06	91.57±0.26	95.50±0.00
SPCA-WE	9.19±3.64	10.47±3.38	21.69±2.08	38.78±0.28	74.81±6.50	88.97±0.52
SPCR-EE	11.5±1.30	19.41±0.00	35.16±0.52	59.52±0.26	86.63±0.26	85.89±0.78
SPCR-WE	0.00	0.00	1.90±0.54	2.67±1.08	8.20±0.81	11.45±1.08

WE- Water Extract, EE-Ethanol Extract

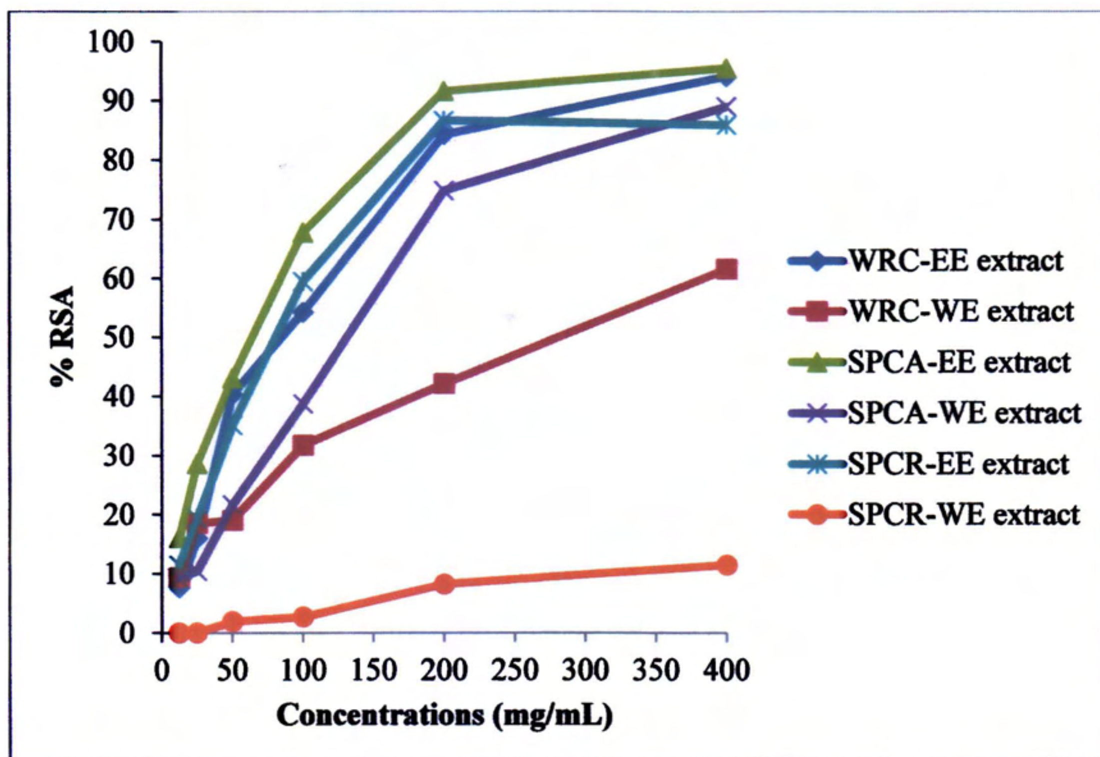


Figure 3.73 **% RSA of EtOH and water crude extracts of the whole plant of *C. repens* (WRC), aerial parts and rhizomes of *B. rotunda* (SPCA and SPCR)**

Table 3.39 Radical Scavenging Activity (IC_{50}) of EtOH and Water Crude Extracts and the Whole Plant of *C. repens* and Aerial Parts and Rhizomes of *B. rotunda*

Sr. No.	Sample	IC_{50} (μg/mL)	
		EtOH extract	Water extract
1.	WRC	84.82	281.01
3.	SPCA	63.65	131.24
2.	SPCR	80.49	> 400
4.	Gallic acid	0.91	
5.	Vitamin C	0.53	

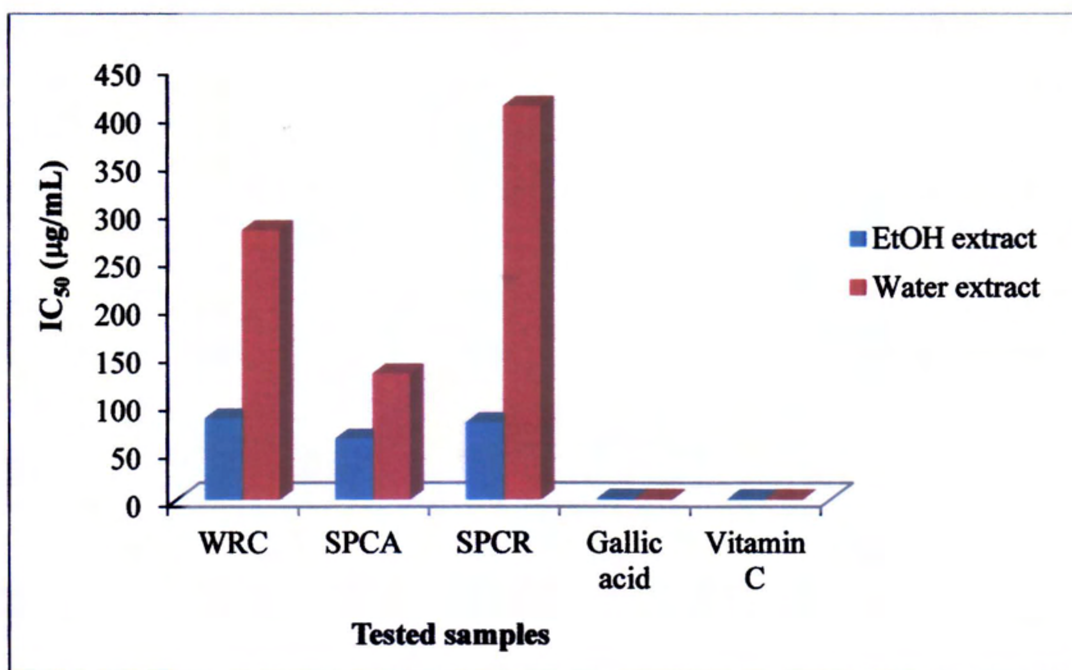


Figure 3.74 A bar graph IC_{50} (µg/mL) of EtOH and water crude extracts of WRC, SPCA, SPCR compared with standards

Table 3.40 % RSA (Radical Scavenging Activity) and IC_{50} Values of Isolated Compounds

Tested sample	% RSA (mean ±SD) in different concentration (µg/mL)						IC_{50} (µg/mL)
	6.25	**12.5**	**25**	**50**	**100**	**200**	
Friedelin	33.64 ±0.69	38.38 ±0.46	42.46 ±0.46	44.10 ±0.28	46.78 ±0.53	50.43 ±0.11	191.11
Friedelinol	28.71 ±0.53	33.88 ±0.94	39.11 ±0.86	42.34 ± 0.48	45.86 ±0.28	47.69 ±0.59	> 200
β-sitosterol-β-D-glucoside	45.07 ±0.48	48.72 ±0.32	50.49 ±0.11	51.95 ± 0.28	54.62 ±0.69	57.36 ±0.56	21.63
Vitamin C	25.20 ±1.40	53.58 ±0.88	65.53 ±1.13	74.82 ±0.59	83.32 ±0.78	91.21 ±0.48	11.7

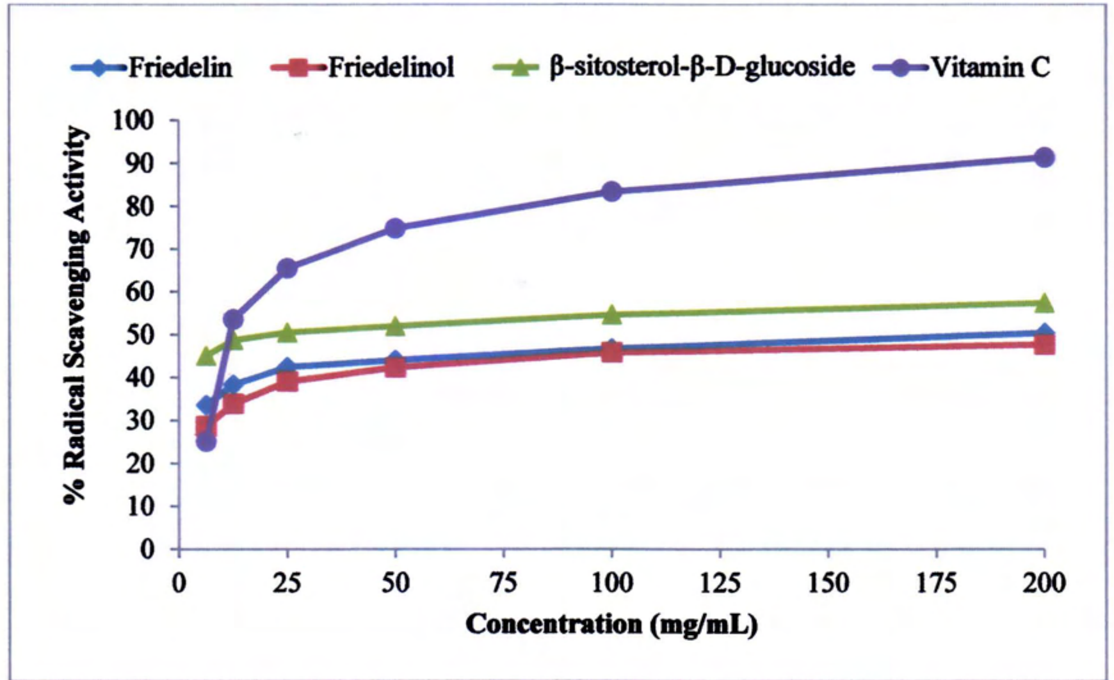


Figure 3.75 Radical scavenging activity of different concentrations of isolated compounds

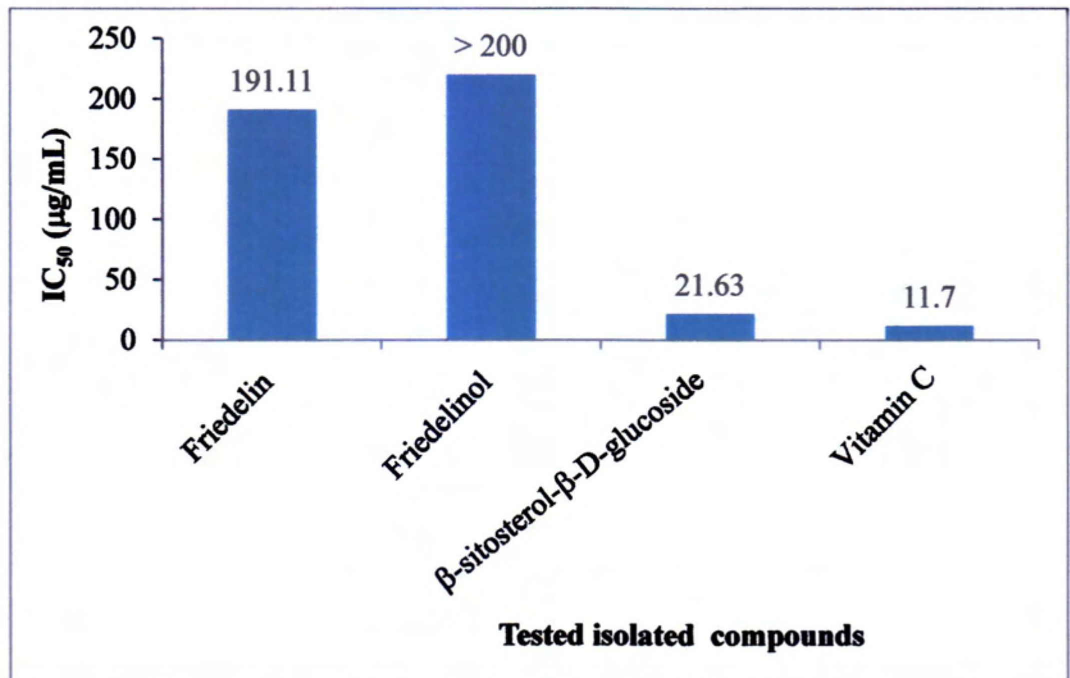


Figure 3.76 A bar graph of IC_{50} values of isolated compounds (A, B, D)

3.7.3 Cytotoxicity of ethanol and water crude extracts of the whole plant of *C. repens* (WRC) and aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR) and some isolated compounds

The cytotoxicity of WRC, SPCA and SPCR were investigated by brine shrimp cytotoxicity bioassay (Dockery and Tomkins, 2000) (Section 2.10.4). The organisms used were brine shrimp *(Artemia salina).* The cytotoxic effect was expressed at LD_{50} values (50% Lethality dose). The cytotoxicity of ethanol and water crude extracts of two selected plants evaluated in this study are reported in Table 3.41. One plant extracts, out of four tested was found to possess cytotoxic in the brine shrimp bioassay. As shown in Table 3.41, the most cytotoxic extract was the SPCR-EtOH [LD_{50}:370.50 μg/mL] whereas other extracts (WRC-EtOH,WRC-H_2O, SPCA-EtOH, SPCA-H_2O, SPCR-H_2O) were not cytotoxic to brine shrimp up to maximum does of 1000 μg/mL. These result suggested that SPCR-EtOH was more cytotoxic, and the other extracts were not cytotoxic to brine shrimp. All of these samples are lower than standards $K_2Cr_2O_7$ (LD_{50} 44.19 μg/mL) and caffeine (LD_{50} 1000 μg/mL) in cytotoxicity.

It is worthy to mention that two selected plants were used to prepare one of the traditional foods and are used to in folk medicine as anticancer. The reported active (cytotoxic) plant in the study are worth of further pharmacological and medical studies in order to define what kind of antitumor activity they have (if any) and to isolate the natural active constituents, which are responsible for the activity.

Table 3.41 Cytotoxicity of Different Doses of Crude Extracts of WRC, SPCA and SPCR

Sample	Survival Brine Shrimp (Mean±SEM) in Various Concentration (μg/mL)				LD_{50} (μg/mL)
	1000	100	10	1	
WRC-EtOH	7.00	10.00	9.67±0.33	10.00	> 1000
WRC-H_2O	8.67±0.67	10.00	10.00	10.00	> 1000
SPCA-EtOH	9.33±0.67	10.00	10.00	10.00	> 1000
SPCA- H_2O	8.00±1.00	10.00	10.00	10.00	> 1000
SPCR-EtOH	1.67±1.67	6.00±1.00	10.00	10.00	370.50
SPCR-H_2O	10.00	10.00	10.00	10.00	> 1000
*$K_2Cr_2O_7$	0	0	8±2.00	9.33±0.67	44.19
*Caffeine	5±1.15	7±0.57	7.66±1.20	10	1000

*Used as Cytotoxic Standards

3.7.4 Antitumor activity of the whole plant of *C. repens* (WRC), aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR)

In this study, tumor producing bacteria, *Agrobacterium tumefaciens* was firstly isolated from the gall tissues of *Sandoricum koetjape* Merr. (Thitto) leaf and cultured for use in the Patato Crown Gall (PCG) test with plant extracts and isolated compounds.

The infected Thitto (*Sandoricum koetjape* Merr.) leaves were collected from Yangon University Campus. The manifestation of leaves-tumor is knobs on one side and dimples on the other side. The bacteria were extracted from dimples side of the leaves by Tween-80 and isolated by serial dilution method. They were grown on YEP (Yeast extract peptone) medium of pH 7.2 at 80 °C. After 24 hours, the bacteria were appeared as creamy coloured colonies on this medium. It was observed that there was a rapid rate of growth on meat extract or YEP medium within 24 hours at 80 °C which is faster than other media.

The culture was examined by a Microscope Nikon, Japan for their morphology. It was observed as rod-shaped and motile under the microscope. From the result of Gram-staining method, it was Gram-negative. The staining procedure is great values in the identification of bacteria. Due to the cell wall structure of the bacteria, the Gram-negative bacteria are easily decolourized but the Gram-positive bacteria resist decolourization. The isolated bacteria were also identified by some biochemical tests such as Motility test, Gelatin liquefaction test, Nitrate reduction test, Indole test, Urease test and Voges-proskauer test. According to the results of morphology, gram staining procedure and the biochemical tests; the isolated bacteria were almost identical with the literature data of Bergey's Manual. Therefore, the isolated bacteria were duced as *A. tumefaciens.*

The antitumor activity of ethyl acetate, ethanol and methanol extracts of WRC, SPCA and SPCR and the isolated compounds **A** to **D** (friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside) were investigated by using PCG test with the isolated bacterium *A. tumefaciens* (Section 2.10.5). For inoculation of the potato disc, 48 hour broth cultures containing 5×10^9 cells/ mL were used. The tested sample were dissolved in DMSO, diluted and mixed with the bacterial culture for inoculation. After preparing the inoculums, the bacterial suspension was inoculated on the cleaned

and sterilized potato discs, and incubated for 12 days at room temperature. The tumors on potato disc were checked by staining the Knob with Lugol's (I_2-KI) solution. In control disc, formation of white knob on the blue background was observed that indicated the presence of tumor cells as no protein is present in tumor cells. If the test sample has antitumor activity, no tumor on the potato disc was observed and its surface remained blue. The active test samples did not form any tumor on the potato discs and its surface remained blue as shown in Figure 3.77.

From this experiment, it was found that ethyl acetate, ethanol and methanol extracts of WRC, SPCA and SPCR were effective in preventing the tumor formation with the doses of 0.4, 0.2 g/disc *in vitro* potato disc assays. In addition, the isolated compounds **A** and **C** were significantly inhibited the formation of tumor with the dose of 0.1 μg/disc. But tumor formation was not prevented by isolated compound **B** and **D** (friedelinol and β-sitosterol-β-D-glucoside) with the dose of 0.1 μg/disc. These results are shown in Table 3.43.

Therefore, it can be concluded that ethyl acetate, ethanol and methanol extracts of WRC, SPCA, SPCR and isolated compounds **A** (fredelin) and **C** (β-sitosterol) have antitumor activity considerably but isolated compounds **B** (friedelinol) and **D** (β-sitosterol-β-D-glucoside) did not have antitumor activity with the dose of 0.1 μg/disc.

Table 3.42 Comparison on the Properties of Tumor Producing Bacteria Isolated from *Sandorium koetjape* Leaf and Reported Properties of *Agrobacterium tumefaciens*

Properties	Isolated bacteria	Standard *A. tumefaciens* *
Growth nature	Aerobic, motile	Aerobic, motile
	Temp. 28°C	Temp. 25-30°C
	pH 7.2	pH 6.0 – 9.0
	YEP agar medium	meat or Yeast extract peptone agar medium
Morphology	Rod shaped	Rod shaped
	Gram negative	Gram negative
	Non – Sporing	Non – Sporing
Biochemical tests		
(a) Gram staining	-	-
(b) Motility	+	+
(c) Gelatin	+	+
(d) Nitrate	+	+
(e) Indole	+	+
(f) Urease	+	+
(g) Voges-Proskauer	-	-

(+) Positive test ,(-) Negative test ; * (Buchanan *et al.*, 1974)

According to these results, the bacteria isolated from *Sandorium koetjape* leaf was *Agrobacterium tumefaciens*.

Table 3.43 Antitumor Activity of Crude Extract from WRC, SPCA, SPCR and Some Isolated Compounds by PCG Test

No.	Test sample	Concentrations (g/disc)	Tumor inhibition
1.	Control	-	-
2.	Ethyl acetate extract of WRC	0.4	+
3.	Ethyl acetate extract of WRC	0.2	+
4.	Ethanol extract of WRC	0.4	+
5.	Ethanol extract of WRC	0.2	+
6.	Methanol extract of WRC	0.4	+
7.	Methanol extract of WRC	0.2	+
8.	Ethyl acetate extract of SPCA	0.4	+
9.	Ethyl acetate extract of SPCA	0.2	+
10.	Ethanol extract of SPCA	0.4	+
11.	Ethanol extract of SPCA	0.2	+
12.	Methanol extract of SPCA	0.4	+
13.	Methanol extract of SPCA	0.2	+
14.	Ethyl acetate of SPCR	0.4	+
15.	Ethyl acetate of SPCR	0.2	+
16.	Ethanol extract of SPCR	0.4	+
17.	Ethanol extract of SPCR	0.2	+
18.	Methanol extract of SPCR	0.4	+
19.	Methanol extract of SPCR	0.2	+
20.	Compound **A** (Friedelin)	0.1 μg/disc	+
21.	Compound **B** (Friedelinol)	0.1 μg/disc	-
22.	Compound **C** (β-sitosterol)	0.1 μg/disc	+
23.	Compound **D** (β-sitosterol-β-D-glucoside)	0.1 μg/disc	-

(+) Presence of antitumor activity

(-) Absence of antitumor activity

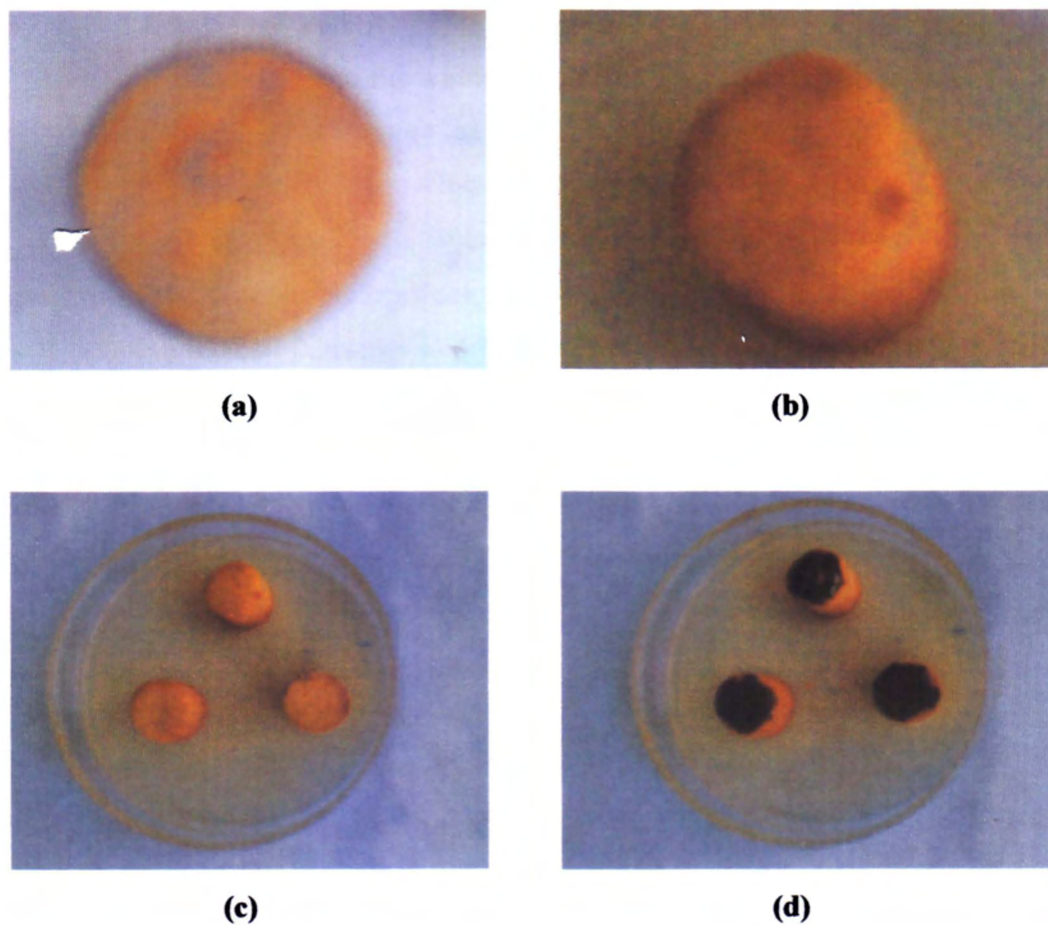


Figure 3.77 Antitumor screening on test sample

(a) Control potato without test sample

(b) Potato disc containing test sample

(c) Before treating with Lugol's solution and

(d) After treating with Lugol's solution

3.7.4.1 Antitumor activity of some crude extracts and some isolated compounds from the whole plant of *C. repens* (WRC), aerial parts and rhizomes of *B. rotunda* (SPCA, SPCR) by agar well diffusion method

Screening of antitumor activity of crude extracts such as ethyl acetate, methanol and ethanol and some isolated compound **A-D** (friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside) from WRC, SPCA, SPCR was done by agar well diffusion method (Ghanney and Rhouma, 2015) according to the procedure as mentioned in Section 2.10.5.4. In this investigation, the extracts and some isolated compounds were tested against. *Agrobacterium tmnefaciens*. The inhibition zone diameter, including the filter paper showed the degree of antitumor activity. The inhibition zones of crude extracts and some isolated compounds against *A.tumefaciens* tested are shown in Figures 3.78, 3.79, 3.80 and 3.81 and the observed data are summarized in Tables 3.44 and 3.45.

According to the results, ethyl acetate extract of WRC showed strong antitumor activity against *A.tumefaciens*. (inhibition zone diameter 40 mm). In addition, methanol extract (inhibition zone diameter 20 mm) and ethanol extract (inhibition zone diameter 36 mm) of WRC exhibited antitumor activity against *A. tumefaciens*.

Ethyl acetate extract (inhibition zone diameter 40 mm), methanol extract (inhibition zone diameter 30 mm) and ethanol extract (inhibition zone diameter 40 mm) of SPCR showed antitumor activity against *A. tumefaciens*. Therefore, ethyl acetate extract and ethanol extract were found to possess the highest antitumor activity.

However, ethyl acetate extract (inhibition zone diameter 40 mm), methanol extract (inhibition zone diameter 24 mm) and ethanol extract (inhibition zone diameter 30 mm) of SPCR exhibited antitumor activity against *A.tumefaciens.* Ethyl acetate of SPCR exhibited the highest potency against *A-tumefaciens*.

In addition, it was found that isolated compounds **B** and **D** (friedelinol, β-sitosterol-β-D-glucoside) did not exhibited antitumor activity against *A.tumefaciens*. The isolated compound **A** (friedelin) (inhibition zone diameter 14 mm) and the isolated compound **C** (β-sitosterol) (inhibition zone diameter 13 mm) exhibited antitumor activity against *A.tumefaciens*.

Table 3.44 Results of Antitumor Activity of Various Extracts from WRC, SPCA, SPCR by Agar Well Diffusion Method

No	Type of Samples	Diameter of Inhibition Zone (mm)		
		EtOAc extract	MeOH extract	EtOH extract
1	WRC	40	20	36
2	SPCA	40	30	40
3	SPCR	40	24	30

Agar well diameter = 10 mm

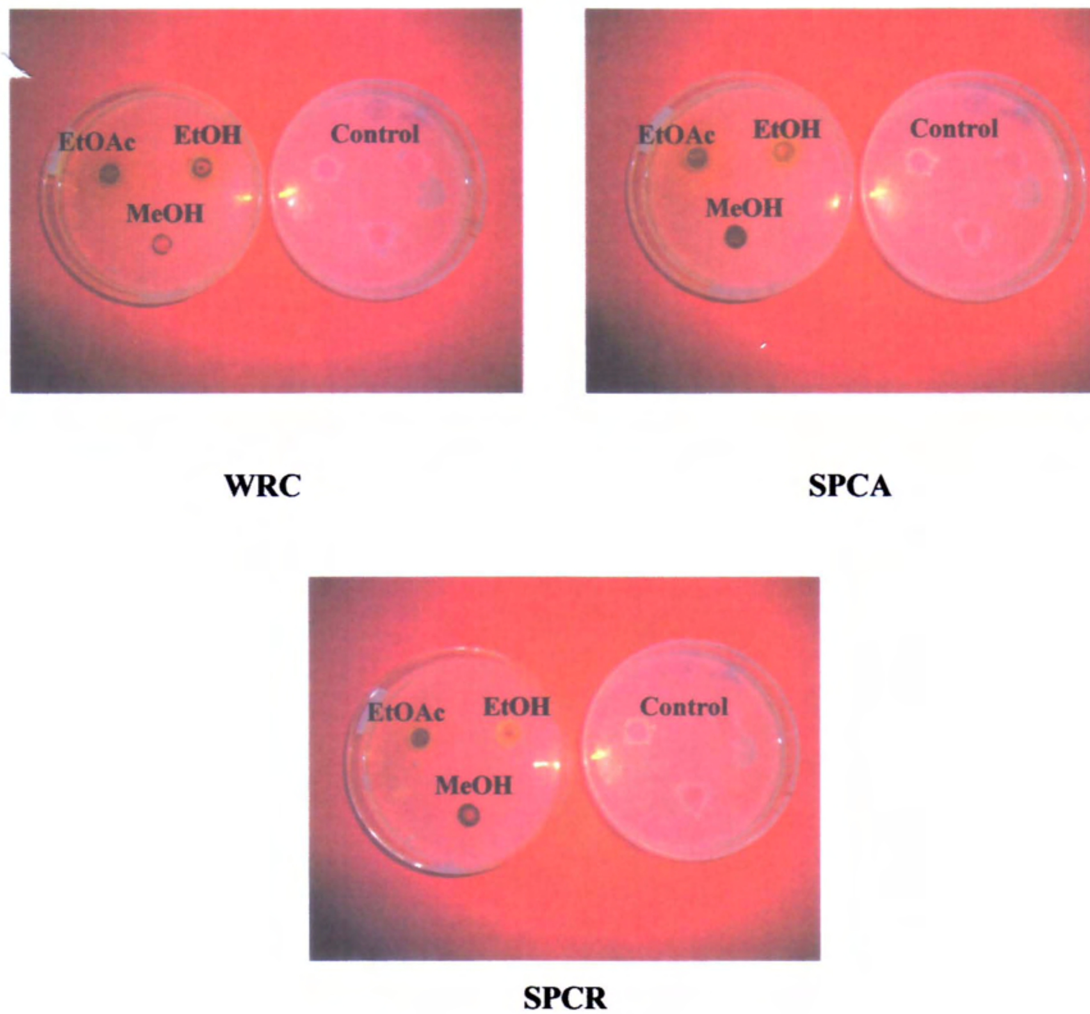


Figure 3.78 Effect of different extracts from WRC, SPCA and SPCR on *Agrobacterium tumefaciens* by agar well diffusion method

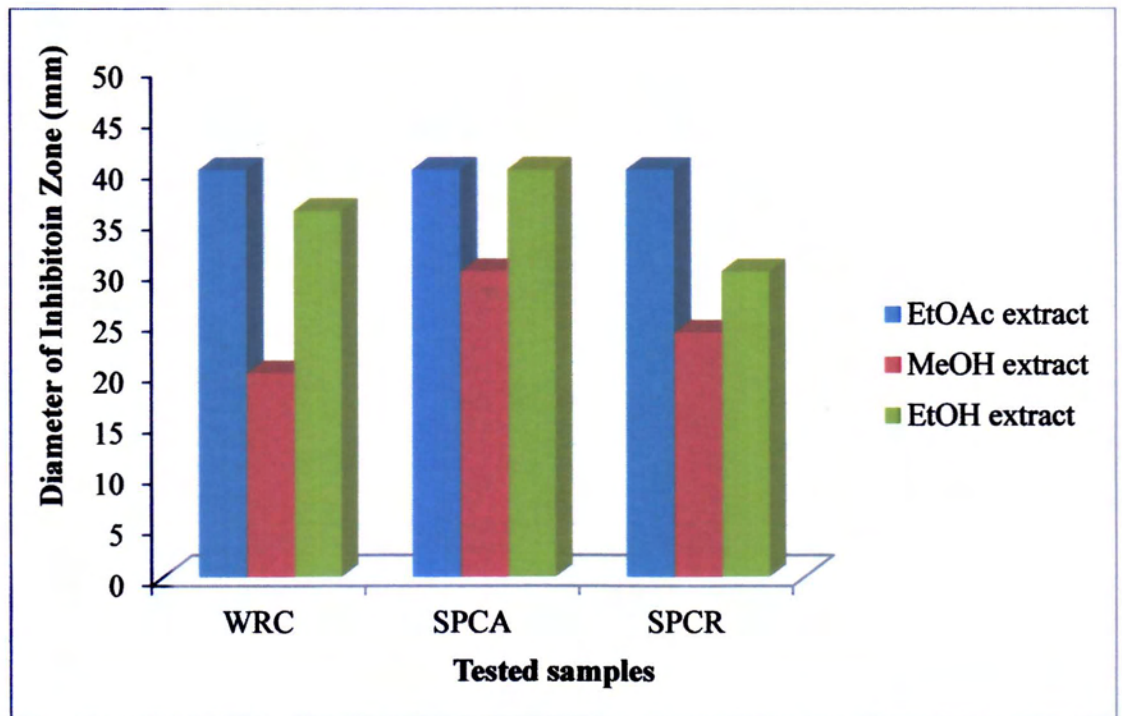


Figure 3.79 Histogram of antitumor activity of different extracts from WRC, SPCA and SPCR on *Agrobacterium tumefaciens* by agar well diffusion method

Table 3.45 Antitumor Activity of Some Isolated Compounds (A-D) against *A. tumefaciens* by Agar Well Diffusion Method

No	Type of Samples	Diameter of Inhibition Zone (mm)
1	Friedelin	14
2	Friedelinol	-
3	β-sitosterol	13
4	β-sitosterol-β-D-glucoside	-

Agar well diameter = 10 mm

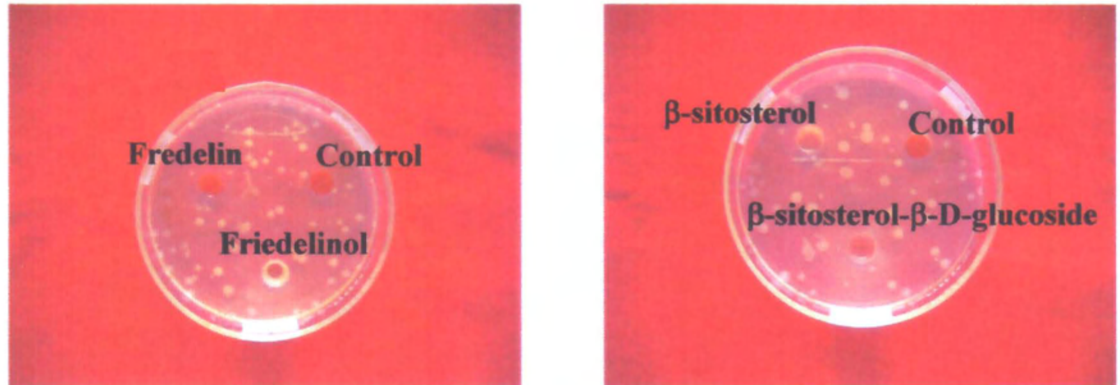


Figure 3.80 Effect of some isolated compounds from A-D on *Agrobacterium tumefaciens* by agar well diffusion method

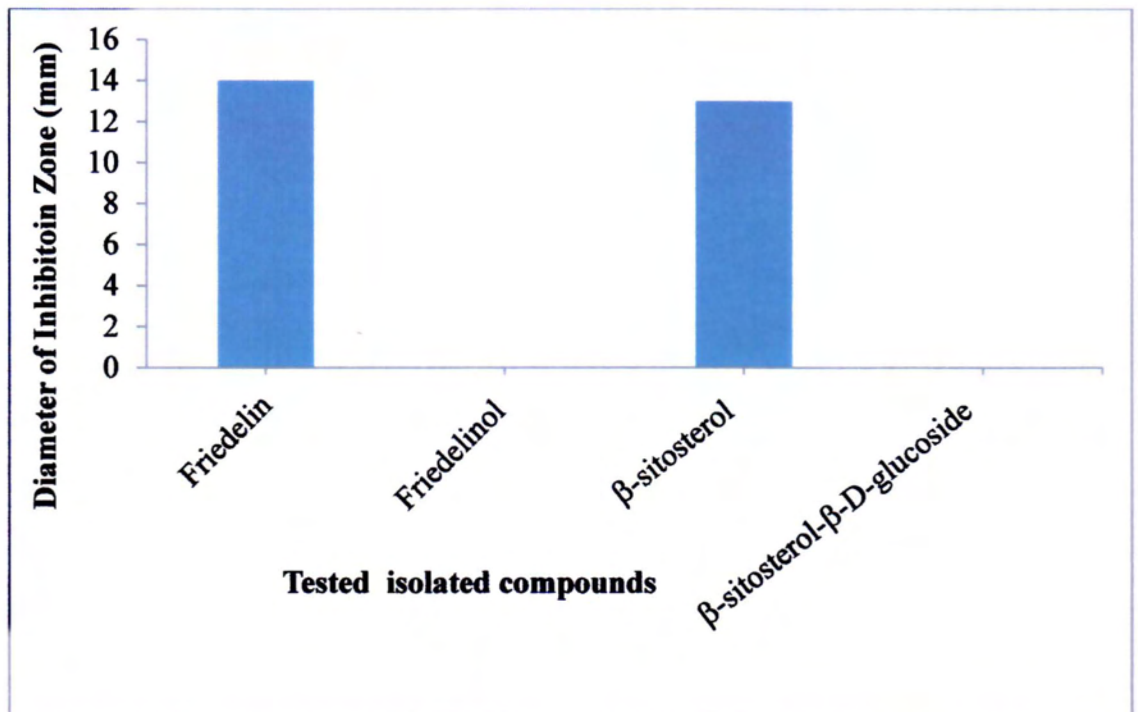


Figure 3.81 Histogram of antitumor activity of some isolated compounds (A-D) on *Agrobacterium tumefaciens* by agar well diffusion method

3.7.5 Screening of antiproliferative activity on cell lines

Cancer is a malignant tumor or malignant neoplasm, is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Many traditional plant treatments for cancer are used throughout the world, and some of these plants have been scrutinized while a good number of them are yet to receive scientific scrutiny. Among them, the whold plant of *C.repens* (WRC), aerial parts and rhizomes of *B.rotunda* (SPCA and SPCR) were selected for this study since they are widely distributed in Myanmar.

Antiproliferative activity is the activity relating to a substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues. Antiproliferative activity were studied in vitro using human cancer cell lines. Screening of antiproliferative activity of methanol extracts of WRC, SPCA and SPCR, chloroform extrat of SPCR and four isolated compounds (pinostrobin, 4′, 7-dimethylkaempferol, galanal A and galanal B) was done by using ten human cancer cell lines according to the procedure as mentioned in Section 2.10.6. Antiproliferative activity was expressed as the IC_{50} (inhibitory concentration) value. 5-Fluorouracil was used as positive control. This experiment was done in Division of Natural Product Chemsitry, Institute of Natural Medicine, University of Toyama, Japan.

The cell lines used were LK-2, A549 (human lung cancer), ECC 4 (human stomach cancer), Colo 205 (human colon cancer), HuH_7 (human liver cancer), Hela (human cervix cancer), K 562 (human leukemia cancer), DU 145 (human prostate cancer), MCF7 (human breast cancer) and WI-38 (normal human fibroblast).

The antiproliferative activity of crude extracts and some isolated compounds are summarized in Table 3.46.

From the results, it was observed that methanol extract of WRC did not show antiproliferative activity. The methanol extract of SPCA were observed to possess higher antiproliferative activity against stomach (ECC4), liver (HuH7), leukemia (K 562), breast (MCF7) human cancer lines and normal human fibroblast (WI-38) than other extracts. The IC_{50} values were found to be 65.43, 56.12, 55.65, 73.74 and 70.24 against lung (LK-2), lung (A 549), stomach, breast human cancer cell lines and normal human fibroblast for chloroform extract of SPCR. The IC_{50} values were found to be 70.02, 57.42, 60.32, > 100, 74.46, > 100, >100, >100, 73.06 and

75.88 μg/mL against lung (LK-2), lung (A549), stomach, colon, liver, cervix, leukemia, prostate, breast human cancer cell lines and normal human fibroblast for methanol extract of SPCR. Since the lower the IC_{50} values, the higher the antiproliferative activity. Therefore, chloroform extract of SPCR was observed to possess higher antiproliferative activity than methanol extract of SPCR. In addition, the antiproliferative activity of four extracts were found to be in order of SPCA (methanol extract) > SPCR (chloroform extract) > SPCR (methanol extract) > WRC (methanol extract).

On the other hand, among the isolated compounds, galanal A (IC_{50} 4.38, 28.20, 32, 38.62, 2.60 μg/ml) and galanal B (IC_{30} 5.75, 8.02, 5.26, 6.79, 5.68 μg/ mL) were found to be more potent than pinostrobin (IC_{50} > 100, 78.74, > 100, >100, >100 μg/ml) and 4′,7-dimethylkaempferol (IC_{50} 8.79, > 100, 35.18, > 100, 40.71 μg/ml) in antiproliferative activity against lung (LK-2), lung (A 549), stomach, breast human cancer cell lines and normal human fibroblast.

It can be inferred that SPCA and SPCR possessed the higher antiproliferative activity than WRC, due to the presence of galanal A and galanal B.

Table 3.46 Antiproliferative Activity of Crude Extracts and Some Isolated Compounds against Various Types of Cancer Cell Lines

Samples	IC_{50} (µg/mL) of Various Samples against Tested Cell Lines									
	LK-2	**A549**	**ECC4**	**COLO205**	**HuH7**	**Hela**	**K562**	**DU145**	**MCF7**	**WI-38**
WRC (MeOH-extract)	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
SPCA (MeOH-extract)	84.88	59.77	24.81	>100	54.91	60.59	66.56	>100	32.49	68.22
SPCR (MeOH-extract)	70.02	57.42	60.32	>100	74.46	>100	>100	>100	73.06	75.88
SPCR ($CHCl_3$-extract)	65.43	56.12	55.65	-	-	-			73.74	70.24
Pinostrobin	>100	78.74	>100	-	-	-			>100	>100
4′,7-Dimethyl kaempferol	8.79	>100	35.18	-	-	-			>100	40.71
Galanal A	4.38	28.20	32	-	-	-			38.62	2.60
Galanal B	5.75	8.02	5.26	-	-	-			6.79	5.68

LK-2, A549 - lung cancer cell lines
ECC4 - stomach cancer cell lines
COLO 205 - colon cancer cell lines
HuH 7 - liver cancer cell lines
Hela - cervix cancer cell lines
K 562 - leukemia cancer cell lines
DU 145 - prostate cancer cell lines
MCF 7 - breast cancer cell lines
WI-38 - normal human fibroblast

CHAPTER IV

4. CONCLUSION

From the overall assessments of the present work concerning with the phytochemical and biomedical investigation of *C. repens* Lam. (Wa-round-chin) and *B. rotunda* (L.) Mansf. (Seik-phoo-chin), the following inferences can be deduced.

1. The preliminary phytochemical tests have revealed that the presence of alkaloids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, saponins, steroids, tannins and terpenoids in the whole plant of *C.repens* (WRC), aerial parts and rhizomes of *B.rotunda* (SPCA and SPCR).
2. Determination of nutritional values of WRC, SPCA and SPCR have also been carried out by AOAC method, resulting 3.11%, 3.28% and 4.49% of moisture, 6.80%, 14.75% and 7.00% of ash, 12.18%, 9.23% and 6.82% of protein, 13.73%, 28.54% and 12.24% of fiber, 7.90%, 5.81% and 3.62% of fat, 56.28 %, 38.39 % and 65.83 % of carbohydrates respectively on the basis of dried samples.
3. The soluble matter contents in some organic solvents such as PE, EtOAc, CH_3COCH_3, EtOH and water were 65.1, 88.0, 77.0, 86.2 and 157.9 mg/g in WRC, 39.4, 17.9, 43.8, 9.0, 171.0 mg/g in SPCA and 1.4, 47.8, 21.7, 12.5 and 127.5 mg/g in SPCR respectively, indicative that all samples were mostly composed of polar constitutes.
4. On silica gel column chromatographic separation, eight compounds were isolated : friedelin (**A**, 0.014%, m.pt. 258-259°C) and friedelinol (**B**, 0.008 %, m.pt. 278-279 °C) from PE extract of WRC, β-sitosterol (**C**, 0.26 %, m.pt. 138-140°C) and β-sitosterol-β-D-glucoside (**D**, 0.9 %, m.pt. 272-274°C) from EtOAc extract of SPCR, pinostrobin (**E**, 0.26 %, m.pt.96-98°C), 4′,7-dimethylkaempferol (**F**, 0.25 %, m.pt. 179-182°C), galanal A (**G**, 0.43 %, m.pt. 165-167°C) and galanal B (**H**, 0.83 %, m.p.t, 134-134.5°C) from $CHCl_3$ extract of SPCR. The isolated compounds were characterized by some physical and chemical properties and structurally identified by the

combination of UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR and ESI MS spectroscopic methods and also by comparing with the reported data.

5. The antimicrobial activity of some crude extracts such as pet-ether, ethyl acetate, ethanol and methanol were tested on six microorganism by agar well diffusion method. In this results, all of the crude extracts except PE extract of WRC exhibited potent antimicrobial activity with inhibition zone diameters ranged between 13 mm ~ 55 mm in WRC, 14 mm ~ 55 mm in SPCA and 13 mm ~ 35 mm in SPCR respectively. Since WRC, SPCA and SPCR were found to possess pronounced antimicrobial activity against both Gram (+) and Gram (-) bacteria and also against fungus.

6. The MIC values of ethyl acetate extracts and some isolated compounds **A-D** (friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside) of WRC, SPCA and SPCR were studied on two species of organisms such as *Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method with different concentrations ranging from 100000 μg/ mL and 100 μg/ mL to 0.1907 μg/ mL and 0.0002 μg/ mL respectively. The MIC values for ethyl acetate extracts of WRC, SPCA and SPCR were found to be 24.4141, 25000, 100000 μg/ mL and 25000, 25000, 100000 μg/ mL against *Staphylococcus aureus* and *Escherichia coli* respectively. Friedelin showed the lowest MIC value, 12.5 μg/ mL and 25 μg/ mL against *S.aureus* and *E. coli*. Friedelinol also exhibited the lowest MIC values of 100 μg/ mL against *Staphylococcus aureus* and *Escherichia coli* while MIC values of β-sitosterol were 25 μg/ mL against *Staphylococcus aureus* and *E.coli*. Therefore ethyl acetate of WRC and friedelin have more pronounced antimicrobial activity compared with other extracts and isolated compounds. Thus, the whole plant of *C. repens* (WRC) possess the highest antimicrobial activity due to the presence of friedelin.

7. The antioxidant activity of ethanol and water crude extracts of WRC, SPCA and SPCR and some isolated compounds **A, B** and **D** (fridelin, friedelinol, β-sitosterol-β-D-glucoside) were evaluated by DPPH free radical scavenging assay. Among the ethanol extracts, the order of radical scavenging activity was observed as SPCA > SPCR > WRC whereas the order of water extracts was SPCA > WRC > SPCR. The ethanol extract of SPCA scavenged 50% of

DPPH free radical with 63.65 μg/mL. The antioxidant potential of isolated compounds were found to be in the order of β-sitosterol-β-D-glucoside > friedelin. Friedelinol did not show % inhibition in the range of concentration 28.71-47.69 μg/ mL indicating that friedelinol did not have the antioxidant property. Ethanol and water extracts of both test sample and some isolated compounds have the lower antioxidant activity than standard gallic acid and vitamic C. Due to these observations, ethanol and water extracts of WRC, SPCA, SPCR and some isolated compounds **A** and **D** (friedelin, β-sitosterol-β-D-glucoside) may be useful for the cure of oxidative stress related diseases.

8. According to the brine shrimp cytotoxicity test, ethanol crude extract of SPCR possessed mild cytotoxicity on brine shrimp. Its LD_{50} was determined to be 370.50 μg/ mL after 24 hours while the other crude extracts did not exhibit their cytotoxic effect up to the optimum dose of 1000 μg/ml.

9. Antitumor activity of ethyl acetate, ethanol and methanol extracts of WRC, SPCA, SPCR and some isolated compounds : friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside were also tested on tumor produced bacteria, *Agrobacterium tumefaciens* isolated from *Sandorium koetjape* Merr. (Thitto) leaves, using PCG (Potato Crown Gall) test. From this experiment, ethyl acetate, ethanol and methanol extracts of WRC, SPCA and SPCR significantly inhibited the formation of tumor with the dose of 0.2 g/disc. In addition, it was observed that some isolated compounds: friedelin and β-sitosterol showed to prevent the tumor formation with the dose of 0.1 μg/disc. But tumor formation was not prevented by isolated compound (friedelinol and β-sitosterol-β-D-glucoside) with the dose up to 0.1 μg/disc.

10 Moreover, antitumor activity of ethyl acetate, methanol and ethanol extracts of WRC, SPCA, SPCR and four isolated compounds : friedelin, friedelinol, β-sitosterol, β-sitosterol-β-D-glucoside were also investigated by employing agar well diffusion method against *Agrobacterium tumefaciens*. According to results obtained, EtOAC extract (40 mm), MeOH extract (20 mm) and EtOH extract (36 mm) from WRC showed antitumor activity against *Agrobacterium tumefaciens*. EtOAc extract (40 mm), MeOH extract (30 mm) and EtOH

extract (40 mm) from SPCA exhibited antitumor activity against *Agrobacterium tumefaciens*, whereas, EtOAc extract (40 mm), MeOH extract (24 mm) and EtOH extract (30 mm) of SPCR exhibited antitumor activity. From this investigation, SPCA extracts were observed to possess higher antitumor activity than WRC and SPCR extracts. Among the crude extracts tested, EtOAc extracts from WRC, SPCA and SPCR showed the most pronounced activity. In the case of compounds, it was found that friedelinol and β-sitosterol-β-D-glucoside did not exhibit antitumor activity against *A.tumefaciens*. Friedelin (14 mm) and β-sitosterol (13 mm) exhibited mild antitumor activity against *A.tumefaciens*.

11. Antiproliferative activity of MeOH extract of WRC, SPCA and SPCR, $CHCl_3$ extract of SPCR and four isolated compounds pinostrobin, 4′, 7-dimethylkaempferol, galanal A and galanal B from SPCR was done by using ten human cancer cell lines. MeOH extract of WRC did not show antiproliferative activity. MeOH extract of SPCA were observed to posses higher antiproliferative activity against stomach (ECC4), liver (HuH7), cervix (Hela), leukemia (K562), breast (MCF 7) human cancer cell lines and normal human fibroblast (WI-18) than other extracts. In addition, the antiproliferative activity of four extracts were found to be in order of SPCA (MeOH extract) (IC_{50} 84.88, 59.77, 24.81, 32.49, 68.22) > SPCR ($CHCl_3$ extract) (IC_{50} 65.43, 56.12, 55.65, 73.74, 70.24) > SPCR (MeOH extract) (IC_{50} 70.02, 57.42, 60.32, 73.06, 75.88) > WRC (MeOH extract) (IC_{50} > 100, > 100, > 100, > 100, > 100) against lung (LK-2), lung (A549), stomach, breast human cancer cell lines and normal human fibroblast. The order of anitproiferative activity of three extracts were observed as SPCA (MeOH extract) (IC_{50} > 100, 54.91, 60.59, 66.56, > 100) > SPCR (MeOH extract) (IC_{50} > 100, 74.46, > 100, > 100, > 100) > WRC (MeOH extract) (IC_{50} > 100, > 100, > 100, > 100, > 100) against colon, liver, cervix, leukemia and prostate human cancer cell lines. Among the test isolated compounds, galanal A (IC_{50} 4.38, 28.20, 32, 38.62, 2.60) and galanal B (IC_{50} 5.75, 8.02, 5.26, 6.79, 5.68) were found to be more potent than pinostrobin (IC_{50} > 100, 78.74, > 100, > 100, > 100) and 4′, 7-dimethylkaempferol (IC_{50} 8.79, > 100, 35.18, > 100, 40.71) in antiproliferative

activity against lung (LK-2), lung (A549), stomach, breast human cancer cell lines and normal human fibroblast. It can be concluded that SPCA and SPCR possessed the higher antiproliferative activity than WRC, due to the presence of galanal A and galanal B.

According to these observations, it can be inferred that since the solvent extracts of the whole plant of *C.repens* (WRC), aerial parts and rhizomes of *B.rotunda* (SPCA and SPCR) and isolated compounds **A, B, C** and **D** showed antitumor activity, antioxidant activity and antimicrobial activity and isolated compounds **E, F, G** and **H** also showed antiproliferative activity, these plant samples may be effectively used in the formulation for treatment of skin diseases, wound infections, diarrhea oxidative stress related diseases, some forms of cancer, some age-related disorders and the diseases infected by the microorganisms tested.

SUGGESTION FOR FURTHER WORK

- The remaining other bioactive phytochemical constituents from WRC and SPCA should be investigated by suitable chromatographic methods followed by modern spectroscopic identification.
- Other pharmacological activities such as hypertension, inflammatory activity etc. should be extended by both *in vitro* and *in vivo* methods.

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APPENDIX

Preparation of Reagent

1. Mayer's Reagent

Mercuric chloride (1.3 g) was dissolved in distilled water (60 cm). It was added to a solution of potassium iodide (5 g) in water (20 mL), mixed and added with sufficient water to made up to 100 mL.

2. Dragendroff's Reagent

(i) Potassium iodide (8.0 g) was dissolved in 20 mL of water.

(ii) Bismith subnitrate (0.885 g) was dissolved in the mixture of 10 mL of glacial acetic acid and 40 mL of water.

(iii) The two solutions were mixed together.

3. Wagner's Reagent

Iodine (1.3 g) was dissolved in 50 mL of water, and mixed with a solution of 2.0 g of potassium iodide in 10 mL of distilled water. And then distilled water added to make up to 100 mL.

4. Sodium Picrate Solution

Picric acid (ca.7.2 g) was quickly dried between filter paper, weighed quickly and treated with about 100 mL distilled water. Solution was then neutralized with 1M solution hydroxide (20 mL). The resulting solution was found to be rather alkaline, this saturated picric acid solution (7.2 g in 100 mL) was added until it was slightly acidic.

5. 10% α-Naphathol Solution

α-Naphthol (10 g) was dissolved in 50 mL of ethanol and the volume was made up to 100 mL in volumetric flask.

6. **1% Ferric Chloride Solution**

Accurately weighed of ferric chloride (1g) was dissolved in distilled water (100 mL).

7. **1% Potassium Ferricyanide Solution**

Accurately weighed of $K_3Fe(CN)_6$ (1g) was dissolved in distilled water (100 mL).

8. **Ninhydrin Reagent**

Ninhydrin (0.2 g) was dissolved in 100 mL of acetone (0.2% w/v).

9. **10% Lead Acetate**

Lead acetate (10 g) was accurately weighed and dissolved in small amount of distilled water and then the volume was made up to 100 mL.

10. **5% H_2SO_4 Solution**

5% H_2SO_4 solution was prepared by adding 5 mL of concentrated H_2SO_4 to 100 mL of distilled water.

11. **1% HCl Solution**

1% HCl solution was prepared by the mixing of 10 mL of HCl of 428.4 mL of distilled water.

12. **Liebermann-Burchard Reagent**

Acetic anhydride (5 mL) and concentrated sulphuric acid (5 mL) were carefully added to (50 mL) absolute ethanol while cooling in ice and freshly prepared solution was used.

13. Benedict's Solution

A mixture of sodium citrate (1.13 g) and anhydrous sodium carbonate (1 g) was heated and then 60 mL of distilled water was added to the above mixture solution while constant stirring. Then, it was cooled and the volume was made to 100 mL in a volumetric flask.

14. Fehling Solution (A)

Copper sulphate crystal ($CuSO_4.5H_2O$, 6.9 g) was dissolved in distilled water and the volume made up to 100 mL in a volume flask.

15. Fehling Solution (B)

Sodium potassium tartarate crystal (Rochell salt, $C_4H_4O_6$ NaK. $4H_2O$, 34.6 g) was dissolved in warm distilled water and 12 g of sodium hydroxide pellet were dissolved in suitable volume of distilled water. The solutions were then mixed, cooled and made up to 100 mL in a volumetric flask with distilled water.

16. Bromocresol Green Indicator

Bromocresol (0.1 g) was warmed with 2.9 mL of 0.05 N NaOH solution and 5 mL of 90% alcohol; after solution was effected, sufficient 20% alcohol was added to produce 250 mL.

17. 1% Gelatin Solution

1 g of gelatin was dissolved in 100 mL of distilled water by prolong heating on a water-bath.

CREDIT SEMINAR COMPLETED

First seminar

Title	:	**Phytochemical Analysis and Nutritional Values of *Cissus repens* Lam. (Wa-round chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin)**
Date	:	5.12.2012
Venue	:	Theatre (S-17), Department of Chemistry, University of Yangon

Second Seminar

Title	:	**Investigation of Cytotoxicity and Antioxidant Activities of Crude Extracts and Isolation of Some Phytoconstituents of *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin)**
Date	:	12.12.2013
Venue	:	Theatre (S-17), Department of Chemistry, University of Yangon

Third seminar

Title	:	**Isolation and Identification of Some Bioactive Phytoconstituents from *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.)Mansf. (Seik-phoo-chin) and Investigation of Antimicrobial Activity and Antitumor Activity**
Date	:	23.12.2014
Venue	:	Theatre (S-17), Department of Chemistry, University of Yangon

Fourth seminar

Title	:	**A Phytochemical and Biomedical Investigation of *Cissus repens* Lam.(Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf.(Seik-phoo-chin)**
Date	:	21.4.2016
Venue	:	Theatre (S-17), Department of Chemistry, University of Yangon

PRELIMINARY PUBLICATION

1. May Mon Kyaw, Nwet Nwet Win, Ni Ni Than, Hnin Hnin Aye, Daw Hla Ngwe, (2016), "A Phytochemical and Biomedical Investigation of *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin)", Chemical Bulletin, Issue No.5, April 2016, Department of Chemistry, University of Yangon.

CURRICULUM VITAE

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Ph.D. Dissertation Title	:	A Phytochemical and Biomedical Investigation of *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf.(Seik-phoo-chin)
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External Examiner Report on

"A Phytochemical and Biomedical Investigation of *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin)"

Submitted by

May Mon Kyaw

(၄ပါရဂူ-ဓ-၅)

The research conducted for Ph.D. dissertation has focused on the evaluation of bioactive principles from locally cultivated two medicinal plants namely, *Cissus repens* Lam. (Wa-round-chin) and *Boesenbergia rotunda* (L.) Mansf. (Seik-phoo-chin).

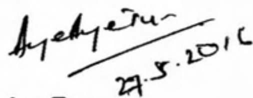
Preliminary studies on phytochemicals; nutritional values; and soluble matter contents of the plants were conducted. The main focuses were isolation and structural elucidation of organic compounds, and biological activities screenings.

Two compounds, friedelin and friedelinol, were isolated from *Cissus repens*. In addition, six compounds, namely, β-sitosterol and its β-D-glucoside, pinostrobin, 4',7-dimethyokaempferol, galanal A and galanal were isolated from *Boesenbergia rotunda*. The structures of all compounds were spectroscopically elucidated.

In addition, biological activities screenings were done for plants extracts and isolated compounds. The antimicrobial activities were screened against six microorganism such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* by agar well diffusion method. Moreover, antioxidant activities via DPPH radical scavenging assay, and cytotoxicity by brine shrimp test were investigated.

In my opinion, the essence and privilege of this dissertation could be at the anticancer activities testing using different assays: potato crown gall test and antiproliferative activity test using different cell lines. *Boesenbergia rotunda* and its compounds have promising activities and need to explore more.

She has done her work well, showed her expertise not only in phytochemistry field but also in biomedical field. She wrote her dissertation well and I hardly noticed topographical and grammar errors. In addition, she showed her good performance on viva voce examination. I satisfied her works and strongly recommended her to be awarded the degree of PhD.



Aye Aye Tun
MSc (YU), DSc (Kyushu Univ.)
Pro-Rector
Dagon University

Referee's Report

for PhD Dissertation submitted by Ma May Mon Kyaw (4PhD-Chem-5)

27-5-2016

I have read carefully PhD Dissertation Title with "Phytochemical and Biomedical Investigation of *Cissus repens* Lam. (Wa-Round-Chin) and *Boesenbergia routunda* (l.) Mansf. (Seik-Phoo-Chin) submitted by Ma May Mon Kyaw (4PhD-Chem-5).

This Dissertation consists of phytochemical investigation, determination of nutritional values and biological activities studies such as antimicrobial activity, acute toxicity and antioxidant activity of the selected medicinal plant from medicinal point of view.

Furthermore, the identification of isolated compounds by tlc method and modern spectroscopic methods including 2D NMR spectroscopy and HR ESI mass spectrometry have also completed for organic chemistry point of view.

The special study which includes the determination of antitumor activity and antiproliferative activity of the selected medicinal plant is very interested for medicinal point of view.

Instead of a little typing error was found in this dissertation, Viva voce examination is also satisfactory.

Therefore, I am glad to recommend Ma May Mon Kyaw (4PhD-Chem-5) for the Degree of Doctor of Philosophy in Chemistry.

Dr Than Soe
Referee
Rector
Myitkyina University