

**ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL AND ANTIOXIDANT  
COMPOUNDS FROM *RICINUS COMMUNIS* LEAVES**

by

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## **DECLARATION**

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Masters in Microbiology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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**Nemudzivhadi, V (Mr)**

**Date**

## **DEDICATION**

I dedicate this work to my mother, brothers, sister, grandmother, late grandfather, uncles and all who supported, encouraged and prayed for me throughout the study.

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### ***Exodus 23:20***

*Behold, I send an angel before you to keep and guard you on the way and to bring you to the place I have prepared for you. Beware of him and obey his voice.*

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## **LIST OF ABBREVIATIONS**

<sup>13</sup> C	Carbon-13
<sup>1</sup> H	Hydrogen-1
A	Acetone
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
ATCC	American type culture collection
BAW	Butanol/Acetic acid/Water
BEA	Benzene/Ethanol/Ammonia hydroxide
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
Bud-8	Human Caucasian skin fibroblast cell line
C	Chloroform
CEF	Chloroform/Ethyl acetate/Formic acid
Cox	Cyclooxygenase
DAPI	4', 6- diamidine-2-phenylindole
DCF	2, 7-dichlorofluorescein
DCM/ D	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMEM	Dubleco's Modified Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPPH	2, 2-diphenyl-1-picrylhydrazyl ()
E	Ethanol
E. F	<i>Enterococcus faecalis</i>
E.C	<i>Escherichia coli</i>
EA	Ethyl acetate
EMW	Ethyl acetate/methanol/water
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
H	Hexane
H <sub>2</sub> DCF-DA	Dihydrodichlorofluorescein diacetate
INT	p-iodonitrotetrazolium violet
Lc <sub>50</sub>	Lethal concentration for 50% of the cells
LPS	Lipopolysaccharides
M	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2- yl)-2, 4-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
NSAIDS	Non-steroidal anti-inflammatory drugs
O <sub>2</sub> <sup>-</sup>	Superoxide radicals
OH <sup>-</sup>	Hydroxyl

P.A	<i>Pseudomonas aeruginosa</i>
PMA	Phorbol 12-myristate 13-acetate
PSN	Penicillin-Streptomycin-Neomycin
R <sub>f</sub> value	Retardation factor Value
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.A	<i>Staphylococcus aureus</i>
TBHQ	Tertiary butylated hydroquinone
TLC	Thin layer chromatography
USA	United States of America
UV	Ultraviolet light
W	Water

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## ABSTRACT

Antioxidants play an important role in living organisms to control level of free radicals and other reactive molecules in the body to reduce oxidative damage. Synthetic antioxidant compounds are used in food industries as food additives to boost our immune systems. These compounds are associated with a number of critical side effects including liver damage and carcinogenesis. Scientists are also concerned about microorganisms that have developed resistant genes against current antibiotics used in hospitals. The aim of the study was to isolate and characterize bioactive compounds from *Ricinus communis* leaves with activity against *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Consequently, medicinal plants are studied and considered for their efficacy and safety, because they possess bioactive compounds with various biological activities.

Leaves of *R. communis* were collected at the University of Limpopo, Turfloop campus in Limpopo province, South Africa. The leaves were dried and milled to a fine powder. A number of trial extraction methods were employed using various solvents of different polarities on a fine powder leaves to identify the best extraction method. Plant extracts were analyzed by thin layer chromatography (TLC) developed in four mobile phases. To detect separated phytochemical compounds, TLC plates were sprayed with vanillin-sulphuric acid in methanol and heated at 110°C for optimal colour development. Qualitative antioxidant activity was determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates. Quantitative antioxidant activity was determined by measuring percentages scavenging activity of DPPH and 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical molecules by plant extracts. Antibacterial activity of all extracts was quantified by a serial microbroth dilution method while bioautography was used in qualitative analysis of the active compounds. Cytotoxicity effect of *R. communis* extracts was evaluated using tetrazolium-based calorimetric assay on human Caucasian skin fibroblast (Bud-8) cell line. Anti-inflammatory activity was assessed using phagoburst kit on Raw 264.7 macrophages cell line. Pure compounds were subjected to nuclear magnetic resonance spectroscopy for 1H, 13C and DEPT experiments to elucidate structures of compounds.

During extraction process, methanol was the best extractant, extracting greater amount of extracts than any of the other solvents. Serial exhaustive extraction method was selected as the best extraction method for extracting compounds from ground plant materials. In quantitative antioxidant assays, chloroform and methanol extracts had highest percentage scavenging activity against DPPH free radicals compared to other extracts and vitamin C. Methanol extract had the highest percentage scavenging activity of ABTS free radicals and minimum percentage scavenging activity was in hexane extract. Acetone, ethyl acetate and ethanol extracts showed strong antioxidant activity against DPPH free radicals in qualitative antioxidant assay on TLC plates. In quantitative antibacterial assay, crude extracts showed lowest minimum inhibitory concentration value of 0.13 mg/ml against all tested organisms and the highest was 1.05 mg/ml. Hexane extracts revealed potent antibacterial activity against all tested microorganisms on bioautograms. Hexane and acetone extracts also revealed anti-inflammatory activity and have ability to reduce oxidative stress. In cytotoxicity effect of plant extracts, Methanol extracts had lethal concentration for 50% of the cells ( $L_{C50}$ ) of 784  $\mu$ g/ml on Human Caucasian skin fibroblast (Bud-8) cell line while hexane extracts had  $L_{C50}$  of 629  $\mu$ g/ml. Plant extracts with high  $L_{C50}$  are low toxic to normal cell line and preferable to work with for drug development. Bioassay-guided fractionations results in successful isolation of three antioxidant and two antibacterial compounds from *R. communis* using column chromatography. Isolated compounds were tested for their biological activities using qualitative DPPH assay on TLC plates for antioxidant activity and bioautography for antibacterial activity. Antioxidant compounds showed strong antioxidant activity after spraying with DPPH in methanol and antibacterial compounds showed less activity compared to the crude extracts. The study suggests the use of crude extracts to fight against pathogenic microorganisms compared to pure compounds. Compound **4** was successful identified as the mixture of stigmasterol and  $\beta$ -sitosterol. The present study recommends the use of *R. communis* leaves as the potential source of antioxidant, antibacterial and anti-inflammatory compounds. The study serves as a scientific proof for use of this plant in traditional medicine for treatment of various ailments.

## **CHAPTER 1**

### **GENERAL INTRODUCTION**

Medicinal plants are defined as any plants that can be used for medicinal or therapeutic purposes (Rekha *et al.*, 2013). They have been employed as a means of primary health care by more than three quarters of the world's population particularly those in rural areas over the years (Subedi *et al.*, 2012). The use of traditional medicinal plant in rural areas is due to lack of both private and public hospitals, poverty, high unemployment rate, low-cost medicines and other health care organizations. The leaves, roots, stems, fruits, barks and flowers are the essential parts of plants used for medicinal purposes (Sravanti-Kota and Manthri, 2011). Different parts of medicinal plants may consist of one or more constituents with immense biological activities. Usually higher plants are used worldwide in various forms as a source of bioactive constituent for the treatment of different ailments (Mahesh and Satish, 2008). The use of medicinal plants is based on preexisting indigenous knowledge of plants which is generally poorly documented and lacks scientific proof of both safety and method of operation, coupled with the fact that the active principles exhibiting these therapeutic potentials are widely unknown (Schmidta *et al.*, 2009).

Traditional healers use plants for treatment of various diseases including microbial infections, wounds, inflammation, diarrhea, dysentery, skin infections and gonorrhea (Mann *et al.*, 2008; Kensa and Yasmin, 2011). About 3 million people in South Africa use herbs prescribed by traditional healers for their primary health (Coopoosamy and Naidoo, 2012). It is important to study medicinal plants for their safety, quality, and dose required for use for optimum beneficial effect because of increased demands for cheap medicines, high unemployment rate, poverty (Shai *et al.*, 2008) and to avoid toxic effects on the consumers. Researchers select indigenous plants based on their use by traditional healer throughout the world. Scientific proof of the use of these traditional medicinal plants has been considered and about 20% of medicinal plants have been studied for their biological activities (Coopoosamy and Naidoo, 2012). Several researches conducted showed that indigenous plants have several biological activities

such as antioxidant, anticarcinogenic, antimutagenic, antitumor, antibacterial, antiviral, anti-inflammatory antifungal, insecticidal, hepatoprotective, antirheumatic, and antivenin (Khalaf *et al.*, 2008; Akrout *et al.*, 2011; Iqbal *et al.*, 2012). These biological activities emanate from the presence of different types of compounds that constitute the various plant parts known as secondary metabolites. Secondary metabolites include flavonoids, tannins, polyphenols, saponins, chromones and tophenones (Akrout *et al.*, 2011).

Microorganisms have developed resistance to a large range of antibiotics used for treatment of infectious diseases caused by bacteria and fungi. As such, there is a great need to screen medicinal plants for their biological activities, with the aim of developing more effective new alternatives to counter the problem of resistance development of microbes against the existing chemotherapies (Verma *et al.*, 2011). Such drugs isolated from medicinal plants may fight against multi-drug resistant bacterial infections (Anam *et al.*, 2010). Many of the modern medicines currently used in different health care organizations such as hospitals have been discovered and established from different natural sources including medicinal plants and microorganisms (Naz and Bano, 2012). About 80% of people in South Africa and other African countries use traditional medicines for health care and only few plants have been commercialized. There is therefore, a greater need to commercialize drugs isolated from medicinal plants for therapeutic purposes (Street and Prinsloo, 2013).

There is also an increased need to screen medicinal plants for antioxidant compounds, because synthetic antioxidants including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) cause liver disorder and other side effects. Reactive oxygen species cause damage on macromolecules and other cell membranes constitute such as lipids, proteins, phospholipids, and lipoproteins. Examples of the reactive oxygen species are superoxide anion, peroxy radicals, hydroxyl radical and singlet oxygen. These molecules are either by-products or unregulated end products of various normal metabolic pathways. The damage caused by excess free radicals results in several diseases such as cancer, cardiovascular diseases, Alzheimer's diseases and Parkinson's diseases (Kratchanova *et al.*, 2010). However, antioxidants have ability to scavenge free radicals or reactive oxygen species and studies have revealed that antioxidant activity of plants is based on their phytoconstituents (Shahwar *et al.*, 2010).

In food industries antioxidants also play a role to prevent deterioration, nutritional losses and off-flavoring food particularly those foods that contain polyunsaturated fatty acids (Kratchanova *et al.*, 2010).

Inflammation is a patho-physiological response of mammalian tissues or cells to a variety of harmful agents including infectious microorganisms and toxic chemical substances. There are various mediators that are responsible for inflammatory process in the living cells such as eicosanoids, prostaglandins from enzyme COX-1/ 2 (Schmidta *et al.*, 2009). Steroidal and non-steroidal are anti-inflammatory drugs currently used for chronic inflammatory diseases. These anti-inflammatory drugs tend to have side effects on the living system. There is a need to screen for nontoxic novel anti-inflammatory drugs from medicinal plants for management of chronic inflammatory diseases (Karawya *et al.*, 2010). Plants are also tested for antitumor activity and more than 50% of drugs used for antitumor activity were isolated from natural source including plants (Selvakumari *et al.*, 2012). Vincristine and taxol are two antitumor drugs isolated from plants and are used as drugs of choice. Medicinal plants are required to be tested for various biological activities through modern scientific methods for their efficacy and safety (Selvakumari *et al.*, 2012).

## CHAPTER 2

### LITERATURE REVIEW

#### **2.1. Medicinal plants**

Plants have been used as a source of food and drugs by rural populations in South Africa and other African countries. People in rural areas use plants for many purposes including timber, wood, non-timber forest products, and medicines. Traditional healers together with native people in Africa and other countries worldwide are aware of the medicinal properties of plants in their surroundings. In India, medicinal plants have been used as medicines for more than 3, 000 years (Rekha *et al.*, 2013). Medicinal plants are either used as crude extracts directly from plants or indirectly as modern medicines such as drugs. It has been stated that traditional healers in India use about 2500 plant species for treatment of various diseases such as wounds, diabetes, fungal infection and 100 species of which are considered as natural medicines (Rekha *et al.*, 2013).

The indigenous knowledge patterning for the preparation and use of medicinal plants for medicinal purposes have been passed on from generation to generation predominantly in African countries. However this knowledge is at risk of being lost forever as the transmission of information between older and young generation is not always guaranteed and accurate. It is very important to document information about each and every medicinal plant from any country globally for their conservation and sustainable use (Betti *et al.*, 2013). Information of the chemical constituents of medicinal plants is required for screening of new therapeutic agents or drugs. The indigenous knowledge of chemical constituents of medicinal plants helps to disclose the importance of traditional remedies (Senthilmurugan-Viji *et al.*, 2013).

#### **2.2. Medicinal plants in South Africa**

South African has a wealth of indigenous knowledge that has been transmitted from older to younger generations. South Africa is a significant focal point of botanical and cultural diversity of medicinal plants. The use of medicinal plants by South African

residence is very significant in their culture. There are more than 4 000 medicinal plants which are used for medicinal purposes by different people from different cultures in South Africa (Van Wyk, 2011). Traditional healers were the only people who exclusively collected and administered medicinal plants to their patients for various diseases. Nowadays everyone has access to buy medicinal plants of their choice from the herbal shops in both rural and urban areas, sold as crude, unprocessed drugs in herbal markets in various parts of South Africa. There are more than 90 promising medicinal plants native to south Africa that have been studied for their potential in commercialization as medicinal products (Van Wyk, 2011).

### **2.3. Medicinal plants as medicines**

Medicinal plants possess many bioactive compounds that can be used to fight against various types of diseases caused by pathogens. They are regarded as a natural source of many drugs, modern medicines, food supplements, pharmaceutical intermediates and chemical units for synthetic drugs (Senthilmurugan-Viji *et al.*, 2013). They have different active compounds based on their geographical location, soil diversity and climate conditions (Rekha *et al.*, 2013; Thite *et al.*, 2013).

These plants play an important role in daily health care of people in rural communities and developing countries. Older people preferred to use local traditional medicines over modern medicines because local medicines are cheap, regarded to be more effective, readily available, safer and rarely have side effects. They have been used for treatment of many diseases such as fever, malaria, diarrhea, skin infectious, and wounds (Betti *et al.*, 2013). It is important to study medicinal plants that are used in developing countries due to insufficient money to buy commercial medicines, unavailability of doctors, hospitals, and other health services in their communities (Senthilmurugan-Viji *et al.*, 2013).

### **2.4. Plant secondary metabolites of therapeutic relevance**

Plants produce a large and diverse range of organic compounds that appear to have no direct functions in growth and development of plants (Karuppusamy, 2009). Such compounds generally have no well-known functions in the process of photosynthesis,

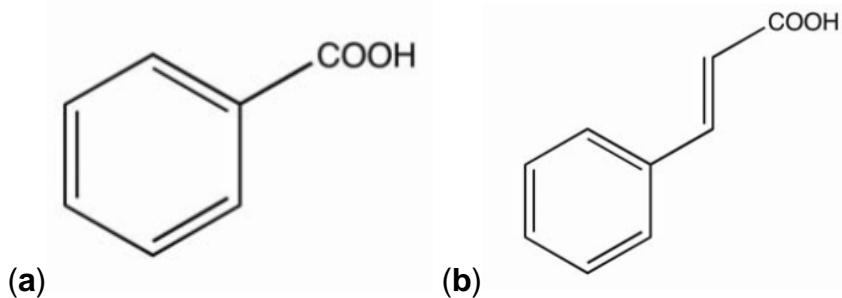
respiration, solute transport, translocation, nutrient assimilation and differentiation (Mazid *et al.*, 2011). Higher plants are a major source of such beneficial secondary metabolites which are useful in the pharmaceutical, agrochemical, flavour and aroma industries and are produced by plants on a continuous basis with no seasonal limitation. Production of secondary metabolites is reliable, predictable, and independent of environmental factors. There are different types of plant derived secondary metabolites such as carotenoids, phytosterols, saponins, alkaloids, tannins, steroids, and flavonoids (Karuppusamy, 2009). Both phenolic compounds and flavonoids are also extensively dispersed in plants and are described to have multiple biological activities including antioxidant, free radical scavenging abilities, anti-inflammatory, antibacterial and anticarcinogenic activities (Patel *et al.*, 2010). Plants produce secondary metabolites as a part of protection mechanisms against pests. Secondary metabolites also play an important role in humans when used to boost the immune system, kill pathogenic microorganisms and also to scavenge free radical molecules. Diets rich in medicinal plants comprise a variety of secondary metabolites that help to protect the body against various illnesses (Karuppusamy, 2009).

#### **2.4.1. Phenolic compounds**

They are produced by plants as protective mechanism against ecological and physiological attack such as pathogens, insects attack, ultraviolet radiation and wound. These compounds have an aromatic ring structure with one or more conjugated hydroxyl groups. They also possess antioxidant properties that play an important role in the prevention of various diseases such as heart diseases, inflammation, diabetes and cancer. Animals and humans consume these compounds from plant products such as fruits and vegetables. Legumes are also rich in phenolic compounds. They occur as either simple or polyphenols, due to the number of phenol units that are available in the molecule. Phenols, coumarins, lignins, tannins and flavonoids are the simple plant phenolic compounds (Khoddami *et al.*, 2013).

There are two major classes of phenolic compounds namely phenolic acids and cell wall phenolics. Phenolic acids are compounds that are found in plants. Phenolic acid exist in the form of esters, glycosides and amide groups. The difference in phenolic acids is on

the number and position of hydroxyls groups located around the aromatic ring of the molecules. These acids have two major structures which are hydroxybenzoic (Figure 2.1a) and hydroxycinnamic acids (Figure 2.1b). Phenolic acids protect cell wall during plant growth against harsh conditions and infections. Cell wall phenolics are associated with other types of cell components. The two major groups of cell wall phenolics are lignins and hydroxycinnamic acids (Khoddami *et al.*, 2013).

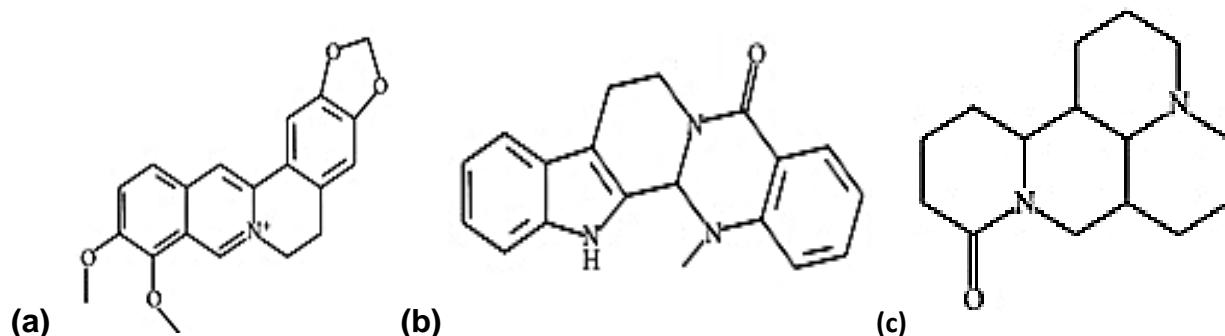


**Figure 2.1:** General structures of phenolic acids (A) hydroxybenzoic acid and (B) hydroxycinnamic acid (Khoddami *et al.*, 2013).

#### 2.4.2. Alkaloids

They are essential compounds used as lead compounds for new drugs discovery. Alkaloids are generally made of a ring structure and a nitrogen atom, located inside the heterocyclic ring structure of the molecule. These compounds are classified in different categories due to their biosynthetic pathways. They are secondary metabolites produced by plants in response to abiotic stress. Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae are families known to have a wide range of alkaloids. They are one of the most effective bioactive compounds from medicinal plants that exhibit essential biological activities (Lu *et al.*, 2012) such as relieving action of ephedrine for asthma, the analgesic actions of morphine and anticancer effects of vinblastine. Some alkaloid compounds have been successfully used as chemotherapeutic drugs for the treatment of cancer. Currently, these compounds are used as anticancer such as camptothecin, topoisomerase inhibitor, and vinblastine which interact with tubulin. They show promising results as anticancer

agents. Examples of natural isolated alkaloids from plants are berberine, evodiamine, matrine (Figure 2.2), piperine, sanguinarine, and tetrandrine (Lu *et al.*, 2012).



**Figure 2.2:** Chemical structures of (a) berberine, (b) evodiamine, (c) matrine (Lu *et al.*, 2012).

#### 2.4.3. Tannins

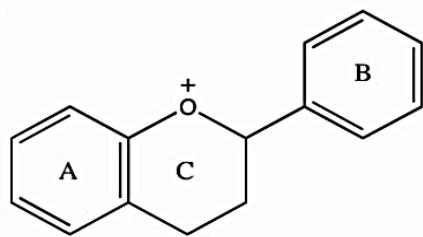
They are diverse group of high molecular weight phenolic compounds joined together with capacity to form reversible and irreversible complexes of proteins, polysaccharides and nucleic acids. These compounds are normally found in traditional medicinal plants, fruits such as grapes and blueberries, tea and chocolate (Saxena *et al.*, 2013). Tannins are secondary metabolites of plants with defensive properties. They are plant phenolic polymers with molecular mass range between 600 to 3000 kda. These compounds are commonly toxic to animals and as such, act as feeding repellents to a great diversity of animals. They cause a sharp, astringent sensation in the mouth of many mammalian herbivores due to their ability to bind salivary proteins. Many herbivores such as cattle, deer and apes do not eat plants with high tannins content (Mazid *et al.*, 2011). They are plant secondary metabolites responsible for different biological activities and have ability to decrease risk of chronic diseases. In India, medicinal plants containing tannins have been used traditionally for treatment of diarrhoea, as diuretics and anti-inflammatory remedy (Saxena *et al.*, 2013).

These compounds have been used in both pharmaceutical and food industries. In the food industry, tannins are used to clarify wine, beer and fruits juices. They are used as antioxidants in fruit juice, beer, and the wine industry. These compounds also play

significant role as caustic for cationic dyes in dyestuff industry and production of inks (Saxena *et al.*, 2013).

#### 2.4.4. Flavonoids

They are phenolic compounds present in plant tissues and play a role in carotenoids and chlorophylls of plants. These compounds mostly have following colours; blue, yellow, orange, purple, and red. Almost all flavonoids are derivatives of aromatic amino acids, phenylalanine and tyrosine. Examples of flavonoids include flavones, flavonols, iso-flavonols, anthocyanins, anthocyanidins, proanthocyanidins and catechins. Flavonoid structures are different based on their scale and form of hydroxylation, prenylation, alkalinisation and glycosylation reactions that change the general structure (Figure 2.3) of flavonoids (Khoddami *et al.*, 2013).



**Figure 2.3:** General structure of flavonoids (Khoddami *et al.*, 2013).

#### 2.4.5. Steroids

They are the most important secondary metabolites of plants which play an essential role in plant cell membranes and plasma membranes as regulatory channels of plant cells. These compounds allow movement of small molecules from outside to the inside of cell by decreasing motion of fatty acid chains. They also play a critical role in the protection of plants from herbivores. For example, milkweeds produce several bitter tasting glucosides (sterols) that protect them against herbivory by most insects and cattle. Triterpene, limnoid are some of the bitter steroids produced by citrus fruits to fight against herbivores (Mazid *et al.*, 2011).

#### **2.4.6. Saponins**

They are secondary metabolites that are found in natural sources including plants. Presence of saponins in plants can be confirmed by stable foam formation in aqueous solutions. There are three groups of saponins namely, glycosylated steroids, triterpenoids and steroid alkaloids. Most of these compounds are known to have antimicrobial activity, inhibit moulds and protect plants from insects attack. They also play an important role in traditional medicinal plants through the exhibition of immunostimulant, hypocholesterolaemic, anticarcinogenic, antifungal, antiviral and antioxidant properties (Saxena *et al.*, 2013).

#### **2.4.7. Terpenoids**

They are groups of secondary metabolites that are responsible for the medicinal activity of some plants. They are derivatives of five carbon isoprene units. Almost all terpenoids molecules have similar structures and differ only in their functional groups and carbon skeletons. These compounds have been used in both food and pharmaceutical industries. In the food industry, terpenoids have been used as flavours and fragrance. Hydrocarbon isoprenes are the building blocks of terpenes. They are classified according to the number of isoprene units, i.e. hemiterpenoids (one isoprene unit), monoterpenoids (two isoprene units), sesquiterpenes (three isoprene units), diterpenes (four isoprene units), triterpenes (six isoprene units) and tetraterperoids (eight isoprene units). This group of compounds is responsible for various biological activities exhibited by plants such as anticacinogenic, antimalarial, antiulcer and antimicrobial activities (Saxena *et al.*, 2013).

### **2.5. Biological activities of plants**

Plants have been used to promote primary health care mostly in rural areas and developing countries, globally. Medicinal plants play an important role in the treatment of various diseases in humans and animals. Medicinal activities of plants are due to secondary metabolites that are produced by plants in response to herbivores and abiotic stress such as harsh conditions, high temperature, ultraviolet light, radiation and low water content. There have been renewed interests in the screening of medicinal

plants with the aim of isolating and characterizing new bioactive compounds that can be used as templates for new drugs or as novel compounds. Since the middle of the 19<sup>th</sup> century, researchers have successfully isolated and characterized different class of bioactive compounds from plants. These bioactive compounds are presently used in the pharmaceutical industries as active ingredients of modern medicines and as leading compounds for discovery of new drugs (Rauf *et al.*, 2013).

Medicinal plants continue to provide valuable therapeutic agents in both modern and traditional medicine. They are a good source of many natural bioactive compounds also known as phytochemicals that are effective and safe to use. Phytochemicals may possess one or more biological activities. Some phytochemicals act in synergistic relationship to exhibit one biological activity. Some of these phytochemicals may act antagonistically i.e. one or more compounds may inhibit activity of other compounds in a crude extracts. However, once they are separated, each compound may exhibit its own biological activity. Bioactive compounds have therapeutic potential and responsible for various biological activities (Molan *et al.*, 2012).

### **2.5.1. Antioxidant activity**

Oxygen plays an important role in biological systems of aerobic forms of life, although its derivatives are highly toxic. Oxygen derivatives are known as free radicals or reactive oxygen species (ROS). Examples of reactive oxygen species are superoxide radicals ( $O_2^-$ ), hydrogen peroxide and hydroxyl ( $OH^-$ ) radicals. Free radicals are produced as either by-products or end products of some biochemical reactions that contribute to the development and maintenance of cellular life. High levels of free radicals in normal systems results in imbalanced between antioxidants and free radicals (Kumar *et al.*, 2012). Free radical molecules possess unpaired electrons which are responsible for highly reactive, uncontrolled, chemical reactions in a cell. The highly reactive nature of free radicals cause a great amount of damage in cells and other cell constituents including lipids, proteins, cell membrane, membrane lipid peroxidation, decrease membrane fluidity, and nucleic acid (DNA and RNA). Oxidative stress is a major cause of many degenerative and chronic conditions. It results from the uncontrolled production of reactive species, which in turn react and disrupt essential cellular components.

Antioxidant compounds fight against oxygen derived reactive species to decrease the risk of all degenerative and chronic diseases (Kumar *et al.*, 2012).

Medicinal plants are a rich source of many bioactive compounds with various biological activities including antioxidant activity. Antioxidants play an important role in the neutralization, inactivation and scavenging of free radicals or ROS. They promote health simply by preventing damage of macromolecules by free radicals. Antioxidant molecules are also used in food and pharmaceutical industries for many purposes. There is a great interest in finding new naturally occurring antioxidant compounds from medicinal plants that are potent and safe to use to scavenge free radicals. Phenolic compounds are the major antioxidants that are able to chelate metal ions, prevent lipid peroxidation, scavenge free radicals and have the potential to protect humans from high levels of free radicals (Guleria *et al.*, 2013). There are a number of methods that are currently used in medicinal plant research laboratory to test for presence of antioxidant activity of traditional medicinal plant extracts. The common procedures involve determination of the ability of plant extracts to scavenge free radicals using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals (Molan *et al.*, 2012).

### **2.5.2. Antimicrobial activity**

Traditional medicinal plants are a natural source of new drugs of high value to mankind. Plants have been used throughout the whole world as raw drugs or traditional medicines. Plants are a rich source of antibacterial compounds. There are several methods used in medical research laboratories to test for bioactive compounds with antimicrobial activity, which includes bioautography and microbroth dilution methods. Pathogenic microorganisms have developed resistance to chemotherapeutics and antibiotics currently used in hospitals. The resistance develops due to long exposure of the pathogenic microorganisms to antibiotics. The microorganisms undergo several genetic adaptive changes until a strain that can survive in the presence of the antibiotic emerges rendering the antibiotic useless. Synthetic drugs are expensive, associated with many side effects and people from developing countries cannot easily access them. Researchers have developed an interest to screen medicinal plants for potent

antimicrobial drugs or compounds to fight against resistant pathogens. These novel drugs if isolated may show promising results in circumventing the problem of drug resistance as they could kill the microorganisms using different pathway/strategies (Renisheya *et al.*, 2011).

In recent years, antibacterial compounds have been screened from a number of medicinal plants based on their traditional use in rural areas for similar purposes. The presence of antibacterial compounds from medicinal plants is a well-known fact and provides information that lead to the development of new antimicrobial drugs. Many of the modern medicines have been derived from natural source including plants and many of these drugs were isolated based on the use of such plants traditionally. About 80% of the world's population depends on traditional medicinal plants for their primary health care (Abraham and Thomas, 2012). Over 50% of all modern clinical drugs are natural product isolated from plants. Natural products play an important role in drug development programs in the pharmaceutical industry (Dey *et al.*, 2010). The antibacterial activity of medicinal plants is due to the presence of secondary metabolites including tannins, alkaloids, flavonoids and other phenolic compounds (Chitemerere and Mukanganyama, 2011).

### **2.5.3. Anti-inflammatory activity**

Inflammation is a series of biochemical reactions that are aimed at alleviating any damage or injury to the human body. Injury may be due to pathogens, chemical or microbiological agents and other stimuli. Symptoms of inflammation include heat, redness, swelling, pain and loss of function. There are number of endogenous mediators which are released during inflammation process such as histamine, serotonin and prostaglandins. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation (Anilkumar, 2010).

Medicinal plants play an important role in development of potent therapeutic agents. Plants have been used as a source of new anti-inflammatory drugs. Medicinal plants possess various secondary metabolites including flavonoids and phenolic compounds. These compounds play a critical role as anti-inflammatory mediators (Anilkumar, 2010). The use of aspirin and Non-steroidal anti-inflammatory drugs (NSAIDS) for treatment of

inflammation has been limited because of the side effects such as severe gastric disorders, nausea, heartburn, stomach pain, allergic reaction and diarrhea. There are a number of medicinal plants that have been used traditional for the treatment of inflammation and related disorder such as rheumatism. There is a great interest to search for novel anti-inflammatory drugs which are safer, potent and have reduced or no side effects (Agnihotri *et al.*, 2010).

#### **2.5.4. Antiproliferative activity**

About 50% of modern drugs in Europe and USA have been derived from natural products. There are 500 000 traditional medicinal plants used in both developing and developed countries for medicinal purposes. Approximately 10% of these plants species have been studied for their therapeutic properties. Medicinal plants are used for treatment of various diseases including ulceration, diabetes, kidney stones, inflammations, rheumatism, and muscle relaxation. The bioactive compounds are responsible for various biological activities from plants such as antitumor and antiproliferative activity. There are a number of plant derived drugs such as vinblastine, vincristine, taxol, and camptothecin which are currently used for treatment of some cancers. There is still a greater interest to screen for new chemotherapeutic or antitumor drugs from medicinal plants (Talib and Mahasneh, 2010). One of the important methods in the search for antitumor agents from medicinal plant, is the selection of plant due to their ethnomedical leads and then test selected plants for their efficacy and safety through modern scientific approaches such as tetrazolium-based colorimetric (MTT) assay (Selvakumari *et al.*, 2012). Plants are major source of several clinically useful antitumor agents. *Ricinus communis*, produces castor beans that contain ricin which has been isolated and found to have strong inhibitory effect on the growth of tumour cells (antiproliferative activity on the cancer cell) (Jung-Yaw and Su-Ying, 1986).

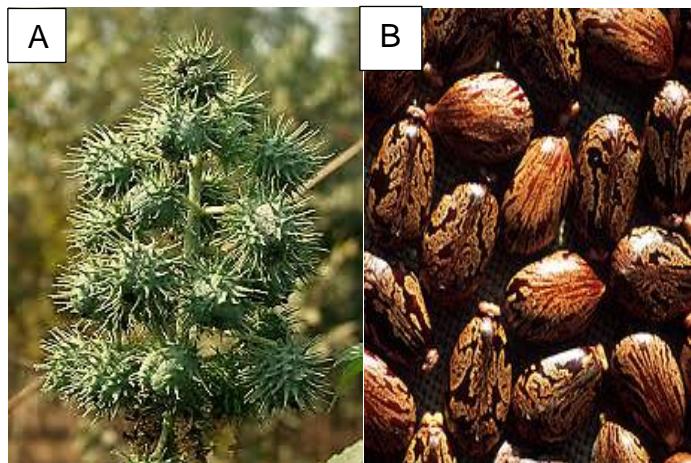
## **2.6. *Ricinus communis***

### **2.6.1. Morphology**

*Ricinus communis* commonly known as Castor oil plant belongs to the family Euphorbiaceae. Castor oil plant is originally from Africa and is now found in all tropical countries. *R. communis* generally grows wild in waste places. Castor oil plant is a wooden tall plant that grows 3.5 to 13.5 m tall with simple hallowed stems. The leaves (Figure 2.4) are long cured, cylindrical with purplish or green petioles. The flowers are monoecious, large, arranged on the thick rachis of an oblong panicle (Figure 2.4). The fruits (Figure 2.5a) of *R. communis* are greenish, deeply grooved, tricoccus capsules, dehiscing longitudinally and septicidally into six valves. Seeds (Figure 2.5b) are ovoid in shape, flattened and smooth shining (Jeyaseelan and Jashothan, 2012). Roots are light in weight almost straight with few rootlets, outer surface dull yellowish brown, nearly smooth but marked with longitudinal wrinkles (Rana *et al.*, 2012). *R. communis* is found in South Africa, India, Brazil and Russia. The plant is non-toxic to most insects, even though small amounts of the toxic protein ricin and alkaloids tricinine are found in vegetative parts of this plant (Singh *et al.*, 2010a). These plants require special treatments if attacked by the insects and other diseases. *R. communis* stems contain Ricinine (1-methyl-3-cyano-4-methoxy-2-pyridine) amino acid. The stems also contain carbohydrates, Saponins, flavonoids and tannins (Singh *et al.*, 2010b). *R. communis* has been traditional used for many purposes throughout the whole world.



**Figure 2.4:** *Ricinus communis* plant (Jena and Gupta, 2012).



**Figure 2.5:** The fruits (A) of *Ricinus communis* and seeds (B) (Jena and Gupta, 2012).

### 2.6.2. Traditional use of *R. communis*

There are different parts of *R. communis* that are used for medicinal purposes including the leaves, barks, seeds, roots and oil. *R. communis* is well known for its biological activities, most important of which are hepatoprotective, laxative, antidiabetic, and antifertility activities. Castor oil from seeds of this plant is also regarded as king oil medicines because it is traditional used for treatment of arthritic diseases. Castor oil is commonly given to children orally for de-worming purposes. In India, castor oils is also used traditionally for many purposes including supporting the growth of hair, combating fungal infections, relieving menstrual pains when applied on the lower abdomen, repairing cracked nipples, softening women's breasts, used in improving eye sight and reducing stretch marks (Rekha et al., 2013). The uses of *R. communis* as medicines have been described in Ayurveda and these include the use of fresh leaves for headache, a decoction of the leaves as an emmenagogue, juice from leaves is used against ringworms, warts, and dropsy. The root bark is purgative and it is also used for skin diseases. *R. communis* seeds contain a lot of oil prescribed for infestation of intestinal worms. The leaf, root and seed oil of castor oil plant have been also used for treatment of inflammation and liver disorders in India (Taur et al., 2011). In Tunisia, *R. communis* is used as a contraceptive herbal drug, treatment of cold tumors, hypoglycemic, laxative, and indurations of mammary glands, corns and moles (Zarai et al., 2012).

### **2.6.3. Research done on *Ricinus communis***

*R. communis* has been studied by various researchers from different countries for antinociceptive activity on methanolic extracts of leaves. This activity were suggested to be due to the presence of saponins, steroids and alkaloids (Taur *et al.*, 2011) and presence of antioxidant activities of methyl Ricinoleate and Ricinoleic acid from seeds extracts (Oloyede, 2012). Essential oils from *R. communis* have been reported to have potential antimicrobial, anticarcinogenic (Zarai *et al.*, 2012), antioxidant (Kadri *et al.*, 2011), and antidiabetic activities (Shokeen *et al.*, 2008). There are several compounds that have been isolated from leaves, roots, seeds of *R. communis* such as quercitin, gallic acid, gentisic acid, Ricinine (1- methyl-3-cyano-4-methoxy-2-pyridine), the disaccharide glycoside, rutin, and two flavonoids compounds; kaempferol-3-O-beta-d-rutinoside and kaempferol-3-O-beta-d-xylopyranoid (Rana *et al.*, 2012). There are number of approaches used to isolate, separate and purify pure compounds from crude extracts.

## **2.7. Separation and purification of compounds**

Extraction of medicinal plants involves the separation of active portions of medicinal plants from inactive components. It employs the use of solvents of varying polarities ranging from non-polar to polar. This includes class of preparations known as decoctions, infusions, fluid extracts, tinctures, and pilular (semisolid) extracts. The products obtained from extraction procedure are impure liquids and mixtures of many compounds. There are a number of extraction procedures used for extraction of compounds from plants such as serial exhaustive extraction, sonification and soxhlet extraction. Different solvents and extraction procedure are used based on the desired outcome. Extraction is considered as the first step of separation and purification of compounds from medicinal plants materials (Sasidharan *et al.*, 2011). There are a number of separation techniques that are used to separate and purify compounds of interest from crude extracts and these include thin layer chromatography, column chromatography and preparative thin layer chromatography plates. The second step of separation and purification of compounds involves the use of column chromatography and preparative thin layer chromatography, to separate components from crude extracts

of plants based on the polarities using one or two solvents of varying polarities into fractions for further purification. The final step involves the use of thin layer chromatography (TLC) plates to confirm the purity of separated compounds (Visht and Chaturvedi, 2012). Pure compounds are further analyzed for structure elucidation and characterization using nuclear magnetic resonance (NMR) and Mass spectrometry (MS).

## **2.8. Structure elucidation of compounds**

Natural products are an essential source of new structures leading to the development of novel drugs in all major disease areas in pharmaceutical industries. *In silico* characterization of natural products and pharmacophore modelling is a challenge, especially on complex and conformationally flexible molecules such as macrolactones or peptides. In the process of characterization of natural products, a particular plant extract is passed through a system which will provide identification of the compound accountable for the binding activity of both known and unknown compounds structures which can be determined by the mass spectrometry (MS) and nuclear magnetic resonance (NMR). NMR determines a true complete structure of the natural compounds based on availability of greater amounts of material or pure compounds isolated. Advances in NMR, MS and High Performance Liquid Chromatography (HPLC) technology have developed hyphenated high performance liquid chromatography (LC)-NMR and LC-NMR-MS for analysis and determination of structure of complex mixture of natural products, avoiding the isolation of individual bioactive compound before structure is elucidated by MS and NMR (Frank and Guy, 2005).

## **2.9. Pathogens**

Various infectious diseases are initiated by pathogenic microorganisms such as bacteria, viruses and fungi. Infectious diseases usually spread through direct contact or indirect contact from a primary individual to another. Pathogenic microorganisms cause premature death throughout the world. The following pathogens have been reported to be resistance to current antibiotics and have also been found to be the most common

infectious pathogens in hospitals namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* (Masoko et al., 2008).

### **2.9.1. *Escherichia coli***

*Escherichia coli* is a Gram negative, rod- shaped bacteria which is mostly found in mammalian intestinal tract. *E. coli* belongs to the family Enterobacteriaceae. Most *E. coli* strains are harmless and beneficial to human health, such as the production of vitamin K2 to prevent development of pathogenic bacteria in the human intestinal tract. However, some *E. coli* strains have the ability to cause severe disease. For example, enterotoxigenic strains produce a toxin in the gut that lead to diarrhea. *E. coli* ATCC 25922 is a nonpathogenic strain of *E. coli*, which is Biosafety level 1 certified to make it useful for various laboratory experiments. *E. coli* ATCC 25922 is mainly used throughout the world as prokaryotic model in both Biotechnology and Microbiology research as the host organism (Pang et al., 2013).

### **2.9.2. *Pseudomonas aeruginosa***

*Pseudomonas aeruginosa* is a common bacterium that can cause various infectious diseases in mammals. *P. aeruginosa* is mostly found in different environments globally such as in soil, water and skin flora. It is a life threatening bacterium in humans and plants (Fang et al., 2012). *P. aeruginosa* ATCC 27853 is commonly used in the fields of Biotechnology and Microbiology for testing of the antimicrobial activity of newly discovered drugs, identification of virulence factors (particularly extracellular enzymes), quality control testing, and drug carrier testing. *P. aeruginosa* has advantages in the invasion of a host and survival under varying environments. It has the potential to produce biofilms and a number of enzymes such as lipase, phospholipase, alkaline phosphatase, exotoxin and proteases. Proteases enzymes that are produced by this organism are assumed to play an essential role during critical *P. aeruginosa* infection (Izrael-Živković et al., 2010). *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections. *P. aeruginosa* infection is a serious problem in patients with cancer, cystic fibrosis, and burns (Kenneth, 2004).

### **2.9.3. *Enterococcus faecalis***

*Enterococcus faecalis* is a Gram-positive lactic acid bacterium commonly found in mammalian intestine and plants. *E. faecalis* causes life threatening diseases such as endocarditis and bacteremia, urinary tract infections, and meningitis (Murray, 1998). *E. faecalis* ATCC 29212 is used for susceptibility testing of pathogens to antibiotics, and commercialized biochemical identification of bacterial species. This strain was isolated from human urine and is heat resistance and has been generally used as a standard control in clinical and food safety tests. These strains are now considered as the adapted pathogens that are hospital associated strains that have developed resistance to clinically relevant antibiotics (Kim *et al.*, 2012).

### **2.9.4. *Staphylococcus aureus***

*Staphylococcus aureus* is a Gram positive bacterium and also known as the major human pathogen. *S. aureus* may easily be carried or attached to the skin for several months in the mucus membranes of nasal passages. People who persistently carry *S. aureus* are at an increased risk of subsequent infection. *S. aureus* mostly causes skin diseases that can result in impetigo or cellulitis which is an inflammation of the connective tissue under the skin, leading to swelling and redness of the area. Mupirocin and neomycin are antibiotics that have been used in hospital for treatment of infections caused by *S. aureus* and to reduce high risk of infection of patients (Carson *et al.*, 2007).

## **2.10. Aim and Objectives**

### **2.10.1. Aim**

The aim of the study was to isolate and characterize bioactive compounds with various biological activities from *R. communis* leaves.

### **2.10.2. Objectives**

- i. To determine the phytochemical spectral profiles of the crude extracts of *R. communis* by thin layer chromatography (TLC) plates.
- ii. To evaluate for the presence of antioxidant constituents in *R. communis* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and quantitative total antioxidant activity assay using ABTS free radicals.
- iii. To evaluate the antibacterial activity of *R. communis* using minimum inhibitory concentration and bioautography.
- iv. To isolate bioactive compounds with antioxidant and antibacterial activity using column chromatography.
- v. To elucidate the structure of isolated compound using Nuclear Magnetic Resonance (NMR) and mass spectrometry (MS).
- vi. To evaluate the effect of plant extracts and isolated compounds for their anti-proliferative and cytotoxic effect on Human Caucasian skin fibroblast (Bud-8) cell line using MTT assay.
- vii. To determine the anti-inflammation activity of the selected plant extracts on macrophages Raw 264.7 cell line.

## CHAPTER 3

### EXTRACTION AND THIN LAYER CHROMATOGRAPHY PROFILES

#### 3.1. INTRODUCTION

There is an increased use of bioactive compounds derived from traditional medicinal plants, used the world over as supplements in foods, chemicals and pharmaceuticals is evident. It is therefore important to establish highly effective extraction methods coupled with the selection of good solvents systems to maximally extract both qualitatively and quantitatively bioactive compounds from medicinal plants. Extraction is the first major step of every research on medicinal plants and plays an important role in isolation of desired bioactive compounds. Extraction methods involved the use of solvents of varying polarities ranging from non-polar to polar solvents. There are several factors that affect extraction of bioactive compounds from plant materials including polarities of solvents, polarity of compounds to solvents, nature of plant matrix, temperature, pressure, time and chemistry of compounds (Azmir *et al.*, 2013).

There are well known procedures that are used to extract bioactive compounds from plant materials such as maceration, infusion, digestion, decoction, hot continuous extraction (soxhlet) and ultrasound extraction (sonication) (Tiwari *et al.*, 2011). During the extraction process, solvents extract compounds of the same polarities from plant materials. The success of extracting bioactive compounds from medicinal plants is highly based on the polarity of solvents used and that of the compounds within the plant. Good solvents that can be employed for the extraction of bioactive compounds should characteristically be easy to evaporate and have low toxicity. There are a number of solvents commonly used in the extraction process of phytochemical compounds including methanol, ethanol, acetone, petroleum ether, diethyl ether and ethyl acetate. Before extraction process may take place, plant materials are ground to a fine powder. The main aim of extracting ground powder materials is to increase the surface area to volume ratio which enhances rate of extraction. The ratio of solvent and plant material in extraction process is 10:1 v/w (Tiwari *et al.*, 2011).

Phytochemical compounds are produced in response to abiotic and biotic stress for protection and survival of plants in harsh conditions such as low water content, high temperature, ultraviolet degradation and exposure to herbivores (Sasidharan *et al.*, 2011). Examples of phytoconstituents are amino acids, sterols, diterpenes, triterpenes, tannin, and alkaloids (Vaghasiya *et al.*, 2011). They exhibit a number of biological activities which are responsible for their therapeutic properties. They are potential precursors for manufacturing new valuable drugs. Quinine, artemisinin, shikonin are examples of modern drugs extracted from traditional medicinal plants (Manjari *et al.*, 2011).

Separation and identification of bioactive compounds is a challenge. Thin layer chromatography (TLC) is one of the separation techniques used. These phytocompounds are visualized on the TLC plates after spraying plates with phytochemical screening reagents or under Ultraviolet light. TLC plates are also used to determine distance travelled, colour and location of desired compounds by their retardation factor ( $R_f$ ) values in the mixture of unknown compounds. TLC plates confirm purity of isolated compounds. Different colours observed on the TLC plates confirm number of phytochemical compounds present in crude extracts (Sasidharan *et al.*, 2011). There are several tests which are used to test for secondary metabolites present in crude extracts namely Dragendorff's and Wagner test for alkaloids, Borntrager's test for anthraquinone, Kellar-Kiliani test for cardiac glycosides, Shanoda test for flavonoids, Fehling test for reducing sugars and frothing test for saponins (Tiwari *et al.*, 2011). The aim was to determine the phytochemical spectral profiles of the crude extracts of *R. communis* by thin layer chromatography (TLC) plates.

## **3.2. METHODS AND MATERIALS**

### **3.2.1. Plant material**

The leaves of *R. communis* were collected at the University of Limpopo (Turfloop campus), Limpopo Province, South Africa during Summer of 2012. Voucher specimen in the garden herbarium and tree label verified the identity of the plant. Plant was

confirmed by Dr Bronwyn Egan (Herbarium). She also provided plant accession details of *R. communis* (UNIN 11352). Leaves were separated from twigs and dried at room temperature. Most scientists have tended to use dried plant material because there are fewer problems associated with large-scale extraction of dried plants rather than fresh plant material (Eloff, 1998). The dried plant materials were ground to fine powder using a blender and stored at room temperature in closed containers in the dark.

### **3.2.2. Extraction procedures**

In this study different solvents and extraction procedures were used (Eloff, 1998; Kotze and Eloff, 2002).

#### *3.2.2.1. Preliminary extraction procedure*

The leaves of *R. communis* were extracted by weighing 1 g of finely ground plant material and extracting it with 10 ml of n-hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol in different polyester centrifuge tubes. Tubes were vigorously shaken for 10 minutes in series 25 shaking incubator machine (New Brunswick Scientific Co., Inc.) at a high speed (200 rpm). There after the extracts were filtered into pre-weighed labelled bottles. The process was repeated three times to exhaustively extract constituents of the plant material and the extracts were combined. The solvent was removed under a stream of cold air at room temperature. The final extracts were reconstituted in acetone to a concentration of 10 mg/ml.

#### *3.2.2.2. Serial exhaustive extraction*

Serial exhaustive extraction of finely ground powder of *R. communis* was done by weighing 5 g of plant materials and dissolved into 50 ml of n-hexane. The bottle was vigorously shaken for an hour using series 25 incubator shaker machine at high speed (200 rpm) and supernatant was filtered into pre-weighed bottle. The same process was repeated three times, to exhaustively extract compounds and supernatants were combined. Same plant residues were extracted in the following order with 50 ml of chloroform, dichloromethane, acetone, ethanol and methanol. Solvents were removed under a stream of cold air at room temperature. The final extracts were reconstituted in acetone to a concentration of 10 mg/ml.

### *3.2.2.3. Extraction enrichment procedures*

There are two methods that were used in this procedure. The first pathway was performed by pre-treating finely ground powder leaves of *R. communis* with hexane followed by extraction of pre-treated plant materials with acetone and ethanol. The second pathway involves the use of different percentages of 20%, 40%, 60%, 80% acetone and ethanol in water for extraction. Tubes were vigorously shaken for 10 minutes and filtered into pre-weighed bottles. The solvents were removed under a stream of cold air at room temperature. The final extracts were dissolved in acetone to a concentration of 10 mg/ml.

#### *3.2.2.3.1. Hexane wash*

The leaves were extracted by weighing one gram of plant material into 10 ml of hexane. Plant residues were dried between subsequent extractions, and then followed by extraction of the same plant materials with acetone and ethanol.

#### *3.2.2.3.2. Acetone and ethanol in water mixtures*

One gram of plant materials of *R. communis* was extracted with 10 ml each of the following: acetone, ethanol, water and 20%, 40%, 60%, 80% acetone and ethanol in water. Solvents were removed under a stream of cold air. The final extracts were dissolved in acetone to a concentration of 10 mg/ml.

#### *3.2.2.4. Optimal extraction method*

Three pre-treatment methods were employed before subsequent extraction of plant materials with acetone and ethanol. One gram of plant materials was extracted with 10 ml of each solvent as outlined in Table 3.1 below.

**Table 3.1:** Table showing solvents used for pre-treatment and extraction procedures

Pretreatment	Extraction
Hexane “wash”	Acetone and ethanol extract
20% acetone and ethanol in water “wash”	80% acetone and ethanol in water extract
Hexane “wash” followed by 20% acetone and 20 % ethanol in water “wash”	Acetone and ethanol extract

### 3.2.2.5. Preliminary Serial exhaustive extraction

#### 3.2.2.5.1. Series 1, 2 and 3

In preliminary serial exhaustive extraction, 10 g of plant materials were serially extracted with 100 ml of solvents of varying polarities from non-polar solvents to polar solvents: (i) Series 1: Hexane, dichloromethane, ethyl acetate, acetone and methanol. (ii) Series 2: Hexane, ethyl acetate, acetone and methanol. (iii) Series 3: Hexane, acetone and methanol. Solvents were removed under a stream of cold air. Extracts were reconstituted in acetone to a final concentration of 10 mg/ml.

### 3.2.3. Preliminary screening for phytochemical compounds

Extracted chemical components (10 mg/ml) were analysed by separation with thin layer chromatography (TLC) using aluminium-backed TLC plates (Fluka, silica gel F<sub>254</sub>). Ten microliter of extracts was loaded on TLC plates and developed in saturated chambers using mobile phases of different polarities, namely benzene/ethanol/ammonia hydroxide (BEA) (non-polar/basic) (18:2:0.2), chloroform/ethyl acetate/formic acid (CEF) (intermediate polarity/acidic) (10:8:2), ethyl acetate/methanol/water (EMW) (polar/neutral) (10:5.4:4) and Butanol/Acetic acid/Water [BAW] (polar) (4:1:5) (Kotze and Elof, 2002). Separated compounds on the TLC plates were examined under ultraviolet light (254 and 365 nm) then sprayed with vanillin-sulphuric acid reagent [0.1 g

vanillin (Sigma ®): 28 ml methanol: 1 ml concentrated sulphuric acid] and heated at 110°C for optimal colour development.

### **3.2.4. Phytochemical screening**

#### *3.2.4.1. Reducing sugars*

The aqueous extract of *R. communis* leaves was obtained by dissolving 0.5 g of plant powder into 5 ml of water. The aqueous solution was added to a boiling Fehling's solution [Fehling A was prepared by dissolving 7 g of hydrated copper (II) sulphate into 100 ml of distilled water]. Fehling B was prepared by dissolving 35 g of potassium sodium tartrate and 10 g of sodium hydroxide in 100 ml of distilled water. Equal volumes of Fehling A and B were mixed to form a deep blue solution of Fehling's solution) in a test tube. The solution was observed for an orange red precipitate (Ayoola *et al.*, 2008).

#### *3.2.4.2. Anthraquinones*

Anthraquinone content in plant extracts was tested by weighing 0.5 g of ethanol extracts of *R. communis* and boiled with 10 ml of 97% sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube to which 1 ml of dilute ammonia was added. The resulting solution was observed for pink colour (Ayoola *et al.*, 2008).

#### *3.2.4.3. Terpenoids*

The ethanol extracts of *R. communis* were tested for terpenoids by weighing 0.5 g of the extract and dissolve into 2 ml of chloroform. Thereafter, 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids (Borokini and Omotayo, 2012).

#### *3.2.4.4. Flavonoids*

The presence of flavonoids were tested in the aqueous extracts of *R. communis* leaves by adding 5 ml of diluted ammonia solution to a portion of the aqueous filtrate of plant extracts, followed by addition of 1 ml of concentrated sulphuric acid. A yellow

colouration that disappears on standing indicates the presence of flavonoids (Borokini and Omotayo, 2012).

#### 3.2.4.5. Cardiac glycosides

The Keller- Killiani test was employed to test for cardiac glycosides by weighing 0.5 g of plant extracts of *R. communis* and dilute to 5 ml of water. The mixture of 2 ml of glacial acetic acid containing one drop of 0.1% ferric chloride solution was added to diluted plant extracts. This mixture was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides (Borokini and Omotayo, 2012).

#### 3.2.4.6. Phlobatannins

Phlobatannin was tested by weighing 0.2 g of powdered leaf sample of *R. communis* into 10 ml of distilled water and filtered. The filtrate was boiled with 2% hydrochloric acid solution. Sample was observed for the formation of red colour of precipitate to draw inference (Borokini and Omotayo, 2012).

#### 3.2.4.7. Saponins

The persistent frothing test was used to test for saponins by weighing 1 g of plant powdered leaf sample of *R. communis* and mixed with 30 ml of tap water. The mixture was vigorously shaken and heated at 100°C. The sample was observed for formation of persistent of froth to draw inference (Odebiyi and Sofowora, 1978).

#### 3.2.4.8. Tannins

The presence of tannins were tested by boiling 0.5 g of powdered leaf of *R. communis* in 5 ml of distilled water in a test tube, then cooled and filtered. A few or three drops of 0.1% ferric chloride was added to 1 ml of the solution in a test tube and observed for brownish green or a blue-black colouration (Trease and Evans, 1989).

#### 3.2.4.9. Alkaloids

Drangendorff's reagent was used to test for alkaloids by weighing 0.2 g of ground powdered leaves of *R. communis* and extract with 95% ethanol using soxhlet extractor.

The extracting solvent was evaporated to dryness using a vacuum evaporator at 45°C. The plant residues were dissolved in 5 ml of 1% hydrochloric acid and 5 drops of drangendoff's reagent was added. Reddish-brown colour change was observed to draw an inference (Harborne, 1973).

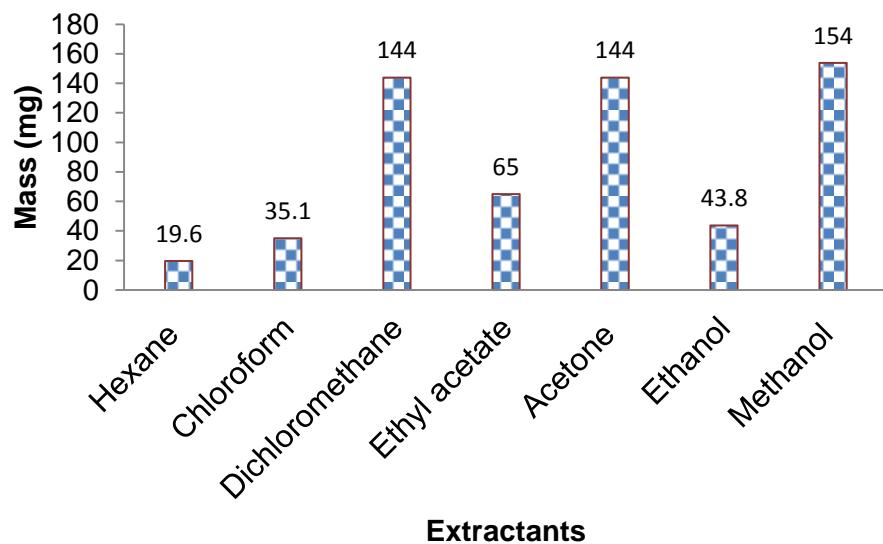
#### 3.2.4.10. Steroids

Steroids were tested by adding 2 ml of acetic anhydride to 0.5 g of *R. communis* plant extract, followed by addition of 2 ml of sulphuric acid to the mixture. Blue or green colour change was observed to draw inference (Borokini and Omotayo, 2012).

### 3.3. RESULTS

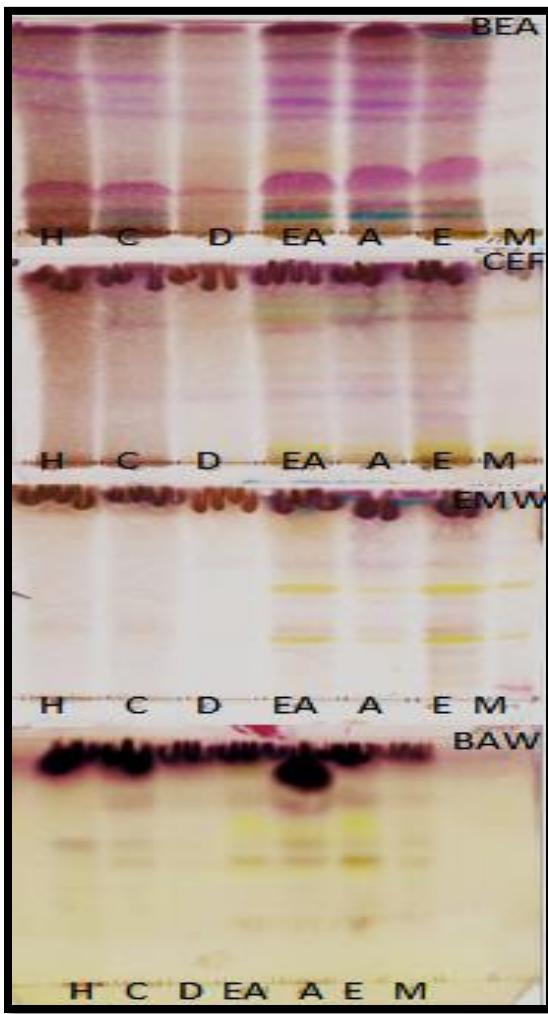
#### 3.3.1. Preliminary extraction procedure

Seven different solvents were used to extract active compounds from *R. communis* leaves. The amount of samples extracted was measured in mg as shown in Figure 3.1. Methanol (154 mg) was the best extractant, followed by acetone (144 mg), dichloromethane (144 mg) and hexane (19.6 mg) in that order.



**Figure 3.1:** The mass of extracts of *R. communis* in mg extracted using different solvents with varying polarity from non-polar solvent to polar solvents; hexane, chloroform, dichloromethane, ethyl acetate, acetone ethanol and methanol.

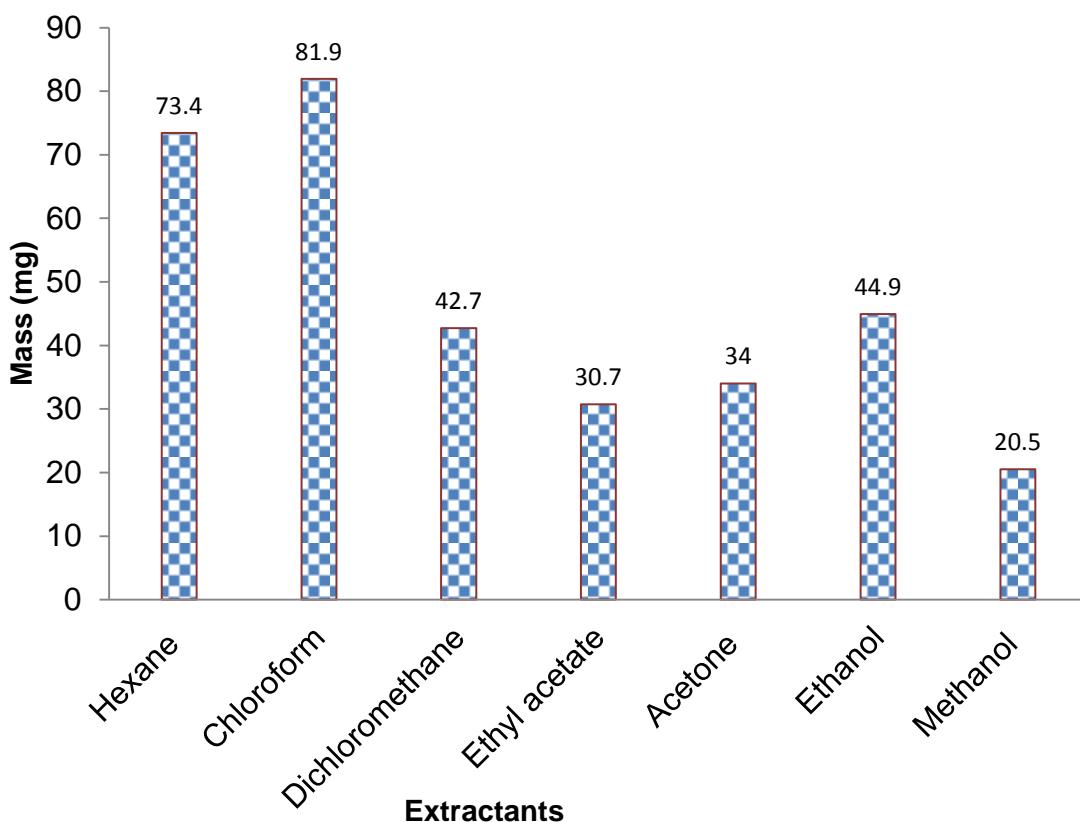
To compare phytochemical profile of the different extracts, aluminum-backed thin layer chromatography plates were used for finger print profile of crude extracts. More bands of phytochemical compounds were observed in BEA solvent system, followed by CEF, BAW and EMW (Figure 3.2). Different colours were observed on TLC plates after spraying with vanillin-sulphuric acid reagent, indicative of the presence of different phytochemical constituents that reacted with spraying reagent.



**Figure 3.2:** Thin layer chromatographic profiles of *R. communis* leaves extracts separated with four solvent systems namely: BEA, CEF, EMW, and BAW and sprayed with vanillin-sulphuric acid reagent. To show compounds extracted with hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**), ethanol (**E**), and methanol (**M**).

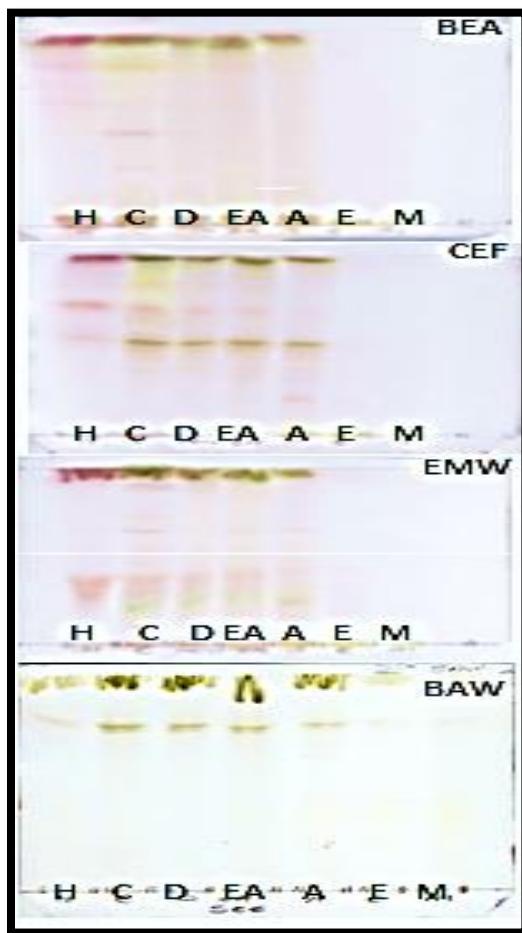
### 3.3.2. Serial exhaustive extraction

In serial exhaustive extraction, chloroform (81.9 mg) was the best extractant compare to other solvents because of the high amount of phytochemical compounds extracted (Figure 3.3). More of non-polar compounds were extracted from plant materials because of a greater mass of constituents in the non-polar solvents.



**Figure 3.3:** The mass of *R. communis* extracts in mg extracted using different solvents with varying polarity from non-polar solvent to polar solvents; hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and methanol.

Crude extracts were analyzed for phytochemical constituents using aluminum-backed TLC-plates developed in four mobile phases and sprayed with vanillin-sulphuric acid reagent. More bands were observed in CEF solvent system, followed by BEA, EMW then BAW (Figure 3.4). Some of the compounds could not move from the base of the TLC plates.

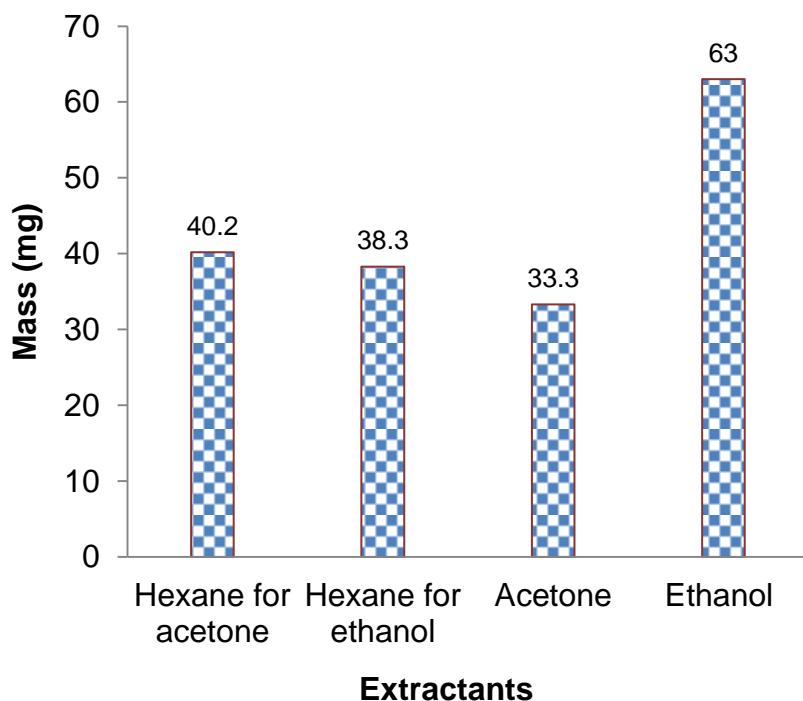


**Figure 3.4:** Thin layer chromatographic profiles of crude *R. communis* leaves extracts eluted in four solvent systems; BEA, CEF, EMW and BAW, and sprayed with vanillin-sulphuric acid reagent. Hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**), ethanol (**E**), and methanol (**M**), in lanes from left to right.

### 3.3.3. Extract enrichment procedures

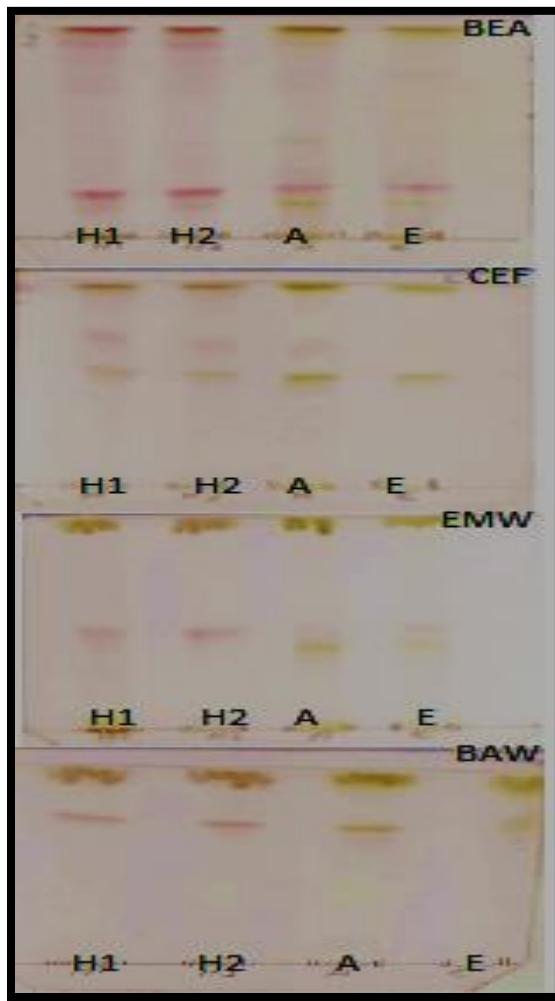
#### 3.3.3.1. Hexane wash

In hexane wash, ethanol extract (62 mg) was the best extractant with greatest mass of extract, followed by hexane for acetone (40.2 mg), and the lowest mass was obtained in acetone extract (33.3 mg) (Figure 3.5).



**Figure 3.5:** The mass of *R. communis* extracts in mg extracted by different solvents with varying polarity; hexane wash for acetone, hexane wash for ethanol, acetone and ethanol.

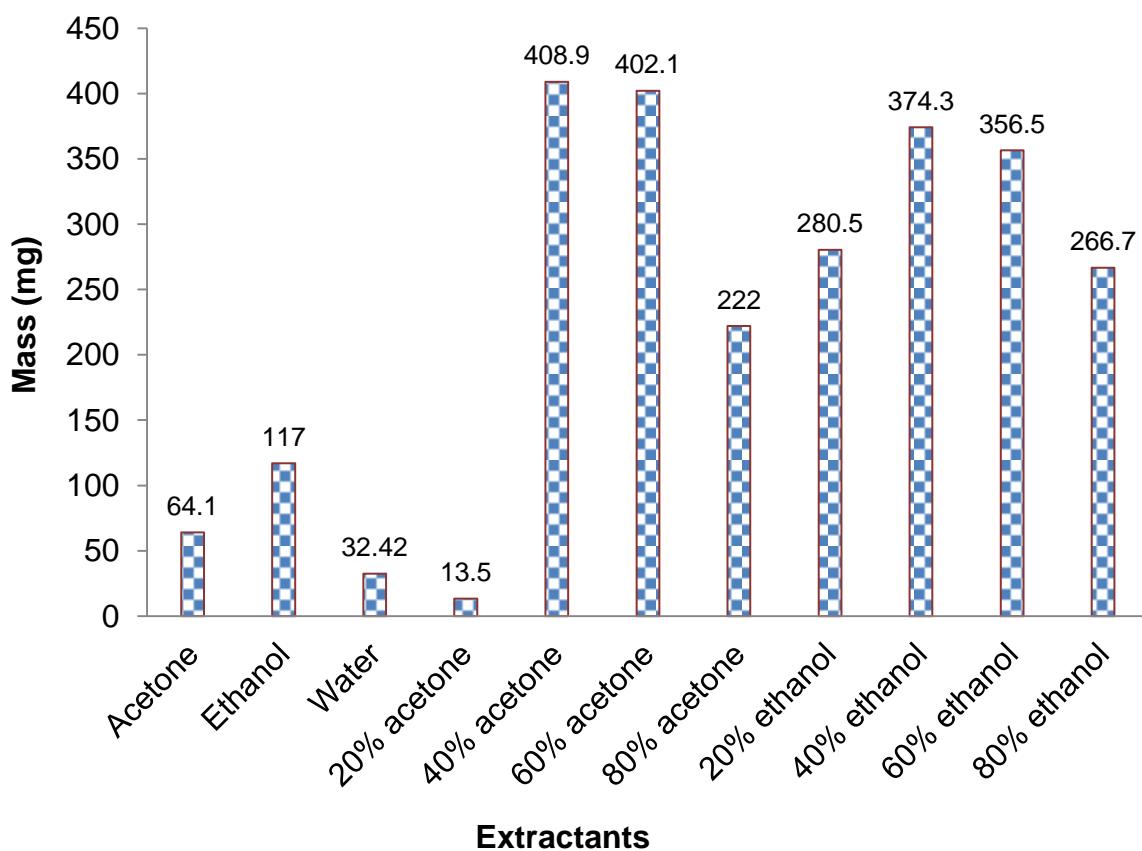
Crude extracts were separated using thin layer chromatography plates developed in four mobile phases and then sprayed with vanillin-sulphuric acid reagent for visualization of compounds that reacts with spraying reagent. More bands were observed in BEA solvent system and the least bands were in BAW (Figure 3.6). Different colours observed on the TLC plates after spraying with vanillin-sulphuric acid reagent confirmed presence of different phytochemical compounds.



**Figure 3.6:** Thin layer chromatographic profiles of *R. communis* leaf extracts eluted in four solvent systems; BEA, CEF, EMW, and BAW and sprayed with vanillin sulphuric acid reagent. Hexane wash for acetone (**H1**), hexane wash for ethanol (**H2**), acetone (**A**), and ethanol (**E**).

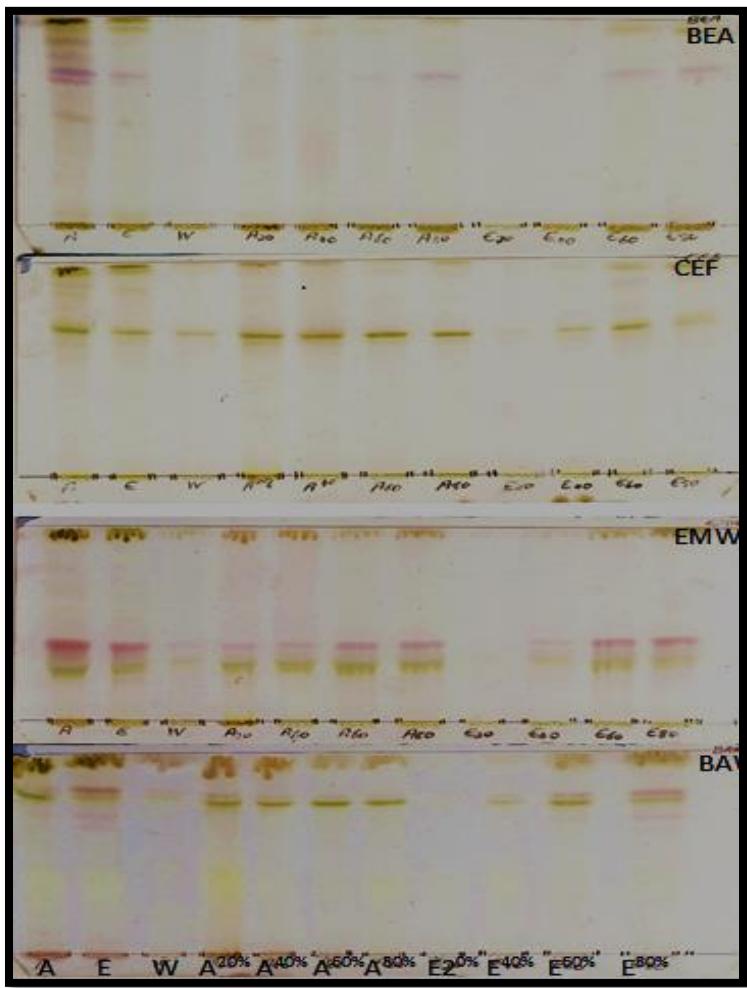
### 3.3.3.2. Acetone and ethanol in water

Acetone, ethanol, water and different percentages of acetone and ethanol in water were used for extraction of phytochemical compounds from *R. communis* leaves. Fraction, 40% acetone in water (408.9 mg) showed greatest mass of extracts compared to ethanol fractions (20%, 40%, 60% and 80%), and the lowest mass was in 20% acetone in water (13.5 mg) (Figure 3.7).



**Figure 3.7:** The mass of *R. communis* extracts in mg extracted by different solvents with varying polarities; acetone, ethanol, water and different percentages (20%, 40%, 60% and 80%) of acetone and ethanol in water.

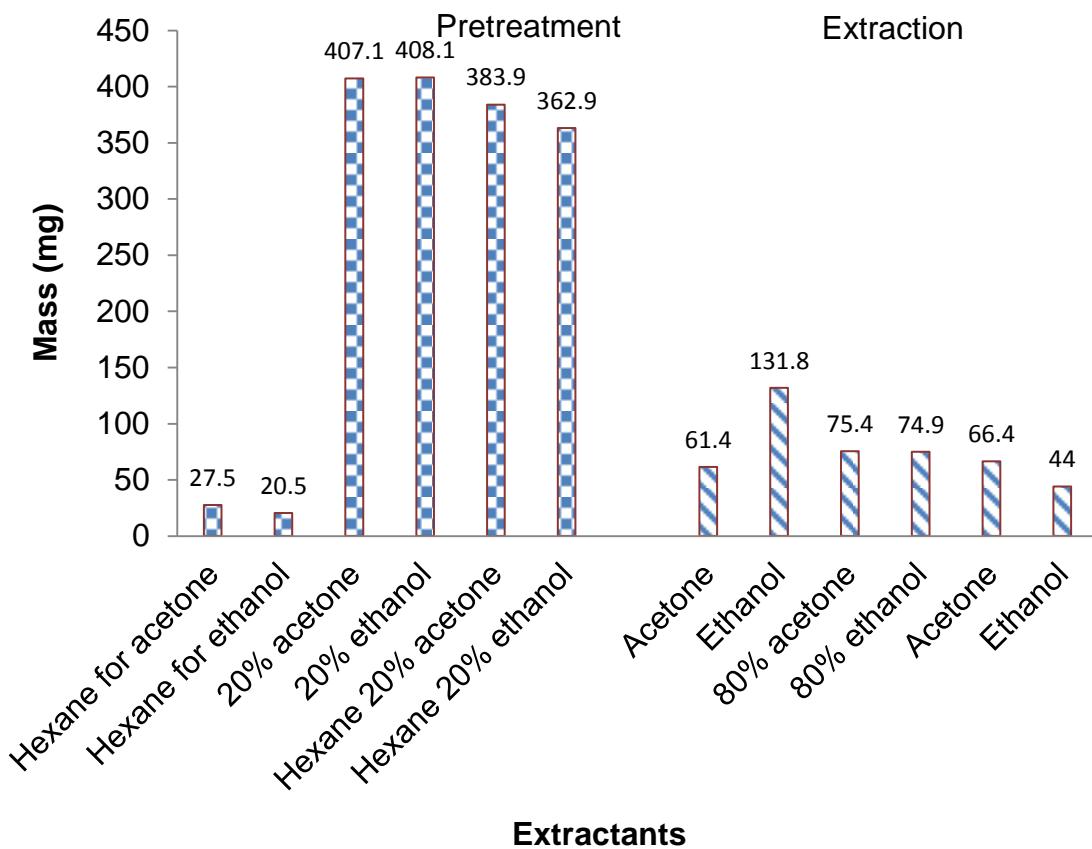
TLC-plates were used for phytochemical analysis of crude extracts and more bands were observed in EMW solvent system, followed by BAW, CEF then BEA. The EMW solvent system was the best separation solvent system with more phytochemical compounds compared to other solvent systems (Figure 3.8). Different phytoconstituents were confirmed by different colours observed on the TLC plates after spraying plates with vanillin-sulphuric acid reagent.



**Figure 3.8:** Thin layer chromatography profiles of *R. communis* extracts were eluted in four solvent systems; BEA, CEF, EMW and BAW and sprayed with vanillin-sulphuric acid reagent. To show compounds extracted with acetone (**A**), and ethanol (**E**), water (**W**), 20% acetone (**A<sup>20%</sup>**), 40% acetone (**A<sup>40%</sup>**), 60% acetone (**A<sup>60%</sup>**), 80% acetone (**A<sup>80%</sup>**), 20% ethanol (**E<sup>20%</sup>**), 40% ethanol (**E<sup>40%</sup>**), 60% ethanol (**E<sup>60%</sup>**), 80% ethanol (**E<sup>80%</sup>**).

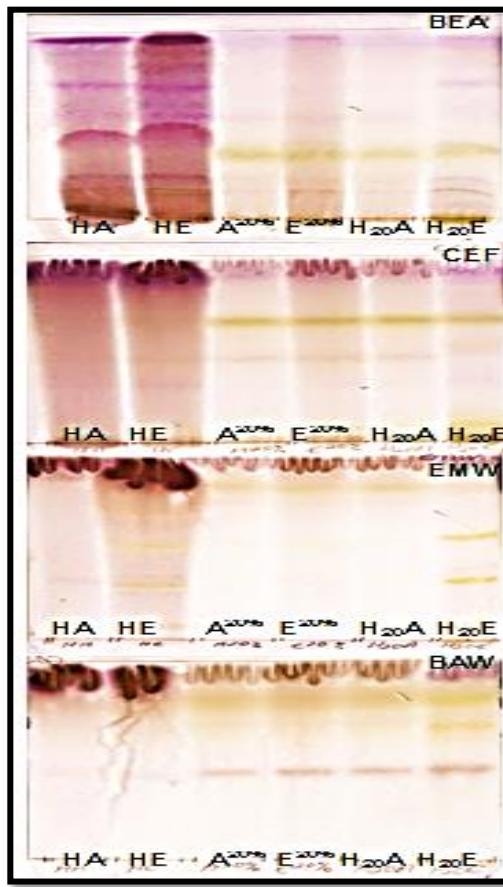
### 3.3.4. OPTIMAL EXTRACTION METHOD

Different quantities of extracts obtained from optimal extraction methods are represented below in Figure 3.9. Optimal extraction methods consist of two steps; pretreatment of plant materials with solvents of various polarities and then extraction of treated materials. In pretreatment, 20% ethanol (407.1 mg) and acetone (408.1 mg) wash had greater mass of extracts and the lowest mass was in hexane for ethanol (20 mg) wash. In extraction, 80% ethanol (131.8 mg) had greatest mass and the least mass was in ethanol (44 mg) used for extraction after plant materials were pretreated with 20% ethanol in hexane wash (Figure 3.9).



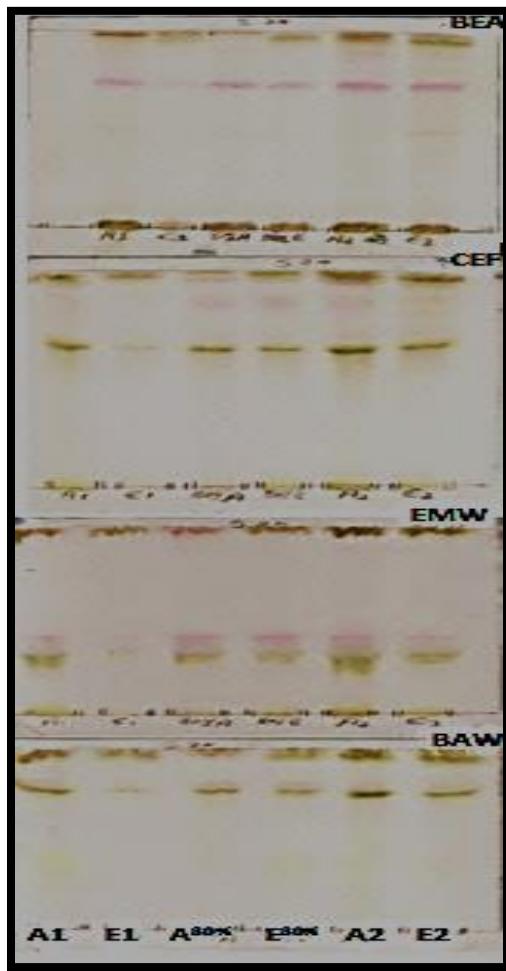
**Figure 3.9:** The mass of *R. communis* extracts in mg extracted by different solvents with varying polarities before and after pretreatment of plant materials with hexane, 20% acetone and ethanol. Pretreated plant materials were extracted with acetone, ethanol, 80% acetone and ethanol in hexane.

Aluminum-backed thin layer chromatography plates were used for finger print profile of crude extracts obtained after pretreatment of plant materials. TLC-plates were sprayed with vanillin sulphuric acid reagent for visualization of phytochemical compounds present. The BEA solvent system showed more bands of phytochemical constituents, followed by CEF, EMW and then BAW (Figure 3.10).



**Figure 3.10:** Thin layer chromatography profiles of *R. communis* extracts eluted in four solvent systems; BEA, CEF, EMW, and BAW and sprayed with vanillin sulphuric acid. hexane wash for acetone (**HA**), hexane wash for ethanol (**HE**), 20% acetone wash (**A<sup>20%</sup>**), 20% ethanol wash (**E<sup>20%</sup>**), hexane wash followed by 20% acetone wash (**H<sub>20</sub>A**), and Hexane wash followed by 20% ethanol wash (**H<sub>20</sub>E**).

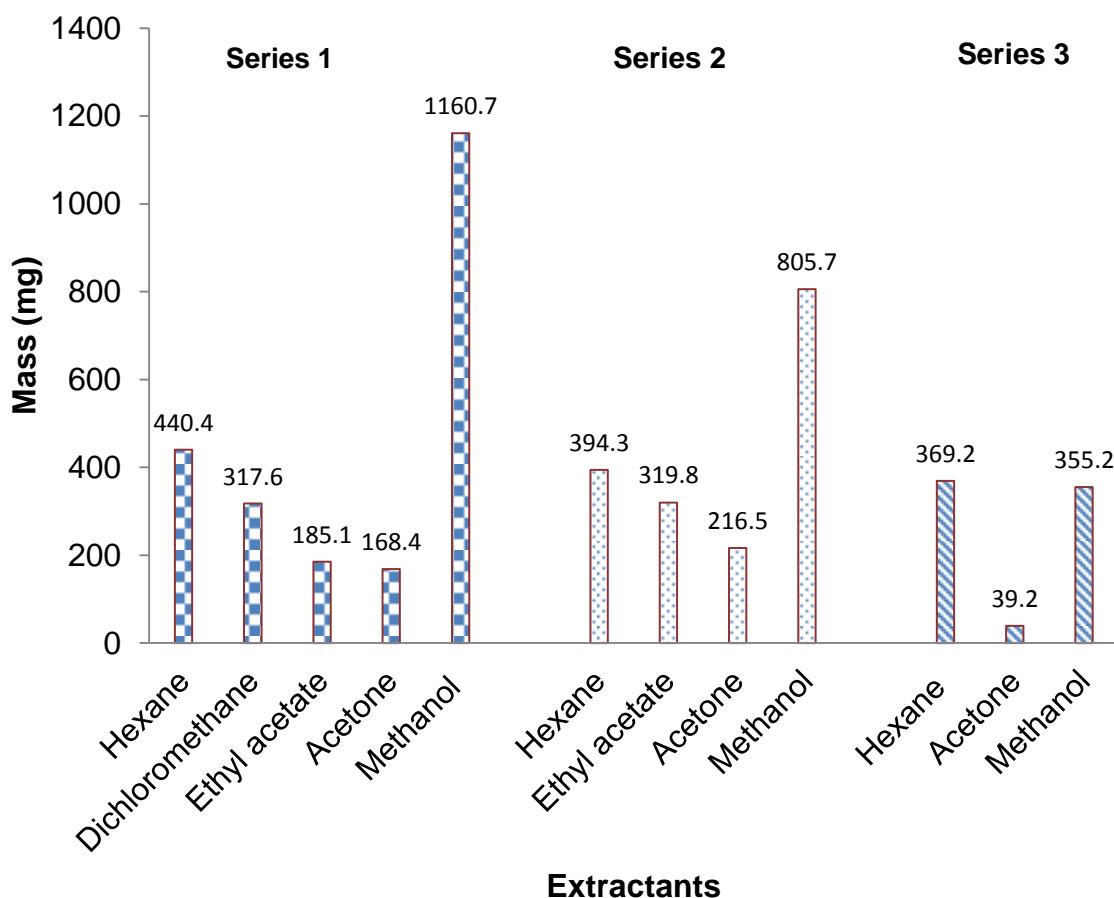
TLC- chromatograms below showed the spectral profile of crude extracts obtained after extraction of pretreated plant materials. Different colours on the aluminum-backed TLC plates were observed after spraying plates with vanillin-sulphuric acid reagent confirmed presence of different phytochemical constituents (Figure 3.11). The EMW solvent system showed more bands of phytochemical constituents followed by CEF, BEA and then BAW.



**Figure 3.11:** Thin layer chromatography profiles of *R. communis* extracts eluted in four solvent systems; BEA, CEF, EMW and BAW from top to bottom and sprayed with vanillin sulphuric acid reagent. To show compounds extracted with Acetone (**A1**), Ethanol (**E1**), 80% acetone (**A<sup>80%</sup>**), 80% ethanol (**E<sup>80%</sup>**), Acetone (**A2**) and Ethanol (**E2**).

### 3.3.5. Serial exhaustive extraction (Series 1, 2, 3)

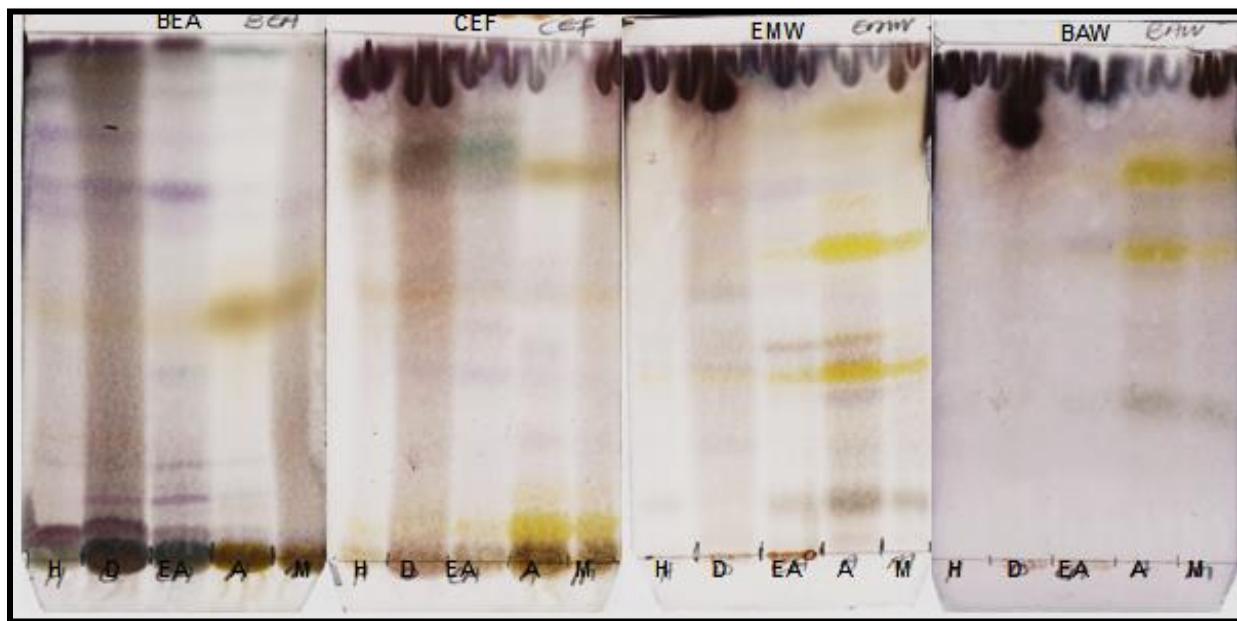
Different quantities of extracts obtained from three series of extraction were performed for preliminary isolation of compounds for large scale extraction (Figure 3.12). Methanol (1160.7 mg and 805 mg) extract had the highest mass of extracts in series 1 and 2, followed by hexanes (440.4 mg and 394.3 mg) and the lowest mass was in acetone extracts, respectively. Hexane (369.2 mg) showed highest mass of extracts in series 3 and the least was in acetone (39.2 mg). Methanol was the best extractant with greater amount of extracts obtained compared to other solvents.



**Figure 3.12:** The mass of *R. communis* extracts in mg extracted using different solvents with varying polarities in three series; hexane, dichloromethane, ethyl acetate, acetone and methanol.

(I). Series 1

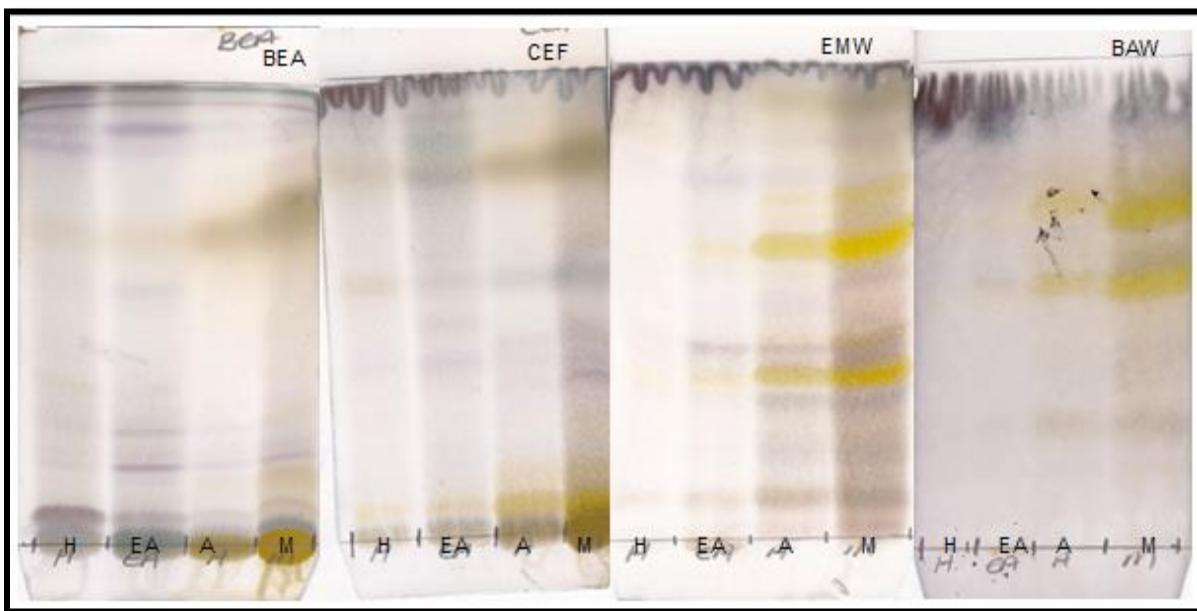
TLC-chromatograms below represent spectral profile of crude extract from series one. Crude extracts were separated with four solvent systems of varying polarities. After spraying TLC plates with vanillin-sulphuric acid reagent, more of phytoconstituent compounds (bands) were observed in BEA, EMW, CEF and then the least in BAW solvent system (Figure 3.13).



**Figure 3.13:** Chromatograms of *R. communis* extracts eluted in four solvent systems; BEA, CEF, EMW, BAW from left to right and then sprayed with vanillin sulphuric acid. To show compounds extracted with Hexane (H), Dichloromethane (D), Ethyl acetate (EA), Acetone (A) and Methanol (M).

(ii). Series 2

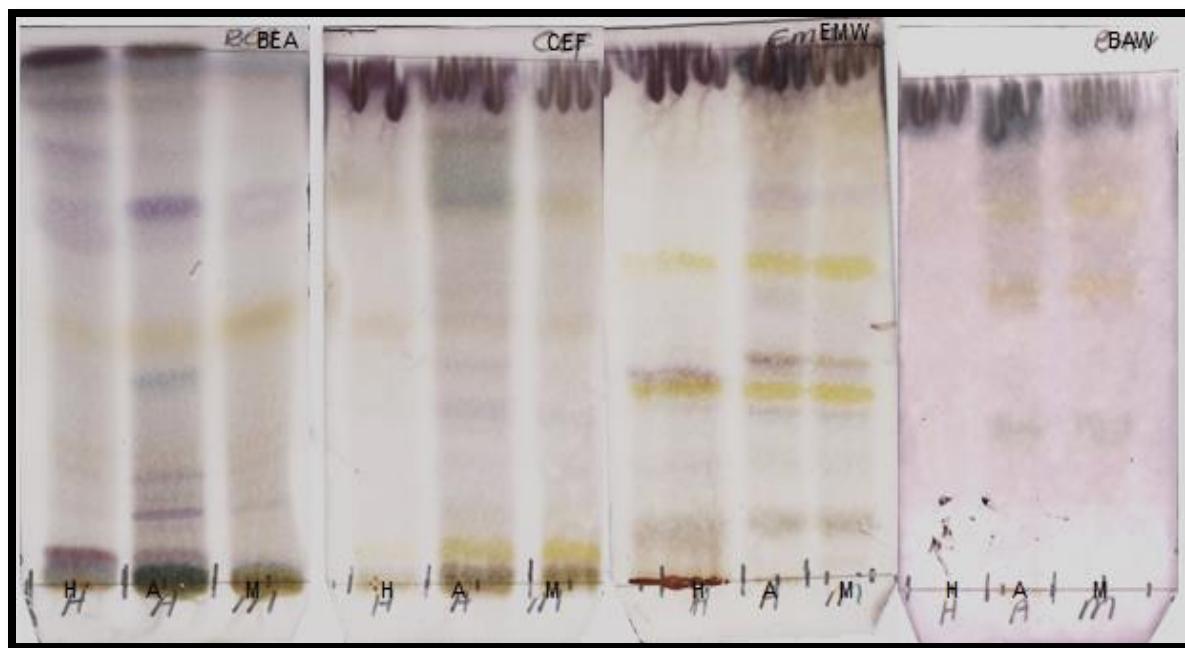
Thin layer chromatography plates were used for phytochemical analysis of crude extracts obtained from series two. Crude extracts were separated with four solvent systems of varying polarities. TLC plates were sprayed with vanillin-sulphuric acid reagent for visualization of phytochemical compounds. More bands were observed in EMW, CEF and the least compounds in BAW solvent system (Figure 3.14).



**Figure 3.14:** Chromatograms of *R. communis* extracts eluted in four solvent systems; BEA, CEF, EMW and BAW from left to right and then sprayed with vanillin sulphuric acid reagent. To show compounds extracted with hexane (**H**), ethyl acetate (**EA**), acetone (**A**) and methanol (**M**).

(iii). Series 3

Crude extracts were separated with four solvent systems of varying polarities and then sprayed with vanillin-sulphuric acid reagent for phytochemical analysis. More of phytochemical compounds were observed in EMW and the least in BAW solvent system (Figure 3.15). The BEA solvent system was the best solvent system for separation of phytochemical compounds present.



**Figure 3.15:** Chromatogram of *R. communis* extracts eluted in four solvent systems; BEA, CEF, EMW, BAW from left to right and then sprayed with vanillin sulphuric acid to show compounds extracted with Hexane (H), Acetone (A) and Methanol (M).

### 3.3.6. Phytochemical screening

Results revealed a number of secondary metabolites present which are responsible for medicinal activity of plants (Table 3.2). Phlabatannins and saponins were absent. Alkaloids, anthraquines, cardiac glycosides, flavonoids, tannins and terpenoids were present in *R. communis* leaves extracts.

**Table 3.2:** Phytochemical constituents of leaf extracts of *R. communis*.

Plants constituents	<i>R. communis</i>
Alkaloids	+
Anthraquines	+
Cardiac glycosides	+
Flavonoids	+
Phlabatannins	-
Reducing sugar	+
Saponins	-
Steroids	+
Tannins	+
Terpenoids	+

+ Presence and - Absence

### **3.4. DISCUSSION**

Extraction is the first major step in the analysis of medicinal plants. It is very important to extract desired chemical components of plant materials for screening of bioactive compounds using biological activity assays and also for isolation and purification of active compounds (Sasidharan *et al.*, 2011). There are a number of extraction procedures employed in this chapter aiming to find the best extraction method to extract a wide range of bioactive constituents in *R. communis* leaves. Traditional healers commonly use water to extract plant compounds, which is limited in its properties of inability to extract non-polar nature. Methanol was the best extractant with a greater mass of extract, followed by dichloromethane and acetone, while the lowest mass was obtained with hexane extract (Figure 3.1). Masoko *et al.* (2008) also found that methanol was a better solvent compared to the others solvents. Extracts were reconstituted in acetone because it has been reported to dissolve both hydrophilic and lipophilic components and not toxic to bacteria (Eloff, 1998).

The analysis of phytochemical revealed that BEA was the best solvent system to separate non-polar compounds. Kotze and Eloff (2002) also reported that BEA was the best solvent system to separate non-polar compounds, CEF separate intermediate compounds, EMW and BAW separate polar compounds. Different colours observed on the TLC chromatograms confirmed the presence of various phytochemicals in *R. communis* leaves. TLC plates developed in BEA solvent system showed the largest numbers of compounds after spraying TLC plates with vanillin-sulphuric acid reagent, followed by CEF and then polar solvent systems (EMW) (Figure 3.2). Obumselu *et al.* (2013) report that a TLC profile study showed only two spots for ethanol and ethyl acetate extracts (*R. communis* leaves) after spraying a plate developed in ethyl acetate: chloroform: methanol: water (15:8:4:1) with iodine. Contrary to findings in this study, larger number of phytochemical compounds extracted from *R. communis* leaves that reacted with vanillin-sulphuric acid reagent than iodine.

Serial exhaustive extraction was the second extraction method used to extract bioactive compounds from *R. communis* leaves and chloroform was the best extractant, followed by hexane, ethanol and methanol. More of non-polar compounds were extracted

because of a greater mass of extracts in non-polar solvents which are hexane and chloroform (Figure 3.3). Chloroform is considered as the best solvent to extract lipophilic compounds while hexane extracts fatty acids from plant materials (Sasidharan *et al.*, 2011). Results suggest that *R. communis* leaves possessed a lot of non-polar and intermediate compounds. The intermediate solvent system (CEF) showed the best resolution of phytochemical constituents present in crude extracts over other solvent systems (Figure 3.4). Phytochemical constituents in methanol and ethanol extracts did not react with vanillin-sulphuric acid reagent hence no bands were observed on the plates. Phytochemical constituents are reported for many biological activities including antibacterial, antioxidant, anti-inflammatory, antioxidant and antitumor activities (Sofowora, 1993).

Chlorophylls and fatty acids were removed from plant materials using hexane wash before extraction because hexane is known to extract chlorophylls and fatty acids (Sasidharan *et al.*, 2011). Ethanol had the largest numbers of phytochemical compounds compare to acetone from washed plant materials of *R. communis* leaves (Figure 3.5). Fingerprint profile for hexane wash extracts showed only three spots of phytochemical compounds reactive to vanillin-sulphuric acid reagent (Figure 3.6). The mass of *R. communis* leaves extracted with various percentages of acetone and ethanol in water are shown in Figure 3.7. The fraction (40% acetone) had the greatest mass of extract and the lowest was in 20% acetone fraction. Enrichment procedure increases the mass of extracts compared to serial exhaustive extraction and preliminary extraction procedures. The important factor is actually not quantity or number of phytochemical compounds present, but the biological activities of plant extracts because phytochemical compounds may be therapeutically active or inactive (Khursheed *et al.*, 2012). TLC plates developed in polar solvent system showed the best resolution of polar phytochemical compounds compared to other solvent systems in phytochemical analysis (Figure 3.8).

In the pretreatment process, 20% ethanol and acetone wash yielded the greatest mass of extracts while the lowest mass was obtained for hexane for ethanol wash (Figure 3.9). Methanol, ethanol and ethyl acetate are polar solvents which are known to extract hydrophilic compounds from plant materials (Sasidharan *et al.*, 2011). Therefore many

hydrophilic compounds were extracted from *R. communis* leaves. Non-polar solvent system showed more bands of phytochemical compounds reacted with vanillin-sulphuric acid over other solvent systems (Figure 3.10). Methanol was the best extractant compare to other solvents because of a greater amount of extract obtained (Figure 3.12). TLC spectral profile of all three series showed that BEA was the best separation system with more bands of phytochemical compounds reacting with vanillin-sulphuric acid reagent (Figure 3.13 to 3.15).

Phytochemical analysis of plant extracts reveals the presence of plant constituent's which are known to exhibit medicinal properties (Sofowora, 1993). *R. communis* extracts revealed the presence of anthraquines, alkaloids, tannins, steroids, terpenoids, flavonoids, cardiac glycosides and reducing sugar. Saponins and phlabatannins were absent. Results in the present study agree with several studies conducted on *R. communis* showed the presence of steroids, flavonoids, tannins and alkaloids (Yadav and Agarwala, 2011; Obumselu *et al.*, 2013; Rao *et al.*, 2013). Other members of Euphorbiaceous that have been reported not to possess saponins and phlabatannins are *Calypha ciliata*, *Croton bonplandianum*, *Euphorbia geniculata*, *Euphorbia hirta L.*, *Jatropha gossypifolia L.*, and *Pedilanthus tithymaloides L.* Alkaloids have been reported in all members of Euphorbiaceae (Kothale *et al.*, 2011). Alkaloids have also been reported as the major compound that exhibit potent antimicrobial activity (Obumselu *et al.*, 2013). Flavonoids have been reported to play a major role in anti-inflammatory, antioxidant and antibacterial activities (Obumselu *et al.*, 2013).

### 3.5. CONCLUSION

A wide range of phytochemical compounds were extracted from *R. communis* leaves. Methanol was the best extractant resulting in a greater yield of plant extract. The most common solvent used by traditional healers is water which is limited by its inability to extract non-polar compounds. The current study recommend the solvents used in this chapter to extract most of non-polar compounds due to the following; BEA was the best solvent system to separate most of phytoconstituents in most of crude extracts. The study revealed that *R. communis* leaves possess greatest number of non-polar phytochemical compounds because of more bands observed in the non-polar solvent

system. Pretreatment of plant materials with hexane resulted in an increase in the quantities of material that were present in the subsequent extracts, however the important factor of medicinal plant research is not quantity but activity of compounds. Serial exhaustive extraction was the best extraction method to separate active part of plant from inactive part of plant for investigation of various biological activities. This chapter recommended serial exhaustive extraction as the best extraction method for isolation of compounds in large scale. The study revealed that various phytochemical compounds (anthraquines, alkaloids, tannins, steroids, terpenoids, flavonoids, cardiac glycosides and reducing sugar) present in *R. communis* leaves are responsible for therapeutic purposes of this plant in the treatment of different ailments. Saponins and phlabatannins are absent in *R. communis* leaves.

## CHAPTER 4

### ANTIOXIDANT ASSAYS

#### **4.1. INTRODUCTION**

From beginning of time, medicinal plants have been studied globally due to their effective bioactive compounds and other medicinal properties (Petal *et al.*, 2010). Plants are a potential source of antioxidant compounds (Huda-Faujan *et al.*, 2009). Antioxidants play an important role to neutralize free radical species which are produced as end or by-products of normal biochemical reactions (Jiménez-Estrada *et al.*, 2013). Reactive oxygen species play an important role in cell physiology (Magama *et al.*, 2013). They have also been reported as carcinogenic and mutagenic agents (Aguirre and Borneo, 2010). High amounts of free radical molecules damage essential macromolecules including DNA, lipids and proteins. The damage to these macromolecules in-turn result in many degenerative conditions such as Parkinson's diseases, atherosclerosis, aging, immunosuppression, ischemic heart disease, diabetes, hair loss membrane lipid peroxidation and decrease membrane fluidity (Kratchanova *et al.*, 2010; Petal *et al.*, 2010; Jiménez-Estrada *et al.*, 2013; Magama *et al.*, 2013).

Different types of compounds that are constitutive of medicinal plants are responsible for free radicals scavenging activity. Compounds that exhibit such activities include flavonoids and terpenoids, phenolic compounds (phenolic acids, quinones, coumarins, lignans, stilbenes, tannins, nitrogen compounds (alkaloids, amines, and betalains), vitamins, carotenoids, and some other endogenous metabolites (Masoko and Elof, 2007; Bichra *et al.*, 2013). Iqbal *et al.* (2012) reported that phenolic compounds are the potent antioxidant compounds.

Antioxidants supplements which are presently used in food industries are vitamin C, vitamin E, selenium,  $\beta$ -carotene, lycopene, lutein and other carotenoids (Jiménez-Estrada *et al.*, 2013). There are two types of antioxidant compounds including natural antioxidants and synthetic antioxidants. Synthetic antioxidants are associated with critical side effects such as liver damage (Parajuli *et al.*, 2012). There is thus, a need to

replace synthetic compounds with newly discovered natural antioxidant compounds isolated from medicinal plants. Antioxidant compounds from plants are safer and decrease risk of many ailments (Bhatt and Negi, 2012).

There are number of methods developed to test for the presence of antioxidant constituents in medicinal plant extracts. The common procedures involve determination of the ability of plants extracts to scavenge free radicals using both qualitative- TLC and quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, ferric reducing antioxidant power (FRAP) assay, ferrous-ion chelating and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radicals assays (Molan *et al.*, 2012). The aim was to evaluate for the presence of antioxidant constituents in *R. communis* using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and quantitative total antioxidant activity assay using ABTS free radicals.

## **4.2. METHODS AND MATERIALS**

### **4.2.1. Qualitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay on TLC**

All crude extracts from Chapter 3 were used for qualitative antioxidant assay on TLC plates. Thin Layer Chromatography (TLC) plates were used to separate extracts as described in Section 3.2.3. The plates were dried in the fume-hood. Chromatograms were sprayed with 0.2% (w/v) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma<sup>®</sup>) in methanol as an indicator. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol as an indicator (Deby and Margotteaux, 1970).

### **4.2.2. Quantitative 2, 2-diphenyl-1-picrylhydrazyl assays**

Based on the results from qualitative antioxidant assay on TLC plates, crude extracts obtained from serial exhaustive extraction were used. Quantitative DPPH assay was done by adding 100 µl of distilled water into each well of 96-welled plate. Plant extracts were prepared at a concentration of 10 mg/ml and 100 µl of plant extracts were transferred into the first well of the 96 plate and extracts in these wells were serially diluted 50%. Vitamin C at a concentration of 2 mg/ml was used as the antioxidant standard (positive control). An additional 15 µl of 0.2% of 2, 2-diphenyl-1-picrylhydrazyl

(sigma®) in methanol was added into each well of microtiter plate and plate was kept in the dark for 15 min. The absorbance was measured at 540 nm using a microtiter plate reader (DTX 880 multimode detector; Beckman coulter, Inc.) (Ayoola *et al.*, 2008). Percentage scavenging activity was calculated as: % scavenging activity (DPPH reduced) = 100% - DPPH oxidized

$$\% \text{ scavenging activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{experiment}}}{\text{Absorbance}_{\text{control}}} \times 100$$

#### **4.2.3. Quantitative total antioxidant activity assay using ABTS free radicals**

Quantitative analyses of antioxidant activity were performed on crude extracts obtained from serial exhaustive extraction using TEAC assay. The method involved generation of radical of the monocation 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS<sup>+</sup>). Blue or green chromophores ABTS<sup>+</sup> were produced through reaction of ABTS and potassium persulphate. Addition of antioxidants to free radicals reduced blue or green chromophores ABTS<sup>+</sup> to colourless ABTS. The extent of decolorisation as a percentage inhibition of ABTS<sup>+</sup> was calculated using a spectrophotometer (Van den berg *et al.*, 1999).

##### *4.2.3.1. Preparation of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS<sup>+</sup>)*

A stock solution of 7 mM of ABTS<sup>+</sup> was prepared by mixing 8 mg of ABTS with 1 ml of water. ABTS free radicals were produced by reacting equal volume of ABTS stock solution with 13.2 mg in 10 ml of (2.45 mM) potassium sulphate. The solution was prepared and incubated for 12-16 hours in the dark at room temperature until the reaction was complete and stored at 4°C until used.

##### *4.2.3.2. Experimental procedure*

Serial dilution method was used to prepare different concentrations of crude extracts hexane, dichloromethane, acetone and methanol. ABTS<sup>+</sup> was diluted with water to equilibrated absorbance of 0.70 ( $\pm 0.02$ ) at 734 nm and mixture of water and acetone was used as a blank. Hundred microliters of ABTS<sup>+</sup> was added to 100  $\mu$ l of 2.50 to

0.039 mg/ml concentrations of crude extracts. The absorbance reading was taken after 6 minutes of reaction. All determinations were carried out in triplicate and calculated as follow:

$$\% \text{ Total antioxidant activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

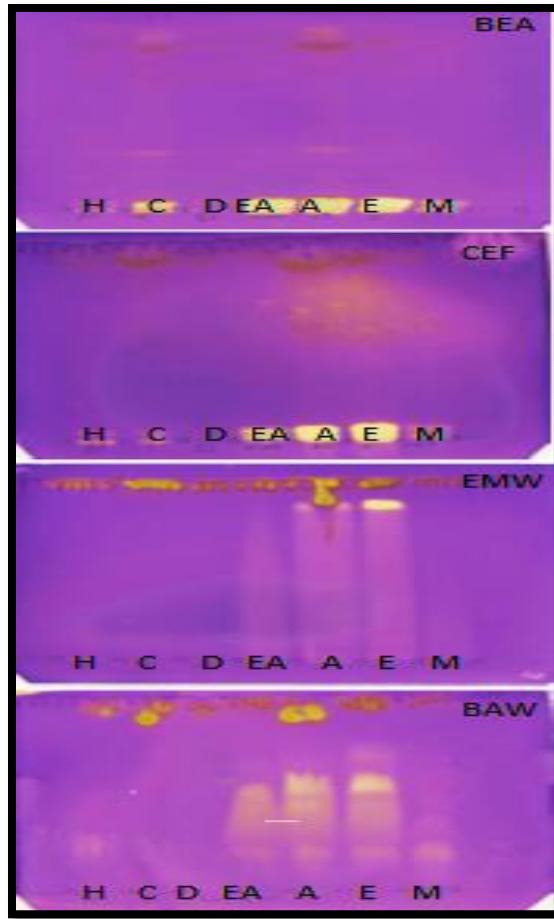
## 4.3. RESULTS

Qualitative antioxidant assay on thin layer chromatography (TLC) plates was used to screen for antioxidant compounds in crude extracts from *R. communis* leaves by spraying chromatograms with 0.2% DPPH in methanol. The presence of antioxidant compounds was detected by yellowish spots against purple background plates sprayed with 0.2% DPPH in methanol (Figure 4.1– 4.9). Most of antioxidant compounds were polar since more of antioxidant bands were observed in EMW and BAW solvent systems. Quantitative antioxidant assays were also performed and percentage scavenging activity against DPPH and ABTS<sup>+</sup> free radicals for each extract was calculated (Figure 4.10– 4.11).

### 4.3.1. Qualitative antioxidant activity assays

#### 4.3.1.1. Preliminary extraction procedure

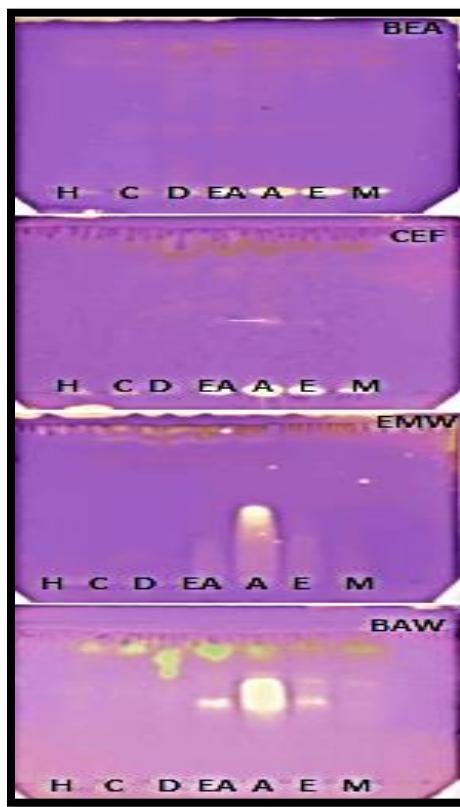
To test for antioxidant activity, chromatograms below were sprayed with 0.2% DPPH in methanol as an indicator and yellowish spots against purple background indicate active compounds with antioxidant activity. Methanol, ethanol, acetone and ethyl acetate extracts had high antioxidant activity at  $R_f$  value of 0.60 in BAW solvent system (Figure 4.1). Most of antioxidant compounds were polar because a number of active bands were observed in EMW and BAW solvent systems.



**Figure 4.1:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, and BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**), ethanol (**E**), and methanol (**M**) in lanes form left to right.

#### 4.3.1.2. Serial exhaustive extraction

Serial exhaustive extraction was the best extraction procedure for isolation of many of polar antioxidant compounds from *R. communis* leaves. Chromatograms below were sprayed with 0.2% DPPH for antioxidant activity. In BAW solvent system, ethyl acetate, acetone and ethanol extracts had very high antioxidant constituents at the  $R_f$  value of 0.60 (Figure 4.2). Acetone extract also revealed active compounds at  $R_f$  value of 0.5 in EMW solvent system.



**Figure 4.2:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW and BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**), ethanol (**E**), and methanol (**M**) in lanes form left to right.

#### 4.3.1.3. Extract enrichment procedure

Extract enrichment procedure consist of two extraction methods; hexane wash, acetone and ethanol in water. Results for both extractions are shown below in Figure 4.3 and 4.4.

##### 4.3.1.3.1. Hexane wash

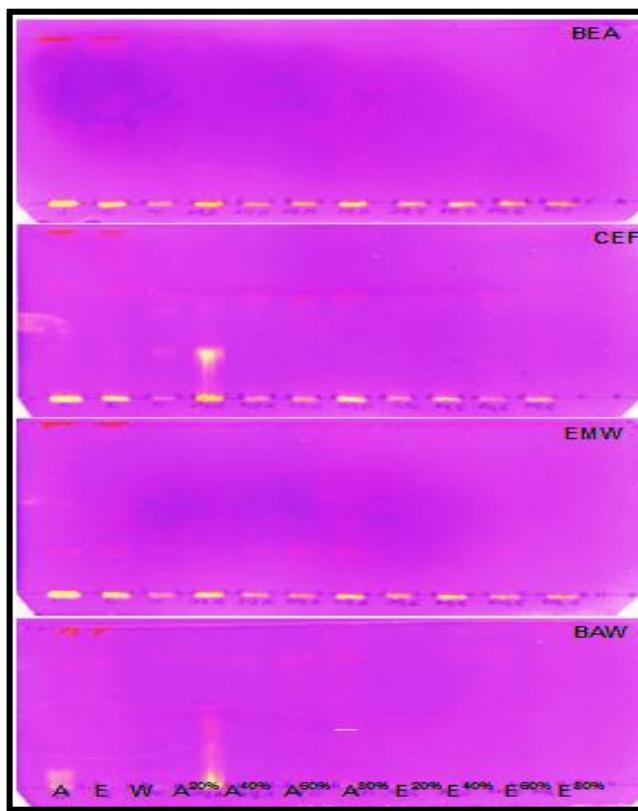
In hexane wash, antioxidant compounds because of their high polarity could not move from the base of the TLC plates in all mobile phases (Figure 4.3). In BAW solvent system, acetone extract was the only extract which revealed high intensity of antioxidant activity after spraying chromatograms with 0.2% DPPH in methanol.



**Figure 4.3:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane wash for acetone (**H1**), hexane wash for ethanol (**H2**), acetone (**A**), and ethanol (**E**), in lanes form left to right.

#### 4.3.1.3.2. Acetone and ethanol in water

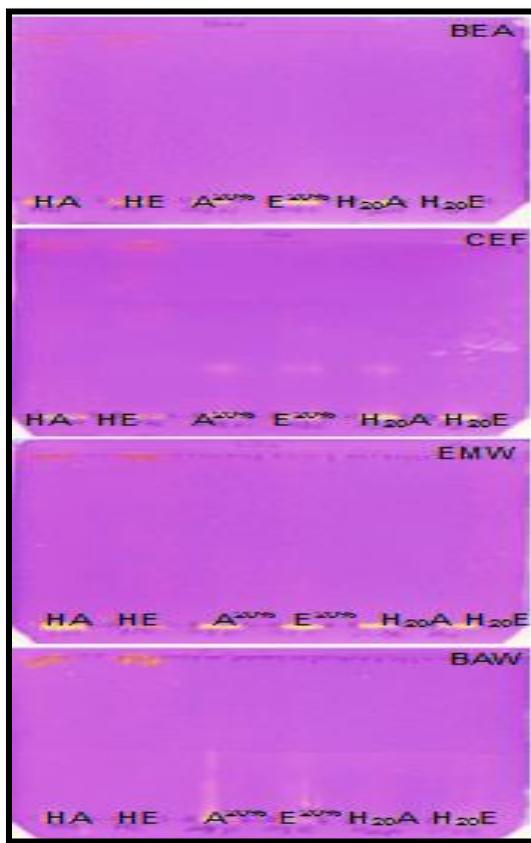
Different percentages of acetone and ethanol in water were used to isolate antioxidant compounds from *R. communis* leaves. TLC plates were sprayed with 0.2% DPPH in methanol as an indicator to test for antioxidant activity. Fraction 20% acetone in water revealed slightly antioxidant activity in CEF solvent system (Figure 4.4). Most of antioxidant compounds could not move from the base of TLC plates sprayed with 0.2% DDPH in all four solvent systems.



**Figure 4.4:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW and BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with acetone (A), and ethanol (E), water (W), 20% Acetone (A<sup>20%</sup>), 40% acetone (A<sup>40%</sup>), 60% acetone (A<sup>60%</sup>), 80% acetone (A<sup>80%</sup>), 20% ethanol (E<sup>20%</sup>), 40% ethanol (E<sup>40%</sup>), 60% ethanol (E<sup>60%</sup>), 80% ethanol (E<sup>80%</sup>), in lanes from left to right.

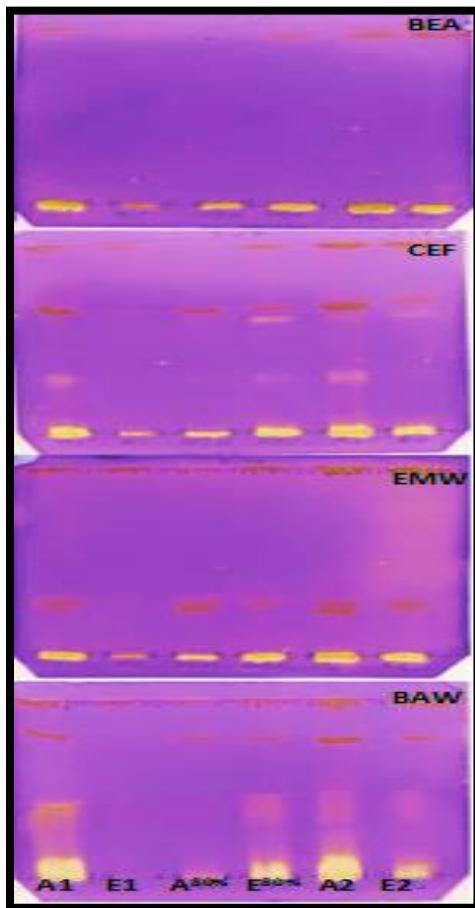
#### 4.3.1.4. Optimal extraction methods

Optimal extraction method involves pretreatment of plant materials with hexane (Figure 4.5) and extraction of pretreated plant materials with acetone and ethanol (Figure 4.6). Most of antioxidant compounds were not separated from the base of TLC plates. CEF solvent system showed slightly antioxidant activity in 20% acetone ( $A^{20\%}$ ), 20% ethanol ( $E^{20\%}$ ) and hexane wash followed by 20% acetone wash ( $H_{20}A$ ) extracts (Figure 4.5).



**Figure 4.5:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane wash for acetone (**HA**), hexane wash for ethanol (**HE**), 20% acetone wash ( $A^{20\%}$ ), 20% ethanol wash ( $E^{20\%}$ ) hexane wash followed by 20% acetone wash ( $H_{20}A$ ), and Hexane wash followed by 20% ethanol wash ( $H_{20}E$ ) in lanes from left to right.

Pretreated plant materials were extracted with acetone, ethanol, 20% ethanol and acetone. To test for antioxidant activity, chromatograms below were sprayed with 0.2% DPPH in methanol as an indicator (Figure 4.6). Antioxidant compounds in BEA solvent system were not separated from base of TLC plates. In CEF solvent system, acetone (**A1**), 20% ethanol and acetone (**A2**) extracts showed high antioxidant activity.

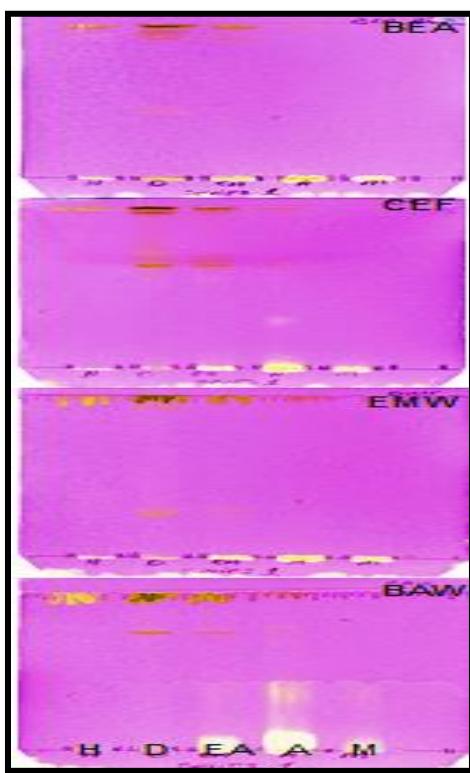


**Figure 4.6:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW and BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with acetone (**A1**), ethanol (**E1**), 80% acetone (**A<sup>80%</sup>**), 80% ethanol (**E<sup>80%</sup>**), acetone (**A2**) and ethanol (**E2**) in lanes from left to right.

#### 4.3.1.5. Serial exhaustive extraction

##### 4.3.1.5.1. Series 1

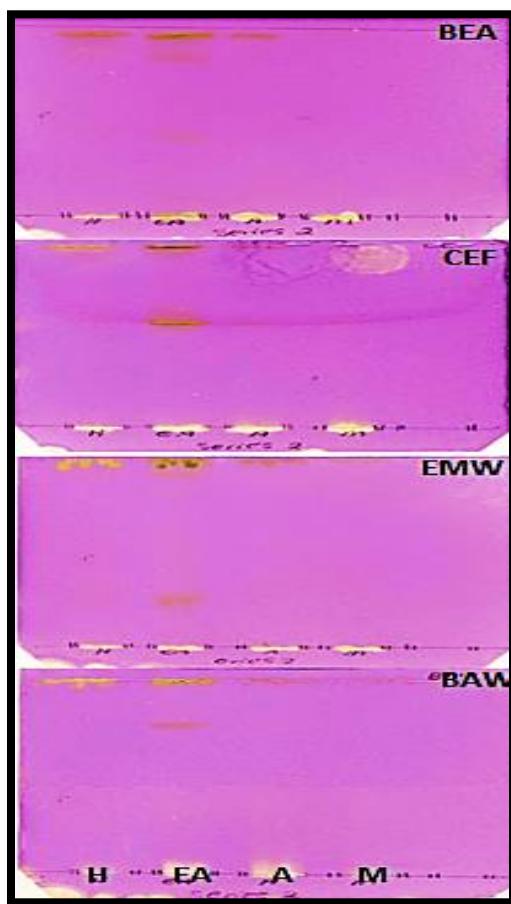
Three series of serial exhaustive extraction were employed using solvents of varying polarities to extract antioxidant compounds from *R. communis* leaves. Plant constituents compounds were separated with four mobile phases. Chromatograms were sprayed with 0.2% DPPH in methanol for qualitative free radical scavenging activity (Figure 4.7 to 4.9). In series one, antioxidant compounds present in BEA, CEF and EMW solvent systems did not move from the base of TLC plates. In BAW solvent system, acetone and ethyl acetate had high antioxidant compounds (Figure 4.7).



**Figure 4.7:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane (**H**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**) and methanol (**M**) in lanes from left to right.

#### 4.3.1.5.2. Series 2

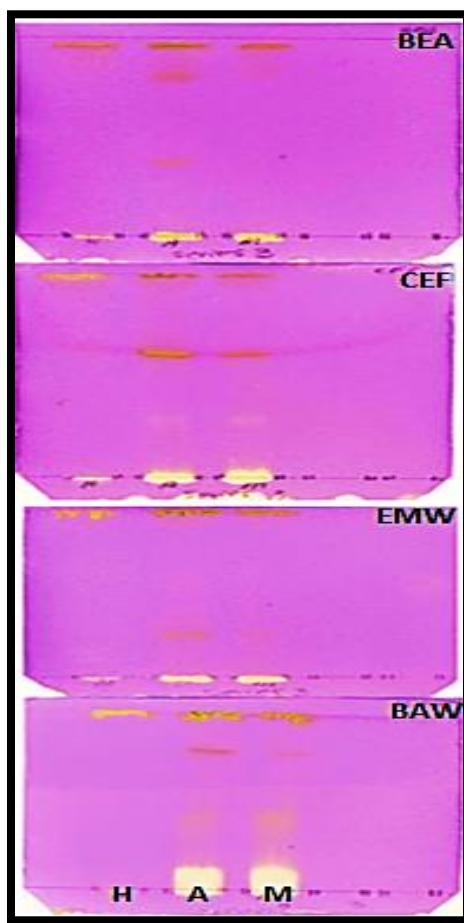
In series 2, chromatograms were sprayed with 0.2% DPPH in methanol as an indicator for qualitative free radicals scavenging activity (Figure 4.8). Antioxidant compounds were not separated from the base of TLC plates in all separation systems (BEA, CEF, EMW, and BAW).



**Figure 4.8:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots indicate antioxidant activity exhibited by compounds extracted with hexane (**H**), ethyl acetate (**EA**), acetone (**A**) and methanol (**M**) in lanes from left to right.

#### 4.3.1.5.3. Series 3

In series three, chromatograms were sprayed with 0.2% DPPH for antioxidant activity. Antioxidant compounds present in BEA, CEF and EMW were not separated from the base of TLC plates (Figure 4.9). Acetone and methanol extracts showed high antioxidant compounds in BAW solvent system.

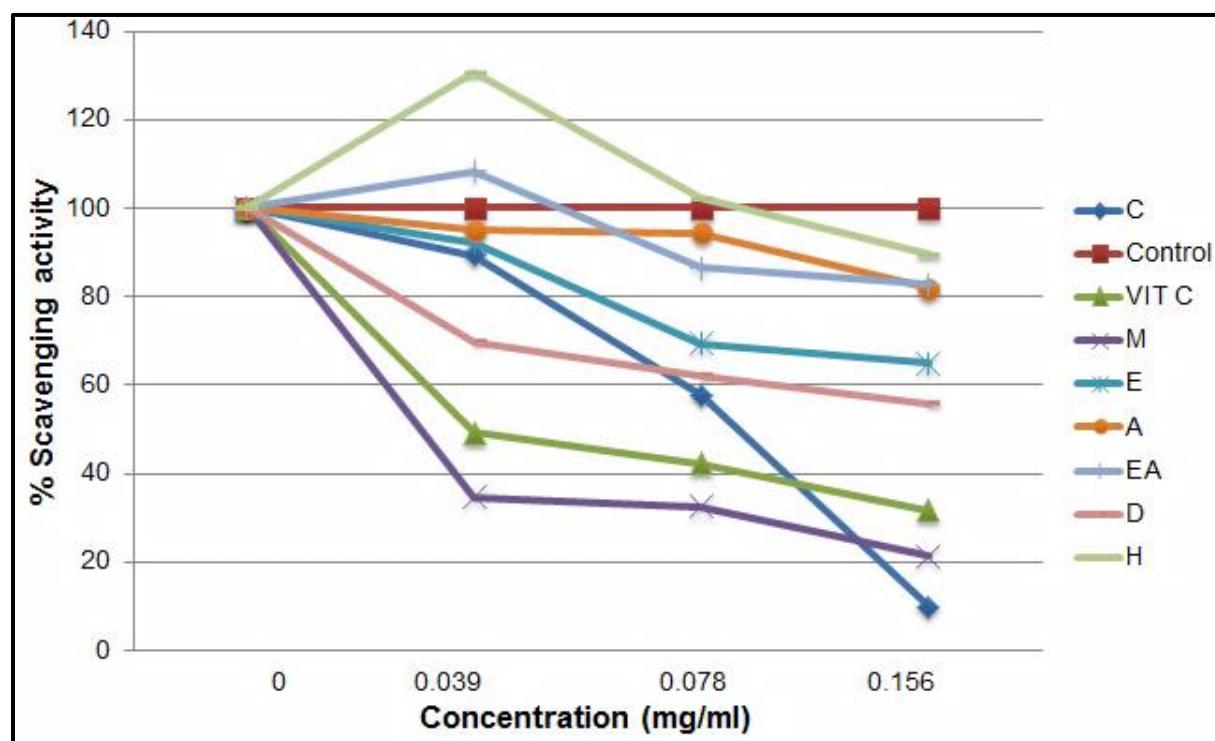


**Figure 4.9:** Chromatograms of *R. communis* crude extracts developed in four solvents systems: BEA, CEF, EMW, BAW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow zones indicate antioxidant activity exhibited by compounds extracted with hexane (**H**), acetone (**A**) and methanol (**M**) in lanes from left to right.

#### 4.3.2. Quantitative total antioxidant activity assays

##### 4.3.2.1. Quantitative 2, 2 – diphenyl-1-pacrylhydrazyl (DPPH) assay

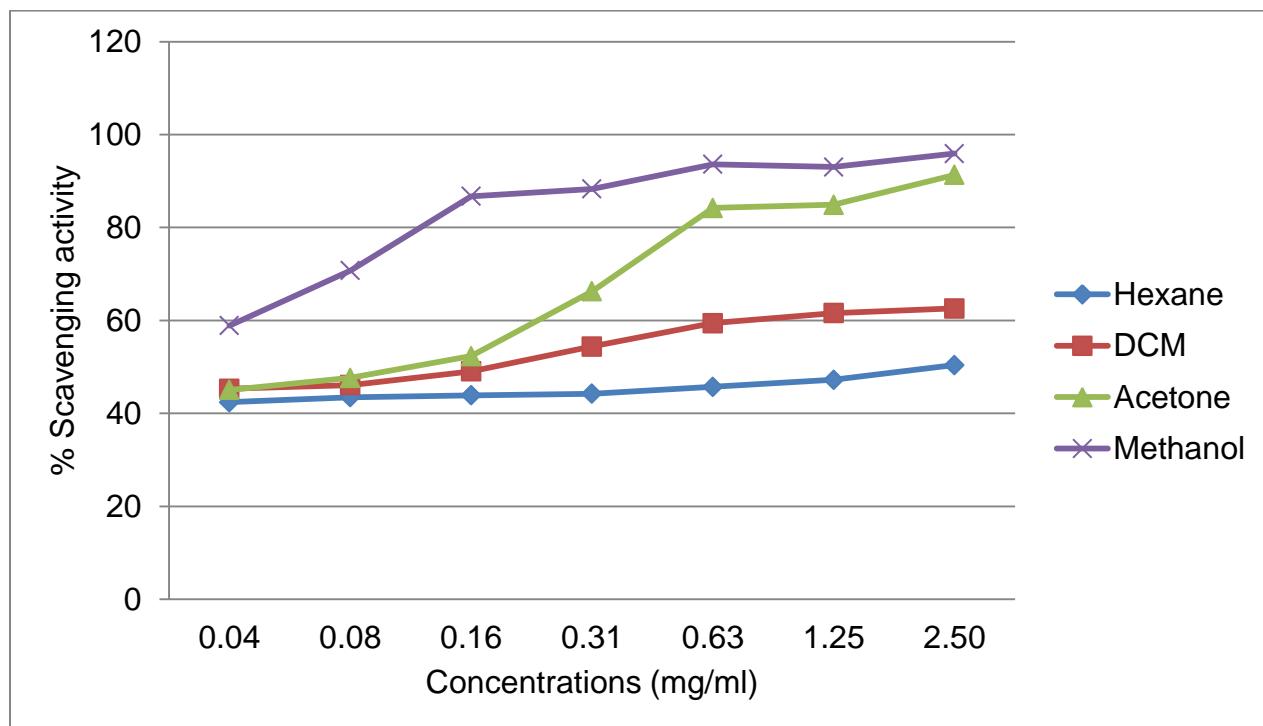
Crude extracts obtained from serial exhaustive extraction were used for quantitative DPPH assay because of large number of antioxidant compounds revealed on the qualitative TLC-DPPH assay. Hexane and ethanol extracts showed to have no antioxidant activity at concentration of 0.039 mg/ml. Dichloromethane, acetone and ethyl acetate extracts showed high free radical scavenging activity; however, the activity was less compared to vitamin C (Figure 4.10). Chloroform and methanol extracts had highest percentage scavenging activity compared to other extracts.



**Figure 4.10:** DPPH scavenging activity (%) of different extracts of *R. communis* at various concentrations at 540 nm. Chloroform (**C**), negative control (**Control**), vitamin C (**VIT C**) positive control, Methanol (**M**), Ethanol (**E**), Acetone (**A**), Dichloromethane (**D**) and Hexane (**H**).

#### 4.3.2.2. ABTS free radicals assay

Antioxidant activity of hexane, dichloromethane, acetone and methanol extracts of *R. communis* was also quantified using ABTS<sup>+</sup> decolorization method. Addition of antioxidants to free radicals reduced the blue chromophores ABTS<sup>+</sup> to colorless. Methanol extract had the highest percentage free radical (ABTS<sup>+</sup>) scavenging activity of 95% at 2.50 mg/ml, followed by acetone, dichloromethane and hexane (42%) at 0.039 mg/ml (Figure 4.11).



**Figure 4.11:** Percentage scavenging activity of ABTS free radicals with respect to increasing concentration of *R. communis* crude extracts; hexane, dichloromethane (DCM), acetone and methanol extracts.

#### **4.4. DISCUSSION**

Traditional medicinal plants are potential source of natural antioxidants that play a role to counteract reactive oxygen species in order to survive (Talukdar *et al.*, 2011). All crude extracts obtained from various number of extraction procedures employed in chapter 3 were tested for free radical scavenging activity using qualitative DPPH-TLC assays. To test for free radical scavenging activity, chromatograms were sprayed with 0.2% DPPH in methanol as an indicator (Deby and Margotteaux, 1970). The degree of antioxidant activity in all crude extracts tested was determined quantitatively from observation of yellow colours intensity on the TLC plates against a purple background.

Scavenging activity of plant extracts may be due to the presence of phytochemical compounds which are flavonoids, alkaloids and tannins. It has also been reported that extracts that have the ability to scavenge free radicals may possess flavonoids (Ilavarasan *et al.*, 2006; Iqbal *et al.*, 2012; Jiménez-Estrada *et al.*, 2013). Results revealed antioxidant compounds in ethyl acetate, acetone and ethanol extracts separated with BAW solvent system (Figure 4.1). Acetone extracts exhibited strong antioxidant activity against DPPH free radicals molecules in polar solvent systems (Figure 4.2). Acetone extracts was the possible extract to isolate potent antioxidant compounds to stabilize free radicals molecules.

The presence of antioxidant activity in *R. communis* leaves may be due to the presence of flavonoids compounds (Chapter 3, Table 3.2). The important role of antioxidant compounds derived from traditional medicinal plants is to neutralize the free radical molecules and terminate the chain reaction before essential molecules are damaged in normal system (Iqbal *et al.*, 2012). Free radical scavenging activity of the methanol extract of *R. communis* L roots in Wistar albino rats have also been also reported (Ilavarasan *et al.*, 2006). The leaves of *R. communis* are the good source of antioxidant compounds. Plates developed in CEF solvent system showed slightly antioxidant compounds that were not separated from the base of TLC plates due to their polarity relative to the solvent system used. Separation of phytochemical compounds on the TLC plates is highly dependent on the polarity of both compounds and separation systems (Visht and Chaturvedi, 2012).

*R. communis* leaves were washed with hexane to remove fatty acids and chlorophyll before exhaustive extraction of antioxidant compounds (Figure 4.3) because hexane is the best solvent to remove fatty acids and chlorophyll from plant materials (Sasidharan et al., 2011). Hexane removed fatty acids constitutive in *R. communis* leaves resulting in rupturing of cellular membrane to expose more of antioxidant compounds present. Although the plant materials were washed, active antioxidant compounds were not separated from the base of TLC plates in all separation systems. In comparison, serial exhaustive extraction is the best extraction method to extract intensive antioxidant compounds from *R. communis* leaves over hexane wash and preliminary extractions. The presence of antioxidant compounds in *R. communis* leaves was also confirmed by Singh et al. (2009). Chromatograms in Figure 4.4 were obtained after testing different percentage extracts of acetone and ethanol in water for antioxidant activity against DPPH free radicals. Since DPPH free radicals is commonly used as a model to screen for the scavenging activity of free radical molecules by crude extracts obtained from traditional medicinal plants (Rajamurugan et al., 2013). Fraction 20% acetone in water extract revealed slightly free radical scavenging activity against DPPH free radicals in a plate developed with intermediate solvent system. To further screen for antioxidant compounds from *R. communis*, the leaves were pretreated with hexane, 20% acetone and ethanol in water to exposed antioxidant compounds (Figure 4.5) before exhaustive extraction (Figure 4.6). Solvent systems used were unable to separate antioxidant compounds from the base of plates, hence the separation of antioxidant compounds was a challenge in some of active compounds.

Series of extractions were developed in order to find the best series of solvents for extraction of active compounds in large scale extraction (Chapter 6) because of high antioxidant activity observed in qualitative TLC-DPPH assays (Figure 4.7- 4.9). Acetone extracts exhibited strong antioxidant activity against DPPH free radical molecules. High degree antioxidant activity of acetone extracts against DPPH free radical molecules was also confirmed in Figure 4.2. Above results suggests that acetone extracts possessed strong antioxidant activity due to high quantity of flavonoid compounds present. Since flavonoids are plants constitutive compounds that are responsible for antioxidant activity (Singh et al., 2009).

Seven crude extracts from serial exhaustive extraction were selected and used for quantitative antioxidant assays using DPPH free radical molecules because of the presence of intensive antioxidant compounds (Figure 4.2). Results are shown in figure 4.10, vitamin C was used as antioxidant standard and acetone as negative control. Scavenging activity of *R. communis* extracts was compared with antioxidant standard. Negative control had 0% scavenging activity against DPPH free radicals. Plant extracts possess hydrogen donating capability and free radical scavenging activity, can therefore act as antioxidants. Hydrogen-donating ability of the antioxidant compounds derived from medicinal plants are responsible for their free radical-scavenging activity (Shahwar et al., 2010). Iqbal et al. (2012) showed high antioxidant activity of aerial part of *R. communis* against DPPH free radicals.

In the study, n-butanol exhibited highest percentage scavenging activity of 61.49% against DPPH and the lowest of 22.37% was in chloroform extract (Iqbal et al., 2012). In the present study, chloroform and methanol extracts had high percentage scavenging activity of DPPH compare to vitamin C. Methanol extract was also found to have effectual antioxidant activity by (Ilavarasan et al., 2006; Rao et al., 2013). Hexane and ethanol extracts showed no antioxidant activity at concentration of 0.039 mg/ml. Dichloromethane, acetone and ethyl acetate extracts also showed antioxidant activity; however, the activity was less compared to vitamin C. Anything above 100% scavenging activity was not active. The highest percentage scavenging activity was observed in chloroform extracts and least scavenging activity was observed in hexane extracts at 0.156 mg/ml. The percentage scavenging activity of DPPH free radicals increases with the increase of concentrations of *R. communis* leaves extracts. Gallic acid, quercetin, gentisic acid, rutin, epicatechin and ellagic acid are the major phenolic compounds found responsible for antioxidant activity of dry leaves of *R. communis* (Singh et al., 2009).

In the present study, quantitative antioxidant activity of hexane, dichloromethane, acetone and methanol extracts was also quantified using ABTS<sup>+</sup> decolorization method (Figure 4.11). ABTS<sup>+</sup> decolorization method is used for the screening of antioxidant activity of plant extracts, is applicable to both lipophilic and hydrophilic antioxidants (Rajamurugan et al., 2013). The maximum free radical scavenging activity was 95% in

methanol extract at the concentration of 2.50 mg/ml, dichloromethane had 62%, acetone 91% and 50% for hexane extract. All crude extracts had more than 50% scavenging activity of ABTS free radicals at 2.50 mg/ml. Results suggests that the leaves of *R. communis* are good source of antioxidant compounds to scavenge both ABTS and DPPH free radicals. Percentage scavenging activity of both ABTS and DPPH free radical molecules increases with the increase of concentrations of the extracts, high concentration of extracts exhibit high percentage scavenging activity.

#### **4.5. CONCLUSION**

The leaves of *R. communis* had high percentage scavenging activity of both DPPH and ABTS free radicals. High degree of free radical scavenging activity was observed in acetone extract. Acetone extract was the possible extract to isolate potent antioxidant compounds, therefore further isolation and purification of antioxidant compounds from acetone extract is required. Antioxidant compounds from plants are quite safe and their side effects are not complicated unlike those of synthetic antioxidants. Antioxidant compounds isolated from acetone extracts of *R. communis* leaves may be used as antioxidant additives or supplements in food industries. The study serves as a scientific proof for use of this plant in traditional medicine for treatment of various ailments.

## CHAPTER 5

### ANTIBACTERIAL ASSAYS

#### **5.1. INTRODUCTION**

Traditionally plants have been employed as a source of medicine for treatment of various diseases globally. Medicinal plants are used traditionally in the form of infusion, decoction, tincture or herbal extracts. In some cases, people from rural areas use plants in combination with two or more plants for treatment of a particular disease (Wendakoon *et al.*, 2012). Currently, there is an increase in the use of plants worldwide because they are readily available and generally considered to be safe with fewer side effects. Plants are considered as the main source of powerful antimicrobial drugs (Rosakutty and Roslin, 2012). Secondary metabolites produced by plants are responsible for antimicrobial activity of plants (Savithramma *et al.*, 2011; Janjua *et al.*, 2013). Approximately 95% of modern drugs have been isolated from traditional medicinal plants (Rosakutty and Roslin, 2012). Medicinal plants are used as a precursor for formulation of many modern antibiotics in pharmaceutical industries (Savithramma *et al.*, 2011).

Recently, pathogenic microorganisms have acquired resistance genes toward current antibiotics used in health care systems. There is therefore a great need to screen medicinal plants for potential and effective antibacterial activity against resistance strains (Shakeri *et al.*, 2012). Over 3 million people in South Africa depend on traditional medicinal plants for primary health care. Some of plants which are used traditionally are not scientifically validated and they possessed compounds that fight against infectious diseases. About 20% of medicinal plants have been tested and scientifically proven; they are now used without fear of toxic substance (Coopooسامy and Naidoo, 2012).

Bioautography is one of the antibacterial assays used to screen for antimicrobial compounds in plant extracts directly on TLC plates. Bioautography is a fast, cheap and effective technique used for detection of bioactive compounds with antimicrobial activity. Bioautography involves separation of compounds in plant extracts using TLC plates in different mobile phases of varying polarities and detection of antimicrobial compounds

directly on the TLC plate. Advantage of bioautography is that it localizes antimicrobial compounds on a TLC plates and retardation factor ( $R_f$ ) value of targeted compound can be easily calculated from plate. There are three different types of bioautography methods known, (i) direct bioautography, where microorganisms are grown on TLC plates and zone of inhibitions are observed directly on the plates (ii) contact bioautography, it involves transferring active compounds from chromatograms to an inoculated agar plate by direct contact (iii) Agar overlay bioautography, where agar medium with grown culture is transferred directly on the chromatograms (Sasidharan et al., 2011). After screening medicinal plant for bioactive compounds, active compounds are extracted, isolated and purified from mixture of compounds using column chromatography. The aim was to evaluate the antibacterial activity of *R. communis* using minimum inhibitory concentration and bioautography.

## **5.2. METHODS AND MATERIALS**

### **5.2.1. Test organisms**

The test organisms were supplied by the Department of Biochemistry, Microbiology and Biotechnology section of the University of Limpopo (Turfloop campus). Two Gram-positive (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) and two Gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) bacterial strains were used in this study. The organisms were sub-cultured on nutrients broth, incubated at 37°C for 24 h and stored at 4°C in the refrigerator as stock cultures.

### **5.2.2. Serial microbroth dilution method**

Minimum inhibitory concentration (MIC) values were determined using the serial microbroth dilution method developed by Eloff (1998). MIC is described as the lowest concentration of the compounds inhibiting the growth of test microorganisms. Dried crude extracts were reconstituted in acetone to a concentration of 10 mg/ml crude extracts. The plant extracts were serially diluted 50% with water in 96 well microtiter plates. Bacterial cultures were sub-cultured and transferred into fresh nutrient broth. Hundred microliters of culture were transferred into each well and acetone was included blanks. Similar dilutions of ampicillin (Sigma) were used as the positive control and

acetone was used as negative control. The microtiter plate was incubated at 37°C for 24 hours. After incubation, 20 µl of 2 mg/ml *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each microplate wells as an indicator of growth. The covered microtiter plates were incubated for 30 min at 37°C and 100% relative humidity. All determinations were carried out in triplicate. Microorganism growth led to the emergence of a purple-red colour resulting from the reduction of INT into formazan. Clear wells indicated the presence of compound in the extracts that inhibited the growth of the microorganisms tested. MIC was recorded as the lowest concentration of the extract that inhibited bacterial growth after 24 hours.

### **5.2.3. Bioautography**

Bioautography described by Begue and Kline (1972) was used to test for antibacterial activity of crude extracts against test microorganisms. Thin layer chromatography plates were separated as described in Section 3.2.3. For bioautographic analysis, 20 µl of each extract (10 mg/ml) was loaded onto the TLC plates. The plates were developed in mobile phases as described earlier. The chromatograms were dried at room temperature for about four days to remove solvents used to develop chromatograms. The chromatograms were sprayed with overnight cultures of bacterial cultures until completely wet and were incubated at 37°C in a humidified chamber for 24 hours. The plates were sprayed with 2 mg/ml of INT (Sigma®) and incubated for a further 2 hours. White areas against pink background indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested bacteria (Begue and Kline, 1972).

## **5.3. RESULTS**

### **5.3.1. Preliminary extraction procedure**

Minimum inhibitory concentration (MIC) values of *R. communis* extracts were calculated and recorded in Table 5.1 below. All crude extracts had good MIC values ranged from 0.13 to 1.05 mg/ml. Hexane extracts exhibited highest MIC value of 1.05 mg/ml against *S. aureus* and lowest of 0.13 mg/ml against *P. aeruginosa* (Table 5.1). Ethanol extracts

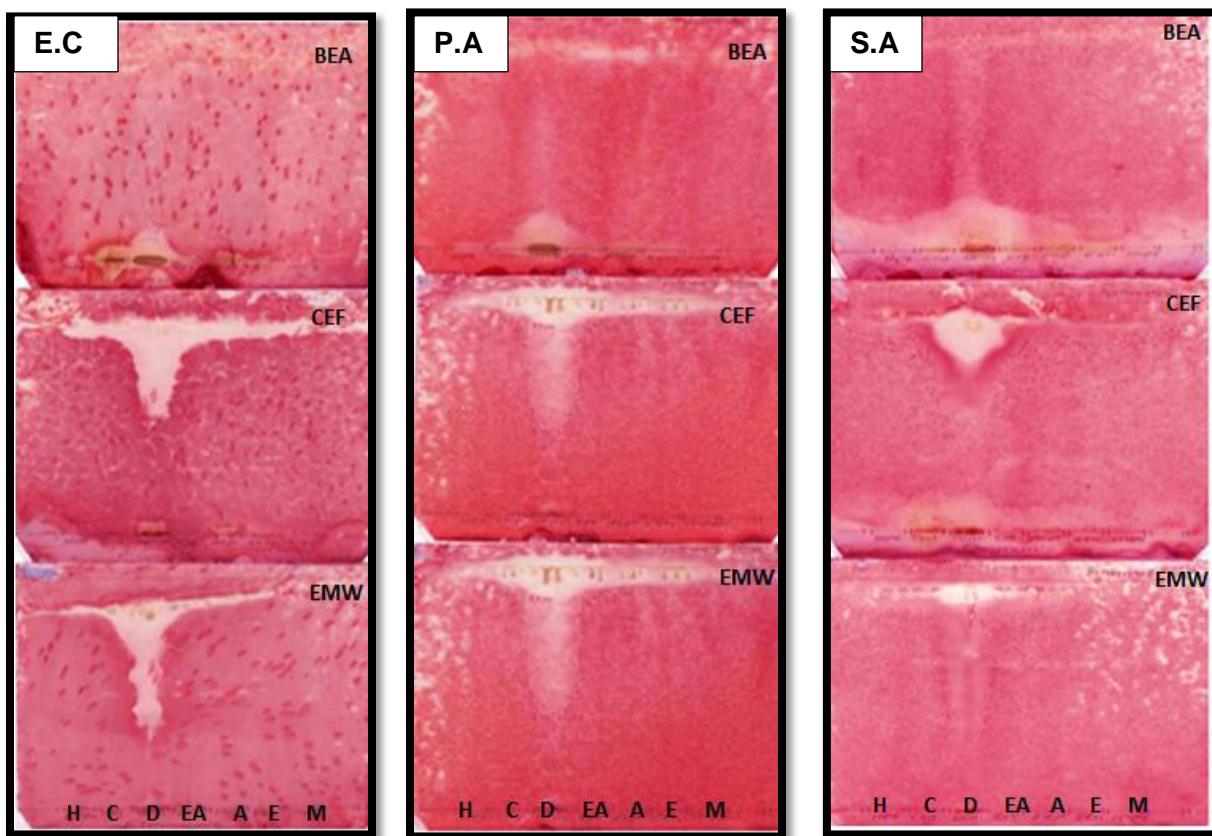
had the lowest average MIC value of 0.31 mg/ml and the highest was in hexane extracts (0.61 mg/ml). Ampicillin had the total average MIC value of 0.16 mg/ml.

**Table 5.1:** Minimum inhibitory concentration (MIC) values (mg/ml) of various extracts of *R. communis* leaves against four tested bacterial species.

	Plant extracts and MIC values (mg/ml)							
Bacteria species	H	C	D	EA	A	E	M	Ampicillin (mg/ml)
<i>E. coli</i>	0.43	0.43	0.27	0.27	0.16	0.32	0.32	0.27
<i>P. aeruginosa</i>	0.13	0.37	0.19	0.21	0.32	0.13	0.32	0.13
<i>S. aureus</i>	1.05	0.32	0.32	0.37	0.32	0.16	0.37	0.08
<i>E. faecalis</i>	0.84	0.74	0.53	0.53	0.53	0.64	0.84	0.16
<b>Averages</b>	<b>0.61</b>	<b>0.47</b>	<b>0.33</b>	<b>0.35</b>	<b>0.33</b>	<b>0.31</b>	<b>0.46</b>	<b>0.16</b>

Solvents used for extraction: hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**), ethanol (**E**), and methanol (**M**).

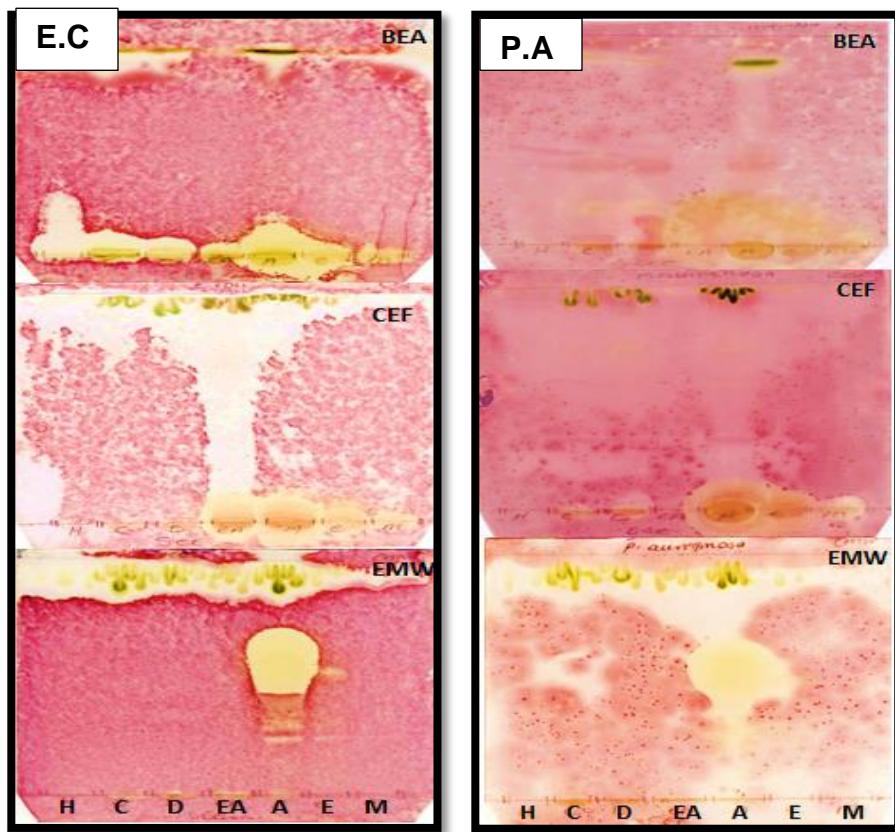
Chromatograms below showed high antimicrobial activity in dichloromethane extract against *E. coli*, *S. aureus* and *P. aeruginosa* (Figure 5.1). Antibacterial compounds were separated in both EMW and CEF solvent systems. In BEA solvent system, active compounds were not separated from the base of TLC plates.



**Figure 5.1:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and then sprayed with *E. coli* (**E.C**), *P. aeruginosa* (**P.A**) and *S. aureus* (**S.A**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), ethanol (**E**), and methanol (**M**) in lane from left to right.

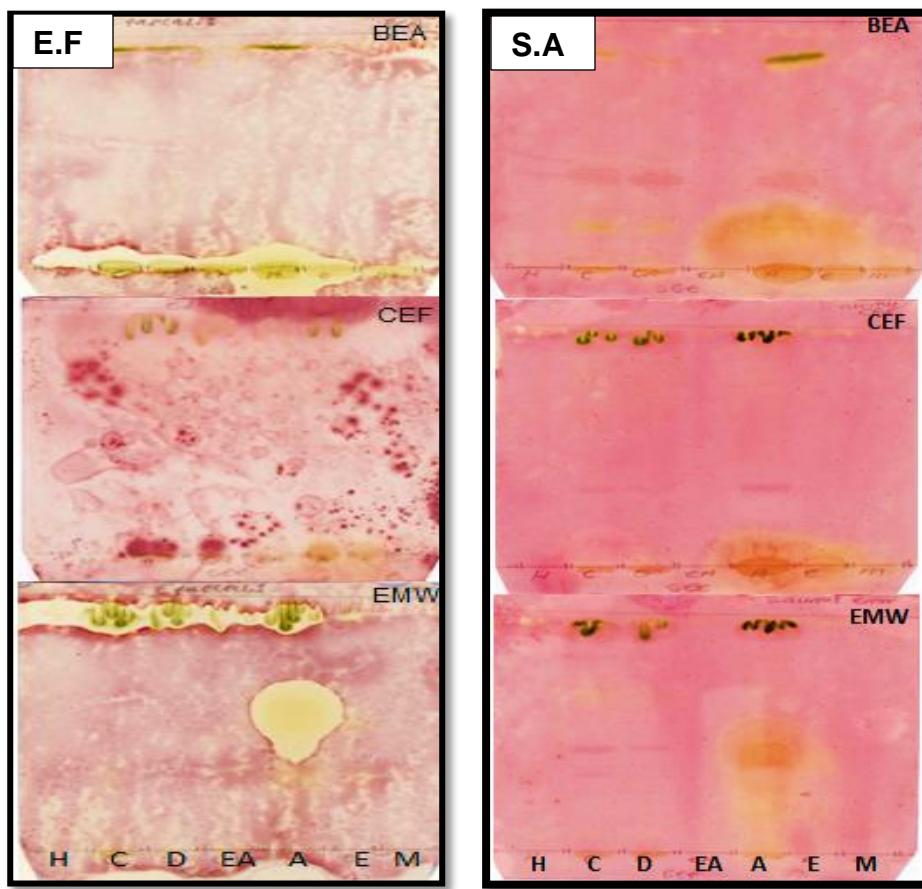
### 5.3.2. Serial exhaustive extraction

Bioautograms were sprayed with *E. coli* and *P. aeruginosa* (Figure 5.2). In EMW solvent system, acetone extract had antibacterial compounds active against both pathogens tested at  $R_f$  value of 0.57. In CEF solvent system, a clear zone exhibited by the ethyl acetate extracts may be due to the presence of formic acid used as mobile phase. In BEA and CEF solvent systems, no antibacterial compounds were observed against *P. aeruginosa*.



**Figure 5.2:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (Centre), EMW (bottom) and sprayed with two Gram negative bacterial species: *E. coli* (**E.C**), and *P. aeruginosa* (**P.A**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), ethanol (**E**), and methanol (**M**) in lane from left to right.

TLC-plates were sprayed with Gram positive bacteria, *E. faecalis* and *S. aureus* (Figure 5.3). Acetone extract had high antibacterial compounds against *E. faecalis* at  $R_f$  value of 0.56 in EMW solvent system. *S. aureus* was less sensitive to all the extracts in all separation systems. Antibacterial compounds in BEA and CEF solvent systems could not move from the base of TLC plates.

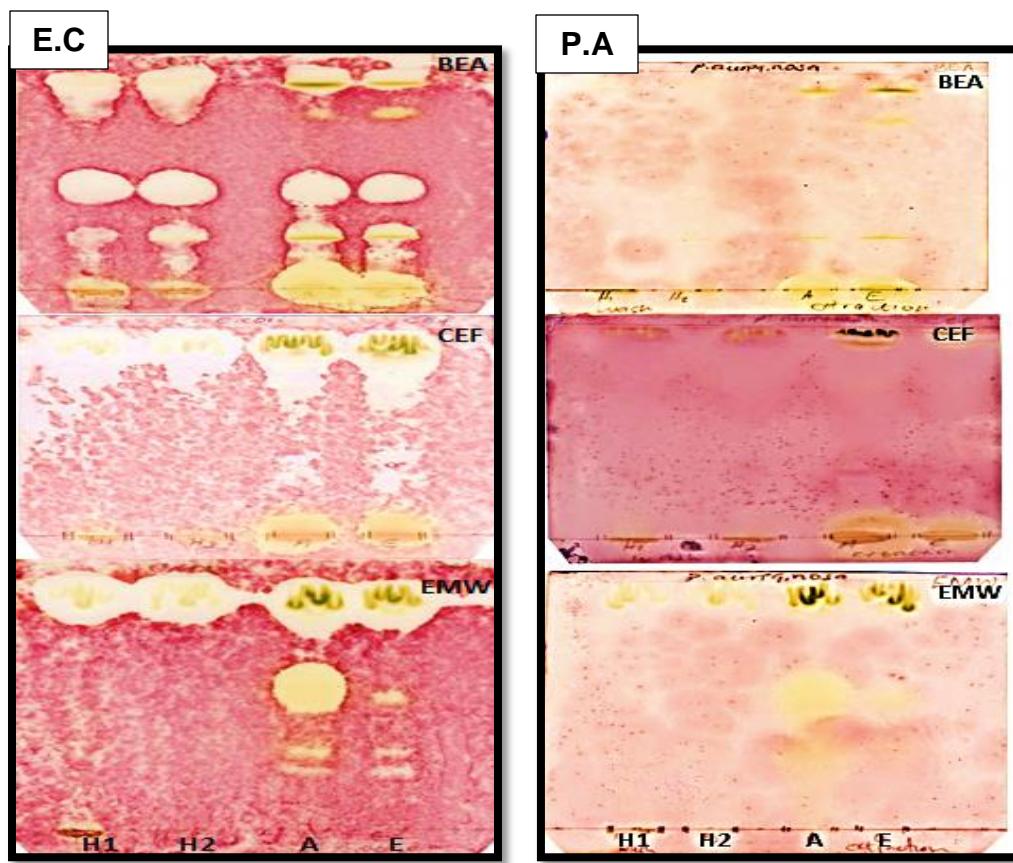


**Figure 5.3:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems: BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacterial species: *E. faecalis* (**E.F.**), and *S. aureus* (**S.A.**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), chloroform (**C**), dichloromethane (**D**), ethyl acetate (**EA**), ethanol (**E**), and methanol (**M**) in lane from left to right.

### 5.3.3. Extract enrichment procedures

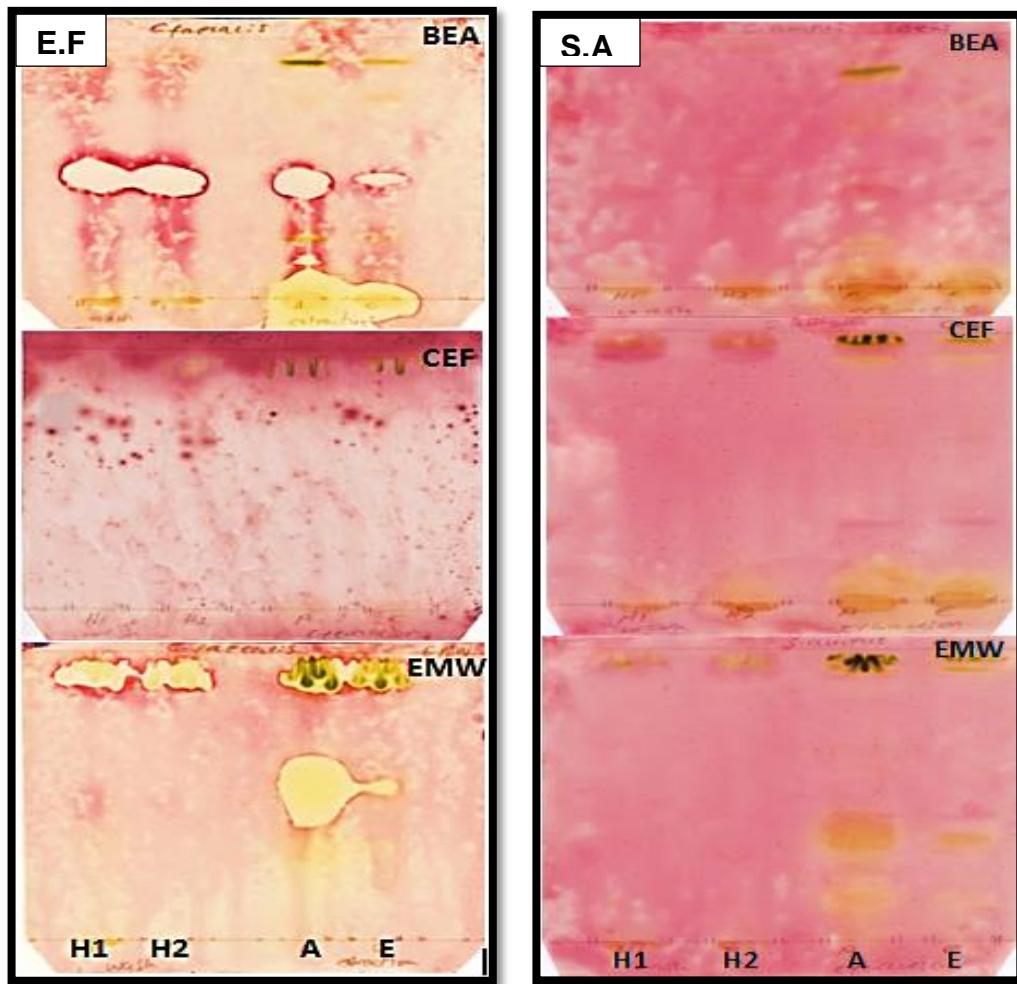
#### 5.3.3.1. Hexane wash

After washing plant materials with hexane, BEA was the best solvent system to separate most of antibacterial compounds present in hexane wash at the  $R_f$  values of 0.27 and 0.40. Most of the bioactive compounds were active against *E. coli*. In EMW, acetone and methanol extracts also contained antibacterial compounds active against *E. coli* and *P. aeruginosa* at the  $R_f$  value of 0.56 and 0.58, respectively (Figure 5.4).



**Figure 5.4:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (Centre), EMW (bottom) and sprayed with two Gram negative bacteria species: *E. coli* (E.C), and *P. aeruginosa* (P.A), white zones indicate active compounds that inhibited growth of tested bacteria species. Hexane wash for acetone (H1), hexane wash for ethanol (H2), acetone (A) and ethanol (E) in lane from left to right.

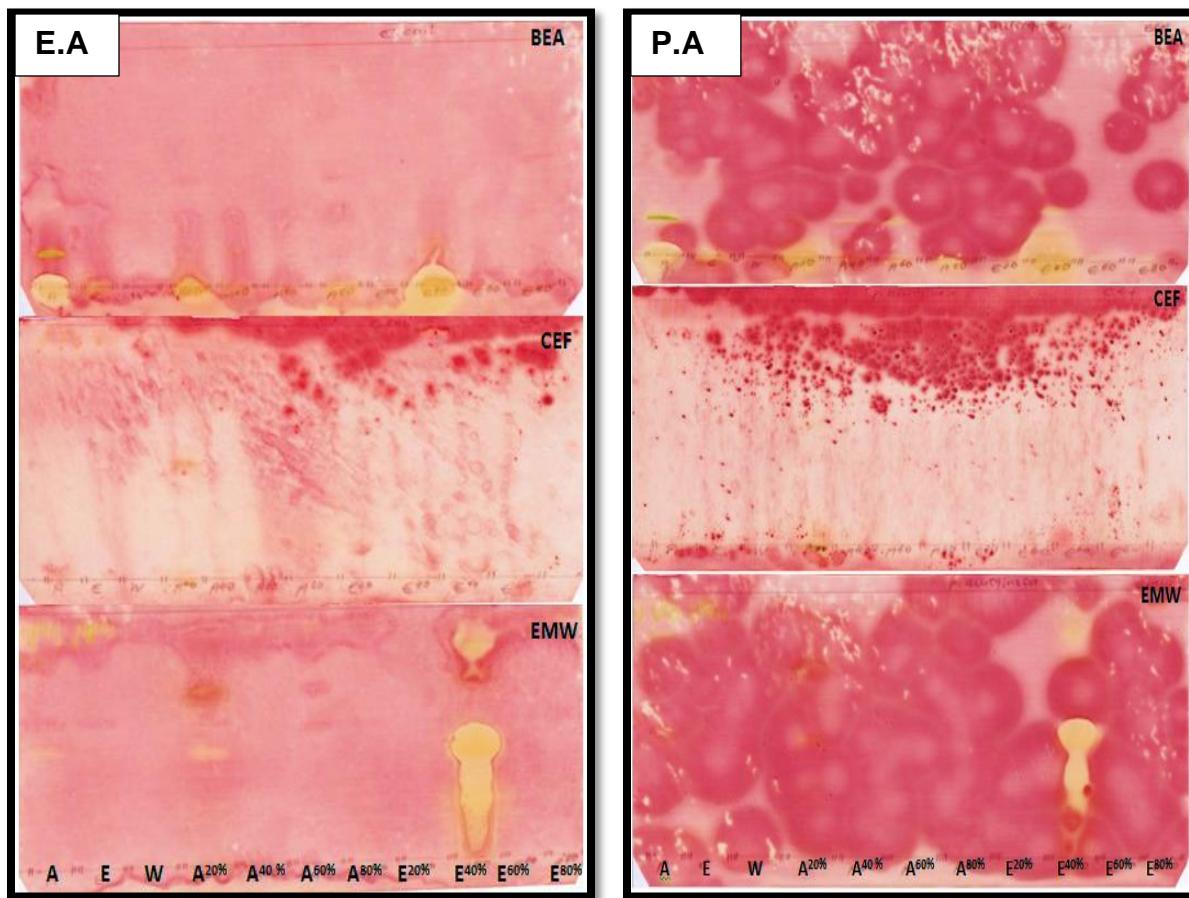
TLC-plates were sprayed with Gram positive bacteria to test for antibacterial compounds. BEA solvent system had antibacterial compounds against *E. faecalis* in all the extracts at the  $R_f$  value of 0.43 (Figure 5.5). Acetone extract also had active compounds against *E. faecalis* at the  $R_f$  value of 0.56 in EMW. *S. aureus* was less sensitive to all the extracts. Some of active compounds were not separated from the base of TLC plates.



**Figure 5.5:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacteria species: *E. faecalis* (**E.F**), and *S. aureus* (**S.A**), white zones indicate active compounds that inhibited growth of tested bacteria species. Hexane wash for acetone (**H1**), hexane wash for ethanol (**H2**), acetone (**A**), and ethanol (**E**) in lane from left to right.

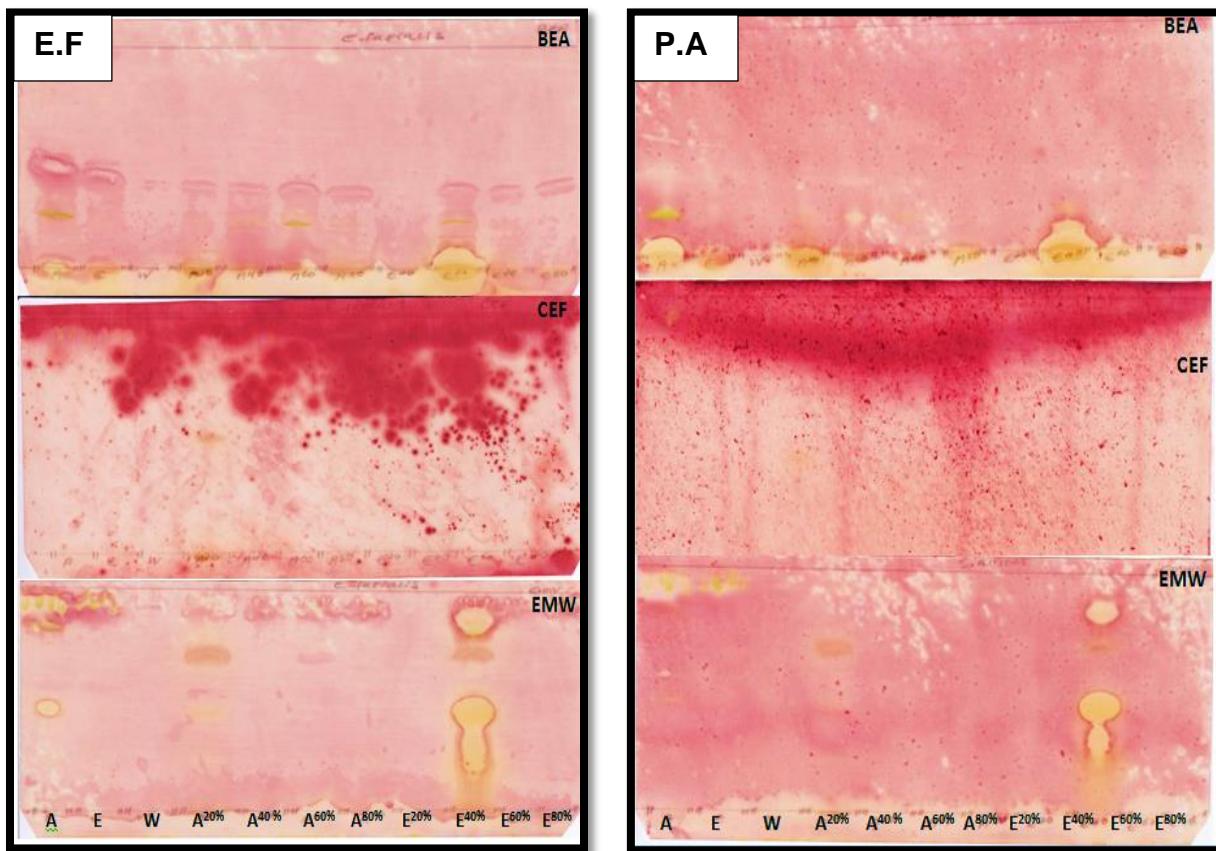
### 5.3.3.2. Acetone and ethanol in water

Bioautograms were sprayed with *E. coli* and *P. aeruginosa* (Figure 5.6) for screening of antibacterial compounds from *R. communis* leaves. In EMW solvent system, 40% ethanol in water fraction had high antibacterial compounds against both Gram negative bacteria at the  $R_f$  of 0.48. Some of antibacterial compounds in BEA and CEF solvent systems were not separated from the base of TLC plates.



**Figure 5.6:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram negative bacteria species: *E. coli* (E.C), and *P. aeruginosa* (P.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Acetone (A), and ethanol (E), water (W), 20% acetone (A<sup>20%</sup>), 40% acetone (A<sup>40%</sup>), 60% acetone (A<sup>60%</sup>), 80% acetone (A<sup>80%</sup>), 20% ethanol (E<sup>20%</sup>), 40% ethanol (E<sup>40%</sup>), 60% ethanol (E<sup>60%</sup>), 80% ethanol (E<sup>80%</sup>) in lane from left to right.

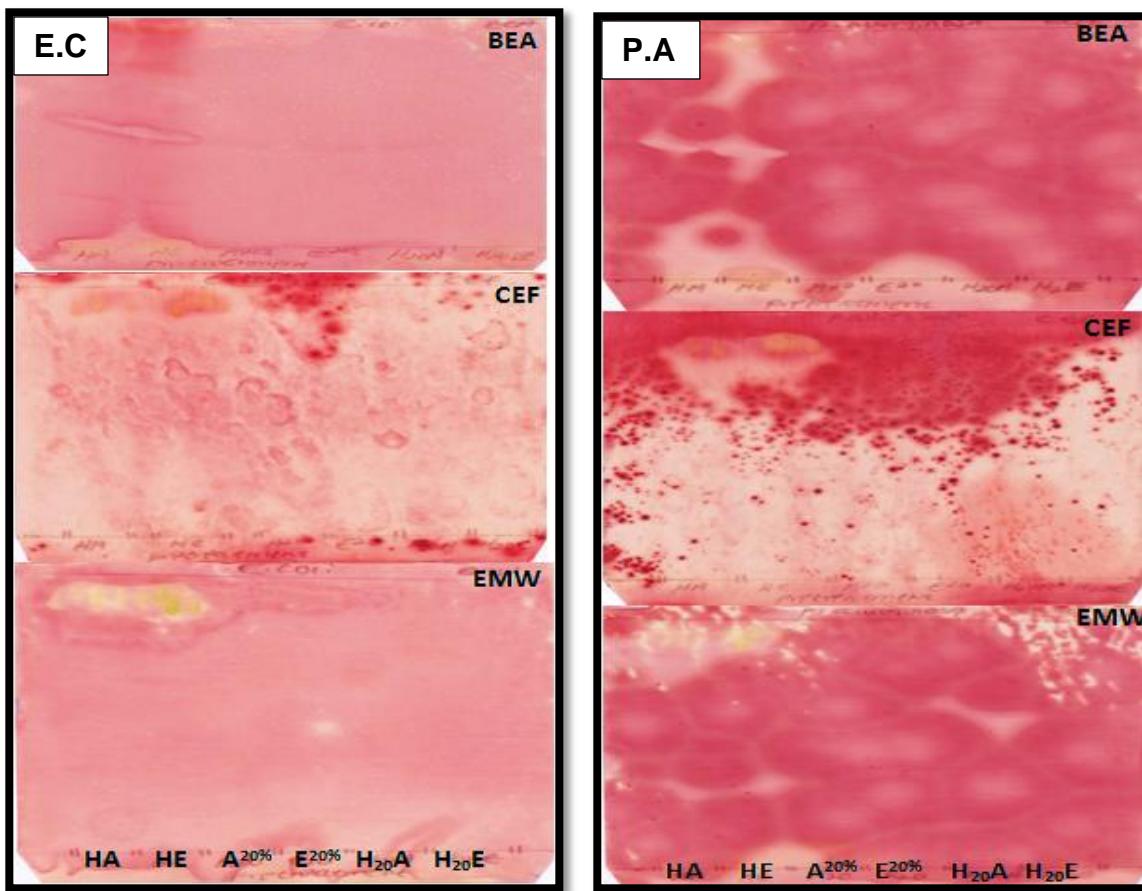
Chromatograms were sprayed with *E. faecalis* and *S. aureus* (Figure 5.7) for screening of antibacterial compounds. BEA was the best solvent system to separate most active compounds against *E. faecalis*. Forty percent ethanol in water fraction ( $R_f$  value of 0.41) had high antibacterial activity against both Gram positive bacteria.



**Figure 5.7:** Bioautograms of *R. communis* leaves extracts separated by three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacterial species: *E. faecalis* (E.F), and *S. aureus* (P.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Acetone (A), and Ethanol (E), Water (W), 20% acetone (A<sup>20%</sup>), 40% acetone (A<sup>40%</sup>), 60% acetone (A<sup>60%</sup>), 80% acetone (A<sup>80%</sup>), 20% ethanol (E<sup>20%</sup>), 40% ethanol (E<sup>40%</sup>), 60% ethanol (E<sup>60%</sup>), and 80% ethanol (E<sup>80%</sup>) in lanes from left to right.

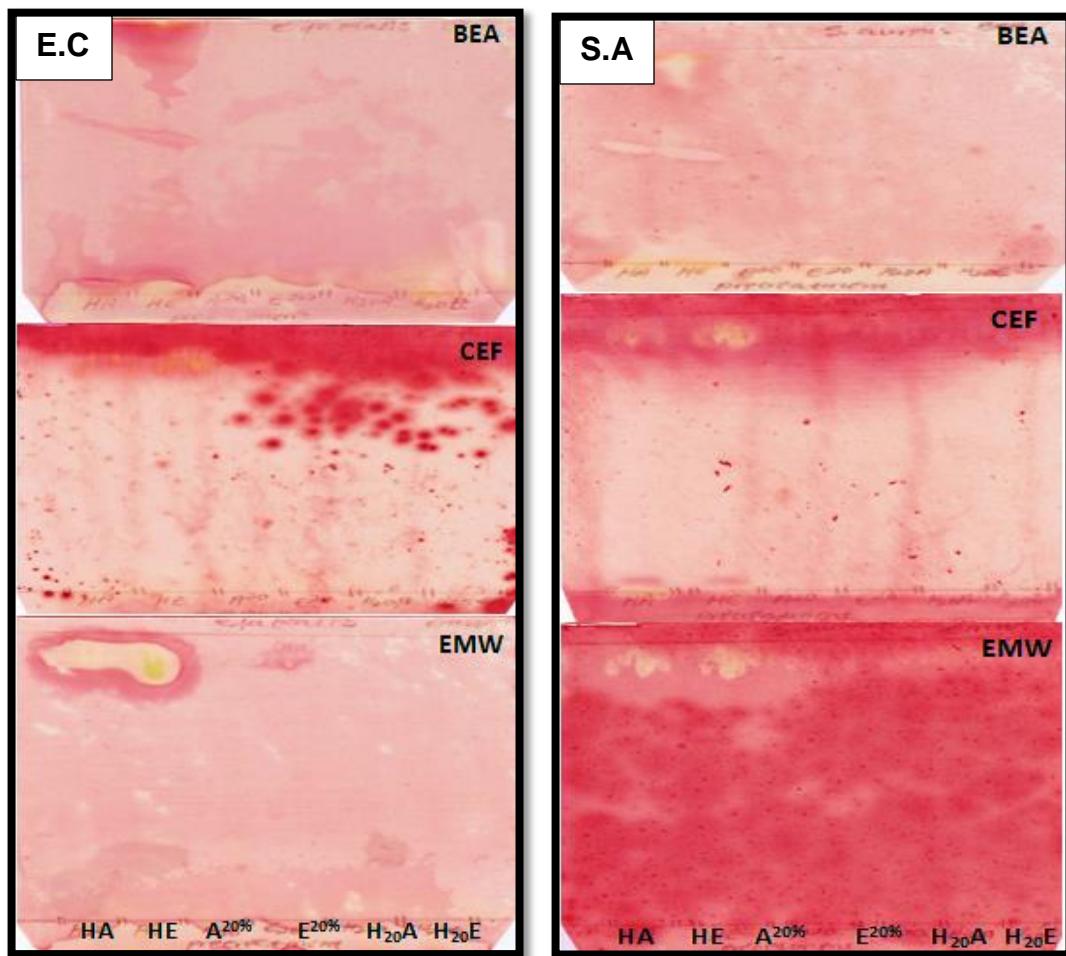
### 5.3.4. Optimal extraction method

Crude extracts of *R. communis* leaves were screened for antibacterial compounds against Gram negative bacteria. BEA solvent system separated active compounds at the  $R_f$  value of 0.5 against *E. coli* and *P. aeruginosa* (Figure 5.8). CEF and EMW solvent system did not have active compounds against tested microorganisms.



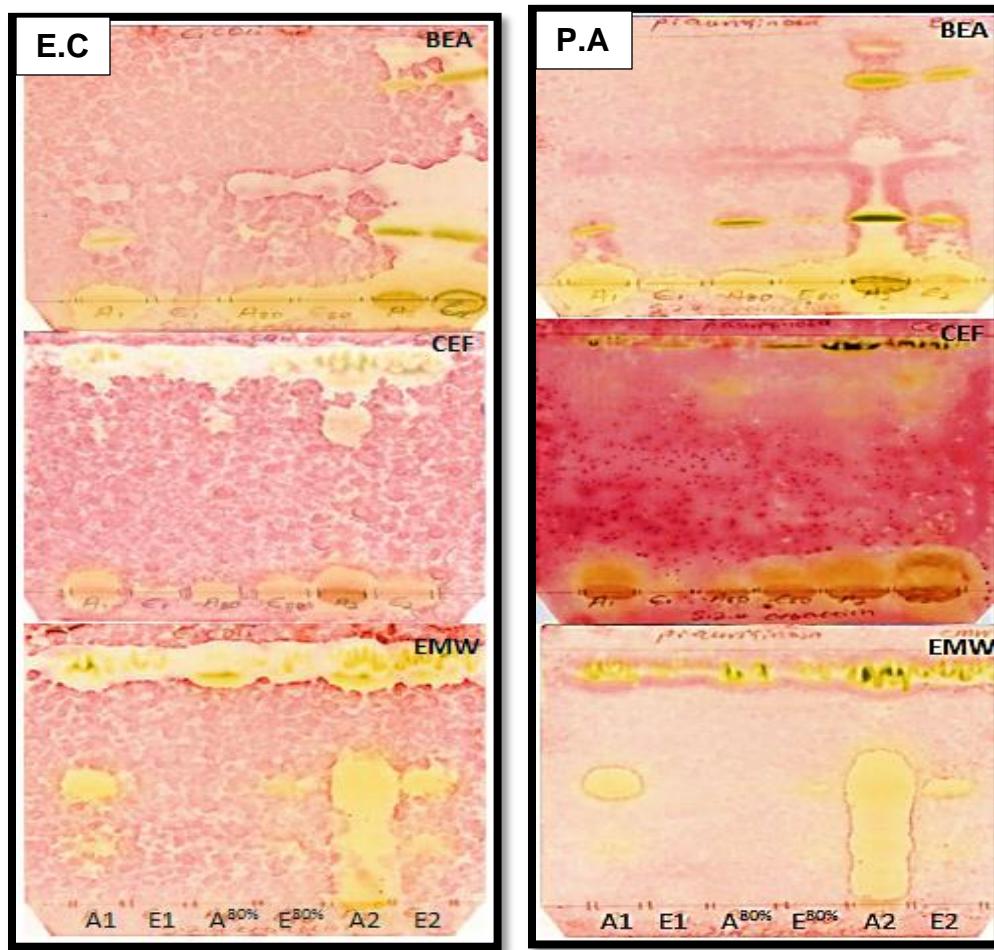
**Figure 5.8:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram negative bacterial species: *E. coli* (**E.C.**), and *P. aeruginosa* (**P.A.**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane wash for acetone (**HA**), hexane wash for ethanol (**HE**), 20% acetone wash (**A<sup>20%</sup>**), 20% ethanol wash (**E<sup>20%</sup>**), hexane wash followed by 20% acetone wash (**H<sub>20</sub>A**), and hexane wash followed by 20% ethanol wash (**H<sub>20</sub>E**) in lane from left to right.

Thin layer chromatography plates were sprayed with *E. faecalis* and *S. aureus* (Figure 5.9). BEA solvent system showed active compounds at the  $R_f$  value of 0.50 against both Gram positive bacteria. Some of the antibacterial compounds could not move from the base of the TLC plates. No active compounds were observed in both CEF and EMW solvent systems.



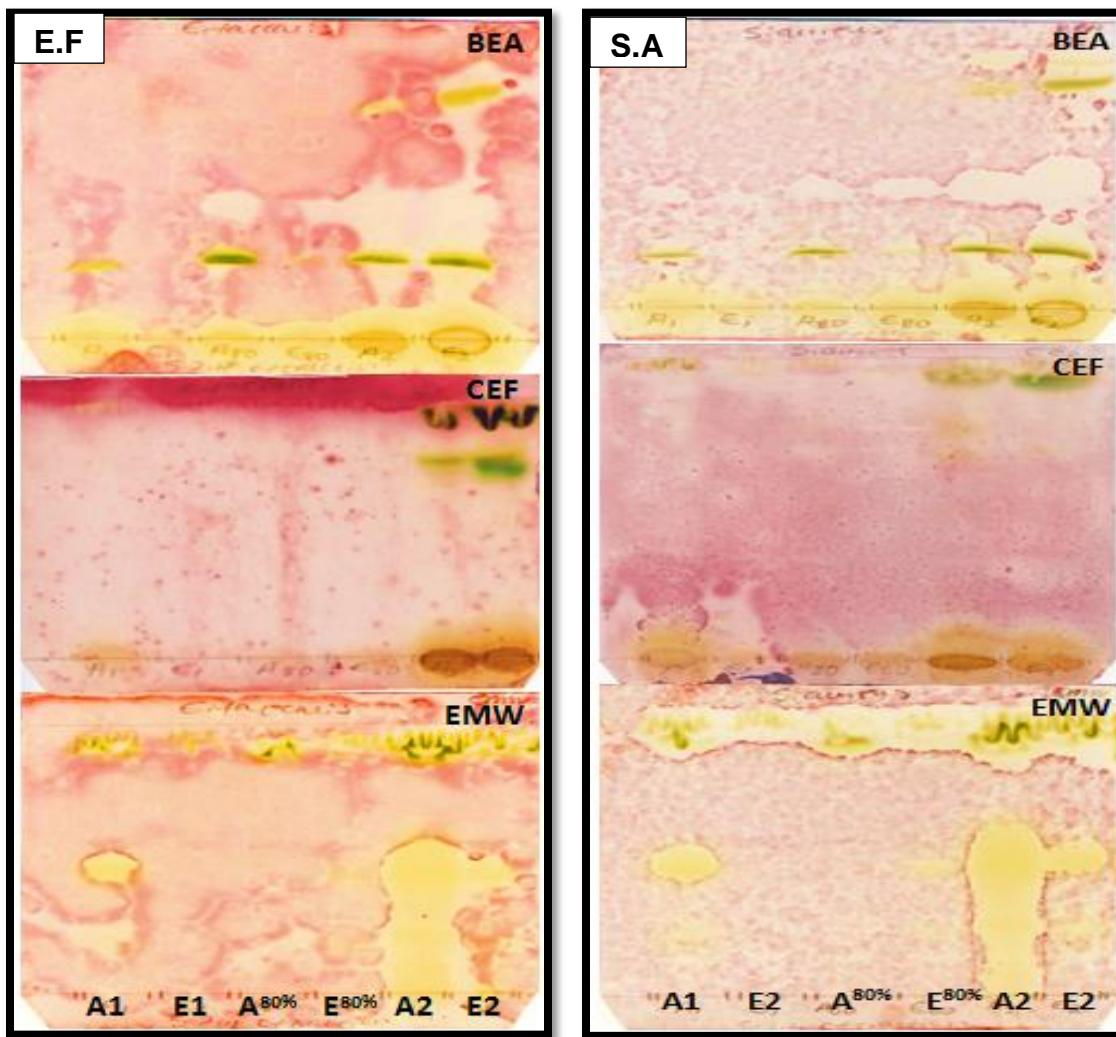
**Figure 5.9:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacterial species: *E. faecalis* (**E.F**), and *S. aureus* (**S.A**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane wash for acetone (**HA**), hexane wash for ethanol (**HE**), 20% acetone wash (**A<sup>20%</sup>**), 20% ethanol wash (**E<sup>20%</sup>**) hexane wash followed by 20% acetone wash (**H<sub>20</sub>A**), and Hexane wash followed by 20% ethanol wash (**H<sub>20</sub>E**) in lanes from left to right.

After pretreatment of plant materials with hexane, extracts were separated with three solvent systems; BEA, CEF and EMW. Chromatograms were sprayed with *E. coli* and *P. aeruginosa* (Figure 5.10) for screening of antibacterial compounds. BEA separated most active compounds in acetone (A2) and ethanol (E2). Acetone (A1), ethanol (E2) and acetone (A2) extracts ( $R_f$  value of 0.45) showed strong antibacterial compounds against both Gram negative bacteria in EMW solvent system.



**Figure 5.10:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram negative bacteria species: *E. coli* (E.C), and *P. aeruginosa* (P.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Acetone (A1), Ethanol (E1), 80% acetone (A<sup>80%</sup>), 80% ethanol (E<sup>80%</sup>), Acetone (A2) and Ethanol (E2) in lanes from left to right.

TLC plates were sprayed with Gram positive bacteria *E. faecalis* and *S. aureus* (Figure 5.11). BEA solvent system also revealed high antibacterial compounds against Gram positive bacteria at  $R_f$  value of 0.45. All four tested microorganisms were inhibited by the same compounds present in acetone and ethanol extracts separated with EMW and BEA solvent systems.



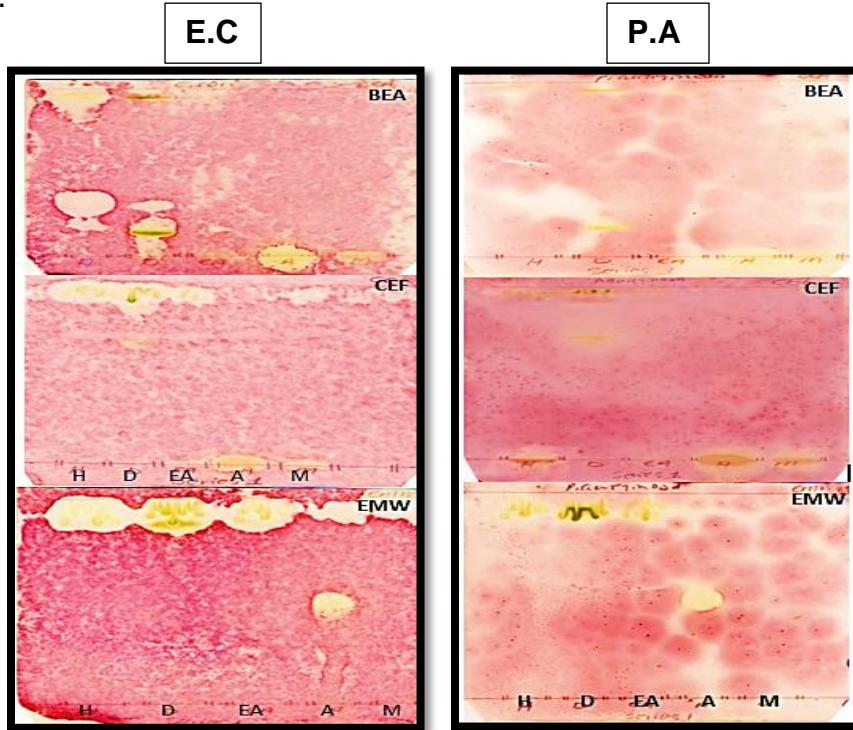
**Figure 5.11:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacteria species: *E. faecalis* (**E.F**), and *S. aureus* (**S.A**), white zones indicate active compounds that inhibited growth of tested bacterial species. Acetone (**A1**), Ethanol (**E1**), 80% acetone (**A<sup>80%</sup>**), 80% ethanol (**E<sup>80%</sup>**), Acetone (**A2**) and Ethanol (**E2**) in lanes from left to right.

### 5.3.5. Serial exhaustive extraction

Three series of extractions were performed using different solvents of varying polarities aiming to find the best series of solvents to use for isolation of antibacterial compounds in large scale isolation.

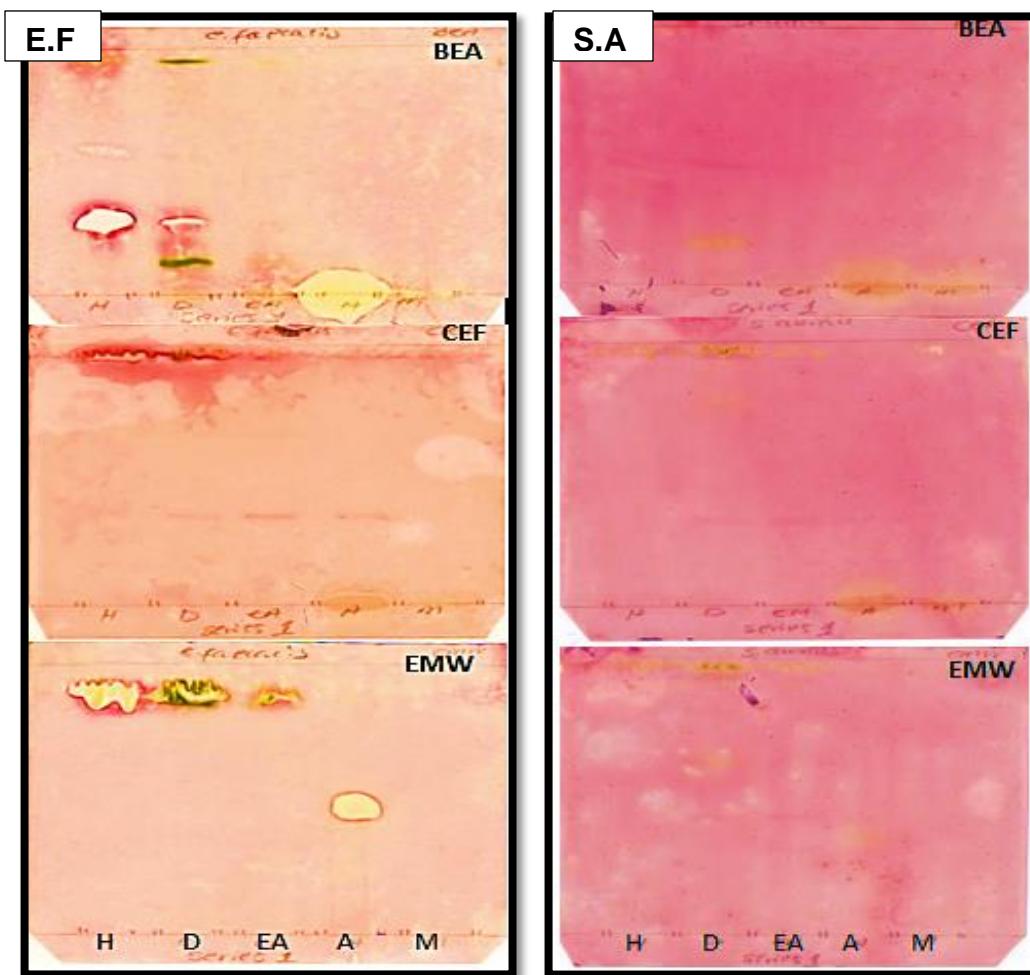
#### 5.3.5.1. Series 1

In series one, BEA solvent system had three antibacterial compounds in hexane and three in dichloromethane extracts against *E. coli*. Acetone extracts had active compounds against both *E. coli*, and *P. aeruginosa* at the  $R_f$  value of 0.47 (Figure 5.12), in EMW solvent system. Some of the active compounds could not move from the base of TLC plates.



**Figure 5.12:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram negative bacteria species: *E. coli* (**E.C.**), and *P. aeruginosa* (**P.A.**), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), dichloromethane (**D**), ethyl acetate (**EA**), acetone (**A**) and methanol (**M**) in lane from left to right.

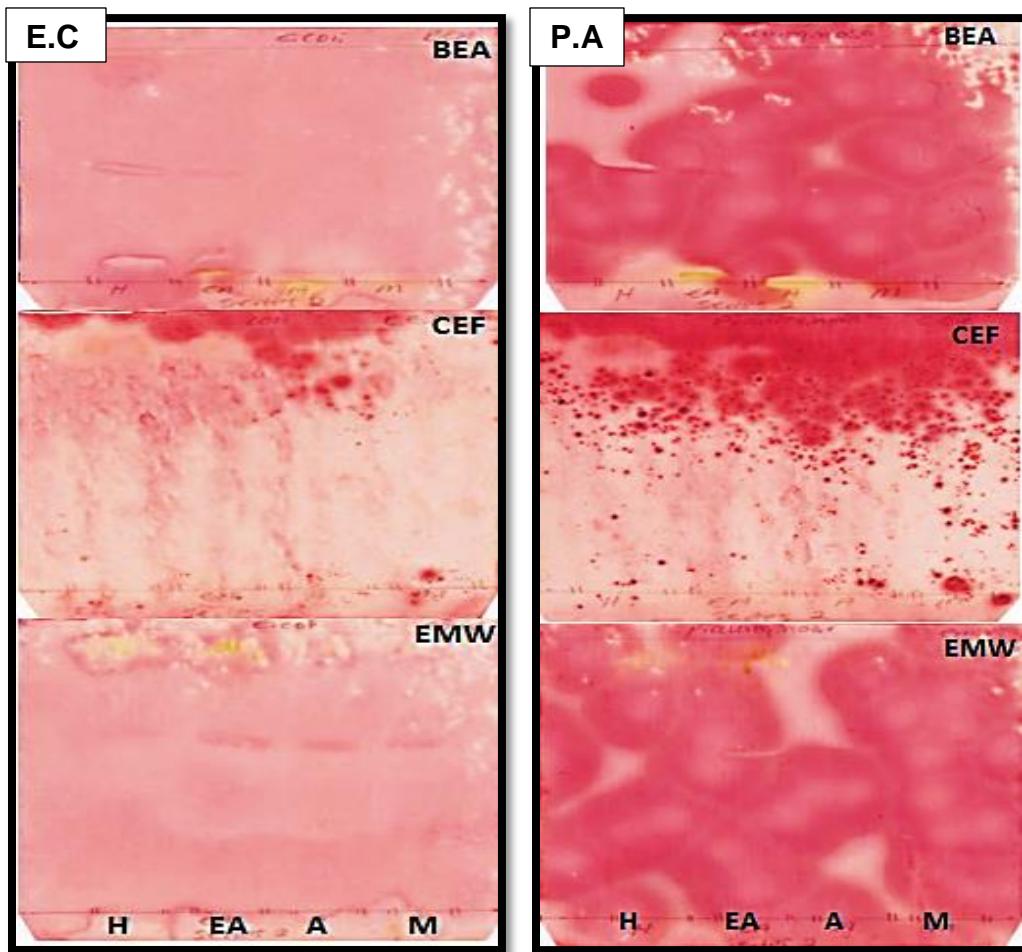
TLC plates developed in BEA solvent system separate antibacterial compounds present in hexane and dichloromethane extracts ( $R_f$  value of 0.29) that inhibited *E. faecalis* growth. *S. aureus* was less sensitive against all the tested crude extracts (Figure 5.13). Some of the active compounds could not move from the base of TLC plates.



**Figure 5.13:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram positive bacteria species: *E. faecalis* (E.F), and *S. aureus* (S.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (H), dichloromethane (D), ethyl acetate (EA), acetone (A) and methanol (M) in lanes from left to right.

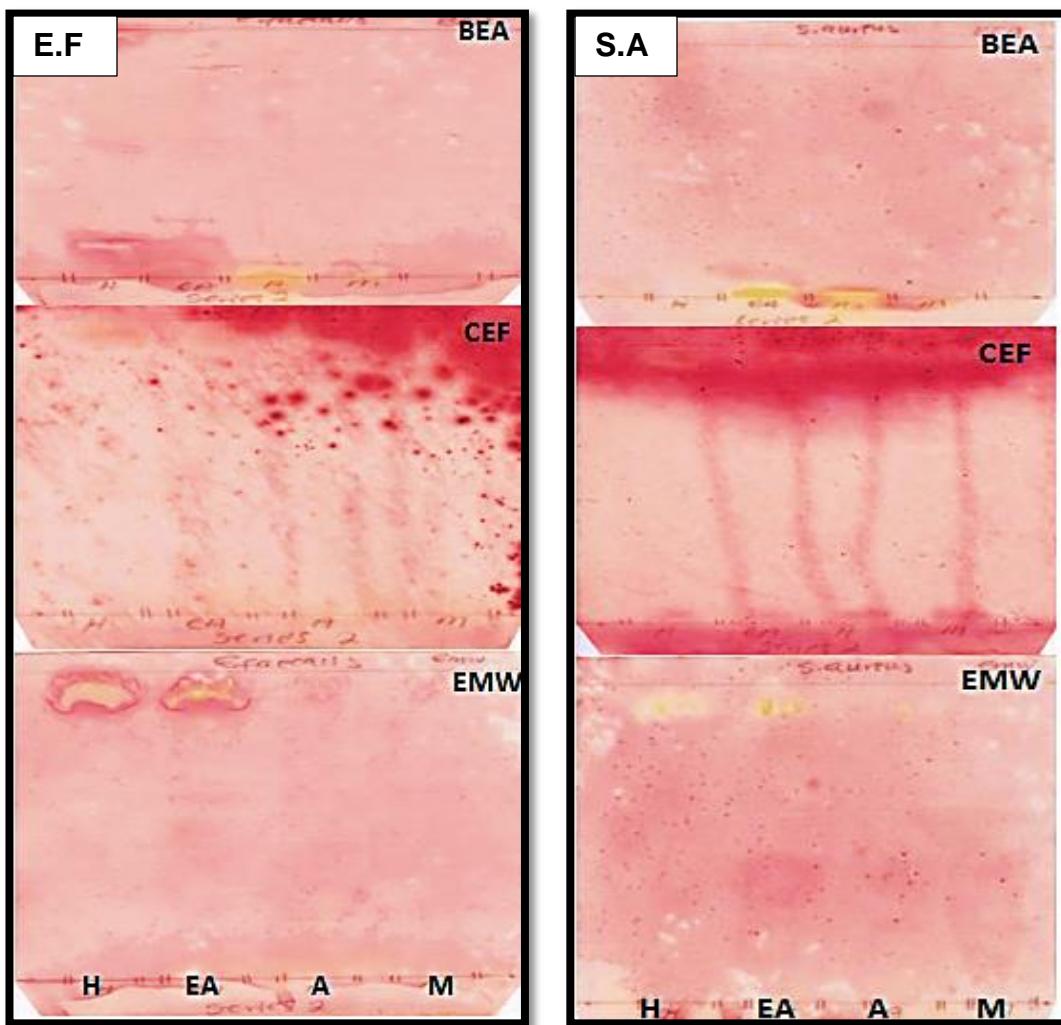
### 5.3.5.2. Series 2

In series two, hexane extracts slightly inhibited both *E. coli* and *P. aeruginosa* (Figure 5.14) in BEA solvent system. Hexane extract showed slight antibacterial activity against tested microorganisms at the  $R_f$  value of 0.47 in BEA solvent system.



**Figure 5.14:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center), EMW (bottom) and sprayed with two Gram negative bacteria species: *E. coli* (E.A), and *P. aeruginosa* (P.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (H), ethyl acetate (EA), acetone (A) and methanol (M) in lanes from left to right.

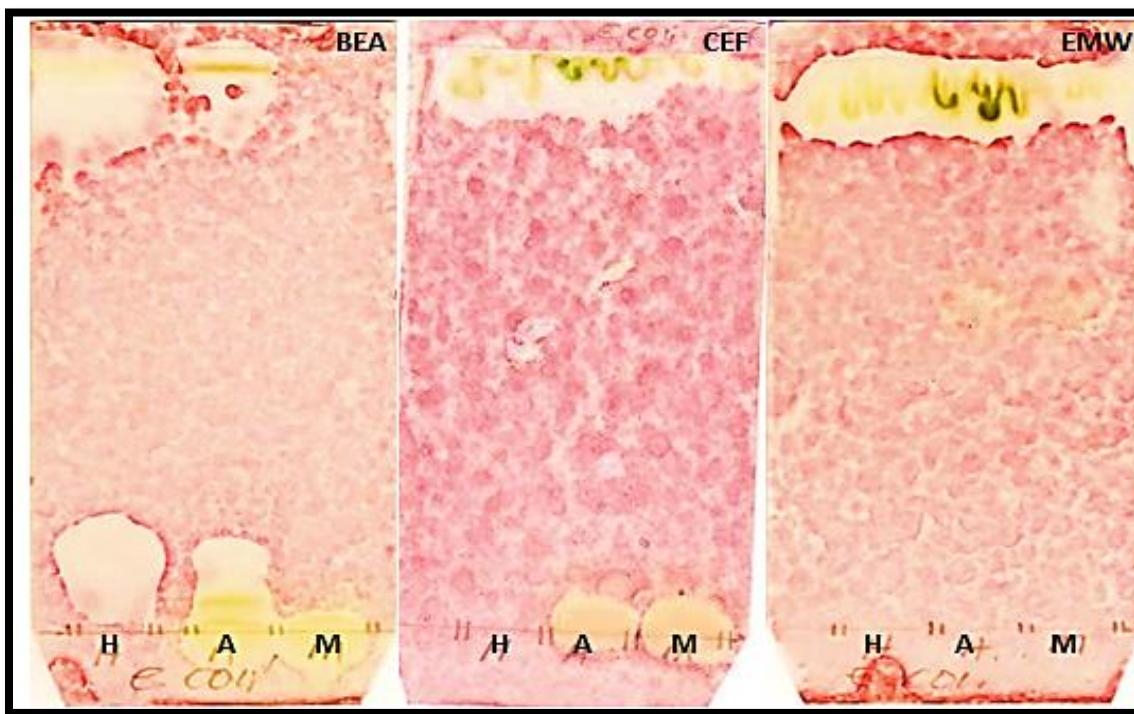
TLC plates eluted in BEA solvent system showed slight antibacterial activity against *E. faecalis*. *S. aureus* was less sensitive to all the crude extracts (Figure 5.15). Some of the active compounds could not move from the base of TLC plates.



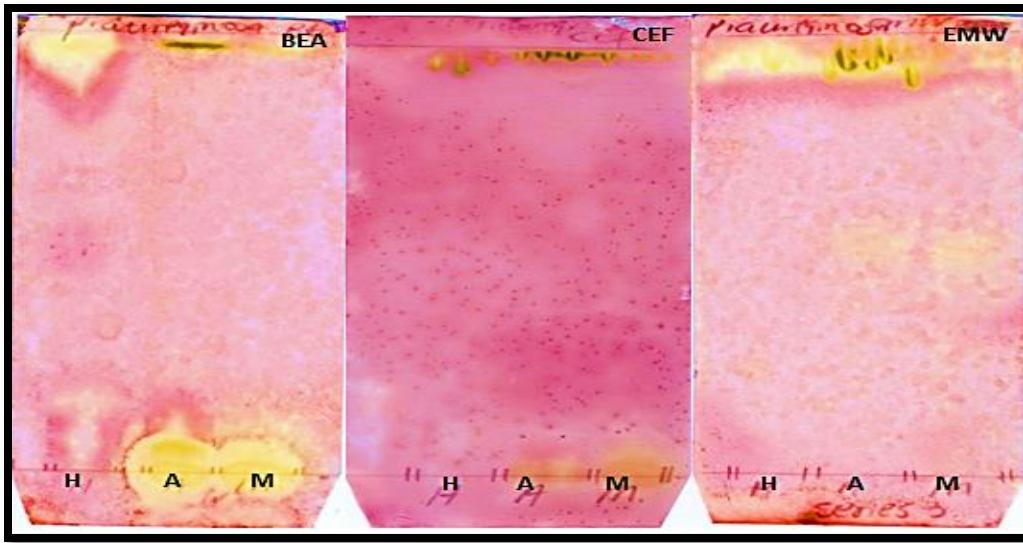
**Figure 5.15:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems; BEA (top), CEF (center) and EMW (bottom) and sprayed with two Gram positive bacterial species: *E. faecalis* (E.F.), and *S. aureus* (S.A), white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (H), ethyl acetate (EA), acetone (A) and methanol (M) in lanes from left to right.

### 5.3.5.3. Series 3

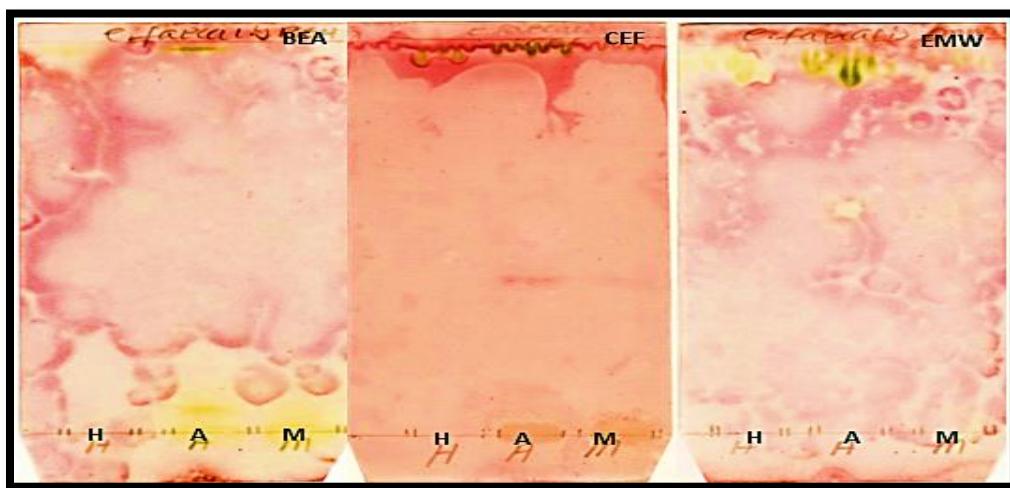
Chromatograms below show the results obtained after screening of antibacterial compounds (Figure 5.16 to 5.19). Hexane and acetone extracts ( $R_f$  values of 0.11) had high antibacterial activity against *E. coli* (Figure 5.16) and *S. aureus* (Figure 5.19). *P. aeruginosa* was less sensitive to all crude extracts (Figure 5.17). BEA solvent system showed high active compounds against *E. faecalis* in all crude extracts (Figure 5.18).



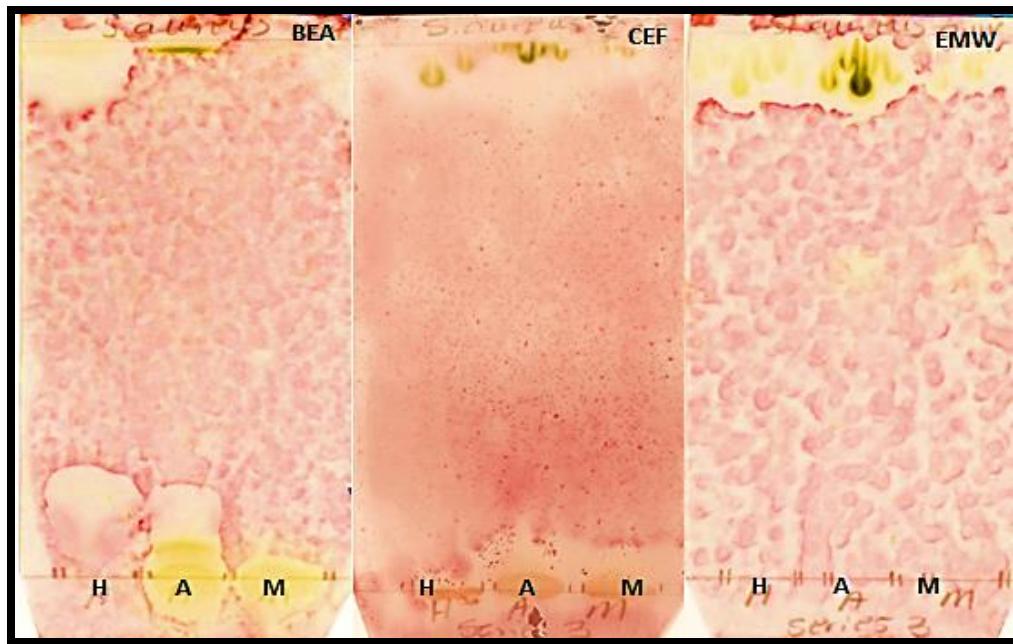
**Figure 5.16:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (left), CEF (center), EMW (right) and sprayed with *E. coli*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (H), acetone (A) and methanol (M) in lane from left to right.



**Figure 5.17:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (left), CEF (center), EMW (right) and sprayed with *P. aeruginosa*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), acetone (**A**) and methanol (**M**) in lane from left to right.



**Figure 5.18:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (left), CEF (center), EMW (right) and sprayed with *E. faecalis*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), acetone (**A**) and methanol (**M**) in lanes from left to right.



**Figure 5.19:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (left), CEF (center) and EMW (right) and sprayed with *S. aureus*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane (**H**), acetone (**A**) and methanol (**M**) in lane from left to right.

## **5.4. DISCUSSION**

Qualitative antibacterial assay on TLC plates was done to revealed and determined number of antibacterial compounds extracted from *R. communis* leaves in all the various extraction methods used in Chapter 3. White spots against pink background on the bioautograms indicate areas of inhibition of bacterial growth by active compounds present in plant extracts (Begue and Kline, 1972). The disadvantage of bioautography is that coloured compounds may mask or hide zone of inhibition of the tested microorganisms. The situation mostly occurs in polar compounds with yellow, green or brown colours on the bioautograms (Sasidharan *et al.*, 2011). There are possibilities that such compounds may inhibit growth of tested microorganisms, however the colour masked zone of inhibition.

The leaves of *R. communis* showed good minimum inhibitory concentration values against all tested microorganisms that range from 0.13 to 1.05 mg/ml (Table 5.1). Ethanol and methanol extracts of *R. communis* leaves have been reported for strong antimicrobial activity against *S. aureus* and *E. coli* (Kumar *et al.*, 2013a). Dichloromethane extracts exhibited high antibacterial activity against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* (Figure 5.1). Antibacterial compounds had  $R_f$  values ranged from 0.29 to 0.88. *E. faecalis* was less sensitive to all crude extracts (results are not shown). Antibacterial activity of *R. communis* leaves extracts may be due to the presence of secondary metabolites such as flavonoids and terpenoids. Polyphenols, flavonoids, triterpenoids and other compounds of phenolic nature or free hydroxyl group have been reported to play an important role in antibacterial activity of medicinal plants (Fawole *et al.*, 2013). The presence of alkaloids signified possession of antimicrobial activity (Obumselu *et al.*, 2013). Therefore, phenolic compounds, alkaloids and other compounds that are responsible for antibacterial compounds of *R. communis* leaves may be classified as active antibacterial compounds.

Extraction of antibacterial compounds from traditional medicinal plants is based on the type of extractant used in the extraction process. Fawole *et al.*, 2013 reported that ethanol, methanol and water are the most common solvents used for extraction of antibacterial compounds from medicinal plants. In the present study more than seven

solvents were used including acetone, hexane, ethyl acetate and dichloromethane to extract antibacterial compounds from *R. communis* leaves. Additionally, methanol and ethanol solvents were reported to extract potent antibacterial compounds by Fawole et al., 2013. In the present study, acetone extracted potent antibacterial compounds against *E. coli*, *P. aeruginosa* (Figure 5.2) and *E. faecalis*. *S. aureus* was less sensitive to all *R. communis* extracts (Figure 5.3). The active antimicrobial compounds in acetone extract also showed strong free radical scavenging activity in Chapter 4 (Figure 4.2), it possesses both antibacterial and antioxidant activity. Kumar et al. (2013a) reported that acetone extracts had a maximum zone of inhibition against *S. aureus* and *E. coli* using disc diffusion methods. They also suggested that the leaves of *R. communis* are possible source of antibacterial compounds. Methanol and ethyl acetate extracts of *Ricinus communis* leaves were found to be substantially active against *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis* (Kumar et al., 2013a). In the present study acetone extracts of *R. communis* leaves was significantly active against *E. coli*, *P. aeruginosa* and *E. faecalis*. White zone appeared on the chromatogram developed in CEF (Figure 5.2) and sprayed with *E. coli* was due to the traces of formic acid left on the chromatogram or toxicity of formic acid used in solvent system because formic acid is toxic to bacteria (Masoko and Eloff, 2005; Masoko et al., 2008).

Even though there is no standard method for extraction of bioactive compounds, traditional doctors use boiling to extract bioactive compounds from medicinal plants. However, it is very important to use appropriate extraction procedure to exhaustively extract active compounds from plant materials. The study showed hexane wash as the best extraction procedure that had extracted largest number of potent antibacterial compounds against *E. coli*, *P. aeruginosa* (Figure 5.4), *E. faecalis* and *S. aureus* were less sensitive (Figure 5.5) to *R. communis* leaves extracts. The main purpose of washing plant materials with hexane was to remove all fatty acids that result in exposing more antibacterial compounds present in *R. communis* leaves (Sasidharan et al., 2011). The leaves of *R. communis* were further extracted with different percentages of acetone and ethanol in water aiming to extract greater number of antibacterial compounds. Twenty percent ethanol extracts in water possessed potent antibacterial compounds against both Gram negative (Figure 5.6) and Gram positive bacteria (Figure 5.7). In

optimal extraction, *R. communis* leaves were pretreated with hexane, hexane extracts showed high antibacterial activity against *E. coli*, *P. aeruginosa* (Figure 5.8) and Gram positive (Figure 5.9) bacteria in BEA solvent system. In most cases hexane is known to extract chlorophylls and fatty acids which are not biologically active, however in the present findings hexane extracts revealed high antibacterial activity against tested pathogens from *R. communis* leaves. Hexane extracts seems to have promising compounds with antibacterial activity which may be isolated and used as precursor for manufacturing new antibiotics to fight against resistance strains. Iqbal *et al.* (2012) also reported about the potent antibacterial activity of hexane extracts of *R. communis* leaves. However, after extracting pretreated plant materials of *R. communis* leaves, results revealed strong antibacterial compounds against four tested microorganisms (Figure 5.10 – 5.11). The study showed the importance of pretreating plant materials with hexane before exhaustive extraction of active compounds that result in removing some of compounds that hinder the activities of other compounds (antagonist effects). Hence large numbers of antibacterial compounds were exposed and extracted from *R. communis* leaves after pretreating process. It can be concluded that *R. communis* leaves possessed large number of antibacterial compounds. Obumselu *et al.* (2013) also reported *R. communis* leaves as a good source of potent antibacterial agent to fight against resistance strains.

Isolation and purification of bioactive compounds from plant materials is a challenge because of large number of active and non-active compounds present. Separation of compounds is highly dependent of the polarity of the solvent system and compounds (Visht and Chaturvedi, 2012). Three series of extractions were performed using solvents of varying polarities aiming to select the best series of solvents for extraction of antibacterial compounds in large scale isolation. Hexane, dichloromethane and acetone extracts showed slight antibacterial activity against *E. coli*, *P. aeruginosa* and *E. faecalis* (Figure 5.12 – 5.15). Hexane and methanol extracts of *R. communis* leaves were also reported for a maximum antimicrobial activity against *E. coli* and *P. aeruginosa* (Jena and Gupta, 2012). Hexane, dichloromethane, acetone and methanol were selected as the best solvents to use for isolation of active compounds in large scale isolation (Chapter 6).

## **5.5. CONCLUSION**

The study showed that the leaves of *R. communis* possessed strong antibacterial compounds against all four tested microorganisms. Hexane wash was the best extraction method to extract most of active compounds. Hexane extracts was the potential extract to isolate antibacterial compounds against tested pathogens. Isolation and purification of active antibacterial compounds from hexane extracts (*R. communis* leaves) is required. *E. coli*, *P. aeruginosa* and *S. aureus* were highly sensitive while *E. faecalis* was slightly resistant to plants extracts. The study serves as a scientific proof for use of this plant in traditional medicine.

## CHAPTER 6

### ISOLATION AND PURIFICATION OF ANTIOXIDANT AND ANTIBACTERIAL COMPOUNDS

#### **6.1. INTRODUCTION**

Traditional medicinal plants are capable of synthesizing bioactive compounds which are beneficial to humankind. Isolation and purification of these compounds is a challenge because of a large number of phytochemical compounds usually present in plants (Sasidharan *et al.*, 2011). This chapter mainly focused on the extraction, isolation and purification of bioactive compounds using column chromatography, preparative thin layer chromatography and thin layer chromatography plates.

Extraction is the most important step in medicinal plant researches, where phytochemical compounds of interest are extracted and analyzed for phytochemical screening assays and other biological activities assays. Extraction involves separation of active parts from inactive parts of the plant using solvents of varying polarities. Serial exhaustive extraction plays an important role in isolation and purification of compounds. It involves the use of solvents from a non-polar solvent to highly polar solvent to ensure exhaustively extraction of different types of compounds present (Tiwari *et al.*, 2011). Solvents of various polarities extract compounds of the same polarity relative to the solvent used in extraction process. Methanol, ethanol and ethyl acetate are polar solvents which are used for extraction of hydrophilic compounds, dichloromethane for lipophilic compounds and hexane for chlorophyll and fatty acids (Sasidharan *et al.*, 2011). Acetone dissolves many of hydrophilic and lipophilic compounds. Solvents used for extraction of bioactive compounds should be nontoxic, inert, easy to remove and not easily flammable (Tiwari *et al.*, 2011). There are several factors that affect rate of extraction such as quantity, nature of target compound, volume and nature of solvents, temperature and polarity of solvents (Visht and Chaturvedi, 2012).

Thin layer chromatography, column chromatography and preparative thin layer chromatography plates are techniques used for separation of phytochemical

compounds. TLC is used as the first step to analyze the fingerprint of the plant extracts. TLC is a fast and inexpensive method that displays a number of compounds present in the plant extracts and distance travelled by the compounds on the plates. An important role of TLC in the isolation and purification process of bioactive compounds is to confirm purity of isolated compound (Sasidharan *et al.*, 2011).

Column chromatography is the second step used for separation of phytochemical compounds using one or two solvents in various ratios for further purification. Based on the number of compounds present, preparative thin layer plates can also be used to further purify targeted compounds. The final step involves the use of thin layer chromatography to confirm purity of isolated compound (Visht and Chaturvedi, 2012). The aim was to isolate bioactive compounds with antioxidant and antibacterial activity using column chromatography.

## **6.2. METHODS AND MATERIALS**

### **6.2.1. Serial exhaustive extraction**

Serial exhaustive extraction was used to extract *R. communis* leaves by weighing 1.125 kg of plant materials and dissolved into 5 litres of *n*-hexane in a bottle. The bottle was vigorously shaken for a day at high speed of 200 rpm. The supernatant was filtered, concentrated using rotary evaporator at 50°C and transferred into pre-weighed labelled bottle. Same process was done three times and supernatants were combined. The same plant residues were extracted in the following order with 5 litres of dichloromethane, acetone and methanol. Solvents were removed under a stream of cold air at room temperature.

### **6.2.2. Phytochemical analysis**

The chemical profile of crude extracts was analysed using aluminium-backed thin layer chromatography plates (Fluka, silica gel F<sub>254</sub>) as described in Section 3.2.3.

### **6.2.3. TLC-DPPH assay**

Qualitative DPPH assay described by Deby and Margotteaux (1970) was done using thin layer chromatography as explained in Section 4.2.1.

### **6.2.4. Bioautography**

Bioautography was done according to Begue and Kline (1972) as described in Section 5.2.3., and bacterial species were maintained in a same manner as in Section 5.2.1.

### **6.2.5. Microbroth dilution method**

Microbroth dilution method described by Eloff (1998) was used to determine minimum inhibitory concentration (MIC) values of crude extracts against four tested bacterial species as explained in Section 5.2.2.

### **6.2.6. Isolation of antioxidant and antibacterial compounds**

#### *6.2.6.1. Open column chromatography*

Column chromatography was used to isolate, separate and purify active compounds with high antioxidant and antibacterial activities from the crude extracts. Open column (40 x 6 cm) was packed with silica gel 60 (particles size 0.063-0.200 mm) (Fluka) using 100% hexane. Crude extracts were dried using rotary evaporator at 50°C and total mass of extracts was determined. Samples were mixed with small amount of silica gel and then subjected to column chromatography. Constituents of crude extracts were eluted through opened column chromatography using solvents in Table 6.1, increasing polarity from non-polar solvent (hexane) to polar solvents (methanol). Collected fractions were further tested for antioxidant using TLC-DPPH assay (Section 6.2.3) and antibacterial compounds using bioautography (Section 6.2.4).

**Table 6.1:** Different percentages of solvents used for elution of open column chromatography.

Elution solvent	Percentages (%)
Hexane	100
Hexane: Ethyl acetate	90:10
	80:20
	70:30
	50:50
	30:70
	10:90
Ethyl acetate	100
Ethyl acetate: Methanol	90:10
	80:20
	70:30
	60:40
	50:50
	40:60
	10:90
Methanol	100

#### 6.2.6.2. *Small column chromatography*

Fractions of above Section 6.2.6.1 with active compounds were combined to make one target fraction. The target fraction was subjected in a small open column chromatography (37 x 3 cm) packed with silica gel 60 for further fractionation. Column was eluted with 20% methanol in chloroform for antioxidant compounds and 90% dichloromethane in ethyl acetate for antibacterial compounds. The eluents were collected in small test tubes and placed under a stream of air to concentrate the fractions for further analysis on the thin layer chromatography plates.

### **6.2.7. Preparative thin layer chromatography**

Active fractions were further separated on TLC silica gel glass plates (Merck Silica gel 60 F<sub>254</sub>) using 20% methanol in chloroform for antioxidant compounds and 90% dichloromethane in ethyl acetate for antibacterial compounds. Plates were visualized under ultraviolet light (254 and 360 nm) to locate targeted bands on the TLC plates. A small part on the side of the plate was sprayed with vanillin-sulphuric acid reagent while the rest of the plates were covered with an aluminium foil. Visualised bands on the side were used as referenced line for scraping off active bands from developed TLC plates. The active compounds in the solvent were filtered and separated compounds from silica gel. For complete removal of silica, filtrates were passed through cotton wool packed in a glass Pasteur pipette. Purity of isolated compounds was confirmed by spraying TLC with vanillin-sulphuric acid and heated 110°C until colour developed.

## 6.3. RESULTS

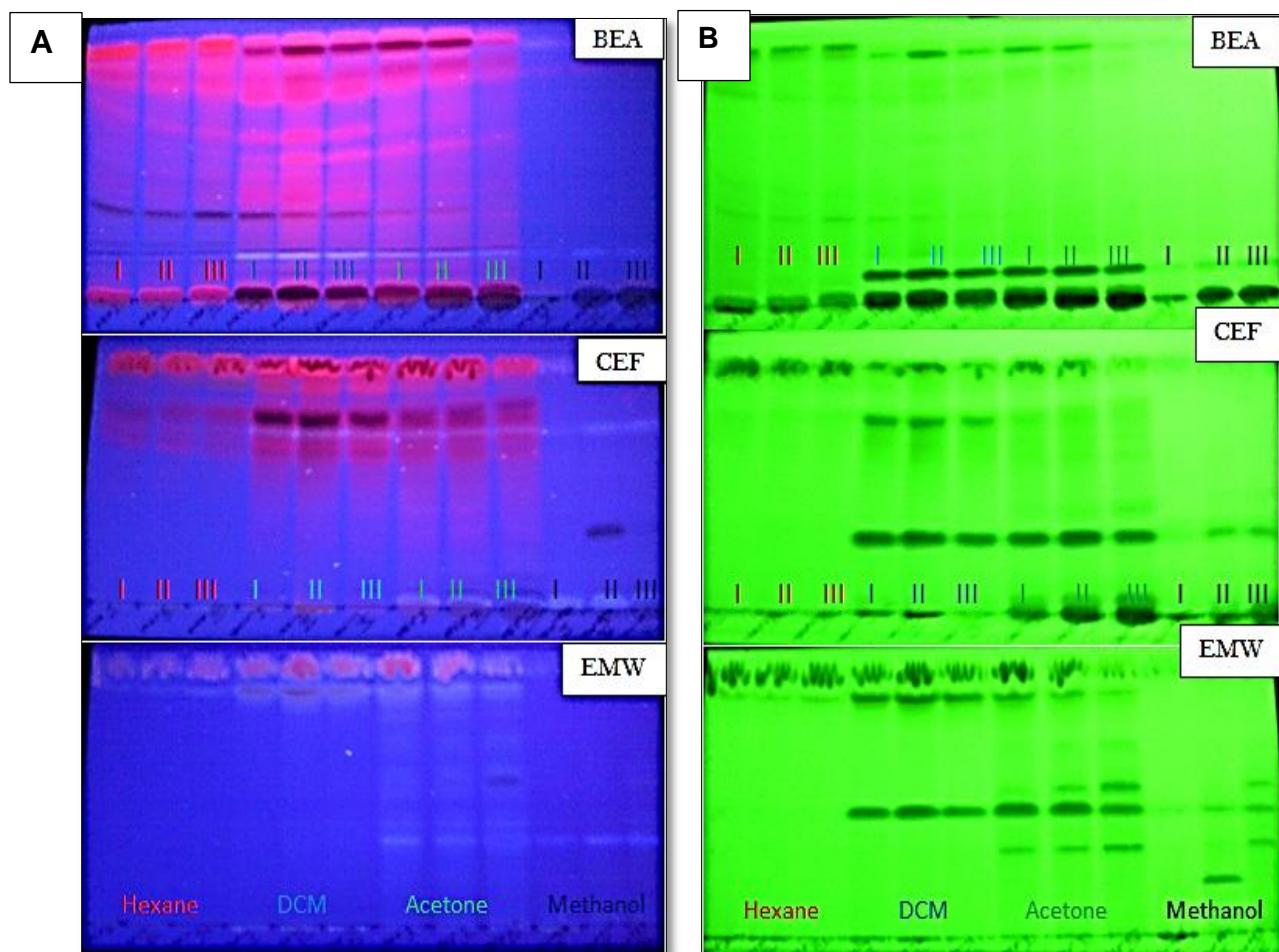
### 6.3.1. Serial exhaustive extraction

Finely ground, dried leaves of *R. communis* were serially extracted with hexane, dichloromethane, acetone and methanol. The total mass of 228.88 g was obtained from 1.125 kg of plant materials (Table 6.2). Methanol was the best extractant with the highest mass of extracts (123.45 g), followed by hexane (53.54 g), dichloromethane (32.28) and the lowest mass of 19.61 g was in acetone extract.

**Table 6.2:** The mass of *R. communis* extracts in grams extracted from 1.125 kg by four solvents with varying polarity.

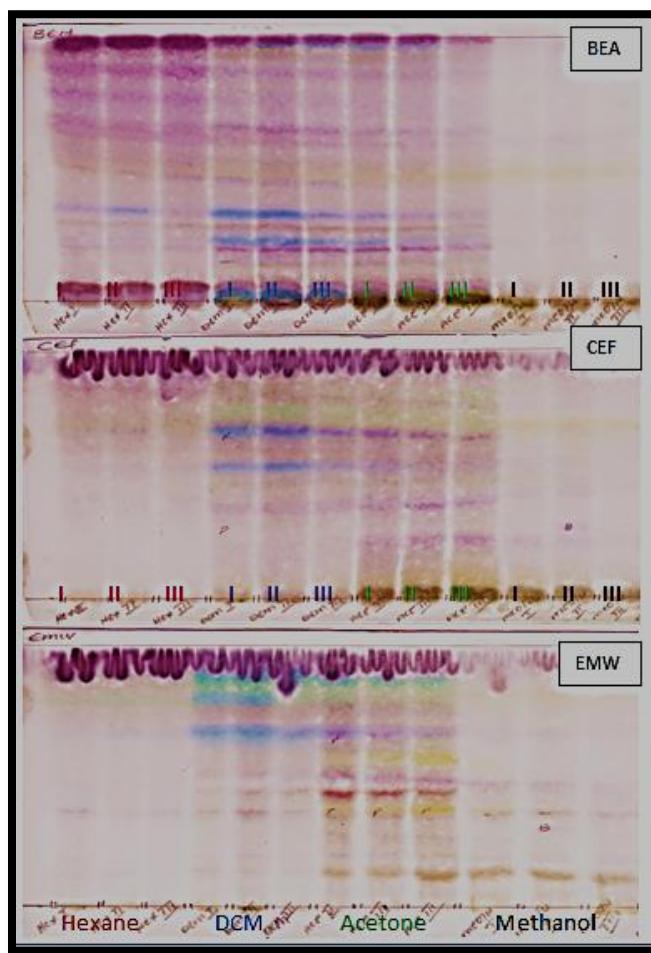
Extractants		Mass residue extracted (g)	
		Mass	Total
Hexane	I	30.01	53.54
	II	15.88	
	III	7.65	
Dichloromethane	I	15.65	32.28
	II	8.94	
	III	7.69	
Acetone	I	9.67	19.61
	II	5.46	
	III	4.48	
Methanol	I	63.93	123.45
	II	34.45	
	III	25.07	
Total			228.88

Crude extracts of *R. communis* leaves were separated with three solvent systems of varying polarities EMW, CEF and BEA. Chromatograms were visualized under ultraviolet light (254 and 365 nm) for the TLC fingerprint of the plant (Figure 6.1). TLC plates developed in BEA solvent system showed a greater number of compounds reacted with UV light 365 nm, followed by CEF and EMW. Phytochemical compounds appeared in red colour under 365 nm and black under 254 nm UV light.



**Figure 6.1:** Thin layer chromatography of *R. communis* leaves extracts separated with three solvent systems; BEA, CEF, EMW from top to bottom and visualized under ultraviolet light 365 (A) and 254 nm (B).

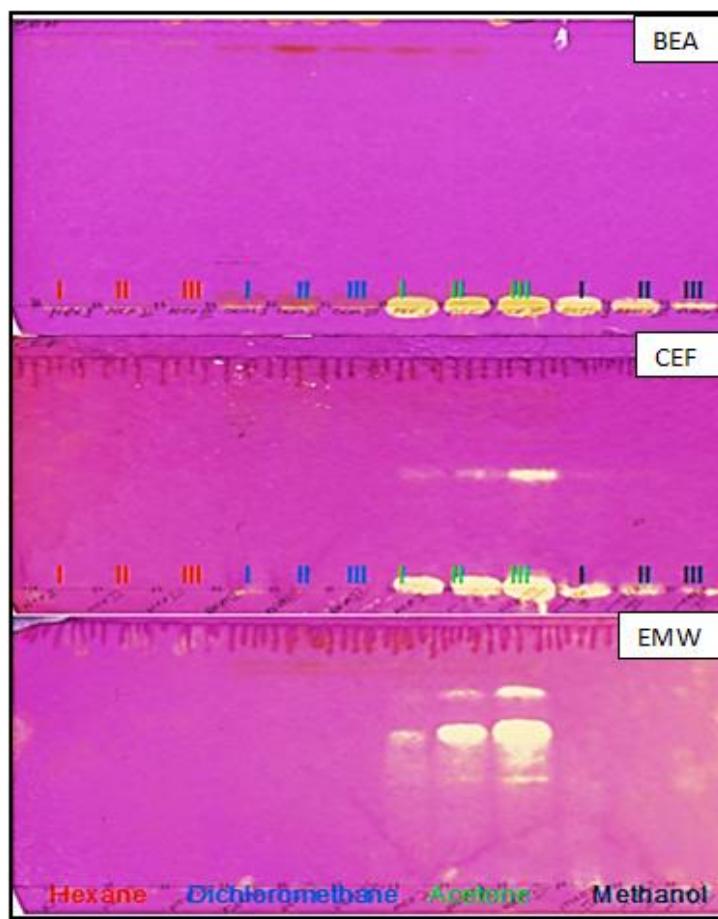
Thin layer chromatography plates were used for spectral profile of hexane, dichloromethane, acetone and methanol extracts of *R. communis* leaves. Crude extracts were separated with BEA, CEF and EMW solvent system then sprayed with vanillin-sulphuric acid reagent for visualization of compounds (Figure 6.2). TLC plate developed in BEA solvent system had good resolution of phytochemical compounds present in *R. communis* leaves, followed by CEF and the least were in EMW. Different colours appeared on the TLC plates confirmed the bioactive compounds of *R. communis* extracts reacted with vanillin-sulphuric acids.



**Figure 6.2:** Chromatograms of *R. communis* leaves extracted hexane, dichloromethane (**DCM**), acetone, and methanol, in lane from left to right, separated with three solvent systems; BEA, CEF, EMW from top to bottom and sprayed with vanillin-sulphuric acid reagent.

### 6.3.2. Antioxidant activity

Hexane, dichloromethane, acetone and methanol extracts were separated with three solvent systems and then sprayed with 0.2% DPPH in methanol as an indicator for free radical scavenging activity. Yellow spots against purple background appeared immediately on the TLC plates after spraying plates with 0.2% DPPH indicate the presence of antioxidant activity. Acetone extracts revealed strong free radical scavenging activity in EMW solvent systems (Figure 6.3). Hexane, DCM and methanol extracts did not exhibit antioxidant activity.



**Figure 6.3:** Chromatograms of *R. communis* crude extracted with hexane, dichloromethane, acetone, and methanol (from left to right), developed in three solvents systems: BEA, CEF, EMW from top to bottom and sprayed with 0.2% DPPH in methanol as an indicator, yellow spots against purple background indicate antioxidant activity exhibited by compounds.

### 6.3.3. Isolation of antioxidant compounds from acetone extract

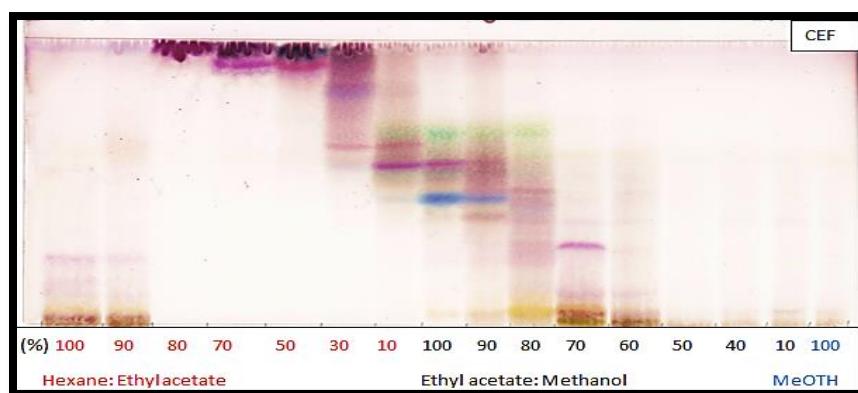
#### 6.3.3.1. Open column chromatography

The column chromatography was eluted with varying percentages of solvents listed (Table 6.3) and mass for each fraction was also recorded. Sixty percent of ethyl acetate in methanol extract had the highest mass of 1.805 g and the lowest mass of 0.015 g was in 90% hexane in ethyl acetate extract. Acetone fractions had the total mass of 20.204 g and hexane (40.253 g).

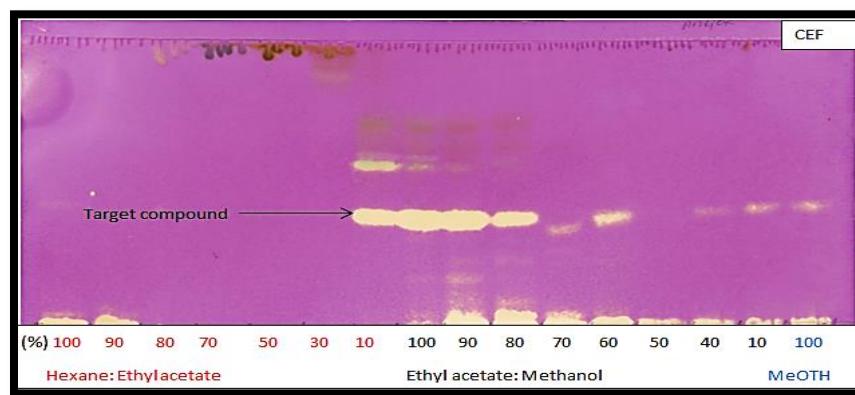
**Table 6.3:** The mass (g) of *R. communis* acetone and hexane extracts fractions collected from column chromatography with different elution solvents.

Elution solvent	Percentages (%)	Mass (g)	
		Acetone	Hexane
Hexane	100	0.364	-
Hexane: Ethyl acetate	90	0.015	16.312
	80	0.882	10.694
	70	0.628	3.528
	50	0.751	2.471
	30	0.519	3.610
	10	0.863	0.806
Ethyl acetate	100	0.422	0.480
Ethyl acetate: Methanol	90	0.176	0.118
	80	6.987	0.891
	70	5.405	0.602
	60	1.805	0.401
	50	0.537	0.027
	40	0.315	0.092
	10	0.280	-
Methanol	100	0.255	0.221
Total		20.204	40.253

CEF solvent system showed a greater separation of all the fractions collected from elution of *R. communis* acetone extracts with different elution systems. Fractions were sprayed with vanillin-sulphuric acid reagent for phytochemical analysis (Figure 6.4). To locate the active antioxidant compounds present in the fractions of *R. communis* acetone extracts, chromatogram was sprayed with 0.2% DPPH in methanol as an indicator (Figure 6.5), yellow spots indicate antioxidant activity of *R. communis* acetone extracts. Active compounds were located at the  $R_f$  value of 0.34

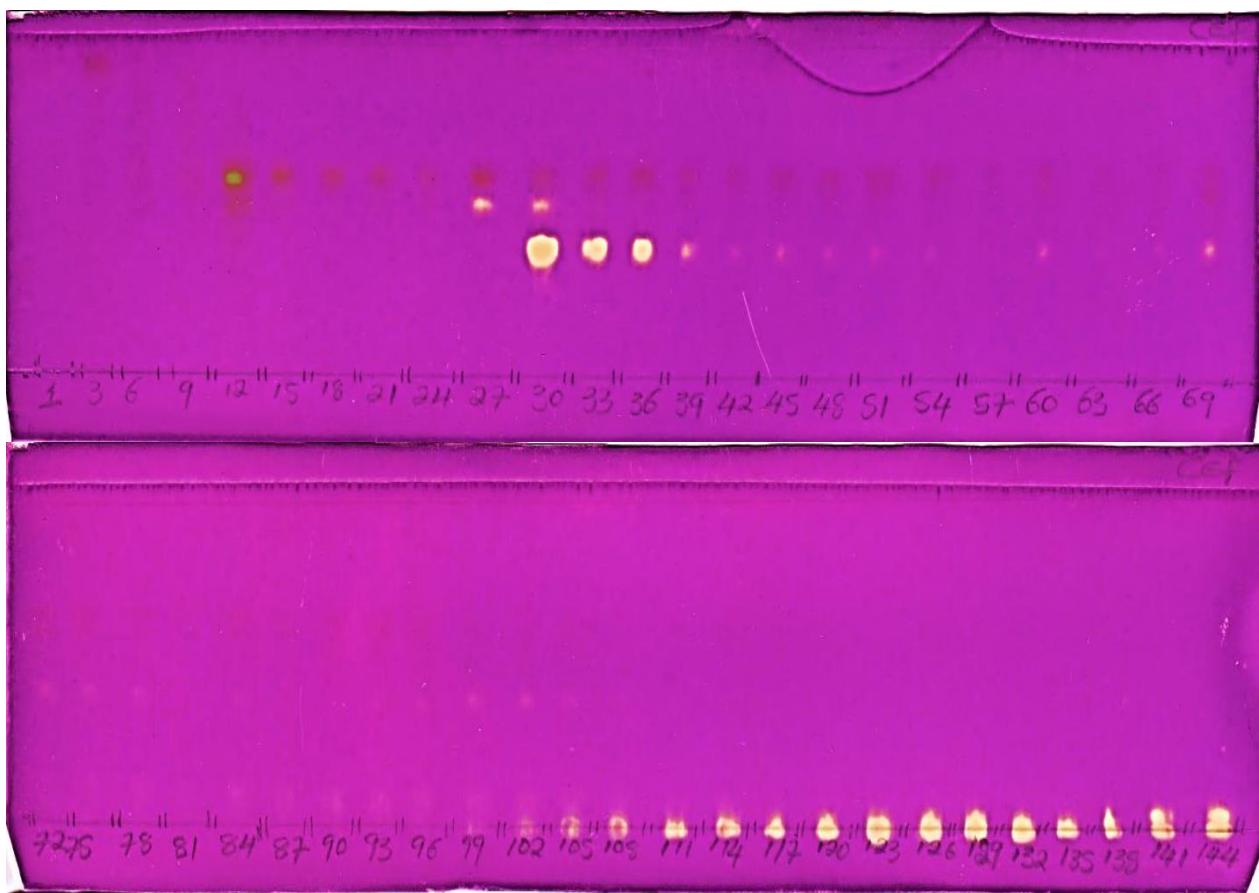


**Figure 6.4:** Thin layer chromatography spectral profile of *R. communis* acetone extracts separated with CEF separation system and sprayed with vanillin-sulphuric acid to reveal compounds isolated with various eluent systems.

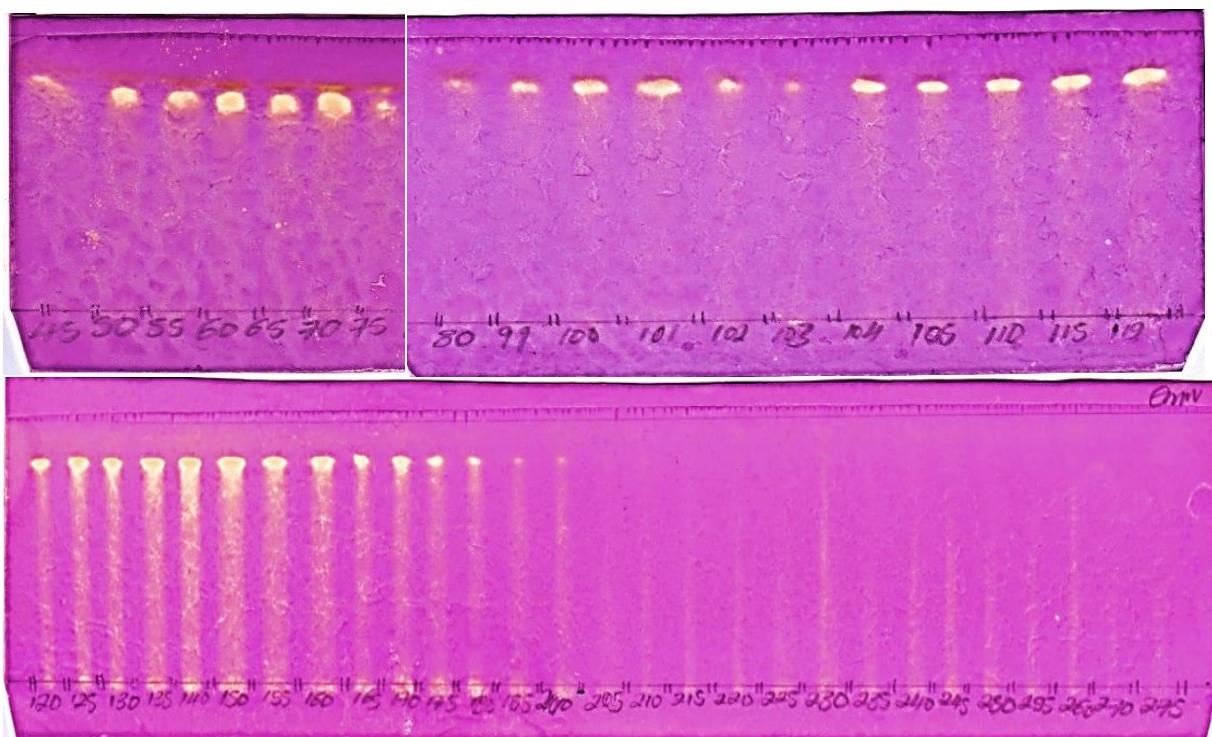


**Figure 6.5:** Chromatograms of *R. communis* acetone extracts separated with CEF separation system and sprayed with 0.2% DPPH to reveal antioxidant compounds isolated with various eluent systems.

Active antioxidant compounds were present in 10% hexane in ethyl acetate, 100% ethyl acetate, and 90 to 80 % ethyl acetate in methanol fractions of *R. communis* acetone extracts (Figure 6.5). Fractions were combined and eluted with 40% hexane in acetone increasing polarity until 100% methanol and 705 fractions were collected. Fractions were separated with CEF (Figure 6.6) and EMW (Figure 6.7) solvent systems and spayed with 0.2% DPPH in methanol to further locate the active antioxidant compounds from *R. communis* acetone extracts. Fraction 30 to 36 revealed antioxidant activity on TLC plates developed in CEF solvent system while fraction 45 to 175 showed slightly antioxidant activity in EMW.

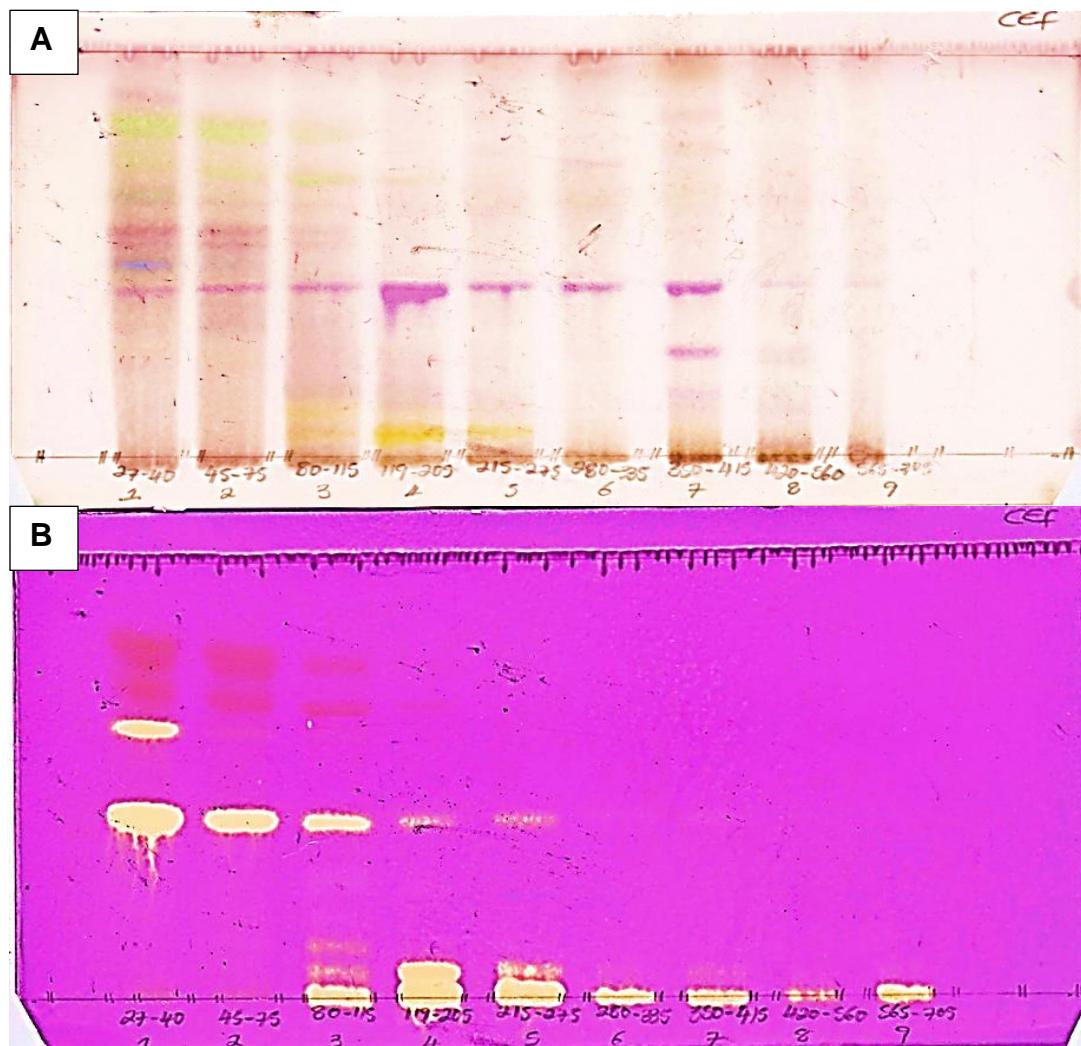


**Figure 6.6:** Chromatograms of *R. communis* acetone extracts isolated with 40% hexane in acetone and developed in CEF separation system and sprayed with 0.2% DPPH to locate isolated antioxidant compounds.



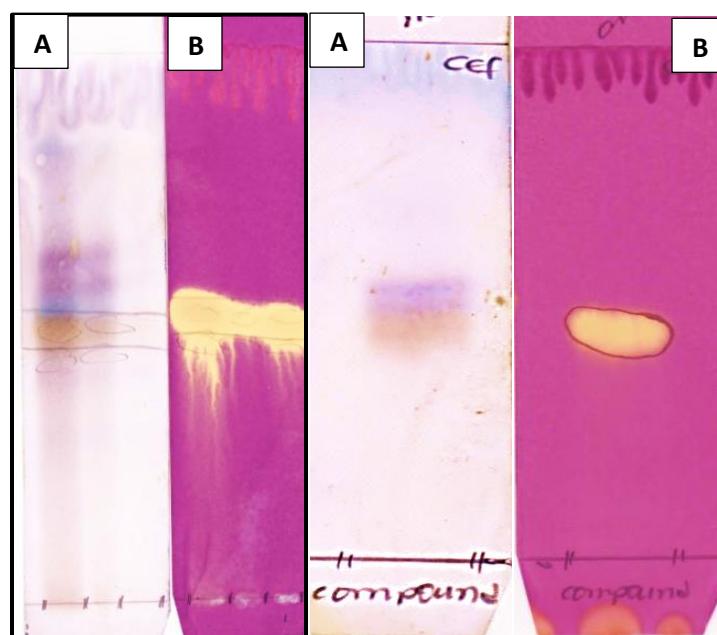
**Figure 6.7:** Chromatograms of *R. communis* acetone extracts isolated with 40% hexane in acetone developed in EMW separation system and sprayed with 0.2% DPPH to show isolated compounds with different eluent systems.

Fractions collected from elution of *R. communis* acetone extracts exhibited antioxidant compounds were pooled together based on the chromatograms sprayed with 0.2% DPPH in Figure 6.6 and 6.7 to a different target fractions. Fractions were separated with CEF solvent system and then sprayed with vanillin-sulphuric acid reagent (Figure 6.8a) for phytochemical analysis and 0.2% DPPH (Figure 6.8b) to locate targeted compounds of interest (antioxidants). Antioxidant compounds present in *R. communis* acetone extracts had the  $R_f$  value of 0.34 in fraction 1, 2 and 3.

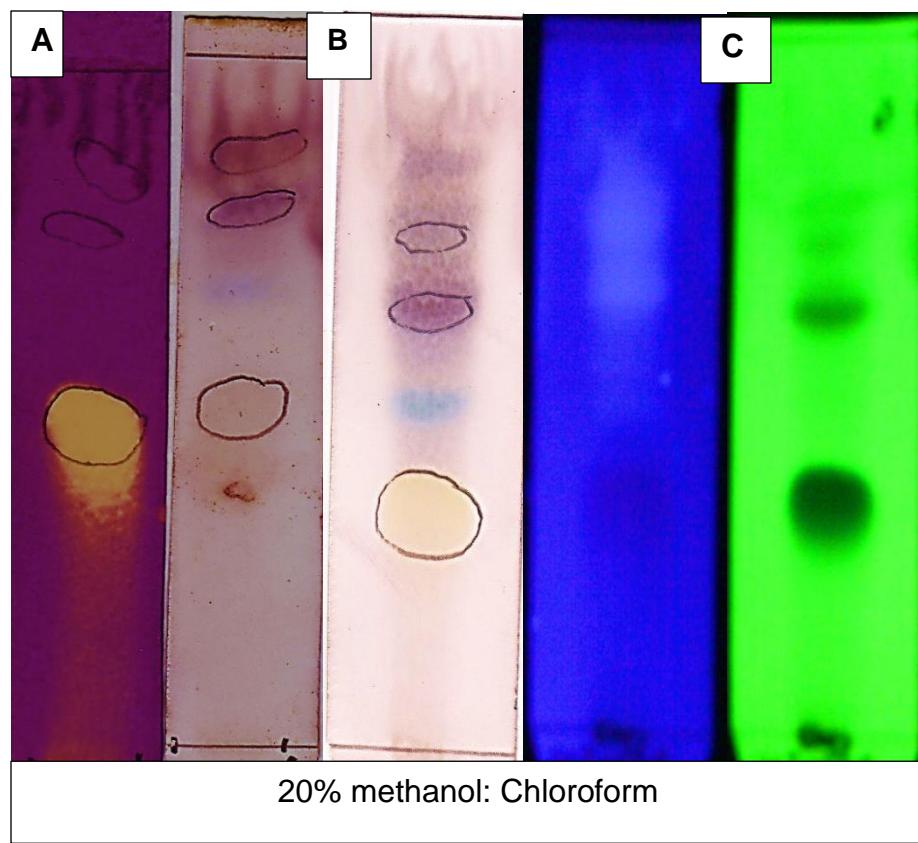


**Figure 6.8:** Chromatograms of *R. communis* acetone extracts isolated with 40% hexane in acetone, developed in CEF separation system and sprayed with vanillin- sulphuric acid (**A**) and 0.2% DPPH (**B**) to show isolated compounds with different eluent systems.

The first three fractions in Figure 6.8b were selected for isolation of antioxidant compounds from *R. communis* acetone extracts. Fractions were combined and purified using preparative thin layer chromatography developed in 20% methanol in chloroform (Figure 6.9). Only active compound was brown in colour at the  $R_f$  value of 0.34 after treating with the vanillin spray reagent (Figure 6.9a) and the other plate was sprayed with 0.2% DPPH for antioxidant activity (Figure 6.9b). In the following experiment the brown compound was consequently targeted for isolation using preparative TLC plates (Figure 6.10), and then sprayed with vanillin spray reagent (Figure 6.10a) and 0.2% DPPH (figure 6.10b). Pure compound confirmed with TLC plates developed in 20% methanol in chloroform.



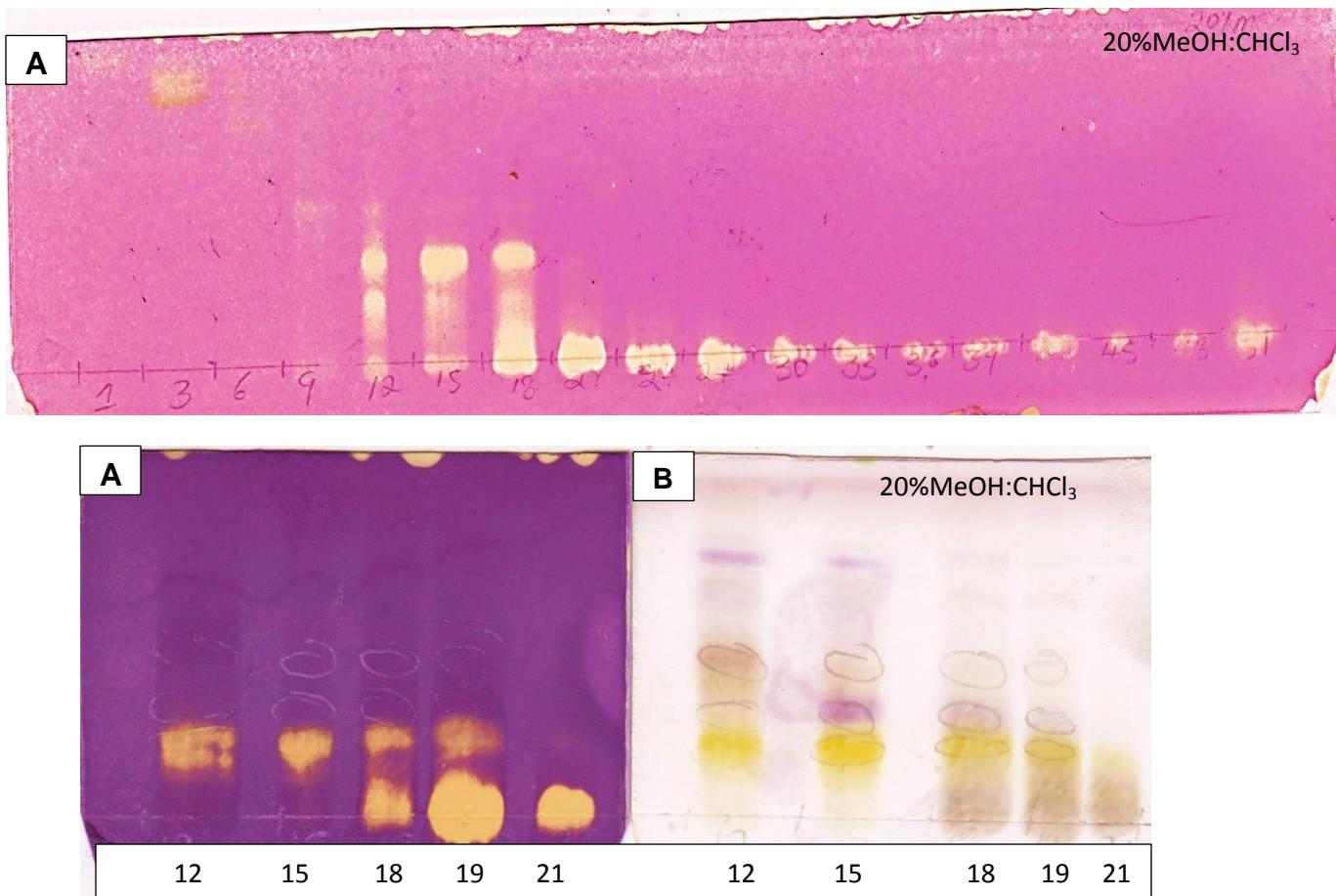
**Figure 6.9:** Chromatograms of *R. communis* acetone extracts isolated with 20% methanol in chloroform, developed in CEF separation system and sprayed with vanillin-sulphuric acid (**A**) and 0.2% DPPH (**B**) to show isolated antioxidant compounds after preparative TLC plates.



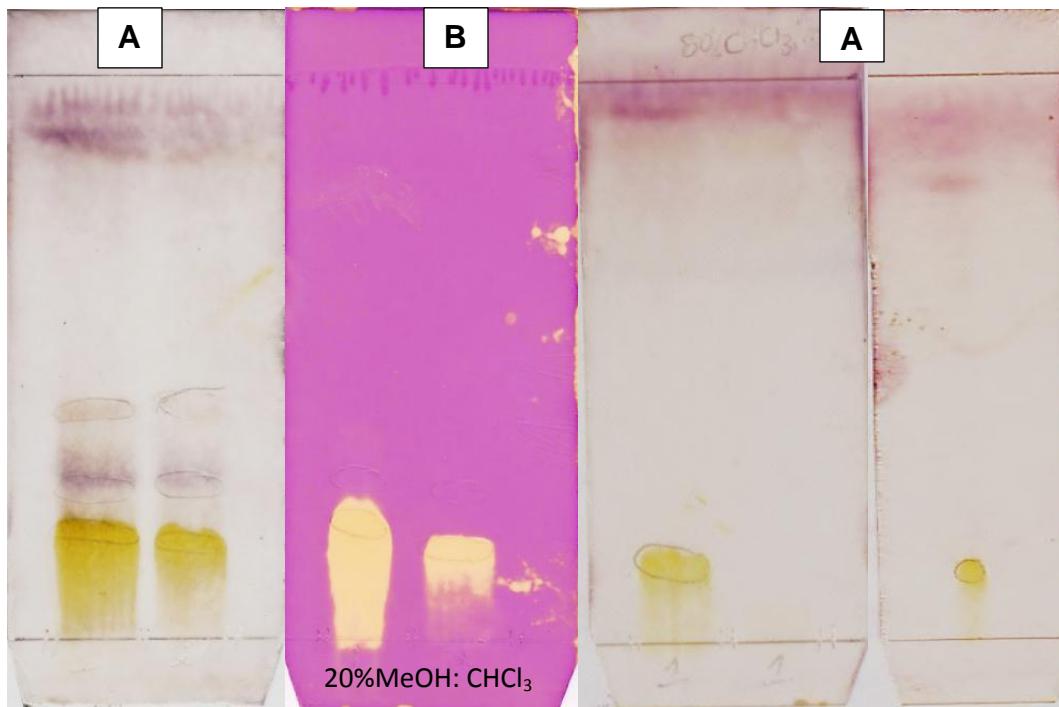
**Figure 6.10:** Chromatograms of *R. communis* acetone extracts isolated with 20% methanol in chloroform, then developed in 20% methanol in chloroform and sprayed with 0.2% DPPH (**A**), vanillin- sulphuric acid (**B**) and under UV light (**C**) to show isolated antioxidant compounds after preparative TLC plates.

Lastly One mg of pure compound was isolated and the structure of the compound was not elucidated because of a less mass of compound. The compound was labeled **Compound 1**.

The fourth fraction in Figure 6.8 was also selected for isolation of antioxidant compounds from *R. communis* acetone extracts. About 0.56 g of fraction 4 was subjected to column chromatography and eluted with 20% methanol in chloroform. Isolates were separated with 80% chloroform in methanol and sprayed with 0.2% DPPH to locate antioxidant compounds (Figure 6.11a) and vanillin spray reagent for phytochemical analysis (Figure 6.11b). Active compounds in Figure 6.11b were yellow in colour after spraying with vanillin spray reagent. Yellow compounds were consequently targeted for isolation using preparative TLC plates (Figure 6.12).



**Figure 6.11:** Chromatograms of *R. communis* acetone extracts isolated with 20% methanol in chloroform, then developed in 20% methanol in chloroform and sprayed with 0.2% DPPH (**A**) and vanillin-sulphuric acid (**B**) to show isolated compounds from column chromatography.



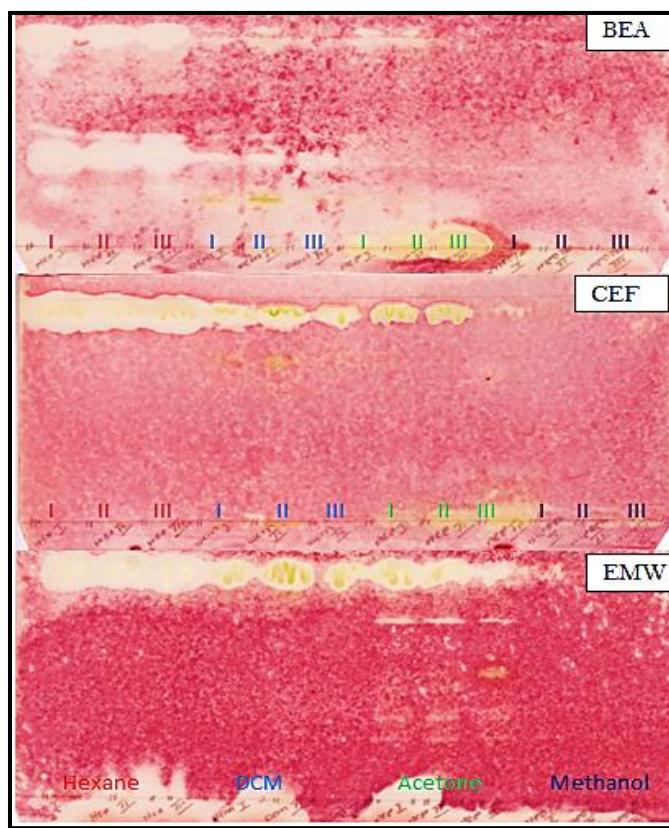
**Figure 6.12:** Chromatograms of *R. communis* acetone extracts isolated with 20% methanol in chloroform, developed in 20% methanol in chloroform and then sprayed with vanillin-sulphuric acid (**A**) and 0.2% DPPH (**B**) to show isolated antioxidant compounds after preparative TLC plates.

About 8.7 mg of a pure antioxidant compound was isolated and labeled **compound 2**. **Compound 3** was also isolated with a total mass of 3.7 mg.

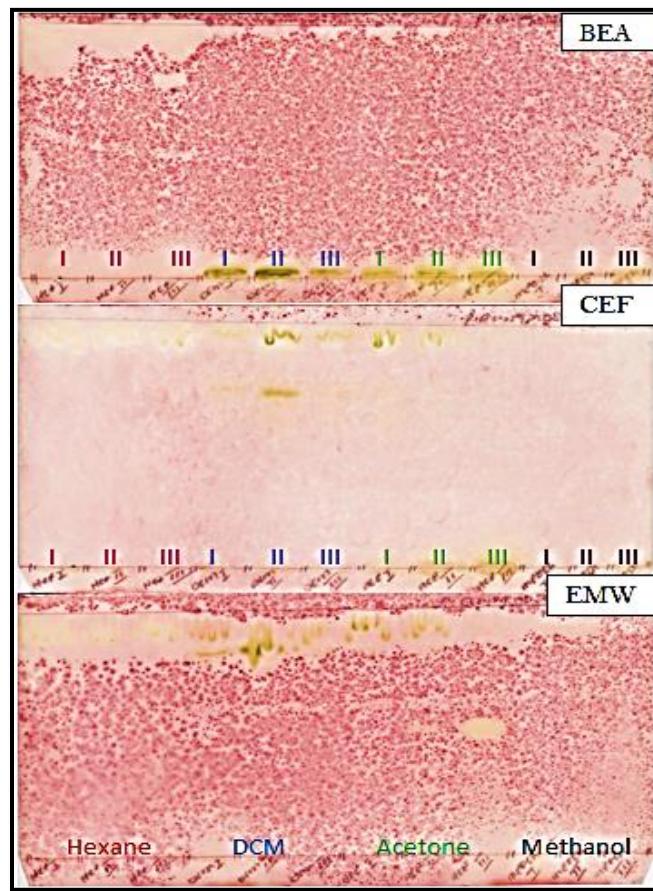
### 6.3.4. Isolation of antibacterial compounds

#### 6.3.4.1. Antibacterial assay

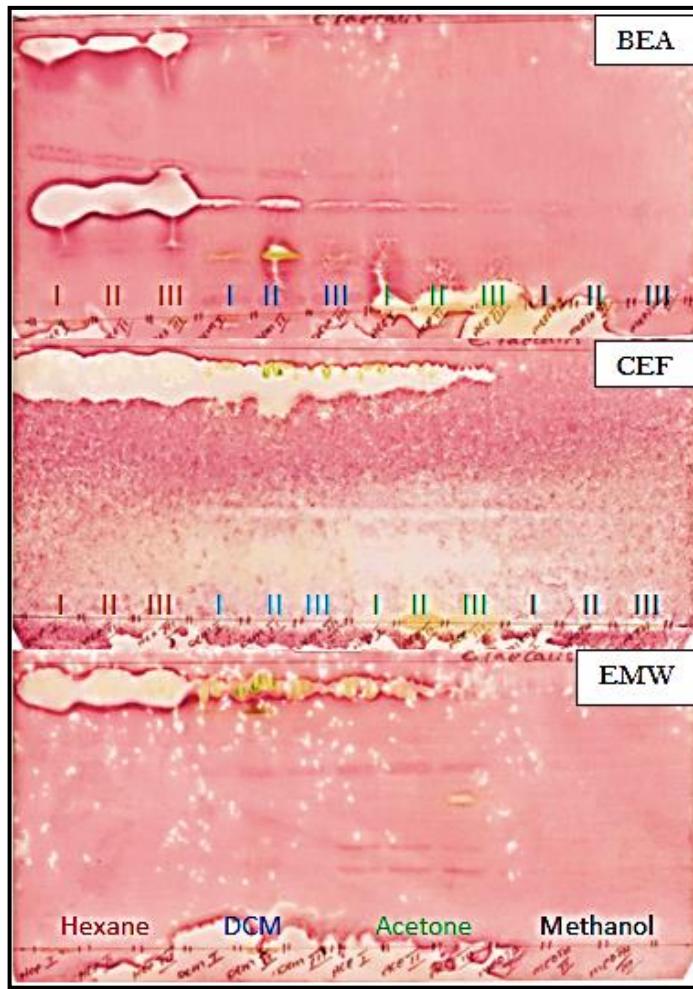
*R. communis* crude extracts were analyzed by bioautography for antibacterial compounds on the chromatograms. Crude extracts were separated with three solvent systems of varying polarities BEA, CEF and EMW. TLC plates were sprayed with *E. coli* (Figure 6.13), *P. aeruginosa* (Figure 6.14), *E. faecalis* (Figure 6.15) and *S. aureus* (Figure 6.16). White zones appear on the chromatograms below indicate zone of inhibition of tested microorganisms by antibacterial compounds present in *R. communis* leaves. Hexane extracts had prominent antibacterial activity against all tested microorganisms compared to dichloromethane, acetone and methanol extracts.



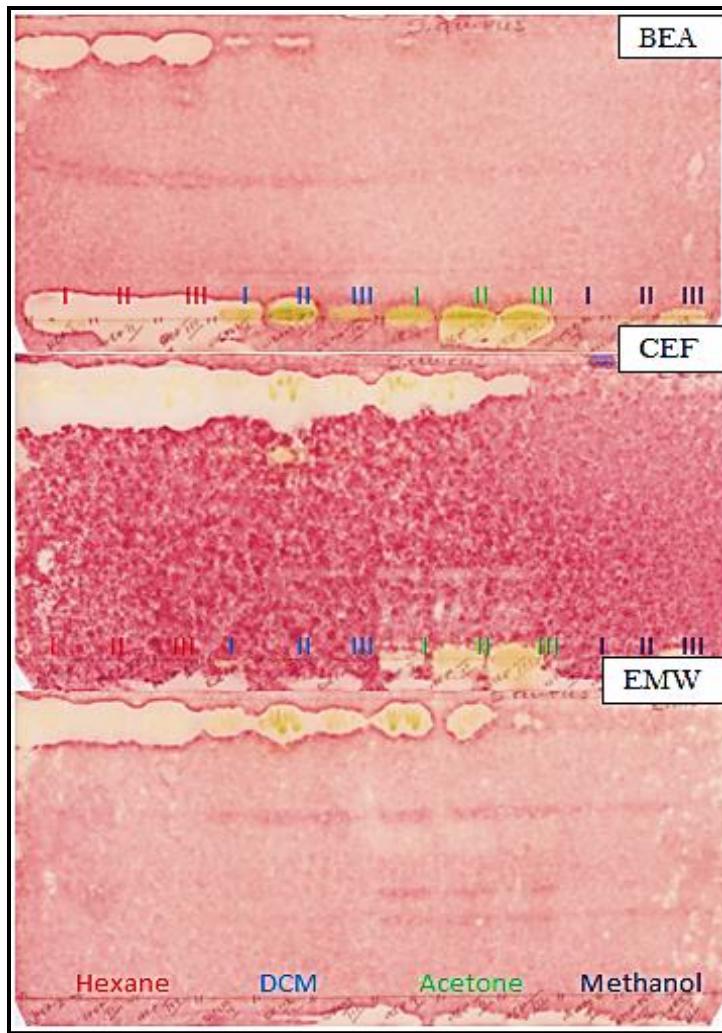
**Figure 6.13:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with *E. coli*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane, dichloromethane (DCM), acetone and methanol in lanes from left to right.



**Figure 6.14:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with *P. aeruginosa*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane, dichloromethane (DCM), acetone and methanol in lanes from left to right.



**Figure 6.15:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (center), EMW (bottom) and sprayed with *E. faecalis*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane, dichloromethane (DCM), acetone and methanol in lane from left to right.



**Figure 6.16:** Bioautograms of *R. communis* leaves extracts separated with three solvent systems BEA (top), CEF (Center), EMW (bottom) and sprayed with *S. aureus*, white zones indicate active compounds that inhibited growth of tested bacterial species. Hexane, dichloromethane (DCM), acetone, and methanol in lanes from left to right.

Crude extracts of *R. communis* leaves showed significant antibacterial activity against tested microorganisms using quantitative microbroth dilution method (Table 6.4). *P. aeruginosa* was the most sensitive microorganism with an average MIC value of 0.50 mg/ml, followed by *S. aureus* (0.86 mg/ml). Acetone extracts had the lowest average MIC value of 0.39 mg/ml against all tested pathogens followed by hexane (0.47 mg/ml),

dichloromethane (0.94 mg/ml) and methanol (1.49 mg/ml) extracts (Table 6.4). Total activity values of all crude extracts were also calculated (Table 6.5). Total activity indicate the largest volume to which crude extracts obtained from one gram of plant materials can be diluted and still inhibit the growth of tested microorganisms. Methanol extracts had the highest average total activity of 46 ml/g, followed by hexane (44 ml/g), acetone (18 ml/g) and dichloromethane (9 ml/g). Extracts with high total activity are preferable to work with (Table 6.5).

**Table 6.4:** Minimum inhibitory concentration (MIC) values of *R. communis* leaves extracts against four tested microorganisms after incubation at 37°C for 24 hours.

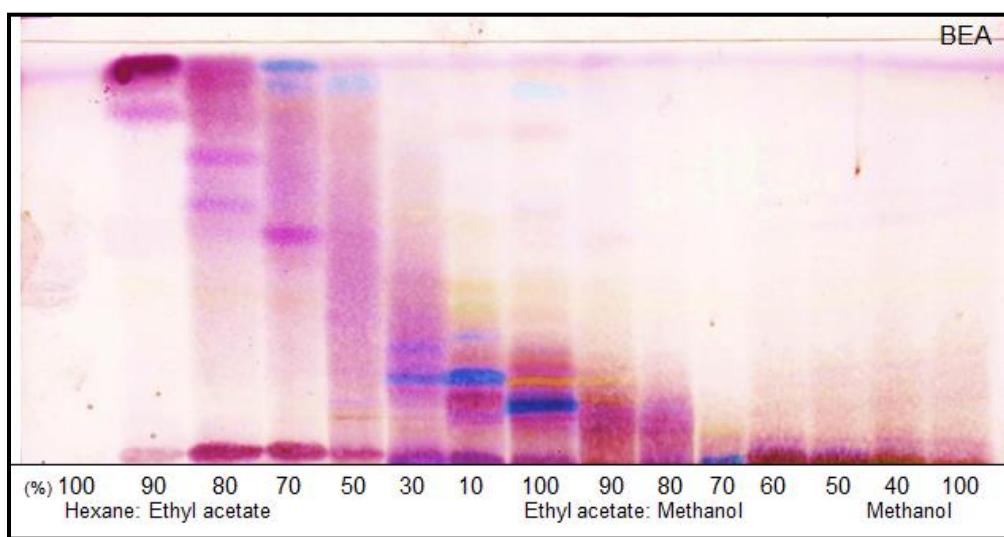
Microorganisms	MIC (mg/ml)												Average	
	Hexane			Dichloromethane			Acetone			Methanol				
	I	II	III	I	II	III	I	II	III	I	II	III		
<i>E. coli</i>	0.31	0.47	0.31	1.25	0.94	0.94	0.31	0.31	0.47	0.94	1.56	3.75	<b>0.96</b>	
<i>P. aeruginosa</i>	0.47	0.47	0.63	0.63	0.63	0.63	0.31	0.31	0.31	0.47	0.63	0.47	<b>0.50</b>	
<i>E. faecalis</i>	0.63	0.63	0.63	1.25	0.63	1.25	0.31	0.47	0.63	1.25	1.88	1.88	<b>0.95</b>	
<i>S. aureus</i>	0.31	0.31	0.31	0.94	0.94	1.25	0.47	0.47	0.31	1.25	1.25	2.50	<b>0.86</b>	
Average	<b>0.43</b>	<b>0.47</b>	<b>0.47</b>	<b>1.02</b>	<b>0.79</b>	<b>1.02</b>	<b>0.35</b>	<b>0.39</b>	<b>0.43</b>	<b>0.98</b>	<b>1.33</b>	<b>2.15</b>		
Total average	<b>0.47</b>			<b>0.94</b>			<b>0.39</b>			<b>1.49</b>				

**Table 6.5:** Total activity (ml/g) of *R. communis* extracts against four tested microorganisms.

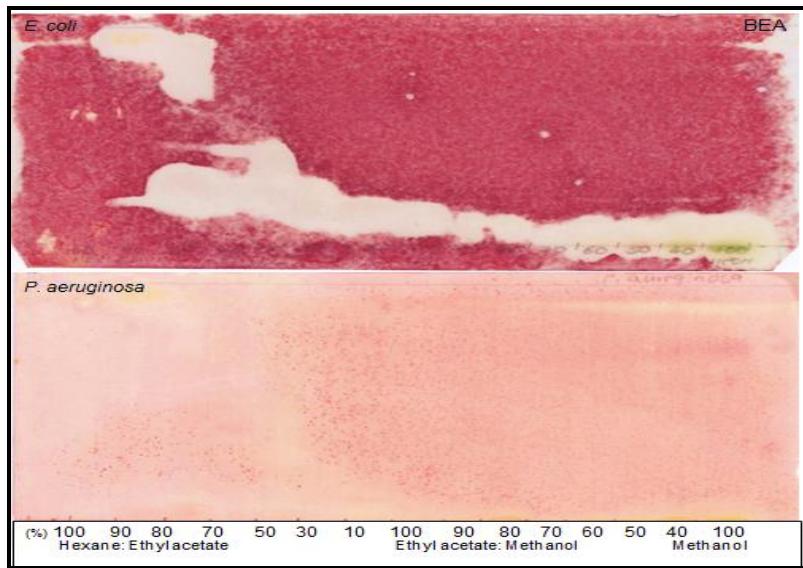
Microorganisms	Total activity (ml/g)												Average	
	Hexane			Dichloromethane			Acetone			Methanol				
	I	II	III	I	II	III	I	II	III	I	II	III		
<i>E. coli</i>	97	34	25	13	10	8	31	18	10	68	22	7	<b>29</b>	
<i>P. aeruginosa</i>	64	34	12	25	14	12	31	18	14	136	57	53	<b>45</b>	
<i>E. faecalis</i>	47	25	12	13	14	6	31	12	7	51	18	13	<b>21</b>	
<i>S. aureus</i>	97	51	25	17	10	6	20	12	14	51	58	10	<b>31</b>	
Average	<b>76</b>	<b>36</b>	<b>19</b>	<b>17</b>	<b>12</b>	<b>8</b>	<b>28</b>	<b>15</b>	<b>11</b>	<b>77</b>	<b>39</b>	<b>21</b>		
Total average	<b>44</b>			<b>9</b>			<b>18</b>			<b>46</b>				

#### 6.3.4.2. Open column chromatography

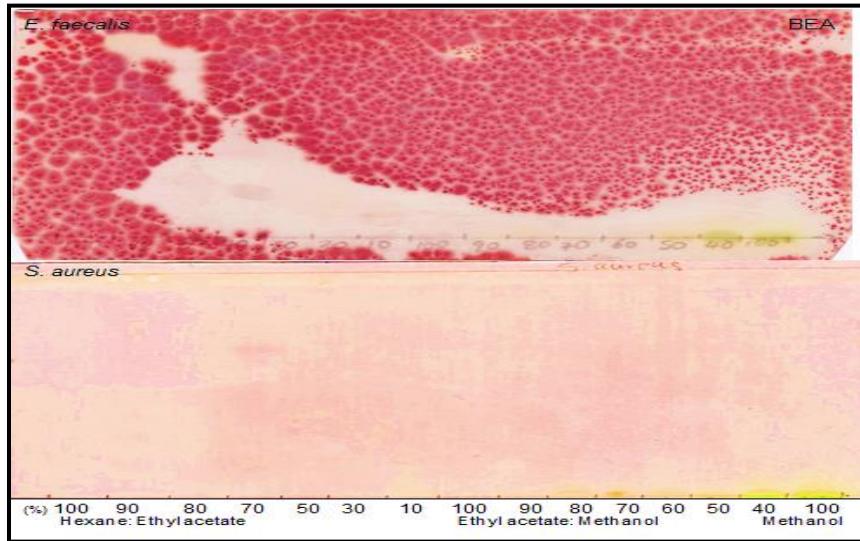
Hexane extracts showed strong antibacterial activity against all tested microorganisms in Figure 6.13 – 6.16. Hexane extracts were combined to the total mass of 53.84 g and subjected into column chromatography (40 x 6 cm). The column chromatography was eluted with varying percentages of solvents listed in Table 6.3 and mass for each fraction was also recorded. Phytochemical analysis of fractions collected was performed (Figure 6.17). Fractions were separated with BEA solvent systems and sprayed with *E. coli*, *P. aeruginosa* (Figure 6.18), *E. faecalis* and *S. aureus* (Figure 6.19) to locate antibacterial compounds. Most active antibacterial compounds were present in ninety to ten (90 –10) percent hexane in ethyl acetate and 100 to 10% ethyl acetate in methanol fractions against *E. coli* and *E. faecalis*. *P. aeruginosa* and *S. aureus* were highly resistant.



**Figure 6.17:** Thin layer chromatography spectral profile of *R. communis* hexane extracts separated with BEA separation system and sprayed with vanillin-sulphuric acid to reveal compounds isolated with various eluent systems.

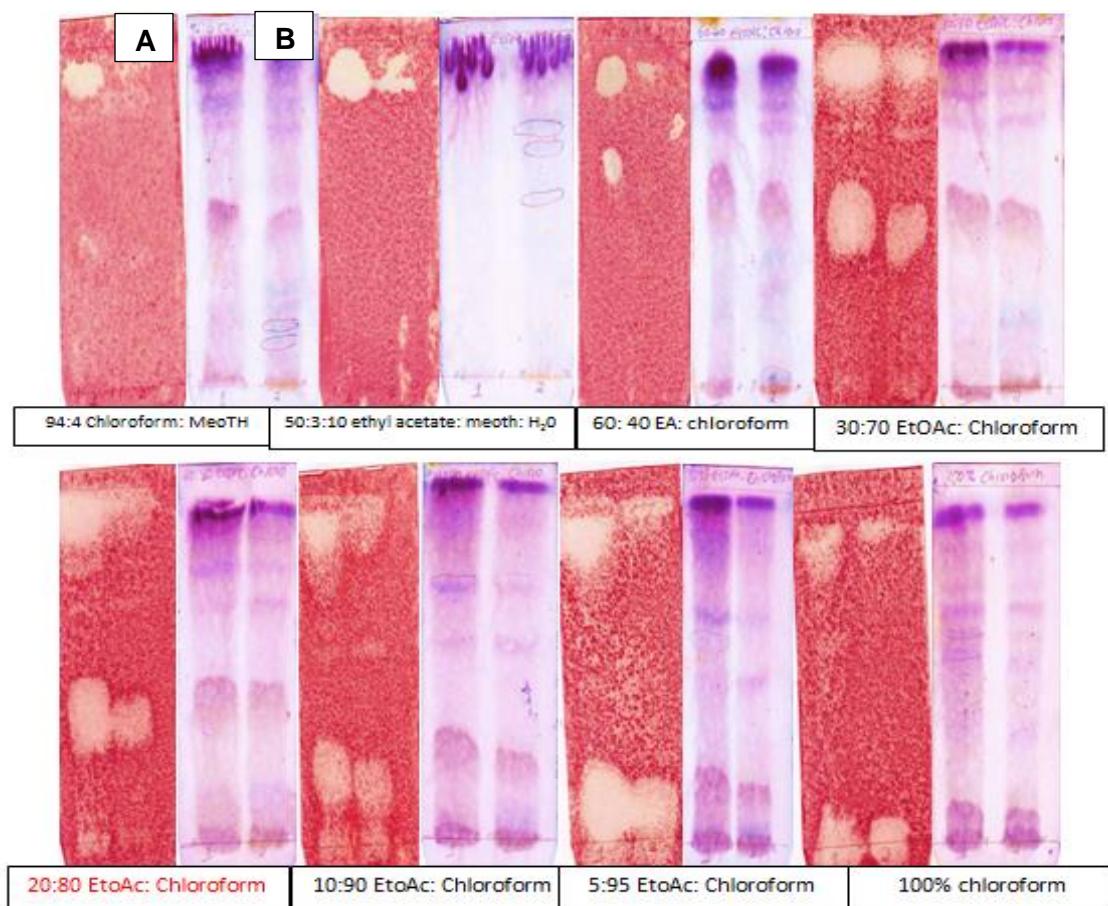


**Figure 6.18:** Bioautograms of *R. communis* hexane extracts separated with BEA separation system and sprayed with two Gram negative bacterial species: *E. coli*, and *P. aeruginosa*, white zones indicate active compounds that inhibited growth of tested bacterial species.



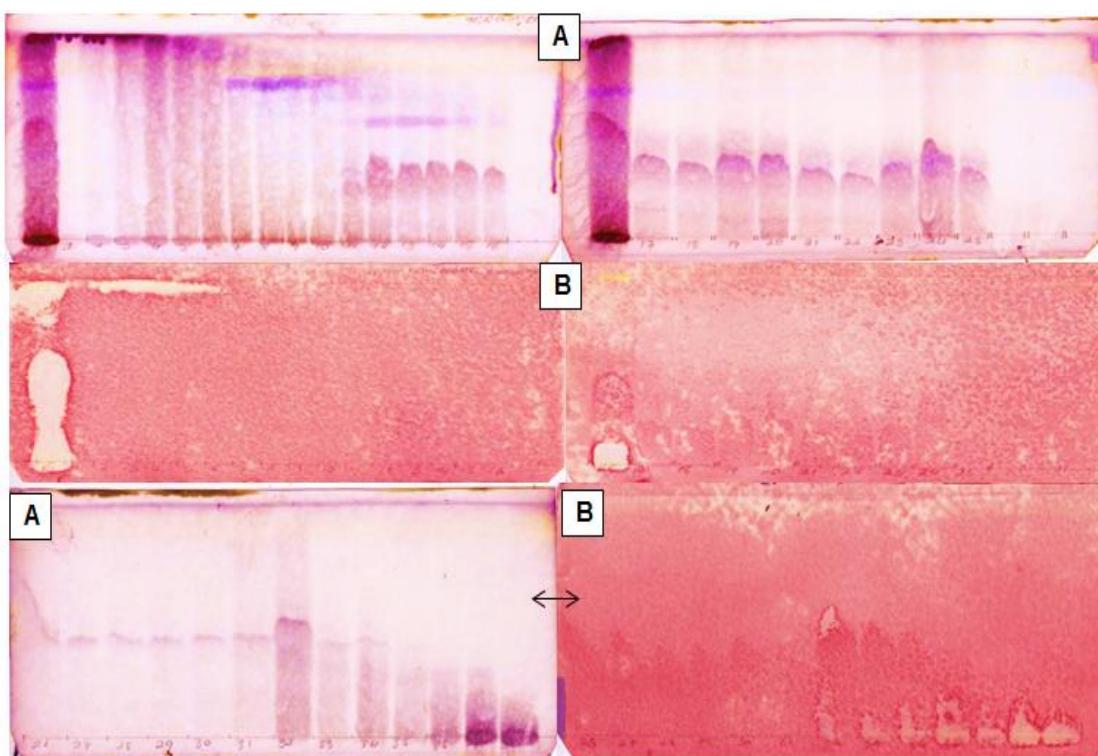
**Figure 6.19:** Bioautograms of *R. communis* hexane extracts separated with BEA separation system and sprayed with two Gram positive bacterial species: *E. faecalis*, and *S. aureus*, white zones indicate active compounds that inhibited growth of tested bacterial species.

Fractions that possessed antibacterial compounds were pooled together (90 –10% hexane in ethyl acetate and 100 to 10% ethyl acetate in methanol fractions) (Figure 6.18 – 6.19). Most active antibacterial compounds were present in 90 – 10% hexane in ethyl acetate fraction. Different solvents were evaluated using TLC plates sprayed with *E. coli* (Figure 6.20a) and vanillin spray reagent (Figure 6.20b) for selection of best solvent to separate active antibacterial compounds present in 90 – 10% hexane in ethyl acetate (Figure 6.20). Twenty percent ethyl acetate in chloroform and 10% ethyl acetate in dichloromethane (not shown) were the best solvents to separate most of active antibacterial compounds. Active antibacterial compounds appeared pink on TLC plates after treatment with vanillin spray reagent (Figure 6.20).



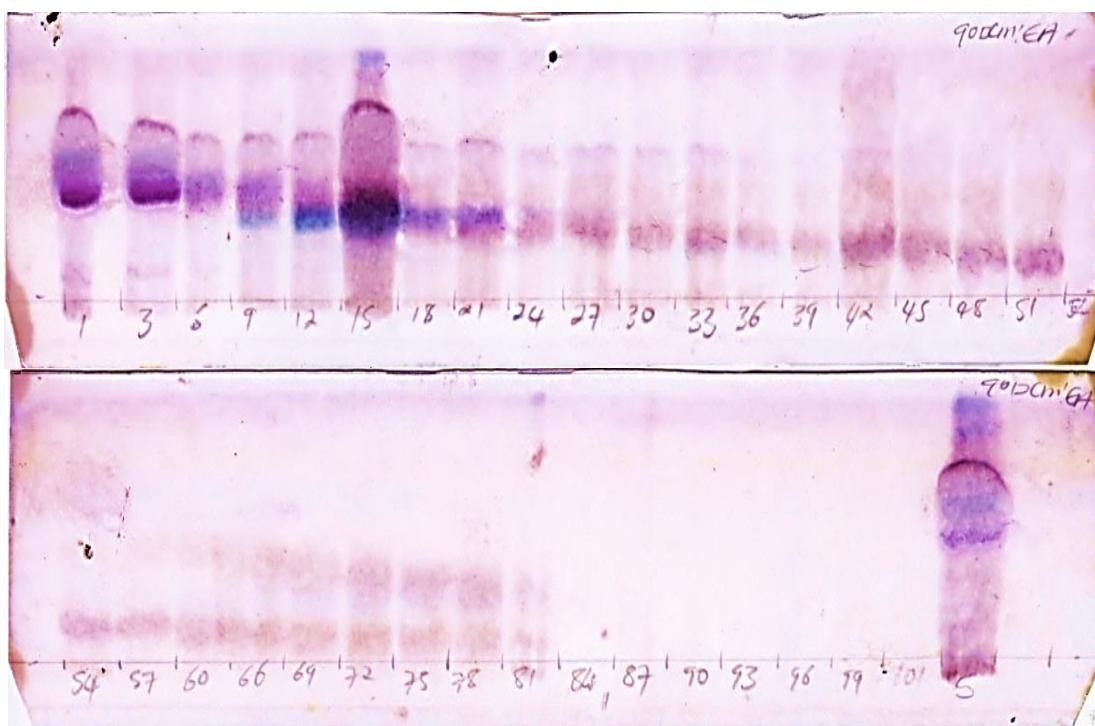
**Figure 6.20:** Chromatograms showed the solvents that were evaluated using TLC plates for selection of best elution system for separation of antibacterial compounds from *R. communis* hexane extracts.

About 27.00 g of 90 – 10% hexane in ethyl acetate fraction of *R. communis* hexane extracts was eluted with 10% ethyl acetate in dichloromethane to separate antibacterial compounds through silica gel in column chromatography. Fractions were separated with 90% dichloromethane in ethyl acetate on a TLC plate then sprayed with vanillin sulphuric acid (Figure 6.21a) and *E. coli* (Figure 6.21b). Antibacterial compounds present in fractions 13 to 25 were pooled together and total mass of 2.303 g was obtained. The active compounds had lost their activity after separation on the TLC plates; the activity was perhaps due to the synergistic effect of compounds present in the extracts (Figure 6.21). Antibacterial compounds were pink in colour on the TLC plates and in the following experiments pink compounds were consequently targeted for isolation.



**Figure 6.21:** Bioautography of fractions obtained from eluting hexane extract from *R. communis* leaves with 10% ethyl acetate in dichloromethane. Chromatograms were sprayed with vanillin sulphuric acid (**A**) and *E. coli* (**B**), white spots against pink background indicates antibacterial compounds present in sub fractions of hexane extracts.

A total mass of 2.303 g, fractions of 10% ethyl acetate in dichloromethane was further subjected into third column chromatography for isolation of antibacterial compounds that had lost the activity. The column was eluted with 90% dichloromethane in ethyl acetate then separated with 10% ethyl acetate in dichloromethane and sprayed with vanillin spray reagent (Figure 6.22). Fractions 1 to 5 were combined and pink compound was targeted and purified using preparative thin layer chromatography. Two pure compounds were isolated; **Compound 4** had the total mass of 102 mg and appeared pink on a plate after treated with vanillin spraying reagent (Figure 6.23). **Compound 5** had a total mass of 4.7 mg (not shown).

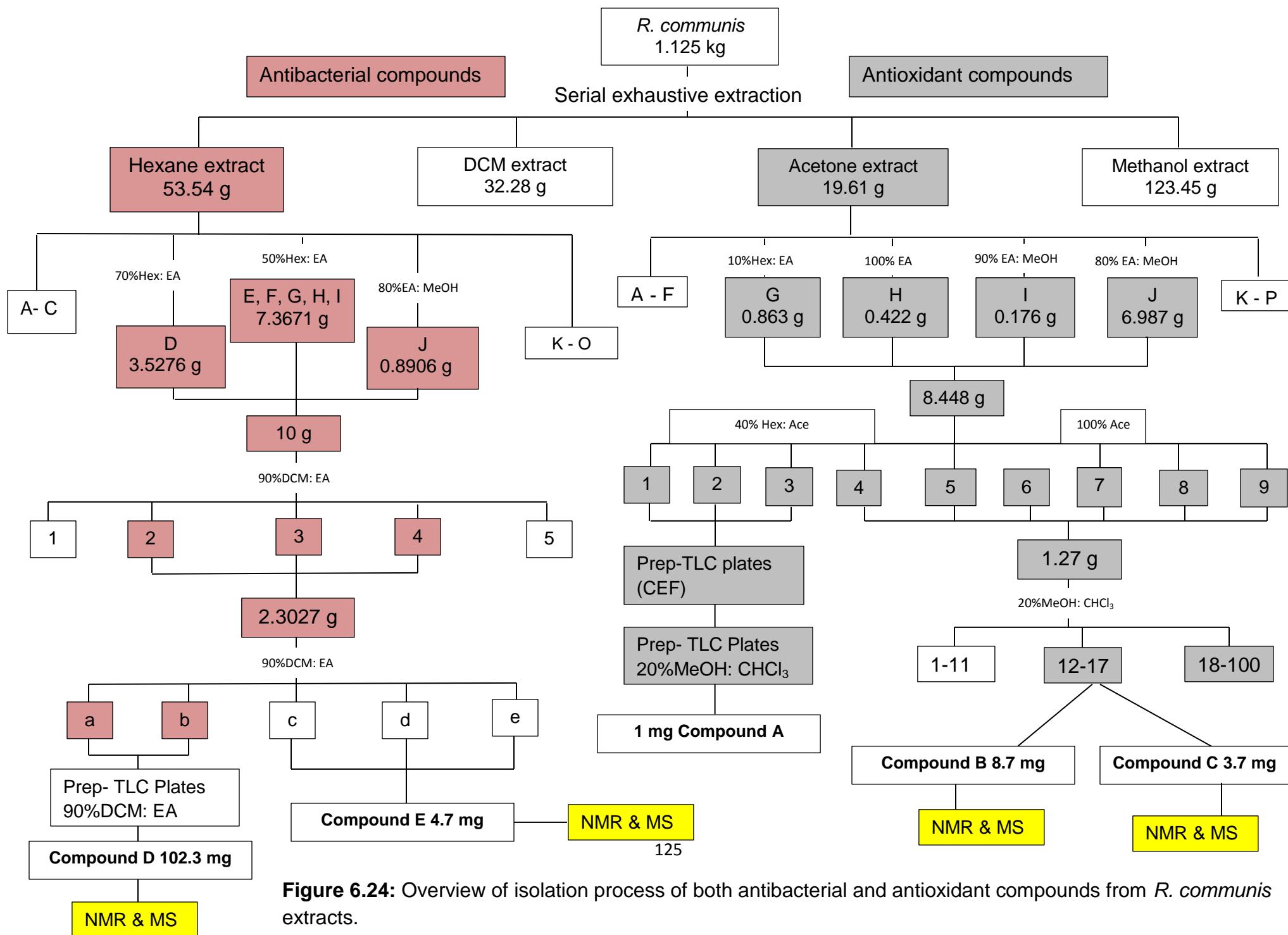


**Figure 6.22:** Chromatograms of *R. communis* hexane extracts isolated with 10% ethyl acetate in dichloromethane developed in 90% dichloromethane in ethyl acetate and then sprayed with vanillin-sulphuric acid to show isolated compounds with different eluent systems.



**Figure 6.23:** Chromatogram of pure compound isolated from *R. communis* hexane extracts with 90% dichloromethane in ethyl acetate and developed in 10% ethyl acetate in dichloromethane and sprayed with vanillin-sulphuric acid. Two pure compounds were isolated with a total mass of 102.3 mg (labeled **compound 4**) and **compound 5** with 4.7 mg.

The overview flow chart of isolation of antioxidant and antibacterial compounds from acetone and hexane extracts respectively is shown in Figure 6.24. Pure compounds were further characterized using nuclear magnetic resonance spectroscopy for structure elucidation.



**Figure 6.24:** Overview of isolation process of both antibacterial and antioxidant compounds from *R. communis* extracts.

## **6.4. DISCUSSION**

Bioassay-guided fractionations on silica gel 60 (particles size 0.063-0.200 mm) in opened column chromatography resulted in the successful isolation of both antioxidant and antibacterial compounds from *R. communis* leaves. Antioxidant compounds were isolated from acetone extracts (Figure 6.3– 6.12) while antibacterial compounds were isolated from hexane extracts (Figure 6.13– 6.23) of *R. communis* leaves. Active compounds were extracted, isolated and purified using column chromatography and preparative thin layer chromatography.

There are a number of problems associated with isolation and purification of active compounds from plant extracts. E.g. losing activity of compounds either antibacterial or antioxidant is a major challenge. Some of the compounds tend to lose activity with each step of the purification process and this may be the proof that some of the compounds work synergistically to produce various biological activities. Antibacterial compounds lost their activity against tested pathogens during the purification process (Figure 6.21). This suggests that two or more compounds were acting collectively to exhibit antibacterial activity of the *R. communis* leaves against tested pathogens. In some other cases, phytochemical compounds may hinder the activity of other compounds, (antagonist effects) and exhibit their own activity once they are separated (Amoo *et al.*, 2009). In the present study antioxidant compound seems to act independently to scavenge free radical molecules (DPPH), because active antioxidant compound maintained its activity throughout the purification process (Figure 6.12). The results are similar with those of Signh *et al.* (2009), where Gallic acid was isolated from *R. communis* and revealed high antioxidant activity. The results are the scientific proof of why people from rural areas use crude extracts from *R. communis* leaves for treatment of infectious diseases and other free radical molecules related diseases. Crude extracts of *R. communis* leaves may be used to fight against tested pathogens at the lowest concentration of 0.31–1.25 mg/ml (Table 6.4). Jena and Gupta (2012) also reported high antimicrobial activity of *R. communis* root extracts against *E. coli* and *P. aeruginosa* at the concentration of 200 mg/ml, however leaf extracts seem to be more potent compared to roots. This study recommends *R. communis* leaves as a good

source of antibacterial compounds against tested pathogens. And it is safe to use crude extracts compared to pure isolated compounds because pathogens may easily developed resistant gene against single pure antibiotic compound unlike many compounds present in crude extracts.

Signh *et al.* (2009) isolated five phenolic compounds from dry leaves of *R. communis* that are responsible for antioxidant activity namely: gallic acid, quercetin, gentisic acid, rutin, epicatechin and ellagic acid. DPPH method was used to screen for antioxidant activity of compounds during the isolation process from various fractions of *R. communis*. Gallic acid has been used as a standard in many antioxidant assays. It has been reported to scavenge a number of free radical molecules including superoxide anions, hydroxyl radicals and singlet oxygen or peroxy radicals. Gallic acid is a phenolic compound that is known to possessed antibacterial activity against *P. aeruginosa* and *S. aureus* (Phiriyawirut and Phachamud, 2011; Kuamwat *et al.*, 2012).

Number of researches done on *R. communis* plant recommended this plant as a good source of antioxidant compounds as a whole (roots, stem, leaves and seeds) (Jena and Gupta, 2012). Methathyl ricinoleate, Ricinoleic acid, 12- octadecadienoic acid and methyl ester are some of the antioxidant compounds isolated from stem and leaves of *R. communis* (Oloyede, 2012). *R. communis* leaves possessed high antioxidant activity and crude extracts of this plant may be used for treatment of diseases connected with oxidative stress. Three antioxidant compounds (Compound 1, 2 and 3) were isolated in low quantities from *R. communis* leaves (Figure 6.10 – 6.12) and two antibacterial compounds (Compound 4 and 5). Compound 1 had the total mass of 1 mg, compound 2 (8.7 mg), compound 3 (3.7 mg), compound 4 (102.3 mg) and compound 5 (4.7 mg). The names and structures of the three antioxidant compounds were not identified due to low quantities of the compounds obtained (because the lowest mass of sample that can be easily detected by nuclear magnetic resonance is 10 mg). A strong antioxidant activity was observed after spraying with DPPH in methanol and antibacterial compounds showed less activity compared to the crude extracts. This study therefore suggests the use of crude extracts to fight against pathogenic microorganisms compare to pure compounds because crude extracts showed potent antibacterial activity against

tested pathogens compared to a single pure compound. Compound 4 was found as the mixture of stigmasterol and  $\beta$ -sitosterol, however compound 5 was also not identified because of its mass. Ghosh *et al.* (2011) reported that stigmasterols are phytosterols natural products and they possess antitumor activity and cholesterol lowering activity. Venkata-Raman *et al.* (2012) reported that stigmasterols have significant antibacterial activity as major components in the leaves of *E. odoratum*.  $\beta$ -sitosterols are also phytosterols and have been reported to possessed strong anti-inflammatory activity and other activities (Saeidnia *et al.*, 2014).

## 6.5. CONCLUSION

The present study showed that the leaves of *R. communis* possess high antioxidant and antibacterial activities. Losing activity of the compound is a disadvantage or drawback of the experiment when isolating and purifying compounds from plant extracts. Although it provides a better understanding that some compounds work together to exhibit one biological activity of the plant. In the present study stigmasterol and  $\beta$ -sitosterol were successful isolated from hexane extracts of *R. communis* leaves. Based on the findings in this study, *R. communis* is highly recommended as a source of potent antibacterial and antioxidant compounds. The study serves as a scientific proof for the use of this plant in traditional medicine for treatment of wounds.

## CHAPTER 7

### CYTOTOXICITY AND ANTI-INFLAMMATION ACTIVITY

#### **7.1. INTRODUCTION**

Medicinal plants have a vital role to preserve healthy human life. From the beginning of time, plants have been known as a source of natural products such as biological active compounds with various activities such as anticancer, antitumor and antiproliferative activity. It has been estimated that 8.2 million people will die because of cancer between 2005 and 2015. Medicinal plants have been used throughout the whole world for treatment of various diseases including cancer. Vinblastine, vincristine and taxol are antitumor drugs that were isolated from medicinal plants and these drugs are currently used for chemotherapy of some cancers (Talib and Mahasneh, 2010).

About 40% of all anticancer drugs that were used for treatment of cancer between 1940 and 2002 were derived from medicinal plants such as Vinca alkaloids, taxus diterpenes, and podophyllumligans. Most anticancer drugs derived from medicinal plants showed fewer side effects as compared to the conventional chemotherapeutic drugs (Belayachi *et al.*, 2013). Researchers are highly interested in exploring medicinal plants to discover and manufacture several novel compounds, since they are rich in bioactive compounds (Talib and Mahasneh, 2010). There is an increased use of medicinal plants in both rural and urban areas, for primary health care because of an increased demand for inexpensive medicines, lack of hospitals and poverty. Therefore, it is important to research medicinal plants for their safety, quality, toxicity, appropriate amount of plant materials to be use and efficacy (Shai *et al.*, 2008).

Inflammation is the body's normal protective responses of cells to an injury caused by various stimulants such as these include pathogens, chemical or microbiological agents, damaged cells and other irritants. Signs of inflammation are swollen joints, joint pain, stiffness and loss of joint functions. Non-steroidal anti-inflammatory drug (NSAIDs) such as ibuprofen and naproxen are currently used for treatment of inflammation. These

drugs are known to cause severe side effects in the body such as heart attacks and stroke (Kumar *et al.*, 2013b).

Medicinal plants are rich in bioactive compounds with anti-inflammatory activity. There is a heightened interest in the screening of medicinal plants for their anti-inflammatory properties which would subsequently lead to the manufacture of novel plant derived anti-inflammatory drugs. Plants have been used traditionally for treatment of various diseases connected with inflammation (Kumar *et al.*, 2013b). The aim was to evaluate the effect of plant extracts cytotoxic effect on Human Caucasian skin fibroblast (Bud-8) cell line using MTT assay and also to determine the anti-inflammation activity of the selected plant extracts on macrophages Raw 264.7 cell line.

## **7.2. METHODS AND MATERIALS**

### **7.2.1. Plant extracts**

Serial exhaustive extraction of *R. communis* was done as in section 6.2.1, using hexane, dichloromethane, acetone and methanol. The plant extracts were reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/ml.

### **7.2.2. MTT assays**

Cell viability was assessed according to Mosaddegh *et al.*, (2012) on the Human Caucasian skin fibroblast (Bud-8) cell line. The proliferation rates of Bud-8 cell line after treatment with plant extracts were determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The yellow compound MTT is reduced by mitochondrial dehydrogenases to the water-insoluble pink compound formazan, depending on the viability of cells. Cells were maintained in the Dubleco's Modified Essential Medium (DMEM) supplemented with 10% of Fetal bovine serum (FBS) and 1x Penicillin-Streptomycin-Neomycin (PSN). Hundred microliters of cells ( $4 \times 10^4$  cells/ml) were seeded in 96 well plates and incubated at 37°C, 5% carbon dioxide for 24 hours. After 24 hours of incubation, the cells were treated with 100 µl of 100, 200, 300, 400 and 500 µg/ml of plant extracts (Hexane, Dichloromethane, acetone and methanol). Plates were incubated at 37°C, 5% CO<sub>2</sub> for 24 and 48 hours. After incubation,

morphology of cells was examined under microscope. Twenty microliters of MTT solution (5 mg/ml) (Sigma) were added to each well. The plates were further incubated for 2 to 4 hours and the medium was removed. Formazan crystals were dissolved with 100  $\mu$ l of Dimethyl sulfoxide (DMSO). The absorbance was measured at 560 nm and percentages of cell viability and LC<sub>50</sub> of cells were calculated:

$$\% \text{ Cell viability} = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Where, Absorbance control is the absorbance of cells treated with DMSO 1% and Absorbance sample is the absorbance of cells treated with test sample.

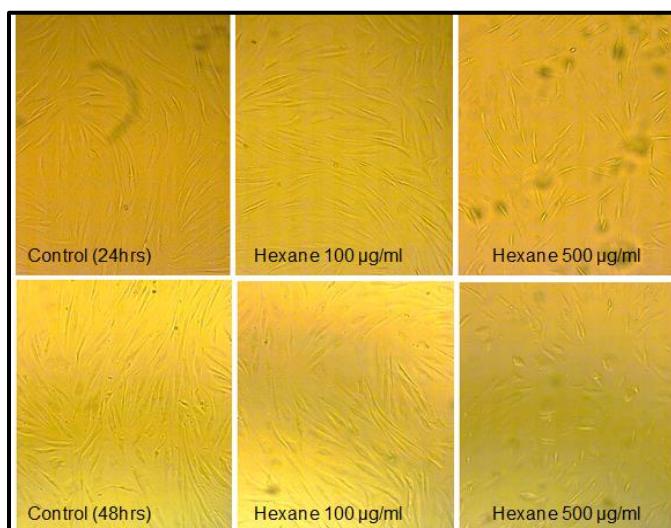
### **7.2.3. Anti-inflammatory activity using phagoburst kit**

Phagoburst assay is a quantitative technique that uses stimulants such as lipopolysaccharide (LPS) and Phorbol 12-myristate 13-acetate (PMA) to induce oxidative stress. Phagoburst assay uses dihydrodichlorofluorescein diacetate (H<sub>2</sub>DCF-DA) to detect the presence of reactive oxygen species in excess. High level of reactive oxygen species tends to attack macromolecules which result to oxidative stress and inflammation response. The H<sub>2</sub>DCF-DA is oxidized to fluorescent 2, 7-dichlorofluorescein (DCF) by the presence of reactive oxygen species. Two hundred microliters of cells (Raw 264.7 macrophages) were transferred to a cover slip in a multi-well plate. The cells were incubated at 37°C, 5% CO<sub>2</sub> for overnight to allow cells to attach. Cells were treated with 100  $\mu$ l of plant extracts (100  $\mu$ g/ml of hexane, dichloromethane, acetone and methanol) and 10 mg/ml of LPS for 24 hours. Two microliters of PMA was also added for 30 minutes. After incubation, the medium was aspirated. Cells were stained with 50  $\mu$ l of 10<sup>-5</sup> M of H<sub>2</sub>DCF-DA and incubated for 20 minutes in the dark. Cells were stained for the second time with 50  $\mu$ l of 20  $\mu$ g/ml, 4', 6-diamidino-2-phenylindole (DAPI) and further incubated for 20 minutes in the dark. Staining solutions were removed and 3.7% paraformaldehyde was added to a cover slip to fix the cells. Coverslips were mounted to a microscope slide and examined under fluorescence microscope.

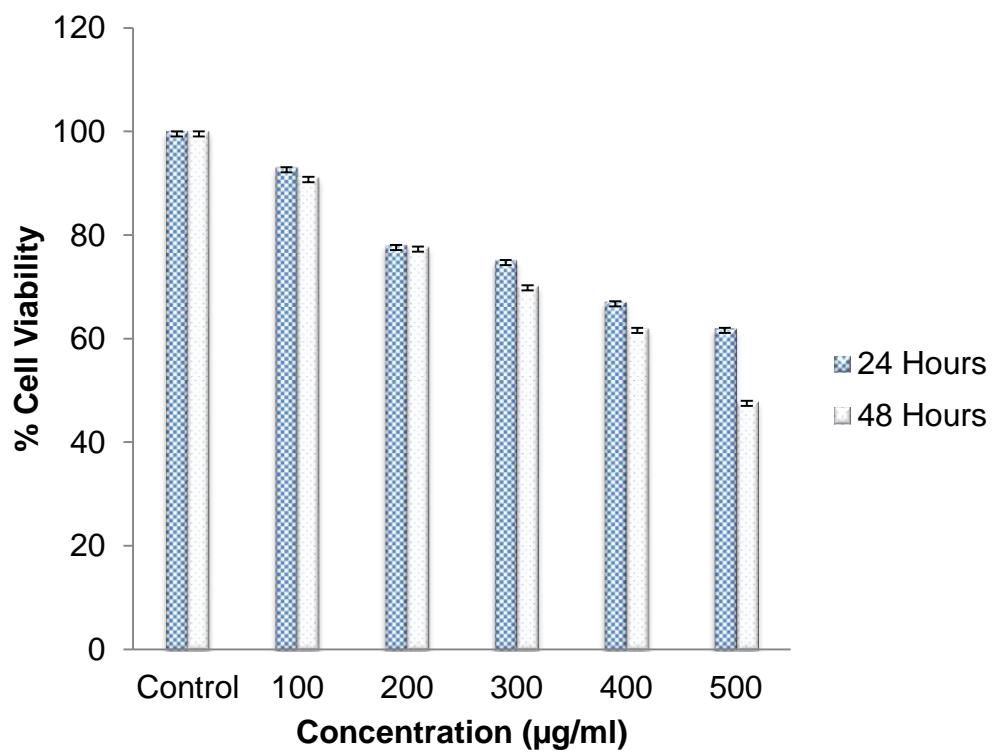
## 7.3. RESULTS

### 7.3.1. Cytotoxicity effects of crude extracts on Bud-8 cell line

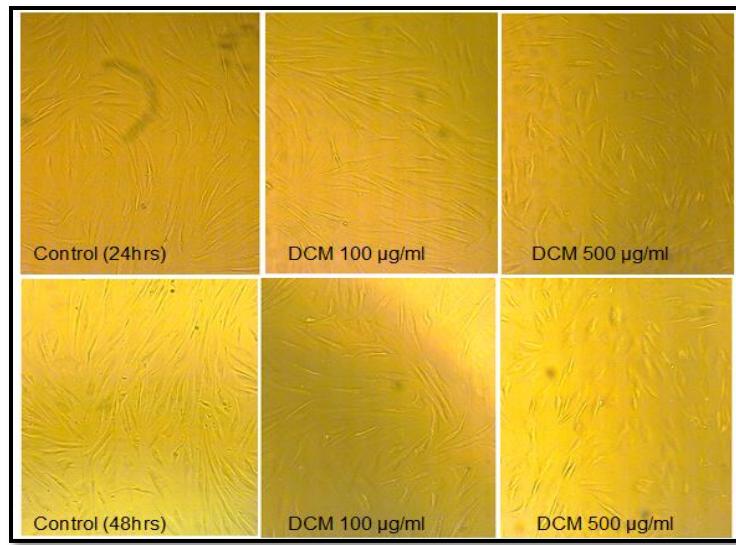
Cytotoxicity effect of hexane, dichloromethane, acetone and methanol extracts of *R. communis* leaves were investigated against Bud-8 cell line. Effect of *R. communis* extracts on Bud-8 cell line was determined after 24 and 48 hours of exposure. Percentage cell viability was calculated by measuring the absorbance of formazan formed from reduction of MTT. Cell morphology of viable cells was also shown for each extract at the highest concentration (500 µg/ml) and lowest concentration (100 µg/ml) (Figure 7.1–7. 8). Bud-8 cell line was treated with varying concentrations of hexane (Figure 7.2) dichloromethane (Figure 7.3), acetone (Figure 7.5) and methanol (Figure 7.7) extracts for 24 and 48 hours. All crude extracts were toxic to Bud-8 cell lines at the highest concentration and the morphology of cells was altered. Hexane and dichloromethane extracts had more than 90% cell viability after 24 and 48 hours of exposure while acetone and methanol had less than 90 % cell viability.



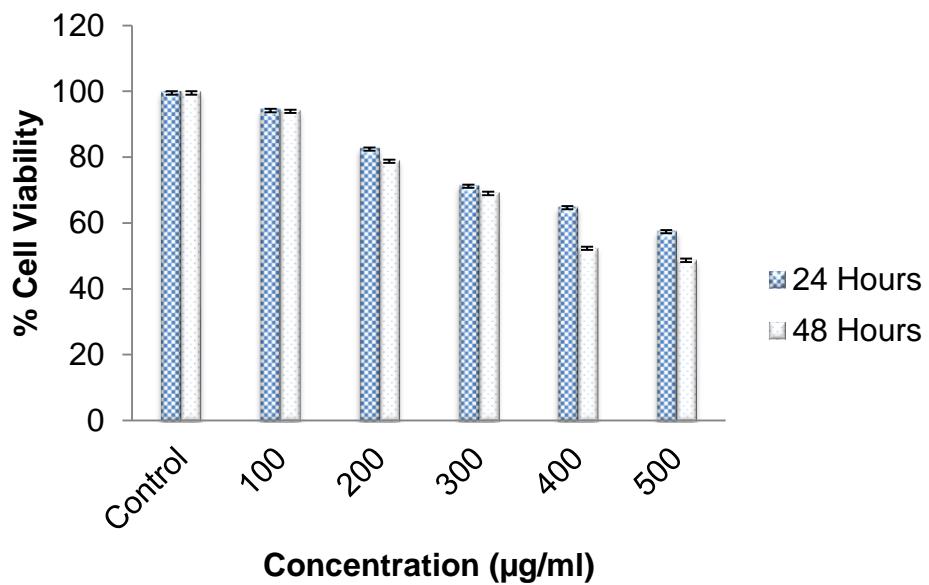
**Figure 7.1:** Cell viability and morphology of Bud-8 cell line after treated with hexane extracts at concentration ranging from 100 and 500 µg/ml.



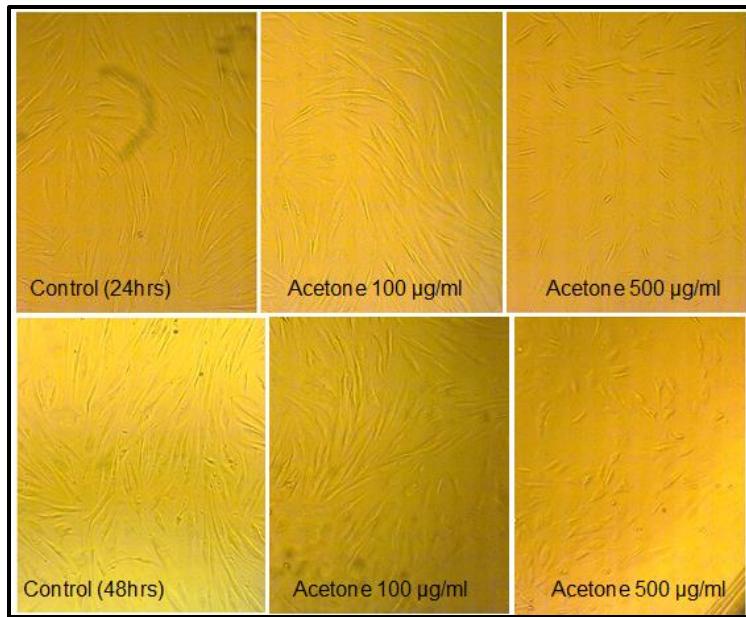
**Figure 7.2:** The cytotoxic effects of *R. communis* hexane extracts against Bud-8 cell line after 24 and 48 hours of exposure using MTT assay at the concentration of 100 – 500 µg/ml.



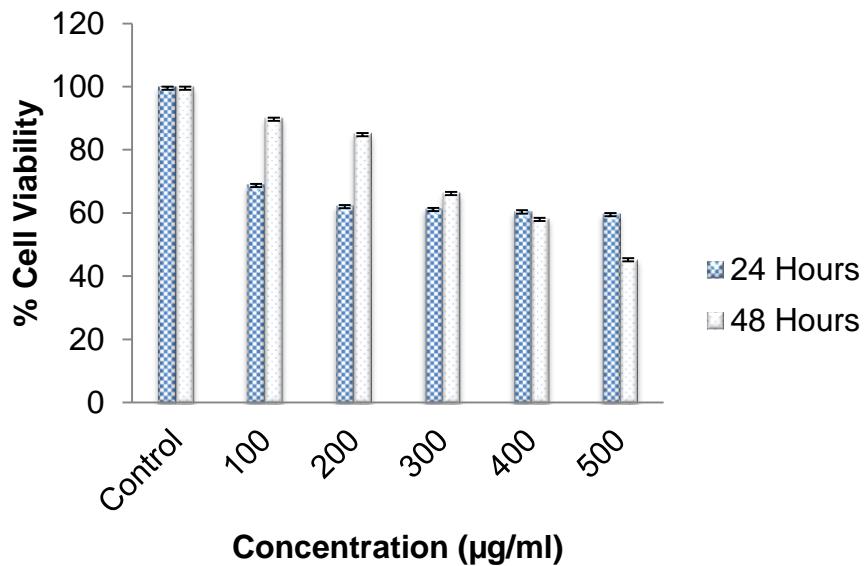
**Figure 7.3:** Cell viability and morphology of Bud-8 cell line after treated with dichloromethane extracts at concentration ranging from 100 and 500 µg/ml.



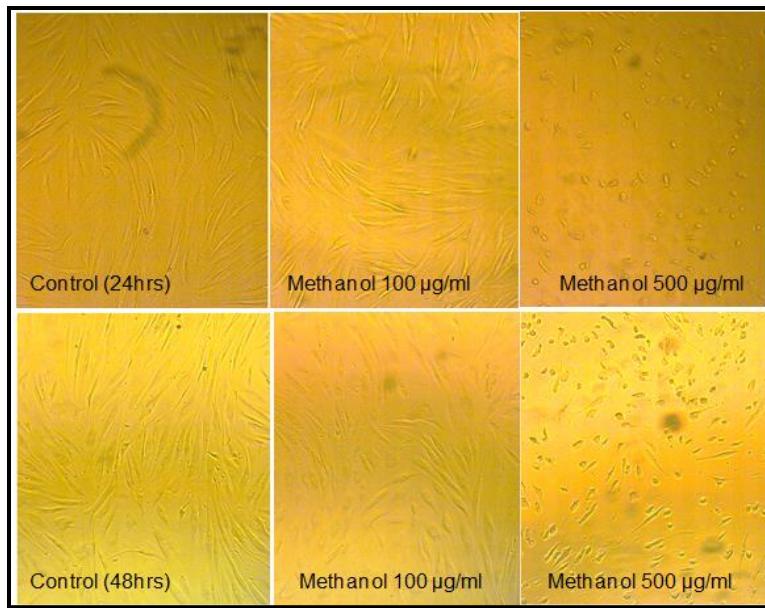
**Figure 7.4:** The cytotoxicity effects of *R. communis* dichloromethane extracts against Bud-8 cell line after 24 and 48 hours of exposure using MTT assay at the concentration of 100 – 500 µg/ml.



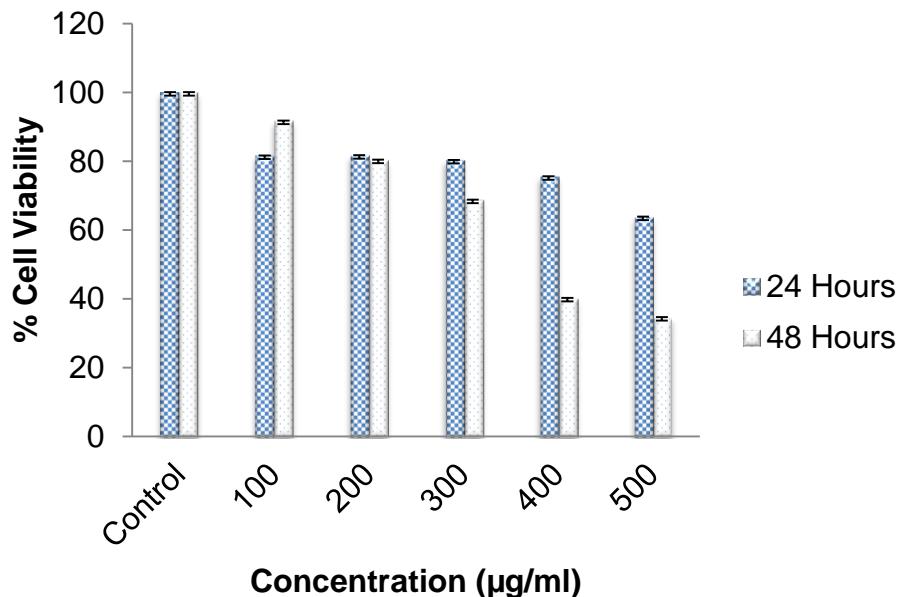
**Figure 7.5:** Cell viability and morphology change of Bud-8 cell line after treated with acetone extracts at concentration ranging from 100 and 500 µg/ml.



**Figure 7.6:** The cytotoxicity effects of *R. communis* acetone extracts against Bud-8 cell line after 24 and 48 hours of exposure using MTT assay at the concentration of 100 – 500 µg/ml.



**Figure 7.7:** Cell viability and morphology change of Bud-8 cell line after treated with methanol extracts at concentration ranging from 100 and 500 µg/ml.



**Figure 7.8:** The cytotoxicity effect of *R. communis* methanol extracts against Bud-8 cell line after 24 and 48 hours of exposure using MTT assay at the concentration of 100 – 500 µg/ml.

The LC<sub>50</sub> of *R. communis* extracts on Bud-8 cell line was calculated for each extract after 24 and 48 hours of exposure (Table 7.1). LC<sub>50</sub> indicate the lowest concentration of plant extracts that inhibits 50% of cells. Methanol extract showed a good LC<sub>50</sub> of 784 µg/ml after 24 hour exposure, followed by hexane (629.3 µg/ml), dichloromethane (573.6 µg/ml) and the lowest was in acetone extract (544.6 µg/ml). The LC<sub>50</sub> for all crude extracts decreased after 48 hours exposure. Hexane had the highest LC<sub>50</sub> of 495 µg/ml and methanol had the lowest LC<sub>50</sub> of 387.1 µg/ml. Extracts with high LC<sub>50</sub> are preferable to work with, because of their lower toxicity effects on the cells (Table 7.1).

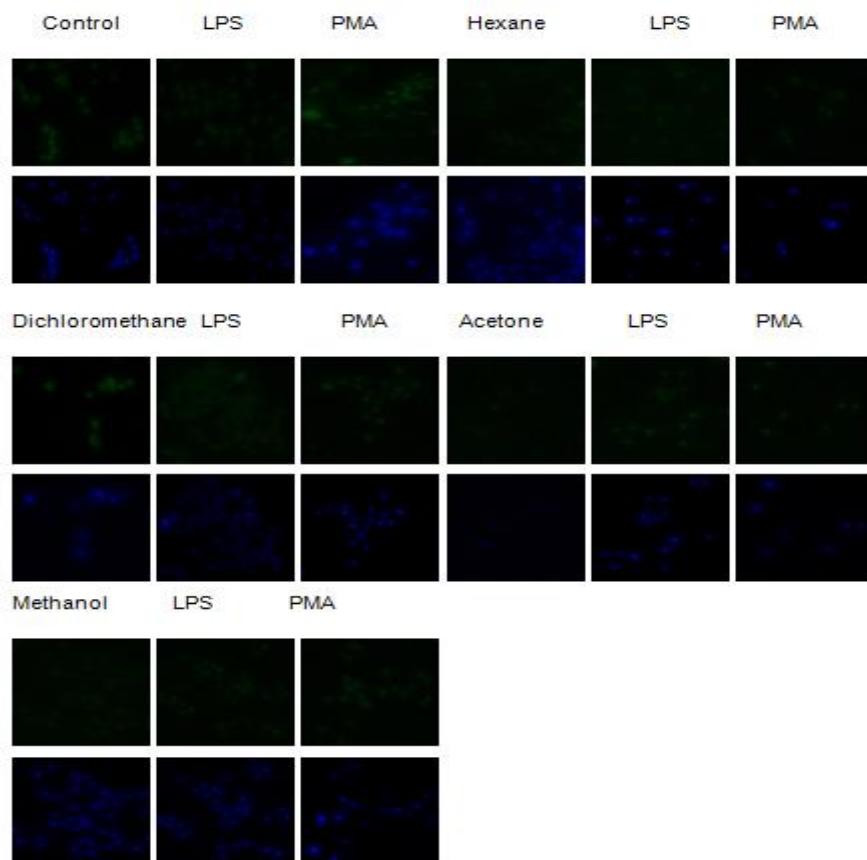
**Table 7.1:** The LC<sub>50</sub> of *R. communis* extracts on BUD-8 cell lines after 24 and 48 hours of exposure.

	LC <sub>50</sub> (µg/ml)	
Extracts	24 hours	48 hours
Hexane	629.3	495
Dichloromethane	573.6	467
Acetone	544.5	471
Methanol	784	387.1

### 7.3.2. Anti-inflammatory activity assay

Hexane, dichloromethane, acetone and methanol were used to extract anti-inflammatory compounds constitutive in *R. communis* leaves. The fluorogenic cell permeant, H<sub>2</sub>DCF-DA (green cells) was used to measure the level of reactive oxygen species in Raw 264.7 macrophage cells induced by LPS and PMA stimulants (Figure 7.9). High level of reactive oxygen species tends to attack macromolecules and this

facilitates cells to undergo oxidative stress and inflammatory response. Antioxidant compounds scavenge the free radical molecules by donating one electron or proton to a molecule. High intensity of green colour is the indication of high level of free radical molecules which can lead cell to undergo oxidative stress and inflammation. A reduced internal H<sub>2</sub>DCF fluorescence intensity is the indication of reduced free radical molecules by antioxidant compounds extracted from *R. communis* leaves. Combination of hexane and acetone extracts with PMA and LPS stimulants depicts low fluorescence intensity as compared to PMA and LPS stimulated cells. Cells were stained with DAPI (blue cells) to locate the nucleus in the cells (Figure 7.9).



**Figure 7.9:** Effect of *R. communis* extracts on Raw 264.7 macrophages cells in respond to oxidative stress and inflammation. Reactive oxygen species were induced by LPS and PMA stimulants. Cells were fixed and stained with H<sub>2</sub>DCF-DA to measure the amount of free radical molecules produced in the cells (green) and DAPI to locate the nucleus in the cells (blue).

## 7.4. DISCUSSION

Medicinal plants have unlimited capacity to synthesis bioactive compounds that are effective and have fewer side effects compared to synthetic drugs. Bioactive compounds from plants revealed various biological activities including anticancer, antibacterial, antioxidant and anti-inflammatory activity. Scientists have developed a greater interest of using these compounds in formulation of new and novel drugs, because of their biological activities and reliability (Vijayarathna and Sasidharan, 2012). Some of these compounds are toxic to our normal system; therefore safety is critical in development of novel drugs (Morobe *et al.*, 2012). In this study cytotoxicity effect of hexane (Figure 7.2), dichloromethane (Figure 7.4), acetone (Figure 7.6) and methanol (Figure 7.8) extracts was investigated on Bud-8 cell line using MTT assay. The experiment was evaluated for 24 and 48 hours of exposure. Percentage cell viability was calculated by measuring the absorbance of formazan formed from reduction of MTT solution by the presence of mitochondrial dehydrogenase in viable cells. Morphology of cells was also shown after treatment of cells with plant extracts at 100 µg/ml and highest concentration of 500 µg/ml. Hexane extracts had the highest percentage cell viability of 93% at the lowest concentration of 100 µg/ml and 62% cell viable at 500 µg/ml (Figure 7.2). Cell viability decreased with the increase in concentrations of plant extracts. Morphology of cells was also altered from its normal shape of fibroblast to oval or circular shapes (Figure 7.3 – 7.8). Higher concentrations of *R. communis* extracts seem to be toxic on Bud-8 cell line. Rana *et al.* (2012) reported the toxicity effect of ricin compound isolated from the seeds of *R. communis*. Ricin kills the cells by disrupting protein synthesis in the cells. *Ricinus communis* agglutinin I (RCA I) have been reported to induce apoptosis on tumor cells and had no toxic effect on normal blood vessels (Rana *et al.*, 2012). Ricin was the only compound isolated from *R. communis* seeds that was reported for high toxic effect on mammalian cells.

Methanol extracts of *R. communis* leaves showed minimal cytotoxicity effect on Bud-8 cell line with the highest LC<sub>50</sub> of 784 µg/ml, followed by hexane, dichloromethane and acetone (544.5 µg/ml) after 24 hours of exposure (Table 7.1). Morobe *et al.* (2012) also reported low toxic effect of methanol extracts on MAGI CCR5+ cell lines. Methanol

extract of *R. communis* seems to be the promising extract to work in formulation of drugs. But methanol extracts showed weak antibacterial activity against tested pathogens (Chapter 5) and strong antioxidant activity (Section 4.3.2). Results suggest methanol extracts as a good source of reliable and nontoxic antioxidant compounds. After 48 hours of exposure hexane had the highest LC<sub>50</sub> of 495 µg/ml and the lowest was 387.1 µg/ml (methanol extract) (Table 7.1).

Roots extracts of *R. communis* have been reported to neutralize free radical molecules and anti-inflammatory activity. The same study suggested that the anti-inflammatory activity of roots extracts of *R. communis* was because of the presence of flavonoids (Ilavarasan *et al.*, 2006). Dihydrodichlorofluorescein diacetate (H<sub>2</sub>DCF-DA) was used to measure the level of reactive oxygen species in activated Raw 264.7 macrophages. Cells were stimulated with LPS and PMA to induce high level of free radicals molecules in the cells. Cells were treated with *R. communis* leaves extracts (hexane, dichloromethane, acetone and methanol) for 24 hours (Figure 7.9). High intensity of green colour in the cell indicates the presence of high level of free radical molecules. H<sub>2</sub>DCF-DA penetrated through the plasma membrane of the cell and then de-esterified to a hydrophilic alcohol dihydrodichlorofluorescein (H<sub>2</sub>DCF). The H<sub>2</sub>DCF is oxidized to fluorochrome DCF (2, 7-dichlorofluorescein) by the presence of reactive oxygen species that fluoresce green when excited with blue light. The brightness of DCF fluorescence reflected the level to which ROS are present in the cell (Karlsson *et al.*, 2010). Macrophage cells that were treated with LPS and PMA stimulants showed high level of ROS in the cells (brightness of green colour). Reactive oxygen species act as the mediator to regulate the cytokines production through activation of the transcription factors, such as NF-kB. This suggests the direct link of ROS and other cytokines to initiate inflammatory response (Zhou *et al.*, 2007). A reduced internal DCF fluorescence (green) intensity indicates the decrease level of ROS by presence of antioxidant compounds from *R. communis* extracts. Hexane and acetone extracts combined with LPS and PMA stimulants depict low fluorescence intensity (green) as compared to cells treated with PMA and LPS stimulants only (Figure 7.9). Therefore, hexane and acetone extracts reduced level of ROS (green) in the macrophages cells either by controlling the pathway responsible for production of ROS or by scavenging the ROS present.

Methanol extract also reduced the level of ROS while dichloromethane extract did not reduce ROS level in the cells. Methanol extracts of *R. communis* have been reported to possessed flavonoid. Flavonoids have also been reported as the anti-inflammatory agent of medicinal plants (Saini *et al.*, 2010). Flavonoids may be responsible for anti-inflammatory activity of *R. communis* leaves. Macrophage cells were stained with DAPI (blue), fluorescent dye that binds to DNA, to locate the nucleus of the cells and also to confirm the viability of the cells (Figure 7.9). The root extracts of *R. communis* were reported for a strong anti-inflammatory compounds (Ilavarasan *et al.*, 2006). This study was the first study to report on the cytotoxicity effect and anti-inflammatory activity of *R. communis* leaves on bud-8 cell lines and macrophage cell line respectively. The present study also agrees with the previous findings concerning the potent anti-inflammatory activity of *R. communis* leaves.

## 7.5. CONCLUSION

The leaves of *R. communis* showed low cytotoxicity effect on Bud-8 cell lines. Methanol extracts seem to be the best extract to isolate compounds from this plant because of its minimal cytotoxicity effect on Bud-8 cell line. Hexane, dichloromethane and acetone extracts were less toxic to Bud-8 cell line compared to methanol extracts. The present study revealed that *R. communis* extracts are safer at low concentrations. Active compounds from this plant may be isolated and used as a precursor in the development of novel drugs. The present study also confidently report on the presence of anti-inflammatory compounds in the leaves of *R. communis*. These compounds may be isolated and used in the formulation of anti-inflammatory drugs. *R. communis* leaves may be used as the natural anti-inflammatory therapeutic agents because of its reliability. The study present the first report on the cytotoxicity effect of *R. communis* leaves extracts on Bud-8 cell line and anti-inflammatory activity of *R. communis* leaves on Raw 264.7 macrophage cell line respectively. The study serves as a scientific proof for use of *R. communis* leaves in traditional medicine for treatment of inflammation diseases.

## CHAPTER 8

### STRUCTURE ELUCIDATION

#### **8.1. INTRODUCTION**

Secondary metabolites such as alkaloids, steroids, terpenoids, and phenolic compounds are responsible for biological activities of plants. It is very important to isolate and elucidate the structure of bioactive compounds for development of new pharmaceuticals drugs (Patra *et al.*, 2012). After isolation and purification of bioactive compounds using various separation techniques, pure compounds are analysed for structure elucidation using various spectroscopic methods such as Nuclear magnetic resonance (NMR) and mass spectroscopy (MS). NMR coupled with MS provides sufficient data of structural elucidation and accurate identification of chemical compounds (Sasidharan *et al.*, 2011).

NMR is a powerful technique that has been used global for quantitative structure elucidation of pure bioactive compounds from natural sources. Compounds which are highly stable and well purified may be used as standards for quantitative NMR calibration. NMR has the following advantages; non-destructive character, common nature of detection and elucidate structure of target compound from the mixture. NMR assays are either developed using internal standards (IS) or external standards (ES) for NMR calibration (Gödecke *et al.*, 2012).

MS is a spectroscopic which plays a role in the structural determination of organic molecules. MS is a highly sensitive method used for structural determination of natural products. MS has the following advantages; it is flexible in the detection. A major characteristic feature of MS is the application of physical principle for sample ionization and separation of the ions produced according to their mass to charge ratio. MS data is also used for determination of molecular mass of a compound. It determines the number of hydrogens and carbons that are present on a compound from  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectrum respectively. MS provides the details of the complete elucidated structure of a compound known as chemical or molecular formula of a compound

(Stobiecki, 2000). The aim was to elucidate the structure of isolated compound using Nuclear Magnetic Resonance (NMR) and mass spectrometry (MS).

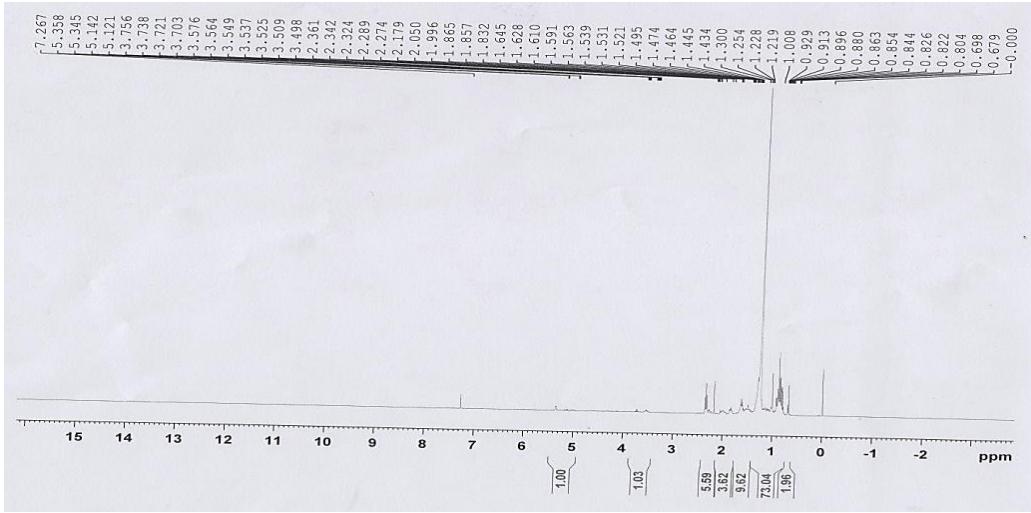
## 8.2. METHODS AND MATERIALS

### 8.2.1. Characterization of pure compounds by nuclear magnetic resonance

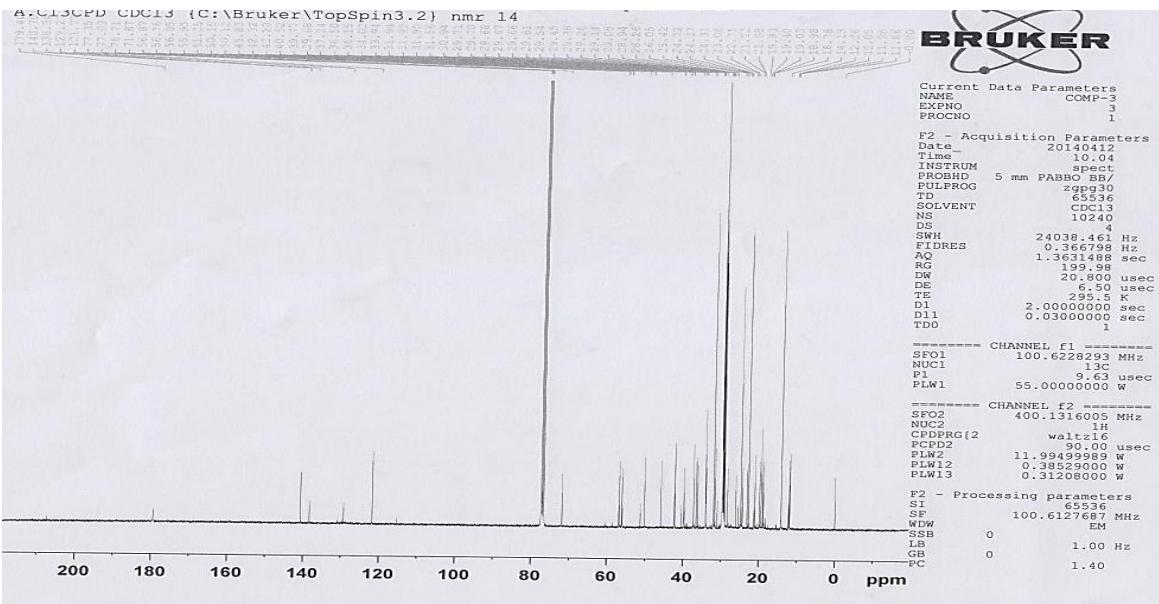
After isolation of compounds from *R. communis* leaves using column chromatography, pure compounds were sent to Dr Mampa, R.M., of the Chemistry Department, University of Limpopo (Turfloop campus) for assistance with NMR. In preparation of NMR samples, compound **2** (8.7 mg) was dissolved in 1 ml of deuterated acetone (Acetone-d) while compounds **3** (3.7 mg), **4** (102.3 mg) and **5** (4.7 mg) were dissolved in deuterated chloroform (Chloroform-d) in  $^1\text{H}$ ,  $^{13}\text{C}$ , and DEPT 135 of the prepared samples were run using 400 MHz NMR Spectrometer (Bruker) at 400 MHz, chloroform-d as a reference signal solvent, number of scans were 10240 and at a temperature of 295.5 K. Dr Mdee, L.K., of the Pharmacy Department, University of Limpopo (Turfloop Campus) assisted with the analysis of the NMR spectrums and structure elucidation of the compounds.

## 8.3. RESULTS

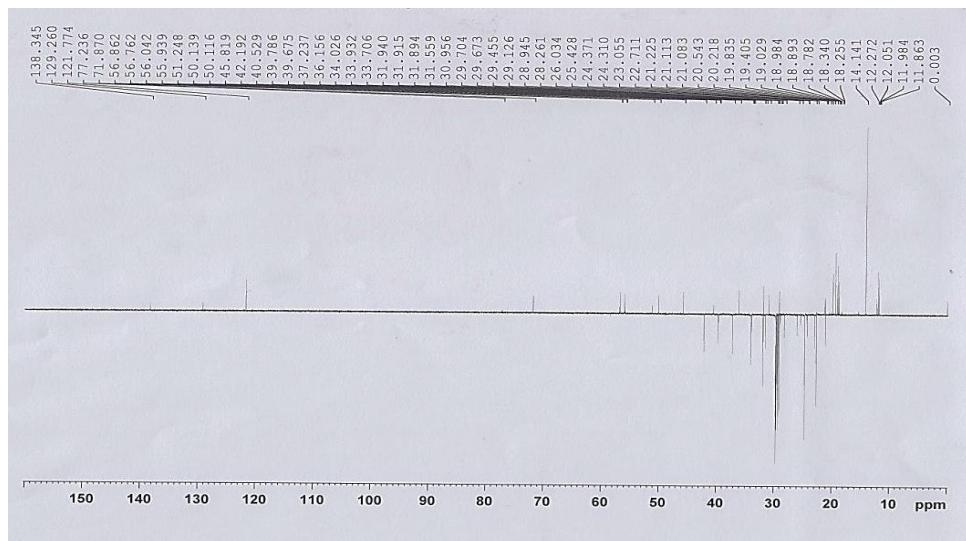
The  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}$ -DEPT 135 NMR spectrums for all compounds subjected to 400 MHz NMR spectrometer are shown in Chapter 11, (Appendix). Due to the small amount isolated of compound **1**(data not shown), **2**, **3** and **5** (Appendix), their structures were not elucidated because observed spectrums suffered from a poor signal-to-noise ratio, samples were too diluted. A spectral analysis of compound **4** was very promising and chemical shifts of compound **4** were recorded and compared with the known chemical shift of Stigmasterol and  $\beta$ -Sitosterol (Table 8.1) (Kpoviessi *et al.*, 2006). Compound **4** was identified as the mixture of Stigmasterol and  $\beta$ -Sitosterol (Figure 8.14). Expansions of the NMR spectrums are shown in Chapter 11 appendix.



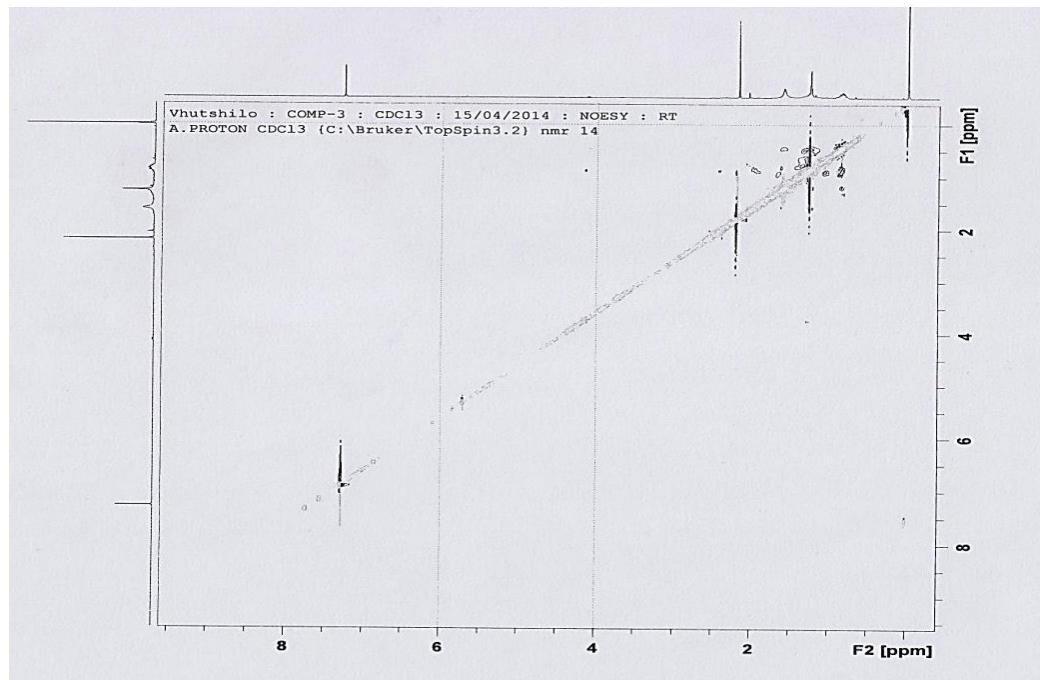
**Figure 8.1:**  $^1\text{H}$  NMR spectrum of **Compound 4**.



**Figure 8.2:**  $^{13}\text{C}$  NMR spectrum of **Compound 4**.



**Figure 8.3:** DEPT 135 -NMR spectrum of **Compound 4**.

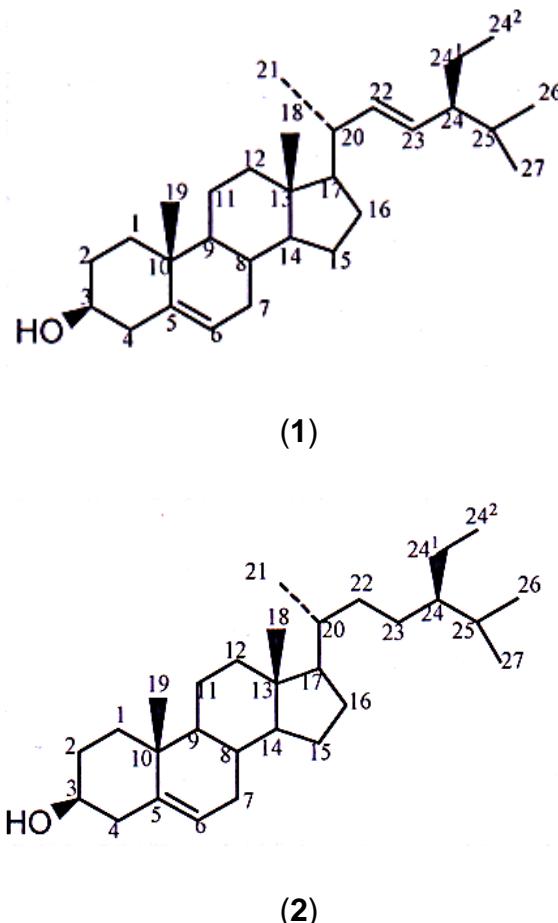


**Figure 8.4:** Noesy spectrum of **Compound 4**.

**Table 8.1:** NMR data of  $^{13}\text{C}$ -DEPT 135 NMR chemical shift values for Stigmasterol (**1**) and  $\beta$ -Sitosterol (**2**) recorded in  $\text{CDCl}_3$ , compound **4** major (Stigmasterol) and minor ( $\beta$ -Sitosterol) were isolated from *R. communis* hexane extracts.

Position	NMR DATA			
	$^{13}\text{C}$ chemical shift (ppm)			
	$^{13}\text{C}$ reference (1) Kpoviessi et <i>al.</i> , 2006	Compound <b>4</b> major ( <b>1</b> )	$^{13}\text{C}$ reference (2) Kpoviessi <i>et al.</i> , 2006	Compound <b>4</b> minor ( <b>2</b> )
1	37.3	<b>37.2</b>	37.3	<b>37.2</b>
2	31.6	<b>31.6</b>	31.9	<b>31.9</b>
3	71.8	<b>71.9</b>	71.8	<b>71.9</b>
4	42.3	<b>42.2</b>	42.3	<b>42.2</b>
5	140.8	<b>140.7</b>	140.8	<b>140.7</b>
6	121.7	<b>121.8</b>	121.7	<b>121.8</b>
7	31.9	<b>31.9</b>	31.9	<b>31.9</b>
8	31.7	<b>31.8</b>	31.9	<b>31.9</b>
9	50.2	<b>50.1</b>	50.2	<b>50.1</b>
10	36.5	<b>36.5</b>	36.5	<b>36.5</b>
11	21.1	<b>21.0</b>	21.2	<b>21.0</b>
12	39.8	<b>39.8</b>	39.7	<b>39.7</b>
13	42.3	<b>42.3</b>	42.2	<b>42.3</b>
14	56.7	<b>56.8</b>	57.0	<b>56.9</b>
15	24.3	<b>24.3</b>	24.4	<b>24.4</b>
16	28.3	<b>28.3</b>	28.9	<b>28.9</b>
17	56.1	<b>56.0</b>	56.8	<b>55.9</b>
18	11.9	<b>11.9</b>	12.3	<b>12.3</b>
19	19.4	<b>19.4</b>	19.4	<b>19.4</b>
20	36.2	<b>36.1</b>	40.5	<b>40.5</b>
21	18.8	<b>18.8</b>	21.2	<b>21.2</b>
22	34.0	<b>34.0</b>	138.3	<b>138.3</b>
23	26.1	<b>26.0</b>	129.3	<b>129.6</b>
24	45.9	<b>45.8</b>	51.3	<b>51.2</b>
25	29.2	<b>29.1</b>	31.9	<b>30.9</b>
26	19.0	<b>19.0</b>	21.1	<b>21.2</b>
27	19.8	<b>19.8</b>	19.0	<b>18.9</b>
24 <sup>1</sup>	23.1	<b>23.1</b>	25.4	<b>25.4</b>
24 <sup>2</sup>	12.3	<b>12.3</b>	12.1	<b>12.0</b>

The bioassay-guided fractionation of hexane extract of *R. communis* leaves by column chromatography coupled with 400 MHz NMR spectroscopy successful led to the isolation and identification of stigmasterol and  $\beta$ -sitosterol (Figure 8.14). Structures of stigmasterol and  $\beta$ -sitosterol were investigated through the analysis of  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{13}\text{C}$ -DEPT spectrums of compound 4. Compound 4 was confirmed as the mixture of stigmasterol and  $\beta$ -sitosterol (Figure 8.14).



**Figure 8.5:** Structure of Stigmasterol (1) and  $\beta$ -Sitosterol (2) isolated from *R. communis* leaves.

#### **8.4 DISCUSSION AND CONCLUSION**

The structures of the isolated compounds were elucidated using spectroscopic analysis, NMR spectra. The structures of compound **1**, **2**, **3** and **5** were not identified due to the poor NMR spectrums from a poor signal to noise ratio. In the present study, compound **4** was isolated from *R. communis* leaves and identified as the mixture of two sterols namely stigmasterol ( $C_{29}H_{48}O$ ) and  $\beta$ -sitosterol ( $C_{29}H_{50}O$ ). Stigmasterol and  $\beta$ -sitosterol are well known compounds and they have been isolated from roots of *R. communis* (Ali and Mitta, 2012; Bayaso *et al.*, 2013). These compounds have been isolated from various indigenous plants worldwide. Kpoviessi *et al.* (2006) also reported isolation of stigmasterol and  $\beta$ -sitosterol from *Justicia ansellian*. Stigmasterol and  $\beta$ -sitosterol were also isolated from chloroform extract of leaves of *Corchorus fascicularis* Lam and their pharmacological activities were also reported including anti-inflammatory activity (Jain and Bari, 2010; Kamboj and Saluja, 2011; Rajput and Rajput, 2012). Compound **4** was not further studied for its biological activities including antibacterial activity due to the lack of sufficient materials. In the literature, stigmasterols and  $\beta$ -sitosterols have been reported to exhibit antitumor activity, cholesterol lowering activity, antibacterial activity and anti-inflammatory activity (Ghosh *et al.*, 2011; Venkata-Raman *et al.*, 2012; Saeidnia *et al.*, 2014). In conclusion, compound **4** was confirmed as the mixture of stigmasterol and  $\beta$ -sitosterol.

## CHAPTER 9

### GENERAL DISCUSSION AND CONCLUSION

The leaves of *Ricinus communis* were screened for antioxidant, anti-inflammatory, cytotoxicity effect and antibacterial activities based on the use of the plant traditionally for medicinal purposes in developing countries. Crude extracts were prepared using number of extraction methods and solvents of varying polarities to maximize the activity of the plant. Methanol extracted a greater quantity of extracts compared to other solvents. The important aspect of the present study was not the quantity, but the activity of the plant. Secondary metabolites of plants are well-known to play a major role in biological activities of traditional medicinal plants. The findings in this study suggest that flavonoids, tannins, terpenoids, steroids and alkaloids were responsible for antioxidant and antibacterial activities of *R. communis* leaves. Phlabatannins and saponins are not responsible for any biological activity present in *R. communis* leaves. Phytochemical compounds are the major elements of medicinal properties of traditional medicinal plants (Saxena *et al.*, 2013).

*R. communis* is one of traditional medicinal plant belongs to family Euphorbiaceace. Plants belong to this family include *Calypha ciliata*, *Croton bonplandianum*, *Euphorbia geniculata*, *Euphorbia hirta L.*, *Jatropha gossypifolia L.*, and *Pedilanthus tithymaloides L* and are known for their pharmacological activity including antioxidant and antibacterial activity (Kothale *et al.*, 2011). In this study, *R. communis* leaves (acetone extracts) showed powerful antioxidant activity against free radical molecules (2, 2-diphenyl-1-picrylhydrazyl) compared to other solvents based on the qualitative DPPH – TLC assays. Quantitative antioxidant activities using DPPH and ABTS free radical molecules also showed high percentage scavenging activity of free radicals by *R. communis* leaves. The findings in this study recommend *R. communis* leaves as the potential source of antioxidant compounds to act against reactive oxygen species to reduce the risk of degenerative diseases and other oxidative stress related diseases.

According to Eloff (1998), acetone was used to reconstitute *R. communis* extracts for quantitative and qualitative antibacterial assays against two Gram positive (*S. aureus*

and *E. faecalis*) and negative bacteria (*E. coli* and *P. aeruginosa*). The leaves of *R. communis* showed slightly antibacterial activity against tested pathogens on qualitative TLC antibacterial assays. After treating plant materials with hexane, to remove chlorophyll and fatty acids, the activity against all tested pathogens was maximized on bioautograms. The serial dilution method was used to quantify the activity of the plant and the lowest concentration which inhibited tested pathogens was 0.31 mg/ml with the highest total activity of 136 ml/g. *E. coli* with average MIC value of 0.96 mg/ml, *P. aeruginosa* (0.50 mg/ml) and *E. faecalis* (0.95 mg/ml) were highly sensitive to all plant extracts. *S. aureus* was less sensitive against *R. communis* leaves on qualitative TLC antibacterial assays. Extracts with low MIC values and high total activity are preferable to work with for development of antibacterial drugs. The findings in the present study suggest that the leaf of *R. communis* was the best source of potent antibacterial compounds to fight against tested microorganisms.

*In vitro* cytotoxicity effect of *R. communis* extracts was also conducted on Bud-8 cell line using MTT assay. Higher concentrations of *R. communis* extracts seem to be toxic on Bud-8 cell even though more than 60% cell viability was obtained. Methanol extract had a good LC<sub>50</sub> of 784 µg/ml after 24 hour exposure and the lowest was in acetone extract (544.6 µg/ml). Plant extracts with high LC<sub>50</sub> are preferable to work with for development of novel drugs. *R. communis* extracts are promising extracts to work with since higher concentration of extracts is required to kill 50% of normal cells. The plant extracts seem to inhibit the cell growth in the first 24 hours of incubation and cell growth increases after 48 hours of incubation, this was observed in acetone extracts. The lowest concentration of 100 µg/ml showed insignificant decrease of cell compared to control after 24 hours of incubation in hexane and DCM extracts (90% cell viability). The lowest concentration that showed insignificant different in cell viability compared to control was used to test for anti-inflammatory activity on Raw 264.7 macrophage cell line using phagoburst assay with two stimulants (LPS and PMA). Free radicals molecules are major cause of inflammation related diseases (Karlsson *et al.*, 2010). Fluorogenic cell permeant H<sub>2</sub>DCF-DA was used to measure the level of reactive oxygen species present in Raw 264.7 macrophage cells induced by LPS and PMA stimulants. Combination of hexane and acetone extracts with PMA and LPS stimulants showed low fluorescence

intensity as compared to cells stimulated PMA and LPS stimulated cells. The results suggest *R. communis* leaves to possess anti-inflammatory activity to reduce risk of ailments caused by oxidative stress. *R. communis* leaves hold promise for isolating anti-inflammatory, antibacterial and antioxidant compounds.

Bioassay-guided fractionation on silica gel 60 (particles size 0.063-0.200 mm) in column chromatography resulted in the successful isolation of antioxidant and antibacterial compounds. Compounds were targeted based on their biological activities using qualitative DPPH assay on TLC plates for antioxidant activity and bioautography for antibacterial activity. Antioxidant compounds showed strong antioxidant activity after spraying with DPPH in methanol and antibacterial compounds showed less activity compared to the crude extracts. Five pure compounds were isolated from *R. communis* leaves compound **1** (1 mg), **2** (8.7 mg), **3** (3.7 mg), **4** (102.3 mg) and **5** (4.7 mg) and subjected into NMR spectroscopy for structure elucidation using following experiments (<sup>1</sup>H, <sup>13</sup>C, DEPT and NOESY). The structures of compound **1**, **2**, **3** and **5** were not identified due to insufficient materials, however compound **4** was successfully identified as the mixture of two phytosterols namely stigmasterol (C<sub>29</sub>H<sub>48</sub>O) and β-sitosterol (C<sub>29</sub>H<sub>50</sub>O). The <sup>13</sup>C-DEPT NMR data and chemical shifts of compound **4** were compared and found similar to those recorded for stigmasterol and β-sitosterol (Kpoviessi *et al.*, 2006; Chaturvedula and Prakash, 2012). These two compounds may be the major elements of anti-inflammatory activity of *R. communis*. Ntandoua *et al.* (2010) reported the anti-inflammatory activity of stigmasterol and β-sitosterol from *Cassia siamea* Lam. stem bark extracts. The stigmasterol and β-sitosterol are well known and they have been previously isolated from *R. communis*. Stigmasterols have been isolated from ether extract of seeds of *R. communis* (Jena and Gupta, 2012). Stigmasterol and β-sitosterol have been isolated from roots of *R. communis* (Ali and Mitta, 2012; Bayaso *et al.*, 2013). Stigmasterols and β-sitosterols are natural products of phytosterols and they possessed antitumor activity, cholesterol lowering activity, antibacterial activity, anti-inflammatory activity and antifertility activity (Ghosh *et al.*, 2011; Venkata-Raman *et al.*, 2012; Saeidnia *et al.*, 2014). Stigmasterol and β-sitosterol were also isolated from the leaves of *Rubus suavissimus*, which has been used to make beverage leaf tea in China (Chaturvedula and Prakash, 2012). β-sitosterol has been

also isolated from petroleum ether extract of *Abutilon indicum* and it revealed moderate larvicidal activity (Saeidnia *et al.*, 2014). Gomes *et al.*, 2007 isolated the mixture of stigmasterol and  $\beta$ -sitosterol from the root extract of *Pluchea indica*, which has been used against snakebite. Saeidnia *et al.*, 2014 reported antibacterial activity of  $\beta$ -sitosterol against *E. coli*, *P. aeruginosa* and *S. aureus* using disc diffusion method with the zone of inhibition ranged 10-14 mm but there was no activity with MICs value above 200  $\mu\text{g}/\text{ml}$ . The study indicates that the leaves of *R. communis* possessed high antioxidant, anti-inflammatory and antibacterial properties. This explains the use of *R. communis* leaves traditionally as medicines for treatment of diverse diseases related to bacterial infections and oxidative stress. The aims of the project have largely accomplished and it appears that there may be scope for continuing work on *R. communis* extracts such as *in vivo* studies.

## CHAPTER 10

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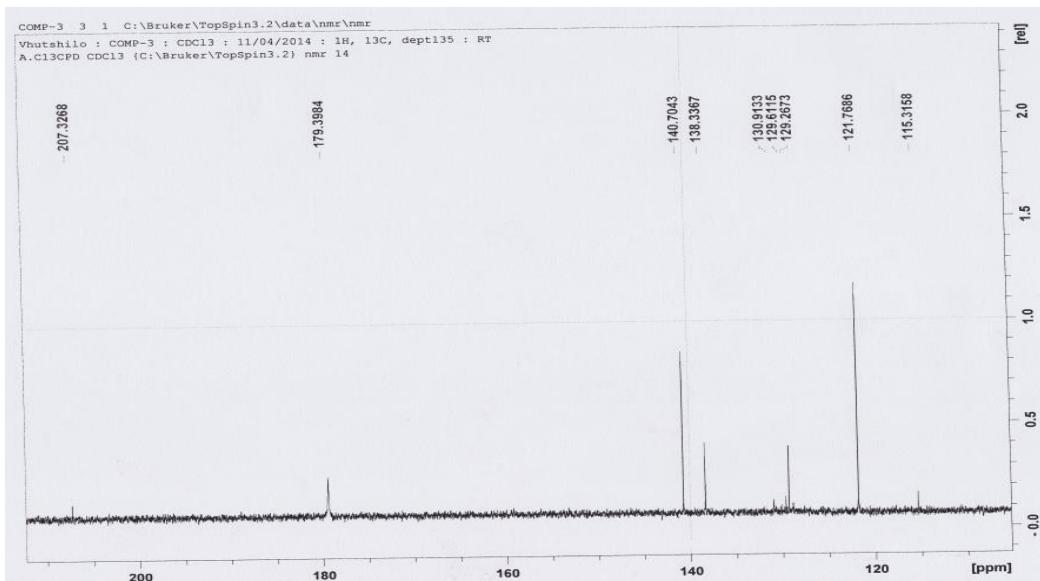
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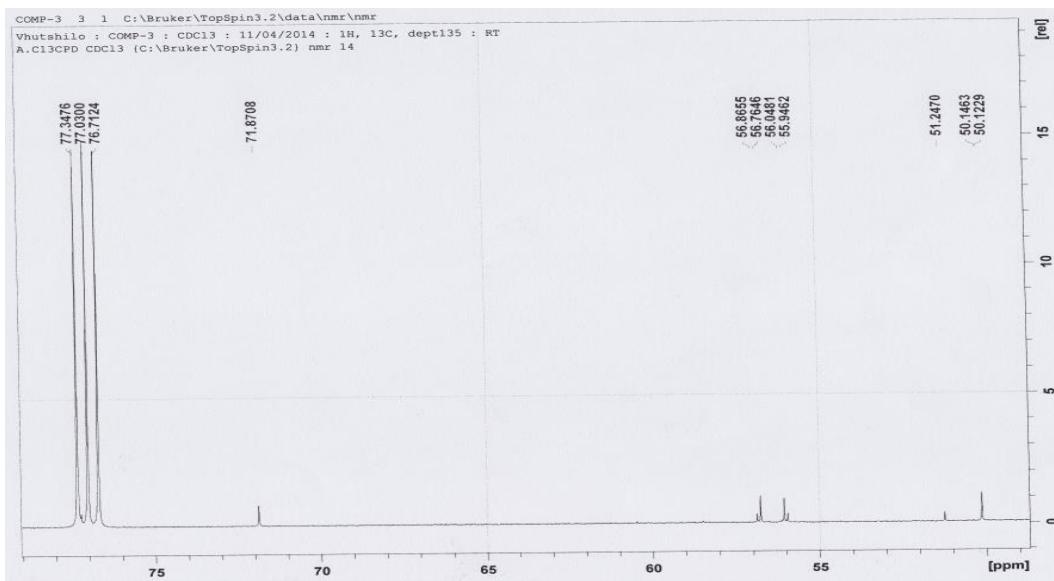
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# CHAPTER 11

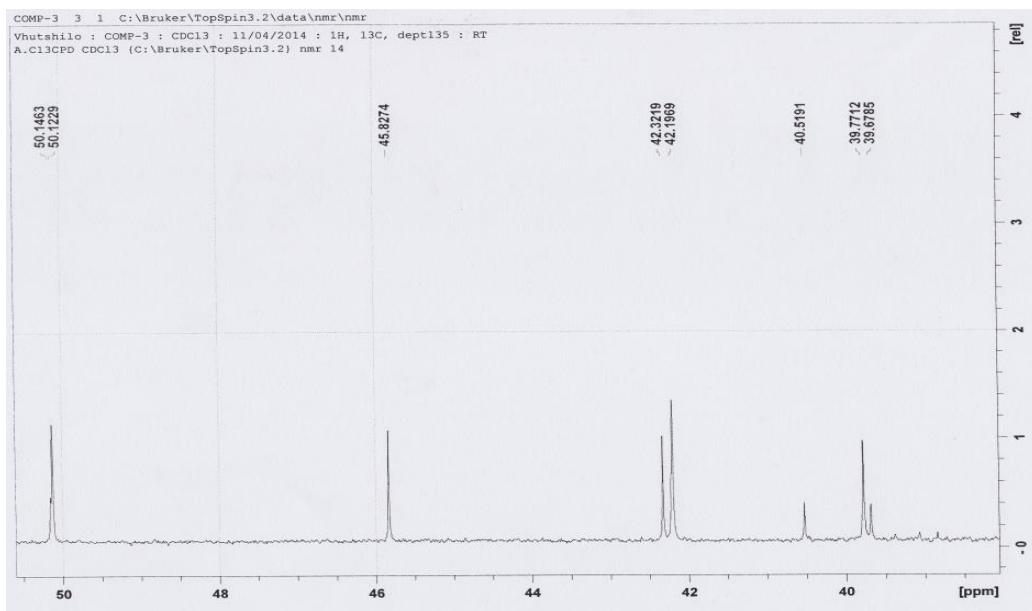
## APPENDIX



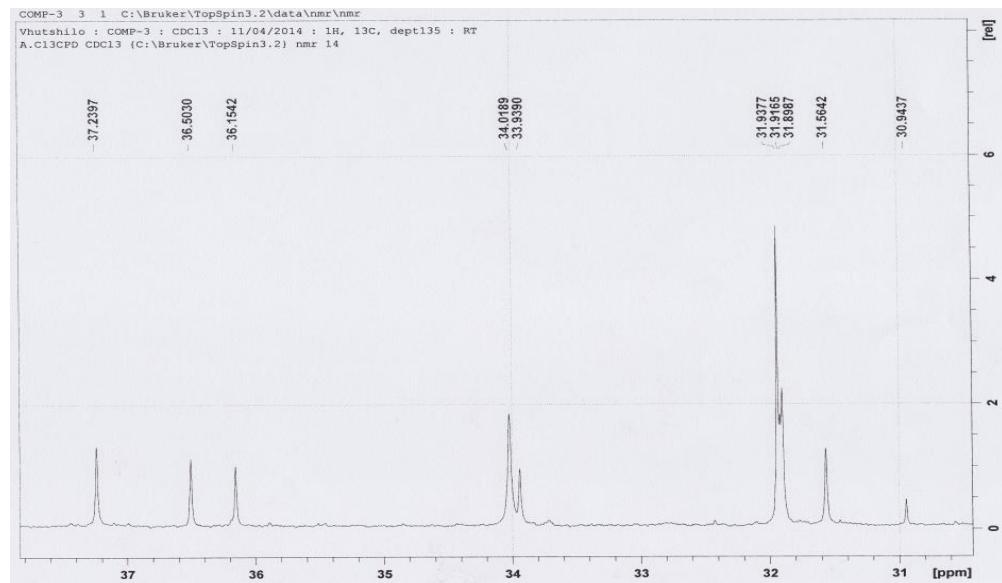
**Figure 11.1:** Expansion of  $^{13}\text{C}$ -NMR spectrum of compound 4 from 200 to 120 ppm.



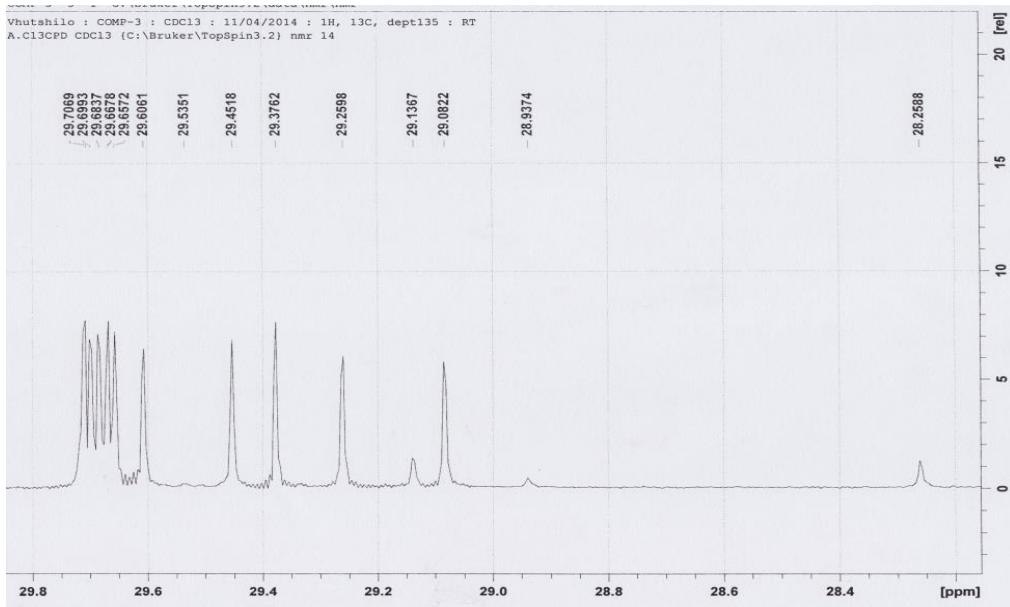
**Figure 11.2:** Expansion of  $^{13}\text{C}$ -NMR spectrum of compound 4 from 75 to 55 ppm.



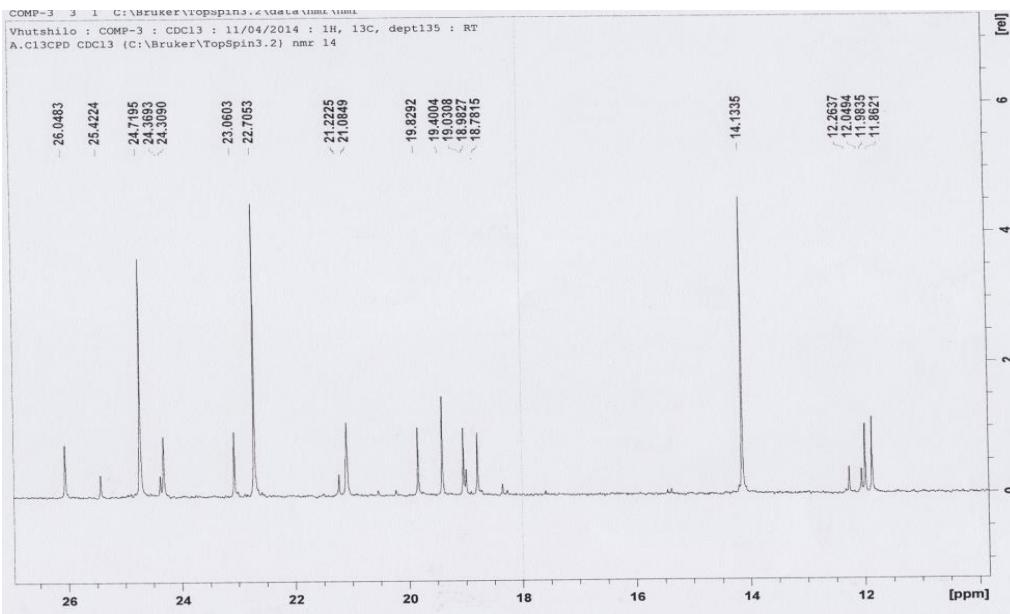
**Figure 11.3:** Expansion of <sup>13</sup>C- NMR spectrum of compound 4 from 50 to 40 ppm.



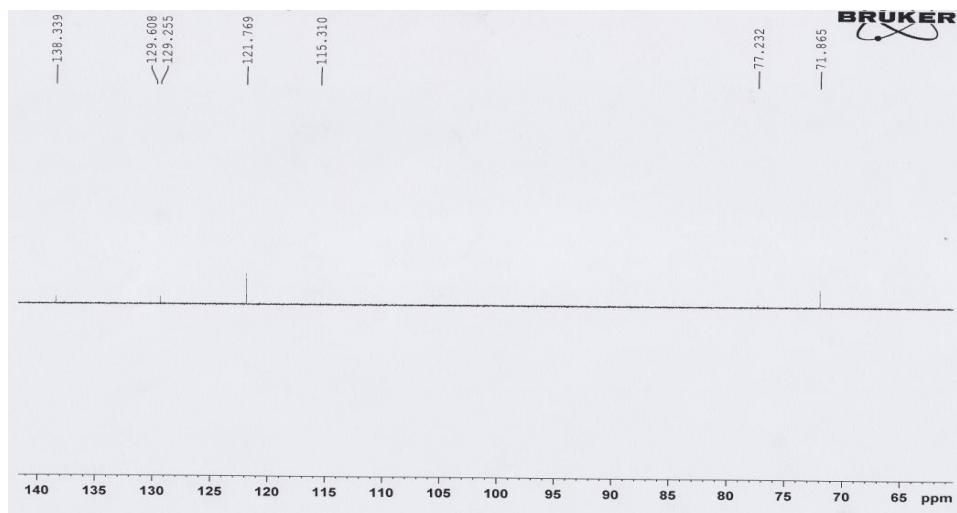
**Figure 11.4:** Expansion of <sup>13</sup>C -NMR spectrum of compound 4 from 37 to 31 ppm.



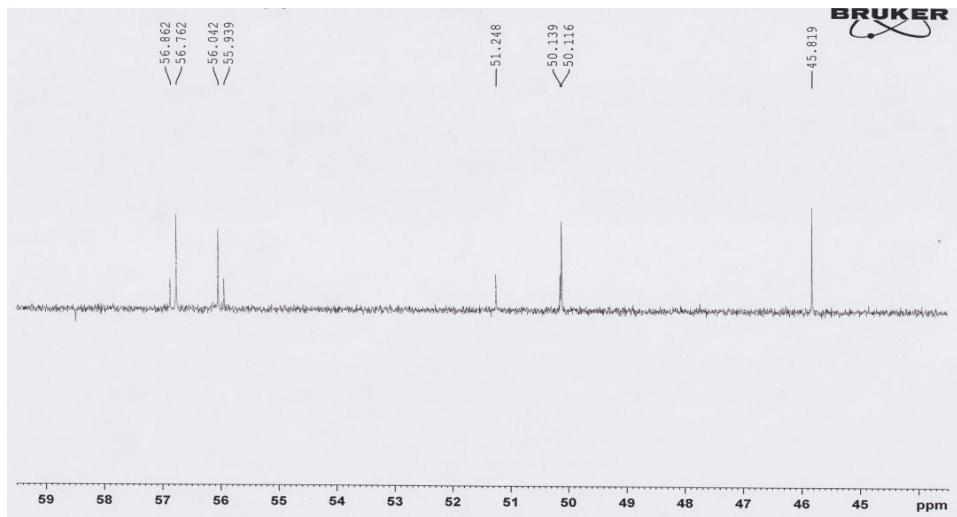
**Figure 11.5:** Expansion of <sup>13</sup>C -NMR spectrum of compound **4** from 29.8 to 28.4 ppm.



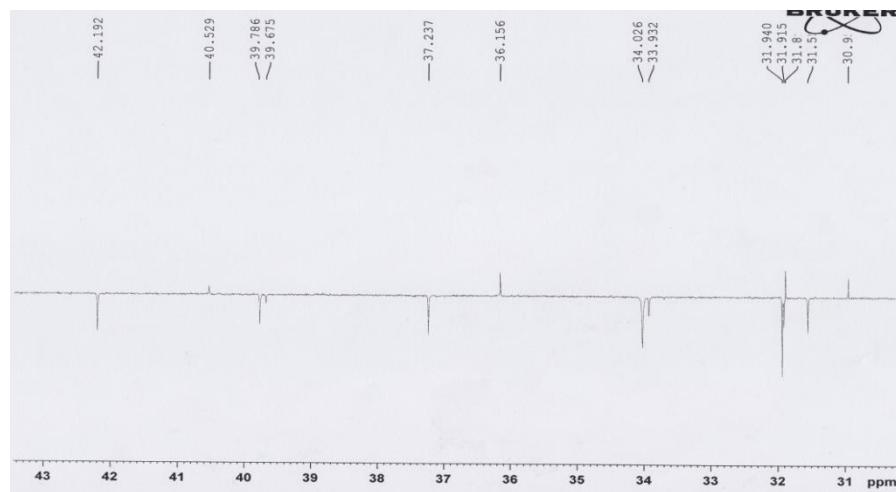
**Figure 11.6:** Expansion of <sup>13</sup>C- NMR spectrum of compound **4** from 26 to 12 ppm.



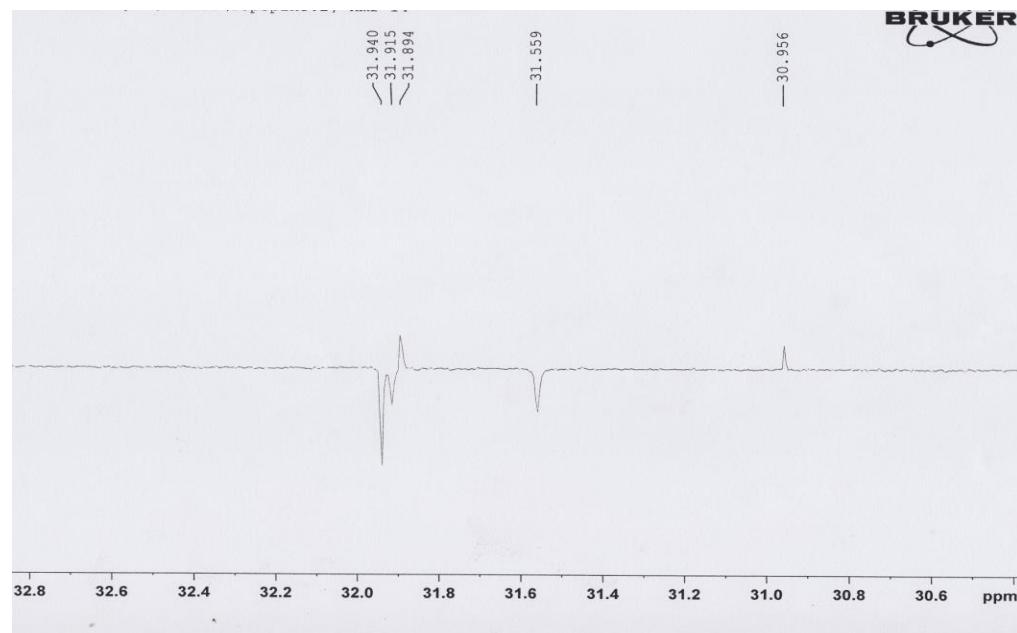
**Figure 11.7:** Expansion of DEPT-NMR spectrum of compound **4** from 140 to 65 ppm.



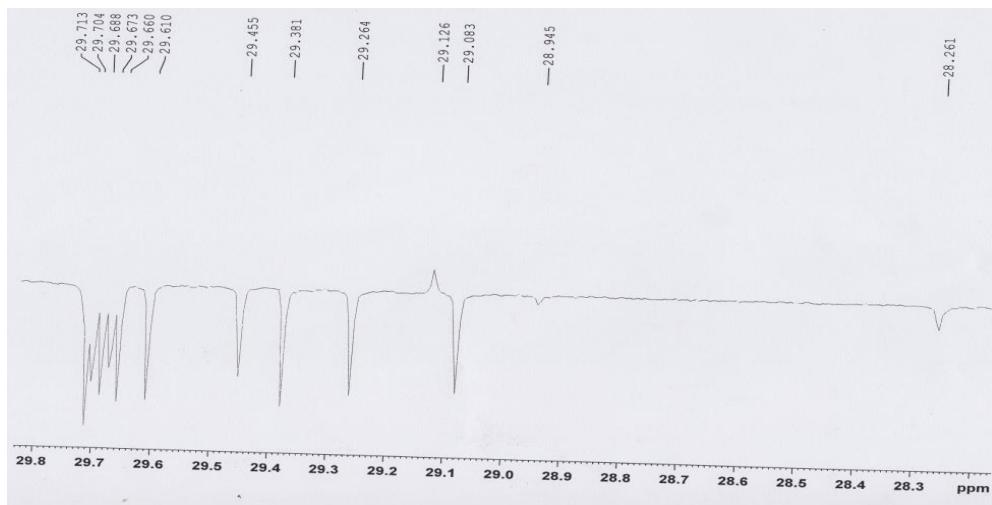
**Figure 11.8:** Expansion of DEPT- NMR spectrum of compound **4** from 59 to 45 ppm.



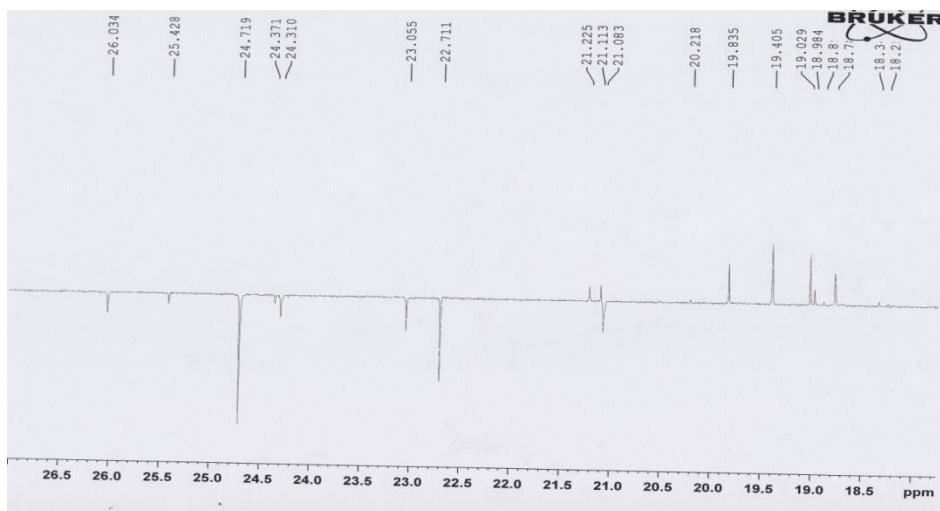
**Figure 11.9:** Expansion of DEPT -NMR spectrum of compound 4 from 43 to 31 ppm.



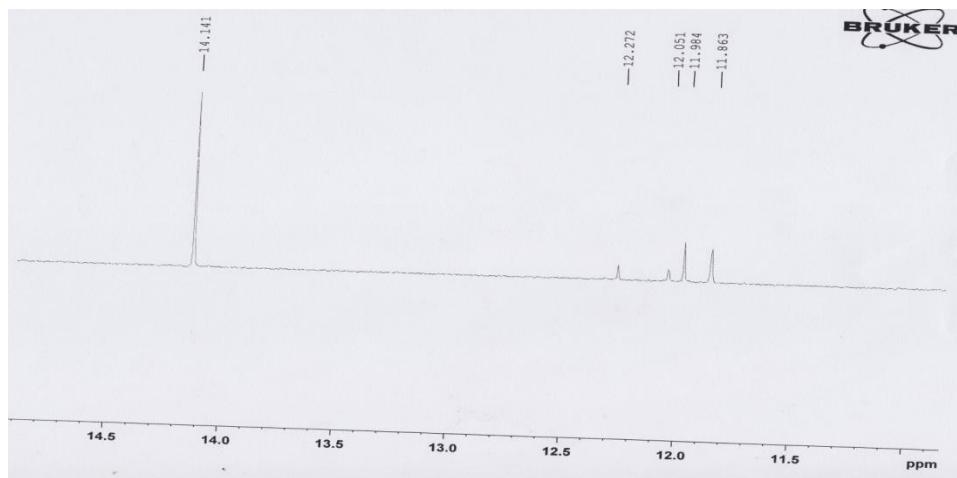
**Figure 11.10:** Expansion of DEPT- NMR spectrum of compound 4 from 32.8 to 30.6 ppm.



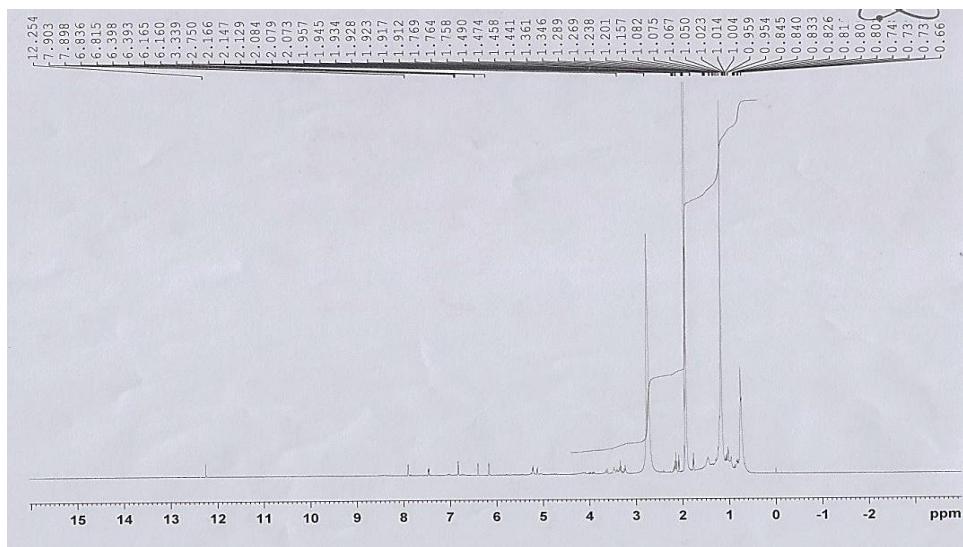
**Figure 11.11:** Expansion of DEPT- NMR spectrum of compound **4** from 29.8 to 28.3 ppm.



**Figure 11.12:** Expansion of DEPT- NMR spectrum of compound **4** from 26.5 to 18.5 ppm



**Figure 11.13:** Expansion of DEPT NMR spectrum of compound **4** from 14.5 to 11.5 ppm



**Figure 11.14:**  $^1\text{H}$ - NMR spectrum of compound 2.

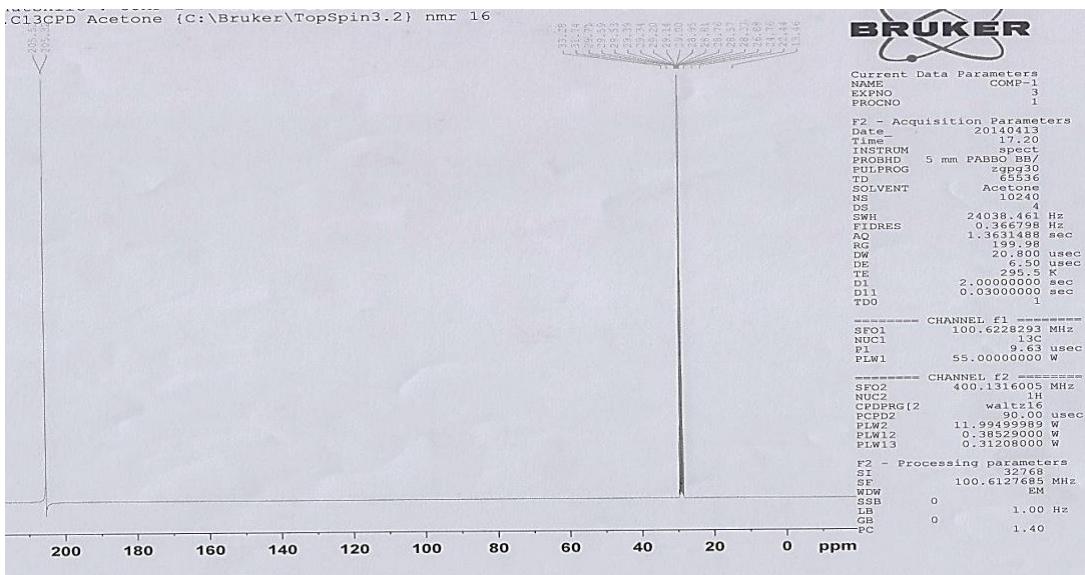


Figure 11.15:  $^{13}\text{C}$ -NMR spectrum of compound 2.

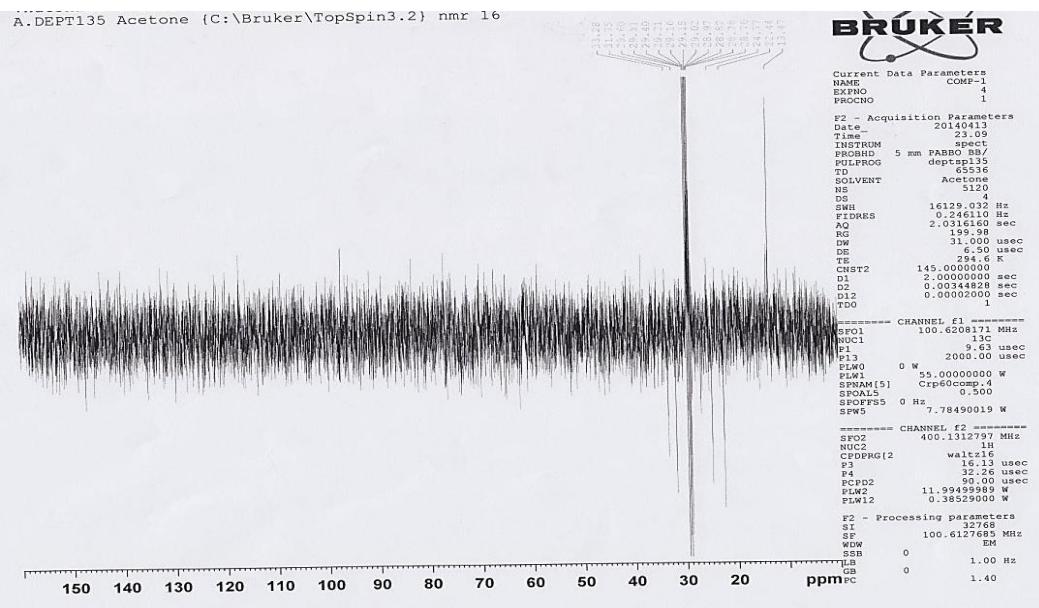
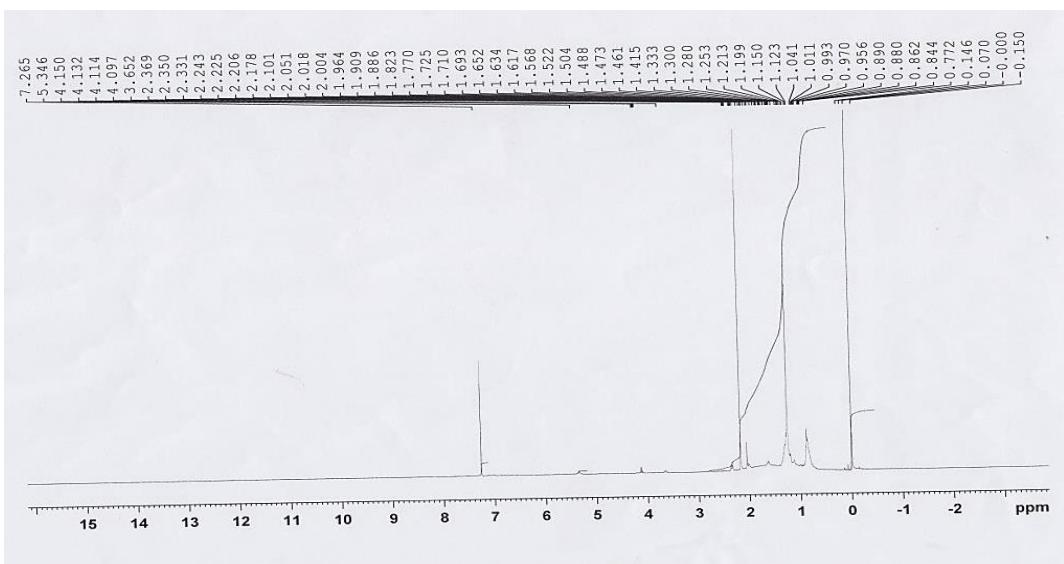
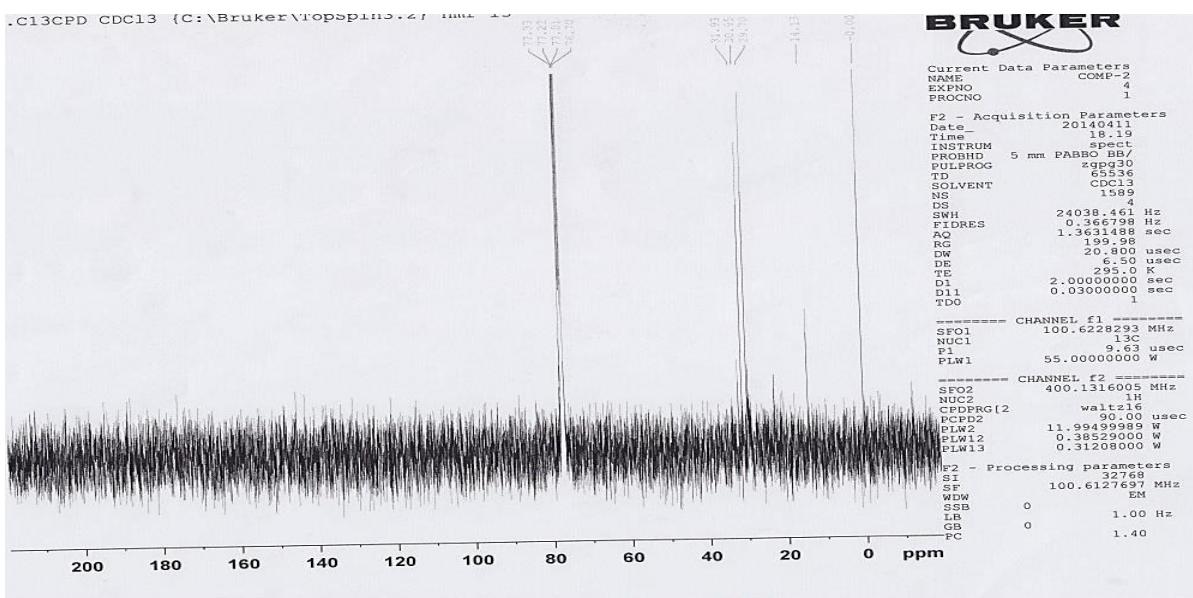


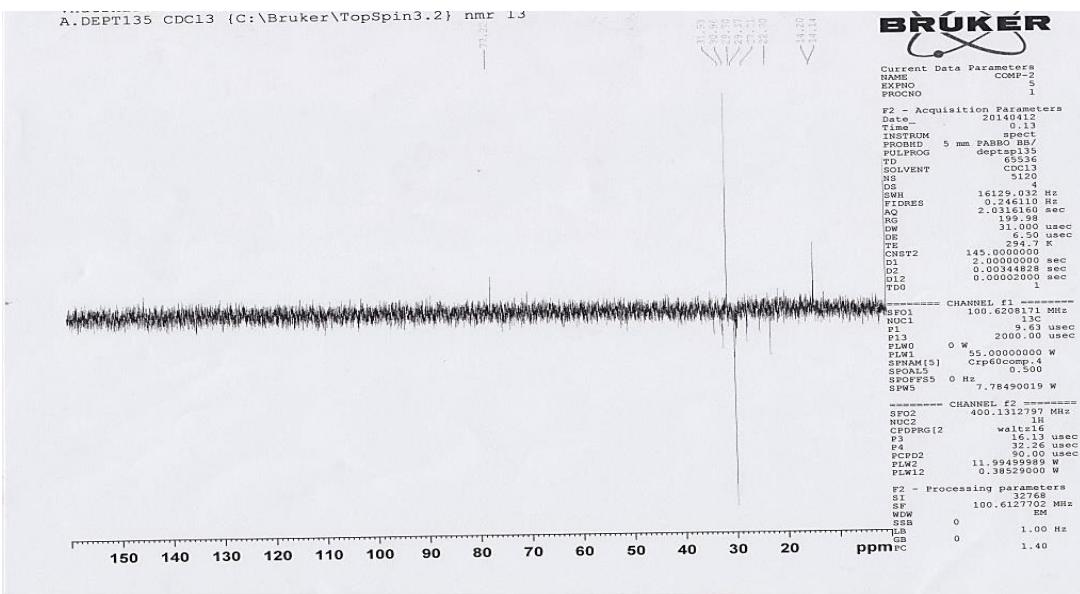
Figure 11.16: DEPT- NMR spectrum of compound 2.



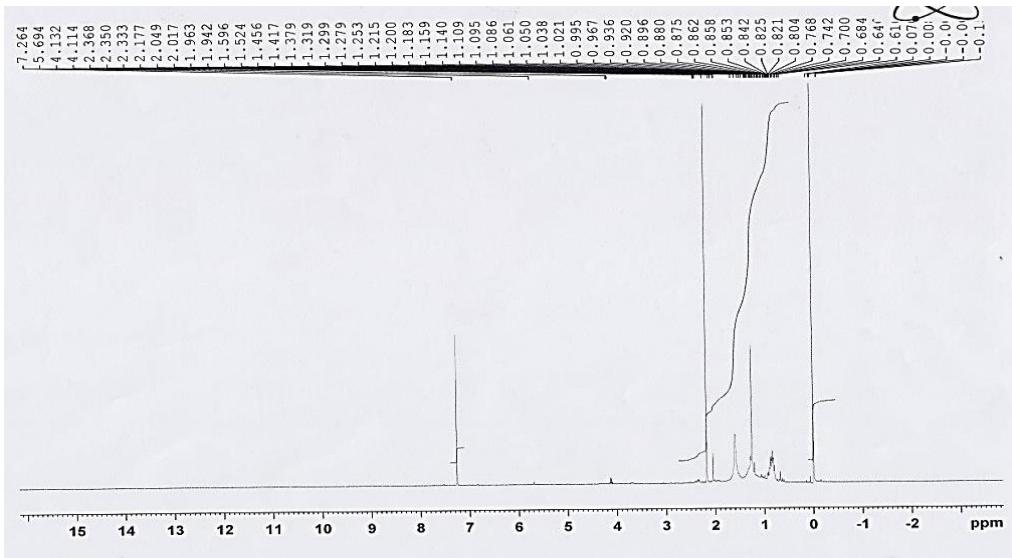
**Figure 11.17:**  $^1\text{H}$ - NMR spectrum of compound 3.



**Figure 11.18:**  $^{13}\text{C}$ -NMR spectrum of compound 3.



**Figure 11.19:** DEPT-NMR spectrum of compound 3.



**Figure 11.20:**  $^1\text{H}$ -NMR spectrum of compound 5.

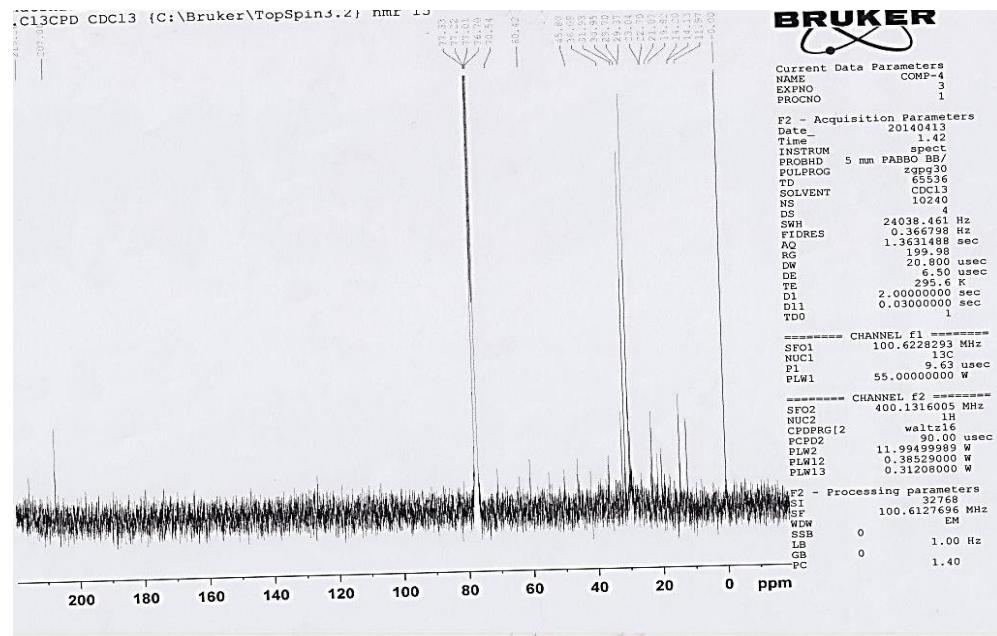


Figure 11.21:  $^{13}\text{C}$ - NMR spectrum of compound 5.

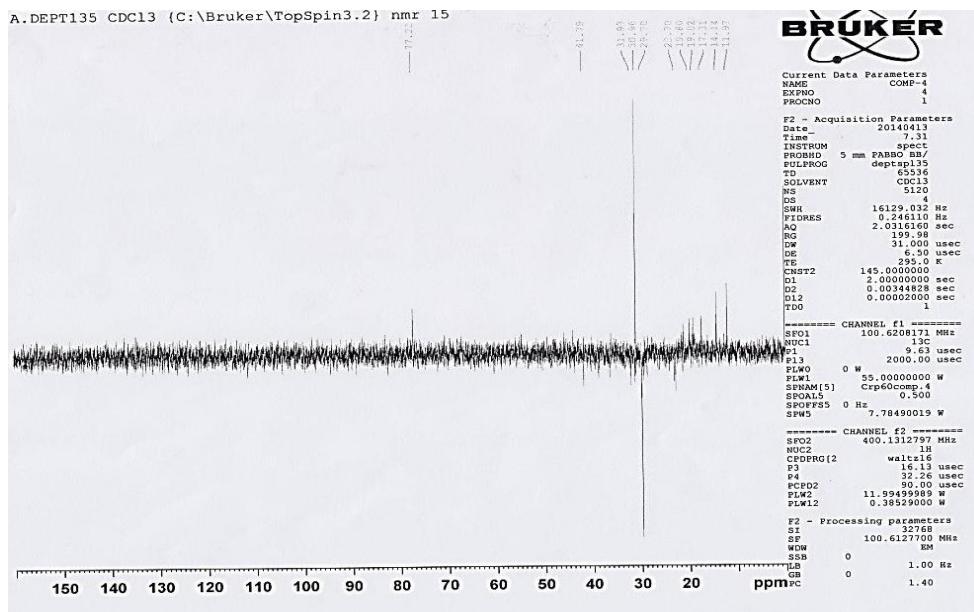


Figure 11.22: DEPT-NMR spectrum of compound 5.