SECONDARY METABOLITES FROM *Juniperus procera* WITH ACTIVITY AGAINST SELECTED MAIZE AND BEAN PATHOGENS

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Chemistry of Egerton University

EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this University or any other for the award of a degree.

Signature…… …………………… Date…………...08.01.2024……

Teresia Wangui Nduati

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Recommendation

This thesis has been submitted with our approval as university supervisors.

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DEDICATION

I dedicate this thesis to my dear dad John Nduati and mum Mary Nduati for their prayers, encouragement, love and support during the research period. To my beloved sister Jane and brother Peter for their encouragement and support.

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ABSTRACT

Maize (*Zea mays*) and beans (*Phaseolus vulgaris* L.) are important food crops in Kenya however, these crops have registered a decline in yields which is partly due to attack by fungal and bacterial pathogens. These pathogens are currently managed by use of synthetic pesticides, whose long term and widespread use has led to negative impacts which has endangered the environment in general. Extracts from *Juniperus procera* have been used as traditional medicine in treatment of various human pathogens. Scientific research has also proved efficacy of its various secondary metabolites towards human pathogens but minimal research has been done on their activity towards phytopathogens. This study, therefore, aimed at isolating secondary metabolites from the bark and leaves of *J. procera* with activity against three important plant pathogens; *Xanthomonas axonopodis* pv. *phaseoli* and *Pseudomonas savastanoi* pv. *phaseolicola* whose host is common bean and a maize pathogen *Fusarium graminearum.* The leaf and bark samples were collected from Mount Elgon National Park Forest, dried under shade and ground into a fine powder. Solvent extraction method was used to extract secondary metabolites from the powdered plant material whereby the powder was first soaked in methanol, filtered and solvent evaporated to obtain methanol extract. The methanol extract was suspended in water and partitioned between hexane and ethyl acetate to obtain ethyl acetate, hexane and aqueous extracts. The ethyl acetate extracts were purified using various chromatographic techniques. The pure compounds were analysed using 1D and 2D NMR and their masses determined using mass spectrometry. A flavonoid was isolated from the bark; epicatechin (**12**) while a biflavonoid and a diterpenoid were isolated from the leaf; podocarpusflavone A (**13**) and juniperolide (**14**) respectively. Disc agar diffusion assay was used to test the extracts and pure compounds against the three phytopathogens. Crude extracts were more active against the bacterial pathogens than the semi-pure fractions. For example, methanol and aqueous crude extracts from leaf had highest inhibition zones of 18.0 and 17.3 mm respectively against *P. savastanoi* pv. *phaseolicola*. Compound **12** had the highest inhibition zone of 21 mm against *P. savastanoi* pv. *phaseolicola*. Both compound **13** and **14** registered inhibition zones of 8 mm against *P. savastanoi* pv. *phaseolicola.* The extracts and the pure compounds did not show activity towards *F. graminearum.* It can therefore, be concluded that this medicinal plant can be a source of compounds to be used in control of important plant bacterial pathogens.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
CIMS	Chemical Ionization Mass Spectrometry
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
EIMS	Electron Impact Mass Spectrometry
ESIMS	Electrospray Ionization Mass Spectrometry
ESI-TOF	Electrospray Ionization-Time of Flight
FABMS	Fast Atom Bombardment Mass Spectrometry
FAO	Food and Agricultural Organisation
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HR-ESMS	High Resolution Electrospray Ionization Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
IR	Infrared Spectroscopy
MALDI	Matrix Assisted Laser Desorption Ionization
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
OECD	Organisation for Economic Co-operation and Development
PDA	Potato Dextrose Agar
pv	Pathovar
SD	Standard deviation
SDG	Sustainable Development Goals
TLC	Thin Layer Chromatography
UV- Vis	Ultraviolet- visible spectroscopy

CHAPTER ONE

INTRODUCTION

1.1 Background information

Maize (*Zea mays*) is an important food crop in Kenya as well as Sub-Saharan Africa that plays a key role in food security (Ranum et al., 2014; Witwer et al., 2012). Maize consumption in Kenya is estimated to range between 88-103 kg per person per year (Abate et al., 2015; Short et al., 2019). It has nutritional benefits as it contains 72% starch, 10% protein, 4% fat and micronutrients (Nuss & Tanumihardjo, 2010). Maize accounts for about 40% of cultivated land in Kenya (Abate et al., 2015) but despite this, Kenya heavily relies on importation to meet the local demand (Abodi et al., 2021). Its consumption is anticipated to rise globally by 16% by the year 2027 both for human and animal consumption as a result of the population growth and expansion of livestock sector (Organisation for Economic Co-operation and Development [OECD]/Food and Agricultural Organisation [FAO], 2018).

Pests and diseases have a major impact on yields. Gibberella ear rot is a fungal infection in maize caused by *Fusarium graminearum* that has been reported to be a major cause for reduction of the grain yield and quality (Leslie & Summerell, 2006). The pathogen is associated with production of mycotoxins majorly deoxynivalenol (DON) before and after harvest (Abbas et al., 2017; Sweeney & Dobson, 1998; Vesonder et al., 1981). Upon consumption of the mycotoxin, symptoms like fever, diarrhoea, headache and vomiting can occur. It has been reported that the mycotoxin cannot be degraded by the normal cooking temperatures (Sobrova et al., 2010). It is, therefore, evident that consumption of maize infected with *F. graminearum* is a threat to human health. The pathogen is also a threat to the income earned by farmers and also those involved in production of secondary goods as a result of reduction of grain yield. Fungicides are mainly used to control the pathogen but their high costs make them unaffordable to the farmers and also lead to increase in costs of production hence reduced profits. The fungicides are also not environment friendly (Nega, 2014).

Common bean (*Phaseolus vulgaris* L) is the most consumed grain legume worldwide and it is a major source of protein in Kenya especially to those who cannot afford other sources of protein like meat (Kiptoo et al., 2016; Munyasa, 2013). In Kenya, common bean is the third most important staple food nationally after maize and wheat (Mohajan, 2014). It is a nutritious food because it is rich in protein, folic acid, complex carbohydrates and iron (Gichangi et al., 2012). Three parts of the common bean plant are highly consumed in Southern and Eastern Africa; the leaves, pods and grains (Katungi et al., 2009). The grains are consumed in fresh or in dried form. Kenyans produce it both for home consumption and for sale (Katungi et al., 2009) and therefore, plays an important role in food security and a source of income to households in Kenya (Kabutbei, 2014). Despite the importance, a decline in yield production has been registered over years (Katungi et al., 2009). The major causes of the yields decline are due to attacks by bacterial, fungal and viral diseases. Some of the bacterial diseases that affect the common bean yield include common bacterial blight and halo blight. Common bacterial blight is a bacterial disease caused by *X. axonopodis* pv. *phaseoli*. It is a seedborne disease and it is an economically important disease in Kenya. It can result to yield losses between 10 and 40%. For instance, a research carried out in farms in Uasin Gishu county reported 13.2 to 35% of disease incidence (Jemeli et al., 2019). Development of the disease is usually attributed to use of uncertified seeds from previous harvest or seeds from the local market (Gicharu et al., 2013). Halo blight is also a bacterial disease caused by *P. savastanoi* pv. *phaseolicola* and causes yield loss of 21 to 40% in Kenya (Makini, 1995).

The most common methods used to control these bacterial diseases are chemical methods. They include, use of antibiotics for example streptomycin which is mainly used as seed treatment by killing external contamination (Schwartz, 2011). Copper-based bactericides are also used. The bactericide is sprayed on the pods and leaves to reduce spread of the pathogen to the healthy parts of the bean plant (Schwartz, 2011). These methods pose negative effects to the farmers, consumers and the environment. The methods are also not affordable to most farmers and increase the cost of production hence lead to less profits for the farmers (Belete & Bastas, 2017).

The plant kingdom is a rich source of diverse secondary metabolites that are synthesized by the plants mainly for defense against pests and diseases (Raja, 2014).These plant based natural products have been used traditionally by communities for various uses for instance; used as human medicine, food additives and also used in agricultural applications (Bulugahapitiya, 2013). Over 57% of medicines are estimated to originate from natural products mainly plants and that only 5-15% of 250,000 higher species of plants have been investigated for natural products(Cragg & Newman, 2005). Pesticides obtained from natural products have been found to be eco-friendly alternatives since they are biodegradable and they don’t leave toxic residues (Lai et al., 2006). Therefore, exploration of more natural products from plants is vital for aid in discovery of more medicines, biopesticides and more applications for the wellness of the society (Bulugahapitiya, 2013).

In efforts to increase yields by controlling the diseases and safeguarding the environment, alternative methods that are affordable and environmentally friendly need to be adopted. This study therefore, aimed at isolating secondary metabolites from *J. procera* with activity against maize pathogen *F. graminearum* and bean pathogens *P. savastanoi* pv. *phaseolicol*a and *X. axonopodis* pv. *phaseoli*.

1.2 Statement of the problem

Maize and beans are staple foods in Kenya. Their demand grows as the population grows but their production has been declining. For instance, an economic survey by Kenya National Bureau of Statistics (KNBS) in 2023 reported gradual reduction of maize production in the past five years. Maize production reduced from 44.6 million bags in 2018 to 44.0 million bags in 2019 which further reduced to 42.1 million bags in 2020. Higher losses were experienced in the consecutive years where 36.7 and 34.3 million bags were produced in the year 2021 and 2022 respectively. According to the same report, reduction in bean production was reported since the year 2020. A reduction was reported from 8.6 million bags in 2020 to 7.4 million bags in 2021 and 5.7 million bags in 2022. As a result, Kenya relies on imports to satisfy the local demand. The deficit is partly due to bacterial and fungal diseases. Halo blight, caused by *P. savastanoi* pv. *phaseolicola* and common bacterial blight caused by *X. axonopodis* pv. *phaseoli* are some of the common bacterial infections that affect the common bean and cause about 10-40% yield losses. Gibberella ear rot which is caused by *F. graminearum* is a common fungal infection that affect maize. This pathogen produces mycotoxins which are a threat to human and livestock health when consumed. These diseases are mostly treated with synthetic pesticides which are considered harmful to the farmer, consumer and the environment.

1.3 Objectives

1.3.1 General objective

To investigate the activity of secondary metabolites from *J. procera* against maize and bean pathogens.

1.3.2 Specific objectives

i. To determine the activity of extracts from the leaves and bark of *J. procera* against *F. graminearum* affecting maize and *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola* affecting common bean.
ii. To isolate and identify the bioactive secondary metabolites from *J. procera.*
iii. To determine the structures of the isolated antifungal and antibacterial secondary metabolites from *J. procera.*

1.4 Research questions

i. Do the extracts from leaves and bark of *J. procera* have activity against maize pathogen *F. graminearum* and bean pathogens *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*?
ii. Can bioactive secondary metabolites from *J. procera* be isolated and identified?
iii. Can the structures of antifungal and antibacterial secondary metabolites from *J. procera* be determined?

1.5 Justification

Maize and common bean are the most widely consumed staple foods in Kenya. This is due to their nutritional benefits, availability and affordability. Maize is rich in starch while common bean is rich in protein and therefore consumed highly by the low-income earners who cannot afford other sources of protein like meat. However, their productivities are low and are gradually declining partly due to bacterial and fungal diseases. The current chemical methods of managing the infections like the synthetic foliar sprays, antibiotics and fungicides have shown negative impacts to the farmers, consumers and environment and are also expensive. Use of compounds from natural products especially from plant origin is a friendly alternative in managing the bacterial and fungal diseases. This is because they are affordable as they freely occur in nature, environmentally friendly and support sustainable development in agriculture. Since there is increasing demand for environment protection with purposes of protecting human lives and health, search for secondary metabolites with antiphytopathogenic activity is important. *Juniperus procera* extracts have been found to possess important secondary metabolites that are active against human fungal and bacterial pathogens but research on their activity towards phytopathogens is limited. This leaves a gap that is important to fill. In addition, this project is in line with sustainable development goal (SDG) number two of zero hunger.

CHAPTER TWO

LITERATURE REVIEW

2.1 Secondary metabolites

Natural products refer to chemical compounds that are produced by living organisms and possess unique biological and pharmacological effects (Sarker & Nahar, 2012). They can be obtained from microorganisms, plants and also from animals. These compounds are classified into two; primary and secondary metabolites. Primary metabolites play a key role in growth and development of the organism. Examples include amino acids, glucose, nucleic acids and fatty acids (Croteau et al., 2000). Secondary metabolites are organic compounds or phytochemicals that are not directly involved in the normal growth, reproduction and development of an organism. Some of the functions of the secondary metabolites in their own organism is to maintain a healthy immunity system and to protect the organism against infections (Tiwari & Rana, 2015). These metabolites are not usually strictly necessary for organism survival but play a key role for the wellness of other organisms like humans. Unlike primary metabolites, their absence do not cause immediate death of the organism (Anulika et al., 2016). Their occurrence is usually limited and unique to particular species. They are produced from few key intermediates of primary metabolism and have also been found to possess important pharmacological properties (Stanforth, 2006).

Secondary metabolites are important to humans since they are a source of medicine, pesticides, herbicides, can be used as food additives and also to promote health (Bulugahapitiya, 2013). They have therefore attracted attention of researchers due to their biological effect on humans and other organisms.

2.1.1 Secondary metabolites in plants

Plants usually produce a large and diverse number of organic compounds which are potential sources of medicines for humans and also pesticide sources for agricultural application. Ethnobotanical information of the medicinal plants has been the leading factor towards discovery of compounds which are potential medicines and for other different applications like in the agricultural sector (Bulugahapitiya, 2013; Karunamoorthi et al., 2009; Karunamoorthi et al., 2008). About 57% of medicines are estimated to originate from natural products mainly plants.

Example of drugs used worldwide from plant origin include Taxol (used in cancer treatment), morphine (used as an analgesic), quinine (for malaria treatment), menthol (as local anaesthetic), vincristine (used in cancer treatment), obtained from *Taxus brevifolia*, *Papever somniferum*, *Cinchona officinalis, Mentha arvensis* and *Catharanthus roseus* respectively (Ataei-Azimi et al., 2008; Bharadwaj et al., 2018; Krishnamurti & Rao, 2016; Makkar et al., 2018; Wani et al., 1971). Statistics have showed that only less than 20% of higher plants have been explored for natural products. It is therefore evident that the plant kingdom is a rich source of secondary metabolites which require further scientific exploration to discover more metabolites and their usefulness (Choudhary et al., 2021).

The metabolites can be found in the leaf, stem, root, flower or the bark depending on the type of the secondary metabolite being produced. Plant secondary metabolites are divided into three distinctive groups namely; terpenes, phenolic compounds and nitrogen containing compounds (Anulika et al., 2016). Recently, studies have reported antifungal and antibacterial activities of phenolic compounds from natural sources (Ecevit et al., 2022). The activity of the phenolic compounds is partly dependent on the position and number of hydroxyl groups (Arif et al., 2009; Vila et al., 2013). In addition, studies have also shown that phenolic compounds are an important group of naturally occurring products from plants which are responsible for the many therapeutic properties in humans as antioxidant, antimicrobial and enzyme inhibitors (Kumar & Pandey, 2013). Their biological activity extends to plants where they protect them against microbial diseases and pests (Antolovich et al., 2000; Servili & Montedoro, 2002). In general, the major antimicrobial compounds from plants comprise of phenolic compounds and some of the examples include saponins (**1**), quinones (**2**), coumarins (**3**), flavonoids (**4**) and xanthones (**5**) as shown in Figure 2.1.

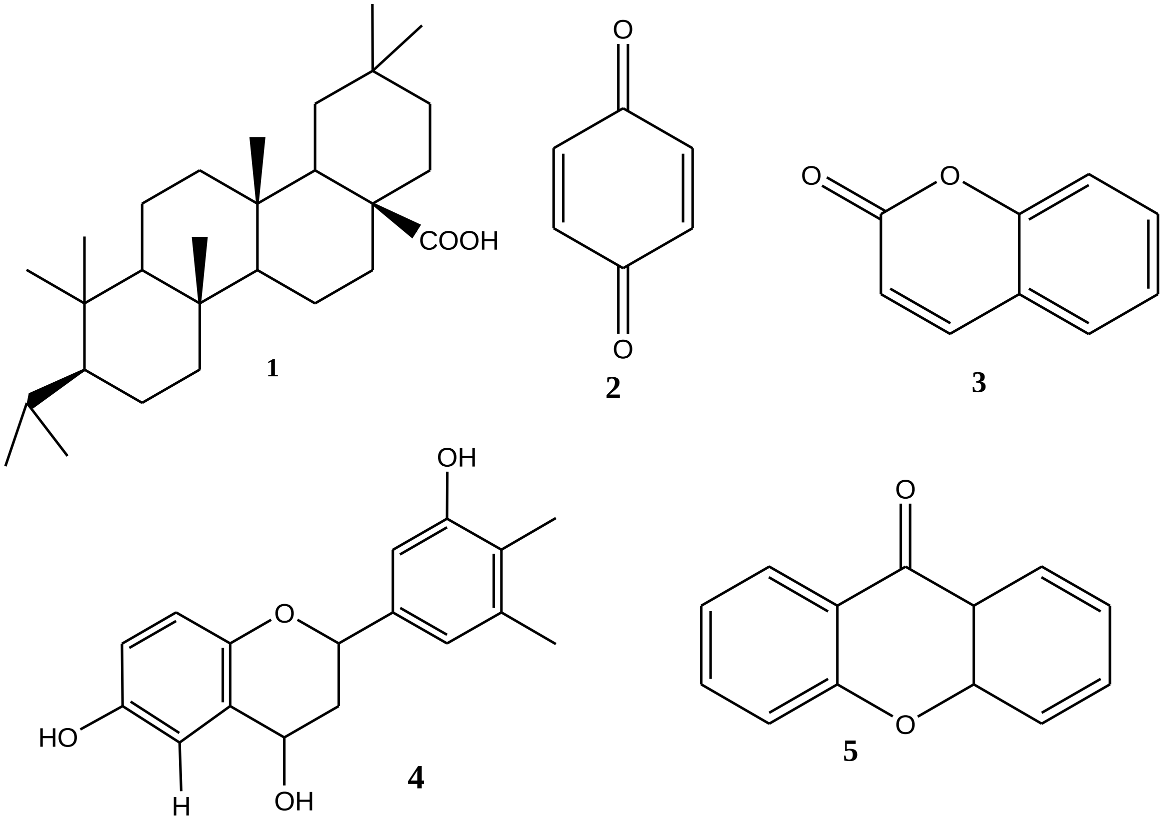


Figure 2. 1: Bioactive secondary metabolites from natural sources

1) saponins, **2**) quinones, **3**) coumarins, **4**) flavonoids, **5**) xanthones

2.1.2 Extraction of secondary metabolites from plants

Extraction involves separation of potential metabolites from their sources. The extraction method used is usually dependent on the extraction target which can be; to isolate a particular compound or class of compounds and to isolate all compounds or unknown compounds from the plant sample. Some of the methods used include; solvent extraction, supercritical fluid extraction, steam distillation and sublimation (Visht & Chaturvedi, 2012).

Solvent extraction is the major technique used for extraction of secondary metabolites from plant sources. This is because most of the organic compounds in plants are of different polarities and therefore the compounds dissolve in solvents with matching polarities. Solvents of different polarities are therefore used. This is according to the concept “like dissolves like” (Zhuang et al., 2021). Several factors are considered while selecting solvents to be used for the extraction; the boiling temperature of the solvent should be as low as possible for easier removal of the solvent after extraction, solvent power of the solvent should also be considered that is, the solvent should have the ability to dissolve desired compounds, the solvent should not react chemically with the compounds or decompose while standing or upon heating, the solvent should also be less viscous, have low vapor pressure and cost friendly (Bulugahapitiya, 2013). Solvent extraction technique constitutes methods like maceration, Soxhlet extraction, solvent- solvent partitioning, plant tissue homogenization, microwave assisted solvent extraction and sonication extraction method (Azwanida, 2015; Bulugahapitiya, 2013).

2.1.3 Isolation/ purification of secondary metabolites

Purification of extracts from natural products is a prime step since the extracts usually contain mixture of compounds and highly complex compounds. This is to enhance structure elucidation of structures of biologically active compounds which are potential biopesticides or drugs. There are various techniques that are used in purification of extracts depending on their nature. These techniques include chromatography, crystallization, distillation and sublimation (Bulugahapitiya, 2013).

Chromatography is a physical method that is mostly used for the purification and analysis of crude extracts in research involving natural products. It is based on the affinity of the compounds to the stationary phase and mobile phase. The technique is used to determine the number of compounds present in an extract and can also be used to separate compounds from a mixture. The common chromatographic methods that are used in natural product research include; Thin layer chromatography (TLC), column chromatography, gas chromatography which is used to separate volatile compounds and high-performance liquid chromatography (HPLC). Other methods include size exclusion chromatography, ion exchange chromatography and gel permeation chromatography (Bulugahapitiya, 2013; Coskun, 2016).

2.1.4 Structure elucidation

Characterization and identification of the isolated pure compound is carried out by conclusive structure elucidation methods. There exist various spectroscopic methods that give useful information about chemical structure of compounds. Some of these methods include; infrared spectroscopy (IR) which is helpful in identifying functional groups like amino group ($-NH_2$), hydroxide group (-OH), carbonyl group (-C=O) among others. Ultra-violet- visible spectroscopy (UV-Vis) which gives information about the chromophores present in the molecule. Mass spectrometry (MS) is a technique that aids in determining the molecular mass and molecular formular of the isolated compound. Some of the techniques under mass spectrometry include chemical ionization mass spectrometry (CIMS), electron impact mass spectrometry (EIMS), electrospray ionization mass spectrometry (ESIMS), matrix assisted laser desorption ionization (MALDI) and fast atom bombardment mass spectrometry (FABMS) (Sarker & Nahar, 2012).

Nuclear Magnetic Resonance (NMR) spectroscopy is also used in structure elucidation and gives information on types and number of carbon atoms and protons or other elements like fluorine and nitrogen present in the compound. It also gives information on the relationship among the atoms (Van de Ven, 1995). It is classified into two categories; one dimensional and two-dimensional NMR techniques. One-dimensional methods include; ^{13}C NMR, ^{1}H NMR, ^{13}C Distortionless Enhancement by Polarization Transfer (DEPT) among others. The two-dimensional involves ^{1}H-^{1}H Nuclear Overhauser Effect Spectroscopy (NOESY), ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC), ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) (Sarker & Nahar, 2012).

2.2 *Juniperus procera*

Juniperus procera (Figure 2.2) belongs to Cupressaceae family. It is commonly known as pencil cedar or East African cedar. In Kenya, it is locally known as “Mutarakwa” by Kikuyu and Meru communities, “Oltarakwai” and “Entarakwai” by Maasai community, “Mukuu” by Kamba community, “Kumutarakwa” by Luhya (Bukusu) community, “Eminet” by Turkana community and “Tarokwa” by Marakwet and Tugen communities (Maundu & Tengnäs, 2005). It is an evergreen tree, grows up to 40 m tall and has a straight trunk (Figure 2.2 A). The tree is almost similar to cypress and their main difference is on the foliage whereby the foliage of *J. procera* is more open and finer than that of cypress (Maundu & Tengnäs, 2005). The leaves of juvenile plants are small and prickly while those of mature plants are scale-like, triangular and overlap closely on the branchlets. The tree is dieocious although some plants produce both sexes (Dharani, 2011) .

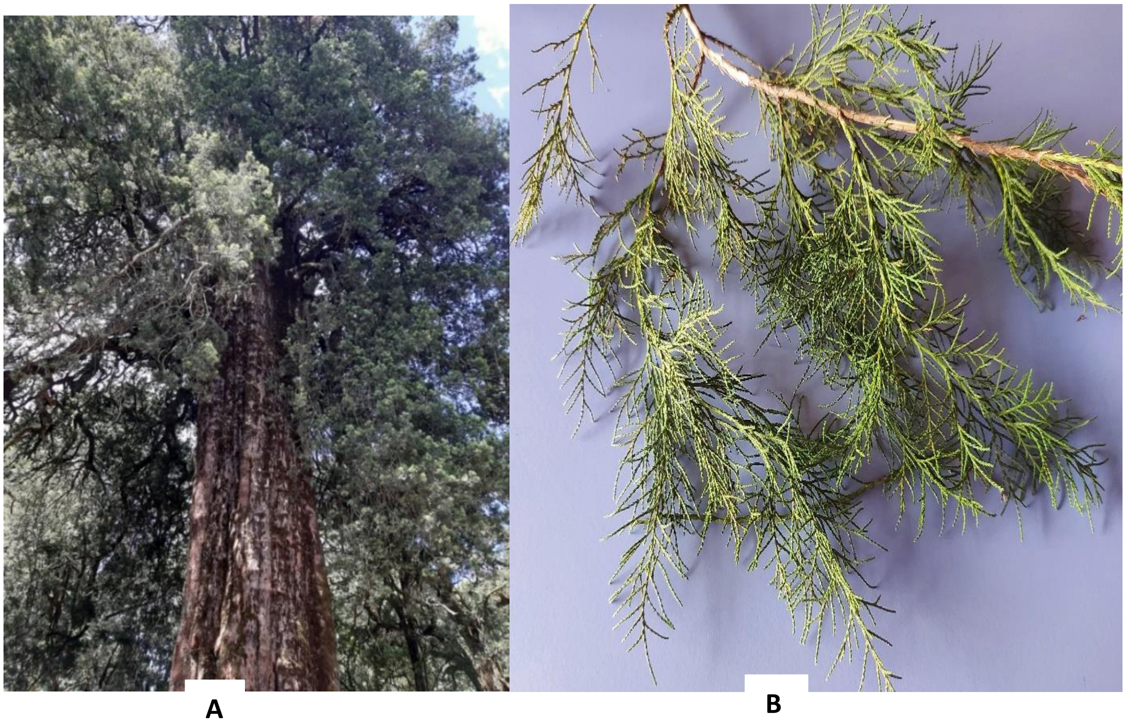


Figure 2. 2: *Juniperus procera* plant

A) *Juniperus procera* tree **B)** *Juniperus procera* leaves

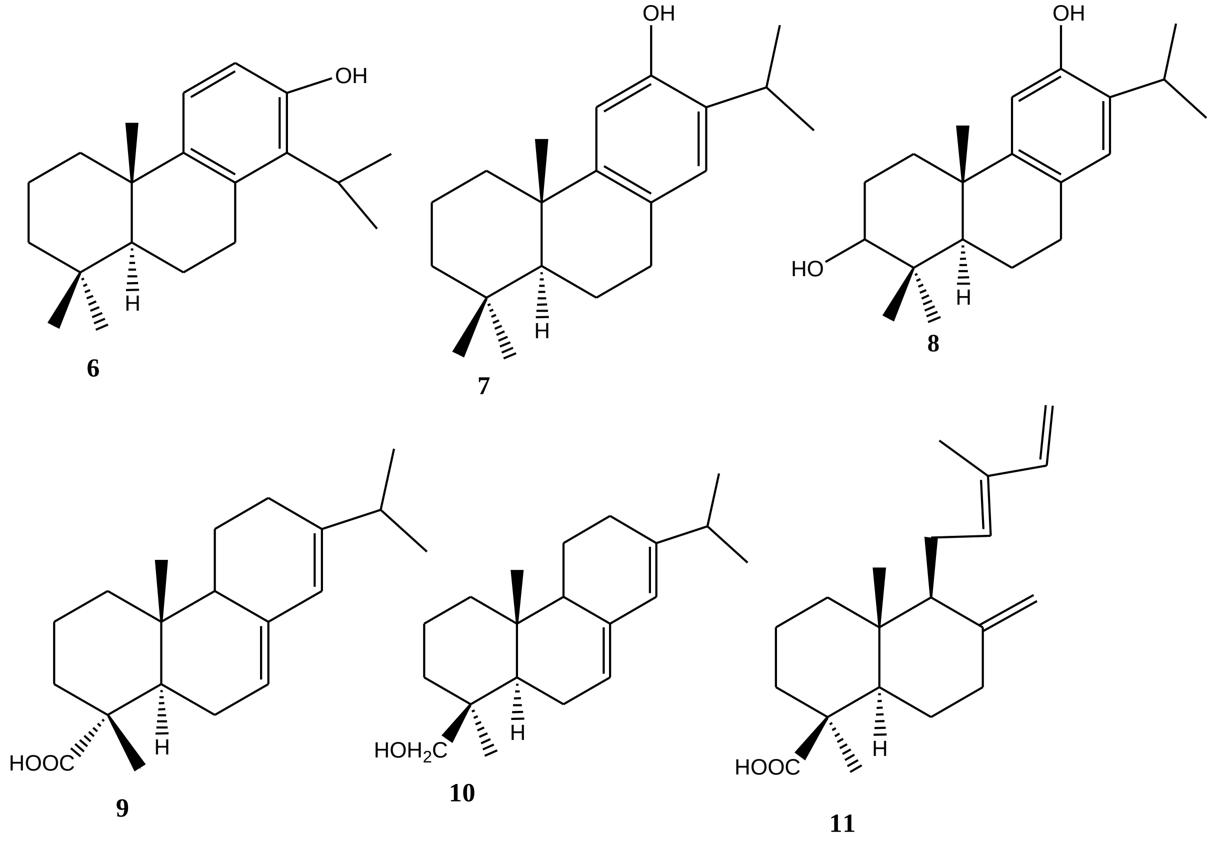
The tree is highly distributed in East African highland forests for instance in Kenya it can be found on the lower slopes of Mt. Kenya, Mt. Elgon, Aberdares, Tugen Hills and Loita Forest. The trees grow at around altitudes of 1050- 3000 m but mainly above 1800 m (Maundu & Tengnäs, 2005).

2.2.1 Traditional uses of *Juniperus procera*

Juniperus procera has a wide range of uses for instance, it is used as a source of firewood, charcoal, used to make poles and posts that are long-lasting in the ground, bee-hives and also used to make pencils. Timber from *J. procera* is the most useful in Kenya since its wood is resistant to termites hence has wide application in construction work (Maundu & Tengnäs, 2005). In addition, the bark, twigs, buds and leaves have been used traditionally for medicinal purposes. For instance, aqueous extract from buds and young twigs are used as treatment for intestinal worms (Dharani, 2011). The stem bark is used as a remedy for gum bleeding by applying a spoonful of the charcoal on teeth and gums (Ngari et al., 2014).

2.2.2 Secondary metabolites isolated from *Juniperus procera*

Some of the compounds isolated from *J. procera* with bioactivity include; Totarol (**6**), extracted from the bark which was found to possess antibacterial activity (Mossa et al., 2004) and from the berries which demonstrated strong antifouling and nematicidal activity (Samoylenko et al., 2008). Ferruginol **(7**), extracted from the bark (Mossa et al., 2004), from the berries that demonstrated antileishmanial activity (Samoylenko et al., 2008) and from aerial parts (Alqasoumi & Abdel-Kader, 2012). Hinokiol (**8**), extracted from berries (Samoylenko et al., 2008) and from aerial parts (Alqasoumi & Abdel-Kader, 2012). Abeta-7,13-diene (**9**) from the berries which was found to be an antimalarial compound (Samoylenko et al., 2008). 4- Epi-abietinol (**10**), extracted from aerial parts was found to possess antimicrobial activity and also demonstrated strong hepatoprotective activity (Alqasoumi & Abdel-Kader, 2012). Communic acid (**11**) , extracted from the berries (Samoylenko et al., 2008) and from aerial parts (Alqasoumi & Abdel-Kader, 2012). Their structures are as shown in Figure 2.3 below.



6 **7** **8** **9** **10** **11**

Figure 2. 3*:* Bioactive secondary metabolites isolated from *Juniperus procera*.

6) Totarol, **7**) ferruginol, **8**) hinokiol, **9**) abeta-7,13-diene, **10**) 4-epi-abietinol, **11**) communic acid.

From the leaves, antioxidant activity attributed to high content of flavonoid and phenolic compounds (Samaha et al., 2017) and antidiarrheal activity (Tafesse & Mekonnen, 2012) have been reported. Phytochemical screening of extracts of *J. procera* leaves have revealed presence of flavonoids, tannins, triterpenoids, alkaloids and saponins (Ali & Suleiman, 2018).

It was noted that most of the research focused on the activity of the extracts from this plant against human pathogens while minimal research had been done on test against phytopathogens thus the importance of this research work.

2.3 Maize and bean pathogens

2.3.1 *Xanthomonas axonopodis* pv. *phaseoli*

This pathogen causes common bacterial blight of beans. It is a warm weather disease and causes the greatest damage to plants especially when the temperatures are between 28^{o}C to 32^{o}C. The primary host of this pathogen is the common bean (Zanatta et al., 2007). This bacterium affects the foliage, pod and the seed of the common bean. The foliage symptoms begin with small water-soaked spots on the bottom of the leaves. They then enlarge, merge, dry and then become brown. Narrow, bright lemon-yellow border of tissue often develops around the dried necrotic lesions (Figure 2.4A, B**)**. Severe infection results in leaves remaining attached to plants giving a burned appearance to foliage (Harveson et al., 2005; Schwartz et al., 2005). The pod symptoms consist of generally sunken, circular and dark brown lesions (Figure 2.4C) that are usually covered with yellow masses of bacteria under high humidity (Schwartz et al., 2005). The seed symptoms involve a brown or butter-yellow discolouration on the seeds (Figure 2.4D). The seeds may also be small and characterized by poor germination and vigour after emergence (Buruchara et al., 2010). Common bacterial blight causes yield losses of up to 40% (Fininsa & Tefera, 2001; Gillard et al., 2009).

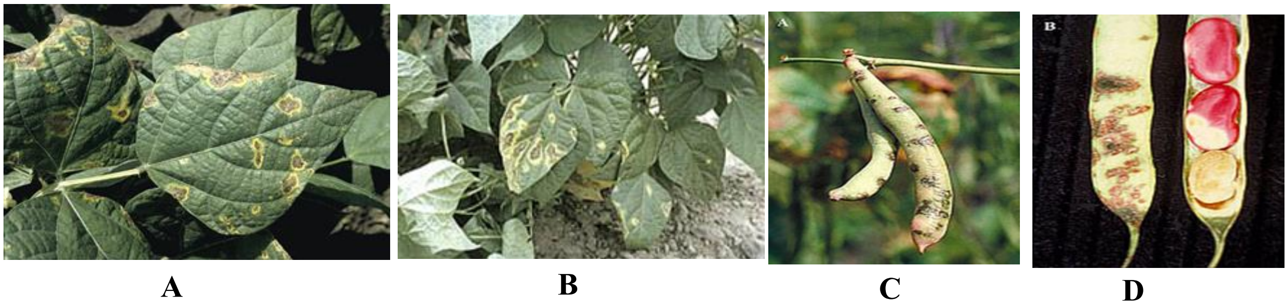


Figure 2. 4: Common bacterial blight symptoms in common bean

A) Large necrotic lesions on leaves with thin blight yellow halos**.** **B**) Common bacterial blight exhibiting both interveinal and marginal lesions on leaves. **C**) Pod symptoms. **D**) Seed symptoms (Harveson & Schwartz, 2007).

2.3.2 *Pseudomonas savastanoi* pv. *phaseolicola*

This pathogen causes halo blight of beans. It is a low temperature disease that is most destructive in areas where temperatures are moderate. The yield loss potential is considered high at temperatures of 18^{o}C to 20^{o}C (Schwartz et al., 2005). The disease mostly affects the leaves and pods. The early symptoms of halo blight occur on leaflets as small water-soaked spots which eventually die and turn tan to straw-coloured. A broad yellow-green halo then develops around necrotic spots, these spots remain small **(**Figure 2.5A) and this distinguishes it from the common bacterial blight (Harveson et al., 2006). At temperatures of 18-23^{o}C, yellow-green chlorosis become conspicuous as a result of production of phaseolotoxin by the pathogen but it decreases when the temperatures are above 23^{o}C and the chlorotic symptoms become less noticeable (Schwartz et al., 2005; Schroth & Hildebrand, 1988). Severe cases of chlorosis also develop in infected plants (Figure 2.5B) (Harveson & Schwartz, 2007). The symptoms in the pods begin as water-soaked circular spots or streaks along pod suture. Bacterial ooze in centre of spots is a cream to silver colour in contrast with the yellow exudate found with *X. axonopodis* pv. *phaseoli* (Harveson et al., 2005). The pod symptoms are characterized by discolouration or they may shrivel if lesions expand into pod sutures or penetrate young pod walls **(**Figure 2.5C) (Schwartz et al., 2005). The seeds in the infected pods can be small, shrivelled and discoloured. Halo blight causes yield loss of 21 to 40% in Kenya (Makini, 1995).

Figure 2. 5: Halo blight symptoms on common bean

A) Small necrotic lesions with thick, yellow-green halos. **B**) General systemic chlorosis from severe halo blight infection. **C**) Halo blight pod symptoms (Harveson & Schwartz, 2007).

2.3.3 *Fusarium graminearum*

Fusarium graminearum is also known as *Gibberella zeae*. It is the main pathogen that cause Gibberella ear rot and Gibberella stalk rot in maize (McMullen et al., 2012; Munkvold, 2003). The fungus produces fruiting bodies known as perithecia which then produce ascospores that infect cereal heads during flowering especially under favourable climatic conditions like high humidity during flowering. The ear infection begins as white mycelium moving down from the tip of infected kernels which later turns to reddish pink (Figure 2.6) (Jeffers, 2004). The infection by this pathogen results in reduction of grain yield and mycotoxin contaminated grains that are not suitable for human and animal consumption (McMullen et al., 2012; Pestka, 2010).

Source: https://www.pioneer.com/us/agronomy/gibberella_ear_rot_cropfocus.html

Figure 2. 6: Maize infected by *Fusarium graminearum*

2.4 Methods of plant disease management

Some of the methods used include; cultural practices, chemical control methods and biological control methods (Ravichandra, 2013)

2.4.1 Cultural practices

This refers to human activities that are carried out with an aim of disease control on plants. These practices include; use of pathogen- free seeds, crop rotation, deep ploughing , weed control to get rid of other hosts, irrigation and water management (Katan, 2010). These methods are useful as they help to reduce buildup of inoculum or reduce inoculum levels. However, they cannot control the diseases completely but can be considered as complementary methods of plant disease management (Katan, 2002).

2.4.2 Chemical control methods/conventional methods

This refers to use of pesticides that are produced or formulated using a chemical process. It is the most used method by the farmers to protect the crops from pests and diseases (Nkechi et al., 2018). However, continuous use of synthetic pesticides has been reported to have some limitations. For instance, they lead to environmental pollution that result from constituent compounds that are non-biodegradable (Kekuda et al., 2016). They cause toxicity and poisoning (Damalas & Koutroubas, 2016) as well as resistance due to continuous use (Halimatunsadiah et al., 2016; Rhoda et al., 2006). They can also lead to chronic health problems that result from accumulated toxic chemical residues as crops take up the constituent compounds and retain them (Jantasorn et al., 2016).

2.4. 3 Biological control methods

Biological control of plant diseases refers use of biologically or naturally occurring compounds to control damage caused by harmful organisms that affect plants (Kumar et al., 2021). Examples include use of microbial pesticides; these are usually obtained from microorganisms (Parker et al., 2019). Biochemical pesticides; these are obtained from plants to control the harmful organisms through non-toxic or safe mechanisms example being plant extracts/oils (Magierowicz et al., 2020). Use of genetically modified organism (GMO) products is also an example of biological control method whereby plant- incorporated protectants (PIPs) are used as a source of pesticidal compounds (Gomiero, 2018).

Biological control has been considered a potential control strategy because chemical control results in accumulation of harmful chemical residues which might lead to ecological problems. As a result, in the western world, a large number of synthetic fungicides have been banned due to their undesirable attributes such as high and acute toxicity (Nega, 2014). Biological control of plant pathogens is therefore a safe alternative strategy for the control of plant diseases.

The plant kingdom has been found to be the storage centre for diversified secondary metabolites which are synthesized by the plants and used as defensive weapons against pest attack. The plants contain a wide spectrum of secondary metabolites such as phenols, flavonoids, terpenoids, quinones, tannins, alkaloids, saponins, coumarins and sterols which show their efficacies against pest species (Raja, 2014).

2.5 Control methods of common bacterial blight and halo blight

Due to the seed-borne nature of common bacterial blight and halo blight, they are difficult to control and are therefore considered important bean diseases worldwide. Different methods are usually applied to manage the disease. Cultural, chemical and biological control methods are usually used to control common bacterial blight and halo blight. Use of resistant bean varieties is also a suitable method which has been reported to have the ability to control the damage caused by the diseases (Olle et al., 2015). The main aim of these methods is to reduce the initial inoculum to prevent an epidemic of the disease (Buruchara et al., 2010; Schwartz et al., 2005).

2.5.1 Cultural control methods

Some of the cultural disease control practises used include use of pathogen free seeds (Gillard et al., 2009; Tar'An et al., 2001), elimination of weeds and other potential hosts of *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*. Crop rotation and intercropping of beans with other plants like sorghum and maize is considered to be effective as the other plants create a physical barrier and hinder the movement of bacteria from one bean plant to the other (Moreno & Mora, 1984; Sharaiha et al., 1989). However, these methods do not guarantee total control of the disease. For instance, use of pathogen free seeds does not guarantee clean crops since even certified seeds can still be colonized by the pathogen. Also, crop rotation with crops like onions has been found not to be effective as they can be a source of inoculum by asymptomatic epiphytic colonization (Gent et al., 2005).

2.5.2 Chemical control methods

Use of chemicals like potassium methyl dithiocarbamate, copper hydroxide and copper sulphate are some of the methods used to control common bacterial blight and halo blight. Copper based bactericides aid in reducing the population of the bacteria (Fininsa, 2003; Selamawit, 2004). Use of the chemical control methods are, however, considered uneconomical for subsistence farmers because of their high costs (Belete & Bastas, 2017). Use of natural products is a safer and cheaper method as they freely occur in nature.

2.5.3 Biological control methods of common bacterial blight

Several biological control methods of bean common bacterial blight have been studied recently and found to be effective. For example, bacterial isolates from bean rhizosphere have been found to inhibit the growth of common bacterial blight in *in vitro* condition. The isolates have also been found to reduce the disease symptoms when applied to seeds before sowing in *in vitro* and greenhouse pathogenicity assay (Giorgio et al., 2016). *Rhizobium leguminosarum* pv. *phaseoli*, applied as a seed treatment has been found to reduce common bacterial blight in both greenhouse and field conditions (Osdaghi et al., 2011).

2.5.4 Biological control methods of halo blight

Extracts of *Allium sativum* (garlic) have shown inhibitory effect on growth of *P. savastanoi* pv. *phaseolicola* in *in vitro* assay (Eman & Afaf, 2014). Also extracts from fungal endophytes from *Zanthoxylum gilletti* and *Markhamia Lutea* were found to have activity against *P. savastanoi* pv. *phaseolicola* in *in vitro* assay (Wanga et al., 2018). More studies are thus necessary to enhance the number of biological control agents for halo blight.

2.6 Control methods of Gibberella ear and stalk rot

The cultural practises used to control this disease include crop rotation with non- host crops and use of resistant hybrid maize seeds (Mesterhazy et al., 2012). However, these methods have been proven to be ineffective (Nerbass et al., 2015). Fungicides sprays are also used to control Gibberella ear rot. However, most of the available fungicides target leaf diseases (Paul et al.,

2011) and are mostly not applied during flowering to control *F. graminearum* since its infections are prevalent during flowering (Reid et al., 2002).

Zealexin, a sesquiterpenoid phytoalexin isolated from maize, has shown inhibitory activity against *F. graminearum*. This phytoalexin is produced in response to attack by *F. graminearum* (Huffaker et al., 2011). Purified zealexin was found to inhibit the fungus growth in physiologically active concentrations which indicate that transgenic enhancement of the levels of this phytoalexin can be beneficial to reduce ear rot in maize.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and preparation

The leaf and bark samples of *J. procera* were collected from Mt. Elgon National Park Forest located at 1.1493°N, 34.5930° E. The park is located in the western part of Kenya, part of it in Trans- Nzoia County and the other part in Bungoma County. The samples were collected after identification by a taxonomist from the Department of Biological Sciences, Egerton University. They were put in sterilized bags and transported to Chemistry Department, Egerton University. They were dried for three weeks under shade and at room temperature to avoid loss of labile compounds as well as to ensure they retained their natural active compounds. Upon drying they were ground into a fine powder using a grinder and the samples were ready for extraction.

3.2 Extraction of secondary metabolites

Solvent extraction technique was used to extract secondary metabolites from the powdered leaf and bark samples. This was carried out using GPR grade solvents with 99% purity however, the solvents were first distilled before use. The leaf (1kg) and bark (700 g) samples were soaked separately in methanol for 24 hours at room temperature for total extraction. The mixture was filtered using Whatman filter paper No 1. The filtrate was evaporated under vacuum in a rotary evaporator at 40°C to obtain the methanol crude extract.

Solvent-solvent partitioning was carried out on the methanol crude extract. This was done by suspending the crude extract in water and partitioning between hexane and ethyl acetate. From this, aqueous, hexane and ethyl acetate extracts were obtained.

3.3 Purification of secondary metabolites

3.3.1 Thin Layer Chromatography

The ethyl acetate extract from the leaf sample was subjected to Thin Layer Chromatography (TLC). This was to determine the mobile phase to be used in column chromatography, that is to obtain the solvent mixture that would lead to optimum separation of the compounds. A TLC plate (1cm x 7cm) coated with silica gel (0.20 mm, Macherey-Nagel) was used. The sample was dissolved in ethyl acetate and a spotter was used to seed the sample in the TLC plate. The sample was spotted 1 cm from the base of the TLC plate, the plate was then placed in a development tank containing solvent mixture (mobile phase) and was allowed to develop. A UV lamp at 254 and 365 nm was used to visualize the developed TLC plate and a pencil was used to draw the visualized compounds. Ethyl acetate, hexane and methanol solvent mixture in the ratio of 6:3:1 respectively, brought about optimum separation and was therefore used in column chromatography as the mobile phase.

The ethyl acetate extract from the bark was also subjected to TLC but optimum separation was not achieved on the different mobile phase used, hence gradient elution was opted in column chromatography.

3.3.2 Column chromatography

The dry ethyl acetate extracts from both the leaf and the bark were re-dissolved in methanol and adsorbed in silica gel (0.063 – 0.2 mm, Macherey-Nagel). The adsorbed samples were carefully loaded on an evenly packed silica gel column. The mobile phase was then loaded. Isocratic type of elution was used in the column with extract from the leaf whereby the mobile phase was the mixture of solvents obtained from TLC throughout the column. Seven different semi-pure fractions were obtained from the column (Fraction 1- 7).

Gradient elution was used for the column with the extract from the bark. Different solvents (hexane, ethyl acetate and methanol) were used with increasing polarity. Six semi-pure fractions (Fraction 1- 6) were obtained from the column. The semi-pure fractions were further purified using HPLC to obtain pure compounds.

3.3.3 High Performance Liquid Chromatography

Analytical HPLC of the semi pure fractions was first carried out to identify the constituents in the samples. Based on the amount and the analytical HPLC profile, fraction 3 from the bark proceeded to further purification while fraction 2 from the leaf was purified further. The solvents used were HPLC grade solvents with 99.8% purity.

Fraction 3 (mobile phase-100% ethyl acetate) from the bark was purified using HPLC. Gemini C18 column (10 × 250 mm, 10 μm particle size, Phenomenex) was used for reverse-phase preparative HPLC. Double distilled water with 0.1% formic acid (A) and Methanol (B) were used as a mobile phase. The gradient elution used 45−70% solvent B over 18 min and thereafter isocratic conditions at 100% solvent B for 7 min, the system returned within 0.5 min to initial conditions of 45% B and was equilibrated for 10 min. The UV monitoring was carried out at 230, 254, 275, 320 and 370 nm, and the flow rate was 3 mL/min. Three fractions (JPB 1- 3) were collected. Fraction JPB 2 was further purified by a reverse-phase preparative HPLC, VP 125/10 NUCLEODUR PolarTec column (10×125 mm, 5 µm, Macherey-Nagel) was used as a stationary phase and flow rate of 3 mL/min: 35% isocratic conditions for 20 min, to afford compound **12** (12 mg).

Fraction 2 from the leaf was also further purified using HPLC. The Gemini C18 column was used. The gradient elution used 45−80% solvent B over 20 min and thereafter isocratic conditions at 100% solvent B for 10 min, the system returned within 0.5 min to initial conditions of 45% B and was equilibrated for 10 min. The UV monitoring was carried out at 254, 275, 320 and 370 nm and the flow rate was 3 mL/min. Eight fractions (JPL 2A- JPL 2H) were collected. Upon collection and evaporation of fraction JPL 2H, a yellow powder was formed which was identified as compound **13** (3.8 mg). Fraction JPL 2D was further purified by a reverse-phase preparative HPLC, VP 125/10 NUCLEODUR PolarTec column (10×125 mm, 5 µm, Macherey-Nagel) was used as a stationary phase and flow rate of 3 mL/min: 55% isocratic conditions for 20 min, to afford compound **14** (3.5 mg).

3.4 Nuclear magnetic resonance spectroscopy

The NMR spectroscopy was carried out on the three pure compounds that were obtained from HPLC. Some of the spectra measured were ^{1}H, ^{13}C, COSY, HSQC, NOESY and HMBC. The spectra were recorded on a Bruker Avance III 700 MHz spectrometer equipped with 5 mm TCI cryoprobe (1H;700MHz, 13C:175 MHz) locked to the respective deuterium signal of the solvent. The readings for compound **12** and compound **14** were done in deuterated methanol while those of compound **13** were done in dimethyl sulfoxide (DMSO). The chemical shifts were given as δ (ppm) and tetramethylsilane was used as an internal standard. The spectra were analyzed using MestReNova software.

3.5 Mass spectrometry

High resolution electrospray ionization mass spectrometry (HR-ESIMS) data was recorded using MaXis electrospray ionization-time of flight (ESI-TOF) mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples of concentration 1mg/mL were used for mass analysis. The data was analysed using Bruker Compass DataAnalysis 4.4 software.

3.6 Media preparation

Potato Dextrose Agar (PDA) was used during the isolation of *F. graminearum*. It was prepared by dissolving 39 g of the powder in 1000 mL of distilled water. The mixture was heated to boiling to dissolve the medium completely. It was then sterilized by autoclaving at 121°C for 15 minutes. It was allowed to cool to about 45-50°C. The medium was amended with streptomycin sulphate (33 mg per liter) after cooling and finally poured into sterile petri dishes.

Nutrient Agar was used during the isolation of *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*. The media was prepared by dissolving 28 g of the powder in 1000 mL of distilled water. The mixture was sterilized by autoclaving at 121°C for 15 minutes. It was allowed to cool and was poured into sterile petri dishes.

Mueller Hinton Agar was used during the bioassays. It was prepared by dissolving 38.0 g of the powder in 1000 mL of distilled water. The mixture was sterilized at 121°C for 15 minutes. It was allowed to cool and was poured in sterile petri dishes.

Nutrient broth was used to activate the bacteria for use in bioassays. It was prepared by dissolving 13.0 g of the powder in 1000 mL of distilled water. It was sterilized by autoclaving at 121°C for 15 minutes.

3.7 Isolation of bacterial pathogens

Infected bean leaf samples were collected from farms in Egerton University. They were first washed using running tap water and then sterilized in 2% sodium hypochlorite for two minutes. The samples were rinsed three times using sterile distilled water for one minute each rinse. The samples were then blot dried in sterile paper towels. The infected parts from three bean leaf samples were cut using sterile scalpel and crushed using sterile pestle and mortar in 6 ml of 0.9% sodium chloride. Dilutions of 10^{-} 1 and 10^{-}2 of the stock solution were prepared and 10µl of the 10 $^{-}$2 dilution was plated onto a culture plate containing sterile nutrient agar media and was spread on the plate using a swab. The plates containing the inoculum were incubated for 24 hours at 37^{o}C. They were monitored for bacterial growth and single colonies were sub cultured to obtain pure cultures. The bacteria were confirmed through pathogenicity experiments.

3.8 Isolation of fungal pathogens

Maize samples (grains) infected by *F. graminearum* were collected from farms in Egerton University. They were first washed using running tap water. They were sterilized in 2% sodium hypochlorite for two minutes and rinsed three times using sterile distilled water for one minute each rinse. The samples were then blot dried in sterile paper towels. The symptomatic areas were cut into small pieces and were placed on plates containing sterile PDA media. The plates were incubated at 25^{o} C for 7 days and monitored for fungal growth, after which the cultures were purified through subculturing. The pathogen was further confirmed through microscopy followed by pathogenicity experiments.

3.9 Pathogenicity test for bean pathogens

Healthy bean seeds (Rosecoco GLP 2) were soaked in sterile distilled water for 30 minutes. A small hole of about 2 mm was made using a sterile needle. Inoculum was prepared by suspending 48 hours old bacterial culture in sterile distilled water and the pricked seeds were soaked in the inoculum for four hours after which the inoculum was drained. Seeds soaked in sterile distilled water were used as the negative control. The seeds were placed under high humidity conditions between moist sterile paper towels in a tightly sealed container overnight for the bacterial infection to take place. The bean seeds were then sown in pots (3 seeds per pot) filled with steam sterilized soil in the greenhouse. Watering was done daily to maintain soil moisture. Occurrence of symptoms was monitored daily upon germination.

3.10 *In vitro* antimicrobial bioassay

Disc diffusion method was used to determine the susceptibility of bacterial and fungal pathogens to the plant extracts. The selected fungal pathogen (*F. graminearum*) and bacterial pathogens, *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*) were seeded in petri dishes containing Mueller- Hinton agar. The pathogens were spread over the entire agar surface using swabs. Blank sterilized discs of 6 mm in diameter were impregnated with 100 µl of the extracts dissolved in DMSO. The discs were then placed on the inoculated plates. Discs containing chloramphenicol and nystatin were used as the positive control for the bacteria and fungus respectively. Discs soaked with DMSO were used as the negative control for the fungus and for the bacteria.

The plates were refrigerated overnight to allow the extracts to diffuse through the media and then incubated for 24 hours at 37^{o} C for bacterial pathogens while those containing the fungus were incubated for 14 days at 25^{o} C. At the end of the incubation period, the diameter of the zones of inhibition were measured in millimetres and their average and standard deviations calculated. The experiment was done in triplicate and repeated once to confirm results. The data was analysed using statistical package for social sciences (SPSS) software. One-way ANOVA was used to determine the difference in the mean inhibitory effect of each extract.

3.11 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined for the crude extracts from the leaf (methanol, ethyl acetate and aqueous extracts) against *P. savastanoi* pv. *phaseolicola*. This was done by dilution of the extract using double fold serial dilution following a modified method used by Sule and Agbabiaka (2008). A stock solution of concentration 10 mg/ml was prepared by measuring 20 mg of the extracts and dissolving them in 2 mL of DMSO. Then 5 mg/ml concentration was prepared by diluting 1mL of the stock solution to 2 mL using DMSO. The same process was repeated to make 2.5, 1.25,0.626, 0.3125 and 0.1563 mg/ml. Sterile discs of 6 mm in diameter were impregnated with the prepared extracts of different concentrations. These discs were then placed in well labelled inoculated culture plates. The samples were allowed to diffuse and the plates incubated at 37^{o}C for 24 hours. The experiments were done in triplicates. The average and standard deviations of the inhibition zone diameters were calculated.

3.12 Pathogenicity test for *Fusarium graminearum*

Toothpick method of inoculation was used to test pathogenicity of *F. graminearum* of causing stalk rot in maize under greenhouse conditions. Round wooden toothpicks were first sterilized by autoclaving at 121°C for 15 minutes. Fourteen-day old fungal culture was applied on the sterile toothpicks. This was done by rolling the sterile toothpicks over the fungal culture. The toothpicks were then used to inoculate the pathogen in the maize by pricking the maize stem until the tip of the toothpick came out on the other end of the stem. The toothpick was then removed carefully in a rotating manner. Toothpicks dipped in sterile distilled water were used as the negative control. Occurrence of symptoms was monitored daily.

3.13 Data Analysis

The NMR spectra obtained were analyzed using MestReNova NMR analysis software. The data obtained from mass spectrometer was analysed using Bruker Compass DataAnalysis 4.4 software. The means of the inhibition zones obtained from antimicrobial bioassays were calculated and the data analysis was done using the statistical package for social sciences (SPSS) software. One-way ANOVA was used to determine the difference in mean inhibitory effect of each extract. The significant mean differences were separated by Tukey’s Honestly Significant Difference (HSD) at 95% confidence level.

CHAPTER FOUR

RESULTS

4.1 Pathogenicity results

The symptoms (halos) were observed on the primary leaves about four weeks after planting on bean plants infected with *P. savastanoi* pv. *phaseolicola* (Figure 4.1). On the other hand, the symptoms for the bean plants infected with *X. axonopodis* pv. *phaseoli* were observed when the beans were seven weeks old on the leaves (Figure 4.2).

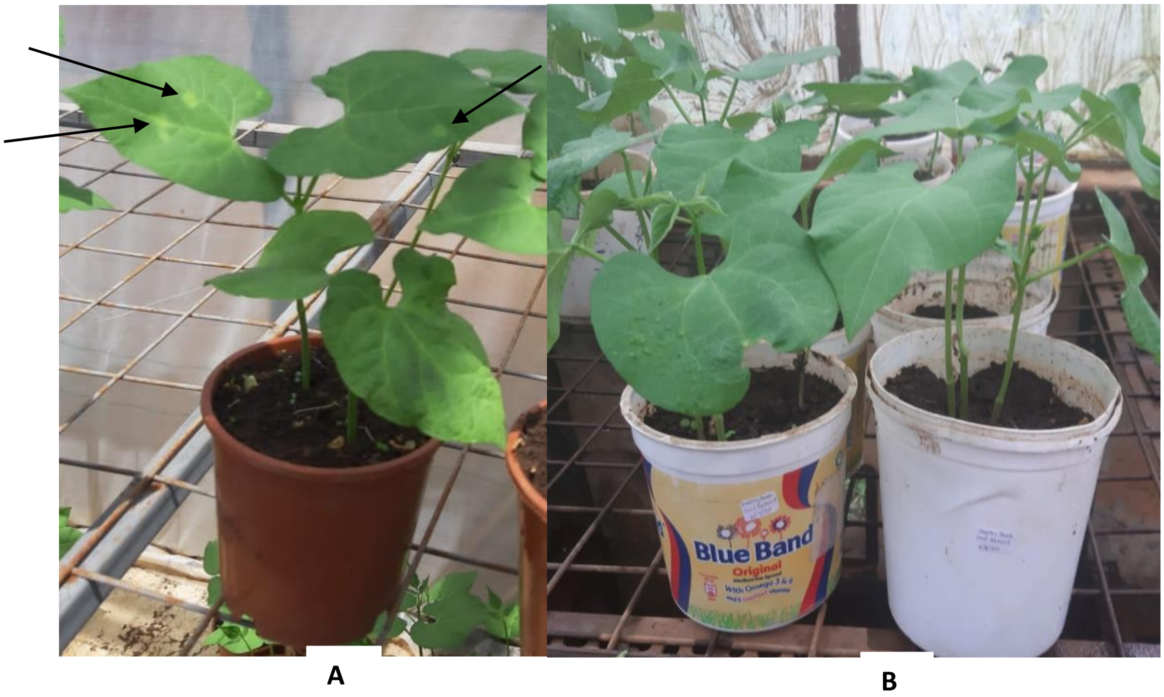


Figure 4. 1: Halo-blight symptoms observed during pathogenicity test of *Pseudomonas savastanoi* pv. *phaseolicola*

A) Broad yellow-green halo around necrotic spots on bean plant infected with *Pseudomonas savastanoi* pv. *phaseolicola*: B) Negative control

Figure 4. 2: Common bacterial blight symptoms observed during pathogenicity test of *Xanthomonas axonopodis* pv. *phaseoli*

A) Necrotic lesions surrounded by a narrow lemon-yellow border on bean plant infected with *Xanthomonas axonopodis* pv. *phaseoli*: B) Negative control

Stalk rot was observed around the pricked regions inoculated with *F. graminearum* while the pricked region of the negative control did not show signs of stalk rot as illustrated in Figure 4.3 below.

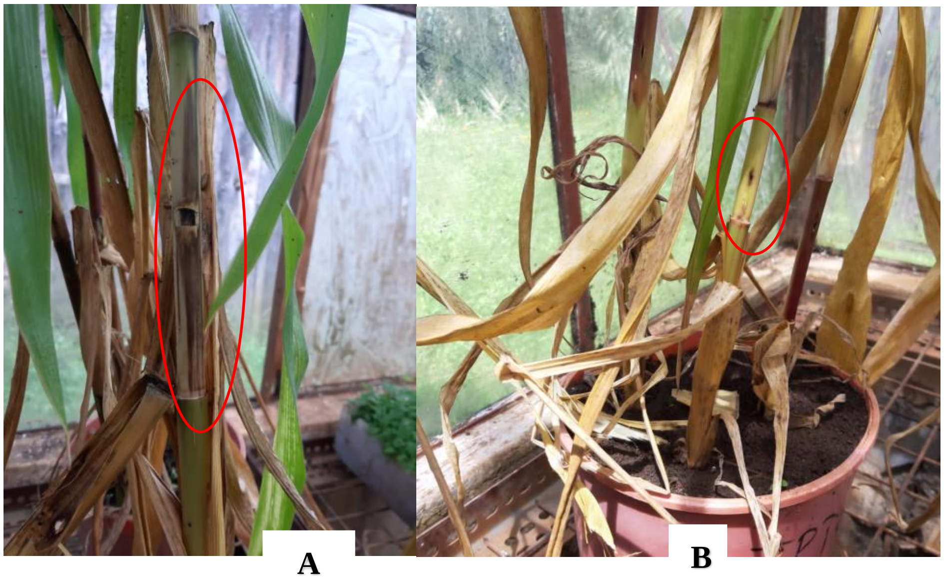


Figure 4. 3: Maize stalk infected with *Fusarium graminearum* versus negative control

A) Stalk rot on maize stem infected with *Fusarium graminearum*: B) Negative control

4.2 Antimicrobial activity of the extracts from *Juniperus procera*

The bioassay data for the extracts from *J. procera* bark and leaf is as shown in Table 4.1, Table 4.2 and Table 4.3. The crude extracts from the leaf and bark of *J. procera* were more active against the bacterial pathogens *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli* than the semi-pure fractions obtained from column chromatography. The crude extracts from the leaf were more active against *P. savastanoi* pv. *phaseolicola* than they were with *X. axonopodis* pv. *phaseoli*. For instance, the methanolic extract from the leaf registered the highest inhibition zone of 18.0 mm followed by the aqueous extract with 17.3 mm while ethyl acetate extract showed an inhibition zone of 14.3 mm against *P. savastanoi* pv. *phaseolicola* (Table 4.1 and Figure 4.4). The inhibition zones for these extracts were significantly different ($P< 0.001$) from the positive control which produced an inhibition of 49.0 mm. On the other hand, these crude extracts showed inhibition zones of less than 10 mm against *X. axonopodis* pv. *phaseoli.* The methanolic and ethyl acetate extracts from the leaf showed inhibition zones of 8.0 mm and 7.3 mm respectively against *X. axonopodis* pv. *phaseoli* whereas hexane and aqueous extracts produced inhibition zones of 7.0 mm and 8.7 mm respectively.

On the other hand, bioassay of the crude extracts from the bark showed higher activity against *X. axonopodis* pv. *phaseoli* compared to their activity against *P. savastanoi* pv. *phaseolicola.* Methanolic extract from the bark had the highest inhibition zone of 13.7 mm while ethyl acetate extract had an inhibition zone of 11.3 mm against *X. axonopodis* (Table 4.2). Hexane and aqueous extracts had inhibitions of 8.3 mm and 7.0 mm respectively. The methanolic and ethyl acetate extracts from the bark showed inhibition zones of 10.0 mm and 10.7 mm respectively against *P. savastanoi* pv. *phaseolicola*. Hexane and aqueous extracts had least inhibitions of 6.0 mm and 6.3 mm, respectively.

In both experiments involving the bark and leaf extracts, ethyl acetate extract produced a higher activity than the hexane extract (Table 4.1 and Table 4.2). It is due to this that the ethyl acetate extract proceeded to further purification. The hexane extracts also had the least activity against both bacterial pathogens compared to the other crude extracts.

All the extracts did not show activity against the fungal pathogen *F. graminearum.*

Table 4.1: Inhibition zones (mm) of *Juniperus procera* leaf extracts against bacterial pathogens

	Test organism (Inhibition zone in mm)	
***Juniperus procera* leaf extracts**	***P. savastanoi* pv. *phaseolicola***	***X. axonopodis* pv. *phaseoli***
Methanol extract	18.0 ± 3.5^{c}	8.0 ± 0^{bc}
Ethyl acetate extract	14.3 ± 2.5^{c}	7.3 ± 0.6^{bc}
Hexane extract	6.7 ± 0.6^{b}	7.0 ± 0^{bc}
Aqueous extract	17.3 ± 2.5^{c}	8.7 ± 1.2^{cd}
Fraction 1	6.0 ± 0^{b}	6.7 ± 0.6^{b}
Fraction 2	6.0 ± 0^{b}	6.7 ± 0.6^{b}
Fraction 3	7.0 ± 0^{b}	7.3 ± 0.6^{bc}
Fraction 4	7.3 ± 1.2^{b}	7.3 ± 0.6^{bc}
Fraction 5	8.7 ± 1.2^{b}	7.0 ± 1.0^{bc}
Fraction 6	6.0 ± 0^{b}	7.3 ± 0.6^{bc}
Fraction 7	8.0 ± 1.7^{b}	7.3 ± 0.6^{bc}
Chloramphenical	49.0 ± 0^{d}	10.0 ± 0^{d}
Negative control	0 ± 0^{a}	0 ± 0^{a}

Inhibition zones of extracts sharing the same letter(s) within a column are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test). The values are the mean of three replicates ± S.D. of the replicates.

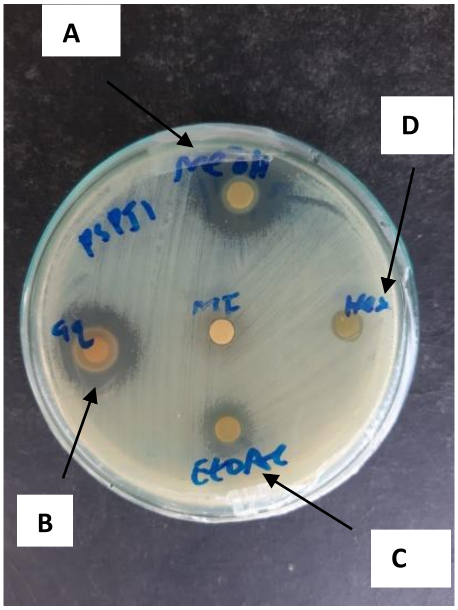


Figure 4. 4: Inhibition of crude extracts from leaf against *Pseudomonas savastanoi* pv. *phaseolicola*

A) Methanol extract. **B**) Aqueous extract **C**) Ethyl acetate extract **D**) Hexane extract

Table 4.2: Inhibition zones (mm) of *Juniperus procera* bark extracts against bacterial pathogens

	Test organism (Inhibition zone in mm)	
***Juniperus procera* bark extracts**	***P. savastanoi* pv. *phaseolicola***	***X. axonopodis* pv. *phaseoli***
Methanol extract	10.0 ± 3^{b}	13.7 ± 1.5^{d}
Ethyl acetate extract	10.7 ± 4.0^{b}	11.3 ± 2.1^{cd}
Hexane extract	6.0 ± 0^{b}	8.3 ± 1.2^{bc}
Aqueous extract	6.3 ± 0.6^{b}	7.0 ± 0^{b}
Chloramphenical	52 ± 0^{c}	29.0 ± 0^{e}
Negative control	0 ± 0^{a}	0 ± 0^{a}

Inhibition zones of extracts sharing the same letter(s) within a column are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test). The values are the mean of three replicates ± S.D. of the replicates.

Table 4.3: Inhibition zones (mm) of *Juniperus procera* bark semi- pure fractions against bacterial pathogens

***Juniperus procera* bark column fractions**	**Test organism (Inhibition zone in mm)**	
	P. savastanoi* pv. *phaseolicola	***X. axonopodis* pv. *phaseoli***
Fraction 1	6.0 ± 0^b	8.0 ± 0^b
Fraction 2	7.7 ± 0.6^c	7.3 ± 0.6^b
Fraction 3	7.0 ± 0^c	8.3 ± 1.5^b
Fraction 4	7.0 ± 0^c	7.0 ± 1.0^b
Fraction 5	7.7 ± 0.6^c	7.3 ± 0.6^b
Fraction 6	7.7 ± 0.6^c	7.0 ± 1.0^b
Chloramphenical	40.0 ± 0^d	25 ± 0^c
Negative control	0 ± 0^a	0 ± 0^a

Inhibition zones of extracts sharing the same letter(s) within a column are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test). The values are the mean of three replicates ± S.D. of the replicates.

Based on the data in Table 4.1, it was observed that *P. savastanoi* pv. *phaseolicola* was more susceptible to the crude extracts than *X. axonopodis* pv. *phaseoli*. Due to this, the minimum inhibitory concentration of crude extracts (methanol, ethyl acetate and aqueous extracts) from leaf was determined against *P. savastanoi* pv. *phaseolicola.* The data was as presented in Table 4.4. It was noted that the activity of the extracts reduced as the concentration decreased.

Table 4.4: Minimum Inhibitory Concentration data for *Juniperus procera* methanol, ethyl acetate and aqueous extracts from the leaves against *Pseudomonas savastanoi* pv. *phaseolicola*

	Inhibition zone (mm)		
Concentrations (mg/ml)	**Methanol extract**	**Ethyl acetate extract**	**Aqueous extract**
10	12.3±0.6^{d}	17.3±2.5^{d}	13.7±2.1^{e}
5	11± 1^{cd}	15±1.7^{d}	10±0^{d}
2.5	10.7±1.5^{cd}	11.7±0.6^{c}	9±0^{cd}
1.25	10.3±1.5^{cd}	11±1^{c}	9±1^{cd}
0.625	8.7±1.2^{c}	7.3±1.2^{b}	7.6±0.6^{bc}
0.3125	6±0^{b}	6±0^{b}	6±0^{b}
0.1563	6±0^{b}	6±0^{b}	6±0^{b}
Chloramphenicol	47±0^{e}	47±0^{e}	47±0^{f}
DMSO	0±0^{a}	0±0^{a}	0±0^{a}

Inhibition zones of extracts sharing the same letter(s) within a column are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test). The values are the mean of three replicates ± S.D. of the replicates.

Bioassay of the three isolated compounds was also carried out against the two pathogens Compound **12** isolated from the bark showed the highest activity against *P. savastanoi* pv. *phaseolicola* with an inhibition zone of 21.7 mm (Figure 4.5), which was significantly different (P< 0.001) from that of the positive control (47 mm) (Table 4. 5). This was a higher activity compared to that of fraction 3 of the bark which had an inhibition zone of 7 mm, from which it was isolated. Both compound **13** and compound **14** showed inhibition zones of 8 mm. These two compounds were isolated from fraction 2 of the leaf and their inhibition zone s was slightly higher than that of fraction 2 which had 6 mm inhibition zone. Compound **13** showed an inhibition of 7 mm against *X. axonopodis* pv. *phaseoli* while compound **14** showed an inhibition zone of 6 mm. Compound **12** did not show any activity against *X. axonopodis* pv. *phaseoli*. Compound **13** showed an inhibition of 7 mm while compound **14** showed an inhibition zone of 6 mm.

All the three compounds did not show activity against the fungal pathogen *F. graminearum.*

Table 4.5: Inhibition zones (mm) of *Juniperus procera* pure compounds against bacterial pathogens

	Test organism (Inhibition zone in mm)	
Treatment	***P. savastanoi* pv. *phaseolicola***	***X. axonopodis* pv. *phaseoli***
Compound **12**	21.7±1.2^{c}	0±0^{a}
Compound **13**	8±1.7^{ab}	7±0^{a}
Compound **14**	8±2^{b}	6±0^{a}
Chloramphenicol	47±0^{d}	37±0^{b}
Negative control	0±0^{a}	0±0^{a}

Inhibition zones of extracts sharing the same letter(s) within a column are not significantly different while those with different letters are significantly different (α =0.05, Tukey's test). The values are the mean of three replicates ± S.D. of the replicates.

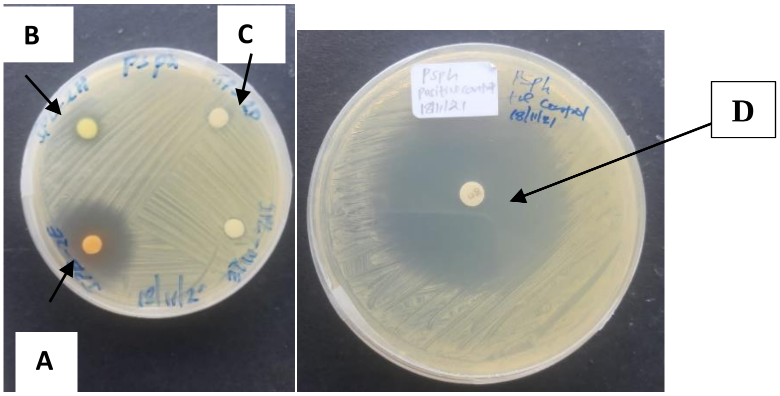


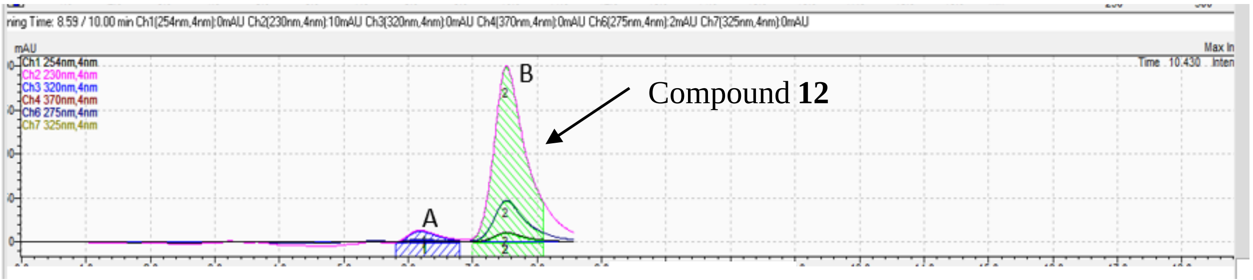
Figure 4. 5: Inhibition of the pure compounds against *Pseudomonas savastanoi* pv. *phaseolicola*

A) Compound **12**. **B**) Compound **13**. **C**) Compound **14**. **D**) Positive control

4.3 Structure elucidation of the isolated compounds

4.3.1 Structure elucidation of compound 12

Purification of ethyl acetate extract from the bark using column chromatography yielded six semi- pure fractions (Fraction 1- 6). Further purification of fraction 3 using HPLC afforded compound **12** (Figure 4.8). The compound belongs to flavonoid class of compounds. It was collected between 7-8 minutes during HPLC purification at 35% isocratic conditions (Figure 4.6). The molecular mass was determined using HR-ESIMS and was found to be 290.07883 which corresponded to molecular formula $C_{15}H_{14}O_6$. This was supported by molecular ion peak m/z 313.06820 $[M+Na]^+$ and 603.14703 $[2M+Na]^+$ as shown in the figure 4.7.



Time (min)

Figure 4. 6: HPLC chromatogram of compound **12**

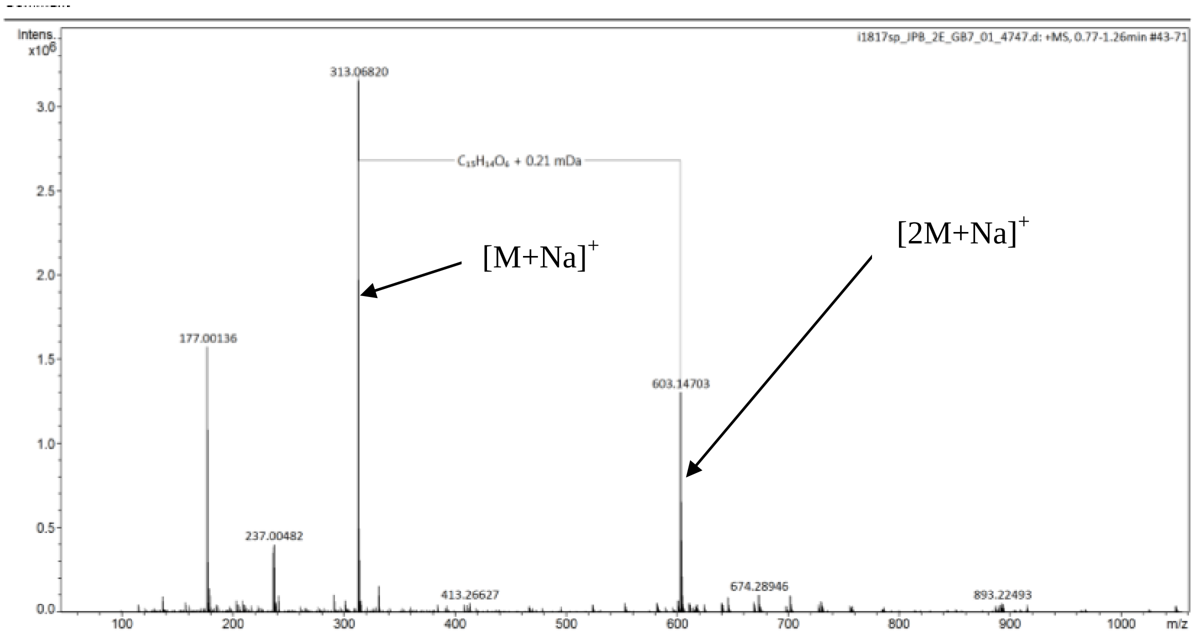


Figure 4. 7: Mass spectrum of compound **12**

The ^{1}H NMR spectrum of compound **12** was also measured. From the spectrum five peaks observed down field above δ_H 5.08 corresponded to the five aromatic protons. The chemical shifts of the protons are δ_H 5.94 (H-6), 5.87 (H-8), 6.86 (H-2$^{\prime}$), 6.78 (H-5$^{\prime}$) and 6.74 (H-6$^{\prime}$). The peaks absorbing at δ_H 2.52 and 2.87 corresponded to protons attached to methylene carbon-4. The doublet peak at δ_H 4.58 corresponded to the proton attached to carbon-2 while the quartet at δ_H 3.99 corresponded to the proton attached to carbon-3.

The ^{13}C NMR spectrum supported presence of fifteen carbon atoms. The carbon atoms consisted one methylene, seven methine and seven quaternary carbon atoms. The chemical shift of the methylene carbon was observed at δ_C 27.1 (C-4). The chemical shifts of the methine carbon atoms were observed at δ_C 81.5 (C-2), 67.4 (C-3), 94.9 (C-6), 94.1 (C-8), 113.9 (C-2$^{\prime}$), 114.7 (C-5$^{\prime}$) and 118.6 (C-6$^{\prime}$). The quaternary carbon atoms had chemical shifts at δ_C 99.4 (C-10), 155.5 (C-5), 156.5 (C-7), 156.2 (C-9), 130.8 (C-1$^{\prime}$), 144.9 (C-3$^{\prime}$) and 144.8 (C-4$^{\prime}$). The chemical shifts of the quaternary carbon atoms are in the aromatic region and therefore they are all in an aromatic ring system.

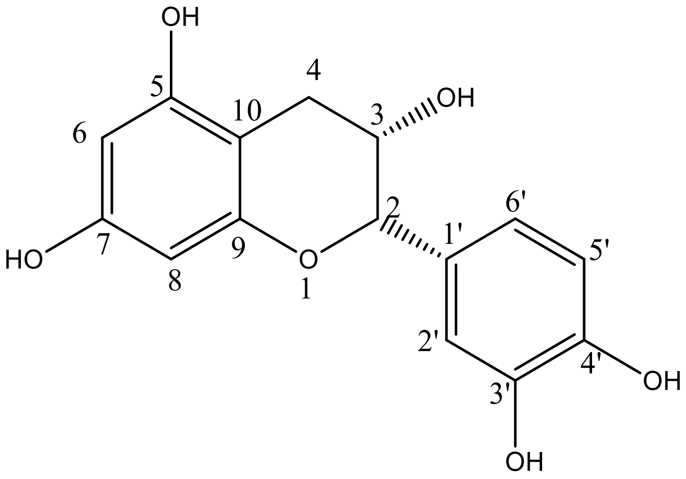


Figure 4. 8: Structure of compound **12**

The HSQC spectrum assisted in assigning protons to carbon atoms that they were directly attached to. From the spectrum, there was correlation between δ_H 4.58 with C- 2, 3.99 with C-3, 2.52/2.87 with C-4, 5.94 with C-6, 5.87 with C-8, 6.86 with C- 2$^{\prime}$, 6.78 with C- 5$^{\prime}$ and 6.74 with C- 6$^{\prime}$.

The ^{1}H- ^{1}H COSY correlation was also determined and gave information on protons that were attached to adjacent carbon atoms. The proton δ_H 4.58 attached to C-2 had a correlation with proton absorbing at δ_H 3.99 which was attached to C-3. Also, the proton with chemical shift δ_H 3.99 showed a correlation with protons δ_H 2.57 and δ_H 2.87 which were both attached to C-4 (Figure 4.9).

The NOESY spectrum was determined. The spectrum shows interactions through space within a molecule. The proton at δ_H 4.58 (H-2) had NOESY correlations with protons absorbing at δ_H 6.86 (H-2′), 6.74 (H-6′), 3.99 (H-3) and 2.52 (H-4). The proton at δ_H 3.99 (H-3) showed correlation with protons at δ_H 6.86 (H-2′), 4.58 (H-2), 6.74 (H-6′) and 2.52/2.87 (H-4). Both protons attached to the methylene carbon (δ_H 2.52 and 2.87) showed NOESY correlations towards each other.

The HMBC spectrum was also obtained. It gives information about proton- carbon connectivity up to three bonds away. From the spectrum, the proton absorbing at δ_H 4.58 (H-2) showed correlations with C-3, C-4, C-1′, C-2′, C-5′ and C-6′ while the proton δ_H 3.99 (H-3) showed correlation with C-2, C-4, C-10 and C-1′. The protons absorbing at δ_H 2.52 and 2.87 (H-4) had correlations with C-2, C-3, C-10, C-5, C-6, C-9 and C-1′. Proton at δ_H 5.94 (H-6) showed correlation with C-10, C-7 and C-8 while proton absorbing at δ_H 5.87 (H-8) showed correlations with C-10, C-6, C-7 and C-9. The proton at δ_H 6.86 (H-2′) had correlations with C-2, C-1′, C-3′ and C-6′. The proton absorbing at δ_H 6.78 (H-5′) showed correlations with C-1′, C-3′, C-4′ and C-6′ while the proton δ_H 6.74 (H-6′) had correlations with C-2, C-3′ and C-5′.

The compound was identified as epicatechin. The NMR data for compound **12** is summarized in table 4.6 and the COSY and HMBC correlations are illustrated in figure 4.9. The data closely corresponded to that obtained by Yusuf et al. (2019).

Table 4.6: NMR data of compound **12** in CD_3OD

Carbon	**^{13}C(δ)**	**HSQC(δ)**	**TYPE**	**COSY**	**NOESY**	**HMBC**	**^{13}C(δ)***
2	81.5	4.58	CH	3	2′,3,4,6′	3, 4,1′,2′,5′,6′	78.5
3	67.4	3.99	CH	2,4	2′,2, 4,6′	2,4,10,1′	66.1
4	27.1	2.52	CH$_2$	3	2,3	2,3,5,10,6,9,1′	27.9
		2.87			3		
5	155.5	-	C	-			155.9
6	94.9	5.94	CH	-		7, 8, 10,	95.1
7	156.5	-	C	-			156.6
8	94.1	5.87	CH	-		6,7,9, 10	94.5
9	156.2	-	C	-			156.3
10	99.4	-	C	-			98.7
1’	130.8	-	C	-			130.9
2’	113.9	6.86	CH	-	2, 3	2,1′,3′,6′	113.9
3’	144.9	-	C	-			144.6
4’	144.8	-	C	-			144.4
5’	114.7	6.78	CH	-		1′,3′,4′,6′	114.6
6’	118.6	6.74	CH	-	2, 3	2,3′,5′	118.0

^{13}C(δ)*- Reference data for ^{13}C (Yusuf et al., 2019)

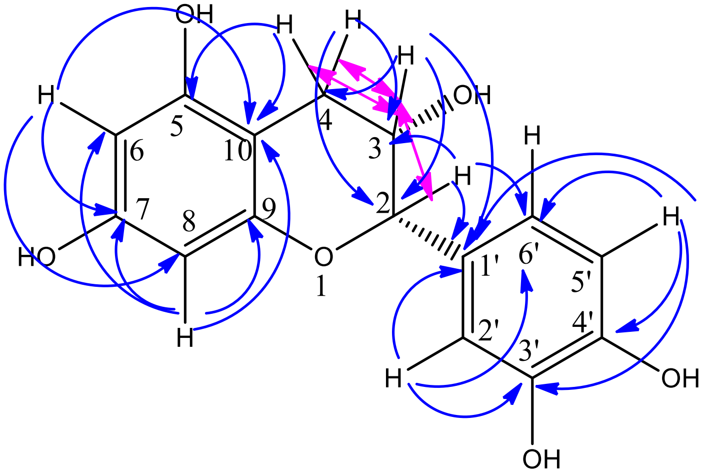


Figure 4. 9: Compound **12** structure showing COSY and HMBC correlations

4.3.2 Structure elucidation of compound 13

Purification of ethyl acetate extract from the leaf using column chromatography yielded seven semi- pure fractions (Fraction 1- Fraction 7). Further purification of fraction 2 using HPLC afforded compound **13** (Figure 4.11). The compound is a biflavonoid and its molecular mass was determined using HR-ESIMS. The molecular mass was found to be of 552.0985 which corresponded to molecular formula $C_{31}H_{20}O_{10}$. This was supported by molecular ion peak at m/z 551.0985 $[M-H]^-$ and m/z 1103.2021 $[2M-H]^-$ as shown in the figure 4.10.

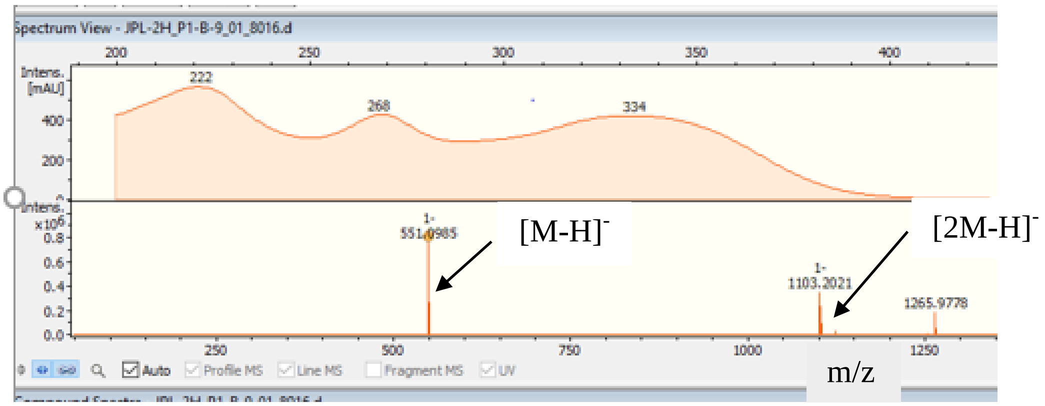


Figure 4. 10: The mass spectra of compound **13**

The ^1H NMR spectrum of compound **13** was measured. The signals between δ_H 6.0- 8.0 were aromatic protons and this supported the presence of an aromatic ring system suggested by the ^{13}C data. These peaks include δ_H 6.82 (H-3), 6.18 (H-6), 6.43 (H-8), 8.01 (H-2′), 7.14 (H-5′), 8.00 (H-6′), 6.88 (H-3″), 6.38 (H-6 ″), 7.69 (H-2 ‴ and 6 ‴) and 6.91 (H-3 ‴ and 5 ‴). The strong singlet signal at δ_H 3.74 supported the protons of the methoxy group.

The carbon atoms were assigned based on the 2D NMR data. That is, COSY, HSQC and HMBC. The compound was found to have 31 carbon atoms with one methoxy group at δ_C 55.2, twelve methine carbon atoms and eighteen quaternary carbon atoms. The methine carbon atoms absorbed at δ_C 102.6 (C-3), 98.5 (C-6), 93.7 (C-8), 131.2 (C-2 ′), 116.1 (C-5 ′), 127.5 (C-6 ′), 102.9 (C-3 ″), 98.6 (C-6 ″), 127.7 (C-2 ‴ and C- 6 ‴) and 114.1 (C-3 ‴ and C-5 ‴). These carbon atoms were in an aromatic ring system. The quaternary carbon atoms absorbed at δ_C 163.6 (C-2), 181.5 (C-4), 161.2 (C-5), 163.8 (C-7), 157.1 (C-9), 103.5 (C-10 and C-10 ″), 120.6 (C-1′), 120.1 (C-3′), 159.6 (C-4′), 162.9 (C-2 ″), 181.9 (C-4 ″), 160.3 (C-5 ″), 162.0 (C-7 ″), 104.1 (C-8 ″), 154.5 (C-9″), 122.8 (C-1 ‴) and 161.9 (C-4 ‴). Based on the chemical shifts of the quaternary carbon atoms, it can be suggested that all the carbon atoms are in an aromatic ring system. Twelve carbon atoms with chemical shifts ranging between δ_C 150- 182 should be oxygenated while the other six quaternary carbon atoms could be at the joints of the aromatic rings. In the molecule, the carbon atoms C-2 ‴ and C- 6 ‴ are equivalent while C-3 ‴ and C-5 ‴ are both equivalent.

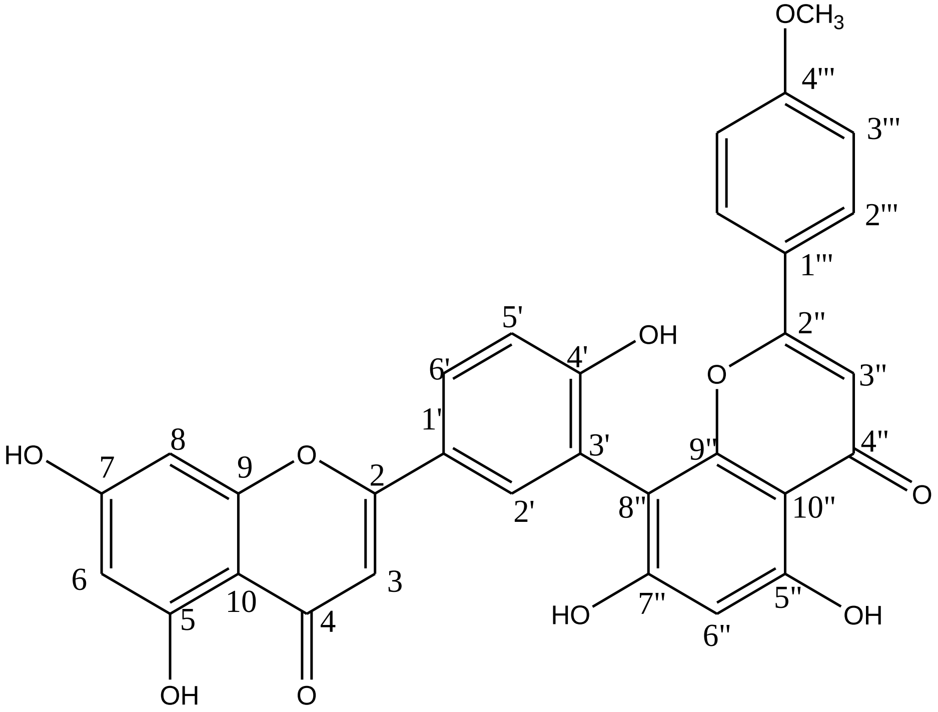


Figure 4. 11: Structure of compound **13**

The HSQC spectrum assisted in assigning protons to carbon atoms that they were directly attached to. From the spectrum, there was correlation between δ_H 6.82 with C- 3, 6.18 with C-6, 6.43 with C-8, 8.01 with C-2′, 7.14 with C-5′, 8.00 with C- 6′, 6.88 with C- 3″, 6.38 with C-6″, 7.69 with C- 2 ‴ and C-6 ‴, 6.91 with C- 3 ‴ and C-5 ‴and 3.74 with CH_3O-4 ‴.

The ^1H-^1H COSY spectrum was also determined and two important correlations were observed. The proton at δ_H 7.14 (H-5′) correlated with proton at δ_H 8.00 (H-6′) and the proton δ_H 7.69 (H-2 ‴) correlated with the proton at δ_H 6.91 (H-3 ‴). These correlations supported the positions of the methine group pairs adjacent to each other.

The proton- carbon HMBC was also determined. The proton at δ_H 6.82 (H-3) showed correlations with C- 2, C-4, C-10 and C-1′. The proton at δ_H 6.18 (H-6) correlated with C-5, C-7, C-8 and C-10 while the proton at δ_H 6.43 (H-8) showed correlations with C-6, C-7, C-9 and C-10. More correlations were observed proton δ_H 8.01 (H-2′) with C-2, C-4′, C-6′ and C-8″. The correlation of H-2′ with C-8″ was an important correlation because it supported the fact that the molecule is a biflavonoid connected at C-3′ and C-8″. Further correlations were observed between the proton at δ_H 7.14 (H-5′) with C-3′ and C-4′ while δ_H 8.00 (H-6′) showed correlation with C-2 and C-2′. There were also correlations between proton δ_H 6.88 (H- 3″) with C-2″, C-4″, C-10″ and C-1 ‴. The proton at δ_H 6.38 (H-6″) showed correlations between C- 5″, C- 7″and C-10″. The proton at δ_H 7.69 (H-2 ‴) showed correlations with C-4 ‴ and C- 6 ‴ while the proton at δ_H 6.91 (H-3 ‴) showed correlations between C-1 ‴, C-4 ‴ and C- 5 ‴. Lastly, a correlation between proton at δ_H 3.74 (CH_3O-4 ‴) with C- 4 ‴. This correlation was vital because it assisted in identifying the carbon atom at which the methoxy group was attached.

The compound was identified as podocarpusflavone A. The NMR data for the compound is summarized in table 4.7 and the COSY and HMBC correlations are illustrated in figure 4.12. This data closely corresponded to that obtained by Carbonezi et al. (2007).

Table 4.7: NMR data of compound **13** in DMSO

Carbon	**^{13}C(δ)**	**HSQC(δ)**	**TYPE**	**COSY**	**HMBC**	**^{13}C(δ)***
2	163.6	-	C	-	-	163.8
3	102.6	6.82	CH	-	2,4,10,1′	103.0
4	181.5	-	C	-	-	181.7
5	161.2	-	C	-	-	161.5
6	98.5	6.18	CH	-	5,7,8,10	98.8
7	163.8	-	C	-	-	164.1
8	93.7	6.43	CH	-	6,7,9,10	94.0
9	157.1	-	C	-	-	157.4
10	103.5	-	C	-	-	103.7
1’	120.6	-	C	-	-	121.1
2’	131.2	8.01	CH	-	2,4′,6′,8″	131.4
3’	120.1	-	C	-	-	119.9
4’	159.6	-	C	-	-	159.5
5’	116.1	7.14	CH	6’	3′,4′	116.2

6’	127.5	8.00	CH	5′	2, 2′	127.9
2”	162.9	-	C	-	-	163.2
3”	102.9	6.88	CH	-	2″,4″,10″,1 ‴	103.2
4”	181.9	-	C	-	-	182.2
5”	160.3	-	C	-	-	160.6
6”	98.6	6.38	CH	-	5″, 7 ‴,10″	98.7
7”	162.0	-	C	-	-	161.9
8”	104.1	-	C	-	-	104.0
9”	154.5	-	C	-	-	154.5
10”	103.5	-	C	-	-	103.7
1’”	122.8	-	C	-	-	123.0
2’”	127.7	7.69	CH	3 ‴	4 ‴,6 ‴	128.0
3’”	114.1	6.91	CH	2 ‴	1 ‴,4 ‴,5 ‴	114.5
4’”	161.9	-	C	-	-	162.2
5’”	114.1	6.91	CH	-	-	114.5
6’”	127.7	7.69	CH	-	-	128.0
CH_3O-4’”	55.2	3.74	CH_3	-	4 ‴	55.5

^{13}C(δ)*- Reference data for ^{13}C (Carbonezi et al., 2007)

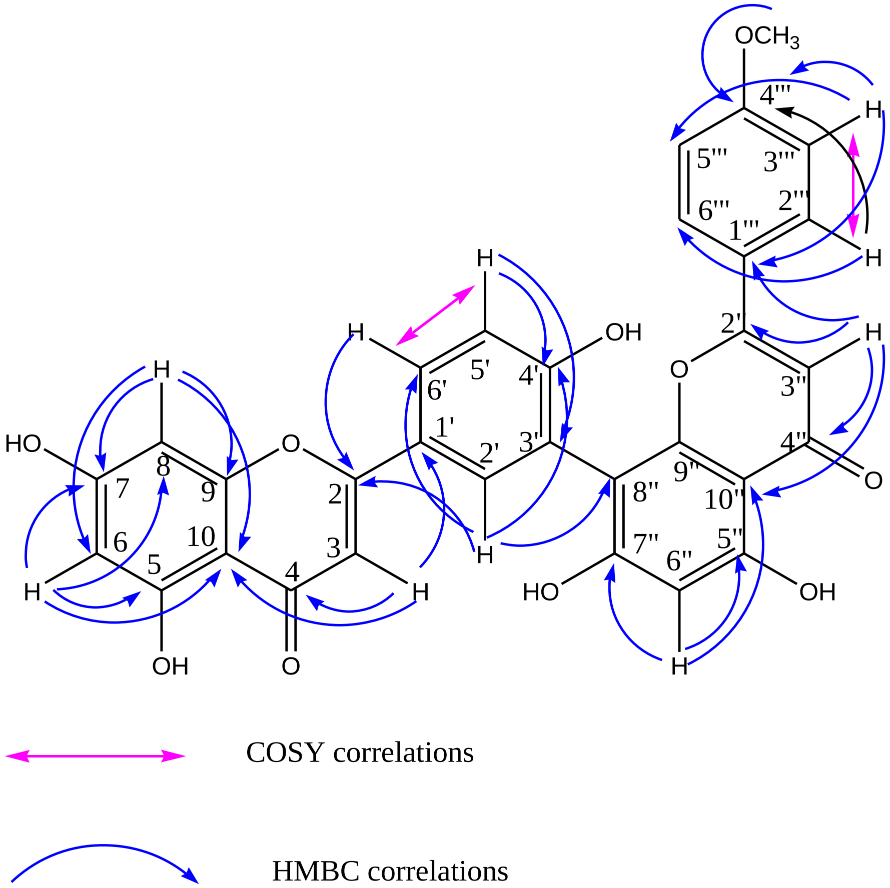


Figure 4. 12: Compound **13** structure showing COSY and HMBC correlations

4.3.3 Structure elucidation of compound 14

Compound **14** (Figure 4.15) was also isolated from the leaf through purification of fraction 2 using HPLC. The compound is a diterpenoid that was collected around 15-16 minutes during HPLC purification at 55% isocratic conditions (Figure 4.13). It was found to have a molecular mass of 332.1915 using HR-ESIMS that corresponded to molecular formula $C_{20}H_{28}O_4$. This was supported by a molecular ion peak at 331.1915 $[M-H]^-$ as shown in figure 4.14.

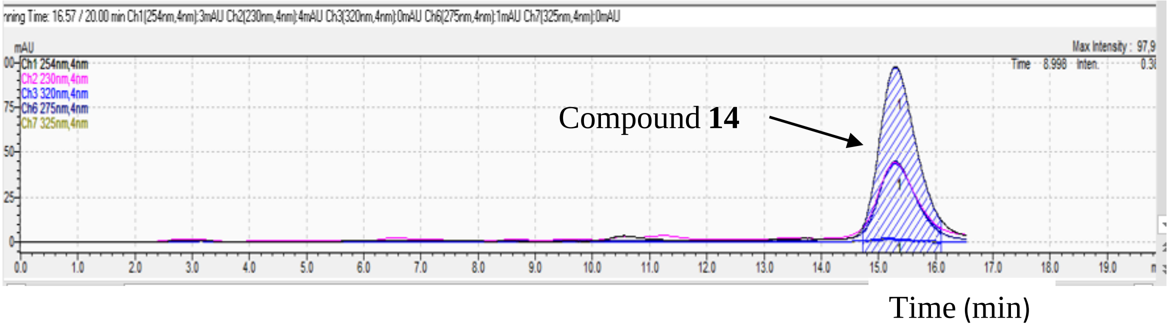


Figure 4. 13: HPLC chromatogram of compound **14**

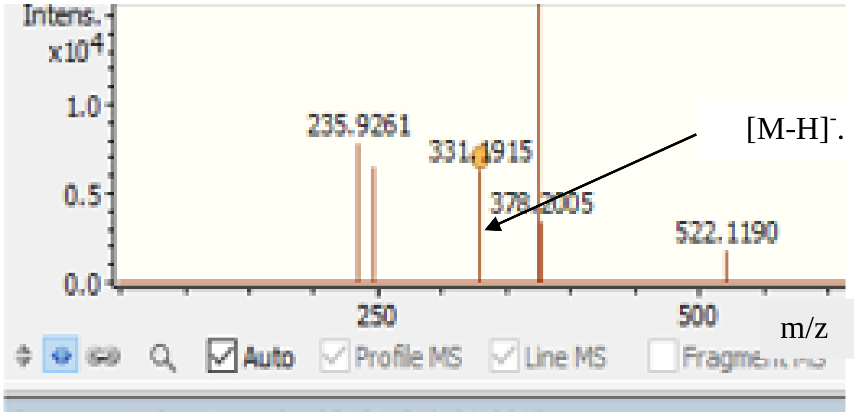


Figure 4. 14: Mass spectrum of compound **14**

The ^{1}H NMR spectrum was measured whereby most of the signals were observed between δ_H 0.5 and 5 ppm. In this region, some of the distinct peaks observed were two strong doublets at around δ_H 0.99 (H-17) and δ_H 0.88 (H-16) which were the methyl protons in the isopropyl group. Two strong singlet peaks were also observed at δ_H 0.73 (H-20) and δ_H 1.33 (H-18). A doublet peak was observed at around δ_H 2.34 (H-5) and also at 4.98 (H-6). Above 5 ppm a distinct peak at δ_H 6.86 (H-14) which corresponds to the proton attached to the double bond.

The carbon atoms were assigned on the basis of the 2D NMR data (COSY, HSQC and HMBC). From the data, twenty carbon atoms were found to be present. These were four methyl, five methylene, five methine and six quaternary carbon atoms. The methyl carbon atoms absorbed at δ_C 16.4, 15.1, 24.2 and 15.8 assigned to C-16, C-17, C-18 and C-20 respectively. The methylene carbon atoms C-1, C-2, C-3, C-11 and C-12 absorbed at δ_C 32.0,17.3,28.1,18.5 and 29.5 respectively. The methine carbon atoms absorbed at δ_C 51.8, 76.8, 48.9, 142.8 and 37.5 corresponding to C-5, C-6, C-9, C-14 and C-15 respectively. The quaternary carbon atoms C-4, C-7, C-8, C-10, C-13 and C-19 absorbed at δ_C 41.9, 194.1, 136.9, 32.8, 71.7 and 181.6 respectively.

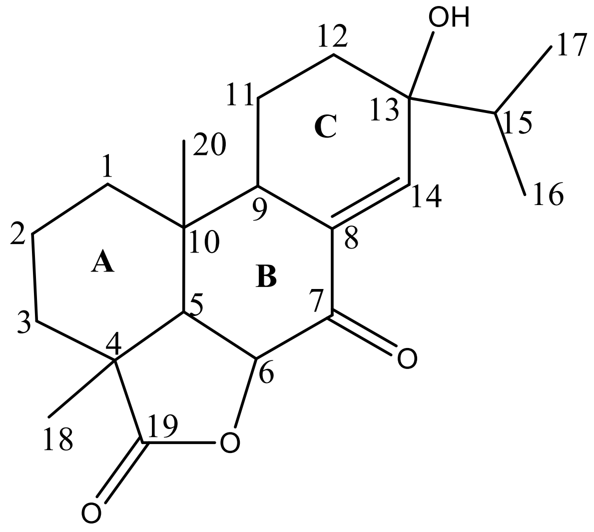


Figure 4. 15: Structure of compound **14**

The ^1H-^1H COSY spectrum was measured and three important correlations were obtained. The proton absorbing at δ_H 2.34 (H-5) had a correlation with the proton absorbing at δ_H 4.98 (H-6). The proton absorbing at δ_H 1.81 (H-15) showed correlations with protons absorbing at δ_H 0.88 (H-16) and δ_H 0.99 (H-17). This correlation was important it assisted in assigning the carbon atoms that made the isopropyl group.

The HSQC spectrum assisted in assigning protons to carbon atoms that they were directly attached to. From the spectrum, there was correlation between δ_H 1.37/1.65 with C- 1, 1.66 with C-2, 1.52/2.12 with C-3, 2.34 with C-5, 4.98 with C-6, 2.22 with C- 9, 1.47/1.73 with C- 11, 1.63/1.76 with C-12, 6.86 with C- 14, 1.81 with C-15, 0.88 with C- 16, 0.99 with C-17, 1.33 with C- 18 and 0.73 with C- 20.

The proton- carbon HMBC spectrum was also measured. It gave information about proton bond connectivity up to three bonds away. Proton resonating at δ_H 1.37/1.65 (H-1) showed correlations with two methylene carbons C-2 and C-3, methine carbon C-9, quaternary carbon-10 and methyl carbon-20. The proton resonating at δ_H 1.6 6 (H-2) had correlations with two quaternary carbon atoms carbon-4 and C-10, methylene carbon- 3, methine carbon-5 and methyl carbon- 20. The methylene carbon proton δ_H 1.52/2.12 (H-3) resonated with C-1, C-2, C-4, C-5, C-18 and C-19.

The methine proton resonating at δ_H 2.34 (H-5) showed correlations with three quaternary carbon atoms C-4, C-10 and C-19, two methyl carbon atoms C-18 and C-20 and methine carbon C-9. The proton resonating at δ_H 4.98 (H-6) correlated with carbonyl carbon-7, quaternary carbon-10 and methine carbon-5. The proton resonating at δ_H 2.22 (H-9) showed correlations with methine carbon atoms -5 and 14, quaternary carbon atoms-8 and 10, methylene carbon atoms-11 and 12 and lastly the methyl carbon-20. Proton resonating at δ_H 1.47/1.73 (H-11) correlated with C-8, C-9, C-12 and C-13. The proton resonating at δ_H 1.63/1.76 (H-12) showed correlations with C-8, C-9, C-13, C-14 which is the methine carbon of the alkene group and C-15. The proton resonating at δ_H 6.86 (H-14) correlated with C-7, C-8, C-9, C-12 and C-15 which was methine carbon of the isopropyl group. Proton resonating at δ_H 1.81 (H-15) correlated with C-12, C-13, C-14, C-16 and C-17. The proton δ_H 0.88 (H-16) showed a correlation with C-13, C-15 and C-17 while proton resonating at δ_H 0.99 (H-17) showed correlations with C-13, C-15 and C-16. The correlations of H-15, H-16 and H-17 aided in assigning the carbon skeleton around the isopropyl group. The methyl protons resonating at δ_H 1.33 (H-18) correlated with C-3, C-4, C-5 and C-19 which was the carbonyl carbon on the lactone ring. The methyl proton at δ_H 0.73 (H-20) correlated with C-5, C-9 and C-10.

The compound was identified as juniperolide. A summarized table of this data is as illustrated in table 4.8. The COSY and HMBC correlations are as illustrated in figure 4.16. The data closely corresponded to that obtained by Jim-Min et al. (1993).

Table 4.8: NMR data of compound **14** in CD_3O

Carbon	**13C(δ)**	**HSQC(δ)**	**Type**	**COSY**	**HMBC**	**13C(δ)***
1	32.0	1.37,1.65	CH_2	-	2,3,9,10,20	32.5
2	17.3	1.66	CH_2		3,4,5,10,20	17.7
3	28.1	1.52,2.12	CH_2		1,2,4,5,18,19	28.3
4	41.9	-	C	-	-	41.8
5	51.8	2.34	CH	6	4,9,10,18,19,20	52.6
6	76.8	4.98	CH	5	5,7,10	76.0
7	194.1	-	C	-	-	193.1
8	136.9	-	C	-	-	136.8
9	48.9	2.22	CH	-	5,8,10,11,12,14,20	49.5
10	32.8	-	C	-	-	33.1
11	18.5	1.47,1.73	CH_2	-	8,9,12,13	18.9
12	29.5	1.63,1.76	CH_2	-	8,9,13,14,15	29.8
13	71.7	-	C	-	-	72.3
14	142.8	6.86	CH	-	7,8,9,12,15	143.0
15	37.5	1.81	CH	16,17	12,13,14,16,17	37.6
16	16.4	0.88	CH_3	15	13,15,17	17.3
17	15.1	0.99	CH_3	15	13,15,16	16.1
18	24.2	1.33	CH_3	-	3,4,5,19	25.3
19	181.6	-	C	-	-	180.0
20	15.8	0.73	CH_3	-	5,9,10	16.6

$^{13}C(\delta)$*- Reference data for ^{13}C (Jim-Min et al., 1993)

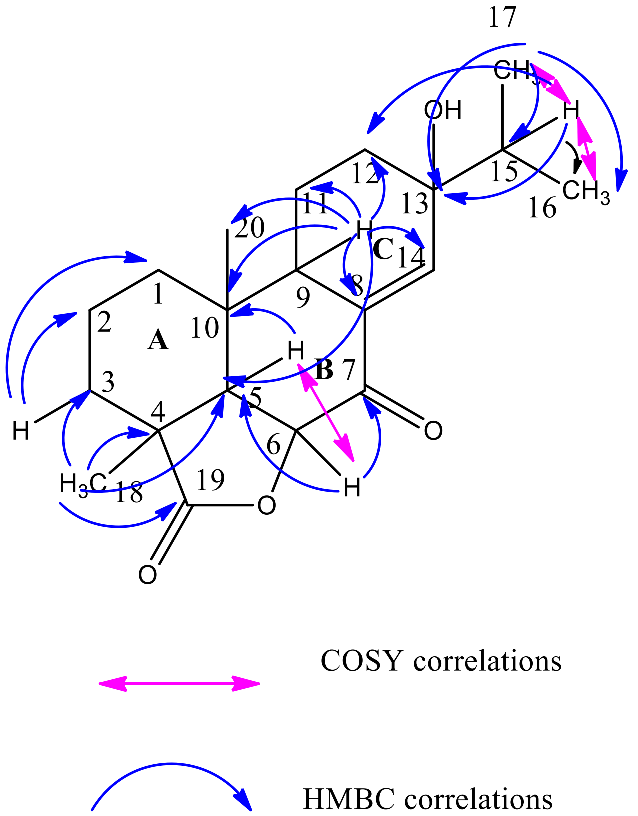


Figure 4. 16: Compound **14** structure showing COSY and HMBC correlations

CHAPTER FIVE

DISCUSSION

5.1 Antimicrobial activity of crude extracts and isolated compounds

From the bioassay experiments, the bacterial test pathogens (*X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*) were found to be more susceptible to the crude extracts compared to the semi-pure fractions. This can be attributed to the synergistic effect of crude extract components. Wagner & Ulrich (2009) stated that crude extracts contain a mixture of bioactive compounds which work in synergy to inhibit the growth of microbes. The synergy effect is usually produced if the components of the crude extracts interrelate with one another with an objective of boosting the solubility hence improving the bioavailability of the various constituent of the extracts. The effect can also occur if the components of the crude extract act on distinct targets (Wagner & Ulrich, 2009). The synergy effect is, therefore, responsible for the enhanced effectiveness of the crude extracts compared to that of semi pure compounds. This trend was also reported by Chepkorir et al. (2018) whereby the crude extracts from *Hagenia abyssinica* and *Withania somnifera* showed higher activity against fungal and bacterial plant pathogens than the isolated pure compounds.

It was also noted that the crude extracts from the leaf were more active against *P. savastanoi* pv. *phaseolicola* while crude extracts from the bark showed higher activity against *X. axonopodis* pv. *phaseoli*. This indicates that the two different parts of the plant have different phytochemical composition. The variation of the chemical composition of extracts from different parts of *J. procera* has previously been reported. In the report, GC- MS analysis showed that extracts from the leaf and the fruit had different chemical composition. In addition, the leaf extract was found to have a better antifungal activity compared to that of fruit extract against *Aspergillus fumigatus* and *Fusarium chlamydosporum* (Bakri et al., 2020). This scenario can be attributed to genetic factors whereby the genes of a plant tend to code more phytochemicals on one part of the plant compared to the others (Andrew et al., 2010). This can also be supported by a study carried out on ferruginol, a compound isolated from *J. procera*, whereby it was found to be present in significantly greater amount in the root extract compared to the leaf and seed extracts (Salih et al., 2022). It can also be attributed to the exposure of the part of the plant to pathogens (Bakar et al., 2015). Plants produce compounds known as phytoalexins in response to attack by pathogens for self-protection. Therefore, if the leaf and the bark are attacked by different pathogens, distinct phytoalexins will be produced hence the difference in phytochemical composition. This difference in phytochemical composition had also been reported previously, whereby the leaves, roots and bark of *Artocapus heterophyllus* plant were found to have different phytochemicals (Ojwang et al., 2017).

Hexane extract was found to have the least activity compared to the other crude extracts tested. Hexane being the least polar solvent amongst the other solvents used during extraction dissolves non-polar compounds. Based on the activity of the hexane extract it can be concluded that the non-polar compounds were not as active as the polar and mid-polar compounds present. Studies have reported that the activity of extracts is dependent on the solvents used during extraction because different solvents extract compounds with different polarities. A study carried out on *Mentha* species extracts showed that non polar extracts had weak antioxidant activity than the polar extracts (Barchan et al., 2014).

Compound **12** was isolated from fraction 3 from the bark extract while compound **13** and **14** were isolated from fraction 2 from leaf extract. All the three compounds registered a higher activity against *P. savastanoi* pv. *phaselicola* compared to the semi pure fractions from which they were isolated (Table 4.1, 4.2 and 4.5). This can be attributed to the increase in concentration of the active compounds. In addition, the compounds contains polyhydroxyl groups which can be active sites responsible for the activity (Vila et al., 2013).

It was noted that the extracts and the three pure compounds isolated were not active against *F. graminearum*. This could be as a result of isolation of a resistant strain of the fungus that was used in the bioassays. Studies have reported resistance of the fungus to antifungal drugs. For instance, it has been reported to be resistant to azole drugs (Zhao et al., 2021). However, it cannot be concluded that the extracts are not fungicidal as the extracts have shown antifungal activity towards other fungal pathogens. For instance, extracts from the leaf and fruit have shown antifungal activity against *Aspergillus fumigatus* and *Fusarium chlamydosporum* (Bakri et al., 2020). Studies have also reported good inhibitory effects of the extracts against *Fusarium oxysporium* and *Aspergillus flavus* (Ghany & Ghany, 2014).

5.2 Structure elucidation of the isolated compounds

Three pure compounds were isolated from the leaf and bark of *J. procera*. From the bark, a flavonoid (**12**) was isolated from ethyl acetate extract and was identified as epicatechin. The identification was through comparison of the data obtained with that from previous literature. The molecule has two chiral centers that is on carbon-2 and carbon-3. The molecule can therefore exist in either cis or trans form (Braicu et al., 2013; Rosen, 2012; Velayutham et al., 2008). The NOESY correlation observed between protons attached to the two carbon atoms that is δ_H 3.99 (H-3) and δ_H 4.58 (H-2) confirmed the cis form of the molecule hence identified as epicatechin. The presence of the compound had previously been reported from Cupressaceae family. For instance, study for investigation of phenolic composition from four *Juniperus* species (*Juniperus communis*, *Juniperus excelsa*, *Juniperus foetidissima* and *Juniperus oxycedrus* subsp. *oxycedrus*) through HPLC-MS analysis reported that catechin was one of the main phenolic compound present (Yaglioglu & Eser, 2017). Catechin had also been reported to be one of the compounds in *Juniperus phoenicea* (Mansour et al., 2018). The compound had been isolated from other different sources and is commonly found in green tea and cacao seeds and their products like dark chocolate (Shay et al., 2015). It has been isolated from stem bark of *Neocarya macrophylla* (Yusuf et al., 2019). The compound has been reported to have more pharmacological activities for instance, it has been reported to have the ability to lower blood pressure and also control cardiovascular diseases (Corti et al., 2009; McCullough et al., 2006). Antioxidant activity of epicatechin has been reported and the catechol moiety of the molecule has been associated with the activity (Ruijters et al., 2013). The compound has also been reported to have anti-inflammatory activity (Dickerhof et al., 2014).

Ethyl acetate extract from the leaf afforded a biflavonoid (**13**) and was identified as podocarpusflavone A. The compound had previously been isolated from different plants. It had been isolated from *Sciadopitys verticillata* and from epiticular material of *Sequoiadendron giganteum* (Wollenweber et al., 1998). It had also been isolated from *Podocarpus parlatorei* (Dávila et al., 2014), *Podocalyx loranthoides* where it was reported to be moderately active against *Leishmania mexicana* promastigotes (Suárez et al., 2003) and from *Ouratea multiflora* where it was reported to have a weak activity against gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* at concentrations 0.5 and 10 µg/ml (Carbonezi et al., 2007). It was also noted that the compound is commonly found in the *Podocarpus* species which had been reported to grow on the same region with *J. procera* (1050-3000m) (Maundu & Tengnäs, 2005). It is therefore evident that it is possible for plants that grow on the same region to produce similar metabolites. From Cupressaceae family, podocarpusflavone A had been isolated from berries of methanolic extract of *Juniperus communis* (Hiermann et al., 1996).

A diterpenoid (**14**) was also isolated from ethyl acetate extract from the leaf. The diterpenoid had previously been isolated from different plants in Cupressaceae family. It was identified as juniperolide and was first isolated from ethyl acetate extract of *Juniperus chinensis* leaves. The NMR data obtained during this study closely corresponded to that obtained previously by Jim-Min et al. (1993). The reading in this study was done in deuterated methanol (CD_3OD) while the reference was done in deuterated chloroform ($CDCl_3$). The slight difference in chemical shift can be attributed to the difference in solvents used (Abd El-Razek, 2007). The compound had also been isolated from *Juniperus tibetica* (Zeng et al., 2021) and from *Juniperus sabina* (Janar et al., 2012).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the results of this study, the following conclusions were deduced:

1. From the *in-vitro* bioassays of this study, it is evident that extracts from the leaf and bark of *J. procera* are active against the bacterial test pathogens (*P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*). The crude extracts showed higher activity than the semi-pure compounds which can be attributed to synergy effect of their components. On the other hand, the extracts did not show activity against the fungal pathogen *F. graminearum*.
2. The extracts from both the leaf and the bark contain numerous compounds as evident in chromatography data. Amongst them, three compounds were successfully isolated. Compound **12**, which was isolated from the bark extract, compound **13** and **14** which were isolated from the leaf extract.
3. Compound **12** was identified as epicatechin while compound **13** was identified as podocarpusflavone A and compound **14**, juniperolide. This is the first time the three compounds were isolated from *J. procera* although they had been isolated previously from other species from Cupressaceae family. Epicatechin **(12)** exhibited the highest activity against *P. savastanoi* pv. *phaseolicola*.

6.2 Recommendations

The following were the recommendations from this research work:

1. Bioactivity of the extracts from *J. procera* against other maize and beans pathogens should be determined to establish whether they can be used to control a wide range of plant pathogens.
2. More compounds should be isolated from *J. procera* and their bioactivity determined.
3. Efficacy of the identified compounds should be further tested under greenhouse and field conditions to confirm their potential for use in management of the target maize and bean pathogens.

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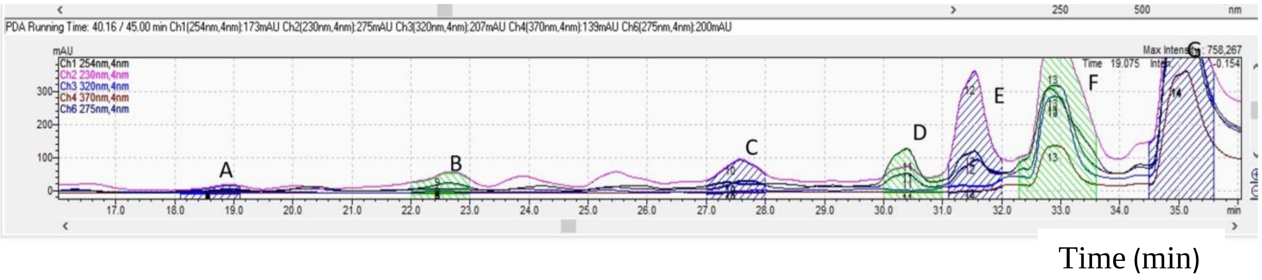
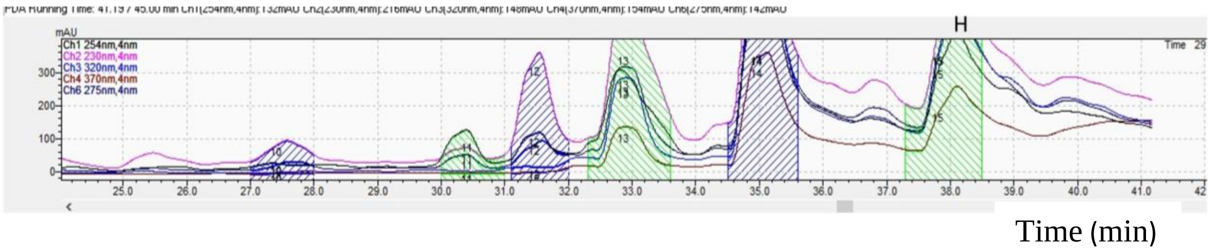
APPENDICES

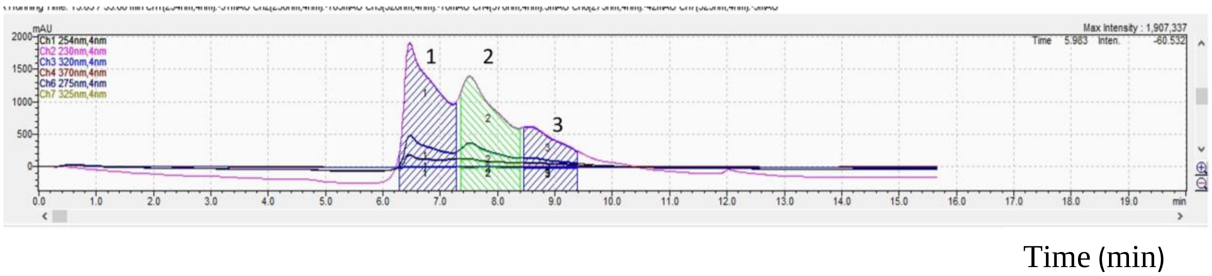
Appendix A: Key data from HPLC analysis

HPLC profile for fraction 2 from the leaf



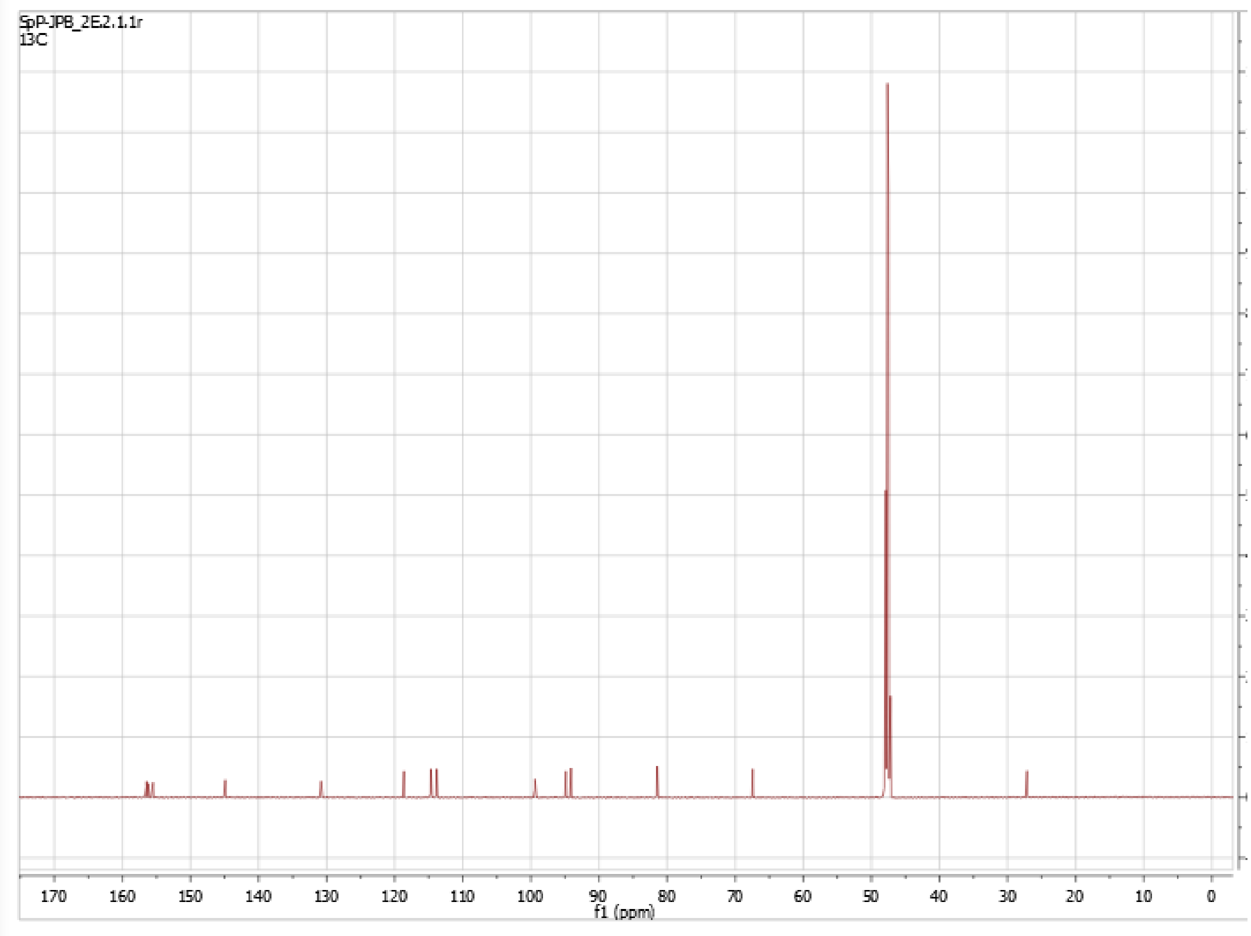


HPLC profile for fraction 3 from the bark

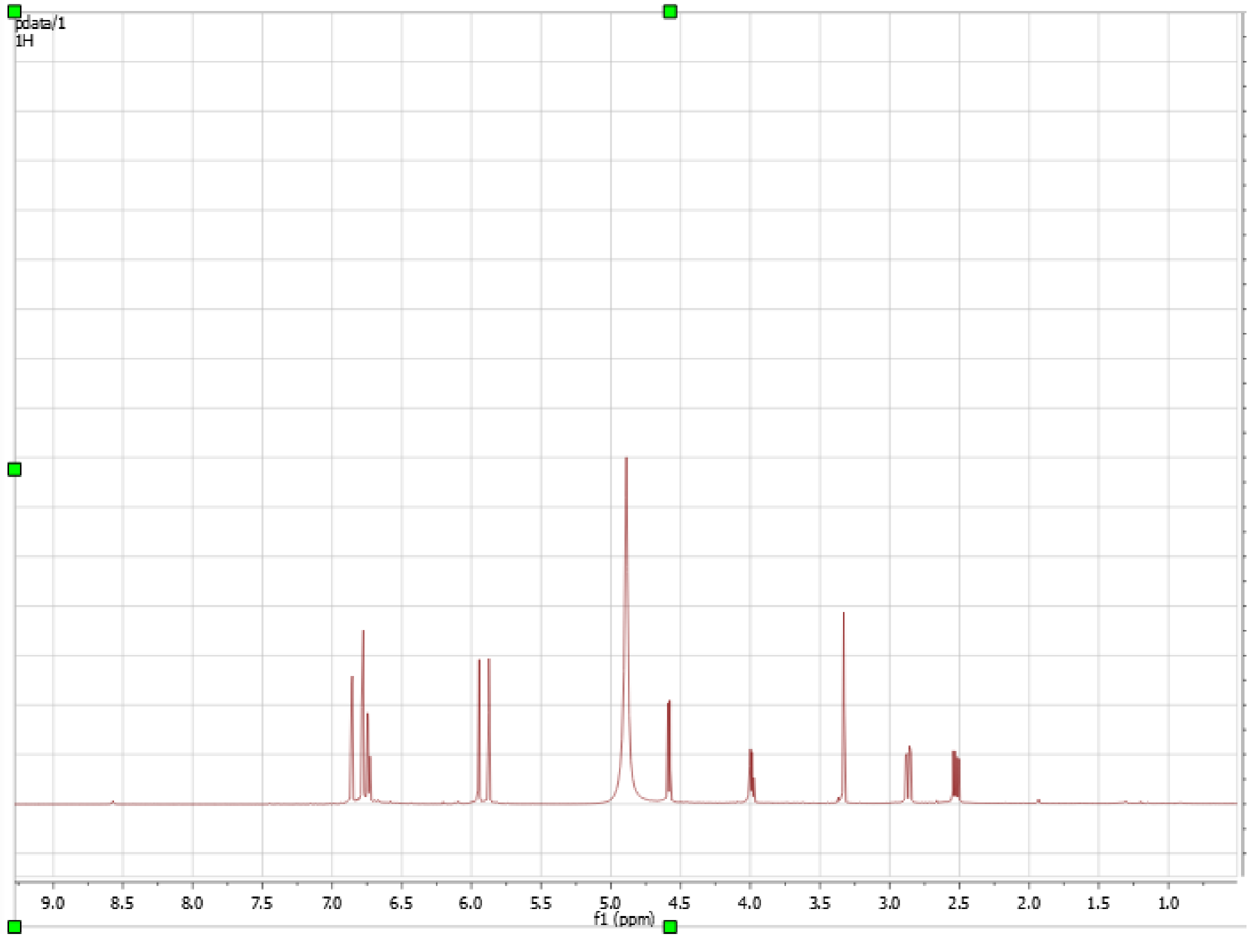


Appendix B: Key data from Nuclear Magnetic Resonance analysis

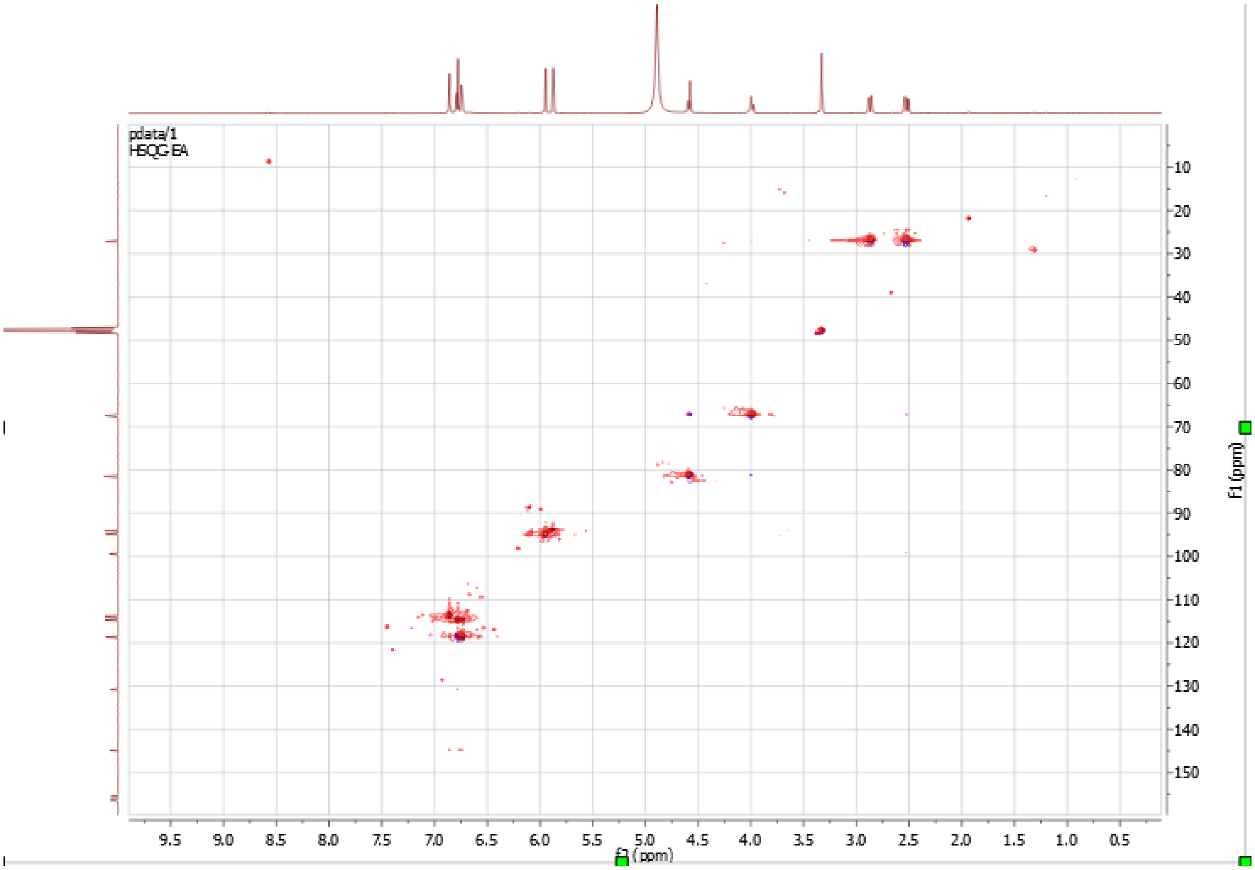
^{13}C NMR spectrum of compound 12



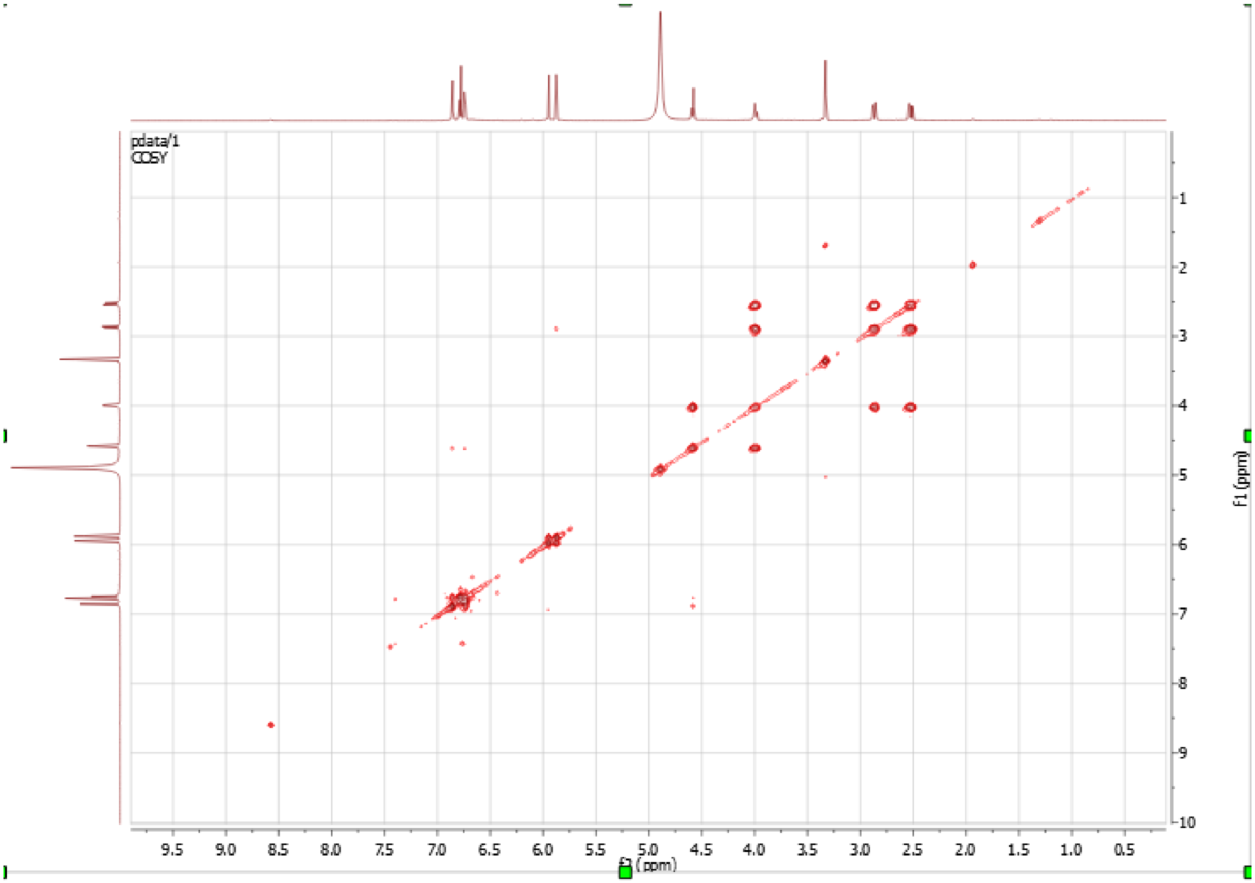
^{1}HNMR spectrum of compound 12



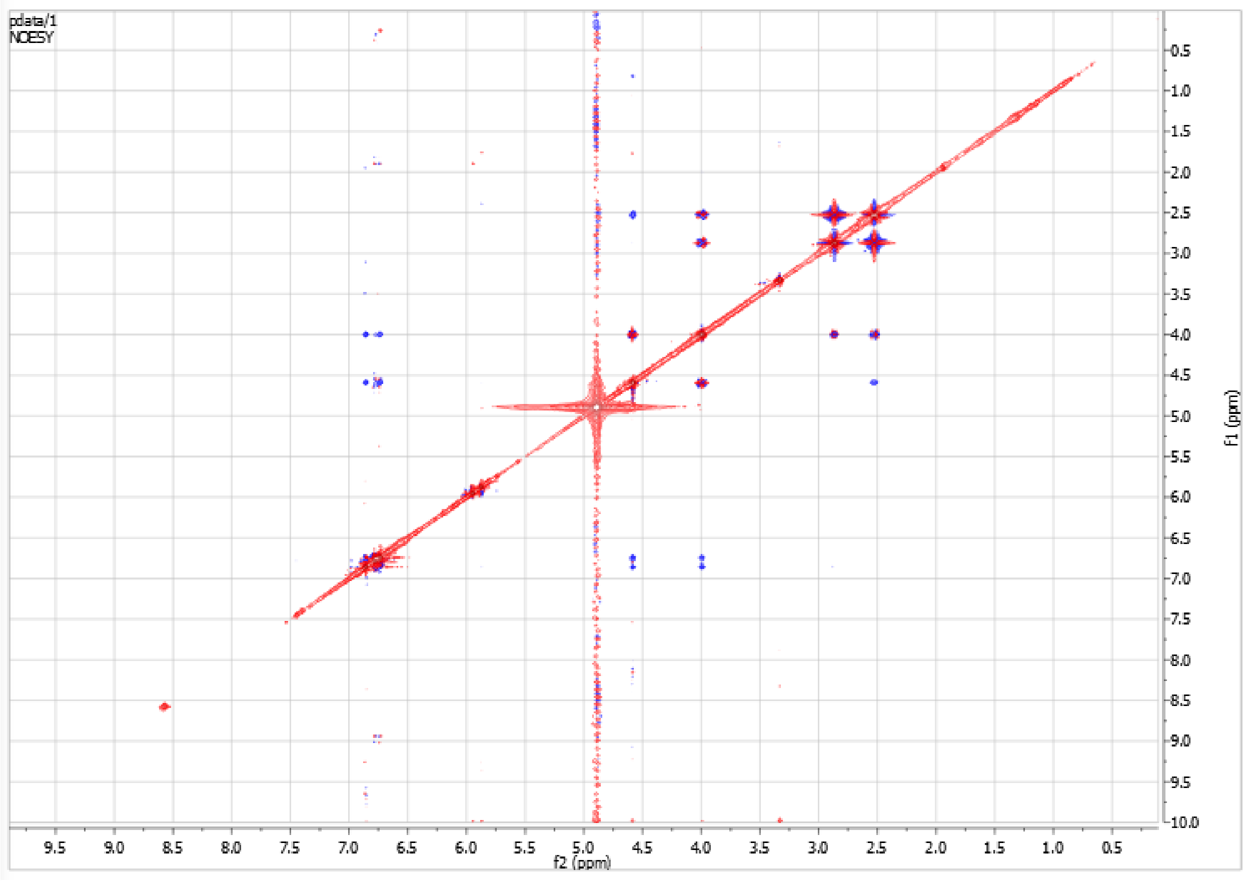
HSQC spectrum of compound 12



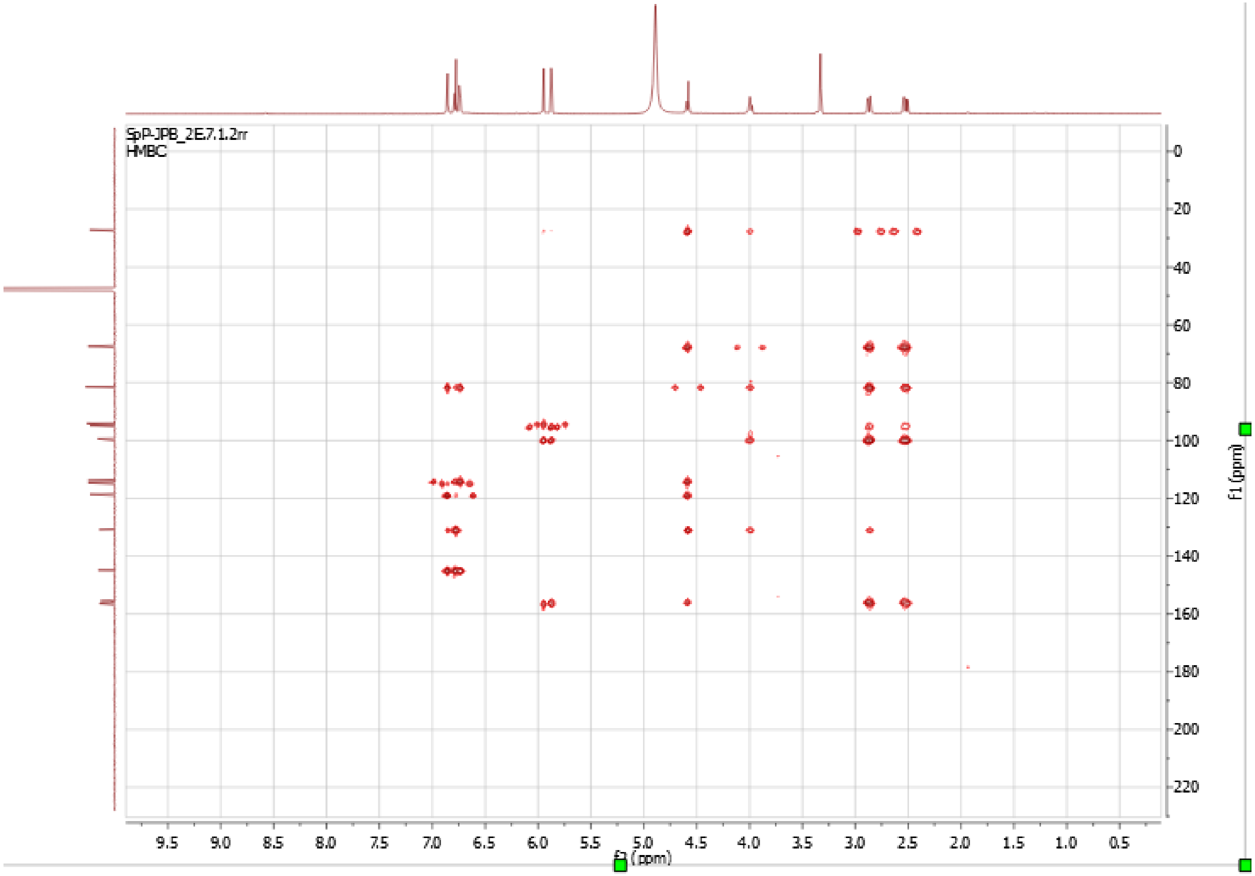
COSY spectrum of compound 12



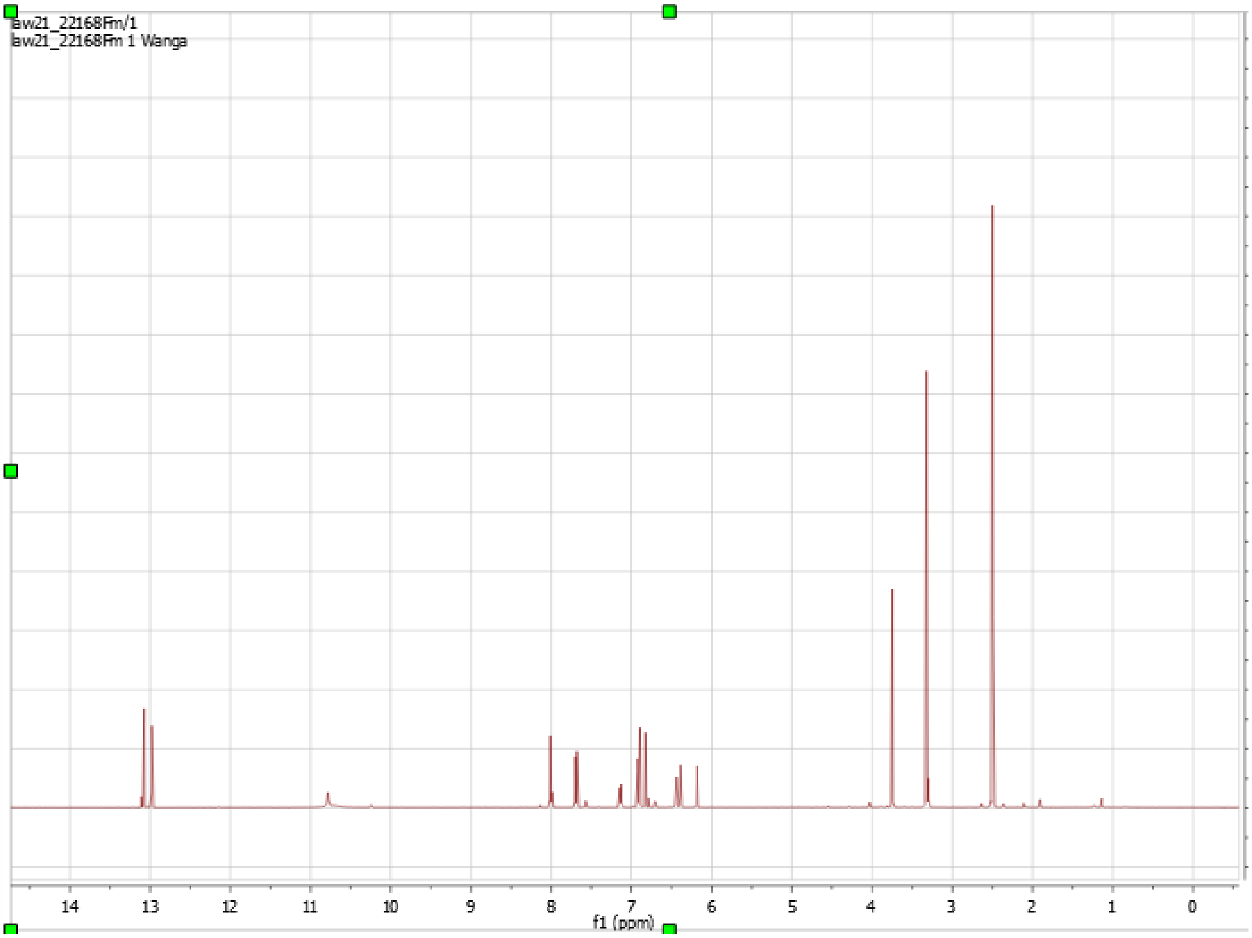
NOESY spectrum of compound 12



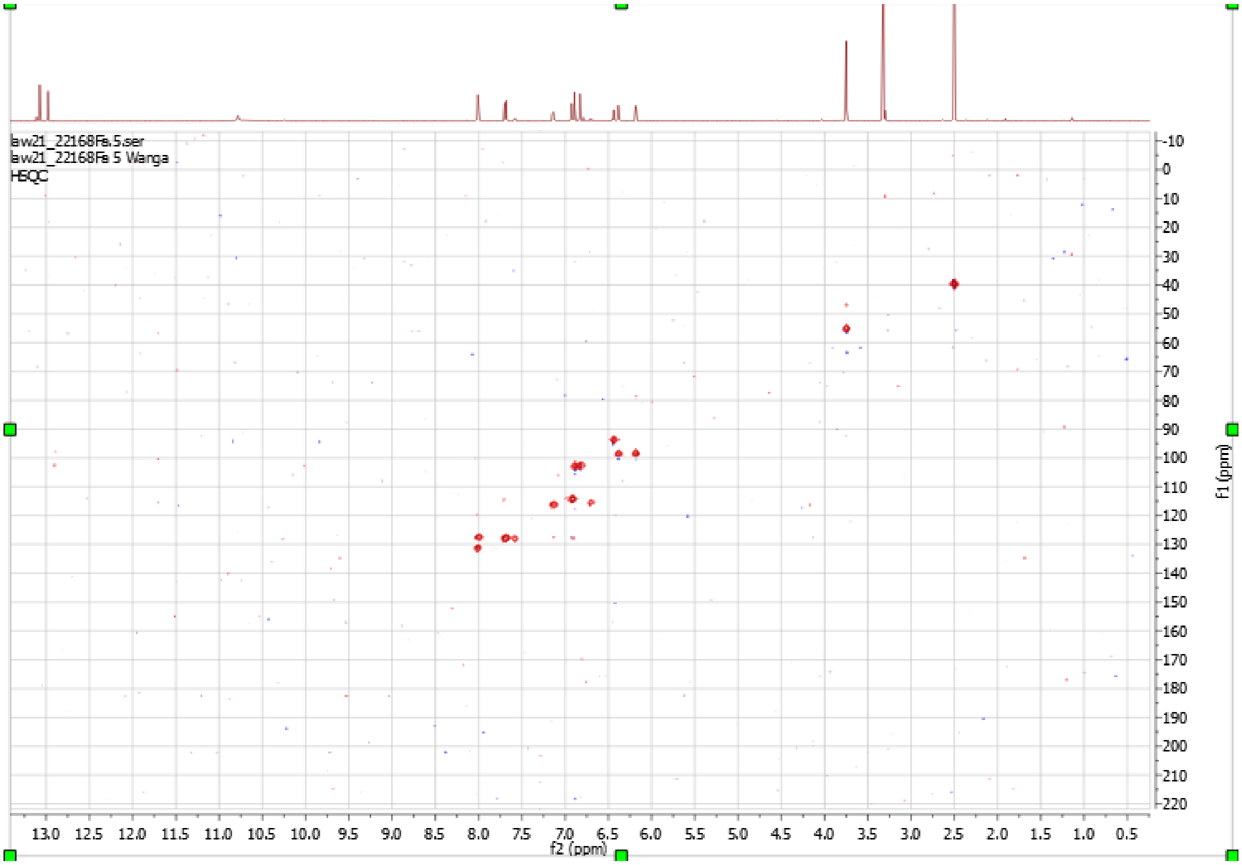
HMBC spectrum of compound 12



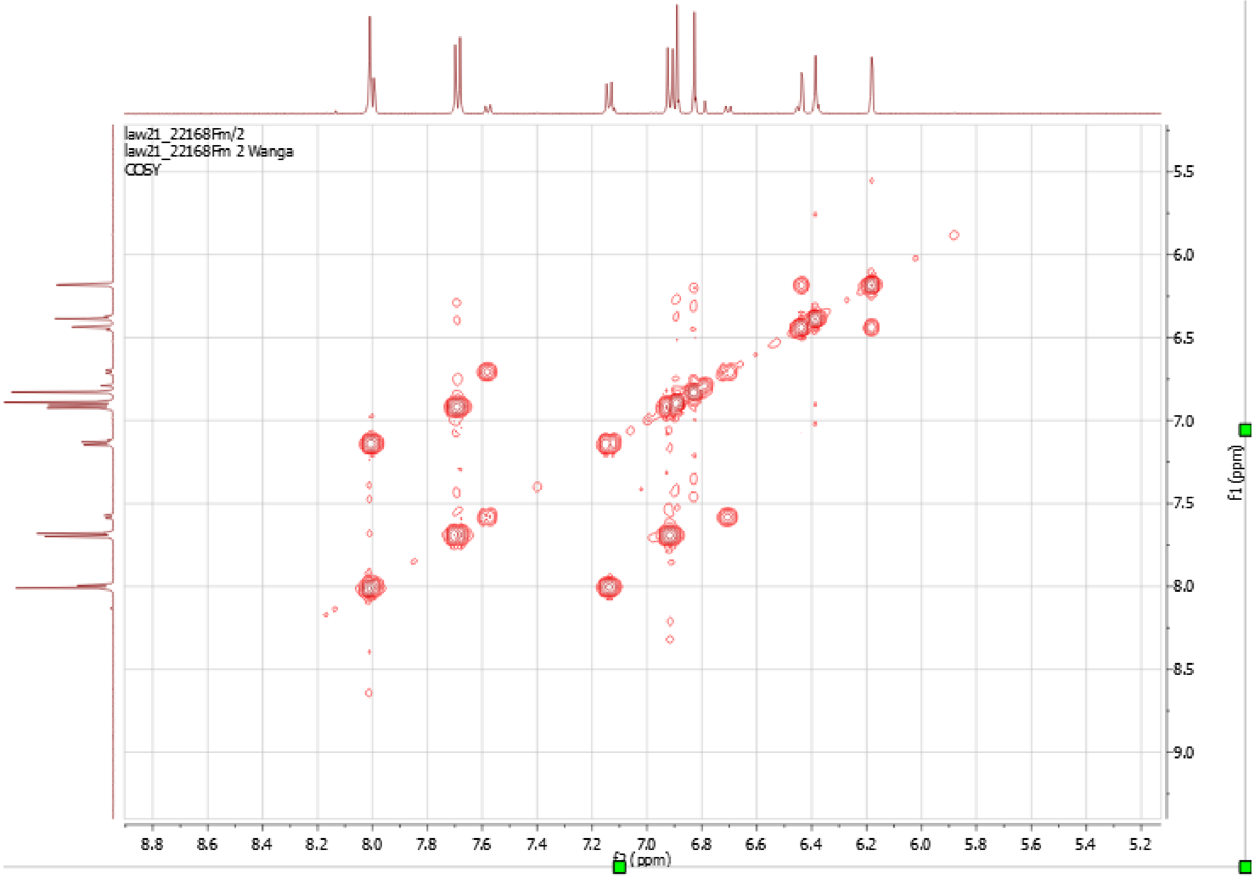
^{1}HNMR of compound 13



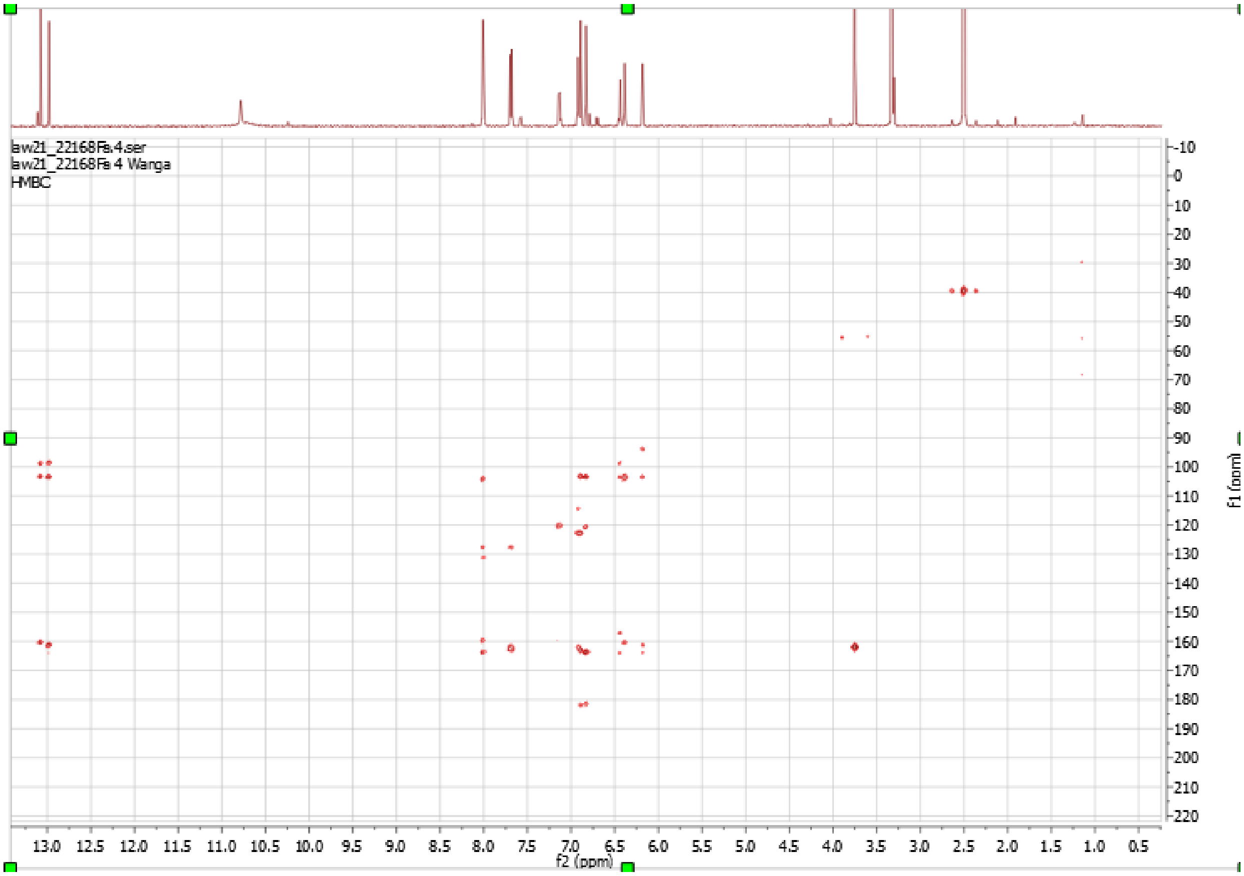
HSQC spectrum of compound 13



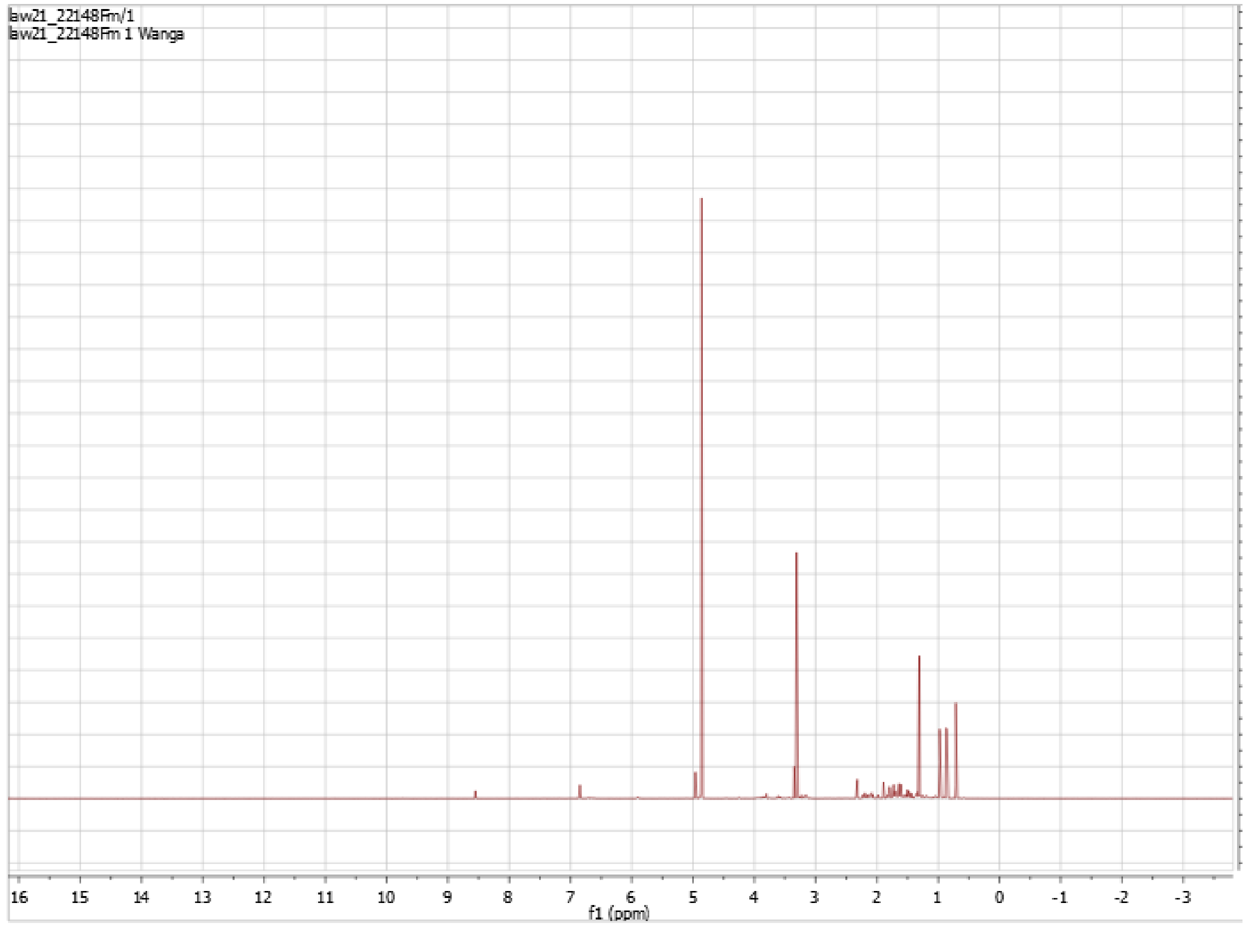
COSY spectrum of compound 13



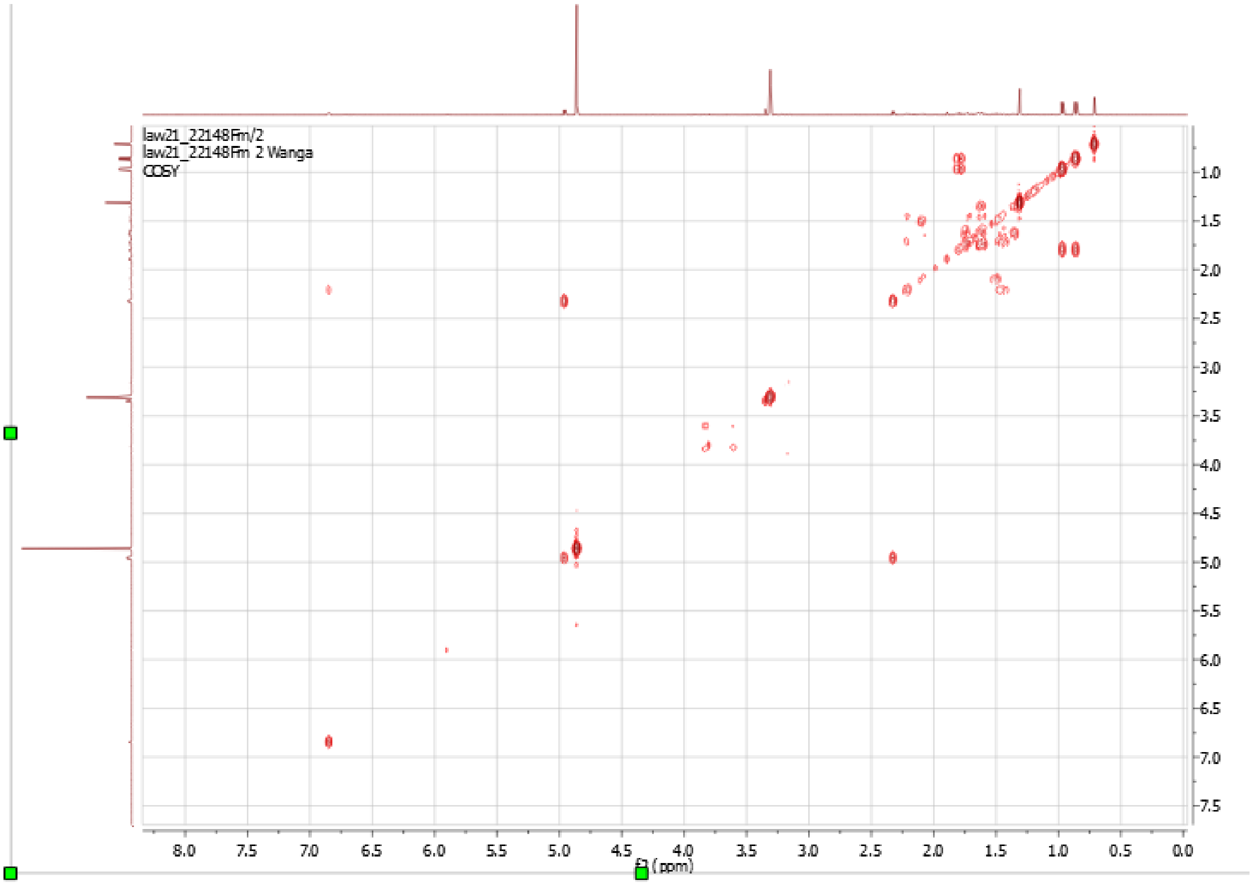
HMBC spectrum of compound 13



^{1}HNMR of compound 14



COSY spectrum of compound 14



HSQC spectrum of compound 14

HMBC spectrum of compound 14

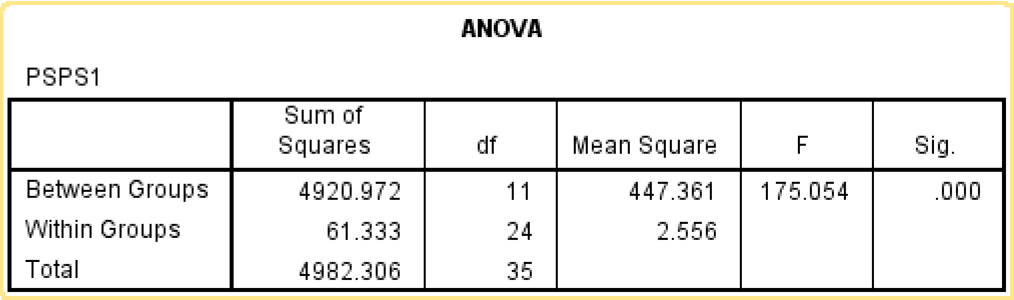
Appendix C: Key data from statistical analysis

ANOVA summary of inhibition zones of crude extracts from leaves of *Juniperus procera* **against** *Pseudomonas savastanoi* pv. *phaseolicola*

```
ONEWAY PSPS1 BY JPL
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

```
[DataSet0]
```



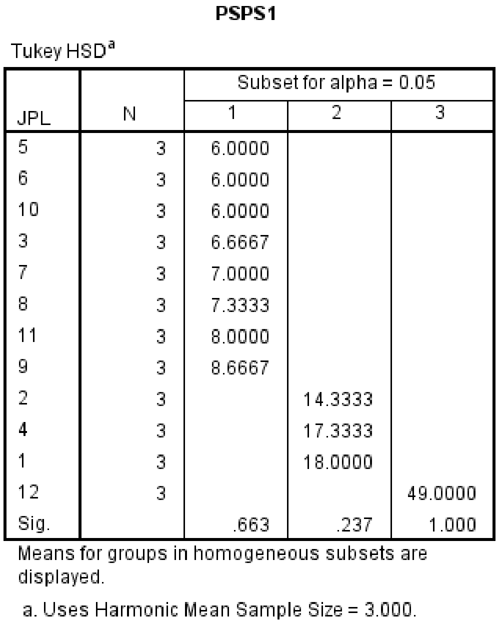
ANOVA

PSPS1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4920.972	11	447.361	175.054	.000
Within Groups	61.333	24	2.556		
Total	4982.306	35			

Post Hoc Tests

Homogeneous Subsets



PSPS1

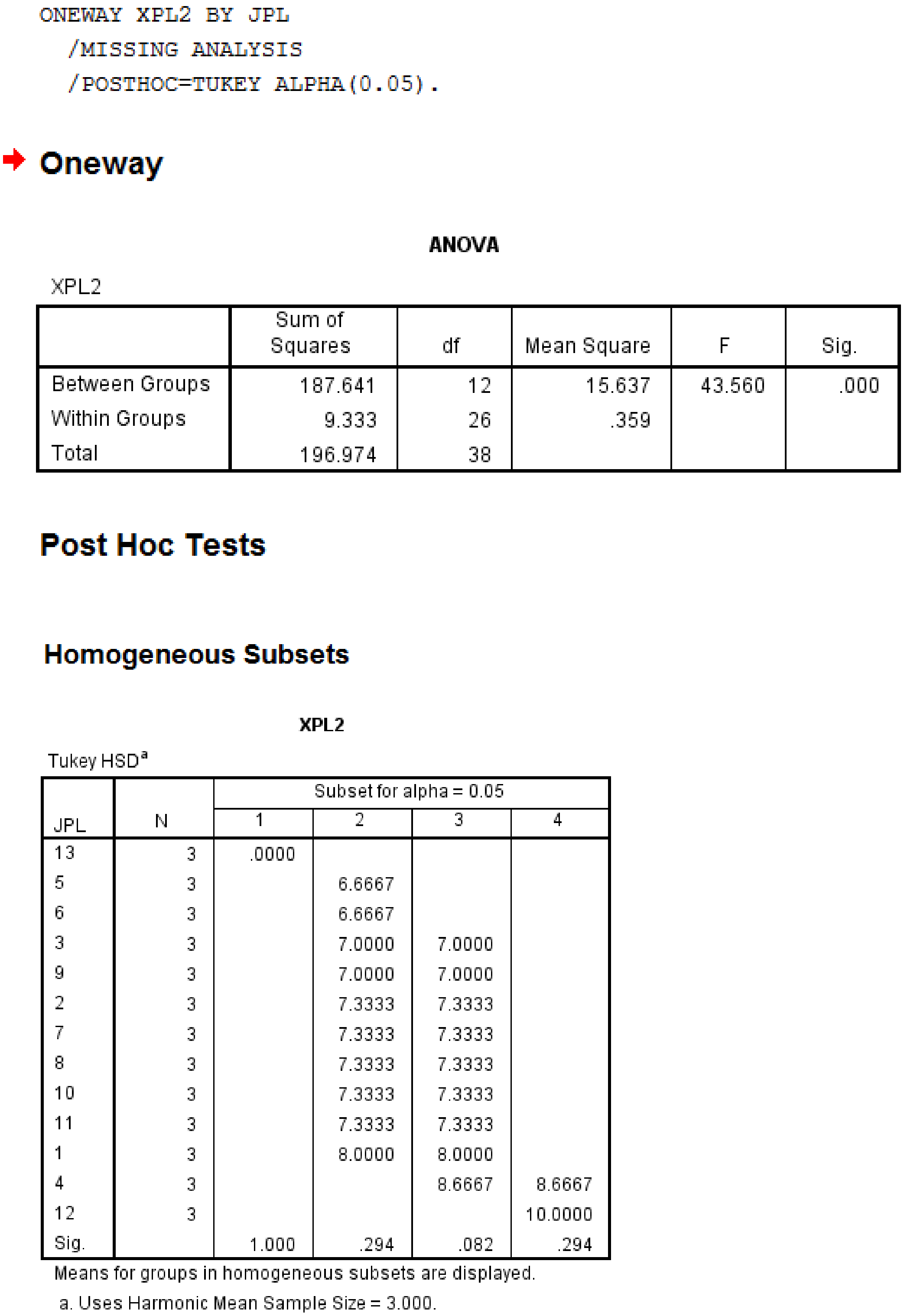
Tukey HSD[a]

JPL	N	Subset for alpha = 0.05		
		1	2	3
5	3	6.0000		
6	3	6.0000		
10	3	6.0000		
3	3	6.6667		
7	3	7.0000		
8	3	7.3333		
11	3	8.0000		
9	3	8.6667		
2	3		14.3333	
4	3		17.3333	
1	3		18.0000	
12	3			49.0000
Sig.		.663	.237	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of crude extracts from ***Juniperus procera*** **leaves against** *Xanthomonas axonopodis* pv. *phaseoli*



```
ONEWAY XPL2 BY JPL
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

ANOVA

XPL2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	187.641	12	15.637	43.560	.000
Within Groups	9.333	26	.359		
Total	196.974	38			

Post Hoc Tests

Homogeneous Subsets

XPL2

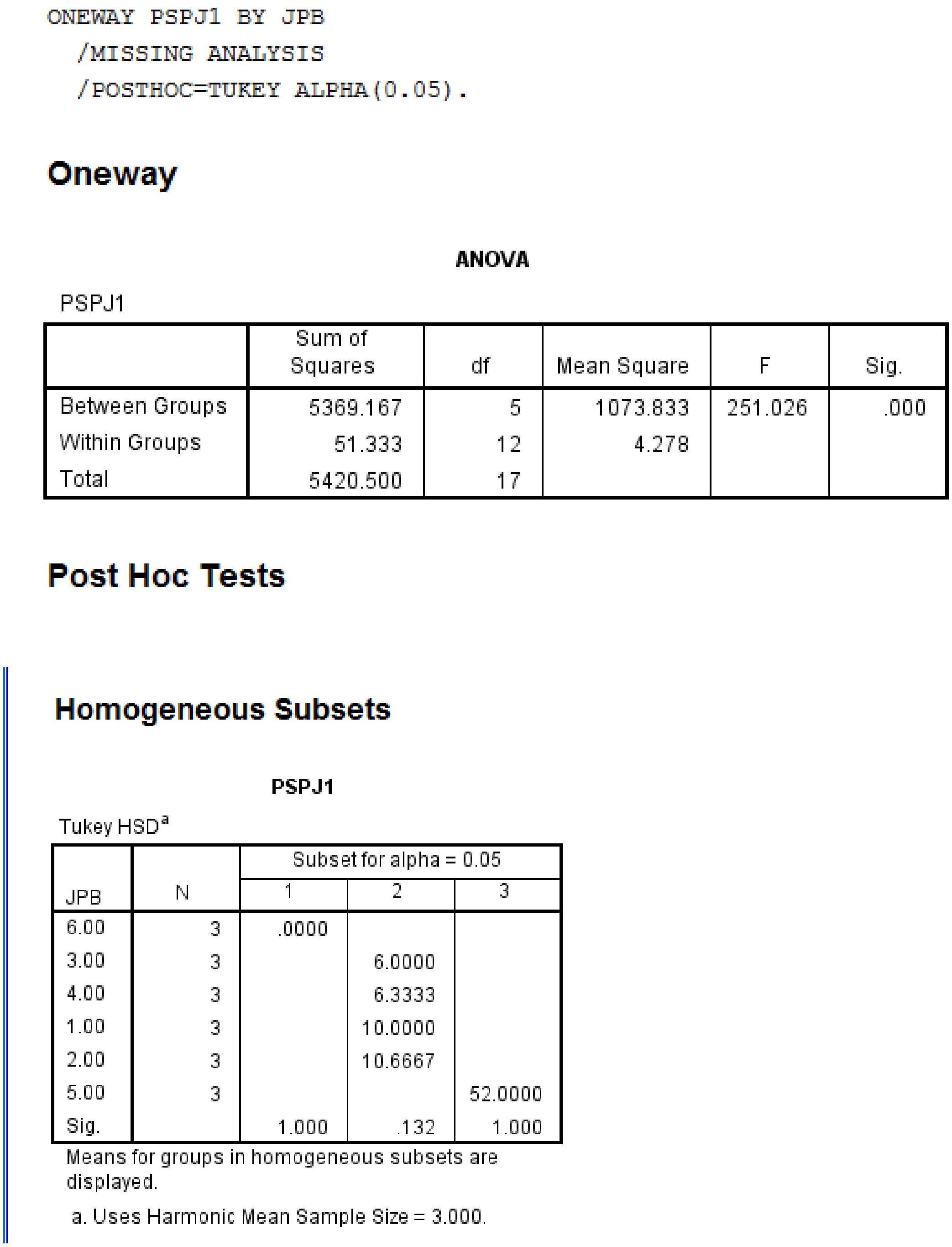
Tukey HSD[a]

JPL	N	Subset for alpha = 0.05			
		1	2	3	4
13	3	.0000			
5	3		6.6667		
6	3		6.6667		
3	3		7.0000	7.0000	
9	3		7.0000	7.0000	
2	3		7.3333	7.3333	
7	3		7.3333	7.3333	
8	3		7.3333	7.3333	
10	3		7.3333	7.3333	
11	3		7.3333	7.3333	
1	3		8.0000	8.0000	
4	3			8.6667	8.6667
12	3				10.0000
Sig.		1.000	.294	.082	.294

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of crude extracts from *Juniperus procera* bark against *Pseudomonas savastanoi* pv. *phaseolicola*



```
ONEWAY PSPJ1 BY JPB
  /MISSING ANALYSIS
  /POSTHOC=TUKEY ALPHA(0.05).
```

Oneway

ANOVA

PSPJ1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5369.167	5	1073.833	251.026	.000
Within Groups	51.333	12	4.278		
Total	5420.500	17			

Post Hoc Tests

Homogeneous Subsets

PSPJ1

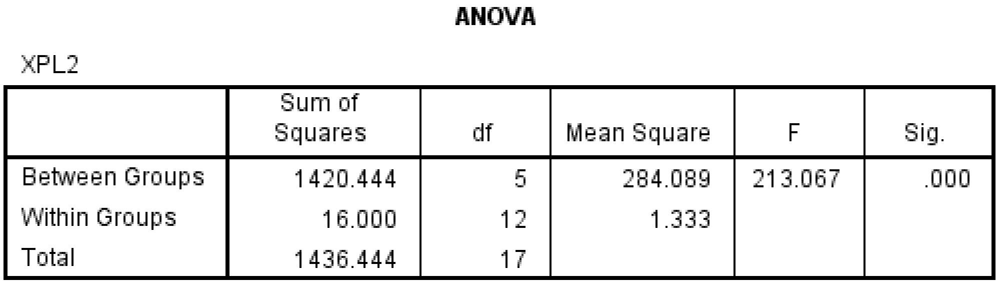
Tukey HSD[a]

JPB	N	Subset for alpha = 0.05		
		1	2	3
6.00	3	.0000		
3.00	3		6.0000	
4.00	3		6.3333	
1.00	3		10.0000	
2.00	3		10.6667	
5.00	3			52.0000
Sig.		1.000	.132	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

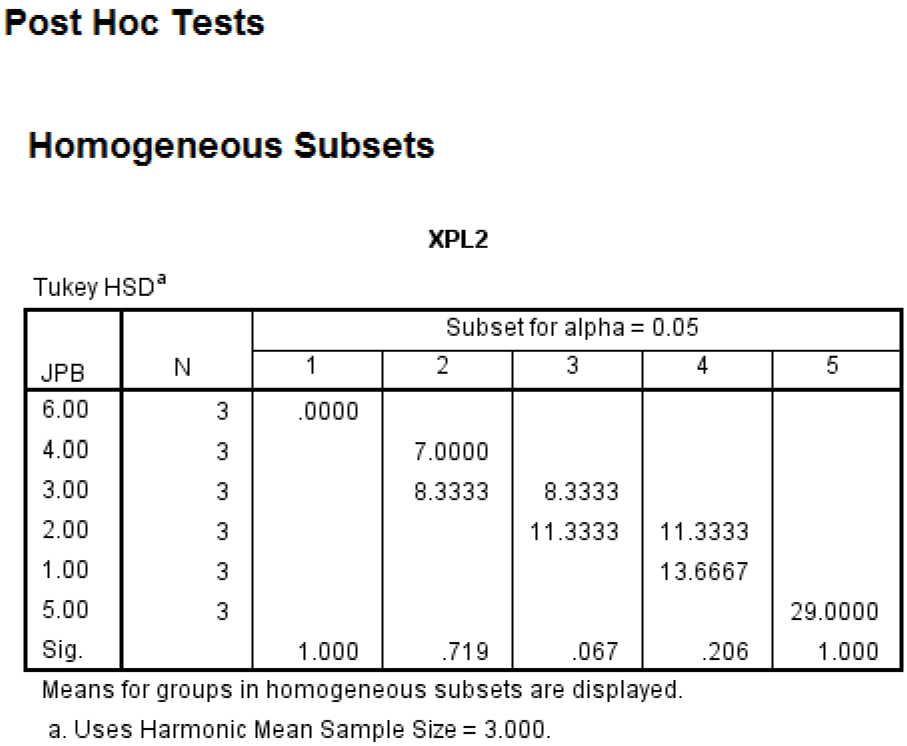
ANOVA summary of inhibition zones of crude extracts from *Juniperus procera* bark against *Xanthomonas axonopodis* pv. *phaseoli*



ANOVA

XPL2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1420.444	5	284.089	213.067	.000
Within Groups	16.000	12	1.333		
Total	1436.444	17			



Post Hoc Tests

Homogeneous Subsets

XPL2

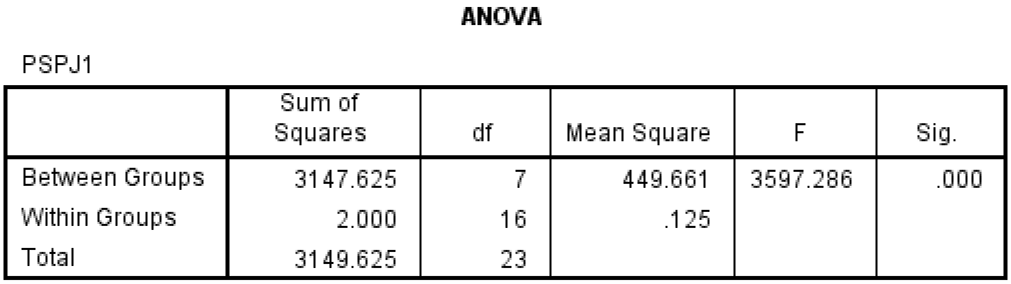
Tukey HSD[a]

JPB	N	Subset for alpha = 0.05				
		1	2	3	4	5
6.00	3	.0000				
4.00	3		7.0000			
3.00	3		8.3333	8.3333		
2.00	3			11.3333	11.3333	
1.00	3				13.6667	
5.00	3					29.0000
Sig.		1.000	.719	.067	.206	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

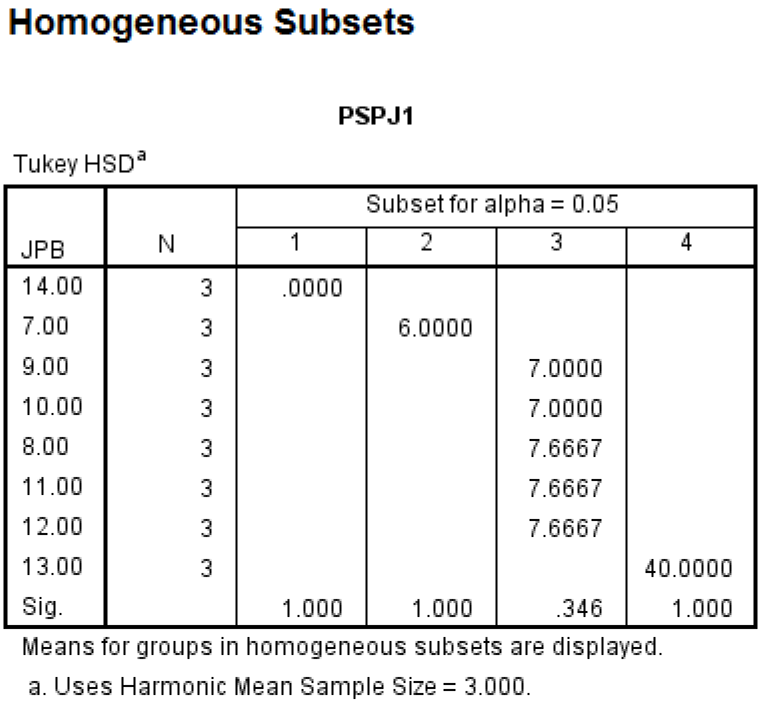
ANOVA summary of inhibition zones of semi-pure fractions from *Juniperus procera* **bark against** *Pseudomonas savastanoi* pv. *phaseolicola*



ANOVA

PSPJ1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3147.625	7	449.661	3597.286	.000
Within Groups	2.000	16	.125		
Total	3149.625	23			



Homogeneous Subsets

PSPJ1

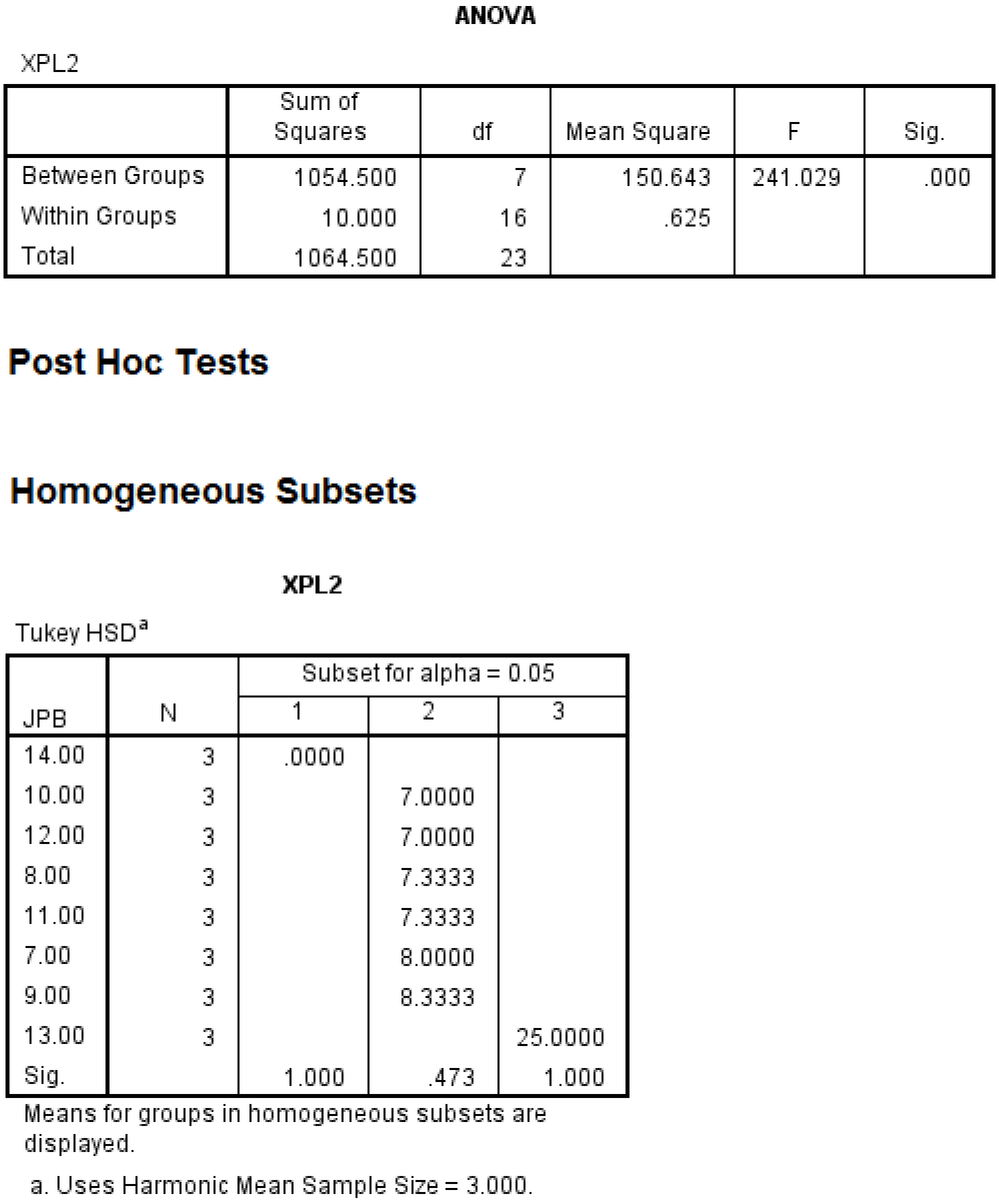
Tukey HSD[a]

JPB	N	Subset for alpha = 0.05			
		1	2	3	4
14.00	3	.0000			
7.00	3		6.0000		
9.00	3			7.0000	
10.00	3			7.0000	
8.00	3			7.6667	
11.00	3			7.6667	
12.00	3			7.6667	
13.00	3				40.0000
Sig.		1.000	1.000	.346	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of semi-pure fractions from *Juniperus procera* bark against *Xanthomonas axonopodis* pv. *phaseoli*



ANOVA

XPL2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1054.500	7	150.643	241.029	.000
Within Groups	10.000	16	.625		
Total	1064.500	23			

Post Hoc Tests

Homogeneous Subsets

XPL2

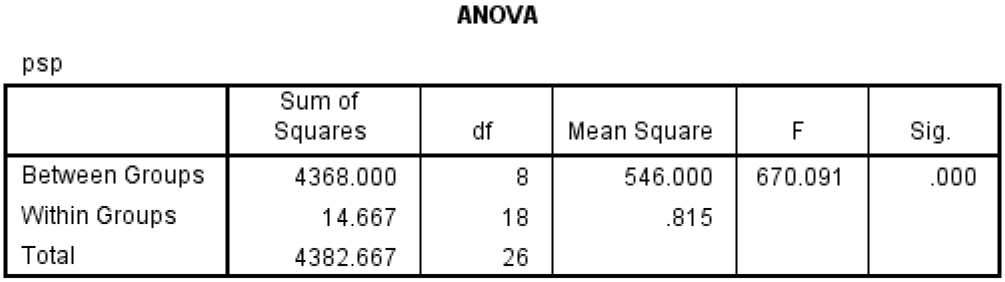
Tukey HSD[a]

JPB	N	Subset for alpha = 0.05		
		1	2	3
14.00	3	.0000		
10.00	3		7.0000	
12.00	3		7.0000	
8.00	3		7.3333	
11.00	3		7.3333	
7.00	3		8.0000	
9.00	3		8.3333	
13.00	3			25.0000
Sig.		1.000	.473	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of MIC data of methanol extract from *Juniperus procera* **leaves against** *Pseudomonas savastanoi* pv. *phaseolicola*



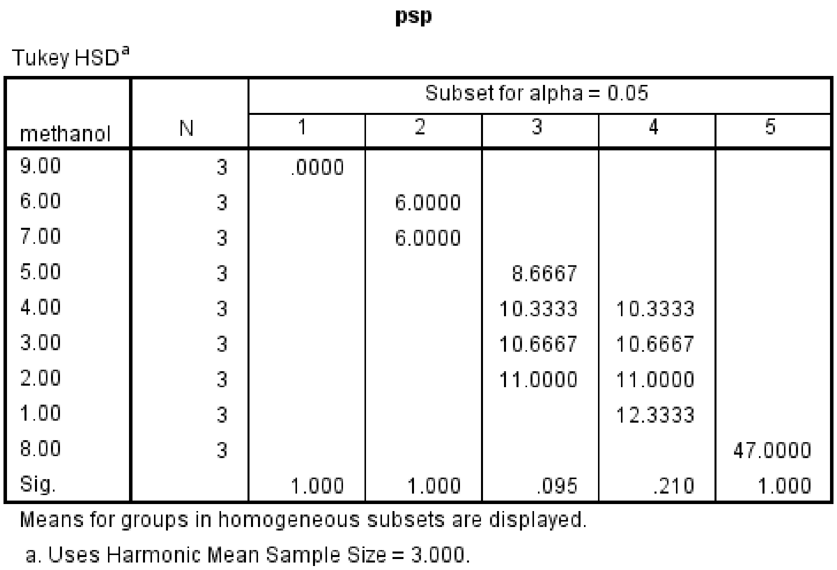
ANOVA

psp

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4368.000	8	546.000	670.091	.000
Within Groups	14.667	18	.815		
Total	4382.667	26			

Post Hoc Tests

Homogeneous Subsets



psp

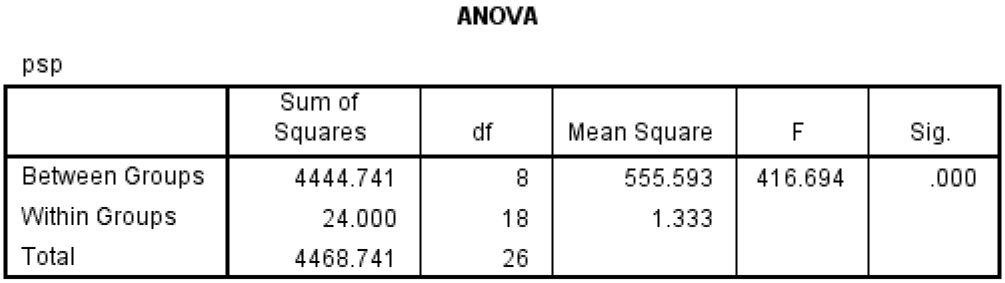
Tukey HSD[a]

methanol	N	Subset for alpha = 0.05				
		1	2	3	4	5
9.00	3	.0000				
6.00	3		6.0000			
7.00	3		6.0000			
5.00	3			8.6667		
4.00	3			10.3333	10.3333	
3.00	3			10.6667	10.6667	
2.00	3			11.0000	11.0000	
1.00	3				12.3333	
8.00	3					47.0000
Sig.		1.000	1.000	.095	.210	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of MIC data of ethyl acetate extract from *Juniperus procera* **leaves against** *Pseudomonas savastanoi* pv. *phaseolicola*



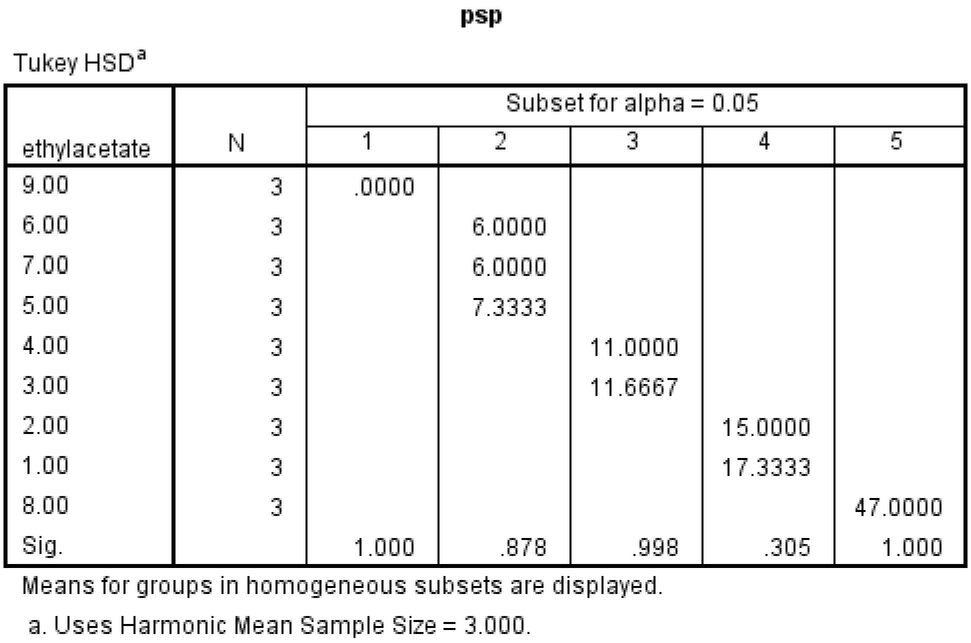
ANOVA

psp

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4444.741	8	555.593	416.694	.000
Within Groups	24.000	18	1.333		
Total	4468.741	26			

Post Hoc Tests

Homogeneous Subsets



psp

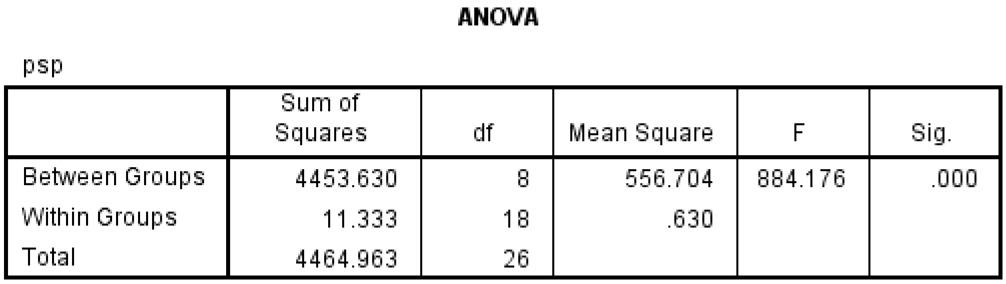
Tukey HSD[a]

ethylacetate	N	Subset for alpha = 0.05				
		1	2	3	4	5
9.00	3	.0000				
6.00	3		6.0000			
7.00	3		6.0000			
5.00	3		7.3333			
4.00	3			11.0000		
3.00	3			11.6667		
2.00	3				15.0000	
1.00	3				17.3333	
8.00	3					47.0000
Sig.		1.000	.878	.998	.305	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of MIC data of aqueous extract from *Juniperus procera* **leaves against** *Pseudomonas savastanoi* pv. *phaseolicola*

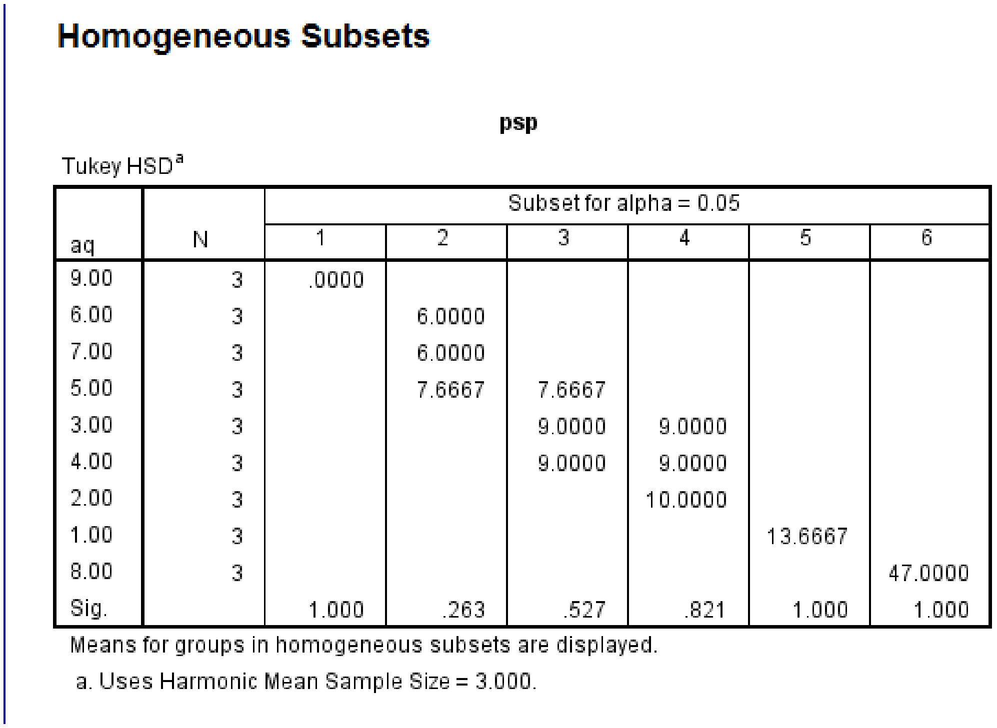


ANOVA

psp

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4453.630	8	556.704	884.176	.000
Within Groups	11.333	18	.630		
Total	4464.963	26			

Post Hoc Tests



Homogeneous Subsets

psp

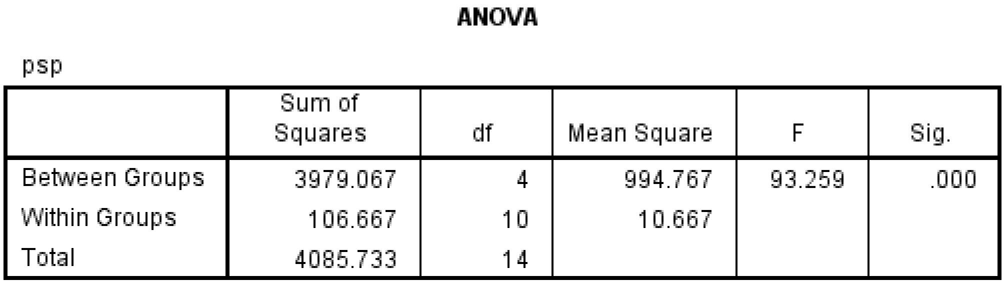
Tukey HSD[a]

aq	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
9.00	3	.0000					
6.00	3		6.0000				
7.00	3		6.0000				
5.00	3		7.6667	7.6667			
3.00	3			9.0000	9.0000		
4.00	3			9.0000	9.0000		
2.00	3				10.0000		
1.00	3					13.6667	
8.00	3						47.0000
Sig.		1.000	.263	.527	.821	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

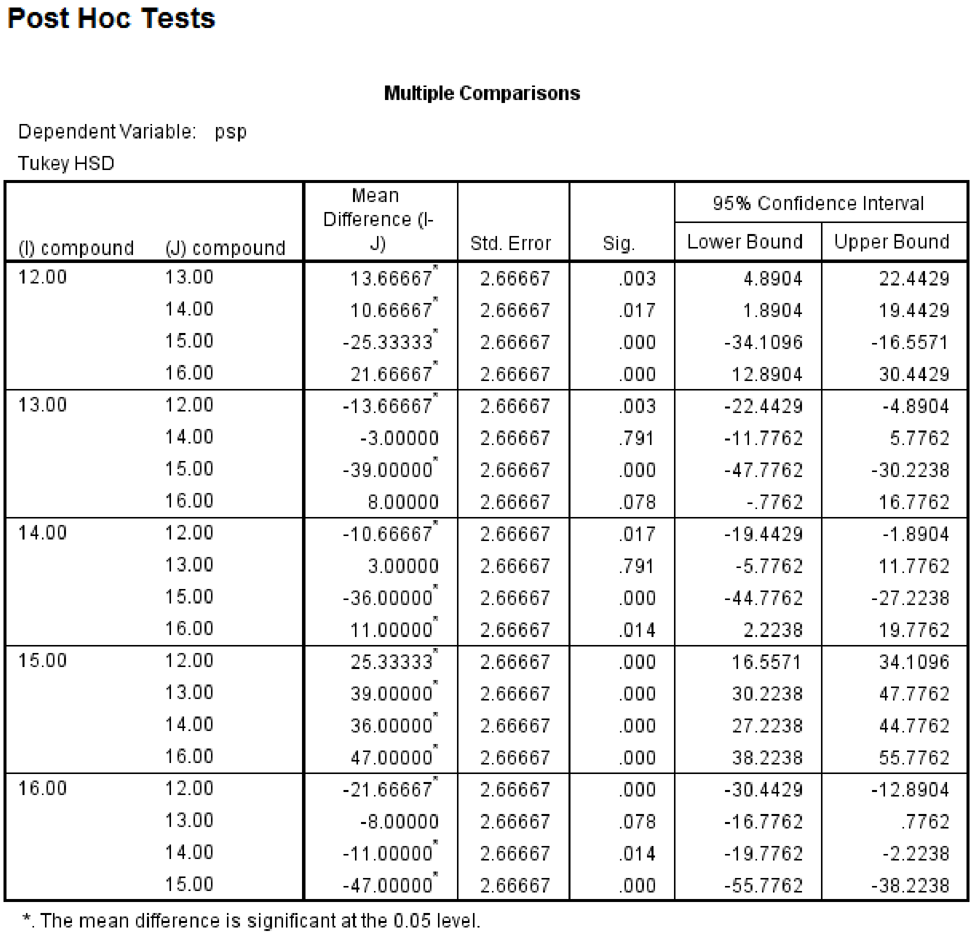
ANOVA summary of inhibition zones of pure compounds from *Juniperus procera* against *Pseudomonas savastanoi* pv. *phaseolicola*



ANOVA

psp

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3979.067	4	994.767	93.259	.000
Within Groups	106.667	10	10.667		
Total	4085.733	14			



Post Hoc Tests

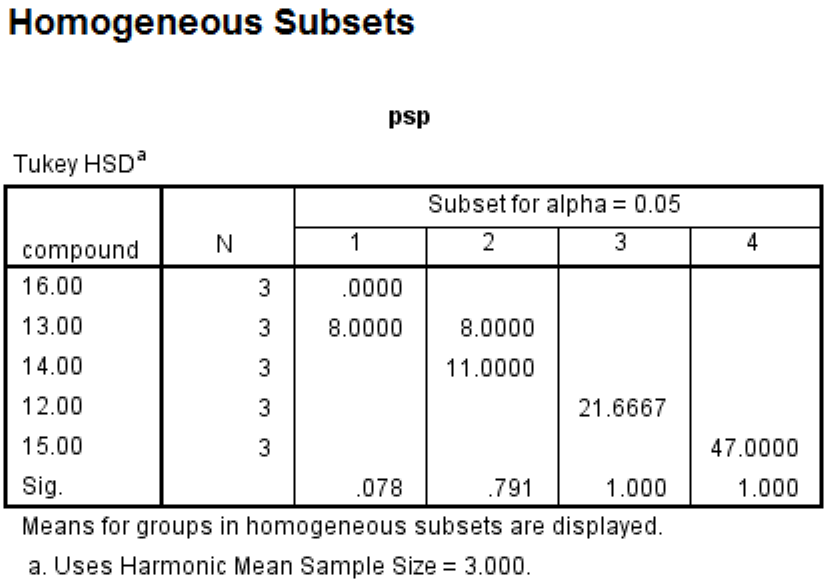
Multiple Comparisons

Dependent Variable: psp

Tukey HSD

(I) compound	(J) compound	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
12.00	13.00	13.66667*	2.66667	.003	4.8904	22.4429
	14.00	10.66667*	2.66667	.017	1.8904	19.4429
	15.00	-25.33333*	2.66667	.000	-34.1096	-16.5571
	16.00	21.66667*	2.66667	.000	12.8904	30.4429
13.00	12.00	-13.66667*	2.66667	.003	-22.4429	-4.8904
	14.00	-3.00000	2.66667	.791	-11.7762	5.7762
	15.00	-39.00000*	2.66667	.000	-47.7762	-30.2238
	16.00	8.00000	2.66667	.078	-.7762	16.7762
14.00	12.00	-10.66667*	2.66667	.017	-19.4429	-1.8904
	13.00	3.00000	2.66667	.791	-5.7762	11.7762
	15.00	-36.00000*	2.66667	.000	-44.7762	-27.2238
	16.00	11.00000*	2.66667	.014	2.2238	19.7762
15.00	12.00	25.33333*	2.66667	.000	16.5571	34.1096
	13.00	39.00000*	2.66667	.000	30.2238	47.7762
	14.00	36.00000*	2.66667	.000	27.2238	44.7762
	16.00	47.00000*	2.66667	.000	38.2238	55.7762
16.00	12.00	-21.66667*	2.66667	.000	-30.4429	-12.8904
	13.00	-8.00000	2.66667	.078	-16.7762	.7762
	14.00	-11.00000*	2.66667	.014	-19.7762	-2.2238
	15.00	-47.00000*	2.66667	.000	-55.7762	-38.2238

*. The mean difference is significant at the 0.05 level.



Homogeneous Subsets

psp

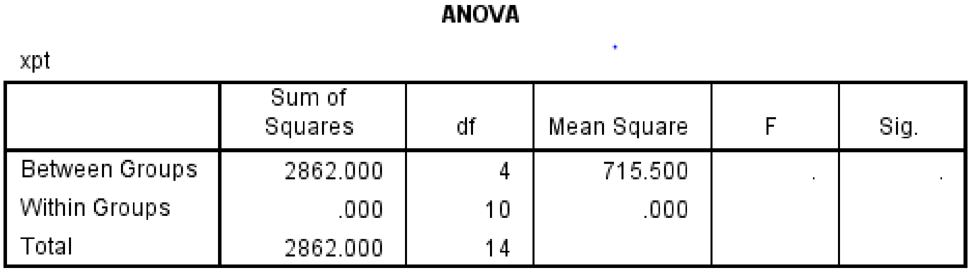
Tukey HSD[a]

compound	N	Subset for alpha = 0.05			
		1	2	3	4
16.00	3	.0000			
13.00	3	8.0000	8.0000		
14.00	3		11.0000		
12.00	3			21.6667	
15.00	3				47.0000
Sig.		.078	.791	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA summary of inhibition zones of pure compounds from *Juniperus procera* **against** *Xanthomonas axonopodis* pv. *phaseoli*



ANOVA

xpt

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2862.000	4	715.500	.	.
Within Groups	.000	10	.000		
Total	2862.000	14			

Appendix D: Abstract of paper published

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African Journal of Biotechnology

Full Length Research Paper

Bioactive compounds from *Juniperus procera* (Cupressaceae) with activity against common bean bacterial pathogens

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Common bean is an important source of protein in sub-Saharan Africa but its production is constrained by various factors especially bacterial diseases. The aim of this study was to evaluate compounds from *Juniperus procera* for bioactivity against common bean pathogens, *Pseudomonas savastanoi* pv. *phaseolicola* and *Xanthomonas axonopodis* pv. *phaseoli.* Solvent extraction method was used to obtain crude extracts from the stem bark and leaf of *J. procera* that underwent fractionation and purification using various chromatographic techniques, leading to the isolation of three compounds, namely: epicatechin (1), podocarpusflavone A (2) and juniperolide (3). Structures of the compounds were elucidated based on NMR and HRESIMS analyses. The bioactivity of the compounds was determined by disc diffusion assay. The compound epicatechin showed the highest activity against *P. savastanoi* pv. *phaseolicola* (21.7±1.2 mm). On the other hand, podocarpusflavone A and juniperolide showed weaker activity of 8.0±1.7 and 8.0±2.0 mm, respectively against the same pathogen. The three compounds showed weak or no activity against *X. axonopodis* pv. *phaseoli* of 6.0±0.0, 7.0±0.0 and 6.0±0.0 mm, respectively. Therefore, epicatechin can be used for the development of biopesticides for the control of *P. savastanoi* pv. *phaseolicola.*

Key words: Antibacterial compounds, *Juniperus procera, Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas axonopodis* pv. *phaseoli*.

Appendix E: Research permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **634178**

Date of Issue: **14/October/2022**

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