ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY ELUCIDATION OF BIOACTIVE COMPOUNDS FROM *CITRUS SINENSIS* PEEL EXTRACT

RaeyYohanes

A Thesis Submitted to the Department of Applied Biology

For partial fulfillment of the requirement for the degree of master's in Applied Microbiology

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

June, 2022

Adama, Ethiopia

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Advisors: Teshome Geremew (PhD)

Milkyas Endale (PhD)

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Declaration

I hereby declare that this Master Thesis entitled "**Antibacterial and Antioxidant Activity Elucidation of Bioactive Compounds from *Citrus sinensis* Peel Extract**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Name of student	Signature	Date

Recommendation

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Antibacterial and Antioxidant Activity Elucidation of Bioactive Compounds from *Citrus sinensis* Peel Extract**" prepared under our guidance by Raey Yohanes submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Biology. Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

Approval page

We, the advisors of the thesis entitled "**Antibacterial and Antioxidant Activity Elucidation of Bioactive Compounds from *Citrus sinensis* Peel Extract**" and developed by Raey Yohanes hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Raey Yohanes have read and evaluated the thesis entitled "**Antibacterial and Antioxidant Activity Elucidation of Bioactive Compounds from *Citrus sinensis* Peel Extract**" and examined the candidate during the open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology.

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Internal Examiner	Signature	Date
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Finally, approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate committee (DGC) and School Graduate Committee (SGC).

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School Dean	Signature	Date
Office of Postgraduate Studies,	Signature	Date

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LIST OF ACRONYMS/ABBREVIATIONS

ADMET: Adsorption, distribution, metabolism, excretion and toxicity

DFT: Density functional theory

DMSO: Dimethylsulphoxide

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

FTIR: Fourier transforms infrared

GC-MS: Gas chromatography mass spectroscopy

IR: Infrared spectroscopy

MIC: Minimum inhibitory concentration

NMR: Nuclear magnetic resonance

OSIRIS: Optical, spectroscopic and infrared remote imaging system

TLC: Thin layer chromatography

UAE: Ultrasonic assisted extraction

UV-Vis: Ultraviolet Visible

ABSTRACT

*Increasing cases of drug resistance, unwanted side effects of existing antibiotics, and the reappearance of earlier known infections have demanded the need for new, safe and effective antimicrobial agents. The objective of this study was to determine the antibacterial and antioxidant activity of Citrus sinensis peel extracts against selected human pathogenic bacterial strains and examine the molecular interaction of isolated compounds with selected protein targets. Using maceration and ultrasonic assisted extraction methods, the peel of Citrus sinensis (300g, 150g) was extracted with ethanol to yield 20.99 g and 11.5 g with (7%, 7.5%) yields, respectively. Phytochemical analysis conducted on Citrus sinensis peel ethanol extract revealed the presence of alkaloid, flavonoid, tannins, steroids, phenol and terpenoid. Silica gel column chromatographic separation of the ethanol extract obtained from maceration afforded four compound **46**, compound **47**, compound **48** and compound **49**. The structures of the isolated compounds were characterized using NMR spectroscopic techniques (^{1}H NMR, ^{13}C NMR, DEPT-135).GC-MS analysis of essential oil revealed the presence of 7 chemical components accounted for 99.84 % of the total compositions. The crude extract and isolated compounds were evaluated in vitro for their antibacterial activities using disc diffusion method against six bacterial strains. A promising zone of inhibition values were observed by crude (12.67±0.58mm), compound **49** (15.67±2.88mm) and compound **46** (12.00±0.00mm) against E. faecalis , S. typhimurium and P. aeruginosa respectively compared to gentamicin (13.00±1.73, 18.00±1.00 and 16.67±1.15 respectively).Also the essential oil showed a good zone of inhibition against S. aureus (14.33±1.52 mm) compared to gentamicin (18.67±0.58 mm). DPPH radical scavenging activity revealed that compound **46** showed IC_{50} value of 0.05 mg/mL compared to ascorbic acid 0.016 mg/mL. Using autodock, a molecular docking study was conducted. It was discovered that, among isolated compounds, compounds **48** had the highest and best scoring poses (lowest energy) against the E. coli gyrase B enzyme (-6.9 Kcal.mol), Human Peroxiredoxin 5 (-7.1 Kcal.mol), and S. aureus Pyruvate Kinase (-8.2 Kcal.mol).The findings of this study support the traditional uses of the Citrus peels for the treatment of infectious diseases especially Gram-positive strains.*

Keywords *Citrus sinesis*, antimicrobial activity, agar disk diffusion, minimum inhibitory concentration, minimum bactericidal concentration, antioxidant activity.

1. INTRODUCTION

1.1. Background

Microbial drug resistance is a major global concern right now. Multidrug-resistant (MDR) microbial strains have emerged as a serious threat to worldwide public health (Ayukekbong *et al*., 2017).Bacteria are becoming increasingly resistant to antibiotics, posing a significant problem in the treatment of a number of well-known illnesses. As a result, new antibacterial chemicals must be developed to be used against these microbes (Sapna *et al*., 2016). Through several mechanisms of action, phytochemicals have shown potential antibacterial activity against sensitive and resistant microorganisms (Rahman *et al*., 2011). Plants have been utilized to treat a variety of diseases and preserve health in various cultures around the world since ancient times (Abalaka *et al*., 2006). The World Health Organization (WHO) estimates that around 80% of the population still uses herbal remedies to treat various ailments because of its ease of access, low cost, and lack of adverse effects (WHO, 2004), traditional medicine is used as a primary source of health care by the majority of Ethiopians because it is culturally rooted, accessible, and economical (Koehn *et al*., 2005; Elias *et al*., 2011).

Food wastes, particularly fruit and vegetable wastes, have increased in recent years as a result of rising food consumption and the expansion of the food business. These wastes are one of the most common sources of municipal solid trash (MSWs). Systems for reducing food waste are being improved, specifically by using fruit and vegetable wastes as a source of bioactive compounds (Ajila *et al*., 2007). These bioactive substances include phytochemical and phenolic compounds, both of which are important in the development of antimicrobial drugs. Fruits have a protective quality due to the presence of phytoconstituents like polyphenolic chemicals (Diankov *et al*., 2011; Siddique *et al*., 2011). Citrus fruits are popular all over the world due to their distinct flavors and nutritional benefits (Yao *et al*., 2004). Citrus fruits are mostly used in the juice processing industry, with the peels being discarded. Citrus juice output is less than half of the fruit weight, while by-product wastes (peels) are produced in enormous quantities (Manthey and Grohmann, 2001).

Antibiotic resistant microorganisms are becoming more common as a result of widespread antibiotic use. As a result, current antimicrobials are ineffective against at least certain

bacterial illnesses (Riffel *et al*., 2002). Several investigations on Citrus peels have indicated the existence of significant components that can be employed in pharmacological or medicinal applications. Citrus peels are nutrient-dense and contain several phytochemicals; they can also be employed as medications or food supplements (Nand, 1998). Numerous components with antibacterial, antioxidant, and anti-inflammatory properties were isolated from the peels of various citrus fruits by researchers (Friedman *et al*., 2002). Orange peel has also been shown to have antibacterial properties in studies. Mehmood *et al.* (2015) discovered that extract from orange peels has significant antibacterial action against enteric pathogens. This study aims to determine the antibacterial of *Citrus sinensis* (sweet orange) peel extracts against selected human pathogenic bacterial strains, evaluate antioxidant activity and molecular interaction of isolated compounds with selected protein targets.

1.2. Statement of the problem

Bacterial infections have become increasingly difficult to treat in the last two decades, particularly in developing nations, and are associated with high morbidity and mortality rates, as well as prolonged hospital stays (Singh *et al*., 2017).*Enterobacteriaceae* include *Klebsiella pneumoniae*, *Escherichia coli* and non-lactose fermenting bacteria such as *Pseudomonas aeruginosa* have been found as main cause of multi-drug resistant bacterial infections (Rossolini *et al*., 2007; De Angelis *et al*., 2014). Despite the introduction of several types of antibiotics by pharmaceutical companies in recent decades, antibiotic resistance has risen considerably in many bacterial infections.

Citrus fruits are the primary source of key phytochemical components and have long been prized for their nutritional and antioxidant characteristics (Crowell, 1999).*Citrus sinensis* peel has many benefits including; health benefits (improve oral health), beauty treatments, cleaning uses, nutritional properties and other uses. Most of the people love to eat oranges but a lot of peels will end up being thrown in the trash. This is unfortunate because there are numerous unique applications that we may not have considered. *Citrus sinensis* peel contains numerous components having antimicrobial, antioxidant and anti-inflammatory activities which can be used in pharmacological or pharmaceutical industries (Friedman *et al.*, 2002).The discovery of natural drugs from natural sources is highly important because many isolated molecules are far more complex (Dhanik *et al*., 2017).

Despite a broad spectrum of ethnomedicinal uses, there are limited scientific studies done on peels of *Citrus sinensis* in Ethiopia. Thus, this study is aimed to search for bioactive chemical constituents from the peel extracts and examine their antibiotic, antibacterial and antioxidant activity along with computational study to know the molecular interaction of active compounds against selected protein targets.

1.3. Objective of the study

1.3.1. General objective

➢ The overall objective of the study was to determine *in vitro* antibacterial and antioxidant activity of the *Citrus sinensis* peel extracts and examine molecular interaction of isolated compounds with selected protein targets.

1.3.2. Specific objectives

- To elucidate bioactive compounds from *Citrus sinensis* peel.
- To characterize the isolated compounds.
- To evaluate the antimicrobial activity of *Citrus sinensis* peel, essential oil and bioactive compounds.
- To evaluate antioxidant activity of *Citrus sinensis* peel oils and extracts.
- To investigate interaction of active compounds against DNA gyrase B, Pyruvate Kinase and peroxiredoxin 5 protein targets.

1.4. Significance of the study

This research is expected to:

- ❖ Provide information on the chemical constituents of *Citrus sinensis* peel extracts that are found in Ethiopia.
- ❖ Provide antimicrobial activity information of *Citrus sinensis* peel extracts and bioactive compounds against selected microbial strains to provide scientific support to traditional uses of the plants to treat infectious diseases.
- ❖ Provide information of essential oils composition of *Citrus sinensis* peel extract and examine their antimicrobial activity.
- ❖ Provide scientific data to have a better understanding of the molecular mechanisms of interaction between the compounds with protein domains of selected bacterial strains using computational analysis.
- ❖ Provide baseline information for future related scientific studies on the plants

2. LITERATURE REVIEW

2.1. Citrus fruit

Citrus fruits, which belong to the family Rutaceae and are known as oranges, mandarins, limes, lemons, grapefruits, and citrons, come in a variety of shapes and sizes (García-Salas *et al*., 2013**).**In Ethiopia smallholders and commercial farmers grow citrus (Citrus spp.) as one of the most commercially important fruit crops (Yesuf,2007). The total area covered and yearly citrus production were expected to be 5165.28 hectares and 38486.5 million tons, respectively (CSA, 2018).

Citrus fruits including oranges, tangerines, limes, grapefruits, bitter oranges, and lemons are consumed on a regular basis, either raw or processed, since they are high in vital nutrients like vitamins, fibers, minerals, organic acids, and phytochemicals. As a result, they provide a variety of nutritional and health benefits and are consumed as whole fruits or as processed products such as peels, pulps, juice, and other extracts that are commercialized and used as food additives (Usman *et al*., 2021). Citrus fruits are high in secondary metabolites such as terpenoids and phenolics. Citrus fruit contains up to 170 antioxidant chemicals, according to Zhou (2012). Carotenoids and limonoids are the most common terpenoids found in citrus fruits, whereas flavonoids (naringenin, naringin, hesperidin, quercetin, and rutin), phenolic acids, and coumarins are the most common phenolic chemicals (Zou *et al*., 2016). The citrus peel is a rich source of flavonone and many polymethoxylated flavones, which are rarely found in other plants (Anitha *et al*., 2016).

Citrus fruits are well-known for other active substances such as flavonoids, vitamins, carotenoids, and minerals, which have been shown to reduce the risk of a variety of chronic diseases (e.g., cardiovascular diseases and age-related macular degeneration). Antioxidant, anticancer, and antitumor capabilities are among the therapeutic effects of these bioactive substances. Antimicrobial, anticancer, antidiabetic, antiplatelet aggregation and anti-inflammatory properties are all present in these substances (Sharma *et al*., 2017).

The most important citrus by-products commonly collected from the peels are the citrus essential oils, which are deposited within the glands in the outer layer of the fruit skin. They are widely used in pharmaceutical preparations (Ferhat *et al*., 2006; Hosni *et al*., 2010); they are also widely used in aromatherapy (Yavari Kia *et al*., 2014; Tadtong *et al*.,

2015) Citrus essential oils' volatile chemical constituents are among the most unique components for identifying and evaluating the many variations (Njoroge *et al*., 2005).

Antimicrobial medications either kill or stop microorganisms from growing (Mathur *et al*., 2011). Citrus fruit products are antimicrobial, meaning they fight bacteria and fungi. Because of its commercial significance in the culinary and pharmaceutical industries around the world, the citrus product plays a significant and physiological role (Mathur *et al*., 2011). Citrus fruits and juices are a good source of bioactive metabolite, which contains antioxidants such ascorbic acid, phenolic compounds, flavonoids, and pectins that are vital in human nutrition (Hegazy *et al*., 2012).

2.2. Citrus *sinensis* (sweet orange)

Citrus sinensis is the most widely grown citrus cultivar group in the world, accounting for over 70% of total annual Citrus species production (Flamini *et al*., 2003). *Citrus sinensis* is a flowering evergreen tree. Orange trees grow to a height of 9–10 meters, with prominent spines on the branches. The leaves are alternate and have narrowly winged petioles (3–5 mm wide, 6.5–15 cm long); the blades are elliptical, oblong, or oval in shape, bluntly toothed, and emit a strong citrus odor due to the presence of copious oil (Goldhamer *et al*., 2012). Axillary flowers are borne individually or in whorls of six (5 cm wide) There are five white petals and 20–25 yellow stamens on this flower. The pericarp, also known as the peel, skin, or rind, and the endocarp, or pulp, with juice sac glands, are anatomically separate parts of the fruit (Orwa *et al.*,2009; Han , 2008).The epidermis of the skin contains epicuticular wax, with numerous little aromatic oil glands that give it its distinct odor. The outer flavedo or epicarp, which is mostly made up of parenchymatous cells and cuticle makes up the pericarp (Goudeau *et al*., 2008).

Oranges are said to have originated in Southeast Asia and were cultivated in China by 2500 BC (Nicolosi *et al*., 2000). Because of its excellent nutritional content, source of vitamins, and other purposes, it is now produced practically everywhere in the world as a human food source (Etebu and Nwauzoma, 2014). Phytochemicals are found in edible fruits and vegetables, and when consumed, they may help to regulate human metabolism and avoid chronic and degenerative diseases (Tripoli *et al*., 2007). Orange fruits are the primary source of key phytochemical elements and have long been prized for their healthy nutritional and antioxidant characteristics (Tripoli *et al*., 2007).

Oranges include powerful natural antioxidants, folate, dietary fiber, and other bioactive components, such as carotenoids and flavonoids, which help to prevent cancer and degenerative disorders (Ejaz *et al*., 2006). Hesperetin and narigenin are two phytochemicals found in sweet orange. Naringenin acts as an antioxidant, free radical scavenger, anti-inflammatory, and immune system modulator in humans (Etebu and Nwauzoma, 2014).

In several regions of the world, the *Citrus sinensis* peel has been used as a traditional medicine to treat stomach discomfort, skin irritation, ringworm infections, neuroprotection, and heart health (Ghasemi, *et al*., 2009). Citrus peels include a number of strong antioxidants that have antioxidant properties such as free radical scavenging and metal chelation. Exploring the active phytochemicals in *Citrus sinensis* peel is exciting since reactive oxygen species play a key role in a variety of disorders; including cancer, cardiovascular dysfunction, neurological diseases, and the aging process (Rafiq *et al*., 2018).

Several investigations were conducted on the composition and biological activity of essential oils extracted from the leaves and peels of *Citrus sinensis* and its hybrids, including antifungal, antioxidant, anti-aflatoxigenic, and antibacterial effects (Njoroge *et al*., 2005; Singh *et al*., 2010; Kamal *et al*., 2013; Hasija *et al*., 2015). Hydrocarbons, alcohols, and esters are among the constituents of the citrus essential oil. As well as aldehydes Monoterpene hydrocarbons are abundant in citrus oils (70–95%), with d-limonene dominating in all of the sweet orange oils observed. Most sweet orange peel oils include low quantities of sesquiterpene hydrocarbons, which are responsible for the distinctive flavor. Aldehydes are a crucial indicator of essential oil quality and contribute to the total concentration of oxygenated compounds (Mitiku *et al*., 2000; Minh Tu *et al*., 2002; Njoroge *et al*., 2005; Ahmad *et al*., 2006).

2.3. Ethno botanical uses of *Citrus sinensis*

For digestive problems, neurological disorders, fever, asthma, blood pressure, general weariness, and vomiting, a leaf decoction with salt is administered orally. Itching is relieved by massaging crushed leaves or fruit juice into the skin. For urethritis, macerated root, leaf, or fruit mesoderm is taken orally; for liver diseases, macerated fruit mesoderm or bark decoction is taken orally. Colds and loss of appetite are treated with fruit juice or leaf decoction with sugar, while headaches and rheumatism are relieved by bathing in crushed

leaf decoction. Roasted fruit is used to treat broken bones. Carminative, antispasmodic, and sedative qualities are all present in leaf oil (Orwa *et al*., 2009).

2.4. Chemical composition of *Citrus sinensis*

Citrus sinensis has a large number of secondary metabolites, which contribute to the plant's pharmacological properties. *Citrus sinensis* fruits, peel, leaves, juice, and roots have been shown to contain a variety of chemical compounds, including the following groups; flavonoids (Gattuso *et al*., 2007), Steroids hydroxyamides, alkanes and fatty acids (Rani *et al*., 2009), coumarins (Li *et al*., 2007), peptides (Matsubara *et al*., 1991), carbohydrates (Kolhed, and Karlberg, 2005), carbamates and alkylamines (Soler *et al*., 2006), carotenoids (Aschoff *et al*., 2015), volatile compounds (Gómez *et al*., 2004), and nutritional elements such as potassium, magnesium, calcium and sodium (Niu *et al*., 2009). The chemical structures of constituents isolated and characterized in *Citrus sinensis* are shown below (Figure 1-7).

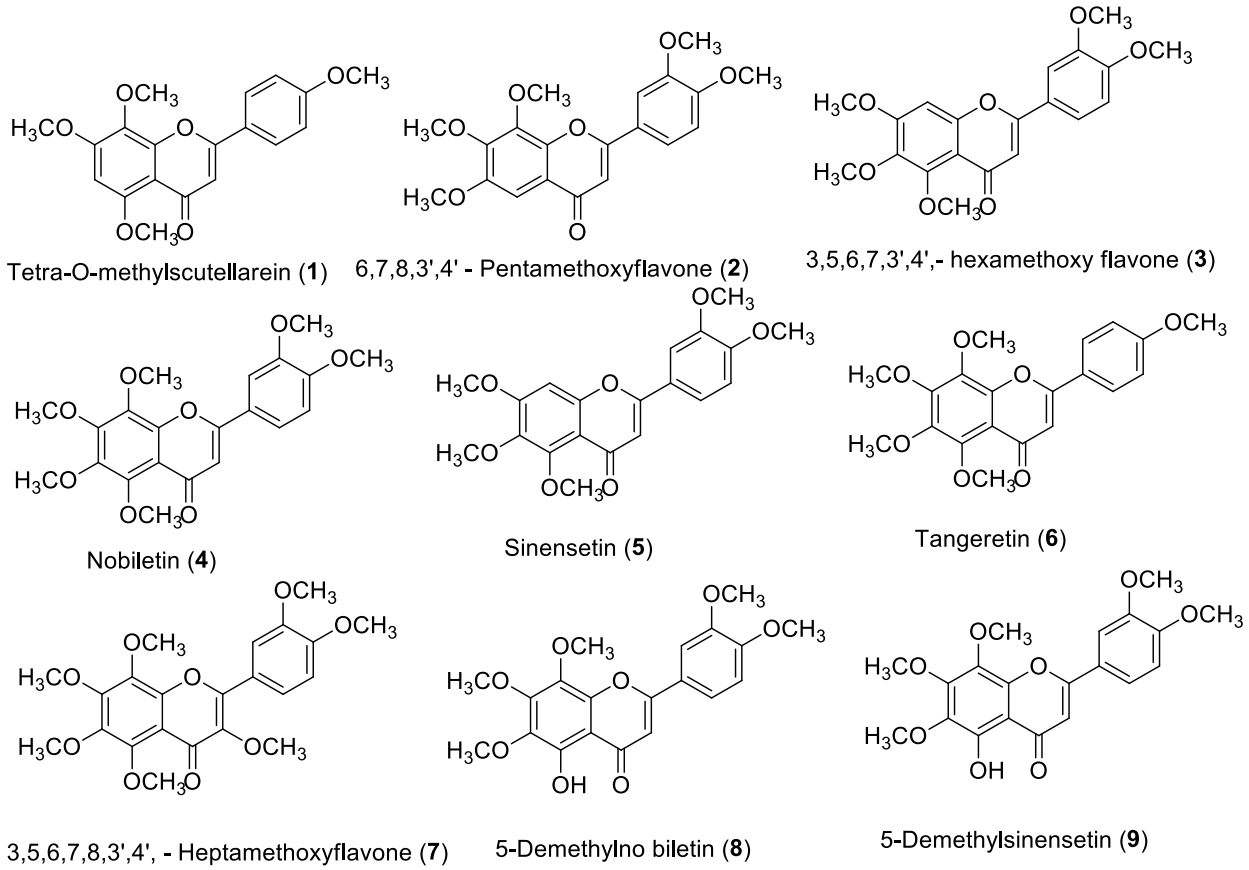
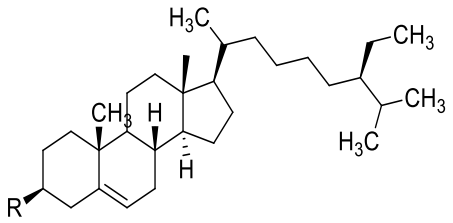
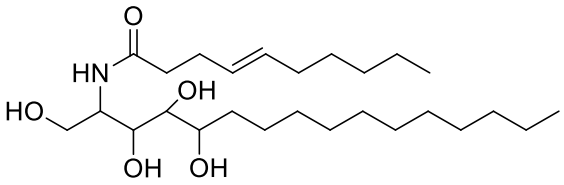


Figure 1: Structures of the flavonoids 1–9 isolated from *Citrus sinensis* peel. (Benito *et al*., 2015)

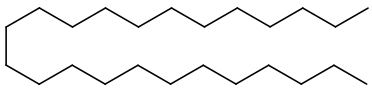


R = OH = Beta-Sitosterol (**10**)
R = O-Glu = Beta-Sitosterol-3-O-Beta-D-glucopyranoside (**11**)

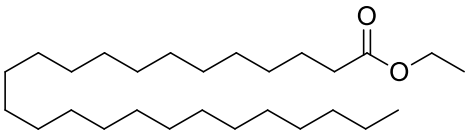
Figure 2: Chemical structure of steroid isolated from *Citrus sinensis* peel. (Rani *et al*., 2009)



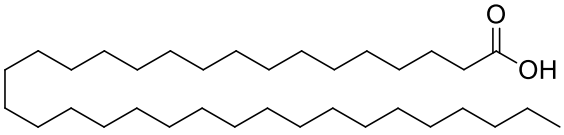
(E)-N-(1,3,4,5- Tetrahydroxyhexadecan-2-yl)dec-4-enamide (**12**)



Tetracosane (**13**)

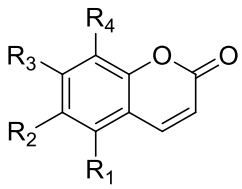


Ethyl pentacosanoate (**14**)

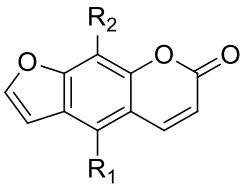


Tetratricoctanoic acid (**15**)

Figure 3: Chemical structures of hydroxyamides, alkanes and fatty acids. (Rani *et al*., 2009)



	R_1	R_2	R_3	R_4
Scoparone (**16**)	H	OCH_3	OCH_3	H
Limettin (**17**)	H	H	OCH_3	OCH_3
Osthol (**18**)	H	H	OCH_3	$CH_2CHC(CH_3)_2$



	R_1	R_2
Xanthotoxin (**19**)	H	OCH_3
Bergapten (**20**)	OCH_3	H
Isopimpinellin (**21**)	OCH_3	OCH_3
Bergaptol (**22**)	OH	H

Figure 4: Chemical structures of coumarins. (Li *et al*., 2007)

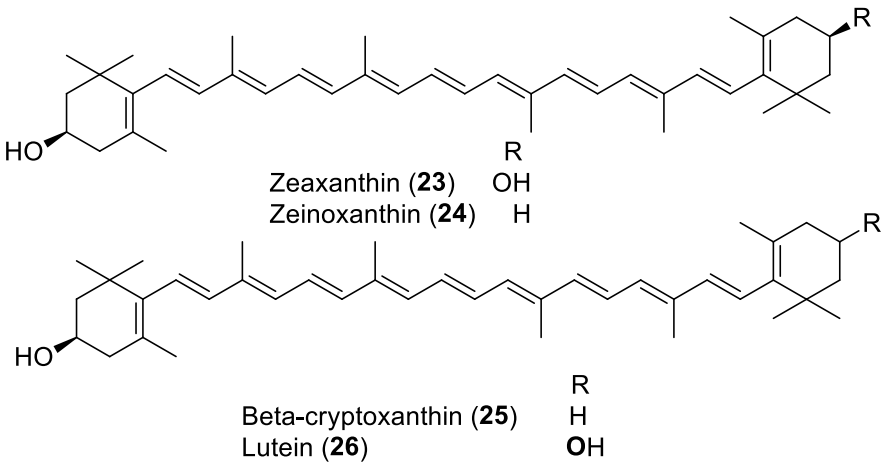


Figure 5: Chemical structure of carotenoid. (Matsubara *et al*., 1991)

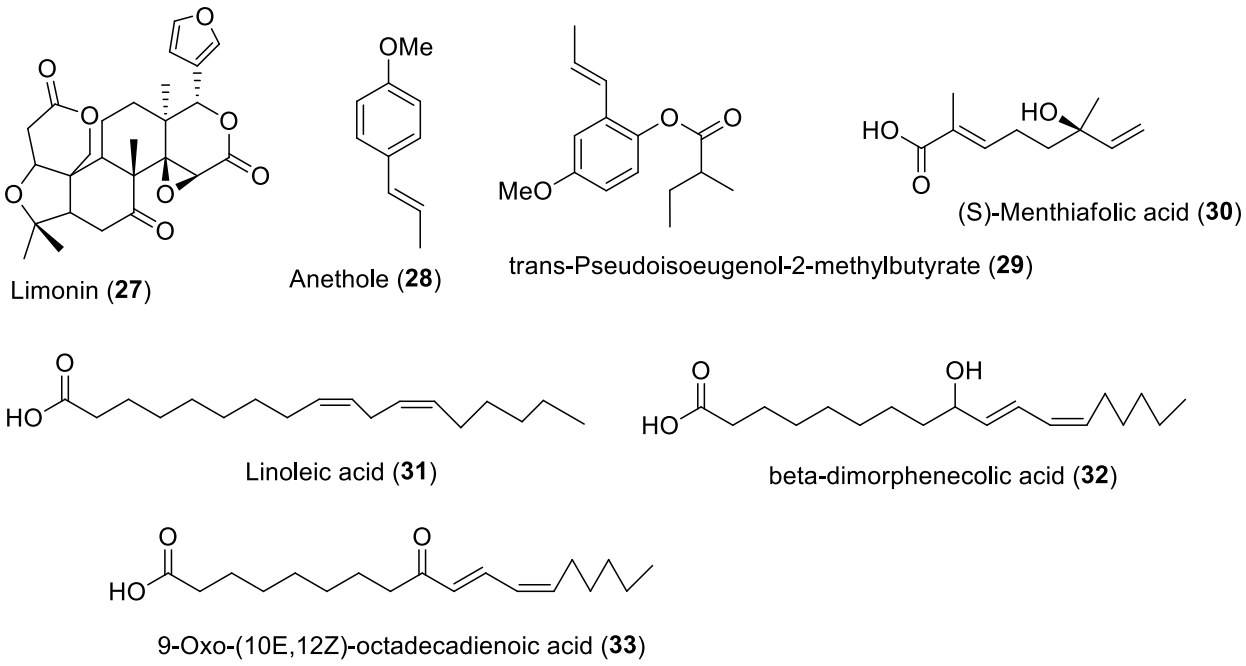


Figure 6: Structures of limonoid10, phenylpropanoids 11 and 12, and acids 13–16 isolated from *Citrus sinensis* peel (Benito *et al*., 2015)

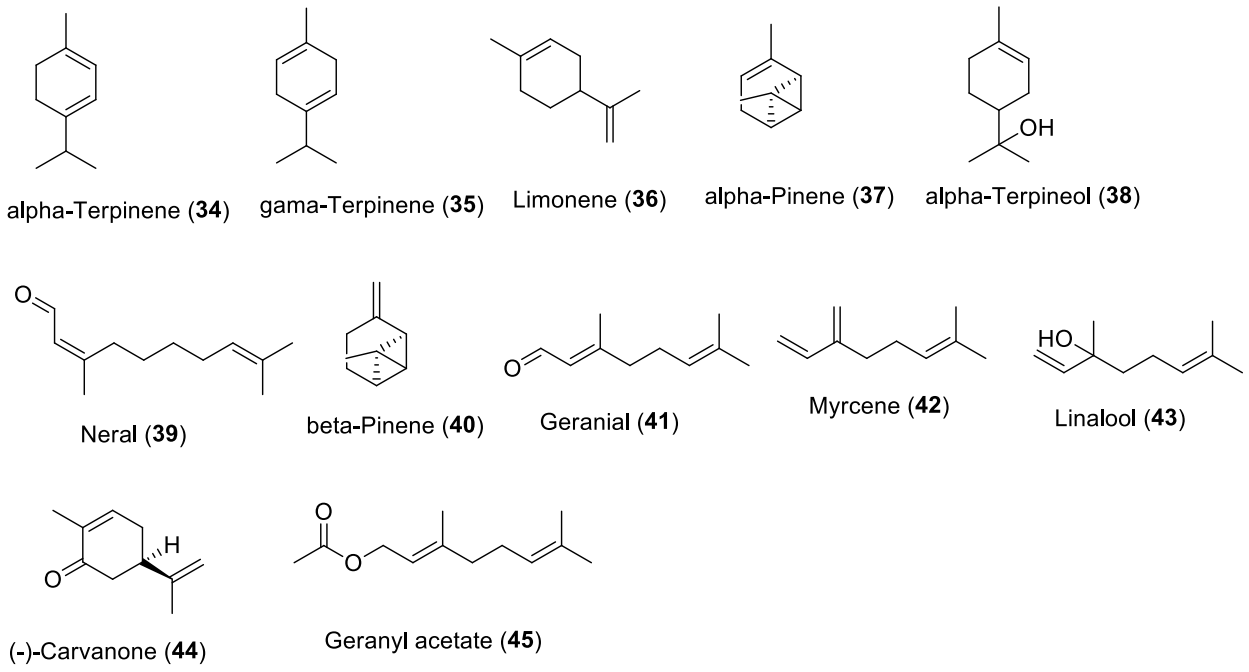


Figure 7: Chemical structures of volatile compounds (Gómez *et al*., 2004)

2.5. Biological activity of Citrus peel extracts and essential oils

2.5.1. Antibacterial activity

Using the agar well-diffusion method, silver nanoparticles generated at 25°C and 60°C using *Citrus sinensis* peel aqueous extract revealed a wide zone of inhibition against *Escherichia coli* (25°C 12.5 mm, 60°C 16.0 mm), *Pseudomonas aeruginosa* (25°C 11.7 mm, 60°C 13.4 mm), and *Staphylococcus aureus* (Kaviya *et al*., 2011). *Listeria monocytogenes* had MICs of 0.3 percent and 0.25 percent v/v in cold-pressed terpene less (CPT) *Citrus sinensis* oil dissolved in ethanol or dimethylsulphoxide (DMSO), and *Salmonella typhimurium* had MICs of 1 percent v/v in cold-pressed terpeneless (CPT) *Citrus sinensis* oil dissolved in ethanol or dimethylsulphoxide (DMSO) *Lactobacillus plantarum* had an intermediate MIC of 0.75 percent v/v in both ethanol and DMSO oil dispersion solutions (Chalova *et al*., 2010). Another study found that 0.1 mL of *Citrus sinensis*oil inhibited *E. coli* (18± 2 mm), *L. monocytogenes* (27± 2 mm), *B. cereus* (19 ±2 mm), and *S. aureus* (14± 3 mm) with different inhibition diameters (Fisher and Phillips, 2006).

Sweet orange oil and its major constituents decanal (73.36 percent), octanal (78.12 percent), and linalool (90.61 percent) obtained by molecular distillation and column

chromatography inhibited the growth of *E. coli* (MIC 100-25 g/mL; MBC 200–50 g/mL), *S. aureus* (MIC 100–50 g/mL; MBC 200–100 g/mL) (Liu *et al*., 2012). For the sensitive strain, streptomycin had a MIC of 0.50 g/mL, while the resistant strain had a MIC of >8 g/mL. These findings show that *Citrus sinensis* has a broad antibacterial spectrum, validating its usage as an antibacterial agent (Juna *et al*., 2016).

2.5.2. Antioxidant activity

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay and the Co(II)/EDTA-induced luminol chemilumine scence experiment were used to assess the antioxidant activity of the orange peel methanolic extract fractions. The antioxidant activity of orange peel methanolic extracts was found to be moderate when compared to the activity of the aglyconesdiosmetin (EC50 71.79 ±13.58 mg) and hesperetin (EC5029.18± 2.80 7 mg). The positive control was quercetin (EC50 = 0.06 mg quercetin/mg DPPH), and the examined aglycones had much better hydroxyl scavenging activity than quercetin (Kanaze *et al*., 2009). The antioxidant activity of methanol and ethanol extracts of *Citrus sinensis* peel revealed considerable free radical scavenging activity generated by ABTS of 55.8% and 60.7%, respectively, as well as DPPH scavenging activity based on its aptitude as a hydrogen donor of 70% and 80%, respectively (Tounsi *et al*., 2011).

The ethanol extract of sweet orange peel had the highest antioxidant capacity in the DPPH testing (79.321.05 percent). In fact, independent of extraction solvent, sweet orange peel extract demonstrated the best antioxidant activity in all experiments. These findings support prior research, which indicated that orange peel extracts have significantly higher activity than other peels in all assays (Hegazy and Ibrahium, 2012). Methanol extracts (70.301.35%) derived from sweet orange peels had the maximum activity, followed by ethanol extracts (66.561.46%) obtained from this fruit peel.

2.6. *In-silico* molecular docking

Molecular docking is a technique for analyzing the conformation and orientation of molecules into the binding site of a macromolecular target (Morris & Lim-Wilby, 2008). Docking is a computational process for finding a suitable ligand that matches the protein's binding site both energetically and geometrically. In other words, it is the study of how two or more molecules, such as a ligand and a protein, interact with one another (Azam & Abbasi, 2013). It predicts the favored structural orientation, binding affinity, and interaction between ligand and protein. It is an effective and competent tool for structural

molecular biology and rational computer-aided (*in-silico*) drug design (Kuntz, 1992). Docking can be used to do virtual screening on large libraries of compounds, rank the result, and provide structural theories about how the ligands block the target, which is extremely useful for lead optimization.

3. MATERIAL AND METHODS

3.1. Sample Collection and identification

Citrus sinensis sample was collected in February 2, 2022 from the Oromia region, Metehara (Merti) farm. The plant material was identified and voucher specimen number RY-0001 was deposited at the National Herbarium of Ethiopia, Addis Ababa University, Ethiopia. *Citrus sinensis* sample (10 kg) was peeled, chopped in to small pieces, and washed by distilled water. The peels were allowed to dry at room temperature (30°C) for six days, powdered using a sterilized mixer grinder, and stored in an air-tight container

3.2. Materials and chemicals used

3.2.1. Materials used

The apparatus and instruments used in this research were: electrical grinder, drying oven, measuring cylinders, bottles, shaker, ruler, filter papers, and funnels, Petridish, round bottom flasks, rotary evaporator, vials, test tubes, TLC Chamber, TLC plates, capillary tubes, UV-lamp, column chromatography,For structural elucidation of pure isolated compounds ^{1}H-NMR, ^{13}C-NMR, DEPT-135 (BrukerAvance 400 MHz spectrometer), were used and for molecular dockingNMR- Spectrometer,Chemdraw Ultra 8 Molecular graphics laboratory (MGL) tools, AutoDockVina, Pymol, Discovery Studio visualizer 2021 were used.

3.2.2. Chemicals

The chemicals and reagents used in this research were: ethyl acetate, *n*-hexane, dichloromethane, chloroform, methanol, silica gel, Wagner's reagent, distilled water, ferric chloride, vanillin, DMSO,Muller-Hinton agar, blood agar, nutrient broth and normal saline.

3.3. Extraction

The extraction of *Citrus sinensis* peel was done by using maceration (by ethanol) and ultrasonic assisted extraction (UAE) methods. For the maceration the powder of the peel (300 g) was soaked in 1L ethanol (99.9%) for 72 hours at room temperature with occasional shaking. The extract was filtered using Whatman filter paper number 1. The extract was concentrated using Rota evaporator at 40 ^{O}C. The ultrasonic assisted extraction was done by initially taking the powdered sample (150 g) and soaking it in 500 mL ethanol (99.9%). Then it was placed in to the ultrasonic bath and sonicated for 30min at

temperature of 45^0 C, after the solution was filtered with Whatman filter paper number 1, concentrated using Rota evaporator at 40 ^{O}C and kept in sterile vials in refrigerator until further use.

3.3.1. Essential Oil Extraction

The Essential oil was extracted by hydro distillation in a modified Clevenger apparatus for 2 hours from 80 g of powdered *Citrus sinensis* peel in 500 mL of distilled water. The condensate (mixture of essential oil and water) was collected in a 100 mL separatory funnel. The essential oil was consecutively separated from the aqueous layer, dried using anhydrous magnesium sulphate, transferred into GC-MS vial and stored at 4°C (Lemes *et al*., 2018).

3.4. Isolation of compounds

3.4.1. Column chromatography

Citrus sinensis peel crude extract (15 g) obtained from maceration was adsorbed on 15 g of silica gel (mesh size 60-120), subjected to silica gel column chromatography (150 g of silica gel) and eluted with increasing gradient of ethyl-acetate in *n*-hexane followed by methanol in dichloromethane. A total of 87 fractions each 100 mL (Table 1) were collected. Fractions that showed similar Retention fraction values and the same characteristic color on Thin-Layer Chromatography were combined. Fraction 40 afforded Compound **46** (34.4mg) greenish compound with single spot on TLC (eluted with 100% EtOAc).Fraction 32 (eluted with 35% EtOAc in *n*-hexane) afforded Compound **47** (11.3mg) yellowish compound. From Fraction 41-46 obtained from fractionation of the crude ethanol extract (eluted with 30% EtOAc in hexane) afforded Compound **48** (10.1mg) yellowish compound. Fraction 19 (eluted with 25% EtOAc in *n*-hexane) afforded Compound **49** (34.7mg) greenish compound.

Table 1: Summary of fractionation of ethanol extract

Solvent system	Ratio	Fractions	Solvent system	Ratio	Fractions
n-hexane	10:0	F_1-F_2	Dichloromethane	1.0:0	F_{51}
n-hexane /EtOAc	9.8:0.2	F_3	Dichloromethane/ Methanol	9.8:0.2	F_{52}
	9.5:0.5	F_4-F_6		9.6:0.4	F_{53}-F_{57}
	9.2:0.2	F_7-F_8		9.4:0.6	F_{58}-F_{59}
	9:1	F_9-F_{12}		9:1	F_{60}-F_{64}
	8.7:1.3	F_{13}		8.5:2.5	F_{65}
	8.5:1.5	F_{14}-F_{15}		8:2	F_{66}
	8:2	F_{16}-F_{17}		7.5:2.5	F_{67}
	7.5:2.5	F_{18}-F_{25}		7:3	F_{68}- F_{71}
	7:3	F_{26}- F_{29}		6:4	F_{72}
	6.5:3.5	F_{30}-F_{32}		5:5	F_{73}
	6:4	F_{33}-F_{34}		4:6	F_{74}
	5:5	F_{35}		3:7	F_{75}
	4:6	F_{36}		2:8	F_{76}- F_{79}
	3:4	F_{37}		1:9	F_{80}- F_{84}
	2:8	F_{38}	Methanol	10:0	F_{85}- F_{87}
	1:9	F_{39}			
EtOAc	10:0	F_{40}-F_{50}			

3.5. Characterization of samples

The NMR spectroscopic technique was employed to elucidate the structure of the isolated compounds with the help of ^{1}H NMR, ^{13}C NMR and DEPT-135 spectra at Addis Ababa University.

3.6. GC-MS analysis of Essential oil extract

GC-MS analysis of essential oil was performed by a GC (7890B, Agilent Technologies, USA) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP-5MS column (30mm × 250 μm internal diameter (i.d.) and 0.25 μm). Helium was used as a carrier gas (flow rate 1 mL/ min).The initial oven temperature was 100°C for 2 min and raised up from 100 to 280ºC at the speed of 10ºC/min (inlet 250 °C; detector 280° C;

splitless injection/purge time 1.0 min), solvent delay 4.00 min. Mass spectra was recorded in electron-impact mode, with ionization energy of mode at 70 eV, scanning the 33-550 m/z range. The volatile compounds in the oil were identified by comparing the mass spectra of the compounds in the oils with those in the database of NIST11 GC-MS libraries (Hanuš *et al*., 2008).

3.7. Phytochemical screening of crude extract

Phytochemical examinations were carried out for *Citrus sinensis* ethanol extract as per the standard methods as described by Sofowara (1993), Trease and Evans (1989), Kokate *et al*. (2001), Kokate *et al*. (2001) and Harborne (1973).

3.7.1. Test for steroid

Two milliliter of acetic anhydride was added to the *Citrus sinensis* peel crude extract which was dissolved in 2 ml of sulphuric acid. A color changes from violet to blue to green indicates the presence of steroids.

3.7.2. Test for terpenoid

Five milliliter of the peel crude extract was mixed with 2ml of chloroform. Then 3ml of concentrated sulphuric acid was added to form a layer (Salkowski test). A reddish brown coloration at the interface indicated the presence of terpenoids.

3.7.3. Test for saponins

Citrus sinensis peel crude extract was dissolved in five milliliter of water and the tubes were shaken vigorously, formation of 1 cm layer of foam indicates the presence of saponins (Kokate *et al*., 2001).

3.7.4. Test for flavonoids

Three milliliter of peel crude extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

3.7.5. Test for tannins

The peel crude extract was boiled in 20ml of water in a test tube. The solution was filtered and a few drops of 0.1% Iron III chloride were added. The appearance of green/bluish black color indicates the presence of tannins.

3.7.6. Test for alkaloids

One milliliter of 1% HCl was added to 3ml of the peel crude extract. The mixture was heated for 20 min, cooled and filtered. Then 1 ml of the filtrate was tested with 0.5 ml Mayer's, reagent (Potassium Mercuric Iodide).Formation of a yellow color precipitate indicates the presence of alkaloids.

3.7.7. Test for phenol

Ferric chloride test: the peel crude extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

3.8. Biological activity

3.8.1. Antibacterial activity

In vitro antibacterial activities of *Citrus sinensis* peel crude extract, oil extract and isolated compounds were examined. The antibacterial activities were determined against six pathogenic bacterial strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853),*Klebsiella pneumonia* (ATCC 700603) *and Salmonella typhimurium* (ATCC 13311)by the agar disk diffusion method. The four sets of dilutions (150, 75, 37.5, and 18.75mg/ml) of *Citrus sinenses peel* crude extract, oil extract (95, 47.5, 23.7, 11.8mg/ml) and isolated compounds (6, 3, 1.5, 0.75 mg/ml) were prepared. The strains of bacteria cells were adjusted at 1.5×10^8 colony forming units (CFU/ml). To determine the amount of bacteria present, a 0.5 McFarland standard was prepared by combining 0.05ml of 1 percent BaCl2 and 9.95ml of 1 percent H_2SO_4 in distilled water. To contrast a bacterial solution, the preparation was stored in a flask. Mueller-Hinton sterile agar plates were inoculated with indicator bacterial strains.

Sterile filter paper disks (Whatman No. 1, diameter = 6 mm) were placed on the inoculated Mueller-Hinton agar plates. From each dilution 20µL were taken and dispensed on the filter papers and allowed to stay at 37°C for 24 hours. Control experiments were carried out under similar condition by using gentamicin as positive control and DMSO as negative

control. The zones of growth inhibition around the disks were measured after 18 to 24 hours. The sensitivities of the bacterial strains to the peel extracts were determined by measuring the sizes of inhibitory zones starting from the edge of disk to the edge of the clear zone on the agar surface around the disks, and values <7 mm were considered as not active against microorganisms.

3.8.2. Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Broth dilution method was adopted to find minimum inhibitory concentrations (MICs) of active extracts. *Citrus sinensis* peel crude extract, oil and isolated compounds that exhibited antibacterial activity in the preceding test were chosen. The initial concentration of the crude (5mg/ml), essential oil (5 mg/ml) and isolated compounds (1.00 mg/ml) were diluted using two-fold serial dilution by transferring 1 ml of crude into 1 ml of sterile nutrient broth and mixed it into a vial, and then it was serially diluting it into 5 vials. Each concentration was inoculated with 0.02 ml of the standardized bacterial cell suspension and incubated for 24 hours at 37^0 C. The turbidity or cloudiness of the broth was indicator of bacteria's growth in the broth (Abou, 2010).

3.8.3. Determination of Minimum Bactericidal Concentrations (MBC)

An MBC (Minimum Bactericidal Concentration) is the lowest concentration of an antibacterial that prevent the growth of an organism after subculture onto media. The broth dilution method was used to determine *Citrus sinensis* peel crude extract, essential oil and isolated compounds minimum bactericidal concentration. Fresh nutrient broth medium was used to transfer samples from the MIC determination set. A loop full of all the tubes used in the MIC, which did not show bacteria growth inoculated and sub-cultured onto sterile nutrient agar media streak plating by a sterile wire loop. Following that, all agar media were incubated at 37°C for 24 hours after the transfer is completed. The test tubes were examined for growth at the end of the incubation period. The MBC of the crude extract against the particular tested strain were recorded at the lowest concentration that exhibited no microbial growth.

3.8.4. Antioxidant activity

Antioxidant activity of the crude extract, oil extract and isolated compounds were measured based on their free radical scavenging activity, which determined by the 1,1-

diphenyl-2-picryl-hydrazyl (DPPH) method. By this method, it is possible to determine the radical scavenging power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517nm.As a result of the color changing from purple to yellow, the absorbance was decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Nishibe *et al*., 1995).Lower absorbance of the reaction mixture indicated higher free radical scavenging activity (Andrzejewska-Goleck *et al*., 1986).

DPPH solution (0.1 mM) was prepared in methanol by dissolving 0.04 g DPPH in 100 ml methanol. The solution was kept in darkness for 30 min to complete the reaction. Crude extract of the peel were diluted in four test tubes 1000, 500, 250, 125 µg/ml. from this concentrations 1 ml of each were mixed with 4ml of 0.04% DPPH. For the compounds the same procedure was performed. The samples were diluted 100, 50, 25, 12.5 µg/ml. from each concentration 1ml of the concentration was mixed with 4ml 0.04% DPPH. The resulting solutions were subjected to UV-Vis spectrophotometer to record absorbance at 517 nm. The percentage DPPH inhibition was calculated according to the following formula.

$$\% \text{ of radical scavenging activity} = \frac{Ab_{standared} - Ab_{analyte}}{Ab_{standard}} \times 100$$

3.9. *In-silico* molecular docking study of isolated compounds for antibacterial activity

3.9.1. Preparation of protein and ligand

3d crystal structures of targeted proteins, *E. coli* Gyrase (PDB ID: 6F86), *S. aureus* Pyruvate Kinase and Human Peroxiredoxin 5 (PDB ID: 1HD2) was chosen as the protein model and retrieved from the Protein Data Bank (http://www.rcsb.org/). The coordinates of the structures were complexed with water molecules and other atoms which are responsible for increased resolution.

Crystal Structure of *E. coli* Gyrase B 24kDa with resolution 1.90 Å has one chain co-crystallized with native ligand 4-(4-bromo-1H-pyrazol-1-yl)-6-[(ethylcarbamoyl) amino]-N-(pyridin-3-yl) pyridine-3-carboxamid (C_{17} H_{16} Br N_7 O_2).

Crystal structure of *S. aureus* Pyruvate Kinase in complex with a naturally occurring bis-indole alkaloid with resolution: 3.30 Å contains three chains, A, B, C, D and one native ligand and four Po4 groups. (3S, 5R)-3,5-bis(6-bromo-1H-indol-3-yl)piperazin-2-one (C_{20} H_{16} Br_2 N_4 O)found on chain Band D and Po4 on chain A, B, C, D.

Crystal structure of Human Peroxiredoxin 5, a Novel Type of Mammalian Peroxiredoxin at 1.5 A Resolution (1HD2) has one chain (A) co-crystalize with benzoic acid (C_7 H_6 O_2) bromide ion (Br).

Therefore het-atoms (native ligand/ PO_4 groups) to make active site free for docking and water molecules which may interfere with ligand/receptor bonding (Wong & Lightstone, 2011) were removed using discovery studios visualizer and saved in pdb format. Then polar hydrogen atoms and Kollman charges were added for proper optimization (Pandey, 2019) usingMGL tool and saved in PDBQT format.

The structures of test compounds were drawn using chemdraw ultra 8. Energy minimizations of the ligand/compounds were done using chemdraw3D ultra 8 and stored in .pdb format. Then PDB format of the compounds were treated using MGL, gasteiger charges were added, non-polar hydrogen's are merged out and torsion angle of the ligand was adjusted automatically. Finally the prepared compound file was saved as PDBQT in working directory.

3.9.2. Grid map preparation and docking

Compound 46 and 48 were docked to target proteins *i.e E. coli* DNA gyrase, *S. aureus* Pyruvate Kinase (PDB ID: 3t07) and Human Peroxiredoxin 5. The active site was identified by studying the interaction between native ligand and the enzyme. Docking was performed on the active site of the protein. The search was carried out by building a grid box with volume that is big enough to cover the active site of the protein.Grid box size of 50 ×50× 54Å points with a grid spacing of 0.375 Å was considered.

The docking of proteins was carried out using the Autodockvina program. After the preparation of configuration file which contains;receptor-name.pdbqt, ligand-name.pdbqt, grid dimension, and exhaustiveness (run repetition) autodockvina run through command prompt.

Docked structure (ligand-protein complex) was prepared using Pymol and structure analysis was done visualized using Biovia Discovery Studio 2021.

3.10. Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data was subjected the data will subjected to SPSS 16 to one-way analysis of variance (ANOVA) and the results were presented by using tables and graphs. P values less than 0.05 was considered statistically significant. Microsoft Excel 2010 statistical package was used for all analyses.

4. RESULT AND DISCUSSION

4.1. Extract yield

Citrus sinensis peel was extracted using ethanol 99.9% by maceration (300 g) and ultrasonic extraction (150 g) methods and yielded brownish (20.99 g, 7%) and yellowish (11.5 g, 7.5%) extract, respectively. Differences in the yields between the extraction protocols were observed. The yield (7%) from maceration extraction was lower than the yield obtained from ultrasonic extraction (7.5%). From these results one can deduce that a better extractive yield can be obtained from the ultrasonic extraction method in agreement with Saini *et al*. (2019). Muhammad *et al*. (2017) reported that UAE had a comparatively higher extraction yield at all solvent concentration levels compared with the maceration technique.

4.2. Thin-layer Chromatography profile of extracts

Ethanol extract of *Citrus sinensis* peel by maceration (B) and ultrasonic-assisted (A) extraction analyzed for their TLC profile using different solvents as a mobile phase and observed 365 nm. Their TLC profile (Figure 8) revealed similarity between maceration and ultrasonic-assisted extractions in furnishing similar compounds. The results of the TLC profile of the crude extract demonstrated the presence of different spots which confirmed the existence of different compounds in the crude extract. The extract obtained from maceration was subjected to silica gel column chromatography for further isolation of compounds.

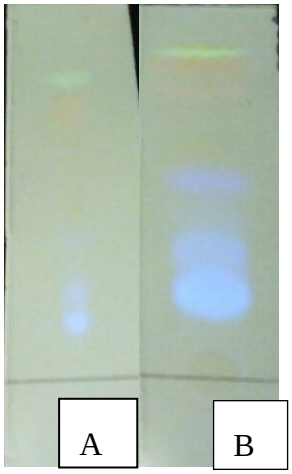


Figure 8: TLC profile of *Citrus sinensis* peel crude extract obtained from ultrasonic assisted extraction (A) and maceration extraction (B) observed under 365nm.

4.3. Phytochemical screening

Following conventional experimental techniques, phytochemical screening tests were performed to determine the class of chemicals present in the crude extracts. The phytochemical screening tests conducted on *Citrus sinensis* peel crude ethanol extract obtained by maceration showed the presence of alkaloid, flavonoid, tannins, steroids, phenol and terpenoid (Table 2).

These findings show that the detected phytochemical substances are the peel's bioactive elements, indicating that the *Citrus sinensis* peel has a valuable reservoir of bioactive compounds with significant medical value. For instance, Thakur *et al*. (2020) reported that terpenoids, flavonoids, saponins, tannins, and phenols are the most common phytochemicals that are found in orange peels. According to Rabab *et al*. (2021) Preliminary qualitative phytochemical analysis made for orange peel revealed the presence of alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids and triterpenoids. These secondary metabolites are reported to have many biological and therapeutic properties (Gul *et al*., 2017),

Plant antimicrobial properties are thought to be caused by phytochemicals such as tannins, flavonoids, saponins, phenolic compounds, and essential oils (Rahman *et al*., 2011). Flavonoids have been related to a variety of biological functions, including antibacterial, antioxidant, and anti-inflammatory effects. They are known to serve a protective role in plants against diseases (Hafidh *et al*., 2011; Dhiman *et al*., 2012). Pathogens are known to be inhibited by the synergistic action of tannins, flavonoids, alkaloids, and saponins (Nwankwo *et al*., 2014).

Table 2: Phytochemical screening results of *Citrus sinensis* peel crude extract.

Secondary metabolites	**Observation**
Alkaloid	+
Tannins	+
Flavonoid	+
Steroid	+
Phenol	+
Terpinoid	+
Saponins	-

+ shows presence, -shows absence

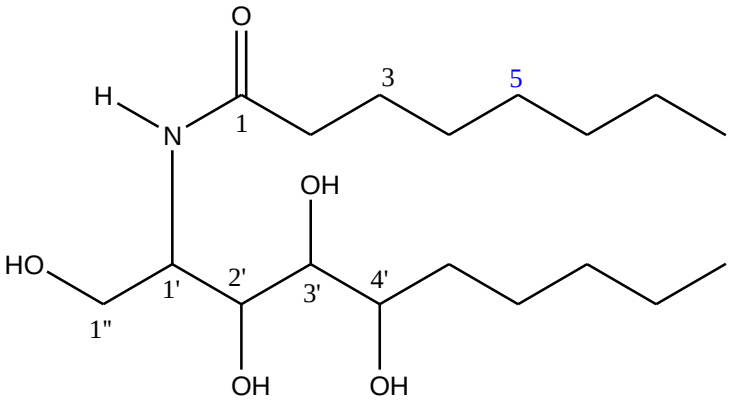
4.4. Characterization of the compounds

In the present work, four compounds (Compound **46**, Compound **47**, Compound **48** and Compound **49**) were isolated from the Ethanol peel extracts of *Citrus sinensis*. The detailed characterizations of these compounds are presented below.

4.4.1. Compound 46

Compound **46** (Figure 9) was isolated as a greenish solid with an Rf value of 0.44 (eluted with 100% EtOAc). Its ^{1}H NMR spectrum (Appendix1.1) revealed the existence of amide protons at δ 8.02 (s, 1H), and hydroxyl protons at δ 3.9 integrated for two protons. Four methylene protons were observed at δ 2.1 (H-4 to H-7). Terminal methyl groups were observed at δ 0.9 (H-9' and H-8).

In the ^{13}C NMR (Appendix 1.2), a carbonyl group (-CO-) was observed at δ 179.3 attributed to amide carbonyl. In addition, carbon signals resonating at δ 65.3 (C-1"), 33.7 (C-2), 31.8 (C-5'), 29.5, 29.4, 29.2, 28.9, 24.8, and 22.4 ppm are corresponding to the methylene groups. These methylene signals pointed down in DPET-135 spectrum. The presence of three sp^3 oxygenated methine carbons were observed at δ 73.9 (C-3'), 62.9 (C-2'), and 63.1 (C-4'). The terminal methyl groups appeared at δ_C 10.1 and 13.5 ppm. The above spectral data is in good agreement with the ^{13}C NMR spectral data of N-(1,3,4,5-tetrahydroxydecan-2-y1)octanamide (46, Fig 9).



46

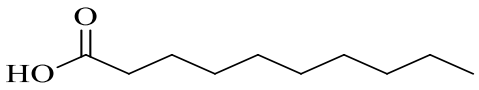
Figure 9: Chemical structure of Compound **46**, N-(1,3,4,5-tetrahydroxydecan-2-y1) octanamide

4.4.2. Compound 47

Compound **47** (Figure 10) was obtained as a greenish solid with Rf value of 0.45 (eluted with 75% EtOAc in n-hexane). It's ^{1}H NMR (Appendix 2.1) spectral data showed the corresponding signals of the methylene protons with chemical shifts between δ 1.3 and 2.4 ppm. Terminal methyl protons appeared at δ 0.9 (t, 3H). In the ^{13}C-NMR (Appendix 2.2), the carbonyl group (-CO-) of the carboxylic acid appeared at δ 178.7. In addition, peaks at δ 22.7-33.9 correspond to the methylene groups (9 in number) of which the most deshielded one that appeared at δ 33.9 suggests methylene next to carbonyl (C-2). Terminal methyl group appeared at δ 14.1. The above spectral data suggest that the compound is decanoic acid fatty acid (**47,** Fig 10)

Table 3: ^{1}H NMR and ^{13}C-NMR spectral data of compound **47** (CDCl3, δ in ppm)

Position	Compound **47**		
	^{1}H NMR	^{13}C NMR	Multiplicity
1		178.7	Carboxyl
2	2.4 (2H),	33.9	CH_2
3	1.6 (2H),	24.7	CH_2
4-7	1.3 (8H),	29.7,29.4, 29.2, 29.1	CH_2
8		31.9	CH_2
9		27.7	CH_2
10	0.9 (t, 3H)	14.1	CH_3



47

Figure 10: Structure of Compound **47**, Decanoic acid

4.4.3. Compound 48

Compound **48** (Figure 11) was isolated as yellowish solid with R_f value of 0.14 (eluted with 30% EtOAc in n-hexane). Analysis of the ^{1}H-NMR spectrum ($CHCl_3$) (Appendix 3.1) demonstrated the presence of six methyl signals at δ 1.0 (s, 1H), 0.9 (d, J = 6.4 Hz, 1H), 0.89 (d, J = 6.5 Hz, 1H), 0.86 (s, 3H), 0.8 (d, J = 1.9 Hz, 1H), 0.8 (s, 3H). The singlet signal observed at δ_H 2.4 integrating for two protons is accounted to methylene protons adjacent to a carboxyl. The intense broad singlet signal at δ_H 1.3 is characteristics of the presence of many overlapping methylene protons. The olefinic proton was observed at *δ* 5.4 (2H, *s*).

Its ^{13}C-NMR (400 MHz, $CHCl_3$) (Appendix 3.2) spectrum analyzed with the aid of DEPT-135(Appendix 3.3) revealed the presence of four quaternary carbon signals observed at δ_C174.6, 140.3,42.3,and 36.7 of which the former suggest the presence of ester carbonyl whereas the second one suggest the presence of sp^2 quaternary carbon. Methine signals were observed at δ_C56.8, 56.0, 55.5, 50.2, 45.8, 36.1 and 31.9 along with one sp^3 oxygenated methine at δ 70.2 which is a characteristic peak of C-3 methine of steroids. The carbon signals exhibited at δ 39.8, 38.9, 37.3, 34.2, 31.9, 29.7,28.2, 25.00 and 22.7were due to methylene carbons. The presences of six methyl signals were apparent at δ_C19.8, 19.3, 19.0, 18.8, 12.0, and 11.9. The presence of one olefinic methine was observed at δ 122.1. This coupled with the aforementioned sp^2 quaternary carbon at δ 140.3 suggest a characteristics features of sterols with Δ^5 bond. The presence of one glucopyranose moiety is evident from the appearance of only one anomeric carbon signal at δ 101.2 along with series of carbon signals at δ 79.6, 76.1, 73.9, 73.6 and 63.3 of which the latter suggest oxygenated methylene (C-6') of the glucopyranose moeity. The spectrum revealed a benzoyl moiety peak resonating at δ 174.6, 127.8, 114.5, and 111.0 of which the former suggest ester carbonyl.

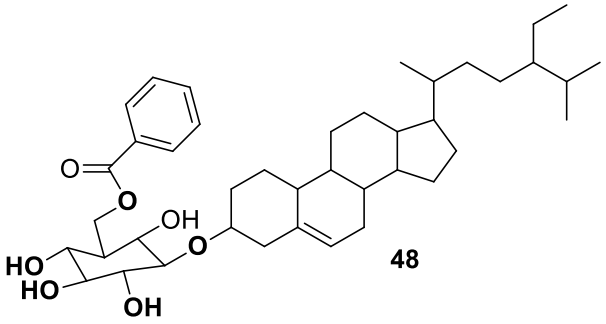


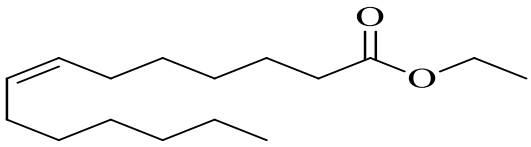
Figure 11: Chemical structure of Compound **48**, glycosylated steroid

4.4.4. Compound 49

Compound **49** (Figure 12) was obtained as greenish solid with an Rf value of δ 0.38 (eluted with 25% EtOAc in *n*-hexane).The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, Table 4) (Appendix 4.1) of compound **49** showed the presence of an olefinic proton signal at δ 5.4 (s, 1H)**.** The presence of oxygenated methylene signal was observed at δ 4.2 (m, 2H). The signal at δ 1.3 (*brs*,6H) is a characteristic signal for many methylene protons in the compound which was supported by the appearance of an intense carbon signal at δ 29.7 in the ^{13}C-NMR spectrum (Appendix 4.2).The triplet signal observed at δ 2.3 is ascribed to methylene protons attached to a carboxyl group. The signal at δ 2.0 (s, 2H) are integrated for the allylic methylene protons. An up field proton signal at 0.9 (s, 6H) is evident for the presence of terminal methyl proton in the compound. The ^{13}C NMR (100 MHz, $CDCl_3$) spectral data with the aid of DEPT-135(Appendix 4.3) revealed a total of 14 carbon signals of which two olefinic methine signals at δ 130.0, and 129.7, and ten methylene signals at δ 34.0, 31.9, 29.7, 29.4, 29.4, 29.3, 29.1, 27.2, 24.7 and 22.7 are clearly evident. Oxygenated methylene was observed at δ 65 (C-1"). The most up field carbon signal at *δ* 14.1 accounts for the presence of terminal methyl protons. The existence of ester carbonyl carbon was observed at δ 179.6 (C-1). The above spectral data of the compound is in good agreement with ethyl tetradec-7-enoate (**49**) which was previously isolated from several plants including chloroform extract of stem of *Turraea vogelii* (Abdulmumeen *et al*., 2019).

Table 4: ^{1}H NMR and ^{13}C-NMR spectral data of compound **49** (CDCl3, δ in ppm)

Position	NMR data of compound **49**		
	^{1}H-NMR	^{13}C-NMR	Multiplicity
1		179.6	Carboxyl
2	2.3 (t, J = 6.8 Hz, 2H),	34.0	CH_2
3	1.6 (brs, 2H),	24.7	CH_2
4		29.4	CH_2
5		29.7	CH_2
6	2.0 (brs, 2H)	27.2	CH_2
7	5.4 (brs, 1H)	130.0	= CH
8	5.4 (brs, 1H)	129.7	= CH
9	2.0 (brs, 2H)	27.2	CH_2
10		29.4	CH_2
11		29.1	CH_2
12		31.9	CH_2
13		22.7	CH_2
14	0.9 (s, 3H)	14.1	CH_3
1'	4.2 (m, 2H)	65.0	CH_2O-
2'	0.9 (s, 3H)	14.3	CH_3



49

Figure 12: Structure of Compound **49**, (Z)-ethyl tetradec-7-enoate (**49**).

4.5. GC-MS analysis of essential oils

The GC-MS analysis is the valuable method which has been increasingly applied for the analysis of medicinal plants for nonpolar components, volatile essential oil, fatty acids and alkaloids. The essential oils of *Citrus sinensis* peel (yield of 0.89% (v/w)) was analyzed by GC-MS and it was found that Limonene (87.5 %) was the major constituent followed by 1,6-Octadien-3-ol, 3,7-dimethyl (4.35 %), Isopulegol (2.46%), pinene (1.93%), trance-p-

Mentha-2,8-dienol (1.45%), Carveol P294 (1.25%) and 2-Cyclohexen-1-one,2-methyl-5-(1-methylethenyl) (0.98%), sequentially. The findings of the present work are with a good agreement with that of Simon *et al*., (2009) which reported limonene (87.9-92.5 %) as a major component of *Citrus sinensis* peel. In a related study, it is confirmed that the peels of citrus species contain more than 70% limonene by Dugo and his co-workers (2002). Previous study by Choike *et al*. (2017) on ethanol extraction and GC-MS analysis of *Citrus sinensis* peel oil reported fifteen compounds and the result revealed that limonene is the major component of the oil. The aforementioned literature reports are in a good agreement with the results obtained in this work.

According to Wahab *et al*. (2013) limonene is responsible for the oil's antibacterial and antifungal properties. Limonene now is known as a significant chemo preventive agent with potential value as a dietary anti-cancer agent in humans (Prashant *et al*., 2012). It is an effective, environmentally friendly, and generally safe solvent that is used in a variety of applications, including adhesive stain removers, cleansers of various kinds, and strippers (Akhilesh *et al*., 2012). Limonene is also important in agriculture since it repels insects (Ngele *et al*., 2014). The variation in *Citrus sinensis* extracts could be explained by differences in plant parts, extraction solvents, and geographical location.

Uraku *et al.* (2020) reported five compounds from GC-MS analysis of methanolic extract of *Citrus sinesis* peel such as Pyridine-2-carbaldehyde, 1Methyl-1*H*-pyrrole-2-carbaldehyde (40%), Glutamic acid(25%), 2Ethyl-5-methyl-1*H*-pyrrole (14%) and Pyrrolidin-2-one (12%).Compared to the present study differences in extraction solvents and geographical location could explain the disparity in these peel extracts.(Appendix 5).

Table 5: Chemical composition of *Citrus sinensis* peel essential oils analyzed by GC-MS

No	Name	RT		Molecular Formula	%
1	Limonene	3.74		$C_{10}H_{16}$	87.5
2	2(10)-Pinene	3.261		$C_{10}H_{16}$	1.93
3	1,6-Octadien-3-ol, 3,7-dimethyl-	4.404		$C_{10}H_{18}O$	4.35
4	trans-p-Mentha-2,8-dienol	4.883		$C_{10}H_{16}O$	1.45
5	Isopulegol	5.704		$C_{10}H_{18}O$	2.46
6	Carveol P294	6.05		$C_{10}H_{16}O$	1.25
7	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-	6.322		$C_{10}H_{14}O$	0.98

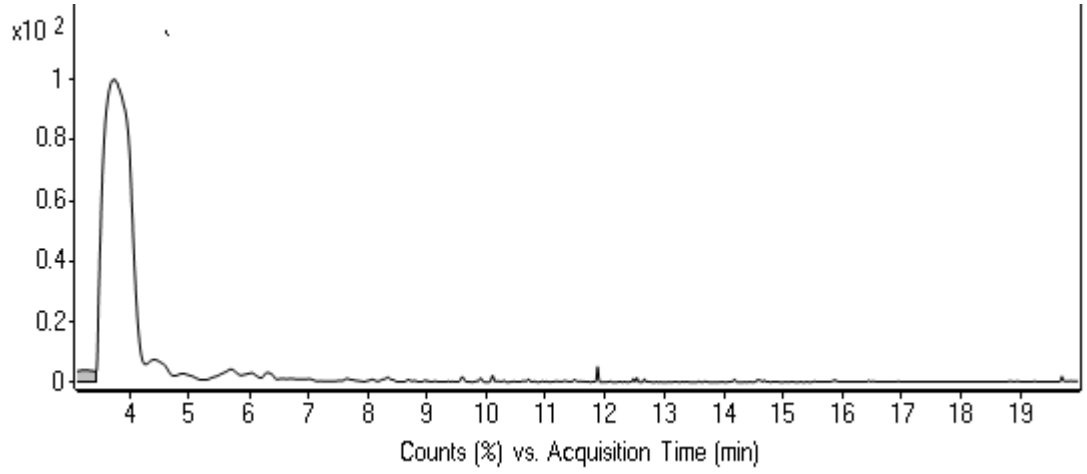


Figure 13: GC chromatogram of *Citrus sinensis* peel essential oil

4.6. Antibacterial activity

The antimicrobial activity of *Citrus sinensis peel* ethanol extract, essential oil and isolated compounds were tested for their antibacterial activity in different concentrations against six pathogenic bacterial strains, (*E. coli, S. aureus, E. faecalis, P. aeruginosa, K. pneumonia and S. typhimurium*) compared to gentamicin as positive control and DMSO as negative control. The mean zone of inhibition values indicated that all the compounds exhibited antibacterial activities ranging from 8.00 mm to 15.67 mm. Activity increased in a dose dependent manner.

Citrus sinensis peel crude extract revealed a promising zone of inhibition against *E. faecalis* (12.67±0.58mm), *S. typhimurium* (11.33±0.58) and *E. coli* (10.33±0.58) at 150 mg/mL compared to gentamicine (13.00±1.73, 18.00±1.00 and 16.33±1.15 respectively) with concentration 10µg/mL. Similarly with this finding Najimu Nisha *et al*. (2013) tested ethanolic extract of *Citrus sinensis* peel against enteric bacteria such as *E.coli* (10-16mm), *K. pneumonia* (11-14mm), *P. aeruginosa* (9-21mm), *S. typhi* (9-18mm).

The essential oil showed the best activity against *S. aureus* (14.33±1.52mm), *E. coli* (12.33±0.58mm), *S. typhimurium* (12.00±0.00mm), *K. pneumonia* (12.00±1.00mm), *E. faecalis* (12.67±0.58) and *P. aeruginosa* (10.67±0.58) at 95mg/mL compared to genamicine (18.67±0.58 mm, 16.33±1.15mm, 18.00±1.00mm, 16.67±2.08mm, 13.00±1.73 and 16.67±1.15 respectively) at concentration of 10µg/mL. Previous studies by Hashem *et al*. (2009) and Edogbanya *et al.* (2019) revealed higher activity of the essential oil against *S. aureus* among the tested bacterial strains which is in a good agreement with the present

study. This finding is supported by the results of Burt *et al*. (2004) which showed that Gram-positive bacteria are generally more sensitive to citrus essential oil than Gram-negative. According to Burt *et al.* (2004), the resistance of Gram-negative bacteria to essential oils is attributed in part to the structures complexity of these microorganisms conversely to those of Gram-positive bacteria. A similar scenario was also reported by Djenane (2015) for other essential oils.

Compound **48** (6 mg/ml) showed good activity against *S. typhimurium*(12.00±1.00mm) compared to gentamicine (18.00±1.00) at 10µg/mL and no zone of inhibition against *E. faecalis, P. aeruginosa* and *S. aureus* compared at all concentrations. Compound **49** (6mg/ml) showed a good antibacterial activity against *S.typhimurium* (15.33±2.89mm), *E.coli* (11.33±0.58mm), *P.aeruginosa* (11.00±1.00), *K.pneumonia* (11.33±0.58) compared to gentamicine (18.00±1.00, 16.33±1.15, 16.67±1.15 and 16.67±2.08 respectively) at 10µg/mL and no zone of inhibition were observed against *E.faecalis* at all concentration. Compound **46** showed a good zone of inhibition against *P.aeruginosa* (12.00±0.00mm) moderate antibacterial activity against *E.coli (*10.67±0.58mm), *K. pneumonia* (10.00±0.00mm) *and S. typhimurium*(10.00±0.00mm) at 6mg/mL concentration. Also compound **47**(6mg/mL**)** exhibited promising zone of inhibition against *P.aeruginosa* (11.67±0.58) *and K.pneumonia* (11.00±0.00mm) compared to gentamicine (16.67±1.15 and 16.67±2.08 respectively).

The antibacterial activity of plants is believed to be due to secondary metabolites such as; tannins, terpinoid, phenol, steroid, alkaloid and flavonoids. These chemicals are recognized to be biologically active, which aids the plants' antibacterial capabilities (Oikeh *et al*., 2013).In the present study phytochemical analysis of *Citrus sinensis* peel extract showed the presence of the above chemicals which can exert antimicrobial activity through different mechanisms. Tannin observed in *Citrus sinensis* peel extract has been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis (Shimada, 2006). Flavonoids have antibacterial and antioxidant characteristics (Hodek *et al*., 2002). Terpenoids present in ethanolic extract of *Citrus sinensis* are thought to play a role in membrane disruption caused by lipophilic substances (Alams *et al*., 2005). The presence of various secondary metabolites in the *Citrus sinensis* peel could explain for their function as antibacterial agents.

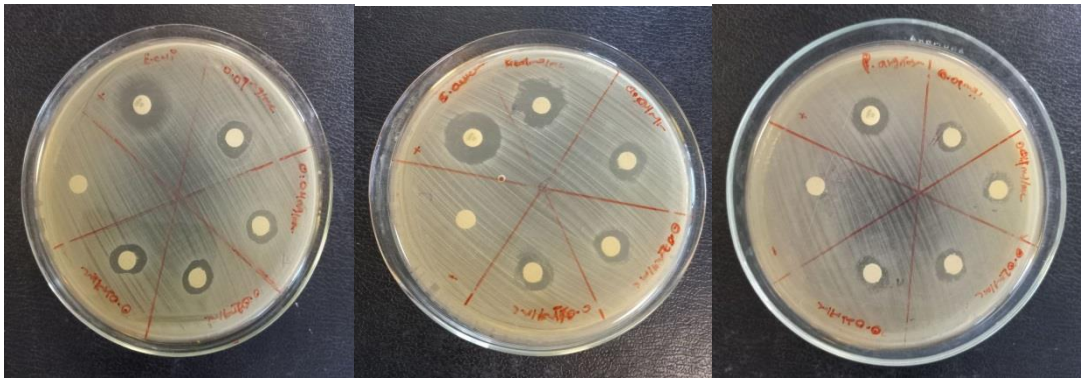
The results of the antibacterial activity showed by *Citrus sinensis* peel ethanol extract, essential oil extract and isolated compounds displayed good antibacterial activity against

all the tested bacterial strains. Concentration of all extracts was significantly associated with mean zone of inhibition (in mm). This study demonstrated an increase in zone of inhibition in a dose dependent manner with increase in concentration which is in good agreement with the report of Shetty *et al.* (2016) and Edogbanya *et al.* (2019).

P. aeruginosa is generally known to be resistant to many antibacterial agents (Suja *et al*., 2017), but inhibited by the ethanol extracts of the *Citrus sinensis* peel this finding agrees with Mohammed *et al*. (2018).The work of Osarumwense (2017) revealed that the methanolic extract of *Citrus sinensis* peel is highly active against Gram positive, Gram negative bacteria and fungi at concentrations of 100 mg/mL, 150 mg/mL and 200mg/mL respectively. Omodamiro and Umekwe (2013) reported that ethanolic extracts at different concentrations (250, 125, 62.5, 31.25, and 15.5 mg/ml) exhibited antibacterial activity against *S. aureus, S.pneumoniae, E. coli, P. mirabilis* and *P. aeruginosa*, in a dose-dependent manner.

Table 6: Antibacterial activity of *Citrus sinensis* peel crude extract and essential oil

	Conc (mg/ml)	Inhibition Diameter (mm)±SD					
		E. coli	*S. aureus*	*K. pneumonia*	*S. typhimurium*	*E. faecalis*	*P. Aeruginosa*
Crude	150	10.33±0.58	9.00±1.00	11.33±0.58	10.00±0.00	12.67±0.58	8.67±1.15
	75	10.33±0.58	7.67±0.58	10.67±0.58	8.67±1.52	11.00±0.00	8.33±0.58
	37	10.00±1.00	7.33±0.58	9.67±1.15	7.67±0.58	8.33±1.52	8.00±1.00
	18	9.67±0.58	7.00±0.00	9.33±0.58	8.67±1.15	6.33±0.58	7.00±0.00
Oil	95	12.33±0.58	14.33±1.52	12.00±1.00	12.00±0.00	12.67±0.58	10.67±0.58
	47	12.00±0.00	12.67±0.58	11.67±0.58	11.33±1.15	11.33±0.58	10.67±0.58
	23	11.33±0.58	11.33±0.58	11.33±0.58	11.00±1.00	11.67±0.58	10.33±1.15
	11	10.67±0.58	10.67±0.58	10.33±0.58	10.67±0.58	10.67±1.15	9.67±1.52
Gentamycin	10µg/mL	16.33±1.15	18.67±0.58	16.67±2.08	18.00±1.00	13.00±1.73	16.67±1.15

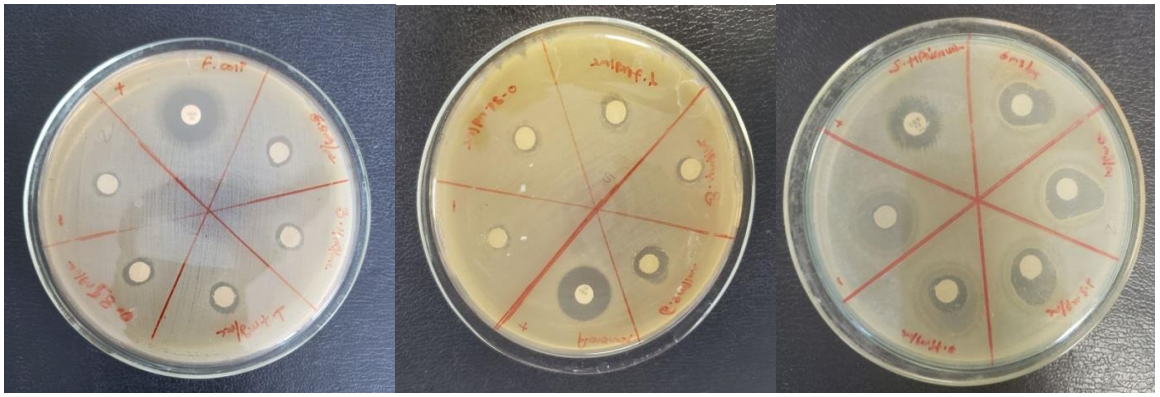


E.coli *S.aureus* *P.aeruginosa*

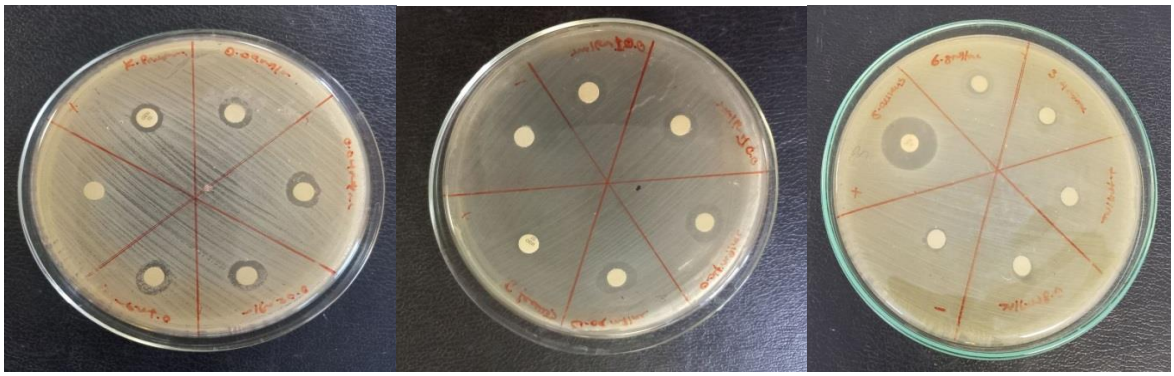
0Figure 14: Inhibition zone of *Citrus sinensis* peel crude extract and essential oil on tested bacterial strains

Table 7: Antibacterial activities of isolated compounds

	Conc (mg/mL)	Inhibition Diameter (mm)±SD					
		E. coli	*S. aureus*	*K. Pneumonia*	*S. typhimurium*	*E. faecalis*	*P aeruginosa*
Compound 46	6	10.67±0.58	9.67±1.53	10.00±0.00	9.67±1.15	10.00±0.00	12.00±0.00
	3	10.00±0.00	9.00±0.00	9.67±0.58	9.00±0.00	10.00±0.00	11.33±0.58
	1.5	10.67±0.58	8.00±1.00	10.33±0.58	9.33±0.58	10.00±0.00	11.00±00
	0.75	10.00±1.00	8.00±1.00	10.67±0.58	9.67±0.58	10.00±0.00	10.00±1.00
Compound 47	6	9.00±1.00	9.33±1.15	11.00±0.00	10.67±0.58	NA	11.67±0.58
	3	9.00±1.00	9.00±0.00	10.00±0.00	9.67±0.58	NA	9.67±0.58
	1.5	8.33±1.53	8.00±1.00	9.00±0.00	8.67±0.58	NA	9.33±0.58
	0.75	8.33±1.15	8.00±1.00	9.00±0.00	8.00±0.00	NA	9.00±0.58
Compound 48	6	10.33±0.58	NA	10.00±0.00	12.00±1.00	NA	NA
	3	9.00±1.00	NA	10.00±0.00	9.00±1.00	NA	NA
	1.5	9.33±0.58	NA	10.00±0.00	9.33±0.58	NA	NA
	0.75	8.33±1.15	NA	11.00±0.00	8.33±1.15	NA	NA
Compound 49	6	11.33±0.58	9.00±2.00	11.33±0.58	15.33±2.89	NA	11.00±1.00
	3	11.00±1.00	8.33±2.08	11.00±0.00	15.67±1.15	NA	9.00±1.00
	1.5	11.00±0.00	8.33±1.52	9.67±1.15	13.00±1.73	NA	8.33±0.58
	0.75	10.67±0.58	8.00±1.15	10.67±0.58	15.67±2.08	NA	8.67±0.58
Gentamycin	10µg/mL	16.33±1.15	18.67±0.58	16.67±2.08	18.00±1.00	13.00±1.73	16.67±1.15



E.coli *P.aeruginosa* *S.typhimurium*



K.pneumonia *E.fecalis* *S.aureus*

Figure 15: Inhibition zones of isolated compounds of *citrus sinensis* peel on tested bacterial strains in different concentration.

4.6.1. Minimum Inhibitory Concentration (MIC)

In the present study, the MIC value obtained from *Citrus sinensis* peel crude extract, essential oil and isolated compounds showed a difference in the inhibitory concentrations against test bacteria. The MIC value of the crude extract has been found to be 1.25mg/ml against *E.coli*, *K.pneumoniae and P. aeruginosa* and 2.5mg/ml against *S.aureus* which is similar result with Baba *et al*. (2018) report with MIC of 1.25mg/ml for *E. coli* and 2.5mg/ml for the *P. aeruginosa*. The essential oil showed MIC value 2.5mg/ml against *P. aeruginosa* and *S. aureus.* Compound **48** showed MIC value (0.25mg/ml, 0.5) against *S. typhimurium* and *E.coli* respectively. Compound **46** and Compound **47** had the same MIC value 0.25mg/ml and 0.5mg/ml against *P. aeruginosa* and *S.aureus* respectively. Compound **49** has showed MIC value of 1mg/ml against *P.aeruginosa.*

4.6.2. Minimum bactericidal concentration (MBC)

MBC values of *Citrus sinensis* peel crude extract (Figure 18) were the same for *P. aeruginosa, E.coli* and *K.pneumoniae* with 2.5mg/ml value and 5mg/ml for *S.aureus* which is a better finding compared to Baba *et al*. (2018), which reported MBC value of 10mg/ml, 5mg/ml against *S.aureus* and *E.coli* respectivly and no antibactericidal activity against *P. aeruginosa*. MBC value of the essential oil was similar against *P. aeruginosa* and *S.aureus* with 0.005mg/ml value. Compound **46**, Compound **47** and Compound **48** have similar MBC value (0.5mg/ml) against *P.aeruginosa* and *E. coli*. Compound **46** and Compound **48** showed MBC value (1.00 mg/ml) against *S.typhimurium* and *S.aureus*. Compound **49** showed MBC value (2.00mg/ml) against *P.aeruginosa* and *S.aureus*.

Table 8: comparison between MIC and MBC value of the synergistic antibacterial activity of the extracts

Bacterial strains	MIC and MBC mg/mL											
	Crude		Essential oil		Compound **46**		compound **47**		compound **48**		Compound **49**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	1.25	2.5							0.25	0.5		
S.aureus	2.5	5	2.5	5	0.5	1	0.5	1			1	2
K.pnumonia	1.25	2.5										
S.typhimurium									0.5	1		
P.arognosa	1.25	2.5	2.5	5	0.25	0.5	0.25	0.5			1	2
E.fecalis	1.25	2.5										

4.7. Antioxidant activity

Crude extract, oil extract and isolated compounds of *Citrus sinensis* peel were tested for their free radical scavenging activities in the DPPH scavenging assay by reacting with stable DPPH radicals. The decrease in absorbance at 517 nm was used to track the DPPH reduction. The DPPH radical scavenging activities (%) of ethanol extract, essential oil and isolated compounds were found to be 55.69% (Compound **46)**, 36.15% (Compound **47**) 20.7% (Compound **48**),23.8% (Compound **49**), 39.94% (Crude) and 19.84% (oil) at 100 and 1000 µg/mL for the compounds and crude, respectively. It was observed that the DPPH scavenging activity increased with increasing concentration of the samples in the assay. For the various concentrations, compound **46** exhibited highest percent inhibition of the DPPH as compared to the other extracts and isolated compounds. Ascorbic acid, positive control (µg/ml), showed maximum scavenging effect at very low and high concentration 78, 83, 91% at 25, 50 and 100 µg/mL.

Antioxidants have the capability to slow down or prevent the oxidation process of other molecules. In general when compared to standard ascorbic acid, the DPPH radical scavenging activity of *Citrus sinensis* peel extracts was found to be lower (Table 8-9), nevertheless, the activity displayed by the extract increased in a dose dependent which is in a good agreement with Suhartomi *et al*. (2019) investigation. In the present study the essential oil showed relatively low antioxidant activity than those reported data. This difference in the results is probably due to the methods used in extraction, the plant variety, and the methodology for the evaluation of the antioxidant activity. Singh *et al*. (2010) found that the antioxidant nature of the essential oil in terms of free radical scavenger may be due also to limonene which is mostly present in the oil. In a related study, Omodamiro and Umekwe (2013) reported the higher scavenging effect observed in peels extract is because of the higher concentration of flavonoids present in them.

Based on data in (Table 9) the lowest IC_{50} value was shown by ascorbic acid (0.016mg/ml), followed by Compound **46** (0.5 mg/ml), Compound **47** (0.212mg/ml), Compound **49**(50 mg/ml) and Compound **48** (2000 mg/ml).

Table 9: DPPH radical scavenging activities (%) of the isolated compounds

Conc (µg/mL)	Control	Compounds								+ve control	
		(Compound **49**)		(Compound **48**)		(Compound **47**)		(Compound **46**)		Ascorbic Acid	
		A	%RSA	A	%RSA	A	%RSA	A	%RSA	A	%RSA
100	1.029	0.784	23.81	0.657	36.15	0.816	20.7	0.702	55.69	0.085	91.74
50	1.029	0.804	21.87	0.736	28.474	0.834	18.95	0.631	49.95	0.168	83.67
25	1.029	0.831	19.24	0.744	27.7	0.839	18.46	0.572	31.2	0.22	78.62
12.5	1.029	0.873	15.16	0.778	24.39	0.883	14.19	0.667	25.56	0.67	34.89
IC50 (mg/mL)		50		0.212		2000		0.05		0.016	

Conc-Concentration, A- Absorbance, %RSA- Radical Scavenging Activity (%)

Table 10: DPPH radical scavenging activities (%) of Citrus sinensis peel crude extract

Conc (µg/ml)	Control	Ethanol Extract		Positive control Ascorbic Acid	
		A	%RSA	A	%RSA
1000	1.029	0.618	39.94	0.04	96.11
500	1.029	0.71	31	0.045	95.63
250	1.029	0.745	27.6	0.047	95.43
125	1.029	0.825	19.83	0.05	95.14
IC50		3251.785			

Conc-Concentration, A- Absorbance

Table 11: DPPH radical scavenging activities (%) of Essential oil extract

Conc (µg/ml)	Control	Essential oil		Positive control Ascorbic Acid	
		A	%RSA	A	%RSA
100	1.275	1.022	19.84	0.028	97.80392
50	1.275	1.095	14.12	0.028	97.80392
25	1.275	1.095	14.12	0.032	97.4902
12.5	1.275	1.101	13.65	0.035	97.2549
IC50		843.07			

4.8. *In-silico* Molecular docking

Two compounds were docked in this study against three organism protein targets proteins in order to predict their orientation and binding affinity at the active site of the receptor. For each compound nine poses were generated with different binding energy and RMSD value. But the dock pose with least binding energy and RMSD value has the highest affinity and considered as the best docked conformation (Azam & Abbasi, 2013). Therefore, from current two protein-ligand dockings the first poses of each compound were chosen for further study as best conformation.

4.8.1. Docking of against *E.coli DNA* gyrasee B enzyme.

4.8.1.1. Docking score

Compound 46 and **48** were docked to evaluate their binding affinity against *E.coli* gyrase B enzyme. Nine poses (mode) were generated with different binding energy and RMSD value ranging from -6.2 up to -4.9 Kcal/mol (Table 11). Maximum binding affinity was observed - 6.2 Kcal.mol and - 6.9 Kcal/mol for compound **46** and **48**, respectively, compared to gentamicine with binding affinity ranging from -7.3 to-6.2 Kcal/mol

Table 12: Compound **46**, **48** and gentamicin binding energy with *E. coli* DNA gyarase B

	Compound 46			**Compound 48**			**Gentamicine**		
Mode	Affinity	Distance from best mode		Affinity	Distance from best mode		Affinity	Distance from best mode	
	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B
1	-6.2	0	0	-6.9	0	0	-7.3	0	0
2	-5.4	1.645	7.106	-6.4	2.267	4.893	-7.1	3.357	6.719
3	-5.3	2.539	4.133	-6.3	1.725	4.034	-7.1	3.181	6.649
4	-5.2	2.587	3.405	-6.3	4.353	10.14	-6.6	1.802	3.323
5	-5.2	2.627	3.613	-6.2	18.718	23.273	-6.3	1.731	2.227
6	-5.2	2.723	5.05	-6.2	5.336	8.281	-6.3	3.296	5.424
7	-5	2.086	3.216	-6.2	6.23	10.467	-6.3	2.892	6.142
8	-5	1.529	-6.407	-6.1	2.407	4.628	-6.2	2.172	5.927
9	-4.9	2.66	7.675	-6.1	18.452	21.849	-6.2	3.219	5.828

4.8.1.2. Interactions between Compounds 46 and 48 against *E. coli* DNA gyrase B enzyme

Compound 46 (Figure 18) interacted with A: VAL-71, A: VAL-167, A: THR-165, A: ASP-73, A: ASN-46, A: PRO-79, A: ASP-49, and A: ARG-76 by Vander Waals interaction. It also has polar interaction with A: GLU-50 and A: GLY-77 by forming conventional carbon hydrogen bond respectively. Hydrophobic interaction formed with A: ALA-47, A: VAL-43, A: ILE-97 and A: ILE-94 by forming alkyl and pi-alkyl bonds.

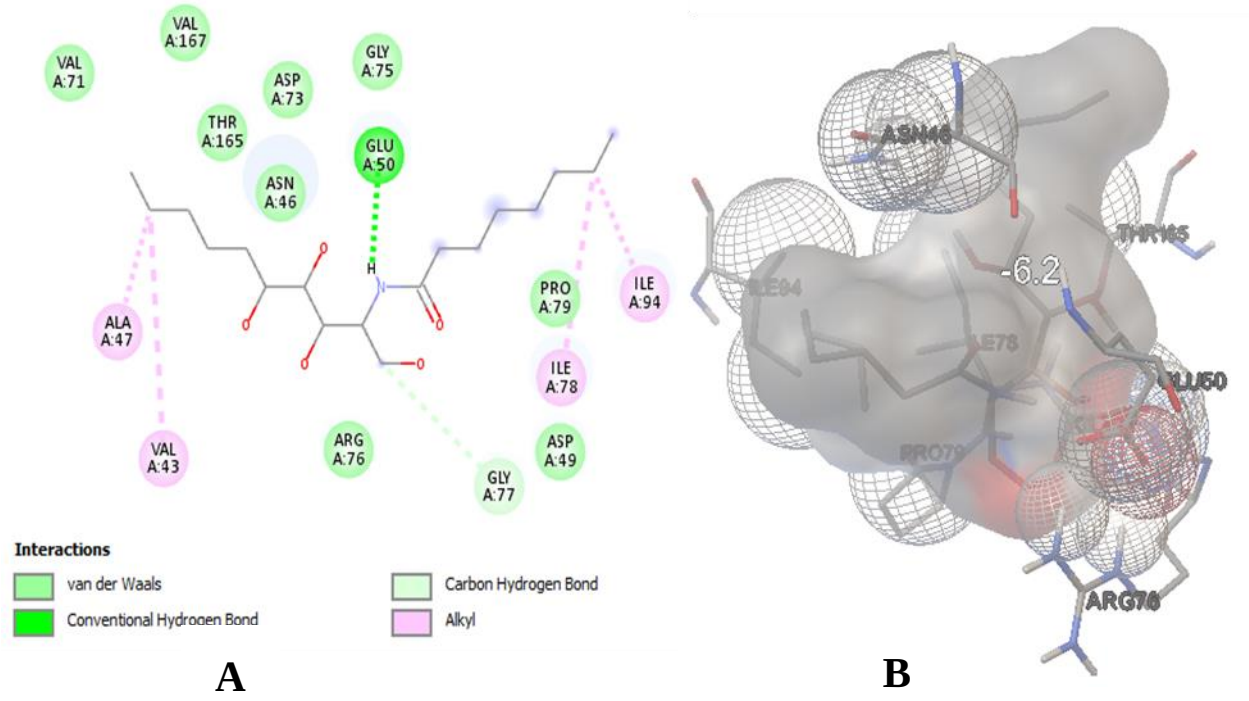


Figure 16: Interaction of Compound **46** on *E.coli* DNA gyrase ((A) 2D interaction, (B) 3D interaction)

Compound 48 (Figure 19) interacted with A: ASP-73, A: GLU-50 by forming hydrogen bond. And shown Vander Waals interaction with A: VAL-93, A: VAL-120, A: VAL-97, A: LEU-98 A: SER-121, A: VAL-188, A: GLY-119, A:-117, A: GLY-77, A: THR-165, A: ALA-47, A: PRO-79 and A: ASN-46. Compound also has Electro static and hydrophobic interaction with A: ARG-76 and A: ALA-53, and A: ILE-94 by pi-cation, alkyl and pi-alkyl bonds respectively.

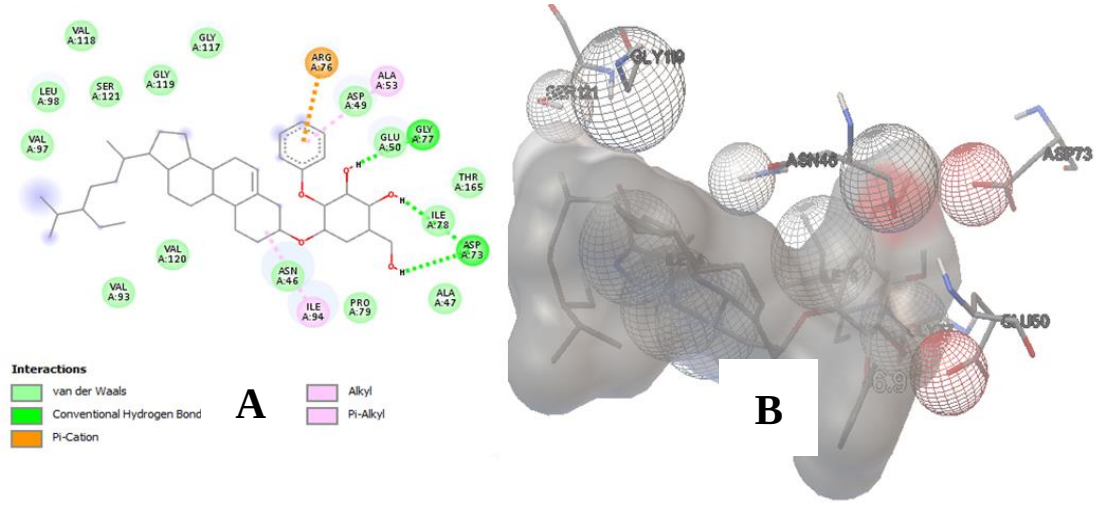


Figure 17: Interaction of Compound **48** on *E.coli* DNA gyrase ((A) 2D interactions (B) 3D interactions)

4.8.2. Docking against Human Peroxiredoxin 5

4.8.2.1. Docking score

Compound 46 and **48** are docked against human Peroxiredoxin 5. For both compound the nine poses (modes) were generated as result with different binding energy value ranging from -5 to -4.6 Kcal/mol for Compound **46** and -7.1 to -6.4 kcal/mol for Compound **48**. But the first one was considered as best conformation/pose for further interaction analysis compared to Ascorbic acid with binding energy value ranging from -5.6 to -4.5 kcal/mol.

Table 13: Different binding energy and RMSD value of compound **46**, **48** and Ascorbic acid

Compound 46				**Compound 48**			**Ascorbic acid**		
Mode	Affinity	Distance from best mode		Affinity	Distance from best mode		Affinity	Distance from best mode	
	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B
1	-5	0	0	-7.1	0	0	-5.6	0	0
2	-4.9	1.423	2.183	-6.9	0.787	11.716	-5.2	1.715	3.599
3	-4.9	8.447	12.385	-6.8	4.232	10.912	-5.1	1.401	2.855
4	-4.9	15.793	19.151	-6.8	5.08	11.208	-5	1.733	3.275
5	-4.7	3.165	9.901	-6.7	3.16	5.089	-4.7	1.921	3.476
6	-4.7	3.293	8.73	-6.6	6.623	12.246	-4.7	2.142	2.880
7	-4.7	2.54	8.529	-6.6	10.802	17.623	-4.7	1.531	2.995
8	-4.7	7.634	9.793	-6.6	4.381	10.328	-4.5	21.034	21.731
9	-4.6	7.718	10.734	-6.4	6.703	10.664	-4.5	17.744	19.150

4.8.2.2. Interactions between compound 46 and 48 with that of human peroxiredoxin 5

Compound 46 (Figure 20) interacted with A: ASN-76, and 78 A: GLU-50 by Vander Waals interaction. With A: VAL-75, A: ARG-124, A: ASN-122 and A: ASP-77 by hydrogen bonds, electrostatic and hydrophobic interaction formed with A: PHE-15, A: VAL-80, A: PRO-19 and A: PRO-100 by pi-sigma, alkyl and pi-alkyl bonds.

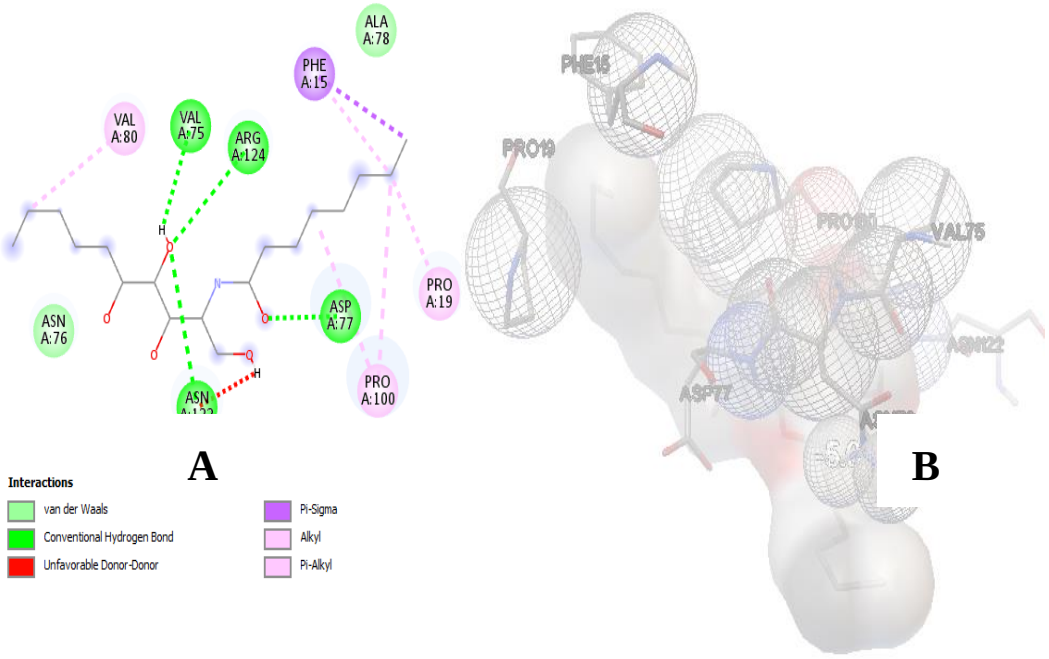


Figure 18: Interaction of compound 46 on peroxiredoxin 5 ((A) 2D interactions (B) 3D interactions)

Compound 48 (Figure 21) interacted with A: THR-147, A: LEU-116, A: ARG-127, GLY A: 46, A: THR-44 and A: ASN-76 and A: ALA-42 by Vander Waals interaction. Compound also has unfavorable, electro static and hydrophobic interaction with A: ARG-124, A: A-43, A: VAL-80, A: PRO-40, A: PRO-45 an A: VAL-80 by pi-pi sigma, alkyl and pi-alkyl bonds

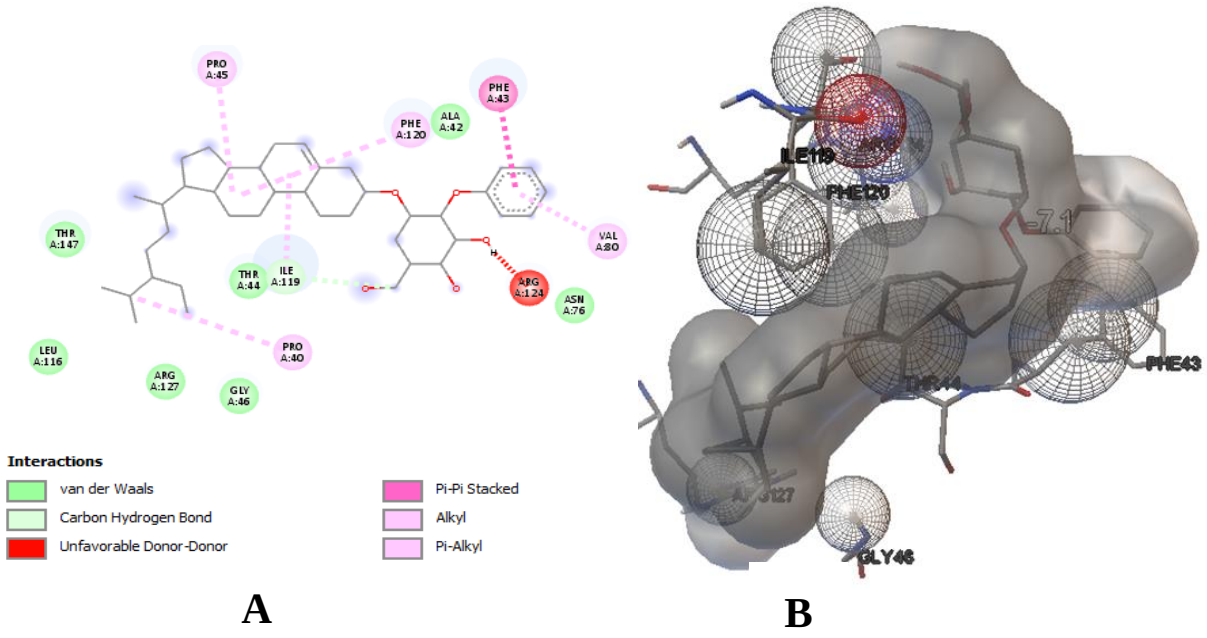


Figure 19: Interaction of compound 48 on peroxiredoxin 5 ((A) 2D interactions (B) 3D interactions)

4.8.3. Docking against of *S. aureus* Pyruvate Kinase

4.8.3.1. Docking score

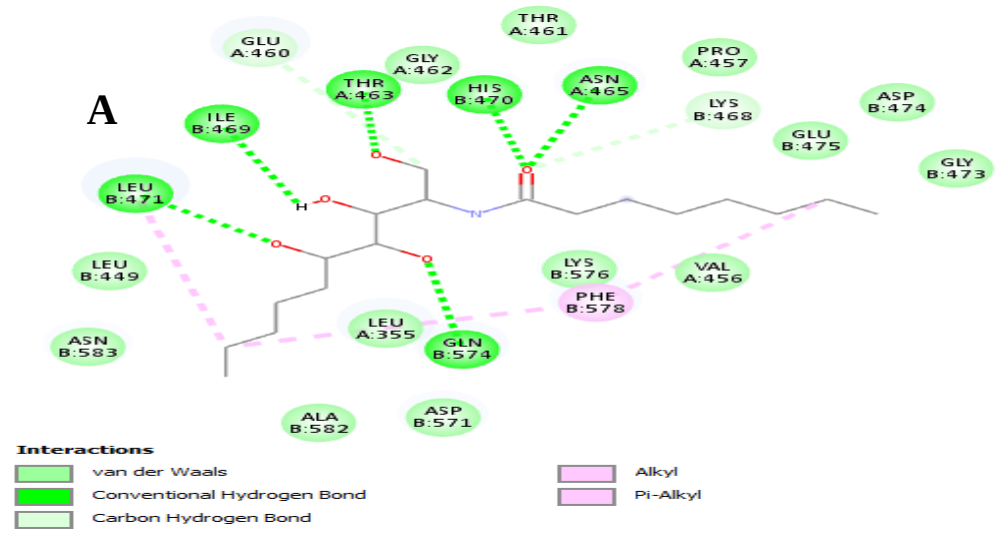
Compounds 46 and **48** are docked against *S. aureus* Pyruvate Kinase enzyme. Nine modes were generated for each compound has minimum binding energy ranging from -6.6 to -5.2 Kcal/mol of compound **46** and -8.2 to -7.7 kcal/mol. On comparative basis Compound **48** revealed minimum binding energy of, -8.2 kcal/mol compared to gentamicine with binding energy ranging from -7.7 to -7.2 kcal/mol (Table 13).

Table 14: Different binding energy and RMSD value of compound **46**, **48** and gentamicine

Compound 46				**Compound 48**			**Gentamicine**		
Mode	Affinity	Distance from best mode		Affinity	Distance from best mode		Affinity	Distance from best mode	
	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B	(kcal/mol)	RMSD L.B	RMSD U.B
1	-6.6	0	0	-8.2	0	0	-7.7	0	0
2	-6.2	2.508	5.622	-8.1	4.26	9.621	-7.6	3.115	5.737
3	-5.7	18.238	21.436	-8.1	38.581	42.21	-7.5	37.866	43.513
4	-5.3	18.934	21.468	-8.1	39.466	43.018	-7.5	2.621	7.179
5	-5.3	25.917	27.584	-8	37.058	40.389	-7.5	37.862	43.204
6	-5.3	15.731	18.067	-7.9	6.331	9.741	-7.4	2.828	5.799
7	-5.3	29.891	32.43	-7.8	4.654	10.549	-7.4	2.702	6.733
8	-5.3	30.741	33.016	-7.8	36.785	39.667	-7.4	2.665	4.271
9	-5.2	35.17	37.833	-7.7	4.894	6.089	-7.2	3.360	6.352

4.8.3.2. Interactions of compound 46 and 48 with *S. aureus* Pyruvate Kinase Enzyme and

Compound 46 (Figure 22) Showed Vander Waals interaction with A: GLU-460, A: GLY-462, A: THR-461, A: PRO-457, A: VAL-456, A: LEU-355, B: ASP-571, B:-ALA-582, B: ASN-583, B: LEU-449, B: GLU-475, A: GLY-773 and B: LYS-576. Compound has multiple hydrogen bonds with B: GLN-574, B: LEU-471, B: ILE-469, A: THR-463, B: HIS-470 and A: ASN-465 by conventional hydrogen bonds and with B: LYS-468 by carbon hydrogen bonds. Hydrophobic interaction formed B: PHE-578 and B: LEU -471 by alkyl and pi-alkyl bonds respectively.



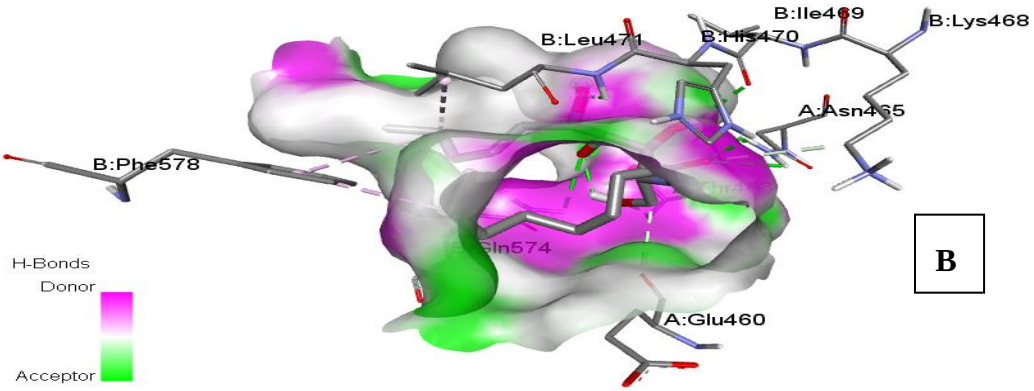
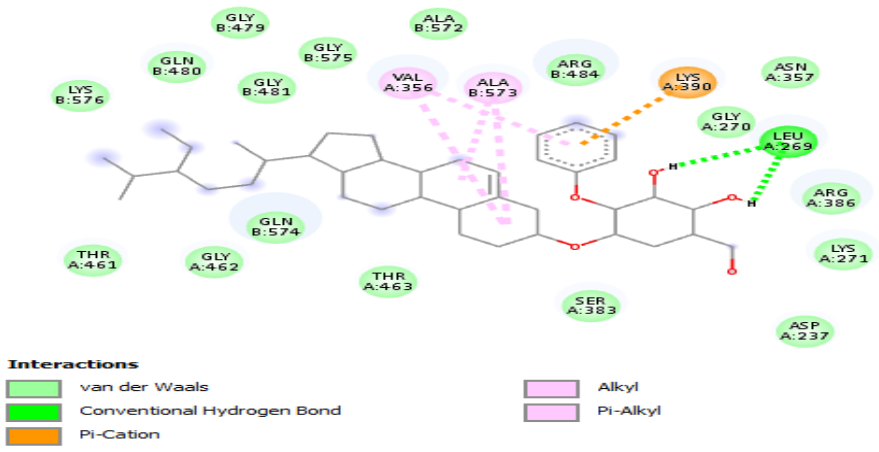


Figure 20: Interaction of Compound **46** on *S. aureus* Pyruvate Kinase Enzyme ((A) 2D interactions (B) 3D interactions)

Compound 48 (Figure 23) interacted with A: THR-461, A: GLY-462, A: THR-463, A: SER-383, A: ASP-237, A: LYS-271, A: ARG-386, A: ASN-357, A: GLY-270, B: ARG-484, B: GLY-575, B: GLY 481, B: GLY 479, B: GLN-480 and B: LYS-576 by Vander Waals interaction. A: LEU-269 showed polar interaction by forming conventional hydrogen bond. Electrostatic and hydrophobic interaction formed with A: LYS-390, B: ALA-573 and A: val-356 by pi-cation charge, alkyl and pi-alkyle bonds respectively.



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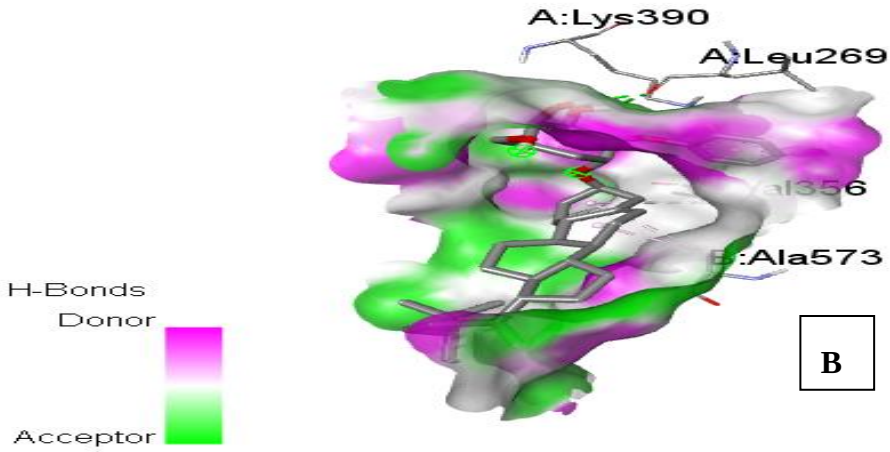

Figure 21: Interaction of compound **48** on *S. aureus* Pyruvate Kinase Enzyme ((A) 2D interactions (B) 3D interactions).

5. CONCLUSION

In the present study, extraction of *Citrus sinensis* with ethanol (99.9%) by maceration (300g) and ultrasonic extraction (150g) resulted yield of 7% (w/w) and 7.5% (w/w) crude extracts, respectively. Phytochemical screening tests conducted on *Citrus sinensis* peel crude ethanolic extract showed the presence of alkaloid, flavonoid, tannins, steroids, phenol and terpinoid. Silica gel column chromatographic separation of the ethanoilc extract furnished four compounds identified as *N*-(1,3,4,5-tetrahydroxydecan-2-y1)octanamide (**46**), decanoic acid (**47**), glycosylated steroid (**48**) and (z)-ethyl tetradec-7-enoate (**49**).

The chemical components of *Citrus sinensis* peel essential oil were analyzed by GC-MS revealed presence of a total of 7 chemical components, accounting for 99.84 % of the total compositions among which Limonene (87.5%) was the major constituent followed by 1,6-Octadien-3-ol,3,7-dimethyl- (4.3%), Isopulegol (2.46%), pinene (1.9%), and trance-p-Mentha-2,8-dienol (1.45%), respectively.

All the crude, essential oil and isolated compounds of *Citrus sinensis* peel extract had promising Antibacterial activity against all the tested strains; *S. aureus* (ATCC 25923),*E. fecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853),*E.coli* (ATCC 25922), *S. Thyphimurium* (ATCC 13311) *and K. Pnumonia* (ATCC 700603).Essential oil showed a good zone of inhibition with 95mg/mL against *S. aureus* (14.33±1.52 mm) and *E. faecalis* (12.67±0.58 mm) compared to gentamicin (18.67±0.58 mm and 13.00±1.73 mm, respectively) at concentration of 10mg/mL. Compound **49** showed highest activities against *S.typhimirium* (15.33±2.89) compared to gentamicin (18.67±0.58 and 18.00±1.00) with 6mg/mL.

Thus, the ethanol extract, essential oils and isolated compounds of *Citrus sinensis* peel had promising antibacterial activity against all the tested strains with special emphasis to *S. aureus* and *E. faecalis strains*. The findings of this study support the traditional uses of the Citrus peels for the treatment of infectious diseases especially Gram-positive strains.

The radical scavenging activities of *Citrus sinensis* peel ethanol extract, essential oil and isolated compounds were 39.94% (crude), 19.84% (oil), 55.69% (compound **46**), 36.15% (compound **47**), 20.7% (compound **48**) and 23.81% (compound **49**). DPPH radical scavenging activity revealed that compound **46** showed IC_{50} value of 0.05 mg/mL

compared to ascorbic acid 0.016 mg/mL indicating the potential of the plant as herbal remedies.

Compound **46** and **48** were docked against three organism protein targets protein in order to predict their orientation and binding affinity at the active site of the receptor. compounds **48** had the highest and best scoring poses (lowest energy) against the *E. coli* gyrase B enzyme (-6.9 Kcal.mol), Human Peroxiredoxin 5 (-7.1 Kcal.mol), and *S. aureus* Pyruvate Kinase (-8.2 Kcal.mol).

The finding of pharmacologically important secondary metabolites, biological evaluation and molecular docking from *Citrus sinensis* peel extracts should bring the attention of experts to look more on the medicinal importance of the plant.

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6. RECOMMENDATIONS

The peels of fruits of *Citrus sinensis* are generally treated as wastes and our study suggest that this underutilized resource can serve as an effective and economical antimicrobial agent. In future, *In-vivo* studies must be undertaken to determine their efficacy in whole-organism systems, including toxicity studies as well as a study of their impacts on beneficial normal microbiota, the margin of safety, and the benefits of generating commercially viable items especially hand disinfectants from the essential oils of the peels. Additional *in vitro* antibacterial studies are also recommended other resistant clinical isolate pathogenic bacteria such as methicillin-resistant *S. aureus*, vancomycin-resistant Enterococcus and multidrug-resistant *Mycobacterium tuberculosis* and fungal test organisms. Future studies need to be done on a combination of the compounds isolated from the peel extract as well as essential oils with standard first line drugs *in vitro* and *in vivo* to examine the potential of their antibacterial and antioxidant activity. Further feasibility studies of industrial preparation of hand disinfectants and herbal hand sanitizers formulations are recommended from the essential oils of the peel extracts in both alcohol mixed and alcohol free formulations.

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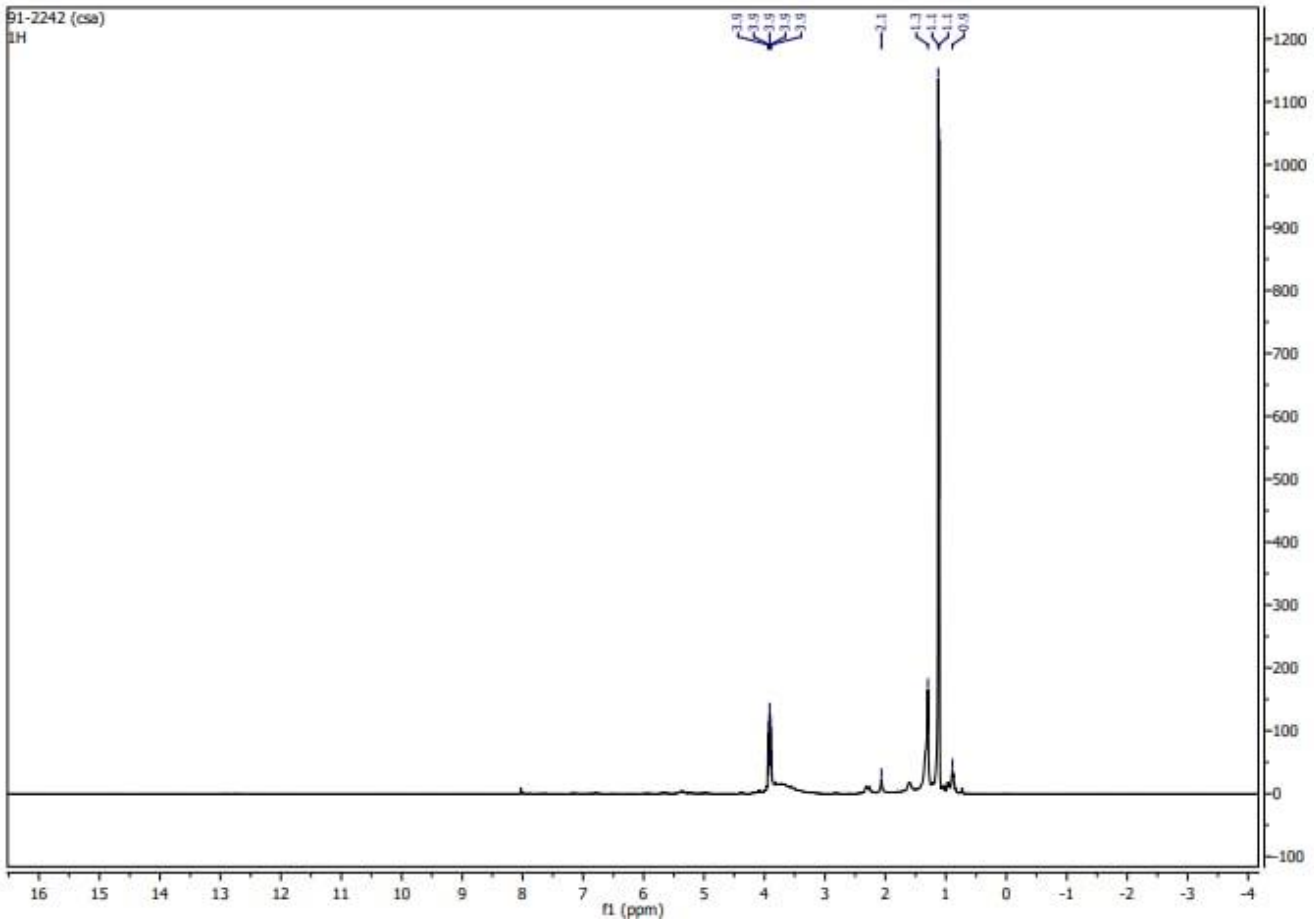
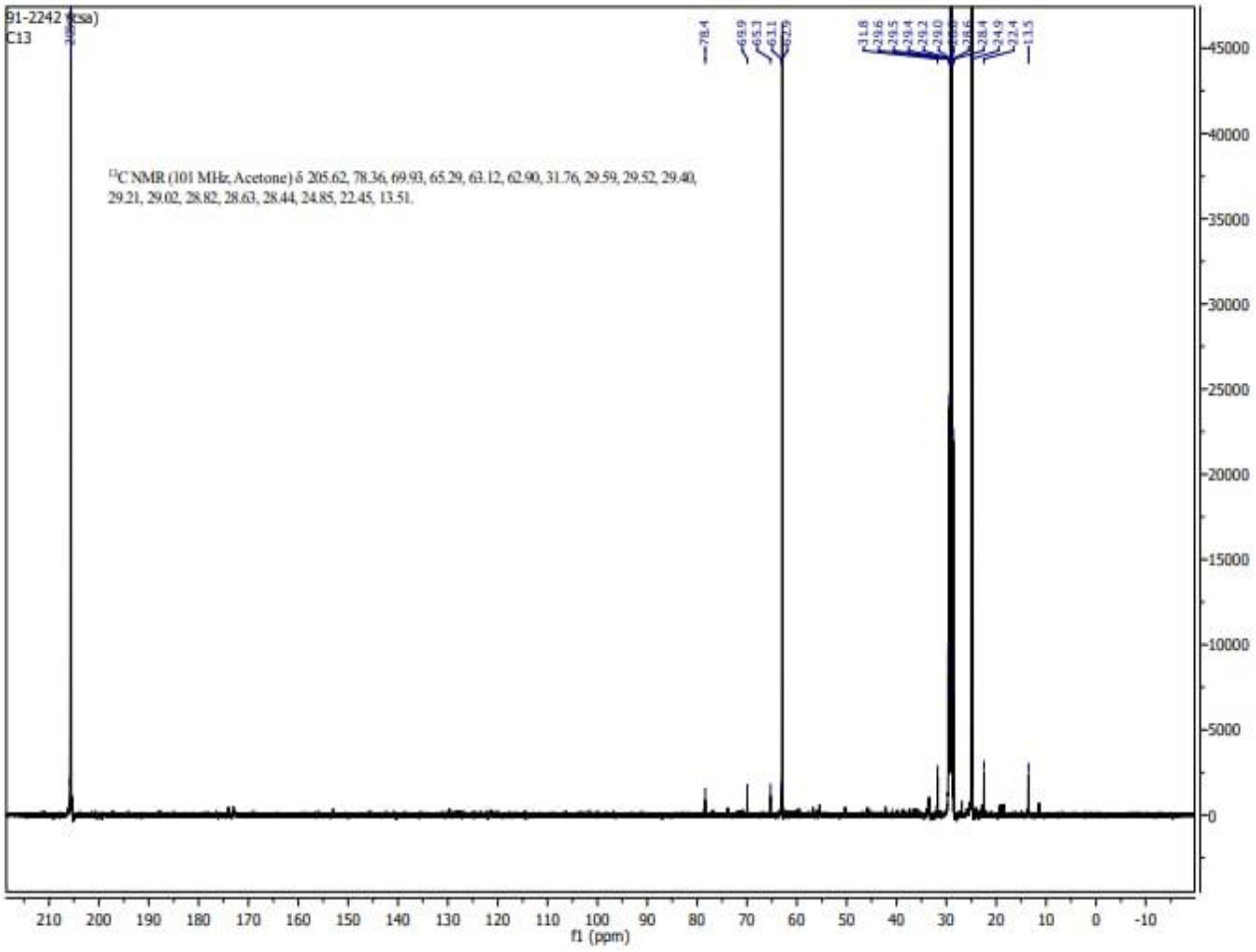
APPENDIX

Appendix1. Spectra of compound 46

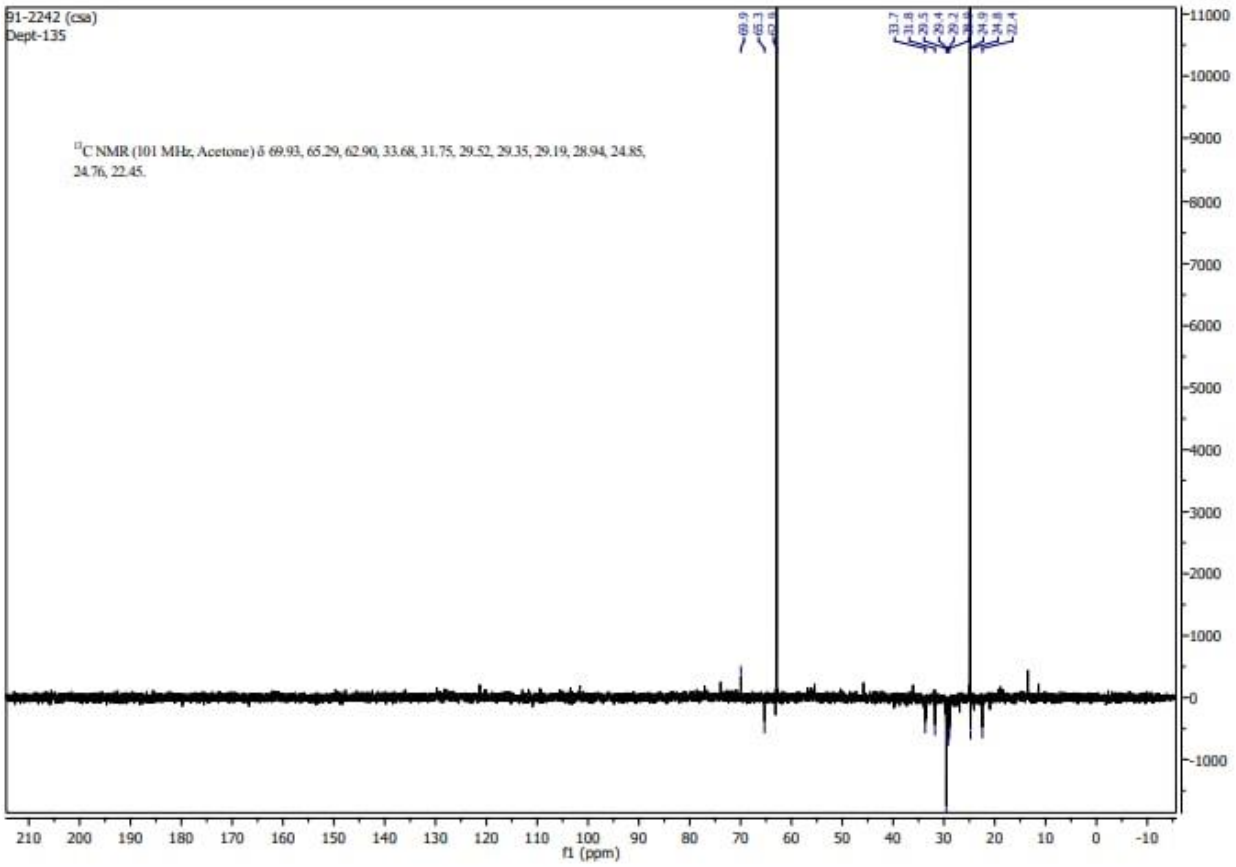
Appendix1.1 The ^{1}H-NMR spectrum of compound 46



Appendix1.2 The ^{13}C-NMR spectrum of compound 46

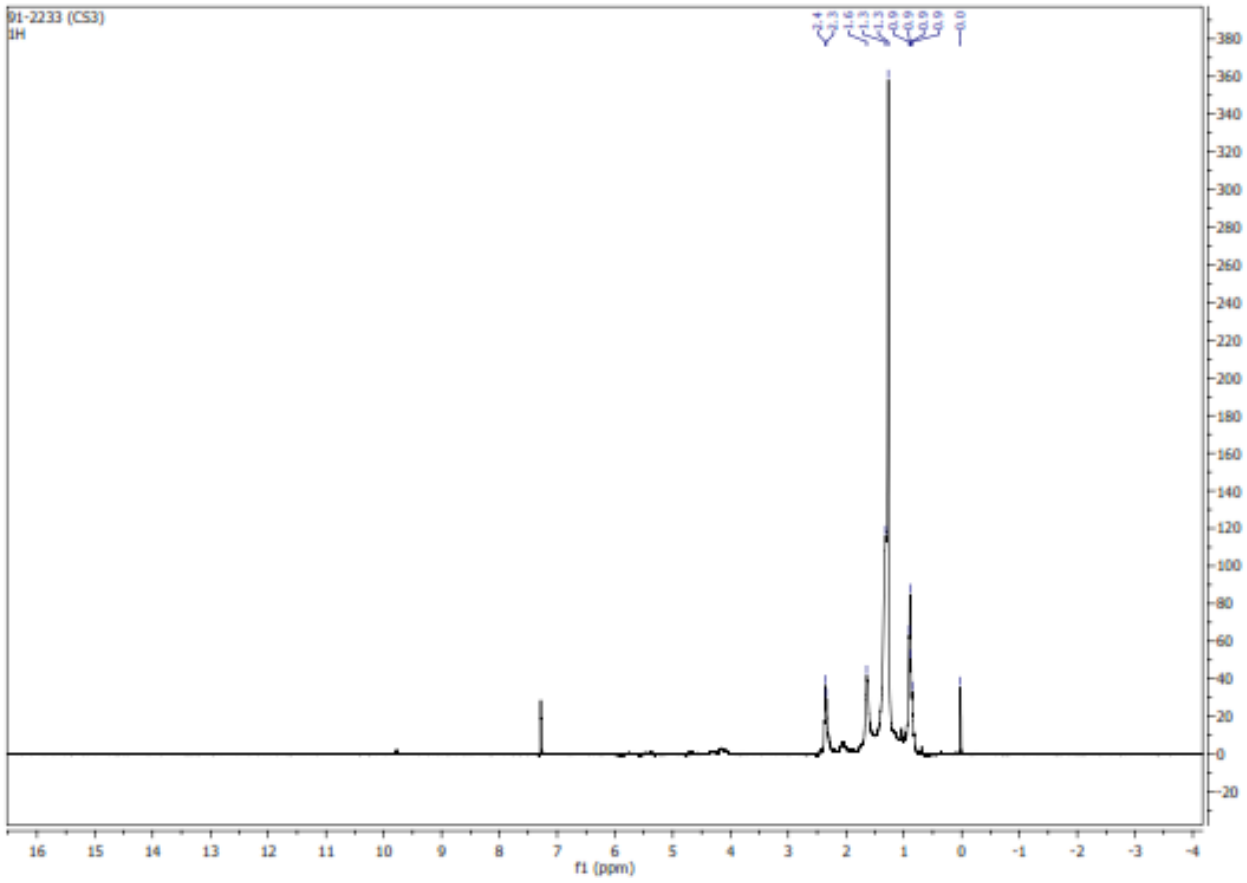


Appendix1.3 The DEPT-135spectrum of Compound 46

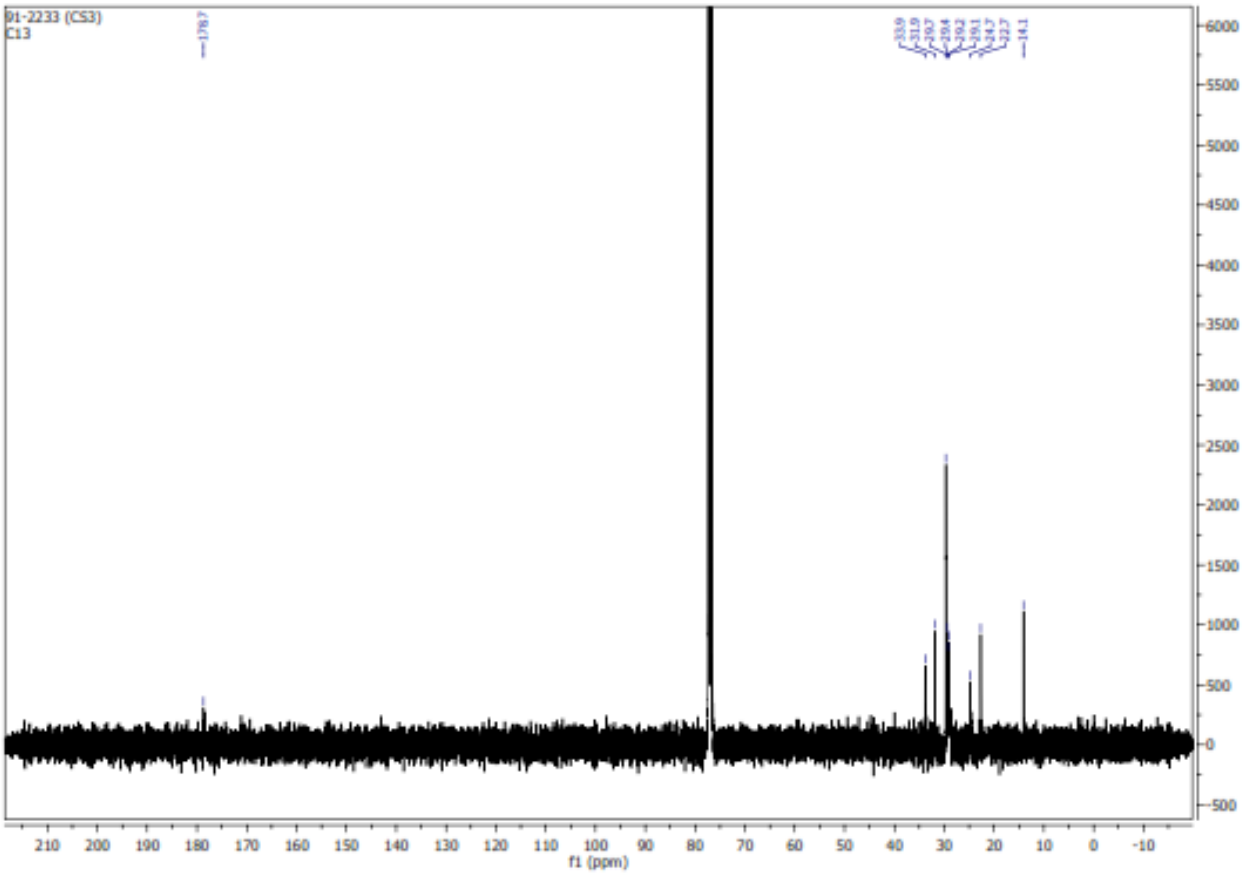


Appendix 2 the spectrum of compound 47

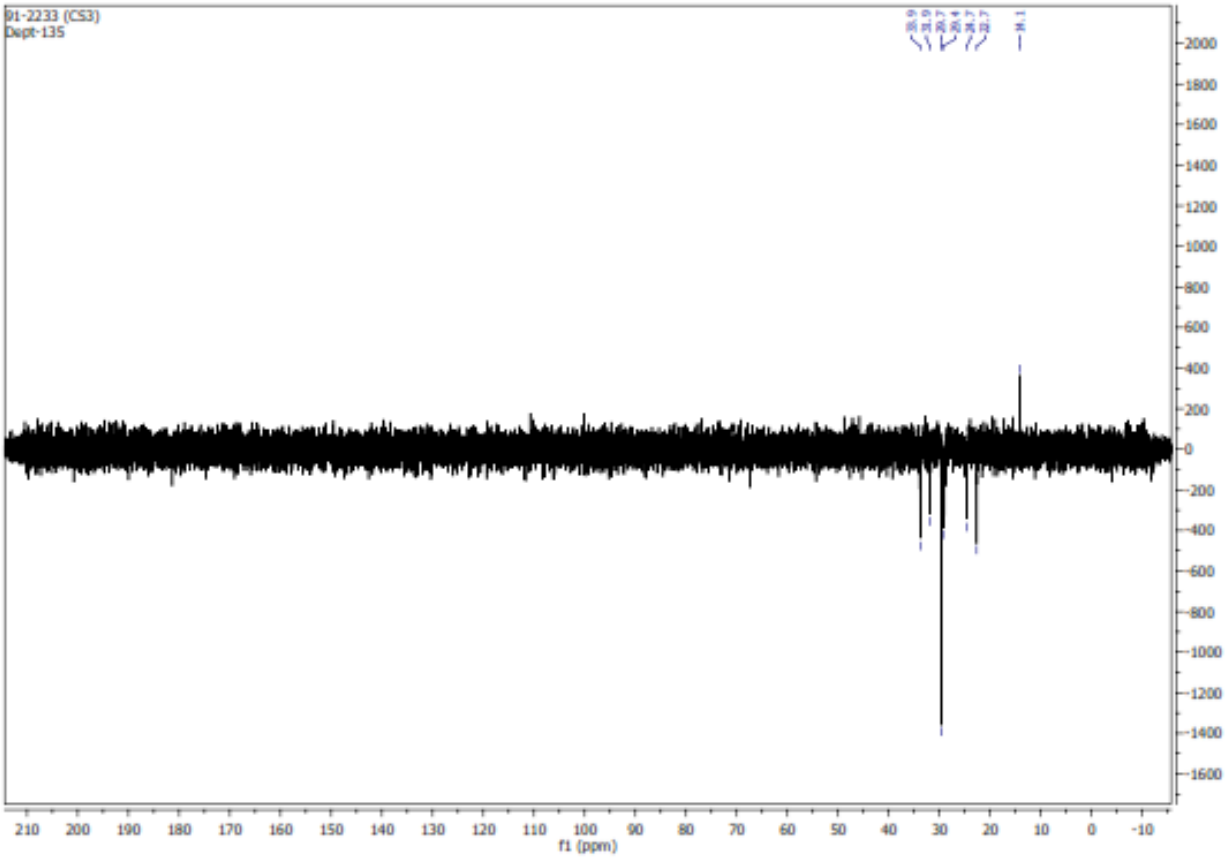
Appendix2.1 the ^{1}H-NMR spectrum of compound 47



Appendix2.2 The ^{13}C-NMR spectrum of Compound 47

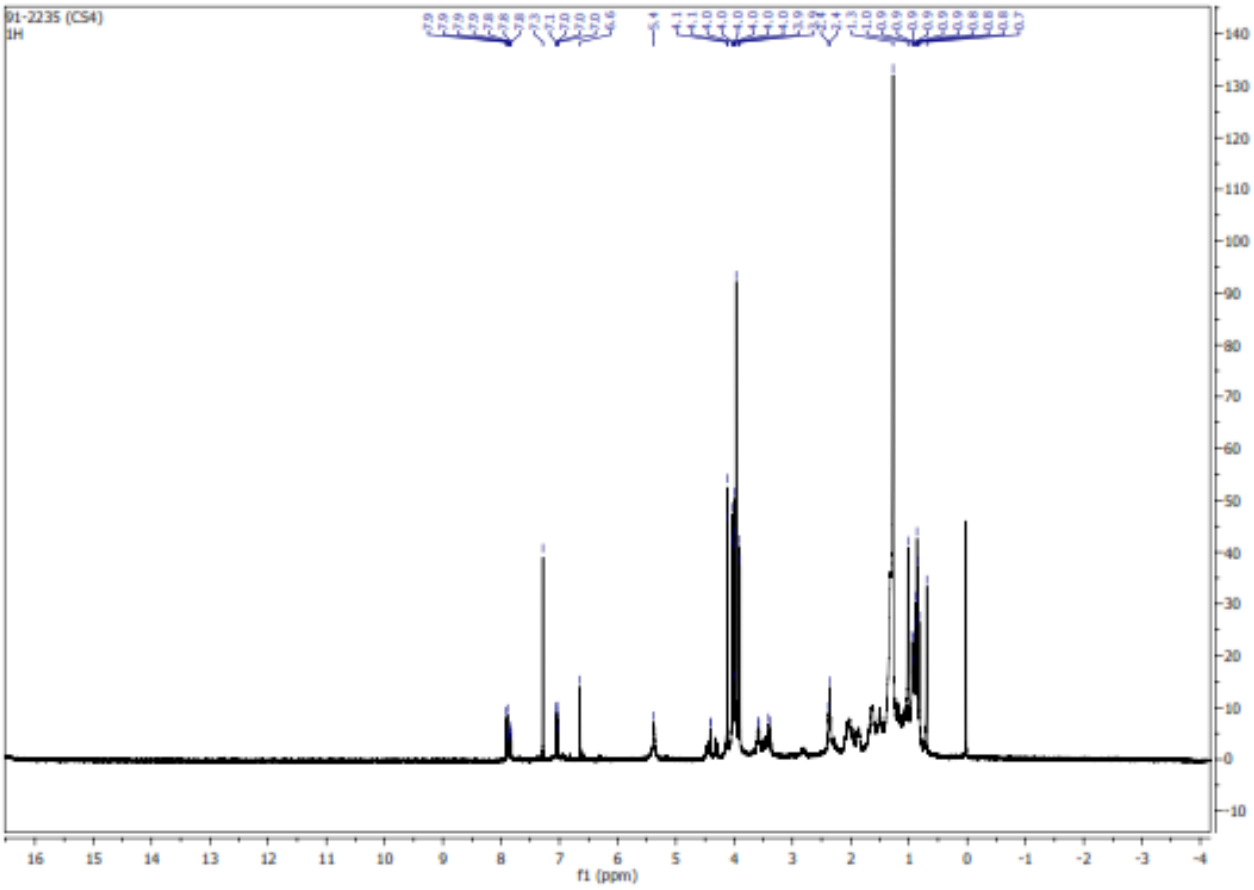


Appendix2.3 The DEPT-135 spectrum of Compound 47

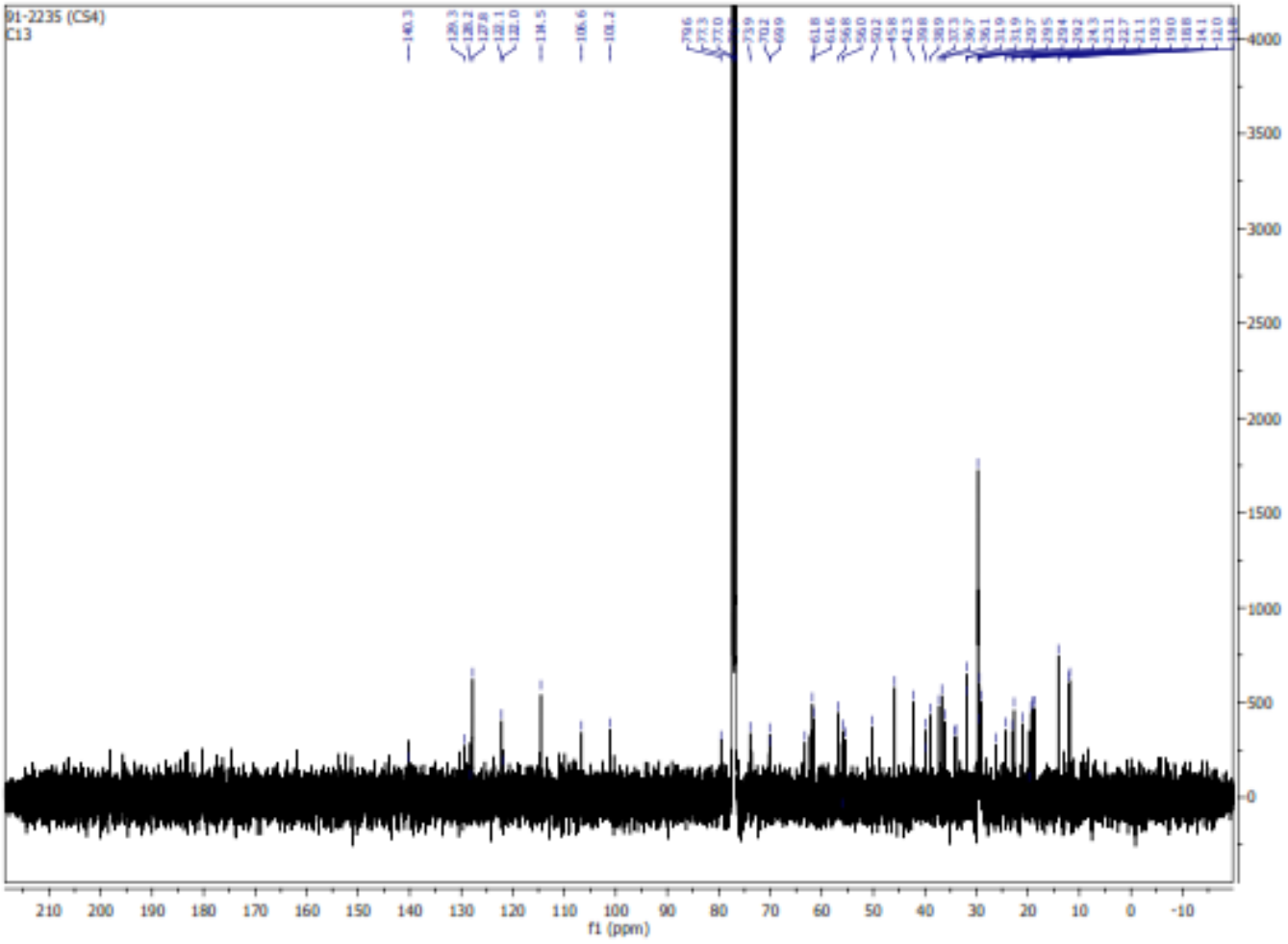


Appendix3 the spectrum of compound 48

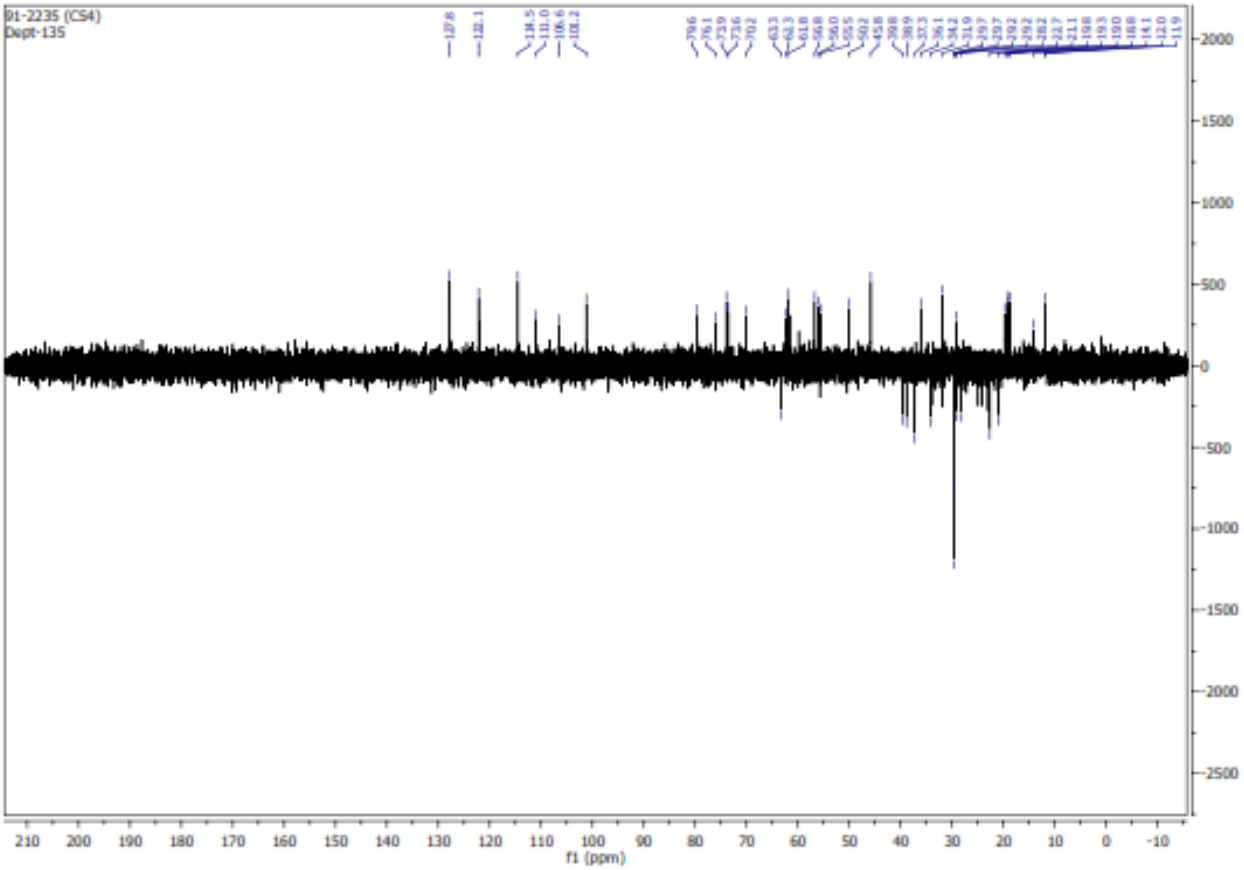
Appendix3.1 The ^{1}H-NMR spectrum of compound 48



Appendix3.2 The ^{13}C-NMR spectrum of compound 48

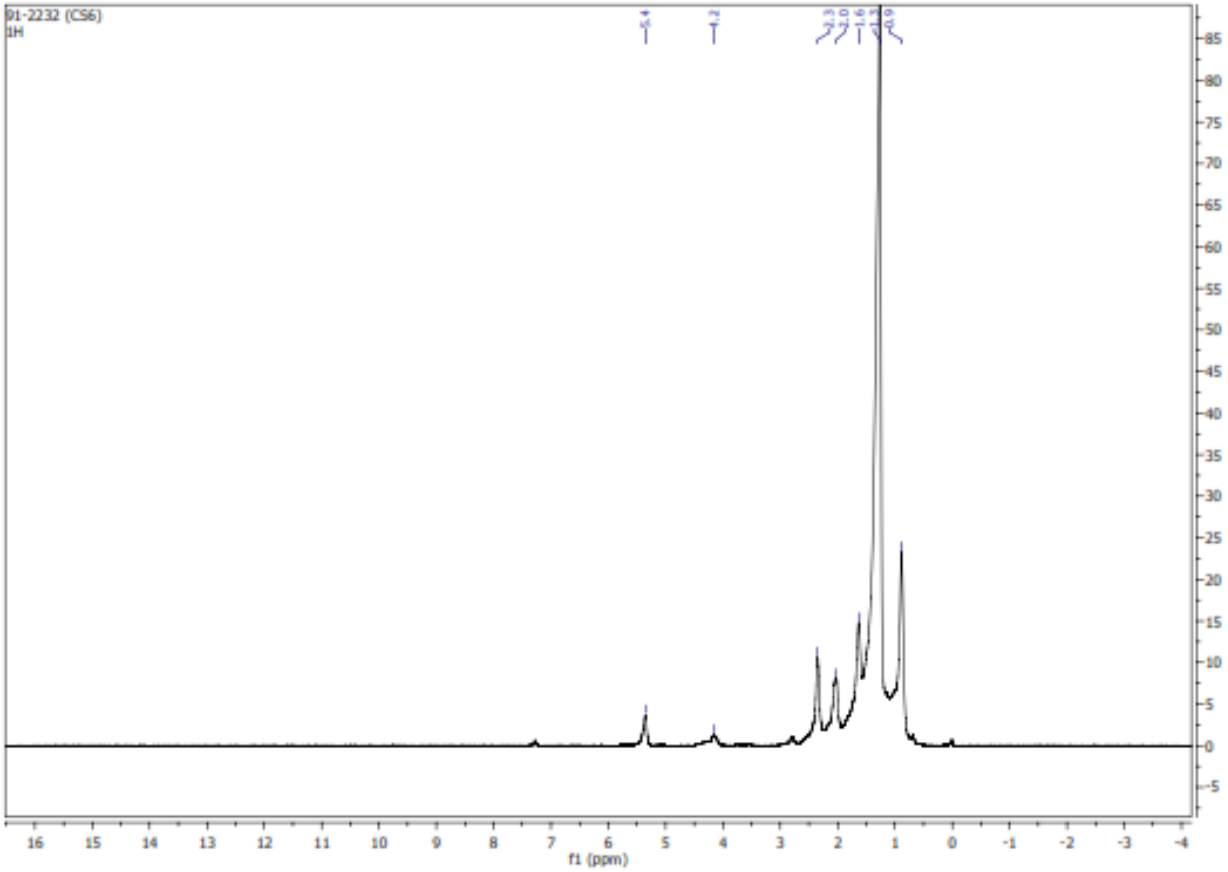


Appendix3.3 The DEPT-135 spectrum of compound 48

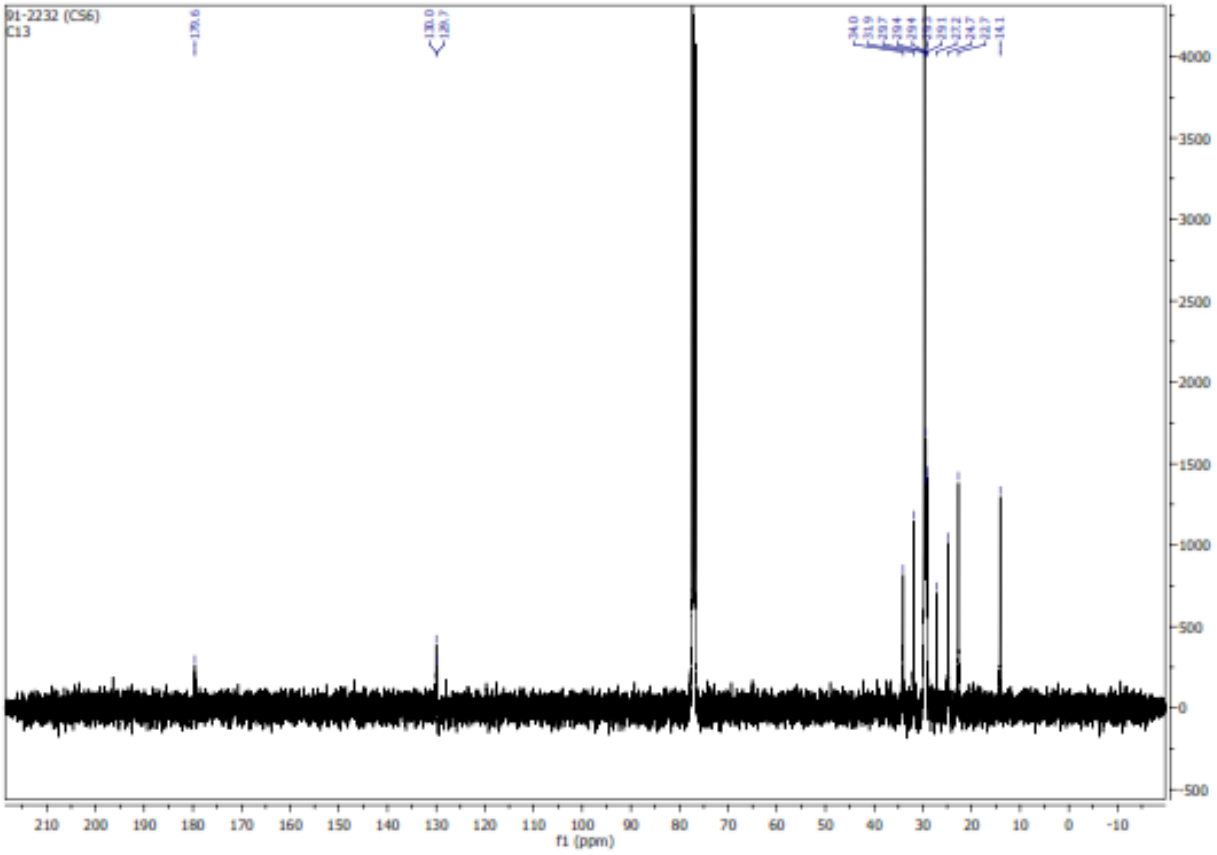


Appendix4 The spectrum of compound 49

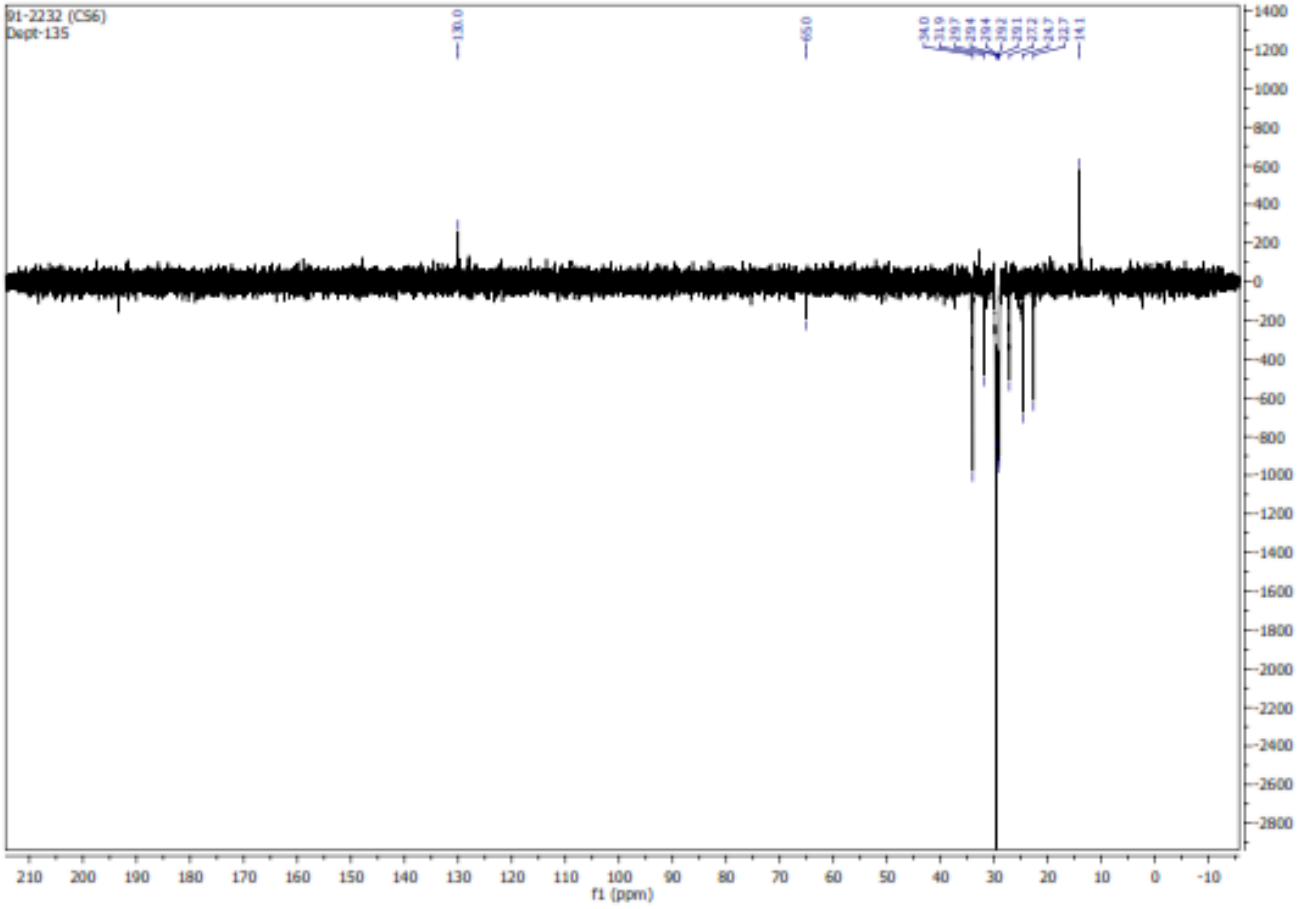
Appendix4.1 The[1] H-NMR spectrum of compound 49



Appendix4.2 The ^{13}C-NMR spectrum of compound 49

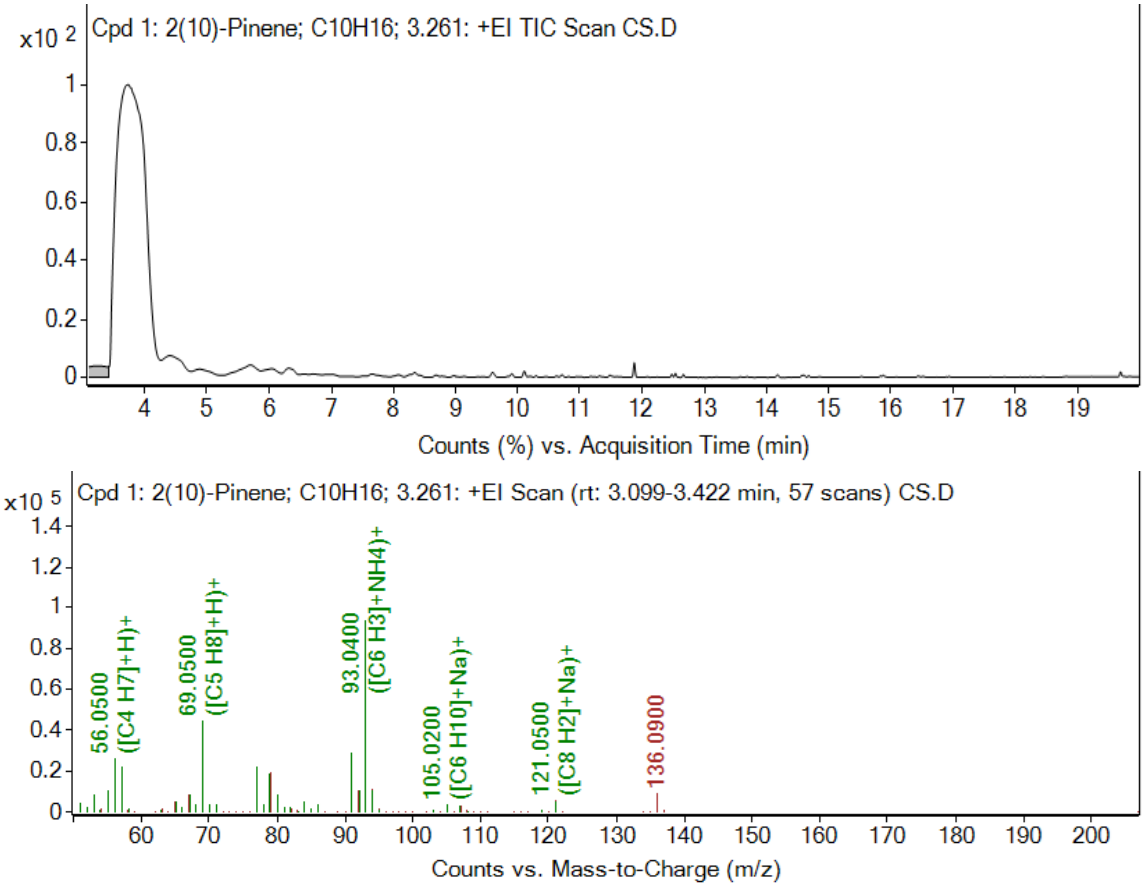


Appendix4.3 The DEPT-135 spectrum of compound 49

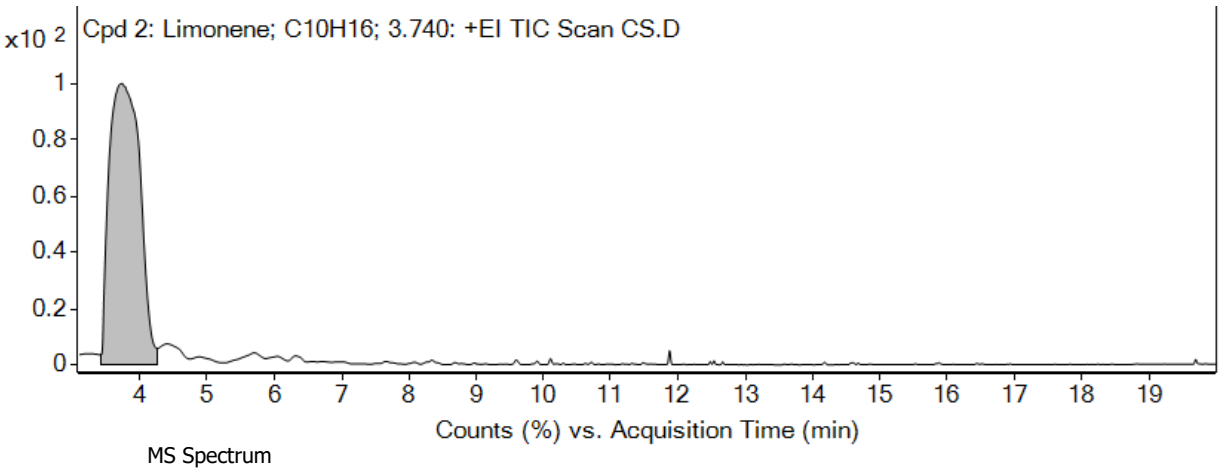


Appendix5. GC-MS chromatogram of the essential oil components

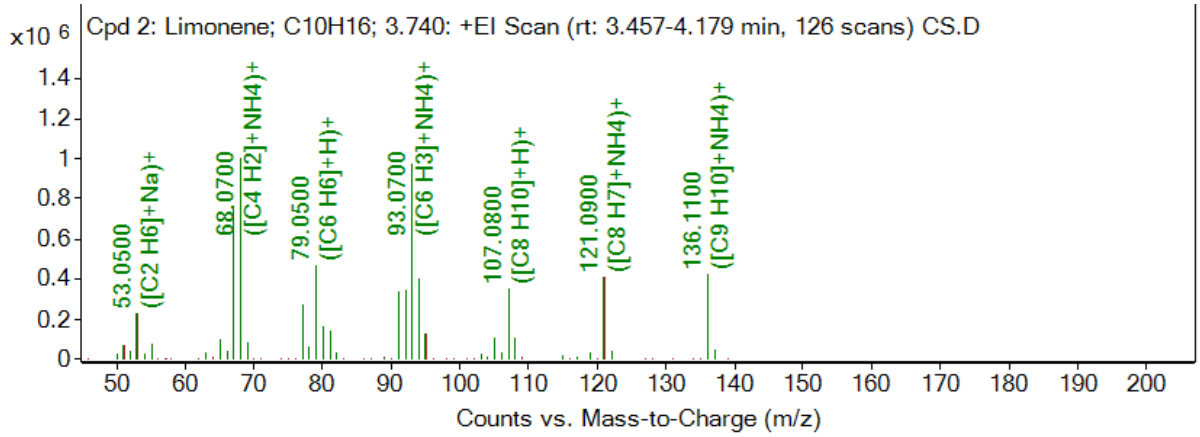
Appendix5.1 GC-MS chromatogram of 2(10)-pinene



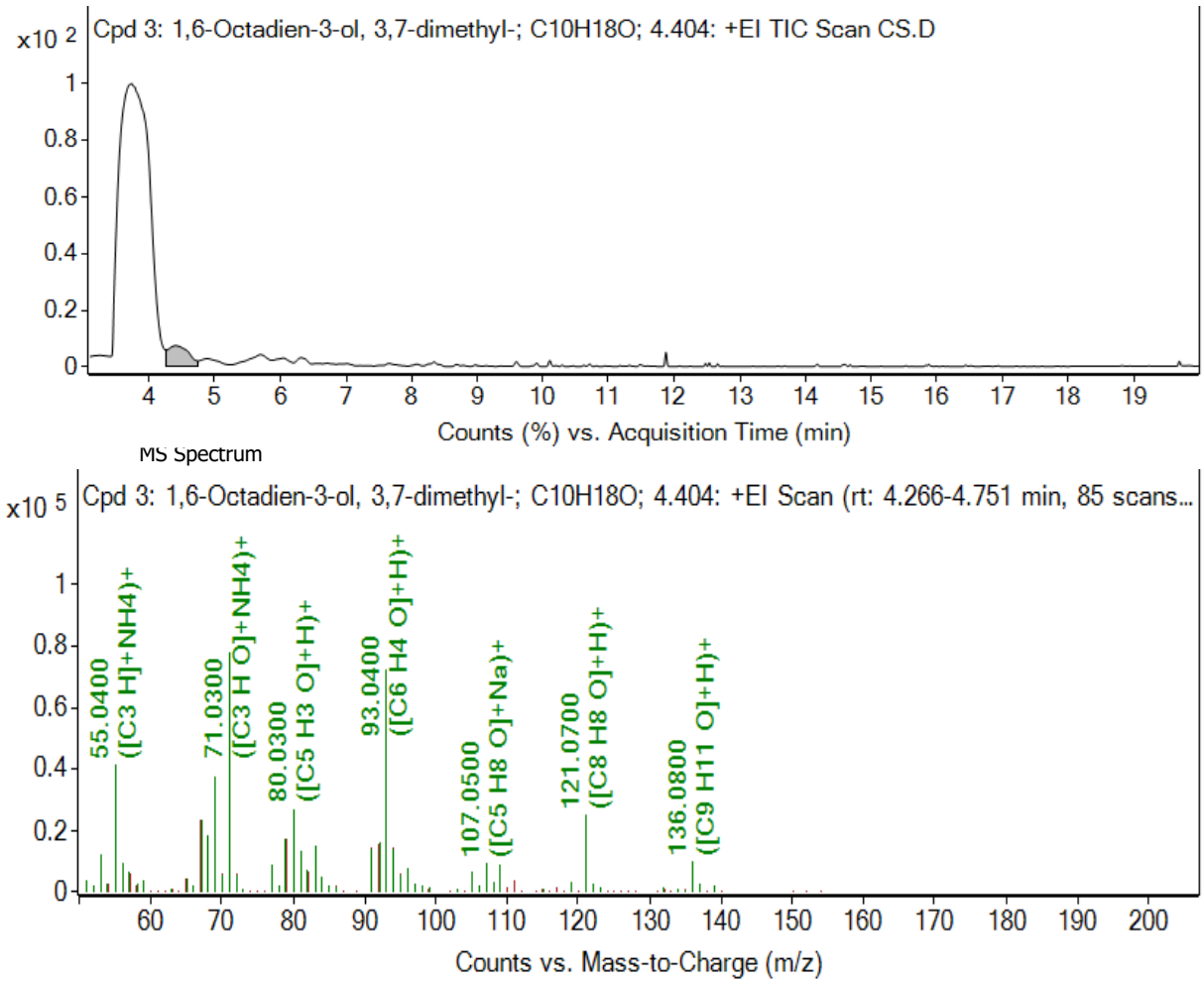
Appendix5.2 GC-MS chromatogram of Limonene



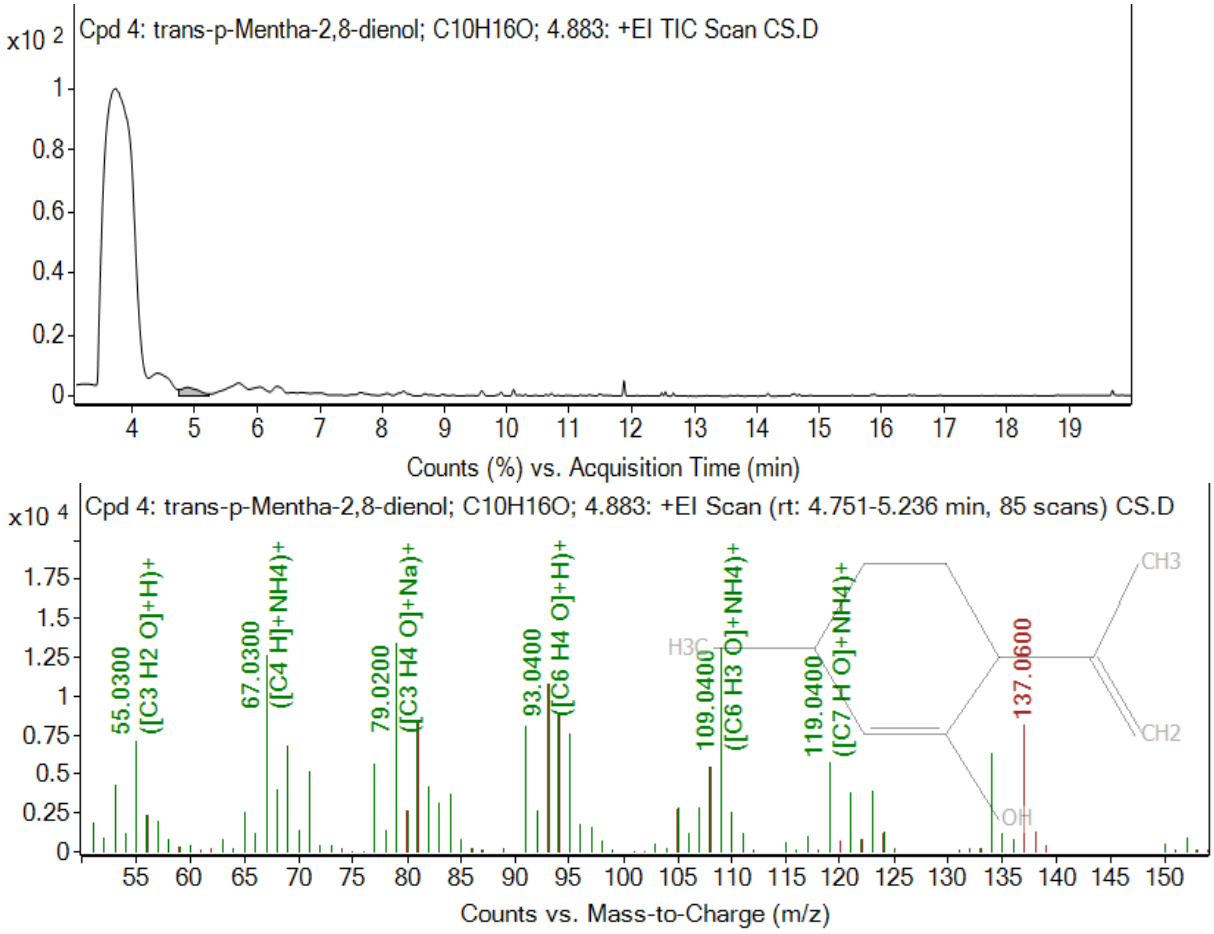
MS Spectrum



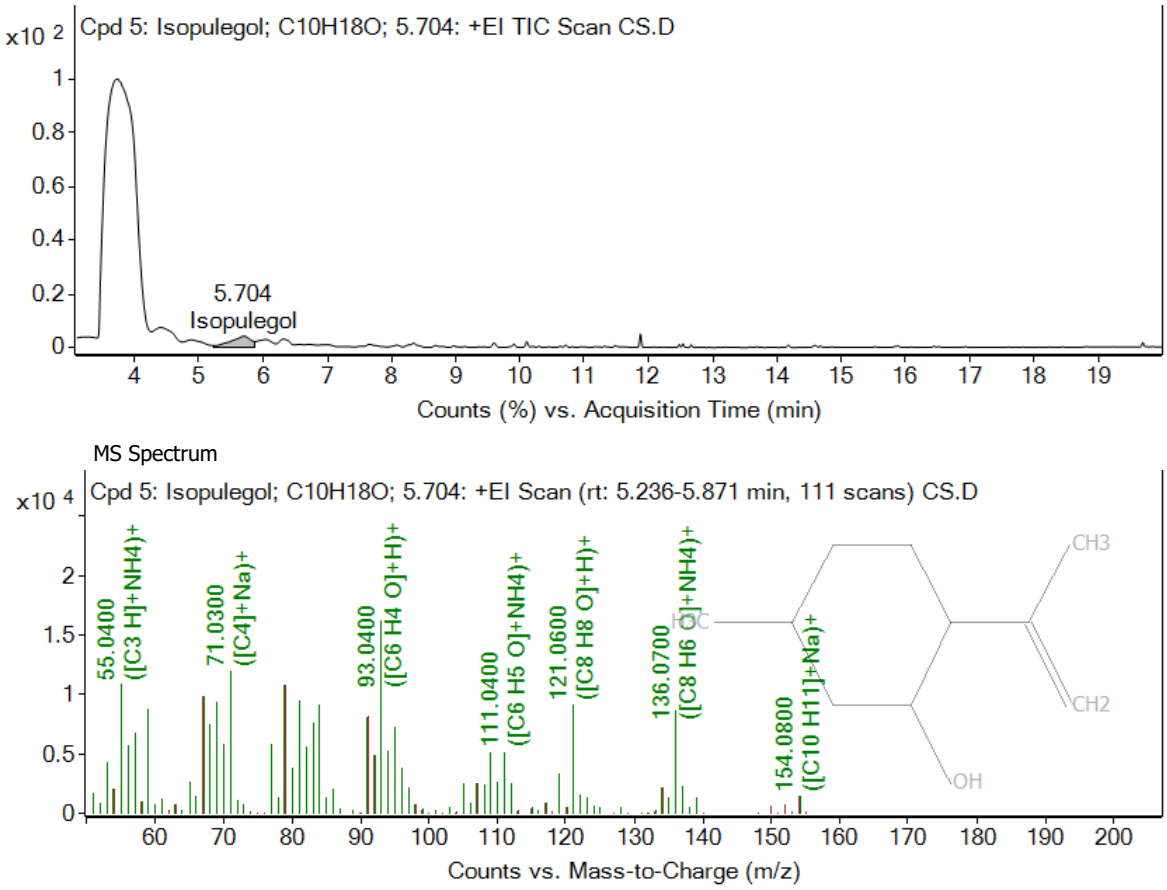
Appendix 5.3 GC-MS chromatogram of 1,6-Octadien-3-ol,3,7-dimethyl-



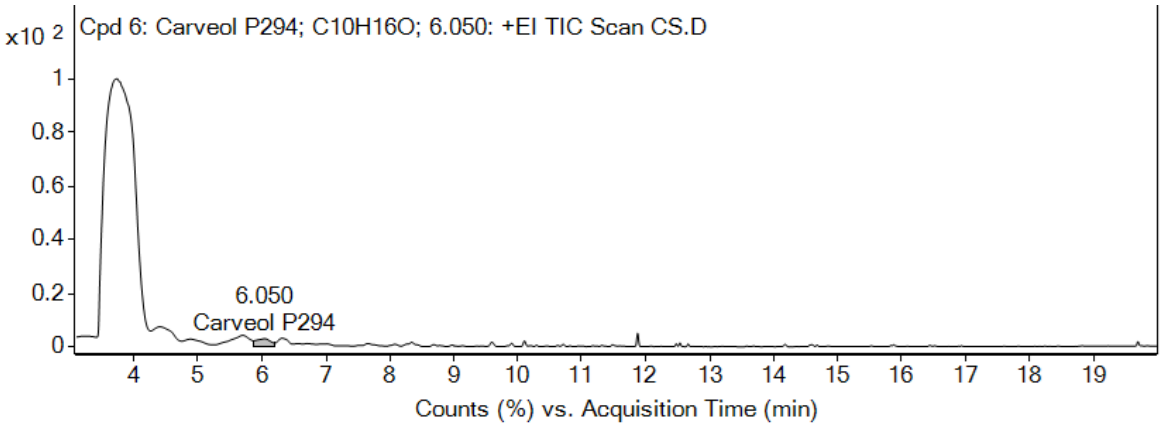
Appendix5.4 GC-MS chromatogram of trans-p-Mentha-2,8-dienol



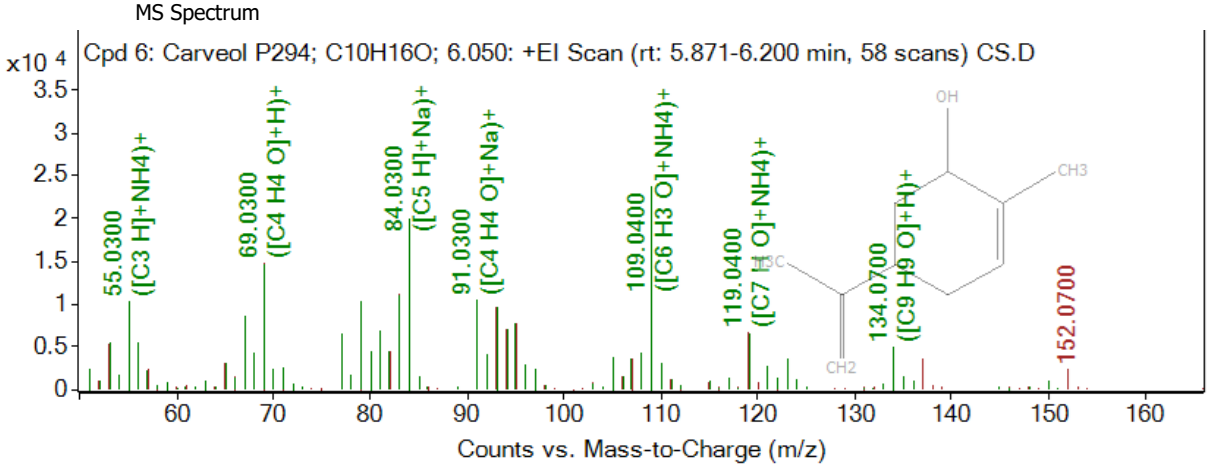
Appendix5.5 GC-MS chromatogram of Isopulegol



Appendix5.6 GC-MS chromatogram of Carveol



MS Spectrum



Appendix5.7 GC-MS chromatogram of 2-Cyclohexen-1-one,2-methyl-5-(1-methylethenyl)