Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents

Yalew Amare Lemma

A Thesis Submitted to Department of Applied Chemistry

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Masters in Medicinal Chemistry

Office of Graduate Studies

Adama Science and Technology University

March/2023

Adama, Ethiopia

"Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents"

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Principal Advisor: Dr. Hailemichael Tesso (PHD)

Co Advisor: Dr. R.Venkatesha Perumal (PHD)

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DECLARATION

I hereby declare that this thesis entitled "**Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents**" 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 acknoeledged through citation.

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RECOMMENDATION OF ADVISORS/ SUPERVISORS

We, the major/co-advisor/supervisor of this thesis, hereby certify that we have read the revised of this thesis entitled "**Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents**" prepared under our guidance by **Yalew Amare Lemma**. Submitted in partial fulfillment of the requirements for the degree of Masters of Science in Medicinal Chemistry. Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

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APPROVAL PAGE

We, the advisors of the thesis entitled "**Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents" and** developed by Yalew Amare Lemma, hereby certify that the recommendation and suggestion made by the board of examiners are appropriately incorporated into the final version of the thesis.

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_____	_____	_____
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APPROVAL OF BOARD OF EXAMINERS

We the undersigned, members of the Board of Examiners of the thesis by Yalew Amare Lemma have read and evaluated the thesis entitled "**Phytochemical Investigation and Biological Evaluation of Roots of *Verbascum sinaiticum* and Computational Studies of its Constituents**" and examined the candidate during 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 Medicinal Chemistry.

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ACRONYMS AND ABBREVIATIONS

MHB	Muller Hinton Broth
DPPH	2, 2-Diphenyl-1-Picryldrazyl
DMSO	Dimethyl Sulfoxide
WHO	World Health Organization
EtOAc	Ethyl Acetate
MeOH	Methanol
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
ADME	Absorption, Distribution, Metabolism, Excretion
d	Doublet
s	Singlet
t	Triplet
TMS	Tetra Methyl Silane
MRSA	Methicillin Resistant *Staphylococcus aureus*
PRDX5	Peroxiredoxin 5
BBB	Blood Brain Barrier
P-gp	Glycoprotein-P

ABSTRACT

*Verbascum sinaiticum Benth is a species of perennial herb in the family Scrophulariaceae. It is one of the significant traditional medicinal plants in Ethiopia and is used in the treatment of tumor, impotency, stomach ache, joint pain, viral infection, cancer, sunstroke fever, abdominal colic, diarrhea and hemorrhage. The present study was aimed at organic solvent extraction of the roots of V. sinaiticum, isolation and structural elucidation of its secondary metabolites, in-vitro and in-silico investigation of their biological activities including prediction of their pharmacokinetics and pharmacodynamics properties. The roots of V. sinaiticum were successively extracted with organic solvents of increasing polarity, namely n-hexane, ethyl acetate, and methanol. The extracts were subjected to extensive chromatographic analysis and a number of compounds were isolated. The structures of the isolated compounds were elucidated by using ^{1}H NMR, ^{13}C NMR, and DEPT-135 spectra. Proposed structures **31-34** are hitherto not reported and are thus new compounds for this plant species. The crude extracts and the isolated compounds were evaluated in-vitro for antibacterial activity by using broth microdilution method, against six bacterial strains and four fungi strains. Furthermore, antioxidant power of the crude extracts and the isolated compounds were evaluated by using the radical scavenging activity of DPPH. The MIC of 0.5 mg/ml was recorded for the methanol extract against S. epidermidis and P. aeruginosa. Similarly MIC of 0.5mg/ml was recorded for Compound **31** and **32** against P. aeruginosa. Similarly 1 and 2 mg/ml of MIC was recorded for compound **34** and **32** against S. epidermidis respectively. Therefore compound **31**, **32** and **34** showed promising activity against P. aeruginosa and S. epidermidis. MIC of 2mg/ml was noted both for methanol and ethyl acetate crude extract against C. albicans and T. mentagrophytes respectively. The results of DPPH activity showed the n-hexane and ethyl acetate roots extracts of V.sinaiticum inhibited the DPPH radical by 69.23% and 64.1% respectively, whereas compound **32** displayed 72.25 % of inhibition. The drug likeness analysis exhibited that compound **32** obeyed Lipinski's rule of five with zero violation. Compound **32** and **33** displayed a binding affinity of -7.8 & -8.3 kcal/mol against receptor LasR of P. aeruginosa. Whereas, compound **31, 33**, **34** and **32** showed a binding affinity of -9.2 and -9.8 kcal/mol against FtsZ receptor of S. epidermidis respectively. Hence, the activities herein indicated as the plant has great potential as a remedy for diseases caused by bacteria, fungi and radicals.*

Key words: V. sinaiticum, antibacterial, antioxidant, antifungal, ADMET, Molecular docking

CHAPTER ONE

1. INTRODUCTION

1.1 Background of the Study

Natural products are those chemical compounds or substances that are isolated from the living organisms. They can be in form of primary or secondary metabolites. Primary metabolites are directly involved in normal growth, development and reproduction. These include class of biomolecules such as carbohydrates, proteins, lipids, nucleic acids, etc.. Secondary metabolites are not directly involved in growth, development and reproduction of an organism, but they have an ecological function (Anulika *et al.,* 2016). Plant secondary metabolite can be found in the leaves, stem, root or the bark of the plant depending on the type of secondary metabolite that is been produced. The most known bioactive secondary metabolites are the alkaloids, tannins, flavonoids and phenolic compounds (Hill, 1952). Many of these secondary metabolites have been used since ancient times for human health care as drugs, spices and food additives (Pengelly, 2020).

Natural products and their derivatives have been recognized for many years as a source of therapeutic agents and chemical scaffolds, especially for the treatment of cancer and infectious disease (Mouhssen *et al.,* 2013, Atanasov *et al*., 2021). Most of the phytochemicals from plant sources such as phenolics and flavonoids have been reported to have positive impact on health and cancer prevention (Venugopal, & Liu, 2012).

As a traditional medicinal plant, the community of Ethiopia uses the root part of *V.sinaiticum* for treatment of different types of diseases including tumor (Teklehaymanot, 2009); impotency, stomach ache and joint pain (Teklehaymanot *et al.*, 2007); viral infection, cancer (Teklehaymanot, 2009); sunstroke fever, abdominal colic, diarrhea, hemorrhage and anthrax (Weldegerima *et al.*, 2008); hepatitis and as potent hepatoprotective medication (Yineger *et al.*, 2007, Umer *et al.*, 2010). Moreover, powdered leaf of *V. sinaiticum* mixed with water is given orally (Teklehaymanot *et al.*, 2007, Fullas, 2010,) and the filtrate is added to left ear and nose of animals (Weldegerima *et al*., 2008) for the treatment of animal trypanosomiasis. Ethiopia is remarkably rich in history, culture and biological diversity. The country is also recognized for its diverse habitats of medicinal plants that results in a high diversity of traditional medicinal knowledge and practices of the people in using medicinal plants (Nofouzi, 2017).

Even though the medicinal plants play critical role in discovery of lead compounds for therapeutics usage currently multi drug resistance strains were common for microbes. The bacteria most likely to cause bacteremia include *Staphylococcus, Streptococcus, Enterococcus, Escherichia, Klebsiella, Pseudomonas, Enterobacter, Haemophilus, and Neisseria genera* (Abebaw *et al*., 2017). Therefore, in recent years, there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. A greater attention has been paid to antimicrobial activity screening and evaluating methods. Several bioassays techniques were used such as disk-diffusion, well diffusion and broth or agar dilution are well known and commonly used to study antimicrobial activity (Balouiri *et al.,* 2015).

Moreover, the modern era of drug designing and development involves a thorough understanding and estimation of the receptor-ligand binding affinity, using molecular docking analysis (Seeliger, & de Groot, 2010). In this case this study was investigated biological activity and receptor-ligand binding affinity of each isolated compounds from root part of *Verbascum sinaiticum*. Hence this work may play significant role in finding of lead compound in drug discovering process for the treatment of bacterial and fungal diseases and may contribute candidate bioactive compound which help to solve the current issues of bacterial resistance.

1.2. Statement of the Problem

Biologically active compounds are important springboard both for the search of new promising substances and for the further development of new drugs (Lavecchia, and Di Giovanni, 2013; Tripathi, and Misra, 2017). Major problem for extensive use of herbs are the lack of information concerning mechanisms of their action (Prokopenko, 2019).

Ethiopia have a diverse habitats of medicinal plants, which results in a high diversity of traditional medicinal knowledge and practices of the people in using them. However, there is limited or no realistic attempt in identifying bioactive molecules of therapeutic potential, elucidating their chemical structures and their pharmacological mechanisms of actions which may lead to discovery of lead compounds for development of clinically useful drugs. *Verbascum sinaitcum* is one of the plant which has been used traditionally to treat different disease in different part of the country. Few researches have hitherto reported on *in-vitro* antimicrobial (Yeabyo *et al*., 2018); antitrypanosomal (Terefe *et al*., 2014); analgesic as well as anti-inflammatory activities of the

plant (Asefa *et al*., 2022). But none has so far attempted to isolate and study the major bioactive constituent(s) of this plant in Ethiopia.

Therefore the aim of this study is to identify the bioactive chemical constituents from the root part of the *V. sinaiticum.* Additionally, Computational or in silico study were undertaken to assess the binding affinity to target proteins and drug like properties of isolated compounds. Therefore, this study may also help in fast prediction of pharmacodynamics and pharmacokinetics properties of isolated compounds.

1.3. Significance of the Study

The outcome of this study was expected to provide basic information on the main bioactive chemical constituents in the root of *V. sinaiticum* that can be evaluated for the treatment of bacterial and fungal diseases. Furthermore it also provide basic information on potential antioxidant activity of crude extract and isolated compounds of root of *V.sinaiticum*. Therefore, the results of the biological activities, bioactive compounds and molecular docking of the isolated compounds of the root of *V.sinaiticum* may help to arrive at finding lead compound for drug discovery and substantiate the observed promising activity with molecular docking analysis on target protein of selected strain of microorganism. Moreover, the study provides baseline information for future pharmacological studies for researchers who are interested to conduct further work on the area.

1.4. Objectives of the Study

1.4.1. General Objectives

- The main objective of this research is to analyses the chemical composition of the roots extracts of *Verbascum sinaiticum* and in vitro their biological activities as well as *in-silico* analysis of their pharmacodynamics and pharmacokinetics properties.

1.4.2. Specific Objectives

- To extract the root of the *Verbascum sinaiticum* successively using n-hexane, EtOAc and MeOH.
- To carry out phytochemical screening of the roots of crude extracts of the plant.
- To fractionate and purify the methanol crude extract of plant root using column chromatography.
- To characterize the structures of isolated compounds using NMR spectroscopic techniques

- To determine the antibacterial and antifungal effect of the crude extract and isolated compounds against *E. coli, P.aeruginosa, K. pneumoniae, S. auras, S. epidermidis, S. agalactiae and C. albicans, Penicillium species, T.mentagrophytes, A. niger respectively.*
- To evaluate the pharmacokinetics properties of the bio active molecules by SwissADME and binding affinity with the targets proteins (LasR (PDB ID: 3JPU) for *P. aeruginosa;* FtsZ (PDB ID: 4M8I) for *S. epidermidis;* CYP51 (PDB ID: 5V5Z) for *Candida albican*; human peroxidoxins (PDB ID: 1HD2) for antioxidants by using Autodock vina 1.2.0.)

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Medicinal Plants Uses

Medicinal plants have been used since ancient times for human health care as drugs and food additives. They also play significant role in prevention and treatment of various disease like cancer, osteoporosis and Coronary Heart Disease (CHD) (Sofora *et al.,* 2013). The African continent is naturally gifted with various plant species with nutritional and medicinal benefits. About 80% of the people in developing countries rely on folk medicines to treat different diseases because of indigenous knowledge, availability, and cost-effectiveness of traditional medicines (Kunle, &Oluwafemi, 2021). Traditional Healers still play a great role in the primary health care systems in Ethiopia. The sparsely distributed forests were important resources of healers and repositories of medicinal plants gene pools (Tesfahuneygn & Gebreegziabher *et al.,* 2019).

Use of medicinal plants and traditional medicine linked to social life, culture, and daily activities of the African people (Olufunke *et al.,* 2017). According to World Health Organization, traditional medicine refers to the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses (WHO, 2012). Thus, traditional medicine remains popular for both historical and cultural reasons. It is estimated that 80% of the African people depend on traditional medicine to meet up their care needs (Andarge *et al.,* 2015). Like other parts of sub-Saharan countries, 70% of human and 90% of livestock population of Ethiopia rely on traditional medicine for primary health care (Kidane *et al.,* 2014). Traditional healers in Ethiopia utilize the herbal resources available in nature for various disease treatments. As reported before in Ethiopia approximately 800 species of the medicinal plants grown in Ethiopia are used for treating about 300 medical conditions (Deffar, 1998). Therefore medicinal plants play significant role in the treatment of different infections that likely caused by a number of pathogens.

Plant derived natural products also represent an important source of antimicrobial agents since they exist naturally and affordable, especially for rural societies in poor developing countries (Ghosh *et al.,* 2008). In addition, active chemical constituents extracted from all parts of plants, from roots to seed and flowers, can act as the source of lead compounds. Examples of some of the

drugs in clinical use obtained from plants include **taxol** and **vincristine**, the highly important anticancer alkaloids isolated from *Taxus brevifolia* and *Vinca rosea* Linn, respectively. **Pilocarpine** is used to treat glaucoma and was obtained from *Pilocarpus jaborandi*. **Morphine**, which is used as an important analgesic, is isolated from the *Opium poppy, Papaver somniferum* (Gareth Thomas, 2007). **Digoxin** which is used to treat atrial fibrillation in heart failure was extracted from *fox glove, Digitalis purpurea*. In addition to these, **nicotine** and **salicylic acid** were extracted from *Tobacco* and from bark of willow tree respectively (Cooper and Nicola, 2014). Therefore, natural products offer an opportunity to discover new compounds that can be converted into drugs.

2.2. The Family Scrophulariaceae

Scrophulariaceae is commonly known as the figwort family. The family Scrophulariaceae is cosmopolitan family with 300 genera and 5400-5500 species. *Verbascum L*. is the largest genus of the family Scrophulariaceae, with about 2500 species worldwide (Grieve, 1995). The Scrophulariaceae have a cosmopolitan distribution, with the majority found in temperate areas including tropical mountains (Hedberg *et al.,* 2006).

2.3. The Genus *Verbascum*

The *Verbascum* is in the family of Scrophulariaceae in the major group flowering plant. (Hedberg *et al.,* 2006). In Europe, Asia and North America, various *Verbascum* species are used as antiseptic, antimalarial, astringent, demulcent, emollient, sedative, narcotic effects and also, they are used for the treatment of tumors, inflammations, migraine, asthma and spasmodic coughs. The species are recorded to treat hemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhea, and have inhibitory activities against some influenza viruses (Nofouzi *et al.,* 2017, Dimitrova *et al*., 2013, Aysu and Durak, 2016). Some of the species which are found in Europe, Asia and Africa are *V. nigrum, V. phlomoides, V. lychnitis, V. Thapsus, V. fruticulosum, V. roripifolium V. sinaiticum (*Tatli & Akdmeri, 2006*).*

2.4 Ethnobotanical Uses of *Verbascum sinaiticum*

Verbascum sinaiticum locally known by the Afan Oromo 'Gurra harree'; Amharic name 'Qetetina' or ye Ahya joro and 'Estedebetra 'in (Ge'ez). It is a perennial plant, 60-150 cm tall (Hedberg *et al.,* 2006). The whole plant is densely shaggy, grayish and its leaves are simple and broadly ovate-oblong (Abebe *et al*., 2003) (Figure 5). Ethno pharmacological studies of medicinal plants have a

great importance in the development herbal medicines and current therapeutic system (Suntar, 2019). Thus, ethnobotanical data of *V. sinaiticum* in different part of Ethiopia is summarized in the table below (Table 1).

Table 1. Ethnobotanical Data about the *Verbascum sinaiticum*

Plant scientific name, (Family)	**Region**	**Parts used**	**Traditional and/or medicinal use**	**Mode of use**	**Reference**
Verbascum sinaiticum (Scrophulariaceae)	**Amhara:** West Gojjam Zone	Root	Treatment of Esophagus, Swelling and Loss of weight	Crushed taken orally	(Alemneh, 2021).
		Root	To treat Asthma and Nose bleeding (epistaxis)	Pounding taken Orally and Pounding take through Nasal	
		Root and leaf	Used for the treatment of Poisoning and diarrhea	Chewing	
	Tigray: Eastern Zone in Ganta Afeshum District,	Fresh root	For Retained placenta for humans	Root is crushed, mixed with water, filtered and drink for Retained placenta	(Kidane *et al.*, 2018)
	Amhara : Chilga District,	Fresh root	For the treatment of impotency , stomach ache and joint pain	Drinking 1 cup decoction	(Mekuanent *et al.*, 2015)
	Oromia : Arsi Zone,	Leaf	For the treatment of diarrhea and hemorrhoid		(Wondimu *et al.*, 2007).

2.5. The Reported Biological Activities of the Genus *Verbascum*

The *Verbascum* species, the largest genus of the family Scrophulariceae. Different part of the plant have been used for the traditional medicines for the centuries in almost all over the part of the

world because of its wide range of biological activities (Kahraman *et al*., 2012). In subsection below a few of studies were reviewed on the plant biological activities that done previously.

2.5.1 Antibacterial Activity

Previous studies have demonstrated that the methanol extract of *V. thapsus* had more growth inhibitory effects on *E. coli* and *S. pyogenes* than the aqueous and ethanol extracts (Ghasemi *et al.*, 2015). Previous studies reported about the antibacterial activity of flowers extract of *V. sinaiticum* by using solvents like methanol and n hexane against *Methicillin resistant S. aureus (MRSA); Bacillu cereus, Escherichia coli ,Pseudomonas aeruginosa* and *Salmonella typhimurium* using microdilution method. The data have shown the methanol extract had the higher activity with MIC (1000 µg/ml) against both *Bacillus cereus* and *Salmonella typhimurium while* n-hexane extract have shown MIC of (>1000 µg/ml) compared to the standard drug tetracycline with MIC (8, 8 and 4 µg/ml) *against Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium respectively* (Talib &Mahasneh, 2010).

Previous studies have proven that ethanol extracts obtained from the leaves of *Verbascum sinuatum w*ere evaluated against clinically isolated Urinary tract pathogens (*Enterococcus faecalis, Escherichia coli, Klebsiella pnemoniae, Pseudomonas aeruginosa*, and *Proteus mirabilis*) by using microdilution method. The documented data have demonstrated that the extracts showed strong antibacterial activity against *Enterococcus faecalis*, and *Proteus mirabilis* with MIC's of 4.0 and, 8.0 µg/mL, compared to the standard drug streptomycin(2, 4) and ampicillin (1, 0.5) µg/ml respectively (Alper & Basaran 2009).

Previous study done here in Ethiopia also demonstrated the antibacterial activity of root extracts *V.sinaiticum* using diethyl ether, chloroform, acetone and ethanol by Agar well-diffusion method against multidrug-resistant enterobacteriaceae both on Gram negative and Gram-positive bacteria. The report were revealed that the acetone root extracts of *V. sinaiticum* (VSRAC) were exhibited broad-spectrum antibacterial activity with zone of inhibition against tested Gram-positive *bacteria B. subtilis* (7–8 mm) and Gram negative bacteria *E.aerogenes* (7–10 mm), *E.coli* (8–10 mm), and *V. cholera* (7–10 mm) at concentration range between 250 µg/ml and 500 µg/ml compared with standard drug ciprofloxacin (*B. subtilis* (37mm),*E.aerogenes* (29mm), E. coli (29mm), *V. cholera* (31mm) at concentration 20 µg/ml (Yeabyo *et al*.,2018).

2.5.2 Antifungal Activity

The antifungal activity was reported on the ethanol leaves extracts obtained from the *Verbascum sinuatum L*. against *Candida albicans* using microdilution method. The report were investigated that the extract had a comparable activity with MIC's 8.0 µg/ml, in comparison to standard drug nystatin 8.0 µg/ml (Alper& Basaran 2009). Previously reported data also suggest methanolic extract of aerial part of *V. speciosum* displayed effective antifungal activity against all the studied species (*Candida albicans, Candida tropicalis, Candida parapsilosis, Candida krusei, and Candida dubliniensis, A.flavus, A.niger, Penicillium and alternaria*). The documented data were revealed that the highest antifungal activities were against *C. parapsilosis* and *Alternaria with MIC of 0.09 and* 0.78 µg/mL respectively (Nofouzi, 2015)

2.5.3 Antioxidant Activity

As has been reported previously the antioxidant activity of 70% aqueous methanol extract and the four successive fractions of (diethyl ether, ethyl acetate, chloroform and methanol) of *Verbascum letourneuxii* were studied. The report were showed that antioxidant (DPPH assay (100 µg/mL) differential scavenging properties as ordered in descending: methanol > aqueous methanol > diethyl ether > chloroform > ethyl acetate with % values of 85.7, 80, 76, 64 and 23, respectively, compared to ascorbic acid (98.4%) (Farid *et al.,* 2020).

The previous studies were also confirmed the antioxidant activities of *V. euphraticum and V. oocarpum* using methanol, n-hexane and ethyl acetate extracts The documented data suggest that the methanolic extract of both plants had highest antioxidant potential (DPPH assay) 48.32 ± 0.11mg^{TE}/g (*Verbascum euphraticum*), 48.14 ± 0.08 mg^{TE}/g (*V. oocarpum*) respectively (Zengin, *et al.*,2021).

2.5.4 Cytotoxic Activity

Previously reported data showed that cytotoxic activity of 70% aqueous methanol extract and the four successive fractions (diethyl ether, ethyl acetate, chloroform and methanol) of *V.sinaiticum* flowers were examined against Vero cell line (Heo-2 and MCF-7) using the MTT assay. The documented data revealed that methanol extract had IC_{50} of 367.11 ± 2.70 (µg/ml) (Talib & Mahasneh, 2010). However, the diethyl ether and the chloroform fractions were described as they were showed significant cytotoxic effects (diethyl ether: IC_{50} 52.5 µg/ml against HepG2, while the chloroform fraction revealed (IC_{50} 70.1, 63.6 and 73.4 (µg/ml) against HepG2, MCF-7 and PC3 compared to doxorubicin with IC_{50} 27.4, 28.2 and 28.7 (µg/ml), respectively. There were also

reported that four compounds isolates from leaves part of *V. sinaiticum* were evaluated in the P-388 lymphocytic leukemia exhibited ED_{50} values of 7.7, 1.2, 1.9, and 1.4 µg/ml respectively (Afifi *et al.,* 1993).

2.5.5 Antiprotozoal Activity

There was a study reported that *V. sinaiticum* had trypanocidal activity. The documented data suggest that aqueous and methanol leaf extracts of *Verbascum sinaiticum* on motility of *Trypanosoma congolense* revealed that ceased motility at concentration of 4 mg/ml within 60 and 50 min respectively compared to standard drug Diminazine aceturate at 4 mg/ml which takes 20 min to cease the motility of Trypanosoma congolense. The crude extracts were partially eliminated trypanosomes in a dose-dependent manner (Terefe *et al*., 2014).

2.5.6 Anti-inflammatory Activity

A study done vitro test was reported that 80% methanol root extract of *V. sinaiticum* possesses peripheral and central analgesic as well as anti-inflammatory activity (Asefa *et al.,* 2022)

2.6 Phytochemistry of Some *Verbascum species* and *Verbascum sinaiticum*

Phytochemicals are a large group of plant-derived compounds which are responsible for the protection of disease. They can be obtained from fruits, vegetables, beans, cereals and plant-based beverages (Arts and Hollman, 2005). Phytochemical can be either primary or secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents whereas; terpenoids, alkaloids, anthraquinones, stilbenoid, naphthalenes and phenolic acid compounds are secondary metabolites (Krishnaiah *et al.,* 2007). Previously reported data suggest aqueous and methanol leaf extracts of *V. sinaiticum* were investigated for the presence of secondary metabolites. The reported phytochemical screening assay were revealed the presence of alkaloids, flavonoids, glycosides, phenolic compounds, saponins, steroids and tannins (Terefe *et al.,* 2014).

Several species of *Verbascum* have important medicinal properties and they have been the subjected of several pharmacological investigation. *Verbascum* species mainly contain groups, such as: Iridoid glycosides, iridoid, saponins, phenylethanoid glycosides, monoterpene glucoside, neolignan glucosides, flavonoids, steroids and spermine alkaloids (Bileflmi, 2004, Thabeta *et al.,* 2022).

There were previous reports on isolation and characterization of wide range of compounds from the plant genus and from different parts of *V. sinaiticum*. The reported compounds were

summarized in (Table 2). In the previous study the docking score were also reported after the compounds were isolated from the flowers of *Verbascum sinaiticum.* Docking experiments were carried out using Glide, firstly using the scoring function Standard Precision (SP) and then extra Precision (XP) on selected compounds (Rutin (**26**), 5-caffeoylquinic acid (**27**), Vanillic acid (**28**) and Hyperoside (**29**) and docking score showed (-8.355, -7.070, -6.845 and -6.780 kcal/mol then the obtained docking poses have been submitted to the binding energy estimation by the Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) showed that (-30.813,-17.867, -37.107, -27.335 kcal/mol) (Zengin *et al.,* 2021).

Table 2. Summary of Some Chemical Constituents of *Verbascum* Species and *V.sinaiticum*

Compound name (NO.)	**Plant Species**	**Extracted (Part)**	**Reference**
Iridoid glucosides 6-O- β-D glucopyranosylcatalpol (**1**), 6-O-(6″-O-trans-p-hydroxycinnamoyl)- β-D-glucopyranosylaucubin(**2**), 6-O- β-D-glucopyranosylaucubin (**3**), 6-O- α-L rhamnopyranosylcatalpol (**4**), pulverulentoside I (**5**) and buddlejoside A4 (**6**)	***V. salviifolium*** *V. undulatum* *V. sinuatum L.*	Methanol (aerial parts) Methanol (aerial parts) Aerial	(Akdemir *et al.*,2005) (Magiatis *et al*.,2000) (Bianco *et al.,* 1981)
Iridoid diglycoside 6-O-glycosyliridoids (Sinuatol I) (**30**)			
Flavonolignans Sinaiticin (***7***) hydnocarpin (**8)**	*V. sinaiticum*	Leaves	(Afifi *et al.,* 1993).
Flavones chrysoeriol (**9**) luteolin (**10**)	*V. sinaiticum*	Leaves Aerial parts	(Afifi *et al.,* 1993).
Iridoids ajugol (**11**) aucubin (**12)**	*Verbascum sinaiticum Benth.*	Aerial part	(Mahmoud, S.M. *et al.,* 2007).
Flavonoids orbol (**14**)	*V.sinaiticum*	Aerial part	(Elgindi. *et al.,* 1999).

Orbol 7-O-β-D glucoside(**15**) 5,3',4'-trihydroxy-8-methyl isoflavone 7-O-β-D-glucoside(**16**) 5-hydroxy-3', 4' dimethoxyisoflavone7- O-α-Lrhmnoside (**17**).			
phenylethanoid glycosides Verbascoside (**18**), Eukovoside (**19**), Martynoside (**20**), Jionoside D (**21**), Campneoside I (**22**) and Campneoside II (**23**),	*V. sinaiticum*	Aerial part	(Gondal *et al.*, 2020).
Saponin SaikosaponinA **(24)** Verbascosaponin B (**25**).	*V. sinaiticum, V. thapsiforme*	Aerial part	(Miyase *et al.*,1997)
Flavonoids Rutin (**26**), 5-caffeoylquinic acid (**27**), Vanillic acid (**28**) and Hyperoside (**29**	*V. sinaiticum*	Flowers	

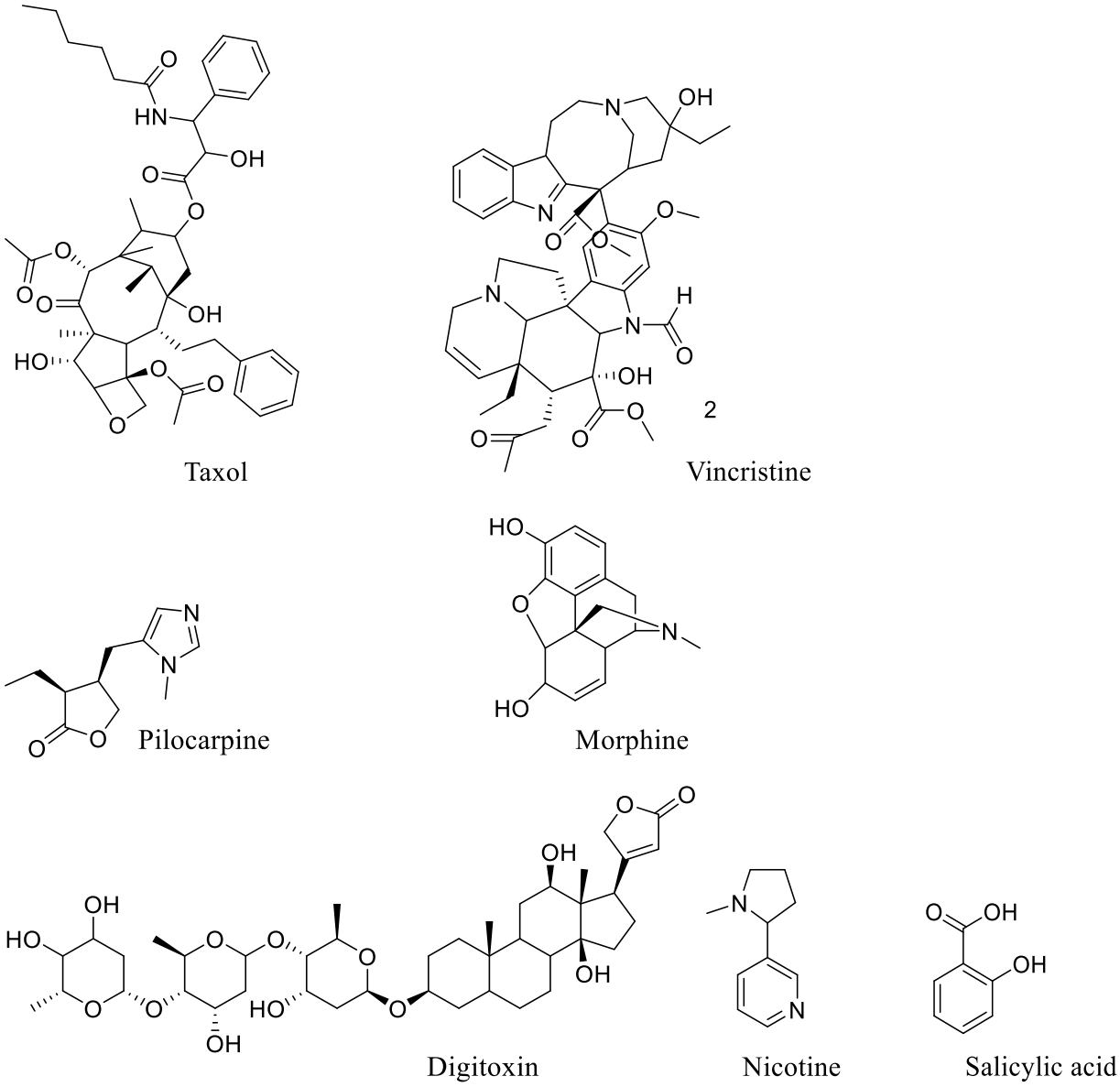

Figure 1. Some of the drugs in clinical use obtained from plants

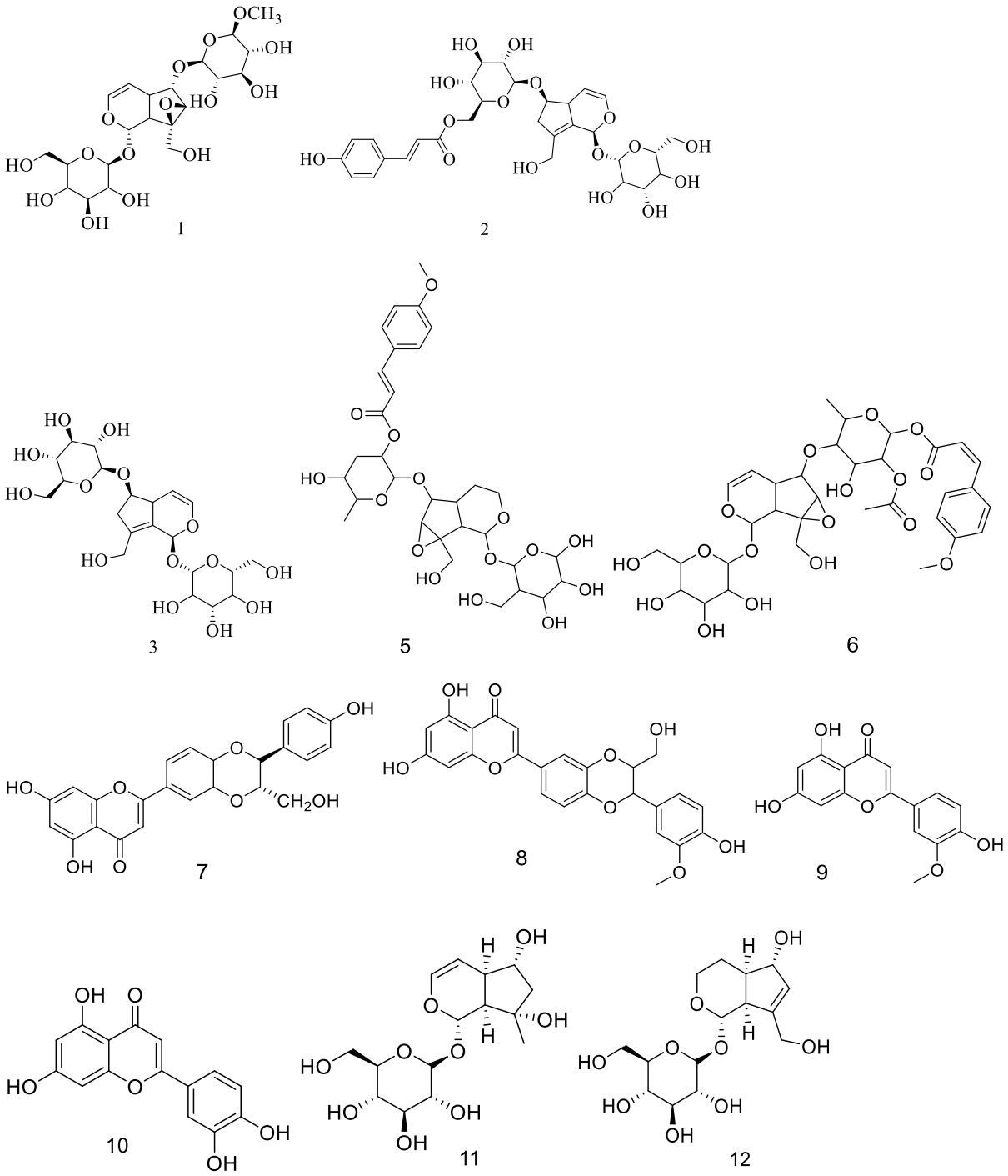

Figure 2. Some chemical constituents *Verbascum* Species and *V. sinaiticum*

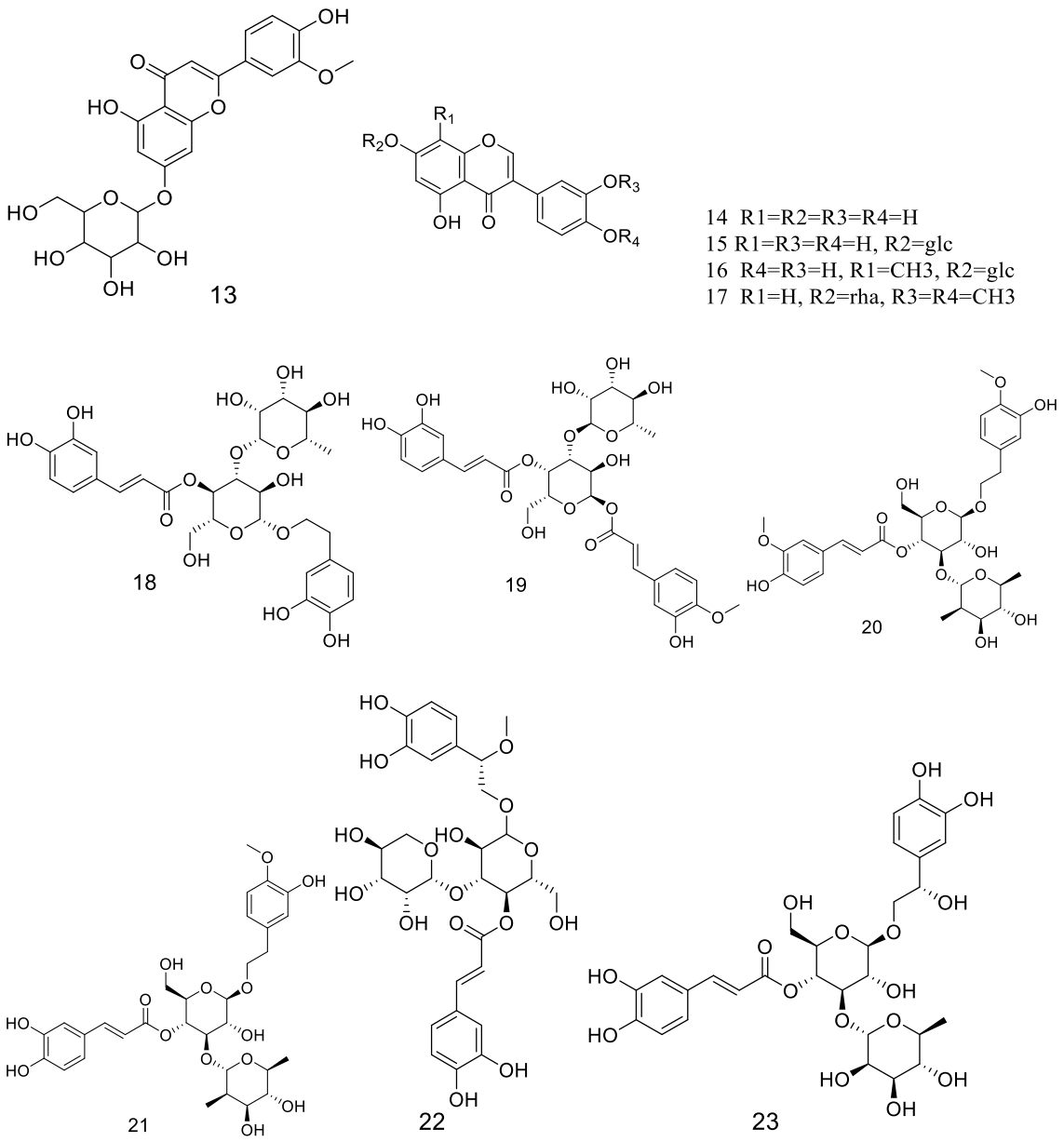

Figure 3. Some chemical constituents *V. sinaiticum*

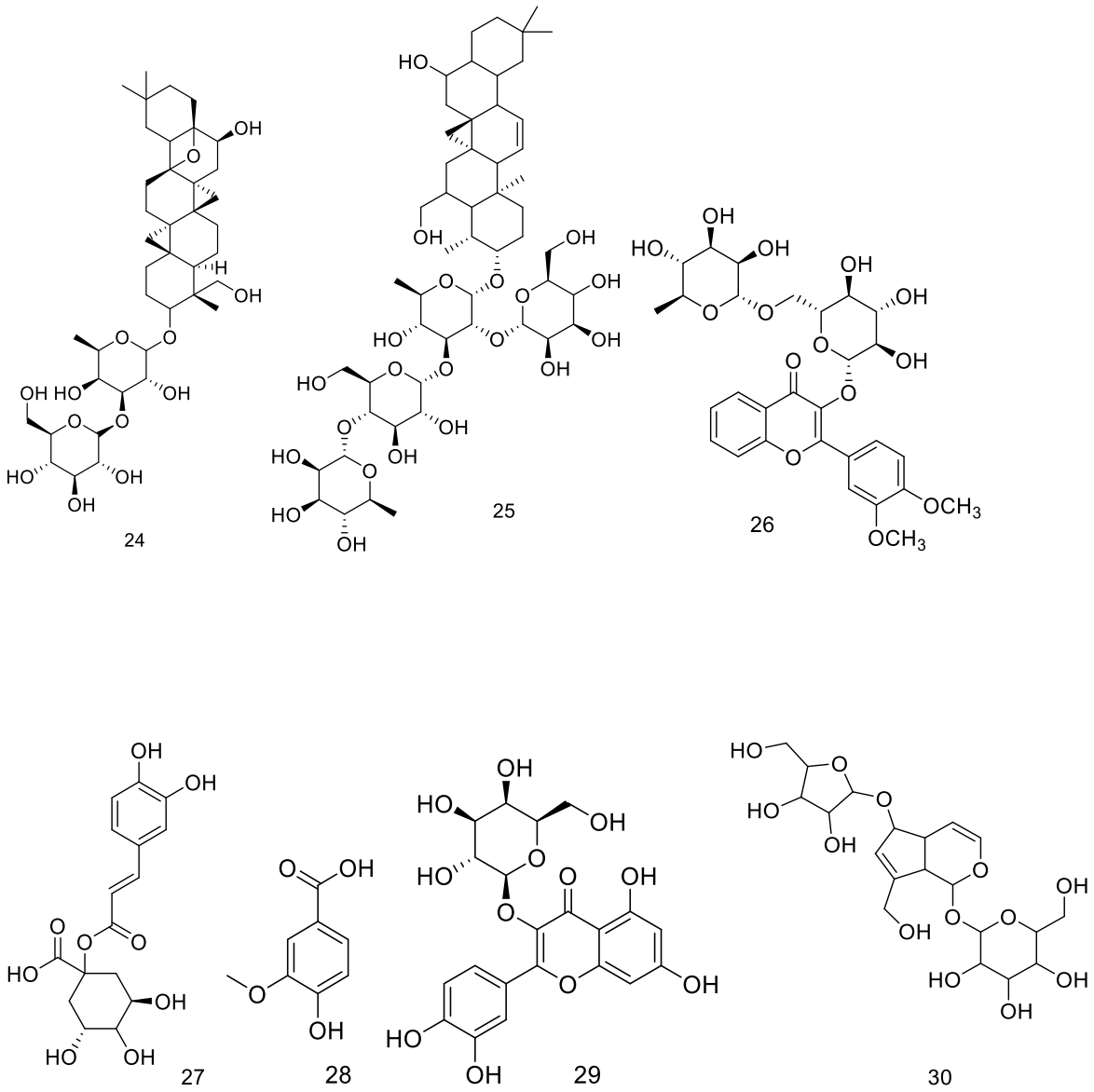

Figure 4. Some chemical constituents *V. sinaiticum*.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant Materials

The fresh roots of *V. sinaiticum* was collected from the Inkolo Wabe Woreda, Bokoji mirt, Kebele, in Arsi zone Oromia region, Ethiopia. The area was located around 274.1 km from the capital of Ethiopia and lies at about 2622 m (8021.65ft.) above the sea level altitude of 7.443340º N longitude 39. 418571º. The average annual temperature in Arsi Zone is 15.05°C and the rainfall averages are 1020 mm. The plant material was authenticated by a botanist Prof. Sabesebe Shibru of the Department of Biology, National Herbarium Center of Ethiopia, Addis Ababa University. The voucher specimen (YA/001) was deposited at the Herbarium for future reference.

3.2. Instruments and Chemicals

3.2.1 Instruments

Column chromatography requires column for packing (3.9 to 4.6 mm) diameter and (25 to 30 cm) length was used. Analytical thin layer chromatography used was a 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminum plate. NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz's. The other materials that were used in this study: TLC plate, TLC chamber, rotary evaporator, reagent bottles, capillary tube, round bottom flasks, beakers (different size), conical flask, funnels, vials, glass wares, refrigerator, Whatman No.1 filter papers, grinder, drying oven, and measuring cylinders.

3.2.1 Chemicals and Reagents

The chemicals and reagents which were used in the study include: silica gel, solvents (n-hexane, ethyl acetate and methanol) for extraction and column elution. In addition to these, anhydrous Sodium sulphate, Sulphuric acid, Vanillin, DMSO, Distilled water, Mueller–Hinton Broth, 2% T ween 80, nutrient agar, and Sabouraud Dextrose Broth (SDB) were used.

3.3. Preparation of Plant materials

The roots of *V. sinaiticum* were washed thoroughly 2-3 times with running tap water to remove dirt and chopped into pieces then allowed for shade dried at room temperature and ventilated room

for 15 days. About 2 kg of the root of plant was air dried. The dry root was ground into fine powder using an electric grinder and then obtained 1kg of coarsely powdered form of plant materials.

A  B 

Figure 5. A photos of Ariel (A) and roots (B) Parts of *V. sinaiticum* picked by Yalew Amare

3.4 Extraction

600 g powdered plant material was divided into two equal parts (300 g each) and each was put in a separate clean bottle and they were sequentially extracted by maceration method with equal volumes (2 L) of *n* –hexane, EtOAc and MeOH (3:10 ratio) for 72 h at room temperature with occasional shaking. At the end of the 72 h maceration, each soaked sample was subjected to final enough agitation before the solution was filtered using filter paper (Whatman, No 1). The filtrate was concentrated under reduced pressure and a temperature range of 40 ºC – 49 ºC, over rotary evaporator to give the crude extracts. The subsequent dried extract of each solvent was weighed and stored in refrigerator below 4°C until needed for further works.

3.5. Isolation and Identification of Bioactive Compounds.

After a preliminary thin layer chromatography analyses of the crude extracts, the separation step on silica gel column was first conducted. Based on the TLC analyses result, the methanol crude extract was selected for column chromatography to further isolation and characterization of its bioactive compounds (Appendix 5). The column chromatography was performed using 100-200 mesh silica gel (150 g) to elute out individual compounds from the root parts of methanolic extract. After loading with the plant extract (25 g) adsorbed onto 25 g of silica gel and subjected to column chromatography with increasing solvent polarity the column was run with varying solvent polarities with different ratios like n-hexane (100%), n-hexane: ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9), ethyl acetate (100%), ethyl acetate: methanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) and methanol (100%). A total of 461 fractions were collected (each 10-15 ml). The fractions were collected and analyzed by thin layer chromatography (TLC) and visualized and spri reagents UV for their composition. Fractions that showed similar R_f values and the same characteristic color on TLC were combined together and coded accordingly: 21-26, 30-37, 38-49,

51-59, 60-73, 74- 87, 90-139, 140-149, 169-178, 179- 188, 189- 198, 199- 208, 209-218, 219-228, 229-238, 239-248, 249-259, 300-309, 310-319, 320-329, 330-339, 340-349, 350-359, 360-369, 370-379, 380-389, 390-403, 404-439, 440- 448, 449-461.

Among all fractions collected, fraction ranges 390 – 403 and 404- 439 eluted with (EtAOc/MeOH (ratio 7:3) were further purified by column chromatography (Appendix 5). Fractions ranges 390-403(1g) subjected to the second chromatographic separation (repack A_1) which was performed with 20% methanolic elution for the weight of 1g using 30.0 g of silica gel (ratio 1:30). The eluting system was a mixture of EtAOc/MeOH (80:20 v/v), then 18 sub fractions were collected. Sub fraction 4 (Fr 4) gave compound **31** (15 mg**)**, Fr 12 gave compound **32** (20 mg**)** (Appendix 5). Again fraction ranges 404- 439 (1.5 g) was subjected third chromatographic separation (repack A_2) using silica gel (35 g) for further separation of compounds with eluting system of EtAOc/MeOH (9:1, 8:2), then totally of 40 sub fractions were collected, again sub fractions that showed similar R_f value were combined together (1-8, 9-17, 18- 31, and 32- 40). Further purification using column chromatography was done on sub fraction 32-40 (300 mg) (repack A_{2a}) using 25 g of silica gel then totally 17 sub fraction were obtained. Sub fraction (Fr 6) gave compound-**33** (20 mg) and Sub fraction (Fr 12), sub fraction (Fr 13) gave compound-**34** (15 mg) (Appendix 5).

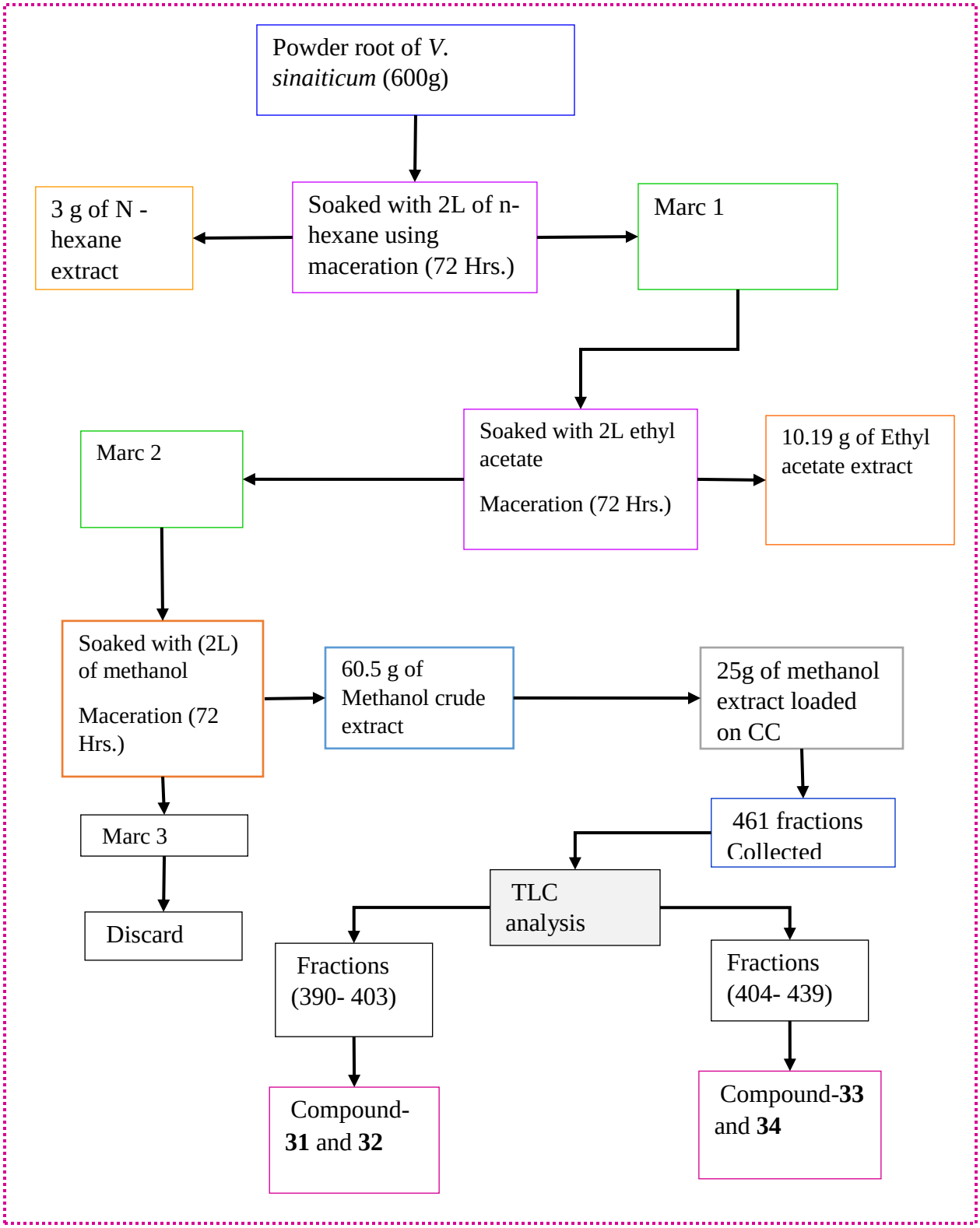

Figure 6. Flow Chart of Extraction and Isolation of Root of *V.sinaiticum*

3.6. Phytochemical Screening on the Crude Extracts of the Roots of *V.sinaiticum*

The preliminary phytochemical analysis of the plant extracts was performed using the standard protocol, to identify the presence of alkaloids, flavonoids, glycoside, saponins, steroids, and terpenes.

1. TEST for alkaloids: this was carried out as described by Rafuf and Sofora.

   Wagner s test: first Wagner's reagent was prepared. 2.5g of iodine was dissolved with 12.5g potassium iodide then add 250ml of distill water. 1ml of HCL and 3 drops of Wagner's reagent was added to each extract solution. The formation of a brown precipitate indicated the presence of alkaloids.

2. Test for flavonoids : this was carried out as described by Dermarderosian and lebrti

   Ferric chloride test: few drops of ferric chloride (5g of ferric chloride was dissolved in 100ml of water), then, it was added to the extract test solution. Formation of blackish red color indicated the presence of flavonoids

3. Test for glycosides : this was carried as described by Evans

   Keller killiani test: the extract test solution was treated with few drops of glacial acetic acid and ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Formation of lower reddish brown layer and upper acetic layer which turns blueish green was taken as an indication for the presence of glycosides.

4. Test for saponin: foam test 1ml of extract was diluted with distilled water to 20ml and shaken for 15 minutes. Development of stable foam Suggests presence of saponins

5. Test for steroids and terpenes

Liebermann Burcherd test: extract solution was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from sides of test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of upper indicated the presence of steroids while the formation deep red color in the lower layer indicted a positive for terpenes.

3.7 Biological Activities

3.7.1Antibacterial and Antifungal Activity Tests

3.7.1.1 Preparation of Test Solution, Bacterial and Fungal Strains for Determination of MIC.

The test solutions were prepared by dissolving 8 mg from each crude extracts and compounds in 2% of Tween 80 dissolved in distilled water to achieve final stock concentration of 4mg/mL solution of the test sample. Minimum inhibitory concentrations (MICs) of extracts and compounds were determined by using micro broth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 2021). For this purpose, serial two-fold dilutions of extracts and compounds ranges from 0.02 to 2 mg/ml was prepared in Mueller-Hinton Broth (MHB) and Sabouraud Dextrose Broth (SDB) for bacteria and fungi respectively. The MIC was defined as the lowest concentration of compound or extract giving complete inhibition of visible growth.

The antibacterial activity of the crude extracts and isolated compounds were tested against three gram-positive bacterial strains *Streptococcus agalactiae* (ATCC 12386), *Staphylococcus aurous* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and three gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 700603) (MIC Results in milligram/ml). The antifungal activity was tested against four fungi strains: *Trichophyton mentagrophytes* (ATCC 18748), *Aspergillus niger* (ATCC 10535) Clinical isolate *Candida albican* and *Penicillium spp*.

Active culture of each bacterial and fungi strains were prepared from stored stock culture at 4°C sub-cultured at 37°C overnight on Mueller Hinton Agar and up to 7 days at 25°C on Sabouraud Dextrose Agar (SDA) respectively.

3.7.1.2 Preparation of Fresh Inoculums for Bioactive Test of Crude Extracts and Compounds

Using sterile disposable inoculating loop, few bacterial colonies and fungal growth were transferred from sub-cultured plate to tubes containing sterile MHB for bacteria and Sabouraud Broth (SBD) for fungi. For standardization of test organism, diluted inoculum was prepared in accordance with the CSLI recommendation. Using Spectrophotometer adjusted to 625nm with OD value range from (0.08 to 0.1) which is equivalent to 1.0×10^8 CFU/ml for bacteria and 1.0×10^7 CFU/ml for fungal species, respectively. From the above standardized inoculum 5×10^5 CFU/ml and 5×10^4 CFU/well test organism's suspensions were prepared by dilution in MHB and SDB respectively. To verify the standardization of test procedure, as negative control, 100 µl 2% Tween

80 was used, and as positive control ciprofloxacin and fluconazole was used as reference substances for bacteria and *fungi*, respectively.

3.7.2. DPPH Radical Scavenging Activity Assay

The free radical scavenging activity of root extracts *V. sinaiticum* and isolated compounds were measured using 1, 1 -diphenyl-2-picrylhydrazyl (DPPH) as described by (Amutha& Lalitha, 2015). 10mg of each crude extracts and 5mg of isolated compounds were dissolved in 10 and 5ml of methanol respectively, to have stock concentration of 1mg/ml of both extracts and compounds. For each extracts and compounds 5 sterilized vials were used to prepare serial dilution using the following formula as described by Gerg Anderson:

$$C_1V_1 = C_2V_2$$

V_1= the volume of stock we start with=?

C_1= 1mg/ml in stock concentration

V_2= total volume needed at new concentration =5ml

C_2= the new concentration= 100µg/ml

By algebraic rearrangement: $V1 = \frac{V2 \times C2}{C1}$ it gave us 0.5ml of volume, this volume measured from stock concentration (1mg/ml) in 10ml or 5ml of volume then added in to 4.5ml of methanol in the first vial to get 100µg/ml concentration, by using same formula above, we had prepared the next serial dilution C_2= 50, 25 and 12.5 µg/ml. Briefly, these different concentrations (12.5, 25, 50 and 100 µg/mL) of plant extracts and compounds were prepared. Then to each concentration, 1 mL of DPPH (0.004g in 100ml methanol) was added. It was protected from light by covering the container with aluminum foil. Corresponding blank sample was prepared and ascorbic acid was used as reference standard. Mixture of 1 mL methanol and 1 mL DPPH solution (without plant extract) was used as control. The decrease in absorbance was measured at 517nm after 30 minutes in dark using UV-VIS spectrophotometer. The percentage of inhibition was calculated using the following formula:

$$\textbf{Inhibition \%} = \frac{AC - AS \times 100}{AC},$$ Where,

Ac = is the absorbance of the control reaction

As = is the absorbance of the sample.

3.8 Statistical Data Analysis

Statistical calculation was done using graph pad prism 8 by using nonlinear regression and inhibitor vs dose response parameters.

3.9 Docking and Pharmacokinetics Properties Analysis

Auto Dock Vina 1.5.6 version was used for molecular docking. The stepwise instructions for docking was provided as follows: Using the Chem Office programme, the 2D structures of the test compounds and the standard compounds, ciprofloxacin and ascorbic acid were sketched, converted to 3D structures (.PDB), and then each molecule's energy was minimized. The energy-minimized ligands were then input into the docking procedure (Butt *et al.,* 2020). The targets, *P. aeruginosa* LasR (PDB ID: 3JPU), *S. epidermidis FtsZ* (PDB ID: 4M8I), *C. albicans* CYP51 (PDB ID: 5V5Z) and Human peroxiredoxin 5 (PDB ID: 1HD2) were retrieved from the Protein Data Bank. Bio via Discovery Studio 2020 was used to prepare the targets. The protein complex's water molecules were removed, and the bound ligand was chosen to determine the characteristics of the binding site before being removed from the complex. The target was augmented with polar hydrogen atoms and the necessary charges. Using Auto Doc Vina (MGLTools-1.5.6), the target and ligand were generated in the necessary format (.pdbqt) for docking, and docking was performed (Trott & Olson 2009). Nine conformers and their associated binding energies were generated for each ligand during the docking procedure. Using Biovia Discovery Studio 2020, the conformation with the lowest binding energy was chosen to determine the interactions between the receptor and ligand.

In order to understand the pharmacokinetics and pharmacodynamics properties of a given substance it is better to have understanding how drugs kill or inhibit the growth of microorganisms. In particular the classification of antibiotics is based on their mechanism of action, and the main groups include inhibitors of cell wall synthesis, inhibitors of protein synthesis, inhibitors of nucleic acids synthesis and antimetabolites. Antibiotics inhibit these routes by interacting with specific cell proteins, usually responsible for defined activity (Bertram *et al.,* 2012).

For example, bacterial two-component systems (TCS) and quorum-sensing (QS) systems have been proposed as promising targets for the discovery of novel antibacterial with a new mechanism of action and with lower potential of resistance development in comparison with conventional antibiotics (Velikova *et al.,* 2013, Soto-Aceves *et al.,* 2021, Bem *et al.,* 2015). Histidine kinase (HKs) as sensor part of bacterial TCS can regulate various process such as: growth, vitality,

antibiotic resistance and virulence. Walk/walR which is also known as YycG/YycG are the most remarkable examples of essential TCS (Velikova *et al.*, 2013). Two quorum-sensing systems (las and rhl) regulate virulence gene expression in *Pseudomonas aeruginosa*. The las system consists of a transcriptional activator, LasR, and LasI, which directs the synthesis of acyl-homoserine lactone (AHL), which is one of the signaling molecules in most Gram-negative bacteria such as *P. aeruginosa*. This communication mechanism enables bacteria to act as a community in the coordinated regulation of gene expression. This regulated expression of virulence genes is vital for the organism's pathogenesis (Smith *et al.,* 2003, Soto-Aceves *et al.,* 2021). Therefore targeting these drug target proteins in a specific organism could possible help to combat current bacterial drug resistance problem.

Additionally, inhibiting the bacterial cell division has been also recognized as one of the drug target. A Cytoplasmic protein knowns as FtsZ, is a key molecule in *Staphylococcus epidermidis* bacterial cell division. It has also been recognized as a novel target for antibacterial drugs (Adamas & Errington, 2009). Candidiasis is a fungal infection caused by a yeast (a type of fungus) called *Candida*. Some species of Candida can cause infection in people; the most common is *C. albicans*. (Sahu *et al.,* 2022).

Thus, for the purpose of this research four proteins target with a particular microorganisms were identified, such as: LasR (PDB ID: 3JPU) for *P. aeruginosa;* FtsZ (PDB ID: 4M8I) for *S. epidermidis;* CYP51 (PDB ID: 5V5Z) for *Candida albican*; human peroxidoxins (PDB ID: 1HD2) for antioxidants activity were used as target proteins for docking of identified compounds. Computer models have been nurtured as a valid alternative to experimental procedures for prediction of Absorption, Distribution, Metabolism and Excretion (ADME) (Hay *et al.,* 2014). In this case the pharmacokinetics properties analysis was evaluated using ADMETlab 2.0, new version online tools. Ciprofloxacin, ketoconazole and Ascorbic acid were used as standard drugs while analyzing the conformations of compounds and pharmacokinetics properties.

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1. Extraction Yield

The extraction yield was measured the solvent effectiveness to extract specific components from the original material. In the present study, the dried roots of *Verbascum sinaiticum* was extracted sequentially with n-hexane, ethyl acetate and methanol respectively and the percentage yields of crude extract in respective solvent were listed in Table 3. The highest crude extract was obtained from methanol mixture extract (10%) which followed by EtOAc (1.7%) extract. However, the lowest yield was obtained from n-hexane extract (0.3%). Dried extract of methanol was highly sticky in nature. However, ethyl acetate crude extract was solid in nature and n hexane crude extract was oily. The percentage of yield was calculated using the following formula:

$$percentage\ of\ yeild = \frac{mass\ of\ crude\ extract}{mass\ of\ materials} X\ 100\%$$

Table 3. Calculated Percentage of Yield of Each Crude Plant Materials

No	Solvent used (each 2 L)	Weight of plant materials in gram	Weight of each solvent extract in (g)	Percentage of yield (%)
1	n- hexane	600 g	3	0.5
2	Ethyl acetate		10.19	1.7
3	Methanol		60	10

4.2 Phytochemical Screening

Phytochemical tests were done to determine the class of compounds present in the each crude extracts that could be responsible for activity and/or cytotoxicity. They were identified by characteristic color changes based on standard procedures as described previously. The results were reported as (+) for presence, and (−) for absences in (Table 4). Methanol, ethyl acetate and n- hexane root extracts of *V. Sinaiticum* was screened for the presence of active substance such as alkaloids, flavonoids, glycosides, saponins, steroids and terpenoids.

Table 4. Phytochemical Screening Test for Each Crude Extracts

No		**Crude Extracts**		
	Type of phytochemical screening	**n-hexane**	**Ethyl acetate**	**Methanol**
1	Alkaloids	+	-	+
2	Flavnoids	-	+	+
3	Glycosides	+	-	+
4	Saponins	+	-	+
5	Steroids	+	-	+
6	Terpenoids	+	+	+

+ → phytochemical present

- → Phytochemical absent

The n hexane root extracts of *V. sinaiticum* were screening its phytochemical constituents as shown in Table 4; the result indicated that the presence of alkaloids, glycosides, saponins, steroids and terpenoids but it indicated the absence of flavnoids. The methanol root extracts of *V. sinaiticum* were screening its phytochemical constituents as shown in Table 4; the result indicated that the presence of alkaloids, flavonoids, glycosides, saponins, steroids and terpenoids. This result in agreement with the previous study done on ethanol root extracts of *V. sinaiticum* screening phytochemical constituents by Yeabyo *et al.*, 2018. The ethyl acetate root extracts of *V. sinaiticum* were screening its phytochemical constituents as shown in the Table 4; the result indicated that the presence of flavonoids and terpenoids remaining phytoconstituents such as alkaloids, glycosides, saponin and steroids were showing absent. These secondary metabolites are known to be biologically active and play noteworthy roles in bioactivity of medicinal plants, because the medicinal values of medicinal plant lies in these phytochemical compounds which produce a firm and specific action on the human body.

4.3 Characterization of Isolated Compounds

Silica gel column chromatoaphic separation of methanol root extract of *V. Siniaticum* afforded four iridiod glycosides derivatives compounds named as Pulveruleutoside I (compound-**31)**, harbagosides (compound-**32)**, sinuatol III (compound-**33** and compound-**34**). The structures of

the isolated compounds were characterized by ^{1}H NMR, ^{13}C NMR and DEPT-135 and Comparisons with data in the literature as discussed here below.

4.3.1 Characterization of Compound-31 (Vs 4)

Compound-**31** (15mg) was isolated as crystalline yellowish powder, with *Rf* value of 0.675 in (70% EtOAc: MeOH) as eluent from methanol root extract of *V. sinaiticum*. When the thin layer chromatography was dipping in 1% vanillin with concentrated H_2SO_4 (sulphuric acid) followed by heating at 80-100 ºC for 1-2 min, it visualized as blackish brown color (Appendix 5).

The ^{1}H-NMR δ H (400MHz, Acetone-d6, Appendix 1a, and Table 5) showed resonances of four aromatic protons at δ 7.70 (d, J = 8.6 Hz, 2H) and 7.02 (d, J = 8.7 Hz, 2H), corresponding to the di substituted pattern of phenyl ring. It also exhibited signals corresponding to broad singlet at δ 7.47 (br s, 1H) and doublet at δ 6.42 (1H, d, J=4.02), with the vicinal coupling constant (J=4.02 Hz) is indicated a cis geometry, and the signals were assigned to H-8‴ and H-9‴ respectively. These spectral features observed in the aromatic region in combination with the cis double-bond protons, which are peri to the carbonyl group, were a clear indication of the presence of the cinnamic acid moiety. Whereas, intense signal was observed up field as singlet at δ 3.88, (s) corresponds to the presence of methoxyls group attached to C-4‴ of phenyl ring. The presence of the methoxyl was also confirmed by resonances peak at δ 54.9 in the ^{13}C NMR. Thus, 2 cis olefinic protons at δ 7.47 (br s, 1H) and 6.42 (1H, d, J=4.02Hz); as well as 2 pairs of ortho-coupled aromatic protons at δ 7.70 (d, J = 8.6 Hz, 2H) and 7.02 (d, J = 8.7 Hz, 2H) and 1 aromatic methoxy group at δ (3.88, s) in the ^{1}H NMR spectrum, showed clearly that the presence of a cis-p-methoxycinnamoyl unit. Therefore, the presence of methoxy group at para position of benzene ring causes resonances of aromatic protons, attached at C-3‴ and C-5‴ less de shielded at δ 7.02 (d, J = 8.7 Hz, 2H), than the protons attached at C-2‴ and C-6‴ observed at δ 7.70 (d, J = 8.6 Hz, 2H), because of the lone pair on oxygen are delocalized to C-3‴ and C-5‴ of aromatic ring then each of carbon atom will have a partial negative charge suggested that more electron density at those position than the protons attached C-2‴ and C-6‴ (Simonnin *et al.*, 1972) .

The spectrum also revealed the presence of olfenic protons signals at δ 6.51 (brs, H-3) and 4.86 (1H, d, J = 8.0 Hz,H-4), correlated to the presence of unsaturated aliphatic protons at C-3 and C-4 of aglycone moiety, of which the downfield chemical shift value of the former is in agreement with its attachment to the oxygen atom. Signals were observed at δ 5.16 (m, 2H), and 5.11 (d,

J=9.4, 2H) correlated with the presence of oxygenated methines of anomeric carbon. These data also appeared at δ 93.9 (C-1), 98.7 (C-1′, C1″) in ^{13}C NMR, corresponding to the presence of three anomeric carbon. While, a signal was appeared at δ 5.37 (brs, 1H), corresponding to the presences of oxygenated methines at C-2″ of rhamnose sugar moiety, which esterified with cinnamoyl group. Whereas signal appearing at δ 5.11 (d, J = 9.4, 2H) attributed to the anomeric protons of C-1 of aglycone (iridoid) moiety, which was esterified with glucose sugar molecule suggested that the presences of two sugar units attached to the aglycone (iridoid) moiety of compound **31**. The protons signal at δ 4.07 (1H d, J = 7.1 Hz, H-6) accounted for the presence of oxygenated methine of aglycone, which esterified with rhamnose sugar moiety. Whereas the protons signals were observed at δ 3.68 (br s) and 3.86 (brs,) accounted to the presence of oxygenated methylene (CH_2O-) attached to C-8 and C-5′ of aglycone and sugar moiety respectively.

Moreover, signal appearing at δ 2.88 (brs, C-7) correlated to the presence of oxygenated methines of the epoxide group, this data also supported by the ^{13}C NMR spectra appeared at δ 57.68. The two signals were appeared at δ 1.96 (2H, d, *J*=2.3Hz), and 2.53 (m) correlated to the aliphatic methine protons observed at C-9 and C-5 of the aglycone moiety group. In addition, signal appeared at δ 1.98 (s, 3H) pointed to the protons of methyl's attached to acetyl group, which substituted to the sugar moiety and the up field signal was observed at δ 1.35 (m) corresponding to methyl's attached to the C-5″ of sugar unit, suggested that a sugar unit was substituted rhamnose sugar.

The ^{13}C-NMR (101 MHz, Acetone-d6, Appendix1b, and Table 5) with the help of DEPT 135 confirmed 6 typical signals at δ (96.6, 73.85, 77.2, 76.7, 71.9, and 61.9 ppm) which corresponding to β – glucose and 6 signals at δ (98.7, 71.89, 70.14, 70.6, 69.07, and 17.19 ppm) for substituted rhamnose sugar moiety. The presence of these sugar units were confirmed by the ^{1}H-NMR spectrum. The remaining signals correlated to those reported for P-methoxycinnamoyl ester 10 signals at δ (129.9, 130.1, 114.4, 161.9, 114.4, 130.1, 115.9, 145.3, 165.8 and 54.9 ppm) and iridoid group 9 signals at δ (93.9, 140.9, 102.4, 35.9, 83.1, 57.7, 65.2, 42.3 and 60.5 ppm) as well as 2 signals at δ (169.7 and 20.0) for acetyl group. The most downfield and up field signals appearing at δ 169.7, 165.8 and 20.0, 17.2, attributed to the carboxyl esters group and methyl's, respectively.

The ^{13}C-NMR in combination with DEPT 135 spectra, confirmed the presence of well resolved resonance of 33 carbons. The multiplicity of each carbon atom was determined by using DEPT-

135 spectrum (Appendix 1c), which showed the presence of two oxygenated quaternary carbon was observed at δ 65.2(C-8), and 161.8 (C-4″), whereas ten (10) signals attributed to oxygenated methines appearing at δ 93.9 (C-1), 98.7 (C-1′), 96.6 (C-1″), 83.1 (C-6), 77.7 (C-5′), 73.8 (C-2′), 76.7 (C-2″), 70.6 (C-3″), 71.8 (C-.4″) and 70.1 (C-4′). The spectrum also revealed six (6) aliphatic methines at δ 145. 3 (β-C), 141.0 (C-3), 115.9 (α-C), 102.4 (C-4), 42.3 (C-9) and 35.9 (C-5). Four (4) aromatic methines were observed at δ 114.4 (C-3″, C-5″) and 130.1ppm (C-2″, C-6″).

The most downfield signals appearing at δ 165.8 and 169.7 were attributed to the carboxyl ester of the cinnamoyl and actyl moiety respectively. The highly downfield-shifted carbon signal at δ 165.8 and the two olefinic carbon atoms at δ 145.7 and 115.9 were evident for the presence of the α,β-unsaturated carbonyl group of the cinnamoyl moiety. Whereas signal at δ 161.8 (C-4″) correlated to the oxygenated quaternary carbon at (C-4″) of phenyl ring, at which oxygenated methyl group attached to it. Weak signal was observed at δ 129.9 (C-1″) attributed to aliphatic quaternary carbon at C-1″ of phenyl ring, while a sharper signal observed at δ 65.22(C-8) attributed to the aliphatic quaternary carbon due the presence of epoxy group of alicyclic structure of aglycone (iridoid) moiety. The two signals appearing at δ 61.9 (C-6′) and 60.6 (C-10) were due to the presence of oxygenated methylene (also supported by DEPT-135 pointing down, appendix 1c) whereas two signals appeared at δ 20.0 (C-8″) and 17.1 (C-7′) ppm attributed to the presence of two methyl (-CH_3) of compound **31**. Therefore, the above spectral data is in good agreement with data reported for pulveruleutoside I, previously reported from roots of *Verbascum lasianthum,* same genus of plant (Akdemir *et al.,* 2004, Alaniya *et al.,* 2014) (Table 5). The proposed structure of compound **31** depicted below in (Figure 7).

Table 5. ^{1}H-NMR (400 MHz, Acetone-D6), ^{13}C and DEPT-135 (101 MHz, Acetone- D6) spectral data of compound-**31** and reported literature (Pulveruleutoside I) in CD_3OD 500 MHz).

Positon	Carbon Atom	Compound-**31**		(Pulveruleutoside I) (Akdemir *et al.*, 2004)	
		^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	CH	5.11 (d, J=9.4 2H)	93.9	5.01 †	94.0
3	CH	6.51 (brs,1H)	141.0	6.43 d	142.0
4	CH	4.86 (d, J = 8.0 Hz, 1H)	102.4	5.12 d	103.0
5	CH	2.53 (m),	35.9	2.31 m	36.3
6	CH	4.07 (d, J = 7.1 Hz, 1H)	83.1	3.95 d	83.0
7	CH	2.88 (brs)	57.7	2.43 m	58.2
8	C	…	65.2	----	66.3
9	CH	1.98 (s)	42.3		42.8
10	CH_2	3.68 (brs)	60.6	3.66 †	59.5
β- D- Glu					
1′	CH	5.16 (m, 2H)	98.7	4.59 d	98.7
2′	CH	3.34 (m)	73.8	3.03 †	74.3
3′	CH	3.40 (d, J=7.6Hz, 1H)	76.7	3.16 †	77.3
4′	CH	3.32 (t, J=8.3Hz)	71.9	3.25 (t, 9.1)	71.1
5′	CH	3.34 (m)	77.2	3.14 †	78.3
6′	CH_2	3.86 (brs)	62.0	3.73 †	62.2
Rha					
1″	CH	5.16 (m, 2H)	96.6	5.00 brs	96.7
2″	CH	5.37(brs)	69.1	5.22 †	72.4
3″	CH		70.6	4.98 †	70.2
4″	CH	3.46 (s)	70.1	3.46 †	70.1
5″	CH	3.68 (brs)	69.1	3.78 †	69.6
6″	CH_3	1.35 (d, J=6.2,1H)	17.2	1.24 d	18.5
Acyl moiety					

1‴	C	…..	129.9	….	127.3
2‴	CH	7.70 (d, J = 8.6 Hz)	130.1	7.73 d	131.3
3‴	CH	7.02 (d, J = 8.1 Hz	114.4	6.97 d	115.3
4‴	C	…	161.9	…	162.2
5‴	CH	7.02 (d, J = 8.7 Hz)	114.4	6.97 d	115.3
6‴	CH	7.70 (d, J = 8.6 Hz)	130.1	7.73 d	131.3
8‴α	CH	6.42 (1H, d, J=4.02Hz)	115.9	6.56 d	115.4
9‴β	CH	7.47 (brs)	145.3	7.65 d	146.0
10‴C=O	C	…	165.9	...	162.7
7‴O**CH₃**	CH_3	3.88 (s)	54.9	3.80 s	56.2
O**C**OCH_3	C	……	169.7	…….	170.8
OCO**CH₃**	CH_3	1.96 (s)	20.0	1.94 s	21.6

†= unclear

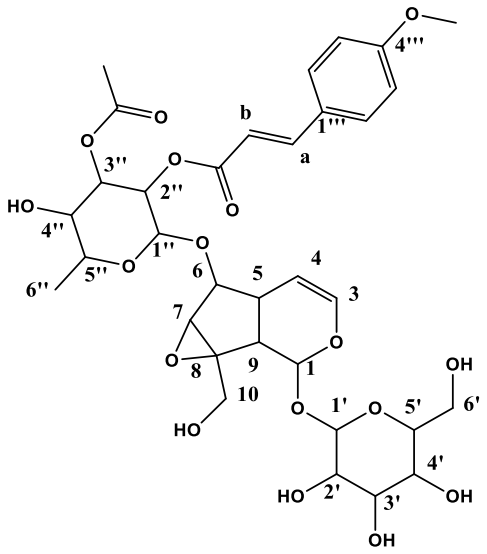

Figure 7.The proposed structure of compound-**31** (Vs-4)

4.3.2 Characterization of Compound-32 (Vs-12)

Compound-**32** (20 mg) was obtained as amorphous brown powder with *Rf* of 0.594 in 70% EtOAc/ MeOH as eluent isolated from methanol extracts. When the sample was developed on thin layer chromatography and then dipped in 1% vanillin with concentrated sulphuric acid (H_2SO) followed by heating at 80-100 ºC for 1-2 min, it visualized as pink color (Appendix 5).

The ^{1}H-NMR δ H (400MHz, acetone-d6, Appendix 2a, Table 6) indicated the presence of five protons in the aromatic region at δ 7.70 (3H, d, J = 10.2 Hz) and 7.45 (2H, d, J = 5.3 Hz), suggested that the presence of a mono substituted phenyl ring. It also exhibited signals corresponding to broad singlet at δ 7.78 (br s, 1H) and doublet at δ 6.52 (2H, d, J=16), with the vicinal coupling constant (J=16Hz) is indicated a trans geometry, and the signals were assigned to H-7 and H-8, respectively. These spectral features observed in the aromatic region in combination with the trans double-bond protons, which are peri to the carbonyl group, were a clear indication of the presence of the cinnamoyl moiety. Additionally, the presence of signal with AB multiplicity pattern at δ 6.43 (2H, d, J = 6.4 Hz, H-3) and δ 4.66 (2H, d, J = 7.8 Hz, H-4) were due to the presence of unsaturated aliphatic protons attached to C-3 and C-4 of pyran ring of iridoid (aglycone moiety). The spectrum also revealed the presence of two peaks at δ 4.94 (d, J = 6.6 Hz) and 5.11 (d, J = 10.1 Hz, H-1,) were attributed to H-1 of iridoid and H-1′, H-5′ of glucose respectively, suggested that the presence of two anomeric protons. These data, also confirmed by ^{13}C-NMR at δ 98.44 (C-1′) and 93.95 (C-1). The protons signals at δ 3.43 (1H, dd, J = 18.3, 9.3 Hz, H-2′), 3.34 (1H, t, J = 8.5 Hz, H-4′) and 3.24 (1H, t, J = 8.5 Hz, H-3′) accounted for the presence of oxygenated methine protons connected to oxygen whereas, the signals appeared at δ 2.96 (s) and 1.97 (d, J = 8.3 Hz, 2H) attributed to aliphatic methines protons at C-9 and C-7 of the aglycone moiety of compound **32.** Whereas a signal appering at δ 2.29 (1H, d, J = 15.1 Hz) attributed to H-5 of pyran ring of aglycone moiety (iridoid). Moreover, signals appeared at δ 1.53, (s, 3H) and 1.30 (s, 4H), ascribed to the protons of two methyls group attached at C-8 and C-7 of the aglycone moiety (iridoid).

The ^{13}C-NMR (101Mz, Acetone-D6, Appendix 2a, Table 6) spectrum in combination with DEPT-135 showed well resolved resonance of 23 carbon atoms including nine for aglycone moiety (iridoid), five for one sugar units and the remaining nine for an aromatic acid moiety with their interpretations. Which showed the presence of three quaternary carbon was observed at δ 166.5 (C =O), δ 134.6 (C-1″) and 87.1 (C-8), six oxygenated methines appears at δ 98.4 (C-1′), 93.9 (C-, C-5′), 76.9 (C-2′), 73.6(C-4′) and 71.2 (C-3′). The spectrum also showed six aliphatic methines at δ 144.0(C-8β), 142.2 (C-3), 119.4 (C-7α), 102.45 (C-4), 54.5 (C-9) and 44.7 (C-7), while five aromatic methines were observed at δ 130.2 (C-4″), 128.9 (C-3″, C-5″) and 128.2 (C-2″, C-6″). The methine carbons of the pyran moiety were observed at δ 142.2 (C-3) and 102.4 (C-4), of which the downfield chemical shift value of the former is in agreement with its attachment to the oxygen atom. The most downfield signals appearing at δ 166.5 are attributed to the carbonyl ester.

Therefore, The highly downfield-shifted carbon signal at δ 166.5 and the two olefinic carbon atoms at δ 144.0 and 119.5 were evident for the presence of the α,β-unsaturated carbonyl group of the cinnamic acid moiety. Signals appearing at δ 134.5 (C-1″) and 87.1 (C-8) correlated to the presences of aliphatic quaternary carbon, whereas the up field signal at δ 18.5 and 21.9 suggests the two methyl group attaced to C-7 and C-8 of aglycone moiety of compound **32**. Thus, the above spectral data is in good agreement with data reported for Harpagoside, previously reported from roots of *Verbascum laxum*, same genus of plant, where the oxygenated methylene group at C-3′ is reduced to hydroxyl group and a proton at C-7 substituted with methyl group in case of proposed structure of compound **32** (Alaniya *et al.*, 2014, Akdemir *et al.*, 2005,) (Figure 8, Table 6)**.** The proposed structure of compound **31** depicted below in (Figure 8).

Table 6. ^{1}H-NMR (400 MHz, Acetone-D 6), ^{13}C and DEPT-135(101 MHz, Acetone-D 6) spectral data of compound **32** and reported literature (Harbagoside) in CD_3OD on an Avance DRX 500 FT instrument (Bruker).

Positon	Compound **32**		Reported Literature (Harbagoside) (Aslaniya *et al.*, 2014, Akdemir *et al.*, 2005,)	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	5.11 (1H, d, J = 10.1 Hz)	93.9	6.21 (1H, d, J = 4.5, H-1)	93.2
3	6.43 (1H, d, *J* = 6.4 Hz,)	142.2	6.44 (1H, d, J =6.4, H-3)	142.4
4	4.66 (1H , d, J = 7.8 Hz,)	106.4	4.96 (1H, d, J = 6.4, H-4)	105.4
5	2.29 (1H, d, *J* = 15.1 Hz)	71.2	2.30 (1H, d, J = 14.5, H-7)	72.1
6	3.67 (brs)	76.9	3.79 (1H, d, J = 4.0, H-6)	78.14
7	1.97 (2H, d, *J* = 8.3 Hz)	44.7	2.03 (1H, dd, J = 14.5, 4.0, H7α)	44.7
8	…	87.2	…	87.1
9	2.96(1H, brs)	54.5	2.97 (1H, d, J = 4.5, H-9)	54.1
10	1.53 (3H, s)	21.9	1.56 (3H, s)	21.4
11	1.30 (4H, brs)	18.5	---	….
Glucose				
1′	4.94 (d, J = 6.6 Hz)	98.4	4.66 (1 d, J = 8.0, H-1)	98.5
2′	3.43 (dd, J = 18.3, 9.3 Hz, 1H)	76.9	3.25–3.38 (4H, m)	76.2

3′	3.24 (1H, t, J = 8.5 Hz)	71.2	3.25–3.38 (4H, m)	70.1
4′	3.34 (1H, t, J = 8.5 Hz)	73.6	3.25–3.38 (4H, m)	76.6
5′	5.11 (1H, d, J = 10.1 Hz)	93.95	…	…
Acyl Moiety				
1″	…	134.6	…	134.0
2″	7.70 (d, J = 10.2 Hz, 2H)	128.3	7.61 (2H, d, J = 8.4)	127.7
3″	7.45 (d, J = 5.3 Hz, 2H)	128.9	7.42 (2H, d, J = 8.4)	128.5
4″	---	130.2	…	…
5″	7.45 (d, J = 5.3 Hz, 2H)	128.9	7.42 (2H, d, J = 8.4)	128.5
6″	7.70 (d, J = 10.2 Hz, 2H	128.3	7.61 (2H, d, J = 8.4)	127.7
H-α	6.52 (d, *J* = 16.0 Hz, 1H)	119.4	6.52 (1H, d, J = 16.0, H-α)	118.8
H-β	7.78 (brs,1H)	144.0	7.68 (H, d, J = 16.0, H-β)	145.0
C=O	…	166.5	…	157.1

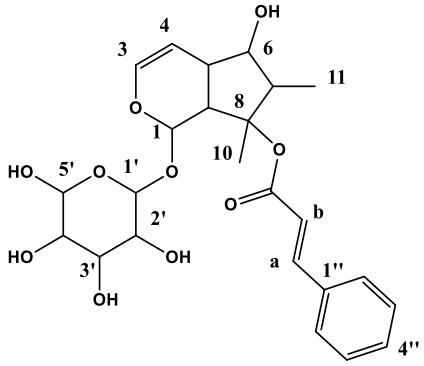

Figure 8. The proposed structure of compound-**32** (Vs-12)

4.3.3 Characterization of Compound-33 (Vs 20)

Compound-**33** (20 mg) was isolated as amorphous brown powder with R_f of 0.378 in (70% EtOAc: MeOH) as eluent from methanol extracts. When the thin layer chromatography was dipping in 1% vanillin with concentrated sulphuric acid H_2SO_4 followed by heating at 80-100 ºC for 1-2 min, it visualized as reddish brown color (Appendix 5).

The ^{1}H- NMR (400 MHz, DMSO- D6, Appendix 3a, Table 7) showed two olefinic protons signals at δ 6.29 (*brs*) and 4.32 (m) attributed to H-3 and H-4 the pyran ring of aglycone moiety

respectively, of which the former is down field in agreement with its attachment with oxygen atom. Signal observed at δ 5.63 (1H, brs, H-7) attributed to additional olefinic protons attached to C-7 of the iridoid moiety, suggesting that aglycone was aucubin type of iridoid (Thabeta *et al.*, 2022). Signal observed at δ 4.91 (m) and 4.32 (m), corresponds to the presence of anomeric protons of glycone and aglycone molecule attached to C-1, C-1′ and C-1″ of compound **33,** respectively. The corresponding anomeric carbon resonances peak at δ 97.3 (C-1′, C-1), 98.42 (C-1″) and 92.6 (C-3″) respectively also confirmed the presences of two sugar moiety which, attached to C-1 and C-6 of iridoid moiety. Very intense peak appearing at δ 3.81(brs) and 3.64 (m) accounted to the protons of oxygenated methines and oxygenated methylene respectively. A peak at δ 3.81 (brs), accounted to the protons of oxygenated methines attached to C-6″, 6′ and signal appearing at δ 3.64 (m) attributed the protons of oxygenated methines attached to C-4″, C-4′ of the two glycone moiety of compound **33**. While, signals observed at δ 3.64 (m) were correlated to the presence of oxygenated methylene, these signals were also confirmed by DEPT 135 NMR data pointing down at δ 63.37, 61.63 and 59.90. The two signals appearing as broad singlet at δ 2.69 (brs, H-5) and doublet at 3.07 (1H, d, J=3.07, H-9), were corresponds to the aliphatic methines at C-5 and C-9 of the aglycone of the compound-**33**.

The ^{13}C-NMR (101Mz, DMSO D-6, Appendix 3a, Table 7) with the aid of DEPT-135 spectra, confirmed the presence of well resolved resonance of 21 carbons, including 9 for iridoid aglycone moiety, twelve for two sugar units with their interpretations. The spectrum at δ 140.42 (C-3) and 129.52 (C-7) is indicate the presence of two pair of unsaturated aliphatic carbons within the iridoid molecule, suggestive that the iridoid was aucubin type. These spectral data also confirmed by ^{1}H NMR. The multiplicity of each carbon atom was determined by using DEPT-135 spectrum from the data (Appendix 3c), which exhibited the presences of one quaternary at δ 145.5 (C-8) whereas, oxygenated methines were observed at δ 97.3 (C-1″, C-1′), 92.6 (C-1), 77.1 (C-5′,C-5″), 71.0 (C-4′), 70.7 (C-4″) 75.3 (C-3″) 73.5 (C-3′). The spectrum also showed the aliphatic methines at δ 140.42 (C-3), 105.4 (C-4), 129.5 (C-7), 46.8 (C-9) and 44.9 (C-5). The methine carbons of the pyran ring were observed at δ 140.4 (C-3) and 105.4 (C-4), of which the downfield chemical shift value of the former is in agreement with its attachment to the oxygen atom. Whereas olfenic carbon of cyclopentane was observed at δ 129.5 (C-7). The presences of three oxygenated methylene was appeared at δ 63.4 (C-6″), 61.6 (C-6′) and 60.0 (C-10) (Appendix 3c, pointed down). Thus, the above spectral data is in good agreement with data reported for Sinuatol-III, previously reported

from aerial part of *Verbascum sinuatum*, same genus of plant, where the methyl group at C-8 is oxidized to hydroxymethyl group (-CH_2OH) group in case of compound **33** (Bianco *et al*., 1981, Thabeta *et al.,* 2022) (Table 7, figure 9. The proposed structure of compound **33** depicted below in (Figure 9).

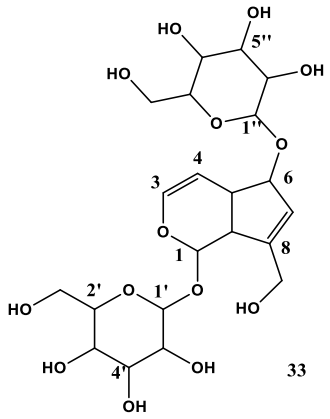

Figure 9. The proposed structure of compound-**33** (Vs-20)

4.3.4 Characterization of Compound-34 (Vs 28)

Compound-**34** (15 mg) was obtained as amorphous brown powder with an R_f value of 0.324 in (70% EtOAc: MeOH) as eluent isolated from methanol extracts. When the thin layer chromatography was dipping in 1% vanillin with concentrated sulphuric acid (H_2SO_4) followed by heating at 80-100 ºC for 1-2 minutes, it visualized as pink color (Appendix 5).

The ^{1}H- NMR (400 MHz, DMSO- d6, Appendix 4a, Table 7) indicated the presence of olfenic protons at δ 6.25 (1H, d, J = 16.7, 6.4 Hz) and 4.26 (2H, d, J=8.4Hz), attributed to the H-3 and H-4 of pyran ring of algycone moiety. The two signals were observed at δ 5.54 (1H, brs) and 4.38 (1H, d, J = 7.8 Hz) corresponding to the presence of anomeric protons of the glycone moiety. The corresponding anomeric carbon resonances at δ 97.30 (C-1′), 97.91 (C-1″) and 92.65 (C-1) where confirmed the presences of glycone molecule, suggested that the presence of two sugar molecule of compound-**34**. Very broad intense peak appearing at δ 3.60 (brs) accounted to the protons of Oxygenated methines of two glycone moiety of compound **34**. Whereas signals observed as broad singlet at δ 3.54 (brs) were correlated to the presence of oxygenated methylene, these signals were also confirmed by intense peak pointing down at δ 61.63 (confirmed by DEPT 135 NMR 4c), suggested the presences of two oxygenated methylene. Signals appearing as triplet at δ 2.88 (1H, t, J = 8.2 Hz, H-5) and 3.07 (3H, dd, J = 20.7, 8.9 Hz, H-9), were corresponds to protons of the

aliphatic methines at C-5 and C-9 of the aglycone moiety. The peak appearing at δ 1.70 (d, J = 5.0 Hz, 2H) accounted for the protons of aliphatic methylene at C-7 of aglycone moiety. Whereas a observed at δ 1.07 (3H, s) accounted to the presence of methyl group.

The ^{13}C-NMR (101Mz, DMSO d-6, Appendix 3a, Table 7) with the aid of DEPT-135 spectra, confirmed the presence of well resolved resonance of 21 carbons, including 9 for iridoid aglycone moiety, twelve for two sugar units with their interpretations. A signal appearing at δ 140.42 corresponding to the presence an aliphatic unsaturated carbons at C-3 of aglycone moiety (iridoid), indicative that the iridoid was ajugol type (Thabeta *et al.*, 2022). The multiplicity of each carbon atom was determined by DEPT-135 spectrum (appendix 4c) which showed the presences of one quaternary carbon at δ 76.7 (C-8). It also exhibited the presences of oxygenated methines at δ 97.9 (C-1″), 97.3 (C-1′), 92.6 (C-1), 75.3 (C-3′, C-3″), 77.2 (C-5′, C-5″), 73.5 (C-2′), 72.8 (C-2″), 70.7 (C-4″), 71.0 (C-4′). The spectrum also displayed the presence of the aliphatic methines at δ 140.4 (C-3), 109.0 (C-4), and 58.8 (C-7). The methine carbons of the pyran ring structure were observed at δ 140.4 (C-3) and 109.0 (C-4), of which the downfield chemical shift value of the prior is in agreement with its attachment to the oxygen atom. Very intense peak pointing down observed at δ 61.6 (C-6″, C-6′) correlated to the two oxygenated methylene group. Whereas, up field signals appearing at δ 47.4 (C-7) (Appendix 4c, pointed down) attributed to aliphatic methylene. The most up field signal appearing at δ 25.21 is indicated to methyl group attached at C-8 of the aglycone moiety.

Moreover, ^{1}H, ^{13}C NMR and DEPT-135 data of compound-**33** and compound-**34** showed signals very similar to each other the only difference was the presence of additional signals δ 145.5 and 129.5 in case of compound-**33,** attributed to the presence of aliphatic quaternary carbon at C-8 and an olifenic proton at C-7 of aglycone moiety. Suggested that the aglycone moiety of compound **33** was acuabin type of iridoid structure. But, aglycone moiety of compound **34** was ajugol type of iridoid because of the absence of an olfenic proton at C-7 of compound **34.** Additionally, compound-**34** showed signals at δ 75.73 and 25.21 attributed to the quaternary carbon at C-8 and methyl group attached at same position of aglycone molecule of its structure. Furthermore, both compounds were showed signals very similar to those of acuabin and ajugol type of iridoid respectively, with additional signals arising from glucose (Mahmoud, S.M. *et al.*, 2007, Thabeta *et al.*, 2022). The above spectral data is in good agreement with data reported for Sinuatol-III,

previously reported from aerial part of *Verbascum sinuatum*, same genus of plant, where the methine group at C-7 is reduced to methylene and hydroxyl group (-OH) group attached at C-8 in case of compound **33.** (Bianco *et al*., 1981) (Table 7, Figure 10). The proposed structure of compound **34** depicted below in (Figure 10).

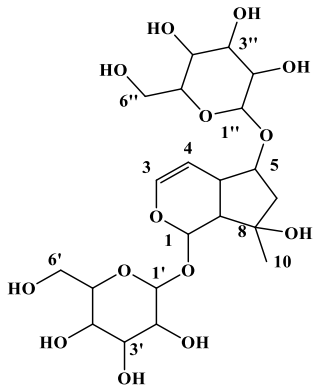

Figure 10. The proposed structure of compound-**34** (Vs-28)

Table 7. ^{1}H-NMR (400MHz, DMSO-D6), ^{13}C and DEPT-135(100Mz, DMSO-D6) spectral data of compound-**33** and Compound-**34** in comparisons with Literature (Sinuatol III) in D_2O on 20MHz Varian CFT-20 instrument.

Position	Compound **33**		Compound **34**		(Sinuatol III)(Bianco *et al*., 1981,)	
	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR
1	4.32(m, 3H)	97.3	4.38 (1H ,d, J=7.8, H-4	92.65	….	96.69,
3	6.29 (brs)	140.4	6.25 (2H, d, J = 6.2,Hz, H-3)	140.4	6.38, dd J = 6.0, 1.6 Hz)	140.84
4	4.32(m)	105.4	4.26 (2H, d, J=8.4Hz))	109.0	---	105.76
5	2.69(brs)	44.9	2.88 (1H, t, J = 8.2 Hz, H-5)	40.1	2.98, brs	41.61
6		92.6		91.9		88.27
7	5.63(brs)	129.5	1.70 (d, J = 5.0 Hz, 2H)	47.5	5.98, brs	126.86
8	---	145.5	----	75.7	---	149.23
9	3.07(d, J=25.8HZ)	46.8	3.07 (3H, dd, J = 20.7, 8.9 Hz, H-9)	58.7	3.10, brd	47.38
10	3.54 (brs)	61.7	1.07 (3H, s)	25.2	---	60.37

Glucose						
1´	4.91 (m)	98.41	5.54 (1H, brs)	97.3		99.32
2´		73.78		73.5		73.56
3´		77.14	3.60 (brs)	77.7		76.97
4´	3.64(m)	70.97	3.60 (brs)	70.9		70.40
5´	3.81(brs),	77.56		77.7		76.49
6´		63.37	3.54 (brs)	61.6		61.45
Glucose						
1″	4.91 (m)	102.39	5.54 (1H, brs)	97.9		100.40
2″		72.76		72.8		71.20
3″		72.37	3.60 (brs)	72.4		71.01
4″	3.64(m)	73.78		73.5		72.87
5 ″	3.81(brs),	70.47		70.1		69.81
6″		…	3.54 (brs)	61.6		17.40

4.4 Antibacterial and Antifungal Activity of the Extracts and Isolated Compounds

The MIC of the plant *V. sinaiticum* extracts was determined for all the organisms using broth dilution assay and all the antibacterial and antifungal experiment was performed in triplicate. Using different sterilized vials the n- hexane, ethyl acetate, methanol extracts and four isolated compounds (each 8mg) were dissolved in 2% Tween 80 in distilled water V/V dilution preparations, with stock concentration of 4mg/ml. 100 µl Mueller Hinton Broth (MHB) and Sabouraud Dextrose Broth (SDB) were pipetted to each pre-labelled 96- well micro titter- plate and prepared in triplicate form for bacteria and fungi respectively.

The first row (Row-A) of the 96- well plate from well- 1 to 7, (well-1, methanol extract, well-2, ethyl acetate extract, well -3 n- hexane extract , well-4 compound-**31** (Vs 4), well – 5 compound-**32** (VS-12), well -6 compound-**33** (VS-20), well -7 Compound-**34** (VS-28)) were filled with 100 µl dissolved extracts and compounds in 2% Tween 80 from each 4mg/ml vials then resulted with

2mg/well concentration in each well. Similarly, well- 8 and 9 were filled with 100 µl dissolved 25µg/ml Ciprofloxacin standard, serves as a positive control whereas, (Well- 9 were filled with 100 µl dissolved 100µg/ml Ketoconazole standard drug for fungus. Likewise, Well- 10 negative control filled with 100 µl 2% Tween 80. Micro Well-11, left freely for growth control to be filled after standardization of organisms, and Well-12 pipetted with 100 µl broth medium for sterility control (Figure 11).

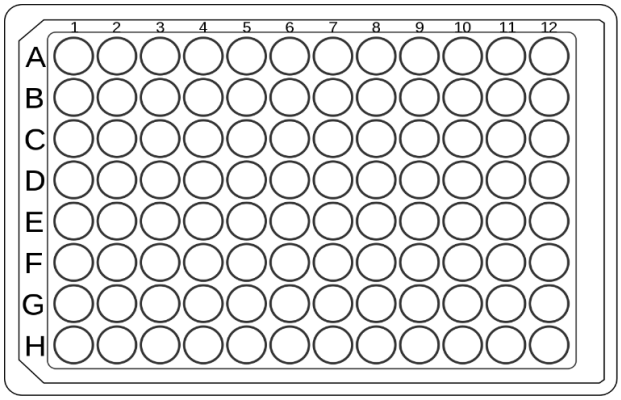

Figure 11. 96- Well micro titter plate

Then all the **(**Row-A) wells having 200 µl volumes which is ready for serial dilution. Within 15 minutes of standardization 100 µl, 5 x10^5 CFU bacterial and 5 × 10^4 CFU fungal test suspensions were applied to each well, except the sterility control (column 12**).** After 18-24 hr incubation at 37^{o}C, all the microtiter plate was examined for visible bacterial growth by adding 40 µl of a 0.2 mg/ml solution of 2, 3, 5-Triphenyltetrazolium chloride (TTC; Tetrazolium chloride) to each well as an indicator of microbial growth and incubated at 37 °C for 30 min. After 30 min incubation, the MIC values were determined visually by observing the presence or absence of pink color. Growth of bacteria was detected by formation of a pink-red coloration while inhibition of growth was signaled by persistence of a clear coloration. The lowest concentration that exhibited color change was considered as the MIC (Figure 13).

Methanol extract of root of *V- sinaiticum* was showed a promising antibacterial activity with lowest MIC of 0.5 mg/ml against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. This study showed a better result in compared to a previous study done on the methanolic flower extracts of *V.sinaiticum* which showed MIC of (1000 µg/ml) against *Pseudomonas aeruginosa* (Talib &Mahasneh, 2010). This is suggestive that the root part of *V. sinaiticum might* contains several bioactive compounds, that can used for the treatment disease caused by Gram positive or

Gram negative bacteria, this data also supported by phytochemical screening data of this study, which indicate the presence bioactive compounds responsible for such antibacterial activity (Table 1).

The ethyl acetate crude extract exhibited interesting antibacterial activity with MIC of 2mg/ml against *Staphylococcus epidermidis*. Whereas, methanol and ethyl acetate extracts displayed remarkable antibacterial activity with MIC of 0.5 and 1 mg/ml against *Pseudomonas aeruginosa* respectively. It is generally expected that more plant extracts would be active against Gram positive bacteria compared with Gram negative bacteria (McCutcheon *et al.,* 1992). However, in the present study, plant extract showed activity against both Gram positive and Gram negative bacteria. The lowest MIC of (>2 mg/ml) was recorded for n- hexane extracts against all tested organism. This is in agreement with previous studies that the n-hexane flower extracts of *V.sinaiticum* had MIC of (>1000 µg/ml) against *(Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella typhimurium*) (Talib &Mahasneh, 2010). Therefore, the result of this study showed that the antibacterial activity is mainly concentrated in the methanol extracts, indicating that the potential antimicrobial compounds were in the high polarity fractions. However, the positive control drugs in all cases had higher activity than the plant crude extract.

The lowest MIC of 0.5 mg/ml and 2 mg/ml was recorded in Compound **31** against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* respectively. Compared to methanol extract compound **31** had better activity against *K. pneumoniae*. It is suggestive that, there is possibility of antagonism effect of various antibacterial active compounds when taken together as in crude extracts (Ambassa *et al.*, 2011). This is therefore indicative that, fractions are the best candidates for the treatment of diseases associated with the tested microorganisms than crude extracts.

Compound **32 and 31** were showed promising antibacterial activity against *Pseudomonas aeruginosa* with MIC of 0.5 mg/ml. Likewise, compound **34** and **33** demonstrated interesting antibacterial activity with MIC of 1 mg/ml against *P. aeruginosa.* Besides, compound **34** and **32** indicated the MIC of 1 mg/ml and 2 mg/ml against *Staphylococcus epidermidis,* in compared to methanol extract with MIC of 0.5 mg/ml (Table 8) of compound **33 and 34** showed lower activity against *Staphylococcus epidermidis*. This may imply that, constituents act together in a synergistic manner and that is why crude methanol extract had higher activity against both *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Therefore, explaining for low antibacterial activity in fractions extracts. Generally, the three organic roots extracts of *V. sinaiticum* shown moderate

broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria compared to standard drug ciprofloxacin (Table 8). This is in agreement with previous studies done on root and leaf extracts of *Verbascum sinaiticum* were active against Gram positive bacteria and Gram negative bacteria (Yeabyo *et al*., 2018, Afifi *et al.,* 1993). Furthermore, the positive control drugs (ciprofloxacin) in all cases had higher activity than the isolated compounds (Table 8).

Table 8. MIC for *V. sinaiticum* Crude Extracts and Isolated Compounds against Both three Gram Negatives and three Gram positive Bacteria.

Crude extracts and compounds	**MIC in mg/ml**					
	Gram negative bacteria			**Gram positive bacteria**		
	E. coli	*P. aeruginosa*	*K. pneumoniae*	*S. aureus*	*S. epidermidis*	*S. agalactiae*
Methanol Ext.	2	0.5	>2	2	0.5	2
Ethyl acetate Ext.	>2	1	>2	>2	2	>2
n hexane Ext.	>2	>2	>2	>2	>2	>2
Compound **31**	>2	0.5	2	>2	>2	>2
Compound **32**	>2	0.5	>2	>2	2	>2
Compound **33**	>2	1	>2	>2	>2	>2
Compound **34**	>2	1	>2	>2	1	>2
Ciprofloxacin (µg/ml)	0.39	0.78	0.089	0.19	0.19	0.19
2% Tween 80	NA	NA	NA	NA	NA	NA

Ext. extracts, Comp. =Compound, MeOH =Methanol, EtAOc = ethyl acetate, n-Hex = normal hexane, MIC= Minimum Inhibitor Concentration, NA= no activity

The MIC of the plant *V. sinaiticum* extracts and isolated compounds was determined for antifungal activity. The lowest MIC of 2 mg/ml was noted both for methanol crude extract and compound-

32 against *C. albicans.* The lowest MIC of 2 mg/ml was determined for ethyl acetate extract against *T.mentagrophytes.* Thus, methanol and ethyl acetate root extracts of *V. siniaticum* might contain potential antifungal compounds. (Table 9).

Table 9. MIC for *V. sinaiticum* Crude Extracts and Isolated Compounds against Four Fungi Strains

Crude extracts and compounds	**MIC in mg/ml**			
	Types of fungi strains			
	C. albicans	*Penicillium species*	*T.mentagrophytes*	*A. niger*
Methanol	2	>2	>2	>2
Ethyl acetate	>2	>2	2	>2
n hexane	>2	>2	>2	>2
Compound **31**	>2	>2	>2	>2
Compound **32**	2	>2	>2	>2
Compound **33**	>2	>2	>2	>2
Compound **34**	>2	>2	>2	>2
Ketoconazole (µg/ml)	50	50	25	100
2% Tween 80	NA	NA	NA	NA

4.5 Radical Scavenging Assay

In the present study, root extracts of *V. sinaiticum* in three different solvents (n-hexane, ethyl acetate and methanol) and four isolated compounds of methanol extracts were tested for their free radical scavenging ability by using DPPH assay and it was observed that the plant extracts and compounds were showed good potency for scavenging free radicals as shown in (Table 10).

Table 10. Free Radical (DPPH) scavenging activity (%) of Root Extract of *V. sinaiticum* and Isolated Compounds at Different Concentrations

Tested samples	**Concentration in (µg/ml)**				**IC_{50}**
	12.5	**25**	**50**	**100**	
n- hexane	61.53	64.1	66.66	69.23	1.75
Ethyl acetate	53.84	56.41	61.53	64.1	2.88
Methanol	22.82	43.58	51.28	58.97	20.98
Compound **31**	56.41	59.97	61.53	66.66	3.79
Compound **32**	58.97	64.53	69.28	72.25	3.33
Compound **33**	50.41	61.53	64.1	66.66	4.58
Compound **34**	53.81	56.41	58.97	69.28	3.84
Ascorbic acid	84.50	85.45	87.75	89.56	0.72

Both extracts and compounds were tested on a concentration ranges (12.5-100 µg/mL) and it was found that the activity altogether increased with increase in concentration of plant root extracts and compounds. The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. In all cases, n-hexane extracts demonstrated to be better antioxidants than the corresponding methanol and ethyl acetate extracts. The results showed that the DPPH free radicals were scavenged by all plant extracts in a concentration dependent manner. Moreover, the IC_{50} values for DPPH radicals with methanol, ethyl acetate and n-hexane extract of the *V. sinaiticum* were found to be 20.98, 1.75 and 2.88 µg/ml, respectively. Interestingly, the IC_{50} value of n-hexane extract (1.75 µg/ml) was less than methanol and ethyl acetate, indicating that n-hexane extract was a more potent scavenger of free radicals than methanol and ethyl acetate extracts. However, it is greater than the standard drug ascorbic acid with IC_{50} (0.72 µg/ml). This result, in agreement with the previous study done on one of the plant species *Verbascum letourneuxii* DPPH assay at concentration of (100 µg/mL) suggested di ethyl ether and chloroform

extract had 70% and 64 % scavenging inhibition respectively (Farid *et al.,* 2020). As Farid *et al.,* explained methanol crude extract have the highest percentage of scavenging activity (85.5%) compared to the corresponding di ethyl ether and chloroform extract, which contradict the present study. Thus, the n-hexane crude extract of *V. sinaiticum* has a mixture of potential antioxidant compounds which also confirmed by the phytochemical investigation of this study. Taken together, our results suggested that n-hexane root extract of *V. sinaiticum* might contain potential antioxidant compounds.

The observed IC_{50} value of compound-**32** exhibited promising antioxidant activity (3.33 µg/ml) followed by compound-**31** (3.79 µg/ml) (Table 10). The lower the IC_{50} value, the higher antioxidant activity of substances. The two isolated compounds demonstrated a promising antioxidant activity with IC_{50} values of compound-**32** (72.25%, IC_{50} value of 3.33 µg/ml) and compound **31** (66.66%, IC_{50} value of 3.79 µg/ml) were showed promising antioxidant potential as compared to ascorbic acid (89.56%, IC_{50} value of 0.72 µg/ml) used as standard antioxidant drug at concentration of 100µg/ml. The graphs were plotted against percent inhibition vs concentration of root extracts, compounds and standard drug ascorbic acid (Figure 12 and 13). IC_{50} value (the amount of antioxidant required to decrease the initial DPPH concentration by 50%). IC_{50} value for each extract, compounds and ascorbic acid was calculated automatically using graph pad prim version 8.

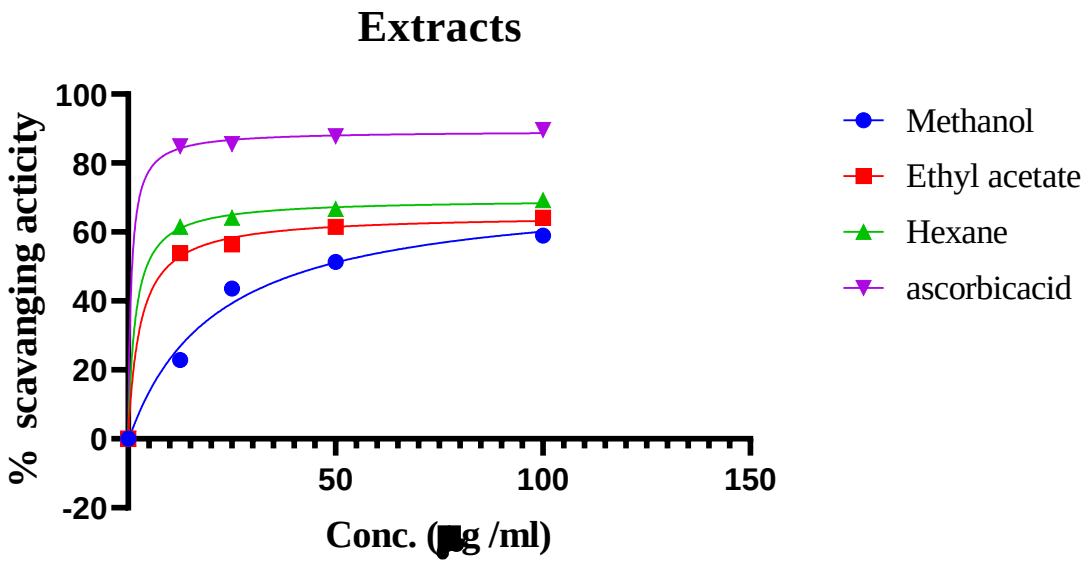

Figure 12.Percent scavenging (DPPH) activity of plant extracts at concentration range of 12.5-100 µg/mL *V. sinaiticum* and standard curve of ascorbic acid

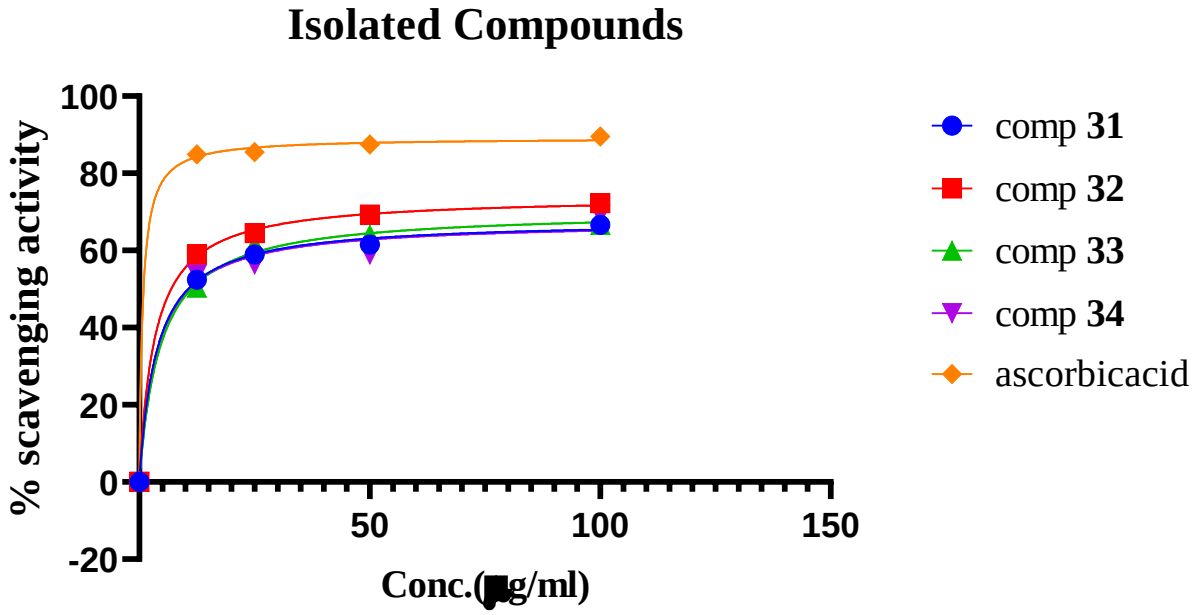

Figure 13.Percent scavenging (DPPH) activity of isolated compounds at concentration range of 12.5-100 µg/mL *V. siniaticum* and standard curve of ascorbic acid

4.6 Molecular Docking Studies

For extra support of the in vitro antibacterial, antifungal and antioxidant activities of the compounds, molecular docking studies of the isolated compound **31, 32, 33** and **34** with binding sites of *P. aeruginosa* LasR (PDB ID: 3JPU), *S. epidermidis* FtsZ (PDB ID: 4M8I), *C. albicans* CYP51 (PDB ID: 5V5Z) and Human peroxiredoxin CYP51 (PDB ID: 1HD2) were performed in order to predict the protein-ligand (each compound) interactions.

In the present study, the molecular docking analysis of the isolated compounds **31, 32, 33** and **34** was carried out to investigate their binding pattern with LasR and the results were compared with the standard antibacterial drug ciprofloxacin. The binding affinity, H-bond, and residual interaction of compounds **31, 32, 33** and **34** along with ciprofloxacin are presented in (Table 11). The result showed a binding pocket of LasR of isolated compounds (**31- 34**) were found to have minimum binding energy ranging from –6.8 to –8.3 kcal/mol (Table 11). Compound **31–34** showed better docking competence with LasR within the binding pocket. In comparison to ciprofloxacin, all the isolated compounds have shown similar amino acids binding interactions with Thr-115 (hydrogen bond) and compound **31** and **32** have shown similar residual amino acid binding interaction with Ala-127 (hydrophobic bond). Compound **32** forms additional residual interaction with Val-76 (hydrophobic bond) in active site of the target protein with docking score -7.8 kcal/mol.

Compound **33** and **34** have shown the docking score -8.3 and -7.5 kcal/mol with no additional residual interactions within the binding pocket. All compounds were not formed an additional residual Van dar Waals interaction with residual amino acids. Whereas, Compound **33** showed interesting binding affinity (-8.3 kcal/mol) followed by compound **32 (-**7.8kcal/mol**),** but lesser than the standard, Ciprofloxacin (-10.2 kcal/mol), which showed hydrogen bonding interactions with Thr-115, Tyr-47. Compound **31** and **32** displayed similar hydrogen bonding interactions with more additional residual amino acid interaction (hydrophobic).Therefore, this additional hydrophobic interaction groups, together with sufficient hydrogen bond interaction, may help them to achieve partition into lipid bilayers. This data also confirmed by log p value of this two compounds described in (Table 15) below. Hence, this docking analysis in agreement with the in vitro experimental data, of compound **31** and **32** of this study, that both compounds inhibit *P. aeruginosa* with MIC of 0.5mg/ml compared to others two isolated compounds.

Binding affinity and interactions with different amino acids are presented in the (Table 11) and 3D and 2D binding interactions is depicted in (Fig 14).

Table 11. Binding affinity and interaction with the target of isolated compounds against *P. aeruginosa* LasR receptor (PDB ID: 3JPU)

Comp.	Affinity (kcal/mol)	H-bond	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
31	-6.8	Thr-115*, Tyr-47*, Trp-60*, Thr-80**, Val-76**, Gly-38**	Ala-127, Try-64, Trp-88, Arg-61, Cys-79, Leu-125	-
32	-7.8	Thr-115*, Trp-60*, Ser-129*, Asp-73**	Ala-127, Val-76, Cys-79, Tyr-47, Leu-125	-
33	-8.3	Thr-115*, Ala-127*, Gly-38*, Asp-73**, Tyr-64**	-	-
34	-7.5	Thr-115*, Gly-126, Asp-73**, Ala-127**	-	-
Ciprofloxacin	-10.2	Thr-115*, Tyr-47*, Try-64**	Ala-127, Val-76, Try-64, Ala-70, Tyr-56, Leu-36, Trp-60	Trp88, Thr-75, Ser-129, Ile-52, Ala-50, Arg-61

(*Conventional Hydrogen bond; **Carbon Hydrogen bond)

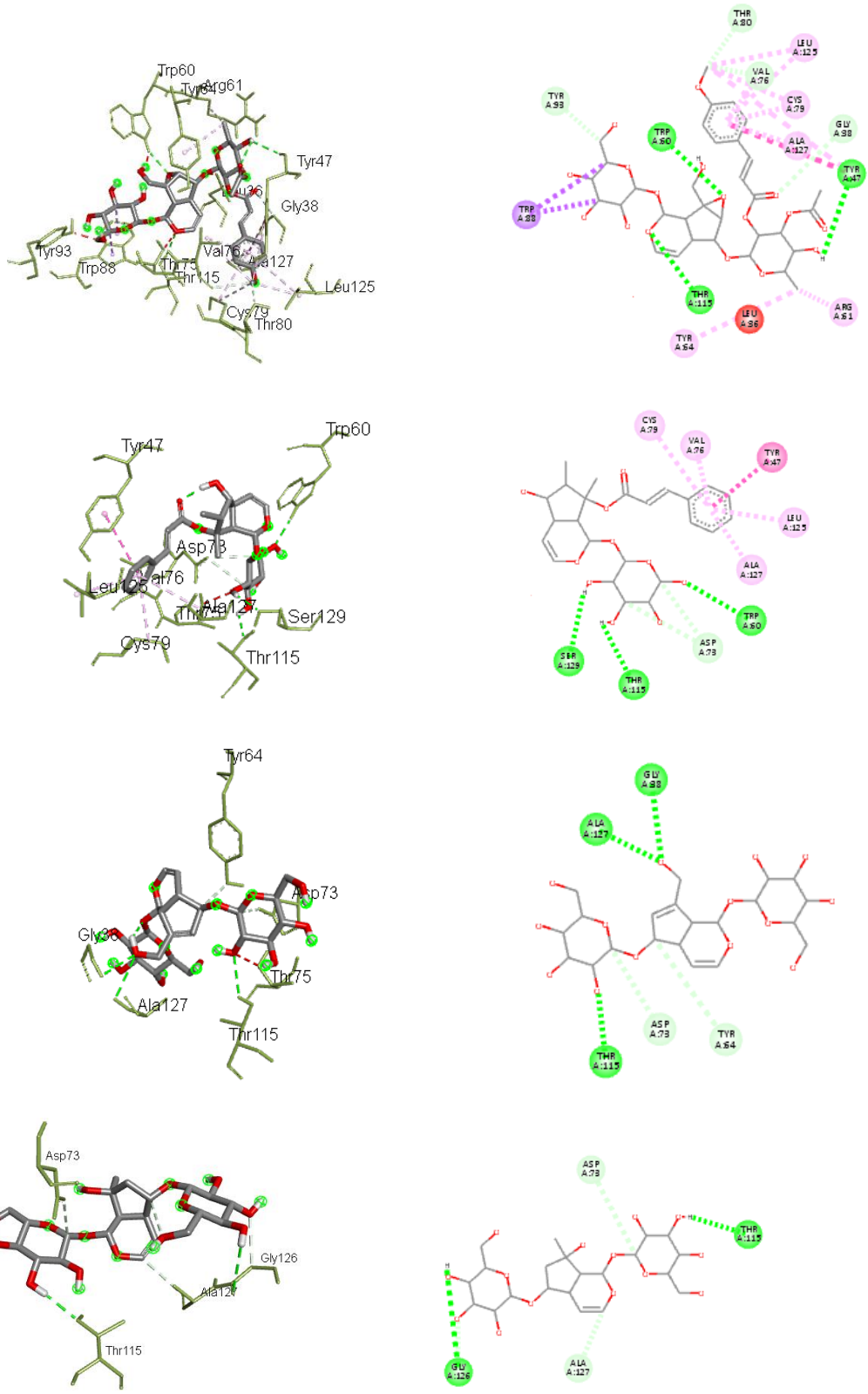

Figure 14. Binding interactions (3D and 2D) of compound **33** and **34** against LasR (PDB ID: 3JPU) receptor.

A cytoplasmic protein known as FtsZ, is a key molecule in *S. epidermidis* bacterial cell division. It has also been recognized as a novel target for antibacterial drugs (Adamas & Errington, 2009). In this study *S. epidermidis* FtsZ (PDB ID: 4M8I) was used as a target and Ciprofloxacin used as reference control. The binding affinity, H-bond, and residual interaction of compounds **31**, **32**, **33** and **34** along with ciprofloxacin are described in (Table 12). The result showed a binding pocket of FtsZ of isolated compounds (**31- 34**) were found to have minimum binding energy ranging from –9.2 to –9.8 kcal/mol, respectively (Table 12). Compounds **31–34** showed interesting docking efficiency with FtsZ within the binding pocket. In comparison to ciprofloxacin, all the isolated compounds have shown similar residual amino acids binding interactions with Gly-108 (hydrogen bond). However, compared to ciprofloxacin all the isolated compounds have shown no residual amino acids binding interactions (Van-der Walls interactions**)**. Compound **32** showed the highest binding affinity score (-9.8 kcal/mol), whereas compound **31, 33 and 34** exhibited similar binding affinities score (-9.2 kcal/mol). Therefore this in silico pharmacodynamics prediction of isolated compounds support in vitro data of this study, in particular compound **32** have a promising antibacterial activity against *S. epidermidis* with binding affinities of (-9.8kcal/mol). Binding affinity and interactions with different amino acids are presented in the (Table 12) and 3D and 2D binding interactions is depicted in (Fig 15).

Table 12. Binding affinity and interaction with the target of isolated compounds against *S. epidermidis FtsZ* receptor (PDB ID: 4M8I).

Comp.	Affinity (kcal/mol)	H-bond	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
31	-9.2	Gly-108*, Thr-109*, Ala-71*, Ala-73*, Asn-166*, Gly-21**	Thr-133, Ala-26, Leu-190	-
32	-9.8	Gly-108*, Ala-73*, Gly-21*, Asn-44*, Asp-46*, Gly-107**	Pro-135	-
33	-9.2	Gly-108*, Thr-109*, Arg-143*, Asn-166*, Gly-107*, Thr-102*, Gly-104*, Gly-104**	-	-
34	-9.2	Gly-108*, Arg-143*, Asn-166*, Thr-133*, Ala-103*, Thr102*, Phe-183*, Gly-104*, Gly-107**	Phe-183	
Cipro.	-8.1	Gly-108*, Thr-109*, Arg-143*, Ala-73*, Gly-72*, Thr-133*, Gly-104*, Gly-110*, Ala-71*, Gly-20**	-	Gly-70

*Conventional Hydrogen bond; **Carbon Hydrogen bond

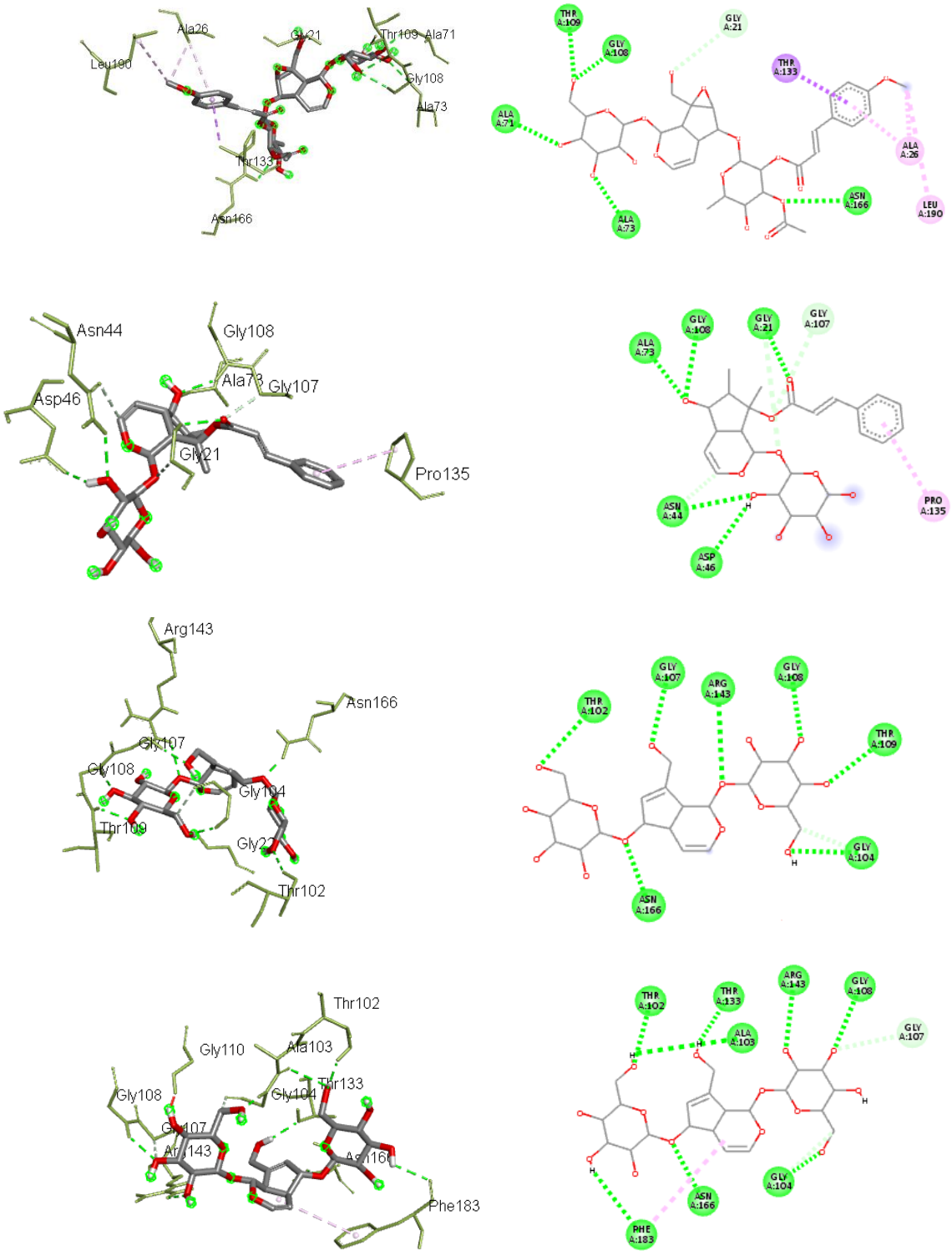

Figure 16. Binding interactions (3D and 2D) of compound **31, 32, 33** and **34** against FtsZ (PDB ID: 4M8I) receptor.

In this study *C. albican* CYP51 (PDB ID: 5V5Z) was used as a target receptor. The result showed a binding pocket of CYP51 of isolated compounds (**31- 34**) were found to have minimum binding energy ranging from -7.8 to -9.7 kcal/mol, (Table 13). The binding affinity, H-bond, and residual interaction with different amino acids of compound **31, 32, 33** and **34** along the drug ketoconazole were used as reference controls presented in (Table 13). In compared to Ketoconazole, all the isolated compounds have shown similar hydrogen bond amino acids binding interaction with His-377. Among all tested compounds, Compound **32** showed interesting binding affinity score (-9.7 kcal/mol). Binding affinity and interactions with different amino acids are presented in the (Table 13) and 3D and 2D binding interactions is depicted in (Fig 15).

Table 13. Binding affinity and interaction with the target of isolated compounds against *C. albican* CYP51 (PDB ID: 5V5Z)

Compd	**Affinity (kcal/mol)**	**H-bond**	**Residual Amino acid Interactions**	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	**Van-der Walls interactions**
31	-8.1	Ser-378*, His-377*, Ser-506*, Ser-378**, His-377**	Tyr-118, , Tyr-132	-
32	-9.7	Ser-378*, Tyr-118*, Tyr-64*, Phe-233**, Met-508**, His-377**	Tyr-118	-
33	-7.8	Ser-378*, His-377*, Ser-507*, Pro-230*	-	-
34	-7.8	Ser-378*, His-377*, Tyr-132**	-	-
KCZ	-10.2	Ser-378*, His-377*	Phe-233, Phe-380, Tyr-118, Tyr-132, Leu-376	Gly-307, Ile-379, Cys-470, Lys-143, Ile-131, Met-508

KCZ = ketoconazole

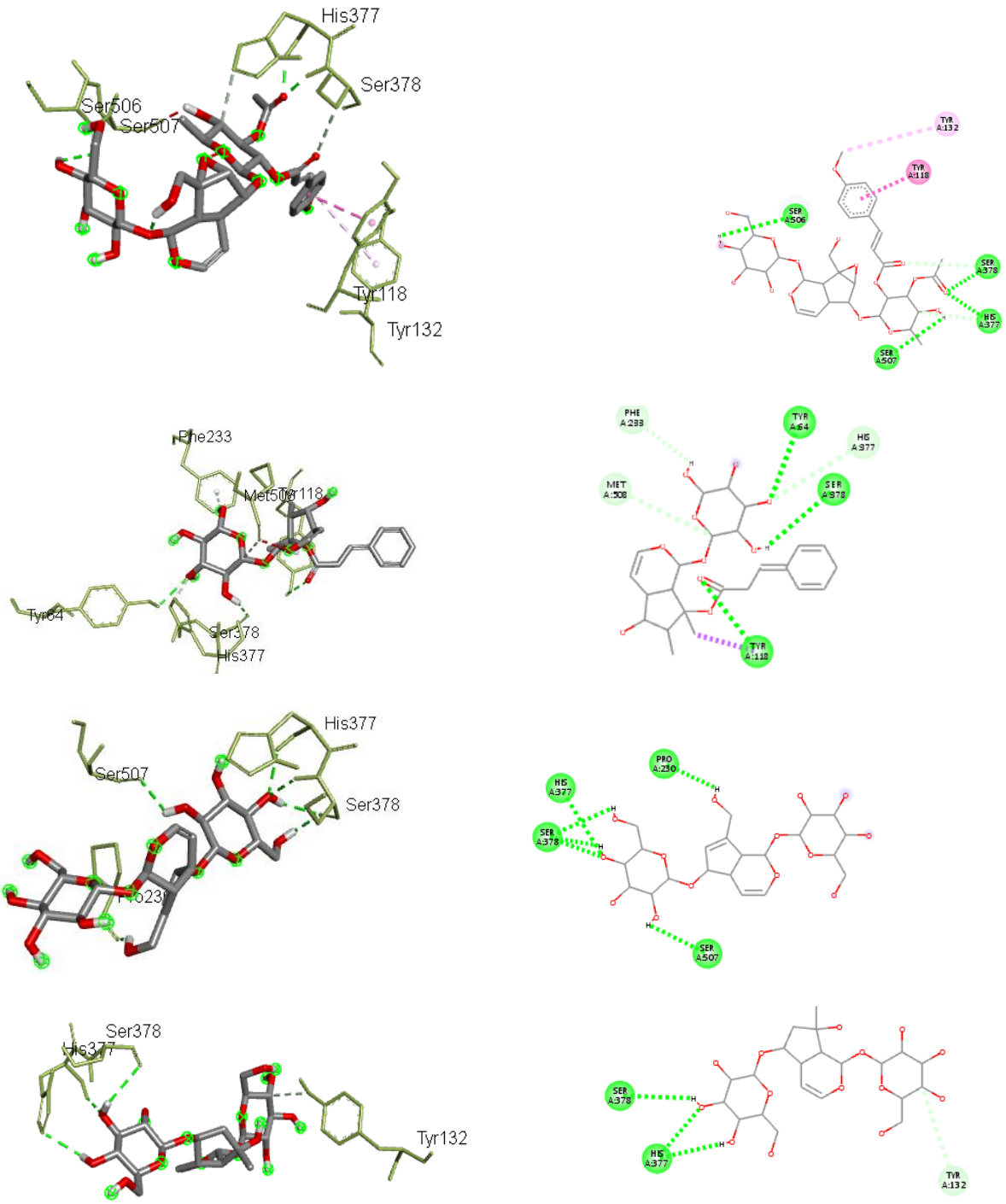

Figure 15. Binding interactions (3D and 2D) of compound 33 and 34 against CYP51 (PDB ID: 5V5Z) receptor.

Human peroxiredoxin 5 (PRDX5) a member of the peroxiredoxin family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydro peroxides. The encoded protein interacts with peroxisome receptor 1 and plays an antioxidant protective role in different tissues under normal conditions and during inflammatory processes (Declercq *et al.*, 2001). In the present study *human peroxidoxins* (PDB ID: 1HD2) enzyme is used as a target. The native co-crystallized ligand benzoic acid and the drug ascorbic acid was utilized as a reference control for docking with PRDX5.

The result showed a binding pocket of PRDX5 of isolated compounds (**31- 34**) were found to have minimum binding energy ranging from -4.8 to -6.1 kcal/mol, (Table 14). The binding affinity, H-bond, and residual interaction with different amino acids of compound **31, 32, 33** and **34** along the native co-crystalized ligand benzoic acid, and standard drug ascorbic acid were used as reference controls presented in (Table 14). In compared to benzoic acid and ascorbic acid, all isolated compounds have shown better antioxidant activity, this might be due to the presence of large amount of hydroxyl group within the structures (Al-Mamary & Moussa, 2021). Compound **31, 33** and **34** in comparison to benzoic acid and ascorbic acid have shown similar hydrogen bond amino acids binding interaction with Cys-47. Except that compound **32** formed similar additional hydrogen bond interaction with residual amino acid Arg-127 compared to native co-crystalized ligand benzoic and standard drug ascorbic acid. The study reviewed by Al-Mamary & Moussa, 2021, showed that small molecule with more than two hydroxyl group has a potent free radical scavenging ability. Therefore, owing this fact, compound **32,** which showed smaller molecular weight (478.28Da) and highest binding affinity score (-6.1 kcal/mol) has Showed a potent free radical scavenging activity. Thus, may act as novel antioxidant activity, which also might considered as remedy for the treatment of diseases caused as a result of oxidative stress. This in silico study of compound **32** also supportive of in vitro experiment study of this data.

Table 14. Binding affinity and interaction with the target of isolated compounds against *human peroxidoxins* (PDB ID: 1HD2)

Comp.	Affinity (kcal/mol)	H-bond	Residual Amino acid Interactions	
			Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions	Van-der Walls interactions
31	-4.8	Cys-47*, Arg-127*, Gly-46*, Thr-147*, Pro-45**	Arg-124, Val-80	
32	-6.1	Arg-127*, Thr-147*	Leu-116	-
33	-5.4	Cys-47*, Gly-46*, Thr-147*, Thr-44*, Asp-145*	-	Pro-40, Phe-120, Ile-119
34	-5.4	Cys-47*, Gly-46*, Thr-147*, Thr-44*, Asp-145*, Gly-148**	-	
Benzoic acid	-4.1	Cys-47*, Arg-127*, Thr-44*	Phe-120	-
Ascorbic acid	-4.2	Cys-47*, Arg-127*, Thr-44*, Gly-46*	-	-

*Conventional Hydrogen bond; **Carbon Hydrogen bond

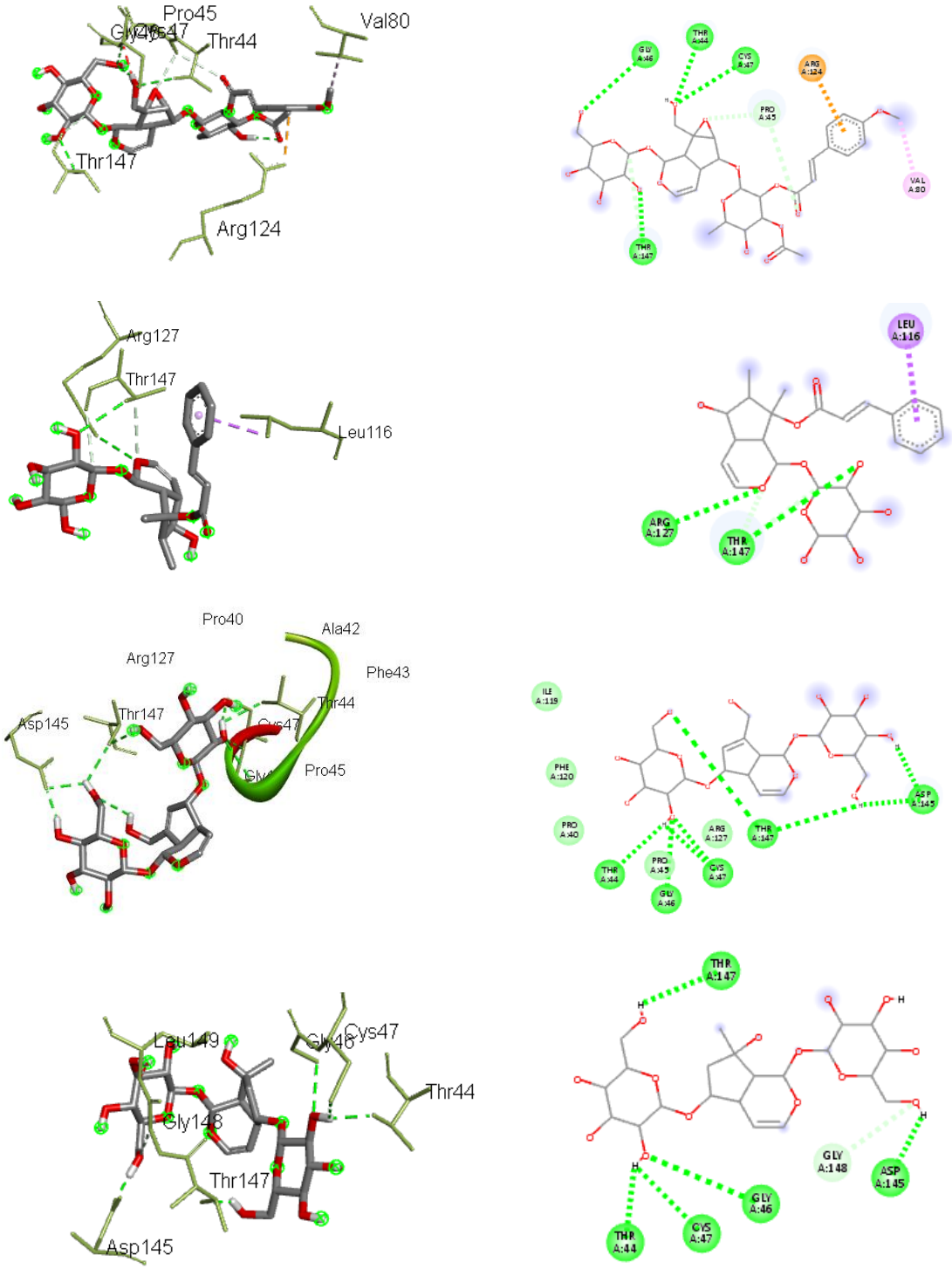

Figure 16. Binding interactions (3D and 2D) of compound 31, 32, 33 and 34 human peroxidoxins (PDB ID: 1HD2) enzyme.

4.6. In silico Pharmacokinetics (ADME), Drug-Likeness and Toxicity Studies Analysis

Pharmacokinetic properties (ADME), Physicochemical properties, drug-likeness and boiled-egg model of compounds **31-34** were determined using the Swiss ADME Web tool. Percentage of absorption (%Abs) was calculated using the formula: % Abs = 109 - (0.345 x TPSA) (Zhao *et al.*, 2002). The toxicity profile of the compounds was predicted using the Pro Tox-II Web tool. Drug-likeness is a prediction that screens whether a particular organic molecule has steady properties with being an orally active drug. The drug-likeness of the isolated compounds was characterized according to "Lipinski's rule." According to Lipinski rule, the potential molecules should have the following physicochemical properties such as, (i) not more than 5 hydrogen bond donors; (i) not more than 10 hydrogen bond acceptors; (iii) A molecular mass less than or equal to 500 Da; (iv) An octanol-water partition coefficient log P not greater than 5 (Lipinski *et al.,* 1997). In the present investigation, only compound **32** obeyed Lipinski's rule of five and are likely to be orally active.

Low molecular weight (MW) indicates that the molecules are light and can easily pass through the cell membrane and preferred for oral absorption, whereas Compounds with MW > 500 Da are absorbed via an alternate route, such as: Intranasal, buccal/sublingual, pulmonary and transdermal routes (Mathias & Hussain, 2010). The present study revealed that all of the molecular weight of the studied compounds (Table 15) were greater than 500 Da except compound **32.** Thus, compound **31, 33** and **34** are predicted to be orally inactive. Thus, these compounds might be given through alternate route of drug administration. Ideally good bioavailability range of lipophilicity (log P) is between 0 and 3, which refers to the state of a good balance between solubility and permeability (Arnott & Planey, 2012). The distribution coefficients (log P) of the isolated compounds (Table 15) were in the range of -3.46 to 0.24. The log (P) of isolated compound **31, 33** and **34** found to be less than zero as foretold in (Table 15) which might be due to the large number of hydroxyl groups present in all compounds. A negative value for log P means the compound has a higher affinity for the aqueous phase (Braeken *et al.*, 2005, Bhal 2007). Thus, those three compounds (**31**, **33** & **34**) are more hydrophilic. Therefore, only Compound **32,** has been shown to have better pharmacokinetics and bioavailability characteristics. The % Abs analysis of the isolated compounds and control showed that all the isolated compounds have the lowest percent absorption than the control except compound **31**, which has the highest value than all compounds and comparable value to standard drug (Table 16).

Table 15. Drug-likeness predictions of compounds **31- 34**, computed by Swiss ADME

Comp.	Molecular Formula	Mol. Wt. (g/mole)	NHD	NHA	Log P(c Log P)	Lipinski's rule of Five Violation	NRB	TPSA ($A^{\circ 2}$)	Veber's rule violation
Comp.**31**	$C_{33}H_{42}O_{17}$	710.38	6	17	-0.61	3	13	241.89	2
Comp.**32**	$C_{24}H_{30}O_{10}$	478.49	5	10	0.24	0	6	155.14	1
Comp.**33**	$C_{21}H_{32}O_{14}$	508.47	9	14	-3.46	3	7	228.22	1
Comp.**34**	$C_{21}H_{34}O_{10}$	510.49	9	14	-3.33	3	6	228.22	1
Cipro.	$C_{17}H_{18}FN_3O_3$	331.34	2	5	1.1	0	3	74.57	0

NHD = Number of Hydrogen donor, NHA = Number of Hydrogen acceptor, NRB = Number of rotatable bonds, TPSA = total polar surface area, Comp. = Compound and Cipro. = Ciprofloxacin

The Swiss ADME prediction parameters have shown that the GIA exhibits low oral absorption for all compounds. The results of the BBB permeability test performed on studied compounds (Table 16) established that all compounds lack BBB permeability. The P-gp is an efflux transporter that pumps foreign substance out of cells, decreased accumulation in multidrug resistance substance and influences the absorption and distribution of a variety of compounds (Elmeliegy *et al.*, 2020, Srivalli & Lakshmi, 2012). As a result of this, detecting permeability glycoprotein substrates and inhibitors is vital for identifying potential medicines and enhancing them. The results showed that, except for compound **31**, all compounds were substrates of permeability glycoprotein (P-gp). Which suggests that, those three compounds (**32**, **33**, and **34**) have induction effect P-gp glycoproteins, which means that they either reduce the total bioavailability of the therapeutic P-gp substrate or substantiate the role of P-gp glycoprotein (Elmeliegy *et al.*, 2020).

Table 16. ADME predictions of compounds **31-34**, computed by Swiss ADME and Pre ADMET.

Comp.	log Kp cm/s	% GI ABS	BBB Permeability	P-gp substrate	Inhibitor Interaction (Swiss ADME/Pre ADMET)				
					CYP 3A4	CYP2D6 inhibitor	CYP2C9 inhibitor	CYP2C19 inhibitor	CYP1A2 inhibitor
31	-11.42	83.45	No	No	No	No	No	No	No
32	-8.89	53.52	No	yes	No	No	No	No	No
33	-12.68	78.73	No	Yes	No	No	No	No	No
34	-12.1	78.73	No	yes	NO	No	No	No	No
Cipro.	-.9.09	83.27	no	yes	No	No	No	No	No

Comp. = compound, GI = Gastro-Intestinal, ABS = Absorption, BBB = Blood Brain Barrier, P-gp = P-glycoprotein, CYP = Cytochrome-P, Cipro. = ciprofloxacin

Cytochrome P450 (CYP) enzymes play an important role in the phase I metabolism of many xenobiotics. Most drug interactions (DDIs) associated with CYP are caused by either CYP inhibition or induction. The early detection of potential DDIs is highly desirable in the pharmaceutical industry, because of DDIs can cause serious adverse events, which can lead to poor patient health and drug development failures (Kato, 2020). In this case, all the screened compounds not showed inhibitor effect against five representative of CYP isoforms enzymes (Table 16).

The c Log P and TPSA values of the compounds were plotted to predict human intestinal absorption (HIA) and blood brain barrier (BBB) entree (Figure 22). The egg-shaped plot is divided into 3 parts including a grey region (no HIA or BBB access), a white area (HIA) and a yolk (BBB access). In this case all compounds were out of ranges, indicating that the molecules probably not permeated the BBB, this is might be due to large number of hydroxyl group of compounds (Patil *et al.,* 2022). This boiled-egg model also predicted whether those compounds were substrates of P-glycoprotein (PGP). Red dots (PGP) represent compounds that are not substrates of the PGP CNS efflux transporter, while, blue dots (PGP +) represent compounds that are substrates of PGP and predicted to pass through the CNS. In this wok only compound **32** was predicted to pass through the CNS.

The organ toxicity (hepatotoxicity) and toxicological endpoints (carcinogenicity, immune toxicity, mutagenicity and cytotoxicity) of the isolated compounds and their toxicity class and LD_{50} were predicted using Pro Tox II server (Table 17). Toxicological endpoints prediction analysis indicated median lethal dose (LD_{50}) values ranging from 2000–5000 mg/Kg. All Compounds were predicted not to be carcinogenic, hepatotoxic, mutagenic and cytotoxic. However, all Compounds were predicted to be immunotoxin. Therefore, all compounds have good profile of toxicity effect apart from that, they demonstrated immunotoxin effect. These data also confirmed by large value of LD_{50}, which refers to an estimate of the amount of poison that, under control conditions, will be a lethal dose to 50% of a large number of test animals of a particular species.

Table 17. Toxicity prediction of compounds **31-34**, computed by Pro-Tox II

Compound	LD_{50} (mg/kg)	Toxicity	Organ Toxicity				
			Hepatotoxicity	Carcinogenicity	Immune toxicity	Mutagenicity	Cytotoxicity
31	5000	5	Inactive	Inactive	Active	Inactive	Inactive
32	2000	4	Inactive	Inactive	Active	Inactive	Inactive
33	2000	4	Inactive	Inactive	Active	Inactive	Inactive
34	2000	4	Inactive	Inactive	Active	Inactive	Inactive
Cipro.	1190	4	Active	Inactive	Active	Inactive	Inactive

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In the present study, the dried roots (600g) of *Verbascum siniaticum* was extracted sequentially with n-hexane (2 L), EtOAc (2 L) and methanol (2 L). The highest crude extract was obtained from methanol mixture extract 60 g (10%). TLC analysis of the methanol crude extract revealed that the compounds isolated are not the only compounds present in the plant material. The chemical components in MeOH root extract of *Verbascum siniaticum* by column chromatographic analysis clearly showed the presence of more than 4 compounds (Appendix 5).

A major outcome of the current study is identification of four iridoid glycosides and microbial test against three gram positive bacteria (*S. auras, S. epidermidis, S. agalactiae*) and three gram negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae*) of the plant extract and isolated compounds. The extracts and isolated compounds also tested against four fungi strains. All of the three crude extracts have a considerable antibacterial activity on most of the bacteria. Accordingly, methanol extract showed the interesting antibacterial activity with MIC (0.5 mg/ml) against *P. aeruginosa* and *S. epidermidis* compared to standard drug ciprofloxacin (0.78 µg/ml and 0.19µg/ml) respectively. Compound **31** and **32** displayed the promising antibacterial activity with MIC of (0.5mg/ml) against *P. aeruginosa* compared to standard drug ciprofloxacin (0. 78 µg/ml). Additionally, Compound **32** and **34**, showed interesting antibacterial activity against *S. epidermidis* with MIC of 2 mg/ml and 1 mg/ml respectively. The results of DPPH activity showed that the n-hexane and ethyl acetate root extracts of *V. siniaticum* inhibited the DPPH radical by 69.23% and 64.1% at a concentration of 100 µg/mL, respectively, whereas compound-**31** and compound-**32** displayed 66.66% and 72.25% of inhibition, respectively, at 100 µg/mL in comparison to standard drug, ascorbic acid (89.56% at 100 µg/mL).

Compound **32** (-7.8 kcal/mol) and **33** (-8.3 kcal/mol) showed a remarkable docking binding affinity of against *P. aeruginosa.* Whereas compound **34** (-9.2kcal) and **32** (-9.8 kcal/mol) showed promising docking binding affinity against *S. epidermidis.* The drug-likeness analysis showed that compound **32** satisfy Lipinski's rule of five with zero violations. The LD_{50} and toxicity class values obtained in silico toxicity profile analysis of the compounds suggested that none of the compounds have hepatotoxicity, mutagenicity, carcinogenicity and cytotoxicity. Therefore, the in vitro antibacterial, radical scavenging activity along with the molecular docking analysis suggest the

potential use of the extracts of *V. sinaiticum* and compounds **32, 31** and **34** as promising antibacterial agents whereas compounds **32** can be considered as promising free radical scavenger.

Generally, the result of the current study used to confirm the traditional practice of this medicinal plant for treatments of some antibacterial infection and other diseases. Therefore, the plant root extracts showed interesting potential for a vast number of bioactive compounds which justified its use for various ailments by traditional practitioners. Our findings demonstrates also to the best of our knowledge that, *V. siniaticum* plant has very high selective potential as a source of novel lead for antioxidant and antibacterial activity. Of particular importance is its high activity against *P. aeruginosa*, *S. epidermidis* and *K. pneumoniae*, which are currently posing great public health challenge due to drug resistance development and as major sources of community and hospital based infections.

5.2 Recommendation

- ✓ The study has demonstrated that there is need for further investigation and isolation of other compound from *Verbascum siniaticum*.
- ✓ The crude and the pure extract of *V.siniaticum* need to be subjected to further tests on other disease causing bacteria.
- ✓ Moreover, there is need to carry out isolation studies of the plant species n hexane extract and all other fraction that were not analyzed in this study.
- ✓ Most importantly, the isolated compounds that showed the highest activity could be subjected to more studies such as in vivo test, in order to be used as antibacterial or templates for the synthesis of drugs used in the treatment of diseases caused by bacteria, fungi and radical.

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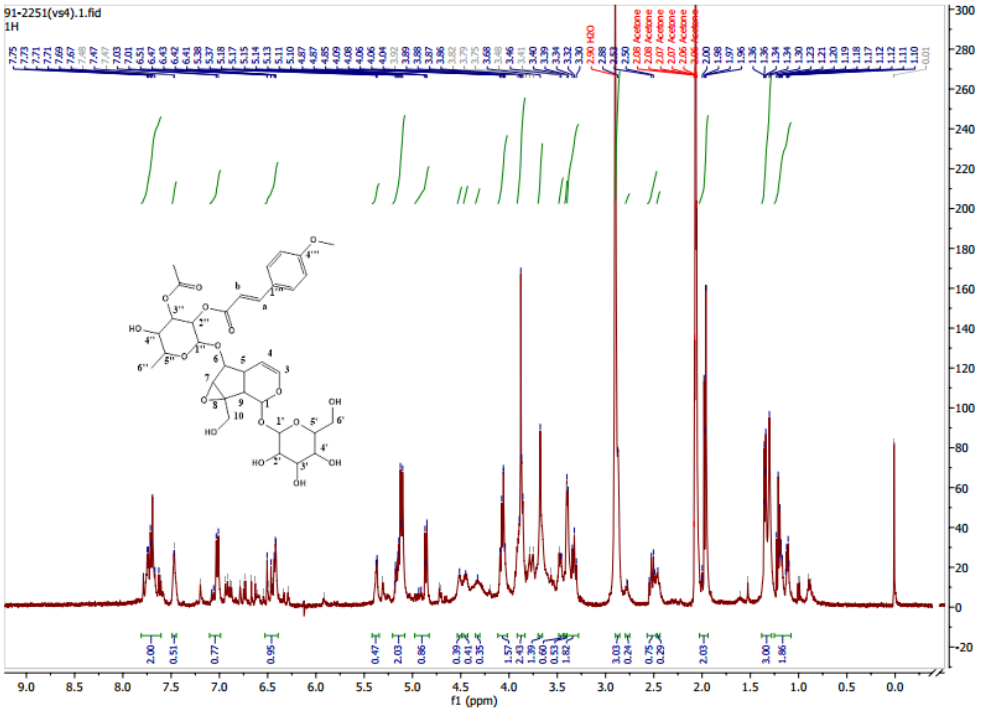
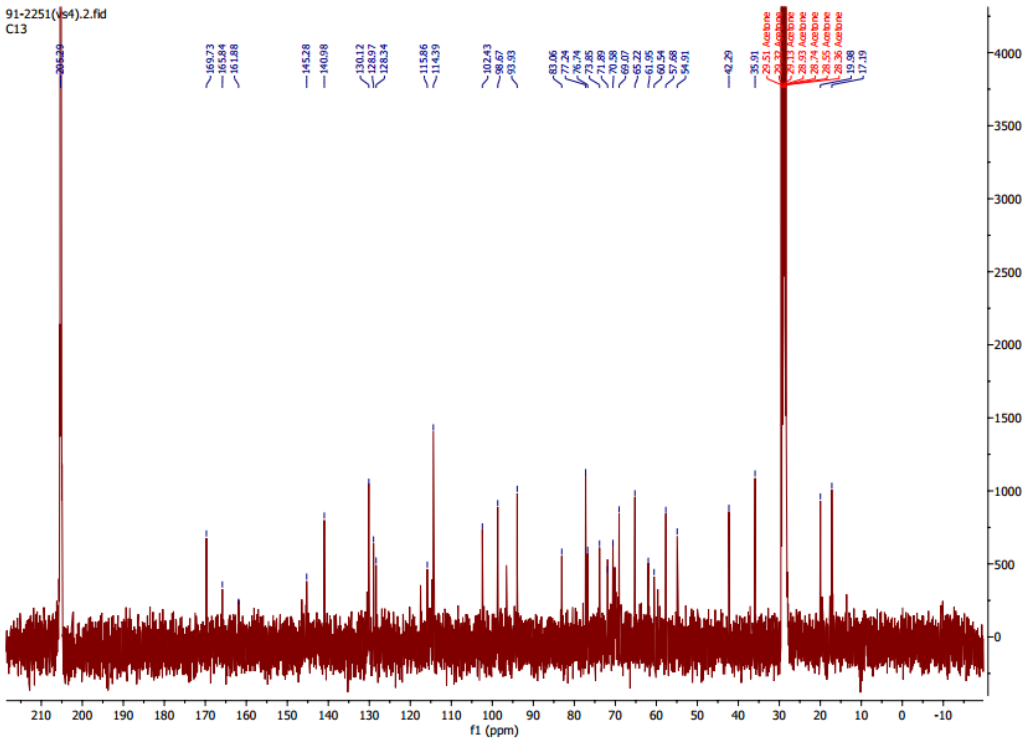
APPENDIXES

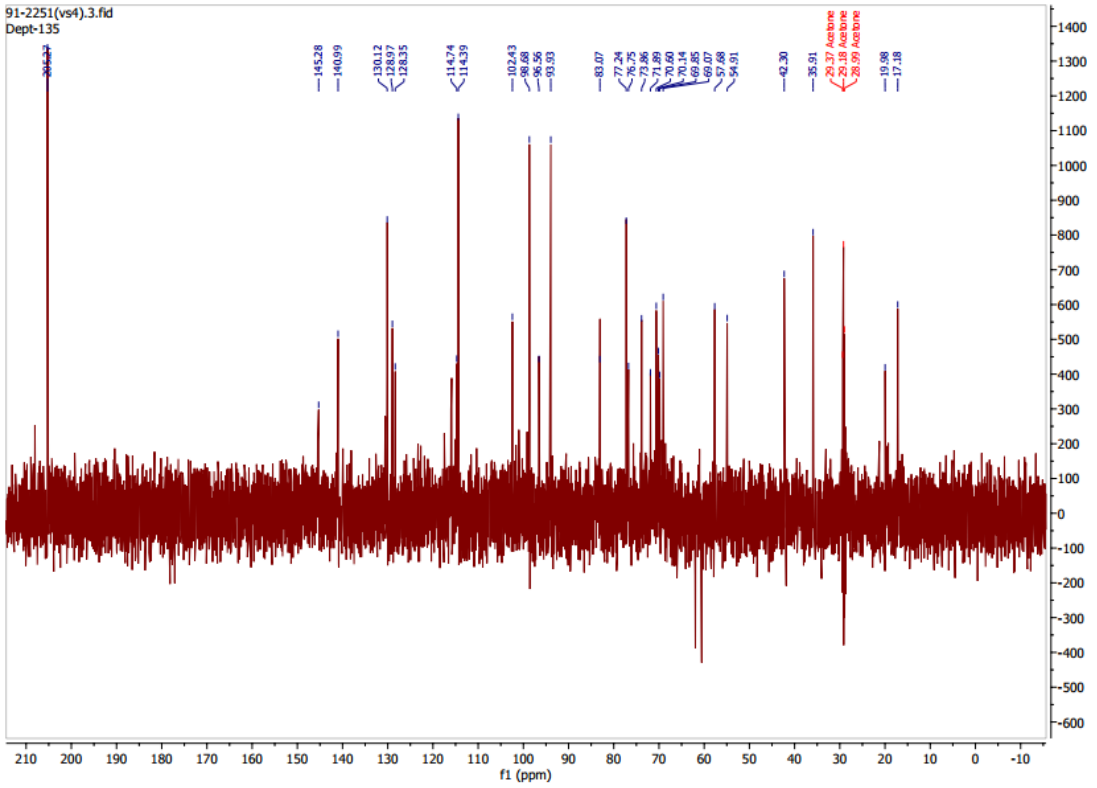
Appendix 1a ^{1}H-NMR for compound-**31** (Vs-4)



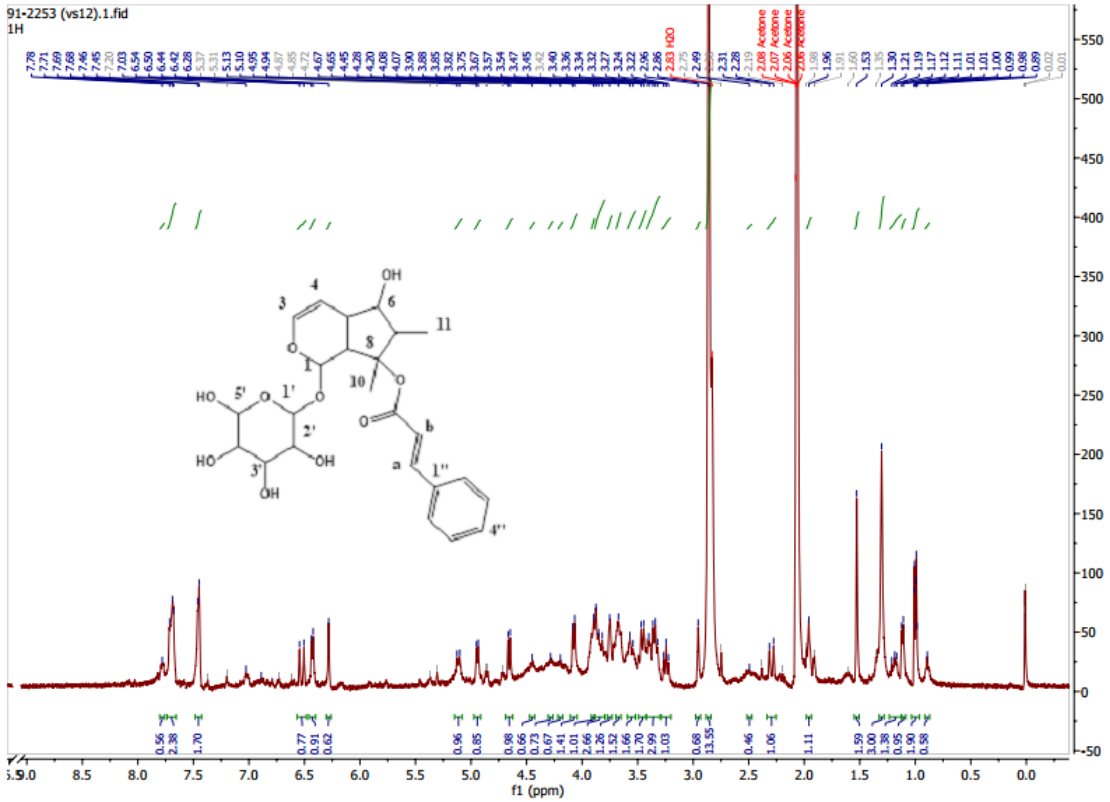
Appendix 1b ^{13}C-NMR for compound **31** (Vs-4)



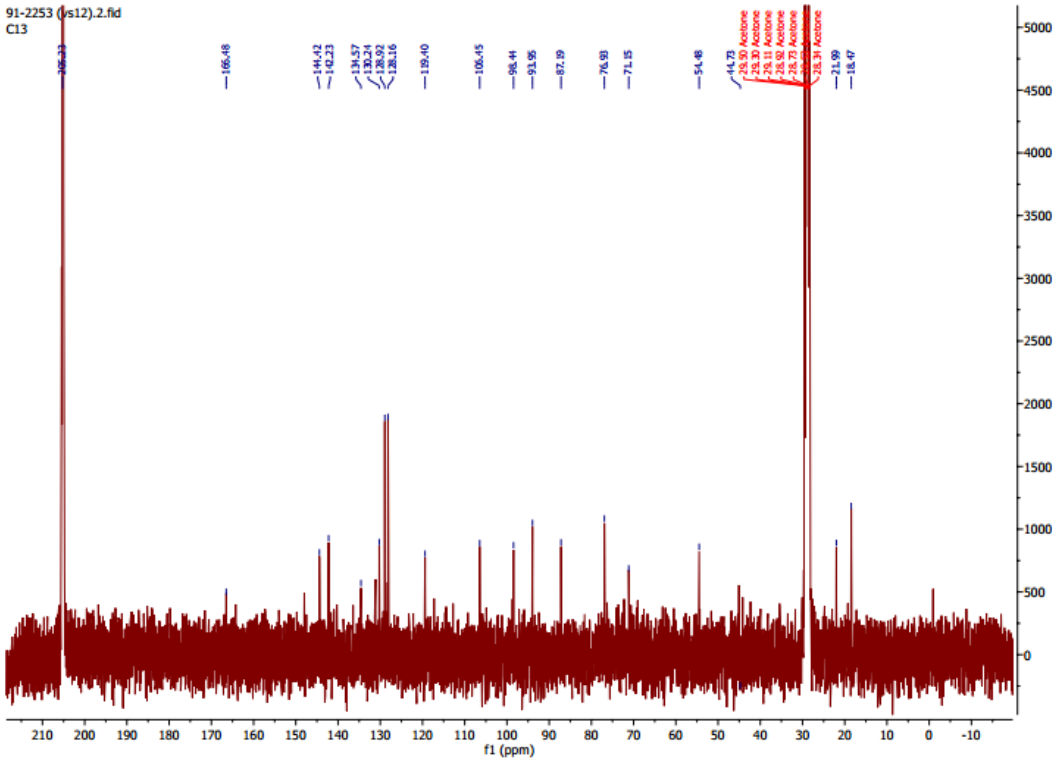
Appendix 1c DEPT-135 for compound **31** (Vs-4)



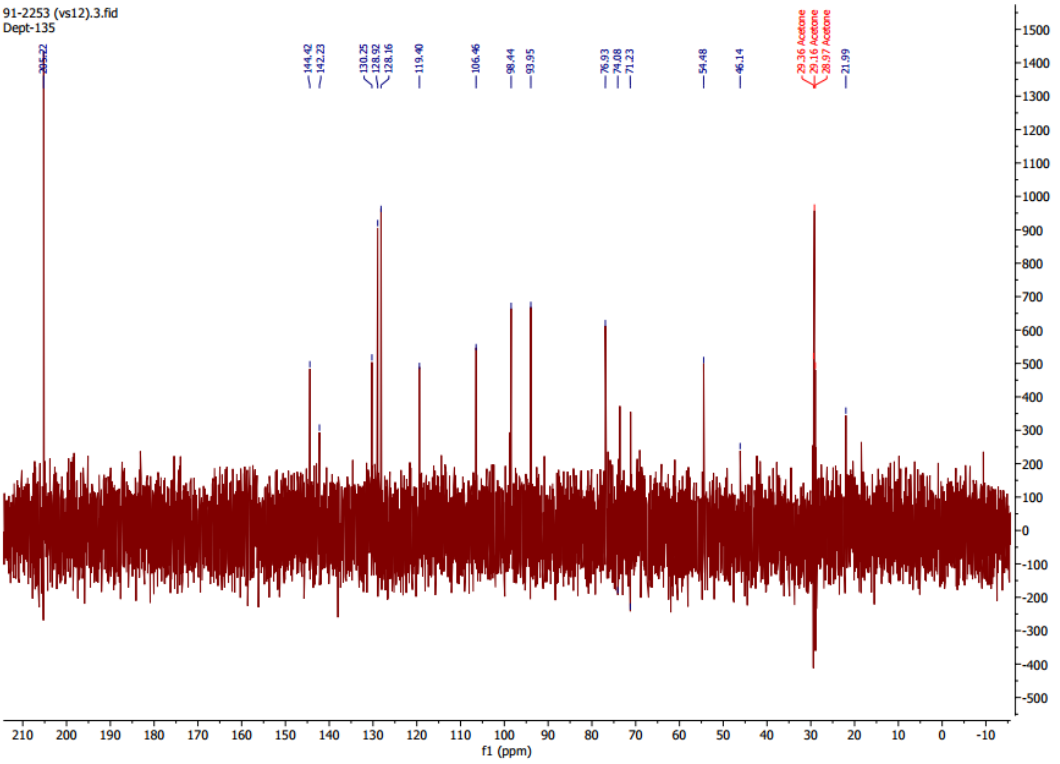
Appendix 2a ^{1}H-NMR for compound **32** (Vs-12)



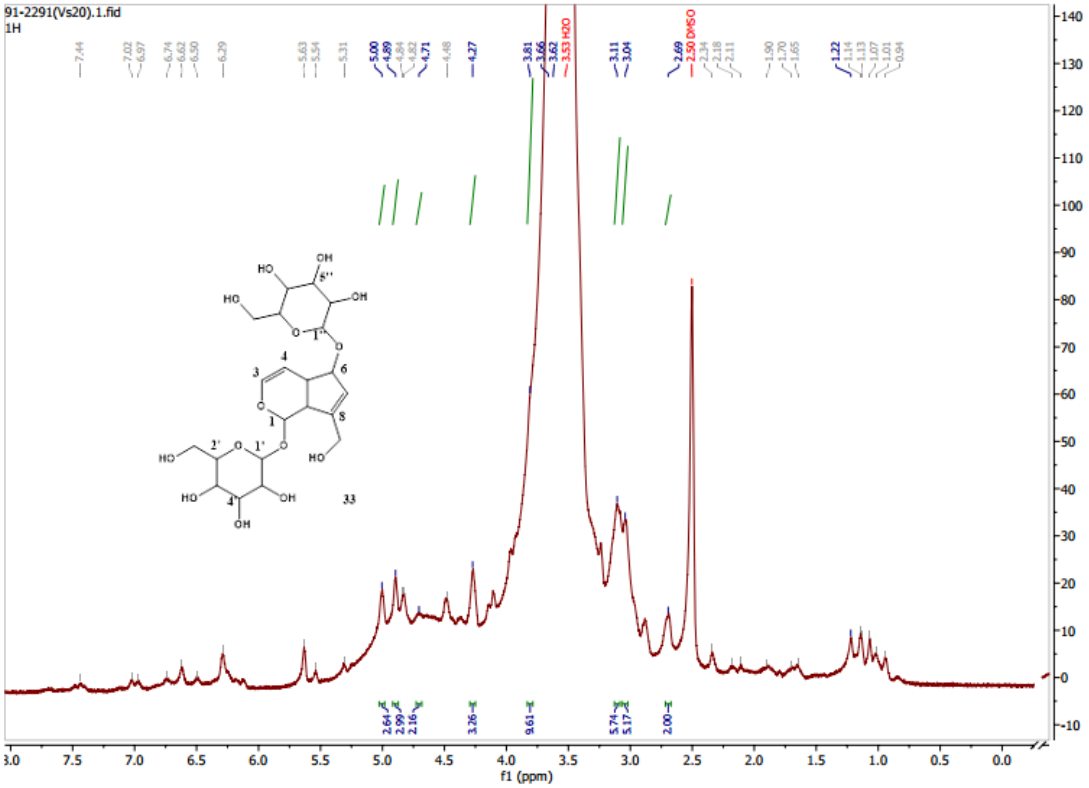
Appendix 2b ^{13}C-NMR for compound **32** (Vs-12)



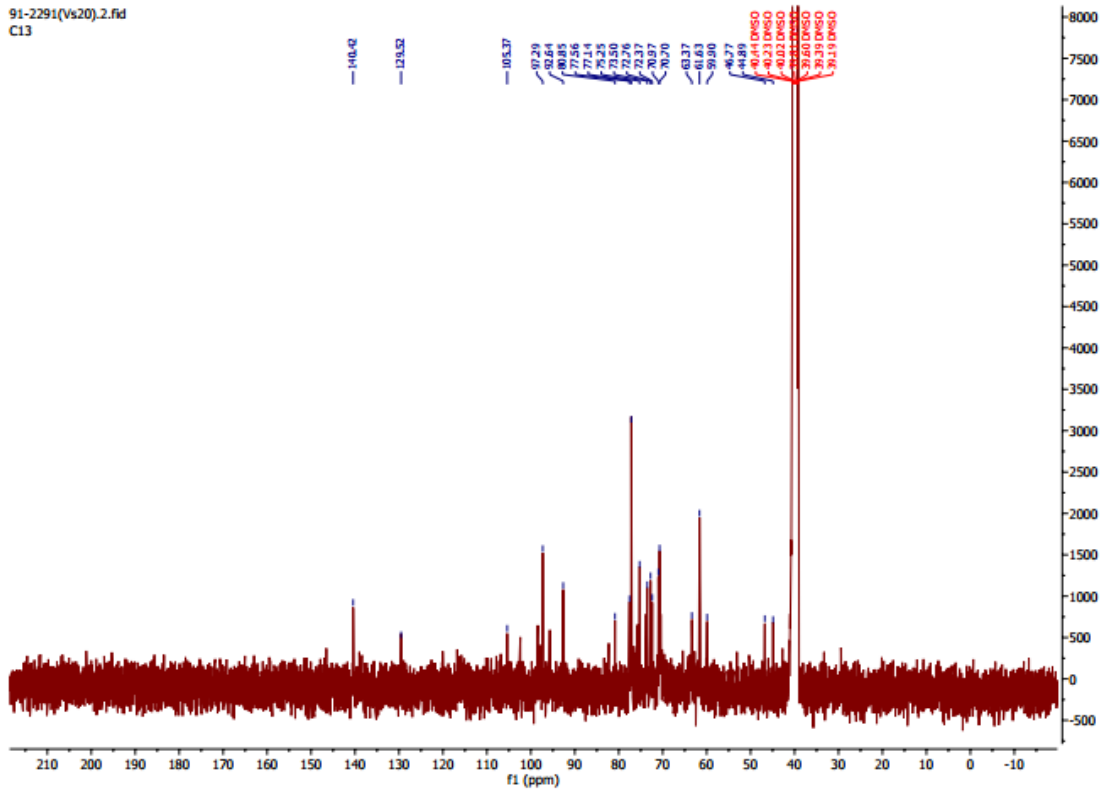
Appendix 2c DEPT-135 for compound **32** (Vs-12)



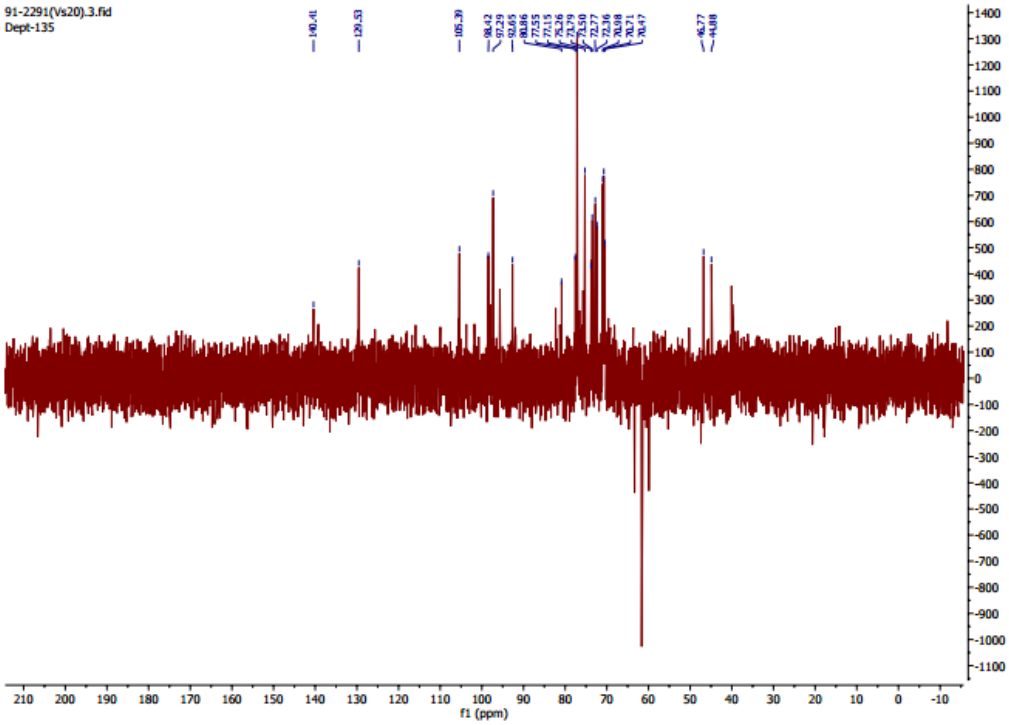
Appendix 3a ^{1}H NMR for compound **33**(Vs-20)



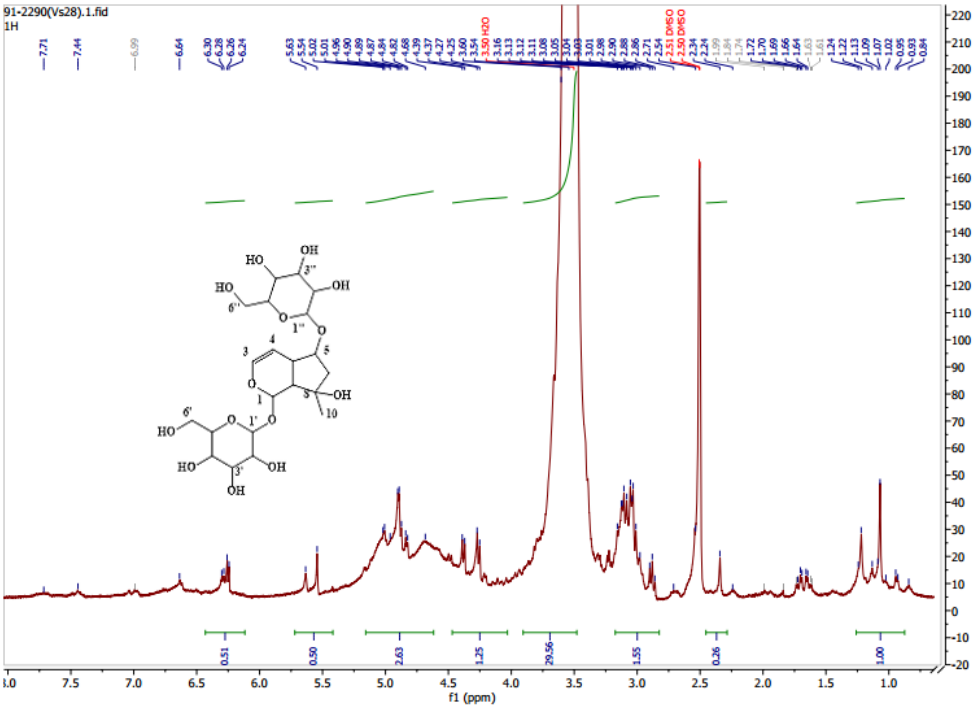
Appendix 3b ^{13}C NMR for compound **33** (Vs-20)



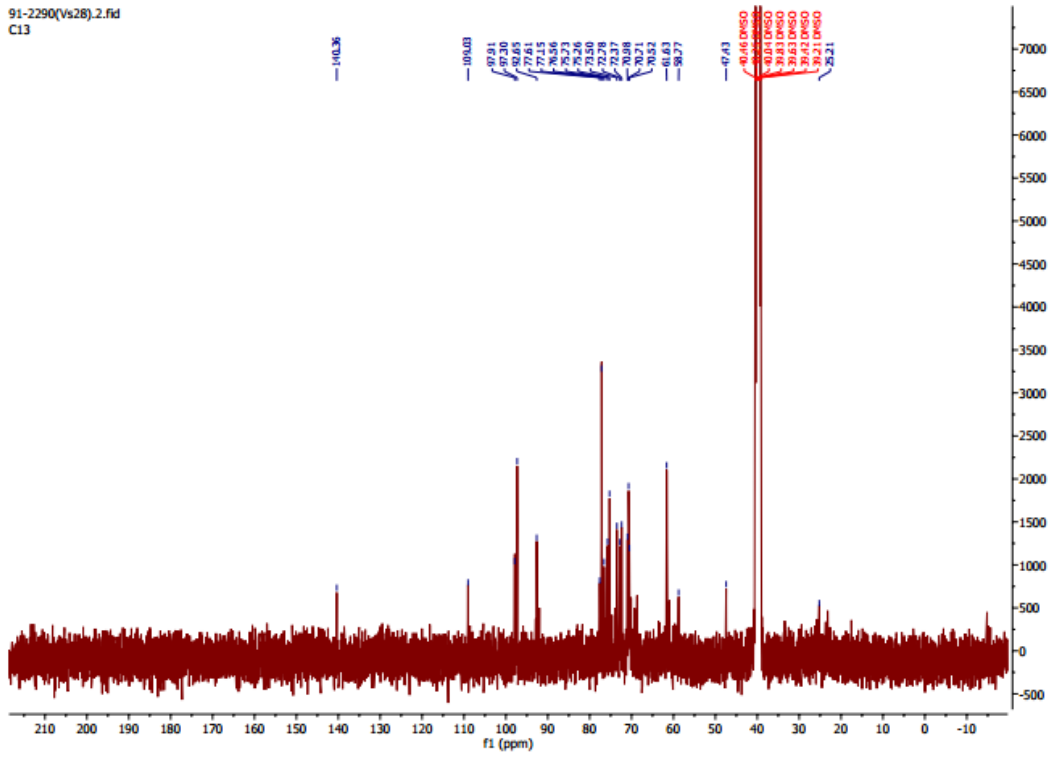
Appendix 3c DEPT-135 NMR for compound **33** (Vs-20)



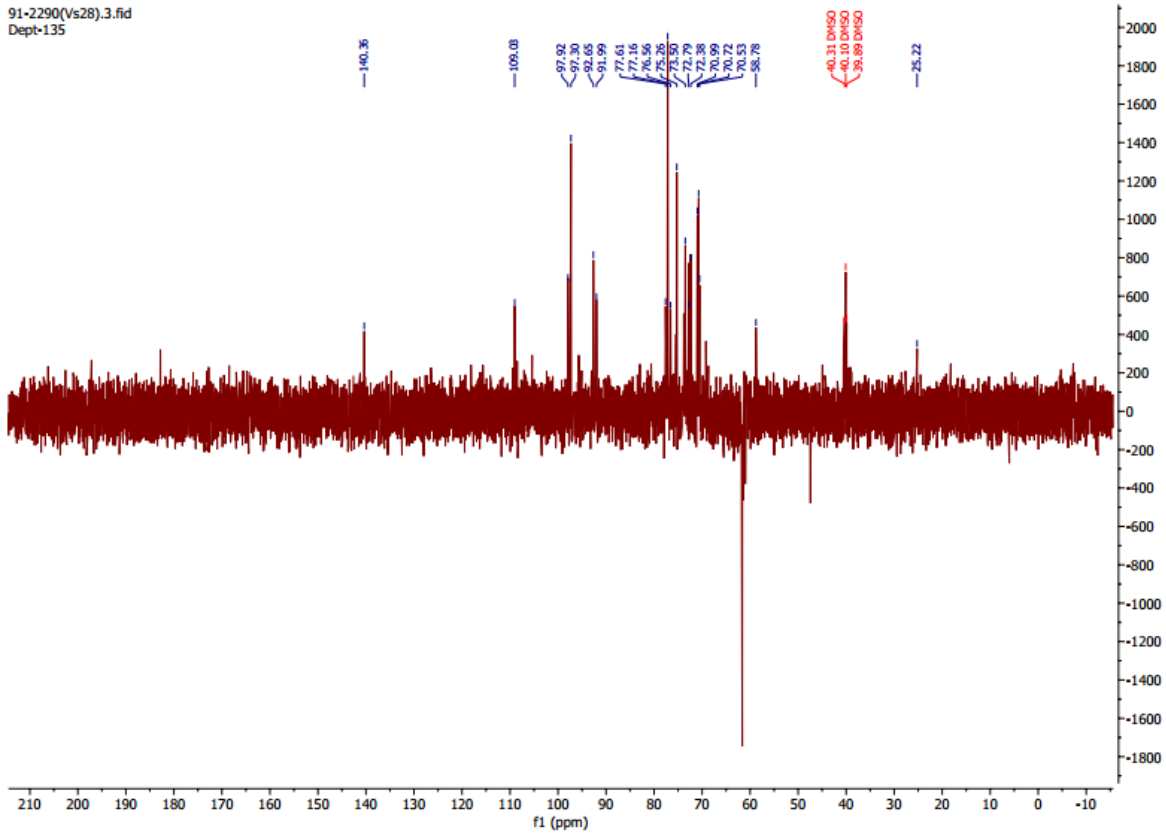
Appendix 4a ^{1}H NMR for compound **34** (Vs-28)



Appendix 4b ^{1}H NMR for compound **34** (Vs-28)

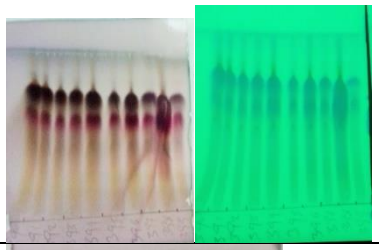
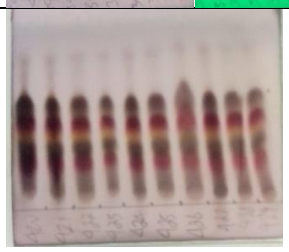
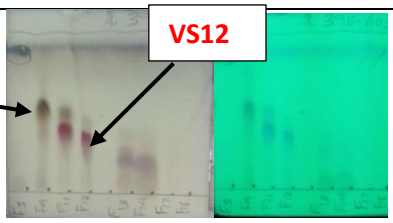
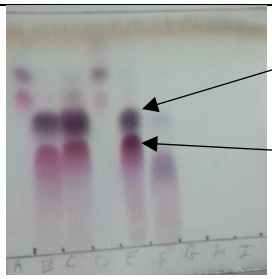


Appendix 4c DEPT-135 for compound **34** (Vs-28)



Appendix 5

Table 18. TLC profile of crude extract and some fractions

No	Fraction	Eluent (ratio)	TLC profile
1	Methanol and ethyl acetate crude	EtAOc:MeOH(7:3)	
2	**Fr 390-403**	EtAOc:MeOH(7:3)	
4	**Fr420-429**		
	Sub fraction(Fr4) or Vs4 and Sub fraction Fr 12 (Vs12)	EtAOc:MeOH(8:2) VS-4	
	Sub fraction (32-40) or B and C (VS-20 and VS28)		
		Vs 20