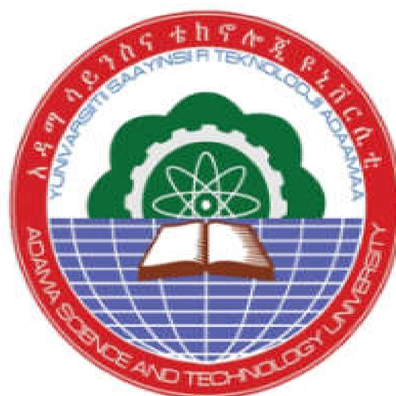
PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL STUDIES OF ROOT EXTRACT OF *GLADIOLUS CANDIDUS.*

BY: **MEGERSA MEKONNEN ADDISSE**



A THESIS SUBMITTED TO THE DEPARTMENT OF APPLIED CHEMISTRY
SCHOOL OF APPLIED NATURAL SCIENCE
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OFFICE OF GRADUATE STUDIES ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

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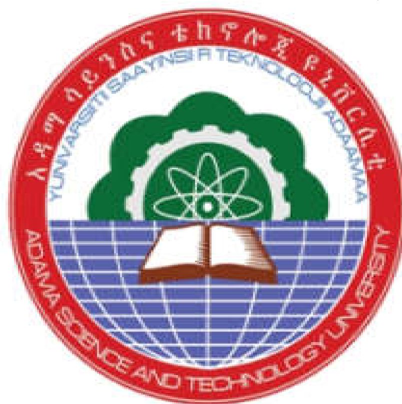

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Advisors approval sheet

To: Applied Chemistry Program

Subject: Thesis Submission

This is to certify that the thesis entitled "Phytochemical Investigation and Antibacterial Studies of *Gladiolus candidus* Root Extract *"submitted* in partial fulfillment of the requirements for the degree of Master of Science in Chemistry has been carried out by Megersa Mekonnen Id. No. GSU/ 0502/06 at Applied Chemistry Program under our supervision. Therefore, we recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis for examination.

Hailemichael Tesso (PhD)	________________	________________
Name of major Advisor	Signature	Date
Yadessa Melaku (PhD)	________________	________________
Name of co-Advisor	Signature	Date

DECLARATION

I hereby declare that this MSc Thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been duly acknowledged.

Name: Megersa Mekonnen

Signature: ____________

This MSc Thesis has been submitted for examination with our approval as thesis Advisors

Name: Hailemichael Tesso (PhD)

Signature: ____________

Date of submission: _________

Name: Yadessa Melaku(PhD)

Signature: ____________

Date of submission: _________

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LIST OF ABBREVATIONS

^{13}C-NMR	Carbon13-Nuclear Magnetic Resonance
CC	Column Chromatography
DEPT-135	Distortion less enhancement by polarization Transfer at angle 135°
EtOAc	Ethyl acetate
IR	Infra-Red
1D-NMR	One Dimensional-Nuclear Magnetic Resonance
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
UV	Ultra Violet

ABSTRACT

Plants have been used to treat a wide range of diseases throughout the history of human beings. The genus Gladiolus (Iridaceae*), with nearly 300 species confined mainly to Africa, has proved to be one of the most important sources of biologically active compounds. In Ethiopian the root of Gladiolus candidus (local name dallo in Afan Oromo and milas golgul in Amharic) is used for the treatment of several diseases including infectious diseases and cancer (nekersa). This study focused on extraction, isolation and characterization of the chemical constituents using CC and spectroscopic method from the roots of Gladiolus candidus and testing of antibacterial activities of the extracts and purified compounds. The roots were successively extracted withn-hexane, ethyl acetate and methanol. The ethyl acetate extracted, followed by separation using silica gel column chromatography. That afforded two compounds characterized as , isomerofisoflavenes*3-(3,4-dihydroxyphenyl)-2H-chromene-7,8-diol*and3-(6-isopentylcyclohex-3-enyl)2'methylpropan-1'-ol).Phytochemical screening of the methanol and EtOAc crude extracts of root of Gladiolus candidus revealed the presence of flavonoids, tannins, alkaloids and saponins. The present study was used gravity column with ethyl acetate and n-hexane as an eluent. However, using the solvent system like n-hexane: EtOAc: $CHCl_3$: MeOH extracts, more phytochemical analysis needs to be carried out on these polar extracts of the plant. This study proved that the crude EtOAc extract, compound* ***30*** *and compound* ***31*** *have promising activity (12, 11 and 14 mm respectively) against, Pseudomonas aeruginosa, which might give a clue about the traditional use of the plant against various infectious diseases. Biological assay on other strains needs to be conducted on future work of extracts of the plant to establish the traditional uses of the plant.*

Key words: Traditional medicines, *G.candidus,* characterization of compounds, antibacterial

1. INTRODUCTION

1.1. Background

Plants have been used to treat a wide range of diseases throughout the history of human beings. Even today, it plays a major role in primary health care as therapeutic remedies in many developing countries. This is mainly because most of these herbals are available, affordable and have little or no side effects [1, 2].

In the last decade, studies have also focused on new group of bioactive components in some foods, which have protective effects against cell oxidation. However, the emergency of resistance pathogens limits the therapeutic use of many of the drugs that are in the market. Some of the compounds obtained from natural sources have also been used as precursors and that can be modified synthetically to improve their therapeutic activities. There are several reasons that necessitate isolation and characterization of bioactive compounds. Some of the reasons are the distribution is not uniform throughout the world to be used by people. Most medicinal plants are under threat of extinction due to climate changes and population pressure [2, 3].

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans [4]. Hence, a great deal of interest has been generated recently in isolation, characterization and biological activity of these phytochemicals [5]. Natural products such as alkaloids, flavonoids, tannins, resins, glycosides, and oils among others reported to be bioactive against a vast range of pathogens and have therapeutic activities (like that of antibacterial, antiviral, anti-oxidants and antifungal) [6]. Many ethnobotanical reports was indicated the use of Ethiopian medicinal plants as “anticancer” remedy and anthrax. These include *Gladiolus candidus* (*Rendle*) plant [7]. In many developing countries, traditional medicines are still the mainstay of health-care, and most of the drugs and cures come from natural sources, such as, plants [8].

Botanical medicines or phytomedicines refer to the use of seeds, berries, leaves, bark, root or flowers of many plants for medicinal purposes by significant number of people [9].

Phytochemistry or natural product chemistry research is the backbone of herbal industry and for novel drug compounds. Plant derived medicines have made large contributions to human health and well-being [10].

Gladiolus candidus is one of the more than 300 species that belongs to the family *Iridaceae*. It mostly grows in moderately moist soil and in rain condition [11, 12]. The genus is found in Asia, Southern Europe, Sub-Saharan Africa, South Africa and Eastern Africa (Ethiopia). In Ethiopia, the species is popularly known as *dallo in Afan Oromo and milas golgul* in Amharic, which has been used in traditional medicine for treatment of some diseases, which are included infectious diseases, 'nekersa' cancerous tumor, edema, wound and anthrax [13].

Despite the tremendous progress in medicine, infectious diseases caused by bacteria, fungi, viruses and parasites continue to pose a threatening challenge to public health [14]. The family *iridaceae* has a wide range of chemical compounds. The most common secondary metabolites are flavonoids and isofl avonoids. The second most common group of secondary metabolites are flavones, quinones and xanthones to threat challenge to public health and therapeutic activities [15]. The present study focuses on isolation and characterization of compounds using CC and spectroscopic methods and evaluation of antibacterial activities of the extracts of *Gladiolus candidus* root.

1.2. Statement of the Problem

Infectious diseases caused by bacteria, fungi, viruses and parasites continue to pose a threatening challenge to public health [16].

These diseases account for all deaths in both developed and developing countries. Mainly in developing countries due to poverty, high mortality rate of the patients, unavailability of medicines, safety of medicinal plants limited by inadequate knowledge of bioassay tests and the emergence of widespread resistance of pathogens to the available drugs. The high cost of synthetic drugs also makes them unaffordable to many people in this country.

In Ethiopia, the *Gladiolus* species is popularly known as *dallo in Afan Oromo and* milas golgul in Amaharic, which has been used as traditional medicine for treatment of diseases, nekersa (cancerous tumor), edema and anthrax [17,18].

Traditionally, as treating cancer (nekersa), the root is powdered and applied directly on the external tumor (wound) or the powder is mixed with water and drunk [19]. Because of this plant, selection was based on traditional medicine.

Therefore, this study was to identify compounds from root of *Gladiolus candidus* plant and testing some selected bacteria strains.

1.3. Significance of the Study

Plant derived medicines have made large contributions to human health and well-being. This study was focused on the phytochemicals investigation and antibacterial studies on the roots of *G. candidus*. This study was isolated and reported two compounds for the first time from this plant. There was provided important information about the structures of the secondary metabolites from the roots of this plant. The chemical constituents of this plant provide baseline information for future pharmacological and bioactive compounds studies. Furthermore, it will provide initial information for the researchers who are interested to conduct further studies on this area. Identified compounds were used as antibacterial agents in this plant. Moreover, crude extracts and pure compound **30** obtained from the plant was exhibited pronounceable antibacterial activities against *Pseudomonas aeruginosa* compared to standard drug.

1.4. Objectives of the Study

1.4.1. General Objective

The main objective of this research is to conduct phytochemicals investigations and antibacterial studies of the root extracts of *G. candidus*.

1.4.2. Specific Objectives

1. To extract the roots of the *G. candidus* successively with *n*-hexane, EtOAc and MeOH;
2. To conduct preliminary phytochemicals screening on the *n*-hexane, EtOAc and MeOH extracts of the root of *G. candidus*;
3. To isolate compounds from extracts of the root of *G. candidus* by using CC;
4. To characterize the isolated compounds by employing spectroscopic methods such as IR, ^{1}H-NMR, ^{13}C-NMRand DEPT-135;
5. To evaluate the antibacterial activities of the roots of crude extracts and isolated compounds of *G. candidus* against two Gram-negative bacterial strains: *p. aeruginosa* and *E. coli* and two Gram-positive bacterial strains: *S. aureus and B. subtilis* using agar well diffusion method.

2. LITERATURE REVIEW

2.1. The Family Iridaceae

Gladiolus candidus is an herbaceous annual climbing plant belongs to the family Iridaceae [18]. The Iridaceae is a large family with over 2000 species, many of which are endemic to South Africa [19]. It is a family of perennial plant [20]. Some species of *Gladioli* have various ethnomedicinal uses such as in the treatment of a variety of diseases including cancer (nekersa), anthrax, edema, ear ache, stomach ache, wounds and eye diseases [21].

2.2. Scientific Classification

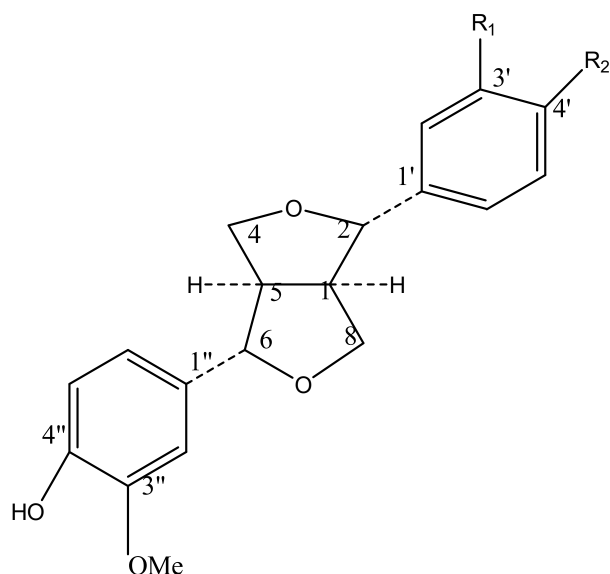
Gladiolus (from Latin, the diminutive of *gladiolus*, a sword) and classified as:
Kingdom: Plantae;
Class: Liliopsida;
Order: Liliales;
Family: Iridaceae;
Genus: *Gladiolus;*
Species: *candidas* [21, 22].

2.3. Phytochemistry

The genus *Gladiolus* contains about 300 species [23]. The family Iridaceae has a wide range of chemical compounds such as flavonoids, isoflavones, anthocyanins, quinonoid, xanthone, saponins, sterols, the O-glycosides of the flavonols quercetin, kaempferol, flavones , glycosides, nonprotein aminoacids and biflavones have been reported [24]. The unusual free amino acids, carboxyphenylalanine and carboxyphenyglycine have distinctive distribution in this family. In the genus *Gladiolus*, various compounds have been reported in various parts of family. However, amongst the species studied here, only *G. dalenii* has phytochemicals reports to have alkaloids [25]. Other *Gladiolus* species such as *G. gregasius* was reported to have saponins, glycosides, steroids, alkaloids, triterpenes and phenols [26]. *G. imbricatus* contain flavonones, quinines and carbohydrates [27].

2.4. *Gladiolus Segetum*

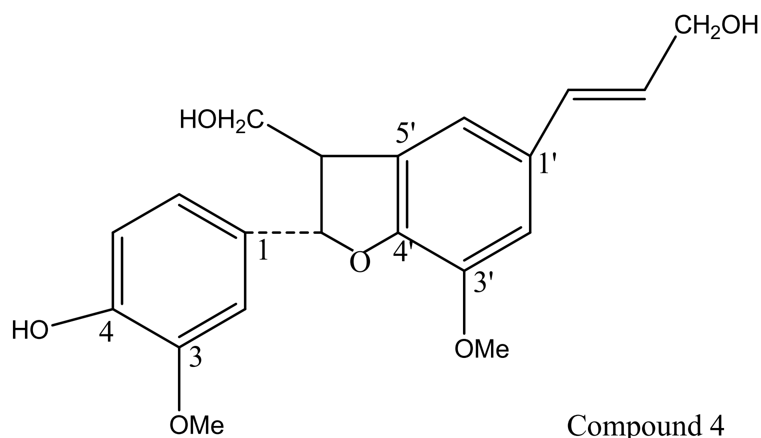
From *Gladiolus segetum* nine compounds were reported and structurally elucidated. These compounds were methanolic extract from the chloroform soluble fraction of the corms of *Gladiolus segetum*, using different chromatographic techniques and various tools of NMR spectral analysis as well as MS spectral analysis.The isolated compounds (1-9) were identified as the lignans (+)-demethoxypinoresinol (**1**), (+)-pinoresinol (**2**) and (+)-pinoresinol monomethylether (**3**); the neolignan (–)-dehydrodiconiferyl alcohol (**4**) and the anthraquinones deoxyerythrolaccin (**5**), physcion (**6**), laccaic acid methylester (**7**), 6'-O-Palmiitoyl-3-O-β-sitosterol glucoside (**8**) and β-sitosterol-3-O-glucoside (**9**) [28].



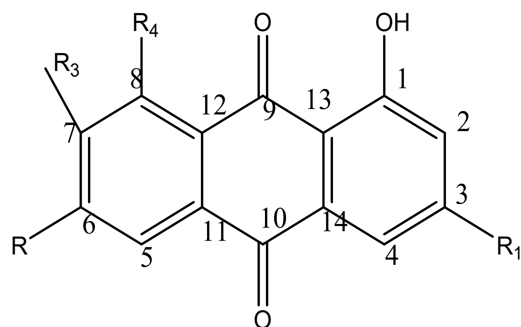
Compound (1): R1= H, R2= OH

Compound (2): R1= OMe, R2= OH

Compound (3): R1, R2= OMe



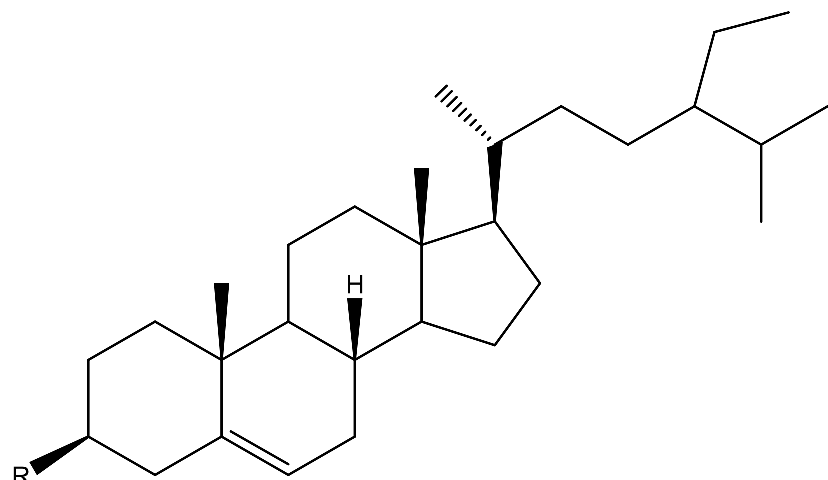
Compound 4



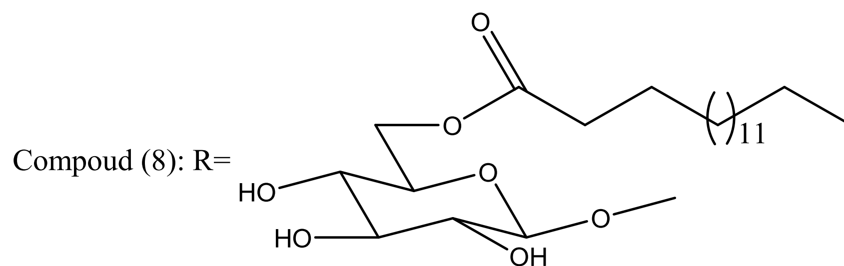
Compound (5): R1, R2= OH, R3= H, R4= Me

Compound (6): R1= Me, R2= OMe, R3= H, R4= OH

Compound (7): R1, R2= OH, R3= COOMe, R4= Me



Compoud (8): R=



Compound (9): R= OH

Figure 1: Chemical structures of compounds reported from *Gladiolus segetum*.

2.5. *Candidas* Species

From candida species 22 chemical constituents were reported. These are 3’-methyl nor-dihydroguaiaretic acid (21) and meso 7S, (8S, 7’R, 8’R)-3, 4, 3’, 4’-tetrahydroxy-7, 7’epoxylignan 22 showed strong activity against standardized strains [29].

R
R3
R2
R1
O
OH
O

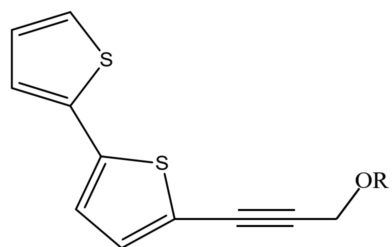
8. R = OCH3; R 1, R3 =H; R 2= OH

9. R, R 2 =OCH3; R 1, R3 = OH

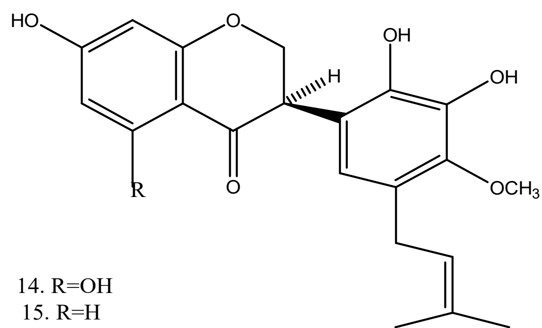
10. R, R 2 =OH; R1 =OAc; R 3 =H

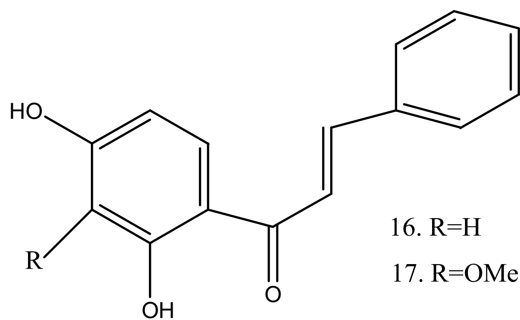
O
O
O
O

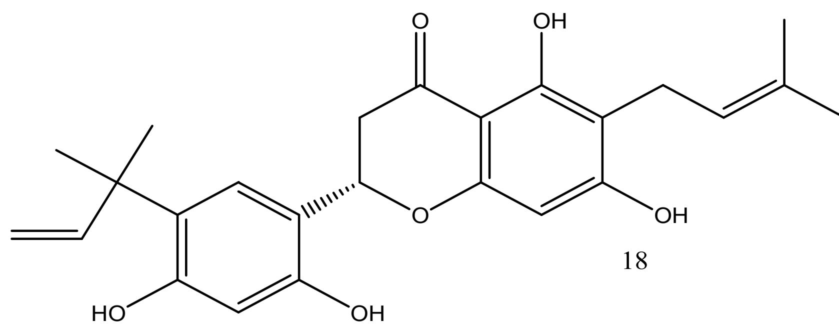
11

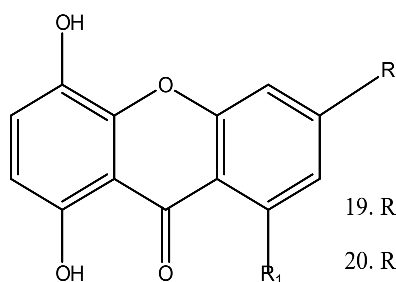


12. R=H
13. R=Ac







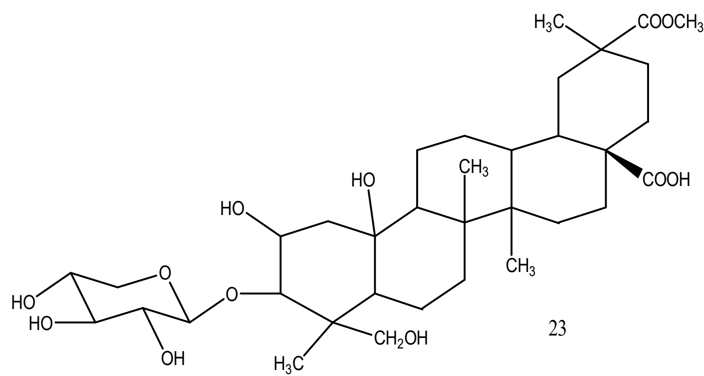


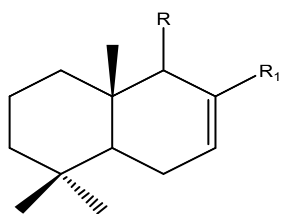
19. R, R1 = CH3

20. R, R1 =H

21. R=H; R1= CH3

22. R = CH3; R1=H

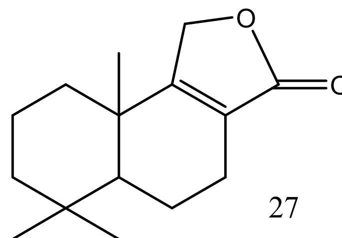




24. R=b-CHO; R1= CHO

25. R=a-CHO; R1= CHO

26. R=b-CH2OH; R1 =CH3



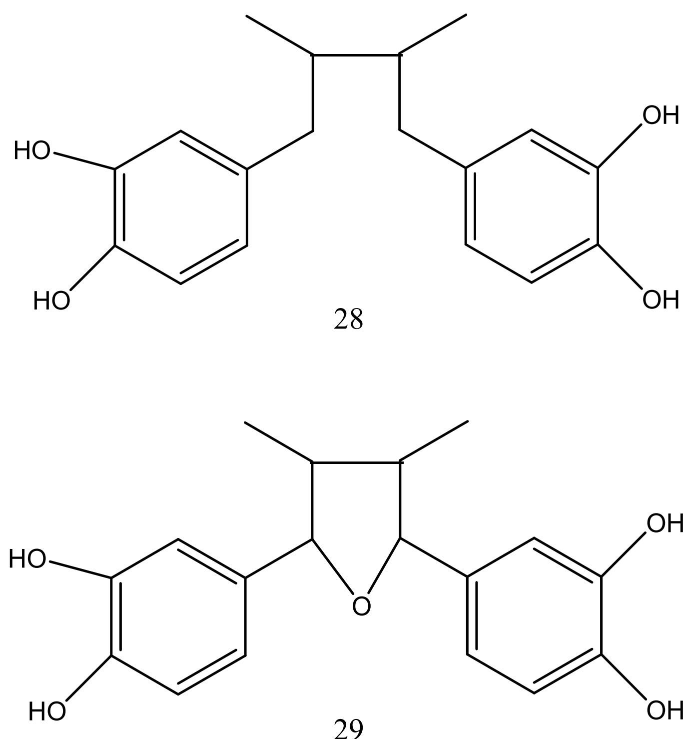


Figure 2: Chemical structures of compounds reported from *Candidus* species.

2.6. Biological Activities of the *Gladiolus* Species

Infectious diseases are considered a major threat to human health, because of unavailability of vaccines or limited chemotherapy. The various parts of *Gladiolus* family have been reported to have antibacterial, anti-inflammatory, insecticidal, antifungal, anti-tuberculosis properties. Pharmacological activities of the crude extracts of *G. gregasius* have been reported to have anti-inflammatory, anti-bacterial, and insecticidal activities [30,].This species is active against the bacteria, *Bacillus subtilis* and *Staphylococcus* aureus [31, 32]. Another bioactivity study on the *Gladiolus* species also reported moderate activity against *Aspergilus niger* [32].

Various bioassay tests were conducted on some *Gladiolus* species. The dichloromethane extracts of bulb of *Gladiolus* were reported to have antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, while ethanol extracts of the plant showed antifungal activity against *Candidas albicans* and *A. niger* [33]. Anti-amoebic activity was also reported [33].

Other *Gladiolus* species *G. gregasius* reported to have alkaloids, flavonoids, triterpenes and coumarins that are the main classes of compounds encountered in this plant. *G. gregasius* tested as antibacterial and anticandidal agents and could be used in the treatment of various gastrointestinal ailments caused by resistant microbial agents. Compounds isolated from this plant was reported active against *A. niger*, *Candida* species and *Psuedomonas aeruginosa* [34].

Eleutherine americana merr (iridaceae family) a medicinal plant, for treating diabetes, breast cancer and stroke. *E. americana* was reported that, it show good antibacterial, anti-inflammatory, and antiviral (inhibited HIV replication) activities. Ethanol extract of *E. americana* inhibited bacteria like *staphylococcus aureus.* Compounds (eleutherol, eleutherol and isoeleutherine) isolated from *n*-hexane extract of *E. americana* were reported potential as antihypertation [35].

Iris germanica (*iridaceae*) were reported to have medicinal importance and used in the treatment of cancer, inflammation, bacterial and viral infections. The methanolic extract or isolated flavonoids were reported to have potent anti-inflammatory effects and showed highest anti-microbial effect against *S. aureus* and *E.coli* [36].

The ethanolic extracts of the aerial parts of *Gladiolus illyricus* (*iridaceae* family) were reported to exhibited antioxidant properties that used to prevent or reduce free radicals from attacking DNA in cell. And reported that extracts showed moderate antimicrobial activity only on *Bacillus subtilis* [37].

Irises (iridaceae) used as the ornamental plants, due to their colorful flowers or in the perfume industry. This species were also used in many part of the world as medicinal plants for healing of a wide diseases. The genus *iris* rich in isoflavonoids which have a wide range of biological activity including anti-inflammatory, antifungal activity, antioxidant and anticancer preventive properties. The occurrence of isoflavonoids in plants of traditional medicine has promoted the increasing interest in search for biologically active constituents specially in iridaceae family. The antifungal activity of methanolic extracts of *Iris nigricans* reported as that of the leaves extract stronger antifungal activity than other part of the plant [38].

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

All solvents and chemicals used were of analytical grade (solvents used are 99% *n*-hexane, 97% ethyl acetate and 97% methanol). The other chemicals which were used for phytochemical screening are 50%NH_3, 5%$FeCl_3$, 10%NaOH, KBr, HCl, HNO_3, H_3PO_4, Wagner's reagent, and Fehling's solution. Vanillin in H_2SO_4 spraying reagent was used for visualizing spots invisible to naked eyes on thin layer chromatography plates. All organic solvents were purchased from Alchem private trading limited company, Addis Ababa (sub-city Kirkos).

3.2. Apparatus and Instruments

The materials that were used in this study are grinder, TLC plate, Rotary evaporator, UV lamp, measuring cylinder and others. Analytical thin layer chromatograms that was used was a 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminum plate. Column chromatography was performed using silica gel (250-400 mesh) Merck. The Infrared using infrared spectrometer at 400-4000 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz, using $CDCl_3$ as solvent. ^{1}H NMR and ^{13}C NMR chemical shifts were referenced to the solvent residual signals of $\delta 1_{H}$7.26 and $\delta^{13}{}_{C}$77.0 and methylene and quaternary carbons signal showed using DEPT-135.

3.3. Plant materials

The fresh roots of *Gladiolus candidus* (*Iridaceae*) were collected and packed in polythelene bag from Gojamma Habuba village, Sudie Woreda, Oromia, Ethiopia, which is 214 km to the East of Addis Ababa in December 2017. The plant material was authenticated by botanist Shamble Alemu, and a voucher specimen (Voucher no: GW 008) was deposited in the National Herbarium, Department of Botany/Biology, Addis Ababa University, Addis Ababa, Ethiopia,.

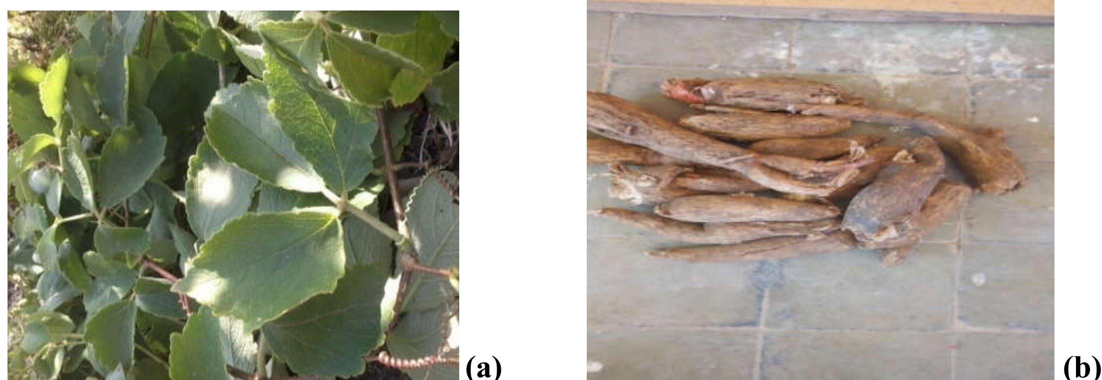


Figure 1: Aerial parts (a) and roots (b) of *G. Candidus* (pictures taken by Megersa Mekonnen at Gojamma Habuba Village, December 2017).

3.4. Preparation of Plant Material and Extraction Methods

The roots were grinded to powder using electric blender. The powdered plant material was stored in polythelene bag until used for extraction.

The powdered roots (500 g) were macerated for 72 hours through shaking and extracted with the solvent *n*-hexane using 500 g:1.5 L sample to solvent ratio at room temperature. The extract was filtered by Whatmann no. 1 filter paper and concentrated by rotary evaporator at 40°C. The process yielded 2.057 g (0.41%) *n*-hexane crude extract.

The marc left was further extracted by same procedure using ethyl acetate, and then concentrated and yielded 6.18 g (1.24%) crude extract. The marc from EtOAc extraction was further extracted by same procedure using MeOH, filtered, concentrated and yielded 100.11 g (20.02%) crude methanol extract. The crude extracts (*n*-hexane, EtOAc and methanol) that showed best TLC profile (Appendix 1) and better preliminary screening result (Table 3, EtOAc extract) was subjected to chromatographic separation (silica gel 250-400 mm mesh size) to isolate compounds. Analytical TLC was run on a 0.2 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Column chromatography was performed using silica gel (250-400 mesh size) Merck. Structural elucidations of the pure isolated compounds carried out by using IR spectra of compounds were recorded using a Perkin-Elmer BX Spectrometer (400-4000 cm^{-1}) as KBr pellets. And NMR spectra that were recorded using Bruker Avance 400 spectrometer operating at 400 MHz and DEPT-135 spectroscopic method.

3.5. Phytochemical Screening Test

Phytochemical screening test were done to identify the type of secondary metabolites present in the crude extract.

Screening procedure

Phytochemical screening test were done to identify the type of secondary metabolites present in the crude extract. Chemical tests were conducted on organic extracts plant root part [38].

Test for Phenols

Ferric Chloride Test: The extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color were confirmed the presence of phenols.

Test for saponins

Distilled water (5 mL) was added to the extracted plant material (0.5 g) in a boiling tube. The mixture was shaked and heated in water bath for 2 minutes. The presence of a stable froth was indicated the presence of saponins [39].

Test for tannins

Distilled water (10 mL) was added to extracted plant material (0.5 g) in a test tube. It was boiled for 3 minutes and filtered using Whatmann filter paper No. 1.

Ferric chloride (0.2 g) was added and the mixture observed a dark or dirty green precipitate which was indicated the presence of tannins [39].

Test for terpenoids

A portion of powdered plant material (0.5 g) was added in a boiling tube and chloroform (2 mL) carefully adds, concentrated sulphuric acid (3 mL) was added drop wise. Presence of a reddish brown coloration at the interface was showed positive, presence of terpenoids [39].

Test for alkaloids

A 0.5 g of the extract was evaporated and the result was heated on boiling water bath with 2 N of HCl (5 mL of HCl) after cooling the mixture was filtered and treated with Wagner's reagent (a mixture of solid iodine, potassium iodide and distilled water). The formation of precipitation was indicated alkaloids [39].

Test for flavonoids

A portion of the extract (0.5 g) was heated with 10 mL ethyl acetate over a steam bath for 3 minutes, the mixture was filtered and 4 mL of the filtrated was shaked with 1 mL dilute ammonia (50%). A yellow coloration was indicated the presence of flavonoids [40].

Test for glycosides

A portion of the ground plant material (0.5 g) was added to a boiling tube. Distilled water (10 mL) was added and stirred. These were filtered and a portion of the filtrated (2 mL) was hydrolyzed with few drops of concentrated hydrochloric acid; a few drops of ammonia solution were then added to the mixture. Five drops of this solution were put aside in a separate test tube and then 2 mL of Benedict's reagent added and boiled. Reddish to brown precipitate were indicated of the presence of glycosides [40].

Test for cardiac glycoside

Keller-Killani Test: To 2 mL of extract, glacial acetic acid, one drop 5% ferric chloride and concentrated sulfuric acid was added. Appearance of reddish brown color at the junction of the two layers was indicated the presence of cardiac glycosides.

Test for anthraquinones glycosides

To 3 mL extract, dilute sulfuric acid was added, boiled and filtered. To the cold filtrate equal volume chloroform was added. The organic layer was separated and ammonia was added. Ammonical layer was turned pink or red and indicated the presence of anthraquinones glycosides [41].

Test for reducing sugar

To 0.5 mL of extract solution, 1 mL of water and 5-8 drops of Fehling's solution were added and observes brick red precipitate, showed the presence of reducing sugar.

Test for phytosterol

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulfuric acid was added. Formation of brown ring at the junction was indicated the presence of phytosterol.

Test for coumarins

NaOH test: 10%NaOH was added to the extract, and chloroform was added. Formation of yellow color showed the presence of coumarins.

3.6. Evaluating Antibacterial Activities of the Roots Extracts of *G. candidus*

The activity of the plant extracts tested against four American Type Culture Collection (ATCC) bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633) *Pseudomonas aeruginosa* (ATCC 7553) and *Escherichia coli* (ATCC 25922). Antibacterial activities of the root extracts of *Gladiolus candidus* were investigated *in vitro* using agar well diffusion method against test bacterial.

The EtOAc crude extracts and isolated pure compounds of *Gladiolus candidus* roots were evaluated for antibacterial assay by using agar well diffusion method. Agar well diffusion test method used as to determine whether a bacterium is resistant to the crude extracts and pure compounds. This method was selected due to its simplicity, capacity to analyze multiple samples simultaneously and its ability to work well with defined inhibitors [42-49].

The antibacterial activities of the two samples were tested against two Gram-negative bacterial strains: *Pseudomonas aeruginosa* and *Escherichia coli* and two Gram-positive bacterial strains: *Staphylococcus aureus* and *bacillus subtilis* using agar well diffusion method.

The bacteria were obtained from Adama Science and Technology University Biology department, Adama, Ethiopia.

The selection of the test micro-organisms were based on their roles for causing infectious diseases such as, external cancer and edema.

The Mueller-Hinton agar was used as nutrient medium for initiating the growth of bacterial by swabbing each test strain on it.

The standard antibiotic drug *Gentamicin* 35 μg/mL were used as positive control and DMSO were used as negative control. Subsequently, about 15, 25 and 35 μg of each extracted and purified samples of *Gladiolus candidus* root extracts were dissolved separately in 1 mL of dimethyl sulfoxide (DMSO) and stirred for 10 minutes. Then, the wells were made on each petri-plates by using sterile cork borer having a diameter of 6 mm.

About 100 μL of each sample of the four different prepared solutions and positive control were poured into wells on all plates with the help of micropipette. The plates were then turned up side down and incubated at 37°C for 24 hours in an incubator.

Finally, after incubation, the zones of inhibition formed around the wells against the test organisms the diameter were measured in millimeter (mm) by using a ruler and recorded, including the well diameter. The reading were taken in three different fixed directions in all three concentrations.

3.7. Isolation and Purification of Compounds

A glass column of 34 cm diameter with 65 cm length was packed with the 150 g silica gel in *n*-hexane. The slurry crude extract (5 g) adsorbed on 5 g silica gel was applied to the top of the packed silica gel column using a spatula.

The column was first eluted with *n*-hexane and the polarity of the solvent system was increased by increasing the percentage of ethyl acetate as shown in Table 1.

The column eluted starting with 100% of pure *n*-hexane, 90%:10% (*n*-hexane:EtOAc), followed by increasing gradient of ethyl acetate in *n*-hexane as an eluent of the solvent system. Totally, 86 fractions were collected each with 50 mL volume.

Table 1. Solvent systems and fractions collected from EtOAc crude extract.

Fraction number	Eluent	Ratio	Volume (mL) collected
1	*n*-hexane	100%	50mL
2-4	*n*-hexane:EtoAc	9:1	»
5-20	»	8:2	»
21-37	»	7:3	»
38-50	»	6:4	»
51-61	»	1:1	»
62-68	»	4:6	»
69-75	»	3:7	»
76-80	»	2:8	»
81-83	»	1:9	»
84	EtOAc	100%	»
85	EtOAc:MeOH	1:9	»
86	EtOAc:MeOH	1:9	»

The spots developed were visualized under UV light at 254 and 365 nm and then by exposure to vanillin reagent. The fractions that showed the same TLC profiles (color and R_f value) were combined and repacked.

Fraction 1-10 did not show any spot and were colorless on TLC. Fraction 11 eluted with the solvent system *n*-hexane: EtOAc (8: 2) showed one clear yellow spot.

On standing for 24 hours (*n*-hexane insoluble) precipitate formed was a yellow gelatin like substance which gave **(**15.2 mg, coded as GGcE-11, R_f = 0.7, 20% ethyl acetate in *n*-hexane) but it is not characterized because of long chain hydrocarbon .

Fraction 12-15 which showed similar spots on TLC were combined and further purified by small sized column chromatography.

However, in purification, the yield became very small for spectroscopic analysis and hence it was left out. Fraction 16 eluted with the solvent system *n*-hexane: EtOAc (8: 2) showed one clear purple spot.

On standing for 24 hours a precipitate (*n*-hexane insoluble) formed was a purple gelatin like substance which gave compound **30** (21.1 mg, coded as GGcE-16, R_f = 0.61, 20% ethyl acetate in *n*-hexane). The spectroscopic analyses were conducted but the ^{13}C NMR spectrum no peak was observed for this sample and was not characterized.

Fraction 17-22 which showed more than three spots on TLC hence it was left out.

Fraction 23 which showed three separable spots was further purified by small column chromatography and eluted with the solvent system *n*-hexane : EtOAc (7 : 3) (30% ethyl acetate in *n*-hexane). From the collected fractions, fraction 23^{1st}, 23^{2nd} and 23^{3rd} showed one clear spot on TLC with similar R_f value. These fractions were combined, and dried to get yellow powdered (*n*-hexane insoluble) compound **30** (19.8 mg, coded as GGcE-23, R_f = 0.44 in 30% EtOAc in *n*-hexane).

Fraction 24-50 which showed similar spots on TLC were combined and further purified by column chromatography but in purification the yield became very small for spectroscopic analysis and hence it was left out.

Fraction 51 eluted with the solvent system *n*-hexane: EtOAc (1:1) showed one clear purple spot.

On standing for 24 hours a precipitate (*n*-hexane insoluble) formed was a dark gelatin like substance which gave compound **31** (16.1 mg, coded as GGcE-51, R_f = 0.35, 50% ethyl acetate in *n*-hexane).

Fraction 59 eluted with the solvent system *n*-hexane: EtOAc (1:1) showed one clear yellow spot. On standing for 24 hours a precipitate (*n*-hexane insoluble) formed was a yellow gelatin like substance which gave 13.2 mg (R_f = 0.3, 50% ethyl acetate in *n*-hexane). The spectroscopic analyses were conducted but the ^{13}C NMR spectrum so much peak was observed for this sample and was not characterize.

Each fraction, starting from fraction 52-58 and 60-86 when monitored by TLC even by changing the ratio of eluent exhaustively and visualized using vanillin, spots do not separate rather form tails and became difficult to isolate them.

4. RESULTS AND DISCUSSION

4.1. Extract Yield

The root of *Gladiolus candidus* were successively extracted with *n*-hexane, EtOAc and methanol with the results presented in Table 2. The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. The *n*-hexane extract yields a deep yellow (2.06 g), the EtOAc extract yields a reddish crude extract (6.18 g) and the methanol extract yields a purple crude extract (100.11 g). The percentage yields of crude extract in respective solvent were listed in (Table 2). The MeOH extract yielded the highest amount of extract while, the *n*-hexane extract result was the lowest yield.

Table 2. Percent yield of each crude extract.

Solvent	Root of *Gladiolus candidus*	
	weight of crude extract (g)	% yield
n-hexane	2.06	0.41
EtOAc	6.18	1.24
Methanol	100.11	20.02

$$\% \text{ yield} = \frac{\text{Weight of crude extract}}{\text{Weight of the sample}} \text{x}100$$

Weight of sample used = 500 g of powdered dried roots of *Gladiolus candidus*.

From this table the yield of methanol extract is higher than *n*-hexane and EtOAc extract. Therefore, this gave a clue that the root part of this plant was found to be rich with polar constituents. In this study, the aim was achieved on amount or yield of extract but not excessive yield of extract.

4.2. Phytochemical Screening Tests

The phytochemical screening tests on the root of *G.candidus* were tested the presence of phenols, alkaloids, anthraquinone glycosides, flavonoids, cardiac glycosides, glycosides, terpenoids, phytosterol, tannins, coumarins, saponins and reducing sugar.

Table 3. Result of phytochemical screening tests of the crude extract of root of *Gladiolus candidus*.

Secondary metabolities	Test/method	Observation		
		n-hexane	EtOAc	Methanol
Phenols	Ferric chloride test	_	_	+
Tannins	Dirty precipitate formation	+	+	+
Alkaloids	Wagner's reagent /test	_	+	+
Anthraquinones glycosides	Ammonical layer to junction	_	+	_
Flavonoids	Yellow coloration	_	+	+
Glycosides	Benedict's test	+	+	_
Cardiac Glycosides	Keller-killani test	_	+	_
Saponins	Forthing test	_	_	+
Coumarins	NaOH test	_	+	_
Terpenoids	Brown coloration	+	+	+
Reducing sugar	Fehling's test	_	_	_
Phytosterol	Brown ring junction	_	_	_

Key: Present (+), Absent (-), *n*-hexane-normal hexane, EtOAc-Ethyl acetate, MeOH-Methanol

The *n*-hexane extract showed that absence of flavonoids, alkaloids, saponins and phenols were observed. This is due to the non-polar *n*-hexane extract defat and dissolves non-polar compounds.

Methanol and EtOAc extracts showed the presence of flavonoids, tannins, alkaloids and saponins among major class of compounds.

However, in the root of *Gladiolus candidus* phytosterol and reducing sugar were not present in *n*-hexane, EtOAc and MeOH extractions in Table 3. Therefore, the phytochemical constituent testing result revealed that the presence of phytochemical constituents in the plant extract. These constituent could be possess antibacterial properties and responsible for curing various ailments and play significant roles in bioactivity of medicinal plants, which produce a definite and specific action on the human body.

Generally, *iridaceae* family reported as rich sources of secondary metabolites, in which flavonoids are predominant, that used as antioxidant and anticancer [49].

4.3. Characterization of isolated compounds

In this work, three compounds have been isolated from the roots extract of *G. candidus.* Described herein is the characterization of these compounds one by one.

4.3.1. Characterization of Compound 30

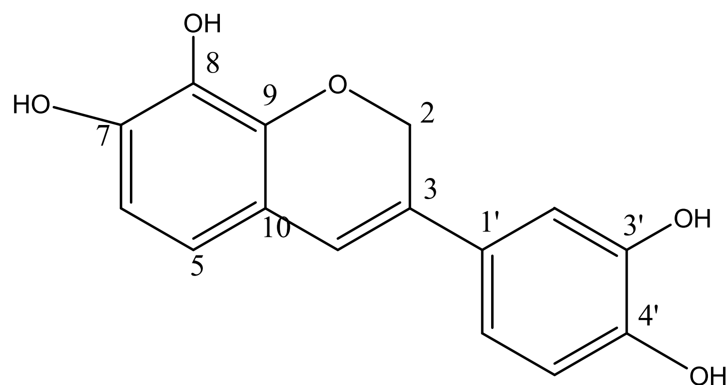
Compound **30** was isolated as yellow powdered solid with R_f = 0.44 (30% EtOAc in *n*-hexane). The IR spectrum (Appendix 9) of GGcE-23 showed vibration at 3428 cm^{-1} (aliphatic O-H stretching), 2915 cm^{-1} (aliphatic C-H stretching), 1614 cm^{-1} (aliphatic C=C stretching) and 1262 cm^{-1} (C-O bond stretching of ether moiety).

The ^1H NMR spectrum ($CDCl_3$, 400 MHz, Table 5, Appendix 10) displayed the presence of two singlet peaks at δ_H 7.38 (1H, *s*) and 4-OH at 5.35 (4H, s). Four doublet peak observed at δ_H 3.75(1H, d, J=7.2), δ_H 3.78(1H, *d*, J=7.2), δ_H 6.33 (2H, *d, J*=2.4 Hz) and δ_H 6.98 (3H,*d, J*=8.4Hz).

The ^{13}C NMR spectrum (Appendix 11) shows the presence of eight quaternary carbons at δ_c (113.39, 114.75, 119.55, 120.32, 150.74, 151.65, 154.46 and 155.18).

There are six methines carbons at δ_c (128.39, 127.61, 122.75, 122.44, 107.92 and 103.12). And one methylenes at carbons δ_c (69.95) and supported by DEPT-135, pointing dawn in DEPT-135 (Appendix 12).

Based on the spectral data the structure of compound **30** was found to be 3-(3,4-dihydroxyphenyl)-2H-chromene-7,8-diol.



3-(3,4-dihydroxyphenyl)-2*H*-chromene-7,8-diol

Figure 3: Structure of proposed compound 30.

Table 4. Spectral data of compound **30**.

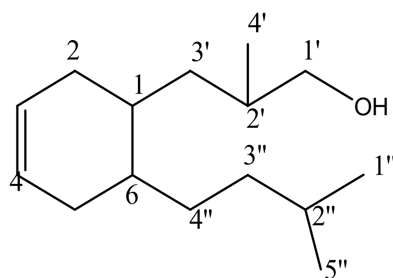
Position	^{1}H NMR of compound **30**	^{13}C NMR of compound **30**	DEPT-135 of compound **30**
2	3.78(d,1H,J=7.2Hz), 3.75(d,1H, J=7.2Hz)	69.95	69.95
3	---	114.75	---
4	6.98 (*d*,2H, *J*=8.4Hz)	128.39	128.39
5	6.33 (*d*,1H, *J*=2.4 Hz)	127.60	127.60
6	6.98 (*d*,1H, *J*=8.4 Hz)	103.12	103.12
7	---	154.47	---
8	---	119.55	---
9	---	155.19	---
10	---	113.79	---
1’	---	120.32	---
2’	7.38 (*s*,1H)	107.92	107.92
3’	---	151.65	---

4'	---	150.74	---
5'	6.98 (*d*,1H, *J*=8.4 Hz)	122.44	122.44
6'	6.33 (*d*,1H, *J*=2.4 Hz)	122.76	122.76
	C-OH 5.35, (s,4H)		

4.3.2. Characterization of Compound 31

Compound **31** was isolated as purple amorphous solid with R_f = 0.55 (50% ethyl acetate in *n*-hexane).The IR (Appendix 13) spectrum of **31** showed vibration at 3427 cm^{-1} (aliphatic O-H stretching), 2928 cm^{-1} (aliphatic C-H stretching) and 1626 cm^{-1} (aliphatic C=C stretching).
The ^{1}H NMR spectrum ($CDCl_3$,400 MHz, Table 6, Appendix 14) displayed the presence of one doublet peak observed at δ_H 1.27 (9H, *d*, *J*=8 Hz). One singlet peak observed at δ_H2.07 (1H, *s*) due to C-OH bond. One doublet of doublet peak observed at δ_H4.17 (*dd,*1H,*J*=6.8 Hz and J=7.2 Hz) and δ_H 3.77(*dd,*1H,*J*=6.8Hzand J=14.4 Hz) . Two multiplet peak at δ_H 1.74 (14H, *m*) and 5.26 (2H, *m*)).

The ^{13}C NMR spectrum (Appendix 15) shows the presence of one hydroxyl aliphatic carbons at δ_c 60.58. Six methines carbons at δ_c (25.79, 21.08, 17.95, 17.87, 122.81 and 122.46).
Six methylenes carbons at δ_c (22.71, 29.95, 29.68, 29.38, 31.95 and 60.53) and also supported by DEPT-135, pointing down in DEPT-135 and three methyl group at δ_c (14.13, 14.20, and 17.84) and also supported by DEPT-135, pointing up in DEPT-135 (Appendix 16).
Based on the spectral data the structure of compound **31** was found to be 3-(6-isopentyl cyclohex-3-enyl)-2-methylpropan-1-ol.



3-(6-Isopentylcyclohex-3-enyl)-2-methylpropan-1-ol

Figure 4: Structure of proposed compound 31

Table 5. Spectral data of compound **31**.

Position	^{1}H NMR of compound **31**	^{13}C NMR of compound **31**	DEPT-135of compound **31**
1	1.74 (*m*,1H)	17.95	17.95
2	1.74 (*m*,2H)	31.95	31.95
3	5.28 (*m*, 1H)	122.81	122.81
4	5.28 (*m*, 1H)	122.46	122.46
5	1.74 (*m*,2H)	22.71	22.71
6	1.74 (*m*,1H)	25.79	25.79
3’	1.74 (*m*,2H)	29.78	29.78
2’	1.74 (*m*,1H)	21.07	21.07
4’	1.27 (*d*,3H,*J*=8Hz)	17.84	17.84
1’	4.17 (*dd,*1H,*J*=6.8 Hz and J=7.2 Hz)	60.53	60.53
	3.77(*dd,*1H,*J*=6.8Hzand J=14.4 Hz)		
4’’	1.74 (*m*,2H)	29.38	29.38

3’’	1.74 (*m*, 2H)	29.68	29.68
2’’	1.74 (*m*,1H)	17.87	17.87
5’’	1.27 (*d*,3H,*J*=8 Hz)	14.20	14.0
1’’	1.27 (*d*,3H,*J*=8 Hz)	14.13	14.13
	C-OH 2.07 (*s*,1H)		

4.4. Antibacterial Activities Evaluation

The EtOAc crude extracts and isolated pure compound **30** and compound **31** of *Gladiolus candidus* roots were evaluated for antibacterial assay by using agar well diffusion method.
Agar well diffusion test method often used as qualitative methods to determine whether a bacterium is resistant, intermediately resistant or susceptible to the crude extracts and pure compound. The results *in vitro* antibacterial test were summarized below.

Table 6. Results of antibacterial activities evaluation.

Sample	Inhibition zone (mm) (Diameter)											
	Gram positive bacteria						Gram negative bacteria					
	S. aureus			*B. subtilis*			*S. aeruginosa*			*E. coli*		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
EtOAc	6	6	6	6	8	9	12	12	12	6	6	6
Cpd 30	8	8	8	8	10	11	14	14	14	6	6	6
Cpd 31	6	6	6	6	7	7	8	8	8	6	7	7
Gentamicine	16			15			16			14		

Where C_1 = concentration 1, C_2 = concentration 2 and C_3 = concentration 3

From the Table 7 (Appendix 2), 9 and 12 mm found to be the zone of inhibition breakpoint for crude extract against *Bacillus subtilis and Pseudomonas aeruginosa* and standard Gentamicin 16 and 15 mm respectively.

Compound **30**, 8, 10 and 14 mm were found to be the zone of inhibition breakpoint against *Bacillus subtilis*, *Staphylococcus aureus and Pseudomonas aeruginosa* and 16, 16 and 15 mm standard standard (Gentamicin), respectively. Where as 7 and 8 mm were found to be zone of inhibition breakpoint for compound **31**and standard (Gentamicin) respectively against *E.coli* and *Pseudomonas aeruginosa*. Overall evaluation of compound **30**and crude extract showed no activity compared to the standard drug against *Escherichia coli*. Compound **31**showed low activities compared to the standard drug against all bacteria. Ethyl acetate crude extracts and compound **31**showed no response against *S. aureus*. Nevertheless, the crude EtOAc, compound **30** extract have promising activity against *S. aeruginosa* compared to standard drug.

5. CONCLUSSIONS AND RECOMMENDATIONS

5.1. Conclusions

The qualitative phytochemical screening of the *n*-hexane crude extract revealed the presence of tannins, glycosides and terpenoids, while EtOAc crude extract revealed the presence of tannins, alkaloids, flavonoids, anthraquinones glycosides, cardiac glycosides, coumarins, glycosides and terpenoids and methanol crude extract revealed the pressnce of phenols, tannins, alkaloids, flavonoids, saponnins and terpenoids of root of *Gladiolus candidus* extract. Silica gel column chromatography separation afforded compound **30** chemical skeletons derived from isoflavenes, 3-(3,4-dihydroxyphenyl)-2H-chromene-7,8-diol and compound **31** long chain hydrocarbon 3-(6-isopentylcyclohex-3-enyl)-2'-methylpropan-1'-ol. These compounds isolated and reported for the first time from root part of *Gladiolus candidus* Thathasisoflavenes and long chain hydrocarbon in carbon skeleton. The scientific findings of the present study proved that the compound **30** isoflavenes nature have promising activity against *pseudomonas aeruginosa* which give a clue about the traditional use of the plant against various infectious diseases (i.e. wound, edema).

5.2. Recommendations

Based on the results of the study, the following recommendations are forwarded:

-by considering the TLC profile of the crude extract, there are still many unisolated secondary metabolites present in the plant and may need to be screened which initiates further phytochemical analysis of the plant.

- the result show that, it is better to use solvent system (like *n*-hexane: EtOAc: $CHCl_3$: MeOH) for more separation.

-future phytochemical work is recommended with quantitative of the plant extraction for better extraction yield.

-more detailed testing needs to establish against various bacterial activities of this plant.

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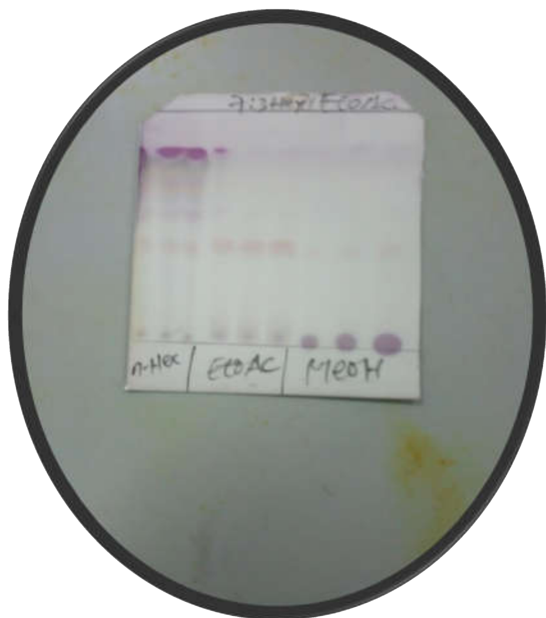
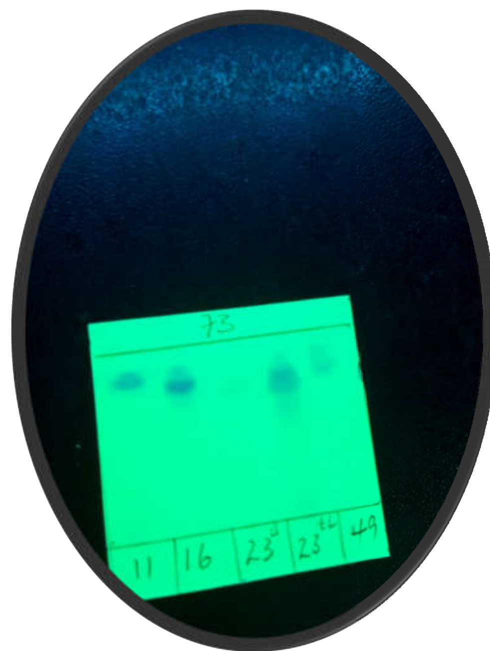
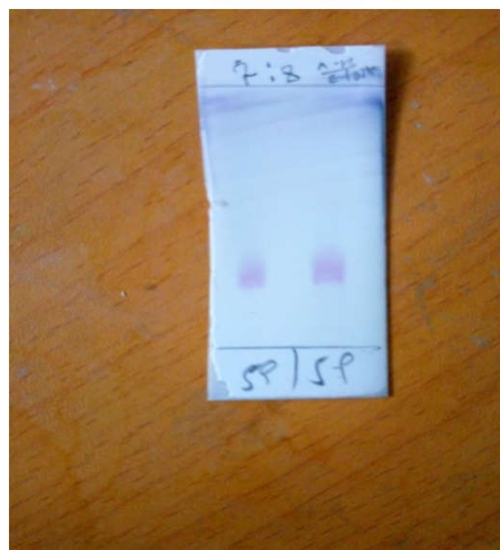
APPENDICES

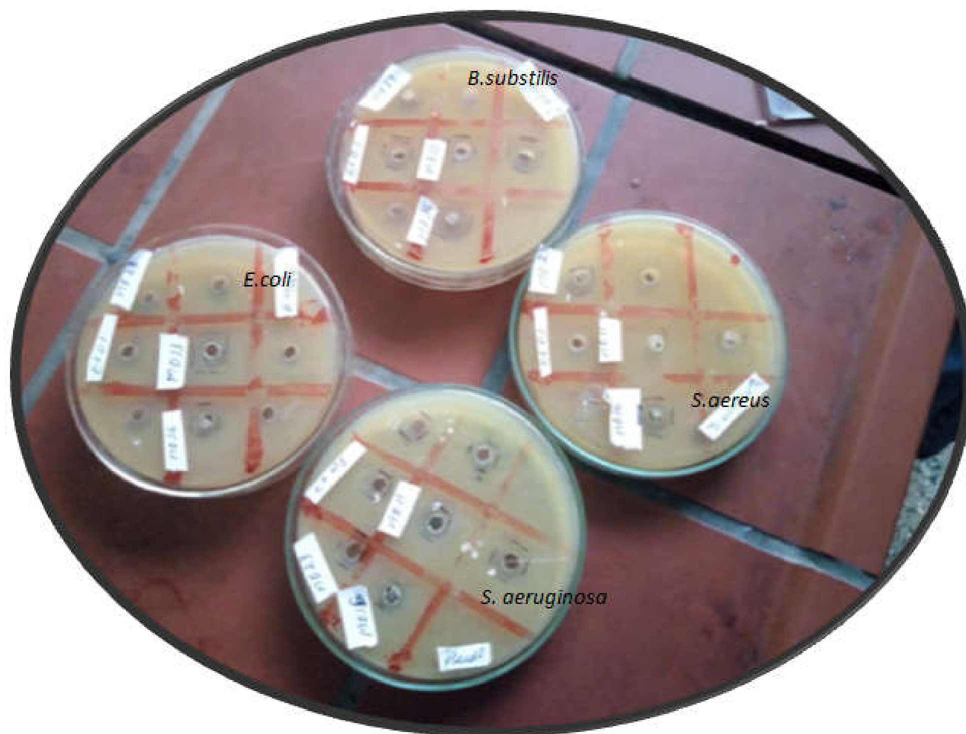
(1). A phtographs of TLC of preliminary test and isolated of fractions.







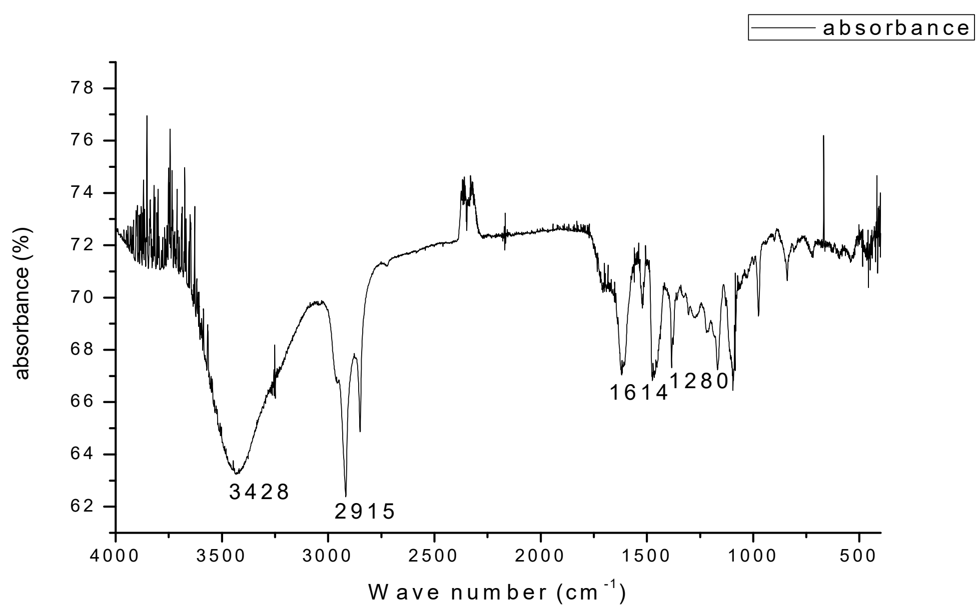
(2). Photgraphs of plates of antibacterial cultures in presence of various tested of EtOAc crude extract and purified fractions.



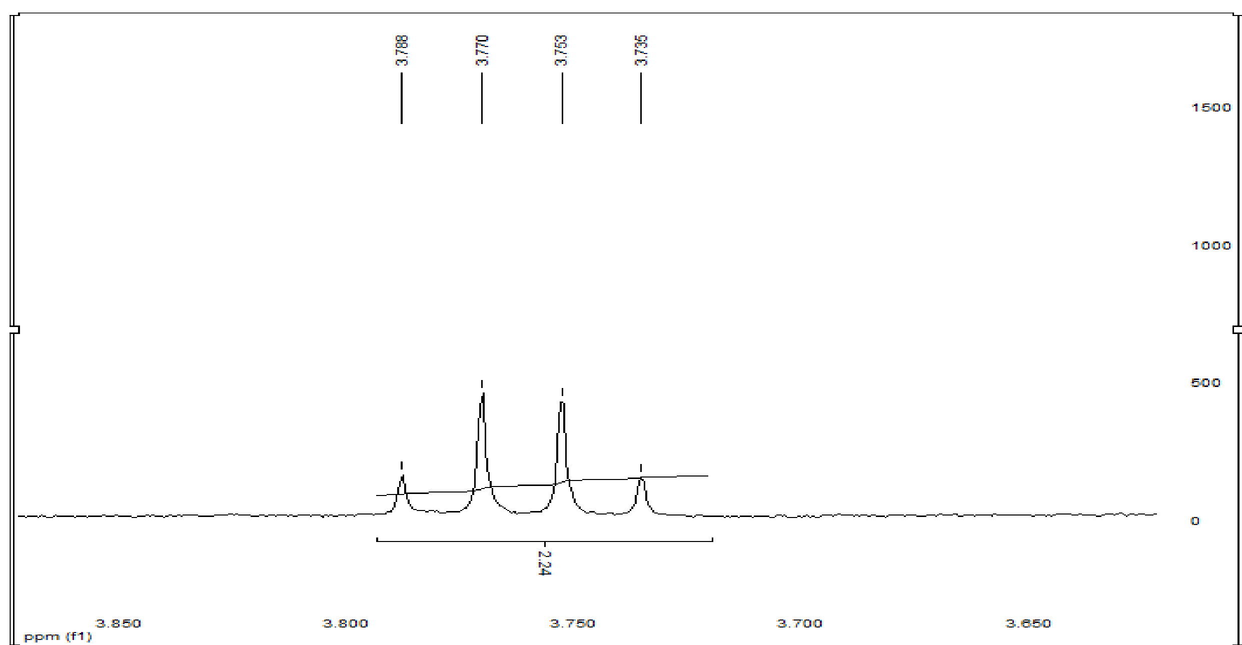
(3). Representative photographs of phytochemical in the active crude extracts of *n*-hexane, EtOAc and methanol successively.

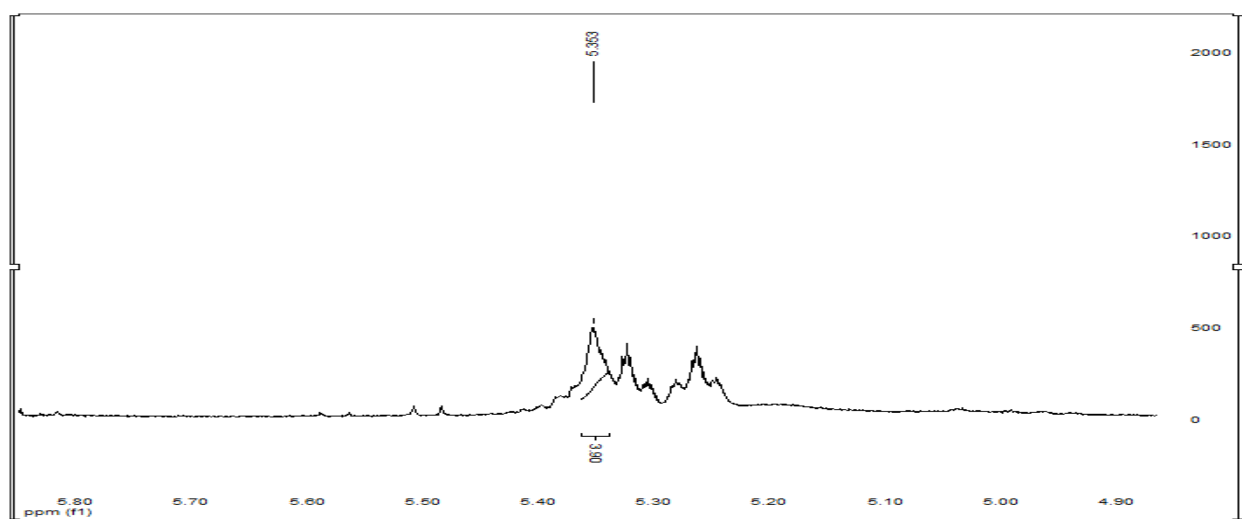
(4). Photographs of isolated fraction (compound).

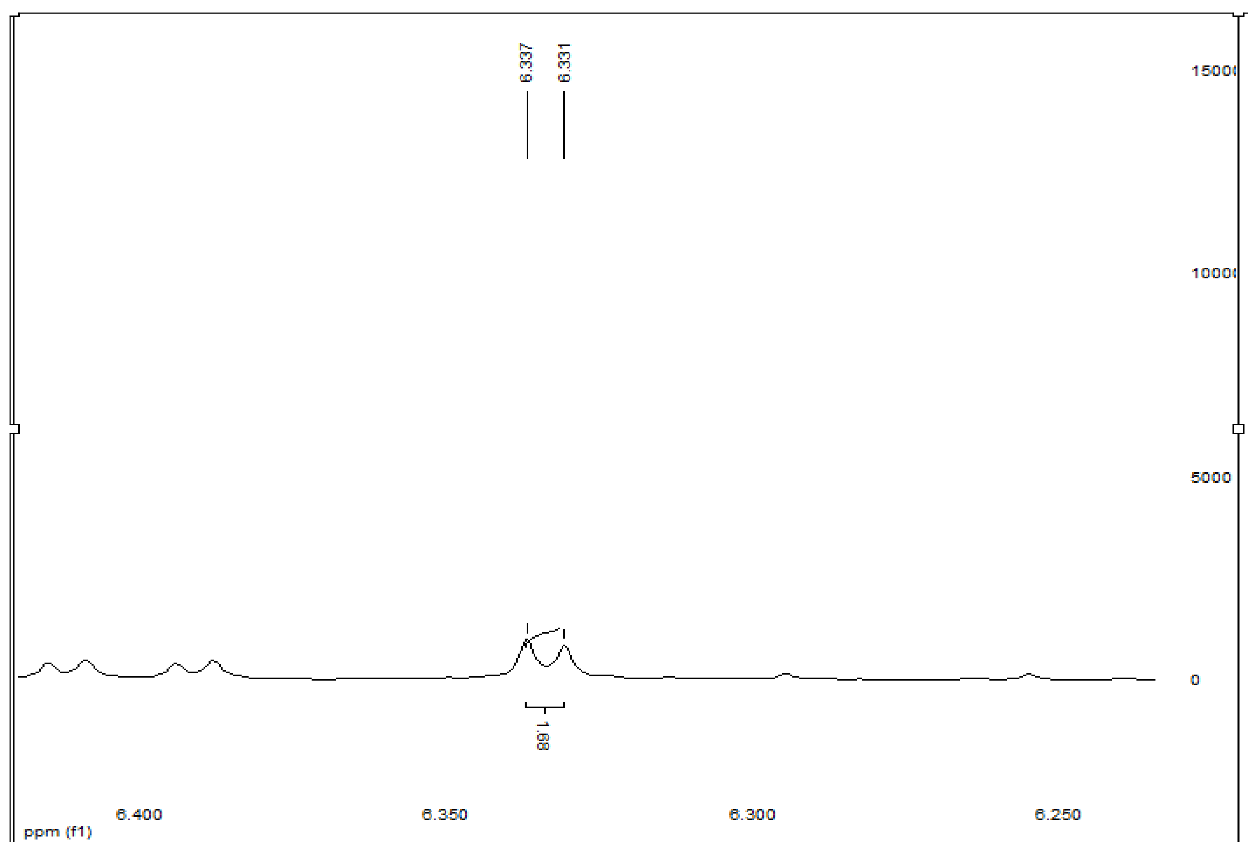
(5). FT-IR spectrum of compound 30.



(6). ^{1}H NMR spectrum of compound 30.



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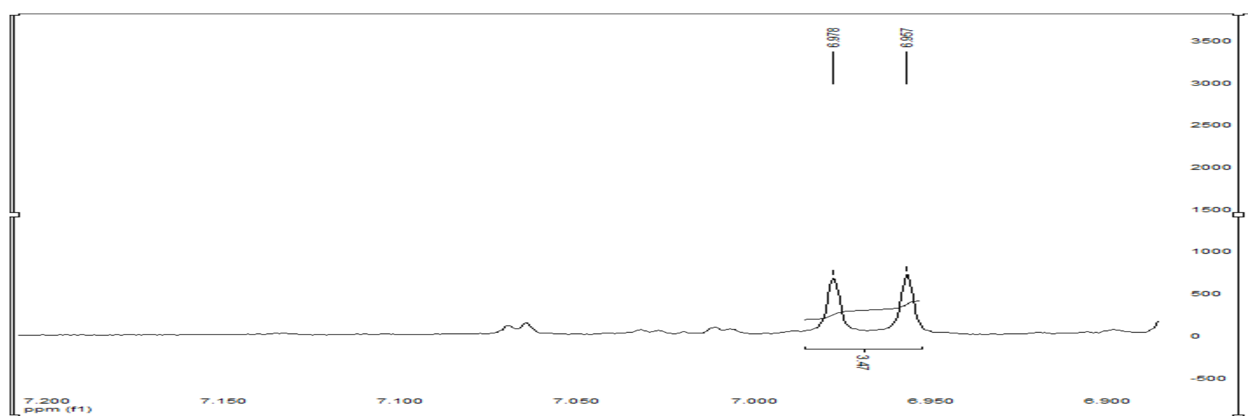
6.331
15000
10000
5000
0
1.68
6.400
6.350
6.300
6.250
ppm (f1)


6.978
6.967
3500
3000
2500
2000
1500
1000
500
0
-500
3.47
7.200
ppm (f1)
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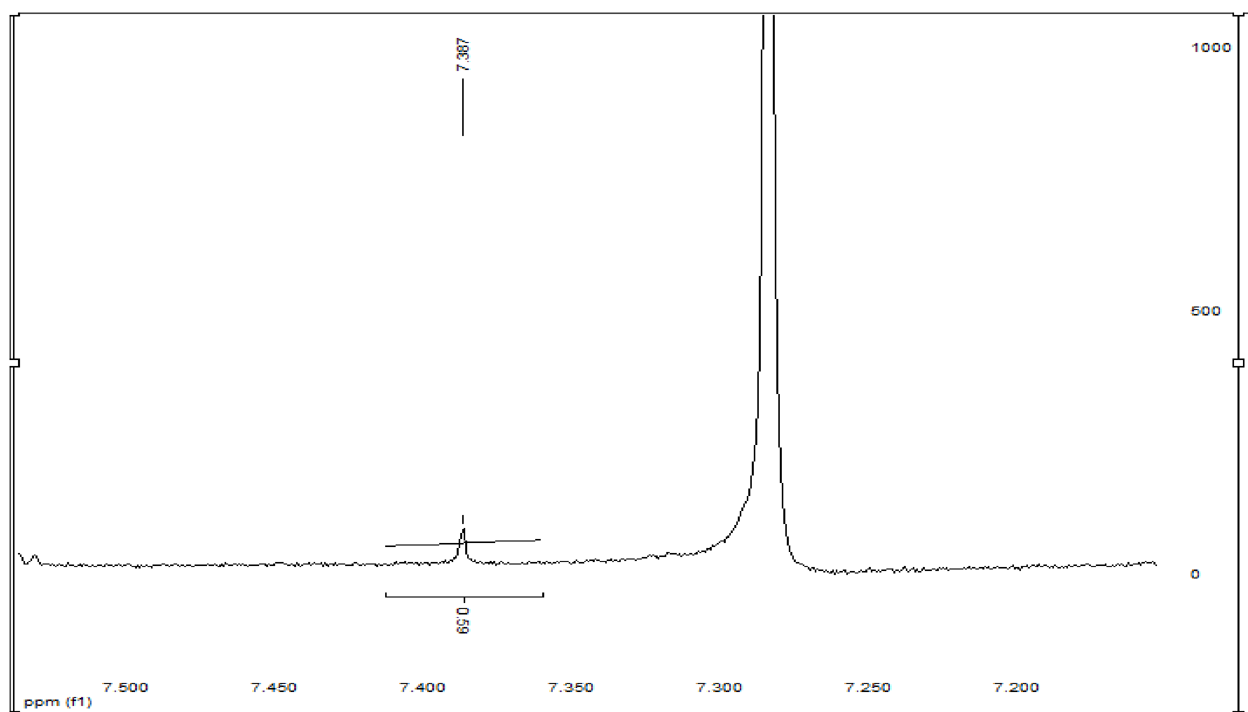
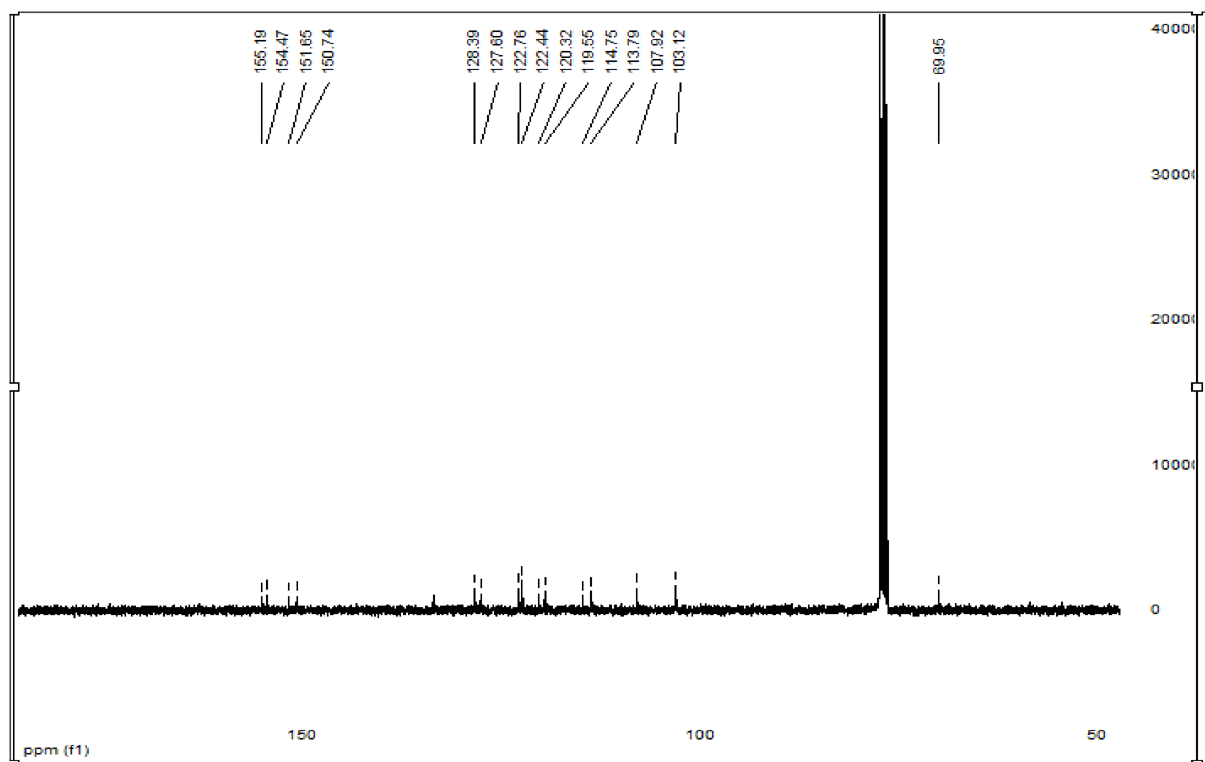
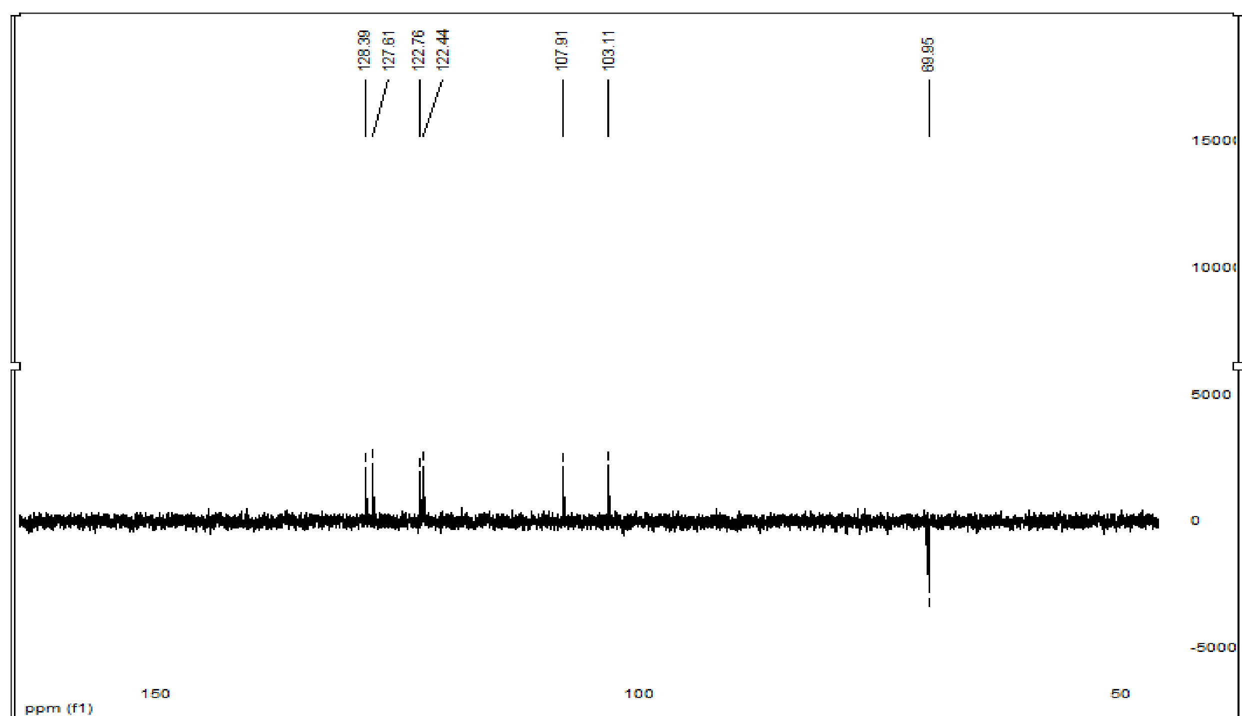
7.100
7.050
7.000
6.950
6.900



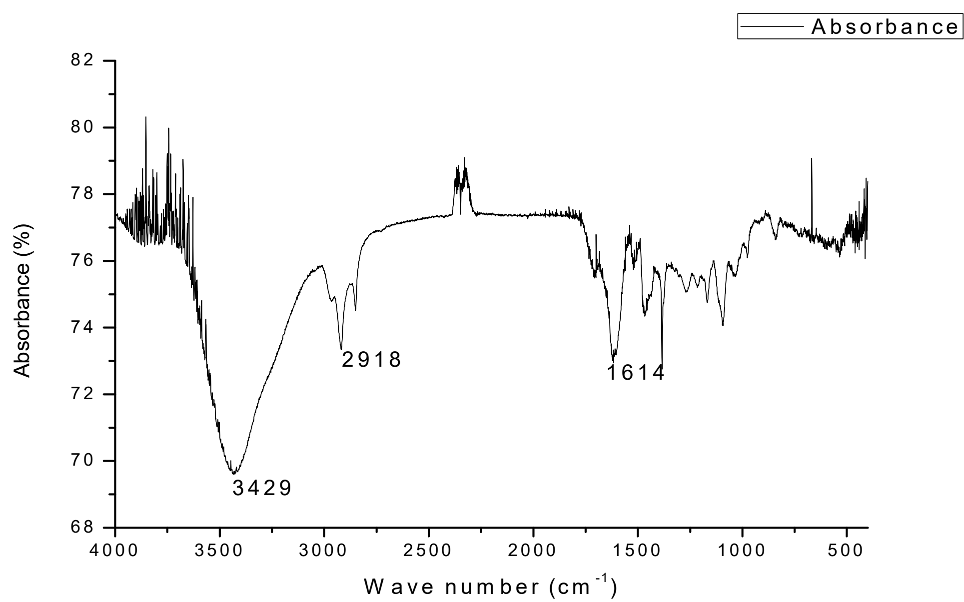
(7). ^{13}C NMR spectrum of compound 30.



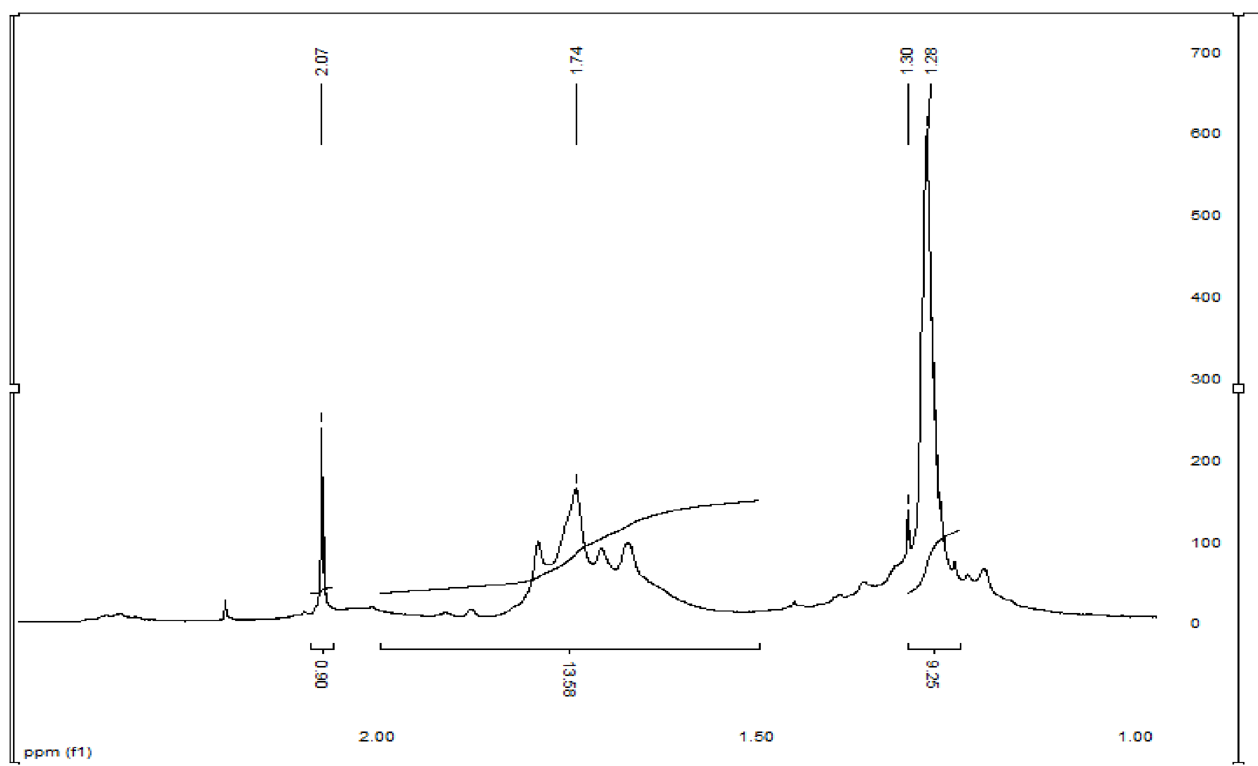
(8). DEPT-135 spectrum of compound 30.

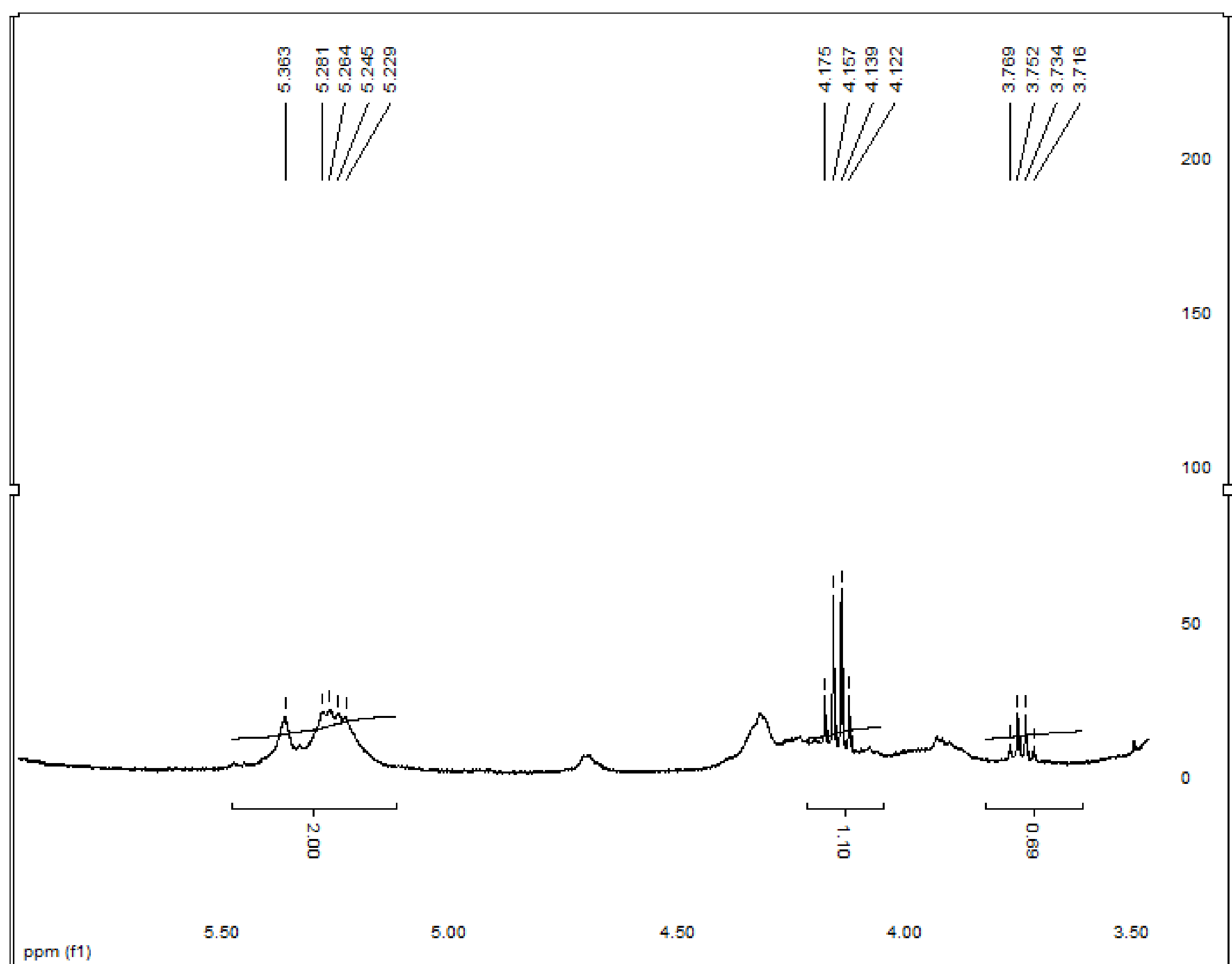


(9). FT-IR spectrum of compound 31.



(10). ^{1}H NMR spectrum of compound 31.


2.07
1.74
1.30
1.28
700
600
500
400
300
200
100
0
0.90
13.58
9.25
2.00
1.50
1.00
ppm (f1)



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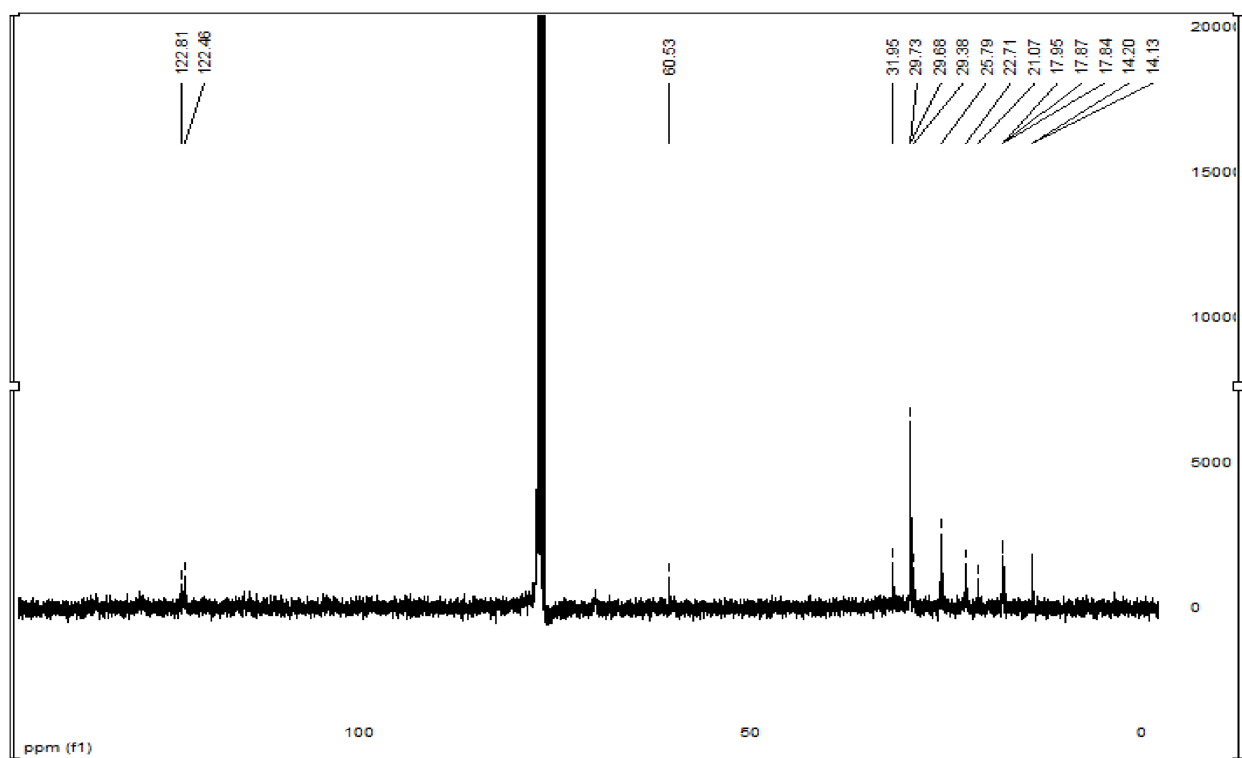
5.281
5.264
5.245
5.229
4.175
4.157
4.139
4.122
3.769
3.752
3.734
3.716
200
150
100
50
0
2.00
1.10
0.69
5.50
5.00
4.50
4.00
3.50
ppm (f1)

(11). ^{13}C NMR spectrum of compound 31.



(12). DEPT-135 spectrum of compound 31.