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M.Sc. THESIS

ON

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF THE ROOT BARK OF *Stereospermum kunthianum* Cham

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PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL EVALUATION OF THE ROOT BARK OF *Stereospermum kunthianum Cham*

A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES JIMMA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTEROF SCIENCE IN CHEMISTRY (ORGANIC).

BY

SISAY TESSEMA

ADVISORS

TSEGAYE GIRMA (PhD)

AND

MUDIN JEMAL (M.SC)

Declaration

I declare that Phytochemical investigation of roots bark of *Stereospermum kunthianum* and evaluation of its antimicrobial activities is my original work, except where reference is made, and has never been submitted anywhere for award of any degree or diploma in any university.

Sisay Tessema

This M.Sc. Thesis has been submitted with our approval as supervisors

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Tsegaye Girma (PhD)

Chemistry Department

Jimma University

Mudin Jemal (M.Sc.)

Chemistry Department

Jimma University

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CC	Column Chromatography
DEPT	Distortion less Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
d	doublet
dd	doublet of doublet
MHz	Mega Hertz
1DNMR	One Dimensional Nuclear Magnetic Resonance
J	coupling constant
^{1}H-NMR	Proton-Nuclear Magnetic Resonance Spectroscopy
^{13} C-NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
HPLC	High Performance Liquid Chromatography
s	singlet
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet /Visible
WHO	World Health Organization.

Abstract

The traditional medicinal system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care. The genus of *Stereospermum* is known to possess medicinal properties in every part of the plant. *S. kunthianum* is one of the medicinal plants that has been claimed to be used traditionally to treat several illnesses such stomachaches, toothache, snake bite, gonorrhea, evil eyes, diarrhea, skin diseases, and headaches. Therefore, this study was focused on the phytochemical isolation and antimicrobial activities of the root bark of *S. kunthianum* Cham. The dried and powdered root bark of the plant was exhaustively extracted with methanol/chloroform (1:1, v/v) by maceration at room temperature. The extract was then filtered and evaporated using a rotary evaporator. Phytochemical screening of the extract was carried out and lead to presence of some secondary metabolites, such as glycosides, alkaloids, tannins and anthraquinone. The isolation of pure compound was made by column chromatography controlled by TLC using petroleum ether in increasing amount of ethyl acetate for polarity. The chemical study of the root bark extract of *S. kunthianum* afforded one pure compound whose structure were established as 6-trans-p-methoxycinnamoyl catalpol using spectroscopic data (^{1}H NMR, ^{13}C NMR) and literature reports. The crude extracts and isolated compounds were subjected to biological evaluation against four bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli)* and one fungus (*Candida albicum).* The zone of growth inhibition of extract and isolated compounds were compared with standard drugs like gentamycin and clotrimazole. The result showed that the isolated compound more activity against all the tested strains, compared to the crude extract. Therefore, biological activities displayed by the root extract and constituents of the root of *S. kunthianum Cham* confirm the traditional uses of this plant against various bacterial ailments.

KEYWORDS: *Stereospermum kunthianum*, phytochemical, antimicrobial activity.

1. INTRODUCTION

1.1. Background of the study

Medicinal plants are very vital in their uses for medication, besides providing ecological, economic, and cultural services. The world primary means of treating diseases and fighting infections have been based on the use of medicinal plants. From ancient times, plants have been rich sources of effective and safe medicines [1]. The uses of medicinal plants for human and animal treatments are practiced from time of immemorial. The local practitioners provided various traditional medications to their patients' diseases such as stomachaches, asthma, dysentery, malaria, evil eyes, cancer, skin diseases, and headaches. Medicinal plants used for traditional medicine play a significant role in the healthcare of the majority of the people in Ethiopia [2]. Medicinal plants are highly demanded in Ethiopia due to the trust of communities on medicinal values of traditional medicines, culturally associated traditions, and their relatively low cost [3]. According to the World Health Organization (WHO), nearly 3.5 billion people in developing countries including Ethiopia believe in the efficiency of plant remedies and use them regularly [4].

The major sources of traditional medicine are medicinal plants. Based on the knowledge accumulated over centuries, plant extracts continue to be used for the treatment of various infectious and chronic diseases in many societies. Plant-based traditional medicine plays a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drug discovery [5]. Various modern drugs were extracted from traditional medicinal plants through the use of plant material following the ethno- botanical leads from indigenous cures used by traditional medical systems [6]. Microorganisms change their metabolism and genetic structures to acquire resistance against the drugs being used in the treatment of common infectious disease. The excessive use of antibiotic was contributed to the emergence and spread of antibiotic resistant bacteria in communities. Drug resistance of microorganisms leads to, high mortality rate, particularly in developing countries and become a great challenge in the pharmaceutical and health care industries. To overcome such challenges, scientists look forward for the development of alternative and novel drugs. Failures of chemotherapeutics and antibiotics of some drugs exhibited by pathogenic microbial agents has led to other alternatives of several medicinal plants for their potential antimicrobial activities [7].

A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds as an alternative that can potentially be effective in the treatment of these pathogenic bacterial infections [8].Plants contain wide range of secondary metabolites, which have been used as pharmaceuticals, agrochemicals, flavours, fragrances, colors, bio-pesticides and food additives [9]. Phytochemicals can be classified as primary and secondary metabolites, Chlorophyll, proteins and common sugars are categorized as primary metabolic while, secondary metabolites include terpenoid, alkaloids and phenolic compounds [10].

The use of crude plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant [11]. Bignoniaceae families are rich in active principles and have high economics and medicinal values. Its plants are particularly abundant in northern South America, central and East Africa [12]. The phytochemicals found in leaves, stem barks, and roots of *Stereospermum* genus such as alkaloids, tannins, saponin, flavonoids, coumarins, anthroquinones, phenols, terpenoids, terpenes, and sterols seemed to be the most active phytochemicals reported in the species. The species under *Stereospermum* genus are known for various bioactivities led by their traditional uses such as to cure toothache, bronchitis, ulcers, cough, gastritis, leprosy and diarrhea [13–15]. *S. kunthianum* Cham is one of medicinal plants practiced by societies. It is a shrub that belongs to family bignoniaceae. Itis a multipurpose plant of significant importance to local communities. It is small slender tree of Africa, different parts of *S. kunthianum* used as traditional medicine to treat human ailments[16]. The phytochemical constituent and antimicrobial activity of natural compounds could be influenced by number of factors including botanical source, time of harvesting, stage of development, and method of extraction in addition to the composition, structure, and functional groups of the natural compounds [17]. Therefore, the study was done to investigate phytochemical constituent and evaluation of antimicrobial activities of its root bark.

1.2. Statement of the problem

Plants and plant-based products have been consumed in medicine to treat infectious diseases and to improve human's health. Traditionally, many plants with medicinal features are used to treat pathogenic microorganisms[18]. A large number of the plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the people throughout the world. Traditionally, people use *S. kunthianum* for medicinal purposes such as antidiarrheal, anti-inflammatory, antibacterial and toothache treatment [19–21]. Phytochemicals are known to vary with variation in climate, weather, soil conditions as well as time of collection, methods of extraction, types of plant use and their parts. In the study area people are known to use *S. kunthianum* traditionally to relief patients' from diseases such as stomachaches, toothache, snake bite, gonorrhea, evil eyes, diarrhea, skin diseases, and headaches [22–24]. There are few documents concerning phytochemical analysis and antimicrobial activity of the leaves and stem bark part of the plant. And also, there are no studies concerning phytochemicals of root of the *S. kunthianum*. Based upon the above evidences, phytochemical pertaining this plant and its antibiotic activities have not been studied exhaustively. Therefore, the present study was focused on the isolation and identification of compounds from this plant and evaluation of its antimicrobial activities.

1.3. Objectives

1.3.1. General objective

- To isolated phytochemical constituents of *S. kunthianum* and evaluate their antimicrobial activities

1.3.2. Specific objectives

- To extract phytochemicals from the root bark of *S. kunthianum* using maceration techniques.
- To isolate secondary metabolites from the crude extract of *S. kunthianum* using chromatographic techniques.
- To evaluate *in vitro* antimicrobial activities of the crude extracts and isolated compounds on bacterial and fungal strains.
- To elucidate structure of isolated compounds using 1D (^{1}H-NMR, ^{13}C NMR and DEPT) spectroscopic techniques.

1.4. Significance of the study

The study has the following significance: -

- Give information about the presences of various phytochemicals in the species.
- Identify bioactive compounds that are used in drug development.
- Put base line information for other researchers who wish to carry out further study on similar plant

2. LITERATURE REVIEW

2.1. Botanical description of the plants

2.1.1. The family Bignoniaceae

The Bignoniaceae family comprising of about 100 genera and 800 species is a family of flowering plants, commonly known as the Trumpet Creeper family, Jacaranda family, Bignonia family, or the Catalpa family. Plant species belonging to this family are distributed worldwide, but most of them occur in the tropical and sub-tropical countries. However, a number of temperate species also grow in North America and East Asia. Although the family is small, the Bignoniaceae plants are important for their reported bio-active constituents and diverse pharmacological [19].

Bignoniaceae family plants are also widely used as traditional medicinal systems of a number of countries of the world including in Africa. Species belonging to this family have been extensively studied in regard to their pharmacological properties (as extracts and isolated compounds). Particular groups of natural products from bignoniaceae have been shown to have potential healing uses as antimicrobial activity isolated from *S. kunthianum* species [25]. It was observed that the traditional medicinal practitioners use a total of seven Bignoniaceae family species for treatment of ailments like cancer, snake bite, skin disorders, gastrointestinal disorders, respiratory tract disorders, gynecological disorders, hepatic disorders, epilepsy, cholera, pain, urinary problems, malaria, heart problems, and sexually transmitted diseases. The seven species of bignoniaceae family plants in use were *Crescentia cujete, Heterophragma adenophyllum, Oroxylumindicum, Stereospermum kunthianum, Tabebuia argentea, Tecomaga udichaudi and Tecoma*[19, 26].

2.1.2. The genus of *stereospermum*

Stereospermum belongs to the family Bignoniaceae is a genus of trees and many plants have been studied so far for various biological potentials. Twenty four known species *stereospermum* are widely distributed in tropical and sub-tropical Asia and Africa[27]. The most studied species included *S. kunthianum, S. colais, S. suaveolens, S. chelonoides, S. personatum, S. tetragonum* and *S. acuminatissimum*[14]. The genus of *stereospermum* are contain phytochemical in different parts of plants. However, flavonoids, saponin, glycosides, quinones and tannin seemed to be the most abundant phytochemical constituents reported in all species of *stereospermum*. The species

under *Stereospermum* genus are known for various bioactivities lead by their traditional uses such as cure toothache, bronchitis, cough, gastric and diarrhea [14, 25].

2.1.3. *Stereospermum kunthianum* Cham species

S. kunthianum is a deciduous shrub or tree found in the dry areas of deciduous forest, woodland, bush, rocky outcrops, termite mound and margins of evergreen forests. Geographic distributions include Nigeria, Democratic Republic of Congo, Djibouti, Eritrea, Ethiopia, Kenya and Mozambique. There are some 30 species with a Central African and Asian distribution [28].

It is a small woody tree of about 5 or 15 m high. It has thin, grey-black bark, smooth or flaking in patches, the trunk is rarely straight, with twisted branches with abundant, fragrant, precocious, pink or purplish flowers, making the tree a spectacular sight. The tree in flower is very showy and well worth cultivation as an ornamental. The seeds however have very poor germination and propagation by suckers is recommended. The scaly bark confers some degree of resistance to fire damage. The wood is whitish tinged with yellow or pink and is fairly hard. Grows well on light silty and sandy soils. Pods are chewed with salt for coughs and are used in treatment of ulcers, leprosy, skin eruptions and venereal diseases; also used to cure flatulence in horses. Leaf is used for washing wounds infusion; macerated leaves are used to treat asthenia and exhaustion. Bark is used as a hemostatic and for treating wounds, and a stem-bark decoction is used to cure bronchitis, pneumonia and coughs. Venereal diseases, respiratory ailments and gastritis are treated using roots and leaves [28, 29]. The plant is known locally as ‘Botoroo’ and is widely used by traditional medical practitioners in Southwestern Ethiopia for the treatment of various human diseases[22, 24].

Figure 1. Picture of *S. kunthianum* plant taken from habitat photography taken from study area Atnago. (August 15/2022)

2.2. Ethno-medicinal uses of *S. kunthianum Cham*

Medicinal plants are the basis for treatment of various diseases in African traditional medicine as well as other forms of treatment from diverse cultures of the world. About 80% of the world's population still depends solely on traditional or herbal medicine for treatment of diseases, mostly in Africa and other developing nations [30]. Traditional medicines remained as the most affordable and easily accessible sources of treatment in the primary healthcare system among diverse communities in Ethiopia [31]. Traditional medicine plays an important role in the daily lives of people living in rural parts of the country. Even though the detail that Ethiopia has an elongate times past by means of long established medicinal plants as a substitute medicine source [2]. The local practitioners provided various traditional medications to their patients' diseases such as stomachaches, toothache, snake bite, gonorrhea, evil eyes, diarrhea, skin diseases and sexually transmitted diseases [19, 22, 24]. The twigs are chewed to clean teeth and to treat toothache. Stem bark powder mixed with water and one cup of tea taken for three days treating stomachache. Dried bark put on fire and the smoke inhaled evil eye [22]. Dried leaf powder mixed with butter is applied to treat gonorrhea [23].

Scientific investigations of ethno-medicinal claims of *S. kunthianum* have been carried out pharmacologically. Some of the pharmacologic parameters investigated using crude extracts or isolated compounds were antibacterial, anti-inflammatory and analgesic, ant-plasmodia, antidiarrheal and antioxidant activities [20, 32–34].

2.3. Phytochemical constituents of *S. kunthianum*

Phytochemicals are various biologically active compounds that occur naturally in plants, which provide potential medicinal benefits for humans. These chemicals accumulate in several parts of the plant including the stems bark, roots and leaves. This genus is also well known for producing a wide variety of metabolites including flavonoids[35], anthraquinones[36], Glycosides[37], phenylpropanoids[38] and steroids[15].Three phytochemicals of utmost interest in this species are glycosides, flavonoids and Quinones. Glycosides like iridoid glycosides (**1)** and phenyl propanoid glycoside (**2**and **3**). Flavonoids like 5, 7, 3', 4'-tetra hydroxyl-3-O-α-rhamnophyransyl-(1– 6) –β-D-glucopyranosyl flavones (**4**) and quercetin (**5**) Steroid likeβ-sitosterol (**6**), Friedelan **(7)** and Quinone's like naphthoquinones; pinnatal, (**8**) sterekunthals A, (**9**) sterekunthal B, (**10**) pyrankunthone A, (**11**) pyrankunthone B, (**12**) and one anthraquinone;

anthrakunthone (**13**) were isolated. The structures of isolated constituents of various genus *Stereospermum* are given as below.

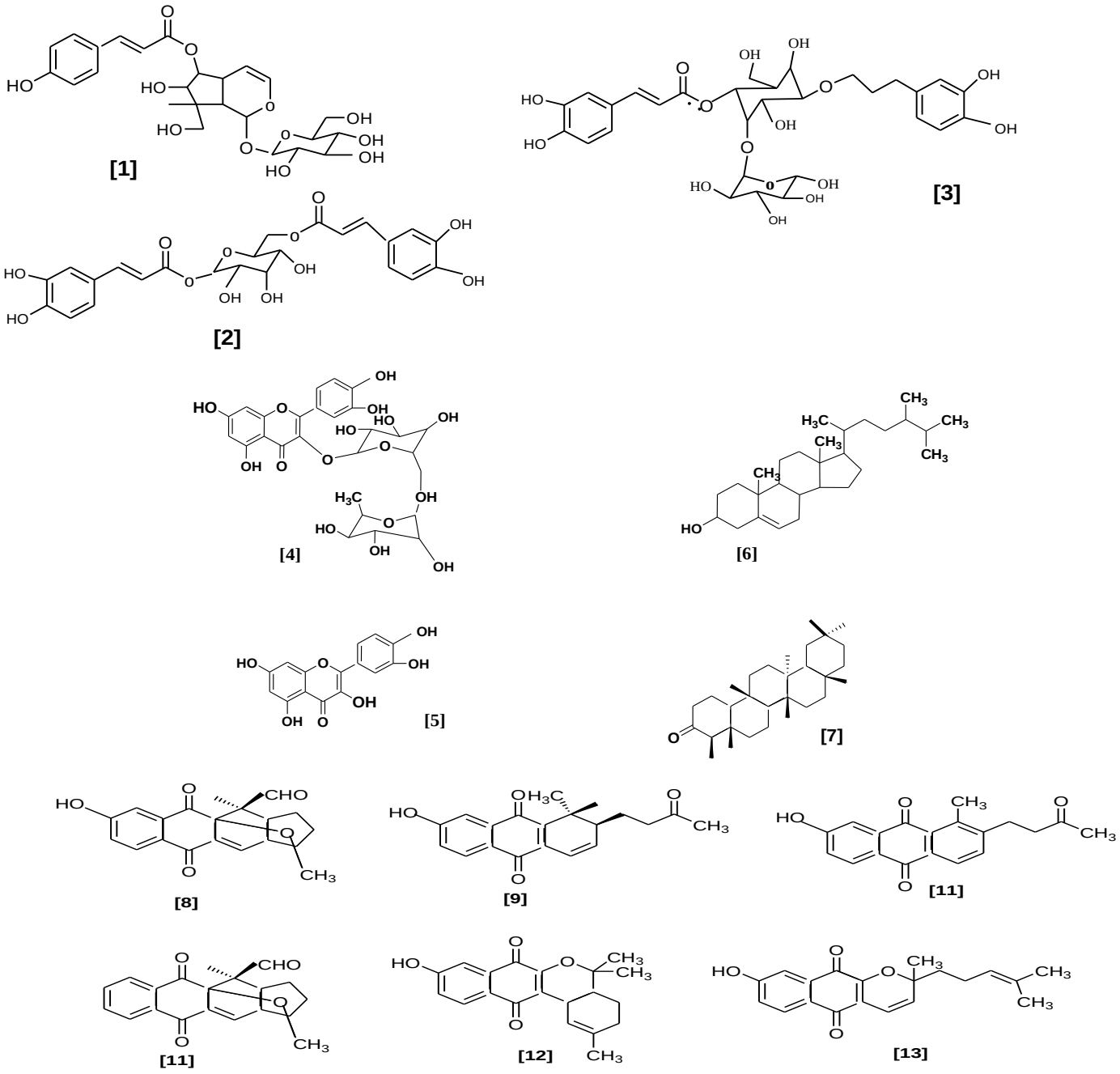

Figure 2. Chemical structures of selected secondary metabolites isolated from *Stereospermum genus*.

2.4. Biological activity of *S. kunthianum Cham*

The biological activity of the plant is attributed to the presence of several secondary metabolite compounds. Some of these compounds have been reported to be present in *S. kunthianum* species possesses antimicrobials activities. Secondary metabolites such as terpenoids, flavonoids, coumarins, glycosides and steroids have been shown *in vitro* antimicrobial activities [40, 41].The pharmacological shreds of evidence reported in the literature have proven that flavonoids have shown anti-cancer, anti-microbial, anti-oxidant, anti-inflammatory, anti-fungal, and anti-ulcer [42]. The presence of tannins in the plant aqueous extract assumed to be responsible for the antidiarrheal activity[33]. A lipophilic extract of the root bark of *S. kunthianum* also revealed in *vitro* antiplasmodial activity. Bioassay-guided fractionation the extract led to the isolation of four novel naphthoquinones (sterekunthals A and B and pyranokunthones A and B) and one novel anthraquinone (anthrakunthone) together with the known naphthoquinone pinnatal[34]. The aqueous extract of the steam bark of *S. kunthianum* possesses antidiarrheal activity. This is a possible reason for its antidiarrheal use in traditional medicine [43]. The stem bark of *S. kunthianum* report the analgesic and anti-inflammatory activities of the previously isolated and characterized iridoid and phenylpropanoid glycosides [32]. Antibacterial activity of leaf concentrate of *S. kunthianum* was performed on *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus Klebsiella spp, Aeromonas hydrophila Salmonella spp* utilizing agar well dispersal system[20].

3. MATERIALS AND METHODS

3.1. Chemicals

Solvents such as, n-hexane, petroleum ether, chloroform, ethyl acetate and methanol are all from (LOBA CHEMIE PVT, LTD, Mumbai, India) were used for extraction and column elution, Silica gel 60-120 mm size, from (LOBA CHEMIE PVT, LTD Mumbai, India) and silica gel coated TLC, iodine for visualization of spots on TLC. DMSO, gentamicin, Mueller Hinton and nutrient agar were used during antimicrobial test. All the chemicals used were analytical grade reagent.

3.2. Apparatus and instruments

Rotary evaporator (HEIDOLPH LABORATA 4000) to concentrate the crude, TLC papers for detection of spots under UV-TECHC(JENWAY 6705, UK), round bottom flask (100 , 250 and 500) mL for extraction and filtration purpose , measuring cylinder for measuring of solvent, grinding machine for grinding of the sample, Whatman no 1 filter paper(diameter 110mm) for filtration of extract, weighing balances for weighing mass of sample, crude extract and isolated compounds, glass columns for column chromatography (500 mL), capillary tube, chamber (UV-TECHC) for detection of spots on TLC, petri dishes were used for anti-microbial tests. Bruker 400 MHz advance NMR spectrometer.

3.3. Collection and preparation of plant materials

Root bark *S. kunthianum Cham* was collected from home garden at Atnago town, Limmu Seka woreda Jimma zone, Oromia Regional State, Ethiopia which was about 110 Km from Jimma town on March, 2021. The plant was authenticated by botanist Melaku Wondafarash, Addis Ababa University (voucher number ST001, (ETH)). The collected plant material was washed rinsed with distilled water, shade dried at room temperature and ground into smaller pieces.

3.4. Extraction and isolation of compounds

3.4.1. Extraction

500g of the powdered sample was soaked into 3 L equal volume of $CHCl_3/CH_3OH$ (1:1, v/v), using cold maceration technique three times for 24h at room temperatures. The extract was filtered first through cotton plug and followed by Whatman filter paper. The filtrate was concentrated using rotary evaporator (HEIDOLPHLABORATA - 4000) at 40 °C under reduced pressure. The resulting brown-jelly extract was stored in desiccators until dry. The dried crude extract was weighed 44 gm, which was of red brown. It was stored in suitable container until further purified and evaluate its antibacterial activity tests (Bioassay).

3.4.2. Phytochemical screening Test

The preliminary qualitative phytochemical screening tests of the $CHCl_3/CH_3OH$ (1:1) root bark extract of *S. kunthianum* were performed for the presence of different chemical groups such as alkaloids, flavonoids, phenols and tannins, saponins, terpenoids, steroid, glycoside's and anthraquinones by using following standard procedures[44, 45].

I. Test for alkaloids: Wagner's Test**:** A fraction of 0.2 g extract was diluted with 5 mL of distilled water and treated with 3-4 drops of Wagner's test reagent [1.27 g of iodine and 2 g of potassium iodide in 100 mL of water] and the formation of a reddish-brown color indicated the presence of the alkaloids.

II. Test for flavonoids: Sodium Hydroxide Test**:** Plant 0.2 g extract is treated with 5 mL dilute NaOH, followed by addition of 3 mL dilute HCl. A yellow solution with NaOH turns colorless with dilute HCl, which shows the presence of flavonoids.

III. Test for saponin: About 0.2 g of extracts was shaken in test tubes with 5mL of distilled and heated on water bath to boiled. Formation of strong and stable foam (1.7cm height) was taken as indication for the presence of saponins.

IV. Test for tannins: The plant 0.5 g extract was diluted with 5mLdistilledwater and 3-4 drop of 10% ferric chloride solution was added. Appearance of the blue-green or black color indicated the presence of phenol and tannins.

V. Test for glycosides: A total mixtures of 0.2 g crude extract and 3 mL glacial acetic acid containing 3-4drops of 2% solution of ferric chloride was poured into another test tube

containing 2 mL of concentrated sulfuric acids side-wise. Appearance of brown ring at the junction of two layers indicates the presence of glycosides.

VI. Anthraquinone: Borntrager's test; 2 drops of dilute $H_2SO_4$2 mL of each extract, boiled and filtered. Filtrate added 3 mL chloroform. Chloroform extractsadded1 mL of ammonia; red color on the ammoniacal layer was observed indicating the presence of anthraquinone glycosides.

VII. Test for terpenoids: 5 mL of extract was mixed with 2 mL of $CHCl_3$ in a test tube. 3 mL of concentrated H_2SO_4 was carefully added along the wall of the test tube to form a layer. An interface with a reddish-brown coloration was confirmed the presence of terpenoids.

3.4.3. Isolation of compound

The crude extract was employed to TLC analysis in various combinations of solvents increasing polarity to identify appropriate solvent system for column elation. Petroleum ether: ethyl acetate combination of different polarity was found to be suitable for elution of column. The crude extract 20 g was adsorbed on 16 g of silica gel (60- 120 mm mesh) and then loaded on to previously packed column with 340 g activated silica gel in petroleum ether (100%). The column was eluted with petroleum ether and ethyl acetate with increasing polarity until, ethyl acetate (100%). Finally, the adsorbed sample was elated using ethyl acetate and methanol up to 10% with increasing polarity. (Table **1)** showed the solvent system used in the column chromatography of crude *S. kunthianum* extract and fractions collected. At the beginning 100 mL of fractions were collected until colored fractions were begins to elute, where 50 mL of fractions were collected. A total of 59 fractions were collected, 40 fractions each with 100 mL and 19 fractions with 50 mL were collected and the solvent was removed using rotary vapor at 40 °C. Then, each fraction was applied on TLC in appropriate solvent system and the various spots were visualized under UV light at 254 and 365 nm and iodine chamber. Fractions with similar TLC profiles were combined together. The fraction eluted up to 10% methanol in ethyl acetate was collected, kept aside to allow the precipitate settled at the bottom and precipitate was washed with n-hexane repeatedly to obtain compound. Based on TLC profile, which show single spot similar TLC are merged into 5 fraction and two pure fractions obtained. Based on TLC profile, fraction 44-45(2%methanol in ethyl acetate with a R_f. Value 0.62), were combined and further purified with ethyl acetate and n-hexane respectively, to give 28 mg of yellow amorphous solid which is labeled as compound **2**. Among the fractions, 46-47 (2.5% methanol in ethyl acetate

with R_f. Value 0.61) were combined and further purified with ethyl acetate and n-hexane respectively, to give 24 mg of yellow amorphous solid which is labelled as compound (**1**) and F34-40(up to 20% petroleum ether in ethyl acetate with $R_{f.}$ Value 0.64) were combined and further purified with ethyl acetate and n-hexane respectively, to give 31 mg of yellow amorphous solid which is labeled as compound **3**. The isolated pure compounds obtained were identified using spectral (^{1}H-NMR, ^{13}C-NMR and DEPT-135) analyses.

Table **1.** Solvent system used in the Column chromatography of crude *S. kunthianum* extract and collected fractions.

No	Solvent system	Ratio in%	Fraction (100mL)	No	Solvent system	Ratio in%	Fraction (50mL)	Compound
1	PE	100	1	25	PE/EA	45:55	28	
2	PE/EA	99:1	2	26	PE/EA	40:60	29	
3	PE/EA	98:2	3	27	PE/EA	35:65	30	
4	PE/EA	96:4	4	28	PE/EA	30:70	31-32	
5	PE/EA	94:6	5	29	PE/EA	25:75	33	
6	PE/EA	92:8	6	30	PE/EA	20:80	34-35	
7	PE/EA	90:10	7	31	PE/EA	15:85	36	
8	PE/EA	88:12	8	32	PE/EA	10:90	37-38	F34-40 Compound **3**
9	PE/EA	86:14	9	33	PE/EA	5:95	39	
10	PE/EA	84:16	10	34	EA	100	40	
11	PE/EA	82:18	11	35	EA/ ME	99.5:0.5	41	
12	PE/EA	80:20	12	36	EA/ME	99:1	42	41-43
13	PE/EA	78:22	13	37	EA/ME	98.5:1.5	43	
14	PE/EA	76:24	14-15	38	EA/ME	98:2	44-45	Compound **2**
15	PE/EA	74:26	16	39	EA/ME	97.5:2.5	46-47	Compound **1**
16	PE/EA	72:28	17	40	EA/ME	97:3	48-49	
17	PE/EA	70:30	18-19	41	EA/ME	96.5:3.3	50-51	
18	PE/EA	68:32	20-21	42	EA/ME	96:4	52-53	48-53
19	PE/EA	66:34	22	43	EA/ME	95:5	54	

20	PE/EA	64:36	23	44	EA/ME	94:6	55	
21	PE/EA	62:38	24	45	EA/ME	93:7	56	
22	PE/EA	60:40	25	46	EA/ME	92:8	57	
23	PE/EA	55:45	26	47	EA/ME	91:9	58	
24	PE/EA	50:50	27	48	EA/ME	90:10	59	

3.5. Structural elucidation

Structural elucidation of the compounds was done based on the data which is obtained from ^{1}H-NMR and ^{13}C-NMR spectra.

3.6. Bioassay Activity

3.6.1. Preparation of test organism and bioassay test

Standard bacterial strains and fungal strains for the test were obtained from the Microbiology Research Lab., Department of Biology Jimma University. The antibacterial activity of crude extract and isolated compound of root bark of *S. Kunthianum* was tested against four bacteria strains (*Staphylococcus aureus* (ATCC-25923), *Bacillus subtilis* (ATCC-6633), *Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (A TCC 27853) and one fungal strain *Candida albicans* (ATCC-14053) by using disc diffusion method. The disc measuring 6mm diameter was prepared from Whatman No.1 filter paper sterilized by dry heat at 180 °C for 1h. The stock solution was prepared by dissolving the crude extract and isolated compounds in1mL DMSO. The antimicrobial activity test was done using disc diffusion method standard procedures [46, 47]. Stocked bacterial and fungal strain were cultured on Muller Hinton agar. Bacterial and fungal strains were grown and maintained on an agar slant at controlled temperature (4 °C). Then strains activation was done for experiment at 37 °C for 24 h for bacterial strains and 48 h for fungal strain on nutrient agar. The culture media was boiled in distilled water to dissolve the media and autoclaved at 121°C for 2 h. and poured into sterile petri dishes refrigerator until use. After the culture media had solidified, organisms were uniformly seeded with it. Four well-isolated colonies of bacterial strains and one fungal strain were used for an agar plate culture and the top of each colony was touched with a loop, and the growth was transferred into a tube containing 4.5 mL of a suitable nutrient broth medium. Then, bacterial and fungal strains were spread on the solid plates with a sterile swab moistened with the bacterial suspension.

250 mg/mL and 20 mg/mL concentration of the sample plant extracts, isolated compound and 1 mL DMSO for negative control was impregnated using Whatman No.1 filter 5paper disc (diameter 6 mm) with the help of micropipette, respectively. Positive control using gentamicin for bacteria strains and clotrimazole for fungal strains was applied simultaneously. Plates were left for 10 minutes till the extract diffuse in the medium and incubated at 37°C for 24 h for bacteria and 48 h for fungal strains. After incubation, the plates were observed for the zone of inhibition and the diameter of the inhibition zone was measured using transparent ruler[46, 47].

4. RESULTS AND DISCUSSION

4.1. Extraction Yield

The air dried and grounded powdered of 500 g the root bark sample was soaked using chloroform/methanol (1:1, v/v), the obtained result of the crude extract weight 44g and the percentage yields of these crude extracts are given below. It was calculated by the formula;

$$\% \text{ yield} = \frac{\text{Weight of the crude extract (g)}}{\text{weight of the dried sample used(g)}} \times 100$$

Yield = 8.8 %

4.2. The preliminary phytochemical screening of the extract of *S. kunthianum*

The preliminary phytochemical screening of plants showed the presence of secondary metabolites and the result of phytochemical test has been summarized in (**Table 2**). These tests were based on visual observation of color formation after addition of specific regents.

Table 2. Phytochemical screening of crude root bark extract of *S. kunthianum*

Phytochemical classes	**Results**
Alkaloids	+
Flavonoids	_
Anthraquinone	+
Iridoid Glycosides	+
Tannins	+
Saponins	_
Terpenoids	_

NB: + sign indicate the presence, whereas - sign indicate the absence

4.3. Characterization of the isolated compound

4.3.1. Characterization of compound 1

Compound **1**(24 mg) was obtained as a yellow amorphous solid with R_f. value of 0.61(2.5% methanol in ethyl acetate). The ^{1}H and ^{13}C-NMR spectroscopic (400 MHz, MeOD) (Appendix 1A-C) data showed the presence of one β-glucopyranosyl unit from the anomeric proton signal at $\delta_{H\ 4.83}$ ppm (1H, d, J=8.0Hz, H-1') and from the carbon signals at δ_C 98.3 ppm, 73.4 ppm, 77.2 ppm, 70.3 ppm, 76.2 ppm and 61.5 ppm, in addition to the signals of the aglycon moiety. The vicinal coupled olefin proton at δ_H 6.39ppm (dd, J=8.0 Hz, 4.0 Hz, H-3) and δ_H 5.02 ppm (dd, J=8.0 Hz, 4 Hz H-4), amethine proton assigned to H-1(δ_H 5.18 ppm); H-5 (δ_H 2.63 ppm); H-6 (δ_H 4.84 ppm); H-7 (δ_H 3.37 ppm) and H-9 (δ_H 2.65 ppm,) and methylene proton assigned to H-10(δ_H 4.20 ppm and δ_H 5.02 ppm) for the aglycone moiety. The downfield signal (δ_H 4.84 ppm) of C-6 indicating that acetyl attachment through an ether linkage[48]. DEPT experiments indicated that compound **1** contained one quaternary carbon (δ_C 65.5 ppm), seven methines (δ_C 141.0 ppm, 101.6 ppm, 93.7 ppm, 79.9 ppm, 58.9 ppm, 41.7 ppm and 35.3 ppm) and one methylene ($\delta_{C\ 61.5}$ ppm) for the aglycone moiety. Further signals in the ^{1}H and ^{13}C-NMR spectra confirmed the presence of an iridoid skeleton (**Table 3**).

In additional, the ^{1}H- NMR spectrum was assigned to a p-methoxy cinnamoyl group by NMR: AA'BB' system of four aromatic protons at δ_H 7.50 ppm (2H) and δ_H 6.39 ppm (2H), one trans double bond at δ_H 7.68 and 6.83 ppm (each 1H, J=16.0 Hz H-7''and H-8''), respectively, and one methoxy group at δ_H 3.90 ppm. The ester carbonyl group at δ_C 167.6 ppm (C-9'') involved in linkage formation with aglycone unit. The ^{13}C NMR spectroscopic data showed the presence of one β- glcopyranosyl and one cinnamoyl moiety, addition to the nine carbon signals for aglycone moiety. The ^{1}H and ^{13}C NMR spectroscopic data were closely related to those of specioside 6'-O-α-D-galactopyranoside[48], except for signals up field for β- glcopyranosyl unit difference due to the additional signals of α -L-rhamnopyranosyl in the literature. In general, the ^{13}C NMR (DEPT) spectrum of appendix (**1** B&C) displayed signals for two methylenes (δ_C 61.5 ppm, C-10, δ_C 59.9 ppm, C-6') and eighteen methines, three quaternary carbons and one methoxy at (δ_C 55.3 ppm and one carbonyl carbon (Table 3). Therefore based on the above spectroscopic evidence and comparison with literature, compound **1** was possibly identified as 6-trans-p-methoxy cinnamoyl catalpol (Figure **3**) has been previously reported from *Heterophragma Sulfureum* and *Stereospermum Cylindricum* species (Bignonianceae family)[38, 48].

Table 3. ^{1}H-NMR (400 MHz, MeOD) and ^{13}C-NMR DEPT-135 (100MHz, MeOD) data of compound **1** with reported data of ^{1}H- NMR) 500 MHz and ^{13}C -NMR 300 MHZ (2.5% methanol in ethyl acetate).

Positions	^{13}C NMR of compound **1**	Observed data ^{1}H NMR compound **1**	^{13}C NMR of compound [48]	Reported data ^{1}H NMR of compound [48]	DEPT
Alcyone	93.7	5.18 (d, J=8.0Hz)	94.8	5.53(1H, d, J= 9.5Hz)	CH
3	141.0	6.39 (dd, J=8,4Hz H-3)	141.5	6.45(1H, d, J=5.9 Hz)	CH
4	101.6	5.02(dd, J=8.0,4Hz H-4)	102.5	5.09(1H,dd,J=5.9,4.2Hz)	CH
5	35.3	2.63m	36.1	2.92 m	CH
6	79.9	4.84 (d, J = 8.0 Hz)	80.5	5.40(1H, brd, J=8.5Hz)	CH
7	58.9	3.37m	84.3	4.48 d (2.1Hz)	CH
8	65.5	-	80.7	-	C
9	41.7	2.65m	43.0	2.90 (1H)	CH
10	59.9	a,4.20, b,3.94(d,J=12Hz)	68.0	a,4.48,b,4.45(d,J=9.5Hz)	CH_2
Glucose1	98.3	4.83 (d, J =8.0Hz)	100.0	5.39(1H, d, J=8.5Hz)	CH
2’	73.4	3.32 m	74.5	4.10(1H, d, J=8.5Hz)	CH
3’	77.2	3.45m	78.0	4.22(1H,dd,J=9.0,8.8Hz)	CH
4’	70.3	3.47m	71.5	4.00(1H,dd,J=9.3,9.0Hz)	CH
5’	76.2	4.10 q (1H, J=4,8Hz)	76.3	4.01(1H, d, J=3.6Hz)	CH
6’	61.5	a,3.73 b,4.13(d,J=12Hz)	61.5	4.30(1H,d,J=9.0,4.45Hz)	CH_2
Cinnamoyl moiety	125.7	-	125.7	-	C
2’’	130.0	7.50(d J=8.0Hz, H-2’’)	130.6	7.58(1H, d, J=8.3Hz)	CH
3’’	115.5	6.83 (d J=8. 0Hz.H-3’’)	116.7	7.21(1H, d, J=8.3Hz)	CH
4’’	160.0	-	161.5	-	C
5’’	115.5	6.83 (d J=8. 0Hz.H-5’’)	116.7	7.21(1H, d, J=8.3Hz)	CH
6’’	130.0	7.50(d J=8.0 Hz, H-6’’)	130.6	7.58(1H, d, J=8.3Hz)	CH
7’’	145.9	7.68(1H,J,16.0Hz,H-7’’)	145.9	7.65(d,16.0, H-7’’)	CH
8’’	113.9	6.41(1H,J,16.0Hz H-8’’)	114.1	6.58(d,16.0, H-8’’)	CH

9''	167.6	-	167.3	-	C
MeOH	55.1	3.90(s, MeO-4'')	94.8	3.79(s, MeO-4'')	OCH_3

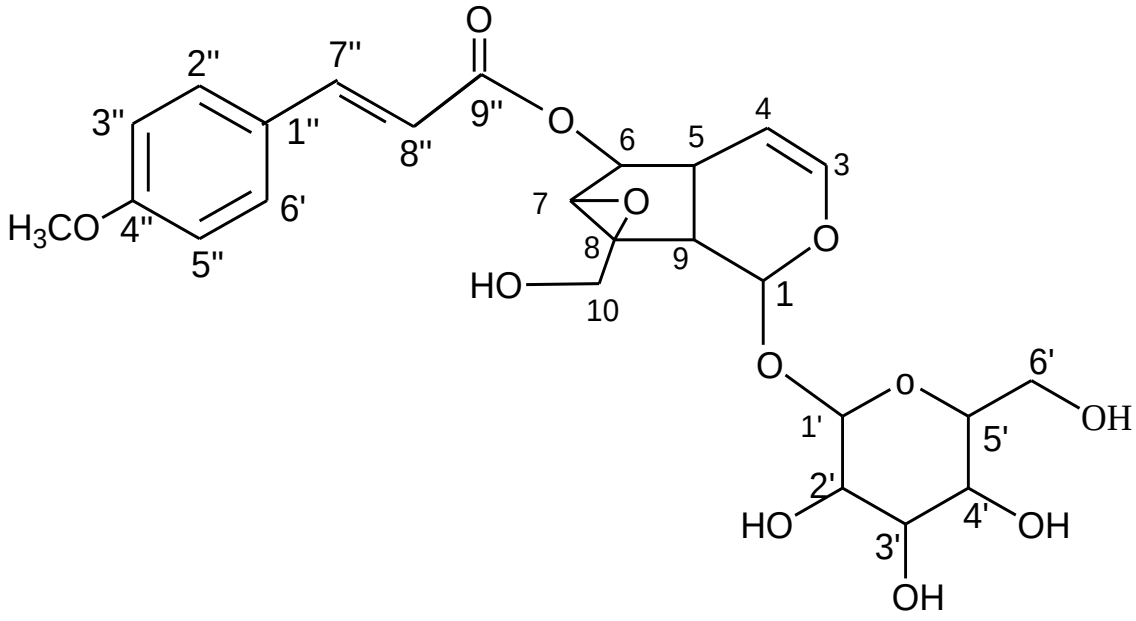

Figure 1. The proposed structure of compound 1.

4.4. Evaluation of antimicrobial activities of the crude and isolated compounds

The crude extracts (chloroform and methanol) and the isolated compounds (**1, 2** and **3**) were evaluated against four bacterial strains (*Staphylococcus aureus (*ATCC-25923), *Escherichia coli (*ATCC-25922); *Pseudomonas aeruginosa (*A TCC 27853)*; Bacillus cereus* (ATCC-6633) and one fungal strains *Candida albicum* (ATCC-14053). The disk diffusion method was used and the zones of growth inhibition of isolates were measured in millimeter (mm) zones of inhibition more than 6 mm were taken into consideration. The zones of growth inhibition (in mm) of each test samples are given in (**Table 4**).

Table 4. Zone growth of inhibition of the extracts and isolated compounds in concentration of 250 mg/mL crude extracts and 20 mg/mL of isolated compounds in mm.

Test sample	Conc. mg/mL	Diameter of zone inhibition of each tested organism (mm)				
Bacterial and fungal strains		*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Candida albicum*
Compound 1	20	11	18	25	16	12
Compound 2	20	13	14	30	12	8
Compound 3	20	10	12	18	12	12
Crude extract	250	10	12	16	11	8
Gentamicin	1Ml	23	22	18	20	*
Clotrimazole	25	*	*	*	*	15
DMSO	1mL	Ni	Ni	Ni	Ni	Ni

N.B Gram-negative (-), bacteria Gram-positive (+), bacteria Not inhibited (Ni), * =not tested.

The zone of growth inhibitions in the above Table (**4**) are different based on the bacterial strains and test samples. Table **4** (Appendix 3A) illustrates that isolated compounds were showed considerable bacterial inhibitions zone comparing to the crude extract. The crude extract and isolated compounds show good inhibition zone on gram positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) when compared with a negative bacterium. The obtained results also indicated that compound **2** exhibit relatively higher zone of inhibition against two bacterial strains namely, *Bacillus subtilis* (30 mm) and *Staphylococcus aureus* (14 mm) with the comparison of standard drug gentamicin (18 mm and 22 mm) respectively. Regarding gram strain negative; *Escherichia coli* on isolated compound (**1**, **2** and **3)** have shown fewer activities to most antimicrobial agent one when compared to gentamicin. In the case of fungus strains crude extract and compound **2** shows less inhibition zone (8 mm) than isolated compound**1**(12 mm) and compound **3** (12mm). Generally isolated compounds showed more activity against all the tested strains, compared to the crude extract.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

In the present study, three compounds were isolated from root bark of *S. kunthianum* Cham. These compounds are **1, 2** and **3** which shows signal spot on TLC. These compounds were fractionated and purified using column chromatographic technique with increasing gradient of ethyl acetate in petroleum ether and methanol in ethyl acetate ether. Compound **1** was isolated and characterized using spectroscopic data (^{1}H-NMR, ^{13}C-NMR and DEPT-135) and by comparing the observed spectral data with literature reports. Phytochemical screening was carried out and lead to presence of some secondary metabolites, the root bark of *S. kunthianum Cham* was contain glycosides, alkaloids, tannins and anthraquinone. The crude extract and isolated compounds exhibited antimicrobial properties with respect to the zone of inhibition against four bacterial strains (*Staphylococcus aureus (*ATCC-25923), *Escherichia coli (*ATCC-25922), *Pseudomonas aeruginosa (*A TCC 27853), *Bacillus cereus* (ATCC-6633) and one fungal strains *Candida albicum* (ATCC-14053). Isolated compounds were showed more antimicrobial activity than crude extracts when used against the bacterial pathogens and fungus Candida albicans. Based on our result it can be concluded that the study plant root bark of *S. kunthianum Cham* contain bioactive compounds that are effective against the tested bacterial and fungal strains.

5.2. Recommendations

Based on the current finding, further studies on the plant are recommended as:

- Further phytochemical investigation on *S. kunthianum Cham* should be done using HPLC on the polar extracts of the plants.
- On this research methanol and chloroform extracts were used extraction of *S. kunthianum.*

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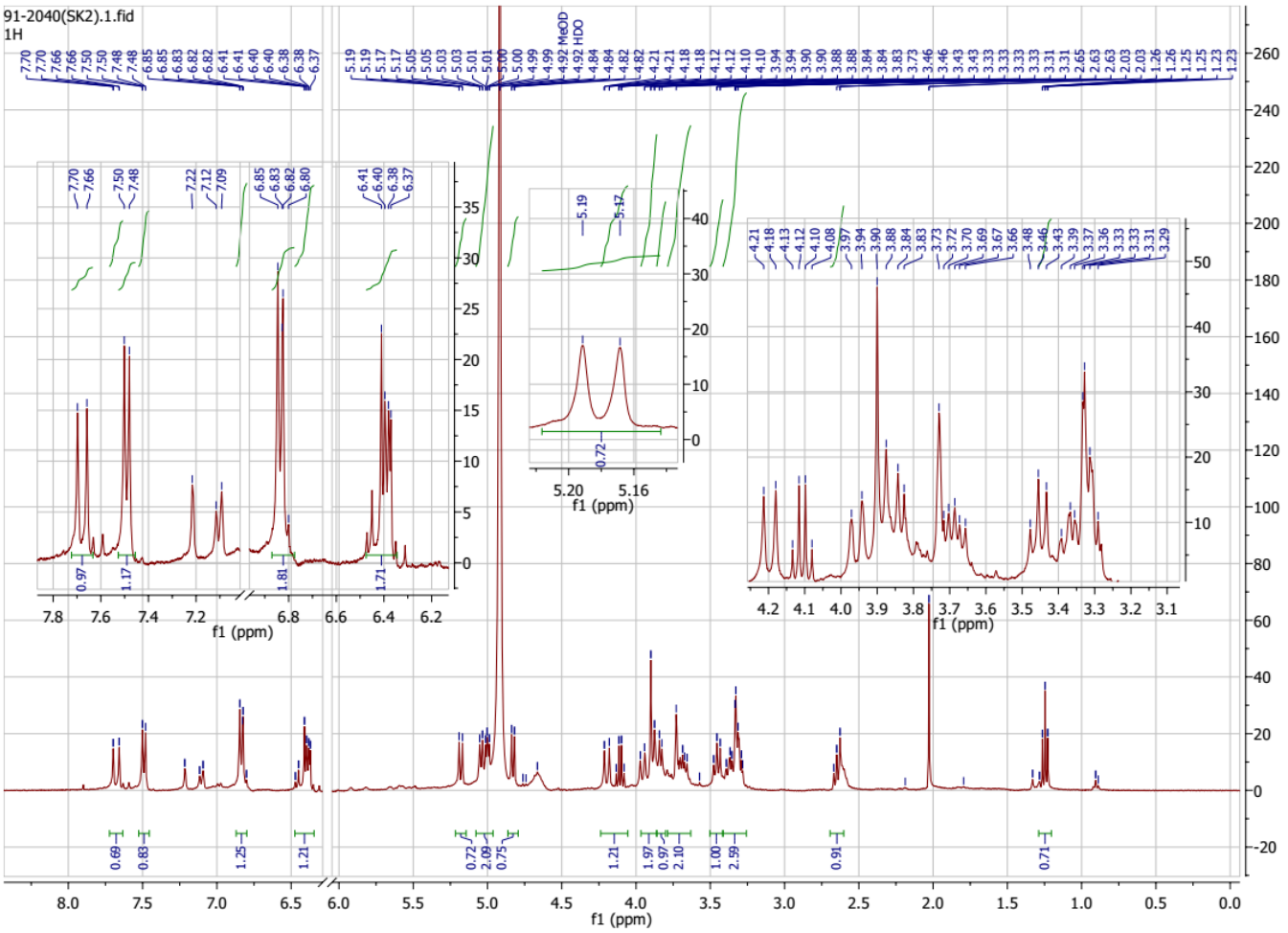
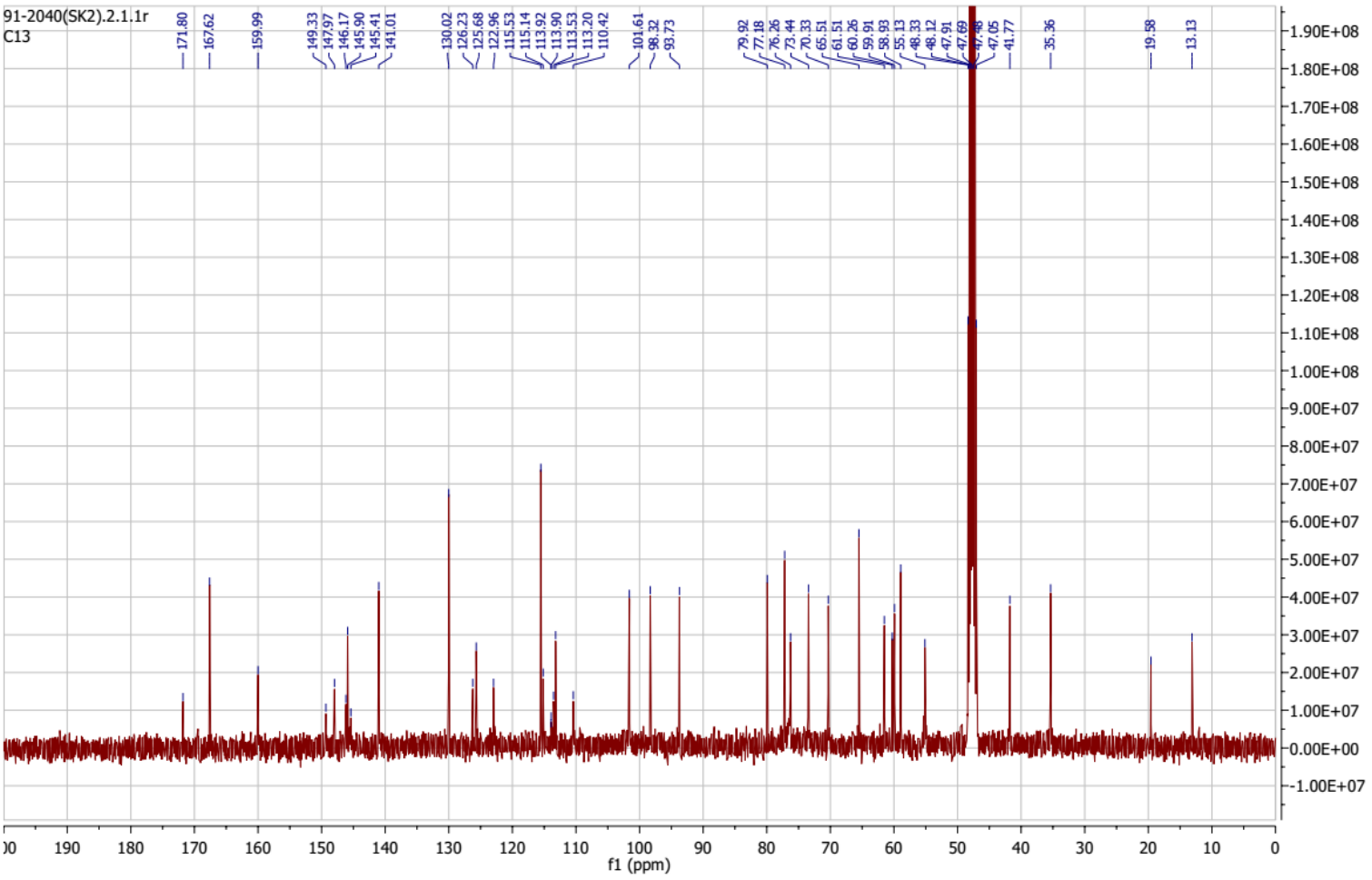
APPENDICES

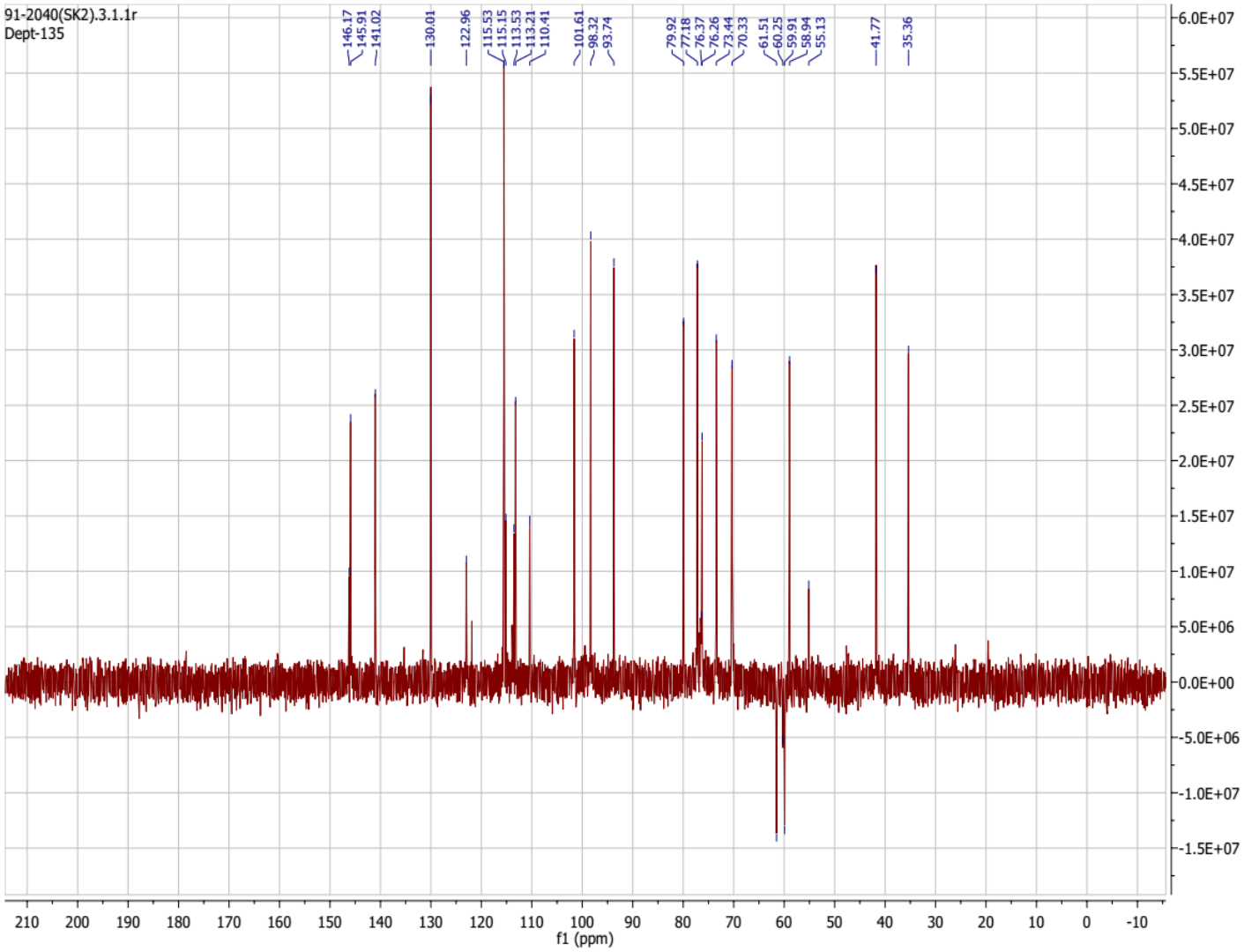
Appendix1A. ^{1}H-NMR spectra of Compound **1**(2.5% methanol in ethyl acetate)



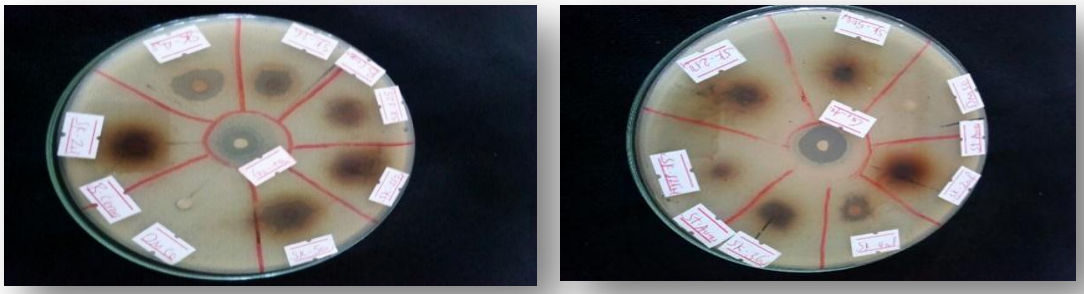
Appendix 1B. ^{13}C-NMR spectra of Compound **1**(2.5% methanol in ethyl acetate)



Appendix 1C. Dept-135 spectra of Compound **1**(2.5% methanol in ethyl acetate)

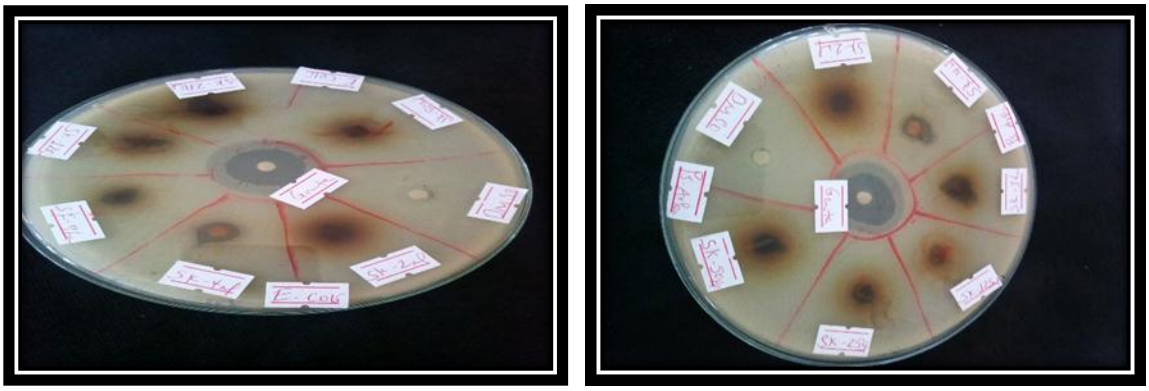


Appendix **3A.** Bioassays tests of crude extract and isolated compounds zone of growth inhibition



A = *Bacillus subtilis* **B=** *Staphylococcus aureus*

Plate **1**: Antibacterial activity of *S. Kunthianum* root bark against two-gram positive bacterial organisms with different concentrations of chloroform/ methanol extract and isolated compounds.



C= *Escherichia coli* **D**= *pseudomonas aeruginosa*

Plate **2**: Antibacterial activity of *S. Kunthianum* root bark against two-gram negative bacterial organisms with different concentrations of chloroform/ methanol extract and isolated compounds.