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DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION OF ROOTS OF *Kniphofia insignis* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITIES

BY

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PHYTOCHEMICAL INVESTIGATION OF ROOTS OF *Kniphofia insignis* AND EVALUATION OF ITS ANTIMICROBIAL ACTIVITIES

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

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Declaration

I declare that Phytochemical investigation of roots of *Kniphofia insignis* and evaluation of its antimicrobial activities this 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.

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

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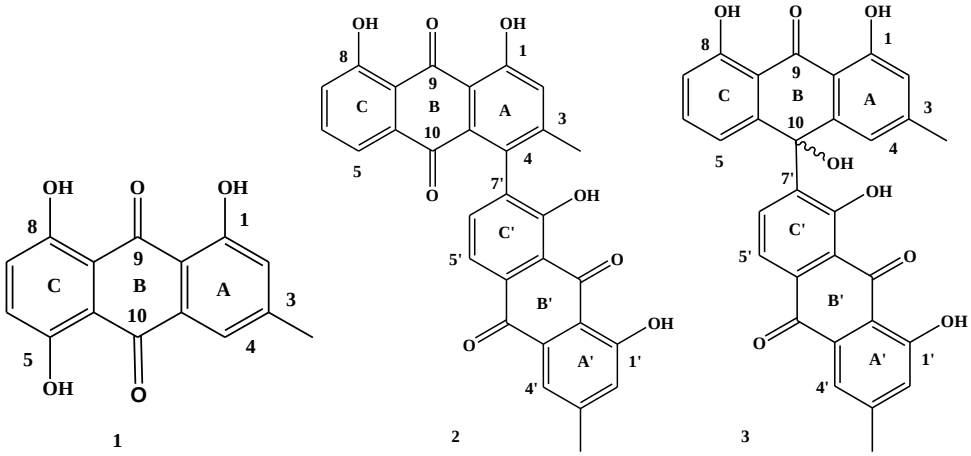
Abbreviations

WHO	World Health Organization
HPLC	Higher Pressure Liquid Chromatography
CC	Column Chromatography
TLC	Thin Layer Chromatography
DMSO	Dimethyl Sulfoxide
NMR	Nuclear Magnetic Resonance
1D NMR	One Dimensional Magnetic Resonance
DEPT	Distortion less Enhancement by Polarization Transfer
IR	Infra-Red
UV	Ultraviolet Visible
J	coupling constant
s	singlet
d	doublet
dd	doublet of doublet
*M*Hz	Mega Herz

Abstract

Kniphofia is a medicinal plant that is commonly used for treatment of different ailments including menstrual pains, infertility, abdominal cramps, wounds, malaria, chest complaint, gonorrhea and hepatitis B. in Ethiopia. Therefore, the aim of this study was to isolate, characterize and evaluate antimicrobial activities of the crude extract and compounds from the root of *Kniphofia insignis*. Meanwhile the root part of the plant was air dried, grinded and subjected to sequential extraction using *n*-hexane, chloroform, acetone and methanol by cold maceration method resulted 10.0(1%), 27.5(2.75%), 17.0(1.7%) and 45.0 (4.5%) g crude extract of each. The concentrated crude extracts were evaluated for their antibacterial and antifungal activities against four bacterial strains (*Escherichia coli, Staphylococcus auraus, Bacillus subtilus and Pseudomonas aeruginosa*) and one fungus strain (*Fusarium spp*) and the extract with the better activity was then subjected to further purification by column chromatography on silica gel. It results three compounds; a monomeric anthraquinone (**1**) and two dimeric anthraquinones (**2** & **3**). The structures of these isolated compounds were characterized by NMR and IR spectroscopy techniques. The isolated compounds were also evaluated for their *in vitro* antimicrobial activity as described above. The compounds exhibited marginal activity against the bacterial strains with the highest activity observed for compound **1** with diameter of inhibition (14 mm) against *P. aeruginosa*. Whereas compound **2** showed better activity (13 mm) against *fusarium spp* fungal strain which supports the validity of the plant used traditionally as a medicine.



1 2 3

1. Introduction

1.1. Background of the Study

Medicinal plants remain the main sources of traditional medicine for human beings and livestock. According to WHO, traditional medicine is "the sum total of all knowledge and practice, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalances, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" [1]. The history of development of medicinal plants broadly be classified into a number of eras. Early period involving the Indian, Chinese, Sumerians, Egyptian, Assyrian and Babylonian civilizations followed by the Greek, Roman, Persian, Eastern and Medieval era and finally, the modern period [2]. Observations from animals evidenced that, even chimpanzees use a number of plant species for their medicinal value [3]. Use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed. Furthermore, the use of medicinal plants in the modernized societies has been focused on extraction for cosmetic applications and development of several drugs, chemotherapeutic from these plants as well as from traditionally used rural herbal remedies [4, 5]. About 80% of the total population of developing countries health care and wellbeing are mainly depending on traditional medicine [6, 7], particularly in Ethiopia about 80% of the total population health care depend on traditional medicine because of its availability, affordability and acceptability [1, 8].

Plants produce different types of products with highly different structures. These products are commonly termed as primary metabolites and secondary metabolites. Primary metabolites are responsible for the growth and development of plants, for example; sugars, protein, lipids, starch. Secondary metabolites on the other hand, appear to function mainly in defense against predators and pathogens and in providing reproductive advantage as intra-specific and inter-specific attractants. They may also act to create competitive advantage as poisons of opponent species. Some of the most important secondary metabolites (bioactive phytochemical) constituents that the plants usually produces are alkaloids, essential oils, flavonoids, tannins, terpenoids, Saponins, and phenolic compounds [7, 9-10], that are empirically responsible for the treatment of different alignments and take part in drug development. Due to the ability of plants to biosynthesize a plethora of compounds,

whose structural diversity is often beyond the dream of even the most imaginative chemists, plants remain important source of medicine for treatment of various diseases including microbial infections. Microbial diseases are causing huge burden throughout the world, and kills many millions of people. This is because of the increased prevalence of resistant microbes such as, bacteria, fungus, etc together with the lack and high cost of new generation drugs has increased infection resulted in morbidity and mortality particularly in developing countries. Moreover, the burden inclined towards children with 46% of all deaths were children aged less than 15 years, whereas only 20% were people aged 60 years and over which mainly caused by microbial diseases [11-12].

1.2. Natural Products as Sources of Drug Discovery

The use of pure compounds as drug substances started in the mid-19th century. Compounds emerged from the study of ethno botanic extracts became important as medicines and were used as pharmacologic tools in the elucidation of disease mechanisms. Those new natural drug substances were the main sources for entire therapeutic areas and in stimulating the formation of the modern pharmaceutical industry [13]. Before mid-19th century, crude and semi pure extracts from plants, microbes and minerals were the only medications available for the treatment of humans and animals illnesses. Today, it is estimated that about 40% of all medicines are either natural products or their semi synthetic derivatives [14]. For instance, digitoxin (**4**) from *Digitalis purpurea* (foxglove), used to treat arrhythmia and congestive heart failure, ephedrine (**5**) from *Ephedra sinlica* for treatment of heart patient; morphine (**6**) from *Papaver somniferum* L. and epibatidine (**7**), from *Epipedobates tricolor*, to treat pain; aspirin (**8**), fever and pain reducing from willow tree *Salix alba L*., penicillin (**9**) from a fungus *Penicillium notatum*, to treat bacterial infection [13,15], artemisinin (**10**) from *Artemisia annua*, and quinine (**11**) from the *Cinchona officialis* are most known antimalarial drugs [13,15-16], Camptothecin (**12**) from *Camptotheca acuminate*, used as anticancer drugs[16] (Figure 1).

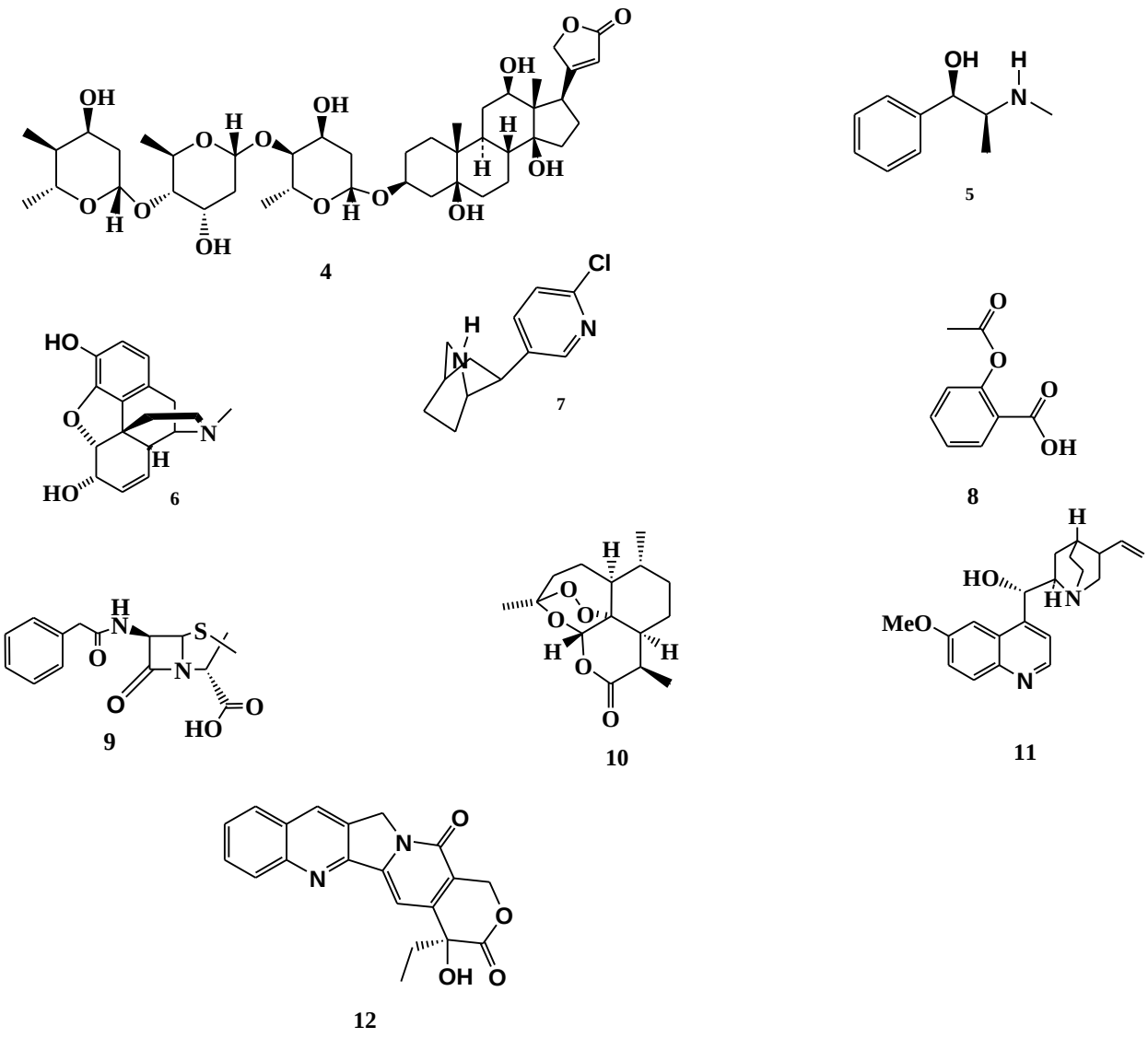


Figure 1. The chemical structure of selected drugs derived from natural products

Many plants of the genus *Kniphofia* are used in traditional medicine and their secondary metabolites are of pharmaceutical interest. However, only little work has been done on the pharmacological activities of compounds from species of the genus including *Kniphofia insignis*. Thus, further research needs to be undertaken on *Kniphofia insignis* in order to identify compounds having significant antimicrobial activities.

1.3. Statement of the Problem

The emergence of various drug-resistant organisms limited therapeutic efficiency of many of currently available antimicrobial drugs. The existence of structurally varied compounds from medicinal plants mainly from those which have documented traditional uses cannot be over emphasized. About 80% of the Ethiopian populations are based on medicinal plants (traditional medicine) for treating various illnesses [1]. However, there is a need for scientific support for plants used in the traditional medicine before these could be recommended for treatment of illness and to get candidate compounds for drug discovery. Medicinal plants have a promising future, because there are about half million plants around the world, and most of their medicinal activities have not been investigated, that their medicinal activities could be decisive in the treatment of present or future studies. This indicates the necessity of scientific investigation of medicinal plants that have not yet investigated. One of the plant species in this regard is *Kniphofia insignis*. Plant of the genus *Kniphofia* contains large number of chemically complex bioactive compounds. Even though the plant including *Kniphofia insignis* is known for its ornamental purpose, it has wide traditional application for treatment of several human illness such as, menstrual pains, infertility, abdominal cramps, wounds, malaria, chest com-plaint, gonorrhea and hepatitis B [11]. However, the phytochemical investigation and the antimicrobial activities pertaining to this plant has not been reported. Therefore, the study was aimed to extract, isolate, characterize compounds from the root of *Kniphofia insignis* and evaluate their antimicrobial activities.

1.4. Objectives

1.4.1. General Objective

- The main objective of this study was to carryout phytochemical investigation of root of *Kniphofia insignis* and evaluate their antimicrobial activities.

1.4.2. Specific Objectives

- To isolate compounds from the root of *Kniphofia insignis* using chromatographic technique;
- To evaluate antimicrobial activity of the crude extracts and isolated compound against four bacterial strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilus*) and a fungi (*Fusariun spp).*

- To characterize the structure of the isolated compounds using spectroscopic techniques such as ^{1}H, ^{13}C NMR and IR;

1.5. Significance of the study

Phytochemical information and bioactive molecules from the root of *Kniphofia insignis*, which has been widely practiced by the local community for its medicinal role has not been reported so far. Therefore, findings of this research would,

- ❖ Provide information about the chemical profile of root of *Kniphofia insignis*.
- ❖ Provide information about antimicrobial activities of the phytochemicals isolated from root of *Kniphofia insignis.*
- ❖ Serve as baseline information for further studies on the same plant genus.

2. Review of Related Literature

2.1. Infectious Diseases

Infectious diseases are the most serious health problems in the world. They are caused by microorganisms such as bacteria, viruses, fungi or parasites and are responsible for greater than 25% of the global disease. They remain public health and economic problems because of the emerging of resistance pathogens that limit the therapeutic uses of many of the drugs that are in the market. In this era of emerging of resistance pathogens, the discovery of new lead compounds with novel mechanism of action from natural source cannot be over emphasized, especially from plants which have documented traditional uses to treat these diseases [17, 18]. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance as well as new emerging infections [19]. The era of antibiotics till the early 1970s led to optimism that infectious disease can be controlled and prevented by modern medicines. However, infections are still the second-leading cause of death worldwide, causing over 13 million deaths each year. This fact may be the result of the emergence of new diseases, the re-emergence of diseases once controlled and more specifically of the development of antimicrobial resistance. This type of resistance may result from changes in the bacterial genome due to mutation [20].

2. 2. Botanical Information *Kniphofia* species

Plant in the genus *Kniphofia* Moench (family *Asphodelaceae,* with 16 genera and about 780 species are widely distributed in the temperate, tropical and subtropical regions of the world), which is commonly named as ‘Red hot pokers’, contain about 71 species. It is characterized by evergreen, perennial, rhizomatous, usually acquiescent and herbaceous habit, whose leaves are linear and usually keeled grass like. Florescence are sub-spicate racemes with a bract at the base of pedicels. Its flowers are tubular rising above the foliage with different colours such as white, yellow to various shades of red which are more conspicuous at the apex of the inflorescence. There are about seven species of *Kniphofia* occur in Ethiopia, of which five; including *Kniphofia foliosa*, *Kniphofia hildebrandtii*, *Kniphofia isoetifolia*, *Kniphofia insignis* and *Kniphofia schimperi* are endemic [21, 22].

The species *K. insignis* commonly named as "*shube*" or '*liche*" (Afan Oromo) clearly distinguished from the other species by the white perianth, which is unusual in the genus, and also by the fusiform roots (figure 2). Leaves 30–100 × 0.3–1.5 cm, linear, glaucous, keels and margin papillate. Peduncle (including raceme) 20–65 cm long, sometimes up to 100 cm long in cultivation. Raceme 8–22 cm long, at flowering, lax. Bracts white, cuspidate, 12–17 × 2–3 mm. Perianth white, pendulous, cylindrical, 24–28 mm long, widening at the mouth and constricting at the base; perianth lobes 2–3 × 1–2 mm long. Pedicel slender 2.5–4 mm long, elongating to 5 mm long in fruit. Stamens and style only shortly exserted, up to 3 mm long, stamens eventually withdrawn. Fruit ovoid and pointed, 8–9 × 6 mm. The species often grows in water-logged or flooded meadows between 2500 and 3100 m. It is so far only known from the Shoa and Arsi Zone. The main flowering period is from June to September. The plant occurs scattered, and it is commonly seen in the "Sululta" plains between "Addis Ababa" and "Chancho" during its flowering period [22].

Figure 2. Picture of the study plant (photo taken by Asnakew A, Semtember 2017.)

2.3. Ethnomedicinal Uses of *Kniphofia* species

Use of the genus *Kniphofia* in traditional medicine is limited to few species. Infusions of the root of *K. uvaria* [23] and *K.linearifolia* is used to treat women infertility, the infusion of rhizomes of *K. laxiflora, K. rooperi, K. parviflora and K. buchanani* are used to treat chest ailments and snake deterrent. The roots *K. foliosa* is used to treat abdominal cramps and wound healing, the root of *K. isoetifolia* is used to treat Gonorrhea and Hepatitis B. and *K. ritualis* is used to treat shoulder pains [11,21,24]. The roots and Rhizome of *K. drepanophylla* is used to treat ring worm, wounds, pimples, acne and eczema [25].

2.4. Phytochemical Information of *Kniphofia* species

Kniphofia is a rich source of Quinones, mainly, monomeric and dimeric anthraquinones, anthrones, monomeric and dimeric phenylanthraquinones, [24] and naphthoquinone [11] (Table 1, Figure 3).

Table 1. Examples of compounds isolated from different parts of *Kniphofia* species.

Compounds	Species (Part of plant)	Reference
Naphthoquinone		
3,5,8-trihydroxy-2-methyl naphthalene-1,4-dione (**13**)	*K.isoetifolia* (R)	[11]
Monomeric Anthraquinones		
Chrysophanol (**14**)	*K. ensifolia*	[26]
	K. foliosa (R)	[21, 24]
	K. foliosa (Rh, L, F), *K. isoetifolia* (Rh, L, F), *K. pumila* (Rh, F)	[27]
	K.isoetifolia (R, F)	[11]
	K. thomsonii (R)	[28]
Aloe-emodin acetate (**15**)	*K. foliosa* (l), *K. isoetifolia* (F)	[27]
	K. thomsonii(R)	[28]
Chrysophanic acid (**16**)	*K. caulescens* (R), *K. foliosa* (R, Fr), *K. linearfolia*(R),	[29]
	K. foliosa (L), *K. foliosa* (Rh), *K. isoetifolia* (Rh, L, F), *K. pumila*(Rh), *K. reynolds* (R)	[27]
Islandicin (**17**)	*K. foliosa*(Rh, R), *K. linearifolia* (R), *K. reynolds* (R),	[29]
	K. foliosa (Rh, L, F), *K. pumila* (Rh), *K. isoetifolia* (Rh)	[27]
	*K. thomsonii (*R*)*	[28]
Dimeric Anthraquinone		
Asphodeline (**2)**	*K.isoetifolia* (R)	[11]
	K. ensifolia	[26]

	K. albescens (R), *K. linearifolia* (R), *K. ensifolia* (R), *K. evansii* (R), *K. northae* (R), *K. tysonii* (R)	[30]
Chrysalodin (**18**)	*K. foliosa* (L)	[31]
Chryslandicin (**19**)	*K. ensifolia*	[26]
	K. foliosa (L)	[31]
	K. foliosa (R)	[21,24, 29, 32]
	K. brachystachy (R), *K. evansii* (R), *K. typoides* (R), *K. tysonii*(R)	[33]
Kniphofine (**20**)	*K. foliosa* (Rh), *K. isoetifolia* (Rh), *K. Pumila* (Rh)	[27]
Chryslandicin-10-methyl ether (**21**)	*K. foliosa* (Rh)	[34]
10-Hydroxy-10-(Chrysophanol-7'-yl)-chrysophanol anthrone (**3**)	*K. ensifolia*	[26]
	K. foliosa (R)	[32, 35]
	K.isoetifolia (R)	[11]
	*K. thomsonii(*R*)*	[28]
Phenyl anthraquinones and anthrones		
Knipholone anthrone (**22**)	*K. foliosa* (R)	[21, 3]
Knipholone (**23**)	*K. ensifolia*	[26]
	K. albescent (R), *K. brachystachy* (R), *K. brerifolia* (R), *K. citrina* (R), *K. ensifolia*(R), *K. evansi*(R), *K. gracilis* (R), *K. linearifolia* (R), *K. tysonii* (R), *K. umbrina* (R)	[33]
	K. foliosa(R)	[21, 24, 32, 35, 36]
Dimeric phenylanthraquinones		
Joziknipholone A (**24**), Joziknipholone B (**25**)	*K.foliosa* (R)	[24, 37]
Miscellaneous compounds		

Flavoglucin (**26**), 5"', 6"'-Dehydroflavoglucin (**27**)	*K.thomsonii* (R)	[28]
3,4-Dihydroxy benzoic acid (**28**)	*K.foliosa* (R)	[24]

Keys R=root, Rh=rhizome, F=flower, Fr= fruit, L=leave

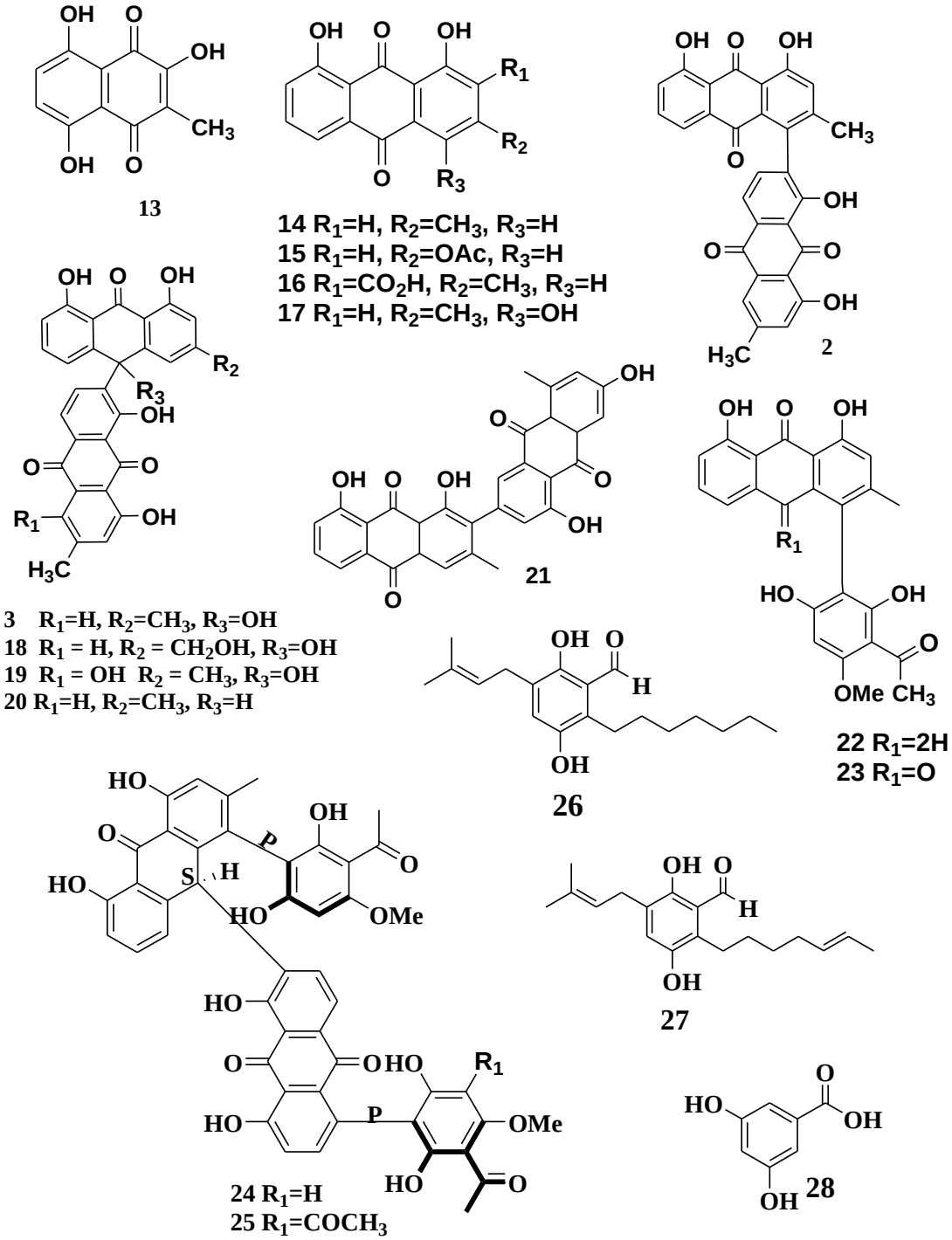


Figure 3. The chemical structure of Compounds isolated from plants in the genus *Kniphofia*.

2.5. Biological activities of some compounds isolated from *Kniphofia* species

There are several literature report that reveal biological test of compounds isolated. Some of the compounds isolated from the genus *Kniphofia* that have been tested and have shown promising biological activities, which are summarized below (Table 2).

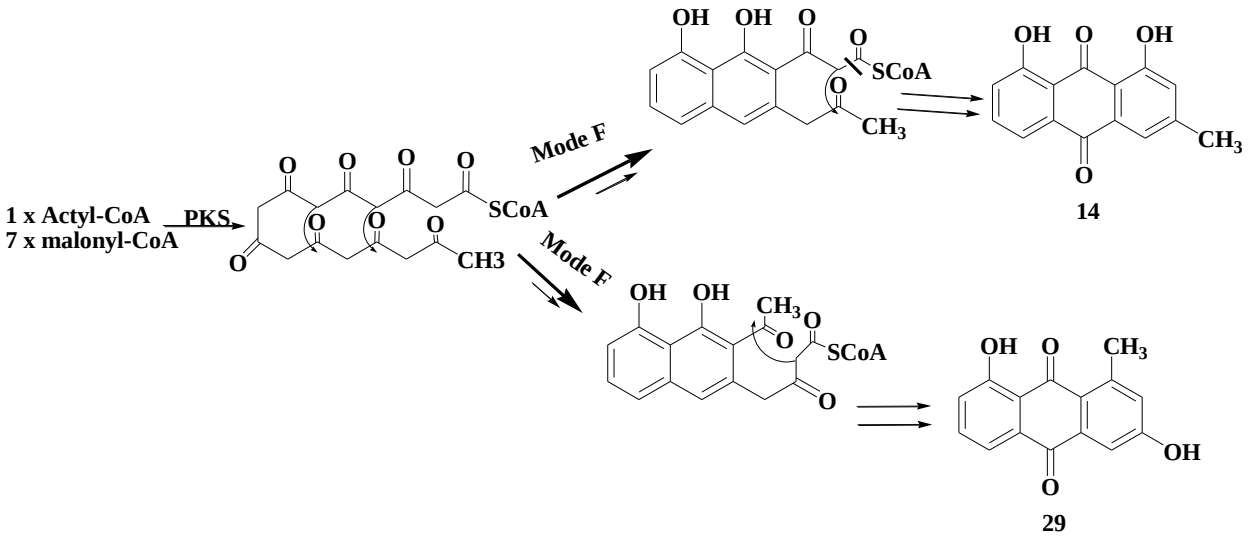
Table 2. Biological activity of some of compounds isolated from the genus *Kniphofia.*

Compounds	Biological activity	References
Asphodeline (**2**), 10-Hydroxy-10,7'-(chrysophanol anthrone)chrysophanol (**3**), 3,5,8-Trihydroxy-2-methyl naphthalene-1,4-dione (**13)** and Chrysophanol (**14**)	Antibacterial activity (*S.aureus, E. faecalis, P. aeruginosa*, and *E. coli*)	[11]
Knipholone anthrone (**22**) and Knipholone (**23**)	Antioxidant activity	[36]
Knipholone anthrone (**22**), Knipholone (**23**), Joziknipholone A (**24**), Joziknipholone B (**25**), Chryslandicin (**19**)	Antiprotozoal activity	[36]

2.6. Biosynthesis of Anthraquinones in *Asphodelaceae*

The metabolic pathways leading to the synthesis of anthraquinones is a polyketide pathway, which is governed by the polyketide synthase enzyme that generates an array of natural products by catalyzing condensation of a number of acetyl units from malonyl-CoA into a growing polyketide chain. The large number of natural products produced by this pathway may be due to the number of acetyl units used, variation in the folding of the polyketide chain and mechanism of ring formation. The acetyl unit condenses stepwise with molecules of malonyl-CoA to give a linear octaketide intermediate which can be folded in two different ways. These are 1,8-dihydroxy-3-methylanthraquinone such as chrysophanol (**12**) are obtained through the customary folding (Scheme 1a) whereas the rare type of folding (Scheme 1b) which is mainly observed in the *Aloe* species (*Asphodelaceae*) results in the formation of 3,8-dihydroxy-1-methylanthraquinones with the

typical example being aloesaponarin II (**29**). In both case, the ring at the center of the fold is formed first, followed by the next two rings, after the polyketide chain has folded [21, 24, 38-39].



Scheme 1. Biosynthesis Pathway of selected Anthraquinones.

3. Material and Methods

3. 1 Chemicals

n-hexane, chloroform, acetone, methanol and ethyl acetate were used for extraction and column elution; Silica gel 60-120 mm mesh size, silica gel coated TLC plate for detection of spots, $CDCl_3$ was used for recording NMR spectra, DMSO was used for sample preparation for antimicrobial susceptibility test, whatmann filter paper for filtration and disc preparation. All chemicals and reagents used were analytical grade, Sephadex LH-20 was also used for further purification.

3.2 Apparatus

Pistil and mortar, weighing balance (Model no WT100001X) , glass column (300 mm (B-14/23,B-19/26) and 500 mm, B-34/35) for column chromatography, Rotary Evaporator (Heidolph, Germany, laborota 4000, No, 519-0000-00-2) were used for extraction and purification, autoclave (Astell, model no: AMA44DBT), Incubator (Genelab Incubator), Hood (CLB-201-04, VERTICAL LAMINAR CABINET), Infrared (IR)(Perkin Elmer IR spectrophotometer), 1D (^{1}H and ^{13}C, 400 MHz, Bruker ultra shield TM 400) NMR were used for characterization.

3.3. Collection and preparation of plant material

The root of *Kniphofia insignis* was collected from North Shoa zone, Oromia regional State, Jida district which is about 116 km away from Addis Ababa on September 2017. It was collected, air dried, grounded and stored in appropriate container in Organic research laboratory Jimma University. The plant material was identified by a Botanist Dr. Dereje Denu, and the voucher specimen (voucher number CH1) has been deposited in Jimma University Herbarium.

3.4. Extraction and isolation

About 1 kg of powdered plant material was sequentially extracted by equal volume of about 5 L of *n*-hexane, chloroform, acetone and methanol, using cold maceration technique three times for 24 hr each at room temperature. The crude extract of each solvent was filtered first through fresh cotton plug and then through whatmann (No. 1) filter paper. Then filtrates were concentrated using rotary evaporator at 45 ^{0}C and all of the dried extract were kept in a desiccator until use. Then, the extract which showed better bacterial activities test and TLC profile, was adsorbed on 23 g silica gel (60-120 mm mesh) and subjected to column chromatography (50 mm diameter) loaded with 165 g silica gel. The appropriate solvent for isolation was made after carrying out TLC analysis of the crude

extracts in various combination of solvents with increasing polarity. Since, *n*-hexane in ethyl acetate was found to show good separation. The column was first eluted with 100% *n*-hexane, and then with increasing gradient of ethyl acetate in *n*-hexane (1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60 and up to 100%) was used as eluent during separation. A total of 126 fractions each with 50 mL were collected. The fractions were concentrated under reduced pressure using a rotary evaporator at 45°C. The identities of the fractions were examined by TLC. The spots developed were visualized under UV light at 254 nm. The fractions that showed the same TLC profiles (*Rf* value) were combined and concentrated, the column chromatography led to isolation of three compounds (Scheme 2). The isolated compounds were characterized by spectroscopic techniques: IR and 1D (^{1}H-NMR and ^{13}C-NMR) for compound **1** and ^{1}H-NMR for compound **2** and **3.** All the spectral analysis were carried out at the Department of chemistry, Addis Ababa University.

3.5. Antimicrobial Assay

The crude extracts and isolated compounds were evaluated for *in vitro* anti-microbial activities against four bacteria strain (*Escherichia coli* ATCC 35218, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilus* ATCC 6633 and *Pseudomonas aeruginosa* ATCC 27853) and one fungus strain (*Fusarium spp*) in microbiology laboratory, Department of Biology Jimma University, by disc diffusion method following the standard procedures reported in literature [15,17,40]. The strains were activated for the experiments at 37°C for 24 hours on nutrient broth for bacterial strain and potato dextrose broth for fungal strain. The culture media was boiled in distilled water to dissolve the media and autoclaved at 121^{o}C for 2 hr. then, it was poured into sterile Petri dishes refrigerator until use. After the culture media had solidified, organisms were uniformly seeded with it using a sterile swab. 100 mg/mL, 50 mg/ml and 25 mg/ml of crude extracts, compound **1** and **2** respectively and the same volume DMSO (negative control) was impregnated using Whatman No.3 filter paper disc (diameter 6 mm) with the help of micropipette, respectively. Positive controls, Gentamicin (for bacteria) and Chltrimazole (for fungus) were assayed simultaneously. Plates were left for 10 minutes till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 hr for bacteria and 48 hr for fungus. After incubation, the diameter of the inhibition zone was measured in millimeter using ruler and mean was recorded.

4. Results and Discussion

4.1. Extraction Yield

One Kilogram of plant material was sequentially extracted with *n*-hexane, chloroform, acetone and methanol results, 10.0, 27.5, 17.0 and 45.0 g of *n*-hexane, chloroform, acetone and methanol crude extract were obtained respectively and it yields totally 119.5 g which is higher crude extract yield as compared with reported (98 g) [11], the percentage yields of these crude extracts are given below (Table 3). As the polarity of the solvent increases the mass (percentage yield) of the extract increase, which, showed that most secondary metabolites in a *K. insignis* root are polar because "like dissolves like". It was calculated by the formula;

$$\%\ yield = \frac{weight\ of\ the\ crude\ extract\ (g)}{weight\ of\ the\ draied\ sample\ usd\ (g)}\ x\ 100$$

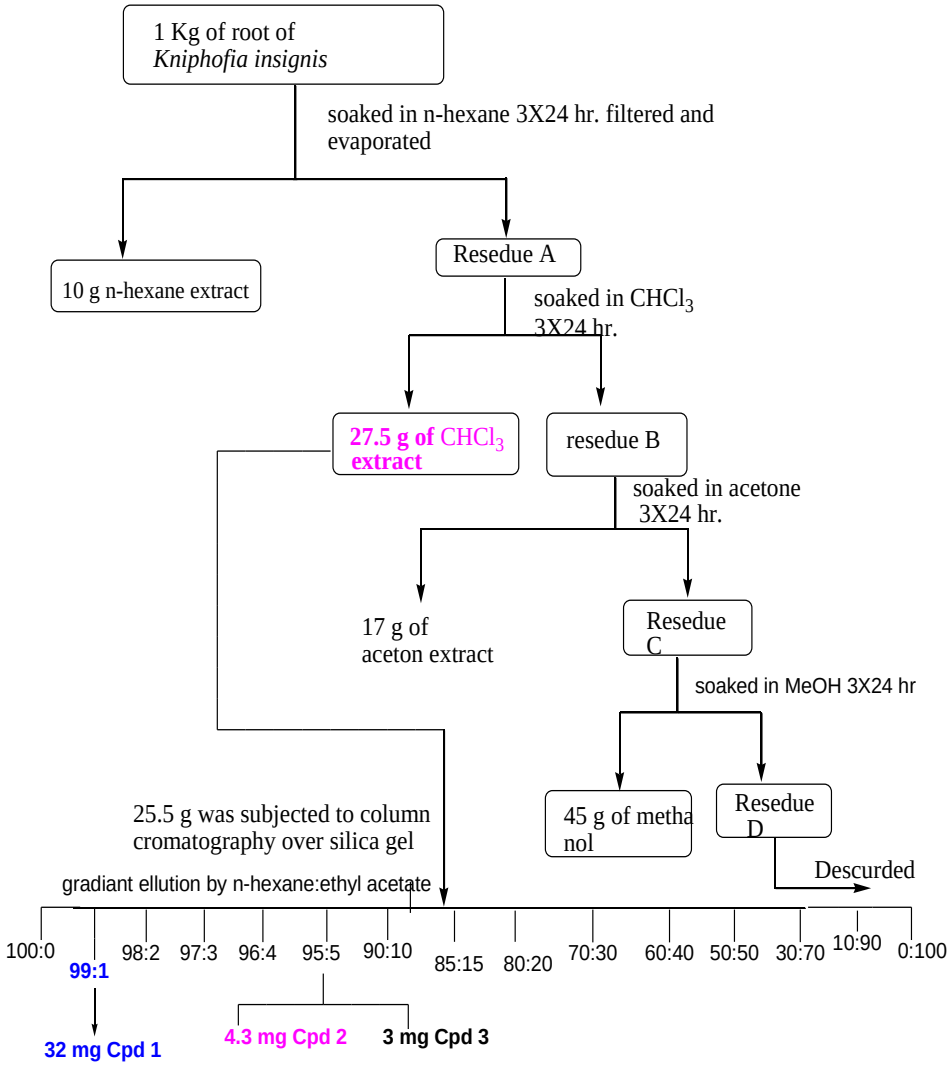
Table 3. Percentage yield of extracts.

Solvent	Mass in (g)	% yield
n-Hexane	10.0	1
Chloroform	27.5	2.75
Acetone	17.0	1.7
Methanol	45.0	4.5

4.2. Results for Isolation of Compounds

Based on superior activity against bacteria strain and TLC profile, chloroform extract was subjected to column chromatography for isolation of compounds. Selection of appropriate solvent for column elution was made after carrying out TLC analysis of the crude extracts. Hence *n*-hexane in ethyl acetate was found to show good separation. It was first eluted with 100% of *n*-hexane and then with increasing gradient of ethyl acetate in *n*-hexane (1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60 and up to 100%) 400 ml each. During the isolation of compounds by using gradient elution of ethyl acetate in *n*-hexane a total of 126 fractions each with 50 mL were collected. Among the fractions, 11-19 (1% ethyl acetate in *n*-hexane) were combined and further purified to give 32 mg of red amorphous solid which is labeled as AA2 (**1**), Fractions 48-61 (5% ethyl acetate in *n*-hexane) combined and re columned using the same solvent gives 4.3 mg of red amorphous solid labeled as A2 (**2**) and 3 mg of red amorphous solid labeled as A3 (**3**) which is summarized in Scheme 2.

The characterization of the isolated compounds were by spectroscopic technique IR and NMR (^{1}H and ^{13}C) for compound **1** and only ^{1}H NMR for compound **2** and **3**, which have been discussed below.



Scheme 2. The summary of procedures used for extraction and isolation of compounds from root of *Kniphofia insignis*.

4.3. Characterization of Isolated Compounds

4.3. 1. Characterization of Compound 1

Compound **1** (32 mg) was isolated as red amorphous solid. It was isolated with 1% of ethyl acetate in *n*-hexane and its Rf value was 0.87 in 9.5:0.5 solvent ratio of *n*-hexane and ethyl acetate. Its IR spectral analysis (Appendix **1A**), showed a strong band in the region of 3426 and 2917 cm^{-1} for the hydroxyl (OH) stretching and aromatic (C-H) stretching vibrations, respectively. Other characteristic signals for carbonyl (C=O) bond vibrations and strong C-O bond stretching were also observed at 1616 and 1268 cm^{-1} respectively.

^{1}H NMR spectrum (400 MHz, $CDCl_3$) (Appendix **2A**) displayed three hydroxyl protons at δ_H 13.04, 12.36 and 12.17 involved in hydrogen bonding and were assigned to hydroxyl groups at C-5, C-8 and C-1 respectively of an anthraquinone skeleton. In ring A, two *meta*-coupled aromatic protons at δ_H 7.14 (1H, *brs*, H-2) and 7.73 (1H, *d*, *J* = 4 Hz, H-4) were observed in addition to the biosynthetically expected methyl group (δ_H 2.51; δ_C (22.3) at C-3 (δ_C 149.1). Whereas in ring C, two *ortho*-coupled equivalent aromatic protons resonating at 7.32 (2H, *d*, *J* = 8.0 Hz) which were assigned to H-6, H-7 confirming the presence of chelated hydroxyl group (δ_H 13.04) being at C-5 (δ_C 158.3) (Table 4).

The ^{13}C NMR spectral data (Appendix **2C**) revealed the presence of carbon signals for fifteen carbon atoms including two carbonyl (δ_C, 190.6 and 186.6) at C-9, C-10; four aromatic methine carbons (δ_C, 129.6, 129.5, 124.6 and 120.8) at C-7, C-6, C-2, C-4, three oxygenated aromatic quaternary carbons (δ_C, 162.9, 158.3 and 157.6) at C-1, C-5, C-8, five aromatic quaternary carbons (δ_C, 149.1, 133.2, 114, 112.8 and 112.5) at C-3, C-4a, C-1a, C-8a, C-5a, respectively, and a methyl carbon (δ_C, 22.3), 3-CH_3 (Table 3). Therefore based on the above spectroscopic evidence and comparison with literature, compound **1** was identified as 1,5,8-trihydroxy-3-methylanthraquinone, trivial name helminthosporin (**1**) (Figure 4) which has been previously reported from *Aloe dawei* and *Aloe lateritia* Subspecies *graminicola* [21, 41, 42] but it is reported for the first time from the genus *Kniphofia*.

Table 4 ^{1}H and ^{13}C (400 MHz, $CDCl_3$) NMR data of compound **1** and reported in ^{1}H (500) and ^{13}C (125) (acetone-d_6) in MHz.

Position	δ_H (*J*, Hz)	δ_C	Reported δ [21]	
			δ_H (*J*, Hz)	δ_C
1	-	162.9	-	162.8
1a	-	114.0	-	114.0
2	7.14 (1H, *s*)	124.6	7.05 (1H, *d* , 1.8)	124.6
3	-	149.1	-	149.1
4	7.73 (1H, *d*, 4)	120.8	7.63 (1H, *d*, 1.6)	120.8
4a	-	133.2		133.2
5	-	158.3		158.2
5a	-	112.5		112.5
6	7.32 (1H, *d*, 8.0)	129.5	7.23 (1H, *d*, 8.0)	129.5
7	7.32 (1H, *d*, 8.0)	129.6	7.23 (1H, *d*, 8.0)	129.6
8	-	157.6	-	157.6
8a	-	112.8	-	112.8
9	-	190.6	-	190.6
10	-	186.6	-	186.6
3-CH3	2.51 (3H, *s*)	22.3	2.43 (3H, *s*)	22.3
1-OH	12.17 (1H, *s*)	-	12.07 (1H, *s*)	-
5-OH	13.04 (1H, *s*)	-	12.94 (1H, *s*)	-
8-OH	12.36 (1H, *s*)	-	12.25 (1H, *s*)	-

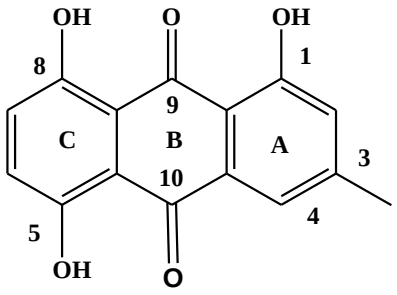


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Figure 4. The chemical structure of compound **1**.

4.3.2. Characterization of Compound 2

Compound **2** (4.3 mg) was isolated as a red crystal from 5% ethyl acetate in *n*-hexane. The ^{1}H NMR spectrum (Appendix **3A**) showed the presence of two aromatic methyl groups resonating at δ_H 2.33(3H, *s*, 3-CH3) and 2.53(3H, *s*, 3'-CH3), eight aromatic protons and four chelated hydroxyl groups at δ_H (12.00, 12.09, 12.45, and 12.53) which, confirmed that this compound is dimeric anthraquinone. In one half of the molecule, the ^{1}H NMR spectrum showed three mutually coupled aromatic protons in ABX spin system at δ_H 7.88 (1H, *dd, J*=8, 4 Hz), 7.73 (1H, *t, J=8* Hz), and 7.34 (1H, *d, J*=8 Hz) assigned for H-5, H-6 and H-7 respectively of the ring C and singlet proton resonated at δ_H 7.73 (1H, *s*, H-2) (Table **5)** with the biosynthetically expected methyl group at position C-3 of ring A, leaving C-4 for point of attachment to the other chrysophanol moiety.

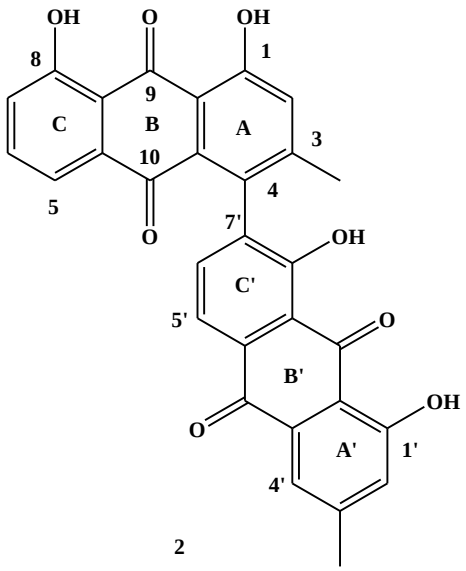

Figure 5 The chemical structure of compound **2**

Similarly, in the other half of the molecule, (ring A'B'C') the ^{1}H NMR spectrum displayed that a broad singlet proton at δ_H 7.16 (1H, *s*), δ_H 7.87 (1H, *s*) in ring A' were assigned to H-2' and H-4' respectively with biosynthetically expected methyl group being at C-3'. In ring C', two *ortho* coupled aromatic protons with AX spin system resonating δ_H 8.0 (1H, *d J*=8 Hz) and 7.65 (1H, *d, J*=8) for H-5' and H-6' respectively leaving C-7' for point of attachment to first chrysophanol moiety (ring ABC). Based on these spectroscopic evidence and comparison with litrature, the structure of compound **2** was found to be 4, 7′-bichrysophanol, trivial name asphodeline (**2**).

The compound is the common metabolites from the genus *Kniphofia* including *K.albescen, K. linearfolia, K.ensifolia, K. evansii, K. northae, K. foliosa K.tysonii, K.isoetifolia* [11, 21, 26, 42].

Table 5. ^{1}HNMR ($CDCl_3$, 400 MHz) data for compound **2** and the reported data in ^{1}HNMR (500 MHz, $CDCl_3$).

Position	δ_H (J in Hz)	δ_H (J in Hz) reported [21]
2	7.73(1H, *s*)	7.32(1H, *s*)
5	7.88 (1H, *dd, J*=8, 4 Hz)	7.62 (1H, *dd,J*=7.4, 1.2 Hz)
6	7.73 (1H, *t, J=8* Hz)	7.60 (1H, *t, J*=7.40 Hz)
7	7.34 (1H, *d, J*=8 Hz)	7.28 (1H, *dd, J*=7.4, 1.2 Hz)
2'	7.16 (1H, *s*)	7.14 (1H, *d,* 1.4 Hz)
4'	7.87 (1H, *s*)	7.73 (1H, *d, J*=1.4 Hz)
5'	8.0 (1H, *d, J*=8)	7.98 (1H, *d, J*=8 Hz)
6'	7.65 (1H, *d, J*=8)	7.40 (1H, *d, J*=8 Hz)
3-CH_3	2.33 (3H, *s*)	2.17 (3H, *s*)
3′-CH_3	2.53 (3H, *s*)	2.52 (3H, *s*)
1-OH	12.53 (1H, *s*)	12.60 (1H, *s*)
1′-OH	12.45 (1H, *s*)	12.40 (1H, *s*)
8-OH	12.09 (1H, *s*)	12.05 (1H, *s*)
8'-OH	12.00 (1H, *s*)	12.03 (1H, *s*)

4.3.3. Characterization of Compound 3

Compound **3** (3 mg) was isolated as red crystal. The ^{1}H NMR data of **3** (appendix **4A**) confirmed that this compound is also dimeric anthraquinone. However, its spectral data was similar to compound **2** with one additional aromatic proton due to the difference position of dimerization. Two aromatic methyl signals at δ_H 2.28 (3H, *s*, 3-CH_3) and 2.47(3H, *s*, 3'-CH_3) were observed for the two aromatic methyl groups and four chelated hydroxyl groups at δ_H (12.48, 12.38, 12.18, and 11.81). Three mutually coupled aromatic protons in ABX spin system at δ_H 6.80 (1H, *d, J*=4 Hz, H-5), δ_H 7.43 (1H, *t, J*=8 Hz H-6) and δ_H 6.98 (1H, *d, J*=8 Hz, H-7) were observed**.** Two *meta* coupled aromatic protons at δ_H 6.83 (1H, *d, J*=4, H-2) and 6.63 (1H, *brs,* H-4) were observed.

The remaining spectral data is for the other half of the molecule ring A′B′C′ system of **3** is similar to that of **2**, two *meta* coupled proton signals at δ_H 7.06 (1H, *d*, *J*=4 Hz, H-2') and δ_H 7.66 (1H, *d*, *J*=4 Hz, H-4'), two *ortho* coupled aromatic protons with AX spin system at δ_H 8.03 (1H, *d*, *J*=8 Hz, H-5') and δ_H 8.67 (1H, *d*, *J*=8 Hz, H-6') were observed.

Unlike the ABX spin system of the first chrysophanol moiety of H-5, H-6 and H-7, the second chrysophanol moiety showed an AX spin system for H-5′ and H-6′ and leaves H-7′ for point of attachment to the first chrysophanol moiety. Highly de-shielded H-4′ (δ_H 7.66) and H-5′ (δ_H 8.03) (ring A'B'C') in contrast to H-4 (δ_H 7.06) and H-5 (δ_H 6.80) (ring ABC) indicates, the second moiety has carbonyl group located at C-10'. Therefore, compound **3** was characterized and identified as 10-Hydroxy-10, 7'-(chrysophanol anthrone)-chrysophanol (**3**) which was previously reported from other *Kniphofia* species from *K. isoetifolia* [11], *K. ensifolia* [26] from *K. foliosa* and from *K. thomsonii* [21, 28, 42-43].

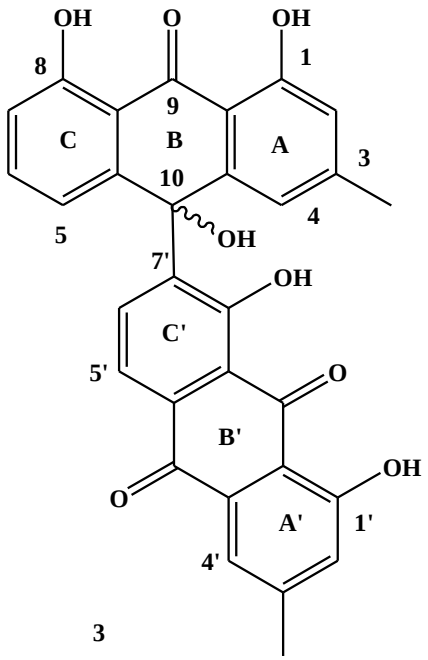

Figure 6 The chemical structure of compound **3.**

Table 6. ^{1}H NMR (400 MHz, CDCl3) data of compound **3** and the reported data in ^{1}H (500 MHz, acetone-*d6*).

Position	δH (m, *J* in Hz)	Reported δH (*m*, *J* in Hz) [21]
2	6.83 (1H, *d*, *J*=4)	6.79 (1H, *d*, *J*=1.2)
4	6.63 (1H, *brs*)	6.64 (1H, *d*, *J*=1.2)
5	6.80 (1H, *d*, *J*=4)	6.76 (1H, *d*, *J*=8.4)
6	7.43 (1H, *t*, *J*=8)	7.51 (1H, *t*, *J*=8.4)
7	6.98 (1H, *d*, *J*=8)	6.92 (1H, *d*, *J*=8.4)
2′	7.06 (1H, *d* ,*J* =4)	7.04 (1H, *d*, *J*=1.4)
4′	7.66 (1H, *d*, *J=4*)	7.37 (1H, *d*, *J*=1.4)
5′	8.03(1H, *d*, *J*=8)	7.85 (1H, *d*, *J*=8.2)
6′	8.67(1H, *d*, *J*=8)	8.66 (1H, *d*, *J*=8.2)
3-CH_3	2.28 (3H, *s*)	2.23 (3H, *s*)
3′-CH_3	2.47 (3H, *s*)	2.32 (3H ,*s*)
1-OH	11.81 (1H, *s*)	11.62 (1H, *s*)
1′-OH	12.48 (1H, *s*)	12.26 (1H, *s*)
8-OH	12.38 (1H, s)	12.18 (1H, *s*)
8′-OH	12.18 (1H, *s*)	12.06 (1H, *s*)

4.4. Evaluation of Antimicrobial Activities of isolated compounds.

The disk diffusion method was used and the zone of growth inhibition of isolates was measured in millimeter (mm) compared to the standard positive controls Gentamycin, Chltrimazole and negative control DMSO. The antimicrobial activity of 100 $mgml^{-1}$ of extracts and isolated compounds 50 $mgml^{-1}$ of compound **1** and 25 $mgml^{-1}$ compound **2** were evaluated by measuring the zone of growth inhibition surrounding the discs in millimeter by ruler and it has been presented below in (Table 7 and appendix **5A)**.

Table 7. Zone of inhibition of the extracts, compound **1** and compound **2** (in mm).

Test organism	Hex extract	Chl extract	Ac extract	Meth extract	1	2	Gent	DMSO	Chlotrimazole
E. coli	16	22	18	16	14	13	32	NI	-
P. aeruginosa	12	15	14	12	15	12	22	NI	-
S. aureus	13	13	15	14	12	14	33	NI	-
B. subtilis	13	16	18	15	13	11	31	NI	-
Fusarium spp	8	12	18	14	12	13	-	NI	20

*Key: Hex= hexane, Chl = chloroform, Ac = Acetone, Meth = Methanol**,** Gent = Gentamicin and NI = no inhibition, -=not applied.*

The crude extracts showed considerable activity on both Gram-positive and Gram-negative bacterial strains with zone of inhibition ranging from 12-22 mm, with the highest activity (22 mm) observed for chloroform extract against *E. coli* (Table 7). However, the inhibitions displayed on both Gram-negative and Gram-positive bacteria for the isolated compounds (**1** and **2**) that have been tested were good with variable degree of potency between the tested compounds. The better activity of the crude extracts over the isolated compound could be accounted to the synergistic interactions of several compounds present in the extract, which cannot be the case when single compounds are evaluated. Alone. Acetone extract showed the highest inhibition (18 mm) against the fungal strain, *Fusarium spp* and lowest by hexane extract (8 mm), whereas, compound **2** showed higher inhibition (13 mm) as compared to compound **1** against the same strain (Table 7). Hexane extract showed the lowest activity against all the tested strain, compared to positive control (gentamycin and Chlotrimazole), other extracts**.** The low activity of hexane extract could be related to the absence of polar bio active secondary metabolites, since hexane interact only with nonpolar organic molecules. Compound **2** showed moderate activities against bacterial strain which showed less inhibition zone as compared from reported [11].

4.5. Summary of Spectroscopic Data for Isolated Compounds

Helminthosporin (**1**) (32 mg): red amorphous solid. IR, 3426 (OH), 2917 (ArC-H), 1616 (C=O) and 1268 (C-O) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$), δH, 13.04 (1H, *s*, 5-OH), 12.36 (1H, *s*, 8-OH), 12.17 (1H, *s*, 1-OH), 7.14 (1H, *s*, H-2), 7.73 (1H, *d*, *J* = 4 Hz, H-4), 7.32 (1H, *d*, *J* = 8.0 Hz, H-6) and 7.32 (1H, *d*, *J* = 8.0 Hz, H-7), 2.51(3H, *s*, 3-CH_3)S. ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta^{13}C$ 190.6 (C-9), 186.6 (C-10), 162.9 (C-1), 158.3 (C-5), 157.6 (C-8), 149.1 (C-3), 133.2 (C-4a), 129.6 (C-7), 129.5 (C-6), 124.6 (C-2), 120.8 (C-4), 114.0 (C-1a), 112.8 (C-8a), 112.5 (C-5a) and 22.3 (3-CH3).

Asphodeline (2): red crystal (4.3 mg). ^{1}H NMR δH (400 MHz, $CDCl_3$) 2.33(3H, s, 3'-CH_3), 2.52 (3H, *s*, 3-CH_3) 7.16 (1H, *s*, H-2'), 7.34 (1H, *d*, *J*=8, Hz, H-7), 7.65(1H, *d*, *J*=8, H-6'), 7.73 (2H, *t*, *J=8* Hz, H-6, H-2)), 7.87 (1H, *s*, H-4'), 7.88 (1H, *dd*, *J*=8, 4 Hz, H-5), 8.00(1H, *d*, *J*=8 Hz, H-5'), 12.00(1H, *s*, 8'-OH),12.09(1H, *s*, 8-OH), 12.45(1H, *s*, 1'-OH) and 12.53(1H, *s*, 1-OH).

10-Hydroxy-10, 7'-(chrysophanol anthrone)-chrysophanol (3): red crystal (3 mg). ^{1}H NMR δH(400 MHz, $CDCl_3$) 2.28 (3H, *s*, 3-CH_3), 2.47 (3H, *s*, 3'-CH_3) ,6.63 (1H, *brs*, H-4), 6.83 (1H, *d*, *J*=4 Hz, H-2), 6.98 (1H, *d*, *J*=8 Hz, H-7), 7.06 (1H, *d*, *J*=4 Hz, H-2'),7.43(1H, *t*, *J*=8 Hz H-6), 7.66 (1H, *d*, *J*=4 H-4'), 8.03 (1H, *d*, *J*=8 Hz, H-5'), 8.67 (1H, *d*, *J*=8 Hz, H-6'), 11.81 (1H, *s*, 1-OH), 12.18(1H, *s*, 8'-OH), 12.38(1H, *s*, 8-OH) and 12.48(1H, *s*, 1'-OH).

5. Conclusion and Recommendation

5.1. Conclusion

The main objective of this study was phytochemical investigation of roots of *Kniphofia insignis* and its antimicrobial activities. The sequential extraction of roots of *Kniphofia insignis* yielded 10 g, 27.5 g, 17 g and 45 g of *n*-hexane, chloroform, acetone and methanol crude extract respectively and elution of chloroform extract gave three compounds namely, 3-methyl-1,5,8-trihydroxyanthracene-9,10-dione (**1**), asphodeline (**2**) and 10-Hydroxy-10,7'-(chrysophanol anthrone)-chrysophanol (**3**).

The antimicrobial activity investigation revealed that the crude extracts and isolated compounds of *Kniphofia insignis* roots showed marginal antimicrobial activities which explains its use as a traditional medicine. The antimicrobial analysis showed that the chloroform extract showed the highest inhibition against *E. coli* which could be accounted to the synergistic interactions of several compounds present in it.

The compounds could be of pharmaceutical interest for therapeutical application as complementary antimicrobial agents for infectious disease, supported the scientific validity of the plant being used traditionally as a medicine in the treatment of microbial infections.

5.2. Recommendation

- Further work is recommended on crude extracts and bioactive compounds to investigate its structures and indicate their exact potential to inhibit several microbes and to develop a novel antimicrobial herbal formulation.
- The present study use only chloroform extract for isolation of pure compounds, Therefore, further isolation and characterization of bioactive compounds from the hexane, acetone and methanol extracts is recommended.
- Further phytochemical investigation on *Kniphofia insignis* is recommended to isolate and characterize more novel compounds with biological activities.
- It is also recommended for antiplasmodial activity of the crude extracts and isolated compounds to be tested because of the chemical structure and traditional claim of the plant.

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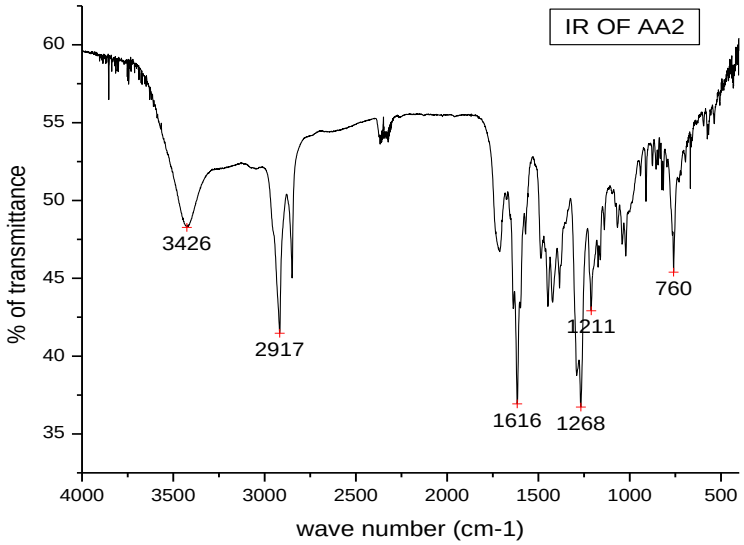
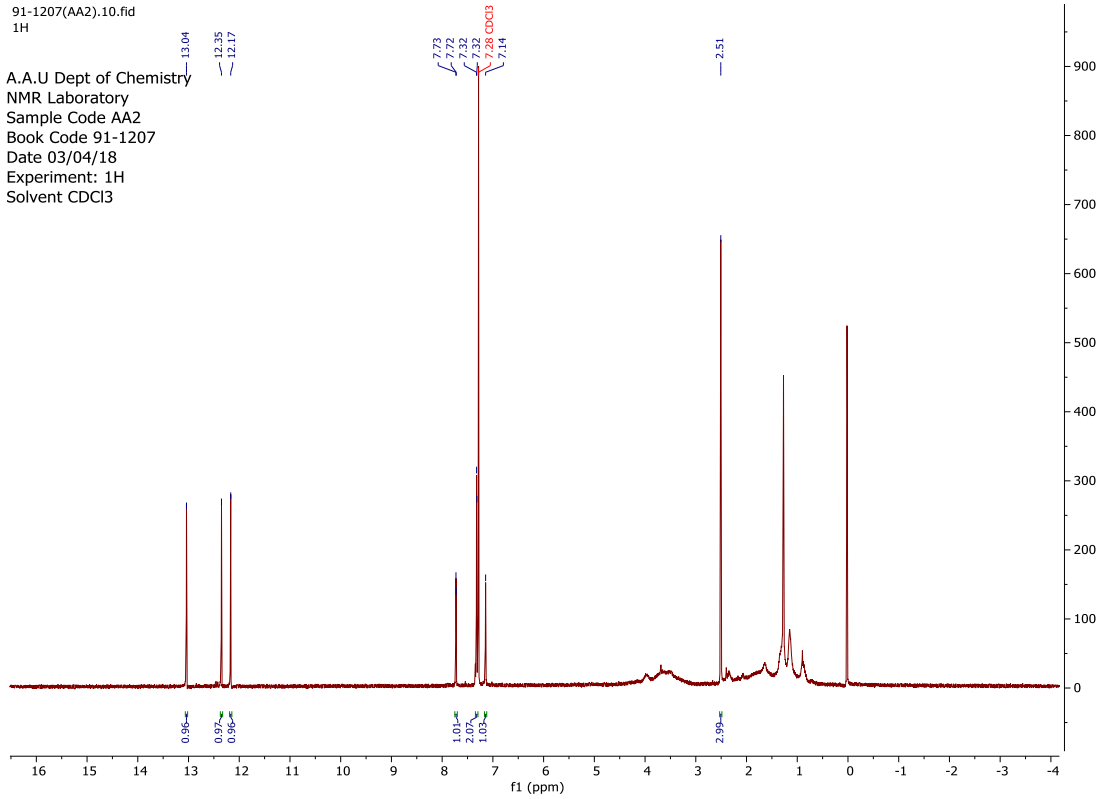
Appendix

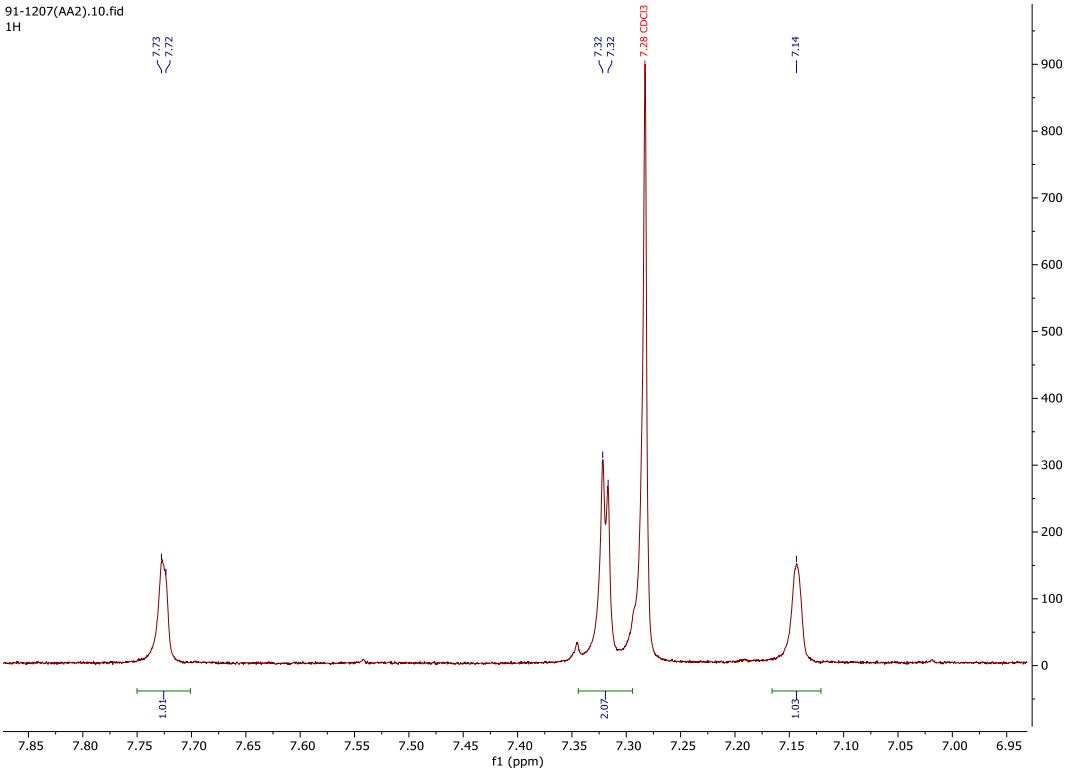
Appendix 1A. IR spectra of compound **1**



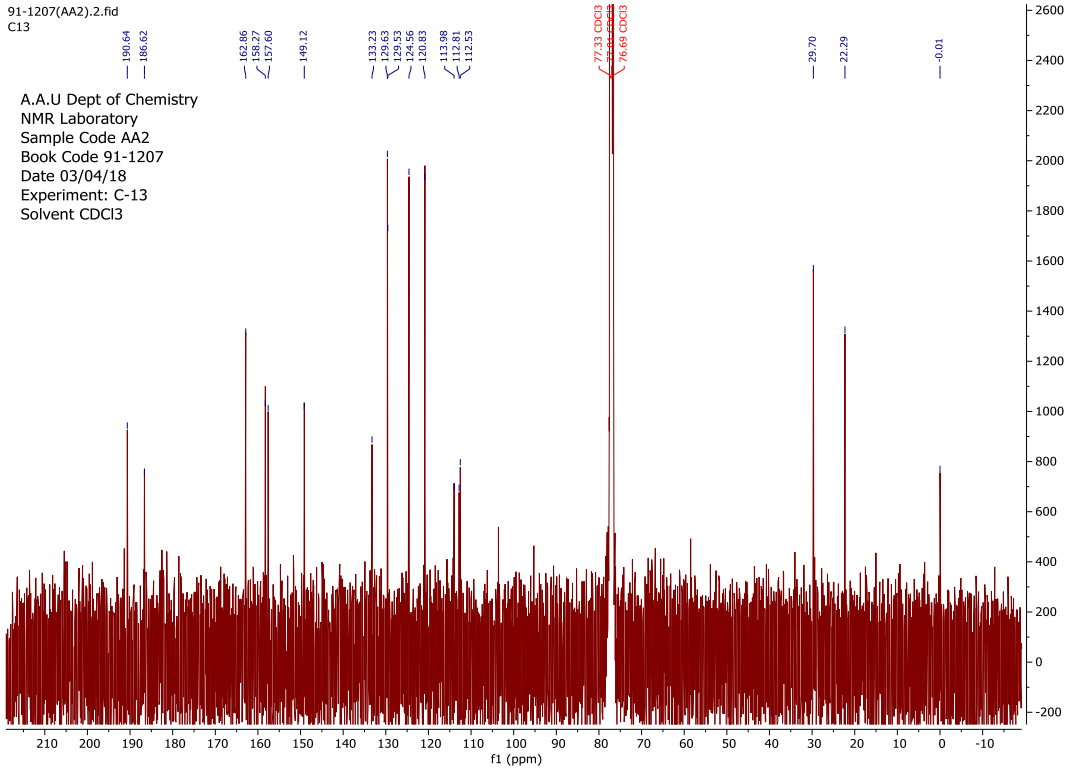
Appendix 2A. ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **1**.



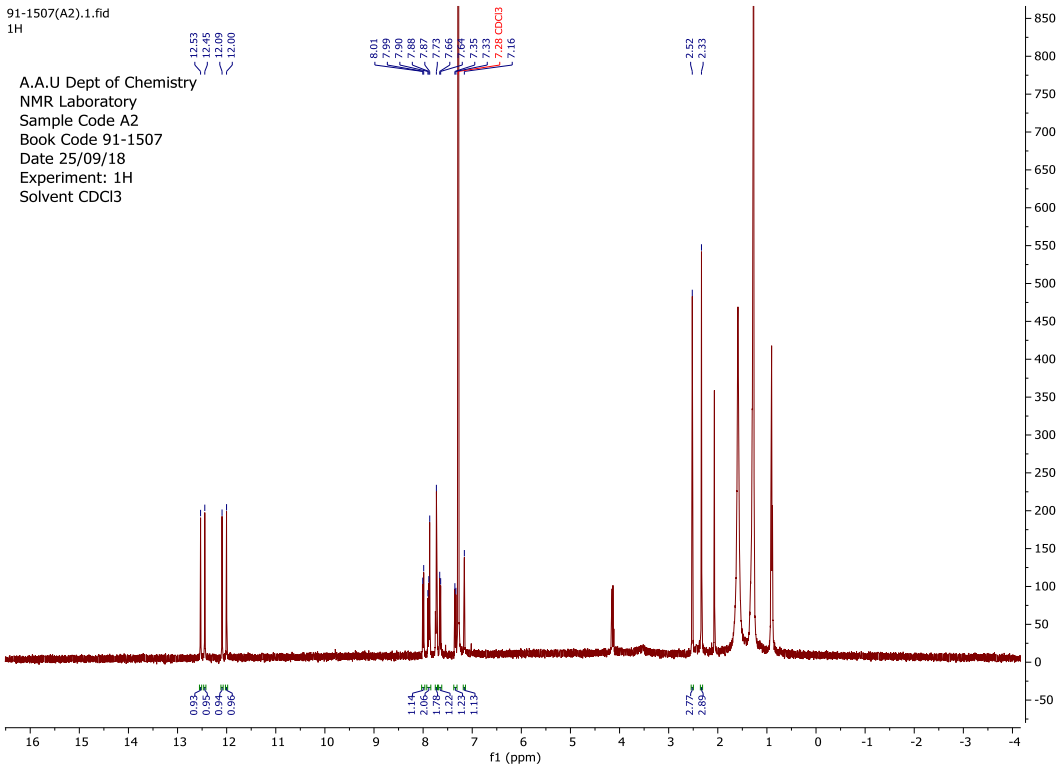
Appendix 2B. Expansion of ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **1**.



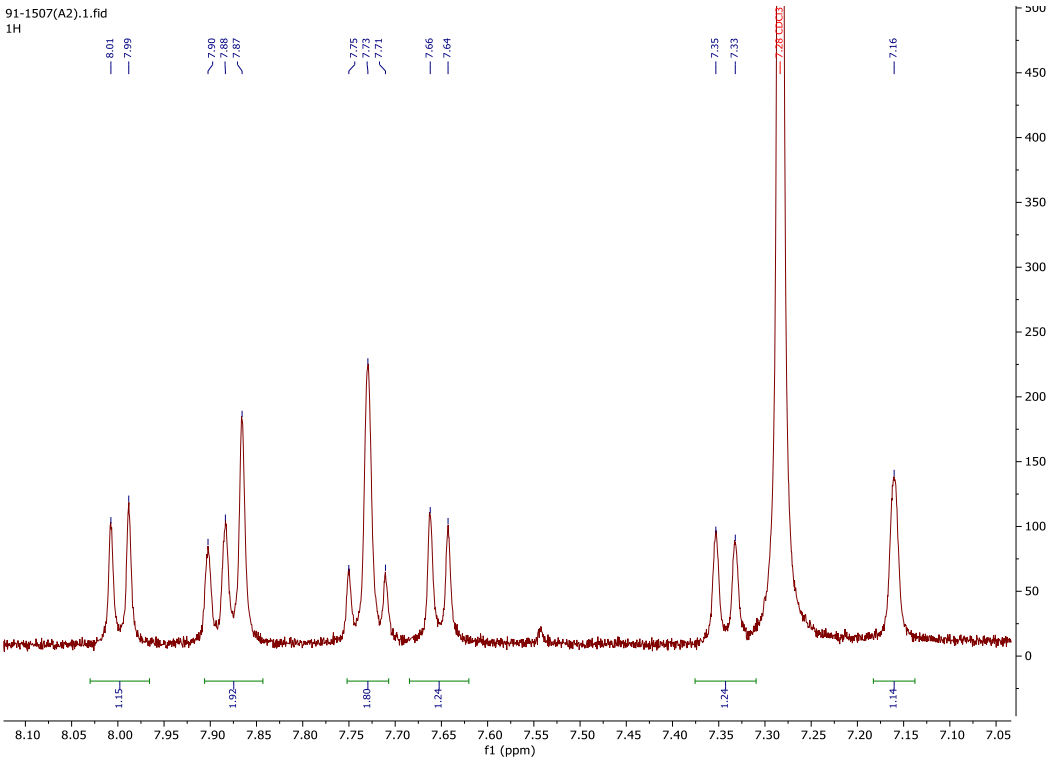
Appendix 2C. ^{13}C NMR (400 MHz, $CDCl_3$) spectra of compound **1**.



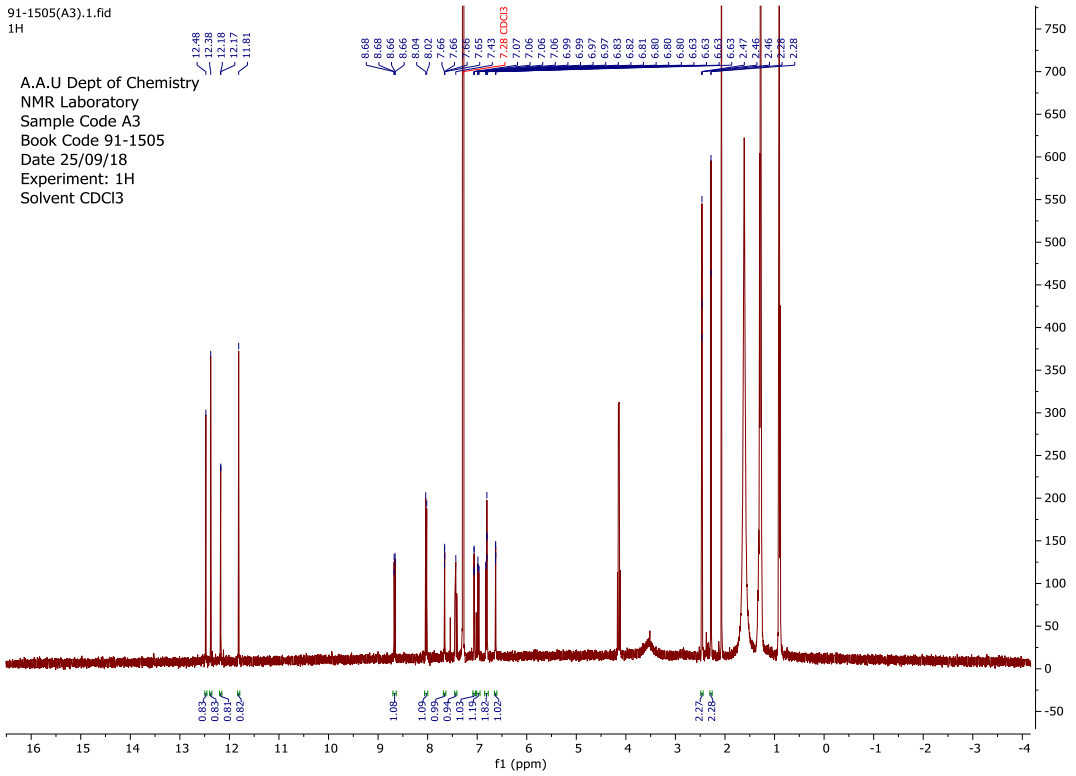
Appendix 3A. ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **2**.



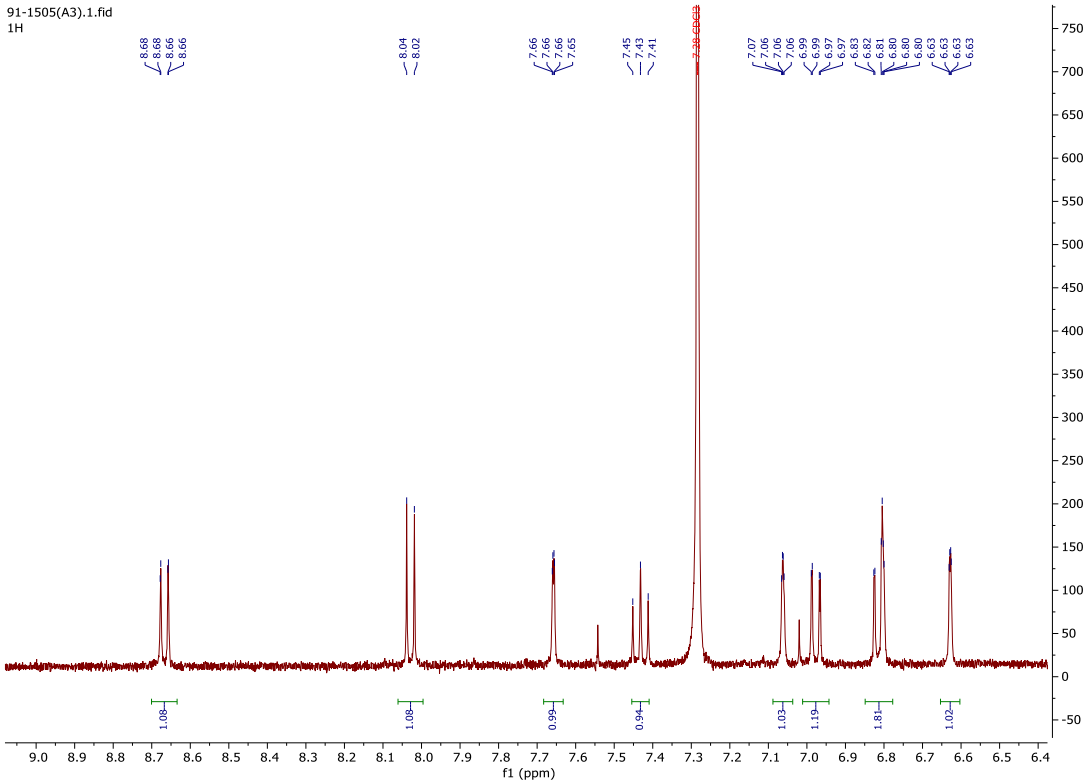
Appendix 3B. Expansion of ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **2**.



Appendix 4A. ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **3**.



Appendix 4B. Expansion of ^{1}H NMR (400 MHz, $CDCl_3$) spectra of compound **3.**



Appendix 5A. Bioactivity result of extracts and isolated compounds.