PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITIES OF EXTRACT OF LEAF OF *WITHANIA SOMNIFERA*

MSC THESIS

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Phytochemical Investigation and Antimicrobial Activities of Extract of Leaf of *Withania somnifera*

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POSTGRADUATE PROGRAM DIRECTORATE

As thesis research advisor, I there by certify that I have read and evaluated this thesis prepared under my guidance, by Dagnachew Tatek entitled: *"***Phytochemical Investigation and Antimicrobial Activities of extract of leaf of *Withania somnifera"***. I recommend it to be submitted as fulfilling the thesis requirement.

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As a member of the Board of Examiners of the MSc thesis open defense examination, we certify that we have read and evaluated the thesis prepared by Dagnachew Tatek and examined the candidate. We recommend that the thesis be accepted as fulfilling the thesis requirements for the Degree of **Master of Science in Chemistry.**

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DEDICATION

This thesis manuscript is dedicated to all my family and my beloved father who departed from this world when I was attending this program.

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ACRONYMS AND ABBREVIATIONS

CC	Column Chromatography
GC-MS	Gas Chromatography-Mass spectrometry
NIST	National Institute of Standard and Technology
PDA	Potato Dextrose Agar
RT	Retention Time
SD	Standard Deviation
WHO	World Health Organization
WS	Withania somnifera

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES IN THE APPENDIX

LIST OF TABLE IN THE APPENDIX

Appendix Table **Page**

Phytochemical Investigation and Antimicrobial Activities of Extract of Leaf of *Withania somnifera*

ABSTRACT

Withania somnifera, locally known as 'gezawa' is traditionally used for the treatment of various human ailments (the leaf part) including gastric, ulcers, colds and skin rashes in Ethiopia. In the present study, W.somnifera (Solaneceae) leaf extracts were investigated for their phytochemicals and antimicrobial activities. The qualitative analysis of n-hexane, chloroform: methanol (1:1), and methanol leaf extracts of the plant revealed the presence of flavonoids, terpenoids and phenols. The n-hexane extract of the leaf of the plant was esterified in order to convert fatty acid to methyl ester fatty acid. The esterified oil was characterized by GC-MS and identified twenty three compounds. From this there twelve are major compounds. These are benzyl nitrile (1.21%), dodecanioc acid (1.23%), decanoic acid (1.54%), hexadecanoic acid (1.80%), methyl tetra decanoate (2.75%), 7,10,13-hexadeca trienoic acid (3.15%), methyl 18-methyl nonadecanoate (3.69%), methyl stearate (4.10%), bis (2-ethylhexyl) phthalate (7.56%), penta decanoic acid, 13-methyl (13.44%), E,Z, 1,3,12-nonadecatriene (26.885) and 9-octadecenoic acid (Z) (27.095%). The crude extracts were tested against four bacteria species (two Gram positive bacteria, Staphylococcus aureus and Streptococcus agalactia; and two Gram negative bacteria, Escherichia coli and Salmonella typhi) and two fungal species (Aspergillus niger and Fusarium oxysporum) using paper disc diffusion method. The maximum antibacterial activity was shown in chloroform: methanol (1: 1) crude extract against salmonella typhi and E. coli (inhibition diameter 21.50 mm and 21.00 mm respectively). Whereas maximum antifungal activity was observed by chloroform: methanol (1: 1) crude extract against A. niger (23.50 mm) than Fusarium (18.60 mm). Therefore *chloroform: methanol extract was significant in antimicrobial activities. Thus the present study supported the traditional claims of the plant.*

Key words: Antimicrobial, Crude extracts, Disc diffusion method, Phytochemicals, *Withania somnifera*.

1. INTRODUCTION

Traditional medicine is defined by the WHO as "the sum total of all knowledge and practice, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or imbalances, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" (WHO, Geneva, 2001). Experts agree that this definition applies to the practice of traditional medicine in Ethiopia. In common with its implementation in many other regions of the developing world, Ethiopian traditional medicine incorporates various "specialties", including spiritual healing, disease prevention measures, surgery, midwifery, water therapy and herbal therapy. Up to 80% of the Ethiopian population has been reported to rely on traditional medicine as a major provider of health care (Kassaye, 2006).

The different forms of specialized areas, herbal therapy appears to play important role in Ethiopian traditional medicine. Ethiopia is considered the home the most diverse plant species in Africa that serve as of many traditional medicinal plants. For instance *Withania somnifera* is a shrub in the *Solanaceae* family. It is common in open grass lands and it is widely found in Ethiopia, where it is more commonly known by the local name '*gezawa'* (Teklehaymanot *et al.,* 2007). As a traditional medicinal plant, *Withania somnifera* is used in Ethiopia as a remedy for antimicrobial activities.

Withania somnifera is a well known Indian medicinal plant widely used in the treatment of many clinical conditions. It is an important drug which has been used either single or in combination with other drugs in Unani as well as Ayurvedic system of medicine for centuries. In Unani system of medicine, roots of *Withania somnifera* used for the medicinal activities. However, leaves of the plant are also reported to be used medicinally (Anonymous, 1982). The fresh roots are collected during January to March and dried under shade for several days. The drug retains its therapeutic efficacy for less than 2 years. It is prone to decomposition and loses its potentials within 2 years. So the fresh dried roots are preferred for medicinal uses.

Further, the compounds, *withaferin* A and *withanolides* isolated from *Withania somnifera* have also been shown to possess tumor inhibitory effect (Worku, 2016). Medicinal plants have regained a wide recognition due to an escalating faith in herbal medicine in the last few decades

contributed by its lesser side effects compared to allopathic medicine. *Withania somnifera* is the most important herbs in Ayurvedic indigenous medical systems for over 3000 years and is commonly used in Indian traditional health care systems. It is a perennial plant belonging to the order *Solanaceae.* Biogenesis of *withanolides* appears to be highly restricted to a few genera of *Solanaceae* and *Withania somnifera* produces the largest number of *withanolides* contains highly diversified functional group and stereo forms of the C_{22} and C_{26} δ lactonizedergostane skeleton (Chaurasiya *et al*., 2008). Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants which provide more health benefits to humans than those attributed to macronutrients and micronutrients. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are known as phytochemicals. They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. Phytochemicals accumulate in different parts of the plants, such as in the roots, stems and leaves. These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation, modulation of hormone metabolism and anti-cancer properties (Saidulu *et al*., 2014).

The most important medicinal properties of *Withania somnifera* have been attributed to the presence of unique classes of steroidal lactones called *withanolides*. These are ergostane based phytochemicals of triterpenoidal metabolic ancestry and include pharmaceutically active molecules like *withaferin* A, *withanolides* D, *withanolide* A and *withanone etc.* The plant selected for this study is endemic to northern Africa, specially the north part of Ethiopia and important in traditional medicine for the treatment of various disease, such as diarrhea, cough, common cold, and general muscular pain, internal wound, loss of appetite malaria, syphilis, gonorrhea, stomachache, toothache, asthma, throat and chest infection. There are some reports on methanol extracts further isolated active compounds *WS*-1 and tested for antimicrobial activity of the root part of *Withania somnifera* (Prakash *et al*., 2011). However, to the best of our knowledge, there are no reports on the chemical composition and antimicrobial activities of the leaf extract of this plant in Ethiopia. Therefore, this work was intended to fill this gap.

1.1. Objectives of the Study

1.1.1. General Objective

- To study the chemical composition, phytochemical constituents and antimicrobial activities of crude extracts of the leaf of *Withania somnifera*.

1.1.2. Specific Objectives

- To extract the leaves of *Withania somnifera* by n-hexane, chloroform/methanol (1:1) and methanol using the Soxhlet extraction method.
- To determine the chemical composition of esterified crude oil extract of n-hexane by using Gas Chromatography – Mass Spectrometry (GC-MS).
- To screen for phytochemical constituents of crude extracts of leaf with hexane, chlorofo rm: methanol (1:1) and methanol.
- To evaluate antimicrobial activities of crude extracts of leaf of *Withania somnifera* against selected bacterial and fungal species using the disc diffusion method.

2. LITERATURE REVIEW

2.1. Overview of Traditional Medicine in Ethiopia

Ethiopia is one of the oldest nations of the world and has a rich history of traditional medicine and indigenous knowledge practices. Ethiopian traditional remedies are originated from locally grown plants, animal products and minerals. Other traditional treatments also include a variety of medical practices such as purging, bleeding and cupping, steam baths and immersion in hot, often thermal, water, and counter-irritation. The knowledge on traditional medicine were mainly orally based, the information on healing practice were passed down by practicing healers from generation to generation, often with considerable secrecy. Hence, the antiquity of Ethiopian Indigenous or traditional medicine could not be established with any certainty due to the lack of adequate written documents. The cultural and indigenous knowledge of medicinal plants in Ethiopia is unevenly distributed among each community members. Peoples in different geographical location with different religious, linguistic and cultural backgrounds have their own specific knowledge which in part has gradually entered wide circulation in the country. In Ethiopian traditional health care system, traditional health practitioners are categorized as herbalist-healers (*Kitelbetash*), spiritual or faith based healers, bone settlers (*waggesha*). The various literature available show the significant role of medicinal plant in primary health care delivery in Ethiopia where 80% of human and 90% of livestock population depend on traditional medicine similar to many developing countries particularly that of Sub-Saharan African countries (Asfaw *et al*., 2015).

Traditional medicine plays an important role in the healthcare of the majority of the people in developing countries, including Ethiopia, and medicinal plants serve as valuable sources of natural therapeutic agents. There have been recent efforts to assess the use of Ethiopian traditional medicinal plants for treatment of various diseases including cancer. He reported that 30 species of plants were used for treating human cancer, with most of them belonging to different plant families. In addition to cancer, a large majority of the plants were also used against various types of other diseases. For most of the plants reported (73%), there was some kind of independent experimental/clinical evidence supporting their claimed anticancer activity (Worku, 2016).

Ethiopia is one of the six centers of biodiversity in the world with several topographies, climatic conditions and various ethnic cultures. Ethno botanical study is a real and encourageable in rich biological resource areas for medicinal plant identification, documentation, ranking, conservation and sustainable usages. The study revealed a total of 49 medicinal plant species (belonging to 31 families and 46 genera) used to treat various human ailments, the majority of which 40 (81.6%) species were collected from wild while the rests from home garden. Herbs constituted the largest growth habit (18 species, 37%) followed by trees (16 species, 32%) and shrubs (15 species, 31%). Leaf `17 (35%) is the plant part widely used followed by root 13 (27%), leafy-stem 5 (10%), and seed 6 (12%). Oral administration was the dominant route (63%), followed by dermal route (22%) and nasal (11%) (Balcha, 2014).

2.2. *Withania somnifera* Species

Withania somnifera also known as '*Gezawa*' in Ethiopia belongs to *Solanaceae* family. Yadav *et al.* (2016) reported that a detail review on *Solanaceae* family. This family is called night shades family which is a family of flowering plants. The most economically important genus of the family is *solanum.* Most members of *solanaceae* are erect or climbing, annual or perennial herbs. There are few trees like *Solanum, Lycianthes*, *Cestrum, Nolana, Physalis*, *Lyceum, Nicotiana*, brubfelsia contain more than 60% of the species. From these some are toxic and some cases used as staple foods. Others also provide as medicinal and ornamental values. The Genus *Withania* is one of the sharp plants classified in the family *Solenaceae* and the species name *Somnifera* means sleep bearing in lain indicating its potent action as a sedative (*Bilal et al.,* 2012*).* The *Solanacea* family is comprised of 84 genera that include about 3,000 species, scattered throughout the world.

Withania somnifera is a small shrub to 2 m high and to 1m across. Almost the whole plant is covered with short, fine, silver-grey, branched hairs. The stems are brownish and prostrate to erect, sometimes leafless below. The leaves are alternate (opposite on flowering shoots), simple, margins entire to slightly wavy, broadly ovate, obovate or oblong, 30–80 mm long and 20–50 mm broad, narrowed into 5–20 mm long petioles, almost hairless and green above, densely hairy below. In Ethiopia it is found in Amhara (Gojjam), in Tigray, Oromia (Shewa) and southern Ethiopia Regions *(*Hepper, 1991). Table 1 shows description of the plant.

Table 1. Description of *withania somnifera*

Family	Species	Genu
Solanaceae	*Somnifera*	*Withania*

2.3. Botanical Description

W. somnifera (shown in Figure 1) is an erect, greyish, slightly hairy evergreen shrub that grows to about 1.5 m in height and has fairly long tuberous roots. The small and greenish-yellow flowers can be single or in clusters. The fruit is smooth, round, and fleshy, with many seeds; it is orange-red when ripe and enclosed in a membranous covering (Evans, 2009).

Figure 1. Image of *Withania somnifera* plant

2.4. Geographical Distribution

Withania somnifera is the most common and widespread species in the genus and occurs naturally, mainly in the drier regions, from the Mediterranean through tropical Africa to South Africa and from the Canary and Cape Verde Islands to the Middle East and Arabia, India, Sri Lanka and southern China. It is cultivated in gardens in the warmer parts of Europe and has become a naturalized weed in South Australia and New South Wales. It is grown in India and elsewhere as a medicinal crop plant, mainly for its fleshy roots.

Withania somnifera is widespread but not common in all provinces of South Africa and also Namibia, Botswana, Swaziland and Lesotho. It is, however, absent from the western halves of the Northern and Western Cape Provinces. It grows in a large number of vegetation types in dry areas to areas with a fairly high rainfall such as coastal vegetation, grassland (also on termite mounds), savanna, scrubland, woodland, often in margins of forests and thickets, also near water, such as on river banks. It is found in light shade as well as full sun, often among rocks where the roots are kept cool. Unfortunately it can become a weed in disturbed areas, cultivated lands and overgrazed pasture. In southern Africa this plant grows at altitudes of 15 - 2300 m (Schmelzer and gurib-Fakim, 2008).

The scientific name of the plant is *Withania somnifera*, (synonym *Physalis somnifera* L.). It belongs to the Family Solanaceae [Evans, 2009]. The common names of *Withania somnifera* are Withania , aswaganda , winter cherry , Indian ginseng. This plant has different local names in Ethiopia. . Some common name of *Withania somnifera* shown below in table 2.

Table 2. Common Names of *Withania somnifera*

Language	*Vernacular name*
English	*Winter cherry*
Oromigna	*Kumo*
Amharic	*Gezawa*

2.5. Methods of Extraction of Medicinal Plants

2.5.1. Maceration

2.5.2. Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent in flask A is heated, and its vapors condense in condenser. The condensed extracting drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube, the liquid contents of chamber siphon into flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effect tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

2.5.3. Solvent Extraction

Most flowers contain too little volatile oil to undergo cold pressing, but their chemical components are too delicate and easily denatured by high heat used in steam distillation instead solvent extraction is used to extract the oil (Wilson and Robert, 2016).

2.6. Phytochemistry

The principal bioactive compounds of *W. somnifera* reported in literature are withanolides, which are triterpene lactones. More than 40 withanolides and approximately 12 alkaloids and several sitoindosides have been isolated and identified from *W. somnifera*. The chemical constituents for the roots, fruits, seeds, and stem include withanone; withaferin A; withanolides A, D, and G; and sitoindosides VII, VIII, IX and X (Ganzera *et al.,* 2003; Kulkarni *et al.,* 2008).

2.7. Phyto Isolates from *Withania somnifera* and their Antimicrobial Activity

The local people use this plant species as source of fuel, for cooking and heating during winter as they lack the natural gas facility. It is also widely used as a fodder plant (Bano *et al.,* 2013).
Umadevi *et al.* (2012) reported that the traditional and medicinal uses of *Withania somnifera*. *Withania somnifera* has long been considering as an excellent general health tonic and cure for a number of health complain. It is sedative, diuretic, anti-inflammatory and an anti-stress agent. *Withania somnifera* is taken for treating cold and coughs, ulcers emaciation, diabetes, conjunctivitis, epilepsy, insomnia, eniledementia, *etc*. According to World Herbal system *Withania somnifera* is considered as one of the most important herbs and the best adaptogenic. This is because of the present of bioactive compounds like ahygrine, tropine, anaferine, glycosides, *withenolides* with starches and amino acids which are stimulates the immune system, combats inflammatory, increases memory and helps maintaining general health and wellness.

Parvinder *et al.* (2001) extracts roots with water and further isolated *withaferin* A **(1)**. And the isolated compound was tested for anti-stress activity. They also isolated *Sitoindosid*-Ix-H **(2)** and *Sitoindiside*-x-Palmitoyl.

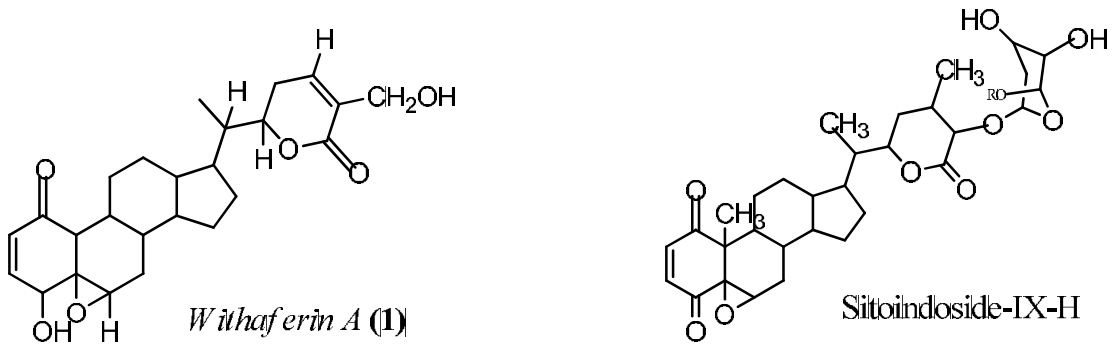


Figure 2. Structure of *Withaniferin* A and *Sitoindoside*-Ix-H

Yadav *et al.* (2010) reported that extracts of the root, stem and leafs of *Withania somnifera* and *in vitro* anticancer activity against various human cancer cell lines. The report showed that ethanolic extract of the leaf is more significant than ethanolic extract of stem and root against anticancer activity in fly ash amended soil.

Budhiraja *et al.* (2000) reported that *withanolides* are isolated from *solanaceous* plants, mainly *withaferin*-A **(3)** and *withanolide* D (4) E are useful biological effects such as anti-cancer, radio sensitizing, antibacterial, adaptogenic, antioxidant, anti inflammatory activity. However none of the *withanolide* has so far been evaluated for pharmacokinetics and pharmacodynamic pr operties.

Figure 3. *Withanolides* isolated from *Withania somnifera* plant

Satish *et al.* (2012) evaluated the immunomodulatory activity of *Withania somnifera*. Experimen ts were conducted in *vivo* in Swiss mice. *Withania somnifera* ethanolic extract was found to enhance immune response as to compare with standard drug. So ethanolic extract of the plant is significant for immune modulatory activity.

Prakash *et al.* (2011*)* reported that extraction of roots of *Withania somnifera* with methanol and further isolated active compounds *WS*-1**(5)** and tested for antimicrobial activity. The initially extracted methanol fraction (150 g) was re-extracted successively by hexane, chloroform and methanol to get the final extracts in the amount of 4g, 3.5g and 100 g respectively.

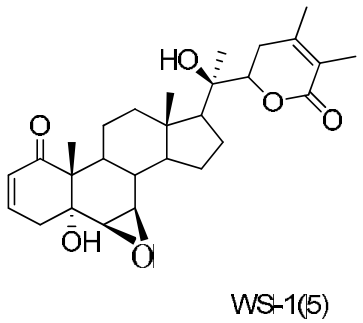


Figure 4. Structure of *Withania somnifera*-1

Ipseeta *et al.* (2003) myocardial infarction is the most lethal manifestation of cardiovascular diseases and it is one of the most important subjects of intense investigation by scientists. Now a days there is an increased realization that herbs can maintain the balance of body and can influence heart diseases and its treatment by providing nutritional substances. Although the therapeutic properties of this plant like immunomodulatory, adaptogenic, antioxidant, hypoglycemic and anti-cancerous are well known, but very few studies are available, which assess its cardio protective potential.

Geeta, (2014) reported that most susceptible microorganisms in the present study were *R. planticola* and *A. tumefaciens*, which had shown susceptibility for almost all the extracts tested. Antibacterial activity of flavonoids of *Withania somnifera*. Antimicrobial activity of flavonoids extracts of *Withania somnifera* were carried out to validate the use of traditional medicinal herb and the results of this study tend to give credence to the common use of *Withania sominfera* plant.

Punum, (2014) reported that the antimicrobial potential of leaf extract of *Withania somnifera* against Gram positive *cocci*. The use of leaf extracts of *Withania somnifera* in the treatment of multidrug resistant pathogen by alternative systems of medicine. The aqueous root extracts of *Withania somnifera* hold an excellent potential as an antibacterial agent against *E. coli* and ascertains the value of medicinal plants used in Ayurveda, which could help in the development of an alternative drug.

Salil *et al.* (1997) investigated about antioxidant activity of glycowithanolides from *Withania somnifera*. So that the oxidative free radical scavenging activity of *Withania somnifera* may be responsible, at least in part, for the antistress, immunomodulatory, anti-inflammatory and anti-aging effects of *Withania somnifera* and its active principles, for drugs with anti-oxidative stress functions.

Ratan *et al.* (2016) determined that the bioactive constituent of *in vitro* Antioxidant Activity of *Withania somnifera* root which is correlated with antioxidative activity. This is due to the presence of ferrous reducing power, nitric oxide radical scavenging activity, Fe^{2+} chelating activity assay, and superoxide anion and hydrogen peroxide radical scavenging activities. The imported root extracts which indicate *Withania somnifera* indigenous root show higher

antioxidant activity. So the isolation of bioactive compounds from *Withania somnifera* will definitely serve as a good phyto therapeutic agent.

Bharathi *et al.* (2015) reported that traditionally, *Withania somnifera* is used to stabilize the mood of patients having behavioral disturbances and experimentally, it has also known to produce more anti-depressant and anti-anxiety effects as compared to drugs imipramine and lorazepam (anti-anxiety). *Withania somnifera* widely used as tranquillizer, improving reproductive and nervous system, rejuvenating body, improving vitality and recovery after chronic illness, so it hold an important position similar to ginseng in China. Anti-stress activity of *Withania somnifera* was conducted in rats using cold water swimming stress treatment and it was found that the drug treated animals show better stress tolerance. Similarly, a *withanolide* free aqueous extract of roots show dose dependent anti-stress activity in mice.

Mahrous *et al*. (2017) reported that ethanolic extracts of the different parts of Egyptian *Withania somnifera* exhibited high acetyl chroline sterase inhibitor effect as well as significant antioxidant activity. *Withanolide s* ***(6)*** was isolated from its leaf extract, which is an interesting drug candidate for treatment of Alzhemer's.

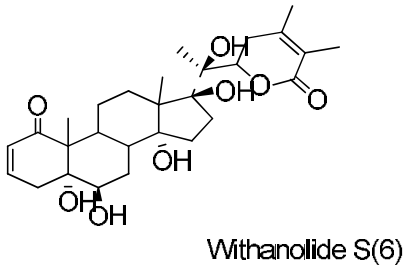


Figure 5. Structure of *Withanolide* S isolated from *Withania somnifera*

2.8. Phytochemical Investigation of *Withania Somnifera* by GC-MS

The oils extracted from the fruit (berry) of *Withania somnifera* were (Ghias *et al*., 2013) evaluated for their chemical composition. The gas chromatography-mass spectrometry (GC-MS) analysis shows the presence of various saturated and unsaturated fatty acids present in the roots of *Withania somnifera*. The total seven fatty acid compounds were reported by GC-MS with the

library searches scale with varying percentage such as linoleic acid (11.247%), palmitic acid, (2.842%) and tetracosanoic acid (0.880%), palmitic acid (0.42%), lenoleic acid (0.23%), oleic acid (0.14%), elaidic acid (0.01%). As shown in the following figure 6.

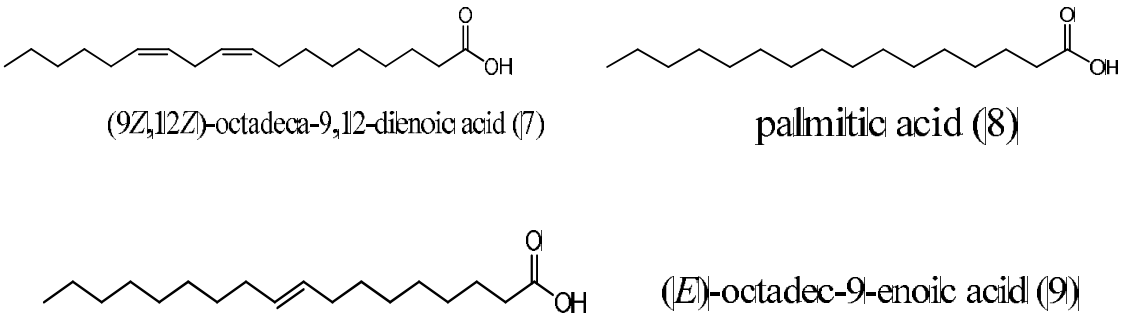
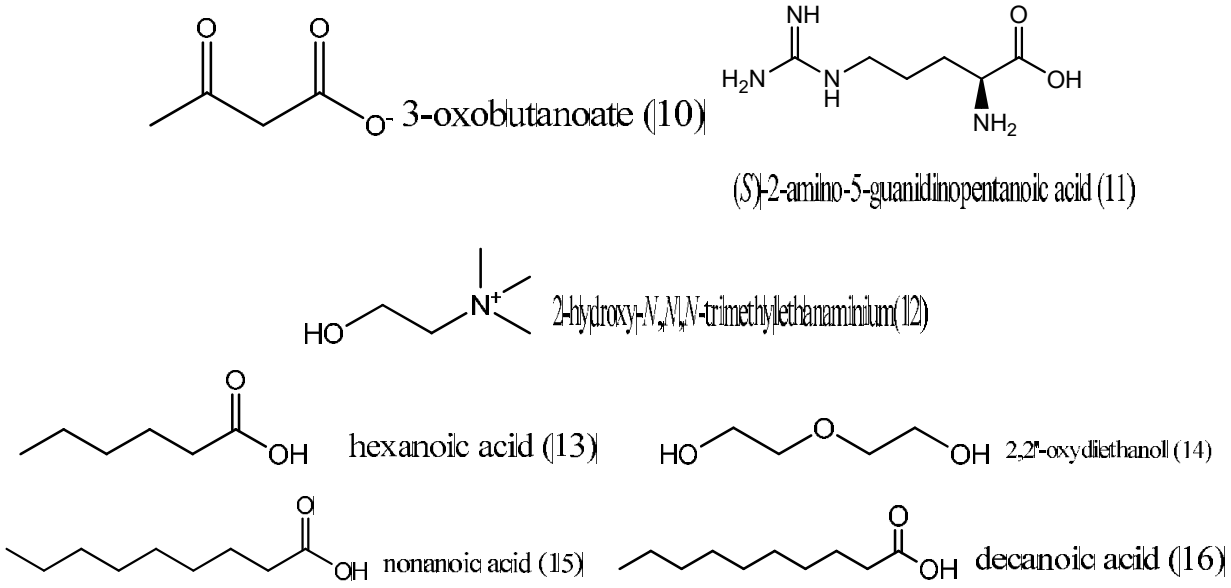


Figure 6. Structure of saturated and unsaturated fatty acids present in the roots of *Withania somnifera* by GC-MS

Bhatia *et al*. 2013 metabolic profiling was performed by GC–MS and NMR spectroscopy on the fruits obtained from four chemotypes of *Withania somnifera*. A combination of ^{1}H NMR spectroscopy and GC–MS identified 82 chemically diverse metabolites consisting of organic acids, fatty acids, aliphatic and aromatic amino acids, polyols, sugars, sterols, tocopherols, phenolic acids and *withanamides* in the fruits of *Withania somnifera*. The range of metabolites identified by GC–MS and NMR of *Withania somnifera* fruits showed various known and unknown metabolites as shown in figure 7.



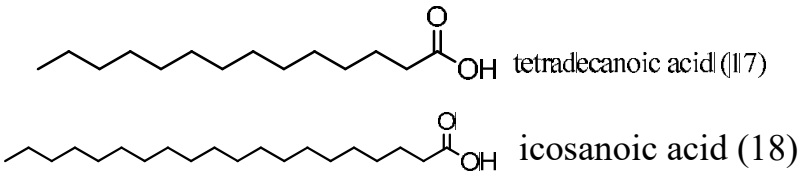
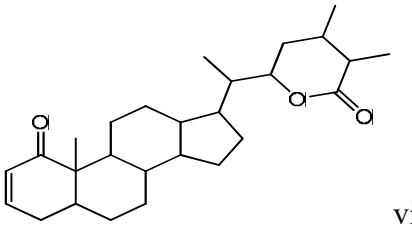


Figure 7. Structure of metabolites identified by GC–MS and NMR of fruits of *Withania somnifera*

Mahenda *et al.* 2017 reported that Assessment of the Consciousness Energy Healing Treated *Withania somnifera* root extract using LC-MS, GC-MS, and NMR Spectroscopy and isolated *withanolides* such as sitoindoside IX, viscosa lactone B, 24, 25-dihydrowithanolide D, *withanolide* A, *withanone*, *withaferin* A, *withanolide* D, ixocarpalactone A, *withanolide* S, *withanolide* sulfoxide, *etc*.



viscosa lactone B, 24, 25-dihydrowithanolide D **(19)**

24, 25-dihydro*withanolide* D, *withanolide* A **(20)**

Figure 8. Structure of *Withania somnifera* root extract using GC-MS and NMR Spectroscopy

3. MATERIALS AND METHODS

3.1. Sample Collection

Leaves of *Withania somnifera* were collected from local area of Eastern Hararge Zone Gende Mude in October 2017 and were identified by Herbarium of Haramaya University. The collected leaves were washed in tap water and kept in shade to dry for 10 days for proper grinding.

3.2. Materials and Apparatus

3.2.1. Apparatus and Instruments

The apparatus and instruments which were employed in this study were polyethylene bag, separatory funnel, grinder, different capacities of beakers, measuring cylinders, flasks, conical flasks, rotary evaporator, round bottom flask, Soxhlet's apparatus, Rotatory evaporator (Büchi Rotovapour R-400, Germany), Whatmman No 1 filter paper, refrigerator, heating mantle, spatula, petridish, incubator, water bath, electronic balance, GC-MS.

3.2.2. Chemicals and Reagents

The chemicals and reagents which employed in this study were solvents like n-hexane (98% AR), chloroform (99.8%; Analytical reagents, Atico house 5309 Grain market ATICO India), methanol (99.8%; Analytical reagents, Abron exports-133001 India), tap water, anhydrous sodium sulphate (Na_2SO_4 99% Blulux Labratories pvt Ltd-121001) $HgCl_2$, KI, $FeCl_3$, H_2SO_4, NaOH (98% BDH Chemicals Ltd Poole, England), boiling chips, and appropriate media for the antimicrobial assay Muller-Hinton agar (Blulux laboratories Pvt Ltd, India), Potato dextrose agar (Blulux laboratories Pvt Ltd., India), and reference chemicals.

3.3. Experimental Sites

Most of the experimental processes were done in the Postgraduate Laboratory of Chemistry Department, Haramaya University. The antimicrobial activity tests were done in the School of Plant Science (Plant Pathology laboratory), Haramaya University. However, GC-MS characterization of esterified oil was done in the Department of Chemistry, Addis Ababa University.

3.4. Extraction Procedures

3.4.1. Powder Preparation

The air dried *Withania somnifera* leaves sample (143 g) were ground with electrical grinder. The powder was packed in air tight polyethylene bag and kept in a refrigerator for further use.

3.4.2. Preparation of Plant Extract

The powder of a dried leaves of *Withania somnifera* 60 gram were extracted with 250 mL of hexane 68℃ in Soxhlet extractor at a temperature not exceeding the boiling point of the solvent. The solvent was recovered under low pressure to obtained dark greenish oil which was labeled n-hexane extract and kept in refrigerator. The resulting marc was air dried and then extracted successively with a mixture of 300 mL of chloroform and methanol (1:1) at 65℃ and the resulting marc was air dried and then extracted with 250 mL methanol at 70 ^{0}C temperature. Then the solvent was evaporated by rotary evaporator (Büchi Rotovapour R-400, Germany) at 40 ℃ temperature with 90 rpm (revolution per minute) and condensed for further studies (Brusotti *et al*., 2014). The overall processes described in figure 9.

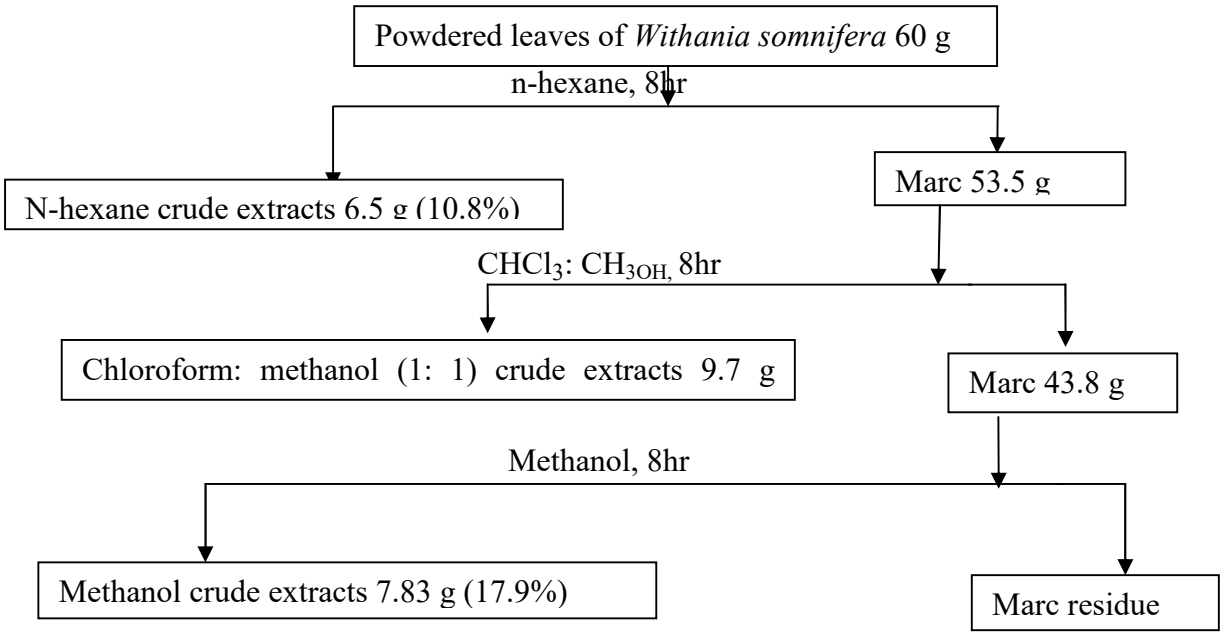


Figure 9. Successive Soxhlet Extraction of Leaves of *Withania somnifera* Plant

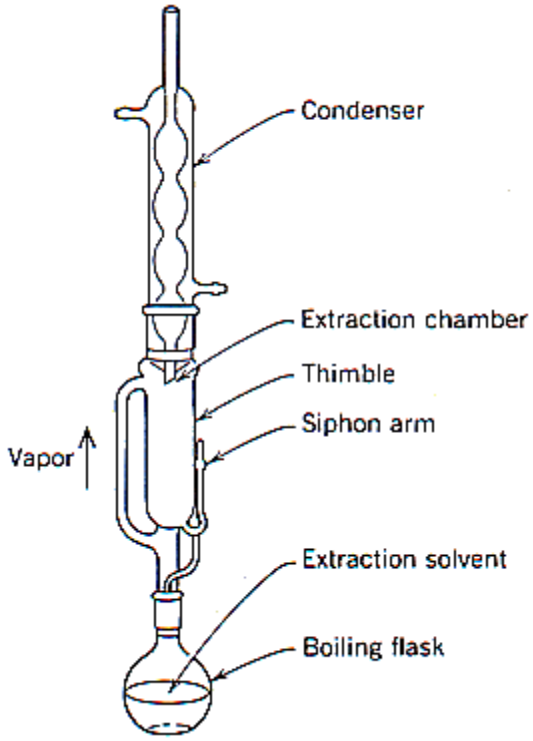


Figure 10. Soxhlet apparatus

3.5. Chemical Analysis of the Esterified n-hexane Leaf extract Oil by GC-MS

3.5.1. Esterification of n-hexane Leaf Extract of *Withania somnifera*

1 gram of n-hexane crude extract of the leaf of *Withania somnifera* was taken. Then esterified using 2% of KOH in methanol and reflux for 30 min at 50 ℃ to converting fatty acid into methyl ester fatty acid. Then subsequently picked up in n-hexane and the solvent was evaporated with rotator evaporator to yield concentrated oil of sample and dehydrated by adding Na_2SO_4 which was used for GC-MS characterization. This can be expressed in flow chart in figure 11.

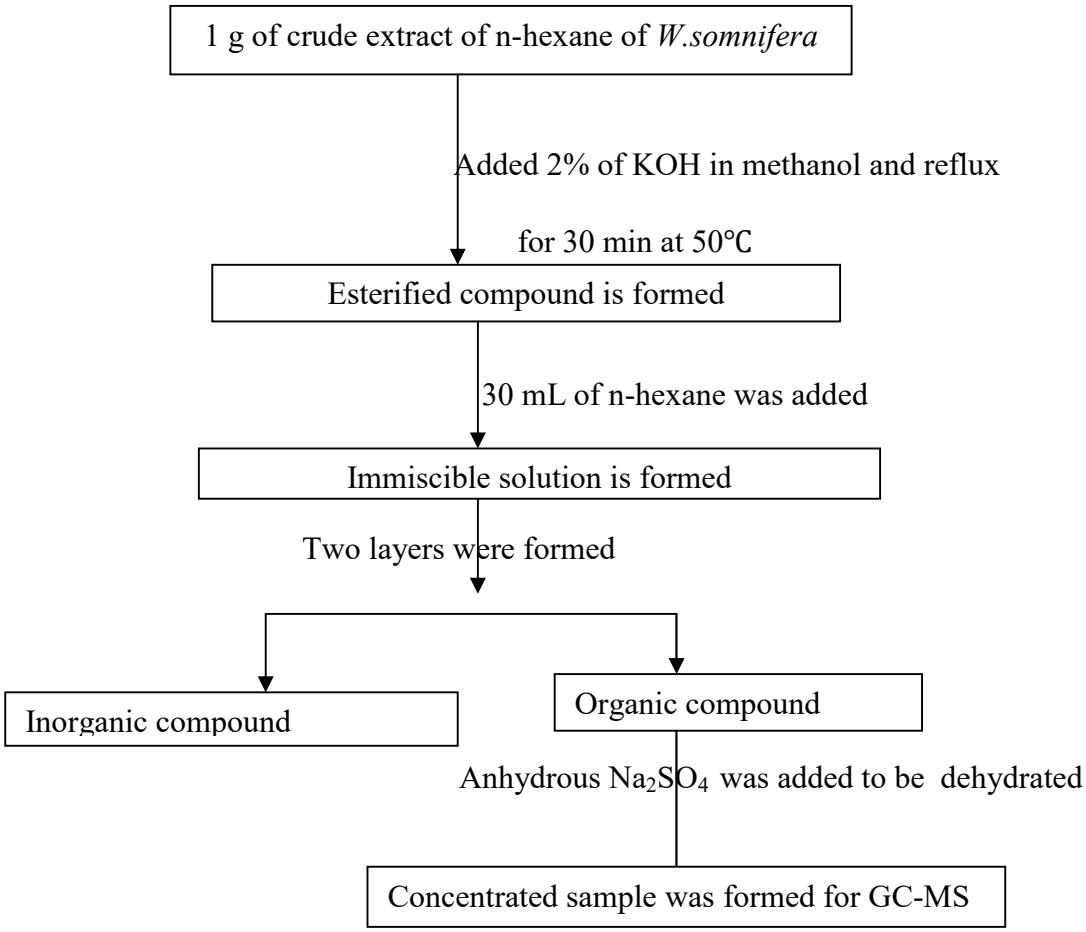


Figure 11. Esterification of n-hexane extract of *Withania somnifera*

3.5.2. Chemical Composition Analysis of the Esterified Oil with GC-MS

Derivatized fatty acid methyl esters were analyzed by using Agilent Technologies 78204 GC equipped with a mass selective detector (5977E) with DB -1701 capillary column (30 m x 0.25 mm, film thickness 0.25 μm). The initial column oven temperature was 100 ℃, kept for two minutes programmed at 20 ℃/ min to 200 ℃ and at 3 ℃/ min to 240℃. The inlet temperature was 275℃. The ion source was an electron ionization type with ionization energy of 70 eV. Ion source temperature was 230℃ and interface temperature was 250℃. The components were identified by comparing very relative retention time and mass spectra with NIST library.

3.6. Preliminary Phytochemical Screening of Leaves of *Withania Somnifera*

The crude extracts of leaves of *Withania somnifera* were tested for the presence of active constituent, such as flavonoid, alkaloids, Phenol, tannins and terpenoids. The process is presented in (table 3.)

Table 3. Procedures for phytochemical Constituent tests

Phytochemical Test	Procedure
Alkaloid	Add 2 mL filtrate with 1% HCl steam. Then add 1 mL of the solution with 6 drops of Wagner's reagent. Alkaloids are present, Brownish-red precipitate formed (Chanda *et al*., 2006).
Flavonoids	To 2-3 mL of the extract, add a piece of magnesium ribbon and 1mL of concentrated hydrochloric acid and formed Pink red or red coloration of the solution (Kumar *et al*., 2007).
Phenol	Spot the extract on a filter paper. Add a drop of phoshomolybdic acid reagent and expose to ammonia vapors and forms blue coloration of the spot (Kumar *et al*., 2007).
Terpenoids	To 1 mL of the extract, add 1ml of chloroform, 2-3 mL of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid and Pink or red coloration(Kumar *et al*., 2007).

3.6.1. Disc Preparation

The 6 mm (diameter) discs were prepared from Whatmann No.1 filter Paper the discs were sterilized by autoclave at 12℃. After the sterilization the moisture discs were dried on hot air oven at 50℃. Then various solvent extract discs and control discs were prepared.

3.7. Antimicrobial Test

N-hexane, chloroform/methanol (1:1) and methanol crude extracts of leaves of *Withania somnifera* were evaluated *in vitro* for antimicrobial assay by using the paper disc diffusion method against two gram positive bacteria (*Staphylococcus aureus and Streptococcus agalactia*) and two gram negative bacteria (*Escherichia coli and Salmonella typhi*) and two fungi,

Aspergillus niger (*A. niger*) and *Fusarium oxysporum.* The bacterial cultures were inoculated into the Muller Hinton Agar (MHA) and incubated at 37℃. Fungal cultures were inoculated into Potato Dextrose Agar (PDA) and incubated at 27℃. All the microbial were obtained from Plant Pathology laboratory of the School of Plant Science, Haramaya University and Chloramphenicol was used as standard drug against bacteria whereas Bavistin was used against fungi. Respective solvents were used as a negative control. From inhibition zone data, antimicrobial activities of crude extracts of the leaves were critically examined by comparing the mean inhibition diameters at 10 $\mu g/mL$ and 20 $\mu g/mL$ concentrations and relating them to the control.

3.7.1. Preparation of Inoculums

The test bacteria strains from the stock cultures were streak on Muller Hinton plates to incubated for 24 hours at 37 ℃. The bacteria colonies were separated and used as inoculums. The inoculums were transferred with bacteriological loop to autoclaved Muller Hinton molten agar cooled to 45℃ in water bath. The contents were mixed by swirling the flasks gently. The medium was poured to sterile Petri plate, allowed to solidify for bio-test uses (Hutchinson, 1986; Ejigu, 2012). For the fungi test, mycelia plugs from stock cultures were transferred to PDA plates and incubated for 5-7 days at 27℃. Then spores of Aspargillus Niger was harvested with washing the surface of the colony with using 10 mL sterile distilled water and transferred in to 300 mL autoclaved PDA cooled to 45℃ in a water bath (Hutchinson, 1986; Ejigu Bayu, 2012).

3.7.2. Preparation of Test Solution

The samples were used to test both antibacterial and antifungal activities was 100% crude. Each of the crude extracts was dissolved in respective solvents.

3.7.3. Testing for Antibacterial and Antifungal Activity of *Withania somnifera*

The antibacterial and antifungal activity studies were carried out in-*vitro* by disc diffusion technique (Newall *et al.,* 1996). The sterile nutrient agar plates and potato dextrose agar plates were prepared. The bacterial test microbial was two Gram-positive bacteria and two Gram negative bacteria like *Staphylococcus aureus*, *Striptococcus agalactia* and *Escherichia coli, Salmonella typhi* respectively and were spread over the nutrient agar plates by using separate

sterile cotton buds. While the fungal test organism like *Aspergillus Niger* and *Fusarium oxisporium* were spread over the potato dextrose agar plates after the microbial lawn preparation three different extracts of plant disc were placed on the organism inoculated plates with equal distance. Positive and negative control discs were also prepared (Chloramphinicol for bacteria and Bavistin for fungi). All bacterial plates were incubated at 27°C for 24 hrs and fungal plates at 24°C for 72hrs. The diameter of the minimum zone of inhibition was measured in mm. For each test, three replicates were performed.

4. RESULTS AND DISCUSSION

4.1. Percentage Yield of the Solvent Extract

The air dried powdered leaves of *Withania somnifera* (60 g) was pulverized and extracted with n-hexane at 60℃ for 8 h by Soxhlet apparatus and yielded a dark green extract (6.5 g, 10.8%). After n-hexane extract mass of the marc was weighed as 53.5 g and extracted with chloroform: methanol (1:1) ratio at 50℃ for 8 h with similar processes and yielded a green extract (9.7 g, 16.16%). Again, after extraction with chloroform: methanol (1:1) mass of the marc was 43.8 g and extracted with methanol at 50℃ with Soxhlet apparatus for 8 h to yield bright green extract (7.83 g, 13.05%). The leaves of *Withania somnifera* constituent more polar components since the yield of n-hexane extract was small to compared to the other. The percentage yield of crude extracts was calculated using the following formula (Mehani and Segni, 2013) and the quantity of the extract obtained by each solvent system is presented in (table 4).

$$\%\text{yield} = \frac{weight\ of\ crude}{weight\ of\ sample} \times 100$$

Table 4. Yield of each crude extract by the method of Soxhlet extraction

Solvent system	Weight of sample marc (g)	Weight of crude extracted (g)	(%) yield
n-hexane	60	6.5	10.8
Chloroform: methanol (1:1)	60	9.7	16.16
Methanol	60	7.83	13.05

As Praksh *et al.*2012 reported that root extract of *W.somnifera* from 150 g with *n*-hexane, chloroform and methanol gives 4 g, 3.5 g and 100 g respectively. To compare with the present study most compounds extracted by chloroform: methanol while in root extract most compounds identified by methanol.

4.2. Phytochemical Screening Test of Crude Extract of *Withania somnifera*

The qualitative analysis of the phytochemical constituents were carried out on n-hexane, chloroform: methanol (1:1) and methanol crude extracts of leaves of *Withania somnifera* plant showed the presence or absence of some bio active compounds. Phytochemical constituents are responsible for medicinal activity of plants. Therefore, each extracts of *Withania somnifera* were tested for the presence of secondary metabolites (phytochemicals) such as, alkaloids, phenols, flavonoids, tannins and terpenoids. Many reports revealed that polar compounds are much easier to be extracted from plant parts compared to the non-polar and semi-polar compounds (Razak *et al*., 2012, Widyawati *et al*., 2014). The results obtained from the present study showed that in methanol extract, flavones, phenols and terpenoids were presented but not alkaloids. In n-hexane extracts showed the presence of alkaloids, flavonoids, phenols and terpenoids and in chloroform: methanol (1: 1) extracts showed the presence of flavonoids, phenols and terpenoids but not alkaloids. The various phytochemicals detected in leafs extract of *Withania somnifera* were known to have beneficial importance in medicinal science. For instance, flavonoids have been referred to as nature's biological response modifiers because of their inherent ability to modify the body's reaction to allargies and they showed their anti-inflammatory, anti-microbial and anti-cancer activities (Aiyelaagbe and Osamudiamen, 2009). It is important to note that solvent polarities play important roles in determining the types of plant compounds with different properties; hence may influent the quality and properties of the extracts obtained (Emmanuel *et al*., 2014) as shown in table 6. According to this test there is no difference between chloroform: methanol (1: 1) and methanol extracts.

Table 5. Preliminary phytochemical screening of leaves of *Withania somnifera* under investigation

No	Phytochemicals	Crude Extracts		
		n-hexane	**Chloroform:methanol (1: 1) ratio**	**Methanol**
1	Alkaloids	+	-	-
2	Phenols	+	+	+
3	Flavonoids	+	+	+
4	Terpenoids	+	+	+

Note: + means present and - means absent

4.3. GC-MS Analysis of Esterified n-hexane Extracted Oil of Leaves of *Withania somnifera*

The oil quality was assessed through analysis by combined gas chromatography – mass spectrometry. Mass spectrometry was run in electron impact ionization (EI) at 70 eV. Identification of the chemical constituents of the oil was determined by their retention time, peak area, molecular formula and interpretation of their mass spectra and confirmed by mass spectral library search using the National Institute of Standards and Technology (Adams, 2007). The gas chromatogram of n-hexane extracted and esterified oil of the leaf of the plant showed the presence of 23 compounds. The components of n-hexane extracted oil were presented in table 6. The major constituents of n-hexane extracted oil were Benzyl nitrile (1.21%), Decanoic acid (1.54%), Dodecanoic acid (1.23%), methyl tetradecanoate (2.75%) pentadecanoic acid, 13-methyl (13.44%), hexadecanoic acid (1.80%), 9-octadecanoic acid, (E) (27.095%), E,Z-1,3,12-nona decatriene (26.88%), methyl stearate (4.10%), 7,10,13-hexadecatrienoic acid (3.15%), bis (2-ethyl hexyl) phthalate (7.56%) and methyl 18-methyl nonadecanoate (3.69%). This compound consists of different functional groups like, alkene, carboxylic acid (saturated and unsaturated),

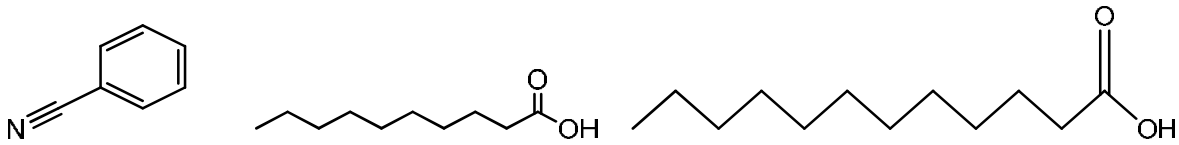
ester *etc*. The various compounds detected in n-hexane leaf extract of *Withania somnifera* by GC-MS were known to have beneficial importance in medicinal science.

Table 6. GC-MS analysis result of *Withania somnifera* n-hexane extracted Oil

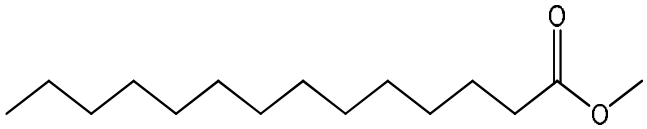
Peak	Compound identified	RT	CAS#	M.Wt	MF	Area %
1	Terpinen-4-ol	5.406	000562-74-3	154.25	$C_{10}H_{18}O$	0.24
2	Benzyl nitrile	6.048	000140-94-4	117.15	$C_6H_5CH_2CN$	1.21
3	Decanoic acid	6.546	000110-42-9	186.295	$C_{10}H_{20}O_2$	1.54
4	(+)-4-carbene Alpha-terpinyl acetate	6.958	029050-33-7 000080-26-2	196.2860	$C_{12}H_{20}O_2$	0.39
5	Caryophyllene	7.352	000087-44-5	204.357	$C_{15}H_{24}$	0.45
6	Benzene,1,4 dimethoxy-2-methyl-5-isopropyl	7.663	014753	194.27	$C_{12}H_{18}O_2$	0.65
7	Naphtalene, 1,2,3,4, 4a, 5, 6, 8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)	7.952	039029-4-9	204.35	$C_{15}H_{24}$	0.58
8	Dodecanoic acid	8.321	000111-82-0	200.32	$C_{12}H_{24}O_2$	1.23
9	Nerolidol	8.882	000142-50-7	222.37	$C_{15}H_{26}O$	0.48
10	Benzene, 1,2,3trimethoxy-5-(2-propenyl)	9.206	000487-11-1	208.25	$C_{12}H_{16}O_3$	0.36
11	Docosanoic acid	9.670	000929-77-1	340.58	$C_{22}H_{44}O_2$	0.91
12	Methyl tetradecanoate	9.878	000124-10-7	242.4	$C_{15}H_{30}O_2$	2.75
13	9-Hexadecenoic acid (z)-	11.399	001120-25-8	254.4	$C_{16}H_{30}O_2$	0.58
14	Pentadecanoic acid, 13-methyl	11.477	000112-39-0	256.24	$C_{16}H_{32}O_2$	13.44

15	Hexadecanoic acid	12.062	000628-97-7	256.42	$C_{16}H_{32}O_2$	1.80
16	9-octadecenoic acid, (E)	13.392	001937-62-8	282.46	$C_{18}H_{34}O_2$	27.09
17	E,Z-1,3,12-Nonadecatriene	13.464	056599-58-7	262.27	$C_{19}H_{34}$	26.88
18	Methyl stearate	13.518	000112-61-8	298.50	$C_{19}H_{38}O_2$	4.10
19	7,10,13-Hexadecatrienoic acid	13.639	000301-00-8	250.38	$C_{16}H_{26}O_2$	3.15
20	6-octen-1-ol, 3,7-dimethyl acetate	13.781	000150-84-5	282.46	$C_{18}H_{34}O_2$	0.50
21	Bis(2-ethyl hexyl)phthalate	14.961	000117-81-7	388.54	$C_{24}H_{36}O_4^{-2}$	7.56
22	Cis-11-Eicosenoic acid	15.987	1000333-63-8	310.50	$C_{20}H_{38}O_2$	0.42
23	Methyl 18-methylnonadecanoate	16.184	1000352-20-6	326.56	$C_{21}H_{42}O_2$	3.69

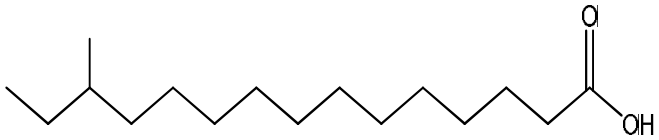
The major constituents of esterified n-hexane extract of *Withania somnifera* identified by GC-MS were represented in (figure 12.)



Benzoic nitrile Decanoic acid Dodecanoic acid



Methyl tetradecanoate



Pentadecanoic acid, 13-methyl

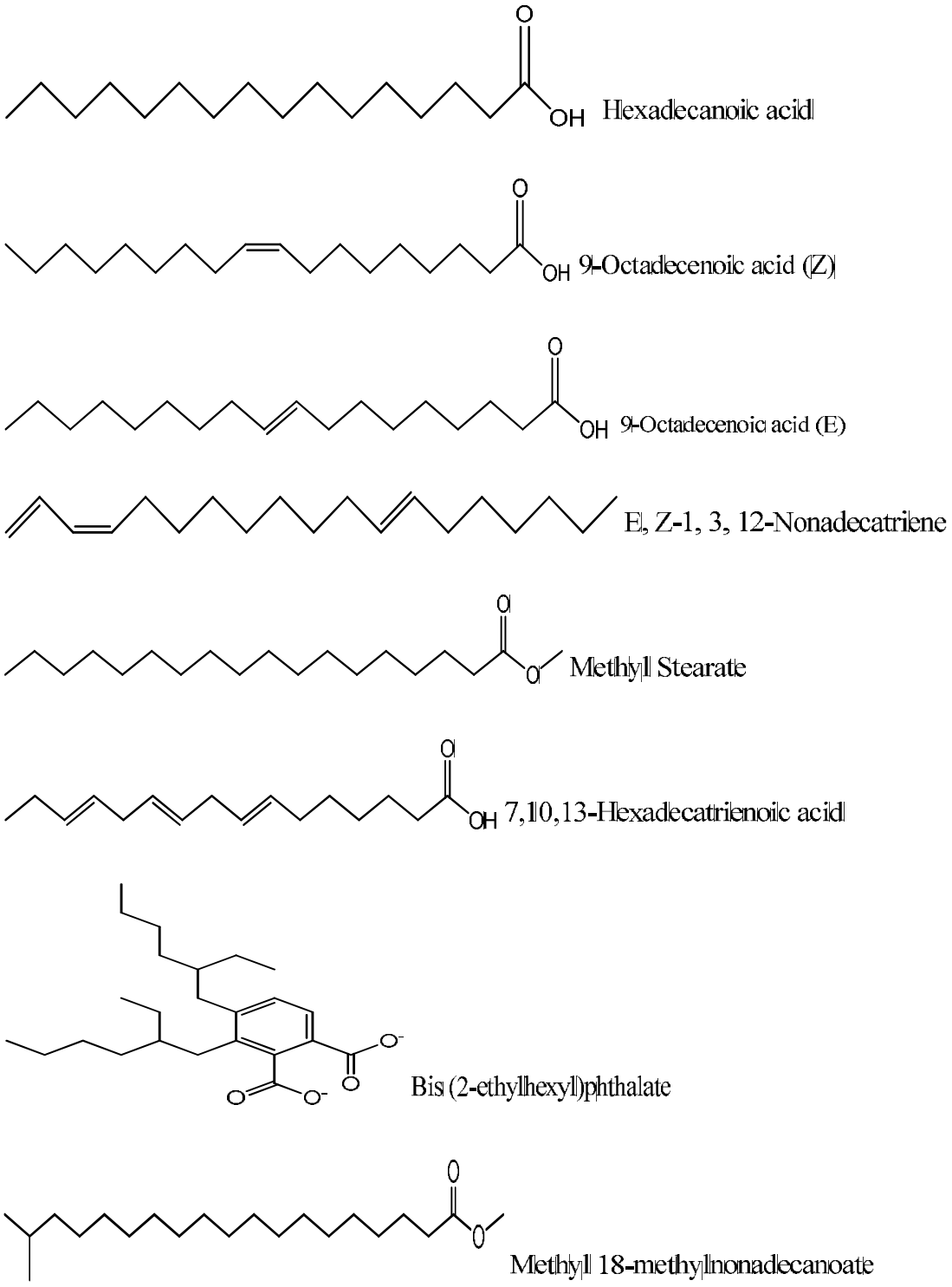


Figure 12. Structures of the major compounds that were obtained n-hexane oil extracts of *Withania somnifera* by GC-MS analysis

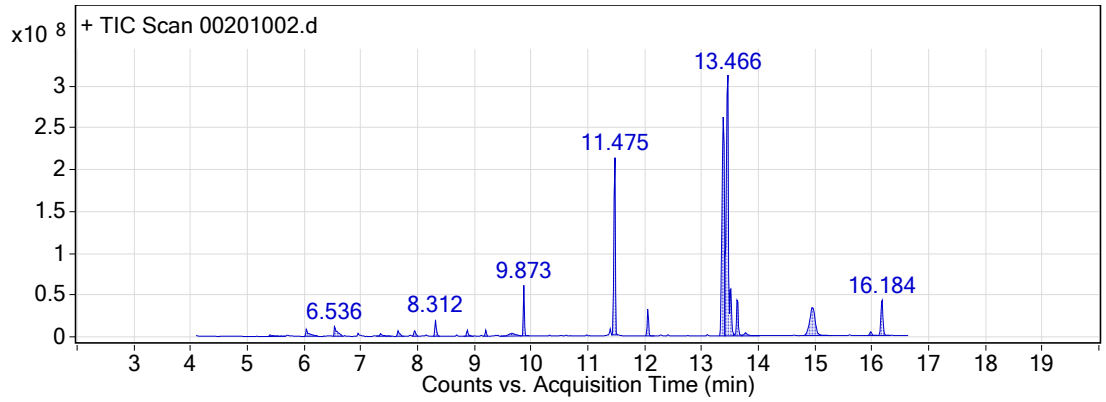


Figure 13. GC chromatogram of esterified *n*-hexane extract of *Withania somnifera* plant

4.4. Analysis of Antimicrobial Activities of *Withania somnifera* Leaf Extract

Antimicrobial activity (assessed in terms of inhibition zone in mm) of the leaves of *Withania somnifera* extracts in three solvents, tested against selected microorganisms were recorded (Table 7). In the present study all crude leaf extracts of *Withania somnifera* were investigated for their potential antibacterial and antifungal activities of the plant and was found to have appreciable antibacterial and antifungal activities. The inhibition zones were measured and compared with the standard reference antibiotics.

4.4.1. Antibacterial Activity

Two Gram positive **(***Staphylococcus aureus* and *Streptococcus***)** and two Gram negative bacteria **(***Escherichia coli* and *Salmonella typhimurium***)** were used to evaluate the antibacterial activity of the crude leaf extract of *withania somnifera.* The leaf extracts exhibited appreciable inhibitory effect against the tested bacterial pathogens at a dose of 10 *µg/mL* and 20 *µg/mL* (Table 7). Among the tested bacterial species, *Salmonella typhi*, which is a Gram-negative bacteria, was found to be the most susceptible to the n-hexane, chloroform: methanol (1:1) and methanol crude extracts of *withania somnifera* leaf, with zone of inhibition of 25 ± 0.04, 21.5 ± 0.04 and 18 ± 0.04 mm, respectively. However, the n-hexane extract of the leaf showed the least antibacterial activity against Gram-positive bacteria, *Streptococcus*, with zone of inhibition of 13.6±0.024 mm. The negative control (n-hexane, chloroform methanol (1:1) and methanol solvents) did not show any inhibition zone against all the test species. The positive control, chloramphenicol,

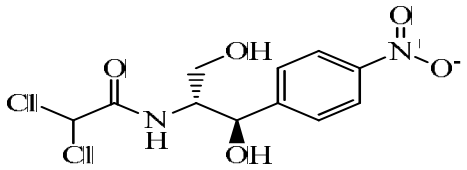
showed an average inhibition zone of 39, 32, 36.3 and 37.6 mm against species of *Staphylococcus aureus*, *Streptococcus, Escherichia coli* and *Salmonella typhimurium* respectively.

Table 7. Zone of bacterial growth inhibition (mm) for crude extracts of from leaf of *Withania somnifera*

Sample extract	Dose in µg/mL	Types of bacteria with mean inhibition zone in diameter (mm)			
		Gram positive bacteria		Gram negative bacteria	
		Staphylococcus aureus	*Streptococcus*	*Escherichia coli*	*Salmonella typhimurium*
n-hexane	10	10.5 ± 0.04	6.5± 0.02	9.4 ± 0.4	13 ± .04
	20	19 ± 0.04	13.6 ± 0.024	17.6 ± 0.40	25 ± 0.04
Chloroform:methanol	10	10 ± 0.005	11.5 ± 0.024	13.5± 0.01	12 ± 0.04
	20	19.5 ± 0.00	20.3 ± 0.024	21 ± 0.01	21.5 ± 0.04
Methanol	10	9.5 ± 0.04	8.5 ± 0.04	9.4± 0.04	10.5 ± 0.04
	20	17 ± 0.04	16.2 ± 0.047	16.8 ± 0.04	18 ± 0.04
Positive control	10	22±0.04	20±0.04	21.3±0.13	21.5±0.06
	20	39±0.04	32±0.04	36.3±0.13	37.6±0.06
Negative control	10	-	-	-	-
	20	-	-	-	-

NB: Value represents mean of triplicates, ± SD, - Stands for no inhibition

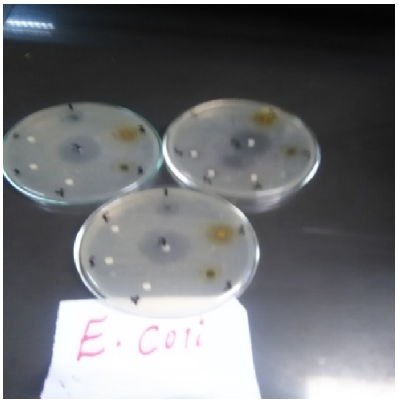
Positive control: chloramphenicol, Negative control: (n-hexane, chloroform and methanol (1:1) and methanol solvents). The chemical structure of chloramphenicol as follow:



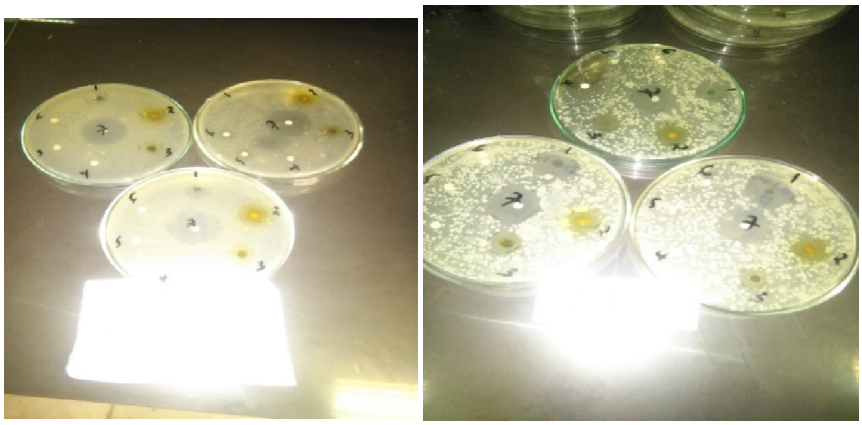
chloramphenicol
2,2-dichloro-*N*-((1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl)acetamide

Figure 14. Chemical structure of chloramphenicol

All crude extracts have higher inhibition effect against the tested bacteria at 20 *µg/mL* dose level as compared to 10 *µg/mL* dose level. As revealed from the results presented in table 7, the antibacterial activities of the tested leaf extracts of the plant were more pronounced on the Gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) than the Gram positive bacterium (*Staphyloccus aureus* and *Streptococcus*). Gram positive cell walls consist of many layers of peptidoglycan and do not possess a lipid outer membrane. Gram negative cell walls on the other hand have only one or a few layers of peptidoglycan but posses an outer membrane consisting of various lipid complexes. In addition to that Gram-positive spores develop as a new structure inside the protective interior of an existing cell wall, Gram negative cells morph directly into spores while maintaining their cell wall structural integrity (Liu *et al*., 2004).



Staphyloccus aureus *Escherichia coli*



Streptococcus agalactia *Salmonella typhimurium*

Figure 15. Antibacterial activities on the leaves of *Withania somnifera*

4.4.2. *An*tifungal Activity

Two fungal species (*Aspergillus niger* and *Fusarium oxisporium*) were used to evaluate the antifungal activity of the crude leaf extracts of *Withania somnifera*. According to the data obtained from this study n-hexane, chloroform:methanol (1: 1) mixture and methanol extracts of the leaves shown inhibition zone against the two fungal species. Chloroform: methanol (1: 1) extract exhibited relative appreciable inhibition zone as shown in table 8.

Table 8. Zone of fungal growth inhibition (mm) for crude extracts from leaves of *Withania somnifera*

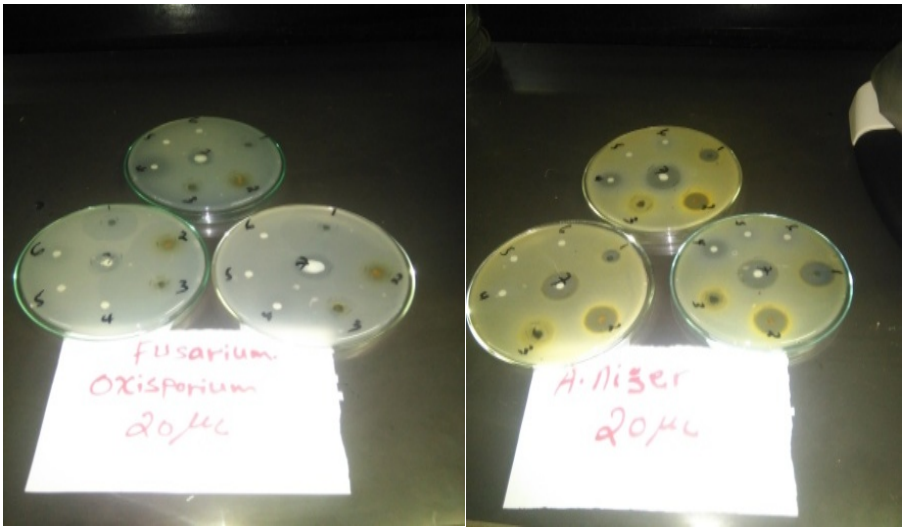
Sample extract	Dose in µg/mL	Types of fungal with mean inhibition zone in diameter (mm)	
		Aspergillus niger	*Fusarium oxisporium*
n-hexane	10	13.5 ± 0.04	6.5 ± 0 .04
	20	20.5±0.041	12 ±0.041
Chloroform: methanol (1:1)	10	14.5 ± 0.04	12.6 ±0.042
	20	23.5±0.071	18.6±0.085
Methanol	10	10.6 ± 0.04	8.25 ± 0.045
	20	18.5±0.11	14.8±0.047
Positive control	10	20.5 ±0.08	12.3±0.02
	20	32.2±0.08	25.8±0.02
Negative control	10	-	-
	20	-	-

NB: Value represents mean of triplicate, ± SD, - Stands for no inhibition

Positive control: Bavistin, Negative control: (n-hexane, chloroform and methanol (1:1) and methanol solvents)

For all samples the inhibition zone against the tested fungi increases with doses (10 *µg/mL*) to dose (20 *µg/mL*). The commercial standard drug (Bavistin) shows higher inhibition zone against antifungal activity compared with all the tested samples. The antifungal activity of these extracts was found to be appreciable. The largest inhibition zones were recorded with chloroform:

methanol (1:1) crude extract against *A. Niger* (23.5±0.07 mm). However the n-hexane extrac of the leaf showed the least antifungal activity against *Fusarium oxisporium* (12±0.041 mm). As shown in the above table 8.



Fusarium oxisporium *Aspergillus niger*

Figure 16. Antifungal activities on leave extracts of *Withania somnifera*

5. SUMMARY, CONCLUSION AND RECOMMENDATION

5.1. Summary

The present study was designed to investigate phytochemicals and antimicrobial activity in leaf extracts of *Withania somnifera*. Firstly *Withania somnifera leaves* were collected and extracted by different organic solvents using Sohxhlet apparatus. Then, the collected *Withania somnifera* leaf crude extracts were concentrated using rotary evaporator. A phytochemical screening test of crude oil extract was done to confirm the presence of phytochemical constituents of the crude extract. From n-hexane crude extract of leaves of *Withania somnifera* 23 organic compounds were characterized by GC-MS based on by comparing their mass spectra with NIST Library. Additionally, the crude extracts were tested for their antimicrobial activities against two Gram positive bacteria, two Gram negative and two fungal species.

5.2. Conclusion and Recommendation

In the present study, the phytochemical screening for leaves of crude extracts of *Withania somnifera* showed the presence of active components like flavonoids, phenols and terpenoids from n-hexane, chloroform: methanol (1:1) and methanol extracts. The n-hexane extracted leaves of esterifies oil of *Withania somnifera* were characterized by GC-MS which have 11 major constituents. These were benzyl nitrile (1.21%), Decanoic acid (1.54%), Dodecanoic acid (1.23%), Methyl tetradecanoate (2.75%), Pentadecanoic acid, 13-methyl (13.44%), Hexadecanoic acid (1.8%), 9-octadecanoic acid, (E) (27.095%), E, Z-1,3,12-nonadecatrien (26.88%), Methyl stearate (4.10%), 7,10,13-hexadecatrienoic acid (3.15%), Bis (2-ethylhexyl), phthalate (7.56%) and Methyl 18-methylnonadecanoate (3.69%). In addition, the crude extracts were tested for their antimicrobial activities against two Gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) and two Gram positive bacteria (*Staphyloccus aureus* and *Streptococcus*) and two fungi (*Fusarium oxysporum* and *Aspergillus niger*). Results showed that all crude extracts were active towards the four bacterial and two fungal species. The study has showed that the observed antimicrobial effect of *Withania somnifera* leaf crude extracts on the bacterial and fungal isolate, though in vitro appear interesting and promising.

Based on the result of the present study, the following recommendations are forwarded.

- To carry out practicality studies in the use of crude extracts of the plant in the cosmetic and health care industries (soap, skin care products and perfume).
- This plant should be studied more extensively to explore its activity on other organisms.
- Toxicity studies of the plant should also be done to determine the safety index of the extracts.
- Further studies should also be carried out to determine the effects of agro-ecological and pest infestation on the active compounds in *Withania somnifera.*

6. REFERENCES

Adams, R.P. 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. *Allured Publishing: Carol Stream, IL, USA*.

Aiyalaagbe, O.O. and Osamudiamen, P.M. 2009. Phytochemical screening for active compounds in mangiferaindica. *Plant science research*, 2(1): 11-13.

Ane, O. and Sandy, V. 2016. Commercial essential oils as potential antimicrobials to treat skin diseases. *Evidence-Based Complementary and Alternative Medicine,* 92.

Anonymous. 1982. The health of India, publications and information directorate. *Council of Scientific and Industrial Research*, 580- 585.

Asfaw Debella, Negero Gemeda, Frehiwot Teka, Biruktawit Girma, Mulugeta Guta, Bekesho Geleta and Ashenif Tadele. 2015. Proceeding of the Workshop on "Ethiopian Traditional Medicine: Past, Current and Future", Adama, Ethiopia. *Modern Medicine Research Directorate Ethiopian Public Health Institute,* 24-25.

Balcha Abera. 2014. Medicinal plants used in traditional medicine by Oromo people, Ghimbi District, Southwest Ethiopia. *Journal of Ethno biology and Ethnomedicine.*

Bano, A., Ayub, M., Rashid, S., Sultana, S. and Sadia, H. 2013. Ethno botany and conservation status of floral diversity of himalayan range of azad jammu and kashmir-pakistan. *Pakistan Journal of Botany*, 45: 243-251.

Bharathi, V., Seshayamma, G., HariJagannadharao, N. and Sivakumar. 2015. Evaluation of anti depressant activity of aqueous extract of *Withania somnifera* roots in albino mice. *Journal of Pharmacy and Biological Sciences,* 10(1).

Bhatia, A., Santosh, K., Bharti , Shri, K., Tewari , Sidhu, P. and Raja Roy. 2013. Metabolic profiling for studying chemotype variations in *Withania somnifera* (L.) Fruits using GC–MS and NMR spectroscopy. *Phytochemistry*, 93: 105–115.

Bilal, A.M. and Jabeena, K. 2012. Botanical, chemical and pharmacological review of *Withania somnifera*. *Indian Journal of Drug and Diseases,* 1: 6.

Brusotti, G., Cesari, I., Dentamaro, A., Caccialanza G. and Massolini G. 2014. Isolation and characterization of bioactive compounds from plant resources: The role of analysis in the ethnopharmacological approach. *Journal of Pharmaceutical and Biomedical Analysis,* 87(18): 218-228.

Budhiraja, Pawan, Krishan and sudhir. 2000. Biological activity of *withanolides. Journal of Scientific and Indusrtial Research,* 59: 904-911.

Chanda, S.V., Parekh, J. and Karathia, N. (2006). Evaluation of antibacterial activity and phytochemical analysis of *Bauhinia variegate* L. bark. *African Journal Biomedicine Research*, 9: 53-56.

Danuta, K., Martyna, M. and Anna, S. 2012. Antimicrobial activities of essential oils, 157-183.

Derwich, E., Benziane, Z., Chabir, R. and Taouil, R. 2011. *In Vitro* Antibacterial Activity and Analysis of the Essential Oil Extract of Leaves of *Rosmarinus officinalis* grown in Morocco. *International Journal of Pharmacy and Pharmacological Science,* 3(3): 89-95.

Ejigu Bayu. 2012. Phytochemical and antimicrobial investigations of leaves of fennel herb *foeniculum vulgare* extracts of MSc Thesis. Haramaya University, Haramaya, Ethiopia.

Emmanuel, S.A., Olajide, O.O., Abubakar, S., Idowu, I.D. , Orishadipe A.T. and Thomas S.A. 2014. Phytochemical and antimicrobial studies of methanol, ethyl acetate, and aqueous extracts of *Moringa oleifera* seeds, *American Journal of Ethnomedicine,* 1(5): 346-354.

Evans, W.C. 2009. Trease and Evans‟ Pharmacognosy. 16th Edition. London: WB Saunders Company Ltd. p 37-8, 83-121, 331-2, 442, 459-470, 504.

Ganzera, M, Choudhary, M.I, Khan, I.A. 200]. Quantitative HPLC analysis of withanolides in *Withania somnifera* . *Fitoterapia*, 74(1-2): 68-76.

Geeta, S. and Padma, K. 2014**.** Antibacterial activity of flavonoids of *Withania somnifera*. *International Journal of Green Pharmacy*.

Ghias Uddin, Abdur Rauf, Sumaira Gul, Muhammad Saleem, Salma Umar and Ajmal Khan. 2013. Proximate chemical composition and biological profile of fatty acids of *Withania somnifera*. *Journal of Medicinal Plants Research*, 7(27): 2034-2039.

Hutchinson, C.R. 1986. Biological methods for studying biosynthesis of natural products. *Journal Home page*.

Ipseeta Mohanty. 2003. Mechanisms of cardioprotective effect of *withania somniferain* experimentally induced myocardial infarction. *Basic Clinical Pharmacol Toxicol,* 94(4):184-190.

Kassaye Kebede Deribe, Alemayehu Amberbir, Binyam Getachew, Yunis Mussema. 2006. A Historical Overview of Traditional Medicine Practices and Policy in Ethiopia. *Ethiopian journal of health development,* 20(2): 127-134.

Kulkarni, S.K, Dhir, A. 2008. *Withania somnifera*: An Indian ginseng. Prog Neuropsychopharma col Biol Psychiatry. 32(5):1093-1105.

Kumar, G.S., Jayaveera, K.N., Kumar, C.K.A., Sanjay, U.P., Swamy, B.M.V. and Kumar, D.V.K. 2007. Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. *Tropical Journal of Pharmastical Research*, 6: 717-723.

Liu,Y., Fu, X., Shen, J., Zhang, H., Hong, W. and Chang, Z. 2004. Periplasmic proteins of Escherichia coli are highly resistant to aggregation: reappraisal for roles of molecular chaperones in Periplasmic. *Biochemistry and Biophysics Research Community*, 316: 795-801.

Mahendra, K.T., Branton, A., Trivedi, D., Nayak, G., Wellborn, B. D., Smith, D.L., Panda, P. and Jana, S. 2017. Assessment of the Consciousness Energy Healing Treated *Withania Somnifera* Root Extract Using LC-MS, GC-MS, and NMR Spectroscopy. *American Journal of Physical Chemistry*, 6(2): 20-30.

Mahrous, R.S.R., Doaa, A., Ghareeb, M., Fahy, Rashan, Abu EL-Khair and Abdallah Aomar. 2017. The protective effect of Egyptian *Withania somnifera* against Alzeheimer's. *Medicinal and Aromatic Plants,* 6: 285.

Mehani, M. and Segni, L. 2013. Antimicrobial effect of essential oil of plant *Schinus molle* on Some Bacteria Pathogens. *World Academy of Science, Engineering and Technology International Journal of Chemical, Nuclear, Metallurgical and Materials Engineering*, 7(12): 34-38

Mishra, L.C., Singh, B.B. and Dagenais, S. 2000. Scientific basis of for the therapeutic use of *withania somnifera,* 5(4): 334-346

Newall, C.A., Anderson L.A. and Phillipson J.D. 1996. *Herbal medicines*, 25.

Parvinder, K., Sheenu, M., Meenakshi, S., Manish, T. and Ramesh, C. 2001. Biologically active constituents of *Withania somnifera* with anti-stress activity. *Indian Journal of Clinical Biology,* 16(2): 195-198.

Prakash, k., Mangala, D., Manandhar, S., Awale and Janaki, B. 2011. Isolation, identification, and antimicrobial activity of *withanolide* from the roots of *Withania somnifera*. *Nepal Journal of Science and Technology,* 12: 179-186.

Punum, B. and Vinita, R. 2014. Antibacterial activity of *Withania somnifera* against Gram-positive isolates from pus samples. *International Quartery Journal of Research in Ayurved*, 35(3): 330-332.

Raimondo, D., Von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. and Manyama, PA. 2009. Red list of South African plants, (eds.) strelitzia 25. South *African National Biodiversity Institute*.

Ratan, K.P. 2016. In vitro antioxidant activity of *Withania somnifera* root. *International Journal of Advanced Research in Chemical Science. Avicenna Journal Phytomedicine*, 6(4): 399-409.

Razak, M.F.A., Yong, P.K., Shah, Z.M., Abdullah, L.C., Yee, S.S. and Yaw I.T.C.S. 2012. The effects of varying solvent polarity on extraction yield of *Orthosiphon stamineus* leaves. *Journal of Applied Sciences,* 12(11): 1207-1210.

Saidulu, C.h., Venkateshwar, C. and Gangadhar Rao, S. 2014. Preliminary phytochemical studies of medicinal plant drug: *Withania somnifera*. *Bio life*, l 2(1): 306-312.

Salil, K.B., Kalkunte, S. and Shibnath, G. 1997. Antioxidant activity of glycol *withanolides* from *withania somnifera*. *Indian Journal of Experimental Biology,* 35: 236-239.

Satish, K., Verma, A., Simashaban, Reena, P., Madhvil, C., Geeta, R. and Prakash, V. 2012. Immunomodulatory activity of *Withania somnifera. Journal of Chemical and Pharmaceutical Research*, 4(1): 559-561.

Schmelzer, G.H. and Gurib-Fakim, A. (Editors). 2008. Plant resources of tropical Africa 11(1). Medicinal plants 1. *PROTA foundation, Wageningen, Netherlands/ Backhuys publishers Leiden, Netherlands/CTA, Wageningen, Netherlands*, 791.

Teklehaymanot Tilahun, Giday Mirutse, Medhen Girmay and Yared Mekonnen. 2007. Knowled ge and use of medicinal plants by people around Debre Libanos monastery in Ethiopia. *Journal of Ethnopharmacol,* 11: 77-83.

Umadevi, M., Rajeswari, R., Sharmila, C., Selvavenadesh, S., Pushpa, Ssampath, K.P. and Kumari. 2012. Traditional and medicinal uses of *Withania somnifera,* 1(9).

WHO. 2001. Legal status of traditional medicine and complementary/alternative medicine: a worldwide review.

Widyawati, P.S., Budianta, T.D.W., Kusuma F.A. and Wijaya, E.L. 2014. Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indica* Less

leaves extracts. *International Journal of Pharmacognosy and Phytochemical Research,* 6(4): 850-855.

Wilson and Roberto. 2016. "The essential guide to essential oils: The secret to vibrant health and beauty."

Worku Abebe. 2016. An overview of Ethiopian traditional medicinal plants used for cancer treatment. *European Journal of Medicinal Plants,* 14(4): 1-16.

Yadav, A., Bajaj, M., Saxena and Saxen, A.K. 2010. In vitro anticancer activity of the root, stem and leafs of *Withania somnifera* against various human cancer cell lines. *Indian Journal of Pharmaceutical Sciences,* 72(5): 659-663.

Yadav, M., Rathi, A., Pednekar and Rewachandani,Y. 2016. Detail review on *Solanaceae* family. *European Journal of Pharmaceutical and Medicinal Research,* 3(1): 369-378.

7. APPENDIX

Table 1. Raw data of Zone of bacteria growth inhibition (mm) of crude extracts of *withania somnifera* 20 µg/mL of the sample

No	Type of bacteria	Triplicate	Sample													
			S1		S2		S3		S4		S5		S6		S7	
1	*Escherichia*		V	H	V	H	V	H	V	H	V	H	V	H	V	H
	coli	1	17	18	20	19	17	16	-	-	-	-	-	-	34	35
		2	18	18	22	21	17	17	-	-	-	-	-	-	37	38
		3	18	17	22	22	18	16	-	-	-	-	-	-	38	36
2	*Salmonella*	1	24	25	20	22	19	18	-	-	-	-	-	-	37	38
	typhimurium	2	25	25	22	21	18	17	-	-	-	-	-	-	38	36
		3	26	25	22	22	19	17	-	-	-	-	-	-	39	38
3	*Staphylococcus*	1	19	19	20	19	17	16	-	-	-	-	-	-	40	39
	aureus	2	20	19	21	18	16	18	-	-	-	-	-	-	40	38
		3	19	18	20	19	19	16	-	-	-	-	-	-	39	38
4	*Streptococcus*	1	13	14	20	20	17	16	-	-	-	-	-	-	32	31
		2	14	14	20	21	16	15	-	-	-	-	-	-	32	32
		3	14	13	21	20	17	16	-	-	-	-	-	-	33	32

NB: S1= Crude oil of n- hexane S2= Crude oil of chloroform: methanol

S3= Crude oil of methanol S4= pure solvent hexane, S5= chloroform: methanol solvent

S6= methanol solvent

Table 2. Raw data of Zone of fungal growth inhibition (mm) of crude extracts of *withania somnifera* 20 µg/mL of the sample

No	Type of fungal	Sample													
	Aspergillus niger	Triplicate	S1		S2		S3		S4		S5		S6		S7	
			V	H	V	H	V	H	V	H	V	H	V	H	V	H
		1	20	20	23	22	20	20	-	-	-	-	-	-	32	34
		2	20	19	25	23	18	17	-	-	-	-	-	-	30	32
		3	22	22	25	23	18	18	-	-	-	-	-	-	33	32
	Fusarium oxisporium	1	12	13	17	18	15	14	-	-	-	-	-	-	25	26
		2	12	11	18	20	16	15	-	-	-	-	-	-	27	25
		3	13	11	19	20	15	14	-	-	-	-	-	-	26	26

NB: S1= Crude oil of n- hexane S2= Crude oil of chloroform: methanol

S3= Crude oil of methanol S4= pure solvent hexane, S5= chloroform: methanol solvent

S6= methanol solvent

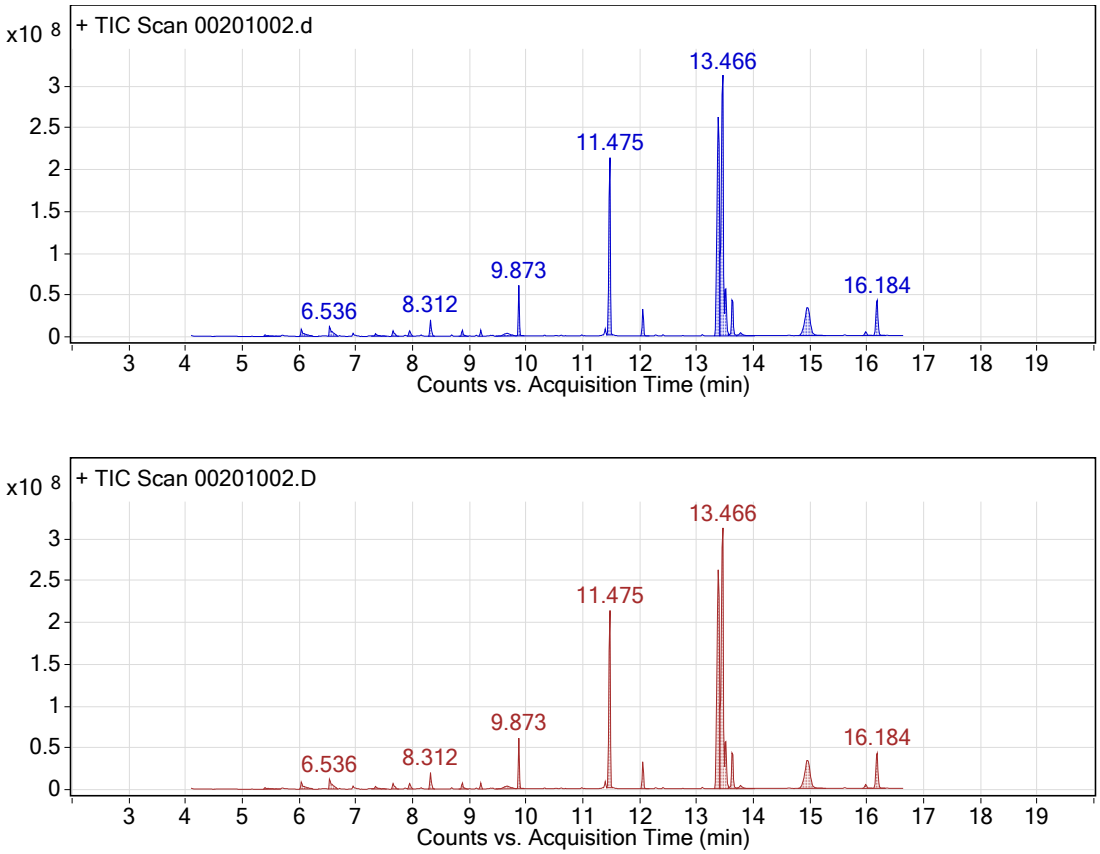
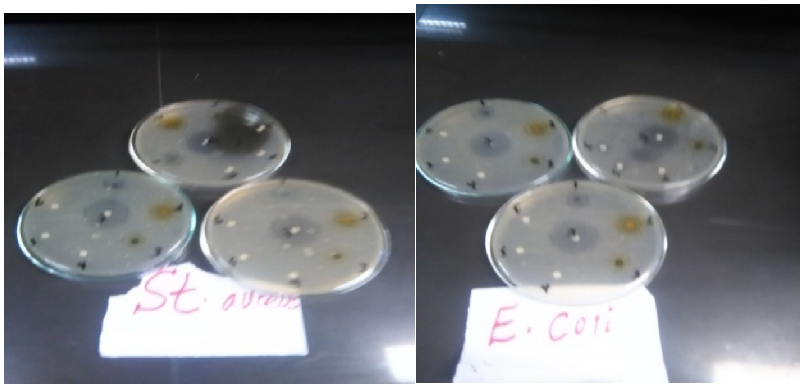
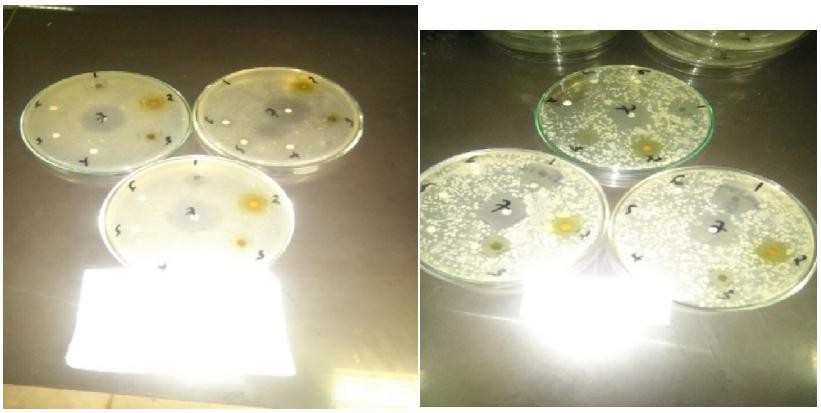


Figure 1. GC chromatogram of esterified *n*-hexane extract of *Withania somnifera* plant



Staphyloccus aureus *Escherichia coli*



Streptococcus *Salmonella typhi*

Figure 2. Antibacterial activities on the leaves of *withania somnifera*

Fusarium oxisporum *Aspergillus niger*

Figure 3. Antifungal activities on leave extracts of *withania somnifera*