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THE ANTIBACTERIAL, ANTIOXIDANT AND TOXICITY STUDIES OF CRUDE AERIAL PART EXTRACTS OF *SATUREJA PUNCTATA* (BENTH.) BRIQ.

BY

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L I S T OF ABBREVATIONS

A A U −Addis Ababa University

ATCC − American Type Culture Collection

CFU − Colony forming unit

DPPH–2, 2-diphenyl-1-picrylhydrazyl

EPHI –Ethiopian Public Health Institute

FDA- US Food and Drug Administration

G −Gram

Hrs- Hours

L − Litre

Ltd− Limited

MBC−Minimum Bactericidal Concentration

MDR– Multiple drug resistant

Mg–Milligram

MHA− Mueller Hinton Agar

MIC−Minimum Inhibitory Concentration

ML–Milliliter

mm− millimeter

SEM −Standard Error of the Mean

NaCl− Sodium chloride

NA−Nutrient Agar

NCCLS− National Committee for Clinical Laboratory Standards

nm–Nanometre

^{o}C– Degree Celsius

OECD −Organization for Economic Co-operation and Development

RNS– Reactive Nitrogen Species

ROS–Reactive Oxygen Species

Rpm – Revolution per minutes

SPSS–Statistical package for social science

T-80 – Teween80

Tet−Tetracycline

V/V –Volume per Volume

W/V– weight per Volume

W/W-Weight per Weight

WHO– World Health Organization

µg–Microgram

ABSTRACT

The ever-increasing demand for antimicrobial products against resistant infectious (microbial) agents necessitates their search from microorganisms and medicinal plants. The *Satureja punctata* (locally known as "lomishet", Amharic) is one of such plants used as herbal medicine in different parts of Ethiopia. The objective of this study is to evaluate the antimicrobial and antioxidant activities of the crude aerial part extract of the plant against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* and its toxicity on animal mouse model. The aerial parts were collected from Entoto and Sendafa and extracted using absolute ethanol, water and n-hexane. The Antibacterial activity was assessed using disc diffusion method, and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extracts were determined by the agar dilution method. The radical scavenging activity of the extracts was evaluated by DPPH assay. The result indicated that the solvent extracts exhibited antibacterial activities on the test organisms, except the n-hexane and the activities were dependent on solvent type and concentration. Consequently, the maximum antibacterial activity was displayed by the aqueous extract against *S. aureus*(10.33±0.25mm), *E. coli* (9.83±0.19mm) and *P.aeruginosa* (8.53±0.12mm) and by ethanol extract against *S. aureus* (10.33±0.41mm), *E. coli*(9.37±0.18mm) and *P.aeruginosa* (10.17±0.46mm) at the concentration of 600mg/ml. Furthermore, the MIC values for both ethanol and water extracts were from 75 to 100 mg/ml; whereas the MBC values for ethanol and water extracts were from 100 to 150mg/ml and100mg/ml, respectively indicating that the ethanol-extract was slightly more effective than the water extracts. The *in vitro* free radical scavenging activity showed that the aqueous, ethanol and n-hexane extracts showed maximum antioxidant activity of 87.89%, 78.68% and 47.18% at 25mg/ml concentration, respectively. Thus, the plant showed interesting antioxidant activity that could be useful in the management of oxidative stress. Oral administration of crude ethanol extract at the dose of 2000 mg/kg did not show any mortalities or evidence of adverse effects on the Swiss albino mice, implying that *S. punctata* is non-toxic to the animal model.

Keywords: Albino mice, Disc diffusion, MBC and MIC,

1. INTRODUCTION

Infectious diseases are caused by bacteria, viruses, fungi and parasites which are a major threat to public health, despite the tremendous progress in human medicine (Cosa *et al*., 2006). The use of antibiotics in agriculture, livestock and poultry has accelerated the development of antibiotic resistant strains of microbial pathogens, potentially complicating treatment for plants, animals and humans (White *et al*., 2002).

There has been a dramatic increase in microbial resistance to antimicrobial agents for the last three decades (Chopra *et al*., 1996). The overuse and abuse of antibiotics in the treatment of bacterial infections has led to the emergence of multiple drug resistant bacteria (MDR) has become a major cause of failure of the treatment of infectious disease (Gibbons, 2005)

Multi-drug resistance by Gram-positive (*Staphylococcus aureus*), Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) and other bacteria like *Mycobacterium tuberculosis* has been reported from all over the world (Westh *et al*., 2004). These multi-drug resistant bacteria have also created additional problems in cancer and AIDS patients. As a result, the human society is facing one of the most serious public health dilemmas over the emergence of infectious bacteria displaying resistance to many antibiotics (Kapil, 2005).

Resistance development to antibiotics and other problems such as high cost, side effects and others has resulted in an increased interest in plants and plant products as antimicrobial agents. Such situation stimulates the development of new anti-microbial agents in order to treat the infectious disease in an effective manner (Doughari *et al*., 2007).

Traditional societies in Africa and elsewhere have always used herbs to promote healing (Bussmann, 2006).According to Okoli *et al*. (2007), traditional medical practices on the African continent date as far back as 4000 years and were the sole medical system for health care before the advent of modern medicine. There is an increasing awareness and interest in medicinal plants and their preparations commonly known as herbal medicines (Steve *et al*., 2009). Accessibility and affordability of the medicinal herbs have made them as fundamental part of many people's

life all over the world. The selection of medicinal plant is a conscious process, which has led to an enormous number of medicinal plants being used by the numerous cultures of the world (Heinrich *et al*., 2004).

Many plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria. The edible plants that are used for traditional medicine contain a wide range of substance to treat infectious diseases with reduced side effects (Duraipandiyan *et al.,* 2006).

Apart from antagonistic activity against infectious disease, plants have also antioxidant property. The term "antioxidant" refers to numerous vitamins, minerals and other phytochemicals to protect against the effects caused by reactive oxygen species (Robinson *et al*., 2007). Free radicals and other ROS are derived by two processes namely normal metabolic activities in the human body and external sources such as exposure to X-rays, ozone, cigarette smoking, air-pollutants and industrial chemicals. The ROS are highly capable of damaging nucleus and cell membranes by reacting with various vital intracellular molecules like DNA, protein, carbohydrates and lipids.

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants. This imbalance leads to damage of important biomolecules and organs with potential impact on the whole organism. It is associated with pathogenic mechanisms of many diseases including atherosclerosis, neurodegenerative diseases, cancer, diabetes and inflammatory diseases, as well as aging processes.

Antioxidants can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress (Reuter *et al*., 2010).Natural antioxidants have been studied extensively for decades in order to find compounds protecting against a number of diseases related to oxidative stress and free radical-induced damage. To date, many plants have been claimed to pose beneficial health effects such as antioxidant properties (Kaur and Arora, 2009).

In Ethiopia herbal medicine is common and prepared for the treatment of different diseases. Ethnobotanical studies revealed that a wider range of Ethiopian plants are being used in the treatment of wounds and other diseases in the traditional health care system of the country (Mirutse *et al.*, 2007; Fisseha *et al.*, 2009; Tilahun and Mirutse ,2010). It is a source of medical treatment for more than 80% of the population in the country use plant based traditional medicine by indigenous knowledge as their major primary health care system (Dawit , 2001). Crude extracts of Ethiopian medicinal plants and others used elsewhere (Haile , 2005) revealed strong antibacterial activities indicating that these plants can serve as sources of effective drugs against bacterial infections.

The Genus *Satureja* (family Lamiaceae) contain more than 30 species that are widely distributed in Europe, North America and Tropical Africa (Chagonda and Chalchat, 2005). The plant also occurs in South Africa (Sebsebe, 1993). In Ethiopia, the genus is represented by eight species (Hedberg, 2006). Preparations made from the aerial parts of several of these species are used in Ethiopian traditional medicine to treat headache, stop menstruation, relieve stomach pains and improve the quality of milk (Hedberg, 2006).

Satureja punctata (Benth.) Briq. (Lamiaceae), locally known as “Lomishet” (Amharic), is an erect perennial herb having purple (sometimes violet) flowers with a pleasant fragrance. It grows in Ethiopia on dry and often on rocky ground, highly grazed grassland occurring at altitudes of 1800- 3700 m in Shoa, Gondar, Tigray, Wello, Gojam, GamoGofa, Bale, Sidamo, Harerge, Arssi and Keffa Regions (Sebsebe, 1993).

In the traditional health care system of Ethiopia, the aerial parts of *S.punctata* (Benth.) Briq. are used for the treatment of diabetes mellitus and various other ailments (Getahun, 1989; Workneh *et al.,* 2010). The leaves of this plant are macerated and the extract is drunk for the treatment of liver disorders (Dawit, 1993; Hailu *et al.*, 2007; Tesfaye *et al.,* 2010). The preliminary phytochemical analysis of the methanol fraction of S. *punctata* revealed the presence of flavonoids, alkaloids, tannins and poly-phenols (Tesfaye *et al.*, 2010).

The use of plants for medicinal purpose requires preliminary study of extracting plants with different solvents and evaluating their inhibitory effects on test organisms and their toxicity on experimental animals. Although antioxidant activity of methanol extract of *Satureja punctata* was tested (Tesfaye *et al*., 2010). Hence, in this study different extraction solvents of varied polarity (aqueous, ethanol and n-hexane) of crude extracts of aerial part of *Satureja punctata* were evaluated for antibacterial and antioxidant activity test and the toxic properties of the plant extract using animal model.

1.1.Objective

1.1.1. General objective

- ❖ To test the antibacterial, antioxidant and acute toxicity properties of the crude extract of the aerial part of *Satureja punctata* (Benth.) Briq.

1.1.2. Specific objectives

- ➢ To evaluate the antibacterial effect of the crude extract of the aerial parts of *Satureja punctata* on specific pathogenic bacteria.
- ➢ To determine the antioxidant properties of the crude extracts *in vitro* system.
- ➢ To evaluate the acute toxicity of the crude extracts of *Satureja punctata* using mice mode

2. LITERATURE REVIEW

2.1. Disease infection and Test microorganisms

2.1.1. Mechanism of disease infection

Pathogenicity is the ability of a microorganism to infect and cause disease by overcoming the defenses of the host (Spicer, 2000). Virulence refers to a property of the pathogen that enables it to multiply and cause harm to its host (Spicer, 2000). The pathogenicity of an organism depends on its ability to adapt to tissue environment and to withstand the lytic activity of the host defenses and it is aided by virulence factors, either acting together or individually at different stages of infection (Wu *et al.,* 2008).

Although a lot of study has been done on the virulence factors of different pathogens, the virulence and mechanism of pathogenicity of some pathogens are yet to be understood. The discovery of microbial virulence factors plays an important role in understanding microbial pathogenesis and also in the identification of targets for novel drugs (Wu *et al*., 2008). A number of approaches have been made to the discovery of microbial virulence factors and these include comparative genomics, transcriptomics and proteomics (Khan *et al*., 2010; Wu *et al*., 2008).

Generally, four crucial steps are necessary for microbial infections. These are (1) entry and adherence to the host tissue, (2) invasion of the host tissue, (3) multiplication, colonization and dissemination in tissues and (4) evasion of the host immune system and damage to tissues (Khan *et al*., 2010). Virulence factors play different roles in these stages of infection, and can be divided into several groups on the basis of the mechanism of virulence. These include (1) membrane proteins, which plays a role in adhesion, colonization and invasion of host cells; promote adherence to host cell surfaces and are responsible for resistance to antibiotics and promote intercellular communication; (2) polysaccharide capsules that surround the bacterial cell and have anti-phagocytic properties and (3) secretory proteins, such as toxin, which can modify the host cell environment and are responsible for some host cell-bacterial interactions (Wu *et al*., 2008).

Some microorganisms are inhabitants on our skins and play a protective role by keeping pathogenic microorganisms from invading our skin. However, if the microbial balance is disrupted, these microorganisms can cause infections ranging from simple infections to serious conditions depending on the length and depth of tissue exposure as well as on the pathogenicity of the invading microorganism. Disruption of the skin barrier either by cuts or by surgery may introduce the otherwise harmless microorganisms into deeper tissues. The use of broad-spectrum antimicrobials also disturbs the microbial balance of microorganisms resulting in fungal overgrowth. Microbial infections by otherwise harmless microorganisms are also high among people with compromised immunity, people using immunosuppressive agents after organ transplant and cancer chemotherapy patients among others (Spicer, 2000).

Microorganisms may also invade traumatic wounds, surgical wounds, cuts, and burns. Traumatic wound infections are best classified as those infected from skin flora, perforated viscus, water and animal and those infected by soil pathogens. *S. aureus* is of the skin flora that causes wound infections resulting in infections such as erysipelas and cellulitis. Surgical wound infections are caused by infections with *S.aureus*, *E. coli*, and *P. aeruginosa*. Burns can be infected by *S. aureus*, *E. coli* and *P. aeruginosa*. Microbial wound infections may cause wound sepsis, injury to the tissue and consequently interference with the normal functioning of the host and if untreated it may lead to chronic wounds (Spicer, 2000).

2.1. 2. Test microorganisms

2.1.2.1. Escherichia coli

Escherichia coli are a common component of aerobic bowel flora. Depending on the antigens they produce, some *E. coli* strains are pathogenic. *E. coli* strains pathogenic to humans are grouped into six pathotypes, the most important being the enteroadhesive, enteroinvasive, enterotoxogenic and enterohaemorrhagic, of which the last two are considered to be the most severe strains. *E. coli* has been reported to cause a number of infections including urinary tract, wound, lung, meningeal and septicaemic infections. Some *E. coli* strains are also identified as a cause of traveller’s diarrhoea and the uraemic syndrome (Spicer, 2000; Mittal *et al.*, 2009).

The pathogenicity of a particular *E. coli* strain is primarily determined by specific virulence factors which include adhesins, invasions, haemolysins, toxins, effacement factors, cytotoxic necrotic factors, capsules and siderophores (Mittal *et al.,* 2009).

2.1.2.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic gram-negative rod, which is widespread in soil and water. *P. aeruginosa* is an opportunistic and a serious pathogen, especially to patients with burns and genetic lung disease, cystic fibrosis (Spicer, 2000). It was also reported to cause skin diseases Pseudomonas dermatitis and otitis externa, ear infections, wound infections, acute pneumonia and urinary tract infections especially catheter-associated urinary tract infections (Mittal *et al*., 2009).

P. aeruginosa produce endotoxins and exotoxins, though the later accounts for much of its pathogenicity. It also has the ability to form a biofilm and for this reason it is a common cause of hospital-acquired infections due to its ability to form biofilms on medical devices (Spicer, 2000).

Other virulence factors of *P. aeruginosa* include cell-associated factors like alginate, lipopolysaccharide, flagellum, pilus and non-pilus adhesins, as well as secretory virulence factors such as protease, elastase, phospholipase, pyocyanin, hemolysins and siderophores. Depending on the site of infection, different amount of virulence factors are produced. A quantitative study for the production of four virulence factors, elastase, phospholipase C, toxin A and exoenzyme S among strains isolated from wound infections, respiratory tract infections and urinary tract infections showed that, although all four virulence factors were produced in all strains, the amounts differed depending on the site of infection. Wound and urinary tract isolates were found to produce higher levels of elastase and phospholipase C compared to isolates of the respiratory tract infections. Wound isolates also produced significantly higher amounts of toxin A (Mittal *et al*., 2009).

2.1.2. 3. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-spore forming spherical bacterium that belongs to the *Staphylococcus* genus. The *Staphylococcus* genus is subdivided into 32 species and subspecies. *S. aureus* produces staphylococcal enterotoxin (SE) and is responsible for almost all staphylococcal food poisoning (FDA 2012).

Staphylococcus intermedius, a *Staphylococcus* species which is commonly associated with dogs and other animals, can also produce SE and has been rarely associated with staphylococcal food poisoning (Le Loir *et al.,* 2003). *Staphylococcus aureus* is a bacterium that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. *S. aureus* is commonly found in the environment (soil, water and air) and is also found in the nose and on the skin of humans.

Staphylococcus aureus is a leading cause of hospital-acquired infections. It is the primary cause of lower respiratory tract infections and surgical site infections (Richards *et al*., 1999) and the second leading cause of nosocomial bacteremia (Wisplinghoff *et al*., 2004), pneumonia, and cardiovascular infections (Richards *et al*., 1999).

Infections with *S. aureus* are especially difficult to treat because of evolved resistance to antimicrobial drugs. Resistance to penicillin and newer narrow-spectrum β-lactamase–resistant penicillin antimicrobial drugs (e.g., methicillin, oxacillin) appeared soon after they were introduced into clinical practice in the 1940s and 1960s, respectively (Lowy, 2001*)*. Penicillin resistance was initially confined to a small number of hospitalized patients, but resistance spread as use of penicillin increased, first to other hospitals and then into the community(Chambers,2001).By the late 1960s, >80% of community- and hospital-acquired *S. aureus* isolates were resistant to penicillin((Lowy, 2001).

2.2. Natural products

Natural products can either be primary or secondary plant or animal metabolites. Primary metabolites unlike secondary metabolites are essential for biochemical pathways necessary for the normal growth and development of plants or animals (Martin, 1995). Secondary metabolites are derived from primary metabolites through biosynthetic processes, and in most cases, they are restricted to certain taxonomic groups. Secondary metabolites have no role in the normal growth of the plant but, may play important ecological roles depending on the conditions under which they were produced, though some may be just mere waste products from physiological processes. Ecological roles played by plant secondary metabolites include attracting pollinators, acting as chemical defense against disease-causing microorganisms and insects, as well as facilitating survival under environmental stresses (Balandrin *et al*., 1985). In addition, secondary

metabolites are responsible for the characteristic smells, colours, flavours and medicinal properties of plants (Martin, 1995).

Generally, primary metabolites are produced in large amounts compared to secondary metabolites. Due to this, primary plant products constitute a large portion of raw materials in scientific, technological and commercial applications. An example of a scientific application of a metabolite is the study of the effects of a metabolite on the growth of organisms. Commercial applications on the other hand include the use of plant extracts as flavourants, fragrances, and pesticides as well as in pharmaceuticals (Balandrin *et al*., 1985). Because of their various applications, natural products have the potential to alleviate most global crises such as malnutrition, poverty and disease outbreaks (Potier *et al*., 1996).

Plants produce a wide range of secondary metabolites. The medicinal properties of plants are attributed to the presence of secondary metabolites such as terpenoids, steroids, saponins, tannins, flavonoids, alkaloids and phenolic compounds (Mdlolo, 2009).

During the last two decades, more than 50% of new drugs introduced to the market for the use against different ailments were of natural origin (Wuyang, 2008). These drugs are important as they are believed to have the potential to prevent and cure diseases. This has intensified the interest in the study of biological effects, isolation and structure determination of natural compounds. Though studies of this nature were initially complicated, they are made much easier by the establishment of new and comprehensive screening methods (Sarkar *et al*., 1996).

2.3. Medicinal plants

Throughout the ages, humans have relied on Nature for their basic needs for the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours and fragrances, and, not the least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. A plant is said to be medicinal if it produces active compounds which are therapeutically effective (Wuyang, 2008; Khaleeliah, 2001).

In addition to the use of plants as medicine by the Sumerians and Akkaidians (2600 BC), other ancient literature on the use of plants as medicine include the Egyptian Ebers Papyrus, dated

1500 BC upwards, with records of over 700 drugs and the Chinese Materia Medica dating 1100 BC, recording over 600 medicinal plants. The Indian Ayurvendic system dating 1000 BC and Greek about 100 BC are also other records on the ancient use of plants as medicine (Shoeb, 2006).

Traditionally, medicinal plants were used in the treatment of various diseases. Plant parts such as leaves, stems, roots, barks, twigs, tubers, bulbs, exudates, flowers and fruits were all used in the treatment of different ailments. These plant materials are used to prepare enemas, extracts, infusions, teas, snuffs and in many other forms which are administered in different ways (van Wyk and Gericke, 2000).

Enemas are oily or aqueous suspensions introduced rectally. Extracts are preparations containing active principles of a crude drug, prepared by extracting the plant material with a suitable solvent such as water or alcohol. Infusions on the other hand are prepared by soaking of the plant material. Teas are prepared by soaking the plant material in hot water for a few minutes. Snuff constitutes finely powdered medicinal plant material which can be inhaled through the nostrils (van Wyk *et al*., 1997).

Epilepsy, malaria, dysentery, pneumonia, inflammations, ulcers, wounds, cancer and sexually transmitted diseases among others, are some of the conditions and diseases reported to have been treated traditionally with plants (Khaleeliah, 2001; van Wyk and Gericke, 2000).

The use of traditional medicine in primary health care is common place, especially in developing countries (Shoeb, 2006; Chinsembu and Hedimbi, 2010). (Mdlolo, 2009) estimated that up to 80% of the population in most developing countries may be using traditional medicine in primary health care. Developed countries have also developed interest in the use of plants as medicine due to their reduced toxicity, availability and affordability compared to manufactured drugs (Khaleeliah, 2001).

2.4. Antimicrobial Agents

Due to their disease treatment and microorganism elimination features, antimicrobials are very important chemicals. There are a great variety of antimicrobial agents currently available. Before choosing a particular antimicrobial agent to employ against a disease or a particular microorganism, its selective toxicity must be taken into account, due to the fact that it is more important to eliminate the bacteria without harming the host organism. Antibiotics are important biochemicals produced by microorganisms and widely employed in current medical use for a long time in semi-synthetic forms. Unfortunately, uncontrolled use of antibiotics, caused from either patients or prescriptions made without cell cultures analyses, increased resistance of bacteria. Increment in resistance and some other problems caused an increasing interest in antimicrobial plant extracts (Freidman, 2007).

Each and every class of antimicrobial agents represents a unique mode of action against a particular microorganism. These actions are mostly dependent on the type of microorganism, which can be related to the cell structure. As an example membrane structures of gram negative and gram positive have essential differences which totally affect their antimicrobial resistance mechanisms (Holley and Patel, 2005).

Antimicrobial agents from natural products are also used to keep safety and quality of foods, especially in meat products, which led numerous developments in meat preservation. Although, synthetic preservatives have usually been employed for this purpose but their use is limited due to their side effects. That is why, in order to overcome the microbial contamination in meat, the use of bioactive phytochemicals as natural preservatives are more preferred by both customers and food industry (Ahn *et al.,* 2004).

2.5. Plant Derivatives as Antimicrobials

The antimicrobial compounds found in plants are of interest because antibiotic resistance is becoming a worldwide public health concern especially in terms of food-borne illness and nosocomial infections (Hsueh *et al.*, 2005; Mora *et al.*, 2005).

Naturally occurring antimicrobials are being sought as replacements for synthetic preservatives such as parabens (ethyl,methyl, butyl and propyl parabens), butylated hydroxytoluene (BHT) and

butylated hydroxyanisole(BHA) that are under scrutiny as suspected cancer causing agents (Bergfeld *et al*., 2005).

Plants produce a multitude of organic compounds that have antimicrobial activity. The compounds are found in various plant parts such as stems, roots, leaves, bark, flowers or fruits and seeds and include alliin/allicins, isothiocyanates, plant pigments (Cutter, 2000), hydrolytic enzymes, proteins, essential oils (Smid and Gorris, 1999), and phytoalexins or phenolic compounds (Cutter, 2000).

2.6. Free Radicals

Free radicals can be defined as high energy atoms with an extra unpaired electron; any molecule containing one or more unpaired electrons; unstable and highly reactive molecules (due to unpaired electron) and they attack stable molecules and steal an electron-creating a new radical chain reaction. Oxidation metabolism is an essential process for survival of living things, drugs, and foodstuff and yet causes formation of free radicals (Antolovich *et al*., 2002).

Free radicals, as they are unstable, have a tendency of being stabilized in a way of reducing their energy level by transferring their excess electron to nearby substances. As an example, when they are formed within body, they attack nearby tissues by oxidizing membrane lipids, cellular proteins, DNA that causes complete shutdown of cellular activities such as respiration and terminates the cell (Stadtman, 1992).

All radical groups formed during oxidation processes, are primarily responsible for certain diseases involving many organs. For example and unpredictable effect of oxidation in DNA can lead to cancers. Also the following disorders are related to free radical chain reactions: Heart and cardiovascular diseases, lung diseases, alcohol-related diseases, all types of cancers, aging, skin diseases, eye disorders, immune system related diseases, central nervous system diseases, radiation injury, kidney diseases, gastrointestinal diseases (Valko *et al*., 2006; Ali *et al*., 1996).

Free radicals and other reactive species are derived either from normal essential metabolic processes or from external sources, such as exposure to x-rays, ozone, cigarette smoking, air pollutants, industrial chemicals (Bagchi and Puri, 1998).

2.7. Oxidative stress

The term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable (Rock, 1996). Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids (Rock, 1996).

Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g., xanthine oxidase, lipogenase, cyclooxygenase) activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS (Ali *et al*., 1996).

The initiation, promotion, and progression of cancer, as well as the side-effects of radiation and chemotherapy, have been linked to the imbalance between ROS and the antioxidant defense system. ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative diseases such as Parkinson's disease (Rao, 2006).

The negative effects caused by free radicals can be observed not only in the body but also in food materials and drugs. Antioxidants are protective substances against these negative effects and can be found in many natural and synthetic sources (Simone, 1992).

2.8. Antioxidants

According to literature, these are "substance that when present in low concentration compared to those of the oxidisable substrates significantly delay or inhibit the oxidation of that substance (Murthy, 2001). The antioxidant can also be defined as "A compound capable of inhibiting oxygen mediated oxidation of diverse substances from simple molecule to polymer and complex bio-systems (Chitang Ho, 1994).

Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers, and helps in converting the radicals to less reactive species. A

variety of free radical scavenging antioxidants is found in dietary sources like fruits, vegetables and tea, etc. (Mandal *et al*, 2009)

2.9. Plant Derivatives as Antioxidants

Antioxidants have an essential role in body defense system against Reactive Oxygen Species (ROS). Natural antioxidants that are present in the food increase the resistance toward oxidative damages and they may have an essential impact on human health. Therefore, consumption of food that is containing phytochemical with potential antioxidant properties can decrease the danger of human diseases. Chain breaking antioxidants are highly reactive with free radicals and form stable compounds that do not contribute to the oxidative chain reaction (Akira *et al*., 2012).

In other way an antioxidant can be defined as a molecule that is capable of slowing or preventing the oxidation of other molecules. Antioxidants are often reducing agents such as thiols or polyphenols. They are believed to play an important role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, and cataracts (Swati *et al*., 2012).

2.10. DPPH free radical scavenging assay

The antioxidant activity can be measured using several methods. Among them DPPH free radical scavenging assay (DPPH) was used in this study. The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants (Kuma *et al*., 2012; Suma and Urooj, 2012).

This method is simple, rapid and measures the capacity of herbal extract to bleach the DPPH radical. The method is sensitive and requires small amount of samples (Junaid *et al*., 2013; Pavithra *et al*., 2013).In recent years, various plant species have been tested for antioxidants activity using DPPH assay (Ho *et al*., 2012; Belmekki and Bendimerad, 2012)

2.11. Use of traditional medicine in Ethiopia

Traditional medicine refers to any ancient, culturally based healthcare practice different from scientific medicine and it is commonly regarded as indigenous, unorthodox, alternative or folk and largely orally transmitted practice used by communities with different cultures (Cotton, 1996). WHO also defined traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises applied to treat, diagnose and prevent illnesses or maintain well being (WHO,2003).

Knowledge of the medicinal plants of Ethiopia and their uses provide wide and vital contribution to human and livestock healthcare needs throughout the country (Mirutse and Tilahun, 2013). These wide and vital uses of traditional medicine in the country could be attributed to cultural diversity and acceptability, psychological comfort, economic affordability, and perceived efficacy against certain type of diseases as compared to modern medicines (Ketema *et al.,* 2013; Wolde *et al.,* 2011).

In Ethiopia, 80% of the people use medicinal plants and plant remedies selected over centuries. Moreover, medicinal plants remain the most important and sometimes the only source of therapeutics (Dawit, 2001). Medicinal plants play a key role in the development and advancement of modern studies by serving as a starting point for the development of novelties in drugs (Pramono, 2001). The knowledge and use of plants is an integral part of many ethnic rural cultures in Ethiopia, the extent of which has not yet been studied in depth (Abbink, 1995).

2.12. *Satureja punctata*

The genus *Satureja***,** in the family Lamiaciaece has about 30 species distributed in tropical Africa, Europe and North America (Mabberky, 1987*)*. *Satureja* species are well known medicinal plants of *Lamiaceae* family. Due to presence of secondary metabolites such as flavonoids, steroids, terpenoids and tannins they are known for their healing properties for a long time and have been used as traditional folk remedies to treat various ailments such as cramps, muscle pains, nausea indigestion, diarrhoea and Infectious diseases (Bezi *et al*., 2009).

Essential oils obtained from the leaves and flowers of different *Satureja sp*. are commonly used in various industrial applications as flavoring material, medicine, and by perfumers (Solomon *et*

al., 1998). The essential oil of *S.punctata* (locally known as “Lomishet”, Amharic) has been shown to contain 67 compounds, of which geranial and neral are the main components with significant antiprotozoal activities (Yinebeb *et al*., 2010). The total hydroalcoholic extract of *S. punctata* and its methanolic and aqueous fractions are also reported to possess hypoglycaemic activity (Workneh *et al*., 2009). The lemon-scented leaves of S. *punctata* are used to flavor fish dishes (Sebsebe, 1993*)*

3. MATERIALS AND METHODS

3.1. Plant material Collection and Authentication

The plant material, *Satureja punctata* were collected in December 2014 from Entoto, which is located at an altitude of 2720m above sea level (Latitude $09^0$04'48.7" and Longitude 038^0 45' 08.7") and Sendafa which is located at an altitude of 2586m above sea level (Latitude $09^0$09'41.1" and Longitude $039^0$01'08.7"). The plant was authenticated by a botanist and representative sample with the specimen number MM01 was kept at the Natural Herbarium of Addis Ababa University (AAU), Addis Ababa, Ethiopia.

3.2. Preparation and Extraction of crude extract

Laboratory based experimental study was conducted in biomedical laboratory, College of Natural Sciences, Addis Ababa University. The collected plant materials were washed thoroughly with tap water to remove dust particles and spread over newspaper and then kept in an open shady area for 14 days in order to avoid loss of volatile compounds by direct sun light. After complete dryness, the plant samples were grounded using a coffee grinder into a fine powder and then sieved using a mesh of 0.5mm mesh size and about 500g of powder was obtained and stored in an airtight closed bottle for further use.

The crude extracts were prepared by cold maceration technique (O'Neill *et al.*, 1985). The dried powder (100g) was extracted by soaking with ethanol (1000ml), n-hexane (1000ml) and aqueous (1000ml) separately(1/10 W/V) in conical flask plugged with cotton wool and wrapped with the aluminum foil using orbital shaker at 120rpm for 72hrs at room temperature. The extracts were filtered through a cotton plug followed by a qualitative filter paper (Whatman filter paper No. 1, 150mm size with retention down to 0.7 µm in liquids; Whatman Ltd., England).

After filtration, the n-hexane and ethanol extracts of plants were subjected to partial concentration using a rotary evaporator (Buchi, Switzerland) attached to a vacuum pump and set in a water bath at 45°C. The partially concentrated extracts contained in screw capped bottle were placed in oven at 40ºC to dry completely. The aqueous extracts of plants were placed in deep-freeze at -20ºc for 24 hr and then allowed to lyophilization to obtain fine crude extract. The

powder (dry residues) of n-hexane, ethanol and aqueous extracts were weighed and after that, the yield of the extracted samples was calculated using the following equation:

$$\text{Percent of yield extraction} = \frac{\text{Final weight(g)}}{\text{Initial weight(g)}} \text{X100s}$$

The yield of n-hexane 2.11gm (4.63%), aqueous 5.10gm (10.19 %) and ethanol 6.28gm (12.56%) were obtained, labeled and stored in refrigerator at 4ºC for further use. All extracts were redissolved with their respective solvent for antibacterial test and they were dissolved in methanol for antioxidant activity tests.

3.3.Test Microorganisms

Standard bacteria culture of gram positive *Staphylococcus aureus (*ATCC 25923*),* and Gram-negative *pseudomonas aeruginosa (*ATCC 27853*), Eschechia coli* (ATCC 25922*)* were obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia.

3.4.Preparation of inoculums

All the test strains were transferred into sterilize nutrient broth and incubated at 37ºc for 24hrs and in the next day, using sterilize loop, streaked in 90mm petridishes which contained sterilized selective media and incubated at 37ºc for 24 hrs and the purity was checked. Four to five well-isolated colony of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 10ml of 0.85% sterile normal saline to dilute the suspension. The turbidity of the 0.5 McFarland standard was prepared by mixing 0.5 ml of 1.75% (w/v) Barium Chloride dehydrate with 99.5 ml 1% (v/v) Sulfuric Acid (H_2SO4) (Lalitha, 2008). The turbidity of the actively growing bacteria suspension was adjusted with sterile saline to obtain turbidity comparable to that of the 0.5 McFarland standards. This results in a suspension containing approximately1.5 x 10^8 CFU/ml.

3.5. Determination of Antibacterial Activity

The antibacterial activity of ethanol and aqueous extracts was evaluated using disc diffusion method (NCCLS, 1997). 25ml of sterilized Muller Hinton Agar (Oxoid Ltd. Basingstoke, Hampshire England) was poured in 90mm Petridishes and was allowed to solidify. The plates were seeded with suspension of test bacteria adjust to 10^8cells/ml using sterile cotton swab and

allowed to set for 10mins. Sterile, 6mm diameter filter paper discs were soaked each within plant extracts at different concentration (600mg/ml, 400mg/ml and 300mg/ml) and placed on the surface of inoculated media agar plates using sterile forceps and then gently pressed down onto the agar surface. Disk soaked with the solvents and Tetracycline 30µg/disc (Oxoid Ltd. Basingstoke, Hampshire England) were used as negative and positive controls, respective. All plates were incubated at 35-37ºC for 24h. Clear inhibition zones around the discs indicated the presence of antibacterial activity. Diameter of inhibition zones was measured in millimeters. Each experiment was carried out in triplicates and the mean diameter of the inhibition zones was recorded for each test organism.

3.6.Determination of MIC (Minimum inhibitory concentration) values

The minimum inhibitory concentration of ethanol and aqueous extract of the plant was determined by agar dilution technique of CLSI (CLSI, 2002). Series of two folds dilution of ethanol and aqueous (300mg/ml- 37.5mg/ml) extracts each was mixed with 19ml of sterilized and molten nutrient agar medium and 1ml of each concentration was poured into pre-labeled sterile petridishes. Plates were dried at 35ºC for 30minutes prior to spot inoculation with bacterial suspension (adjusted to 0.5 Mac Farland standards) containing approximately $1.5x10^{8}$ CFU/spot using a sterilized inoculating loop. Nutrient agar with solvent was used as positive control. The inoculum spots were allowed to dry at room temperature and plates were incubated at 35-37ºC for 24h. Each test was done in triplicate. Growth inhibition was judged by comparison with growth in control plates placed without the extracts.

3.7. Determination of MBC (Minimum bactericidal concentration) values

The MBC of the extracts on the test isolates was determined according to (Mishra *et al.*, 2008). Fresh nutrient agar medium was poured into Petriplates and allowed to solidify. Inoculum from various concentration plates of MIC experiment showing no growth was subcultured on freshly prepared plates. The lowest concentration at which the test bacteria were did not recovered on fresh medium was the MBC.

3.8. *In Vitro* Antioxidant Activity

The radical scavenging activity of plant extracts was determined on the basis of the radical scavenging effect on the DPPH free radical (Blois, 1958). 1ml of different concentrations (1.5625, 3.125, 6.25, 12.5 and 25mg/ml) of each extract was mixed with 2ml of DPPH 0.1mM solution in methanol in labeled tubes. The tubes were incubated in dark for 30 minutes at room temperature and the absorbance was measured at 517nm using UV-Vis spectrophotometer 201215(Single Beam, India). Ascorbic acid at concentration of (1.5625, 3.125, 6.25, 12.5 and 25mg/ml) was used as standard control. The control was prepared by mixing 2 ml of DPPH solution with 2 ml Methanol. The experiment was done in triplicates. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation

$$\%\ \text{DPPH radical scavenging capacity} = \frac{\mathbf{A}\ \text{control} - \mathbf{A}\ \text{sample}}{\mathbf{A}\ \text{Control}}\ \text{X}100$$

Where; **A** control is the absorbance of DPPH radical + methanol reaction;

A sample is the absorbance of DPPH radical + sample extract /standard

The antioxidant activity of the different extract was expressed in % inhibition

3.9. Toxicity Test

Experimental animals

Female Swiss albino mice weighing 20-25g and 8-12 weeks old were obtained from the animal house of the College of Natural Sciences, Addis Ababa University. A total of 15 female animals were randomly selected, marked and distributed to permit individual identification, and kept in their cages for at least 5 days prior to dosing. All animals were housed in an air-conditioned room and were allowed to acclimatize for at least 5 days prior to dosing. They were fed with pellet diet and water *ad libitum*. The animals were kept at room temperature and were exposed to a 12-h light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996).

Acute toxicity study

The acute oral toxicity of the crude ethanol extracts of *Satureja punctata* was evaluated in mice using OECD guideline 420(2001). Animals were divided into three dosage groups with 5 animals per dose. The control (first) group received 0.2ml of the 3% Tween 80 vehicle. The second and third groups were given 0.2ml of a single dose of 300 mg/kg body weight and 2000 mg/kg body weight of *S. punctata,* respectively. Gavage dosing was performed using a curved, ball-tipped intubation needle affixed to a 1 ml syringe. All solutions were prepared just prior to dosing and were kept chilled and tightly capped. Body weight, food, and water consumption were monitored daily. Animals were left to fast approximately 3-4hr hours prior to dosing and were left to fast 1-2 hours after dosing. Following administration of a single dose of plant extracts, the animals were observed for behavioural changes and general toxicity signs. The result was recorded for the first 30 minutes and at hourly intervals for 4 hours and thereafter for a total of 14 days. Body weight was recorded on Day 0 (before dosing), Day 7 and Day 14.

3.10. Data Analysis

The data obtained for antibacterial and antioxidant tests were analyzed with Microsoft office Excel 2007. Results were expressed as mean ± SEM. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Post Hoc Multiple Comparison Tests using statistical software (SPSS) package version 20.0 for windows and P values < 0.05 were considered as significant.

4. RESULTS

4.1. Yield of Extraction

The yields in grams and percentage (w/w) of n-hexane, ethanol and aqueous extracts of the aerial parts of *S. punctata* were 2.11gm (4.63%), 6.28gm (12.56%) and 5.10gm (10.19%), respectively (Table 1)

Table 1. Yield of *S. punctata* extracted by ethanol, n-hexane and aqueous

Extract	Yield(g)	Yield (%)
Ethanol	6.28	12.56
Aqueous	5.10	10.19
n-hexane	2.11	4.63

4.2. Determination of Antibacterial Activity

The result of the antibacterial activity of different solvent extracts of *S. punctata* at various concentrations against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* is given in (Table 2). The evaluation of antimicrobial potential by disc diffusion method indicated that all the bacterial tested showed growth inhibition toward the plant extract.

The data showed that the ethanol extract at a concentration of 600mg/ml more or less had no significant difference antibacterial activity when compared to aqueous extract at the same concentration against *S. aureus*. The ethanol extract showed maximum antibacterial activity against *S. aureus* (10.33±0.41), *E. coli* (9.37±0.18) and *P. aeruginosa* (10.17±0.46) at the concentration of 600mg/ml. At the concentration of 400mg/ml it showed 9.04±0.22, 8.54±0.29 and 8.43±0.28mm zone of inhibition against *S. aureus, E. coli* and *P. aeruginosa,* respectively.

The crude aqueous extract showed minimum antibacterial activity against *S. aureus* (8.01±0.29), *E. coli* (7.79±0.23) and *P. aeruginosa* (7.72±0.27) at the concentration of 300mg/ml when compared with the concentration of 600mg/ml and 400mg/ml of ethanol extract indicating that antibacterial activity of the extract was increased as the concentration increased. The inhibitory

activity of the crude ethanol extract of the aerial part of *S. punctata* was not as potent as the positive control (Tetracycline) except ethanol extract at the concentration of 600mg/ml against *P. aeruginosa* with inhibition zone of 10.17±0.46mm. The results obtained from crude ethanol extracts of aerial part of *S. punctata* showed that there was a significance difference (at $P<0.05$) in the inhibition of all test organisms at the concentration of 300mg/ml, 400mg/ml and 600mg/ml indicating that antibacterial activities was a concentration dependent

.

The data showed that the zone of inhibition for aqueous and ethanol extracts of the aerial part of *S. punctata* against *S. aureus* increase to some extent with increasing the concentration. The crude ethanol extract was more potent than that of crude aqueous extract against *P. aeruginosa* at the concentration of 600mg/ml with the zone of inhibition 10.17±0.46mm but less potent against *E. coli* at a concentration of 400mg/ml with the zone of inhibition 8.54±0.29mm.

The aqueous extract showed maximum antibacterial activity at a concentration of 600mg/ml against *S. aureus* (10.33±0.25), *E. coli* (9.83±0.19) and *P. aeruginosa* (8.53±0.12). The inhibition of crude aqueous extract of the aerial part of *S. punctata* was less potent against *S. aureus, E. coli* and *P.aeruginosa* compared to positive control (Tetracycline) which mean that positive control was highly growth inhibitor but the negative control did not show any zone of inhibition against *S. aureus, E. coli* and *P.aeruginosa* (table 2).

At the concentration of 400mg/ml the crude aqueous extract showed zone of inhibition against *S. aureus* (9.07±0.13), *E. coli* (8.67±0.11) and *P. aeruginosa* (7.83±0.15) indicating that *S.aureus* was more sensitive than the other bacteria. The aqueous extract showed minimum antibacterial activity against *S. auerus* (8.01±0.29), *E. coli* (7.79±0.23) and *P. aeruginosa* (7.27±0.27) at the concentration of 300mg/ml when compared with the concentration of 600mg/ml and 400mg/ml of aqueous extract.

The n-hexane extract of *S. punctata* at various concentrations showed no antibacterial activity against the tested pathogenic microorganisms. On the other hand, crude ethanol and aqueous extracts at various concentrations showed different antibacterial activity with different zone of inhibition (Table 2). Among the tested bacteria, the growth of *P. aeruginosa* showed the

minimum zone of inhibition (7.27±0.27) when comparing with the other bacteria strains at a concentration of 300mg/ml while *S.aureus* showed maximum zone of inhibition at a concentration of 600mg/ml of aqueous and ethanol extracts 10.33±0.25mm and 10.33±0.41mm, respectively. The antibacterial potential of crude aqueous and ethanol extracts of aerial part of *S. punctata* was slightly dose-dependent against all tested pathogenic microorganisms.

The standard drug tetracycline showed the inhibition zone of 25.00±0.00, 22.33±1.4 and 10.17±0.17mm for *S aureus, E. coli and P. aeruginosa,* respectively. Inhibition of test bacteria by tetracycline was 2-3 times higher when compared to ethanol and aqueous extracts (Table 2). Negative controls did not show inhibitory action on any of the test organisms, while positive controls significantly inhibited growth of all test organisms except *P. aeruginosa* at the concentration of 600mg/ml of crude ethanol extract.

Table 2**:** Antibacterial activity of *S. punctata* at different concentration

Plants	Types of solvent/extracts	Concentration(mg/ml)	Zone of inhibition(mm)		
			S. aureus	*E.coli*	*P. aeruginosa*
Satureja Punctata	Aqueous	300	8.01 ± 0.29^{c}	7.79 ± 0.23^{c}	7.27 ± 0.27^{c}
		400	9.07 ± 0.13^{b}	8.67 ± 0.11^{b}	7.83 ± 0.15^{b}
		600	10.33 ± 0.25^{a}	9.83 ± 0.19^{a}	8.53 ± 0.12^{a}
	Ethanol	300	8.17 ± 0.39^{c}	7.93 ± 0.17^{c}	7.73 ± 0.32^{c}
		400	9.04 ± 0.22^{b}	8.54 ± 0.29^{b}	8.43 ± 0.28^{b}
		600	10.33 ± 0.41^{a}	9.37 ± 0.18^{a}	10.17 ± 0.46^{e}
	n-hexane	300	-	-	-
		400	-	-	-
		600	-	-	-
+ve control (Tet)		30 µg/disc	25.00 ± 0.00^{d}	22.33 ± 1.45^{f}	10.17 ± 0.17^{e}
-ve Control			-	-	-

Data represented as mean±SEM. (N=3). Values followed by different letters indicate statistically significant

(-) in the Table showed no inhibitory effects

4.3. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC values of the different bacteria are shown in (Table 3). Ethanol extract of *S. punctata* had MIC values of 100mg/ml, 75mg/ml and100mg/ml against *S. aureus, E. coli* and *P. aeruginosa*, respectively. The MBC values, which were determined by sub-culturing the samples having dilution values of greater or equal to MIC values, were described in (Table 3). The MBC values of the ethanol extract against *S. aureus, E. coli* and *P. aeruginosa* were 150mg/ml, 100mg/ml and 150mg/ml, respectively.

The MIC values of aqueous extract of *S. punctata* against *S. aureus, E. coli* and *P. aeruginosa* were 75mg/ml, 75mg/ml and 100mg/ml, respectively. The MBC values of the aqueous extract ranged from 100mg/ml for *S. aureus* and *P. aeruginosa* to 150mg/ml of *E. coli* (Table 3).

Table 3: MIC and MBC (mg/ml) of Ethanol and Aqueous Extract of *S*`. *Punctata.*

Test organisms	Ethanol Extract (mg/ml)		Aqueous Extract(mg/ml)	
	MIC	MBC	MIC	MBC
S. aureus	100	150	75	100
E. coli	75	100	75	100
P. aeruginosa	100	150	100	150

4.4. *In vitro* antioxidant activity test

4.4.1. DPPH Free radical scavenging activity

The result of *in-vtro* antioxidant activity was presented in (Table 4 and Figure 1).Accordingly to solvent extracts of *Satureja Punctata* exhibited an antioxidant activity in relation to standard ascorbic acid at varying concentration tested (1.5625, 3.125, 6.25, 12.5, 25mg /ml).

The data showed that the aqueous extract at a concentration of 1.5625mg/ml showed a percentage inhibition of 26.05% and whereas at a concentration of 25mg/ml it was 87.89% (Table 4 and Figure 1) indicating an increase in percentage of inhibition was observed at the increase in the concentration of aqueous. The percentage inhibition of the aqueous extract at the concentration of 3.125mg/ml and 25mg/ml was lower than the standard ascorbic acid whereas at the concentration of 1.5625mg/, 6.25 and 12.5mg/ml the percentage inhibition of aqueous extract was higher than the standard ascorbic acid at the same concentration. The aqueous extract exhibited highest DPPH radical scavenging activity as compared to the ethanol and n-hexane extracts at the concentration of 25mg/ml that was 87.89% (Table 4 and Figure 1).

The result of the study showed that the ethanol extract at a concentration of 1.5625mg/ml showed a percentage inhibition of 32.05% and 78.67% at a concentration of 25mg/ml. An increase in percentage of inhibition was observed as a function of increase in the concentration of ethanol extract. The percentage inhibition of ethanol extract at a concentration of 25mg/ml was lower than the standard ascorbic acid at the same concentration (Table 4 and Figure 1).

The n-hexane extract at a concentration of 1.5625mg/ml showed a percentage inhibition of 25.1% and 47.18% at a concentration of 25mg/ml (Table 4 and Figure 1). When comparing percentage inhibition of n-hexane extract with the standard ascorbic acid in all concentration, it was showed that lower percentage of inhibition but there was an increased percentage of inhibition was observed as the concentration increased.

The highest radical scavenging effect was found in aqueous extract followed by ethanol extract and then n-hexane at the concentration of 25mg/ml 87.89%, 78.67% and 47.18%, respectively. There was a dose dependant increase in the percentage antioxidant activity for all concentrations tested.

Table 4:Antioxidant activity of Aqueous, Ethanol and n-hexane extracts of *Satureja punctata* and Standard Ascorbic acid

Concentration(mg/ml)	% inhibition of Aqueous	% inhibition of Ethanol	% inhibition of n-hexane	% inhibition of Ascorbic Acid
1.5625	26.05	32.05	25.13	24.36
3.125	32.41	39.48	32.31	37.95
6.25	57.28	58.36	41.54	50.77
12.5	75.23	67.49	43.08	65.64
25	87.89	78.67	47.18	96.00

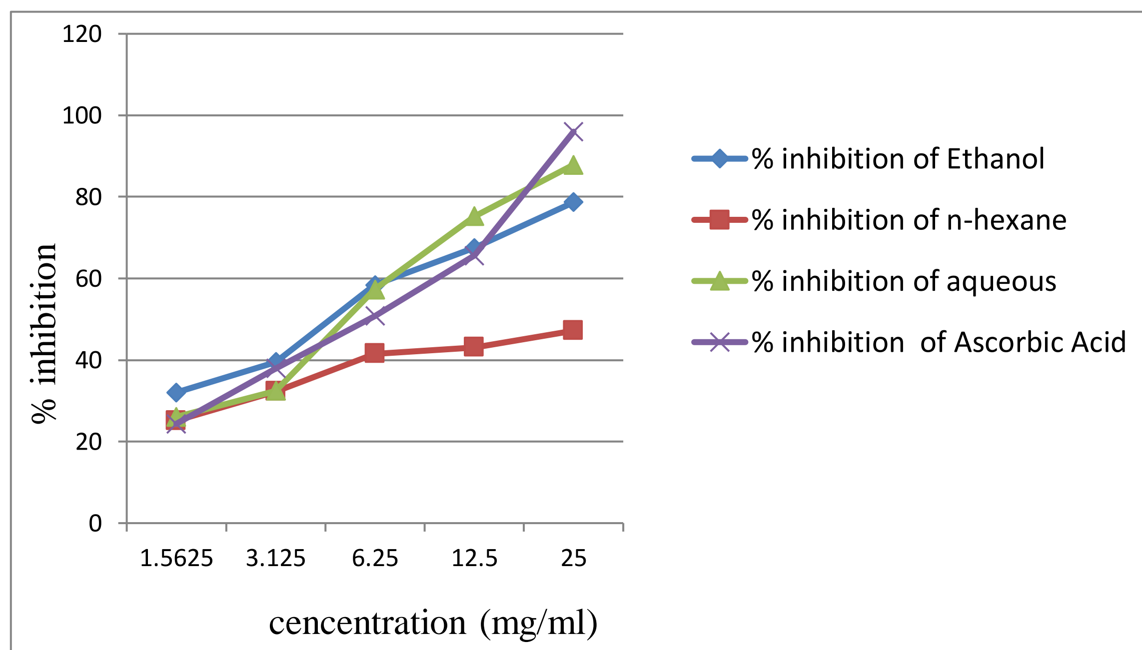

Figure 1. Antioxidant activity of aqueous,ethanol and n-hexane extract of *S.punctata*

4.5. Acute toxicity study

The mice did not show any signs of toxicity or change in general behavior or other physiological activities like grooming, hyperactivity, sedation, respiratory arrest, convulsions, motor activity when observed initially after every one hour for 4 hrs, then 24, 48, 36 and 72 hours after administration of 300mg/kg and 2000mg/kg body weight of the extract. A dose up to 2000 mg/kg of ethanol extract of *Satureja punctata* did not cause any mortality in mice during 72 hour observation period. The mice did not show any signs of toxicity or change in general behavior or other physiological activities during this period.

5. DISCUSSION

In this study the solvent type and the extract concentration showed the different zone of inhibition on the three test bacteria. The result indicated that the crude aqueous and ethanol extracts showed statistically significant zone of inhibition (at $P<0.05$) against *S. aureus, E. coli* and *P. aeruginosa* at all the tested concentration but slightly in a concentration dependent manner (Table 2). The ethanol extract in general showed slightly higher growth inhibition than aqueous extract against all test bacteria.

The results showed that the mean zone of inhibition produced by crude ethanol extract of the aerial part of *S.punctata* against all test organisms except *P. aeruginosa* at the concentration 600mg/ml was lower than that produced by positive control (Tetracycline).This may be attributed to the fact that the plant extracts being in crude form contain smaller concentrations of bioactive compounds (Chew *et al*., 2012).

In this study the aqueous extract inhibited the growth of *E. coli* (Table 2) which is contrary to the findings of Serrano *et al.,* 2011 where aqueous extracts of the aerial part of *Satureja Montana* L. did not show any effect on *E. coli* using disc diffusion method. Similarly, the MIC of the crude ethanol extract of the same plant on *E. coli* (75mg/ml) was much higher than that of the report (15.10mg/ml) Serrano *et al.,* 2011.

Likewise, the inhibitory activity of the lowest concentration of ethanol and aqueous extracts of *Satureja punctata* on *Pseudomonas aeruginosa* at 300mg/ml was 7.27±0.21mm and 7.73±0.32mm, respectively. This was much lower than, the antimicrobial effect of 40mg/ml ethanol and aqueous extracts of *Satureja bachtiarica* with zones of inhibitions of 8.8±0.28 mm 6.4±0.5mm against *Pseudomonas aeruginosa*, respectively reported by Sureshjani *et al.,* (2013).

Similarly, Stanojkovic *et al.,* (2013) reported the antimicrobial activity of methanol extract of aerial part of *Satureja kitaibelii* Wierzb. Ex heuff with MIC and MBC values against on *S. aureus*(0.625/2.5mg/ml), on *E.coli* (0.313/2.5mg/ml) *and* (1.25/2.5mg/ml against *P.aeruginosa*, respectively . The MIC values obtained in the present study were much higher

than the MIC values of ethanol extract of *Agave picta* against *E. coli* (6 mg/mL) and S. aureus (7 mg/mL) reported by Veràstegui *et a.l,* 2007 showing that the solvents extract of *S. punctata* were much less effective (12 times). In general, the MBC values were found to be higher than corresponding MIC values. In some other studies, the MBC values of methanol extract of aerial parts of *Lythrum salicaria* (Becker *et al*., 2005) and aerial parts of *Zuccagania punctata* (Zampini *et al*., 2005) were identical to the corresponding MIC values.

Sahin *et al.,* 2003 also tested MIC values of methanol and hexane extracts of aerial part of *Satureja hortensis* L. against *E. coli* using micro dilution assay and found that the MIC values of methanol extract was 250-500 µg /ml but hexane extract did not show any inhibition at all tested concentrations. Based on these results, they conclude that methanol extract has strong antimicrobial activity as compared to hexane extract. Their observation confirmed the evidence in a previous study reported that methanol is a better solvent for extraction of antimicrobial substances from medicinal plants than water, ethanol and hexane (Ahmad *et al.,* 1998).

Amanlou *et al.,* 2004 compared the antibacterial activity of crude methanol extract of wild and cultivated *Satureja Khuzistanica Jamzad* (SKJ) and found the extract of wild plant to be stronger probably due to the presence of isoeugenol. Gulluce *et al*., (2003) assayed zone of inhibition of crude methanol extract and also determined the MIC values of crude methanol extract of *Satureja hortensis* against *P. aeruginosa* and was found that zone of inhibition for crude methanol extract was 11mm, while MIC values of crude methanol extract was 250mg/ml.

According to Sujana *et al*., (2013), antibacterial activity of ethanol and hexane extract of leaves of *Mentha piperta* L. against *S.aureus* and *E.coli* showed that ethanol extract with zone of inhibition against *S. aureus* and *E. coli* 10.3mm and 11.2mm, respectively, while hexane extract showed zone of inhibition against *S. aureus* (7.2mm) and *E. coli* (10.6mm). This is in contrast with the present study in which the crude n-hexane extract of aerial part of *Satureja punctata* did not show any zone of inhibition against *S. aureus*, *E.coli* and *P.aeruginosa*.

As Sureshjani *et al.,* 2013 reported that, antimicrobial effect of ethanol and aqueous extracts of *Satureja bachtiarica* against *Pseudomonas aeruginosa* at a concentration of 40mg/ml showed

that the zones of inhibition for ethanol extract 8.8±0.28mm and for aqueous extract 6.4±0.5mm against *Pseudomonas aeruginosa*. When compared with this report, in this study crude aqueous extract of aerial part of *S. punctata* was evaluated at the concentration of 300mg/ml, 400mg/ml and 600mg/ml against *Pseudomonas aeruginosa* using disc diffusion method and was showed that zone of inhibition 7.27±0.21mm, 7.83±0.15mm and 8.53± 0.12mm against *P. aeruginosa,* respectively. At the same time, crude ethanol extract of aerial part of *S. punctata* was evaluated against *P. aeruginosa* at the concentration of 300mg/ml, 400mg/ml and 600mg/ml and was found that the zone of inhibition7.73±0.32mm, 8.43±0.28mm and 10.17±0.46mm, respectively.

In this study crude n-hexane extract of aerial part of *S.punctata* did not show any zone of inhibition against test organisms which is similar to the report of Mann, 2012 where hexane fraction of *Ocimum gratissimum* L. did not showed that zone of inhibition for *S. aureus* and *E. coli.* Goyal and Kaushik, (2011) reported that hexane extract of leaves of *Ocimum Sanctum* against *S. aureus* and *E. coli* was found completely inactive against test bacteria which is similar to the present study in which crude n-hexane extract of aerial part of *S. punctata* did not show any zone of inhibition against test bacteria

Sahin *et al.,* 2003 evaluated the antimicrobial activities of hexane extracts of aerial parts of *Satureja hortensis* L. against *E. coli*, *P. aeruginosa* and *S. aureus* using disc diffusion assay at the concentration of 300 µg/disc and was found that hexane extract did not show any zone of inhibition for three test microorganisms. This agrees with the present study that crude n-hexane extracts of aerial part of *Satureja punctata* at the concentration of 300mg/ml, 400mg/ml and 600mg/ml did not show any zone of inhibition against *E. coli*, *P. aeruginosa* and *S. aureus*.

In this study, antioxidant activity of the crude aqueous, ethanol and n-hexane extracts of aerial part of *S. punctata* was determined using DPPH and showed that the free radicals scavenging activity in a concentration dependent manner and the minimum and maximum percentage inhibition of DPPH at the concentration of 1.56mg/ml and 25mg/ml were 26.05% and 87.89%, 32.05% and 78.67%, 25.13% and 47.18% for aqueous ,ethanol and n-hexane, respectively indicating that the percentage inhibition of DPPH was concentration dependent. Tesfaye *et al.,* 2010 also studied free radical scavenging activity of methanol fraction of aerial parts of *S.*

punctata using DPPH and found that *S. punctata* scavenge free radicals in a concentration dependent manner and the maximum percentage inhibition of DPPH was 88.1% at a concentration of 10mg/ml.

At the concentration of 1.5625mg/ml, 6.25mg/ml and 12.5mg/ml crude aqueous extract of the aerial part of *S. punctata* showed better antioxidant activity than that of ascorbic acid at the same concentration. Also, the crude ethanol extract of the aerial part of *S. punctata* showed better antioxidant activity than that of ascorbic acid at the concentration of 1.5625mg/l, 3.125mg/ml,6.25mg/ml and 12.5mg/ml.

The results obtained from the antioxidant activity of crude ethanol extracts of *Satureja hortensis* indicated that the extract showed antioxidant activity in a dose dependent manner by different capability(Bahramikia *et al.,* 2008).

Rosa and Yoia, 2010 evaluated the antioxidative potential of the methanol extract of leaves of *Satureja macrostema* using DPPH assay and also compared the DPPH radical scavenging activity of the methanol extract of leaves of *S. macrostema* with that of ascorbic acid and reported that the DPPH radical scavenging abilities of the methanol extract (89.78%) which was less than that of ascorbic acid (97%) at the concentration of 10mg/ml.

In this study, crude ethanol extract of the aerial parts of *S.punctata* was evaluated for acute toxicity study using Swiss albino mice as per OECD guideline 420 and showed that *S. punctata* did not show toxic effect in mice model. This finding confirmed the evidence in a previous study reported that evaluation of acute toxicity effect of crude aqueous extract of the aerial parts of *S. punctata* in a dose of 2000mg/kg body weight did not produce changes in behavior (Tesfaye *et al*., 2010). According to Rosa and Yoia, 2010, evaluation of acute oral toxicity of *Satureja macrostema* using Swiss albino mice as per guideline 420 did not produce any mortality up to 4000mg/kg body weight.

6. CONCLUSION

The study showed that the aqueous and ethanol extracts of the aerial part of *Satureja punctata* have got significant antibacterial and antioxidant effect but n-hexane did not show antibacterial activity.

Ethanol was a better solvent for extraction of antibacterial and antioxidant substances for the plant compared to the other solvents by providing slightly higher yields, antibacterial and antioxidant activities. The extracts obtained using a high polarity solvent (aqueous) were considerably more effective radical scavengers than those using less polarity solvent (n-hexane), indicating that antioxidant or active compounds of different polarity could be present in the aerial part of *S. punctata.* The free radical scavenging property may be one of the mechanisms by which this plant is effective in its ethno-pharmacological uses against different ailments.

This study supported the view that certain medicinal plants are promising sources of potential antibacterial and antioxidants and may be effective as preventive agents in the pathogenesis of some diseases.

7. RECOMMENDATION

- Antibacterial tests are recommended to be done on more strains of bacteria since in this study only three bacteria were tested
- The antibacterial activity test was conducted by the disc diffusion method for all the solvent extracts. It is recommended to carry out antibacterial tests by other methods such as the agar well diffusion method for all the extracts to check if there are differences in activity.
- Evaluation for antioxidant activity using other methods (e.g. various biochemical assays both *in vivo* and *in vitro*) are essential to characterize them as effective antioxidants.
- Further study on fractionation and possibly isolating active compounds justifies the effectiveness of the plant as antibacterial and antioxidant.

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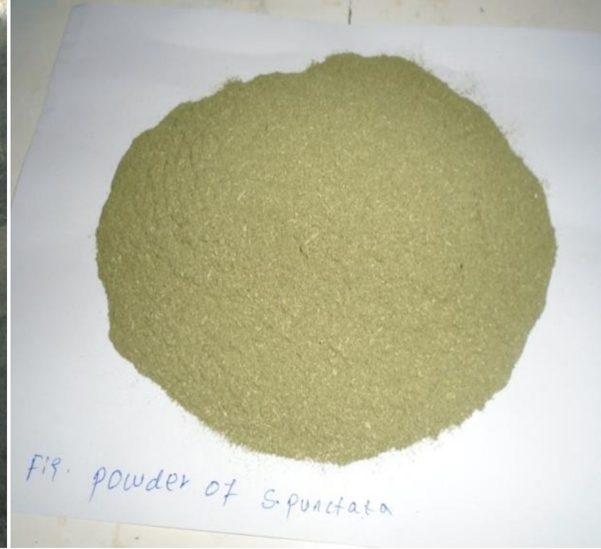
9. APPENDICES

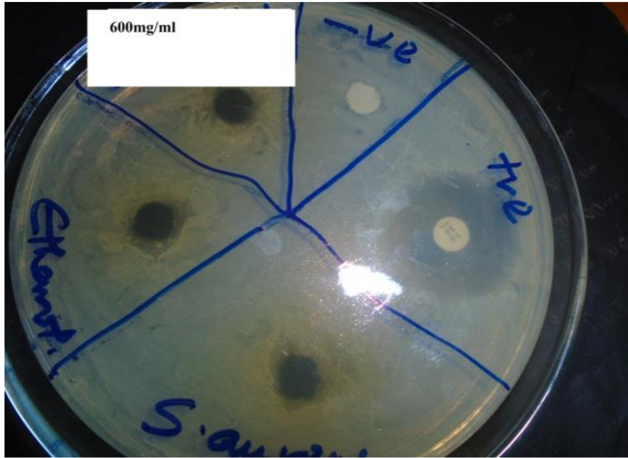
Appendix 1. *Satureja punctata*

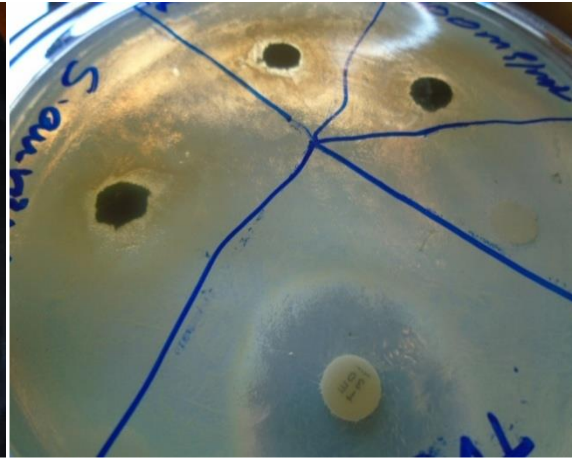


Appendix 2. Powder of *Satureja punctata*

Appendix 3. Filtering the extracts

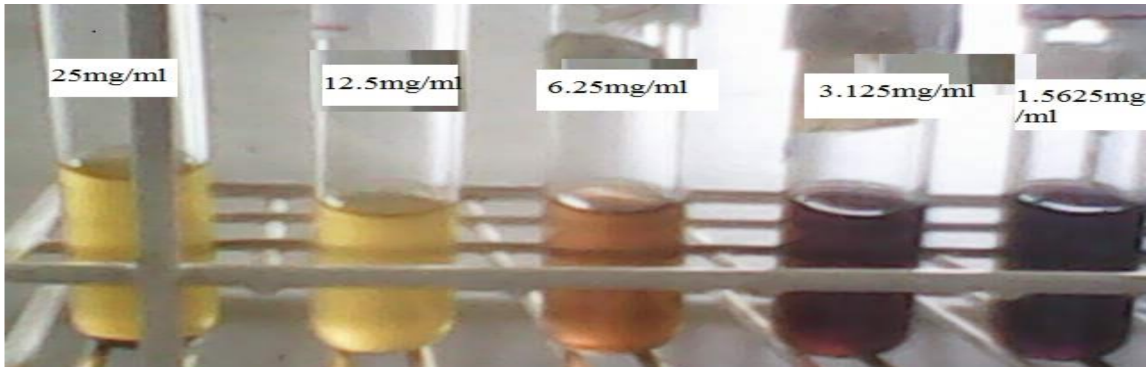
Appendix 4. Adjusting rotary evaporator



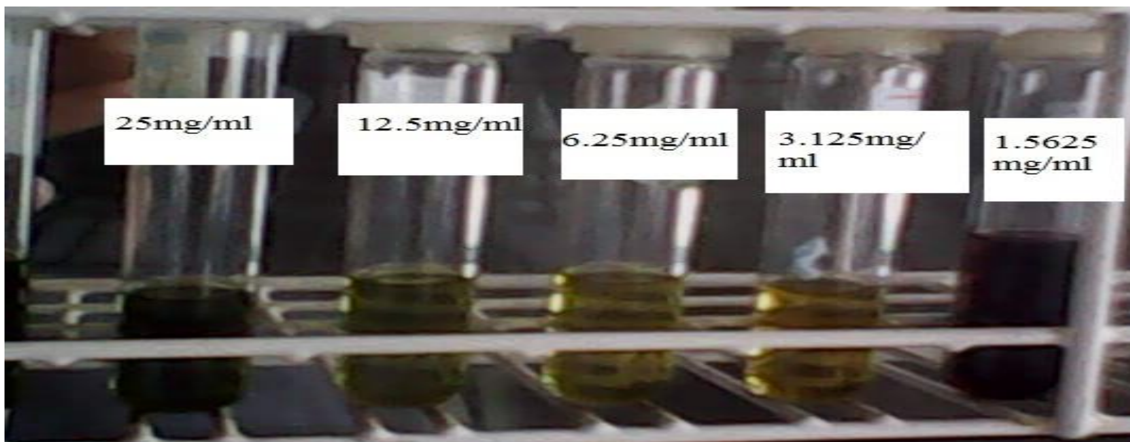


Appendix 5. Antibacterial activity of ethanol extract

Appendix 6. Antibacterial activity of aqueous extract



Appendix 7. Antioxidant Activity of Aqueous Extract



Appendix 8. Antioxidant Activity of Ethanol Extract

DECLARATION

I, the undersigned, declare that this thesis is my own original work. It has not been presented in other university or colleges, seeking for similar degree or other purposes. All sources of materials used for the thesis has been duly acknowledged.

Name: Mesfin Melaku

Signature: ______________________

Date:____________________________

Advisor: Prof. Yalemtsehay Mekonnen

Signature: _____________________

Date:_________________________