"Antioxidant and antimicrobial activities of medicinal plant Arnebia Benthamii"

A Dissertation

Submitted By

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TO

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Under the guidance of

DR. TABARAK MALIK

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DISSERTATION TOPIC APPROVAL PERFORMA

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Signature of Supervisor

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*Supervisor should finally encircle one topic out of three proposed topics and put up for approval before Project Approval Committee (PAC)

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ABSTRACT

Arnebia benthamii is a medicinal plant belonging to family boraginaceae, which has Anti-inflammatory, Antibacterial, wound-healing and the Antifungal properties. Different methods were followed to determine the presence of phytochemical constituents, antioxidant and antimicrobial properties. The alkaloids are present in methanol(M), and ethanol, while absent in butanol, ethyl acetate, and water extracts of both stem part and leaf part. Terpenoids are present in all the solvent extracts of stem part and leaf part except butanol and ethyl acetate extracts. The highest phenolic and flavonoid content have been found in methanol extract of stem and leaf as well as in Eethanol and Ethyl Acetate. The highest DPPH scavenging activity was found to be in the methanol and ethyl acetate extract of leaf and the least in ethanol and water. And the superoxide scavenging activity was found to be highest in the aqueous and ethanol extract of leaf and the least in ethyl acetate of stem part. Each strains of bacteria and fungi was tested for susceptibility tests against different solvent extracts of leaf part & stem part of A. benthamii. The result showed that the plant extracts of leaf part and stem part both exhibited significant antimicrobial activity. But the Aq. extract was completely ineffective against all the tested bacterial and fungal strains except Staphylococcus aureus. The fungal strains of A. solani were also exhibited significant antifungal activity by both leaf part & stem part extracts of A. benthamii.

CERTIFICATE

This is to certify that SHILPI SHREYA bearing registration no.11010869 has completed M.tech dissertation **entitled "Antioxidant and antimicrobial activities of medicinal plant Arnebia Benthamii"** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of the dissertation has been submitted to any other University or Institute for the award of any degree or diploma. The dissertation is fit for submission and the partial fulfilment of the condition for the award of Master of technology in Biotechnology.

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Last but not the least, I would like to thank my beloved parents for their endless and unwavering love which were undeniably the bedrock upon which the past years of my life ***have been built. I'm also grateful for the strength and enthusiasm which they have given m****e to achieve success in every spheres of my life. Without their care and support, the present work would have been a mere dream.*

SHILPI SHREYA

DECLARATION

I hereby declare that the dissertation entitled **"Antioxidant and antimicrobial activities of medicinal plant Arnebia Benthamii"** submitted for M.Tech degree is entirely my original work and all ideas and references have been duly acknowledged. It does not contain any work for the award of any other degree or diploma.

Date: 5-may-2015 *Shilpi Shreya (11010869)*

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER-1

INTRODUCTION

From the thousand of years, nature has been the main source of all types of medicinal agents and from the natural sources of the traditional medicine ,a great number of modern drugs have been isolated. Medicinal plants are always playing an important role in the development of all types of mankind's health. In most of the developing countries, a huge part of populations near about 80% are totally dependent only on medicinal plants for their primary health care service. Most of the medicines are derived directly or indirectly from medicinal plants (**Newman *et al.*, 2000**). Medicinal plants can be used in the development of various drugs which used for certain disorders and infectious diseases.

Arnebia benthamii is a medicinal plant belonging to family boraginaceae, the perennial Herb was selected for the present study. It is very high-value Himalayan medicinal plant, it is ranking second in the list of the medicinal plants. It is occurring in the alpine and subalpine Himalaya at altitude of 3000-3900 m asl .The morphology is erect, herbaceous perennial, and 30 to 90 cm in Height. This species is a major ingredient of the commercial drug available, Gaozaban, which has Anti-inflammatory, Antibacterial, wound-healing and the Antifungal properties **(Sumit Manjkhola *et al.*,2002)**.The roots of this plant yield a red pigment, called Shikonin (dye), which has several medicinal properties and it's availability is also marketed under the name of Ratanjot **(Kirtikar *et al*., 1984).**

It also possess the Anticancerous and Anti-HIV activity. The flowering shoots are used in preparation of sherbet (syrup) and jam which is used in various type of diseases of tongue, throat, cardiac disorders and fever **(Kashiwada *et al.*,1995)**.Free radicals play a key role in affecting human health by causing various types of diseases, such as cancer and cardiovascular diseases by the cell degeneration. These free radicals are generated during normal body function, or acquired from the environment. Human body has multiple mechanisms of enzymatic and non enzymatic antioxidant systems to protect the cellular molecules against the reactive oxygen species (ROS) which induced damage **(D.Anderson,1999).**

Oxygen radicals can cause damage to various types of the biomolecules (eg.- lipids, protein, DNA etc), and leading to many chronic diseases such as cancer, the cardiovascular-diseases, atherosclerosis, diabetics, rheumatoid arthritis, reperfusion injury, and the other degenerative diseases in humans **(F.Yun-Zhong *et al*.,2002).** In the human body the innate defense may not be enough for severe or continued oxidative stress. Thus, certain amounts of exogenous antioxidants are constantly required to maintain a sufficient amount of antioxidants to balance the ROS in the human body. There are several types of synthetic antioxidants are available like butylated hydrotoluene (BHT) butylated hydroxyanisole (BHA), and the tertiary-butylhydroquinone (TBHQ), which is widely used in the foods to prevent the oxidation. The used of this synthetic antioxidant in food are not good to our health because of their toxicity and carcinogenicity .Hence, the various types of medicinal plants are used for antioxidant that capable to protect from damage from ROS and cure from diseases.

1.1. PLANT PROFILE.

Fig.1. *ARNEBIA BENTHAMII*

Classification

Domain : *Eukaryota- Whittaker & Margulis,1978 eukaryotes*

Kingdom : *Plantae-Haeckel, 1866 – Plants*

Subkingdom : *Viridaeplantae*- Cavalier-Smith, 1981

Phylum : *Tracheophyta*- Sinnott, 1935 ex Cavalier-Smith, 1998

Subphylum : *Euphyllophytina*

Infraphylum : *Radiatopses*- Kenrick& Crane, 1997

Class : *Magnoliopsida*-Brongniart, 1843 –Dicotyledons

Subclass : *Asteridae-Takhtajan, 1967*

Superorder : *Solanacae-R. Dahlgren Ex Reveal, 1992*

Order : *Boraginales*- Dumortier, 1829

Family : *Boraginaceae*- A.L. de Jussieu, 1789**.**

Subfamily : *Boraginoideae*

Tribe : *Lithospermeae*

Genus : *Arnebia–Forsskl,1775*

Source: http:// Plants.gov/java/Arnebiabenthamii/Classification Servel.

1.1.1. Botanical Description

Plants annualor perennial of 30–90 cm height.Roots are frequently containing purple dye. Its Stems are erect or prostrate. Flowers often heterostylous. Calyx 5-parted to base, slightly enlarged and sometimes is hardened. Corolla are in funnelform, usually with hairs outside. Ovary 4-lobed. Style 2- or 4-branched, with each 1 stigma terminating 1 branch. The long-styled flowers which has stamens inserted at middle of corolla tube; style long. Short-styled flowers with stamens inserted at throat.

1.1.2. Medicinal properties

The **"Gule Khazaban**" from ***Arnebia benthamii*** is very costly medicine **(Kaul, 1997)** used in the treatment of heart problems. This plant is useful in the treatment of diseases of the tongue and throat. The species is a major ingredient of the commercial drug available under the name "Gaozaban", which has antibacterial preoperties as well as antifungal and wound healing properties. A red pigment is yielded by the roots known as Shikonin (a dye), which has several medicinal properties and is marketed under the trade name Ratanjot. The species also consists properties of stimulant, tonic, diuretic and expectorant. The flowering shoots have various applications including sherbet (syrup) preparation and jam for diseases of tongue,fever and cardiac disorders. Secondary metabolites, Arnebin 1 and Arnebin 3 obtained from the other species of this genus are reported to possess anti cancerous property **(Harborne and Baxter, 1996).** *Arnebia euchroma* exhibits potent anti-HIV activity **(Kashiwada *et al*.,1995).** As a result of over exploitation of its rhizomes for medicinal purposes, *A.benthamii* L. has been listed in the Indian Red Data Book **(Sastry and Jain, 1984).**

1.2. PHYTOCHEMICALS.

Phytochemicals ,the non-nutritive plant chemicals have protective or disease preventive properties. More than thousand known phytochemicals produced from plants which provides them protection from various stresses but recent research explains that they can also protect humans against diseases. Lycopene in tomatoes, flavonoids in fruits and isoflavones in soy are few well-known phytochemicals which are not essential nutrients and are not necessary by the human body for sustaining life. Plant sterols, flavonoids and sulfur-containing compounds are three classes of micronutrients present in fruits and vegetables. The risk of atherosclerosis, which are the result of fatty deposits build up in artery walls are reduced due

to these compounds. Within these categories there are many possible compounds, which are not well described and whose modes of action are not defined. Various plant products may also be linked to various atherosclerotic process, like antioxidant vitamins, phytoestrogens and trace minerals. Many of the plants micronutrients will clearly be the topic of further future research. As research developes on these different compounds, many components in plants that are still unrecognized will also be identified that may help in reducing risk of cardiovascular disease.

1.2.1. Mechanism of action

The phytochemicals are present in plants which works differently with some of the following possible actions:

1.2.1. **Hormonal action:** Isoflavones found in soy, imitate human estrogens and reduces menopausal symptoms and osteoporosis.

1.2.2. **Interference with DNA replication:** Saponins found in beans involves with the DNA cell replication, thus preventing the multiplication of cancer cells. The compound found in hot peppers, Capsaicin, provides protection to DNA against carcinogens.

1.2.3. **Anti-bacterial effect:** The phytochemical allicin from garlic hasanti-bacterial properties.

1.2.4. **Physical action:** Some phytochemicals bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls. Pro-anthocyanidins are responsible for the anti-adhesion properties of cranberry. Cranberry consumption will reduce the risk of urinary tract infections and will improve dental health.

1.3. ANTIOXIDENT.

Antioxidants ,a type of complex compounds found in our diet act as a protective shield for our body against certain disastrous enemies (diseases) such as cardiac diseases, arthritis, aterial diseases, cataracts and also premature ageing along with several chronic diseases. Most phytochemicals possess antioxidant activity and provide cell protection against oxidative damage which reduces the risk of cancer development. Some of the phytochemicals with antioxidant activity are allylsulfides (onions, leeks, garlic), carotenoids (fruits, carrots), flavonoids (fruits, vegetables), polyphenols (tea, grapes) **(Helmut, 1997).**

The free radicals play very important role in human health and beneficial in combating against several diseases. During a chemical reaction (oxidation), one reactant loses an

electron and is called oxidant or free radical, while the other undergoes electron gain. Oxygen in unstable form is the most common free radical in living organisms. This is called Reactive Oxygen Species (ROS) and is generated during metabolic activities. Change of a molecule into a free radical can be due to contaminants in the environment as well as normal metabolism of a cell. Few examples of ROS are OH, O2, H2O2, O3, HOCI, RO2, and RO. Any molecule can become a free radical by either losing or gaining an electron **(Balz, 1997).** Once initiated these free radicals get involved in chain reaction with stable types. The formed compounds have longer stability and in body and increase the potential for damage of cell. Cell damage is often caused by the free radicals at the site of their operation causing serious disorders. The accumulation of plaque in arteries can occur due to oxidation. LDL Cholesterol functions as free radical and damages the free artery lining. It hampers the blood circulation which may lead to heart attack. The neutralization or termination of free radicals o is achieved by antioxidants or enzymatic mechanism **(Halliwell, 1994)** after the radicals are propagated. It is required for balancing the fee radical & antioxidant activity.

Some of the effects of excess of free radicals are as follows:

i. DNA, RNA, proteins, enzymes can be damaged.

ii. Lead to the formation of tumours and cause cancers.

iii. Cardiovascular Diseases

iv. Nervous disorders

v. Premature ageing

vi. Parkinson's and Alzheimer's Diseases vii. Rheumatic and Pulmonary Disorders

A number of processes are taking place in our body like breathing, breaking up of protein in the body or exposure of body to air pollution or UV radiation leads to the formation of free radicals that aid in the process of oxidation which further leads to several health problems. If the body is healthy it is capable enough to break down these free radicals before they become harmful. If formation of free radicals exceeds in the body, it can cause damage to the cells and tissues which can be counteracted by antioxidants which prevent the free radical formation and if the formation occurs it helps in their removal from blood stream. Thus antioxidants play potentially beneficial role in the risk reduction and treatment of diseases.

1.4. ANTIMICROBIAL ACTIVITY.

Antimicrobial activity refers to any substance that hinders the growth of microorganisms. The use of antibiotics has revolutionized the treatment of various infections from bacteria. However, their excessive use has led to an alarming increase in antibiotic resistance among microorganisms **(Hart and Karriuri, 1998)** thus necessitating the need for development of novel antimicrobials **(Chopra *et al*., 1997).**

1.4.1. Microbial substances

Flavonoids are known to be synthesized by plants in response to microbial **infection (Dixon *et al*., 2005)** and are effective anti-microbial substances against a wide array of micro-organisms. Antimicrobial flavonoids have been reported from *Erythrina latissima* **(Wanjala *et al*., 2002)**. The activity is probably due to their ability to form a complex with extra-cellular and soluble proteins, which then binds to bacterial cell wall. More lipophilic flavonoids may also disrupt microbial membranes **(Tsuchiya *et al*., 1996).** Flavonoids lacking hydroxyl groups on their β-rings are more active against micro-organisms and the microbial target is the membrane with –OH groups (**Chaurasia and Vyas, 1997).**

Towards phenolics, different sensitivities are exhibited by phenolic compounds specially the anti-microbial properties(Finnish berries). Food preservation and food development are those properties which can be utilized by these. **(Puupponen Pimia *et al*., 2001)** Phenols are toxic to micro- organisms because of the sites and numbers of hydroxyl groups present on the phenol groups, which can be related to their relative toxicity to microorganisms. There is evidence that highly oxidized phenols possess inhibitory action **(Urs and Dunleavy, 1975).** Polyphenols which can form heavy soluble complexes with proteins may bind to bacterial adhesions there by disturbing the availability of receptor on the cell surface.

Tannins (tannic acid) are water-soluble polyphenols that are present in many plant foods **(Scalbert, 1991).** The anti-microbial activities of tannins are well documented. Tannins inhibit the growth of many fungi, yeasts, bacteria and viruses. The mode of anti-microbial action of tannins and their ability to inactivate enzymes, cell envelop transport proteins as well as microbial adhesins also have been studied.

CHAPTER-2

REVIEW OF LITERATURE

2.1. OXIDATION

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Free radicals are produced by the oxidation reactions and these free radicals damage the cells by starting some chain reactions. Antioxidants are oxidized themselves to inhibit these chain reactions, so antioxidants are the reducing agents such as polyphenols (**Seis and Helmut, 1997**).

Oxidation reactions are necessary for living but they can also cause damage, therefore plants and animals possess antioxidants. Antioxidants prevent oxidative damage to proteins, lipids and DNA by either preventing the free radicals from being formed or remove them from the cell. Iron is an essential metal for normal cellular physiology, but iron if present in excess can cause cell injury. That's why it plays a catalytic role in the initiation of free radical reactions.

2.2. REACTIVE OXYGEN SPECIES.

Reactive oxygen species (ROS) are produced as a consequence of aerobic metabolism. In plants, they are always formed as a byproduct of various metabolic pathways occuring in different compartments of the cell (**C. H. Foyer, 1997; L. A. Del R´ıo *et al.*, 2006;**).

Environmental stresses such as salinity, chilling, drought, and UV-B radiations as well as pathogens attack lead to increased production of reactive oxygen species in plants due to disruption of cellular homeostasis (**K. Shah *et al.*, 2001; R. Mittler, 2002; P. Sharma and R. S. Dubey, 2005; W. H. Hu et al, 2008; C. Han *et al.*, 2009; R. Maheshwari and R. S. Dubey, 2009; G. Tanou *et al.*, 2009; S. Mishra *et al.*, 2011; S. Srivastava and R. S. Dubey, 2011; P. Sharma and R. S. Dubey, 2007**). When the level of ROS exceeds the defense mechanisms, a state of **"oxidative stress"** is said to have occurred

2.3. FREE RADICALS.

Free radicals which are define as independent chemical species with one or more unpaired electrons can be formed from a diverse group of chemicals and are generally considered to be highly reactive **(By Cheeseman, 1993).** Reactive oxygen species (ROS) are produced as a consequence of aerobic metabolism. In plants, they are always formed as a byproduct of various metabolic pathways occuring in different compartments of the cell (**C. H. Foyer, 1997; L. A. Del R. *et al.*, 2006; O. Blokhina and K. V. Fagerstedt, 2010**). Environmental stresses such as salinity, chilling, drought, and UV-B radiations as well as pathogens attack lead to increased production of reactive oxygen species in plants due to disruption of cellular homeostasis (**K. Shah *et al.*, 2001; R. Mittler, 2002; P. Sharma and R. S. Dubey, 2005; W. H. Hu et al, 2008; C. Han *et al.*, 2009; R. Maheshwari and R. S. Dubey, 2009; G. Tanou *et al.*, 2009; S. Mishra *et al.*, 2011; S. Srivastava and R. S. Dubey, 2011; P. Sharma and R. S. Dubey, 2007**). When the level of ROS exceeds the defense mechanisms, a state of **"oxidative stress"** is said to have occurred

2.3.1. Sources of Production of Free Radicals in human body

- Internal sources
- External source
- Physiological Factors.

Internal sources

These can be enzymatic reactions, which serve as a source of free radicals. These include those reactions involved in the respiratory chain, in prostaglandin synthesis and in the cytochrome P450 system **(By Liu T., 1999)**. Some internal sources of generation of free radicals are mitochondria, xanthine oxidase, phagocytes,reactions involving iron and other transition metals, peroxisomes, Arachidonate pathways, exercise, ischaemia/reperfusion, inflammation.

External sources

These includes non-enzymatic reactions of the oxygen with organic compounds. Free radicals also arise in reactions, which are initiated by ionizing radiations. Some external sources of

free radicals are cigarette smoke ,environmental pollutant ,radiations ,ultraviolet light ,ozone , certain drugs, pesticides, anesthetics and industrial solvents **(By Bagchi, 1998)**.

Physiological Factors

Mental status like stress, emotion etc. and disease conditions are also responsible for the formation of free radicals.

2.3.2. Types of Free Radicals.

- Superoxide radical
- Hydroperoxyl radical
- Hydrogen peroxide
- Triplet oxygen
- Active oxygen

Superoxide ($O_2^{\cdot}$)

The body makes another oxygen radical (i.e. unpaired electrons is located on oxygen), superoxide. Adding one electron to the oxygen molecule makes superoxide, which is generally a poor reactive radical. Some superoxide is made by "accidents of chemistry", in that many molecules in the body react directly with oxygen to make superoxide. Examples include the catecholamine and some constituents of mitochondria electron transport chains. Such superoxide generation is unavoidable. In addition, some soperoxide is made deliberately For example activated phagocytes generate large amounts of superoxide as part of the mechanism by which foreign organisms are killed. Hydrogen peroxide (H_2O_2) is the most stable reactive oxygen metabolites. H_2O_2 may be generated directly by divalent redacting of O_2 or indirectly by univalent reduction of $O_2^{\cdot}$. Hydrogen peroxide is the primary product of the reduction of O_2 by numerous oxidases. H_2O_2 is very sensitive to decomposition by redox-active metal complexes, of which catalase and peroxidase are the most effective exponents. Metal ions have a strong effect on the chemistry of O_2 and its reduction products. The well known Fanton reaction is initiated when Fe^{2+} comes in contact with H_2O_2 to product $^{\cdot}OH$.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$$

H_2O_2 also reacts with $O_2^{\cdot}$ to initiate Haber-Weiss reaction producing $^{\cdot}OH$ in the presence of Fe^{2+}

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + OH^{-}$$

Hydroxyl radical ($^{\cdot}OH$)

Radiation can split water in the body to generate the hydroxyl radicals. This is a highly reactive radical, which once generated, attacks wherever it is next to. Its lifetime in vivo is very short because the hydroxyl radical reacts at its site of information usually leaving behind a legacy in the form of a propagating free-radical chain reaction.

Nitric oxide ($NO^{\cdot}$)

Another physiological free radical is nitric oxide, which is produced by the vascular endothelium as a relaxing factor and also by phagocytes and in the brain. Nitric oxide has many physiological functions, but excess nitric oxide can be toxic.

Hydrogen peroxide

The univalent reduction of superoxide produces hydrogen peroxide, which is not a free radical because all its electrons are paired. The main damages caused by this are breaking up of DNA, resulting in single strand breaks and formation of DNA protein crosslink. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecule.

Singlet oxygen ($^1O_2$)

It is not a free radical but it can be formed in some radical reactions and can trigger off others. This arises from hydrogen peroxide molecules. Singlet oxygen on decomposition generates superoxide and hydroxyl radicals.

2.4. OXIDATIVE DAMAGE.

Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. An imbalance between oxidants and antioxidants leading to damage called 'oxidative stress'. Oxidants are product of aerobic metabolism but when it produced at very high rates under the pathophysiological conditions, cause damage. The antioxidant molecules add significantly to protect this damage by the various types of enzymes such as superoxide dismutase, glutathione peroxidases and catalase **(By Sies H.,1997).** The chemical nature of reactive oxygen species (ROS) are very harmful to cells. At high concentrations, the ROS

activate genetically programmed cell suicide events **(Christine, H. Foyer and Graham Noctor,2003).**

The various types of infectious diseases in farm animals, such as pneumonia and enteritis, are directly or indirectly related to oxidative stress, i.e. various types of chemical phenomenon involving an imbalance in the redox reaction of the individual animal. The oxidative stress should be easily prevented with antioxidants. **(Jens Lykkesfeldt and Ove Svendsen**,**2007)**The oxidants or cellular oxidative metabolism are the agent which induces the apoptosis. Many inhibitors of apoptosis have antioxidant activities and it enhance the cellular antioxidant defenses.

2.5. ANTIOXIDENT .

Plants possess a complex antioxidative defence system having enzymatic and non-enzymatic components for scavenging the ROS. In plant cells, specific ROS scavenging systems are found in many cell organelles such as chloroplasts, mitochondria, and peroxisomes. In order to avoid the oxidative damage higher plants increase the level of intracellular anti-oxidative defense.

Different components of anti-oxidative defense system involved in ROS scavenging are overexpressed or downregulated to add to the present understanding of the role of the antioxidant systems.

2.5.1. Role of Antioxidants

Antioxidants play an important role in our body. They are intimately involved in the prevention of cellular damage, the common pathway for cancer, aging, and a variety of diseases. They act as the scavengers that scavenge the free radicals that are generated in the body when oxygen interacts with certain molecules. Superoxide O_2 is one of the main reactive oxygen species in the cell. Naturally in our body, free radical is dismissed by certain enzyme such as Superoxide dismutase (SOD) that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Hence SOD serves as a key antioxidant role in our body.

Antioxidants also play an important role in preventing the food from deterioration or rancidity. In industries, antioxidants are widely used to prevent the oxidative degradation of

polymers such as rubbers, plastics and adhesives that causes a loss of strength and flexibility in those materials.

2.6. ANTIMICROBIAL ACTIVITY.

In **2009, Anjali *et al*.** studied that true form of Rajanjot was extracted from *A. nobilis* which is imported from Afganistan and revealed that root extracts are used to colour food stuffs, oils and fats. Besides, imparting pleasing colours it is also known as an antiseptic, anthelmintic, antipyretic etc. Similarly, El-Syed (2010) reported the effects of *A. hispidissima* and *Echium rauwolfii* root extracts (ethanolic) on the growth of two rhizospheric soil fungi of pigeonpea. The results indicated that both root extracts reduced the number of colonies of rhizospheric fungi *Fusarium oxysporum* and *Thanatephorus cucumeris.*

In **2010, Abdulameer and Al-Mussawi** has investigated the antibacterial activity of shikonin that showed greatest antibacterial activity i.e. (50mm) against *P. aeruginosa*, (45mm) against *E. coli*, (40 mm) against *S. aureus*, and (38 mm) against *K. pneumonia.*

In **2010, Fangl *et al*.** has evaluated the antibacterial activity of *A. euchroma* (Royle) Johnst (hairy roots) against the *P. syringae*, *P. melonis*, *E. coli*, *S. aureus*, *F. oxysporum*, f.sp. *vasinfectum* and *Verticillium dahliae* by filter paper method and mycelium growth rate method. The results divulged that ethanol extract had significant antibacterial action on three kinds of bacteria and the best inhibitory effect on *S. aureus*. Acetone extract had bacteriostatic action on *P. syringae*, whose inhibitory effect was greater than the ethanol extract of 26.5%.

In **2011, Ramakrishnan *et al*.,** studied, the antimicrobial activity of the petroleum ether, chloroform, methanol and aqueous extracts of the leaves of *Ageratum conyzoides* Linn.(Asteraceae), *Argemone Mexicana* Linn (Papaveraceae), *Heliotropium indicum* (Boraginaceae), *Alistona scholaris* L. R Brown (Apocynaceae), were screened against *S. aureus, E. coli, P. aureginosa, A. Niger* and *C. albicans.* The results indicated that the chloroform, methanol and aqueous extracts of all tested plant materials are active against both Gram positive and Gram negative bacteria. Of all the extracts, methanol was found to be more effective than other. Phytochemical screening of the methanolic extracts of selected plants revealed the presence of alkaloids, tannins and flavonoids.

In the same year **2011, Haghbeen *et al*.** has analysed dried root of the plant *A. euchroma* for shikalkin pigment which was found to be 8.5(w/w) also the root explants of the young

plantlets, obtained from the germinated seeds for the antimicrobial activity of shikalkin pigment on some fungi and Gram-positive and negative bacteria.

In **2011, Amin and Khan *et al*.** also evaluated the antimicrobial activities of methanol and aqueous extracts of 5 medicinal plants of a traditional medicine were against 6 human gram positive (*S. aureus, Micrococcos luteus*) and gram negative (*E. coli, P. aeruginosa, Enterobacter, K. pneumoniae*) pathogens. The most active extract found was *Azadirachta indica* leaves which represented widest zone of inhibition of 16(±0.05) mm and minimum inhibitory concentration 0.19 mg/ml against *K. pneumoniae. Calotropis procera* leaves was found least active representing lowest Zones of inhibition 3.13(±0.05) mm and highest minimum inhibitory concentration value (20mg/ml) against test microorganisms.

In **2012, Moghaddam *et al*.** studied the antimicrobial and antioxidant activity of *Onosma dichroanthum* Boiss. (boraginaceae) by agar dilution and well diffusion methods. Results showed that Gram positive bacteria were more sensitive than Gram negative bacteria, especially to acetone extract in the well diffusion method. *Bacillus cereus* with minimum inhibitory concentration (0.078 mg/ml) was the most sensitive bacterium. These results indicated that the use of *O. dichroanthum* root can be a confirmation for using this plant in traditional medicine, as antiseptic and antioxidant effect.

2.6. ARNEBIA BENTHAMI (PHYTOCHEMISTRY)

A phytochemical is a natural bioactive compound found in plant foods that works with nutrients and dietary fiber to provide protection against disease. The phytochemicals working together with nutrients found in fruits, vegetables and nuts, may help in slowing the aging process and reduce the risk of diseases like cancer, heart disease, stroke, high blood pressure, cataracts, osteoporosis, and urinary tract infections. **Prabhaker *et al*. (1981)** has isolated vitexin a flavonoid from the flowers of *Arnebiahispidissima* which showed potent hypotensive, anti-inflamatory and antispasmodic properties.

Alantolactones and iso-alantolactones were obtained from the roots of *Inula racemosa* and Arnebins isolated from the roots of *Arnebia nobilis* were found to be poorly effective against ringworm infection. Arnebins have shown promising *in vitro* and *in vivo* activity while lactones show good activity in vitro but poor efficacy in vivo **(Wahab et al., 1982).** In the same year **Khan et al. (1983)** isolated arnebin-7, alkanin acetate, alkanin isovalerate, alkanin

betasitostrol and a new isohexanyl napthazarin, alkanin beta-hydroxy-isovalverate from the roots of A. hispidissma . **Lu et al. (1983)** also isolated deoxyshikonin, acetyleshikonin, shikonin and beta-hydroxy-sovaleryl shikonin napthoquinones from the petrol ether extract of Arnebia guttata roots**.**

Afzal and Oriquat (1986) as a part of their search for new natural sources of shikonin, alkanin and their derivatives reported that the petroleum extract of air dried roots of Arnebia decumbens contained stigma-sterol, shikonin acetate and shikonin. They further reported that the extract yielded a compound characterized as 5,8, dihydroxy-2-(14-methylpent-13-enyl) 1,4-napthoquinone (i.e. reduced shikonin) and shikonin isovalerate. Similarly, **Tanaka et al. (1986)** has reported that shikalkin isolated from A. euchroma roots exhibited anti-inflammatory activity on the odema caused by thermal injury to the skin of rats.

Luo and Mai (1987) separated and determined 5-napthoquinones shikonin β-actetoxymethyl butylalkanin, deoxyshikonin acetylshikonin and β-dimethylacryl alkanin from *A. euchroma* using high performance TLC and in the same year,**Wassel *et al*.(1987)** screened methanol extracts of *A. Hispidissima* for pyrrolizidine alkaloids a nd anti-tumor activity detected saturated or 1,2 unsaturated pyrrolizidine alkaloids.The extracts were found to have significant cytotoxic activity against *Ehrlich ascites* carcinoma *in vitro*.

Guirong and Jing (1990) detected the anticancer activity of napthoquinone pigment L-III from *A.euchroma* that inhibit the proliferation of stomach cancer cell lines and esophagus cancer cell lines. They further reported that the anti- cancer effect of napthoquinones pigment L-III might be related to its role of influencing the amount of RNA and ultrastructure of cancer cells

.

Yao *et al*. (1991) isolated three phenolic compounds from the roots of *A. euchroma* which inhibitors of *in vitro* prostaglandin biosynthesis. Two known compounds were identified shikono-furans and des-methyllasiodeplodin and the other new compound was named as arnebinol and its structure was elucidated as a novel type of mono-terpenylbenzenoid.

After that **Yang *et al*. (1992)** isolated tormentic acid and 2 alpha-hydroxyursolic acid from the dried leaves of *Arnebia eu chroma* and **Rodder and Rengel (1993)** isolated two pyrrolizidine alkaloids from the MeOH extract of *A. euchroma* (commercially called Ziaco in china a nd Nanshikon in Japan). Also in the same year, **Khatoon *et al*. (1993)** have reported the chemical analysis of commercial Ratanjot by TLC fluorescence finger printing to find out the adultrants through comparison of napthoquinone contents and all the market samples

investigated were found to be mixtures of two or three species (*A. nobilis, A. benthamii, A. euchroma*) except for the sample from Amritsar which was identified as *A. nobilis*. The acted as shikonin an active component from the roots of *A. euchroma* clinically used to treat phlebitis and vascular purpura was reported by **Wang *et al*. (1994).**Shikonin significantly inhibited inflammation induced by croton oil in mice (Ear oedema Test) and yeast induced paw swelling in rats.

In the next year, **Kashiwada *et al*. (1995**) reported that bioactivity directed fractionation of *A. euchroma* roots (obtained from a commercial source in China) resulted in the isolation of 3-monosodium and 3-monopotassium salts of isomeric caffeic acid tetramers. Mixtures of dipotassium and disodium salts of a caffeic acid tetramer glucoside were also isolated from the active fraction. **Gaddipati *et al*. (2000)** stated that shikonin analogue 93/637 derived from extracts of roots of *A. nobilis* has inhibitory effect on cellular growth of IGFS (Insulin-like growth factors)and potential therapeutic treatment of human prostate cancer.The hexane extract of *A. Hispidissima* yielded a mixture of naphthaquinones viz. Arnebin-1, Arnebin-7, Tiglic acid (ester of dihydroxy alkannin), alkannin, arnebinol and cycloarnebin-7 and arnebin-1 was the major naphthaquinone found both in vivo (0.62%) and in vitro (0.27%) cell cultures **(Singh et al.,2004).** In **2006, Yani et al.** evaluated the napthoquinone derivatives of Boraginaceous herbs by high performance liquid chromatography. The good quantity of napthoquinone was found in A.euchroma and A. guttata.

CHAPTER 3

AIMS and OBJECTIVES

- Analysis of Phytochemicals studies.
- Evaluation of in-vitro antioxidant potential.
- Evaluation of antimicrobial activity.

CHAPTER-4

RATIONAL/SCOPE OF STUDY

Oxidative stress contributes to the development of various diseases including, Alzhiemer's, Parkinson's, diabetes and rheumatoid arthritis. Oxidative damage in DNA can also cause cancer. Several antioxidant enzymes such as catalase, glutathione reductase, glutathione S-transferase, superoxide dismutase, glutathione peroxidise, etc. protect DNA from oxidative stress. *Arnebia Benthamii,* can be used as an alternative to reduce the oxidative damage caused by free radicals. It can also be used as an antimicrobial supplement to prevent the growth of food borne pathogens. This study provides scientific insight to further determine the antimicrobial and antioxidant properties of *Arnebia Benthamii*. On the basis of the present finding, it will be revealed that whether or not Arnebia Benthamii can be used as a natural antimicrobial agent and a potential antioxidant against infections and/or diseases.

CHAPTER-5
METHODOLOGY

5.1. INSTRUMENTATION.

- Pestle mortal
- Water bath
- Glass wares
- Digital balance
- Heating oven
- Digital balance
- Spectrophotometer
- Refrigerator
- Incubator
- Laminar Flow

5.2. CHEMICALS AND REAGENTS.

Extraction	Methanol, Ethanol, Butanol, Ethyl acetate,
Phytochemical screening	Chloroform, Sulphuric acid, Ferric chloride, Glacial acetic acid, Folin- Ciocalteau reagent, Wangner's reagent, Sodium hydroxide, Methanol, Aluminium chloride, Potassium acetate.
Antioxidant activity	1,1-diphenyl 2- picrylhydrazyl (DPPH), Ascorbic acid, DMSO, Methenol, riboflavin, EDTA, nitro-blue tetrazolium (NBT), phosphate buffer,

Antimicrobial activity	Nutrient agar, potato dextrose agar, Escherichia coli strain, Bacillus subtilis strain, Staphylococcus aureus strain, salmonella strain, A. solani strain, Gentamicin disc, fluconazole disc.

5.3. IDENTIFICATION AND SAMPLE COLLECTION.

The plant *Arnebia Benthamii* was collected as a whole plant from Duksum Kashmir Himalaya, J&K, India in July-September 2014. Plant was identified at Kashmir University Herbarium (KASH), Centre of Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar.

5.4. PROCESSING PREPARATION OF PLANT EXTRACTION.

Fig. 2. Sample of Stem Part

Fig. 3. Sample of Leaf Part

a. The sample was washed and shade dried.

b. The leaves were taken separately and grinded to powder form with the help of pestle mortal. The sample was weighed and 10 g of the sample was mixed in each beaker containing 100 ml of (70%) methanol, (70%) ethanol, (70%) butanol, (70%) ethyl acetate, and aqueous respectively. Then it all left for 48 hours at room temperature.

c. The stem was separately grinded to powder form with the help of pestle mortar. The sample was weighed and 5 g of the sample was mixed in each beaker containing 50 ml of (70%) methanol, (70%) ethanol, (70%) butanol, (70%) ethyl acetate, and aqueous respectively. Then it all left for 48 hours at room temperature.

Fig. 4. Stem extract preparation.

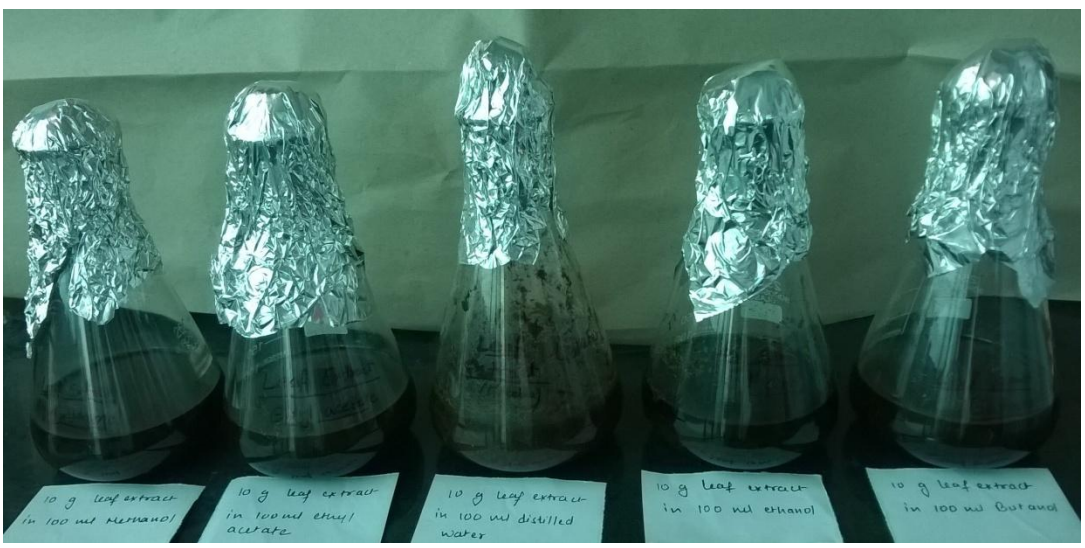


Fig. 5. Leaf extract preparation.

d. The different extracts were then filtered with Whatman filter paper, and the filtrate was evaporated by rotary evaporator and made stock solution with 10% DMSO

e. The extracts was collected and then dried and weighed and stored in 250ml clear plastic laboratory chemical conical flask storage bottle under 4 degrees Celsius in fridge.

Fig. 6. Stem extracts.

Fig. 7. Leaf extracts.

5.5. EXPERIMENTAL METHODOLOGY.

5.5.1. PHYTOCHEMICAL SCREENING.

5.5.1.1. Qualitative Phytochemicals Screening.

5.5.1.1.1 Test for Alkaloids

2 ml of extract is mixed with some dropes of Wagner's reagent and leave for 5 min. A reddish brown precipitate is formed. It indicates the presence of alkaloids.

5.5.1.1.2. Test for Terpenoids (Salkowski Test)

2 ml of extract is mixed with 2 ml of chloroform. Then adding 3ml of concentrated sulfuric acid to form a layer. A reddish-brown colouration of the interface indicated the presence of terpenoids.

5.5.1.1.3. Test for Flavonoids

1 ml of extract is added with 2 ml of sodium hydroxide (NaOH) and 1 ml of hydrochloric acid (Hcl). Yellow to colourless appearance was observed. It indicate the presence of flavonoids.

5.5.1.1.4. Test for Tannins

A few drops of 0.1% ferric chloride was added in the 1ml of plant extract and observed for yellow precipitate which indicate the presence of tannins.

5.5.1.1.5. Test for Glycoside

Extract was mixed with 2ml of glacial acetic acid containing 2 drops of 2% Ferric chloride ($FeCl_{3)}$. The mixture was poured into another tube containing 2ml of concentrated sulfuric acid. A brown ring at the interphase indicates the presence of glycosides.

5.5.1.1.6. Test for Phenols (Ferric chloride test)

A small quantity of extract was dissolved in the distilled water, and a few drops of 10% aqueous ferric chloride was added. After few minutes a dark blue-green or black colouration indicate the presence of phenols.

5.5.1.2. Qualitative Phytochemicals Screening.

5.5.1.2. Determination of total phenolic content:

The amounts of total phenolics in extracts was determined according to the Follin-Ciocalteau procedure **(Padmaja et al., 2011).** The samples was introduced into the test tubes and then the Folin-Ciocalteau reagent and sodium carbonate was added. The tubes was mixed and allowed to stand for few min. Absorption was measured. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g tissue as calculated from standard gallic acid graph. A standard calibration curve was prepared by plotting absorbance Vs concentration and it was found to be linear over this concentration range.

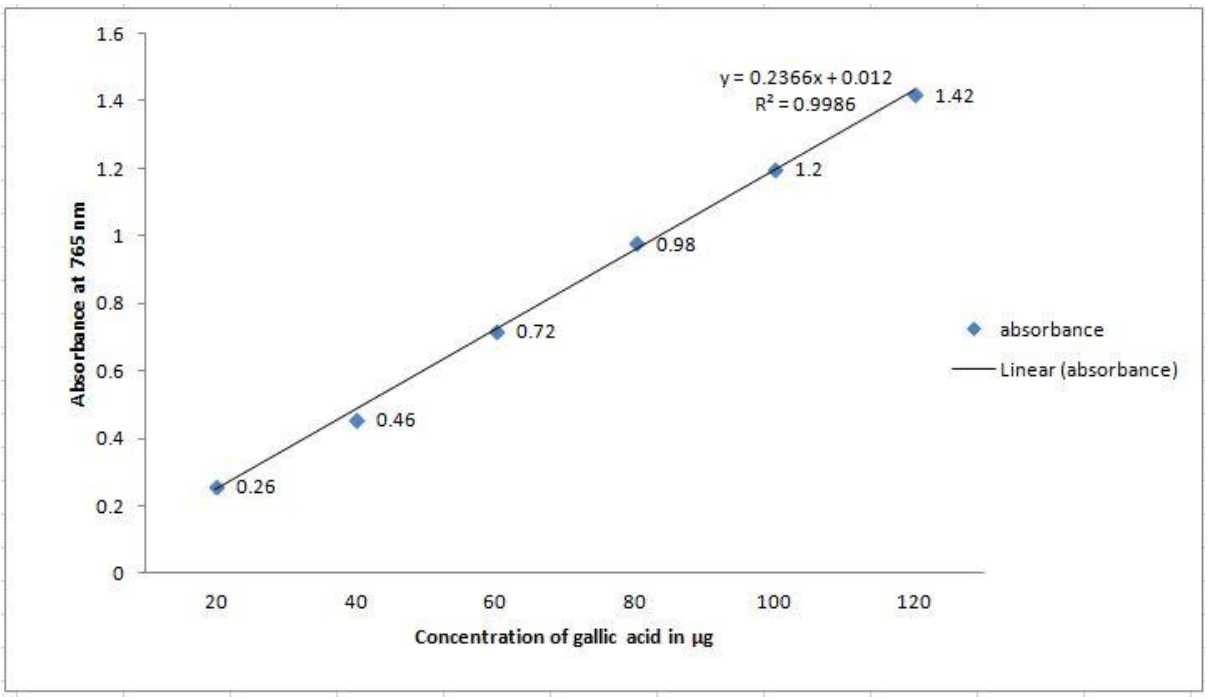


Fig. 8. Standard Gallic acid curve for estimating total phenolic content Gallic Acid in µg

5.5.1.3. DETERMINATION OF TOTAL FLAVONOIDS CONTENT:

Colorimetric aluminium chloride method is taken for flavonoid determination **(Ebrahimzaded et al., 2008a. B; Nabavi et al., 2008).** 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water and is stored at room temperature for 30 minutes. The absorbance is recorded at 415 nm spectrophotometrically. The total flavonoid content is calculated as quercetin with the help of calibration curve. The

quercetin solution has been prepared so far as stock solution as concentration of 1mg/ml in methanol.

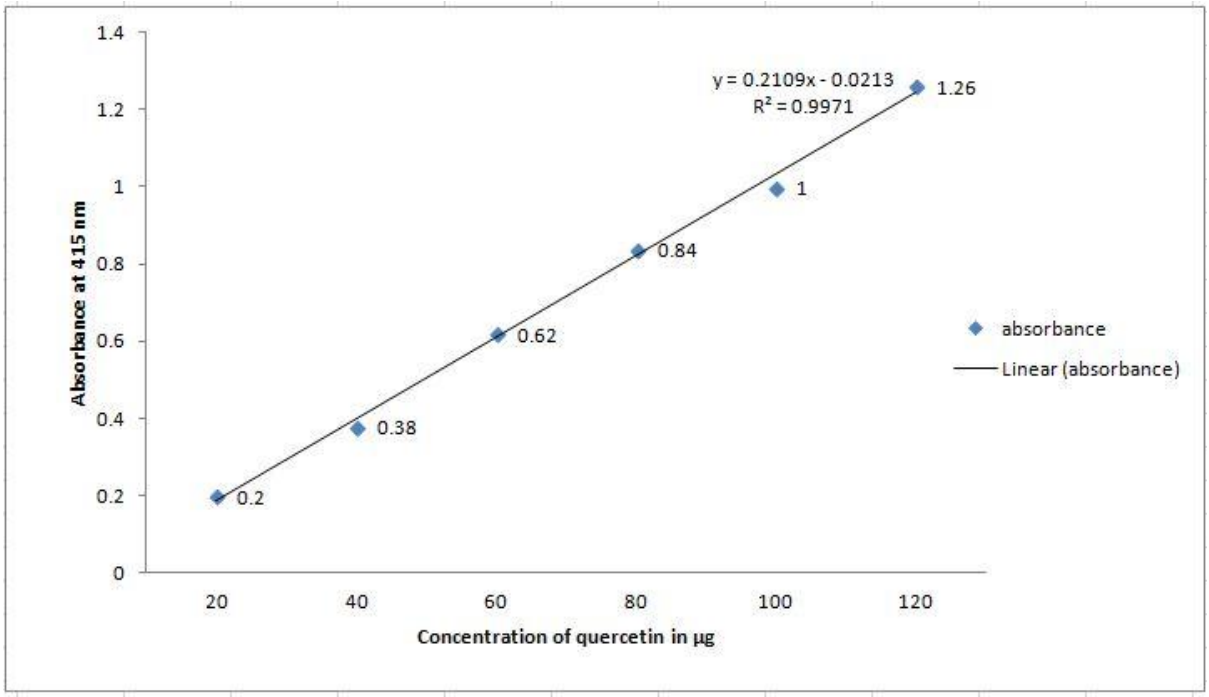


Fig. 9. Standard Quercetin curve for estimating total Quercetin in µg

5.5.2. ANTI-OXIDANT ACTIVITY.

For evaluation of antioxidant activity of all plant extracts (aerial part and root), different methods followed were as:

5.5.2.1. DPPH assay

The antioxidant activity of all plant extracts was measured with 1,1-diphenyl-2- picryl hydrazyl radical (DPPH) spectrophotometrically. **(Parray *et al.*, 2011).** The stock solution of plant extract was prepared by dissolving known amount of dry extract in 10 % Aq. DMSO. The working solutions of the extracts were prepared from the stock solution using suitable dilution. A solution of 0.005% DPPH was also prepared in methanol. Different concentrations was added to DPPH solution. The scavenging activity was observed by bleaching of DPPH solution from violet colour to light yellow. For control,ascorbic acid was used.

inhibition = {Ac As/Ac} x 100%

Where 'Ac' is the absorbance of the controlled reaction and 'As' is absorbance of sample.

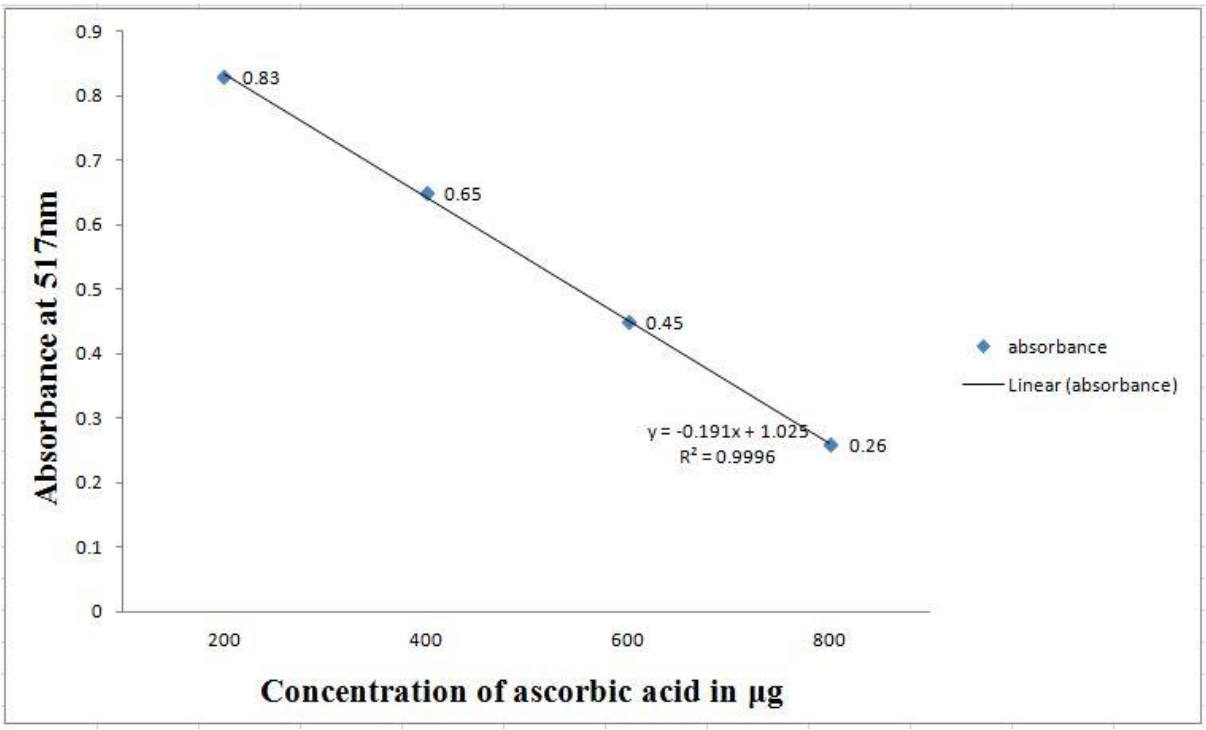


Fig. 10. Standard Ascorbic acid curve in µg

5.5.2.2. Superoxide anion radical scavenging activity.

Measurement of superoxide anion scavenging activity of the all extracts based on the method described by Liu *et al*. (1997). 100µl riboflavin solution (20µg), 200µl EDTA solution (12mM), 200µl mµl ethanol and 100µl nitro-blue tetrazolium (NBT) solution (0.1mg) were mixed in test tube and reaction mixture was diluted upto 3ml with phosphate buffer(50mM). The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5 minutes. This is taken as control. Then different concentrations (50 µl) i.e. 200µg, 400µg, 600µg, 800µg, of plant extracts were taken and diluted upto 150µl with methanol. To each of these add 100µl riboflavin, 200µl EDTA, 200µl methanol and 100µl NBT was mixed in test tube and further diluted upto 3ml with phosphate buffer. Absorbance was measured after illumination for 5 minutes at 590nm. Decreased absorbance of the reaction mixture indicates increased super oxide anion scavenging activity. The % inhibition of super oxide anion generation was calculated using the following formula:

SOD scavenged (%) = Ac- As/Ac x 100

Where 'A_c' is the absorbance of the blank and 'A_s' is absorbance of sample.

5.5.3. ANTI-MICROBIAL ACTIVITY.

5.5.3.1. Preparation of Glassware

All the glass ware was washed with tap water and then rinsed 2-3 times with distilled water. After drying in an oven, the all glass ware was autoclaved at 121ºC and 15lb pressure for 20 min and was kept for further use.

5.5.3.2. Test Microorganisms

Escherichia coli, Bacillus subtilis, Staphylococcus aureus, salmonella, Alternaria solani.

5.5.3.3. Antimicrobial Susceptibility Testing

The two principal methods are used for the antimicrobial susceptibility testing.

5.5.3.3.1. Disc diffusion method

The method of Kirby-Bauer (Bauer *et al*., 1966) was followed for the disc diffusion method. In this method, firstly 6mm sterilized filter paper disks were taken and soaked into the desired concentration of plant extract. Then these disks were placed on the surface of solidified agar in plate which was pre-inoculated with test organisms. These plates were then incubated at 37 ^{o}C for 24 hrs (bacteria) and at 27^{o}C for 48 hrs (fungi). After the incubation, the zone diameter was measured.

5.5.3.3.2. Well diffusion method

The principle of agar well diffusion is similar to that of agar disk diffusion assay. In this method, the sterile cork borer was used to make 6 to 8 mm diameter wells in the agar plates which was pre-inoculated with test organisms. Than the desired volume of the plant extract were introduced into the agar well. These plates were then incubated at 37 ^{o} C for 24 hrs (bacteria) and at 27 ^{o} C for 48 hrs (fungi). After the incubation, the zone diameter was measured.

CHAPTER-6

RESULTS AND DISCUSSION

6.1. PLANT EXTRACTION

Table 1. characteristics of the crude extracts of leaf part and root part of Arnebia benthamii

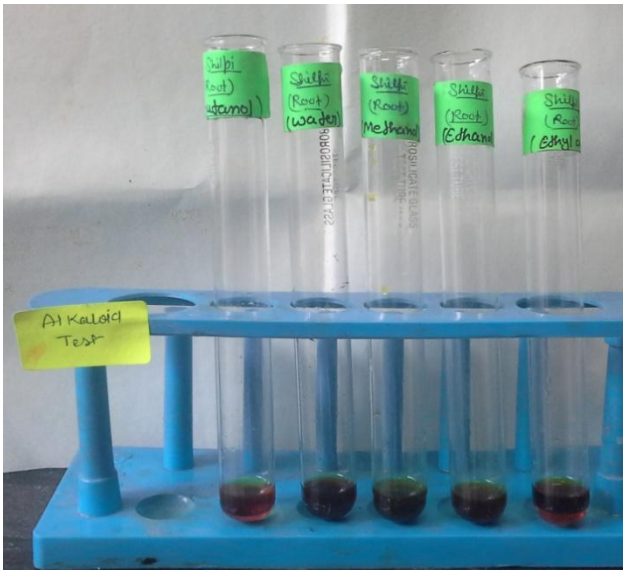
Sr. no.	Solvent	Plants parts used	Colour	Odour
1	Butanol	Leaf part	Light Green	Characteristic
2	Methanol		Brownish Green	Characteristic
3	Ethanol		Deep Green	Characteristic
4	Ethyl acetate		Deep Green	Characteristic
5	Aqueous		Deep Brown	Characteristic
6	Butanol	Stem Part	Light Green	Characteristic
7	Methanol		Deep Green	Characteristic
8	Ethanol		Deep Green	Characteristic
9	Ethyl acetate		Light Green	Characteristic
10	Aqueous		Deep Brown	Characteristic

6.2. PHYTOCHEMICAL SCREENING.

6.2.1. Qualitative Phytochemicals Screening.

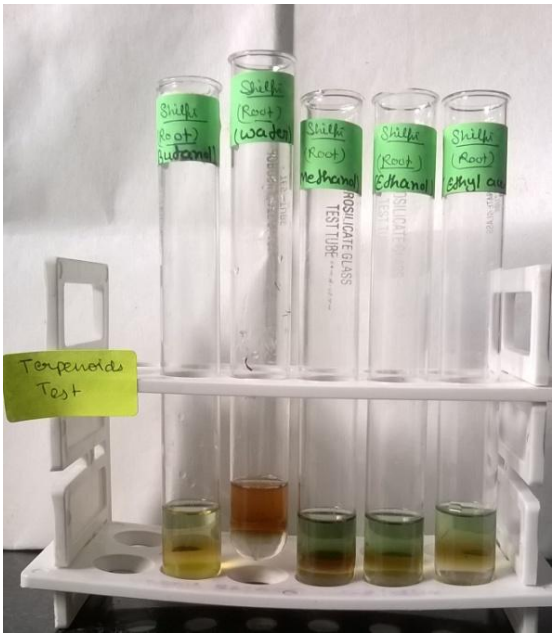
Different methods were followed to determine qualitatively the presence of phytochemical constituents present in the plant extracts.

Fig.11 Test for Alkaloids



In Stem Extracts **In Leaf Extracts**

Fig.12 Test for Terpenoids (Salkowski Test)



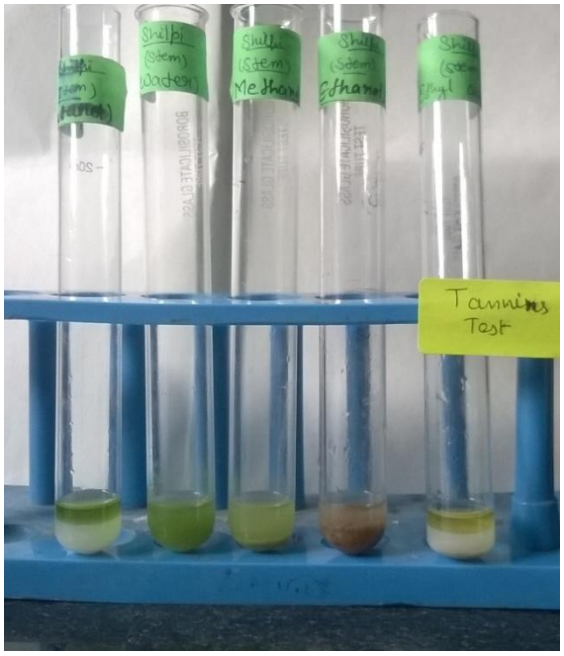
In Stem Extracts **In Leaf Extracts**

Fig.13 Test for Flavonoids

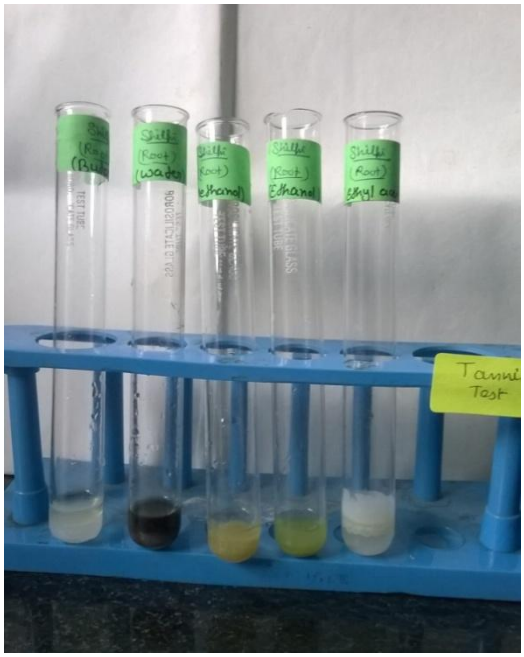
In Stem Extracts

In Leaf Extracts

Fig.14 Test for Tannins

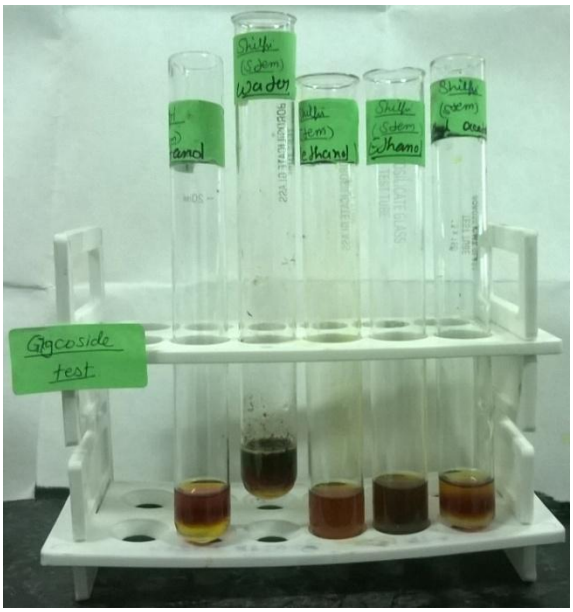


In Stem Extracts



In Leaf Extracts

Fig.15 Test for Glycosides

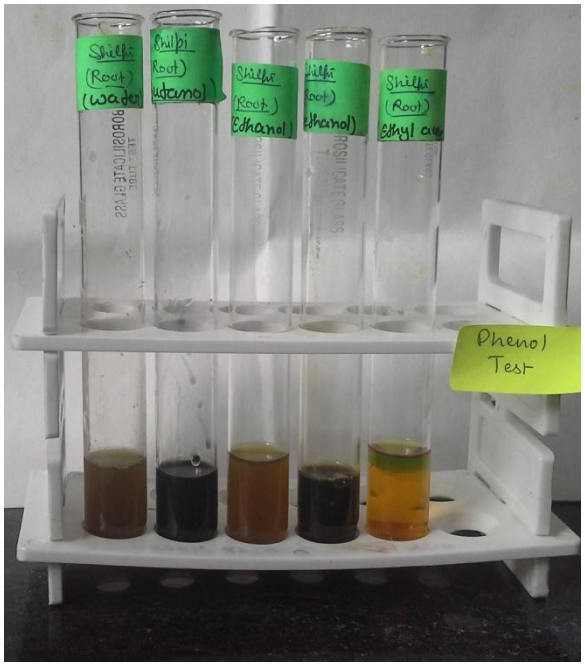


In Stem Extracts

In Leaf Extracts

Fig.16 Test for Phenols (Ferric chloride test)

In Stem Extracts



In Leaf Extracts

Table 2. Qualitative analysis for various secondary metabolites in leaf part (LP) extracts of **A. benthamii**

Sr. no.	Phytochemical constituents	B	M	Eth	EA	Aq
1	Alkaloids	_	+	+	_	_
2	Terpenoids	_	+	+	_	+
3	Flavonoids	+	_	_	+	+
4	Tannins	_	_	_	_	_
5	Glycosides	+	+	+	+	_
6	Phenols	_	+	+	+	_

(+) = present; (-) = Absent; EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous.

Table 3. Qualitative analysis for various secondary metabolites in stem part (SP) extracts of **A. benthamii**

Sr. no.	Phytochemical constituents	B	M	Eth	EA	Aq
1	Alkaloids	_	+	+	_	_
2	Terpenoids	_	+	+	_	+
3	Flavonoids	+	+	_	+	+
4	Tannins	_	_	_	_	_
5	Glycosides	+	_	_	+	+
6	Phenols	+	+	+	+	_

(+) = present; (-) = Absent; EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous.

As illustrated in Table- 2 and 3, the alkaloids are present in methanol(M), and ethanol (Eth), while absent in butanol (B), ethyl acetate (EA), and aqueous (Aq) extracts of both stem part and leaf part. Terpenoids are present in all the solvent extracts of SP and LP except B and EA extracts. It is present in more amount in Aq as compare to another extracts. Glycoside present in more amount in B and EA of SP and LP both but it is absent in Aq of LP and M , Eth of SP extracts. Tannin is absent in both SP and LP extracts. Phenol is absent in Aq of both SP, and LP extracts.

6.2.2. Qualitative Phytochemicals Screening

6.2.2.1. TOTAL PHENOLIC CONTENT

The total phenolic contents of solvent extract was determined and expressed in terms of Gallic acid equivalents (mg/g).

Total Phenolic Content = (Absorbance of Test/ Absorbance of Standard) Concentration of Standard.

Table 4. Total Phenolic content in stem and leaf extracts.

Sr. no.	Solvent	Total Phenolic content in stem (mg/g)	Total Phenolic content in leaf (mg/g)
1	Butanol	50.17 ± 1.10	55.4 ± 0.65
2	Methanol	122.63 ± 0.78	116.63 ± 0.78
3	Ethanol	83.4 ± 0.71	87.42 ± 0.91
4	Ethyl-acetate	77.47 ± 0.57	71.43 ± 0.94
5	Aqueous	35.47 ± 1.06	30.57 ± 0.98

Data presented as mean ± standard deviation.

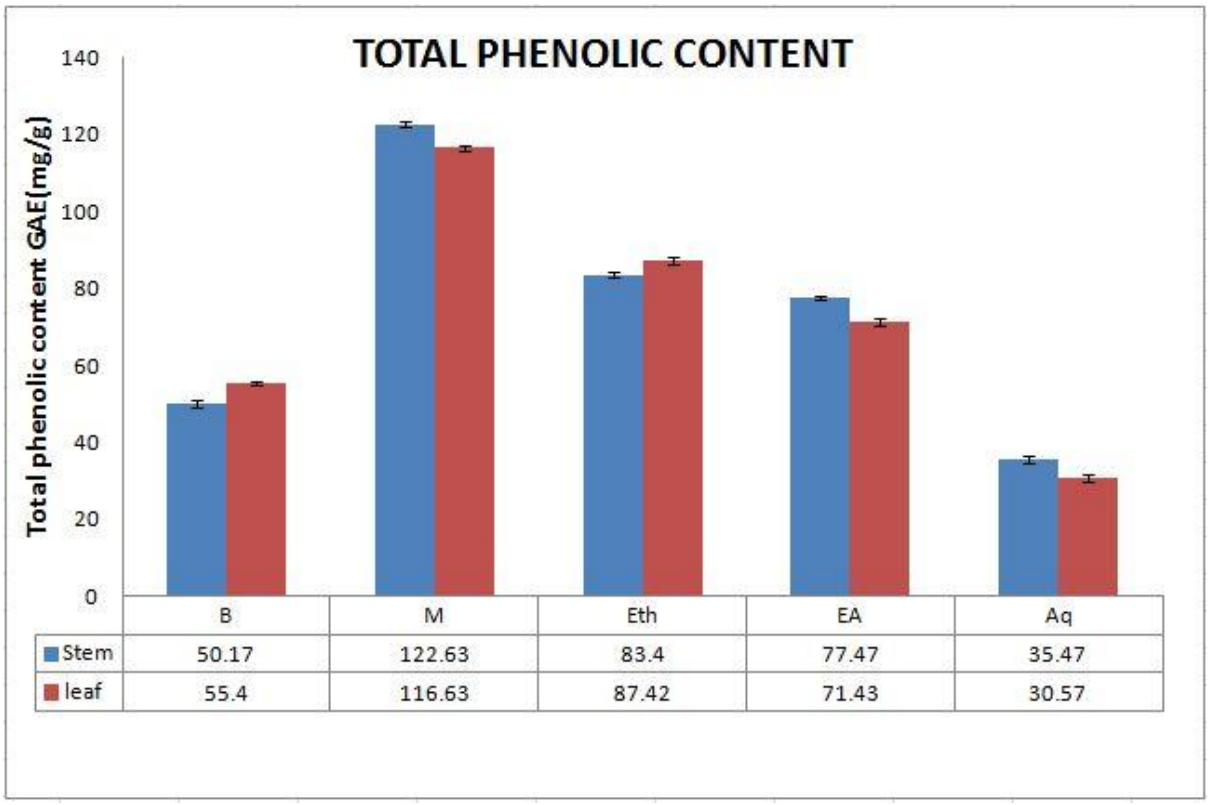


Fig. 17 Total Phenolic Content of Stem parts (SP) and Leaf part (LP) extracts of *A. Benthamii.*

In the Table-4 and given above it is shown that The highest phenolic content have been found in methanol extract of stem (122.63 ± 0.78) and leaf (116.63 ± 0.78). But also as good amount in leaf extract of Ethanol (87.42 ± 0.91), and stem extract of Ethyl Acetate (77.47 ± 0.57) . The least amount of phenolic content was found in aqueous extract of both leaf (30.57 ± 0.98) and stem (35.47 ± 1.06) extract as illustrated in Table-4 and Fig.-17.

6.2.2.2. TOTAL FLAVONOIDS CONTENT

The total flavonoid contents of solvent extract was determined and expressed in terms of Quercetin equivalents (mg/g).

Total Flavonoid Content = (Absorbance of Test/ Absorbance of Standard) Concentration of Standard.

Table 5. Total Flavonoid content in stem and leaf extracts.

Sr. no.	Solvent	Total Flavonoid content in stem extracts(mg/g)	Total Flavonoid content in leaf extracts(mg/g)
1	Butanol	63.55 ± 0.76	66.5 ± 0.57
2	Methanol	108.4 ± 0.97	114.53 ± 0.99
3	Ethanol	86.57 ± 1.06	81.47 ± 0.77
4	Ethyl-acetate	75.57 ± 0.94	70.42 ± 0.74
5	Aqueous	36.16 ± 0.46	31.41 ± 1.1

Data presented as mean ± standard deviation.

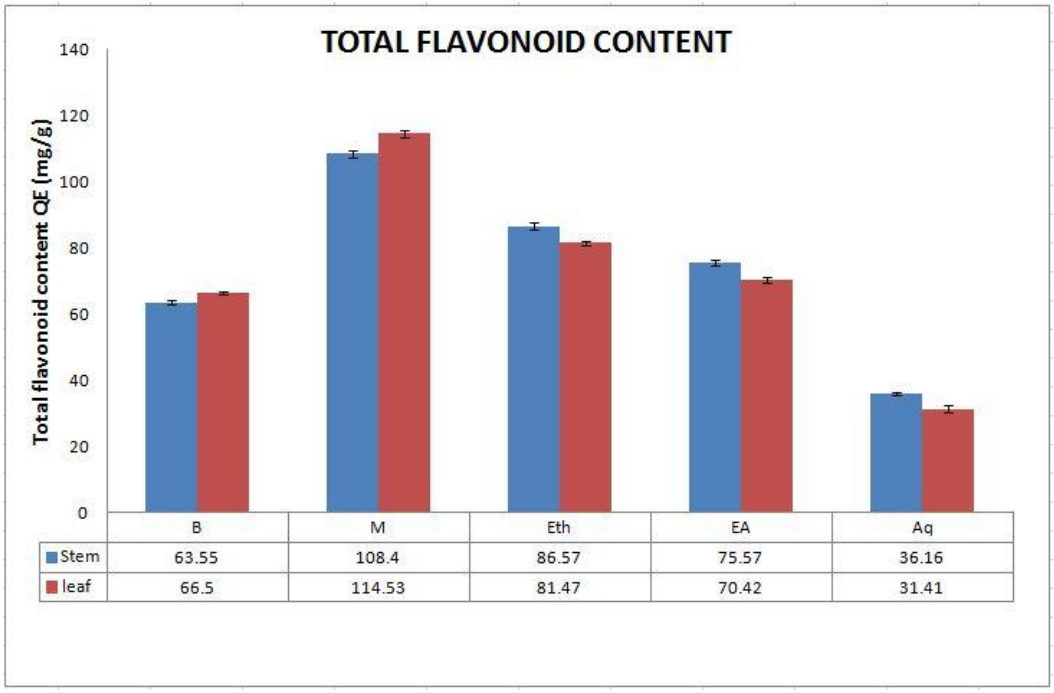


Fig. 18 Total Flavonoid Content of Stem parts and Leaf part extracts of *A. Benthamii.*

In the Table-5 and Fig.-18 given above it is shown that the highest flavonoid content have been found in methanol extract of stem (108.4 ± 0.97) and leaf (114.53 ± 0.99) as well as in stem extract of Eethanol (86.57 ± 1.06) and Ethyl Acetate (75.57 ± 0.94) . The least amount of phenolic content was found in aqueous extract of leaf (31.41 ± 1.1) and stem (36.16 ± 0.46) both.

6.3. ANTIOXIDENT ACTIVITY.

6.3.1. DPPH Assay.

Table 6. Radical Scavenging activities of extracts of SP & LP extracts of *A. benthamii*. as measured by DPPH method at different concentrations

Parts used	Extracts	% scavenging activity			
		200 (µl/ml)	400 (µl/ml)	600 (µl/ml)	800 (µl/ml)
SP	B	35.28 ± 0.77	46.63 ± 0.96	57.66 ± 1.17	68.70 ± 1.24
	M	37.62 ± 0.87	48.39 ± 0.94	59.23 ± 0.71	70.32 ± 1.25
	Eth	36.41 ± 0.92	47.22 ± 0.95	57.44 ± 0.7	68.47 ± 0.72
	EA	36.40 ± 0.64	47.32 ± 0.77	58.42 ± 0.73	68.26 ± 1.11
	Aq	35.38 ± 0.83	46.56 ± 1.01	57.27 ± 0.81	68.39 ± 0.87
LP	B	41.52 ± 0.9	51.52 ± 1.4	63.06 ± 1.05	71.78 ± 0.73
	M	41.36 ± 0.74	51.60 ± 1.3	62.97 ± 0.95	74.45 ± 1.23
	Eth	39.41 ± 0.73	50.22 ± 0.8	61.49 ± 0.74	71.02 ± 1.3
	EA	38.46 ± 0.71	49.59 ± 1.28	60.43 ± 1.6	74.14 ± 1.5
	Aq	40.52 ± 0.75	50.41 ± 0.62	61.44 ± 0.7	71.72 ± 1.01
Control	Aa	39.16 ± 0.55	51.61 ± 0.46	66.54 ± 0.5	81.41 ± 0.53

Values are represented as Mean ± SD, All experiments were repeated thrice; SP = stem part; LP=leaf part, EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous, Aa= ascorbic-acid

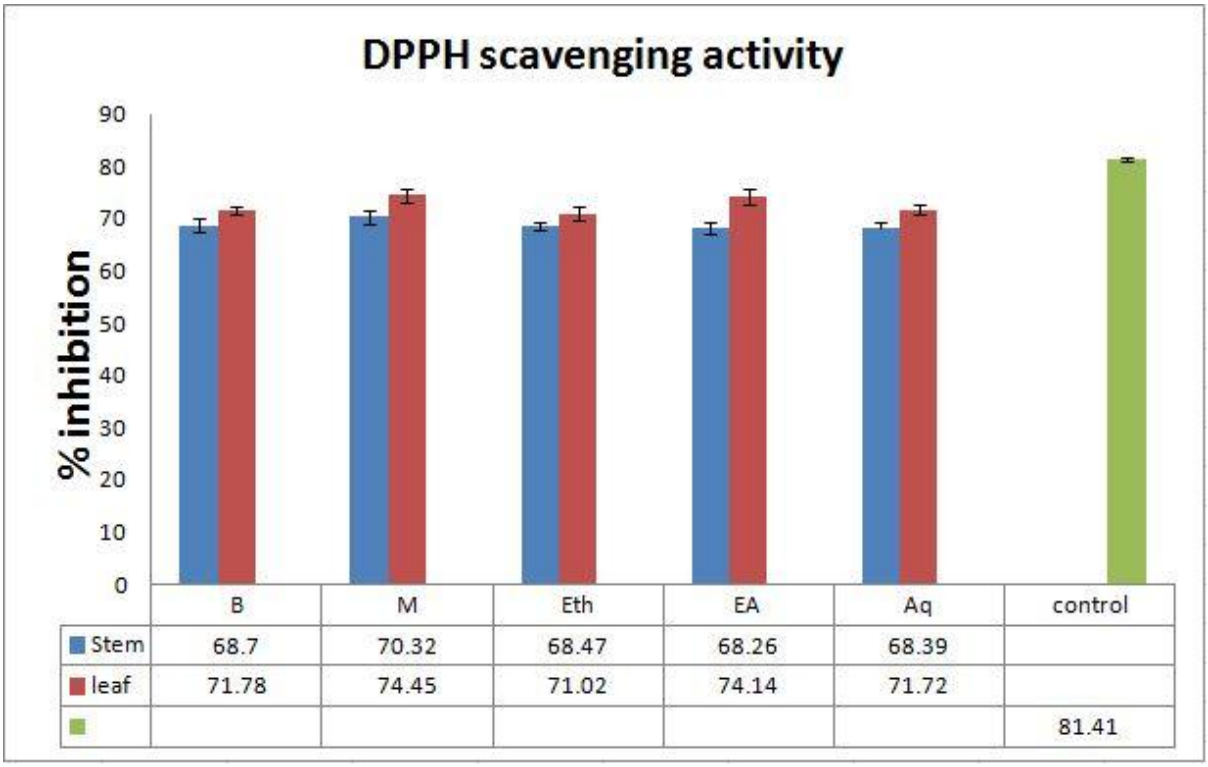


Fig. 19 DPPH Scavenging activity (%).

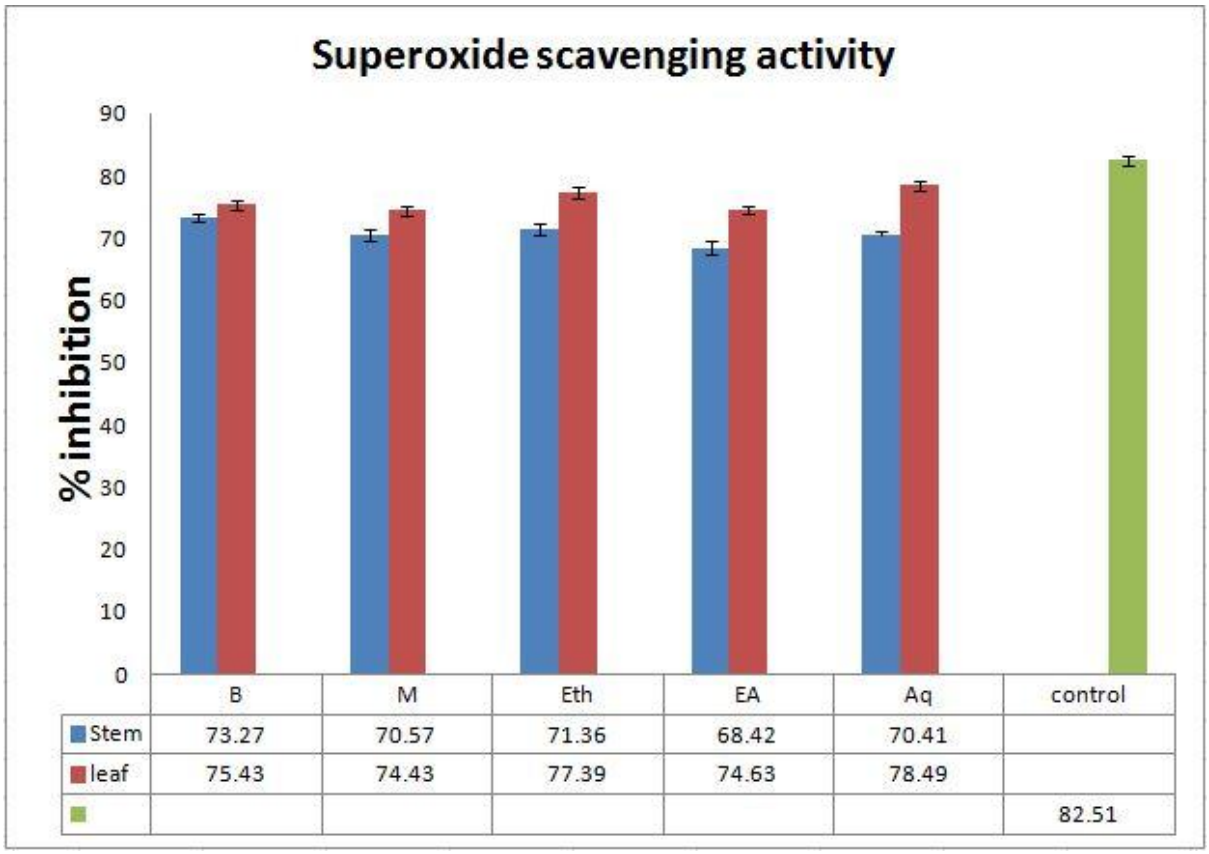
As illustrated in Table-6 and Fig.-19, the highest DPPH scavenging activity was found to be in the methanol and ethyl acetate extract of leaf and the least in ethanol and aqueous. In stem extract, the highest % inhibition was found in methanol and others are almost equal.

6.3.2. Superoxide anion radical scavenging activity.

Table 7. Superoxide dismutase (SOD) activity of extracts of aerial & root parts of A. benthamii measured by riboflavin photo-oxidation method at different concentrations presented as % of inhibition rate.

Parts used	Extracts	% scavenging activity			
		200 (µl/ml)	400 (µl/ml)	600 (µl/ml)	800 (µl/ml)
SP	B	37.32 ± 0.690	48.51 ± 0.758	58.22 ± 0.905	73.27 ± 0.669
	M	34.46 ± 0.993	49.36 ± 0.847	59.14 ± 0.993	70.57 ± 1.02
	Eth	36.49 ± 0.624	49.45 ± 0.756	57.57 ± 0.598	71.36 ± 0.876
	EA	35.55 ± 1.047	46.54 ± 0.869	57.53 ± 1.091	68.42 ± 1.097
	Aq	37.63 ± 1.016	46.42 ± 0.883	58.41 ± 0.645	70.41 ± 0.835
LP	B	43.55 ± 0.629	52.54 ± 0.805	64.26 ± 0.844	75.43 ± 0.765
	M	40.41 ± 0.996	49.58 ± 0.490	61.27 ± 0.914	74.43 ± 0.770
	Eth	42.15 ± 0.759	53.48 ± 0.939	65.54 ± 0.921	77.39 ± 0.926
	EA	41.62 ± 0.965	53.22 ± 0.673	64.3 ± 0.694	74.63 ± 0.589
	Aq	40.27 ± 0.894	52.63 ± 0.978	66.38 ± 1.062	78.49 ± 0.731
	Aa	41.58 ± 1.041	54.47 ± 0.875	68.46 ± 0.703	82.51 ± 0.724

Values are represented as Mean ± SD, All experiments were repeated thrice; SP = stem part; LP=leaf part, EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous, Aa= ascorbic acid



	B	M	Eth	EA	Aq	control
Stem	73.27	70.57	71.36	68.42	70.41	
leaf	75.43	74.43	77.39	74.63	78.49	
						82.51

Fig: 20 Superoxide scavenging activity(%)

As illustrated in Table-7 and Fig.-20, the highest superoxide scavenging activity was found to be in the aqueous and ethanol extract of leaf and the least in ethyl acetate of stem part. Extracts of leaf part exerting more inhibition than stem part.

6.4. ANTIMICROBIAL ACTIVITY

Table 8. Antimicrobial activity of different solvents of Leaf part (LP) extracts of *A. benthamii*.

Strain	Extract	Leaf Part	Standard
Escherichia coli	B	0	11.33 ± 0.942
	M	7.33 ± 0.471	11.67 ± 1.24
	Eth	6 ± 0.667	11.67 ± 0.942
	EA	0	11.33 ± 1.24
	Aq	0	12.67 ± 0.471
Bacillus subtilis	B	6.33 ± 0.471	16 ± 1.414
	M	5.67 ± 0.471	16.33 ± 0.471
	Eth	4.33 ± 0.471	16.33 ± 1.247
	EA	0	15.67 ± 1.247
	Aq	0	16 ± 1.632
Staphylococcus aureus	B	0	11 ± 0.816
	M	0	11.33 ± 0.942
	Eth	0	11 ± 0.816
	EA	4.33 ± 0.471	11.33 ± 0.942
	Aq	7.33 ± 0.471	11 ± 0.816
salmonella	B	0	10.67 ± 1.24
	M	4.33 ± 0.471	11.33 ± 0.942
	Eth	4 ± 0.667	10.67 ± 1.24
	EA	0	11.33 ± 0.942
	Aq	0	11.67 ± 1.24
A. solani	B	7.67 ± 0.471	10.33 ± 0.471
	M	3.67 ± 0.471	9.67 ± 0.471
	Eth	0	10.33 ± 0.471
	EA	2.33 ± 0.471	9.67 ± 0.471
	Aq	0	10.33 ± 0.471

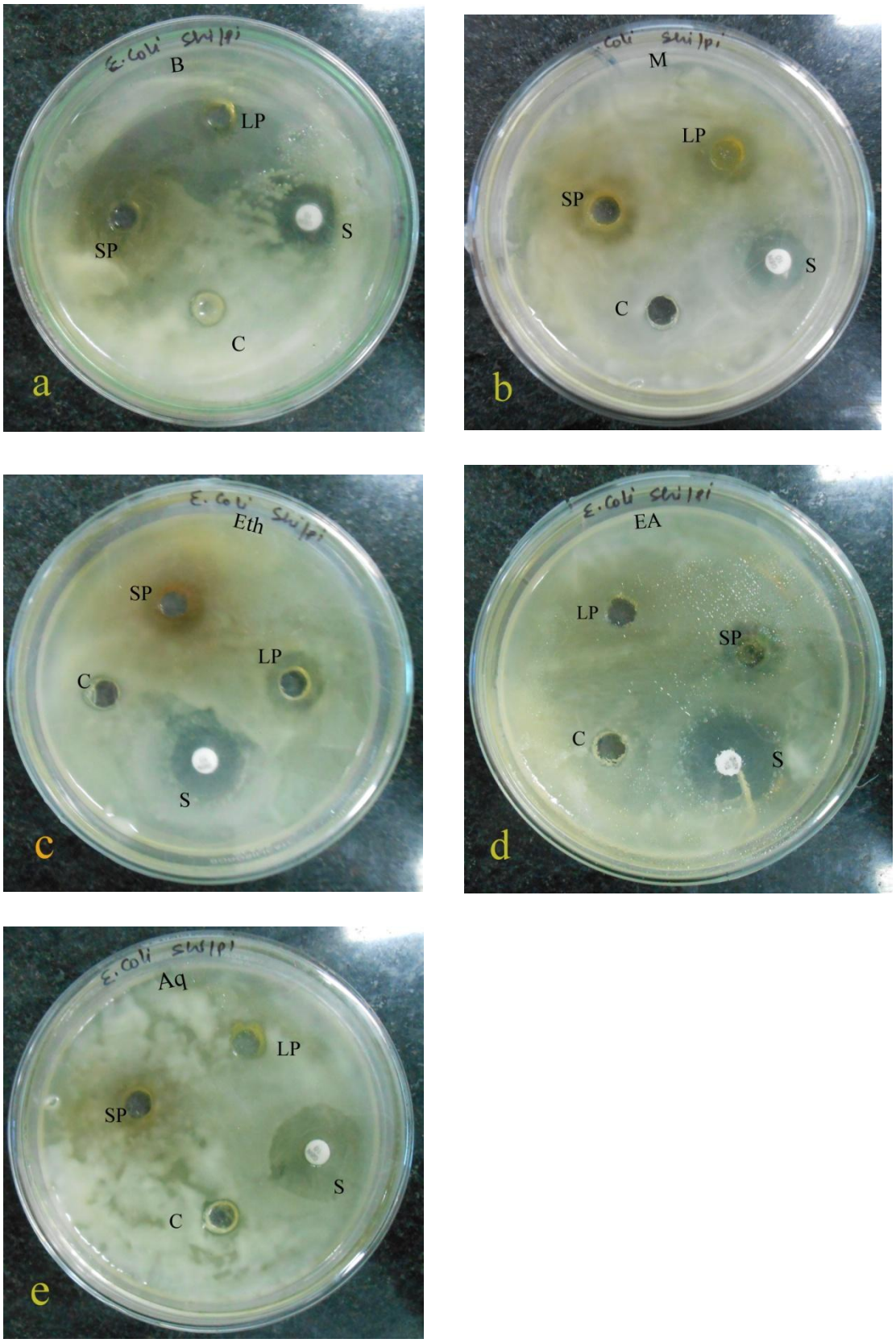
Values are represented as Mean ± SD, All experiments were repeated thrice; SP = stem part; LP=leaf part, EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous. DMSO was used as negative control and was found resistant in all strains.

Table 9. Antimicrobial activity of different solvents of Stem part (SP) extracts of *A. benthamii*

Strain	Extract	Stem Part	Standard
Escherichia coli	B	0	11.33 ± 0.942
	M	6 ± 0.667	11.67 ± 1.24
	Eth	5.67 ± 0.942	11.67 ± 0.942
	EA	5.67 ± 1.24	11.33 ± 1.24
	Aq	0	12.67 ± 0.471
Bacillus subtilis	B	6.67 ± 1.24	16 ± 1.414
	M	0	16.33 ± 0.471
	Eth	0	16.33 ± 1.247
	EA	0	15.67 ± 1.247
	Aq	0	16 ± 1.632
Staphylococcus aureus	B	0	12 ± 0.816
	M	0	11.33 ± 0.942
	Eth	0	12 ± 0.816
	EA	7 ± 0.667	11.33 ± 0.816
	Aq	0	12 ± 0.816
salmonella	B	0	12.33 ± 0.471
	M	3.67 ± 0.471	11 ± 0.667
	Eth	3.67 ± 0.471	12.33 ± 0.471
	EA	0	11 ± 0.667
	Aq	0	12.33 ± 0.471
A. solani	B	0	10.67 ± 0.942
	M	5.33 ± 0.471	10.67 ± 0.471
	Eth	0	10.67 ± 0.942
	EA	4.67 ± 0.471	10.67 ± 0.471
	Aq	0	10.67 ± 0.942

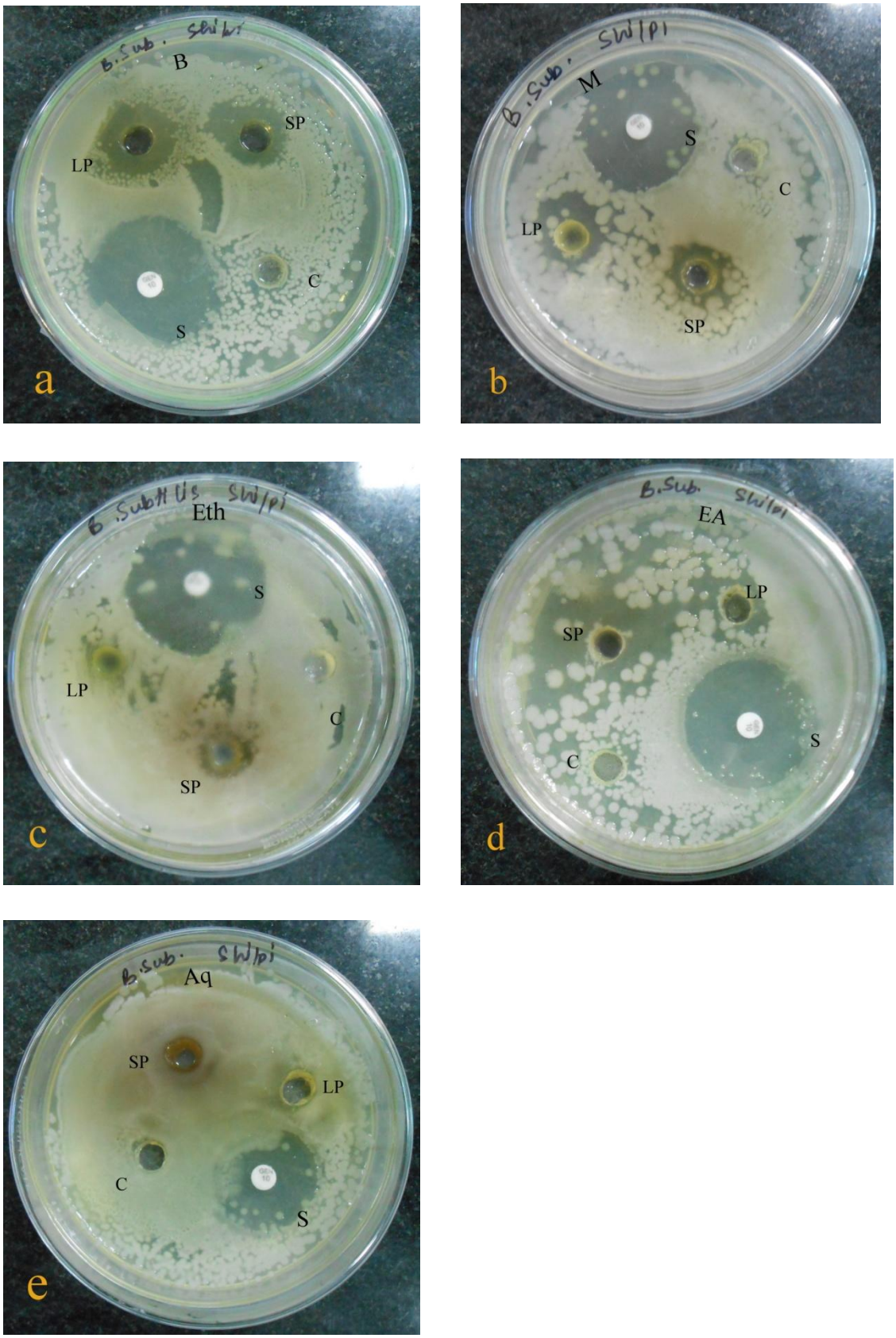
Values are represented as Mean ± SD, All experiments were repeated thrice; SP = stem part; LP=leaf part, EA=Ethyl acetate, B= Butanol, Eth=Ethanol, M=Methanol, Aq=Aqueous. DMSO was used as negative control and was found resistant in all strains.

Fig.21 Antimicrobial activity against E. coli



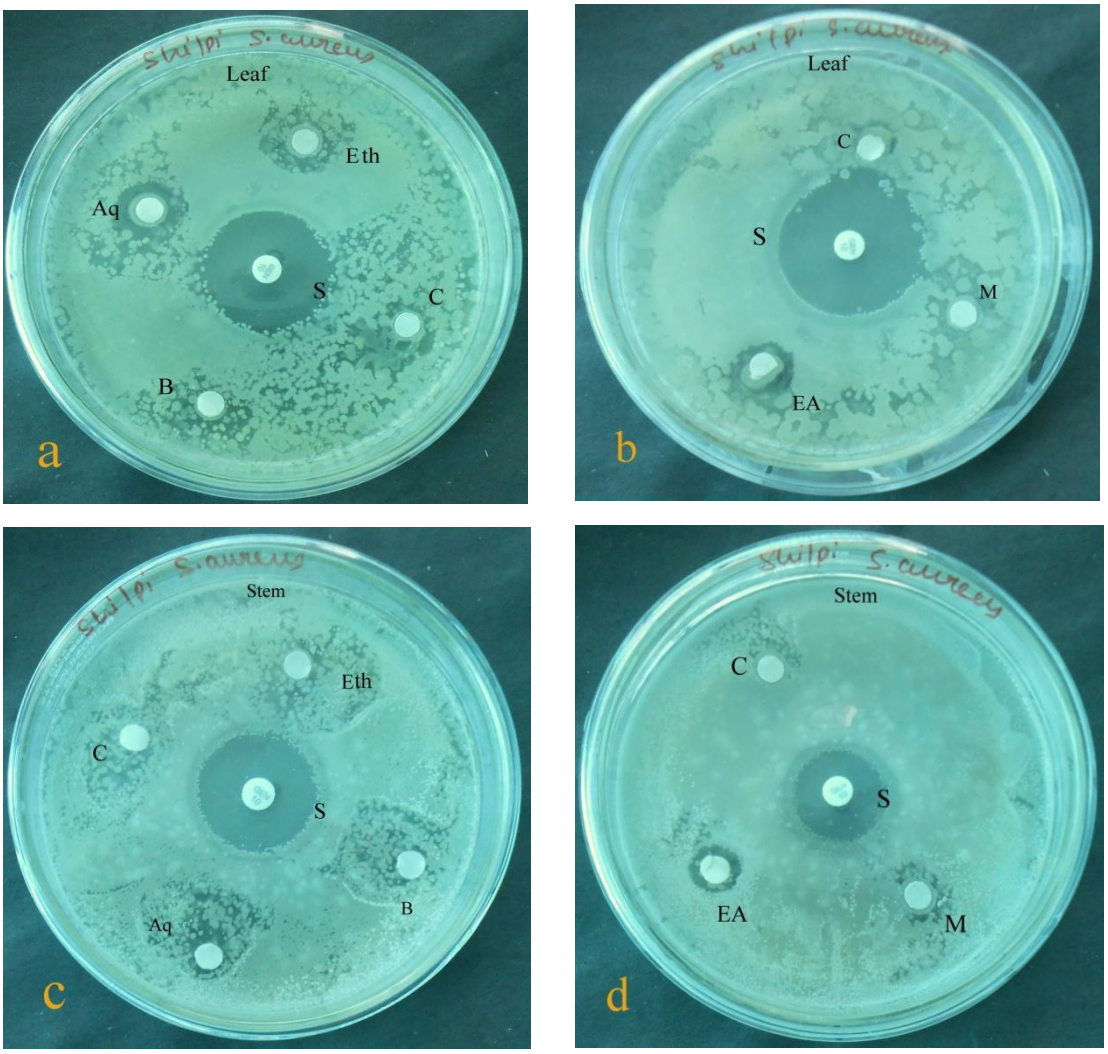
a = butanol, b = methanol, c = ethanol, d = ethyl acetate, e = aqueous.

Fig.22 Antimicrobial activity against Bacillus subtilis



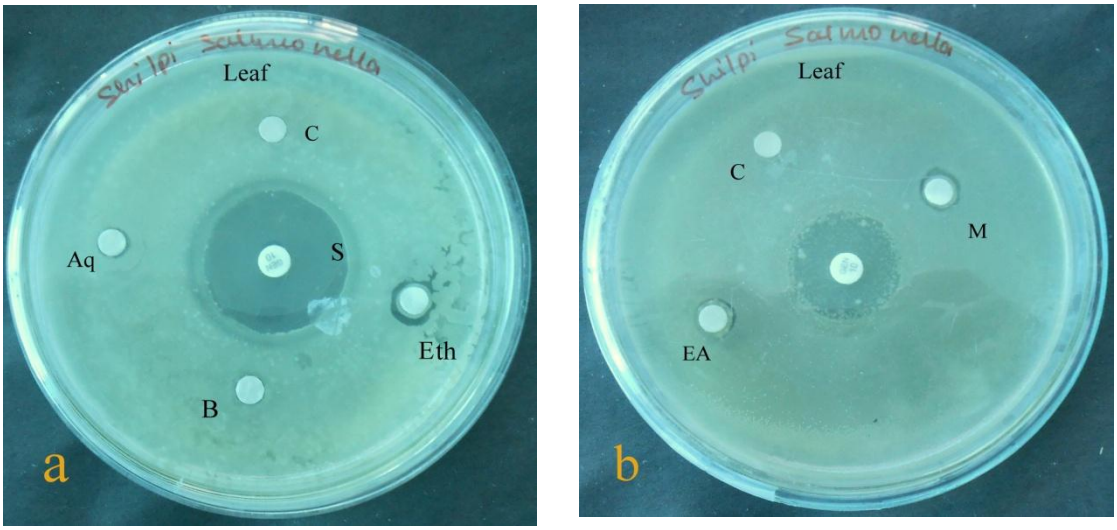
a = butanol, b = methanol, c = ethanol, d = ethyl acetate, e = aqueous.

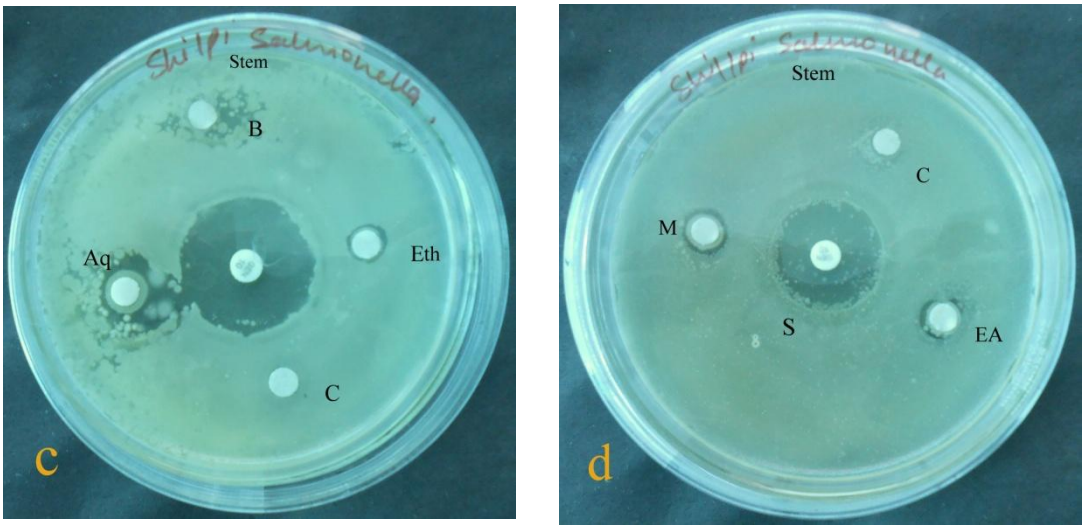
Fig.23 Antimicrobial activity against Staphylococcus aureus.



a and b are represented aii the extract of leaf part and the c and d are represented aii the extract of stem part.

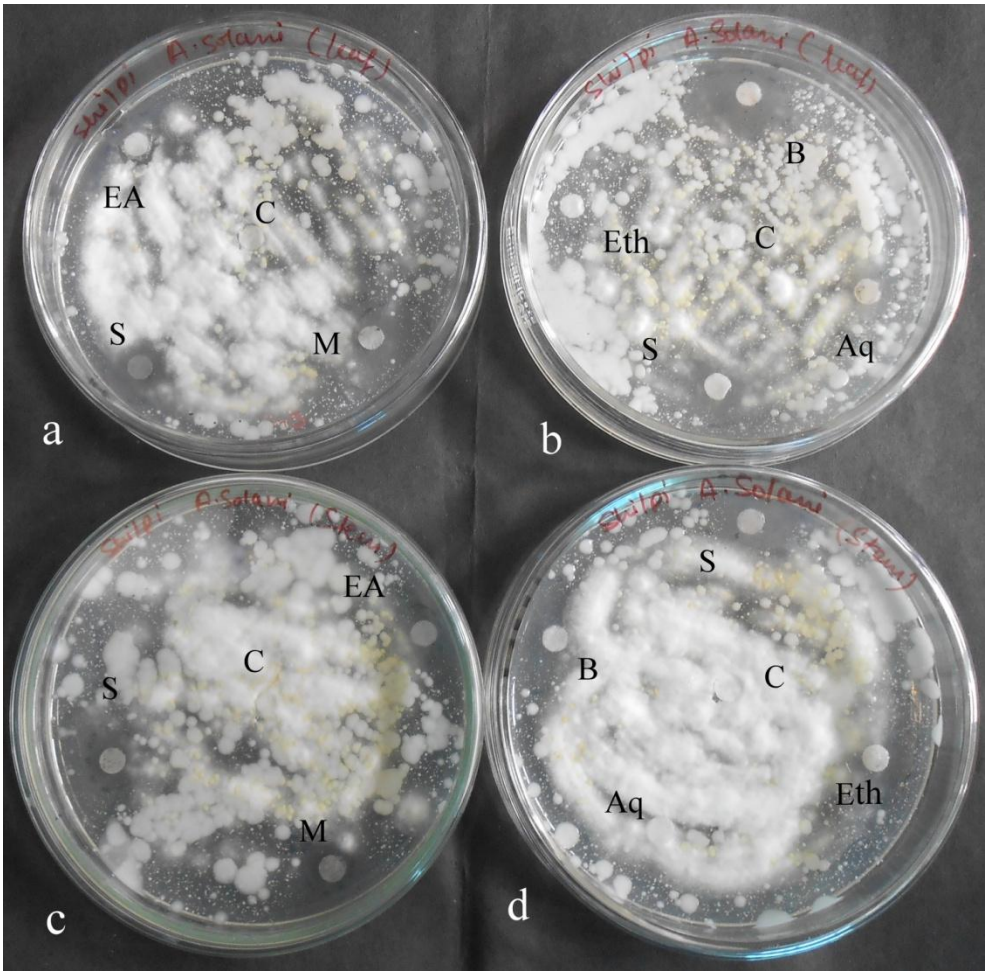
Fig.24 Antimicrobial activity against salmonella.





a and b are represented aii the extract of leaf part and the c and d are represented aii the extract of stem part.

Fig.25 Antimicrobial activity against A. solani



a and b are represented aii the extract of leaf part and the c and d are represented aii the extract of stem part.

Each strains of bacteria and fungi was tested for susceptibility tests against different solvent extracts of LP & SP of A. benthamii using the disc diffusion and well diffusion method. Antibiotics were used as positive controls on each tested strains while as DMSO was used as negative control and was found resistant in all strains. The result showed that the plant extracts of leaf part and stem part both exhibited significant antimicrobial activity. Comparative analysis reveals that the MeOH extract of LP were found to exhibit highest antibacterial activities against all tested bacterial strains except *S. aureus* which was found resistant. After MeOH extract, the highest IZD (7mm) was recorded for ethanol against *E. coli.* The EA extract showed least inhibitory activity against *Escherichia coli* and no activity against salmonella however the highest IZD (8mm) of EA extract was recorded for S. aureus and A. solani. All the strains except Bacillus subtilis were found resistant against B extracts. The Aq. extract was completely ineffective against all the tested bacterial and fungal strains except Staphylococcus aureus. The fungal strains of A. solani were exhibited significant antifungal activity by both LP & SP extracts of A. benthamii. However, the Aq. and Eth extract of both LP & SP did not show any antifungal activity. The M extract of SP and B extract of LP showed highest inhibitory activity against A. solani with IZD (5mm) and IZD (8mm) respectively.

CHAPTER-7
CONCLUSION

From the results of our study we concluded that the different methods were followed to determine qualitatively the presence of phytochemical constituents present in the plant extracts. Different methods were followed to determine qualitatively the presence of phytochemical constituents present in the plant extracts. The alkaloids are present in methanol(M), and ethanol (Eth), while absent in butanol (B), ethyl acetate (EA), and water (W) extracts of both stem part and leaf part. Terpenoids are present in all the solvent extracts of SP and LP except B and EA extracts. It is present in more amount in W as compare to another extracts. Glycoside present in more amount in B and EA of SP and LP both but it is absent in W of LP and M , Eth of SP extracts. Tannin is absent in both SP and LP extracts. Phenol is absent in W of both SP, and LP extracts. The highest phenolic content have been found in methanol extract of stem and leaf as well as in Eethanol and Ethyl Acetate . The least amount of phenolic content was found in water extract of leaf and stem both extract. The highest flavonoid content have been found in methanol extract of stem and leaf as well as in Eethanol and Ethyl Acetate . The least amount of phenolic content was found in water extract of leaf and stem both extract.

To obtain information about the mechanisms of the anti-oxidative effects of the different extracts, two *in-vitro* tests, the DPPH and Superoxide anion radical scavenging activity were used to know the antioxidant properties of plant extracts of *A. benthamii*. Together both the methods provide a better assessment of antioxidant properties and results show inhibitory activity that was concentration dependent. The highest DPPH scavenging activity was found to be in the methanol and ethyl acetate extract of leaf and the least in ethanol and water. In stem extract, the highest % inhibition was found in methanol and others are almost equal. And the superoxide scavenging activity was found to be highest in the aqueous and ethanol extract of leaf and the least in ethyl acetate of stem part. Extracts of leaf part exerting more inhibition than stem part. The plant *A. benthamii* if properly extracted can be used for preparation of various new drugs to cure various epidemiological diseases. Also the antioxidant activity results of the plant roots reveals that it seems to be good prospect for developing antioxidant substances and is now expected that screening of this plant for antimicrobial/antioxidant activity will be helpful in obtaining broad spectrum herbal

formulation as well as new antimicrobial and antioxidant substances. Each strains of bacteria and fungi was tested for susceptibility tests against different solvent extracts of LP & SP of A. benthamii. The result showed that the plant extracts of leaf part and stem part both exhibited significant antimicrobial activity. Comparative analysis reveals that the MeOH extract of LP were found to exhibit highest antibacterial activities against all tested bacterial strains except *S. aureus* which was found resistant. But the Aq. extract was completely ineffective against all the tested bacterial and fungal strains except **Staphylococcus aureus.** The fungal strains of **A. solani** were also exhibited significant antifungal activity by both LP & SP extracts of A. benthamii. However, the Aq. and Eth extract of both LP & SP did not show any antifungal activity.

CHAPTER-8

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