A DISSERTATION ON

Identification of Bioagents of *Andrographis paniculata* (Kalmegh) and their Antimicrobial Effects

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IN BIOTECHNOLOGY

BY
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M. Tech Biotechnology (IV Semester)

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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, Hina Khatoon, a student of M. Tech Biotechnology (**2nd**Year / **4th**Semester), Integral University have completed my six months dissertation work entitled **"Identification of Bioagents of *Andrographis paniculata* (Kalmegh) and their Antimicrobial Effects"** successfully from Integral University under the able guidance of Dr. Mohammad Haneef and Dr. Salman Akhtar. I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

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CERTIFICATE BY SUPERVISOR

Certificate that Ms **Hina Khatoon** (EnrolmentNumber:1600102664) has carried out the research work presented in this thesis entitled **"Identification of Bioagents of *Andrographis paniculata* (Kalmegh) and their Antimicrobial Effects"** for the award of **M. Tech (Biotechnology)** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **M. Tech (Biotechnology)**.

I wish her good luck and bright future.

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This is to certify that **Hina Khatoon,** a student of **M. Tech (Biotechnology)** (**2nd**Year / **4th**Semester), Integral University has completed her six months dissertation work entitled **"Identification of Bioagents of *Andrographis paniculata* (Kalmegh) and their Antimicrobial Effects"** successfully. She has completed this work from Integral University under the guidance of Dr. Mohammad Haneef, Assistant Professor, Department of Bioengineering. The dissertation was a compulsory part of her **M. Tech (Biotechnology).**

I wish her good luck and bright future.

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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

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Date: 19th JULY 2022 **HINA KHATOON**

LIST OF ABBREVIATIONS

AP-*Andrographis paniculata*

DMSO-Dimethyl sulphooxide

CIMAP –Central Institute of Medicinal and Aromatic Plants

LAI – Leaf Area Index

IU – International unit.

mM – mili molar.

$Mgcl_2$ – Magnesium chloride.

NAA – Napthalene acetic acid.

NaOH – Sodium hydroxide.

NADH – Nicotinamide adenine dinucleotide

PPM – Parts per million.

V – Volume.

W – Weight.

W/V – Weight/volume.

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1. INTRODUCTION

In many developing countries, it is estimated that about two third population relies heavily on traditional practitioners and medicinal plants to meet primary healthcare needs (Farnsworth *et al*., 1991). As a result of the numerous problems associated with orthodox drugs, many plant species are now been revalued by researchers based on variation in plant species and their therapeutic chemical principles. Therefore, the need to do a thorough literature search on some species with a view to update the current state of knowledge is imperative. One of such plant species is *Andrographis paniculata (Andrographis paniculata)* used in ancient oriental and ayurvedic medicine. The genus Andrographis which belongs to the Acanthaceae family comprises of about 40 species. Only a few are popular for their use in folk medicine for assorted health concerns. Of these few, *Andrographis paniculata* is the most important and *Andrographis paniculata,* commonly known as King of Bitters or kalmegh, is an annual, branched, erect handsome herb running half to one meter in height. It is native to peninsular India and Srilanka and is also distributed in different regions of Southeast Asia, China, America, West Indies and Christmas Island. It is cultivated because of its well-known medicinal value and it grows well in most soil types thus it is widely distributed (Latto*et al*., 2006). The aerial parts and roots of the plant have been widely used as traditional medicine in China, India, Thailand and other Southeast Asian countries to treat many maladies.

It is known as King of Bitters (English), Mahatikta (Sanskrit), Kiryato (Gujarati), Mahatita (Hindi), Kalmegh (Bengali), or Fah TalaiJone (Thai) (Li, *et al*., 2007). A wide array of studies has been conducted by researchers, especially in Asia, following reports about the medicinal properties possessed by this plant mostly according to traditional medical practitioners in ayurvedic medical system. Phytochemical studies have revealed that *Andrographis paniculata* contains diverse compounds including labdane diterpenoid lactones, flavonoids and miscellaneous compounds. It has been shown to possess wide spectrum of pharmacological properties (Mishra *et al*., 2007 and Khare*et al*., 2007). This review is focused on its medicinal properties, phytochemistry and the pharmacological effects of its various extracts and compounds including anti-microbial, cytotoxicity, anti-protozoan, antiinflammatory, antioxidant, immunostimulant, anti-diabetic, anti-infective, anti-angiogenic, hepato-renal protective, sex hormone modulatory, liver enzymes modulatory and insecticidal activities. Furthermore, this review also discusses some toxicological aspects of this species.

Kalmegh (*Andrographis paniculata*) commonly known as “king of bitter” belonging to family Acanthacea, is an important annual medicinal herb widely distributed in Madhya Pradesh, India. It is hardy and erect herb which grows mainly as undershrub in tropical, moist deciduous forest. It is one of the most widely used plant in Ayurvedic formulations (Okeke, *et al*. 2001). It is used to overcome sannipata type of fever, difficulty in breathing, hemopathy burning sensation, cough, skin diseases, fever, ulcer and worms. It is also useful in acidity and liver complaints (Aiyer and Kolammal, 1962).

Andrographis paniculata grows erect to a height of 30– 110 cm in moist, shady places. The slender stem is dark green, squared in cross-section with longitudinal furrows and wings along the angles. The lance shaped leaves have hairless blades measuring up to 8 centimeters long by 2.5 wide. The small flowers are borne in spreading racemes. The fruit is a capsule around 2 centimeters long and a few millimeters wide. It contains many yellow brown seeds. It is also known as Bhui-neem, meaning "neem of the ground", since the plant, though being a small annual herb, has a similar strong bitter taste as that of the large Neem tree.

Medicinal plants contain physiologically active principles that over the years have been exploited in traditional medicine for the treatment of various ailments (Akbarsha, *et al*. 1990). *Andrographis paniculata* contains diterpenes, lactones and flavonoids. Flavonoids mainly exist in the root, but have also been isolated from the leaves. Aerial parts contain alkanes, ketones, and aldehydes and the bitter principles in the leaves were due to presence of the lactone andrographolide named kalmegin. Four lactones Chuaxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide) and D (14-deoxy-11, 12- didehydroandrographolide) were isolated from the aerial parts. The leaf and stem extracts were assayed for the presence of glycosides, flavonoids, gums, steroids, terpenoids, tannins, saponins and phenolic compounds. The ethanol, acetone, methanol, petroleum ether and chloroform extracts of Kalmegh were screened for the presence of secondary metabolites (Goodman and Gilman, 2000).

Plants have been used as a source of medicine across the globe in almost every culture, since ancient times. As per the reports of WHO, almost 80% population of the world depends upon the conventional system of medicine for maintaining their health (Dubey *et al*, 2004 and Singh *et al*, 2012). Rural communities and tribal populations depend upon plants to fulfil their basic health needs. They have knowledge regarding the traditional use of plants which have verbally been passed from one generation to another generation. Kalmegh is one of the

significant herbs used in the conventional medicine system. Asian and European countries are using leaves and roots of the Kalmegh plant since ancient times for treating a wide range of health issues. For instance, Tribal people of Sonebhadra District of Uttar Pradesh and member tribe of Arunachal Pradesh use roots and leaves of this plant against varieties of diseases like cholera, colic disease, diarrhea, dysentery, fever, filaria, malaria, stomach complaints, tonic. Tribal people of Sonebhadra uses the leaves extract with milk against snake bites. Memba tribe also utilizes Kalmegh as an antihelmintic agent (Singh *et al*, 2012; Rethy*et al*, 2010; Kakulte*et al*, 2014). Rural community of Tryambakeshwar hill of Nashik district, Maharashtra, uses Kalmegh plant powder in treating warts. They use Kalmegh leaf in insect bites and the whole plant is used in hepatitis and inflammation (Akbar, 2011). As it has blood purifying property, therefore it is used for leprosy, gonorrhea, scabies, boils, skin eruptions, and chronic and seasonal fever in many conventional systems (Deng, 1978 and Dymock *et al*, 1972). Kalmegh juice or infusion treats irregular bowel conditions, enhances appetite, and relieve griping in infants (Chopra *et al*, 1982 and Borhanuddin*et al*, 1994). In the conventional medicine system of Malaysia, Kalmegh is used in diabetes and hypertension (Jarukamjorn*et al*, 2008). In the Scandinavian and Japanese and Thai traditional medicine system, it is used against common cold and fever (Chaudhari *et al*, 2010).

1.1 AIMS AND OBJECTIVES OF THE STUDY

- To collect, isolate and identify the various parts of *Andrographis paniculata* (Kalmegh)
- To prepare crude extract of selected parts of *Andrographis paniculata* (Kalmegh)
- Antimicrobial and Phytochemical Analysis of crude extract of selected *Andrographis paniculata* (Kalmegh) and their effects on *Bacillus subtilis*
- *Insilico* guided identification of bioagents from *Andrographis paniculata* (Kalmegh)

2. REVIEW OF LITERATURE:

Geetha *et al*. (2017) obtained from this study, indicated that, the plant extracts showed the strongest antimicrobial activity than the control. Further studies are needed for these potent plant extracts to evaluate the other parameters of antimicrobial activity (e.g., toxicity, in vivo efficacy, antiviral and antiparasitic and antimycobacterial activity).

Soma Roy *et al*. (2010) studied *Andrographis paniculata* chloroform extract showed antimicrobial activity against all the tested pathogenic clinical strains of bacteria. An interesting point to note is that the extract showed better inhibitory action against the gram-negative bacteria, and this highlights its future to be exploited as a potentially powerful antimicrobial agent against the gram-negative bacteria that have made treating noscomial infections increasingly difficult. The antimicrobial activity results were also comparable to that of the antibiotic (amikacin) used as a standard reference. GC-MS analysis of the extracts indicated that phenols, aromatic carboxylic acids and esters are the active antimicrobial principles present in *Andrographis paniculata*. The results also indicated that chloroform was a suitable organic solvent for extraction of the active compounds responsible for antimicrobial activity of *Andrographis paniculata*.

Anurag Singh *et al*. (2017) studied the leaf extract of *Andrographis paniculate* Nees have great potential as antimicrobial compounds against bacteria. *Andrographis paniculate* Nees contains large number of secondary metabolites like flavonoids, terpenoids, alkaloids, tannins, saponins and phenolic compound with antibacterial activities and thus are sources of natural bioactive molecules to control pathogens that cause disease in humans. Based on the results we concluded that methanolic extract of the leaves of *Andrographis paniculata* were exhibited pharmacological phytoconstituents and antimicrobial property. Thus, they can be used in the treatment of infectious diseases caused by the different microbes.

S.Gurupriya *et al*. (2016) studied the threat of bioterrorism with multi-drug resistant pathogens emphasized the need for continued development of new antibiotics. Currently, the ongoing battle against bacteria and fungi prevails certainty of evolving resistance. Plants may be an important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vitro analysis of plant extracts for their biological activity. The present study, stem extract of *Andrographis paniculata* have tested against clinical pathogens. The number of bacterial and fungal strains was determined in accordance with their cell wall structure. Based on this analysis, out of the

five bacterial and five fungal pathogens against chloroform stem extract of *Andrographis paniculata* exhibited the highest antibacterial activity against E. coli and highest antifungal activity against Candida tropicalis. The present study opens a new era in correlating the Ayurveda and Siddha with modern microbiology. The promising result obtained in this study may lead to the development of a potential antibiotic from the stem extract of *Andrographis paniculata* against bacteria and fungi. Further it also encourages the young researchers to test other medicinal plants for their biological activities.

Suparna *et al*. (2014) showed the presence of many phytochemicals amongst which flavonoids were the predominant which could be responsible for the antioxidant property of the same. The phytochemical analysis of the extracts of *Andrographis paniculata* leaves. The extracts of *Andrographis paniculata* leaves showed considerable anti-oxidant activity by DPPH scavenging assay and Total reducing capacity. The best results were obtained with DCM extract showing the lowest IC50 value of 69.32 µg/ml. The IC50 value can be further reduced by using purified extracts. The total reducing capacity increased in dose dependent manner for all the three extracts. The DCM extracts of leaves of *Andrographis paniculata* shows a better antibacterial activity against the gram-positive organisms. The present study of antioxidant and in-vitro antimicrobial evaluation of *Andrographis paniculata* forms a primary platform for further phytochemical and pharmacological studies.

2.1 Biological diversity of Kalmegh

2.1.1 Origin and distribution

Kalmegh is native to Taiwan, mainland, China, India and Sri Lanka. It is commonly found in the tropical and subtropical Asia, Southeast Asia, and some other countries including Cambodia, Caribbean islands, Indonesia Laos, Malaysia, Myanmar, Sri Lanka, Thailand.

2.1.2 Taxonomy

Andrographis paniculata one of the most important medicinal crop, belonging to the family Acanthaceae and order personales (table: 1). A total number of species of this crop varied in different reports, which comprises either 19 (USDA, 2014). 28 (Parixit *et al*., 2012; Valdiani*etal*., 2012). 40 (Mishra *et al*., 2007; Sharma and Sharma 2013), or 44 (Borhanuddin*et al*., 1993) species. However, the genus Andrographis consists of 40 species and about 19 species are reported to be available in India, out of which *Andrographis paniculata* and *Andrographis paniculata* have medicinal properties. The exact numbers of

species of *Andrographis* genus are not validated yet. Total number of chromosomes of *Andrographis paniculata* is 25 and 50 in gametophytic (Madav *et al*., 1995) and sporophytic (Sheeja *et al*., 2007) count, respectively. In addition, genotypic differences are important considerations to find out high yielding germplasms of kalmegh for enhancing productivity and profitability.

Table 2.1: Taxonomic classification

1	Domain	Eukaryotic
2	Kingdom	Plantae
3	Subkingdom	Tracheobionta
4	Division	Angiosperm
5	Class	Dicotyledonae
6	Subclass	Gamopellatae
7	Series	Bicarpellatae
8	Order	Personales
9	Family	Acanthaceae
10	Subfamily	Acanthoideae
11	Tribe	Justiciae
12	Subtribe	Andrographideae
13	Genus	Andrographis
14	Species	*Andrographis paniculata* (Burm. f.) Wall ex. Nees

Reddy *et al*., 2005; She *et al*., 2008; Sheeja and Kuttan 2007).

2.1.3 Vernacular names of Kalmegh

Kalmegh is well known under different vernacular names such as Kirta, Kiryata, Kalpnath, Create, Green Chirata and King of Bitters. It is also called as maha-tita or bhui-neem because of its similarity in appearance and bitter taste as that of neem (Azadirachta indica A. Juss). According to different languages the vernacular names of Kalmegh are listed in Table: 2

Table 2.2: Different names of kalmegh

1	Arab	Quasabhuva
2	Bengali	Kalmegh
3	English	The Creat, King of bitters
4	Gujarati	Kariyatu
5	Hindi	Kirayat
6	Kannad	Nelaberu
7	Malayalam	Kiriyattu
8	Oriya	Bhuinimo
9	Marathi	Oli-kiryata, Oriya
10	Sanskrit	Kalmegha
11	Tamil	Nilavembu
12	Telugu	Nilavembu

2.1.4 Botanical characteristic of Kalmegh

Kalmegh is an annual, branched, herbaceous plant growing to a height of 30– 110 cm in moist and shady places having stem acutely quadrangular, slender, much branched, easily broken fragile texture which is dark green in colour, squared in cross section with

longitudinal furrows and wings along the angles. The leaves are simple, opposite, lanceolate, glabrous, lanceshaped, 2–12 cm long, and 1–3 cm wide with hairless blades measuring up to 8 centimeters long by 2.5 wide. Margin of the leaves are acute and entire or slightly undulated and upper leaves often bract form with short petiole (Table: 3).

The flowers are small in size, borne in spreading racemes which possess botanical features of calyx 5-particle, small, linear; corolla tube narrow, about 6 mm long; limb longer than the tube, bilabiate; upper lip oblong, white with a yellowish top; lower lip broadly cuneate, 3-lobed, white with violet markings; stamens 2, inserted in the throat and far exserted; anther basally bearded. Superior ovary, 2-celled; style far exerted. The fruit of the plant is a capsule which is erect, linear-oblong, 1–2 cm long and 2–5 mm wide, compressed, longitudinally furrowed on broad faces, acute at both ends, thinly glandular-hairy which contains many yellow brown seeds which are very small in size and sub quadrate (Kumar *et al*., 2012). Inflorescence of the plant is characterized as patent, terminal and axillary in panicle, 10– 30 mm long; bract small; pedicel short.

Table 2.3: Morphological details of the Kalmegh

S.No.	Traits Value	Characteristics
1	Plant height	30-110 cm
2	Stem	Dark green
3	Length	30-100 cm
4	Diameter	2-6 mm
5	Shape	Quadrangular with longitudinal furrows and wings on the angles of the young parts, slightly enlarged at the nodes
6	leaves	Glabrous
7	length	2-12 cm
8	width	1-3 cm

9	Arrangement	Lanceolate
10	Shape	Pinnate, acute apex, entire margin
11	flowers	White with rose-purple spots on the petals
12	Size	Small, in lax spreading axillary and terminal racemes or panicles
13	Seed	capsule linear-oblong, acute at both ends
14	Size	1.9 cm × 0.3 cm
15	Colour	Yellowish brown
16	Shape	Subquadrate, numerous

2.1.5 Important varieties

There are very limited varieties of Kalmegh released, however, plant Breeder, Dr. H. O. Mishra from CIMAP, Lucknow developed and released a variety named CIM-Megha during 1980 which is one of the most important varieties of Kalmegh being used for cultivation in northern India. Some of the other important varieties released for commercial cultivation are as under: ♣ AK-1 (Anand Kalmegh-1)

♣ IC-111286

♣ IC-111287

♣ IC-111289

♣ KI- 5

♣ IIIM (J)- 90

2.2 STUDIES ON KALMEGH:

2.2.1 Phytochemical studies

Phytochemicals are defined as bioactive non-nutrient plant compounds found in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major

chronic diseases (Liu 2004). Medicinal plants contain some organic compounds which provide definite physiological action on the human body. These compounds are synthesized by primary or rather secondary metabolism of living organisms. Secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas. A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro. Plant products have been part of phytomedicines since time immemorial. This can be derived from barks, leaves, flowers, roots, fruits, seeds. Knowledge of the chemical constituents of plants is desirable because such information will be value for synthesis of complex chemical substances (Yadav and Agarwala 2011). Based on their biosynthetic origin, phytochemicals can be divided into several categories: Phenol, Alkaloids, Steroids, Terpenoids, Saponins, etc. Phytochemicals could also exhibit other bioactivities such as antimutagenic, anticarcinogenic, antioxidants, and antimicrobial and anti-inflammatory properties (Chew 2011). Phenolic is one of the major groups of phytochemicals that can be found ubiquitiously in certain plants. Phenolic compounds are potent antioxidants and free radical scavenger which can act as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers (Chew 2009).

2.2.2 Antimicrobial studies

In the developing countries the mortality rate is mainly due to infectious bacterial diseases. The bacterial organisms including gram positive and gram negative like different species of Bacillus, Staphylococcus, Salmonella and Pseudomonas are the main source to cause severe infections in humans. Because these organisms have the ability to survive even in harsh conditions due to their multiple environmental habitats. The synthetic antibiotics have the following limitations: Firstly, these are costly and are out of range from the patients belonging to developing countries. Secondly, with the passage of time microorganisms develop resistance against antibiotics. Therefore, after some time these antibiotics are not effective against the microbes (Bibi *et al*., 2011). The world-wide emergence of multi drug resistant Escherichia coli and many other β-lactamase producers has become a major therapeutic problem. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. However, the past record of rapid widespread emergence of resistance to newly introduced antimicrobial

agents will have a short life expectancy. For this reason, researchers are increasingly turning their attention to herbal products for new resistance microbial stain (Singh *et al*., 2008).

2.2.3 Gene expression

Traditional medicinal systems have always contributed chemical entities with attractive scaffolds for drug discovery and the plant biodiversity holds for us in the area of anti-cancer research, plant-derived molecules have shown wonders as chemotherapeutic agents. Andrographolide (Andro) is one such molecule which has been shown to possess inhibitory effect on cancer cell growth and andrographolide is a labdane diterpene isolated from the leaves of *Andrographis paniculata*. Researchers have analyzed the effect of andrographolide on the activation of NF-kappa B induced by a N-formylmethionylleucyl-phenylalanine (fMLP) and platelet-activating factor (PAF) in HL-60 cells differentiated to neutrophils. Andrographolide has been clearly shown to inhibit the NF-kappa B luciferase activity induced by PAF. On other hand, andrographolide also reduced the DNA binding of NF-kappa B in whole cells and in nuclear extracts induced by PAF and fMLP (Hidalgo *et al*., 2005). Andrographolide is known to exert several biological properties including analgesic, antipyretic and antiinflammatory effects. Previous research in vitro model studies in biological processes shows incubation of endothelial cell with non-toxic concentrations of andrographolide attenuated the tumour necrosis factor-α (TNF)-induced intercellular adhesion molecule-1 (ICAM-1) expression and reported that the concentration ranges of andrographolide also inhibited the TNF induced endothelial-monocyte adhesion in a concentration-dependent manner (Habtemariam*et al*., 1998). The stimulatory effect of andrographolide and *Andrographis paniculata* extract and on cytotoxic T lymphocyte (CTL) production study was clearly showed that *Andrographis paniculata* extract and andrographolide stimulate the CTL production through enhanced secretion of IL-2 and IFN-gamma by T cells, inhibiting tumor growth in vivo. Andro attenuates endothelial cell motility and tumor-endothelial cell interaction and andro suppresses breast tumor growth in orthotopic NOD/SCID mice model. The anti-tumor activity of Andro in both in vitro and in vivo model was studied (Sheeja and Kuttan 2007) with down regulation of PI3 kinase/Akt activation and inhibition of pro-angiogenic molecules, OPN and VEGF expressions and results of experimental studies evidently observed that andro may act as an effective anti-tumor and anti-angiogenic agent for the treatment of breast cancer (Kumar *et al*., 2012).

2.2.4Antioxidant studies

The oxidation is the transfer of electrons from one atom to another and represents an essential part of both aerobic life and our metabolism, since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP. However, problems may arise when the electron flow becomes uncoupled (transfer of unpaired single electrons), generating free radicals (Pietta 2000). Antioxidants are those substances which possess free radical chain reaction breaking properties. Recently there has been an upsurge of interest in the therapeutic potential medicinal plants as antioxidants in re-antioxidants in reducing oxidative stressinduced tissue injury (Pourmorad*et al*., 2006). Among the numerous naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective. They are known to inhibit lipid peroxidation (by inactivating lipoxygenase), to scavenge free radicals and active oxygen species by propagating a reaction cycle and to chelate heavy metal ions (Veeru*et al*., 2009). These are important in living organisms as well as in food because they may delay or stop formation of free radical by giving hydrogen atoms or scavenging them. Oxidative stress is involved in the pathology of cancer, atherosclerosis, malaria and rheumatoid arthritis (Teran *et al*., 2008). Reactive oxygen species are responsible for variety of pathological conditions. Innate defense system of the human body may not be sufficient for curing the damage caused by continued oxidative stress. Thus, there is a need to supply the antioxidants exogenously to balance their levels in the human body. Many synthetic antioxidants such as buthylated hydroxyl toluene (BHT), butylated hydroxyanisole and tertiary butyl hydroquinone (TBHQ) are commonly used for preservation of fats and oil foods. Recently there is an upsurge of interest in natural products as antioxidant as they can inhibit the free radical reactions (Guleria, *et al*., 2010).

2.2.5 Anticancer studies

Cancer causes of more than six million deaths each year in the world. In 2001, about 1,268,000 new cancer cases and 553,400 deaths were reported in the United States. For a long time, plants are being used in the treatment of cancer. According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products. The search for anticancer agents from plant sources started in the 1950s and resulted in the discovery and development of the vinca alkaloids, vincristine, and the isolation of the cytotoxic podophyllotoxins. More than 60% of currently used anticancer agents are derived in one way or another from natural sources. Breast cancer is one of the main life-threatening diseases that

a woman may have to face during her lifetime. The increasing incidence of breast neoplasia reported over the last a few decades has led to development of new anticancer drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of enormous pool of synthetic, biological, and natural products (Angelopoulos *et al*., 2004 and Mukherjee *et al*., 2001).

Cancer is a complex genetic disease that is caused primarily by environmental factors. The cancer-causing agents (carcinogens) can be present in food, water, air, in chemicals and sunlight that people are exposed to. Modern medicine attributes most cases of cancer to changes in DNA that reduce or eliminate the normal controls over cellular growth, maturation, and programmed cell death. These changes are more likely to occur in people with certain genetic backgrounds and in persons infected by chronic viruses (e.g., viral hepatitis may lead to liver cancer; HIV may lead to lymphoma). The ultimate cause, regardless of genetic propensity or viruses that may influence the risk of the cancer, is often exposure to carcinogenic chemicals (including those found in nature) and/or to radiation (including natural cosmic and earthly radiation), coupled with a failure of the immune system to eliminate the cancer cells at an early stage in their multiplication. The immunological weakness might arise years after the exposure to chemicals or radiation (Block 1991a). More significantly, a globalization of unhealthy lifestyles, particularly cigarette smoking, the adoption of many features of the modern estern diet (high fat, low fibre content), tobacco smoking, alcohol consumption, excess use of caffeine and other drugs, sunshine, infections from such oncogenic virus like cervical papillomaviruses, adenoviruses Karposis sarcoma (HSV) or exposure to asbestos will increase cancer incidence. Tobacco use and diet each account for about 30% of new cancer cases, with infection associated with a further 15%; thus, much of cancer is preventable. These obviously are implicated as causal agents of mammalian cancers. However, a large population of people is often exposed to these agents. Consequently, cancer cells continue to divide even in situations in which normal cells will usually wait for a special chemical transduction signal. The tumour cells would ignore such stop signals that are sent out by adjacent tissues. A Cancer cell also has the character of immortality even in vitro whereas normal cells stop dividing after 50-70 generations and undergoes a programmed cell death (Apoptosis). Cancer cells continue to grow invading nearby tissues and metastasizing to distant parts of the body. Metastasis is the most lethal aspect of carcinogenesis (Block 1991b). The plants represent a vast potential resource for anticancer compounds. As with all areas of phytomedicine, the value of these plants lies in

the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory. The antitumor activity of medicinal-plant-derived compounds may result from a number of mechanisms, including effects on cytoskeletal proteins that play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulition of the immune system, etc. These plants continue to be subject to extensive screening worldwide, in an attempt to develop still more effective anticancer treatments (Dixit *et al*., 2010). Epidemiological studies suggested that antioxidant supplements might reduce the risk of breast cancer recurrence or breast cancer-related mortality and consuming food and beverages rich in Polyphenols is associated with a lower incidence of cancers. Experimental investigations demonstrated that many naturally occurring agents and plant extracts have shown antioxidant and anticancer potential in a variety of bioassay systems and animal models, having relevance to human disease, e.g., Crude methanolic extract (CME) from the pericarp of Garcinia mangostana (Family: Guttiferae) has antiproliferative, apoptotic, and antioxidative activities against human breast cancer cell line in vitro. The antioxidant and anticancer activity of the extracts from medicinal plants and herbs was associated with their components of Phenolic compounds; the major types of phenolic compounds included phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids (Wang *et al*., 2006).

Cancer initiation and progression and a number of human diseases including cardiovascular, metabolic, inflammatory, and neurodegenerative diseases are related to reactive oxygen species (ROS) and reactive nitrogen species (RNS), which once accumulated inside the cell, can attack proteins, lipids, and DNA, causing a state of oxidative stress. Living organisms are equipped with an antioxidant defense system that regulates the toxic impact of ROS and RNS. However, a disturbance in the balance between the production of ROS or RNS and antioxidant defenses may lead to cell molecule or tissue injury (Carvalho *et al*., 2012). There is strong evidence that the antioxidants prevent carcinogenesis, and natural products have proven to be an important source of new and effective antioxidant and anticancer agents (Milaeva, 2011). Secondary plant metabolites, such as phenolic compounds, have been found to be strong antioxidants, which can scavenge or suppress ROS and RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production, and up-regulate or protect antioxidant defense, thereby preventing carcinogenesis (Halliwell, 2007 and Huang *et al*., 2010). Breast cancers exhibit remarkable heterogeneity not only with respect to estrogen (ER), progesterone (PR), and human epidermal growth factor-2 (HER-2)

receptor expression but also with respect to tumor size, grade, and nodal status. Thus, breast carcinoma is a mixture of diverse phenotypes, which raises different treatment needs and at the present moment there is no cure for metastatic breast cancer whereby the necessary search for new drugs to treat and control this disease is much needed (Jatoi, *et al*., 2008 and Pagani *et al*., 2010).

The extremely bitter and characteristic taste of *Andrographis paniculata*, of the Acanthaceae family, gives it the term ―King of bitters‖. *Andrographis paniculata* is a well-known medicinal plant in South and Southeast Asia and is traditionally used for treating a variety of ailments. Several recent studies have validated some of the medicinal properties of this plant and its use in traditional medicine. Such properties include its antimicrobial activity, hepatoprotective capacity, and antidiarrhoeal potential (Radha *et al*., 2011). It was recommended in Charaka Samhita dating to 175BC for treatment of jaundice along with other plants in multi plant preparations. It has also been used traditionally for sluggish liver as antidote in case of colic dysentery and dyspepsia. It is used as bitter tonic, antispasmodic, antiperistatic, stomach and also an antihelmintic. It has been employed with benefit in case of general debility in convalescence after fever, disorders of liver and advanced stages of dysentery. The juice of fresh leaves is a domestic remedy in the treatment of colic pain, loss of appetite, irregular stools and diarrhea. Long known in traditional Asian medicine as an immune system booster, Andrographis has demonstrated significant activity in fighting common cold, flu and upper respiratory infections (Rafat*et al*., 2010).

2.3 Traditional use

A plenty of bibliographical evidence is available throughout Asia about the medicinal properties of *Andrographis paniculata*. It is a well-known ingredient used in the preparation of drugs in Sidha and Ayurveda. Therapeutically important active principle of *Andrographis paniculata* found in aerial parts is Andrographolide. It's a colourless, crystalline, bitter in taste and known as diterpene lactone. Other reported compounds include 14-deoxy11, 12 - didehydroandrographolide / andrographolide D; 14-deoxy andrographolide; non bitter compound neoandrographolide; homoandrographolide; andrographosterol; andrographane; andrographone; andrographosterin; andrograpanin; alpha-sterol; stigmasterol; apigenin-7,4'-di-O- methyl ether; 5 hydroxy 7, 8, 2', 3'-tetramethoxyflavone; monohydroxy trimethyl flavones; andrographin; dihydroxy-di-methoxy flavones; panicolin; andrographoneo; andrographoside; andropaniculosin A; andropani-culoside A and number of compounds were

identified by previous researchers (Wu *et al*., 2008). It is reported that inflammation caused by histam in, dimethyl benzene and adrenaline was significantly reduced by neoandrographolide and andrographolide (Deng *et al*., 1978).

The anti-inflammatory action of dehydroandrographolide was due to its effect of increasing the synthesis and release of adrenocorticotropic hormone (ACTH) of the pituitary gland of brain (Yin *et al*., 1993). ACTH signals the adrenal gland to make cortisol, a natural anti-inflammatory agent. Other research group found andrographolide to inhibit edema by 60% at a concentration of 200 mg/kg body weight and 62.7% at 400 mg/kg body weight in three hours (Madav*et al*., 1995 and Xia *et al*., 2004). The expression of inducible nitric oxide synthase was suppressed by andrographolide. The diterpenoid also had inhibitory effect on the production of oxygen free radical by human neutrophils (Levita*et al*., 2010). The administration of methanolic extract of *Andrographis paniculata* produced complete inhibition of carageenan induced inflammation compared with the control models (Sheeja, *et al*., 2006). The antioxidant activity of *Andrographis paniculata* was observed due to the presence of 14- deoxyandrographolide which were isolated from the plant (Koteswara*et al*., 2004). Verma and Vinayak studied the effect of the aqueous extract of *Andrographis paniculata* on antioxidant protection system in lymphoma bearing mice (Verma and Vinayak 2007.). A significant increase in catalase, superoxide dismutase and glutathione S transferase activities were observed by the different doses of the aqueous extracts of the medicinal plant which indicated the antioxidant properties of *Andrographis paniculata* and might reduce the oxidative stress. On other hand, the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase were increased by the hydro alcoholic extract of the medicinal plant (Ojha *et al*., 2009). The plant powder of *Andrographis paniculata* can prevent or stop diarrhoea on animal model (Gupta *et al*., 1990).

The components of the plant like andrographolide and neoandrographolide showed similar activity to loperamide (Imodium), the most common antidiarrhoeal drug. The plant was used to treat 1,611 cases of diarrhea with overall effectiveness of 91.3% (Madav*et al*., 1995). Plant extract and partially purified fractions when orally administered to mice experimentally envenomed with rattle snake venom s.c injection showed potent neutralizing effect against the venom. The isolated fractions effectively inhibited the toxic effect of snake venoms invitro than in-vivo (Verma, and Vinayak, 2007). Methanolic extracts showed antimalarial activity against Plasmodium berghei, one of the parasites that transmit malaria. The extract considerably shows inhibition of multiplication of the parasites. The extract was also

effective in killing filarial worms that obstruct lymph channels in the body leading to gross swelling termed elephantiasis (Misra*et al*., 1992 and Kapil *et al*., 1993). Inhibition for anti-malarial activity towards plasmodium falciparum (in vitro) using the lactate dehydrogenase (LDH) assay is also reported (Siti *et alet al*., 2002). Extracts of *Andrographis paniculata* in different solvents could increase the time taken for forming blood clots, thus decreasing the risk of subsequent closing of blood vessels (restinosis), seen after angioplasty procedures (Wang *et al*., 1993). An extract of the plant produced antihypertensive effects as it relaxed the smooth muscle in the walls of blood vessels and prevented the blood vessel from constricting and limiting the blood flow to the heart, brain and other parts of the body (Huang 1987). The andrographolide given to animals produces a significant increase in the bile flow which facilitates the digestion; recently many researchers concluded that the plant was a useful remedy for treatment of infective hepatitis. Andrographolide exhibits protective effects in carbon tetrachloride induced hepatopathy in rats. The inhibitory effect of the plant extract and andrographolide on hepatic cytochrome p450 (CYPS) activities using rat and human liver chromosomes has also been reported (Pekthong*et al*., 2008).

Plant with combinations of other natural products may increase cytotoxic activity of Natural Killer Cells (NK) and Tumour Necrosis Factor (TNF-alpha) while decreasing DNA damage in patients with late-stage cancer (See *et al*., 2002). The anticancer activity has been reported in B16F0 melanoma syngeneic and HT-29 xenographt models (Rajagopal *et al*., 2003). In vivo of *Andrographis paniculata* indicated that the plant have good prevented hexachlorocyclohexane induced increase in activities of γ-glutamyl transpeptidase, glutathione-S-transferase and lipidperoxidation in mouse liver indicates antioxidant potential and hepatoprotective effect of *Andrographis paniculata* (Trivedi, and Rawal, 2000) it is reported that andrographolide is able to efficiently block T cell activation in vitro as well as in vivo, a feature that could be useful for interfering with detrimental T cell responses (Iruretagoyena*et al*., 2005). The plant has been reported as a potent stimulator of the immune system (Kumar *et al*., 2004).

3. MATERIALS AND METHODS

3.1 Collection and sampling of plant Kalmegh:

The different parts of *Andrographis paniculata* such as leaf, root, stem and aerial part were collected from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India and were collected for the following experiments. The plant materials were dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in airtight container. The powder obtained was subjected to successive Soxhlet extraction with the organic solvents with increasing order.

Fig. 3.1 Sampling view of *Andrographis paniculata* plant from CIMAP, Lucknow

3.1.1 Preparation of extract from leaf using Soxhlet

Firstly, fresh *Andrographis paniculate* leaves were washed thoroughly with tap water followed by distilled water. Kept all the leaves for shade dried until all the water content was lost completely. Dried leaves were crushed and powdered using blender. Fine powder was obtained after sieving and stored in airtight container until further use.

Fig. 3.2 Wet, dry and powdered leaf extracts of *A. paniculata*

3.1.2 Preparation of extract from stem

Fresh *Andrographis paniculate* stems were washed thoroughly in tap water followed by distilled water and were then shade dried until all the water content was lost completely. Dried stems were crushed and powdered using blender. Fine powder was obtained after sieving and stored in airtight container until further use.

Fig. 3.3 Wet, dry and powdered stem extracts of *A. paniculata*

3.1.3 Preparation of extract from root

Fresh *Andrographis paniculata* roots were washed thoroughly in tap water followed by distilled water and were then shade dried until all the water content was lost completely. Dried roots were crushed and powdered using blender. Fine powder was obtained after sieving and stored in airtight container until further use.

Fig. 3.4 Wet, dry and powdered root extracts of *A. paniculata*

3.1.4 Preparation of extract from aerial part

Fresh *Andrographis paniculata* plants were washed thoroughly in tap water followed by distilled water and were then shade dried until all the water content was lost completely. Dried plants were crushed and powdered using blender. Fine powder was obtained after sieving and stored in airtight container until further use.

Fig. 3.5 Wet and dry aerial part of *A. paniculata*

3.2 SOXHLET EXTRACTION

Fig. 3.6 Soxhlet extraction view of *A. paniculata* plant

3.2.1 Soxhlet Extraction of leaf

The leaves of *Andrographis paniculate* plants were shade dried and pulverized. 4.5 g of powdered material was packed in Soxhlet apparatus and subjected to continuous percolation for 8 h using 150 ml, methanol as solvent. The methanol extract was concentrated under vacuum and dried in desiccators and then submitted to lyophilization in order to remove the solvent completely to produce powdered form of extracts. Lyophilization removes the water and stabilizes the extract so that it can retain satisfactory pharmacological activity during long term storage. The weight of the dried mass is recorded (1.19 gm) and used for experimental studies. The yield was 5.9% with respect to dry starting material with characteristic odour and greasy consistency.

3.2.2 Soxhlet Extraction of stem

The stems of *Andrographis paniculate* plants were shade dried and pulverized. 2.14 g of powdered material was packed in Soxhlet apparatus and subjected to continuous percolation for 8 h using 150 ml, methanol as solvent. The methanol extract was concentrated under vacuum and dried in desiccators and then submitted to lyophilization in order to remove the

solvent completely to produce powdered form of extracts. Lyophilization removes the water and stabilizes the extract so that it can retain satisfactory pharmacological activity during long term storage. The weight of the dried mass is recorded (0.30 gm) and used for experimental studies. The yield was 5.9% with respect to dry starting material with characteristic odour and greasy consistency.

3.2.3 Soxhlet Extraction of root

The roots of *Andrographis paniculate* plants were shade dried and pulverized. 4.8 g of powdered material was packed in Soxhlet apparatus and subjected to continuous percolation for 8 h using 150 ml, methanol as solvent. The methanol extract was concentrated under vacuum and dried in desiccators and then submitted to lyophilization in order to remove the solvent completely to produce powdered form of extracts. Lyophilization removes the water and stabilizes the extract so that it can retain satisfactory pharmacological activity during long term storage. The weight of the dried mass is recorded (0.23 gm) and used for experimental studies. The yield was 5.9% with respect to dry starting material with characteristic odour and greasy consistency.

3.2.4 Soxhlet Extraction of aerial part

The aerial part of *Andrographis paniculata* plants were shade dried and pulverized. 9.5 g of powdered material was packed in Soxhlet apparatus and subjected to continuous percolation for 8 h using 150 ml, methanol as solvent. The methanol extract was concentrated under vacuum and dried in desiccators and then submitted to lyophilization in order to remove the solvent completely to produce powdered form of extracts. Lyophilization removes the water and stabilizes the extract so that it can retain satisfactory pharmacological activity during long term storage. The weight of the dried mass is recorded (6.39 gm) and used for experimental studies. The yield was 5.9% with respect to dry starting material with characteristic odour and greasy consistency.

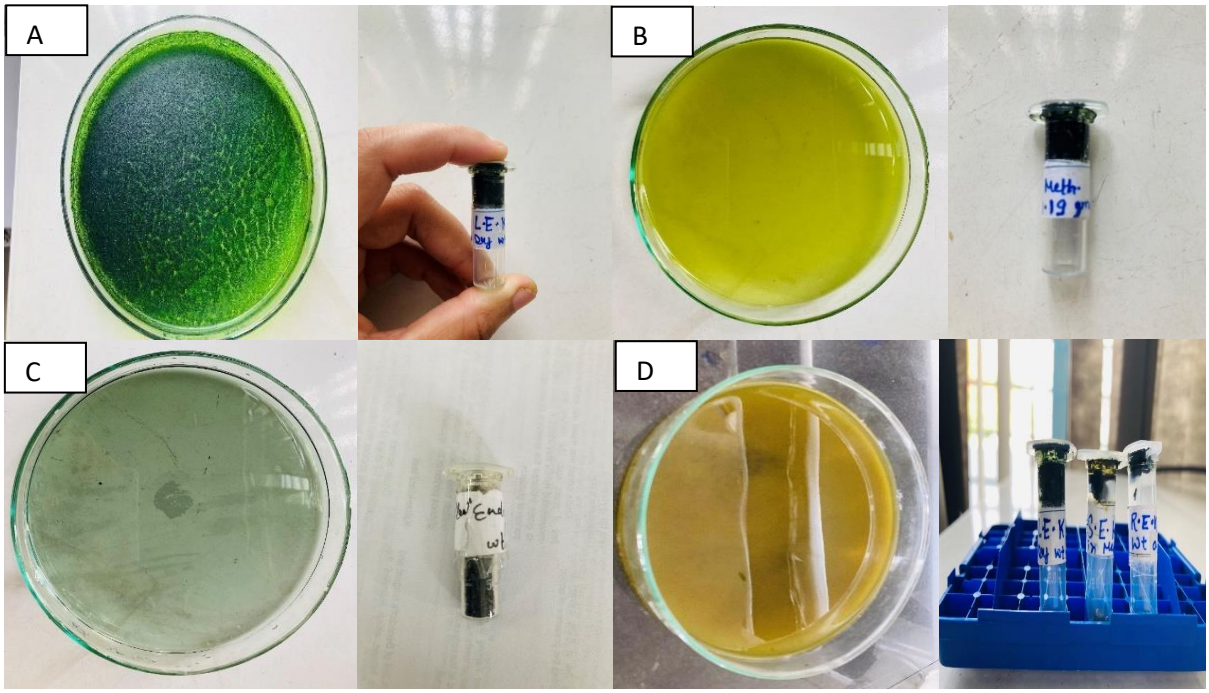


Fig. 3.7 Prepared Leaf, stem, root and aerial extracts by Soxhlet method

3.3 Test microorganisms

To evaluate the antimicrobial activity of *Andrographis paniculata* extracts, strain of microorganism was selected, namely *Bacillus subtilis*. The bacterial strain was collected from clinical lab and sub cultured in nutrient agar medium and used for antimicrobial susceptibility test.

3.4 Preparation of media and Nutrient Agar plates

The media used for antibacterial test were Nutrient Agar/Broth. The media were obtained from Integral University, India. The test bacterial strain was inoculated into nutrient broth and incubated at 35C for 24hrs. After the incubation period, the culture tubes were compared with the turbidity (opacity) standard.

3.5 Preparation various concentration of crude extracts of different parts of the plant

The crude extract was dissolved in sterile distilled water to prepare appropriate dilution to get required concentrations of about 50µl, 75µl, 100µl and125µl. Whatman filter paper (No:1) was used to prepare discs approximately 6 mm in diameter and sterilized in hot air oven.

After sterilization, the discs were loaded with different concentrations of prepared plant extract solutions and it was kept under refrigeration for 24 hrs. Above discs were dispensed onto the surface of the inoculated agar plates. Each disc was pressed down firmly to ensure completely contact with the agar surface. Then the plates were incubated at 5°C for 1 hr to permitted good diffusion and transferred in to incubator at 37°C for 24 hrs. After completion of 24hrs, the plates were inverted and placed in an incubator set to respective temperature for 24 hrs.

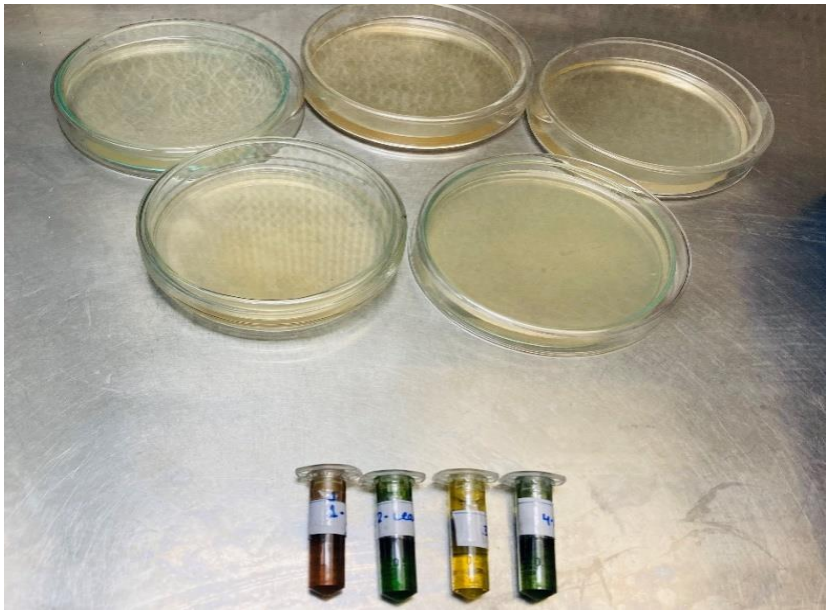


Fig. 3.8 Prepared crude extracts with different concentrations in DMSO.

3.6 Antibacterial assay

Bioassay was carried out by Agar well diffusion method. Fresh bacterial culture of 0.5 ml having 108 CFU was spread on nutrient agar plate with glass spreader. A well of 6mm diameter was punched off into agar medium with sterile cork borer and filled with 50µl, 75µl, 100µl and 125µl of aqueous and methanol extracts by using micro pipette in each well in aseptic condition. Plates were then kept in a refrigerator to allow pre-diffusion of extract for 30minutes and further incubated in an incubator at 35C for 24hrs. The antibacterial activity was evaluated by measuring the zone of inhibition. The experiment was done in triplicate and the mean diameter of the inhibition zone was calculated. Antibiotic Tetracycline at a concentration of 30 µg/ml as positive control and 100% DMSO (Dimethyl sulphoxide) as a negative control were used.

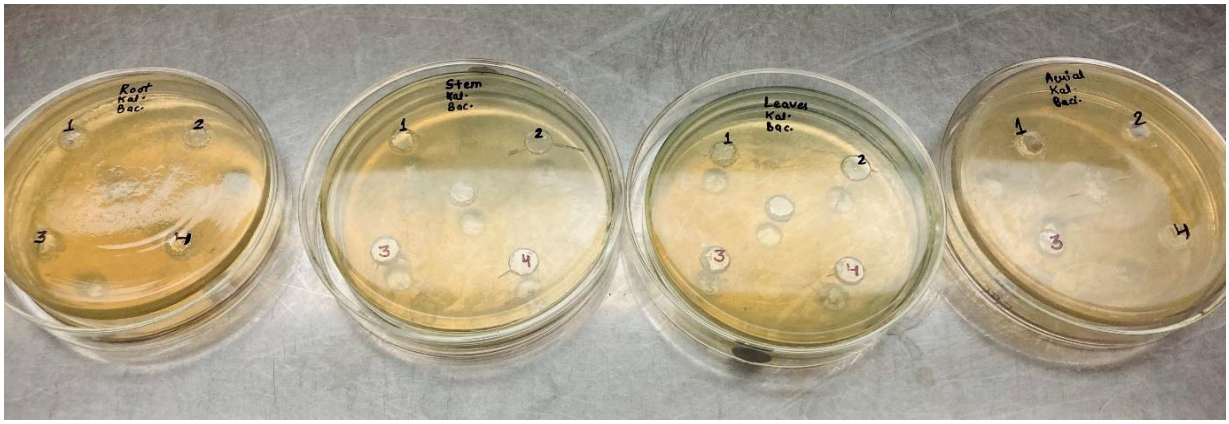


Fig. 3.9 Culture plates were prepared followed by agar well diffusion method

3.7 ANTIMICROBIAL ACTIVITY

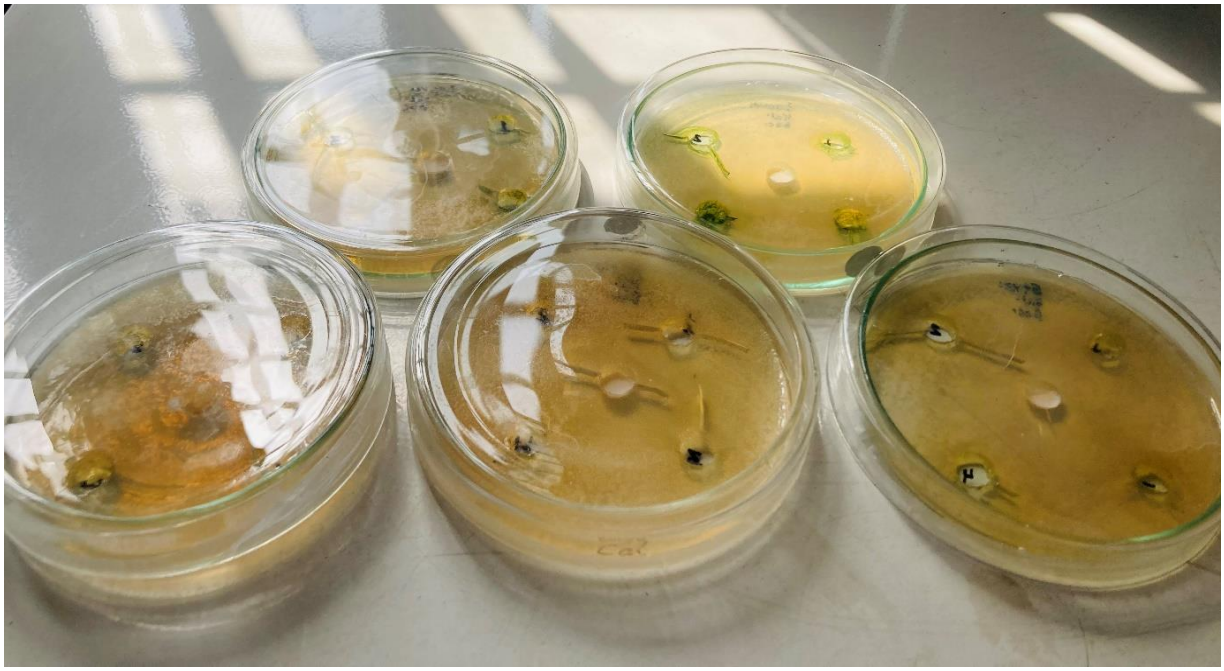


Fig. 3.10 Culture plates shows antibacterial activity of leaf, stem, root and aerial part.

3.7.1 Antimicrobial Activity of leaves

The methanolic leaf extracts of *Andrographis paniculata* were tested by Agar well diffusion method. Different concentration of the *Andrographis paniculate* crude extractsAP50 µl (0.05gm/ml), AP75µl (0.075gm/ml), AP100µl (0.10gm/ml) and AP125µl (0.125gm/ml) were prepared in Double distilled water. Petri plates were prepared by pouring 25 ml of nutrient medium for bacteria. The surfaces of media were inoculated with bacteria from a broth culture. A sterile cotton swab is dipped into a standardized bacterial test suspension and used

to evenly inoculate the entire surface of the Nutrient Agar plate. After solidification well (6 mm in diameter) created on petriplate and the crude extractsAP50µl (0.05gm/ml), AP75 µl (0.075gm/ml), AP100µl (0.10gm/ml) and AP125µl (0.125gm/ml) were placed on test organism-seeded plates. The antibacterial assay plates were incubated at 35 C for 24h. The diameter of the inhibition zone was measured in millimetres (mm).

3.7.2 Antimicrobial Activity of stem

The methanolic stem extracts of *Andrographis paniculata* were tested by Agar well diffusion method. Different concentration of the *Andrographis paniculata* crude extractsAP50µl (0.05gm/ml), AP75µl (0.075gm/ml), AP100µl (0.10gm/ml) and AP125 µl (0.125gm/ml) was prepared in Double distilled water. Petri plates were prepared by pouring 25 ml of nutrient medium for bacteria. The surfaces of media were inoculated with bacteria from a broth culture. A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the Nutrient Agar plate. After solidification well (6 mm in diameter) created on petriplate and the crude extractsAP50 (0.05gm/ml), AP75 (0.075gm/ml), AP100 (0.10gm/ml) and AP125 (0.125gm/ml) were placed on test organism-seeded plates. The antibacterial assay plates were incubated at 35 C for 24h. The diameter of the inhibition zone was measured in millimetres (mm).

3.7.3 Antimicrobial Activity of root

The methanolic root extracts of *Andrographis paniculata* were tested by Agar well diffusion method. Different concentration of the *Andrographis paniculata* crude extracts AP50 µl(0.05gm/ml), AP75 µl(0.075gm/ml), AP100µl (0.10gm/ml) and AP125µl (0.125gm/ml) was prepared in Double distilled water. Petri plates were prepared by pouring 25 ml of nutrient medium for bacteria. The surfaces of media were inoculated with bacteria from a broth culture. A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the Nutrient Agar plate. After solidification well (6 mm in diameter) created on Petri plate and the crude extractsAP50 (0.05gm/ml), AP75 (0.075gm/ml), AP100 (0.10gm/ml) and AP125 (0.125gm/ml) were placed on test organism-seeded plates. The antibacterial assay plates were incubated at 35 C for 24h. The diameter of the inhibition zones was measured in millimetres (mm).

3.7.4 Antimicrobial Activity of aerial part

The methanolic aerial extracts of *Andrographis paniculata* were tested by Agar well diffusion method. Different concentration of the *Andrographis paniculate* crude extractsAP50µl (0.05gm/ml), AP75µl (0.075gm/ml), AP100µl (0.10gm/ml) and AP125µl (0.125gm/ml) was prepared in Double distilled water. Petri plates were prepared by pouring 25 ml of nutrient medium for bacteria. The surfaces of media were inoculated with bacteria from a broth culture. A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the Nutrient Agar plate. After solidification well (6 mm in diameter) created on petriplate and the crude extractsAP50 (0.05gm/ml), AP75 (0.075gm/ml), AP100 (0.10gm/ml) and AP125 (0.125gm/ml) were placed on test organism-seeded plates. The antibacterial assay plates were incubated at 25 C for 24h. The diameter of the inhibition zone was measured in millimetres (mm).

3.8 PHYTOCHEMICAL TESTS

The extract was tested for the presence of bioactive compounds by using following standard methods.

3.8.1 Test for proteins

3.8.1.1 Millon's test

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

3.8.1.2 Ninhydrin test

Crude extract when boiled with 2ml of 0.2% solution of Ninhydrin, violet colour appeared suggesting the presence of amino acids and proteins.

3.8.2 Test for carbohydrates

3.8.2.1 Fehling's test

Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

3.8.2.2 Benedict's test

Crude extract when mixed with 2ml of Benedict's reagent and boiled, a reddish-brown precipitate formed which indicated the presence of the carbohydrates.

3.8.2.3 Molisch's test

Crude extract was mixed with 2ml of Molisch's reagent and the mixture was shaken properly. After that, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. Appearance of a violet ring at the interphase indicated the presence of carbohydrate.

3.8.2.4 Iodine test

Crude extract was mixed with 2ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

3.8.3 Test for phenols and tannins

Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

3.8.4 Test for flavonoids

3.8.4.1 Shinoda test

Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

3.8.4.2 Alkaline reagent test

Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicated the presence of flavonoids.

3.8.5 Test for saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

3.8.6 Test for glycosides

3.8.6.1 Liebermann's test

Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was added. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

3.8.6.2 Salkowski's test

Crude extract was mixed with 2ml of chloroform. Then 2ml of concentrated H2SO4 was added carefully and shaken gently. A reddish-brown colour indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.

3.8.6.3 Keller-kilani test

Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of FeCl3. The mixture was then poured into another test tube containing 2ml of concentrated H2SO4. A brown ring at the interphase indicated the presence of cardiac glycosides.

3.8.7 Test for steroid

Crude extract was mixed with 2ml of chloroform and concentrated H2SO4 was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H2SO4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

3.8.8 Test for terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H2SO4 was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

3.8.9 Test for alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer's And Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

3.9 PHYTOCHEMICAL ANALYSIS

3.9.1Phytochemical Analysis of leaves

The phytochemical screening of the leaf extract of *Andrographis paniculata* was done quantitatively and qualitatively to reveal the presence of phytoconstituents like secondary metabolites such as flavonoids, terpenoids, alkaloid, tannins, saponins and phenolic

compound according to phytochemical methods accounted by Harborne (1973), Evans (2002), and Sofowora (1993).

3.9.2 Phytochemical Analysis of stem

The phytochemical screening of the stem extract of *Andrographis paniculata* was done quantitatively and qualitatively to reveal the presence of phytoconstituents like secondary metabolites such as flavonoids, terpenoids, alkaloid, tannins, saponins and phenolic compound according to phytochemical methods accounted by Harborne (1973), Evans (2002), and Sofowora (1993).

3.9.3 Phytochemical Analysis of root

The phytochemical screening of the root extract of *Andrographis paniculata* was done quantitatively and qualitatively to reveal the presence of phytoconstituents like secondary metabolites such as flavonoids, terpenoids, alkaloid, tannins, saponins and phenolic compound according to phytochemical methods accounted by Harborne (1973), Evans (2002), and Sofowora (1993).

3.9.4 Phytochemical Analysis of aerial part

The phytochemical screening of the leaf extract of *Andrographis paniculata* was done quantitatively and qualitatively to reveal the presence of phytoconstituents like secondary metabolites such as flavonoids, terpenoids, alkaloid, tannins, saponins and phenolic compound according to phytochemical methods accounted by Harborne (1973), Evans (2002), and Sofowora (1993).

4. RESULTS AND DISCUSSION

4.1 ANTIBACTERIAL ASSAY OF LEAF, STEM, ROOT AND AERIAL PART

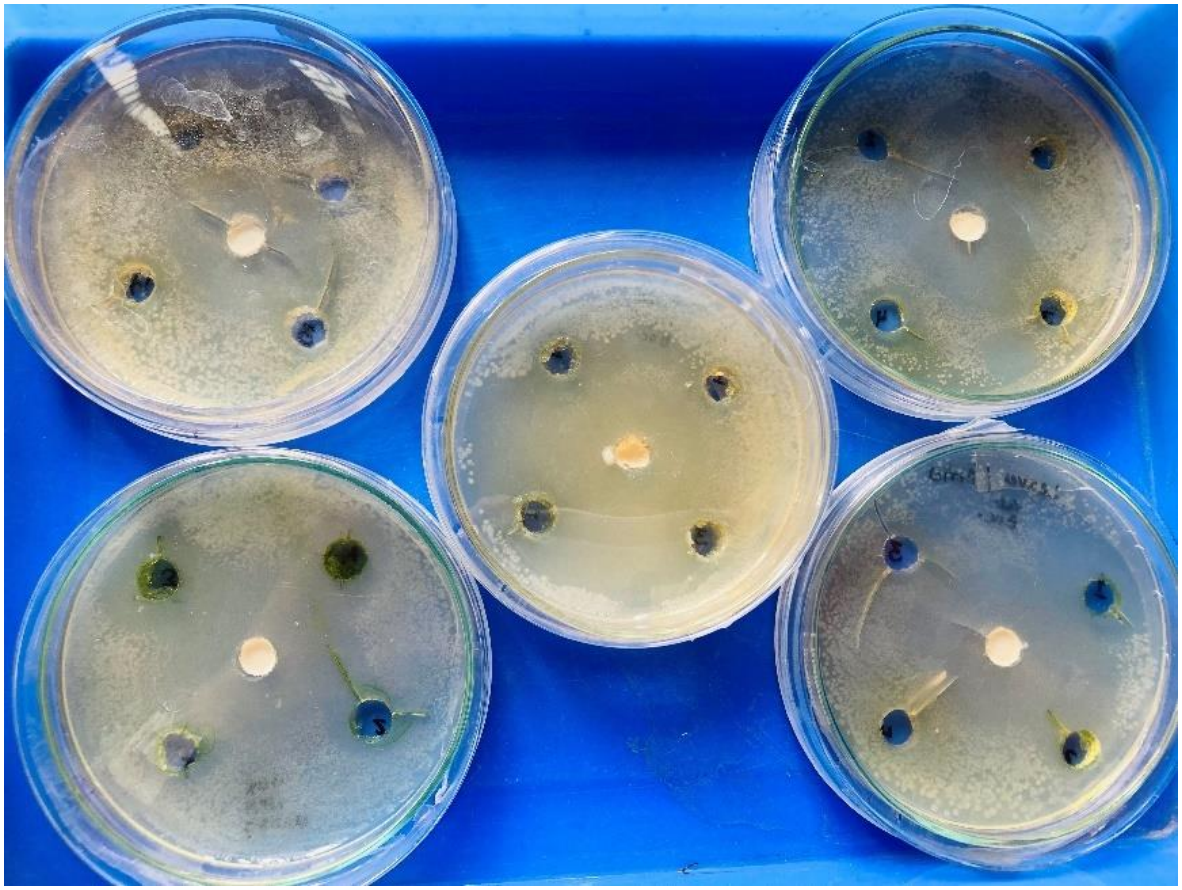
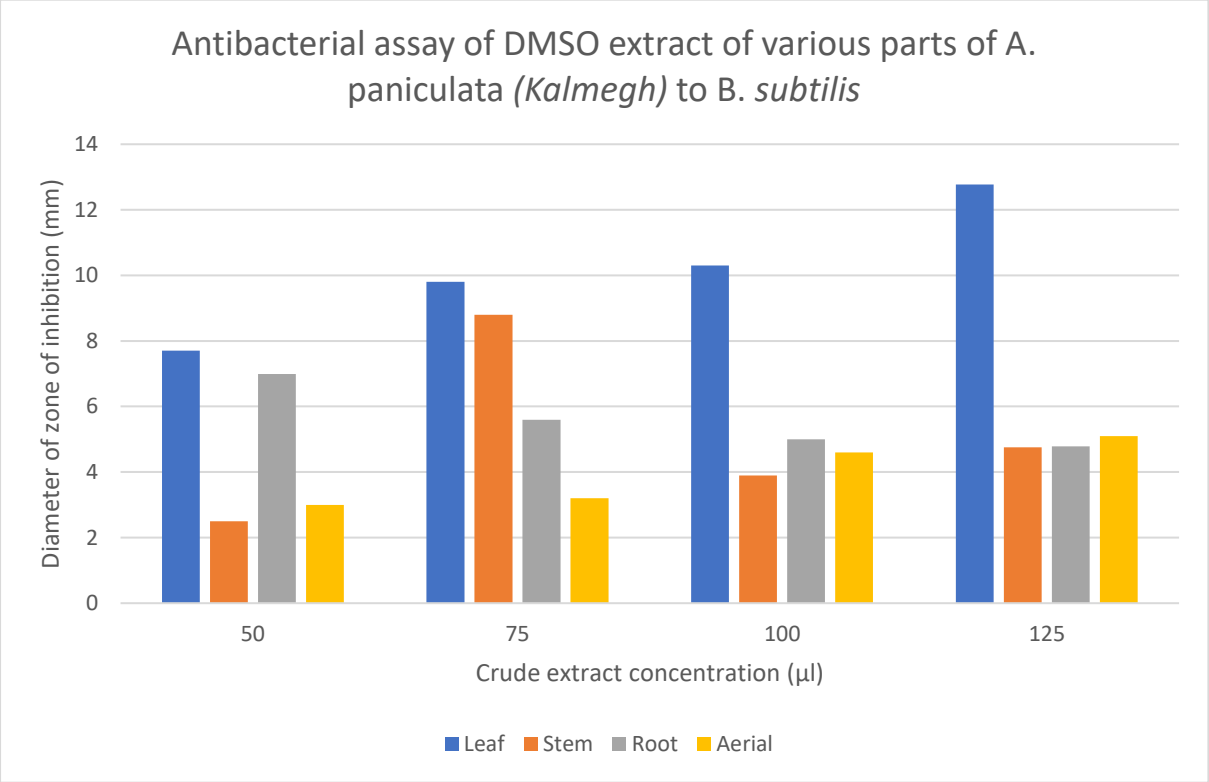


Fig. 4.1 Culture plates shows antibacterial assay of leaf, stem, root and aerial part.

Table 4.1: Antibacterial activity assay of DMSO extract of various parts from *A. paniculata* to *B. Subtilis*

S.No.	Crude extract Concentration (µl)	Diameter of zone of inhibition (mm)			
		Leaf	Stem	Root	Aerial
1	50	7.7	2.5	6.99	3.0
2	75	9.8	2.8	5.59	3.2
3	100	10.3	3.9	5.0	4.60
4	125	12.77	4.75	4.78	5.1



4.2 PHYTOCHEMICAL ANALYSIS:

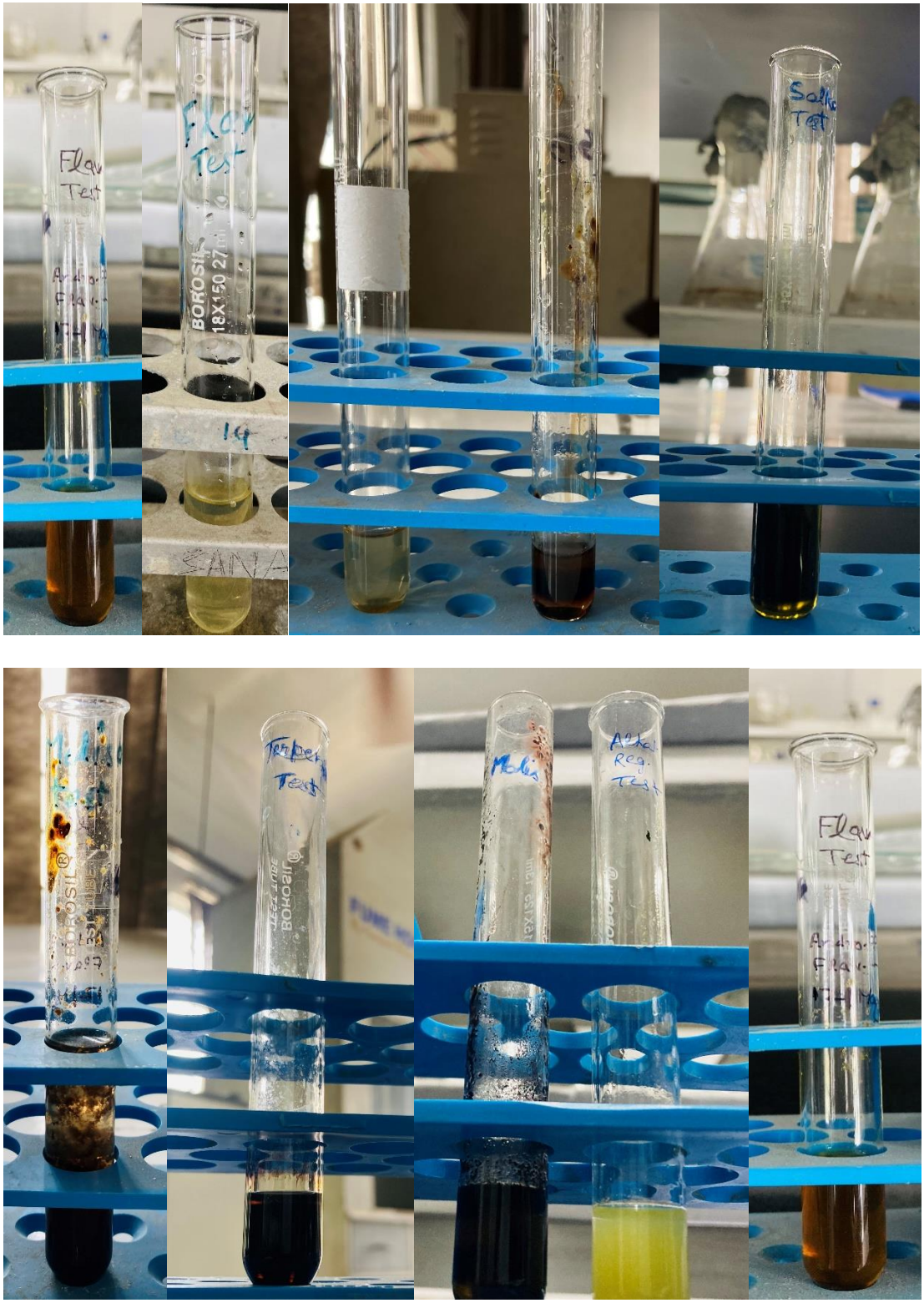


Fig 4.2 Phytochemical tests of different extracts of *A. paniculata*

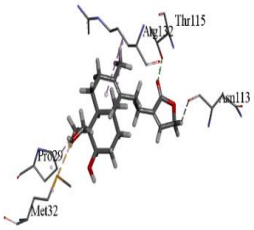
Table 4.4: Phytochemical tests of *A. paniculata* extracts

S.No.	TEST	LEAF	STEM	ROOT	AERIAL PART
1.	**Test for proteins**				
	Millon's test	+	+	+	+
	Ninhydrin test	+	+	+	+
2.	**Test for carbohydrates**				
	Fehling's test	+	+	+	+
	Benedict's test	+	-	+	+
	Molisch's test	+	+	+	+
	Iodine test	+	+	-	+
3.	Test for phenols and tannins	+	+	+	+
4.	**Test for flavonoids**				
	Shinoda test	+	+	+	+
	Alkaline reagent test	+	+	+	+
5.	Test for saponins	+	-	-	+
6.	**Test for glycosides**				
	Liebermann's test	+	+	+	+
	Salkowski's test	+	+	+	+
	Keller-kilani test	+	+	-	+
7.	Test for steroids	+	+	+	+
8.	Test for terpenoids	+	+	+	+
9.	Test for alkaloid	+	+	+	+

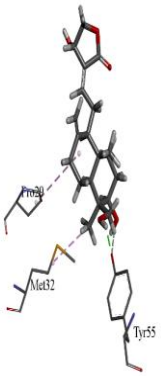
4.3 INSILICO GUIDED IDENTIFICATION:

Table 4.5 Insilico Guided Identification of Bioagents from *Andrographis paniculata* (Kalmegh)

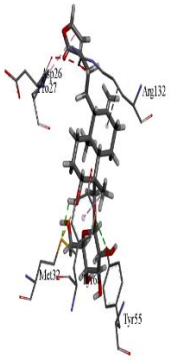
S.NO.	COMPOUND NAME (COMP ID)	SCORE	HYDROGEN BOND	OTHER INTERACTIONS
1	14-Deoxyandrographoside (21679042)	3950	THR A:115	MET A:32 ARG A:132 PRO A:29
2	Andropanolide (7067324)	3878	TYR A:55	MET A:32 PRO A:29
3	Andropanoside (44575270)	4914	MET A:32 TYR A:55	PRO A:27 TYR A:68
4	Procumbid (12314528)	3726	PRO A:27 THR A:199 ASN A:133 TYR A:68 TYR A:68	PRO A:29 ARG A:132 PRO A:27 PRO A:27
5	Kaempferol (5280863)	3244	ASP A:146	THR A:167 LYS A:145



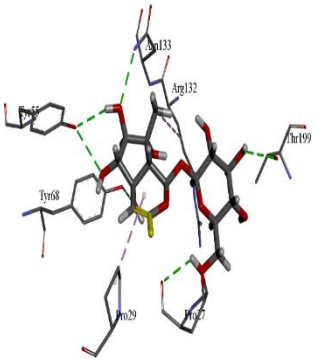
14-Deoxyandrographoside



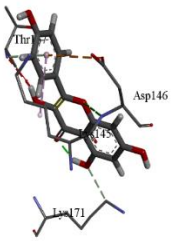
Andropanolide



Andropanoside



Procumbid



Kaempferol

Fig. 4.3 Insilico Guided 2-D Structures of different compounds of Andrographis paniculata (Kalmegh)

5. CONCLUSION

The antibacterial activity of methanol extract of different parts of Andrographis paniculate were observed using Agar well diffusion method by measuring the diameter of the growth inhibition zone. The results are depicted. In total of 4 extracts belonging to different plant parts of *Andrographis paniculata* were tested in the present investigation. In case of leaf extract, methanol extracts showed significant and highest antibacterial activity against *Bacillus subtilis*. The significant and higher antibacterial activity of the methanol extracts of leaf extracts of *Andrographis paniculata* is probably due to the presence of the Andropanoside.

The root extracts of methanol showed a positive significant antibacterial activity against *Bacillus subtilis*. This may be due to the presence of bicyclic diterpenoid lactone (andrographolide), deoxyandrographolide, which possess antibacterial, antifungal, and anti-insect activities. Further, the presence of antibacterial activity in leaf and root extracts also implies that are would be possibilities of substituting leaves for roots and stem which utilizing this plant species for bacterial related infections. However, of leaves for medicinal purposes is more sustainable compared to harvesting of plant parts such as stem and roots. Similarly, methanolic extracts of stem and aerial extracts exhibited moderate degree of bacterial activity against bacteria. This is interesting in that the traditional method of treating a bacterial infection was by administrating a decoction of the plant or a part there of by boiling it in water, whereas according to our results an organic solvent is better, hence this may be more beneficial. The standard drug Azithromycin (50µg/ml) showed high degree of inhibition against *Bacillus subtilis*.

From the above results it can be concluded that plant extracts have great potential as antibacterial compounds against microorganisms and that they can be used in the treatment of infectious diseases caused by resistant microorganisms. *Andrographis paniculata* showed maximum antibacterial activity and so this plant can be used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither to unmet therapeutic needs. However, further investigation on isolation and characterization of the active principle(s) of the plant extracts responsible for the antibacterial activity is necessary and it would give comprehensive evidence of bioactive potential of medicinal plants.

The phytochemical characteristics of *A. paniculata* plant tested were summarized in the table-4.4. The results revealed the presence of medically active compounds in the *Andrographis paniculata* plant. From the table, it could be seen that, proteins, carbohydrates, phenols and tannins, flavonoids and saponins were present in the plant.

Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities. Analysis of the plant extracts revealed the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids, and alkaloids.

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