PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Andrographis echioides*(L.) Nees.(ACANTHACEAE)

A dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University
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In partial fulfilment of the requirements
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MASTER OF PHARMACY
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MADURAI - 625 020

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Dr. A. ABDUL HASAN SATHALI, M.Pharm., Ph.D.,
PRINCIPAL (i/c)
College of Pharmacy,
Madurai Medical College,
Madurai-625020

CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Andrographis echioides(L.) Nees.* (ACANTHACEAE)''** submitted by **Mrs. S. NATHIYA (Reg. No. 261220707)** in partial fulfilment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr.M.G.R. Medical University is a bonafide work done by her during the academic year 2013-2014 under the guidance of **Dr.(Mrs). AJITHADAS ARUNA, M.Pharm.,Ph.D.,** Joint Director of Medical Education (Pharmacy), in the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625 020.

The dissertation is forwarded to the Controller of Examinations, The Tamil Nadu Dr.M.G.R.Medical University, Chennai.

(A. ABDUL HASAN SATHALI)

Station:

Date :

Dr. (Mrs). AJITHADAS ARUNA, M.Pharm., Ph. D.,
Joint Director of Medical Education (Pharmacy)
Former Principal
College of Pharmacy,
MaduraiMedicalCollege,
Madurai-625020

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Madurai- 625 020.

(AJITHADAS ARUNA)
Project Supervisor

Dr. (Mr.) D. STEPHEN., M.Sc., Ph.D.,

ASSISTANT PROFESSOR,

DEPARTMENT OF BOTANY,

THE AMERICAN COLLEGE,

MADURAI - 625002.

CERTIFICATE

This is to certify that the specimen brought by **Mrs. S.NATHIYA** II.M. Pharmacy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai is identified as ***Andrographis echioides(L.) Nees*** belonging to the family ***Acanthaceae.***

SSTATION : Madurai.

(Dr. D. STEPHEN)

DATE :

Dr. D. STEPHEN, Ph.D.,
LECTURER IN BOTANY
THE AMERICAN COLLEGE
MADURAI-625 002
TAMILNADU INDIA

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DEDICATED TO MY PARENTS AND FAMILY

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Introduction

CHAPTER-1

INTRODUCTION

Herbal drugs have been used as medicines for the treatment of various ailments since time immemorial and hence plants always have an ever green role in the health care system. Each and every plant is similar to a factory which has a ability to synthesize a sizable amount of unusual phytoconstituents which are highly complex in structures [1].

Plants are the only economic source of a member of well-established and important drugs. In spite of tremendous advances made in the modern system of medicine, there are still certain disease conditions for which suitable drugs are not available in the Allopathic system. Moreover, the modern system of medicine produces lot of side effects to the patients [2]. Nowadays herbs have been the main source of medicine throughout the human history. About 25 to 30% of today's prescription drugs contains chemical derived from herbs [3].

The factors responsible for the continued and extensive use of herbal drugs in India are their effectiveness, easy availability, low cost, comparatively less toxic effects and storage.

Herbal drugs are having complex molecular architecture and they show amazing arrangements of functional group, ring system and other structural attributes. The reasons for interest in herbal drugs are [4]

- Serve as a lead compounds for newer drugs
- Give information about the bio mechanism
- Natural products are permanent challenge with respect to synthesis and stimulate the development of new reagents and reactions.

- Collection of natural products as well as their derivatives and analogues are valuable starting points for drug discovery [5]

The main limitation of the plant is the lack of standardization of raw materials, processing methods, the final products, dosage, formulations and non-existence criteria for quality control.

The plant kingdom still hold many species of plants containing substance of medicinal value which have yet to be discovered and large numbers of plants are constantly being screened for their pharmacological value. So that we have to make an attempt to identify the medicinal herbs used for mankind.

Urolithiasis

Nephrolithiasis term indicates that the disease is characterized by the formation of stone in the kidneys or urinary tract. K**idney stone**, also known as **renal calculus** (from the Latin *rēnēs*, "kidneys," and calculus, “pebble”), is a solid concretion or crystal aggregation formed in the kidneys from dietary minerals in the urine**.**[6]

Epidemiology

Nearly 4-15% of the human populations are suffering from urinary stone problem in all over the globe. 13% of men and 75% of women will develop kidney stone during their life time in the US. The area of high incidence of urinary calculi are British islands, Scandinvian countries, Central Europe, Northern Australia, Northern India, Pakistan, Mediterranean countries. So they are known as **Stone belts.**

Mode of formation

Excretion of stone substances (eg: oxalate) + Calcium

Supersaturated solution of salt

Bacteria (eg: nano bacteria)

Solution

↓

Inhibiting nidus of calcium

Lack of substances keeping calcium salts

(Magnesium, phosphate)

Lack of inhibitors of crystal formation

Citrate, inorganic pyrophosphate & few

Uroepithelial glycoproteins

↓

Precipitation and salt formation

Pre disposing factors

Urinary pH

Dehydration- causes increased urinary concentration.

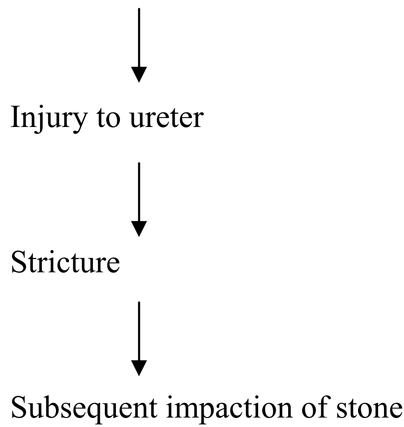
Stasis- obstruction to urine flow encourages salt precipitation

Renal disease

Metabolic factors- Hyper calcuria, hyper phosphaturia, oxaluria, urate excess

EFFECT & COMPLICATION:

Passage of small size stone ⟹ Colic

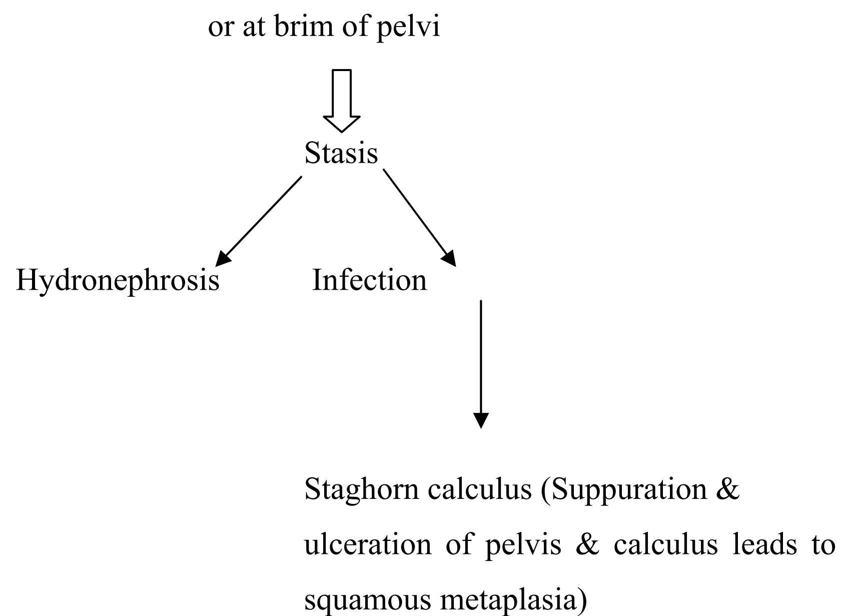


Injury to ureter

Stricture

Subsequent impaction of stone

Passage of larger stone ⟹ Impaction at upper & lower ends of ureter or at brim of pelvi



Stasis

Hydronephrosis

Infection

Staghorn calculus (Suppuration & ulceration of pelvis & calculus leads to squamous metaplasia)

Types:[106]

1. Calcium stones:

   Calcium oxalate (50%), Calcium phosphate (50%), mixture of calcium oxalate & calcium phosphate (45%)

2. Mixed (struvite) stones

   Magnesium-ammonium-calcium phosphate

3. Uric acid stone (5-10%)

4. Cystine stones (1%)

Hereditary xanthinuria, xanthine stones.

Treatment [7]

- Calcium stones are treated with Thiazide diuretics & allopurinol.
- Antimicrobial agents, judicious surgery are used for the treatment of struvite stones.
- Alkali & allopurinol are useful in uric acid stones.
- Cystine stones are treated with allopurinol & penicillamine

Herbal drugs used for the treatment of Urolithiasis

The plants which find use in the treatment of urolithiasis are *Coleus aromaticus* (Labiatae), *Cucurbita pepo* (Cucurbitaceae), *Mallotus philippensis* (Euphorbiaceae), *Tribulus terrestris* (Zygophyllaceae) and *Bergenia ligulata* (Saxifragaceae).

Diabetes mellitus[8]

Diabetes mellitus is a metabolic disease characterized by dysregulation of carbohydrate, protein, and lipid metabolism. The primary feature of this disorder is elevation in blood glucose levels (hyperglycemia), resulting from either a defect in insulin secretion from the pancreas, a change in insulin action or both. Sustained hyperglycemia has been shown to affect almost all tissues in the body and is associated with significant complications of multiple organ systems, including the eyes, nerves, kidneys and blood vessels.

These complications are responsible for the high degree of morbidity and mortality seen in the diabetic population.

Epidemiology

About 16 million Americans have diabetes (between 6 and 7% of the total US population). Around the world, the prevalence of diabetes is expected to double between

1994 and 2010, at which time about 240 million people will have the disease. In the United States, the incidence of diabetes rises as the population ages and as the prevalence of obesity increases. There are 60 to 70 diabetic individuals for every 1,000 patients and 30 to 35 of these patients are undiagnosed.

Classification

1. Type 1 diabetes was previously called insulin-dependent diabetes or juvenile diabetes. results from beta cell destruction, leading to absolute insulin deficiency.
2. Type 2 diabetes was formerly known as non-insulin-dependent diabetes or adult-onset diabetes resulting from a progressive insulin secretion defect on a background of impaired insulin function
3. Gestational diabetes occurs during pregnancy and usually resolves after delivery. Other types of diabetes may occur in individuals with certain genetic disorders, pancreatic diseases, infections, injuries to the pancreas and endocrine diseases. Drug therapy with certain agents may also induce a diabetic state.

Symptoms of diabetes

The symptoms include intense thirst, polyphagia, polyuria (frequent urination with increased amount of urine), acute hyperglycemic crisis: diabetic ketoacidosis (DKA) and hyperglycemica, hyperosmolar coma (HHS), blurred vision (osmotic swelling of lenses), dizziness, extreme tiredness, genital itching, nausea, vomiting, slow healing of wounds

Treatment

The major components of the treatment of diabetes are:

1. Diet (combined with exercise if possible).
2. Oral hypoglycaemic therapy.
3. Insulin treatment.

Herbal drug used in the treatment of Diabetes mellitus

Some of the plants used in the treatment of Diabetes include *Andrographis paniculata* (Acanthaceae), *Ficus benghalensis* (Moraceae), *Ficus glomerata* (Moraceae), *Psidium guajava* (Myrtaceae), *Syzygium cumini* (Myrtaceae), *Posidonia oceanic* (Posidoniaceae), *Bruguiera gymnorrhiza* (Rhizophoraceae), *Aegle marmelos* (Rutaceae), *Salvadora oleoides* (Salvadoraceae), *Phyllanthus reticulates* (Phyllanthaceae) and *Selaginella tamariscina* (Selaginellaceae)

Infectious diseases [9]

Infectious diseases are caused by microorganisms such as bacteria, viruses, fungi or parasites. Some of these organisms constitute the "normal flora" or micro biota present and are participating in the metabolism of food products, the protection against pathogenic microorganisms and the development and stimulation of the immune system. However, some of them can become pathogenic, for instance when introduced in normally sterile environments such as the blood or in case of suppression of the immune system. Besides these opportunistic pathogens (e.g., *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans)*strict pathogens exist that do not belong to the normal flora and can cause disease (e.g., *Mycobacterium tuberculosis*, *Vibrio cholera, Neisseria gonorrhoeae*). Clinical symptoms associated with infectious diseases are mostly not disease-specific or not microorganism-specific and often include general signs such as fever, loss of appetite, fatigue and muscle aches. Therefore, laboratory tests of different body fluid samples (e.g., blood, urine, cerebrospinal fluid (CSF)) are used for the detection and determination of the causative agent. Traditional culture-based methods in the clinical microbiology laboratory are time-consuming and are associated with a low sensitivity in case of slow-growing or fastidious microorganisms.

An infection is the colonization of a host organism by parasite species. Infecting parasites seek to use the host's resources to reproduce, often resulting in disease. Colloquially infections are usually considered to be caused by microorganism or micro parasites like viruses, bacteria, viroids, though larger organism like macro parasites and fungi can also infect.

Hosts normally fight infections themselves via their immune system. Mammalian hosts react to infections with an innate response, often involving inflammations followed by an adaptive response. Pharmaceuticals can also help to fight infections.

Infections may be divided into following

- Bacterial infections
- Fungal infections
- Parasitic infections
- Protozoan infections
- Viral infections
- Worms infestation

Bacterial infections

Pathogenic bacteria are bacteria which causes bacterial infections. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. The highly pathogenic bacteria are classified in to two types "gram positive bacteria" and "gram negative bacteria". The pathogenic bacteria contribute to globally important diseases such as pneumonia, which are caused by bacteria such as *Streptococcus* and *Pseudomonas* and food borne illness such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy. [10]

Some of the bacteria that are highly pathogenic include *Staphylococcus aureus Basillus substilis, Streptococcus viridians S. pyogens* (Gram positive bacteria) *Esherichia*

coli, Klebsiella pneumonia, Proteus albus, Salmonella typhi, Proteus mirabilis and *Pseudomonas aeruginosa* (Gram negative bacteria).

Staphylococcus aureus can cause a range of illness ranging from minor skin infections such as pimples, boils, cellulitis, and folliculitis, scaled skin syndrome to life threatening diseases such as pneumonia, meninigitis, endocarditis, osteomyelitis, toxic shock syndrome, bacterimia and sepsis. It affects the skin, soft tissue, respiratory tract, bones, joints etc. It is one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections.

Pseudomonas aeruginosa is another common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world and thrives not only in normal atmospheres but also in hypoxic atmospheres and hence has colonized many natural and artificial environments. The organism generally infects damaged tissues or those with reduced immunity. The symptoms of infections are generalized inflammation and sepsis. If such colonization occurs in critical body organs, such as the lungs, urinary tract and kidneys, the results can be fatal. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment including catheters, causing cross infections in hospitals and clinics. It also causes hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills.[11]

Fungal infections

Pathogenic fungi are those that cause disease in humans or other organisms. The study of pathogenic fungi is referred to as medical mycology. Although fungi are eukaryotic organisms many pathogenic fungi are also microorganisms. A **mycosis** is a fungal infection of animals, including humans. Mycoses are common and a variety of

environmental and physiological conditions can contribute to the development of fungal diseases. Inhalation of fungal spores or localized colonization of the skin may initiate persistent infections; therefore, mycoses often start in the lungs or on the skin.

People are at risk of fungal infections when they are taking strong antibiotics for a long period of time because antibiotics kill not only damaging bacteria, but healthy bacteria as well. This alters the balance of microorganisms in the mouth, vagina, intestines and other places in the body and results in an overgrowth of fungus. Individuals with weakened immune system are also at risk of developing fungal infections. This is the case of people with HIV/AIDS, people under steroid treatments and people taking chemotherapy. People with diabetes also tend to develop fungal infections. Very young and very old people also, are groups at risk.

Mycoses are classified according to the tissue levels initially colonized

- Superficial mycoses
- Cutaneous mycoses
- Subcutaneous mycoses
- Systemic mycoses due to primary pathogens
- Systemic mycoses due to opportunistic pathogens

Candida species are important human pathogens that are best known for causing opportunist infections in immune compromised hosts (e.g. transplant patients, AIDS sufferers and cancer patients).

The most common pathogenic fungi include *Aspergillus fumigates* and *Aspergillus flavus*. *Aspergillus flavus* produces aflatoxin, a toxin and a carcinogen and which can potentially contaminate foods such as nuts etc. *Aspergillus fumigatus* and *Aspergillus clavatus* can cause allergic diseases. Aspergillosis is the group of diseases caused

by *Aspergillus*. The symptoms include fever, cough, chest pain or breathlessness. Usually, only patients with weakened immune systems or with other lung conditions are susceptible.

Cryptococcus neoformans is another major human and animal pathogen. *Cryptococcus laurentii* and *Cryptococcus albidus* have been known to occasionally cause moderate to severe disease in human patients with compromised immunity.

Viral Infections

Viruses are tiny organisms that are made up of the genetic material known as DNA or RNA which the virus uses to replicate. It invades and attaches itself to a living cell in order to survive and then multiply and produce more virus particles. Viruses can be transmitted in numerous ways, such as through contact with an infected person, swallowing, inhalation, or unsafe sex. Poor hygiene and eating habits increases the risk of contracting a viral infection.

Types of Viral Infections

Viruses affect any part of the body and cause infections such as the common cold, flu, gastroenteritis, chicken pox or herpes. The most common type of viral infections involves the respiratory tract.

The common cold is a frequently occurring viral infection and symptoms are sneezing, stuffy nose, sore throat and coughing. A cold can last from two days to two weeks though it is a minor infection of the nose and throat. They are highly contagious and are spread by fluids from sneezing or coughing, which contain the infection.

Influenza, also known as the "flu", is a respiratory infection that is caused by viruses. The flu differs in several ways from the common cold. Symptoms of the flu include body chills, fever, headache, muscle ache and sore throat. Unlike many other viral respiratory infections, the flu can cause severe illness and life-threatening complications in many people. The flu is contracted in the same airborne manner as the common cold. The viruses are easily transmitted especially in highly populated areas.

Herpes is caused by the herpes simplex virus (HSV). This infection can infect the mouth, genitals and anus. Oral herpes causes sores around the mouth and face, while genital herpes affects the genitals, buttocks and anus. Genital herpes is known as a sexually transmitted disease (STD) and it is transmitted through sexual contact through the mouth and genitals. This virus can be spread even when sores are not present. Like chicken pox, this virus will remain in the body forever. The symptoms include fever, muscle aches, coughing, sneezing, runny nose, headache, chills, diarrhoea, vomiting, rashes and weakness. More severe symptoms include personality changes, neck stiffness, dehydration, seizures, paralysis of the limbs, confusion, back pain, loss of sensation, impaired bladder and bowel function, sleepiness that can progress into a coma or death.

Protozoan infections

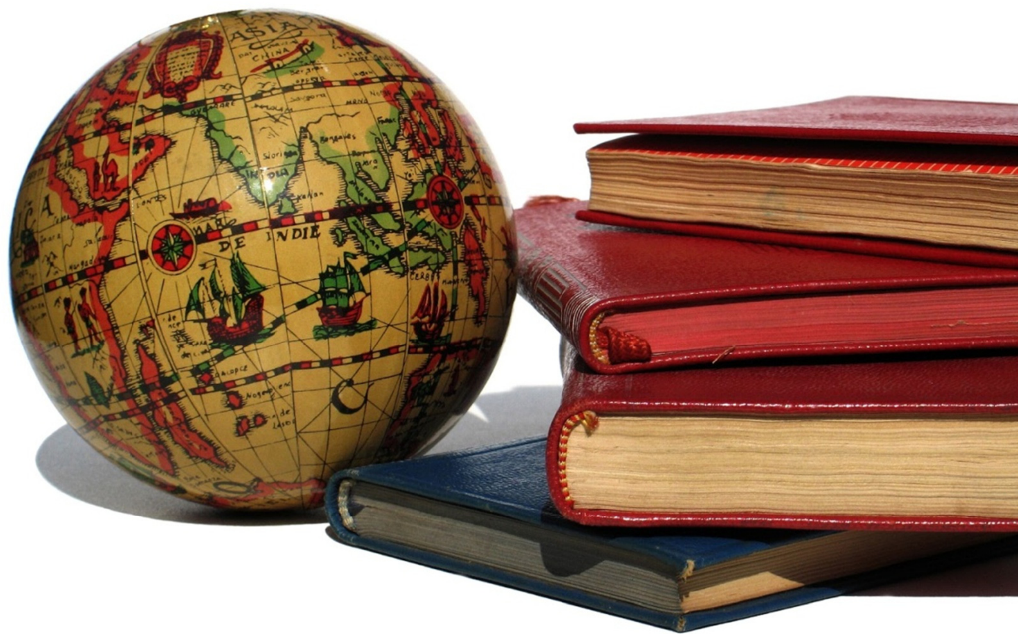
Protozoan infections are parasitic diseases caused by organisms, formerly classified in the Kingdom Protozoa. They include organisms classified in Amoebozoa, Excavata and Chromalyeolata The species traditionally collectively termed "protozoa" are not closely related to each other and have only superficial similarities (eukaryotic, unicellular, motile, though with exceptions.) The term "protozoa" is discouraged in the modern biosciences but is still encountered in medicine. This is partially because of the conservative character of medical classification and partially due to the necessity of making identifications of organisms based upon appearances and not upon DNA. Examples include *Entamoeba histolytica, Plasmodium* (some of which cause malaria), and *Giardia lamblia, Trypanosome brucei* transmitted by the fly and the cause of African sleeping sickness.

Tropical diseases

Tropical diseases are infectious diseases that either occurs uniquely in tropical and subtropical regions (which is rare) or more commonly, either more widespread in the tropics

or more difficult to prevent or control. Since the advent of air travel, people more frequently visit these regions and contract many of these diseases, most notably malaria and hepatitis.

Andrographis echioides is a very commonly seen herb and since vast work has been carried out on *Andrographis paniculata* and other species, an attempt has been made in this study to scientifically screen the herb for possible pharmacological activity.



LITERATURE REVIEW

CHAPTER-2

REVIEW OF LITERATURE

This chapter deals with a detailed review of the literature from secondary and tertiary sources on the pharmacognosy, phytochemistry and pharmacology of the various species of *Andrographis.* This chapter helps us to find out the various activities and chemicals isolated from various species of *Andrographis.*

Andrographis echioides

Jayaprakasam B *et al*., (2001) have studied the isolation of new chalcone glucoside, androechin from *Andrographis echioides*. The flavone glucoside, echioidinin 5-O-glucoside were isolated from ethyl acetate extract. The Androechin was characterized as 2, 2', 6'-trihydroxy-4'-methoxy chalcone, 2'-O-β-D-glucopyranoside by spectral and chemical studies. [12]

Qadrie ZL *et al*., (2009) have evaluated antibacterial activity of ethanolic extract of *Indoneesiella echioides* (L.) Nees, by the filter paper disc method against *Staphylococcus aureus* and *E. coli* bacteria. The organisms maintained on nutrient agar slants were using nutrient broth. One loop full of the respective culture was taken in slants and maintained below 40°C and incubated at 37°C for 24h which were observed for the growth of organism and compared with that of sterile broth. The presence of turbidity indicates growth and suitability of the culture.[13]

Radha R *et al.,* (2011) have studied the phytochemical screening and antimicrobial activity of various extracts of *Andrographis echioides* (L.) Nees. The petroleum ether, chloroform, acetone, methanol extracts of leaves and stem of *Andrographis echioides* against Gram +ve, two gram (-) bacteria and two fungi using disc diffusion method. The phytochemical screening results revealed the presence of alkaloids, flavonoids, glycosides,

phenols, tannins and saponins in leaf and stem. The highest activity was observed against Gram (+) bacteria in leaf and stem extract. The anti -fungal activity against *Candida albicans* was observed. [14]

OTHER SPECIES

Andrographis paniculata

Viswanathan *et al*., (1981) have isolated the flavone, apigenin 7, 4'-dio –o-methyl ether from *Andrographis paniculata* ,which showed significant antiulcer activity in Shay rats,histamine induced ulcer in guinea pig and aspirin ulcers in rats.[15]

Huc Q and Zhou BN (1982) have isolated the two diterpenoid glycoside from *Andrographis paniculata*. Two new glucosides are 14-deoxy andrographolide-19-β glucoside and andrographolide-19β glucoside.[16]

Dutta A and Sukul NC (1982) have studied the filaricidal properties of *Andrographis paniculata*. The water decoction of these leaves killed the microfilaria of dipetalonema within 40min by *in vitro* method.[17]

Zhang CY (1996) has studied cardiovascular activity of aqueous extract of *Andrographis paniculata* in anesthetized rats. The extract significantly produce decreased blood pressure and heart rate and exhibited anti-hypertensive effect in chronic and acute hypertensive rats.[18]

Burgos RA e*t al.*, (1997) have evaluated the testicular toxicity of dried extract of *Andrographis paniculata* in male Sprague rats at dose level 20, 200, 1000μg/kg daily for 60 days and observed the reproductive organ weight and testicular histology and ultra-structural analysis and testosterone level. The *Andrographis paniculata* extract did not produce subchronic testicular toxicity effect in male rats.[19]

Gupta PP (1998) has evaluated the anti-allergic activity of diterpene andrographolide and neo andrographolide from *Andrographis paniculata,* these were tested Anti-PCA

(passive cutaneous anaphylaxis) and mast cell stability activity in rats. These isolates possessed mast cell stability activity against compound 48/80 and sensitized mast cells, against egg albumin induced egg albumin.This activity was comparable to disodium chromoglycate.[20]

Date S *et al.*,(1999) have reported the hepatoprotective activity of various plants. *Phyllanthus species, Picrrorhiza kurroa, G.glabra, Andrographis paniculata, Eu microphylla, Tridax procumbens, Coccinia indica, Cassia fistula are* used as hepatoprotective plants.[21]

Zafar R and Mehta P (1999) have reported andrographolide in root culture of *Andrographis paniculata* Nees. The static culture contained MS medium supplemented with BA and 2,4D for the production andrographolide and the andrographolide in root callus extract was found to be 0.04mg% and plant root extract 0.29mg %.[22]

Amrogan E *et al*., (1999) have studied the inhibition effect of andrographolide from *Andrographis paniculata* on PAF-induced platelet aggregation. It influenced the biosynthesis of eicosanoids and platelet activating factor. These results of andrographolide showed that the mechanism of action differs from NSAIDS drugs.[23]

Hamgain RK (2000) has evaluated the treatment of acne vulgaris by herbomineral forms SK-34, SK-235 using student T tests. The study was for 8 weeks. At the end of 8th week none of the patients showed any skin reaction. SK-34 containing plants - *Triphala, A. indica, Rubia cordifolia, Curcuma longa* and *Andrographis paniculata.* SK-235 containing plants-*Berberi saristatic Prones amygdalus* and *Aloe vera*[24]

Akbarsha MA and Murugaian P (2000) have studied the male reproductive toxicity and male anti fertility property of andrographolide in Wistar albino rats. On the testis and cauda epididymidal spermatozoa at two dose level for 48 days. It showed that sperm count decreased, spermatozoa were not motile. Andrographolide could affect spermatogenesis by

preventing cytokinese of the dividing spermatogenic cell lines, possible prospective use of andrographolide in male contraception have been suggested.[25]

Rajini M *et al*., (2000) have isolated the andrographolide from the leaves of *Andrographis paniculata* by cold maceration (dichloromethane and methanol 1:1). The andrographolide from the extract were recrystallized and the compound was confirmed through IR, UV, MS, melting point and co-chromatography with reference standard.[26]

Garg SC., (2000) has conducted an ethnobotanical survey on some medicinal plants for snake bite. More than 100 species like *Artemisa vulgaris, Andrographis paniculata, Aristolochia bracteolate, Cissampelos pareria, Collocarpus epigaeus,Cynodon dactylon* were used as antidotes for snake bite. These plants were competitive inhibitors of three basic phospholipase A_2iso enzymes and adenosine triphosphotases enzyme for snake bite.[27]

Mandal SC *et al*., (2001) have studied the psychopharmacological activity of the extract of *Andrographis paniculata* on experimental model. The extract exhibits alteration in behavior pattern and reduction in spontaneous motility and also produce prolongation of the pentobarbitone induced sleeping time and lower the temperature in animal model.[28]

Trivedi NP and Rawal VM (2001) have studied the hepatoprotective and anti-oxidant property of *Andrographis paniculata* (Nees) in BHC induced liver damage in mice. The administration of *Androgaphis paniculata* showed protective effect in the activity of superoxidase, glutathione peroxidase, glutathione reductase and lipid peroxidase .The results indicates that the plant possess anti-oxidant and hepatoprotective action.[29]

Zaridah MZ *et al.,* (2001) have studied *in vitro* anti-filarial effect of various plant species against adult worm of *Subperiodic brugiamalayi*. Five aqueous extract from 3 plant species (dried husk, dried seed, dried leaves of *Xylocarpus granatum,* dried stem of *T. crispa* and dried leaves of *Andrographis paniculata)* were tested in vitro against adult worm of

Subperiodic brugiamalayi over the 24h observation period, as a measure of anti -filarial activity.[30]

Arya KR and Agarwal SC (2001) have reported the ethnobotanical survey of herbal folk remedy for piles used by patients of chronic piles during 1999-2001.[31]

Lalla *et al.,* (2002) have studied standardization of homeopathic mother tincture of *Andrographis paniculata and* investigated the organoleptic, physical, chemical property, HPTLC, using reference compound..[32]

Kamdom RC *et al*., (2002) have studied the mechanism of superoxide scavenging activity of neo andrographolide from *Andrographis paniculata*. The neo andrographolide might scavenge free radical by donating the allyl dehydrogen of unsaturated lactone ring.[33]

Ashok *et al*., (2002) have studied the effect of aging on andrographolide content in *Andrographis paniculata* by HPLC method. The best harvesting time was observed as 120 days after sowing, to get higher biomass containing maximum andrographolide content.[34]

Kumaran*et al*., (2003) have estimated the isolated andrographolide from *Andrographis paniculata* in rabbit serum by HPLC and UV-Vis spectrophotometric detector. Octa decylsilane column, methanol used as mobile phase and eluate were monitored at 223nm. The absolute recovery of andrographolide ranged between 97.24-99.4%.[35]

Singha PK *et al*., (2003) have evaluated antimicrobial activity of aqueous extract of *Andrographis paniculata*. The aqueous extract showed significant antimicrobial activity due to the combined effect of isolated arabinogalacton protein and andrographolide.[36]

Katyal M., (2003) have studied the phytochemical and pharmacological potential of *Andrographis paniculata* and this review provides consolidated information on chemical and biological profile of *Andrographis paniculata.*[37]

Ha X *et al., (2003)* have isolated six new metabolites M-5-M-10 of andrographolide of *Andrographis paniculata* from rat urine. Metabolites are 14-deoxy-12(R)-

sulfoandrographolide 3-sulfate(M-5),14-deoxy-12(S)-sulfoandrographolide 3-sulfate (M-6), 14-sulfo isoandrographolide, 14-deoxy-11,12-di dehydroandrographolide (M-8), isoandrographolide (M-9) and 14-deoxy andrographolide (M-10), based on respective chemical evidence and spectroscopic analysis.[38]

Saxena and UpadhyS., (2004) have reported the isolation and identification of flavonoidal glycoside from *Andrographis paniculata*. [39]

Rao YK *et al.*,(2004*)* have identified two flavonoids and andrographolide from the whole plant of *Andrographis paniculata*.5,7,2',3'-tetra methoxyflavonone and 5-hydroxy -7,2',3'-tri methoxy flavone, andrographolide, diterpenoids, poly phenol were obtained from the whole plant of *Andrographis paniculata.*[40]

Reddy BA *et al*., (2005) have isolated the two new flavonoids from whole plant of *Andrographis microbotrye*. The two flavonoids are 5,7,8,2'-tetramethyl flavone (1)and 2-hydroxy,2,3,4'-trimethylchalcone(3). The three flavones are 5-hydroxy-7-methoxy flavone,5,2',6'-tri-hydroxy-methoxy flavone and5,7,2',6' tetra hyroxy flavone. The structure of new compounds 1 and 3 have established by extensive 2D NMR.[41]

Reddy VLN *et al.*, (2005) have evaluated anti-HIV activity and isolated bisandrographolide from *Andrographis paniculata*. Six compounds of andrographolide,14-deoxy-11,12-didehydroandrographolide, andrographanin,14-deoxy-11,12-didehydro andrographolide, 5-hydroxy-7,3-dimethoxy flavone, 5-hydroxy 7,8-dimethy flavone were isolated and tested for anti-HIV and cytotoxic activity.[42]

Burgose*et al*., (2005) have isolated 14-deoxy andrographolide from *Andrographi spaniculata* and observed platelet activating factor antagonistic activity in bovine neutrophils.14-DAP concentration between 10-100μg reduces the extracellular acidification rate and intracellular alkalination. 14-DAP reduced PAF-induced calcium influx in the presence of extracellular calcium and tyrosine phosphorylation ofa 44 KDA protein

corresponding to the MAPK. It was concluded that 14-DAP is an effective antagonist of PAF mediated process in bovine neutrophils.[43]

Maiti K *et al.*, (2006) have reported the therapeutic effect of andrographolide from the *Andrographis paniculata*. The andrographolide is a diterpene lactone which is responsible for various pharmacological activities like antioxidant, hypoglycemic etc.[44]

Tan ML *et al* (2006) have evaluated cytotoxic activity of major diterpenoid consitituent of *Andrographis paniculata* in human cell lines. Seven compounds like andrographolide,14-deoxy andrographolide, andrographiside, deoxyandrographolide, 14-deoxy-12-methoxy-andrographolide, neoandrographolide, 14-deoxy-11,12-didehydro andrographolide were evaluated in human tumor cell line. 14-deoxy andrographolide, 14-deoxy-11,12-didehydro andrographolide exhibited cytotoxic activity.[45]

Shariff A *et al.*, (2007) have evaluated hepatoprotective activity of alcoholic extract of *Andrographis paniculata*. The extract was entrapped into micro pellets of calcium alginate for masking bitter taste and the formed pellets were evaluated for hepatoprotective activity in paracetamol induced hepatotoxicity in rats. 22.5mg/kg body wt of alcoholic extract was given orally for 9 days and the activity was observed. This study showed that the extract successfully inhibit the paracetamol induced hepatotoxicity which was indicated by the decrease in AST, ALT, ALP.[46]

Chander R *et al.*, (2008) have studied the Antidyslipedemic and Antioxident activity of SBC herbal formulation. It contains *Terminalia bellirica, Terminelia chubula, Andrographis paniculata and Gymnema sylvertre*. The administration of this drug at the dose 200mg/kg causes lowering in the level of cholesterol, phospholipid. Antidyslipedimic effect was compared with gemfibrozil. SBC also inhibit the formation of lipid peroxides in microsomes induced by non enzymic as well as enzymic system. [47]

Tomar A., (2008) collected the ethnobotanical survey of gastric disorder cured plants. Five species like *Adenocalymna alliaceum, Andrographis paniculata, Barleria prionitis, Carica papaya, Cyperus rotundus* were used as folk medicine in gastric pain by the rural people.[48]

Kamble MB *et al*., (2008) have studied the hepatoprotective activity of aqueous and ethanolic extract of herbal formulation. Aqueous extract of *Acacia catechu, Allium sativum and* ethanolic extract of *Andographis paniculata, Azadirachta indica, Boerhavia diffusa, Curcuma longa, Eclipta alba, Luffa echinata, Emlica officinalis, Phyllanthus amarus, Picrorrhiza kurroa* were present in the formulation. The hepatoprotective activity was studied on acute liver toxicity models of CCl_4 and paracetamol induced liver damage methods.49]

Zhao F *et al*., (2008) have studied in-vitro method of anti-tumor activity of andrographolide from *Andrographis paniculata* by inducing Apoptosis and inhibiting VEGF level .It induced apoptosis of prostate cancer cells via the activation of caspase 3, up-regulation of bax and down regulation of bcl-2.Its inhibitory activity on the level of vascular endothelial growth factor also verified by ELISA.[50]

Bansel YK and Singh S., (2009) have developed the in-vitro propagation of *Andrographis paniculata* which was achieved through tissue culture. Different plant growth regulators were involved for regeneration. Explant was obtained from 20-25 days old seedlings. Cytokinin and auxin were used for multiple shoot formation through MS medium with BAP.[51]

Dua VK *et al.,* (2009) have isolated four xanthones from the root of *Andrographis paniculata* which were tested for, in-vitro antiprotozoal activity against *Trypanosome brucei* and *Leishmaniain fantum*. The compound TDR 13011 showed significant activity against *T. b. brucei* and *L. infantum* with a 50% inhibitory concentration of 4.6µg/mL and 8µg/mL respectively.[52]

Chen JX ***et al.,*** **(2009)** have studied in-vitro and in-vivo antiviral activity of andrographolide isolated from *Andrographis paniculata* against avian influenza virus.100-200mg/kg dose level for 7 days. AL-1 was effective against avian influenza A and human influenza A (H1N1) virus in-vitro.AL-1 directly interfering with viral hemoagglutinin to block binding to cellular receptors.[53]

Lin FL et al., (2009) have studied the anti-oxidant, antiedema, analgesic activity of aqueous and ethanolic extract of *Andrographis paniculata*. This plant was used as folk medicine for inflammation, fever, diarrhoea. The aqueous extract showed high concentration of total flavonoid but lower phenol content than the ethanolic extract. The aqueous extract showed more anti-oxidant activity and also potent anti-edema, analgesic activity.[54]

Chao HP ***et al*****., (2010)** have studied that the andrographolide exhibits anti invasive activity against colon cancer cells via inhibition of MMP2 activity. Andrographolide produced growth suppression, apoptosis, and promotion anti-angiogenesis and anti transformation. The invasion ability of CT 26 cells in matri gel based invasion assays.[55]

Tanwar BS ***et al*****., (2010)** have evaluated phytochemical and mollucicidal activity of *Andrographis paniculata*. The powder and ethanolic extract showed LC50 at295.55mg/l.[56]

Lima TB et ***al.,*** **(2010)** have studied the hepatoprotective activity of Liv- 52 (*Eclipta alba, Andrographis paniculata, Tinospora cordifolia, Boerhaavia diffusa, Berberis aristata)* against carbon tetrachloride induced hepatotoxicity in albino rats.. The CCl_4 (1ml/kg, i.p) was administered to animals for inducing the hepato toxicity, silymarin (25mg/kg. p.o) was used as standard. The total protein, glutathione were measured. The histopathological evaluation was also done and SGPT, SGOT, ALP, bilirubin level was also measured. The extract exhibited protection of liver tissue and hepatoprotective activity.[57]

Pannerselvam S ***et al.,*** **(2011)** have evaluated the gastric protective efficacy of andrographolide from *Andrographis paniculata* in rats with duodenal ulcer induced by

cystamine administration (3mg/kg) for 30 days. The ulcer score measured in duodenal tissue. The study revealed that the andrographolide has potent antiulcer properties that was likely caused by minimum inflammatory changes counteracting free radical formation and maintain thiol redox status in the duodenum.[58]

Alagesaboopathi C and Balu S (2000) have studied anti-fungal activity of leaves of some species of *Andrographis wallich ex Nees on Helminthosporium oryzae Breda dehaan* by spore germination and agar cup bioassay methods. The plant species evaluated are *Andrographis paniculata, Andrographis alata, Andrographis microbotrys, Andrographis neesiana.* All the five extracts inhibited the test fungi and the *Andrographis microbotrys* was found to be more effective.[59]

Andrographis elongate

Jeyakrishna G *et al.,* (2001) have isolated new 2'-oxygenated flavones from *Andrographis elongate*. 5,2',6'-tri-hydroxy 7-methoxy flavone & skull cap flavone 1,2'-O-β-D-(4'-E-cinnamyl glucopyranoside, 7-methy wogonin, skull cap flavones 1,2'-O-β-D-glucopyranoside. The structures were elucidated by FAB-MS and (1D and 2D)-NMR studies.[60]

Andrographis viscosula

Rao YK *et al*., (2002) have isolated 5, 7, 2'-trimethoxy flavones (1) and 5, 7, 2', 4', 6'-penta methoxy flavone from the whole plant of *Andrographis viscosula.* The three flavones are echioidinin, 5, 2', 6'-tri hydroxyl-7-methoxy flavone and echioidin. These compounds were elucidated on the basis of ID and 2D NMR spectral studies.[61]

Andrographis alata

Das B *et al*., (2006) have isolated the five acylated 5, 7, 2', 6',-oxygenated flavone glycosides from the whole plant of *Andrographis alata*.5,2', 6'-tri-hydroxy- 7-methoxy

flavone-2'-o-beta-D-gluco pyranoside. The structure of the compounds were established from spectral (1D and2D NMR) and chemical studies.[62]

Andrographis stenophylla

Thangavel N and Gupta JK., **(2007)** have studied anti- inflammatory and anti – snake venom activity of methanolic extract of *Andrographis stenophylla* using carrageenan-induced acute pedal paw edema model and Freund's adjuvant induced chronic inflammatory model. The dose level of 50 and 100mg/kg extract showed anti -inflammatory activity and the leaf extract was found to potentiate the polyvalent snake venom anti serum action in experimental animal.[63]

Andrographis serphyllifolia

Ravishankar K and Rao BG., **(2008)** have evaluated the anti-inflammatory activity of the ethanolic root extract of *Andrographis serphyllifolia* in experimental rat. The dose level of 200mg/kg of the ethanolic extract shows maximum inhibition (38-95%) in carageenan- induced rat paw oedema as compared with the standard diclofenac sodium(57-80%) after 3hr of carrageenan injection. Ethanolic extract significantly inhibit the histamine induced rat paw edema.[64]

The review of literature revealed that fragmentary information was available on *Andrographis echioides* while much information was available on *Andrographi spaniculata.* Hence the present study was planned to explore the pharmacognostic, phytochemical and pharmacological activity of the whole plant of *Andrographis echioides* (L) *Nees.*

CHAPTER 3

PLANT PROFILE [65-67]

BOTANICAL SOURCE : *Andrographis echioides*(L.) Nees

FAMILY : Acanthaceae

SYNONYMS : *Indoneesiella echioides* (L) Sreemadh

Justicia echioides L.

Andrographis echioides is an erect herbs growing to a height of 10-50cm. The whole plant is villous with the leaves being opposite decussate, elliptic, oblong, and obtuse to round at apex, attenuate at base and 7.5cm long, 2.4cm wide. The flowers appear during March-June and October-December.

SYSTEMATIC POSITION

Kingdom	:	Plantae
Subkingdom	:	Trachiobionta
Infra kingdom	:	Streptophyta
Phyllum	:	Tracheophyta
Class	:	Magnoliatae
Subclass	:	Asteridae
Order	:	Scrophulariales
Suborder	:	Asteranae
Family	:	Acanthaceae
Subfamily	:	Acanthoideae
Tribe	:	Ruellieae
Subtribe	:	Andrographinae
Division	:	Magnoliophyta

Subdivision	:	Spermatophyta
Genus	:	AndrographisWall.exNees
Species	:	Andrographis echioides

COMMON NAME

English	:	False water willow

VERNACULAR NAMES

Tamil	:	Gopuramthangi, Malaithangi
Malayalam	:	Pituma
Telungu	:	Tellavemu
Gujarathi	:	Kalukariyatun
Marathi	:	Ranchimani
Oriya	:	Lavalata
Others	:	False water willow

Geographical Distribution and Occurrence

Andrographis echioides is distributed in the tropical India and Srilanka. It is found in several regions in Tamil Nadu and Kerala like Kannur, Kottayam, Alappuzha, Kollam, Palakad, Thiruvananthapuram and Madurai.

Habit and Habitat:

Andrographis echioides is an annual herb found in South India.

Ethnomedical Information for whole plant:

1. The whole plants are used for scorpion sting, snake bite and stomach ache.
2. The leaves are used for cuts and fever.
3. The herb is used for the treatment of dysentery, diabetes, swellings, liver damage and jaundice.
4. The juice of the leaves is used for hair falling and hair growth.

AIMS AND SCOPE

CHAPTER 4

AIM AND SCOPE OF THE STUDY [68, 69]

The ethnomedical information revealed that almost whole plant is used in various indigenous system of medicine against several diseases.

Andrographis (Acantaceae) is a genus of about 40 species. Several *Andrographis* species have been used in the treatment of dyspepsia, influenza, malaria, respiratory infections, antidote for poisonous stings, diabetes and urinary tract infections.

Andrographis echioides (L) Nees is a procumbent annual herb, the plant have properties similar to those of *Andrographis paniculata*. The herb is useful for the treatment of dysentery, diabetes, swelling and also used for liver damage,jaundice, cuts and fever.

The present study has been designed to carry out the pharmacognostical, phytochemical and pharmacological evaluation of whole plant of *Andrographis echioides.*

1. Detailed pharmacognostical studies of whole plant of *Andrographis echioides.*
2. Preliminary phytochemical studies on the extracts in order to confirm the presence of primary and secondary metabolites like phenol, flavonoids tannins.
3. Estimation of the total phenol, flavonoid, tannins and vitamin content in *Andrographis echioides* extract.
4. Phytochemical evaluation of the extract by means of the TLC and HPTLC studies.
5. Determination of Mineral (or) heavy metals using X-Ray fluorimetry.
6. Evaluation of the extracts of the plant *Andrographis echioides* for the following pharmacological studies.

    (a) Determination of *in-vitro* antioxidant potential by,

1. DPPH Assay
2. Hydrogen peroxide scavenging activity
3. Ferric reducing Antioxidant power (FRAP) Assay
4. Reducing power Assay
5. Total Antioxidant activity by phospho molybdenum method.

(b) To study *in-vitro* calcium oxalate crystallization inhibition by ethanolic extract of *Andrographis echioides.*

(c) To study *in-vitro* anti-diabetic activity of *Andrographis echioides* extract.

(d) To study the *in-vitro* anti-bacterial activity of *Andrographis echioides*.

(e) To study the *in-vitro* anti-fungal activity of *Andrographis echioides.*

PHARMACOGNOSTICAL STUDIES

CHAPTER - 5

PHARMACOGNOSTIC STUDIES

Pharmacognostical evaluation gives valuable information regarding the morphology, microscopical and physical characteristics of the crude drugs and the resulting observations have been incorporated in various pharmacopoeias. There are number of plants whose identity, pharmacognostic studies and medicinal properties have not yet been scientifically studied. Hence pharmacognostic study gives the scientific information regarding the purity and quality of the plant drugs.

MATERIALS AND METHODS

SECTION A - MACROSCOPICAL STUDIES

Morphological studies include aspects of outward appearance (shape, structure, colour, and pattern) as well as the form and structure of the various parts like leaves, flowers etc. Some of these gross morphological characters of the drugs such as shape, size, margin, apex and venation are identification features of the drugs. These features give valuable information about the drugs.

Collection of Plant Material

The plant specimens were collected from Madurai Medical College campus. The plant was identified and authenticated by Dr. L. Stephen, Lecturer, American College, Madurai. The authenticated herbarium sheet has been placed at the Dept. of Pharmacognosy, College of Pharmacy, Madurai Medical College. A copy of the herbarium has been presented in **Fig.1**A special care was taken to select healthy plants and normal organs for macroscopical studies. The macroscopical features of the plant are presented were in **Fig.2.1 to 2.4.** The

whole plant material was washed thoroughly and dried in shade. The shade dried plant material were powdered and used for further studies.

SECTION B - MICROSCOPICAL STUDIES

The microscopical study of organized crude drugs is an important parameter for evaluation. The microscopicalevaluation allow more detailed examination of the plant material to identify the organized drug by its histological character. It provides detailed information about the crude drugs by virtue of its property to magnify the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drug under evaluations. It can also be used in the determination of the optical as well as micro chemical properties of the crude drug.

Material and Methods.

Collection of Specimen

The leaves, root, stem of the plant were subjected tomicroscopicalevaluation.The samples of the leaf were cut and removed from the plant and fixed in FAA (formalin-5ml+acetic acid-5ml+70%ethyl alcohol-90ml). After 24h of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol [70]**.** The infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were then cast into paraffin blocks.

Sectioning

The paraffin embedded specimen was sectioned with the help of rotary microtome. The thickness of the sections was 10-12µm, de-waxing of the sections was carried out by customary procedure [71]. The sections were then stained with toludine blue, [72] Since toludine blue is a polychromatic stain, the staining results were remarkably good; and some

cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. The sections were also stained with saffranin and fast-green and iodine wherever necessary.

For studying the stomata morphology, venation pattern and the trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) was used. The clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid were also prepared for studying stomatal morphology. Glycerin mounted temporary preparations were made for macerated/ cleared material. The powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell components were studied and measured.

Photomicrographs

Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observation bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. The magnification of the figures was indicated by the scale bars on the photomicrographs. The microscopic features observed for the leaves of the plant are presented in **Fig. 3 to 8.**

SECTION C - QUANTITATIVE MICROSCOPY

Quantitative microscopy or determination of some pharmacognostic parameters is useful for setting standards for crude drugs. This is used for evaluation, identification, characterization and standardization of crude drugs. A clear idea about the identity and characteristic features of the drug can be obtained after several number of determinations. The characteristic's number obtained is noted and compared with a standard value to find out whether it is within the range and standard deviation.

DETERMINATION OF LEAF CONSTANTS [73-77]

The stomatal number, stomatal index, vein islet number and vein termination number were determined on fresh leaves by using standard procedures.

Stomatal number and stomatal index

The stomatal number (SN) and stomatal index (SI) is a very specific criterion for identification and characterization of leaf crude drugs.

Stomatal number

The average number of stomata present in one square millimeter area of each surface of a leaf epidermis is termed as stomatal number**.**[74].

Stomatal index

The stomatal index is the percentage of the number of stomata to the total number of the epidermal cells, each stomata being counted as one cell. The stomatal index was calculated using the formula S.I = (S/E+S) × 100; where S = Number of stomata per unit area; E = Number of epidermal cells in the same unit area.

Procedure

The upper and lower epidermis was peeled out separately by means of forceps. The cleared leaf was placed on a slide and mounted in glycerin. A camera lucida and drawing board was placed and a stage micrometer was inserted for making the drawing scale. A square of 1mm was drawn by means of stage micrometer. The slide with cleared leaf (epidermis) was placed on the stage of the microscope and examined under 45X objective and 10X eye piece. The epidermal cells and stomata was traced. The numbers of stomata present in the area of 1 sq. mm. including the cell if at least half of its area lies within the square was counted. The result for each field was calculated and the average number of stomata per sq. mm. The results obtained are tabulated in **table 1.**

For stomatal index, the glycerin mounted leaf peeling as mentioned above was made and circle (O) like mark for each stomata and a cross (X) like mark for each epidermal cells was marked on the chart paper. The results obtained are tabulated in **table 1.**

Vein islet number and vein termination number

Vein islet number

It is the average number of vein-islets per square mm of the leaf surface. It is determined by counting the number of vein-islets in an area of 4sq.mm of the central part of the leaf midway between the midrib and the margin.

Vein termination number [75]

Vein termination number is the average number of veinlet termination present in per sq.mm of the leaf surface. It is determined by counting the number of vein terminations in an area of 4sq.mm of the central part of the leaf midway between the midrib and the margin.

Procedure:

The fragments of leaves, about 5x5 mm in size, from middle and lamina portion was placed in a test tube containing about 5 ml of chloral hydrate and heated on water bath for about 15 minutes or until the fragments are transparent. The cleared fragments were stained with saffranin solution and a temporary mount was prepared with glycerol solution. The stage micrometre placed on the microscopic stage, examined under 10X objective and 6X eye piece and an area of 1 sq mm square was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and vein terminals included in the square was drawn. The number of vein islets and terminals within the square were counted. The results obtained in the number of vein islets and terminals in 1sq.mm. The results obtained are tabulated in **table1.**

SECTION D - POWDER ANALYSIS AND MICROSCOPY

Powder analysis

The behavior of the powder with different chemical reagents was carried out as per standard procedure [74] and the results are presented in **table 2**.

Fluorescence analysis

The fluorescent analysis of the drug powder as well as the plant extracts of *Andrographis echioides* were carried out and the observations are tabulated in **table 3.**

Powder microscopy

The powder was observed under a microscope and the various characteristic features observed under the microscope is presented in **Fig. 9.**

SECTION E - STANDARDIZATION PARAMETERS [77]

The determination of the ash values, loss on drying, foreign organic matter and extractive values etc. gives a clear idea about the specific characteristics of crude drug under examination, besides its macro-morphological or cyto-morphological, microscopical nature in both its entire and its powder form. These diagnostic features enable the analyst to know the nature and characteristic of crude drugs. The procedures recommended in Indian Pharmacopoeia were followed to calculate total ash, water-soluble ash, acid-insoluble ash, sulphated ash and loss on drying at 110°C. The percentage of extractive values for different solvents was also calculated.

Determination of foreign organic matter

The part of organ or organs other than those specified in the definition or description of the crude drugs is defined as foreign organic matter. An accurately weighed 100g of air dried coarse drug and spread out in a thin layer. The sample drug was inspected with the unaided eye or with the use of 6X lens and the foreign organic matter was separated manually as completely as possible and weighed. The percentage of foreign organic matter was calculated with reference to the weight of the drug taken. The results obtained are presented in **table 4.**

Determination of Moisture Content (Loss on Drying)

An accurately weighed 10g of powdered drug was placed in a tared weighing bottle. Then the bottle was dried at 105°C for 5h and weighed. The drying and weighing was continued at one hour intervals until the difference between the two successive weighing is not more than 0.25%. The loss on drying was calculated with reference to the amount of powder taken. The results obtained are presented in **table 4.**

Determination of Ash values

Ash Content [76]

The residue remaining left after incineration of the crude drug is designated as ash. The residue obtained usually represents the inorganic salts naturally occurring in the drug and adhering to it. It varies within definite limits according to the soils where the plant material is generally collected. It may also include inorganic matter deliberately added for the purpose of adulteration. Hence, an ash value determination furnishes the basis for judging the identity and cleanliness of any drug and gives information relative to its adulteration/contamination with inorganic matter, thus ash values are helpful in determining the quality and purity of drug.

Procedure

Determination of Total Ash

An accurately weighed about 2g of air dried powdered drug was taken in a tared silica crucible and incinerated at a temperature not exceeding 450°C, upto 6h until free from carbon the cooled and weighed, repeated for constant value. Then the percentage of total ash was calculated with reference to the air-dried drug. The results obtained are presented in **table 4.**

Determination of Acid Insoluble Ash

The ash obtained as directed under total ash was boiled with 25mL of 2M hydrochloric acid for 5min on a water bath, and then the insoluble matter was collected on an ashless filter paper (Whatmann) and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450°C, cooled in a dessicator and weighed. The percentage of acid insoluble ash with reference to the air-dried drug was calculated. The results obtained are presented in **table 4.**

Determination of Water Soluble Ash

The ash obtained as directed under total ash was mixed with 25mL of water and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450°C, cooled in a dessicator and weighed. The insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug. The results obtained are presented in **table 4.**

Determination of sulphated Ash

An accurately weighed 2g of air dried powdered drug in a tared silica crucible which was previously ignited and cooled before weighing at a temperature not exceeding 450°C.

The residue was moistened with 1mL of concentrated sulphuric acid, ignited at 800 ± 25°C until all black particles have disappeared. It was then cooled; again sulphuric acid was added and ignited. It was cooled and the percentage of sulphated ash was calculated with reference to air dried drug. The results obtained are presented in **table 4.**

Extractive values [76]

Extractive values help to determine the amount of soluble active constituents in a given amount of medicinal plant material, when extracted with solvents. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of drug and solvent used. The use of single solvent can also be used for providing preliminary information on the quality of a particular drug sample and hence is an important tool for analysis of plant crude materials for its identity, purity and quality.

Procedure

Determination of ethanol soluble extractive

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of ethanol in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of ethanol. Then evaporate 25mL of the filtrate to dryness in a tared china dish dry at 105°C and weighed. The percentage of ethanol soluble extractive was calculated with reference to the air dried drug. The results obtained are presented in **table 4.**

Determination of water soluble extractive

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of chloroform water in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of

chloroform water. Then evaporate 25mL of the filtrate to dryness in a tared china dish dry at 105°C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried drug. The results obtained are presented in **table 4.**

Determination of petroleum ether soluble extractive

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent. The results obtained are presented in **table 4.**

Determination of hexane soluble extractive

The procedure adopted under ethanol soluble extractive was followed using hexane as a solvent. The results obtained are presented in **table 4.**

Determination of chloroform soluble extractive

The procedure adopted under ethanol soluble extractive was followed using chloroform as a solvent. The results obtained are presented in **table4.**

Determination of benzene soluble extractive

The procedure adopted under ethanol soluble extractive was followed using benzene as a solvent. The results obtained are presented in **table 4.**

Determination of ethyl acetate soluble extractive

The procedure adopted under ethanol soluble extractive was followed using ethyl acetate as a solvent. The results obtained are presented in **table 4.**

Determination of acetone soluble extractive

The procedure adopted under ethanol soluble extractive was followed using acetone as a solvent. The results obtained are presented in **table 4.**

Determination of Foaming Index

Some plant materials when shaken with water cause persistent foam which may be attributed to the presence of saponins in that material. The foaming ability of an aqueous solution of plant materials and their extracts is measured in terms of foaming index.

Procedure

An accurate quantity of about 1g of the coarse plant material was weighed and transferred into an Erlenmeyer flask containing 100mL of boiling water. The flask was boiled at moderate heat for 30min. The solution was cooled and filtered into a 100mL volumetric flask and sufficient distilled water was added to dilute to volume. The solution was poured into ten stoppered test tubes in successive portions of 1mL, 2mL, etc. upto 10mL, and the volume of the liquid in each tube was adjusted with water upto 10mL. The tubes were then stoppered and shaken in a length wise motion for 15sec (two shakes/sec) and allowed to stand for 15min. The height of foam was measured. If the height of the foam in every tube was less than 1cm the foaming index was less than 100. If a height of foam of 1cm was measured in any test tube, the volume of the plant material decoction in this tube (a) was used to determine the index. If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case, the determination was repeated using a new series dilution of the decoction in order to obtain a result. The foaming index was calculated by using the following formula 1000/A where A was the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The results obtained are presented in **table 4.**

Determination of volatile oil

Volatile oils are characterized by their odour, oil like appearance and also it has ability to volatilize at room temperature. Chemicaly they are mixture of monoterpenes,

sesquiterpenes and their oxygenated derivatives. Volatile oils are estimatedby hydro-distillation method using a standard procedure.

RESULTS AND DISCUSSION

SECTION A - MACROSCOPICAL STUDIES

Observations and discussion

Leaves (Fig.2.2 & 2.3)

Andrographis echioides is an erect herb growing to a height of 10-50cm. The leaves are hairy on both sides. The whole plant is villous with the leaves being opposite decussate, elliptic oblong, obtuse to round at apex, attenuate at base and 7.5cm long, 2.4cm wide. It is a small herb, four sided, it looks like temple tower and the leaves are greenish with red patches.

Flowers (Fig.2.4)

Flowers are borne in spike-like racemes, up to 2cm long. The stalk carrying the raceme is densely hairy. Flowers are erect. Sepal tube is 2mm long, with thread-like sepals up to 9mm long. Flowers have a 4mm long tube, opening into two lips. Upper lip is oblong, up to 5.5 x 2mm, 2-lobed above. Lower lip is up to 7mm long, with 3 oblong-lance shaped lobes, marked with purple. Stamen filaments are flattened. The flowers appear during March-June and October-December.

Seeds

The seeds are 0.15-0.2 cm long, black, pitted without retinacula spiny.

Organoleptic evaluation

- Nature - Coarse Powder
- Colour - Light green

Fig.1: Herbarium of the plant

HERBARIUM

NAME S. NATHIYA

Reg.No. 261220707

COLLEGE / ~~SCHOOL~~ COLLEGE OF PHARMACY MADURAI MEDICAL COLLEGE

NAME	:	ANDROGRAPHIS ECHIOIDES(L.)NEES
FAMILY	:	ACANTHACEAE
GUNUS	:	ANDROGRAPHIS
SPECIES	:	ECHIOIDES
LOCALITY	:	MADURAI
DATE	:	

Date :

R. Gomis.

Professor / Teacher-in-charge

Dhanalakshmi

Fig.2.1: Habitat of the plant

Fig.2.2: Leaf of the plant

Fig.2.3: Leaf - ventral and dorsal view

Fig.2.4: Flower of the leaf

FIG: 2.5 DIAGRAMETIC REPRESENTATION OF *Andrographis echiodes (L.) Nees*

- Odour - Characteristic
- Taste - Bitter

SECTION B - MICROSCOPICAL STUDIES

Midrib of the leaf (Fig.3.1, 2)

The leaf has a thick and prominent midrib **(Fig.3.1)**.The midrib has thick, dorsiventral lamina, rectangular adaxial part and wide expanded abaxial part **(Fig 3.2)**. The midrib is 600µm thick and the adaxial part is 300µm wide, the abaxial part is 600µm wide. The midrib is thick and comprises of rectangular thin walled cells. The ground tissue is parenchymatous, the cells are angular thin walled and compact. The mesophyll tissue is horizontally trans current along adaxial part of the midrib.**(Fig.3.2).** The vascular strand is single, concave disc, measuring 150µm thick and 350µm wide. The vascular strand is collateral with adaxial, several parallel lines of xylem elements and abaxial phloem elements. The phloem elements are in small discrete nests **(Fig.3.2)**.The xylem elements are narrow, angular in outline, thick walled and are arranged in long multiples.

The lateral vein (Fig.4.1)

The lateral vein is planoconvex with flat adaxial side and thick conical adaxial part **(Fig.4.1)**. The lateral vein has a single, small and collateral vascular bundle. The vein is 300 µm thick **(Fig.4.1)**.

Lamina (Fig.4.2, 3)

The lamina is 110µm thick. The adaxial epidermis fairly thick and the cells are rectangular and thin walled. Multicellular, uniseriate, un-branched non-glandular trichomes are seen arising from adaxial epidermis **(Fig.4.2).** The abaxial epidermis comparatively thin and the cells are cylindrical. The mesophyll tissue consists of adaxial, single row of loosely arranged palisade cells and abaxial spongy parenchyma cells of lobed and less compact

tissue. The leaf margin is thick, bluntly conical **(Fig.4.3)**. The epidermal cells along the marginal part are lightly dilated into large rectangular or squarish cells with prominent cuticle. The palisade and spongy mesophyll tissues are retained in the marginal lamina.

Stomata and epidermal cells (Fig.5.1, 2, 3)

Some of the epidermal cells of the lamina are modified into elongated wide cells called lithocysts. Within the lithocyst occurs thick, cylindrical cystolith. The surface of the cystolith is echinate. **(Fig. 5.1).**

The epidermal cells have wavy thick anticlinal walls. **(Fig.5.2).**The stomata are diacytic type. There are two subsidiary cells, lying on the ends of the guard cells, the common wall of the subsidiary cells is at right angles to the guard cells.**(Fig.5.2, 3)**. The guard cells of the stomata are 10x20µm in size.

Venation Pattern (Fig.6.1, 2)

The veins are thin and straight, they form wide vein-islets of rectangular outline. The islets are more or less parallel to each other. The vein-terminations are branched long, slender undulate **(Fig.6.2)**.

Stem (Fig.7.1, 2)

The stem is four angled with thick conical ridges at the corners **(Fig.7.1)**.The stem is 2mm thick. The stem consists of a thick and distinct epidermal layer of squarish thin walled cells. The cortical zone is narrow and includes about six layerof angular thin walled parenchyma cells. Secondary phloem occurs in the continuous layer on the outer circumference of the secondary xylem cylinder. Secondary xylem cylinder is thicker along the corner of the stem and thin in between the corners **(Fig.7.2)**. Xylem consists of numerous solitary, diffuse vessels. The vessels are circular and thick walled. The vessels are both

Fig.3.1: T.S of Leaf through midrib

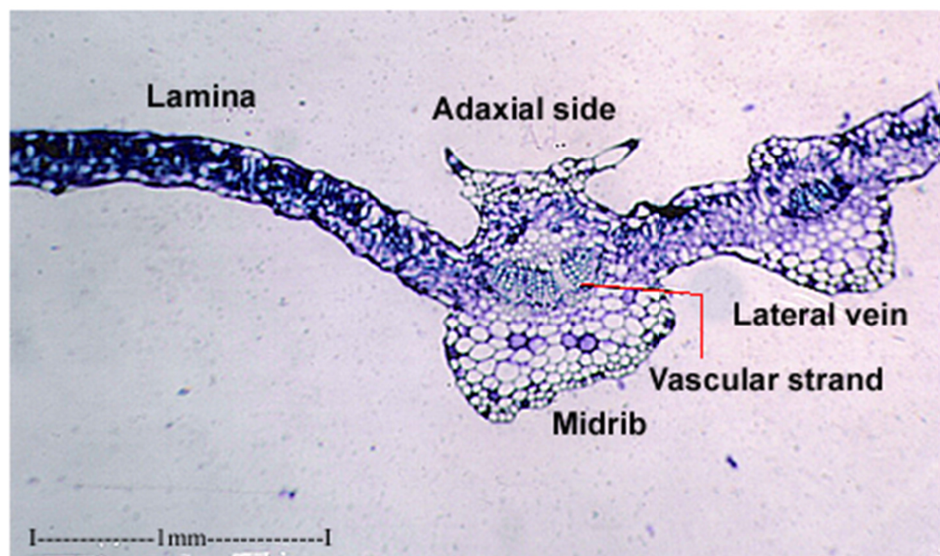
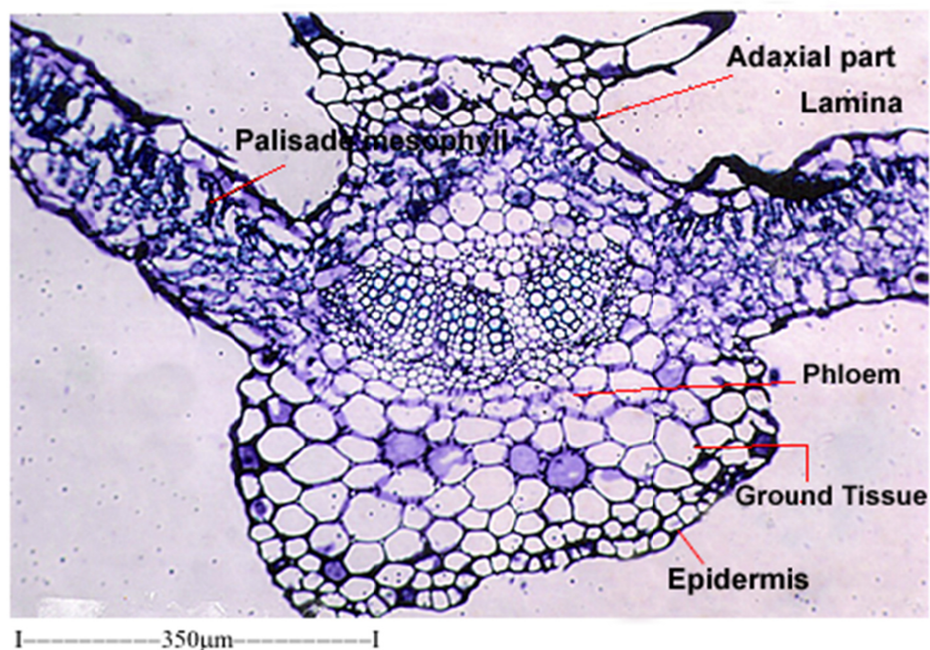


Fig.3.2: T.S of Midrib



I——————350μm——————I

Fig.4.1: T.S. of leaf through lateral vein

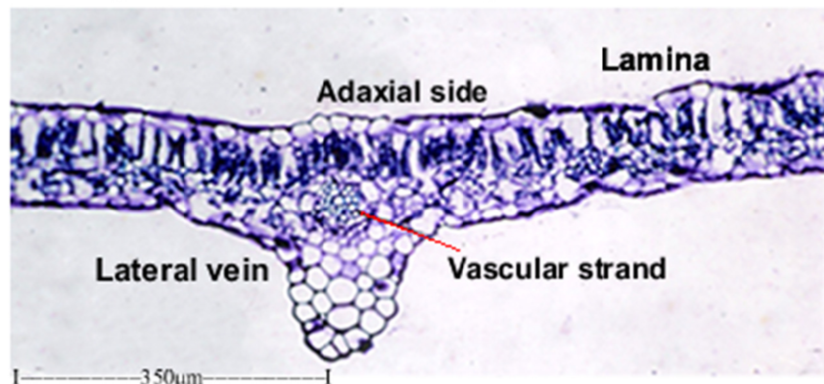


Fig.4.2: T.S. of lamina bearing trichomes

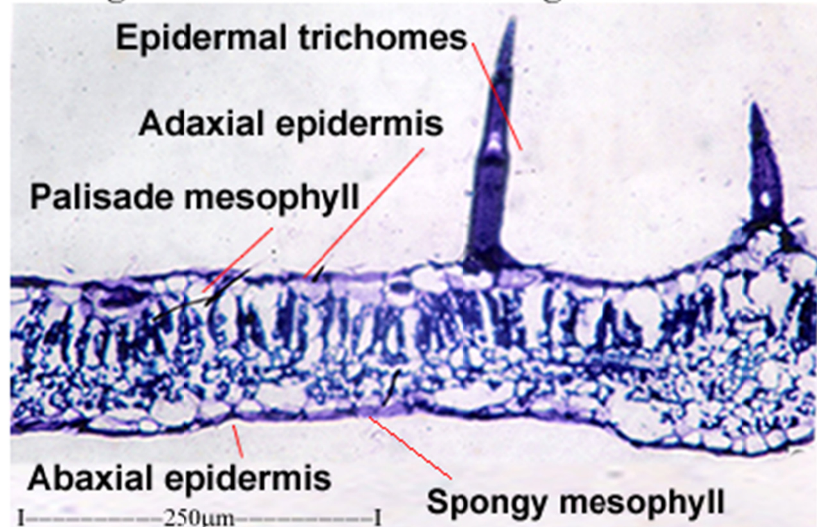


Fig.4.3: T.S. of lamina through margin

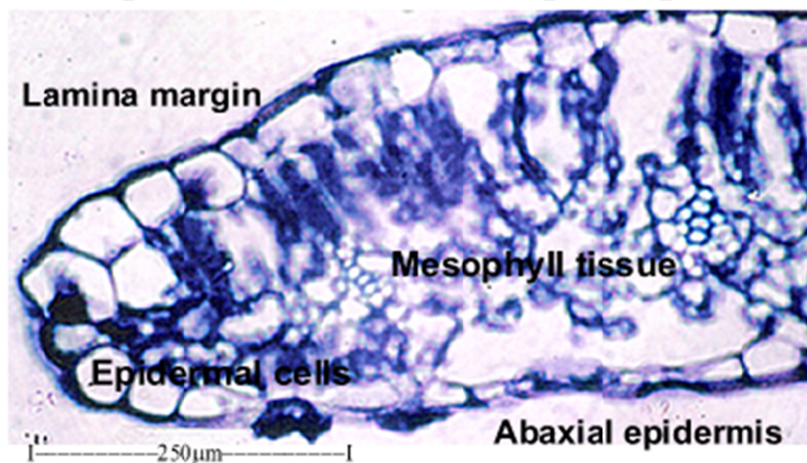
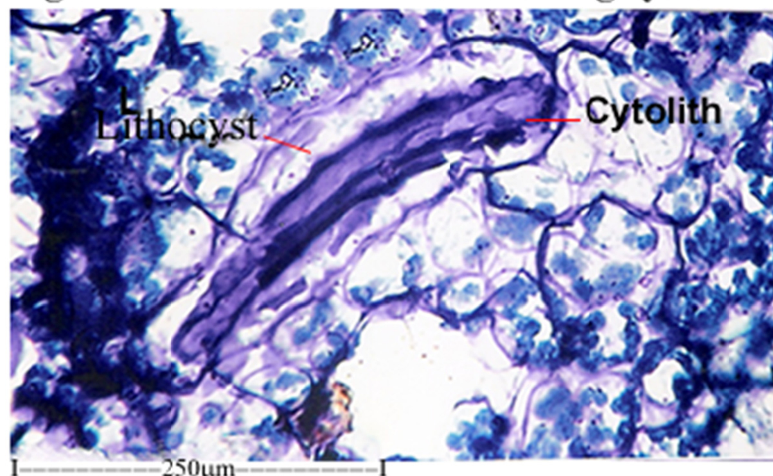
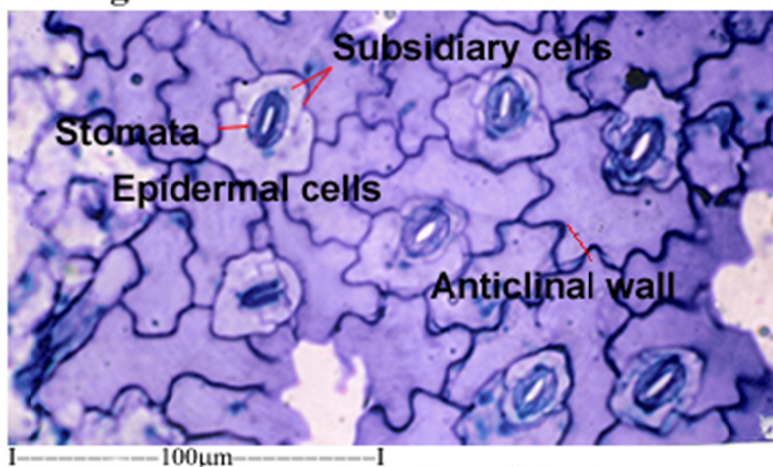


Fig.5.1: Paradermal section showing cystolith



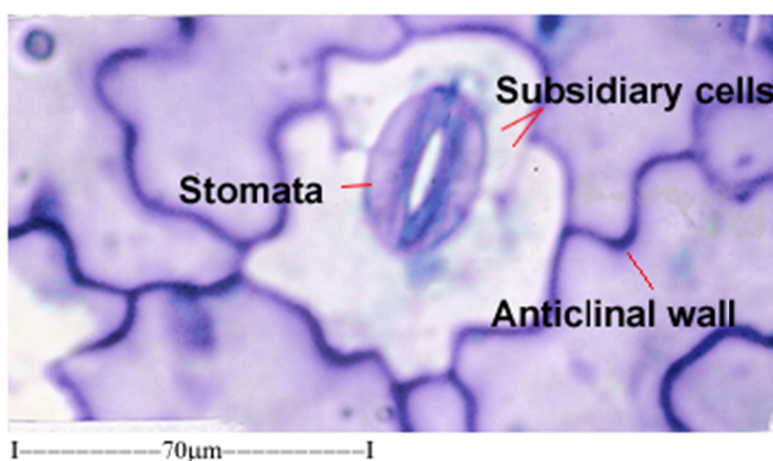
I——————250µm——————I

Fig.5.2: Paradermal section of stomata



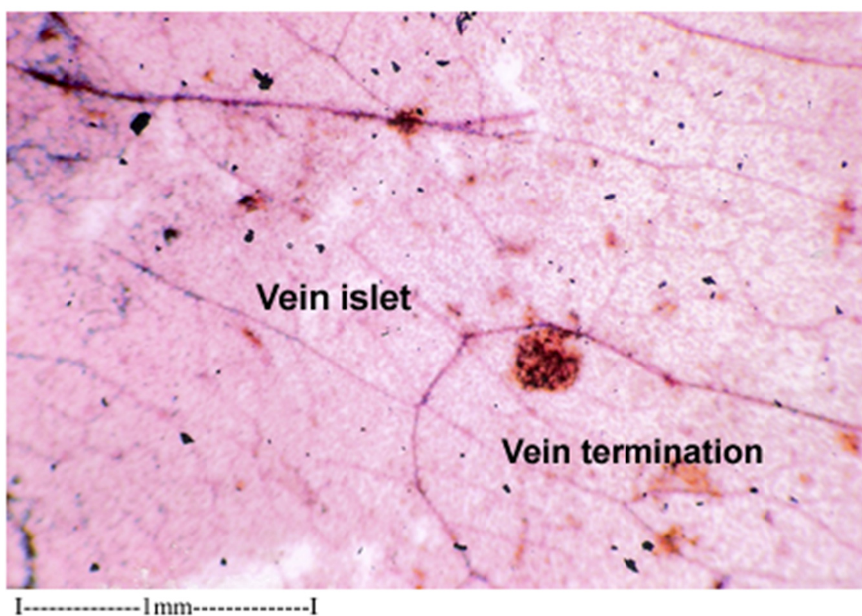
I——————100µm——————I

Fig.5.3: Paradermal section of single stomata



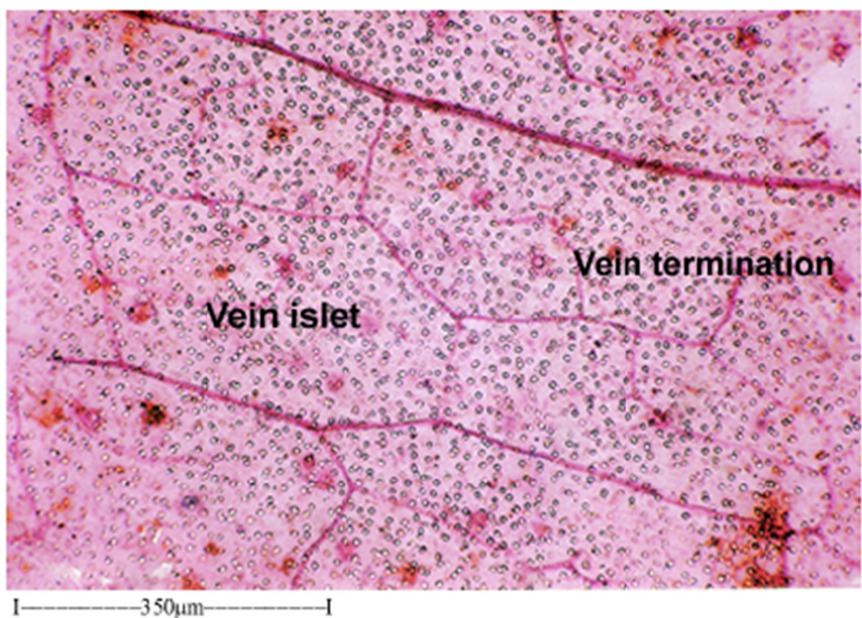
I——————70µm——————I

Fig.6.1: Venation pattern



I--------------1mm--------------I

Fig.6.2: Venation pattern



I------------350µm------------I

Fig.7.1:T.S of stem entire view

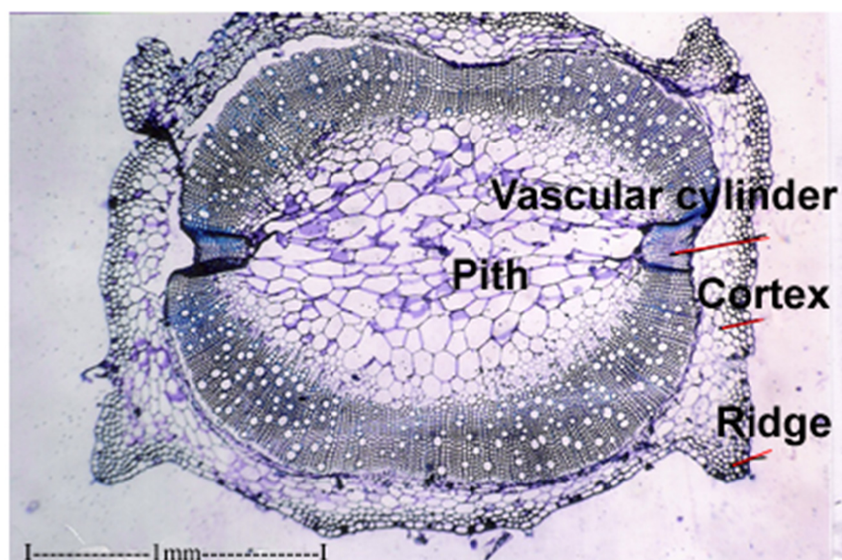


Fig.7.2:T.S of stem a sector enlarged

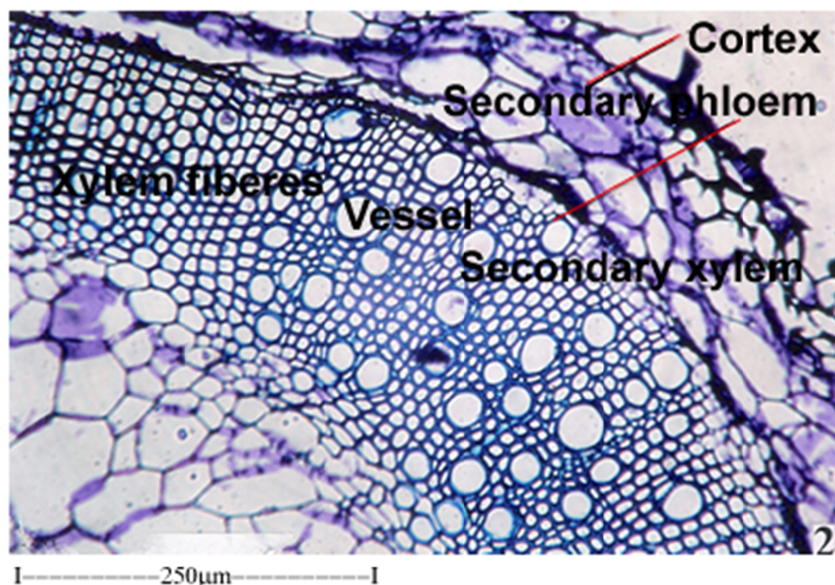
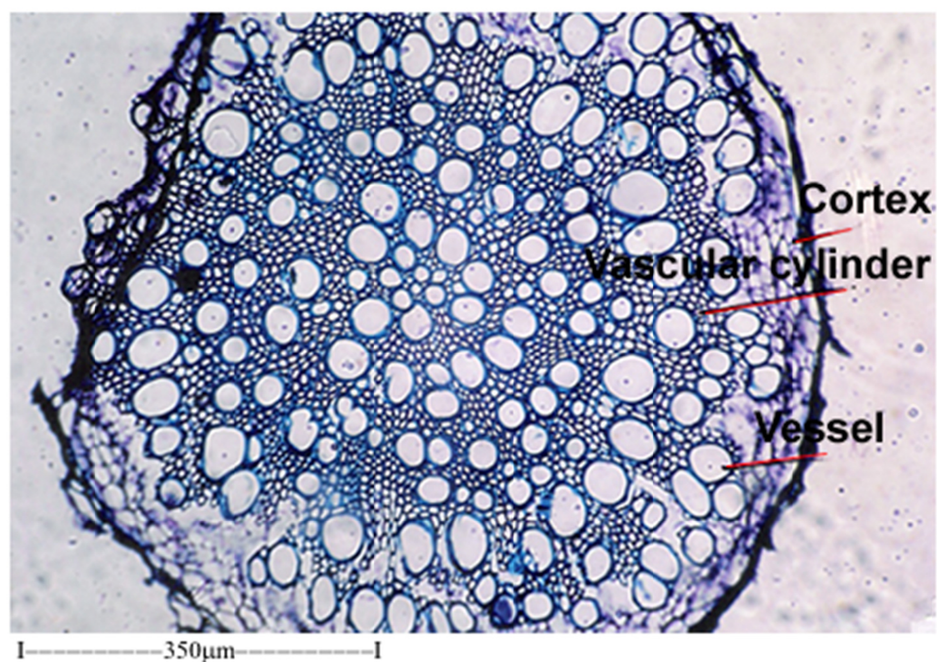
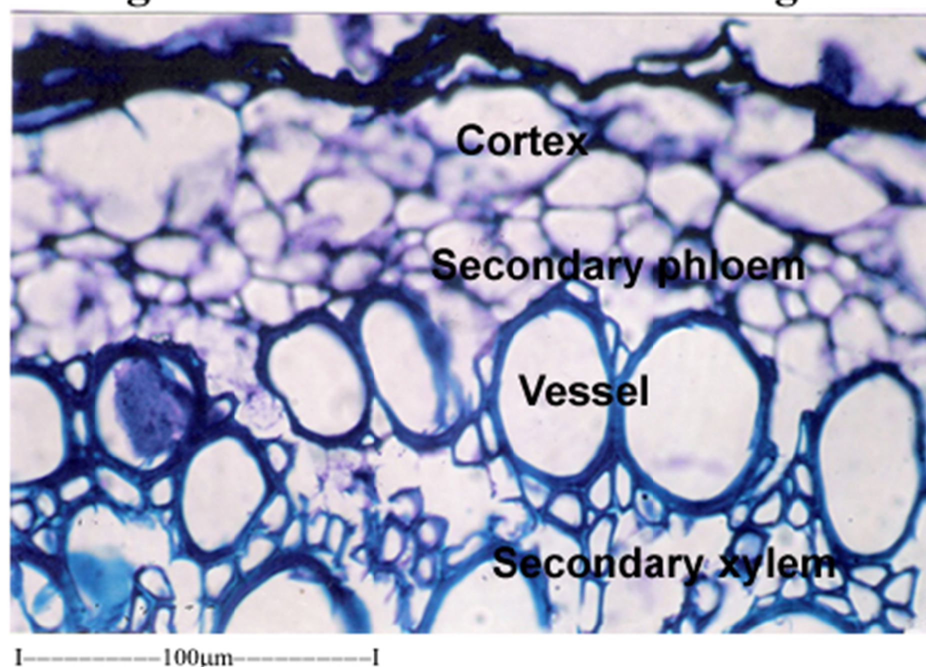


Fig.8.1:T.S of root



I-----------350µm-----------I

Fig.8.2:T.S of root a sector enlarged



I-----------100µm-----------I

narrow and wide type. Xylem includes xylem fibre,which are thick walled and lignified. The vessels are 20-40µm wide. The pith is wide and consists of compact, thin walled parenchyma cells.

Root (Fig.8.1, 2)

The root is circular in sectional view, measuring 850µm thick. The epidermis is crushed and obliterated forming a thick dark line on the surface the root. **(Fig.8.1)**. One or two large thin walled cortical layers of cells which are intact. Secondary phloem occurs in small, isolated masses distributed along the peripheral part of the xylem.

Secondary xylem occupies the major portion of the root. It consists of dense distribution of vessels which are circular to elliptical, thick walled and mostly solitary. The ground tissue of the secondary xylem cylinder is the fibres which are narrow and dense. The vessels in the central part of the root are narrow and they become wider towards the pericarp. The vessels are 20-60µm in diameter.

SECTION C - QUANTITATIVE MICROSCOPY

The results obtained for various leaf constants like stomatal number, stomatal index, vein islet and vein termination number were presented in **table 1.**

Table 1 : Quantitative microscopical parameters of the leaf of *Andrographis echioides*

S. No.	Parameters*	Values obtained*
1	Stomatal number in upper epidermis	35.34 ± 1.47
2	Stomatal number in lower epidermis	53.42 ± 2.12
3	Stomatal index in upper epidermis	16.51 ± 0.11
4.	Stomatal index in lower epidermis	18.96 ± 0.60
5.	Vein islet number	8.90 ± 0.42
6.	Vein termination number	21.00 ± 1.08

*** Values are mean of 10 readings ± SEM**

From the **table 1,** it can be observed that the stomatal number was 35.34 ± 1.47 and 53.42 ± 2.12, while the stomatal index was 16.51 ± 0.11 and 18.96 ± 0.60 for upper and lower epidermis respectively. The vein islet number and vein termination number was found to be 8.90 ± 0.42 and 21.00 ± 1.08 respectively.

SECTION D - POWDER ANALYSIS AND MICROSCOPY

POWDER ANALYSIS

The observations of the powder in day light and in UV light with various chemicals and reagents were presented in **table 2.**

Table 2: Behavior of the *Andrographis echioides*(L) Nees.powder with various chemicals and reagents

Drug powder + reagent	Colour in day light (Visible)	Colour in UV light	
		254nm	365nm
Powder	Pale green	Green	Black
Powder + 1M sodium hydroxide	Pale green	Dark green	Brown
Powder + Iodine	Green color	Dark green	Dark green
Powder + 10% potassium hydroxide	Pale green	Green	Greenish black
Powder + 1M Hydrochloric acid	Brown	Dark brown	Black
Powder + Glacial acetic acid	Brownish green	Brown	Orange
Powder + 50% sulphuric acid	Brown	Dark brown	Dark brown
Powder + 50% nitric acid	Brownish green	Green	Bluish black
Powder + 50% hydrochloric acid	Pale yellow	Brown	Dark brown

Note :-Colour reactions are viewed under natural light by naked eye

From the **table 2,** it can be seen that when the powder was treated with alkalis like sodium hydroxide and potassium hydroxide was pale green in daylight and green at 254nm and greenish black at 365nm and when treated with hydrochloric acid and sulphuric acid, the

powder appeared brown to brownish yellow in day light and brown to dark brown under UV. The powder appeared orange under UV light at 365nm when treated with glacial acetic acid.

FLUORESCENCE ANALYSIS

The fluorescence analysisof the extracts in day light and in UV light were presented in **table 3.**

Table 3: Fluorescence analysis of extracts of *Andrographis echioides* (L)Nees

Extract	Consistency	Colour in day light	Colour in UV light	
			254nm	365nm
Ethanol	Semisolid	Green	Dark green	Orange
Pet ether	Semisolid	Pale yellow	Fluorescent green	Green
Chloroform	Semisolid	Brownish green	Greenish black	Orange
Ethyl acetate	Semisolid	Green	Brown	Orange
Water	Semisolid	Pale yellow	Dark green	Fluorescent green
Benzene	Semisolid	Yellow	Green	Orange
Hexane	Semisolid	Green	Green	Brown
Acetone	Semisolid	Green	Dark green	Orange

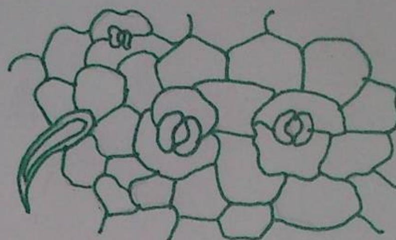
From the **table3,** it can be observed that the aqueous extract and petroleum extract were fluorescent green in UV light at 365nm and 254nm respectively. The chloroform, ethyl acetate, benzene and acetone extracts appeared orange in UV light at 365nm. These observations will be useful for determining the purity of the sample in future and helps in preparing standards for this plant.

POWDER MICROSCOPY

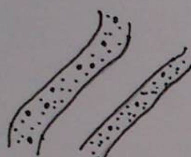
The various characters seen under the microscope are presented in **Fig. 9.** The characteristic features observed included stomata, trichomes (multicellular, uniseriate,

Fig.9: Powder Microscopy of Andrographis Echioides

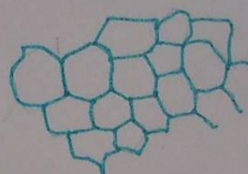
Covering Trichomes



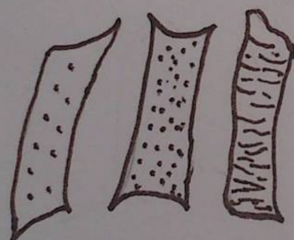
Diacytic Stomata



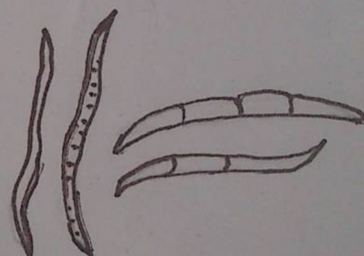
Cystoliths



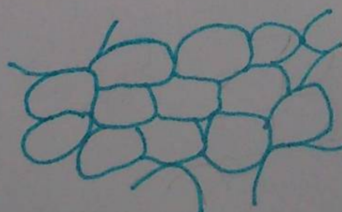
Epidermal cells



Xylem Vessels



Xylem fibres



Parenchyma cells

unbranched), cystoliths, xylem vessels, xylem fibres, parenchyma cells, epidermal cells and palisade cells

SECTION E - STANDARDIZATION PARAMETERS

The results obtained for the various standardization parameters like foreign organic matter, moisture content, ash values, extractive values, volatile oil and foaming index were presented in **table 4.**

Table 4: Standardization parameters of *Andrographisechioides*(L) Nees.

S. No	Parameters*	Values*expressed as %
1	**Foreign organic matter**	0.01 ± 0.02
2	**Moisture content**	5.73 ± 0.066
3	**Ash values**	
	Total ash	14.38 ± 0.003
	Acid insoluble ash	2.96 ± 0.006
	Water soluble ash	4.83 ± 0.010
	Sulphated ash	7.74 ± 0.010
4	**Extractive Values**	
	Petroleum ether	24.68 ± 0.001
	Chloroform	7.46 ± 0.008
	Ethyl acetate	5.88 ± 0.010
	Ethanol	5.76 ± 0.008
	Acetone	5.97 ± 0.010
	Water	22.07 ± 0.010
	Hexane	3.87 ± 0.008
	Benzene	6.24 ± 0.003
5	**Foaming index**	100
6	**Volatile oil**	Nil

From the results in **table 4,** it can be seen that the moisture content was found to be 5.73 ± 0.066 which is within the prescribed limits. If the moisture content was more, then it

would lead to microbial growth and hence loss of active principles thus leading to loss of pharmacological activity.

The total ash was found to be 14.38 ± 0.003 while the acid insoluble ash, water soluble ash and sulphated ash was found to be 2.96 ± 0.006, 4.83 ± 0.010 and 7.74 ± 0.010 respectively. The determination of ash values helps in the determination of quality and purity of the crude material in powder form at a future date.

The water soluble extractive value was found to be 22.07 ± 0.010 while the petroleum ether soluble extractive value was found to be 24.68 ± 0.001.

The standardization parameters determined will add weightage to the pharmacognostic parameters and will be useful for identification of adulterants, determination of quality of the plant material in crude form in future.

PHYTOCHEMICAL STUDIES

CHAPTER- 6

PHYTOCHEMICAL EVALUAITON

A phytochemical is a compound found in plants that works with nutrients and dietary fiber to protect against disease. They also slow the aging process. Phytochemical screening is a process of tracing plant bioactive principles of plant to discover their medicinal benefits.

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as **secondary metabolites,** often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. The **primary metabolites** such as phyto-sterols, acyl lipids, nucleotides, amino acids, and organic acids found in all plants and perform metabolic roles that are essential and are evident. Secondary metabolites are responsible for medicinal activity of plants and hence phytochemical screening and identification of bio active principle in medicinal plants were carried out in extract using standard procedure. [78-82]

MATERIALS AND METHODS

Preparation of Extract

About 500g of the dried whole plant powder was defatted with 1.5L petroleum ether (60-80^0 C) by maceration. The solvent was removed by filtration and the marc was dried. To the dried marc 1.5L of 70% ethanol was added and the extraction was performed by triple maceration (72h process). It was then filtered and the combined filtrate was evaporated to a cohesive mass using rotary evaporator.

SECTION A - PRELIMINARY PHYTOCHEMICAL SCREENING

Qualitative chemical tests are carried out for the purpose of specific identity of the substances in the crude extracts. The color reaction or precipitate usually observed can identify a class of compound. Chemical tests can be useful for the investigation of the chemical compounds and to observe the efficiency of an extraction process.

The ethanolic extract was subjected to preliminary phytochemical screening. The various chemical tests performed on the extracts were for steroids, triterpenoids, flavonoid, carbohyrates, glycosides, alkaloids, phenols, tannins and saponins and the results were recorded.

1**. Test for sterols**

The powder was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

a. Salkowski's Test: A few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turning red indicates the presence of sterols.

b. Liebermann – Burchard's Test: To the chloroform solution a few drops of acetic anhydride and 1mL of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring and the upper layer turning green indicates the presence of sterols.

2. **Test for carbohydrates**

a. Benedict's test: The aqueous extract of the powdered was treated with Benedict's reagent and boiled on water bath and cooled. Orange precipitate indicates the presence of carbohydrates.

b. Fehling's Test: The aqueous extract of the powdered leaf was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour. A red precipitate indicates the presence of free reducing sugars

3. Test for glycosides

The substance was mixed with a little anthrone on a watch glass. One drop of concentrated sulphuric acid was added, made into a paste and warmed gently over a water bath. No dark green coloration indicates the absence of glycosides.

4. Test for Cardiac Glycosides

a. Keller Kiliani Test: The substance was boiled with 10% alcohol for 2min, cooled and filtered. To the filtrate, lead sub acetate was added and filtered. The filtrate was then extracted with chloroform. The chloroform layer was separated and evaporated to dryness. The residue was dissolved in glacial acetic acid with traces of ferric chloride. To this few drops of sulphuric acid was added slowly along the sides of the test tube. No reddish brown layer changes to green colour on standing indicate the absence of cardiac glycosides.

b. Legal test: The substance was dissolved in pyridine; sodium nitroprusside solution was added to it and made alkaline. No pink or red colour indicates the absence of cardiac glycosides.

c. Baljet test: To the substance sodium picrate solution is added. No yellow to orange colour indicates the absence of cardiac glycosides.

4. Test for Proteins and free amino acids

a. Millon's Test: A small quantity of aciduous – alcoholic extract of the powdered drug was heated with Millon's reagent. White precipitate indicates the presence of proteins.

b. Biuret Test: To the alcoholic extract of powdered drug, one ml of dilute sodium hydroxide(10%) solution was added followed by this one drop of very dilute copper sulphate solution was added. No Violet colour indicates the absence of proteins.

5. Test for Mucilage

A few mL of aqueous extract was prepared from the powdered drug was treated with ruthenium red. No Pinkish red colour indicates the absence of mucilage.

6. Test for Flavonoids

a. Magnesium turning- con HCl test: A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added and Boiled for five minutes. A red colour indicates the presence of flavonoids.

b. Alkali Test: To the small quantity of test solution 10% aqueous sodium hydroxide Solution was added. A yellow orange color indicates the presence of flavonoids.

7. Test for terpenoids

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and residue obtained was dissolved in small amount of chloroform and the chloroform solution tin and thionyl chloride were added. A pink color indicates the presence of terpenoids.

8. Test for Tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract, few drops of ferric chloride solution were added. A bluish black color indicates the presence of tannins.

10. Test for Alkaloid

About 2g of the powdered material was mixed with 1g of calcium hydroxide and 5mL of water into a smooth paste and set aside for 5min. It was then evaporated to dryness in a porcelain dish on a water bath. To the residue, 20mL of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To the residue, 5mL of dilute hydrochloric acid was added. The solution was

divided into four parts and 2mL of each of the following reagents were added and the colour noted below indicates the presence of alkaloids.

a) Mayer's Reagent - Cream precipitate

b) Dragendorff's Reagent - Reddish brown precipitate

c) Hager's Reagent - Yellow precipitate

d) Wagner's Reagent - Reddish brown precipitate

Test for purine group (Murexide test)

The residue obtained after the evaporation of chloroform as described above was treated with 1mL of hydrochloric acid in a porcelain dish and 0.1gm of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapour of dilute ammonia solution. Purple colour indicates the presence of purine group of alkaloids.

Test for carbazole alkaloids

To the alcoholic extract of the leaf powder, conc. H_2SO_4 was added. No Violet color indicates absence of carbazole alkaloids.

10. Test for saponins

Foam test: About 0.5g of the powdered drug was boiled gently for 2min with 20mL of water and filtered while hot and allowed to cool. 5mL of the filtrate was then diluted with water and shaken vigorously. Frothing indicates the presence of saponins.

The above chemical tests were carried out using whole plant ethanolic extract of *Andrographis echioides* (L.) *Nees* and the results were tabulated in **Table 5.**

SECTION B - QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENT [83-89]

The quantitative estimation of therapeutically active phytoconstituents in medicinal plant is very essential for their identification and quantification. A particular group of compound like flavanoids, polyphenols, tannin and alkaloids are the widest spread secondary metabolites in plant kingdom. These compounds have received much attention as potential natural therapeutic properties. Therfore, it is worthwhile to determine the total amount of flavonoid ,tannin and vitamin c present in the crude extracts can be quantified by means of using standard procedure with reference marker compound and then reporting them as equivalent to that much amount of compound present in that extract as per standard compound.

1. Determination of total phenolic content [83]

Principle

The total phenol content of *Andrographis echioides (L) Nees*., was determined by the Folin-Ciocalteu colorimetric method**.** The Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. The method measures the amount of substance needed to inhibit the oxidation of the reagent.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% sodium carbonate

1N Folin-Ciocalteu reagent (diluted with equal volume of water and made 1N)

Procedure

The compound gallic acid was used as standard and was weighed and dissolved in distilled water to produce 1mg/mL stock solution. The stock solution was further diluted to

get concentrations ranging from 2-10µg/mL. To these solutions, 0.5mL of Folin-Ciocalteu reagent and 0.5mL of sodium carbonate was added and the final volume was made up to 10mL with distilled water. The absorbance was measured at 760nm after incubation at room temperature for 30min. The 70% ethanolic extract (0.5mL of 50µg/mL and 1mL of 100µg/mL) was mixed with 0.5mL of Folin-Ciocalteu reagent and 0.5mL of 10% sodium carbonate and final volume was made up to 10mL with distilled water, and the absorbance was measured at 760nm after incubation at room temperature for 30min. A calibration curve was constructed by plotting concentration versus absorbance of gallic acid (**Fig 10.).** A linear regression equation was formed and the amount of phenolic compounds was determined using this equation. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of extract. The results are tabulated in **Table 6.**

Determination of total flavanoid content [84-86]

Principle

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation.[85. Aluminum ions form stable complexes with C4 keto group and either to C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavanoids. These complexes showed a strong absorption at 415nm which is used for the estimation of flavanoids.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

10% aluminium chloride

1M potassium acetate

Procedure

A known quantity of quercetin was dissolved in ethanol to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 20-100μg/mL. 1ml of the above standard solutions were taken in different volumetric flasks, 0.1mL of aluminium chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminium chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of quercetin (**Fig.11)**. 1mL of ethanolic extract at concentrations 50μg/mL and 100μg/mL were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table 7.** The amount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg querecetin equivalents /g of extract.

3. Determination of total tannin content [88, 89]

Principle

Total tannin content of ethanolic extracts *Andrographis echioides (L) Nees* was determined by Folin Denis reagent method. Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly blue coloured solution. The intensity is measured in a spectrometerically at 700nm

Reagents

a) Folin Denis Reagent (sodium tungstate 100g and phosphomolybdic acid 20gm were dissolved in distilled water 750ml along with phosphoric acid 50ml. The mixture was refluxed for 2 hr and volume was made up to 1 litre with distilled water)
b) Sodium carbonates solution (10%)

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure

An aliquot of 0.2ml and0.4ml of (1mg/ml) ethanolic extracts of *Andrographis echioides (L)Nees* was taken in separate test tubes and mixed with distilled water to made up to the volume of 1ml. Then add 0.5ml of Folin Denis reagent and allowed to stand for 15 mins, then 1ml of 10% sodium carbonate solution was added and the mixture was mixed with distilled water and made up to 10ml, allowed to stand for 30min at room temperature and the tannin content was determined spectrophotometrically at 700nm and the readings were tabulated in **Table 8.**

The calibration curve was generated by preparing tannic acid at different concentration (2 -10µg/mL) (**Fig 12)**. The reaction mixture without sample was used as blank. The total tannin content in the ethanolic extract of was expressed as milligrams of tannic acid equivalent per gm of extract.

Estimation of vitamin C [87]

Principle

The estimation of Vitamin C was carried out using the method of *Subasini et al.,* with slight modifications. The keto group of ascorbic acid undergoes a condensation reaction with 2, 4 dinitro phenyl hydrazine to form a hydrazone which is orange yellow and has an absorbance of about 520nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

0.2% dinitro phenyl hydrazine

85% sulphuric acid

Procedure

Ascorbic acid was weighed and dissolved in water to get stock solution of 1mg/mL. Further dilutions were made to get the concentrations ranging from 40-200µg/mL. To 1mL of sample 0.5mL of dinitro phenyl hydrazine solution was added and incubated for 3h at 37ºC. After 3h, 2.5mL of 85% sulphuric acid was added and the absorbance was measured after 30min at 520nm. A calibration curve was constructed by plotting concentration versus absorbance of ascorbic acid **(Fig 13).** The procedure was repeated for the plant extract (200 µg/mL.) as above and the absorbance was measured at 520nm after 3h and the readings were tabulated in **Table 9.** The amount of vitamin C present can be determined by linear regression analysis. The vitamin C content was expressed as mg/g of extract.

SECTION C - DETERMINATION OF METALS AND MINERALS BY X-RAY FLUORIMETRY [90]

The herbal products or extracts can have high level of heavy metals and elements.Which shows the need to study their level in important medicinal plants. In ancient days, Indian medicinal system recommended most of the herbal drugs in the form of decoction (hot water extract), where the possibility of heavy metal toxicity is minimized or detoxified by other medicinal plants. It has been reported that whatever is taken as food could cause metabolic disturbance subject to the allowed upper& lower limits of trace metals. The deficiency & excess of essential micronutrients & trace toxic metals can cause serious effects on health. Some metals are also essential nutrients (Cu, Fe, Zn etc.) & only become toxic at higher concentrations, while others (Pb, Cd, Ni etc.) don't have beneficial properties.

X-ray fluorescence (XRF) analysis is one of the most common non-destructive methods for qualitative as well as quantitative determination of elemental composition of materials. It is suitable for solids, liquids as well as powders. There are two main methodological techniques that are wavelength dispersive analysis (WD-XRF) and energy dispersive analysis (EDXRF) where the spectra are collected simultaneously in a wide energy

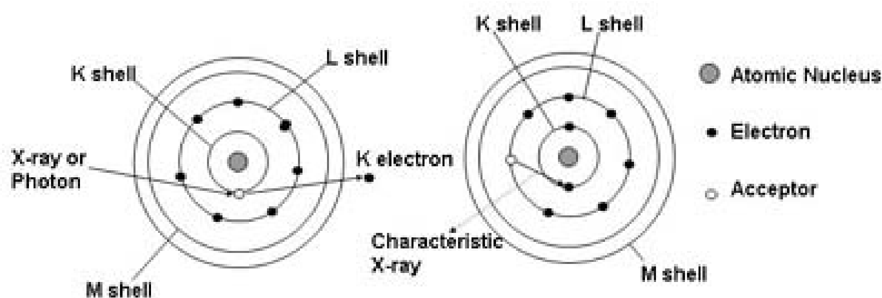
range. XRF has the advantage of being non-destructive, multi-elemental, fast and cost-effective. Furthermore, it offers a fairly uniform detection limit across a large portion of the Periodic Table and is applicable to a wide range of concentrations from 100% to a few ppm.

Principle

X-ray is a type of electromagnetic waves such as visible light ray, but the difference is its extremely short wavelength, measuring from 100A to 0.1A. X-ray fluorescence analysis is a method that uses the characteristic X-ray (fluorescent X-ray) that is generated when X-ray is irradiated on a substance. The fluorescent X-ray is the excess energy irradiated as electromagnetic field, which is generated when the irradiated X-ray forces the constituent atom's inner-shell electrons to the outer shell and the vacant space (acceptor) falls on the outer-shell electrons. The generation of fluorescent X-ray. These rays possess energy characteristic to each element, and qualitative analysis and quantitative analysis using the energy's X-ray intensity (number of photons) are possible. X-ray fluorescence analysis can be considered as spectrochemical analysis of an X-ray region. It has the same characteristics as atomic absorption

For example, in flameless atomic absorption spectrometry (FLAAS), elements in the sample are atomized in 2000 to 3000C flame and in ICP atomic emission spectrometry (ICP-AES), sample is excited in 6000 to 9000C plasma flame. X-ray fluorescence likewise excites the sample using X-ray to obtain information.

Fig. 14 X-ray generation



Instrument

Instrument : Bruker S4-Pioneer

Standard Anode material : Rhodium

Detector : Scintillation counter and proportional counter

Software : SPECTRA plus software

Version : 1.6

Sample preparation (Pressed pellet technique)

The sample used for XRF analysis was prepared as a flat disc (pellet), with a diameter of 20-50nm. The pellet was made with the sample powder in the ratio of 1:4, i.e., 2mg of whole plant of *Andrographis echioides* powder was mixed with 8mg of diluents like boric acid or stearates (calcium.magnesium). The pellet looked like a bilayer tablet which consists of drug one side and diluents on the other side.

Procedure

The sample pellet was then subjected to X-ray fluorimetric analysis for the period of 1 hr approximately. The elements which were present in the sample were analysed and the results were interpreted automatically by the software package of the computer in terms of parts per million. The results obtained are presented in **Table 10.**

SECTION D - CHROMATOGRAPHY

Herbal drugs are accepted as important therapeutic agents for the treatment of many diseases. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Pharmacognostical analysis of medicinal herbs remains challenging issues for analytical chemists, as herbs are a complicated system of mixtures. Analytical separation techniques for example

chromatography , and mass spectrometry (MS), etc. among the most popular methods of choice used for quality control of raw material and finished herbal product.[91,92]

Chromatography represents the most versatile separation technique and readily available. Plant materials are separated and purified by using various chromatographic techniques. Herbal medicine is a complicated system of mixtures. Thus, the methods of choice for identification of “botanical drug” are mainly intended to obtain a characteristic fingerprint of a specific plant that represent the presence of a particular quality defining chemical constituents. For such purposes, chromatographic techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography – mass spectrometry (GC-MS) and thin layer chromatography (TLC) were used widely as reported in numerous publications.

Chromatography is the separation of two or more compounds or ions from mixture of compounds by the equilibrium distribution between two phases, one which is moving and the other which is stationary. These two phases can be solid-liquid, liquid-liquid or gas-liquid.

THIN LAYER CHROMATOGRAPHY

Principle

The principle of separation is adsorption. The components move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travel slower and the components with less affinity towards the stationary phase travel faster. Thus the components are separated on a thin layer chromatographic plate based on the affinity of the components towards the stationary phase.

Preparation of TLC Plates

Slurry of the adsorbent (silica gel G) was prepared in water (1: 2). Dry, clean glass plates (20cm x 5cm) were laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25mm thickness and coated in a single passage of the

spreader over them. These plates were left on the template for air drying until the transparency of the layer disappeared and dried at 110°C for 30min and kept in a dessicator. The plates were used when required. Aluminum plates coated with silica gel G F_{254} (Merck) were also used.

Application of Extract

Adrographis echioides extract was mixed with methanol and taken in a capillary tube and it was banded on TLC plate 2cm above the bottom end.

Development of chromatogram

TLC plates were developed in a chromatographic tank by using the mobile phase Toluene: Ethyl acetate: Methanol (5:3:2). The plates were allowed to develop 3/4 of the length and then removed. The solvent front was immediately marked and the plates were allowed to dry and then it was examined by visually, under UV light at 254 and 366nm. The results are presented in **table 11.**

Stationary phase - Silica Gel G

Mobile phase - Toluene: Ethyl acetate: Methanol (5: 3: 2)

Detecting agent - Visual & UV light

The TLC plates were examined and the spots were viewed under UV light and in day light. The R_f value of the spots obtained were calculated using the formula - [Distance travelled by solute / Distance travelled by solvent]. The photographic representation of the plate under UV light and in day light is presented in **Fig 15** and the R_f value of the spots are presented in the **table 11.**

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY [93-94]

HPTLC method is advanced method of Thin Layer chromatography (TLC). It has more advantages like better resolution, faster development of spots and also easy detection and quantification of separated compounds over TLC. HPTLC is useful method for

identification of plants and extracts. It is used as a powerful quality control tool when the similarities and differences between the test samples and the standard markers are compared. HPTLC is the most reliable method for quantification of phytoconstituents even present in nanogram level. This finger print analysis can be used to check and monitor the purity of drugs rapidely. It can also be used for the detection of adulterants.

Isolation of Andrographolide [26]

200g of whole dried crushed *Andrographis echioides* was extracted, twice with a 1:1 mixture of dichloromethane and methanol by cold maceration (1 × 1L and 1 × 0.2L). Each extraction was conducted for 6h with constant stirring. The extracts were filtered and the filtrates were concentrated at a temperature below 70°C to produce a green mass. The green mass was washed with toluene several times until most of coloured matter was removed from the residue. The toluene was completely removed from the residue by evaporation. The residue was dissolved in 60-70°C hot ethanol and filtered while hot. The filtrate was cooled in a refrigerator for crystallization.

Instrument

CAMAG TLC Scanner 3 "Scanner3-070408"S/N 070408(1.41.21) was used for detection and CAMAG Linomat 5 sample applicator was used for the application of the track. Twin trough plate development chamber was used for development of chromatogram. Software used was winCATS 1.4.3

Sample

The *Andrographis echioides* was dissolved in 1ml of methanol and 2μL of this solution was used for taking HPTLC fingerprint.

Stationary Phase

Aluminium sheets pre-coated with silica gel Merck G F_{254}, 0.2mm layer thickness were used as the stationary phase.

Mobile phase

Toluene: ethyl acetate: methanol (5: 3: 2) was used as the mobile phase for development of chromatogram. The mobile phase was taken in a CAMAG twin trough glass chamber.

Detection wavelength

The developed plates were examined at wavelength 254nm and 366nm in Densitometry TLC scanner3. The TLC visualization, 3D display of the finger print profile and peak display at 254nm and 366nm are presented in **Fig 16 to 19.** The R_f values and area under curve for each peak of are presented in **Table 12.**

RESULTS AND DISCUSSION

SECTION A - PRELIMINARY PHYTOCHEMICAL SCREENING

The results obtained for the preliminary phytochemical screening is depicted in

Table 5.

Table 5: Preliminary phytochemical screening for the 70% ethanolic extract of *Andrographis echioides* (L.)Nees

S. No.	TEST	RESULTS
1.	**TEST FOR STEROLS**	
	a. Salkowski's test	+
	b. Libermann- burchard's test	+
2.	**TEST FOR CARBOHYDRATES**	
	a. Molisch's test	+
	b. Fehling's test	+
	c. Benedict's test	+
3.	**TEST FOR PROTEINS**	
	a. Millon's test	_
	b. Biuret test	_
4.	**TEST FOR ALKALOIDS**	
	a. Mayer's reagent	+
	b. Dragendroff's reagent	+
	c. Hager's reagent	+
	d. Wagner's reagent	+
5.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	_
	i) Borntrager's test	_
	ii) Modified Borntrager's test	_
	b. Cardiac glycosides	
	i) Keller Killiani test	-
6.	**TEST FOR SAPONINS**	
	Foam test	+
7.	**TEST FOR TANNINS**	
	$Fecl_3$ test	+
8.	**TEST FOR FLAVONOIDS**	
	a. Shinoda test	+
	b. Alkali test	+
	c. Acid test	+
9.	**TEST FOR TRITERPENOIDS**	+
10.	**TEST FOR VOLATILE OILS**	-

(+) indicates positive reaction (-) indicates negative reaction

The 70% ethanolic extract of whole plant of *Andrographis echioides* showed the presence of sterols, alkaloids, carbhydrates, Triterpenoids, flavonoids, phenols, tannins and saponins.

SECTION B - QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

The results obtained for estimation of various phyto constituents are discussed in this section.

Estimation of total phenol content

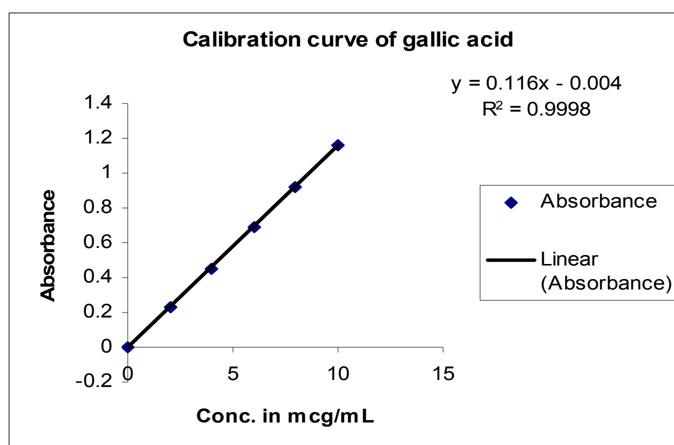
The results for the total phenolic content of 70% ethanolic extract of *Andrographis echioides (L.)Nees* were tabulated in **table 6** and the calibration curve for standard gallic acid is represented in **Fig 10.**

Table 6: Total phenolic content in 70% ethanolic extract of in terms of gallic acid equivalents

S. No.	Conc. of gallic acid in µg/mL	Absorbance at 760nm	Conc. of ethanol extract in µg/mL	Absorbance at 760nm*	Amt. of phenolic content in terms mg GAE/g of ext.*
1	2	0.229 ± 0.010	50	0.22 ± 0.002	39.84 ± 0.35
2	4	0.452 ± 0.006	100	0.48 ± 0.001	43.10 ± 0.95
3	6	0.695 ± 0.005		**Average**	**41.47±0.65**
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

*** mean of three readings ±SEM**

Fig 10. Calibration curve of gallic acid



The linear regression equation was found to be y = 0.116x - 0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the extract in terms mg GAE/g of extract was found to be **41.47±0.65** by using the above linear regression equation.

Estimation of total flavonoid content

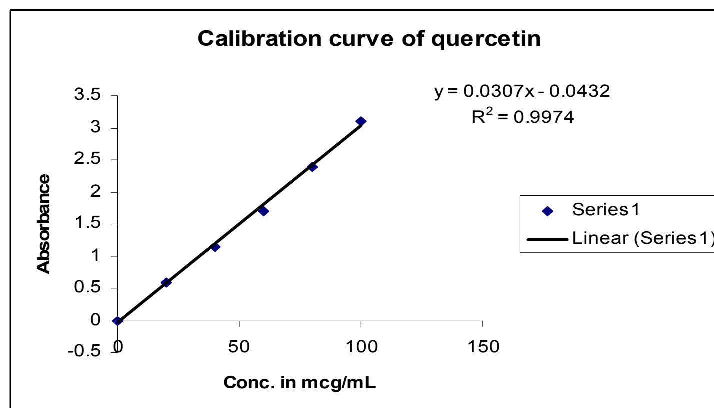
The results for total flavonoid content of 70% ethanolic extract of *Andrographis echioides (L) Nees* were presented in **table 7** and the calibration curve for quercetin is presented in **Fig 11.**

Table 7: Total flavonoid content per gram of extract in terms of quercetin by aluminium chloride method

S. No.	Conc. of quercetin in µg/mL	Absorbance at 415nm	Conc. of ethanol extract in µg/Ml	Absorbance at 415nm	Amt of flavonoid content in terms mg quercetin equivalent/ g of ext.
1	20	0.589 ± 0.01	50	0.024±0.0006	41.87±0.23
2	40	1.151 ± 0.04	100	0.116±0.003	53.16±0.01
3	60	1.710 ± 0.09		**Average**	47.515±0.12
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

***mean of three readings** ± SEM

Fig 11. Calibration curve of quercetin



The linear regression equation was found to be y = 0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the ethanolic

extract of *Andrographis echioides(L)Nees* in terms mg quercetin equivalent/g of extract was found to be 47.515±0.12mg/g of extract by using the above linear regression equation.

Estimation of total tannin content

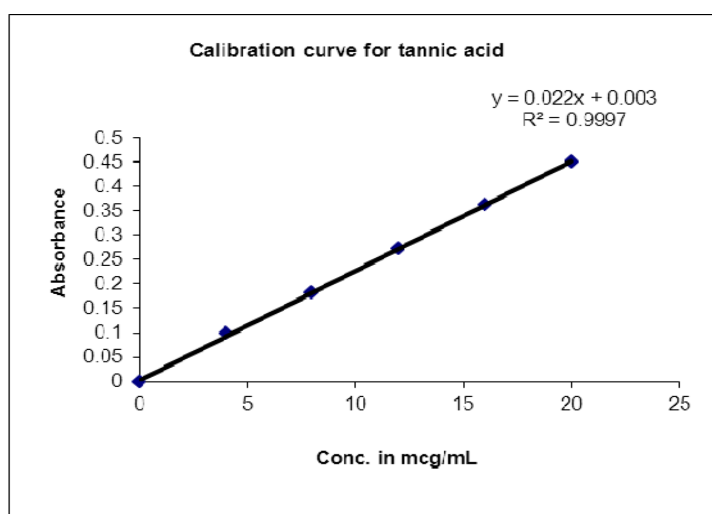
The results for total tannin content of 70% ethanolic extract of *Andrographis echioides* (L.)Nees are presented in **Table 8** and the calibration curve for tannic acid is presented in **Fig 12.**

Table 8: Total tannin content in 70% ethanolic extract of *Andrographis echioides* (L) Nees of tannic acid equivalents

<table>
<tr><th>S. No.</th><th>Conc. of tannic acid in µg/mL</th><th>Absorbance at 415nm</th><th>Conc. of ethanolic extract in µg/mL</th><th>Absorbance at 415nm</th><th>Amt of total tannic acid content in terms mg quercetin equivalent/ g of extract</th></tr>
<tr><td>1</td><td>2</td><td>0.581 ± 0.01</td><td>20</td><td>0.024 ± 0.001</td><td>42.5 ± 0.12</td></tr>
<tr><td>2</td><td>4</td><td>1.151 ± 0.04</td><td>40</td><td>0.065 ± 0.001</td><td>63.9 ± 0.09</td></tr>
<tr><td>3</td><td>6</td><td>1.710 ± 0.09</td><td></td><td rowspan="3">Average</td><td rowspan="3">53.2 ± 0.105</td></tr>
<tr><td>4</td><td>8</td><td>2.390 ± 0.03</td><td></td></tr>
<tr><td>5</td><td>10</td><td>3.112 ± 0.03</td><td></td></tr>
</table>

***mean of three readings** ± SEM

Fig 12: **Calibration curve for tannic acid**



The linear regression equation was found to be y = 0.022x + 0.003.while the correlation was found to be 0.9997. The amount of tannin content present in the extract in terms mg equivalent /g was to be 53.2±0.105 mg/gm of extract by using above linear equation.

Estimation of Vitamic C content

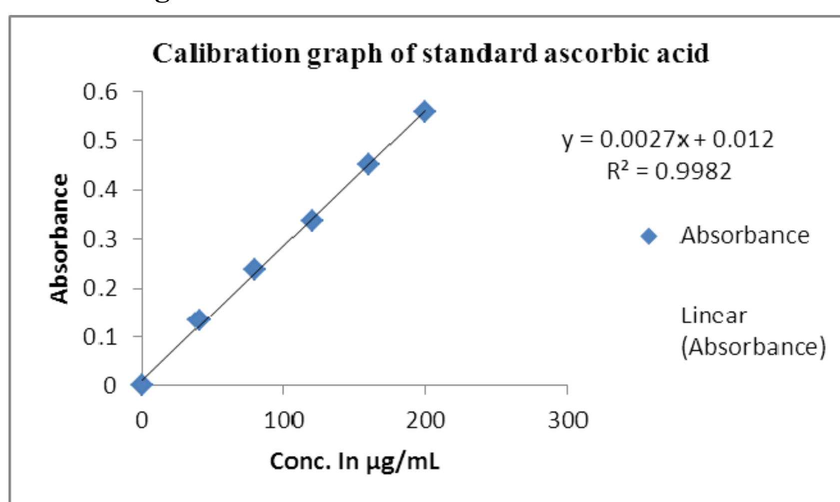
The results for vitamin C content of 70% ethanolic extract of *Andrographis echioides* are presented in **Table 9** and the calibration curve for ascorbic acid is presented in **Fig 13.**

Table 9: Estimation of Vitamin C in *Andrographis echioides* (L) Nees

S. No.	Conc. of ascorbic acid in µg/mL	Absorbance at 520nm	Conc. of methanol extract in µg/mL	Absorbance at 520nm	Amt of vitamin C present / g of extract
1	40	0.135 ± 0.000	200	0.087±0.0006	222.76±0.370
2	80	0.265 ± 0.015			
3	120	0.346 ± 0.010			
4	160	0.468 ± 0.011			
5	200	0.525 ± 0.010			

***mean of three readings** ± SEM

Fig. 13: Calibration curve of standard ascorbic acid



The linear regression equation was found to be y = 0.0027x + 0.012 and a correlation co-efficient of 0.9982. The amount of vitamin C content present in the 70% ethanolic extract of *Adrographis echioides* was found to be 222.76 ± 0.37 mg/g of fresh leaves by using the above linear regression equation.

SECTION C - DETERMINATION OF MTALS AND MINERALS BY X-RAY FLUORIMETRY

The various elements present in elemental form and in oxide form are presented in **table 10.**

Table 10: Elemental analysis of whole plant powder of *Andrographis echioides* (L) Nees

Oxide form	AN-EC	Elemental form	AN-EC
Formula	**Conc %**	**Formula**	**Conc %**
Al_2O_3	0.178	Al	0.094
Br	0.037	Br	0.037
CaO	16.690	Ca	11.930
Cl	3.389	Cl	3.389
CuO	0.015	Cu	0.012
Fe_2O_3	0.337	Fe	0.236
K_2O	9.650	K	8.011
MgO	1.716	Mg	1.035
Na_2O	0.169	Na	0.126
		O	8.870
P_2O_5	0.888	P	0.388
SO_3	2.052	S	0.822
SiO_2	1.050	Si	0.493
SrO	0.182	Sr	0.154
ZnO	0.022	Zn	0.018

SECTION D - CHROMATOGRAPHY

Thin layer chromatography *Andrographis echioides* (L.) Nees extract

The photographic visualization of the TLC plate is represented in **Fig 15.**

Fig 15: Thin layer chromatography of *Andrographis echioides* (L) Nees extract

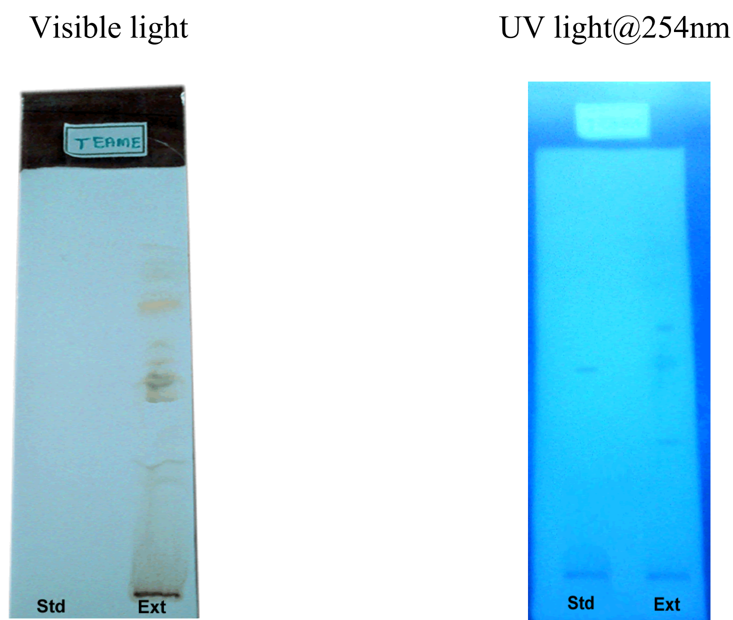


Table 11: TLC of *Andrographis echioides* (L) Nees extract

S. No	Solvent system	Detecting agent	No of spots	Color of spots	R_f values of extract	R_f values of std
1.	Toluene: ethyl acetate : methanol (5: 3: 2)	Visible light	**8**	Light brown	**0.29**	
				Brown	**0.52**	
				Dark brown	**0.57**	
				Yellow	**0.61**	
				Light yellow	**0.68**	
				Orange	**0.80**	
				Light brown	**0.84**	
				Brown	**0.89**	
		Under UV light at 254nm	**3**	Light green	**0.38**	**0.57**
				Green	**0.57**	
				Light green	**0.65**	

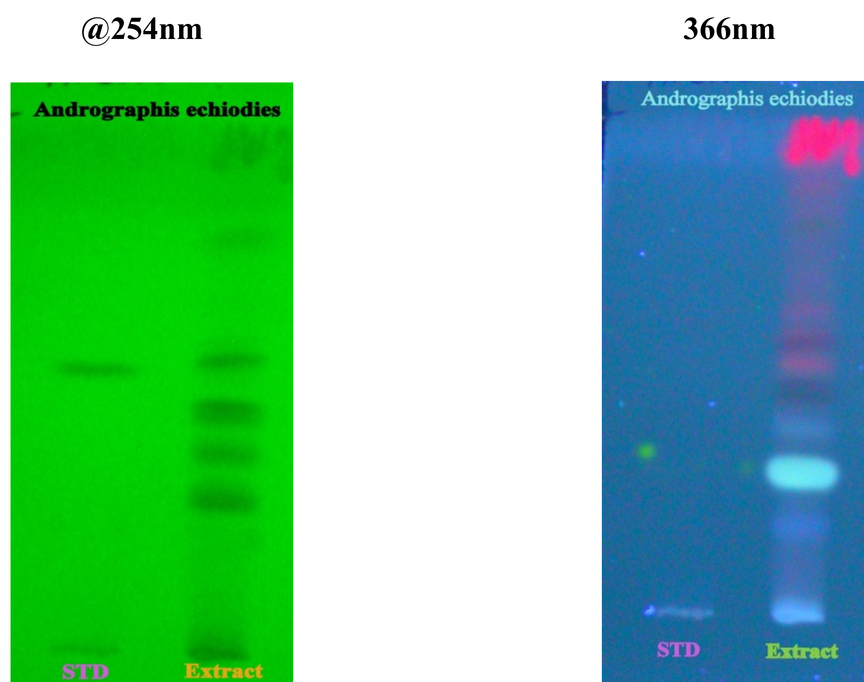
The extract when viewed in day light showed 8 spots at R_f value of 0.29, 0.52, 0.57, 0.61, 0.68, 0.80, 0.84 and 0.89 and when viewed under visible light, 3 spots at R_f value of 0.38, 0.57 and 0.65 after development in the mobile phase namely Toluene: Ethyl acetate: Methanol(5:3:2) were seen.

The Rf value obtained for the extract was also compared with a standard Andrographolide. The Rf value of the extract at 0.57 matched with that of the standard and hence andrographolide may be present in the extract. Further confirmation must be done using HPTLC.

High Performance Thin Layer Chromatography

The visualization of the TLC plate of *Andrographis echioides* extract at 254nm and 366nm is presented in **Fig 16.** The photo of plate at 254nm shows the presence of 8 spots in extract and 1 spot in standard, while at 366nm shows the presence 6 spots.

Fig 16: Visualization at 254nm and 366nm



The 3D display of the fingerprint profile and the peak display of Andrographis echioides (L) Nees and standard andrographolide at 254nm and 366nm is presented in **Figs 17, 18 & 19.**

Fig 17: 3D Display of the fingerprint profile at 254nm and 366nm

@ 254nm **@ 366nm**

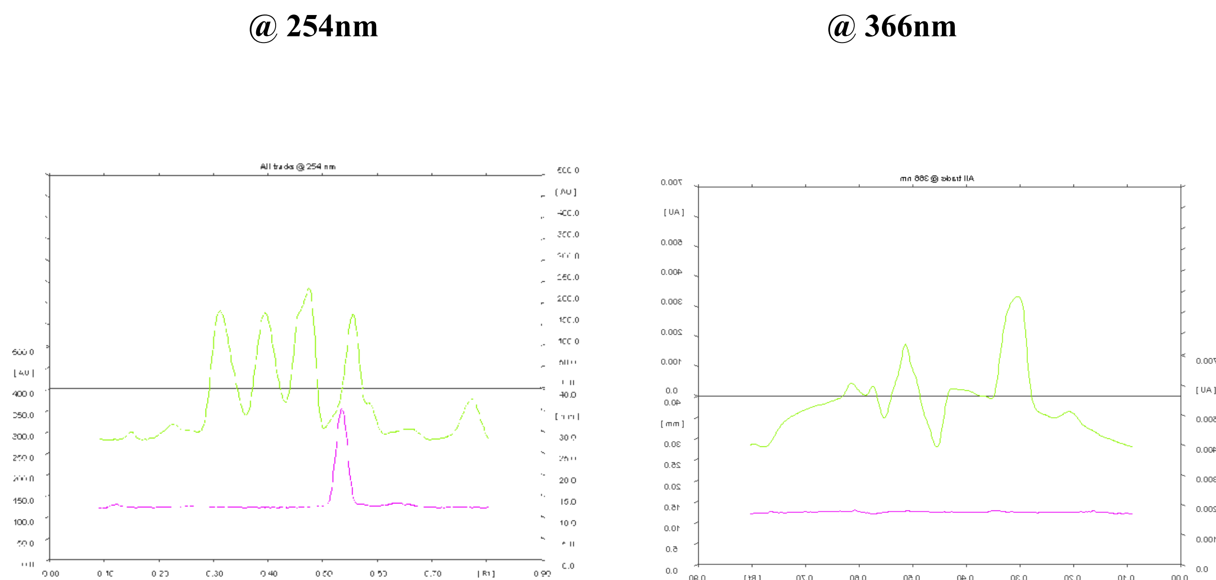
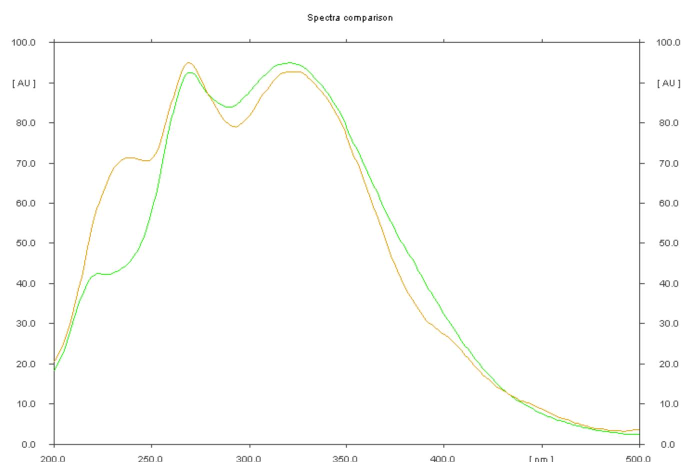


Fig 18. Overlay Spectral Display

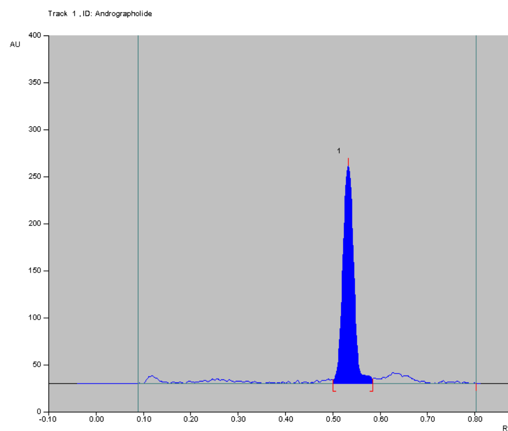


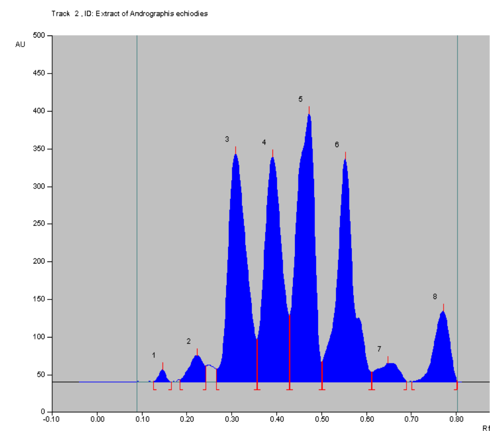
The display at 254nm shows the presence of 8 peaks and standard shows the presence of 1 peak while at 366nm shows the presence of 6 peaks for the sample and no peaks for standard. The Rf values of peaks along with the area under the curve for each peak at 254 and 366 nm are tabulated in **Table 12.**

Fig 19: Peak display of standard Andrographolide and *Andrographis echioides* extract at 254nm and 366nm

Peak Display @ 254nm

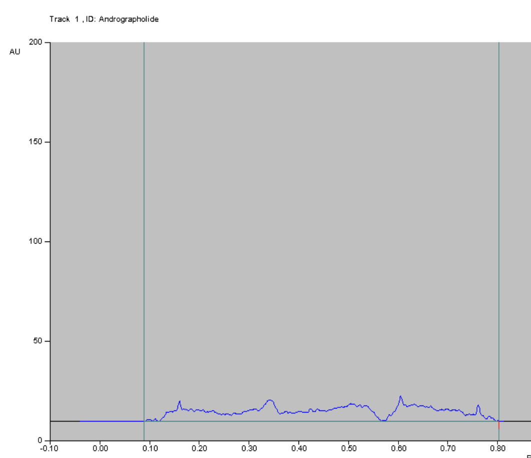
STD **Extract**





Peak Display @ 366nm

STD **Extract**



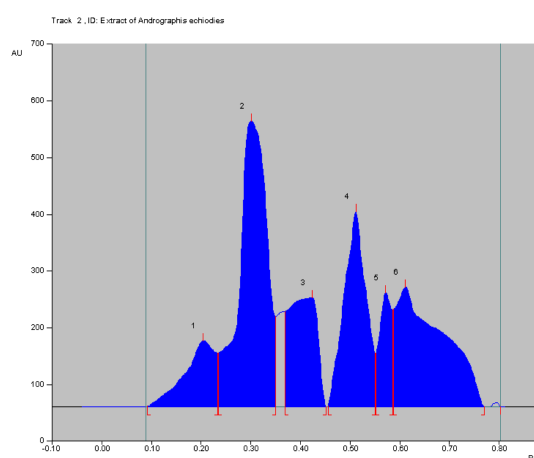


Table 12: R_f **values and area under the curve for each peak at 254 and 366nm**

S.No	@ 254nm				@ 366nm			
	STD		Sample		STD		Sample	
	Rf value	Area (AU)	Rf value	Area (AU)	Rf value	Area (AU)	Rf value	Area (AU)
1	**0.54**	**5050.2**	0.15	236.8			0.21	6806.9
2			0.23	1052.3			0.30	26810.8
3			0.31	11527.8			0.43	10707.2
4			0.39	10950.3			0.51	14427.3
5			0.48	12872.3			0.57	4704.4
6			**0.56**	**10315.1**			0.61	19172.9
7			0.65	1099.7				
8			0.77	3001.7				

From the table 12, it can be seen that the Rf value of spot number 5 of the sample coincided with that of the standard andrographolide and hence may be concluded that andrographolide is present in the sample. Isolation and characterization has to be carried out in future.

PHARMACOLOGICAL STUDIES

CHAPTER-7

PHARMACOLOGICAL STUDIES

The ethanolic extract of *Andrographis echioides* was subjected to pharmacological screening and the following activities were carried out namely *in vitro* antioxidant activity, *in vitro* anti lithiatic, *in vitro* anti diabetic, antibacterial and antifungal activities.

SECTION A - *IN VITRO* ANTIOXIDANT ACTIVITY

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfersan electron from a substance to an oxidizing agent. Oxidation reaction can produce free radicals which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reaction by being oxidized themselves.The cellular anti-oxidant status determines the susceptibility to oxidative damage and usually altered inresponse to oxidative stress. [96]

The role of antioxidants are protecting against certain conditions such as heart disease, stroke and cancer, anemia, Parkinson's diseases, mongolism, neuro degeneration. It has been proposed that the mechanisms leading to these diseases may be promoted by free radicals and that antioxidants may oppose the action of these molecules. In addition to the well known antioxidants such as Vitamins C and E, there is growing research demonstrating the potentially beneficial effects of plant-derived antioxidants,polyphenols, found in fruits, vegetables, nuts, cereals and drinks such as tea and red wine.

Free radicals

Free radicals are unstable molecules that include the hydrogen atom, nitric oxide(NO) and molecular oxygen (O_2). These naturally occur in the body as a result ofchemical reactions during normal cellular processes. They can also be formed inresponse to excess pollution, too much UV sunlight and exposure to cigarettesmoke. In an attempt to stabilize, they attack other

molecules in the body potentially leading to cell damage and triggering the formation of another free radical resulting in a chain reaction. This type of free radical action has been implicated in certain chronic and aging diseases such as cancer, heart disease, stroke, rheumatoid arthritis, cataracts and Alzheimer's disease.

Protective mechanisms of antioxidants

Antioxidants are compounds that help to inhibit the many oxidation reactions caused by free radicals thereby preventing or delaying damage to the cells and tissues. Their mechanisms of action include:-

- Scavenging reactive oxygen and nitrogen free radical species.
- Decreasing the localised oxygen concentration thereby reducing molecular oxygen's oxidation potential.
- Metabolising lipid peroxides to non-radical products.
- Chelating metal ions to prevent the generation of free radicals.

Types of antioxidants

Synthetic antioxidants:

Butylated hydroxyl toluene (BHT), butylated hydroxyl anisole(BHA), tertiary butylated hydroxyl quinine and gallic acid ester.

Plant derived antioxidants:

Tannins, lignans, stilbens, coumarins, quinones, xanthones, phenolic acids, flavones, flavonols, catechin, anthocyanins and proanthocyanins prevent the onset of degenerative diseases because of their redox properties which allow them to act as hydrogen donors, reducing agents, hydroxyl radical scavengers and nitric oxide scavengers.

Methods for screening antioxidant activity

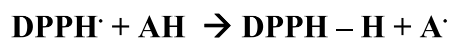
Some of the *in vitro* models for the evaluation of antioxidant activity are enumerated below

- DPPH method
- Hydrogen peroxide method
- Superoxide radical scavenging activity
- Hydroxyl radical scavenging activity
- Nitric oxide radical inhibition assay
- Reducing power assay
- Phosphomolybdenum method
- Peroxy nitrile radical scavenging activity
- Xanthine oxidase method
- Ferric reducing ability of Plasma
- Thiobarbituric acid assay etc.

Method 1: Free radical Scavenging activity using diphenylpicrylhydrazyl (DPPH) free radical[95-97]

Principle

The free radical scavenging activity of the extracts is evaluated by assessing their ability to reduce the colour of DPPH in ethanol according to Jananie *et al.,* 2011. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of specific compound or plant extracts. DPPH is a stable free radical with a distinctive ESR signal. Its reaction with antioxidants can be followed by the loss of absorbance at 518nm. It is widely accepted that DPPH accept an electron or hydrogen radical and become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of DPPH (purple colour solution) shows a strong absorption at 517nm. DPPH radicals react with suitable reducing agents where the pairing of electrons takes place and the solution loses colour stochiometrically with the number of electrons taken up.



$$\mathbf{DPPH^{\cdot} + AH \rightarrow DPPH - H + A^{\cdot}}$$

Instrument

Shimadzu UV Visible spectrometer, Model 1800

Reagents

0.1mMdiphenylpicrylhydrazylradical in ethanol

Procedure

A stock solution of 1mg/mL concentration of 70% ethanolic extract of *Andrographis echioides* was prepared. To 1mL of various concentrations of test sample, 4mL of DPPH was added. Control was prepared without sample in an identical manner. DPPH was replaced by ethanol in case of control. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance was measured at 517nm. Vitamin C was used as standard. The percentage scavenging was calculated using the formula [(Control-Test)/Control] x 100. A graph was constructed by plotting concentration versus percentage inhibition **(Fig. 24)** and a linear regression equation calculated. The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. A triplicate reading was taken and average was calculated. The results obtained are presented in **table 13.**

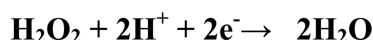
Method 2: Determination of scavenging activity against hydrogen peroxide [98, 99]

Principle

The free radical scavenging activity of plant extract against hydrogen peroxide was determined by using the method of Janani *et al,* 2011. The principle was based on the capacity of the extracts to decompose the hydrogen peroxide to water.H_2O_2 in the presence of O_2-can generate highly reactive $^{\cdot}OH$ hydroxyl radicals via the metal, the scavenging of H_2O_2 in cells is critical to avoid oxidative damage. Thus the scavenging of hydrogen peroxide is an important antioxidant defence mechanism.

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

The decomposition of hydrogen peroxide to water involves the transfer of electrons



$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$$

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

6% hydrogen peroxide diluted with water in the ratio of 1:10

0.1M phosphate buffer pH 7.4

Procedure

To the 70% ethanolic extract of *Andrographis echioides* dissolved in ethanol to get a stock solution containing 1mg/mL. Varying quantities of the stock solution were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then 0.2mL of hydrogen peroxide solution was added and the absorbance was measured at 230nm after 10min. The reaction mixture without sample and reagent was used as blank. The hydrogen peroxide solution was used a control. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula = $(A_{control} - A_{sample}) / A_{control} \times 100$. A graph was plotted using concentration versus percentage inhibition **(Fig. 25)**. A linear regression equation was calculated using the above graph. The concentration of extract to produce 50% inhibition was found using linear regression analysis. The results obtained are presented in **table 14.**

Method 3: Ferric Reducing Antioxidant Power (FRAP) Assay][100]

Total antioxidant activity is measured by FRAP assay of Benzie *et al.,* 1999. The ferric reducing antioxidant power assay measures the potential of antioxidants to reduce the Fe^{3+} and 2,4,6 tripyridyl-s-triazyne (TPTZ) complex present in stoichiometric excess to the blue coloured Fe^{2+} complex which increases the absorption at 593nm.

Principle

At low pH, reduction of ferric tripyridyltriazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

FRAP Reagent

a) Acetate buffer 30mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add 16 ml of glacial acetic acid and make the volume to 1 L with distilled water.

b) TPTZ (2, 4, 6-tripyridyl-s- triazine) (M.W. 312.34) 10mM in 40mM HCl

c) $FeCl_3$. $6H_2O$ (M.W. 270.30) 20mM

The working FRAP reagent was prepared freshly by mixing a b & c in the ratio of 10:1:1 at the time of use.

Procedure

A stock solution of 1mg/mL concentration of ethanolic extract of *Andrographis echioides* was prepared. To the 1mL of various concentrations of test samples and mixed with 3mL of working FRAP reagent and absorbance was measured at 0min after vortexing at 593nm. Thereafter samples were placed at 37ºC in water bath and absorption was again measured after 4min. Ascorbic acid was used as standard. The FRAP value of the sample was calculated using this equation:[Change in absorbance of sample from 0-4min/ change in

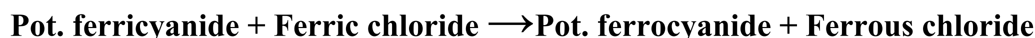
absorbance of standard from 0-4min] * Frap value of standard.The result obtained for the FRAP assay are presented in the **Table 15** and **Fig. 26.**

Method 4: Reducing power assay [101]

Principle

Reducing power assay is a spectrophotometric method and is based on the principle that increases in absorbance of the reaction mixture indicates the increases in the reducing power of the sample. Anti -oxidant activity may be due to a variety of mechanism viz., the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the reductive capacity and free radical scavenging. The assay is based on the reduction of ferric in potassium ferricyanide to ferrous to form potassium ferrocyanide by the sample and the subsequent formation of Prussian blue colour with ferric chloride. The absorbance of the blue complex is measured at 700nm.

Antioxidant



Pot. ferricyanide + Ferric chloride ⟶Pot. ferrocyanide + Ferrous chloride

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Materials required

Ethanolic extracts of *Andrographis echioides*

1% w/v potassium ferricyanide

10% w/v trichloro acetic acid

0.2M, phosphate buffer (pH 6.6)

0.1% w/v ferric chloride

Procedure

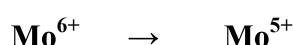
The reducing power ability of plant extracts was screened as mentioned by Oyaizu *et.al.,* 1986 by assessing the ability of the test extract to reduce $FeCl_3$solution). 0.2 to1 mL of

plant extract solution (1mg/mL) was mixed with 0.75mL phosphate buffer and 0.75mL of 1% potassium ferricyanide [$K_3Fe(CN_6)$] and incubated at 50°C for 20min.0.75mL of 1%trichloro acetic acid was added to the mixture ,allowed to stand for 10min. The whole mixture was then centrifuged at 3000rpm for 10min. Finally 1.5mL of the supernatant was removed and mixed with 1.5mL of distilled water and 0.1mL of 0.1%ferric chloride solution and the absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The results obtained are presented in **table 16** and **Fig. 27.**

Method 5: Total antioxidant activity by Phosphomolybdenum Method [102-104]

Principle

Total antioxidant capacity was measured by spectrophotometric method of Nilgum *et al.,* 2009. Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex at acidic pH and the absorbance was measured at 695nm. This method is used to determine the total antioxidant activity of samples.



$$\mathbf{Mo^{6+} \rightarrow Mo^{5+}}$$

Instruments

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

Procedure

An aliquot of 0.3mL of different concentrations of sample solutions was combined with 2.7mL of the reagent solution (H_2SO_4, sodium phosphate and ammonium molybdate). In

case of blank, 0.3mL of ethanol was used in place of sample. The tubes were incubated for 95°C for 90min. After the mixture was cooled to room temperature, the absorbance was measured at 695nm against blank. The standard ascorbic acid was treated in a similar manner. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid. The results obtained are presented in **Table 17** and **Fig. 28.**

SECTION B - *INVITRO* CALCIUM OXALATE CRYSTALLIZATION INHIBITION

Kidney stones are one of the painful, common disorders of the urinary tract. Men tend to be affected more frequently than women. Urinary stones are polycrystalline aggregate consisting varying amount of crystals are formed from dissolved urinary minerals. Kidney stones contain calcium, oxalate, phosphate, magnesium, uric acid.[105]

The formation of urinary calculi involves a crystallization process that includes nucleation, growth and aggregation of crystals.

Nucleation: Nucleation is the formation of a solid crystal phase in a solution. It is an essential step in stone formation.

Homogeneous nucleation: The term super saturation refers to a solution that contains more of the dissolved material than could be dissolved by the solvent under normal circumstances. The point at which saturation of a solution is reached, and crystallization begins is commonly known as thermodynamic solubility product. Urine contains inhibitors of crystallization and can hold large concentrations of solute above the metastable state. If the concentration of solute increases further and a point is reached where it cannot be held in solution, which is the point of formation of product in urine. The process of nucleation in a pure solution is known as homogeneous nucleation

Heterogeneous nucleation: New crystals deposits on pre-existing crystal surface of similar type. Secondary nucleation results in the mass production of crystals. Epitaxy is a process

whereby material of one crystal type is precipitated upon the surface of another whose lattice dimensions are almost identical. Epitaxy is clinically important in the formation of calcium oxalate stones. These two processes are closely related to heterogeneous nucleation.

Growth

After nucleation, crystal growth is the next major step in stone formation. Crystal growth is determined by the molecular size and shape of the molecule, the physical properties of the material, supersaturated solution level, pH and defects that may form in the crystal structure. Crystal growth is one of the prerequisites for particle formation.

Aggregation

Crystals in solution stick together and form a large particle. Aggregation of particles in solution is determined by a balance of forces, some with aggregating effects and some with disaggregating effects. Interparticle distance increases attractive force and favours particle aggregation. In various steps of stone formation, crystal aggregation is an important factor than nucleation and growth because aggregation occurs within seconds.

Type of stones

Calcium stone: This is most common type of calcium stones are calcium hydrogen phosphate dihydrate (Brushite crystals) and calcium oxalate. Calcium is a normal part of a healthy diet. Calcium that is not used by the bones and muscles goes to the kidneys. In most people, the kidneys flush out the extra calcium with the rest of the urine. For some people this calcium gets accumulated and aggregates with other waste products to form stones.

Struvite stone: These stones normally develop after an infection in the urinary system. These stones contain the mineral magnesium and the waste product ammonia.

Uric Acid Stone: These stones form when there is too much acid in the urine. Metabolic disorders that cause improper acidification of the urine mostly end in the formation of uric acid stones. This can be prevented by reducing the amount of meat in diet.

Cystine stone: Their occurrence is mostly rare. Cystine is one of the building blocks that make up muscles, nerves, and other parts of the body. Excessive accumulation of cystine due to improper dietary factors can result in cystinuria, which ultimately ends up in stone formation.

Symptoms of kidney stone

1. Extreme pain due to acutelyblocks the flow of urine
2. Person feels a sharp, crampingpain in the back and side in the area of kidney or lower abdomen.
3. Nausea and vomiting

Diagnosis of kidney stone

1. X- ray or sonogram
2. CT (computed tomography) scan or an IVP (intravenous pyelogram).

These tests help to determine the proper treatment.

Treatment of kidney stones

1. Disintegrate the stone at a microscopic level.
2. Completely flush out all dissolved particles painlessly.
3. Prevent bacteria from collecting in the urinary tracts.
4. Keep kidneys from forming stones again.

MATERIALS AND METHODS [106, 107]

Chemicals

10Mmcalciumchloridedihydrate ($CaCl_2.2H_2O$)

4mM sodium oxalate ($Na_2C_2O_4$)

0.15M sodium chloride

Principle

The effect of calcium oxalate crystallization was determined by the time course

measurement of turbidity changes due to the crystal nucleation and aggregation in the calcium chloride dehydrate solution on the addition of 0.15M sodium oxalate. The precipitation of calcium oxalate at 37°C and pH-6.5 has been studied by the measurement of turbidity at 620nm. A spectrophotometer UV/Visible (Shimadzu 1800) was employed to measure the turbidity of the formation of calcium oxalate

Preparation of calcium chloride and sodium oxalate solution

0.15M Sodium chloride solution was prepared by standard procedure.10mM of calcium chloride dihydrate and 4mM sodium oxalate was prepared by 0.15M sodium chloride solution as solvent.

Procedure

Study without inhibitor

About 1.5mL of calcium chloride dihydrate was transferred in to cell and the blank reading was recorded. Then 1.5mL of sodium oxalate was added to above solution and the absorbance was recorded at 620nm within 10min of the addition of this solution.

Study with inhibitor

Various concentration of ethanolic extract of *Andrographis echioides* was prepared by using 0.15M sodium chloride solution 1mL of calcium chloride dihyrate and 1mL of various concentration (1mg/ml, 2mg/ml, 3mg/ml) plant extract (inhibitor solution) were taken in the cell and the blank reading was recorded. About 1mL of 4mM sodium oxalate was added to above solution and the absorbance was recorded at 620nm within 10min of the addition of this solution. The percentage of the inhibition was calculated by using the following formula - **Percentage of inhibition = [1-(T_I /T_C)] X100;** where T_i – Tutbidimetric slope with inhibitor and T_c – Turbidimetric slope without inhibitor. The results are presented in **Table 18** and the photomicrographs of the crystals are presented in **Fig. 29.**

Microscopic study

A drop of crystallizable solution as well as a drop of mixture of inhibiting solution with crystallizable solution was placed on the microscopic slide and the crystals were observed by research microscope. The growth of crystals in different stages (t_1 and t_2) with and without plant extracts were observed research microscope. The effects of plant extracts on crystallization were noted and photograph of crystallization are given **Fig. 30** and **Table 19.**

SECTION C - *IN VITRO* ANTIDIABETIC ACTIVITY [108-112]

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar level that results from defects in insulin secretion or action or both. Diabetes was known even in ancient times which are characterized by excessive flow of urine, insatiable thirst, and extensive disturbances of carbohydrates, protein, and lipid metabolism. This disease was coined by Graeco-Roman physician Aretaeus of Cappadocia (80-130A.D) and derived from Greek word ***Diabainein***-“To flow through” and ***Mellitus***-“Honey sweet” which comes from Latin word by German physician Johann Peter Frank (1745-1821) in order to distinguish *Diabetes mellitus.* It is a disorder caused by the low levels of or the absence of the insulin hormone which is necessary to take glucose into the cells where they can be converted into energy or stored as glycogen for later use. The use of glucose in the body to provide energy is important as the Central Nervous System, the brain and spinal cord require glucose in order to make their energy or ATP. Hypoglycemia is the overproduction of or the hypersensitivity to the insulin produced. Insulin is produced in the pancreas. Without insulin, the body cannot maintain proper blood sugar levels.[8]

Types of *Diabetes mellitus*

There are two types of *Diabetes mellitus*. Type I diabetes (IDDM) is caused due to insulin insufficiency because autoimmune destruction of the islet β cells of pancreas. Patients are totally dependent on exogenous source of insulin. This type of diabetes constitutes less

than 10% of the diabetic population. Type II diabetes (NIDDM) is caused due to a metabolic derangement. Patients are unable to respond to insulin and it can be treated with dietary changes, exercise and medication. Type II diabetes is the more common form of diabetes constituting 90% of the diabetic population. A problematic aspect of type II diabetes is that it often begins insidiously and it is recognized when serious long-term complications occur.

Symptoms of both type I and type II Diabetes

- High levels of sugar in the blood
- Unusual thirst
- Frequent urination
- Extreme hunger and loss of weight
- Blurred vision
- Nausea and vomiting
- Extreme weakness and tiredness
- Irritability, mood changes etc.

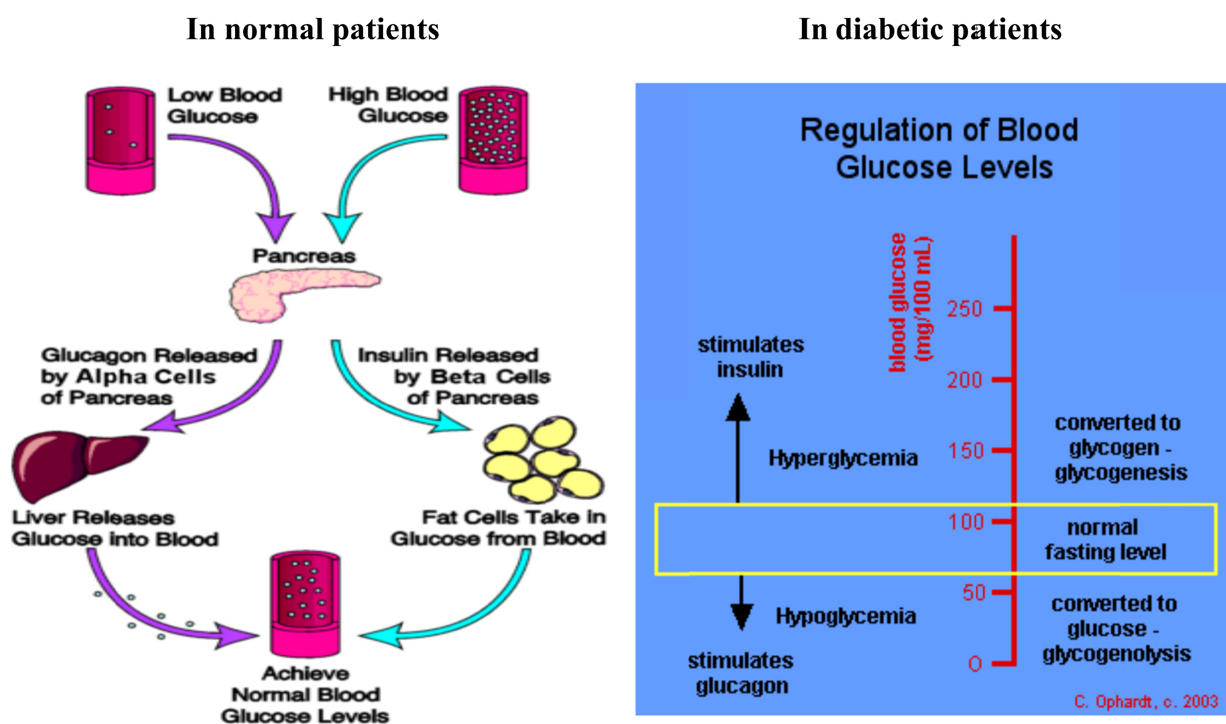
The international diabetes foundation and WHO describe the dramatic increase in *Diabetes mellitus* that is occurring throughout the world today and the prevalence is a rising global concern. Recent statistics report that the disease had affected 347 million adult worldwide by the year 2008. An estimated 3.4 million people died from diabetes related consequences in the year 2004, and this number is projected to double between 2005 and 2030. The long–term un-regulation of blood glucose and lipid contributes directly to the serious life threatening complications of diabetes including nephropathy, retinopathy, neuropathy, cardiovascular disease and stroke.

The herbal drugs with anti-diabetic activity are yet to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine. The plants provide a potential source of hypoglycemic

drugs because many plants and plant derived compounds have been used in the treatment of diabetes. Many Indian plants have been investigated for their beneficial use in different types of diabetes and reports occur in numerous scientific journals. Ayurveda and other traditional medicinal system for the treatment of diabetes describe a number of plants used as herbal drugs. Hence, they play an important role as alternative medicine due to less side effects and low cost. The active principles present in medicinal plants have been reported to possess pancreatic beta cells re-generating, insulin releasing and fighting the problem of insulin resistance. Hyperglycemia is involved in the etiology of development of diabetic complications. Hypoglycemic herbs increase insulin secretion, enhance glucose uptake by adipose or muscle tissues and inhibit glucose absorption from intestine and glucose production from liver.

Fig 20: Regulation of blood glucose in normal &diabetic patients

In normal patients **In diabetic patients**



IN-VITRO **ANTI -DIABETIC METHODS**

The anti-diabetic activity was carried out by the following methods

- Non-enzymatic glycosylation of Haemoglobulin assay
- Glucose uptake by yeast cells
- Alpha-amylase inhibition assay
- Alpha-glycosidase inhibition assay

METHOD 1: Non-enzymatic glycosylation of Haemoglobulin assay [108,110]

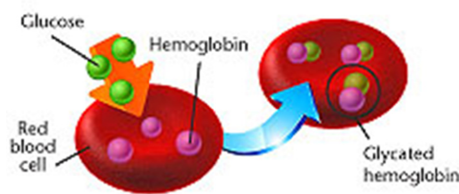
Glycated hemoglobin (Hemoglobin A1c, A1C, or Hb_{1c}; sometimes also **HbA1c**) is a form of haemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time The amount of glycated hemoglobin should not be more than 12%.In *Diabetes mellitus*, higher amounts of glycated hemoglobin, indicating poorer control of blood glucose levels, have been associated with cardiovascular disease, nephropathy, and retinopathy. Monitoring HbA_{1c} in type 1 diabetic patients may improve outcome of treatment.

Principle

Glycation of proteins is a frequent occurrence, but in the case of hemoglobin, a non-enzymatic reaction occurs between glucose and the N-end of the beta chain. This forms a Schiff base which is itself converted to 1-deoxyfructose. This rearrangement is known as Amadori rearrangement. When blood glucose levels are high, glucose molecules attach to the hemoglobin in red blood cells. The longer hyperglycemia occurs in blood, the more glucose binds to hemoglobin in the red blood cells and higher the glycated hemoglobin **Fig. 21.** Glucose levels are intermittently raised in portal vessels carrying absorbed glucose to the liver for regulation. Passing red cells will have increased glycation after sugary food intake. Once a hemoglobin molecule is glycated, it remains that way. A buildup of glycated

hemoglobin within the red cell, therefore, reflects the average level of glucose to which the cell has been exposed during its life-cycle. Measuring glycated hemoglobin assesses the effectiveness of therapy by monitoring long-term serum glucose regulation. The HbA1c level is proportional to average blood glucose concentration over the previous four weeks to three months. Some researchers state that the major proportion of its value is weighted toward the most recent 2 to 4 weeks.This is also supported by the data from actual practice showing that HbA1c level improved significantly already after 20 days since glucose-lowering treatment intensification.

Fig.21: Glycatedhaemoglobin



Reagents

Glucose (2%W/V)

Haemoglobulin (0.06%)

Gentamycin (0.02%)

0.01M phosphate buffer (pH- 7.4)

Procedure

The ethanolic extract of *Andrographis echioides* was dissolved in DMSO reagent to get stock solution containing 1mg/mL. 1mL of various concentrations of stock solution (20-100µg/mL) of the extract was added to 1mL of glucose (2%), haemoglobin (0.06%) and gentamycin (0.02%) solutions were prepared in 0.01M phosphate buffer solution (pH- 7.4) . The reaction mixture was incubated in dark at room temperature for 72h. After that the

absorbance was measured at 520nm by using colorimeter. The reaction mixture without sample was used as control. Water used as blank. Alpha-tocopherol (Trolax) was used as standard. The percentage inhibition was calculated using the formula - (**As-Ac)/As X 100**where**A_S** – Absorbance of sample and **A_C** – Absorbance of Control. The results obtained are presented in **Table 21** and the graphical representation is presented in **Fig. 31.**

METHOD 2: Glucose uptake in Yeast cells method [109.110]

Reagents

10% v/v Baker's yeast suspension

Glucose solution (5mM,10mM)

Glucose oxidase solution

Preparation of yeast suspension

2g of commercial Baker's yeast was mixed with distilled water and centrifuged at 3,000rpm for 5min and the supernatant fluid was removed and again centrifuged till supernatant fluid was clear then 10%v/v yeast suspension was prepared by dissolving the yeast with distilled water.

Procedure

To the ethanolic extract of *Andrographis echioides* was dissolved in ethanol to get stock solution containing 1mg/mL. 1mL ofvarious concentrations of stock solution (20-100µg/mL) of the extract was added to 1mL of 5mM, 10mM of glucose solution in separate tubes and then incubated at 37°C, and 100µL of 10%v/v yeast suspension was added. It was vortexed and further incubated at 37°C for 1h, and then the tubes were centrifuged for 5min. The supernatant fluid was separated and 50µL of supernatant fluid was added to 1mL of glucose oxidase solution and kept aside for 20min. The absorbance of the colour developed was measured at 520nm by using a colorimeter. Water used as blank and the reaction mixture without sample used as control. Acarbose used as standard. The increase is glucose uptake

was calculated using the formula - **Increase in glucose uptake (%) = (As-Ac)/As X 100**where**A_S** – Absorbance of sample and **A_C** – Absorbance of Control. The results are presented in **table 21**and **22** the graphical representation in **Fig 32 & 33.**

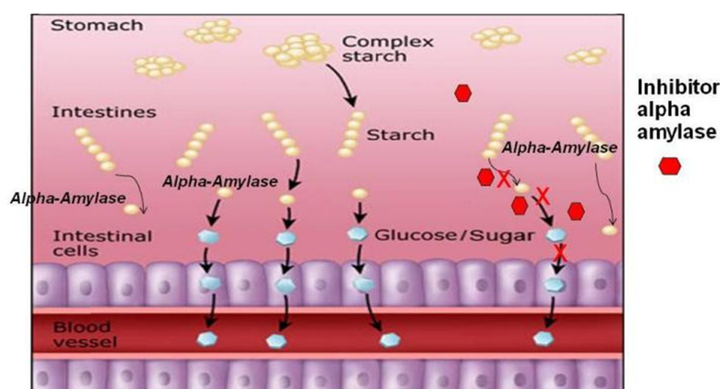
METHOD 3: Alpha amylase inhibition assay [109,111]

Pancreatic α-amylase belongs to the class of α 1, 4- glucono hydrolases and is one of the important target enzymes for the conventional treatment of diabetes. It catalyzes the initial step in hydrolysis of starch to maltose and maltotriose which are then acted upon by α-glucosidases, broken down in to glucose and it enters the blood stream. Naturally available α-amylase inhibitors from medicinally important plants are shown to be very effective in managing post prandial hyperglycemia which is a major concern in type 2 diabetes.

Principle

Pancreatic α-amylase is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. α-amylase inhibitors are agents which inhibit the amylase activity **Fig.22.** In which results in the delay of carbohydrate digestion and prolong overall carbohydrate digestion time causing reduction in the rate of glucose absorption and consequently reducing the post prandial plasma glucose rise**.**

Fig. 22: Inhibition of α-amylase enzyme



Reagents

Glucose or potato starch(1% w/v in sodium acetate buffer)

Alpha- amylase enzyme (1% w/v in sodiumacetate buffer)

0.1M sodium acetate buffer(pH 7.2)

Iodine –iodide indicator

Acarbose (1mg/mL in sodiumacetate buffer)

Preparation of sodium acetate buffer

820.3mg of sodium acetate and 18.7mg sodium chloride was dissolved in 100mL of distilled water.

Preparation of iodine-iodide indicator

635mg of iodine and 1g of potassium iodide was dissolved in 250mL of distilled water.

Procedure

To the ethanolic extract of *Andrographis echioides* was dissolved in sodium acetate buffer to get stock solution containing 1mg/mL.1mL of various concentrations of stock solution (100-1000µg/mL) of the extract was added to 1mL of 1%w/v of glucose solution, 1mL of alpha-amylase (1%w/v) and 2mL of sodium acetate buffer(0.1M, pH 7.2) and the reaction mixture was incubated for 1h. 0.1mlLof iodine-iodide indicator was added. The absorbance was measured at 565nm of the resulting chromophore by using UV-Visible spectrophotometer. Sodium acetate buffer was used as blank. The reaction mixture without sample was used as control and acarbose used as standard. The percentage inhibition was calculated using the formula - **Percentage inhibition of α-amylase = (As-Ac)/As X 100,** where $\mathbf{A_S}$ – Absorbance of sample and $\mathbf{A_C}$ – Absorbance of control. The results are presented in **table 23** and the graphical representation in **Fig. 34.**

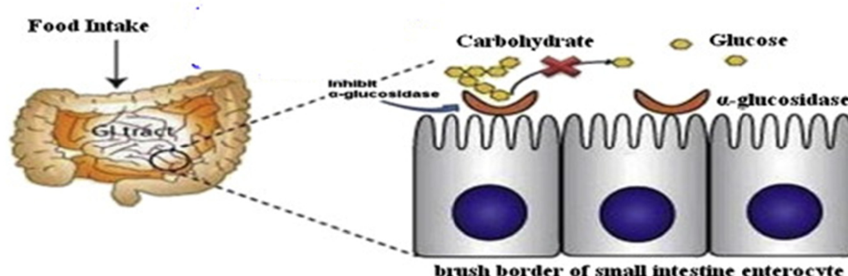
METHOD 4: Alpha-glucosidase inhibition assay [110,112]

α-glucosidase inhibitors are used to establish greater inhibitors are used to establish greater glycemic control over hyperglycemia in Diabetes type 2 particularly with regard to postprandial hyperglycemia. They may be used as monotherapy in conjunction with an appropriate diabetic diet and exercise, or they may be used in conjunction with other anti-diabetic drugs. α-glucosidase inhibitors may also be useful in patients with Diabetes type 2.

Principle

Alpha glucosidase inhibitors act as competitive inhibitors of enzymes needed to digest carbohydrates specifically α-glucosidase enzymes in the brush border of the small intestine. The membrane bound intestinal α-glucosidases hydrolyze oligosaccharides, trisaccharides and disacchardies to glucose and other monosaccharides in the small intestine **Fig. 23.** Inhibition of these enzyme systems reduces the rate of digestion of carbohydrates. Less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In patients with diabetes, the short-term effect of these drugs therapies is to decrease current blood glucose levels: the long term effect is a small reduction in haemoglobin level.

Fig.23: Inhibition of α-glucosidase enzyme



Reagents

α-glucosidase enzyme (1U/ml)

0.2 M Tris buffer (pH -8)

Glucose (2%w/v)

Glucose oxidase solution

Procedure

To the ethanolic extract of *Andrographis echioides*was dissolved in ethanol to get stock solution containing 1mg/mL. 1mL of various concentration of stock solution (200-1000µg/ml) of the extract was added to 1mL of 2%w/v of glucose with 1mL of 0.2M Tris buffer (pH -8) and incubated for 5min at 37°C. The reaction was initiated by adding 1mL of α-glucosidase enzyme (1U/mL) followed by incubation for 10min at 37°C. Then the reaction mixture was heated for 2min in boiling water bath to stop the reaction. The amount of liberated glucose is measured by glucose oxidase solution. The absorbance was measured at 540nm by using UV-Visible spectrophotometer. Water was used as blank and the reaction mixture without sample was used as control. Acarbose used as standard. The percentage inhibition was calculated using the formula - **Percentage inhibition of Alpha-glucosidase =(Ac-As)/Ac X 100,** where $\mathbf{A_C}$ – Absorbance of control and $\mathbf{A_S}$ – Absorbance of sample. The results obtained are presented in **table 24** and the graphical representation in **Fig. 35.**

SECTION D - ANTI MICROBIAL ACTIVITY [103-118]

Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals, but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man.

Human infections particularly those involving micro-organisms are bacteria, fungi, viruses, nematodes cause serious damages in tropical and subtropical countries of the world. In recent years, multiple drug resistance in human pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of such diseases.

The World Health Organization estimated that 80% of the populations of developing countries still rely on traditional medicines, mostly plant drugs for their primary health care needs. Herbs are supposed to be safe but many unsafe and fatal side effects have recently reported. Hence, there is an urgent need to study the screening of antimicrobial properties of herbs, which will be helpful in the treatment of several diseases caused by microorganisms.

Many plant families represent reservoir of effective chemotherapeutics and can provide valuable source of natural antimicrobials. Thus for many thousands of years, plant extracts have been used because of their antimicrobial traits, which are due to the compounds synthesized in the secondary metabolism of the plant. Anti- microbial properties of plant components were first documented in the late 19th century. Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen substituted derivatives. Most of the secondary metabolites, which at least 12,000 have been isolated. These substances serve as plant defence mechanisms and herbivores.

Antibacterial activity

The ethanolic extract of *Andrographis echioides* was screened for antibacterial activity on five bacterial strains and the zone of inhibition, minimum inhibitory concentration was determined.

Preparation of extract

The ethanolic extract of *Andrographis echioides* was dissolved in DMSO solution and to produce a stock solution of 2mg/mL.

Preparation of MH agar medium

Muller Hinton agar (MH, HI media) was used for culture of bacterial strains.It consists of Beef 2g, casein acid hydrolysate 17.5g, starch 1.5g and agar 17g (pH 7.4 ± 0.2). MH agar (38g) was weighed and dissolved in 1000ml of distilled water and adjusted to pH 7.3 ± 0.2, sterilized by autoclaving at 121°C for 15min at 15psi pressure and was used for sensitivity tests. This medium was used for screening *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella spp.* and *Escherichia coli.*

Preparation of Blood agar medium

Blood agar medium (BAM) contains mammalian blood usually at a concentration of 5–10%. Blood agar medium are enriched, differential media used to isolate fussy organisms and detect hemolytic activity. β-hemolytic activity will show lysis and complete digestion of red blood cell contents surrounding colony.

The media contains nutrient substrate (heart extract and peptones) 20.0; sodium chloride 5.0: agar-agar 15.0. 40g of the above media was suspended in 1000mL of distilled water and autoclaved for 15min at 121°C and cooled to 45-50°C. The prepared medium was clear and yellowish-brown and then 5-8% defibrinated blood was added.

The blood agar medium was used for the antibacterial activity against the bacterial strains namely *Streptococcus pyogens*.

Preparation of bacterial cultures

The various bacterial strains like *E.coli, Klebsilla spp, Pseudomonas aeruginosa*, *Streptococcus pyogens, Staphylococcus aureus* etc. were utilized for screening antibacterial activity. A few colonies of the bacterial strains picked from the agar slopes and were inoculated into 4mL peptone water in a test tube. They were incubated for 24h to form suspensions. The suspension was diluted with saline if necessary.

The visual density equivalent to standard prepared by adding 0.5ml of 1% barium chloride to 99.5mL of 1%sulphuric acid. These suspensions were then used for seeding.

Disc diffusion technique

The MH media was poured aseptically into sterilized petridishes and the petridishes were swirled to settle the agar and allowed to cool .The bacterial strains were the seeded on the MH agar media by streaking the plate with a sterile swab containing the strain. The plain sterile discs were impregnated with various volumes of the extract, l and DMSO (negative control) and dried. Amikacin (30µg/disc) was used as standard. The discs were then placed on the plate with the help of sterile needle and the plates were incubated at 37°C for 24h.

Similarly the blood agar medium was used for antibacterial activity against the *Streptococcus* species and the above procedure was adopted.

The results were read and the zone of inhibition was then measured and they are presented in **Tables 27.**The photographic representations of the antibacterial activity are presented in **Figs 36.**

Anti –fungal activity [119-122]

Disc diffusion method

Inoculum preparation

The fungal colony to be tested was grown in potato dextrose agar slant at 35° C to induce the conidium and sporangiospore formation. After 7-10 days of incubation with well grown spores, the culture was taken for testing. 5ml of 0.85% sterile saline was added to the culture tube and the suspension were made by gently probing the colonies with the tip of Pasteur pipette. With the help of sterile pipettes, the saline with conidia was transferred in to a sterile screw cap tube. The tube was then vortexed for 30sec to 1min and allowed to stand at room temperature for 5 to 10min for the heavier particles to settle down. The upper

homogenous suspensions were collected and the densities of the conidial suspensions were read and adjusted the optical density (OD) to be between 0.09 and 0.11 for *Aspergillus* species, 0.15 to 0.17 for *Fusarium* species by using UV Visible spectrophotometer at 530nm. The suspensions were diluted 1:50 in RPMI 1640 medium. The final concentration of the conidia was 0.2 – 1 X 10^4 cfu/mL.

Preparation of media

Sabourand Dextrose Agar (Hi- media) media (SDA) was used for cultivation of fungi and particularly pathogenic fungi associated with skin infections. Formula gm/litre Peptone - 10g, dextrose 40g and agar 15 g; pH 5.6 $\pm$ 0.2. SDA (65g) was dissolved in 1000ml of distilled water. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure.

Preparation of extract

A stock solution of 400mg/mL of the ethanolic extract of the plant *Arographis echioides* was prepared by dissolving the required quantity of the extract in DMSO. 10, 20 and 30µL were impregnated on the plain sterile disc and dried.

Procedure

The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swap dipped into the inoculum suspension. The plate was allowed to dry for 20min. using a pair of flame sterilized forceps the extract containing discs were applied on to the surface of the inoculated plates. The plates were incubated at 35°C for 48h. The plates were read at 24h and 48h. Itraconazole used as standard. (30µl/mL). The results obtained are presented in **Table 29** and **Fig. 38.**

RESULTS AND DISCUSSION

SECTION A - *IN VITRO* ANTIOXIDANT ACTIVITY

Method I: Free radical Scavenging activity using diphenylpicrylhydrazyl (DPPH) free radical

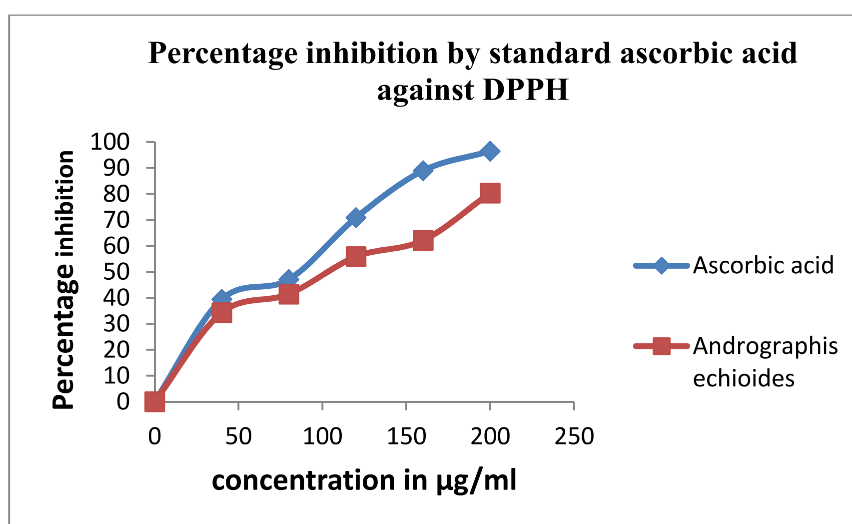
The results obtained for the free radical scavenging activity against DPPH radical is presented in**Table 13.**

Table 13: Percentage inhibition of ethanolic extract of *Andrographis echioides* and standard ascorbic acid against DPPH at 517nm

S. No.	Conc. in µg/mL	Percentage inhibition by ascorbic acid	Percentage inhibition by *A. echoiodes*
1	40	39.36±0.03	34.3±0.01
2	80	47.06±0.008	41.46±0.02
3	120	70.84±0.02	55.90±0.06
4	160	88.94±0.02	62.12±0.01
5	200	96.47±0.01	80.33±0.06
	IC_{50}	**84.77µg/mL**	**112.090µg/mL**

***mean of three readings ± SEM**

Fig 24: Free radical scavenging assay of ascorbic acid and 70%ethanolic extract of *Andrographis echioides* against DPPH at 517nm



From the **table 13**, it can been seen that the 70%ethanolic extract of *Andrographis echioides* showed a percentage inhibition of 80.33±0.06 while ascorbic acid showed a percentage inhibition of96.47±0.01 at a concentration of 200µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be**112.090µg/mL** and **84.77µg/mL**for70% ethanolic extract and ascorbic acid respectively. The extract possessed a good radical scavenging capacity

Method 2: Determination of scavenging activity against hydrogen peroxide

The results obtained for the scavenging activity against hydrogen peroxide are presented in **Table 14.**

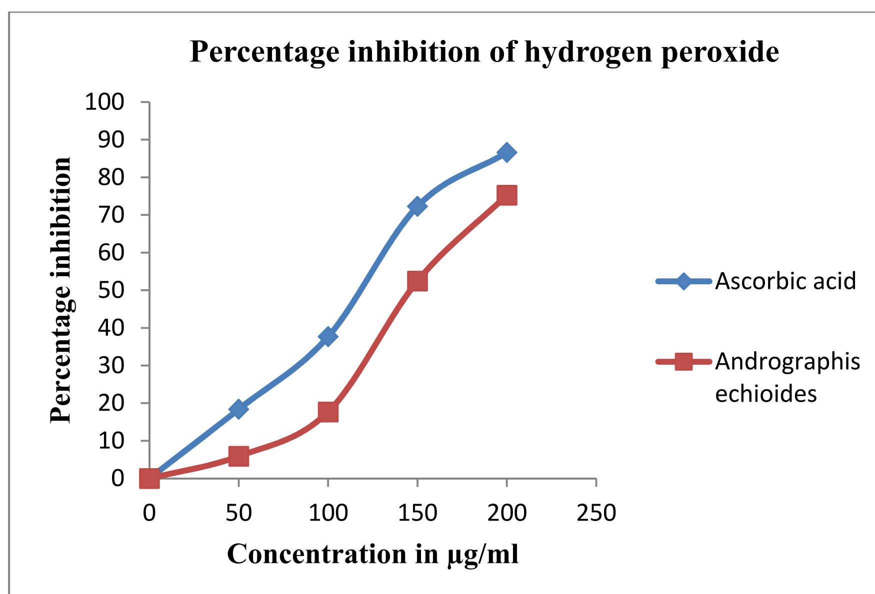
Table 14: Percentage inhibition of hydrogen peroxide of ascorbic acid and 70%ethanolic extract of *Andrographis echioides*

S. No.	Conc. in µg/mL	Percentage inhibition by ascorbic acid	Percentage inhibition by *Andrographis echioides*
1	50	18.2±0.20	5.89±0.02
2	100	37.68±0.003	17.67±0.06
3	150	72.29±0.10	52.45±0.02
4	200	86.58±0.05	75.27±0.03
	IC_{50}	**115.441µg/mL**	**150.086µg/mL**

***mean of three readings ± SEM**

From the **table 14**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of75.27±0.03 while ascorbic acid showed a percentage inhibitionof86.70±0.05 at a concentration of 200µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be **150.086µg/mL** and **115.441µg/mL** for ethanolic extract and ascorbic acid respectively. The extract possessed a better hydrogen peroxide scavenging activity comparable to that of ascorbic acid.

Fig 25: Percentage inhibition of hydrogen peroxide by ethanolic extract of *Andrographis echioides*



Method 3: Ferric reducing anti -oxidant assay

The results obtained for the ferric reducing antioxidant assay is presented in **Table 15.**

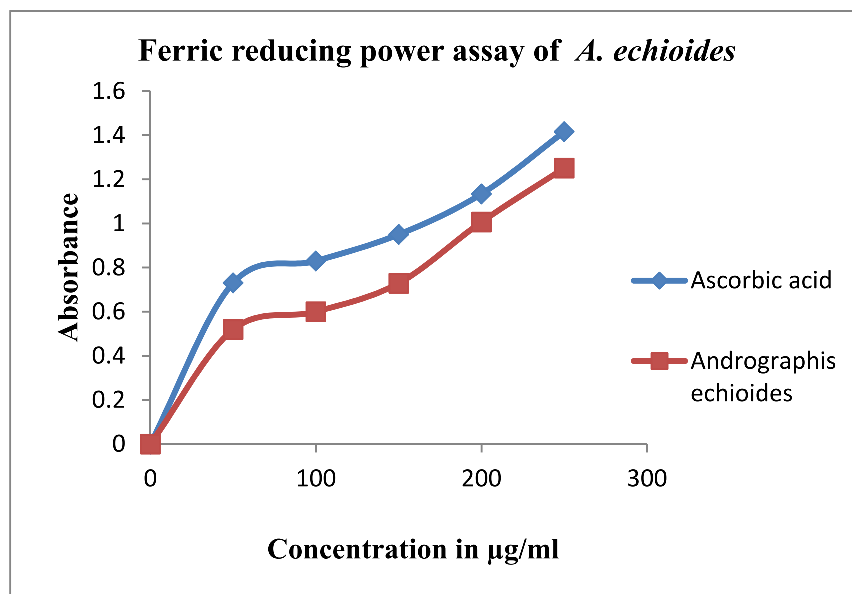
Table 15: Ferric reducing anti-oxidant assay of ascorbic acid and ethanolic extract of *Andrographis echioides*

S. No	Conc. in µg/mL	Absorbance of ascorbic acid	Absorbance of ethanolic extract of *A. echioides*
1	50	0.736±0.0008	0.520±0.001
2	100	0.831±0.0008	0.610±0.003
3	150	0.954±0.0006	0.730±0.000
4	200	1.130 ±0.0003	1.010±0.001
5	250	1.410 ±0.0008	1.251±0.001

***Mean of three readings ± SEM**

From the **table 15**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a stoichometric increase in absorbance with increase in concentration. A maximum absorbance of **1.251 ± 0.001** and **1.41 ± 0.0008** was seen for the extract and ascorbic acid at a concentration of **250µg/mL** respectively.

Fig.26: Ferric reducing anti-oxidant assay of 70%ethanolic extract of *Andrgraphis echioides*



Method 4: Reducing power assay

The results obtained for the reducing powerassay is presented in**Table 16.**

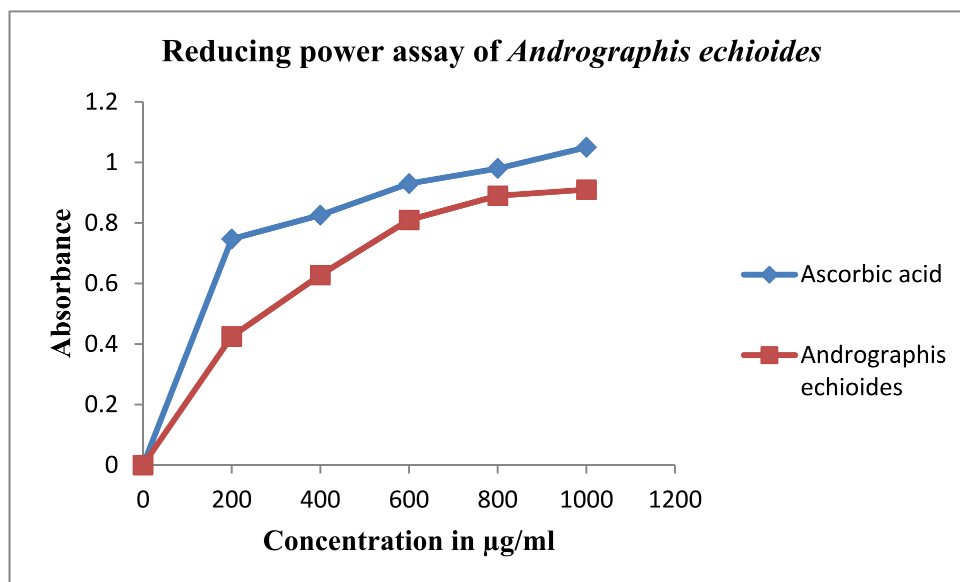
Table 16: Reducing power assay of ascorbic acidand ethanolic extract of *Andrographis echioides*

S. No	Conc. in µg/mL	Absorbance of ascorbic acid	Absorbance of ethanolic extract of *A. echioides*
1	200	0.754±0.008	0.42±0.001
2	400	0.825±0.001	0.62±0.001
3	600	0.930±0.001	0.81±0.001
4	800	0.980 ±0.003	0.89±0.001
5	1000	1.060 ±0.005	0.91±0.001

***mean of three readings ± SEM**

From the **table16**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a stoichometric increase in absorbance with increase in concentration. A maximum absorbance of **0.91 ± 0.001**and **1.06 ± 0.005** was seen for the extract and ascorbic acid at a concentration of 1mg/mL respectively.

Fig. 27: Reducing power assay of *Andrographisechioides*



Method 5: Total antioxidantactivity by Phosphomolybdenum Method

The results obtained for total antioxidant activity by phosphomolybdenum is presented in **Table 17.**

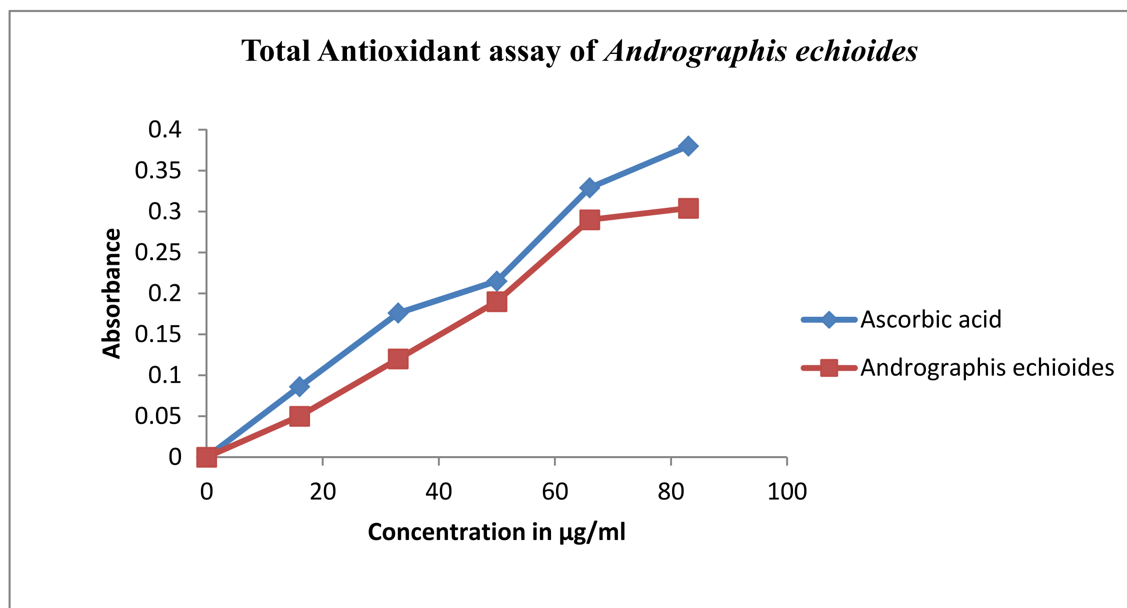
Table 17: Total anti-oxidantassay of ascorbic acid and ethanolic extract of *Andrographis echioides*(L.)Nees

S. No	Conc. in µg/mL	Absorbance of ascorbic acid	Absorbance of ethanolic extract of *A. echioides*
1	16	0.086±0.003	0.05±0.003
2	33	0.170 ±0.003	0.12±0.003
3	50	0.220 ±0.000	0.19±0.001
4	66	0.320 ±0.001	0.29±0.001
5	83	0.380 ±0.003	0.30±0.001

***mean of three readings ± SEM**

From the **table 17**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a stoichometric increase in absorbance with increase in concentration. A maximum absorbance of **0.30 ± 0.001** and **0.38 ± 0.003** was seen for the extract and ascorbic acid at a concentration of **83µg/mL** respectively.

Fig. 28: Total Anti-oxidant assay of ascorbic acid and ethanolic extract of *Andrographis echioides* (L.)Nees



SECTION B - *IN VITRO* CALCIUM OXALATE CRYSTALLIZATION INHIBITION

This study was designed to evaluate the inhibiting potential of ethanolic extract of *Andrographis echioides* on *in vitro* calcium oxalate crystallization. The concentration of inhibitor (EAE), turbidimetric slope relating to the curves of crystallization with and without inhibitor and percentage of inhibition were tabulated. The results of the percentage inhibition of the extract on the calcium oxalate crystallization are presented in **Table 18.**

The effect of ethanolic extract of *Andrographis echioides* on various phases of calcium oxalate crystallization was determined by time course measurement of turbidity in the calcium chloride solution.

In this experiment, initial detectable increase in the turbidity after induction of the crystallization with sodium oxalate was observed. In the control experiment, there was an initial steep rise in turbidity in the nucleation phase and attained maximum, followed by a decrease in the aggregation.

Table 18: Effect of 70% ethanolic extract of *Andrographis echioides* (L)Nees on calcium oxalate crystallization

Conc. of Inhibitor in mg/mL	Slope	Percentage inhibition
0	0.0432	0
1	0.0395	14.58
2	0.0243	43.75
3	0.012	72.2

The ethanolic extract of *Andrographis echioides* inhibited the slope of turbidity in a concentration dependent manner and followed by very slow decrease. Crystallization by the addition of calcium chloride and sodium oxalate was inhibited by ethanolic extract of *Andrographis echioides* at concentration of 1mg/mL, 2mg/mL; 3mg/mL inhibit calcium oxalate crystallization at the percentage of **14.58%, 43.75%,** and **72.2%** respectively.

Microscopy study

The photographs indicating the different stages of growth of Calcium oxalate (t1 and t2) with and without inhibitor were presented in **Figs 29** and**Table 19.**

Table 19: Time corresponding to the stage of crystallization with and without inhibitor

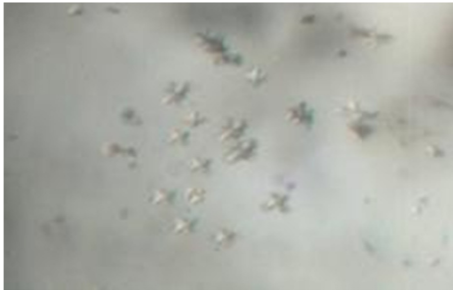
Number of Photographs	Stages ofCrystallization	Concentration of inhibitor	Time(seconds)
1	Growth	00	20
2	Aggregation	00	50
3	Growth	3mg/ml(EAE)	60
4	Aggregation	3mg/ml(EAE)	120

Identification test for calcium oxalate crystals

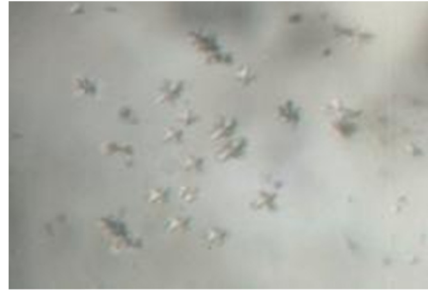
1. A drop of crystallizable solution was mixed with a drop of hydrochloric acid and viewed under the microscope. Solublilization of crystals was observed.

Fig 29: Photographs of crystallization for without and with inhibitor

Control (Growth)



Control (Aggregation)

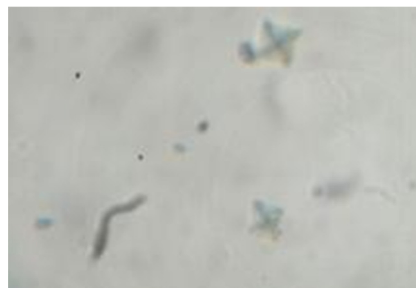


2mg/mL EAE (Growth)

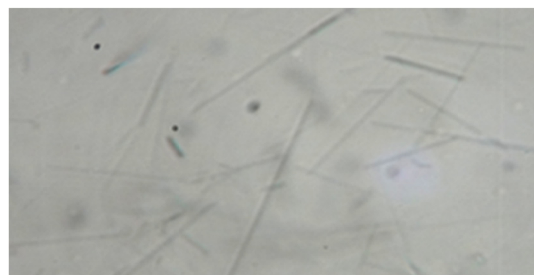
2mg/mL EAE(Aggregation)

3mg/mL EAE(Growth)

3mg/mL EAE (Aggregation)



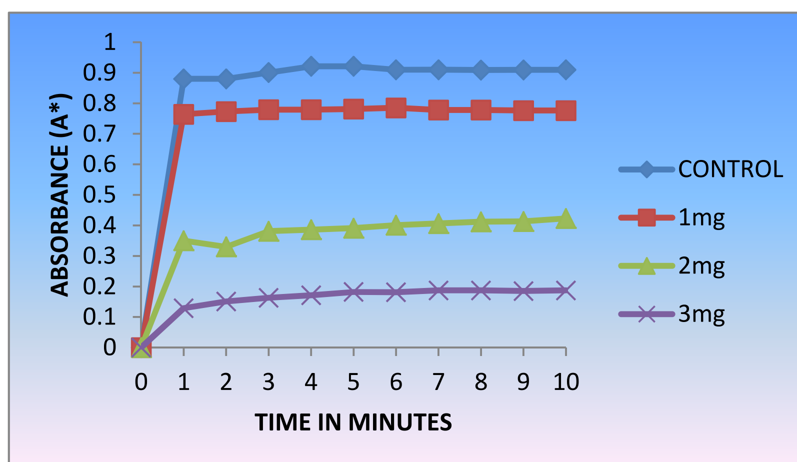
Identification of calcium oxalate crystals with 50% sulphuric acid



2. A drop of acetic acid was mixed with drop of crystallizable solution and viewed under the microscope. No change was observed in the crystallizable solution.
3. 50% sulphuric acid was added to the crystallizable solution, a gradual separation of needle like crystal of calcium oxalateat the site of original crystal.

Crystals of calcium oxalate are primary constituent of more than 60% of the majority of human kidney stones. *In vitro* crystallization systems are used for variety of purposes in urolithiasis research. Since kidney stone formation is a complex process it includes super saturation, nucleation, growth, aggregation and retention within renal tubules. Initial events of nucleation of crystals occur in the first few minutes, the graphs were re-plotted within the first three minutes for each concentration of extract as well as control. The initial positive slope of the turbidity curve which is mainly due to increase in the particle number resulting from crystal nucleation, after a plateau is achieved in this negative slope (i.e) a progressive decrease of absorbance reflected from the decrease in the particle number due to the crystal aggregation.

Fig. 30: Variance of absorbance according to time with inhibitor (EAE)



In this study ethanolic extract of *Andrographis echioides* inhibited the nucleation and aggregation of calcium oxalate in a concentration dependent manner. Physiological inhibitors

of urolithiasis found in urine include inorganic (eg. magnesium, pyrophosphate) and organic citrate, uro-epithelial glycoproteins, prothrombin fragment and glycosamino glycones, Organic compounds adsorb to the surface of crystal and inhibit the crystal nucleation, growth, aggregation are possible therapeutic strategy.

The medicinal plants contain chemical compounds which possess an inhibitor effect in the crystallization of calcium oxalate. Calcium oxalate inhibitors prevent crystal growth and aggregation by coating the surface of the growing calcium crystals or by complexing with calcium and oxalate. Chemical constituent like higher carboxylic acid like citrate chelates calcium and form soluble chelates and excrete through urine. Macromolecules of higher weight of plant extract exert their mode of action similar to natural urinary inhibitors.

Several studies are carried out using microscope to validate the results obtained by the turbidimetric method. Microscope observations revealed that the extract visibly reduces the size of crystals with significant reduction of crystal number. Phenol, flavonoids, tannins were present in *Andrographis echioides* extract. Phenolic compounds have been reported for urolithiasis. Hence the calcium oxalate crystallization inhibition of ethanolic extract of *Andrographis echioides* may be due to the presence of phenolic compounds present in the extract.

SECTION C - IN VITRO ANTIDIABETIC ACTIVITY

Method 1: Non enzymatic glycosylation of haemoglobin

The results obtained for non-enzymatic glycosylation of haemoglobin is presented in **Table 20**and the graphical representation in **Fig 31.** From the **table 20**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of **68.5±0.11** while α-tocopherol showed a percentage inhibition of **79.95±0.088** at a concentration of 100µg/mL. The IC_{50} value calculated using the linear regression analysis

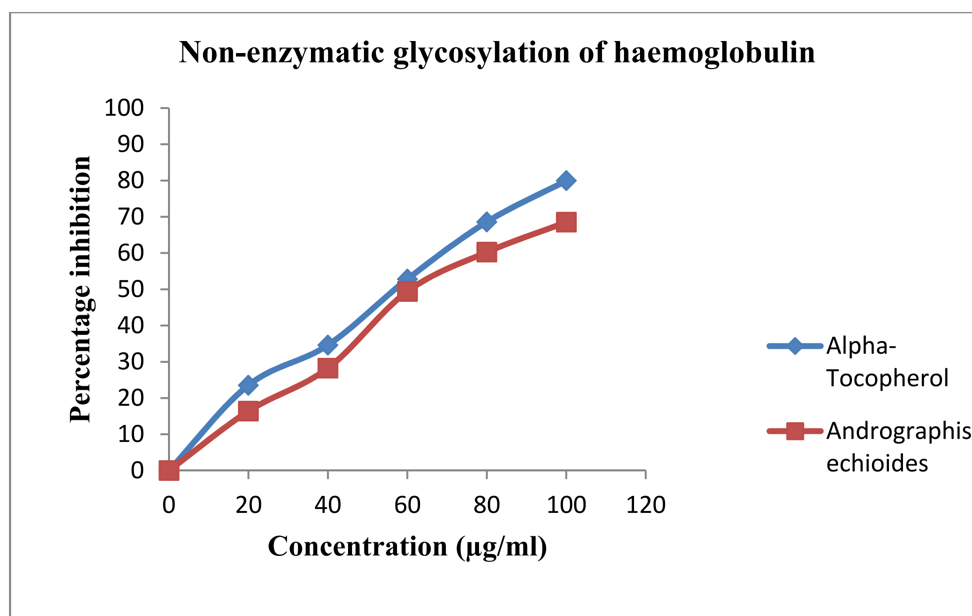
was found to be **68.19µg/mL** and **58.56µg/mL** for ethanolic extract and α-tocopherol respectively.

Table 20: *In vitro* non enzymatic glycosylation of haemoglobin

S. No.	Conc. in µg/mL	α-Tocopherol		*Andrographisechioides*extract	
		Absorbance	% inhibition	Absorbance	% inhibition
1	20	0.159±0.0034	23.53±0.20	0.14±0.0008	16.38±0.080
2	40	0.187±0.0029	34.60 ±0.47	0.17±0.0014	28.24±0.066
3	60	0.258±0.0045	52.79±0.05	0.24±0.0028	49.41±0.147
4	80	0.386±0.0041	68.57±0.45	0.31±0.0026	60.25±0.021
5	100	0.588±0.0043	79.95±0.09	0.39±0.0008	68.50 ±0.110
		IC_{50}	**58.56µg/mL**	**IC_{50}**	**68.19µg/mL**

***mean of three readings ± SEM**

Fig 31:*In vitro* non enzymatic glycosylation of haemoglobulin



METHOD 2: Glucose uptake in yeast cells method

The results obtained inhibition of glucose (5mM & 10mM) uptake in yeast cells are presented in **Table 21** and the graphical representation in **Fig32, 33.** From the **Table 21**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of 73.13±0.03 while acarbose showed a percentage inhibition of 78.66±0.033 at a

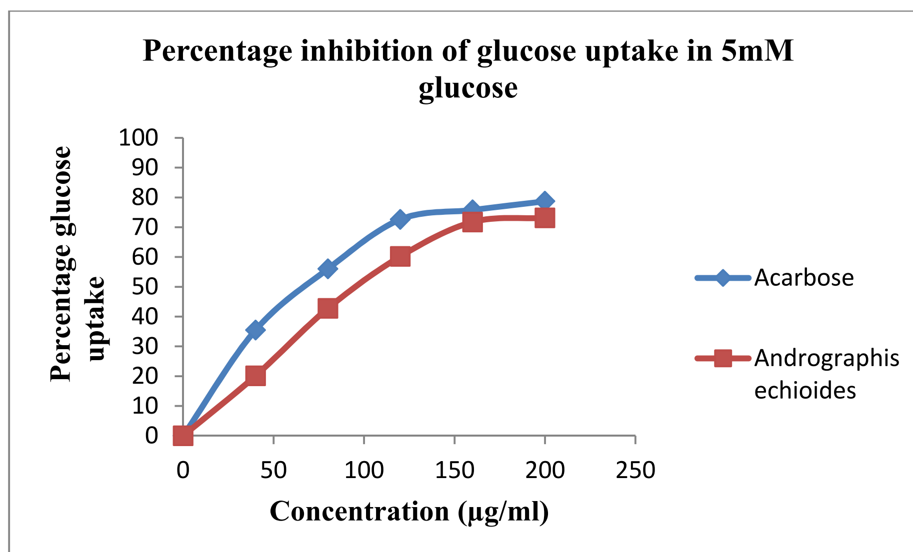
concentration of 100µg/mL. The IC_{50} value calculated using the linear regression analysis was found to be **91.83µg/mL** and**113.87µg/mL** respectively for ethanolic extract and acarbose.

Table 21: Percentage inhibition of glucose uptake in 5mM concentration

S. No.	Conc. in µg/mL	Acarbose		*Andrographisechioides*extract	
		Absorbance	**% inhibition**	**Absorbance**	**% inhibition**
1	20	0.06±0.0003	35.47±0.003	0.051±0.001	20.13±0.03
2	40	0.09±0.0003	56.03±0.003	0.070 ±0.003	42.76±0.03
3	60	0.14±0.0003	72.56±0.030	0.100 ±0.003	60.2±0.05
4	80	0.16±0.0005	75.73±0.030	0.143±0.003	71.36±0.03
5	100	0.19 ±0.0003	78.66±0.033	0.150 ±0.003	73.13±0.03
		IC_{50}	**91.83µg/mL**	**IC_{50}**	**113.87µg/mL**

***mean of three readings ± SEM**

Fig. 32: Percentage inhibition of 5mM glucose uptake



From the **Table 22**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of **63.57±0.039** while acarbose showed a percentage inhibition of **74.13±0.033**at a concentration of **100µg/mL.** The IC_{50} value

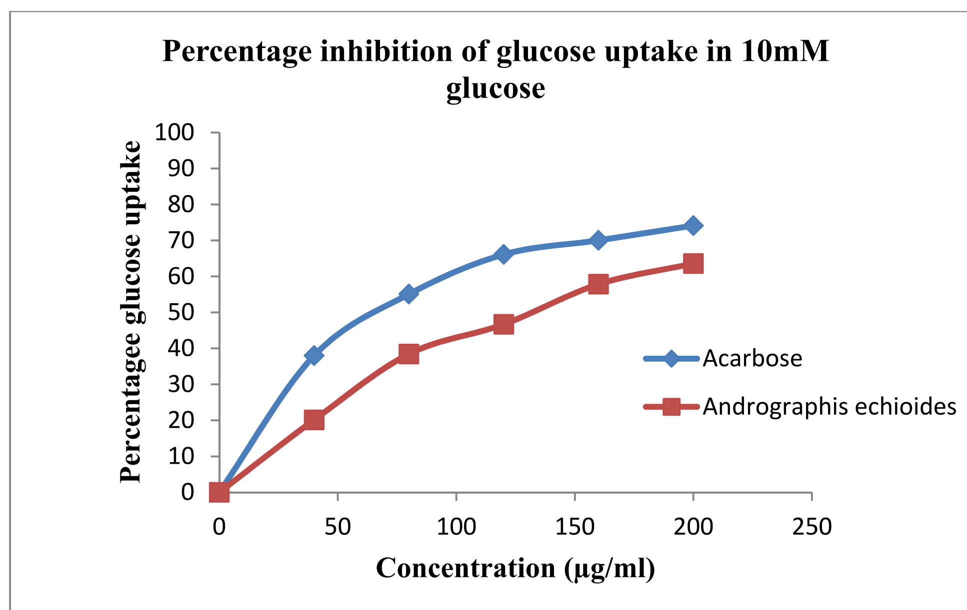
calculated using the linear regression analysis was found to be **53.57µg/mL** and **98.37µg/mL** for ethanolic extract and acarbose respectively.

Table 22: Percentage inhibition of glucose uptake in 10 mM concentration

S. No.	Conc. in µg/mL	Acarbose		*Andrographisechioides* Extract	
		Absorbance	% inhibition	Absorbance	% inhibition
1	20	0.129±0.001	37.95±0.023	0.10±0.003	20.13±0.03
2	40	0.181±0.003	55.08±0.040	0.13±0.003	38.44±0.01
3	60	0.230 ±0.001	66.13±0.033	0.15±0.003	46.70 ±0.05
4	80	0.260 ±0.001	70.03±0.030	0.19±0.003	57.83±0.03
5	100	0.300 ±0.001	74.13±0.033	0.23±0.005	63.57±0.04
		IC_{50}	**98.37µg/mL**	**IC_{50}**	**53.57µg/Ml**

***Mean of three readings ± SEM**

Fig 33: Percentage inhibition of glucose uptake in 10mM concentration



METHOD 3: Alpha amylase inhibitionassay

The results obtained inhibition of α-amylase is presented in **table 23**and the graphical representation in **Fig 34.**

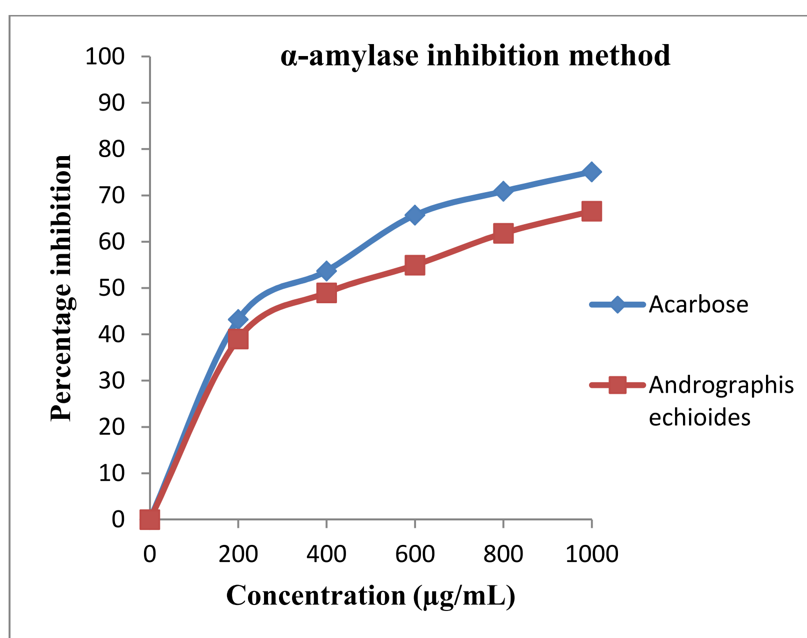
Table 23: α-amylase inhibitory activity of acarbose and *Andrographisechioides*extract

S. No	Conc.in µg/mL	Acarbose		*Andrographis echioides* extract	
		Absorbance	% inhibition	Absorbance	% inhibition
1	200	0.097±0.0006	43.20 ±0.057	0.083±0.0008	39.03±0.005
2	400	0.107±0.0003	53.70 ±0.050	0.098±0.0003	48.97±0.005
3	600	0.146±0.0003	65.76±0.008	0.110 ±0.0005	54.96±0.005
4	800	0.188±0.0007	70.89±0.005	0.130 ±0.0006	61.83±0.005
5	1000	0.220±0.0003	75.26±0.120	0.157±0.0005	66.60 ±0.057
		IC_{50}	**478.80µg/mL**	**IC_{50}**	**581.95µg/Ml**

***mean of three readings ± SEM**

From the **Table 23**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of **66.6±0.057** while acarbose showed a percentage inhibition of **75.26±0.12** at a concentration of **1mg/mL.** The IC_{50} value calculated using the linear regression analysis was found to be **581.95µg/mL** and **478.80µg/mL** for ethanolic extract and acarbose respectively.

Fig. 34: α-amylase inhibitory activity of acarbose and *Andrographis echioides* extract



METHOD 4: Alpha glucosidase inhibition assay

The results obtained inhibition of α-glucosidase is presented in **table 24** and the graphical representation in **Fig. 35.**

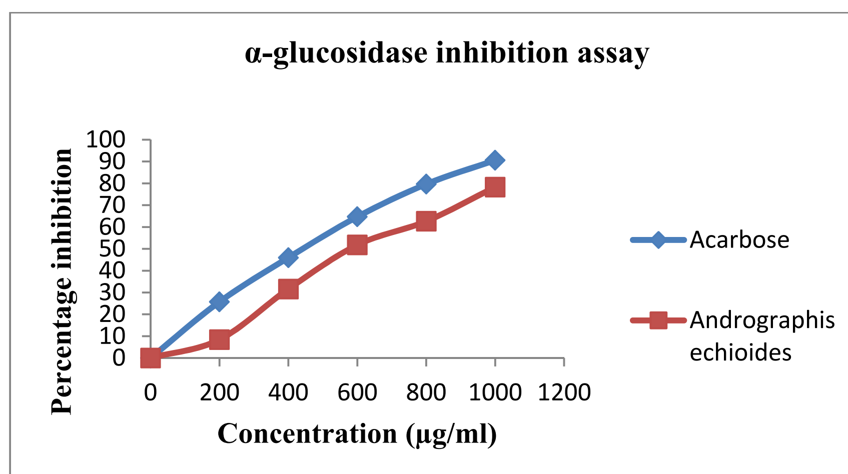
Table 24: α-glucosidase inhibitory activity of acarbose and *Andrographis echioides* extract

S. No.	Conc. in (µg/ml)	Acarbose		*Andrographis echioides* extract	
		Absorbance	% inhibition	Absorbance	% inhibition
1	200	0.83 ±0.001	25.74±0.07	0.98±0.002	8.43±0.24
2	400	0.52±0.001	45.97±0.30	0.79±0.002	31.6±0.25
3	600	0.37±0.007	64.70±0.38	0.51±0.003	51.8±0.28
4	800	0.21±0.001	79.63±0.10	0.40±0.001	62.7 ±0.21
5	1000	0.10±0.001	90.61±0.47	0.21±0.001	78.3±0.46
		IC_{50}	**487.71µg/mL**	**IC_{50}**	**636.94µg/Ml**

***mean of three readings ± SEM**

From the **Table 24**, it can be seen that the 70% ethanolic extract of *Andrographis echioides* showed a percentage inhibition of **78.3±0.46** while acarbose showed a percentage inhibition of **90.61±0.47** at a concentration of **1mg/mL.** The IC_{50} value calculated using the linear regression analysis was found to be **636.94µg/mL** and **487.71µg/mL** for ethanolic extract and acarbose respectively.

Fig.29: α-glucosidase inhibitory activity of acarbose and *Andrographis echioides* extract



SECTION D - ANTIMICROBIAL ACTIVITY

Antibacterial activity

The results obtained for susceptibility tests for extracts against various microorganisms are presented in **Table25** and the photographs of the plates are presented in **Fig. 36.** From the **table 25,** it can be seen that there is growth of organism for extract at the concentration of 200µg/disc itself except *Streptococcus pyogens* and no growth of organism in all concentration of extract and microorganism.

Table 25: Susceptibility test against microorganisms

S. No.	Name of the sample	Conc.in µg/disc	1	2	3	4	5
1	Control	-	+	+	+	+	+
2	Standard	30	-	-	-	-	-
3	Ethanolic extract of *Andrographis echioides*	200	+	+	+	-	+
		400	-	-	-	-	-
		600	-	-	-	-	-

Note: (+) indicate growth (-) indicate no growth;

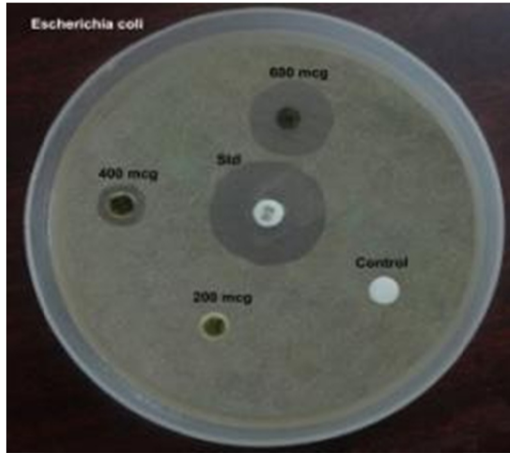
1. *E.coli,* 2.*Klebsilla spp,* 3.*Pseudomonas aeruginosa,* 4. *Streptococcus pyogens*
5. *Staphylococcus aureus.*

Minimum Inhibitory Concentration (MIC)

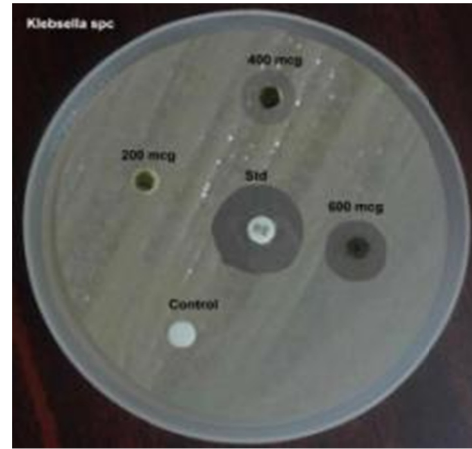
The minimum inhibitory concentration was defined as the lowest concentration of the extract that allows no more than 20% growth of microbes after incubation on agar at 37ºC for 18-48h. The minimum inhibitory concentrations for the ethanolic extract against various organisms are presented in **Table 26.** The minimum inhibitory concentration was found to be less than 200µg/disc for the all organism except *Streptococcus pyogens* was found to be less than 100µg/disc.

Fig 36: Anti-bacterial activity of ethanolic extract of *Andrographis echioides*

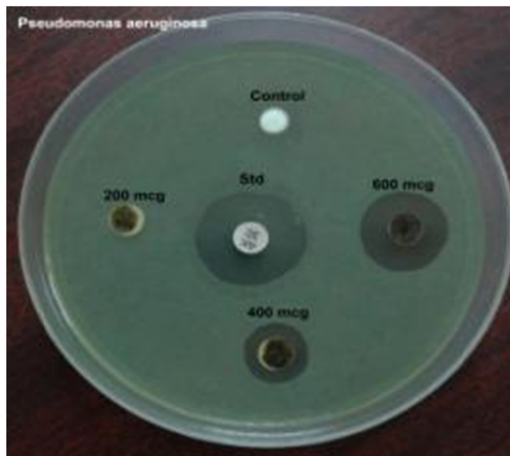
Escherichia coli



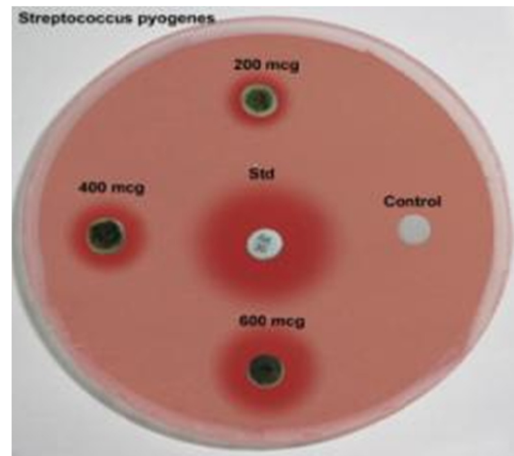
Klebsiella spp.



Pseudomonas aeruginosa



Streptococcus pyogenes



Staphylococcus aureus

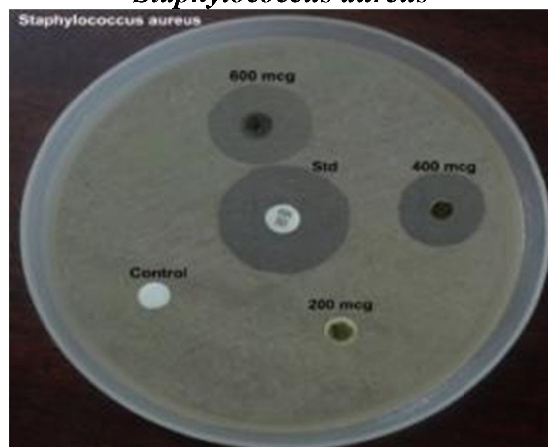


Table 26: MIC of the extract *a*gainst various microorganisms

S. No.	Name of the organism	MIC in µg/disc
1	*Escherichia coli*	< 200
2	*Klebsiellaspes*	<200
3	*Pseudomonas aeruginosa*	<200
4	*Streptococcus pyogens*	< 100
5	*Staphylococcus aureus*	< 200

Zones of inhibition

The zones of inhibition obtained for various concentrations of the ethanolic extract of *Andrographis echioides* are presented in **Table 27.** The zones of inhibition of the extract were compared with that of standard drug amikacin. The pictorial representations of the same are presented in **Fig 37.**

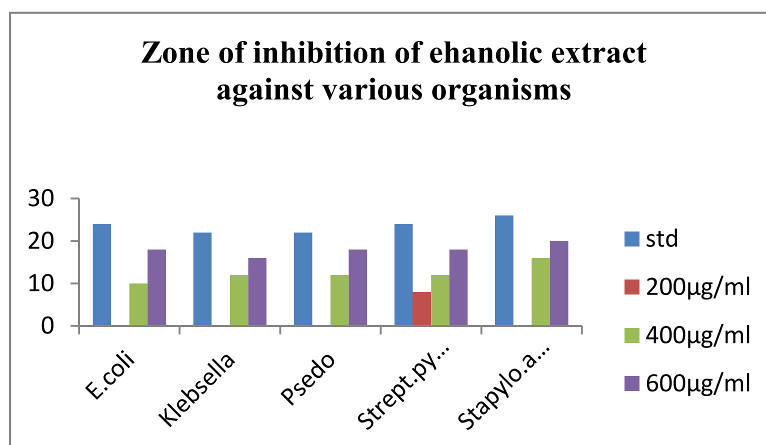
Table 27: Zone of inhibition against various microorganisms

S. No.	Name of the organism	Zone of inhibition(in mm)			
		StandardAmikacin30 µg/disc	Ethanolic extract of *Andrographis echioides*		
			200	400	600
1	*Escherichia coli*	24.0±0.5	_	10.0±0.0	18.0±0.0
2	*Klebsiellaspp*	22.0±0.0	_	12.0±0.5	16.0±0.0
3	*Pseudomonas aeruginosa*	22.0±0.0	_	12.0±0.0	18.0±0.0
4	*Streptococcus pyogens*	24.0±0.5	8.0±0.0	12.0±0.5	18.0±0.0
5	*Staphylococcus aureus*	26.0±0.0	_	16.0±0.0	20.0±0.5

***mean of 2 readings ±SEM**

The zone of inhibition of ethanolic extract of *Andrographis echioides* at **600µg** against *E. coli ,Klebsilla spp, Pseudomonas aeruginosa, Streptococcus pyogens*, *Staphylococcus aureus* was 18, 16, 18,18 and 20mm respectively.

Fig 37: Zone of inhibition of ethanolic extract against various organisms



The results showed that the extract has exhibited significant antibacterial activity against *E. coli, Pseudomonas aeruginosa, Streptococcus pyogens* and *Staphylococcus aureus* when compared to standard Amikacin. *E.coli, Klebsilla spps, Pseudomonas aeruginosa* are the chief causative organisms to produce urinary tract infection. Since the ethanolic extract of *Andrographis echioides* showed significant activity and hence could be used in the treatment of urinary tract infections.

Anti fungal activity

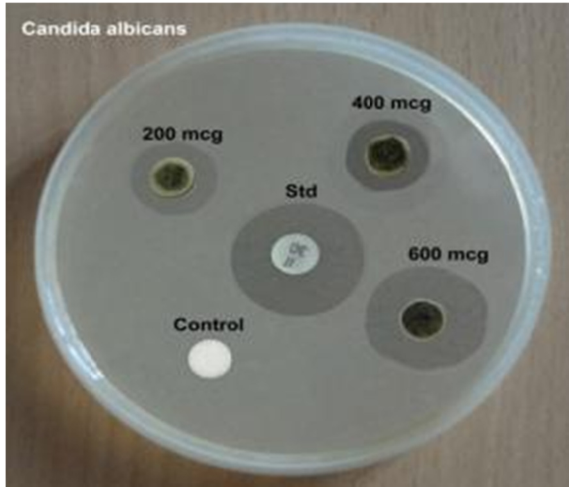
The results obtained for susceptibility tests for extracts against various microorganisms are presented in **Table 28** and photograph of the plate are presented in **Fig. 38.**

Table 28: MIC of ethanolic extract of *Andrographis echioides* against various fungal isolates

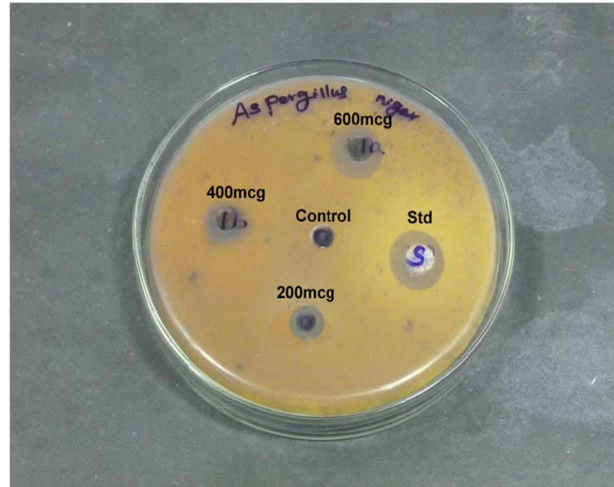
S. No.	Name of the fungal isolate	MIC of Itraconazole(µg/disc)	MIC of ethanolic extract (µg/disc)
1.	*Candida albicans*	30	< 100
2.	*Aspergillusniger*	30	< 100
3.	*Aspergillusflavus*	30	200-400

Fig 38: Antifungal activity of *Andrographis echioides*

Candida albicans



Aspergillus niger



Aspergillus flavus

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was defined as the lowest concentration of the extract that allows no more than 20% growth of microbes after incubation on agar at 37°C for 18 to 48h. The minimum inhibitory concentration against various microorganismsis presented in **Table 28**. The minimum inhibitory concentration of ethanolic extract of *Andrographis echioides* against the fungi *Candida albicans, Aspergillus niger,* found to be less than100µg/disc while for *Aspergillus flavus* it was found to be **200-400µg/disc.**

Zone of inhibition

The zone of inhibition by antibiotic disc diffusion method of ethanolic extract of *Andrographis echioides* is presented in **Table 29.** The zone of inhibition of the extract was compared with the standard drug itraconazole. The photograph of zones of inhibition obtained is presented in **Fig 38** and graphical representation present in **Fig 39.**

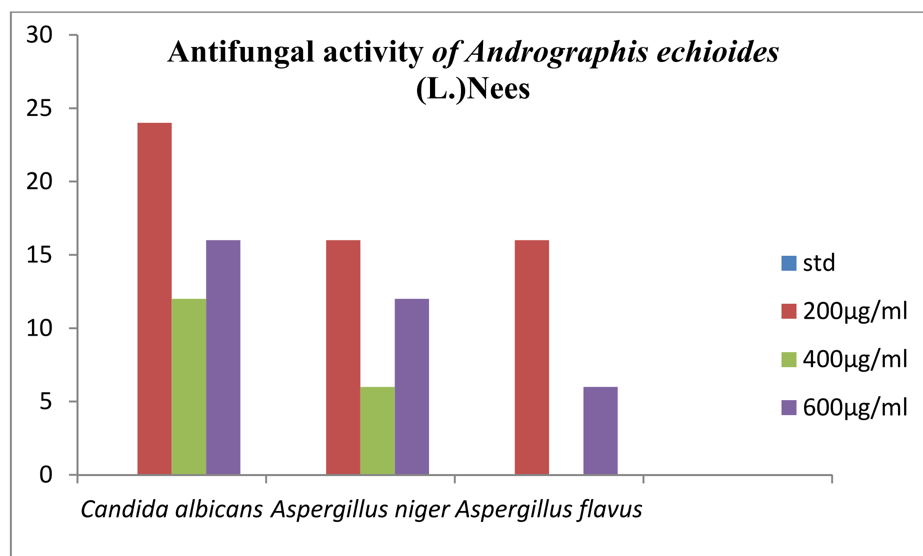
Table 29: Zone of inhibition of ethanolic extract against various micro organisms

S. No.	Name of the organism	Itraconazole	Zone of inhibition(mm)*		
			Conc.of extract in mcg/disc		
			200	400	600
1	*Candida albicans*	24.0 ± 0.0	12.0±0.10	16.0±0.1	20.0±0.2
2	*Aspergillusniger*	16.0±1.0	6.0±0.02	12.0±0.0	14.0±0.0
3	*Aspergillusflavus*	16.0±0.0	--	6.0±0.0	14.0±0.2

*** mean of 2 readings ± SEM**

From the **Table 29** the zone of inhibition of extract of *Androgrphis echioides* against various fungi tested was less than that produced by the standard drug itraconazole. The activity against fungi was moderately good.

Fig 39: Zone of inhibition of *Andrographis echioides* and Itraconazole against various organisms



SUMMARY

AND CONCLUSION

CHAPTER 8

SUMMARY AND CONCLUSION

There are many unknown plants with high medicinal value still have not recognized their importance. They have not been brought to the light scientific world. The pharmacopoeial standards in Ayurvedic Pharmacopoeia of India are not adequate enough to ensure the quality of plant materials since the material received in the manufacturing premises are not in a condition that effective microscopic examination alone will help. Recently there has been a revival of interest on medicinal plants.

In the present dissertation entitled "**Pharmacognostical, Phytochemical, and Pharmacological studies *Andrographis echioides* (L) Nees. (Acanthaceae)", a** commonly available plant has been explored for its pharmacognostic, phytochemical and pharmacological potential.

The chapter on **Pharmacognostical evaluation** deals with various parameters in order to substantiate and identify the plant for future work. This study establishes not only pharmacognostic and characterization of leaves but also microscopic and fluorescence analysis of the powder. These characteristics can be used as identification, authentication parameters of the plant.

The chapter on **Phytochemical evaluation** deals with preliminary phytochemical screening which revealed the presence of phenols, flavonoids, saponins and triterpenoids in the plant extract.

- The determination of total phenolic (**41.47mg/g**), total flavonoids (**47.515mg/g**), and total tannin content (**53.2mg/g**), total vitamin C content (**222.76mg/g**)

confirmed the significant concentration of these phytoconstituents in the ethanolic extract of *Andrographis echioides.*

- The **TLC** analysis and **HPTLC** finger print profile on the ethanolic extract of *Andrographis echioides* will help us in the identification of the plant in future.
- The determination of **elemental analysis** showed that the powder were free from heavy metal**s** like **mercury, arsenic, and cadmium and lead.** Hence the *Andrographis echioides* was free from the toxic elements and safe for use.

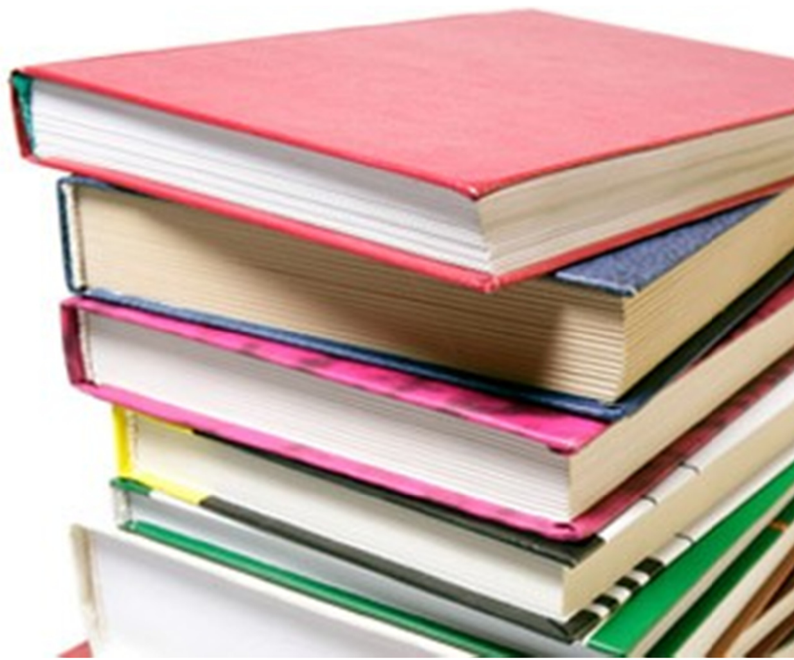
The chapter on **Pharmacolgical evaluation** deals with antioxidant activity, in vitro inhibition of calcium oxalate crystallization, in vitro antidiabetic activity and anti microbial activity.

- The Section A of this chapter deals with the anti oxidant activity. The ethanolic extract of *Andrgraphis echioides* has exhibited radical scavenging activity by **DPPH assay and Hydrogen peroxide** method shown potent anti oxidant activity.
- The Section B of the chapter deals with the effect of extract on inhibition of in vitro calcium oxalate crystallization. The extract had exhibited inhibition of **invitro calcium oxalate** crystallization at 3mg/mL.
- The Section C of this chapter deals with in vitro anti diabetic activity. The effect of the extract of *Andrgraphis echioides* is as follows
  - Significant ***in-vitro*** **Anti-diabetic activity** was exhibited by70% ethanolic extract of *Andrographis echioides* in **Non-enzymatic glycosylation of Haemoglobulin assay.** The extract *s*howed a percentage inhibition of **68.5 ± 0.11** while α-tocopherol showed a percentage inhibition of **79.95 ± 0.088** at a concentration of 100µg/mL.

- **In Glucose uptake by yeast cells method,** the percentage inhibition of extract was **73.13 ± 0.03** while acarbose showed a percentage inhibition of **78.66 ± 0.033** at a concentration of 100µg/mL at **5mM glucose concentration**.
- **In Alpha-amylase inhibition assay,** the extract showed a percentage inhibition of **66.6 ± 0.057** while acarbose showed a percentage inhibition of **75.26 ± 0.12** at a concentration of **1000µg/mL.**
- **In Alpha-glucosidase inhibition assay,** the extract showed apercentage inhibition of **78.3± 0.46** while acarbose showed a percentage inhibition of **90.61 ± 0.47** at a concentration of **1000µg/mL.**

- It can be concluded from the above that the extract of *Andrographis echioides* possess potent anti-diabetic activity.
- The Section D of this chapter deals with antimicrobial activity of the extract. A significant **anti-bacterial activity** was exhibited by ethanolic extract (**400µg/disc, 600µg/disc**) against ***Escherichia coli, Klebsella spp, Pseudomonas aeruginosa, Streptococcus pyogens*** and ***Staphylococcu saureus.*** The extract also showed significant **anti- fungal activity (400µg/disc, 600µg/disc)** against ***Candida albicans, Aspergillus niger*** and ***Aspergillus flavus***.

The ethanolic extract of *Andrographis echioides* may serve as a lead medicinal plant to synthesize various semi-synthetic drugs to treat various life threatening disease like *Diabetes mellitus,* Kidney stone, bacterial and fungal infections.



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