PHARMACOGNOSTICAL, PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Rhynchoglossum notonianum* (Wall.) B.L. Burtt - AN UNDERUTILIZED NATURE'S TREASURE

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CHAPTER – I

INTRODUCTION

Herbs have formed the basis of sophisticated traditional medicine systems throughout human history. The nature has gifted abundant plant wealth for all living creatures, which possess medicinal virtues. Present trend of wide usage of herbs is not a throwback to the Dark Ages but an indication that herbs are a growing part of modern and high-tech medicine.

Modern medicine usually aims to develop a unique single compound or a 'magic bullet' to treat a specific disease condition. Herbal medicine is a major component in all indigenous traditional medicine systems as well as in ayurvedic, homeopathic, naturopathic, oriental and Native American Indian medicine. Nowadays the herbal products symbolize safety in contrast to the synthetics that are regarded as unsafe to the human as well as the environment. A phytomedicine may be a food (including nutraceuticals) or a drug or a medical device or a cosmetic. The World Health Organization (WHO) estimates that 80% of the world populations make use of herbal medicine for some aspect of primary healthcare. [1]

IMPORTANCE AND SCOPE

Herbs are staging a comeback and herbal renaissance is happening all over the globe. The blind dependence on synthetics is over and people are returning to the naturals. Even the modern allopathic system of medicine has adopted a number of plant-derived drugs which form an important segment of the modern pharmacopoeia. Phytotherapy serves as a bridge between the traditional and modern medicine.

Some important chemical intermediates obtained from plants are needed for the manufacture of modern drugs [2] (Eg : Diosgenin in *Dioscorea deltoidea* used in the synthesis of steroidal hormones). Green plants synthesize large number of commercially important secondary metabolites and find use in a number of pharmaceutical compounds. Examples are vinblastine, taxol, podophyllotoxin, camptothecin, digitoxigenin, tubocurarine, and artemesin among others.

Plants can provide biologically active molecules and lead structures for the development of modified derivatives with improved activity and/or reduced side effects and toxicity [2]. Apart from isolated compounds, the crude extract of medicinal plants may also be used as medicaments. The isolation, identification of active principles and screening of the mode of action of a drug is of prime importance. About 121 major plant drugs have been identified for which there is no other synthetic alternative is currently available [2].

A multidisciplinary approach combining natural product with synthetic and biosynthesis methods may prove very effective. The traditional knowledge-inspired Reverse Pharmacology related to reversing the routine laboratory-to-clinic progression of new drugs to a clinic-to-laboratory progression. In this process, ***safety*** becomes as starting point and ***efficacy*** becomes a matter of validation. [3]

As believed by many analysts that the present ‘one drug fits all’ approach may be unsustainable in the future. In the management of polygenic clinical conditions, rationally designed multiherbal formulation is being developed.

ANCIENT LITERATURES [4]

The first written records documented the use of herbs in the treatment of diseases are the Mesopotamian clay tablet writings and the Egyptian Papyrus, which contains 876 prescriptions made up of more than 500 substances which includes many herbs. The Middle Eastern era is followed by the Greco-Roman era that saw the writing of the De Materia Medica, which contains about 600 plant products. The Arab medicine was built on Greco-Roman and the Text of Jami of Ibn Baiar lists 2000 substances of which many are plant products. Charak Samhita of Indian ayurveda describes 582 herbs. The classic of the materia medica of China focus on the description of individual herbs which includes 252 botanical substances. Chinese-influenced Korean medicine was adapted by the Japanese during the dynasty of Emperor Ingyo (411–453 AD).

REGULATORY STATUS

Despite a long history of successful application in treating illnesses, phytomedicine are still considered to be anecdotal and ineffective by the some regulatory agencies and allowed only as food supplements. This is because of lack of elaborative safety and efficacy studies.[4] The United States Food and Drug Administration (US FDA) released its first botanical guideline to evaluate and approve phytomedicine as both prescription and over-the-counter medicine. The WHO has released Pharmacopeial monographs on herbal medicines and Guidelines to define basic criteria for evaluation of herbal medicines.

STANDARDIZATION [4]

Herbs contain various concentrations of hundreds of constituents. The source and quality of raw crude materials play a pivotal role in guaranteeing the quality and stability of herbal preparations. Other factors includes the use of fresh/dried plant material, age and part of the plant, method of collection, period and time of collection, temperature, light exposure, water and nutrient availability, drying, packing, storage, and transportation of raw material, etc can explain why frequently the composition, quality and therapeutic value of herbal drugs varies.

The standardization of the herbal drugs is not merely analytical operations that conclude with the identification and assay of the phytoconstituent but also it expedite the total information viz., passport data of raw material, quality parameters and process controls. Apart from these factors, the method of extraction and contamination with microorganisms, heavy metals, pesticides, etc., can also modify the quality, safety, and efficacy of herbal drugs. So, pharmaceutical industries prefer using cultivated plants which show only smaller variation in their constituents instead of using wild harvested one.

Reproducible assays of the phytomedicine generate confidence in the mind of the user and prescriber. With rapid advancement of instrumentation in the industry, the chemical standardization of herbal products by hyphenated techniques (HPTLC, HPLC, LC/MS and GC/MS) has become more useful in the standardization.

ANTIINFLAMMATORY DRUGS

INFLAMMATION

Inflammation is a complex immune response to vascular tissues to a injury or infection caused by pathogens, irritants or damaged cells which clinically characterized by signs of edema, redness, pain, warmth and loss of function. The cellular mechanisms of inflammation fall into four distinct categories[5]. They are as follows:

A) Vasodilatation
B) Alterations in vascular permeability
C) Migration of WBCs to the site of inflammation
D) Phagocytosis

CLASSIFICATION OF INFLAMMATION [6, 7]

Inflammation may broadly classified into three categories. They are -

(1) Acute inflammation
(2) Chronic inflammation
(3) Miscellaneous kind of inflammation

ACUTE INFLAMMATION

Tissue injury is produced by a single exposure to non-replicating antigen, the protective phenomena results in inflammation and restoration of affected tissue to its normal healthy state. Acute inflammation is distinguished by local vasodilatation and infiltration of leukocytes from circulating blood. It lasts from few minutes to 1 or 2 days. The cardinal signs are redness, heat, swelling and pain.

CHRONIC INFLAMMATION

Chronic Inflammation is distinguished by the infiltration of mononuclear cells, proliferation of fibroblasts, collagen fibers and formation of a connective tissue which results in tumor like swelling.

MISCELLANEOUS KIND OF INFLAMMATION

This group of disorders mainly involves allergic and dermatological disorders like pemphigus, pemphigoid, discoid lupus and contact dermatitis.

PROBABLE INFLAMMENOGENIC FACTORS

1. Lysosomal enzymes
2. Prostaglandin (PGS)
3. Complement system
4. Protein breakdown process
5. Calcium influx
6. Cyclic nucleotides

MEDIATORS OF INFLAMMATION

1. Histamine
2. Bradykinins
3. Prostaglandin
4. Thromboxane A2
5. Prostcyclin
6. The Leucotrienes
7. Platelet activating factor
8. Interleukin – I

MECHANISM OF INFLAMMATION

The mechanism of inflammatory pathways classified as follows:

1. Arachidonic acid (AA) dependent pathway which include cyclooxygenase (COX), lipoxygenase (LOX) and phospholipase A2 (PLA2) as mediators

   and

2. AA-independent pathway which include nitric oxide synthase (NOS), NF-*k*B, peroxisome proliferator activated receptor (PPAR) and NSAID activated gen-1 (NAG-1)

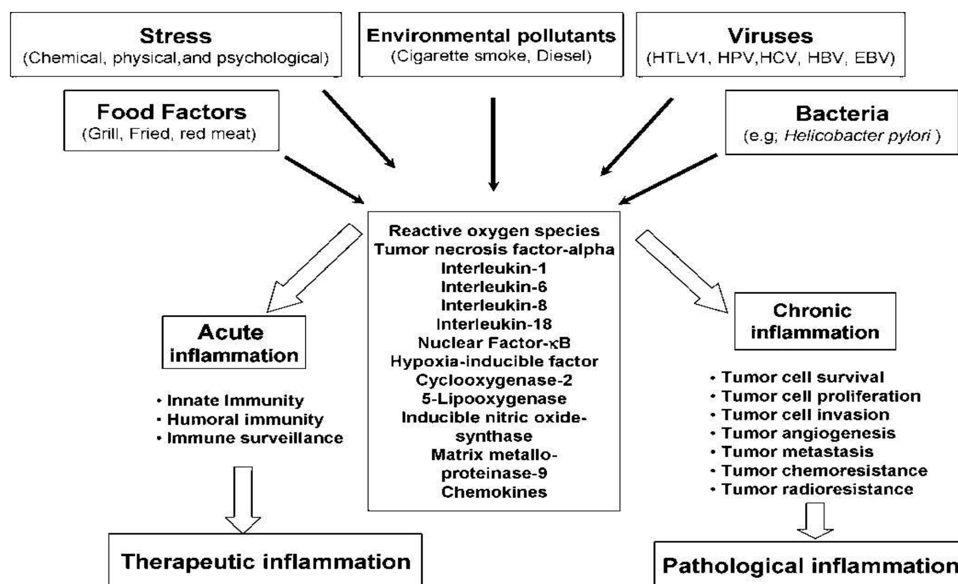
DIAGNOSIS

i) Increased total leukocyte count

ii) Increased differential leukocyte count

iii) Elevated erythrocyte sedimentation rate

iv) Elevated C-Reactive protein



Flow chart showing types of inflammation and its triggering factors [8]

PHARMACOLOGICAL MANAGEMENT

a) Non selective COX inhibitors

b) COX-2 Selective inhibitors

c) Dual inhibitors of 5-LOX and COX

d) Steroidal anti-inflammatory drugs

e) Disease modifying antirheumatic drugs

f) Herbal formulations

NON PHARMACOLOGICAL MANAGEMENT

a) Dietary manipulation

b) Ice and heat treatment

ANTIINFLAMMATORY HERBAL REMEDIES

1) *Boswellia serrata*

2) *Curcuma longa*

3) *Ficus racemosa*

4) *Ocimum sanctum*

5) *Hypericum perforatum*

6) *Artemesia species,*

Etc.,

INFLAMMATION MEDIATED DERMATOLOGICAL INFECTIONS [9-12]

It was known by detailed studies that those plant with some pharmacological activity often have a significant level of some constituents. To name few –

Pharmacological activity	Responsible phytoconstituent
Anti-inflammatory	Flavonoids
Firming and toning of the skin	Tannins
Wound healing (Cicatrization)	Sterols

Often the skin healing process is interrupted by opportunistic infections. In such cases, the use of the above phytoconstituent-rich plants can provide a solution for a complex array of microbial and fungal infections. [9]

The inflammatory skin diseases produce marked variations which generally of papulosquamous in nature. Inflammation and erythema are common in acne, lichen planus, psoriasis, and pityriasis rosea. [10, 11]

Free radical oxidative stress is involved in the pathogenesis of a wide array of human diseases including inflammatory skin diseases. These conditions treated with the traditional medicine are partly justified by detailed studies. [12]

RHEUMATOID ARTHRITIS [13-15]

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory autoimmune disease preferably affecting the synovial joints and periarticular tissues. Systemic manifestations of RA include severe crippling deformities and functional disabilities. The joints generally affected were neck, shoulder, elbows, hip, knees and ankles.

RA is classified as an inflammatory arthritis, the disease comprises of three basic inter linked processes [16] like

1. Inflammation
2. Synovial proliferation and
3. Joint tissue destruction.

EPIDEMIOLOGY [17]

About 1% of the world's population is affected by rheumatoid arthritis, out of which women three times more often than men. 60% of the patients showed functional disability after ten years of the onset of the disease and more than 50% of the patients showed severe disability or even death after 20 years of onset of disease.

ETIOLOGY

- Etiology is not well known
- Due to the effect of microbial agents in immunogenetically predisposed individuals
- Due to the firm existence of infectious agents viz., *Mycoplasma, Epstein Barr Virus (EBR), Cytomegalovirus (CMV) or Rubella.*

SIGNS AND SYMPTOMS

- Pain and swelling of joints in symmetrical fashion
- Onset of disease begins with fatigue, weakness, morning stiffness
- Arthralgia, myalgia, redness and difficult in movement
- Rheumatoid nodules close to the synovial joints

DIAGNOSIS

- Rheumatoid Arthritis Factor (RAF) in blood
- Radiological abnormalities like narrowing of joint space and deviation of fingers and wrist are seen in x-ray diagnosis
- Laboratory findings include mild normocytic, normochromic anaemia, elevated ESR, mild leucocytosis and hypergamma globulinemia
- CCP (Anticyclic citrullinated peptide) antibody test.

TREATMENT

- For symptomatic relief, Non-steroidal anti-inflammatory drugs (NSAID) were used.
- Disease modified anti arthritic drugs (DMARD) includes – Gold compounds, d-Pencillamine, Chloroquine, Sulfasalazine, Leflunomine, and Immunosuppressant.

HERBAL REMEDIES FOR RHEUMATOID ARTHRITIS

- *Aconitum napellus*
- *Alpinia officinarum*
- *Ficus benghlensis*

- *Menthe arvensis*
- *Capsicum annum*
- *Trachyspermum roxburghianum*

  Etc.,

STREPTOCOCCAL DISEASES

Streptococcal disease is a common bacterial infection caused by group - A (beta-haemolytic) streptococcus. These streptococcal diseases can occasionally lead to serious complications like rheumatic fever and kidney disease (glomerulonephritis).

Streptococcus pyogenes

1. Pyogenic infection
    - Respiratory tract - acute tonsillitis or pharyngitis and scarlet fever
    - Skin infection - infection of wound, burns etc.
    - Deep infection - bone and joint infection, septicemia, abscess in internal organs.

2. Non-suppurative complications
    - Rheumatic fever
    - Acute glomerulonephritis.

RHEUMATIC FEVER [18-20]

Rheumatic fever is a systemic, post-streptococcal non-suppurative inflammatory disease, principally affecting the heart, joints, central nervous system, skin and subcutaneous tissues. The chronic stage of rheumatic fever involves all the layers of the heart (pancarditis) leading to rheumatic heart diseases (RHD).

EPIDEMOLOGY

Streptococcal infection appears more frequent in children between 5 and 15. It prevails in developing countries like India, Pakistan, parts of Africa and South America. About three-fourth of rheumatoid fever patients have arthritis and two-third of patients have carditis.

ETIOLOGY

Certain strains of beta hemolytic streptococcus cause pharyngitis and provoke the necessary antibodies for the causation of rheumatic fever.

ANTIOXIDANTS

Antioxidants are any substance which, when present in lower concentrations compared to those of oxidisable substrates, significantly delay or inhibit oxidation of these substrates.

REACTIVE OXYGEN SPECIES [21]

Life on the earth survives only due to the presence of essential element, oxygen which gives us energy by oxidation of food products which is essential to perform biological functions. During this oxidation process, highly reactive and

potentially damaging Reactive oxygen species **(ROS)** and reactive nitrogen species **(RNS)** are continuously generated in the human body. Increased production of ROS and RNS formed during metabolism or by the action of ionizing mediators, can interact with biomolecules and leads to an onset of variety of diseases including cancers, inflammation, neurodegeneration, Parkinson's disease, atherosclerosis and premature ageing, cardiovascular diseases and other illnesses.

MECHANISM OF ROS TOXICITY [21]

ROS are the mediators of inflammation. ROS, on interaction with platelets, neutrophils, macrophages and other cells results in the synthesis of eicosanoids followed by the activation and release of various cytokines, which in turn propagating the inflammatory process.

The molecular mechanism is as follows:

- Oxidation of vital thiol compounds to disulphides
- Loss of tissue GSH
- Impairment of energy generation
- Inhibition of calcium influx and electrolyte homeostasis
- Oxidation of cytochromes
- DNA strand cleavage
- Initiation and promotions of mutations and carcinogenesis

ROS mediated inflammation is involved in the pathogenesis of infectious disease including tuberculosis, septic shock, rheumatoid arthritis, Inflammatory bowel disease, cancer, atherosclerosis, hepatitis and even Alzheimer's dementia.

TYPES OF REACTIVE OXYGEN SPECIES

Reactive oxygen species	Types	Symbol
Radicals	Superoxide Hydroxyl Alkoxyl Peroxyl Nitric oxide Thioyl radical	O_2^{*-} $^{*}OH$ RO^{*} ROO^{*} NO^{*} $R\text{-}S^{*}$
Non-radicals	Hydrogen peroxide Hypochlorous acid Ozone Singlet oxygen Peroxynitrite Lipid peroxide	H_2O_2 HOCl 0_3 $^{1}O_2$ $ONOO^{-}$ LOOH

INFLAMMATION AND CANCER [22-24]

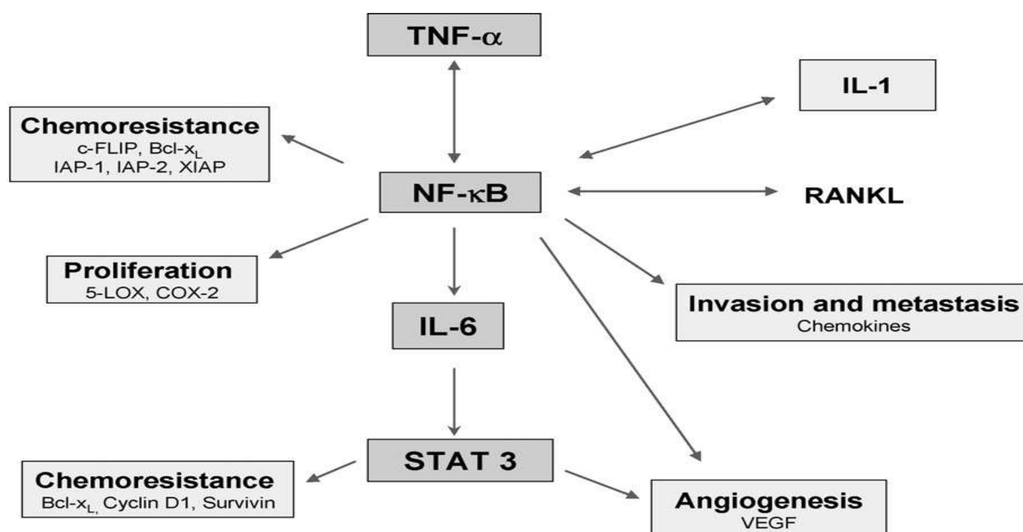
Chronic inflammation has been found to propagate a wide variety of illnesses, including cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer’s disease, pulmonary diseases, and autoimmune diseases, etc.,[22]. Chronic inflammation has been linked to various steps involved in the formation of tumor including cellular transformation, promotion, survival, apoptosis, proliferation, invasion, angiogenesis, and metastasis [23, 24]. There has been new realization provide evidence for a strong link between this type of inflammation and many type of cancers. The various

inflammatory mediators are responsible for the steps leading to tumorogenesis, their growth and metastasis.

Table showing Relationship between inflammation and cancer

INDUCER	INFLAMMATION	CANCER
Tobacco smoke	Bronchitis	Lung cancer
Helicobacter pyroli	Gastritis	Gastric cancer
Hepatitis B & C virus	Hepatitis	Hepatocellular carcinoma
Gastric acid & Alcohol	Oesophagitis	Oesophageal cancer
Asbestos fibers	Asbestosis	Mesothelioma
UV Light	Sunburn	Melanoma

Flow chart of inflammatory networking in cancer[7]



HEPATIC DISEASE [25, 26]

Hepatic disease is a collection of clinical conditions, diseases, and infections that target the cells, tissues, structures, or functions of the liver.

TYPES OF LIVER DISEASE

a) Necrosis

b) Cirrhosis

c) Hepatitis - Viral, toxic or of deficiency type.

d) Hepatic failure

e) Chemical or Drug induced hepatotoxicity

- Hepatitis
- Jaundice

  and
- Carcinogenesis.

g) Liver disorders due to altered metabolic function. It associated with fat and bilirubin metabolisms.

SIGNS AND SYMPTOMS OF THE LIVER DISEASE

- Yellowing of the skin and eyes
- Darkened urine and light colored stools
- Nausea and vomiting with loss of appetite
- Drastic changes in weight
- Diarrhoea
- Abdominal swelling and pain
- Malaise, fatigue
- Generalized prolonged itching

- Mental confusion
- Varicose veins
- Hypoglycemia
- Low grade fever with muscle pain

THERAPY/TREATMENT

(A) Therapy for liver disease

(1) Allopathic treatment

(i) Ursodeoxycholic acid

(ii) Penicillamine

(iii) Other drugs include alpha interferon, ribavirin, lamivudine, steroids and antibiotics

(2) Herbal treatment:

1. Anti hepatotoxic agents
2. Hepatotropic agents
3. Hepatoprotective agents

SOME HERBAL HEPATOPROTECTIVE DRUGS [27]

a) Aegle marmelos

b) Annona squamosa

c) Chamomile capitula

d) Coccinia grandis

e) Ficus carica

f) Flacourtia indica

g) Phyllanthus niruri

Etc.,

CHAPTER-II

LITERATURE SURVEY

The literature survey of the ***Rhynchoglossum notonianum* (Wall.) B.L. Burtt** (Synonyms: *Wulfenia notonianum, Klugia notoniana*), Family: Gesneriaceae carries high potential uses especially for the treatment of polio and inflammation, but the proper scientific studies have not been reported for this plant. As there is no pharmacognostic, phytochemical and pharmacological work of this traditionally much valued drug, the present work was taken up with a view to lay down the standards, which could be very useful to detect the authenticity of this underutilized medicinally useful plant.

ETHANOMEDICAL USES

AERIAL PARTS

1. Dr. R. Sathyavathi et al (2007) recorded the usage of aerial parts of *Rhynchoglossum notonianum* (Synonym : *Klugia notoniana, Wulfenia notonianum*) as a remedy to treat ailments like polio and swelling in the folklore medico-botanical investigation.[28]
2. Vishal Gupta (2005) documented the utilization of leaf juice of *Rhynchoglossum lazulinus* A.S. Rao & J. Joseph as an appetizer by the tribes of Arunachal Pradesh.[29]

ROOTS

1. Chinese website reported the usage of dried roots of *R. lazulinu*s for softening of hardmasses, elimination of nodulation and in the treatment of goiter.[30]

PHARMACOGNOSY

1. Mayer V (2003) analyzed the systematic position and generic differentiation of the morphologically and geographically outstanding tribe of Epithemateae (Gesneriaceae). [31]
2. Moller M (2003) evaluated the current taxonomic treatments, phylogenetic relationships and a website has been introduced for access to the data pertaining to the Cytology on Gesneriaceae. [32]

PHYTOCHEMISTRY

1. G. M. Fu et al (2008) summarizes the major development in research and structural activity relationship analyses of Phenylethanoid glycosides. [33]
2. Ulrike W. Arnold et al (2002) extracted, isolated and elucidated the structure for three Phenylpropanoid and three Iridoid glycosides from the methanolic extract of *Wulfenia carinthiaca.* [34]

PHARMACOLOGY

1. Rinaldo Cervellati et al (2004) reported the three phenylpropanoid glycoside from *Wulfenia carinthiaca* showed a very high antioxidant activity and confirmed the antinociceptive and antiedematogenic activity in the adopted models like acetic acid – induced writhing and paw edema induced by carrageenan, respectively. [35]
2. G. M. Fu et al (2008) analyzed the structural activity relationship of phenylethanoid glycosides obtained from *Wulfenia carinthiaca,* which pharmacologically possess a broad array of biological activities including antibacterial, antitumor, anti-inflammatory, antioxidant, hepatoprotective, antiviral, neuroprotective, immunomodulatory and tyrosinase inhibitory actions. [33]

CHAPTER-III

AIM AND SCOPE OF THE PRESENT STUDY

***Rhynchoglossum notonianum* (Wall.) B.L. Burtt** (Gesneriaceae) is a succulent herb belonging to the family Gesneriaceae native to Asia. This plant is used in Indian traditional medicine as a underexplored remedy to treat some ailments.

The ethnomedical information revealed that aerial parts of this plant are employed in the treatment of polio and swelling. The pharmacognostical, phytochemical and pharmacological activity screening studies have not yet been reported so far on the leaves and stems of *Rhynchoglossum notonianum.* Hence, the present work has been designed in such a way as to carry out the following studies on 70% ethanolic extract of the leaves and stems of *Rhynchoglossum notonianum* **(ERN)**.

- Pharmacognostical studies of the leaves and stems
- Preliminary phytochemical screening of ERN
- Estimation of total pheonlic content
- Estimation of total flavonoid content
- Estimation of flavonol content
- Estimation of total tannin content
- Estimation of proanthocyanidins
- Estimation of Lycopene and chlorophyll
- Phytochemical evaluation of extract by Thin Layer Chromatography (TLC) using various mobile solvent systems to record the chromatogram for the future studies.

- Phytochemical evaluation of extract by High Performance Thin Layer Chromatography (HPTLC) finger printing.
- Screening of the ERN extract of leaves and stems for the following pharmacological activities.
  - ✓ *Invitro* antioxidant activity using following tests -
    - DPPH radical scavenging activity
    - Peroxide ion scavenging activity
    - Nitric oxide scavenging activity
    - Ferric reducing power assay
  - ✓ *Invitro* antiarthritic activity
  - ✓ *Invitro* anti-inflammatory activity using HRBC membrane stabilization
  - ✓ *Invitro* hepatoprotective activity by amelioration of Arsenic trioxide induced hepatotoxicity in chicken livers
  - ✓ Anticancer activity by following methods :
    - *Invitro* anti-mitotic assay using green gram model
    - *Invitro* cytotoxic activity screening by MTT assay using HeLa cell lines
  - ✓ *Invitro* antibacterial activity against following microorganisms using agar well diffusion method
    - *Streptococcus pyogenes*
    - *Pseudomonas aurogenosa*
    - *Stapylococcus aureus*
    - *Escherichia coli*

CHAPTER-IV

PHARMACOGNOSTICAL STUDIES

Evaluation of crude drug means evidence of its identity, determination of its purity, quality and detection of nature of adulteration. The reasons for the evaluation are biochemical variations in the drug due to various agro climatic conditions, deterioration of drug due to treatment, storage, substitution and adulteration as a result of carelessness, ignorance or fraud [36]. Evaluation of crude drugs can be classified into morphological, microscopical, physical, chemical and biological evaluation. The process of evaluation is termed as standardization [37].

Organoleptic evaluation [38]

It refers to the evaluation of drug by means of using our sense organs for detecting the color, odour, size, shape, taste and special features including touch, texture etc., of the plant organs, since the majority of the information on the identity, purity, quality of the material can be drawn from these observations.

MORPHOLOGICAL STUDIES

Morphology is the study of the form of object, whilst **morphography** is the description of that form. Macro or gross morphology is the study and description of the whole drug. **Cytomorphology** is the study of morphological characteristics of particular cells.

Microscopical evaluation: It is essential because botanical identity of the phytodrug is essential prerequisite for undertaking the analysis of medicinal properties of any plant. 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 there by

confirm the structural details of the plant drugs under evaluations. It can also be used in the determination of the optical as well as micro chemical properties of the crude drug specimens under study.

SECTION – A
PLANT PROFILE

BIOLOGICAL SOURCE : *Rhynchoglossum notonianum* (Wall.) B.L. Burtt

FAMILY : Gesneriaceae

Rhynchoglossum notonianum is a succulent herb belonging to the family Gesneriaceae.[39]

SYSTEMATIC POSITION [40]

Kingdom	:	Plantae
Phylum	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Scrophulariales
Family	:	Gesneriaceae
Genus	:	*Rhynchoglossum*
Species	:	*notonianum*

SYNONYMS [41]

East Indian Klugia

Klugia notoniana

Wulfenia notoniana

VERNACULAR NAMES [40, 41]

English	:	East Indian klugia
Hindi	:	Neel
Malayalam	:	Verilla
Marathi	:	Neel
Sinhala	:	Diya – nilla, Nari Nakuta
Tamil	:	Neer Sambirani

GEOGRAPHICAL DISTRIBUTION [39]

Rhynchoglossum notonianum is distributed in countries like India (northern and north-western region), Srilanka, South China to New Guinea and Central America which includes mainly Mexico, Colombia and Venezuela.

HABIT AND HABITAT OF PLANT [42]

Rhynchoglossum notonianum is an annual succulent herb, with a creeping habit **(Fig. 1.1)**. It is found along the sides of the streams and shady, wet rocks (preferably limestone) of evergreen and semi evergreen forest is the natural habitat. It also occurs in shady, wet and open places usually in the lowlands.

MORPHOLOGICAL STUDIES ON *Rhynchoglossum notonianum*

PLANT DESCRIPTION [39-41]

Rhynchoglossum notonianum is perennial or annual monocarpic herbs. The whole plant is more or less hairy (**Fig. 1.4**). It has sparse indumentum often branched, multi-celled, non-glandular hairs.

Stems are terete, fleshy, succulent and usually branched **(Fig. 1.3)**. Leaves are alternate, shortly petiolate and leaf bases are unequal, one half cordate and the other half is sinuate-attenuate **(Fig. 1.5** and **1.6)**. The texture of leaf is thin and delicate. Lamina is obliquely ovate-cordate. Inflorescences of *R. notonianum* are terminal on main and the side branches.

Deep blue colored flowers **(Fig. 1.2)** are borne in unilateral racemes with two rows of flowers and pointed downwards. Flowers of *R. notonianum* are more than an inch long and large, very unequally two-lipped in which upper lip is shorter and 2-toothed whereas lower lip is much larger, round or elongate, with two cavities near the base. Four stamens are present. Ovary is globose-ovoid and immersed in a fleshy cup. Fruits are globose or ovoid; capsule was included in the calyx, dehiscing loculicidally by two valves.

Flowering season : August-November.

SECTION - B

MICROSCOPICAL STUDY OF THE LEAF AND STEM [42, 43, 44]

MATERIAL AND METHODS:

COLLECTION OF SPECIMEN

Plants were collected from Palaruvi in Pothigai hills near Sengottai, Tirunelveli District of Tamil Nadu and identified by Dr. Sasikala Ethirajulu, Botanist, Siddha Central Research Institute, Chennai – 106. Care was taken to select healthy plants and normal organs for the study. The required samples of stem, petiole and leaf were cut and removed from the plant, washed and fixed in FAA solution (Formalin: Acetic acid: 70% ethanol in the ratio of 5: 5: 90). After 72 hours of fixing, they were washed in water and dehydrated with graded series of tertiary butyl alcohol (TBA) as per the schedule given by Sauss, 1940. Paraffin wax (melting point 58°-60°C) was infiltered gradually till the super saturation of TBA solution and the specimens were embedded in wax for sectioning.

SECTIONING

The paraffin embedded specimen was sectioned with the help of rotary microtome. The thickness of the sections was 10-12µm, dewaxing of the sections was carried out by the customary procedure.[43] The sections were stained with toluidine blue.[45] Since toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with safranin and fast-green and iodine.

For studying the stomata morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid [43] were prepared. Glycerin mounted temporary preparations were made for macerated/ cleared material. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerin medium after staining. Different cell component were studied and measured.

Sections were stained with toluidine blue. All slides, after staining in toluidine blue were dehydrated by employing graded series of ethyl alcohol (70 %, 90%, 100% alcohol) and xylol-alcohol (50-50) and passed through xylol and mounted in DPX mountant[44].

Clearing of leaves for studying stomatal number and stomatal index was done by using 5% sodium hydroxide along with chlorinated soda solution supplemented with gentle heat. Quantitative microscopy was carried out and values were determined [46, 47]. Photomicrographs were taken with the help of Nikon Eclipse E200 Microscope. Leaf impression method was also carried out to study the type and density of stomata adopting the following procedure.

STOMATAL STUDY BY LEAF IMPRESSION METHOD [48, 49]:

Both sides of the leaf were painted with a thick patch of clear nail polish and it was allowed to dry. Clear package sealing tape of $1cm^2$ dimensions was stuck on the nail polish patch and peeled gently. A cloudy leaf impression was observed on the tape piece. Leaf impression thus obtained was kept on a clean glass slide and excess tape was trimmed out using scissors. Leaf impression was observed under a light

microscope to atleast 400X power and the stomata were observed. Microphotographs were taken to record the type and density of the stomata on either surface of the leaf.

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. Magnification of the figures was indicated by the scalebars.

STEM:

Transverse section of stem (**Fig. 2.1**) is circular to oval in shape. Epidermis is madeup of single layer of rectangular cells. Hypodermis is composed of 3 to 5 rows of radially elongated parenchyma cells of which the outermost layer is larger in size. Medullary bundles are scattered in the ground tissue. The ground tissue is parenchymatous and madeup of large, thin walled parenchyma cells (**Fig. 2.2**).

The ground tissue is finely divided by an ill-defined endodermis and pericycle (**Fig. 2.3 and 2.4**) which are small parenchymatous cells. These two layers are not distinguishable in mature stems. Secretory canals of schizogenous origin with resinous contents are present accompanying the xylem in the medullary bundles.

PETIOLE:

Transverse section of petiole is oval in shape (**Fig. 3.1**). Epidermis is single layered and made up of small rectangular cells. It is followed by hypodermis composed of 3 to 5 layers of parenchyma cells. The ground tissue is made up of thin walled, parenchyma cells. A large number of separate vascular strands are scattered in the ground tissue (**Fig. 3.2**). Secretory canals of variable diameter with resinous

contents accompany the xylem of the vascular bundles. Secretory canal is lined by a single layer of epithelial cells (**Fig. 3.2**).

T.S. OF LEAF:

Midrib:

Transverse section of leaf shows a hump on the adaxial surface and convexity on the abaxial surface (**Fig. 4.1**). The hypodermal region is composed of 2 to 3 rows of collenchyma cells. The ground tissue is parenchymatous and madeup of thin walled polygonal cells. 5 meristeles are situated at the central region of the ground tissue. Larger veins have 2 collateral vascular bundles, with the xylem groups directed towards one another (**Fig. 4.2**).

Lamina:

Leaf is dorsiventral in nature (**Fig. 4.1** & **4.2**). Epidermis is single layered, made up of small rectangular cells. Palisade tissue **(Fig. 4.3**) is composed of a single row of columnar closely arranged cells. It is followed by 3 or 4 rows of loosely packed spongy tissue. Cystoliths are confined to the lower epidermis. They vary in shape either spherical or ellipsoidal. Stomatal index for lower epidermis was found to be 8 – 11; palisade ratio was calculated as 2 to 3 and the vein islet number was found to be in the range of 2 - 4 (**Fig. 6.1**).

Epidermis in surface view

Adaxial foliar epidermis is polygonal in shape with slightly wavy walls and (**Fig. 5.1**) possess a little number of stomata (**Fig. 7.1**).

Abaxial foliar epidermal cells have wavy contour and perforated by numerous cruciferous stomata (**Fig. 7.2**). Cystoliths are seen (**Fig. 6.2**) in abaxial foliar epidermal cells.

SECTION – C

POWDER MICROSCOPY

POWDER PREPARATION:

The dried leaves and stems were pulverized in a dry mortar and pestle, followed by sieving with a nylon mesh to remove larger particles. The fine powder thus obtained was observed under microscope. The following were the dominant components in the powder analysis.

VASCULAR ELEMENTS:

1) **The xylem vessels:**

   Xylem vessels **(Fig. 8.1)** are the predominant elements in the leaf powder. They are characteristic of having spiral thickenings with horizontal slits connecting the adjacent cells. The spiral thickening gives the striking appearance in the leaf powder.

2) **The xylem fibers:**

   Next to the vessels, the fibers appear in large number. They are relatively longer than the vessels but thinner. Often the fibers occur as a multiple strands **(Fig. 8.2)**. Extraxylary (phloem) fibers of lignified and non-lignified nature also found (**Fig. 13).**

TRICHOMES:

There are predominantly larger trichomes in the leaf powder. They are characteristic in having angular shape resulting in a pyramid like appearance in the shorter trichomes with one or two cells. There are two types of trichomes viz., the

glandular and non-glandular. The non-glandular trichomes **(Fig. 10)** are showing continuous growth as the top most cells are sharp in shape. The glandular trichome **(Fig. 9)** terminates its growth on its tip cell which gets converted to a round shaped gland.

STOMATA:

The stomatal remains are also found in the leaf powder which is accompanied by the epidermal and stomatal guard cells **(Fig. 11)**. The stomatal guard cells are typical kidney shaped with starch content within them, whereas other epidermal peeling does not show any starch content.

MESOPHYLLS:

The mesophyll tissue **(Fig. 12.1)** is also found in bunches of varying size according to their degree of dryness and powdering.

SECTION - D

QUANTITATIVE MICROSCOPY [50]

DETERMINATION OF LEAF CONSTANTS:

The vein islet number, vein termination number, stomatal number and stomatal index were determined on fresh leaves using the standard procedures laid down for the purpose.

A. Vein islet number and vein termination number [38, 50]

The term "vein islet" is used to denote the minute area of photosynthetic tissue encircled by the ultimate division of the conducting strands. The number of vein islets per square mm area is called vein- islet number.

Vein termination number may be defined as the number of vein terminations present in one square mm area of the photosynthetic tissue.

Determination of vein islet number and vein termination number:

The fragment of leaf lamina with an area of not less than 1 sq. mm excluding the midrib and the margin of the leaf was taken. The fragments of leaf lamina were cleared by heating in a test tube containing chloral hydrate solution on a boiling water bath until clear. The cleared fragments were stained with safranin solution and a temporary mount was prepared with glycerol solution. The stage micrometer 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 including those over lapping on two adjacent sides and excluding those intersected by others two sides were counted. The results obtained in the number of vein islets and terminals in one sq. mm were tabulated in **Table - 1**.

A. Stomatal Number:

The average number of stomata per square mm area of epidermis of the leaf is called stomatal number [50].

Determination of Stomatal Number:

To study the stomatal morphology (type of stomata), stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling by partial maceration employing the Jeffrey's maceration fluid [43].

A fragment was transferred in to microscopic slide and the mount of lower and upper epidermis was prepared with a small drop of glycerol solution at one side of the cover slip to prevent the slide from drying. The slide was examined under 45X objective and 10X eye piece to which a microscopical apparatus was attached. Circle (O) like mark was marked on the drawing paper for each stoma. The average number of stomata/mm^2 for each surface of the leaf was calculated and their values are tabulated in **Table - 1.**

B. Stomatal Index:

It is defined as the percentage which the numbers of stomata form to the total number of epidermal cells, each stoma being counted as one cell.

Stomatal index,

$$S.I = \frac{S}{S + E} \times 100$$ ……▸ **Formula** (I)

Where,

S = Number of stomata per unit area

E = Number of epidermal cells in the same unit area

Determination of Stomatal Index:

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 drawing paper. The stomatal index was calculated by using the **Formula – 1** mentioned above. The values are tabulated in **Table – 1.**

C. Determination of Palisade Ratio

Palisade ratio is the average number of palisade cells under one epidermal cell. It is another important criterion for identifications and evaluations for crude drugs. Since it is constant for a plant species which is useful to differentiate the species and does not altered based on geographical variation [38, 50].

Epidermal peeling was done by partial maceration by Jeffery's maceration fluid [43] were prepared. A fragment was transferred into a microscopical slide and the mount of upper epidermis was prepared with a small drop of glycerol on one side of the cover slip to prevent the preparation from drying. The same was examined under 45X objective and 10X eye piece.

Four adjacent epidermal cells were traced; focusing gently downward to bring the palisade cells into view and sufficient palisade cells to cover the outlined four epidermal cells were then traced. The palisade cells under the epidermal cells were counted and calculate the palisade ratio by using the following formula and the results were tabulated in **Table - 1.**

$$\text{Palisade ratio} = \frac{\text{Average number of palisade cells beneath the 4 epidermal cells}}{4}$$

TABLE - 1 : QUANTITATIVE MICROSCOPICAL PARAMETERS OF THE LEAF OF *Rhynchoglossum notonianum*

S. No.	Parameters	Values obtained*
01	Stomatal number in upper epidermis	6.6 ± 0.37
02	Stomatal number in lower epidermis	83.9 ± 2.28
03	Stomatal index in upper epidermis	18.26 ± 0.79
04	Stomatal index in lower epidermis	29.17 ± 1.23
05	Vein islet number	2.5 ± 0.22
06	Vein termination number	2.3 ± 0.15
07	Palisade ratio	2.5 ± 0.26

***Mean of 10 readings ± SEM**

SECTION - E

PHYSICAL PARAMETERS [46, 50-53]

STANDARDIZATION PARAMETERS

The evaluation of ash values, determination of moisture content, 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 and further evaluation of different parameters indicate their acceptability. The procedures recommended in Indian Pharmacopoeia, 1996 and WHO guidelines, 1998[46, 53] were followed to calculate the total ash, water-soluble ash, acid-insoluble ash and loss on drying at 110°C. The percentage of extractive values for different solvents was also determined.

I. ASH VALUES [46, 53]

The ash values were determined by using air dried powdered leaves as per the official method.

(A) Total ash :

2 grams of the crude leaf powder was accurately weighed in a tarred nickel crucible. The ground drug was scattered in a fine even layer on the bottom of the crucible and incinerated by gradually increasing the heat not exceeding 450˚C [dull red heat] until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried drug was calculated. The results were represented in **Table - 2**.

(B) Acid insoluble ash:

The ash obtained from the total ash was boiled for 5 minutes with 25ml of 2M hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, ignited to constant weight, cooled in a desiccator and weighed. The percentage of acid insoluble ash with reference to the air – dried drug was calculated. The results were represented in **Table - 2**.

(C) Water soluble ash:

For water soluble ash, ash obtained from the total ash was boiled with 25mL of distilled water. The insoluble matter was collected in a Gooch crucible, washed with hot water ignited to a constant weight. Cooled in a desiccator and weighed. The weight of the insoluble matter was subtracted from the weight of the total ash. The differences gave the weight of the water soluble ash. It was calculated with reference to the air-dried powder. The results were represented in **Table - 2**.

II. Loss on drying [46, 53]

For the determination of loss on drying, the method described by Wallis was followed. 2 grams of the powdered crude drug was accurately weighed in a tarred dish and dried in an oven at 100˚ C. It was cooled in desiccators and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken and presented in **Table - 2**.

III. Determination of Volatile Oil [53]

Volatile oils are characterized by their odour, oil like appearance and also it has ability to volatilize at room temperature. Chemically they are mixtures of monoterpenes, sesquiterpenes and their oxygenated derivatives. Volatile oils can be estimated by hydro distillation method.

An accurately weighed 100g of plant material was crushed and added to the flask containing distilled water upto one third of the plant material was immersed and few pieces of porcelain beads were added. The flask containing liquid was heated until it boils. After 3hrs, heating was stopped and the collected oil was measured on the graduated receiver tube. Oil content of the plant material was calculated in mL/100g of plant materials. The result was presented in **Table - 2**.

IV. Determination of foreign organic matter [53]

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 was 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 result was presented in **Table - 2**.

The physical parameters such as Total ash, Acid insoluble ash, Water soluble ash, Extractive values and Loss on drying were determined separately for air dried powdered leaves of this plant as per the official method.

V. Determination of Foaming Index [53]

Plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

An accurately weighed 1g of the coarse plant material was transferred into a 500mL conical flask containing 100mL of boiling water. The flask was maintained at moderate boiling for 30min. The solution was cooled and filtered into a 100mL volumetric flask and sufficient distilled water was added to dilute to volume. The decoction was poured into 10 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 up to 10mL. The tubes were 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 this tube was first or second tube in a series, an intermediate dilution was prepared in a similar manner to obtain a more precise result.

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 using the following formula,

Foaming index = 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 result obtained was presented in **Table - 2**.

TABLE – 2 : ANALYTICAL PARAMETERS OF *Rhynchoglossum notonianum*

S. No.	Parameters*	Values* expressed as %
01	**Ash values**	
	Total ash	14.205 ± 1.11
	Water soluble ash	7.167 ± 0.16
	Acid insoluble ash	0.149 ± 0.02
02	Volatile oil content	Nil
03	Foreign organic matter	0.41 ± 0.04
04	Loss on drying	9.085 ± 0.57
05	Foaming index	< 100

* Mean of six readings (n = 6)

DETERMINATION OF EXTRACTIVE VALUES [46, 53]

(I) Alcohol (Ethanol) soluble extractive:

5 gm of the air –dried drug, coarsely powdered, was macerated in 100mL of ethanol in a closed flask for 24 hours, shaking frequently during 6 hrs and allowed to stand for 18 hrs, filtered rapidly, taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105˚C, to constant weight. The percentage of alcohol soluble extractive with references to the air - dried drug was calculated and presented in **Table - 3**.

(II) Determination of Water-Soluble Extractive:

5 gm of the air dried drug coarsely powdered, was macerated in 100 mL of chloroform water in a closed flask for 24 hours, shaking frequently during 6 hours, and allowed to stand for 18 hours. Filtered rapidly, taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred-flat-bottomed shallow dish and dried at 105˚C to constant weight. The percentage of water – soluble extractive with reference to the air-dried drug was calculated and depicted in **Table-3.**

(III) Determination of Petroleum Ether soluble Extractive:

5 gm of the air –dried drug, coarsely powdered, was macerated in 100mL of ethanol in a closed flask for 24 hours, shaking frequently during 6 hrs and allowed stand for 18 hrs. Thereafter it was filtered rapidly, taking precautions against loss of petroleum ether. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105˚ c, to constant weight. The percentage of petroleum ether soluble extractive with references to the air - dried drug was calculated and presented in **Table - 3**.

(IV) Determination of Chloroform, Methanol, Ethyl acetate, 70% ethanol and Hexane soluble extractives:

The procedure followed for the determination of alcohol soluble extractive value was adopted for the determination of benzene soluble extractive, Chloroform soluble extractive, methanol soluble extractive, ethyl acetate soluble extractive and acetone soluble extractive. Instead of alcohol, respective solvents were used for the determination of their extractive values. The percentage of chloroform, methanol, ethyl acetate, 70% ethanol and hexane soluble extractives with reference to air dried drug was calculated and presented in **Table - 3**.

TABLE – 3: EXTRACTIVE VALUES OF *Rhynchoglossum notonianum*

S. No.	Solvent	Extractive values* expressed as %
1	Petroleum ether	9.261 ± 0.055
2	Chloroform	7.392 ± 0.149
3	Ethyl acetate	6.555 ± 0.096
4	Methanol	12.63 ± 0.167
5	Ethanol	14.484 ± 0.054
6	70% Ethanol	17.21 ± 0.078
7	Aqueous	13.273 ± 0.041
8	Hexane	11.387 ± 0.048
9	Ether	9.619 ± 0.028

* Mean of three readings

POWDER ANALYSIS

The behavior of the ***Rhynchoglossum notonianum*** powder with various chemical reagents is tabulated in **Table – 4** and that of fluorescence analysis of the powder and the extracts of ***Rhynchoglossum notonianum*** are presented in **Table – 5** and **Table – 6,** respectively. The powder showed the presence of phytosterols, tannins, proteins, flavonoids, carbohydrate, glycoside and phenolic compounds.

TABLE – 4 : BEHAVIOUR OF THE POWDER OF *Rhynchoglossum notonianum* WITH VARIOUS CHEMICAL REAGENTS

Powder + Reagents	Color / Precipitate	Presence of active principle
Picric acid	Yellow precipitate	Protein present
Conc. Sulphuric acid	Reddish brown color	Phytosterols present
Libermann Burchard reagent	Reddish brown color	Phytosterols present
Aqueous ferric chloride	Greenish black color	Tannins present
Iodine solution	Blue color	Presence of starch
Mayer's reagent	No cream color	Absence of alkaloids
Spot test	No stain	No fixed oil present
Sulfosalicylic acid	White precipitate	Protein present
Aqueous sodium hydroxide	Yellow color	Flavonoids present
Mg - HCl	Magenta color	Flavonoids present
Aqueous lead acetate	White precipitate	Flavonoids present

Note : *Color reactions are viewed under natural day light by naked eye*

Table – 5 : FLUORESCENCE ANALYSIS OF POWDER OF *Rhynchoglossum notonianum*

Powder + Reagent	Day light	UV light (254 nm)	UV light (366 nm)
Drug powder	Green	Dark green	Brown
Drug powder + aqueous 1M sodium hydroxide	Yellowish green	Fluorescent green	Black
Drug powder + 50% Nitric acid	Light brown	Green	Black
Drug powder + Iodine	Reddish brown	Dark green	Violet
Drug powder + 10% Potassium hydroxide	Yellowish green	Fluorescent green	Greyish black
Drug powder +1M hydrochloric acid	Light brown	Dark green	Black
Drug powder + glacial acetic acid	Light green	Fluorescent green	Greyish black
Drug powder + 50% sulphuric acid	Light green	Fluorescent green	Greyish black
Drug powder + 50% nitric acid	Light brown	Green	Black
Drug powder + 50% hydrochloric acid	Light brown	Light green	Grey

Table – 6 : FLUORESCENCE ANALYSIS OF EXTRACTS OF

Rhynchoglossum notonianum

Extracts	**Consistency**	**Color in daylight**	**Color under UV Lamp 366nm**
Petroleum Extract	Semisolid	Dark green	Reddish orange
Ether extract	Semisolid	Green	Brown
Chloroform extract	Semisolid	Greenish brown	Orange
Aqueous extract	Semisolid	Greenish brown	Green
Methanolic extract	Semisolid	Dark green	Reddish orange
Hexane extract	Semisolid	Green	Greenish brown
Ethanolic extract	Semisolid	Dark green	Reddish orange
70% ethanolic extract	Semisolid	Blackish green	Reddish orange
Ethyl acetate extract	Semisolid	Green	Orange

CHAPTER –V
PHYTOCHEMICAL STUDIES

Phytochemistry has developed into a distinct discipline and is closely related to natural product organic chemistry and plant biochemistry and deals with a variety of secondary metabolites that are produced by plants, their chemical structures, biosynthesis, metabolism, natural distribution and biological functions. For these operations, methods are needed for separation, purification and identification of the many different constituents present in plants. Thus, advances in our understanding of phytochemistry are directly related to successful exploitation of known techniques and the continuing development of new techniques to solve outstanding problems as they come. One of the challenges of phytochemistry is to carry out all the above operations on small amounts of material. [52]

The stems and leaves of *Rhynchoglossum notonianum* were collected hilly terrains near Courtallam, Tirunelveli District of Tamil Nadu situated in Pothigai hills. Collected plant materials were authenticated by the Taxonomist.

The plant material were washed very thoroughly and dried in shade. The shadow dried leaves and stems were powdered and then subjected to the following preliminary phytochemical tests.

SECTION - A
ORGANOLEPTIC EVALUATION

1. **Nature** - Coarse powder
2. **Colour** - Dark green in colour
3. **Odour** - Characteristic odour
4. **Taste** - Bitter

Powdered plant material and extracts were subjected to the following chemical tests and the results were presented in the **Table No. 7 and 8.**

SECTION - B

QUALITATIVE PHYTOCHEMICAL EXAMINATION OF THE LEAF AND STEM POWDER OF *Rhynchoglossum notonianum* [38, 50, 51]

The chemical nature, specific identity, polarity, etc of the substances in the crude extract can be determined by a number of ways including wet chemical tests. In that a color reaction or precipitate is response to specific compound usually a class of compound. Such test can be useful for the investigation of the chemical compounds and to monitor the effectiveness of an extraction process. The petroleum ether, ether, ethyl acetate, methanol, ethanol and aqueous extracts were subjected to qualitative chemical analysis. The various tests performed on the extracts to identify steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols, tannins and saponins.

1. Test for sterols

The powdered plant material 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 was added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turned red in color indicating the presence of sterols.

b. Liebermann – Burchard's Test

To the chloroform solution a few drops of acetic anhydride and 1 ml 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 was formed. The upper layer turned green indicating the presence of sterols.

2. Test for Terpenoids

Noller's test :

A little of the powdered crude drug was extracted with chloroform and filtered. The filtrate was warmed gently with tin and thionylchloride. The colour of the solution turned to pink indicates the presence of terpenoids.

3. Test for carbohydrates

a. Molisch's Test:

The aqueous extract of the powdered crude drug when treated with alcoholic solution of α-naphthol in the presence of sulphuric acid gave purple color indicates the presence of carbohydrates.

b. Fehling's Test:

The aqueous extract of the powdered crude drug was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour. Red precipitate was formed indicating the presence of free reducing sugars.

4. Test for Flavonoids

a. Magnesium turning- con HCl test:

A little of the powdered crude 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. Red or magenta color thus obtained indicates the presence of flavonoids.

b. Alkali Test

To the small quantity of test solution 10% aqueous sodium hydroxide solution was added. Yellow orange coloration indicates the presence of flavonoids.

C. Acid Test

To the small quantity of test solution, few drops of concentrated sulphuric acid was added. Yellow to crimson coloration indicates the presence of flavonoids.

5. Test for Proteins

a. Millon's Test

A small quantity of aciduous – alcoholic extract of the powdered drug was heated with Millon's reagent. White precipitate turned red on heating indicates the presence of proteins.

b. Biuret Test

To another portion of aciduous – alcoholic extract of the powdered drug one ml of 10% sodium hydroxide solution was added, followed by this one drop of dilute copper sulphate solution was added. Violet color was obtained indicating the presence of proteins.

6. Test for Alkaloids

a. About 2gm of the powdered material was mixed with 1gm of calcium hydroxide and 5ml of water into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. To this 200ml 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 this 5ml of dilute hydrochloric acid was added followed by 2ml of each of the following reagents.

a) Mayer's Reagent - No Cream precipitate

b) Dragendorff's Reagent - No Orange brown precipitate

c) Hager's Reagent - No Yellow precipitate

d) Wagner's Reagent - No Reddish brown precipitate.

These negative results indicate the absence of alkaloids.

b. Test for Purine group (Murexide test)

The residue obtained after the evaporation of chloroform as described in (a) was treated with 1ml of hydrochloric acid in a porcelain dish and 0.1gm of Potassium chlorate was added and evaporated to dryness on a water bath. Then the residue was exposed to the vapour of dilute ammonia solution. No purple color was obtained indicating the absence of purine group of alkaloids.

7. Test for Glycosides

a. Borntrager's Test

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filterate, benzene was added and shaken well. The organic layer was separated to which ammonia solution was added slowly. Pink color was not observed in ammonical layer showing the absence of anthraquinone glycosides.

b. Modified Borntrager's Test

About 0.1g of the powdered drug was boiled for 2minutes with dilute hydrochloric acid and few drops of ferric chloride solution, filtered while hot

and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract.

Pink color was observed in ammonical layer showing the presence of anthraquinone glycosides.

8. Test for Cardiac Glycosides (for Deoxysugar)

a) Keller Kiliani Test

About 1g of the powdered leaf was boiled with 10ml of 70% alcohol for 2 minutes, cooled and filtered. To the filtrate 10ml of water and 5 drops of lead acetate solution was added, filtered and evaporated to dryness. The Residue was dissolved in 3 ml of glacial acetic acid. To this, 2 drops of ferric chloride solution was added. Then 3 ml of concentrated sulphuric acid was added to the sides of the test tube carefully and observed.

Reddish brown layer was not observed indicating the absence of deoxy sugars of cardiac glycoside

b) Test for Cyanogenetic Glycosides

Small quantity of the powder was placed in a stoppered conical flask with just sufficient water, to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place. No brick red color was produced on the paper indicating the absence of cyanogenetic glycosides.

9. Test for saponins

About 0.5g of the powdered drug was boiled gently for 2 minutes with 20ml of water and filtered while hot and allowed to cool. 5 ml of the filtrate was then

diluted with water and shaken vigorously. Frothing was not produced indicating the absence of saponins.

10. Test for Tannins

A small quantity of the powdered drug was extracted with water. To the aqueous extract few drops of ferric chloride solution was added. Bluish black color was produced indicating the presence of tannins.

11. Test for the presence of Volatile oil

Weighed quantity (250 gm) of fresh leaves was subjected to hydrodistillation using volatile oil estimation apparatus (BP 1980). Oil was not collected indicate the absence of volatile oil in fresh leaves and stems.

12. **Test for mucilage**

Few ml of aqueous extract prepared from the powdered crude drug was treated with ruthenium red. Red colouration indicates the presence of mucilage.

TABLE – 7

PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE STEM AND LEAF POWDER OF *Rhynchoglossum notonianum*

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	-
	e. Test for Purine group (Murexide test)	-
5.	**TEST FOR GLYCOSIDES**	
	a. Anthraquinone glycosides	
	i) Borntrager's test	-
	ii) Modified Borntrager's test	+
	b. Cardiac glycosides	
	i) Keller Killiani test	-
	c. Cyanogenetic glycosides	-
	d. Coumarin glycosides	-
6.	**TEST FOR SAPONINS**	-
7.	**TEST FOR TANNINS**	
	$FeCl_3$ test	+
8.	**TEST FOR FLAVONOIDS**	
	a. Shinoda test	+
	b. Alkali test	+
	c. Acid test	+
9.	**TEST FOR TERPENOIDS**	+
10.	**TEST FOR VOLATILE OILS**	-
11.	**TEST FOR MUCILAGE**	+

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

TABLE- 8
PRELIMINARY PHYTOCHEMICAL SCREENING OF LEAF AND STEM POWDER EXTRACT OF *Rhynchoglossum notonianum*

Tests	EXTRACTS						
	PET	$CHCl_3$	ETOAc	ACET	MeOH	EtOH	AQUA
I. Test for Sterols							
a. Salkowski's test	+	+	+	+	+	+	-
b. Libermann- Burchard's test	+	+	+	+	+	+	-
II. Test for Carbohydrates							
a. Molisch's test	-	-	-	-	+	+	+
b. Fehling's test	-	-	-	-	+	+	+
c. Benedict's test	-	-	-	-	+	+	+
III.Test for Proteins							
a. Millon's test	-	-	-	-	+	+	+
b. Biuret test	-	-	-	-	+	+	+
IV.Test for Alkaloids							
a.Mayer's reagent	-	-	-	-	-	-	-
b. Dragendroff's reagent	-	-	-	-	-	-	-
c. Hager's reagent	-	-	-	-	-	-	-
d. Wagner's reagent	-	-	-	-	-	-	-
e. Murexide test	-	-	-	-	-	-	-
V.Test for Glycosides a. Anthraquinone glycosides							
i) Borntrager's test	-	-	-	-	-	-	-
ii) Modified Borntrager's test	-	-	+	-	+	+	+
b. Cardiac glycosides							
i) Keller Killiani test	-	-	-	-	-	-	-
c. Cyanogenetic glycosides	-	-	-	-	-	-	-
d. Coumarin glycosides	-	-	-	-	-	-	-
VI.Test for Saponins	-	-	-	-	-	-	-
VII.Test for Tannins							
$Fecl_3$ test	-	-	-	+	+	+	+
VIII.Test for Flavonoids							
a. Shinoda test	-	-	-	+	+	+	+
b. Alkali test	-	-	-	+	+	+	+
c. Acid test	-	-	-	+	+	+	+
IX.Test for Terpenoids	-	-	-	-	-	-	-
X.Test for Mucilage	-	-	-	+	+	+	+

{Abbreviations used : (+) indicates positive reaction (-) indicates negative reaction PET = Pet.ether Extract; $CHCl_3$ = Chloroform; ETOAc = Ethylacetate; ACET = Acetone; MeOH = Methanol; EtOH = 70% Ethanol; AQUA = Aqueous Extracts}

PREPARATION OF EXTRACTS

EXTRACTION PROCEDURE

The shade dried and coarsely powdered leaves and stems of *Rhynchoglossum notonianum was* defatted with petroleum ether (60-80ºC) by cold maceration. Defatted marc was extracted with 70% Ethanol (AR grade) by cold maceration and filtered. The filtrate was concentrated under reduced pressure. A dark green residue was obtained. The extract was subjected to quantitative estimation for total phenolic content, tannin content, lycopene, carotenoids, proanthocyanidine, flavonol and flavonoid contents.

SECTION - C

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

In quantitative estimations, a particular group of compound present in the crude extracts can be quantified by means of using standard or reference marker compound and then reporting them as equivalent to that much amount of compound present in that extract as per standard compound.

ESTIMATION OF TOTAL PHENOLIC COMPOUNDS [54-58]

Principle

The total phenolic content of the *Rhynchoglossum notonianum* extract was determined by **Folin – Ciocalteu** Reagent method. This regeant consists of a mixture of phosphotungstate and phosphomolybdate which is reduced, during oxidation of the phenolic substances, into a mixture of blue molybdenum and tungsten oxides. The intensity of colour is proportional to the amount of oxidized phenolic compounds and it can be estimated as gallic acid equivalents at 765nm.

Materials Required

1. 70% ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* **(ERN)**
2. Gallic acid
3. 10% w/v sodium carbonate solution
4. Folin–Ciocalteu reagent solution : 1N dilute commercial reagent 2N with an equal volume of distilled water was prepared immediately before use.

Procedure

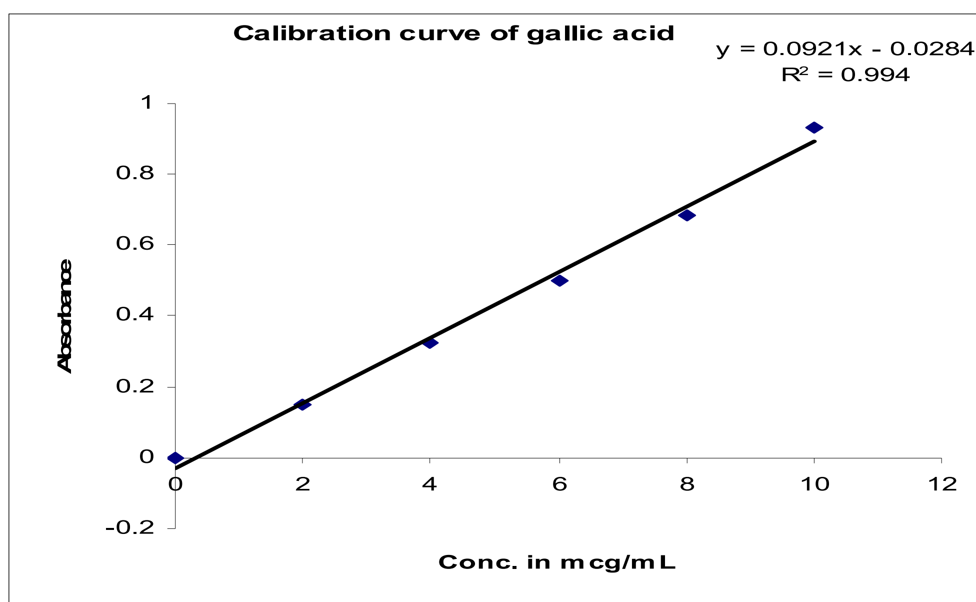
The total phenolic content of ERN was estimated using **Folin–Ciocalteu** reagent method. 0.05 and 0.1ml of ERN (1mg/ml) was transferred into separate test tube. To this solution, Folin–Ciocalteu Reagent 0.5ml and 1ml of sodium carbonate were added and final volume was made up to 10ml with distilled water. The mixture was allowed to stand for 1 hour with intermittent shaking. The absorbance of the reaction mixture was measured at wavelength 765nm. A calibration curve was generated **(Fig. 14)** using absorbance reading of gallic acid at different concentrations (2, 4, 6, 8, 10µg/ml). The reaction mixture without sample was used as the blank. The total phenolic content in the ERN was expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g) and the results were presented in **Table - 09.**

TABLE – 09 : ESTIMATION OF TOTAL PHENOLIC CONTENT OF *ERN*

S. No	Concentration of gallic acid in µg/mL	*Absorbance at 760nm	Concentration of ERN in µg/mL	*Absorbance at 760nm	*Total phenolic content (mg GAE/g of extract)
1	2	0.229 ± 0.010	50	0.624±0.014	108.36 ± 2.54
2	4	0.452 ± 0.006	100	1.279±0.074	110.56 ± 6.44
3	6	0.695 ± 0.005	**Average = 109.46 ± 1.10 mg GAE/g of ERN**		
4	8	0.918 ± 0.031			
5	10	1.162 ± 0.028			

(n = 3) *Mean of three readings ± SEM

FIG. 14 : CALIBRATION CURVE OF GALLIC ACID



ESTIMATION OF TOTAL TANNIN CONTENT [59,60]

Principle

The total tannin content is determined by Folin - Denis method. This method is based on the oxidation of molecules containing a phenolic hydroxyl group. The tannin and tannin like compounds reduce phosphotungustomolybdic acid in alkaline solution to produce a blue colored solution, the intensity of which is proportional to the amount of tannin and can be estimated as tannic acid equivalents at wavelength of 700nm.

Materials Required

1. 70% ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* **(ERN)**
2. Tannic acid
3. 10% W/V sodium carbonate solution
4. **Folin-Denis** reagent: Sodium tungustate 100gm and phosphomolybdic acid 20gm were dissolved in distilled water 750ml along with phosphoric acid 50ml. The mixture was refluxed for 2hr and volume was made up to 1lit with distilled water

Procedure

The total tannin content of ERN was estimated using **Folin - Denis** reagent method. 0.2ml of ERN from 100µg/ml stock was transferred into a test tube. To this solution, Folin - Denis reagent 0.5ml and 1ml of sodium carbonate were added and final volume was making up to 10ml with distilled water. The mixture was allowed to stand for 1hour with intermittent shaking. The absorbance of reaction mixture was measured at wavelength 700nm. A calibration curve was generated using absorbance

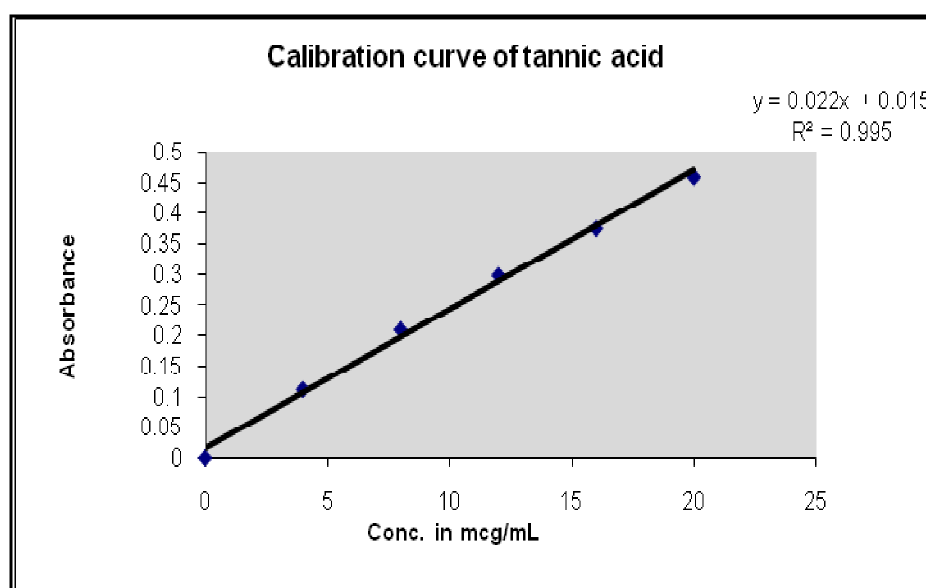
reading of tannic acid **(Fig. 15)** at different concentrations (20, 40, 60, 80 and 100µg/ml). The reaction mixture without sample was used as the blank. The total tannin content in the ERN extract was expressed as milligrams of tannic acid equivalent per gram of extract (mg TAE/g) and results were presented in **Table : 10.**

TABLE – 10 : ESTIMATION OF TOTAL TANNIN CONTENT IN ERN

S.No.	Conc. of Tannic acid in µg/ml	Absorbance at 700nm *	Conc. of ERN in µg/ml	Absorbance at 700nm *	Amount of total Tannin content in terms mg TAE/g *
1	20	0.589 ± 0.01	20	0.125±0.016	249.5 ± 0.77
2	40	1.151 ± 0.04			
3	60	1.710 ± 0.09			
4	80	2.390 ± 0.03			
5	100	3.112 ± 0.03			

(n = 3) * Mean of three readings ± SEM

FIG. 15 : CALIBRATION CURVE FOR TANNIC ACID



ESTIMATION OF TOTAL FLAVONOID CONTENT [61-63]

Principle

The total flavonoid content of plant extract was estimated by aluminum chloride colorimetric assay method. In this method, aluminum chloride complexes with standard and test sample (aluminum chloride complexes with flavonoids of C_3-C_5 hydroxyl group) and to produce intense colour in acidic medium. The intensity of the colour is proportional to the amount of flavonoids and can be estimated as quercetin equivalents at wavelength of 415 nm.

Materials Required

1. 70% Ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* **(ERN)**
2. 10% W/V aluminum chloride
3. 1M potassium acetate
4. 95% V/V ethanol

Procedure

0.5ml of ERN (1mg/ml) was transferred into a test tube. To this solution, 0.1 ml of aluminum chloride, 0.1ml of potassium acetate and 1.5ml of 95% ethanol were added. The final volume was made up to 5ml with distilled water. The mixture was allowed to stand for 30min with intermittent shaking. The absorbance of reaction mixture was measured at 415nm. A calibration curve was generated using absorbance readings of quercetin **(Fig. 16)** at different concentrations (5-50µg/ml). The reaction mixture without aluminium chloride was used as the blank. The total flavonoid

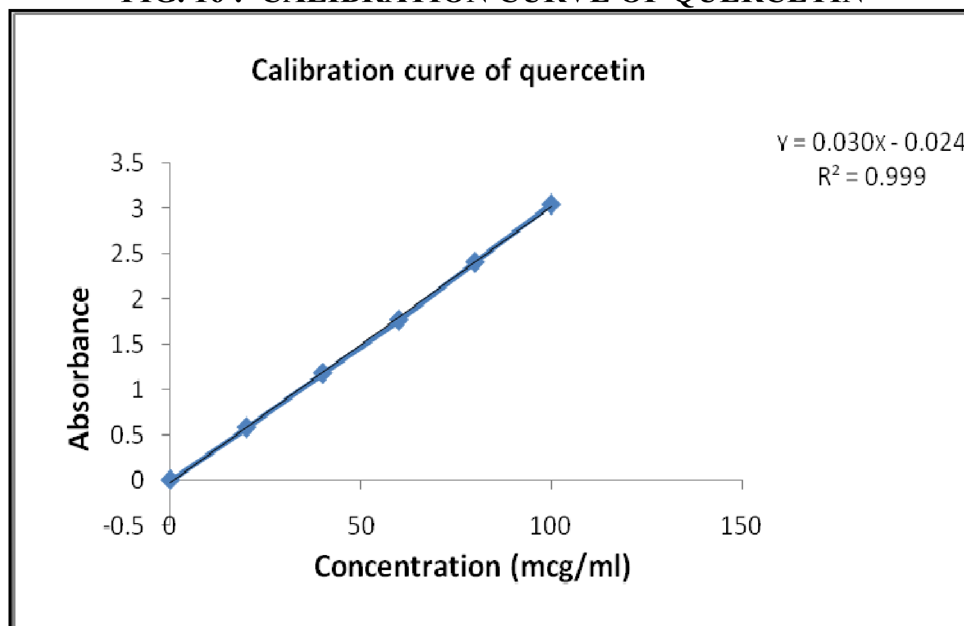
content in ERN was expressed as milligrams of quercetin equivalent (QE) per gram of extract and the results were presented in **Table – 11.**

TABLE – 11 : ESTIMATION OF TOTAL FLAVONOID CONTENT OF ERN BY ALUMINIUM CHLORIDE METHOD

S. No.	Conc. of quercetin in µg/ml	Absorbance at 415nm *	Conc. of methanolic extract in µg/ml	Absorbance at 415nm *	Amt of total flavonoid content in terms mg QE/ g of extract *
1	20	0.581 ± 0.01	50	0.152 ± 0.01	117.33 ± 1.13
2	40	1.240 ± 0.06	100	0.321 ± 0.01	115.22 ± 1.11
3	60	1.766 ± 0.03	**Average = 116.28 ± 0.75 mg QE/ g of extract**		
4	80	2.352 ± 0.02			
5	100	3.137 ± 0.03			

*Mean of three readings ± SEM

FIG. 16 : CALIBRATION CURVE OF QUERCETIN



ESTIMATION OF TOTAL FLAVONOLS CONTENT [64-66]

Principle

A quantitative estimation of flavonols in 70% ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* is possible by colorimetric assay. The aluminium chloride method is used to determine the flavone and flavonol content. The aluminium chloride method for the flavonol estimation is based on the formation of a complex between the aluminum ion Al (III) and the hydroxyl groups of the flavonol.

Materials Required

1. Methanolic plant extract (10 mg/ml) of *Rhynchoglossum notonianum*
2. Aluminum trichloride (20mg/ml) solution
3. Sodium acetate (50mg/ml) solution
4. Standard rutin in methanol (0.5 mg/ml)

Procedure

The total flavonols content was also estimated by using the aluminium chloride method with some minor modifications. This procedure is based on the formation of complex which absorbed maximum at the wavelength of 440 nm. 1 ml of methanolic plant extract (10 mg/ml) was mixed with 1 ml of aluminum trichloride (20 mg/ ml) and 3ml of sodium acetate (50 mg/ ml). The absorbance was read at 440 nm in UV spectrophotometer after incubation of the reaction mixture for 2.5 hours. The absorption of standard rutin in methanol (0.5 mg/ml) was measured under the similar conditions.

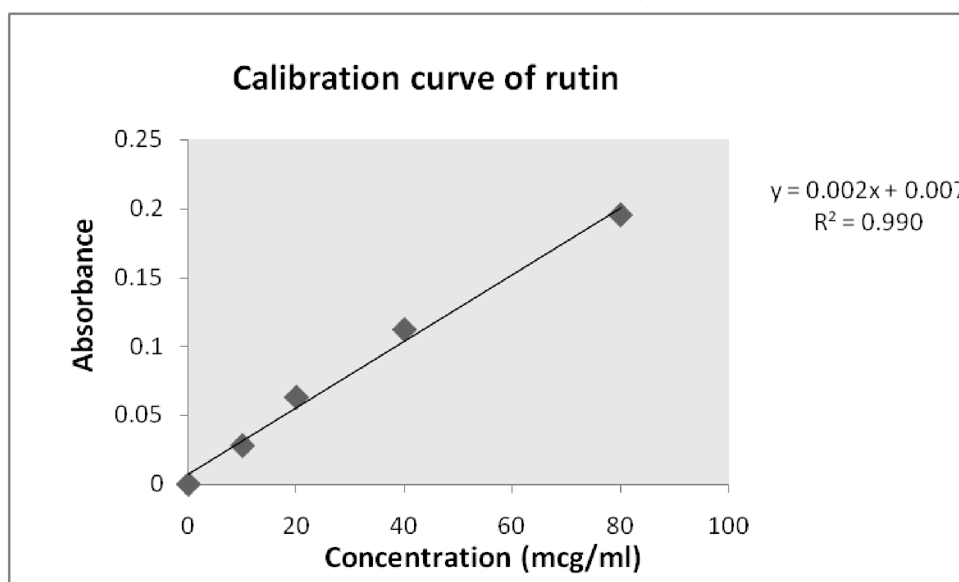
All the determinations were carried out in triplicates. The amount of flavonols present in the plant extracts in rutin equivalents (RE) was calculated using the calibration curve **(Fig. 17)** of standard rutin. The findings were presented in **Table - 12.**

TABLE – 12 : ESTIMATION OF TOTAL FLAVONOLS CONTENT OF ERN

S. No.	Conc. of rutin in µg/ml	Absorbance at 440 nm *	Conc. of methanolic extract in µg/ml	Absorbance at 440 nm *	Amt of total flavonols content * (mg rutin equivalent/ g of extract)
1	100	0.074 ± 0.01	100	0.034 ± 0.03	0.491 ± 0.02
2	200	0.109 ± 0.02	200	0.051± 0.02	0.505 ± 0.03
3	400	0.145 ± 0.01	**Average = 0.498 ± 0.01 mg RE/ g of extract**		
4	800	0.195 ± 0.03			
5	1000	0.237 ± 0.03			

*Mean of three readings ± SEM

FIG. 17 : CALIBRATION CURVE OF RUTIN FOR ESTIMATION OF TOTAL FLAVONOLS



DETERMINATION OF TOTAL PROANTHOCYANIDINS [66]

Principle

Spectrophotometric determination methods such as Vanillin-HCl assay, butanol-HCl assay and Folin-Ciocalteau assay have been generally used for the estimation of proanthocyanins. The Vanillin-HCl assay is specific for a narrow range of flavonols and dihydrochalcones. In this Vanillin-HCl assay, vanillin is protonated in an acid environment results in a weak electrophilic carbocation which reacts at the 6 or 8 position of the flavonoid ring. This intermediate compound is dehydrated to form a coloured compound.

Materials Required

1. 70% Ethanolic plant extract (0.1 mg/ml) of *Rhynchoglossum notonianum*
2. 4% Vanillin in methanol solution
3. Hydrochloric acid
4. Standard rutin in methanol

Procedure

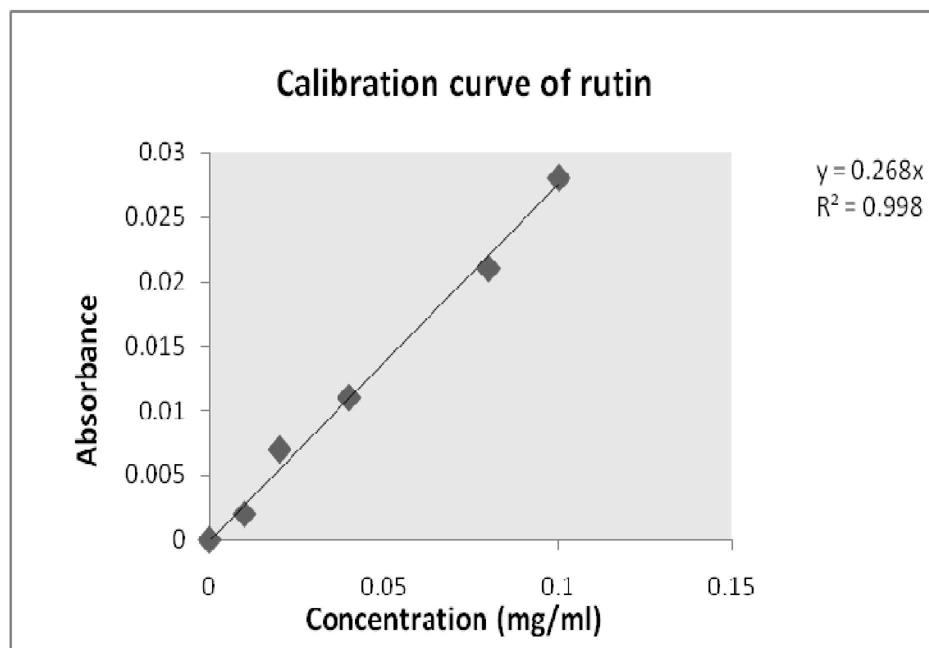
Proanthocyanidin content was estimated using the method adopted by Sun *et al*. To 0.5 ml of 0.1 mg/ml of extract solution, 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid was added. This reaction mixture was allowed to stand for 15 min. The absorbance was measured at wavelength of 500 nm in UV spectrophotometer. All the determinations were carried out in triplicates. The total proanthocyanidin content was expressed as rutin equivalents (mg/g). The results were presented in **Table - 13.**

TABLE – 13 : ESTIMATION OF PROANTHOCYANIDIN CONTENT OF ERN

S.No.	Conc. of rutin (µg/ml)	Absorbance at 500 nm	Conc. of ERN (µg/ml)	Absorbance at 500 nm	Amount of proanthocyanidin content in terms mg RE/ g of extract
1	100	0.002	100	0.004	149.25
2	200	0.006	200	0.008	167.91
3	400	0.011	**Average = 158.58 ± 6.59 mg RE/ g of extract**		
4	800	0.021			
5	1000	0.027			

*Mean of two readings ± SEM

FIG. 18 : CALIBRATION CURVE OF RUTIN FOR PROANTHOCYANIDIN ESTIMATION



ESTIMATION OF BETACAROTENE AND LYCOPENE [67-69]

Materials Required

1. Dried petroleum ether plant extract of *Rhynchoglossum notonianum*
2. Acetone
3. Hexane
4. Whatman No.4 filter paper

Procedure

β - Carotene and lycopene were estimated according to the methodology of Nagata and Yamashita (1992). 100 mg of the dried petroleum ether extract was shaken vigorously with 10 ml of acetone–hexane mixture (in the ratio of 4 parts: 6 parts) for 1 min and filtered through Whatman filter paper (No. 4). The absorbance of the filtrate was measured at 453, 505 and 663 nm. Amount of **β -**carotene and lycopene present in the plant extract was calculated using the following equations:

Lycopene (mg/100 ml) = -0.0458 A663 + 0.372 A505 - 0.0806 A453

β- Carotene (mg/100 ml) = 0.216 A663 - 0.304 A505 + 0.452 A453

Where, A663, A505 and A453 are absorbance at 663nm, 505nm and 453nm, respectively. All the determinations were carried out in triplicate and the results were mean values ± standard deviations which were expressed as mg of carotenoid per gram of the extract. The results were presented in **Table – 14.**

TABLE – 14 : ESTIMATION OF BETACAROTENE AND LYCOPENE

Wavelength	Absorbance *	*Amount of β-carotene (mg of β-carotene/g of extract)	*Amount of Lycopene (mg of lycopene/g of extract)
663 nm	2.575 ± 0.09	1.729 ± 0.02	0.182 ± 0.02
505 nm	1.439 ± 0.08		
453 nm	3.583 ± 0.13		

*Mean of three readings ± SEM

SECTION-D
CHROMATOGRAPHY

Chromatography comprises a group of methods for separating molecular mixtures that depends on the differential affinities of the solutes between two immiscible phases. One of the phases is a fixed bed of large surface area called the stationary phase, while the other is fluid, which moves through or over the surface of the fixed phase called the mobile phase.

Chromatographic methods can be classified according to the nature of the stationary and mobile phases. If the stationary phase is a solid, the process is called as adsorption chromatography and if the stationary phase is a liquid, it is termed as partition chromatography.

The various types of chromatography include paper chromatography (PC), thin layer chromatography (TLC), column chromatography (CC), gas chromatography (GC), high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC).

Thin Layer Chromatography [54,70]

Thin layer chromatography (TLC) is an easy technique to adopt for separation and identification of organic compounds. The principle involved is adsorption. The solute competes with the solvent for the surface sites on the adsorption. Depending on the distribution coefficients, the compounds are distributed on the surface of the adsorbent. The compound, which is readily soluble but not strongly adsorbed, moves up along with the solvent and that not so soluble but more strongly adsorbed move up less readily leading to the separation of compounds.

Preparation of TLC Plates

A 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.

Sample application

The 70% ethanolic extract of *R. notonianum* (ERN) was dissolved in ethanol to get 5mg/mL. A small quantity of the sample was drawn and spotted as a spot with the help of capillary tube.

Development of the chromatogram

After drying of the spot, the plates were developed in a chromatographic tanks containing various combination of solvents as a mobile phase. After one third of the plate was developed, the plates were taken outside and dried. The TLC plates were **(Fig. : 20 & 21)** visualized with one of the following techniques

(a) Sprayed with suitable detecting reagent

(b) Under UV light at 254 and 366nm

The R_f values were calculated using the formula,

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

The results were presented in **Table - 15.**

TABLE-15 : THIN LAYER CHROMATOGRAPHY OF EXTRACT OF STEMS AND LEAVES OF *Rhynchoglossum notonianum*

Solvent System	Stationary phase	Mobile phase	CHROMATOGRAM		
			No. of spots	R_f value	Detecting agent
I	Silica gel-G	Chloroform : Methanol : Water (61:32:7)	2	0.93	Vanillin-H2SO4 spray / UV light **(Fig. 19)**
				0.59	
II	Silica gel-G	Chloroform : Methanol : Isopropanol : Water (5:6:1:4)	3	0.78	Visible / UV light **(Fig. 20)**
				0.65	
				0.33	
III	Silica gel-G	Toluene : Acetic acid (4:2)	8	0.92	UV light (366 nm) **(Fig. 21)**
				0.84	
				0.79	
				0.74	
				0.68	
				0.63	
				0.53	
				0.29	

SECTION-E

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY ANALYSIS OF 70% ETHANOLIC EXTRACT OF LEAVES AND STEMS OF *Rhynchoglossum notonianum* [70,71]

High performance thin layer chromatography (HPTLC) is a modern adaptation of TLC with improved versatility, separation efficiency and detection limits. HPTLC is useful for identification of plants and their extracts because each plant species produces a distinct chromatogram, with unique marker compounds used for plant identification. It is used as a quality control tool since comparison of chromatograms of different lots can demonstrate the similarities and differences between the test samples and the standard chemical markers. HPTLC is a reliable method for quantitation of nanogram level even when present in complex formulation. HPTLC finger print analysis is used for rapid identity check, for monitoring purity of drugs, for detection of adulterants, for determining whether a material is derived from a defined botanical species and also to known whether the constituents are clearly characterized.

Development of HPTLC fingerprint

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 70% ethanolic extract of *R. notonianum* **(ERN)** was dissolved in ethanol to get a concentration of 10mg/mL 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 [71]

Toluene: Acetic acid (4 : 2) was used as the mobile phase for developing the chromatogram. The mobile phase was taken in a CAMAG twin trough glass chamber.

Detection wavelength

The developed plates were examined at wavelength 254 and 366nm.

The TLC visualization, 3D display of the finger print profile and peak display at 254 and 366 nm were presented in **Table – 16 & 17** and **Fig. 22 to 27.**

Development of HPTLC Finger Print

About 2µl of ERN was applied as 8mm band for each spot using **CAMAG Linomet V** sample applicator on Aluminum sheets pre-coated with **silica gel 60GF 254 HPTLC plates** used as a stationary phase. The plates were developed in the mobile phase, **Toluene: Acetic acid** (4 : 2) to a distance of 8cm in **CAMAG-Twin trough glass chamber**. Then the track were scanned using **densitometry TLC Scanner 3** equipped with **Win CATS 1.4.3 Software** at a wavelength of 254 nm and

366nm using deuterium lamp and the finger print profiles were recorded and presented in **Table No. – 16 & 17.**

R_f VALUES AND AREA OF SEPERATED COMPOUNDS

TABLE – 16 :

HPTLC chromatogram at 254nm

Peak	Rf value	Area (AU)
01	0.08	1553.6
02	0.21	2547.1
03	0.30	3347.3
04	0.49	648.3
05	0.69	1713.6
06	0.76	5339.3
07	0.81	8374.5
08	0.86	5083.0
09	0.91	299.7

TABLE – 17 :

HPTLC chromatogram at 366nm

Peak	Rf value	Area (AU)
01	0.07	349.7
02	0.19	1136.4
03	0.38	692.2
04	0.62	3211.2
05	0.67	3009.0
06	0.69	3736.9
07	0.72	3850.2
08	0.77	6197.6
09	0.85	5262.6
10	0.91	1344.7

FINGERPRINTING:

3D DISPLAY

FIG. 22 : 3D DISPLAY AT 254nm

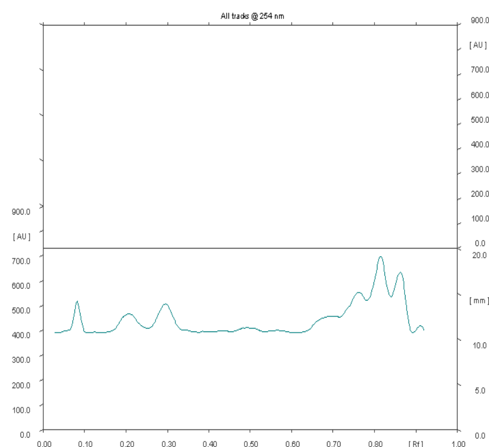
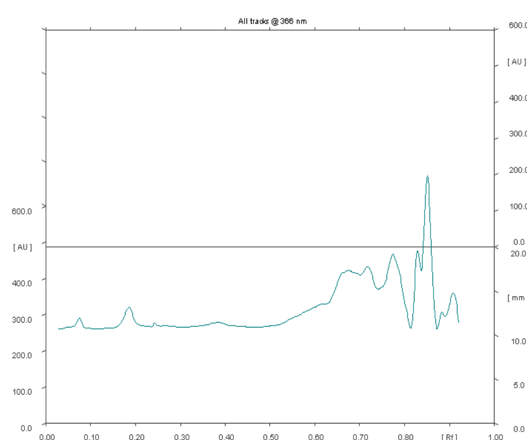


FIG. 23 : 3D DISPLAY AT 366 nm



CHAPTER-VI

PHARMACOLOGICAL EVALUATION

Systematic pharmacological screening is the hallmark of a robust study. It is therefore imperative to explore every possible biological activity of the plant. The 70% ethanolic extract of *Rhynchoglossum notonianum* **(ERN)** was screened for antioxidant activities, antiinflammatory, anti arthritic, hepatoprotective, antibacterial and anticancer activities.

PART – I : INVITRO ANTIOXIDANT ACTIVITY [54, 72-75]

Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. Oxidative stress causes various disease conditions such as ageing, anemia, arthritis, asthma, inflammation, ischemia, neurodegeneration, Parkinson's disease, and perhaps dementia. Phenolic hydroxyl group compounds may exert powerful antioxidant activity *in-vitro* by lipid peroxidase inhibitors or scavenging the free radicals. To name few, the antioxidant activity can be performed by following methods.

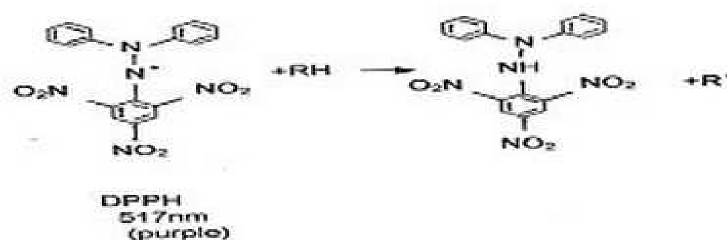
1. DPPH method
2. Superoxide radical scavenging activity
3. Hydroxyl radical scavenging activity
4. Nitric oxide radical inhibition assay
5. Reducing power method
6. Phosphomolybdenum method,

   Etc.,

DPPH (2, 2-DIPHENYL-1-PICRYLHYDRAZYL) FREE RADICAL SCAVENGING ASSAY [76-78]

Principle

The free radical scavenging activity is measured by using DPPH assay. DPPH molecules are stable free radical that contain unpaired electron. The quantitative estimation of free radical scavenging activity is determined according to Blois *et al* method[78]. When the solution of DPPH is mixed with substance that can donate a hydrogen atom, it is converted to its reduced form which is indicated by the color changes from deep violet to pale yellow color. The result of the antioxidant activity is expressed as EC_{50} or IC_{50}.



Materials required

1. 70% Ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* (ERN)
2. 0.1 mM 2, 2-diphenyl-1-picrylhydrazyl (DPPH)
3. 95% v/v ethanol
4. Ascorbic acid

Procedure

The DPPH solution (0.1mM) in ethanol was prepared and 4.0mL of this solution was added to 1.0 mL of extract solution (or standard) in water of different

concentrations (10-50 µg/mL). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Scavenging of DPPH radical was calculated using the following equation.

$$\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where,

A_0-the absorbance of the control reaction

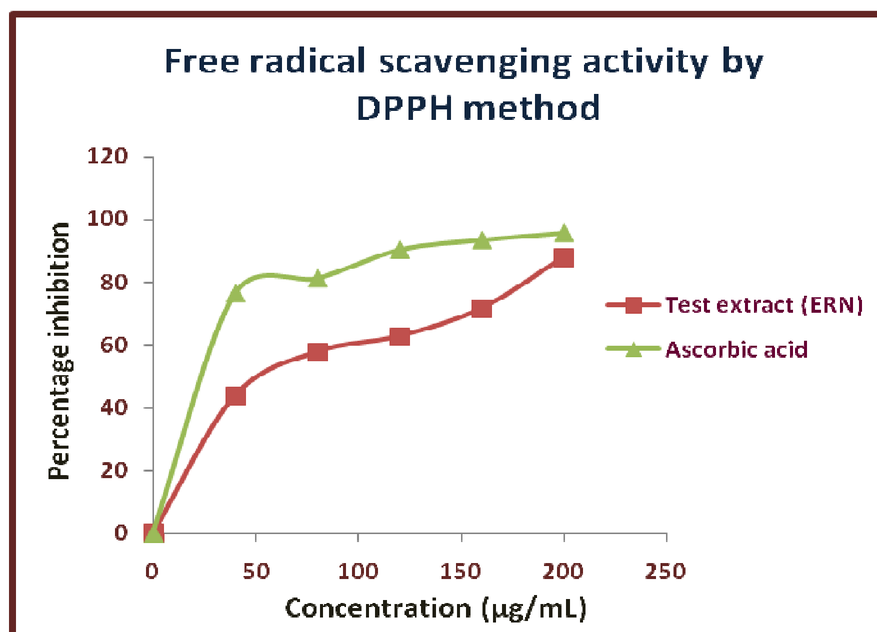
A_1- the absorbance of the test sample

IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve by plotting the extract concentration Vs % inhibition **(Fig. 28)**. The mean values were obtained from triplicate experiments and presented in **Table - 18**. Antioxidant activity of different concentration of ERN and ascorbic acid by DPPH scavenging assay was compared by plotting a bar diagram. **(Fig. 29)**

TABLE - 18 : FREE RADICAL SCAVENGING ACTIVITY OF *ERN* BY DPPH METHOD

S.No	Conc in µg/mL	Percentage Inhibition	
		Ascorbic acid	**70 % Ethanolic extract of *Rhynchoglossum notonianum***
01	40	76.44 ± 1.73	48.06 ± 1.68
02	80	81.22 ± 1.13	53.68 ± 1.22
03	120	90.33 ± 2.38	57.51 ± 0.15
04	160	93.45 ± 1.47	74.36 ± 1.37
05	200	95.68 ± 2.02	80.22 ± 0.95
IC50		**40.68**	**93.51**

FIG. 28 : FREE RADICAL SCAVENGING ACTIVITY OF *ERN* AGAINST DPPH



DETERMINATION OF SCAVENGING ACTIVITY AGAINST HYDROGEN PEROXIDE [79]

Principle:

The radical scavenging activity against hydrogen peroxide of plant extract was determined by using the method of Ruch *et al* [79]. The principle is based on the capacity of the extracts to decompose the hydrogen peroxide to water.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Materials required

70% Ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* (ERN)

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

0.1M, pH 7.4 phosphate buffer

Procedure

The ERN was dissolved in ethanol to get a stock solution of 1mg/mL. Varying quantities of the stock solution (40 – 200 µg/mL) were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then mixed with 0.2mL of hydrogen peroxide solution. The absorbance of the reaction mixture was measured at 230nm after 10min. The reaction mixture without sample was used as blank. Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula

$$\textbf{\% inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

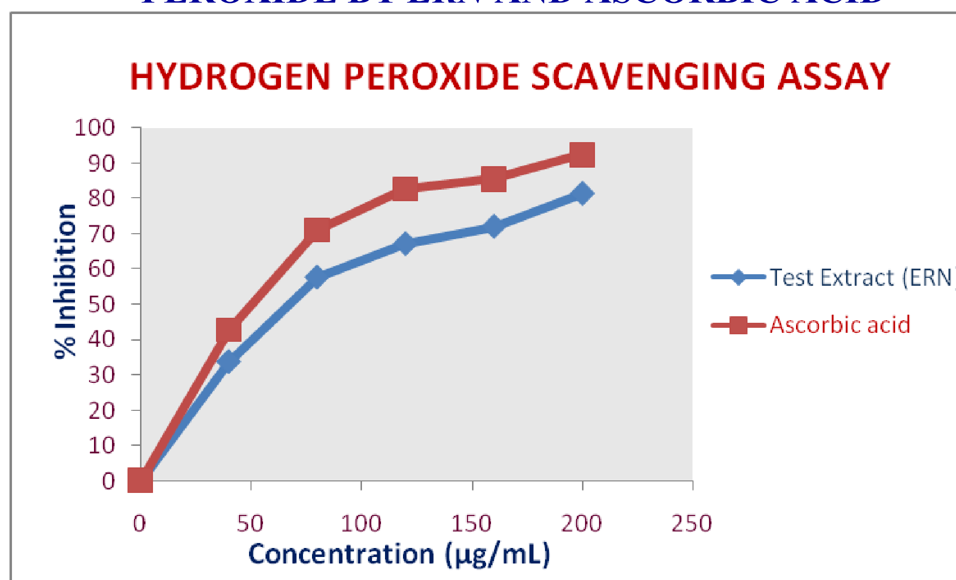
The results are presented in **Table - 19 and Fig. - 30.**

TABLE - 19. PERCENTAGE INHIBITION OF *ERN* AND ASCORBIC ACID AGAINST HYDROGEN PEROXIDE

S.No.	Conc. in µg/mL	Percentage inhibition*	
		Ethanolic extract of *Rhynchoglossum notonianum*	Ascorbic acid
01	40	33.71 ± 0.41	42.72 ± 0.68
02	80	57.64 ± 0.56	71.08 ± 0.89
03	120	67.11 ± 1.02	82.7 ± 0.96
04	160	71.99 ± 0.82	85.45 ± 0.86
05	200	81.34 ± 0.69	92.42 ± 0.65
IC_{50}		**94.91 µg/mL**	**71.33 µg/mL**

***Mean of three readings ± SEM**

FIG. 30 : PERCENTAGE INHIBITION OF HYDROGEN PEROXIDE BY *ERN* AND ASCORBIC ACID



NITRIC OXIDE SCAVENGING ACTIVITY ASSAY [80, 81]

Principle

Nitric oxide scavenging activity was determined according to the method reported by Green *et al.,* 1982 [80]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions. These nitrite ions can be determined by Griess Illosvoy reaction. The nitrite ions produced diazotizes sulphanilamide and the diazonium salt thus obtained reacts with N,N naphthyl ethylene diamine dihydrochloride to give a pink colour chromophore which has a maximum absorption at 546nm.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

70% Ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* (ERN)

10mM sodium nitroprusside

Phosphate buffered saline pH 7.4

2% sulphanilamide in ortho phosphoric acid

0.1% naphthyl ethylene diamine dihydrochloride

Procedure

To 1mL of sodium nitroprusside, 2.5mL phosphate buffered saline (pH 7.4) was added. 1mL of extracts at various concentrations was added to the above solution and the mixture was incubated at 25°C for 30min. To 1.5mL of the incubated mixture add 1mL of sulphanilamide in phosphoric acid and 0.5mL of naphthyl ethylene diamine dihydrochloride. The absorbance was measured at 546nm. Ascorbic acid was used as a standard. The percentage inhibition of nitric oxide radical generated was calculated using the following formula:

% inhibition = [(Control-Test)/Control] x 100

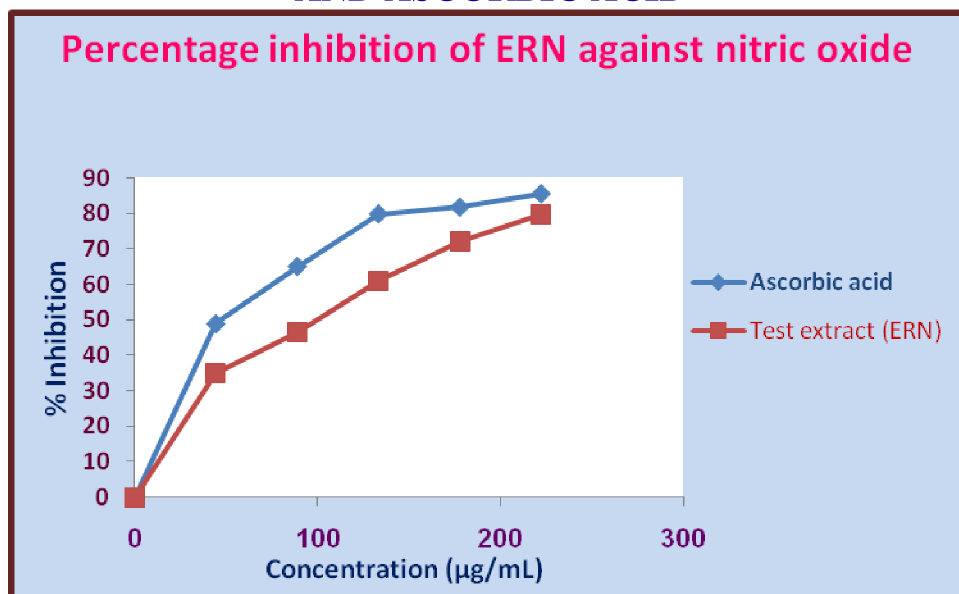
The IC_{50} was calculated using linear regression analysis. The results were presented in **Table - 20** and **Fig. – 31.**

TABLE – 20 : PERCENTAGE INHIBITION OF *ERN* AND ASCORBIC ACID AGAINST NITRIC OXIDE

S. No.	Concentration (µg/mL)	Percentage inhibition*	
		Ascorbic acid	ERN
1	44.44	48.95 ± 0.62	36.06 ± 0.31
2	88.89	65.09 ± 0.74	46.54 ± 0..53
3	133.33	79.87 ± 0.44	60.91 ± 2.12
4	177.78	81.76 ± 0.53	72.12 ± 1.59
5	222.22	85.53 ± 0.65	79.66 ± 0.17
IC 50		**81.96**	**114.19**

*Mean of three readings ± SEM

FIG. - 31: NITRIC OXIDE RADICAL SCAVENGING BY *ERN* AND ASCORBIC ACID



REDUCING POWER ASSAY [75, 82-85]

Principle

Reducing power assay is a spectrophotometric method and is based on the principle that increases in absorbance of the reaction mixture indicates the increase in the reducing power of the sample. This reducing capacity of the test extracts may serve as the antioxidant activity. Antioxidant activity may be due to a variety of mechanisms viz., the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and free radical scavenging. The assay is based on the reduction of ferric in potassium ferricyanide to 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.

Potassium ferricyanide + Ferric chloride

Potassiumferrocyanide + Ferrous chloride

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Materials required

70% Ethanolic extract of leaves and stems of *Rhynchoglossum notonianum* (ERN)

Ascorbic acid

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 the plant extracts was screened by assessing the ability of the test extract to reduce $FeCl_3$ solution as mentioned by Oyaizu *et al*.[85] 0.1-0.5mL of plant extract solution (final concentration 100-500 mcg/mL) was mixed with 0.75mL phosphate buffer and 0.75mL of 1% potassium ferricyanide [$K_3Fe(CN_6)$], then mixture was incubated at 50°C for 20min. 0.75mL of 1% trichloro acetic acid was added to the mixture, allowed to stand for 10 minutes. 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 was measured at 700nm in UV-Visible spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean ± standard error of the mean. The antioxidant activity was expressed as equivalents of ascorbic acid (µg/g). The results obtained are presented in **Table – 21 & 22** and **Fig. 32 & 33.**

TABLE - 21: REDUCING POWER OF *ERN* AND ASCORBIC ACID ON POTASSIUM FERRIC CYANIDE

S.No.	Ascorbic acid		70% Ethanolic extract of *Rhynchoglossum notonianum*	
	Conc. in µg/mL	**Percentage increase in reducing power**	**Conc. in µg/mL**	**Percentage increase in reducing power**
1	10	20.87 ± 4.81	100	20.39 ± 3.83
2	15	35.44± 4.35	200	43.2 ± 0.92
3	20	55.02 ± 5.86	300	57.93 ± 5.20
4	25	79.61 ± 6.36	400	83.01 ± 5.49
5	30	97.41 ± 0.61	500	96.44 ± 1.15
	IC 50	**17.25 µg/mL**	**IC 50**	**250.01 µg/mL**

TABLE – 22 : TOTAL REDUCING POWER OF ASCORBIC ACID AND ERN

Ascorbic acid		ERN	
Concentration (µg/mL)	**Absorbance***	**Concentration (µg/mL)**	**Absorbance***
10	0.249 ± 0.01	200	0.248 ± 0.01
15	0.279 ± 0.01	400	0.295 ± 0.01
20	0.319 ± 0.01	600	0.325 ± 0.01
25	0.370 ± 0.01	800	0.377 ± 0.01
30	0.407 ± 0.01	1000	0.405 ± 0.01

*** Mean of three readings ± SEM**

Fig. 32.1 : Reducing power of ascorbic acid

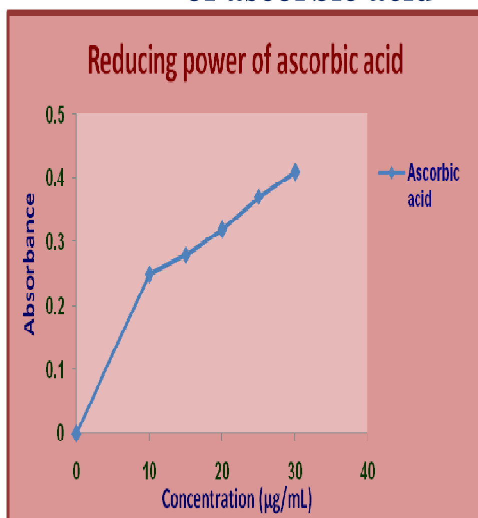


Fig. 32.2 : Reducing power of ERN

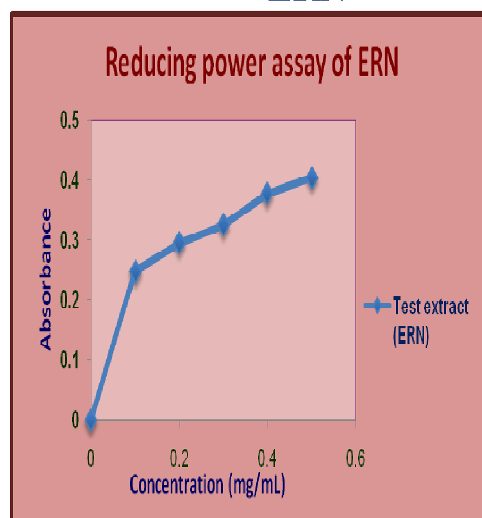


Fig. 33.1 : Percentage increase in reducing power of ascorbic acid

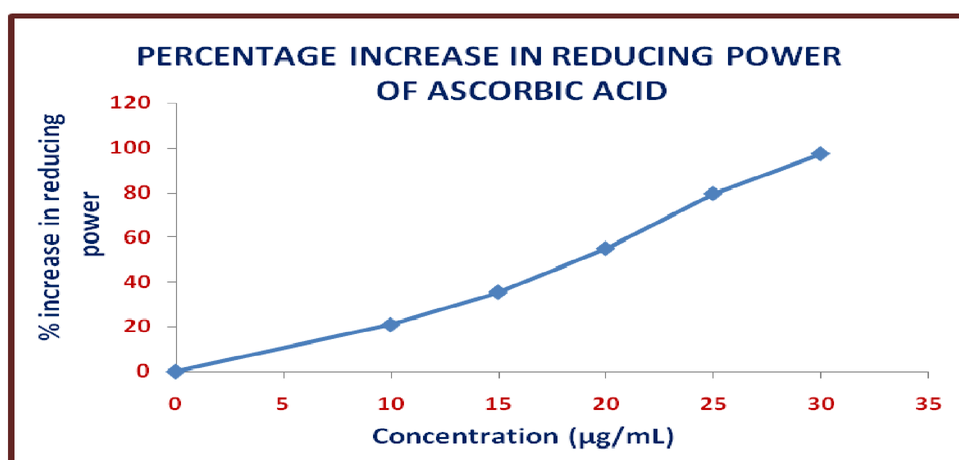
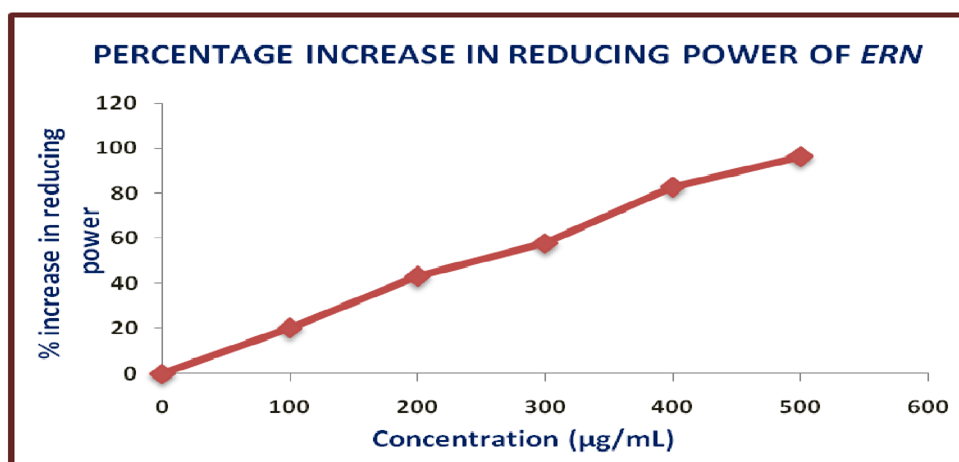


Fig. 33.2 : Percentage increase in reducing power of *ERN*



Part – II

IN VITRO ANTI-INFLAMMATORY ACTIVITY SCREENING BY MEMBRANE STABILIZATION STUDY [86-88]

Principle:

The method of Sadique et al (1989) and modified by Oyedapo and Famurewa (1995) and Oyedapo *et al.,* (2004) was employed in the membrane stabilizing activity assay. When RBCs are subjected to heat and treatment with hyposaline they release haemoglobin which has a maximum absorbance at about 560nm. The capacity of the extract to reduce hyposaline and heat induced lysis is basis of the assay.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Materials required

70% Ethanolic extract of leaves and stems of *R. notonianum* **(ERN)**

0.2M Sodium phosphate buffer (pH 7.4)

0.36% w/v Hyposaline

10% v/v HRBC suspension in isosaline

Preparation of HRBC suspension in isosaline

The human erythrocytes suspension was used for the *in-vitro* membrane stabilization assay. Blood was collected from healthy volunteers who had not consumed any NSAIDs for two weeks prior to the experiment. The blood was mixed with equal volume of Alsever's solution (2% dextrose, 8.0% sodium citrate, 0.5%

citric acid and 0.42% sodium chloride) and centrifuged at 3000rpm. The packed cells were washed with isosaline and a 10% v/v erythrocyte suspension in isosaline was prepared.

Procedure

The assay mixture consist of 2mL of hyposaline and 1mL of phosphate buffer and varying volumes (0.1 to 0.5mL) of the ERN (1mg/mL) and 0.5mL HRBC suspension in isosaline, then the final volume were made up to 4.5mL with isosaline. The control was prepared as mentioned above without the test extract, while drug control was also prepared similarly but without HRBC suspension. The reaction mixture was incubated at 56ºC for 30min in a water bath, then the tube was cooled under running water. Then the absorbance of the released hemoglobin was measured at 560nm. Diclofenac 50µg/mL was used as a reference standard. The percentage membrane stabilisation activity of the compounds were determined by the formula

$$\textbf{\% Membrane stabilization} = \frac{[A_{control} - (A_{test} - A_{product\ control})]}{A_{control}} \times 100$$

Where,

$A_{control}$ - Absorbance in control

A_{test} - Absorbance in test

$A_{product\ control}$ - Absorbance in product control

The results obtained for the *in-vitro* membrane stabilization effect were presented in **Table – 23, Fig. 34.1** and **Fig. 34.2**

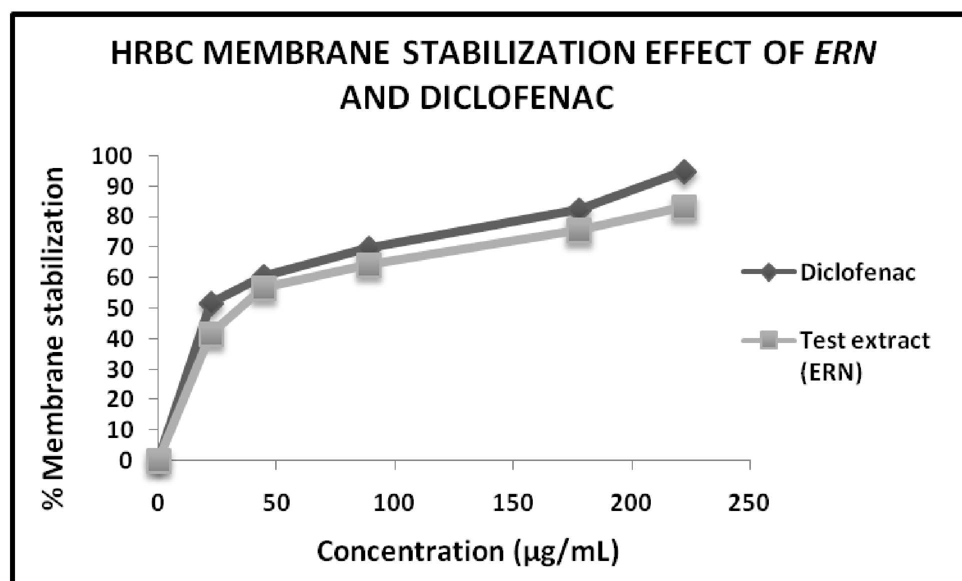
TABLE - 23 : PERCENTAGE OF MEMBRANE STABILIZATION BY DICLOFENAC AND *ERN*

S.No.	Conc. in µg/mL	Percentage membrane stabilization of	Percentage membrane stabilization of extract *

		Diclofenac*	
1	22.22	51.51 ± 1.42	41.56 ± 1.48
2	44.44	60.28 ± 0.88	56.55 ± 1.78
3	88.89	69.7 ± 1.95	64.35 ± 1.09
4	177.78	82.26 ± 1.17	75.53 ± 1.32
5	222.22	94.82 ± 1.07	83.17 ± 2.07

* Mean of three readings ± SEM

FIG. 34.1 : PERCENTAGE OF MEMBRANE STABILIZATION BY DICLOFENAC AND *ERN*



Part – III : INVITRO ANTIARTHRITIC ACTIVITY BY PROTEIN DENATURATION METHOD [89-94]

Rheumatoid arthritis is an autoimmune disorder. One among the cause for the disease is due to the denaturation of the protein. Antiarthritic activity was studied by inhibition of protein denaturation method .

Materials required

70% Ethanolic extract of leaves and stems of *R. notonianum* **(ERN)**

Diclofenac sodium

Bovine serum albumin (5% w/v aqueous solution)

Phosphate buffer (P^H 6.3)

Instrument : UV/ Visible spectrophotometer at 416 nm.

EXPERIMENTAL PROTOCOL

The following four solutions were prepared

1. **Test solution** (0.5mL)

   The test solution consists of 0.45mL bovine serum albumin (5% w/v aqueous solution) and 0.05mL of ERN (31.25, 62.5, 125, 250 and 500µg/mL concentrations).

2. **Test control solution** (0.5mL)

   The test control solution consists of 0.45mL bovine serum albumin and 0.05mL distilled water.

3. **Product control** (0.5mL)

   The product control consists of 0.45mL distilled water and 0.05mL of ERN (31.25, 62.5, 125, 250 and 500µg/mL concentrations).

4. **Standard solution** (0.5mL)

   Standard solution consists of 0.45mL of bovine serum albumin and 0.05mL of Diclofenac sodium solution.

All the above test samples was adjusted to p^H 6.3 using a small amount of 1N hydrochloric acid. They were incubated at 37^0C for 20 minutes and heated at 57^0 C for 3 minutes. Allow to cool and about 2.5mL of phosphate buffer (p^H 6.3) was added to all the above solution. The absorbance was measured using UV spectrophotometer at 416nm. The percentage inhibition of protein denaturation was calculated using the formula :

$$\textbf{Percentage inhibition} = 100 - \left\{ \frac{\text{OD of test solution-OD of product control}}{\text{OD of test control}} \right\} \times 100$$

The control represents 100% protein denaturation. The results were compared with the standard drug diclofenac sodium treated sample. The results were presented in **Table – 24** and **Fig. 35.1.**

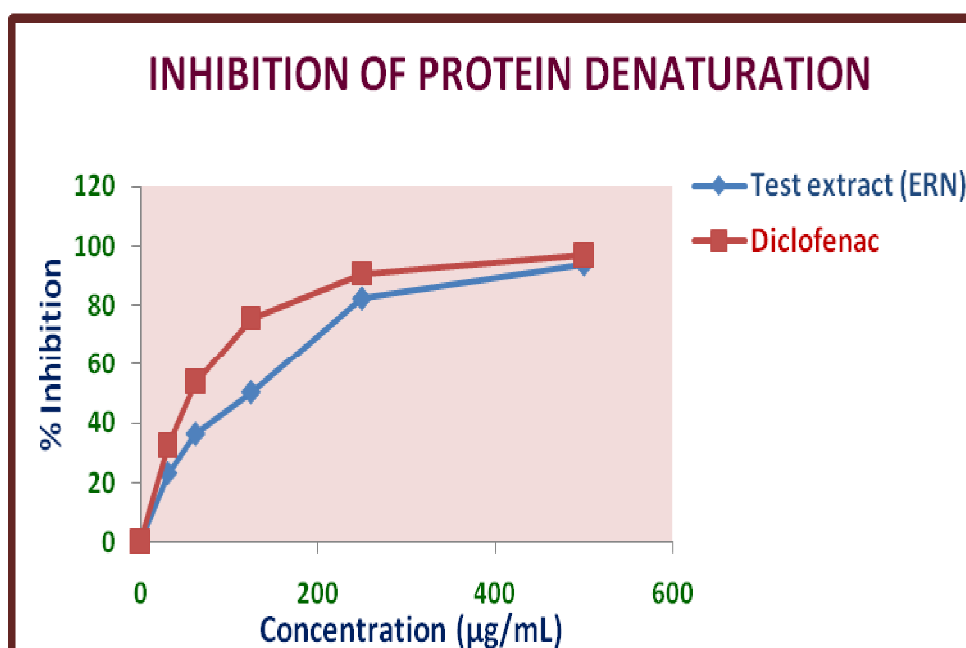
TABLE – 24 : EFFECT OF ERN AND DICLOFENAC SODIUM ON INHIBITION OF PROTEIN DENATURAION

S. No.	Concentration in µg/mL	% Inhibition*	
		ERN	Diclofenac Sodium
01	31.25	23.08 ± 1.60	32.48 ± 2.44
02	62.5	36.32 ± 2.12	53.85 ± 1.11

03	125	50.43 ± 1.94	75.64 ± 1.21
04	250	82.48 ± 1.85	90.60 ± 2.12
05	500	94.02 ± 2.28	98.01 ± 1.39

* Mean of three readings ± SEM

FIG. – 35.1 : EFFECT OF ERN AND DICLOFENAC SODIUM ON INHIBITION OF PROTEIN DENATURAION



PART – IV

SECTION - A

IN VITRO ANTI-MITOTIC ACTIVITY SCREENING BY SPROUTING GREEN GRAM MODEL [95-97]

Principle:

Invitro anti-mitotic activity screening by sprouting green gram model was carried out by the methods adopted by Kumar and Singhal[95] to evaluate the response of germinating green gram in the presence of antimitotic drugs. Growth of inactive embryo in the seed begins as a result of a triphasic progression of water imbibitions in the seeds to protrusion (radicle growth) through shedding of the seed coat. Synthetic as well as herbal anticancer drugs were resulted in the consequences of reduction of water imbibitions, failure of seed germination, growth retardation and radical decay. Quantification of antimitotic activity of the plant extracts can be determined by observing the water imbibition and morphological examination of green gram after treating with the test drugs.

Materials required :

Green gram seeds of uniform weight

70% Ethanolic extract of leaves and stems of *R. notonianum* **(ERN)**

Cisplatin

Procedure :

Green gram were purchased from local grocery shop, sorted for healthy seeds of equal weight (weighing 22.03 ± 0.01 mg) and used for this study. They were kept aside in tap water or in a ERN for 24 hrs at room temperature for imbibitions of water, considering former as a control and later as a test group. After 24 hrs treatment, they were drained off to remove water or the drug solution and the seeds were weighed after drying them on a dry tissue paper. For studying morphological characters, the sprouting time can be extended to 72 hrs and the datas were recorded as photographs. The weight of seedlings, percentage germination and length of the radicle was

recorded and compared with standard drug Cisplatin **(Table – 25 to 27** and **Fig. 36.1 to 36.4).** Percentage inhibition was calculated using the following equation :

$$\textbf{\% inhibition} = \frac{(W_c - W_t)}{(W_c - W_s)} \times 100$$

Where,

W_c = Weight of control (Tap water treated) seeds

W_t = Weight of test drug treated seeds and

W_s = Weight of the seed producing maximum inhibition (i.e., for *R. notonianum*, 10mg/mL concentration and for Cisplatin-treated group 2.5mg/mL concentration were taken as W_s which produces the maximal inhibition)

TABLE – 25 : DOSE DEPENDENT REDUCTION IN IMBIBITION BY *ERN*

(Volume = 1 mL, n = 10, Time = 24 hrs)

Concentration of ERN	**1 mg/mL**	**2 mg/mL**	**4 mg/mL**	**8 mg/mL**	**10 mg/mL**	**Control (Tap water)**
Average seed weight# (mg)	197 ± 1.11	171 ± 2.47	142.3 ± 1.39	112.3 ± 2.15	104.7 ± 2.71	214.7 ± 1.86
% inhibition* (% Reduction in water imbibitions)	21.19 ± 3.75	47.02 ± 6.45	68.11 ± 2.89	94.45 ± 2.86	100	0

* Mean of ten readings ± SEM

TABLE – 26 : DOSE DEPENDENT REDUCTION IN IMBIBITION BY CISPLATIN

(Volume = 1 mL, n = 10, Time = 24 hrs)

Concentration of Cisplatin	**0.5 mg/mL**	**1.0 mg/mL**	**1.5 mg/mL**	**2.0 mg/mL**	**2.5 mg/mL**	**Control (Tap water)**
Average seed weight# (mg)	161.3 ±	134.7 ±	127.6 ±	99.4 ±	103.2 ±	214.7 ±

	2.27	1.98	2.18	1.39	2.11	0.69
% inhibition* (% Reduction in water imbibitions)	47.89 ± 2.11	71.15 ± 3.38	78.12 ± 3.17	93.18 ± 5.54	100	0

* Mean of ten readings ± SEM

TABLE - 27 : EFFECT OF *R.notonianum* AND CISPLATIN ON THE PERCENTAGE GERMINATION AND RADICLE LENGTH OF GREEN GRAM SEED

Concentration (mg/mL)	**% Germination***		**Radicle length# (%)**	
	Cisplatin	***R.notonianum***	**Cisplatin**	***R.notonianum***
0.1	78	88	76.67	93.33
1.0	43	64	33.33	80
5.0	07	31	6.67	43.33
10.0	0	12	0	6.67

(* : n = 100 and # : n = 20)

Effect of different concentrations of *R. notonianum* and Cisplatin on sprouting of green gram seeds after 24 hours of sprouting in aqueous media was shown in **Fig. 36.5**.

SECTION - B

IN VITRO ANTI CANCER ACTIVITY - MICROCULTURE TETRAZOLIUM (MTT) ASSAY [98-100]

Principle

3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. MTT assay is based on the capacity of mitochondrial enzyme (mitochondrial succinate dehydrogenase) in living cells to reduce the yellow water soluble MTT substrate into an insoluble, purple colored formazan product. The intensity of colored formazan product was measured at 570 nm spectrophotometrically. As reduction of MTT can only occur in metabolically active cells and the amount of formazan produced is directly proportional to the number of viable cells, the level of activity is considered as a measure of the cell's viability.

Materials required

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, India, and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). All cells were kept in incubator which is maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/mL. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 hrs, the cells were treated with serial concentrations of the extracts and fractions. They were

initially dissolved in neat dimethylsulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 500, 250, 125, 62.5 and 31.25 µg/mL. The final volume in each well was 200 µl and the plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 48h. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

After 48h of incubation, 15µL of MTT (5mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µL of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

$$\textbf{\% Cell Inhibition} = 100 - \frac{\textbf{Absorbance of sample}}{\textbf{Absorbance of Control}} \times 100$$

The findings were presented in the **Table – 28** and **Fig. 38.1** to **Fig. 38.6.** Further, nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using GraphPad Prism software **(Fig. 37)**.

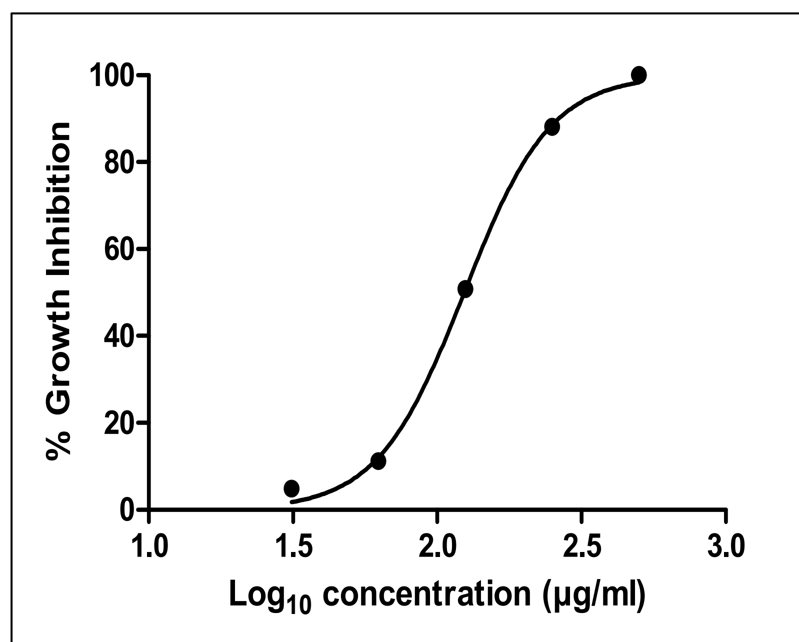
Table – 28 : MTT assay for evaluation of percentage cell growth inhibition in HeLa Cell lines

S. No.	Concentration (µg/mL)	Percentage cell inhibition	IC_{50}

1	31.25	4.89	**124 µg/mL**
2	62.5	11.18	
3	125	50.77	
4	250	88.17	
5	500	100	

* Mean of three readings ± SEM

Fig. 37 : MTT assay for evaluation of percentage cell growth inhibition in HeLa Cell lines

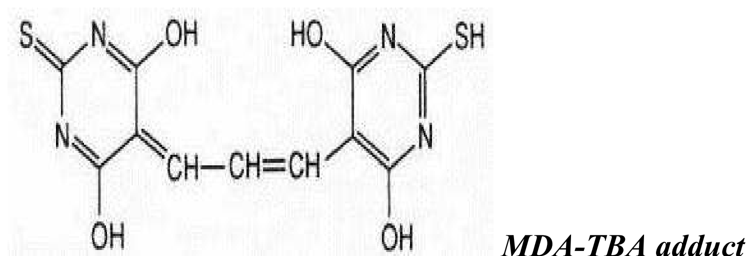


PART - V

INVITRO AMELIORATIVE EFFECT OF ARSENIC TRIOXIDE INDUCED HEPATIC INJURY BY ERN [101-108]

Being a principle metabolic organ, liver is a major target for arsenic toxicity. Exposure of arsenic induces lipid peroxidation which helps in the initiation of oxidative stress by forming reactive oxygen species (ROS). Oxidative stress (which leads to hepatic diseases) has been characterized by oxidative injury to the cellular components viz., protein, lipid, and nucleic acid. Arsenic exposure reduces total hepatocyte protein content and gradually suppresses the proliferation of hepatocytes in a time dependent manner. Lipid peroxidation and proteins were quantified to prove the ameliorative effect of plant extract on arsenic induced hepatic damage.

Lipid peroxidation is screened by Thiobarbituric Acid Reactive Substances (TBARS) assay. In this assay, malondialdehyde forms a 1:2 (MDA-TBA) adduct with thiobarbituric acid and it can be measured as malondialdehyde equivalence standard, colorimetrically.



MDA-TBA adduct

Oxidative stress has been characterized by oxidative injury to the cellular components viz., protein, lipid, and nucleic acid. TBARS is a well-established assay to quantify the lipid peroxidation. Malondialdehyde forms a 1:2 (MDA-TBA) adduct with thiobarbituric acid which can be measured colorimetrically.

In protein assay, cupric ions (Cu^{2+}) chelate with the peptide bonds are reduced into cuprous ions (Cu^{+}), under alkaline conditions. Cuprous ion (Cu^{+}) reduction of Folin Ciocalteu Reagent (sodium tungstate molybdate and phosphate) produces a blue purple color that can be read at 650 – 750 nm.

MATERIALS & REAGENTS REQUIRED:

- Analytical grade arsenic trioxide (As_2O_3)
- 70% Ethanolic extract of leaves and stems of *R. notonianum (ERN)*
- Fresh liver sample of healthy adult chicken (*Gallus domesticus*)
- 0.005% v/v Hydrochloric acid
- Distilled water
- 2M Phosphate buffer solution (pH 7.4)

PREPARATION OF REAGENTS AND TISSUE HOMOGENATE

Arsenic trioxide solution:

40 mg of arsenic trioxide was dissolved in 1 mL of 0.005% V/V hydrochloric acid and final volume was made up to 100 mL using distilled water. 1 mL of the above solution was made up to 10 mL with distilled water and used for the experiment.

Alkaline copper reagent:

1mL of 1% w/v copper sulphate, 1mL of 2% w/v sodium tartrate and 98mL of 2% w/v sodium carbonate solution.

TBA reagent :

200 mg of thiobarbituric acid was dissolved in 60 mL of 50% V/V acetic acid.

Liver sample:

Fresh liver sample of healthy adult chicken (*Gallus domesticus*) weighing about 1.5kg was obtained from local slaughter house which was

transferred to laboratory instantaneously under frozen condition and processed immediately as follows.

TISSUE HOMOGENATE PREPARATION

For protein estimation:

2.5 g of fresh liver of healthy adult chicken (*Gallus domesticus*) was homogenized in 100 mL of previously chilled distilled water.

***For estimation of Lipid peroxidation* (LPO):**

25 mg of liver was homogenized in 100 mL of previously chilled phosphate buffer solution.

EXPERIMENTAL PROTOCOL

This study comprise of two steps. To carry out these two steps, following test groups were prepared.

Control group : 0.2 mL of liver homogenate

STEP – I : DETERMINATION OF THE EFFECT OF As_2O_3 EXPOSURE ON CHICKEN LIVER HOMOGENATE

1) **Toxin treated group**

0.1 - 0.5 mL of As_2O_3 solution were mixed with 0.2 mL of liver homogenate.

STEP – II : DETERMINATION OF AMELIORATIVE EFFECT OF ERN ON As_2O_3 INDUCED CHANGES ON LIVER HOMOGENATE

2) **As_2O_3 treated group**

0.3 mL of As_2O_3 was mixed with 0.2 mL of liver homogenate.

3) **Test extract treated group**

0.5 mL of test extract was mixed with 0.2 mL of homogenate.

4) **Toxin + test extract treated group**

0.3 mL of As_2O_3 solution and 0.1 to 0.4 mL of test extract were mixed with 0.2 mL of liver homogenate.

The final volume in all the test groups were made up to 1.0 mL with double distilled water (DDW) / phosphate buffer saline (PBS) was added for the estimation of protein and LPO, respectively. All the test groups were incubated for 30 minutes at 37ºC.

PROTEIN ESTIMATION

To 1mL of tissue homogenate, 6mL of alkaline copper reagent was added. Mixed well and allowed to stand at 37ºC for ten minutes. To this mixture, 0.3mL of the Folin-Ciocalteu reagent was added, mixed and incubated in the dark for 30 minutes at 37ºC. After incubation period the absorbance was read at 500nm using a UV-visible spectrophotometer.

A standard calibration curve **(Table – 30** and **Fig. 39)** was also generated in the similar manner, by only replacing tissue homogenate with 1mL Bovine Serum Albumin (0-300 µg/mL).

Arsenic trioxide induced changes in protein content (in mg protein/100mg fresh liver) and amelioration by *ERN* was given in the **Table – 29** and **Table – 32**, respectively.

ESTIMATION OF LIPID PEROXIDATION

The TBARS levels were determined by the method adopted by Ohkawa *et al.* To the tissue homogenate (Drug treated, toxin treated, and drug + toxin treated groups), 0.5 mL of normal saline, 1 mL of 20% trichloroacetic acid and 0.25 mL of thiobarbituric acid were added. The test tubes were kept for boiling at 95ºC for one hour. To this, 3 mL of n-butanol was added and mixed well followed by the centrifugation at 3000 rpm for 10 minutes. After the centrifugation, the separated butanol layer was collected and absorbance was recorded in a spectrophotometer at 535 nm.

A standard calibration curve **(Table – 31** and **Fig. 40)** was also generated in the similar manner, by only replacing tissue homogenate with malondialdehyde (10-50 nM/mL). Concentration of thiobarbituric reactive substances (TBARS) was expressed in terms of nM of malondialdehyde/mL of tissue homogenate.

Arsenic trioxide induced changes in lipid peroxidation (in nM malondialdehyde/mg protein/hr) and amelioration by *ERN* was given in the **Table – 29** and **Table – 32**, respectively.

Table - 29 : Arsenic trioxide induced changes in protein content and lipid peroxidation of chicken liver homogenate *invitro*

Concentration of Arsenic (µg/mL)	Protein content* (mg protein/100mg fresh liver)	Lipid peroxidation* (nM malondialdehyde/mg protein/hr)	Significant at p <
0	13.03 ± 0.32	4.85 ± 0.17	-
1	9.39 ± 0.15	5.45 ± 0.62	0.001

2	9.65 ± 0.82	5.82 ± 0.09	0.001
3	10.12 ± 0.76	5.97 ± 0.35	0.001
4	10.58 ± 0.47	6.22 ± 0.06	0.001
5	11.64 ± 0.91	6.43 ± 0.21	0.001

* Mean of three readings ± SEM

Table - 30 : Standard calibration of Bovine serum albumin for protein estimation

S. No	Conc. of bovine serum albumin (µg/mL)	*Absorbance at 500 nm
1	60	0.092 ± 0.01
2	120	0.185 ± 0.02
3	180	0.276 ± 0.01
4	240	0.369 ± 0.02
5	300	0.451 ± 0.02

* Mean of three readings ± SEM

Fig. 39 : Standard calibration curve for Bovine serum albumin

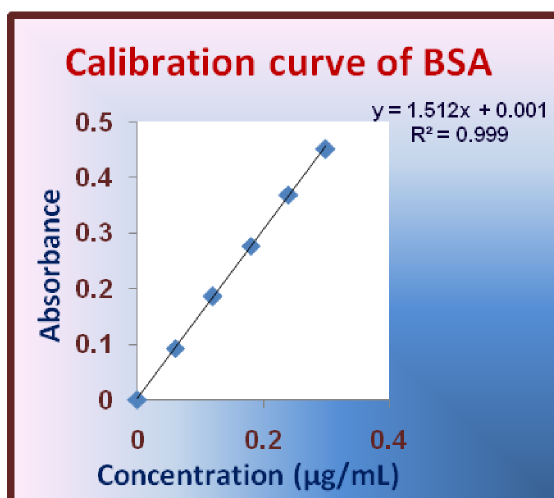


Fig. 40 : Standard calibration curve for Malondialdehyde

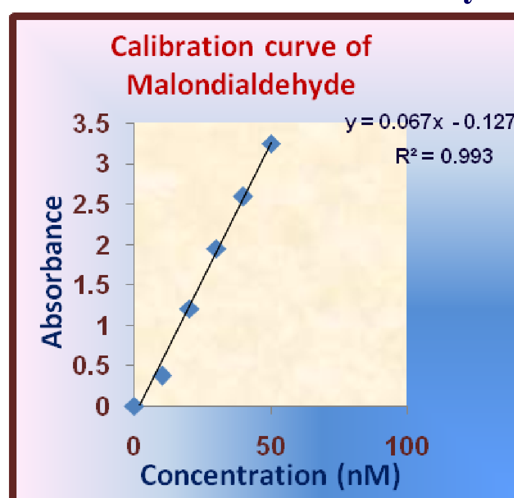


Table - 31 : Standard calibration of Malondialdehyde for estimation of lipid peroxidation

S. No	Conc. of malondialdehyde (nM/mL)	*Absorbance at 535 nm
1	10	0.374 ± 0.01
2	20	1.206 ± 0.03

3	30	1.954 ± 0.01
4	40	2.597 ± 0.14
5	50	3.251 ± 0.11

* Mean of three readings ± SEM

Table – 32 : Arsenic trioxide induced changes in protein content and lipid peroxidation of chicken liver homogenate and its amelioration by ethanolic extract of *Rhynchoglossum notonianum* (ERN)

Conc. of Arsenic (µg/mL)	Conc. of ERN (µg/mL)	Protein content* (mg protein/100mg fresh liver)	Significant at p <	Lipid peroxidation* (nM malondialdehyde/mg protein/hr)	Significant at p <
0	0	13.03 ± 0.34	-	4.49 ± 0.09	-
3	0	11.24 ± 0.29	-	5.97 ± 0.04	-
0	200	2.65 ± 0.02	-	6.21 ± 0.07	-
3	40	7.14 ± 0.41	0.001	5.18 ± 0.26	0.001
3	80	5.09 ± 0.03	0.001	4.81 ± 0.11	0.001
3	120	4.17 ± 0.25	0.001	4.49 ± 0.02	0.001
3	160	3.77 ± 0.06	0.001	4.05 ± 0.38	0.001
3	200	3.04 ± 0.13	0.001	4.01 ± 0.02	0.001

* Mean of three readings ± SEM

PART - VI

ANTIBACTERIAL ACTIVITY [109-111]

The 70% ethanolic extract of *Rhynchoglossum notonianum* was screened for antibacterial activity.

MINIMUM INHIBITORY CONCENTRATION (MIC)

Bacteria

The various organisms used in the present study include *Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli.* These organisms were confirmed by biochemical tests.

Preparation of media

Muller Hinton Agar (MH, Hi media) was used. The formula of the medium: (gm/litre) Beef - 2 g, caesin acid hydrolysate 17.5g, starch 1.5g and agar 17 g; 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 15 minutes at 15psi pressure and was used for sensitivity tests.

Preparation of bacterial cultures

Few colonies of the bacterial strains selected for study were picked from the agar slopes and inoculated into 4mL peptone water in a test tube. These tubes were incubated for 2-4 hours to produce suspensions. The suspensions were then diluted, if necessary with saline to a density visually equivalent to that of standard prepared by adding 0.5mL of 1% barium chloride to 99.5mL of 1% sulphuric acid. These suspensions were used for seeding.

Preparation of the extracts

The plant extract was dissolved in DMSO to get a concentration of 10mg/mL.

Preparation of agar plates

The media (20mL) was introduced aseptically into sterilized petridishes [110] and the petridishes were swirled until the agar begins to set.

Disc Diffusion technique

The pathogenic strains were then seeded on the MH agar media in a petridish by streaking the plate with the help of a sterile swab. Care was taken for the even distribution of culture all over the plate. The seeded plates were allowed to dry.

Test Procedure

The plain sterile discs of 6mm diameter were obtained from Hi Media. The discs were then impregnated with different concentrations of the 70% ethanolic extract of *Rhynchoglossum notonianum* and solvent DMSO. Amikacin discs were used as a standard. Each disc contained 30µg. The standard, extract and DMSO discs were then placed on the seeded medium plates. The plates were then incubated at 37°C for 24h. The results were read by the presence or absence of zone of inhibition. The zone of inhibition was then measured. The results were tabulated in **Table – 33** and **Fig. : 43 – 46.**

TABLE – 33 : ANTIBACTERIAL ACTIVITY OF ERN

S. No.	*Microorganism*	Amikacin		ERN	
		Concentration	Zone of	Concentration	Zone of

		(µg)	**inhibition (mm)**	**(µg)**	**inhibition (mm)**
01	*Streptococcus pyogenes*	30	26	500 1000 1500	- - 20
02	*Pseudomonas aurogenosa*		21	500 1000 1500	- - 19
03	*Staphylococcus aureus*		21	500 1000 1500	- - 18
04	*Escherichia coli*		26	500 1000 1500	- - 20

CHAPTER-VII

RESULTS AND DISCUSSION

This dissertation covers the works on pharmacognostic, phytochemical and pharmacological studies on the leaves and stems of *Rhynchoglossum notonianum*, belongs to the family Gesneriaceae.

CHAPTER-I

Introduction of this dissertation includes an information about the herbal medicine, need for standardization, inflammation and its relationship with reactive oxygen species, cancer and inflammatory skin diseases. Apart from this, inflammation -mediated diseases like rheumatoid arthritis, hepatic diseases and *Streptococcal* infection were also discussed in this chapter.

CHAPTER-II

Literature survey pertaining to the pharmacognostic, phytochemical and pharmacological studies and ethanomedical information of *Rhynchoglossum notonianum* and its related species has dealt in this chapter. Ethnomedical information revealed that the plant is used for the treatment of polio and inflammation.

CHAPTER-III

Aim and scope of the present study have been discussed in this part considering the ethnomedical information and chemical nature of phytoconstituents present in this plant.

CHAPTER-IV

Pharmacognostical studies

In this chapter, an attempt has been made to fix certain pharmacognostic standards for the leaves and stems of *Rhynchoglossum notonianum* which includes the macroscopical, microscopical, quantitative microscopy and physical standards.

Section-A

Scientific classification, synonym, vernacular names, habit and habitat of the plant and morphological characters of leaves and flowers of ***Rhynchoglossum notonianum*** were described in detail to identify the plant with the support of photographs **(Fig. 1.1** to **Fig. 1.4)**

Salient features of the macroscopy of leaves were observed, such as leaves are alternate, shortly petiolate and leaf bases are unequal, one half cordate and the other half is sinuate-attenuate **(Fig. 1.5** and **Fig. 1.6)**. The texture of leaf is thin and delicate. Lamina is obliquely ovate-cordate. Inflorescences of *R. notonianum* are terminal on main and the side branches. The leaves are shiny dark green in colour.

Section-B

It deals with the microscopical studies of the leaves and stems to find out the arrangement of tissues. **(Fig. : 2 – 7).** Review of literature revealed that no detailed work has been carried out regarding morphological and microscopical studies of *Rhynchoglossum notonianum* since these characters play an important role in the authentication of crude drug and is also useful for the detection of adulteration.

The important anatomical features observed in the leaves and stems of *Rhynchoglossum notonianum* are as follows -

- Leaf is dorsiventral in nature. Transverse section of leaf shows a hump on the adaxial surface and convexity on the abaxial surface.
- Epidermis is single layered, made-up of small rectangular cells. Palisade tissue is composed of a single row of columnar closely arranged cells. It is followed by 3 or 4 rows of loosely packed spongy tissue.
- Adaxial foliar epidermis is polygonal in shape with slightly wavy walls and possesses a little number of stomata. Abaxial foliar epidermal cells have wavy contour and perforated by numerous cruciferous (anisocytic) stomata.
- Cystoliths are confined to the lower epidermis. They vary in shape either spherical or ellipsoidal.
- The hypodermal region of midrib is composed of two to three rows of collenchyma cells. The ground tissue of the midrib is parenchymatous and made-up of thin walled polygonal cells. Five meristeles are situated at the central region of the ground tissue.
- Larger veins have two collateral vascular bundles, with the xylem groups directed towards one another.
- **T.S. of the stem:** It shows the epidermis followed by hypodermis, ground tissue which is finely divided by an ill-defined endodermis and pericycle which are small parenchymatous cells. These two layers are not distinguishable in mature stems. A large number of separate vascular strands are scattered in the ground tissue. Secretory canals of variable diameter with resinous contents accompany the xylem of the vascular bundles. Secretory canal is lined by a single layer of epithelial cells.

- **T. S. of petiole:** It is oval in shape. Epidermis is followed by hypodermis composed of 3 to 5 layers of collenchyma cells which is followed by ground tissue of parenchyma cells with scattered vascular bundles and secretory canals.

Section-C

The following anatomical characters were observed with powder microscopy of leaves and stems revealed the presence of -

- ❖ Xylem vessels with spiral thickenings and horizontal slits.
- ❖ Large numbers of xylem fibers which are longer than the vessels but thinner in appearance. It often occur as a multiple strands
- ❖ Phloem fibers of lignified and non-lignified nature
- ❖ There are two types of trichomes viz., the glandular and non-glandular. Angular shaped, pyramid like shorter trichomes with one or two cells.
- ❖ Cruciferous stomata
- ❖ The typical kidney shaped stomatal guard cells with starch content
- ❖ The mesophyll tissue found in bunches of varying size
- ❖ Epidermal fragments with thick, polygonal, slightly wavy anticlinal walls

The observed macroscopical, microscopical, cytomorphological features has added more weightage to the authenticity of the plant. Moreover, microscopical assessment also offer supporting evidence when united with other analytical parameters based upon their cell types and cell inclusion details etc.

Section-D

It deals with the quantitative microscopy such as vein-islet number, vein termination number, stomatal number, stomatal index and palisade ratio. Quantitative microscopy gives a constant value or range of values for stomatal number, stomatal index, vein termination number, vein islets number and palisade ratio which are the basic criterion for identification and evaluation of the medicinal plant. It can be used for the authentication of crude drug as these values remains constant for a particular plant species and these may not be altered by any geographical variation. The observation obtained from the present study were tabulated as follows:

S. No.	Parameters	Values obtained*
01	Stomatal number in upper epidermis	6.6 ± 0.37
02	Stomatal number in lower epidermis	83.9 ± 2.28
03	Stomatal index in upper epidermis	18.26 ± 0.79
04	Stomatal index in lower epidermis	29.17 ± 1.23
05	Vein islet number	2.5 ± 0.22
06	Vein termination number	2.3 ± 0.15
07	Palisade ratio	2.5 ± 0.26

*Mean of 10 readings ± SEM

Section-E

The physical parameters like ash values, extractive values, moisture content, foaming index and swelling index are very valuable for the development of standardization parameters for *Rhynchoglossum notonianum*. To assess the purity and quality of the herbal drugs, standardization plays a major role in herbal drug manufacture. Physical parameters like profile of ash values, loss on drying and

various extractive values have been determined for this plant which is summarized below.

S. No.	Parameters*	Values* expressed as %
01	**ASH VALUES**	
	Total ash	14.205 ± 1.11
	Water soluble ash	7.167 ± 0.16
	Acid insoluble ash	0.149 ± 0.02
02	Volatile oil content	Nil
03	Foreign organic matter	0.41 ± 0.04
04	Loss on drying	9.085 ± 0.57
05	Foaming index	< 100

* Mean of six readings (n = 6)

Highest extractive value (17.21%) was noted in 70% ethanolic extract and the lowest extractive value (6.555%) was observed in ethyl acetate extract. The results were tabulated as follows:

EXTRACTIVE VALUES

S. No.	Solvent	Extractive values* expressed as %
1	Petroleum ether	9.261 ± 0.055
2	Ether	9.619 ± 0.028
3	Hexane	11.387 ± 0.048
4	Chloroform	7.392 ± 0.149
5	Ethyl acetate	6.555 ± 0.096
6	Methanol	12.63 ± 0.167
7	Ethanol	14.484 ± 0.054
8	70% Ethanol	17.21 ± 0.078
9	Aqueous	13.273 ± 0.041

* Mean of three readings (n = 3)

Section – F

The fluorescence analysis of the powdered drug of *Rhynchoglossum notonianum* in various solvents were performed under normal and UV light. All the leaf and stem extracts are examined in short (254nm) and longer wavelengths (366 nm) to detect the fluorescent compounds. The coloured fluorescence obtained for the leaf and stem powder as well as extracts are presented in following table.

Reaction of powdered drug with various chemical reagents

Powder + Reagents	Color / Precipitate	Presence of active principle
Picric acid	Yellow precipitate	Protein present
Conc. Sulphuric acid	Reddish brown color	Phytosterols present
Libermann Burchard reagent	Reddish brown color	Phytosterols present
Aqueous ferric chloride	Greenish black color	Tannins present
Iodine solution	Blue color	Presence of starch
Mayer's reagent	No cream color	Absence of alkaloids
Spot test	No stain	No fixed oil present
Sulfosalicylic acid	White precipitate	Protein present
Aqueous sodium hydroxide	Yellow color	Flavonoids present
Mg - HCl	Magenta color	Flavonoids present
Aqueous lead acetate	White precipitate	Flavonoids present

Fluorescence Analysis of powder of *Rhynchoglossum notonianum*

Powder + Reagent	Day light	UV light (254 nm)	UV light (366 nm)
Drug powder	Green	Dark green	Brown
Drug powder + 1M NaOH	Yellowish green	Fluorescent green	Black
Drug powder + 50% HNO_3	Light brown	Green	Black
Drug powder + I_2	Reddish brown	Dark green	Violet
Drug powder + 10% KOH	Yellowish green	Fluorescent green	Greyish black
Drug powder +1M HCl	Light brown	Dark green	Black
Drug powder + CH_3COOH	Light green	Fluorescent green	Greyish black
Drug powder + 50% H_2SO_4	Light green	Fluorescent green	Greyish black
Drug powder + 50% HCl	Light brown	Light green	Grey

Fluorescence analysis of extracts of *Rhynchoglossum notonianum*

Extracts	Consistency	Color in daylight	UV light (366 nm)
Petroleum Extract	Semisolid	Dark green	Reddish orange
Ether extract	Semisolid	Green	Brown
Chloroform extract	Semisolid	Greenish brown	Orange
Aqueous extract	Semisolid	Greenish brown	Green
Methanolic extract	Semisolid	Dark green	Reddish orange
Hexane extract	Semisolid	Green	Greenish brown
Ethanolic extract	Semisolid	Dark green	Reddish orange
70% ethanolic extract	Semisolid	Blackish green	Reddish orange
Ethyl acetate extract	Semisolid	Green	Orange

CHAPTER-V

Section-A

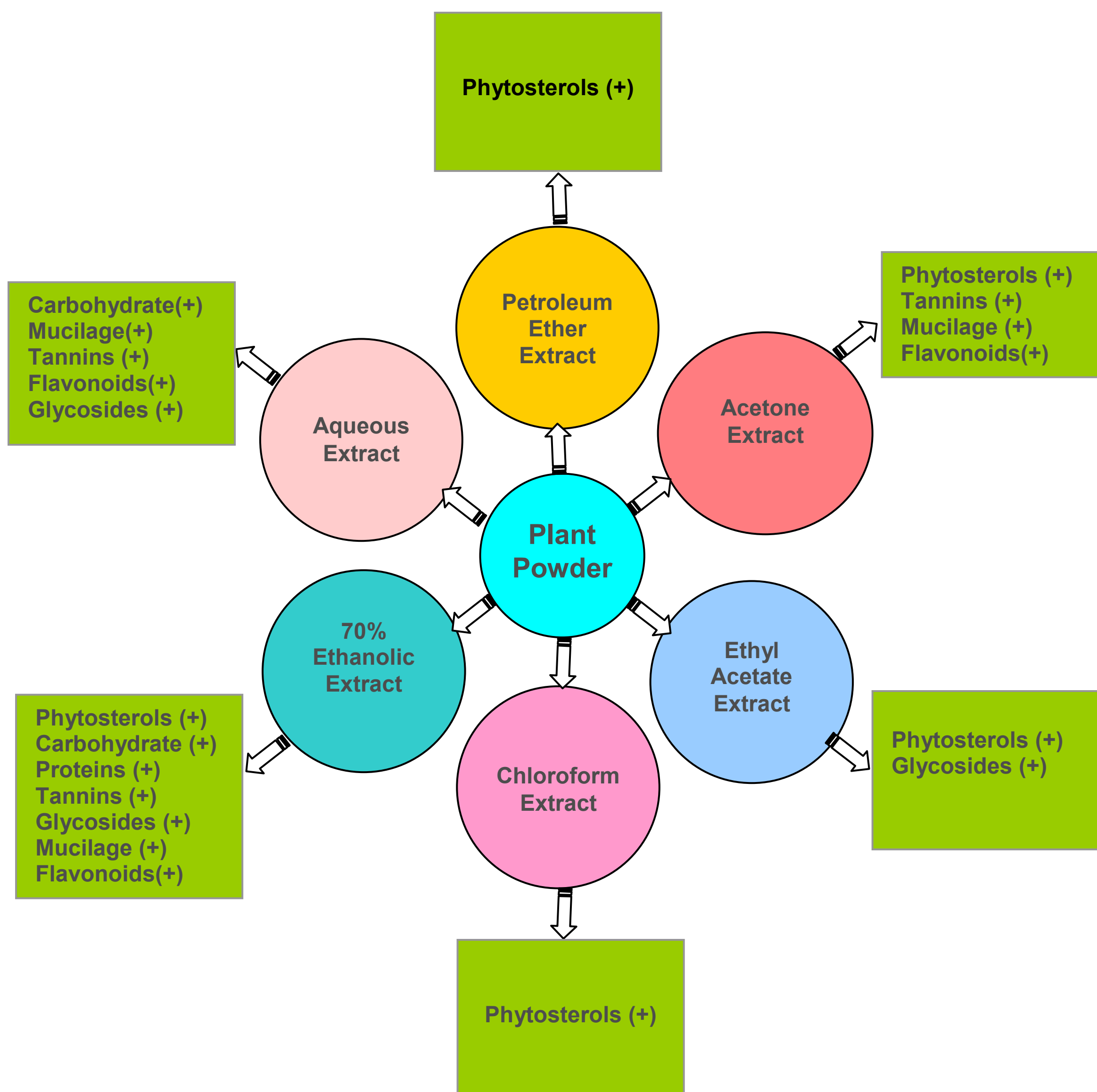
This part deals with the organoleptic evaluation of the *Rhynchoglossum notonianum* which indicates that the powder material is dark green in color, bitter in taste with characteristic odour.

Section-B

This part deals with preliminary qualitative phytochemical examination of the powdered leaves and stems of *R. notonianum*. The results were tabulated in **Table No: 7**. This study indicates the presence of sterols, carbohydrates, proteins, glycosides, flavonoids, terpenoids, tannins, phenols and mucilages.

Constituents present	Constituents absent
Carbohydrates, flavonoids, glycosides, mucilages, phenols, proteins, sterols, tannins and terpenoids.	Alkaloids, fixed oil, gums, saponins and volatile oils

Preliminary phytochemical screening was also carried out with various extracts such as petroleum ether, chloroform, ethylacetate, acetone, methanol, 70% ethanol and aqueous extracts of the leaves and stems of *R. notonianum*. The results were tabulated in the **Table No: 8** and diagrammatical representation is as shown below:



Diagrammatic Scheme of presence of various phytoconstituents in the various extracts of *R. notonianum*

Section-C

This part deals with preparation of extract and was subjected to the estimation of total phenolic content, tannin content, flavonols content, proanthocyanidine content, flavonoid content, betacarotene and lycopene contents. Preliminary phytochemical studies revealed the presence of secondary metabolites like flavonoids, phenols and tannins in this extract. Hence the extract was estimated quantitatively for total phenolic, total tannin and total flavonoid content and the results were shown in the following table.

Phytoconstituents	**Total amount present (mg/gram of extract)**
Total phenolics	109.46 ± 1.10
Total tannins	249.50 ± 0.77
Total flavonoids	116.28 ± 0.75
Total flavonols	0.498 ± 0.01
Proanthocyanidin	158.58 ± 6.59
Betacarotene	1.729 ± 0.02
Lycopene	0.182 ± 0.02

Section-D

TLC STUDIES

This part deals with TLC analysis of ethanolic extract of stems and leaves of *Rhynchoglossum notonianum* (ERN). Preliminary phytochemical studies revealed the presence of secondary metabolites like flavonoids and phenolics by chemical test.

Hence these extracts were subjected to TLC analysis to evaluate the secondary metabolites by using various solvent systems and the results were tabulated in the **Table No.15.**

This study revealed that ERN exhibited two spots in solvent system – I ((Chloroform : Methanol : Water in the ratio of 61:32:7) and three spots in solvent system-II (Chloroform : Methanol : Isopropanol : Water in the ratio of 5:6:1:4). The intensity of colour of the spot was seen under visible light. ERN showed eight spots in solvent system-III (Toluene : Acetic acid in the ratio of 4:2). This TLC analysis study revealed that ERN exhibited significant number of spots in solvent system - III.

THIN LAYER CHROMATOGRAPHY OF ERN

Solvent System	Stationary phase	Mobile phase	CHROMATOGRAM		
			No. of spots	R_f value	Detecting agent
I	Silica gel-G	Chloroform : Methanol : Water (61:32:7)	02	0.93	Vanillin-H2SO4 spray / UV light **(Fig. 19)**
				0.59	
II	Silica gel-G	Chloroform : Methanol : Isopropanol : Water (5:6:1:4)	03	0.78	Visible / UV light **(Fig. 20)**
				0.65	
				0.33	
III	Silica gel-G	Toluene : Acetic acid (4:2)	08	0.92	UV light (366 nm) **(Fig. 21)**
				0.84	
				0.79	
				0.74	
				0.68	
				0.63	
				0.53	
				0.29	

Section-E

HPTLC STUDIES

HPTLC fingerprint profile of the ethanolic extract of the *R. notonianum* showed the presence of different bioactive principles which are responsible for its biological activity. The fingerprint profile may serve as a quality control tool since comparison of chromatograms of different batches can exhibit the similarities and differences between the test samples. Hence HPTLC fingerprint profile is very useful for rapid identity check, monitoring purity of drugs and for the detection of adulterants, if any.

HPTLC chromatogram was recorded for ERN using the mobile phase – III (Toluene : Acetic acid in the ratio of 4:2), which is the same solvent system used for TLC. This study revealed that ERN exhibited ten peaks in longer wavelength (366nm) at the R_f values 0.07, 0.19, 0.38, 0.62, 0.67, 0.69, 0.72, 0.77, 0.85, 0.91 and nine peaks in shorter wavelength (254nm) at the R_f values 0.08, 0.21, 0.3, 0.49, 0.69, 0.76, 0.81, 0.86 and 0.91 were obtained in solvent system-III.

CHAPTER-VI

This chapter deals with the pharmacological screening of 70% ethanolic extract of *Rhynchoglossum notonianum* (ERN) for *invitro* antioxidant, anti-inflammatory, anti- arthritic, anticancer, hepatoprotective and anti-bacterial activity.

Part – I : ANTIOXIDANT SCREENING

Oxygen is important for life, but free radicals generation from several physiological and biochemical processes provokes development of degenerative diseases such as inflammation, diabetic complications, liver cirrhosis, nephrotoxicity,

cancer etc. Reactive oxygen species (ROS) inactivate several enzymes and damage chief cellular components resulting in tissue injury through covalent binding and lipid peroxidation. Natural antioxidant molecules like phenolic acids, polyphenols and flavonoids trap free radicals and leads to inhibition of the oxidative mechanisms. Plants are the key resource for free radical scavenging molecules whereas a variety of synthetic antioxidants were suspected to be carcinogenic.

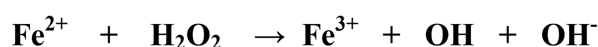
DPPH, peroxide, nitric oxide scavenging activity and ferric reducing power assay screening of *ERN* were summarized and tabulated below.

DPPH radical ion scavenging activity

DPPH is a stable lipophilic free radical which readily accepts an electron or hydrogen radical from the antioxidant compound and converts its color from violet to yellow colored stable diamagnetic molecule[78]. The intensity of the color is detected at 517nm. Lower the absorbance under UV-Visible light indicates higher the free radical scavenging activity. Free radical scavenging activity of ERN (200 µg/mL) was found to be 80.22 ± 0.95% when compared **(Fig. 29)** to standard drug ascorbic acid which was found to be 96.78 ± 2.02%. IC_{50} value of ERN and ascorbic acid was found to be 93.51µg/mL and 18.04µg/mL, respectively. This finding indicates the significant antioxidant activity of ERN.

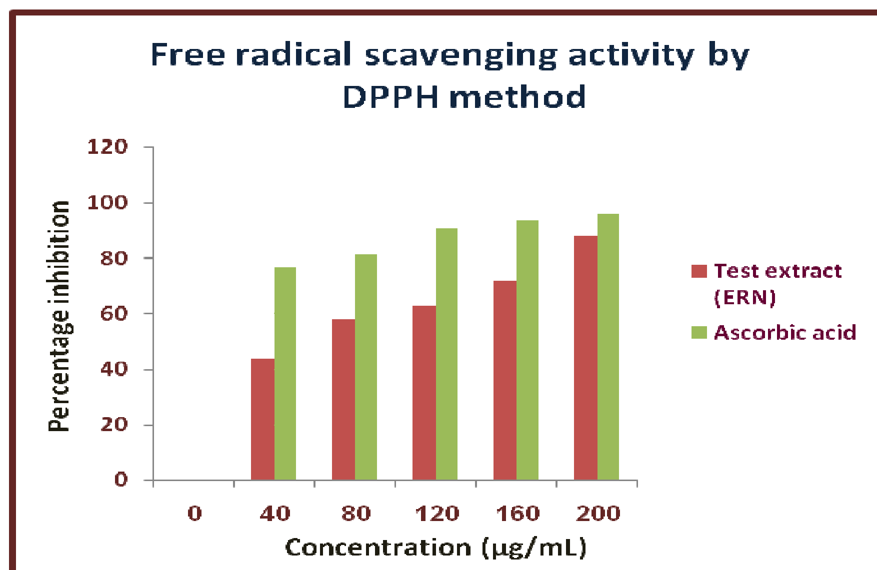
Hydrogen peroxide scavenging activity

The scavenging of hydrogen peroxide by ascorbic acid and ERN was increased with increase in concentration. Hydrogen peroxide by itself is not very reactive [112] but it is capable of generating a highly reactive hydroxyl radical through the Fenton reaction.[113 & 114]



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

Fig. 29 : Antioxidant activity of ERN and ascorbic acid in DPPH scavenging assay



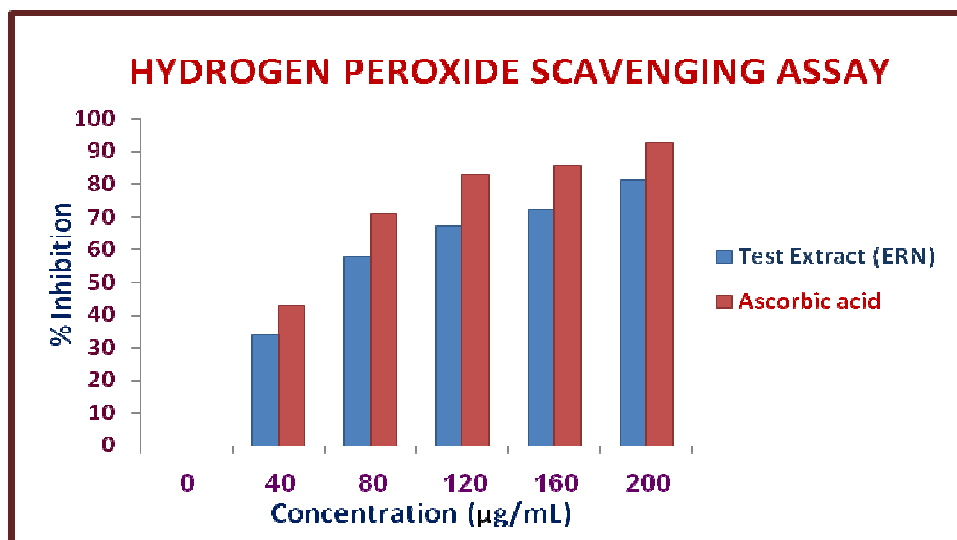
Hence the scavenging of hydrogen peroxide is an important defense mechanism.[115] Hydrogen peroxide is decomposed to water by the transfer of electron as given in the equation below [116]

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

The inhibitory effect of the extract on hydrogen peroxide may be attributed to the phenolic compounds present due to their electron donating capacity.

Addition of hydrogen peroxide to cells in culture can lead to transition of metal ion dependent hydroxyl mediated oxidative DNA damage. Levels of hydrogen peroxide at or below about 20-50mg seem to have limited cytotoxicity to many cell types. Since phenolic compounds present in the plant extract are good electron donor, they may accelerate the conversion of hydrogen peroxide to water.[79] Hydrogen peroxide free radical scavenging activity of ERN (200 µg/mL) was found to be 81.34 ± 0.69% when compared to standard drug ascorbic acid which was found to be 92.42 ± 0.65%.

Fig. 30.2 : Percentage inhibition of hydrogen peroxide by ERN and ascorbic acid



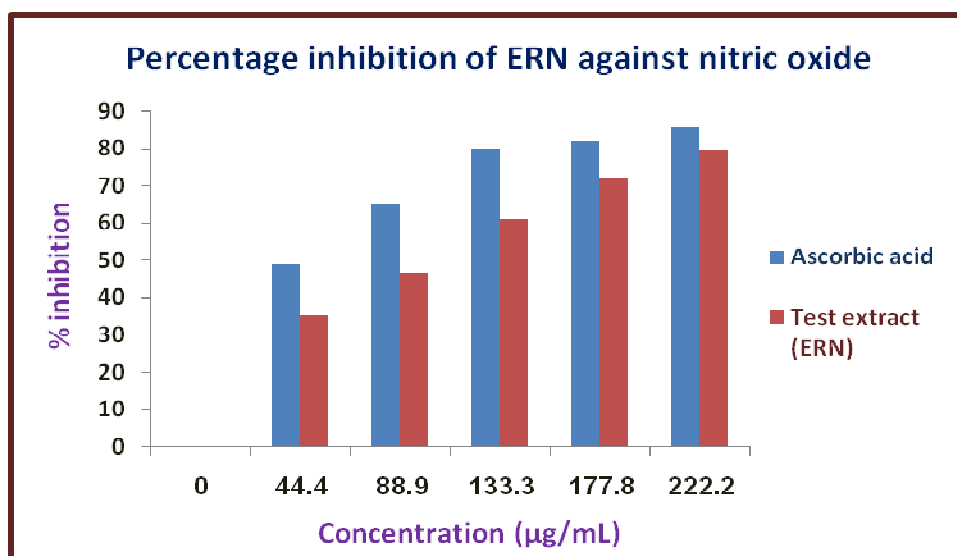
IC $_{50}$ value of ERN and ascorbic acid was found to be 94.91µg/mL and 71.33 µg/mL, respectively. This finding **(Fig. 30.2)** indicates the significant antioxidant activity of ERN.

Nitric oxide scavenging activity

Nitric oxide is a very unstable diffusible free radical. It reacts with oxygen to produce the stable product nitrates and nitrites which plays various roles as an effector molecule in cancer and various inflammatory processes. Antioxidants compete with oxygen and reduce the production of nitric oxide, thereby, reduces the burden of nitric oxide[81]. Higher levels of these radical are toxic to tissues resulting in the vascular collapse.

At 222µg/mL percentage inhibition of ERN and ascorbic acid were found to be 79.66% and 85.53% respectively in nitric oxide scavenging activity. ERN and ascorbic acid showed the IC50 value of 114.19 µg/mL and 81.96 µg/mL respectively. This study revealed that ERN showed significant nitric oxide scavenging inhibition assay when compared to ascorbic acid which is shown in the **Figure 31.2**.

Fig. – 31.2 : Nitric oxide radical scavenging by ERN *and ascorbic acid*



Ferric reducing power assay

Presence of antioxidants in the test extract reduce Fe^{3+}/ferricyanide complex to Fe^{2+} by donating electrons results in formation of various shades of green and blue[82]. By measuring the absorbance of Perl's Prussian blue at 700 nm, it is easy to monitor the Fe^{2+} concentration. Higher the absorbance indicates the higher reducing power[85]. The reducing power of a compound may be used as a significant indicator of its potential antioxidant activity with various mechanisms like prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging[84].

Ferric reducing power assay of ERN was revealed that 200 µg/mL of this extract showed absorbance of 0.248 where as 0.249 for standard drug ascorbic acid at the concentration of 10 µg/mL. ERN at 1000 µg/mL showed the absorbance of 0.405, where as the absorbance of standard drug was 0.407 at the concentration of 30 µg/mL. An increase in absorbance indicates that enhanced reducing potential of ethanolic extract of *R. notonianum.*

PART - II

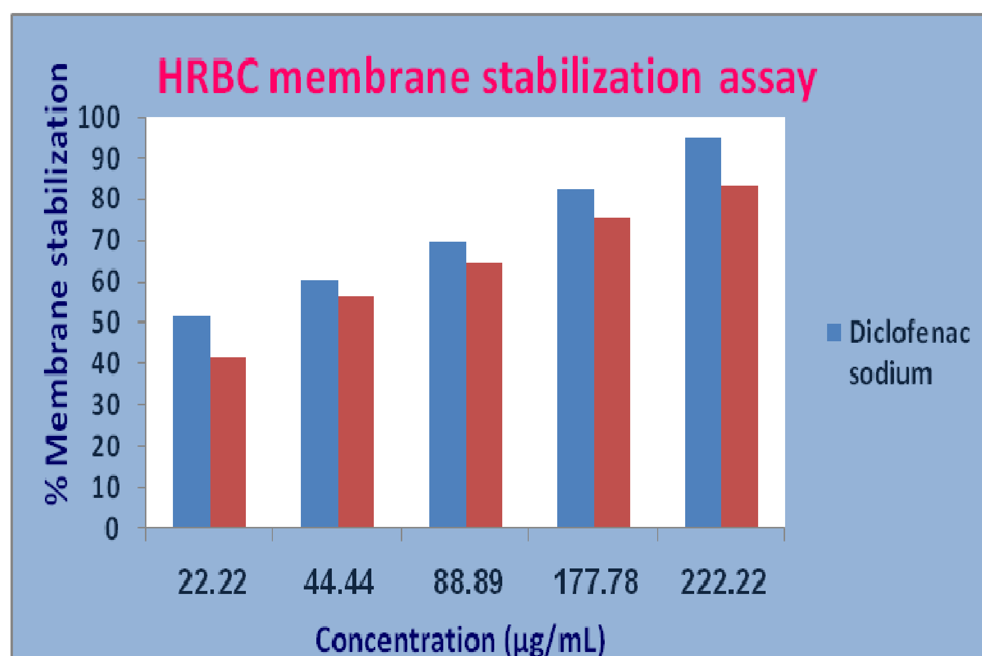
In vitro membrane stabilization study

Lysosomes are intracellular particles which contain most of the lytic and digestive enzymes of the tissue. The rupture of the lysosomes results in injury or death to surrounding tissues and also acute inflammation. The membranes of lysosomes and erythrocytes are destroyed by similar agents; hence a test was developed to measure the ability of compounds to stabilize erythrocyte membrane to heat hemolysis. RBCs when exposed to various injurious substances such as methyl salicylate, phenyl hydrazine, and hypotonic medium or over heat will cause lysis of membrane accompanied by heamolysis and oxidation of hemoglobin[117]. RBCs membranes are easily susceptible to free radical mediated lipid peroxidation by break down of biomolecules. Due to it has rich source of iron and high oxygen partial pressure. RBCs membranes are similar to lysosomal cells. All NSAIDs inhibited hemolysis while other type of compounds had no effect [118]. Hence prevention of hypotonic and heat mediated RBCs membrane lysis taken as measure of anti inflammatory activity of drugs.

A study has reported that the flavanoids exert membrane stabilizing effect on lysosomes both *in-vitro* and *in vivo* in experimental animals [86 &119]. Another report has suggested that tannins and saponins have the ability to bind cations and other bio-molecules and are able to stabilize the erythrocyte membrane [120, 121]. From the **Table - 23**, it can be seen that extract is highly potent on human erythrocyte and thus adequately protecting it against heat and hypotonicity induced lysis. The ERN and standard diclofenac sodium at the concentration of 222.22 µg/mL exert the membrane stabilization of 83.17% and 94.82%, respectively. This can be compared by using the bar diagram which is shown in Fig. 34.2.

The phytochemical analysis showed that the ERN has flavonoids and tannins. Hence the HRBC membrane stabilizing capacity may be due to the presence of the above mentioned constituents which will prevent the oxidation of hemoglobin[122] and also due to its antioxidant property.

FIG. 34.2 : COMPARISON OF PERCENTAGE OF MEMBRANE STABILIZATION BY DICLOFENAC AND ERN



PART - III

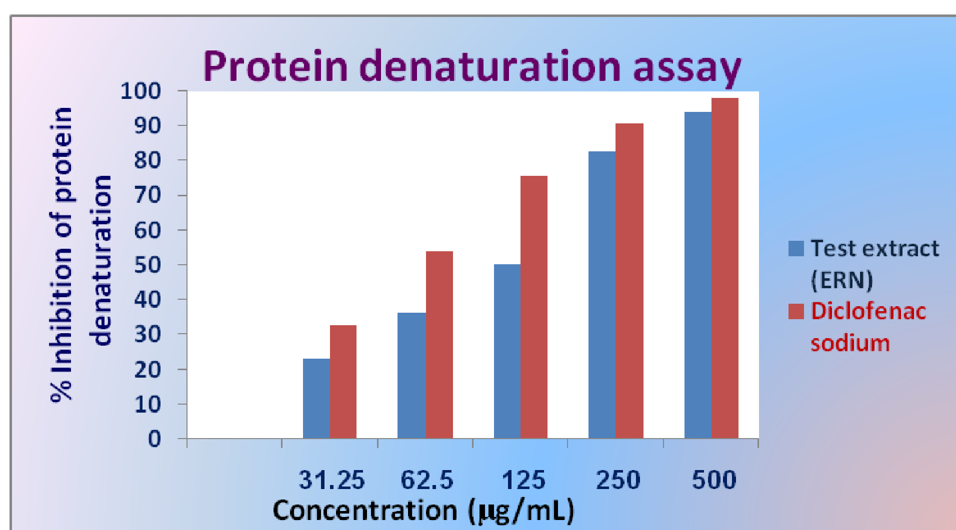
Invitro antiarthritic activity by protein denaturation method

The principle involved is the inhibition of protein denaturation. Denaturation of protein was found to be one of the causes of rheumatoid arthritis[93]. In rheumatoid arthritis, the production of autoantigen may be due to protein denaturation which involves the alteration of electrostatic hydrogen, hydrophobic and disulphide bonding[91].

The protein used in this study is bovine serum albumin. Denaturation of protein is carried out by heating. The aim of this activity is to inhibit denaturation and to

exhibit protective effect against rheumatoid arthritis. The percentage inhibition of protein denaturation by ERN was compared with standard drug (Diclofenac sodium). The results were presented in the **Table - 24.** It has been reported that anti-arthritic activity may be due to the presence of phenolic compounds. The inhibition of protein denaturation by ERN may be due to the presence of flavanoids and tannins.

FIG. – 35.2 : COMPARISON OF EFFECT OF ERN AND DICLOFENAC SODIUM ON INHIBITION OF PROTEIN DENATURAION



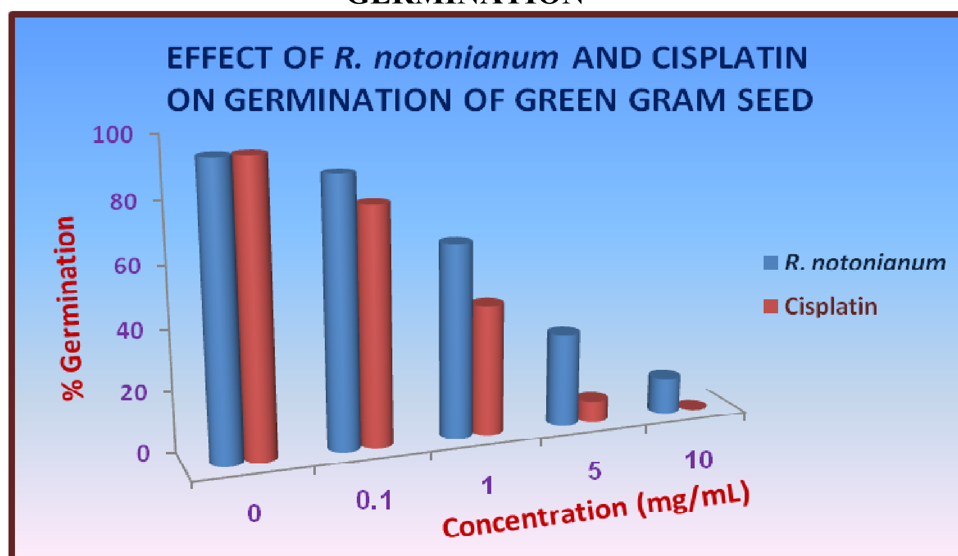
The ERN showed 94.02 % inhibition at 500 µg/mL where as standard drug diclofenac sodium showed 98.01% inhibition at 500 µg/mL.

PART – IV
SECTION – A

Invitro anti-mitotic activity screening by sprouting green gram model

Ninety five percent of green gram seeds germinated in the control group under the test conditions. 70% Ethanolic extract of leaves and stems of *R. notonianum* and Cisplatin produced a dose-dependent inhibitory effect on seed germination **(Table - 27** and **Fig.36.1).**

Fig. 36.1 : DOSE DEPENDENT INHIBITORY EFFECT ON SEED GERMINATION



The extent of water imbibition and seedling growth was indicated by an increase in seedling weight at 24 hrs as compared to the weight of dry seeds **(Table – 25, 26 and Fig. 36.2, 36.3).** The gain in radicle length after 48hrs was markedly affected by most of the concentrations in a dose-dependent manner **(Table – 27 and Fig. 36.4).**

There is a rising need to develop simple, economical and novel *in-vitro* methods for the preliminary screening of anticancer drugs that could be followed by the validation in animal model. [123]

FIG. 36.2 : REDUCTION IN WATER IMBIBITION BY *R. notonianum*

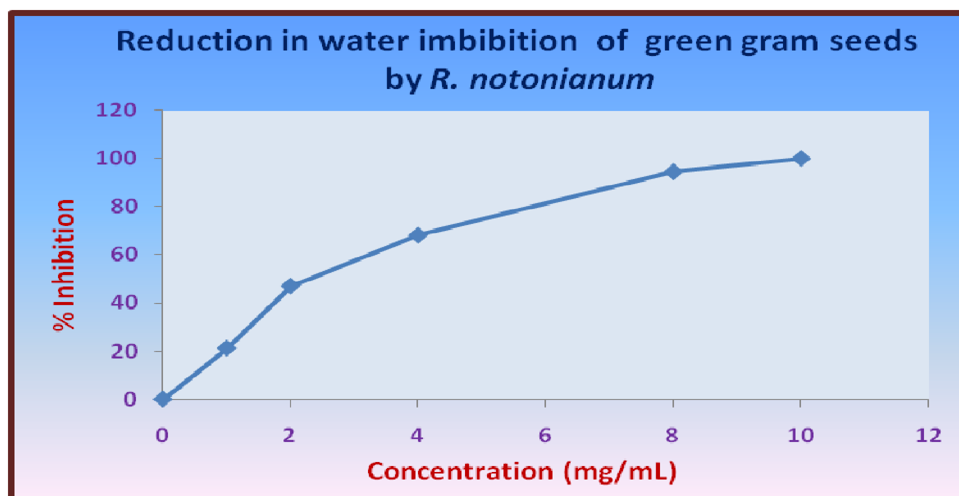


FIG. 36.3 : REDUCTION IN WATER IMBIBITION BY CISPLATIN

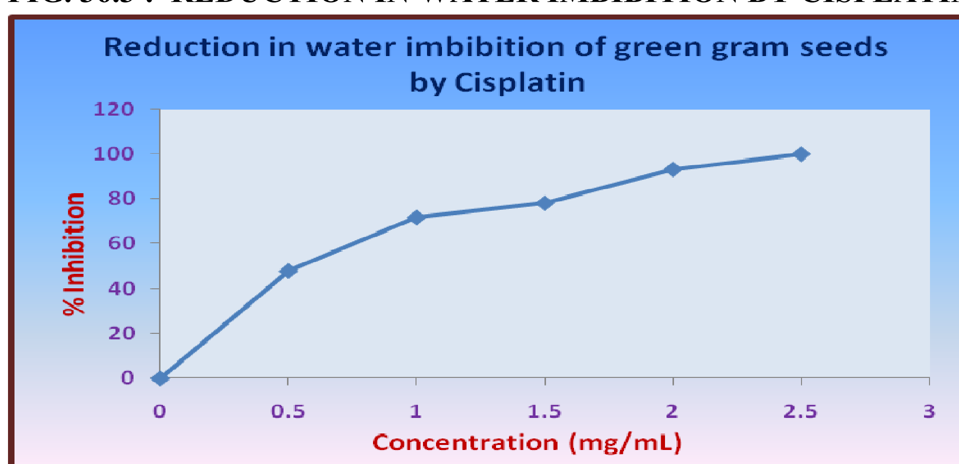
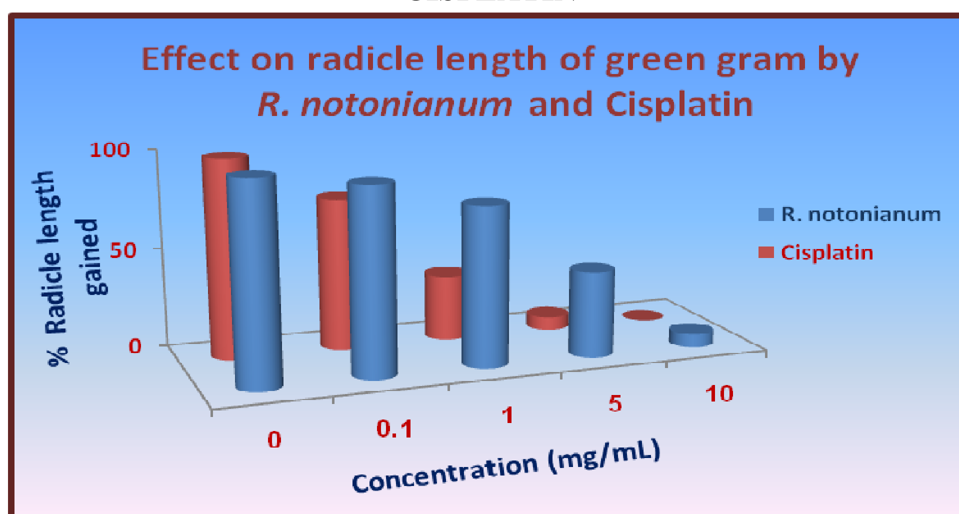


FIG. 36.4 : EFFECT OF RADICLE LENGTH BY *R. notonianum* AND CISPLATIN



Screening of anti-mitotic activity using mung bean developed by Kumar and Singhal[95] has established inhibition of the following parameters: (a) Percentage sprouting of seeds, (b) Imbibitions of water, (c) Length of radical in 24 hrs and (d) growth (cytotoxicity).

In the present study, the response of germinating green gram seeds to *R. notonianum extract* and Cisplatin were evaluated. Water imbibition was allowed to some extent at low concentrations while higher concentrations of *R. notonianum* extract completely inhibited the weight gain. Due to this reduction in water imbibitions failed to trigger the seed germination. Further, soaking the seedlings in higher drug concentrations not only produced a growth retarding effect, but it also produced radicle decay. The growth retardation with *R. notonianum* could have also resulted from the inhibition of cell division and radical protrusion brought about by osmotic stress.[124-126] In present study the growth retardation brought about by *R. notonianum* was found to be associated with a significant reduction in mitotic index in the root tip meristematic tissue.

Whether the inhibition is specific for cancer or is incidental to treatment with *R. notonianum* was found by checking the inhibitory activity of known anticancer drug like Cisplatin. False positive results are possible when imbibitions alone is measured, but when both imbibitions and morphological observation methods are used together, false positive can be avoided.

SECTION : B

In-vitro anti cancer activity by MTT assay

A *HeLa* cell is an immortal cell line derived from cervical cancer cells. Absorbance values lower than the control cell's absorbance indicates decline in the rate of cell proliferation. On the other hand, a higher absorbance rate indicates an increase in cell proliferation which may be compensated by cell death. The confirmation of cell death may be inferred from morphological changes.

The ethanolic extract of *R. notonianum* stems and leaves was evaluated for its cytotoxicity against *HeLa* cell lines at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds steadily upto 250 µg/mL which is presented in **Table – 28** and graphically represented in **Fig. 37.**

IC_{50} value on *HeLa* cell line was found to be 124 µg/mL and R^2 value was 0.9982. MTT assay also shows significant effect on *HeLa* cell line. The present study evaluated that *R. notonianum* has potential activity on *HeLa* cell and has tremandous anticancer activity on cervical cancer.

PART - V

In-vitro ameliorative effect of arsenic trioxide induced hepatic injury

Arsenic is a well known toxicant and its exposure results in increased free radical level which acts as an effector molecule of various illnesses viz., hematological, hepatic, renal and neurological disorders. The present study was planned to estimate lethal effect of arsenic trioxide (As_2O_3) by a biochemical parameters indicative of oxidative stress and its amelioration by ERN, *in vitro*.

Table - 26 show the effect of increased concentration of arsenic trioxide results in increased production of thiobarbaturic acid reactive substances (TBARS) and decreased protein content ($p<0.001$) in fresh chicken liver homogenate. Table - 29 show that simultaneous addition of sub lethal concentration (3 µg/mL) of As_2O_3 and various concentrations (40-200 µg/mL) of ERN to liver homogenate caused significant amelioration ($p<0.001$).

The maximum retardation of As_2O_3 induced toxicity by ERN at 200 µg/mL concentration *in vitro*, in turn, revealed that ERN may have better hepatoprotective activity against As_2O_3 induced hepatic damage, *in-vitro*.

Fig. 41 : Changes in protein content with ERN

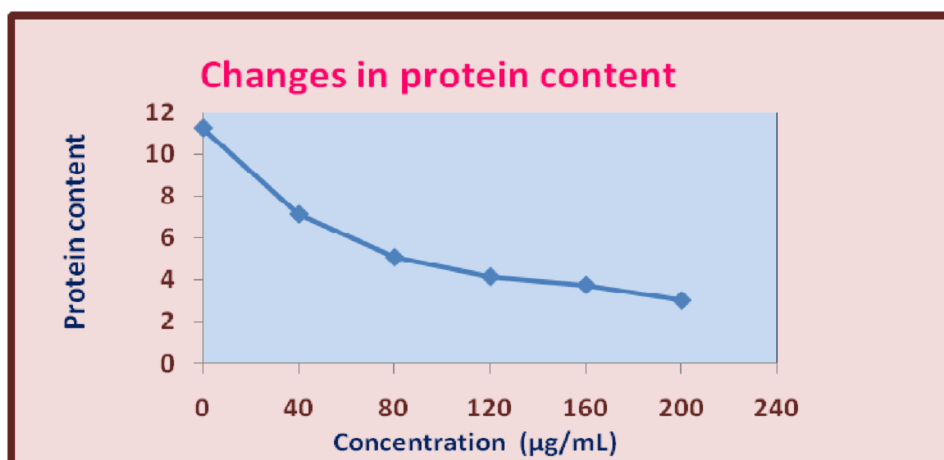
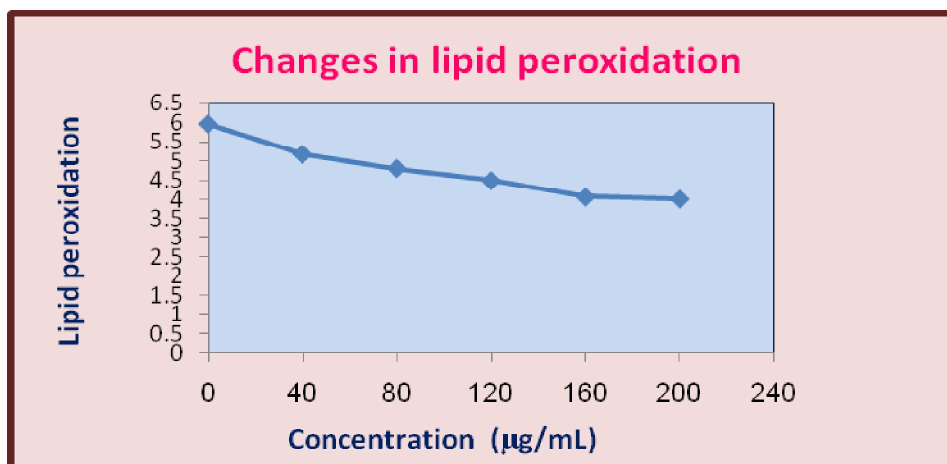


Fig. 42 : Changes in lipid peroxidation with ERN



PART - VI

Invitro anti-bacterial activity

This part of the pharmacological study deals with the anti-bacterial activity against various microorganisms including one which is the causative organism (*Streptococcus pyogenes)* for rheumatic fever and endocarditis. The most widespread killer disease in the developing countries is of microbiological origin. Hence the research and development of antimicrobial therapeutics from plant origin could be more precious [127]. The necessity of new antimicrobial agent has become apparent especially for the treatment of infections where microbial resistance to antibiotics has developed [128]. As resistance towards established antitbiotics has become widespread among bacteria and fungi, new class of antimicrobial substances is required [129]. Plant based antimicrobial agents have enormous therapeutic potential. They are effective in the treatment of infections and simultaneously mitigating many of the side effects, which are generally associated with synthetic antimicrobials.

The zones of inhibition by the disc diffusion technique indicated that the extract had significant effect on all the microorganisms tested. ERN was subjected to anti-bacterial activity against *Streptococcus pyogenes, Pseudomonas aurogenosa, Staphylococcus aureus* and *Escherichia coli.* Zone of inhibition of ERN was found to be *Streptococcus pyogenes* (20mm), *Streptococcus pyogenes* (19mm), *Pseudomonas aurogenosa* (18mm), *Staphylococcus aureus* (20mm) compared to amikacin. These indicate that ERN has exerted significant anti-bacterial activity against all the four bacterial pathogens at 1500 µg/mL concentration, which is comparable with 30µg amikacin.

CHAPTER- IX

CONCLUSION

There are many unknown plants with high medicinal value still have not been recognised their importance. They have not been brought to the light of scientific world. This dissertation covers pharmacognostical, phytochemical and pharmacological studies on the underexploited medicinal plant *Rhynchoglossum notonianum* belonging to the family Gesneriaceae.

Pharmacognostical parameters have been determined on the leaves and stems in order to authenticate and recognize the plant for future work.

Preliminary phytochemical screening on the leaves of *R. notonianum* confirms the presence of flavonoids, phenols, tannins, glycosides and carbohydrate.

Preparation of extract was carried out; dry extract of the same was used for the quantitative phytochemical and pharmacological study.

Determination of total phenolics (109.46 mg/g), total tannins(249.5 mg/g), total flavonoid content(116.28 mg/g), total flavonols (0.498 mg/g), proanthocyanidin (158.58 mg/g), betacarotene (1.729 mg/g) and lycopene (0.182 mg/g) confirms the major concentration of these phytoconstituents in the 70% ethanolic extract of *R. notonianum* (ERN), by which it exhibited significant invitro antioxidant activity. Evaluation of phytoconstituents was performed by TLC and HPTLC studies on ERN and fingerprint profile was recorded.

Pharmacological screening confirms,

- ERN have exhibited radical scavenging activity by DPPH, peroxide and nitric oxide scavenging assays and it also exerted significant antioxidant potential by ferric reducing power assay method.

- Significant hepatoprotective activity at 200 μg/mL of ERN ($p<0.001$) against arsenic trioxide induced hepatotoxicity in fresh chicken liver homogenate was observed and the *in-vitro* ameliorative activity of these extracts on arsenic trioxide induced hepatic damage may be ascribed for this hepatoprotective activity which is confirmed by changes in lipid peroxidation ($p<0.001$) and protein content ($p<0.001$).

- At the site of inflammation, ERN may possibly inhibit the release of lysosomal content of neutrophils (i.e., bactericidal enzymes and proteinases), which upon extracellular release cause further tissue inflammation and damage (Chou, 1997). In the present study, results indicate that the ERN possesses significant anti-inflammatory properties which may be due to the strong occurrence of polyphenolic compounds such as flavonoids, tannins and phenols.

- 500μg/mL of ERN showed significant antiarthritic activity by inhibition of protein denaturation. Rheumatiod arthiritis (RA) being a common inflammatory disease affects about 1% of the adult population worldwide. It occurs in immunogenetically predisposed individuals. Protein denaturation was found to be one of the causes of RA. ERN has shown significant anti-

arthritic activity and the phenolic constituents may be responsible for this activity.

- Antimitotic activity screening by using *invitro* green gram model revealed the inhibitory potential of water imbibitions, decreased seed germination and lesser radicle length which inturn showed the inhibition of mitotic cell division. Hence it could be concluded that the *R. notonianum* exhibited the statistically significant ($p<0.05$) antimitotic activity which further can be studied by invitro cell lines.

- The extract of *R. notonianum* stems and leaves was evaluated for its cytotoxicity against *HeLa* cell lines at different concentrations to determine the IC_{50} by MTT assay. IC_{50} value of ERN was found to have a potential activity on *HeLa* cell and has tremandous anticancer activity on cervical cancer.

- Significant anti-bacterial activity exhibited by ERN (1500 µg/mL) against *Streptococcus pyogenes* which is a causative organism of rheumatic fever. *Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli* are also sensitive to ERN. Anti-bacterial activity of this extract against these microorganisms was comparable with standard drug amikacin, thus can be used in the treatment of infectious disease caused by these bacteria.

- ERN has also shown significant anti-bacterial activity against the pathogens causing rheumatic fever. Phenolic phytoconstituents may be responsible for this activity.

- Phenolic secondary metabolite responsible for invitro antioxidant, hepatoprotective activity, anti-arthritic activity and anti bacterial activity may be isolated and evaluated for further studies.

- As *R. notonianum* is rich in multiple phytoconstituents, further elaborate studies have to be conducted on the isolation, identification and characterization of the effective moiety responsible for the activity. This pharmacologically potent moiety can be used as lead compound for designing a potent anti-inflammatory drug which can be used for treatment of various diseases such as inflammation, cancer, neurological disorder, aging and inflammation-mediated diseases.

- Future aim of this study is to isolate the pharmacologically potent phytochemical constituents responsible for the above activities and also to carry out the *invivo* investigations.

FIG. 1.1 : HABITAT OF *Rhynchoglossum notonianum*

FIG. 1.2 : FLOWER OF *R.notonianum*

FIG. 1.3 : STEM OF *R.notonianum*

Fig. 1.4 : ***Rhynchoglossum notonianum***

LEAF OF *Rhynchoglossum notonianum*

FIG. 1.5 : DORSAL VIEW

FIG. 1.6 : VENTRAL VIEW

FIG. 3.1 : T.S. OF PETIOLE

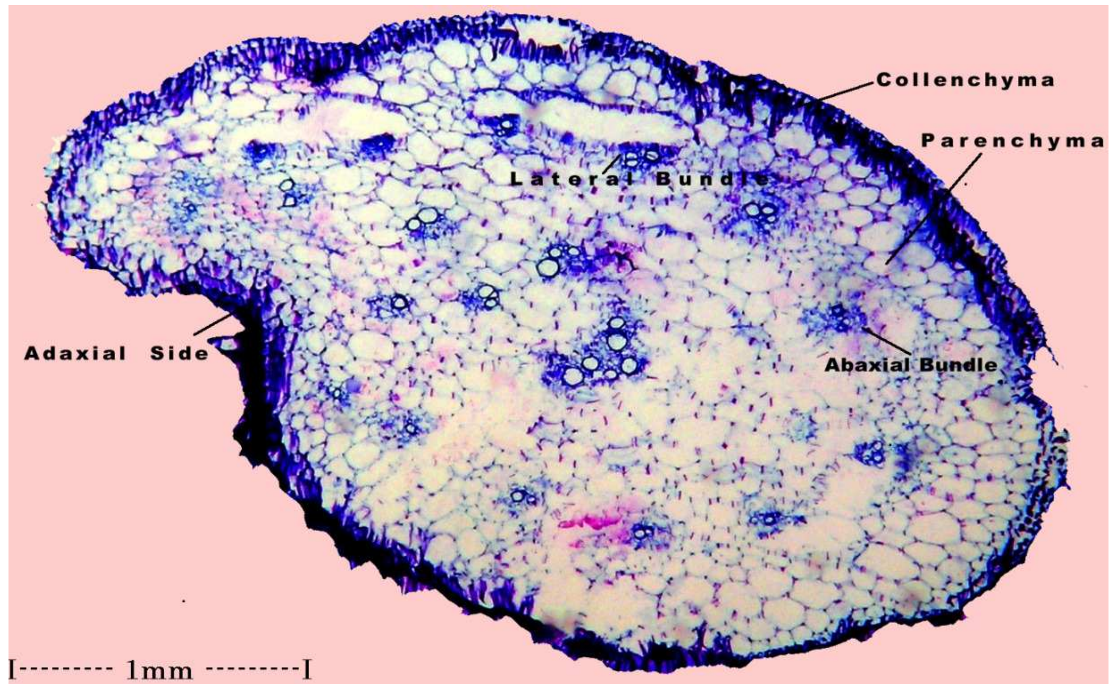


FIG. 3.2 : T.S. OF PETIOLE ENLARGED VIEW

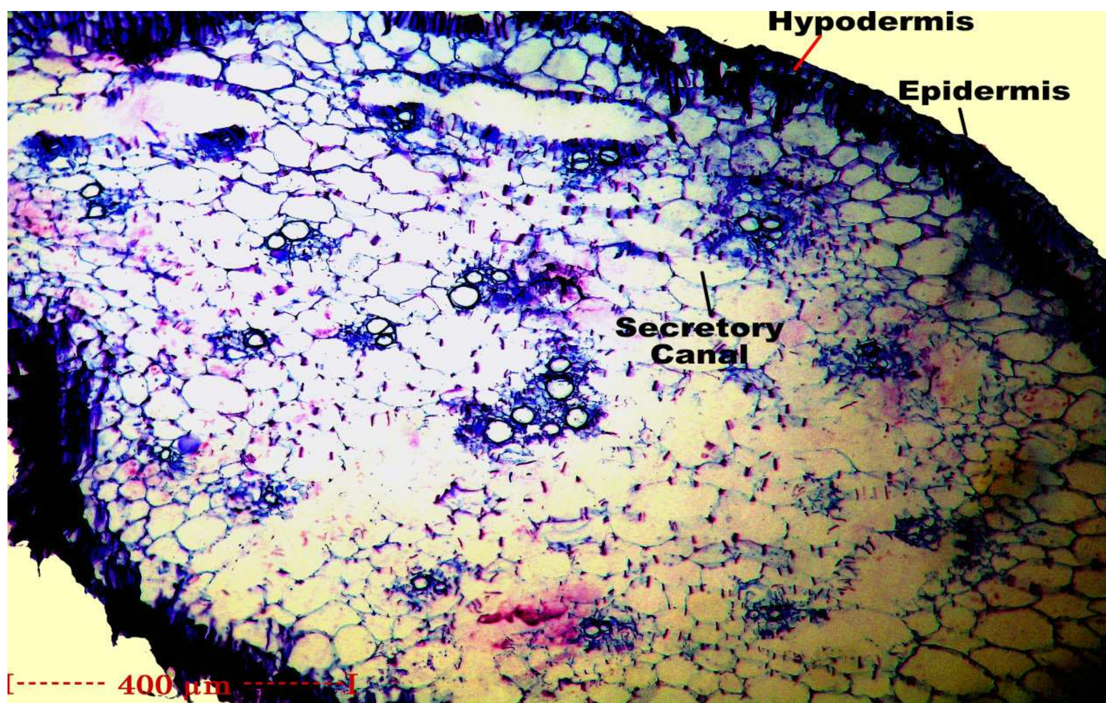


FIG. 2.1 : T.S. OF STEM

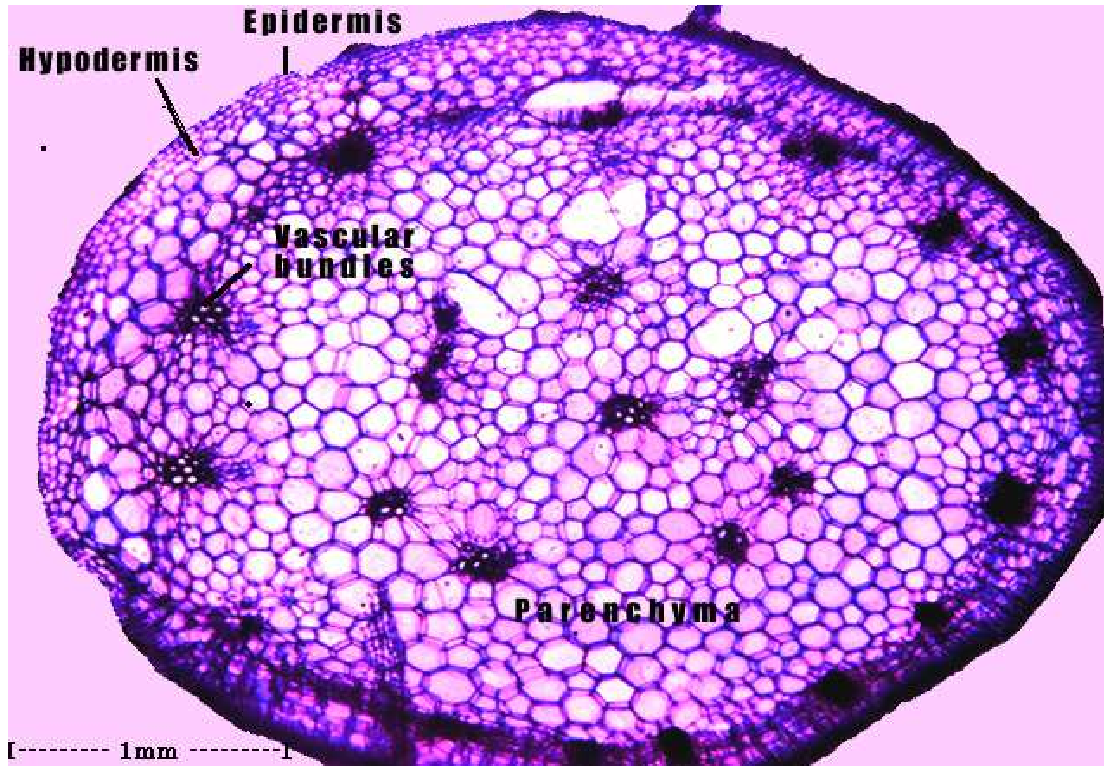


FIG. 2.2 : T.S. OF STEM – INNER REGION ENLARGED

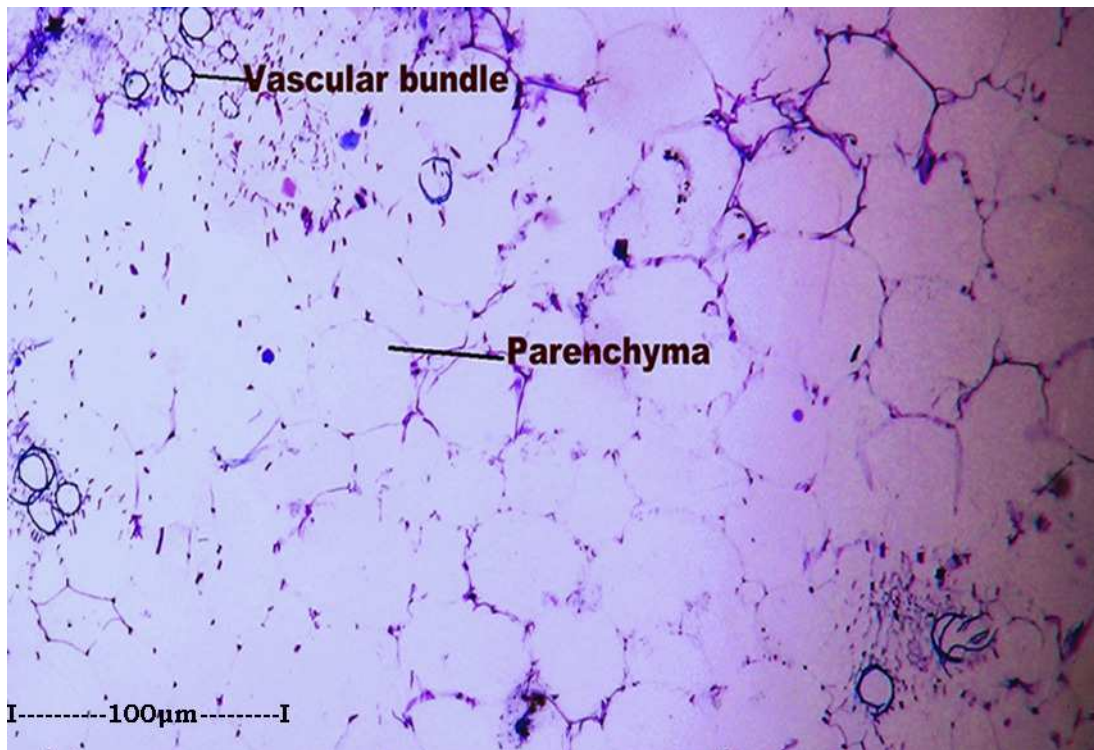


FIG. 2.3 : T.S. OF STEM SHOWING ILL-DEFINED ENDODERMIS AND PERICYCLE LAYER

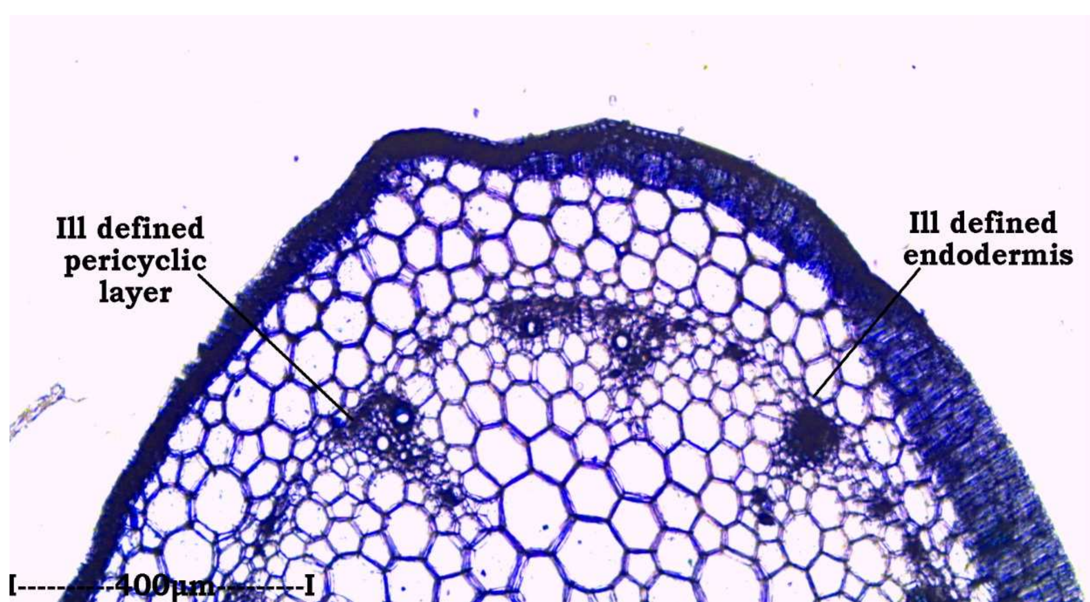


FIG. 2.4 : ILL-DEFINED ENDODERMIS AND PERICYCLE LAYER ENLARGED VIEW

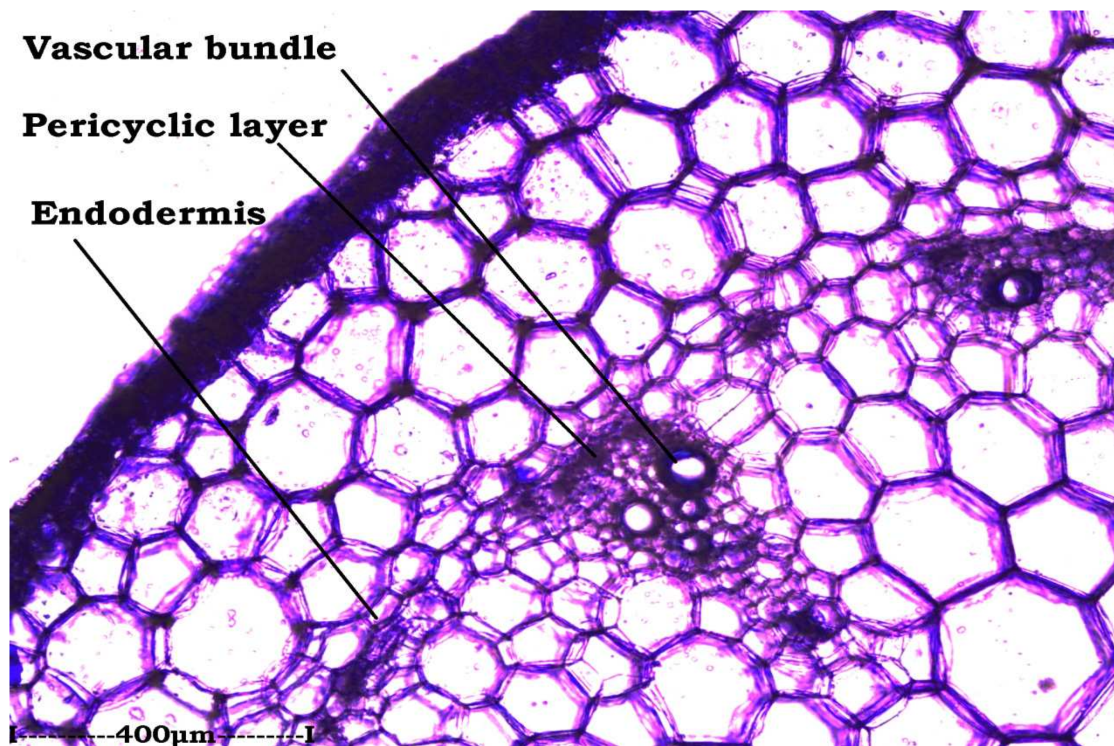


FIG. 4.1 : T.S. OF LEAF THROUGH MIDRIB

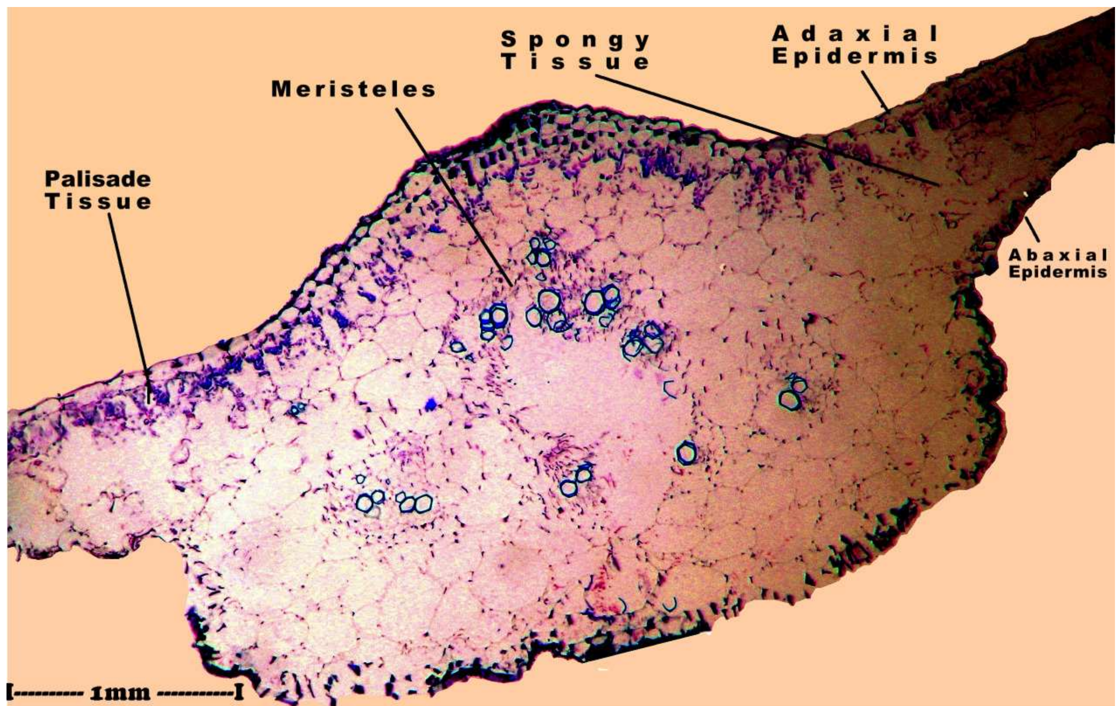


FIG. 4.2 : T.S. OF LARGER VEIN ENLARGED VIEW

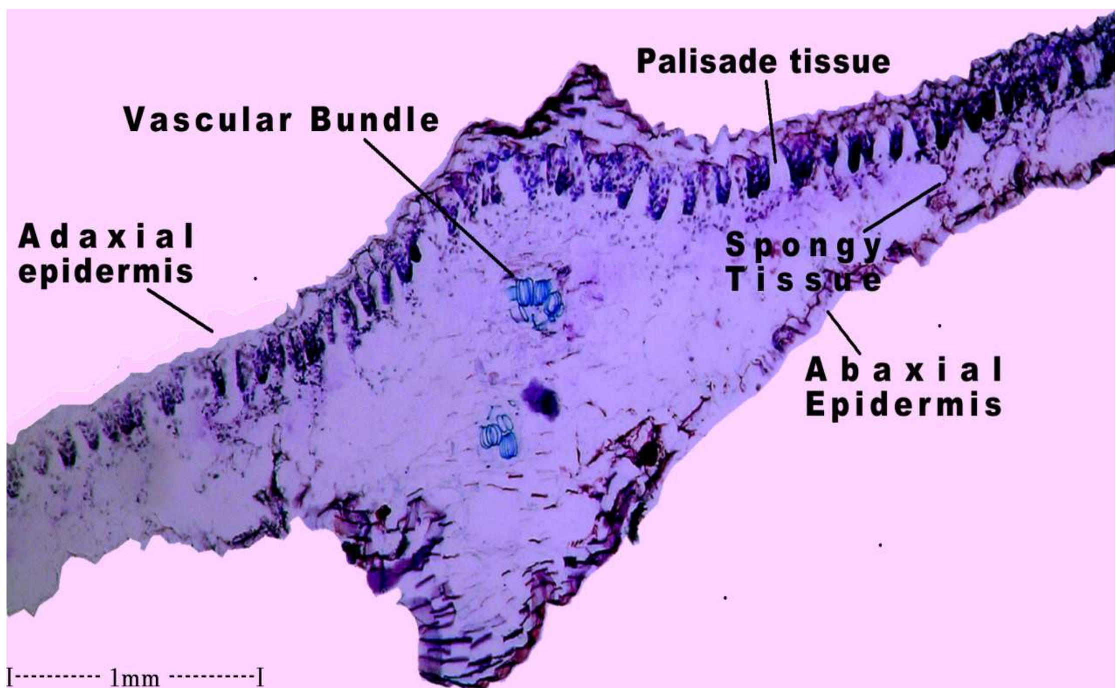


FIG. 4.3 : T. S. OF LAMINA SHOWING MESOPHYLL TISSUE

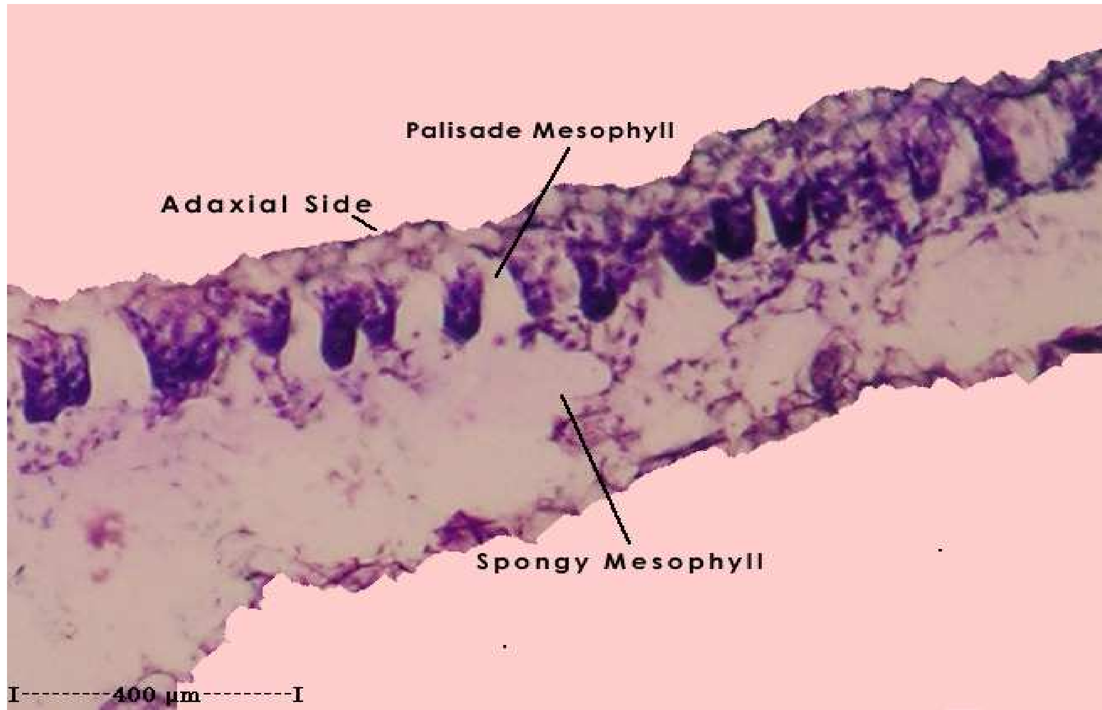


FIG. 5.1 : ADAXIAL FOLIAR EPIDERMIS

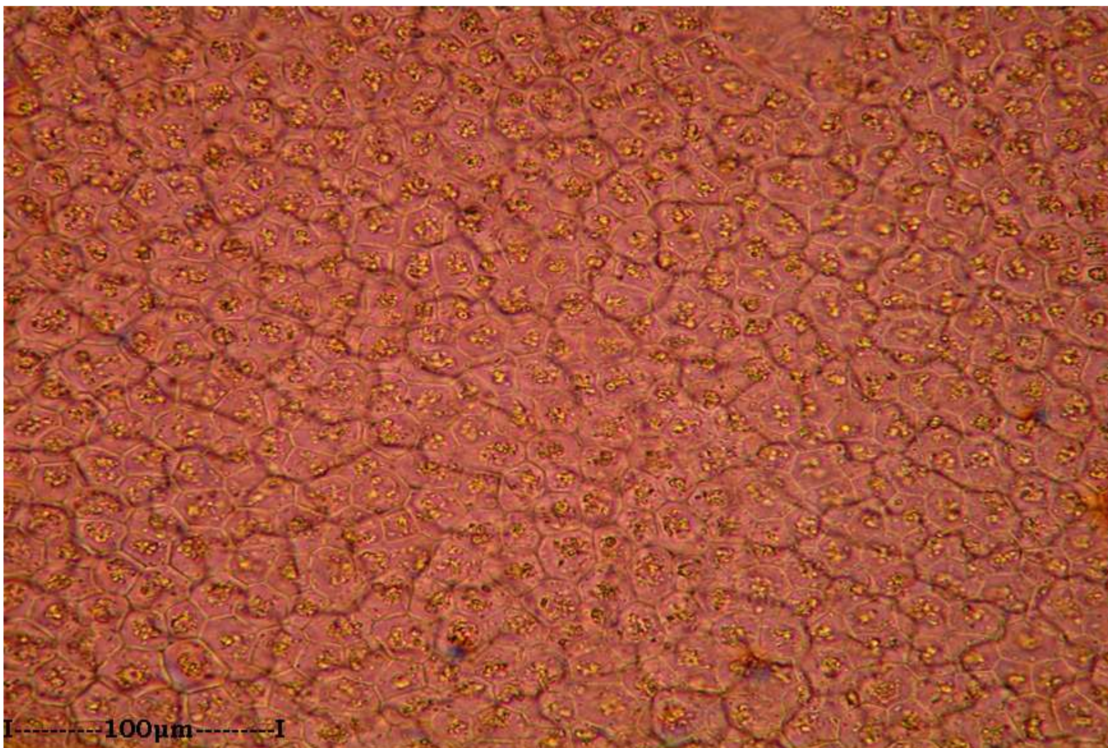


FIG. 5.2 : ABAXIAL FOLIAR EPIDERMIS

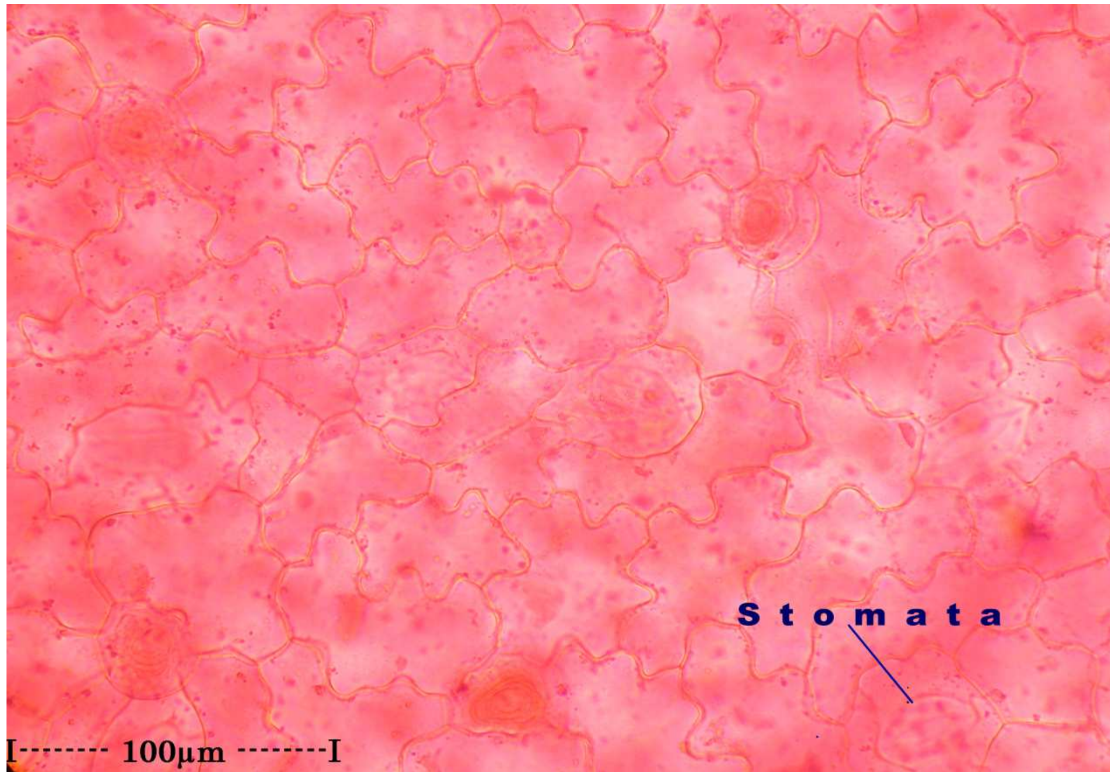


FIG. 6.1 : VEIN ISLETS

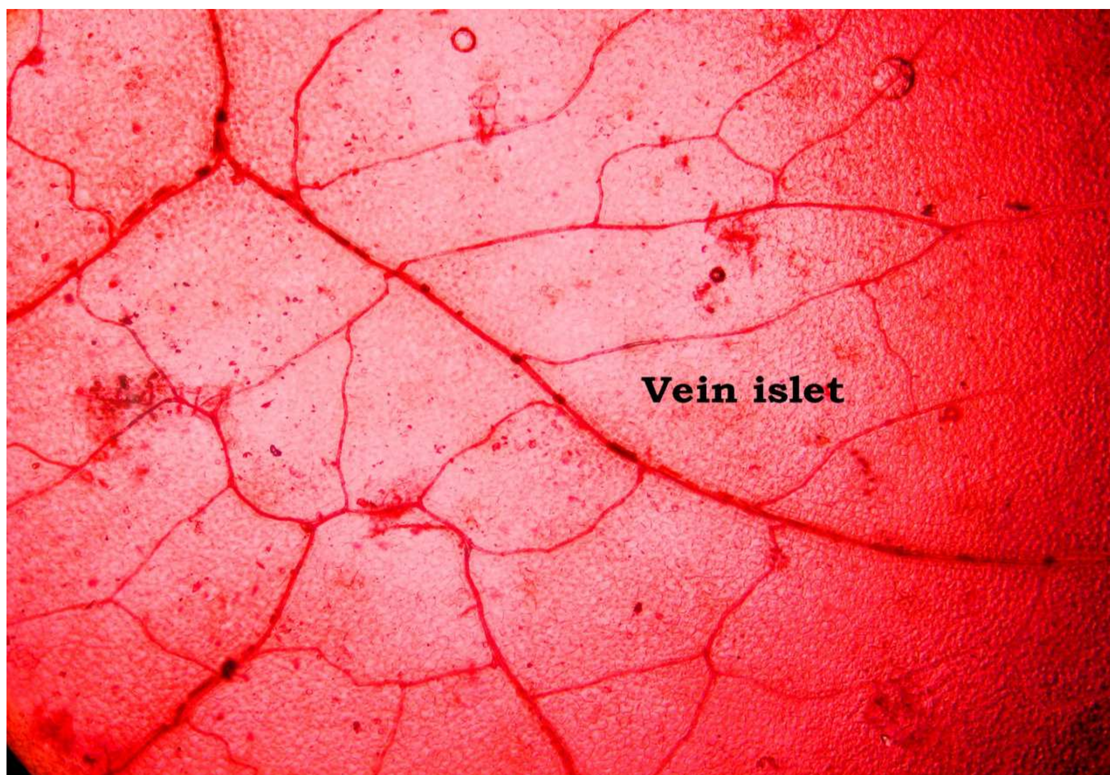
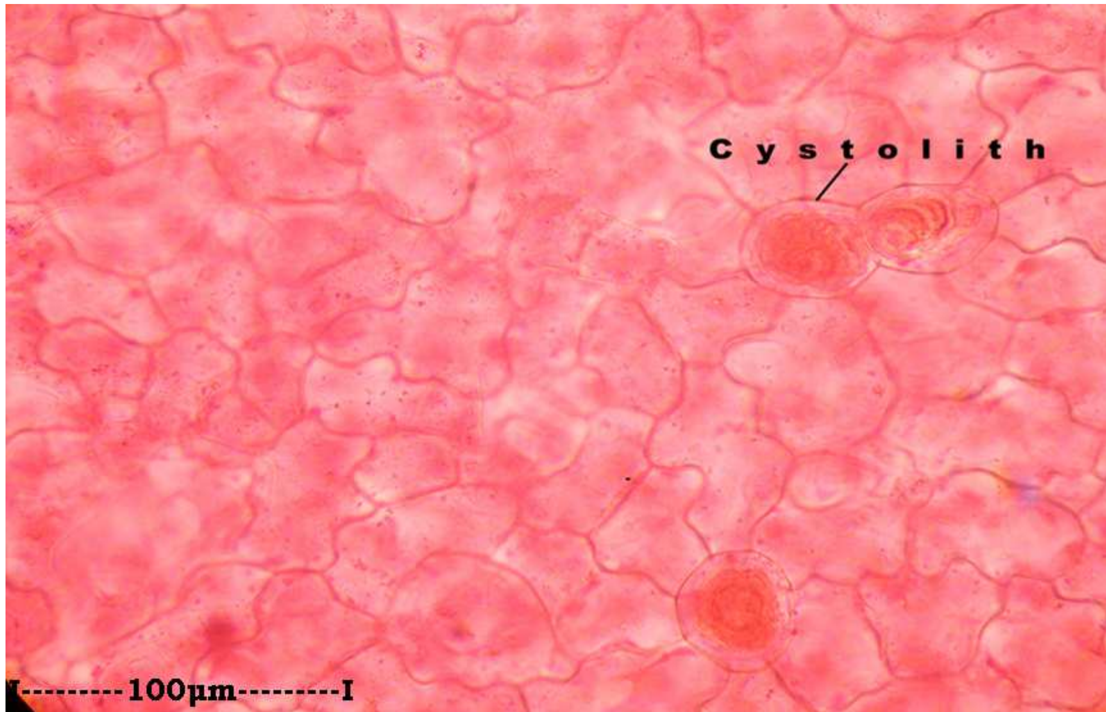


FIG. 6.2 : ABAXIAL FOLIAR EPIDERMIS SHOWING CYSTOLITH



STOMATAL ARRANGEMENT STUDY BY LEAF IMPRESSION METHOD

FIG. 7.1 :UPPER EPIDERMIS

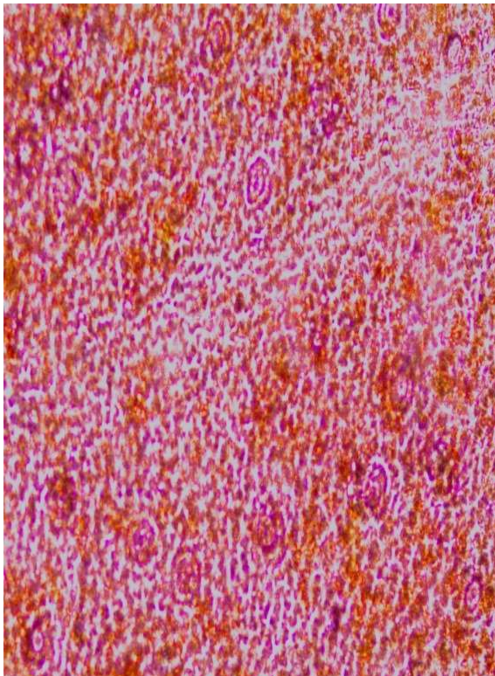
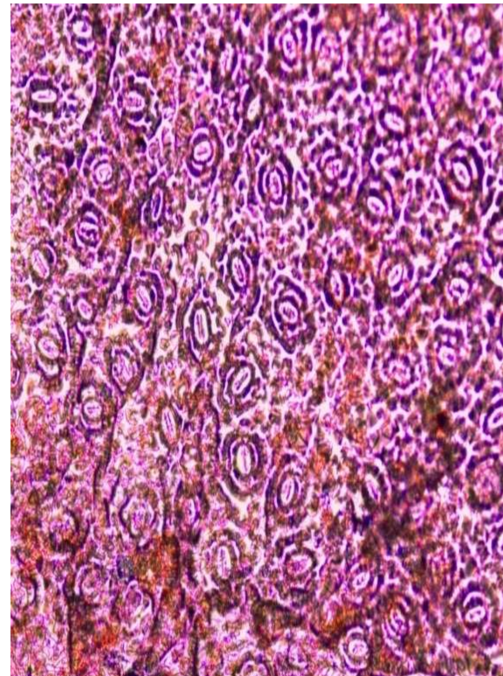


FIG. 7.2 :LOWER EPIDERMIS



MICROSCOPICAL CHARACTERS OF POWDER OF *Rhynchoglossum notonianum*

FIG. 8.1 : XYLEM VESSELS

FIG. 8.2 : XYLEM FIBRES

FIG. 9 : GLANDULAR TRICHOMES

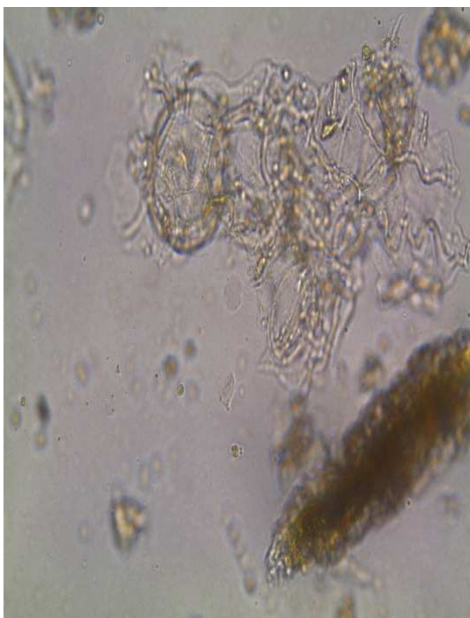


FIG. 10 : NON-GLANDULAR TRICHOMES

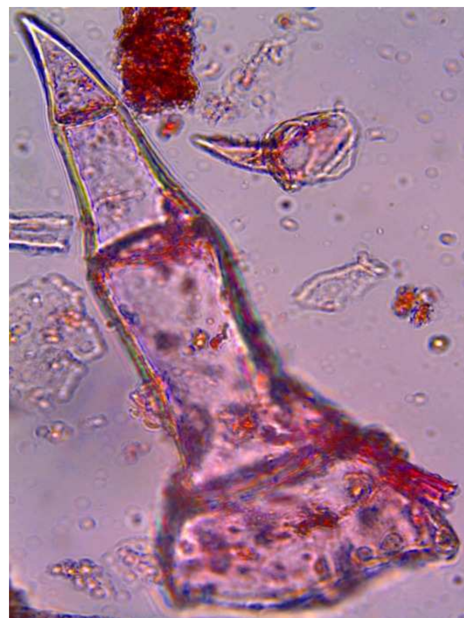


FIG. 11 : STOMATA

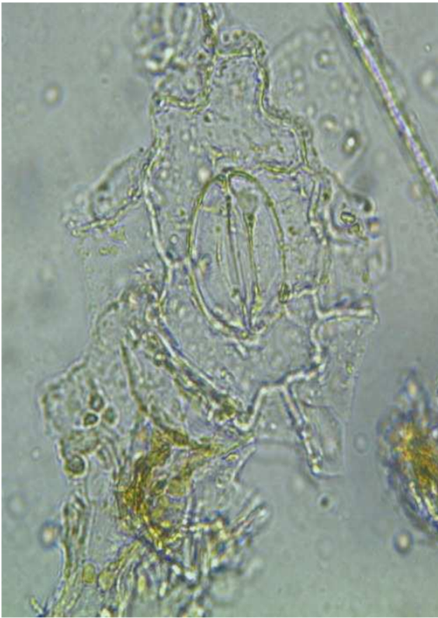


FIG. 12.1 : MESOPHYLL

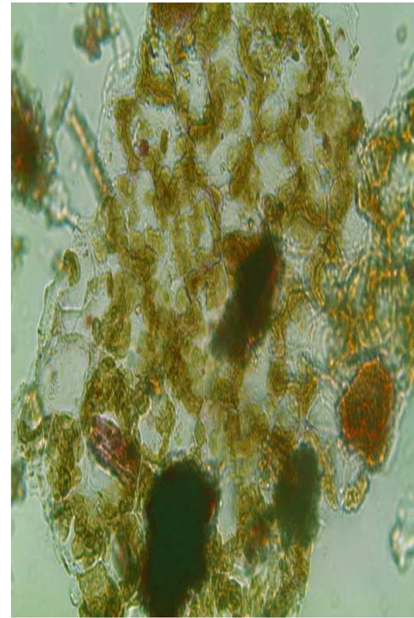


FIG.12.2 : SCALARIFORM PARENCHYMA CELLS

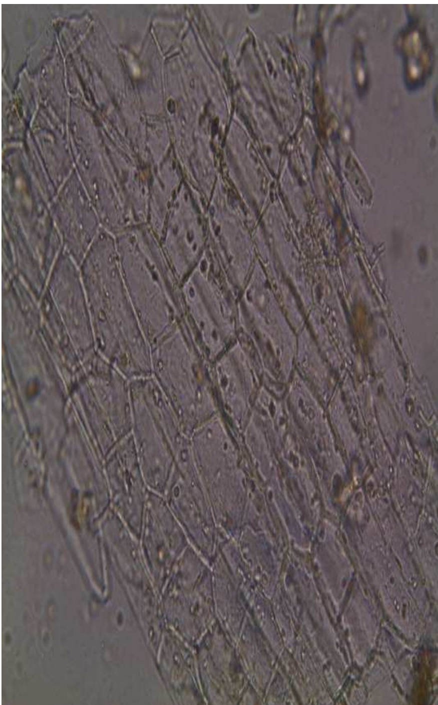
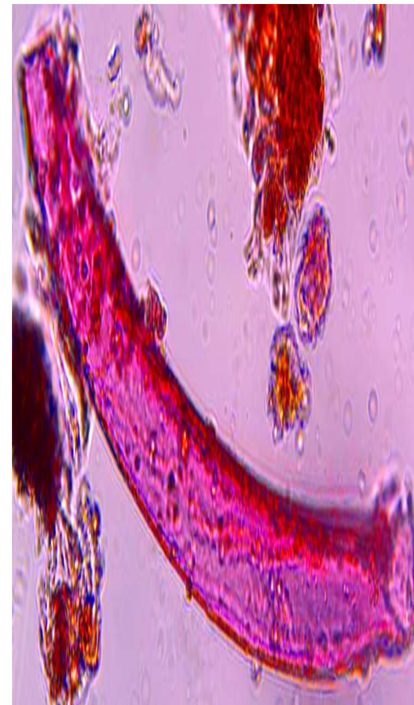


FIG. 13 : PHLOEM FIBERS



TLC CHROMATOGRAM

Fig. 19

Mobile Phase :

Chloroform : Methanol : Water (61:32:7)

Fig. 20

Mobile Phase :

Chloroform : Methanol : Isopropanol : Water (5:6:1:4)

Fig. 21

Mobile Phase :

Toluene : Acetic acid (4:2)

VISUALIZATION

Fig. 25 : Visualization at 366nm

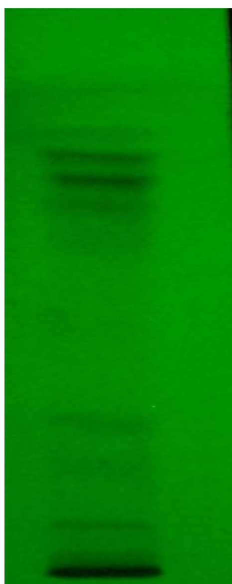
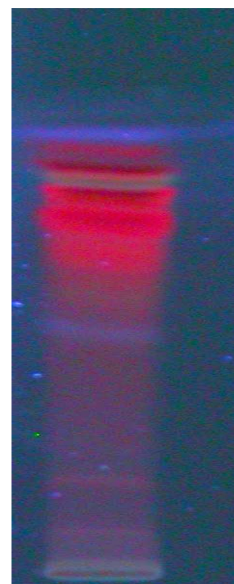


Fig. 24 : Visualization at 254nm



PEAK DISPLAY

Fig. 27 : Peak display at 366nm

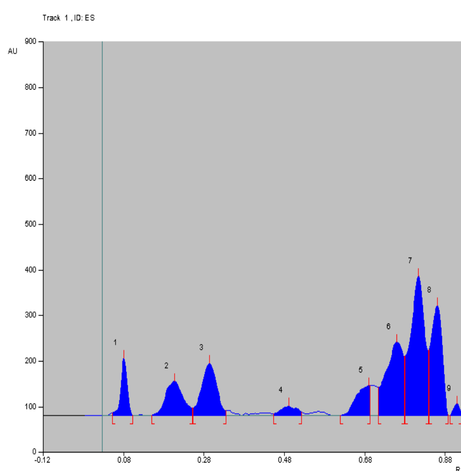


Fig. 26 : Peak display at 254nm

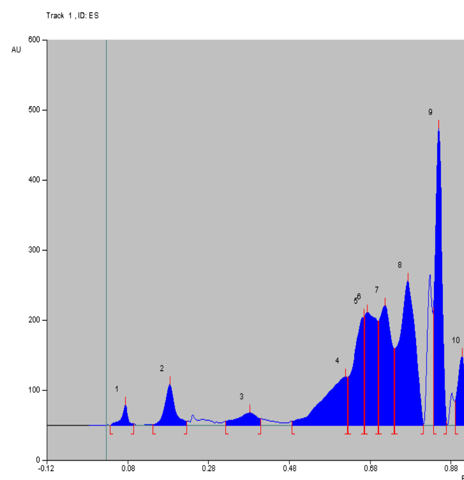
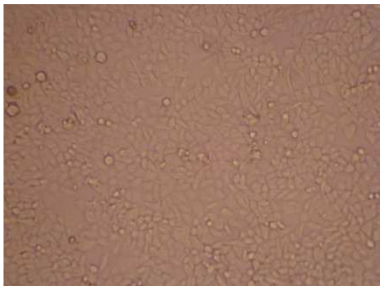


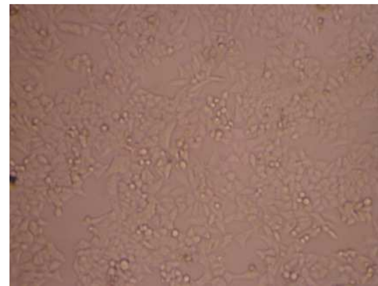
FIG. 36.5 : EFFECT OF DIFFERENT CONCENTRATIONS OF *R. notonianum* AND CISPLATIN ON SPROUTING OF GREEN GRAM SEEDS

FIG. 38 : *INVITRO* CYTOTOXICITY ACTIVITY OF ETHANOLIC EXTRACT OF *Rhynchoglossum notonianum* BY MTT ASSAY

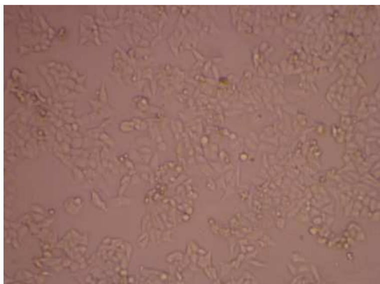
Control



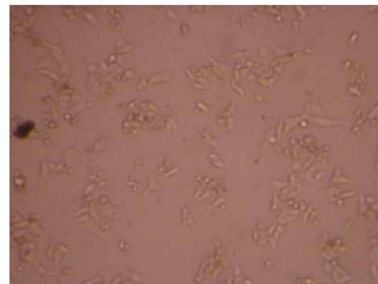
31.25 µg



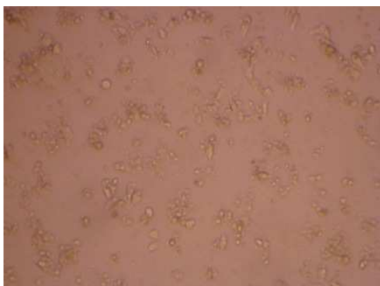
62.5 µg



125 µg



250 µg



500 µg

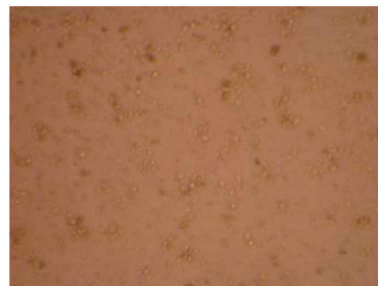


FIG. 43 : EFFECT OF ERN AGAINST *Streptococcus pyogenes*

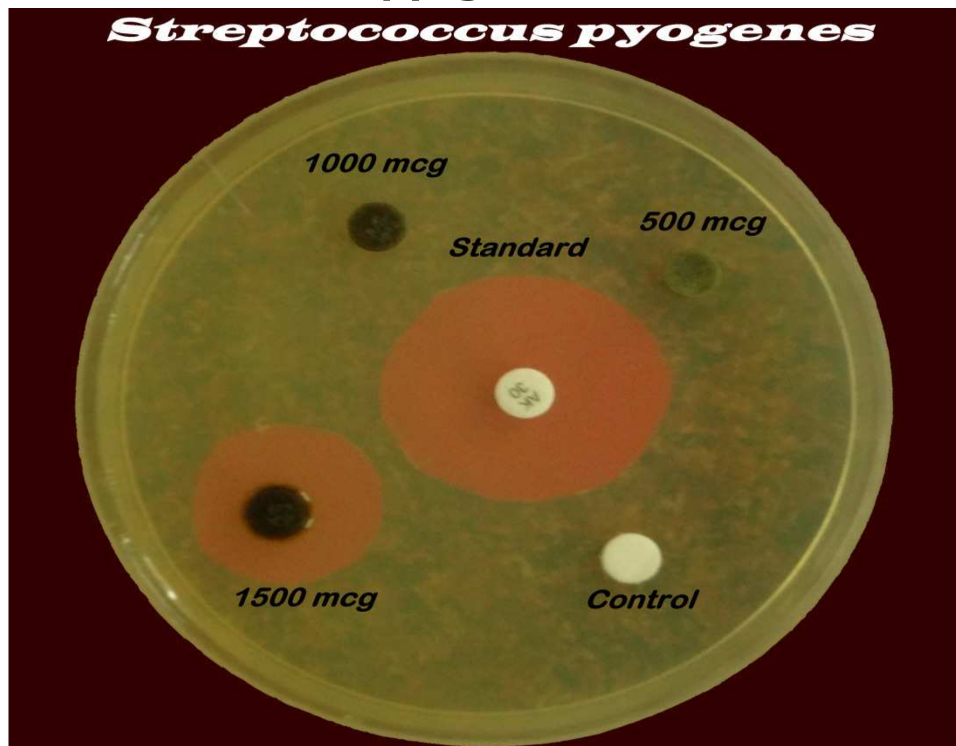


FIG. 44 : EFFECT OF ERN AGAINST *Pseudomonas aeruginosa*

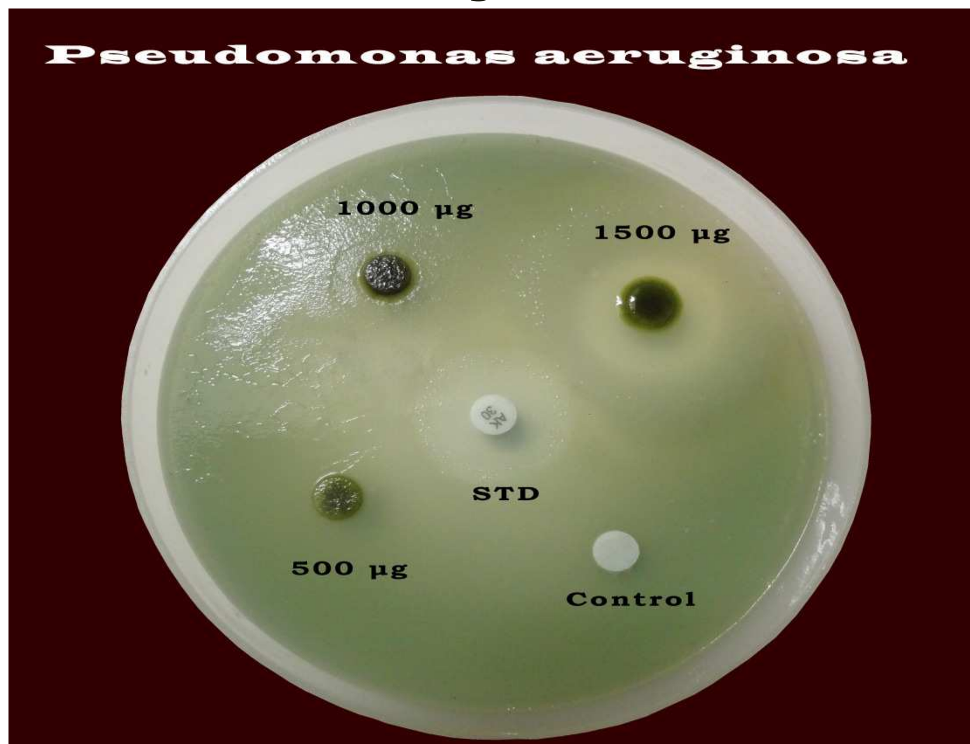
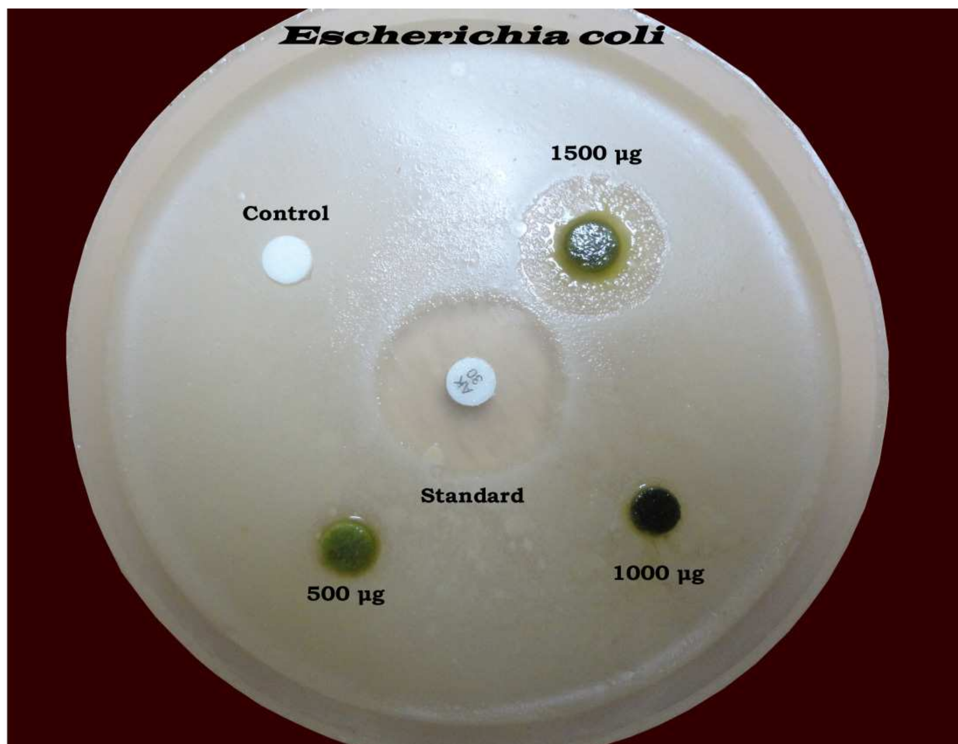


FIG. 45 : EFFECT OF ERN AGAINST *Staphylococcus aureus*

FIG. 46 : EFFECT OF ERN AGAINST *Escherichia coli*



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