PHARMACOGNOSTIC, PHYTOCHEMICAL, ANTITHYROID, ANTIOXIDATIVE AND ANTIHYPERGLYCEMIC EVALUATION OF *Artemisia nilagirica* LEAVES,C.B.clarke

A dissertation submitted to
The Tamil Nadu Dr. M.G.R. MedicalUniversity
Chennai-600 032

In partial fulfillment of the requirements
for the award of the degree of
MASTER OF PHARMACY
IN
PHARMACOGNOSY

Submitted by
26118670

DEPARTMENT OF PHARMACOGNOSY

COLLEGE OF PHARMACY

MADURAI MEDICAL COLLEGE

MADURAI - 625 02

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CERTIFICATE

This is to certify that the dissertation entitled **"PHARMACOGNOSTIC, PHYTOCHEMICAL, ANTITHYROID, ANTIOXIDATIVE AND ANTIHYPERGLYCEMIC EVALUATION OF *Artemisia nilagirica* LEAVES C.B. Clarke"** submitted by **Mrs. B.SOBHA** in partial fulfillment of the requirement for the award of the degree of **MASTER OF PHARMACY** in **PHARMACOGNOSY** by The Tamil Nadu Dr. M.G.R. Medical University is a bonafide work done by her during the academic year 2012-2013 at the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai-625020.

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(Miss.GOWRI)
Project Supervisor

PARC PLANT ANATOMY RESEARCH CENTRE

Dr.P. Jayaraman, Ph.D.
Director, PARC,
Retd. Professor, Presidency College

Herbal PARC

AUTHENTICATION CERTIFICATE

Based upon the ~~Organolep~~tic /macroscopic /~~microsco~~pic examination of fresh /~~market~~ sample, it is certified that the specimen given by B.SOBHA (M.Pharm), Dept. of Pharmacognosy, Madurai Medical College. is identified as below:

Binomial: Artemisia nilagirica (clarke) Pamp.

Family: Compositae.

Synonym(s): Artemisia vulgaris L. var. nilagirica clarke.

Regional names: Tam: Machipatri.

Reg.No of the certificate: PARC/2013/2007.

References: Nair, N.C & Henry, A.N. Flora of TamilNadu, India I: ——— .1983.

Henry, A.N. *et al.* Ibid. II: 89:31 .1987.

Ibid. III: ——— .1989.

Date: 01/03/13.

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Introduction

INTRODUCTION

The word Pharmacognosy is derived from the Greek words, pharmakon (drug) and gnosis (knowledge). The term Pharmacognosy was used by the Austrian physician Schmidt in 1811 and in 1815 by C.A.Seydler in a work titled "Anelecta Pharmacognostica". Pharmacognosy is the study of natural product molecules (typically secondary metabolites) that are useful for medicinal, ecological, gustatory or other functional properties.[1]

The contemporary study of Pharmacognosy can be divided into the fields of

1. Medical ethnobotany

The study of the traditional use of plants for medicinal purposes.

2. Phytotherapy

The study of medicinal uses of plant extract.

3. Phytochemistry

The study of chemicals and the identification of new drug derived from plant sources.

4. Zoo pharmacognosy

The study of animals self medicate, by selecting and using plants, soils and insects to treat and prevent diseases.

5. Marine pharmacognosy

The study of chemicals derived from marine organisms

ANTITHYROID ACTIVITY [2]

Thyroid gland

The thyroid is a butterfly shaped endocrine gland located in the lower front of neck. The thyroid gland secretes thyroid hormones and carried to every tissue in the body .Thyroid hormones help the body use energy stay warm and other organs as they work normally.

Hyperthyroidism

The term hyperthyroidism refers to any condition in which there is too much of thyroid hormone produced in the body. In other words, the thyroid gland is overactive. The thyroid gland secretes excessive thyroid hormones triiodothyroxine (T_3) and tetraiodothyroxine (T_4).

Hyperthyroidism is a type of thyrotoxicosis ,a hyper metabolic clinical syndrome. Graves disease is another type of hyperthyroidism. Thyroid hormone is important to a cellular level, affecting nearly every type of tissue in the body. The thyroid makes the right amount of hormones T_3 and T_4 in healthy people. Shortly, **the thyroid runs our metabolism.**

Causes [3]

1. Getting too much of iodine
2. Graves disease
3. Inflammation of the thyroid because of viral infections.
4. Non cancerous growth of thyroid gland.
5. Some tumors of testes or ovaries.
6. Taking large amounts of thyroid hormone.

Symptoms

- Difficulty in concentration
- Fatigue
- Frequent bowel movements
- Enlarged thyroid gland(GOITER)
- Intolerance of heat
- Increased appetite
- Increased sweating
- Irregular menstruation in women
- Nervousness
- Restlessness
- Weight loss (rarely weight gain)
- Breast development in men
- Clammy skin
- Diarrhoea
- Loss of hair
- Tremor in hands
- High blood pressure
- Itching
- Protruding of eyes
- Irregular heart beats
- Difficulty in sleeping

Diagnosis

Diagnosis of hyperthyroidism is confirmed by laboratory tests

1. To measure the amount of thyroid hormones T_3 andT_4
2. Measure TSH in the blood
3. Thyroid scan will find out the over activeness or goiter or inflammation.
4. A thyroid uptake is the test to measure the ability of the gland to collect iodine.

Treatment[4]

Antithyroid medications

Antithyroid drugs

Drugs used for antithyroid category are Methimazole and Propyl thiouracil (PTU) Methimazole is preferred due to less side effects and quick control of overactive thyroid and do not cause any damage to thyroid gland. PTU is used only in rare conditions.

Beta blockers

The beta adrenergic blocking agents block the action of thyroid hormone in our body, but it does not change the high levels of thyroid hormone in our blood. Propanolol was the first drug to be developed. Atenolol, Metoprolol, Nadolol and Inderal as twice a day dosage.

Radioactive iodine

Radioactive iodine is most widely used for the permanent treatment of hyperthyroidism. Radioactive iodine damage or destroys the thyroid hormone.

When it is taken, the level of thyroid nodules shrink in size and the level of thyroid hormone in blood returns to normal.

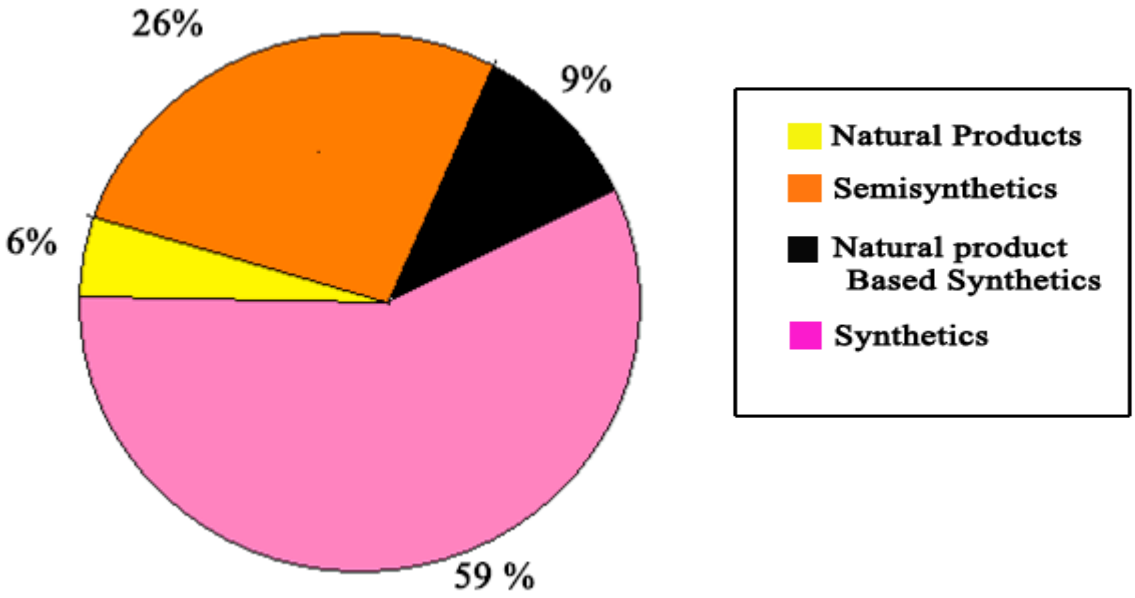
Surgical removal

It is another permanent cure for hyperthyroidism. The important case is such that the patient has hyperthyroidism due to a hot nodule in the lower aspect of the may need to remove the entire lobe which contains the hot nodule .If the thyroid must be removed with surgery, the patient must take the thyroid hormone by pills for rest of the life.

Natural products in modern medicine [6]

A Natural product is a chemical compound or substance found in living organism in nature having pharmacological and biological activity. WHO reveals that study about 80% of the world's population still relies on traditional medicine. Natural products still play a very important role in the medicine in remain 20%of the world's population. Between 1983 and 1994, the new approved drugs have natural products as their source as 41%.

The role of natural products in modern medicine



Medicinal plants in modern medicine [7]

The widely held belief that modern medicines are all the product of complex obtained from higher plants, resulting in sales exceeding $12 billion annually in the United States alone, and about 25% of all prescriptions contain one or more active ingredients from plants. Indeed, pharmaceutical firms that once shunned natural-products research are now beginning to study the possibilities of this resource. Around the world, numerous companies have been formed to explore the potential of plants as modern medicines. Many names could be cited of plants, long in use by indigenous peoples, that have found their way into modern medicine.

Example: **Pilocarpus jaborandi**, a shrubby tree of northeastern Brazil that is the source of **pilocarpine,** a compound used to reduce the intraocular pressure resulting from glaucoma. In Brazilian folk medicine it was originally used to increase salivation and cause swelling. The leaves were introduced into Europe in 1873, and by 1877 the compound pilocarpine, isolated from the leaves, began to be employed in

ophthalmology. More recently the drug has also been approved, by the U.S. Food and Drug Administration, for the treatment of dry-mouth syndrome (a side effect of certain cancer treatments and a disease of aging).Recent domestication of Pilocarpus species as crop plants is taking the pressure off the wild sources.

Not all plant-derived drugs have backgrounds in traditional and folk medicine. Some of them have instead been developed as the result of a broad-scale, random search of the plant kingdom. One example is the discovery of taxol, which is derived from the bark of the Pacific yew**, Taxus brevifolia**. Recently introduced into the war on cancer, this drug is now used either alone or in combination with other therapies for the treatment of breast and ovarian cancer. Current Problems and Prospects as new screening systems are developed, even well-known plants are subjected to reanalysis in hopes of identifying new pharmacological actions. Even so, fewer than one percent of the world's known 265,000 species of higher plants have as yet been exhaustively examined for their chemical composition and medical potential.

The pharmacological search of the balance of the plant kingdom is truly a race against time, as deforestation and species extinction, coupled with the loss of knowledge about the traditional medicinal uses of plants, are reducing this vast phytochemical reservoir with increasing speed.

Clinical studies with plant extracts are being initiated, and the results with many species are encouraging. For example, the prickly pear cactus, Opuntiaficus-indica, has been studied for use by patients with benign prostatic hypertrophy, a disease found in middle-aged and older men. Dried flowers of the species were given to patients, and results were so promising that further clinical trials are now under way. Many plants deserve evaluation for clinical application in modern medicine.

This appears tobe a promising direction for future studies aimed at improving human health.

Natural products in treating hyperthyroidism

Thyroid hormones play an important role in development, metabolism, thermoregulation, and growth[8] . However, under several pathological conditions like Graves' disease, tumors of thyroid and pituitary gland stimulate thyroid cells to produce more hormones, which results in a hyperthyroid state [9]. Alterations in the level of these hormones lead not only to altered basal metabolic rate but also to many health problems. Particularly, hyperthyroidism, if not treated properly, sometimes ends up with the common health problems such as diabetes mellitus and cardiovascular diseases.[10] Resemble to the human hyperthyroidisms were easily achieved in rodents by continuous treatment of l-thyroxine (LT4), a synthetic form of thyroid hormone.[10,11]Hyperthyroidism leads to oxidative damage of liver [12], osteoporosis [13], heart failure[14] and increased risk of heart attack [15]

Herbal medicine derived from plant extracts are used for the treatment in wide variety of clinical diseases. Plants derived natural products such as flavonoids, terpenoids, steroids, coumarins, alkaloids etc have diverse pharmacological properties showing antioxidant and antithyroid activity.[16]

Lemon balm

It is approved by the German Commission for nervous sleeping disorders and gastrointestinal complaints. The free dried extractsof lemon balm show the antithyroid activity by binding to TSH and prevents subsequent thyroid hormone production and release.

Bugle weed

Aqueous extract of bugle weed binds with TSH and TSH like immunoglobulin inhibits the enzymatic reaction that converts peripheral $T_3$and T_4.

Grom well

Cabbage turnips and rutabags, soybeans, peanuts, pine nuts millets interfere with thyroid iodine uptake.

Motherwort

It is traditional heart tonic and uterine stimulant. It is adjuvant for thyroid hyper function.

Ayurveda

Traditional study reveals that the fruit of amla for hyperthyroidism to reduce the T_3 and T_4 .

Chinese medicine

Brown sea weed, Chinese yam (Dioscoreaoppositifolia), Fritillaria, Scrophularia and Rhemanniaglutinosa are traditionally given as combinations rather than single herbs .It is sold in the form of supplement. They are target for treatment of hyperthyroidism.

Antioxidant activity [17]

Antioxidant is a molecule that inhibits the oxidation of other molecules.Antioxidant means the activity of numerous vitamins, minerals and other phytochemicals to protect the damage caused by reactive oxygen species .Antioxidants are widely used in dietary supplement and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness. Although initial studies suggested that antioxidant supplements might

promote health, later large clinical trials with a limited number of antioxidants detected no benefit and even suggested that excess supplementation with certain putative antioxidants may be harmful. Antioxidants also have many industrial uses, such as preservatives in food and cosmetics and to prevent the degradation of rubber and gasoline.

Oxidative stress

Oxidative stress is a modulation of thiol redox reactions, involved mainly in signalling pathways. Therefore, non-radical oxidants (enzymatically generated hydrogen peroxide, other peroxides, quinones, etc.) play a basic role in the oxidation of thiols for the sake of signalling, without the necessityof formation of free radical intermediates (Ghezzi*et al.,*2005; Jones, 2006; 2008). As oxidative stress appears to be an important part of many human diseases, the use of antioxidants in Pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. Moreover, oxidative stress is both the cause and the consequence of diseases.

Functions[17]

The main function of antioxidant system is keep the oxidants at optimum level not to remove oxidants entirely. It defences system scavenges and minimizes the free radical formation. It is suggested for an inverse relationship between dietary intake of antioxidant rich food and incidence of human diseases.

Natural product s having antioxidant activity[18]

Plant based antioxidants are replaced by synthetic ones for safety concerns. The plants have antioxidant properties which protect our cells from oxidative damage, which is further linked to many diseases. These antioxidant properties of plants are due to various phytochemicals present in the plants.

Examples are

Emblica officinalis

Andrographi spaniculata

Curcuma domestica

Trigonella foenum

Aloe vera

Zingiber officinalis

Moringa oleifera

Anti hyper glycemic activity[19]

Hyper glycemia occurs when the glucose level is higher than the usual level of our blood.

Causes

People who are older, overweight, have family members with diabetes or form certain ethnic groups are also develops hyperglycemia.

Is hyper glycemia same as diabetes

Hyperglycemia is a symptom of diabetes .we can have hyperglycemia without diabetes.

Synthetic drugs used for hyperglycemia[20,21,22,23,24]

Insulin

Insulin injections are used to provide the necessary insulin for the body which needs but lacks in some cases. Rapid acting brands are Novolog,Apidra and Humalog . Short acting brands Humulin R and NovalinR .

Alpha glucosidase inhibitors

It slowdowns the breakdown of complex sugars to glucose in the intestine.Examples are Precose and Glycol.

Biguanides

It reduces the amount of glucose produced by the liver and reduces the resistance to insulin in the cells. Metformin (Glucophage), Riomet, GlucophageXR

Meglitinides

It stimulates the pancreas to release insulin after meals Eg Starlix and Prandin.

Thiazolidinediones

It reduces insulin resistance, production of glucose by the liver and liver and glucose uptake by the muscles. Eg Rosiglitazone and Pioglitazone.

Dpp-4 inhibitors

It stimulates the insulin production and reduces glucose release in the liver.

Sulphonyl ureas

It stimulates the insulin production. Eg Chlorpropamide, Glyburide, Glipizide, Glimepride, Tolbutamide and Tolazamide.

Natural products in treating hyperglycemia[25,26,27]

The antihyperglycemic effect of the plants are for their ability to restore the functions of pancreatic tissues by increasing output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes.

Examples; Allium sativum, Allium cepa, Coccinia indica, Ceasalpinia bonducella, Eugenia jambolana etc.,

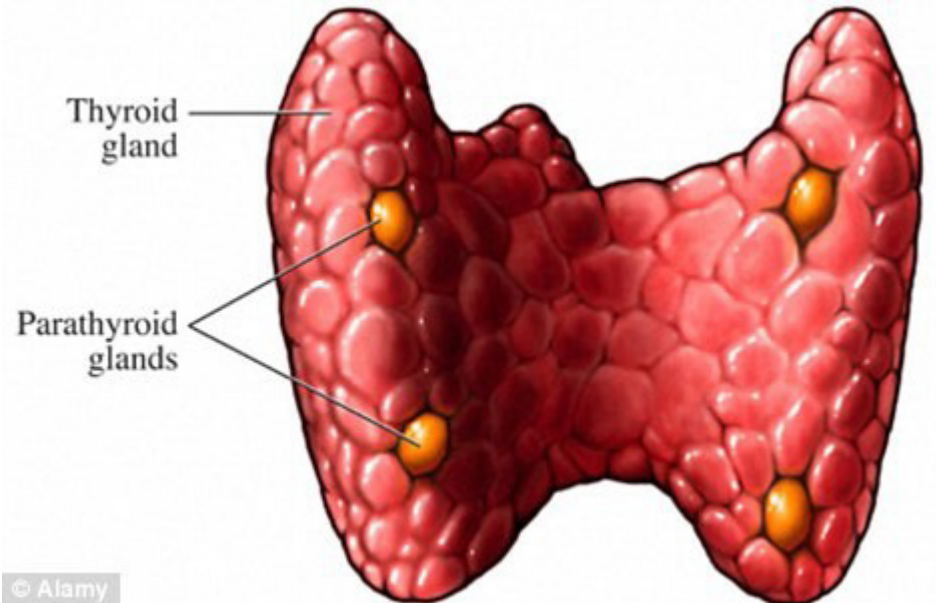
Reason for selecting *Artemisia nilagirica*

Recent studies of thyroid inhibiting compound of plant origin have yielded an impressive assay of novel structure. In my study, methanolic extract of Artemisia nilagirica is used.

Traditionally Artemisia nilagirica is used for the treatment of poor appetite, indigestion, constipation, irregular menstruation, epilepsy, typhoid, insomnia, gout, measles, cancer, antiseptic, antioxidant, anthelmentic, expectorant, diuretic, diaphoretic, cholagogue and insect repellant.[28]

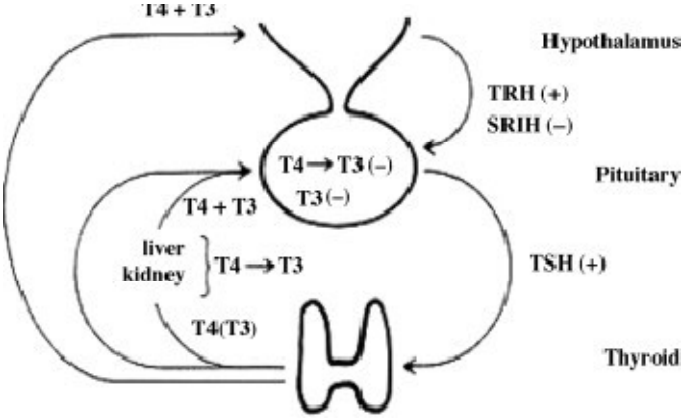
In this study, Artemisia nilagirica is evaluated for antithyroid, antioxidative, antihy perglycemic activity

THYROID GLAND

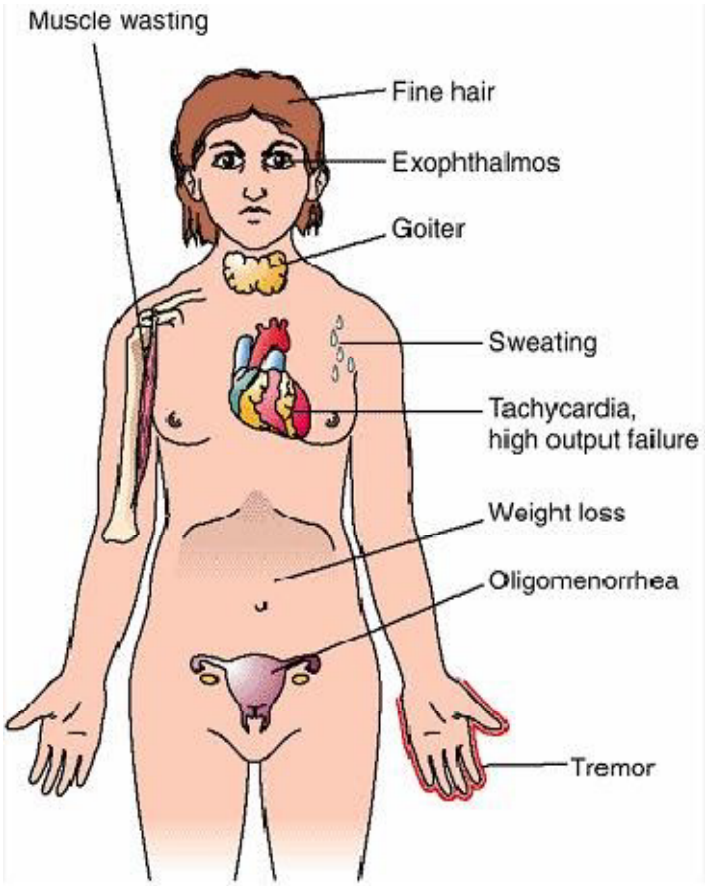


FACTORS THAT MAY CAUSE HYPERTHYROIDISM

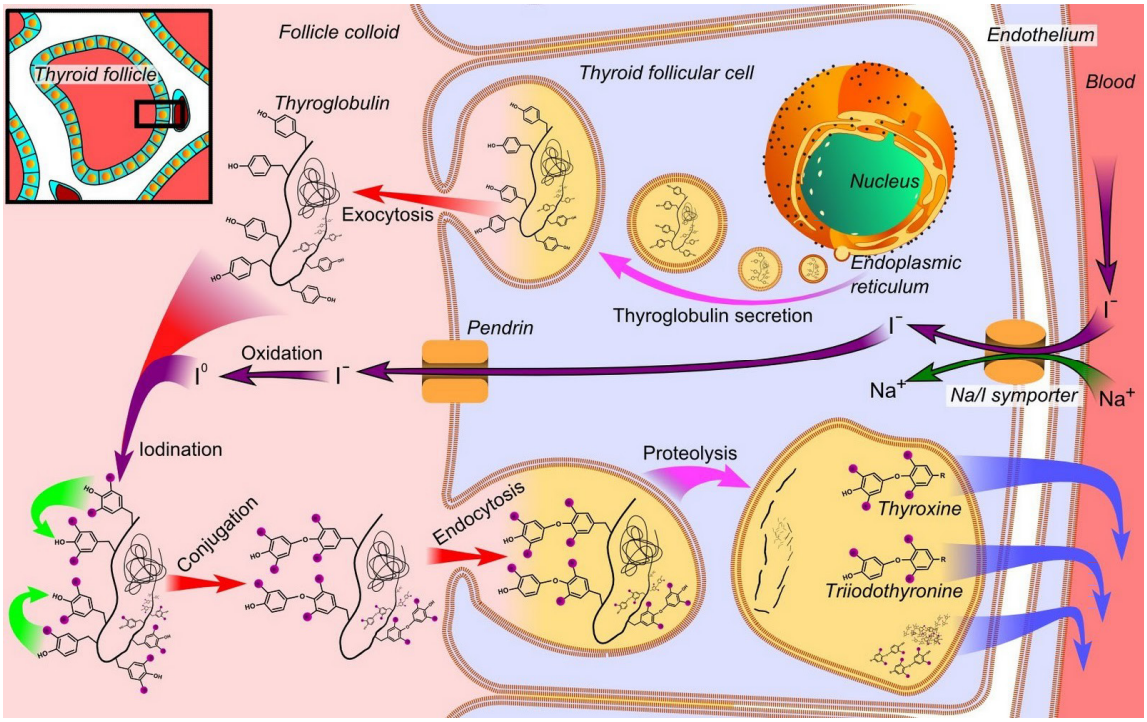
REGULATION OF THYROID GLAND

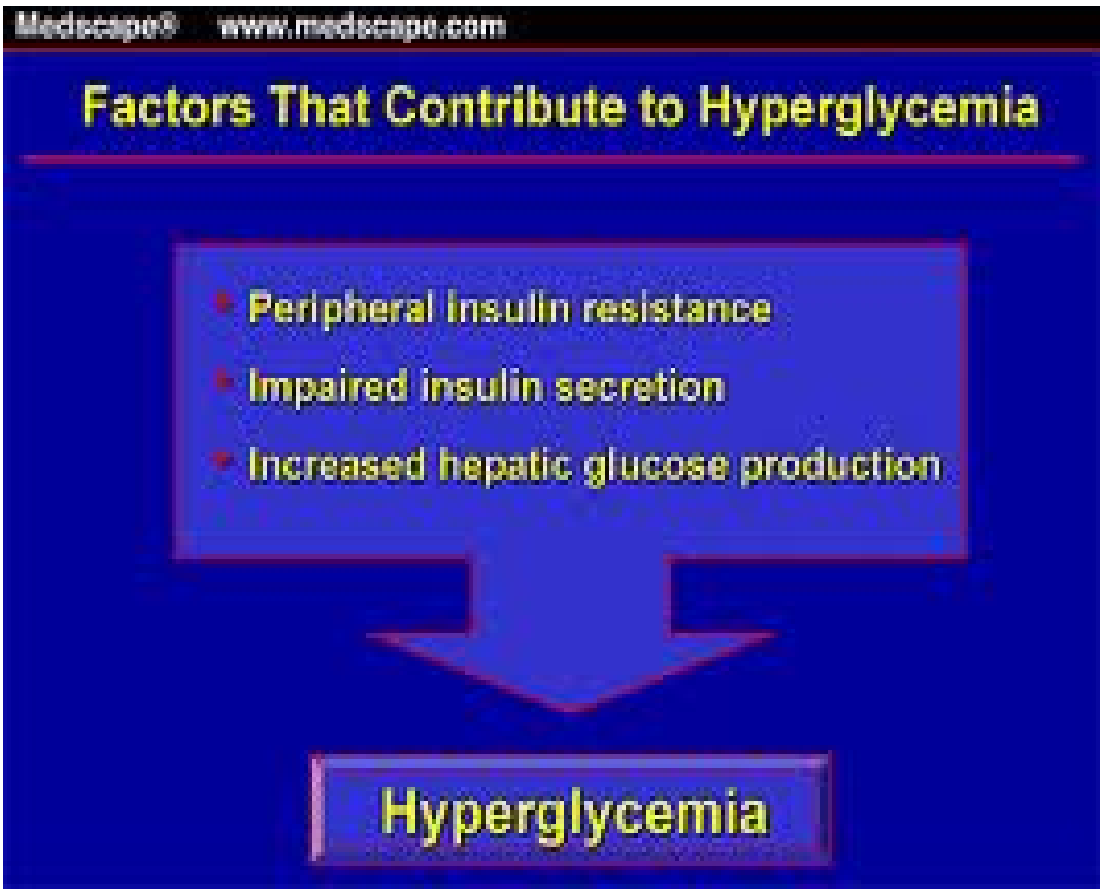


COMPLICATIONS IN HYPERTHYROIDISM



PRODUCTION OF THYROID HORMONE IN THYROID GLAND






Chronic Kidney Disease
Renal Graft, Nephritis
Glomeruloneph
Cateracts
Retinal degeneration
Macular degeneration
Skin Aging, Sunburn
Psoriasis, Dermatitis
Melanoma
Kidney
Eye
Skin
Endotoxin Liver Injury
Ischemic Bowel
Gastro-Intestinal
Heart
Angioplasty, CHD,
Keshan disease
(selinium deficiency)
Cardiac Fibrosis
Hypertension
Ischemia
Myocardial infarction
IBD, MS,
Cancer, Lupus,
Auto immune disorders
Chronic Inflammations
Immune System
Oxidative Stress
Joint
Vessel
Vasospasm
Endothelial Dysfunction
Atherosclerosis
Restenosis
Hypertension
Rheumatoid Arthritis
Osteo-Arthritis
Psorisis
Multi-organ
Lung
Brain
Cancer, Diabetes,
Radiation, Aging,
Chronic Fatigue
Infammatory-immune
Injury Ischemia-Reflow
Stroke, Trauma
Cancer, Migrane
Neurotoxins
Parkinson's Disease
Alzheimer's Disease
OCD, ADHD, Autism
Asthma, COPD,
Allergies, ARDS,
Hyperoxia, Cancer

Aim and Scope of the Study

AIM AND SCOPE OF THE STUDY

In recent years, much effort has been applied to the synthesis of potential anthyroid, antihyperglycemic and antioxidative drug. However a vast amount of herbal drug has given relatively small improvements over the synthetic drug. There is a continued need for new templates to use in the design of potential drug. Natural products provide such templates.

Artemisia nilagirica is used in traditional medicine for the treatment of various ailments such as poor appetite, indigestion, constipation, travel sickness, parasitic infection, irregular menstruation, menstrual pain, cramp, cold, epilepsy, typhoid, tuberculosis, urinary calculi and fever. The purpose is to link the traditional concepts and uses of herbal drugs, herbal products and certain phytochemicals for potential phytomedicine using modern scientific approaches.

The phytochemical studies on the leaves have been reported the presence of coumarins, flavonoids, tannins, terpenoids, volatileoil, carbohydrates and sterols. The present research is focused to investigate the antithyroid, anti hyperglycemic and antioxidative effects of Artemisia nilagirica.

This work has been designed for the following studies on the leaves of Artemisia nilagirica.

- Pharmacognostical studies on the leaves
- Preliminary phytochemical screening on the extracts of Artemisia nilagirica.
- Estimation of total phenols, total tannins and total flavonoids.
- Identification of the active principle in methanolic extract of A.nilagirica by TLC

- ❖ Isolation of active principle from methanolic extract of A.nilagirica using column chromatography.
- ❖ Structural elucidation of the active principle by using analytical processes such as spectroscopy (UV, FTIR & NMR).
- ❖ Quantification of the active principle in the methanolic extract of A.nilagirica by HPLC
- ❖ Pharmacological activities

  Invitro antioxidants activity

  - DPPH radical scavenging activity
  - Hydrogen peroxide scavenging activity
  - Antioxidant activity using cod-liver oil

1. In vivo antioxidant activities
   - ➢ Lipid peroxidation (LPO)
   - ➢ Super oxide dismutase (SOD)
   - ➢ Catalase activities (CAT)
   - ➢ Glutathione content (GSH)

2.In vivo antithyroid activity

- ➢ Estimation of T_3 and T_4 by Radioimmunoassay

3.Invivo antihyperglycemic activity

- ➢ Estimation of serum glucose concentration.

Review of Literature

REVIEW OF LITERATURE

Literature review refers to any collection of materials on a topic and a summary of sources. It provides with a handy guide to a particular topic. The literature review of Artemisia nilagirica and other varieties reveals that the following works have been already carried out on those plants.

Artemisia nilagirica

PHARMACOLOGICAL STUDIES

1.Samaiya.G.C,.et al [1986] have proved the Antimicrobial efficiency of essential oil of the leaves of Artemisia nilagirica. The essential oil from the leaves of Artemisia nilagirica showed a strong zone of inhibition against Bacillus substilis, k. Pneumonia, S.faecalis, B.cereus and staphylococcus aureus[29]

2..**Shafid.D.M,et al.,[2004]**have described the Composition and Antifungal activity of the oil of Artemisia nilagirica on the plant pathogens phytophtheracapsici [30]

3.**Verma.P.R et al.,[2007}** have described the Larvicidal activity of Artemisia nilagirica (Clarke) pamp and Ocimum sanctum Linn a preliminary herb.[31]

4.**Chowdary.H et al.,[2003]**have evaluated the Invitro Antifungal activity of two essential oils on Drechslerasorokiniana causing spot blotch of Wheat .[32]

5.**Suseela.V, et al [2009**] have described the Free radical scavenging potential of aqueous extract of Artemisia nilagirica (Clarke) pamp leaves.[33]

6.**Abdul R Ahmeethunisha , et al., [2010]** have evaluated the Antibacterial activity of Artemisia nilagirica leaf extracts against Clinical and Phytopathogenic bacteria.[34]

7.**Vijayalakshmi A et al.,[2011]** have described about the Anticonvulsant activity of Artemisia nilagirica leaves .[35]

8.**Perumal.P et al.,[2010]** have studied the Antitumor and Antioxidant activity of *Artemisia nilagirica* Ehrlich's Ascites carcinoma in the Swiss Albino mice.[36]

9.**Devamurari.V.P., et al[2010] have** proved the Anticancer activity of Artemisia nilagirica. [37]

PHYTOCHEMICAL STUDIES

1.**Haider,F, et al.,[2007]** have studied the Oil constituents of Artemisia nilgirica variety septentrionales during different growth phases at subtropical conditions of North Indian plain.[38]

2.**Devmurari V.P., et al [2010]** have evaluated the Antibacterial activity of Ethanolic Extract of Artemisia nilagirica.[39]

PHARMACOGNOSTICAL STUDIES.

1.**N.Pavalarani,et al [2012] have** studied the Pharmacognostic and Phytochemical screening of Artemisia nilagirica leaves found in Nilagiris District of Tamilnadu.[40]

OTHER VARIETIES.

Artemisia vulgaris

PHYTOCHEMICAL STUDIES

1.**Bagci.E,et al [2010]** have studied the Essential oil composition of the aerial parts of the two Artemisia species (A.vulgaris and A.absinthium) from East Antolian region [41]

2.**Lohani.H,et al.,[2007**] have described the chemical composition of Essential oil of Artemisia vulgaris from three different areas of Uttaranchal at two periods (June and November)[42]

3.**Bagchi.G.D.et al[2004]** have studied the effect of winter and summer seasons on essential oil quality of Artemisia vulgaris grown in north Indian plains.[43]

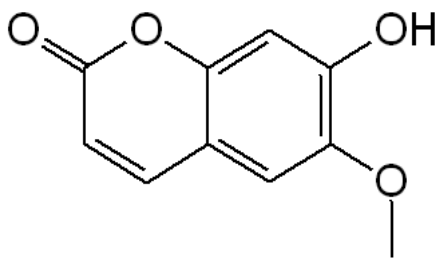
4.**Thao.N.T.P et al [2004]** have described the Artemisia vulgaris L.from Vietnam ;the chemical variability and composition of the oil along the vegetative life of the plant.[44]

5.**Carnat.A.et al[2000]** have isolated two Major dicaffeoylquinic acids fromthe flowering tops of Artemisia vulgaris.[45]

6.**Natmuller.R. et al [1981]** have described about the New irregular monoterpenes in the oil of Artemisia vulgaris.[46]

7.**Ikhsanova.M.A.et al[1986]** have described about the Coumarins like (esculin, esculetin, umbelliferone and scopoletin) from Artemisia vulgaris.[47]

STRUCTURE OF SCOPOLETIN[48]



7-hydroxy-6-methoxychromen-2-one **or** 7-Hydroxy-5-methoxycoumarin [20]

PHARMACOLOGICAL STUDIES

1.**Villasenor.I.M,Landrid [2006]** have compared the anti hyperglycaemic potentials of medicinal plants like Artemisia vulgaris, Solanumnigrum etc.,[48]

2.**Gilani A.H.et al[2005]** have described the Hepatoprotective activity of aqueous-methanol extract of the aerial parts of Artemisia vulgaris.[49]

PHARMACOGNOSTICAL STUDIES

Artemisia nilagirica (C.B.Clarke) is an aromatic plant belonging to the family Compositae

Synonym [50]

Artemisia vulgaris L.variety nilagirica C.B.Clarke

Common Name [51]

Titapati

Systematic position [51]

Kingdom - Plantae

Subkingdom - Angiosperms

Division - Magiliophyta

Class - Rosopsida

Order - Asterales

Genus - Artemisia

Species - nilagirica

Vernacular names [52]

English - Indian wormwood

Hindi - Dona, Majpatri, Nagdona

Kannada - Majepatri

Malayalam - Masipatri, Makkippuvu

Sanskrit - Nagadaman

Tamil - Masipathri, Makkipoo

Telugu - Machipathri, Davanamu

Geographical distribution [53]

It is found in Asia, Europe, Northern Africa, Alaska and North America

Habit and Habitat of the plant [54]

Indian wormwood is an aromatic shrub, 1-2m high, yellow or dark red small flowers, grows throughout India in hills up to 2400 elevation. This medicinal herb is erect, hairy, often half woody. It is a tall, aromatic, pubescent or villous shrub like herb found throughout hilly regions of India.

Description of the plant [52]

Leaves

The leaves are simple leaves, arranged oppositely one another, lobed, laciniate or pinnatipartite and 5-4 long.

Stem

Red purplish angular grooved stem, paniculately branched and leafy.

Flowers

Flower heads are small, ovoid or globosely in panicle racemes. It is many petaled and pale yellow.

Fruits

The fruits are achenes.

Seed

Radicle is short.

Root

The root system is fibrous.

MACROSCOPICAL AND MICROSCOPICAL STUDY

Materials and methods

Fresh leaf was used for microscopical examination .The cut portion of the leaf was first fixed using FAA (Formalin-5ml+ Acetic acid -5ml+70% ethanol-90ml).After 24hrs of fixing ,the specimens were dehydrated with graded series of tertiary butyl alcohol, then infiltration was carried by gradual addition of paraffin wax (melting point 58-600 C) Then the specimens were cast into paraffin blocks.The paraffin embedded specimens were sectioned with the help of Rotary microtome. The sections were stained with toluidine blue.

Leaf

The transverse section of the leaf exhibits thick and prominent midrib and thin lamina (fig1).

Midrib

The midrib consists of thick and wide adaxial conical hump & wide and large abaxial midrib part. The epidermis of the midrib is very thin, darkly stared and the cells are not much distinct. There is a sub epidermal layer of small, elliptical, thin walled cells. The ground tissue is parenchymatous, the cells being polyhedral, thin walled, angular and compact. The midrib is 900µm thick, the adaxial hump is 450µm wide and the abaxial part is 1mm wide .The vascular system of the midrib consists of larger, semicircular main strand and 2 smaller accessory bundles located on the upper lateral part of the main bundle. The vascular bundles are collateral with long radial lines of wide, thick walled, angular xylem elements and a thick abaxial arc of phloem elements .(Fig2.1)

Lateral vein

The lateral vein is flat on the adaxial side and broadly conical on the abaxial side . It is 320µm thick and 200µm wide. There is a single collateral vascular bundle which includes a few short, xylem strands and an arc of phloem elements on the abaxial side. The ground tissue including the epidermis is fairly larger, thin walled and compact. There is a small, collateral, vascular bundle situated in the middle of the lateral vein (Fig2.2)

Lamina

The lamina of the leaf is dorsiventral and bifacial with much dilated fairly thick walled adaxial epidermal cells which are 20µm thick .The outer tangential walls of the cells are slightly conical. The abaxial epidermal cells are very thin, cylindrical and thick walled. There is a single, row of cylindrical less compact palisade layer of cells and about 4 layers of spherical and lobed spongy parenchyma cells.(Fig 2.3)

Petiole

The distal part of the petiole has 2 prominent lateral wings and central thick and broad petiole proper. The lower part of the petiole is 3 lobed and the upper part is semi circular. The petiole is 800µm thick, the adaxial part is 500µm wide and abaxial lobed part is 750µm wide. The epidermis is very thin comprising small, squarish thick walled cells. The ground tissue is parenchymatous, thin walled polygonal and compact. There are 2 large, unequal, vascular bundles placed in the lower part of the petiole. The vascular bundles are collateral with dense, compact rows of small, xylem elements and thick band of compact phloem elements.

The proximal part of the petiole is slightly different from the distal part. The petiole has wide, distinct wings and the petiole proper is somewhat circular with

slightly raised adaxial hump .The petiole is1.8mm thick and 1.55mm wide. The epidermis is thin; the cells are small and thick walled. The ground tissue is homogenous and parenchymatous (Fig 3.2).The vascular system consists of an arc of 4 independent vascular bundles of which the median bundle is the largest and lateral bundles are collateral. The vascular bundles have long, parallel rows of xylem elements which are more compact. The xylem elements are wide, angular and thin walled. Phloem occurs on the lower part of the xylem strand comprising small groups of sieve elements. The lower part of the phloem band includes compact, small, angular parenchymatous cells.(Fig 4.1)

Stomata

The stomata occur on the abaxial side of the lamina. They are located within pits which are enclosed by dense epidermis trichomes. The guard cells are widely elliptical measuring 25×20µm in size .The epidermis cells are thin walled, slightly wavy and polyhedral in outline (Fig 4.2).

Venation pattern

The major and minor veins become gradually thin forming dense, reticulate network. The vein-islets are wide and vary in shape from rectangular to polygonal in outline (Fig 5.1).The islets have distinct boundaries which are slightly wavy or straight .The vein-islet terminations are present in most of the islets. The terminations are thin, long and slightly wavy (Fig 5.2).

Glandular trichomes

Within the islets there are seen prominent 2 celled, elliptical, subsessile glandular trichomes (Fig 5.3).The trichomes have thin walls and cytoplasmic content. The glands are 45×20µm in size. The glands are random in distribution.

QUANTITATIVE MICROSCOPY [55,56]

Quantitative microscopy or determinations of some pharmacognostical parameters are useful for setting standards for crude drugs. Quantitative microscopy is used for evaluation of crude drugs. It includes vein islet number, vein termination number, stomatalnumber, stomatal index and palisade ratio.

Stomatal number and stomatal index

Stomatal number

The average number of stomata per square millimetre of epidermis is termed as stomatal number.

Stomatal index

The percentage proportion of the ultimate divisions of the epidermis of a leaf which have been converted into stomata.

Determination of stomatal number and stomatal index

The replicas of leaf surface may be made which are satisfactory for the determination of stomatal number and stomatal index using fresh leaves. The 50% gelatin and water gel is liquefied on a hot slide. The fresh leaf is placed on it. Then the slide is inverted and cooled under tap water and after about 15-30 minutes the specimen is stripped off. The imprint on the gelatin gives clear outline of the epidermal cells, stomata and trichomes. The slide was examined under 45x objective and 10x eye piece to which a microscopical apparatus was attached. The stomatal index was calculated by using the formula

Stomatal index S.I.= $\frac{S}{E+S} x100$

Where

S = Number of stomata in 1sq mm area of leaf.

E=Number of epidermal cells (including trichomes) in the same area of leaf.

Vein islet number

The number of vein islets per mm^2 calculated from four contiguous square millimeters in the central part of the lamina, midway between the midrib and the margin.

Vein termination number

The number of vein islet terminations per mm^2 of leaf surface. An ultimate free termination of a veinlet or branch of a veinlet.

Detemination of vein islets and vein termination number

The fragment of leaf lamina with an area of not less than 1sq.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 a temporary hydrate solution on a boiling water bath until clear. The clear fragments were stained with safranin solution and mount was prepared with glycerol solution. The stage micrometer placed on the microscopic stage, examined under10x objective lens and 6x eyepiece 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 overlapping on two adjacent sides and excluding those intersected by results obtained in the number of vein islets and terminals in 1sq.mm.were tabulated in table

Palisade ratio

The average number of palisade cells beneath each upper epidermal cell is termed as palisade ratio.

Determination of palisade ratio

Epidermal peeling was done by partial maceration by Jeffery‘s maceration fluid, were prepared. A fragment was transferred into a micro scopical 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 and10x eyepiece. Four adjacent epidermal cells were traced. Focusing gently downward to bring the palisade cells into view and sufficient palisade cells to cover the outline . Four epidermal cells were then traced. The palisade cells under the epidermal cells were counted and the palisade ratio were calculated by using the following formula and the results were tabulated in **table1**

Powder analysis [57,58]

The behaviour of the powder with different chemical reagents was carried out as per standard procedure mentioned by Kay(1938) and Johansen(1940) the observations are tabulated

Flourescence analysis

The fluorescent analysis of the crude drug of A.niagirica were carried out by usig the method of chase and pratt (1949)[5].The observations are tabulated in **table 2**

Standardisation parameters

The physical evaluations like the ash values, loss on drying, foreign organic matter and extractive values gives the clear idea about the crude drug under examination

Determination of volatile oil [59]

Volatile oil apparatus was thoroughly washed and the glycerin was applied at both the ends. An accurately weighed 100g of plant material was crushed and

introduced into the flask containing distilled water until one third of the plant material was immersed and few pieces of porcelain bits were added. Small quantity of glycerin is also added. The flask containing liquid was heated until it boils. After 3hrs heating was stopped and the collected oil was recorded on the graduated receiver tube. Oil content of the plant material was calculated in ml/100g of plant material.

Determination of foreign organic matter [59]

10gms of a drug powder was weighed and spread in a thin layer on a paper. It was examined under6X magnification .The foreign material was picked out and weighed. Then the percentage was calculated.

Determination of moisture content [60]

Loss on drying was noted for this drug .1gm of powdered drug was taken in a tarred petridish and it was weighed. The weighed drug powder was kept in hot air oven at 100-105^{0}C for 1hr. Then it was cooled in a desiccator and again weighed. The loss on drying was calculated with reference to the air dried powder.

Ash content [61]

The residue remaining after incineration is the ash content of crude drug, which simply represents inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration.

Total ash

2gms of the dried coarsely powdered drug was taken in a tarred crucible. The crucible was kept in a incinerator at 450^{o}C .The drug becomes ash within one or two hrs and It was weighed.

Water soluble ash

Total ash obtained by previous procedure was boiled with 25ml of distilled water. The insoluble matter was filtered in ash less filter paper, ignited to constant weight, cooled in a dessicator and weighed. The weight of the insoluble matter was subtracted from the weight of the ash taken. The difference gave the weight of the water soluble ash.

Acid insoluble ash

Total ash obtained from the previous procedure was mixed with25ml of dilute hydrochloric acid and boiled for five minutes. The ash was filtered in a ashless filter paper. The filter paper with ash in a crucible is kept in an incinerator for one or two hours. Then the ash was weighed.

DETERMINATION OF EXTRACTIVE VALUES [60,61]

Ethanol extractive values

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of ethanol in a closed conical flask for 24hrs.It was frequently shaken for 6hrs,then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant weight and weighed. The percentage was calculated with reference to the air dried powder.

Methanol extractive values

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of methanol in a closed conical flask for 24hrs. It was frequently shaken for 6hrs, then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant weight and weighed. The percentage was calculated with reference to the air dried powder.

Petroleum ether extractive value

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of petroleum ether in a closed conical flask for 24hrs.It was frequently shaken for 6hrs, then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant weight and weighed. The percentage was calculated with reference to the air dried powder.

Chloroform extractive value

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of chloroform in a closed conical flask for 24hrs.It was frequently shaken for 6hrs,then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant weight and weighed. The percentage was calculated with reference to the air dried powder.

Ethyl acetate extractive value

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of ethyl acetate in a closed conical flask for 24hrs.It was frequently shaken for 6hrs,then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant weight and weighed. The percentage was calculated with reference to the air dried powder.

Water soluble extractive value

5gms of the dried coarsely powdered drug was weighed and macerated with 100ml of chloroform water in a closed conical flask for 24hrs. It was frequently shaken for 6hrs, then it was allowed to stand for 18 hrs. It was filtered rapidly. 25 ml of the filterate was taken in a tarred china dish and it was dried at 105^0C to constant

weight and weighed. The percentage was calculated with reference to the air dried powder.

Determinations of foaming index [60]

Plant materials contain saponins that can cause persistent foam when it was shaken as a aqueous decoction. The foaming ability of plant materials and their extracts was 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 30 minutes .The solution was cooled and filtered into a100ml 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 15 minutes .The height of foam was measured .If the height of the foam in every tube was less than 1cm the foaming 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 than1cm 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 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.

Determination of swelling index [60]

Swelling index is the volume in ml taken up by the swelling of 1g of plant material under specified conditions .Its determination is based on the addition of water. Using a glass stoppered measuring cylinder, the material is shaken repeatedly for 1hr and then allowed to stand for required period of time. The volume of the mixture (in ml) is read.

About 1g of weighed powder was introduced introduced into a25ml glass stoppered measuring cylinder,25ml of water was added and the mixture was shaken thoroughly every 10minutes for 1hr and allowed to stand for 3hrs at room temperature. The volume in ml occupied by the plant material including the sticky mucilage was measured. The mean value of the individual determination was calculated related to 1g of plant material and the result was tabulated **in table 4.**

Fig.2. Dorsal view

Fig.3. Ventral view

Fig. 15. Habit and Habitat of Plant

Fig. 16. Herbarium of *Artemisia nilagirica*

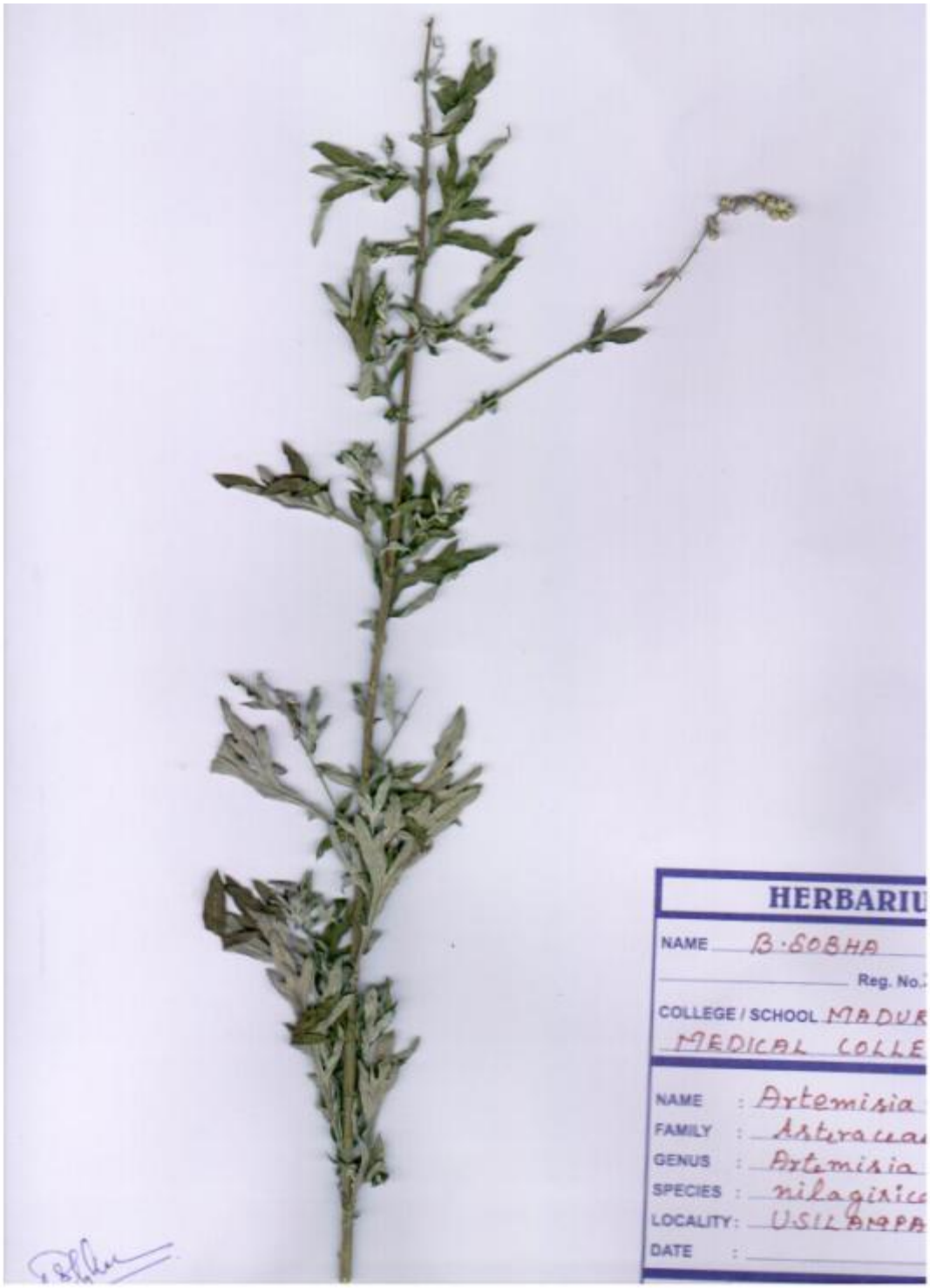


Fig. 4. Fruits of Artemisia nilagirica

Fig. 5. Flower of Artemisia nilagirica

Fig. 5. Seeds

Fig. 6. T.S.of Leaf through Midrib

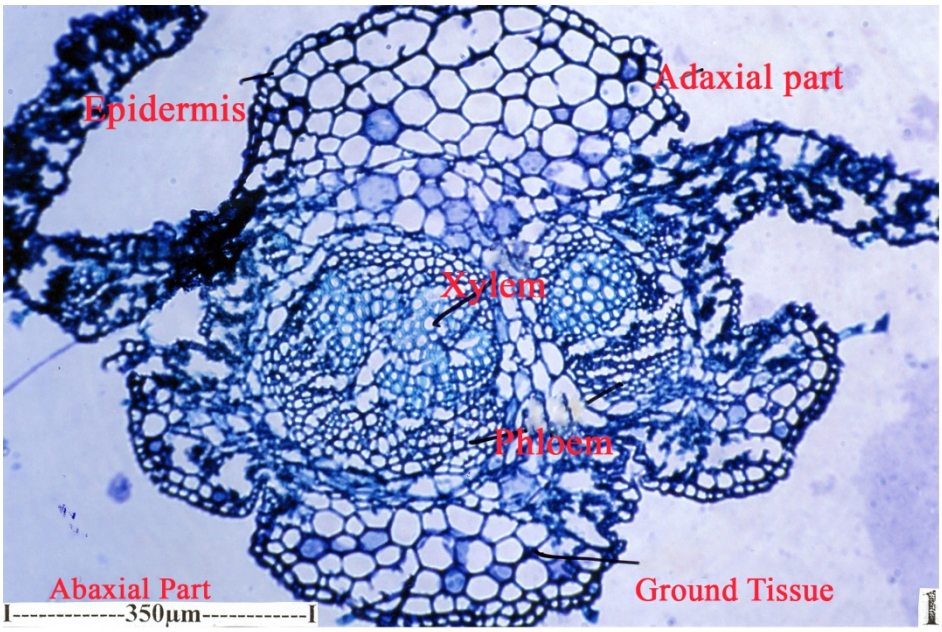


Fig. 7. T.S. of Midrib enlarged

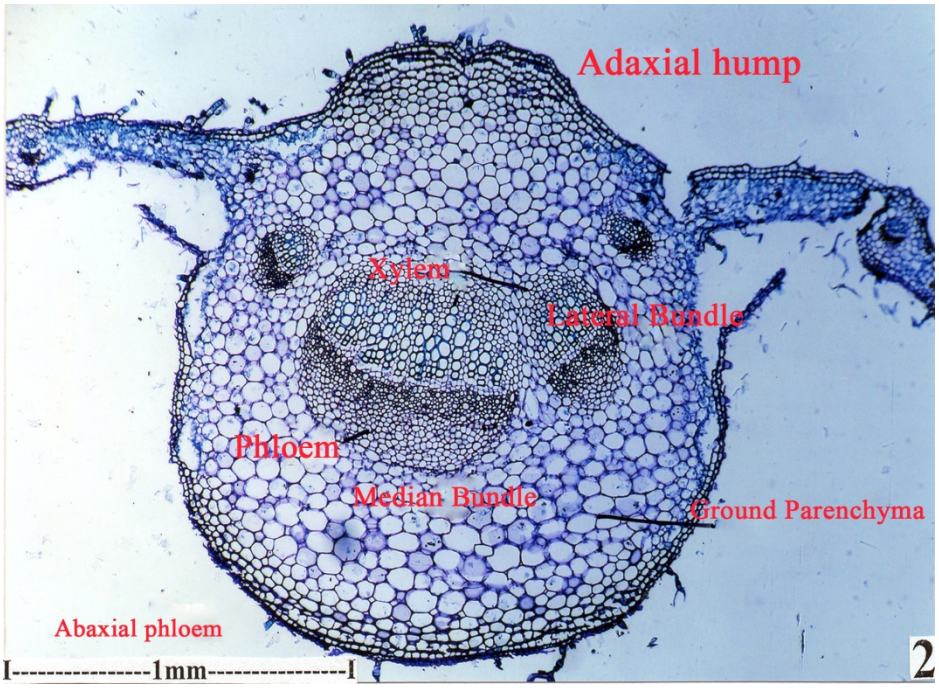


Fig. 8. T.S. Median and Lateral Veins on the Midrib enlarged

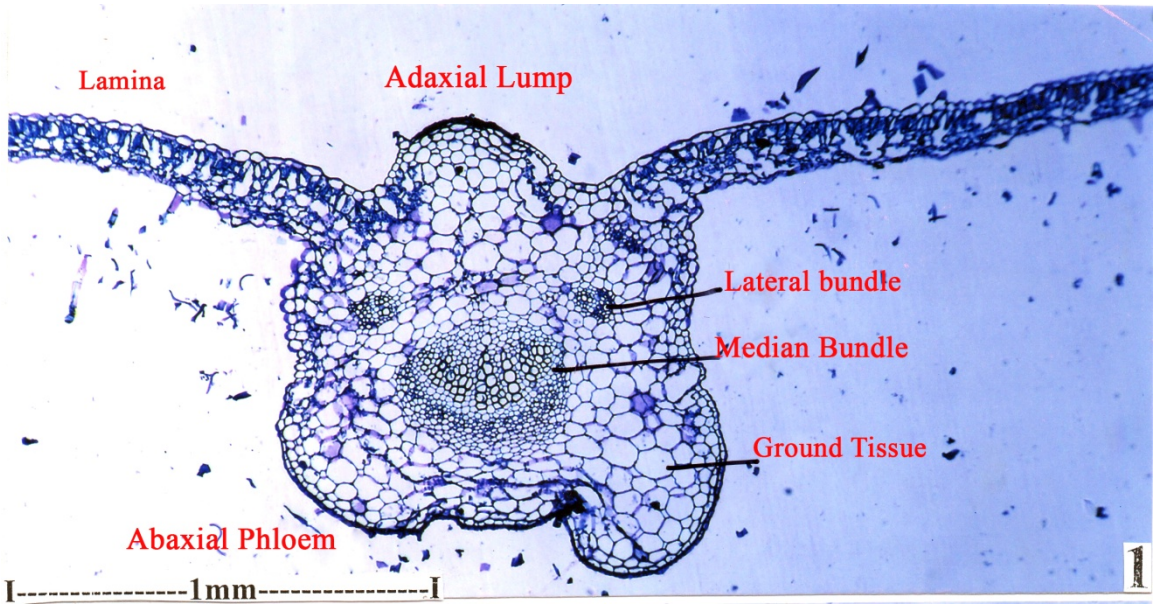


Fig. 9. T.S of lamina through lateral vein

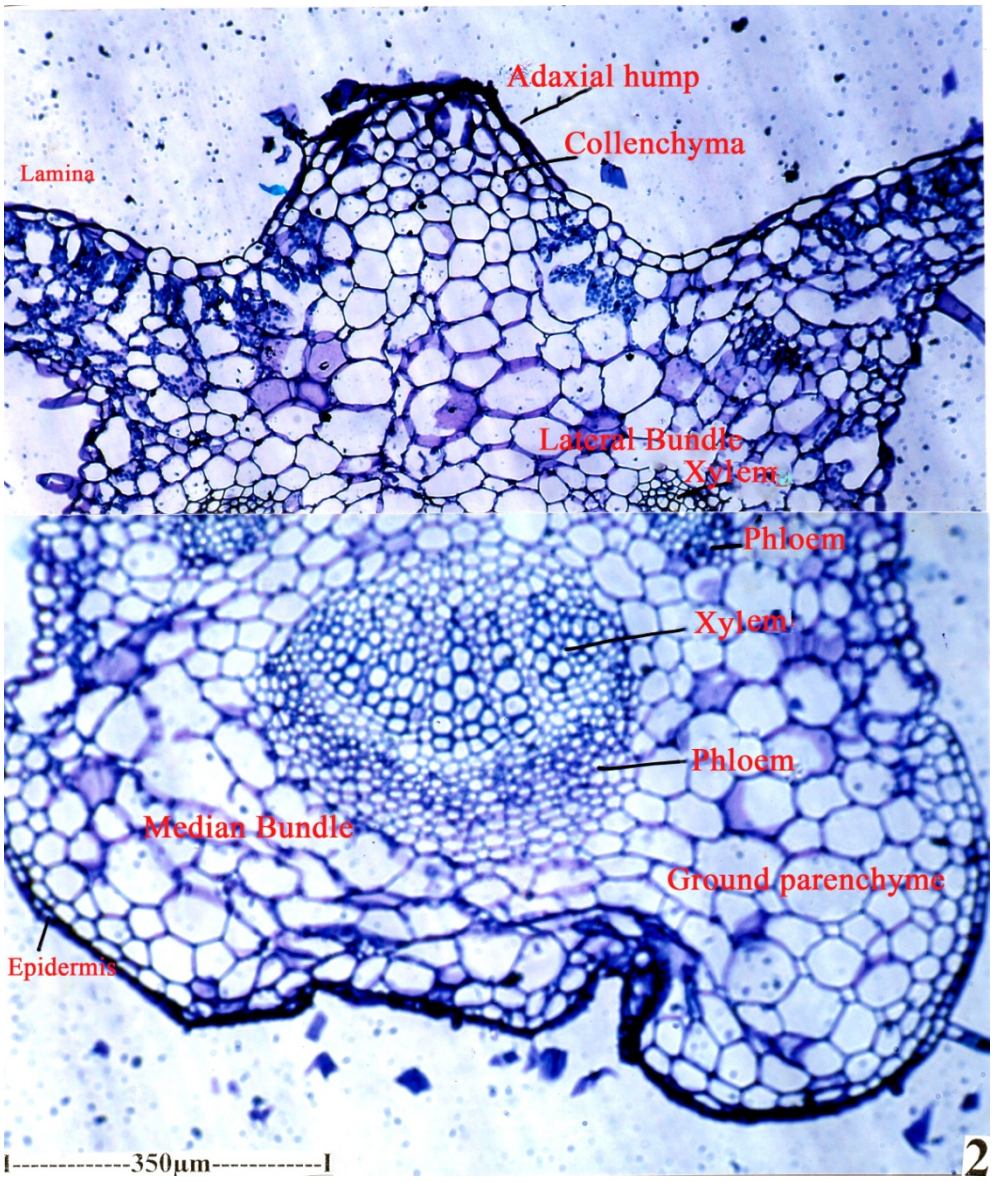


Fig. 10. T.S. of Lamina

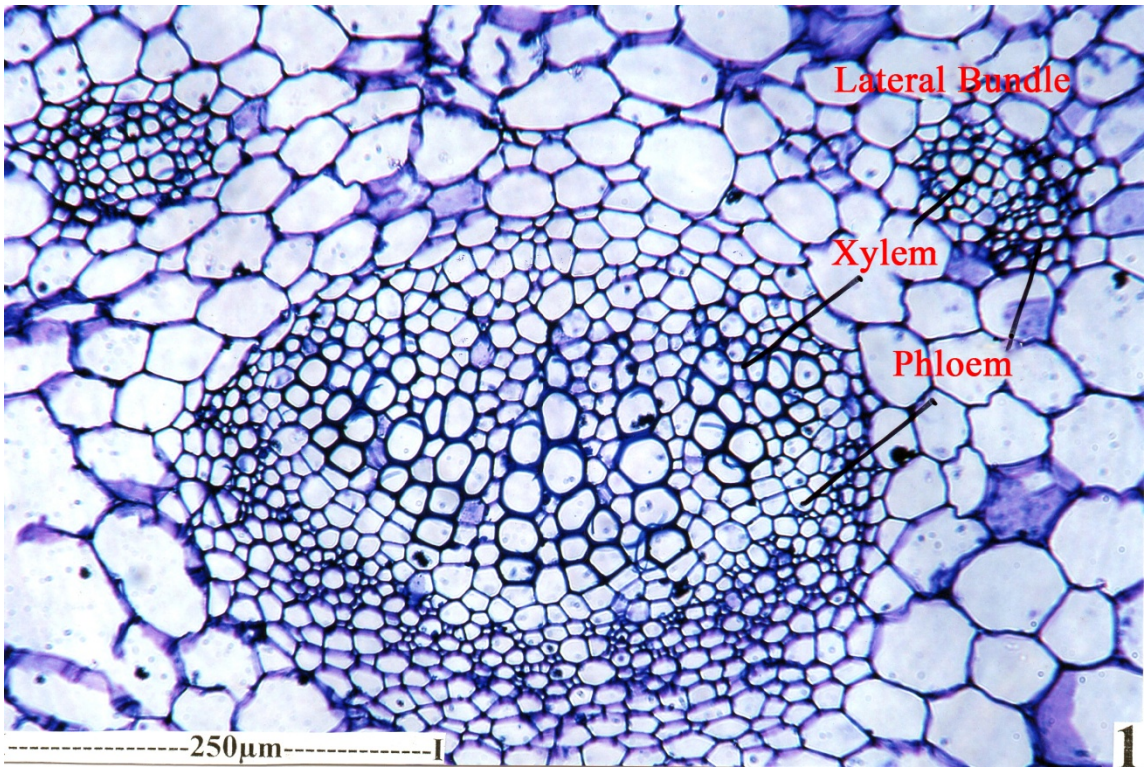


Fig.11. T.S. of Petiole through distal part

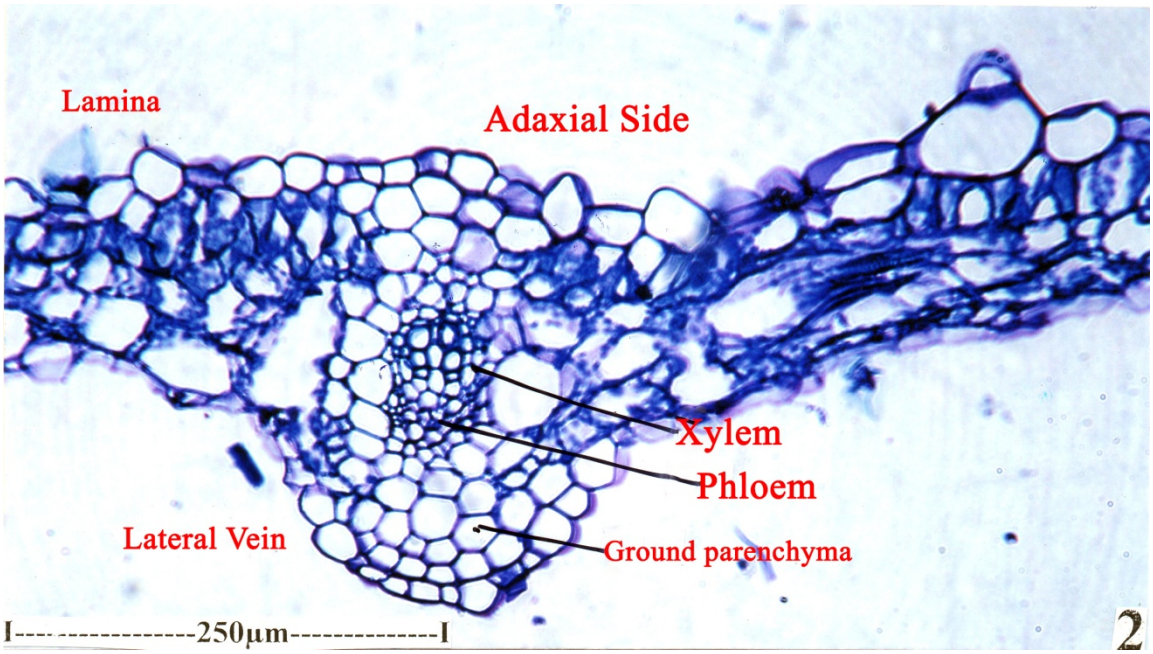


Fig. 12. T.S. of Petiole through Proximal part

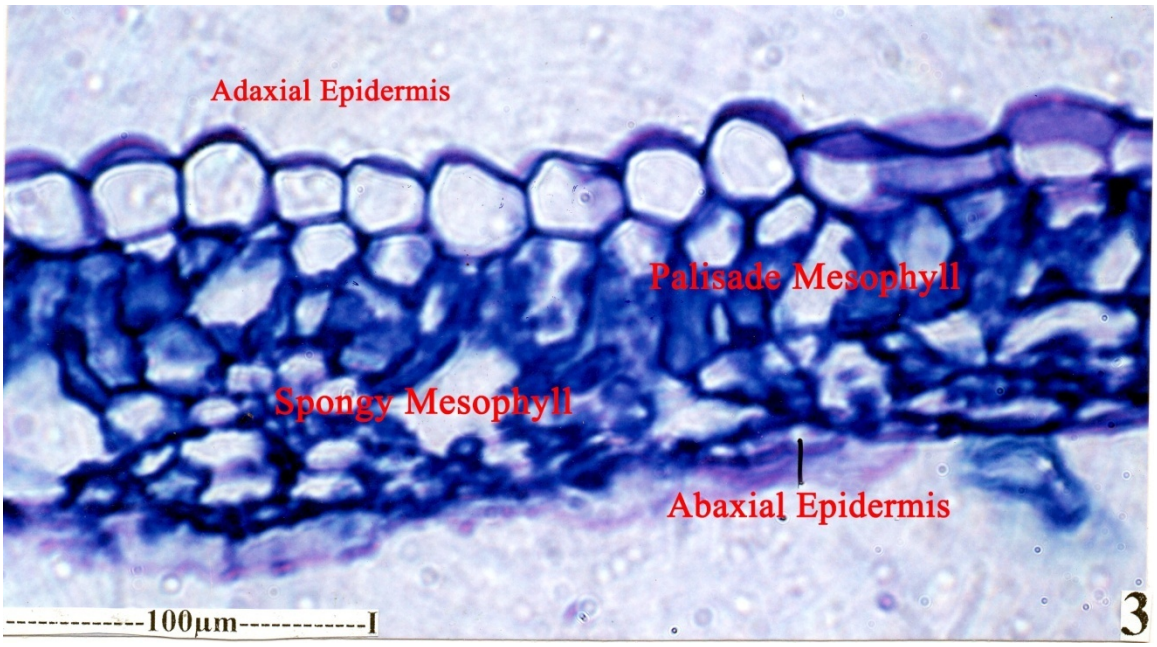


Fig. 13. Venation pattern

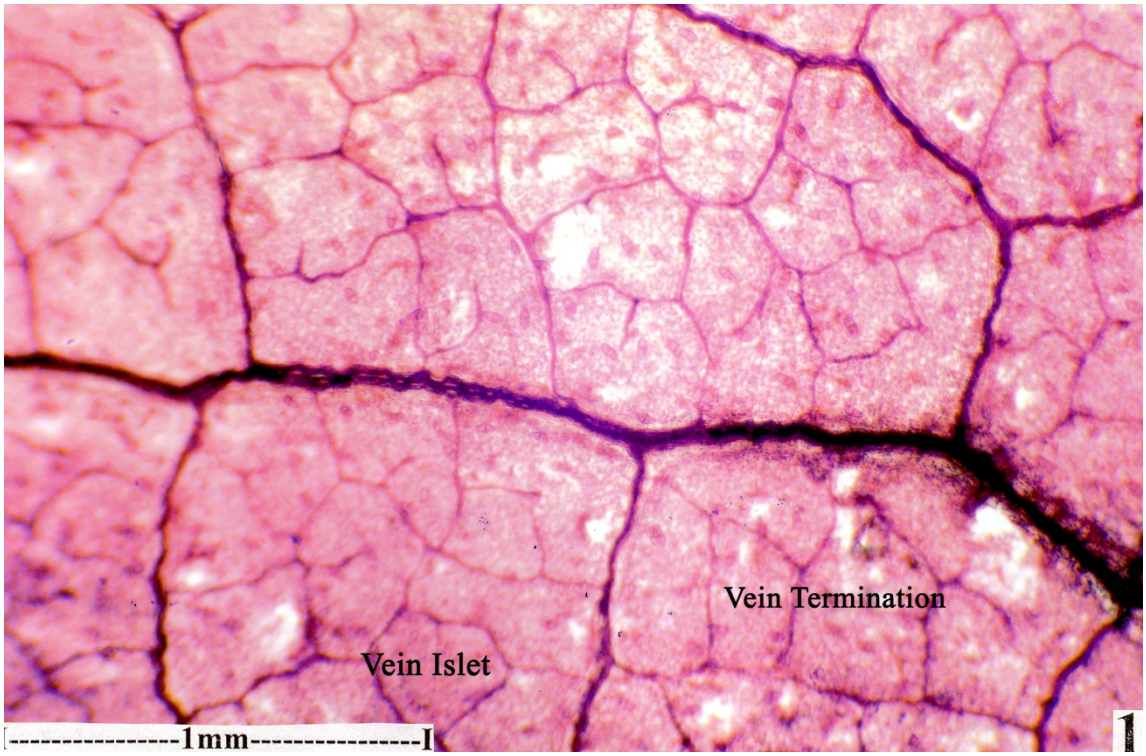
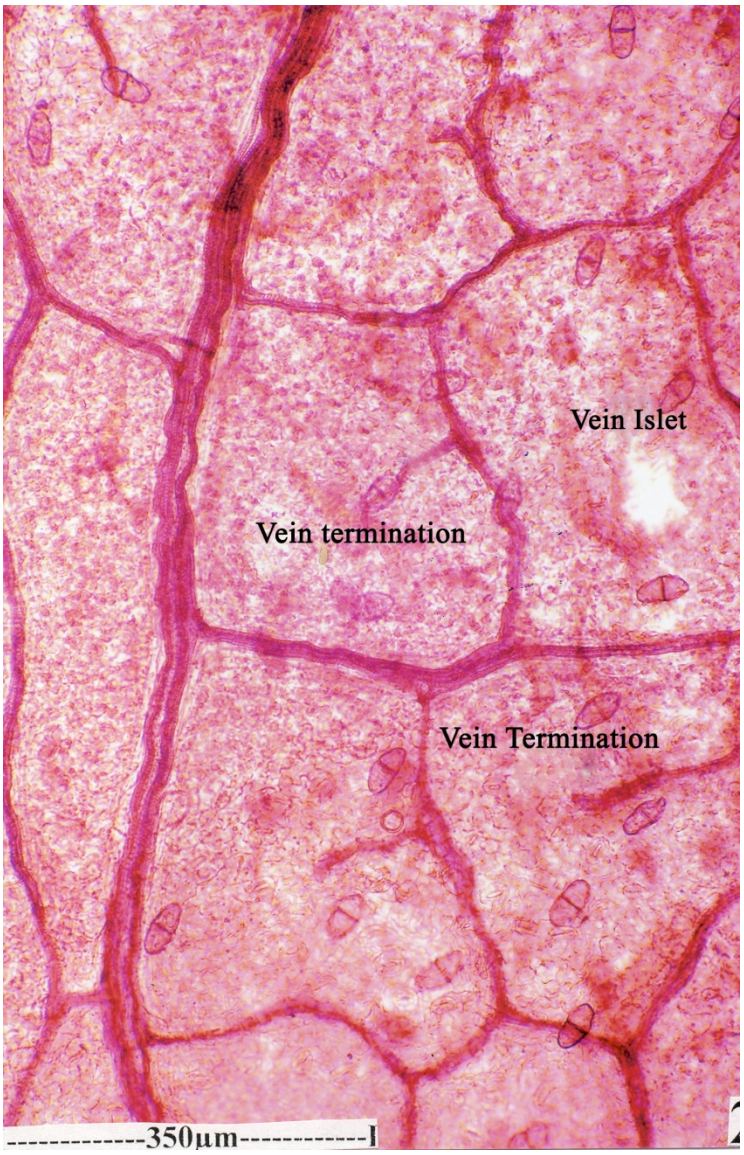
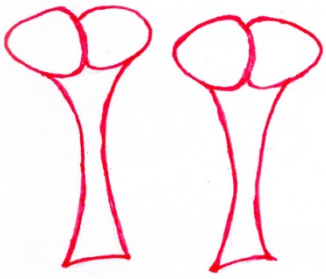


Fig. 14. Vein islet and vein termination enlarged



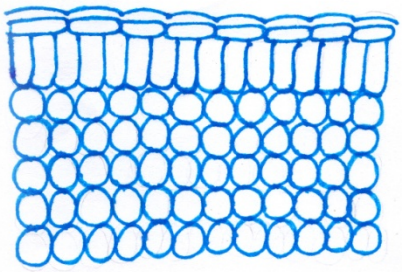
POWDER MICROSCOPY

GLANDULAR TRICHOMES

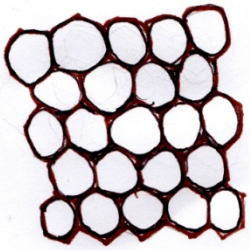


ANAMOCYTIC STOMATA

PALISIDE MESOPHYLL



COLLENCHYMA CELLS



Phytochemical Studies

PHYTOCHEMICAL EVALUATION

Phytochemistry deals with natural product organic chemistry and plant biochemistry .It also 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.

The shadow dried leaves of Artemesia nilagirica were powdered and then subjected to the following preliminary phytochemical studies.

Organoleptic evaluation

Nature - Coarse powder

Colour - Green

Odour - Aromatic odour

Taste - Aromatic sweet

QUALITATIVE CHEMICAL EVALUATION[55,56]

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. The chemical test can be useful for the investigation of the chemical compounds. The various tests for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, phenols and tannins were performed on the extracts and the results were tabulated **in table 5**

Test for sterols

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

a.Salkowski 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 colour indicating the presence of sterols.

b.Libbermann-burchard's test

To the chloroform solution a few drops of acetic anhydride and 1ml of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring was formed. The upper layer turned green indicating the presence of sterols.

2.Test for carbohydrates

a)Molisch's test

The aqueous extract of the powdered leaf was treated with 2-3 drops of 1% alcoholic α-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube .A purple colour indicates the presence of carbohydrates.

b)Fehling's test

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

c)Benedict's test

The aqueous extract of the powdered leaf was treated with equal volume of Benedict's reagent. A red precipitate was formed indicating the presence of reducing sugar.

3. Test for proteins

a). Millon's test

A small quantity acidulous-alcoholic extract of the powdered drug was heated with Millon's reagent. A white precipitate turning red on heating indicates the presence of proteins.

b).Biuret test

To one portion of acidulous-alcoholic extract of the powdered drug, one ml of 10% solution of copper sulphate and sodium hydroxide was added. Violet colour was obtained indicating the presence of proteins.

Test for alkaloids

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 and extracted with chloroform. 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 test

To small quantity of the extract add Mayer's reagent. No cream colour precipitate indicates the absence of alkaloids.

b)Dragendroff's test

To small quantity of the extract dragendroff's reagent was added .No orange brown precipitate indicates the absence of alkaloids.

c)Wagner's test

To small quantity of the extract Wagner's reagent was added. No reddish brown precipitate indicates the absence of alkaloids.

d)Hager's test

To small quantity of extracts Hager's reagent was added. No yellow precipitate indicates the absence of alkaloids.

5.Test for glycosides

a.Borntrager's test

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The organic layer was separated to which ammonia solution was added slowly. No pink colour in the ammoniacal layer indicates the absence of anthraquinone glycosides.

b.Modified borntragers 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. No pink colour was observed in ammoniacal layer showing the absence of anthraquinone glycosides.

c.Test for cardiac glycosides (for deoxysugar)

i) 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 solution of leads subacetate were added and filtered, evaporated to dryness. The residue was dissolved in 3ml of glacial acetic acid. To these 2 drops of ferric chloride solution was added. Then concentrated sulphuric acid was added to the test tube carefully and observed. No reddish brown layer was observed indicating absence of deoxysugars of cardiac glycosides.

ii)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 hrs in a warm place. No brick red colour was produced on the paper indicating the absence of cyanogenetic glycosides.

6.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. 5ml of the filtrate was then diluted with water and shaken vigorously. Frothing was produced indicating the presence of saponins .

7.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. A bluish black colour was produced indicating the presence of tannins.

8 Test for flavonoids

Shinoda'stest

A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes. Red colour was obtained indicating the presence of flavonoids.

Alkali test

To the small quantity of test solution, 10% aqueous sodium hydroxide solution was added yellow orange colour was produced indicating the presence of flavonoids.

Acid test

To the small quantity of test solution, few drops of concentrated sulphuric acid were added yellow to crimson colour was obtained indicates the presence of flavonoids.

9.Test for terpenoids

A little of the powdered leaf was extracted with chloroform and filtered. The filtrate was warmed gently with chloroform and thionylchloride. Pink colour solution appeared which indicated the presence of terpenoids.

10.Test for the presence of volatile oil

Weighed quantity (250 gm) of fresh leaves were extracted and subjected to hydrodistillation using volatile oil estimation apparatus. Volatile oil was obtained indicating the presence of volatile oil .

11.Test for coumarins

a) $FeCl_3$ Test

To the alcoholic extract of the drug , few drops of alcoholic $FeCl_3$ were added. The formation of deep green colour turning yellow on the addition of concentrated nitric acid indicates the presence of coumarins.

b) Flourescence test

The alcoholic extract of crude extract was mixed with 1N NaOH solution. A blue green fluorescence indicates the presence of coumarins.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Preparation of methanolic extract of Artemisia nilagirica

The shade dried and coarsely powdered leaves of Artemisia nilagirica was defatted with petroleum ether for three days by triple maceration. The defatted marc was extracted with methanol by triple maceration and filtered. The filtrate was concentrated under reduced pressure to obtain a solid residue which was dark green in colour.

Estimations:

Total phenol determination[63,64,65]

Phenols comprise the largest group of secondary metabolites. They are defined as compounds that have at least one hydroxyl group attached to benzene ring. Phenolic compounds are commonly found in plants and they have been reported to have multiple biological effects, including antioxidant activity

Principle

The total phenolic content of the various concentrations of extract of Artemisia nilagirica was determined by Folin-Ciocalteu reagent method. All the phenolic compounds are oxidized by the Folin-Ciocalteureagent. This reagent is reduced during oxidation of the phenolic substances, into a mixture of blue molybdenum and tungsten oxides.

The blue colour reduced has a maximum absorption at about 750-760nm.The absorption is proportional to the quantity of oxidized phenolic compounds.

Instrument

Shimadzu UV Visible spectrophotometer model 1800.

Reagents required

a) Folin-Ciocalteu Reagent (1N)

The Folin-cioacalteu reagent is a mixture of phosphomolybdate and phospho tungstate. Commercially available Folin-Ciocalteu reagent (2N) was diluted with an equal volume of distilled water. The resultant solution was kept in a brown colour bottle and stored in refrigerator at 4^0C.

b) Sodium carbonate solution (10%)

Procedure

To the 1ml(1mg/ml and 0.5 mg/ml) of methanolic extracts of Artemisia nilagirica, 0.5ml of Folin Ciocalteu reagent(1N) was added and allowed to stand for 15 minutes. Then 1ml of 10% sodium carbonate solution was added to the above solution. Finally the mixtures were made upto 10ml with distilled water and allowed to stand for 30 minutes at room temperature and total phenols were determined spectrophotometrically at 760 nm.

The calibration curve generated by preparing gallic acid at different concentration (2,4,6,8,10µg/ml).The reaction mixture without sample was used as blank. Total phenol content of various leaf extracts was expressed in terms of mg of gallic acid equivalent per gm extract(mg GAE/g).

2. TOTAL FLAVONOID DETERMINATION[66,67]

The word 'flavonoid' is derived from the latin word, flavus meaning yellow and many flavonoids are indeed yellow in colour. It consists of a single benzene ring joined to a benzo gamma pyronestructure. These include catechins, lecoanthocynanins, flavonones, flavones, flavonols, chalcones, aurones and isoflavones.

Principle

The aluminium chloride colorimetric technique was used for estimation of total flavonoid estimation. Aluminium ions form stable complexes with C_4ketogroup and either to C_3 or C_5 hydroxyl groups of flavones and flavonols in acidic medium .It also forms acid labile complexes with ortho hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavonoids. These complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents required

10% aluminium chloride

1M Potassium acetate

Procedure

1ml of methanolic extracts of Artemisia nilagirica 50µg/ml and 100µg/ml were taken and 0.1ml of aluminium chloride, 0.1ml of potassium acetate solution and 2.8 ml of ethanol were added and the final volume was then made up to5ml with distilled water. After 20minutes, the absorbance was measured at 415nm.

A calibration curve was constructed by plotting absorbance reading of quercetin at different concentrations. The sample without aluminium chloride was used as a blank. The total flavonoid content in the extract was expressed as milligrams of quercetin equivalent per gram of extract.

TOTAL TANNIN CONTENT DETERMINATION[69]

Principle

Total tannin content of extract was determined by folindenis reagent method. Tannin like compounds reduces phosphor tungsto molybdic acid in alkaline solution to produce a highly blue coloured solution and the intensity of which is proportional to the amount of tannins and estimated by spectrometer at 700nm

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents

a) Folin Denis reagent

b) Sodium carbonate 10%

Procedure

0.2ml of (1mg/ml) methanolic extracts of Dalbergiasissoo, was made upto 1ml with distilled water. Then add 0.5ml of Folin Denis reagent and allowed to stand for 15minutes, then 1ml of sodium carbonate solution was added to the mixture and it was made up to 5 ml with distilled water. The mixture was allowed to stand for 30minutes at room temperature and the tannin content was determined spectrophotometrically at700nm.

The calibration curve was generated by preparing tannic acid at different concentration (4,8,12,16,20µg/ml) The reaction mixture without sample was used as blank .The total tannin content in the leaf extract was expressed as milligram of tannic acid equivalent per gram of extract.

THIN LAYER CHROMATOGRAPHY [70]

In 1958,Stahl developed standard equipment for analyzing compound mixture by thin layer chromatography .It combines the advantages of paper and column chromatography. It is a simple equipment. It has a short development time. It has a wide choice of stationary phase. It is possible to early recovery of separated components. It possess easy visualisation of separated components.

Principle

The principle of separation is adsorption. A thin layer of adsorbent coated on a chromatographic plate is spotted by one or more components. The mobile phase solvent flows through because of capillary action. According to their affinities, the components move towards the adsorbent. The components with more affinity move slower. The component with less affinity move faster. Thus the components are separated on the basis of affinities.

Procedure

Preparation of TLC plates

It is based on pouring technique. The slurry is prepared by mixing the Silica Gel G with water at the ratio of 1:2 .It is poured on the glass plate having dimensions of 20X 20cm which is maintained on levelled surface. The slurry is uniformly spreaded on the plate. The plates are dried in a hot air oven at 105^0C . Then it is kept in a dessicator.

Application of the sample

The methanolic extract of Artemisia nilagirica 2-5 of a 1% solution spotted on a plate using a capillary tube. The spot was kept 2cm above the base of the plate.

Mobile phase

Ethyl acetate: Methanol: Water 100:6:4

Development of chromatogram

The glass plate is kept in the development glass tank which has mobile phase. The lower end above the surface of the solvent front has reached a suitable height. The glass plate is removed and dried in room temperature. The spot is identified in UV light at 365nm.

Rf Value

The Rf Value is calculated by the formula,

$$\text{Rf value} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

ISOLATION OF ACTIVE PRICIPLE BY COLUMN CHROMATOGRAPHY [79]

The isolation of active principle from the methanolic extract of Artemisia nilagirica by Column chromatography. The column chromatography was first developed by the American petroleum chemist D.T.Day in 1900, M.S.Tswett and the Polish botanist in1906.

Principle

The rate of adsorption varies with a given adsorbent for different materials. This is the principle of selective adsorption. The mixture to be separated is dissolved in a suitable solvent and allowed to pass through a tube containing the adsorbent. The component which has greater affinity is adsorbed in the upper part of the column and the component which has lesser affinity is adsorbed in the lower part of the column.

Materials Required

Column

Silica gel (230-400mesh)

Cotton

Ethyl acetate AR grade

Hexane AR grade

Methanol AR grade

Precoated TLC plates

Procedure

Preparation of the column

The glass column was placed in a ring stand in a vertical position. A plug of glass wool was pushed down to the bottom of the column. 22 g of silica gel was dissolved in approximately 50 ml of hexane to make a slurry. Slurry of silica gel

(230-400 mesh, column chromatography grade) was prepared with a suitable solvent and poured gently into the column. The stop cock was opened and some solvent was allowed to drain out. The layer of solvent always covered the adsorbent to avoid development of cracks in the column.

Pre-elution of the column

Hexane was added to the top of the silica gel. The solvent level was monitored, both as it was flowing through the silica gel and the level at the top. When the bottom solvent level was at the bottom of the column, the pre-elution process was completed and the column was ready to load.

Sample loading

The sample to be isolated was dissolved in a small amount of solvent, Hexane and loaded into the column. Once the sample was in the column, fresh eluting solvent was added to the top and hence the column was ready to begin the elution process.

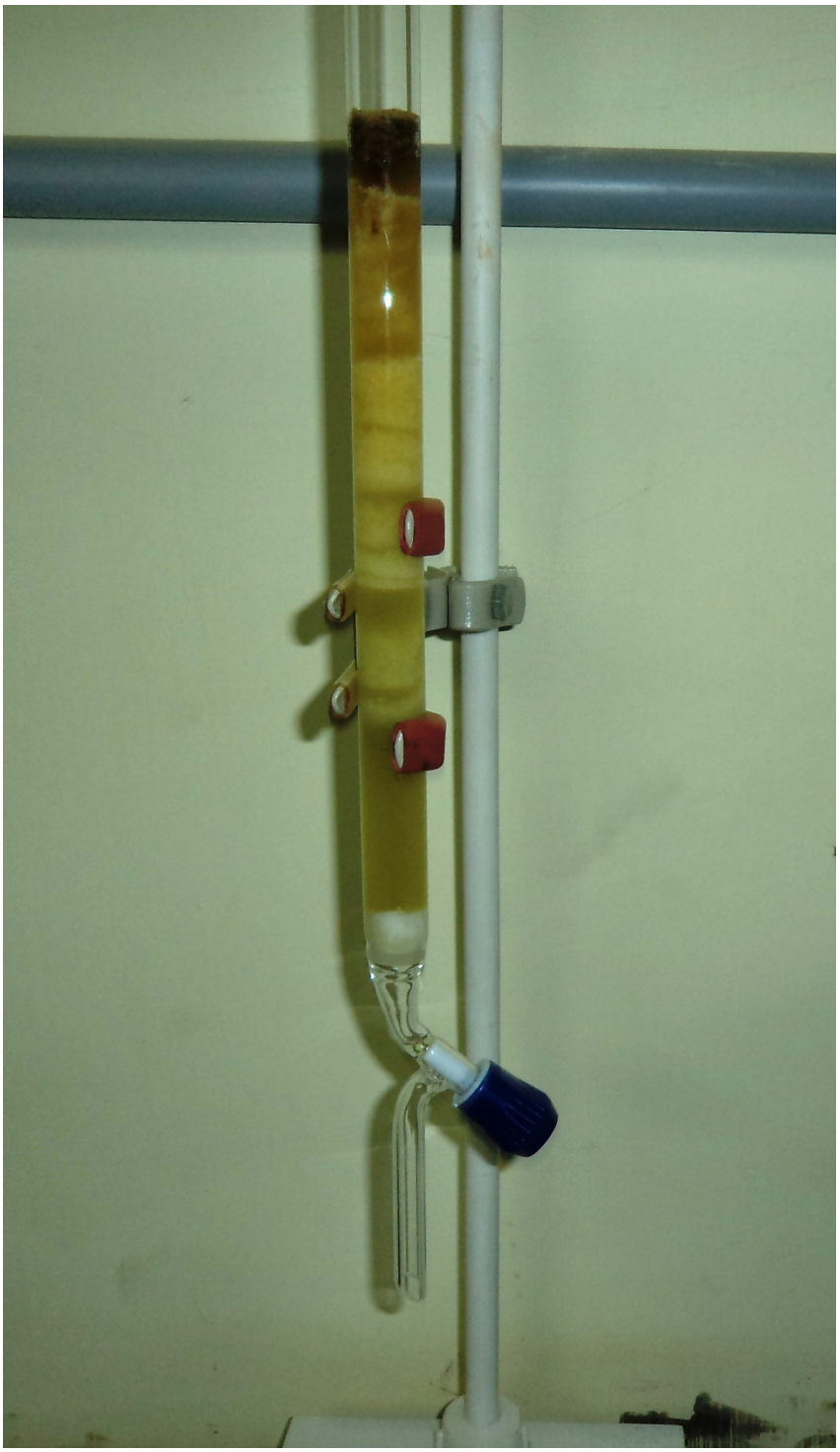
Elution of the column

The solvent Hexane was allowed to flow through the column and fresh solvent was added as necessary. The coloured bands travelled down the column as the compound was being eluted. As soon as the coloured compound began to elute, the collection beaker was changed.

Elution of the column with the second elution solvent

As a mixture of compounds was being separated, the eluting solvent was changed to a more polar system by using an 80:20 mixture of Hexane and Ethyl acetate. All the collected fractions were subjected to analysis

Fig. 1. Column Chromatography



FRACTIONS	ELUTES	NATURE OF RESIDUE	No. OF SPOTS
1-4	Hexane 100ml	No residue	-
5-10	Hexane: pet ether 90:10	Green brown	3
9-14	Hexane: Pet ether 80:20	Green brown	2
5-18	Hexane: pet ether 70:30	Brown	2

STRUCTURAL ELUCIDATION OF ACTIVE PRINCIPLE

The structural elucidations of the active principle were performed by spectroscopical studies (UV, FTIR&NMR).The spectroscopic studies are based on the electromagnetic spectrum.

ULTRA VIOLET ESTIMATION [71,72]

UV spectroscopy is the study of absorption of Ultra violet radiation which ranges from 200nm to 400nm. It is based on Beer-Lamberts law.

Principle

The bonding and non bonding electrons absorb the radiation and undergo transition from ground state to excited state. By the characteristic absorption peaks, the nature of the electrons present and the molecular structure can be elucidated.

Instrument

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure

The methanolic extract of Artemisia nilagirica 1mg/ml concentration was taken in the test tube. The wavelength was set as 360nm in a spectrophotometer. It

was adjusted to auto zero by methanol. The sample solution taken in a cuvette and placed in the UV and connect UV probe for observing peak.

FOURIER TRANSFORM INFRARED SPECTROSCOPY [73]

Infrared spectroscopy is one of the most powerful analytical techniques which offers possibility of structure elucidation and functional group determination. This technique coupled with intensity measurements is used for quantitative analysis

Range of Infrared radiation

- The photographic region-visible to 1.2μ
- The very Near infrared region-1.2 to2.5μ
- The near infrared region-25 to 25μ
- The far infrared region-25 to 300-400.

Principle [74]

The atoms or groups of atoms are connected by bonds. These bonds are analogous to springs and not rigid in nature. It maintains some vibrations with some frequency, characteristic to every portion of the molecule, because of the continuous motion of the molecule. This is called the natural frequency of vibration.

If, Applied infrared frequency = Natural frequency of vibration,

Absorption of IR radiation takes place and a peak is observed.

Instrument

Alpha T Bruker optics FTIR spectrophotometer.

Procedure[75]

1/8” solid sample was taken in a micro spatula with about0.25-0.50 mg ofKBr, It was mixed and grinded thoroughly with mortar and pestle. Sample was kept in a pellet press. It was pressed at 5000-10000 psi. The sample was carefully removed

from the die and placed in the sample holder of FTIR. Then the instrument was connected to the computer and the spectrum was run for observing peaks.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance is a branch of spectroscopy in which radio frequency waves induce transitions between magnetic energy levels of nuclei of a molecule. The first observations of NMR signals were observed by Purcell and Bloch in 1945. NMR is a technique that enables us to study the shape and structure of molecules.

Principle [76]

Any proton or nucleus with odd mass number spins on its own axis. When energy in the form of Radiofrequency is applied and when,

Applied frequency = Precessional frequency

Absorption of energy occurs and a NMR signal is recorded. Application of magnetic field and radio frequency is necessary to cause a NMR Spectra.

Instrument

1H NMR Bruker Advance III 500MHz multinuclear FT-NMR Spectrometer instrument at CLRI was used for NMR analysis.

Procedure for 13C NMR[77]

The no. of transients for a ^{13}C experiment is higher than that of a regular proton NMR. A minimum of 128 transients to obtain a good spectrum. Line broadening was used to obtain clear spectrum .Then the sample was placed for acquisition .Then the spectrum was run for the observing peaks.

Procedure for proton NMR

This important and well established application of nuclear magnetic resonance will serve to illustrate some of the novel aspects of this method. To begin with , the NMR spectrometer must be tuned to a specific nucleus, in this case of proton .The actual procedure for obtaining the spectrum varies,but the simplest is referred to as the continuous wave (CW) method. A typical CW-spectrometer is shown in the diagram. A solution of the sample in a uniform 5mm glass tube is oriented between the poles of a powerful magnet, and is spun to average any magnetic field variations ,as well as tube imperfections .Radio frequency radiation of appropriate energy is broadcast into the sample from an antenna coil (colored red) .A receiver coil surrounds the sample tube , and emission of absorbed RF energy is monitored by dedicated electronic devices and a computer. An NMR spectrum is acquired by varying or sweeping the magnetic field over a small range while observing the RF signal from the sample. An equally effective technique is to vary the frequency of the RF radiation while holding the external field constant.

HIGH PERFORMANCE (PRESSURE) LIQUID CHROMATOGRAPHY

HPLC is the speed at which separations take place. The diffusion of the column is reduced and resolution is improved, because of the decrease in time. This technique has improved performance when compared to column chromatography.

Principle [78,80]

The principle of separation in HPLC is adsorption. The resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length. The numbers of theoretical plates are related to the surface area

of the stationary phase. The better resolution is formed by the smaller particle size of the stationary phase.

Procedure[81]

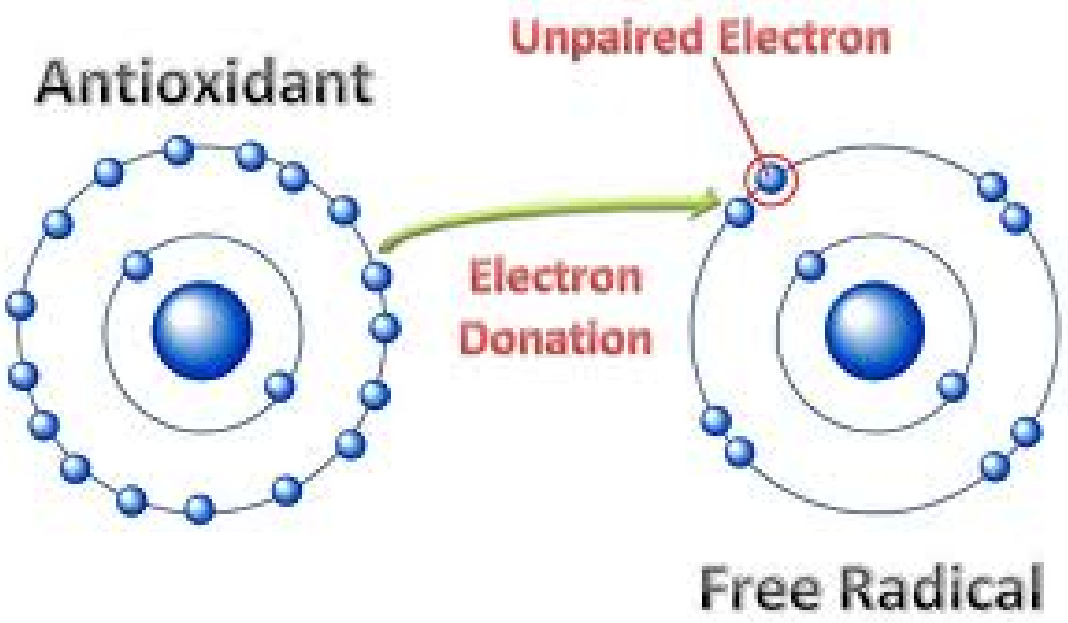
The sufficient solvent from the reservoir ensure to run was checked. The screw on the right hand side of the RCM was used to pressurize the column to 800psi. the solvent selector on the inlet manifold at the front of the pump was switched for running the solvent. The power to the pump was switched on and slowly increases the flow rate. The UV detector was switched on and set as zero .The Data moduler was switched on to set the date and time. The injector lever was switched on. The needle was wiped with tissue paper and sample was injected to the injector and at the same time press "inject" on the data module to start the data collection. The data module plots a real time chromatogram, and at the end of the run time (15 min) replots the chromatogram with details of retention time (RT), peak area (A or H) and relative areas of the peaks (conc). The flow is stopped slowly. The solvent selector was changed to flushing solvent and shut down.

Pharmacological activities

PHARMACOLGICAL ACTIVITIES

INVITRO ANTIOXIDANT ACTIVITIES [82]

A variety of in-vitro chemical methods are being used to determine the antioxidant activity of products and ingredients, but questions regarding whether the results have any bearing on effectiveness in the human body are leading to development of additional methods that may be more appropriate for screening potential antioxidant ingredients.



DPPH RADICAL SCAVENGING ACTIVITY[83]

Principle

The DPPH assay is the measurement of the free radical scavenging activity. DPPH molecules are stable free radical that contain unpaired electron. According to Blois et al method, the quantitative estimation of free radical scavenging activity is determined. When the solution of DPPH is mixed with a substance that can donate a hydrogen atom , it is converted to its reduced form The result was expressed in IC_{50}

Materials required

1.Methanolic extract of Artemisia nilagirica

2.2,2-diphenyl-1-picryl hydrazyl (DPPH) assay

3.95% Ethanol

4.Ascorbic acid

Instrument

Shimadzu UV Visible spectrophotometer, model 1800

Procedure

The stock solution of DPPH was prepared in ethanol (4mg/100ml).To 1ml of test samples of different concentrations 4ml of DPPH was added. Control without test compound was prepared in an identical manner. Blank was prepared in the similar way, where DPPH was replaced by ethanol. The reaction was allowed to completed in the dark for about 30 minutes.Then the absorbance of test mixtures was read at 517 nm. The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical Ascorbic acid was used as standard.

The percentage scavenging was calculated using the formula

% inhibition $=(A_{control}- A_{sample})/A_{control}\times 100$

The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using linear regression analysis. The results obtained were tabulated.

HYDROGEN PEROXIDE METHOD[84]

Principle

The radical scavenging activity of plant extract against hydrogen peroxide was determined by using the method of Janani et al. The principle is based on the

capacity of the extracts to decompose the hydrogen peroxide to water.H_2O_2 in the presence of O_2 - can generate highly reactive OH hydroxyl radicals via the metal, the scavenging of hydrogen peroxide in cells is to avoid oxidative damage. Thus the scavenging of hydrogen peroxide is an important antioxidant defence mechanism.

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

The decomposition of hydrogen peroxide to water involves the transfer of electrons as in equation

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

Reagents

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

0.1M,pH7.4 phosphate buffer

Instrument

Shimadzu UV Visible spectrophotometer, model 1800

Procedure

To 1ml of test solutions at different concentrations, 3.8ml of 0.1M phosphate buffer solution(pH 7.4) was added and then mixed with 0.2ml of hydrogen peroxide solution. The absorbance of the reaction mixture was measured at 230nm after 10 minutes .The reaction mixture without sample was used as blank .Sample blank was also prepared without reagents. Ascorbic acid was used as standard. The% inhibition of hydrogen peroxide was calculated using the formula

$$\% \text{ inhibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

The concentration of the sample required 50% reduction in absorbance (IC_{50}) was calculated using regression analysis

ANTIOXIDANT ACTIVITY USING COD LIVER OIL [85,86]

The in-vitro antioxidant activity using cod liver oil was determined by the method of Lee et al, 2002.According to Diplock 1997, the antioxidant action may be attributed to various mechanisms, some of which may be prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging action.

Principle

Antilipid peroxidation activity was evaluated using cod liver oil as substrate and effects of methanolic extract of Artemisia nilagirica were found to be dose dependent. When the ferrous ion is added, it induces the lipid peroxidation in oil. Per oxidation induction by ferrous ions takes place by the formation of ferry -per ferryl complex through Fenton's reaction

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

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + .OOH + H^+$

Hydroxyl radicals were generated by Fenton's reaction using an ammonium thiocyanate assay system.

Reagents

Cod liver oil

Ammonium thiocyanate

50 m M Phosphate buffer 7.4

70% ethanol

Ammonium thiocyanate

Ferrous chloride

Methanolic extract of Artemisia nilagirica

Procedure

The antioxidant activity of methanolic extract of Artemisia nilagirica against lipid per oxidation was measured by ammonium thiocyanate assay. The sample solution was 400µl of methanolic extract of A.nilagirica (6µg to 10µg) ,200 µl of diluted cod liver oil(25mg/ml of 99% Ethanol), 400µl of 50m M phosphate buffer (pH7.4) incubated for 15 minutes at 40^0C . From this solution 100µl of aliquot was mixed with 3ml of 70% ethanol, 100µl of ammonium thiocyanate (300mg/ml in distilled water) and 100µl of ferrous chloride (2.4 mg/ml in 3.5%HCl) solution is incubated at room temperature for 3 minutes. Absorbance is measured at 500nm. Control was measured without sample.

The % inhibition was calculated by

% inhibition = $(A_{control} - A_{Sample})/A_{control}X$ 100

The concentration of the sample required for 50% reduction in absorbance (IC_{50}) was calculated using regression analysis.

INVIVO ANTIOXIDANT ACTIVITIES

LIPID PEROXIDATION[87]

Lipid peroxidation can be defined as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds Lipid peroxidation is a well established mechanism of cellular injury in cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a compounds including reactive carbonyl compounds. A large number of toxic products formed during lipid peroxidation. These have effect on site away from area of their generation. Hence they behave as a toxic ‘second messengers’. Membrane lipids are particularly susceptible to lipid peroxidation. since

membrane from the basis of many cellular organelles like mitochondria, plasma membranes, endoplasmic reticulum, lysosomes, peroxisomes, etc., damage caused by the LP is highly detrimental to functioning of the cell and its survival.

Principle

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and isused as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsatured fatty acid peroxides generate malondialdehyde (MDA) upon decomposition. The measurement of MDA is used as an indicator of lipid peroxidation. The lipid peroxidation assay is based on the reaction of a chromogenic reagent,N-methyl-2-phenylindole with MDA at 450C.One molecule of either MDA reacts with 2 molecules of reagent to yield a stable chromophore with maximal absorbance at525 nm.

Procedure

Separated liver tissues were weighed and homogenized in ice-cold 0.01M Tris-HCl (pH 7.4), and then centrifuged, at 12,000 g for 15 min as described by Kavutcu et al. [115]. The concentrations of liver LPO were determined by estimating malondialdehyde (MDA) using the thiobarbituric acid test at absorbance 525 nm, as nM of MDA/mg protein [116].

GSH ASSAY[88-93]

Glutathione (GSH) is a tripeptide (y – glutamylcysteinylglycine) widely distributed in both plants and animals. GSH serves as a nucleophile co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydro peroxides. GSH involved in amino acid transport and maintenance of protein sulfhydryl reduction status. Concentration of GSH ranges from a few micromolar in plasma to several millimolar

Principle

The Glutathione Reductase Assay Kit enables the spectrophotometric measurement of glutathione reductase activity by the increase in absorbance caused by the reduction of DTNB [5,5¢-dithiobis(2-nitrobenzoic acid)] at 412 nm (Colorimetric assay) .This assay is based on the reduction of glutathione (GSSG) by NADPH in the presence of glutathione reductase. In addition, 5, 5¢-dithiobis(2-nitrobenzoic acid) [DTNB] reacts with the reduced glutathione (GSH) formed:

GR

NADPH + H+ GSSG → NADP + 2 GSH

The reduced glutathione can then spontaneously react with DTNB:

GSH + DTNB → GS-TNB + TNB

TNB = 5-thio(2-nitrobenzoic acid)

The reaction is measured by the increase in absorbance at 412 nm using an extinction coefficient (emM) of 14.15 for TNB.

Colorimetric assay - One unit will cause the reduction of 1.0 mmole of DTNB to TNB at 25 °C at pH 7.5.

The concentration of enzyme can be calculated using the formula:

units/ml = (DAsample - DAblank) x (dilution factor)/

εmM x (volume of sample in ml)

For TNB6

εmM = 14.15 mM-1cm

Assay Procedure

1. Assay Buffer and 2 mM Oxidized Glutathione Solution at 25 °C for at least 10 minutes was equilibrated before starting the assay.

2. The kinetic program in the spectrophotometer wassetted. The following programs are recommended:

Colorimetric assay

Wavelength: 412 nm

Initial delay: 60 seconds

Interval: 10 seconds

Number of readings: 11

3. The spectrophotometer with a cuvette filled with water was made autozero.

4. The following solution volumes in a 1 ml quartz cuvette in the order shown in table were placed.

S.No.	SOLUTIONS	COLORIMETRIC ASSAY
1	2 mM Oxidized Glutathione	500 µl
2	Assay Buffer	50-150 µl
3	Sample	0-100µl
4	3 mM DTNB	250 µl
5	2 mM NADPH	50 µl
	Total volume	1.00 ml

For both (UV and Colorimetric) assays perform a positive control by adding 10 to 20 ml of the Glutathione Reductase Positive Control Solution per control reaction 2 mM NADPH Solution - Dissolve a portion of the b-Nicotinamide adenine dinucleotide phosphate, reduced (NADPH) at 1.85 mg/ml in Assay Buffer to prepare a working solution of 2 mM. Store at 4 °C. Prepare the NADPH solution fresh every day

5. Liver tissue homogenates were mixed with 0.1mL of 25% trichloroacetic acid (Merck, CA, USA), and then centrifuged at 4,200 rpm for 40 min at 4◦C. Glutathione (GSH) contents were measured at absorbance 412nm using 2-nitrobenzoic acid

6. The reaction with Assay Buffer instead of enzyme sample solution was run as a blank.

7. The amount of enzyme in the sample was calculated

SUPER OXIDE DISMUTASE ASSAY

Principle[94]

The SOD activity is based on inhibition of nitrobluetetrazolium(NBT) reduction. Illumination of riboflavin in the presence of O2 and electro donor like methionine generates superoxide anions and this has been used as a basis of SOD assay. The reduction of NBT by superoxide radicals to blue colouredformazan was followed at 560nm .

One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under specified conditions.

$$2O2- + 2H+ \rightarrow O2+H2O2$$

Calculation

Units/mg=1000/µg enzyme resulting in ½ maximum inhibition

Reagents

- 0.067 M Potassium phosphate buffer, pH 7.8
- M Ethylene diaminetetraacetic acid (EDTA) containing 0.3 mM sodium cyanide
- 0.12 mM Riboflavin (store cold in a dark bottle)
- 1.5 mMNitrobluetetrazolium (NBT) (store cold)

Procedure

A series of samples ranging from 0.1-10 micrograms is recommended. A tube containing approximately 100 micrograms will generally produce maximum inhibition. the tubes in a light box providing uniform light intensity were placed (A foil-lined box approximately 4' long X 8" X 6" with an internally mounted 40 W fluorescent bulb has been used successfully). The tubes were incubated for 5-8 minutes to achieve a standard temperature. The0.05 ml riboflavin was added atzero time and at time intervals. All tubes were incubated in the light box for 12 minutes and at timed intervals read A560. The percent inhibition of NBT reduction was determined. SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitrotetrazolium blue to form formazan dye. SOD activity was then measured at 560nm by the degree of inhibition of this reaction, and was expressed as U/mg protein.

CATALASE ACTIVITY

Principle

Decomposition of H2O2 in the presence of catalase was followed at 240nm [24]. Catalase activity was defined as the amount of enzyme required to decompose

1nM of H2O2 per minute, at 25◦C and pH 7.8. Results were expressed as U/mg protein.

Calculation

Units/mg =ΔA_{240}/min X 1000/ 43.6 Xmg enzyme /ml reaction mixture

Reagents

- 0.05 M Potassium phosphate, pH 7.0
- 0.059 M Hydrogen peroxide (30%) in 0.05 M potassium phosphate, pH 7.0

Procedure

- The spectrophotometer was adjusted to 240 nm and 25°C.
- The following reagents were pipetted

Reagent grade water	1.9 ml
0.059 M Hydrogen peroxide	1.0 ml

It was incubated in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. The ΔA240/min from the initial (45 second) linear portion of the curve was calculated

ANTITHYROID ACTIVITY

Thyroid hormones play an important role in development, metabolism, thermoregulation, and growth [96]. However, under several pathological conditions like Graves' disease, tumors of thyroid and pituitary gland stimulate thyroid cells to produce more hormones, which results in a hyperthyroid state [97]. Alterations in the level of these hormones lead not only to altered basal metabolic rate but also to many

health problems. Particularly, hyperthyroidism, if not treated properly, sometimes ends up with the common health problems such as diabetes mellitus and cardiovascular diseases [98]. Resemble to the human hyperthyroidisms were easily achieved in rodents by continuous treatment of l-thyroxine (LT4), a synthetic form of thyroid hormone [97,98] .Hyperthyroidism leads to oxidative damage of liver [99], osteoporosis [100], heart failure [101] and increased risk of heart attack [102]

1.**Materials and Methods**

Animals and Experimental Design.

Adult Female Sprague-Dawely rats (6-wk old) weighing 190–240 g were used in the experiments after allowing 28 days acclimatization. The animals were allocated four per polycarbonate cage in a temperature (20–25◦C) and humidity (40–45%) controlled room. The light : dark cycle was 12 hr : 12 hr and normal rodent pellet diet and water were supplied during acclimatization, free to access. After acclimatization, hyperthyroidism was achieved by daily subcutaneous injection of LT4 (Sigma, MO, USA) at a dose of 0.3mg/kg for 12 consecutive days according to the previous established method [98], and animals were randomly divided into 5 groups of 6 rats each after 12th LT4 treatment; intact control, LT4 control, PTU (Sigma, MO, USA) 10 mg/kg, MEAN extracts 150 and 250mg/kg treated groups. PTU 10 mg/kg was also selected based on the previous in vivo efficacy test on the LT4-induced hyperthyroidisms in rodents [97,98]. MEAN extract was orally administered once a day for 15 days from 12th LT4 treatment, in a volume of 5 mL/kg, dissolved in 1% CMC, and PTU was intraperitoneally injected, in a volume of 5 mL/kg, dissolved in saline. All animals were overnight fasted before first LT4 and test material treatment with sacrifice. Equal volume of saline was subcutaneously

treated in intact control rats instead of LT4, and equal volume of distilled water was orally administered in intact and LT4 control rats, instead of MEAN extracts or PTU.

ESTIMATION OF TSH, T3 AND T4 BY RADIOIMMUNOASSAY

Hyperthyroid state is accompanied with an increase in pro oxidant to antioxidants ratio, that promotes accumulation of oxidatively damaged molecules which leads to oxidative stress [103]. Among them liver is a major target organ for thyroid hormone with important biological and medical implications [104], and the atrophic changes and decreases of body fat masses were accompanied with bodyweight decreases [105–106]. These hyperthyroidisms with relative organ damages have been ameliorated by treatment of various antioxidants [97, 103 108, 109].

Propylthiouracil (PTU) is a thioamide drug used to treat hyperthyroidism by decreasing the amount of thyroid hormone produced by the thyroid gland [110], and it also inhibited the enzyme deiodinase, which converts thyroxine (T4) to the active form triiodothyronine (T3) [111].Therefore, PTU has been selected as a potential reference drug for developing a new agent to treat hyperthyroidisms, and it potential inhibited LT4-induced hyperthyroidisms in rats and showed constant antioxidant effects at 10 mg/kg levels [3, 4]. However, the usages of PTU have been limited because of notable side effects include a risk of a granulocytosis in clinics [112].

Principle

This assay is based on the competition between TSH, T3, T4and (T3 analog bound to biotinylated carrier protein) for a limited number of binding sites on 125I-labelled monoclonal anti-triiodothyronine antibodies (tracer). Allowing to react a fixed amount of conjugate and antibody with different amounts of ligand the radioactivity measured on the solid phase will be inversely proportional to the

concentration of ligand. During a 2-hour incubation period with continuous agitation immuno-complex is immobilized on the reactive surface of test tubes. Decanting the supernatant from all tubes the radioactivity in tubes can be measured in a gamma counter.

The average counts per minute (CPM) for each pair of assay tubes was calculated the percent B0 / T for zero standard (S1) was calculated by using the following equation:

$$B0 / T \% = \frac{S1\ (cpm)}{T\ (cpm)} \times 100$$

Calculate the normalised percent binding for each standard, control and sample respectively by using the following equation:

$$B / B0 = \frac{S2\text{-}6\ [C, Sx]\ (cpm)}{S1\ (cpm)\ T\ (cpm)} \times 100$$

For simplicity, these values are uncorrected for non-specific binding (NSB). This is enabled by low NSB being less than 1.5% of total count (using magnetic separator).

Contents of the kit

1 vial 125I-TRACER(55ml), containing about 300 kBq 125I-labelled monoclonal antibody in buffer with 0.1% NaN3.

6 vials STANDARDS

0.5 ml per vial, containing 0 (S1), 2 (S2), 5 (S3), 10 (S4), 20 (S5) and 40 (S6) pmol/l FT3 in human serum with 0.1% NaN3

1 vial CONJUGATE(55ml),ready to use containing conjugate in buffer with 0.1% NaN3. Do not expose to direct sunlight.

1 vial CONTROLSERUM

Lyophilized human serum with 0.1%NaN3.The concentration of the control serum is specified in the quality certificate enclosed.

2boxes COATED TUBE, ready to use. 2 x 50 reactive test tubes, 12x75 mm, packed in plastic boxes.

Equipment required

Test tube rack

Precision pipettes with disposable tips (100 and 500 µl)

Vortex mixer

Centrifuge

Plastic foil

Absorbent tissue

Gamma counter

Preparation of reagents

Add 500 µl distilled water to the lyophilised control serum. Mix gently with shaking or vortexing (foaming should be avoided). Ensure that complete dissolution is achieved, and allow the solution to equilibrate at room temperature for at least 20 minutes.

Specimen collection and storage

Serum samples can be prepared according to common procedures used routinely in clinical laboratory practice. Sera can be stored at 2-8 °C for two days after collection. For later analysis they should be stored deep-frozen (-20 °C). Repeated freezing and thawing should be avoided

Procedure

6mL of blood samples were collected into evacuated tubes, and serum was separated by centrifugation at 3000 rpm for 10 min at 4◦C. Separated serum was

stored at −20◦C before analysis .Count each tube for at least 60 seconds in a gamma counter. The serum FT3,FT4, and FTSH were analysed by RIA KIT

ANTIHYPERGLYCEMIC ACTIVITY

Glucose oxidase method for serum glucose estimation

Principle

Glucose oxidase catalyses oxidation of glucose to gluconic acid. The formed hydrogen peroxide is detected by chromogenic oxygen acceptor phenolaminophenazone in the presence of peroxidase

α-D-glucose → β-D- glucose

β-D-glucose+H2O+O2→D-gluconic acid +H2O2

H2O2+4-amino phenazone+phenol→Quinonemine+4H2O

The intensity of the colour formed is proportional to the glucose concentration in the sample.

Materials and methods

Blood serum

Buffer phenolaminophenazone

Instrumentation

Shimadzu UV Visible spectrophotometer model 1800

Procedure

The wavelength is adjusted to 505nm at 370C. The instrument is set to auto zero. The sample, standard and control were incubated for 10 minutes at room temperature. The absorbance is readed.

Calculations

(A) sample/(A) standard X 100(Standard concentration)= mg/dl glucose in the sample.

Results and Discussions

RESULTS AND DISCUSSION

Morphological evaluation

Interpretations of its morphological characters based on organoleptic and wide morphological characters showed that the leaf arrangement is opposite to one another, lobed, laciniate or pinnatipartite and 5-14cm long. The flowers are many petaled which are small pale yellow and have a pleasant aroma. The fruits are achenes.

Microscopical evaluation

Microscopical evaluation helps magnifying the fine structure of minute objects and there by confirm the structural details of plant drug and thus offer supporting evidence when combined with other analytical parameters based upon their cell types and cell inclusion details etc.,

a) The leaf has thick midrib and thin lamina

b) The epidermis of the midrib is very thin and the cells are not much distinct.

c) The lateral vein is 320µm thick and 200µm wide

d) The lamina is bifacial and the adaxial epidermal cells are 20µm thick.

e) The petiole is 800µm thick and adaxial part is 500µm wide and abaxial part is 750µm wide. The petiole is lobed and the upper part is semicircular.

f) The stomata are anamocytic which are enclosed by dense epidermal trichomes.

g) The venation is reticulate, vein islets are wide and the terminations are thin, and wavy

h) The evaluation of powder microscopy showed two celled glandular trichomes, collenchymas cells and palisade mesophyll.

S.No.	Parameters	Values obtained
1.	Stomatal number in lower epidermis	59.8±0.416
2.	Stomatal index in lower epidermis	18.41±0.140
3.	Vein islet number	11.583±0.1332
4.	Vein termination number	14.120±0.1910
5.	Palisade ratioin upper epidermis	9.25±0.70

Mean of 10 readings ±SEM

Fluorescence analysis

The organic molecules absorb light usually over a specific range of wave length and many of them such radiations So if the powder is treated with different chemical reagents and seen under UV light, different colors was produced.

FLUORESCENCE ANALYSIS OF POWDER OF A. nilagirica Table2

S.No	Powder + analysis	Daylight	UV 254nm	UV365nm
1	Drug powder	Green	Green	Yellow
2	Drug powder+ aqueous 1 M NaOH	Green	Green	Green
3	Drug +alcoholic 1M NaOH	Green	Green	Yellow
4	Drug powder+ iodine	Green	Green	Green
5	Drug powder+ 10%KOH	Green	Green	Yellow
6	Drug powder+1MHCl	Green	Green	Green
7	Drug powder +glacial acetic acid	Green	Yellow	Yellow
8	Drug powder+ 50%H_2SO_4	Green	Green	Yellow
9	Drug powder+50%HNO_3	Green	Green	Yellow
10	Drug powder + 50% HCl	Green	Yellow	Yellow

Therefore it can be used for the identification of the drug .The fluorescent characteristic of the drug powder with different chemical reagent was studied by observing under UVlight 254and365 nm .The test and observations were recorded .It suggested that a non- fluorescent compound may fluorescent if it mixed with impurities . So the results obtained from the present fluorescent studies help to check any impurities present in leaf powder of Artemisia nilagirica.

Determination of moisture content

The loss on drying of plant materials should be determined and the water content should be controlled. This is especially important for materials that absorb the moisture easily and quickly deteriorate quickly in presence of water. The test determines both water and volatile matter.

Foreign organic matter

Drugs should be free from moulds, insects, animal faecal matter and other contaminations such as earth materials, stone and extraneous materials. The amount of foreign matter shall not be more the percentage prescribed in the monograph.

Determination of volatile oil

The fresh leaves of A.nilagiria were used for determination of volatile oil using Clevenger apparatus. It was calculated in ml and tabulated.

ASH VALUE

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

The total ash of a crude drug reflects the care taken in its preparation. The acid insoluble in dilute hydrochloric acid. A higher limit of acid insoluble ash is imposed; especially the calcium oxalate content of the drug is higher. Water soluble ash is the water soluble portion of the total ash. These ash values are important quantitative standards.

PHYSICAL PARAMETERS Table3

S.No	PARAMETERS	Values expressed as%
1	Volatile oil	0.5ml
2	Foreign organic matter	0.2 ±0.0001
3	Moisture content	0.891±0.0617
4	ASH VALUES	
a	Total ash	14.07±0.0052
b	Water soluble ash	6.87±0.0091
c	Acid insoluble ash	1.91±.0035

EXTRACTIVE VALUES

The extractive values were calculated for different extracts like methanol, ethanol, pet ether, aqueous, ethyl acetate and chloroform. Aqueous extract has higher value because of contamination methanol extract was preferred.

Table 4

S.No.	Parameters	Values expressed as %
1	**Extractive values**	
	Methanol extract	21.648±0.71234
	Ethanol extract	11.68±0.092376
	Pet ether extract	19.8933±1.2327
	Chloroform extract	11.12±1.1199
	Ethyl acetate extract	9.7066±1.6591
	Aqueous extract	31.0933±1.8511
	Foaming index	More than 100

PHYTOCHEMICAL SCREENING OF Artemisia nilagirica

The results obtained for investigation on phytochemical analysis of various extracts are presented in table. The methanolic extract showed the presence of a flavonoids, tannins, saponins and coumarins.None of the extract shows alkaloids, amino acids and glycosides

Table 5

S.No	TEST	ETHANOL	AQUEOUS	METHANOL	PET ETHER
1	Alkaloids	-	-	-	-
2	Amino acids	-	-	-	-
3	Carbohydrates	-	+	-	-
4	Flavonoids	+	+	+	+
5	Glycosides	-	-	-	-
6	Coumarins	+	+	+	+
7	Tannins	+	+	+	-
8	Saponins	+	+	+	-
9	Terpenoids	-	-	-	+
10	Volatile oil	-	+	-	+
11	Sterols	-	-	-	+

ESTIMATION OF PHENOLIC CONTENT

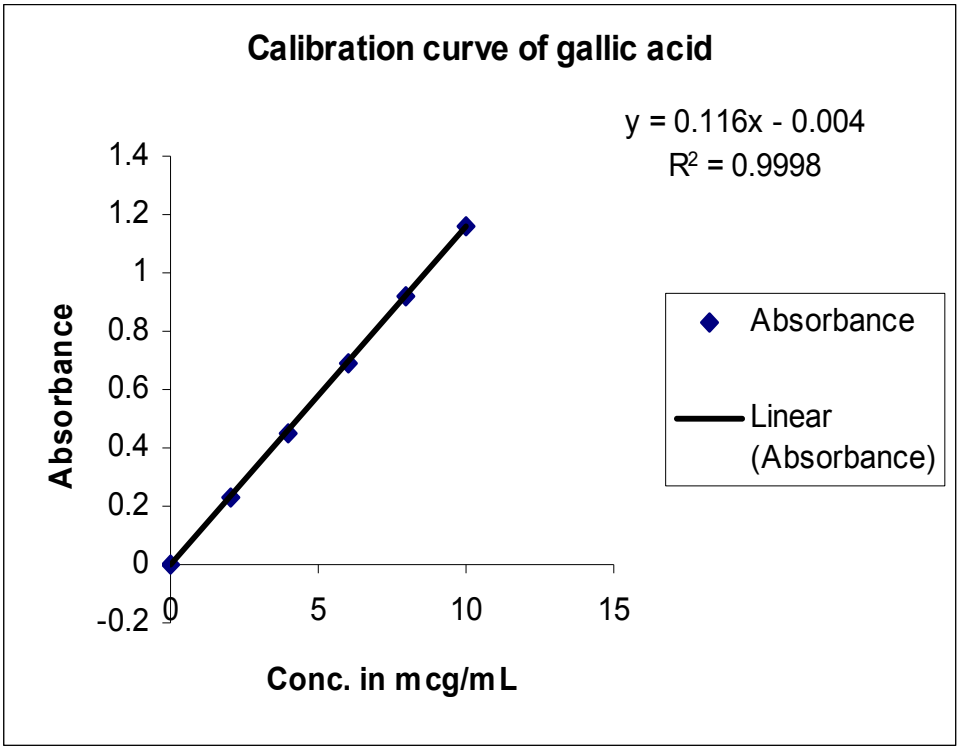
In the present study, total phenolic content in extract was estimated using modified Folinciocalteu method. Values are expressed as gallic acid equivalents and the results are tabulated. The total phenolic content for methanolic extract of Artemisia nilagirica were found to be117.4525mg/g of extract.

The linear regression equation was found to be y=0.116x-0.004 while the correlation was found to be 0.

998.The amount of phenolic content present in the extract were found to be by using above linear regression analysis

Table 6

S.No.	Conc.of gallic acid µg/ml	Absorbance at 760nm	Conc.of methanolic extract µg/ml	Absorbance at 760nm	Amount of total phenolic content in terms mg GAE/g of extract
1	20	0.229±0.010	50	0.694±0.099	108.746±1.216
2	40	0.452±0.006	100	1.190±0.0751	126.159 ±0.8160
3	60	0.695±0.005			
4	80	0.918±0.031			
5	100	1.162±0.028			
				Average	**117.45±1.016**



Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants.1-3 Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers.4 They have also metal chelation properties.

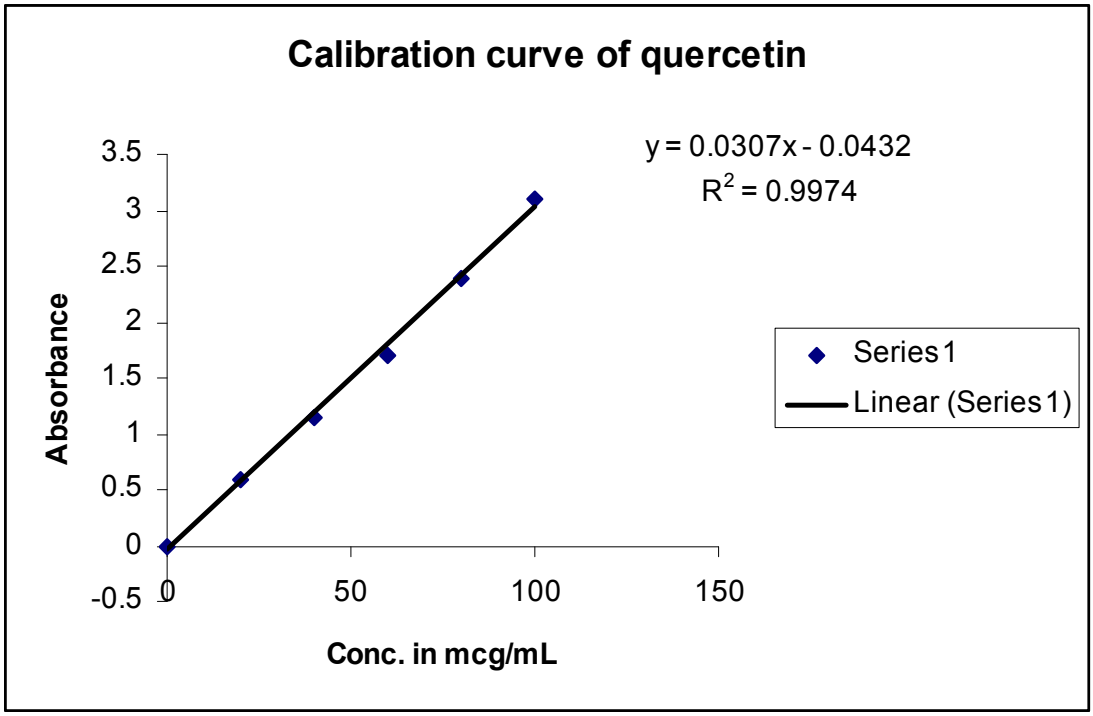
ESTIMATION OF FLAVONOID CONTENT

Flavonoids are a group of poly phenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. some evidence suggests that the biological actions of these compounds are related to their antioxidant activity.the total flavonoid content of methanolic extract was found to be 84.299 mg/g of extract.

Table 7

S.No.	Conc.of quercetin µg/ml	Absorbance at 415nm	Conc. of methanolic extract in µg/ml	Absorbance at 415nm	Amount of flavonoid content in terms mg quercetin equivalent/g of extract
1	20	0.589±0.01	50	0.0753±0.0021	77.22±00711
2	40	1.151±0.04	100	0.237±0.0093	91.378±0.3046
3	60	1.710±0.09			
4	80	2.390±0.03			
5	100	3.112±0.03			
				Average	**84.299±0.18**

the linear regression equation was found to y=0.0307x+ 0.0432 while the correlation was found to be 0.9974the amount of phenolic content present in the extract were found to be by using above linear regression 84.299±0.1878 mg/g of extract.



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Flavonoids are known for their free radical scavenging and anti- oxidant activities, the positions of the substituents also affect the physiological properties of different flavonoids. The flavonols having orto or para hydroxyl group in the 2- phenyl ring are known.

ESTIMATION OF TANNIN CONTENT

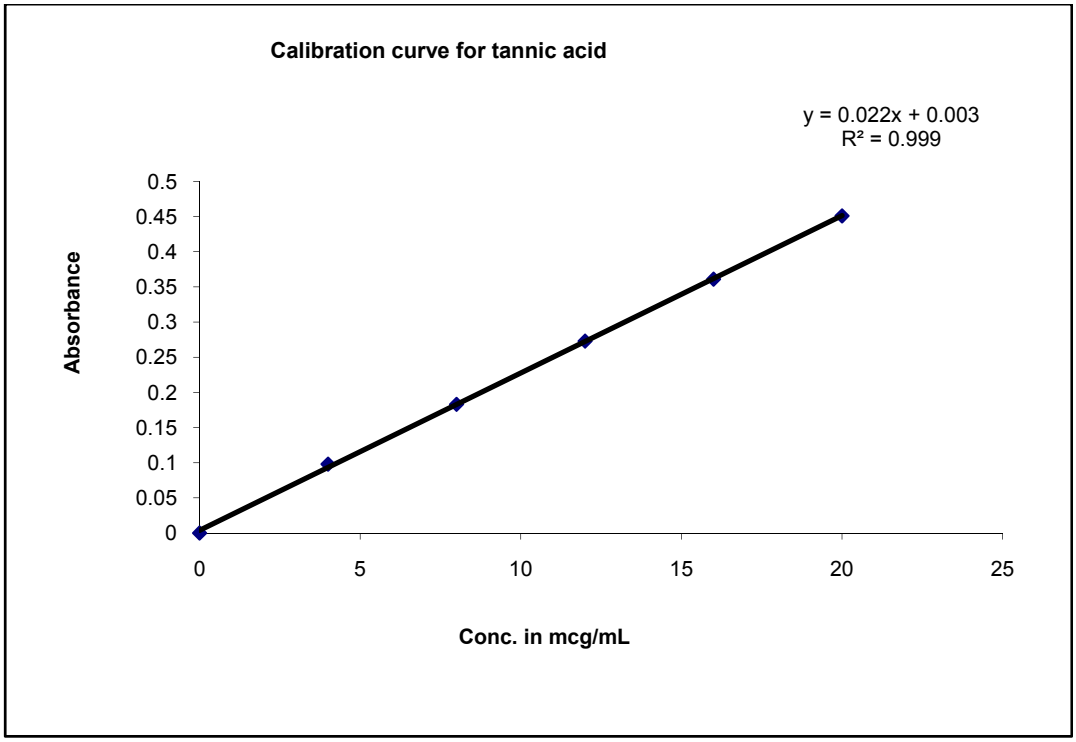
Total tannin determination was carried out by spectrophotometry after oxidation of the analyte with a theFolindenis reagent in alkaline medium. This method is based on the redox reactions and reducing agents in sample.

Total tannin content for methanolic extract of leaves of Artemisia nilagirica was found to be 757 .50 ±3.8420 mg of tannic acid equivalent/g of extract

The linear regression equation was found to be y=0.022x+0.003while the correlation was found to be 0.999. The amount of tannin content present in the methanolic extract of A.nilgiricawas found to be 757.50±3.8420mg/g

Table 8

S.No.	Conc. of tannic acid in µg/ml	Absorbance at 700nm	Conc. ofmethanolic extract in µg/ml	Absorbance at 700nm	Amount of tannin content in terms mg of tannic acid equivalent/g of extract
1	20	0.589±0.01	20	0.343±0.0860	757.50±3.8420
2	40	1.151±0.04			
3	60	1.710±0.09			
4	80	2.390±0.03			
5	100	3.112±0.03		**Average**	**757.50±3.8420**



Tannins are generally defined as naturally occurring polyphenolic compounds of high molecular weight to form complexes with the proteins. Tannins are important source of protein in animals but unfortunately the amounts of tannins that they contain vary widely and largely unpredictably, and their effects on animals range from beneficial to toxicity and death

THIN LAYER CHROMATOGRAPHY

SOLVENT SYSTEM

Ethyl acetate : methanol : water =100 : 6 : 1

Distance travelled by solute =7.5

Distance travelled by solvent=8.6

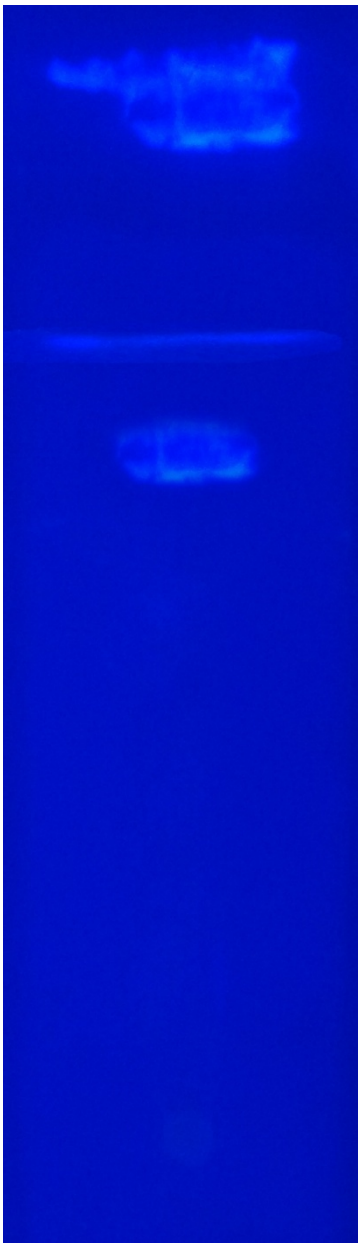
Rf = Distance travelled by the solute/Distance travelled by the solvent

= 7.5/8.6

= 0.8720

Rf value of scopoletin matches the sample of methanolic extract of A.nilagirica .It may be scopoletin

Fig. 15. Thin layer chromatography of MEAN



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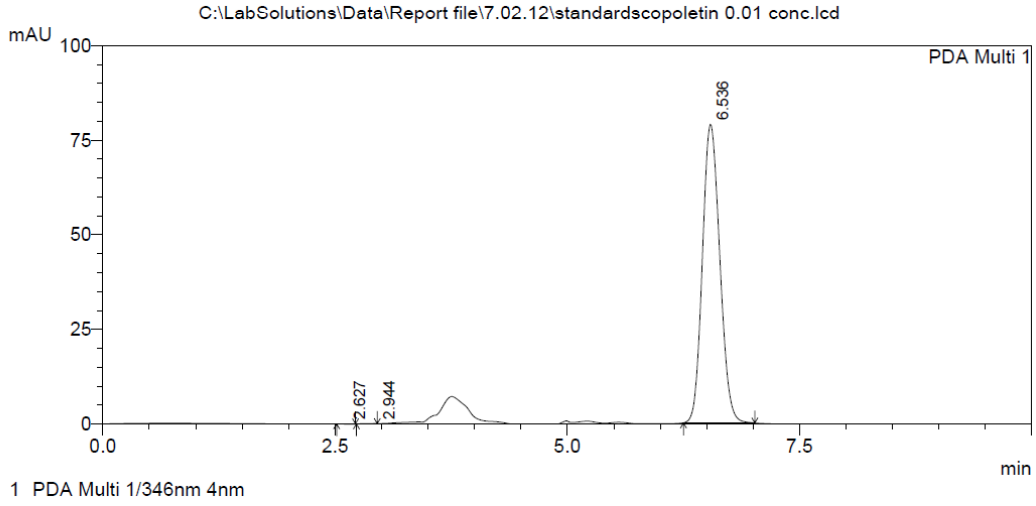

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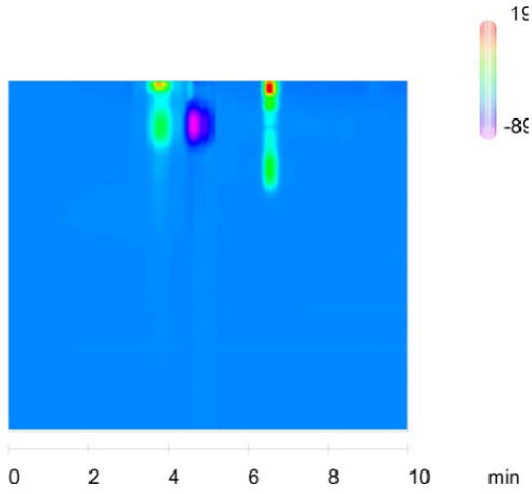
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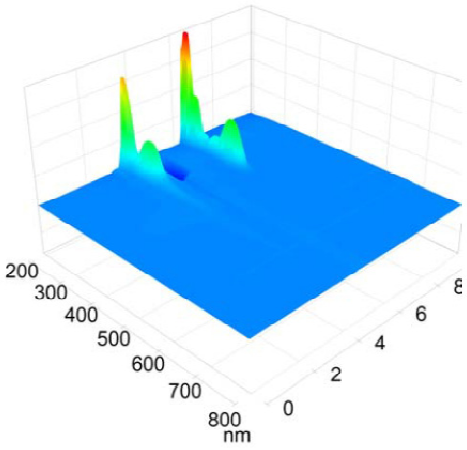
0
2
4
6
8
10
min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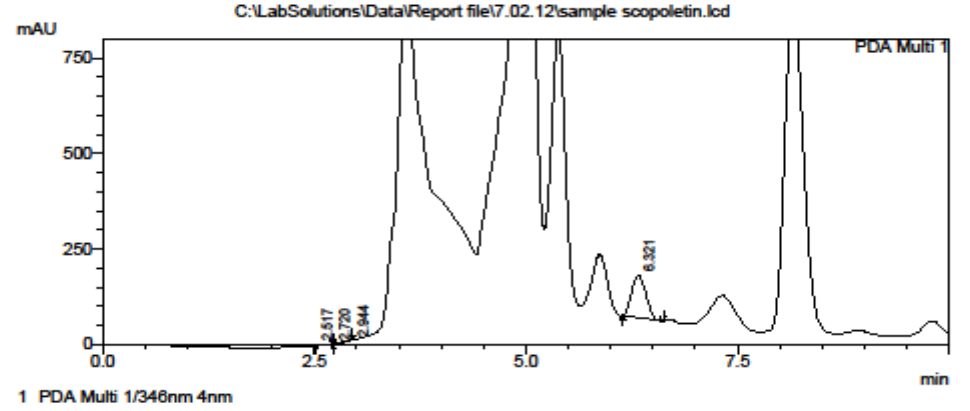

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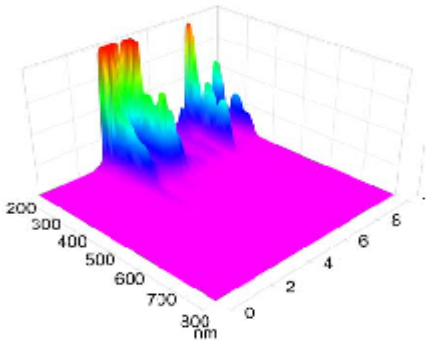

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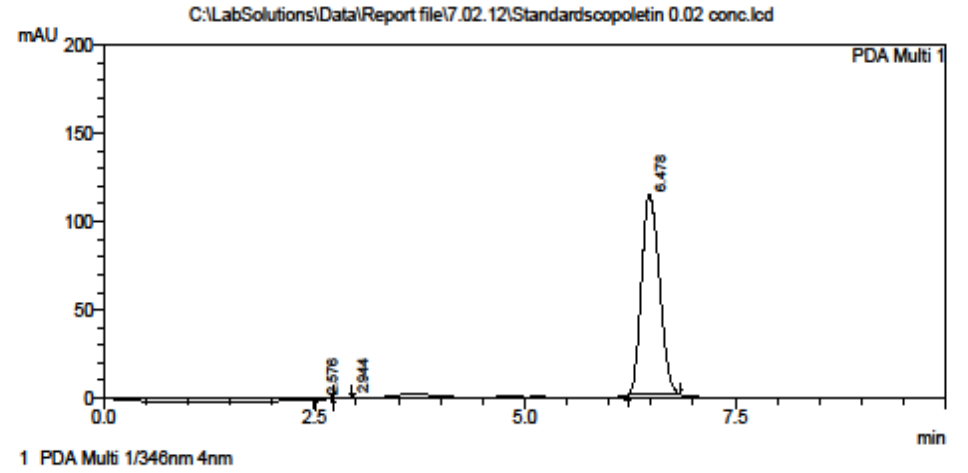

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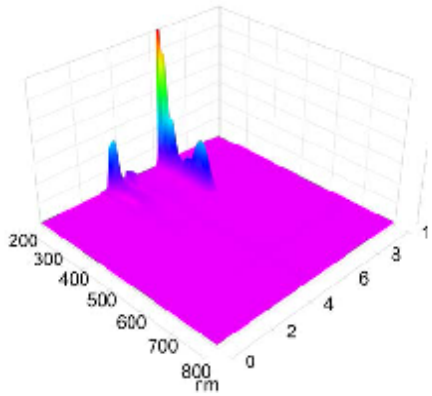

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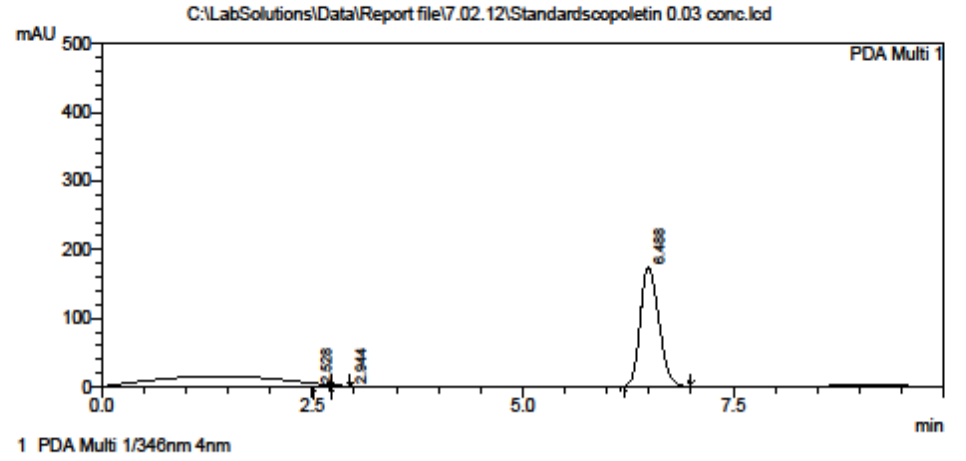

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3D Graph C:\...\Data\Report file\7.02.12\Standardscopoletin 0.(

2/7/2013 13:04:54 1 / 3

Periyar TBI HPLC Analysis Report

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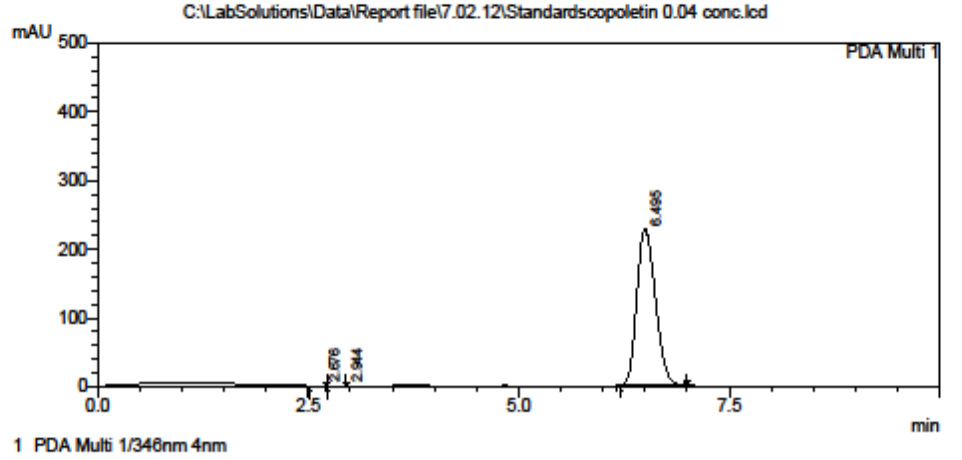

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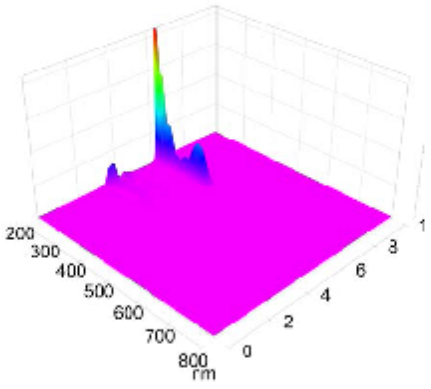

Periyar TBI HPLC Analysis Report

C:\LabSolutions\Data\Report file\7.02.12\standardscopoletin 0.05 conc.lcd

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Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.05 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
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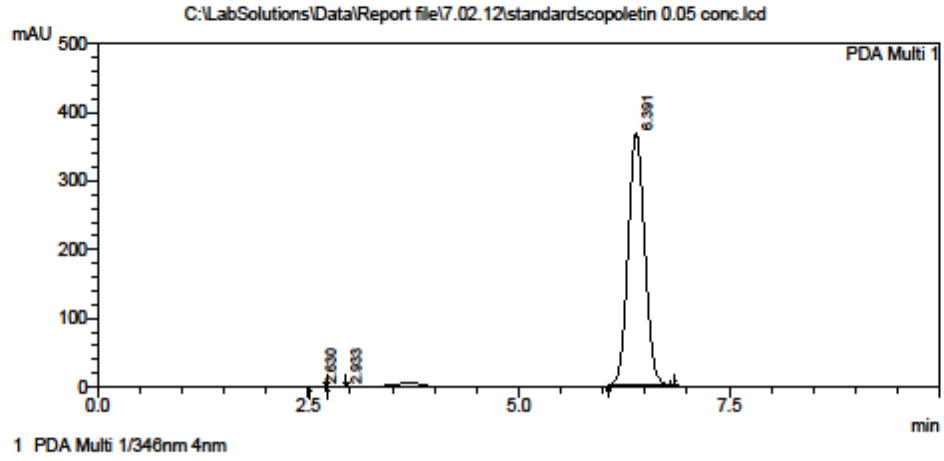

1 PDA Multi 1/346nm 4nm

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3D Graph C:\...\Data\Report file\7.02.12\standardscopoletin 0.(

Periyar TBI HPLC Analysis Report

C:\LabSolutions\Data\Report file\7.02.12\standardscopoletin 0.06 conc.lcd

Acquired by	: Admin
Sample Name	: standard scopoletin 0.06 conc
Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.06 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
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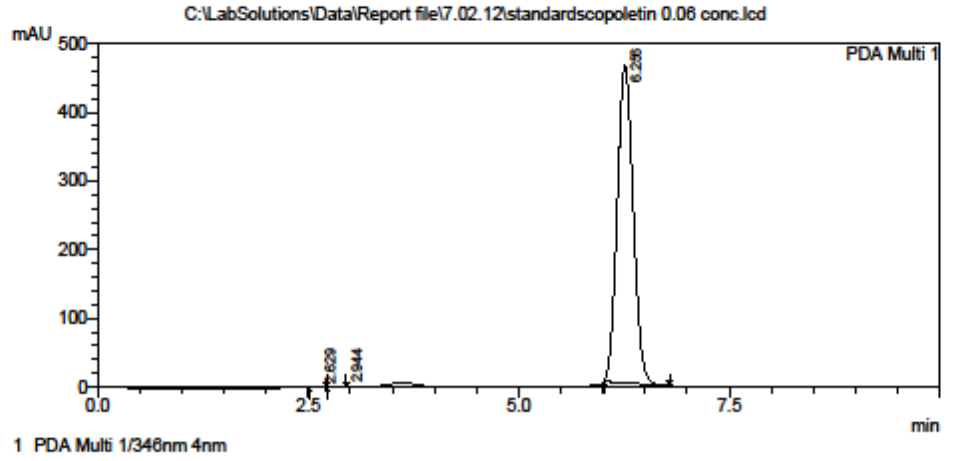

1 PDA Multi 1/346nm 4nm

C:\...\Data\Report file\7.02.12\standardscopoletin 0.06 conc.lcd

3D Graph C:\...\Data\Report file\7.02.12\standardscopoletin 0.(

2/7/2013 13:08:38 1 / 3

Periyar TBI HPLC Analysis Report

C:\LabSolutions\Data\Report file\7.02.12\standardscopoletin 0.07 conc.lcd

Acquired by	: Admin
Sample Name	: standard scopoletin 0.07 conc
Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.07 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
Data Acquired	: 2/7/2013 11:34:52 AM
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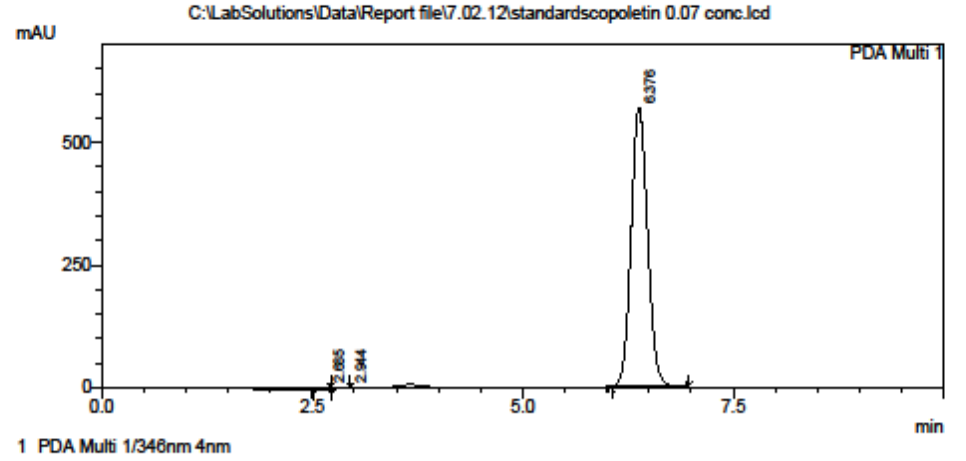

1 PDA Multi 1/346nm 4nm

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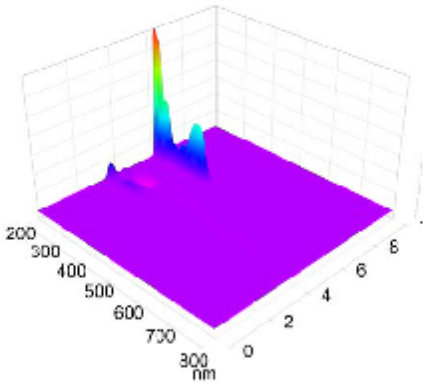

Periyar TBI HPLC Analysis Report

C:\LabSolutions\Data\Report file\7.02.12\standardscopoletin 0.08 conc.lcd

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Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.08 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
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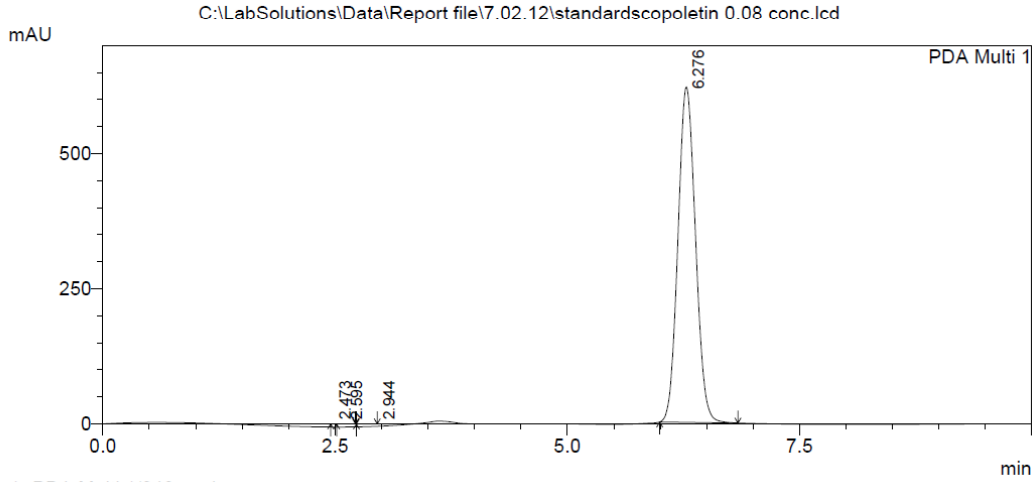

1 PDA Multi 1/346nm 4nm

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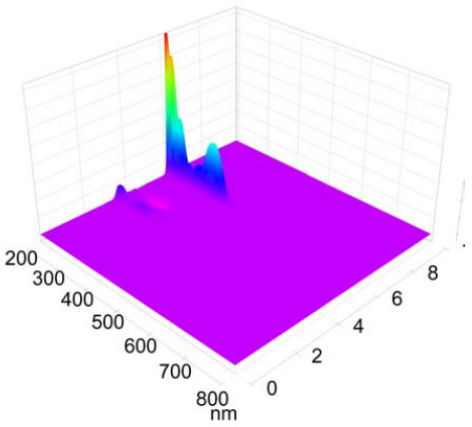
3D Graph C:\...\Data\Report file\7.02.12\standardscopoletin 0.08 c



Periyar TBI HPLC Analysis Report

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Acquired by	: Admin
Sample Name	: standard scopoletin 0.10 conc
Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.10 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
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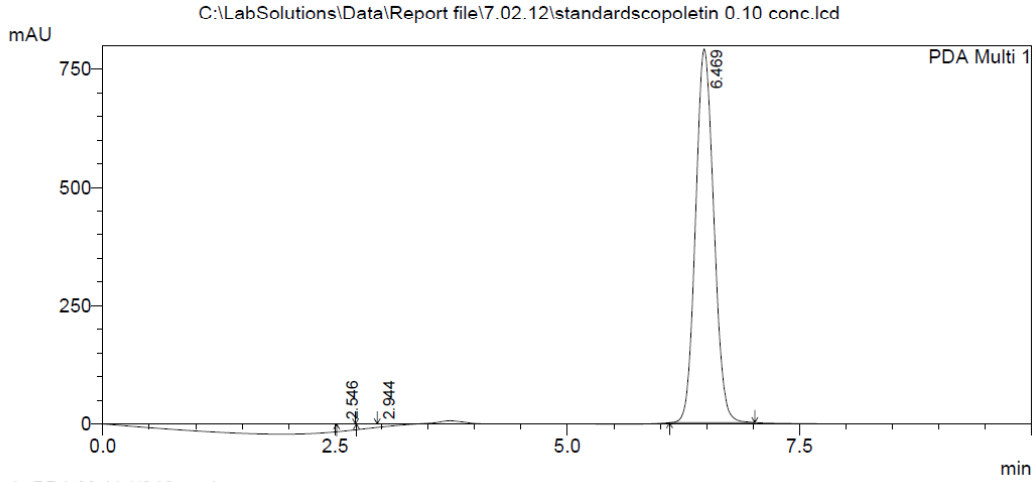
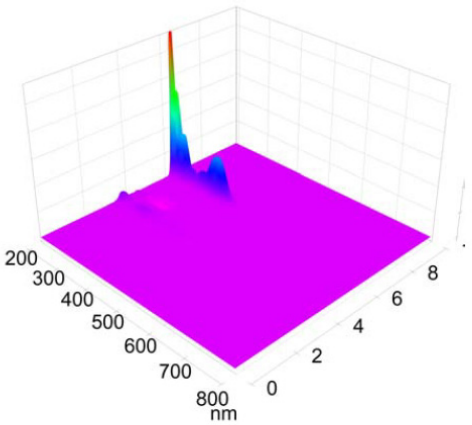


1 PDA Multi 1/346nm 4nm

C:\...\Data\Report file\7.02.12\standardscopoletin 0.10 conc.lcd

3D Graph C:\...\Data\Report file\7.02.12\standardscopoletin 0.10 c



S

2/7/2013 13:11:01 1 / 3

Periyar TBI HPLC Analysis Report

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Acquired by	: Admin
Sample Name	: standard scopoletin 0.09 conc
Sample ID	: standard scopoletin
Injection Volume	: 20 uL
Data File Name	: standardscopoletin 0.09 conc.lcd
Method File Name	: Scopoletin 01 02 2013-final.lcm
Report File Name	: Default.lcr
Data Acquired	: 2/7/2013 12:18:36 PM
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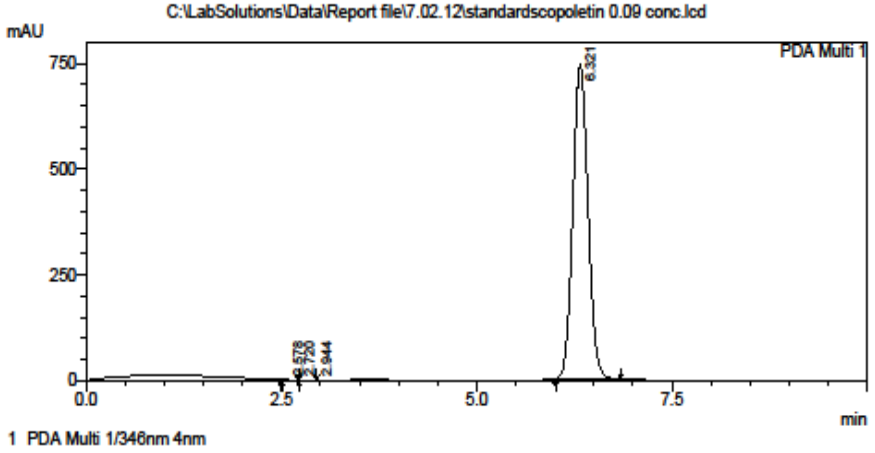
C:\LabSolutions\Data\Report file\7.02.12\standardscopoletin 0.09 conc.lcd



1 PDA Multi 1/346nm 4nm

C:\...\Data\Report file\7.02.12\standardscopoletin 0.09 conc.lcd

3D Graph C:\...\Data\Report file\7.02.12\standardscopoletin 0.09 c

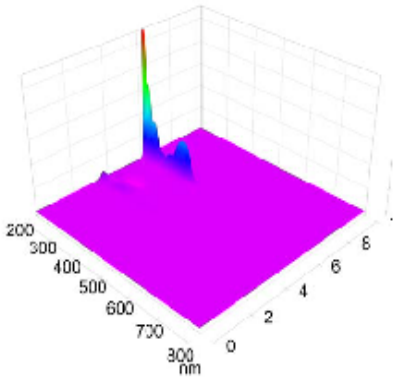
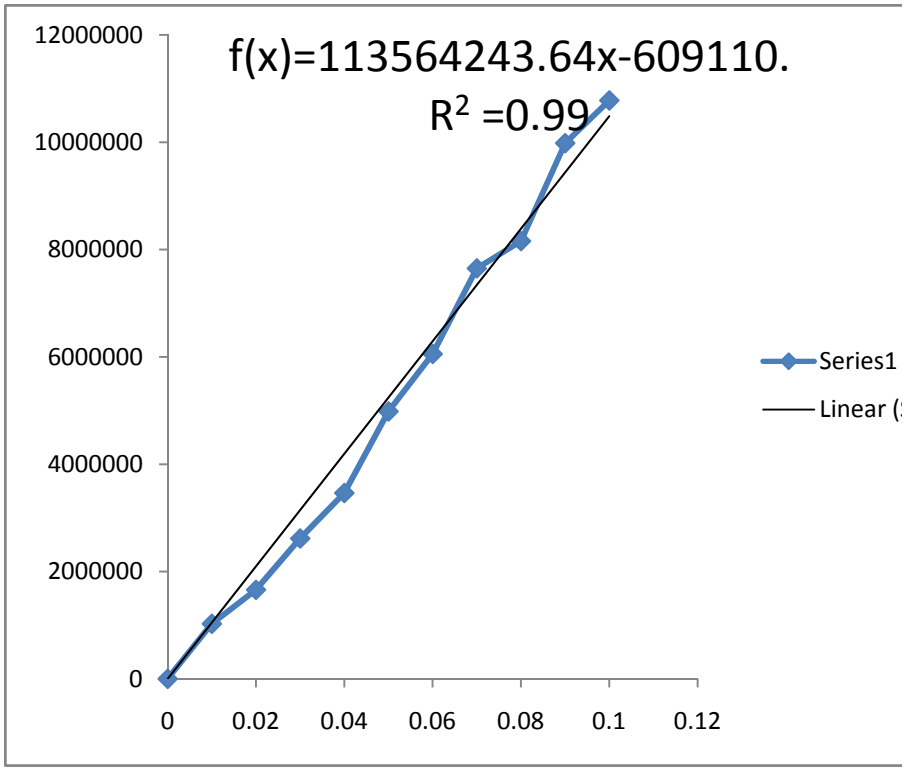


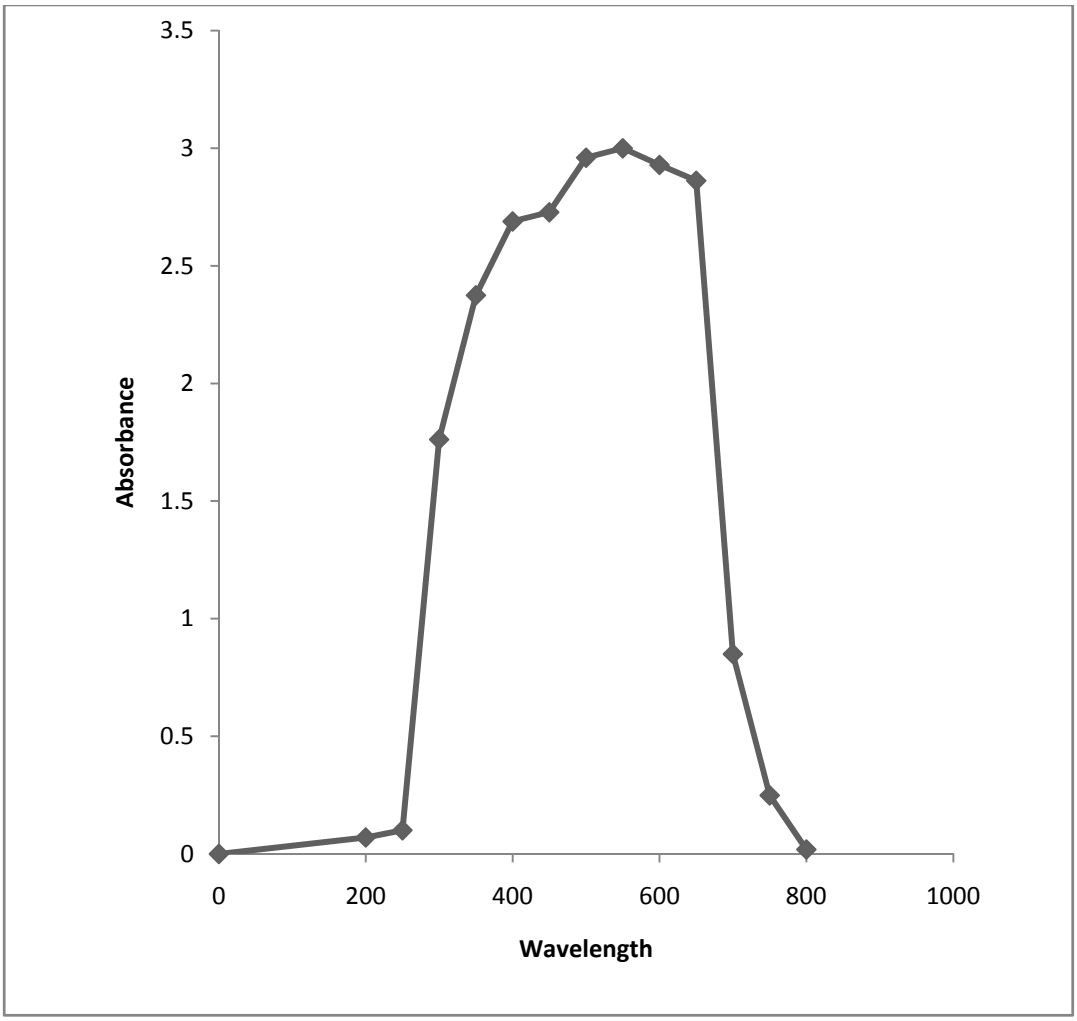
Table 9

S.No.	CONC.µl	AUC
	0.01	1025794
	0.02	1659509
	0.03	2616277
	0.04	3462711
	0.05	4982576
	0.06	6053406
	0.07	7649223
	0.08	8157746
	0.09	9982937
	0.10	10779049

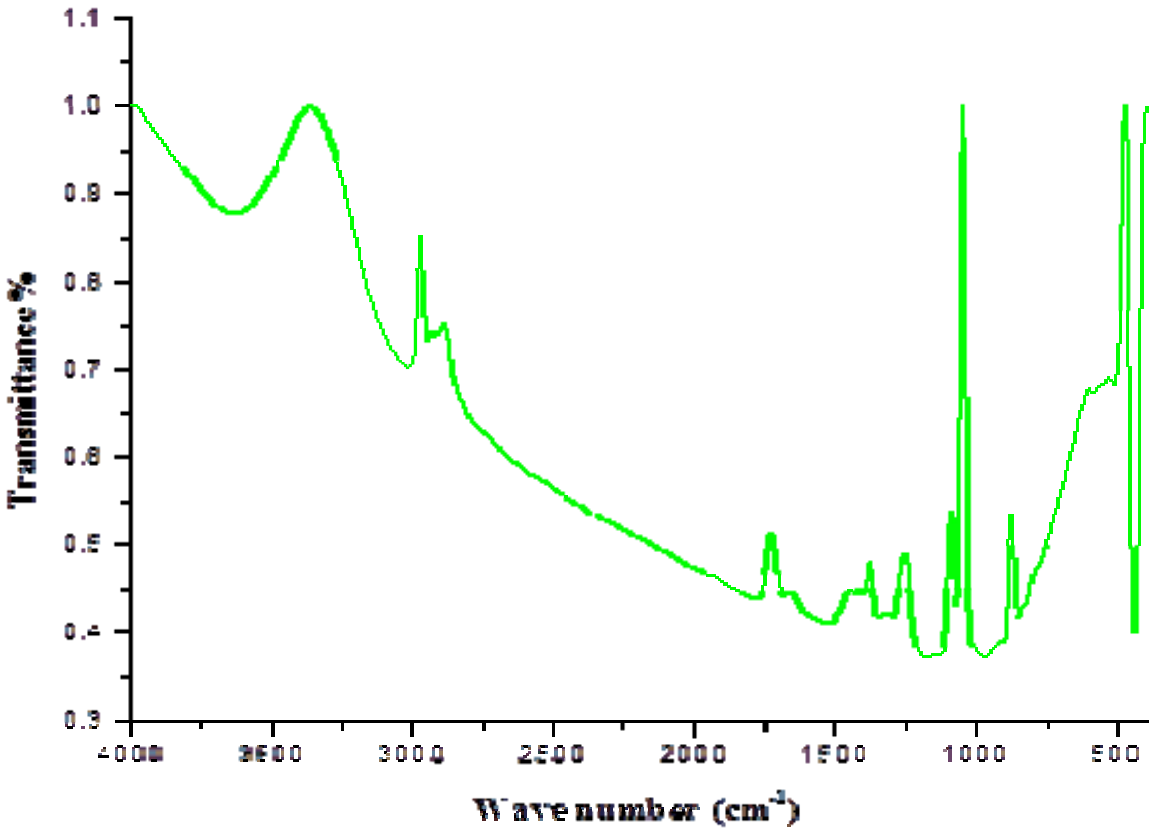


Sample area	1328692
Sample concentration	0.016013
	0.012953
Final concentration of sample	0.014483

UV ESTIMATION OF ACTIVE PRINCIPLE FROM METHANOLIC EXTRACT OF *Artemisia nilagirica*



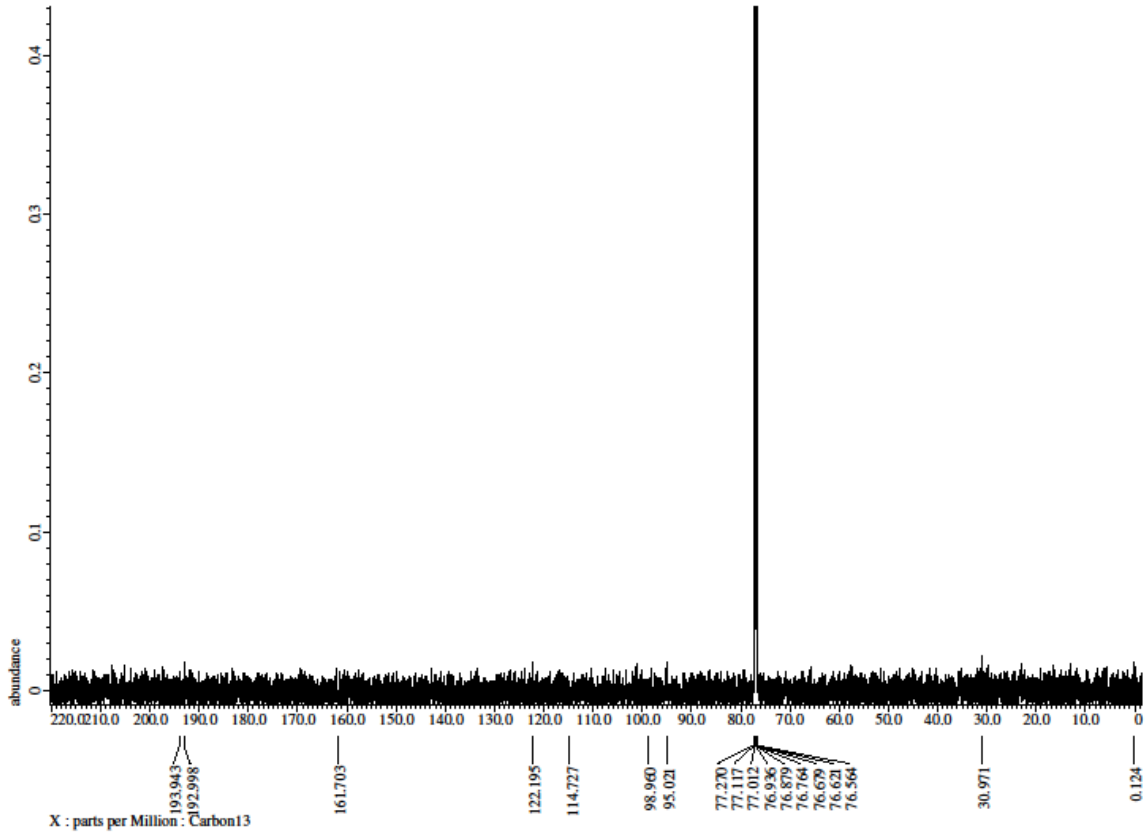
STRUCTURAL ELUCIDATION USING FTIR OF ACTIVE PRINCIPLE



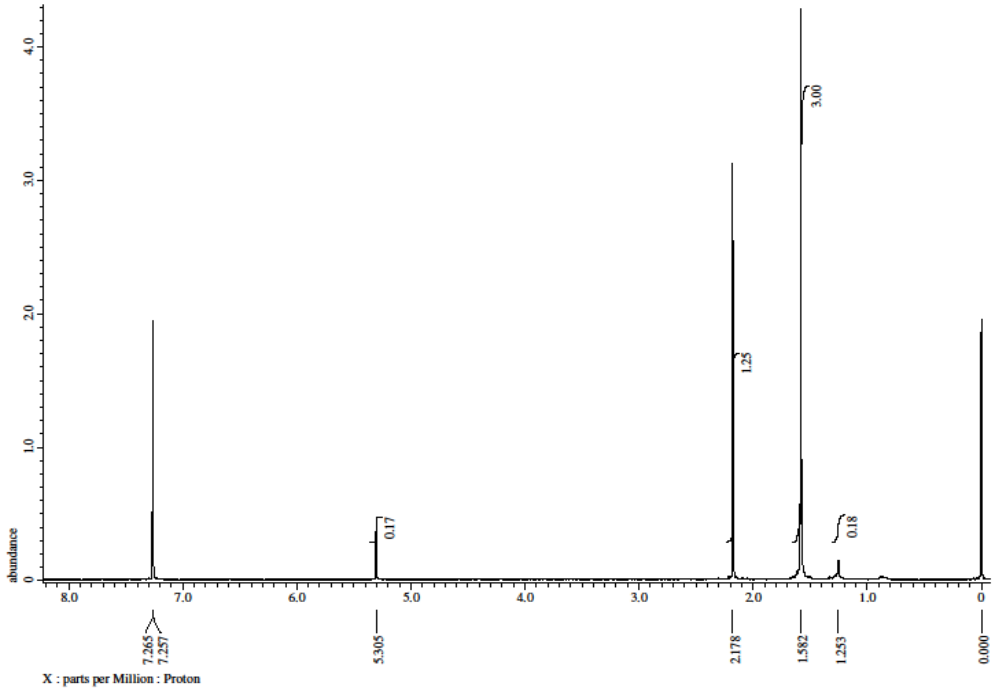
C- Aromatic C-H stretching vibration at 3020 cm-1 appear merely as a shoulder on the stronger alkane C-H stretching bands. The presence of peak at 1771 cm-1 was due to the carbonyl group in the structure of the compound which overlapped the weak overtones and combination bands of C-H out of plane bending vibrations which in turn is a function of the substitution pattern on the ring. The band at 1516 cm-1 due to semi-circle stretching vibration of the ring appears only for phenyl and o- and m- substituted phenyls and the intensity will be weak for rings conjucated with carbonyl groups. The band resulting from out-of-plane bending vibration by quadrants occurs at 444 cm-1 confirms the o- disubstituted benzene (C=O and phenyl group), whereas, the band at 975 cm-1 confirms the C-H inplane

bending vibration of phenyl ring. The band at 866 cm-1 confirms the presence of two adjacent hydrogen in the structure. Phenols in non-polar solvents line n-hexane exhibit a sharp rather weak O-H stretching absorption due to non-bonded or free OH groups. These non-bonded O-H stretching bands appear near 3630 cm-1 in case of phenols. The C-H asymmetric stretching bands generally appear for methyl group near 2947 cm-1 and for C-H inplane bending vibration it appears at 1346 cm-1. The band at 1175 cm-1 confirms the O stretching vibration in phenols. The band at 1088 cm-1 confims the C-O-C symmetric stretching vibration present in the compound structure.

13C NMR DETERMINATION OF ISOLATED ACTIVE PRINCIPLE FROM A.nilagirica



PROTON NMR DETERMINATION OF ISOLATED COMPOUND FROM A.nilagirica



The NMR spectra shows the elucidation of the sample of A.nilagirica. From the Ppm values the structure of the sample can be interpreted. The observing peaks are interpreted and values are tabulated

INTERPRETATION OF PROTON NMR Table 10

S.No.	Ppm(Value)	Groups assigned
1	7.265,7.257	May be due to aromatic proton
2	5.305	May be H atom attached to alkenes
3	2.178	May be double bonded functional group is present . (ketones, acids,aldehydes, esters and amides)
4	1.582,1.253	May be non functional groups
5	0.000	May be alcohol or carboxylic acid

INTERPRETATION OF ^{13}C NMR Table 11

S.No.	Ppm values	Groups assigned
1	193.943,192.998	C=O (in aldehydes)
2	161.703	C=O
3	122.195	C=C
4	114.727	C=C in alkenes
5	98.960,95.021	C-O
6	77.270,77.117,77.012,76.936, 76.879,76.764,76.679,76.621,76.564	C-O
7	30.971	C-C

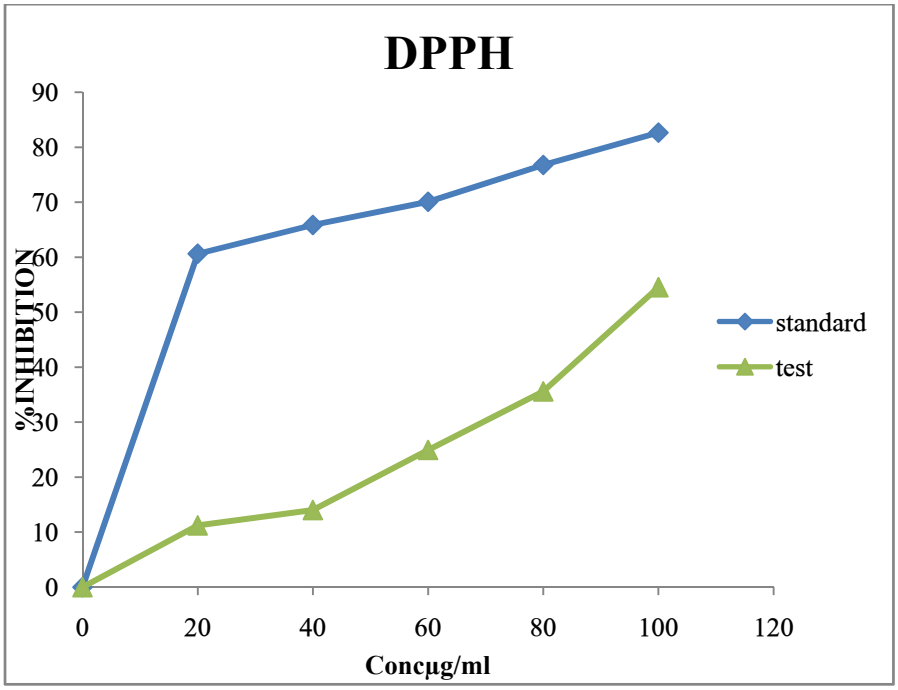
PHARMACOLOGICAL ACTIVITIES

DPPH RADICAL SCAVENGING ACTIVITY

This assay measures by spectrophotometer the ability of antioxidants to reduce 2,2- diphenylpicrylhydrazyl (DPPH), another radical not commonly found in biological systems. The IC50 of the standard ascorbic acid was16.96mg/ml and the IC50 of the methanolic extract of A.nilagirica was 97.633mg/ml.

Table 12

S.No	**Conc. in µg/ml**	**% inhibition of Ascorbic acid**	**%inhibition of A.nilagirica**
1	20	60.64 ± 0.314	11.23±3.815
2	40	65.86± 0.075	14.03±4.713
3	60	70.08±0.0513	24.95±7.793
4	80	76.77±0.0913	35.59±7.753
5	100	82.66±0.325	54.53±2.354
	IC50	**16.96mg/ml**	**97.633mg/ml**

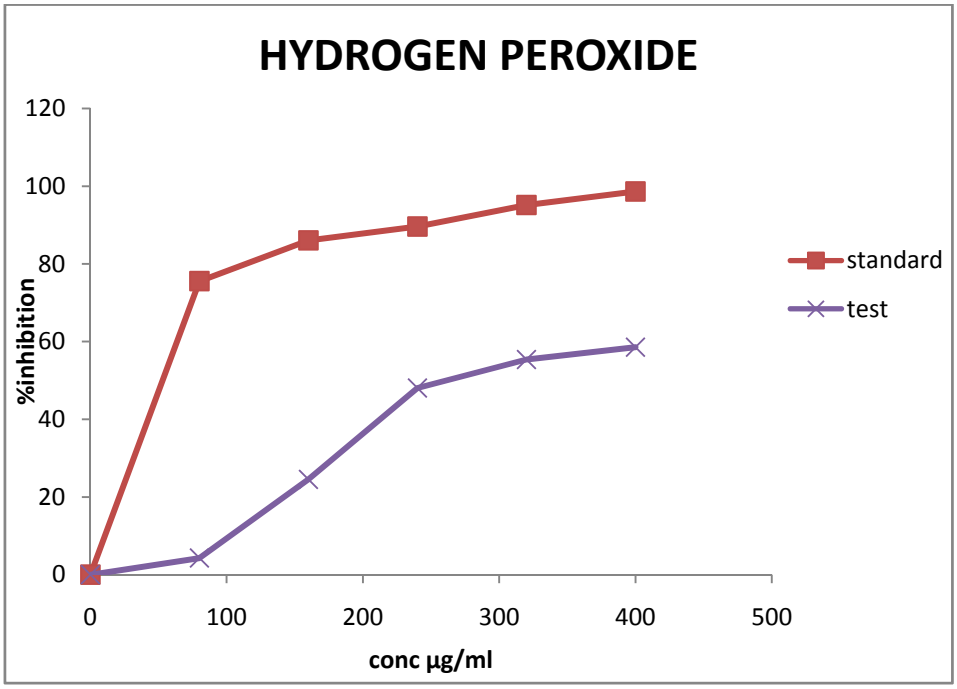


HYDROGEN PEROXIDE METHOD

H2O2 scavenging activity is one of the methods for the estimation of reactive oxygen scavenging ability in biological material. It cannot be evaluated as the total antioxidant activity due to the fact that antioxidants can act directly, for example by scavenging reactive oxygen species (O2• −, H2O2,•OH) or by inhibiting their generation, or indirectly by regulating endogenous antioxidant defenses. H2O2 is easily and sensitively measured by using peroxidase-based assay systems. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H2O2 is very important throughout food systems. (Nabavi et al., 2008a) The IC50 of the standard ascorbic acid was 19.99mg/ml and the IC50 of the methanolic extract of A.nilagirica was 158.8mg/ml.

Table 13

S.No	Conc.µg/ml	%inhibition of ascorbic acid	%inhibition of A.nilagirica
1	40	75.54±0.58	4.30±0.673
2	80	86.05±0.47	24.52±2.38
3	120	89.24±0.21	48.04±3.66
4	160	95.42±0.29	55.38±0.87
5	200	98.63±0.31	58.55±0.900
	IC50	**16.99mg/ml**	**158.8mg/ml**

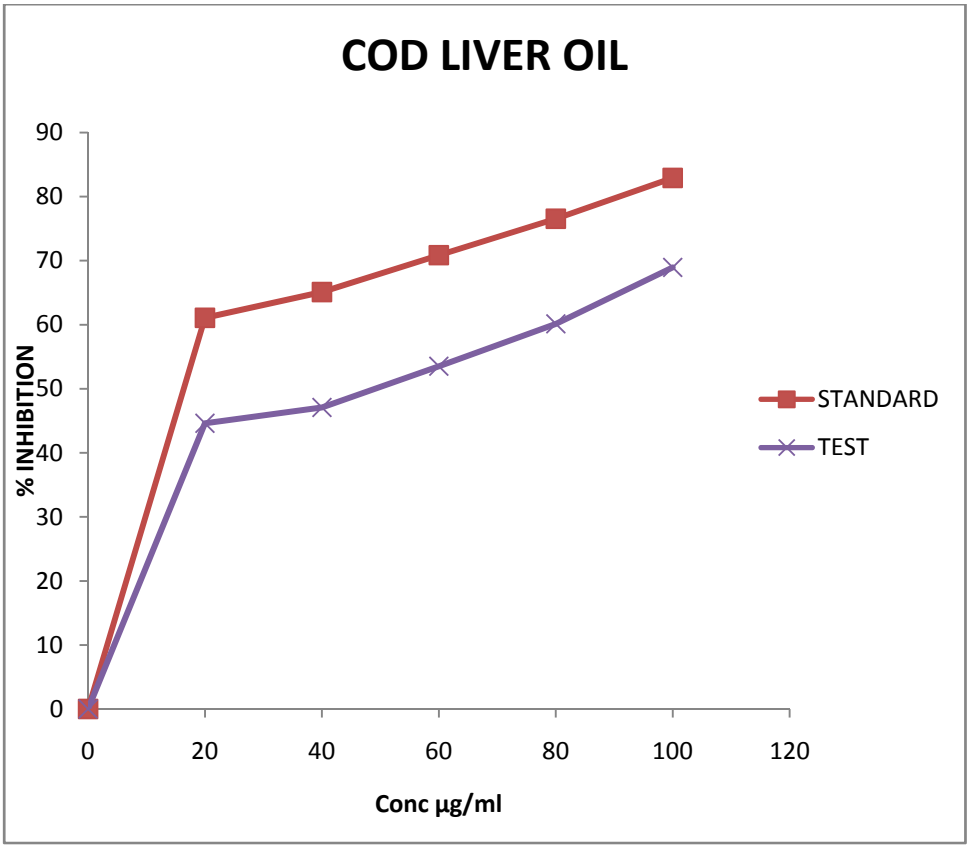


ANTIOXIDANT ACTIVITY USING COD LIVER OIL

The results obtained for methanolic extract of A. nilagirica may be attributed to several reasons viz. the inhibition of ferry-per ferryl complex formation, scavenging of OH- or superoxide radical or by changing the ratio of Fe+++ to Fe ++, reducing the rate of conversion ferrous or ferric by chelation of the ion itself . The IC50 of the standard ascorbic acid 16.81mg/ml and the IC50 of the methanolic extract of A.nilagirica was 50.52mg/ml.

Table 14

S.No.	**Conc µg/ml**	**% inhibition of Ascorbic acid**	**% inhibition of A.nilagirica**
1	20	61.08±0.199	44.61±0.86
2	40	65.1±0.153	47.08±0.66
3	60	70.86±0.080	53.52±0.721
4	80	76.55±0.31	60.13±0.755
5	100	82.92±0.055	68.95±0.38
	IC50	**16.81mg/ml**	**50.52mg/ml**



ANTITHYROID ACTIVITY

Thyroid hormones (T3 and T4) are involved in the regulation of numerous body functions including lipid and carbohydrate metabolism, oxygen consumption, and several physiological functions such as development, reproduction, and growth [117]. Alterations in their normal levels cause some biochemical and clinical abnormalities such as hypothyroidism and hyperthyroidism [118]. Extended exposure to the treatment with exogenous LT4 may alter thyroid activity by interfering with thyroid hormones synthesis, which provokes the disruption of thyroid axis, resulting in numerous abnormalities [119]. Hyperthyroidism simply defined as increases of serum T3 and T4 with decrease of serum TSH, a pituitary hormone that regulated thyroid functions [120.121]. In the present study, LT4-induced increases of serum T3

and T4 levels, and decreases of serum TSH concentrations were significantly and dose-dependently inhibited by treatment of MEAN extracts.

SERUM THYROID HORMONE LEVELS IN THE LT_4 AND TEST MATERIALS TREATED RATS

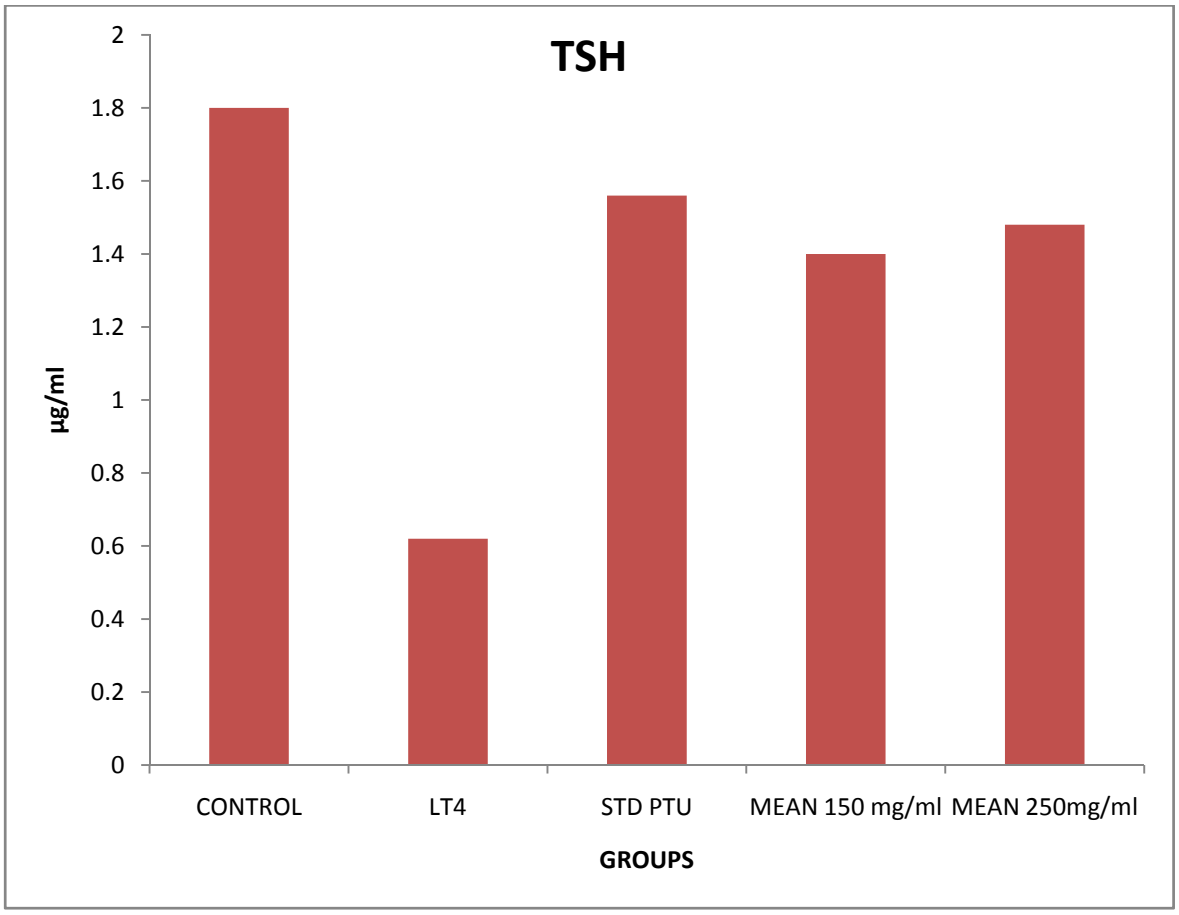
Table 15

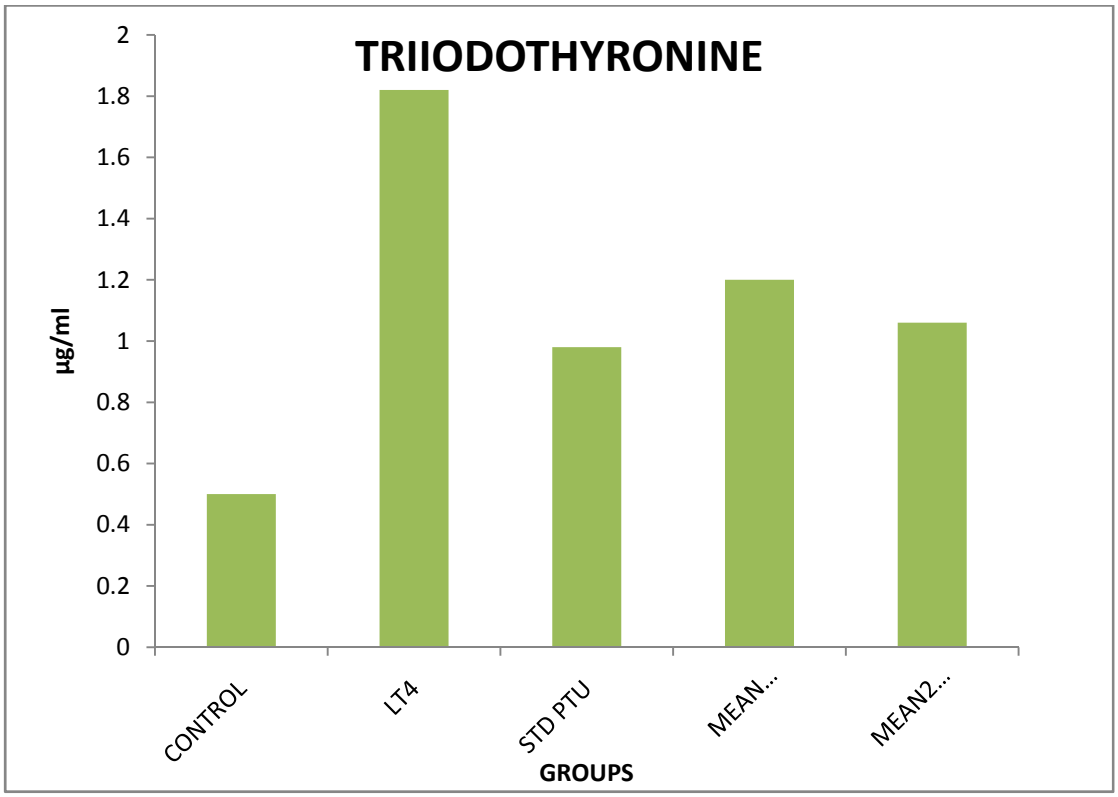
GROUPS	Thyroid stimulating hormone(µg/ml)TSH	Triiodothyronine (µg/ml) T_3	Thyroxine(µg/ml) T_4
Normal control 10ml/kg 1% CMC	1.80±0.20	0.50±0.17	42.30±4.15
LT4 treated animals (Hyperthyroid control)	0.62±0.15	1.82±0.21	152.40±7.40
STD control PTU 10mg/kg	1.56±0.18Δa	0.98±0.19Δa	68.90±5.60Δa
MEAN 150mg/kg	1.40±0.12Δb	1.20±0.20Δb	82.50±6.15Δb
MEAN 250mg/kg	1.48±0.14Δb	1.06±0.18Δb	73.40±5.80Δb

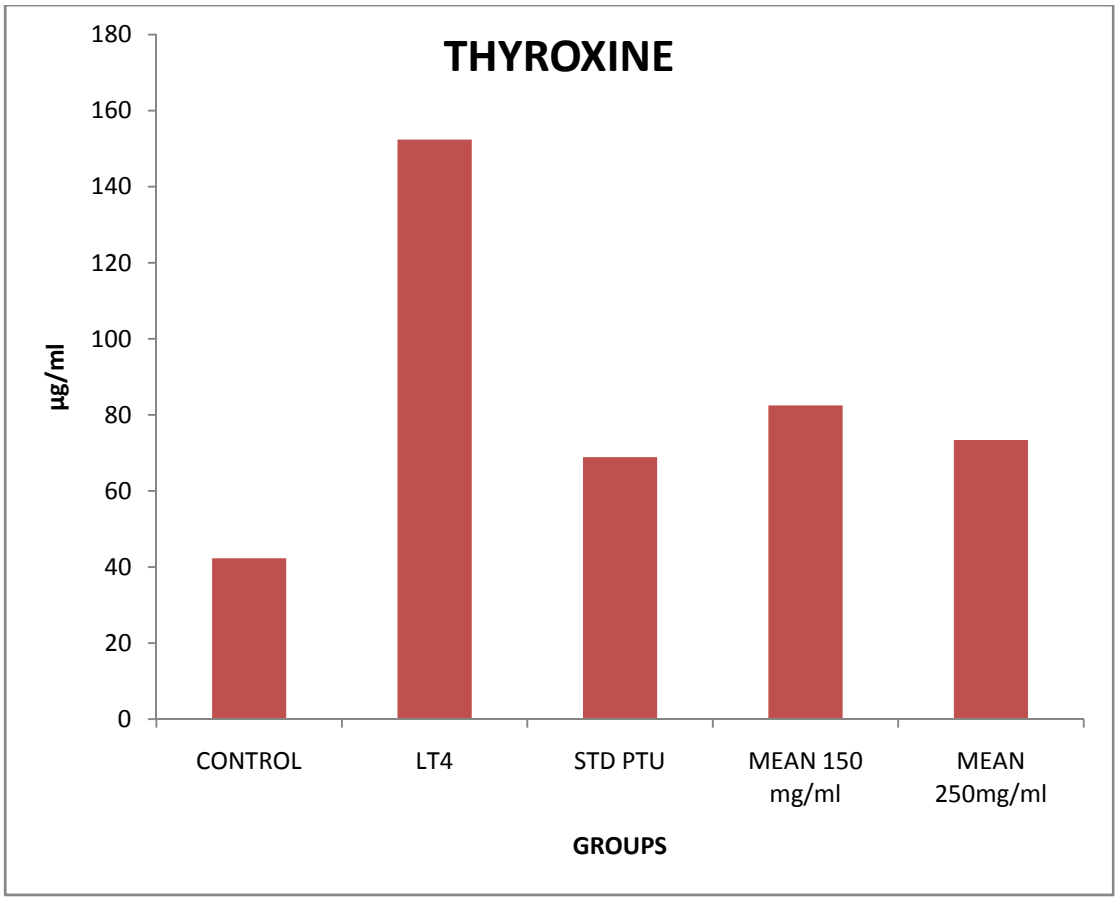
Values are expressed as Mean ± SEM

ΔaValues are significantly different from normal control at $P < 0.01$

ΔbValues are significantly different from Toxic control at $P<0.01$


TSH
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
µg/ml
CONTROL
LT4
STD PTU
MEAN 150 mg/ml
MEAN 250mg/ml
GROUPS


TRIIODOTHYRONINE
µg/ml
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
LT4
STD PTU
MEAN...
MEAN2...
GROUPS


THYROXINE
180
160
140
120
100
80
60
40
20
0
µg/ml
CONTROL
LT4
STD PTU
MEAN 150 mg/ml
MEAN 250mg/ml
GROUPS

INVIVO ANTI OXIDANT ACTIVITY

The activity has been well documented that thyroid dysfunctions increases LPO reactions and reactive oxygen species (ROS) [118]. LPO is an autocatalytic mechanism leading to oxidative destruction of cellular membranes [122]. Such destruction can lead to cell death and to the production of toxic and reactive aldehyde metabolites called free radicals, where MDA is the most important [123]. It is known that ROS would lead to oxidative damage of biological macromolecules, including lipids, proteins, and DNA [118], and oxidative stress also influenced to the body adipocyte results in decreases of body fat masses and related body weight decreases . MDA is a terminal product of LPO. So the content of MDA can be used to estimate the extent of LPO [118], and marked increases of liver MDA contents have been observed in hyperthyroid animals [, 124,125]. GSH is representative endogenous antioxidants; prevent tissue damage by keeping the ROS at low levels and at certain cellular concentrations,and accepted as protective antioxidant factors in tissues [126]. SOD is one of the antioxidant enzymes that contribute to enzymatic defense mechanisms, and catalase is an enzyme catalyzes the conversion of H2O2 to H2O [36]. The increase of some antioxidant enzymes activities such as SOD and catalase may be indicative of the failure of compensating the induced oxidative stress [127]. In hyperthyroidism, it is well known that marked decreases of tissue GSH contents were induced, represent the decreases of antioxidant defense systems [129.130]. Controversially, SOD and catalase activities were increase to remove over-produced ROS as of indication of the failure of compensating the induced oxidative stress [127,]. LT4-induced oxidative stresses and related organ damages were ameliorated by treatment of MEAN extracts in the present study like other previously tested antioxidants [3, 9, 14, 15], as direct evidenced that MEAN have potent antioxidant

effects enough to inhibited hyperthyroidisms.. In the present study, we only focused on the *in vivo* protective effects to hyperthyroidism of crud extract itself not on the active compounds. Thus, these active compound searches should be proceeding in future.

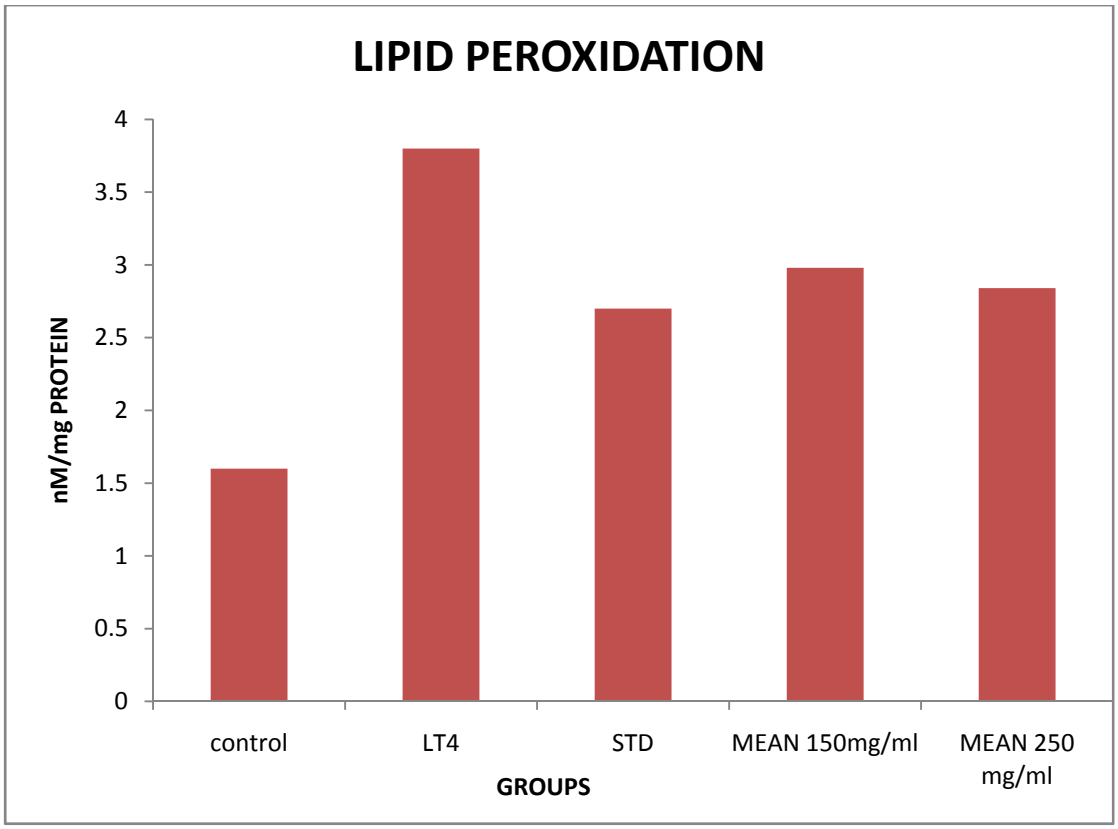
LIVER LIPIDPEROXIDATION AND ANTIOXIDANT DEFENSE SYSTEM IN THE LT_4 AND TEST MATERIALS TREATED RATS Table 16

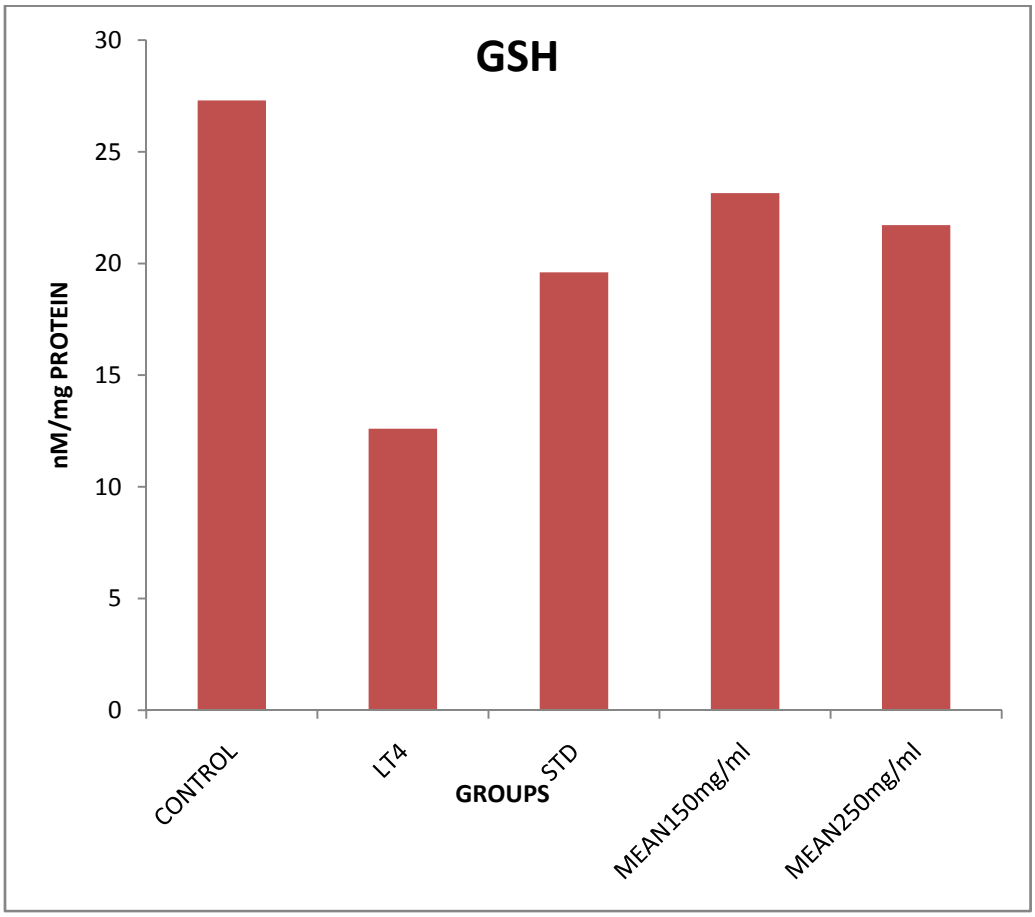
Groups	Lipid Peroxidation (nN/mg protein)	GSH (mN/mg protein)	SOD(U/mg protein)	Catalase(U/mg protein)
Normal control 10 mg/kg 1%CMC	1.60±0.25	27.30±2.60	19.00±2.80	21.15±1.20
LT4 treated animals (Hyperthyroid control)	3.80 ± 0.45Δa	12.60 ±1.75Δa	40.15 ± 5.25Δa	39.30 ±3.10Δa
STD control PTU 10mg/kg	2.70 ± 0.68Δb	19.60 ± 2.10Δb	24.10 ±3.40Δb	26.45 ±2.45Δb
MEAN 150mg/kg	2.98 ± 0.76Δb	23.15 ± 2.30Δb	31.26 ± 4.45Δb	31.30 ± 2.70Δb
MEAN 250mg/kg	2.84±0.70Δb	27.72 ± 2.05Δb	26.35 ± 3.80Δb	28.50 ± 2.60Δb

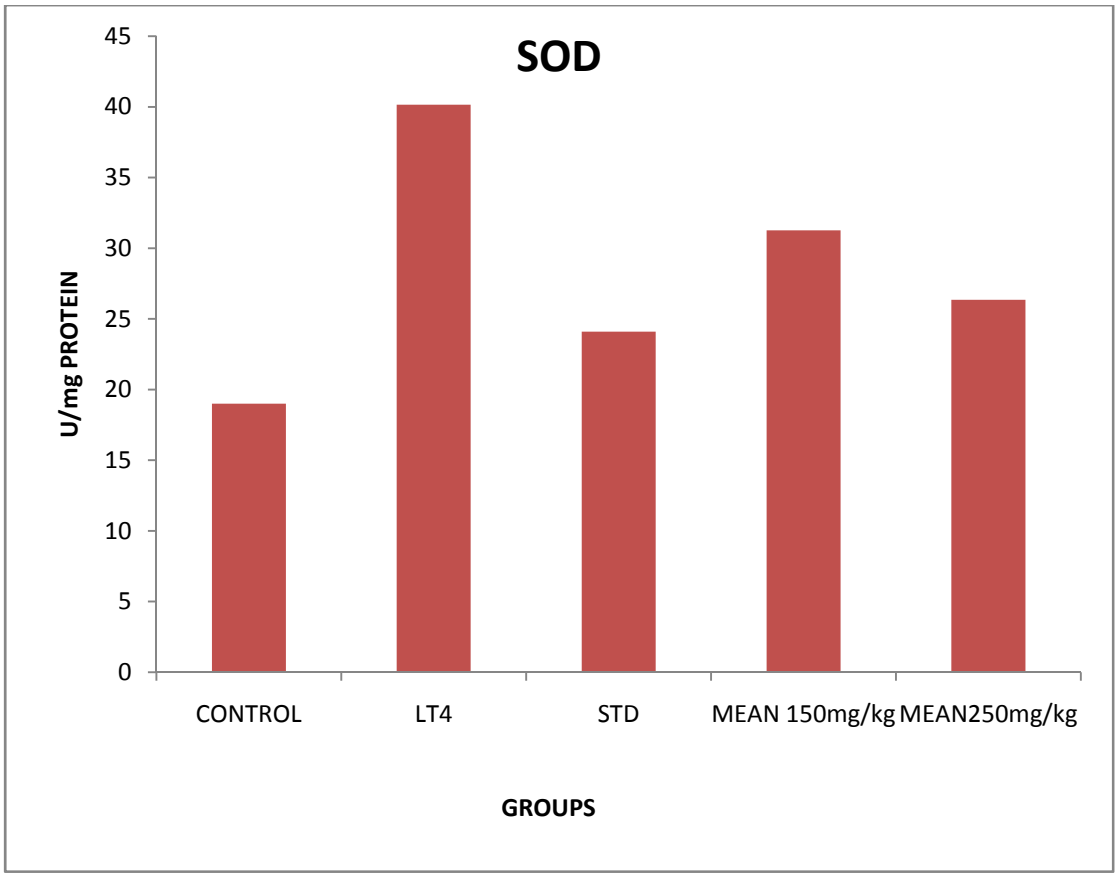
Values are expressed as Mean ± SEM

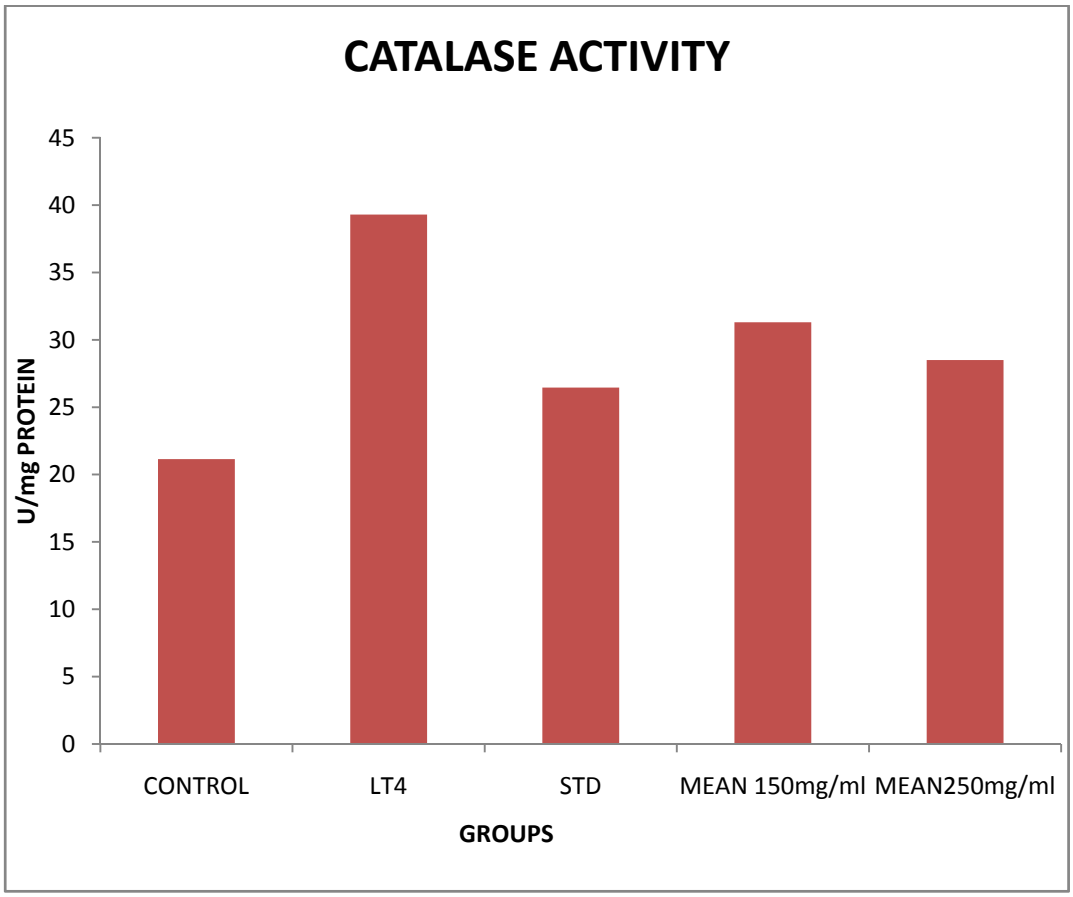
Δa Values are significantly different from normal control at P<0.01

Δb Values are significantly different from toxic control at P<0.01


LIPID PEROXIDATION
nM/mg PROTEIN
4
3.5
3
2.5
2
1.5
1
0.5
0
control
LT4
STD
MEAN 150mg/ml
MEAN 250 mg/ml
GROUPS


GSH
30
25
20
15
10
5
0
nM/mg PROTEIN
CONTROL
LT4
STD
MEAN150mg/ml
MEAN250mg/ml
GROUPS

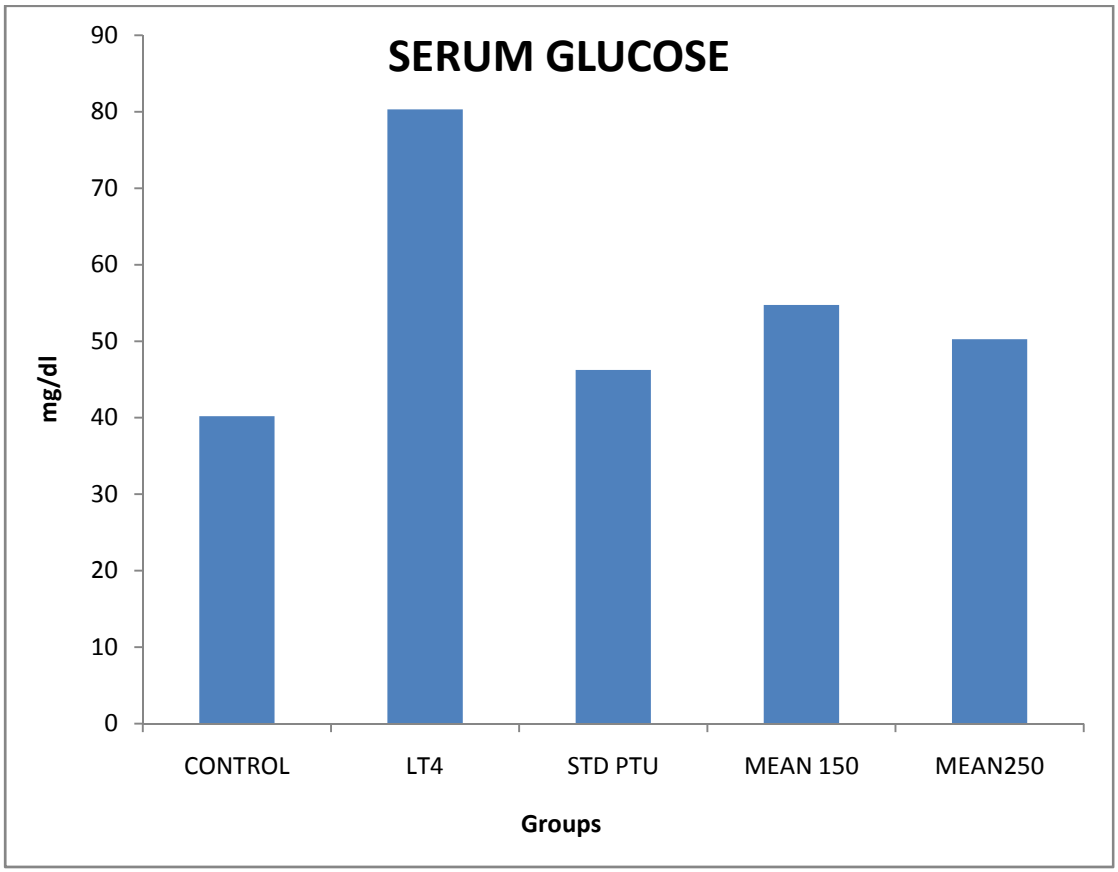

SOD
45
40
35
30
25
20
15
10
5
0
U/mg PROTEIN
CONTROL
LT4
STD
MEAN 150mg/kg
MEAN250mg/kg
GROUPS


CATALASE ACTIVITY
U/mg PROTEIN
45
40
35
30
25
20
15
10
5
0
CONTROL
LT4
STD
MEAN 150mg/ml
MEAN250mg/ml
GROUPS

SERUM GLUCOSE CONCENTRATION IN HYPERTHYROID RATS Table 17

Groups	Serum glucose concentration (mg/dL)
Normal control 10 mg/kg 1%cmc	40.19333 ± 0.597
LT4 treated animals (Hyperthyroid control)	80.30333 ± 0.490725
STD control PTU 10mg/kg	46.19333 ± 0.166966
MEAN 150mg/kg	54.73667 ± 0.139682
MEAN 250mg/kg	50.26 ± 0.2381

LT_4 induced hyperthyroid rats has hyperglycemia because of stress. Since thyroid hormones are also gluconeogenic as well as glycolgenolytic in nature, the changes in serum glucose concentrations could be the result of methanolic extract of A. nilagirica induced alterations in the status of thyroid functions in animals


SERUM GLUCOSE
90
80
70
60
50
40
30
20
10
0
mg/dl
CONTROL
LT4
STD PTU
MEAN 150
MEAN250
Groups

Summary and Conclusion

SUMMARY AND CONCLUSION

This dissertation is entitled **Pharmacognostic, Phytochemical,Antithyroid, Antioxidative and Antihyperglycemic evaluation of *Artemisia nilagirica*.C.B.clarke(Compositae)** The review of literature revealed very little information on the pharmacognostic and pharmacological work of the leaves of ***Artemisia nilagirica***.Hence it was decided to carry out the work on macroscopic and microscopic studies.It revealed the following characters which will play an important role in the authentication of crude drug and will also be useful for the detection of adulteration.

a) The leaves are simple ,oppositely arrangedand lobed
b) The microscopy of leaves shows that the midrib is thick and wide abaxial part &wide adaxial part.
c) The stomata is anomocytic with epidermal cells and the glandulartrichomes are two celled.
d) The reticulate venation are seen and rectangular to polygonal in shape.
e) . The vascular bundles are collateral with dense, compact rows of small, xylem elements and thick band of compact phloem elements in the petiole part.
f) Evaluation of powder microscopy showed two celled glandular trichomes.

The macroscopy and microscopy features has added more weightage to the authenticity of the plant.The quantitative analytical microscopy and standardisation parameters gives values which are constant and will be useful for quality and purity of the crude drug.

Preliminary phytochemical screening and quantitative estimation of phytoconstituents throws light on the type of secondary metabolites present in

theplant.The isolation of the active priciple in the methanolic extract by coloumnchromatography.HPLC shows the quantification of the active principle for determining the amount of secondary metabolites which may responsible for the pharmacological activity.

The isolated activate principle shows the structural elucidation by the spectroscopical methods UV , FTIR and NMR

The various pharmacological activities carried out included invitro and invivo antioxidant activities, antithyroid and antihyperglycemic activities. The antithyroid activities showed that the amount of TSH, T_3 and T_4 present in the serum samples. Then the antioxidant defense system are LPO which measures antioxidative stress and other antioxidative defense systems are CAT,SOD and GSH. The antihyper glycemic activity by estimation of serum glucose concentration . Hence the presence of the secondary metabolites collaborates a wide range of biological activities such as antithyroid, anti oxidative and antihyper glycemicavctivity.

In conclusion the pharmacognostic features, physicochemical constants, phytochemical constants, preliminary phytochemical studies, column chromatography, spectroscopical methods (UV, FTIR and NMR) HPLC shows that the quality and quantity of the secondary metabolites. It may be concluded that Artemisia nilagirica could be a potential source of medicine exhibiting wide range of biological activity.

LT4-induced hypothyroidism and liver damages were inhibited by oral treatment of MEAN 150 and 300mg/kg. In addition, they also enhanced the liver antioxidant defense systems—they dose-dependently inhibited LT4-induced increases of LPO and changes on the GSH contents, SOD, and catalase activities as direct evidences that MEAN have favorable ameliorating effect on the hyperthyroidisms

and related organ damages induced by LT4 through antioxidant effects. MEAN showed comparable effects on the LT4-induced rat hyperthyroidism as compared with PTU 10mg/kg. These effects of MEAN may help the improvement of hyperthyroidisms and accompanied various organ damages, but active compound searches should be proceeding in future.

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