



# **“PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTICANCER ACTIVITY OF THE LEAVES OF ASPARAGUS RACEMOSUS WILLD., (LILIACEAE)”**

**Dr. Babasaheb Ambedkar Technological University, Lonere**

Submitted by

**Miss. SWAMI KIRTI SHIVANAND**

Under the supervision of

***Dr. CHETAN BHUTADA J***

(M.Pharm, Pharmacognosy)

**ADITYA PHARMACY COLLEGE , Beed – 431122**

## **1. INTRODUCTION**

Cancer is a disease characterized by uncontrolled multiplication and spread of abnormal form of the body's own cells. It is one of the major causes of death in the developed Nations. Cancer is also responsible for approximately one quarter of all death in the world with lung and bowel cancer comprising the largest category closely followed by breast and prostate cancer <sup>(1)</sup>.

### **TYPES OF CANCER <sup>(2-3)</sup>**

Cancer is classified in two ways: by the type of tissue in which the cancer originates (histological types) and by primary site, or the location in the body where the cancer first developed.

#### **Carcinoma**

Cancers derived from epithelial cells. This group includes the most common cancers, particularly in the aged and include nearly all those developing in the breast, prostate, lung, pancreas and colon.

## Sarcoma

Cancer arising from connective tissues (i.e. bone, cartilage, nerve), each of which develops from cells originating in mesenchymal cells outside the bone marrow.

## Lymphoma and leukemia

These two classes of cancer arise from hematopoietic (blood-forming) cells that leave the marrow and tend to mature in the lymph nodes and blood respectively.

Leukemia is the most common type of cancer in children accounting for about 30%.

## Germ cell tumor

Cancer derived from pluripotent cells, most often presenting in the testicle or the ovary (seminoma and dysgerminoma respectively).

## Blastoma

Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common in children than in adults.

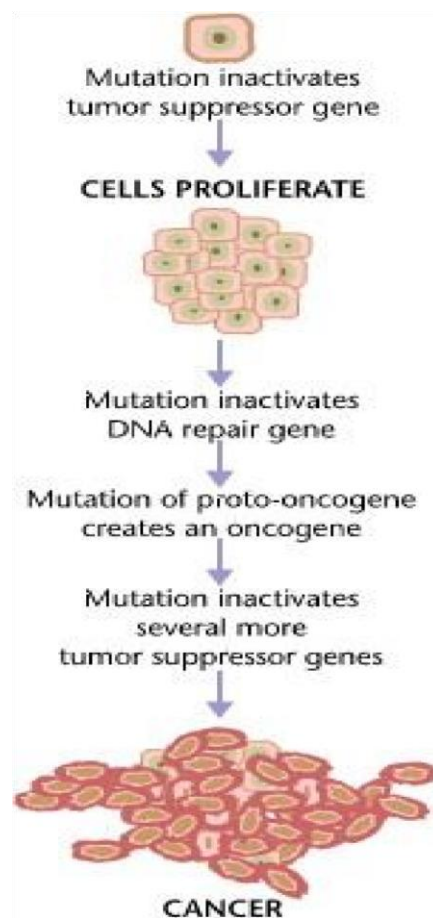


Fig. 1 Cancer cell proliferation

## SINGS AND SYMPTOMS

The signs and symptoms of cancer will depend on where the cancer is, how big it is, and how much it affects the organs or tissues. If a cancer has spread (metastasized), signs or symptoms may appear in different parts of the body <sup>(4)</sup>.

As cancer grows, it can begin to push on nearby organs, blood vessels and nerves. This pressure causes some of the signs and symptoms of cancer. If the cancer is in a critical area, such as certain parts of the brain, even the smallest tumor can cause symptoms.

But sometimes cancer starts in places where it won't cause any signs or symptoms until it has grown quite large. Cancers of the pancreas, for example, usually don't cause symptoms until they grow large enough to press on nearby nerves or organs (this causes back or belly pain). Others may grow around the bile duct and block the flow of bile. This causes the eyes and skin to look yellow (jaundice). By the time a pancreatic cancer causes signs or symptoms like these, it's usually in an advanced stage. This means it has grown and spread beyond the place it started – the pancreas<sup>(5)</sup>.

A cancer may also cause symptoms like fever, extreme tiredness (fatigue), or weight loss. This may be because cancer cells use up much of the body's energy supply, or they may release substances that change the way the body makes energy from food. Cancer can also cause the immune system to react in ways that produce these signs and symptoms.

Sometimes, cancer cells release substances into the bloodstream that cause symptoms that are not usually linked to cancer. For example, some cancers of the pancreas can release substances that cause blood clots in veins of the legs. Some lung cancers make hormone-like substances that raise blood calcium levels. This affects nerves and muscles, making the person feel weak and dizzy<sup>(6)</sup>.

### Typical symptoms of cancer includes <sup>(7)</sup>

- ❖ Presence of an unusual lump in the body
- ❖ Changes in a mole on the skin
- ❖ A persistent cough or hoarseness
- ❖ A change in bowel habits, such as unusual diarrhoea or constipation
- ❖ Difficulty in swallowing or continuous indigestion
- ❖ Any abnormal bleeding, including bleeding from the vagina or blood in urine or faeces
- ❖ A persistent sore or ulcer
- ❖ Difficulty in passing urine
- ❖ Unexplained weight loss
- ❖ Unexplained pain
- ❖ Unexplained tiredness or fatigue
- ❖ Skin changes such as an unexplained rash or unusual texture

- ❖ Unexplained night sweats
- ❖ Abdominal pain

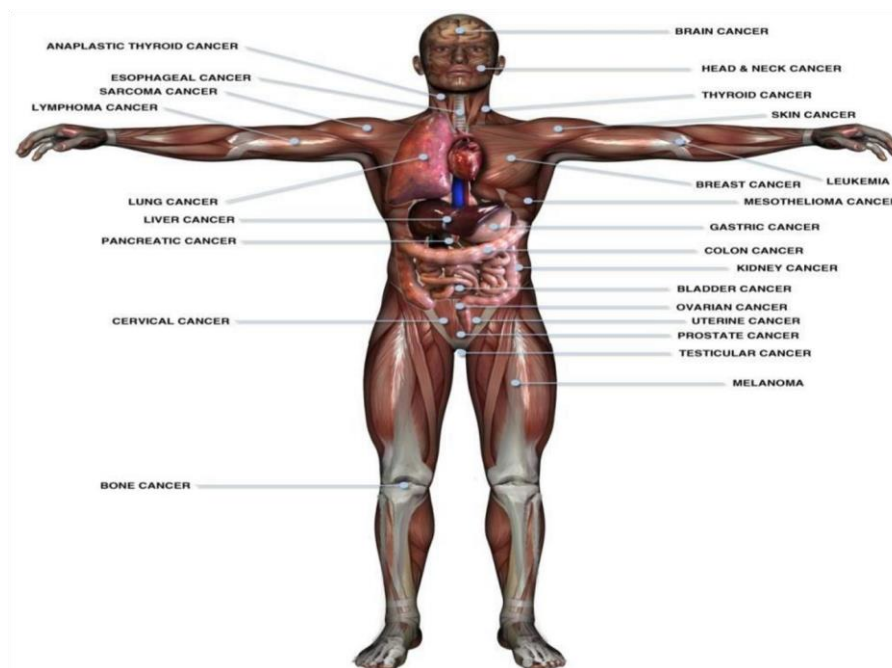
## PARTS AFFECTED

Cancer affects various parts of the body like brain, liver, bladder, thyroid, skin, pancreas, bone, lung, kidney, brain, cervical, lymphoma etc.,

## TYPES OF TREATMENTS

The aim of cancer treatment is to cure the cancer. In some cancers that are very slow-growing or that have spread beyond the original area of the body, the aim may be to control the cancer and delay its progress.

The main treatments for cancer are surgery, radiotherapy and chemotherapy. Other treatments, such as hormonal therapy and targeted therapy may also be used for certain cancers. Often, a combination of more than one type of treatment is used.



**Fig.2 Cancer and the human body: An inside look**

## Surgery

Surgery aims to remove all or part of a tumor. Some operations are done as day surgery, so the person only needs to take a short time off work. Others are more complex and spending a few weeks, or even months away from work.

## Radiotherapy

This treatment uses high-energy x-rays to destroy cancer cells, while doing as little harm as possible to normal cells. Radiotherapy that aims to cure the cancer will often mean a person needs to go to the hospital for several weeks <sup>(8, 9)</sup>.

## Chemotherapy

Chemotherapy drugs interfere with the process of cell division. They affect normal cells as well as cancer cells. As a result, they often cause side effects. The drugs are often given as a liquid through a drip into a vein (intravenously). They circulate in the blood stream and reach the cancer cells wherever they are in the body. Some chemotherapy drugs are given as tablets or capsules, which can be taken at home.

Intravenous chemotherapy may take minutes, hours or a few days. The treatment is followed by a few weeks of rest to allow the body to recover from any side effects. Together, the treatment and the rest period are known as a cycle of chemotherapy. Usually 4–6 cycles of treatment are given, which take 4–8 months<sup>(10)</sup>. Some treatments for particular types of cancer last much longer than this, while others may be shorter. Sometimes, a drug is given continuously into the vein by a small portable pump over the course of a few months.

## Hormonal therapies

These are drugs that can stop or slow the growth of cancer cells by either changing the level of particular hormones in the body, or preventing hormones affecting the cancer cells. Most hormonal therapies are given as tablets, but some are given as injections every few weeks or months and they do still have side effects.

## Targeted therapies

These are part of a newer group of drug treatment that works by targeting the growth of cancer cells. They generally have little effect on normal cell growth, so they usually have less troublesome side effects than chemotherapy. Targeted therapies may be given as a drip (intravenous infusion) or as tablets. Tiredness and other side effects may sometimes make this difficult<sup>(11)</sup>.

## DISADVANTAGES OF CANCER TREATMENTS

Side effects and how long they last will depend on the treatment being given and also vary from person to person. Some people will be able to work during their treatment, while others will need to be off for a few weeks or months. Common side effects of treatments include fatigue, risk of infection, nausea (feeling sick), vomiting (being sick), hair loss, sore mouth, diarrhoea or constipation, numbness or tingling in the hands or feet and body changes.

Cancer and its treatments can cause physical changes. These may be temporary or permanent. Body changes depends on the individual's situation, but other changes includes; Hair loss, changes in complexion or skin tone, altered appearance after surgery, weight loss or gain. Tiredness for many months or sometimes years, pain or lack of movement in an arm after breast surgery and can able to eat little and often after stomach surgery.

**Disadvantages of Radiotherapy and chemotherapy**

Radiotherapy not only destroys the cancer cells but it damages the normal cells also which is the major side effect of this therapy. In case of pregnancy radiotherapy may injure the fetus. Radiotherapy equipment is expensive so the treatment is also highly expensive.

Lowered a WBC, RB and Platelet count which increases the risk of infection, anemia, bleeding and bruising. Damage to the nerve system that may further weaken a person mental capacity, increased memory loss and cause fatigue<sup>(12)</sup>.

It is well recognized that allopathic drugs are not without danger as they produce side effect to the normal cell and the chemotherapeutic agents causes non selective damage to the normal cell, therefore worldwide research is now focusing on the investigation of best antitumor agents from herbal sources.

**HERBAL DRUGS IN THE TREATMENT OF CANCER <sup>(13-16)</sup>**

Plant and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of chemoprevention drug discovery and development<sup>(13)</sup>. Around 60% of currently used anticancer agents are derived in one- way or another from natural sources, including plants, marine source and micro organisms.

Herbal medicines are plant derived materials or preparations, which contain raw or processed ingredient from one or more plants with therapeutic value and used as dietary supplements to fight or prevent common diseases in various systems of medicine such as Ayurveda, Unani and Siddha. Plant products have been used throughout human history for various purposes including medicine<sup>(14)</sup>. Herbs can be viewed as biosynthetic chemical laboratories, producing a number of chemical compounds. Herbal drugs ranges from parts of plants to isolated purified active constituents. They may come from mostly leaves, roots, barks, seeds and flower. They are eaten, swallowed, drunk, inhaled or applied to the skin. Several secondary metabolites are produced by the higher plants as natural defense against disease and infections. The Indian system of medicine known as Ayurveda uses mainly plant based drug or formulation to treat various disease including cancer: and around. Approximately 877 small molecule drugs are introduced worldwide between 1981- 2002, 61% can be traced back to their origin in natural products. Recent surveys suggest that one in three persons use medicinal natural products daily and possibly one in two cancer patients use them as well.

A brief review on Japanese anticancer plants proves that various phyto constituents viz., terpenoids, saponins, polyphenols, polysaccharide, flavonoids, glycoside, alkaloids have been reported to inhibit tumor. In recent years, extensive research has been focused on the anticarcinogenic potential of triterpenes, flavonoids and polyphenols<sup>(15, 16)</sup>.

Table 1. Few plants which are likely to possess anticancer activity are listed below

S.NO	BOTANICAL SOURCE	PARTS USED	CONSTITUTION	USE
1	<i>Abrus precatorious</i>	Seeds	Abrin, Lectin	Suppress DLA and EAC
2	<i>Catharanthus roseus</i>	Herbs	Alkaloids- Vincristine, Vinblastin	Leukemia, lymphoma, lung and breast cancer
3	<i>Camptotheca acuminata</i>	Herbs	Camptothecin	Colorectal, ovarian and lungs cancer
4	<i>Plumbago rosea</i>	Herbs	Napthoquinones- Plumbagin	EAC
5	<i>Taxus brevifolia</i>	Bark	Taxol	Lung, breast and neck cancer
6	<i>Cissus quadrangularis</i>	Climber	Triterpenoids	Tumor

Although many plants are used to treat tumors in Indian traditional systems of medicine, most of these plants are not scientifically evaluated. If a systemic and intensive ethno pharmacological study is carried on one or more plants used in traditional system, are sure to provide effective anticancer drugs. In view of the wide spread interest on *Asparagus* the present work has been undertaken for the investigation of anticancer activity of the leaves of *Asparagus recemosus* Willd.,

## 2. REVIEW OF LITERATURE

The literature review encompasses the systematic information on pharmacognosy, phytochemistry and pharmacological studies of the leaves of *Asparagus racemosus* Willd., It was found that very little information was available on the leaves of this species.

### PHARMACOGNOSTICAL REVIEW

❶ No pharmacognostical work has been done on the leaves.



## PHYTOCHEMICAL REVIEW

- Verma A et al., (2014) studied the isolation, purification and spectral analysis of pure compound obtained from leaf extract of *Asparagus racemosus* <sup>(17)</sup>.
- 
- Shashi Alok et al., (2013) conducted a study in plant profile, phytochemistry and pharmacology of *Asparagus racemosus* (Shatavari)<sup>(18)</sup>.
- Asha Verma and N.Singh (2012) studied the chromatographic separation of ethyl acetate crude extract of Shatavari : A ethnomedicinal plant<sup>(19)</sup>.
- V.K.Karmakar et al., (2012) carried out the phytochemical investigation and antibacterial and antioxidant potentials of *Asparagus racemosus* <sup>(20)</sup>.

## PHARMACOLOGICAL REVIEW

- Shiv Prakah Varma et al., (2014) studied the inhibitory effects of *Asparagus racemosus* leaf extract on renal cell carcinoma cell line<sup>(21)</sup>.
- Battu GR and Kumar BM (2013) conducted a study in anti-inflammatory activity of leaf extract *Asparagus racemosus* Willd. <sup>(22)</sup>.
- Asha Verma et al., (2013) investigated *invitro* antioxidant activity and phytochemical screening of ethyl acetate extract of leaves of *Asparagus racemosus*<sup>(23)</sup>

## 3. PLANT PROFILE



**Fig. 3** *Asparagus racemosus* Willd.,



**PLANT PROFILE** (24-29)

Botanical name: *Asparagus racemosus* Willd.,

Common name : Satavari

Tamil name : Thannirvitan Kelangu, Ammaikodi

Family : Liliaceae

**TAXONOMICAL STATUS**

Kingdom : Plantae- plant

Division : Magnoliophyta- flowering plant

Class : Liliopsida- monocotylendons

Order : Liliaces

Family : Liliaceae

Genus : *Asparagus*

Species : *racemosus*

**Plant decription**

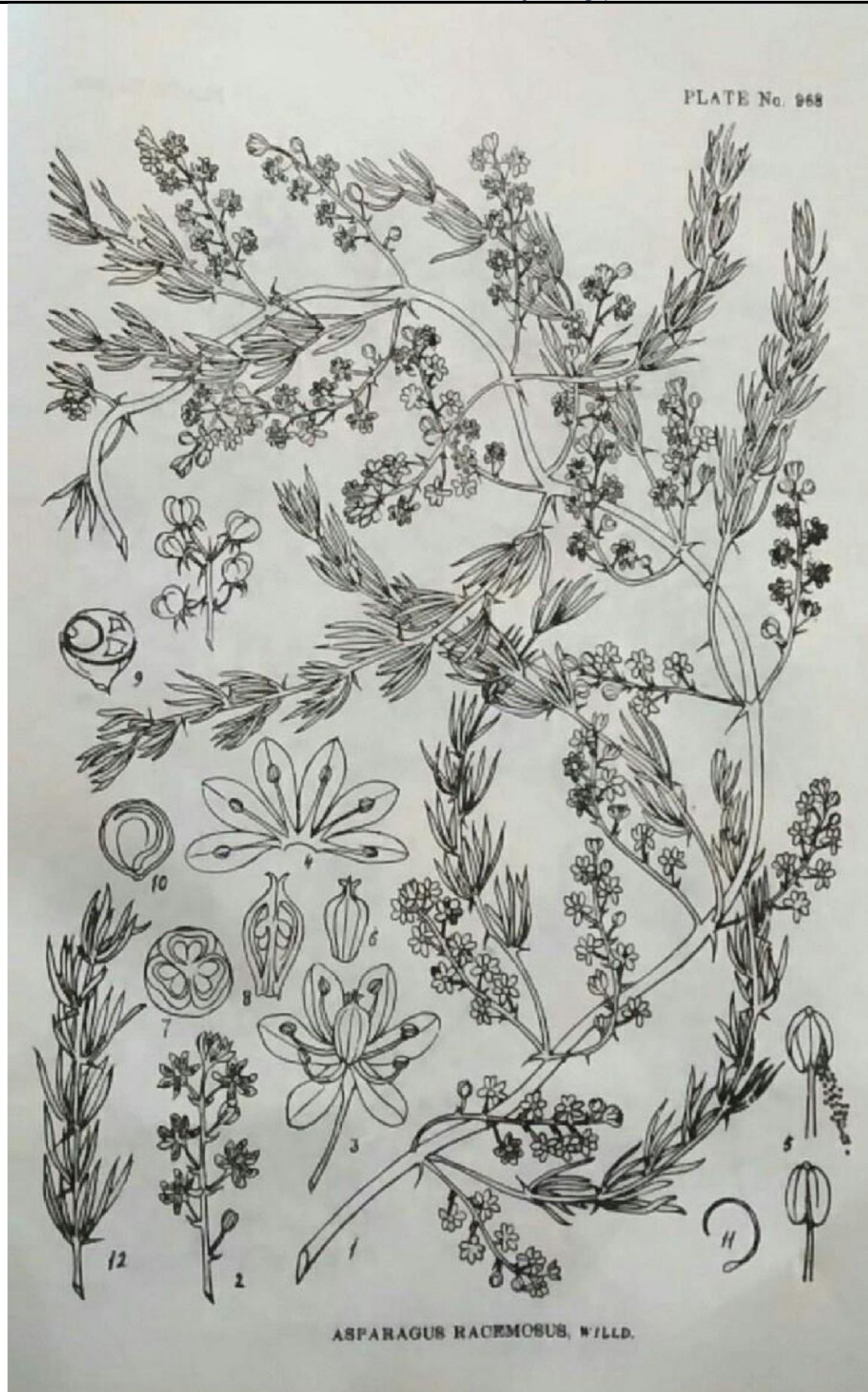
*Asparagus racemosus* Willd., is a perennial shrub, with a tuberous root-stock, stems covered with recurved spines, linear leaves arranged in a tuft, white flowers and sweet-scented appears in October.

**Habitate**

It grows in a variety of habitats including open forest found wild in tropical and subtropical parts of India including the Andamans and ascending in the Himalayas to 1500 meter.

**Ethnomedicinal uses**

Used in the treatment of rheumatism, cough, fertility problem, dysentery, diarrhoea, gastric complaints, gonorrhea, leucorrhoea, impotency, stomach problem, dyspepsia, anemia, general debility, typhoid, retention of urine, tonic, fever, gout and used to increase lactation, sperm count and tonic. The paste of fresh leaves is used on the burning sensation of the skin in smallpox.



#### 4. RATIONALE FOR SELECTION OF THE PLANT

Now a day's lots of herbal plants are being upgraded by validating the traditional claims and establishing its medicinal value. Various plants are yet to be scientifically proven for their therapeutic efficacy. *Asparagus racemosus* Willd., is one such plant belonging to the family Liliacea. This plant is commonly known as Satavari.

The plant has a long history of numerous traditional and ethno botanical applications in diverse cultures. *Asparagus racemosus* Willd., (Liliaceae) is traditionally used in the treatment of dysentery, epilepsy, inflammation, leprosy, night blindness, tuberculosis, snake bite, cancer, spasms, gonorrhea, piles, diabetes, rheumatism, cough, diarrhoea, gastric troubles, head ache and it increases the lactation. The aerial parts are used as spasmolytic, anticancer, anti arrhythmic, antibacterial and anti fungal <sup>(30-32)</sup>. Traditionally the aerial part has been used for the treatment of cancer but there is no scientific proof. So, the present study was undertaken to investigate the effects of *Asparagus racemosus* Willd., leaves on anticancer activity.

#### 5. AIM AND OBJECTIVES

##### AIM

The main aim of the present work is to study the anticancer activity of the leaves of *Asparagus racemosus* Willd.,

The main objectives of the present study are

- To standardize the leaves of *Asparagus racemosus* Willd., by carrying out the pharmacognostical and phytochemical studies.
- To evaluate the *invitro* anticancer activity of various extracts
- To evaluate the *invivo* anticancer activity
- Isolation and identification of R u i n

#### 6. PLAN OF WORK

Collection of the plant material

Authentication

#### PHARMACOGNOSTICAL STUDIES

Macroscopy

Microscopy

Physicochemical constants

Qualitative analysis of heavy metals and inorganic elements

Quantitative estimation of heavy metals

Pesticide residue analysis

#### 4.PHYTOCHEMICAL STUDIES

Preparation of extracts

Preliminary phytochemical screening and fluorescence analysis of powder and extracts

Quantitative estimation of phytoconstituents

Thin layer chromatography of extracts

High Performance Thin Layer Chromatography

#### SELECTION OF ACTIVE EXTRACT

*Invitro* anticancer activity by MTT Assay

#### ACUTE TOXICITY STUDIES

#### INVIVO ANTICANCER ACTIVITY

#### ISOLATION AND IDENTIFICATION OF RUTIN

### 7. PHARMACOGNOSTICAL STUDIES <sup>(33, 34)</sup>

Pharmacognostical studies basically deals with the identification, authentication and standardization of herbal medicinal plants through macroscopical, microscopical characters and physicochemical observations as prescribed by an authoritative source such as World Health Organization (WHO). This should be carried out before any tests are undertaken, because lack of proper identification leads to the usage of improper drug. Thus, in recent years there has been an emphasis in pharmacognostical standardization of medicinal plant of therapeutic potential which will help in the correct identification of the plant.

#### 7.1 MATERIALS AND METHODS

##### Collection and authentication of the plant

The fresh and healthy leaves of *Asparagus racemosus* Willd., were collected from Ariyaperumbakkam Village, Kanchipuram Dist., Tamil Nadu, India during the month of August 2014. The plant was identified

and authenticated by Dr. Jayaraman, Director, Plant Anatomy and Research Centre, Thambaram (PARC/2014/2316). A voucher specimen has been reserved in the Department of Pharmacognosy, COP, MMC, Chennai. The leaves were immediately fixed using FAA (formalin: acetic acid: ethyl alcohol) as fixative agent for anatomical studies.

## Macroscopy

Macroscopical characters include organoleptic characters and morphological features of the leaves were studied. **Microscopical studies** (35-38)

## Staining method

The collected leaves were fixed in FAA solution (formalin-5ml+ Acetic acid- 5ml + 70% Ethyl alcohol-90ml). After 24 hrs of fixing, it was dehydrated with graded series of tertiary butyl alcohol as per the schedule given by Sass 1940. Infiltration of the leaves was carried by gradual addition of paraffin wax (melting point 58-60° C) until TBA solution attained super saturation. The leaves were cast into paraffin blocks.

### Sectioning (39, 40)

The paraffin embedded leaves were sectioned with the help of Rotary Microtome. The thicknesses of the sections were 10-12µm. The sections were stained with Toluidine blue. Since, toluidine blue is a polychromatic stain different colours of the cells were obtained depending upon the nature of the cells.

## Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon lab photo 2 microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books.

### Powder microscopy (41, 42)

The shade dried, powdered leaves were used for powder microscopic analysis. The organoleptic characters were observed and to identify the different characteristic features, staining reagents were used. Powder was stained with 1% phloroglucinol in conc. hydrochloric acid and observed under microscope. All the lignified cells stained with pink colour.

### Physicochemical constants (43-45)

Shade dried powdered leaves of *Asparagus racemosus* was used for the determination of physicochemical constants in accordance with WHO guidelines.

## Ash values

Ash values are helpful in determining the quality and purity of a crude drug in the powdered form. The residue remaining after incineration is the ash content of the drug, which simply represents inorganic salts, naturally occurring to the drug or adhering to it or deliberately added to it, as a form of adulteration.

### Total ash

Total ash is designed to measure the total amount of ash remaining after incineration. This includes both physiological ash which is derived from plant tissue itself and non-physiological ash which is the residue of the extraneous matter adhering to the plant surface.

### Procedure

Silica crucible was heated to red hot for 30min and it was allowed to cool in a desiccator. About 2gm of powder was taken in to the silica crucible and it was ignited at a temperature not exceeding 600°C until free carbon, cooled in a desiccator for 30min and weighed. The percentage of ash was calculated with reference to the air dried drug.

### Water soluble ash

The total ash was boiled with 25ml of water for 5min. The insoluble matter was collected in an ashless filter paper, washed with hot water and ignited for 15min at a temperature not exceeding 450°C, cooled and weighed. The percentage of water soluble ash was calculated with reference to air dried drug.

### Acid insoluble ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

### Procedure

To the total ash 25ml of dil.HCl was added drop wise and ignited. The insoluble matter was collected in an ashless filter paper and washed with hot water until the filtrate is neutral. The insoluble matter was transferred into a crucible. The residue was cooled in a desiccator for 30min and weighed. The content of acid insoluble ash was calculated with reference to air dried drug.

### Sulphated ash

### Procedure

Silica crucible was heated to red hot for 30min and it was allowed to cool in desiccator. 2gm of substance was taken into the crucible, ignited gently at first until the substance is thoroughly charred. Cooled and the residue was moistened with 1ml of sulphuric acid, heated gently until fumes are no longer evolved.



Ignited at  $800 \pm 25^\circ\text{C}$  until all black particles disappeared. Cooled the crucible and few drops of sulphuric acid was added and heated again. The operation is repeated until two successive weighing do not differ by more than 0.5gm.

### **Determination of extractive values**

Extractive values are useful for the evaluation of phytoconstituents especially when the constituents of a drug cannot be readily estimated by any other means. Further these values indicate the nature of the active constituents present in a crude drug.

### **Determination of water soluble extractive**

5gm of the leaf powder was weighed and macerated with 100ml of chloroform water (95ml distilled water and 5ml chloroform) in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. The percentage of water soluble extractive value with reference to the air dried drug was calculated.

$$\text{Water soluble extractive value} = \text{weight of the dried extract} / \text{weight of the sample taken} \times 100$$

### **Determination of alcohol soluble extractive**

5gm of the powder was weighed and macerated with 100ml 90% ethanol in a closed flask for 24 hours. It was shaken frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly, taking precautions against loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. It was dried at  $105^\circ\text{C}$  for 1 hour in a hot air oven. The dish was cooled in desiccator and weighed. The process was repeated till the constant weight was obtained. The percentage of alcohol soluble extractive value with reference to the air dried drug was calculated.

$$\text{Alcohol soluble extractive} = \text{weight of the dried extract} / \text{weight of the sample taken} \times 100$$

### **Determination of non volatile ether soluble extractive (fixed oil content)**

A suitably weighed quantity of the drug was transferred to an extraction thimble and extracted with petroleum ether (Boiling Point  $40 - 60^\circ\text{C}$ ) in a Soxhlet apparatus for 6 hours. The extract was filtered into a tarred evaporating dish, evaporated and dried at  $105^\circ\text{C}$  to constant weight. The percentage of non volatile ether soluble extractive value with reference to the air dried drug was calculated.

### **Determination of volatile ether soluble extractive**

2gm of powdered drug was accurately weighed and extracted with anhydrous diethyl ether in a continuous extraction apparatus for 20 hours. The ether solution was transferred to tarred porcelain dish and evaporated spontaneously. Then it was dried over phosphorous pentoxide for 18 hrs and the total ether extract

was weighed. The extract was heated gradually and dried at 105°C to constant weight. The loss in weight represents the volatile portion of the extract.

### **Loss on drying <sup>(46)</sup>**

Specified quantity of the substances was taken in a previously ignited and cooled silica crucible and the substance was evenly distributed by gentle side wise shaking. The crucible with the contents and the lid were weighed accurately. The loaded crucible and the lid were placed in the drying chamber (105°C). The substance was heated for a specified period of time to a constant weight. The crucible was covered with the lid and allowed to cool in a dessicator at room temperature before weighing. Finally the crucible was weighed to calculate the loss on drying with reference to the air dried substance.

### **Determination of foaming index**

1gm of the coarsely powdered drug was weighed and transferred to 500ml conical flask containing 100ml boiling water. The flask was maintained at a temperature of 80-90°C for about 30min. It was then cooled and filtered into a volumetric flask and sufficient water was added through the filtrate to make up the volume to 100ml. The decoction was poured into 10 stopper test tubes (height 16cm, diameter 16mm) in successive portions of 1ml, 2ml, 3ml, 4ml up to 10ml and the volume of the liquid in each tube was adjusted with water to 10ml. The tubes were stoppered and shaken in a length wise motion for 15 seconds, two shakes per second. They were allowed to stand for 15min and the height of the foam was measured. The results were assessed as follows: If the height of the foam in every tube is less than 1cm, the foaming index is less than 100. If a height of 1cm is measured in any tube, the volume of the plant material decoction in the tube (a) is used to determine the index. If this tube is the first or second tube in a series, prepare an intermediate dilution in a similar manner to obtain a more precise result.

If the height of the foam is more than 1cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilution of the decoction in order to obtain a result. Calculate the foaming index using the following formula.

$$\text{Foaming index} = 1000/a$$

Where, a = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed.

### **Determination of swelling index <sup>(47)</sup>**

The swelling index is the volume in ml occupied by the swelling of 1gm of plant material under specified conditions. A specified quantity of the plant material previously reduced to the required fineness was accurately weighed and transferred into a 25ml glass stoppered measuring cylinder. The internal diameter of the cylinder should be about 16mm, the length of the graduated portion about 125mm, marked in 0.2ml divisions from 0 to 25ml in an upward direction. 25ml of water was added and the mixture was shaken thoroughly every 10min for 1hour, allowed to stand for 3 hours at room temperature. The volume in ml

occupied by the plant material was measured including any sticky mucilage. The mean value of the individual determination, related to 1gm of plant material was calculated.

### Foreign organic matter

100g of the drug sample was weighed and spread out as a thin layer. The foreign matter was detected by inspection with the use of a lens. Foreign matter found were separated and weighed and the percentage was calculated.

Foreign organic matter =  $\frac{\text{Weight of the sample after inspection}}{\text{Weight of the sample before inspection}}$

### Crude fiber content <sup>(48)</sup>

About 2g of the accurately weighed drug sample was extracted with ether. Then 200ml of 1.25% sulphuric acid was added to the extracted drug and the whole mixture was boiled for 30mins under reflux in a 500ml flask. The whole mixture was then filtered through a hardened filter and the residue was washed with boiling water until free of acid. The entire residue was rinsed back into the flask with 200ml boiling 1.25% sodium hydroxide solution and again boiled under reflux for 30mins. The liquid was then quickly filtered through a tarred filter and the residue on the filter was washed with boiling water until neutral, dried at 110°C to constant weight. The difference between the weight of the dried residue and that of the incinerated residue represents the weight of the crude fibre and expressed as percentage of the original weight of the material.

### ESTIMATION OF HEAVY METALS AND INORGANIC ELEMENTS <sup>(49)</sup>

Plant minerals play a vital role in metabolism and osmolality. Presence of elements vary with the soil, climate conditions etc. There are essential and non essential elements which may be beneficial or harmful to living things. Non essential elements like lead, arsenic, cyanide, chromium, cadmium, aluminium, silver bring about toxic effects resulting in intoxicification. Hence, qualitative estimation of inorganic elements in the powdered leaves of *Asparagus racemosus* Willd. were carried out.

### QUALITATIVE ANALYSIS OF INORGANIC ELEMENTS AND HEAVY METALS

To the ash of the drug material 50% v/v hydrochloric acid was added and kept for 1 hour. It was filtered and the filtrate was used for the following tests.

**Aluminium:** White gelatinous precipitate of aluminium hydroxide is formed on addition of ammonia solution. It is slightly soluble in excess of the reagent. The precipitate dissolves readily in strong acid and base, but after boiling it becomes insoluble.

**Arsenic:** Arsenous salts in neutral solution react with solution of copper sulphate to form green precipitate (Scheele's green) which on boiling gives a red precipitate of cupric oxide.

**Borate:** The mixture obtained by the addition of sulphuric acid and alcohol (95%) to a borate when ignited, burns with flame tinged with green.

**Calcium:** Solution of calcium salts, when treated with ammonium carbonate solution, yield a white precipitate after boiling and cooling the mixture (it is insoluble in ammonium chloride solution).

**Carbonate:** Carbonate, when treated with dilute acid, effervescence liberating carbon dioxide which is colourless and produces a white precipitate in calcium hydroxide solution.

**Chlorides:** Chlorides, when treated with silver nitrate solution, yield a white crude precipitate which is insoluble in nitric acid, but soluble after being well washed with water, in diluted ammonia, from which it is re precipitated by the addition of nitric acid.

**Copper:** An excess of ammonia, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue coloured solution.

**Iron:** Solution of ferric salts, when treated with potassium ferrocyanide solution, yields an intense blue precipitate which is insoluble in dilute HCl.

**Lead:** Strong solution of lead salts, when treated with HCl, yield a white precipitate which is soluble in boiling water and is deposited as crystals when the solution is cooled.

**Magnesium:** Solution of magnesium salts, when treated with ammonium carbonate solution and boiled, yield a white precipitate, but yield no precipitate in the presence of ammonium chloride solution.

**Mercury:** Solution of mercury salts, when treated with sodium hydroxide solution, yields a yellow precipitate.

**Nitrate:** With solution of ferrous sulphate no brown colour was observed but if sulphuric acid is added (slow from the side of the test tube), a brown colour is produced at the junction of two liquids, indicating the presence of nitrates.

**Phosphate:** Solution of phosphate when treated with silver nitrate, dilute ammonia solution and in dilute nitric acid yield yellow precipitate of normal silver ortho phosphate (distinction from meta and pyrophosphate) solution.

**Potassium:** Moderately strong potassium salts, which have been previously ignited to remove ammonium salts, when treated with perchloric acid (60%) yield a white crystalline precipitate.

**Silver:** Solution of silver salts, when treated with potassium iodide solution yield a cream coloured precipitate which is insoluble in dilute ammonia solution and in nitric acid.

**Sulphates:** Solution of sulphates, when treated with lead acetate solution yields a white precipitate which is insoluble in ammonium acetate solution and in sodium hydroxide.

**QUANTITATIVE ANALYSIS OF HEAVY METALS** <sup>(50)</sup>

The heavy metals are quantitatively determined by using Inductive coupled plasma-Optical emission spectroscopy. **Instrumentation parameters:**

**Instrument name:** Inductive coupled plasma-Optical emission spectroscopy.

**Instrument Model:** PE Optima 5300DV ICP-OES; Optical system Dual view-axial or radial.

**Detector system:** Charge coupled detector, (UV-Visible detector which is maintaining at -40° C) to detect the intensity of the emission line.

**Light source (Torch):** Positioned horizontally in the sample compartment along the central axis of the spectrometer optics. Changing from axial to radial viewing is a simple software command and is accomplished by computer control of a mirror located in the optical path. The torch assembly of this system comprises of two concentric quartz tubes. **Standard alumina injector:** 2.0mm inner diameter.

**Spray chamber:** Scott type

**Nebulizer:** Cross flow gem tip.

**Preparation of sample by acid digestion method:**

50mg of powder was treated with acid mixture of sulphuric acid: water in the ratio of 4:1 in the Kjeldahl flask and heated continuously till the solution is colourless. The sample mixture was then transferred in a 25ml volumetric flask and made up to the volume with distilled water. Blank solution was prepared as above without sample.

The standards of arsenic, lead, mercury and cadmium were prepared as per the protocol and the calibration curve was developed for each of them.

**PESTICIDE RESIDUE ANALYSIS (BY GC-MS)** <sup>(51, 52)</sup>

Medicinal plant materials may contain pesticide residues, which accumulate as a result of agriculture practices such as spraying, treatment during cultivation and administration of fumigants during storage. Furthermore, similar to other agriculture products, there could be contamination from microorganism, heavy metal and pesticides. The contamination of medicinal plants by pesticides may be a consequence of the use of pesticides during cultivation, migration from neighboring culture or due environmental contamination.

Since many medicinal preparation of plant origin are taken over long periods of time, limits for pesticide residues should be established, following recommendation of the food and agriculture organization (FAO) of the United Nation and World Health Organization (WHO) which have already established the safety index of these residues in food and animal feed. This guideline includes the analytical methodology of pesticide residues.

Out of various pesticides available only chlorinated hydrocarbon and related pesticides like aldrin, BHC, chlordane, dieldrin, DDT and few organo-phosphorous pesticides like carbophenothion retain a long residual action.

Chromatography (column and GC) is the principle method for the determination of pesticides residue. Samples are extracted by standard procedures, impurities are removed by partition or adsorption and moderately broad spectrum of pesticides are measured in single determination.

#### Instrument parameters and conditions

<b>Instrument</b>	Gas chromatography with mass spectroscopy
<b>Make and Model</b>	Hewlett Packard
<b>Specification</b>	Electron ionization source with Quadra pole analyzer
<b>Mass range</b>	10-425 atmosphere
<b>Detector</b>	Electron ionization detector

#### Procedure

Extraction of the pesticides from the solid sample was carried out by solvent extraction. About 2.0g of the powder was treated with 15ml of n-hexane: dichloromethane (4:1). Sample was blended for 3mins and the solution mixture was transferred to 50ml tubes and centrifuged for 12mins. The supernatant was concentrated at 40°C to a volume approximately to 1ml. The cleanup of the extract was carried out by solid- phase extraction on a column packed with silica gel. The column was conditioned with 6ml dichloromethane and not allowed to dry. The extract (1ml) was applied to the column and eluted with 45ml of n-hexane: dichloromethane (3:2 v/v). The elute was concentrated almost to dryness and diluted with n-hexane to a volume of 1.0ml. 1µl volume was injected automatically into the GC-MS.

## 7.2 RESULTS AND DISCUSSION

### MACROSCOPY

The leaves (cladodes) are like pine needles, small and uniform and flowers are white and have small spikes.

Leaf blade - triangular in transverse section

Margin -not visible.

-Sweet and bitter.

Taste



Colour	- Dark green
Size	-1-5mm long
Shape	-Pine needles (phylloclade's)
Odour	- Characteristic



**Fig 4 Whole plant of *Asparagus racemosus* Willd.,**



**Fig. 5 Leaves of *Asparagus racemosus* Willd.,**

## MICROSCOPY

The plant bears thin cylindrical climbing stem which bears thin membranous scaly leaves and a cluster of curved finger like greenish photosynthetic organs called cladodes or phyllodades.

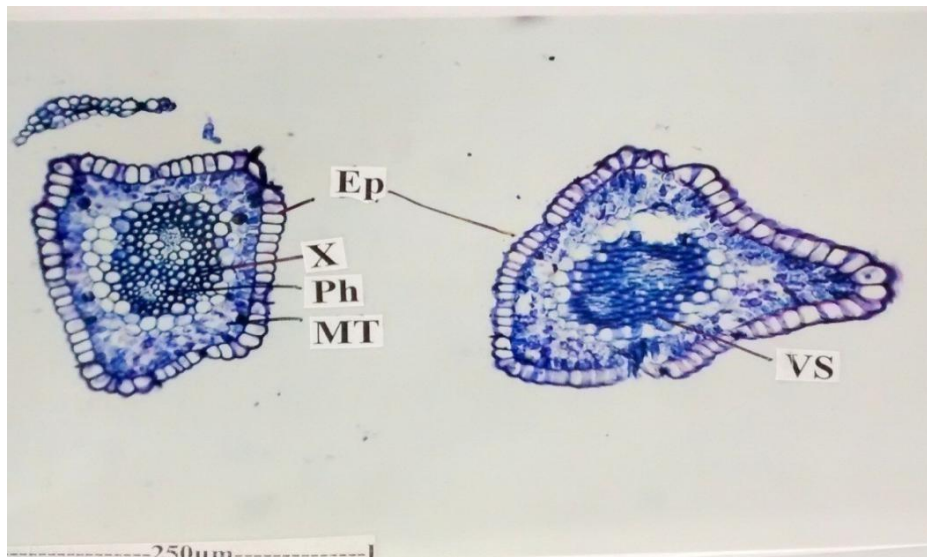
In cross section view the phyllodades appear 4 angled or 3 angled with lateral projection (Fig 6). The rectangular phyllodade is about 400µm thick. It consists of thick and wide radially oblong epidermal cells with fairly prominent cuticle. The epidermal cells are 40µm thick. Inner to the epidermis is 2 or 3 layer of circular, chlorophyllous palisade cells are seen. Inner to the palisade zone occurs 1 or 2 layer of thin walled parenchyma cells. The vascular systems consist of a thick circular cylinder of fibres enclosing two small vascular elements (Fig 7& 9). The vascular strands have small cluster of 2 or more wide, angular xylem elements and a thick mass of phloem elements (Fig 9). The bundle sheath fibres are heavily thick walled and lignified with narrow lumen. The xylem elements are 30µm in diameter. The fibro vascular system is 220µm in diameter.

The stomata are sunken in the epidermis in section. There are 2 conical stomatal ledges on the outer and inner side of the stoma (Fig 8).

**SCALE LEAF**

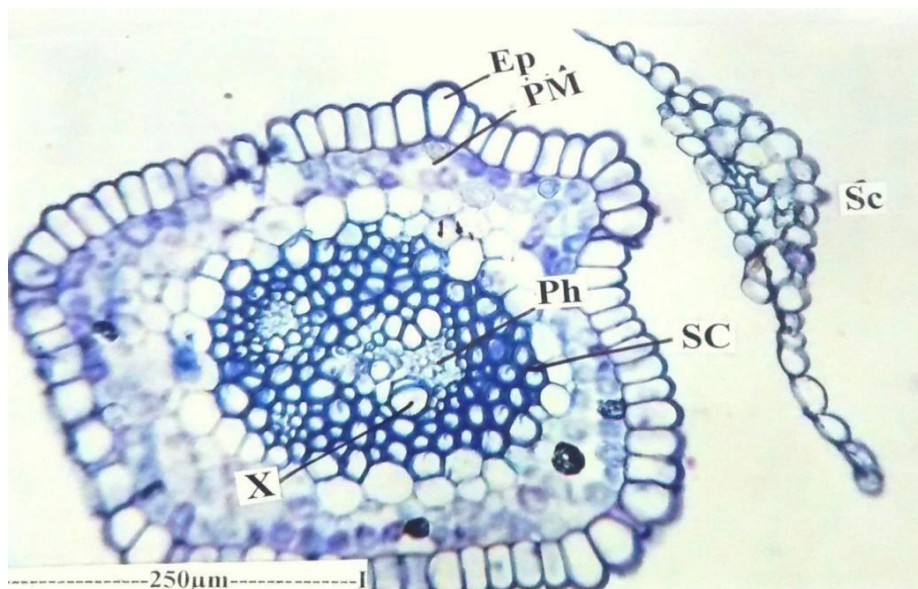
The scale leaf is thick and spindle shaped in the middle measuring 70µm thick. It consists of 3 layer of cell with central core of some 7-8 thick walled cells.

The marginal part of the scale is uniseriate; the cell being spindle shaped.



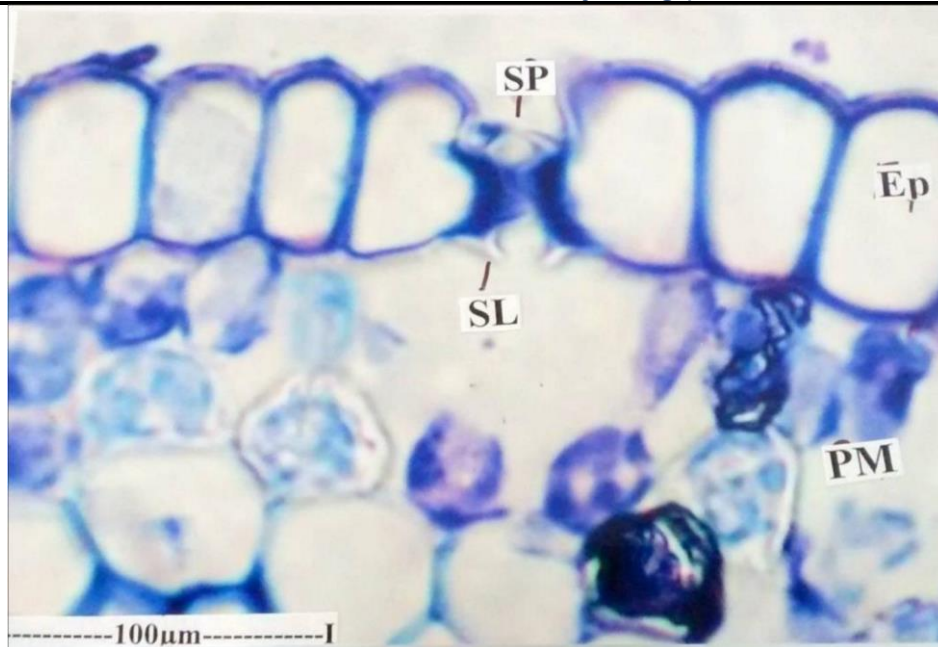
**Fig 6.T.S of Phyllodades entire view.**

(Ep-Epidermis, MT-Mesophyll tissues, Ph-Phloem, X- Xylem)



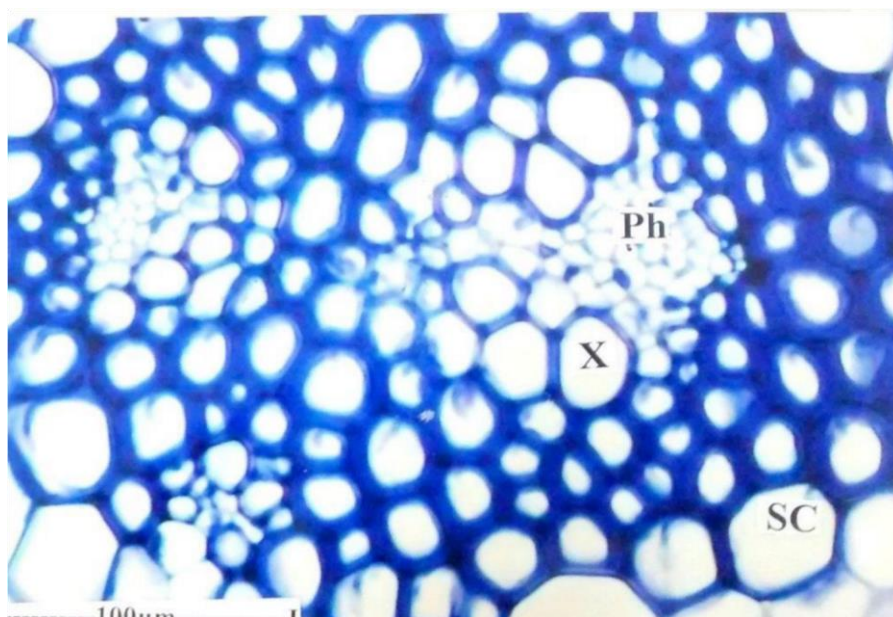
**Fig 7. T.S of a Phyllodade and scale leaf**

(Ep-Epidermis, PM-Palisade mesophyll, SC-Sclerenchyma, Sc-Scale, SL- Stomatal ledge, X- Xylem)



**Fig 8. T.S of Epidermis of the phyllode showing sunken stoma and stomatal ledges.**

(Ep-Epidermis, PM-Palisade mesophyll, SL- Stomatal ledge, SP-Stomatal pore)



**Fig 9. T.S of a fibro vascular system with two masses of phloem and xylem.**

(Ph-Phloem, SC-Sclerenchyma, X- Xylem)

## POWDER MICROSCOPY

### Organoleptic characters

Color	- Light greenish colour
Odour	- Characteristic
Taste	- Bitter



## Microscopical characters

The powder of the leaves showed the presence of lignified fibres, epidermis with palisade cells and stomata when examined under microscope which are of diagnostic importance



**Fig 10. Lignified fibre**



**Fig 11. Epidermis with palisade cells**



**Fig 12. Epidermis with Stomata**

## PHYSICOCHEMICAL PARAMETERS

Various physicochemical parameters were evaluated and the observed values for the physicochemical constants are given in Table 2.

**Table No: 2. Physicochemical Constants**

S. No.	Parameters	Percentage w/w
<b>I.</b>	<b>ASH VALUES</b>	
1.	Total ash	10.47±1.12
2.	Water soluble ash	05.20±0.45
3.	Acid soluble ash	01.34±0.38
4.	Sulphated ash	06.62±0.51

<b>II.</b>	<b>Extractive values</b>	
1.	Ethanol soluble extractive	11.42±0.27
2.	Water soluble extractive	38.86±0.47
3.	Ether insoluble non-volatile	21.74±0.35
4.	Ether insoluble volatile	11.23±0.73
<b>III.</b>	<b>LOSS ON DRYING</b>	04.68±0.44
<b>IV.</b>	<b>CRUDE FIBRE CONTENT</b>	51.32±1.42
<b>V.</b>	<b>SWELLING INDEX</b>	03.62±0.76
<b>VI.</b>	<b>FOAMING INDEX</b>	Less than 100

## INORGANIC ELEMENTS AND HEAVY METAL ANALYSIS

Qualitative estimation of inorganic elements were analyzed and the results were tabulated in Table 3.

**Table 3. Qualitative estimation of Inorganic elements**

S.No	Inorganic elements	Observation
1	Aluminum	+
2	Arsenic	-
3	Calcium	+
4	Carbonate	-
5	Chloride	+
6	Copper	+
7	Iron	-
8	Lead	-
9	Magnesium	+
10	Mercury	-
11	Phosphate	+

12	Sulphates	+
----	-----------	---

### Quantitative estimation of Heavy metals by ICP-OES method

The quantification of the individual heavy metals were done for the powdered leaves of *Asparagus racemosus* Willd., by ICP-OES technique, the following metals like arsenic, lead, cadmium and mercury were detected and quantified. The results are given in the Table 4.

**Table 4. Quantitative estimation of Heavy metals**

S.No	Element	Results (ppm)	Specification
1.	Mercury	0.0013	Not more than 0.5ppm
2.	Arsenic	0.0083	Not more than 5.0ppm
3.	Lead	Not detected	Not more than 10 ppm
4.	Cadmium	0.0037	Not more than 0.3ppm

The above observation showed that the heavy metal is within the limits as per WHO standard, so the leaves are safe to consume internally.

### DETERMINATION OF PESTICIDAL RESIDUE BY GC-MS METHOD

The powdered leaves of *Asparagus racemoss* Willd., was tested for the presence of any pesticide residues by Gas chromatography- Mass spectroscopy method using reference standards. The results obtained from the analysis were given in Table 5.

**Table 5. Determination of Pesticidal residues**

S.No	Pesticide groups	Pesticides
1	Organophosphorus	Absent
2	Carbamates	Absent



## DISCUSSION

Pharmacognostical studies play a key factor in establishing the authenticity of the plant materials. The botanical identity of the leaf was established by examining its morphological, anatomical features as well as the WHO recommended physicochemical studies. The results of this standardization may throw immense light on the botanical identity of the leaves of *Asparagus racemosus* which may be useful in judging the authenticity of the plant and also to differentiate the leaves from its adulterants and substitutes.

The macroscopy of the leaves were examined. The microscopical characters showed the presence of epidermis, mesophyll tissues, phloem, palisade mesophyll, sclerenchyma, scale, stomatal ledge, stomatal pore, vascular stands and xylem.

Powder microscopy showed the presence of lignified fibres, epidermis with palisade cells and epidermis with stomata. These features can be employed for inter specific identity of the drug.

Physicochemical parameters are mainly used in judging the purity and quality of the powdered drug. Ash values of a drug give an idea of the earthy matter or inorganic elements and other impurities present along with the drug. The ash values of a drug are used for detecting low grade product and exhausted drug and hence it proves to be important criterion to judge the purity of crude drugs. A high ash value is the indication of substitution, contamination and adulteration.

The total ash usually consists of carbonate, phosphate and silicates. Total ash was found to be  $10.47 \pm 1.12\%$  w/w. The acid insoluble ash indicates contamination with siliceous materials like earth and sand. The value was found to be  $1.34 \pm 0.38\%$  w/w. The value of water soluble ash was found to be  $5.20 \pm 0.45\%$  w/w. Sulphated ash is obtained by treatment with dil. sulphuric acid where the oxides are converted to sulphates. The value was found to be  $6.62 \pm 0.51\%$  w/w.

The parameter which is useful for the prediction of the nature of constituents is the extractive value. The alcohol soluble and water soluble extractives were found to be  $11.42 \pm 0.27\%$  w/w and  $38.86 \pm 0.47\%$  w/w respectively. Ether insoluble non-volatile and ether insoluble volatile extractive were found to be  $21.74 \pm 0.35\%$  w/w and  $11.23 \pm 0.73\%$  w/w respectively.

The alcohol soluble and water soluble extractive value indicates the presence of considerable amount of polar compounds. These constants would help to identify and to standardize the plant by future researchers.

The crude fiber content which is used as a distinguishing factor between similar drugs was found to be  $51.32 \pm 1.42\%$  w/w. Loss on drying determines the amount of volatile matter of any kind that can be driven off under conditions specified and was found to be  $04.68 \pm 0.44\%$  w/w. The swelling index and foaming index were found to be  $3.63 \pm 0.76$  ml/gm and less than 100 respectively.

The qualitative analysis of heavy metals and inorganic elements were carried out. The quantitative estimation of heavy metals and pesticide residue were carried out and it showed only trace amount of heavy metals (within the limits) when compared to beneficial elements and absence of pesticide residue.

These detailed pharmacognostical studies on leaves of *Asparagus racemosus* provides information on the identification of the drug and also used to differentiate the plant from its adulterants and substitutes.

## 8. PHYTOCHEMICAL STUDIES

Phytochemical evaluation is used to determine the nature of phytoconstituents present in the plant. These constituents are essential for the pharmacological activities of the plant. Therefore a complete investigation is required to characterize the phytoconstituents qualitatively and quantitatively.

### 8.1 MATERIALS AND METHODS

#### Preparation of Extracts <sup>(53)</sup>

Extraction is the preliminary step involved in the phytochemical studies. It brings out the metabolites into the extracting solvent depends upon its polarity.

#### Extraction

The shade dried, coarsely powdered leaves of *Asparagus racemosus* Willd., (500gm) was successively extracted with n-Hexane (60-80°C), chloroform, ethyl acetate and ethanol in a soxhlet apparatus. Each extract was concentrated using rotary vacuum evaporator. The percentage yield, colour and consistency of these extracts were recorded and used for further detailed phytochemical studies.

#### Preliminary phytochemical screening <sup>(54-56)</sup>

The chemical tests for various phytoconstituents in the dried powder and extracts of leaves of *Asparagus racemosus* Willd., were carried out as described below and the results were recorded.

#### Alkaloids

##### Dragendorff's test

To the sample, few drops of potassium bismuth iodide solution was added (Dragendorff's reagent), formation of reddish brown color indicates the presence of alkaloids.

##### Mayer's test

The substance was mixed with little amount of dilute hydrochloric acid and Mayer's reagent and examined for the formation of white precipitate.

##### Wagner's test

The test solution was mixed with Wagner's reagent and examined for the formation of reddish brown precipitate.

**Carbohydrates:****Molisch's test**

In a test tube containing sample, 2 ml of distilled water and 2 drops of freshly prepared 20% alcoholic solution of alpha naphthol were added and mixed well. 2ml of concentrated sulphuric acid along the side of the test tube was added. Formation of violet ring at the junction of two layers, which disappears on addition of excess alkali solution, indicates the presence of carbohydrates.

**Fehling's test**

The test solution was mixed with Fehling's A and B, heated and examined for the appearance of red coloration for the presence of sugar.

**Glycosides****Borntrager's test**

The sample was boiled with 1ml of sulphuric acid in a test tube for five minutes. Filtered while hot, cooled and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour in the ammonical layer indicates the presence of anthroquinone glycosides.

**Modified Borntrager's test**

The test material was boiled with 2ml of the dilute sulphuric acid. This was treated with 2ml of 5% aqueous ferric chloride solution (freshly prepared) for 5 minutes and shaken with equal volume of chloroform. The lower layer of solvent was separated and shaken with half of its volume of dilute ammonia. A rose pink to red colour is produced in the ammonical layer.

**Phenols**

Small quantity of sample was dissolved in 2 ml of distilled water, added a few drops 10% aqueous ferric chloride solution. Formation of blue or green colour indicates presence of phenols.

**Proteins (Biuret's test)**

To 1 ml of sample solution, 5 to 8 drops of copper sulphate solution (10%) was added. Formation of violet colour indicates the presence of proteins.

**Saponins**

To 5 ml of sample, added a few drops of sodium bicarbonates solution. Shake the mixture vigorously and left for 3 minutes. Formation of honey comb like froth indicates the presence of saponins.

## **Tannins**

### **Lead acetate test**

The test solution was mixed with basic lead acetate solution and examined for formation of a white precipitate.

### **Ferric chloride test:**

A few drops of 5% aqueous ferric chloride solution was added to 2ml of an aqueous extract of the drug and examined for the appearance of bluish black colour.

## **Detection of Steroids and Triterpenoids**

### **Libermann Burchards test**

The sample was treated with few drops of acetic anhydride, boiled and cooled. Conc.sulphuric acid was added from the sides of the test tube; brown ring is formed at the junction of two layers and upper layer turns green which shows presence of steroids and formation of deep red colour indicates presence of tri terpenoids.

### **Salkowski test**

The sample was treated with few drops of conc. sulphuric acid, red colour at lower layer indicates the presence of steroids and formation of yellow coloured lower layer indicates presence of tri terpenoids.

## **Detection of Flavonoids**

### **Shinoda's test**

To the sample, few piece of magnesium turnings and conc.HCl was added drop wise, pink to crimson red, occasionally green to blue colour appears after few minutes indicates the presence of flavonoids.

### **Alkaline reagent test**

To the test sample few drops of sodium hydroxide solution was added, intense yellow colour is formed which turns to colorless on addition of few drops of dilute acid indicates the presence of flavonoids.

## **Detection of Gums and Mucilages**

Small quantities of test substances was dissolved in 5 to 10ml of acetic anhydride by means of heat, cooled and add 0.05ml of conc. sulphuric acid; it is examined for the formation of bright purplish red colour.

## **Detection of fixed oils and fats**

Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oils and fats.

**QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS****Total phenolic content (Folin – Ciocalteu's assay) <sup>(57)</sup>**

Total phenolic content of the extracts were determined using Folin – Ciocalteu's assay. 0.5ml extracts were mixed with 2.5ml of 10 fold diluted Folin Ciocalteu's reagent and 2.5ml of 7.5% sodium carbonate. After incubation at 40°C for 30 minutes, the absorbance of the reaction mixtures were measured at 765nm in a spectrophotometer. Three replicates were made for each test samples. Gallic acid was used as a standard and total phenolic content of the extracts were expressed in mg of Gallic acid equivalents (mg GAE/g extract).

**Total flavanoid content <sup>(58)</sup>**

Total flavanoid content was determined by colorimetric method, using quercetin as a standard. The test samples were individually dissolved in DMSO. Then the sample solution (150 µl) was mixed with 150 µl of 2% aluminium chloride. After 10min of incubation at ambient temperature, the absorbance of the supernatant was measured at 435nm using spectrophotometer. Three replicates were made for each test samples. The total flavanoid content was expressed as quercetin equivalent in mg/gm of extract (mg QRT/gm extract).

**Total tannin content <sup>(59)</sup>**

0.1g of the extracts were transferred to a 100ml flask, 50ml of water was added and boiled for 30min. After filtration with cotton filter, filtrate was transferred to a 500ml flask and the volumes were made up to the mark with distilled water. 0.5 ml aliquots were transferred to vials, 1ml of 1%  $K_3Fe(CN)_6$  and 1 ml of 1%  $FeCl_3$  were added and the volume was made up to 10ml with distilled water. After 5 min absorbance was measured at 510nm against a reagent blank spectrophotometer and concentration of tannins in the test sample was determined and expressed as mg equivalent of tannic acid per gram of sample.

**FLUORESCENCE ANALYSIS <sup>(60, 61)</sup>**

Fluorescence analysis was carried out in day light and in UV light. The leaf powder was treated with different solvents and the fluorescence was observed in day light and in near and far UV light. The extracts were also observed under UV light.

**CHROMATOGRAPHY <sup>(62-64)</sup>**

Chromatographic methods are important analytical tool in the separation, identification and estimation of components present in the plant.

**THIN LAYER CHROMATOGRAPHY****Principle**

Thin layer chromatography is a technique used for the separation, identification of single or mixture of components present in various extracts. It is a reliable technique in which solute undergoes distribution between two phases, stationary and mobile phase. The separation is mainly based on the differential migration that occurs when a solvent flows along the thin layer of stationary phase.

This may be achieved by partition and adsorption depending on stationary phase used.

**Selection of mobile phase**

Solvent mixture was selected on the basis of the phytoconstituents present in each extracts from the vast analysis, best solvent was selected which showed good separation with maximum number of components.

**HPTLC- FINGERPRINT PROFILE** <sup>(65, 66)</sup>

HPTLC is one of the versatile chromatographic method which helps in the identification of compounds and thereby authentication of purity of herbal drugs. The time required in this method for the demonstration of most of the characteristic constituents of a drug is very quick and short. In addition to qualitative detection, HPTLC also provides semi-quantitative information on major active constituents of a drug, thus enabling an assessment of drug quality.

HPTLC serves as a convenient tool for finding the distribution pattern of phyto constituents which is unique to each plant. The fingerprint obtained is suitable for monitoring the identity and purity of drugs and for detecting adulteration and substitution. HPTLC technique is helpful in order to check the identity, purity and standardize the quantity of active principles present in the herbal extract.

**Instrument Conditions**

<b>Sample used</b>	Ethyl acetate
<b>Instrument</b>	CAMAG HPTLC
<b>HPTLC Applicator</b>	CAMAG LINOMAT IV
<b>HPTLC Scanner</b>	CAMAG TLC SCANNER II
<b>Sample dilution</b>	100mg of sample extracted with 1ml of Ethyl acetate
<b>Volume of injection</b>	20µl
<b>Mobile phase</b>	Toluene:Ethylacetate:Methanol:Formicacid (6:3:1:0.2)
<b>Lambda max</b>	254nm
<b>Lamp</b>	Deuterium



Stationary phase	Aluminum coated Silica Gel – MerkF254
------------------	---------------------------------------

## Equipment

A Camag HPTLC system equipped with a sample applicator Linomat IV, Twin trough plate development chamber, TLC Scanner II.

## Chromatographic conditions

The estimation has been done using the following chromatographic conditions. Chromatography was performed on a 10 × 10cm pre-activated HPTLC silicagel 60 F254 plate. Samples were applied to the plate as 6mm wide band with an automatic TLC applicator Linomat IV with nitrogen flow (CAMAG, Switzerland), 8mm from the bottom. Densitometric scanning was performed on CAMAG scanner II. The plates were pre-washed with solvent ethyl acetate.

## 8.2 RESULTS AND DISCUSSION

The results of phytochemical studies are as follows,

### Extraction

The percentage yield of successive extracts of leaves of *Asparagus racemosus* Willd., tabulated in Table 6

**Table 6. Percentage yield of successive extracts of leaves of**

*Asparagus racemosus* Willd.,

S.No	Extract	Method of Extraction	Physical nature	Colour	Percentage yield (% W/W)
	n-Hexane	Continuous extraction using Soxhlet apparatus	Semi solid	Yellowish brown	3.5%
	Chloroform		Semi solid	Green	4.89%
	Ethyl acetate		Semi solid	Yellowish brown	3.9%
	Ethanol		Semi solid	Brown	4.3%

**Qualitative phytochemical analysis**

Qualitative phytochemical analysis for the leaf powder and various extracts were carried out and the results are tabulated in Table 7.

**Table .7. Preliminary phytochemical screening of powdered leaves and extracts**

S.NO	Test	powdered leaf	n-Hexane	Chloroform	Ethyl acetate	Ethanol
1	Alkaloids	-	-	-	-	-
2	Carbohydrates	+	-	-	-	+
3	Flavanoids	+	+	+	+	+
4	Glycosides	-	-	-	-	-
5	Phenolic compounds	+	-	+	+	+
6	Proteins	-	-	-	+	+
7	Quinones	-	-	-	-	-
8	Saponins	+	-	+	+	+
9	Steroids	+	-	+	-	+
10	Tannins	+	-	-	+	+
11	Terpenoids	+	-	-	+	-
12	Volatile oils	-	-	-	-	-

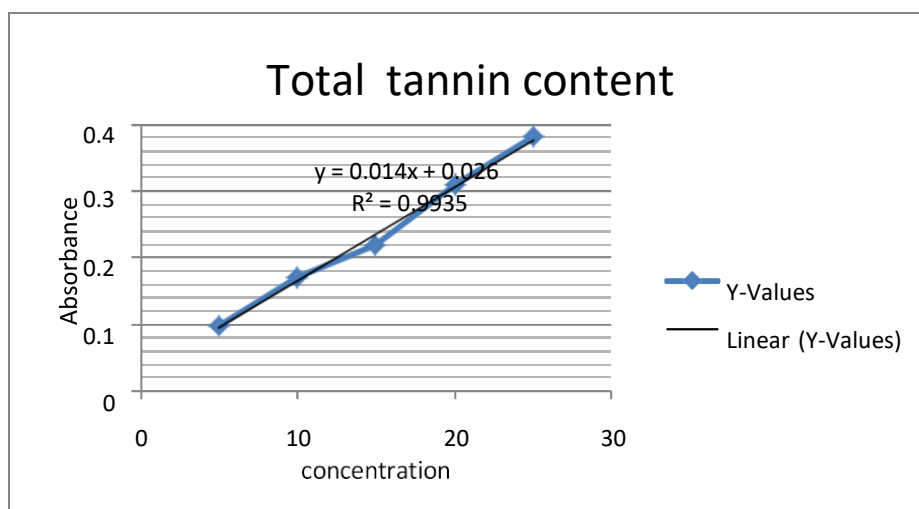
**Note:** + ve indicates positive result, whereas – ve indicates negative result

**QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS**

The phytoconstituents like tannin, flavanoid and phenolic compounds were estimated quantitatively and tabulated in Table 8, 9 and 10.

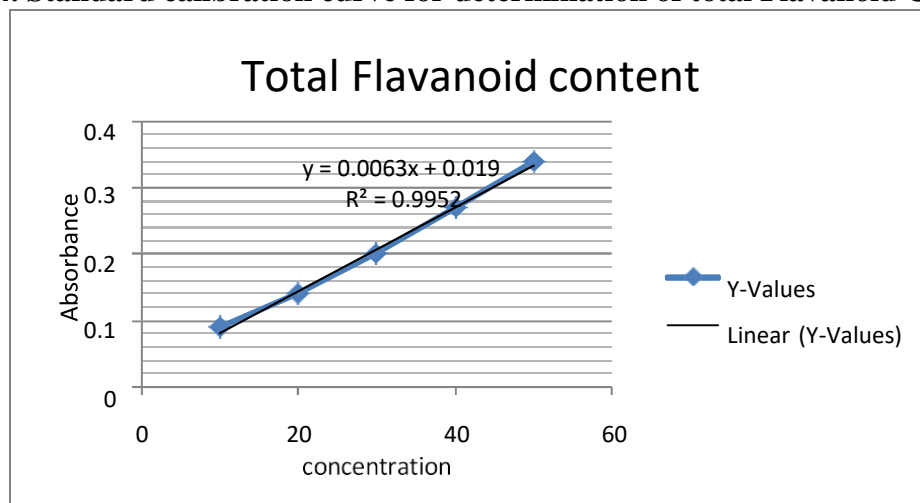
**TOTAL TANNIN CONTENT****Table 8. Determination of total tannin content**

S.No	Concentration of standard solution( $\mu\text{g/ml}$ )	Absorbance(765nm)
1.	5	0.10
2.	10	0.17
3.	15	0.22
4.	20	0.31
5.	25	0.38
6.	Chloroform	$0.20 \pm 0.019$
7.	Ethyl Acetate	$0.34 \pm 0.038$
8.	Ethanol	$0.37 \pm 0.046$

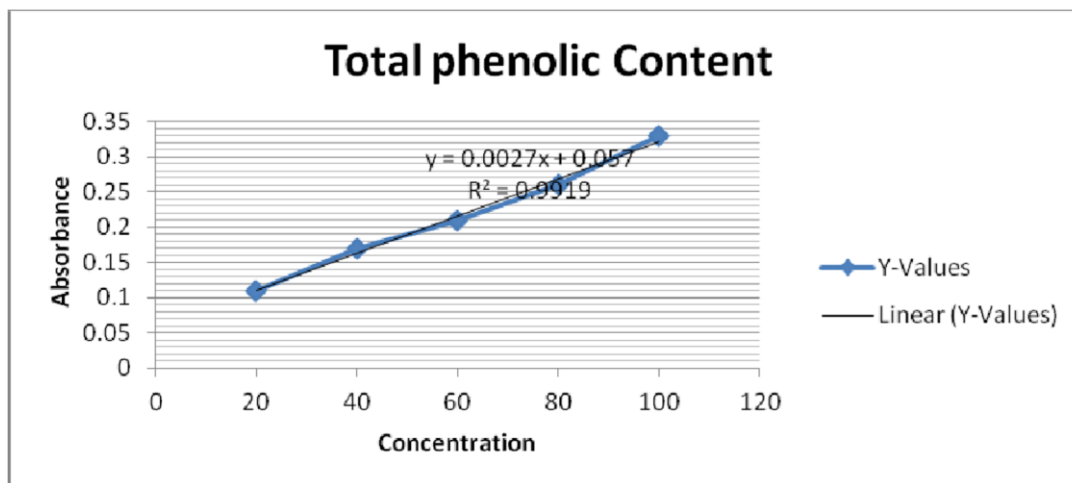
**Fig 13 Standard calibration curve for determination of total tannins Content****TOTAL FLAVANOID CONTENT****Table 9. Determination of Total Flavanoid content**

S.No	Concentration of standard solution( $\mu\text{g/ml}$ )	Absorbance(765nm)
1.	10	0.09
2.	20	0.14
3.	30	0.20
4.	40	0.27

5.	50	0.34
6.	Chloroform	$0.17 \pm 0.015$
7.	Ethyl Acetate	$0.19 \pm 0.037$
8	Ethanol	$0.29 \pm 0.028$

**Fig 14. Standard calibration curve for determination of total Flavanoid Content****TOTAL PHENOLIC CONTENT****Table 10. Determination of Total Phenolic Content**

S.No	Concentration of standard solution( $\mu\text{g/ml}$ )	Absorbance(765nm)
1.	20	0.11
2.	40	0.17
3.	60	0.21
4.	80	0.26
5.	100	0.33
6.	Chloroform	$0.18 \pm 0.027$
7.	Ethyl acetate	$0.22 \pm 0.038$
8	Ethanol	$0.26 \pm 0.034$

**Fig 15 Calibration curve for determination of total Phenolic content****Total 11. Amount of Tannins, Flavanoid and Phenolic compounds present in extracts**

S.No	EXTRACTS	TOTAL TANNIN CONTENT	TOTAL FLAVANOID CONTENT	TOTAL PHENOLIC CONTENT
1.	Chloroform	14.73±0.37µg/mg	4.67±0.56 µg/mg	40.31±0.49 µg/mg
2.	Ethyl acetate	16.58±0.45µg/mg	7.98 ±0.48µg/mg	60.85±0.29 µg/mg
	Ethanol	19.34±0.33µg/mg	34.44 ±0.62µg/mg	73.78±0.53 µg/mg

**FLUORESCENCE ANALYSIS**

The Fluorescence analysis of leaf powder and various extracts were carried out and given in Table 12 and 13.

**Table 12. Fluorescence characteristic of powdered leaves**

S.NO	TREATMENT	DAY LIGHT	SHORT UV (254nm)	LONG UV (366nm)
1.	Powder	Light green	Green	Dark green
2.	Powder + water	Yellowish green	Yellowish brown	Green
3.	Powder + NaoH	Yellowish brown	Dark brown	Dark green

4.	Powder + HCl	Yellowish brown	Dark brown	Dark green
5.	Powder + Acetic acid	Dark brown	Brown	Yellowish green
6.	Powder + Alc.KOH	Brown	Brownish green	Green
7.	Powder + KOH	Light green	Brown	Dark green
8.	Powder + Sulphuric acid	Dark brown	Dark black	Dark green
9.	Powder + Nitric acid	Yellowish green	Light brown	Yellowish green
10.	Powder + Iodine	Yellowish green	Brown	Green
11.	Powder + Fecl <sub>3</sub>	Brown	Dark brown	Green
12.	Powder +Ammonia	Light green	Greenish black	Dark green
13.	Ethanol	Greenish black	Brown	Green

Table 13. Fluorescence analysis of various extracts

S.NO	Extracts	Day light	Short UV	Long UV
1	n-Hexane	Yellowish brown	Brown	Dark brown
2	Chloroform	Green	Yellowish brown	Dark green
3	Ethyl acetate	Yellowish brown	Brown	Dark black
4	Ethanol	Brown	Green	Dark green

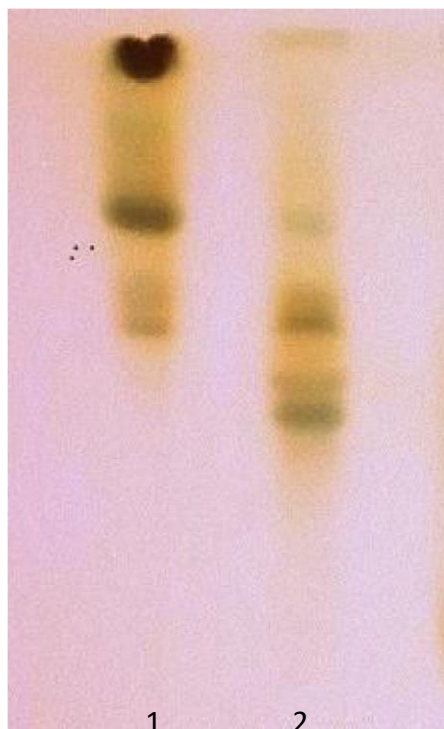


**THIN LAYER CHROMATOGRAPHY OF EXTRACTS**

Thin layer chromatography of n-Hexane, Chloroform, Ethyl acetate and Ethanol extracts were carried out and the results are tabulated in Table 14.

**Table. 14 Thin layer chromatography studies**

S.No	Extracts	Solvent system	No. of Spots	R <sub>f</sub> value
1.	n-Hexane	Ethyl acetate: Chloroform:Ethanol (5:3:2)	3	0.48 0.51 0.53
2.	Chloroform	Ethyl acetate: Chloroform:Ethanol (5:3:2)	4	0.36 0.39 0.46 0.49
3.	Ethyl acetate	Ethyl acetate: chloroform(6:4)	4	0.45 0.50 0.52 0.53
4.	Ethanol	Ethyl acetate: chloroform(6:4)	3	0.29 0.31 0.32

**Fig 16 TLC of extracts**

n-hexane extract

Chloroform extract

**Fig 17 TLC of extracts**

Ethyl acetate extract

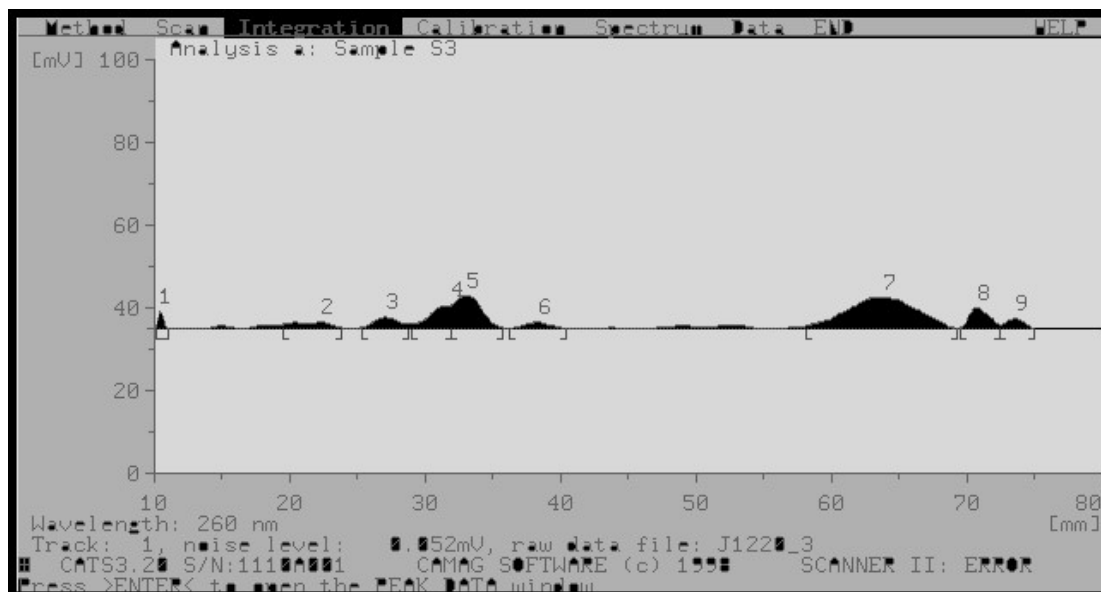
Ethanol extract

## HPTLC

High performance thin layer chromatography (HPTLC) finger printing was performed with the ethyl acetate extract of *Asparagus racemosus* Willd., The chromatographic conditions were carried as detailed in material and method of this study. There were 9 peaks observed with different  $R_f$  values and different heights. Percentages of areas were also obtained from the chromatogram.

**Table 15 HPTLC profile of ethyl acetate extracts**

S.NO	Extract	Solvent system	mber of spots	$R_f$ values
		Toluene:Ethylacetate:Methanol:Formicacid (6:3:1:0.2)	9	10, 22, 27, 32, 33, 38, 63, 70, 73

**Fig 18 HPTLC FINGER PRINT FOR ETHYL ACETATE EXTRACT OF***Asparagus racemosus* Willd.,

## DISCUSSION

In India most of the traditional knowledge on medical plants is in the oral form carried over generations to generations without any standard inventory. Necessary steps are needed for proper documentation, systematic regulation and widespread application. Since herbal medicines are prepared from materials of plant origin, they are prone to contamination, deterioration and variation in composition. Hence, before proceeding to clinical studies, scientists need to authenticate plants and also to detect their potency. A lot of analytical techniques have been developed for quality control of drug from plant origin.

Therefore it is very important to undertake phytochemical investigation along with biological screening to understand therapeutic dynamics of medicinal plant and also to develop quality parameter.

In this analysis, different polarity of phytoconstituents were sorted out from the coarsely powdered leaves of *Asparagus racemosus* Willd., by using solvents of increasing polarity like n-Hexane, Chloroform, Ethyl acetate and Ethanol in soxhlet apparatus. Successive extractive values revealed the solubility and polarity particulars of the metabolites in the powder. Percentage yield of various extracts were as follows, n-hexane (3.5%w/w), chloroform (4.89w/w), ethyl acetate (3.9w/w) and ethanol (4.35%w/w). Chloroform extract showed high extractive yield compared to other extracts.

Qualitative preliminary phytochemical analysis were performed with different chemical reagents to detect the phytoconstituent's nature and their presence in each extract and powder. The n-hexane extract showed the presence flavanoids. The chloroform extract showed the presence of flavanoids, phenolic compound, saponins and steroids. Ethyl acetate extract showed the presence of flavanoids, phenolic compounds, proteins, tannins and terpenoids. The ethanolic extract showed the presence of carbohydrates, steroids, flavanoids, phenols, proteins, saponnis, and tannins.

The quantitative phytochemical analysis for chloroform, ethyl acetate and ethanol extracts were performed. The total phenolic content was found to be  $40.31 \pm 0.49$ ,  $60.85 \pm 0.29$  &  $73.78 \pm 0.53 \mu\text{g/ml\%w/w}$ . The total tannin content was found to be  $14.73 \pm 0.37$ ,  $16.58 \pm 0.45$  &  $19.34 \pm 0.33 \mu\text{g/ml\%w/w}$ . The total flavanoid content was found to be  $4.67 \pm 0.56$ ,  $7.98 \pm 0.48$  &  $36.44 \pm 0.62 \mu\text{g/ml\%w/w}$ .

Fluorescence analysis for leaf powder and extracts were carried which is an important qualitative diagnostic tool to detected the presence of chromophore in the drug under UV and day light. There was no characteristic florescence observed with either in the powder or in the extracts.

Thin layer chromatographic analysis of all the extracts were carried out using different solvent systems. The hexane extract showed 3 spots, where as 4 spots were found in chloroform extract. Ethyl acetate extract showed 4 spots and ethanol extract showed 3 spots. TLC was performed for the identification of different components in the extracts qualitatively.

HPTLC was done for ethyl acetate extract and the finger print of the extract showed 9 spots with the  $R_f$  value of 0.10, 0.22, 0.27, 0.32, 0.33, 0.38, 0.63, 0.70 and 0.73.

Since secondary metabolites are responsible for the therapeutic activity, this study will be helpful in the selection of extract for pharmacological activity.

## SELECTION OF ACTIVE EXTRACT

### INVITRO ANTICANCER ACTIVITY

#### 9.1 MATERIALS AND METHOD

##### Method : MTT Assay

##### Extracts used: Chloroform, ethyl acetate and ethanol extracts

##### Cell line used

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

##### Cell treatment procedure

The monolayer cells were binding with trypsin-ethylen diaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a heamocytometer and diluted with medium containing 5% FBS to give final density of  $1 \times 10^5$  cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in dimethylsulfoxide (DMSO) and an aliquot

of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity<sup>(67)</sup>. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

### MTT assay

#### Principle

This colorimetric assay is based on the capacity of a mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the 3- [4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) into a insoluble purple formazan product which is measured spectrophotometrically. Only viable cells with active mitochondria reduces the MTT, the amount of formazan produced is directly proportional to the number of viable cells<sup>(68)</sup>.

#### Procedure

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using 96 well plate counter <sup>(69)</sup>.

The % cell inhibition was determined using the following formula. % Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC<sub>50</sub> was determined using GraphPad Prism software.

## 9.2 RESULTS AND DISCUSSION

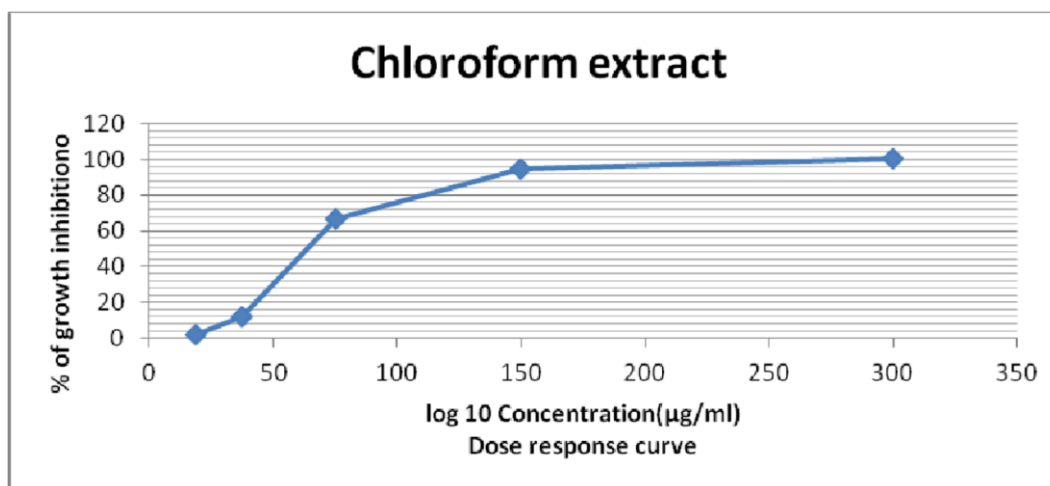
The *invitro* anticancer activity of the chloroform, ethyl acetate and ethanol extracts were given in Table 16, 17 and 18.

**Table 16. MTT Assay of Chloroform extract**

Plant extract	Conc.µg/ml	Absorbance	% inhibition	IC <sub>50</sub> µg/ml	R <sub>2</sub>
Chloroform extract of	18.75	0.4053	2.1721	62.587µg/ml	0.9993
	37.50	0.3633	12.3089		

<i>Asparagus racemosus</i>  Willd.,	75	0.1376	66.7739		
	150	0.2366	94.2880		
	300	0	100		

**Fig 19 DOSE RESPONSE CURVE OF CHLOROFORM EXTRACT OF *Asparagus racemosus* FOR HELA CELL LINE BY MTT ASSAY**

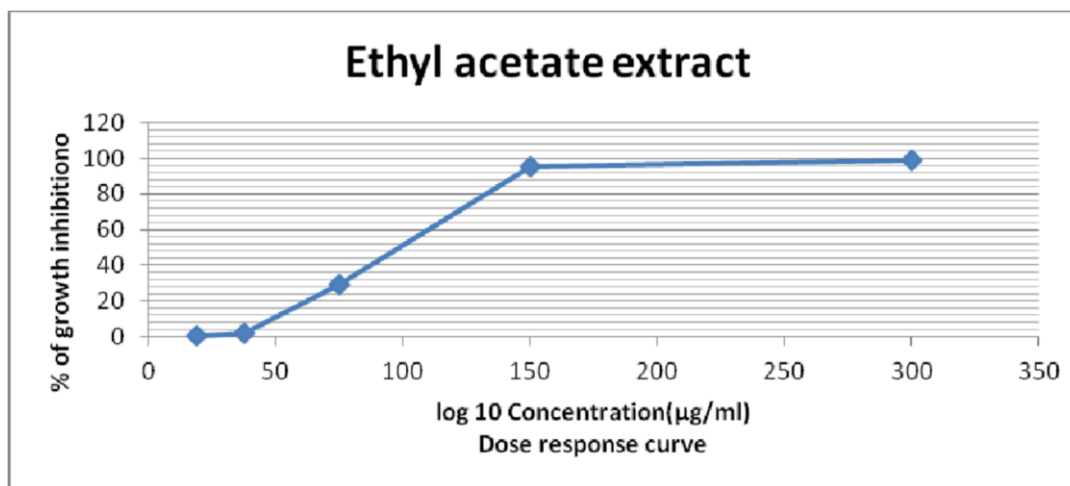


**Table 17. MTT Assay of Ethyl acetate extract**

Plant extract	Conc.µg/ml	Absorbance	% inhibition	IC <sub>50</sub> µg/ml	R <sub>2</sub>
<i>Ethyl acetate extract of Asparagus racemosus</i>  Willd.,	18.75	0.4133	0.2413	87.837µg/ml	0.9999
	37.50	0.4073	1.6894		
	75	0.2930	29.2839		
	150	0.0193	95.3338		
	300	0.0036	99.1150		



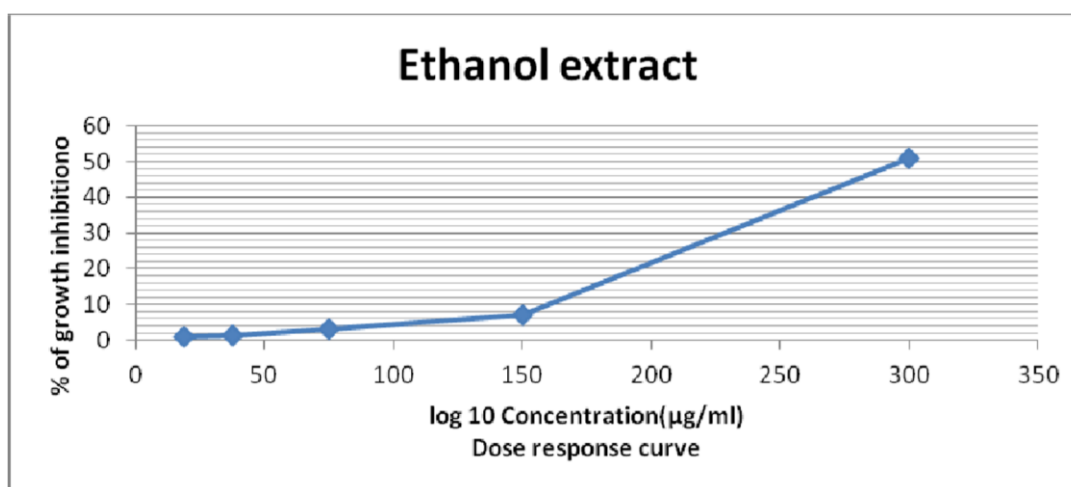
**Fig 20 DOSE RESPONSE CURVE OF ETHYL ACETATE OF *Asparagus racemosus* FOR HELA CELL LINE BY MTT ASSAY**



**Table 18. MTT Assay of Ethanol extract**

Plant extract	Conc.µg/ml	Absorbance	% inhibition	IC <sub>50</sub> µg/ml	R <sub>2</sub>
Ethanol extract of <i>Asparagus racemosus</i> Willd.,	18.75	0.4110	0.8045	297.7µg/ml	0.9953
	37.50	0.40833	1.4481		
	75	0.4016	3.0571		
	150	0.3836	7.1600		
	300	0.2040	50.764		

**Fig 21 DOSE RESPONSE CURVE OF ETHANOL OF *Asparagus racemosus* FOR HELA CELL LINE BY MTT ASSAY**



## DISCUSSION

The *invitro* anticancer study for chloroform, ethyl acetate and ethanol extracts were carried out by MTT assay. The extracts were screened for its cytotoxicity against HeLa cell line at different concentration to determine the IC<sub>50</sub> value.

The results are tabulated and graphically represented. The percentage growth inhibition was found to be increased with the increasing concentration of test compound. The  $IC_{50}$  value of Chloroform, Ethyl acetate and Ethanol extracts on the HeLa cell line were found to be 62.587, 87.837, 297.9  $\mu\text{g/ml}$  and  $R^2$  values were 0.9993, 0.9999, 0.9953 respectively. The chloroform and ethyl acetate extract showed significant *invitro* anticancer activity against HeLa cell line when compared to the ethanol which was taken for further *invivo* anticancer studies.

## 10. PHARMACOLOGICAL STUDIES

### 10.1 ACUTE TOXICITY STUDY <sup>(70 , 71)</sup>

#### 10.1.1 MATERIALS AND METHOD

Acute toxicity study was designed as per the OECD guidelines 423. (Acute toxic class method).

#### Principles and purposes

Acute toxicity testing determines the toxicity of a chemical or drug substances after single administration. The main purpose of acute toxicity study is to evaluate the degree of toxicity in a quantitative and qualitative manner.

The method of determination has changed in the last three decade mainly for animal welfare reasons producing mortality in animals in order to determine  $LD_{50}$  is no longer the main purpose of acute toxicity testing. The test is based on stepwise procedure with use of minimum number of animals per step. Sufficient information is obtained on the acute toxicity of the substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined dose. The substance is tested using a stepwise procedure, each step using three animals or a single sex (normally females). Absence or presence of compound related mortality of the animals dosed at one step will determine the next step i.e.

- No further testing is needed
- Dosing of three additional animals with the same dose
- Dosing of three additional animals with the next higher (or) the next lower dose levels

#### Selection of dose level and administration of dose

The traditional herbal medicine the mortality was unlikely at the highest starting dose level (2000mg/Kg body weight). Hence a limit test at dose level of 2000mg/Kg body weight was conducted in all three animals.

#### Experimental animals

Healthy Swiss Albino Mice either sex weighing between 20-25g were selected for the study. For all the three animals water was provided *ad libitum* and food was withheld overnight prior to dosing.

#### Observation

The animals were observed individually after dosing once during the first 30 minutes, periodically for the first 24 hours, with special attention given during the first 4 hours, and daily thereafter for a total of 14 days. The following clinical observation were made and recorded.

**Toxic signs**

All mice were observed for any toxic signs.

**Body weight**

Individual body weight was recorded for all the animals.

**Cage side observation**

The faeces colour, faeces consistency, change in skin and fur, eyes, mucus membrane (nasal) of the animals were observed.

**Physical Examination**

Physical observation included changes in respiratory system (rate), cardiovascular system (heart rate), autonomic nervous system (salivation, lacrimation, piloerection, urinary incontinence and defecation), central nervous system (drowsiness, convulsions, motor activity, writhing, motor in coordination, righting reflex, pinna reflex, corneal reflex and tremors were recorded.

**10.1.2. RESULTS AND DISCUSSION**

Behavioural and physical observation of the animals treated with ethyl acetate and chloroform extracts was noted of and tabulated in Table 19.

**Table 19. Acute toxicity studies**

OBSERVATION	30mins	4 hrs	24 hrs	14th day
Body weight	No change	No change	No change	No change
Preterminal deaths	Absent	Absent	Absent	Absent
Cage side observation	Normal	Normal	Normal	Normal
Motor activity	Normal	Normal	Normal	Normal
Convulsions	Absent	Absent	Absent	Absent
Piloerection	Absent	Absent	Absent	Absent
Righting reflex	Present	Present	Present	Present
Lacrimation	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal
Respiration	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal
Diarrhoea	Absent	Absent	Absent	Absent
Loss of corneal reflex	Normal	Normal	Normal	Normal
Loss of pinna reflex	Normal	Normal	Normal	Normal
Grooming	Absent	Absent	Absent	Absent
Sedation	Normal	Normal	Normal	Normal
Excitation	Normal	Normal	Normal	Normal
Aggression	Normal	Normal	Normal	Normal

The acute toxicity studies were done by OECD guidelines 423. No mortality was observed following the oral administration of leaves extracts even with the highest dose 2000mg/kg body weight. So, 1/5<sup>th</sup> of this dose (400mg/kg) was selected for the *invivo* anticancer activity.

## 10.2 INVIVO ANTICANCER ACTIVITY

### EHRlich'S ASCITES CARCINOMA <sup>(72, 73)</sup>

Ehrlich's Ascites Carcinoma (EAC) used in the present investigation has been maintained in the ascitic form in Albino Swiss mice by serial intraperitoneal transplantation. The tumor originally appeared in the mammary gland of a white mouse in 1907. The discovery was credited to Dr. P.Ehrlich. An ascitic form of this tumor was produced subcutaneously by Lowenthal and Jahn by intraperitoneal injection of tumor cell suspension. The ascitic fluid contained about  $2.5-10 \times 10^7$  tumor cells/ml at day 10 after transplantation (i.p.) of  $1 \times 10^6$  cells. The host animals survived approximately 3 weeks after tumor implantation.

#### 10.2.1. MATERIALS AND METHOD

##### Materials

EAC cell line, hemocytometer, hemoglobinometer, Thoma pipettes, tuberculin syringes, needle, sterile scalpel, forceps and scissors.

##### Extracts used

Chloroform and ethyl acetate extracts

##### Cell line

Ehrlich Ascites Carcinoma (EAC) cells were obtained through the courtesy of National Cancer Institute, Chennai, India. The EAC cells were maintained in Swiss Albino mice, by intraperitoneal (i.p.) transplantation of every 9 days<sup>(74)</sup>. The ascitic fluid was collected by syringe and the tumor cell count was performed in a Neubauer hemocytometer and  $2 \times 10^7$  cells/per ml was obtained by dilution with normal saline<sup>(75)</sup>. EAC cells 9 days old were used for the screening study.

##### Animals

Swiss Albino mice of either sex of 6-8 weeks age and weighing  $20 \pm 2$  g were used throughout the study. The animals were kept in polypropylene cages on sawdust beds, which were changed twice a week and maintained under standard laboratory conditions (temperature  $25 \pm 2^\circ\text{C}$ ) with dark/ light cycle (14/10 hrs). They were allowed free access to food and tap water *ad libitum*. The mice were acclimatized to laboratory condition for 1 week before the commencement of experiment.

**Table 20. Grouping of animals**

S.No	Groups	Description	Number of animals
1	Group I	Disease control	10
2	Group II	Chloroform extract (400mg/kg /p.o)	10
3	Group III	Ethyl acetate extract (400mg/kg/ p.o)	10
4	Group IV	5-fluorouracil(20mg/kg/i.p)	10

### Treatment schedule

Swiss Albino mice were weighed ( $20 \pm 2$  g) and divided into 4 groups (n=10) and given food and water *ad libitum*. EAC cells ( $2 \times 10^6$  cells/mouse) were injected intraperitoneally to each mouse of each group. This was taken as day 0. On the first day groups II and III were administered with chloroform and ethyl acetate extracts orally and group IV received standard drug 5-fluorouracil (20mg/kg) for 14 subsequent days. On 15<sup>th</sup> day, 18 hr after the last dose and 24 hr fasting five mice were sacrificed from each group and the rest of the animal of each group were kept to observe the life span of the tumor hosts.

The groups and the design of the experiment were as follows:

- Group I : EAC ( $2 \times 10^6$  cells /mice) + Normal saline (5ml of 0.9% NaCl / kg)
- Group II : EAC ( $2 \times 10^6$  cells /mice) +Chloroform Extract (400 mg/ kg/p.o)
- Group III : EAC ( $2 \times 10^6$  cells /mice) + Ethyl acetate extract(400 mg/ kg/p.o)
- Group IV : EAC ( $2 \times 10^6$  cells/mice) + 5-fluorouracil (20mg/kg/i.p)

### Ehrlich ascitic carcinoma tumor growth response

The ascitic fluid was completely harvested from each animal in all the groups and ascitic tumor volume, viable and nonviable tumor cell count by using 0.4% trypan blue dye exclusion assay method was performed.

### Tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume determined by centrifuging at 1000g for 5 min.

### Viable and nonviable tumor cell count

The cells were then stained with trypan blue dye (0.4% in normal saline). The cells that did not take up the dye were viable and those that took the stain were nonviable. These viable and nonviable cells were counted.

No. of cells x Dilution

Cell count = .....

Area x Thickness of liquid film

Mean survival time (MST), percentage of mean survival time (%MST) and percentage increased life span (% ILS) was evaluated to all the groups. MST of each group animals were monitored by recording the mortality daily and calculated using the following equation<sup>(76)</sup>.

The results are expressed as percentage of mean survival time of treated animals over mean survival time of the control group (Vs control, T/C %). The percentage of lifespan (ILS %) was calculated according to the formula:

$$\text{ILS\%} = (\text{T}-\text{C}) / \text{C} \times 100$$

Where T represents mean survival time of treated animals; C represents mean survival time of control animals. By National Cancer Institute criteria, T/C exceeding 125% and ILS exceeding 25% indicate that the drug has significant antitumor activity<sup>(77)</sup>.

At the end of experimental period, half of the mice were killed the next day after an overnight fasting by decapitation. Blood was collected retro orbital fluxes and used for estimation of heamoglobin (Hb) content, red blood cell count (RBC), white blood cell count (WBC) and WBC differential count.

0.1-Ml of heparinized blood was taken in Sahli's Heamoglobinometer and diluted with 0.1N HCl until the color matched with standard. The reading was then taken from the graduated cylinder and expressed as gm/100ml of blood.

The blood sample was diluted (1:200) with diluting fluid by using Thoma pipette.

Neutral Glycerine	20.0ml
Sodium Sulphate	8.0g
Sodium Chloride	1.0g
Methyl Violet	25.0mg
	160.0ml

After vigorous mixing, a drop of resultant mixture was discharged under the cover glass of Neubauer haemocytometer and the corpuscles were allowed to settle for 3 minutes. The number of erythrocytes in 80 small squares were counted under light microscope. The number of cells in 1  $\mu$ l of undiluted blood was calculated by:



RBC count/  $\square$ l = Number of cells in 80 small squares  $\times$  (1.0/0.02)  $\times$  200

### Total count of leukocytes

Blood was diluted 1:20 with a diluting fluid containing 3% acetic acid and a few drops of aqueous solution of methylene blue. The Neubauer hemocytometer was filled similarly with the mixture as in case of erythrocyte counting and the number of cells in four corner blocks (each block subdivided into 16 squares) were counted. The total leukocyte count per  $\square$ l of blood was calculated from multiplying the average number of cells in four blocks by 200.

### Differential count of leukocytes

Freshly drawn blood without anticoagulant was used to prepare blood films on perfectly clean grease free slides.

The smears were air dried and stained with Leishman's stain at pH: 6.4-6.8 (One gram of dry Leishman's-eosin-methylene blue was placed in a clean, dry, dark bottle and 500 ml of acetone free methyl alcohol was added. The mixture was filtered, kept in a dark place for at least a week and shaken daily for five minutes. A buffer solution of pH: 6.4 was used during staining to maintain the pH of the stain around (6.4-

6.8). The blood film was covered with undiluted stain and left for one minute.

- The stain was washed with two volumes of buffer solution until a metallic scum appears.
- The mixture was allowed to remain for 8-10 min; the slides were then flooded with tap water and were washed. The slides were air dried and observed under oil immersion objective of a light microscope. The cells were identified and counted according to their nuclear-cytoplasmic characteristics. The percentage distribution of each type of white blood cell (WBC) was calculated after counting at least 100 leukocytes.

### Statistical analysis

The experimental data were expressed as mean $\pm$ SEM. The data were analyzed using ANOVA and Dunett's test. The results were considered statistically significance if  $p < 0.05$ .

## 10.2.2 RESULTS AND DISCUSSION

The invivo anticancer activity for chloroform and ethyl acetate extract were carried out and tabulated in Table 21 and 22.

**Table 21. Effect of Chloroform and Ethyl acetate extract of *Asparagus racemosus* on the tumor growth parameter**

Groups	Body weight (gm)	Mean survival time (days)	%ILS	Tumor volume (MI)	Viable cell count (10 <sup>7</sup> cells/ml)	Nonviable cell count (10 <sup>7</sup> cells/ml)
I Group EAC control	25.35 $\pm$ 0.31	18.64 $\pm$ 1.25	-	6.92 $\pm$ 0.13	11.64 $\pm$ 0.09*	3.23 $\pm$ 0.73
Group II EAC+ (Chloroform Extract 400 mg/kg.b.w)	25.35 $\pm$ 0.31*	23.53 $\pm$ 0.40*	26.23*	6.44 $\pm$ 0.15*	6.25 $\pm$ 0.03*	0.77 $\pm$ 0.02*
Group III						

(Ethylacetate Eaxtract 400 mg/kg.b.w)	22.57±0.43*	27.34±0.32*	46.67*	5.47±0.16*	4.21±0.03*	1.55±0.10*
Group IV EAC+(5-FU, 20mg/kg.b.w)	21.34±0.12*	34.9 ± 0.41*	87.32*	3.62±0.14*	3.34±0.02*	6.91±0.05*

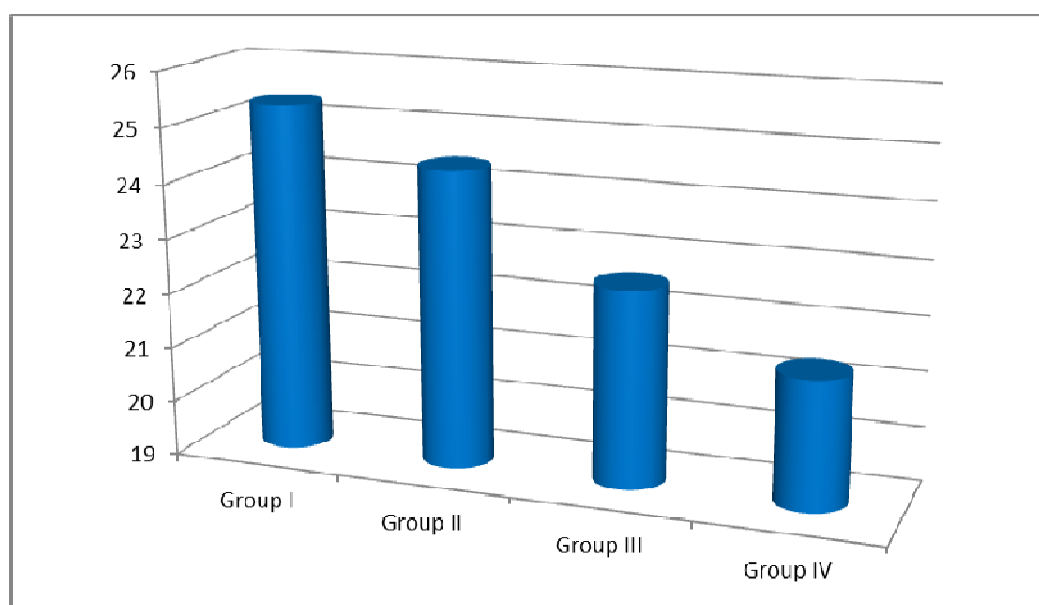
Values are as mean± SEM. Number of mice in each groups (n=10),\*P<0.05 as compared with group I, \*P <0.05 considered as significant.

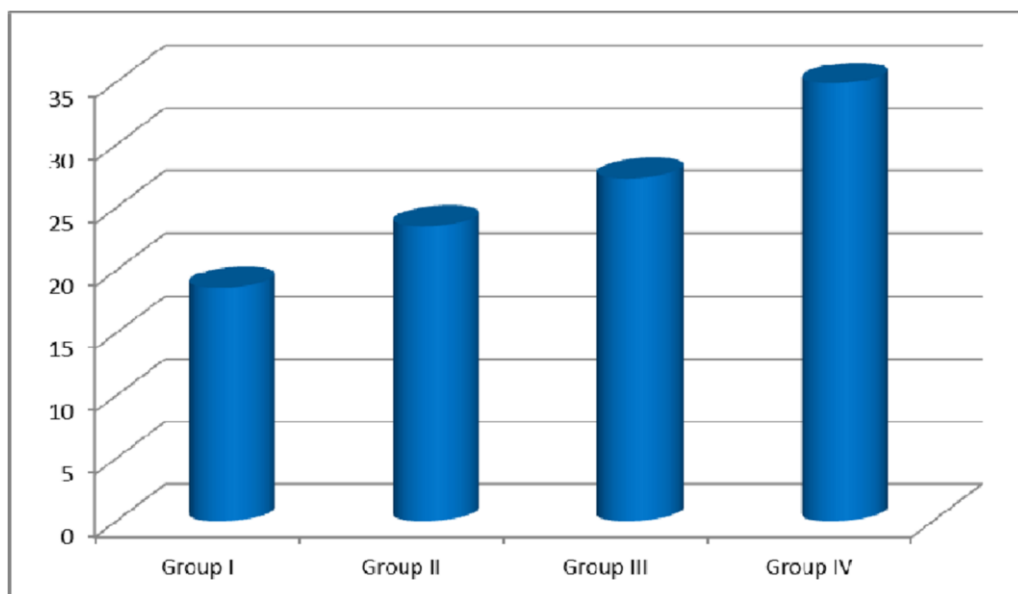
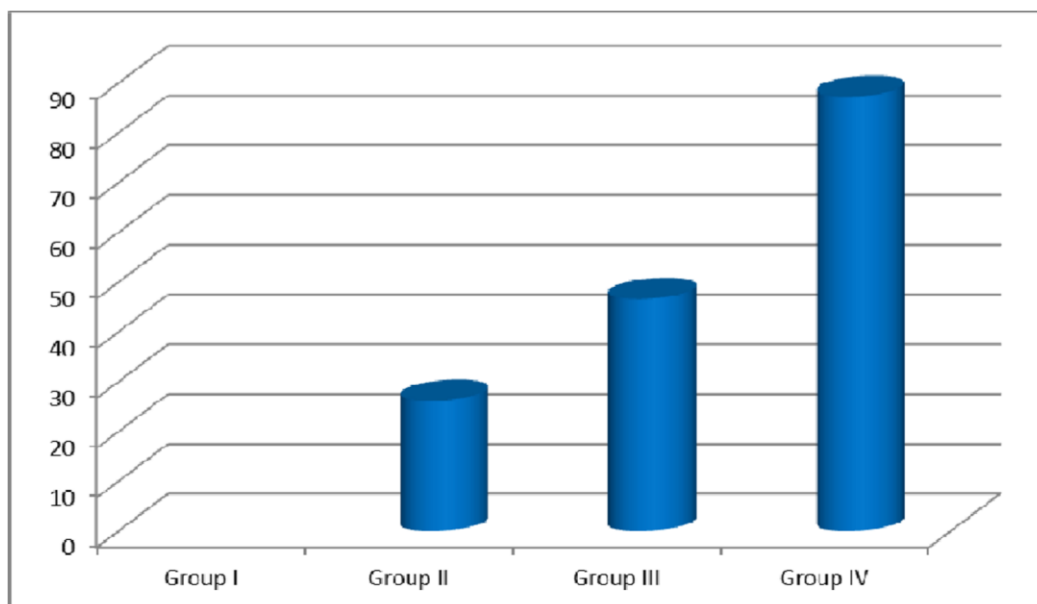
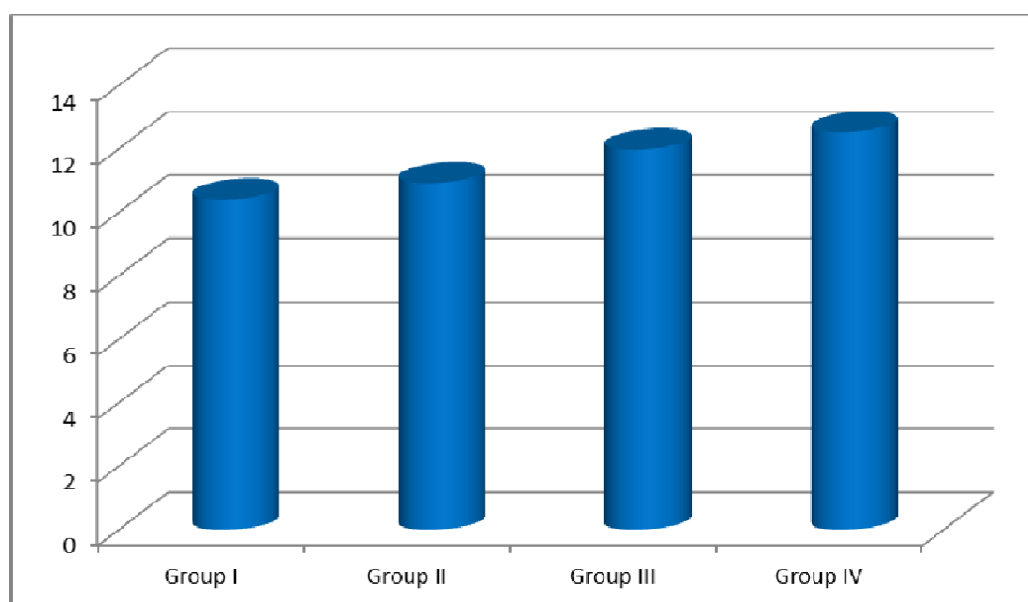
**Table 22. Effect of Chloroform and Ethyl acetate extract of *Asparagus racemosus* on heamatological parameters**

Groups	Heamoglobin (g/dl)	RBC (Count x 10 <sup>8</sup> )	WBC (Count x 10 <sup>8</sup> )	Lymphocyte (%) <sup>b</sup>	Monocyte (%) <sup>b</sup>	Neutrophil (%) <sup>b</sup>
I Group EAC control	10.40±1.23	3.26±0.58	5.31±0.30	40.72±0.82	2.32±0.15	26.17±0.20
Group II EAC+ (Chloroform Extract 400 mg/kg.b.w)	10.92±0.82***	3.76±0.03***	8.21±0.58***	63.05±0.46 ***	16.61±0.21***	4.62±0.18 ***
Group III EAC+(Ethylacetate Eaxtract 400 mg/kg.b.w)	11.97±0.17**	6.10±0.26 **	9.37±0.20 **	76.47±0.61**	5.05±0.33 **	17.95±0.21 * *
Group IV EAC+(5-FU, 20mg/kg.b.w)	12.52±0.31**	5.62±0.31**	11.75±0.17**	77.60±0.50**	4.20±0.27**	17.37±0.30**

Vales are expressed as means± SEM. Number of mice in each groups (n=10), \*\*\*P<0.001, \*\*P<0.01 when compared to EAC control group, P<0.05 is considered as significant

**Fig 22 GRAPHICAL REPRESENTATION OF BODY WEIGHT(gm)**



**Fig 23 GRAPHICAL REPRESENTATION OF MEAN SURVIVAL TIME(days)****Fig 24 GRAPHICAL REPRESENTATION OF % INCREASE IN LIFE SPAN****Fig 25 GRAPHICAL REPRESENTATION OF HEAMOGLOBIN(%)**

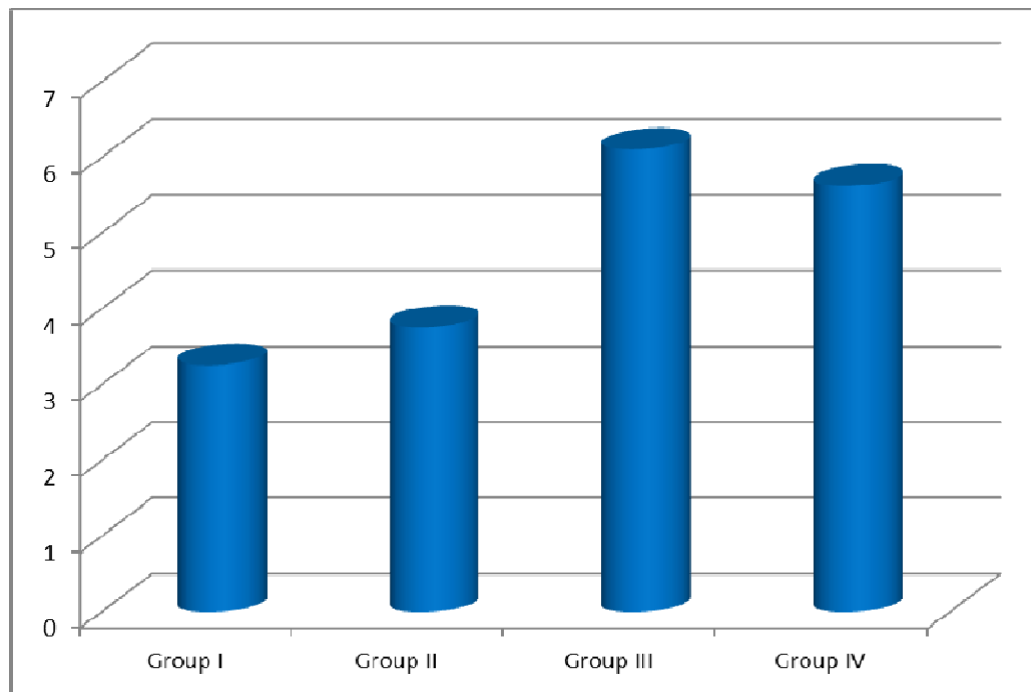
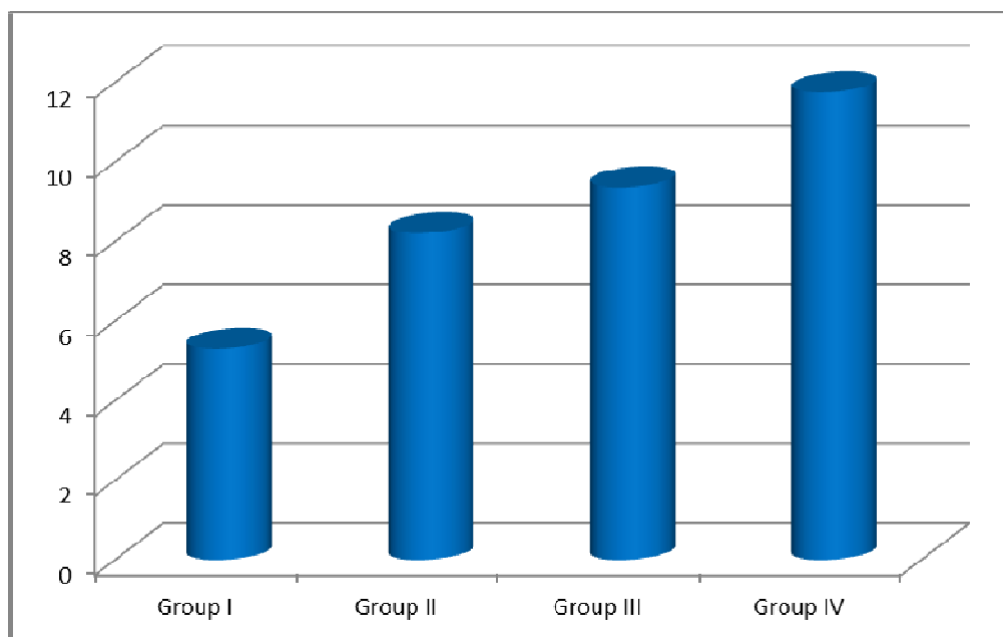
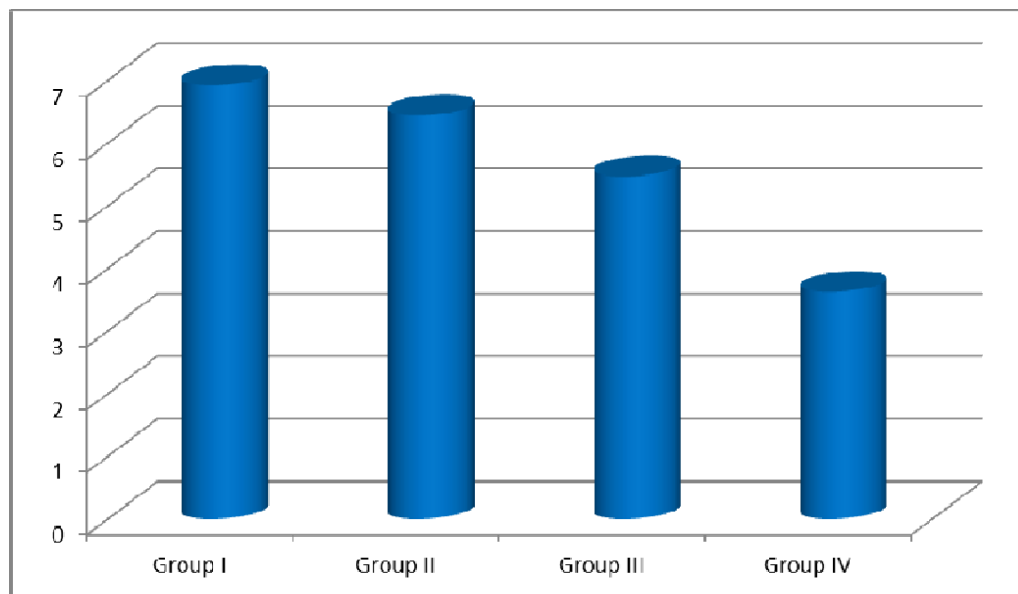
**Fig 26 GRAPHICAL REPRESENTATION OF RBC COUNT ( $1 \times 10^6$  cells/mm<sup>3</sup>)****Fig 27 GRAPHICAL REPRESENTATION OF WBC COUNT ( $1 \times 10^3$  cells/mm<sup>3</sup>)**

Fig 28 GRAPHICAL REPRESENTATION OF CELL VOLUME(ml)



### 10.2.2 RESULTS AND DISCUSSION

The result suggests that the chloroform and ethyl acetate extract of *Asparagus racemosus* showed significant antitumor activity in EAC bearing mice. The effect of *A. racemosus* extracts at the dose of 400mg/kg on biological parameters are discussed below.

#### Effect of *Asparagus racemosus* on mean survival time and tumor growth

The present investigation indicates that the Chloroform and ethyl acetate showed significant antitumor activity in EAC bearing mice. The effects of chloroform and ethyl acetate (400 mg/kg) at on the body weight, tumor volume, viable and nonviable cell count, survival time and ILS, were shown in Table 21 and 22. Administration of chloroform and ethyl acetate extract reduces the tumor volume, packed cell volume and viable tumor cell count when compared to EAC control mice. In EAC control mice the mean survival time was  $18.64 \pm 1.25$  days. Whereas, it was significantly increased when treated with chloroform, ethyl acetate and standard drug ( $23.53 \pm 0.40$ ,  $27.34 \pm 0.32$ ,  $34.9 \pm 0.41$  days) respectively.

The increase in the life span was observed with the mice treated with chloroform, ethyl acetate and 5-fluorouracil ( $P < 0.01$ ) as compared to the control group.

#### Effect on heamatological parameters

The heamoglobin content in the EAC control mice (9.8g %) was significantly decreased where as in extract treated group the percentage of heamoglobin was increased to  $10.92 \pm 0.82$  g % and  $11.97 \pm 0.17$  g %. Moderate changes in the RBC count were also observed in the extract treated mice. The total WBC counts were significantly higher in the EAC control mice whereas, extract treated mice shows significant reduction in the WBC counts as compared to that of control mice. The differential leukocyte count, the percentage of neutrophils was increased while the lymphocyte count was decreased in the extract treated mice when compared with EAC control mice.

The present study was carried out to evaluate the anticancer effect of *Asparagus racemosus* on EAC bearing mice. The ethyl acetate extract of *Asparagus racemosus* showed significant anticancer activity when compared to chloroform extract against the transplantable tumor. The reliable criteria for judging the value of any anticancer drug

are the prolongation of life span of animals. A reduction in the number of ascitic tumor cells may indicate either an effect on peritoneal macrophages or other components of the immune system, therefore increasing their capacity of killing the tumor cells, or a direct effect on tumor cell growth. The ethyl acetate extract of leaves of *Asparagus racemosus* inhibited significantly the tumor volume, viable cell count and enhancement in survival time of EAC bearing mice and thereby acts as anti-neoplastic agent.

Myelo suppression is a frequent and major complication of cancer chemotherapy. The treated and subsequent tumor inhibition resulted in appreciable improvements in hemoglobin content, RBC and WBC counts. These observations assume great significance, as anemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis and thereby limiting the use of these drugs. The improvement in hematological profile of the tumor bearing mice following the treatment with extract could be due to the action of the different phytoconstituents present in the extract.

## 11. ISOLATION AND IDENTIFICATION OF RUTIN

Since rutin was found to possess anticancer activity an attempt was made to isolate and identify the rutin from the Ethyl acetate extract of *Asparagus racemosus*. Rutin, also called as rutoside, quercetin-3-rutinoside and sophorin, is the glycoside between the flavonol quercetin and the disaccharide rutinose ( $\alpha$ -L-rhamnopyranosyl- (1 $\rightarrow$ 6))- $\beta$ -D glucopyranose). Rutin is that the rhamno glucoside of the flavonoid quercetin, and located in several plants and used for treatment of various diseases associated with the vascular. The molecular formula  $C_{27}H_{30}O_{16}$ <sup>(80)</sup>

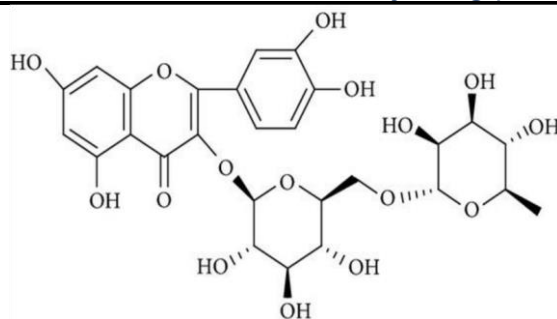
### Occurrences

*Asparagus* leaves and flower have been found to contain more rutin (about 2.5% dry weight)<sup>(82)</sup>. Rutin is one of the phenolic compounds found in the invasive plant species *Carpobrotus edulis*. Rutin is a citrus flavonoid glycoside found in many plants including buckwheat, the leaves and petioles of *Rheum* species. Rutin is also found in the fruit of the *Fevadanta* tree, fruits and flowers of the pagoda tree, fruits and fruit rinds (especially the citrus fruits orange, grape fruit, lemon and lime) and apple; berries such as mulberry, ash tree fruits, aronia berries and cranberries<sup>(84)</sup>.

### Chemical relatives

Rutin (quercetin rutinoside), like quercetin, is a glycosides of the flavonoid quercetin. As such, the chemical structure of both are very similar, with the difference existing in the hydroxyl functional group. Both quercetin and rutin are used in many countries as medication for blood vessel protection, and are ingredients of numerous multivitamin preparation and herbal remedies<sup>(83, 84)</sup>.





**Fig 29 Structure of Rutin**

### Uses of rutin

Anti-inflammatory, antioxidants inhibit platelet aggregation and decrease the capillary permeability, making the blood thinner and improving the circulation, strengthens the capillaries and can be used to treat hemorrhoids, varicose vein and unpleasant- looking venous oedema of the legs, lowered LDL cholesterol and the risk of atherosclerosis and heart beats<sup>(85)</sup>. Rutin having a VEGF inhibitor (angiogenesis inhibitor) that can help to prevent some cancer. Moreover rutin has been used clinically as a therapeutic medicine because it's strong antioxidant properties<sup>(86)</sup>. So the present study is aimed to isolate and study the anticancer activity of rutin present in *Asparagus racemosus*.

## 11.1 MATERIALS AND METHOD

**Extract used : Ethyl acetate extract**

**Method : Partition separation**

### Procedure <sup>(87)</sup>

To 3gm of extract, 25 ml of H<sub>2</sub>O was added and extracted with petroleum ether (50ml x 3), then with chloroform (50 ml x 3). After extraction, the aqueous layer was collected and left to stand during a cold place for 72 hours; a yellow precipitate separated out of the solution. The precipitate was filtered and washed with a combination of chloroform: ethyl acetate: ethanol (50:25:25). The undissolved part of the precipitate was dissolved in hot methanol and filtered, the filtrate was evaporated to dryness. The yellow powder of Rutin obtained.

### Identification of Isolated Rutin

#### Melting point

Melting point of the isolated compound was determined by using melting point apparatus.

#### Chemical test

To the isolated compound copper acetate was added, formation of emerald green colour indicates the presence of rutin.

## TLC

The isolated compound was subjected in to TLC studies

Stationary phase: Pre-coated aluminum sheet with silica gel G

Mobile phases: Butanol: acetic acid: water (5:3:5)

## SPECTRAL STUDIES

The isolated compound was subjected to IR spectral analysis

### INFRA-RED SPECTROSCOPY

It is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. It is carried out for the determination of chemical functional group in the sample. The commonly used sampling techniques for solid include pressed pellet technique and for liquid sample Nujol mull technique. The most useful IR region lies was between  $4000\text{-}670\text{ cm}^{-1}$ . Vibration (stretching and bending) that cause changes in dipole moment and bond length when IR radiation of suitable frequency is applied, then the vibration are infra-red active.

## 11.2 RESULTS AND DISCUSSION

### Physical and chemical properties of isolated compound

<b>Colour</b>	Greenish-yellow
<b>Nature</b>	Powder
<b>Melting point</b>	188- 190 $^{\circ}\text{C}$
<b>Solubility</b>	Soluble in water
<b>Chemical test</b>	Emerald green colour was obtained with copper acetate test its indicates presence of Rutin
<b>TLC solvent system</b>	Butanol: acetic acid: water (5:3:5)
<b>R<sub>f</sub> value</b>	0.82



Fig 30 TLC Rutin

## IR SPECTROSCOPY

IR  $\text{Vcm}^{-1}$  : KBr disk method

## IR SPECTRUM

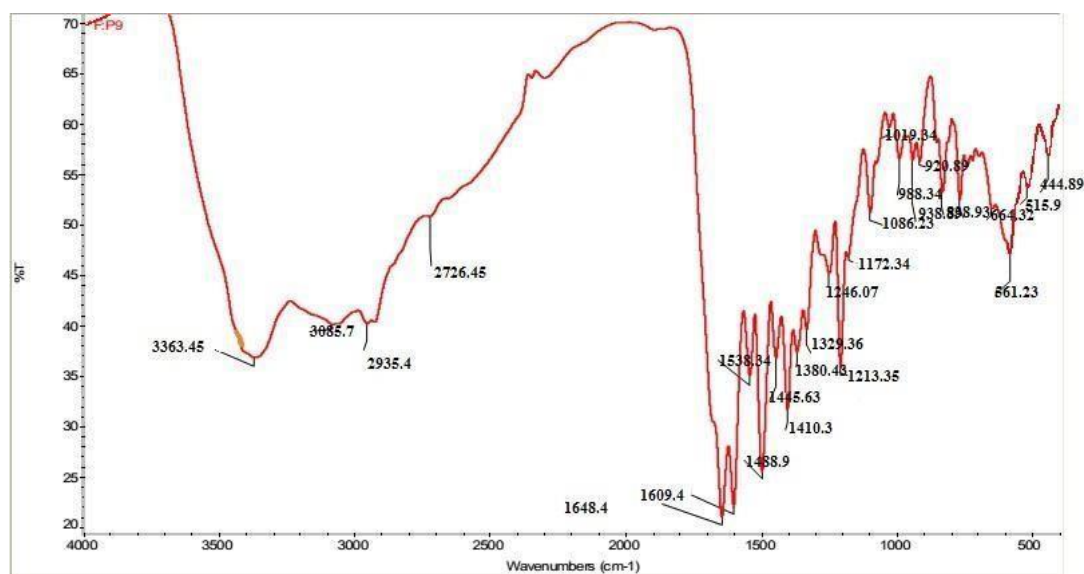


Fig 31 IR spectrum of isolated Rutin

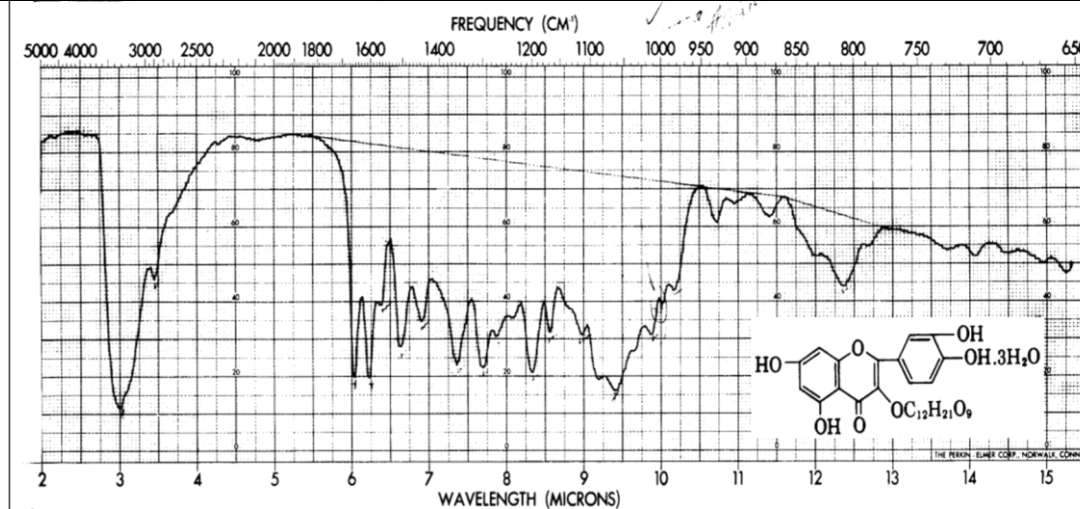


Fig 32 IR spectrum of previously isolated Rutin

Table 23 IR Spectra of isolated compound

S.NO	Obervation	Observed wave number (cm <sup>-1</sup> )
1	OH streching	3363.45
2	C-H streching Alkene	3085.7
3	C-H streching Alkane	2935.4
4	C-H streching Aldehyde	2726.45
5	C=C streching Alkene	1648.4, 1609.4
6	C=C streching Alkene	1538.34
7	C-H bending	1488.9

The ethyl acetate extract was used for the isolation of Rutin. The compound was isolated and identified by melting point, chemical test, TLC and IR spectrum. The results observed matches with the previously isolated rutin. Further study is focused on other spectral analysis and anticancer activity of Rutin.

## 12. SUMMARY AND CONCLUSION

Cancer is one of the leading causes of death worldwide. Currently available allopathic drugs for treating cancer causes number of side effects hence, people are now looking towards the herbal medicine. This paved a vital necessity for finding natural anticancer drug from herbal source. *Asparagus racemosus* Willd., is one such plant which is traditionally used in the treatment of cancer was selected for the present study.

The literature survey showed only scrappy information on the leaves of this plant. With this scanty of information on the leaves, consistent expectation of unexplored phytochemical profile and pharmacological efficacy forms the rationale for the study.

### Pharmacognostical studies

A perusal of literature showed paucity of pharmacognostical information on the leaves of this plant. Hence, it was carried out and reported for the first time.

In pharmacognostical studies macroscopy, microscopy, determination of physicochemical constants, analysis of inorganic elements, heavy metals and pesticide residue of the leaves were carried out.

Macroscopical study showed that the leaves are pine needle (phylloclades) shaped, sweet and bitter taste and dark green in colour. The various distinguishing features of leaves observed through anatomical studies were,

- Phyllodades 3-4 angled with lateral projections
- Radially oblong epidermal cells with prominent cuticle
- Circular chlorophyllous palisade cells
- Vascular strands with angular xylem and thick mass of phloem elements

The microscopical analysis of powder showed the presence of lignified fibres, epidermis with palisade cells and stomata.

Various physicochemical constants such as ash values, extractive values, loss on drying, crude fibre content, swelling index and foaming index were carried out. These values will help in confirming the identity and purity of the plant. Any significant deviation in the percentage of any parameters reported in this work may indicate adulteration or substitution in the drug.

Qualitative estimation of inorganic elements, quantitative estimation of heavy metals and pesticide residue were carried out and it showed only trace amount of heavy metals (within the limits) and absence of pesticide residue.

These pharmacognostical details observed from the present study might offer reliable clues for the correct identification of the leaves of this plant in crude as well as fragmentary form and also ensures its differentiation from its substitutes and adulterants. This is first report on the pharmacognostical standardization on the leaves of *Asparagus racemosus* Willd.,

### Phytochemical studies

Phytochemical evaluation deals with chemical analysis of the extracts used for pharmacological screening.

The successive solvent extraction was carried out with solvents like n-Hexane, chloroform, ethyl acetate and ethanol. Chloroform extract showed more percentage yield when compared to other extracts. Qualitative preliminary phytochemical analysis was aided in identifying the phytoconstituents present in

different extracts. Quantitative estimation of flavanoid, tannin and phenolic content were carried out, for the extracts. Fluorescence analysis was carried out, to detect the fluorescent chromophores present in the powdered drug as well as in the extracts. No fluorescence was observed in powder and the extracts.

Thin layer chromatography for the extracts was performed to identify the single or mixture of constituents in the extract. High Performance Thin Layer Chromatography- Finger print profile was carried out to detect the number of constituents present in the ethyl acetate extract.

### ***Invitro studies***

The therapeutically active extract to carry out the *invivo* anticancer activity was selected based on *invitro* anticancer activity. The chloroform, ethyl acetate and ethanol extracts were subjected to *invitro* anticancer studies using HeLa cells line by MTT assay. Chloroform and ethyl acetate extracts showed significant anticancer activity by MTT assay and these two extracts were selected for *invivo* anticancer studies.

### **Acute toxicity studies**

Acute toxicity study was carried out for chloroform and ethyl acetate extracts according to OECD guidelines 423. The extracts are non-toxic up to 2000mg/kg body weight indicating the safety of the extracts.

### ***Invivo anticancer studies***

*Invivo* anticancer activity of chloroform and ethyl acetate extracts were carried out on Ehrlich's Ascites Carcinoma (EAC) bearing mice. The ethyl acetate extract showed significant anticancer activity at the dose of 400mg/kg body weight when compared to chloroform extract and the effects were comparable with the standard drug.

Since, rutin was found to possess anticancer activity, an attempt was made to isolate rutin by partition separation method. The isolated compound was subjected to melting point, chemical test, TLC and IR spectroscopic studies.

From the above mentioned studies, it can be concluded that the pharmacognostical standards generated for the leaves will be useful for the proper identification of the plant. With the support of phytochemical and *invitro* cytotoxic activity the chloroform and ethyl acetate extracts were selected and subjected to *invivo* anticancer activity. The ethyl acetate extract at the dose level of 400mg/kg showed significant anticancer activity. Further studies are focused on detailed spectral analysis of isolated rutin and isolation of other phytoconstituents which is responsible for anticancer activity of the leaves.



## 13. REFERENCES

1. Rantg and Dales. Pharmacology. 6<sup>th</sup> ed. New york. Elsevier. 2010. p.718-721.
2. Macmillan Cancer Support. Cured – but at what cost? Long-term consequences of cancer and its treatment. 2013. Macmillan Cancer Support. The road to recovery: getting back to work. 2007. <http://www.macmillan.org.uk>.
3. Annucchi AM, Guglielmelli P and Tefferi A. Advances in understanding and management of myeloproliferative neoplasms. *CA Cancer J Clin*. 2009; 59:171-191.
4. Hultcrantz M, Kristinsson SY, Andersson TM, et al. Patterns of survival among patients with myeloproliferative neoplasms diagnosed in Sweden from 1973 to 2008: a population-based study. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*. 2012; 30:2995-3001.
5. Keohane C, Radia DH, and Harrison CN. Treatment and management of myelofibrosis in the era of JAK inhibitors. *Biologic: Targets & Therapy*. 2013; 7:189-198.
6. Arndt CAS and Crist WM. Common musculoskeletal tumors of childhood and adolescence. Medical Progress. *New England Journal of Medicine*. 1999; 341:242-352.
7. Krasin MJ, Rodriguez-Galindo C, Davidoff AM, et al. Efficacy of combined surgery and irradiation for localized sarcoma family of tumors. *Pediatric Blood Cancer*. 2004; 43:229-236.
8. Amir Z, Neary D and Luker K. Cancer survivors' views of work 3 years post diagnosis: a UK perspective. *European Journal of Oncology Nursing*. 2008; 12(3):190–197.
9. Chartered Institute of Personnel and Development (CIPD). Manager support for return to work following long-term sickness absence. [www.cipd.co.uk/hr/resources/guides/manager-support-return-work-following-long-term-sickness-absence.aspx](http://www.cipd.co.uk/hr/resources/guides/manager-support-return-work-following-long-term-sickness-absence.aspx) (accessed October 2013).
10. Samuel Joshua L, Vipin Chandra Pal, Senthil Kumar K L, Ram Kumar Sahu and Amit Roy. Antitumor activity of the ethanol extract of *Amaranthus spinosus* leaves against EAC bearing Swiss Albino mice. *Der Pharmacia Lettr*. 2010; 2(2): 10-15.
11. Sivakumar P, Sunil Mengani, Vijayabaskaran M, Sambath Kumar R, Perumal P and Jayakar B. Antitumor and Antioxidant Activities of *Triumfetta rhomboidea* against Ehrlich Ascites Carcinoma bearing Swiss Albino mice. *RJPBCS*. 2010; 1(4): 489-494.
12. Vijayabaskaran M, Amol K. Badkhal, Babu G, Sivakumar P, Perumal P, Sivakumar T, Sampathkumar R and Jayakar B. Antitumor Activity and Antioxidant Status of *Symplocos racemosa* Roxb Against Ehrlich Ascites Carcinoma in Swiss Albino Mice. . *RJPBCS*. 2010; 1(3): 306-314.
13. Srivastava J, Lambert J and Vietmeyer N. Medicinal plants:An expanding role in development. *World Bank Technical*. 1996; p.320.
14. Goel A, Kunnumakkara AB and Aggarwal BB. Curcumin as “ Curecumin”: from kitchen to clinic. *Biochem Pharmacol*. 2008; 75:787-809.
15. Gupta M, Mazumder U K, Rath N and Mukhopadhyay DK. Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich ascites carcinoma. *Journal of Ethanopharmacology* 2000; 72:151-156.

16. Gupta M, Anti mazumder UK, Haldar P, Kander CH, et.al. Anticancer activity of *Indigofera aspalathosides* and *Wedelia calendulaceae* in Swiss albino mice. *Iranian Journal of Pharmaceutical Research*. 2009; 6(2):141-145.
17. Asha Verma, Sudhanshudhar Dwivedi and Namrata Singh. Isolation, purification and spectral analysis of pure compound obtained from leaves extract of *Asparagus racemosus*. *World Journal of Pharmaceutical Research*. 2014; 3(1): 45-51.
18. Shashi Alok and Sanjay Kumar Jain. Plant profile, phytochemistry and pharmacology of *Asparagus racemosus* (Shatavari). *A review of Asian Pacific Journal of Tropical Disease*. 2013; 3(3): 242-251
19. Asha Verma and singh N. Chromatographic separation of ethyl acetate crude extract of Shatavari : A Ethnomedicinal plant. *International Journal of Pharmaceutical Science and Health care*. 2012; 2 (6): 97-102.
20. Karmakar UK, Biswas, Chowdhury A, Raihan et.al. Phytochemical Investigation and evaluation of antibacterial and antioxidant potential of *Asparagus racemosus*. *International Journal of Pharmacognosy*. 2012; 8: 53- 57.
21. Shiv Prakash Verma and Vikash Chandra Tripathi. *Asparagus Racemosus* Leaf Extract Inhibits Growth of UOK 146 Renal Cell Carcinoma Cell Line: Simultaneous Oncogenic PRCCTFE3 Fusion Transcript Inhibition and Apoptosis Independent Cell Death. *Asian Pacific Journal cancer prevention*. 2014; 15(5):1937-1941.
22. Asha Verma, Namrata Singh and Arvind Kumar. Investigation of antioxidant activity and *invitro* phytochemical screening of ethyl acetate leaves extract of *Asparagus racemosus* (family-*Asparagaceae*). *International Journal of Universal Pharmacy and Bio Sciences*. 2013; 2(3): 50-55.
23. Battu GR and Kumar BM. Anti-inflammatory activity of leaf extract of *Asparagus racemosus* Willd. *Int. J. Chem. Sci*. 2010; 8(2):1329-1338.
24. Deshpande DJ .A Hand book of Herbal Remedies. Jodhapur: Abrobios; 2008. p. 387.
25. Dinesh Jadhav. Medicinal Plants of India. Jodhapur: Scientific; 2008: vol I p. 41.
26. The wealth of India. New Delhi: The controller of publication; 2010: vol I p.470-472.
27. Nagamani , Suresh , Ahuja J and Reddy V. Comparative phytochemical screening of Vatashunga, Shatavari and Shatapushpa claimed for Prajasthapana activity. *Annals of Biological Research*. 2012; 3 (3):1294-1304.
28. Shashi Alok and Sanjay Kumar Jain. Plant profile, phytochemistry and pharmacology of *Asparagus racemosus* (Shatavari). *A review Asian Pacific Journal of Tropical Disease*. 2013; 3(3): 242-251.
29. Indian Pharmacopoeia. 4<sup>th</sup>ed. New Delhi: the controller of Publication; 1996: Vol IV p. 105-108.
30. Battu GR and Kumar BM. Anti-inflammatory activity of leaf extract of *Asparagus racemosus* Willd. *Int. J. Chem. Sci*. 2010; 8(2):1329-1338.
31. Shiv Prakash Verma and Vikash Chandra Tripathi. *Asparagus racemosus* leaf Extract Inhibits Growth of UOK 146 Renal Cell Carcinoma Cell Line: Simultaneous Oncogenic PRCCTFE3 Fusion Transcript Inhibition and Apoptosis Independent Cell Death. *Asian Pacific Journal Cancer Prevention*. 2014; 15(5):1937-1941.

32. Raval PK, Nishteshwar K Patel BR and Shukla J. *Asparagus racemosus* Wild. – A Comparative Phytochemical analysis of fresh dried roots of Shatavari. *International Journal of Pharmaceutical & Biological Archives*. 2012; 3(6):1458-1461.
33. Claus P. Pharmacognosy. 6<sup>th</sup> ed. London: Henry Kempton; 1970. p.15-33.
34. Johansen DA. Plant Microtechnique. New York: Mc Graw Hill Book Co; 1940. p. 523.
35. Easu K. Plant Anatomy John Wiley and sons. New York: 1964. p.767.
36. Gamble J S. Flora of the Presidency of Madras. Botanical Survey of India. Calcutta: India. 1935. 2014; 4(7): 3337-3342.
37. Henry AN, Kumari GR and Chitra V. Flora of Tamilnadu, India. Botanical Survey of India, Southern Circle. Coimbatore: India; 1987. Vol.3 p-258.
38. Johansen DA. Plant Microtechnique. New York: Mc Graw Hill Book Co; 1940. p.523.
39. Mathew KM. The Flora of Tamil Nadu Karnatic. Tiruchirappalli: India; 1983. Vol 1. p.688.
40. Gamopetalae and Monochlamydae. The Ranipat Herbarium, St.John's College. Tiruchirappalli: India; 1983. Vol 3. p.689-1540.
41. Kokate CK. Practical Pharmacognosy. 1<sup>st</sup> ed. New Delhi: Vallabh Prakashan; 1986. p.15-30.
42. Tyler VE, Brady LR and Robbers JE. Pharmacognosy. 9<sup>th</sup> ed. New Delhi: Philadelphia USA: Lea and Febiger; 1998. p.78.
43. The Ayurvedic Pharmacopoeia of India. New Delhi: The controller of publications; 2005. p.143.
44. Indian Pharmacopoeia. New Delhi: The controller of publications; 1996. p. A47-60.
45. Mukarjee PK. Quality control of herbal drugs. New Delhi: Business Horizons; 2002: p.188-399.
46. British Pharmacopoeia. General Medical Council, London: Pharmaceutical press; 1968: p.1276-1283.
47. Khandelwal KR. Practical Pharmacognosy. Pune: Nirali Prakashan; 1998. p.20.
48. Kokate CK. Practical Pharmacognosy. 1<sup>st</sup> ed. New Delhi: Vallabh Prakashan; 1986. p.123-124.
49. Kokate CK, Purohit AP and Gokahle S B. Pharmacognosy. 24<sup>th</sup> ed. Pune: vallabh prakashan; 2003. p.108-109.
50. Lira, Sergio, Peter Brush, Laurence Senak, Chi San Wu and Edward Malawer. The use of Inductively coupled plasma-optical emission spectroscopy in the determination of heavy metals in crospovidone and povidone as a Replacement for the concomitant visual comparison test. *Pharmacopoeial Forum*. 2008: (3)4, 6-10.
51. Marili VN, Rodrigues, Felix GR, Reyes, Pedro M, Magalhaes and Susame Rath. GC-MS determination of organochlorine pesticides in medicinal plants harvested in Brazil. *Journal Brazilian Chemistry Society*. 2007; 18(1):135-142.
52. Vinod D Rangary. Text book of Pharmacognosy and Phytochemistry. 2<sup>nd</sup> ed. 2009. Vol 2. p.136-38.
53. Harborne J B. Phytochemical methods. A guide to modern techniques of plant analysis. 2<sup>nd</sup> ed. London: Chapman and Hall; 1973. p.4-34.
54. Tyler VE, Brady L R and Robbers JE. Pharmacognosy. 9<sup>th</sup> ed. Philadelphia USA: Lea and Febiger; 1998. p.78.
55. Khandelwal KR. Practical Pharmacognosy. Pune: Nirali prakashan; 1998. p.20-42.
56. Wallis TE. Textbook of pharamacognosy. New delhi: CBS Publishers and Distributers; 1985. p.133-248.

57. Edeiga HO, Okwu DE and Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*. 2005; 4(7): 685- 688.
58. Singleton V L, Orthofer R and Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and Anti-oxidants by means of Folin- ciocalteu reagent *Methods Enzymol*, 199; 152-178.
59. Shivakumar B S. Quantitative Determination of total content of phenol, flavonoid and tannin in leaf extract of *Barlaria buxifolia* Linn. *Am. J. PharmTech Res*. 2012; 2(5):417-422.
60. Chase CR and Pratt RJ. Fluorescence of powdered vegetable drugs with particular reference to development of a system of identification. *American Pharmaceutical System Science*. 1949: 28. 324-333.
61. Kokosi CJ, Kokoski RJ and Slama FT. Fluorescence of powdered vegetable drug under ultraviolet radiation. *Journal of American pharmaceutical association*. 1958: 47. 715-717.
62. Beckett AH and Stenlake JB. Practical Pharmaceutical Chemistry. 2<sup>nd</sup> ed. CBS publishers: Newdelhi; 2001. p.115-126.
63. Stahl E. Thin layer chromatography. 2<sup>nd</sup> ed. Newyork (heidenberg): springerverlag; 1969. p.30-160.
64. Wagner H. Plant drug analysis. A Thin layer chromatography Atlas. 2<sup>nd</sup> ed,( Heidenberg): springer-verlag Belgium; 2002. p.227-305.
65. Gurdeep R, Chatwal, Sham K and Anand. Instrumental methods of chemical analysis. Mumbai: Himalaya publishing house; 2007. p.615.
66. Asha Verma and Singh N. Chromatographic separation of ethyl acetate crude extract of Shatavari : A Ethnomedicinal Plant. *International Journal of Pharmaceutical Science and Health care*. 2012; 2(6): 97-102.
67. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983; 65: 55-63.
68. Monks A, Leilane Hesporte Icoamoto, Deborabarbosa, Vendramini, Costa, et al., Feasibility of high flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. *Journal of the National Cancer Institute*. 1991; 83: 757-766.
69. Eike Reich and Anne Schibli. High-Performance Thin Layer Chromatography for the Analysis of Medicinal Plants. 2006. P. 67-114.
70. OECD. Guideline for testing of chemicals 423 (online); 2001 [cited on 2010 Feb 11]. Available from URL: <http://iccvam.Niehs.Nih.Gov/SuppDocs/OECD/OECD GL423.pdf>.
71. OECD. Guideline for testing of chemicals 425 (online); 2001 [ cited on 2010 Feb 11]. Available from URL: <http://iccvam.Niehs.Nih.Gov/SuppDocs/OECD/OECD GL425.pdf>.
72. Dacie JV and Lewis SM. Practical Hematology. 2nd ed. London: J and A Churchill; 1985. p. 38-48.
73. D'Armour FE, Blood FR. and Belden DA. The manual for laboratory work in mammalian physiology. 3<sup>rd</sup> ed. Chicago: The University of Chicago Press; 1965. p. 4-6.
74. Dagli MLZ, Guerra JL and Saldiva PHN. An experimental study on the lymphatic dissemination of the solid Ehrlich tumor in mice. *Brazilian Journal of Veterinary Research and Animal Science*. 1992; 29: 97-103.
75. Gupta M, Mazumder U K, Kumar R S, Sivakumar T and Vamsi M L M. Antitumor activity and antioxidant status of *Caesalpinia bonducella* against Ehrlich ascites carcinoma in Swiss albino mice. *J. Pharm. Sci*. 2004; 94: 177-184.

76. Gupta M, Mazumder UK, Kumar RS, Sivakumar T and Vamsi MLM. Antitumor activity and antioxidant role of *Bauhinia racemosa* against Ehrlich ascites carcinoma in Swiss albino mice. *Acta Pharmacol. Sin.* 2004b; 25: 1070-1076.
77. Orsolic N, Kosalec I and Basic I. Effects of local administration of propolis and its polyphenolic compounds on tumor formation and growth. *Bio. Pharm. Bull.* 2005; 28(10): 1928-1933.
78. Plowman J, Dykes DJ, Hollingshead M, Simpson-Herren L and Alley MC. Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval, Humana.T.B. Eds. Totowa. NJ. 1995. p.101.
79. Wintrobe MM, Lee GR, Boggs DR, Bithel TC, Athens JW and Foerester J. Clinical Hematology. 5<sup>th</sup> ed. Philadelphia; 1961. p. 326.
80. Martina Medvidovic-Kosanovic, Mirela Samardzic, Nela Malatesti and Milan Sak-Bosnar. Int. J. Electrochem. Electroanalytical Characterization of a Copper(II)-Rutin Complex. *International Journal of Electrochemical Science.* 2011; 6: 1075 – 1084.
81. Sanjay Jain, Champalal Dhanotiya and Neelesh Malviya. Physicochemical characterization and determination of rutin-phospholipid complex. *IJPSR.* 2012; 3(3): 909-913.
82. Jaganath IB, Millen W, Lean M, Edward CA and Crozier. In vitro catabolism of rutin by human facial bacteria and the antioxidant capacity of its catabolites. *Free Radic Biol Med.* 2009; 47(8): 1189-1193.
83. Meng F, Liu R, Bal H, Liu BW and Liu Y. Inhibitory effect of quercetin, rutin and puerarin on HDL oxidation induced by Cu<sup>2+</sup>. *Sichuan Da Xue Xue Bao Yi Xue Ban.* 2004; 35(6): 836-838.
84. Naruamol Vachirapatama and Busaya Chamnankid. Separation and determination of rutin in apple by High performance liquid chromatography. *Thammasat International Journal of Science and Technology.* 2012; 17(3); 27- 32.
85. Deshpande DJ. A Hand book of Herbal Remedies. Jodhapur: Abrobios; 2008. p. 387
86. Charles E Sando and John Uri Lloyd. The isolation and identification of rutin from the flowers of elder (*Sambucus canadensis* L.). *J. Biol. Chem.* 1924; 58:737-745.
87. Praveen Kumar Ashok and Bhawana Saini. HPLC Analysis and Isolation of Rutin from Stem Bark of *Ginkgo biloba* L. *Journal of Pharmacognosy and Phytochemistry.* 2013; 2(4):68-71.