EVALUATION OF THE ANTIOXIDANT ACTIVITY OF EXTRACTS OF *Achyranthes aspera*

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MAY2010

Submitted in partial fulfillment of the Degree of Bachelor of Technology

DEPARTMENT OF
BIOTECHNOLOGY & BIOINFORMATICS
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WAKNAGHAT, SOLAN, HP, INDIA

Certificate

This is to certify that the project report entitled "Evaluation of the Antioxidant activity of extracts of "Achyranthes aspera", submitted by Ketan Mahajan and Vandita Aggarwal in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

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Certified that this work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma

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ACKNOWLEDGEMENT

Although there were mutual efforts put in the project, but the success of our work could have never been accomplished without the constant help and support of many people, and to them we would like to express our sincerest gratitude.

First and foremost, it is our proud privilege to have the opportunity to thank our Project Guide, ***Assistant Prof.*** **(Dr.) S. Tandon**. It was an honor to carry out our project work under her expert guidance and supervision, without which our work would never have been possible. We appreciate her vast knowledge and skills in many areas and her assistance in writing reports, making presentations, etc. always making us do work efficiently.

We would also like to show our gratitude for **Anshu Aggarwal** of the Department of Biotechnology, whose constant suggestions played a significant role in the completion of our work .We would also like to thank the members of the panel evaluating our work and constantly giving suggestions to perform better - **Dr. C. Tandon, Dr. Hemant Sood , and Dr. Manu Sharma.**

We would also like to express our gratitude for **Dr. Y. Medury,** the Vice Chancellor of our university, and **Brig. (Retd.) Balbir Singh,** the Registrar of our university and **Prof. R. S. Chauhan Head of the Department** for having provided all kinds of facilities to carry out research. It was through their persistence, understanding and kindness that we are encouraged to apply for graduate training.

We would also like to thank staff of the biotechnology lab of our university for their constant support. It was a pleasure to work with them. We would also like to thank our parents whose constant support gave us the courage and zeal to complete this work.

Ketan Mahajan

Date: 22/05/10

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List of abbreviation

S.No.	TITLE	Full form
1.	AA	ABSORBENCE
2.	BSA	BOVINE SERUM ALBUMIN
3.	DCM	DICHLOROMETHANE
4.	DMSO	DIAMETHYLSUFOXIDE
5.	D.W.	DISTTILED WATER
6.	O.D.	OPTICAL DENSITY
7.	PBS	PHOSPHATE BUFFERED SALINE
8.	TPC	TOTAL PHENOLIC CONTENT

Abstract

The main purpose of our project is to Evaluate the antioxidant activities of the root sample of Achyranthes aspera by making different extracts and further fractions. Our methodology includes firstly identifying the phytochemicals present in the root sample and then evaluating the reductive potential of extracted phytochemicals.

This was done by crushing the roots sample of Achyranthes aspera in the Mortar and Pestle assembly and making Ethanolic and Aqueous extract of the crushed root sample by dissolving the roots in ethanol and water respectively. Further this extraction procedure was carried out in order to collect various phytochemicals in a lawyer wise order extracting Hexane then Alkaloids and Tannins and in the end Saponins.

The antioxidant free radical scavenging activity of aqueous and ethanolic extracts were estimated by performing DPPH assay[J. D. Habila1*, I. A. Bello1, A. A. Dzikwi3, H. Musa2 and N. Abubakar2]

Also total protein content of aqueous and ethanolic extracts were quantified by Lowry's method [Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J.Biol.Chem 193: 265]

The extract showing the maximum reductive potential will have the maximum antioxidant scavenging activity and is therefore selected to use for future work. After all the observations and calculations the aqueous extracts of the root sample was found to have the maximum reductive potential and therefore maximum free radical scavenging activity. These results are helpful in estimation of optimal dose of the root sample.

Further scope of this successful demonstration would include the determination of antiproliferative effect of the extract having maximum free radical activity on various cell lines in the laboratory. The particular extract can be further inspected on model organisms in the animal house to ensure the safety of the drug. This would lead to opening of new horizons in development of biology and will help all those people who suffer heavy losses due to cancer.

CHAPTER 1

INTRODUCTION

1.1 Cancer:-

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display *uncontrolled growth* (division beyond the normal limits), *invasion* (intrusion on and destruction of adjacent tissues), and sometimes *metastasis* (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize. Most cancers form a tumor but some, like leukemia, do not. The branch of medicine concerned with the study, diagnosis, treatment, and prevention of cancer is oncology.

Cancer affects people at all ages with the risk for most types increasing with age. Cancer caused about 13% of all human deaths in 2007 (7.6 million).Cancers are caused by abnormalities in the genetic material of the transformed cells. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents.

Cancers are classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. These are the histology and the location, respectively. Examples of general categories include:

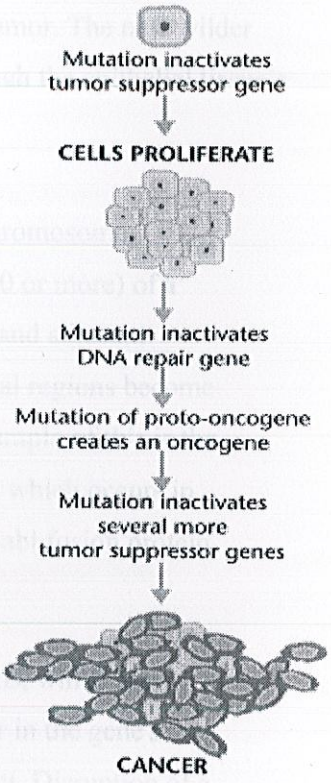
- **Carcinoma:** Malignant tumors derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer.
- **Sarcoma:** Malignant tumors derived from connective tissue, or mesenchymal cells.
- **Lymphoma** and **leukemia:** Malignancies derived from hematopoietic (blood-forming) cells

Cancer is fundamentally a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered. Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide.

There are two broad categories of genes which are affected by these changes. Oncogenes may be normal genes which are expressed at inappropriately high levels, or altered genes which have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell. *Inactivation of Tumor suppressor genes* in cancer cells results in the loss of normal functions in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.

There is a diverse classification scheme for the various genomic changes which may contribute to the generation of cancer cells. Most of these changes are mutations, or changes in the nucleotide sequence of genomic DNA. Aneuploidy, the presence of an abnormal number of chromosomes, is one genomic change which is not a mutation, and may involve either gain or loss of one or more chromosomes through errors in mitosis.

Development of cancer starts when a mutation in DNA occurs either through miscopying or damaging effects of exposure to radiation or a chemical carcinogen.



Cancer

Figure no. 1

The mutation causes cells either to proliferate even if tissue doesn't need a replacement cells or keep cell from self destructing. After many divisions a third mutation may arise and if the mutation gives the cells a further advantage then the cell will grow more faster than precedent and thus speed up the growth of the tumor .

As their presence of blood supply so tumor grows much faster. Individual cells from the tumor use blood vessels as highway by which they move to other body parts which is called metastasis of tumor. To further form the secondary tumor the cells leave the blood vessels and invade the tissue. The survivability rate of such cells of low but of invasion is done once then there is rise of secondary tumor.

Because of this the chances of occurrence of more mutation will increase and the next mutation paves the way to the development of more aggressive tumor. The new wilder cells created by another mutation are able to push their way through the epithelial tissue's basement membrane and angiogenesis takes place.

Large-scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains many copies (often 20 or more) of a small chromosomal locus, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. A well-known example of this is the Philadelphia chromosome, or translocation of chromosomes 9 and 22, which occurs in chronic myelogenous leukemia, and results in production of the BCR-abl fusion protein, an oncogenic tyrosine kinase.

Small-scale mutations include point mutations, deletions, and insertions, which may occur in the promoter of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product. Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, and such an event may also result in the expression of viral oncogenes in the affected cell and its descendants.

Various other mutations involve:-

1. Ionization radiation.

2. Infection.
3. Immune dysfunction.
4. Hereditary.

Ionization radiation:-

Sources of ionizing radiation, such as radon gas, can cause cancer. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies. It is estimated that 2% of future cancers will be due to current CT scans.

Infection:-

Some cancers can be caused by infection. This is especially true in animals such as birds, but also in humans, with viruses responsible for up to 20% of human cancers worldwide. These include human papillomavirus (cervical carcinoma), human polyomaviruses (mesothelioma, brain tumors), Epstein-Barr virus (B-cell lymphoproliferative disease, Human T-cell leukemia virus-1 (T-cell leukemias).

Immune dysfunction:-

HIV is associated with a number of malignancies, including Kaposi's sarcoma, non-Hodgkin's lymphoma, and HPV-associated malignancies such as anal cancer and cervical cancer. AIDS-defining illnesses have long included these diagnoses. The increased incidence of malignancies in HIV patients points to the breakdown of immune surveillance as a possible etiology of cancer

Other cancer-promoting genetic abnormalities may randomly occur through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers is usually affected by complex interactions between carcinogens and the host's genome.

Anything which replicates (living cells) will probabilistically suffer from errors (mutations). Unless error correction and prevention is properly carried out, the errors will survive, and might be passed along to daughter cells. Normally, the body safeguards against cancer via numerous methods, such as: apoptosis, helper molecules (some DNA polymerases), possibly senescence, etc. However these error-correction methods often fail in small ways, especially in environments that make errors more likely to arise and propagate.

For example, such environments can include the presence of disruptive substances called carcinogens. Cancer is thus a *progressive* disease, and these progressive errors slowly accumulate until a cell begins to act contrary to its function in the organism.

The errors which cause cancer are often *self-amplifying*, eventually compounding at an exponential rate. For example:

- A mutation in the error-correcting machinery of a cell might cause that cell and its children to accumulate errors more rapidly
- A mutation in signaling (endocrine) machinery of the cell can send error-causing signals to nearby cells
- A mutation might cause cells to become neoplastic, causing them to migrate and disrupt more healthy cells
- A mutation may cause the cell to become immortal causing them to disrupt healthy cells forever

Thus cancer often explodes in something akin to a chain reaction caused by a few errors, which compound into more severe errors. Errors which produce more errors are effectively the root cause of cancer, and also the reason that cancer is so hard to treat: even if there were 10,000,000,000 cancerous cells and one killed all but 10 of those cells, those cells (and other error-prone precancerous cells) could still self-replicate or send error-causing signals to other cells, starting the process over again. This rebellion-like scenario is an undesirable survival of the fittest, where the driving forces of evolution work against the body's design and enforcement of order. In fact, once cancer has begun

to develop, this same force continues to drive the progression of cancer towards more invasive stages, and is called clonal evolution.

Cancer gives most people no symptoms or signs that exclusively indicate the disease. Unfortunately, every complaint or symptom of cancer can be explained by a harmless condition as well. If certain symptoms occur, however, a doctor should be seen for further evaluation. Some common symptoms that may occur with cancer are as follows:

In case of Persistent cough or blood-tinged saliva :

i. These symptoms usually represent simple infections such as bronchitis or sinusitis.

ii. They could be symptoms of cancer of the lung, head, and neck. Anyone with a cough that lasts more than a month or with blood in the mucus that is coughed up should see a doctor.

In case of Breast lump or breast discharge:

2 Most breast lumps are noncancerous tumors such as fibroadenomas or cysts. But all breast lumps need to be thoroughly investigated.

i. A negative mammogram result is not usually sufficient to evaluate a breast lump. So doctor needs to determine the appropriate X-ray study which might include an MRI or an ultrasound of the breast.

ii. Discharge from a breast is common, but some forms of discharge may be signs of cancer. If discharge is bloody or from only one nipple, further evaluation is recommended.

In case of Obvious change in a wart or a mole :

i. Multicolored moles that have irregular edges or bleed may be cancerous.

ii. Larger moles are more worrisome and need to be evaluated, especially if they seem to be enlarging.

iii. Removing a mole is usually simple. You should have your doctor evaluate any suspicious mole for removal. The doctor will send it for examination under a microscope for skin cancer.

Most cancers are initially recognized either because signs or symptoms appear or through screening. Neither of these led to a definitive diagnosis, which usually requires the opinion of a pathologist, a type of physician (medical doctor) who specializes in the diagnosis of cancer and other diseases. People with suspected cancer are investigated with medical tests. These commonly include blood tests, X-rays, CT scans and endoscopy.

Definitive diagnosis requires the histologic examination of a biopsy specimen, although the initial indication of malignancy can be symptomatic or radiographic imaging abnormalities. Most cancers can be treated and some cured, depending on the specific type, location, and stage.

Once diagnosed, cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, treatments are becoming more specific for different varieties of cancer.

There has been significant progress in the development of targeted therapy drugs that act specifically on detectable molecular abnormalities in certain tumors, and which minimize damage to normal cells. The prognosis of cancer patients is most influenced by the type of cancer, as well as the stage, or extent of the disease.

1.2 Achyranthes aspera-

1.3. i) Physical Characteristics:-

- Prickly Chaff-flower is an erect or prostrate, annual or perennial herb, often with a woody base, which grows as wasteland herb everywhere. Since time immemorial, it is in use as folk medicine.
- It holds a reputed position as medicinal herb in different systems of medicine in India. Stems 0.4-2 m, pilose or puberulent. Leaf blades elliptic, ovate, or broadly ovate to orbiculate, obovate-orbiculate, or broadly rhombate, 1-20 × 2-6 cm, adpressed-pubescent abaxially and adaxially.
- Inflorescences to 30 cm; bracts membranous; bracteoles long-aristate, spinose; wings attached at sides and base. Flowers: tepals 4 or 5, length 3-7 mm; pseudostaminodes with margins fimbriate at apex, often with dorsal scale.
- It is hardy to zone 0. It is in flower from July to September, and the seeds ripen in October. The flowers are hermaphrodite (have both male and female organs)
- The plant prefers light (sandy), medium (loamy) and heavy (clay) soils. The plant prefers acid, neutral and basic (alkaline) soils. It can grow in semi-shade (light woodland) or no shade. It requires moist soil.

1.3. ii) Classification:-

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons

Subclass *Caryophyllidae*

Order *Caryophyllales*

Family *Amaranthaceae – Amaranth family*

Genus *Achyranthes L. – chaff flower*

Species *Achyranthes aspera L.*

Variety *Achyranthes aspera L. var. aspera*

Characters	*A. aspera* var. *argentea*	*A. aspera* var. *perphyristachya*
Plant	Erect, branched herbs or under shrubs, upto 15 cm high	An erect or subscandent annual herb upto 1m high
Stem	--	Angular, ribbed, thickened above the node, more or less densely hairy
Leaves	Ovate-elliptic, acute, silky oboxillary	Opposite, elliptic or obovate, form an acute or obtuse base, acuminate or roundec apex
Flowers	--	Deflexed, congested near the apex of the axis, perfect, bracts and bracteoles subequal
Fruits	--	An utricle, oblong-cylindric, truncate at apex, rounded at base
Seeds	--	Subcylinderic, truncate at apex, reddish brown
Flowering & Fruiting	Aug.-Dec.	Sept. - Dec.

TABLE 1: Botanical differences between *Achyranthes* subspecies

1.3. ii) Morphological description:-

- It is an annual or perennial herb. Stem erect, 0.5-2.0m in high, base woody, angular or ribbed, simple or branched, often tinged with pink color; nodes bulged.
- Seeds are 2 to 3 mm long, 1 to 1.5 mm wide, truncate above, reddish to dark brown and shiny, enclosed in chaffy calyx parts that remain attached. This species

is readily distinguished by the opposite leaves, branched stem and spiny bracts that are erect before flowering.

- Stem is 0.5 to 2 m tall, angularly-ribbed, generally square, more or less densely hairy and thickened above the nodes
- Leaves opposite, petiolate, ovate-elliptic-obovate-rounded, in various sizes, apex usually rounded, finely or softly pubescent on both sides.
- Leaves are 2 to 10 cm long, 0.7 to 5 cm wide; petiole 0.5 to 1.5 cm long; inflorescence terminal spikes, rigid, 10 to 50 cm long excluding the peduncle, with paired branches below;
- Flowers are hermaphrodite and in auxiliary or terminal spikes, which are more than 50 cm in long, greenish white, bracteate and bracteolate.
- Perianth lobes 4-6, glabrous, shining, ovate-oblong and pointed. Stamens 5 in number, staminodes are truncate, fimbriate, ovary oblong, sub-compressed and ovule solitary.
- Flowers are small, green, perfect, densely arranged at top of spike, less clustered in the center, scattered and often in pairs near the base; subtended by long-acuminate bracts or bracteoles, 2 to 3.5 mm long, stiff and spiny, erect.

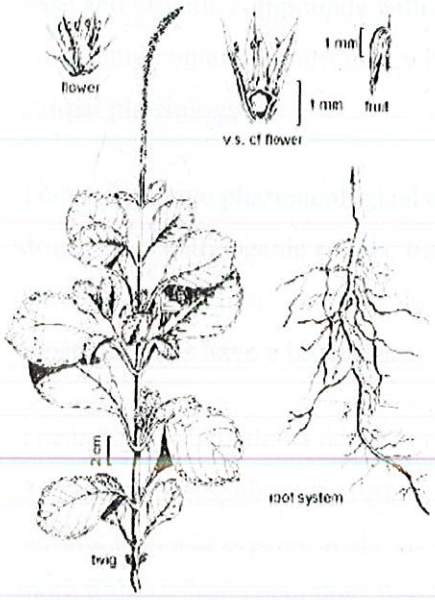


Figure no. 2

Figure no. 3

1.3. iii) Phytochemical constituents:-

- The plant contains triterpenoid saponins possessing oleanolic acid as aglycone, viz. A, B, C and D as major chemical constituents. (Dwivedi et al.) It also contains alkaloids and tannins. Saponins and Oleic acid are found in the seeds which have been defatted. Moreover Saponins C and D are found in the unripe fruits.
- Other constituents of the plant are ecdysterone, long chain alcohol, viz. 17-penta triacontanol, 27-cyclohexyl heptaeosan-7-ol, 16-hydroxyl 26-methyl heptacosan-2one and 36, 47-dihydroxy hen-pentacontan-4one.
- It also contains a water soluble base, betaine Achyranthine, Galactose, Glucose Sapogensis ,Xylose .

1.3. iii) a.) Alkaloids:-

Alkaloids are naturally occurring chemical compounds containing basic nitrogen atoms. The name derives from the word alkaline and is used to describe any nitrogen-containing base and organic compounds with one or more of the following features: a heterocyclic compound containing nitrogen, with an alkaline pH and a marked physiological action on animal physiology.

They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine, the stimulant caffeine, nicotine, the analgesic morphine, or the antimalarial drug quinine. Most alkaloids have a bitter taste.

The basicity of alkaloids depends on the lone pairs of electrons on their nitrogen atoms. As organic bases, alkaloids form salts with mineral acids such as hydrochloric acid and sulfuric acid and organic acids such as tartaric acid or maleic acid. These salts are usually more water-soluble than their free base form.

With the exception of caffeine, alkaloids are one of several classes of compound which are generally precipitated from aqueous solutions by tannins, a fact which is important in herbalism.

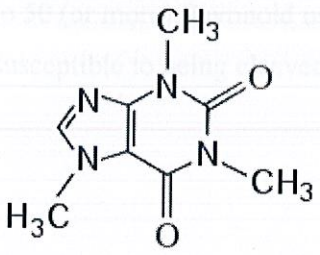


Fig. no. –4 Caffeine, a Purine alkaloid

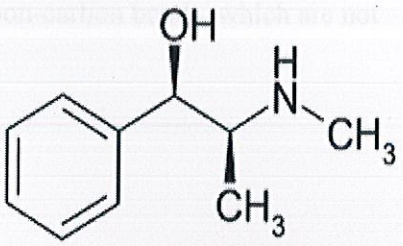


Fig. no. –5 Chemical structure of ephedrine, phenethylamine alkaloid

1.3. iii) a.) Tannins:-

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins and various other organic compounds including amino acids and alkaloids. The astringency from the tannins is what causes the dry and puckery feeling in the mouth following the consumption of unripened fruit or red wine.

Tannins have molecular weights ranging from 500 to over 3,000.Tannins are incompatible with alkalis, gelatin, heavy metals, iron, lime water, metallic salts, strong oxidizing agents and zinc sulfate, since they form complexes and precipitate in aqueous solution. Tannins are usually divided into hydrolyzable tannins and condensed tannins.

Hydrolyzable Tannin:-

They are tannins on heating with hydrochloric or sulphuric acids yield gallic or ellagic acids. At the center of a hydrolyzable tannin molecule, there is a carbohydrate (usually D-glucose).

Condensed Tannin:-

They are tannins on heating with hydrochloric acid yield phlobaphenes like phloroglucinol. Condensed tannins, also known as proanthocyanidins, are polymers of 2 to 50 (or more) flavonoid units that are joined by carbon-carbon bonds, which are not susceptible to being cleaved by hydrolysis.

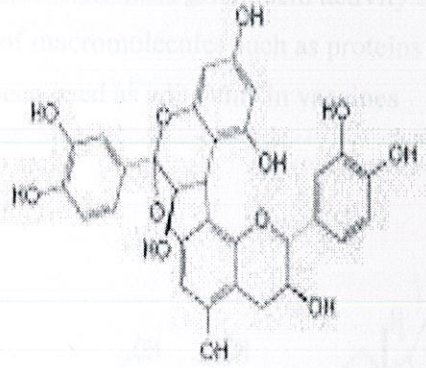


FIG. NO. 6 -- Proanthocyanidins

1.3. iii) c.) Saponins:-

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, with saponins found in particular abundance in various plant species.

Specifically, they are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions, and structurally by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. A ready and therapeutically relevant example is the cardio-active agent digoxin, from common foxglove.

The aglycone (glycoside-free portion) of the saponins is termed sapogenins. The number of saccharide chains attached to the sapogenin/aglycone core can vary – giving rise to

another dimension of nomenclature (monodesmosidic, bidesmosidic, etc.) – as can the length of each chain.

Saponins seem to react with the cholesterol rich membranes of cancer cells, thereby limiting their growth and viability.In plants, saponins may serve as anti-feedants and to protect the plant against microbes and fungi. Some plant saponins (e.g. from oat and spinach) may enhance nutrient absorption and aid in animal digestion.

The amphipathic nature of the class gives them activity as surfactants that can be used to enhance penetration of macromolecules such as proteins through cell membranes. Saponins have also been used as adjuvants in vaccines

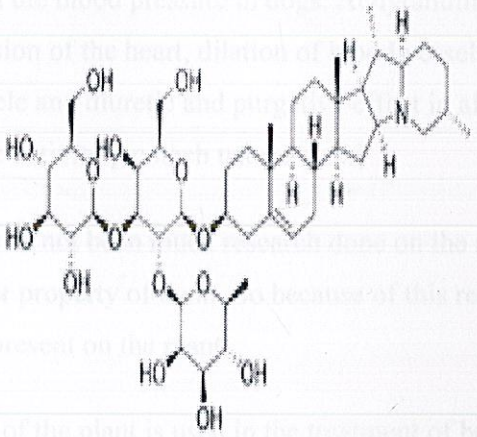


FIG. NO. 7: Chemical structure of the saponin solanine

1.3. iv) Earlier research:-

According to earlier research done on Achyranthus aspera it is thought to be one of the more important mdicinal herbs of Nepal. According to scientists the whole plant can be

used medicinally, but the roots are generally considered to be more effective. They contain triterpenoid saponins[Nguyen Van Dan & Doan Thi Nhu].

The root is astringent, diuretic and antispasmodic.[Lassak. E. V. and McCarthy. T. & Stuart. Rev. G. A. & Manandhar. N. P.]

It is also thought to be used in the treatment of dropsy, rheumatism, stomach problems, cholera, skin diseases and rabies.[Lassak. E. V. and McCarthy. T. & Manandhar. N. P.]

The juice extracted from the root of this plant, mixed with the root of Urena lobata and the bark of Psidium guajava, is used in the treatment of diarrhoea and dysentery.[Manandhar. N. P.]

Alcoholic and aqueous extracts of the roots cause fall in blood pressure but the chloroform extract raised the blood pressure in dogs. Achyranthine produced hypertension and depression of the heart, dilation of blood vessels in dogs, spasmogenic effect in frog rectus muscle and diuretic and purgative effect in albino rats. Alcoholic extract has hypoglycemic activity.[ecotech nature care.]

But unfortunately there has not been much research done on the roots of plant in direction to the anticancer property of them. So because of this reason there is not much availability of literature present on the plant.

Apart from that the juice of the plant is used in the treatment of boils, diarrhoea, dysentery, haemorrhoids, rheumatic pains, itches and skin eruptions. The ash from the burnt plant, often mixed with mustard oil and a pinch of salt, is used as a tooth powder for cleaning teeth.It is believed to relieve pyorrhea and toothache. The leaf is emetic and a decoction is used in the treatment of diarrhoea and dysentery. A paste of the leaves is applied in the treatment of rabies, nervous disorders, hysteria, insect and snake bites [Manandhar. N. P.].

1.3. v) IC-50:-

The **half maximal inhibitory concentration (IC_{50})** is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. Often, the compound in question is a drug candidate.

This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}).

It is commonly used as a measure of antagonist drug potency in pharmacological research. Sometimes, it is also converted to the **pIC_{50}** scale (-log IC_{50}), in which higher values indicate exponentially greater potency. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro*. It is comparable to an EC_{50} for agonist drugs. EC_{50} also represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo*.

The IC_{50} of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. IC_{50} values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

IC_{50} values are very dependent on conditions under which they are measured. In general, the higher the concentration of inhibitor, the more agonist activity will be lowered. IC_{50} value increases as enzyme concentration increases. Furthermore depending on the type of inhibition other factors may influence IC_{50} value; for ATP dependent enzymes IC_{50} value has an interdependency with concentration of ATP, especially so if inhibition is all of it competitive. IC_{50} values can be used to compare the potency of two antagonists.

CHAPTER 2

MATERIALS AND METHODS

2.1 Obtaining and crushing of Achyranthes aspera roots:-

The samples of Achyranthes aspera roots were obtained from the company "Natural remedies –BANGLORE"

Natural Remedies provides Plant Extract, Nutraceutical Extracts, Food and Beverage Ingredients, Animal Feed Supplements for Poultry, Herbal Products for Human and Animal Health Care, Plant Extract, Feed Supplements.

After obtaining of the samples the roots were crushed fine using the mortar and pestle assembly. The crushing was done for 30-45 mins.in order to make a fine powder of the roots.

2.2 Preparation of aqueous extract of Achyranthes aspera roots:-

Materials required:-

- Roots.
- Distilled water.
- Centrifuge.
- Weighing balance.
- Filter paper.
- Beakers.
- Rotary evaporator.

Methodology:-

- The sample of crushed roots was taken and there weights were measured.
- After weighing the roots Distilled water was added in 10% w/v ratio in the pre-weighed root sample in order to make a crude mixture of roots and water.
- Using proper size filter paper the filtration of the crude mixture was done in which the supernatant was taken and crude part was recycled for use again.
- In order to eliminate the cell debris at narrower scale in the supernatant the centrifugation of the supernatant was carried out at 10,000 rpm for 20 minutes at 4 degree Celsius.
- The supernatant obtained was dried using rotary evaporator for overnight in order to remove all the solvents left in extract and concentrate the extract.
- After drying the residue was collected and it was freeze dried and stored at -70^{0}C.

2.3 Preparation of ethanolic extract of Achyranthes aspera roots:-

Materials required:-

- Roots.
- Ethanol.
- Centrifuge.
- Weighing balance.
- Filter paper.
- Beakers.
- Rotary evaporator.

Methodology:-

- The sample of crushed roots was taken and there weights were measured.
- After weighing the roots ethanol was added in 10% w/v ratio in the pre-weighed root sample in order to make a crude mixture of roots and water.
- Using proper size filter paper the filtration of the crude mixture was done in which the supernatant was taken and crude part was recycled for use again.
- In order to eliminate the cell debris at narrower scale in the supernatant the centrifugation of the supernatant was carried out at 10,000 rpm for 20 minutes at 4 degree Celsius.
- The supernatant obtained was dried using rotary evaporator for overnight in order to remove all the solvents left in extract and concentrate the extract.
- After drying the residue was collected and it was freeze dried and stored at -70^{0}C.

2.4 Extraction of various phytochemicals from Achyranthes aspera roots:-

Methodology:-

Achyranthes aspera (pickly chaff flower)

Roots weighed (35 gms.)

Roots crushed (with the help of mortar and pestle assembly)

Powdered (preserved *at* -20 degree)

70% ethanol added to roots (10% w/v for preparation of ethanol extract)(350ml.)

Centrifugation (1000 rpm, 20mins, 4^0c)

Supernatant taken

Dried (Rotary evaporator)

Dried powdered ()

Aq. Ethanolic extract (layer 1)

(min. amt. of water added)

Separating funnel used

Hexane added (Helps in defatting)

Hexane layer (collected at the top) (layer 2)

+ Aq. Ethanolic layer

Dichloromethane added to Aq. Ethanolic layer

(Helps in removal of alkaloids)

Alkaloids layer (collected at the top) (layer 3)

+ Aq. Ethanolic layer

Ethyl acetate added to Aq. Ethanolic layer

(Helps in removal of taminns)

Tannins layer (layer 4) +Aq. Ethanolic layer

Butanol added to Aq. Ethanolic layer

(Helps in removal of saponins)

Saponins layer (layer 5)

+ Aq. Ethanolic layer

Left over Aq. Ethanolic layer (layer 6)

2.4 Estimation of protein content in Achyranthes aspera by the method of Lowry et. al (1951):-

Principle:-

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.

The Lowry method is sensitive to low concentrations of protein. The concentrations range from 0.10 - 2 mg of protein per mL. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate.

Materials required:-

- Test tubes.
- Distilled water.
- Pipettes.
- Folin's reagent.
- Spectrophotometer.

Reagents required:-

- **A.** 2% Na2CO3 in 0.1 N NaOH
- **B.** 2% NaK Tartrate in H_2O
- **C.** 1% CuSO4 in H_2O
- **D.** 49 mL of A, 0.5 mL of B, 0.5 mL of C
- **E.** Phenol Reagent - 1 part Folin-Phenol [2 N] : 1 part water

[Reagents A, B and C may be stored indefinitely]

- BSA Standard - 1 mg/ mL

Bovine Serum Albumin: 5 mg in 5 mL of water [1 µg / µl].
Freeze 1 mL aliquots.

Methodology (for standard plot):-

1. Set up four test tubes in rack.
2. Add BSA [25, 50, 100,200 ug. /µl.] to these tubes.
3. Add 3 mL of solution D to each test tube.

4. Mix in cyclomixer.
5. Incubate for 10 minutes at room temperature.
6. Add 0.3 mL of dilute Folin-phenol solution to each tube.
7. Vortex each tube immediately.
8. Incubate at room temperature for 30 minutes.
9. Determine absorbance of each sample at 670 nm.
10. Plot absorbance *vs* mg protein to obtain standard curve.

Methodology (for test sample):-

1. Set up five test tubes in rack.
2. Add .1 ml. of the sample to these five tubes.
3. Dilute the first test tube with a dilution ratio of 1:2, second test tube with a dilution ratio of 1:5, third test tube with a dilution ratio of 1:10, fourth test tube with a dilution ratio of 1:20.
4. Add 3 mL of solution D to each test tube.
5. Mix in cyclomixer.
6. Incubate for 10 minutes at room temperature.
7. Add 0.3 mL of dilute Folin-phenol solution to each tube.
8. Vortex each tube immediately.
9. Incubate at room temperature for 30 minutes.
10. Determine absorbance of each sample at 670 nm.
11. Plot absorbance *vs* mg protein to obtain the curve.

12. In order to know the conc. Of protein content in sample plot the sample readings in standard curve of Lowry to get the protein conc. in the sample.

Calculations:-

- BSA stock solution-10mg/10ml
- 2% Na2CO3-2gm of Na2CO3 in 100ml of water
- 0.1N NaOH-0.4gm of NaOH in 100ml of water
- 2% NaK Tartrate in H_2O-2gm of NaK Tartrate in 100ml of water
- 1% CuSO4 in H_2O-1gm of CuSO4 in 100 ml of water

2.5 Estimation of phenolic content in Achyranthes aspera by the Folin- Ciocalteu method:-

Principle:-

Phenol is when mixed with Folin-Ciocalteu reagent and sodium carbonate solution it gives bluish color and that bluish color absorbed the light. The intensity of bluish color is directly proportional to the amount of phenol in sample.

Materials required:-

- Test tubes.
- Distilled water.
- Pipettes.
- Folin's reagent.
- Spectrophotometer.

Reagents required:-

1. *Folin Ciocalteu Reagent:-*

- This is usually purchased as the 2N reagent. The preparation of the reagent is from sodium tungstate, sodium molybdate, lithium sulfate, bromine, and some acids.

2. *Gallic Acid Stock Solution:-*

- In a 100-mL volumetric flask, dissolve 0.500 g of dry gallic acid in10 mL of ethanol and dilute to volume with water. Can be opened daily, but to store, keep closed in a refrigerator up to two weeks.

3. *Sodium Carbonate Solution:-*

- Dissolve 200 g of anhydrous sodium carbonate in 800 mL of water and bring to a boil. After cooling, add a few crystals of sodium carbonate, and after 24 hr, filter and add water to 1 L.

Methodology:-

- To prepare a calibration curve, add 0, 1, 2, 3, 5, and 10 mL of the above phenol stock solution into 100 mL volumetric flasks, and then dilute to volume with water. These solutions will have phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid, the effective range of the assay.
- From each calibration solution, sample, or blank, pipet 20 µL into separate cuvettes, and to each add 1.58 mL water, and then add 100 µL of the Folin-Ciocalteu reagent, and mix well.
- Wait for between 30 sec and 8 min, and then add 300 µL of the sodium carbonate solution, and shake to mix.

- Leave the solutions at 20°C for 2 hr and determine the absorbance of each solution at 765 nm against the blank (the "0 mL" solution) and plot absorbance vs. concentration. Alternatively, they can be left at 40°C for 30 min before reading the absorbance.

- Create a calibration curve with the standards and determine the levels in the samples. Do not neglect to multiply the observed concentrations by any dilution factor of the original sample. Results are reported at Gallic Acid Equivalent, GAE, because the phenols in wine contain mostly other phenols, and only small amounts of Gallic acid.

Calculations:-

- Gallic acid stock solution - 0.5gm in 10ml of ethanol.
- Stock solution of sample - 0.5gm in 10ml of ethanol.

2.6 Estimation of reductive potential of aqueous and ethanolic extracts and of various phytochemicals extracted from Achyranthes aspera:-

Principle:-

Reduction potential (also known as redox potential, oxidation / reduction potential or ORP) is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced. Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced.

The phytochemicals have tendency to scavenge the free radicals in vivo or in vitro and get reduced. So effective will be the phytochemical better will be the scavenging activity.

Materials required:-

- Test tubes.
- Distilled water.

- Pipettes.
- Spectrophotometer.

Reagents required:-

- Phosphate buffer :- 0.2M ,pH 6.6
- Potassium ferricyanide :-1%
- Trichloroacetic acid :-10%
- Ferric chloride:-0.1%

Methodology:-

- Different conc. of ethanolic extract (20, 40, 60 and 100 ug. /ml.) in 1 ml. of distilled water was made.
- Different conc. of ethanolic extract was then mixed with 2.5 ml. of phosphate buffer and 2.5 ml.of potassium ferricyanide.
- The mixture was incubated at 50oc for 20 mins.
- After that 2.5 ml. of Trichloroacetic acid was added to the mixture
- A portion of resulting mixture was mixed with 0.5 ml.of FeCl3
- Determine absorbance of at 700 nm.

Calculations:-

- Phosphate buffer :- 0.2M , pH 6.6

Weigh Monosodium phosphate monohydrate-1.788gm and Disodium phosphate heptahydrate-1.886gm.Both dissolved in 100ml of water.

- Potassium ferricyanide :-1%:-

1 gm of Potassium ferricyanide in 100ml of water.

- Trichloroacetic acid :-10%:-

  10gm of anhydrous TCA in 100ml of water.

- Ferric chloride:-0.1%:-

  0.1gm of ferric chloride in 100ml of water.

2.7 Various methodologies for screening of phytochemicals present in Achyranthes aspera:-

The ethanolic extracts were subjected to various chemical tests in order to determine plants secondary constituents by following methods:-

- ***Test for presence of saponins:-***

  0.5 gm. of extract dissolved in 10 ml. of D.W. in test tube. It was stoppered with cork and shaken vigorously for 30 sec. then allowed standing for 45 min. appearing of froth confirms presence of saponins

- ***Test for flavanoids:-***

  The alcholic extract was treated with a few drops of conc. HCL and magenisum turnings . The presence was indicated by development of pink or magenta color within 3 min.

- ***Test for tannins:-***

  0.5 gm. Of extract was dissolved in 5 ml. of water followed by few drops of 10% ferric chloride. a blue black ,green ,precipitate. would indicate presence of tannins.

- ***Test for alkaloids :-***

  0.5 gm. Of ethanol extract with 1% aq. HCL. On a stem bath;1 ml. of filtrate with a few drops of mayers reagent and a second 1 ml. portion was treated with dragendorff reagent . Turbidity would indicate alkaloids.

2.8 Determination of antioxidant activity of aqueous and ethanolic extract from Achyranthes aspera using DPPH free radical scavenging activity :-

Principle:-

DPPH (1,1-Diphenyl-2-picrylhydrazyl) is a stable free radial with red color (absorbed at 517nm). If free radicals have been scavenged, DPPH will generate its color to yellow. This assay uses this character to show herbs free radical scavenging activity.

Materials required:-

- Test tubes.
- Distilled water.
- Pipettes.
- Spectrophotometer.

Reagents required:-

- Methanol
- DPPH

Methodology:-

- Sample stock solutions (1.0 mg. /ml.)were diluted to final concentration of 250,125,50,25,10,5ug./ml. in methanol.
- 1 ml. of a 0.3 mM DPPH methanol solution was added to 2.5 ml. solution of the extract or standard and allowed to react at room temperature for 30 mins.

- The absorbance of resulting mixture was measured at 518 nm. and converted to % antioxidant activity(AA%) using the formula :-

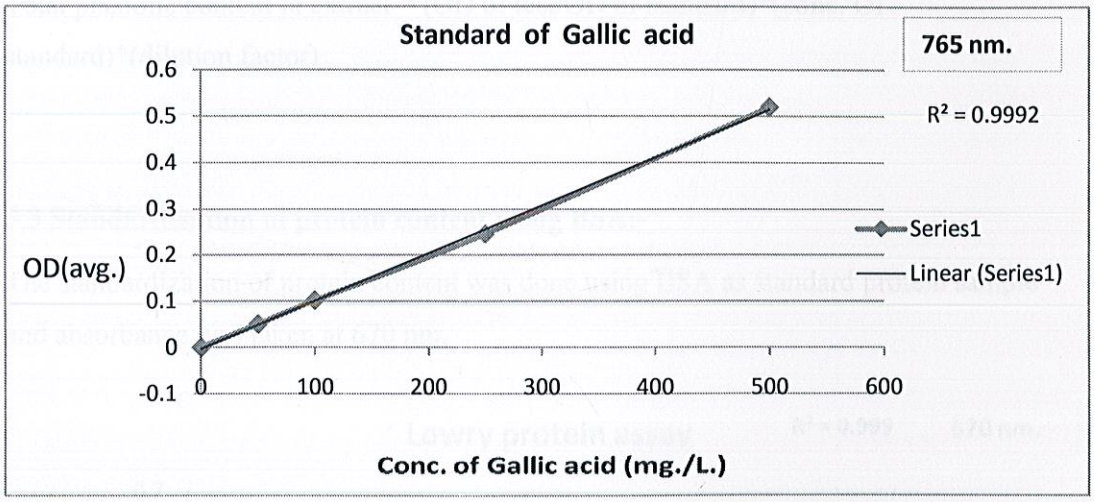
  - ✓ {AA%=100 – {(abs of sample-abs blank)*100}/abs control}.

CHAPTER 3

RESULTS AND ANALYSIS

3.1 Gallic acid standardization for total phenolic content of Achyranthes aspera in ethanolic extract:-

Gallic acid is used as a standard for determining the phenol content of various analytes by the Folin-Ciocalteau assay because it is relatively stable and cheap. In this assay the absorbance was taken at 765 nm.



Graph no. 1

Readings of Gallic acid standard:-

Conc. Of Gallic acid in std. (mg. /l.)	OD (avg.) at 765 nm.
50	0.05
100	0.101
250	0.246
500	0.520

Table no. 2

3.2 Total phenolic content of achyranthes aspera in ethanolic extract:-

OD (avg.) at 765 nm.	Dilution ratios	Total phenolic content (mg./l.)
.006	1:10	29.70
.013	1:5	64.35
.034	1:3.3	114.02
.081	1:2	155.76

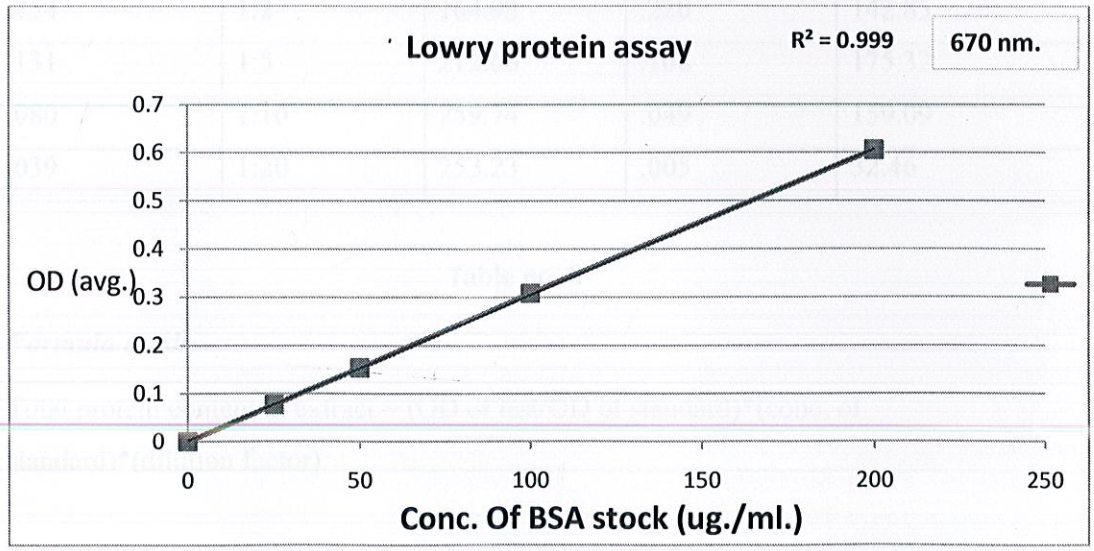
Table no. 3

Formula used: -

Total phenolic content in extract = (OD of test/OD of standard)*(conc. Of -standard)*(dilution factor)

3.3 Standardization of protein content using BSA:-

The standardization of protein content was done using BSA as standard protein sample and absorbance was taken at 670 nm.



Graph no. 2

Readings of Lowry protein assay:-

Conc. Of Lowry protein assay (ug. /ml.)	OD (avg.) at 670 nm.
25	0.077
50	0.154
100	0.308
200	0.616

Table no. 4

3.4 Total protein content of Achyranthus aspera in aqueous and ethanolic extract:-

OD (avg.) (ethanolic extract) at 670 nm.	Dilution ratios	Conc. Of total protein content in ethanolic extract (ug./ml.)	OD (avg.) (aqueous extract) at 670 nm.	Conc. Of total protein content in aqueous extract (ug./ml.)
.254	1:2	164.93	.220	142.85
.131	1:5	212.66	.108	175.32
.080	1:10	259.74	.049	159.09
.039	1:20	253.23	.005	32.46

Table no. 5

Formula used:-

Total protein content in extract = (OD of test/OD of standard)*(conc. of standard)*(dilution factor).

3.5 Phytochemical screening of Achyranthus aspera:-

1. ***Saponins:*** - formation of froth confirms the presence of saponins.

FIG. NO.8 - ETHANOLIC EXTRACT

FIG. NO.9 - AQUEOUS EXTRACT

2. ***Alkaloids:*** - Turbidity on a hot steam bath confirms presence of alkaloids.

FIG. NO.10 - ETHANOLIC EXTRACT

FIG. NO.11 - AQUEOUS EXTRACT

3. ***Tannins:*** - A Blue, Black or Green precipitate tells presence of tannins.

FIG. NO.12 - ETHANOLIC EXTRACT

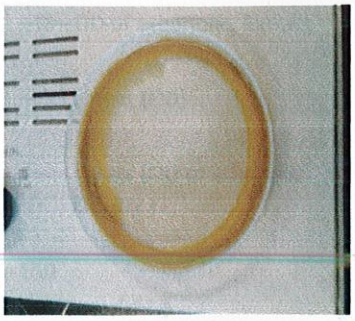
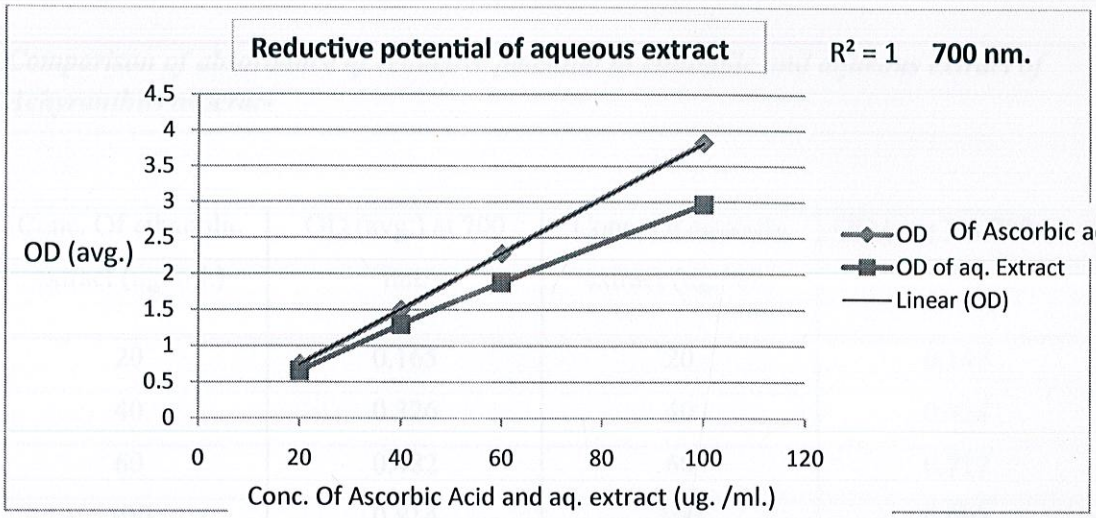


FIG. NO.13 - AQUEOUS EXTRACT

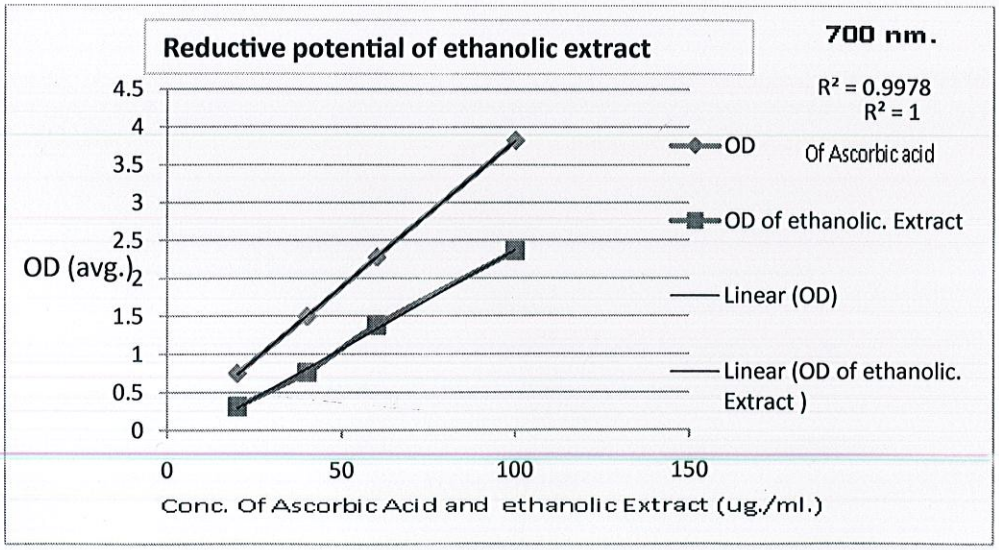
3.6 Reductive potential of aqueous and ethanolic extract of Achyranthus aspera:-

3.6.i) Reductive potential of aqueous extract of Achyranthus aspera:-



Graph no. 3

3.6.ii) Reductive potential of ethanolic extract of Achyranthus aspera:-



Graph no. 4

Results:-

From above graph we can say that the reductive potential of aqueous extract is much higher than ethanolic extract as the curve of aqueous extract is rising with standard curve of ascorbic acid. So aqueous extract is preferred.

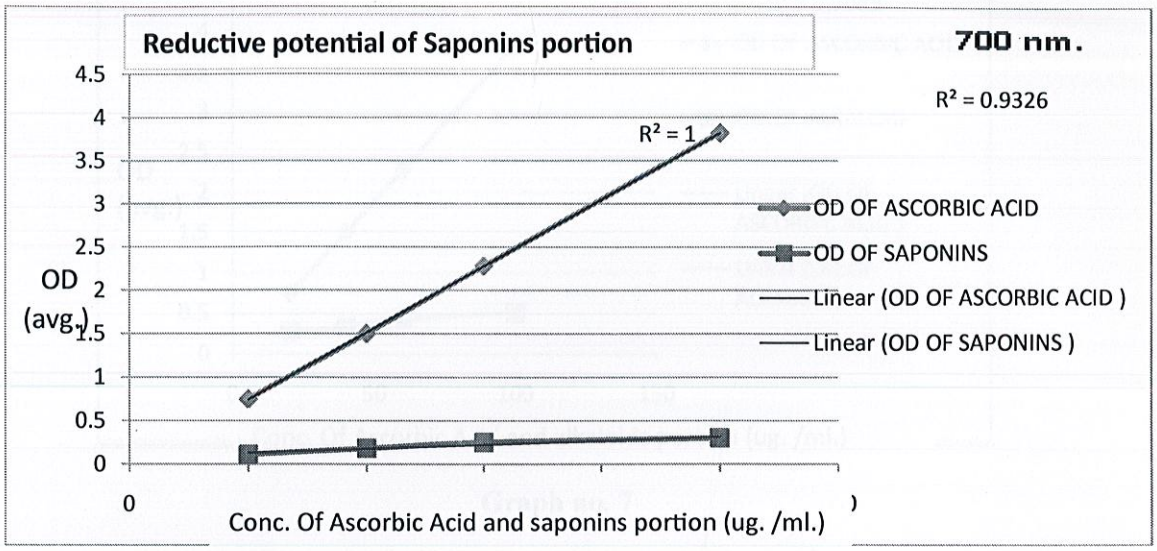
Comparison of absorbance of reductive potential in ethanolic and aqueous extract of Achyranthus aspera:-

Conc. Of ethanolic extract (ug./ml.)	OD (avg.) at 700 nm.	Conc. Of aqueous extract (ug./ml.)	OD (avg.) at 700nm.
20	0.165	20	0.147
40	0.326	40	0.424
60	0.482	60	0.717
100	0.814	100	0.993

Table no. 6

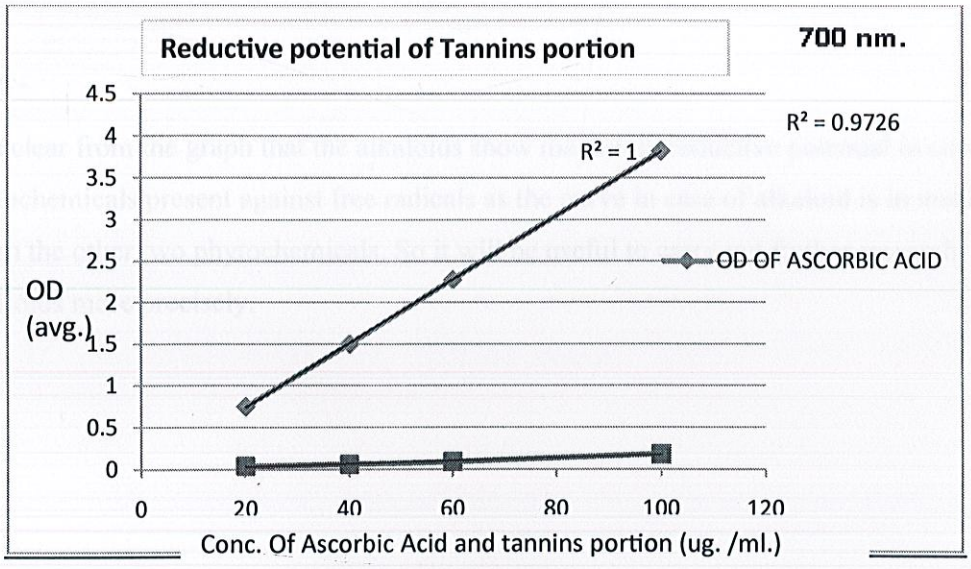
3.7 Estimation of reductive potential of the various phytochemicals extracted from Achyranthus aspera.-

SAPONINS:-



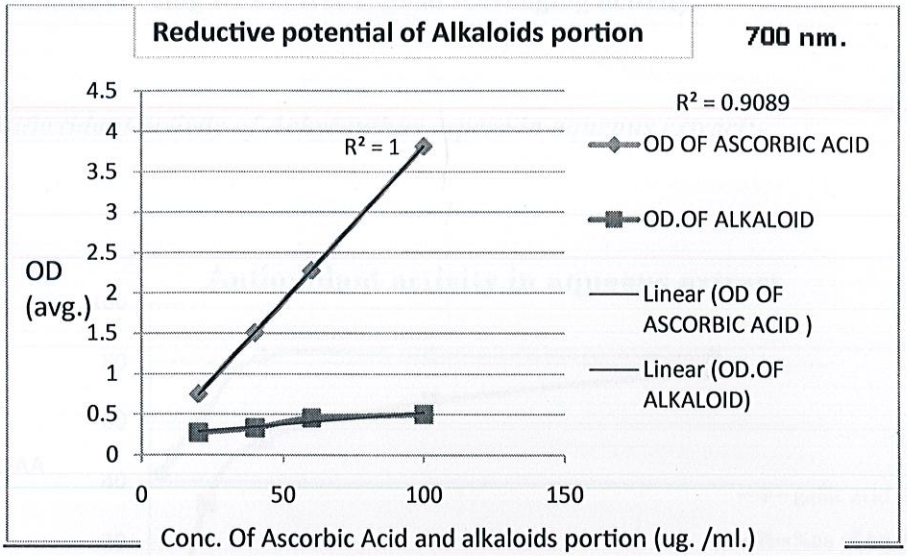
Graph no. 5

TANNINS:-



Graph no. 6

ALKALOIDS:-



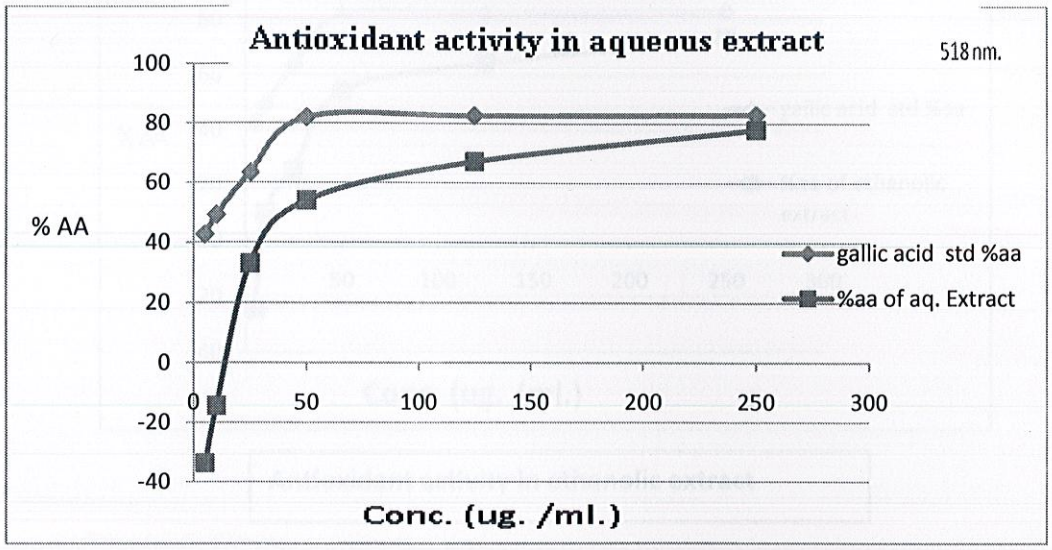
Graph no. 7

Results:-

As it is clear from the graph that the alkaloids show maximum reductive potential in case of phytochemicals present against free radicals as the curve in case of alkaloid is in much rise than the other two phytochemicals. So it will be useful to carry out further research on alkaloids more precisely.

3.8 Estimation of Antioxidant activity of Achyranthus aspera in aqueous and ethanolic extract using DPPH free radical scavenging activity.

3.8 i) Antioxidant activity of Achyranthus aspera in aqueous extract:-



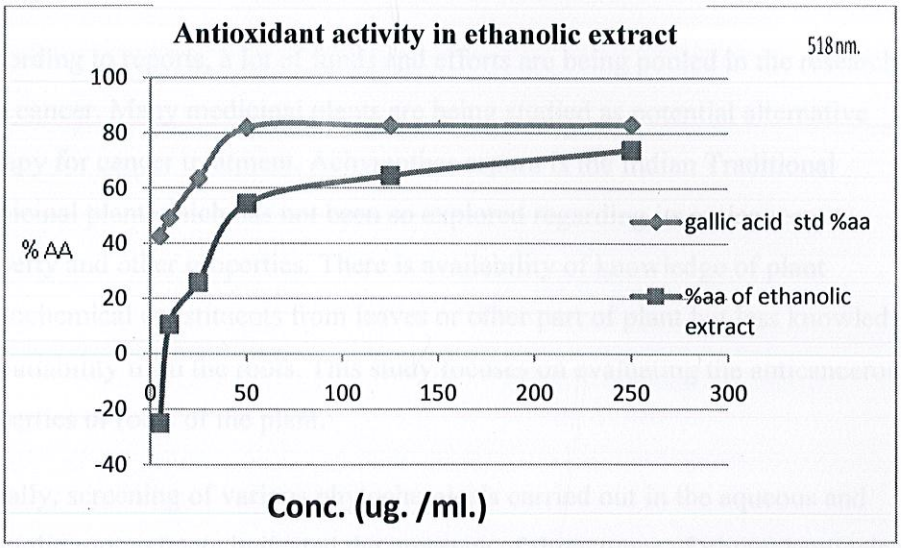
Antioxidant activity in aqueous extract

Graph no. 8

Formula used:-

AA%=100 – {(abs of sample-abs blank)*100}/abs control.

3.8ii) Antioxidant activity of Achyranthus aspera in ethanolic extract:-



Antioxidant activity in ethanolic extract

Graph no. 9

Formula used:-

AA%=100 – {(abs of sample-abs blank)*100}/abs control.

Results:-

From above graphical representation it is clear that the aqueous extract is more effective in free radical scavenging activity as the % of absorbance is much higher in case of aqueous extract.

CHAPTER 4

DISCUSSION

According to reports, a lot of funds and efforts are being pooled in the research to cure cancer. Many medicinal plants are being studied as potential alternative therapy for cancer treatment. Achyranthes aspera is the Indian Traditional Medicinal plant which has not been so explored regarding its anticancerous property and other properties. There is availability of knowledge of plant phytochemical constituents from leaves or other part of plant but less knowledge is availability from the roots. This study focuses on evaluating the anticancerous properties of roots of the plant.

Initially, screening of various phytochemicals carried out in the aqueous and ethanolic root extracts indicated the presence of three types of phytochemicals which were: Alkaloids, Tannins,and Saponins. The extracts were prepared using extraction method described in chapter 2.

Total protein content and polyphenols of aqueous and ethanolic extracts were quantified by Lowry's and gallic acid estimation respectively. The presence of high total phenolic content in aqueous extract gave us insight into the antioxidant properties of Achyranthes aspera. Bio activity guided successive solvent extraction was used as extraction procedure for obtaining different phytochemical constituents from the root sample. Alkaloids, saponins and tannins were collected as separate layer using the separating funnel.

Moreover the capability of aqueous and ethanolic extract in reducing the free radicals was also seen and was found to be best in aqueous extract showing a good indication of future. Estimation of antioxidant activities of reducing the free radicals using various layers was also done by evaluating reductive potential.

The antioxidant properties of Achyranthes aspera was also checked again using DPPH free radical scavenging assay. Comparison of antioxidant activities to

scavenge free radicals was done and similar to the results of reductive potential aqueous extract here also showed the best result leading to again a better indication of attaining amazing results.

CHAPTER 5

CONCLUSION

Comparison of antioxidant activities of different extracts and various phytochemical constituents extracted from Achyranthes aspera root sample revealed that the free radical scavenging activity of aqueous extracts of root sample is more than that of ethanolic extracts. So conclusion could be withdrawn that aqueous extract is showing more potential to cure cancer compared to ethanolic extract.

Moreover reductive potential in case of phytochemicals extracted was found out to be best in alkaloid fraction was found to be greatest amongst the three phytochemical fractions of the sample. However the aqueous extracts were found to have the highest reductive potential as compared to ethanolic extract and various other fractions.

So on a concluding note it would not be wrong to say that through extensive research done on medicinal plants the deadliest disease of current time can be terminated.

CHAPTER 6

FUTURE DIRECTION

Several avenues for further work are open based on the observations present in this study such as the role played by the scavenging radicals in antioxidant system of Achyranthes aspera, reductive potential of different extracts, phenolic contents, etc.

Further the effect of antioxidants on MCF-7 cell lines can be checked. Also an overall comparative study can be carried out for different medicinal plants with Achyranthes aspera for the antioxidant activity. Achyranthes aspera can also be studied for its effect on various types of cancer and also on different stages by using model animals in the animal house of the laboratory such as mice, rabbits.

CHAPTER 7

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