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Vidyavihar

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"A Comparative Study: Phytochemical Analysis Of Plants From Polluted And Unpolluted Areas"

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26th March 2014

CERTIFICATE

This is to certify that Miss Ankita Rajendra Parab Studying in M.Sc Part II from K J Somaiya College has satisfactorily completed the dissertation work entitled **"A Comparative Study: Phytochemical Analysis Of Plants FromPolluted And Unpolluted Areas"** towards the partial fulfillment of Master's degree in Biotechnology as prescribed by the University of Mumbai during the academic year 2013-2014.

This work was completed at Srujan Biotech (Dombivli) under the guidance of Mr. Vaibhav Jawalekar. This dissertation represents independent work carried out by her.

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Dissertation work Entitled

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AnkitaRajendraParab

Abstract

The plants *Vincarosea .Linn* and *Asteracanthalongifolia (K. Schum)* are used for their medicinal properties. Vinca contains alkaloids Vinblastine and Vincristine which are having anti-cancer properties. While Asteracantha contains compound Betulin which is having antidiuretic properties. These biochemicals play a very important role in treatment of hazardous diseases. Phytochemicals such as saponins, terpenoids, glycosides, etc. are suggested to be present in plants. Both these plants are very significant medicinal plants. However, the constant exposure to the environmental pollution, affects the formation of these useful alkaloids, and other biochemicals thereby affecting the medicinal value of these plants. Although these biochemicals are present in very low quantities in the entire plant, they are in increasing demand. Hence, it is essential to study the effect of pollutants on these compounds.

We conducted a comparative study of these plants growing in both polluted and unpolluted areas. This study was conducted with the objective that it will reveal the biochemical and phytochemical alterations occurring and also affecting the alkaloid production in these plants due to the environmental pollutants. We used techniques such as colorimetric analysis and RP-HPLC (Reverse Phase - High Performance Liquid
Chromatography) for the determination and quantification of the different biochemicals.

Key words: *Vincarosea .Linn,Asteracanthalongifolia (K. Schum),*alkaloids, RP - HPLC, phytochemicals, colorimetric analysis.

Index

I. List of Abbreviations…….……..…….I

1. Introduction…….1

2. Review of Literature

2.1 Principles……...…….4

2.1.1 Sugars…….4

2.1.2 Proteins…….....…..6

2.1.3 Phytochemicals……...7

2.1.4 Soxhlet extraction……...7

2.1.5 HPLC……..…..…..8

2.2 Selected plants…….….10

2.2.1 Review of literature of*Vincarosea .Linn*……11

2.2.2 Review of literature of*Asteracanthalongifolia (K.Schum)*…….17

2.2.3 Comparative studies on other plants……..…..23

3. Aims and Objectives……..25

4. Materials and methods

4.1 Materials……...26

4.1.1 Samples……26

4.1.2 Glasswares……26

4.1.3 Instruments and Equipments……27

4.1.4 Chemicals for phytochemical analysis……..…28

4.1.5 Chemicals for Sugar estimation (DNSA Method)……...28

4.1.6 Chemicals for Protein estimation (Folin-Lowry's method)…….28

4.1.7 Requirements for RP-HPLC analysis……..……..…28

4.2 Methods……30

4.2.1 Soxhlet extraction……...…..31

4.2.2 Water extracts……..…….32

4.2.3 Estimation of total sugar content……..……32

i. Reducing sugars…….……..……..…32

ii. Non-Reducing sugars…….…….……..…32

4.2.4 Estimation of protein content……..…….…….……33

4.2.5 Phytochemical tests……..…..…….…..33

i. Test for Tannins ……..……..…33

ii. Test for Saponins……...…..……..33

iii. Test f Phlobatannins…….…...……33

iv. Test for Flavonoids…….…….....34

v. Test for Terpenoids……...…….…..….34

vi. Test for Cardiac Glycosides…….……34

vii. Test for Steroids……...…34

viii. Test for Alkaloids……..…34

5. Observations and Calculations

5.1 Observations and calculations for Total Sugar Content……...35

5.2 Observations and calculations for Protein content……...……52

5.3 Observations for Phytochemical tests……..61

6. HPLC chromatograms

6.1 RP-HPLC analysis for alkaloids in Vinca (Unpolluted) methanolic extracts..82

6.2 RP-HPLC analysis for alkaloids in Vinca (Polluted) methanolic extracts….83

6.3 RP-HPLC analysis for alkaloids in Asteracantha (Unpolluted) methanolic extracts.....84
6.4 RP-HPLC analysis for alkaloids in Asteracantha (Polluted) methanolic extracts.....85

7. Results and Discussions

7.1 Results……..…….…..86
7.1.1 Comparison of results for Total sugar content and Protein content estimation……...……..86
7.1.2 Comparison of results for HPLC chromatograms…….…...86

7.2 Discussions…….……..87

8. Conclusion……..……89

9. Future Prospects…….…….90

10. Bibliography……..…….[i]

11. Appendix……..…….….[v]

List of Tables

Sr. No.	Description	Page no.
	ΔOD for the variour parts of the plants – Sugars	
1	Table 5.1 Standardization of sugars using glucose stock solution	35
2	Table 5.2 Reducing sugars – Leaf, stem and root of Vinca unpolluted	36
3	Table 5.3 Non-Reducing sugars – Leaf, stem and root of Vinca unpolluted	38
4	Table 5.4 Reducing sugars – Leaf, stem and root of Vinca polluted	40
5	Table 5.5 Non-Reducing sugars – Leaf, stem and root of Vinca polluted	42
6	Table 5.6 Reducing sugars – Leaf, stem and root of Asteracantha unpolluted	44
7	Table 5.7 Non-Reducing sugars – Leaf, stem and root of Asteracantha unpolluted	46
8	Table 5.8 Reducing sugars – Leaf, stem and root of Asteracantha polluted	48
9	Table 5.9 Non-Reducing sugars – Leaf, stem and root of Asteracantha polluted	50
	ΔOD for the variour parts of the plants – Proteins	
10	Table 5.10 Standardization of proteins using BSA stock solution	52
11	Table 5.11 Proteins – Leaf, stem and root of Vinca unpolluted	53
12	Table 5.12 Proteins – Leaf, stem and root of Vinca polluted	55
13	Table 5.13 Proteins – Leaf, stem and root of Asteracantha unpolluted	57
14	Table 5.14 Proteins – Leaf, stem and root of Asteracantha polluted	59
	Observations for phytochemical analysis	
15	Table 1: Phytochemical analysis done on water extract of leaf of Vinca (unpolluted)	61
16	Table 2: Phytochemical analysis done on water extract of stem of Vinca (unpolluted)	62
17	Table 3: Phytochemical analysis done on water extract of root of Vinca (unpolluted)	63
18	Table 4: Phytochemical analysis done on whole plants' methanolic extract and water extracts of Vinca (Unpolluted)	64
19	Table 5: Phytochemical analysis done on water extract of leaf of Vinca (Polluted)	66
20	Table 6: Phytochemical analysis done on water extract of stem of Vinca (Polluted)	67
21	Table 7: Phytochemical analysis done on water extract of root of Vinca (Polluted)	68
22	Table 8: Phytochemical analysis done on whole plants' methanolic extract and water extracts of Vinca (Polluted)	70
23	Table 9: Phytochemical analysis done on water extract of leaf of Asteracantha (unpolluted)	72
24	Table 10: Phytochemical analysis done on water extract of stem of Asteracantha (unpolluted)	73

25	Table 11: Phytochemical analysis done on water extract of root of Asteracantha (unpolluted)	74
26	Table 12: Phytochemical analysis done on whole plants' methanolic extract	75
	and water extracts of Asteracantha (Unpolluted)	
27	Table 13: Phytochemical analysis done on water extract of leaf of Asteracantha(Polluted)	77
28	Table 14: Phytochemical analysis done on water extract of stem of Asteracantha(Polluted)	78
29	Table 15: Phytochemical analysis done on water extract of root of Asteracantha (Polluted)	79
30	Table 16: Phytochemical analysis done on wholeplants' methanolic extract and water extracts of Asteracantha (Polluted)	80
	Results for HPLC chromatograms	
31	Table 6.1: Results of RP-HPLC for alkaloids of Vinca Unpolluted methanolic extracts	82
32	Table 6.2: Results of RP-HPLC for alkaloids of Vinca Polluted methanolic extracts	83
33	Table 6.3: Results of RP-HPLC for alkaloids of Asteracantha Unpolluted methanolic extracts	84
34	Table 6.4: Results of RP-HPLC for alkaloids of Asteracantha Polluted methanolic extracts	85
	Comparision of results for Total sugar content and Protein content estimation	
35	Table 7.1: Comparision of biochemicals from Vinca - polluted area and unpolluted area	86
36	Table 7.2: Comparision of biochemicals from Asteracantha - polluted area and unpolluted area	86
	Comparision of results for HPLC chromatograms	
37	Table 7.3: Comparision of Number of alkaloids found inVincafrom polluted area and unpolluted area	86
38	Table 7.4: Comparision of Number of alkaloids found inAsteracanthafrom polluted area and unpolluted area	86

List of Figures

Sr. No.	Description	Page No.
1	Fig 1.1 Reducing sugar containing hemiacetal group and its reaction with water	5
2	Fig. 1.2 Non reducing sugar	6
3	Fig 1.3 *Vinca rosea .Linn*, flower *and* showing a few flowers	11
4	Fig 1.4 Asteracantha longifolia (K.Schum) with flower and whole plant showing a few flowers	17
	Materials and Methods	
5	Fig 4.1 Soxlet apparatus used for extraction of methanolic extracts of the selected plants	27
6	Fig 4.2 HPLC unit with its specifications	29
7	Fig 4.3 Soxlet apparatus showing its individual elements	31
	Observations and Calculations	
8	Fig 5.i Standard Graph of proteins using standard BSA stock solution	35
9	Fig 5.ii Standard Graph of sugars using standard glucose stock solution	52
	Observations for Phytochemicals	
10	Fig 5.1 Leaf of Vinca unpolluted showing presence of saponins and flavonoids respectively	61
11	Fig 5.2 Stem of Vinca unpolluted showing presence of saponins and flavonoids respectively	62
12	Fig 5.3 Root of Vinca unpolluted showing presence of saponins, flavonoids and alkaloids respectively	63
13	Fig 5.4 Methanolic extracts of Vinca unpolluted showing presence of alkaloids and saponins respectively	64
14	Fig 5.5 Methanolic extracts of Vinca unpolluted showing presence of flavonoids	64
15	Fig 5.6 Water extracts of Vinca unpolluted showing presence of alkaloids and saponins respectively	65
16	Fig 5.7 Water extracts of Vinca unpolluted showing presence of flavonoids	65
17	Fig 5.8 Leaf of Vinca polluted showing presence of saponins and flavonoids	66
18	Fig 5.9 Stem of Vinca polluted showing presence of saponins and flavonoids	67
19	Fig 5.10 Roots of Vinca polluted showing presence of saponins and flavonoids	68

20	Fig 5.11 Roots of Vinca polluted showing presence of Steroids by test i and ii respectively	69
21	Fig 5.12 Roots of Vinca polluted showing presence of alkaloids	69
22	Fig 5.13 Methanolic extracts and water extracts of Vinca polluted showing the presence of saponins and methanolic extracts of Vinca polluted showing the presence of steroids by test ii	70
23	Fig 5.14 Methanolic extracts and water extracts of Vinca polluted showing the presence of Flavonoids	71
24	Fig 5.15 Methanolic extracts and water extracts of Vinca polluted showing the presence of alkaloids	71
25	Fig 5.16 Leaf of Asteracantha Unpolluted showing the presence of Saponnins and flavonoids respectively	72
26	Fig 5.17 Stem of Asteracantha Unpolluted showing the presence of saponins and flavonoids respectively	73
27	Fig 5.18 Root of Asteracantha Unpolluted showing the presence of saponins and alkaloids respectively	74
28	Fig 5.19 Root of Asteracantha Unpolluted showing the presence of flavonoids respectively	74
29	Fig 5.20 Methanolic extrcts of Asteracantha unpolluted showing the presence of saponins and flavonoids respectively	75
30	Fig 5.21 Methanolic extrcts of Asteracantha unpolluted showing the presence glycosides and alkaloids respectively	76
31	Fig 5.22 Water extrcts of Asteracantha unpolluted showing the presence saponins, flavonoids and alkaloids respectively	76
32	Fig 5.23 Leaf of Asteracantha polluted showing the presence of saponins and flavonoids respectively	77
33	Fig 5.24 Stem of Asteracantha polluted showing the presence of saponins and flavonoids respectively	78
34	Fig 5.25 Root of Asteracantha polluted showing the presence of saponins, flavonoids and alkaloids respectively	79
35	Fig 5.26 Methanolic and water extracts of Asteracantha polluted showing the presence of saponins and alkaloids respectively	80
36	Fig 5.27 Methanolic and water extracts of Asteracantha polluted showing the presence of glycosides	81
37	Fig 5.28 Methanolic extracts of Asteracantha polluted showing the presence of terpenoids	81
38	Fig 5.29 Methanolic and water extracts of Asteracantha polluted showing the presence of flavonoids	81

	RP-HPLC Chromatograms	
39	Fig 6.1 Chromatogram for Vinca (Unpolluted) methanolic extracts	82
40	Fig 6.2 Chromatogram for Vinca (Polluted) methanolic extracts	83
41	Fig 6.3 Chromatogram for Asteracantha (Unpolluted) methanolic extracts	84
42	Fig 6.4 Chromatogram for Asteracantha (Polluted) methanolic extracts	85

List of Abbreviations

Sr. No.	Abbreviations	Names
1	AAS	Atomic Absorption Spectroscopy
2	BSA	Bovine Serum Albumin
3	CO	Carbon monoxide
4	CO_2	Carbon dioxide
5	–CO-NH-	Peptide bond
6	CAT	Catalase
7	DNA	Deoxyribonucleic acid
8	DNSA	3,5 - Dinitrosalicyclic acid
9	DPPH	2,2 – Diphenyl – 1 – picrylhydrazyl
10	D/W	Distilled Water
11	EDTA	Ethylenediaminetetraacetic acid
12	(γ) GT	Gammaglutamyltranspeptidase
13	GC – MS	Gas Chromatography – Mass Spectrometry
14	GPx	Glutathione peroxidase
15	HPLC	High Performance Liquid Chromatography
16	K^+	Potassium ions
17	LCD	Liquid Crystal Display
18	LOD	Limits Of Detection
19	LOQ	Limits Of Quantification
20	Mg^{++}	Magnesium ions
21	MIDC	Maharashtra Industrial Development Corporation
22	MS	Murashige and Skoog
23	NH^{4+}	Ammonium ions
24	NO_X	Nitrogen oxides
25	OD and ΔOD	Optical Density
26	PAPL	PadmajaAerobiologicals Private Limited (Laboratory)
27	PCR	Polymerase Chain Reaction
28	RAPD	Random Amplified Polymorphic DNA
29	RFLP	Restriction Fragment Length Polymorphism
30	ROS	Reactive Oxygen Species
31	RP – HPLC – UV	Reverse Phase - High Performance Liquid Chromatography – Ultra Voilet
32	RT	Retention Time
33	SGD	Strictosidine β – D –glucosidase
34	SO_X	Sulphur oxides
35	TIA	Terpenoidindole alkaloids
36	TLC	Thin Layer Chromatography
37	VBL	Vinblastine
38	VCR	Vincristine
39	VDS	Vindesine
40	VRL	Vinorelbine
41	WHO	World Health Organization

Plant is a living organism of the kind exemplified by trees, shrubs, herbs, grasses, ferns, and mosses, typically growing in a perpetual site. It's an Auxotroph which makes its own food. Many plants have special substances in their roots, leaves, flowers, or seeds that help them to survive. Since the early times, people have gathered these substances to create herbal medicines to treat certain diseases. Many of the powerful drugs used in modern medicines originated in plants. Today's plant-based drugs are used in treatment of a range of diseases, from headaches to cancer.

Medicinal plants have been acknowledged and used throughout human history. Plants synthesize compounds which perform important biological functions, and defend against attack from insects, fungi and herbivorous mammals. At least 12,000 such compounds have been isolated so far. (Tapsell LC, Hemphill I,*et al*, 2006). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs. Thus, herbal medicines do not differ greatly from conventional drugs in terms of their function.

The indigenous systems of medicines, developed in India for centuries, make use of many medicinal herbs. In one of the study of the WHO, it is estimated that 80 per cent of the population of developing countries relies on traditional plant based medicines for their health requirements (Dr. Badri M. *et al*, 1978). Because of increasing demand and wider use, it is essential that the quality of plant-based drugs should be assured prior to use.

From past few decades the increasing environmental pollution has been a major concern.It is affecting most of the living beings including the human and the plants. The plants are more affected to the environmental pollutants since the plants are directly exposed to them for maximum of their life span. This causes deleterious effects on the plants. It's been observed that the environmental pollutants have increased rapidly in India which is affecting the different plant species of this country. It has been observed that in the state of Maharashtra there was difference in the behavior of the plants depending on their exposure to the different climatic conditions.Thus same plants growing in the minimal polluted rural areas exhibit different properties as compared to those growing in the highly polluted urban areas.

Environmental pollution is caused by vehicular emissions consisting of gases such as CO2, CO, NOX and SOX. Plants absorb the atmospheric carbon dioxide to carry out

photosynthesis. When plants are grown in such a polluted atmosphere the absorption of such toxic gases will cause both genetic and physiological changes. The genetic changes will be in the form of mutations affecting the normal development of the plant and even affecting the biochemical pathways. The physiological changes will be seen in form of dwarfing of the plant, yellowing and wilting of leaves and roots appearing fragile. Thus the overall development and behavior of the plant will be affected due to the polluted environment.

When pollution in the air or water contaminates the plants from which herbal drugs are derived, they affect both plant physiology and biochemical characteristics. Plants exposed to pollution stress show changes in production of secondary metabolites. High levels of contamination in medicinal or other plants may either suppress or uncontrollably increase secondary metabolite production thereby affecting production of the biochemicals.

Alternatively, the presence of heavy metals, due to pollution, in medicinal plants may stimulate production of bioactive compounds in many plant species. Moreover, some research results suggest that heavy metals may play an important role in triggering plant genes to alter the titers or nature of secondary plant metabolites, although the exact mechanism by which this happens remains unclear. In particular, reactive oxygen species (ROS), generated during heavy metal stress, may cause lipid peroxidation that stimulates formation of highly active signaling compounds capable of triggering production of bioactive compounds (secondary metabolites) that either enhances the medicinal value of the plant or toxifies it. (Nasim SA, Dhir B, 2010)

These different physiological and biochemical modifications taking place within a plant can be deliberated by performing a phytochemical analysis. This can be done by performing comparative analysis of the plants obtained from unpolluted and polluted regions. The analysis will include the observation for the phenotypic, genetic and biochemical changes in the plants of both these categories. The genetic changes can be studied using modern molecular biology techniques. While the biochemical changes can be studied by phytochemical analysis using HPLC, GC-MS techniques. The later analytical methods will enable to quantify the amount of proteins and sugars present in these plants.

There are studies which have performed comparative analysis of the medicinal plants from the unpolluted and polluted regions. The products with medicinal value were affected by pollutants. However, such comparative studies are yet to be performed in *Vinca rosea .Linn* and *Asteracantha longifolia (K. Schum)* which are of very high medicinal value.

2.1 Principles

Screening or Preliminary Test is the first thing to be done before major discoveries of molecules or drug entities are known. It is used to provide concrete knowledge and research to what plant active constituents have potential to benefit mankind.

2.1.1Sugars:

Carbohydrates are of fundamental importance as they are the main source of energy in the cell and are the most abundant biomolecules on earth. They are required for various metabolic reactions in a living cell. They are also the structural components of living organisms.

Certain sugars can be estimated based on their ability to reduce certain compounds like potassium ferricynide, 3,5-dinitrosalicyclic acid and copper sulphate. Hence they are called reducing sugars. These compounds are generally reduced by reducing sugars in the presence of alkali. An alkaline solution of DNSA is reduced by sugars to 3-amino-5-nitro salicyclic acid. The chemistry of the reaction is complicated and different sugars give different color yields. Thus determination of complex mixtures of reducing sugars would require some different analytical technique. A pre-preparation for non-reducing sugars to convert them to reducing sugars and then estimate it by the DNSA method.

A **reducing sugar** is a carbohydrate that is oxidized by a weak oxidizing agent (an oxidizing agent capable of oxidizing aldehydes but notalcohols, such as the Tollen's reagent) in basic aqueous solution. The characteristic property of reducing sugars is that, in aqueous medium, they generate one or more compounds containing an aldehyde group.

Hemiacetals and hemiketals are compounds that are derived from aldehydes and ketones respectively. The Greek word"hemi" means half. These compounds are formed by formal addition of an alcohol to the carbonyl group.When the alcohol group is replaced by a second alkoxy group, an acetal or a ketal, respectively, is formed.

eg. 1: α-D-glucose, which contains a hemiacetal group and, therefore, reacts with water to give an open-chain form containing an aldehyde group.

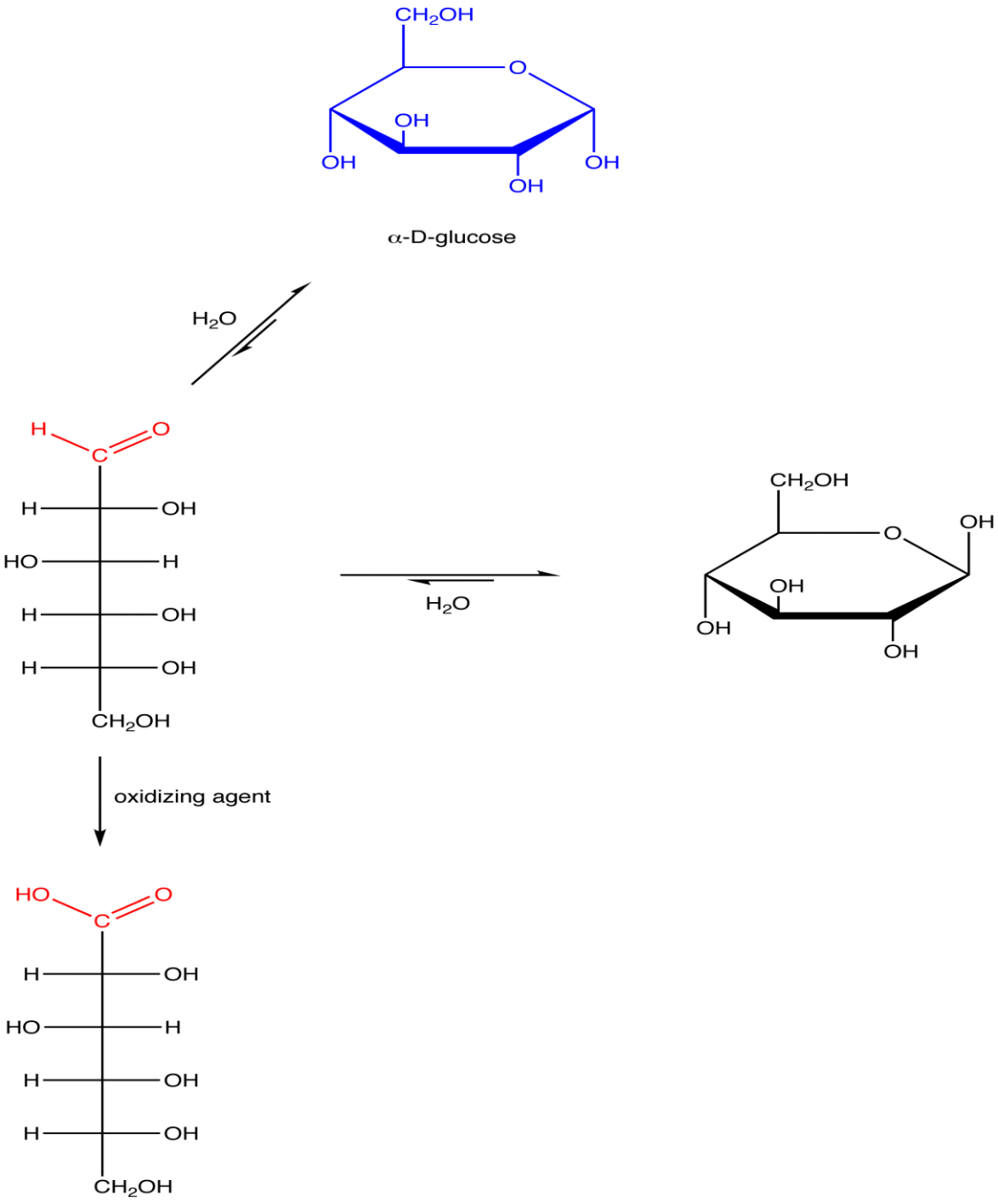

Fig 1.1Reducing sugar containing hemiacetal group and its reaction with water

A **non-reducing sugar** is a carbohydrate that is not oxidized by a weak oxidizing agent (an oxidizing agent that oxidizes aldehydes but notalcohols, such as the Tollen's reagent) in basic aqueous solution. The characteristic property of non-reducing sugars is that, in basic aqueous medium, they do not generate any compounds containing an aldehyde group.

eg: sucrose, which contains neither a hemiacetal group nor a hemiketal group and, therefore, is stable in water.

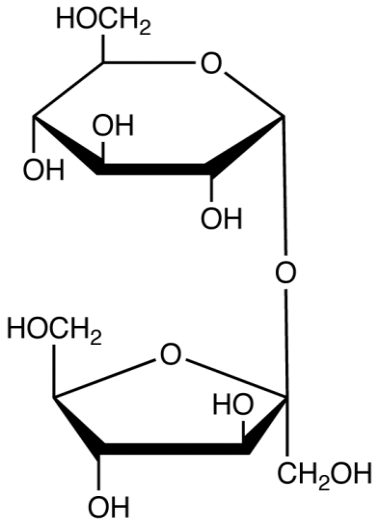

Fig 1.2 Non reducing sugar

2.1.2 Proteins:

Proteins are the most abundant intracellular macromolecules and constitute over half of the dry weight of most of the living things. These are the complex organic nitrogenous substances which play an important role in the functioning of a cell. The proteins with catalytic activity are largely responsible for determining the properties of a cell in a particular environment. Proteins are involved in a variety of functions such as building of the cell wall and membrane structure assisting in the transport of small molecules across the membranes, acting as stored form of nitrogen in the seeds, and catalyzing biochemical reactions. In some plants large amount of stored proteins serves as reserves of energy and nitrogen to be utilized during periods of rapid growth.

Folin Lowry's method is the most commomly used method for determination of proteins in the extracts because of high sensitivity and quantity as low as 20µg of proteins can be measured the –CO-NH- (peptide bonds) in poly peptide chain reacts with copper in an

alkaline medium to give blue colored complex. In addition, tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contributes towards enhancing the sensitivity of this method. Care should be taken that substances like EDTA, Tris, carbohydrates, $NH4^{+}$, K^{+}, Mg^{++} should not be present in the sample preparation as they interfere with the color development.

2.1.3 Phytochemicals:

Phytochemicals are a large group of plant-derived compounds hypothesized to be responsible for much of the disease protection conferred from diets high in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine. Hundreds of phytochemical compounds, with several different biological functions, have been identified in plant-based foods. Therefore, consuming a variety of plant-based foods helps to ensure that individuals receive the optimum benefits from the fruits and vegetables consumed. More research is needed to fully explain the actions of phytochemical compounds in the human body (Heneman K. and Zidenberg-CherrS., 2008).

2.1.4 Soxlet Extraction:

A Soxhlet extractor is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet . Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance.

Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The extraction solvent to be used is taken into a distillation flask and the Soxhlet extractor is now placed onto this flask. The Soxhlet is then equipped with a condenser.

The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.

The chamber containing the solid material is slowly filled with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillationflask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

2.1.5 HPLC:

High-performance liquid chromatography (formerly referred to as high-pressure liquid chromatography), HPLC, is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Normal Phase HPLC uses a very non-polar environment, hydrophilic molecules tends to associate with each other (like water drops on an oily surface). The hydrophilic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of a particle if that surface is also hydrophilic. Increasing the polarity of the mobile phase will subsequently decrease the adsorption and ultimately cause the sample molecules to exit the column. This mechanism is called Normal Phase Chromatography. It is a very powerful technique that often requires non-polar solvents.

Reversed Phase HPLC is the opposite of normal phase, results from the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction

between the solute and the solid support resulting in de-sorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote de-sorption.

2.2 Selected plants

The two plants species selected for this research project are as following,

1. *Vinca rosea.Linn (Catharanthus roseus G. Don)*
2. *Asteracantha longifolia (K. Schum) (Hygrophila auriculata(L.) Ness)*

These plants are medicinal shrubs having many useful secondary metabolites mainly alkaloids but are in very small quantities. Vinblastine, vincristine and other such alkaloids which are found in *Vinca rosea .Linn* are used for formulating anticancer drugs. Whereas lupeol, stigmasterol, etc in *Asteracantha longifolia(K. Schum)* which have antipyretic, hepatoprotective, antioxidant, anticancer and other such properties.

These alkaloids are in high demand because of their anticancer properties. However they are given in very low yields by these plantsthus they are high in demand but.

2.2.1 Review of literature of*Vinca rosea .Linn*:

Vinca rosea .Linn:

Botanical Classification:

Fig 1.3 *Vinca rosea .Linn*, flower andshowing a few flowers

Domain: Eukarya
Kingdom: Plantae
Phylum: Magnoliophyta
Class: Magnoliopsida
Order: Gentianales
Family: Apocynaceae
Genus: Catharanthus
Species: *Catharanthus roseusG. Don*
Synonym: *Vinca rosea .Linn*

Vinca rosea .Linn also goes by other botanical name *Catharanthus roseusG. Don* commonly known as the Madagascar rosy periwinkle, is a species of Catharanthus native and endemic to Madagascar. Other English names occasionally used include Cape periwinkle, rose periwinkle, rosy periwinkle, and "old-maid".

The word Catharanthus derives from the Greek language meaning "pure flower." While roseus means red, rose or rosy thus, resulting in how the Madagascan Periwinkle has also been given the name the "rosy" periwinkle.

Habitat: It is a short plant found generally in any gardens and can grow even on the road side. It can grow up to 1 m tall. Its leaves are oblong, glossy green and can grow as long as 9cm and 3.5 cm broad with a very short petiole. It has two types of flowering, white flowers and pink flowers both with dark red centers. A short basal tube around 2.5cm to 3 cm long with a corolla of about 2.5cm diameter, with five petal like lobes. Its fruit is a 2.5 cm to 3 cm long and 3mm broad pair of follicles.

Uses: The records indicate that Vinca has been used as a medicinal herb for centuries. Although native to Madagascar, the plant has naturalized throughout subtropical Asia, Africa and the Americas and has been used both ornamentally and medicinally. All parts of the plant have been used in regional herbal medicine, including the dried root, leaves, flowers and stalks. Alkaloids used in modern medicine are extracted from the whole dried plant. To help preserve the plant in the wild, it is cultivated for medicinal use in many areas of the world.

Indian Ayurvedic medicine and other traditional herbal systems use Vinca for the treatment of diabetesand certain cancers, such as Hodgkin's disease and acute leukaemia. The drug for treating Hodgkin's disease has increased patients' chances of survival from one-in-five to nine-in-ten. Insect stings are relieved using a juice from the leaves. Herbal use in the Caribbean includes using extracts from the flowers as eyewash for infants. The flowers are also used for treating asthma and excess gas. Other traditional herbal treatments include using the plant for painful menstruation, tuberculosis and rheumatism.

A lot of studies have been done on *Vinca rosea .Linn*plants such as phytochemical analysis, vinca alkaloids, secondary metabolite study, study of enzymes in *C. roseus*, etc.

Vinca (Madagascar periwinkle) has more than 400 known alkaloids!Some are used by the pharmaceutical industry for the treatment of childhood leukemia, Hodgkin's disease, testicular cancer and cancerous tumors.Taken as a daily supplement, it improves the blood supply to the brain, increases oxygen and glucose for the brain to use, helps prevent abnormal coagulation

of blood, and it raises brain levels of the neurotransmitter serotonin. There are two classes of active compounds in Vinca: alkaloids and tannins.The major alkaloid is known as vincamine.A closely related semi-synthetic derivative of vincamine is widely used as medicine is known as ethyl-apovincaminate or vinpocetine.

It has vasodilating, blood thinning, and memory-enhancing actions. It has been shown in double-blind studies to help alleviate a type of dementia known as vascular dementia, in which the arteries supplying blood to the brain develop atherosclerotic plaques.Extracts of Vinca have significant anticancer activity against numerous cell types.The greatest activity is seen against multi-drug resistant tumor types which suggest that there are compounds in Vincathat are synergistic or additive with anti-neoplastic elements by inhibiting resistance to them.

In India, leaf extracts are also used for treating external wounds. The hypoglycemic activity of alkaloids isolated from Vincahave been studiedpharmacologically and a remedy derived from the plant has been marketed under the proprietary name Vinculin as a treatment for diabetes.Alcoholic whole plant extracts at high dose (500 mg/kg) exhibited significant antihyperglycemic activity and has absence of acute toxicity. The extract effectively reverses the changes in the blood sugar level and the beta-cell population. The exact phytoconstituents responsible for the antidiabetic effect are not known yet.Two chemotherapy drugs are: Vinblastine (scientific name vincaleukoblastine) and Vincristine (scientific name 22oxovincaleukoblastine). These alkaloids are cytotoxic and target sites in cell division thus preventing mitosis to proceed. Although for a normal person, this action of the vinca alkaloids can be toxic but they are helpful agents in stopping the division of cancerous cells. Vinca alkaloids are extensively used in the prevention and treatment of cancer. The two common cancer treatment drugs (chemotherapy) from Lilly Velban and Oncovin are
produced from vinblastin and vincristine respectively. (Tropilab® Inc,)

Vinca(Apocynaceae) is a medicinal plant better known as Madagascar periwinkle and in Malaysia as Kemuning Cina. The aerial part of the plant contains about 130 different alkaloids from which well-known high value secondary metabolites vincristine and vinblastine are used in chemotherapy to treat diverse cancers, while ajmalicine and serpentine are prescribed for hypertension. A large body of literature documented the activities of
Vincain different ailments. Recently, antioxidant potential was assessed against 2,2-diphenyl1-picrylhydrazyl (DPPH) along with screening of phenolic compounds.

Since more than three decades, different analytical techniques have been used for qualitative or quantitative determination of Vincametabolites. Among them HPLCtechnique is still widely used for the separation and analysis of secondary metabolites from Vinca.

Separation of alkaloids by HPLC analysis is not only essential for plant cell line screening, but also for the design and the validation of product recovery and purification processes at an industrial scale. Therefore, efficiency of the harvesting procedure as well as the accuracy of separation methods relies on the detectors sensitivity highlighted the studies on Vincaalkaloids by HPLC. The major constraint for this type of studies is the lack of sensitive and accurate rapid estimation methods due to complexity in the chemical assay of molecules that occur in low quantities. HPLC system equipped with an auto sampler provides a powerful tool to analyze various samples. The separation of indole alkaloids is based on reverse phase chromatography using C column as a stationary phase. Several mobile phases usually consist of a mixture of buffer solutions like diammonium phosphate or ammonium acetate supplemented with triethylamine along with methanol or acetonitrile. Detection was carried out using a UV detector at fixed wavelength or a fluorescence detector. Recently in one of the study Pereira and associates discuss the metabolite analysis and its biological potential using HPLC analysis for phenolic compounds and amino acids of
Vincaseeds.(SiddiquiM .J. A.,*et al*, 2011)

Vinca alkaloids are a subset of drugs obtained from the Madagascar periwinkle plant. They are naturally extractedfrom the pink periwinkle plant, Vincaand have hypoglycemic as well as cytotoxic effects. They have been used to treat diabetes, high blood pressure and have been used as disinfectants. The vinca alkaloids are also important for being cancer fighters. There are four major vinca alkaloids in clinical use: Vinblastine (VBL), vinorelbine (VRL), vincristine (VCR) and vindesine (VDS). VCR, VBL and VRL have been approved for use in the United States. Vinflunine is also a new synthetic vinca alkaloid, which has been approved in Europe for the treatment of second-line transitional cell carcinoma of the urothelium is being developed for other malignancies. Vinca alkaloidsare the second-most-used class of cancer drugs and will stay among the original cancer therapies.

Molecular cloning and analysis of strictosidine beta-D-glucosidase, an enzyme in terpenoid indole alkaloid biosynthesis in Catharanthus roseus (Geerlings A*et al*, 2000).Strictosidine beta-

D-glucosidase (SGD) is an enzyme involved in the biosynthesis of terpenoid indole alkaloids (TIAs) by converting strictosidine to cathenamine. The biosynthetic pathway toward strictosidine is thought to be similar in all TIA-producing plants. Somewhere downstream of strictosidine formation, however, the biosynthesis diverges to give rise to the different TIAs found. SGD may play a role in creating this biosynthetic diversity.

Vinca produces widely usedalkaloids such as the antihypertensive compounds ajmalicine and serpentine, as well as the anticancer agentsvinblastine and vincristine. The latter compounds bind specifically to tubulin during mitosis and block the ability of the protein to polymerize into microtubules. Both normal and malignant cells exposed to these alkaloids undergo changes characteristic of apoptosis. Earlier reports about Vincacell culture transformation using particle bombardment provided data about promoter activities monitored by gene expression. Analysis of Vincaalkaloids has been reported by variouschromatographic and electrophoretic techniques. (Guttman A.*et al*, 2004)

Vinca alkaloids are a material of a class of organic compounds made up of carbon, hydrogen, nitrogen and oxygenthat is often derived from plants is named alkaloid. Although, the name represents alkali like some do not exhibit alkaline properties. Many alkaloids with having poisonous characteristics have physiological effects too that make them useful as medicines. The oldest group of the plant alkaloids groups that used to treat cancer are the vinca alkaloids.(Moudi M.*et al*, 2013)

A preliminary phytochemical analysis was carried out of Vincaa very useful and much exploited medicinal plant in Tamil Nadu. This study revealed that the methanol, petroleum ether, ethanol, and aqueous extract of leaves of Vincawere analyzed through qualitative and quantitative identification of secondary metabolites. The phytochemical constituents like alkaloids, flavonoids, Sterols, Phenols, Tannins and glycosides were screened through TLC. (GomathiB. and AnuradhaR. , 2013)

One of the studies show antidiabetic activity of Vinca methanolic whole plant extracts in alloxan induced diabetic rats for 14 days. The methanolic whole plant extract at high dose (500 mg/kg) exhibited significant anti-hyperglycemic activity than whole plant extract at low dose (300 mg/kg) in diabetic rats. The methanolic extracts also showed improvement in parameters like body weight and lipid profile as well as regeneration of -cells of pancreas in

diabetic rats. Histopathological studies reinforce the healing of pancreas, by methanolic Vinca extracts, as a possible mechanism of their antidiabetic activity (AhmedM. F. *et al*, 2010)

The main mechanisms of vincaakaloid cytotoxicity is due to their interactions with tubulin and disruption of microtubule function, particularly of microtubules comprising the mitotic spindle apparatus, directly causing metaphase arrest. (Dr. Negi RS. 2011)

2.2.2 Review of literature of*Asteracantha longifolia(K. Schum)*:

***Asteracantha longifolia (K. Schum)*:**

Botanical Classification:

Fig 1.4 *Asteracantha longifolia (K.Schum)* with flowerand whole plant showing a few flowers

Domain: Eukarya

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Asterales

Family: Acanthaceae

Genus: Hygrophila

Species: *Hygrophila auriculataK. Schum*

Synonym: *Asteracanthalongifolia(L.) Ness*

- Asteracantha (Sanskrit: Kokilaksha) is a plant in the acanthus family. It is native to India and commonly known as marsh barbelor gokulakanta.
- In India, its seeds, roots, and panchang (panch = five ang = parts, i.e. root, flower, stem, fruit, and leaves as ash burnt together) are used as a medication in ayurveda.

Habitat: It is a robust, erect, annual herb. The stems are sub-quadrangularwith thickened nodes; the leaves are oblanceolate, with a yellow spine in its axil; the flowers pale, purple blue, densely clustered in axils; the fruits are oblong, glabrous capsules, 4-8 seeded.The seeds contain large amount of tenacious mucilage and potassium salts.

Uses: The roots, leaves and seeds have been used in Indian systems of medicine as diuretics and also employed to cure jaundice, dropsy, rheumatism, anasarca and diseases of the urinogenital tract.Roots are sweet, sour, bitter, refrigerant, diuretic, anti-inflammatory, analgesic, haemopoictic, hepatoprotective and tonic. It is useful in inflammations, hyperdipsia, strangury, jaundice and vesical calculi. It is also used in flatulence and dysentery. Leaves are haemopoictic, hepatoprotective, anti-inflammatory, antioxidant, analgesic, antidiabetic, stomachic, ophthalmic, diuretic and liver tonic. It is used in hepatic obstruction, jaundice, arthritis, rheumatism and diseases of urinogenital tract. It is useful in flatulence and other stomach related diseases. It is useful in anemia and for treating blood diseases. It is used to lower the blood sugar level. It is also used in diarrhoea and dysentery. A decoction of the roots is used as a diuretic and to treat rheumatism, gonorrhoea, and other diseases of the genito-urinary tract, jaundice and anasarca.

Studies on *Asteracantha longifolia (K. Schum)* also involves extraction of secondary metabolites from the plant, antibacterial potential, quantification of the secondary metabolites, etc

The earliest study was carried onAsteracantha, in the year 1887, the entire plant was used in medicine; the practitionersof native medicine in Ceylon considered it as one of the best medicines in dropsy, and was given in the form of a decoction, and locally was used as a fomentation in cases of inflammation and in rheumatism. The ashes of the burnt plant, in doses

of about half a teaspoonful twice or thrice a day, was a form of administration followed by native practitioners of India. Also, they frequently employed it in dropsical cases, and it undoubtedly possesses considerable power as a diuretic. It is also favourably reported by several of the surgeons of India.(Williama A.andJayesingha L.C.M.C, 1887)

A study on rheumatoid arthritis and Asteracanthарevealed that, plant contains a phytosterol essential oil is present in roots diuretic properties of the seeds is due to large amount of mucilage and potassium salts, seeds also contain 23% of an yellow semi drying oil. Diastase, Iipase, protease and a alkaloid were isolated from the seeds. Asterol I,II,III and IV, Asteracanthine and Asteracanthicine were also isolated from the seeds. An alkaloid and lupeol were also isolated from the whole plant. The chemical investigation of the plant showed that it has very high protein content. The higher protein content of the plant may probably help to raise the immunity of the patient. It may help for the destruction of immune aggregate, i.e. antigen antibody complexes which are one of the basic causes of the disease (ThankammaA., 1999).

The compounds identified in Asteracantha are mainly phytosterols, fatty acids, minerals, polyphenols, proanthocyanins, mucilage, alkaloids, enzymes, amino acids, carbohydrates, hydrocarbons, flavonoids, terpenoids, vitamins and glycosides. Some of the reported phytoconstituents are lupeol, lupenone, 25-oxo-hentriacontanyl acetate, stigmasterol, betulin, β- carotene, hentriacontane, apigenin-7-O-glucuronide, apigenin-7-O-glucoside, 3methylnonacosane, 2,3-ethylcholesta, 2,3-dien-3β-ol, luteolin, asteracanthine, asteracanthicine, luteolin-7-rutinoside, methyl-8-n-hexyltetracosanoate, β-sitosterol, histidine, phenylalanine, lysine, ascorbic acid, nicotinic acid, n-triacontane, glucose, mannose, rhamnose, arabinose, xylose, maltose, myristic acid, oleic acid, palmitic acid, stearic acid, linoleic acid etc. Ethanolic extract of the fruits, hydroalcoholic extract of whole plant and crude petroleum ether extract of the plant are having anticancer activity. Antibacterial activity was exhibited by the chloroform and methanol extract of the whole plant, and methanolic extract of the leaves. Antifungal activity against Aspergillus tamari, Rhizopus solani, Mucor mucedo and Aspergillus niger is due to the proteins and peptides present in the plant. Potential in treating liver diseases of the aerial parts, roots and whole plant was studied by various models viz. Carbon tetrachloride induced hepatotoxicity, paracetamol and thioacetamide intoxication, and galactosamine induced liver dysfunction in rats. Seeds, leaves, aerial parts and roots showed

antinociceptive activity which was studied using both chemical and thermal methods of nociception in mice. Some Ayurvedic, Unani and Siddha formulations of the plant are claimed to have anabolic-cum androgenic like activity. The plant was also studied for haematopoeitic, hypoglycemic, anti-inflammatory, antioxidant, hypotensive, diuretic, macrofilaricidal activities etc. Apart from the above established studies the plant is traditionally used for the treatment of anasaraca, diseases of urinogenital tract, dropsy of chronic Bright's disease, hyperdipsia, vesical calculi, flatulence, diarrhea, dysentery, leucorrhoea, gonorrhoea, asthma, blood diseases, gastric diseases, painful micturition, menorrhagea etc. (Patra A. *et al*, 2009)

The aqueous and ethanolic extract of Asteracanthaand *Andrographis paniculata*were shown to have broad spectrum antibacterial activity. The ethanolic extract of both the plants Asteracanthaand *Andrographis paniculata*were analysed by GC-MS. The study indicated the potential usefulness of Asteracanthaand *Andrographis paniculata* in the treatment of various pathogenic diseases.(Abiramii N. *et al*, 2009)

One of the studies was carried out on alkaloids, tannins, saponins, steroid, terpenoid, flavonoids, phenolic compounds and cardic glycoside distribution in five medicinal plants belonging to different families including Asteracantha. The significance of the plants in traditional medicine and the importance of the distribution of the chemical constituents were discussed with respect to the role of the plants in ethnomedicine in India. (DossA. 2009)

A study was conducted to understand the nephrotoxic effects of the alkaloids on rats. The results demonstrated that the ethanolic extract of whole plant of Asteracanthaevinced the therapeutic effect and inhibited gentamicin-induced proximal tubular necrosis (Bibu K J *et al*, 2009)

The antilithiatic effect of Asteracantha (Acanthaceae) was determined on ethylene glycol induced lithiasis in male albino rats. The histological finding showed improvement after treatment with Asteracantha. These observations enable to conclude that the curative and preventive properties of Asteracantha against ethylene glycol induced urolithiasis. (Satish R. *et al*, 2010)

Preliminary Phytochemical and Pharmacognostical Screening of the Ayurvedic Drug Asteracantha was studied. The plant is widely distributed throughout India, Sri Lanka, Burma, Malaysia and Nepal. Following various folk claims for cure numerous diseases, efforts have been made by researcher to verify the efficacy of the plant through scientific biological screenings. The plant contains saponins, alkaloids, steroids, tannins, flavonoids and triterpenoids are the main phytoconstituents. A scrutiny of literature revealed some notable pharmacological activities like anti-nociceptive, anti-tumor, antioxidant, hepatoprotective, hypoglycemic, haematinic, diuretics, free radical scavenging, anthelmintic, anti-inflammatory, antipyretic, anabolic and androgenic activities. This study deals with the preliminary phytochemical screening and detailed pharmacognostical study of leaf, stem and root of Asteracantha, which includes macro and microscopic studies, determination of physico-chemical parameters of the extract using TLC and HPTLC fingerprinting. The aqueous, alcoholic, petroleum ether, chloroform, ethyl acetate and n-butanol fractions separated from the alcoholic extract of Asteracantha were prepared and the total phenolic content was estimated (HussainM. S.*et al*, 2011)

Isolation and characterization of chemical constituents from Asteracantha seeds was studied. Asteracantha is commonly known as "Neermulli".The plant is an important medicinal herb, widely distributed in India and is used for different medicinal purposes. It is also used commercially as an ingredient of some over the counter formulations in liver disorder and those prescribed by general tonic. Many constituents have been reported from the plant Asteracantha.The extracted seeds of Asteracantha were subjected to isolation and purification of phytoconstutients. Isolation of phytoconstutients was done by Column chromatography using gradient elution with different mobile phases and silica gel as stationary phase. (KethaniC. D.*et al*, 2012)

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (ROS) now appears the fundamental mechanism underlying a number of human neurodegenerative disorders, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Antioxidants are the compounds which terminate the attack of reactive species and reduce the risk of diseases. A study was conducted to determine the antioxidant activity of two medicinal plants Asteracantha and *P. daemia*. They exhibited varying degrees of antioxidant activity ranged between 6.41 to 83.90%. The methanolic extract of Asteracantha

showed significantly higher antioxidant activity than the *P. daemia*. These results suggested the potentials of Asteracantha as a medicine against freeradical-associated oxidative damage (Doss A. and AnandS.P. 2013)

2.2.3 Comparative studies on other plants:

A published work on "The Growth of *Chenopodium Murale* Irrigated with Polluted and Unpolluted Water:a Modeling Approach" in which there is a study of random sample of 407 individual plants of *Chenopodium murale* growing in two sitesone irrigated with polluted water (polluted area) and the other irrigated with unpolluted water (unpolluted area) were collected to determine the effects of water pollutants on the growth parametersof this medicinally important plant.(Elkarmi A. *et al*, 2009)

Study has been carried out in selected quarry locations of Bangalore district for the determination of protein contents in *Calotropis gigantia, Muntingia calabora, and Annona squamosa* located in the vicinity near stone crushing units during summer, monsoon, and winter seasons. A significant reduction in protein and chlorophyll contents of the sampled leaves was observed compared to control, which may be attributed to the high emission and leaf deposition of dust, which adversely affects the metabolic activity of the plant. (NaikDP.
et al, 2005)

A study deals with the effect of effluent from electroplating industry. Effluent showed inhibitory effect on seed germination and seedling growth in the test plants. In *Zea mays*about 80% mortality of seedlings followed by leaf necrosis and browning of root tips was recorded after 15 days of growth and in *Oryza sativa* about 60% mortality followed by needle like necrotic tip in lives and necrotic brown tip of roots was recorded after 18 days of growth. Severity of toxicity was reduced after the dilution of effluent to 50% (Pandey S N. 2004)

Study reveals the genotoxic effect of pollutants emitted by Harduaganj thermal power plant on *Solanum melongena*. Due to the effects of various pollutants the chromosomal abnormalities like reduced chiasma frequency, increased number of univalents, multivalents, laggards, stickiness, precocious separation of chromosomes and pollen sterility have been observed. It is suggested that pollution is the major cause of chromosomal/genic variations in
S. melongena growing around thermal power plant. (Siddique I. and Ansari M Y K., 2005)
Plant polyphenols have drawn increasing attention due to their potent antioxidant properties and their marked effects in the prevention of various oxidative stress associated diseases such as cancer. A review provides an updated and comprehensive overview on phenolic extraction, purification, analysis and quantification as well as antioxidant properties of phytoconstituents.

The anticancer effects of phenolics in-vitro and in-vivo animal models were viewed, including recent human intervention studies. Also, possible mechanisms of action involving antioxidant and pro-oxidant activity as well as interference with cellular functions were discussed. (DaiJ. and MumperR. J. 2010)

A paper published in 2012 discussed, the medicinal plants and their potential characteristics when used as a treatment. The following were the characteristics discussed,

- Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.
- Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.
- Preventive medicine- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases.

This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment. (HassanB. A. R. 2012)

An RP-HPLC method with photodiode array detection was developed for the determination of major constituent berberine from *Berberis aristata* (Barberry) and *Berberis tinctoria*. Berberine (an isoquinoline alkaloid, have been reported to possess hepatoprotective and antiinflammatory actions) was isolated from the plant extract on semi-preparative HPLC and separated on HPLC by using an isocratic mode consisting of 0.1% trifloroacetic acid: acetonitrile (60:40, v/v) at a flow rate of 1 mL/min. The berberine content in *B. aristata* and
B. tinctoria was found to be 3.18% and 1.46% respectively. (ShigwanH. *et al*, 2013)

3. Aims and objectives:

Aim: To perform the comparative analysis of biochemicals in plants from polluted vs. those with unpolluted regions.

Objective: To understand the effect of environmental pollutants on the biochemical characteristics of these plants. We performed the comparison between the following: Vinca from unpolluted region (n=3) versus Vinca from polluted region (n=3), Asteracantha from unpolluted region (n=3) versus Asteracantha from polluted region (n=3). We performed preliminary screening tests i.e. estimation of proteins, sugars and the phytochemical analysis on all the plants. Then selected the one sample of plant which showed the optimal results and performed RP-HPLC analysis for alkaloids on that plant.

4.1 MATERIALS

4.1.1SAMPLES:

The selectedplants were acquired fromdifferent both,polluted and unpolluted regions.

Vinca:

Polluted area: This plant was acquired from polluted areas of MIDC area of Kalyan in Maharashtra. (3 plant samples from this area)

Unpolluted area: This plant was acquired from the hilly area located in Thane (W), Maharashtra called the "Yeoor hills". (3 plant samples from this area)

The plants were acquired in the month of December, bright and live with pink flowers.

Asteracantha:

Polluted area: This plant was obtained from the MIDC area located in Palghar, Maharashtra. (3 plant samples from this area)

Unpolluted area: This plant was obtained from the "Yeoor hills" located in Thane (W). Here fresh plants blossomed with flowers were procured. (3 plant samples from this area)

These were used for comparative biochemical analysis.

4.1.2GLASSWARES:

- Test tubes
- Pipettes (1ml, 5ml, 10ml)
- Glass Beakers (150ml, 500ml)
- Conical Flasks (250ml, 500ml)
- Glass rod

4.1.3INSTRUMENTS AND EQUIPMENTS:

- Colorimeter (450nm and 660nm)
- Micropipette (100 ul), micro tips
- Soxhlet apparatus(Consisting of a round bottom flask, jacket and condenser as shown in fig 4.1)
- HPLC unit (Fig 4.2)

Fig 4.1 Soxlet apparatus used for extraction of methanolic extracts of the selected plants

4.1.4CHEMICALS FOR PHYTOCHEMICAL ANALYSIS:

- **10% $FeCl_3$**
- **1% 1M HCl**
- **Chloroform**
- **H_2SO_4 conc.**
- **20% NaOH**
- **Mayer's reagent**
- **HCl conc.**
- **Na_2CO_3**
- **CH_3COOH (Acetic acid)**

4.1.5 CHEMICALS FOR SUGAR ESTIMATION (DNSA method):

- **Standard glucose stock:** 1 mg/ml
- **DNSA (Dinitro salicylic acid)**

4.1.6CHEMICALS FOR PROTEIN ESTIMATION (Folin – Lowry's method):

- **Standard BSA stock Solution** (200 µg /ml)
- **Lowry's solution Folin's reagent**

4.1.7 REQUIREMENTS FOR RP-HPLC ANALYSIS

Methods of HPLC analysis of Vinca

A **reversed phase-HPLC (RP-HPLC)** validated method was used to determine quantitativelyanticancer markers (vindoline, vincristine, catharanthine and vinblastine) using equipment with degasser, an auto sampler, a column heater, quaternary pump and UV detector. Column (Eclipse plus C18, 250 mm x 4.6 mm, 5 µm i.d.) was maintained at 40C and samples (20 µl) were eluted by an isocratic mobile phase consisting of methanol:acetonitrile:ammonium acetate buffer (15:45:40 v/v) with 0.1% triethylamine (25 mM) at 297 nm. (Siddiqui M J *et al*, 2010)

o

HPLC ANALYSIS OF ASTERACANTHA:

The quantitative analysis for alkaloids from Asteracantha was performed by **RP-HPLC** on Luna C18 100 Å, 250 x 4.6 mm column under isocratic elution of acetonitrile and water (80:20, v/v) with a flow rate of 1.0 ml/min and the total run time was 20 min. The column temperature was adjusted at 25°C and the detection wavelength was set at 210 nm. (Maji A K. et al, 2013)

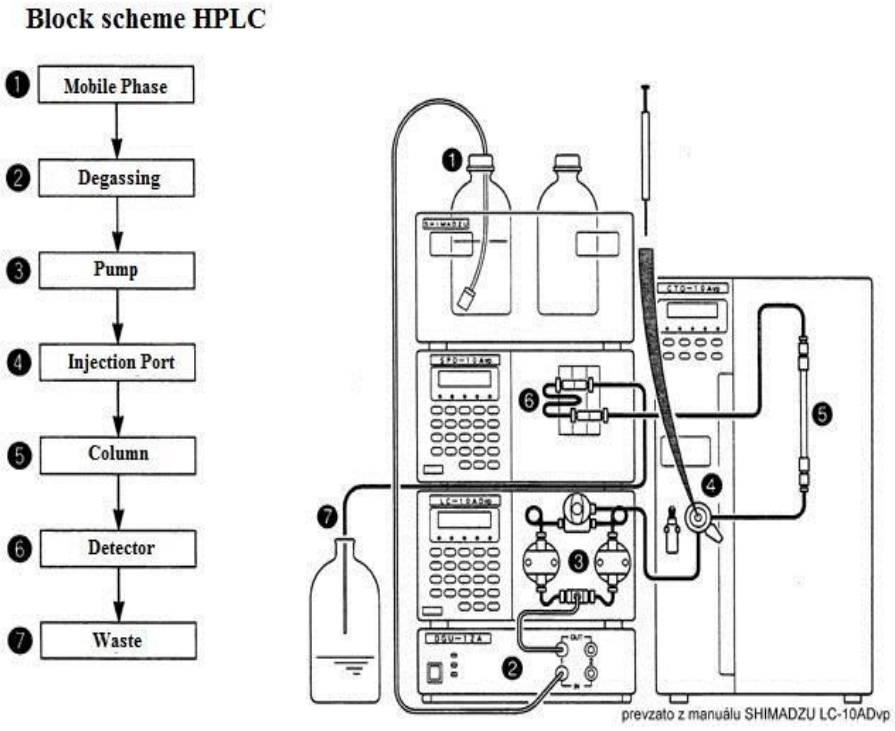

Fig 4.2HPLC unit with its specifications

4.2 METHODS

1. Collecting plants from polluted and unpolluted areas.
2. HPLC grade methanolic extracts were obtained using Soxhlet apparatus.
3. RP-HPLC analysis for alkaloids. **[**The normal phase HPLC analysis originally used a polar stationary phase or column, so that the nonpolar solutewas eluted from the column first and then the polar solute would be gradually eluted. TheRP-HPLC analysis i. e. Reverse Phase HPLCuses nonpolar columns so that the polar soluteis eluted first and the nonpolar soluteis gradually eluted**]**
4. To perform preliminary phytochemical analysis. (of methanolic extracts and water extracts)
5. To estimate Protein and sugar contents by Folin – Lowry's method and DNSA method respectively.

Phytochemical analysis of all the plant samples was performed. The presence of saponins, tannins, alkaloids, flavanoids, steroids or cardiac glycosides would provoke for an in-depth study on the plant. The metabolites are of various pharmacological importances.
Water extracts as well as methanolic extracts were prepared for all the tests.

Preparation of Extracts:

4.2.1Soxhlet extraction:

Weigh 2.5 gms of each, Leaf, stem and roots, of the plant. Place it in a filter paper and place this in the Jacket of the Soxhlet apparatus. Attach a pipe to the bottom outlet of the condenser and connect the other end of the pipe to a tap. Attach the inlet pipe and allow water to continuously flow. Add 75 ml of HPLC grade methanol in the round bottom flask and arrange it as follows,

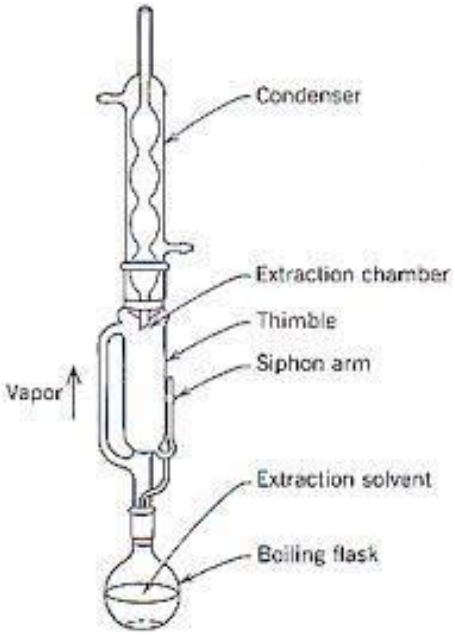

Fig 4.3 Soxlet apparatus showing its individual elements

Switch on the machine after making all the arrangements. As the flask is heated up the methanol will boil and start evaporating and the vapors are condensed in the condenser and falls on the sample placed in the Jacket and the extract falls in the flask again this accounts to one cycle. Around 13 to 15 such cycles yields complete HPLC grade methanolic extracts.
These extracts were further used for phytochemical analysis and HPLC analysis

4.2.2Water extracts:

Water extracts of leaf, stem, roots and whole plant were prepared by the following method.

Take 10 gm of plant material in 20ml distilled water. Grind the plant material in a mortar and pestle in minimum amount of distilled water initially later add remaining to make 20 ml, filter allow it to settle and use the supernatant to perform phytochemical tests

Biochemical parameters:

4.2.3 Estimation of Total Sugar Content:

i) Reducing sugars:

The extract was prepared by crushing 1gm of plant 10 ml of D/W allow it to settle and use supernatant to estimate the reducing sugar concentration

The reaction mixture contains 0.1ml extract + 1.9ml D/W + 2ml DNSA solution. Keep it for boiling in a water bath for 8 mins. Take OD at 420 nm.

ii) Non-Reducing sugars:

The extract was prepared by crushing 1gm of plant 2 ml of D/W. Add 2ml of conc. HCl to it and autoclave it for 45mins. Neutralize this solution using sodium carbonate (until the effervescence stops to obtain a clear solution). Filter this and make volume upto 10ml with D/W.

The reaction mixture contains 0.1ml the above extract + 1.9ml D/W + 2ml DNSA solution. Keep it for boiling in a water bath for 8 mins. Take OD at 420 nm.

The unknown concentrations of the sugars are then calculated by using a standard graph.

4.2.4 Estimation of Protein content:

The extract was prepared by crushing 1gm of plant 10 ml of D/W allow it to settle and use supernatant to estimate the protein concentration.

The reaction mixture contains 0.1ml extract + 0.9ml DW + 2ml Lowry's solution. Keep it in dark for 10 mins. Then add 0.2 ml of Folin's reagent and keep it in dark for 20 mins and take OD at 660 nm.

The obtained value of the absorbance of the unknown is the plotted on the graph to get a concentration

4.2.5 Phytochemical tests:

The phytochemical tests involves tests for the following,

i. **Test for Tannins**:

Add 1 ml of the water extract in 20 ml of distilled water, boil this for 5 minutes. Filter this and add 2ml distilled water and 2 – 3 drops of $FeCl_3$ solution. Formation of green precipitate willindicatethe presence of tannins.

ii. **Test for Saponins**:

Add 2.5 ml of water extract in 10ml distilled water, boil for 10 minutes and then filter. Add 2.5 ml of this filtrate and 10 ml distilled water and shakevigorously. The formation of stable foam willindicate the presence of saponins.

iii. **Test for Phlobatannins**:

Add 1 ml of water extract in1 ml of 1% HCl and then boil for 2 minutes. Deposition of a red precipitate will indicate an evidence for the presence of phlobatannins.

iv. **Test for Flavonoids**:

Take 1ml of water extract andboil with 10 ml of D/W for 5 mins and filtered while hot. Add few drops of 20% NaOH solution to 1ml cooled filtrate. Any color change of precipitation will indicate the presence of flavonoids.

v. **Test for Terpenoids**:

Add 5 ml of thewater extract in 2 ml of chloroform and 3 ml of concentrated sulphuric acid. Reddish brown precipitate at the interface will indicate the presence of terpenoids.

vi. **Tests for Cardiac glycosides**:

Add 5 ml of the waterextract in 2 ml of Glacial acetic acidcontaining a few drops of 10% $FeCl_3$. Then add Sulphuric acid carefully. A colour change from violet to blue to green will indicate the presence of a steroidal nucleus (that is, a glycone portion of glycoside).

vii. **Tests for steroids**:

a) Add 2 ml of organic extract in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it.A red colour produced in the lower chloroform layerindicates the presence of steroids
b) Add 2 ml of the organic extract was dissolved in 2 ml of chloroform and treated with sulphuric and 1 ml acetic acid. Development of a greenish colourwill indicate the presence of steroids.

viii. **Test for Alkaloid**:

Add 3 ml aqueous extract in 3 ml Mayer reagent. Turbidity of the resulting precipitate will betaken as an evidence for the presence of alkaloids.

5.1Observations and Calculations for Total Sugar Content Std. Glucose stock: 1 mg/ml Range: 0.2 mg to 1 mg Total volume: 1ml

Table 5.1(For standardization of glucose)

Tube no.	Concentration (mg)	Std. sugar solution (ml)	Distilled water (ml)	DNSA (ml)	OD at 450 nm
1	0.0	0.0	1.0	2	0.0
2	0.2	0.2	0.8	2	0.14
3	0.4	0.4	0.6	2	0.22
4	0.6	0.6	0.4	2	0.32
5	0.8	0.8	0.2	2	0.41
6	1.0	1.0	0.0	2	0.49

(After adding DNSA keep the tubes in boiling water bath for 10 minutes and then take the OD)

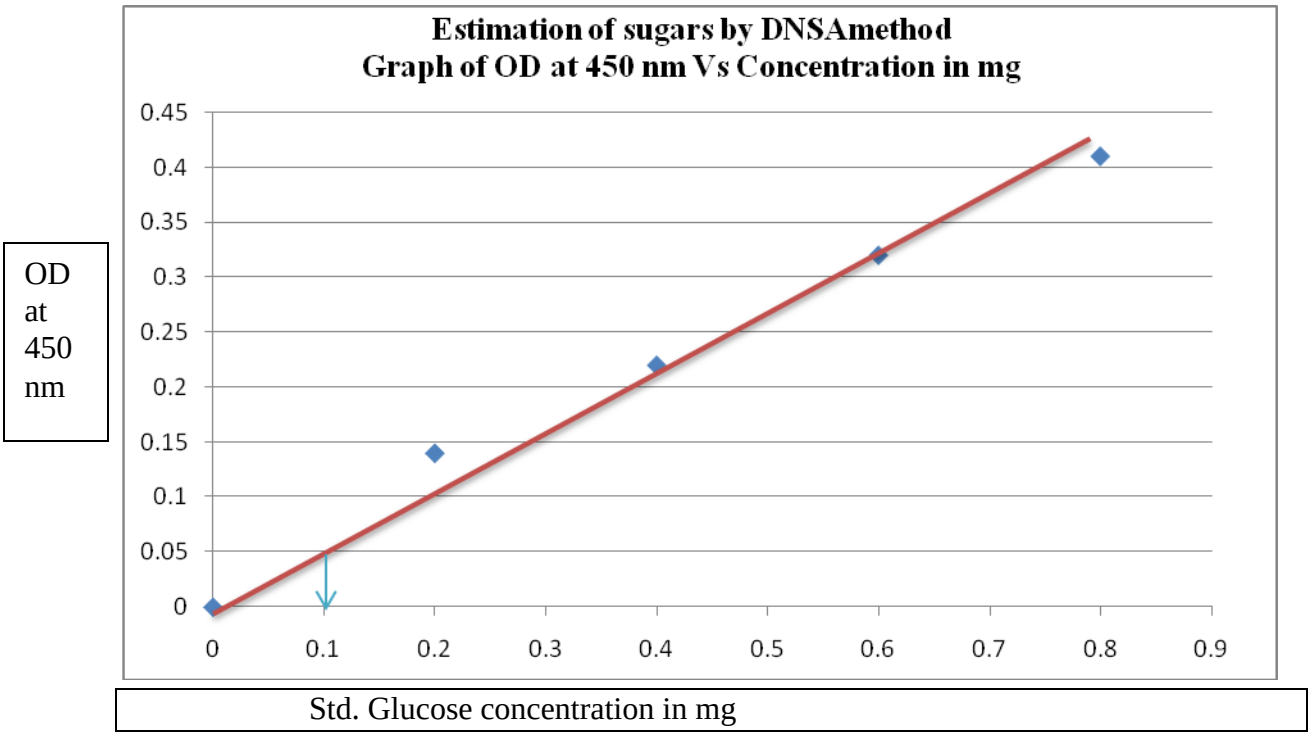

Fig 5.i Standard Graph of proteins using standard BSA stock solution

OD was taken at 450 nm for sample of Vinca (unpolluted)

(OD of Blank= 0.04)

0.05 Δ OD ≅ 0.10 mg of sugars

Table 5.2 (Reducing Sugars)	**OD**	**ΔOD**
Leaf	0.62	0.58
Stem	0.57	0.53
Root	0.69	0.65

i) Calculation for reducing sugars in Vinca (unpolluted)Leaf

∴0.58 Δ OD ≅1.16 mg of reducing sugars

Hence,

0.1 ml contains 1.16mg of reducing sugars

∴1 ml contains 11.6mg of reducing sugars ∴

10 ml contains 116 mg of reducing sugars

∴ 1gm Vinca leaf has 116mg of reducing sugars**.**

ii)Calculation for reducing sugars in Vinca (unpolluted) Stem

∴0.53 Δ OD ≅1.06 mg of reducing sugars

Hence,

0.1 ml contains 1.06mg of reducing sugars

∴1 ml contains 10.6mg of reducing sugars

∴ 10 ml contains 106mg of reducing sugars

∴ 1gm Vinca stem has 106mg of reducing sugars

iii)Calculation for reducing sugars in Vinca (unpolluted) Root

∴0.65 Δ OD ≅1.3 mg of reducing sugars

Hence,

0.1 ml contains 1.3mg of reducing sugars

∴1 ml contains 13mg of reducing sugars

∴ 10 ml contains 130mg of reducing sugars

∴ 1gm Vinca root has 130 mg of reducing sugars

Table 5.3 (Non-reducing Sugars)	OD	ΔOD
Leaf	0.41	0.37
Stem	0.36	0.32
Root	0.48	0.44

i)Calculation for non-reducing sugars in Vinca (unpolluted) Leaf

∴0.37 Δ OD ≅0.74 mg of non- reducing sugars

Hence,

0.1 ml contains 0.74mg of non-reducing sugars

∴1 ml contains 7.4mg of non-reducing sugars

∴ 10 ml contains 74mg of non-reducing sugars

∴ 1gm Vinca leaf has 74mg of non-reducing sugars

ii)Calculation for non-reducing sugars in Vinca (unpolluted)Stem

∴0.32 Δ OD ≅0.64 mg of non-reducing sugars

Hence,

0.1 ml contains 0.64mg of non-reducing sugars

∴1 ml contains 6.4mg of non-reducing sugars

∴ 10 ml contains 64mg of non-reducing sugars

∴ 1gm Vinca stem has 64mg of non-reducing sugars

iii)Calculation for non-reducing sugars in Vinca (unpolluted) Root

∴0.44 Δ OD ≡0.88 mg of non-reducing sugars

Hence,

0.1 ml contains 0.88mg of non-reducing sugars

∴1 ml contains 8.8mg of non-reducing sugars

∴ 10 ml contains 88mg of non-reducing sugars

∴ 1gm Vinca root has 88mg of non-reducing sugars

Calculations for total sugar content in Vinca from unpolluted area:

Total sugar content = Amount of reducing sugars + Amount of non-reducing sugars

∴ Total sugar content in Vinca (unpolluted) Leaf is**190 mg**

∴ Total sugar content in Vinca (unpolluted) stemis**170 mg**

∴ Total sugar content in Vinca (unpolluted) rootsis**218 mg**

OD was taken at 450 nm for sample of Vinca (polluted)

(OD of Blank= 0.04)

0.05 Δ OD ≅ 0.10 µg of sugars

Table 5.4 (Reducing sugars)	OD	ΔOD
Leaf	0.32	0.28
Stem	0.22	0.18
Root	0.25	0.21

i)Calculation for reducing sugars in Vinca (polluted) Leaf

∴0.28 Δ OD ≅0.56 mg of reducing sugars

Hence,

0.1 ml contains 0.56mg of reducing sugars

∴1 ml contains 5.6mg of reducing sugars

∴ 10 ml contains 56 mg of reducing sugars

∴ 1gm Vinca leaf has 56mg of reducing sugars

ii)Calculation for reducing sugars in Vinca (polluted) Stem

∴0.18 Δ OD ≅0.36 mg of reducing sugars

Hence,

0.1 ml contains 0.36mg of reducing sugars

∴1 ml contains 3.6mg of reducing sugars

∴ 10 ml contains 36mg of reducing sugars

∴ 1gm Vinca stem has 36mg of reducing sugars

ii)Calculation for reducing sugars in Vinca (polluted) Root

∴ 0.21 Δ OD ≅0.42 mg of reducing sugars

Hence,

0.1 ml contains 0.42mg of reducing sugars

∴1 ml contains 4.2mg of reducing sugars

∴ 10 ml contains 42mg of reducing sugars

∴ 1gm Vinca root has 42mg of reducing sugars

Table 5.5 (Non-reducing sugars)	OD	ΔOD
Leaf	0.38	0.34
Stem	0.31	0.27
Root	0.28	0.24

i)Calculation for non-reducing sugars in Vinca (polluted) Leaf

∴0.34 Δ OD ≅0.68 mg of non-reducing sugars

Hence,

0.1 ml contains 0.68mg of non-reducing sugars

∴1 ml contains 6.8mg of non-reducing sugars

∴ 10 ml contains 68mg of non-reducing sugars

∴ 1gm Vinca leaf has 68mg of non-reducing sugars

ii)Calculation for non-reducing sugars in Vinca (polluted) Stem

∴0.27 Δ OD ≅0.54 mg of reducing sugars

Hence,

0.1 ml contains 0.54mg of non-reducing sugars

∴1 ml contains 5.4mg of non-reducing sugars

∴ 10 ml contains 54mg of non-reducing sugars

∴ 1gm Vinca stem has 54mg of non-reducing sugars **iii)Calculation for non-reducing sugars in Vinca (polluted) Root**

∴0.24 Δ OD ≅ 0.48 mg of non-reducing sugars

Hence,

0.1 ml contains 0.48mg of non-reducing sugars

∴1 ml contains 4.8mg of non-reducing sugars

∴ 10 ml contains 48mg of non-reducing sugars

∴ 1gm Vinca root has 48mg of non-reducing sugars

Calculations for total sugar content in Vinca from polluted area:

Total sugar content = Amount of reducing sugars + Amount of non-reducing sugars

∴ Total sugar content in Vinca (polluted) Leafis **124 mg**

∴ Total sugar content in Vinca (polluted) stem is **90 mg**

∴ Total sugar content in Vinca (polluted) roots is **90 mg**

OD was taken at 450 nm for sample of Asteracantha (unpolluted)

(OD of Blank= 0.04)

0.05 Δ OD ≅ 0.10 µg of sugars

Table 5.6 (Reducing sugars)	OD	ΔOD
Leaf	0.66	0.62
Stem	0.86	0.82
Root	0.57	0.53

i)Calculation for reducing sugars in Asteracantha (unpolluted) Leaf

∴0.62 Δ OD ≅1.24 mg of reducing sugars

Hence,

0.1 ml contains 1.24mg of reducing sugars

∴1 ml contains 12.4mg of reducing sugars

∴ 10 ml contains 124mg of reducing sugars

∴ 1gm Asteracantha leaf has 124mg of reducing sugars

ii)Calculation for reducing sugars in Asteracantha (unpolluted) Stem

∴0.82 Δ OD ≅1.64 mg of reducing sugars

Hence,

0.1 ml contains 1.64mg of reducing sugars

∴1 ml contains 16.4mg of reducing sugars

∴ 10 ml contains 164mg of reducing sugars

∴ 1gm Asteracantha stem has 164mg of reducing sugars

ii)Calculation for reducing sugars in Asteracantha (unpolluted) Root

∴0.53 Δ OD ≅ 0.53 mg of reducing sugars

Hence,

0.1 ml contains 1.06mg of reducing sugars

∴1 ml contains 10.6mg of reducing sugars

∴ 10 ml contains 106 mg of reducing sugars

∴ 1gm Asteracantha root has 106mg of reducing sugars

Table 5.7 (Nonreducing sugars)	OD	ΔOD
Leaf	0.66	0.62
Stem	0.73	0.69
Root	0.64	0.60

i)Calculation for non-reducing sugars in Asteracantha (unpolluted) Leaf

∴0.62 Δ OD ≅1.24 mg of reducing sugars

Hence,

0.1 ml contains 1.24mg of reducing sugars

∴1 ml contains 12.4mg of reducing sugars

∴ 10 ml contains 124mg of reducing sugars

∴ 1gm Asteracantha leaf has 124mg of reducing sugars

ii)Calculation for non-reducing sugars in Asteracantha (unpolluted) Stem

∴0.69 Δ OD ≅1.38 mg of reducing sugars

Hence,

0.1 ml contains 1.38mg of reducing sugars

∴1 ml contains 13.8mg of reducing sugars

∴ 10 ml contains 138mg of reducing sugars

∴ 1gm Asteracantha stem has 138mg of reducing sugars

iii)Calculation for non-reducing sugars in Asteracantha (unpolluted) Root

∴0.60 Δ OD ≅ 0.60 µg i. e. 1.2mg of reducing sugars

Hence,

0.1 ml contains 1.2mg of reducing sugars

∴1 ml contains 12mg of reducing sugars

∴ 10 ml contains 120mg of reducing sugars

∴ 1gm Asteracantha root has 120 mg of reducing sugars

Calculations for total sugar content in Asteracantha from unpolluted area:

Total sugar content = Amount of reducing sugars + Amount of non-reducing sugars

∴ Total sugar content in Asteracantha (unpolluted) Leaf is**248mg**

∴ Total sugar content in Asteracantha (unpolluted) stem is**302mg**

∴ Total sugar content in Asteracantha (unpolluted) roots is**226mg**

OD was taken at 450 nm for sample of Asteracantha (polluted)

(OD of Blank= 0.04)

0.05 Δ OD ≅ 0.10 µg of sugars

Table 5.8 (Reducing sugars)	OD	ΔOD
Leaf	0.30	0.26
Stem	0.32	0.28
Root	0.38	0.34

i)Calculation for reducing sugars in Asteracantha (polluted) Leaf

∴0.26 Δ OD ≅0.52 mg of reducing sugars

Hence,

0.1 ml contains 0.52mg of reducing sugars

∴1 ml contains 5.2mg of reducing sugars

∴ 10 ml contains 52mg of reducing sugars

∴ 1gm Asteracantha leaf has 52mg of reducing sugars

ii)Calculation for reducing sugars in Asteracantha (polluted) Stem

∴0.28 Δ OD ≅ 0.56 mg of reducing sugars

Hence,

0.1 ml contains 0.56mg of reducing sugars

∴1 ml contains 5.6mg ofreducing sugars

∴ 10 ml contains 56mg of reducing sugars

∴ 1gm Asteracantha stem has 56 mg of reducing sugars

iii)Calculation for reducing sugars in Asteracantha (polluted) Root

∴0.34 Δ OD ≅ 0.68 mg of reducing sugars

Hence,

0.1 ml contains 0.68mg of reducing sugars

∴1 ml contains 6.8mg of reducing sugars

∴ 10 ml contains 68mg of reducing sugars

∴ 1gm Asteracantha root has 68mg of reducing sugars

Table 5.9 (Nonreducing sugars)	OD	ΔOD
Leaf	0.40	0.36
Stem	0.33	0.29
Root	0.41	0.37

i)Calculation for non-reducing sugars in Asteracantha (polluted)Leaf

∴0.36 Δ OD ≅ 0.72 mg of reducing sugars

Hence,

0.1 ml contains 0.72mg of reducing sugars

∴1 ml contains 7.2mg of reducing sugars

∴ 10 ml contains 72mg of reducing sugars

∴ 1gm Asteracantha leaf has 72mg of reducing sugars

ii)Calculation for non-reducing sugars in Asteracantha (polluted) Stem

∴0.29 Δ OD ≅ 0.58 mg of reducing sugars

Hence,

0.1 ml contains 0.58mg of reducing sugars

∴1 ml contains 5.8mg of reducing sugars

∴ 10 ml contains 58mg of reducing sugars

∴ 1gm Asteracantha stem has 58mg of reducing sugars

iii)Calculation for non-reducing sugars in Asteracantha (polluted) Root

∴0.37 Δ OD ≅ 0.74 mg of reducing sugars

Hence,

0.1 ml contains 0.74mg of reducing sugars

∴1 ml contains 7.4mg of reducing sugars

∴ 10 ml contains 74mg of reducing sugars

∴ 1gm Asteracantha root has 74mg of reducing sugars

Calculations for total sugar content in Asteracantha from polluted area:

Total sugar content = Amount of reducing sugars + Amount of non-reducing sugars

∴ Total sugar content in Asteracantha (polluted) Leaf is**124mg**

∴ Total sugar content in Asteracantha (polluted) stem is**114mg**

∴ Total sugar content in Asteracantha (polluted) roots is**142mg**

5.2 Observations and Calculations for Protein content BSA:

200µg/ml

Range: 40µg to 200µg Total volume: 1ml Table 5.10
(For standardization of BSA)

Tube no.	Concentration (µg)	Amount of stock (ml)	D/W (ml)	Lowry's solution (ml)		Foin ciocalteau's reagent		OD at 660nm
Blank	-	0	1.0	2	Incubate	0.2	Incubate at	0.0
1	40	0.2	0.8	2	at	0.2	37° C for	0.05
2	80	0.4	0.6	2	37° C for	0.2	10minutes	0.11
3	120	0.6	0.4	2	10minutes	0.2	Keep in	0.15
4	160	0.8	0.2	2		0.2	dark	0.19
5	200	1.0	0	2		0.2		0.24

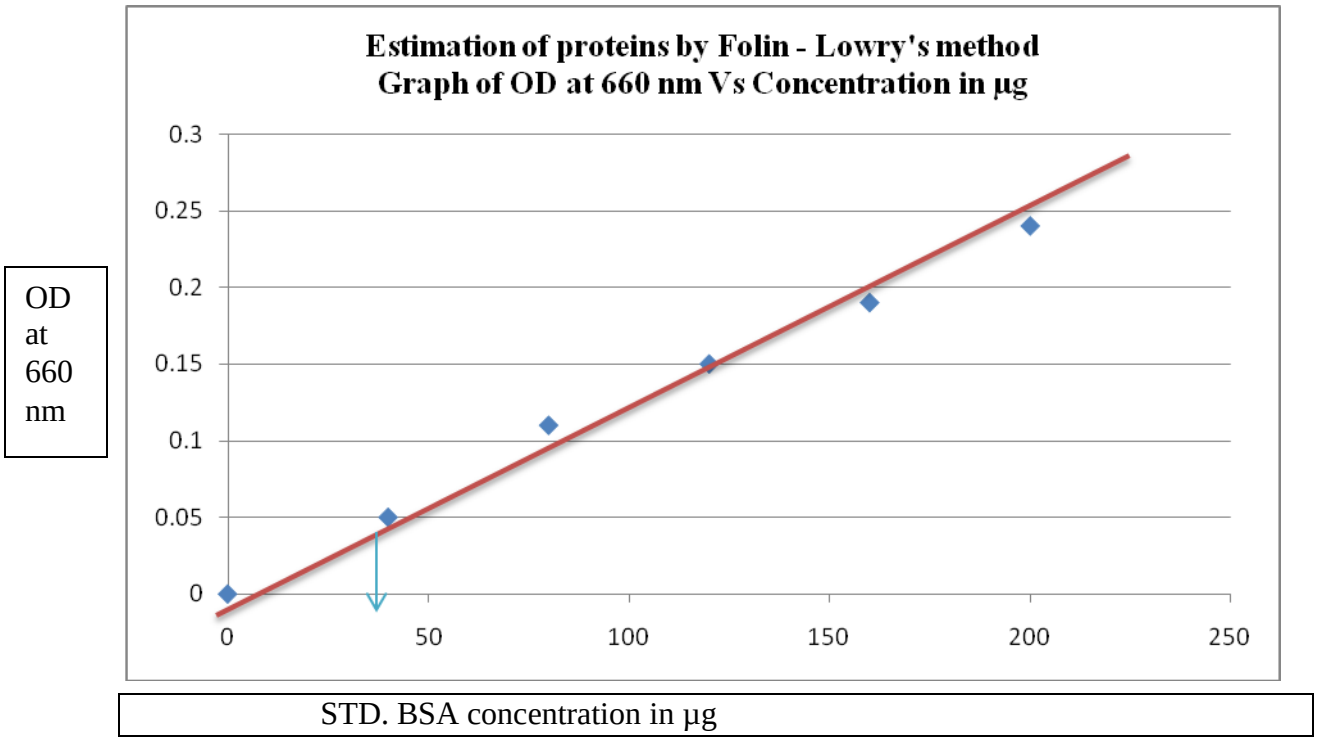

Fig 5.ii Standard Graph of sugars using standard glucose stock solution

OD was taken at 660nm for sample of Vinca (unpolluted)

(OD of Blank= 0.05)

Table 5.11	**OD**	**ΔOD**
Leaf	0.86	0.81
Stem	0.40	0.35
Root	0.62	0.57

0.05 Δ OD ≅ 40 µg of protein

i) Calculations for proteins present in Vinca (unpolluted) Leaf

∴ 0.81 Δ OD ≅ 648 µg i. e. 0.648 mg of proteins

Hence,

0.1 ml contains 0.648 mg of protein

∴1 ml contains 6.48 mg of protein

∴ 10 ml contains 64.8 mg of proteins

∴ 1gm vinca leaf has 64.8 mg of proteins

ii)Calculations for proteins present in Vinca (unpolluted) Stem

∴0.35 Δ OD ≅ 280 µg i. e. 0.280 mg of proteins

Hence,

0.1 ml contains 0.280 mg of protein

∴1 ml contains 2.80 mg of protein

∴ 10 ml contains 28.0 mg of proteins

∴ 1gm Vinca stem has 28.0 mg of proteins

iii)Calculations for proteins present in Vinca (unpolluted) Roots

∴0.57 Δ OD ≅ 456 µg i. e. 0.456 mg of proteins

Hence,

0.1 ml contains 0.456 mg of protein

∴1 ml contains 4.56 mg of protein

∴ 10 ml contains 45.6 mg of proteins

∴ 1gm Vinca root has 45.6 mg of proteins

OD was taken at 660nm for sample of Vinca (polluted)

(OD of Blank= 0.05)

Table 5.12	**OD**	**ΔOD**

Leaf	0.76	0.71
Stem	0.26	0.21
Root	0.39	0.34

0.05 Δ OD ≅ 40 µg of protein

i)Calculations for proteins present in Vinca (polluted) Leaf

∴0.71 Δ OD ≅ 568 µg i. e. 0.568 mg of proteins

Hence,

0.1 ml contains 0.568 mg of protein

∴1 ml contains 5.68 mg of protein
∴ 10 ml contains 56.8 mg of proteins
∴ 1gm Vinca leaf has 56.8 mg of proteins

ii)Calculations for proteins present in Vinca (polluted) Stem

∴0.21 Δ OD ≅ 168 µg i. e. 0.168 mg of proteins

Hence,

0.1 ml contains 0.168 mg of protein

∴1 ml contains 1.68 mg of protein
∴ 10 ml contains 16.8 mg of proteins
∴ 1gm Vinca stem has 16.8 mg of proteins

iii)Calculations for proteins present in Vinca (polluted) Roots

∴0.34 Δ OD ≅ 272 µg i. e. 0.272 mg of proteins

Hence,

0.1 ml contains 0.272 mg of protein

∴1 ml contains 2.72 mg of protein

∴ 10 ml contains 27.2 mg of proteins

∴ 1gm Vinca root has 27.2 mg of proteins

OD was taken at 660nm for sample of Asteracantha (unpolluted)

(OD of Blank= 0.05)

Table 5.13	**OD**	**ΔOD**
Leaf	0.51	0.46
Stem	0.26	0.21
Root	0.32	0.27

i)Calculations for proteins present in Asteracantha (unpolluted) Leaf

∴0.46 Δ OD ≅ 368 µg i. e. 0.368 mg of proteins

Hence,

0.1 ml contains 0.368 mg of protein

∴1 ml contains 3.68 mg of protein

∴ 10 ml contains 36.8 mg of proteins

∴ 1gm Asteracantha leaf has 36.8 mg of proteins

ii)Calculations for proteins present in Asteracantha (unpolluted) Stem

∴0.21 Δ OD ≅ 168 µg i. e. 0.168 mg of proteins

Hence,

0.1 ml contains 0.168 mg of protein

∴1 ml contains 1.68 mg of protein

∴ 10 ml contains 16.8 mg of proteins

∴ 1gm Asteracantha stem has 16.8 mg of proteins

iii)Calculations for proteins present in Asteracantha (unpolluted) Root

0.27 Δ OD ≅ 216 µg i. e. 0.216 mg of proteins

Hence,

0.1 ml contains 0.216 mg of protein

∴1 ml contains 2.16 mg of protein

∴ 10 ml contains 21.6 mg of proteins

∴ 1gm Asteracantha root has 21.6 mg of proteins

OD was taken at 660nm for sample of Asteracantha (polluted)

(OD of Blank= 0.05)

Table 5.14	**OD**	**ΔOD**
Leaf	**0.38**	**0.33**
Stem	**0.13**	**0.08**
Root	**0.26**	**0.21**

i)Calculations for proteins present in Asteracantha (polluted) Leaf

∴0.33 Δ OD ≅ 264 µg i. e. 0.2648 mg of proteins

Hence,

0.1 ml contains 0.264 mg of protein

∴1 ml contains 2.64 mg of protein

∴ 10 ml contains 26.4 mg of proteins

∴ 1gm Asteracantha leaf has 26.4 mg of proteins

ii)Calculations for proteins present in Asteracantha (polluted) Stem

∴0.08 Δ OD ≅ 64 µg i. e. 0.64 mg of proteins

Hence,

0.1 ml contains 0.64 mg of protein

∴1 ml contains 6.4 mg of protein

∴ 10 ml contains 64.0 mg of proteins

∴ 1gm Asteracantha root has 64.0 mg of proteins

iii)Calculations for proteins present in Asteracantha (polluted) Root

∴0.21 Δ OD ≅ 168 µg i. e. 0.168 mg of proteins

Hence,

0.1 ml contains 0.168 mg of protein

∴1 ml contains 1.68 mg of protein

∴ 10 ml contains 16.8 mg of proteins

∴ 1gm Asteracantha root has 16.8 mg of proteins

5.3 Observations for phytochemical analysis

Phytochemical analysis on Vinca(unpolluted) showed the following results;

Table 1: Phytochemical analysis done on water extract of **leaf** of Vinca (unpolluted)

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

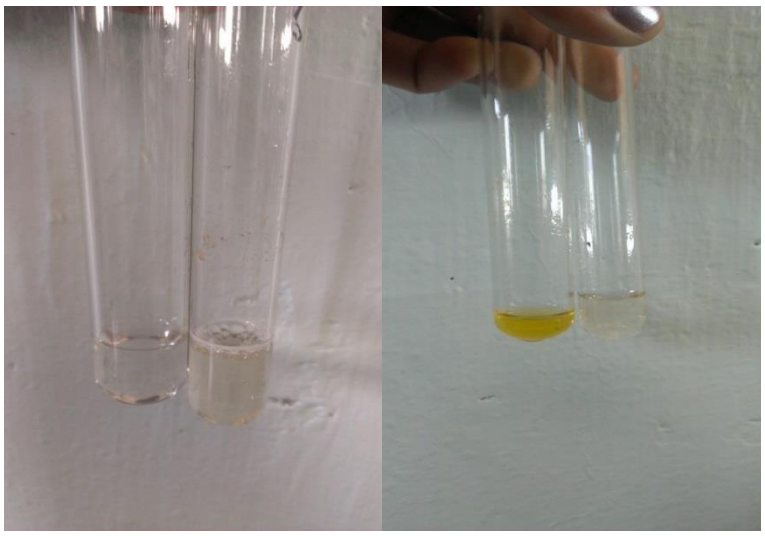

Fig 5.1 Leaf of Vinca unpolluted showing presence of saponins and flavonoids

Table 2: Phytochemical analysis done on water extract of **stem** of Vinca (unpolluted)

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

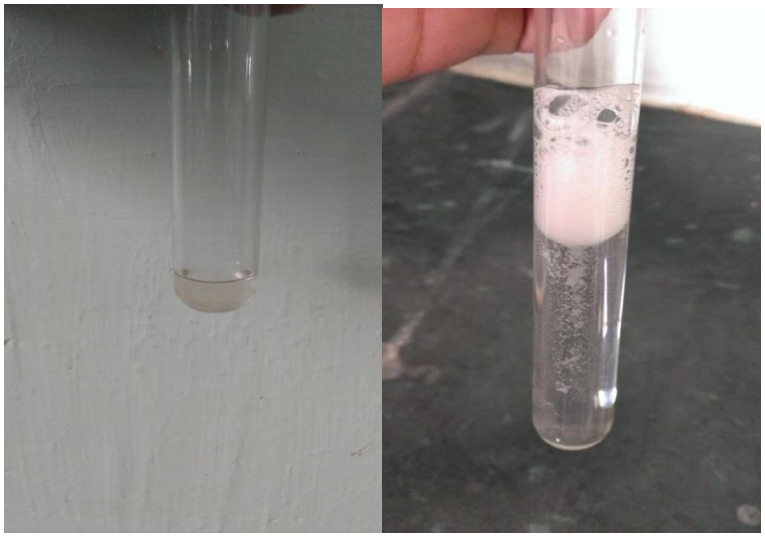

Fig 5.2 Stem of Vinca unpolluted showing presence of saponins and flavonoids

Table 3: Phytochemical analysis done on water extract of **root** of Vinca (unpolluted)

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

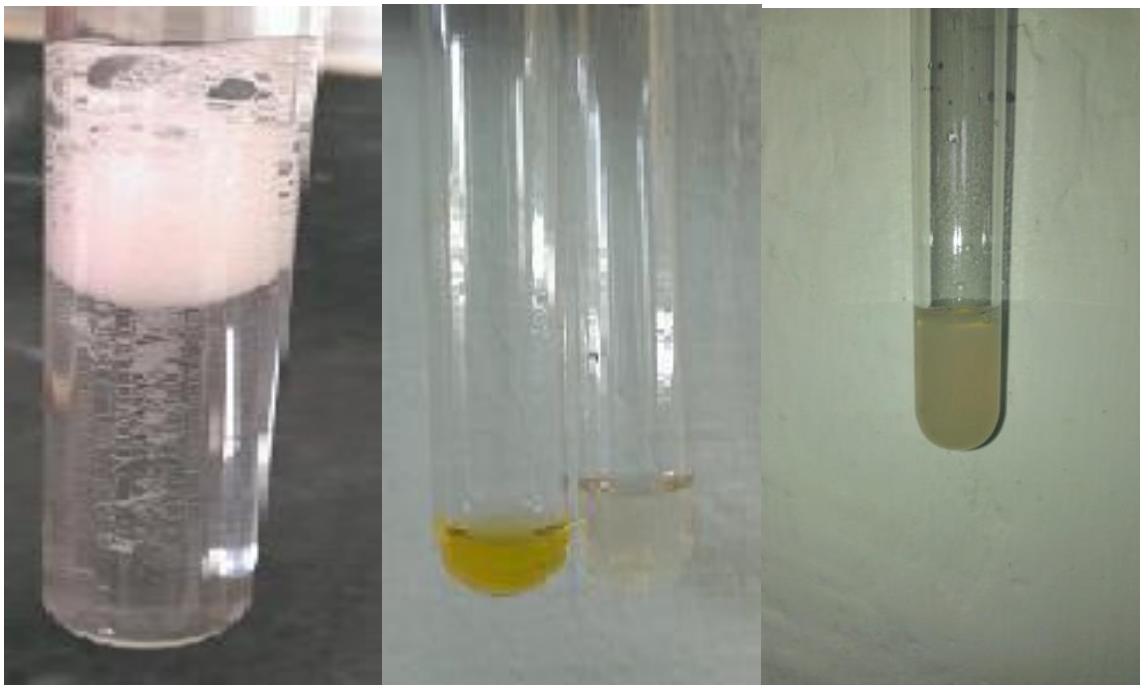

Fig 5.3 Root of Vinca unpolluted showing presence of saponins, flavonoids and alkaloids

Table 4: Phytochemical analysis done on **whole plants'** methanolic extract and water extractsof Vinca (Unpolluted) showed the following results;

Compound	Methanolic extracts	Water Extracts
Tannins	-	-
Saponins	+++	+
Flavonoids	+	+
Phlobatannins	-	-
Glycosides	-	-
Steroids i) ii)	- -	- -
Terpenoids	-	-
Alkaloids	+	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

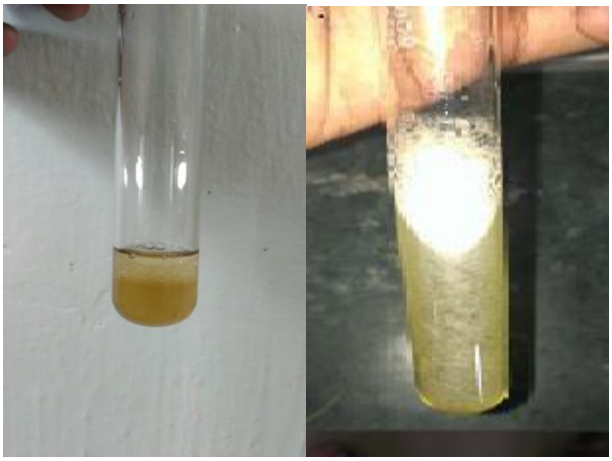

Fig 5.4Methanolic extracts of Vinca unpolluted showing presence of alkaloids and saponins

Fig 5.5Methanolic extracts of Vinca unpolluted showing presence of flavonoids

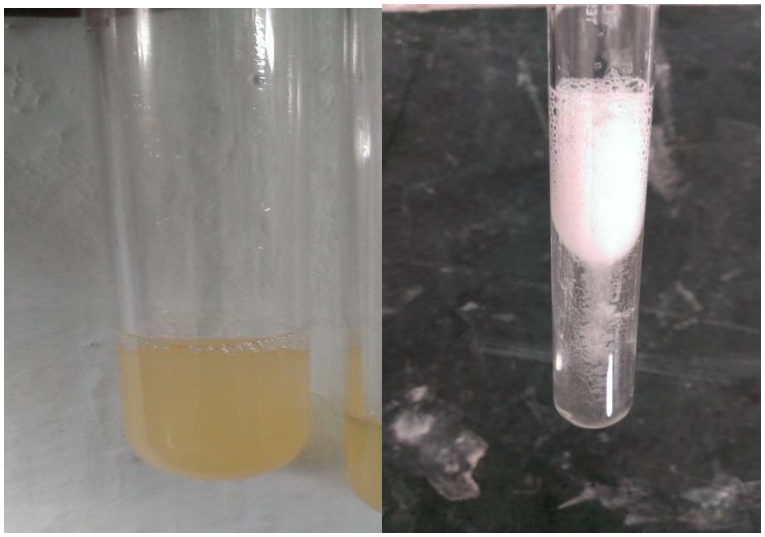

Fig 5.6 Water extracts of Vinca unpolluted showing presence of alkaloids and saponins

Fig 5.7 Water extracts of Vinca unpolluted showing presence of flavonoids Phytochemical analysis on Vinca(polluted) showed the following results;

Table 5: Phytochemical analysis done on water extract of **leaf** of Vinca (polluted)

Compound	Water Extracts
Tannins	-
Saponins	+++
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

Fig 5.8 Leaf of Vinca polluted showing presence of saponins and flavonoids

Table 6: Phytochemical analysis done on water extract of **stem** of Vinca (polluted)

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

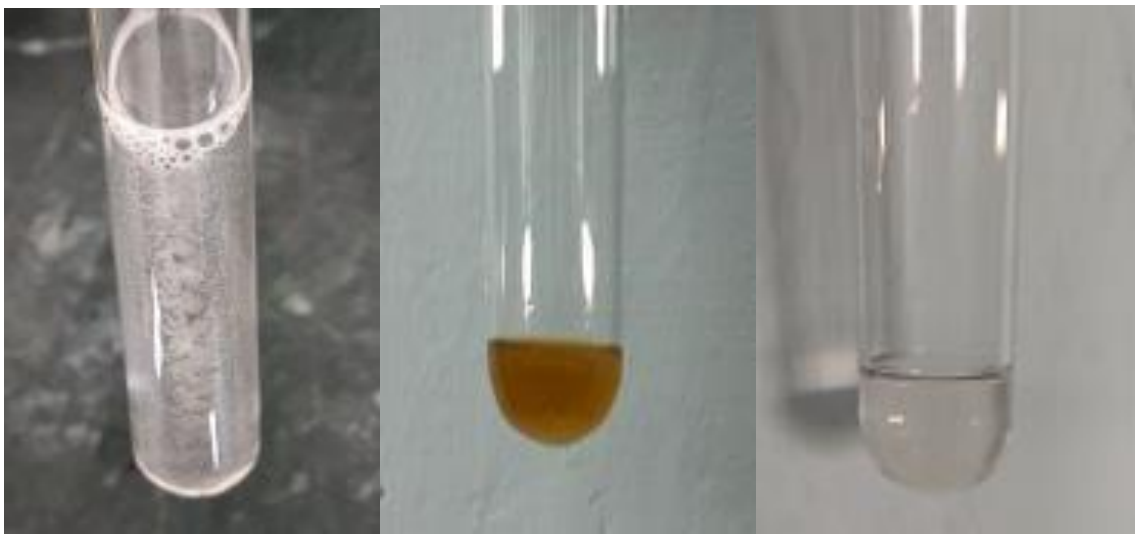

Fig 5.9 Stem of Vinca polluted showing presence of saponins and flavonoids

Table 7: Phytochemical analysis done on water extract of **root** of Vinca (polluted)

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	+ +
Terpenoids	-
Alkaloids	+

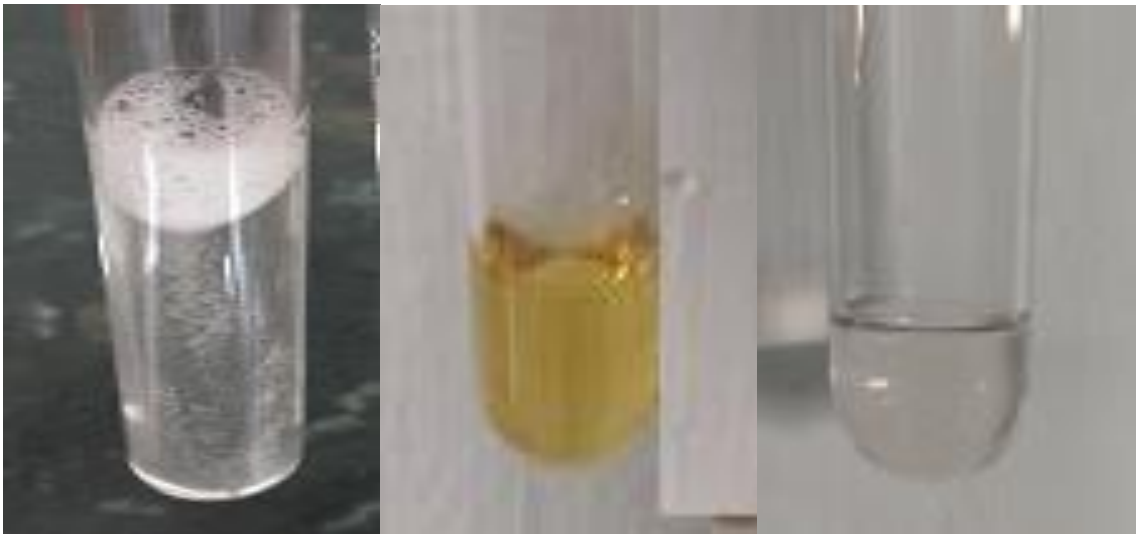

Fig 5.10 Roots of Vinca polluted showing presence of saponins and flavonoids

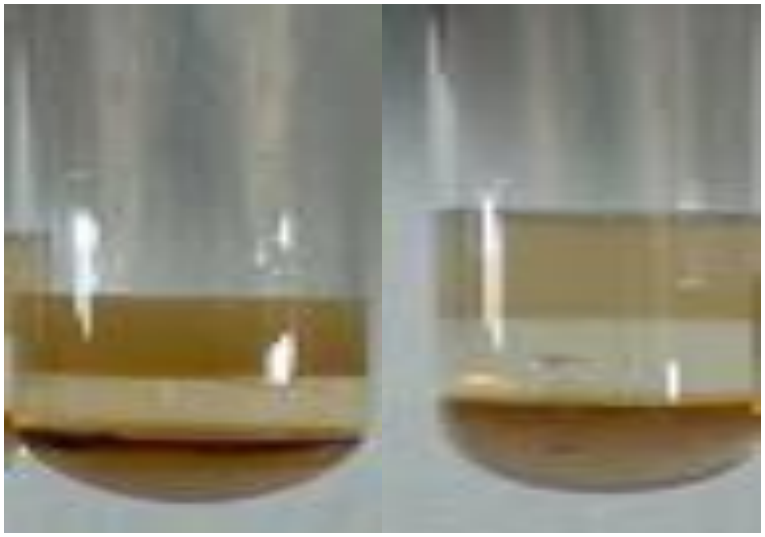

Fig 5.11Roots of Vinca polluted showing presence of Steroids

Fig 5.12Roots of Vinca polluted showing presence of alkaloids **Table**

8:Phytochemical analysis done on **whole plants'** methanolic extracts and water extracts of Vinca (polluted) showed the following results;

Compound	Methanolic extracts	Water Extracts
Tannins	-	-
Saponins	+++	+
Flavonoids	+	+
Phlobatannins	-	-
Glycosides	-	-
Steroids i) ii)	+ +	- -
Terpenoids	-	-
Alkaloids	+	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

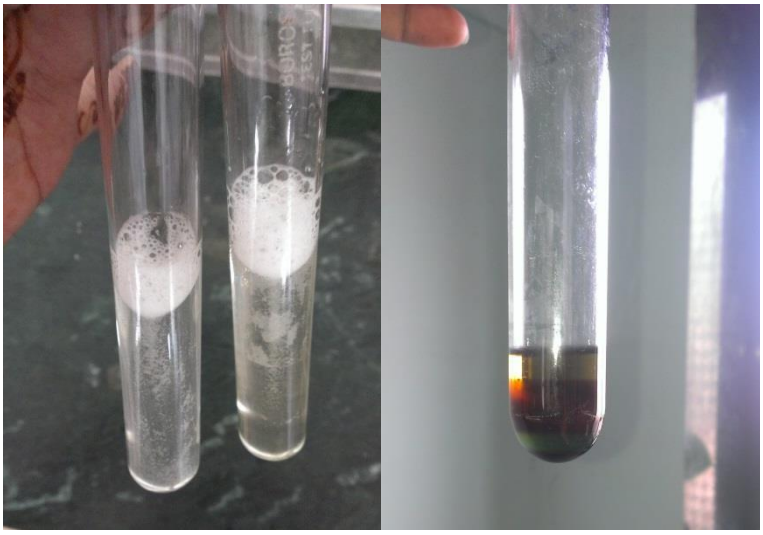

Fig 5.13 Methanolic extracts and water extracts of Vinca polluted showing the presence of saponins and methanolic extracts of Vinca polluted showing the presence of steroids

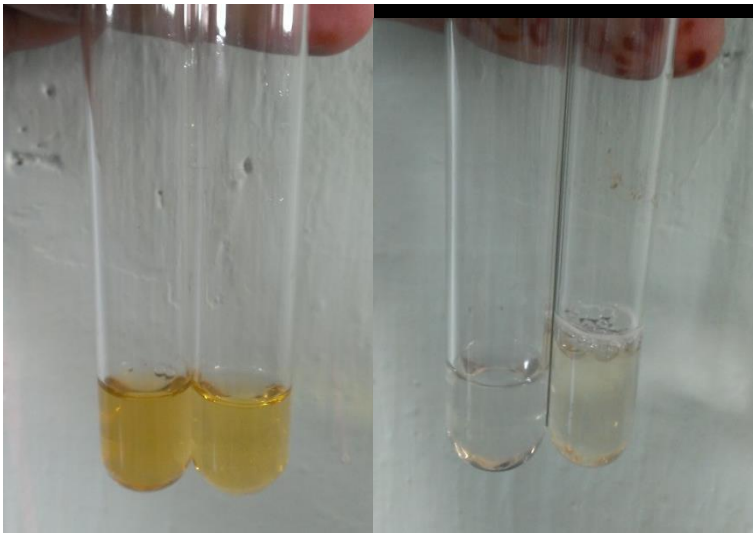

Fig5.14 Methanolic extracts and water extracts of Vinca polluted showing the presence of Flavonoids

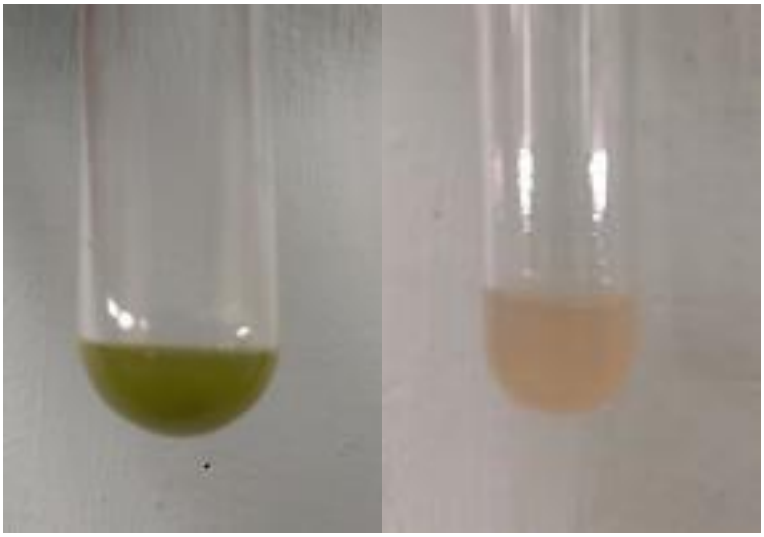

Fig 5.15 Methanolic extracts and water extracts of Vinca polluted showing the presence of alkaloids

Phytochemical analysis on Asteracantha (Unpolluted) showed the following results;

Table 9: Phytochemical analysis done on water extract of **leaf** of Asteracantha (unpolluted)

Compound	Water Extracts
Tannins	-
Saponins	+++
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

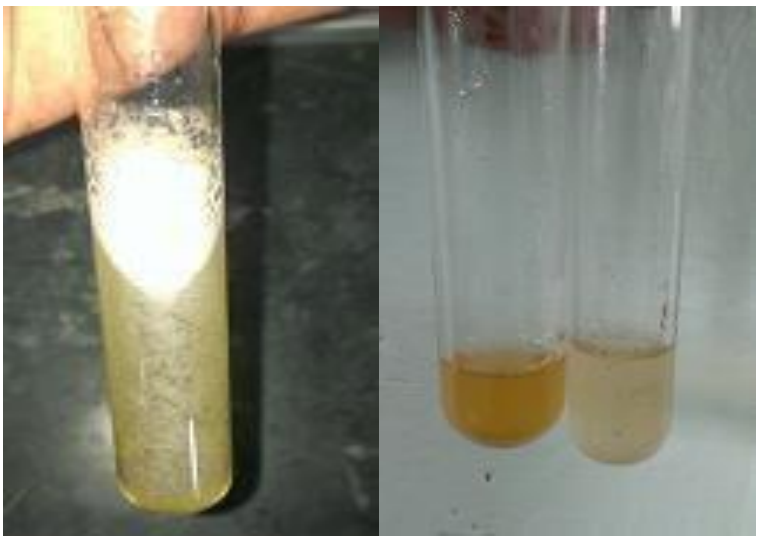

Fig 5.16 Leaf of Asteracantha Unpolluted showing the presence of Saponins and flavonoids

Table 10: Phytochemical analysis done on water extract of **stem** of Asteracantha (unpolluted);

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

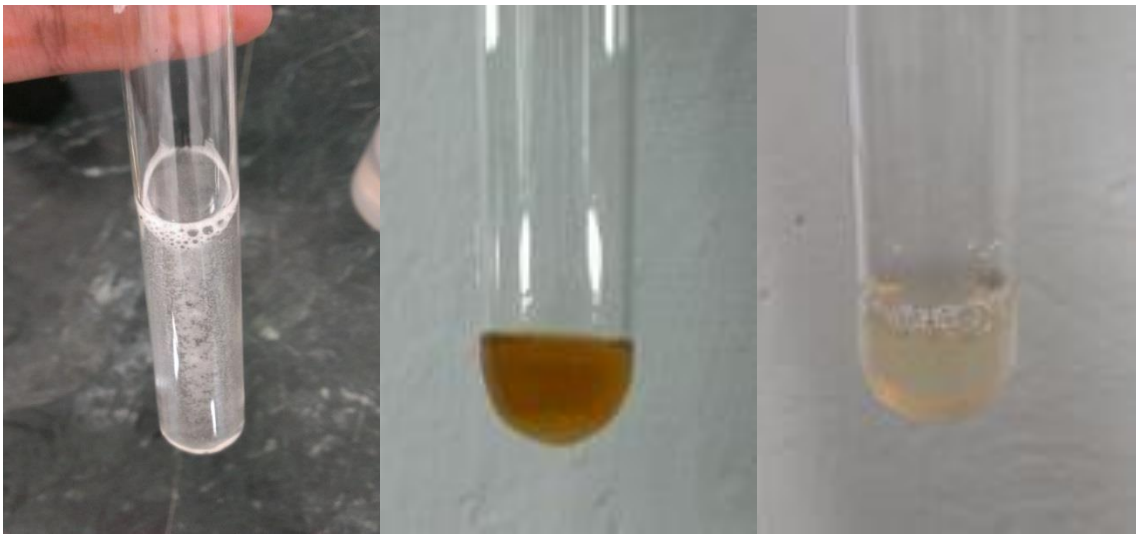

Fig 5.17 Stem of Asteracantha Unpolluted showing the presence of saponins and flavonoids

Table 11: Phytochemical analysis done on water extract of **root** of Asteracantha (unpolluted);

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

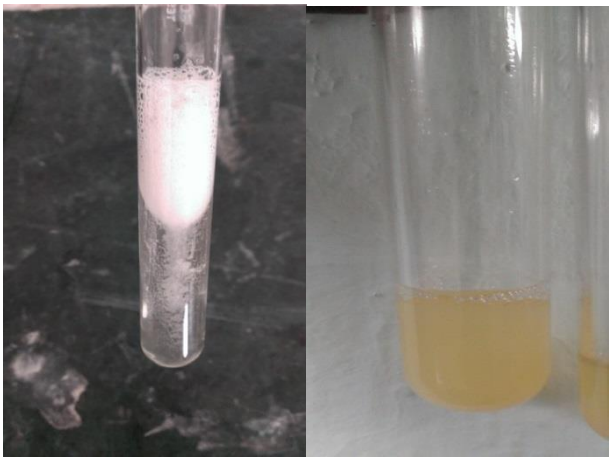

Fig 5.18Root of Asteracantha Unpolluted showing the presence of saponins and alkaloids

Fig 5.19 Root of Asteracantha Unpolluted showing the presence of flavonoids

Table 12: Phytochemical analysis done on **whole plants'** methanolic extract and water extracts of Asteracantha (unpolluted) showed the following results;

Compound	Methanolic extracts	Water Extracts
Tannins	-	-
Saponins	+++	+++
Flavonoids	+	+
Phlobatannins	-	-
Glycosides	+	-
Steroids i) ii)	- -	- -
Terpenoids	-	-
Alkaloids	+	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

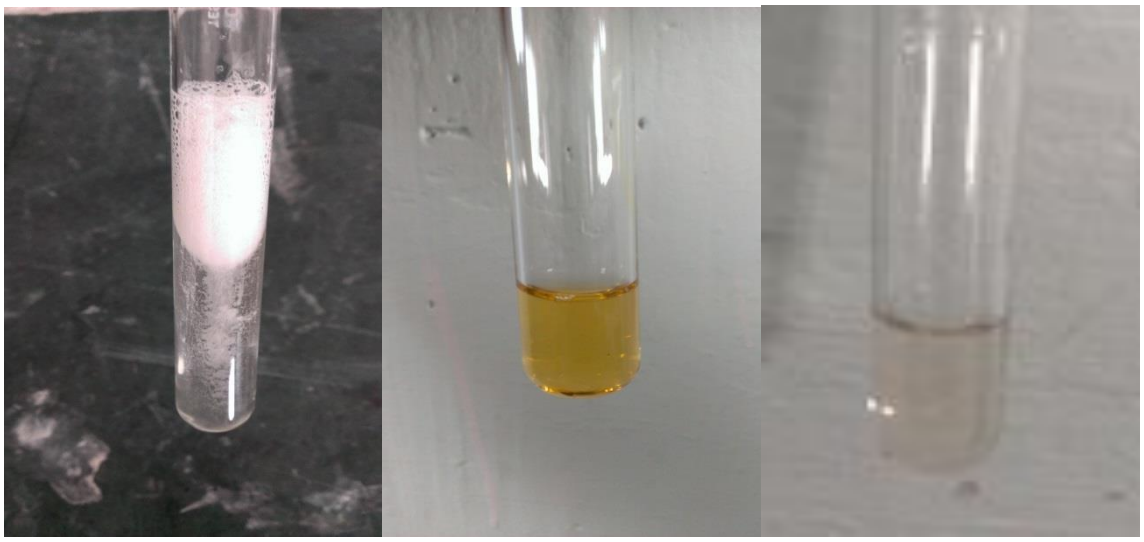

Fig 5.20 Methanolic extrcts of Asteracantha unpolluted showing the presence of saponins and flavonoids

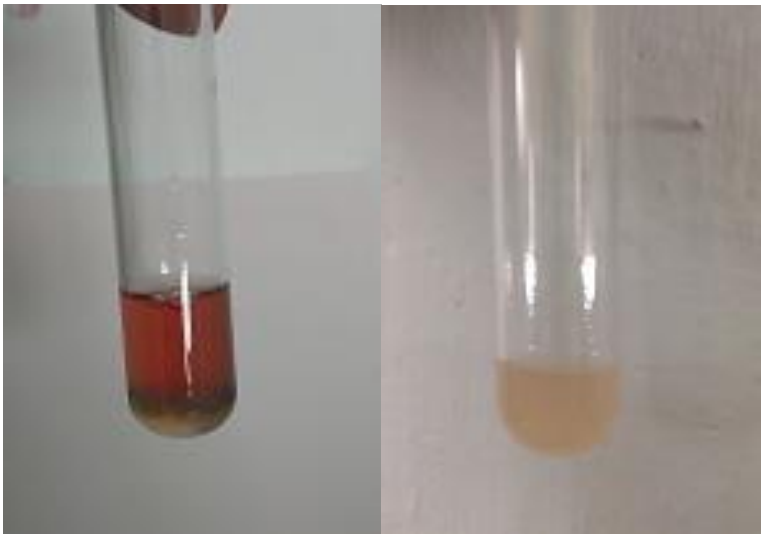

Fig 5.21 Methanolic extrcts of Asteracantha unpolluted showing the presence glycosides and alkaloids

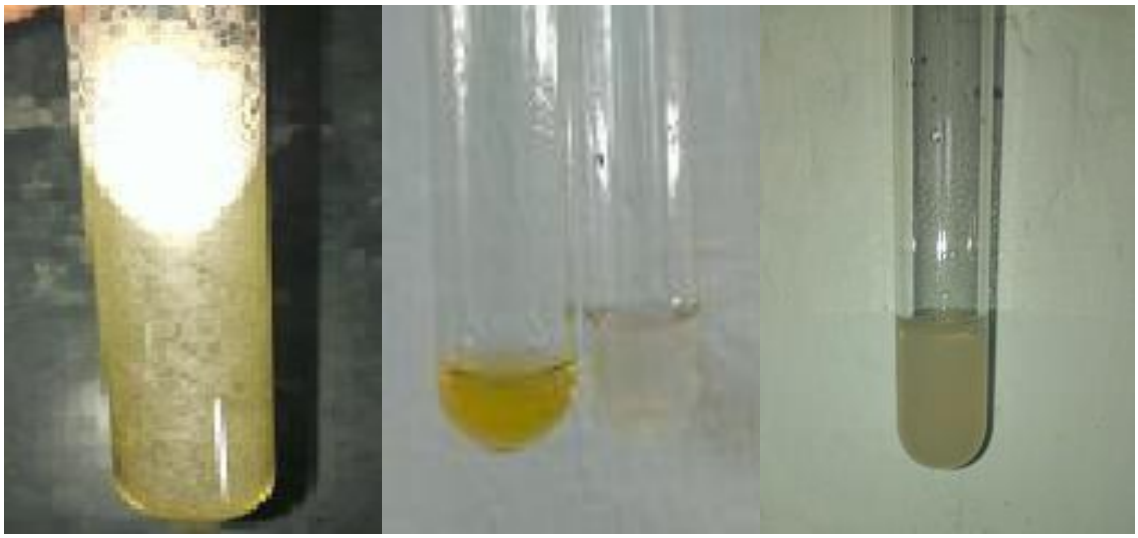

Fig 5.22 Water extrcts of Asteracantha unpolluted showing the presence saponins, flavonoids and alkaloids

Phytochemical analysis on Asteracantha (Polluted) showed the following results;

Table 13: Phytochemical analysis done on water extract of **leaf** of Asteracantha (polluted);

Compound	Water Extracts
Tannins	-
Saponins	+++
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

Fig 5.23 Leaf of Asteracantha polluted showing the presence of saponins and flavonoids

Table 14: Phytochemical analysis done on water extract of **stem** of Asteracantha (polluted);

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	-

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

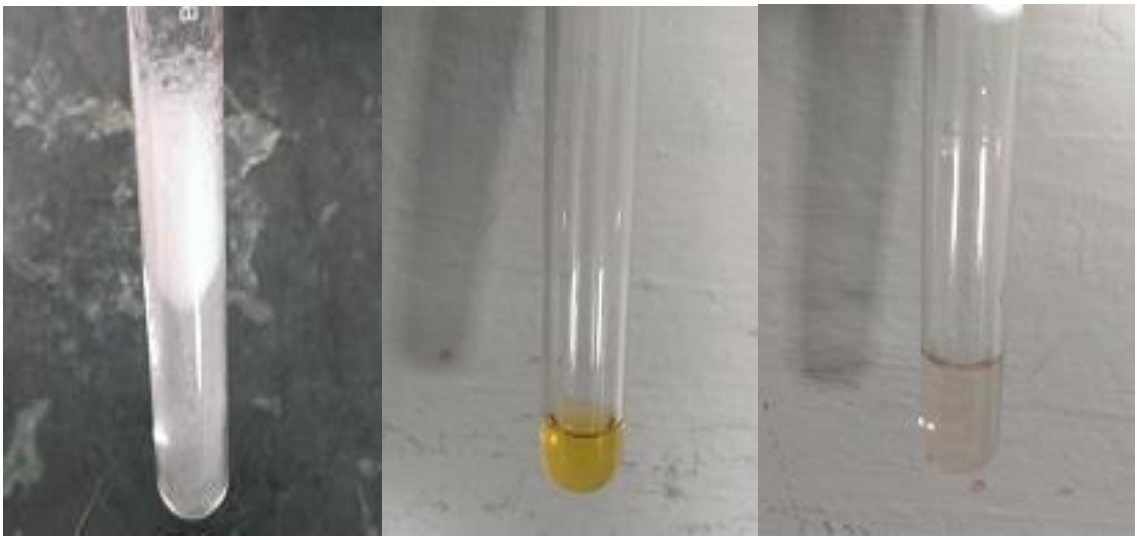

Fig 5.24 Stem of Asteracantha polluted showing the presence of saponins and flavonoids

Table 15: Phytochemical analysis done on water extract of **root** of Asteracantha (polluted);

Compound	Water Extracts
Tannins	-
Saponins	+
Flavonoids	+
Phlobatannins	-
Glycosides	-
Steroids i) ii)	- -
Terpenoids	-
Alkaloids	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

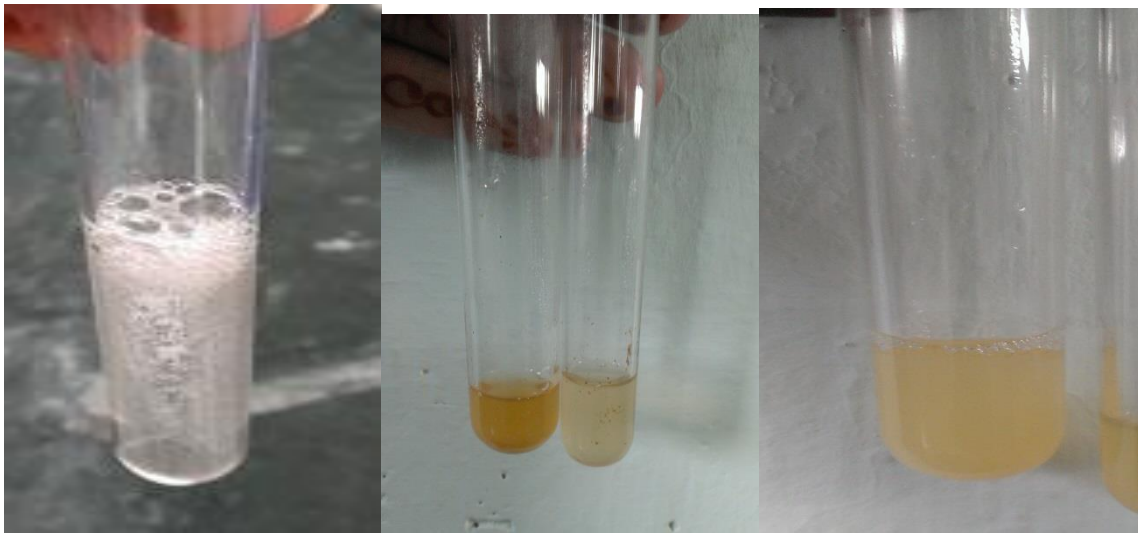

Fig 5.25 Root of Asteracantha polluted showing the presence of saponins, flavonoids and alkaloids

Table 16: Phytochemical analysis done on **whole plants'** methanolic extract and water extracts of Asteracantha (polluted) showed the following results;

Compound	Methanolic extracts	Water Extracts
Tannins	-	-
Saponins	+++	+++
Flavonoids	+	+
Phlobatannins	-	-
Glycosides	+	+
Steroids i) ii)	- -	- -
Terpenoids	+	-
Alkaloids	+	+

[Key: (+) = Present; (+++) = Highly present; (-) = Absent]

Fig 5.26 Methanolic and water extracts of Asteracantha polluted showing the presence of saponins and alkaloids

Fig 5.27 Methanolic and water extracts of Asteracantha polluted showing the presence of glycosides

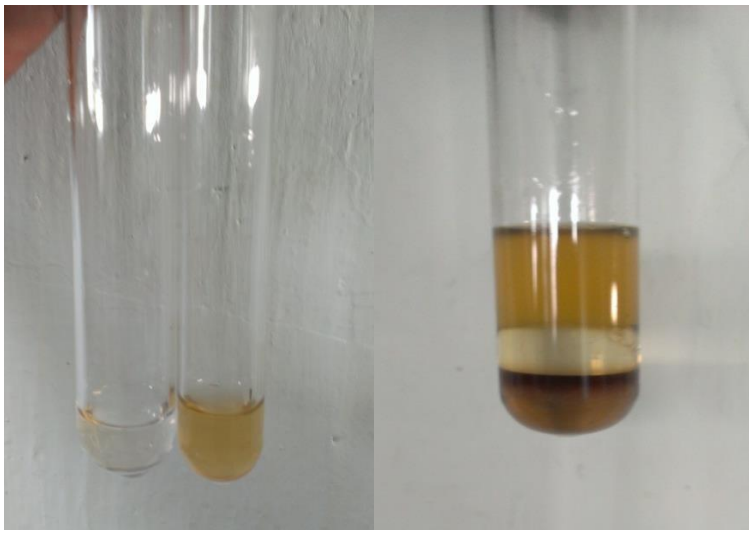

Fig 5.28 Methanolic extracts of Asteracantha polluted showing the presence of terpenoids

Fig 5.29Methanolic and water extracts of Asteracantha polluted showing the presence of flavonoids

6.1 RP-HPLC analysis for alkaloids in Vinca (Unpolluted) methanolic extracts

Sample Name :*Vinca rosea .Linn* (unpolluted} (HPLC grade methanolic extracts)
Injection Volume : 20 uL
Data Filename :RP-HPLC-13mar_3.lcd
Method Filename :RP-HPLC.lcm
Report Filename : Default.lcr
Date Acquired : 3/13/2014

<Chromatogram>

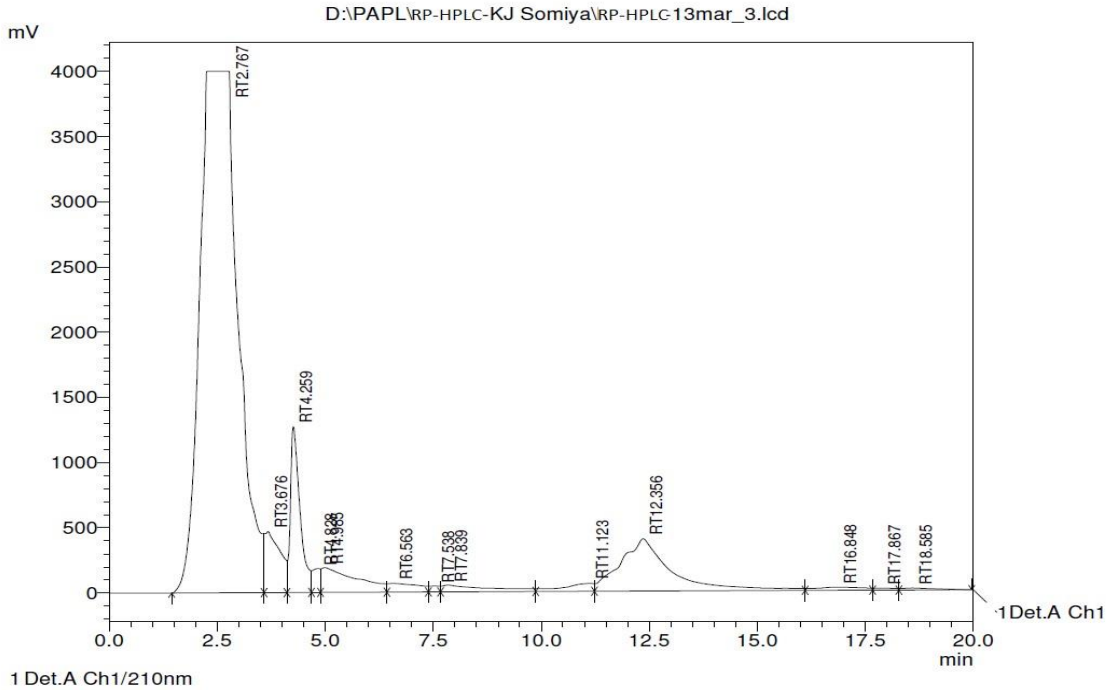

Fig 6.1 Chromatogram for Vinca (Unpolluted) methanolic extracts

Table 6.1: Results of RP-HPLC for alkaloids of Vinca Unpolluted methanolic extracts

ID#	Name	Ret. Time	Area	Area%	Height	Units	Channel
1	RT2.767	2.767	245646045	72.783	3998186	mg/L	Ch1
2	RT3.676	3.676	11743964	3.480	468450	mg/L	Ch1
3	RT4.259	4.259	19768952	5.857	1272171	mg/L	Ch1
4	RT4.828	4.828	2228568	0.660	183144	mg/L	Ch1
5	RT4.985	4.985	10791172	3.197	189654	mg/L	Ch1
6	RT6.563	6.563	3327017	0.986	67324	mg/L	Ch1
7	RT7.538	7.538	732765	0.217	45347	mg/L	Ch1
8	RT7.839	7.839	4157215	1.232	52241	mg/L	Ch1
9	RT11.123	11.123	3059610	0.907	62281	mg/L	Ch1
10	RT12.356	12.356	32768916	9.709	401216	mg/L	Ch1
11	RT16.848	16.848	1821224	0.540	23152	mg/L	Ch1
12	RT17.867	17.867	559198	0.166	16558	mg/L	Ch1
13	RT18.585	18.585	900153	0.267	15769		Ch1

6.2 RP-HPLC analysis for alkaloids in Vinca (Polluted) methanolic extracts

Sample Name :*Vinca rosea .Linn* (polluted} (HPLC grade methanolic extracts)
Injection Volume : 20 uL
Data Filename : RP-HPLC-13mar_3.lcd
Method Filename :RP- HPLC.lcm
Report Filename : Default.lcr
Date Acquired : 3/13/2014

<Chromatogram>

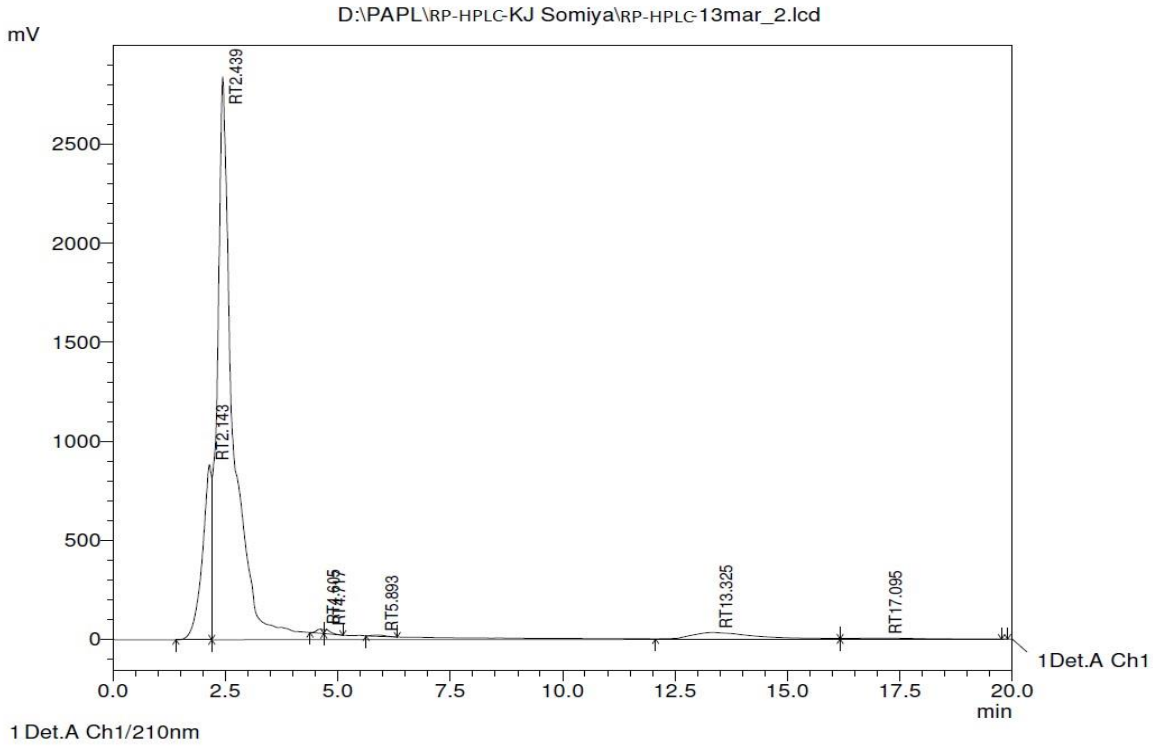

Fig 6.2 Chromatogram for Vinca (Polluted) methanolic extracts

Table 6.2: Results of RP-HPLC for alkaloids of Vinca Polluted methanolic extracts

ID#	Name	Ret. Time	Area	Area%	Height	Units	Channel
1	RT2.143	2.143	12398849	13.589	882931	mg/L	Ch1
2	RT2.439	2.439	74701425	81.873	2840506	mg/L	Ch1
3	RT4.605	4.605	225606	0.247	21300	mg/L	Ch1
4	RT4.717	4.717	210179	0.230	21363	mg/L	Ch1
5	RT5.893	5.893	151984	0.167	7558	mg/L	Ch1
6	RT13.325	13.325	3083613	3.380	31992	mg/L	Ch1
7	RT17.095	17.095	468456	0.513	4775	mg/L	Ch1

6.3 RP-HPLC analysis for alkaloids in asteracantha (Unpolluted) methanolic extracts

Sample Name :*Asteracantha longifolia (K. Schum)* (unpolluted} (HPLC grade methanolic extracts)
Injection Volume : 20 uL
Data Filename : RP-HPLC-13mar_3.lcd
Method Filename :RP- HPLC.lcm
Report Filename : Default.lcr
Date Acquired : 3/13/2014

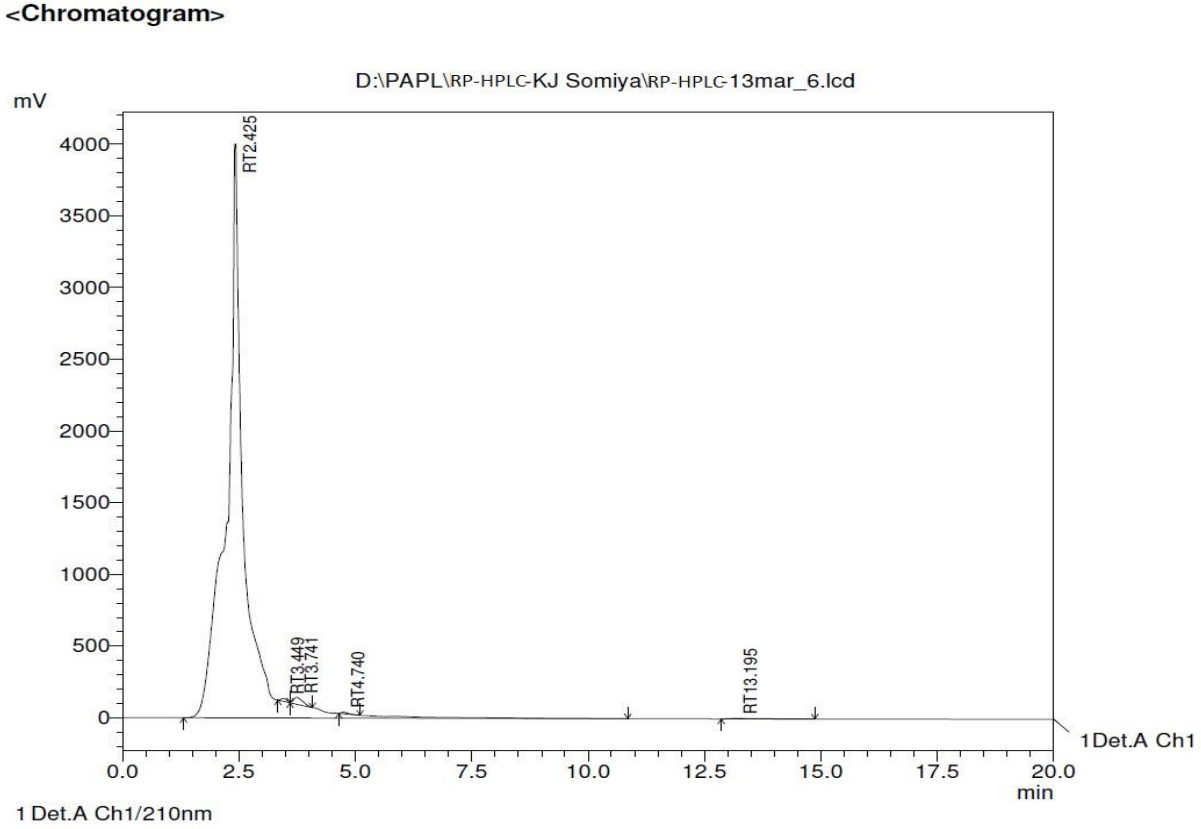

Fig 6.3Chromatogram for Asteracantha (Unpolluted) methanolic extracts

Table 6.3: Results of RP-HPLC for alkaloids of Asteracantha Unpolluted methanolic extracts

ID#	Name	Ret. time	Area	Area%	Height	Units	Channel
1	RT2.425	2.425	105402649	98.946	4001252	mg/L	Ch1
2	RT3.449	3.449	190636	0.179	20585	mg/L	Ch1
3	RT3.741	3.741	677098	0.636	49154	mg/L	Ch1
4	RT4.740	4.740	136805	0.128	12667	mg/L	Ch1
5	RT13.195	13.195	117800	0.111	2750	mg/L	Ch1

6.4 RP-HPLC analysis for alkaloids in Asteracantha (Polluted) methanolic extracts

Sample Name :*Asteracantha longifolia (K. Schum)* (polluted} (HPLC grade methanolic extracts)
Injection Volume : 20 uL
Data Filename : RP-HPLC-13mar_3.lcd
Method Filename :RP- HPLC.lcm
Report Filename : Default.lcr
Date Acquired : 3/13/2014

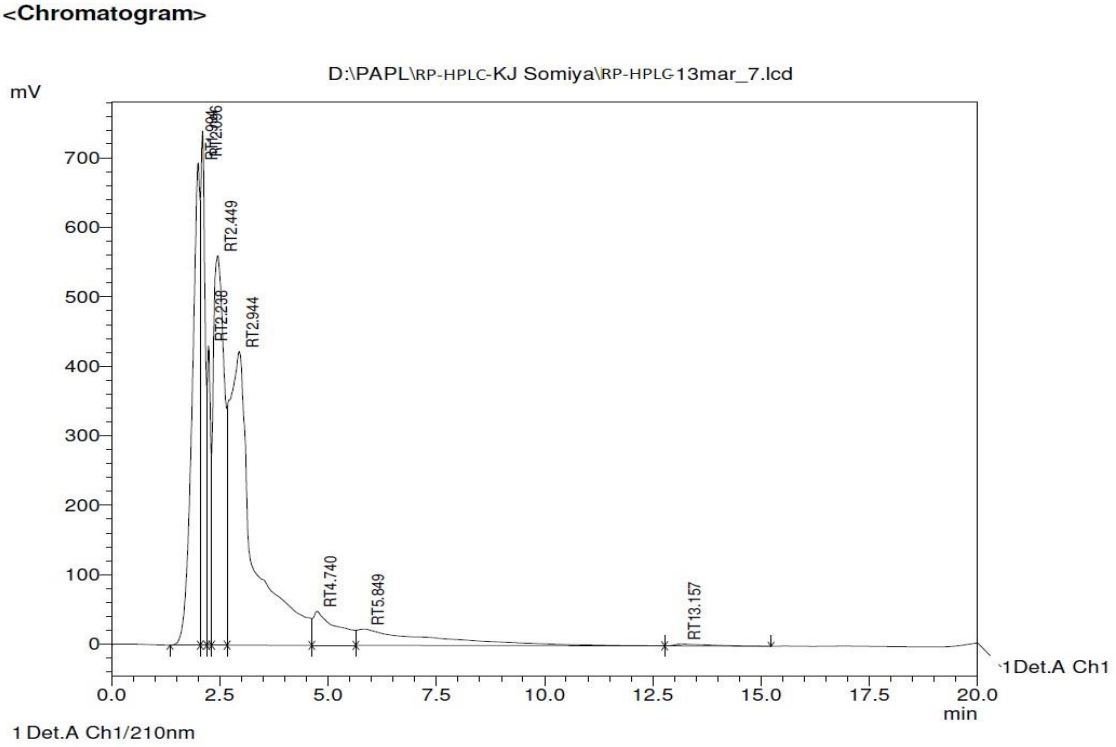

Fig 6.4Chromatogram for Asteracantha (Polluted) methanolic extracts

Table 6.4: Results of RP-HPLC for alkaloids of Asteracantha Polluted methanolic extracts

ID#	Name	Ret. time	Area	Area%	Height	Units	Channel
1	RT1.994	1.994	8785463	18.387	694459	mg/L	Ch1
2	RT2.096	2.096	5289828	11.071	740615	mg/L	Ch1
3	RT2.238	2.238	2371971	4.964	431110	mg/L	Ch1
4	RT2.449	2.449	9845291	20.605	560661	mg/L	Ch1
5	RT2.944	2.944	16574146	34.688	423141	mg/L	Ch1
6	RT4.740	4.740	1929821	4.039	49077	mg/L	Ch1
7	RT5.849	5.849	2836803	5.937	23706	mg/L	Ch1
8	RT13.157	13.157	147417	0.309	2936	mg/L	Ch1

7.1 Results

7.1.1 Comparision of results for Total sugar content and Protein content estimation

Table 7.1: Comparision of biochemicals from Vinca - polluted area and unpolluted area

	Vinca unpolluted			Vinca Polluted		
	Leaf	Stem	Root	Leaf	Stem	Root
TOTAL SUGARS	190 mg	170 mg	218 mg	124 mg	90 mg	90 mg
PROTEINS	64.8 mg	28 mg	45.6 mg	56.8 mg	16.8 mg	27.2 mg

Table 7.2: Comparision of biochemicals from Asteracantha - polluted area and unpolluted area

	Asteracantha unpolluted			Asteracantha Polluted		
	Leaf	Stem	Root	Leaf	Stem	Root
TOTAL SUGARS	248 mg	302 mg	226 mg	124 mg	114 mg	142 mg
PROTEINS	36.8 mg	16.8 mg	21.6 mg	26.8 mg	64 mg	16.8 mg

7.1.2 Comparision of results for HPLC chromatograms

Table 7.3: Comparision of Number of alkaloids found in Vinca from polluted area and unpolluted area

	Vinca unpolluted	Vinca Poluted
NO OF ALKALOIDS	13	7

Table 7.4: Comparision of Number of alkaloids found in Asteracantha from polluted area and unpolluted area

	Asteracantha unpolluted	Asteracantha polluted
NO OF ALKALOIDS	5	8

7.2 Discussions

The selected plants from both the polluted and unpolluted areas showed very much similar allometry, hence phytochemical studies were directly opted for.

The aim of this study was to perform the comparative analysis of biochemicals in plants from polluted vs. those with unpolluted regions. The objective being to understand the effects of environmental pollutants on the biochemical characteristics of these plants. Erstwhile no such study has been done on the selected plants, *Vinca rosea .Linn* and *Asteracantha longifolia (K. Schum)*. Hence, we did a preliminary analysis of pollution on these plants.We usedthree samples for each species of plant from both the polluted and unpolluted area for this study.

On comparing the results we found that the amount of sugars and proteins is decreased in both the selected plant species collected from polluted areas.Thus we can construe that the pollutionmaybe affecting the biochemical properties in these plants.

We performed comparative phytochemical analysis for *Vincarosea Linn* from polluted area versus non-polluted area. The former plant showed the presence of steroids, whereas steroids are absent in the Vinca collected from the unpolluted area. Thus we can infer that steroid production was enhanced when this palnt was exposed to pollution. Further studies are required to know which biochemical pathway/s is upregulated for enhanced steroid production.

The comparative phytochemical analysis on the plant *Asteracantha longifolia (K. Schum)* revealed the presence of terpenoids and glycosides in the plant collected from polluted area. These were absent in plants collected from unpolluted area. This shows that the environmental pollution maybe affecting different phytochemicals of the plants. These results indicate that some of the environmental pollutants may be causing stress in these plants due to which the pathways for the production of these compounds is upregulated in plants exposed to the environmental pollution.

In our study we were interested in knowing the effects of pollutants mainly on the alkaloids, since the medicinal value of the plants is reliant on the alkaloids of that plant. Using RP-HPLC analysis, we studied the plant chromatograms of Vinca and Asteracantha from both polluted and unpolluted areas. In the chromatograms the peaks designated various alkaloids present in the methanolic extracts of the plants.

We compared the chromatograms obtained for the plant *Vinca rosea .Linn* from polluted and unpolluted areas. We observed that the chromatogram of Vinca from unpolluted area showed 13 peaks which indicated that this plant has 13 different alkaloids, whileVinca from polluted area showed only7 peaks which meant that this plant consists of 7 alkaloids. The results allowed us to interpret that the decline in the number of alkaloids in Vincafrom polluted area is because of relentless rise in the environmental pollution in that area. There is a probability that the pollutants may have caused genetic mutations which may have led to the decreased production of the alkaloids. Thus environmental pollution has caused deleterious effects in *Vinca rosea .Linn*.

Comparing the chromatograms obtained for the plant *Asteracantha longifolia (K. Schum)* from polluted and unpolluted areas, we found out that the chromatogram of Asteracantha from unpolluted area is showing 5 peaks which means this plant has 5 different alkaloids, whereas the Asteracantha from polluted area shows 8 peaks which means that this plant consists of 8 alkaloids. There is a rise in the number of alkaloids, because increasing environmental pollution has mutated the DNA of Asteracantha so as to produce new alkaloids in the plant. This can mean that either the three new alkaloids can be of medicinal use or can be toxic. From this we can interpret that upsurge in the number of alkaloids found in Asteracantha polluted plant is because environmental pollution has shown additive effects on the plant *Asteracantha longifolia (K. Schum).*

Thus, the difference in the biochemicals and the phytochemicals found in these plants indicate that Environmental pollution maybe affecting the plants.

8. Conclusion

From the above results we conclude that plants,*Vinca rosea .Linn* and *Asteracantha longifolia (K.Schum)*growing in the polluted areas have become progressively inferior in their biochemical constituents as compared to those growing in the unpolluted areas.

A significant decrease in the biochemical contents may be the result of DNA mutation and it may also be leading to change metabolic pathways.

These plants when screened for alkaloidsusing RP-HPLC, shows that there is modification in the number of alkaloids present in those growing in the polluted areas, which clearly indicates that pollution has affected these plants on a genetic level.

This study was performed on a small number of sample (n=3), due to which the statistical analysis was not performed. Hence this study is a preliminary qualitative analysis which gives slight indication that there is effect of the environmental pollution on the biochemical and phytochemical characteristics of the plants in polluted area, which affects their medicinal value. In future, this data needs to be validated on a large number samples from both *Vinca rosea .Linn* and *Asthercantha longifolia (K.Schum)*. Along with the biochemical parameters, genetic mutations also need to be determined. This will enable us to understand the environmental pollution effects on the molecular level thereby affecting the medicinal value of these plants.

9. Future Aspects

The medicinally important compounds expected to be present in Vinca are **vinblastine** and **vincristine** which are therapeutic against Cancer and in Asteracantha compounds such as**betulin** are having antidiuretic properties.

This study needs to be performed to understand the genetic alteration and protein related pathways affected. Pollutants also affect the plant on a genetic level which can be further determined using molecular biology by PCR and RFLP/RAPD studies. Changes at both the RNA and protein content and quality can be determined by specific molecular biology techniques.

The quantitative determination of different types of pollutants present in the air, water and soil in the polluted areas from where the plants were collected can also be done using AAS (Atomic Absorption Spectroscopy) techniques.

Medicinal plants have a promising future because there are about half million plants around the world, and most of their medicinal activities have not investigated yet, and their medicinal activities could be decisive in the treatment of present or future studies.

By running an HPLC analysis along with **standards** for the useful and biologically important compounds we can get optimized results of the amount of change which is caused on the production of metabolites in the said plants.

Anti-microbial activities of the plants can be compared for the production of future novelty drugs.

Therefore, all this information will help the scientists and researchers to screen the compounds responsible for different bioactivities and to elucidate the mechanism of action.

Appendix

Sr. No.	Requirements	Particulars
1	10% $FeCl_3$	Dissolve 10 gm of $FeCl_3$ in 100ml D/W
2	1% 1M HCl	Dissolve 1.43 ml HCl in 50 ml of D/W
3	20% NaOH	Dissolve 20 gm of NaOH in 100 ml D/W
4	Mayer's reagent	Dissolve 1.368 gm $HgCl_2$ in 60 ml D/W and Dissolve 5 gm of KI in 10 ml D/W. Mix these two and then make volume to 100 ml.
5	Standard glucose stock solution: (1 mg/ml)	Dissolve 100 mg of standard glucose in 100 ml D/W.
6	Standard BSA stock Solution: (200 µg /ml)	Dissolve 200 mg of standard BSA in 1 litre of D/W.
7	Lowry's solution:	Separately prepare the following solutions, Alkaline solution (Solution A – 100ml) 0.572 gmsNaOH 2.862 gms Na_2CO_3 Aqueous $CuSO_4$ (Solution B – 20ml) 0.285 gms $CuSO_4$. 5 H_2O (Solution C – 20ml) 0.571 gms Sodium Tartarate. 2 H_2O Add solutions A, B and C in the ratio 100:1:1 to prepare lowry's solution to add in the reaction mixture during protein estimation.
8	Folin's reagent:	Prepare this reagent while doing the estimation, Add 5ml of 2N FolinCiocalteau'sreagent in 6ml Distilled water (solution is light sensitive, store in amber coloured bottle).
9	DNSA (Dinitro salicylic acid) as follows,	Solution I – dissolve 30 gms of potassium sodium tartarate in minimum amount of distilled water and then make the volume to 50 ml Solition II – dissolve 0.16 gms of 2N NaOH in 20ml D/W Heat the above two solutions and add 0.5 gms of DNSA powder to it while continuously stirring with glass rod. Once clear solution is obtained make the

		total volume to 100 ml by adding distilled water and store in amber coloured bottle.

[i]

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