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ANTIOXIDANT PROPERTIES OF FLAVONOIDS ISOLATED FROM LEAVES AND FLOWERS OF *Tabernaemontana heyneana Wall.*

A PROJECT REPORT

Submitted by

ARAVIND, M.

DEEPTHI SRI, S.

TILAK, S.

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ANNA UNIVERSITY: CHENNAI 600025

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ANNA UNIVERSITY: CHENNAI 600025

BONAFIDE CERTIFICATE

Certified that this project report "**ANTIOXIDANT PROPERTIES OF FLAVONOIDS ISOLATED FROM LEAVES AND FLOWERS OF *Tabernaemontana heyneana Wall.***" is the bonafide work of **ARAVIND,M., DEEPTHI SRI,S., TILAK,S.** who carried out the project work under my supervision.

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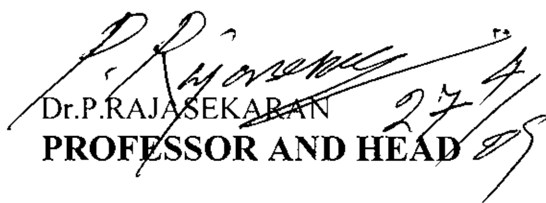


Dr.P.RAJASEKARAN
PROFESSOR AND HEAD

Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore – 641006.

SIGNATURE

Mr.T.SATHISH KUMAR
SUPERVISOR
Lecturer

Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore – 641006.

CERTIFICATE OF EVALUATION

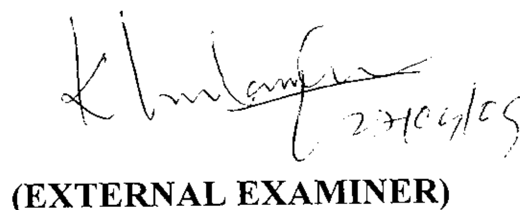
COLLEGE : Kumaraguru College of Technology

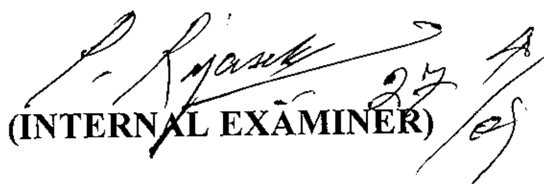
BRANCH : Biotechnology

SEMESTER : Eighth Semester

NAME OF THE STUDENTS	TITLE OF PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
ARAVIND.M Reg.No. 71205214002 **DEEPTHI SRI.S** Reg.No. 71205214008 **TILAK.S** Reg.No. 71205214037	**ANTIOXIDANT PROPERTIES OF FLAVONOIDS ISOLATED FROM LEAVES AND FLOWERS OF *Tabernaemontana heyneana Wall.***	**Mr.T.SATHISH KUMAR** Lecturer, Department of Biotechnology.

The report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was confirmed to be the report of the work done by the above students and then evaluated.





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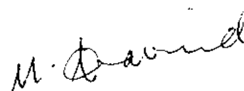

[Aravind.M]

[Deepthi Sri.S]

[Tilak.S]

ABSTRACT

Abstract

The study was carried out to determine the extraction optimisation of flavonoids from the flowers and leaves of *Tabernaemontana heyneana Wall.* The L_{16} orthogonal design involves the effect of single factors such as temperature, extraction time, material ratio,solvent ratio and no. of extractions.The optimal conditions for the extraction of flavonoids from the leaves was found to be 85°C , 2 hours with a material ratio of 1:5 , 85% ethanol and 4 times of extraction , whereas for flowers it was at 85°C ,3 hours with a material ratio of 1:20 ,75% ethanol and 1 time of extraction. The identification of flavonoids using TLC in the optimal extract extract of both leaves and flowers showed the presence of Quercetin , Rutin and Leucoanthocyanin related compounds. The isolation of flavonoids using PTLC from the optimal leaf and flower extracts revealed the presence of Quercetin , Rutin and Leucoanthocyanin related compounds which were visualized at long UV light(365nm). RP-HPLC analysis of PTLC isolated compounds from the leaves and flowers also confirmed the presence of Quercetin , Rutin and Leucoanthocyanin related compounds. Partial purification carried out for the optimal extract of leaves and flowers using silica gel column chromatography yield four different fractions that were identified as Quercetin , Rutin , Leucoanthocyanin and unknown phenolic acids. The isolated compounds from the leaves and flowers possessed appreciable 85-90% of the total antioxidant activity in the plants.

Key words: Antioxidants, DPPH assay, Flavonoids, Free radical scavenging, Tabernaemontana heyneana Wall.

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LIST OF ABBREVIATIONS

UV	-	Ultra Violet
ROS	-	Reactive Oxygen Species
HPLC	-	High Performance Liquid Chromatography
TLC	-	Thin Layer Chromatography
PTLC	-	Preparative Thin Layer Chromatography
BSI	-	Botanical Survey of India
DPPH Assay	-	1, 1-Diphenyl-2-Picryl-hydrazil

INTRODUCTION

1. Introduction

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds.

Flavonoids are members of a class of natural compounds with widespread occurrence in the plant kingdom. They are one of the largest groups of natural products known. Over 4000 flavonoids have been identified to date, widely distributed in the leaves, seeds, bark and flowers of plants. In plants, these compounds provide protection against ultraviolet radiation, pathogens, and herbivores and are anthocyanin copigments in flowers that attract pollinating insects. They are also responsible for the characteristic red and blue colors of berries; wines and certain vegetables.1 Flavonoids are benzo-_-pyrone derivatives consisting of phenolic and pyrane rings and are classified according to substitutions. There are six classes of flavonoids,which differ in their chemical structure – flavanols,

flavones, flavonols, flavanons, isoflavons and anthocyanidins. Most dietary flavonoids occur in food as 3-*O*-glycosides and polymers, but they can also exist in aglycon forms. Many beneficial health effects are attributed to flavonoids, mostly due to their antioxidant and chelating abilities.2 numerous studies have been conducted to prove flavonoids' efficacy as antimycotic, antibacterial, antiviral, anti-inflammatory, antioxidant, immune modulator, and enzyme inhibitor, mutagenic and toxic agents.(Ajay Sharma *et al.*,2007).

One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals (Van Acker *et al.*, 1996). A free radical is molecule containing one or more unpaired electrons in atomic or molecular orbitals that includes super oxide (O_2.−), hydroxyl radicals (OH-) and H_2O_2, collectively known as reactive oxygen species (ROS) (Sathishkumar *et al.*, 2008). These ROS may induce oxidative damage to various macromolecules like polyunsaturated fatty acids in cell membranes, carbohydrates, proteins and DNA which results in homeostatic imbalance.

Tabernaemontana heyneana Wall. (Apocynaceae) known as kundalam paalai in Tamil, is known to possess antimicrobial activity against skin diseases, venereal diseases, respiratory problems, nervous disorders and various other diseases (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.*, 2006). The stembark decoction is used for cleaning cuts and wounds before dressing them (Chandrashekar *et al.*, 1995). The mixture of leaf and stem powder of this plant along with the stem bark of *Ficus racemosa, Ficus benghalensis, Madhuca longifolia,* is heated with coconut oil and applied

externally to cure skin diseases (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.,* 2006). Similarly the same mixture along with the stem bark of *Strychnos nux-vomica* and fruits of *Carica papaya* were taken internally to induce abortion (Ignacimuthu and Ayyanar, 2005; Ignacimuthu *et al.,* 2006).

In many cases, it is difficult to find quickly suitable experimental conditions for a given separation task. Prediction of separation conditions is not yet straightforward. Therefore, good experimental design becomes increasingly important. Orthogonal design which only focuses on the main effects of the factors, allows the number of experiments to be drastically reduced. In separation science, this kind of experimental design has already shown its usefulness in liquid chromatography and capillary electrophoresis (Hu Zhide *et al.,* 2002).

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 *Tabernaemontan*

The genus *Tabernaemontana* (Apocynaceae) has a wide distribution. They occur in tropical as well as subtropical parts of the world. Plants belonging to this genus are known to provide indole alkaloids of unusual structures, with novel bio activity (Halliwell and Gutteridge, 1989). Many species have been used in folk medicine against several diseases like diarrhoea, skin infections, warts, syphilis, Hansen's disease, cancer and insect bites.

2.1. (a) *Tabernaemontana orientalis*

T. orientalis (or *Ervatamia orientalis*) is an under tree growing widely in many parts of Kerala and Tamil Nadu. The existence of considerable amounts of alkaloids in this plant was first reported in 1952 and reference to literature revealed that no further work has been done on this. Many medicinal properties have been ascribed to the plants of the *Ervatamia (Tabernaemontana)* group and since no pharmacological work has been done on this particular plant , a systematic pharmacological screening of the drugs obtained was taken up. The common name 'iodine bush' arose from its reputed use by Aborigines to cure sores and ulcers. It belongs to a family with many toxic members and this one has been suspected of poisoning horses and cows. However, there is no record of human poisoning and it appears that the protein-breaking enzymes, and possibly even antibiotics, in the milky latex may actually have healing properties. It has also been called Bitter Bark and has found use as a quinine substitute in colonial times.

(Patricia I Oteiza et al.,2005). The total alkaloid content from leaves harvested in Queensland (Australia) has been tested at 0.8%. The leaves contain ervatamine and 19-dehydoervatamine (2-acylindole alkaloids), together with ibogaine and other related alkaloids. In another study leaves and bark of different Australian specimens were quantitatively and qualitatively analyzed for their alkaloids. In the leaves the alkaloids isolated (0.22% yield) were ibogaine (~32.5%), iboxygaine (~4.5%), ervatamine (~9%), 19-dehydroervatamine (~18%) and apparacine (~36%). Samples of bark were found to contain between 1.3 and 2.0% alkaloids. The root bark has not been tested yet, but is likely to have high yields of ibogaine and related alkaloids, especially in the rootbark. (Van Beek, 1984).

2.1. (b) *Tabernaemontana divaricata*

Crepe jasmine is a beautifully shaped evergreen shrub which forms symmetrical 6 ft (2 m) high mounds of glossy foliage. The waxy blossoms are white five-petaled pinwheels that are borne in small clusters on the stem tips. (Brouillard and Cheminat,1988). Novel indole alkaloids of the aspidosperma-type, viz., voafinidine and voalenine were obtained in minor amounts from the leaf extract of *Tabernaemontana divaricata* (double flower variety). The structures of these alkaloids were elucidated by spectral methods.(Toh-Seok Kam *et al.*,1996). Bark scrapings are used by the Tikunas to ease the pain after childbirth. Decoctions of the bark are used to alleviate stomach and rheumatic pains, as well as diarrhoea and has a reputation of making women more fertile.(Tammy Jenkins *et al.*,1999). The latex has a cooling effect and is applied to wounds to prevent inflammation. Latex mixed with oil is applied to the head to relieve headaches, eye pressure and corneal inflammation.(Dejan Godjevac *et*

al.,2004) The juice from the flowers is dropped into the eyes in cases of ophthalmia (not advised without medical supervision!!). Chewing the root relieves tooth-ache. Decocted with oil and applied to the head it is said to relieve all indispositions, especially pains, of the head. A water decoction of the root is an efficient wormicide and is used to treat respiratory problems such as asthma. In Malaysia the leaves are pounded with sugar candy and water to give a drink for curing coughs. Yunani practitioners value the flowers as an analgesic. In Indonesia the leaves, bark and twigs may form the main components of an arrow poison used on the Mentawei Islands. *Tabernaemontana divaricata* contains at least 15 alkaloids of the complex indole type.(Ayoola *et al.*,2008).

2.1. (c) Tabernaemontana pandacaqui

In parts of Australia 'banana bush' was seemingly recognized in the nursery trade as E. angustisepala. Studies on carrageenin-induced rat paw edema, yeast-induced hyperthermia in rat and writhing response induced by acetic acid in mice showed that the alcoholic extract of stems of *Tabernaemontana pandacaqui* has significant anti-inflammatory, antipyretic and antinociceptive activities. These activities are due to alkaloidal components since they were also observed when the crude alkaloidal (CA) fraction separated from alcoholic extract was tested in the same models. (T. Taesotikul *et al.*, 2002*). Tabernaemontana pandacaqui* has been shown to contain the indole alkaloids ervatamine and epiervatamine and dimeric alkaloids in a total alkaloid content of 0.46%. Animal testing of these alkaloids produced reserpine-like effects, including decreased activity, piloerection and a marked and sustained decrease in blood pressure. It also showed antispasmodic and anticonvulsant effects.

Bioassays by a particularly curious group of researchers appear to be the only human testing results available. They experienced relaxation, minor intoxication and minor visual effects for several hours after ingesting an unmeasured quantity of a decoction of the whole plant. None reported any "feeling of toxicity". (Van Beek, 1984).

2.1. (d) Tabernaemontana heyneana Wall.

Ervatamia heyneana is a medicinal plant that was given the name of *Tabernaemontana heyneana Wall.* in Botany.In southern India, the species of *Ervatamia heyneana* is used medicinally as a substitute for `*Tabernaemontana divaricata*` which is a closely related and more widely distributed species. The acrid, bitter root is used as a local anodyne and chewed to relieve toothache. Rubbed into a thin paste of *Ervatamia heyneana* with water is often taken internally as a vermicide. This paste of the plant is also applied with limejuice to clear opacity of the cornea. Charcoal is prepared from the root and the milky juice of the leaves and this is vastly used to treat ophthalmia. The juice of the flowers, mixed with oil, is applied externally to relieve the burning sensation in sore eyes. The mixture of the juice of the flowers and oil is rapidly used for relief of some skin diseases. The milky juice of the leaves is used as a cooling application for wounds and it also reduce inflammation. The ethanolic extract and isolated alkaloids heyneanine and voacristine prevented pregnancy when administered during the preimplantation period in Sprague-Dawley rats. These were, however, found to possess significant uterotrophic activity. The roots of *Tabernaemontana heyneana Wall.* were examined and the isolation and identification of additional indole alkaloids and some pharmacological

properties of coronaridine are described. Extraction of the roots yielded the alkaloids coronaridine, voacangine, ibogamine, 19-oxocoronaridine, and the pseudoindoxyl of voacangine. An aqueous ethanolic extract of the roots was found to prevent fertilization of adult female rats when administered orally. After the residue of this extract was treated chromatographic fractionation on silica gel yielded the alkaloid coronaridine. When administered to adult female rats, orally, coronaridine hydrochloride at levels of 5 mg/kg/day or above prevented pregnancy. Data indicated that coronaridine was weakly estrogenic. (Meyer WE *et al.*, 1973).

Table 2.1 (d) Classification of *Tabernaemontana heyneana Wall.*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Gentianales
Family	Apocynaceae
Genus	Tabernaemontana
Species	Heyneana

Fig 2.1 (d) Tabernaemontana heyneana Wall

At present,there are not many scientific reports on the extraction of flavonoids from the leaves and flowers of *Tabernamontana heyneana Wall.* In this study, the optimal conditions to extract flavonoids from the leaves and flowers of *Tabernamontana heyneana Wall.* were investigated systematically in order to explore a proper process to utilize the *Tabernamontana heyneana Wall.* in the area of healthcare.

2.2 FLAVONOIDS

Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. Havsteen, (2002).

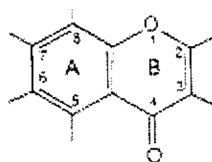

Fig. 2.2(a) Structure of benzo-γ-pyrone

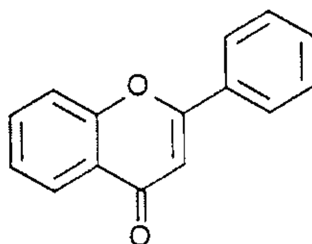

Fig. 2.2(b) The basic structure of flavonoids

2.2.1 CLASSIFICATION OF FLAVONOIDS

According to the IUPAC nomenclature flavonoids can be classified into:

2.2.1(a) Flavonols

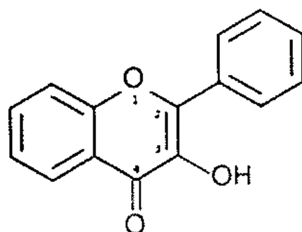

Fig 2.2.1 (a) Structure of flavonols

Ex: Quercetin, Kaempferol, Myricetin, Fisetin, Isorhamnetin, Pachypodol, Rhamnazin

2.2.1(b) Flavones

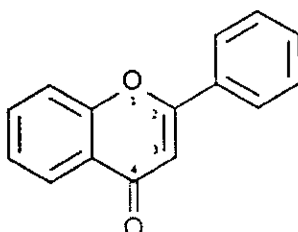

Fig 2.2.1(b) Structure of Flavones

Ex: Luteolin, Apigenin, Tangeritin

2.2.1(c) Flavonones

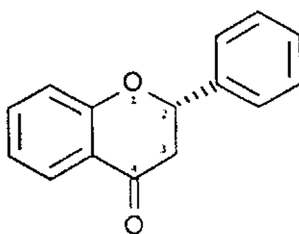

Fig 2.2.1(c) Structure of Flavonones

Ex: Hesperetin, Naringenin, Eriodictyol, Homoeriodictyol

2.2.1(d) Flavanonols

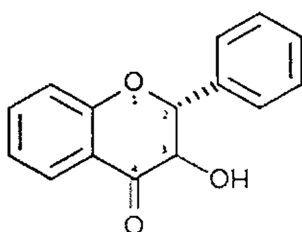

Fig 2.2.1(d) Structure of Flavanonols

Ex: Taxifolin, Dihydrokaempferol

2.2.1(e) Isoflavones

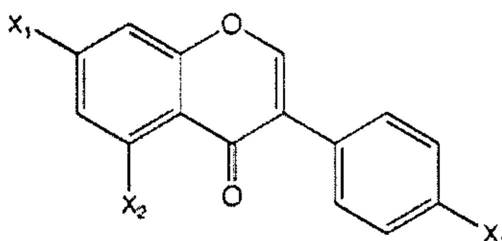

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Fig 2.2.1(e) Structure of Isoflavones

Ex: Genistein, Daidzein, Glycitein

2.2.1(f) Anthocyanidins

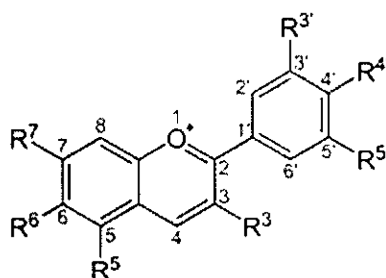

Fig 2.2.1(f) Structure of Anthocyanidins

Ex: Cyanidin, Malvidin

2.2.2. Flavanoids as free radical scavengers

One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals. These highly reactive species arise in the course of many physiological processes, especially due to leakage of electrons in the respiratory chain.(Fazilatun Nessa *et al.*,2004) In the final phase of respiratory chain, the transfer of four electrons to dioxygen generates two molecules of water, i.e., a safe product, but partial reduction leads to the formation of highly toxic compounds, e.g., the superoxide anion ($O_2^{\cdot-}$). This species is also formed by macrophages in the first line of defense against invading foreign cells or virus particles (Geissman *et al.,* 1963). This reaction is desirable, but excess superoxide anion must be scavenged quickly before it destroy too many essential, unsaturated lipids in the membranes, as well as sulfhydryl groups, e.g., in the active sites of key enzymes. Protonation of the superoxide anion yields the hydroperoxide radical HO_2.which spontaneously reacts with another hydroperoxide to produce H_2O_2, whose half life is short and also deleterious (Montanari *et al.,* 1998). Similarly, H_2O_2 is also produced in a reaction catalyzed by the enzyme superoxide dismutase (SOD) while scavenging the superoxide anion.

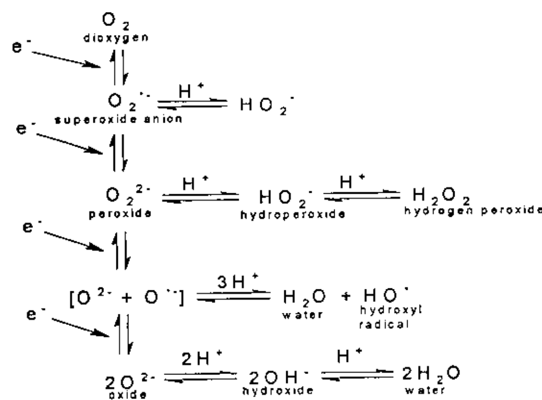

Fig 2.2.2 Formation of Reactive Oxygen Species

2.2.3 Biological effects of flavonoids

Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity.(Sukanda Vichitphan et al.,2007) Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancers and cardiovascular diseases.(Emanuel L. Johnson and Walter F.Schmidt.,2004). The beneficial effects of fruit, vegetables, and tea or even red wine have been attributed to flavonoid compounds rather than to known nutrients and vitamins.

2.3 Flavonoid extraction methods

2.3 (a) Orthogonal design

The Principle of Orthogonal Design says that each relation in a relational database should be unique. Orthogonal design which only focuses on the main effects of the factors, allows the number of experiments to be drastically reduced. (Xiong Hao-ping *et al*, .2005).In separation science, this kind of experimental design has already shown its usefulness in liquid chromatography and capillary electrophoresis.

2.3 (b) Supercritical extractions

The system consists of solvent and feed pumps, refrigeration module, 5 L/50 MPa and 1 L/50 MPa extraction vessels, and 2 L/30 MPa and 1 L/30 MPa absorbent vessels. Light-phase fluid (carbon dioxide) was supplied from the CO2 cylinder by a high-pressure metering pump. Heavy-phase fluid (aqueous ethanol) was supplied to the system by means of a duplex high-pressure pump. CO2 flow was controlled by pump displacement and was monitored with high-pressure mass-flow meter.(Yuanying Qi *et al.*, 2007).Operating temperature was regulated in the extractor and separators by means of three thermo-static baths. A series of valves regulated the pressure in the extractor and separators. (Xiong Hao-ping *et al*,.2005).

2.4 Isolation methods

Thin Layer Chromatography (TLC) plates were activated before use. One dimension TLC was performed with the purified compounds using two different solvent systems; Benzene-ethyl acetate (1:9) and Chloroform-Methanol (1:3) as mobile phase (K.M.Hazra *et al.*, 2007).

The flavonoids were separated using paper chromatography. The flavonoid fractions were then separated using Thin Layer Chromatography on silica gel and visualised under UV light (366nm) (Y.Feng *et al.*, 1988).

Chromatographies of the optimized extracts from the leaves and flowers of *Tabernaemontana heyneana Wall.* were run one dimensionally in the mobile phase solvent (ethyl acetate - ethanol - water, 5:1:5, v/ v/ v) at room temperature of 20-25°C (Sathish kumar *et al.*, 2008).

2.5 Purification methods

2.5 (a) Chromatography

Two Flavonoids were isolated from bran of hard red spring wheat and identified as apigenin-6-C-arabinoside-8-C-hexoside and apigenin-6-C-hexoside-8-C-pentoside. The flavonoids were extracted from bran with dilute NaOH solution (pH 11). They were purified by chromatography on XAD-2 resin and Sephadex G-15 columns, followed by paper chromatography with three different solvents and finally by Thin Layer Chromatography.(Jingren He *et al.*,2006).

Hawthorn fruit extract has been shown to have many health benefits including being cardiovascular protective, hypotensive and hypocholesterolemic. The dry hawthorn fruit was extracted successively with ether, ethyl acetate, butanol and water. The column chromatographic separation led to isolation of eight pure compounds; namely, ursolic acid, hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and protocatechuic acid. (Pauline Guinot *et al.*, 2008).

The HPLC is also used for confirmation of the purity of the compounds to give a better separation. HPLC is used for purifying compounds that cannot be purified by using paper chromatography.

OBJECTIVES

3. Objectives

- To optimize extraction of flavonoids from leaves and flowers using orthogonal design of experiment.
- To detect the presence of flavonoids from optimal extract using thin layer chromatography (TLC).
- To isolate flavonoids using preparative thin layer chromatography (PTLC).
- To investigate the *invitro* antioxidant property of isolated flavonoids.
- To purify flavonoids using silica gel column chromatography.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 PLANT MATERIAL

The plant was collected from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India and the species was identified, confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India and a voucher specimen (No. DBT 001) was deposited at Department of Biotechnology, Kumaraguru College of Technology, and Coimbatore, India .The leaves of *Tabernaemontana heyneana Wall.* dried in a hot air oven for 2-3 days at 50°C and made into powdered form. Fresh flowers were used for the analysis.

OPTIMISATION OF FLAVONOIDS

4.2.1 EXTRACTION OF FLAVONOIDS BY ETHYL ACETATE

Extraction of flavonoids could be obtained by L_9 (3^4) orthogonal design i.e., three levels and four different variables (Tempearature, Extraction time, Solid Liquid ratio, Number of extraction.) Temperature varying from 60 °C to 80 °C. Extraction time varying from 1 to 3 hours and the solid liquid ratio varying from 1:10 to 1:20.

Table 4.2.1(a) 3^4 ORTHOGONAL DESIGN PARAMETERS

A	B	C	D
Temp.(°C)	**Ext.tim.(hrs)**	**Sol : liq (W:V)**	**No.of ext**
60	1	1:10	1
70	2	1:15	2
80	3	1:20	3

Table 4.2.1(b) 3^4 L_9 ORTHOGONAL DESIGN

Expt No	Column			
	A	B	C	D
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

4.2.2 EXTRACTION OF FLAVONOIDS BY ETHANOL

Extraction of flavonoids from leaves and flowers could be determined by L_{16} (4^5) orthogonal design. In this orthogonal design there are four levels for a different five variables (Temperature, Extraction time, Solvent percentage, Solid Liquid ratio, Number of extraction.) Temperature varying from 55 °C to 85 °C. Extraction time varying from 1 to 4 hours. Solvent percentage varying from 65 to 95. The solid liquid ratio varying from 1:5 to 1:20.

Table 4.2.2 (a) 4^5 ORTHOGONAL DESIGN PARAMETERS

Levels	A	B	C	D	E
	Temp. (°C)	Ext.tim. (hrs)	Solvent (%)	Sol : liq (W:V)	No.of.ext
1	55	1	65	1:5	1
2	65	2	75	1:10	2
3	75	3	85	1:15	3
4	85	4	95	1:20	4

Table 4.2.2(b) (4^5) L_{16} ORTHOGONAL DESIGN

Exp.	A	B	C	D	E
1	1	1	2	3	4
2	1	2	1	4	3
3	1	3	4	1	2
4	1	4	3	2	1
5	2	1	1	1	1
6	2	2	2	2	2
7	2	3	3	3	3
8	2	4	4	4	4
9	3	1	3	4	2
10	3	2	4	3	1
11	3	3	1	2	4
12	3	4	2	1	3
13	4	1	4	2	3
14	4	2	3	1	4
15	4	3	2	4	1

16	4	4	1	3	2

4.3. ESTIMATION OF TOTAL FLAVONOIDS

To about 0.1ml of the sample, 2.4ml of distilled water was added. Then, 75µl of 5% $NaNO_2$ was added. After maintaining at room temperature for 5 minutes 150µl of 10% $AlCl_3.6H_2O$ was added. 0.5ml of 1M NaOH was added 6 minutes later and the absorbance was measured at 510nm.

4.4. IDENTIFICATION OF FLAVONOIDS BY THIN LAYER CHROMATOGRAPHY

Chromatographies of the optimized extracts from leaves and flowers were run one dimensionally in the mobile phase solvent (ethyl acetate - ethanol - water, 5:1:5, v/ v/ v) at room temperature of 20-25°C. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 5mm. Authentic markers of flavonol (quercetin) and flavonoid glycoside (rutin) obtained commercially were co-chromatographed. Identification of the flavonoids in the extracts were identified under UV light after the application of ammonia. A similar preparative thin layer chromatography (PTLC) was also performed to confirm the results of TLC.

4.5. ANTIOXIDANT ASSAY

4.5.(a) DPPH radical scavenging assay

DPPH radical scavenging activity was determined according to the (Park *et al.*, 2004). To 0.5ml of DPPH radical solution, 1.0ml of the extract (100-500µg/ml) and the reaction mixture was vortexed for 10s and allowed to stand at room temperature for 30 minutes. The absorbance was recorded

at 517nm by using UV-Vis spectrophotometer and compared with the 75%ethanol which acted as control solution. The percentage of DPPH scavenging activity was expressed in percentage [1-(test sample absorbance/blank sample absorbance)] X 100(%).

4.6. PARTIAL PURIFICATION OF FLAVONOIDS

The extracted flavonoids were used for the partial purification by using a silica-gel column (30 cm x 1.5 cm i.d). The column was eluted with chloroform , 5% methanol in chloroform , 10 % methanol in chloroform and 20% methanol in chloroform (in an order of increasing polarity) to obtain four fractions at a flow rate of 1.0 ml/min.

The fractions were collected and they were run on micro slides and developed with ethyl acetate: ethanol: water (5: 1: 5, v/v/v) as mobile phase. Spots were detected by spraying with Ammonium solution and the plates were observed under long UV light of 365 nm. (Zesheng Zhang *et al.*,2001).

4.7 RP-HPLC ANALYSIS

RP-HPLC analysis was performed using isolated fragments. Separation was carried out at 25°C on a Waters SunFire C_{18} column (250 x 4.6 mm i.d.) protected by a C_{18} pre-column. Twenty microlitres of the isolated compounds from leaves and flowers was injected and eluted isocratically at 1 ml/min with 100% acetonitrile solvent. Peaks were detected by UV absorbance at 360 nm and quantification was performed by interpolation on an external calibration curve using rutin and quercetin standard solutions. All data were analysed using a ChromQuest software system.(Pauline Guinot *et al.*,2008).

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1 EXTRACTION OF FLAVONOIDS USING ETHYL ACETATE

5.1.1 EXTRACTION OF FLAVONOIDS FROM LEAVES USING ETHYL ACETATE

5.1.1(a) Effect of Temperature

The flavonoids were extracted in the temperature range from 60° C to 80° C . It was found that a higher temperature can decrease the fluid density that may reduce the extraction efficiency and at the same time it can accelerate the solvent flow and thus increase the flavonoid content (He Guo-qing *et* al., 2005) . Hence, the figure 5.1.1(a) shows the optimal extraction of flavonoids was obtained at 80° C.

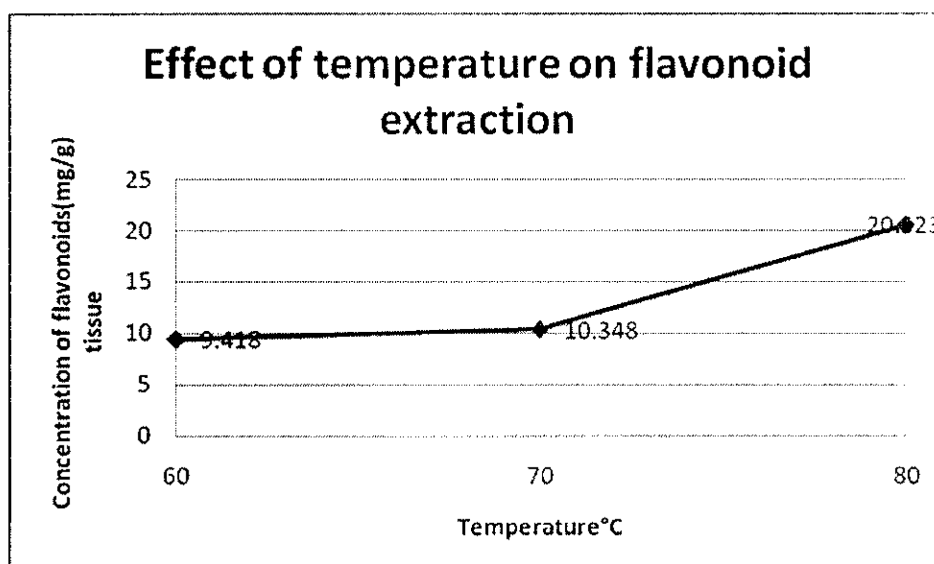

Fig 5.1.1 (a) Effect of temperature on the extraction of flavonoids from leaves using ethyl acetate

5.1.1(b) Effect of Extraction time

The flavonoids were extracted at different time intervals (1 to 3 hrs). The maximum yield was obtained at 1 hr of extraction .An initial increase of content was observed for 1 hr of extraction time and a sudden decrease in the flavonoid content was observed at 2hrs and 3hrs.Thus, the figure 5.1.1(b) showed the contents of flavonoids extracted for 1 hr reached its maxima.

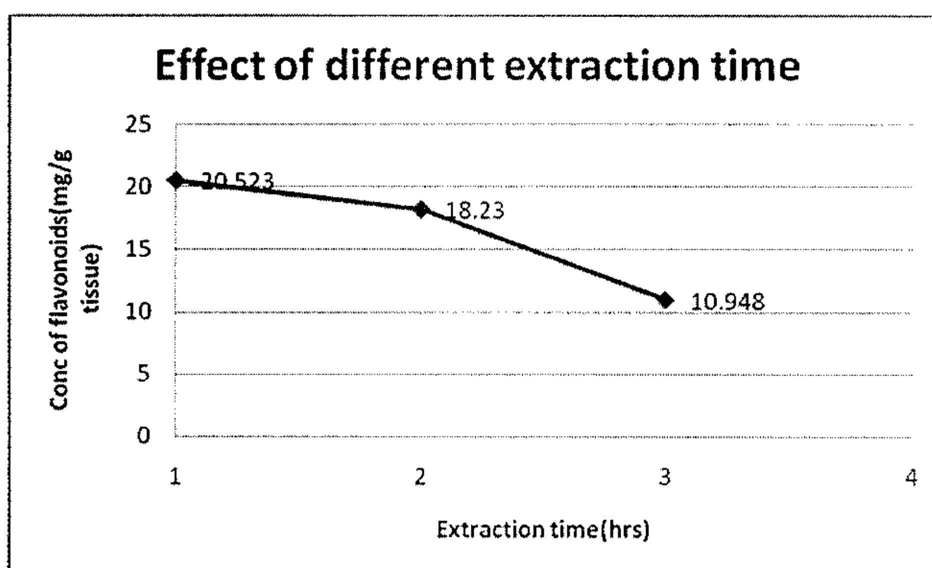

Fig 5.1.1(b) Effect of extraction time on the extraction of flavonoids from leaves using ethyl acetate

5.1.1(c) Effect of material ratio (W/V)

Figure 5.1.1(c) showed the contents of flavonoids extracted were maximum at a ratio of 1:20. A sudden decrease in the flavonoids content was noticed and then a sudden increase in the material ratio. This decrease might be due to the fact that when the material ratio reaches a certain level , the extract has been well dissolved in the solution that the contents of the

extract becomes saturated and thus prevents further increase (Yaqin Xu *et al.*, 2005).

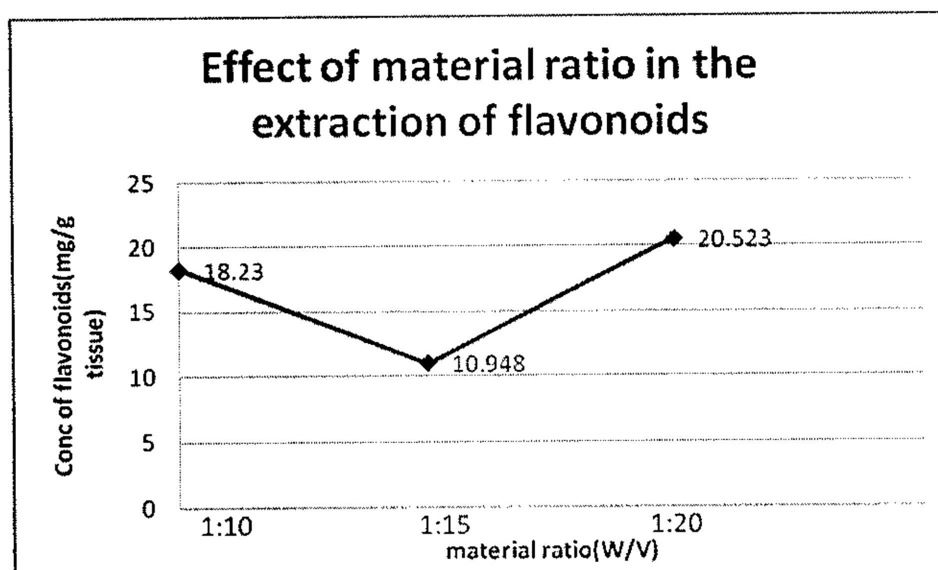

Fig 5.1.1(c) Effect of material ratio on the extraction of flavonoids from leaves using ethyl acetate

5.1.1(d) Effect of number of extractions on flavonoids

The contents of flavonoid extract increases with the no. of extractions i.e., a gradual rise is noticed from 1 time to 4 times.

5.1.2 EXTRACTION OF FLAVONOIDS FROM FLOWERS USING ETHYL ACETATE

5.1.2 (a) Effect of Temperature

The flavonoids were extracted in the temperature range from 60° C to 80° C . It was found that a higher temperature can decrease the fluid density that may reduce the extraction efficiency and at the same time it can accelerate the solvent flow and thus increase the flavonoid content (He Guo-

qing *et* al., 2005) . Hence, the figure 5.1.2 (a) shows the optimal extraction of flavonoids was obtained at 80° C.

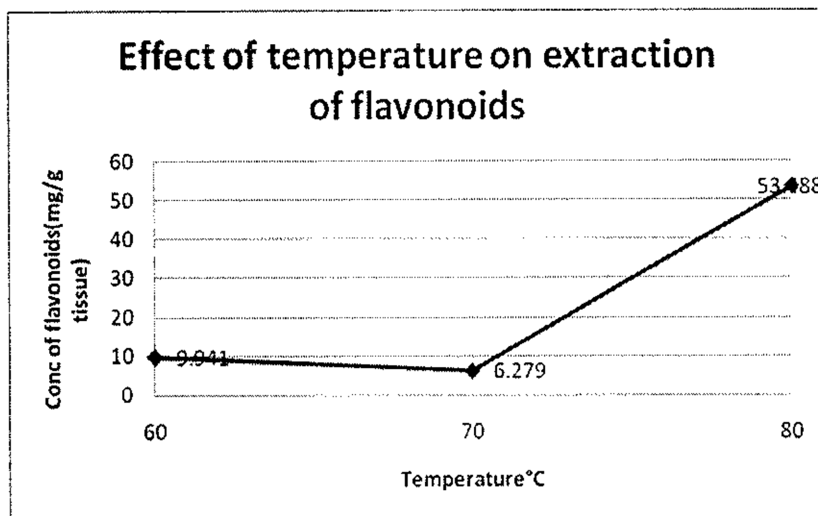

Fig 5.1.2 (a) Effect of temperature on the extraction of flavonoids from flowers using ethyl acetate

5.1.2(b) Effect of Extraction time

The flavonoids were extracted at different time intervals (1 to 3 hrs). The maximum yield was obtained at 1 hr of extraction . A gradual decrease in the flavonoid content was observed at 2hrs and 3hrs . Thus, the figure 5.1.2(b) showed the contents of flavonoids extracted for 1hr reached its maxima .

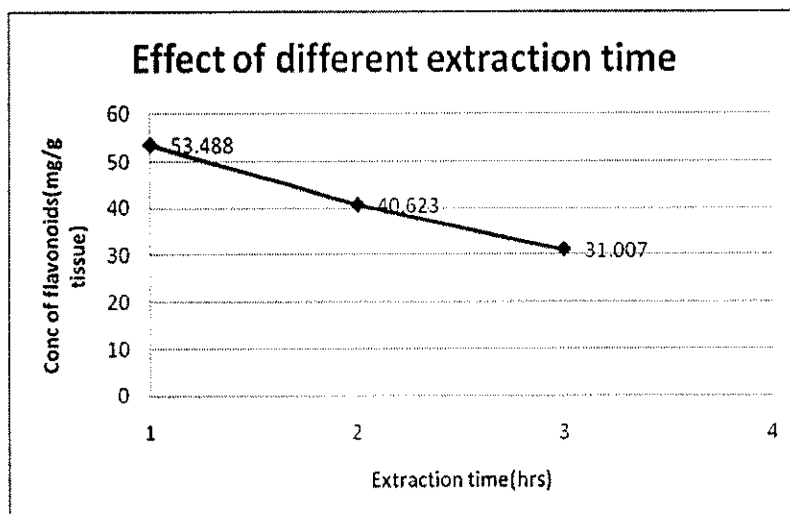

Fig 5.1.2(b) Effect of extraction time on the extraction of flavonoids from flowers using ethyl acetate

5.1.2(c) Effect of material ratio (W/V)

Figure 5.1.2(c) showed the contents of flavonoids extracted were maximum at a ratio of 1:20. An initial decrease in the flavonoids content was noticed and then it increased as there is an increase in the material ratio.

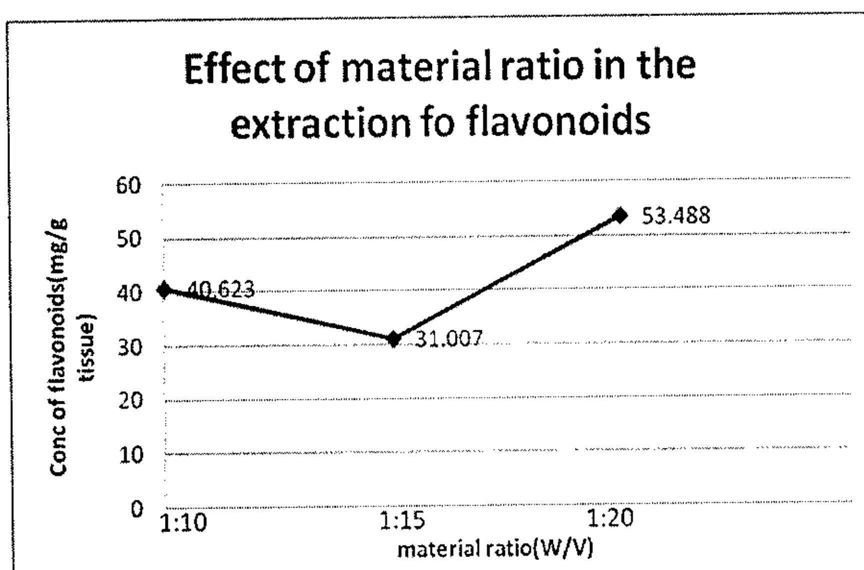

Fig 5.1.2(c) Effect of material ratio on the extraction of flavonoids from flowers using ethyl acetate

5.1.2(d) Effect of number of extractions on flavonoids

The contents of flavonoid extract increases with the no. of extractions i.e., a gradual rise is noticed from 1 time to 4 times.

5.2 EXTRACTION OF FLAVONOIDS USING ETHANOL

5.2.1 EXTRACTION OF FLAVONOIDS FROM LEAVES USING ETHANOL

5.2.1 (a) Effect of Temperature

The flavonoids were extracted in the temperature range from 55° C to 85° C . It was found that a higher temperature can decrease the fluid density that may reduce the extraction efficiency and at the same time it can accelerate the solvent flow and thus increase the flavonoid content (He Guo-qing *et* al., 2005) . Hence, the figure 5.2.1(a) shows the optimal extraction of flavonoids was obtained at 85° C.

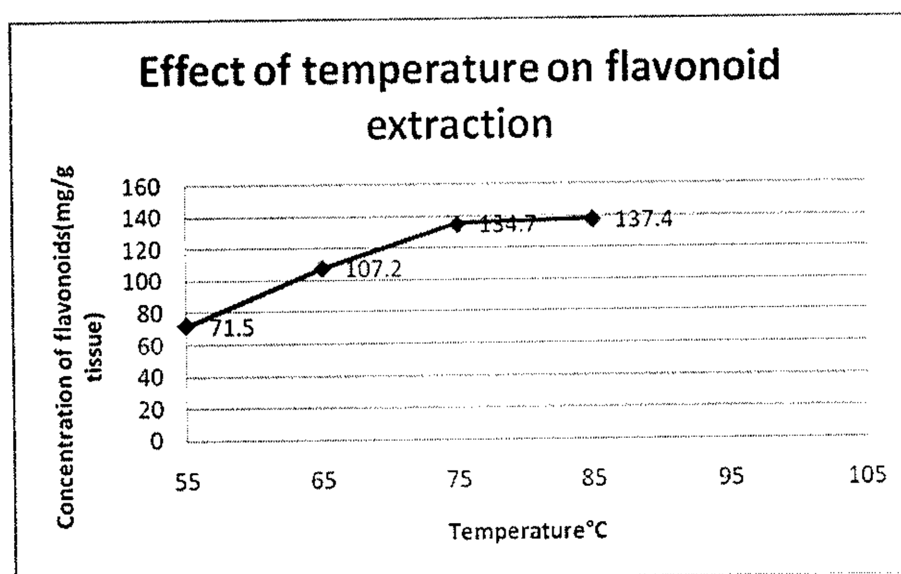

Fig 5.2.1(a) Effect of temperature on the extraction of flavonoids from leaves using ethanol

5.2.1(b) Effect of Extraction time

The flavonoids were extracted at different time intervals (1 to 4 hrs). The maximum yield was obtained at 2 hrs of extraction . A decrease in the

flavonoid content was observed at 1hr and 3 hrs and a sudden increase of content was observed for 4 hrs of extraction time . Thus, the figure 5.2.1(b) showed the contents of flavonoids extracted for 2 hrs reached its maxima.

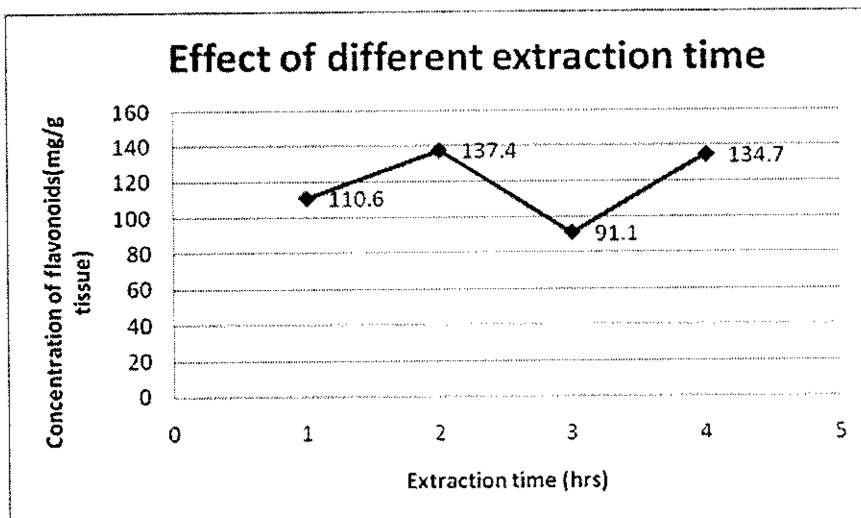

Fig 5.2.1(b) Effect of extraction time on the extraction of flavonoids from leaves using ethanol

5.2.1(c) Effect of material ratio (W/V)

Figure 5.2.1(c) showed the contents of flavonoids extracted were maximum at a ratio of 1:5. A gradual decrease in the flavonoids content was noticed when there is an increase in the material ratio. This decrease might be due to the fact that when the material ratio reaches a certain level , the extract has beenwell dissolved in the solution that the contents of the extract becomes saturated and thus prevents further increase(Yaqin Xu *et al.*, 2005).

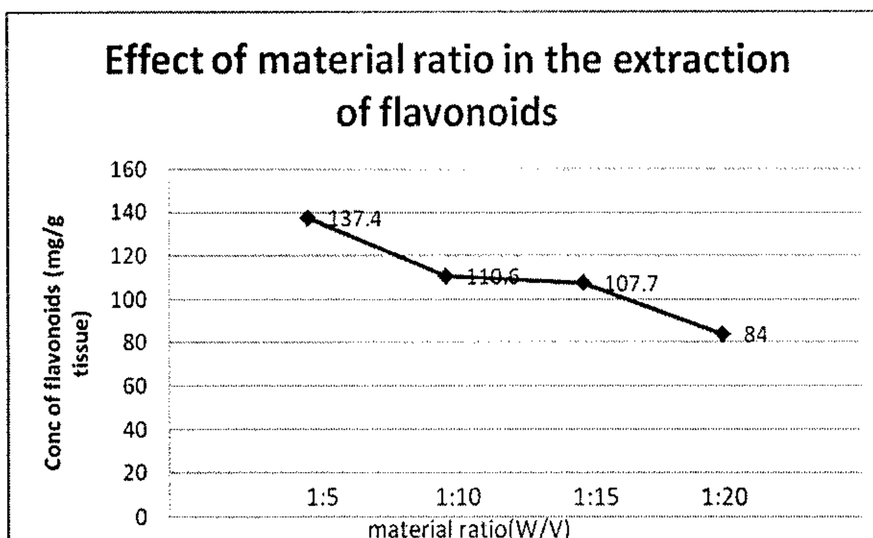

Fig 5.2.1(c) Effect of material ratio on the extraction of flavonoids from leaves using ethanol

5.2.1(d) Effect of extracting agent (ethanol)

The contents of flavonoid extract increases with the concentration of ethanol i.e., 65% to 95%. Figure 5.2.1(d) shows the contents of flavonoids extracted attained maxima at 85%.

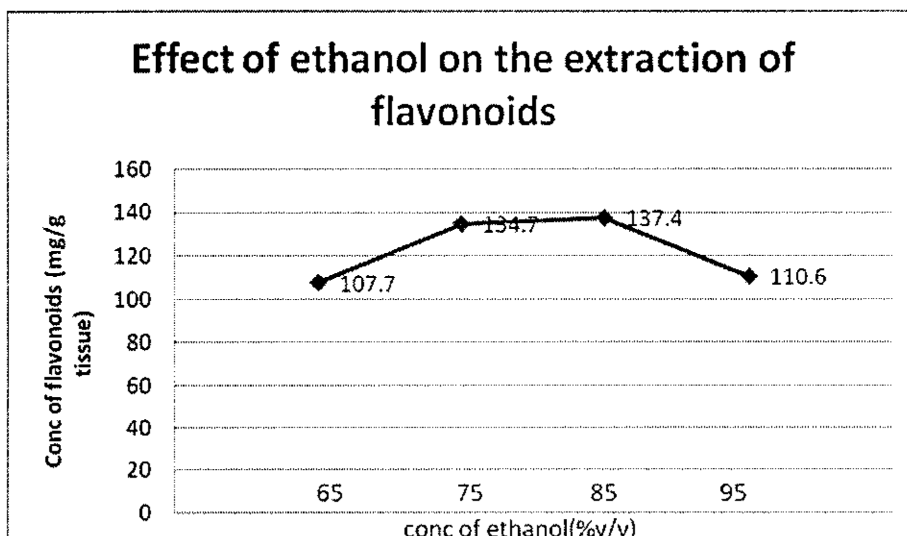

Fig 5.2.1(d) Effect of solvent ratio on the extraction of flavonoids from leaves using ethanol

5.2.1(e) Effect of number of extractions on flavonoids

The contents of flavonoid extract increases with the no. of extractions i.e., a gradual rise is noticed from 1 time to 4 times. Obviously, when the no. of extraction times increased the yield of the respective bioactive principle may be increased (Chen *et al.,* 2007). In this investigation, flavonoid content was increased by 4 times of the extraction.

5.2.2 EXTRACTION OF FLAVONOIDS FROM FLOWERS USING ETHANOL

5.2.2 (a) Effect of Temperature

The flavonoids were extracted in the temperature range from 55° C to 85° C . It was found that a higher temperature can decrease the fluid density that may reduce the extraction efficiency and at the same time it can accelerate the solvent flow and thus increase the flavonoid content (He Guo-qing *et* al., 2005) . Hence, the figure 5.2.2(a) shows the optimal extraction of flavonoids was obtained at 85° C.

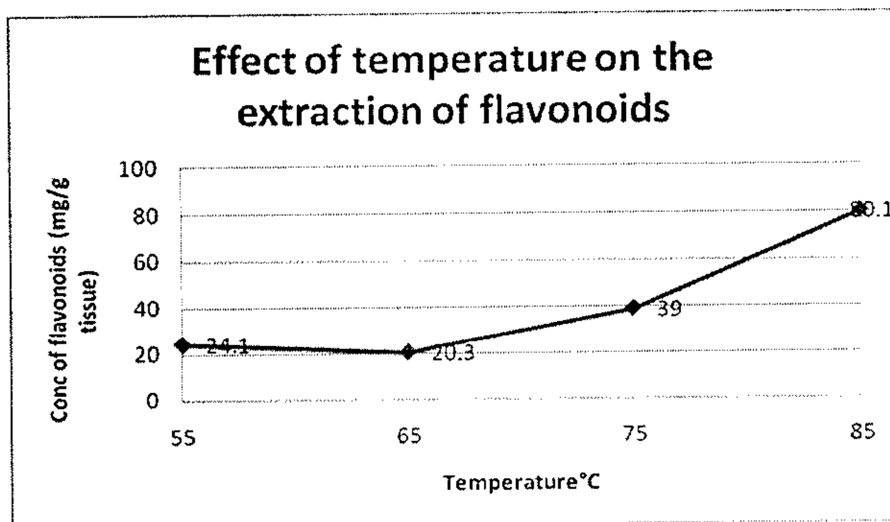

Fig 5.2.2(a) Effect of temperature on the extraction of flavonoids from flowers using ethanol

5.2.2(b) Effect of Extraction time

The flavonoids were extracted at different time intervals (1 to 4 hrs). The maximum yield was obtained at 3 hrs of extraction . A decrease in the flavonoid content was observed at 2hr and 4 hrs . Thus, the figure 5.2.2(b) showed the contents of flavonoids extracted for 3 hrs reached its maxima .

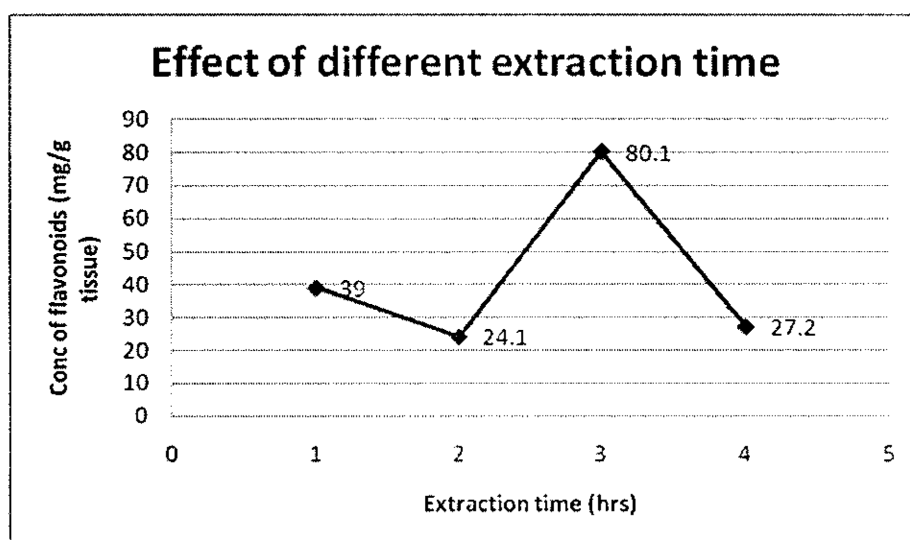

Fig 5.2.2 (b) Effect of extraction time on the extraction of flavonoids from flowers using ethanol

5.2.2(c) Effect of material ratio (W/V)

Figure 5.2.2(c) showed the contents of flavonoids extracted were maximum at a ratio of 1:20. A gradual increase in the flavonoids content was noticed when there is an increase in the material ratio.

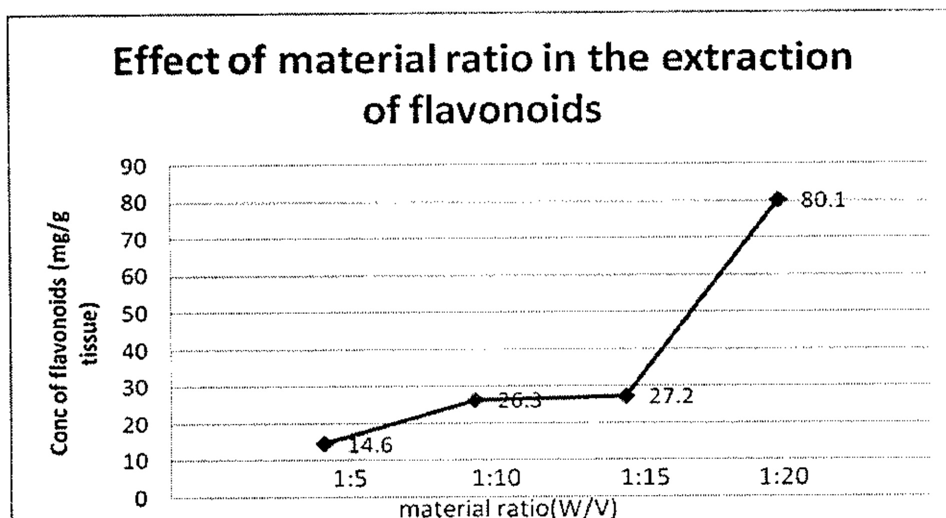

Fig 5.2.2 (c) Effect of material ratio on the extraction of flavonoids from flowers using ethanol

5.2.2(d) Effect of extracting agent (ethanol)

The contents of flavonoid extract increases with the concentration of ethanol i.e., 65% to 95%. Figure 5.2.2(d) shows the contents of flavonoids extracted attained maxima at 75%.

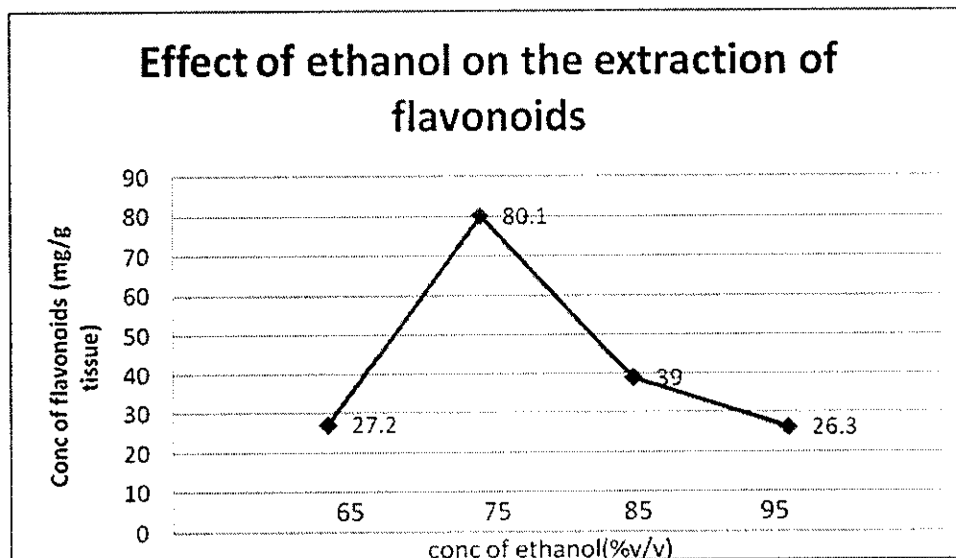

Fig 5.2.2(d) Effect of solvent ratio on the extraction of flavonoids from flowers using ethanol

5.2.2 (e) Effect of number of extractions on flavonoids

The contents of flavonoid extract increases with the no. of extractions i.e., a gradual rise is noticed from 1 time to 4 times. Obviously, when the no. of extraction times increased, the yield of the respective bioactive principle may be increased (Chen *et al.,* 2007). In this investigation, flavonoid content was increased by 4 times of the extraction.

5.3 OPTIMIZATION OF EXTRACTION OF FLAVONOIDS

5.3.1 Optimization of extraction of flavonoids from leaves (Ethyl acetate) using L_9 orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table 5.3.1(a) and Table 5.3.1(b). The order of the effect of factors on flavonoids extraction was A>C>D>B. The temperature had the greatest effect on the extraction procedure and it was found to be significantly different at 5% level. An equivalent effect was observed in the material ratio change, even though it was not proved to be significant difference at 5% level. The other factors such as material ratio, extraction duration and no. of extractions did not play a vital role in extracting the flavonoids to a higher yield. The optimum extraction conditions obtained from the statistical analysis were $A_3B_1C_3D_2$. It means that 80°C, 1hr extraction duration, a material ratio 1:20 and 2 times of extraction was the optimum conditions for flavonoids extraction.

Table 5.3.1(a) Experimental results and range analysis for leaves (Ethyl Acetate)

Expt.	A	B	C	D	OD values	Flav. (mg/g)
1	1	1	1	1	0.05	2.85
2	1	2	2	2	0.04	3.66
3	1	3	3	3	0.08	9.42
4	2	1	2	3	0.07	6.37
5	2	2	3	1	0.08	10.34
6	2	3	1	2	0.15	8.89
7	**3**	**1**	**3**	**2**	**0.18**	**20.52**
8	3	2	1	3	0.15	18.23
9	3	3	2	1	0.09	10.94
K1	5.30	9.91	9.99	8.04		
K2	8.53	10.74	6.99	11.02		
K3	16.56	9.75	13.42	11.33		
k1	1.76	3.30	3.33	2.68		
k2	2.84	3.58	2.33	3.67		
k3	5.52	3.25	4.47	3.77		
R	3.76	0.33	2.15	1.10		

Table 5.3.1 (b) One way ANOVA for leaves (Ethyl Acetate)

Levels	Sum of squares	Degrees of freedom	Mean square	F-values
A	**201.802**	2	**100.901**	**9.638**
B	1.862	2	0.931	0.026
C	62.423	2	31.211	1.118
D	19.955	2	9.977	0.300
		8		

5.3.2 Optimization of extraction of flavonoids from flowers (Ethyl acetate) using L_9 orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table 5.3.2(a) and Table 5.3.2(b). The order of the effect of factors on flavonoids extraction was A>B>D>C. The temperature had the greatest effect on the extraction procedure and it was found to be significantly different at 5% level. An equivalent effect was observed in the material ratio change, even though it was not proved to be significant difference at 5% level. The other factors such as material ratio, extraction duration and no. of extractions did not play a vital role in extracting the flavonoids to a higher yield. The optimum extraction conditions obtained from the statistical analysis were $A_3B_1C_3D_2$. It means that 80°C, 1hr extraction duration, a material ratio 1:20 and 2 times of extraction was the optimum conditions for flavonoids extraction.

Table 5.3.2(a) Experimental results and range analysis for flowers (Ethyl acetate)

Expt.	A	B	C	D	OD values	Flav. (mg/g)
1	1	1	1	1	0.17	9.94
2	1	2	2	2	0.08	7.06
3	1	3	3	3	0.01	2.09
4	2	1	2	3	0.05	4.70
5	2	2	3	1	0.05	6.27
6	2	3	1	2	0.10	6.16
7	**3**	**1**	**3**	**2**	**0.46**	**53.48**
8	3	2	1	3	0.34	40.62
9	3	3	2	1	0.26	31.0
K1	6.36	22.71	18.90	15.74		
K2	5.71	17.98	14.25	22.23		
K3	41.70	13.08	20.62	15.80		
k1	2.12	7.57	6.30	5.24		
k2	1.90	5.99	4.75	7.41		
k3	13.90	4.36	6.87	5.26		
R	11.99	3.208	2.12	2.165		

Table 5.3.2 (b) One way ANOVA for flowers (Ethyl acetate)

Levels	Sum of squares	Degrees of freedom	Mean square	F-values
A	**2544.59**	**2**	**1272.29**	**35.41**
B	138.99	2	69.493	0.437
C	65.244	2	32.622	0.094
D	83.534	2	41.767	0.121
		8		

5.3.3. Optimization of extraction of flavonoids from leaves (Ethanol) using L_{16} orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table 5.3.3(a) and Table 5.3.3(b). The order of the effect of factors on flavonoids extraction was A>D>E>B>C. The temperature had the greatest effect on the extraction procedure and it was found to be significantly different at 5% level. An equivalent effect was observed in the material ratio change, even though it was not proved to be significant difference at 5% level. The other factors such as solvent (%), extraction duration and no. of extractions did not play a vital role in extracting the flavonoids to a higher yield. The optimum extraction conditions obtained from the statistical analysis were $A_4B_2C_3D_1E_4$. It means that 85°C, 2hrs extraction duration, a material ratio 1:5, 75% ethanol concentration and 4 times of extraction was the optimum conditions for flavonoids extraction.

Table 5.3.3(a) Experimental results and range analysis for leaves (Ethanol)

Expt.	**A**	**B**	**C**	**D**	**E**	**Flav. (mg/g)**
1	1	1	2	3	4	71.5
2	1	2	1	4	3	32.6
3	1	3	4	1	2	39.4
4	1	4	3	2	1	69.2
5	2	1	1	1	1	107.2
6	2	2	2	2	2	51.2
7	2	3	3	3	3	51.3
8	2	4	4	4	4	43.7
9	3	1	3	4	2	52.9
10	3	2	4	3	1	100.5
11	3	3	1	2	4	91.1
12	3	4	2	1	3	134.7
13	4	1	4	2	3	110.6
14	**4**	**2**	**3**	**1**	**4**	**137.4**
15	4	3	2	4	1	84.0
16	4	4	1	3	2	107.7
K1	53.2	64.8	84.6	104.7	90.2	
K2	63.3	80.4	85.3	80.5	62.8	
K3	94.8	66.4	77.7	82.7	82.3	
K4	109.9	88.8	73.5	53.3	85.9	
k1	13.3	16.2	21.2	26.2	22.6	
k2	15.8	20.1	21.3	20.1	15.7	
k3	23.7	16.6	19.4	20.7	20.6	

k4	27.5	22.2	18.4	13.3	21.5	
R	14.2	6	2.8	12.9	6.9	

Table 5.3.3 (b) One way ANOVA for leaves (Ethanol)

LEVELS	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F-VALUE
A	**8443.84**	**3**	**2814.6**	**4.89**
B	1168.31	3	389.44	0.37
C	386.99	3	128.99	0.12
D	5316.78	3	1772.26	2.26
E	1761.59	3	587.2	0.58
		15		

5.3.4 Optimization of extraction of flavonoids from flowers (Ethanol) using L_{16} orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table 5.3.4(a) and Table 5.3.4(b). The order of the effect of factors on flavonoids extraction was A>D>E>B>C. The temperature had the greatest effect on the extraction procedure and it was found to be significantly different at 5% level. An equivalent effect was observed in the material ratio change, even though it was not proved to be significant difference at 5% level. The other factors such as solvent (%), extraction duration and no. of extractions did not play a vital role in extracting the flavonoids to a higher yield. The optimum extraction conditions obtained

from the statistical analysis were $A_4B_3C_2D_4E_1$. It means that 85°C, 3 hrs extraction duration, a material ratio 1:20, 75% ethanol concentration and 1 time of extraction were the optimum conditions for flavonoids extraction.

Table 5.3.4(a) Experimental results and range analysis for flowers (Ethanol)

Expt.	A	B	C	D	E	Flav. (mg/g)
1	1	1	2	3	4	21.8
2	1	2	1	4	3	24.1
3	1	3	4	1	2	10.4
4	1	4	3	2	1	19.2
5	2	1	1	1	1	10.7
6	2	2	2	2	2	17.1
7	2	3	3	3	3	18.9
8	2	4	4	4	4	20.3
9	3	1	3	4	2	39
10	3	2	4	3	1	16.2
11	3	3	1	2	4	7.0
12	3	4	2	1	3	14.6
13	4	1	4	2	3	26.3
14	4	2	3	1	4	10.5
15	**4**	**3**	**2**	**4**	**1**	**80.1**
16	4	4	1	3	2	27.2
K1	75.5	97.8	69.0	46.2	126.2	
K2	67.0	67.9	133.6	69.6	93.7	

K3	76.8	116.4	87.6	84.1	83.9	
K4	144.1	81.3	73.2	163.5	59.6	
k1	18.8	24.45	17.25	11.55	31.55	
k2	16.75	16.98	33.4	17.4	23.42	
k3	19.2	29.1	21.9	21.02	20.97	
k4	36.02	20.32	18.3	40.87	14.9	
R	19.28	12.12	16.15	29.32	16.65	

Table 5.3.4 (b) One way ANOVA for flowers (Ethanol)

LEVELS	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARE	F-VALUE
A	**959.35**	**3**	**319.78**	**1.370**
B	239.75	3	97.91	0.348
C	657.76	3	219.25	0.865
D	1942.19	3	647.39	3.85
E	570.65	3	190.21	0.733
		15		

5.4 IDENTIFICATION AND ISOLATION OF FLAVONOIDS FROM OPTIMAL EXTRACT USING THIN LAYER CHROMATOGRAPHY (TLC) AND PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

5.4.1 TLC and PTLC results for leaves

The results of fig 5.4.1(a) TLC and fig 5.4.1(b) PTLC of leaves revealed the presence of flavonoid glycosides, flavonols and leukoanthocyanins in the optimized extracts.

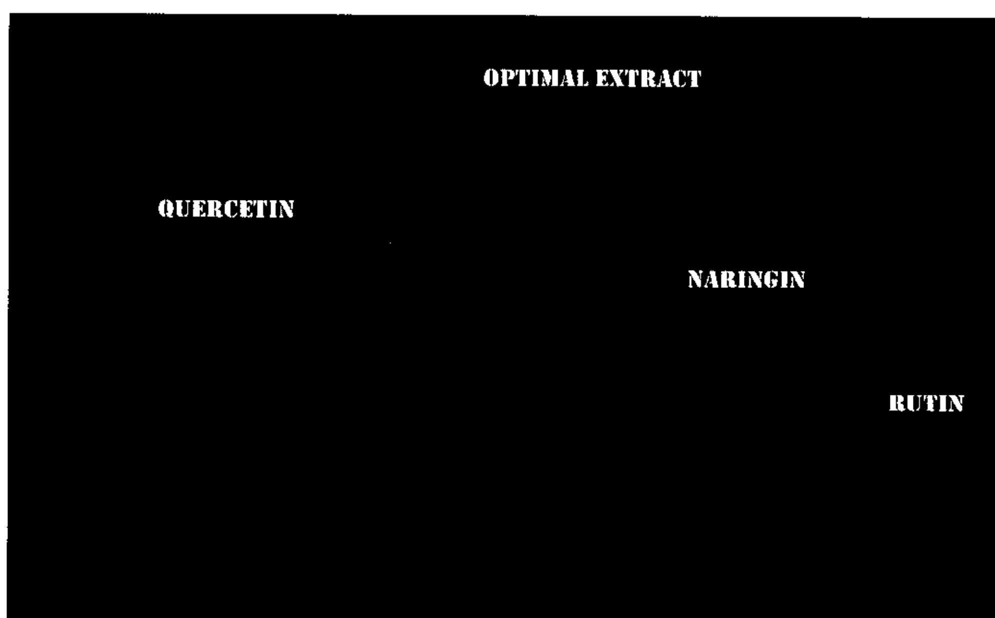

Fig 5.4.1(a) TLC for leaves in long UV light

Fig 5.4.1(b) PTLC of leaves in long UV light

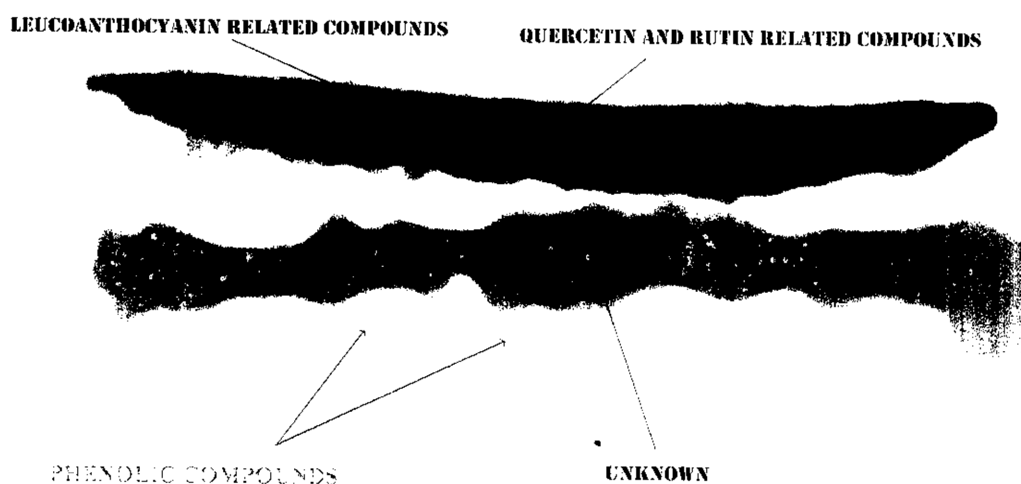

Fig 5.4.1(c) Simulation of PTLC leaves in long uv light

5.4.2 TLC and PTLC results for flowers

The results of fig 5.4.2(a) TLC and fig 5.4.2(b) PTLC of flowers

revealed the presence of flavonoid glycosides, flavonols and leukoanthocyanins in the optimized extracts.

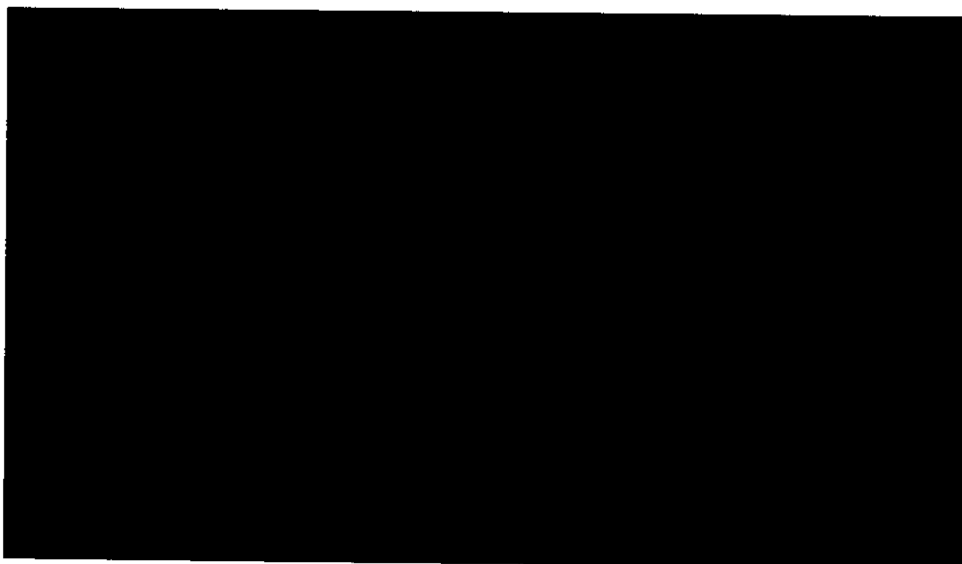

Fig 5.4.2(a) TLC of flowers in long uv light

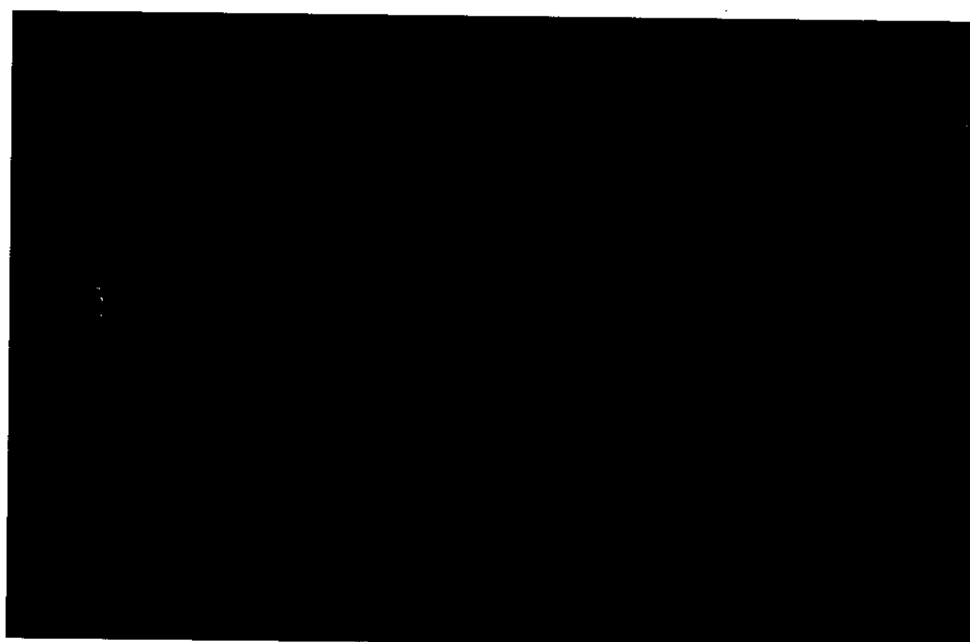

Fig 5.4.2(b) PTLC of flowers in long uv light

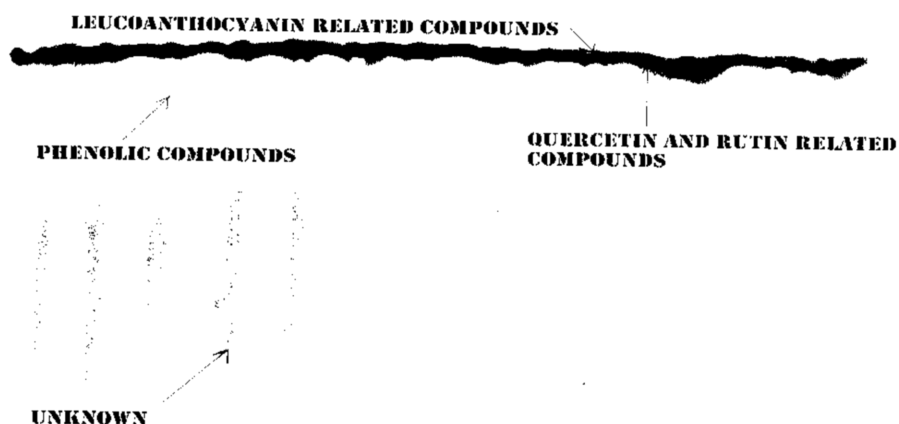

Fig 5.4.2(c) Simulation of PTLC flowers in long uv light

5.5. Partial purification

5.5.1 Partial purification of leaves

In the single step purification, four compounds were isolated from the optimised ethanol extract. The yield of these four fractions was calculated (Table 5.5.1(a)).

Table 5.5.1(a) Pooled fractions from different eluents of leaves (Silica gel column chromatography)

Fractions	Content (mg/g)
1st	113.9
2nd	24.4
3rd	23.06
4th	6.97

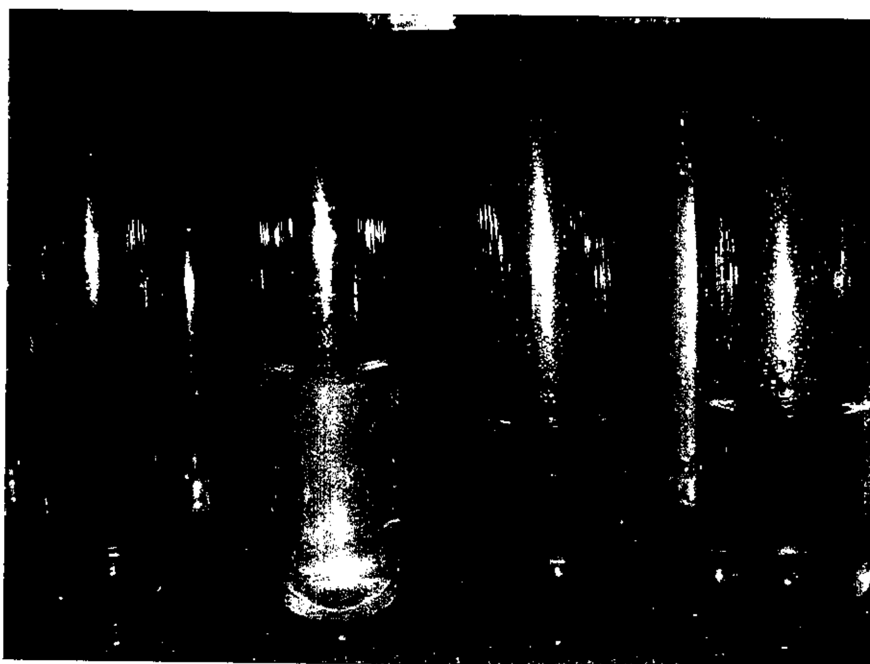

Fig 5.5.1(a) Pooled fractions of different eluents of leaves(Silica gel column chromatography)

Quercetin std	Rutin std	Chloroform(C)	5%Methanol/ Chloroform	10%Methanol/ Chloroform	20%Methanol/ Chloroform

Fig 5.5.1(b)Simulated TLC fractions of different eluents (Leaves)

5.5.2 Partial purification of flowers

In the single step purification, four compounds were isolated from the optimised ethanol extract. The yield of these four fractions was calculated (Table 5.5.2(a)).

Table 5.5.2(a) Pooled fractions from different eluents of flowers (Silica gel column chromatography)

Fractions	Content (mg/g)
1st	48.83
2nd	12.79
3rd	9.3
4th	4.6

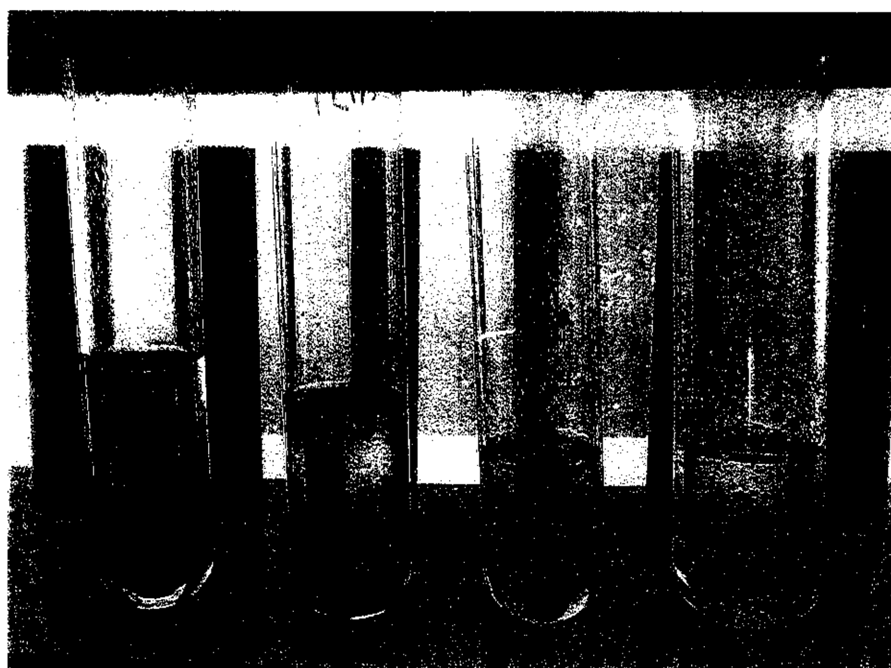

Fig 5.5.2(a) Pooled fractions of different eluents from flowers (Silica gel column chromatography)

Quercetin std	Rutin std	Chloroform(C)	5%Methanol/ Chloroform	10%Methanol/ Chloroform	20%Methanol/ Chloroform

Fig 5.5.2(b) Simulated TLC fractions of different eluents (Flowers)

5.6. Antioxidant Assay

5.6.1 DPPH assay for leaves

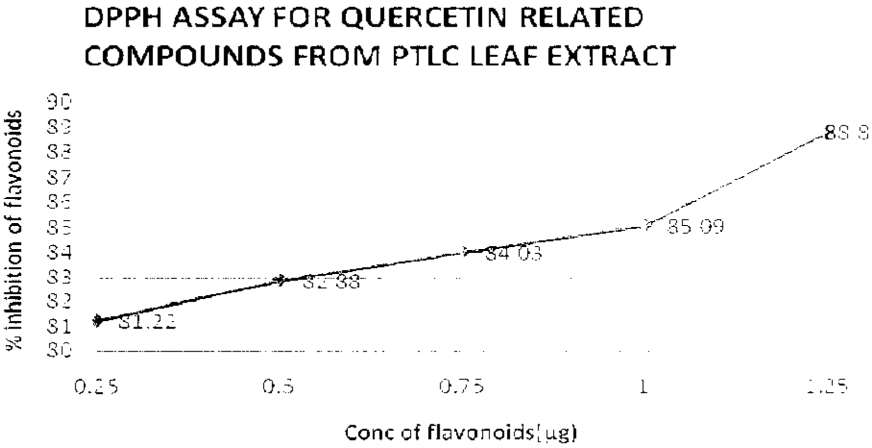

Fig 5.6.1 (a) DPPH assay for Quercetin related compounds from PTLC leaf extract

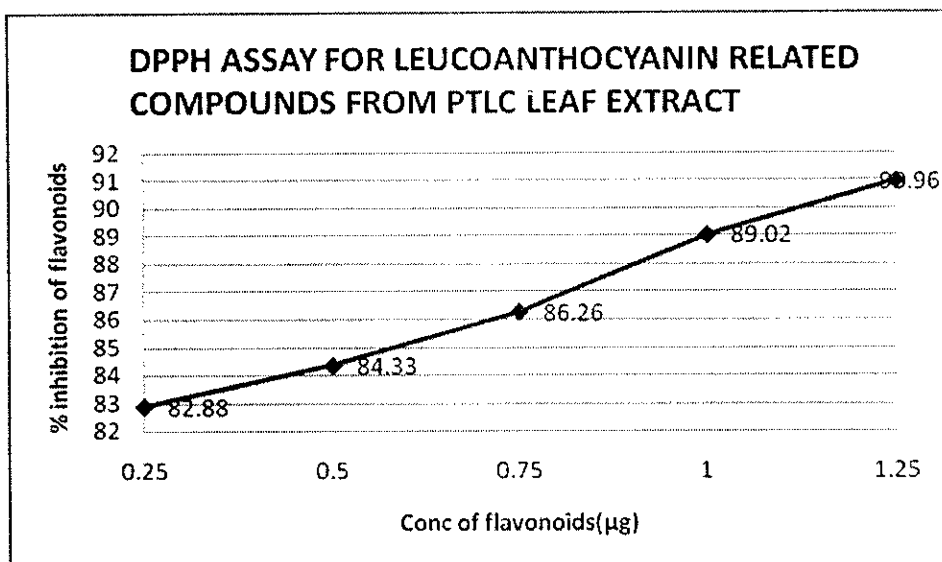

Fig 5.6.1(b) DPPH assay for Leucoanthocyanin related compounds from PTLC leaf extract

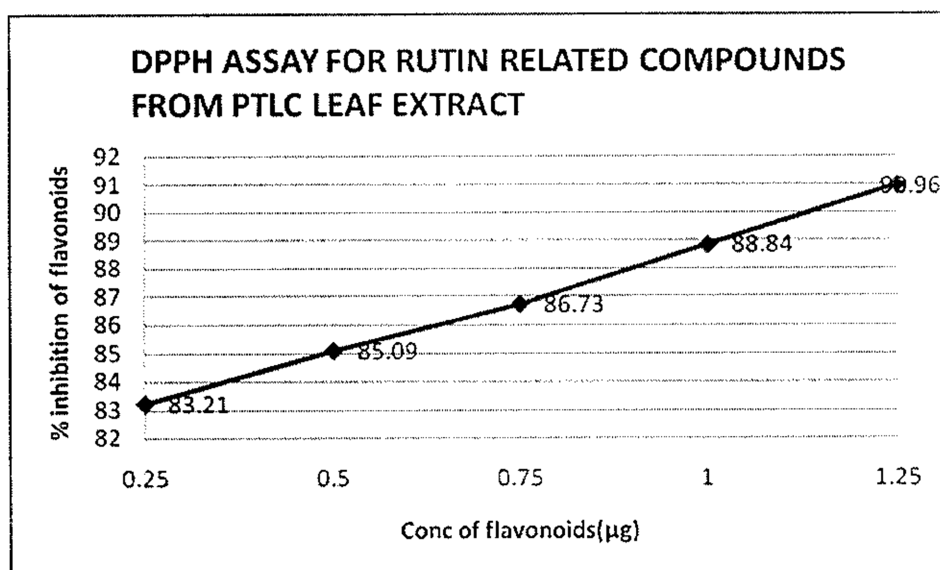

Fig 5.6.1(c) DPPH assay for Rutin related compounds from PTLC leaf extract

Table 5.6.1 Total percentage inhibition of flavonoids from the PTLC leaf extract

PTLC leaf extract	Total % inhibition
Quercetin	84.4
Leucoanthocyanin	86.69
Rutin	86.96

5.6.2 DPPH assay for flowers

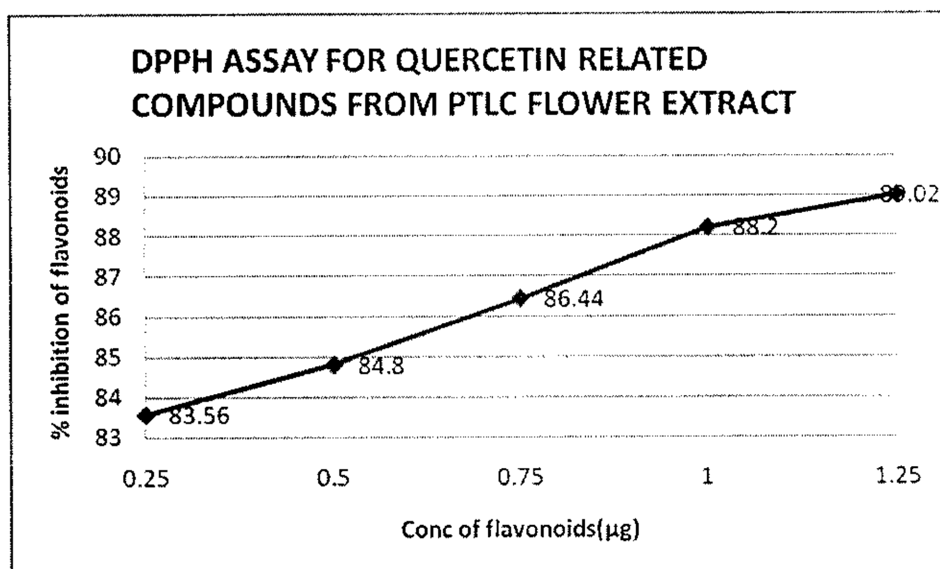

Fig 5.6.2(a) DPPH assay for Quercetin related compounds from PTLC flower extract

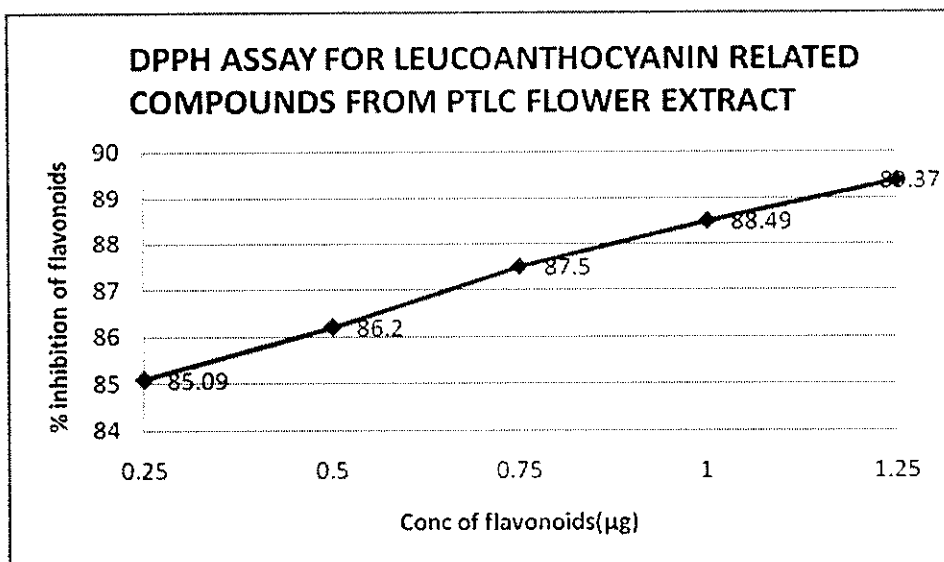

Fig 5.6.2(b) DPPH assay for Leucoanthocyanin related compounds from PTLC flower extract

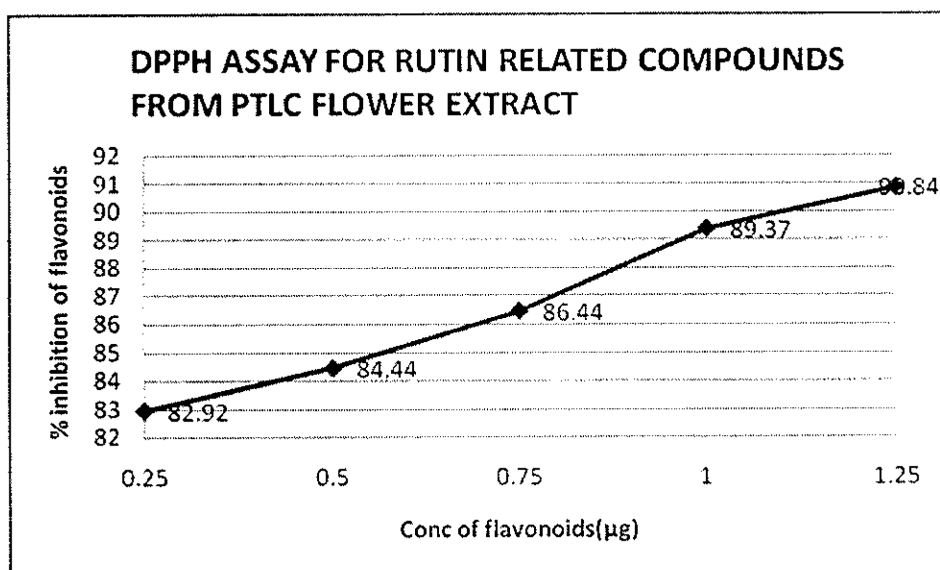

Fig 5.6.2(c) DPPH assay for Rutin related compounds from PTLC flower extract

Table 5.6.2 Total percentage inhibition of flavonoids from the PTLC flowers extract

PTLC flower extract	Total % inhibition
Quercetin	86.4
Leucoanthocyanin	87.33
Rutin	86.8

5.7 Reverse Phase-High Performance Liquid Chromatography

5.7.1 RP-HPLC standards

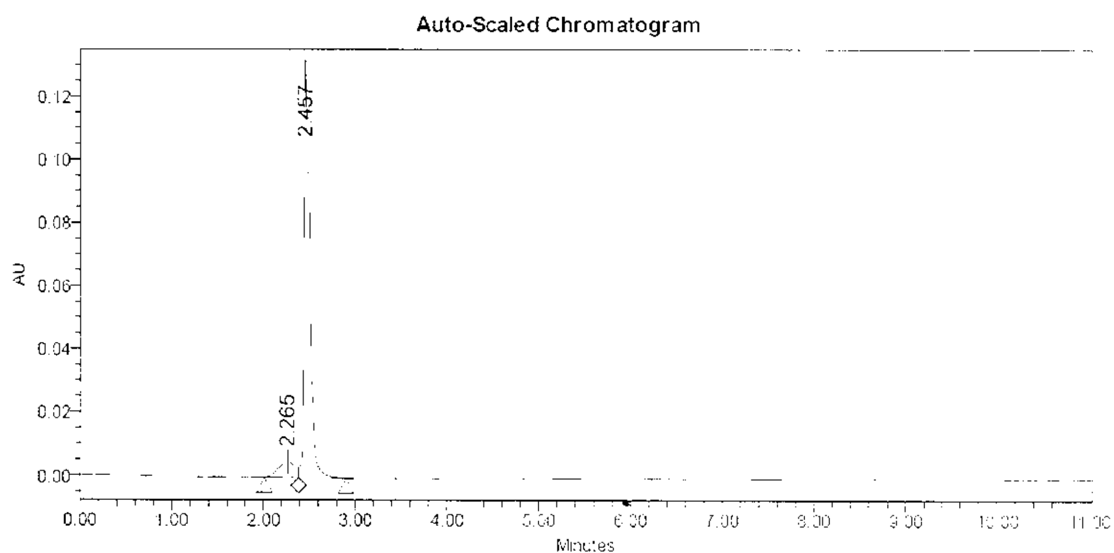

Fig 5.7.1 (a) RP-HPLC analysis of standard Quercetin

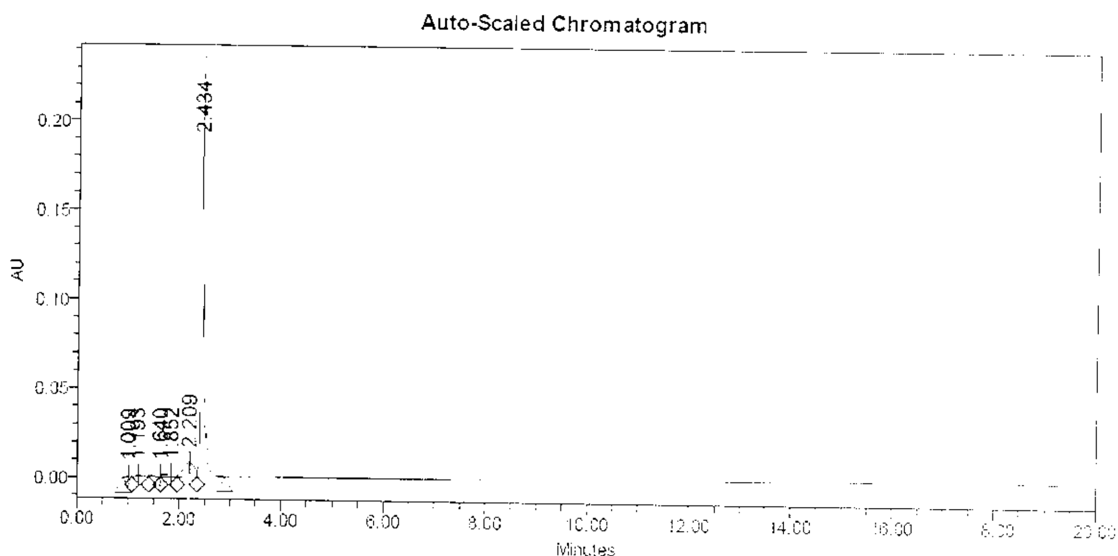

Fig 5.7.1 (b) RP-HPLC analysis of standard Rutin

5.7.2 RP-HPLC analysis for leaves

Figure 5.7.2 (a), fig 5.7.1 (b) and fig 5.7.2 (c) shows typical HPLC chromatograms obtained by analysis of the PTLC fragments. Single peaks were observed at *R*t = 2.417 min for leucoanthocyanins, Rt = 2.418 min for quercetin and rutin related compounds and Rt = 2.435 min for the unknown compound.

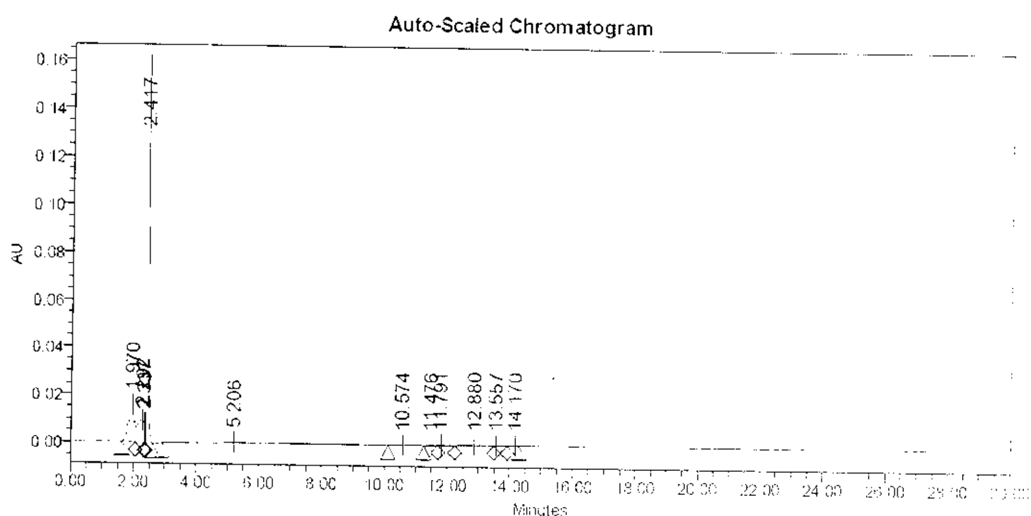

Fig 5.7.2 (a) RP-HPLC analysis of isolated compounds from PTLC leaves (Leucoanthocyanins)

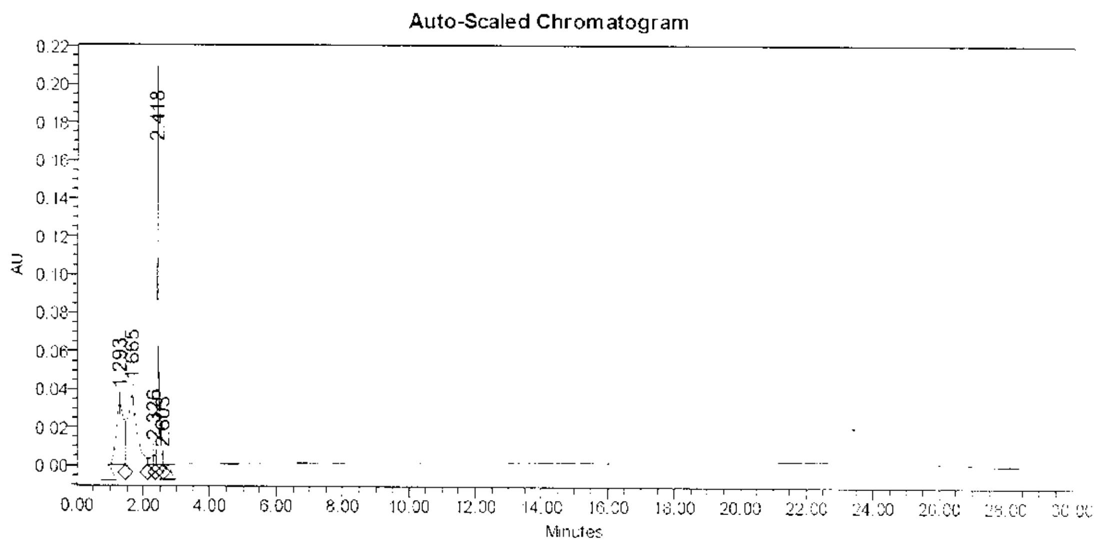

Fig 5.7.2 (b) RP-HPLC analysis of isolated compounds from PTLC leaves (quercetin related and rutin related compounds)

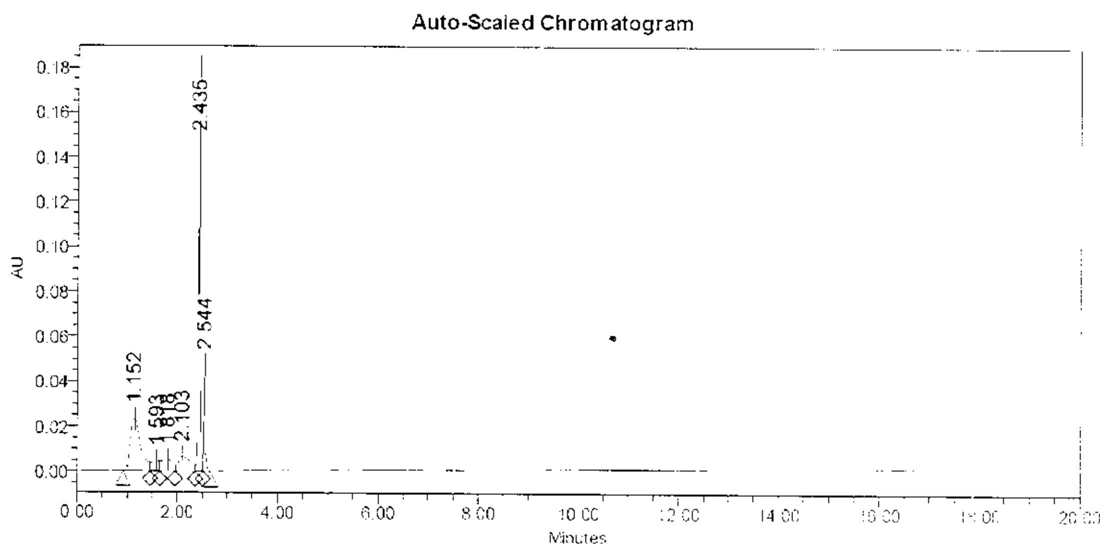

Fig 5.7.2 (c) RP-HPLC analysis of isolated compounds from PTLC leaves (unknown compound)

5.7.3 RP-HPLC analysis for flowers:

Figure 5.7.3 (a), fig 5.7.3 (b) and fig 5.7.3 (c) shows typical HPLC chromatograms obtained by analysis of the PTLC fragments. Single peaks were observed at *R*t = 2.430 min for leucoanthocyanins, Rt = 2.418 min for quercetin and rutin related compounds and Rt = 2.435 min for the unknown compound.

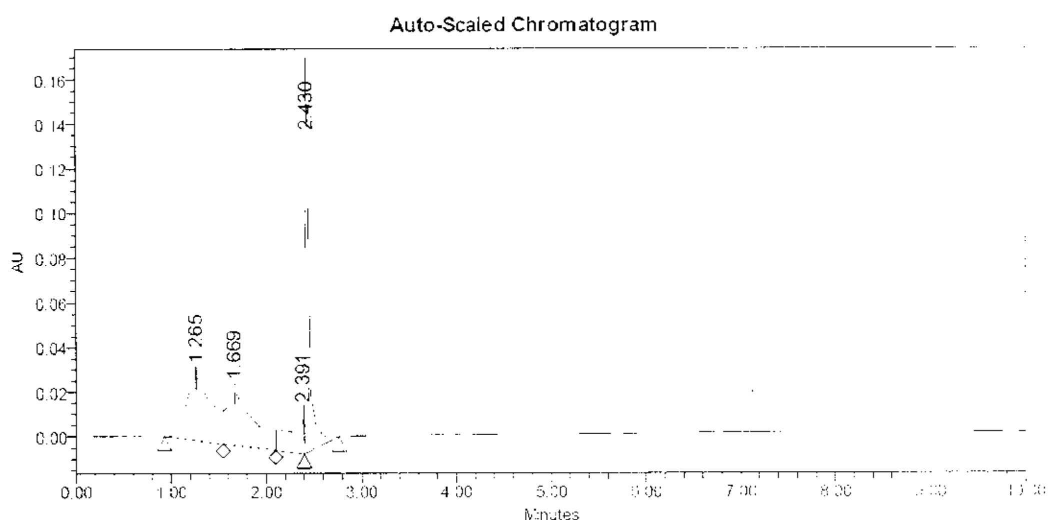

Fig 5.7.3(a) RP-HPLC analysis of isolated compounds from PTLC flowers (Leucoanthocyanins)

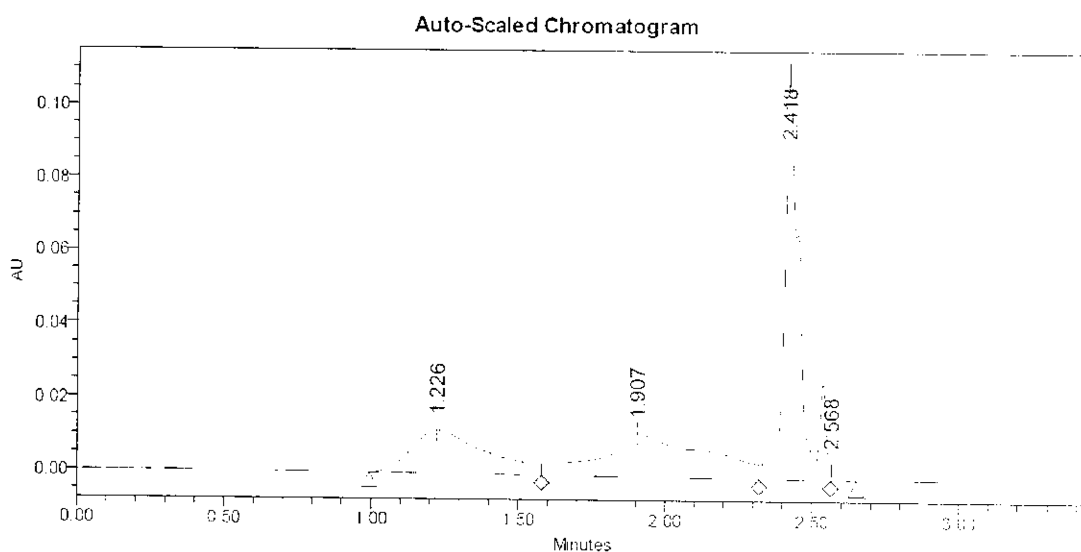

Fig5.7.3 (b) RP-HPLC analysis of isolated compounds from PTLC flowers (quercetin related and rutin related compounds)

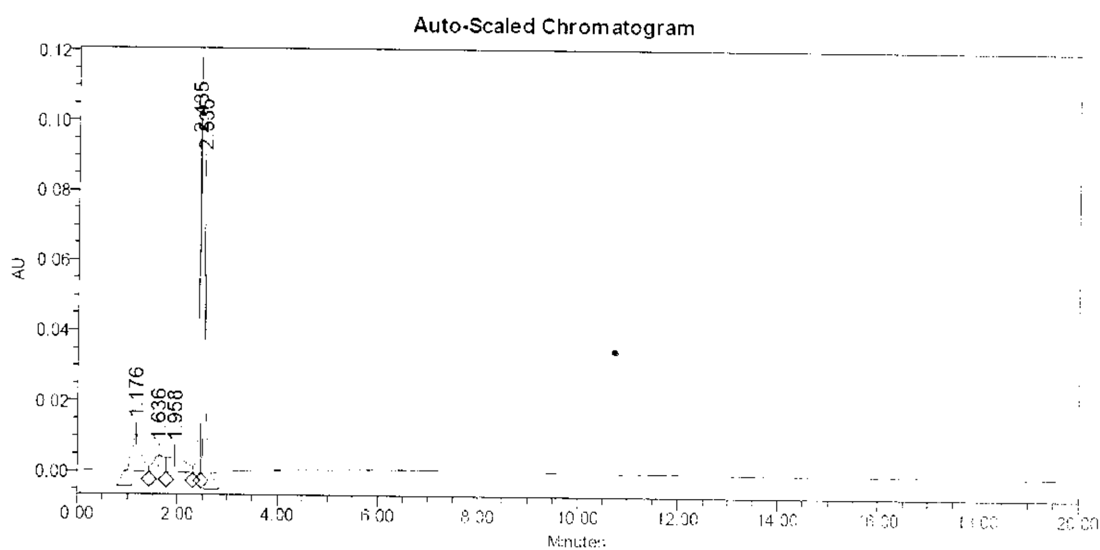

Fig 5.7.3 (c) RP-HPLC analysis of isolated compounds from PTLC flowers (unknown compound)

CONCLUSION

6. CONCLUSION

The study has been focused on the antioxidant activities of *T.heynaena Wall.* in the fresh leaves and flowers by using two different solvents i.e., ethanol and ethyl acetate . Ethanolic extracts proved to have the better yield when compared with the ethyl acetate extracts.

Then the Orthogonal design of experiment shows a significant increase in the flavonoid concentration (~ 31.5 fold increase). The orthogonal design of experiment from leaves proved that at temperature 85°C, 1:5 (sol:liq), 85% solvent and extraction time duration of 2 hours were the optimal conditions to extract the flavonoids . While, flowers were optimised at temperature 85°C, 1:20 (sol:liq), 75% solvent and extraction time duration of 3 hours .

Level A (Temperature,°C) was found to have a significant difference at 5% level showing that it is a strong factor that greatly affects the flavonoid extraction from leaves. The following order may be responsible for an optimized flavonoids extraction: A>D>E>B>C. Level D (solid:liquid ratio , w/v) was found to be the major factor to affect the flavonoids extraction from flowers . The following order may be responsible for an optimized flavonoid extraction: D> A> E> C> B.

The flavonoids from both leaves and flowers were detected using Thin Layer Chromatography (TLC) and were isolated by performing Preparative Thin layer Chromatography (PTLC) which showed three different spots(Leucoanthocyanin related , Quercetin and Rutin related) visualized under long UV light of 365 nm .

The HPLC of the isolated fractions from the PTLC was compared with the standards – Quercetin and Rutin. It was found that Rutin , Quercetin

and Leucoanthocyanin related compounds were present. Quercetin has a strong anti-cancer property, while Rutin has nephroprotective and thromboprotective property.

The isolated fragments from the leaves and flowers were purified by using Silica gel Column Chromatography and investigated for antioxidant property. It was observed that the isolated flavonoids possessed antioxidant property.

APPENDICES

APPENDIX 1

ESTIMATION OF TOTAL FLAVONOIDS

a) 5% Sodium nitrite

Dissolve 0.5g of sodium nitrite in 10ml distilled water.

b) 10% Aluminium chloride

Dissolve 1g of $AlCl_3.6H_2O$ in 10ml distilled water.

C) 1M Sodium hydroxide

Dissolve 0.4g of NaOH in 10ml distilled water.

APPENDIX 2

DPPH ASSAY

Preparation of DPPH solution

Take 4 mg of DPPH and make up to 100 ml with methanol. Keep in the dark for 30 minutes.

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