EXTRACTION OPTIMIZATION, ISOLATION AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF FLAVONOIDS FROM THE LEAVES OF *Tabernaemontana heyneana Wall.*

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

Certified that this project report " **EXTRACTION OPTIMIZATION, ISOLATION AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF FLAVONOIDS FROM THE LEAVES OF *Tabernaemontana heyneana Wall.*** " is the bonafide work of **MANIKANDAN.V** who carried out the project work under my supervision.

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[Manikandan.V]

ABSTRACT

Abstract

Tabernaemontana heyneana wall. belonging to family Apocynaceae, is widely distributed throughout India. In folk medicine it is used to treat several diseases like skin infections, respiratory problems, venereal diseases, nerve complaints, to induce abortion and to cure many other ailments. In this study, antioxidant activities were studied in the fresh and mature leaves by using three different solvents i.e., methanol, ethanol and acetone. The results in triplicates proved that the methanolic extract possess a higher antioxidant power than the other two extracts in both the fresh and matured leaves. Reducing power assay proved a high antioxidant activity in this plant, which is supported by phosphomolybdnenum assay. Concentrations at 100-1000µg/ml showed a high (70.6% to 86.03%) ferrous ion chelation activity in the fresh leaves and (39.92% to 59.21%) in the matured leaves of plant extracts. The hydroxyl radical and $ABTS^{\cdot+}$ assays proved a significant radical scavenging activities (25.46 % to 57.23 % and 42.95 % to 78.53 %) respectively at the same concentrations which confirmed the presence of potent antioxidants and their substantial activities in the plant. The studies also revealed that the fresh leaves had potent antioxidant capacity than the matured leaves. The orthogonal design of experiment proved that at temperature 65°C, 1:5 (sol:liq), 65% solvent and extraction time duration of 1 hour were the optimal conditions to extract the flavonoids from the leaves. Isolated flavonoids by PTLC method showed 5 different bands (Cyanidin related, luteolin related, ellagic acid related, polyphenols and quercetin related compounds) visualized at UV light. The isolated flavonoids possessed approximately 60 – 70% of the total antioxidant activities in the plants.

Key words: Flavonoids, Antioxidants, Free radical scavenging, PTLC.

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LIST OF ABBREVIATION

UV	-	Ultra Violet
ROS	-	Reactive oxygen species
SOD	-	Super oxide dismutase
GPx	-	Glutathione peroxidase
GR	-	Glutathione\ Reductase
HPLC	-	High Performance Liquid Chromatography
TLC	-	Thin Layer Chromatography
PTLC	-	Preparative thin layer chromatography
BSI	-	Botanical Survey of India
FRAP Assay	-	Ferric Ion Reducing/ Antioxidant Power Assay
TCA	-	Tri Chloroacetic Acid
CUPRAC	-	Cupric Ion Reducing Antioxidant Capacity
EDTA	-	Ethidium Diamine Tetra Acetic acid
TBA	-	Thio Barbituric Acid
ABTS	-	Azobis 3- ethyl Benzo Thiazoline-6- Sulfonic acid
TAA	-	Total Antioxidant Activity

INTRODUCTION

1.INTRODUCTION

Humans have gathered food and medical herbs ever since their arrival on earth and were guided then by instinct, followed by experience, and also by rational thought (Abdolbaset Ghorbani, 2005). For millions of years, mankind has fared quite well using this approach, but after the development of science and technology, people felt that the current state of affairs was quite satisfactory and, hence, they failed to support research and education adequately. Therefore, it is time to examine more closely what we are eating, how diseases can be treated more efficiently, and how we can effectively conserve our natural resources(Lev and Amar, 2000). One of our natural resources is the plants in remote forests, some of which may contain compounds of potential medical use.

Basically ,medicinal plants produce two types of constituents viz., primary metabolites (proteins, carbohydrates, nucleic acids etc.,) and secondary metabolites or phytochemicals (alkaloids, flavonoids, polyphenols, tannins, quinines etc.,).These phytochemicals possess antimicrobial and antioxidant activities. They are non-nutrient compounds found in plant-derived foods that have biological activity in the body. They contribute to food taste, aromas, colors and other characteristics(Cook and Samman , 1996). They act as antioxidants, anticancer, antimicrobial agents, mimic like hormones etc.,

One such compound is flavonoids which appears to play a major role in the successful medical treatments of ancient times, and their use has persevered up to now. Flavonoids are a group of polyphenolic compounds possessing low molecular weight that exhibit a common benzo-γ-pyrone structure. (Havsteen, 2002).They are categorized into various subclasses

including flavones, flavonols, flavanones, isoflavanones, flavans, isoflavanoids, anthocyanidins, and catechins.

The average human diet contains a considerable amount of flavonoids and the major dietary sources are fruits (i.e., orange, grapefruit, apple, and strawberry), vegetables (i.e. onion, broccoli, green pepper and tomato), soybeans and different herbs . Among the classes of flavonoids, flavanones have been defined as citrus flavonoids due to their almost unique presence in citrus fruits.

They act as co-pigments. They protect plant from UV damaging effects.They posses pharmacological effects as anti inflammatory, anti infective,antioxidant,antiulcerogic.Example: Quercetin is used for its antidiarrhoel activity(Jorgensen *et al.*, 1998).

ANTIOXIDANTS

Antioxidants are molecules that inhibit or prevent the oxidation of a substrate that evolved to protect biological systems against damage induced by ROS. A sophisticated, co- operative array of antioxidant defense mechanisms, the antioxidant network, is found in biological systems (Torel *et al.,* 1986). The network works in concert in order to avoid oxidative stress through different mechanisms RONS scavenging, reduction of peroxides and metal chelating activity (Vivekananthan *et al*, 2003).

There are two types of antioxidants viz., enzymatic and non-enzymatic. The major non-enzymatic antioxidants are vitamins C, E, carotenoids, and phenolic compounds, especially of flavonoids that includes isoflavones, flavonols, anthocyanins, glycosides etc. These antioxidants can be derived mainly from the plant sources in the form of vegetables, fruits, nuts and cereals. Similarly, enzymatic antioxidants like catalase, peroxidases, Superoxidedismutase(SOD),Glutathioneperoxidase(GPx),and Glutathione\

Reductase (GR) also plays a pivotal role in the removal of free radicals. It has been observed that these enzymes are widely distributed in various organisms.

Even though medicinal plants have innumerable properties, the antimicrobial and antioxidant properties are considered to be significant because of their preservative effects conferred to the tissues.(Bjelakovic *et al.*, 2004).

Recently interest has grown considerably to exploit naturally occurring low molecular antioxidants especially of flavonoids,(Kalemba and Kunicka, (2003)) Which creates some beneficial effects in the food and pharmaceutical industries.[as an alternative for synthetic antioxidants]

Thus, the natural products research is an important part of the drug discovery process and the main advantage of these products are their tremendous molecular diversity with various functions.(Mauro Serafini, 2001)

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. *Tabernaemontana*

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.

2.1.1.*Tabernaemontana orientalis*

T. orientalis (or *Ervatamia orientalis*) is a fairly widespread and possibly very variable shrub, recorded from Papua New Guinea, Melanesia and Indonesia. Habitats include monsoon forest thickets, stabilised sand dunes or sandstone country by streams and vine-thickets on rock outcrops. Appears as a regeneration species after logging operations and along the edges of rainforest and other areas where it can receive full sun. The well known Frangipani (plumeria) and Oleander (nerium) are members of the same family, the flowers of this species also being very fragrant and this shrub may be quite suitable to grow as an ornamental in more tropical gardens. (Pietta, 2000) May be amenable to pruning, and propagation from cuttings as well as fresh seeds. Latex reportedly used by aborigenes to cure sores and ulcers, may have some healing properties, hence the common name Iodine bush.

2.1.2.*Tabernaemontana pubescens*

T. pubescens is sometimes considered a more or less hairy (short fine hairs) type of *T. orientalis* recorded from NT, nth WA and Qld, it's habitat is very similar to that of *T. orientalis*. Habitats include monsoon forest thickets, stabilised sand dunes or sandstone country by streams and vine-thickets on rock outcrops. (Brouillard and Cheminat, 1988) Appears as a regeneration species after logging operations and along the edges of rainforest and other areas where it can receive full sun. Latex also used as for other species and *T. pubescens* reported to contain alkaloids.

2.1.3.*Tabernaemontana divaricata*

Crepe jasmine is a beautifully shaped evergreen shrub which forms symmetrical 6 ft (2 m) high mounds of glossy foliage. The many branches tend to grow almost parallel to the ground giving the shrub an attractive horizontal aspect (the species name, *divaricata*, means "at an obtuse angle"). Like many members of the Apocynaceae family, the stems of crepe jasmine exude a milky latex when broken. The large shiny leaves are deep green and are 6 or more inches (15 cm) in length and about 2 in (5 cm) in width. Crepe jasmine blooms in spring but flowers may appear sporadically all year. The waxy blossoms are white five-pedaled pinwheels that are borne in small clusters on the stem tips. (Brouillard and Cheminat 1988) .A frequently encountered cultivar is 'Flore Pleno', which has double flowers. The reference Hortus Third also lists another cultivar 'Grand folia' with double pedaled flowers and larger leaves. *Tabermontana divaricata* is native to tropical areas of India and is widely grown for its ornamental value in frost free areas around the world.

2.1.4.*Tabernaemontana coronaria*

Coronaria is a spreading, bushy, many-branched shrub with elliptic-oblong, wavy-margined, thin, glossy, mid- to dark green leaves, paler underneath. Produces cymes of 4 to 6 salver form waxy, pure white flowers. Scented at dusk and after dark. In general, this spreading, bushy shrub grows to a height of 6 to 10 feet tall and 5 to 8 feet wide. It bears white, waxy summer flowers and has oblong leaves with wavy margins that are dark green above and pale green beneath. Native to parts of India, China, and Thailand, this plant thrives where temperatures are above 50 degrees F. Soil should be moist, fertile and sun full. (Brown and Rice-Evans 1998). If grown indoors, water sparingly during the winter, but moderately during the growing season. Fertilize monthly during the growing season with a balanced fertilizer.

2.1.5.*Tabernaemontana arborea*

A moderately-sized forest tree, reaching the canopy but not more than about 70 cm in trunk diameter. The trunk is straight, light-colored with greenish spots, and fluted, slightly flared at the base, usually unbranched for most of its length (Calman 1972). Leaves are opposite, shiny, fairly thick to the feel, and drip copious white latex when broken (as does the bark). One of each pair of leaves is nearly always notably larger than the other.

2.1.6.Tabernaemontana pandacaqui

In parts of Australia 'banana bush' was seemingly recognized in the nursery trade as E. angustisepala. This type is identified as naturally occurring in Qld and NE NSW, where it is also cultivated for gardens, and possibly in New Guinea and Melanesia. It is prized for it's fragrant flowers and brightly colored interesting fruit. Seems to like rainforest margins, especially along

streams, and will become an understory plant in rainforest and drier vine forests, it is reported to be meeting with some success in cultivation. May be able to propagate with cuttings or fresh seed (Cai *et al.*, 1999).The latex was (is) reportedly used in the same way as that from *T. orientalis*. The root bark has been used for tropical fever. A recent listing of plants in Old (1993) named only *T. orientalis* and *T. pandacaqui* as being found in Old. Whilst The flora of the Kimberley region (1992) names *E. orientalis* and *E. pubescens* as occurring in that region, but in the notes states that both these species may be synonymous with *T. pandacqui* according to recent taxonomic work. In the latest edition of Australian Rainforest Plants (1994) it is stated that

E. angustisepala is now recognised as *Tabernaemontana pandacaqui*.Other type specimens are recorded from such places as New Caledonia and other areas close to Australia, so the situation is a complex one, as there can also a lot of variation within recognized species.

2.1.7.*Tabernaemontana ventricosa*

This small to medium-sized trees can reach a maximum height of 15 m, but averages between 4 and 8 m. It has a single, slender, straight trunk with smooth grayish-brown bark. The tree is low branching with smooth, dark green young branches turning a pale brown when mature (Briggs and Colebrook 1962). The flowers are salver-shaped, white and sweet smelling. (October - December) The fruits are pods, usually two joined at the base. They are dark green and smooth, sometimes with wrinkles and light green speckles or markings. They split open to reveal fleshy orange pulp with embedded seeds in June to August. This tree grows revering forests and the forest under storey in the coastal areas of KwaZulu-Natal . It is also found in Zimbabwe and Mozambique .

2.1.8.*Tabernaemontana heyneana*

Tabernaemontana heyneana wall. (Apocynaceae) known as kundalam paalai in tamil, possess antimicrobial activity against skin diseases, venereal diseases, respiratory problems, nervous disorders and various other diseases(Savarimuthu *et al.*, 2006). Isolation of Several unusual alkaloids like 15-β-stemmadenine, tabernoxidine, coronaridine, voacangine, and iboganine with antimicrobial activities have been reported previously. The stem bark decoction is used for cleaning cuts and wounds before dressing them. 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 (Savarimuthu *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. However, little is known about its antioxidant activity. Owing to their diverse biological activities and increasing consumer interest, we investigated the antioxidant potential of this plant extract by employing various established in vitro systems in relation to its total phenol, vitamin C and flavonoid content.

` Tabernaemontana heyneana belongs to the systemic position is

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Gentianales

Family : Apocynaceae

Genus : *Tabernaemontona*

Species : *heyneana*

2.1.9.Reactive oxygen species:

Reactive oxygen species (ROS) and their likely involvement in some human physiopathology has attracted growing interest from the health sector over the last few decades. Oxidative stress, caused by an imbalance between antioxidant systems and the production of oxidants, including ROS, seems to be associated with many multi factorial diseases, especially cancers cardiovascular diseases and inflammatory disorders (Langley-Evans ,2000).. The mechanisms by which these pathologies develop generally involve oxidative alteration of physiologically critical molecules, including proteins, lipids, carbohydrates and nucleic acids, along with modulation of gene expression and the inflammatory response. The human organism has developed defense systems to deal with this oxidative stress. These include enzymatic systems, especially superoxide dis mutases, catalases, glutathion peroxidases and thioredoxin systems, which are recognized as being highly efficient in ROS detoxification.The main nonenzymatic antioxidants present in the human

organism are glutathion, bilirubin, estrogenic sex hormones, uric acid, coenzyme Q, melanin, melatonin, _-tocopherol and lipoicacid (Schroeter *et al.,* 2003).Moreover, many studies have now confirmed that exogenic antioxidants, especially supplied by foods, are essential for counteracting oxidative stress. These antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes tocopherols, tocotrienols) ascorbic acid and carotenoids .

2.2.Flavonoids:

The term flavonoids is a collective noun for plant pigments, mostly derived from benzo-γ-pyrone, which is synonymous with chromone. (Harborne , 1988),

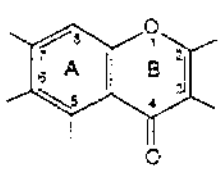

Fig. 2.1. Structure of benzo-γ-pyrone

The number and position of hydroxyl groups attached to the A-ring are controlled by different genes (Fig. 2.2).

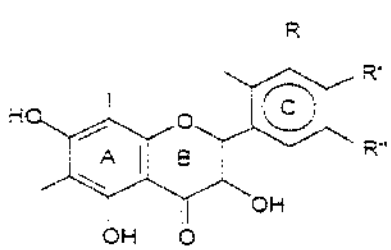

Fig. 2.2. Structure of flavonoles

2.2.1.Distribution of flavonoids

The flavonoids are qualitatively and quantitatively one of the largest groups of natural products known to be present in plants. Since almost all flavonoids are pigments and undoubtedly associated with some important biological functions, they are widely distributed in all geographical zones. Anthocyanins are highly distributed in fruits, flowers and are often used as natural dyes (Dixon *et al* 1998). Flavones, flavonols and flavanones are distributed in fruits, vegetables, nuts, tea (black and green) and in beverages. Flavanones are exclusively found in citrus fruits. Isoflavonoids are found only in the legumes.

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. 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.

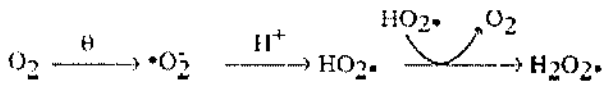

Fig. 2.3. Formation of H_2O_2 from superoxide anion

2.2.3.Flavanoids as UV-filters

A mixture of flavanones, flavones and flavonols in the central vacuole of epidermal cells of leaves serves as a filter, lessening the UV-A and UV-B irradiation that penetrates the earth's atmosphere (KiJhnau 1976). The present day epidermal layer has been shown to absorb over 90% of UV-B radiation administered. While the photosynthetic machinery appears to be the key site of damage in sensitive plants, damage to growth and flowering processes has also been observed. PSII appears to be the most sensitive part of the photosynthetic machinery, but the actual target site is unclear (Middleton and Teramura 1993). Whereas a mixture of flavanones, flavones, and flavonols in high enough concentration in the epidermis would provide a good filter protection in the UV-B region for mesophyll cells containing chloroplasts.

2.2.4.Flavonoids as hormones

Flavonoids can act as hormones in both plants and animals. The action of an active substance silybin, a complex flavonoid was found to simulate the action of estrogen in animal tissues (Wilson *et al.,* 1992). It was found that the hydroxyl groups of the aglycone portion of this flavonoid were positioned in space just like those of an estrogen. Since estrogens have anabolic effects, one might suspect that flavonoids might be able to act as growth hormones in animals also (Lucille *et al.,* 2006). However, so far only few indications of such a function have been found.

2.3.Orthogonal Design

Orthogonal design was used to reduce the number of experiments among the various levels and variables. In this design temperature ,extraction time, solid liquid ratio, solvent percentage, Number of extraction can be taken as a variables (sarawak *et al.,* 2002).The four varies levels could be taken. According to this orthogonal design for this four levels the number of experiments will be 16.This is known as L_{16} Orthogonal design. Among this 16 trials, the highest yield obtaining variables and levels could be identified.

2.4.Isolation of flavonoids

The flavonoids can be used as a taxonomic markers because they possess structural variability, chemical stability, widespread distribution in the plant kingdom and easy and rabid identification (Jumaat *et al.,* 2002). Therefore the flavonoids must be isolated for these medicinal use.(Neal *et al.,* 2007) The HPLC and TLC are recognized 16 different types of phenolic compounds including phenolic acids (protocatechuic acid and ellagic acids), 5 types of hydroxycinnamics acids, 4 types of coumarins, the flavonones narigenin and catechin.

OBJECTIVES

3. OBJECTIVES

1. Initial phytochemical screening and quantification of flavonoids in the leaves extract of *T.heyneana*
2. *In vitro* antioxidant studies in the crude leaf extracts (Fresh and Matured/ old)
3. Extraction optimization of flavonoids using orthogonal design of experiment
4. Detection and isolation of flavonoids using Preparative thin layer chromatography (PTLC).
5. *In vitro* antioxidant studies of the isolated flavonoids

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 EXTRACT PREPARATION

- The plant leaves was collected from the medicinal garden of Kumaraguru College of Technology, Coimbatore, India. The species was identified, confirmed and deposited at Botanical Survey of India (BSI), Southern Circle, Coimbatore, India.
- About 5g of air dried leaves were dissolved in 50ml of methanol or Ethanol or Acetone and kept in an orbital shaker for overnight. The residue was re-extracted under the same conditions. The obtained extracts were filtered with Whatman No.1 filter paper and the filtrate was collected and used for experimental analysis

4.2 FERRIC ION REDUCING/ ANTIOXIDANT POWER ASSAY

(FRAP ASSAY)

PRINCIPLE

The antioxidants present in the sample reduced the oxidant probe and the respective product interacted with some coloring agents to form a colored complex. In this method, the antioxidants reduced the Fe^{3+} to Fe^{2+}. This ion then conjugated with the ferricyanide ion to form a Prussian blue colored product, which was spectrophotometrically measured at 700nm. The presence of SDS prevents the formation of turbidity in the solution

$$Fe^{3+} + \text{antioxidant} \rightleftarrows Fe^{2+} + \text{oxidizedantioxidant},$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$$

or

$$Fe(CN)_6^{3-} + \text{antioxidant} \rightleftarrows Fe(CN)_6^{4-} + \text{oxidizedantioxidant}$$

$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$$

REAGENTS

See Appendix 1

PROCEDURE

About 2 ml of the extract (100-1000µg/ml), added 2 ml phosphate buffer (0.2 M, pH 6.6) and 2 ml of 1% potassium ferricyanide. The mixture was incubated in a 50°C water bath for 20 minutes, then rapidly cooled, mixed with

2 ml of 10% trichloroacetic acid(TCA) and centrifuged at 650g for 10 minutes. Pipetted out 4 ml of the supernatant, and 0.8 ml of 0.1% ferric chloride. Mixed well and allow to stand for 10 minutes. The absorbance at 700 nm was used to measure the reducing power.

4.3. CUPRAC (CURIC ION REDUCING ANTIOXIDANT CAPACITY) ASSAY

PRINCIPLE

Antioxidants especially the hydroxyl groups of phenolic compounds are converted to their respective quinones [oxidized form] in the presence of cupric chloride (Cu^{2+}) which in turn is reduced to Cu^{+}. The formed Cu^{+} then reacted with the chromogen, neocuproine (2,9-dimethyl-1,10-phenanthroline) to form a yellow colored complex which is spectrophotometrically measured at 450nm.

REAGENTS

See Appendix 2

PROCEDURE

About 0.1ml to 1 ml of the extract, added 1ml of cupric chloride, 1ml of ammonium acetate, 1ml of neocuproine and finally, 1ml of absolute ethanol. The mixture was incubated at room temperature for 30 minutes. The increase in the absorbance at 450 nm was used to measure the cupric ion reducing power of the salivary sample. Ascorbic acid and α- tocopherol acetate was used as a positive control.

4.4. FERROUS ION CHELATING ABILITY

Decker and Welch (1990)

PRINCIPLE

The ferrozine (disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, Fz), a chelating agent quantitatively complexes with Fe^{2+} to form a magenta color [$Fe(II)(FZ)_3$] which is colorimetrically measured at 562nm.. A competition occurs between the ferrozine and chelators (antioxidants), present in the plant extract for Fe^{2+} to form the complex and thereby a decrease in the absorbance will be observed.

REAGENTS

See Appendix 3

PROCEDURE

About 2ml of the extract (100-1000µg/ml) was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine solutions and allowed to react for 10 minutes at room temperature. The absorbance at 562 nm of the resulting solutions were measured and recorded. The ferrous ion chelating ability was expressed in percentage [1 – (test sample absorbance/blank sample absorbance)] X 100(%). The control contained $FeCl_2$ and ferrozine solutions.

4.5. HYDROXY RADICAL SCAVENGING ASSAY

PRINCIPLE

Hydroxyl radicals are produced by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction). These damage the substrate deoxyribose, which undergoes degradation to form malondialdehyde. This produces a pink chromogen with TBA. The antioxidants inhibit this reaction and hence there is a decrease in the color intensity of the chromogen as the antioxidant potential increased.

REAGENTS

See Appendix 4

e) Buffer preparation

About 28.39 g of 0.2 M of Na_2HPO_4 were dissolved into 1000ml of distilled water. It is kept as solution A.

About 31.2 g of 0.2 M of NaH_2PO_4 were dissolved into 1000 ml of distilled water.It is kept as solution B.

26.5 ml of solution A and 73.5 ml of solution B were mixed

PROCEDURE

A modified deoxyribose (TBARS) method was used to determine the hydroxyl ion scavenging activity (Res *et al.*, 2006). To a test tube added 1.5 ml of phosphate buffer (pH 7.0), 0.5 ml of 10 mM 2-deoxy-D-ribose, 0.25 ml of 20 mM Na_2-EDTA, 0.25 ml of 20 mM $FeCl_2$ solution, 1.9 ml distilled water, 0.1 ml of sample solution (100-1000µg/ml) and 0.5 ml of 10 mM H_2O_2 rapidly in this order, and the mixture in a total volume of 10 ml was incubated for 4 h at 37°C in a water bath. At the end of the period, the reaction was arrested by

adding 2.5 ml of 2.8% TCA. To this added 2.5 ml of 1% TBA and the reaction mixture was kept at 100°C in a boiling water bath for 10 minutes. The mixture was cooled under running tap water, and the absorbance at 520 nm was recorded. The hydroxyl radical scavenging ability was expressed in percentage [1 – (test sample absorbance/blank sample absorbance)] X 100(%). Mannitol was used as a positive control.

4.6. CERIUM(IV) SULPHATE ASSAY

PRINCIPLE

Cerium(IV) sulphate assay is based on the oxidation of antioxidant compounds with Ce(IV) sulphate and the absorbance of unreacted Ce(IV) ion is measured at 320 nm. The decrease in the absorbance is directly proportional to the concentration of total antioxidants present in sample.

REAGENTS

See Appendix 5

PROCEDURE

1. One milliliter of 2.0×10−3M Ce(IV) solution was added to 1mL of sample of varying concentration(200-1000µl/ml), and the mixture was diluted to 10mL with distilled water.
2. After shaking for a few minutes, the solution was let to stand for 30 min at room temperature.
3. The absorbance of the reaction mixture was measured at 320 nm against a blank composed of distilled water.
4. The decrease in the absorbance at 320 nm was used to measure the unreacted Ce(IV) ion in the sample which indicates the increase in antioxidant power of the sample.

4.7. ABTS CATION RADICAL SCAVENGING ASSAY

PRINCIPLE

The ABTS (2,2- azobis 3- ethylbenzothiazoline-6- sulfonic acid diammonium salt) assay is based on the inhibition of the absorbance of ABTS radical cation, which has a characteristic long wavelength absorption spectrum .

REAGENTS

See Appendix 6

PROCEDURE

Take 0.9 ml of ABTS+ and add 0.1 ml of extract. Then kept at 6 minutes at room temperature.Then greenish colour can be obtained. Take O.D values at 734 nm.

4.8. Extraction of flavonoids

Extraction of flavonoids could be obtained by the orthogonal design. In this orthogonal design there are four levels for a different five variables(Tempearature, Extraction time, Solvent percentage, Solid Liquid ratio, Number of extraction.) Temperature varying from the 65 °C to 95 °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.

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

Table 4.8.1. Factors and Levels

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

Table 4.8.2 L_{16} Orthogonal Design Graph

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

Flavonoids are part of a family of naturally occurring polyphenolic compounds and represent one of the most prevalent classes of compounds in vegetables, nuts, fruits and beverages such as coffee, tea, and red wine (Moon *et al.,* 2001) as well as in medical herbs. The classes of flavonoids include chalcones, flavones, flavonols, flavanones, flavanols, anthocyanins and isoflavones (Hu *et al.,* 2003). The flavonoids exert a wide range of biochemical and pharmacological properties, including cancer preventive activities. This effect has been endorsed by a wide variety of mechanisms, like free radical scavenging, modifying enzymes that detoxify carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters. The amount of total flavonoids in the plant extract was found to range between 4.3 to 4.7mg/g tissue. The standard deviation value was found to be nominal (0.170) and the SEM value was found to be 0.098.

Free radicals are involved in the normal physiology of living organisms. They act as a messenger for signal transduction and also affect gene expression (Caillet *et al.,* 2007). Several biomolecules in the living system may act as free radical scavengers. Further, dietary supplements including vitamins and polyphenolic compounds like flavonoids play a significant role in this matter . In the recent past, several herbal drugs, which have free radical scavenging potential (Antioxidant capacity), have

gained importance in treating a plethora of chronic diseases (Dorman *et al.,* 2003). Many methods like ascorbic acid quantification, total phenolics and flavonoids determination, reducing power capacity, total antioxidant activity, metal chelation power, free radical scavenging activity,Cerium sulphate assay, CUPRAC assay have been proposed to explain and evaluate the antioxidant properties of the extracts. Such methods have been adopted to study the antioxidant properties of the methanolic,ethanolic and acetone leaf extracts of *Tabernaemontana heyneana wall.*

The total antioxidant activity (TAA) of the plant was determined by the method described by Prieto. The TAA of the *T.heyneana wall.* extract was measured at various concentrations (100-1000 µg/ml) and expressed as ascorbic acid equivalents. An increased concentration of antioxidants (73.8 µg to 390 µg) was noticed by increasing the concentration of plant extracts as depicted in Fig.1. Statistical analysis at 1% level was proved to be significant ($p < 0.01$). The standard error was found to have an insignificant deviation (0.00003) proving a high concentration of antioxidants in the extract. A high correlation between the antioxidant level and the extract concentration was observed according to Karl – Pearson correlation of analysis (0.996) and the regression analysis (Absorbance (y) = 0.1472 + 0.001 X concentration (x)).

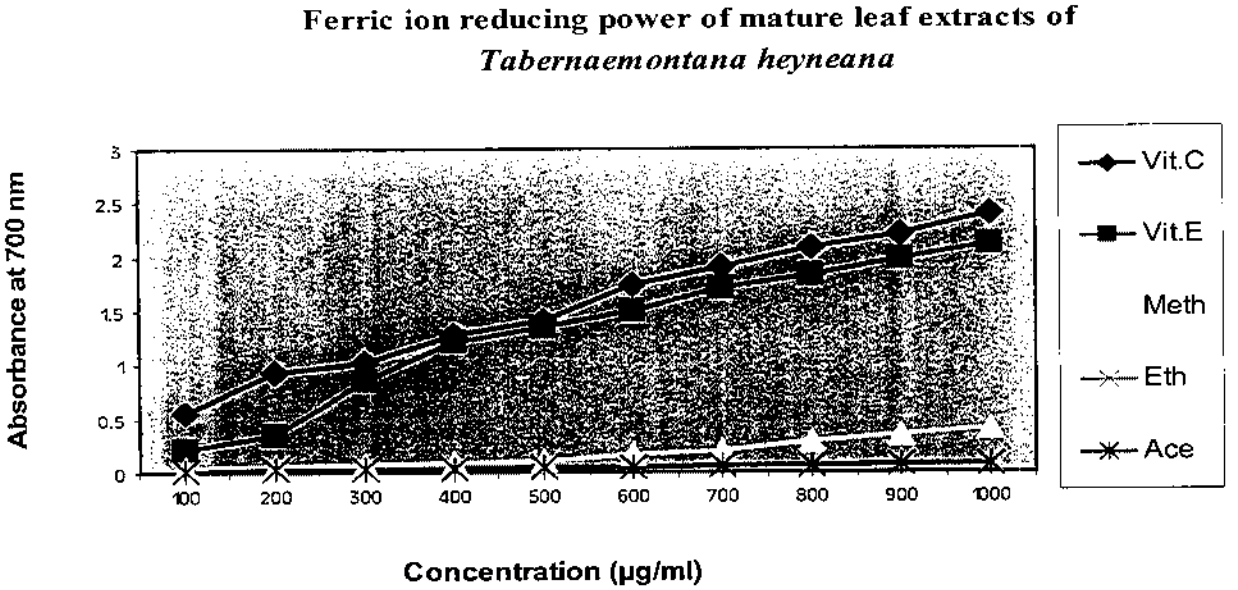

Fig 5.1.1. Ferric ion reducing power antioxidant activity (FRAP) for mature leaves

The reducing capacity of the plant was evaluated by the method proposed by Oyaizu (1988). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$), which is considered to be the primary ROS. The formed $O_2^{\bullet-}$ is responsible to produce H_2O_2, from which a very dangerous, high reactive and short lived (half-life of approx. 10^{-9} s) radical, •OH, was generated through a Fenton mediated reaction catalyzed by Fe^{2+}.

$$2O_2^{\cdot-} + 2H+ \rightarrow H_2O_2 + O_2$$

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

$O_2^{\bullet-}$ is also involved in the regeneration of Fe^{2+} from Fe^{3+} .

$$Fe^{3+} + O_2^{\cdot-} \leftrightarrow Fe^{2-} \text{ --- } O_2 \leftrightarrow Fe^{2+} + O_2$$

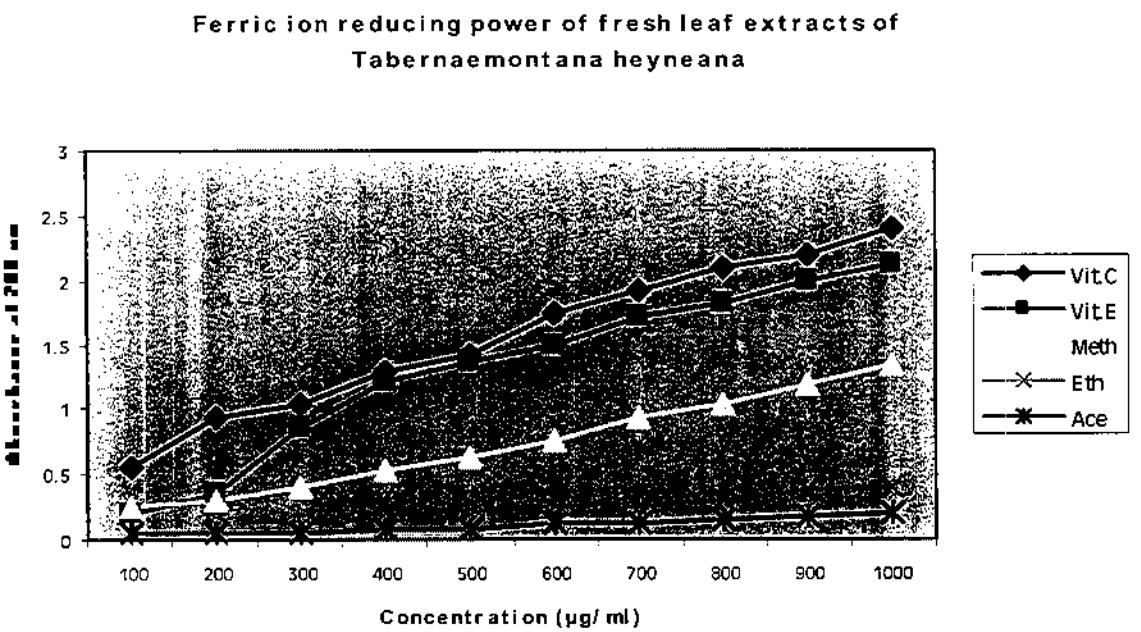

Fig 5.1.2. Ferric ion reducing power antioxidant activity (FRAP) for fresh leaves

Antioxidants in the plant extracts may disrupt the Fe^{3+} to Fe^{2+} transformation by competing with $O_2^{\bullet-}$ and thereby causing a decrease in the formation of hydroxyl radicals . The reducing ability of the *T.heyneana wall.* extract was measured at various concentrations (100-1000 µg/ml). An increased absorbance as a function of extract concentration as depicted in Fig.2, indicated a high reducing potentiality of the extract. Statistical analysis at 1% level was proved to be significant ($p < 0.01$). The standard error was found to have a trifling deviation (0.00004) proving a high reducing power of the extract. A high

correlation between the concentration and the absorbance was observed according to Karl – Pearson correlation of analysis (0.996) and the regression analysis (Absorbance (y) = 0.05 + 0.001 X concentration (x)).

The ferrous ion chelating ability of the plant was determined by the method proposed by Decker and Welch (1990). The most realistic *in vivo* production of hydroxyl radical according to the Fenton reaction occurs when M^{n+} is iron, copper, chromium, or cobalt. However, Rae and co-workers recently reported that the upper limit of so-called "free pools" of copper was far less than a single atom per cell. Although Fenton chemistry is known to occur *in vitro*, its significance under physiological conditions is not clear, noting particularly the negligible availability of "free catalytic iron" due to its effective sequestration by the various metal-binding proteins and other antioxidants . However, organisms overloaded by iron (as in the conditions of hemochromatosis, b-thalassemia, hemodialysis) contain higher amounts of "free available iron" and this can have deleterious effects.

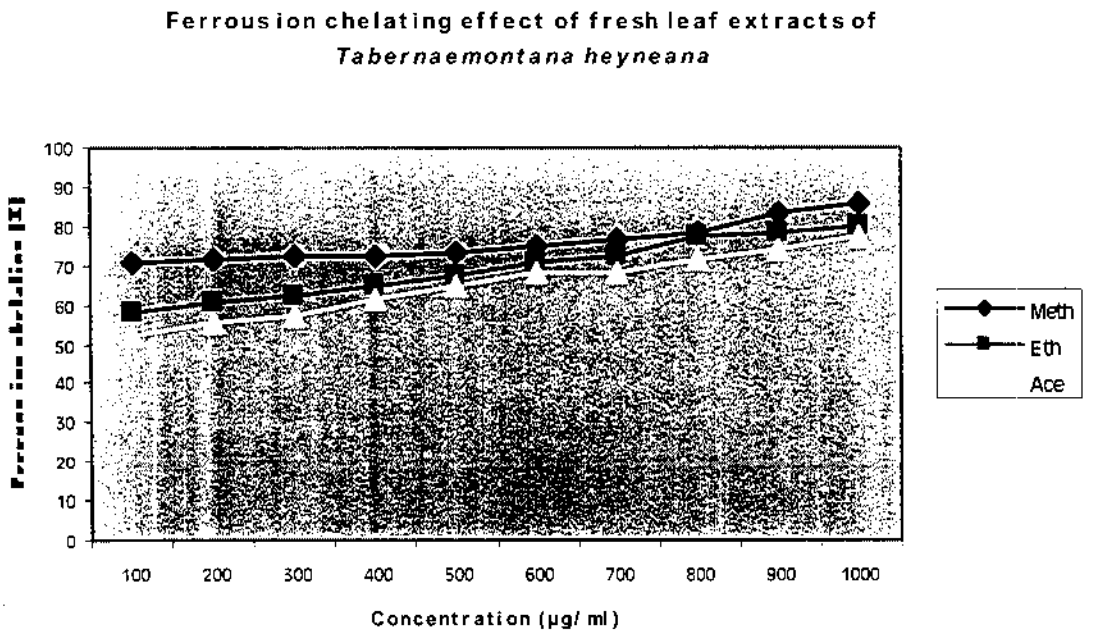

Fig 5.2.1. Ferrous Ion Chelating Effect Of Fresh Leaf Extract

In this method, ferrozine (disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, Fz), a chelating agent quantitatively complexes with Fe^{2+} to form a magenta color $[Fe(II)(FZ)_3]$ which is colorimetrically measured at 562nm.. A competition occurs between the ferrozine and chelators (antioxidants), present in the plant extract for Fe^{2+} to form the complex and thereby a decrease in the absorbance will be observed.

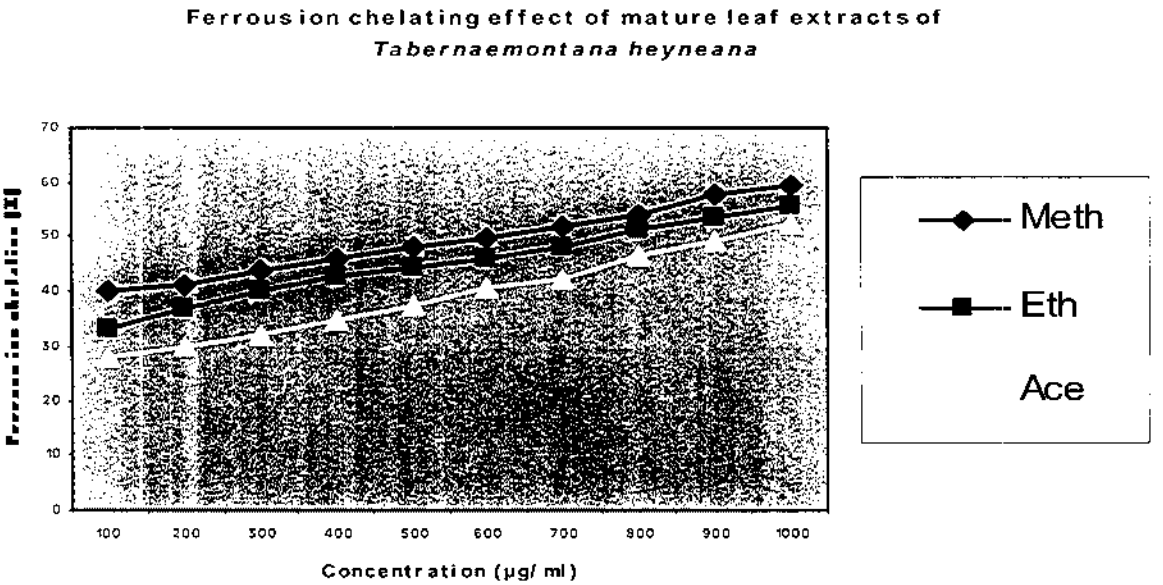

Fig 5.2.2. Ferrous Ion Chelating Effect Of Mature Leaf Extract

The ferrous ion chelating capacity of the *T.heyneana wall.* methanolic extract was measured at various concentrations (100-1000 µg/ml). According to Fig.3, it was observed a decrease in the absorbance as a function of concentration proving a high Fe^{2+} chelation effect by this plant. A high % chelating effect in relation with the plant extract concentration explored its tremendous free radical impeding effect (70.6% to 86.03%) has been shown in Fig.4. Statistical analysis using students t-Test was proved to be significant at 1% level ($p < 0.01$). The standard error was found to have a minimal deviation (0.002) proving a high chelating effect of the extract. A high correlation (0.932) was observed between the concentration and the absorbance. The regression

analysis was also supporting a high chelating activity of the plant (Absorbance (y) = 67.169 + 0.016 X concentration (x)).

The ability of hydroxyl radical scavenging activity was measured by a modified TBARS method described by Res. Peroxisomes are known to produce H_2O_2, but not $O_2^{\cdot-}$, under physiologic conditions (Rapak *et al.*, 2005). Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules. The catalase produced by the organelle decomposes hydrogen peroxide and presumably prevents the accumulation of this toxic compound. When peroxisomes are damaged and the H_2O_2 consuming enzymes were down regulated, H_2O_2 will be released into the cytosol, create an oxidative stress and thereby produce the hydroxyl radicals. Hydroxyl radical is the most reactive radical known in chemistry. It can abstract hydrogen atoms from various biological molecules especially from unsaturated fatty acids thereby leading to lipid peroxidation. Similarly, hydroxyl radicals may lead to the formation of sulfur radicals from thiols that are capable to combine with oxygen to generate oxysulfur radicals, which can also damage several biological molecules (Halliwell, 1991). Hence, removal of free radicals by the antioxidants in biological system gains a significant importance.

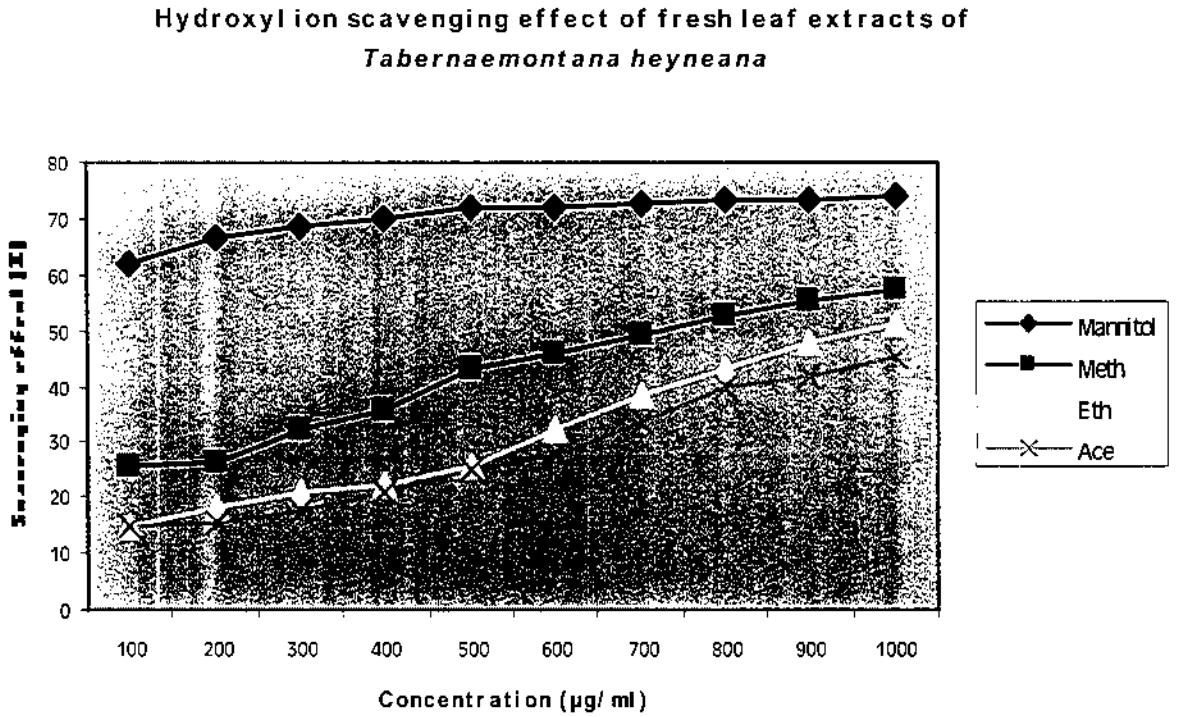

Fig 5.3. Hydroxyl Radical Scavenging Activity Of Fresh Leaf Extract

The hydroxyl ion scavenging activity of the *T.heyneana wall.* methanolic extract was measured at various concentrations (100-1000 µg/ml). It shows a decreased absorbance of plant extract as a function of increased concentration revealing its high scavenging potentiality (25.46 % to 57.23 %).

Statistical analysis using students t-Test was proved to be significant at 1% level ($p < 0.01$). The standard error was found to have a minimal deviation (0.002)

proving a significant hydroxyl ion scavenging effect by the extract. A siginificant correlation (0.989) has been observed between the concentration and the absorbance. The regression analysis showed a high scavenging activity of the plant (Absorbance (y) = 21.046 + 0.38 X concentration (x)).

The potentiality to scavenge the $ABTS^{\cdot+}$ cation was measured by using Re et al., method (1999). have recently reported superiority of $ABTS^{\cdot+}$ assay over $DPPH^{\cdot}$, as $ABTS^{\cdot+}$ assay is operable over a wide range of pH, inexpensive, and rapid with reacting with the extracts (2h) than that of $DPPH^{\cdot}$ (24h) assay. The activity of the plant extract was measured at various concentrations (100-1000 µg/ml). High decolourization of $ABTS^{\cdot+}$ (42.95 % to 78.53 %) by the plant extract as a function of concentration increase has been depicted in Fig.5.4.

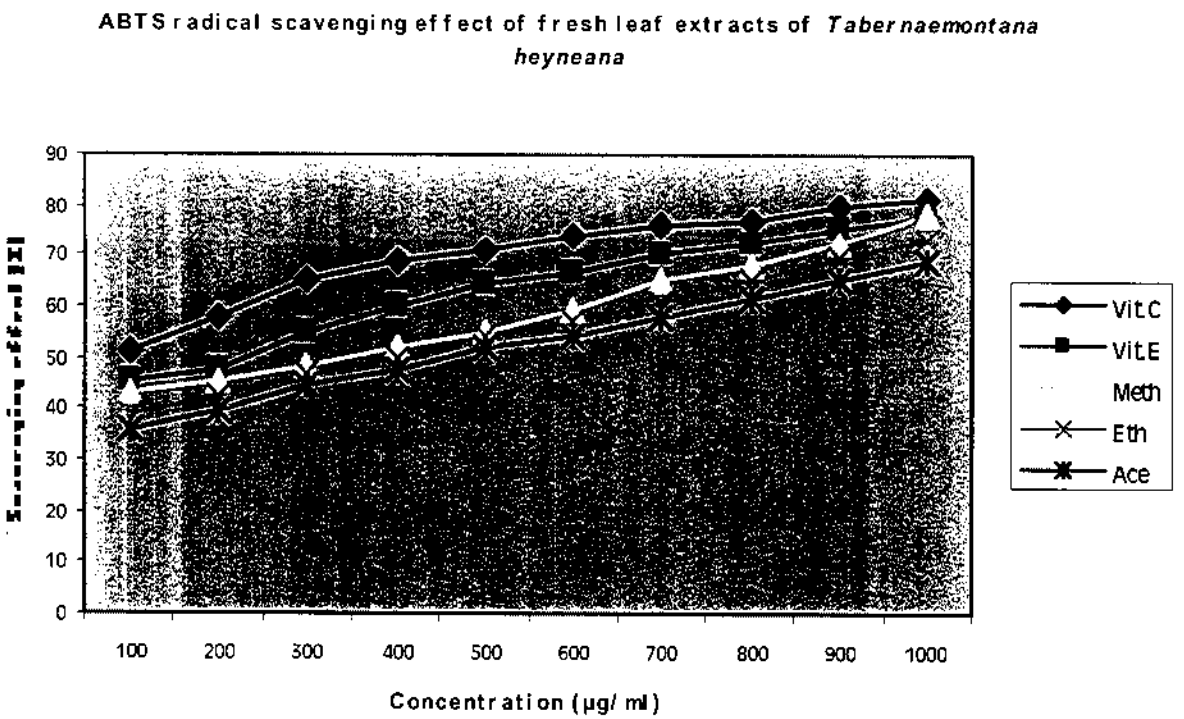

Fig 5.4. ABTS Radical Scavenging Activity Of Fresh Leaf Extract

Statistical analysis performed on the data using t-Test was proved to be significant at 1% level ($p < 0.01$). The standard error was found to have a least deviation (0.002) proving a significant hydroxyl ion scavenging effect by the extract. An elevated correlation (0.994) has been observed between the concentration and the absorbance. The regression analysis performed on the data also proved a high radical scavening activity of the plant (Absorbance (y) = 36.857 + 0.04 X concentration (x))

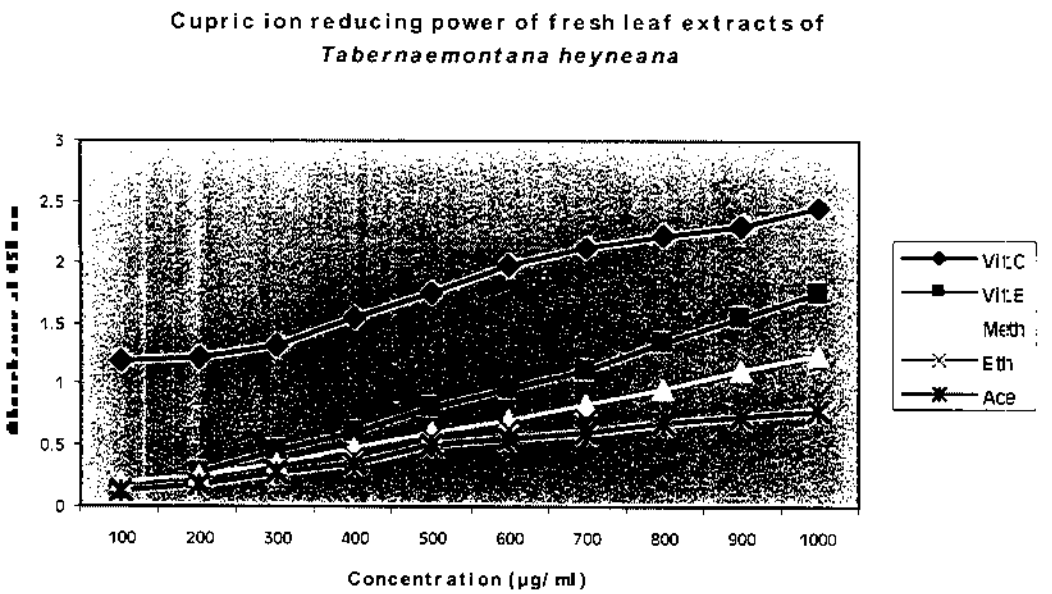

Fig .5.5.1. Modified cupric ion reducing antioxidant capacity (CUPRAC) assay for fresh leaves

An increased absorbance as a function of leaf extract concentration was noticed which indicates the high reducing potentiality.

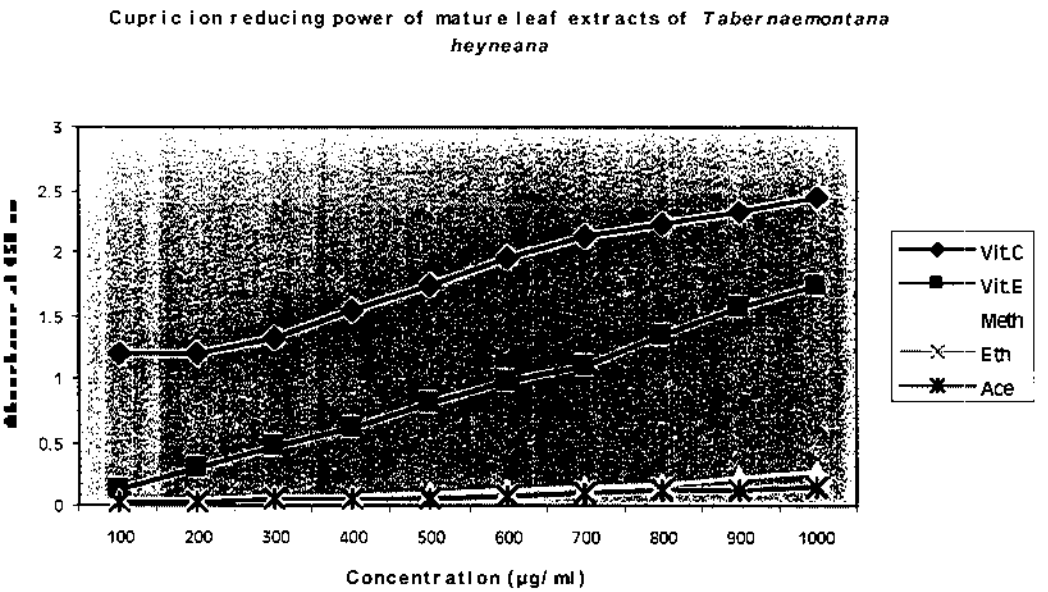

Fig. 5.5.2. Modified cupric ion reducing antioxidant capacity (CUPRAC) assay for Mature leaves

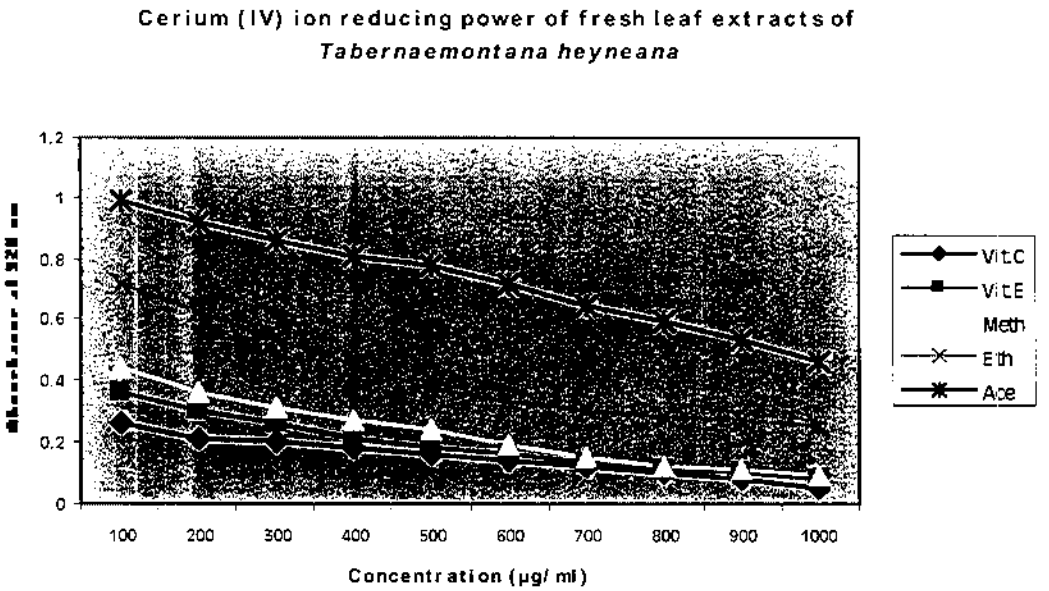

Fig 5.6.1.Cerium (IV) ion reducing power assay for fresh leaves

A decreased absorbance as a function of leaf extract concentration was noticed which indicates the high reducing potentiality

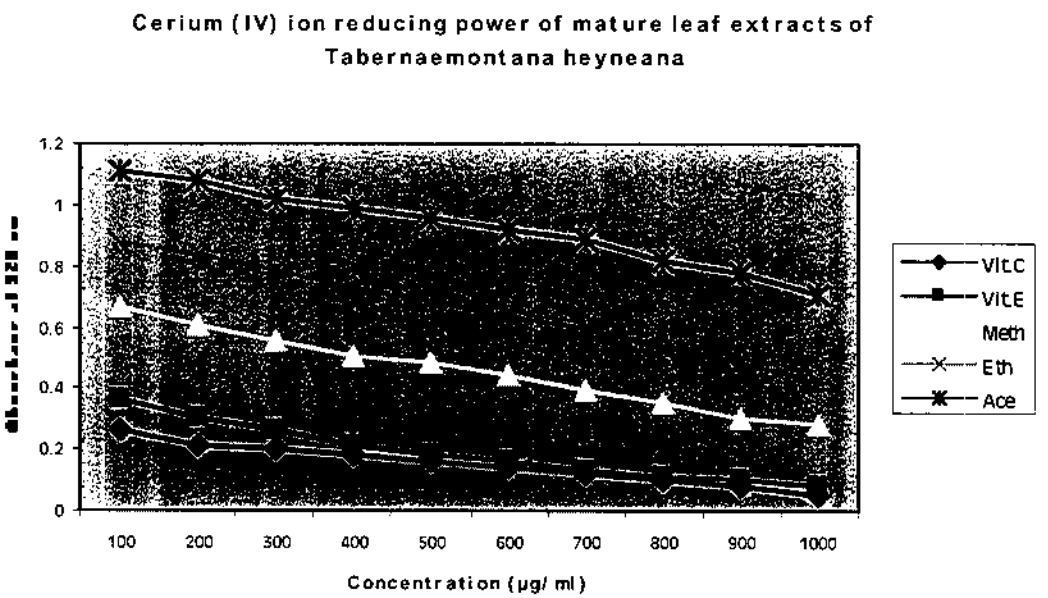

Fig 5.6.2.Cerium (IV) ion reducing power assay for mature leaves

Exp.	A	B	C	D	E	Flav. (mg/g)
1	1	1	2	3	4	46.9
2	1	2	1	4	3	50.5
3	1	3	4	1	2	40
4	1	4	3	2	1	41.6
5	2	1	1	1	1	35.9
6	2	2	2	2	2	59.5
7	2	3	3	3	3	65.8
8	**2**	**4**	**4**	**4**	**4**	**132.3**
9	3	1	3	4	2	58.5
10	3	2	4	3	1	100.6
11	3	3	1	2	4	53
12	3	4	2	1	3	38.9
13	4	1	4	2	3	48.8
14	4	2	3	1	4	33
15	4	3	2	4	1	131.2
16	4	4	1	3	2	109.4

Table 5.1. L_{16} Orthogonal design graph values

One way ANOVA

Levels	Sum of squares	Degrees of freedom	Mean square	F-values
A	2912.32	3	970.77	1.04
B	2478.77	3	826.26	0.86
C	1982.26	3	660.75	0.66
D	8107.01	3	2702.34	4.62
E	1409.89	3	469.96	0.46
		15		

Table 5.2. One way ANOVA

Levels A, B, C and E were found to have no significant difference at 5% level. Level D (Solid:Liquid, W/V) was found to have a significant difference at 5% level proving that it is a strong factor that greatly affects the flavonoids extraction. The following order may be responsible for an optimized flavonoids extraction

D> A> B> C> E

Effect of Temperature

The effect of temperature in the flavonoids extraction could be high in high temperature (Yaqin xu *et al.*, 2005). But the high concentration of the flavonoids could be obtained at the temperature 75°C.

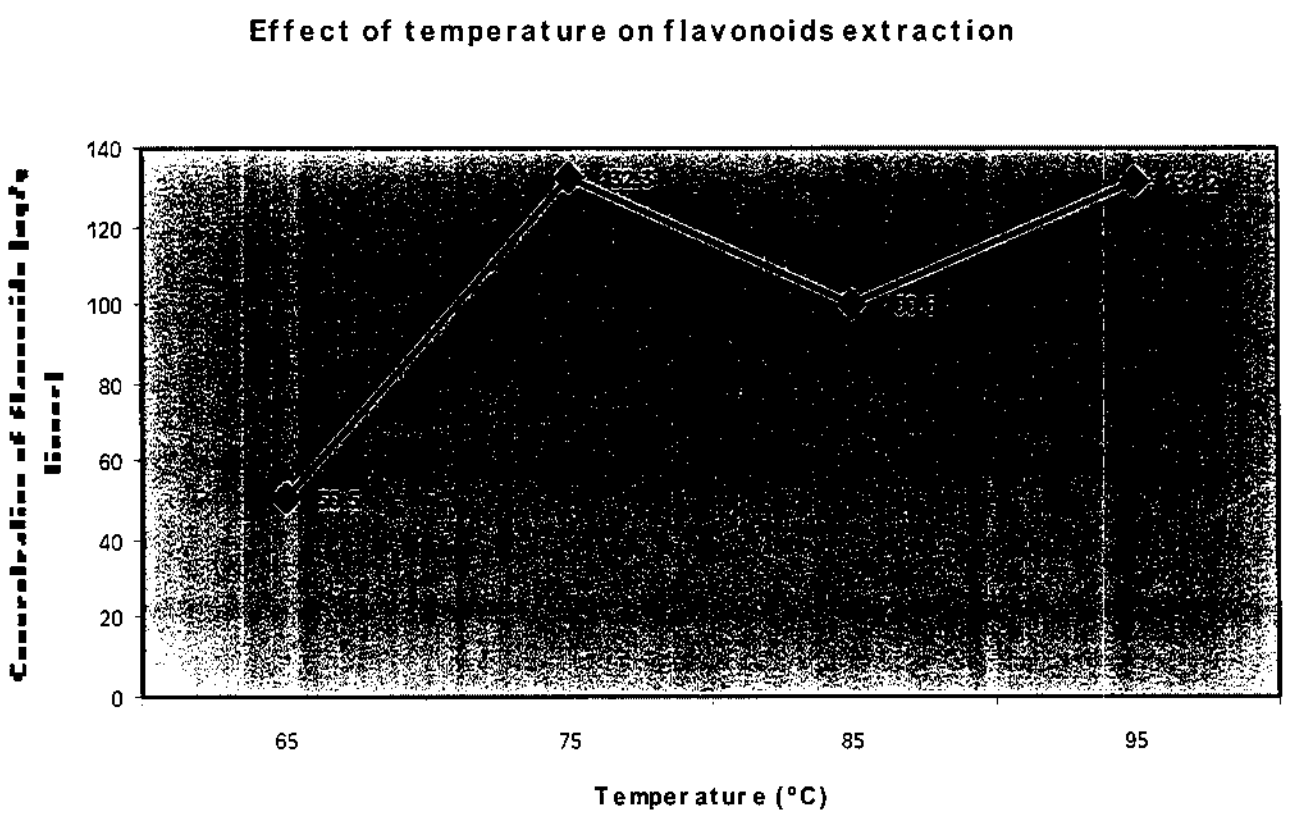

Fig. 5.7.1.Effect of temperature on flavonoids extraction

Effect of different extraction time

When the extraction time increases the flavonoid concentration also increased.The high concentration of the flavonoids is obtained at four hours extraction time.

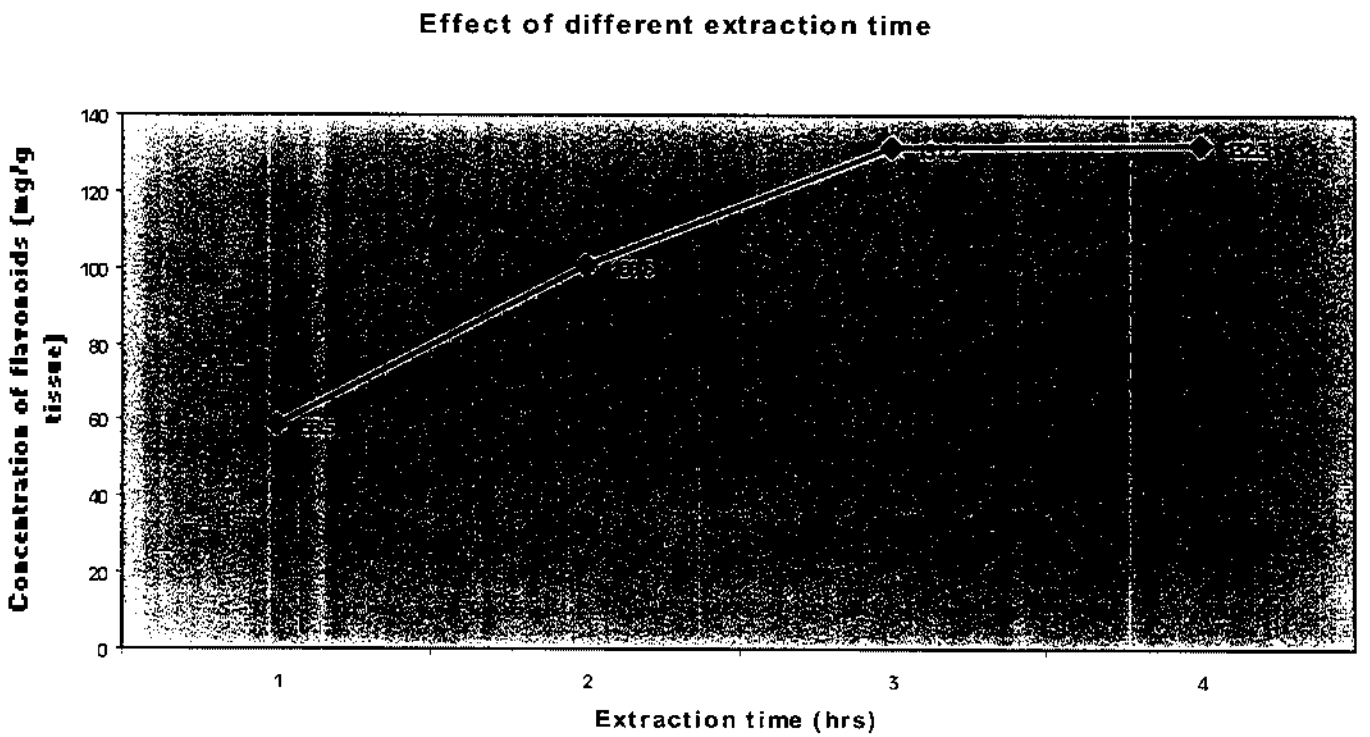

Fig. 5.7.2.Effect of different extraction time on flavonoids extraction

Effect of solvent in the extraction of flavonoids

The solvent percentage plays the major role in the flavonoid extraction. The high concentration of the flavonoid could be obtained at the 95% solvent.

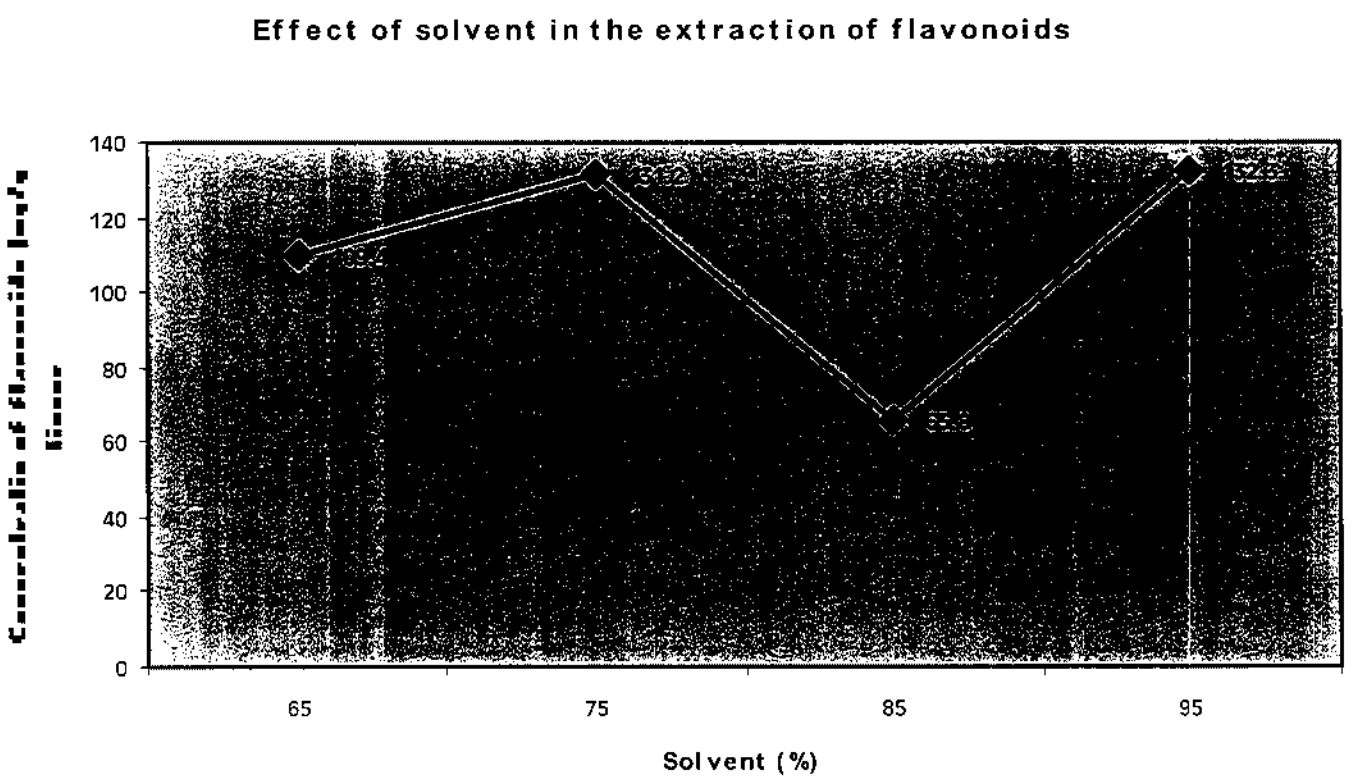

Fig 5.7.3. Effect of solvent in the extraction of flavonoids

Effect of soild: liquid (w/v) in the extraction of flavonoids

Here the solid liquid ratio increases the flavonoids concentration also get increased (Puupponen-pimia *et al.,* 2005). So the high concentration of the flavonoid obtained at the 1:20 ratio of the solid liquid ratio.

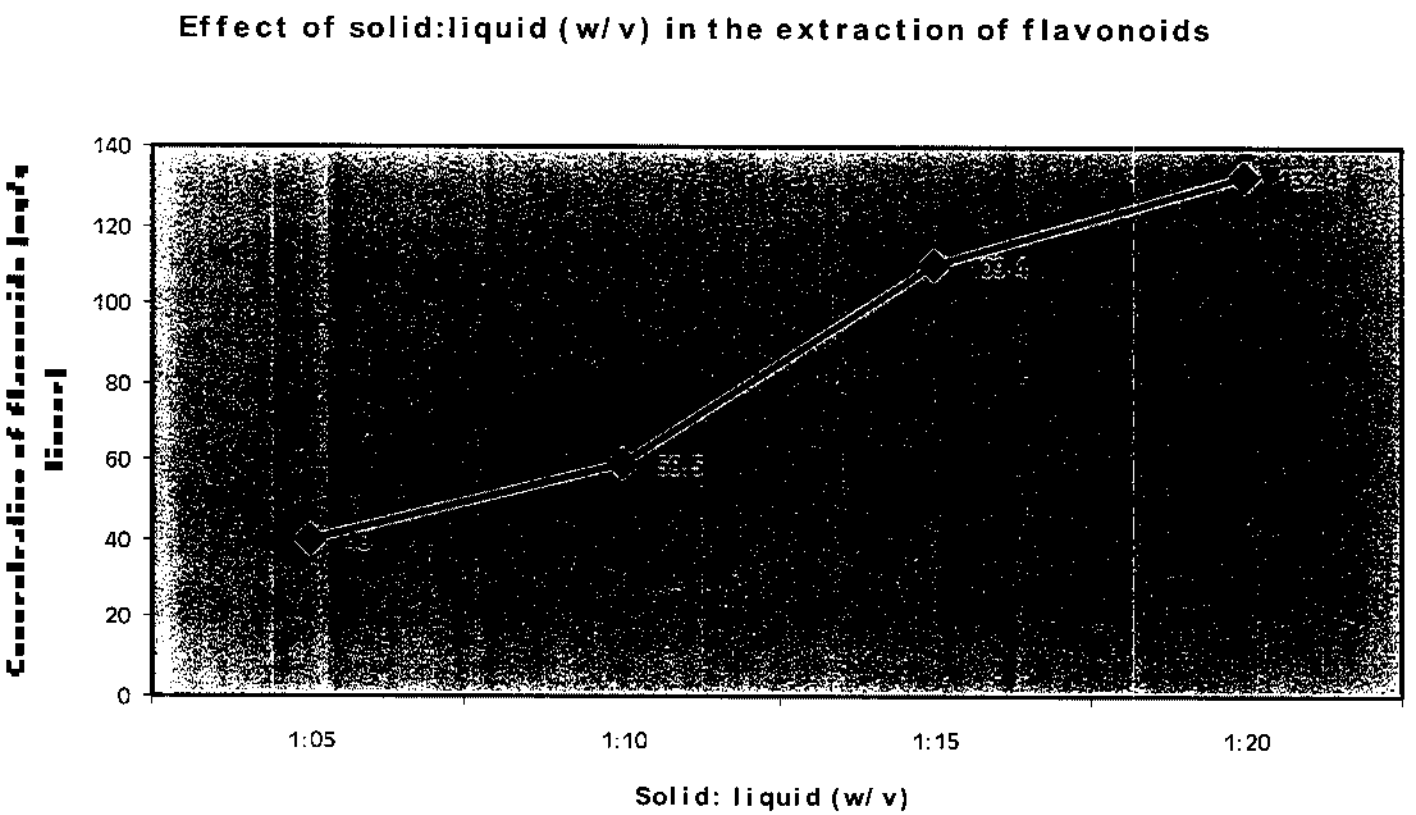

Fig 5.7.4. Effect of soild: liquid (w/v) in the extraction of flavonoids

Detection and isolation of flavonoids.

The detection and isolation of flavonoids was done by using TLC and PTLC method respectively. There are three mixture of solvent could be used as a mobile phase. i.e. Ethyl Acetate, Ethanol, Water (5:1:5).The Spraying Agent was Liquid Ammonia (Zesheng Zhanga *et al.,* 2001).

The standard flavonoids used were rutin and quercetin. These are all the artificial flavonoids with known Rf values. Then sample could be compared with the standard after solvent reached the solvent front.

Then the TLC plate could be inspected under UV light. Fig 5.8.1 shows the 5 different colours under UV light (Magenta, Blue green, Purple, Orange, Yellow,). These 5 different colours denotes the Cyanidin related, luteolin related, ellagic acid related, polyphenols and quercetin related compounds as contained in the sample leaf extracts.

Then the isolation of flavonoids can be done by PTLC method. Here the sample added without any gap about 40 microgram per spot. Around 10-12 spots can be added in the PTLC plate. Then it would be kept in the mobile phase until the solvent reached the solvent front. Fig 5.8.2. shows the the three different bands obtained in the PTLC plate.

Then scrap out those flavonoids occurred in the three bands then the FRAP assay were carried out for those different three extracts. Finally, According to the FRAP assay results,the flavonoid concentration obtained 50 mg, 35mg 15 mg as three bands respectively. the isolated flavonoids possessed approx. 60 – 70% of the total antioxidant activities in the plants.

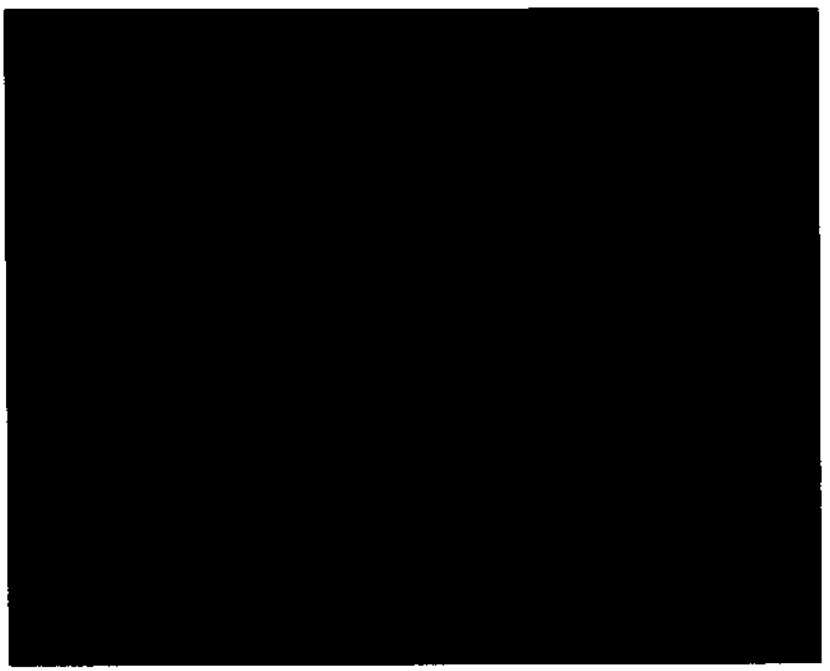

Fig 5.8.1. Identification of flavonoids by using TLC under UV light.

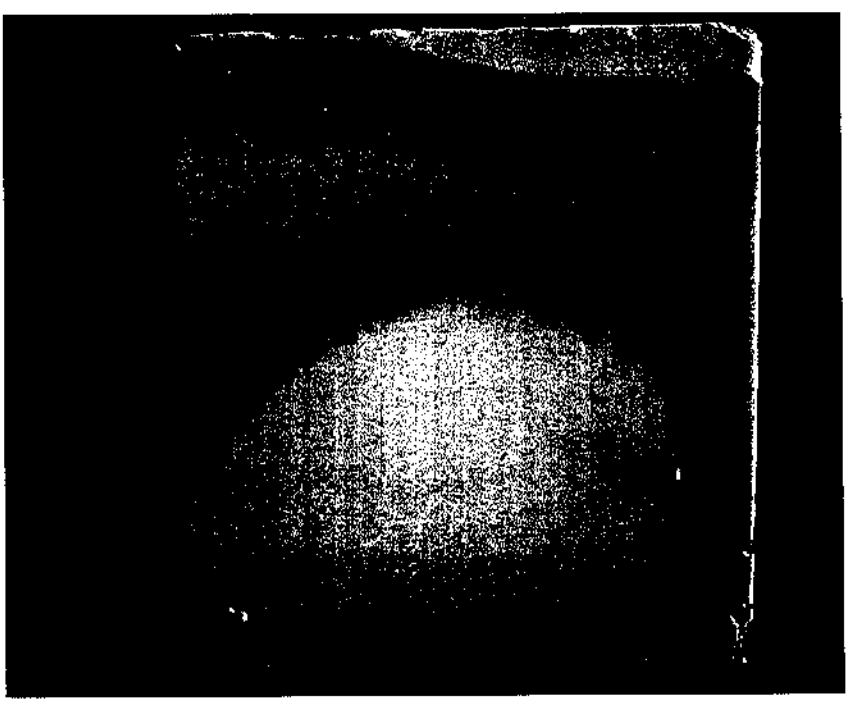

Fig 5.8.2. Isolation of flavonoids by using PTLC.

CONCLUSION

6.CONCLUSION

This study has been focused on the antioxidant activities of *T.heynaena* in the fresh and mature leaves by using three different solvents i.e., methanol, ethanol and acetone. Fresh methanolic leaf extracts proved to have the best antioxidant property than the other solvent extracts. Even though the methanolic extracts having best antioxidant property, it could not be used for medicinal purpose because of its poisonous nature .

When comparing the Fresh and mature leaves, the old/ mature leaves have the least antioxidant power than the fresh/ young leaves. Comparative investigation between the short term (15 days) preserved **(4°C)** leaves and the fresh leaves proved to have no significant difference in their antioxidant property.

Then the Orthogonal design experiment proves a significant increase in the flavonoid concentration (~ 31.5 fold increase). The orthogonal design of experiment proved that at temperature 65°C, 1:5 (sol:liq), 65% solvent and extraction time duration of 1 hour were the optimal conditions to extract the flavonoids from the leaves.

Then level D (solid:liquid, w/v) was found to be the major factor to affect the flavonoids extraction. The following order may be responsible for an optimized flavonoids extraction is D> A> B> C> E,

Isolated flvaonoids by PTLC method showed 5 different bands (Cyanidin related, luteolin related, ellagic acid related, polyphenols and quercetin related compounds) visualized at UV light. The isolated flavonoids possessed approx. 60 – 70% of the total antioxidant activities in the plants.

APPENDICES

APPENDIX 1

FERRIC ION REDUCING/ ANTIOXIDANT POWER ASSAY

(FRAP ASSAY)

a) Stock preparation

About 50 mg of filtrate dissolved into 50 ml of water. Then the concentration varied from 100microgram per 2ml.

b) Buffer preparation

About 28.39 g of 0.2 M of Na_2HPO_4 were dissolved into 1000ml of distilled water. It is kept as solution A.

About 31.2 g of 0.2 M of NaH_2PO_4 were dissolved into 1000 ml of distilled water.It is kept as solution B.

26.5 ml of solution A and 73.5 ml of solution B were mixed.

c) 1% potassium ferric cyanide

Dissolved 1g of potassium ferricyanide in 100ml of distilled water.

d) 10% Tri Chloro Acetic acid (TCA)

Dissolved 10g of TCA into 100 ml of Distilled water.

e) 0.1% Ferric chloride

Dissolved 0.1g of ferric chloride in 100ml of distilled water

APPENDIX 2

CUPRAC (CURIC ION REDUCING ANTIOXIDANT CAPACITY) ASSAY

a) Stock preparation

About 50 mg of filtrate dissolved into 50 ml of water.Then the concentration varied from 100 µg per 2ml.

b) 0.0075M Neocuproine (2,9-dimethyl-1,10-phenanthroline)

Dissolved 0.031g of neocuproine in 20ml of distilled water

c) 0.01M Cupric chloride

Dissolved 0.034g of cupric chloride in 20ml of distilled water

d) 1M Ammonmium acetate

Dissolved 1.54g of ammonium acetate in 20ml of distilled water

e) Absolute ethanol

APPENDIX 3

FERROUS ION CHELATING ABILITY

a) Stock preparation

About 50 mg of filtrate dissolved into 50 ml of water.

Then the concentration varied from 100µg per 2ml.

b)2 mM Ferrous chloride

Dissolve 39.8 mg of $FeCl_2$ into 100 ml of water

c) 5 mM Ferrozine

Dissolve 49.2 mg of ferrozine into 20 ml of water.

APPENDIX 4

HYDROXY RADICAL SCAVENGING ASSAY

a)10 mM Deoxy Ribose

About 13.4 mg of deoxyribose were dissolved into 10 ml of distilled water.

b)20 mM EDTA

About 7.44 g of EDTA were dissolved into 1000 ml of distilled water.

c)20 mM Ferrous chloride

About 3.98 g of ferrous chloride into 1000 ml of distilled water.

d)10 mM Hydrogen Peroxide

About 10 microlitre of hydrogen peroxide were dissolved into 24.9 ml of distilled water

APPENDIX 5

CERIUM(IV) SULPHATE ASSAY

a) 2.0×10−3M Ce(IV) solution

A cerium(IV) sulfate solution containing 2×10−3MCe(IV) was prepared by dissolving 0.0809 g $Ce(SO_4)_2 \cdot 4H_2O$ in 25ml distilled water, adding 17ml concentrated H_2SO_4 and thoroughly mixing by the aid of a magnetic stirrer until total dissolution at room temperature. This solution was totally transferred to a 100 ml flask, and diluted to the mark with distilled water.

APPENDIX 6

ABTS CATION RADICAL SCAVENGING ASSAY

a) ABTS+

5 ml of 14 mM ABTS were added with 5 ml of 4.9 mM $(NH_4)_2S_2O_8$.

b) $(NH_4)_2S_2O_8$

22 mg of $(NH_4)_2S_2O_8$ were dissolved with 20 ml of distilled water.

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8.REFERENCES

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