Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC

PAVITHRA. J

(13PBT006)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore-641043

In Partial Fulfilment of the Requirements for the

Degree of Master of Science in Biotechnology

March, 2015

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Signature of the Head of the Department **Signature of the Supervisor**

ACKNOWLEDGMENT

"GRATITUDE IS THE MEMORY OF HEART"

The author wishes her sincere thanks to **Lord almighty** for the blessings which helps in the completion of this study.

I express my sincere gratitude to the Chancellor **Dr. T.S.K. MeenakshiSundram**, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore for providing her all necessities to undertake this study.

The author expresses her sincere thanks to **Dr. (Mrs) Sheela Ramachandran**, Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore for providing all necessary facilities for the study.

The author records her sincere gratitude to Dr. **(Mrs) Gowri Ramakrishna**, Former Registrar and **Dr.(Mrs)A.Venmathy** present registrar Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore for the immense help rendered.

The author to express her sincere thanks to **Dr. (Mrs) A.Parvathi**, Dean, Professor and Head, Department of Mathematics, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore, for the timely help rendered throughout the course.

The author is extremely thankful to her guide **Dr. (Mrs) S.Annapurani,** M.Sc., Dip.Ed, M.Phil., Ph.D., Professor and Head Department of Biochemistry, Biotechnology and Bioinformatics. Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore, for her constructive criticism and inspiration throughout the study.

The author also extends her thanks to all the staff members of Department of Biochemistry, Biotechnology, and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore, for their support and encouragement to complete the work.

The author express her deep sense of thanks to beloved **Dr. (Mrs) R.Santhi, M.Srilatha, K.Lavanya and family members** and well wishers for their support to the completion of this work.

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1.0 INTRODUCTION

Medicinal plants are a rich source of bioactive phytochemicals or bionutrients. Studies were carried out to find out the phytochemicals and their important role in preventing chronic diseases like cancer, diabetes and coronary heart diseases. The phytochemicals with disease preventing functions are dietary fiber, antioxidants, anticancer agents, detoxifying agents, immunity potentiating agents and neuropharmacological agents. Some of these phytochemicals have more than one function. The activities of phytochemicals and their potential function in protection against different types of diseases were done by various researchers. Biologically active Phytochemicals provide health benefits for humans. Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. Many phytochemicals (~ 4000) have been cataloged and are classified by protective function, physical characteristics and chemical characteristics. About 150 phytochemicals have been studied in detail (Mamta *et al.,* 2013).

Ayurveda, a traditional Indian medical practice using plant drugs has been successful from very early times. These natural drugs have been used for preventing or suppressing various tumors with different lines of treatment. In India, people of different ethnic groups inhabiting various terrains, possess their own distinct culture religious rites, food habit and a rich knowledge of traditional medicine. They practice herbal medicine to cure a variety of diseases for thousands of years. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient times and an impressive number of modern drugs have been developed from them (Sumitra and Krunal, 2013).

The development in the field of modern medicine temporarily subdued the traditional herbal medicine. But it has now staged a comeback and an “herbal renaissance” is blooming across the world. Medicinal plants are nature’s priceless gift to human (Thenmozhi *et al.,* 2013).

Plants which have one or more of its organ containing substances that can be used for the therapeutic purpose are called medicinal plants. Many photochemical surveys have been published including the random sampling approach which involved some plant accessions collected from all parts of the world. The major chemical substances of interest in these surveys have been the alkaloids and steroidal sapogenins (saponins). However, other diverse groups of naturally occurring phytochemicals are flavonoids, tannins, unsaturated sterols, triterpenoids and essential oils (Anubha, 2013).

Tabernaemontana divaricata is a common tropical region plant and it is used for medicinal purposes, particularly the flowers of the plant. The study is conducted to compare and identify the phytochemicals constituents by Qualitative Phytochemical analysis and Thin Layer Chromatography (Vedha *et al*., 2012).

The numbers of studies were aimed at characterizing the health promoting properties of many phenolic compounds which exhibit antioxidant properties. It is used in the treatment and management of cancer, cardiovascular and neurodegenerative diseases. It is also used as in anti-aging or cosmetic products. Phenolic compounds are ubiquitous in plants which collectively synthesize several thousand different chemical structures and are characterized by hydroxylated aromatic ring(s). The compounds enable the plants to adapt to changing biotic and abiotic environments and provide color, taste and health-promoting benefits to plants. Phenolic compounds represent the most studied phytochemicals and have been widely explored as model systems in different areas of plant research. The pharmacological activities of any plant sample are due to the presence of metabolites, secondary metabolites and secretary products in it. These usually consist of the phenolic compounds, alkaloids, tannins, saponins, carbohydrates, glycosides, flavonoids, steroids, etc. Most phenolic compounds such as flavonoids, glycosides, terpenoids, flavonons, carbohydrates and anthraquinones are found distributed throughout the plant kingdom. Similarly, the polyphenolic compounds most commonly found in plant extracts are the phenolic acids, flavonoids and tannins. Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom (Gupta *et al.*, 2012).

Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system, (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some of are still to be explored. Standardization of plant materials is the need of the day. Several pharmacopoeia containing monographs of the plant materials describe only the physicochemical parameters. The modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbals and its formulations. Also, the WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards. HPTLC offers

better resolution; estimation of active constituents can be done with reasonable accuracy in a shorter time (Sushma *et al.,* 2013).

Since not much work has been carried out on the biological activities of the species *Tabernaemontana divaricata* the present study entitled "**Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabernaemontana divaricata* leaf and its characterisation by TLC, HPTLC and HPLC**" has been aimed with the following objectives:

1. To screen the phytochemical constituents and estimation of total flavonoids from the leaves of *Tabarnaemontana divaricata.*
2. To determine the antioxidative potential of *Tabarnaemontana divaricata.*
3. To characterize the flavonoid fractions in the ethyl acetate extract of *Tabarnaemontana divaricata* leaves by TLC, HPTLC and HPLC.

2.0 REVIEW OF LITERATURE

India is a largest producer of medicinal plants and is rightly called as "Botanical garden of the World". Medicinal plants having natural therapeutic values against different type diseases and also provide high quality of food and raw materials for livelihood. Many works have been done on these plants to treat cancer and some plant products have been marketed as anticancer drugs, based on the traditional using and scientific reports. It may promote host resistance against infection by re-stabilizing body equilibrium and conditioning the body tissues. Several reports describe the anticancer activity of medicinal plants is due to the presence of antioxidants in them (Umadevi *et al*., 2013).

The review of literature pertaining to the present study entitled" **Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC"** is discussed under the following headings:

2.1. Importance of medicinal plants

2.2. Phytochemical constituents of medicinal plants

2.3. Free radicals

  2.3.1 Reactive oxygen species

  2.3.2 Reactive nitrogen species

2.4. Flavonoid as antioxidants

2.5. Characterization of flavonoids

  2.5.1 TLC

  2.5.2 HPTLC

  2.5.3 HPLC

2.6. Plant under study

2.1 IMPORTANCE OF MEDICINAL PLANTS

The term of medicinal plants include a various types of plants used in herbalism and some of these plants have a medicinal activities. These medicinal plants consider as a rich resources of ingredients which can be used in drug development and synthesis (Bassam, 2012).

Herbal medicines have a vital role in the prevention and treatment of cancer, it is execute the therapeutic effect by inhibiting cancer activating enzymes and hormones, stimulating DNA repair mechanism, promoting production of protective enzymes then antioxidant action and

enhancing immunity. Siddha system is providing good medicine and it is good base for scientific exploration of potent anti-cancer herbs (Jeeva *et al.*, 2013).

Therefore it is a very important point for the open access journals to encourage researchers and clinicians to work hard in order to clarify the main active ingredients which can be extracted from medicinal plants. Moreover, to clarify their role in the treatment of present diseases, and how they can be used to produce or synthesis more effective drugs (Bassam, 2012).

2.2 PHYTOCHEMICAL CONSTITUENTS OF MEDICINAL PLANTS

Medicinal plants are a group of species that accumulate different active principles, useful in treating various human or animal diseases. The long term use of herbs in medicine is a sure indication of their value and usefulness in the future. Phytotherapy is a source of treating and improving certain diseases by using the beneficial effects of medicinal plants. An important amount of therapeutic products77% for cardiovascular diseases and 74% for digestive diseases are derived from medicinal plants (Oancea *et al.*, 2013).

Phytochemical are very important in medicine and constitute most of the valuable drugs. Alkaloids are rich in medicine and constitute most of the valuable drugs. They have physiological effect on animals. *(*Anubha, 2013)

Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds or secondary metabolites of plants serve as defense mechanism against predation by many microorganism, insects and herbivores. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins and steroids (Sofowara, 1993).

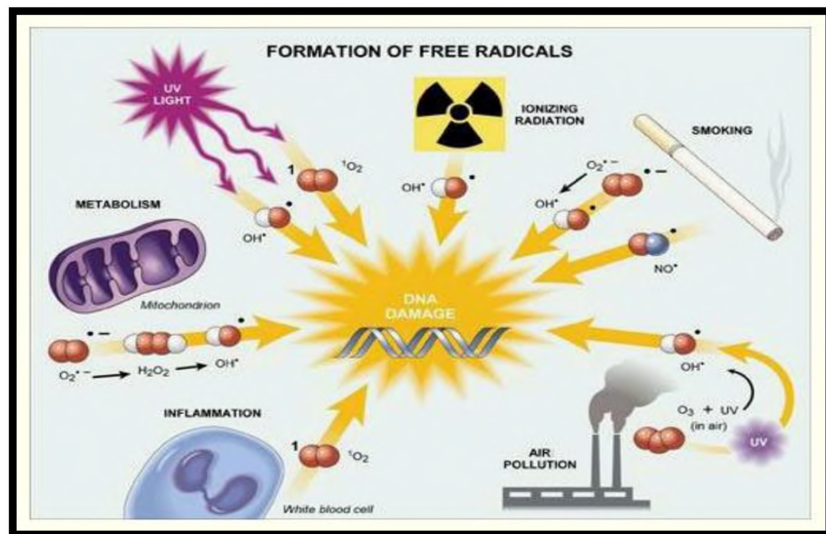
2.3 FREE RADICALS

Free radical have unpaired electron at the outer orbit; as a consequence, they were tending to be unstable and very reactive. The unpaired electron determined the reactivity degree of free radical. Main class of free radicals generated in living organism was derived from oxygen, such as superoxide, hydroxyl, peroxyl ($RO^{2\bullet}$), alkoxyl ($RO^{\bullet}$), and hydroperoxyl ($HO^{2\bullet}$) radicals. They were called as reactive oxygen species (ROS). Another major radicals such as nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($^{\bullet}NO_2$) were free radicals nitrogen and were called as reactive nitrogen species (RNS). Both ROS and RNS were normal products of metabolism processes and could be beneficial or even deleterious for organism. At a low concentration, ROS and RNS defense the body from infectious agents and played roles in a number of cellular

signaling systems. The over production of ROS and RNS could damage and decrease the function of cellular lipid, proteins, and DNA in biological process usually called as an oxidative stress or nitrosative stress (Achmad Fuad Hafid *et al.*, 2014).

FIGURE 1

FORMATION OF FREE RADICALS



(http://www.gojitrees.com/highestantioxidantrate.htm)

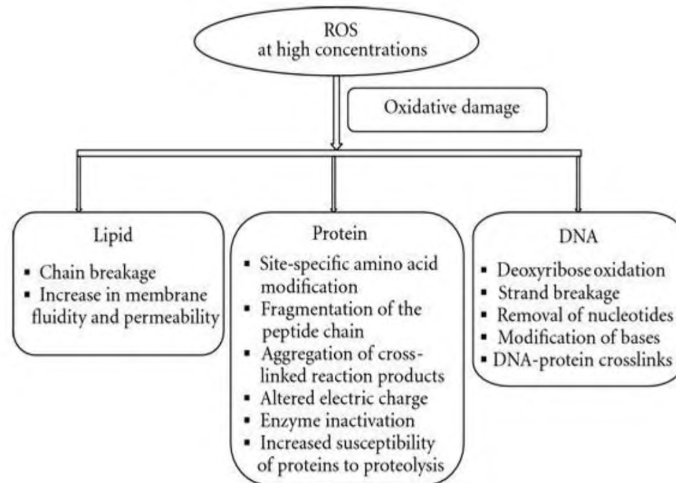
2.3.1 REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species formed *in vivo* , such as superoxideanion, hydroxyl radical and hydrogen peroxide,are highly reactive and potentially damaging trasient chemical species. ROS are regulated by endogens superoxide dismutase, glutathione peroxidase and catalase but due to over prodution of reactive species, induced by exposure to external oxidant substances or afailure in the defense mechanisms, damages the cell structures, DNA, lipids and proteins (Volko *et al.*,2006).

Scavenging of ROS is perturbed under a number of stressful conditions such as salinity, drought, and high light, toxicity due to metals, pathogens, and so forth. Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA (Pallavi *et al.*, 2012).

FIGURE 2

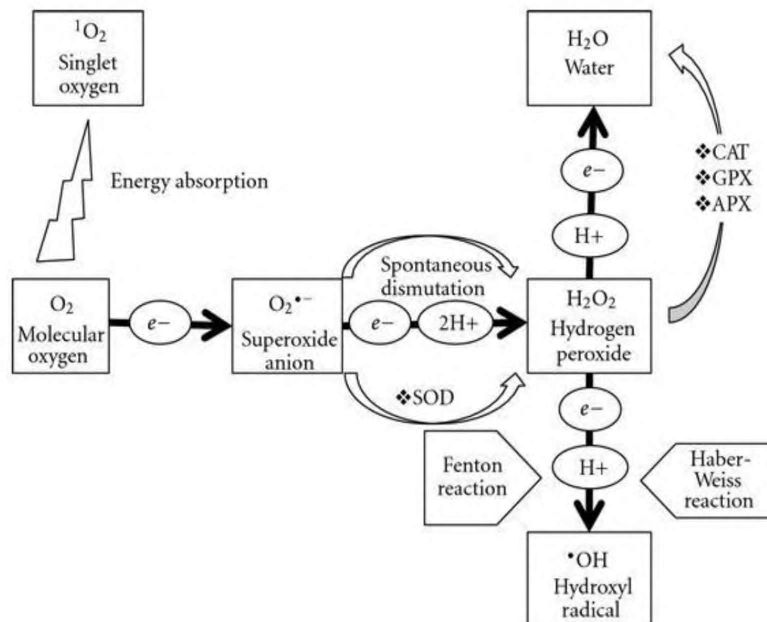
REACTIVE OXYGEN SPECIES (ROS) INDUCED OXIDATIVE DAMAGE TO LIPIDS, PROTEINS, AND DNA.



(Pallavi *et al.*, 2012)

FIGURE 3

GENERATION OF REACTIVE OXYGEN SPECIES



(Pallavi *et al.*, 2012)

2.3.1.1 SUPEROXIDE

Superoxide anion radicals are biologically quite toxic and are deployed by the immune system to kill invading microorganisms. In phagocytes, it is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Superoxide anion radical is an oxygen-cantered radical with selective reactivity. It is produced as a result of the donation of one electron to oxygen *in vivo* (Ilhami, 2011).

2.3.1.2 HYDROGEN PEROXIDE

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} to form hydroxyl radical and this may be the origin of many of its toxic effects (Nishaa *et al.*, 2012).

2.3.2 REACTIVE NITROGEN SPECIES (RNS)

The reactive nitrogen species are a famaily of antimicrobial molecules derived from nitric oxide(NO) and superoxide($O_2^{\cdot -}$) produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS_2) and NADPH oxidase respectively. NOS_2 is expressed primarily in macrophages after induction by cytokines and microbial products (Lovine *et al.*, 2008).

2.3.2.1 NITRIC OXIDE

Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. Suppression of released NO may be partially attributed to direct NO scavenging, as the *M.arundinacea* extract decreased the amount of nitrite generated from the decomposition of SNP *in vitro*. The scavenging of NO by the extract was increased in concentration dependent manner (Nishaa *et al.*, 2012).

2.4 FLAVONOIDS AS ANTIOXIDANTS

Organisms have developed some mechanisms to protect their bodies from free radicals-induced oxidative or nitrosative stress. They produced antioxidant molecules to protect cells from damage which caused by free radicals. At a low concentration in the body, antioxidants could protect the cells and its content like proteins, lipids, carbohydrates, and DNA significantly.

Plants produced some compounds such as polyphenols and flavonoid that tend to have free radical scavenging activity (Achmad *et al.,* 2014).

Antioxidants interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors. The natural antioxidant mechanisms may be insufficient in variety of conditions and hence dietary intake of antioxidant compounds is important. The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases. Plant based antioxidants are preferred to the synthetic ones because of their multiple mechanisms of actions and non-toxic nature. These facts have inspired widespread screening of plants for possible medicinal and antioxidant properties; the isolation and characterization of diverse phytochemicals and the utilization to antioxidants of natural origin to prevent the diseases (Padmanabhan and Jangle, 2012).

2.4.1 FLAVONOIDS

Flavonoid content are the basis for evolution of the preventive role of plant material as compensating factor to oxidative damage caused by free radicals which is a critical etiological aspect responsible for several chronic human ailments, the maximum content of phenol was found in capsicum annum and the richest source of flavonoids in *Lactuca sativa* (Srivastava *et al*., 2013).

Flavonoids are polyphenolic compounds that are ubiquitous in nature. More than 4,000 flavonoids have been recognized, many of which occur in vegetables, fruits and beverages like tea, coffee and fruit drinks. The flavonoids appear to have played a major role in successful medical treatments of ancient times, and their use has persisted up to now (Mamta *et al.,* 2013).

2.5CHARACTERIZATION OF FLAVONOIDS

Flavonoids have gained recent attention because of their broad biological and pharmacological activities. Flavonoids have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The

position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Mamta *et al.,* 2013).

2.5.1 TLC

Many aqueous extract or alcoholic extracts, hydro alcoholic extracts are used in manufacturing Ayurvedic and herbal formulations. If the phytochemical profile of the plant or its part is known an appropriate kind of extract can always be used by selection for a particular purpose. A TLC profile of the phytochemical can be employed for the similarity or dissimilarity or to find out the presence or absence of the certain phytochemicals. TLC has excellent resolution and, therefore, permits simultaneous identification of a wide range of substances in a single run. In this paper, application of TLC methodology in testing, investigation, advantages of HPTLC for analysis of Medicinal plants, General guideline for the analysis by HPTLC, Trouble shooting in HPTLC, Typical tasks in the quality control of medicinal plant (Rahul *et al*., 2013).

2.5.2 High Performance Thin Layer Chromatography

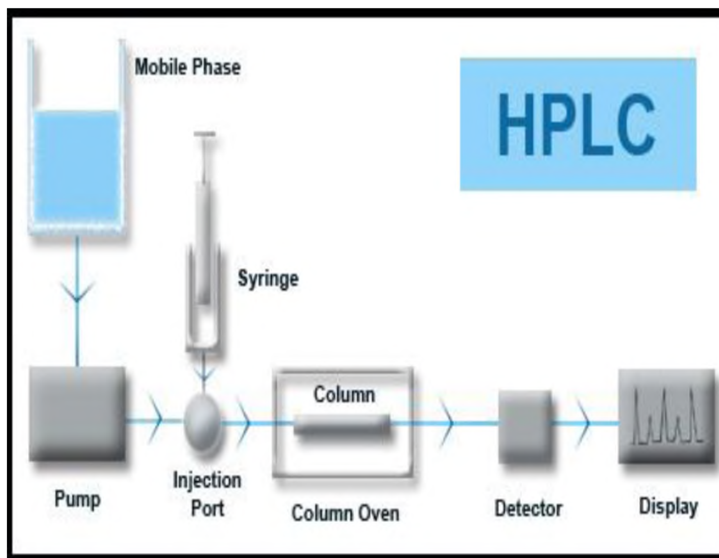
The characterization of the bio-active flavanoid quercetin, quercetin-3-O-rutinoside and kaempferol present in the florets of four different cult had also been determined using High Performance Thin Layer Chromatography (Sajeli, 2015).

2.5.3 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) has been the most widely employed chromatographic technique in flavonoid analysis during the past 20 years (14–18). It has added a new dimension to the investigation of flavonoids in food and plant extracts. The separations are far more rapid than classical methods and provide high resolution and sensitivity. HPLC of flavonoids is a widely used methodology and easily adapted to the quantitation of individual compounds. It has the advantage of generating a chemical fingerprint, which can be used in defining the identity and quality of a given sample (Fazilatun *et al*., 2005)

FIGURE 4

BASICS OF HPLC INSTRUMENTATION



(Lab-traning.com)

2.6 PLANT UNDER STUDY

INTRODUCTION OF *Tabernaemontana divaricata*

Tabernaemontana divaricata belongs to the family Apocynaceae, a common garden plant in tropical countries and also distributed in coast forests of Bengal, Myanmar and mangrove forests of China and Japan (Kanthlal *et al*., 2012). *Tabernaemontana divaricata* (Apocynaceae), commonly called *Pinwheel Flower*, *Crape Jasmine*, East India *Rosebay* and *Nero's Crown* is an evergreen shrub probably native to India and now cultivated throughout South East Asia and the warmer regions of continental Asia. In zones where it is not hardy it is grown as a house/glasshouse plant for its attractive flowers and foliage. The stem exudes a milky latex when broken, whence the name *Milk Flower* in the Bengali language Known as *Wathusudda* (meaning "garden white") in the Sinhalese language. Other Indian names include *Chandni* and *Taggar*.

The plant generally grows to a height of 5–6 feet (1.5–1.8 m) and is dichotomously-branched. The large shiny leaves are deep green and about 6 inches (15 cm) in length and 2 inches (5.1 cm) in width. The waxy blossoms are found in small clusters on the stem tips. The (single) flowers have the characteristic 'pinwheel' shape also seen in other genera in the family Apocynaceae such as *Vinca* and *Nerium*. Both single and double-flowered forms are cultivated, the flowers of both forms being white. The plant blooms in spring but flowers appear

sporadically all year. The flowers of the single form are unscented but the double-flowered form has a pleasing fragrance. The roots have a bitter taste.

They are used in Ayurvedic medicine as an anthelmintic for ascariasis and a treatment for scabies. The young stems are also good for the teeth, the latex being applied, on a cotton pellet, to teeth with dental caries. In mainland South East Asia an infusion of the roots is used as an antipyretic. In the Malay Peninsula a sweetened infusion of the leaves is used as a cough medicine, the pounded roots are applied externally for eye diseases (unspecified) and an unspecified part of the plant is mixed with other herbs to make a medicinal snuff for treating nasal ulcers.

It has been sued as antioxidant, antiinfection, anticancer and analgesia as a folk medicine in India, Sri Lanka, Vietnam, Malaysia and Thailand. The plant grows upto a height of about 6-feet, bears attractive, white colored flowers and its juice posses a reliable anti-bacterial action in eye infection. It has been used in Chinese, Ayurvedic and Thai as traditional medicine for the treatment of fever, pain and dysentery. Alkaloids and non-alkaloids constituents such as terpenoids, steroids, flavonoids, phenyl propanoids, phenolic acids and enzymes are the major constituents present in *Tabernaemontana divaricata* (Kanthlal *et al*., 2012).

3.0 EXPERIMENTAL PROCEDURE

The present research focused on "**Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC"** was conducted in three different phases.

PHASE I

3.1 ASSESSMENT OF PHYTOCHEMICALS OF *Tabarnaemontana divaricata* LEAVES

3.1.1. Collection of plant material

3.1.2. Preparation of different extracts of *Tabarnaemontana divaricata* leaves

3.1.3. Preliminary Phytochemical analysis

3.1.4 Estimation of flavonoids

PHASE II

3.2 EVALUATION OF ANTIOXIDATIVE POTENTIAL OF *Tabarnaemontana divaricata* Flavonoid fraction (TdFf)

3.2.1. DPPH radical scavenging assay

3.2.2. ABTS radical scavenging assay

3.2.3. Superoxide radical scavenging assay

3.2.4. Ferric Reducing Antioxidant Power (FRAP) assay

3.2.5. Hydroxyl radical scavenging assay

3.2.6. Hydrogen peroxide scavenging assay

3.2.7. Nitric oxide scavenging assay

PHASE III

3.3 CHARACTERIZATION OF TdFf BY CHROMATOGRAPHIC TECHNIQUES

3.3.1. Thin Layer Chromatography (TLC)

3.3.2. High Performance Thin Layer Chromatography (HPTLC)

3.3.3. High Performance Liquid Chromatography (HPLC)

PHASE I

3.1 ASSESSMENT OF PHYTOCHEMICALS OF *Tabarnaemontana divaricata* LEAVES

In Phase I, the phytochemical screening of four different extracts of *Tabarnaemontana divaricata* leaves were done. From this, the best extracts with higher flavonoid content were selected for further studies.

3.1.1 Collection of plant material

Fresh leaves of *Tabarnaemontana divaricata* were collected in an area free of pesticides and other contaminants from Coimbatore district, Tamil Nadu. The collected leaves were washed thoroughly in tap water, shade dried and finely powdered.

3.1.2 Preparation of different extracts of *Tabarnaemontana divaricata* leaves

Ten gram of leaf powder of *Tabarnaemontana divaricata* was taken to prepare in aqueous, ethanol, chloroform and ethyl acetate extracts individually. Plugged with cotton wool and then kept on a rotary shaker at 190-220rpm for 24 hours. After 24 hours the supernatant was collected and the solvent was evaporated and stored at 4°C in air tight containers and all the organic extracts were concentrated using flask evaporator. The aqueous extract was concentrated using freeze dryer (Santhi *et al.*, 2011).

PLATE 1

MEDICINAL PLANTS SELECTED FOR THE STUDY

Tabarnaemontana divaricata

Tabarnaemontana divaricata

Kingdom	-	Plantae
Class	-	Angiosperms
Order	-	Gentianales
Family	-	Apocynaceae
Genus	-	*Tabarnaemontana*

Species - *divaricata*

Common name - Crepe Jasmine

3.1.3 Preliminary phytochemical analysis and estimation of flavonoids

Phytochemical screening was performed using standard procedures. The procedures for detection of carbohydrates, cholesterol, protein, amino acids, alkaloids, flavonoids, terpenoids, glycosides, steroids, tannins, saponins and phlobatinins are given in Appendix I. Extractions were used for the estimation of flavonoid (Appendix II). Total flavonoids contents of the selected plant extracts were determined by colorimetric method (Chang *et al.*, 2002).

PHASE II

Phase II involved the assessment of antioxidative potential of *Tabarnaemontana divaricata* leaves.

3.2 EVALUATION OF ANTIOXIDATIVE POTENTIAL OF TdFf

The antioxidative potential of TdFf was evaluated by assessing the scavenging of using different concentrations by DPPH, ABTS, Superoxide radicals, FRAP, Hydroxyl radical Scavenging , Hydrogen peroxide and Nitric oxide assays.

3.2.1 DPPH radical scavenging assay

DPPH radical scavenging activity was measured according to the method of Aquino *et al.* (2001) as given in Appendix III.

3.2.2 ABTS radical scavenging assay

ABTS radical scavenging activity was determined by the method described by Long and Halliwell (2001) as given in Appendix IV.

3.2.3 Superoxide radical scavenging assay

Superoxide radical scavenging activity was determined by the method of McCord and Fridovich (1969) as elaborated in Appendix V.

3.2.4 Ferric reducing antioxidant power (FRAP) assay

FRAP activity was determined by the method of Benzic and Strain (1996) as given in Appendix VI.

3.2.5 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was analyzed according to the method of Elizabeth and Rao (1990) as given in Appendix VII.

3.2.6 Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity was determined according to the method described by Ruch *et al.* (1989) as given in Appendix VIII.

3.2.7 Nitric oxide scavenging assay

Nitric oxide radical scavenging activity was determined by the method described by Garrat (1964) as given in Appendix IX.

PHASE III

3.3 CHARACTERIZATION OF TdFf BY CHROMATOGRAPHIC TECHNIQUES

This phase involved the characterization of TdFf by Thin Layer Chromatography (TLC), High Performance Thin Layer liquid Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC).

3.3.1 Thin Layer Chromatography (TLC)

TheTLC analysis was performed on precoated 20 X 20 cm (0.25 mm thick) TLC plates K6F Silica gel 60 A purchased from Whatman, USA. 10 µl of standard flavonoid (quercetin, myricetin, kaempferol and catechin) solution (concentration 0.1 mg /ml), was applied as spots onto TLC sheets. TdFf were dissolved in 100 ml appropriate solvent and from that 10 µl was taken for the study. Four different mobile phases were selected (according to their polarity) to establish the R_F value for standard. The spotting line was about 0.5 cm from the developing solution. The plates were developed at room temperature in a vertical separating chamber to the height of approximately 18 cm from the start. The chamber was previously saturated for two hours with the appropriate mobile phase.

Standard flavonoid mixtures and TdFf were chromatographed on silica gel plates using the above solvent systems and the retention factor (R_F) was calculated.

$$R_F \text{ value} = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

After drying, visualization was performed without derivatization (under short wavelength-254nm, UV light illumination - 366 nm and White light) (Puri *et al.*, 2010).

3.3.2 High Performance Thin Layer Chromatography (HPTLC)

A densitometry-C of HPTLC analysis was performed for the development of characteristic finger printing profile. The TdFf was dissolved with HPLC grade methanol 100

mg/0.5ml. The solution was centrifuged at 3000rpm for 5 min and used for HPTLC analysis. Then, 2 µl of the samples were loaded as 5 mm band length in the 5 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase (alkaloid compound) and the plate was developed in the respective mobile phase (Chloroform-Methanol-Water 10:1.35:1) up to 90 mm. The developed plate was dried using hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at visible light, UV 254nm and UV366 nm. Finally, the plate was fixed in scanner stage and scanned at 254nm. The Peak table, Peak display and Peak densitogram was identified (Puri *et al.*, 2010).

3.3.3. High Performance Liquid Chromatography (HPLC)

The analysis of flavonoids in the samples was carried out by using HPLC. The HPLC conditions adjusted were as follows:

The samples (in methanol) were filtered through a 0.45µm PTFE syringe tip filter and were injected (10µL) through the BDS Hypersil RP-C18 column (Thermo, 5µm, 120Å, 250mm × 4.6mm) at column temperature 40°C. The liquid chromatography system was equipped with the photo diode array detector (PDA) and a vacuum degasser and analytical data was evaluated by using X-Caliber data processing system (2.0 SR2). The mobile phase composed of methanol (100%) was eluted at a flow rate of 1mL/min and the effluent was monitored at 280nm by UV detector. The peaks were detected and compared with the standards (Puri *et al.*, 2010). The salient findings made in all the phases of the study are discussed in the next Chapter.

4.0 RESULTS AND DISCUSSION

Plants which have one or more of its organ containing substances that can be used for the therapeutic purpose, are called medicinal plants (Anubha, 2013). Medicinal plants are a group of species that accumulate different active principles, useful in treating various human or animal diseases. The long term use of herbs in medicine is a sure indication of their value and usefulness in the future. There are various active principles of spontaneous medicinal plants depending on concentration, quality, climate, area and other characteristics which make difficult their quantitative determination in phototherapy (Oancea *et al.*, 2013).

Medicinal plants are a rich source of bioactive phytochemicals or bionutrients (Mamta *et al.*, 2013). It contain some organic compounds which produce physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoid (Victor and Chidi, 2009).

The results of the study entitled **"Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC".**

In the present research, studies were carried out in three phases. The first two phases constituted the preparation of extracts; phytochemical analysis and isolation of flavonoid fractions. The third phase involved the characterization of flavonoid fractions.

In the first phase different organic extracts were prepared. Assessment of total flavonoid content and the selection of flavonoid fraction were carried out. In the second phase antioxidant potential of TdFf was evaluated by the free radical scavenging activity. In the third phase, selected flavonoid fractions in the ethyl acetate extract of *Tabarnaemontana divaricata* was characterized by chromatographic analysis. The results are presented and discussed under the following headings:

PHASE I

4.1 PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT EXTRACTS OF *Tabarnaemontana divaricata*

4.1.1. Phytochemical constituents of TdFf

4.1.2. Flavonoid contents of three different leaf extracts of *Tabarnaemontana divaricata*

PHASE II

4.2 ANTIOXIDATIVE POTENTIAL OF TdFf

4.2.1. DPPH radical scavenging activity

4.2.2. ABTS radical scavenging activity

4.2.3. Superoxide scavenging activity

4.2.4. FRAP activity

4.2.5. Hydroxyl scavenging activity

4.2.6. Hydrogen peroxide scavenging activity

4.2.7. Nitric oxide scavenging activity

PHASE III

4.3 CHARACTERIZATION OF TdFf BY CHROMATOGRAPHIC TECHNIQUES

4.3.1. TLC

4.3.2. HPTLC

4.3.3. HPLC

PHASE I

4.1 PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT EXTRACTS OF *Tabarnaemontana divaricata*

4.1.1. Phytochemical constituents of TdFf

Phytochemical is a bioactive non-nutrient plant compounds present in fruits, vegetables and other plant parts, whose ingestion has been linked to reductions in the risk of major chronic diseases. The phytochemical analysis of different solvent extracts of the leaves and flowers revealed the presence of alkaloids, flavonoid, tannins, phytosterols, cardiac glycosides, terpenoids, reducing sugars and saponins (Thiyagarajan *et al.*, 2014).

In the present study the leaves of *Tabarnaemontana divaricata* was used to analyze the active constituents such as alkaloids, tannins, flavonoids, carbohydrate, protein, phenol, steroid and saponins. The results are shown in table 1.

TABLE 1

PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT EXTRACTS OF *Tabarnaemontana divaricata*

S. No	Phytochemical constituents	Ethyl acetate	Chloroform	Ethanol	Aqueous
1.	Carbohydrate	-	+	+	+
2.	Cholesterol	+	+	-	+
3.	Protein	+	+	-	+
4.	Amino Acid	+	+	-	+
5.	Alkaloids	+	+	-	+
6.	Flavonoids	+	-	+	+
7.	Terpenoids	-	-	-	-
8.	Cardiac Glycosides	+	+	+	+
9.	Phenols	+	+	+	+
10.	Glycosides	+	+	+	+
11.	Tannins	+	+	+	+
12.	Steroids	+	+	+	+
13.	Saponins	-	+	+	-
14.	Phlobatinins	+	+	-	+

(+) presence **(-) absence**

The phytochemical screening of the ethyl acetate extract showed positive result for cholesterol, protein, amino acid, alkaloids, flavonoid, cardiac glycosides, phenols glycosides, tannins, steroids, and phlobatinins. The chloroform extract showed the presence of carbohydrate, cholesterol, protein, amino acid, alkaloids, cardiac glycosides, phenols, glycosides, tannins, steroids, saponins, and phlobatinins. The ethanol extract showed the presence of carbohydrate, flavanoid, cardiac glycosides, phenols, glycosids, tannins, steroids, saponins and phlobatinins. The aqueous extract showed the all the analyzed constituents except terpenoids and saponins.

Pushpa *et al.* (2012) reported the phytochemical analysis of the dried leaves of *Taebernaemontana coronaria* (Apocynaceae) indicated the presence of a steroid, tannins,

saponins, alkaloids and reducing sugars. Shazid *et al.* (2011) also showed the presence of steroids, tannins, saponins, gums and reducing sugar in the ethanol extract of *T. divaricata (L.)*.

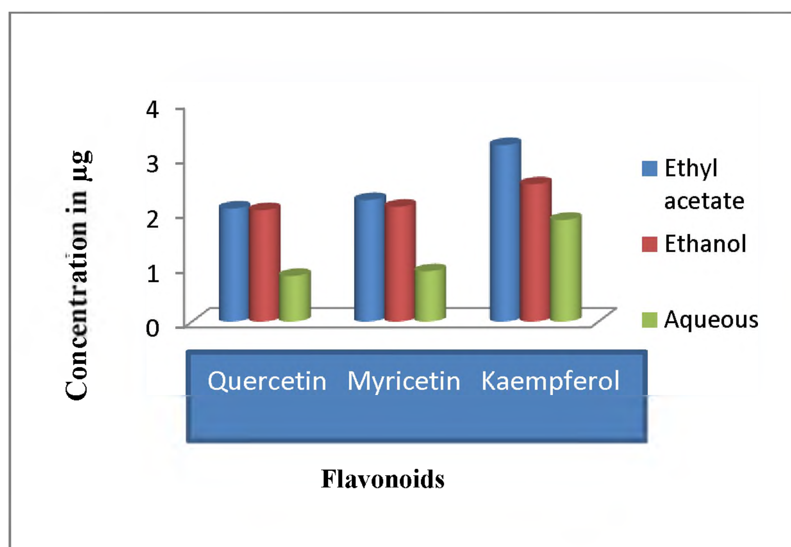
4.1.2. Flavonoid content of the leaf extracts of *Tabarnaemontana divaricata*

The quantitative analysis showed the presence of flavonoids in ethyl acetate, ethanol, and aqueous. The concentration of flavonoids were estimated in the three extracts namely ethanol, ethyl acetate and aqueous against the standard flavonoids quercetin, kaempferol and myricetin.

The figure 5 shows the flavonoid content for the three extracts. In this the highest flavonoid content was present in ethyl acetate extract of *Tabarnaemontana divaricata* leaves. So, that further analysis of free radical scavenging activity was performed in ethyl acetate extract of *Tabarnaemontana divaricata* leaves alone and is denoted as TdFf.

FIGURE 5

QUERCETIN, KAEMPFEROL AND MYRICETIN CONTENT OF TdFf LEAF



Flavonoids are free radical scavengers, super antioxidants which prevent oxidative cell damage and have strong anticancer activity. As antioxidants, flavonoids provide anti-inflammatory actions. In food systems, flavonoids can act as free radical scavengers and terminate the radical chain reaction that occurs during the oxidation of triglycerides (Awogbindin *et al.*, 2014). Sudha *et al.*, 2011 also reported 53.60 mg/g of flavonoid in the raw papino fruit.

PHASE II

4.2 Antioxidative Potential OF TdFf

Antioxidants are agents which scavenge the free radicals and prevent the damage caused by them. They can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Jananie *et al*., 2012).

Free radicals, especially reactive oxygen species (ROS), which are produced during the oxidative burst of polymorphonuclear neutrophils (PMNs), are generated by two key enzymes, namely, NADPH oxidase and myeloperoxidase (MPO). PMNs are thought to produce both extracellular and intracellular reactive oxygen species that may have an important role in the host defense against invading microorganisms. It can oxidize essential biomolecules such as DNA, proteins, and lipids leading to the development of several diseases such as cancer, cardiovascular disease, rheumatism, and neurodegenerative disorders (Rainatou, *et al*., 2015).

Antioxidative potential of the flavonoid fraction was determined by their capacity to scavenge the free radicals such as DPPH, ABTS, superoxide, FRAP, hydroxyl radical scavenging , hydrogen peroxide and nitric oxide activity.

4.2.1 DPPH scavenging activity

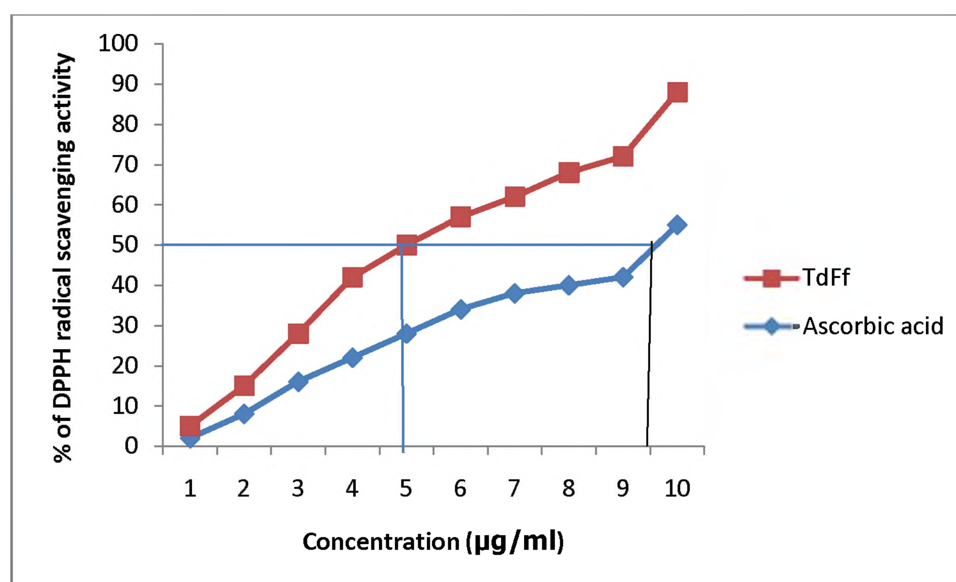
The DPPH (1,1-diphenyl-2 picrylhydrazyl) radical scavenging activity of *Tabarnaemontana divaricata* at various doses was assessed spectrophotometrically by the quantitative estimation of the discoloration of the same. The ethyl acetate extract of *Tabarnaemontana divaricata* concluded that dose dependent DPPH radical scavenging activity. When the concentration of extract increased simultaneously free radical scavenging activity also increased (Figure 6).

$$\textbf{DPPH}^{\cdot} + \textbf{antioxidant}^{-} \longrightarrow \textbf{DPPH}^{-}\textbf{H} + \textbf{antioxidant}$$

Purple color → Yellow color

FIGURE 6

DPPH RADICAL SCAVENGING ACTIVITY OF TdFf



The ethyl acetate extract of *Tabarnaemontana divaricata* showed dose dependent DPPH radical scavenging activity. From the figure 4, the inhibitory concentration of flavonoid fraction of *Tabarnaemontana divaricata* (TdFf) was found to be 4.8µg/ml (EC_{50}) which showed 50 percent scavenging of DPPH radical.

The DPPH radical has been widely used to test the ability of compounds as free radical scavengers or hydrogen donors. Promising natural antioxidant must be able to scavenge DPPH radical (Awogbindin *et al.*, 2014).

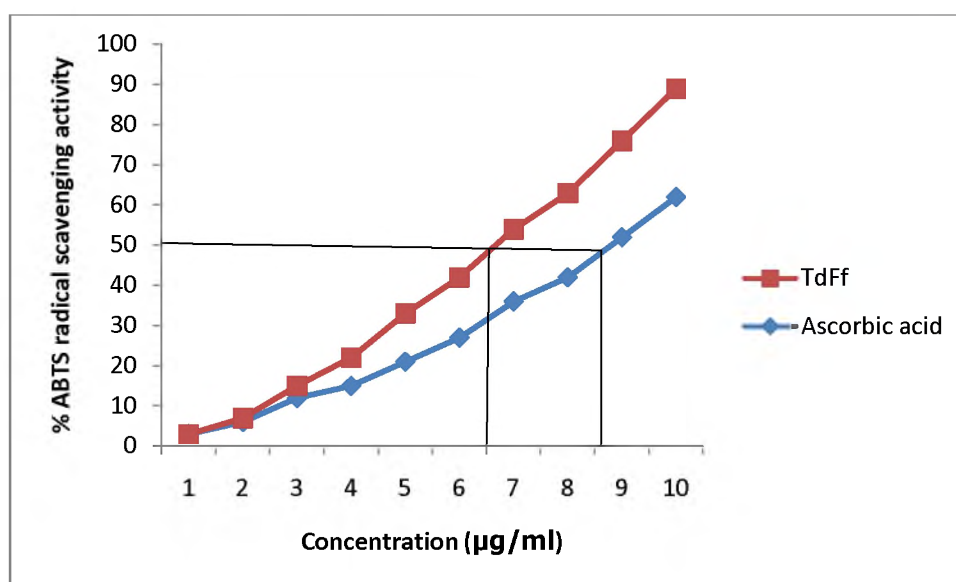
The reactivity of ethanolic extract of *M.arundinacea* extract was analyzed with DPPH, a stable free radical. DPPH picks up one electron in the presence of a free radical scavenger, the absorption decreases and the resulting discoloration is stechiometrically related to the number of electrons gained (Nishaa *et al.*, 2012).

4.2.2 ABTS radical scavenging activity

The ABTS (Azinobis (3-ethylbenzo-thiazoline-6-sulfonate) is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods. The radical scavenging activity of TdFf increased found in a dose dependent manner as shown in figure 7.

FIGURE 7

ABTS RADICAL SCAVENGING ACTIVITY OF TdFf



The radical scavenging activity of TdFf was found to be comparable with its standard ascorbic acid. The ethyl acetate extract of *Tabarnaemontana divaricata* concluded a dose dependent ABTS radical scavenging activity. The EC_{50} value of ABTS as found to be 6µg/ml and 8.2µg/ml for TdFf and standard respectively. The leaf extract was found to be two times more active than that of ascorbic acid.

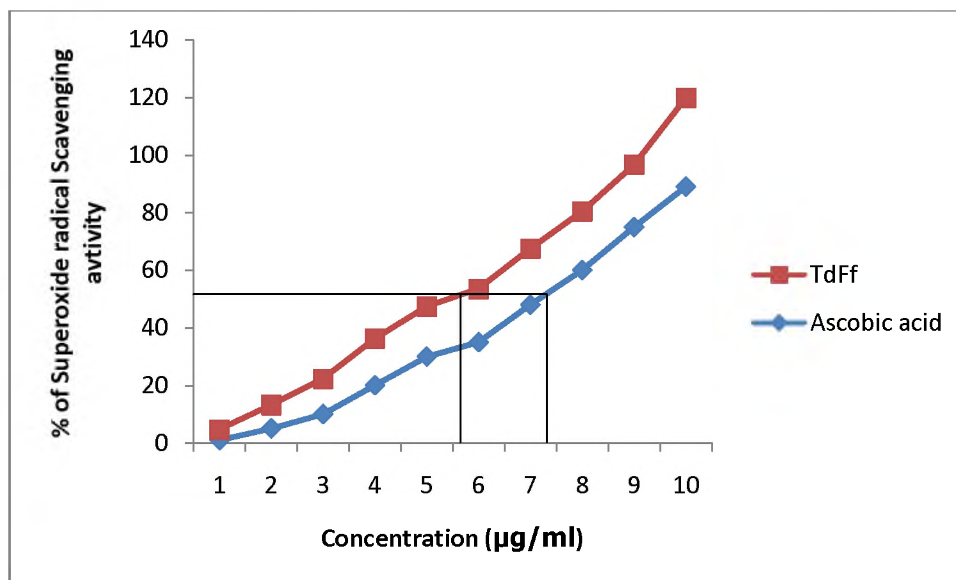
In this assay, ABTS is converted to its radical cation by addition of potassium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and ascorbic acid (Raghavendra *et al.*, 2013).

4.2.3 Superoxide scavenging activity

The ethyl acetate extract of *Tabarnaemontana divaricata* concluded a dose dependent. Superoxide scavenging activity it helps the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Then the free radical scavenging activity was increased.

FIGURE 8

SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF TdFf



The EC_{50} value of superoxide was found to be 5.1µg/ml and 7.8µg/ml for TdFf and standard respectively. Scavenging of superoxide by the leaf extract was found to be comparable to that of ascorbic acid (Figure 8).

Superoxide anion radicals are biologically quite toxic and are deployed by the immune system to kill invading microorganisms. In phagocytes, it is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. Superoxide anion radical is an oxygen-cantered radical with selective reactivity. It is produced as a result of the donation of one electron to oxygen *in vivo* (Ilhami, 2011).

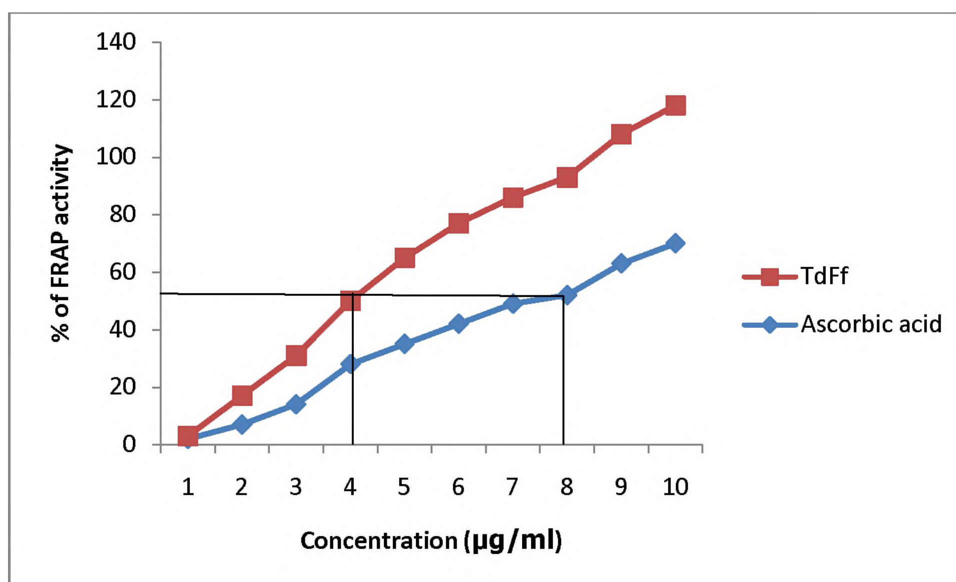
4.2.4 FRAP Activity

The extract was estimated from its ability to reduce TPTZ-Fe (III) complex to a TPTZ-Fe (II) complex. The FRAP values increased with increasing concentrations as shown in Figure 9.

The EC_{50} value of FRAP showed the percentage of scavenging activity and it was found to be 3.5µg/ml and 7.4µg/ml for TdFf and standard respectively and indicating that the leaf extract was more active than that of ascorbic acid.

FIGURE 9

FRAP SCAVENGING ACTIVITY OF TdFf



The FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex and producing a colored ferrous tripyridyltriazine [Fe^{2+}-TPTZ]. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom 26. FRAP assay treats the antioxidants in the sample as a reductant in a redox-linked colorimetric reaction27. Hence they should be able to donate electrons to free radicals stable in the actual biological and food system (Nishaa *et al.*, 2012).

FRAP also followed that the gradual increase in the scavenging activity with increasing concentration of the leaf extract. The ethanolic extract of *M. arundinacea* was found to be an effective scavenger of ABTS, DPPH, H_2O_2, and NO and also possessed a good reducing power and FRAPS activity (Nishaa *et al*., 2012).

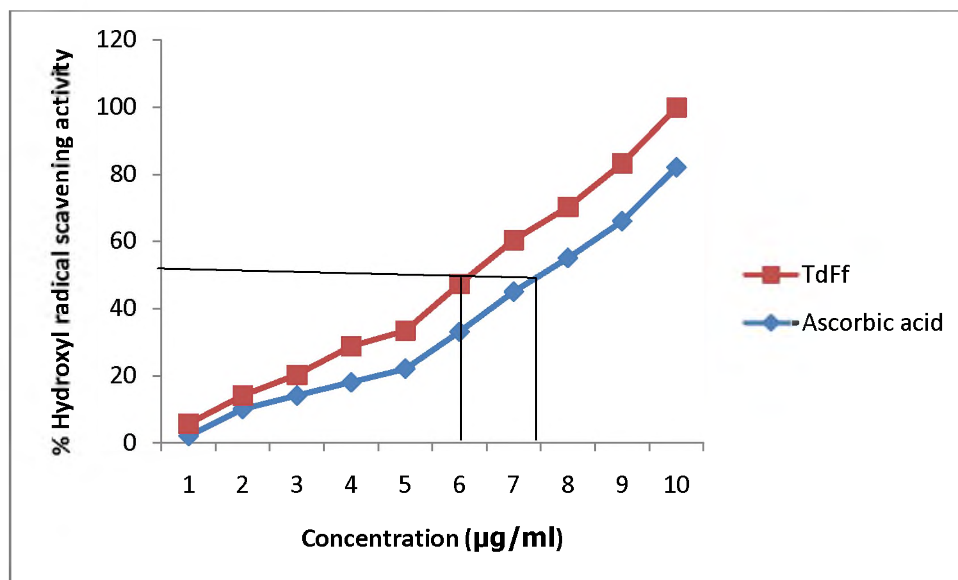
4.2.7 Hydroxyl Scavenging Activity

Hydroxyl radical was generated by ascorbate system which initiated the degradation of flavonoids. The concentration of (10-100µg/ml) TdFf were assessed the radical scavenging activity.

From the figure 10, the 50 percent effective/inhibitory concentrations (EC_{50}) of ethyl acetate extract of *Tabarnaemontana divaricata* was found to be 8.3µg/ml.

FIGURE 10

HYDROXYL RADICAL SCAVENGING ACTIVITY OF TdFf



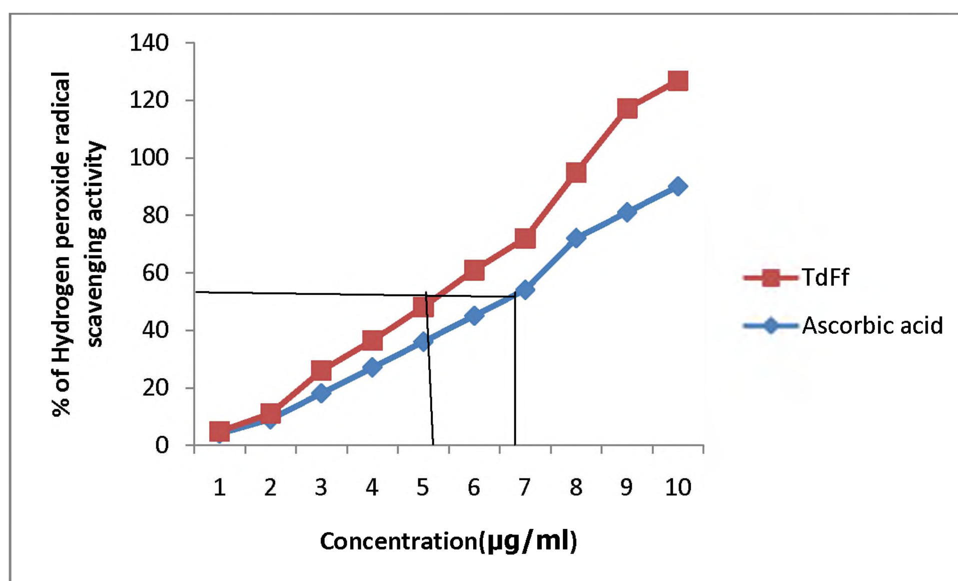
Hydroxyl radical generated by ascorbate or EDTA or H_2O_2 system initiated the degradation of deoxyribose which was inhibited significantly by ACP (Varghese *et* al., 2014).

4.2.6 Hydrogen Peroxide Scavenging Activity

There was a gradual increase in the scavenging activity of *Tabarnaemontana divaricata* extract with increasing concentration as shown in figure 11.

FIGURE 11

HYDROGEN PEROXIDE RADICAL SCAVENGING OF TdFf



The EC_{50} value of TdFf was found to be 4.7µg/ml and 6.3µg/ml for standard respectively. TdFf showed three times more effective scavenging of H_2O_2 than that of ascorbic acid.

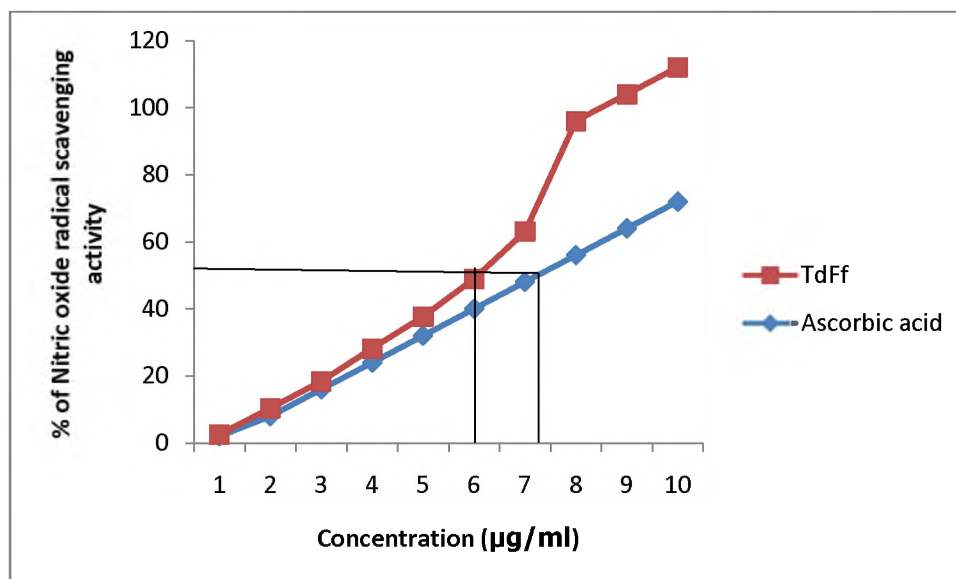
Hydrogen Peroxide radical scavenging and total antioxidant activity of *Crataegus monogyna* water and ethanol extracts of leaves, flowers and fruits. Hawthorn leaves, flowers, and berries are used in traditional medicine in the treatment of chronic heart failure, high blood pressure, arrhythmia, and various digestive ailments, as well as geriatric and anti arteriosclerosis remedies (Serhat *et al.*, 2012).

4.2.5 Nitric Oxide Scavenging Activity

Figure 12 shows a dose dependent nitric oxide scavenging effect of TpFf. Ascorbic acid also possesses dose dependent free radical scavenging activity.

FIGURE 12

NITRIC OXIDE SCAVENGING ACTIVITY OF TdFf



The EC_{50} value of TdFf was found to be 5.5µg/ml and 6.5µg/ml for standard respectively. The scavenging activity of superoxide by the leaf extract was found to be comparable to that of ascorbic acid.

The ethanolic extract of leaves of *Ocimuum basilicum* and *Trigonella foenum graecum* showed significant activity in nitric oxide radical scavenging, indicating their potential antioxidant effects (Meera et al., 2009).

Rumzhum *et al.*, 2012 observed that the extract is likely to have concentration dependent nitric oxide scavenging activity. The leaves may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body.

PHASE III

Characterization of available flavonoid compounds in *Tabarnaemontana divaricata* was carried out using the chromatographic techniques like TLC, HPTLC and HPLC.

4.3. CHARACTERIZATION OF TdFf BY CHROMATOGRAPHIC TECHNIQUES

The characterization of TdFf with the help of Thin Layer Chromatography (TLC), High Performance Thin Layer liquid Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) will provide the information regarding the components of flavonoid fractions of *Tabarnaemontana divaricata.*

4.3.1. TLC

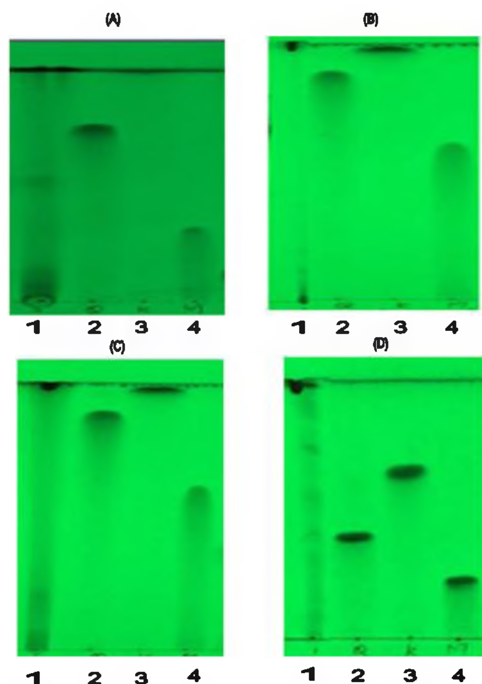
Method development for TLC is an important requirement for establishing the separation pattern of secondary metabolites. In the present study the extracts were subjected to different solvent systems and their thin layer chromatogram was analyzed.

Four different solvent systems (plate 2) were tried with different combination of solvent and composition was given as follows: Solvent system 1- Ethyl acetate: Formic acid: Water (13:3: 4), Solvent system 2- Butanol: Acetic acid: Water (8: 2: 10), Solvent system 3- Ethyl acetate: Ethanol: Water (10:2:10) and Solvent system 4- Chloroform: Ethyl acetate: Acetic acid (12:7:1). When the standard flavonoids (quercetin, myricetin and kaempferol) were spotted, each solvent system showed different RF values.

In the present study, the solvent system 4 (chloroform: ethyl acetate: acetic acid) showed good resolution with dense, compact and well separated spots of all the three standard flavonoids and TpFf (plate 2). TLC profile of ethyl acetate extract of TpFf revealed the Rf values of standard flavonoids quercetin, kaempferol, myricetin was given in the Table 3. TpFf showed well defined spots RF values 0.50, 0.95 and 0.80 which were found to be similar with that of Rf value of standard quercetin, myricetin and kaempferol respectively along with an unknown flavonoid RF as 0.60 (Table 3).

PLATE 2

TLC CHROMATOGRAM of STANDARD FLAVONOID SYSTEMS AND TdFf



Solvent system 1, (B) Solvent system 2, (C) Solvent system 3 and (D) Solvent system 4

Lane 1 – TdFf, Lane 2 - Quercetin, Lane 3 - Kaempferol and Lane 4 – Myricetin

TABLE 2

R_F VALUES OF STANDARD FLAVONOIDS IN DIFFERENT SOLVENT SYSTEMS

S. no	Solvent Systems	Quercetin (R_F)	Myricetin (R_F)	Kaempferol (R_F)
1.	Ethyl acetate: Formic acid: Water (13:3: 4)	0.46 ± 0.125	0.06 ± 0.125	0.74 ± 0.125
2.	Butanol: Acetic acid: Water (8: 2: 10)	1.35 ± 1.774	0.20 ± 0.081	0.55± 0.057
3.	Ethyl acetate: Ethanol: Water (10:2:10)	0.75 ± 0.221	0.43 ± 0.201	0.33 ± 0.955
4.	Chloroform: Ethyl acetate: Acetic acid (12:7:1)	0.95 ± 0.114	0.50 ± 0.173	0.80 ± 0.141

TABLE 3

RF VALUES OF FLAVANOIDS OF TdFf IN CHLOROFORM: ETHYL ACETATE: ACETIC ACID (12:7:1) SOLVENT SYSTEM

Spots	Rf Values
Spot 1	0.50 ± 0.173
Spot 2	0.95 ± 0.114
Spot 3	0.80 ± 0.141
Spot 4	0.60 ± 0.041

TLC profile of ethyl acetate extract of TdFf revealed information about the flavonoids and was given in the Tables 2 and 3. In the present study, the solvent system 4 showed good resolution with dense, compact and well separated spots of all the three flavonoids. The other three systems (1, 2 and 3) may not be useful in many cases, especially where speed and simplicity of procedure are important and hence are not recommended (Plate 4). For the optimization of chromatographic separation, the conditions for separation on a specified coating material can be optimized by the right choice of mobile phase, i.e., its chemical composition. This can aid in achieving satisfactory separation within a minimum migration distance i.e., in shortest possible time. When solvent system 4 was used as a mobile phase, TdFf left spots with R_F values 0.95, 0.50, 0.80 which was equal to R_F value of standard quercetin, myricetin and kaempferol respectively along with an unknown flavonoid R_F as 0.60.

Maobe *et al.*, (2012) reported that *Bidens Pilopsa* showed six spots of flavonoid in the hexane extract, using the mobile phase Hexane:Dichloromethane (50:50) . Two spot were observed in the dichloromethane extract in Hexane:Dichloromethane (90:10) as mobile phase and in ethyl acetate extract two spots were observed using Dichloromethane:Ethyl acetate (90:10) and two spots in the ethanolic extract in Ethanol:Hexane (90:10) as mobile phase whose standard R_f values is equal to 0.13,0.20,0.35,0.45,0.47,0.5,0.70,0.75and 0.85 respectively.

4.3.2. HPTLC

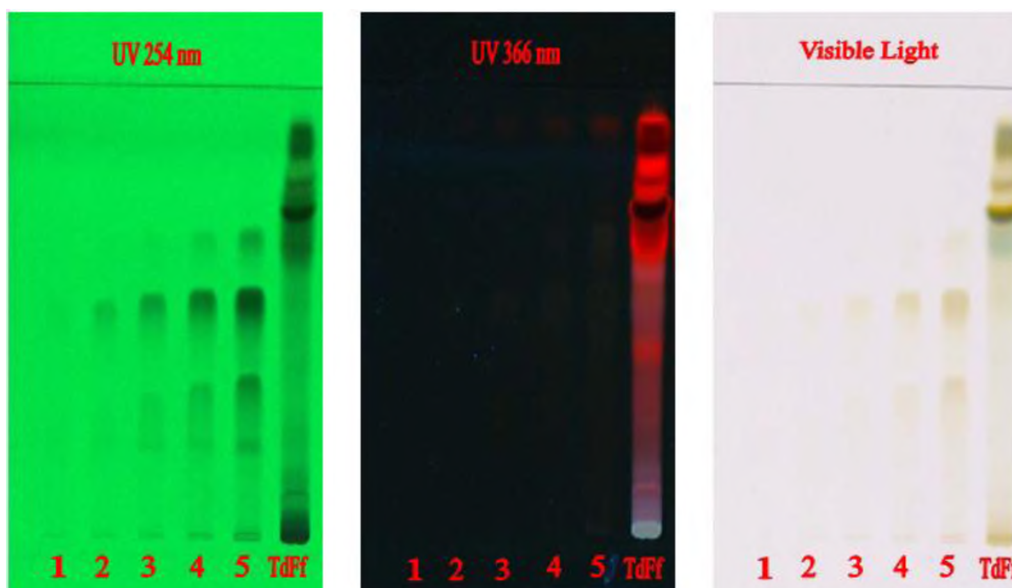
The HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials. It allows the analysis of a broad number of compounds both efficiently and cost effectively. The fractions were characterized against reference standards.

Chromatographic fingerprint is a holistic, valid and rapid method. HPTLC is a valuable quality assessment tool for the evaluation of botanical materials. It allows the analysis of a broad number of compounds both efficiently and cost effectively.

Different compositions of the mobile phase for HPTLC were tested and the desired resolution of compounds, together with symmetrical and reproducible peaks, was achieved using chloroform: ethyl acetate: acetic acid (12:7:1) as the mobile phase. The calibration curves for compounds were linear. Peak purity test were performed by comparing the spectra. The HPTLC analysis was carried out to confirm the presence of flavonoids and their derivatives in TdFf using quercetin, myricetin and kaempferol as standards (Plate 3).

PLATE 3

HPTLC CHROMATOGRAM PATTERN OF STANDARD FLAVANOIDS AND TdFf



Lane 1 to 5 – Standards

Lane 1 – 0.2µg, Lane 2 – 0.4 µg, Lane 3 – 0.6 µg, Lane 4 – 0.8 µg and Lane 5 – 1.0 µg

FIGURE 13

PEAK DENSITOGRAM OF STANDARD FLAVONOID MIXTURE

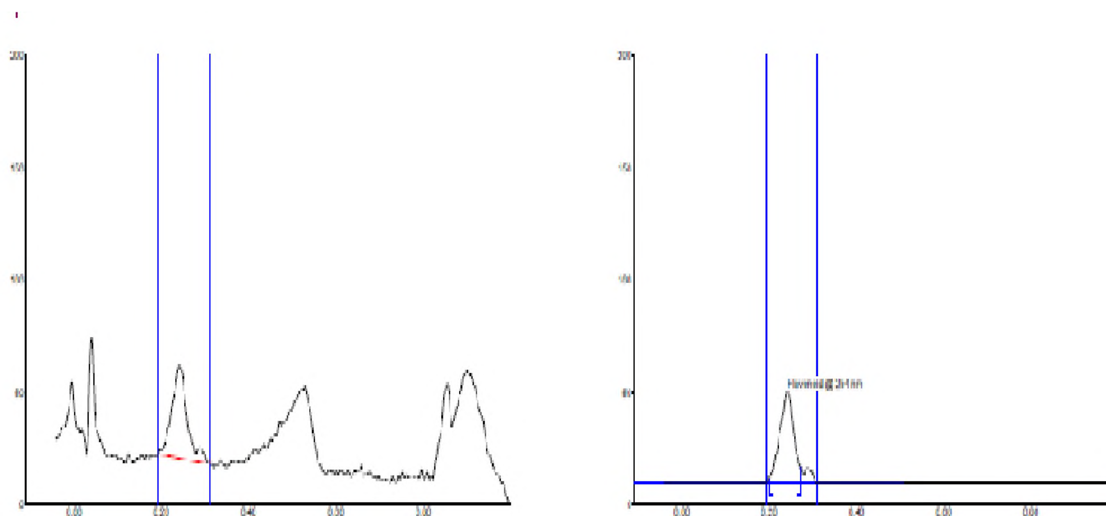


FIGURE 14

PEAK DENSITOGRAM OF FLAVONOID PROFILE OF TdFf

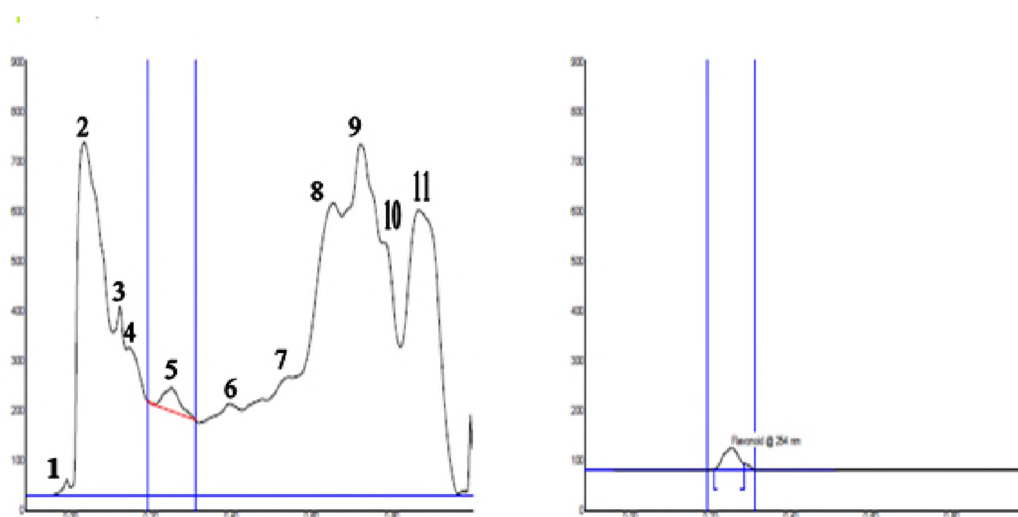


Figure 13 and 14 shows the flavonoid profile of TdFf against the mixture of flavonoids standards. The standard flavonoid Rf value was found to be 0.24. Out of 11 peaks in TdFf, 5th peak was found to be our standard flavonoid mixture as its Rf value corresponds to the Rf value of the standard mixture. The flavonoids profile, UV-visible chromatogram pattern, densitogram displays at 254 nm showed the presence of flavonoids and 10 unknown flavonoids in TdFf.

HPTLC studies have shown that it is more versatile than ordinary TLC methods, as the spots were well resolved (Jirge *et al*., 2011). This technique can be used to rectify many qualitative and quantitative analytical problems in a wide range of fields including medicines, pharmaceutical, chemistry, biochemistry and toxicology (Ramya *et al*., 2010).

The superimposed HPTLC fingerprints (figure 15) also revealed the presence of flavonoids in TdFf. HPTLC fingerprinting is proved to be a linear (figure 16) and precise. HPTLC is an accurate method for herbal formulation and can be used further in quality control of non-established herbals.

FIGURE 15

3D DISPLAY PF HPTLC CHROMATOGRAM OF TdFf and FLAVONOID STANDARD MIXTURE

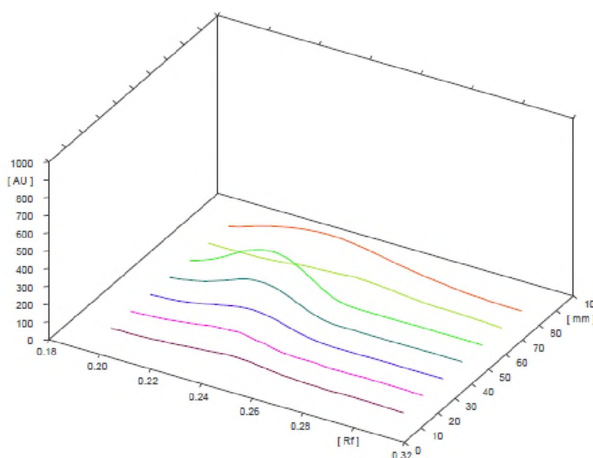
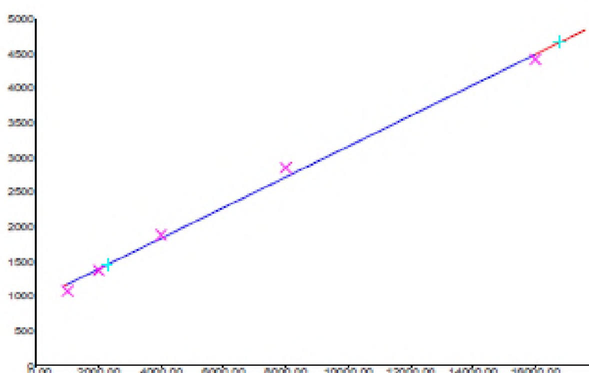


FIGURE 16

CALIBRATION CURVE OF STANDARD FLAVONOID MIXTURE



Calibration mode: Linear Y = 953.9 + 0.2209 * X r = 0.99759 Sdv = 4.66

Methanol mixture of *Clerodendrum viscosum* vent roots showed bands for flavonoid (Prasanth *et al*., 2012) in HPTLC analysis. Lakshmi *et al*., (2012) showed the presence of quercetin and rutin in the Acacia Catechu ethanolic leaf extract. The presence of alkaloids and phenolic compounds (Quercetin) in *Barleria cristata* Linn. leaves, Ferulic acid in *Lycopodium clavatum* in HPTLC fingerprint were reported by Narmadha and Devaki (2012); Srivastava *et al*., (2012) respectively.

4.3.3. HPLC

The HPLC analysis of TdFf was performed against the standard flavonoids quercetin, myricetin and kaempferol. Figure 17 shows the retention time and retention area of the standard flavonoids quercetin, myricetin and kaempferol 2.997: 6209, 3.051: 5990 and 3.061: 2491 respectively.

FIGURE 17

HPLC CHROMATOGRAMS OF STANDARD FLAVONOIDS IN METHANOL AS MOBILE PHASE

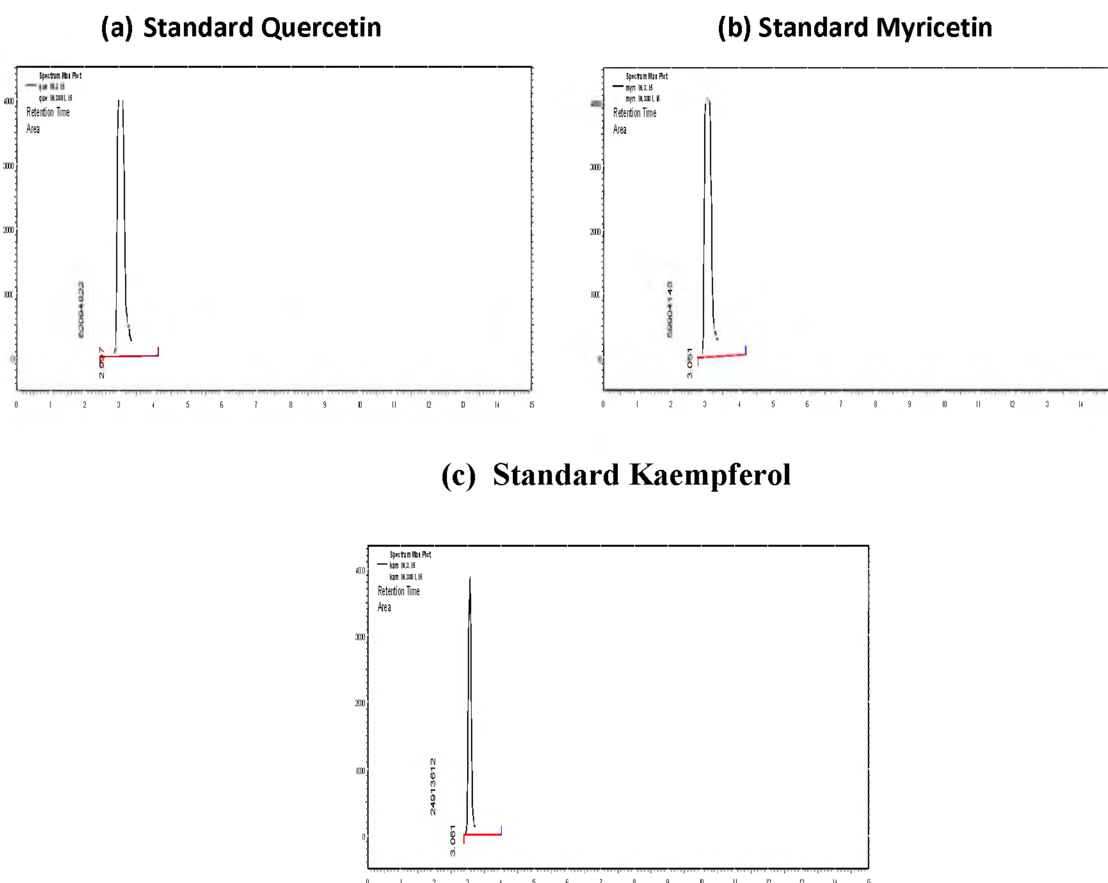
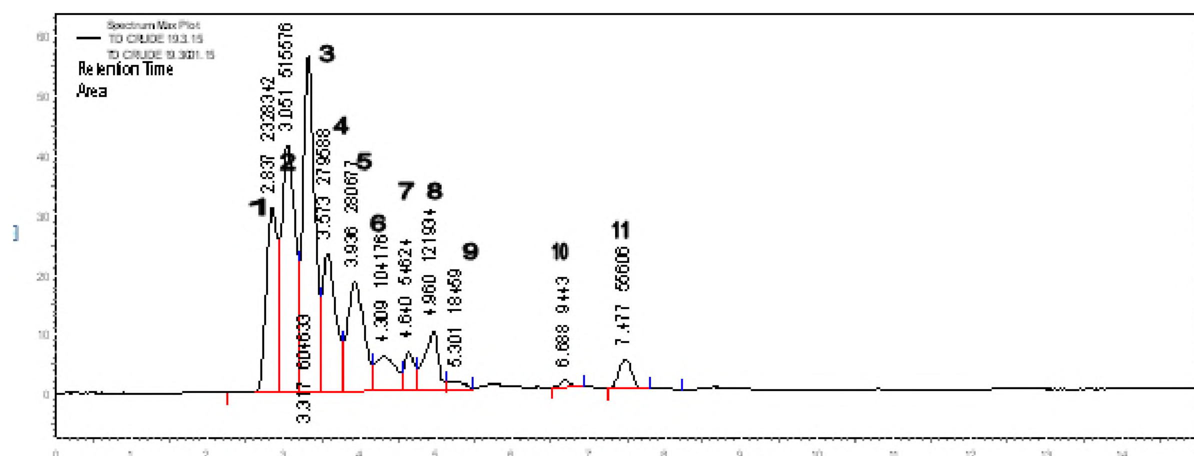


Figure 18 shows the HPLC chromatogram of TdFf with many peaks. The HPLC analysis of TdFf showed 2 major peaks (1, 2, 3) with the retention time and retention area of 2.837: 2328, 3.051:5155 and 3.317:6046 which relatively corresponds to the retention time and retention area of Quercetin, Myricetin and Keampferol. The other peaks showed the presence of unknown flavonoids or other secondary metabolites which require further research.

FIGURE 18

HPLC CHROMATOGRAM OF TdFf IN METHANOL AS MOBILE PHASE



Bisera *et al*., (2009) analyzed flavonoids content in Spanish *Sideritis* using HPLC and quantified as glycosides of isoscutellarein and hypolaetin, and luteolin, chryseriol and apigenin as aglycons, along with the less polar flavones sideritoflavone, xantomicrol, gardenin D, 8-methoxy cirsilineol and desmetilnobiletine. Naeem *et al*. (2009) reported that three flavonoids namely quercetin, kaempferol and myricetin in the methanolic extract of *Euphorbia wallichii*.

Ilina *et al*. (2008) identified the four flavonoids such as hyperoside, vitexin, orientin and homoorientin from the ethyl acetate extract of aerial parts of *Prunus serotina Ehrh* by the HPLC method. Tao *et al*. (2008) separated two major bioactive flavonoids galangin and 3-O-methyl galangin from *Alpinia officinarum* by HPLC.

Apart from the flavonoids Quercetin, Kaempferol, Myricetin, unknown flavonoids were also recorded in TLC, HPTLC and HPLC.

Characterization by TLC, HPTLC and HPLC showed the presence of quercetin, myricetin, kaempferol and unknown flavonoids.

SUMMARY AND CONCLUSION

Medicinal plants are natural source, many plants are used as the traditional medicine is help to rual communities. Plants contain wide range of chemicals constituents such as vitamins, flavonoids. The research entitled, " **Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC**" was carried out and the result were summarized as follows:

The experiments were carried out in three phases. In the first phase, the preliminary phytochemical analysis was carried out with four different solvents namely aqueous, ethanol, ethyl acetate and chloroform. All the extracts showed the presence of phytoconstituents namely phenols, glycosides, tannins and steroids. Ethyl acetate, chloroform, aqueous extract showed the presence of cholesterol, protein, amino acid, alkaloids and phlobatinins. Terpenoids were found to be absent in ethyl acetate, chloroform, ethanol and aqueous. Carbohydrate was found to present of chloroform ethanol and aqueous.

Flavonoids were found to be present in the three solvent extracts namely ethyl acetate, ethanol and aqueous. Quantitative estimation of flavonoids was carried out for the above three solvent extracts. High content of the flavonoids were present in ethyl acetate extract. Highest flavonoid content was present in ethyl acetate extract of *Tabarnaemontana divaricata* leaves. So, further analysis of free radical scavenging activity and characterization were performed in ethyl acetate extract of *Tabarnaemontana divaricata* leaves alone and is denoted as TdFf.

In the second phase, free radical scavenging activities were evaluated by following the DPPH, ABTS, Superoxide, FRAP, Hydroxyl, Hydrogen peroxide and Nitric oxide. The 50 % effective concentrations were found to be 4.8µg/ml; 6.0µg/ml;5.1µg/ml; 3.5µg/ml;8.3µg/ml;4.7µg/ml;5.5µg/ml for DPPH, ABTS, Super oxide, FRAP, Hydroxyl, Hydrogen peroxide and Nitric oxide respectively. The radical scavenging activity of TdFf was found to be comparable with the standard ascorbic acid. TdFf showed a dose dependent free radical scavenging activity confirmed the antioxidative potential.

In the third phase, the characterization of TdFf was carried out by TLC, HPTLC and HPLC. In TLC four different solvent combinations were used in order to find out the best identify the flavonoids using three standard flavonoids namely quercetin, kaempferol and

myricetin. The clear bands were observed only in the chloroform: ethyl acetate: acetic acid solvent system in the ratio12:7:1 respectively

The HPTLC analysis was carried out to confirm the presence of flavonoids in TdFf. The results showed the presence of flavonoids against reference standard flavonoids ($R_f = 0.4$) and 10 unknown flavonoids in TdFf.

The HPLC analysis of TdFf was performed against the standard flavonoids. The results showed the retention time and retention area of standards (quercetin, kaempferol and myricetin) as 2.997; 6209, 3.051; 5990 and 3.061; 2491 respectively. The HPLC analysis of TdFf showed three major peaks which correspond to the standard Quercetin, Myricetin, Kaempferol and unknown flavonoids.

To conclude the ethyl acetate flavonoids fraction of ***Tabarnaemontana divaricata*** are responsible for the antioxidative effect. So, TdFf can be recommended as antioxidant to the individuals under oxidative stress such as inflammations, ageing and other diseases.

FUTURE RECOMMENDATION

1. Study on the enzymic and nonenzymic antioxidants in the leaf extract to know the total antioxidant potential of the leaf.
2. GS-MS and FTIR studies for further characterization of flavonoid.
3. *In vitro* and *in vivo* studies to follow the anticancer activity and the immunomodulatory role of the extract.

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7.0 APPENDICES

APPENDIX I

PHYTOCHEMICAL ANALYSIS

1. Test for Carbohydrates (Deb, 2004)

To 2ml of extract 2 drops of Molisch's reagent was added and shaken well.2ml of Con. Sulphuric acid was added on the sides of the test tube. A reddish violet ring appeared at the junction of two layers indicates the presence of carbohydrates (Molisch's test).

2. Test for Cholesterol (Deb, 2004)

To 2ml of the extract 2ml of chloroform was added in dry test tube. Then 10 drops of acetic anhydride and 2 to 3 drops of Con. Sulphuric acid were added. A red rose colour changed to blue green colour (Liberman Burchard test).

3. Test for Proteins (Deb, 2004)

To 2ml of extract 1ml of 40%sodium hydroxide solution and 1 to 2 drops of 1% coppersulphate solution was added. A violet colour indicated the presence of peptide linkage of the molecule (Biret test).

4. Test for Amino acids (Kumar *et al.,* 2009)

To 2 ml of sample added 2 ml of ninhydrin reagent and kept in water bath for 20 minutes. Appearance of purple colour indicated the presence of amino acids in the sample (Ninhydrin test).

5. Test for Alkaloids (Kumar *et al.,* 2009)

To the extract added 1% HCL and drops of Mayer's reagent and Dragendroff's reagent. Organic precipitates indicate the presence of alkaloids in the sample (Wagner and Dragendorff's test).

6. Test for Flavonoids (Ayoola *et al.,* 2008)

5ml of dilute ammonia solution were added to a portion of aqueous filtrate of each plant extract followed by addition of Con. Sulphuric acid. A yellow coloration is observed which confirms the presence of flavonoids and it disappears on standing.

7. Test for Terpenoids (Ayoola *et al.,* 2008)

5ml of extract added 2 ml of chloroform and 2 ml of con.Sulphuric acid to form a monolayer of reddish brown coloration of the interface was showed to form positive result for terpenoids (Salkowsi test).

8. Test for Cardiac glycosides (Ayoola *et al.,* 2008)

5ml of extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 ml of con.Sulphuric acid. A brown ring of the interface indicated deoxy sugar characteristics of cardenolides. A violet ring might appear below the brown ring whereas the acetic acid layer, a greenish ring might appear below the brown ring whereas the acetic acid layer, a greenish ring might form just gradually throughout thin layer (Keller Kiliani test).

9. Test for phenols (Raaman, 2006)

To 50mg of the sample was dissolved in 5ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicates the presence phenolic compounds (Ferric chloride test).

10. Test for glycosides (Raaman, 2006)

To 50mg of the plant extract was hydrolysed with concentrated hydrochloric acid for 2hours on a water bath, filtered and the hydrolysate was subjected to the following test.
Borntrager's from the filtered hydrolysate, 3ml of chloroform layer was separated and 2ml of 1% ammonia solution was added to it. Pink colour indicates the presence of glycosides.

11. Test for Tannins (Edeoga *et al.,* 2005)

5ml of extract was added to few drops of 1% lead acetate. A yellow precipitated indicated the presence of tannins.

12. Test for Steroids (Edeoga *et al.,* 2005)

2ml of acetic anhydride was added to 0.5g of extract with 2ml of sulphuric acid. The color change from violet to blue or green indicated the presence of steroids.

13. Test for saponins (Kumar *et al.,* 2009)

The extract with 20ml of distilled water was agitated in a graduated cyclinder for 15minutes. The formation of 1cm layer of foam indicated the presence of saponins.

14. Test for phlobatinins (Edeoga *et al.*, 2005)

When an aqueous extract of each plant sample were boiled with 1% aqueous HCL, red precipitate was deposited which was taken as evidence for the presence of phlobatinins.

APPENDIX II

DETERMINATION OF TOTAL FLAVONOIDS

The total flavonoid content of the extracts were estimated by using aluminium chloride colorimetric technique (Chang *et. al.*, 2002) and reported as Standard flavonoids (Quercetin, Myricetin and Kaempferol) equivalents per gram of leaves. Standards in various concentrations (12.5-100µg/ml) were prepared in methanol as standard. 0.5 ml of each methanol extracts (1:10) were taken in test tubes and 1.5 ml methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water were added separately to each tubes. All the tubes were incubated at room temperature for 30 min. Optical density was measured in the spectrophotometer at 415 nm.

APPENDIX III

DPPH RADICAL SCAVENGING ACTIVITY

(Mensor *et al*., 2001)

PRINCIPLE

Disappearance of purple colour of radical DPPH by the antioxidants of the extract at 515nm in a spectrometer shows the scavenging ability.

REAGENTS

1. DPPH – 2, 2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol
3. Tris EDTA buffer,pH 7.2

PROCEDURE

The leaf extract (10-100µg/ml) was prepared in Tris EDTA buffer added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30minutes. Methanol served as the blank and DPPH in methanol, without the leaf extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 515nm in a spectrophotometer. The radical scavenging activity was calculated as follows:

$$\text{Scavenging activity \%} = 100 - \frac{A518\ (\text{sample}) - A518\ (\text{blank})}{A518\ (\text{blank})} \times 100$$

APPENDIX IV

ABTS RADICAL SCAVENGING ACTIVITY

(Shirwaikar *et al*., 2006)

PRINCIPLE:

The antioxidant effect of the leaf extracts depends on the intensity of the blue colour of the was ABTS (2,2'-azino-bis- 3-ethyl benzthiazoline-6-sulphonic acid) radical cation at 745nm.

REAGENT

ABTS Solution (7mM with 2.45mM ammonium persulfate)

PROCEDURE

ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5ml) of the different extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer and the per cent inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

APPENDIX V

SUPER OXIDE RADICAL SCAVENGING ACTIVITY

(Winterbourn *et al.*, 1975)

PRINCIPLE

This assay is based on the inhibition of the production of pink colour nitroblue tetrazolium formazon on the superoxide ion by the plant extracts and is measured spectrophotometrically at 560nm.

REAGENTS

1. EDTA (0.1M containing 1.5mg of NaCN)
2. Nitroblue tetrazolium (NBT – 1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)

PROCEDURE

Superoxide anions were generated in samples that contained in 3.0ml, 0.02ml of the leaf extracts (10-100µg/ml), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm in a spectrophotometer. The tubes were illuminated using a fluorescent lamp for 30 minutes. The absorbance was measured again at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

APPENDIX VI

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

(Benzie and Strain, 1999)

PRINCIPLE

At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) can be monitored by measuring the change in absorption at 593nm. The reaction is non - specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric to ferrous half reaction will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture.

REAGENTS

FRAP Reagent

a) Acetate buffer 300 mM pH 3.6: Weigh 3.1g sodium acetate trihydrate and add16 ml of glacial acetic acid and make the volume to 1 L with distilled water.

b) TPTZ (2, 4, 6-tripyridyl- s- triazine) (M.W. 312.34) 10 mM in 40mM HCl (M.W.36.46)

c) $FeCl_3$. $6H_2O$ (M.W. 270.30) 20 mM

The working FRAP reagent was prepared by mixing a b & c in the ratio of 10:1:1 at the time of use.

Standard Ascorbic Acid (M.W. 176.13) 1000 µ M

PROCEDURE

Sample (100 µl) is mixed with 3 ml of working FRAP reagent and absorbance (593 nm) is measured at 0 minute after vortexing. Thereafter, samples are placed at 37°C in water bath and absorption is again measured after 4 minutes. Ascorbic acid standards (100µM-1000µM) were processed in the same way.

Blank the analyzer/ spectrophotometer with Blank -Measure the OD of Standard and Test at zero minute and again after four minutes at 593 nm. Results were calculated as follows:

FRAP value of Sample (µM) = (Change in absorbance of sample from 0 to 4 minute / Change in absorbance of standard from 0 to 4 minute) X FRAP value of standard (1000 µM) (FRAP value of Ascorbic acid is 2).

APPENDIX VII

HYDROXYL RADICAL SCAVENGING ACTIVITY

(Elizabeth and Rao, 1990)

PRINCIPLE

The principle of the assay is the quantification of 2'-deoxyribose degradation product, malondialdehyde, by its condensation with thiobarbituric acid at 532nm.

REAGENTS

1. Deoxyribose (2.8mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)

PROCEDURE

The reaction mixture contained 0.1ml of deoxyribose, 0.1ml of $FeCl_3$, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH_2PO_4-KOH buffer and 20µl of plant extracts in a final volume of 1.0ml. The mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1.0 ml of TBA was added and heated at 95°C for 20 minutes to develop the colour. After cooling, the TBARS formation was measured spectrophotometrically at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the samples. The percent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative percent TBARS was calculated for the extract treated groups.

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

APPENDIX VIII

HYDROGEN PEROXIDE RADICAL SCAVENGING ACTIVITY

(Ruch *et al.*, 1989)

PRINCIPLE:

The ability of the leaf extracts to scavenge hydrogen peroxide was assessed by the difference in the absorbance at 230nm.

REAGENTS

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 (40mM) in phosphate buffer

PROCEDURE

A solution of H_2O_2 (40mM) was prepared in phosphate buffer. Leaf extracts at the concentration of 10mg-100µg/ml were added to H_2O_2 solution (0.6ml) and the total volume was made up to 3ml. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer. A blank solution containing phosphate buffer, without H_2O_2 was prepared. The extent of H_2O_2 scavenging of the plant extracts was calculated as:

$$\text{\% scavenging of hydrogen peroxide} = \frac{(A_0 - A_1)}{A_0} \times 100$$

A_0 - Absorbance of control

A_1 - Absorbance in the presence of plant extract

APPENDIX IX

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

(Green *et al.*, 1982)

PRINCIPLE

Sodium nitroprusside in aqueous solution, at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that are estimated spectrophotometrically at 546nm.

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% H_3PO_4 and
4. 0.1% naphthylethylene diamine dihydrochloride)

PROCEDURE

The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of leaf extracts (10-100µg/ml) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the extracts. The absorbance was read at 546nm against the reagent blank, in a Spectrophotometer.

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test})}{\text{Control}} \times 100$$

Phytochemical analysis and antioxidative potential of total flavonoids of ethyl acetate extract of *Tabarnaemontana divaricata* leaf and its characterization by TLC, HPTLC and HPLC

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(13PBT006)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

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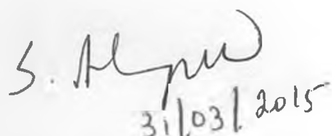
In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

March, 2015



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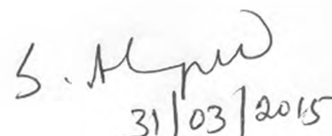

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