Delonix regia – A HEDGEROW SOURCE OF PHYTOCONSTITUENTS

A PROJECT REPORT

Submitted by

T.**PRIYA DHARSHINI (1110204033)**

P.SOWMIYA (1110204046)

D.VEENA VARSHA (1110204057)

in partial fulfillment for the award of the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OFTECHNOLOGY

COIMBATORE-641049

(An Autonomous Institution Affiliated To Anna University, Chennai)

APRIL 2015

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE 641 049

(An Autonomous Institution Affiliated to Anna University, Chennai)

BONAFIDE CERTIFICATE

Certified that this project report **"*Delonix regia* – A HEDGEROW SOURCE OF PHYTOCONSTITUENTS"** is the bonafide work of "**T.PRIYA DHARSHINI (1110204033), P.SOWMIYA (1110204046) and D.VEENA VARSHA (1110204057)"** who carried out the project work under my supervision.

SIGNATURE

Dr. A.Manickam

HEAD OF THE DEPARTMENT

Department of Biotechnology

Kumaraguru College of Technology

Chinnavedampatti

Coimbatore-641049

SIGNATURE

Dr.R.Baskar

SUPERVISOR

Associate Professor

Department of Biotechnology

Kumaraguru College of Technology

Chinnavedampatti

Coimbatore-641049

_____

INTERNAL EXAMINER

_____

EXERNAL EXAMINER

ACKNOWLEDGEMENT

We would like to express our heartfelt gratitude to our guide and class advisor, Dr.R.Baskar, Associate Professor, Department of Biotechnology, KCT for guiding us through the ropes of the subject and motivating us to overcome our hardships.

We thank Dr.A.Manickam, Professor and head, Department of Biotechnology, KCT for his support.

We extend our sincere thanks to Dr. R. S. Kumar, Principal, KCT and the management for providing us with all the required facilities.

We are grateful to Dr. T. Sathishkumar, Assistant Professor(SRG) and Mr. S. Sivamani, Assistant Professor(SRG) for sparing their valuable time and giving us necessary inputs on TLC and RSM, respectively. We extend our thanks to the members of review committee, Dr.P.Ramalingam and Mr.M.Shanmuga Prakash for their invaluable suggestions at the right time.

We thank Dr. G. Sudha, Associate Professor and Head-in-charge, Department of Biochemistry, Periyar University, Salem for helping us with statistical computation.

We earnestly thank Ms. G. Poorani, JRF, DBT Project, and Mr. P. Ram Kumar, II, M.Tech., Department of Biotechnology, KCT for guiding us through the nuances of techniques used.

Thanks to Botanical Survey of India, TNAU campus for authenticating our plant specimen, Dalmia Research Institute for anthocyanin profiling by HPTLC and PSGCAS for helping us with FTIR.

We are indebted to our friends and family for standing by us throughout our academic pursuit.

[T.PRIYA DHARSHINI] [P. SOWMIYA] [D.VEENA VARSHA]

ABSTRACT

The human body is so delicately woven that it requires a fine balance of various constituents for its resistance against diseases. Free radicals are detrimental to the body since they affect this balance. They are produced as a result of oxidation reactions that take place in the body. The activity of free radical intermediates in the chain reactions may be refrained by natural antioxidants present in the body or dietary intake and hence different age-related and other disorders caused by Reactive Oxygen Species (ROS) are averted. The abundant presence of antioxidants in different forms, i.e., enzymatic, non-enzymatic and phytochemical forms is well-known. *Delonix regia*, commonly referred as "Gulmohar" in India, is a rich source of antioxidants. Ten extracts of the leaf sample were prepared using five different solvents, i.e., Acetone, ethyl acetate, hexane, methanol and petroleum ether by two different methods, soxhlet extraction and extraction in shaker at room temperature. A broad spectrum of assays was performed to evaluate *in vitro* and endogenous antioxidant potential of the samples. Characterisation for identifying phytochemicals was also performed using techniques like TLC, HPTLC and FTIR. Further, the phytochemical content in the extract was optimized by Response surface methodology. The overall results proved that the method of extraction and solvent used are highly influential factors in the estimation of antioxidant potential. The tests suggest *D.regia* as a trailblazer among hedgerow sources with massive antioxidant potential.

Keywords: *Delonix regia,* antioxidant, free radicals, phytochemicals, solvents

TABLE OF CONTENTS

4.3.2 DPPH Radical Scavenging Assay

4.3.3 ABTS Radical Scavenging Assay

4.3.4 Reducing Power Assay

4.3.5 CUPRAC Assay

PHASE II

4.4 Phytochemical Analysis

4.4.1 Estimation of Flavonoids

4.4.2 Estimation of Total Phenols

4.4.3 Estimation of Anthocyanins

4.4.4 Estimation of Tannins

4.4.5 Estimation of Alkaloids

4.4.6 Estimation of Sterols

PHASE III

4.5 Thin Layer Chromatography (TLC)
of Leaf Extracts

4.5.1 Bioautography of Leaf Extracts

PHASE IV

4.6 Antimicrobial Activity

PHASE V

4.7 High Performance Preparative Thin Layer Chromatography (HPTLC) of leaf extracts

4.8 Fourier Transform Infrared Spectroscopy (FTIR)

PHASE VI

4.9 Orthogonal Design of Experiments

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
µm	Micrometer
AAE	Ascorbic Acid Equivalence
ABTS	2,2 – Azobis (3- ethlybenzothiazoline-6-sulfonic acid)
ANOVA	Analysis Of Variance
ATP	Adenosine Tri Phosphate
BFI	Brittle Factor Index
BHA	Butylated Hydroxide Anisole
BHT	Butylated Hydroxide Toulene
CAT	Catalase
CSFR/DT	Crushing Strength Friability / Disintegrating Time Ratio
CUPRAC	Cupric Reducing Antioxidant Capacity
DPPH	1,1 – Diphenyl – 2 – Picryl - Hydrazyl
ETC	Electron Transfer Chain
FCR	Folin Ciocalteau Reagent
FRAP	Ferric Reducing Antioxidant Potential
FTIR	Fourier Transfer Infrared Spectroscopy
GPX	Glutathioine Peroxidase
HCl	Hydrochloric Acid
HPTLC	High Performance Preparative Thin Layer Chromatography
MBTH	3-Methyl-2-BenzoThiazolinoneHydrazoneHydrochloride

MDA	Malondialdehyde
mg	Milligram
ml	Millilitre
NaOH	Sodium Hydroxide
Ni	Nickel
PDRA	Pods of *Delonixregia* Ash
PTLC	Preparative Thin Layer Chromatography
PUFA	Poly Unsaturated Fatty Acid
ROS	Reactive Oxygen Species
SOD	Superoxide Dimutase
SPI	Sodium meta Per Iodate
TAC	Total Antioxidant Capacity
TBHQ	Tertiary Butylated Hydroxide Quininone
TCA	Trichloro Acetic Acid
TCA	Trichloro Acetic Acid
TLC	Thin Layer Chromatography

1.0 INTRODUCTION

1.0 GENERAL

Ageing and age related problems are natural phenomena that tend to bother a majority of human population. These are caused mainly due to the accumulation of free radicals in the body. There exists a wide spectrum of bodily disorders/diseases associated with free radicals. They include arthritis, diabetes, arthrosclerosis, neurological disorders like Alzheimer's disease, connective tissue related disorders.

Free radicals, by definition, are molecules or atoms that possess lone pair of electrons in their outermost orbital. Electrons generally exist in pairs in the orbitals of the atoms/molecules and complete the duplet or octet structures. On the contrary, when they exist as unpaired electrons, the atoms/molecules bind to lose or gain an electron from one of their neighboring counterparts to complete the structure.

When electron transfer occurs between atoms, the balance is disturbed and this leads to subsequent chain reactions. The chain reactions continue for a specific period of time resulting in free radicals or Reactive Oxygen Species (ROS) like superoxide, hydrogen peroxide, nitric oxide.

A classic example of the effect of free radicals in the body is malondialdehyde (MDA) formation. When ROS like hydrogen peroxide attack the lipid bilayers of the cells, lipid peroxidation o the PUFA (poly unsaturated fatty acids) occurs. The resulting product is malondialdehyde. Malondialdehyde is considered as a potential mutagen and causes diverse class of diseases. Studies suggest that MDA may be a cause of keratoconus, keratopathy, and arthritis.

The damage caused by free radicals produced in the body may be counteracted by the antioxidants. 'Antioxidants', as the name suggests, act against the radicals produced in the body. The antioxidants are of *in vivo* or dietary in origin. Leaves, fruits and flowers of plants are believed to be rich sources of antioxidants. The natural sources of antioxidants are consumed by the human race to acquire the benefits that the scientific community presents.

Colored fruits, vegetables and flowers have garnered attention for their rich antioxidant potential. Antioxidants may be present in different classes like photochemicals (alkaloids, flavones, iso flavones, flavonoids), enzymes and non-enzymes. Enzymes include catalase, superoxide dismutase while non enzymes include vitamins A, C, E.

Various plants have been studied by the researchers over the world for their antioxidant capacity. *Delonix regia* commonly referred as "Gulmohar" tree is one such plant. It is hedgerow tree found across the world. It is widely found in tropical countries like India, Indonesia, Bangladesh, Pakistan, China, Yemen, Nepal, Hong Kong, etc… The past researches on this angiosperm suggest that it has good prospects of becoming a major natural antioxidant source.

Delonix regia (Gulmohar) belongs to the family, Fabaceae and falls under the sub family, Caesalpiniodaceae. It is also called as "Flame tree" for its flamboyant flowering pattern.The flowers of this tree appears as flame due to their orangish red colour.It is also referred as the Royal Poinciana tree. The tree has bright green foliage as its marked feature.

The trees usually grow up to a height of 5-12 meters, providing shade on the pavements. The pods are generally green in colour when tender and turn dark brown, when mature.

D.regia has been vastly exploited for the health and economic benefits that it provides. *D.regia* has anti-microbial, anti-viral, anti-cancerous, anti-diabetic, gastro protective, hepato protective properties. It has also been studied for environmental purposes like dye dehydration, carbon sequestration and air purification.

Fig 1. Morphological Features of *Delonix regia*

D.regia is believed to contain antioxidants as flavonoids, anthocyanins, phenolic acids and so on. If the antioxidant potential of *D.regia* could be systematically studied in different solvent extracts, it can also be enhanced by Response Surface Methodology.

The antioxidant potential of the *D.regia* leaves could be studied by a variety of antioxidant assays like DPPH(2,2-diphenyl dipicryl hydrazyl) assay, ABTS (2,4 Azobis Benzothiazoline assay, TAC (total antioxidant

capacity assay), Metal chelating assays like CUPRAC (Cupric reducing antioxidant capacity) assay, FRAP (Ferric reducing antioxidant power) assay and Reducing power assay. Phytochemical assays for total flavonoids, alkaloids and phenols also may be performed.

Delonix regia, when studied for its phytochemical content, showed abundant reserve of flavonoids and anthocyanins. (Shah *et al*., 2009). In general, these phytochemicals are believed to possess curative effects for several common health issues and age-related complications. Phytochemical antioxidants, naturally present in dietary consumption are highly active in neutralizing the free radicals produced by redox reactions. The presence of these phytochemical antioxidants can be confirmed by various separation techniques.

D.regia is also proven to possess anti-microbial effects on several microbial species (Parekh *et al*., 2005). The leaf extracts of this plant may be tested for anti-bacterial activity using different species of gram negative and gram positive bacteria. Zones of inhibition are observed on the plate to identify the anti-bacterial effect of different solvent extracts of *D.regia* leaves.

Subjecting antioxidant sources to various lab techniques like Preparative Thin Layer Chromatography (PTLC), High Performance Thin Layer Chromatography (HPTLC) and Fourier Transformed Infrared Spectroscopy (FTIR) offer a clear insight into the structural features of the various constituents present in them.

Preparative TLC can be performed to understand the phytochemical constitution of different solvent extracts of *D.regia* leaves. The

chromatogram may be analysed and the R_f values should be noted to recognize each of the phytochemicals in particular.

High performance TLC is preferred for profiling one or more phytochemicals, in specific, based on the researchers' choice.

Fourier Transformed IR spectroscopy is advisable for identification of different functional groups present in the solvent extracts of leaves.

Once the leaf extracts have been thoroughly studied by the above mentioned techniques, it is wise to enhance the phytoconstituents present in them by orthogonal design of experiments (Nayak and Rastogi, 2013). This leads to increased antioxidant capacity and higher yield.

The antioxidants isolated from *D.regia,* can be incorporated in potions or nutrient syrups. If this could be brought about successfully, the pharmaceutical industry may experience a drastic shift towards natural antioxidants. They may serve as the elixir of life, promising tangible solutions to the most pressing health and environmental issues.

2.0 OBJECTIVES

1. To evaluate and compare the *in vitro* antioxidant potential of leaves of *Delonix regia* in different solvent extracts by both hot and cold extraction methods .
2. To determine and compare phytochemical constituents in the leaves of *Delonix reg*ia in different solvent extracts by both hot and cold extraction methods.
3. To determine the antibacterial effect of leaf extracts of *D.regia.*
4. To identify the phytochemical antioxidants in the leaf extracts by PTLC and HPTLC.
5. To structurally elucidate the phytochemical constituents in the leaf extracts by FTIR.
6. To optimize the phtyochemical antioxidants in leaves by orthogonal design of experiments.

3.0 REVIEW OF LITERATURE

3.1 Free radicals-an Introduction

Free radical simply implies a lone pair of electrons which are produced within the body as a result of metabolic activity. These are highly unstable molecules which are potent enough to damage the lipid bilayer of the cell membrane and thereby causing irreparable cell damage. The implications range from ageing problems to dreadful cancer.

Reactive Oxygen Species (ROS) is the common term used for free radicals and it includes radicals of oxygen, hydrogen peroxide, hydroxyl and super oxide. ROS are formed when the balance between the generation of free radicals and their antagonist antioxidants is disturbed. The generation of ROS is sometimes triggered by exogenous substances. In order to avert the damage caused due to this imbalance, an innate immunological response will be conferred by the immune system (Vaishali *et al*., 2011).

Apart from the defense system within our body, antioxidants play a prominent role in neutralizing the free radicals and thereby reinstating the balance between the formation and the neutralization of free radicals. This phenomenon will prevent the cells from undergoing oxidative stress (Vivek *et al*., 2013).

The oxidative stress will in turn have devastating impact on essential biomolecules like proteins, carbohydrates, lipids and nucleic acids. It is evident that the damage on these bio molecules could prove to be the genesis of serious medical complications like neurological disorder, atherosclerosis, cardiovascular diseases and so on (Duan., 2006).

3.2 Sources of ROS

The generation of ROS is mainly due to the exogenous and endogenous factors.

3.2.1 Exogenous factors

It includes pollutants of air and water, lethal dosage of drugs, constituents present in cigarettes, several classes of antibiotics, radiations, pesticides and fried foods.

The intermediates produced when ionizing radiation interacts with water molecules give rise to radicals of oxygen, hydrogen peroxide, hydroxide or simply oxygen molecule. The process is termed is radiolysis. The fact that the human body contains large volume of water makes it liable to this process when exposed to ionizing radiations and produces ROS through a series of sequential events.

3.2.2 Endogenous factors

This includes several intracellular mechanisms and depends upon the type of cells and tissues. The major sources are NADPH oxidase (NOX) complex, endoplasmic reticulum and mitochondrial peroxisome .

3.3 ROS in mitochondria

It is known that mitochondria convert the energy in the cell and store it in form of Adenosine Tri Phosphate (ATP). The synthesis of ATP by oxidative phosphorylation involves Electron Transport Chain (ETC). It is through this process that protons are transported across the inner membrane of mitochondria where a series of oxidation –reduction reactions takes

place. The final product being oxygen under normal conditions will be reduced to water. But sometimes oxygen will be incompletely or prematurely reduced to super oxide radical. This radical is potent is enough to inactivate specific important enzymes and also will trigger lipid peroxidation. When too much damage is caused to mitochondria, the cell eventually will activate apoptosis or Programmed Cell Death (PCD) (Murphy, 2008).

3.4Pro-oxidants

These are chemical constituents that kindle oxidative stress by producing ROS or by impeding the innate antioxidant mechanism. The damage caused due to the free radicals generated by pro oxidants could prove fatal for cells and tissues (Schwartz,1996).

The fact that a chemical substance could possibly be an antioxidant or a pro-oxidant relies upon certain conditions like concentration of that compound and the presence of oxygen or transition metals like manganese iron and copper. Thermodynamically, reduction of molecular oxygen or other molecules to free radicals is spin forbidden. Initially this results in the formation of singlet oxygen directly or via reduction of transition series metals and finally producing molecular oxygen or hydrogen peroxide.

3.4.1 Metals as pro oxidants

Transition metals acts as pro oxidants will lead to series of chronic conditions the symptoms of which are often relatable with the common symptomology. Some of the conditions include hemochromatosis, Wilson’s disease (increased copper level) and chronic magnesium. It has been

proved that these diseases are linked with human Parkinsonism (Bondy *et al*., 1998).

3.4.2 Vitamins as pro oxidants

Vitamins which are predominantly antioxidants can also reduce metal ions and thereby leading to the synthesis of free radicals. The reduced metal ions can be oxidized and then re-reduced to produce ROS by a process called 'Redox cycling'. However, the pro-oxidant capability of antioxidant vitamins is currently under study (Carr and Frei,1999).

3.5 Relation between MDA and free radicals

Malondialdehyde (MDA) consists of three highly reactive aldehyde groups which are synthesized usually as byproducts during the peroxidation of PUFA and arachidonic acid. MDA has the tendency to readily react with the functional groups of protein, lipids and DNA. This MDA-protein complex will have properties which will deviate from the normal characteristics of a protein.

The significance of MDA is that it can be used to detect the presence or measure the extent of lipid peroxidation. This is conferred by the reactive carbonyl compounds present in MDA which are derived as a result of lipid peroxidation. And hence, MDA levels could be used a marker to reveal the oxidative stress in the cells and tissues.

The adducts formed between MDA and DNA will pave way for mutagenic or carcinogenic complications (de Souza *et al*., 2014).

3.6 Body defense

To sublime the effect of free radicals produced within the body, there are a few metabolic pathways which act as defense against free radical formation. These mechanisms can be brought about with help of enzymes like Superoxide Dismutase (SOD), catalase (CAT) and Glutathioine peroxidase (GPX). Apart from the enzymes, non enzymatic molecules like thiols, disulphide bonding and thioredoxin play an eminent role in antioxidant defense system.

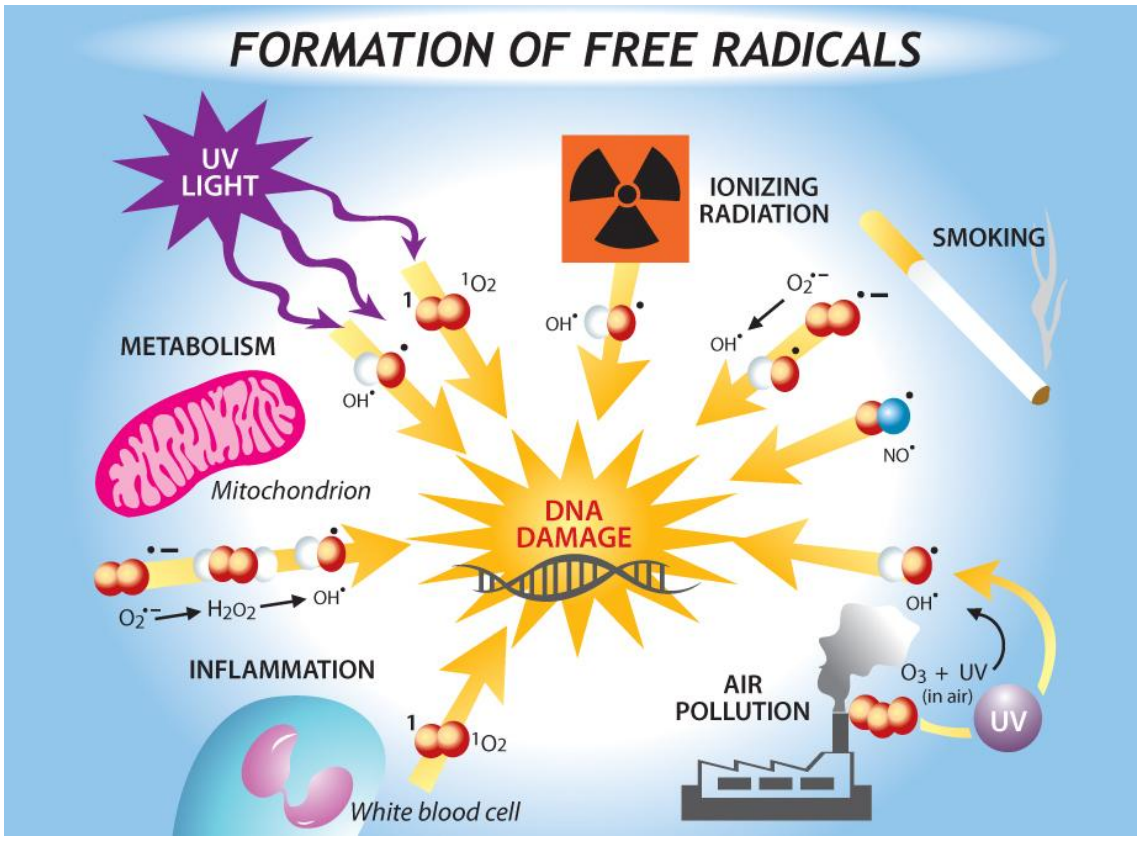
Also, compounds found in diet, prove to be effective against free radical formation. Compounds like β-carotene, ascorbic acid, α-tocopherol exhibit high antioxidant activity (Aqil *et al.*, 2006).

3.7 Role of free radicals

Free radicals at high concentrations are undeniably dreadful for cells. At moderate concentrations, they are observed to function as regulatory factors in several important signaling pathways. The cell responses induced by the ROS protects the cell from undergoing oxidative stress and thereby reinforcing ‘redox homeostasis. Thus ROS have evolved to function as signaling molecules for various physiological functions like vascular tone regulation, production of erythropoietin and signal transduction from membrane receptors.

The ROS involved in physiological functions are typically produced by the isoforms of regulating enzyme, NADPH oxidase. When this enzyme is stimulated in an uncontrollable manner, it will lead to synthesis of large amount of ROS.

There occurs stimulation of β-adrenergic receptors which leads to excessive synthesis of free radicals in the mitochondria, consequently contributing to stronger contraction of respective cells (Alfadda and Sallam, 2012).



3.8 Antioxidants

Antioxidants are molecules which are capable of stabilizing the free radicals and protecting the cell from the adverse effect of oxidative stress. They do this by hindering the initiation or the propagation of oxidation chain reactions. These molecules makes use of free radicals in a constructive manner (Mishra *et al.*,2011).

3.9 Types of antioxidants (Gupta and Sharma, 2006)

3.9.1 Natural antioxidants

There occurs a wide variety of antioxidants in nature which are unique in terms of composition and properties. It is of the following types:

3.9.1.1 Enzymes

Superoxide Dismutase, catalase, glutathione peroxidase found in plasma transform ROS into stable compounds and prevent damage to the cells.

3.9.1.2 High molecular weight compounds

It includes several proteins like albumin, ceruloplasmin and transferrin. These proteins are involved in the metal catalysis of free radicals.

3.9.1.3 Low molecular weight copmounds

It is of two types:

-Lipid soluble antioxidants

Tocopherol, Quinines, Bilirubin and other polyphenols.

-Water soluble antioxidants

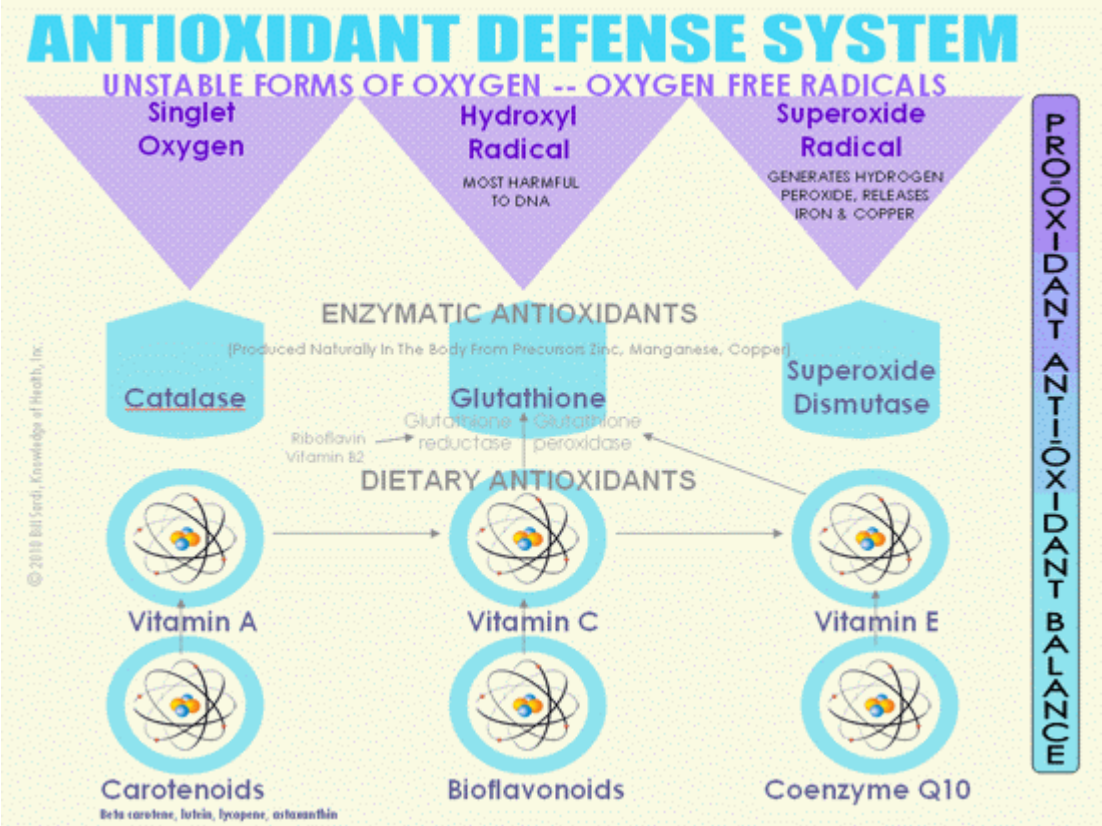
Ascorbic acid and Uric acid.

3.9.1.3.1 Mineral

Selenium, copper, manganese, zinc,etc., Chromium is also widely used in the formulation of drugs the recent days.

3.9.1.3.2 Vitamins

Vitamins A, vitamin C and vitamin E play significant role in inhibiting the lipid peroxidation (Sies *et al*.,1992).



3.9.2 Plants as antioxidants

The antioxidant property of plants is mainly attributed to phytochemical compounds such as flavonoids, phenolic acids, etc... (Subhendu *et al*., 2011).

The antioxidant based drugs derived from plant sources have been widely used in the prevention and treatment of various diseases like stroke,

diabetes, Alzheimer's disease and cancer over the past few decades. Realizing the significance of antioxidants derived from plants, many studies have been conducted on various types of plants. One such study was carried out on Gulmohar tree i.e., *Delonix regia* (Shabir *et al*., 2011).

Various antioxidant assays were performed to determine the anti oxidant property of the plant, *Delonix regia* (Aqil *et al*., 2006).

Based on how significantly the antioxidants delay or prevent the oxidation of bio molecules, it is divided into three categories:

3.9.2.1 First line defense antioxidants

Enzymes like SOD,CAT,GTX , glutathione reductase and minerals like Selenium, Manganese, Copper and Zinc constitue the first line defense antioxidants.

3.9.2.2 Second line defense antioxidants

Glutathione (GSH), Vitamin C, Vitamin E, Uric acid , Bilirubin, Carotenoids, Albumin and Flavonoids fall under this category.

3.9.2.3 Third line defense antioxidants

Complex group of enzymes which are involved in repairing damaged DNA, protein, oxidized lipids i.e., lipases, proteases, methionine sulphoximide reductase, transferase and DNA repair enzymes (Gupta and Sharma, 2006).

3.9.3 Synthetic antioxidants

These are synthetically produced compounds which are approved by FDI and hence can be administered in the form of dietary supplements. Such antioxidants include BHA(Butylated Hydroxide Anisole), TVHQ(Tertiary Butylated Hydroxide Quininone) and BHT (Butylated Hydroxide Toulene) (Shabir *et al*., 2011).

3.10 *Delonix regia* as an antioxidant

A few antioxidant assays were performed for *Delonix regia.*

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was performed and the absorbance was read at 517nm.

ABTS (2, 2′-azino bis [3-ethylbenzothiazoline-6-sulphonic acid]) radical scavenging activity assay was performed to find the efficacy of the scavenging activity of the extracts obtained from the leaves and flowers. The absorbance was read at 730nm with Ascorbic acid as standard (Vaishali *et al*., 2011).

Superoxide anion scavenging active assay involving the reduction of superoxide anion radicals was carried out. To generate the superoxide anion, Phenozine Methosulphate Nictotinamide (PMS-NADPH) was used. The reaction mixture was incubated at 25°C for 5 minutes and the absorbance was read at 560nm against blank samples (Subhendu *et al*., 2011).

Reducing power assay was carried out to assess the reducing capability of the leaf and flower extract of *Delonix regia.* The absorbance was read at 700nm. Ascorbic acid was used as reference standard. Increase

in absorbance value implies increased concentration of the extracts and thereby increased reducing power(Aqil *et al*., 2006).

3.10.1 Quantification of total phenol and flavonoid content of *Delonix regia*

The preliminary phytochemical screening of Delonix regia extract showed that it contains proteins, amino acids, cardio glycoside, alkaloids, flavonoids, tannins and phenolic compound. However the content of flavonoid was higher than that of others (Shanmukha *et al*., 2011)

3.10.2 Total phenolic activity

The phenolic contents in the flowers and leaves of the Gulmohar plant were estimated by Folin-Ciocalteau Reagent (FCR) method. However slight modifications have been done to the original method. The FC reagent was diluted and to the various concentrations of plant sample along with the sodium bicarbonate was incubated at dark for 30 minutes. The optical density was measured colourimetrically at 765nm. A standard graph was plotted between the optical density reading (at 765nm) and varying concentration of the plant extract of *Delonix regia.* The results were expressed as Gallic acid equivalent per gram of dry weight (mg GAE/g dw). (Shewale *et al*., 2011)

3.10.3 Carotenoids from *Delonix regia*

The presence of carotenoids from various floral parts of *Delonix regia* was studied to understand the biogenesis and the role of carotenoids in the flower. The floral parts include petals, sepals, filaments and anthers.

The carotenoids extracted from various floral parts were subjected to quantitative and qualitative analysis. The distribution of carotenoids in floral petals varies considerably; some contain few or no carotenoids, whereas others contain mainly epoxy carotenoids specific to petals. The role of epoxy carotenoids in petals was not known. Indirect evidence suggested that carotenoids play a part in reproduction of cryptogams and various animals. The presence of certain type of carotenoids in the anthers of *Delonix regia* had suggested that 'oxygenated carotenoids' may have a significant role in the reproduction of this plant. (Jungalwala and Cama, 1962*)*

3.10.4 Anthocyanin extract from the extract of flowers of *Delonix regia*

Due to the advancements in the technology, various processes have been developed to extract the phytoconstituents. Pilot scale plant membrane technology was used to manufacture extracts and also to characterize the biomolecular compositions, their activities and functionality. HPLC-DAD technique, three major anthocyanins was identified. The molecular structure was elucidated by mass spectroscopy and also from their UV –Vis spectra (Adje et *al*., 2008).

3.10.5 Isolation and characterization of steroid derivatives from *Delonix regia*

Although *Delonix regia* is extensively used as an ornamental plant, various phytoconstituents were identified in the seeds, flowers, bark, wood and leaves of drugs like β-sitoserol, tannins, saponins, flavonoids, steroids, alkaloids and carotene hydrocarbons. (Kumar *et al*., 2011).

3.11 Antimicrobial effect of *Delonix regia*

In order to prevent the ill-effects of increasing pathological infections, there is a need to find an alternative source of immunization. The rise in the pathological infections has been due to the failure of the chemotherapeutic agents and also the increasing resistance of microorganisms to the antibiotics.

So, in order to tackle the growing needs for the protection of human health, science resorts to plants due to the vast availability and their enormous medicinal properties. Though plants have been used traditionally to treat various medical conditions, the full potency of the plants in preventing and controlling the diseases is still an area to be explored (Sama *et al*., 2012).

One such easily available plant in the local tropical habitat is *Delonix regia.* The anti microbial properties of this plant is mainly attributed to the presence of phenolic compounds like tannins, carotenoids, flavonoids, alkaloids and others (Salem *et al*.,2013).

3.12 Other properties of *Delonix regia*

3.12.1 Anti-inflammatory activity of *Delonix regia*

Anti-inflammatory properties of *Delonix regia* leaves were studied using methanolic extracts and *in-vivo* studies were carried out in Wistar albino rats. Oedema was induced in the rats by the administration of carrageenan in saline. The ethanolic suspension was administered half an hour prior to this. The extent to which the Wistar albino rats developed paw oedema was recorded using a plethysmometer. Paw oedema contracted by the rats was found to be a two-phase event. Histamine and serotonin release contributed to the initial phase. Kinin was found to be responsible for the

intermediate phase maintenance and prostaglandins were for the second phase.

Cotton pellet granuloma method and subsequent statistical analysis by one-way Analysis of Variance (ANOVA) also established the same. Weight of the cotton pellets gave necessary insight into the quantity of the fluid absorbed (granuloma) and dry weight attributed its weight to the degree of granuloma of the granulomatous tissue.

From these studies, it was evident that most of the anti-inflammatory triterpenes isolated, contained lupine, oleanane, ursane and taraxastane (Shewale *et al*., 2011).

3.12.2 Gastro-protective activity of *Delonix regia*

Ethanolic extracts of *Delonix regia* flowers were used to study its gastro-protective effect in ulcer-induced Wistar albino rats. Gastro-protective activity was tested in three modes of ulcer-induced rats:-aspirin induced ulcer model, alcohol induced ulcer model and Pylorus induced ulcer model. All the above models were studied similar model rats, ignoring their sexes. Test and control groups were designated for each category of model.

Phytochemical analysis of the three above mentioned models confirmed the presence of flavonoids and tannins. Tannins, by their precipitating action on proteins, and vasocontrictory effect could prevent ulcer development.

Flavonoids offer gastro-protective effect by offering capillary resistance and enhancing microcirculation.

Delonix regia flower extract was found to possess curative properties against several other gastro-intestinal disorders, apart from ulcer.70% concentration of the extract was found to provide the optimum results (Shiramane *et al* ., 2011)

3.12.3 Anti-fungal activity of *Delonix regia*

In *Delonix regia,* a predominant presence of flavonoids, sterols, tannins and phenolic compounds was witnessed amidst other phytochemicals. These are viewed as potent anti-fungal medicinal sources.

An in-vitro study was conducted to prove the same. The test organisms used were *Aspergillus niger, A.flavus, Rhizosporum bataticola and Fusarium auxisporum* maintained on potato dextrose agar and incubated at 27°C±1°c for 48 hours.

Homogenized flower and seed powders were sequentially extracted using various solvents and stored at 90°C.The solvents used were petroleum ether, acetone, ethanol, benzene and water. Anti-fungal assay was performed by Gould and Bowie method of agar disc diffusion (Gould and Bowie, 1952)

Zones of inhibition were observed after incubation at 37°C for 48 hours. Mean of five triplicates was calculated.

Maximum anti-fungal activity was observed on *A.niger* due to the flavonoids from the leaves and flowers of *D.regia* (Sharma *et al*., 2010).

3.12.4 Nano-silica production

Fuel extraction by the burning of Pods of *D.regia* (PDRA), led to the production of pod ash. This waste material can be used as an alternate source for the production of nanosilica; since the pod ash contains a high quantity of Silica (about 60%).PDRA is amorphous in nature, which makes the production simpler.

Appropriate quantity of PDRA samples were used in the production of pure silica by mixing with distilled water and sodium hydroxide. The solution was boiled and filtered. The residue was again washed with water. The filtrate was cooled, followed by acid wash and base. It was cooled and dried for a day. The product was finally subjected to FTIR.

Pure silica was refluxed with HCl and again, washed with deionised water. The product was again refluxed with NaOH. Silica precipitate was washed several times and dried for storage.

It was concluded that nanosilica with 99% purity could be a valuable product of PDRA. It may be used in paint industry for its unique mechanical and physical properties like scratch and mar resistance, stiffness, flow lines etc... (Indhumathi *et al.*, 2011).

3.12.5 *Delonix regia* seed gum as a non-synthetic tab-binder

Delonix regia seed gum(DRSG) can be used as a good non-synthetic source of pharmaceutical binder, in contrast to the conventional synthetic acacia BP or tragacanth BP. Tensile strength, brittle fracture index(BFI),crushing strength-friability/disintegration time ratio(CSFR/DT) of a formulation are the mechanical properties to be considered while evaluating a new binder for commercial use.

Several existing binders like synthetic acacia BP or tragacanth BP were compared to the seed gum extracted from unripe, yet mature pods of *D.regia.* The swelling capacity of the gums were determined by Bowen and Vadino method.Triplicates were made to ensure precision. Granules and tablets were prepared by any one of the conventional methods. The above mentioned mechanical properties were tested by the available methods.

DRSG was found to be more efficient than ACG or TRG. At lower concentrations, it yielded better results on the parameters taken into consideration (Gbadegsine *et. al*., 2009).

3.12.6 *Delonix regia* as potent glucose inhibitor

Though *D.regia* has been a traditional medicine for diabetes in Bangladesh, it has not been much explored by the scientific fraternity for glucose tolerance properties.

Methanolic extracts of the leaves were prepared and the filtrate was dried. Subsequent phytochemical screening was performed to identify flavonoids, tannins, saponins...Swiss albino mice were bred and acclimatized to standard to standard laboratory conditions, one week, in advance. Standard diet and free access to water were provided to the mice. Joy and Kuttan procedure was performed to test glucose tolerance (Joy and Kuttan ., 1999). Control and test groups were designated. After an hour, all mice were administered with oral glucose. Statistical analysis was performed.

A reasonably good level of hypoglycaemic activity (42.46%) was observed in the mice. From the study, it was established that *D.regia* is a

valuable glucose inhibitor and this pharmacological activity may be attributed to its triterpenoids (Rahman *et al*., 2011).

3.12.7 Adsorption study on *Delonix regia*

The ability of *Delonix regia* to remove nickel from aqueous solution has been extensively studied. Different experimental conditions like effect of pH, contact time, initial concentration and adsorbent dosage on the adsorption of Ni were studied. The reaction kinetics was studied using different models including Langmuir and Freundlich adsorption model was used for mathematical description of the adsorption equilibrium and isotherm constants were evaluated.

Nickel is a comparatively rare metal, but finds wide applications in industries. Nickel is found in traceable amounts in plants, animals and sea water. Numerous methods like chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, membrane separation, electrochemical treatment, evaporation and adsorption. Among these, adsorption was found to the most promising and economically feasible alternative to remove the metal. Due to the easy availability and inexpensiveness, plant material has been widely used for adsorption.

The adsorption capacity of the *Delonix regia* (Gulmohar) tree bark was studied by a batch technique. Equilibrium data fitted very well to the Langmuir and Freundlich model. The pseudo first and second –order kinetic models were also applied to the experimental data and the data

agreed very well with pseudo second order kinetic model (Patil and Shrivastava, 2012).

3.12.8 Carbon dioxide sequestration in *Delonix regia*

As the rate of photosynthesis increases, more carbon dioxide have been converted into biomass and the reducing carbon in the atmosphere and sequestering it in plant tissue above and below ground in the global carbon cycle biomass is of importance and hence carbon sequestration was used to quantify pools and changes of Green House Gases from the terrestrial biosphere to the atmosphere associated with land use and land cover changes. (Chavan and Rasal, 2012).

3.13 HPTLC

High Performance Thin layer Chromatography is widely used in pharmacological sector to structurally elucidate various chemical compounds. The extracts obtained from the plants will contain innumerable compounds and some of these compounds will mutually confer curative activity. Hence, in order to narrow down the best combination of these active compounds that reflects the quality of the original herbal medicinal composistion , several effective and economical methods are being used. One such method is High Performance Thin layer Chromatography (Mariswamy *et al*., 2012).

3.14 FTIR

Fourier Transform Infrared Spectroscopy is a tool which is widely used to identify and characterize the phytochemical compounds present in medicinal plants. This method is immensely employed because it is an economical non-invasive method which produces high resolution results. In the recent years, it is also being used for pharmaceutical analysis.

FT-IR measures the degree of bond vibrations within the functional groups of the chemical compounds and generates a spectrum which are ascertained to be 'biochemical markers' or 'molecular fingerprints'(Ashokkumar and Ramaswamy,2014).

4.0 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Chemicals

1,1-Diphenyl-2-picryl hydrazyl(DPPH), 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid(ABTS), disodium hydrogen phosphate, ammonium persulfate, methanol, acetone, ethyl acetate, hexane, petroleum ether, ethanol, Trichloroacetic acid (TCA), sulphuric acid, hydrochloric acid, sodium carbonate, aluminium chloride, ammonium molybdate , ascorbic acid, acetate buffer, sodium hydroxide, neocuproine , sodium nitrite, catechin, sodium acetate buffer, cupric chloride ,theophylline, acetic acid , sodium meta per iodate (SPI), 3-methyl-2-benzo thizolinone hydrozone hydrochloride (MBTH) , tannic acid , formic acid , cholesterol, sodium nitrate, quercetin ,phosphate buffer, potassium ferricyanide , chloroform .

4.1.2 Plant specimen

Delonix regia leaves were collected from in and around Coimbatore district. And it was authenticated by Scientist In-Charge, Botanical Survey of India, Tamil Nadu Agricultural University (TNAU), Coimbatore. The voucher specimen of the plant sample has been deposited in the herbarium of the department

4.2 METHODS

4.2.1 Sample preparation by hot extraction

The plant sample (leaves) was dried under shade and ground to coarse powder. Hot extraction of the leaves was performed using Soxhlet apparatus using different solvents. 150ml of five different solvents of varying polarity index namely acetone, methanol, ethyl acetate, hexane and petroleum ether were extracted with 10g of powdered sample each. The extracts were obtained after 20 cycles and were dried to obtain dry weight extracts which was stored for further experiments.

4.2.2 Sample preparation by cold extraction

Samples by cold extraction was prepared using 10g of coarsely ground leaves with 150 ml of methanol, acetone, ethyl acetate, hexane and petroleum ether each in shaker at room temperature for 24 hours . After the incubation time, the extracts was filtered and dried. Dried samples were stored for future tests.

4.2. PHASE I

4.2.1 *IN VITRO* ANTIOXIDANT ASSAYS

4.2.1.1 Total antioxidant capacity assay

(Mohammad *et al., 2011)*

Principle

The assay is based on the formation of the green phosphate – molybdenum complex by the process of reduction of Molybdenum (VI) to Molybdenum (V) in the reagent by antioxidant present in the extracts at acidic pH which is measured at 695 nm.

Reagents

1. 28mM disodium hydrogen phosphate, 4mM ammonium molybdate and 0.6M sulphuric acid.
2. Ascorbic acid (standard).

Procedure

- **S**amples were prepared by dissolving in their solvents used for extraction (1mg/ml) both hot and cold extracts.
- 0.1ml of the reagent (disodium hydrogen phosphate+ammonium molbdate+sulphuric acid) was added.
- The tubes were capped and incubated at 95ºc for 90minutes in boiling water bath.
- After the incubation the tubes were cooled to room temperature and the absorbance was measured at 675 nm .
- Ascorbic acid was used as standard.

4.2.1.2 DPPH radical scavenging assay

Principle

The principle behind the assay is the changing of color from purple to yellow color by the reduction of 1, 1-diphenyl1-2-picryl-hydrazyl radical forming reduced DPPH by the scavenging activity of the antioxidants present in the extracts by the donation of protons. The color change is based upon the number of electrons taken up for pairing which is measured by decrease in absorbance at 517 nm.

Reagents

1. 0.02g of DPPH in Methanol.
2. Methanol.

Procedure

- The leaf extracts were prepared by dissolving in their respective solvents used for extraction in 1mg/ml concentration.
- 0.02g of DPPH in 75ml of Methanol was freshly prepared.
- Varying concentrations of sample triplicates (50 to 250µg/ml) were prepared and made up to 2ml with methanol.
- 0.5ml of DPPH reagent was added to all test tubes and mixed well.
- The test tubes were incubated in dark for 30 minutes and the colored products formed were measured spectrophotometrically at 517nm.
- Standards were prepared using Ascorbic acid.
- Ascorbic acid equivalent was used to express the total antioxidant capacity.

4.2.1.3 ABTS radical scavenging assay

(Rakholiya *et al., 2011)*

Principle

In this assay, 2, 2-Azobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt is converted to its radical cation by the addition of ammonium persulfate which results in the reduction of blue color which is read at 745 nm.

Reagents

1. Methanol.
2. ABTS (7mM).
3. Ammonium persulfate (2.45mM).

Procedure

- Equal volumes of ABTS solution (7mM) and ammonium persulfate (2.45mM) was mixed well and was kept in the dark for 16 hours at room temperature to form a dark colored solution.
- The initial absorbance of the solution was measured at 745 nm and was found to be exceeding the visible range (2.5+).
- Final absorbance of 0.700(±0.02) was obtained by diluting the solution with methanol at room temperature.
- Different extracts were dissolved in their respective solvents (1mg/ml) and varying concentrations of the sample triplicates (50 to 250µg/ml) were prepared.
- About 3ml 0f ABTS standard solution was added to 0.5ml of each sample mixture.
- After a period of 6 minutes, the decrease in absorbance of the mixture was read at 745 nm.

- 0.5m of methanol and 3 ml of ABTS solution was taken as control and methanol was set as blank.
- The percentage inhibition of the ABTS radical by the antioxidants in the sample was calculated.

4.2.1.4 CUPRAC assay

Principle

CUPRAC method involves the reduction of cupric ion to cuprous ion by the antioxidants present in the sample. It follows an electron transfer mechanism.

Reagents

1. Cupric chloride solution
2. Ammonium acetate
3. Neocuproine

Procedure

- Solvents used for preparation of extract were used for preparing samples by dissolving 1mg/ml of solvent.
- ml of sample of varying concentration was taken and 1ml of ammonium acetate was added.
- 1ml of cupric chloride and 1ml of neocuproine was added.
- The tubes were incubated for half an hour.
- Absorbance values were noted at 450 nm.

4.2.1.5 Reducing power assay

Principle

Reducing power assay is based on the reduction of Fe^{3+} to Fe^{2+} by direct electron transfer. Prussian blue color complex is formed by the addition of free Fe^{2+} ions to the reduced product, which is read at 700 nm.

Reagents

1. Trichloroacetic acid
2. 1% Potassium ferricyanide
3. 0.2M Phosphate buffer , pH 6.6
4. 0.1% Ferric chloride

Procedure

- Samples of varying concentration (100- 500 µg/ml) were taken and made up to 2.5 ml with methanol.
- To all the reaction mixtures 2.5 ml of 1% potassium ferricyanide and 2.5ml 0f 0.2M phosphate buffer were added and incubated at 50ºC for 20 minutes.
- Excluding the sample, the rest of the reaction mixture was taken as control.
- After the incubation time the reaction was terminated by the addition of 2.5 ml of 10% trichloroacetic acid followed by centrifugation at 3000 rpm for 10 minutes.
- 2.5ml of the supernatant was taken and 2.5 ml of deionized water and 0.4ml of 0.1% ferric chloride solution were added.
- The colored complex formed was read at 700 nm.

4.4 PHASE II

4.4.1 PHYTOCHEMICAL ANALYSIS

4.4.1.1 Estimation of flavonoids

Reagents

1. 5% Sodium nitrate
2. 10%Aluminium chloride
3. 1M Sodium hydroxide

Procedure

- Samples were dissolved in their respective solvents (1mg/ml) to serve as stock.
- 25 µl of the sample was taken and made up to 2.5 ml with distilled water.
- To the sample 75 µl of 5% sodium nitrate solution was added and incubated at room temperature for 5 minutes.
- After incubation 150 µl of 10% Aluminium Chloride was added and incubated at room temperature for 6minutes.
- 0.5 ml of 1M Sodium Hydroxide was added and the colored complex formed was read at 510 nm
- Quercetin was used as a standard to prepare a calibration curve.

4.4.1.2 Estimation of Total phenols

Reagents

1. Folin Ciocalteau reagent
2. Sodium carbonate

Procedure

- Sample was prepared by dissolving the extracts in their respective solvents (1mg/ml).
- 0.1ml of the sample was taken and made up to 2ml with distilled water.
- 0.5 ml of Folin Ciocalteau reagent was added and the tubes were incubated for 3 minutes.
- After the incubation period, 2ml of sodium carbonate was added and the tubes were kept in water bath for one minute at 50ºC.
- Then the absorbance of the colored complex formed was read at 650 nm.
- Catechol was used as standard to draw the calibration curve.

4.4.1.3 Estimation of anthocyanins

Reagents

1. 10% Formic acid

Procedure

- 0.5ml of the sample was taken and 4ml of 10% formic acid was added.
- And the absorbance was read at 530 nm.
- The anthocyanin content was calculated on the basis of the following equation and determined as cyanidin -3 –glucoside equivalent

Anthocyanin content (mg/ 100 g of dry matter) =

A x MW x DF x 100/ ε x W)

Where A = Absorbance

MW = Molecular weight of cyaniding-3-glucoside chloride ($C_{21}H_{21}ClO_{11}$. 449.2)

DW = Dilution factor

ε = Molar absorptivity (26.900)

W = Weight of the sample

4.4.1.4 Estimation of sterols

Reagents

1. Chloroform
2. Lieberman Burchard reagent
3. Cholesterol

Procedure

- 1ml of the sample (1mg/ml) was taken and made up to 5ml with chloroform.
- 2ml of Lieberman Burchard reagent (0.5ml 0f conc. Sulfuric acid in 10ml of acetic anhydride) was added to the mixture.
- The tubes were plugged and kept in the dark for 15 minutes and the green complex formed was read at 640 nm.
- β-sitosterol was used as standard.

4.4.1.5 Estimation of Tannins

Reagents

1. Sodium carbonate
2. Tannic acid

Procedure

- 2ml of the samples dissolved in their corresponding solvents were taken (1mg/ml).
- 2.5ml of sodium carbonate solution was added to all the samples and 2ml of distilled was taken as blank.
- All the tubes were made up to 50ml with distilled water and incubated at room temperature for 90 minutes
- The absorbance was read at 260 nm.
- Tannic acid (tannin) was used to serve as standard.

4.4.1.6 Estimation of alkaloids

Reagents

1. 0.1M Acetic acid
2. 0.01M Sodium meta per iodate (SPI)
3. 0.01M 3-methyl-2-benzo thiazolinone hydrazone hydrochloride (MBTH)
4. Theophylline

Procedure

- 1.5ml of the each sample was taken in boiling tubes and 1ml of 0.01M SPI solution and 0.5 ml 0.1M acetic acid solution was added.

- 10 ml of distilled water was added to all the boiling tubes and was kept in the boiling water bath for 10 minutes.
- After incubation, 2ml of the 0.01M MBTH solution was added to all the tubes and kept in the boiling water bath for 2 minutes.
- The tubes were cooled and made up to 25 ml with distilled water.
- The complex formed was read at 630 nm.
- Varying concentration (5 to 25µg/ml) of theophylline was used to serve as standards.

4.5 PHASE III

4.5.1 Thin Layer Chromatography (TLC) of leaf extracts

Materials

1. Silica gel
2. Acetone
3. Methanol
4. Hexane

Procedure

Silica gel G_{50} (0.2-0.3 mm thick and 32g/60ml of distilled water) was coated on glass plate (20×20 cm) and dried at room temperature. Then the plates were kept in oven at 100ºc for 30 minutes for activation of the plates and were brought back to room temperature. About 1.5cm above the edge of the plate 20 µl of the leave samples was spotted. The one dimensional development of the plate were done by keeping them in an air

tight chromatography chamber which contained the mobile phase solvent mixture, acetone – hexane – methanol (1:2:1, v/ v/ v) about 200ml.The plates were dried and was identified under UV light at 365 nm.

4.5.2 Bioautography of leaf extracts

The spotted and developed TLC plates were sprayed with DPPH solution dissolved in methanol and were dried and viewed under UV light for the specific identification of antioxidants present.

4.5.3 Preparative Thin Layer Chromatography (PTLC) of leaf extracts

Silica gel G_{50}(0.5-1.0 mm thick and 46g/85ml of distilled water) was coated on glass plate (20×20 cm) and was dried at room temperature. Then the plate was activated by placing them in oven at 100ºc for 30 minutes. The procedure was repeated as same for TLC.

4.5.4 High Performance Preparative Thin Layer Chromatography (HPTLC) of leaf extracts

Procedure

Test solution preparation

The given plant sample was centrifuged at 3000rpm for 5min.This solution was used as test solution for HPTLC analysis.

Sample Loading

7.5 µl of the test solutions were loaded as 5mm band length in the 2 x 10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument.
(Samples Concentration – 0.5mg in 7.5µl)

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Anthocyanin) and the plate was developed in the respective mobile phase up to 90mm.

Photo-documentation

The developed plate was dried by 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 UV366nm.

Derivatization

The developed plate was sprayed with respective spray reagent (Anthocyanin) and dried at 100°C in Hot air oven. The plate was photo-documented at Visible light and UV 366nm using Photo-documentation (CAMAG REPROSTAR 3) chamber.

Scanning

After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 500nm. The Peak table, Peak display and Peak densitogram were noted.

ANALYSIS DETAILS

Mobile phase

Ethyl acetate-Glacial acetic acid-Formic acid-Water (10:1.1:1.1:2.6)

Spray reagent

Anisaldehyde sulphuric acid reagent.

4.5.5 Fourier Transformation Infrared Spectroscopy (FTIR)

The hot and cold acetone extracts of the leaves of *Delonix regia* was subjected to FTIR spectroscopic analysis. The analysis was carried out by analyst In-charge in PSG College of Arts and Science, Coimbatore, Tamil Nadu.

PHASE IV

4.6.1 Antimicrobial assay of Solvent extracts of *Delonix regia*

Stock preparation

1mg of both hot and cold different solvent extracts of leaves of *Delonix regia* was dissolved in 1ml of Dimethyl sulfoxide (DMSO).

The microbial strains for the process is as follows:

- *Escherichia coli*
- *Staphylococus aureus*
- *Pseudomonas monteilli*
- *Enterococus faecalis*

4.6.2 Inoculum preparation

Bacterium Inoculum

24 hours old culture of gram positive and gram negative bacteria was used for the preparation of inoculum. Four to five bacterial colonies were taken with sterile loop and was transferred into the flasks containing Nutrient

broth. The flasks were kept in orbital shaker at room temperature for 24 hours.

Test Medium

Nutrient agar medium was used for the well diffusion method.

4.6.3 Antimicrobial Susceptibility testing

Nutrient agar medium was used for the well diffusion method of antimicrobial testing as per the procedure described by Magaldi *et al* (2004).The medium was autoclaved at 120ºC and was cooled to 50-55ºC . The cooled medium was poured into the petriplates to a uniform depth of 4mm into each plates. The culture was inoculated on to the medium after its solidification. Sterile L shaped rod was used for even distribution of the culture on the surface of the Nutrient agar medium. Agar wells were made using a sterile medium. 50µl of each extracts were added to agar wells of different strains.

The plates were incubated for 24 hours at 35-37ºC.

The percentage(%) inhibition was calculated using the formula:

% of inhibiton =I (Diameter of the zone of inhibition in mm)/ (diameter of the petriplates in mm) ×100

4.7 Orthogonal Design of Experiments

Materials

1. Ethyl acetate
2. Powdered sample

Method

To improve the anthocyanin content present in ethyl acetate extract of leaf extract, orthogonal design of experiments were carried out for both hot and cold extraction at different conditions.

Hot Extraction

The sample and the solvent were taken in two different ratios namely 1:15 and 1:20 in Soxhlet apparatus and they were run at 15,20,25 cycles each respectively. The samples were collected from each combinations and test for anthocyanin content and flavonoids was carried out.

Cold Extraction

For cold extraction the sample and the solvent mixture was kept in the shaker at room temperature of varying ratios 1:15 and 1:20. They were kept for varying time duration of 8,16 and 24 hours each for ratios. After the incubation time test for anthocyanin content and flavonoids were calculated by collecting samples from each combinations used.

4.8 Statistical Analysis

The results obtained from experiments were expressed as mean ±SD of the triplicates and two-way ANOVA analysis was carried out for the data obtained. The significant difference obtained between the sample means were calculated by DMRT using IRRISTAT version 3.1. p values<0.05 were regarded as significant.

5.0 RESULTS AND DISCUSSION

Free radicals, also called as Reactive Oxygen Species (ROS) have become a major threat to health in recent times by paving way for many medical conditions dreaded by the humans. These free radicals are potent enough to damage the lipid bilayer membrane and act as a hindrance for various biochemical pathways. It also results in severe nerve disorders like Alzheimer's disease, atherosclerosis, cardiac complications...Moreover, it acts as a key factor in inducing mutation, which ultimately results in cancer. Hence, to protect the human organ systems that are prone to damage by ROS, antioxidants are quiet essential. Antioxidants neutralize the free radicals and impede the chain reaction initiation and propagation (Brewer, 2011).

The human body itself contains an innate immune system to tackle these free radicals by various mechanisms and thus prevent the cells from undergoing oxidative stress.

The medicinal properties of plants are well known, since their legacy dates back to ancient human civilizations where they were being profoundly employed to treat various ailments. Even today, plants are used as a major ingredient in the formulation of various life saving drugs. Hence, it has become a necessity to explore various plant varieties for their medicinal properties (Mariajancyrani *et al*., 2013).

Plant extracts, generally used as flavoring agents are strong proton donors. This property makes them strong antioxidants. The antioxidant

property of plant extracts can be attributed to the presence of phenolic acids, phenolic diterpenes, flavonoids, volatile oils and plant pigments.

Delonix regia (Boj. Ex. Hook) Raf, is a tree widely inhabiting the tropical regions and is communally termed as ‘Gulmohar’ or ‘Flamboyant tree’. It has been extensively used for ornamental purposes. But, recent researches on its antioxidant potential have made it an important medicinal plant. (Veigas *et al*.,2007)

Delonix regia was found to contain various classes of phytoconstituents like phenolics, terpenoids, flavonoids, phytosterols and glycosides. Being a rich source of phytochemicals , it plays a crucial role as an antioxidant and thus prevents various medical complications that leave human lives sabotaged (Mariajancyrani *et al*., 2013).

Once the presence of these phytochemicals has been ascertained, the structural and the chemical properties of various compounds can be elucidated by various techniques like High Performance Thin Layer Chromatography (HPTLC), Gas Column Mass Spectroscopy (GC-MS) and Fourier Transform Infrared Spectroscopy (FT-IR) (Sahu and Saxena, 2013).

Apart from the antioxidant properties of *Delonix regia,* the leaf extracts possess noteworthy antimicrobial activity also. It has been confirmed that the ability of the leaves of *Delonix regia* to potentially inhibit the growth of micro organisms is due to the presence of flavonoids, alkaloids and other phytoconstituents. Also, the barks and flowers of

Delonix regia exhibit broad spectrum of anti viral, anti fungal and anti - inflammatory attributes (Salem *et al*., 2014)

PHASE I

5.1. *In vitro* free radical scavenging assays

5.1.1 Total antioxidant capacity assay

The total Antioxidant Capacity assay of leaves of *Delonix regia* was carried out for different solvent extracts during hot and cold extraction conditions as depicted in t**ables 5.1.1.1 & 5.1.1.2** and **figures 5.1.1.1 & 5.1.1.2** respectively. The total antioxidant assay gives an estimate of overall antioxidant potential of the different solvent extracts of *Delonix regia* leaves. There is a formation of phophomolybdenum complex, the intensity of which indicates the potential of solvent extracts as a scavenger of free radicals. The total antioxidant capacity of different solvent extracts was expressed as number of equivalents of ascorbic acid

From the table and figures, it was found that the antioxidant activity was high in extracts that were prepared using solvents with high polarity index. Thus, methanol and acetone extracts, whose polarity is high showed maximal antioxidant activity when compared to the extracts of ethyl acetate (moderately polar), hexane and petroleum ether (non-polar). The result obtained was in accordance with that obtained from the total antioxidant capacity for the extracts of Sapota peel by Gomathy *et al*.(2013), where methanolic extracts showed maximal antioxidant activity.

Table 5.1.1.1 Total Antioxidant Capacity (TAC) of different solvent extracts of *Delonix regia* leaves under hot extraction conditions expressed as ascorbic acid equivalents

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	25	**50**	**75**	**100**	**125**
ACE	1.227^{Pb} ±0.054	2.308^{Qd} ±0.185	3.040^{Re} ±0.456	3.980^{Se} ±0.050	**4.460^{Te}** ±0.025
EAE	0.230^{Pcb} ±0.045	0.625^{Qc} ±0.010	0.780^{Rc} ±0.025	1.092^{RcD} ±0.130	1.126^{Sc} ±0.125
HEXE	0.073^{Pac} ±0.015	0.103^{Qa} ±0.012	0.167^{Ra} ±0.021	0.227^{Sa} ±0.020	0.320^{Ta} ±0.090
METE	0.360^{Pb} ±0.185	0.642^{Qb} ±0.030	1.3508^{Rb} ±0.007	1.992^{Sb} ±0.060	2.148^{Tb} ±0.050
PETE	0.124^{Pdc} ±0.071	0.239^{Qc} ±0.026	0.309^{Rd} ±0.012	0.390^{Sd} ±0.016	0.461^{Td} ±0.012

Values represent mean ± standard deviation of three replicates.

Means ranked by the common letter across the rows (P-T) (denoting concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a-e) (denoting different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract;

HEXE – Hexane Extract; **METE** – Methanol Extract;

PETE – Petroleum Ether Extract

Table 5.1.1.2 Total Antioxidant Capacity (TAC) of different solvent extracts of *Delonix regia* leaves under cold extraction conditions expressed as ascorbic acid equivalents

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	25	**50**	**75**	**100**	**125**
ACE	1.300^{Pc} ±0.819	1.500^{Qd} ±0.996	2.060^{Re} ±0.105	2.580^{Se} ±0.020	**3.092^{Te}** ±0.040
EAE	0.230^{Pb} ±0.062	0.625^{Qc} ±0.078	0.780^{Rb} ±0.010	1.092^{Sb} ±0.008	1.126^{Tb} ±0.026
HEXE	0.073^{Pa} ±0.094	0.103^{Qb} ±0.037	0.167^{Ra} ±0.100	0.227^{Sa} ±0.082	0.320^{Ta} ±0.100
METE	0.360^{Pc} ±0.008	0.642^{Qe} ±0.010	1.3508^{Rd} ±0.014	1.992^{Sd} ±0.006	2.148^{Td} ±0.016
PETE	0.124^{Pa} ±0.080	0.239^{Pa} ±0.050	0.309^{Qa} ±0.043	0.390^{Ra} ±0.078	0.461^{Sa} ±0.090

Values represent mean ± standard deviation of three replicates.

Means ranked by the common letter across the rows (P-T) (denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a-e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ;

HEXE – Hexane Extract; **METE** – Methanol Extract ;

PETE – Petroleum Ether Extract

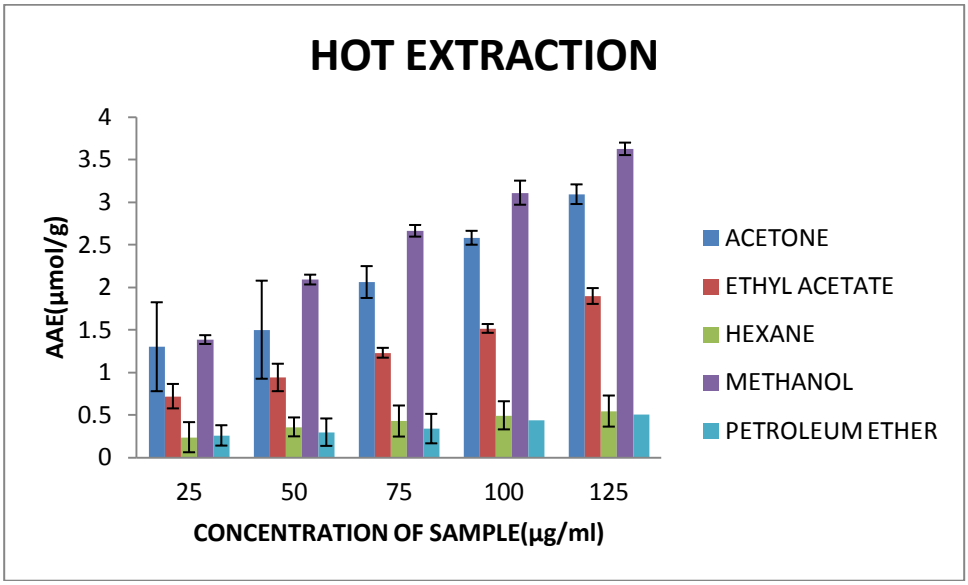


Fig. 5.1.1.1 Total Antioxidant Capacity (TAC) of different solvent extracts of *Delonix regia* leaves under hot extraction conditions expressed as ascorbic acid equivalents

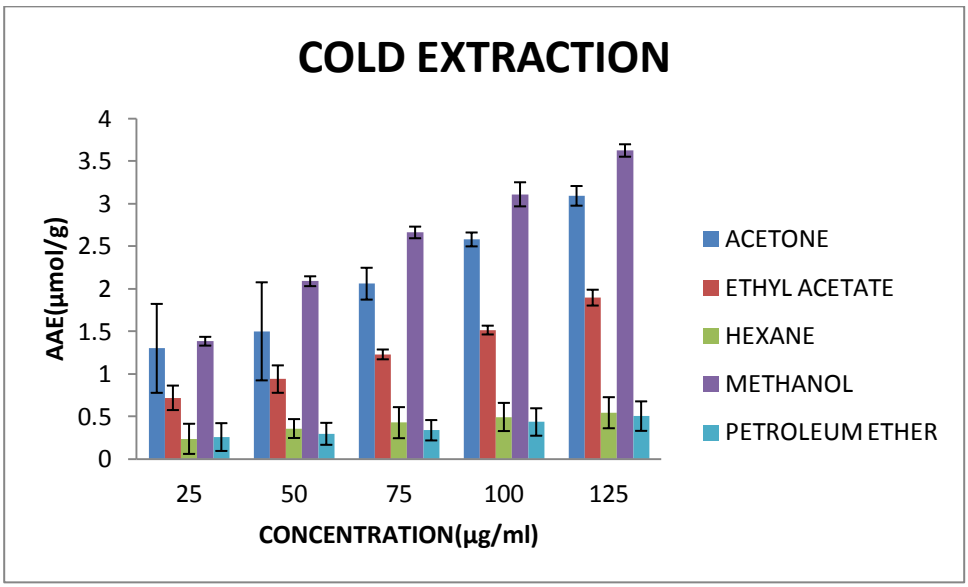


Fig. 5.1.1.2 Total Antioxidant Capacity (TAC) of different solvent extracts of *Delonix regia* leaves under cold extraction conditions expressed as ascorbic acid equivalents

5.1.2 DPPH radical scavenging activity

DPPH is a nitrogen centered radical that shows absorbance at 517 nm. Deep violet colored methanolic DPPH solution changes to yellow colour in the presence of DPPH radical scavengers. DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet colour in the form of EC_{50} values. Lower EC_{50} values represent higher antioxidant activity (Yamguchi *et al*., 1998).

This free radical scavenging assay is based on the ability of the antioxidants present in the leaf extracts of *Delonix regia* to decolorize the DPPH radical.

Free radical scavenging potential (DPPH) of different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions are represented in **tables 5.1.2.1 & 5.1.2.2** and **figures 5.1.2.1 & 5.1.2.2** respectively. The radical scavenging activity increases with increase in the concentration of the sample which was reflected at the decrease in the absorbance. Ascorbic acid was used as standard.

From the table 5.1.2.1 and figure 5.1.2 1, it was inferred that at 250 µg/ml, the hot extracts of acetone and methanol exhibited maximal radical scavenging activity. Statistically, the scavenging activity of antioxidants (hot extraction) was effective in the order of Methanolic extract> Acetone extract>Ethyl acetate extract>Petroleum extract>Hexane extract.

Our results are in concordance with reports made by Shabir *et al*. (2011), Salem (2013) and Patil *et al*.(2014).

Table 5.1.2.1 Scavenging effect (%) on DPPH radical by different solvent extracts of *Delonix regia* leaves under hot extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	50	**100**	**150**	**200**	**250**
ACE	76.84^{Pd} ±0.11	80.79^{Qd} ±0.34	84.06^{Sd} ±0.64	85.09^{Sd} ±0.52	86.08^{Sd} ±0.04
EAE	31.65^{Pc} ±0.85	54.32^{Qc} ±0.64	63.25^{Rc} ±0.40	71.27^{Sc} ±0.44	77.21^{Tc} ±0.54
HEXE	4.80^{Pa} ±0.42	11.49^{Qa} ±0.35	16.97^{Ra} ±0.75	29.57^{Sa} ±0.50	36.87^{Ta} ±0.91
METE	76.89^{Pd} ±0.42	81.25^{Qd} ±0.71	87.96^{Rd} ±0.64	90.10^{Se} ±0.20	**90.95^{Sd}** ±0.32
PETE	26.37^{Pb} ±0.84	44.60^{Qb} ±0.37	48.02^{Rb} ±0.10	55.56^{Sb} ±0.79	70.33^{Sa} ±0.60
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	55.58	73.55	84.56	90.56	96.45

Values represent mean± standard deviation of three replicates.

Means ranked by the common letter across the rows (P-T) (denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a-e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ;

HEXE – Hexane Extract ; **METE** – Methanol Extract ; **PETE** – Petroleum Ether Extract

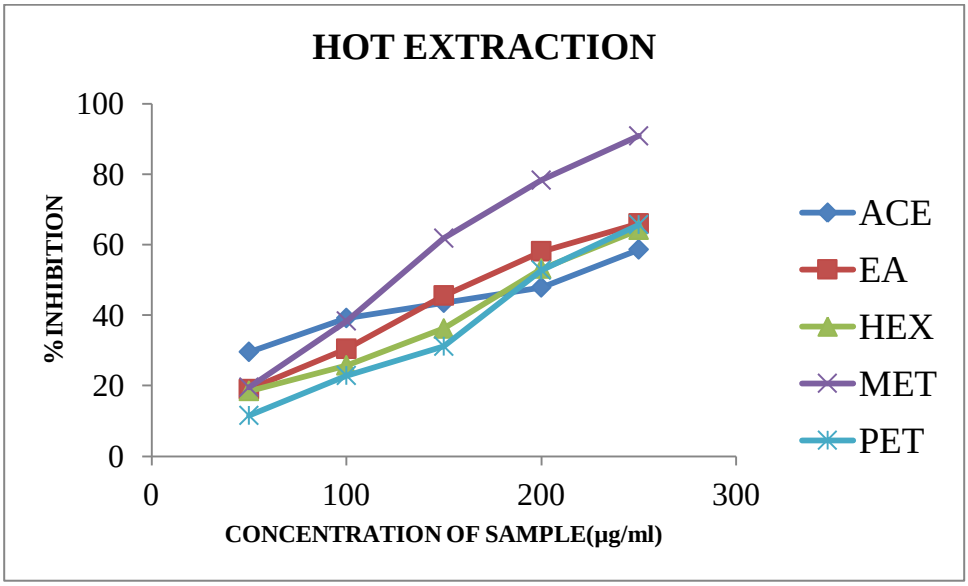


Fig. 5.1.2.1 Scavenging effect (%) on DPPH radical by different solvent extracts of *Delonix regia* leaves under hot extraction conditions

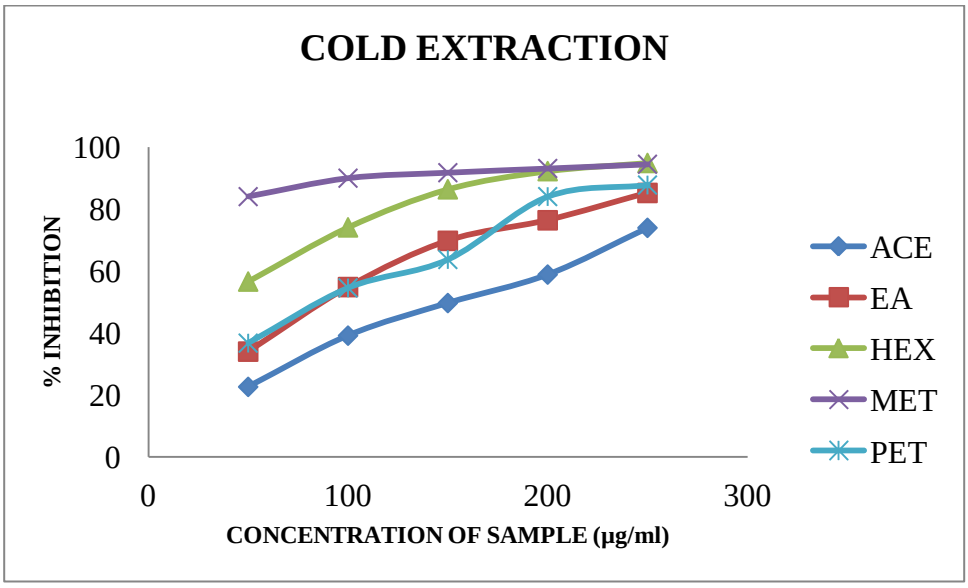


Fig. 5.1.2.2 Scavenging effect (%) on DPPH radical by different solvent extracts of *Delonix regia* leaves under hot extraction conditions

Table 5.1.2.2 Scavenging effect (%) on DPPH radical by different solvent extracts of *Delonix regia* leaves under cold extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	50	**100**	**150**	**200**	**250**
ACE	22.57^{Pa} ±0.25	39.11 ±0.60	49.63 ±0.90	58.83 ±0.60	73.73^{Ta} ±0.36
EAE	33.95^{Pb} ±0.86	54.49^{Qb} ±41	69.72^{Rc} ±0.61	76.36^{Sb} ±0.28	85.19^{Tb} ±0.17
HEXE	56.58^{Pd} ±0.75	74.04^{Qc} ±0.59	86.38^{Rd} ±0.45	92.24^{Sd} ±0.94	**96.88^{Tc}** ±0.54
METE	84.00^{Pe} ±0.36	89.96^{Qd} ±0.45	91.74^{PRe} ±0.53	93.10^{Rd} ±0.66	94.46^{Rc} ±0.02
PETE	36.69^{Pc} ±0.48	54.43^{Qb} ±0.33	63.72^{Rb} ±0.20	84.02^{Sc} ±0.35	87.75^{Tc} ±0.26
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	55.58	73.55	84.56	90.56	96.45

Values represent mean ± standard deviation of three replicates.

Means ranked by the common letter across the rows (P-T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

In contrast, from the table 5.1.2.2 and figure 5.1.2 2, it was inferred that at 250 µg/ml, the cold extracts of hexane and methanol exhibited maximal radical scavenging activity. Statistically, the scavenging activity of antioxidants (cold extraction) was effective in the order of Hexane extract> Methanol extract>Ethyl acetate extract>Petroleum extract>Acetone extract.

5.1.3 ABTS cation radical scavenging activity

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum (Sanchez Moreno, 2002).

This radical scavenging assay is used to determine the efficiency of the leaf extracts of *Delonix regia* to successfully scavenge the ABTS radical. The absorbance of the sample decreases with increase in the concentration of the sample and thus increasing the percentage inhibition. Ascorbic acid was used as standard.

Free radical scavenging potential (ABTS) of different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions are represented in **tables 5.1.3.1 & 5.1.3.2** and **figures 5.1.3.1 & 5.1.3.2.** respectively.

From the table 5.1.3.1 and figure 5.1.3 1, it was inferred that at 250 µg/ml, hot extracts of acetone possess high antioxidant activity which is evident from the maximal scavenging activity followed by methanol extracts. Statistically, the scavenging activity of antioxidants (hot

extraction) was effective in the order of Acetone extract >Methanolic extract> Ethyl acetate extract>Hexane extract> Petroleum extract.

Table 5.1.3.1 Scavenging effect (%) on ABTS radical by different solvent extracts of *Delonix regia* leaves under hot extraction conditions

SOLVENTS USED	**SAMPLE CONCENTRATION (µg/ml)**				
	50	**100**	**150**	**200**	**250**
ACE	81.98^{Pac} ±0.84	87.57^{Pc} ±0.72	92.00^{Rc} ±0.77	94.32^{Rc} ±0.38	**97.87^{Sb}** ±0.49
EAE	64.11^{Pa} ±0.57	54.30^{Qa} ±0.49	63.25^{Qa} ±0.66	71.27^{Ra} ±0.66	77.21^{Ra} ±0.61
HEXE	61.60^{Pa} ±0.66	65.78^{Qb} ±0.47	69.21^{Ra} ±0.77	73.59^{Sa} ±0.70	77.02^{Ta} ±0.99
METE	66.45^{Pb} ±0.79	80.65^{Qb} ±0.77	83.58^{Rb} ±0.23	90.38^{Rb} ±0.64	95.66^{Sb} ±0.42
PETE	63.54^{Pa} ±0.49	65.25^{Pa} ±0.82	68.53^{Qa} ±0.46	72.09^{Sa} ±0.41	75.72^{Ta} ±0.32
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	23.67	41.56	52.69	67.90	87.67

Values represent mean ± standard deviation of three replicates. Means ranked by the common letter across the rows (P-T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a-e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

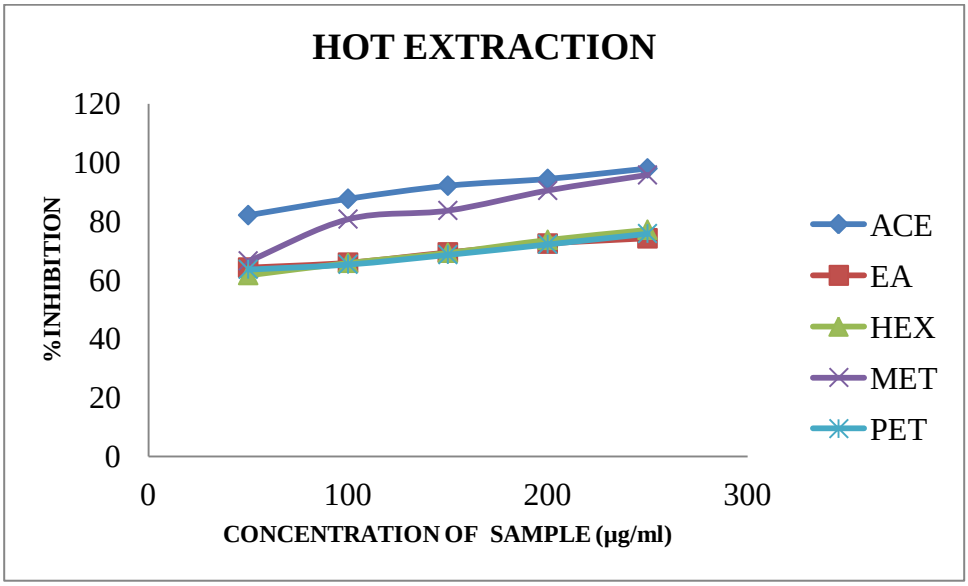


Fig. 5.1.3.1 Scavenging effect (%) on ABTS radical by different solvent extracts of *Delonix regia* leaves under hot extraction conditions

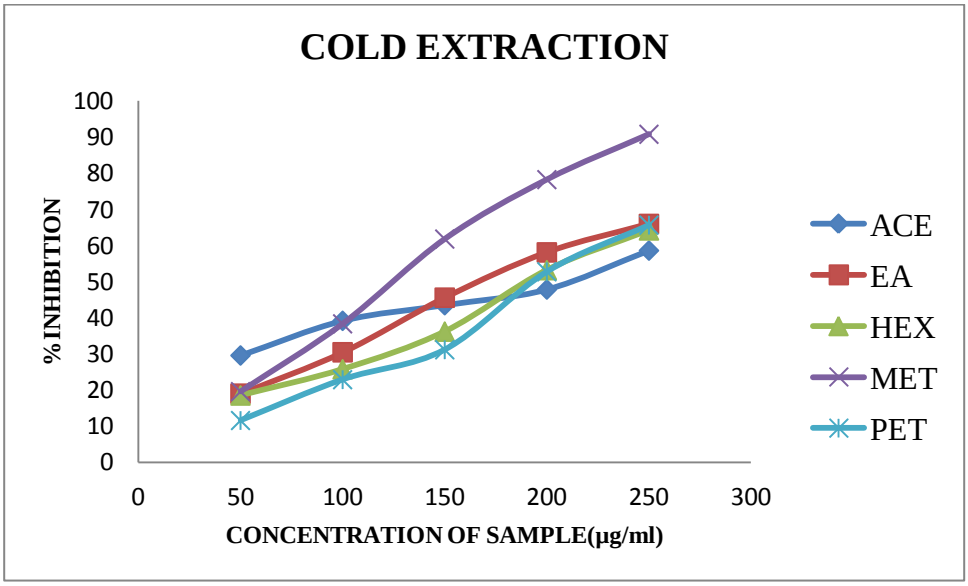


Fig. 5.1.3.2 Scavenging effect (%) on ABTS radical by different solvent extracts of *Delonix regia* leaves under cold extraction conditions

Table 5.1.3.2 Scavenging effect (%) on ABTS radical by different solvent extracts of *Delonix regia* leaves under cold extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	50	**100**	**150**	**200**	**250**
ACE	29.55^{Pc} ±0.785	39.152^{Qd} ±0.717	43.48^{Rc} ±0.678	47.82^{Sa} ±0.760	58.60^{Ta} ±0.380
EAE	18.96^{Pb} ±0.395	30.42^{Qc} ±0.500	45.55^{Rc} ±0.866	58.096^{Sc} ±0.285	65.97^{Tb} ±0.19
HEXE	18.47^{Pb} ±0.249	25.73^{Qb} ±0.915	36.16^{Rb} ±0.473	53.18^{Sb} ±0.605	64.193^{Tb} ±0.271
METE	19.54^{Pb} ±0.874	38.31^{Qd} ±0.605	61.973^{Rb} ±0.540	78.25^{Sd} ±0.812	**90.80^{Tc}** ±0.580
PETE	11.526^{Pa} ±0.679	22.053^{Qa} ±0.892	31.13^{Ra} ±0.725	52.736^{Sb} ±0.866	65.65^{Tb} ±0.937
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	23.67	41.56	52.69	67.90	87.67

Values represent mean± standard deviation of three replicates.

Means ranked by the common letter across the rows (P - T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;
METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

In contrast, from the table 5.1.3.2 and figure 5.1.3 2, it was inferred that at 250 µg/ml, the cold extracts of methanol exhibited maximal radical scavenging activity. Statistically, the scavenging activity of antioxidants (cold extraction) was effective in the order of Methanol extract>Ethyl acetate extract>Petroleum extract>Hexane Extract>Acetone extract.

Similar results were obtained for crude methanolic extracts which was inferred from the work done by Gomathy *et al.,* (2013).

5.1.4 Reducing power capacity

In this method, antioxidant compounds form a coloured complex with potassium ferricyanide, trichloroacetic acid and ferric chloride that was measured at 700 nm. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample (Tenpe *et al*., 2008).

Reducing power capacity of the different solvent extracts of *Delonix regia* leaves under both hot and cold extraction conditions are indicated in **tables 5.1.4.1 & 5.1.4.2** and **figures 5.1.4.1 & 5.1.4.2** respectively.

Reducing power of different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions increases with increase in concentration.

Table 5.1.4.1 Reducing power capacity of different solvent extracts of *Delonix regia* leaves under hot extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	50	**100**	**150**	**200**	**250**
ACE	0.228^{Pb} ±0.005	0.380^{Qc} ±0.003	0.581^{Sd} ±0.003	0.697_{Td} ±0.005	0.778^{Tc} ±0.02
EAE	0.244^{Pb} ±0.004	0.337^{Qc} ±0.002	0.379^{Qc} ±0.004	0.430^{Rc} ±0.004	0.570^{Sb} ±0.007
HEXE	0.032^{Pa} ±0.005	0.040^{Pa} ±0.002	0.057^{Pa} ±0.005	0.097^{Qa} ±0.004	0.145^{Ra} ±0.003
METE	0.758^{Pc} ±0.007	1.029^{Qd} ±0.003	1.281^{Re} ±0.004	1.541^{Se} ±0.011	**1.635^{Td}** ±0.006
PETE	0.043^{Pa} ±0.003	0.073^{Qb} ±0.004	0.103^{Rb} ±0.004	0.131^{Sb} ±0.002	0.158^{Ta} ±0.006
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	0.240	0.390	0.560	0.751	0.942

Values represent mean ± standard deviation of three replicates.

Means ranked by the common letter across the rows (P - T)(denoting between concentration) are not significantly different at 5% level by DMRT.

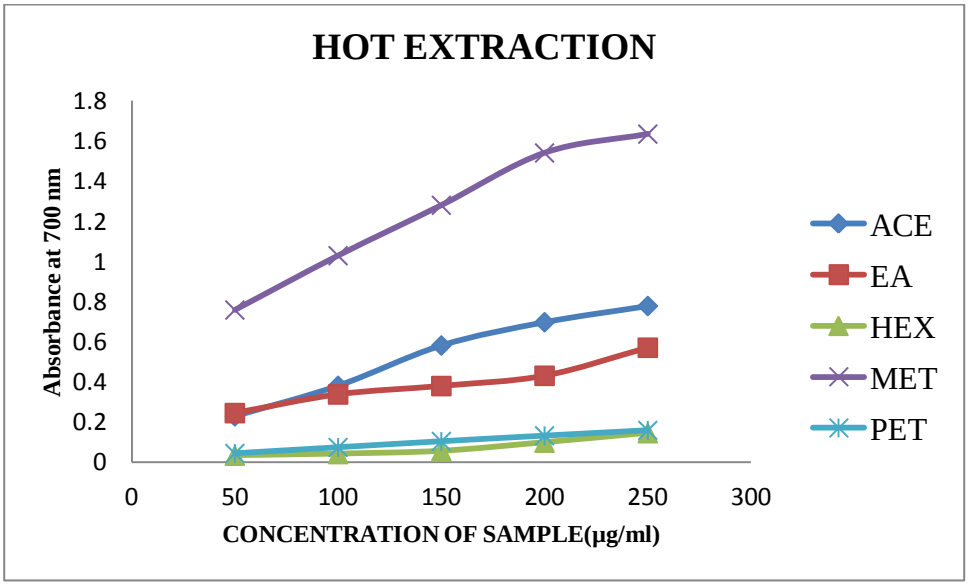
Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

From the table 5.1.4.1 and figure 5.1.4 1, it was shown that at 250 µg/ml, the hot extracts of methanol possess high antioxidant activity which is evident from the maximum reducing power capacity followed by acetone extracts. Statistically, the reducing power of antioxidants (hot extraction) was effective in the order of Methanolic extract>Acetone extract> Ethyl acetate extract> Petroleum extract> Hexane extract.

Fig. 5.1.4.1 Reducing power capacity of different solvent extracts of *Delonix regia* leaves under hot extraction conditions



In, contrast, from the table 5.1.4.2 and figure 5.1.4 2, it was inferred that at 250 µg/ml, the cold extracts of methanol exhibited maximal reducing power capacity. Statistically, the reducing power of antioxidants (cold extraction) was effective in the order of Methanol extract> Ethyl acetate extract> Acetone extract > Hexane Extract >Petroleum extract.

Table 5.1.4.2 Reducing power capacity of different solvent extracts of *Delonix regia* leaves under cold extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	50	**100**	**150**	**200**	**250**
ACE	0.168^{Pb} ±0.006	0.236^{Qb} ±0.003	0.286^{Qb} ±0.003	0.391^{Rc} ±0.005	0.449^{Sc} ±0.003
EAE	0.245^{Pd} ±0.006	0.337^{Qc} ±0.003	0.379^{Qc} ±0.005	0.429^{Rd} ±0.004	0.570^{Sd} ±0.005
HEXE	0.205^{Pc} ±0.003	0.220^{Pb} ±0.005	0.230^{Pb} ±0.001	0.243^{Pb} ±0.004	0.254^{Pb} ±0.004
METE	0.288^{Pe} ±0.005	0.441^{Qd} ±0.005	0.779^{Rd} ±0.006	1.069^{Se} ±0.008	$\mathbf{1.131^{Te}}$ ±0.003
PETE	0.018^{Pa} ±0.002	0.078^{Qa} ±0.005	0.099^{Ra} ±0.002	0.152^{Sa} ±0.007	0.194^{Ta} ±0.006
Standard (µg/ml)	**20**	**40**	**60**	**80**	**100**
Ascorbic acid	0.240	0.390	0.560	0.751	0.942

Values represent mean ± standard deviation of three replicates.

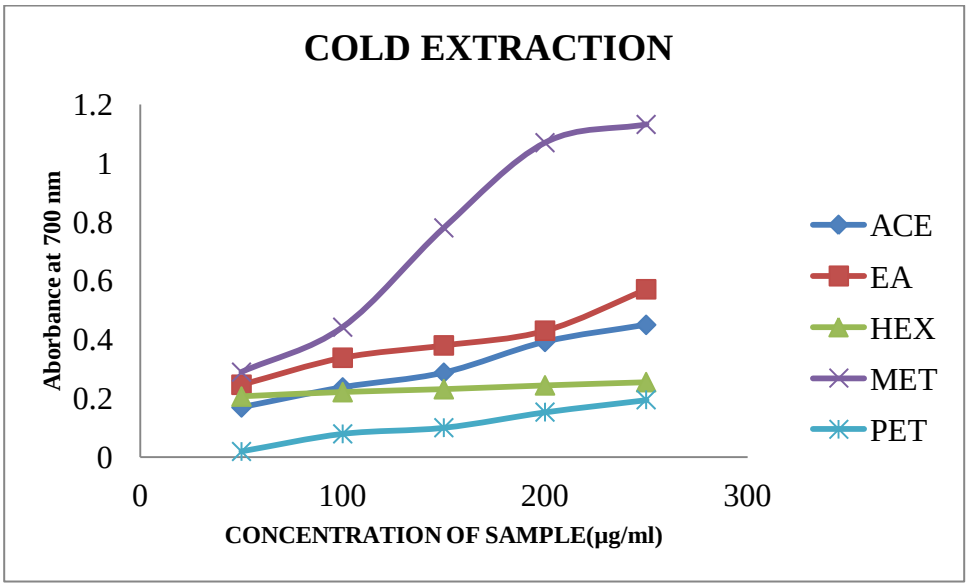
Means ranked by the common letter across the rows (P - T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

Fig. 5.1.4.2 Reducing power capacity of different solvent extracts of *Delonix regia* leaves under cold extraction conditions



The present results are in close coincidence with the reports made by Mariajancyrani *et al* (2013).

5.1.5 CUPRAC ASSAY

The chromogenic redox reagent used for the CUPRAC assay was bis (neocuproine) copper (II) chelate. This reagent works at pH 7, and the absorbance of the Cu (I)-chelate formed as a result of redox reaction with reducing polyphenols was measured at 450 nm. The color was due to the Cu(I)-Nc chelate formed, obtained with reacting varying concentrations of antioxidants with the CUPRAC reagent (Apak *et al*.,2007).

n $Cu(Nc)_2^{2+}$ + n-electron reductant (AO) $\longleftrightarrow$ n $Cu(Nc)_2^{+}$ + n-electron oxidized product + n H^+

This method is based on the ability of the antioxidants present in the leaf extracts of *Delonix regia* to reduce cupric ions to cuprous ions. The standard used is ascorbic acid.

The copper reducing capacity of the different solvent extracts of *Delonix regia* leaves under both hot and cold extraction conditions are represented in **tables 5.1.5.1 & 5.1.5.2** and **figures 5.1.5.1 & 5.1.5.2** respectively.

Table 5.1.5.1 Copper reducing power capacity of different solvent extracts of *Delonix regia* leaves under hot extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	100	**200**	**300**	**400**	**500**
ACE	0.229^{Pd} ±0.007	0.482^{Qe} ±0.013	0.777^{Re} ±0.005	0.978^{Sd} ±0.008	**1.178^{Te}** ±0.007
EAE	0.223^{Pd} ±0.009	0.355^{Qd} ±0.006	0.506^{Rd} ±0.005	0.587^{Rc} ±0.007	0.636^{Sc} ±0.008
HEXE	0.103^{Pb} ±0.003	0.160^{Qb} ±0.004	0.196^{Qb} ±0.005	0.243^{Rb} ±0.008	0.283^{Rb} ±0.011
METE	0.168^{Pc} ±0.003	0.298^{Qc} ±0.002	0.444^{Rc} ±0.005	0.592^{Sc} ±0.003	0.713^{Td} ±0.012
PETE	0.045^{Pa} ±0.007	0.078^{Qa} ±0.002	0.109^{Ra} ±0.005	0.150^{Sa} ±0.006	0.186^{Ta} ±0.005

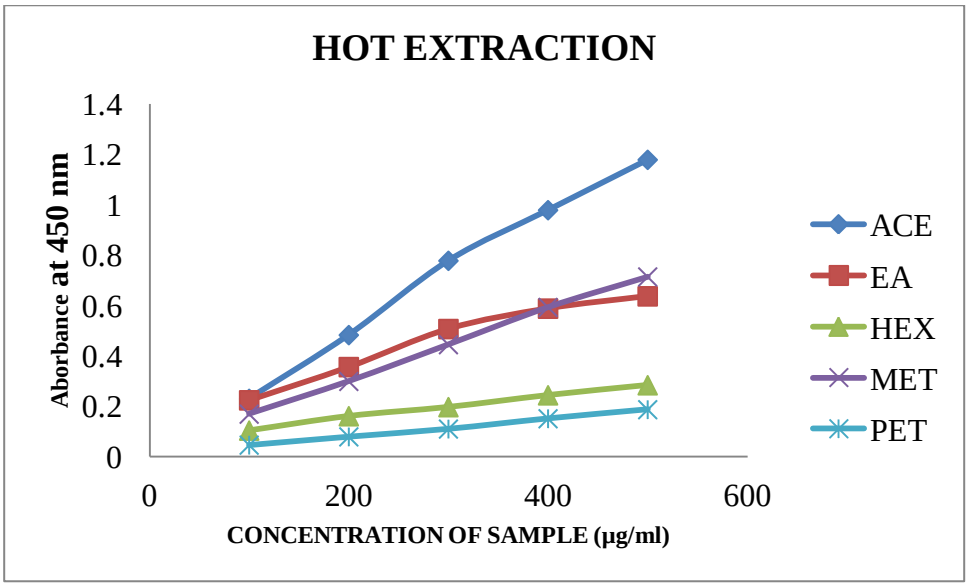
Means ranked by the common letter across the rows (P - T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract From the table 5.1.5.1 and figure 5.1.5 1, it was shown that at 500 µg/ml, the hot extracts of methanol possess high antioxidant activity which is evident from the maximum reducing power capacity followed by acetone extracts. Statistically, the reducing power of antioxidants (hot extraction) was effective in the order of Acetone extract >Methanolic extract> > Ethyl acetate extract> Hexane extract> Petroleum extract.

Fig. 5.1.5.1 Copper reducing power capacity of different solvent extracts of *Delonix regia* leaves under hot extraction conditions



Whereas, from the table 5.1.5.2 and figure 5.1.5 2, it was inferred that at 500 µg/ml, the cold extracts of methanol exhibited maximal copper reducing power capacity. Statistically, the reducing power of antioxidants (cold extraction) was effective in the order of Methanol extract>Acetone extract > Ethyl acetate extract> > Hexane Extract >Petroleum extract.

Table 5.1.5.1 Copper reducing power capacity of different solvent extracts of *Delonix regia* leaves under cold extraction conditions

SOLVENTS USED	SAMPLE CONCENTRATION (µg/ml)				
	100	**200**	**300**	**400**	**500**
ACE	0.166^{Pc} ±0.003	0.311^{Qc} ±0.003	0.336^{Qd} ±0.008	0.389^{Rc} ±0.007	0.434^{Sd} ±0.003
EAE	0.085^{Pb} ±0.006	0.191^{Qb} ±0.009	0.223^{Rc} ±0.006	0.363^{Sc} ±0.004	0.394^{Sc} ±0.005
HEXE	0.029^{Pa} ±0.008	0.051^{Qa} ±0.009	0.097^{Rb} ±0.005	0.122^{Sb} ±0.005	0.179^{Tb} ±0.008
METE	0.252^{Pd} ±0.003	0.535^{Qd} ±0.009	0.568^{Qe} ±0.005	0.698^{Rd} ±0.005	$\mathbf{0.875^{Se}}$ ±0.003
PETE	0.022^{Pa} ±0.004	0.053^{Qa} ±0.005	0.066^{Qa} ±0.002	0.080^{Ra} ±0.008	0.097^{Ra} ±0.004

Values represent mean ± standard deviation of three replicates.

Means ranked by the common letter across the rows (P - T)(denoting between concentration) are not significantly different at 5% level by DMRT.

Means ranked by the common letter across the columns (a - e) (denoting between different solvents) are not significantly different at 5% level by DMRT.

ACE – Acetone Extract; **EAE** – Ethyl acetate extract ; **HEXE** – Hexane Extract ;

METE – Methanol Extract ; **PETE** – Petroleum Ether Extract

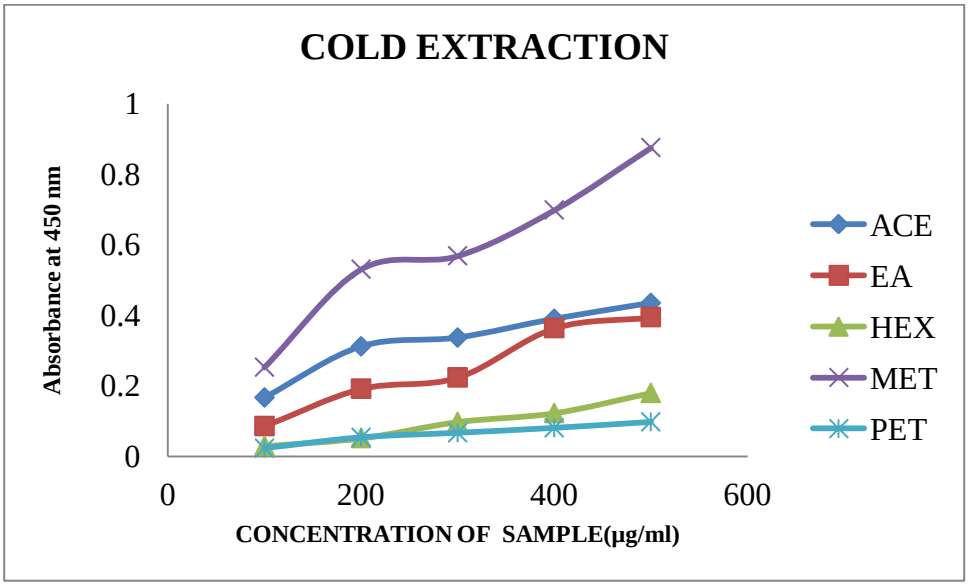


Fig. 5.1.5.2 Copper reducing power capacity of different solvent extracts of *Delonix regia* leaves under hot extraction conditions

Literature survey suggests that the CUPRAC assay is most favourable for samples with high flavonoid content (Apak *et al*., 2011).

PHASE II

5.2 PHYTOCHEMICALS

Table 5.2.1 & **5.2.2** and **figures 5.2.1 – 5.2.6** represents the contents of flavonoids, total phenol , tannins, alkaloids , anthocyanin and sterols in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions respectively.

Phenolic compounds, among other flavonoids and phenolic acids, depict antioxidant and antiradical activities *in vitro*. There is evidence that phenolic compounds can also act as antioxidants and scavengers of free

radicals *in vivo*. The antiradical and antioxidant effects of phenolic compounds *in vivo* may slow down the ageing processes as well as protect the human body against diseases such as atherosclerosis, coronary diseases and cancer.

Flavonoids, a group of polyphenolic compounds, can widely found in fruits and vegetables. Numerous positive health effects of flavonoids have been described. They have been reported to exhibit anti-cancer, anti-viral, and anti-inflammatory effects, and to reduce the risk of cardiovascular diseases. These activities are generally associated with antioxidant or free radical scavenging properties of flavonoids. The number of flavonoid derivatives is more than 4000 and their antioxidant properties are very different. Alkaloids are a class of non-nutritive phytochemical compounds that are synthesized as secondary metabolites by the plant cells. They fight against free radicals and are capable of quenching their activity. The presence of phytoconstituents like flavonoids, alkaloids and others was estimated by various phytochemical assays.

The presence of these phytochemicals simply attribute to the antimicrobial and antioxidant activities of the leaf extracts of *Delonix regia* and it has also been reported that the wood and bark of this tree possess high quantity of polyphenol (Einbond *et al*., 2003). Apart from the phytochemicals, other chemical constituents like Quercetin 3-rhamnoside, afzelin, astragalin, isoquercitin were isolated from the leaf extracts (Azab *et al*., 2013).

Flavonoids, major crusaders in the prevention of cancer also possess anti-viral and anti- bacterial properties. In the present study, a significant

elevation in flavonoid content is in the hot and cold extracts of ethyl acetate respectively. It has been reported that flavonoids were also profoundly present in the bark extracts (Fatmawaty and Astuti, 2013).

Alkaloids are usually synthesized as secondary metabolites in the plants and thus exhibit various health benefits. A significant increase in alkaloid content was observed in the hot and cold extracts of methanol respectively.

Total phenols are involved in the delaying the process of ageing and we have observed a profound elevation in total phenol content in the hot and cold extracts of methanol respectively.

Tannins and anthocyanins also contribute to antioxidant properties of plant extracts. Anthocyanins are representative of plant pigments widely distributed in colored fruits and flowers. Because anthocyanins are widely consumed, finding out additional biological activities related to these compounds would be of great interest (Andreia *et al*., 2007). Anthocyanins are normally obtained by extraction from plants and the extraction methods currently employed are with the use methanol, ethanol, acetone, water or mixtures as solvents. There is considerable anecdotal and epidemiological evidence that dietary anthocyanins and polyphenols confer preventive and therapeutic role in a number of diseases.

In the present study, both anthocyanins and tannins were found to be significantly increased in acetone and petroleum ether extracts (both hot and cold) respectively. It has been demonstrated by Saleh *et al.* 1976 that the floral parts of *Delonix regia* was found to contain two anthocyanins – cyanidin-3-glucoside and cyanidin-3-gentiobioside.

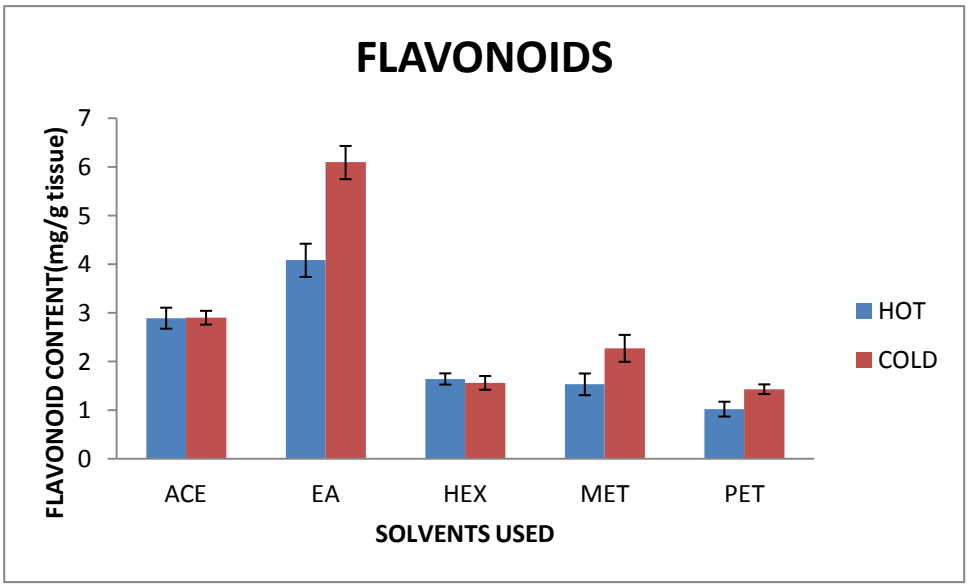


FIG 5.2.1 Flavonoid content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

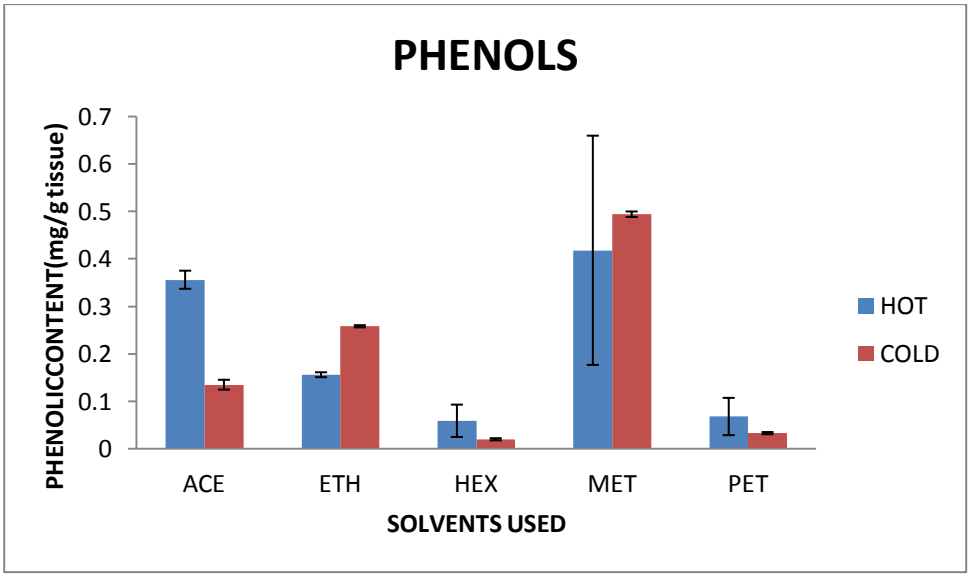


FIG 5.2.2 Total Phenol content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

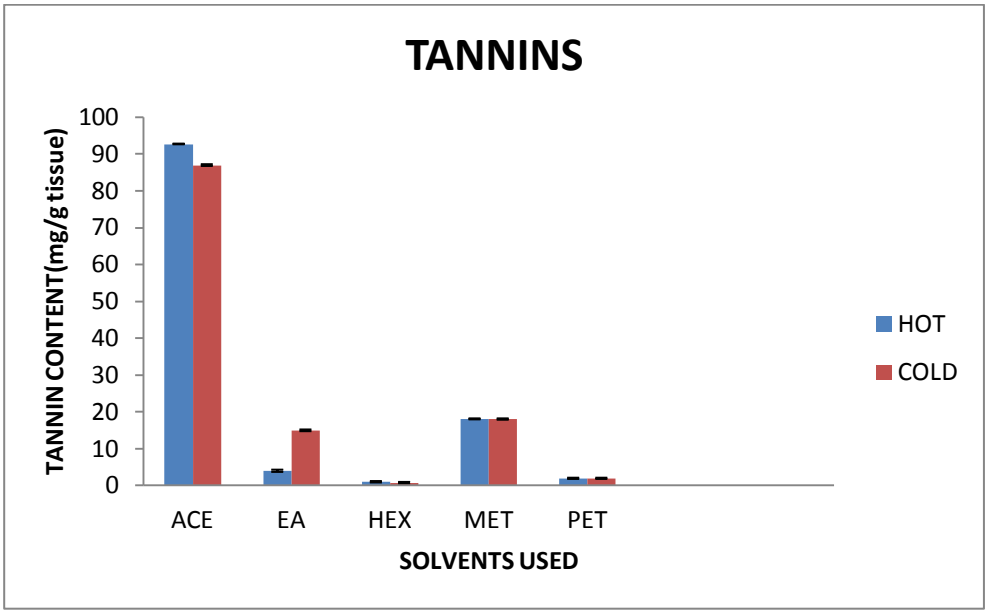


FIG 5.2.3 Tannin content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

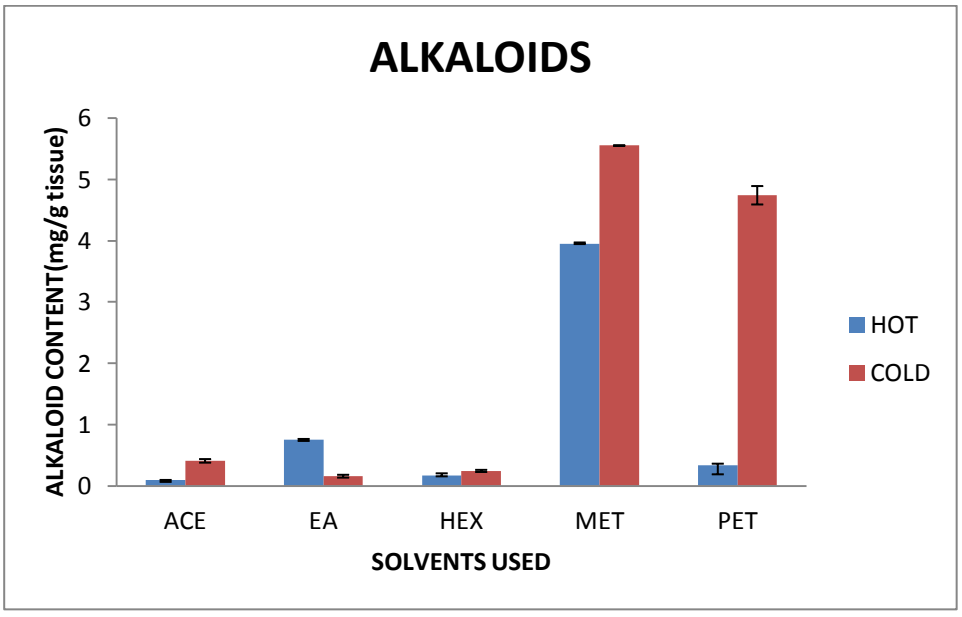


FIG 5.2.4 Alkaloid content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

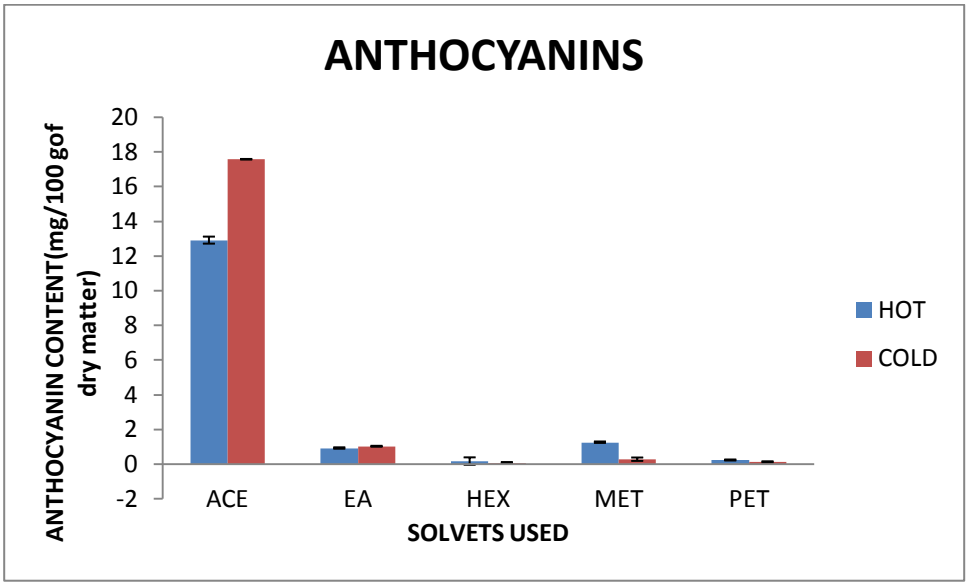


FIG 5.2.5 Anthocyanin content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

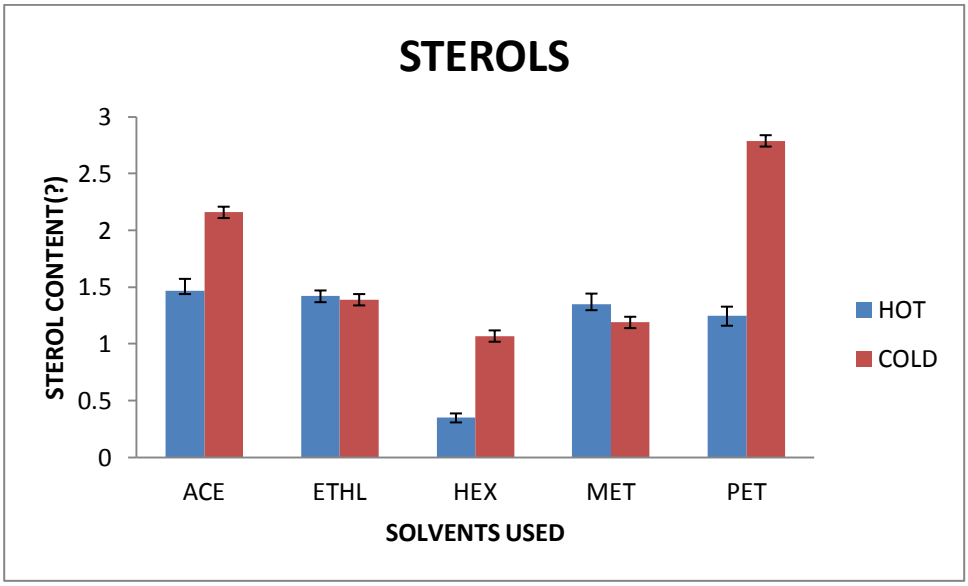


FIG 5.2.6 Sterol content in different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

PHASE III

5.3 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was done for different solvent extracts of *Delonix regia.* The chromatogram obtained was analyzed for the presence of various phytochemicals and 4 compounds were profoundly present. This interpretation was obtained from the band patterns of the chromatogram, which was visualized under UV spectrum.

The retention factor (R_f value) was calculated for each extract and this was based on the length of the bands from its loading point. The R_f values thus obtained were compared with a standard marker and the compounds present in the extracts were finalized.

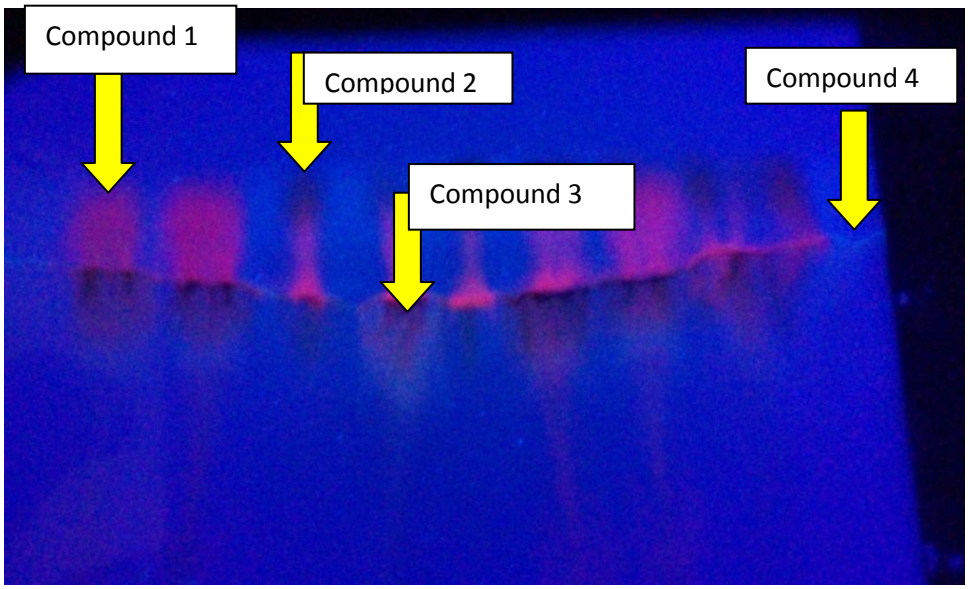


Fig.5.3 Thin Layer chromatogram of different solvent extracts of *Delonix regia* leaves under hot and cold extraction conditions

Compounds Identified by Thin Layer Chromatography

A. **HOT EXTRACTION**

Solvent Extract	**Broad class of Phytochemical**	**Specific class of Phytochemical**
Acetone	Flavone	Hydroxy flavone
Ethyl Acetate	Flavone	Flavonone
Hexane	Flavone	Chrysin
Methanol	Galangin	Flavone
Petroleum Ether	Flavone	Flavonone

B. COLD EXTRACTION

Solvent Extract	**Broad class of Phytochemical**	**Specific class of Phytochemical**
Acetone	Flavone	Naringenin
Ethyl Acetate	Flavone	Flavonone
Hexane	Kaempherol	Flavone
Methanol	Flavone	-
Petroleum Ether	Flavone	3-Hydroxy flavone

(**Ref:** Andreia et al., 2007)

5.4 Bioautography

This is an exclusive method to identify the presence of antioxidants in a sample. A Thin Layer Chromatography was done for the extracts of *Delonix regia* and the TLC plate was sprayed with DPPH solution, which characteristically bind to the antioxidant compounds present in the chromatogram and thereby appears as purple spots when obbserved under UV spectrum.

Fig5.4 DPPH was sprayed over the samples on TLC plate to identify the purple coloured antioxidant spots in the samples.

PHASE IV

5.5 Antimicrobial activity of solvent extracts of *Delonix regia* leaves

The presence of high flavonoid content in the extracts of *Delonix regia* is in strong agreement with the antimicrobial potentiality. Hence, in order to determine the efficacy of the *Delonix regia* in successfully impeding the growth of microorganisms, antimicrobial testing was performed.

The method used was well diffusion type and four different strains of bacteria were used. It includes two gram positive and two gram negative microbes. Pure cultures of *Staphylococcus aureus, Pseudomonas monteilli , Enterococcus faecalis* and *Escherichia coli* were grown under *in vitro* conditions on a petriplate containing agar as growth medium. The ten different extracts of *Delonix regia* were loaded into the wells and antibiotic, chloramphenicol was used as control.

The plates that were incubated for 24 hours were then observed for the presence of zone of clearance. Zone of clearance implies that the growth of the microorganism was aborted by the compounds present in the extracts of *Delonix regia*. The extracts of methanol exhibited maximal antibacterial activity which was evident from the zone of clearance on the plates loaded with the hot and cold methanolic extracts of *Delonix regia.* The zone of clearance was obtained for all the four strains of bacteria.

Shabir *et al*., (2011) obtained similar results for 80% methanolic extracts of leaves, bark and flowers of *Delonix regia* .

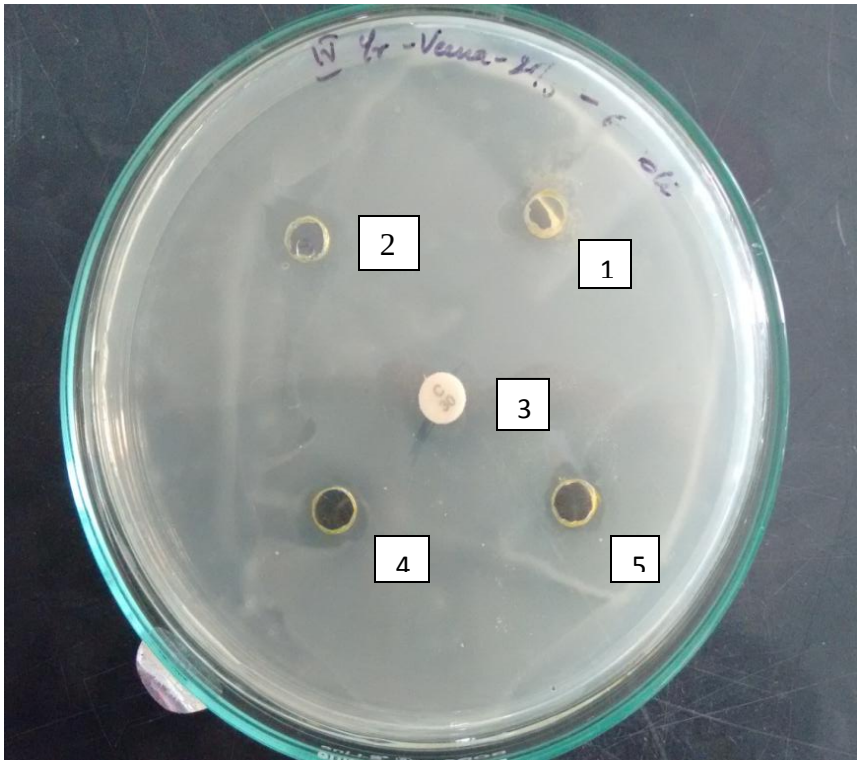


Fig 5.5.1 Antibacterial activity of *Delonix regia* extracts on *E.coli*

1. Acetone extract (cold); 2. Acetone extract (hot); 3. Chloramphenicol (control)
4. Ethyl acetate extract (cold); 5. Ethyl acetate extract (hot)

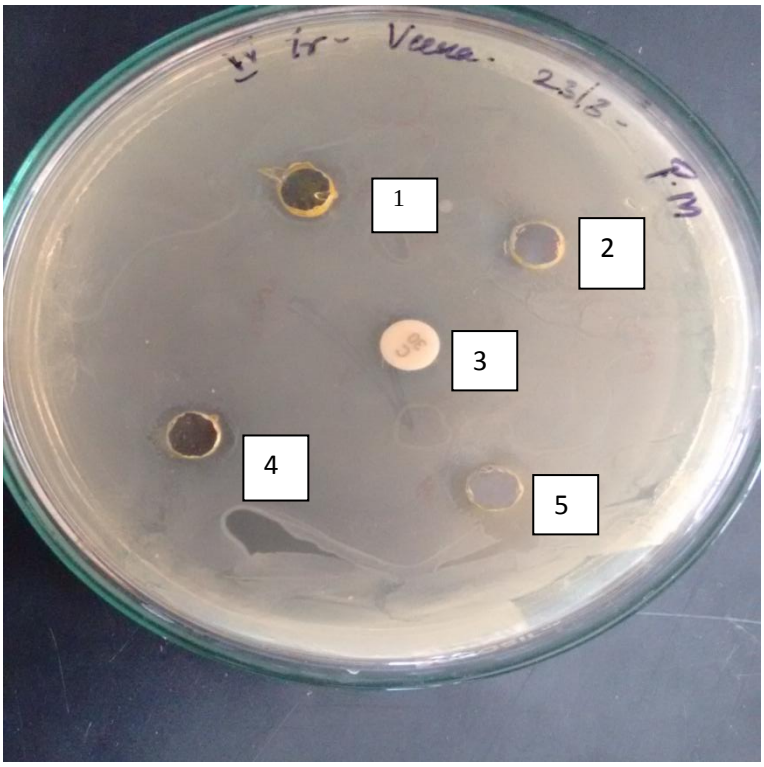


Fig. 5.5.2 Antibacterial activity of *Delonix regia* extracts on *Proteus mirabilis*

1. Hexane extract (hot): 2. Hexane extract (cold); 3. Chloramphenicol (control)
4. Methanol (hot); 5. Methanol (cold)

It has been inferred from the report by Parekh and Chanda, (2007) that methanolic and aqueous extracts of medicinal plants were tested for antimicrobial activity. The results suggest that methanolic extracts were far more effective than the aqueous and it is in accordance with the outcomes obtained by Venkatesan *et al*., (2005).

Hence, it can be well affirmed that methanolic extracts of *Delonix regia* contains compounds like flavonoids, which in turn attribute to the antibacterial activity of *Delonix regia* (Salem, 2013).

PHASE V

5.6 High Performance Thin Layer chromatogram (HPTLC) of solvent extracts of *Delonix regia* leaves

The phytochemical evaluations of plants which have a suitable history of use in folklore have often resulted in the isolation of principles with remarkable bioactivities. Hence, methodologies that can generate a fingerprint of each extract in large collections would be useful to detect stability of the same extract over time. Preferably, the method should be based on electronic storage, retrieval and analysis of the data.. A lot of separating and analytical methods like spectrophotometry, high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), gas chromatography, mass spectrometry (GC –MS) and Fourier Transform Infrared Spectroscopy (FTIR) are being developed for the study about active compounds in plants.

High performance thin layer chromatography (HPTLC) could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to

analyze several samples simultaneously using a small quantity of mobile phase

HPTLC fingerprinting is a highly reliable and advanced method of confirming the presence of phytoconstituents in the plant samples. The area under a peak, and peak height from the densitogram and the Rf values could be used to affirmatively declare the presence of a particular phytochemical.

From the Rf values and heights of peaks mentioned in the above table, it may be inferred that peaks 5-8 of hot acetone extracts of *D.regia* and peaks 2 and 3 of cold acetone extracts of *D.regia* confirm the presence of anthocyanins in the samples. Earlier studies by Chanda *et al* (2013) o*n Cissus quadrangularis* also show similar results for the presence of anthocyanins.

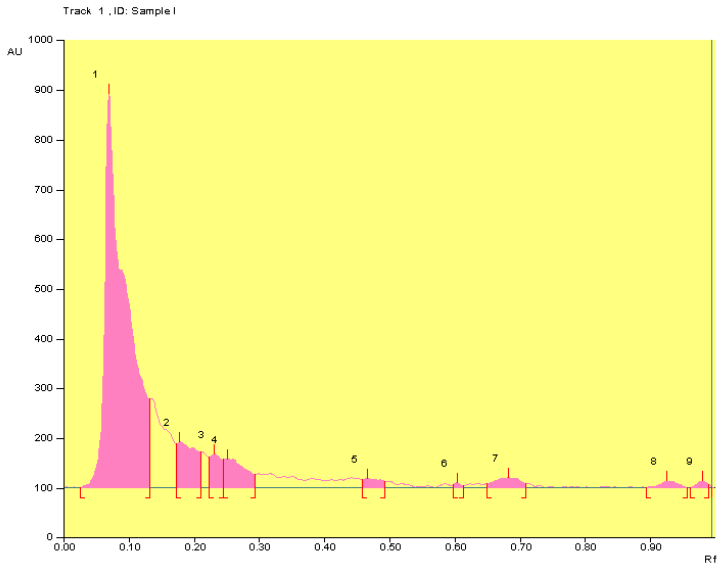


Fig 5.6.1 Hot Acetone extract of *D.regia* leaves densitogram display (Scanned at 500nm)

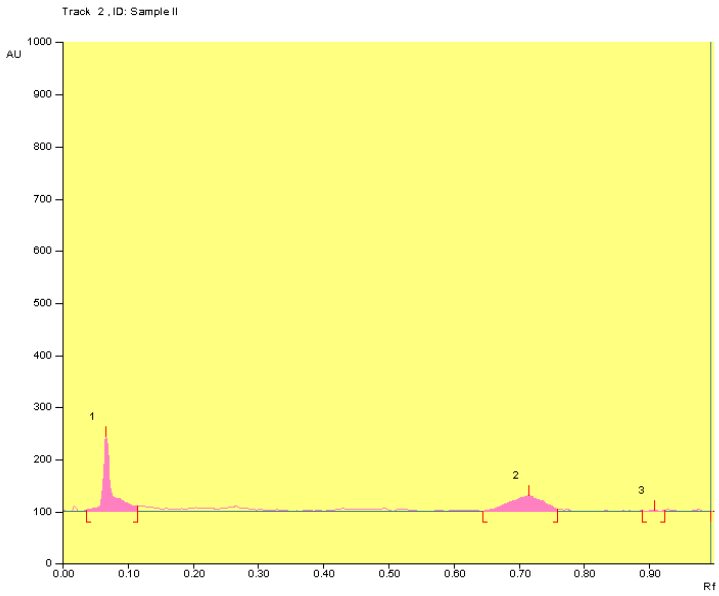


FIG 5.6.2 Cold Acetone extract of *D.regia*-leaves Peak densitogram display (Scanned at 500nm)

Table 5.6.1 Peak Table

	Peak	Rf	Height	Area
Sample I	1	0.07	792	23383.9
Sample I	2	0.18	91.6	2539.6
Sample I	3	0.23	66.9	1122.8
Sample I	4	0.25	57.7	1757.5
Sample I	5	0.47	18.4	472.5
Sample I	6	0.60	10.0	92.7
Sample I	7	0.68	19.9	730.4
Sample I	8	0.93	13.5	324.6
Sample I	9	0.98	13.4	184.8
Sample II	1	0.07	144.1	1773.2
Sample II	2	0.71	30.1	1454.9
Sample II	3	0.91	2.20	29.20

Sample I- Hot acetone extract of *D.regia* leaves

Sample II-Cold acetone extracts of *D.regia* leaves

5.7 Fourier Transfer Infra Red Spectrum (FTIR) of solvent extracts of *Delonix regia* leaves

FTIR is an easy and commonly used method to elucidate the structural features of compounds present in plant samples. Each spectral range corresponds to unique features in a molecular structure and hence they can be called fingerprint regions.

Spectral analysis of both, hot and cold acetone extracts of *D.regia* leaves yielded the following spectral bands. Similar studies on the FTIR

analysis on *Bougainvillea glabra flower* extract (Sahu and Saxena, 2013) and *Aerva lanata* roots (Mariswamy *et al.* 2012)

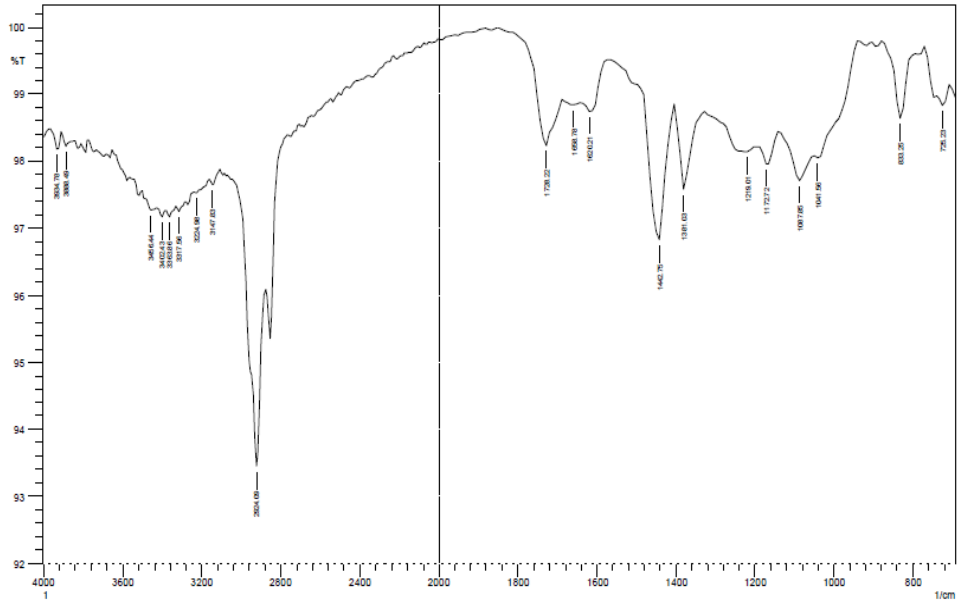


Fig. 5.7.1 Spectral analysis of acetone extract of *Delonix regia* under hot extraction conditions

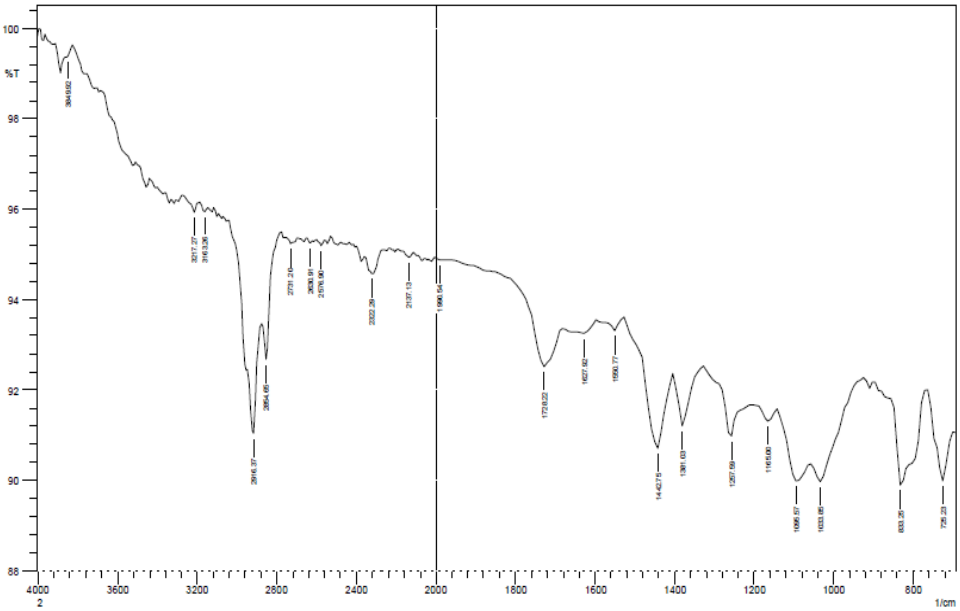


Fig. 5.7.1 Spectral analysis of acetone extract of *Delonix regia* under

cold extraction conditions

Table 5.7.1 Hot Acetone extracts of *Delonix regia* leaves (John Coates)

SPECTRAL RANGE	MOLECULAR ASSIGNMENT
3456.44-3147.83	O-H(H-bonded stretch)
2924.09	-CH_3(C-H symmetrical/asymmetrical stretch)
1728.22	-CHO
1658.78	-NH_3
1620.21	-Quinone or conjugated ketone
1442.75	Carbonate ion
1381.03	Organic sulfates
1219.01	Aromatic phosphates(P-O-C stretch)
1172.72	Sulfonate
1087.85	Silicone
1041.56	Silicone
833.25	Peroxides(C-O-O stretch)
725.23	Aliphatic chloro compounds(C-Cl stretch)

Table 5.7.2 Cold Acetone extract of *Delonix regia* leaves (John Coates)

SPECTRAL RANGE	MOLECULAR ASSIGNMENT
3217.27	Normal polymeric O-H stretch
3163.26	Ammonium ion
2916.37	Methylene(C-H symmetric/asymmetric stretch)
2854.65	Methylene(C-H symmetric/asymmetric stretch)
2731.20	Methyl(C-H symmetric/asymmetric stretch)
2576.90	Thiols(S-H stretch)
2137.13	Thiocyanate(-SCN)
1990.54	Transitional metal carbonyls
1728.22	Ester
1627.92	Secondary amine(N-H bend)
1550.77	Secondary amine(N-H bend)
1442.75	Carbonate ion
1381.03	Phenol or tertiary alcohol(OH-in plane bend)
1257.59+833.25	Epoxy and oxirane rings
1165.00	Aromatic ring (C-H in plane bend)
1095.57,1033.85	Aliphatic fluoro compounds(C-F stretch)
725.23	Aliphatic chloro compounds(C-Cl stretch)

Spectral analysis of hot acetone extracts of *D.regia* depict the presence of amide, conjugated ketone, and carbonate ion(1658.78+1620.21+1442.75 cm^{-1}) in combination, which indicates a possible flavone backbone. Also, a broad spectrum of 3456.44-3147.83 cm^{-1} indicates the presence of O-H bonds (Nirmaladevi *et al*, 2010).

Similarly, the presence of tertiary phenolic tertiary alcohols (1381.03 cm^{-1}) in cold acetone extracts indicates the possible existence of phenolic acids.

5.8 ORTHOGONAL DESIGN OF EXPERIMENTS:

In order to obtain maximum extraction efficiency it is important to optimize the extraction conditions. The optimization mainly concentrate on designing the experiments based on the factors involved in extraction like solid- liquid ratio, number of cycles and time taken for extraction. Since this concept includes large number of experiments , it is significant to chose a method which gives rapid results in an economical manner (Sathishkumar et al., 2009) . One such method is Response Surface Methodology.

TABLE 5.8.1 Orthogonal design parameters

	HOT EXTRACTION		COLD EXTRACTION	
LEVELS	A S/L RATIO	B NO.OF CYCLES	A S/L RATIO	B NO.OF HOURS
1	1 : 15	15	1 : 15	8
2	1:20	20	1:20	16
3	1:25	25	1:25	24

Since , RSM was done to enhance the flavonoid and anthocyanin content, two responses were recorded for each hot and cold extracts.

ANOVA for Response Surface Quadratic model

RESPONSE 1: Anthocyanins

TABLE 5.8.2 Analysis of variance table [Partial sum of squares - Type III]

COLD EXTRACTS						
Source	Sum of Squares	Degrees of freedom	Mean square	F value	P value	
Model	177.56	5	35.51	11.83	0.0026	**SIGNIFICANT**
A	74.69	1	74.69	24.89	0.0016	
B	0.49	1	0.49	0.16	0.6990	
AB	1.88	1	1.88	0.63	0.4550	
A^2	82.26	1	82.26	27.41	0.0012	
B^2	0.24	1	0.24	0.081	0.7837	
Residual	21.01	7	3.00			
Lack of fit	21.01	3	7.00			**INSIGNIFICANT**
Pure error	0.000	4	0.000			
Cor Total	198.57	12				

HOT EXTRACTS						
Source	Sum of squares	Degrees of freedom	Mean square	F value	P value	
Model	342.08	5	68.42	7.64	0.0093	
A	226.32	1	226.32	25.29	0.0015	
B	9.98	1	9.98	1.12	0.3259	
AB	1.89	1	1.89	0.21	0.6597	
A^2	85.68	1	85.68	9.57	0.0175	
B^2	55.81	1	55.81	6.24	0.0412	
Residual	62.64	7	8.95			
Lack of fit	62.64	3	20.88			
Pure error	0.000	4	0.000			
CorTotal	404.72	12				

RESPONSE 2: Flavonoids

TABLE 5.8.3 Analysis of variance table [Partial sum of squares - Type III]

COLD EXTRACTS						
Source	Sum of Squares	Degrees of freedom	Mean square	F value	P value	
Model	40.58	5	8.12	30.01	0.0001	**SIGNIFICANT**
A	31.74	1	31.74	117.36	0.0001	
B	0.44	1	0.44	1.64	0.2415	
AB	0.42	1	0.42	1.54	0.2548	
A^2	5.62	1	5.62	20.78	0.0026	
B^2	0.27	1	0.27	0.99	0.3526	
Residual	1.89	7	0.27			
Lack of fit	1.89	3	0.63			**INSIGNIFICANT**
Pure error	0.000	4	0.000			
Cor Total	42.47	12				
HOT EXTRACTS						

Source	Sum of squares	Degrees of freedom	Mean square	F value	P value	
Model	73.58	5	14.72	46.75	0.0001	**SIGNIFICANT**
A	37.55	1	37.55	119.29	0.0001	
B	0.98	1	0.98	3.10	0.1216	
AB	0.99	1	0.99	3.15	0.1194	
A^2	28.96	1	28.96	92.00	0.0001	
B^2	1.474E-003	1	1.474E-003	4.683E-003	0.9474	
Residual	2.20	7	0.31			
Lack of fit	2.20	3	0.73			**INSIGNIFICANT**
Pure error	0.000	4	0.000			
CorTotal	75.78	12				

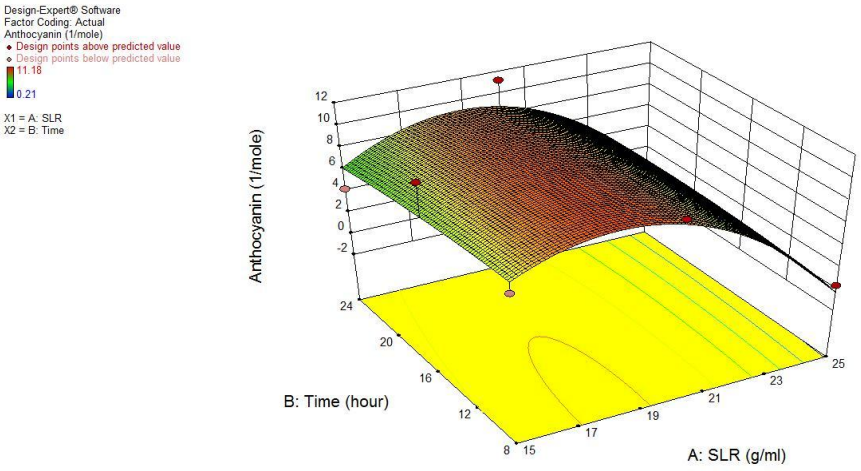


FIG 5.8.1 MODEL GRAPH FOR ANTHOCYANIN – COLD EXTRACTS

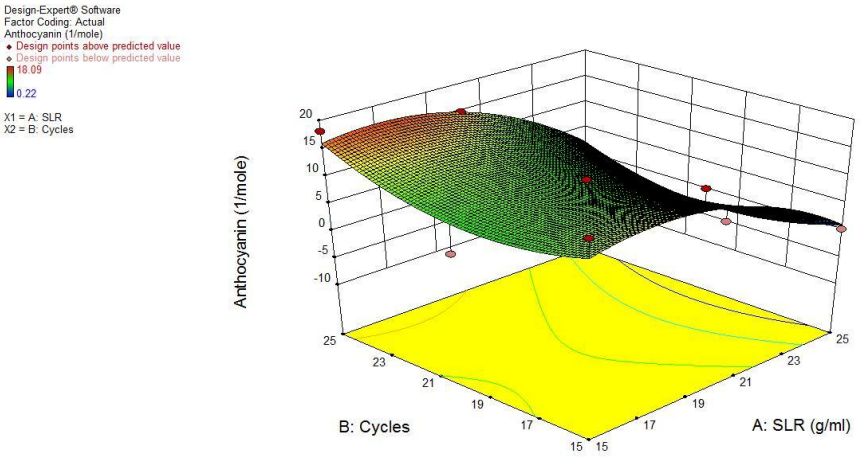


FIG 5.8.2 MODEL GRAPH FOR ANTHOCYANIN –HOT EXTRACTS

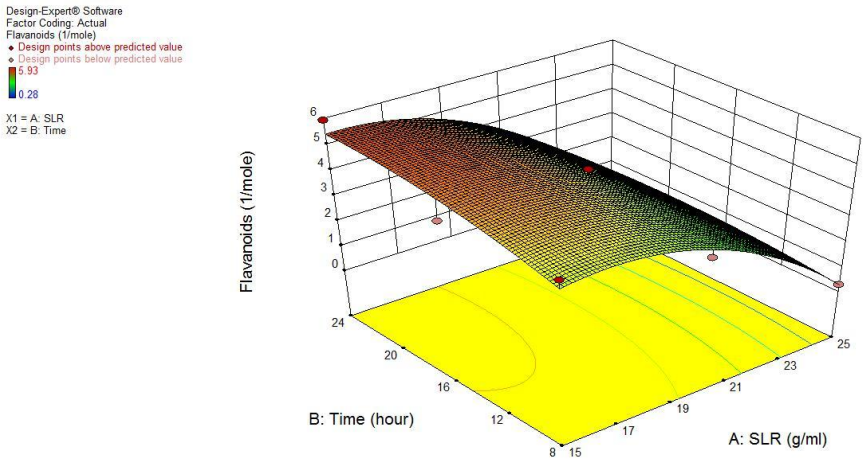


FIG 5.8.3 MODEL GRAPH FOR FLAVONOIDS – COLD EXTRACTS

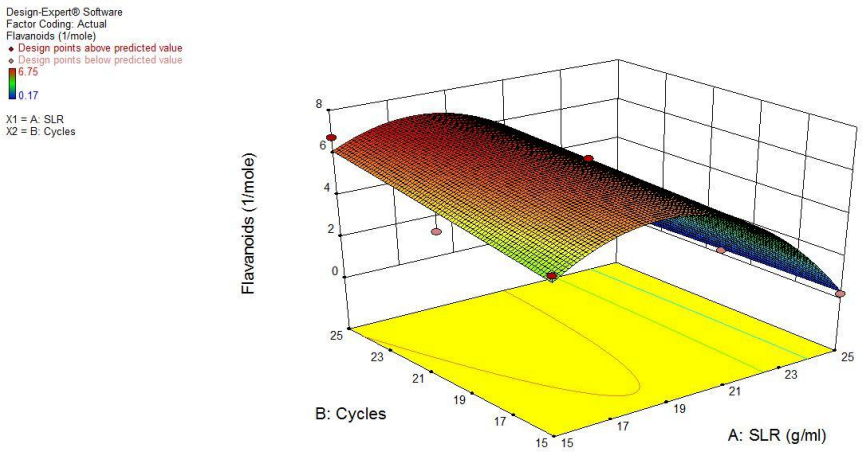


FIG 5.8.4 MODEL GRAPH FOR FLAVONOIDS – HOT EXTRACTS

CONCLUSION

Extensive studies on *D.regia* indicate that it could be a hedgerow treasure with good antioxidant potential besides possessing antimicrobial properties. Characterisation techniques like FTIR and HPTLC have also indicated the same.The flavonoid and anthocyanin content in the extracts could be enhanced successfully by Response surface methodology. Hot methanolic and acetonic leaf extracts have yielded consistently good results hinting that the solvent used and extraction method are highly influential factors in extraction. A general trend that was observed in efficiency (Acetone>Methanol>Ethyl acetate>Hexane>Petroleum ether) may be attributed to the fact that extraction efficiency increases with increasing polarity index of the solvent. Such facts may be put to use in the development of a commercial nutrient potion, which will ultimately become the Elixir of human life-a solution to all health problems of mankind. The development of a commercial product, literally from roadside resource will shift global attention towards Indian pharmaceutical industry and bring about a manifold difference in its economy.

REFERENCES

Adetogun, G.E and Alebeowu, G. (2009), 'Properties of *Delonix regia* seed gum as a tab binder', Acta Poloniae Pharmaceutica n Drug Research, 66 (4), pp. 433-438.

Adje, F., Lozan, Y.F., Meude, E., Lozan, P., Adima, A., N'zi, G.A. and Gaydou, E.M. (2008) 'Anthocyanin Characterization of Pilot Plant Water Extracts of *Delonix regia* Flowers', Molecules, 13(6), *pp*. 1238-1245.

Alfadda, A.A. and Sallam, R.M. (2012) 'Reactive Oxygen Species in Health and Disease', Journal of Biomedicine and Biotechnology, Vol. 2012.

Andreia .A. de Lima, Eliana ,M., Sussuchi, and Wagner F. (2007), 'Electrochemical and Antioxidant Properties of Anthocyanins and Anthocyanidins', Croatica Chemica Acta Ccacaa, Vol.80 (1), pp. 29-34.

Apak, R.,Guclu, K., Demirata, B., Ozyurek, M., Celik, S.E.,Bektasoglu, B., Berker, K.I. and Ozyurt, D. (2007) 'Comparitive Evaluation of Various Total Antioxidant Capacity Assays Applied to Phenolic Compounds with the CUPRAC Assay', Molecules, Vol.12, pp. 1496-1547.

Aqil, F., Ahmad, I. and Mehmood, Z. (2006) 'Antioxidant and Free Radical Scavenging Properties of Twelve Traditionally Used Indian Medicinal Plants', Turkish Journal of Biology, 30 (3), pp. 177-183.

Ashokkumar,R. and Ramaswamy,M. (2014) 'Phytochemical Screening by FTIR Spectroscospic Analysis of leaf extracts of selected Indian Medicinal

Plants', International Journal of Current Microbiology and Applied Sciences, Vol.3(1), pp. 395-406.

Azab, S.S., Abdel-Diam, M. and Eldashan, O.A. (2013) 'Phytochemical, cytotoxic, hepatoprotective and antioxidant properties of *Delonix regia* leaves extract', Medicinal Chemical Research, Vol. 22(9), pp. 4269-4277.

Bondy,S.C. ,Guo-Ross,S.X. and Pien,J. (1998) 'Mechanism underlying the Aliminium-induced potentiation of the pro-oxidant properties of transition metals', Europe Pub Med Central(Neurotoxicology) ,19(1), pp. 65-71.

Brewer,M.S. (2011) 'Natural Antioxidants : Sources, compounds, Mechanism of Actions and Potential Applications', Comprehensive Reviews in Food Science and Food Safety, Vol. 11, pp. 221-247.

Carr, A. and Frei, B. (1999) 'Does Vitamin C act as a pro-oxidant under physiological conditions?' Federation of American Societies for Experimental Biology, Vol. 13, pp. 1007-1024.

Chanda,S., Baravalia, Y. and Nagani, K. (2013) 'Spectral analysis of methanol extract of *Cissus quadrangularis* L. stem and its fractions', Journal of Pharmacognosy and Phytochemistry, Vol. 2(4), pp. 149-157 .

Chavan, B.L. and Rasal, G.B. (2012) 'Comparative Status of Carbon Dioxide Sequestration in *Albizia Lebbek and Delonix Regia'*,Universal Journal of Environmental Research and Technology, 2(1), pp. 85-92.

Chitra, V., Ilango, K., Rajanandh, M.G. and Soni, D. (2010) 'Evaluation of *Delonix regia* Linn. Flowers for Antiarthritic and antioxidant activity in female wistar rats', Annals of Biological Research, Vol.1 (2), pp. 142-147.

Coates,J (2000) 'Interpretation of Infrared Spectra, A Practical Approach', Encyclopedia of Analytical Chemistry R.A. Meyers (Ed.),John Wiley & Sons Ltd, Chichester, pp. 10815 – 10837.

de Sousa,M.C., Vierira,R.B.,Dosantos.,D.S.,Carvalho,C.A.,Camargo,S.E., Mancini,M.N. and de Oliveira,L.D. (2014) 'Antioxidants and biomarkers of oxidative damage in the saliva of patients with Down syndrome', Europe Pub Med Central, Vol. 60(4), pp. 600-605.

Duan, X. (2006) 'Evaluation of antioxidant property of extract and fractionsobtained from red algae *Polysyphonia urceolata'*, Food Chemistry, Vol.95, pp. 37-49.

Einbond, L.S., Reynertson, K.A., Luo, X.D., Basile, M.J. and Kennelly, E.J. (2003) 'Anthocyanin antioxidant from edible fruits', Food Chemistry, Vol. 84(1), pp. 23-28.

Fatmawaty, F. and Astuti, H. (2013) 'Antimalarial activity of *Delonix regia* on mice with *Plasmodium berghei*', Journal of Natural Products, Vol. 6, pp. 61-66.

Gomathy, K., Baskar, R. and Kumaresan, K. 2013 'Comparison of antioxidant potential in pulp and peel extracts of *Manilkara zapota* (L.) P.Royen', Academic Journals, Vol, 12(31), pp. 4936- 4943.

Gould, J.C. and Bowie, J .H. (1952) 'The determination of bacterial sensitivity to antibiotic', Edniberg Medical Journal, 59(26), pp. 178.

Guliyeva, V.B., Gul, M. and Yildirim, A.(2004) ' *Hippophae rhamnoides* L.: Chromatographic methods to determine chemical composition, use in

traditional medicine and pharmacological effects', Journal of Chromatography B, Vol. 812, pp. 291-307.

Gupta, V.K. and Sharma, S.K. (2006) 'Plants a Natural Antioxidants', Natural Product Radiance, Vol. 5(4), pp. 326-324.

Indhumathi, P., Syed Shabhudeen, S.P., Saraswathy, C. P. (2011) 'Synthesis and characterization of nano-silica from the pods of *Delonix regia* ash', International Journal of Advanced Engineering Technology, 2(4), pp. 421-426.

Joy, K.L. and Kuttan, R.J. (1999) 'Anti-diabetic activity of *Picrorrhiza kurroa* extract', Journal of Ethnopharmacology , 67(2), pp.143-148.

Jungalwala, F. B and Cama, H .R. (1962) 'Carotenoids in *Delonix regia* (Gul Mohr) Flower', Biochemical Journal, 85(1), pp. 1-8.

Kalpana,R.,Mital,K and Sumitra,C. (2011) 'Vegetable and Fruit peel as a novel source of Antioxidants', Journal of Medicinal Plants Research, Vol. 5(1), pp. 63-71.

Kumar, S.M., Astalakshmi, N., Aparna, P., Poojitha, K., Karishma, S. K. and Sivajyotsna, P. (2011) 'Isolation and characterization of a new steroidal derivative and evaluation of its antioxidant potential from *Delonix regia* (BOJ. EX. HOOK) RAF', 10(2), pp. 90-94.

Mariajancyrani, J., Chandramohan, G. and Ravikumar, R. (2013) 'Isolation and Identification of Phytoconstituents from *Delonix regia'*, International Journal of Pharmacy and Pharmaceutical Sciences, Vol. 5(4), pp.671-674.

Mariajancyrani,J., Chandramohan,G., Saravanan,P. and Saivaraj,S. (2013) '*In Vitro* Antioxidant potential of *Delonix regia* leaves', Scholars Academic Journal of Pharmacy, Vol.2(6), pp. 468-471.

Mariswamy, Y., Gnanaraj, W.E. and Antonisamy, J.M. (2012) 'FTIR Spectroscopic studies on Aerva Lanata (L.) Juss.Ex.Schult', Asian Journal of Pharmaceutical and Clinical Research, Vol.5, Issue.2, pp. 82-86.

Medic-Saric, M., Jasprica,I., Smolcic- Bubalo, A. and Mocnar,A. (2007) 'Optimization of chromatographic conditions in Thin Layer Chromatography of Flavonoids and Phenolic Acids', Croatia Chema Acta, Vol. 77 (1-2), pp. 361-366.

Mishra, S.S., Patel, .K.K., Raghuwanshi, N., Pathak, A., Panda, P.P., Girhepunje, K. and Patro, N. (2011) 'Screening of Ten Indian Medicinal plant extracts for antioxidant activity', ABRNBW, 2(1), pp.262-270.

Mohammed,Z.M. (2013) 'Evaluation of the Antibacterial and Antioxidant activities of Stem Bark Extracts of *Delonix regia* and Erthrina humeana Grown in Egypt', Journal of Forest Products and Industries, Vol. 2(2), pp. 48-52.

Murphy, M.P. (2009) 'How mitochondria produce reactive oxygen species', Biochemical Journal, Vol.417, pp. 1-13.

Nayak, C and Rastogi, N.K. (2013) 'Characterization of Anthocyaninins from Garcinia indica choisy', Food Chemistry, Vol 118 (3), pp-719-724.

Parekh, J., Jadeja, D. and Chanda, S. (2005) 'Efficacy of Aqueous and Methanol extracts of Some Medicinal Plants for Potential Antibacterial Activity', Turkish Journal of Biology, 29(2005), pp. 203-210.

Patil, A.K. and Shrivastava, V.S. (2012) 'Adsorption of Ni (II) from aqueous solution on *Delonix regia* (Gulmohar) tree bark, Arch', Journal of Applied Sciences Research, 2 (2), pp. 404-413.

Patil, D.D., Mhaske, D.K., Patare, M.A. and Wadhawa, G.C. (2014) 'Antibacterial and Antioxidant, Anti-Inflammatory Study of Leaves and Root of *Delonix regia*', International Journal of Pharmaceutical and Chemical Sciences, Vol. 3(1), pp. 131-135.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1999) 'Microbiology', The McGraw –Hill Companies, Inc., Boston.

Rahman,M.M., Hasan, M.N., Das,A.K., Hossain,M.T., Jahan,R., Khatun,M.A. and Rahmatullah,M. (2011) 'Effect of *Delonix regia* leaf extract on glucose tolerance in glucose induced hyperglycemic mice' African Journal of Traditional, Complement and Alternative Medicine, 8(1),pp.34-36.

Sahu,N. and Saxena, J. (2013) 'Phytochemical Analysis of Bougainvillea Glabra Choisy by FT-IR and UV-Vis Spectroscopic Analysis', International Journal of Pharmaceutical Sciences Review Research, Vol. 21(1), pp. 196-198.

Saleh, N.A.M. and Ishak, M.S. (1976) 'Anthocyanins of some leguminosae flowers and their effect on color variation', Phytochemistry, Vol.15, pp. 835-836.

Salem, M.Z.M., Megeed, A.A. and Ali, H.M. (2014) 'Stem Wood and Bark Extracts of *Delonix regia:* Chemical Analysis and Antibacterial, Antifungal, and Antioxidant Properties', Bio Resources, Vol. 9(2), pp. 2382-2395.

Salem,M.J.M. (2013) 'Evaluation of the Antibacterial and Antioxidant Activities of Stem Bark Extracts of *Delonix Regia* and *Erythrina Humeana* Grown in Egypt', Journal of forest products & industries, 2013, 2(2), pp.48-52.

Sama, K., Raja, X.V.A. and Yadav, H.R. (2012) 'Antibacterial and pharmacognostical evaluation of *Delonix regia* root bark', International Journal of Pharmacy and Life Sciences, 3(4), pp.1628-1630.

Sampath, M. (2013) 'Optimization of the Extraction Process of Phenolic Antioxidant from *Polyalthia Longifolia (Sonn.)* Thawaites', Journal of Applied Pharmaceutical Science Vol. 3 (02), pp. 148-152.

Sanchez- Moreno, C. (2002) 'Methods used to evaluate the free radical scavenging activity in foods and biological system', Food Science Technology International, Vol. 8, pp. 122.

Sathishkumar, T., Baskar, R., Shanmugam, S., Rajasekaran, P., Sadasivam, S. and Manikandan, V. (2009) 'Optimization of flavonoids extraction from the leaves of *Tabernaemontana heyneana Wall.* using L16 Orthogonal design', Nature and Science, 6(3), pp. 1545-0740.

Schwartz, J.L. (1996) 'The dual roles of nutrients as antioxidants and prooxidants; their effects on tumor cell and growth', The Journal of Nutrition, Vol. 126, pp. 122,1S-7S.

Shabir, G., Anwar, F., Sultana, B., Khalid, Z.M. and Afzal, M. (2011) 'Antioxidant and Antimicrobial Attributes and Phenolics of Different Solvent Extracts from Leaves, Flowers and Bark of Gold Mohar [*Delonix regia* (Bojer ex Hook.) Raf.]', *Molecules*, *16*(1), pp. 7302-7319.

Shah, N.M., Morris, M., Grijalva, A. and Chapman, J.M. (2009) 'Characterization of Anthocyanins and Flavonol Glycosides from *Delonix regia* and Ixora Casei Hance by LC-ESI-MS-MS', Molecules, 2(9), pp. 337-341.

Shanmukha, Patel, H., Patel, J. and Riyazunnisa. (2011) 'Quantification of Total Phenol and Flavonoid Content of *Delonix regia* Flowers', ChemTech, 3(1), pp 280-283.

Sharma, R.A., Chandrawat, P., Sharma, S., Sharma, D., Sharma, B. and Singh, D. (2010) 'Efficacy of *Delonix regia* rafin (syn. *Poinciana regia*) (BOJER EX. HOOK) for potential antifungal activity', The Bioscan, 5(3), pp. 441 – 444.

Shewale, W.D., Deshmukh, T.A., Patil, L.S. and Patil, V.R. (2011) 'Anti-Inflammatory Activity of *Delonix regia* (Boj. Ex. Hook)', Hindawi Publishing Corporation, 1, pp.1-4.

Shiramane, R., Basavaraj, S., Manoj, K., Karnakumar, B., Khan, A. (2011) 'Carotenoids *in Delonix regia* (Gul Mohr) Flower', IRJP, 2(5), pp.234-238.

Sies, H.V. and Stahl, W. (1995) 'Vitamins E and C, beta carotenr and other carotenoids as antioxidants', American Journal of Clinical Nutrition, Vol. 62, pp. 1315-1321.

Su, J., Fan, C., Whitaker, J.R., Haard, N.F., Shoemaker, C.F. and Singh, R.P. (1997) Antioxidant activity and mechanism of isolated components from flowers of *Delonix regia*. *Food* for Health in the Pacific Rim, Culinary and Hospitality Industry Publication Services, Texas, USA, pp 243-252.

Tenpe, C.R., Upaganlawar, A.B., Khairnar, A.U. and YeoleAntioxidant, P.G. (2008) 'Antihyperlipidaemic and antidiabetic activity of *Dendrophthoe falcata* leaves-A preliminary study', Pharmacognosy Magazine,vol. 4, pp.16.

Tokusoglu, O., Yildirim, Z. (2012) 'Effects of cooking methods on the Anthocyaninl levels and antioxidant activity of a local Turkish Sweet potato [Ipomoea batatas (L.) LAM]', Turkish Journal of Field Crops, 2012, 17(1):87-90

Veigas,J.M., Narayan,M.S., Kotamballi,N., Chidabaramurthy and Neelwarne,B. (2007) ' Antioxidative Efficacies of Floral Petal Extracts of *Delonix regia* Rafin',International Journal of Biomedical and Pharmaceutical Sciences, Vol.1(1), pp. 73-82.

Venkatesan, M., Vishavanathan, M.B. and Ramesh, N. (2005) 'Antibacterial potential from Indian *Suregada angustifolia*', Journal of Ethnopharmocology, Vol. 99, pp. 349- 352.

Vivek, M.N., Sachidananda Swamy, H.C., Manasa, M, Pallavi, S., Yashodakambar, Asha, M.M., Chaithra, M., Prashith Kekuda, T.R.,

Mallikarjun, N. and Onkarappa, R. (2013) ‘Antimicrobial and Antioxidant activity of leaf and flower extract of Caesalpinia *pulcherrima*, *Delonix regia* and *Peltaphorum ferrugineum*’, Journal of Applied Pharmaceutical Science Vol. 3 (08), pp. 064-071.

Yamaguchi, T., Takamura, H., Matoba, T. and Terao, J. (1998) ‘HPLC method for evaluation of the free radical-scavenging activity of foods by using 1, 1,-diphenyl-2-picrylhydrazyl’, Bioscience, Biotechnology, Biochemistry, Vol. 62, pp.1201– 1204.

Zou, Y., Lu, Y. and Wei, D. (2004) ‘Antioxidant activity of a flavonoid rich extract of *Hypericum perforatum* L. *in vitro*.’ Journal of Agricultural and Food Chemistry, Vol. *52*, 5032-5039.