Comparitive assesment of antioxidant and free radical scavenging activity of few plants used as hair growth promoter

By

RAMYA DEVI P

(16PBO011)

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Signature of the Supervisor

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Introduction

Introduction

Today most of the human beings are very careful about their beauty and hair play an important role in this. From ancient times all over the world people use shampoo to clean their hair. It is the most common form of hair treatment. Shampoos have primarily been products aimed at cleansing the hair and scalp.

The diversity of qualities demanded from a good shampoo by today's consumer goes far beyond this general function. A cosmetic benefit is expected, and the shampoo formulation has to be tailored to all the possible variations associated with hair quality, age, care habit and specific problems relating to the superficial condition of the scalp. Regular shampoo can wreak havoc on not only our hair but also our bodies. Because it contains harmful and synthetic chemicals that are absorbed through the skins pores we are putting ourselves at risk by directly applying carcinogenic chemicals into the scalp.

Among the thousands of naturally occurring constituents so far identified in plants and exhibiting a long history of safe use, there are none that pose - or reasonably might be expected to pose - a significant risk to human health at current low levels of intake when used as flavoring substances. Due to their natural origin, environmental and genetic factors will influence the chemical composition of the plant essential oils. Factors such as species and subspecies, geographical location, harvest time, plant part used and method of isolation all affect chemical composition of the crude material separated from the plant. Phytochemicals are chemicals produced by plants through primary or secondary metabolism. Phytochemicals are naturally occurring primary and secondary compounds in the medicinal plants, leaves, vegetables and roots. They generally have biological activity in the plant host and play a role in plant growth or that has defence mechanism and protect from various diseases, pathogens, or predators.

More than thousands of year, people from all over the world plant materials were used for hair care (Gupta *et al.*, 2010). In Ayurvedic literature there are records of number of herbs, which promote growth of hair, keep them soft and silky and do not allow them to turn grey at an early stage. Besides health care, beautification of the body is also done by herbs, various cosmetics, and colour. Though many Ayurvedic plant products have been replaced by synthetic chemical

compounds, the safety and efficacy of natural products could not find suitable match. There is once again a revival of preference for natural products (Sharma *et al*., 2003).

In India, a variety of herbs and their extracts were used as shampoos since ancient times. Natural herbal rinses are gentle and nourishing for the scalp. They rely upon botanicals, to promote healthy hair and scalp, rather than the synthetic ingredients and chemicals that can damage hair and cause build-up. Herbal infusions have been used for centuries to naturally soften hair, increase manageability, and restore luster, body and bounce. Herbal hair rinses can provide a deep cleansing, lighten, darken or enrich your natural hair color, soothe irritation, prevent dandruff or stimulate the scalp to increase growth (Thomas *et al.*, 2017).

Application of plant leafs, flowers or seeds as shampoos are good as they don't have any adverse effects and are used for centuries to help smooth, soften, shine, and otherwise condition human hair, leading to a healthy attractive appearance (Campo *et al*., 2017). Human beigns in general face various problems like hair, graying, hair fall, dandruff, hair breakage etc. (Singh *et al.*, 2015). The age at which hair problem appears is largely decided by heredity. But premature loss of pigment in adults, hair fall, breakage all are caused by a variety of factors, including illness, certain drugs, wariness, even shock and it is irreversible.

Plant materials can be used as hair growth stimulation, hair colorants and dyes, and in a number of hair and scalp complaints such as dandruff. Literature study revealed many herbs whose leaves are used as shampoo by various people. It is revealed that regular use of herbal shampoos gives a black shininess and softness to the hair. Besides, it provides an anti-agening affect to the hair preventing grayness formation of the hair. Among those four plants were taken for the study, *Hibiscus rosa-sinensis*, *Mussaenda frondosa* and *Albizia amara* , *Derris indica* respectively.

According to traditional texts (Nadkarni, 1954; Kumar *et al*., 1994), it is well accepted that the leaves and flowers of *Hibiscus rosa-sinensis* have hair growth promoting and anti-greying properties. Moreover, in India the herbal products in the market intended for hair growth include the extract of various parts of *Hibiscus rosa-sinensis* (Adhirajan *et al*., 2003).The natives of southern India use the red hibiscus as hair shampoo for the remedy of hair-fall and dandruff by inducing the hair follicles to make protective oils (Rummel, 2005). The leaf extract can be

developed into skin care products because of its high antioxidant activity. (Divya *et al*., 2013). *Hibiscus rosa-sinensis* is used for the treatment of various diseases including alopecia (Upadhyay and Upadhyay, 2011; Upadhyay et al., 2013 and Adhirajan *et al.*, 2003). Traditionally, apart from this it is also used for anti-dandruff agent. Moreover it is useful for hair growth promoting activity, by stimulating hair follicles and improves the blood flow of the hair (Brandon, 2014 and Throat *et al*., 2009). The leaves and flowers of *Hibiscus rosa-sinensis* have hair growth promoting activity (Babu *et al*., 2003).

Albizia amara are used as source for animal fodder and may also be used as firewood. it is folk remedy for curing various diseases viz., dandruff, diarrhea, common cold, wounds and gonnorrhoea. (farnsworth *et al* .,1985). *Albizia amara* is an important medicinal plant found throughout India. The entire plant possesses pharmaceutical constituents of great significance. This plant has been used as an important folk medicine for the treatment of several diseases like diarrhea, gonorrhea, skin diseases, poisonous bites and leprosy. (Indravathi *et al.,* 2014)

Pongamia pinnata is a fast-growing tree which reaches 40 feet in height and spread, forming a broad, spreading canopy casting moderate shade. (Allen, 1981).This plant has long been used in India and neighboring regions as a source of traditional medicines, animal fodder, green manure, timber, fish poison and fuel. Extract of the plant possess significant anti-diarrhoeal, anti-fungal, anti-plasmodial, anti-ulcerogenic, anti-inflammatory and analgesicactivities. Antibacterial activity of various extract of leaves of *P. pinnata* was carried in attempt to support the use by medicinal practitioner for the treatment ofenteric infection. (Arote *et al.,* 2008)

Mussaenda frondosa leaves are used for the treatment of jaundice, asthma, hyperacidity, fever, ulcer, leprosy and used as diuretic (Kumarasamyraja *et al*.,2012)leaves are said to possess anthelmimtic activity (Ashakesari and Bhim charan maiti 2015) hepatoprotective activity against ethanol (sambrekar sudhir *et al.,* 2012) hepatoprotective against isoniazid induced hepatotoxicity (sambrekar sudhir *et al*., 2014), wound healing and antibactrrial activities (patil *et al.,* 2010) antimicrobial activity (jayasinghe *et al*., 2002)

The objectives of the present study are as follows:

1. To investigate the twenty four phytochemicals present in *Hibiscus rosa-sinensis*, *Mussanda frondosa* and *Albizia amara, Derris indica* methanolic leaf extract.
2. To analyse and compare the biochemical parameters carbohydrates, proteins, total phenolics and flavonoids present in *Hibiscus rosa-sinensis*, *Mussanda frondosa* and *Albizia amara , Derris indica* methanolic leaf extract.
3. To investigate the presence of antioxidants present in the extracts by Thin Layer Chromatography.
4. To study the antioxidant activity of *Hibiscus rosa-sinensis*, *Mussanda frondosa* and *Albizia amara, Derisindica* plants.
5. To study and compare the free radical scavenging activities of the methanolic leaf extract of *Hibiscus rosa-sinensis, Mussanda frondosa* and *Albizia amara, Derisindica*.

Review of Literature

REVIEW OF LITERATURE

Hair is an important part of the overall appeal of the human body (Cash, 2001; Messenger, 2000; Stough *et al*., 2005). Hair is one of the external barometers of internal body conditions. It is the protective appendages on the body and considered accessory structure of the integument along with sebaceous glands, sweat glands and nails (Rathi *et al*., 2008). Throughout history and in most of civilizations, scalp hair has been associated with positive signals such as beauty and power. (Rushton *et al*., 2002). The earliest forms of hair cosmetic procedures in ancient Egypt were hair setting by the use of mud and hair coloring with henna. Shampooing is the most common form of hair treatment. The primary function of shampoo is aimed at cleansing of the hair necessitated due to accumulated sebum, dust, scalp debris etc. Various shampoo formulations are associated with hair quality, hair care habit and specific problems such as treatment of oily hairs, dandruff and for androgenic alopecia. Plants have been widely used for hair growth promotion since ancient times as reported in Ayurveda, Chinese and Unani systems of medicine. Indian women use herbals such as shikkakai and reetha that are natural cleansing agents without harmful effects (Patil *et al*., 2015). A survey of global hair care market trends indicates that consumer use of herbal products has significant increased over the past years. There is also a strong market trend towards natural ingredients such as botanical/herbal products.

Ayurveda is the traditional medicinal system of India and believed to have originated over 6000 years ago. It describes the ways to remain healthy as well as methods to treat disease. In Ayurveda, hair is considered as a by product of bone formation. The tissue responsible for building bones is also responsible for the growth of hair. Nowadays, herbal extracts and herbal powders are used in the preparations to enhance beauty and increase the attractiveness of the person. These herbal preparations are used as sunburn, complexion brighter and hair growing. Plant products have been part of phytomedicines and herbo - cosmeceutical products since time immemorial. These can be derived from any part of the plant like bark, leaves, flowers, roots, fruits and seeds (Cragg and David, 2001) i.e., any part of the plant may contain active components. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances. Such Phytochemical screening of various plants is reported by many workers (Majob *et al*., 2003; Parekh and Chanda, 2007; Parekh and Chanda, 2008).

Herbal medicinal system dates back to several billion years to the existence of human civilization and it forms the basis for treatment of human diseases. Indian Ayurvedic medicinal system and traditional Chinese medicinal system are the most ancient yet a living tradition that uses plants as a whole extract which is basis for modern medicine.

1. Medicinal herbs have been used as several forms in various indigenous medicinal system like Siddha, Ayurveda, Unani, Western and Chinese traditional medicine system.
2. The Phytochemical constituents of plants were considered important as it increased the acceptability of traditional medicine.

The substances which act as antioxidants are phenolics, a major secondary metabolite required for the growth and reproduction of plants and are produced as a response for defending injured plants against stress. Likewise, flavonoids are common group of polyphenolic compounds rich in plant leaves, stem, bark. The carbonyl group present in phenols, flavonoids protect from any type of attack.

***Hibiscus rosa-sinenecis* Linn**

Hibiscus rosa-sinensis Linn (Malvaceae) is a glabrous shrub widely cultivated in the tropics as an ornamental plant and has several forms with varying colours of flowers. In medicine, however the red flowered variety is preferred (Adhirajan *et al*., 2003).

Geographical distribution

It is a native of China. It is grown as an ornamental plant in gardens throughout India and often planted as a hedge or fence plant (Sharma *et al*., 2001). About 40 species are found in India is a native of China. It is a common Indian garden perennial shurb (Mudgal VN1974) and often planted as a hedge or fence plant. It is a fancy plant frequently a support or fence plant. (Bhaskar, 2011).

Phytochemistry

Different extract of *Hibiscus rosa-sinensis* plant exposed the occurrence of alkaloids, glycosides, greasy materials, diminishing sugars, resin, sterols and the absence of tannins and Saponins (Jadhav VM, *et al.*,2009). The phytochemical screening demonstrates that the plant is rich in alkaloids, terpenoids, flavonoids, glycosides, fatty materials, saponins, gums and

adhesive.(singh *et al*.,2017). Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more (Edeoga *et al*., 2005). These natural compounds formed the foundations of modern prescription drugs as we know today (Goh *et al*., 1995). Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as an defense system against disease or more accurately, to protect against disease. Phytochemicals are divided into two groups, which are primary and secondary constituents; according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids and phenolic compounds (Krishnaiah *et al*., 2007) and many more such as flavonoids, tannins and so on.

Table: Pharmacological properties of *Hibiscus rosa-sinensis*

Plant parts	Pharmacological Properties	Reference
Dried flowers	Antifertility Activity, AbortifacientActivity, AntiestrogenicActivity, Anti-implantation Activity, AntiovulatoryActivity, Miscellaneous Activity	Batta and Shanthakumari 1970; Singh*et al*., 1982; Kholkuteudupa, 1976; Reddy *et al*.,1997; Prakash *et al*,.1990
Aerial parts, driedleaves, driedplant, flowers	Anticonvulsive activity,Anti-inflammatory Activity,Antipyretic Activity, Antispasmodic Activity,Antifungal Activity,Antiviral Activity, CNS depressant Activity,	Singh *et al*,1978; kasture*et al*., Bhakuni *et al*., 1969; Renu 1983; Van den berghe *et al*.,1978
Flowers, Leaves	Hair growth activity	Adhirajan*et al*.,2003

The leaves and flowers of *Hibiscus rosa-sinensis* promote hair growth and aid in healing of ulcers. It contains taraxeryl acetate, beta-sitosterol, camposterol, stigmasterol, erogosterol, flavonoids, glycosides, lipids, citric and oxalic acids. Leaf extract of Hibiscus rosa-sinensis increases hair length and the anagen/telogen ratio of hair follicles in mice (Adhirajan et al., 2003).

Table: Ethanobotanical profile of *Hibiscus rosa-sinensis*		
Plant Parts	**Ethanobotanical uses**	**References**
Root and flower	Cough and fever	Jadhav*et al* .,2009
leaves	Hair growth ,help in recuperating of ulcers, Skin disease	Ansari 1997; Kurup and joshi 1979; Dwivedi *et al*., 1977 and Singh *et al*., 2017
flower	Blood vessel hypetension, Diabetes ,epilepsy , bronchial catarrh and leprosy	Sethi*et al*., 1986 and Kastur*eet al*., 2000

Albizia amara

The genus *Albizia* is represented by more than 100 species and are mainly confined to tropical and sub- tropical regions of Asia, Africa and Australia. About 16 species of Albizia are indigenous to the Indian subcontinent. *Albizia amara* belongs to the family Leguminaceae, is a valuable medicinal and multipurpose drought tolerant tree commonly found in dry forests of India. *Albizia amara* (Fabaceae) known as "Oil Cake tree" is an endemic plant in dry areas of Tamilnadu, Andhra and Karnataka in India (Rajkumar *et al*., 2012).

Albizia amara grows throughout Southern India and in some parts of Madhya Pradesh (Akilandeswari *et al.,* 2009 and Mar *et al*., 1991). This plant is a small to moderate-sized, much-branched deciduous tree with smooth, dark green, scaly bark. It resembles the acacias but lacks thorns (Prasad and Thippeswamy, 2011). The genus *Albizia* mostly consist of approximately 150 species, most of them are trees and shrubs confined to tropical and subtropical region of Asia, Africa and Australia. Among those about 16 species of *Albizia* are native to Indian subcontinent and have been used as avenue trees and shade trees in tea and coffee plantation.

The dried leaf powder commonly called as Arappu were used to wash hairs, separately and also in combination with shikakai powder as it acts as a natural conditioner, controls hair fall, dandruff and cools the body. The seeds of this plant are used as an astringent, treating piles, diarrhoea, gonorrhea, leprosy, leucoderma, erysipelas and abscesses. The leaves and flowers have been applied to boils, burns, eruptions, swellings and as a remedy for dandruff. The fruit

pods are emetic and are used in treatment of cough and malaria. The tree yield gum which cures ulcer. The bark is used to treat jaundice and mouth inflammation.

MEDICINAL USES

The plant extracts of *A. amara* are used extensively in traditional medicine (Reddy *et al*., 1967). Herbal drugs have been in use by different civilizations in different parts of the world for centuries to fight a large number of diseases. Many of these are in common use even today (Rajkumar and Sinha 2010).

Table: Ethanobotanical profile of *Albizia amara*

Plant Parts	Ethanobotanical uses	References
Whole plant	Used in diarrhea, gonorrhea, skin diseases, poisonous bites and leprosy. Treat piles	Indravathi *et al* .,2014 Rajkumar and Sinha 2010
Seed	Used in Piles, diarrhea ,gonorrhoea	Mar *et al* .,1991
Flowers	Used in Cough ,ulcers, dandruff ,malaria	Mar *et al* .,1991
Bark	Used in Ulcers and molluscidal	Kashyapa and ramesh 1992 Ayoub and yankov 1986
Roots	The roots are used for the various treatments like angina pectoris, ischemic heart disease,anticancer, decreases cholesterol**,** anti bacterial, Asthma, bronchial disease, dyspnea	Sarada, 1974, Tripathi, 1984; Pal, 2010, Zafar; Sualeh, 2010; Mahmood, 2006; Lokhande, *et al* 2009; Sharma, 2010
leaves and flowers	The leaves and flowers have been applied to boils, eruptions, and swellings, also regarded as an emetic and as a remedy for coughs, ulcer, dandruff and malaria	Woongchon *et al*.,1991

PHYTOCHEMICALS:

Investigation on phytochemical analysis of different extracts viz., hydro methanolic, petroleumether, toluene, chloroform, methanol and ethanol extracts of *Albizia amara* was done by Praveen *et al*., 2001. Phytochemical estimation of ethanolic extract of albizia amara revealed presence of alkaloids, glycoside, essetional oil, saponins, triterponoids, flavonoids, aminoacids and absence of steroids, carbohydrates (Khan *et al*., 2010). Analysis of methanolic extract recorded the presence of saponins, tannins, alkaloids, cardiac glycosides, carbohydtrate, flavonoids, terpnoids, quninones, glycosides (Praveen *et al*.,2011)

Derris indica (Pongamia pinnata)

It is widely distributed throughout tropical Asia and the Seychelles Islands, South Eastern Asia, Australia, India and locally distributed throughout the State of Maharashtra (India) along the banks of rivers; very common near the sea-coast in tidal and beach-forests in Konkan; along Deccan rivers (Prajapati, Purohit *et al* 2003).

Pongamia pinnata (L.), locally known as Karanja,is also called as *Derris indica* is a mangrove plant belonging to the family, Fabaceae. *Pongamia pinnata* (L.) Pierre [Synonyms: Derris indica (Lam.) Bennett, Derris pinnata Lour, Millettia novo-guineensis Kane and Hat, Pongamia glabra Vent, Cytisuspinnatus L, *Pongamia pinnat*a Merr.](zahidlqbalsajid *et al*., 2012,Parmar *et al*.,1976). Habitat of *P. pinnata* is in the littoral regions of South East Asia, Australia and Fiji (Chopra *et al*., 1986; Simin *et al*., 2002). Traditionally, its bark is used in pile; leaves are effective as medicated bath and rheumatic pains; and the seeds are used in hypertension, bronchitis, whooping cough, skin diseases and rheumatic arthritis (Ballal, 2005; Tanaka *et al*., 1992; Carcache *et al*., 2003).

Table: Pharmacological properties of *Albizia amara*

Plant parts	Pharmacological Properties	Reference
Seed, Leaf	Anticancer activity, antimicrobial activity	Mar *et al* .,1991;gopinath *et al*., 2013;Praveen et al .,2011

Leaf, bark	Antioxidant activity	Suresh kumar*et al.*, 2008; Rajkumar *et al*., 2012; Mulapalli et al.,2012
Leaf	Larvicidalactivity, Anti inflammatory	Murgan*et al*., 2006; Khan *et al.*, 2010
Bark	Antihyperlipedemic activity, Molluscidal activity	Mulapalli*et al*.,2012

In India, seeds were used for skin ailments. Today the oil is used as a liniment for rheumatism; their juice is used for colds, coughs, diarrhea, dyspepsia, flatulence, gonorrhea, and leprosy. Roots are used for cleaning gums, teeth, and ulcers also effective in fistulous sores and gonorrhea (Rastogi and Malhotra, 2001; Chauhan *et al*., 2002). Ayurvedic medicine described the root and bark as alexipharmic, anthelmintic, and useful in abdominal enlargement, diseases of the eye, skin, and vagina,itch, piles, splenomegaly, tumors, ulcers, and wound

GEOGRAPHICAL DISTRIBUTION

Derris indica is a medicinal plannative to Western Ghats and chiefly found in tidal forests of India (Krishnamurthi, 1969). *Derris indica* grows abundantly along the coasts and riverbanks in Myanmar. The tree is known for its multipurpose benefits and as a potential source of biodiesel (Naik *et al*.,2008).

Table: Phytochemical constituents of *Derris indica*

Plant Name	Chemical Constituents	Reference
plant	flavonoids such as furanoflavones, furanoflavonols, chromenoflavones, furanochalcones and pyranochalcones	Tanaka*et al* ., 1992; Kang*et al* ., 2003; Yadav et al .,2004
Stem bark	pongaflavanol and tunicatachalcone	Yin H.,*et al* .,2006
fruits	furanoflavonoids, pongapinnol A–D, and pongacoumestan	Yadav P.P.,*et al* .,2004

MEDICINAL USES

Pongamia pinnata is a medicinal plannative to Western Ghats and chiefly found in tidal forests of India (Krishnamurthi, 1969). Pongamia pinnata grows abundantly along the coasts and riverbanks in Myanmar. The tree is known for its multipurpose benefits and as a potential source of biodiesel (Naik *et al*., 2008).

Table: Ethanobotanical profile of *Derris indica*		
Plant Parts	**Ethanobotanical uses**	**References**
Whole plant	Used as treating Wounds, inflammations, piles, ulcers and skininfection, contains properties anti-inflammatory and ulcerogenic effect anti-inflammatory, anti-plasmodial, anti-nonciceptive, anti-hyperglycamic, anti-lipidperoxidative, anti-diarrhoeal, anti-ulcer, anti-hyperammonic and antioxidant activity.	Chopad e*et al*., 2009; Srinivasan *et al*,.2001
All parts	used asa crude drug for the treatment of tumours, piles, skin diseases, itches, abscess, painful,rheumatic joints wounds, ulcers, diarrhea.	Meera *et al* .,2003 and Shoba and Thomas 2001
Seed	Used as skin ailments hypertension,bronchitis, whooping cough, skin diseases and rheumaticarthritis.	Ballal, 2005; Tanaka *et al*., 1992 and Carcache *et al*., 2003
Roots	used for cleaninggums, teeth, and ulcers also effective in fistulous sores and gonorrhea .	Rastogi and Malhotra, 2001; Chauhan *et al*., 2002
Leaves	The leaves are anthelmintic, digestive, and laxative, forinflammations, piles and wounds	Brown,1995
fruit and seed	while the fruit and seedused for keratitis, piles, urinary discharges, and diseases of the brain, eye, head, and skin	Brown,1995

Unani uses the ash to strengthen the teeth, the seed, carminative and depurative, for chest complaints, chronic fevers, earache, hydrocele, and lumbago (Ahmad *et al*., 2004; Yadav *et al*., 2004).

***Mussaenda frondosa* Linn**

The rubiaceae family is a rich source of medicinal plants with 500 genus and 5300 species in it. Majority of the family members are trees or shrubs and the plant shows great variations in its habitat. *Mussaenda* is an active member of the family Rubiaceae. This genus includes numerous ornamental plants with 194 species.(Gunasekaran *et al*., 2015). *Mussaenda frondosa* Linn commonly called as Nagavalli reported to possess number of medicinal properties.

Table: Chemical constituents of *Mussaenda frondosa*

Plant Name	Chemical Constituents	Reference
Flower	Quercetin,hypenin,ferulicacid,sinapic acid, βsitosterol,βsitosterol glycoside	Lakshmi *et al* .,1985
Leaves	β- sitosterol, lupeol, mussendoside, mussendoside-M 3-palmitoyl lupeol, sanshide methyl ester	Mahesh and Niharika, 2017

Medicinal uses

Mussaenda roxburghii Hook. f. (family- Rubiaceae) is a perennial shrub grows in the foothills and moist areas of valley. Roots are used in treatment of jaundice (Saha *et al.*, 2011), skin diseases, cuts, wounds and boils etc (Patil and Joshi, 2011). Leaves are used in the ailments of bone fracture (Das *et al.*,2009). The paste obtained from leaf of this plant is applied to treat boils (Rahman, 2010). The genus Mussaenda is a wealthy source of medicinally important phytoconstituents such as terpenes, flavanoids and iridoids. The numerous species of this genus is easy growing and said to possess numerous medicinal properties such asantibacterial, antiinflammatory, antioxidant, antipyretic, antiviral, cytotoxicity, diuretic, etc., (Gunasekaran *et al*., 2015)

Table: Ethanobotanical profile of *Mussaenda frondosa*

Plant Parts	Ethanobotanical uses	References
Whole plants	Depurant, febrifiuge, purgative, asthma, albuminuria, gastroenteritis conjunctivitis and	Poullain *et al* ., 2004

	dermatosis	
Leaves	Fever and inflammation, treatment of jaundice, asthma,hyperacidity, fever, ulcers, leprosy, diuretic, wound, astringent, expectorant, antiinflammatory,cardiotonic, cough bronchitis, swells , antimicrobial	Fortina *et al* ., 2002; Jain, 1991
Stem	Antioxidant and free radical scavenging activity	Poullain *et al* ., 2004
Roots	Hepatic jaundice, skin rashrs in babies	Segawan and Kasenene, 2007

Materials and Methods

MATERIALS AND METHODS

3.1 Collection of plant material:

Plant materials were collected at respective places as given in the table.1. collected plants were authenticated by Botanical survey of India, Coimbatore, Tamil Nadu, India.

Table 1. List of plant materials taken for this study

S.No	Plant Name	Family	Place
1	*Hibiscus rosa-sinencis L.*	Malvaceae	Coimbatore
2	*Mussaenda frondosa L.*	Rubiaceae	Kozhikode
3	*Albizia amara Roxb.*	Fabaceae	Coimbatore
5	*Derris indica L.*	Fabaceae	Coimbatore

Leaves were rinsed with distilled water. Afterwards, the samples were dried under shade,ground and sieved for extraction process.

3.2 Solvent extraction [Farombi *et.al,* 2003]

Powdered samples (10g each) was weighed and soaked separately in 50 ml methanol in a conical flask stoppered with rubber cork and kept in orbital shaker for 24 hours. It was then filtered off using sterile filter paper (What Man No: 1) into a sterile conical flask

3.3 Physiochemical parameters

3.3.1 Determination of moisture content (AOAC, 1990)

A quantity of 5 g of sample was dried in the oven at 105˚C and moisture content was calculated as percentage.

$$\text{Moisture Content} = \frac{W - D}{W} \times 100$$

Where,

W = Wet weight

D = Dry weight

Plate – 1

Classification	
Kingdom	Plantae
Division	magnoliophyta
Class	magnoliopsida
Order	Malvales
Family	Malvaceae
Genus	***Hibiscus***
Species	***rosa-sinensis L.***

Plate -2

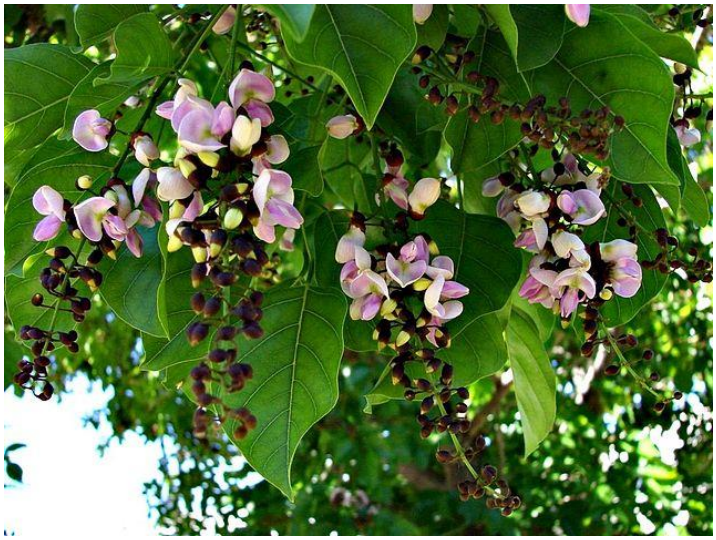
Classification	
Kingdom	Plantae
Division	Magnoliaphyta
Class	magnoliopsida
Order	Gentianales
Family	Rubiaceae
Genus	***Mussaenda***
Species	***Frondosa L.***

Plate 3

Classification	
Kingdom	Plantae
Division	Tracheophyta
Class	Equisetopsida
Order	Fabales
Family	Fabaceae
Genus	***Albizia***
Species	***Amara Rxob.***

Plate 4

Classification	
Kingdom	Plantae
Division	tracheophyta
Class	magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	***Derris***
Species	***Indica L.***



3.3.2 Determination of solubility percentage (Kokate, 1994)

Ethanol

- ✓ About 5 g of powdered material was weighed and macerated with 100 ml of 90% ethanol in a closed flask for 24 hours shaking frequently during the first 6 hours and kept undisturbed for 18 hours.
- ✓ Thereafter, it was filtered rapidly taking precautions against loss of the solvent.
- ✓ About 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed, swallowed dish dried at 105˚ C for 6 hrs and cooled in a desiccator and weighed.
- ✓ The content of extractable matter (% w/w) air-dried material was calculated as follows.

$$\text{soluble extractives (\% w/w)} = \frac{\text{(Weight of residue) x 5 x 100}}{\text{Weight of the sample}}$$

3.4 Phytochemical Screenings

The extracts obtained in the successive extraction process of the four leaves extract were subjected to various preliminary phytochemical screening for the identification of phytoconstituents present.

3.4.1 Test for Carbohydrates

Molisch's test:

To a small amount of the extract few drops of Molisch's reagent was added followed by the addition of conc. H_2SO_4 along the sides of the test tube. The mixture was then allowed to stand for 2 min and then diluted with 5 ml of distilled water. Formation of red or dull violet colour at the inter phase of two layers indicates the presence of carbohydrates.

3.4.2 Test for Reducing Sugar

Benedict's Test

Extract (2 ml) were treated with 2 ml of Benedict's reagent and heated in a water bath for 3 minutes. Presence of green, red or yellow ppt indicates the presence of reducing sugar.

3.4.3 Test for Proteins

Millon's test:

Extract (3 ml) was mixed with 5 ml of millon's reagent. White precipitate is formed. On warming precipitate turn's brick red or the precipitate dissolves giving red colored solution.

Biuret test:

To extract (3 ml) few drops of 10% sodium chloride and 1% copper sulphate was added for the formation of violet or purple colour. On addition of alkali, it becomes dark violet.

3.4.4 Vitamin C

DNPH test:

Sample was treated with Dinitrophenyl hydrazine and sulphuric acid. Formation of yellow precipitate indicates the presence of vitamin C**.**

3.4.5 Test for Alkaloids

Mayer's test:

Sample (2 ml) was treated with few drops of Mayer's reagent. Appearance of white precipitate indicated the presence of alkaloids.

Wagner's test:

Sample (2 ml) was mixed with few drops of Wagner's reagent. Appearance of reddish brown precipitate indicated the presence of alkaloids.

Hager's test:

Sample solution and few drops of Hager's reagent were added. Appearance of yellow precipitate indicated the presence of alkaloids.

3.4.6 Tests for Flavonoids

Shinoda test:

Sample extract was treated with 5 ml of 95% ethanol, few drops of concentrated Hydrochloric acid and 0.5 g of magnesium turnings were also added. Pink colour was observed. Addition of increasing amount of sodium hydroxide to the residue shown yellow coloration, this decolorized after addition of acid indicates the presence of flavones.

Flavanones:

Sample extract (1 ml) was taken and 10 % of sodium hydroxide was added. Yellow to orange colour formation indicates the presence of flavanones.

Alkaline test:

Sample extract (1 ml) was treated with few drops of sodium hydroxide. Yellow colour is formed which turns to be colourless after adding a few drops of diluted acid.

3.4.7 Test for Glycosides:

Legal's test:

Sample extract was mixed with few drops of pyridine and 2 drops of 2 % sodium nitroprusside was added. To the reaction mixture 0.5 ml of 20 % sodium hydroxide was added. Appearance of pink to red color indicated the presence of glycosides.

Bromine water test

Sample (1 ml) was treated with 3 drops of bromine water and the formation of yellow precipitate indicates the presence of glycosides

3.4.8 Test for cardiac Glycosides

Keller-killani test:

Five ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of conc. sulphuric acid, a brown ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

3.4.9 Test for anthroquinone

Borntragor's test

Extract (0.5 ml) was added with 5-10 ml of dilute hydrochloric acid and boiled on water bath for 10 minutes. Solution was filtered and filtrate was extracted with benzene and mixed with ammonia solution. Red color was obtained in ammonia layer that indicated the presence of anthraquinone glycosides.

3.4.10 Test for Terpenoids

Extracts were treated with $CHCl_3$ (0.5 ml) and 1ml of Conc. H_2SO_4. Formation of reddish brown precipitate shows the presence of terpenoids.

3.4.11 Test for Diterpenes:

Extracts (3 ml) are treated with a few drops of copper acetate solution. Formation of emerald green colour indicates presence of diterpenes.

3.4.12 Test for Triterpenoids

Extract (5 ml) was dissolved in chloroform (2 ml) and then acetic anhydride (1 ml) was added to it. Concentrated sulphuric acid (1 ml) was added to the solution. Formation of reddish violet colour shows the presence of triterpenoids.

3.4.13 Test for Saponin

Foam test:

To 1 ml of the extract 5 ml distilled water was added and shaken vigorously. Formation of foam indicated presence of saponins.

3.4.14 Test for phenols

Extract (2 ml) was treated with 5% ferric chloride solution and observed for the formation of deep blue or black colour. To 1 ml of the extract, 2 ml of distilled water, 3 drops of 10% aqueous ferric chloride ($FeCl_3$) and 3 drops of potassium Ferro cyanide were added. Formation of blue or green color showed the presence of polyphenols.

Extract (2 ml) was treated with 3ml of 10 % lead acetate. Formation of precipitate indicates the presence of phenols.

3.4.15 Test for Tannins(Trease and Evans, 1989)

To 1ml of extract solution, 4 ml of water and 1-2 drops of 10 % ferric chloride solution was added. Blue colour indicates gallic tannins and green black catecholic tannins

3.4.16 Test for Phlobatanins:

0.5 g extract was dissolved in distilled water and filtered. The filtrate was boiled with 2M HCl solution. Formation of red precipitate showed the presence of phlobatanins.

3.4.17 Test for Quinones

To 1ml of test solution Alcoholic KOH solution was added separately. Quinones were indicated by colour ranging from red to blue.

3.4.18 Test for Sterols

Extract (2 ml) was treated with 2 ml of trichloroacetic acid. On heating the colour changes from red to violet. This indicates the presence of sterols.

3.4.19 Test for Phytosterols

Salkowski's test:

0.2g of the extract was mixed with 2 ml of chloroform ($CHCl_3$) and concentrated 6M H_2SO_4 (3ml) was carefully added forming a layer. A reddish brown coloration of the interface indicated the presence of phytosterols.

3.4.20 Test for Oxalate

1 ml of extract was mixed with 1 ml of dil. Sulphuric acid and dil. acetic acid and boiled for 3 minutes. 1ml ferrous sulphate was added. Yellow precipitate indicated the presence of oxalate

3.4.21 Test for Anthocyanin

NaOH test:

1 ml of extract was treated with 2 ml NaOH. Blue green colour formation indicates the presence of anthocyanin.

3.4.22 Test for Resin

5 ml of distilled water was added to the 3 ml of the methanol extract for turbidity, which indicates the presence of resins in the plant sample.

3.4.23 Test for Coumarins

The aqueous leaf extract (5 ml) was evaporated to dryness in a water bath. Distilled water (3 ml) was added and the mixture heated on a water bath to boil and the mixture cooled under running water. The solution (0.5 ml, 10%) was added. Both test tubes were observed under Ultra Violet light and presence of coumarins was indicated by (blue or green) fluorescence in test tube containing ammonia solution.

3.4.25 Test for Catechin

Match stick was dipped in plant extract, dried and then moistened with concentrated HCl. Warm near flame, a red or pink wood is produced which shows the presence of catechin

Data collected

The change of colour was observed when the test reagent was added to the prepared sample for the phytochemical test. The result was recorded as present (+) or absent (-) depending on the outcome of the test.

3.4.2 Quantitative test

The biochemical parameters analyzed were

1. Carbohydrates
2. Proteins

3.4.2.1 Biochemicals parameters

3.4.2.1.1 Total Carbohydrates: (Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxyl methyl furfural. This compound forms with anthrone, a green coloured product with an absorption maximum at 630 nm.

Materials

- 2.5N HCl
- Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice cold 95 % H_2SO_4 prepared fresh before use.

Standard glucose (Stock): Dissolved 100 mg in 100 ml of water.

Working standard – 10 ml of a stock solution was diluted to 100 ml distilled water.

Procedure

- About 100 mg of the sample was taken in a boiling tube and it was hydrolysed by keeping it in a boiling water bath for three hours with 5ml of 2.5N HCl and cooled to room temperature.
- Then it was neutralized with solid sodium carbonate until the effervescence created.
- The volume was made up to 100 ml and centrifuged.
- The supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis.
- The standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and '0' served as blank.
- The volume was made up to 1 ml in all the tubes including the sample test tubes by adding distilled water.
- Then, 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath.

- Then it was cooled rapidly and the green colour developed was read at 630 nm.
- A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
- From the graph, the amount of carbohydrates present in the sample tube was calculated.

Calculation

Amount of carbohydrates present in 100mg of the sample is calculated by

$$\frac{\text{mg of glucose}}{\text{volume of test sample}} \times 100$$

3.4.2.1.2 Estimation of protein (Lowry *et al*., 1951)

Principle

The blue colour developed by phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method.

Materials:

- Reagent A - 2 % sodium carbonate in 0.1 sodium hydroxide
- Reagent B - 0.5 % copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate.
- Reagent C - Alkaline copper solution : Mixed 50 ml of A and 1 ml of B prior to use.
- Reagent D - Folin-Ciocalteau Reagent.

Stock standard : 50 mg of bovine serum albumin (Fraction V) was weighed and dissolved in distilled water and the volume was made up to 50 ml in a standard flask.

Working standard: About 10ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contain 200 µg proteins.

Procedure

Extraction of protein from sample

Extraction is usually carried out with buffers used for the enzyme assay. About 50mg of the weighed sample was ground well with pestle and mortar in 5- 10 ml of the buffered centrifuged. The sample was used for protein estimation.

Estimation of protein

- About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard were pipette into a series of test tubes and 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
- The volume was made up to 1 ml in all test tubes. A tube with 1 ml of water severed as the blank.
- About 5 ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 minutes.
- Then 0.5 ml of reagent D was added. Mixed well and incubated at room temperature in the dark for 30 min. Blue colour developed was read at 660 nm.
- A standard graph was drawn and the amount of protein present in the sample was calculated.

Calculation

The amount of protein present in the sample was expressed in

$$\text{mg /g or100g} = \frac{\text{mg of protein}}{\text{volume of the test standard}} \times \text{concentrationof the standard}$$

3.4.2.2 Phytochemical Parameters

- Phenol
- Polyphenol
- Flavonoid
- Tannin

3.4.2.2.1 Estimation of Total Phenol content (Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which can be estimated spectrophotometrically at 650 nm.

Materials

- Ethanol (80 %)
- Folin-ciocalteau reagent

- Na_2CO_3 (20 %)

Stock standard: Gallic acid (100µg/ml in water)

Working standard: Dilute 10 times of stock

Procedure

- Grind 0.5 g of the sample with a pestle and mortar in 10 times volume of 80 % ethanol.
- Centrifuge the homogenate at 10,000 rpm for 20 minutes. Re-extraction is done and the supernatants were pooled and evaporated to dryness.
- Dissolve the residue in a known volume of distilled water (5 ml).
- Pipette out different aliquots (0.2 to 2 ml) into test tubes.
- Make up the volume in each tube to 3 ml with distilled water.
- Add 0.5ml of Folin-Ciocalteau reagent.
- After 3 minutes, add 2 ml of 20 percent Na_2CO_3 solution to each tube.
- Mix thoroughly, place the tube in boiling water for exactly 1 minutes, cool and measure the absorbance at 650nm against a reagent blank.
- Prepare a standard curve using different concentrations of gallic acid.

Calculation

From the standard curve the concentration of phenols in the sample were observed and express as GAE mg of phenols/g of material.

3.4.2.2.2 Determination of total polyphenol (Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrophotometer at 650nm.

Reagents

- Diluted Folin - Ciocalteau reagent (1:10 dilution)
- 20% Sodium carbonate
- Ethanol

Stock solution: 100 mg of catechol was made up with 100 ml distilled water

Working standard: 10 ml of stock standard was diluted to 100 ml. 1.0 ml of this contains 100 µg of catechol.

Procedure

- ✓ A working standard of 0.5 – 2.5 ml catechol solution corresponding to 50 – 250 µg of catechol were pipetted out into a series of test tubes including samples and standard.
- ✓ The volume was made upto 2.5 ml with water. To all the tubes added 0.5ml of diluted Folin – Ciocalteau reagent.
- ✓ After 3 minutes, 2 ml of 20% Na_2CO_3 solution was added to each tube and mixed thoroughly.
- ✓ The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank.

Calculation

The results were expressed as mg / g

3.4.2.2.3 Determination of total flavonoid content (Grubesic *et al*., 2005)

Principle

The content of flavonols was determined by using rutin as a reference compound. This method was based on the formation of complex with maximum absorption at 440 nm.

Reagents:

1. Aluminium chloride solution (20 mg/ml)
2. Sodium acetate (30 mg/ml)

Procedure

- ➢ About 1 ml of each extract was mixed with 1 ml aluminium chloride and 3 ml sodium acetate.
- ➢ After 2.5 hrs the samples were read at 440 nm absorbance.
- ➢ The absorption of standard rutin solution in methanol was measured under the same conditions.
- ➢ A duplicate was carried out for all the determinations.

Calculation:

The amount of flavonoids in plant extracts in rutin equivalents was calculated by the following formula.

$$X = (A - m_0) / (A_0 - m)$$

Where,

X = flavonoid content (μg/ml) in rutin equivalents

A = absorption of plant extraction solution,

A_0 = absorption of standard rutin solution,

m = weight of plant extract (μg)

m_0 = weight of rutin in the solution (μg)

3.4.2.2.4 Estimation of Tannin (Folin and Ciocalteu Method, 1927)

Principle

The principle behind this method is reduction of Phosphotungstomolybdic acid in alkaline solution to produce coloured complex.

Materials

1. Folin- Ciocalteu reagent
2. Sodium carbonate
3. Tannic acid

Procedure

- To 0.1 ml of the sample extract 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent, 1 ml of 35% sodium carbonate solution were added and diluted to 10 ml with distilled water.
- The mixture was shaken well, kept at room temperature for 30 min and was measured at 725 nm.
- Blank was prepared with water instead of the sample.
- A set of standard solutions of Tannic acid is treated in the same manner as described earlier and read against a blank.

Calculation

The results of Tannin are expressed in terms of Tannic acid in mg/g tissue TAE of extract.

3.4.3. Antioxidants assays

3.4.3.1 TLC screening for phytochemical analysis and antioxidant activity

Qualitative screening of the constituents in each of the crude extracts of the selected four species for antioxidant activity was screened by thin layer chromatography (TLC) analysis. For about 10µL of each sample was loaded on the TLC plates. The TLC chromatograms were developed in the following solvent systems.

1. Ethyl acetate/ methanol/water (EMW) 10:1.35:1
2. Chloroform/ethyl acetate/formic acid (CEF) 10:8:2
3. Benzene/ ethanol/ ammonium (BEA) 18:2:0.2

For detection of chemical compounds and antioxidants in the extracts respectively, two spray reagents were separately used.

1. Vanillin in sulphuric acid
2. DPPH 0.2% in NAOH

3.4.3.2 Enzymatic assays

The enzymatic antioxidant analysed in this present study were Polyphenol oxidase and Peroxidase.

3.4.3.2.1. Polyphenol Oxidase (PPO) (Esterbauer *et al*., 1977)

Principle

Polyphenol oxidase was copper protein of wide occurrence in nature,which catalase the aerobic oxidation of certain phenolic substrate to quinines, which are auto oxidized to dark brown pigments generally known as melanins. The polyphenol oxidase (PPO) comprises catechol oxidase and laccase.

Reagents

- Tris-HCl (50 mM, pH 7.2).
- Sorbitol (0.4 M).
- Sodium chloride (10 mM).
- Phosphate buffer (0.1 M, pH 6.5).

- Catechol (0.01 M).

Procedure

- ✓ Into a cuvette, 0.2 ml of the sample extract was added to the reaction mixture containing, 2.5 ml of phosphate buffer and 0.3 ml of catechol solution.
- ✓ The change in the absorbance was recorded every 30 sec up to 5 minute.

Calculation

One unit is equal to the changes in absorbance at 495nm/minute

The activity of PPO can be calculated using the formula

Enzyme units in the sample = $K \times (\Delta A/minute)$

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

3.4.3.2.2. Peroxidase (Reddy *et al*., 1995)

Principle

In the presence of the hydrogen donor pyrogallol or dianisidine, peroxidase converts $H_2O_2$to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a coloured product called purpurogalli can be followed spectrophotometrically at 430nm.

Reagents

- Pyrogallol : 0.05 M in 0.1M phosphate buffer (pH 6.5)
- H_2O_2: 1% in 0.1M phosphate buffer, pH 6.5

Procedure

- ✓ About 3 ml of pyrogallol solution, 0.1ml of the extract was added and the spectrophotometer was adjusted to read zero at 430 nm.
- ✓ To the test cuvette, 0.5ml of H_2O_2 was added and mixed.
- ✓ The change in absorbance was recorded every 30 secondsup to 3 minutes in a spectrophotometer.

Calculation

One unit of peroxidase is defined as the change in absorbance at 430 nm/minute.

3.4.3.3. Non-enzymatic assays

3.4.3.3.1. Ascorbic acid (Roe and Keuther, 1943)

Principle

Ascorbate is converted into dehydroascorbate on treatment with activated charcoal, which reacts with 2, 4-dinitrophenyl hydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in sulphuric acid, whose absorbance can be measured spectrophotometrically at 540nm.

Reagents

- TCA (4%)
- 2,4-dinitrophenyl hydrazine reagent (2%) in 9N H_2SO_4
- Thiourea (10%)
- Sulphuric acid (85%)
- Ascorbic acid (100mg of Ascorbic acid in 100ml of 4% TCA)

Procedure

- ✓ Ascorbic acid solution of 1 mlwere taken and added with 0.1 ml of plant extract.
- ✓ The reaction mixture was made up to 2.0 ml with 4% TCA.
- ✓ To this, 0.5 ml of DNPH reagent was added followed by 2 drops of 10% thiourea solution.
- ✓ The tubes were incubated at 37˚C for 3 h.
- ✓ The osazone formed was dissolved by the addition of 2.5 ml of 85% sulphuric acid.
- ✓ DNPH reagent and thiourea were added to the blank after the addition of sulphuric acid.
- ✓ After cooling the tubes, the absorbance was read spectrophotometrically at 540 nm.

Calculation

The concentration of ascorbate in the sample was calculated and expressed in terms of mg/g .

3.4.3.3.2 Total polyphenol (Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrophotometer at 650nm.

Reagents

- Diluted Folin - Ciocalteau reagent (1:10 dilution)
- 20% Sodium carbonate
- Ethanol

Stock solution : 100 mg of catechol was made up with 100ml distilled water

Working standard: 10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

Procedure

- ✓ A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 – 250µg of catechol were pipetted out into a series of test tubes including samples and standard.
- ✓ The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – Ciocalteau reagent.
- ✓ After 3 minutes, 2 ml of 20% Na_2CO_3 solution was added to each tube and mixed thoroughly.
- ✓ The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank.

Calculation

The results were expressed as mg / g.

3.4.4 Free radical scavenging activity

3.4.4.1.DPPH radical scavenging activity (Mensor *et al.*,2001)

Principle

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

Reagents

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)(0.1mM=39.4 mg in 1000 ml)

2. Methanol

Procedure

The extracts (20µl) were added to 0.5 ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer.

Calculation

The radical scavenging activity was calculated as follows

$$\text{Scavenging activity \%} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

3.4.3.2 Ferric Reducing Antioxidant Power (FRAP) Assay (Pulido et al., 2000)

Principle

Ferric reducing ability of plasma (FRAP) assay is based on the principle of reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by the antioxidants of a sample at low pH. The end product (Fe^{2+}-TPTZ) has blue color with absorption maximum at 593 nm

Procedure

900µL of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed with 90 µL of distilled water and 30 µL of test sample (Benzie and Strain, 1996). Readings were taken at 593 nm, for every 15s, the reaction monitored for up to 30 min.

Calculation

scavenging activity was calculated as follows

$$\text{Scavenging activity \%} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

3.4.3.3 Reducing power assay (Oyaizu, 1986)

Principle

Substances, which have reduction potential, react with potassium ferricyanide (Fe_3+) to form potassium ferrocyanide (Fe_2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

$$\text{Potassium ferricyanide + Ferric chloride} \xrightarrow{\text{Antioxidant}} \text{Potassium ferrocyanide + Ferrous chloride}$$

Reagents

1. Potassium ferricyanide (1%)
2. Phosphate buffer (0.2 M, pH 6.6)
3. Trichloro acetic acid (10%)
4. Ferric chloride (0.1%)
5. Ascorbic acid(1%)

Procedure

0.5 ml of the plant extracts were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). This mixture was kept at 50ºC in water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 minutes whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm.

Calculation

Reducing power was measured by different extract .

scavenging activity was calculated as follows

$$\text{Scavenging activity \%} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

3.4.3.4 Metal chelating activity (Dinis *et al.*, 1994)

Chemicals used

1. 2 mM of $FeCl_2.4H_2O$
2. 5 mM of ferrozine
3. Gallic acid

Procedure

Aliquots (1 ml) of the plant extracts dissolved in the same solvents at concentrations of 1, 2.5 and 5 mg/ml were separately added to 2.8 ml of distilled water, followed by mixing with 50 µl of 2 mM $FeCl_2.4H_2O$ and 150 µl of 5 mM ferrozine. All the above without plant extract served as control. The mixtures were then shaken vigorously and left standing at room temperature for 10 min. Absorbance levels of the solutions were measured using a spectrophotometer at 562 nm. All tests and analyses were run in triplicate and averaged.

Calculation

$$\%\text{Inhibition} = [1 - (A_{sample}/A_{Control})] \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of the plant extracts or standards.

Results and Discussion

4. Result and Discussion

4.1 Physiochemical Parameters

4.1.1 Moisture content

Moisture content differs from climate to climate in same plant. Maximum and minimum moisture content was observed in *Derris indica* and *Mussaenda frondosa* respectively. Moisture content of *Pongamia pinnata* leaf was recorded as 9.16 % w/w by Menpara and Chanda, (2014). According to Udo *et al*., (2016) leaf of *H. rosa-sinensis* had moisture content of about 2.63 %. These findings agree with the report of (Okunade, 2002; Krishnaiah *et al*., 2009 and Anyasor *et al*., 2010)

4.1.2 Solubility test

Methanolic solubility was found to be similar in all the leaf samples which ranged from 4.01 – 4.61 in *H. rosasinensis* and *M. frondosa* respectively. *Pongamia pinnata* showed 7.20 % solubility in the work done by Menpara and Chanda, (2014) in methanol. Alcohol solubility was found to be 9.6 % (w/w) by Rao *et al*., (2014) in *H. rosa-sinensis*.

Table: 4 Physiochemical parameters of four plants

Physiochemical parameters	Moisture content (%)	Methanol Solubility (%)
Hibiscus rosa-sinensi	3.18	4.01
Mussaenda frondosa	2.76	4.61
Albizia amara	3.07	4.05
Derris indica	3.38	4.09

4.2 Preliminary Qualitative tests

4.2.1 Phytochemical Screening

Chemically constituents may be therapeutically active or inactive. Several phytochemical surveys have been carried on for detecting diverse groups of naturally

Table: 5Phytochemical investigation of methanolic extract of four plants

S.No	Name of the Phytochemicals		Name of the test	Methanol Extract			
				Hibiscus rosa-sinensis	*Mussaenda frondosa*	*Albizia amara*	*Derris indica*
1	Carbohydrates		Molisch's	+	+	+	+
2	Proteins		Millon's test	+	-	+	-
			Xanthoprotic test	+	+	+	+
			Ninhydrin test	-	-	-	-
3	Vitamin C		DNPH test	+	+	+	+
4	Alkaloids		Mayer's	+	+	+	+
			Wagner's	-	+	-	+
			Hager's test	+	+	+	+
5	Flavonoids	Flavones	Shinodia test	+	-	-	-
		Flavanones	NaOH test	+	-	-	-
		Alkaline test		-	+	-	+
6	Glycosides		Bromine water test	-	-	-	+
			Legal's test	-	+	-	+
7	Cardiac glycosides		Keller-killani test	+	-	+	+
8	Anthraquinone		Borntragors test	-	-	-	-
9	Terpenoids		Salkowki's test	+	-	+	+
10	Diterpenes		Copper acetate test	-	+	+	+
11	Triterpenoids			+	+	+	+
12	Saponins			+	+	+	+
13	Phenols		Ferric chloride test	+	+	+	+
14	Tannin	catecholic tannin		+	+	+	+
15	Phlobatanins			-	-	-	-
16	Quinones			-	+	-	+
17	Sterols			-	-	-	-
18	Phytosterols		LibermanBurchard	+	+	+	+
19	Oxalate			+	-	+	+
20	Anthocyanin			-	-	-	-
21	Resins			+	+	+	+
22	coumarins			+	-	+	+
-23	Catechnin			-	-	-	-
24	Reducing sugar			+	+	+	+

Key: + = Presence - = Absence

occurring phytochemicals. The phytochemical research approach is considered effective in discovering bioactive profile of plants of therapeutic importance. During the present study four medicinal plants i. e. *Hibiscus rosa-sinensi, Mussaenda frondosa, Albizia amara and Derris indica* which were commonly used in hair washing purposes are taken. The extracts were subjected to phytochemical screening for identification of presences or absence of phytoconstituents. Factors such as species and subspecies, geographical location, harvest time, plant part used and method of isolation all affect chemical composition of the crude material separated from the plant.

Qualitative analysis of phytochemical constituents of methanol extract *H.rosa-sinensis* revealed presence of protein, vitamin C, alkaloids, flavonoids, cardiac glycosides, terpenoids, tritrepenoids, saponins, phenols, tannin, phytosterols, tannin, oxalate, resins, coumarins, reducing sugar and absence of glycosides, sterols, anthocyanin anthraquinone, diterponoids, phlobatamines, quninones, catechin,. Preliminary phytochemical screening for *H. rosasinensis* leaves revealed the presence of carbohydrates and/or glycosides, steroids and/or triterpenes, flavonoids, and tannins while alkaloids and/or nitrogenous bases, saponins, and coumarins were absent (Faten *et al*., 2012). Udo *et al*, (2016) studied the phytochemical constituents of methanolic leaf extracts of *H.rosa-sinensis*, which revealed the presence of alkaloids, tannins, cardiac glycosides and combined anthraquinones and absence of saponins, flavonoids and phlobatannins.. According to Faten *et al*., (2012) *H.rosasinensis* leaves revealed the presence of carbohydrates and/or glycosides, steroids and/or triterpenes, flavonoids, and tannins while alkaloids and/or nitrogenous bases, saponins, and coumarins were absent.

Previous qualitative analysis done by krishnaiah *et al*., (2009) on phytochemical constituents in ethanol extract of *H. rosa-sinensis* revealed presence of Tannins, saponins, flavonoids, terpenoids, alkaloids and absence of phlobatannins, cardiac glycosides. The study conducted by Udo *et al*., (2016) in leaves of *H. rosa-sinensis* revealed that the certain phytochemical constituents showed strong presence (tannins, anthraruinones, cardiac glycosides

while some had less concentration (alkaloids), free hydroxyl anthraquinone were not present. Phytochemical study conducted by Rao *et al.*, (2014) reveals that alkaloids, tannins, saponins, triterponoids, coumarins, steroids, flavonoids were present in the ethanolic leaf extract of *H.rosa-sinensis.*

Research work on phytochemical estimation of ethanolic extract of *Albizia amara* done by Khan *et al*., (2010) revealed presence of alkaloid, glycoside, esstional oil, saponins, flavonoids, aminoacid and absence of steroids and carbohydrates. Investigation on phytochemical analysis of different extracts viz., hydro-methanolic, petroleum, toluene, chloroform, methanol, extracts of *Albizia amara* done by Praveen *et al* (2011) revealed presence of saponins, tannins, alkaloids, cardiac glycosides, carbohydrate, flavonoids, terpenoids, glycosides and quinones.

Khandelwal, (2001) worked on *D. indica* and found that saponins, tannins, carbohydrates, alkaloids, flavonoids, glycosides, steroids, proteins and alkaloids were present. The preliminary phytochemical screening of methanolic leaf extract of *P. pinnata* was done by Dwivedi *et al*, (2017) showed presence of alkaloids, glycosides, steroids, flavonoids, and proteins, while tannins, terpenes were absent.

The preliminary phytochemical investigation of *Mussaenda frondosa* showed the presence of steroids and phenolic compounds, glycosides, carbohydrates etc. (Mahesh and niharika 2017). Aqueous and alcoholic extracts showed the presence of flavanoids, sterols, proteins and glycosides, aqueous extract showed the presence of flavanoids, proteins and glycosides while chloroform and petroleum ether extract showed only presence of carbohydrates and glycosides and no flavanoids and sterols, probably extracts in providing hepatoprotection (Sambrekar *et al*., 2010). Mahesh and Niharika, (2017) worked on petroleum ether, ethyl acetate and butanol extracts of *M. frondosa* leaf which resulted in the presence of steroids, glycosides, flavonoids, phenolic compounds etc.,

4.2.2 Thin Layer Chromatography for phytochemicals

TLC were carried out on the methanolic extract of using two solvents (Table 6) using two different running solvents were studied. The colour bands produced depicts presence of a compound.

Table:6. TLC Screening of various methanolic extracts of four plants

Solvent System Used	Detection Reagent	Observation	Inference	Methanolic Extract			
				1	2	3	4
Ethyl acetate/ methanol/water (EMW) 10:1.35:1	Vanillin in sulphuric acid	Green/ Red/ Pink	Bitter Principle	+	+	+	+
Chloroform/ethyl acetate/formic acid (CEF) 10:8:2		Red/ Pink	Steroid / terpinoids	+	+	+	+

1 – *H. rosa-sinensis*; 2-*M. forndosa;*3-*A.amara; 4 -D.indica*

4.3 Quantitative test

4.3.1 Biochemical parameters

4.3.1.1 Total Carbohydrates

Among the four plants taken for the study maximum was found in *Albizia amara* (161.75 mg/100g), and minimum was registered in Methanolic leaf extracts of *A. amara* (161.75 mg/100g). revealed presence of 31.66 % of carbohydrate (Udo *et al*., 2016).

4.3.1.2 Protein

Protein content among the four plants varied from 88.33 – 225.00 µg/ 100 g. the highest amount of protein was found in *H. rosa sinensis* (225.00 µg/ 100 g) and the lowest was found in

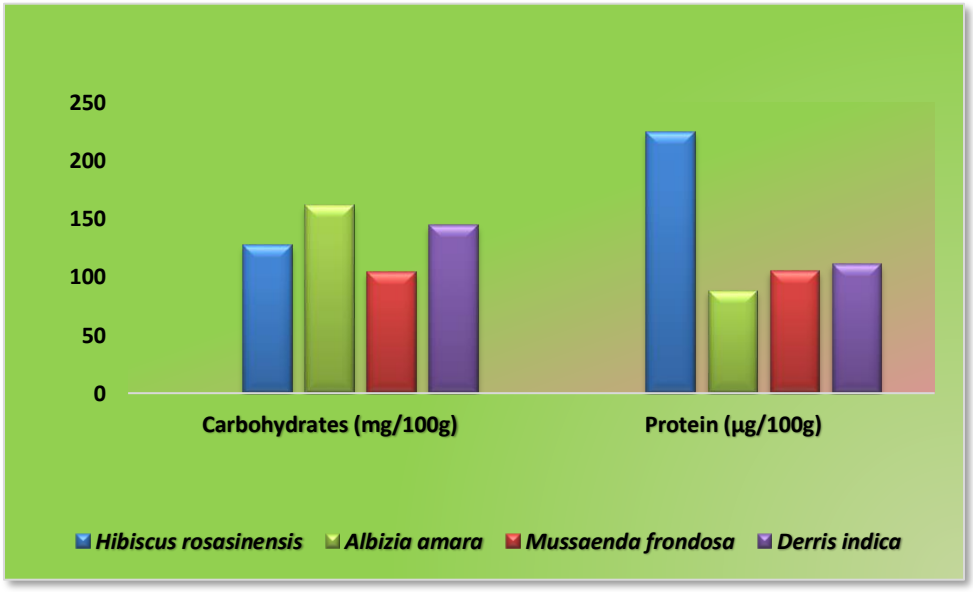
A. amara (88.33 µg/ 100 g). The ranking of plants based on the content of protein was H. rosa-sinensis > D. indica > M. frondosa > A. amara. Udo *et al*., (2016) registered that in his work in methanolic leaf extracts of *H. rosa-sinensis* protein was found to be 7.01 %.

Table: 7 Quantitative tests for Biochemical Parameters – Carbohydrates and Proteins

Plant name	Carbohydrate (mg/100g)	Protein (µg/100g)
Hibiscus rosa-sinensis	**127.07 ± 19.25**	**225.00 ± 0.83**
Mussaenda frondosa	**104.22 ± 11.58**	**105.00 ± 0.83**
Albizia amara	**161.75 ± 13.95**	**88.33 ± 0.84**
Derris indica	**144.33 ± 4.54**	**110.83 ± 0.84**
SEd	**10.9551**	**0.6797**
CD(<0.5)	**25.2629**	**1.5675**

Values are mean ± SD of three triplicates

Fig: 1 Comparison of Carbohydrate and Protein in methanolic extract of *H.rosa-sinensis*, *M.frondosa*, *A.amara* and *D. indica*



4.3.2 Plant Physiochemical parameters

Plant phenolics and flavonoids are a major group of compounds which have the following effects; choleretic and diuretic functions , decreasing blood pressure, reducing the viscosity of the blood and stimulating intestinal peristalsis (Lin *et al*., 2007) , as well as primary antioxidation or free radicals scavenging activities (Shahidi and Wanasundara, 1992 ; Rathee, *et al*., 2007; Pan *et al*., 2010).

4.3.2.1 Total Flavonoids

Present investigation revealed that flavonoids are present maximum in *A. amara* (261.60 µg/g) and minimum in *M. frondosa* (221.47 µg/g). The ranking of plants on the basis of flavonoid content among the three plants were *H. rosa-sinensis* > *A. amara* > *D. indica* > *M. frondosa.*

Previous study done by Sajid *et al*., (2012) flavonoid was found to be 0.38 %in Derris indica. krishnaiah *et al*., (2009) observed that flavonoids present in *Hibiscus rosa-sinensis* and Derris indica was found to be 0.40 % and 8.9 % respectively. Total flavonoids in *Pongamia pinnata* was found to be 3.44 g CE/100 g (Sajid *et al*., 2012)

4.3.2.2 Total Phenolic Content:

Study done by Faten *et al., (2012*) revealed that the phenolic content of *H. rosa-sinensis* extract was found to be 48.40 mg catechol equivalent /g of dry sample while the flavonoid contents was 24.26 mg quercetin equivalent/g of dry sample.

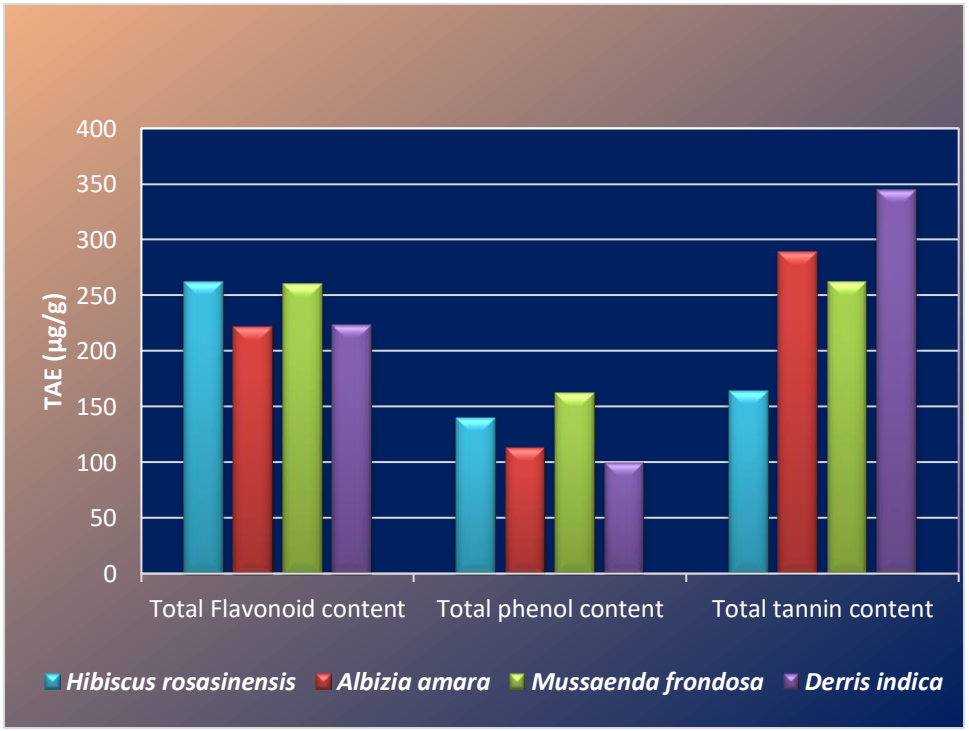
Therefore, in this study, the obtained level of phenolics and flavonoid in *H. rosa-sinensis* extract may be a sign to suggest that the extract may possess antioxidant activity. Our suggestion is in close agreement with previous reports that there is a strong correlation between the total phenolic and flavonoids content and antioxidant activity of extract from plant (Cai *et al*., 2004; Pan *et al*., 2007). According to krishnaiah *et al*., (2009) and Wong *et al*., (2010) the phenolic content in *H. rosa-sinensis* was found to be 0.680 and 735 % respectively. Studies done in *A. amara, D. indica* and *P.pinnata* revealed the presence of 15.90 %, 6.0 % and 2.40 % phenolic compounds respectively (Muchuweti *et al*., 2006; krishnaveni *et al*., 2014 and Sajid *et al*., 2012).

Table: 8 Quantitative test for the phytochemical – Flavonoid, Phenol and Tannin

Plant name	Flavonoid(µg/g)	Total phenol content(µg/g)	Total tannin content(µg/g)
Hibiscus rosa-sinensis	261.60 ± 0.80	140.36 ± 1.20	164 ± 16.90
Mussaenda frondosa	221.47 ± 1.01	113.33 ± 0.90	288.87 ± 6.05
Albizia amara	260.27 ± 1.14	161.95 ± 4.60	261.27 ± 13.57
Derris indica	222.93 ± 1.70	99.47 ± 0.61	344.33 ±2.81
SEd	0.9866	1.9907	9.2567
CD(<0.5)	2.2751	4.5907	21.3463

Values are mean ± SD of three triplicates

Fig : 2 Comparison of Total Flavonoid, Total Phenol and Total Tannin in methanolic extract of *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*



Total Phenol Content of *Albizia amara* in terms of Gallic acid equivalent was found to be 243.37 for methanolic extract Rajkumar *et al*., (2012). Phenolics in the leaves were also higher than those reported previously for methanolic extract of leaves (0.86g GAE /100g) of *P. pinnata* (Gupta and Sharma 2011; Babu and Rao 2010).

Plant based phenol compounds exhibit rich antioxidant activity by scavenging the free radicals generated during the normal metabolism process. This group encompasses a wide diversity of compounds, which mainly includes: flavonoids and proanthocyanidins (condensed tannins) (Shahidi and Naczk, 2004). A positive relationship between total phenols and antioxidant activity has also been found in albiziaamara .(rajkumar *et al* ., 2011)

4.3.2.3 Total Tannin

Total tannin content was found to be more in *Derris indica* and low in *Hibiscus rosa-sinensis* with a range of 344.33 µg/g and 164 µg/g respectively. Previous study done by krishnaiah *et al*., (2009) revealed that Tannin was found to be 8.5%in *Hibiscus rosasinensis*. Total tannin content was found to the more in *Albizia amara* and low in *Derris indica* with a range of 161.95 and99.47 respectively.

Presence of tannins (high molecular weight phenols) in adequate amounts can be advantageous as they are able to quench free radicals very effectively, which in turn depended on the number of aromatic rings, molecular weight,and nature of the hydroxyl group substitution (Cai *et al*., 2006).

4.4 Antioxidant

4.4.2 Enzymatic Antioxidant

4.4.2.1 Polyphenol Oxidase

Poly phenol oxidase activity was found to be maximum in *Hibiscus rosasinensis* (58.00) and minimum in *Derris indica* (11.04).

Table: 9 Levels of various enzymatic antioxidant in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

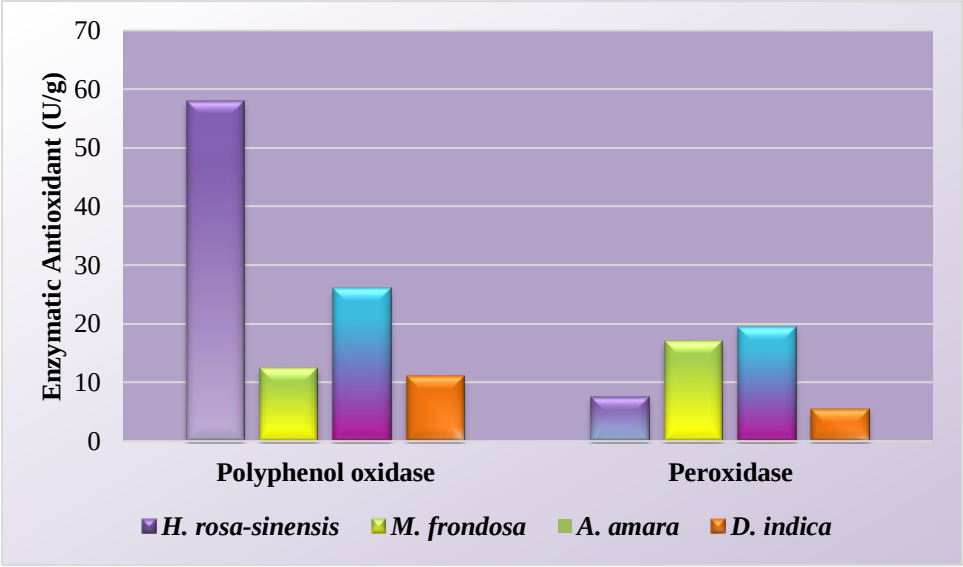
Plant name	Polyphenol oxidase (U*/g)	Peroxidase ($U^{\#}$/g)
H. rosa- sinensis	58.00±0.90	7.50±0.90
M.frondosa	12.52±0.65	17.00±0.90
A. amara	26.01±0.93	19.50±0.57
D. indica	11.04±0.86	5.50±0.90
SEd	**0.6860**	**0.6776**
CD (p<0.05)	**1.5819**	**1.5626**

Values are mean ± SD of three triplicates

*1 Unit – Activity of catechol oxidase which transforms 1 U/mol of dihydro-phenol to quinine/min.

$^{\#}$1 unit –

Fig: 3 Comparison of Enzymatic Antioxidants in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*



4.4.2.2 Peroxidase

Present investigation showed a significant peroxidase activity. The peroxidase activity was found to be maximum in *Albizia amara* (19.50) and minimum in *Derris indica* (5.50).

4.4.3 Non-Enzymatic Antioxidants

4.4.3.1 Ascorbic Acid

In the present investigation non-enzymatic antioxidant ascorbic acid was present between 1.76 – 3.96 µg/ ml when compared with all the four plants. In methanolic leaf extract values were found to be maximum in *Albizia amara* (3.96) and minimum in *Derris indica* (1.76).

4.4.3.2 Total Polyphenol

Total polyphenol estimation revealed that the values of the four plants ranged from 127.13 mg/100g to 302.33 mg/g. Hibiscus rosa-sinensis had the lowest value and Derris indica had the highest value of 127.13 mg/100g and respectively.

4.5 Free Radical Scavenging activity

The main characteristics of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources.These free radicals may oxidize nucleic acids,proteins,lipids and DNA and can initiate degenerative diseases.

Antioxidant compounds like phenolic acids,polyphenols and flavanoids scavenge free radicals such as peroxide,hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Pourmorad *et al*., 2006).

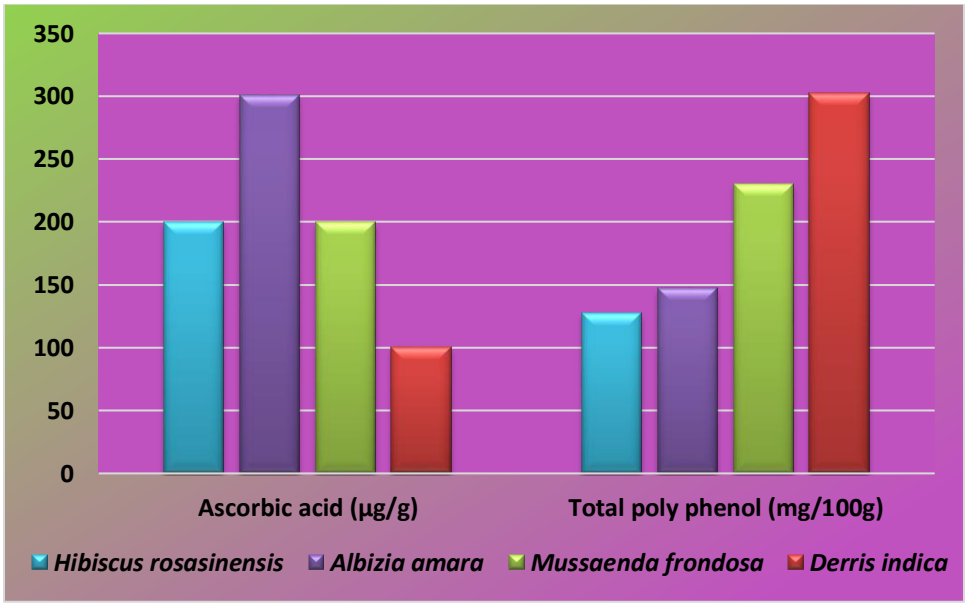
Radical scavenging activity of sample extact were determined based on the inhibition of DPPH and ferric reducing antioxidant activity power (FRAP) assays (Mak *et al*., 2013).

Table: 10 Levels of Non-Enzymatic enzymatic activity in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

Plant name	Ascorbic acid (µg/g)	Total poly phenol (mg/100g)
Hibiscus rosa-sinensis	2.08 ± 0.15	127.13±2.20
Mussaenda frondosa	2.07 ± 0.44	230.47±1.21
Albizia amara	3.96 ± 0.23	147.60±1.04
Derris indica	1.76 ± 0.14	302.33±0.31
SEd	**0.2186**	**1.1165**
CD(<0.5)	**0.5041**	**2.5748**

Values are mean ± SD of three triplicates

Fig: 4 Comparison of Total Non-enzymatic antioxidant Ascorbic acid and Total Polyphenol in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*



4.5.1 DPPH Scavengigng activity

DPPH radical has the advantage of being unaffected by certain side reactions, such as metal ion chelation, and enzyme inhibition, brought about by various additives. A freshly prepared sample of DPPH exhibits a deep purple colour, with maximum absorbance at 517 nm. The purple color generally fades or disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably by free radical attack) and convert them to a pale yellow or bleached product (i.e. 2,2-diphenyl-1-hydrazine or a substitutedanalogous of hydrazine), resulting in a decrease in absorbance at 517 nm (Yamaguchi *et al*., 1998). Hence, the more rapidly the absorbance decreases, the more potent is the antioxidant activity of the extract, in terms of hydrogen-atom-donating capacity (Muchweti *et al*., 2016)(AA)

The DPPH scavenging activity of methanol extract of *H. rosa-sinensis, Mussaenda, A. amara* and D. indica concentration dependent (increasing from 10µg/ml-50µg/ml) and it was able to inhibit the formation of reducing power assay .the antioxidant activity of methanol extract increased in a dose dependent manner with IC_{50}value of 40.62, 29.4, 28.89and 32.88in *H. rosa-sinensis,Mussaenda,A. amara,D. indica* respectively.

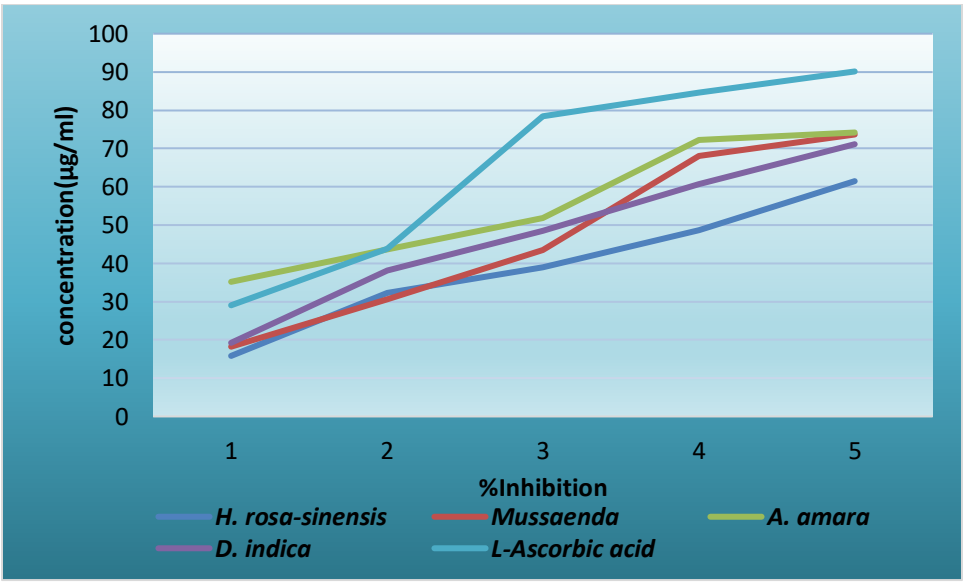
DPPH, a stable free radical was used to study the radical scavenging effects of petroleum ether and methanol extracts by Rajkumar *et al*,. (2011) in *Albizia amara* with DPPH IC_{50} values of 164 and 213 (µg/ml) in methanolic and petroleum ether extract respectively. According to Muchweti *et al.,* (2016) DPPH test is a commonly employed assay in antioxidant studies and offers rapid technique in which to screen for antioxidant activity. In *Albizia amara* the percentage inhibition in *A. amara* was found to be 95.84. DPPH radical scavenging assay is relatively a rapid and sensitive approach to evaluate the antioxidant activity of a specific compound or plant extract (Qureshi., *et al* 2010). The lower IC50 values reflect the greater potency for antioxidant activity of the extracts. It is widely accepted that the, antioxidant activity of a plant material is strongly correlated with the amount of phenolics, as well as the degree of hydroxylation of the phenolics, and other chemicals structural features (Demiray *et al*., 2009) Scvenging activity of free radicals of 1,1diphenyl-1,2-picryl hydrazyl (DPPH) has been widely

Table: 11 DPPH Scavenging activity of *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

Conc.(µg/ml)	*H. rosa-sinensis*	*M.frondosa*	*A. amara*	*D. indica*	L-Ascorbic acid
10	15.82±0.90	18.23±0.62	35.17±0.70	19.23±0.80	29.04±0.78
20	32.24±0.56	30.60±0.17	43.61±0.75	38.15±0.76	43.77±0.86
30	38.91±0.85	43.42±0.77	51.82±0.75	48.44±0.75	78.39±0.79
40	48.73±0.70	68.03±0.60	72.29±0.70	60.72±0.81	84.53±0.91
50	61.47±0.70	73.657±0.71	74.18±0.75	71.12±0.61	90.13±0.55
SEd	0.6143	0.5583	0.5996	0.6130	0.6425
CD(p<0.05)	1.3688	1.2440	1.3361	1.3659	1.4317

Values are mean ± SD of three triplicates

Fig: 5 Comparison of DPPH scavenging activity in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*



used to evaluate the antioxidant activity of plants.The DPPH radical scavenging activity increases with increasing concentration.(Siju *et al*., 2010). DPPH IC50 values in Pongamia pinnata leaves were found to vary from 4.42 to 16. 46 in various solvent study by Sajid *et al*., (2012)

4.5.2 Reducing Power assay

The reducing power assey activity of methanol extract of *H. rosa-sinensis, Mussaenda frondosa, A. amara* and *D. indica* concentration dependent (increasing from 5µg/ml-30µg/ml)and it was able to inhibit the formation of reducing power assay .the antioxidant activity of methanol extract increased in a dose dependent manner with IC_{50}value of 28.76, 31.64, 31.05and 31.28 in *H rosa-sinensis, Mussaenda frondosa, A. amara* and *D. indica* respectively.

It has been reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom Hence, from various results obtained it is concluded that methanolic extract may have the higher amounts of reductones and polyphenol in comparision to the petroleum ether extract of *Albizia amara* leaf (Oyaizu, 1986; Zhang *et al*., 2009)

According to Sajid *et al*., (2012) the reducing power of *Pongamia pinnata* leaf for 10 mg/ml extract concentration in various extracts ranged from 0.43 – 1.4 mg/ ml. in *Albizia amara* reducing power assay was reported by Rajkumar *et al*., (2011) as 0.087 and 0.151 µg/ml in both methanolic and petroleum ether extracts.

4.5.3 Metal Chelating Activity

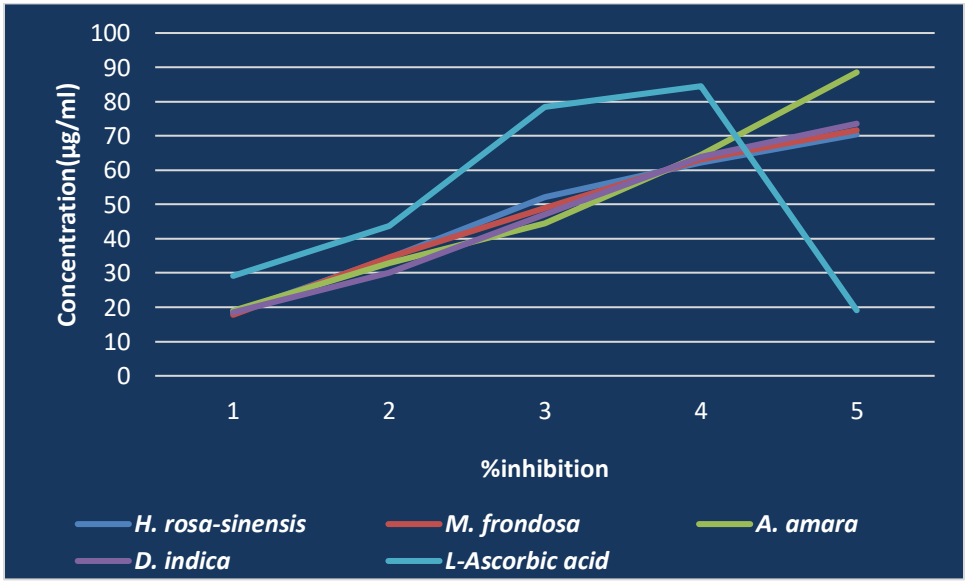
The metal chelating activity of methanol extract of *H. rosa-sinensis, Mussaenda,frondosa, A. amara* and *D. indica* concentration dependent (increasing from 5µg/ml-30µg/ml)and it was able to inhibit the formation of reducing power assay .the antioxidant activity of methanol extract increased in a dose dependent manner with IC_{50} value of 28.99, 30.47, 29.02 and 27.61in *H. rosa-sinensis, Mussaenda,frondosa, A. amara* and *D. indica* respectively.

Table: 12 Reducing power assay of *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

Conc. (µg/ml)	*H. rosa-sinensis*	*M.frondosa*	*A. amara*	*D. indica*	L-Ascorbic acid
10	18.27±0.90	17.79±0.60	18.99±0.63	18.51±0.86	29.11±0.17
20	34.38±0.70	34.62±0.20	32.93±0.81	30.06±0.84	43.77±0.19
30	52.16±0.50	49.01±0.85	44.50±0.76	47.12±0.84	78.49±0.89
40	62.26±0.42	63.22±0.50	64.42±0.60	63.97±0.66	84.49±0.60
50	70.43±0.70	71.63±0.70	88.53±0.71	73.56±0.53	19.12±0.64
SEd	**0.5436**	**0.4984**	**0.5753**	**0.6175**	**0.6179**
CD (p<0.05)	**1.2113**	**1.1106**	**1.2819**	**1.3759**	**1.3767**

Values are mean ± SD of three triplicates

Fig: 6 Comparison of reducing power assay in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*



The free radical scavenging activity of leaves extract in this study was also found to be greater than that of methanolic extract of the leaves (IC50: 192 µg/mL) of *P. pinnata* reported in an earlier study (Gupta, V.; Sharma, M 2011).

It is widely accepted that the, antioxidant activity of a plant material is strongly correlated with the amount of phenolics, as well as the degree of hydroxylation of the phenolics, and other chemicals structural features (Demiray, S *et al*., 2009).

The metel chelating capacity experiment conducted by skrishveni *et al*. ,(2014) on the road side of railway junction and road sides of its residential area in Selam,Tamilnadu showed varied results in aqueous leaf extract of *D. indica* showed the percentage of 45.61µg/ml,81.82 respectively.

4.5.4 FRAP (Ferric Reducing Antioxidant Power) Assay

The FRAP activity of methanol extract of *H. rosa-sinensis, Mussaenda,frondosa, A. amara* and *D. indica* concentration dependent (increasing from 5µg/ml-30µg/ml)and it was able to inhibit the formation of reducing power assay .the antioxidant activity of methanol extract increased in a dose dependent manner with IC_{50} value of 27.01, 28.5, 29.86and 19.12in *H. rosa-sinensis, Mussaenda,frondosa, A. amara* and *D. indica* respectively. Free radicals induce numerous diseases by lipid peroxidation, protein peroxidation and DNA damage. It has been reported that numerous plant extracts have antioxidant activities to scavenge free radicals.

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems

Table: 13 Metal chelating activity of *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

Conc. (µg/ml)	*H. rosa-sinensis*	*M.frondosa*	*A. amara*	*D. indica*	L-Ascorbic acid
10	25.65±0.79	24.62±0.79	23.87±0.79	27.85±0.68	29.09±0.94
20	33.99±0.79	32.70±1.18	44.74±1.15	49.66±0.80	43.64±0.75
30	42.31±0.83	45.40±0.90	51.89±1.33	54.17±0.76	78.46±0.82
40	68.89±0.84	65.46±0.73	66.70±0.71	63.94±0.82	84.62±0.68
50	73.25±0.75	78.37±0.74	80.74±0.56	74.31±0.57	90.12±0.96
SEd	**0.6559**	**0.7224**	**0.7774**	**0.5971**	**0.6817**
CD (p<0.05)	**1.4615**	**1.6094**	**1.7322**	**1.3304**	**1.5190**

Values are mean of three triplicates

Fig: 7 Comparison of Metal chelating activity in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

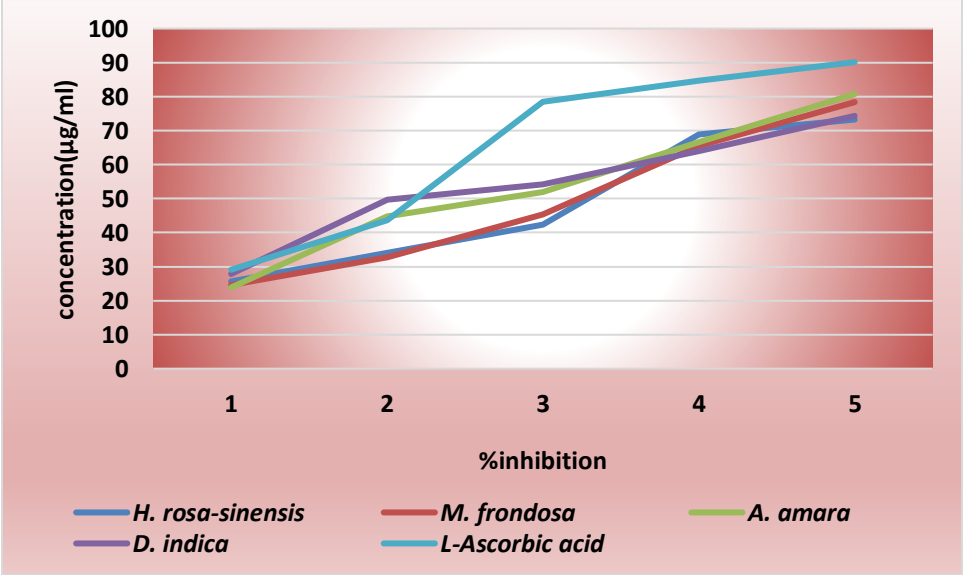


Table: 14 FRAP assay of *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

Conc. (µg/ml)	*H. rosa-sinensis*	*M.frondosa*	*A. amara*	*D. indica*	L-Ascorbic acid
10	13.80 ± 0.84	15.64 ± 0.69	11.48 ± 0.68	20.42 ± 0.99	29.44 ± 1.00
20	31.57 ± 0.77	37.27 ± 0.89	35.52 ± 0.93	40.84 ± 0.65	43.57 ± 0.63
30	55.49 ± 0.90	52.62 ± 0.93	50.53 ± 0.73	51.58 ± 1.21	78.55 ± 1.25
40	57.01 ± 0.60	62.26 ± 0.77	76.65 ± 0.90	77.18 ± 0.85	84.63 ± 1.14
50	60.53 ± 0.71	70.27 ± 1.07	81.43 ± 1.04	82.22 ± 1.02	90.13 ± 0.90
SEd	0.6295	0.7181	0.7077	0.7838	0.8215
CD (P<0.05)	1.4027	1.6001	1.5769	1.7463	1.8304

Fig; 8 Comparison of FRAP assay in *H.rosa-sinensis, M.frondosa, A.amara* and *D.indica*

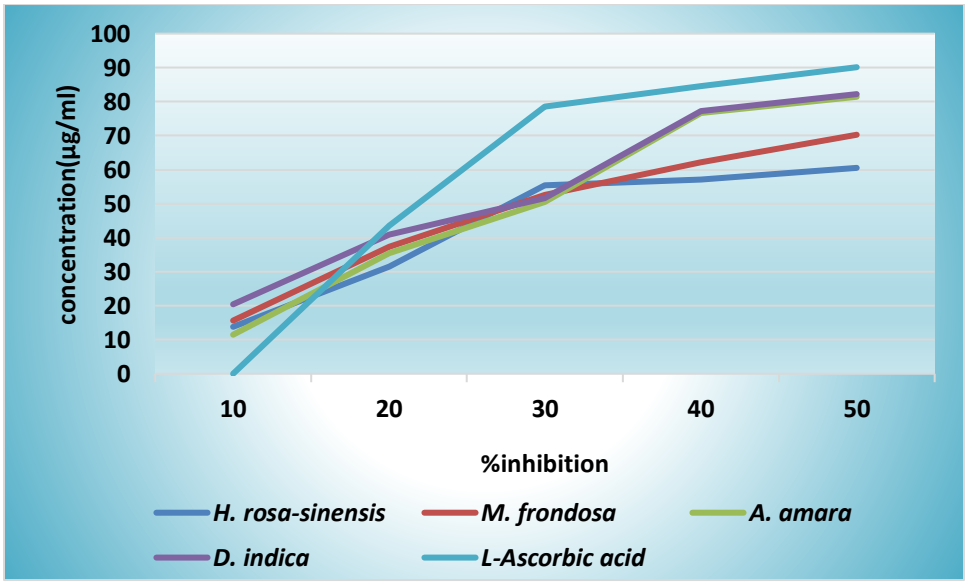
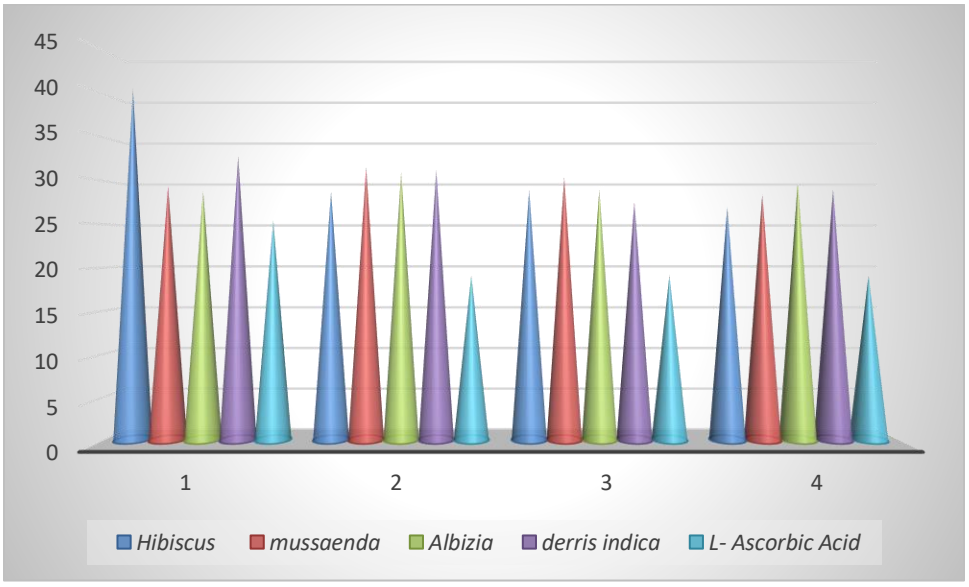


Table: 15 IC $_{50}$ values of various methanolic leaf extracts and standards

Plant Extracts / Standards	IC $_{50}$ (µg/ml)			
	DPPH Assay	Reducing Power Assay	Metal Chelating Assay	FRAP Assay
Hibiscus rosa-sinensis	40.62	28.76	28.99	27.01
Mussaenda frondosa	29.4	31.64	30.47	28.5
Albizia amara	28.89	31.05	29.02	29.86
Derris indica	32.88	31.28	27.61	29.01
L- Ascorbic Acid	25.49	19.12	19.12	19.12

Fig: 9 Graphical representation of IC $_{50}$ values of various free radical scavenging capacity of four plants.



Summary and Conclusion

Summary and Conclusion

Plants have various primary and secondary chemical constituents, which play an important role in curing diseases or enhancing the health. In this study four plants (*Hibiscus rosa-sinensis, Mussaenda frondosa, Albizia amara* and *Derris indica*) which are used by local people for hair cleansing and growth promoting were taken for the study. Various parameters like the presence or absence of phytoconstituents, their antioxidant activity and also its free radical scavenging capacity were carried out.

Phytochemical screening test revealed the presence of 12 compounds (carbohydrates, proteins, Vitamin C, alkaloids, triterpenoids, saponins, phenol, tannin, phytosterol, tannin, resins and reducing sugar) out of 24 tested were present in all the four extracts.

Biochemical parameters like carbohydrate and protein was found to be high in *A. amara* and *H. rosa-sinensis* respectively. The phytochemical parameters of the four plants were analysed. Flavonoid, total phenol and total tannin was present more in *H. rosa-sinensis, A. amara* and *Derris indica* respectively.

Antioxidant properties of the three plants were analysed, and all the three showed a positive result in showing the activity. Enzymatic antioxidants like polyphenol oxidase and peroxidase were found maximum in *H. rosa-sinensis* and *A.amara* respectively. Non-enzymatic antioxidants ascorbic acid and total polyphenol were found more in *A. amara* and *Derris indica* respectively.

DPPH assay, Reducing power assay, FRAP assy and metal chelating assays were done to see the performance of plants in scavenging the free radicals present. The maximum IC_{50} values in DPPH assay, Reducing Power Assay, FRAP Assay and Metal Chelating Assay was found to be near to the standard in *A. amara*, *H. rosasinensis*, *H. rosa-sinensis* and *Derris indica.*

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