Studies on the leaves of *Ficus religiosa* Linn. (Family: Moraceae)

Submitted by

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Exam Roll No. - M4PHA19006

Registration No. - 140830 of 2017-2018

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India

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Thesis Submitted In Partial Fulfillment Of The Requirements For The Degree of Master of Pharmacy

Department of Pharmaceutical Technology

Faculty of Engineering and Technology

JADAVPUR UNIVERSITY

2019

FORWARDING CERTIFICATE

This is to certify that the thesis entitled "Studies on the leaves of Ficus religiosa Linn. (Family : Moraceae)" submitted by Arunava Sen, with Examination Roll No.: M4PHA19006, Registration No.: 140830 of 2017-2018, in the year 2017-2018) for the partial fulfillment of degree of Masters of Pharmacy, Jadavpur University, is absolutely based upon his own research project work under my supervision, in the Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.

He has included his finding into this thesis. His thesis has not been submitted before for any degree/diploma or any other academic award elsewhere. I am satisfied that he carried out his thesis with proper care and confidence to my satisfaction.

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CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled "Studies on the leaves of Ficus religiosa *Linn. (Family : Moraceae)" submitted by Arunava Sen, with Examination Roll No.: M4PHA19006, Registration No.: 140830 of 2017-2018, in the year 2017-2018) for the partial fulfillment of degree of Masters of Pharmacy, Jadavpur University, is absolutely based upon his own research project work under my supervision, in the Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032.*

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Declaration of Originality and Compliance of Academic Ethics.

I hereby declare that the thesis contains literature survey and original research work by me (Arunava Sen), as part of my Master of Pharmacy studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are original to this work.

Name **: Arunava Sen**

Exam Roll No. **: M4PHA19006**

Registration No. **: 140830 of 2017-2018**

Thesis **: Studies on the leaves of *Ficus religiosa* Linn. (Family : Moraceae)**

Signature with Date :

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I wish to thank my friends and all others who have extended their cooperation and helped me immensely during the entire duration of this thesis.

Arunava Sen
Regn. No.-
Exam Roll no.-

Dedicated to my Parents

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List of Abbreviations, Figures and Tables:

List of Abbreviations:

TLC	Thin layer chromatography
TPC	Total phenolic content
TFC	Total Flavonoid content
Vit C	Vitamin C
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl.
HPLC	High Performance Liquid Chromatography.
Rf factor	Retardation Factor
RT	Retention Time

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INTRODUCTION:

Covering a time span of thirty years (1981-2012) in their in-depth review declared that about 26.28% of the 1130 New Chemical Entities were either natural products or were derived from natural product mostly by semi-synthetic modification and with another about 21.76% are created around a pharmacophore from natural product (Mandal *et al*., 2015).

Since early human history, chemical compounds obtained from plants have been investigated and utilised by physicians to treat various diseases. Botanicals have been a prolific supply of new drugs and leads since the Vedic period. World Health Organization postulates that around eighty percent of inhabitants from developing countries around the globe depend upon the traditional system of medication for their primary health care requirements, and about 85% of the traditional medicine involves the use of plant derived extracts. This means that about 3.5-4 billion people throughout the world rely on plants as sources of medicine.

China and India are the two largest consumers of medicinal plants in the world. Over five thousand species of plants are used in Traditional Chinese Medicine, whereas India uses about seven thousand species. According to Export Import Bank, the growth rate of 7% per annum has been seen in the international market for medicinal plant related trade. In the World Herbal market, China's share is USD 6 billion, whereas India's share is merely USD 1 billion. All the major herbal based pharmaceutical companies are showing a constant growth of about USD 80-250 billion in Europe and the United States.

Medicinal plants throughout have played a crucial role in the design and development of potent therapeutic agents. During 1950-1970, approximately a hundred new plant-based drugs were

introduced in the US drug market including vinblastine, vincristine, reserpine, deserpidine and reseinnamine, which are derived from higher plants.

The genus *Ficus* is a group of about 850 species comprising of trees, shrubs, vines. These are commonly known as Figs. They are distributed throughout the world's tropics, though abundantly found in tropical areas of East Asia.

Ficus religosa is a dry season-deciduous tree whose growth is supported by a wide variety of climate ranging from Tropical rainforest climate, Tropical monsoon climate, Tropical savanna climate with dry summer, Wet temperate climate (wet all year) to Warm temperate climate with dry summer. The optimum precipitation may range from 60mm to 100mm. A suitable altitude for its growth, ranges from 10ft to 4990ft and can tolerate a temperature ranging from 0°C to 35°C.

Ficus religiosa L. is the most popular member of the Ficus genus and is addressed by more than a hundred names locally. It is common in areas of the sub-Himalayan tract, Bengal and central parts of India. It has been cultivated extensively throughout the world (McFarland, 1944, Galil, 1984).

It holds a huge significance in mythological and religious sector in Indian culture, since ages.

Ficus religiosa (sacred Fig), colloquially known as Peepal tree or Ashwattha, is habitated all over India starting from the plains to the Himalayas, as avenues or roadside tree around temples.

Ficus religiosa is a popular bodhi tree.

The specific epithet "religiosa" and synonym "bodhi tree" alludes to the religious significance attached to this tree. Since antiquity, *F. religiosa* has got mythological, religious and medicinal

importance in Indian culture. It is the oldest portrayed tree in India. Atharvaveda (sacred text of Hinduism) links it with the third heaven and discusses its medicinal properties along with Soma and Kustha (holy medicinal herbs) (Kala *et al*., 2006, Sitaramam *et al.*, 2009).

Fig species are characterized by their unique inflorescence and distinctive pollination syndrome, which utilizes wasp belonging to the family Agonidae for pollination (Ronsted *et al*., 2008).

The specific identification of many of the species can be difficult, but Figs as a bunch are comparatively easy to recognize. Many have aerial roots and a particular shape or habit, and their fruits are different from other plants. The Fig fruit is an enclosed inflorescence generally termed as a syconium, an urn–like structure with the Fig's tiny flowers, lined on the inside (Ronsted *et al*., 2005).

It starts its life epiphytically. Soon afterwards, its far-growing roots strangle the host and further extend to the ground to establish itself as an independent tree (Pullaiah, 2006).

These can rise up to 30m high with a trunk diameter of up to 3m.

The most suitable soil for it's optimum growth is deep, alluvial sandy loam with good drainage. It can also grow in rock crevices.

All Figs constitute a white to yellowish latex, some in abundant quantities; the twig has paired stipules or a circular stipule scar if the stipules have fallen off; and the lateral veins at the base of the leaf are steep, forming a tighter angle with the midrib than the other lateral veins, a feature noted as "tri-veined".

Figs are keystone species in several tropical forest ecosystems. Their fruit are a key resource for some frugivores including fruit bats, and primates including: capuchin monkeys, langurs,

gibbons and mangabeys. They are even more important for birds such as Asian barbets, pigeons, hornbills, Fig-parrots and bulbuls, which can virtually entirely live on Figs when these are in plethora.

Ficus religiosa has a very long life span of about 1000 years. Of all the members of the Ficus genus, *Ficus religiosa* is the most popular one. It is abundantly found in sub-Himalayan tract, Bengal and central India.

The therapeutic applications of *Ficus. religiosa* have been mentioned in traditional systems of medicine like, Ayurveda, Unani, etc. It has been used in treatment for numerous disorders of the CNS, GIT, Endocrine system, Respiratory system, Reproductive system and infectious diseases.

It's roots, barks, leaves, fruits and latex has been immensely used as Indian traditional medicine for the therapy of various ailments including asthma, diabetes, diarrhea, epilepsy, gastric problems, inflammatory disorders, infectious and sexual disorders.

Ficus religiosa has been listed as an "environmental weed" or "naturalized weed" by the Global Compendium of Weeds (Randall., 2012). It has been assigned an invasiveness high risk score of 7 in a risk assessment prepared for the species' invasiveness in Hawaii by PIER. Such a high score predicts it will become an important pest in suitable climate zones. The key reasons for its invasive behavior are its quick growing nature, tolerance to variety of climate zones and soil varieties, reported lifespan of over three thousand years, and its smothering growth environment because it usually begins life as an epiphytic plant (Stephen Forbes, 25 December 2016).

Rabah tribes of West Bengal use the bark of Ashwatha (*Ficus religiosa)* for hematuria. Inhabitants of Jammu and Kashmir consume the extract of it's bark to alleviate respiratory disorder.

The initial step in the isolation and analysis of secondary metabolites is extraction. This process aims to filter out the compounds from cellular matrix. A elementary knowledge regarding their place of occurrence, nature and characteristics is extremely essential in choosing an extraction technique. Once the chemical nature of the secondary metabolite of interest is known to us, an extraction method ought to be conducted in a highly coordinated manner aiming to obtain a high yield and purity. When the chemical composition is unknown it is advisable to reproduce the extraction methods employed traditionally in order to boost the possibilities of isolating potential bioactive metabolites.

The overall aim of the extraction process is to establish the composition of the natural product under investigation. This can be achieved either by physical assay technique (thin layer chromatography, high performance thin layer chromatography, high performance liquid chromatography, liquid chromatography-mass chromatography, liquid chromatography-nuclear magnetic resonance) by comparing with some standard biomarkers or by applying bioassay techniques. Secondly, the natural products need to be characterized by carrying out various screening techniques in detail. The preparation of extract comes prior to separation, identification and characterization of bioactive compounds.

A key factor in the widespread recognition of natural or alternative medicine by the international society involves the "modernization" of herbal medicine. So the application of standardization and quality control of herbal materials by use of modern science and technology is a very

important issue. Only a standardized and a validated process of extraction alone can assure an extract of consistent quality. In general, standardized extracts are usually tested for a minimum content or range of certain specified markers compounds.

Plants are the foundation of existence on world and are central to people's livelihoods. India is rich in medicinal plants. More than 2500 plant species contain therapeutic values. A huge number of medicinal plants are being utilised from the natural plants for the commercial production of medicine. Our body shows a large number of foreign chemicals daily, most of which are semi-synthetic. Our incapability to properly metabolise them, inversely affects the health by generating free radicals. Free radicals are also produced during normal metabolism of aerobic cells. The oxygen utilization in cell's growth leads to the production of series of oxygen free radicals. Extremely dynamic free radicals and their uninhibited manufacture are responsible for many pathological process such as cell tumor (prostate and colon cancers), coronary heart diseases, bleeding wounds, constipation, dysentery, boils and mumps.

The screening studies for antioxidant activity of medicinal and food plants have been performed extensively for the past few decades with the objective of finding an efficient remedy for several diseases and means to delay aging symptoms. Due to the antioxidant activity it is useful in inhibition of various human diseases. In nature antioxidants present in leafy vegetables and seed, such as Ascorbic acid, Vitamin E and phenolic compound possess the ability to reduce the oxidative damages in diseases like cancer, cardiovascular diseases, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing.

Plant Authentication and Herbarium preparation:

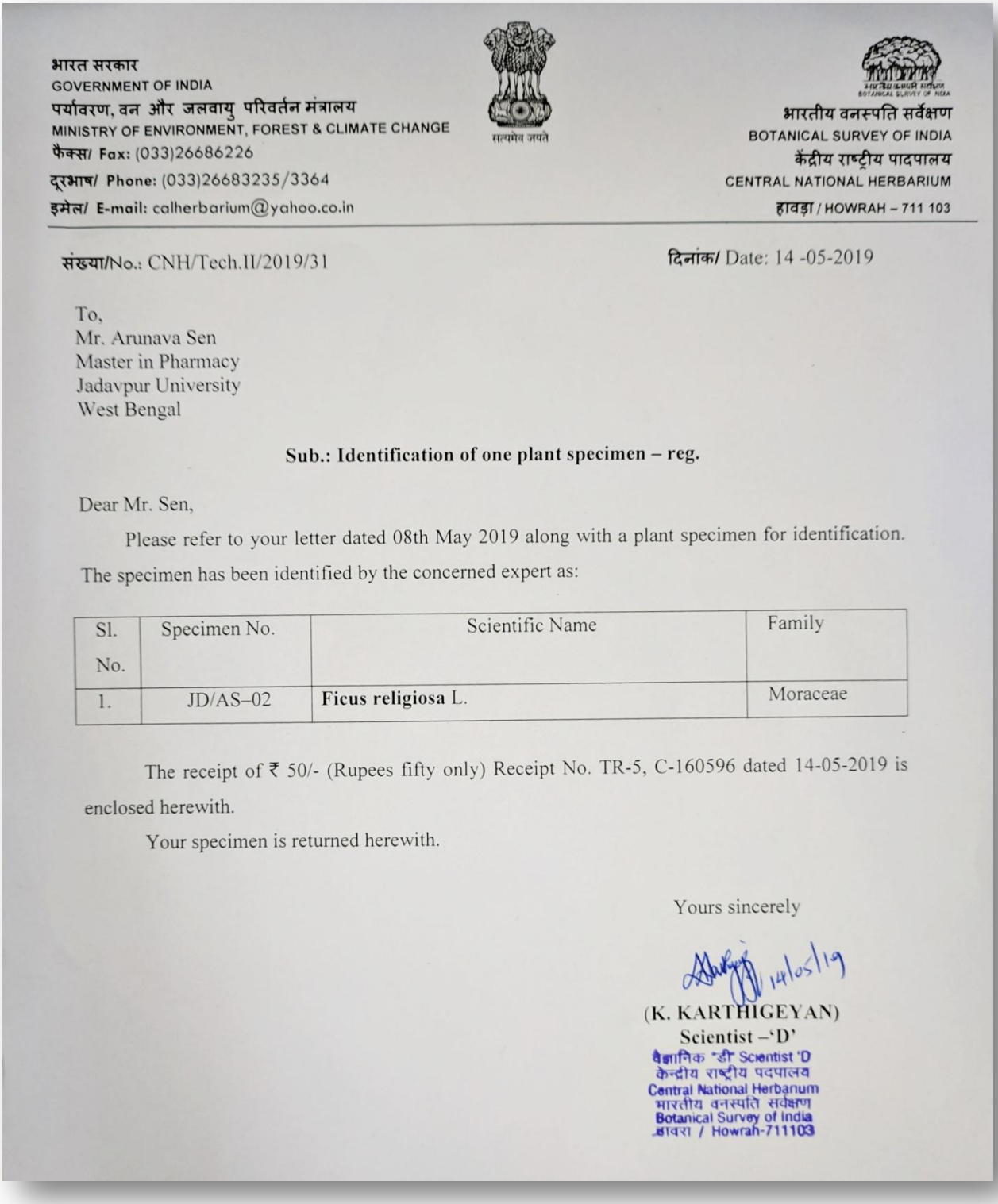
In Pharmacognostic research, the foremost order of work is to establish the taxonomical identity of the plants at the initial stage of research. This plays a very important role which is often skipped by researchers. It is utmost essential to authenticate the plant material prior to reporting new or even known substances from new or unexplored plants.

After collection of the plant part, it is dried and herbarium is prepared. It is then subjected to authentication by a certified taxonomist. For future reference of the studied plant, the herbarium is deposited in a recognized herbarium. On a note card affixed with the voucher specimen, all the relevant details of the plant are noted down ie, plant's origin, altitude of occurrence, climatic and micro-environmental conditions and any other relevant information such as it's local uses, overall health of the plant specimen and other facts that may be useful for future investigation. This aforementioned information is of vital importance when there is a necessity to recollect the plant material and reproduce the work in future.

The plant specimen bearing specimen no. JD/AS-02, was taken for identification to Central National Herbarium, Botanical Survey of India, Howrah. It was identified and authenticated to be *Ficus religiosa* L. belonging to Moraceae family by K. Karthigeyan (Scientist-D, certified taxonomist).


HERBARIUM SPECIMEN
No. JD/AS-02 Date of Collection 20/03/2019
Name of the Plant Ficus religiosa
Family Moraceae
Habitat Terrestrial
Locality Arambagh
Altitude Plainland Local Name: Pipal tree
Remarks Large Tree
Collected & by: Arunava Sen
Determined by: CNH, BSI
NEW DEEPAK, Serampore

Figure 1.



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सत्यमेव जयते

भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA
केंद्रीय राष्ट्रीय पादपालय
CENTRAL NATIONAL HERBARIUM
हावड़ा / HOWRAH – 711 103

संख्या/No.: CNH/Tech.II/2019/31

दिनांक/ Date: 14 -05-2019

To,
Mr. Arunava Sen
Master in Pharmacy
Jadavpur University
West Bengal

Sub.: Identification of one plant specimen – reg.

Dear Mr. Sen,

Please refer to your letter dated 08th May 2019 along with a plant specimen for identification. The specimen has been identified by the concerned expert as:

Sl. No.	Specimen No.	Scientific Name	Family
1.	JD/AS–02	**Ficus religiosa** L.	Moraceae

The receipt of ₹ 50/- (Rupees fifty only) Receipt No. TR-5, C-160596 dated 14-05-2019 is enclosed herewith.

Your specimen is returned herewith.

Yours sincerely

14/05/19

(K. KARTHIGEYAN)
Scientist –'D'
वैज्ञानिक 'डी' Scientist 'D
केन्द्रीय राष्ट्रीय पदपालय
Central National Herbarium
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
हावरा / Howrah-711103

Figure 2.

Description of *Ficus religiosa* plant:

Kingdo: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Rosales

Family: Moraceae

Genus: *Ficus*

Species: *Religiosa*

Scientific Name: *Ficus religiosa*

Other Names: Bodhi tree, Sacred tree, Peepal tree, Ashvattha

Ficus religiosa Linn.

Location: Botanical Survey of India, Howrah

Ficus religiosa is a large deciduous plant. At initial stage it is an epiphyte, but matures into a crown wide. Its falling branches possess dark green coriaceous, stipulate leaves. The leaves are cordate shaped and has a prominent drip tip. The Margin may be entire or undulate with five to seven secondary veins on each side of the mid vein, eight pair lateral veins with finely reticulate venation. Petioles are long and slender with ovate or acute stipules. Male flowers are few in number and are found near apical pore. The calyx has 2–3-lobes with revolute margin. There is a single stamen with a short filament. Gall flowers are pedunculate in nature. The calyx has 3–4-

lobes. The ovary is globose and smooth with short style. The stigma is large with 2-lobes. Female flowers are sessile in nature and the calyx has 4-lobes. It has a thin style and a narrow stigma (Kirtikar and Basu, 1993; Warrier et al., 1995; Zhekun and Gilbert, 2003).

The bark is flat or slightly curved with thin or membranous flakes, often covered with crustose lichen patches. The outer bark is grayish or ash-colored, exfoliated with irregular rounded flakes of 2–2.5cm thickness. The middle bark sections appear as brownish or light reddish brown in color. The inner part consists of the layers of light yellowish or orange brown colored granular tissue. The bark is odorless and its taste is astringent. The plant bears few adventitious roots (Warrier et al., 1995; Koilpillai et al., 2010).

Literature review:

Sawarkar *et al*., 2011 reported that a lot of plants synthesize compounds that aid in human health maintenance.

With an aim to increase the wide range of medicinal uses, now in the present day we attempt to bring in new drugs with more potent and desired activity with less to no side effects against particular diseases (Roy *et al*., 2009).

There are around 800 species and 2000 varieties of *Ficus* genus, occuring in the most tropical and subtropical forests (Hamed, 2011).

All *Ficus* species contain latex-like material within their vasculatures which provide protection and self-healing from physical harm (Sirisha *et al*., 2010).

Numerous studies have suggested that *Ficus* species are highly efficient in managing various types of disorders of the respiratory system, sexual disorders, CNS, CVS, GIT, skin infections and diabetics etc (Sirisha *et al*., 2010; Vinutha *et al*., 2007).

It was found that *Ficus religiosa* exhibited antidiabetic activity by increasing the

serum insulin level, body weight and glycogen content and also exhibited anti lipid peroxidative effect against streptozotocin induced diabetic rats (Pandit *et al*., 2010).

Kunwar and Bussmann, (2006) reported that leaf juice with honey is used for various of diarrhoea, asthma, cough, ear pain, toothache, migraine, in gastric problems.

The bark, powdered and made into paste, is used in cases of anal fistula and as absorbent for inflammatory swellings and also used in burns (Nadkarni, 1954, Warrier *et al*., 1995).

(Khan *et al*., 2011), (Kalyon *et al*., 2000), suggested the presence of antiulcer and wound healing activity of Ficus religiosa leaves.

(Pandit *et al*., 2010) reported it's effectiveness in diabetics, diarrhea, leucorrhoea, anxiety, for vaginal and other urinogenital diseases and to improve the complexion.

It was reported that *Ficus religiosa* is useful as a cardiac tonic and effective in management of diseases of vagina. It is also used to treat vomiting, anorexia and edema (Singh, 2006).

(Sirisha *et al*., 2010) reported that, the fruit extract of *Ficus* species exhibited anti tumour and antibacterial activity.

The hydro alcoholic extract of leaves of *Ficus religiosa* also reported antiulcer activity (Saha and Goswami, 2010).

The methanol extract of stem and bark of Ficus religiosa also reported to have inhibitory effect of the enzyme cycylo oxygenase (COX) leading to inhibition of PG's synthesis, tested on carrageenan-induced inflammation in rats. Further, various studies reported anti-inflammatory effect due to presence of tannin in bark (Sreelekshmi *et al*., 2007).

The aqueous extract of bark of *Ficus religiosa* was reported to possess anti-inflammatory and mast-cell proliferative effect *(*Viswanathan *et al*., 1990).

It has been found that *Ficus religiosa* is used as a national therapy against various infectious disorders. The antibacterial potential of *Ficus religiosa* was studied with chloroform extract of the leaves of *Ficus religiosa* which inhibited the growth of various Salmonella species, *P. vulgaris, E. coli, B. Subtilis* and *K. Pneumonia* etc (Hemaiswarya *et al*., 2009).

(Pandit *et al*., 2010) reported that the aqueous bark extract of *Ficus religiosa* exhibited antidiabetic activity against streptozotocin-induced diabetic rats.

(Yadav *et al*., 2011) has illustrated the nephro protective effect of methanol extract of *Ficus religiosa* latex.

(Sirisha *et al*., 2010) has reported that *Ficus religiosa* has also shown antioxidant activity.

The presence of flavonoids, saponins and tannins was observed in the preliminary phytochemical analysis of the methanol extract of *Ficus religiosa* bark (Uma *et al.,* 2009).

As oxidative stress is the primary cause of diabetes, the antioxidant activity of the aqueous extraction of *F. religiosa* was investigated in streptozotocin-induced to diabetic rats. *Ficus religiosa* is a widely used in the India as medicine for oxidative stress.(Makhija *et al*.,2010).

Sharma and Gupta (2007) investigated the *in vitro* antioxidant effect of the ethyl acetate root extracts of *F. religiosa* by using diphenylpicryl-hydrazyl (DPPH) radical scavenging activity, hydroxyl radical scavenging activity, reducing capacity and hydrogen peroxide scavenging assay.

Pharmacognostical research on *F. religiosa* had led to the isolation of few classes of plant metabolites. However, due to the vast traditional use and pharmacological benefits of *F. religiosa*, there lies a huge scope for its phytochemical study, which may further expand its existing therapeutic potential (Goel *et al*., 2010).

Objective:

- The major objective of the present study was to investigate the *in-Vitro* antioxidant potential of leaf extract of *Ficus religiosa*.
- The aim is to study their Phytochemical compositions, total phenolic content and total flavonoid contents.
- Then study of antioxidant activities with ABTS radical scavenging and reducing power activity.
- Fractionate the Petroleum ether extract into different compounds and attempt to identify the fraction responsible for antioxidant activity.

Materials and methods:

I. Collection of leaves:

Mature leaves of *Ficus religiosa* were collected from Arambagh in Hoogly District of West Bengal during the month of August. Microscopical study was performed on it under a compound microscope.

These leaves were washed properly and then subjected to shed drying for 2 weeks.

The dried leaves were then pulvarised in a grinder mixer to obtain fine powder.

II. Macroscopic study *Ficus religiosa* leaf:

Leaf parts	Characteristics
Duration	Evergreen
Leaf arrangement	Alternate
Petiole color size	Brownish-yellow(dried), 7-12 cm
Lamina shape	Cordate with drip tip
Type	Simple
Venation	Pinnate-reticulate
Apex	Caudate-Acuminate
Base surface	Cordate

Texture	Thin, smooth
Upper epidermis	Shiny, glabrous
Lower epidermis	Coraiceous

Table 1.

III. Extraction of *Ficus religiosa* Linn. leaves by Continuous Hot Percolation Method and Microwave Assisted Extraction:

90 gm of powdered leaf each was weighed and extracted with 1 liter of petroleum ether (60-80°C)(i) and 1 liter of methanol(ii) separately by successive extraction in a soxhlet apparatus for 48 hrs. The extracts were concentrated by evaporating the solvent. The dried extract was weighed and the percentage yields was calculated.

Microwave Assisted Extraction utilizes microwave energy to assist partition of analytes from the sample matrix into the solvent. Microwave radiation interacts with dipoles of polar and polarizable materials (e.g. solvents and sample) causes heating near the surface of the materials and heat is transferred by conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix (Kaufmann et al., 2002). In non-polar solvents, poor heating occurs as the energy is transferred by dielectric absorption only (Mandal *et al., 2015).* Microwave Assisted Extraction can be considered as selective methods that favour polar molecules and solvents with high dielectric constant. Hence, Microwave Assisted Extraction could not be performed on the petroleum ether as it is a non-polar solvent.

10 gm of powdered leaf was also extracted in methanol(iii) by microwave assisted extraction.

Percentage Yields from (i), (ii) and (iii) were also compared.

IV. Phytochemical screening of *Ficus religiosa* Linn. leaves (Mandal *et al*., 2015):

The two extracts obtained, were further subjected to Phytochemical screening

- **Test for Carbohydrates**

Molisch test: All carbohydrates give a purple color when treated with alpha-napthol and conc. Sulphuric acid. With a soluble carbohydrate, this appears as a ring if the sulphuric acid is gently poured in to form a layer below the aqueous solution. However, in case of an insoluble carbohydrate(cellulose), the color will develop only on shaking.

- **Test for steroid**

A few mg of the substance is dissolved in chloroform and acetic anhydride is added followed by sulphuric acid. Green colour indicates the presence of steroids.

- **Test for Reducing sugar**

Fehling's test: To the heated solution of the substance, slowly add a mixture of equal parts of Fehling's solution No. 1 and No. 2. In certain cases, reduction takes place near the boilingpoint and isshown by a brick red precipitate of cuprous oxide. Reducing sugars include all monosaccharides, and some diasaccharides (lactose, maltose, celloboise and gentiobiose). Nonreducing sugars include some diasaccharides (sucrose) and polysaccharides.

Benedict's test: Equal volume of Benedict's reagent and test solution was mixed in a test tube. It was heated in a boiling water bath for 5 mins.

- **Test for Monosaccarides**

Barfoed's test: When sugars are heated with Barfoed's reagent for 12 min, a red color is produced..

- **Test for Proteins**

Millon's Test:To the aqueous extract, add afew drops of Millon's reagent. A white precipitate formed indicates the presence of proteins.

Biuret test: To an aliquot of the aqueous extract, add a few drops of % copper sulfate solution. To tis, add 1 mL ethanol (95%), followed by excess potassium hydroxide solution. A pink color in ethanolic layer indicates the presence of proteins.

- **Test for Steroids**

Liebermann- Burchard Test : A few mg of the substance is dissolved in chloroform and acetic anhydride is added followed by sulphuric acid. Green colour indicates the presence of steroids and pink colour terpenoids.

- **Test for Triterpenoids**

Salkowski's test: 2 mL of the extract, 2 mL of chloroform and 2 mL of conc. H_2SO_4 was added and it was shaken well. The colour was observed.

- **Test for Alkaliods**

The powdered drug is mixed thoroughly with 1 mL of 10% ammonia solution or 10% sodium carbonate solution, and then extracted by shaking for about 5 min with 5 mL methanol at 60°C. the filtrate is cooled and then diluted enough for high-performance thin layer chromatography sample application.

Dragendorff's test: To a few milliliters of the extract, add 2mL of Dragendroff's reagent (potassium bismuth iodide). A reddish brown precipitate confirms the test as positive.

Mayer's test: To a few milliliters of the extract , add a few drops of Mayer's reagent (potasiomercuric iodide). A cream colored precipitate indicates a positive test.

Hager's test: Toa few milliliters of the extract, add a few drops of Hager's reagent (saturated solution of picric acid). A yellow precipitate indicates a positive test.

Wagner's test: To a few milliliters of the extract,add a few drops of Wagner's reagent (solution of iodine in potassium iodide). A reddish brown precipitate indicates a positive test.

- **Test for glycosides**

Legal's test: To 2 mL of extract, add sodium nitroprusside. A pink color indicates the presence of glycosides.

- **Test for Tannins and Phenolic compounds**

Ferric Chloride test: To the aqueous extract solution, add a few drops of neutral 5% ferric chloride. A dark green color indicates the presence of phenolic compounds.

Lead Acetate test: The aqueous extract is dissolved in distilled water, and to this is added 3 mL of 10% lead acetate solution. A bulky white precipitate indicates the presence of phenolic compounds.

- **Test for Flavonoid**

Shinoda Test: To the plant extract, add a mixture containing a piece of magnesium ribbon and concentrated hydrochloric acid. The formation of a red color indicates flavonoids, flavonones and xanthone.

To the test ferric solution, add ferric chloride solution. A change of color from green to black occurs in the presence of flavonoids.

V. Total Phenolic Content:

The total phenolic content of both extracts of *Ficus religiosa* leaf(petroleum ether and methanol) were determined by using Folin Ciocalteu. Gallic acid was used as reference standard for plotting calibration curve. A volume, each 1mL of plant extracts (1mg/mL) were individually mixed with 5mL of Folin ciocalteu reagent (diluted 1:10 with distilled water) and were neutralized with 4 mL of sodium carbonate solution (7.5% w/v) in separate test tubes. The reaction mixture shaken vigorously and incubated for 1 hour. 200µl was pipetted from each test tube into a 96 well plate and absorbance was observed in UV-VIS spectrophotometer (Spectramax) at 765 Nm. The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

The UV-Spectrophotometer used: **SpectraMax M5** manufactured by Molecular Devices.

VI. Total Flavonoid Content:

The Total Flavonoid Content of the petroleum ether and the methanol extract were determined by Aluminum Chloride colorimetric method. Quercetin was used as reference standard for plotting calibration curve. Some minor modifications were made to the standard procedure. In brief, 50 µL of crude extracts (1 mg/mL petroleum ether and methanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution. 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand

for 15 min, and absorbance was measured at 415 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight. The UV-Spectrophotometer used: **SpectraMax M5** manufactured by Molecular Devices.

VII. Anti-oxidant assay by ABTS method:

The ABTS assay is a widely used method for the assessment of antioxidant capacities of natural products. It is a spectrophotometric technique based on quenching of stable colored radicals (ABTS) and show the radical scavenging ability of antioxidants even when present in complex biological mixtures such as plants. The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with a Trolox (water soluble Vitamin E analogue) standard. The ABTS is generated by reacting with a strong oxidizing agent (eg, potassium permanganate or potassium persulfate) with the ABTS salt. Some modifications were made to the standard procedure. $ABTS^{\cdot+}$ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. $ABTS^{\cdot+}$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm. After the addition of 5 µl of plant extracts (petroleum ether and methanol extracts) to 3.995 mL of diluted $ABTS^{\cdot+}$ solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent inhibition of absorbance at 734 nm was calculated using the formula, $ABTS^{\cdot+}$ scavenging effect (%) = $\{(A_B - A_A)/A_B\} \times 100$ (2), where, A_B is absorbance of ABTS radical + methanol; AA is absorbance of ABTS radical + sample extract/standard. Trolox was used as standard substance.

The UV-Spectrophotometer used: **SpectraMax M5** manufactured by Molecular Devices.

VIII. Thin Layer Chromatography:

TLC is a planar chromatography, routinely used in the field of phytochemicals to identify the components in a compound mixture. The identification of phytocompounds by thin layer chromatography (TLC) is the basic technique in several pharmacopoeias. TLC is performed under standard conditions, and the spot of the substance under study , with or without derivation, is compared with that of a similarly developed reference material applied at approximately the same concentration (Mandal *et al*., 2015). Silica gel-G (gypsum) was mixed with water to make thick slurry paste which was then spread on a glass plate to make TLC plates. The resultant plate is dried and activated by heating in an oven for thirty minutes at 120°C. The thickness of the analytical TLC plate adsorbent layer is typically around 100-250µm. Mobile phase optimized was benzene : chloroform (7:3). The mobile phase was poured into separate TLC chambers to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity. Now, the plates prepared with sample spotting is placed in the TLC chambers so that the side of the plates with the sample line is facing the mobile phase. Then the chamber is closed with a lid. The plates are then immersed, such that the sample spots are well above the level of mobile phase for development. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber.

IX. Column Chromatgraphy of the Petroleum Ether extract:

The main principle involved in column chromatography is adsorption of the solutes of a solution through a stationary phase and separates the mixture into individual components. This is based on the affinity towards the mobile phase and stationary phase. Two methods are generally used to prepare a column: the dry method and the wet method. For the dry method, the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry. (Shusterman *et al.*,1997). For the wet method, a slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Eluent is slowly passed through the column to advance the organic material. The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. At the end of the column, they elute one at a time. During the entire chromatography process the eluent is collected in a series of fractions. The column plugged with a cotton at the bottom, was packed by wet method using Alumina as stationary phase and petroleum ether as mobile phase. The mobile phase constantly flushed until the stationary phase level settled. Then the extract is added from the top of the column in such a way that the top level of the stationary phase is not disturbed. By turning on the tap below it is allowed to be adsorbed on the surface of the alumina. The solvent is repeatedly added as many times as needed throughout the process. When the tap is on the compounds in the compound mixture move along with the eluent depending on the polarity of the sample molecule.

X. HPLC of the fractions against Ascorbic Acid as standard:

The fractions obtained, were further dried and redissolved in Acetone. They were stored in refrigerator until sedimentation of the waxy materials occurred. Then the solution was filtered. This process was repeated 3 times until there were no further sedimentation observed under storing.

Unwanted pigments such as chlorophylls and flavonoids are also sometimes present at high concentration and depend on the plant part under use. Although they are not easily removed but use of activated charcoal or activated carbon to decolorize the solutions by a selective adsorption phenomenon has been beneficial. The solution may be percolated through a relatively short charcoal column, or the powder can be mixed with the liquid to be decolorized, left to stand for a period of time, and filtered. (Mandal *et al*., 2015)

10 gm Activated charcoal was added to each solution and boiled for 10 minutes. This process was done to remove the pigments and make the solutions colorless. The solution was then repeatedly filtered with a Wattman filter until there was no trace of charcoal on the filter. The solutions were then concentrated and taken for HPLC.

High Performance Liquid Chromatography (HPLC) is a chromatographic technique used to identify quantify, separate and purify individual compounds present in a mixture. In HPLC the sample mixture is passed along with a liquid solvent under high pressure through a column filled with a solid adsorbent material. The pressure within the system is built up with the help of pumps. The working principle is that each compound in the mixture interacts slightly different the adsorbent material in the column, resulting in varying flow rates for different compounds. This leads to separation of the components as they flow out of the

column. The adsorbent material typically used is typically granular, made up of solid particles such as silica, constituting the stationary phase. The pressurized liquid is the mobile phase. The detector is attached to a digital microprocessor and user software for data acquisition and analysis. The separated compounds are visualized as peaks with the number of peaks corresponding to the number of separated components in the mixture. The area of the peak is proportional to the concentration of the compound present within the mixture.

Ascorbic acid 0.1mg/mL was taken as standard. Mobile phase taken was methanol : water in the ratio 15:85 and made alkaline upto a range of 3.0-3.5 by adding hexane sulphonic acid. C-8 RP column used. UV-visible detector was set at 280nm. 100µg/mL of std was injected with a Hamilton syringe and the Retention time (RT) and the AUC was observed. The same process was repeated for the fraction samples and the RT was compared for the presence of Ascorbic acid. The HPLC instrument used : **EMPOWER 2 BINARY SYSTEM** manufactured by **WATERS (USA)**.

Results and Discussion

Leaf characteristics of *Ficus religiosa*

The leaves of *Ficus religiosa* tree are rubbery; heart shaped, long tipped, extended slim petioles and purple fruits rising in pairs. Leaves also contain campesterol, stigmasterol, isofucosterol, α-amyrin, lupeol, tannic acid, arginine, serine, aspartic acid, isoleucine, leucine, n-nonacosane, n-hentricontanen, hexa-cosanol and n-octacosan. (Wlliamson and Hooper, 2002), (Behari et al., 1984), (Verma and Bhatia, 1986)

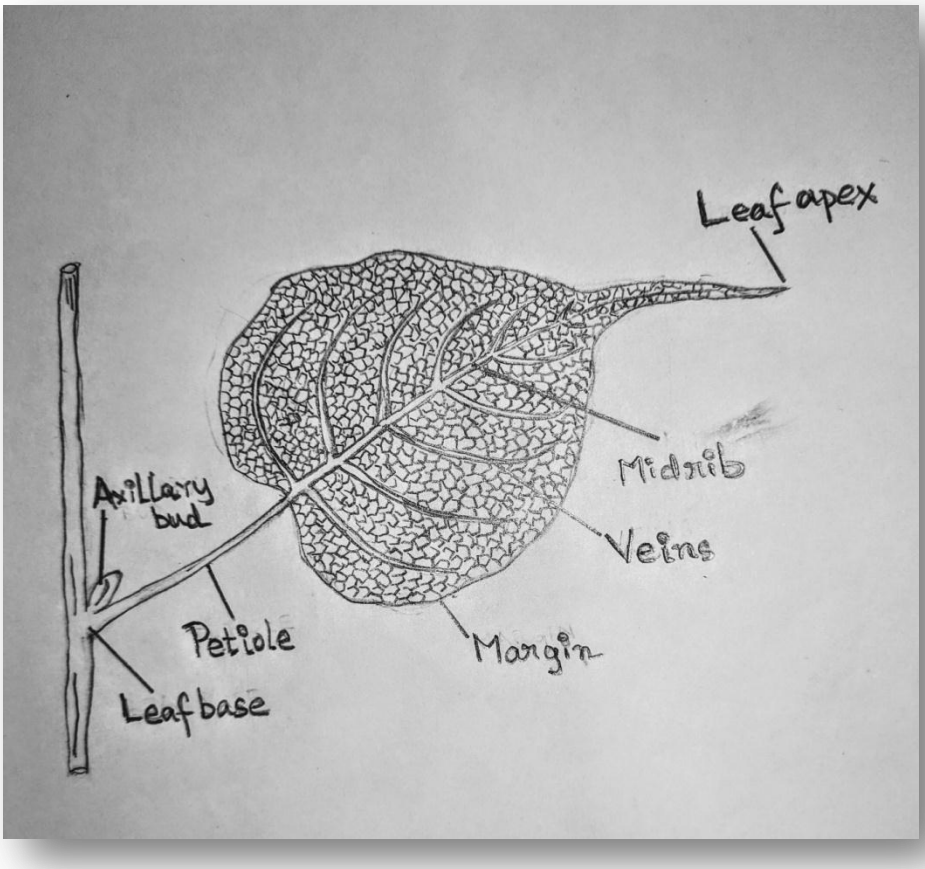


Figure 3. Leaf of *Ficus religiosa* showing various parts of the leaf

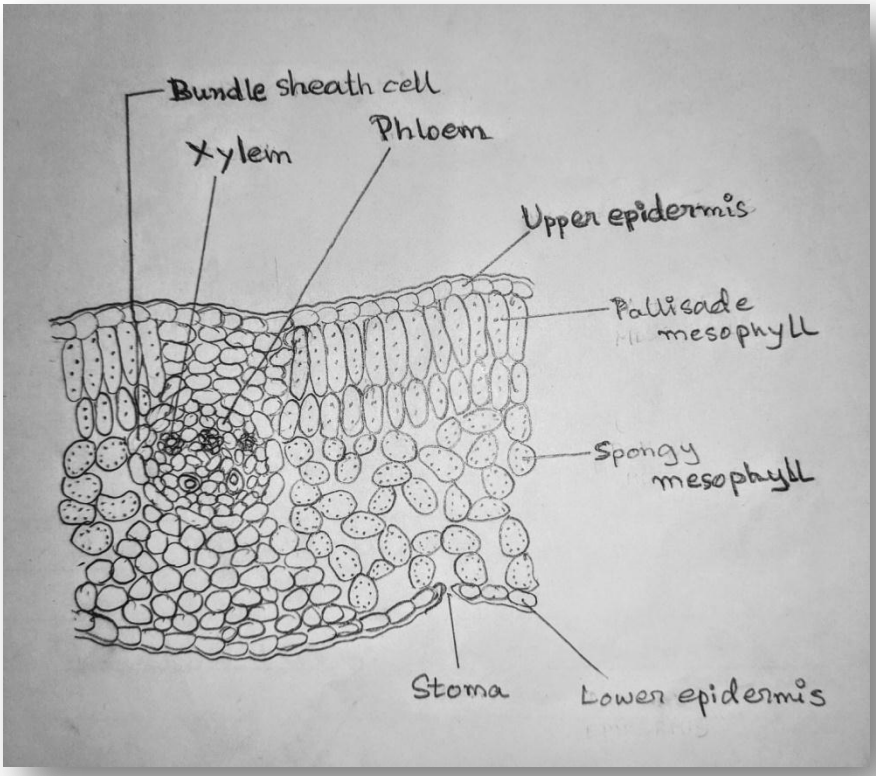


Figure 4. Transverse-section of leaf of *Ficus religiosa* under microscopy

Percentage yield

Hot percolation method	
Extract	Percentage yield
Methanol	4.3 %
Petroleum ether (60-80)	2.5 %
Microwave extraction method	

Methanol	6.3 %

Table 2.

Microwave Assisted extraction (6.3%) was observed to yield a higher percentage of methanol extract than by Soxhlet extraction (4.3%).

Phytochemical Screening

Sl. No.	Name of Test	Petroleum ether	Methanol
Test for Carcohydrates			
	Molisch's Test	-	-
	Fehling'sTest	-	-
	Benedict's Test	-	-
	Barfoed'sTest	-	-
Test for proteins and aminoacids			
	Biuret Test	-	-
	Millon's Test	-	-
Test for triterpenoids			
	Salkowski test	+	+
Test for alkaloids			
	Dragendroff's test	+	+
	Mayer's test	+	+
	Hager's test	+	+
	Wagner's test	+	+
Test for glycoside			

	Legal's test	-	-
Test for flavonoids			
	Shinoda test	+	+
	$FeCl_3$ test	+	+
Test for tannins and phenolic compounds			
	$FeCl_3$ test	+	+
	Lead Acetate test	+	+
Test for steroids			
	Libermann Burchard test	-	-

Table 3.

"+" denotes present**,** **"–"** denotes absent

Both the petroleum ether and methanol extract were found to contain triterpenoids, alkaloids, flavonoids and phenols.

Carbohydrates, proteins, glycosides and steroids were absent in both the extracts.

Total Phenolic Content:

Table 4. Total Phenolic Content of petroleum ether and methanol extracts of leaves of *Ficus religiosa*

Sample Extract	Total Phenolic Content (mg GAE/gm extract)

Petroleum ether(60-80)◦C	2.84
Methanol	5.48

Total Phenolic Content is used to estimate the amount of phenol present in a sample. These phenolic compounds which are present in the plant possess redox properties, which make these capable of antioxidant activity (Shoib and Sahid., 2015; Soobrattee *et al*., 2005). Antioxidant property of many phenolics play a crucial role in managing oxidative balance of the body by protecting against oxidants, oxidative reactions and reactive species (Kala *et al*., 2016). The methanol extract of the leaves of Ficus religiosa exhibited a higher TPC than the petroleum ether extract of the plant leaves (Table 4).

Total Flavonoid Content:

Table 5. Total Flavonoid Content of petroleum ether and methanol extracts of leaves of *Ficus religiosa*

Sample Extract	Total Flavonoid Content (mg Quercetin/gm extract)
Petroleum ether(60-80)◦C	9.3

Methanol	26.7

Flavonoids, comprising of flavones, flavonols and condensed tannins, are plant secondary metabolites, whose antioxidant activity is dependant on the presence of free 3-OH groups. Plant flavonoids have exhibited antioxidant activity in both *in-vitro* and *in-vivo* (Geetha *et al*., 2003; Shimoi *et al*., 1996). Flavonoids are the most diverse and widespread group of natural compounds probably the most important natural phenols. All of these compounds constitute a broad spectrum of chemical and biological activities including radical scavenging and antimicrobial activities. The methanol extract of the leaves of *Ficus religiosa* exhibited a higher TFC than the petroleum ether extract of the plant leaves (Table 5).

Antioxidant Activity:

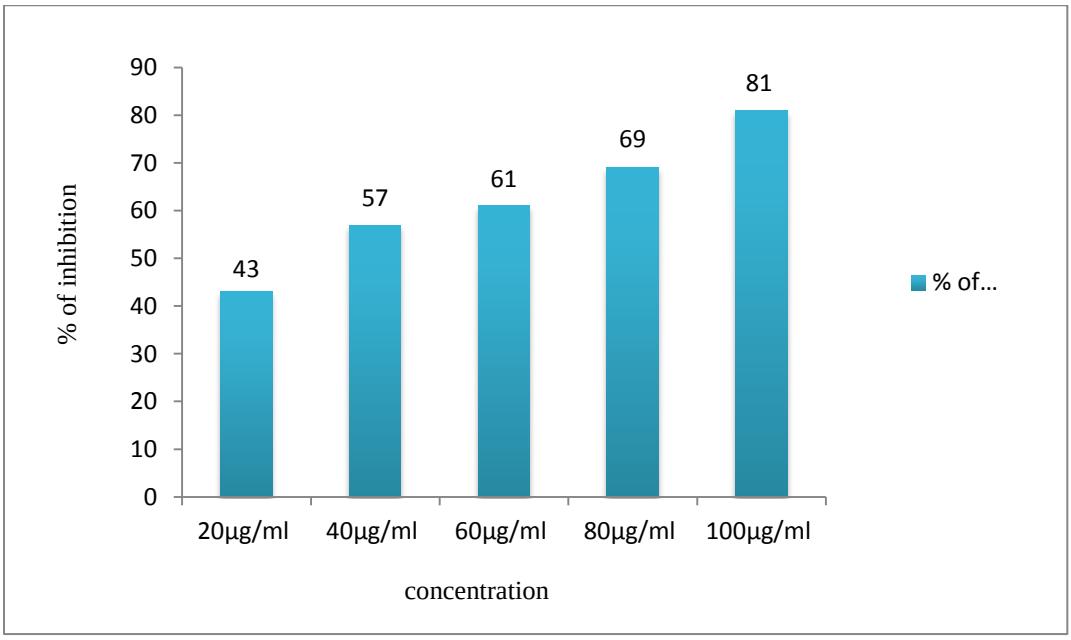


Table 6. Screening results of ABTS radical scavenging activity of petroleum ether extract

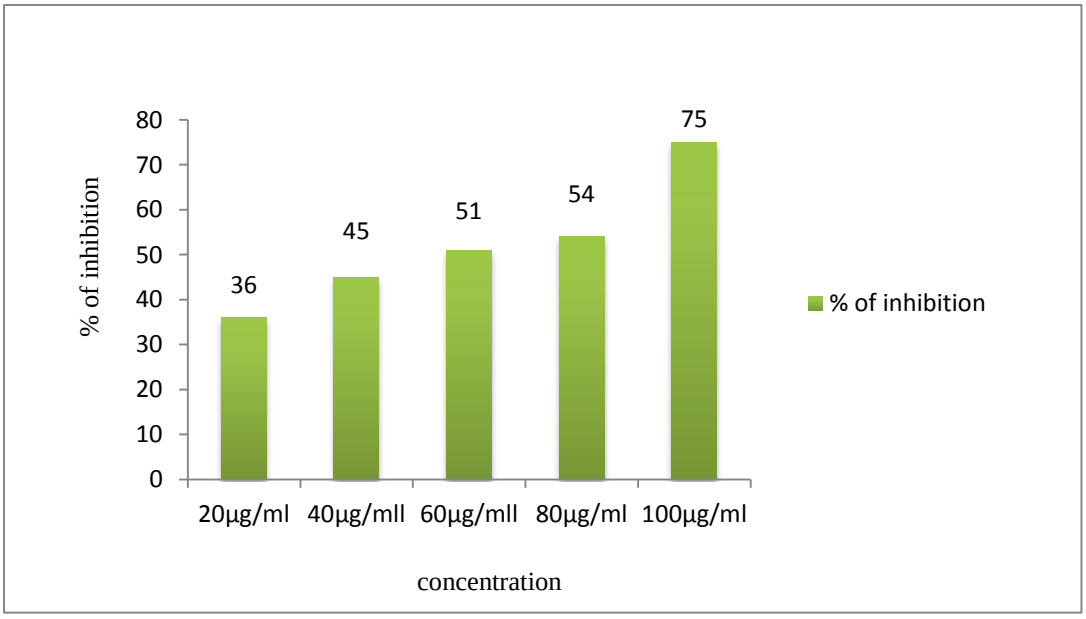


Table 7. Screening results ABTS radical scavenging activity of methanol extract

For both the extracts different concentrations were taken, viz. 20, 40, 60, 80 and 100 µg/mL.

For petroleum ether extract, maximum percent of inhibition (81%) was observed in 100 µg/mL and the minimum percent of inhibition (43%) was observed in the 20 µg/mL concentration. 40, 60, 80 µg/mL had a percent of inhibition of 57%, 61% and 69% respectively.

For methanol extract, maximum percent of inhibition (75%) was observed in 100µg/mL and the minimum percent of inhibition(36%) was observed in 20 µg/mL concentration. 40, 60, 80, µg/mL had a percent of inhibition of 45%, 51% and 54% respectively.

Thin Layer Chromatography

Table 8. TLC analysis of Petroleum ether extract and methanol extract of *Ficus religiosa* leaves.

Extract	Number of spots	Rf value
Petroleum ether(60-80)°C	2	0.068, 0.187
Methanol	3	0.045, 0.092, 0.40

Thin layer chromatography is a very useful process to analyze the progress of a reaction, and for identifying compounds present in a given test drug and for ensuring the purity of a substance. Thin layer chromatography of different extracts of leaves of the plant was carried out using silica gel GF-254 as adsorbent and benzene: chloroform in the ratio of 7:3 as a mobile phase. For the petroleum ether extract, 2 yellow spots were observed under UV light

having Rf values 0.068 and 0.187. For methanol extract, 3 yellow spots were observed under UV light having Rf values 0.045, 0.092 and 0.40.

Column chromatography

The petroleum ether was only taken for further separation by column chromatography. After preparing the column the petroleum ether dry extract resoluted with a few milliliters of petroleum ether and made concentrate. It was then poured into the column followed by petroleum ether as mobile phase. Two prominent layers were observed to form, ie orange and green. The orange layer was eluted with petroleum ether and collected in a beaker. Soon the cotton at the bottom of the column turned white indicating that the remaining analyte has no furher affinity towards petroleum ether. Hence, we select benzene, with a polarity index of slightly higher, as mobile phase. The analyte is further eluted with benzene and collected in a beaker.

Figure 5. Column chromatography showing formation of 2 separate bands.

HPLC of the fractions to identify the presence of Ascorbic Acid

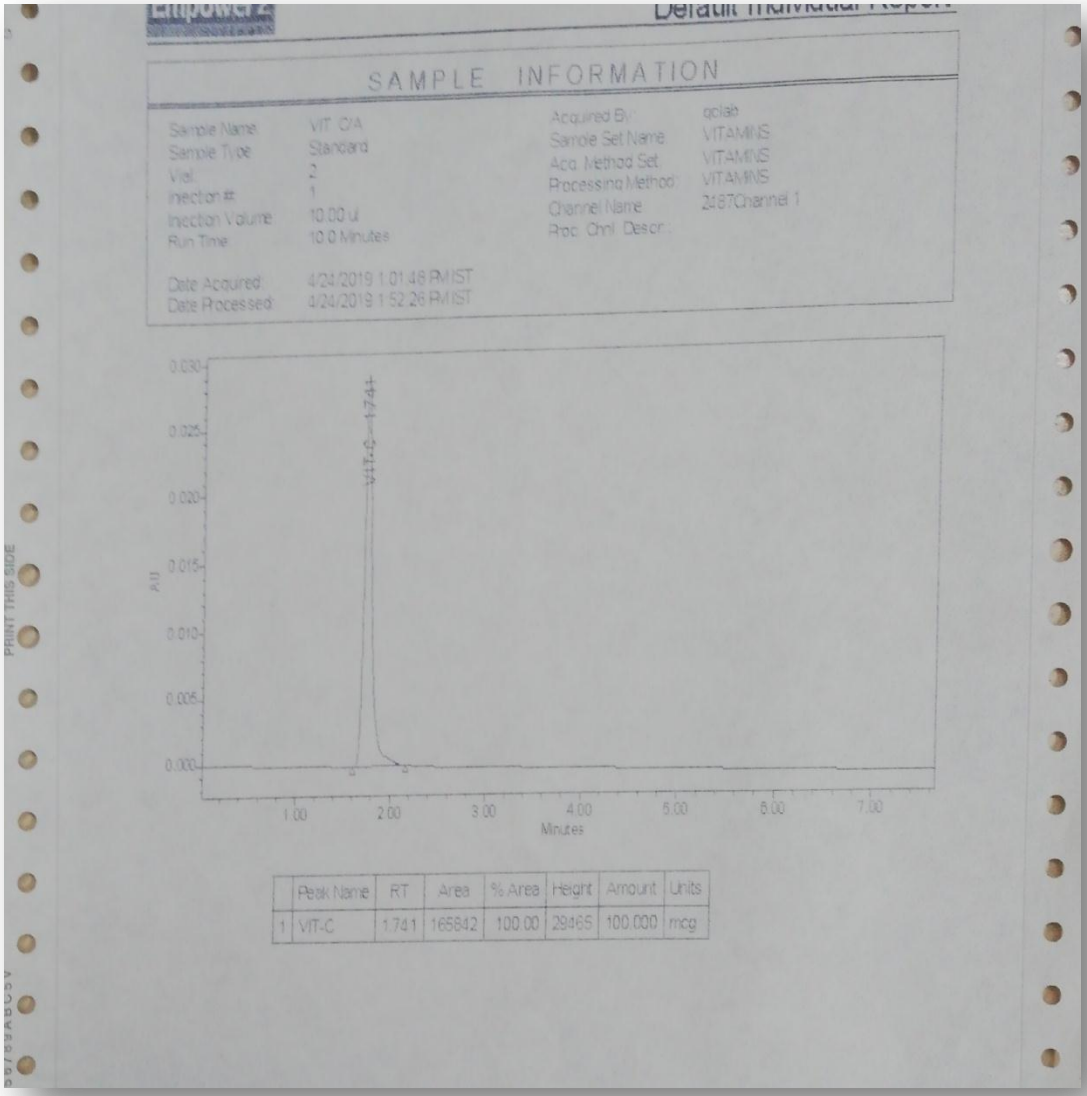
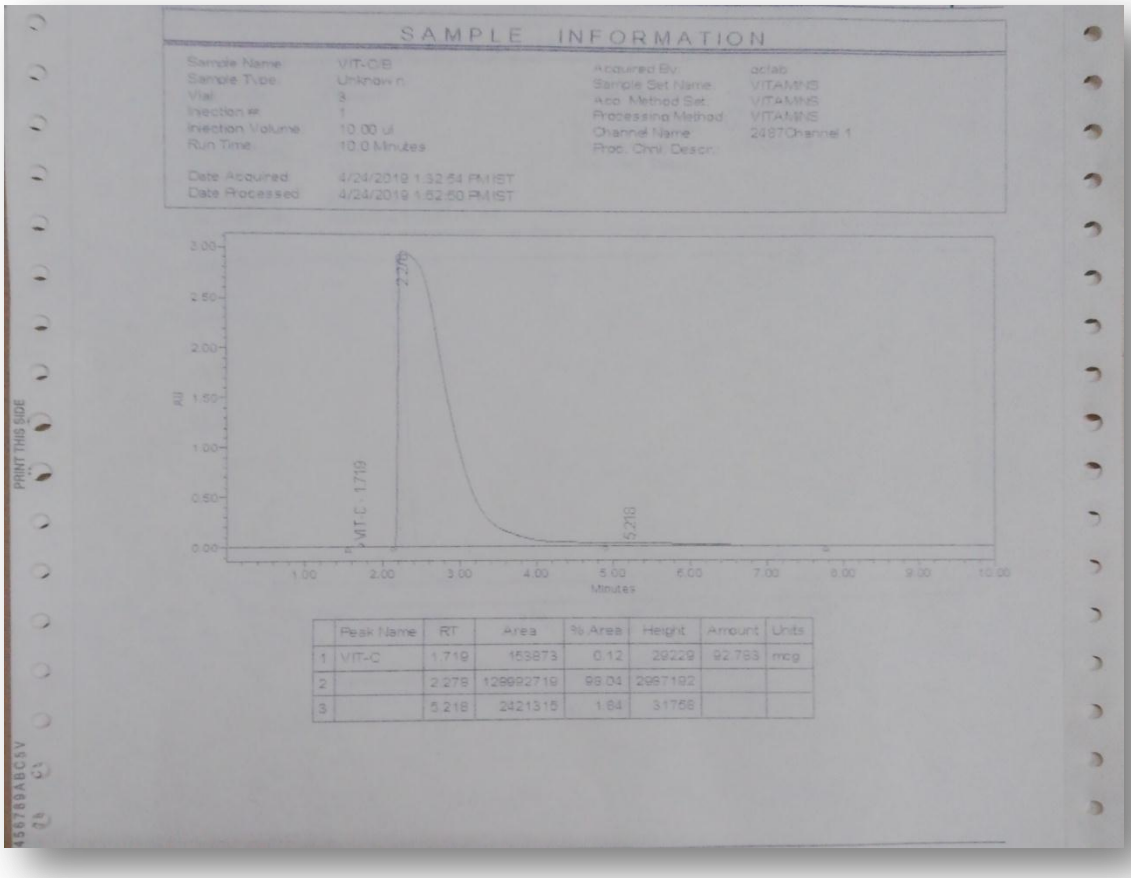
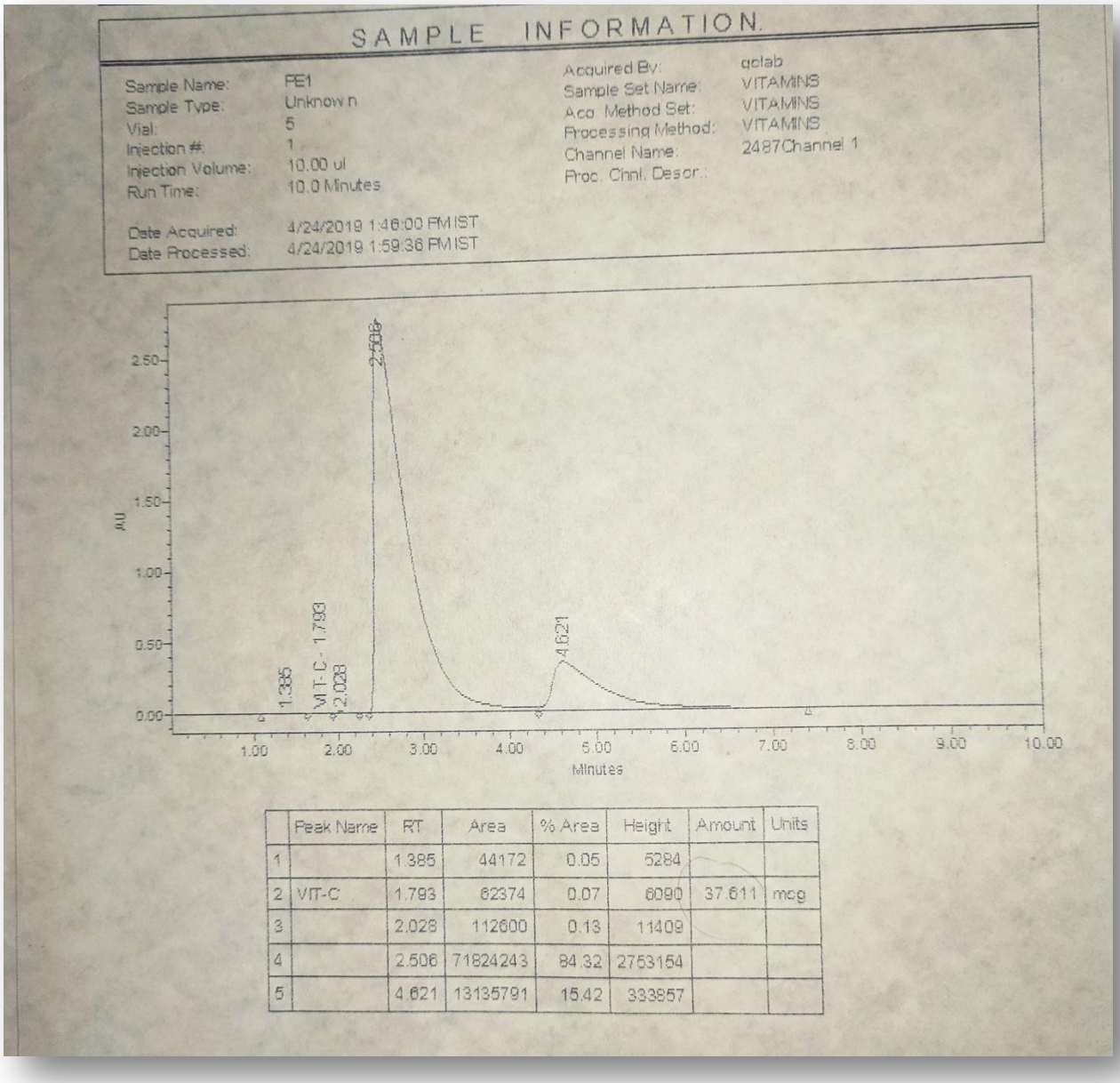

SAMPLE INFORMATION
Sample Name: VIT. C/A
Sample Type: Standard
Vial: 2
Injection #: 1
Injection Volume: 10.00 ul
Run Time: 10.0 Minutes
Acquired By: qclab
Sample Set Name: VITAMINS
Acq. Method Set: VITAMINS
Processing Method: VITAMINS
Channel Name: 2487Channel 1
Proc. Chnl. Descr.:
Date Acquired: 4/24/2019 1:01:48 PM IST
Date Processed: 4/24/2019 1:52:26 PM IST
VIT-C 1.741
AU
Minutes
Peak Name RT Area % Area Height Amount Units
1 VIT-C 1.741 165842 100.00 29465 100.000 mcg

Fig. 6.



	Peak Name	RT	Area	% Area	Height	Amount	Units
1	VIT-C	1.719	153873	0.12	28229	92.783	mcg
2		2.279	128992719	98.04	2987192		
3		5.218	2421315	1.84	31756		

Fig. 7.



	Peak Name	RT	Area	% Area	Height	Amount	Units
1		1.385	44172	0.05	5284		
2	VIT-C	1.793	62374	0.07	6090	37.611	mcg
3		2.028	112600	0.13	11409		
4		2.506	71824243	84.32	2753154		
5		4.621	13135791	15.42	333957		

Fig. 8.

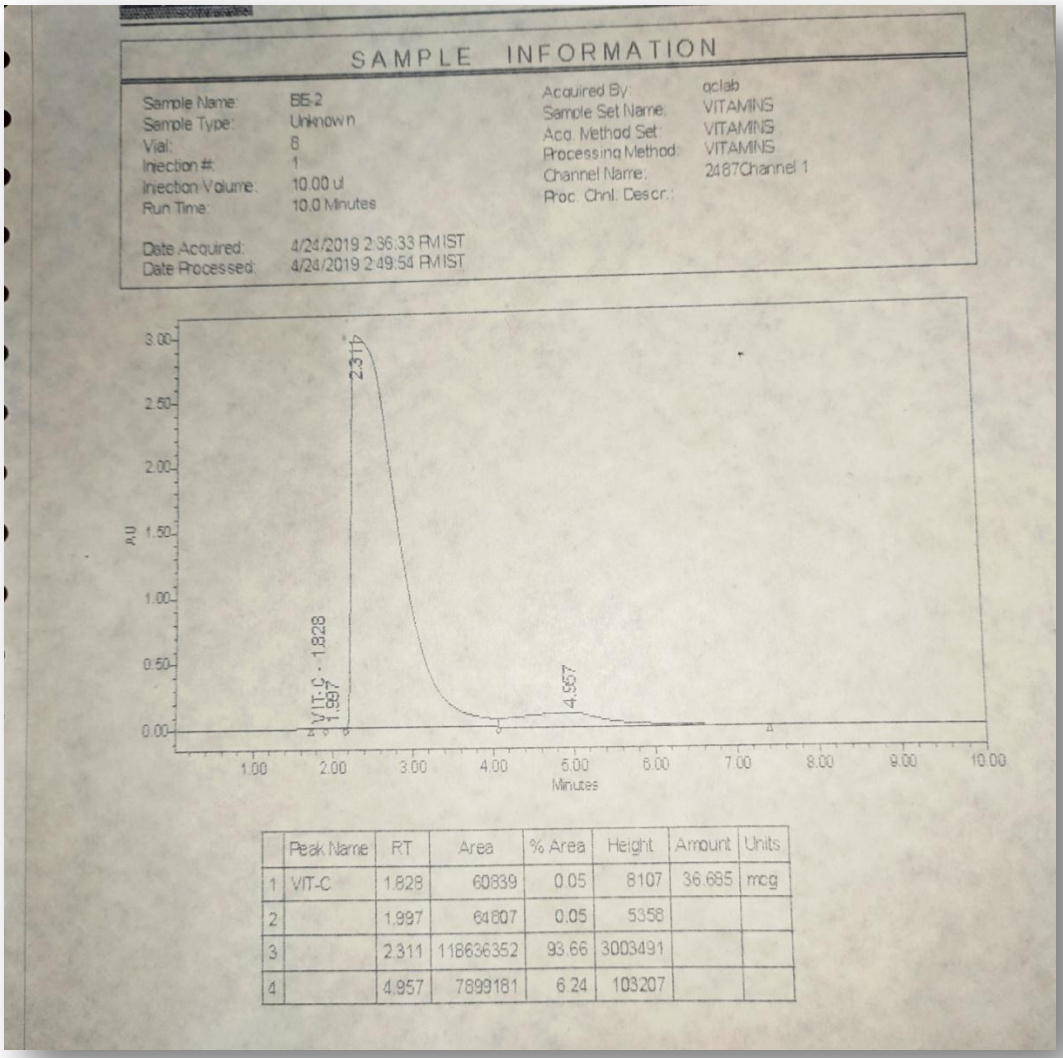


Fig 9.

Retention Time (RT) is a measure of the time taken for a solute to pass through a chromatography column. It is calculated as the time from injection to detection.

Fig 6. Represents the RT of a single peak at 1.74 exhibited presence of Ascorbic acid, sample name denoted by VIT C/A . We have two fractions , one in eluted by petroleum ether, dried,

resolute in acetone and depigmented by boiling in activated charcoal and filtering and another eluted with benzene and subjected to same process. As the samples were taken in Acetone as solvent, we first had to check the solubility of Vitamin C in acetone which is sparingly soluble. Then, Vitamin C in acetone as solvent was run as standard to observe whether the peak of acetone overlaps the peak of Vitamin C. Fig 7 represents the a peak at 1.72 for Ascorbic acid and an peak at 2.27 for Acetone with sample name as VIT C/B. Next, we inject petroleum ether eluent sample, sample name denoted as PE1. In Fig 8. we observe 4 peaks, one of which at 1.79 denoting the presence of Ascorbic acid. After that we inject the benzene eluent sample, denoted by BE2 and observe a similar graph with VIT C producing a peak at 1.82, Fig 9. Hence, we can conclude there is a presence of Ascorbic Acid in petroleum ether eluent and benzene eluent of leaves of *Ficus religiosa* extract.

The amounts of Ascorbic acid found in VIT C/A, VIT C/B, PE1 and BE2 were 100µg/gm, 92.783µg/gm, 37.611µg/gm and 36.685µg/gm respectively.

Conclusion:

This assignment aims to present plant description, leaf characteristics, extraction with a polar and a non-polar solvent, phytochemical constituents, total phenolic content, total flavonoid content, thin layer chromatography, antioxidant activity, column chromatography and HPLC of leaves of *Ficus religiosa* (Moraceae). This plant possesses a variety of phytochemical constituents including flavonoids, phenols, tannins, alkaloids, triterpenoids. Antioxidant activity of petroleum ether and methanol extract of leaves of *Ficus religiosa* has been found by means of free radical scavenging assays. The petroleum ether and methanol extract both contain phenols and flavonoids which indicates the possibility of antioxidant activity, slightly higher in methanol extract than petroleum ether. Further study was conducted only with the petroleum ether extract, ie., Column chromatography and HPLC. In column chromatography fractions were obtained with petroleum ether and benzene respectively. The fractions were subjected to HPLC. Presence of Ascorbic acid was confirmed in both fractions, which suggests its antioxidant activity.

The results of the current study suggests that the specified plant can be screened for pharmacological activity for human ailments related to antioxidants. The results of this study exhibits that *Ficus religiosa* has the promising characteristics of a potential antioxidant and maybe effective in prevention of various diseases.

All the observed data were in accordance with prior researches on the *Ficus religiosa*. Hence, the findings of the presented work support the earlier claims of its pharmacological characteristics.

There is a demand for the standardization of phytochemicals and bioactivity-guided identification of bioactive metabolites. Phytochemical investigation performed on *Ficus religiosa* has resulted in the isolation of few groups of plant metabolites. Nevertheless, the vast traditional application and evident therapeutic benefits of *Ficus religiosa* suggests a vast opportunity for further pharmacognostic

investigation. The results of such phytochemical research may promote further expansion of its current therapeutic potential.

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