Investigation of Antioxidant & Cytotoxic Properties of *Callicarpa attenuata*

A project submitted

by

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This work is dedicated to my parents and my sister to whom I owe my achievements.

Certification statement

This is to certify that, this project titled ‘Investigation of Antioxidant & Cytotoxic Properties of *Callicarpa attenuata*’ submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Shejuti Rahman Brishty, Lecturer, Department of Pharmacy, BRAC University and this project is the result of the author’s original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the project paper itself.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

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Abstract

Present study was performed to find out important phytochemical constituents, and establish the scientific basis of the use of the leaves of *Callicarpa attenuata* as therapeutic agent in traditional medicine in Bangladesh. The leaves of the plant were extracted by using methanol and the crude methanolic extract was subjected to phytochemical screening and *in vitro* biological investigation. The study confirmed mild total phenolic content and DPPH free radical scavenging activity that in turn confirmed mild antioxidant potential of the plant under experiment, and moderate cytotoxicity in brine shrimp lethality bioassay. Nevertheless, further investigation is needed to develop new drugs, especially anti-microbial agents, to treat a wide variety of diseases.

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List of Abbreviations

DPPH - 1, 1-diphenyl-2-picrylhydrazyl

ASA - Ascorbic Acid

DMSO - Dimethylsulphoxide

GAE - Gallic Acid Extract

VS - VincristineSulphate

TPC - Total Phenol Content

WHO - World Health Organization

ME - Methanolic extract

Chapter 1: Introduction

1.1 Phytopharmacology and Phytotherapy

Humans and plants have a much intertwined relationship as they have shared very close quarters throughout history. Due to the nature of this relationship, there are many properties of plants that have been observed and utilized by humans. The utilization of plant properties has therefore constituted a very important branch of pharmacy known as Phytochemistry.

Present day medication is different from that used before when phytochemistry was still a relatively new concept. Technological advancements have given way to various preferences in what can be defined as safe to use as a medication. Modern medicine has seen fewer uses of direct plant extracts and primitive experimental techniques. The ease of use and time efficiency of the modern techniques causes it to be more preferable. Other advantages include steady arrangements of the procedures, use of various unified measurements and ease of reproducibility of the methods. Even through the modern methods are very convenient, all products cannot be synthesized artificially without a starting material, hence the continuing importance of phytochemistry has to be considered and cannot be negated. Teas, herbal teas, and tinctures have been undermined by chemical pharmacology, however it is necessary to use them in daily small-scale production in laboratories. There are many examples of use of modern medicine as a better option, e.g. in hypertension. It would be foolish to depend on mistletoe and garlic instead of using synthetic hypotensive drugs that have recently revolutionized the therapy of this disorder. However, herbal medicine could be a favorable option for the excessive and dangerous use of drugs, detectable especially in more developed societies. The purpose of Phytotherapy is to accept and nurture a new approach to preexisting recovery of nature and its resources, among which medicinal plants are an important part.

New drugs were discovered by traditional, experimentation and molecular methods. The usual methodology makes the utilizing of medication that are found by experimentation over the years by trying out various types of medicine.

Plant derived drugs are widely available in tropical countries. Bangladesh is a nation with a wide variety of species which can be proven to be useful for various ailments. It has a variety of climatic conditions each with its own set of organisms with local uses observed by the locals living there. The variety allows better chances of finding compounds that give positive results in curing diseases. The herbs that grow in these native areas possess high restorative and helpful qualities.

1.2 History of Plants in remedy

In the primordial time, searching for a cure of disease made the people look for drugs in the environment. This led to the discovery of new kinds of medicine from nature. During that period, there was not adequate evidence regarding the reason for the diseases or relating to which herb and how it might be applied as a medicine (*Stojanoski, 1999*). All of it was grounded on practice. Through time, the causes for the utilization of exact curative plants for cure of specific diseases were revealed; and so, usage of plants of medicine progressively uninhibited empiric outline. Plants of medicine have become established with the help of illustrative proofs. Nonetheless, the decreasing ability of artificial medication and the growing contraindications of their use create the practice of nature derived medications prominent (*Kelly, 2009*). The earliest written proof of the use of therapeutic herbs for the research of drugs have been recorded on the Sumerian slab originated in Nagpur which is about five thousand years old. For medicine preparation, 12 formulations were contained into it, which alluded to more than two hundred-fifty different plants, few of these were alkaloids, for example, poppy, mandrake and henbane.

"Pen T'Sao," which is a Chinese book, was written by Shen Nung around 2500 BC, contains 365 medications (therapeutic plant’s dried segments), large portions of these are still utilized these days. For example, great yellow gentian, Rhei rhizoma, camphor, podophyllum, folium, ephedra, cinnamon bark, jimson weed and ginseng (*Wiard, 2006*).

The holy book Vedas from India points at treating diseases using plant based medicine that are found everywhere in the country. Many seasoning plants which are utilized, still now originated from India such as, nutmeg, clove, pepper and so on (*Bottcher, 1965*).

The Ebers Papyrus represented a collection of 800 prescriptions in 1550 BC, which talk about 700 plant species and medicines used for treatment (*Katic, 1980*). For example, aloe, senna, garlic, onion, pomegranate, castor oil plant fig, willow, coriander, common centaury, Juniper etc.

As indicated by information mentioned in the Bible & the heavenly Jewish book the Talmud, during different ceremonies going with a treatment, aromatic plants were used, e.g. Myrtle & incense. The Arabs presented numerous new plants in pharmacotherapy. However, for the most part from the Indian nation used to have trade relations where most of the plants with a genuine therapeutic value that has held on in all pharmacopeias in the world. They are still perceived even now. The Arabs utilized deadly nightshade, coffee, henbane, ginger, strychnine, saffron, cinnamon, rheum, curcuma, pepper senna etc. A few medications with solid activity were supplanted by drugs with mild activity, for example, Sennae was applied as a slight diuretic preparation, contrasted with the laxatives Heleborus odorus utilized until at that point.

In the Middle Ages, European doctors turned to the Arab works “De Re Medica” by John Mesue (850 AD), “Canon Medicinae” by Avicenna (980-1037), and “Liber Magnae Collectionis Simplicum Alimentorum Et Medicamentorum” by Ibn Baitar (1197-1248), where more than 1000 medicinal plants were described (Biljana, 2012).

In Macedonia, St Clement’s work were of exceptional noteworthiness. They alluded to the Nikeian pharmacological references starting from year 850 and transferred his immense learning on curative plants to his pupils & from them to the general individuals.

Mid nineteenth century was recorded to be the defining moment of learning, advancement and utilizing medicinal plants. The discovery, substantiation, and isolation of alkaloids from poppy in 1806, ipecacuanha in 1817, strychnos in 1817, quinine in 1820, pomegranate in 1878, and various other plants. At that point the isolation of glycosides denoted the start of logical drug store. With the progressions and redesigns of the chemical approaches, rest of the active substances from curative plants were found. For example, saponosides, etheric oils, hormones, tannins, vitamins, and so forth.

In late nineteenth and mid twentieth century, there was an incredible risk of termination of therapeutic plants from treatment. Many authors composed that medications gotten from them had numerous downsides because of the dangerous activity of enzymes that cause crucial variations amid the procedure of therapeutic plants drying, i.e. medicinal plants' remedial activity relies upon the method of drying. In the nineteenth century, glycosides, therapeutics and alkaloids isolated in pure form were progressively supplanting the medications from what they have been secluded. In any case, soon it was affirmed that in spite of the fact that the activity of unadulterated alkaloids was snappier, the activity of alkaloid drugs was full and durable. In mid twentieth century, adjustment techniques for new therapeutic plants were proposed, particularly which are with labile medicinal components. Additionally, broad exertion was given in the investigation of manufacture and development of therapeutic plants.

In terms of physiological, chemical, and clinical examinations, various overlooked plants and medications were reestablished to the drug store: Punica granatum, Aconitum, Hyoscyamus, Stramonium, Secale cornutum, Filix mas, Ricinus, Opium Styrax, Colchicum et cetera. The active segments of therapeutic plants were found to be the result of the characteristic, most consistent research center. People endorse the medication acquired from these plants were best in perspective of the way that humans are necessary element of nature. Many cases like this type are there; maybe they will lead genuine research into the old compositions on therapeutic plants, which would not be seen to straighten something up about history but rather as potential wellsprings of contemporary pharmacotherapy.

Right now, all pharmacopeias on the planet: Ph. Eur 6, BP 2007, USP XXXI recommend plant medications of genuine restorative criticalness. There are nations (the, Russia, United Kingdom, Germany) that have isolated herbal pharmacopeias. However, in all actuality, a significantly higher number of informal medications have dependably been utilized. Their application depends on the encounters of famous prescription (customary or well-known solution) or through the new logical research and trial comes about. Many remedial plants are connected through self-drug or at the proposal of a doctor or drug specialist. They are utilized independently or in mix with artificial medications (corresponding drug). For sufficient and effective treatment, knowledge of the precise diagnosis of the sickness and in

addition of therapeutic plants, like the pharmacological impact of their parts is vital. Plant prescriptions and phyto preparations with characterized active parts, verified activity and, at times, remedial productivity, are connected as helpful means. In Germany, rational phytotherapy is utilized, in view of uses of arrangements whose proficiency relies upon the connected dosage and distinguished active components, and their effectiveness has been validated by clinical tests & trial. Those medicines have been made from identical plant extracts, and these meet every one of the necessities for pharmaceutical quality of medications.

1.3 Significance of medicinal plants in humanity’s ailments

The term "medicinal plant" incorporate different sorts of plants utilized as a part of herbalism. It means the utilization of those plants for therapeutic purposes, along with the investigation of such employments.

"Herb" has been derivated from the word of Latin origin, "*herba*" and a French word "*herbe*". Presently day, herb alludes to those pieces of the plant like natural product, seed, bark, stem, bloom, leaf, fruit disgrace or a root, and a woodless plant. In the early days, the expression "herb" was just connected to woodless plants, including those that originated from bushes and trees. These therapeutic plants have additionally been utilized as consumables, flavonoid, solution or fragrance and furthermore in some religious activities.

Utilization of plants to accomplish curative applications began some time before middle ages. Old Unani compositions Chinese and Egyptian works depicted the herbs utilization. Evidence proves that Unani Hakims, Indian Vaids, European, Indian and Mediterranean societies were utilizing herbs for more than four thousand years as prescription. Indigenous societies, for example, Rome, Egypt, Iran, Africa and America utilized herbs in their recuperating customs, while other created usual restorative backgrounds, for example, Unani, Ayurvedic and Medicine from China for treatment of common disorders utilized them efficiently.

Traditional systems of medication keep on being broadly honed on many records. Populace rise, insufficient supply of medications, the restrictive cost of medicines, reactions of a few manufactured medications and improvement of properties of the medications utilized now-a-days for infectious diseases have prompted expanded the utilization of plant derived substances as a wellspring of pharmaceuticals for a broad assortment of human illnesses.

Among ancient human advancements, India has been known to be a rich storehouse of therapeutic plants. The woodland present in India is the essential store of an extensive number of therapeutic and fragrant plants that are to a great extent gathered as crude materials for the fabrication of medications and perfumery items. Around 8,000 natural cures were classified in AYUSH frameworks in India. Unani, Ayurveda, Folk (tribal), and Siddha solutions are the major systems of indigenous prescriptions. Of all these types of medication, Unani & Ayurvedic Medicine are mostly created & generally practiced in Indian subcontinent.

Some time ago, WHO projected that eighty percent of people worldwide are dependent on plant derived medicines for some aspect of their primary health care needs. It further says that around 21,000 plant species have the potential to be used as medicinal plants.

As indicated by information accessible, more than seventy-five percent of the population of the world depends principally on plants and plant extricates for their medicinal service’s needs. Over thirty percent of the whole plant species, at one time or other, were utilized for therapeutic purposes. In developed countries evaluation has been carried out, like the United States, plant derived drugs make as much as 25% of the aggregate medications. While in fast developing nations, for example, India and China, the portion is as much as 80%. In this manner, the economic benefit of therapeutic plants is considerably more to nations, for example, India than to rest of the world. These nations convey two third of the plants utilized as a part of the present day system of medicine and the healthcare system of country populace rely upon homegrown medicinal system.

Treating diseases using medicinal plants has been considered safe since there are no or negligible side effects. These pharmaceuticals are in a state of harmony with nature, which is the greatest advantage. The notable fact is by utilization of home grown medicines is sovereign of all genders & age groups.

Early researchers used to realize that, herbs can only cure different health related difficulties and disorders. They showed an informative report which reached land at correct decisions about the practicality of diverse herbs that have pharmaceutical value. The medications formulated, are generally free of symptoms, allergic responses or side effects. This is the purpose of natural treatment. It is developing in acknowledgment over the world. These herbs having medicinal excellence give rational means to the treatment of numerous internal sicknesses, which are generally viewed as troublesome to cure.

Medicinal plants like *Tulsi*, *Aloe*, *Turmeric*, *Ginger* & *Neem* can curea lot of shared disorders. These are regarded as home medicines in numerous areas of the country. It is a fact that many of clients are utilizing Basil to make medicines, black tea, in religious activities such as pooja and in the everyday life.

In numerous areas of the earth numerous herbs are utilized to honor their royals showing it as an emblem of luck. Ever since the role of herbs as medicine has been found, many consumers started planting basil and various medicinal plants where their garden is.

Therapeutic plants can be reflected as valuable assets of constituents which may be utilized in the development of drug. It may either be pharmacopoeial, non pharmacopoeial or artificial drugs. Aside from that, these plants assume a basic part in the advancement of human standards around the entire globe. In addition, a few plants have been treated as a critical source of nourishment and because of that, they are proposed for their helpful esteems. Some of these plants incorporate green tea, turmeric, aloe, ginger, pepper, and walnuts and so forth. A few plants and the derivatives of these are considered as an imperative source for API which are utilized as a part of aspirin and toothpaste and so forth.

Aside from the therapeutic uses, herbs have been likewise utilized as a part of normal color, bother control, sustenance, fragrance, tea et cetera. In numerous nations, various types of therapeutic plants/herbs are utilized to keep ants, flies, mice and escape far from homes and workplaces. Presently a day’s therapeutic herbs are boss hotspots for pharmaceutical assembling.

Formulas to cure basic sicknesses, for example, hypertension, looseness of the bowels, obstruction, low sperm tally, diarrhea, feeble penile erection, heaps, covered tongue,

menstrual clutters, bronchial asthma, fever and leucorrhea are given by the conventional pharmaceutical doctors effectively.

Over the past two decades, there has been a momentous increment in the utilization of home grown medication; in any case, there is as yet a critical absence of research information in this field. Thusly, since 1999, WHO has distributed three volumes of the WHO monographs on chosen therapeutic plants.

1.4 Trading of medicinal plants

Trading therapeutic plants is difficult to evaluate precisely, in light of the fact that a great part of the local trade is either unrecorded or inadequately ordered and therapeutic plants are likewise utilized as a part of nonmedicinal end-utilizes and not testified. Local exchange, specifically, is inadequately recorded. Rising worldwide consideration in restorative plants has additionally made a persistent and to a great extent "underground" trade of plant materials, large portions of which are being gathered in Least Developed Nations in an unregulated way, bringing about an aimless yield of wild assortments and serious harm to biodiversity. It is, subsequently, impossible to survey worldwide trade every single restorative plant. Also, official exchange insights either don't identify the plants independently or don't separate their restorative use from another utilization. Therapeutic plants contributions to a boundless scope of materials that are utilized as a part of medicinal or curative items. Changed types of various items which contain components of these plants are on-exchange. They are not considered in this record in view of the trouble of recognizing them (e.g. many are exchanged as meds or foods as opposed to as therapeutic plants). Germany, for instance, is one of the central shippers of therapeutic plants and furthermore a noteworthy worldwide producer and exporter of drugs. Tolerating these information limitations, the accompanying proof gives a wide photo of the global exchange in spite of the fact that, for the above reasons, the information demonstrated should just be considered as suggestion.

1.5 Future of medicinal plants

Interest for therapeutic and aromatic plants can be relied upon to hold on for the not so distant future, in spite of the fact that the rate of offer ascents for some therapeutic and fragrant plant materials will likely not coordinate those uncovered in the 1990s. While the worldwide market for medicinal and aromatic plants can be anticipated to be at any rate US $ 60 billion (WHO 2003), correct market figures and market patterns are hard to decide because of home grown materials in a huge number of items being sold through some substantial number outlets, going from business deals over the web to mass market deals in grocery stores and characteristic item supplies. What's more, good or unfavorable press reports about a specific natural item can cause a particularly solid development or a fast decrease in sales. Most market overviews propose just a moderate increment in general request inside the US for medicinal plants, as contrasted and the 1990s. On the off chance that the US medicinal foundation completely acknowledges therapeutic plants as a component of the standard, ordinary medication framework (following the case of Asian and European nations), deals could fundamentally increase.

Rising livelihoods in Asia are probably going to raise the way of life of residents, expanding interest for extra medicinal and aromatic plants as the population experiences the detrimental impacts of maturing, weight pick up, and other restorative issues that much of the time happen in generally prosperous social orders (Gross 2001). The expansion sought after for medicinal and aromatic plants will probably keep on threatening inalienable species in a few areas. Value contrasts amongst wild and developed plants because of a longing for the wild material or the inaccessibility of developed plant material at present encourages unsustainable accumulation practices in a few areas, particularly in financially discouraged locales that need assets for ensuring plants. The monetary profits for gathering and offering neighborhood plant material much of the time speak to a significant offer of total salary for some medicinal plant authorities in a few districts. For instance, a gathering of wild ginseng (esteemed at $2 million of every 2002), in West Virginia can be a significant expansion to spending plans of poor families.

1.6 Perspective of Bangladeshi medicinal plants

Bangladesh is exceptionally rich in biodiversity. It has more than 500 medicinal plants species. Although it’s hugely crowded, but in terms of size, it’s a little nation is somewhat

one of a kind in having broadened genetic resources in an extensive variety of living spaces. Expanding population and diverse anthropogenic activities on the regular environments are posturing extreme and serious dangers to once thick and rich genetically enhanced plant groups of this nation. Loss of environments from the wild woodlands and from the provincial forests, developed fields, and wild terrains are very regular in this nation. A wide genetic base has been substituted by a narrow one, and the old genetic differing qualities is vanishing both inside and outside of the old quality focuses. This pattern is unavoidable with the requirement for remarkably productive and uniform cultivars in cutting edge and refined cultivating frameworks. Presently, we have no safe range for characteristic genetic resources and besides, have no practical approach on protection of biodiversity. In spite of the fact that there are a few quality banks having restricted offices to safeguard some monetary harvests like rice, jute, wheat, beats and so forth in Bangladesh, there is no incorporated association to manage germplasms of the wild relatives of agribusiness, agriculture, medicinal and monetarily less important forest animals. Bangladesh Agricultural Research Council is extremely worried on this issue. Be that as it may, the rich and differing legacy of customary medicinal framework in the Indian sub-landmass including Bangladesh is progressively undermined by the interchange of various factors, for example, quick deforestation and natural surroundings devastation, unselective accumulation and exploitative trade network.

1.7 Literature review of the plant

1.7.1 Plant name

Callicarpa attenuata

1.7.2 Common names

Velvety Beauty Berry

Malayalam- Thinperivelam Vennthekku

Hindi- Bastra, Priyangu

Marathi- Aesar, Kan-phulia

French- Mulberry of Western Ghats

Tamil- Seembakkulthu

Kannada- Ardri

1.7.3 Derivation of Name

The generic name came from the Greek *kalos*, beauty, and *carpos*, fruit; alluding to the brightly colored fruits of the type species. Linnaeus originally published the genus in Act Soc. Reg. Sci. Ups. (1741) 80.

1.7.4 Distribution

Hainan, Sichuan, Guangdong, Guangxi, Yunnan India, Taiwan, Indonesia, Laos, Philippines, Singapore, Malaysia, Myanmar, Thailand, Vietnam.

1.7.5 Description

Shrub is about 5-15 ft. tall. Stem light gray, much branched slender, 4 angular, young parts densely stellate pubescent, nodes annulated, internodes 4-8 cm long, leaves elliptic-lanceolate, obovate-lanceolate, 5-20 x 1.5-5 cm across, base cuneate, margin entire near the base, rest serrate-dentate, apex acuminate or shallow caudate, cretaceous, dark green glabrate on the dorsal side, paler pubescent, dotted with numerous minute glands beneath, stellate-pubescent along the veins, lateral veins 10-13 on either side of the midrib, arcuate at margins, impressed above and prominent beneath, petiole slender, canaliculated, about 0.4-2 cm long, pubescent, estipulate. Inflorescence axillary cyme, divaricately branched, peduncle glandular, about 0.5-1 cm long, bracts linear about 1 mm long. Flowers numerous, bisexual, actinomorphic, pedicel about 1-3 mm long, calyx cupular, 4 toothed, acute glandular teeth, truncate, stellate pubescent outside, corolla infundibular, 4 lobed, lobes rose or purple, subequal, suborbicular, slightly curved, corolla tube about 1 mm long, pubescent, Stamens 4 exerted attached below the corolla tube, filaments filiform about 3 mm long, glabrous, whitish pink, anthers oblong, creamy yellow, Ovary globose, about 0.5 mm long, glandular,

pubescent, style filiform, white, 4-5 mm long, stigma capitate, Fruit drupe, globose about 2-3 mm in diameter, succulent, green and white when ripe.

1.7.6 Classification

Kingdom: Plantae

Phylum: Angiosperms

Order: Lamiales

Family: Verbenaceae

Genus: *Callicarpa*

Species: *Callicarpa attenuata*

1.7.7 Cultivation details

It needs highly fertile and full drained soil with sufficient sunlight. They are often found in the poor, moist, sandy soils of hilly and wild areas.

1.7.8 Edible uses

Japanese people of the twentieth century often had its bark with betel nut and lime as a substitute for piper

Figure 1.1: ***Callicarpa attenuata***

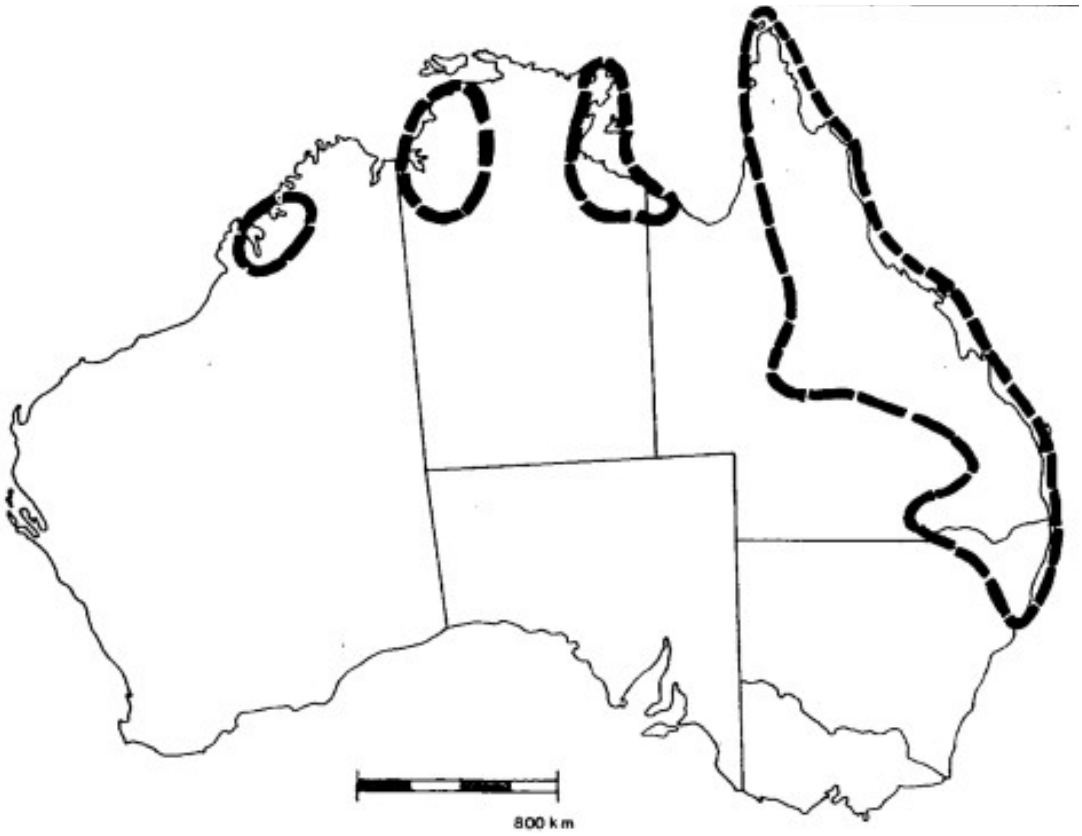

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Figure 1.2: Distribution of genus *Callicarpa attenuata* in Australia

1.7.9 Medicinal use

Leaves can be used to relieve rheumatic pain in rheumatism. The tincture and decoction can be used to treat diarrhea and dysentery. Root is often chewed to prevent rashes. Juice of the root is used to ease digestion. The inner bark of the plant is used to apply on cuts and wounds. The fruit berry is edible and consumed when ripened completely. An assortment of ethnomedical utilization of *Callicarpa* has been recorded in numerous antiquated Chinese books. Phytochemical examination of this class has brought about recognizable proof of more than 200 substance constituents, among which diterpenes, triterpenoids, and flavonoids are the prevalent gatherings. The confines and rough concentrate have shown a wide range of *in vitro and in vivo* pharmacological impacts including mitigating, hemostatic, neuroprotective, against amnesia, antitubercular, cell reinforcement, antimicrobial, and pain relieving exercises. Arrangements containing *Callicarpa* species applied great adequacy on clinical uses of gynecological aggravation, inside and outside drain and in addition, skin breaks out vulgar and endless pharyngitis, and so on. From the poisonous quality viewpoint, just three *Callicarpa* species have been evaluated.

1.8 The *Callicarpa* genus

Linnaeus (1753) described the genus *Callicarpa americana* that originated in North America. He placed it under the name *Tetrandria monogyania* without reference to any family where it was retained by Murray (1797), Flourier (1793), Rexburg (1840, 1820) and so on. In 1810, Robert Brown referred it to the family Verbenaceae where most of the botanists retained it.

Endlicher (1836) grouped the Verbenaceae into 3 tribes: *Lippieae*, *Lantaneae*, and *Aegiphileae*, with *Callicarpa* in the tribe Aegiphileae. This tribe was accepted by Meissner (1840), Endlicher (1841), Bentham and Hooker (1876), Bailey (1883, 1890, 1901) and others.

Briquet reclassified the Verbenaceae and in 1895, upgraded the tribe Vitaceae to a sub family named Viticoideae. The classification was accepted by Dalla Torre & Harms (1904), Junnel

(1994) and later Bricket divided it into 2 groups: 1. Tubular, which is characterized by a tubular calyx and the rim of which is deeply 4 ft. long and often found with foliaceous lobes. Group 2 is named Cyathimorphae which is characterized by cyathiform calyx and also the rim is sub truncate entirely or shortly toothed.

1.9 Objective of determining the antioxidant property

Numerous illnesses are associated with oxidative worry because of free radicals. Cancer preventive agents can meddle with the oxidation procedure when they respond with free radicals, catalytic metals, chelating and furthermore by going as scavengers for oxygen. There has been an expanding interest in the plant derived cancer preventing agents which may assume a part in the treatment of infections and for enhancing wellbeing. The therapeutic properties of various plants were examined alongside their powerful antioxidant properties with no reactions and economic viability. Free radicals or particularly reactive oxygen species are shaped by exogenous chemicals or endogenous metabolic frameworks in the human body. These are set up for oxidizing bio-particles through nucleic acids, proteins, lipids and DNA and can start specific degenerative disorders like a neurological issue, tumor, emphysema, cirrhosis, atherosclerosis, joint bothering and so on.

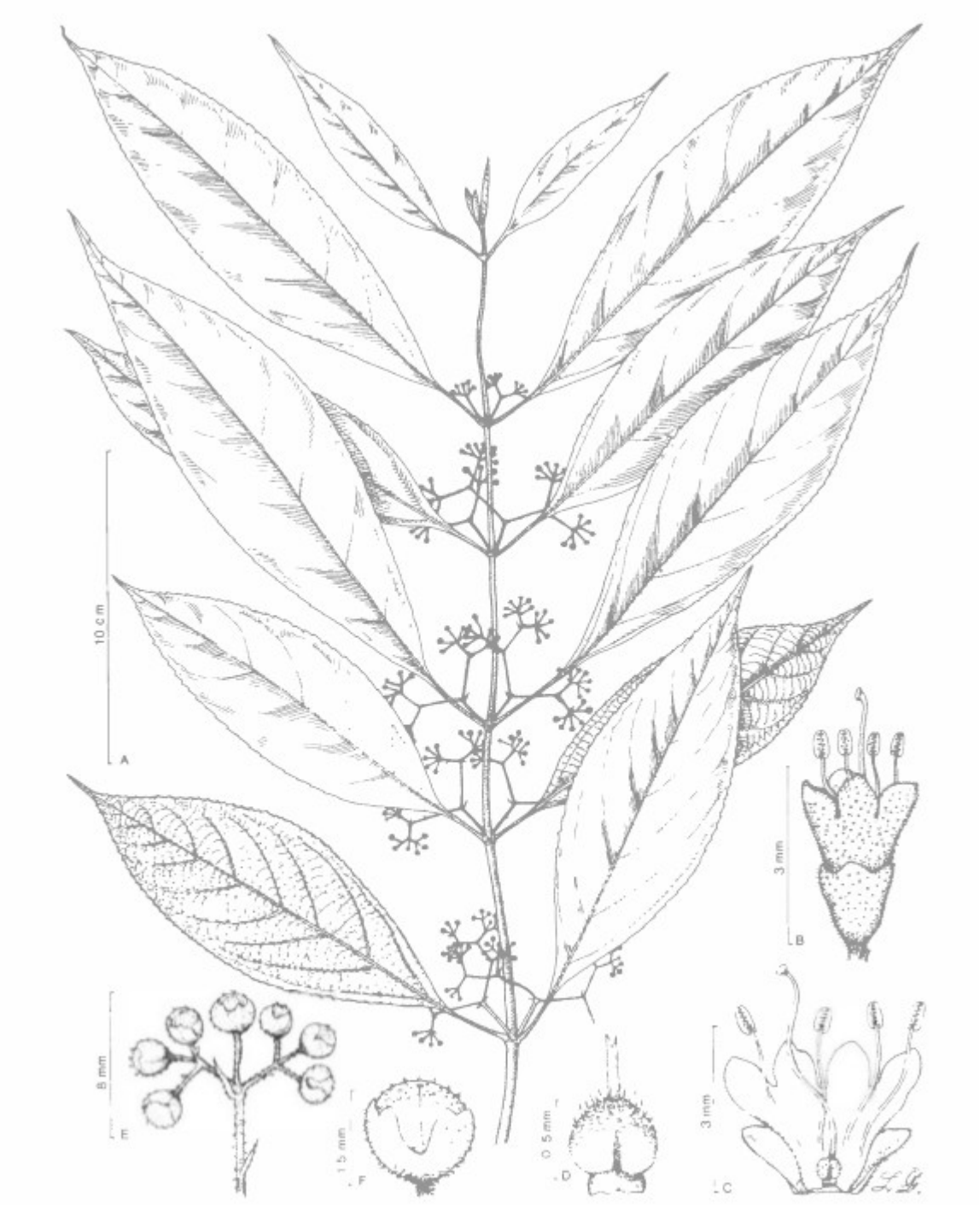


Figure 1.3: A typical diagram of *Callicarpa attenuata*

1.10 Objective of determining the total phenolic content

Polyphenols have turned into an extraordinary focus of research interest due to their apparent health benefits. They happen in a variety of organic products, vegetables, nuts, seeds, blooms, bark, refreshments, and even some processed food, as a segment of the natural ingredients utilized. They have been found to show anticarcinogenic, antiulcer, antithrombotic, calming, safe balancing, antimicrobial, vasodilatory and pain relieving impacts.

Enthusiasm for the research of polyphenols from various normal sources has developed on the grounds that polyphenols can be used as antioxidants in the food business, and they provide advantages to human health in different ways. The advantageous impacts of polyphenols on human health could be because of their free radical scavenging properties, hindering the pernicious activity of these atoms on cells.

1.11 Objective of the cytotoxicity test

At few of the greater concentrations, the compounds which are bioactive are constantly lethal for the body of human & it legitimizes that declaration, which is, 'At the maximum quantity, Pharmacology is considered as toxicology and at the minimum quantity, toxicology is considered as pharmacology'. Biological assessment of the deadliness of Brine shrimp (McLaughlin, 1998) is one of the quick and exhaustive biological assessment to those compound which are biologically active and also from the normal and artificial source. Naturally produced segments, extracts along with compounds which are unadulterated, are examined to know the bioactivity of them through this technique. Into this technique, deadliness of *in vivo* inside one of the basic zoological creature, which is the nauplii of Brine shrimp and that is utilized as the satisfactory monitor to do fractionation and screening for discovering fresh natural compounds that are biologically active.

Cytotoxicity is specified through this biological assessment and also numerous pharmacological activities like, antibacterial, pesticidal, anti-cancer, antiviral and compound’s other activities (Meyer, 1982; McLaughlin, 1998).

Due to the rapidity in technique, economic and simplicity, biological assessment of the deadliness of Brine shrimp is better than other analyzing techniques of cytotoxicity. Huge quantity of living beings and comparatively fewer quantity of specimen are used by it so that

validation can be done statistically. This technique also does not need the serum of animal like other techniques.

Chapter 2: Methodology

2.1 Collection and preparation of the plant extract

Leaves of *C. attenuata* were gathered from Dhaka in September 2016. The plant was identified at the Bangladesh National Herbarium. After washing properly, the leaves were sun dried for 1 week. The leaves were then stove dried for 24 hours at an extensively low temperature (not more than 40°C) for better granulating. The dried leaves were then grounded to a coarse powder utilizing high limit pounding machine.

2.2 Extraction of the plant material

500 gm of the powdered material was taken in a clean, amber glass holder and soaked in 2.5 liters of methanol. The holder with its substance was sealed by aluminium foil and kept for a time of 15 days going with infrequent shaking and stirring. The entire blend was then filtered through a clean cotton plug and at last with filter paper. The volume of the filtrate was then decreased utilizing a Rota-evaporator at low temperature and weight. The mass of the extract was 85 gm.

2.3 Further drying

The extract was then allowed to lose moisture for proper experimental purposes by covering up the mouth of the beaker with a tissue paper for another 2 weeks. After two weeks most of the moisture content was lost and it attained a very dark sticky form. This methanolic extract was then used to carry out the experiments.

2.4 Evaluation of antioxidant activity

Phenolic compounds may assume an essential part in protecting body cells from damage by hydrogen peroxide and from the harm carriedout by unsaturated fats and lipid peroxides, absorbing and neutralizing free radicals (Sroka and Cisowski, 2003).

The phenolic compounds employ their antioxidant properties by redox properties, which enable them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Proesto*et al.* 2017).

Antioxidant compounds can be found in various foods and medicinal plantswhich play animportantpart in the prevention and treatment of chronic diseases which are caused by oxidative pressure. They frequentlyhave strong antioxidant and free radical scavenging abilities and anti-inflammatory action too, which are similarly the basis of other bioactivities and health benefits, such as anti-aging, anticancer, and defensive activity for cardiovascular diseases, diabetes mellitus, obesity and neurodegenerative diseases (An-Na Li *et al.*2014).

A different synthetic cancer preventive agent, for example, tert-butyl-1-hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are regularly utilized as nourishment added substances to expand timeframe of realistic usability. It has been found to cause consequences of carcinogenic effects. Accordingly, the interest for regular cancer preventive agent from plant origin has significantly expanded lately. Plant polyphenols were widely contemplated for the probability that they may underlie the defensive impacts given by foods grown from the ground utilization against tumor and other chronic diseases. The aim of this study was to assess the methanolic extract of *C. attenuata* as a new probable source of plant-derived antioxidants and phenolic components.

The therapeutic properties of plants have already been explored in the current logical advancements all through the world, because of their strong cancer preventing properties, no side effects and financial practicality.

The cancer prevention agent movement tested as:

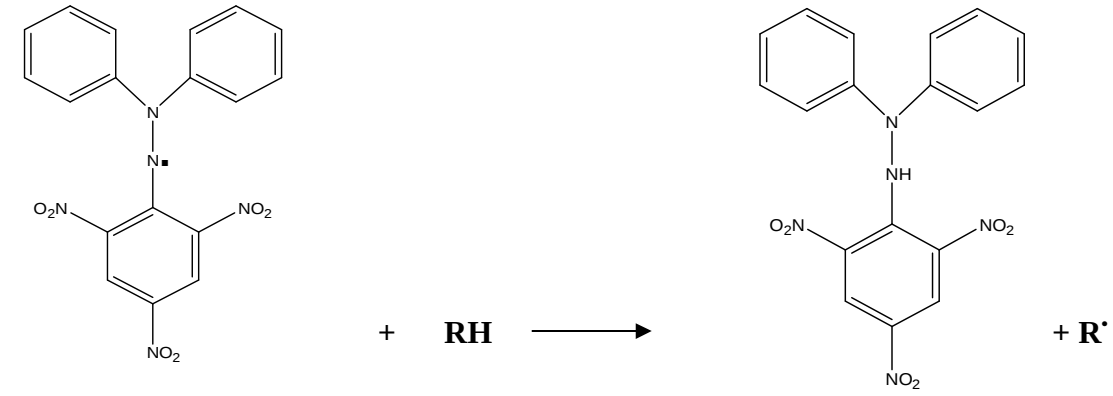
- Determination of antioxidant properties: DPPH assay
- Determination of total phenolic content.

2.4.1 Principle

The free radical scavenging property (cell reinforcement limit) of the plant removes on the steady radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were figured.

2.0 ml of a methanol solution of the concentrate at different concentration was blended with 3.0 ml of a DPPH methanol solution (20 µg/ml). The antioxidant activity potential was assayed from the reduction of a purple shaded methanol solution of DPPH radical by the

plant's methanolic extract when contrasted with that of tert-butyl-1-hydroxytoluene and ascorbic corrosive by UV spectrophotometer.



*DPPH (oxidized form)

DPPH (reduced form)

-picrylhydrazyl

2.

D the free radical scavenging activity also known as antioxidant p ds and medicinal plants.

2.4.3 Materials required

Chemicals:

- Ascorbic acid
- distilled water
- methanol
- n-hexane
- Carbon tetra chloride
- Chloroform
- Tert-butyl-1-hydroxytoluene (BHT)
- 1,1-diphenyl-2picrylhydrazyl

Apparatus:

- Test tube
- UV spectrophotometer
- Beakers (100 and 200ml)
- Amber reagent bottle

- Pipette
- Light proof box
- Micropipette (50-200µl)

2.4.4 Preparation of control to measure antioxidant property

ASA & BHT were utilized as the control of positive. The measured quantity of BHT & ASA were made to dissolve into methanol for getting a stock solution that has a conc. of 1000µg/ml. Utilizing the stock solution, serial dilution has been carried out for getting distinctive fixations extending from 500µg/ml-0.977µg/ml.

2.4.5 Preparation of test specimen

The ascertained volume of various extract has been measured & dissolved in methanol to obtain the stock solution (Conc. 1000 µg/ml). Stock solution has undergone serial dilution to give various concentrates extending from 500.0 to 0.977 µg/ml which have been stored in the designated flasks.

2.4.6 Preparation of DPPH solution

Powdered DPPH weighing 20mg has been measured and has been made to dissolve in methanol to obtain a DPPH solution with a conc. of 20 µg/ml. The solution has been set up within the amber colored container and kept in the light resistant cupboard.

2.4.7 Free radical scavenging activity determination

2.0 ml methanolic solution of the sample (extract control) at various designated concentrations (500 µg/ml to 0.977 µg/ml) has been combined with 3.0 ml of a DPPH methanol solution (20 µg/ml). After a reaction period of thirty minutes at room temperature in a dark cupboard, the absorbance has been measured at 517 nm with respect to methanol being the blank by UV spectrophotometer.

Scavenging of free radical DPPH *(I%)* was calculated following this equation:

$$(I\%) = (1 - A_{sample} / A_{blank}) \; X \; 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except for the test material).

Extract concentration showing 50% inhibition *(IC_{50})* was calculated from the graph was plotted to percentage of inhibition against extract concentration.

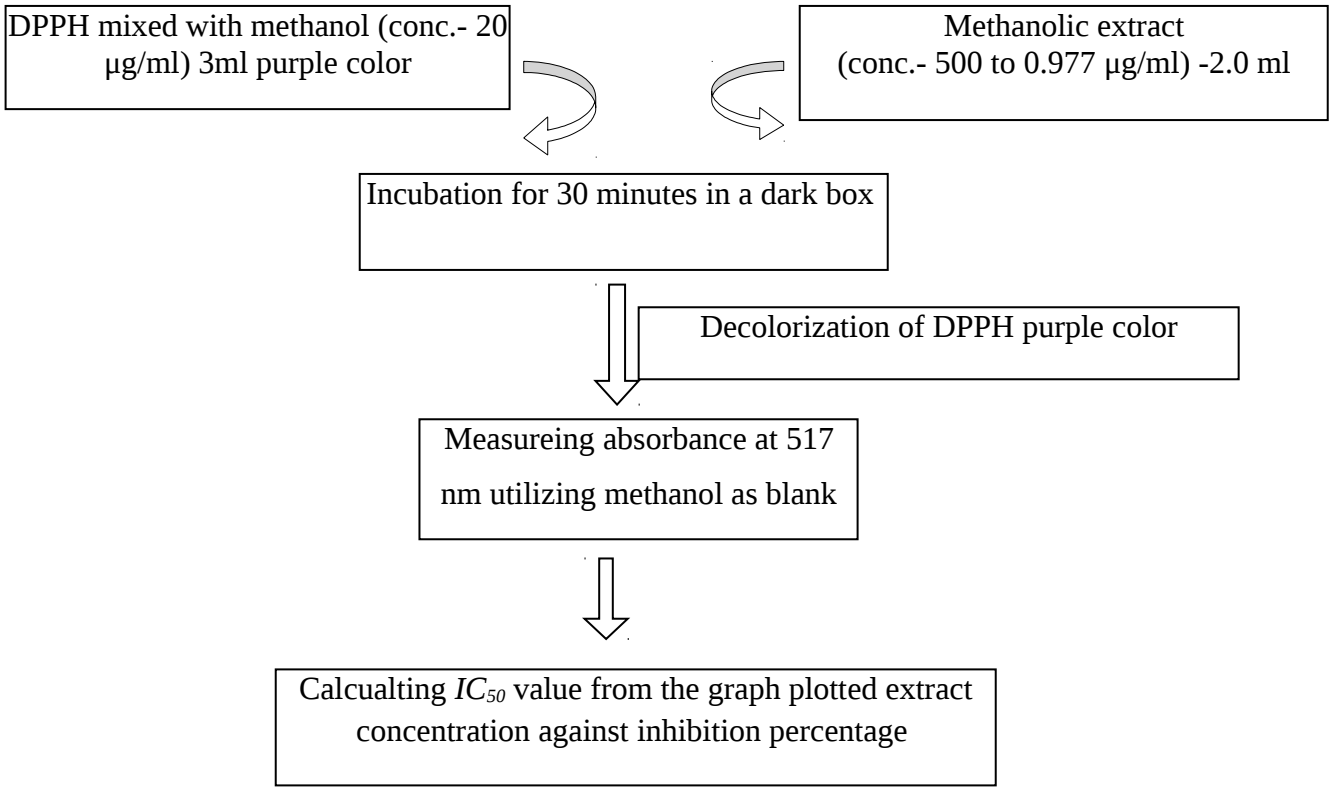


Figure 2.1: Flow chart of the method of assaying DPPH scavenging activity

The experiments have been performed thrice. The results were expressed as mean ± SD in each case.

2.5 Total Phenolic content determination

Phenolic Quantification Assay depends on Folin-Ciocalteu method. The FC reagent contains phosphomolybdic/phosphotungstic complexes. The strategy depends on the move of electrons in antacid medium from phenolic mixes to frame a blue chromophore created by a phosphotungstic/phospho-molybdenum complex in which the maximum absorption relies on

upon the concentration of phenolic mixes. The diminished Folin-Ciocalteu reagent is noticeable with a spectrophotometer in the scope of 690 to 710 nm. The response temperature has been utilized to decrease the time important to achieve the most extreme shading (T= 37ºC). For the most part, gallic corrosive is utilized as the reference standard component and expressed as gallic acid equivalents (mg/ml).

Assay principle The F-C assay has been utilized as a test of total phenolics in natural products, but the basic process is an oxidation/reduction reaction. In the first F-C test, the carbonate support is utilized for pH alteration and the end-purpose of the response was accomplished after 120 min at room temperature, which makes its execution for routine investigation troublesome.

The technique was accomplished in a 96-well microplate arrangement and it was coupled to a few phenolic mixes and nourishment items (wines, brews, juices, implantations).

2.5.1 Steps and situations to obtain reliable and foreseeable data

1. The appropriate volume ratio of Alkali and F-C reagent (1:10 v/v).

2. Optimal reaction time and temperature (T=37ºC) for development of color.

3. Observing of Absorbance at 700 nm.

4. Use of Gallic Acid as the reference standard phenol.

2.5.2 Materials and methods

Total phenolic content of methanolic extract of leaves *C. attenuata* has been calculated by means of Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard.

2.5.3 Materials required

Reagents:

- Na_2CO_3 solution

- Folin-Ciocalteu reagent 10x diluted
- Chloroform
- Methanol
- Carbon tetra chloride
- Pet ether
- Tert-butyl-1-hydroxytoluene
- Distilled water

Apparatus:

- UV spectrophotometer
- Test tube
- Beaker (100 and 200ml)
- Pipette
- Micropipette
- Vial

2.5.4 Total phenolic compound analysis

To 0.5 ml of Sodium carbonate (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (10xdiuted) and 2.0 ml of Na_2CO_3 (7.5 % w/v) reagent was included. The blend was kept for 20 minutes at ambient temperature. Following 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer, and utilizing the standard curve arranged from gallic acid arrangement with various concentration, the compounded phenols substance of the specimen

was measured. The phenolic substance of the specimen was communicated as mg of GAE (gallic acid equal)/gm of the concentrate.

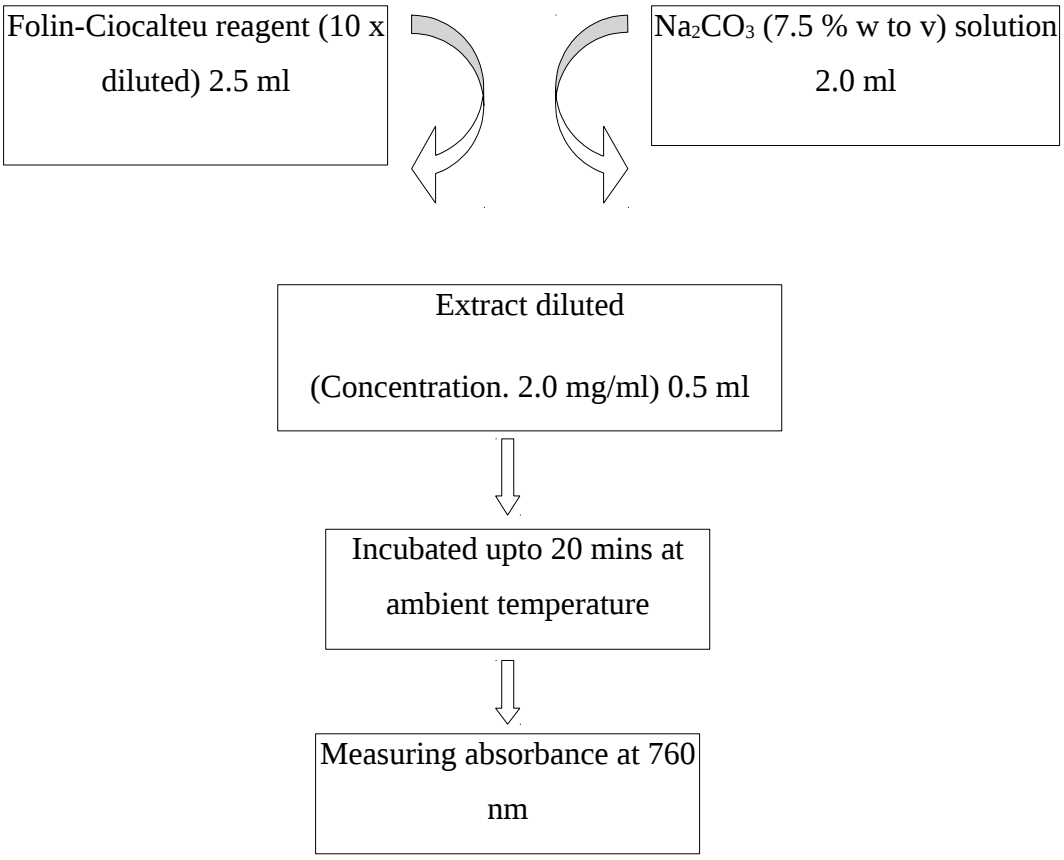


Figure 2.2: Schematic representation of the total phenolic content determination

2.6 Principle of Brine Shrimp Lethality assay

Into the replicated water of ocean, eggs of brine shrimp are produced for getting the nauplii. Preferred amount of test specimen is made through adding a measured quantity of dimethylsulphoxide (DMSO). Calculation of nauplii are done through the visual checkup. Then they are transferred into vials of 5ml replicated water of ocean. After that, addition of various amount of specimen are done by micropipette into vials which are marked previously. Finally, after passing 24 hours, survivors are calculated.

2.6.1 Materials

1. *Artemia Salina* Leach
2. Sodium Chloride
3. Small tank with holed separating dam
4. Lamp
5. Micropipette
6. Pipette
7. Test tubes
8. Glass vials
9. Magnifying glass
10. Test specimens of investigational plants

2.6.2 Experimental Procedure

2.6.3 Ocean water preparation

Weighing was done of 38gm salt of ocean which is unadulterated sodium chloride. Then it was melted into 1L purified H_2O and after that, filtration was done for getting the solution that is clear.

2.6.4 Brine shrimp hatching

From the shop of pet, eggs of brine shrimp named *Artemia Salina* Leach was gathered and it was utilized as test animal. Into a small tank, water of the ocean was poured and into a portion of that tank, eggs of shrimp were taken which was enclosed after that. For hatching of shrimp and for the maturation to get nauplii, 24 hours was indorsed. Between the time of hatching, continuous supply of O_2 was ensured. Attraction happened between hatched shrimps and lamp by holed dam. Then test was done upon them.

Ten alive shrimps were putted to individual test tubes which contain 5ml water of ocean through Pasteur pipette.

2.6.5 Investigational plant's test specimen preparation

Into vials, each and every test specimen were added and melted into unadulterated 100µl dimethyl sulfoxide (DMSO) for getting stock solutions. After that, into the initial test tube that contain 5ml replicated water of ocean with ten nauplii shrimp, solution of 50µl was added. Therefore, 400 µg/ml was that solution's finishing concentration. Next, from that stock solution, different solutions maintaining sequence was made through the technique of serial dilution. Addition of 50 µl specimen and 50µl dimethyl sulfoxide into the test tube and vial respectively was same for each situation. Hence, various concentrations were prepared into distinct test tubes.

Table 2.1: Test specimen with the values of concentration after the serial dilution

Number of Test Tube	Concentration (µg/ml)
1	400
2	200
3	100
4	50
5	25
6	12.5
7	6.25
8	3.125
9	1.5625
10	0.78125

2.6.6 Control group preparation

For the validation of test techniques and for ensuring outcomes, control groups are utilized into the study of cytotoxicity. Outcomes that are found are the effect of test specimen. Impacts of additional probable aspects are abolished. Following groups of control are utilized:

i. Control of positive
ii. Control of negative

2.6.7 Preparation of control group of positive

Into the study of cytotoxicity, control of positive is the generally recognized cytotoxic negotiator. Source of positive control into this investigation was vincristine sulfate. Previously determined quantity of vincristine sulphate was melted into DMSO for obtaining the first concentration, which was 20 µg/ml and then, serial dilutions had prepared from that utilizing DMSO for obtaining 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125µg/ml, 0.15625µg/ml, 0.078125µg/ml & 0.0390µg/ml concentrations. After that, into previously measured vials, which contain 10 alive nauplii of brine shrimp into 5ml replicated water of ocean, solutions of positive control were poured for obtaining groups of positive control.

2.6.8 Preparation of control group of negative

For utilizing as groups of control, dimethyl sulfoxide 100µl was taken in 3 previously marked vials of glass individually that contain 5ml replicated water of ocean and ten nauplii of shrimp. This investigation might be unacceptable if quick rate of death of brine shrimps happen because other causes rather than the compound’s cytotoxicity were responsible for the death of nauplii.

2.6.9 Nauplii counting

Inspection of vials were done through one magnifying glass and survivor quantity was measured after passing 24 hours. Calculation was done for death percentage (%) for individual dilution. Statistical analyzing of the outcomes of mortality and concentration were done through utilizing linear regression with the help of a program of IBM-PC. Value of LC_{50} is used for the expression of efficiency or plant compound’s connection between

concentration and mortality. This characterizes the concentration of the chemical, which is responsible for the mortality of 50% test specimens after specific time of exposure.

Chapter 3: Result and Discussion

3.1 DPPH Assay

The methanolic extract of C. attenuate leaves was subjected to free radical scavenging activity by Brans Williams *et al*., 1995 using ASA and BHT values as standard.

In this study, the methanolic extract showed an IC_{50} value of 428.36 µg/ml, as presented at table 4.1.1. This is much higher than the standards presented by ASA and BHT. So, the methanolic extract of leaves of *C. atteniuata* exhibited mild free radical scavenging activity.

Plant part	**Sample code**	**Test Sample**	**IC_{50} (µg/ml)**
Leaves of *Callicarpa attenuata*	ME	Methanolic extract	428.36
ASA (Ascorbic acid) (standard)			3.01
BHT (*tert*-butyl-1-hydroxytoluene) (standard)			27.5

Table 3.1 IC_{50} values of the standards against methanolic extract of leaves of *Callicarpa attenuata*

Table 3.2: IC_{50} value of Ascorbic acid (ASA)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% inhibition	IC_{50} (µg/ml)
0.325	500	0.005	98.46	3.01
	250	0.006	98.15	
	125	0.015	95.38	
	62.5	0.024	92.61	
	31.25	0.068	79.07	
	15.625	0.098	69.84	
	7.813	0.139	57.23	
	3.906	0.186	42.76	
	1.953	0.175	46.15	
	0.977	0.193	40.61	

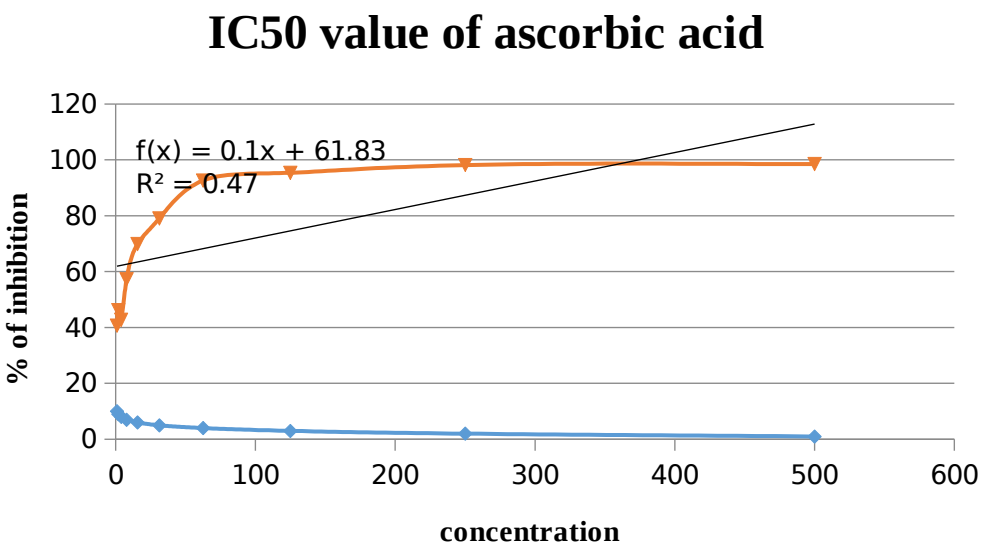


Figure 3.1: IC_{50} value of ascorbic acid

Table 3.3: IC_{50} value of *tert*-butyl-1-hydroxytoluene (BHT)

Absorbance of the blank	Conc. (µg/ml)	Absorbance of the extract	% inhibition	IC_{50} (µg/ml)
0.325	500	0.018	94.46	27.5
	250	0.068	79.07	
	125	0.097	70.15	
	62.5	0.135	58.46	
	31.25	0.159	51.07	
	15.625	0.175	46.15	
	7.813	0.206	36.61	
	3.906	0.225	30.76	
	1.953	0.238	26.76	
	0.977	0.287	11.69	

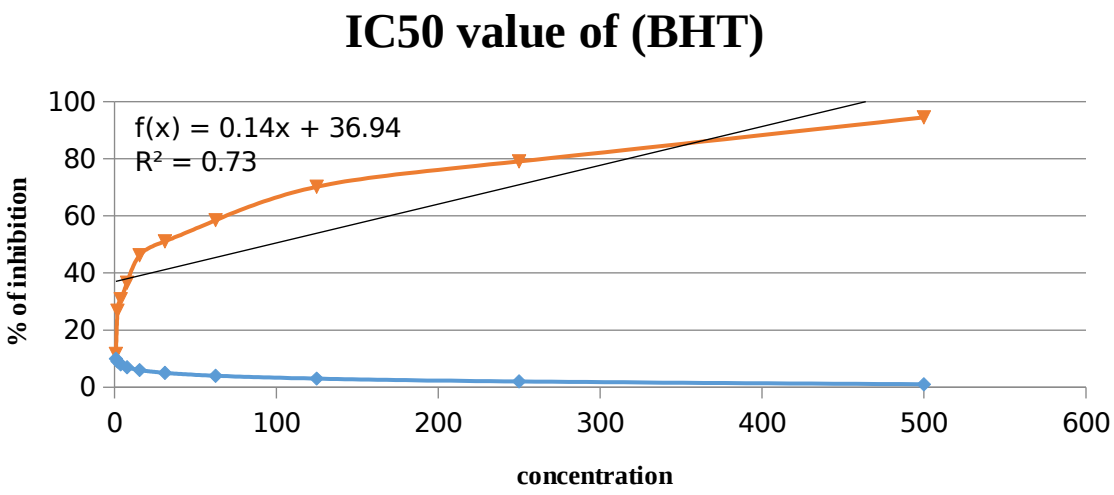


Figure 3.2: IC_{50} value of tert-butyl-1-hydroxytoluene (BHT)

Table 3.4 IC_{50} values of the standards against methanolic extract of leaves of *Callicarpa attenuata*

Conc. µg/ml	Absorbance	% of Scavenging	IC_{50}
500	0.0684	80.3448	
250	0.0751	78.4195	
125	0.0801	76.9827	
62.5	0.0878	74.7701	
31.25	0.0967	72.2126	9.687
15.625	0.126	63.7931	
7.813	0.182	47.7011	
3.906	0.197	43.3908	
1.953	0.238	31.6091	
0.977	0.295	15.2298	
Blank	0.348		

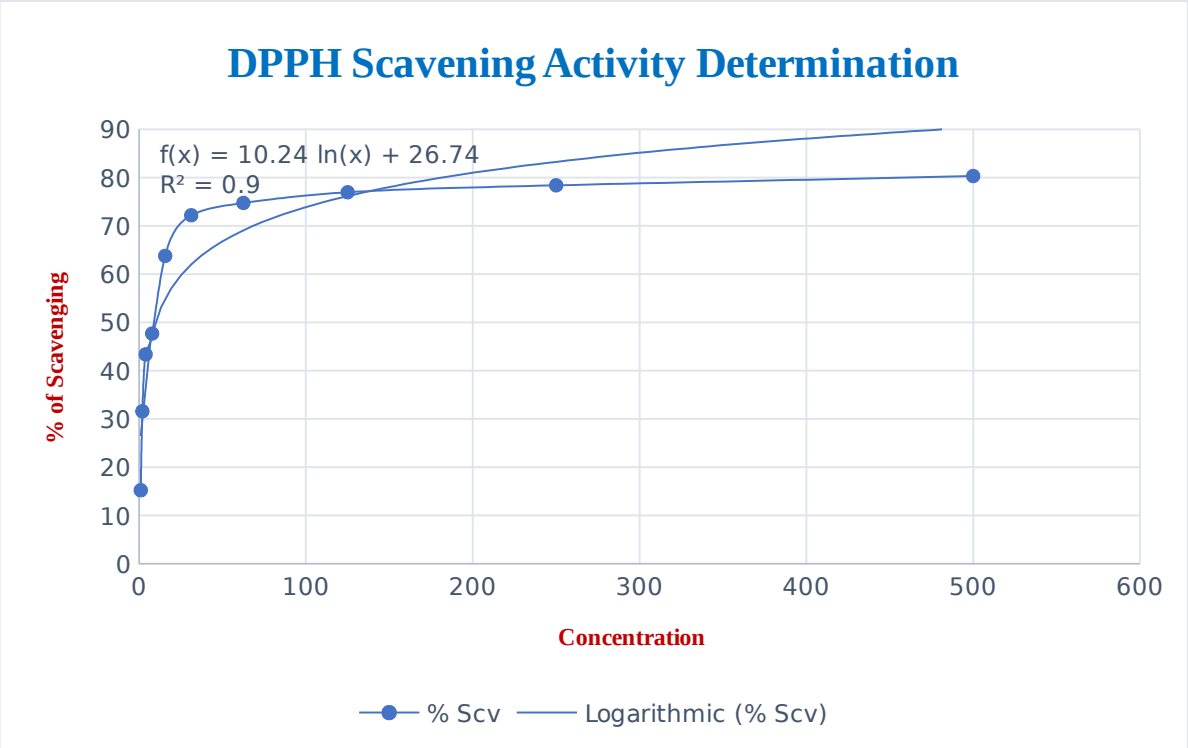


Figure 3.3: Percentage of scavenging vs concentration

3.2 Determination of Total Phenolic Content

The methanolic extract of *Callicarpa attenuata* leaves was used for determining its total phenolic content. For this, Folin Ciocalteeu reagent was used. Using the absorbance value of the extract solutions, the total phenolic content of the extract was found and compared to the standard gallic acid. The total phenolic content of the extract was found to be 24.7125mg of GAE/gm of extract. Assessing the graph and comparing this standard Gallic acid curve to the sample, moderate amount of total phenolic content was found from the leaves extract of *Callicarpa attenutata.*

Table 3.5: Standard curve prepration by using gallic acid

Sl. No.	Conc. Of the Standard (µg / ml)	Absorbance	Regression line	R^2
1	100	0.800	**y = 0.0081x - 0.0007**	**0.9975**
2	50	0.423		
3	25	0.215		
4	12.5	0.123		
5	6.25	0.047		
6	3.125	0.007		
7	1.5625	0.003		
8	0.78125	0.000		
9	0.3906	0.000		
10	0	0.000		

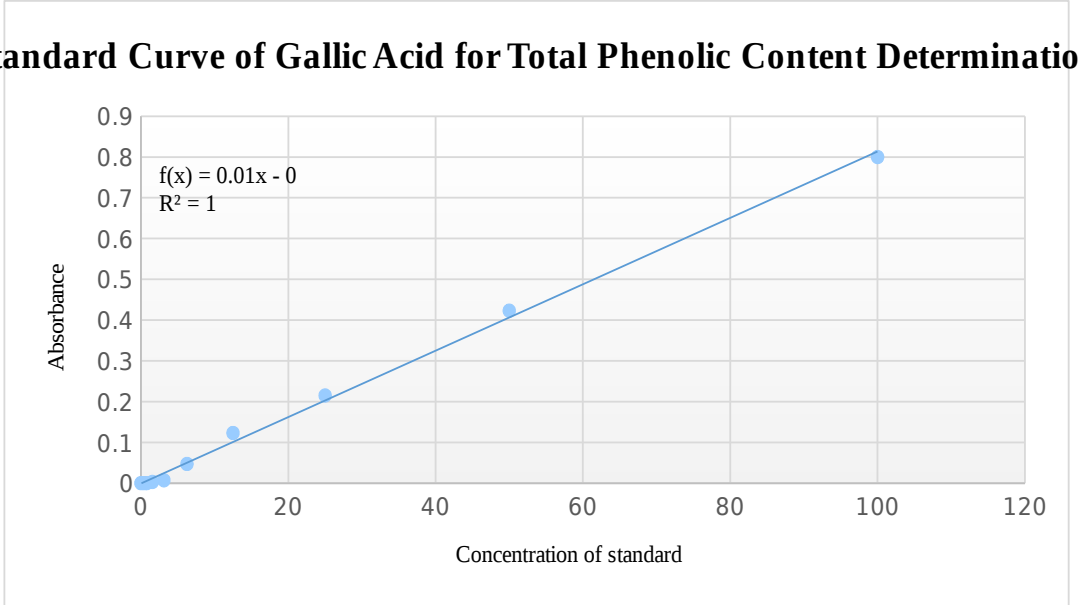


Figure 3.4: Standard curve of Gallic Acid for total phenolic determination

Table 3.6: Test samples for total phenolic content determination

Plant part	Sample code	Test Sample	Total phenolic content (mg of GAE / gm of extract
Leaves of *Callicarpa attenuata*	ME	Methanolic extract	24.7125

3.3 Brine Shrimp Lethality Bioassay

The methanolic extract showed the lethality of 8.343 µg/ml LC_{50} (µg/ml). The methanolic extract (ME) of leaves of *C. attenuata* was inspected for brine shrimp lethality bioassay. The cytotoxicity of the extract to brine shrimp was observed and the results are given in Table 5.2.2. The lethal concentration (LC_{50}) of the test sample was determined by plotting the percentage of mortality rate of shrimps against the logarithm of concentration. The curve of regression analysis helps in gaining the optimum line. Vincristine sulfate (VS) was used as positive control and the LC_{50} was found to be 0.45 µg/ml. The LC_{50} of the methanolic extract of leaves of *C. attenuata* 8.343 µg/ml. This value is indicative of mild cytotoxic activity in leaves of *C. attenuata*.

Table 3.7: LC_{50} values of the test samples of leaves of *Callicarpa attenuata*

Test samples	Regression line	R^2	LC_{50} (µg/ml)
ME	y = 33.748x + 61.653	0.973	0.47

Table 3.8 Impact of extract of the methanol of *Callicarpa attenuata* leaves on the nauplii of shrimp

Conc. (µg/mL)	$Log_{10\ conc.}$	% of mortality	LC_{50}
0	-	0	8.343
0.78125	-1.1072	10	
1.5625	0.19382	20	
3.125	0.49485	20	
6.25	0.79588	30	
12.5	1.09691	40	
25	1.39794	50	
50	1.69897	50	
100	2	60	
200	2.30103	70	
400	2.60206	90	

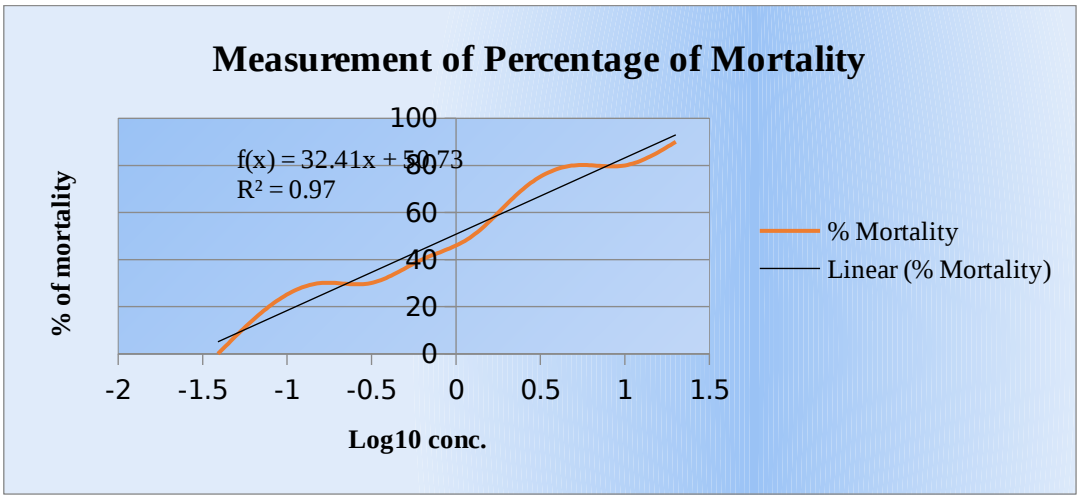


Fig 3.5: Percentage of mortality against log_{10} concentration

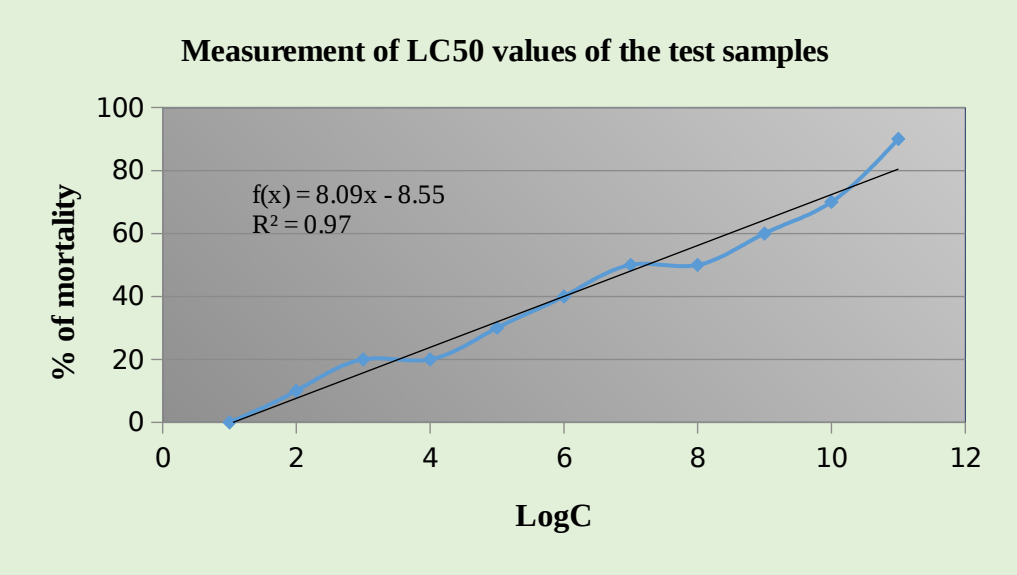


Figure 3.6: Plot of % mortality and predicted regression line of ME

Conclusion

The basic methanolic extract of *Callicarpa attenuata* leaves is used as the medicine of herbal for treating several diseases of human. Bioassay of *Callicarpa attenuata* showed that some antioxidant properties are present in it. A mild cytotoxicity of the methanolic extract of leaves of *Callicarpa attenuata* was also confirmed as compared with the standards.

As a result, the study established that, extract of *Callicarpa attenuata* leaves is capable to become a noteworthy specimen for developing drug. However, chemical investigations and pharmacological studies in animal models should be carried out to further establish the biological effects.

Reference

Aruoma O.I., (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. J Am Oil Chem Soc. 75, 199-212

Auudy B., Ferreira F., Blasina L., Lafon F., Arredondo F., Dajas R. and Tripathi P.C., (2003). Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. J. Ethnopharmacol. 84, 131-138

Ayafor J.F., (1972). Limonoids and phytol derivatives from Cedrela sinensis. Tetrahedron. 28, 9343

Ayatollahi, S.A., Shojaii, A., Kobarfard, F., Nori, M., Fathi, M., and Choudhari, M.I., (2009), Terpenes from aerial parts of Euphorbia splendid, Journal of Medicinal Plants Research Vol. 3(9): 660-665

Ahmad Abid Munir., (2012). A Taxonomic Revision of the Genus *Callicarpa Longfolia*. (Verbenacae) in Australia

Bala, K.R. & Seshadri, T.R. (1971). Isolation and synthesis of some coumarin components of Mesua ferrea seed oil. Phytochemistry 10: 1131

Bandaranayake, W.M., Selliah, S.S., Sultanbawa, M.U.S. & Games, D.E. (1975). Xanthones and 4- phenyl coumarins of Mesua thwaitesii. Phytochemistry 14: 265

British Pharmacopoeia, (2007). British Pharmacopoeia Commission, London

Chow, Y.L. & Quon, H.H. (1968). Chemical constituents of the heartwood of Mesua ferrea. Phytochemistry 7: 1871.

Mayadas, T. N., Tsokos, G. C., & Tsuboi, N. (2009). Mechanisms of Immune Complex-Mediated Neutrophil Recruitment and Tissue Injury. Circulation, 120(20), 2012-2024. doi:10.1161/circulationaha.108.771170

Raven, P. H., Wu, Z., & Hong, D. (1999). Flora of China Illustrations. Beijing: Science Press.

Petrovska, B. (2012). Historical review of medicinal plants′ usage. Pharmacognosy Reviews, 6(11), 1. doi:10.4103/0973-7847.95849
Glesinger L. Medicine through centuries. Zagreb: Zora; (1954). pp. 21–38

Ferguson, R. (2004). Review: Classics in Anthropometric History * Anne Kelly, Anthea Symonds: The Social Construction of Community Nursing. Social History of Medicine, 17(2), 309-310.

Rani, V., & Yadav, U. C. (2015). Free radicals in human health and disease. New Delhi: Springer.

Izzo, A. A., Hoon-Kim, S., Radhakrishnan, R., & Williamson, E. M. (2016). A Critical Approach to Evaluating Clinical Efficacy, Adverse Events and Drug Interactions of Herbal Remedies. Phytotherapy Research, 30(5), 691-700.

Ronald R., (1982). Antbiotics-An Introduction, F. Hoffman La Roche & Co. Basle, Switzerland: 70-71

Steven Foster, et al., (1996). Specializing in Herbs, Medicinal and Aromatic Plants—Photography, Consulting and Publications, Steven Foster Group, Inc.

Stojanoski N. Development of health culture in Veles and its region from the past to the end of the 20th century. Veles: Society of science and art. 1999:13–34

Wang, X. (2011). Cytotoxic activities of some selected medicinal plants of the genus Euphorbia. Journal of Medicinal Plants Research, 5(31). doi:10.5897/jmpr11.1096

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