In vitro Phytochemical and biological Investigation of plant *Lannea coromandelica* (Family: Anacardiaceae)

This Thesis Paper is submitted to the Department of Pharmacy, East West University in Conformity with the Requirements for the Degree of Bachelor of Pharmacy.

Submitted By

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CERTIFICATE

This is to certify that the research work on "In vitro Phytochemical and biological Investigation of plant *Lannea coromandelica* (Family: Anacardiaceae)" submitted to the Department of Pharmacy, East West University in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy was carried out by Md. Arif Wahid (ID:2008-3-70-081) under o guidance and supervision of Kh.Tanvir Ahmed, lecturer, Department of Pharmacy, East West university, Dhaka and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and facilities availed of this connection are duly acknowledged.

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DEDICATED
TO
MY PARENTS

ABSTRACT

Lannea coromandelica (family Anacardiaceae**)** has been investigated for the evaluation of the biological activities. The stem bark and leaf of *Lannea coromandelica* was extracted with ethanol. The concentrated extract was then partitioned with *n*-hexane, Dichloromethane and Ethyl acetate and aqueous fraction.

The crude *n*-hexane, Dichloromethane and aqueous fractions of the ethanolic extract of the plant were subjected to microbiological investigation by the disc diffusion method. Out of all the samples, Dichloromethane soluble partitionates of the ethanolic extract appeared very potent in terms of both zone of inhibition & spectrum of activity. Whereas the *n*-hexane and aqueous soluble partitionates showed low to moderate activity for some microorganisms.

The fractions were subjected to antioxidant test by Total phenolic content, DPPH radical scavenging assay (Quantitative analysis) and IC_{50}, Reducing power assay, Total antioxidant capacity by phosphomolybdenum method, Total flavonoids content.interms of DPPH method we found that potent antioxidant activity with IC_{50} 3.8 µg /ml,8 µg /ml, 6 µg /ml respectively for DCM fraction, n-hexane fraction of bark and DCM fraction of leaf comparison to the standard IC_{50} 24µg/ml.

The fractions were subjected to thrombolytic activity test , we found that The n-hexane fraction of the ethanolic extract of *Lannea coromandelica* bark produces poor activity among other extracts. The average clot lysis activity of the fraction is found to be 28.6 1 for volunteer 1 and 22.80 for volunteer 2. Dichloromethane partitionate of *Lannea coromandelica* bark also produces highest result having average values of clot lysis are 63.383 for volunteer 1 and 58.65 for volunteer 2. The average clot lysis of Dichloromethane partition of *Lannea coromandelica* leaf fraction is found to be 31.6633 for volunteer 1 and 51.0111 for volunteer 2 comparing the average clot lysis by streptokinase is 55.35849 in volunteer 1 and 50.85403 for volunteer 2.

Key words: Anacardiaceae, Extrction, Antioxidant test, Antimicrobial test, Thrombolytic test.

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1. INTRODUCTION

1.1 Rationale And Objective Of The Work

The plant is a biosynthetic laboratory and the remedial phyto-elements produced inside a plant through a cascade of biochemical reactions significantly contribute to the traditional and modern medicines. These alluring active ingredients are nothing but the chemical defence against diseases which can hold back numerous pathological discrepancies and can reset physiological harmony.

Medicinal plants are plants whose extracts can be used directly or indirectly for the treatment of different ailments. Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed (Edward, 2001). Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based on plants (Shinwari and Khan, 1998).

However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants.

The use of medicines from plants in the form of local medicine dates back to 4000-5000 B.C. While the medicinal values of these plants are due to the presence of small doses of active compounds which produces physiological actions in the human and animal body (Zaidi, 1998). Some of the important bioactive compounds found in medicinal plants are alkaloids, glycosides, resins, gums, mucilages etc. (Sack and Forehlich, 1982). It was observed that developed countries mostly imports raw materials of valuable medicinal plants from developing countries. Where they are screened, analyzed and used in drug preparations, and returned as high priced medicines to developing countries (Shinawie, *2002).*

Plants can be considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates.

There are several familiar approaches for lead searching from the plants and the isolated bioactive compounds are utilized in three basic ways (Cox, P.A., 1994):

1. Unmodified natural plant products where ethnomedical uses suggested clinical efficacy, e.g., digitalis.

2. Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g., vincristine
3. Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin

Secondary plant metabolites that results from plant evolution may be equal or superior to that found in synthetic combinatorial chemical libraries. It was estimated that in 1991 in the United States, for every 10,000 pure compounds (most likely those based on synthesis) that are biologically evaluated (primarily *in vitro*), 20 would be tested in animal models, and 10 of these would be clinically evaluated, and only one would reach U.S. Food and Drug Administration approval for marketing. The time required for this process was estimated as 10 years at a cost of $231 million (U.S.) (Vagelos, 1991). Most large pharmaceutical manufacturers and some small biotechnology firms have the ability to screen 1,000 or more substances per week using high throughput *in vitro* assays. In addition to synthetic compounds from their own programs, some of these companies screen plant, microbial, and marine organisms.

The work described in this dissertation is an attempt to evaluate *Lannea coromandelica and his* possible pharmacological and microbiological profiles . No extensive chemical and biological investigations have been carried out on these plants. Therefore, the objective of this work is to explore the possibility of developing new drug candidates from these plants for the treatment of various diseases.

1.2 Necessity Of Studying Of Medicinal Plants

- Medicinal plants have played an essential role in the development of human culture, for example religions and different ceremonies. (E.g. *Dutura has long been associated with the worship of Shiva, the Indian god*).
- Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin.
- Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine.
- Many food crops have medicinal effects, for example garlic.
- Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species.
- Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons.

- Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants.
- Plant resources (E.g. *Angiosperm, Gymnosperm, Seedless vascular plants, Bryophytes*) for new medicine.
- The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry.
- With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60%of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases.
- To identify alternative and complementary medicine.
- To reduce the toxicity of drug therapy especially toxicity reduction of synthetic and semi synthetic drugs.
- To find the lead compound diversification to treat various diseases.

1.3 The Plant Family: Anacardiaceae

The plant under investigation- *Lannea coromandelica* belongs to the family Anacardiaceae.

1.3.1 *Taxonomy*

Anacardiaceae, the cashew family, includes approximately 800 species in 82 genera. Some selected genera include-

Table 1.1 Various generas of Anacardiaceae

Actinocheita	Bouea	Dracontomelon	Haematostaphis	Mangifera
Anacardium	Buchanania	Drimycarpus	Haplorhus	Mauria
Androtium	Campnosperma	Ebandoua	Harpephyllum	Nothopegia
Antrocaryon	Cardenasiodendron	Euleria	Heeria	Ochoterenaea
Apterokarpos	Choerospondias	Euroschinus	Holigarna	Protorhus
Astronium	Comocladia	Faguetia	Koordersiodendron	Pseudoprotorhus
Baronia	Cotinus	Fegimanra	Lannea	Spondias
Bonetiella	Cyrtocarpa	Gluta	Lithraea	Toxicodendron

Members of the family are cultivated throughout the world for their edible fruits and seeds, medicinal compounds, valuable timber, and landscape appeal. Some of the products of Anacardiaceae, including mango (Mangifera indica), pistachio (Pistacia vera), cashew (Anacardium occidentale), and pink peppercorn (Schinus terebinthifolia), are enjoyed worldwide while other notables such as the pantropical Spondias fruits, the marula of Africa (Sclerocarya birrea), and the Neotropical fruits of Antrocaryon are restricted to localized cultivation and consumption and are not generally transported far distances to larger markets.

1.3.2 *Taxonomic History*

The family Anacardiaceae was first proposed by Lindley in 1830 but its members have been variously placed in other families including the Blepharocaryaceae Airy Shaw, Cassuviaceae Juss. ex R. Br., Comocladiaceae Martinov, Julianiaceae Hemsl., Lentiscaceae Horaninow, Pistaciaceae Adans., Podoaceae Baill. ex Franch., Rhoaceae Spreng. ex Sadler, Schinaceae Raf., Spondiadaceae Martinov, Sumachiaceae (DC.) Perleb, Terebinthaceae Durande, and Vernicaceae Link. Three of these families, Podoaceae, Blepharocaryaceae, and Julianiaceae, are still considered by some taxonomists to be distinct but closely related.

Podoaceae, including Dobinea and Campylopetalum, has been separated from the cashew family because the pistillate flowers lack a perianth and are adnate, by their pedicels, to a bract (Takhtajan, 1969, 1980, 1997; Hutchinson, 1973; Willis, 1973; Dahlgren, 1980; Watson and Dallwitz, 1992). Numerous authors recognize a separate Julianiaceae that includes two genera,

Amphipterygium and Orthopterygium, based on the absence of a perianth in the pistillate flowers and those flowers being enclosed in an involucre (Bessey, 1915; Hutchinson, 1926; Wettstein 1935, 1944; Copeland and Doyel, 1940; Gundersen, 1950; Standley and Steyermark, 1949; Barkley, 1957; Melchior, 1964; Stone, 1973; Cronquist, 1988; Watson and Dallwitz, 1992).

The monogeneric Blepharocaryaceae was proposed by Airy Shaw (1965) on the basis of its two species having opposite pinnate leaves and a cupule-like mature pistillate inflorescence. While many authors recognize the affinity of Blepharocarya and Anacardiaceae, placement of the genus within the infrafamilial classification of the family has been problematic due to its aberrant morphology (Engler, 1892; Wannan et al., 1987; Wannan and Quinn 1990, 1991).

1.3.3 *Characteristics*

The Anacardiaceae includes primarily trees and shrubs (rarely lianas or subshrubs), with resin canals and clear to milky exudate. The leaves are estipulate, usually alternate (rarely opposite or whorled), and may be simple or pinnately compound .The flowers are generally not highly conspicuous but are distinctive in having an intrastaminal nectariferous disc and apotropous ovules (an ovule with a raphe that is ventral when ascending and dorsal when descending). Morphological fruit diversity is exceedingly high with a myriad of types found in the family.

Although the majority of the family has drupaceous fruits, many of these are variously modified for different mechanisms of dispersal and several other fruit types are also represented. Two genera, *Anacardium* and *Semecarpus*, have an enlarged edible hypocarp subtending the drupe. One species of *Anacardium*, *A. microsepalum*, lacks the hypocarp and grows in the flooded forests of the Amazon where it may be fish dispersed (Mitchell and Mori, 1987; J. D. Mitchell, pers. com.).

Water dispersal has been reported or purported for three genera, *Mangifera*, *Poupartiopsis*, and *Spondias*. The variety of mechanisms for wind dispersal include subtending enlarged sepals (*Astronium*, *Loxostylis*, *Myracrodruon*, *Parishia*), subtending enlarged petals (*Gluta*, *Swintonia*), trichome-covered margins on a globose fruit (*Actinocheita*), trichome-covered margins on a flattened fruit (*Blepharocarya*, *Ochoterenaea*), elm-like samaras with an encircling marginal wing (*Campylopetalum*, *Cardenasiodendron*, *Dobinea*, *Laurophyllus*, *Pseudosmodingium*, *Smodingium*), samaras with a single wing (*Faguetia*, *Loxopterygium*, *Schinopsis*), dry samara-like syncarps (multiple fruit, *Amphipterygium*, *Orthopterygium*), dry achene-like fruit (*Apterokarpos*), and elongated ciliate pedicles of sterile flowers on a tumbleweed-like infructescence (*Cotinus*).

1.3.4 *Description*

Trees or shrubs each with inconspicuous flowers, highly poisonous, sometimes foul smelling resinous or milky sap. Resin-canals located in the inner fibrous bark of plants fibrovascular system found in the stems, roots and leaves is characteristic of all members of this family; resin-canals located in the pith is a characteristic of many of the cashew family species and several species have them located in the primary cortex or the regular bark. Tannin sacs are also widespread among the family.

The wood of Anacardiaceae has the frequent occurrence of simple small holes in the vessels, occasionally in some species side by side with scalariform holes (in *Campnosperma*, *Micronychia* and *Anaphrenium argenteum*). The simple pits are located along the vessel wall and in contact with the parenchyma.

Leaves are alternate or rarely opposite and without stipule.

Flowers grow at the end of a branch or stem or at an angle from where the leaf joins the stem and have bracts. Often with this family bisexual and male flowers on some plants, and bisexual and female flowers on others or flowers having both stamens and pistils . Calyx with 3 to 7 cleft sepals and the same number of petals, occasionally no petals, overlapping each other in the bud. Stamens twice as many or equal to the number of petals, inserted at the base of the fleshy ring or cup-shaped disk, and inserted below the pistil(s). stamen stalks separate, anthers able to move. Flowers have the ovary free, but the petals and stamen are borne on the calyx. In the stamenate flowers, ovaries are 1-celled. In the pistillate flowers, ovaries are 1-celled or sometimes 4-5-celled. 1-3 styles and 1 ovule in each cavity.

Fruits rarely opening at maturity and are most often drupes.

Seed coats are very thin or are crust like. Little or no endosperm. Fleshy cotyledons. Solitary seeds with no albumen around the embryo.

1.3.5 *Distribution*

Anacardiaceae are found in dry to moist, mostly lowland habitats, primarily in the tropics and subtropics but extending into the temperate zone. The family is native to the western hemisphere (from southern Canada south to Patagonia), Africa, southern Europe, temperate and tropical Asia, tropical and subtropical Australia, and most of the Pacific Islands. The family is absent from northern Europe, temperate and arid Australia, New Zealand, the Galapagos Islands, and extreme desert and high elevation habitats .

1.3.6 *Anacardiaceae Species Available in Bangladesh*

Table 1.2: Anacardiaceae Species Available in Bangladesh

Scientific name	Genus	Local name	Medicinal uses
1. Lannea grandis	*Lannea*	Jikkha	Urinary problems. Leaf is taken as diuretic
2. Mangifera indica	*Mangifera*	Aame/ Theghace	Influenza, helminthiasis. The green fruit is used against influenza. The bark is used against helminthic infections
3. Lannea coromandelica	*Lannea*	Jiga/Jigar	Seminal problems. The bark is aadministered in cases of seminal weakness and excessive seminal emissions.
4. Spondias pinnata	*Spondias*	Amra	Used as fruit
5. Spondias mombin	*Spondias*	Amra	The fruit-juice is used as a febrifuge and diuretic.
6. Mangifera sylvatica	*Mangifera*	Himalayan Mango, Pickling Mango, or Nepal Mango	Influenza, helminthiasis.
7. Anacardium giganteum	*Anacardium*	Kazu badam	Anti-mycoses, antioxidant
8. Anacardium occidentale	*Anacardium*	Kazu badam	Used as fruit
9. Buchanania arborescens	*Buchanania*	Blume	Unknown

10. Schinus polygama	*Schinus*	Morich gach	Anti-inflammatory
11. Buchanania lanzan	*Buchanania*	Chirauli	Unknown
12. Choerospondias axillaris	*Choerospondias*	Lapsi	Unknown
13. Toxicodendron acuminatum	*Toxicodendron*	Bichuti	Anti-inflammatory, Analgesic
14. Spondias dulcis	*Spondias*	Amra	The fruit-juice is used as a febrifuge and diuretic.

1.4 Introduction To *Lannea coromandelica*

1.4.1 *Taxonomic hierarchy of the investigated plant*

Kingdom : Plantae

Phylum : Magnoliophyta

Class : Spermatophyta

Subclass : Rosids

Order : sapindales

Family : Anacardiaceae

Genus : *Lannea*

Species : *L. coromandelica*

1.4.2 *Plant Description*

Genus: *Lannea*

Species: *L. coromandelica.*

Family: Anacardiaceae.

Distribution

Native:

- ASIA-TEMPERATE
  China: China - Guangdong [s.w.], Guangxi [s.], Yunnan [s.]
- ASIA-TROPICAL
  Indian Subcontinent: Bangladesh; Bhutan; India - Arunachal Pradesh, Assam, Bihar, Gujarat, Himachal Pradesh, Jammu and Kashmir, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Meghalaya, Orissa, Rajasthan, Sikkim, Tamil Nadu, Tripura, Uttar Pradesh, West Bengal; Nepal; Pakistan; Sri Lanka
  North Indian Ocean: India - Andaman and Nicobar
  Indo-China: Cambodia; Laos; Myanmar; Thailand; Vietnam

Naturalized:

- naturalized in Malaysia .

Cultivated:

- cultivated in Malaysia .
- **Common names:**

Table 1.3: Local names of *L. coromandelica*

Local name	**Country**
Jiga/Jigar/ kasmala/ bohar/ ghadi/ Kocha.	Bangladesh
Mohin/kiamil /jhingan	India
halonre /thulo dabdabe	Nepal
Baing	Myanmar
Jailbhadi/jhingan /wodier	Pakistan

Category: Deciduous tree.

Stem: Soft Wooded

Roots: Deep roots, Tap roots

It is a deciduous tree, growing up to 14 m tall. Branchlets are minutely covered with starry hairs. Bark thick, ashy-grey. Leaves crowded at the end of branches, imparipinnate, 30-45 cm long; leaflets 7-11, oblong or elliptic, acuminate, 2.5-5 cm long. Alternately arranged leaves are pinnate, with a single terminal leaflet (pinnae) at the end. Flowers small, greenish yellow in compact fascicles of racemes, at the end of the leafless branches. Drupes, reniform, produced in clusters from the end of leafless branches Flowers are unisexual, greenish, the male in compound and female in simple racemes. Sepals 4, about 1 mm long, broad ovate. Petals: 4, 2 mm long, oblong, green yellow. Fruit is ovoid, compressed, in panicles, at the end of leafless branches. Flowering : January-March.

1.4.3 *Growing conditions*

Like the Mango, the tree thrives in humid tropical and subtropical areas growing up to 2 metres in a single growing season. It grows on all types of soil, as long as they are well drained. It has been noted that some trees can suffer from some nutritional disorders if the soil is too alkaline.

Trees are cold sensitive when small and should be protected from serious frost and strong wind. Trees do best in full sun, but will produce some fruit in light shade. As a large and vigorous tree they prefer not be planted underneath other large trees and unlike some mango varieties they are not too fussed on salt spray.

Figure 1.1*: L. coromandelica* leaf

Figure 1.2*: L. coromandelica* leaf, fruit & bark.

1.4.4 ***Medicinal Uses of L. coromandelica***

The bark is considered astringent and stomachic; used as a lotion in impetigenous eruptions, leprous and obstinate ulcers; cures sprains, bruises, skin eruptions, heart diseases, dysentery and mouth sores. Decoction of the bark is used for toothache. Its bark along with the bark of *Aegle mermelos*, *Artocarpus heterophyllus* and *Sygygium cumini* is useful in impotency. Scrapped bark is chewed for 2-3 days to cure glossitis. Boiled leaves are applied as a fomentation for local swelling and pains .

2. LITERATURE REVIEW OF *LANNEA COROMANDELICA*

S.Sankara Subramanian and A.G.R. Nair (Department of Chemistry, Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry-6, India) have isolated Quercetin-3-arabinoside and ellagic acid from the flowers and leaves of *Lannea coromandelica.* From the stem berk they found β-sitosterol, physcion and physcion anthranol B along with the earlier results.(Sankara, Nair 2001)

Md. Tofazzal Islam and Satoshi Tahara (Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Japan) worked on the stem berk of the *Lannea coromandelica* and isolated dihydroflavonols, (2R,3S)-(+)-3′,5-dihydroxy-4′,7-dimethoxydihydroflavonol and (2R,3R)-(+)-4′,5,7-trimethoxydihydroflavonol. Structure elucidation.

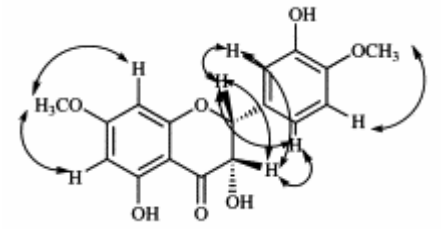


Figure 2.1: NOE correlations observed in the NOESY spectrum

In their electron microscope observation they visualized that both *Lannea* and commercial tannins caused lysis of cell membrane followed by fragmentation of cellular materials. Latterly it has been used as the lead compound. This is the first report of zoosporicidal activity of natural polyflavonoid tannins against an oomycete phytopathogen. (Tofazzal, Satoshi 2002).

Surjeet Singh and G. B. Singh in their article have shown that oral administration of an ethanolic extract of *Lannea coromandelica* (ELC) demonstrated a dose related antiinflammatory activity (AIA) in carrageenan and dextran induced oedema and adjuvant induced arthritis in rats. ELC

reduced the pleural exudate volume and inhibited leucocyte migration in carrageenan-induced pleurisy in rats. It lacked analgesic, antipyretic or ulcerogenic effect and failed to exhibit any effect in cotton pellet granuloma. (Surjeet,2006)

Chellapandian, M. Balachandran, S. in their article "Effect of feeding *Lannea coromandelica* leaves on the reproduction of goats have determined the effect of feeding *L. coromandelica* leaves on the kidding percentage, birth weight, 90-day weaning weight, milk yield and milk fat percentage of does and semen volume, colour, sperm motility, density and mean reaction time of Tellicherry bucks. The authors have concluded that it can feed to goat without any negative impact on the reproductive performance of the goat. (Chellapandian, 2006).

Avinash Kumar Reddy, Jyothi M Joy a, C.K. Ashok Kumara in their work have tried to establish logical representation for the folklore uses of the plant *Lannea coromandelica* like the wound healing activity, hypotensive activity, and zoo sporicidal activity. This article is the review of research works done on the plant L. coromandelica till to date and they have discussed it by the local names, morphology, traditional claims, chemistry, pharmacological activities. (Avinash, Ashok 2011)

Badrul Alam, Sarowar Hossain, Razibul Habib, Julia Rea and Anwarul Islam worked on the "Antioxidant and Analgesic Activities of *Lannea coromandelica* Linn. Bark Extract". They evaluated the antioxidant and analgesic properties. They determined the analgesic activity for its central and peripheral pharmacological actions using hotplate as well as tail immersion method and acetic acid-induced writhing test with formalin induced pain in mice, respectively. To evaluate antioxidant potential of MLCB, total antioxidant activity, scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as total Reactive Oxygen Species (ROS) and assessment of reducing power were used by them. They demonstrated the strong antioxidant and antinociceptive activities of methanolic extract of the bark of *L. coromandelica*. Finally they concluded that, these results suggest that the MLCB could be used as a potential antinociceptive agent along with its antioxidant potentiality.(Badrul 2012)

Table 2.1: Summary of the Studies on *Lannea coromandelica*

Year	Author(S) /Researcher(S)	Work title
2001	S.Sankara Subramanian and A.G.R. Nair	Polyphenols from *Lannea coromandelica*
2002	Md. Tofazzal Islam and Satoshi Tahara.	Dihydroflavonols from *Lannea coromandelica*
2006	Surjeet Singh and G. B. Singh	ethanolic extract of *Lannea coromandelica* (ELC) demonstrated a dose related antiinflammatory activity (AIA) in carrageenan and dextran induced oedema and adjuvant induced arthritis in rats
2006	Chellapandian, Balachandran.	Effect of feeding *Lannea coromandelica* leaves on the reproduction of goats
2011	Avinash Kumar Reddy, Jyothi M Joy a,C.K. Ashok Kumara	uses of the plant *Lannea coromandelica* like the wound healing activity, hypotensive activity, and zoo sporicidal activity
2012	BadrulAlam, Sarowar Hossain, Razibul Habib, Julia Rea and Anwarul Islam	Antioxidant and Analgesic Activities of *Lannea coromandelica* Linn. Bark Extract

3. COLLECTION, PREPARATION AND PHYTOCHEMICAL SCREENING OF PLANT LANNEA COROMANDELICA

3.1. Collection And Preparation Of The Plant Material

Leaf and bark of *Lannea coromandelica* were collected from RAJSAHI in August 2011. The plant was identified using this leaf and bark by Bangladesh National Herbarium. One voucher specimen has been deposited in Bangladesh National Herbarium **(DACB accession no. 36703)** and another one to East West University. Leaf and bark was collected from the main plant and then chopped into small pieces followed by air drying for several days..

3.2 Extraction Of The Plant Material

3.2.1 *Chopping and drying of the plants*

About three kilograms plants were collected. At first these plant was chopping to small pieces with scissor and dried under sun-light for two weeks. After drying, the dried plants were grinded into grinding machine to get fine powder. After grinding the weight was preserved in a glass container covered with aluminum foil papers.480g powder was dissolve in 2litre ethanol solvent and got 23.5g crude extract (total).

3.2.2 *Grinding of dry plants*

About 1 kilogram of fresh plants was collected and they were dried under sun-light for about 8 days. After drying plants were weighed again in Electrical balance and the total weight was about 480.00 grams. Then the dried plants were grinded into grinding machine to get fine powder. After grinding the weight of the grinded plants was measured and the weight was about 480.00 grams. All grinded plants were preserved in an air tight container.

Figure 3.1: Grinding Machine

Figure 3.2: Electrical Balance

3.2.3 *Apparatus used for sample extraction*

- Grinding machine
- Sample plant
- Beaker
- Rotary evaporator (IKA RRV05 Basic, Biometra)
- Electric Balance (SHIMADZU AY220 & SCALTEC SPB31)
- Round Bottle Flask (BOROSIL 500ml & 1000ml)
- Test Tubes (Length- 15cm, dimeter-1.5cm, capacity25 ml)
- Dropper
- Filter paper
- Cotton
- Funnels
- Conical Flasks (100ml, 250ml, 500ml & 1000ml)
- Pipette (2, 10ml)
- Volumetric Flask (250ml & 500ml)
- Aluminum foil paper
- Glass rods

3.2.4 *Reagent used for sample extraction*

We used 2 liters Ethanol as solvent.

3.3 Preliminary Phytochemical Investigations Of Crude Extract

Freshly prepared crude extracts of *Lannea coromandelica* were qualitatively tested to detect the presence of various secondary metabolites of the plant including alkaloids, flavonoids, steroids, terpenoids, reducing sugars, tannins, anthraquinone, cardiac glycoside, and saponins by following standard procedures.

3.3.1 *Apparatus*

Test tube	Filter paper
Conical flask	Bunsen burner
Beaker	Pipette
Electronic balance	Dropper
Test tube holder	Vortex mixer

3.3.2 *Reagents*

Concentrated Sulphuric acid (H_2SO_4)	Methanol
Dragandroff reagent (Solution A & Solution B)	Dilute (10%) ammonia

Solution A: Bismuth Nitrate (0.17g) in AcOH (2ml) & H_2O (8ml)

Solution B: KI (4g) in AcOH (2ml) & H_2O (8ml)

1% HCl	Chloroform
Acetic anhydride	Distilled water
Fehling's solution (Solution A & Solution B)	0.1 % Ferric Chloride
Dilute Sulphuric acid (H_2SO_4)	Glacial acetic acid
Olive oil	

3.3.3 *Methods & results of preliminary phytochemical investigations of crude extract*

Preliminary qualitative chemical tests were carried out on the ethanolic extract of *Lannea coromandelica* (bark and leaf)using standard procedures to identify the constituents. (Sofowara A, 1993; Harborne JB, 1998; Kokate CK, 2001; Trease and Evans, 1989)

Table 3.1: Phytochemical screening result.

Secondary metabolite	**Testing procedure**	**Result**
Alkaloids	10 ml methanol was added to 20 mg plant crude extract. Then it was placed in sonic bath to dissolve. Then it was filtered & 2 ml filtrate was mixed with 1% HCl. 6 drops of Dragandroff reagent was added to 1 ml of the liquid. An orange precipitate indicates the presence of alkaloid.	Alkaloids are present in the crude ethanolic extract of *Lannea coromandelica* bark and leaf
Flavonoids	5 ml dilute (10%) ammonia solution was added to a 4 ml of the filtrate of crude extract. Then 1 ml conc. H_2SO_4 was added. A yellow color indicates the presence of flavonoids.	Flavonoids are present in the crude ethanolic extract of *Lannea coromandelica* bark and leaf.
Steroids		Steroids are present in the crude ethanolic extract of *Lannea coromandelica* bark but not in the leaf..
Terpenoids		Terpenoids are present in the crude ethanolic extract of *Lannea coromandelica* bark

		and leaf.
Reducing Sugars	1 ml distilled water and 5-8 drops of Fehling's solutin (both A & B are added in equal amount) was added to 0.5 ml of extract solution. Then it was heated. A brick red precipitate indicates the presence of reducing sugars.	Reducing sugars are absent in both the crude ethanolic extract of *Lannea coromandelica* bark and leaf.
Tannins		Tannins are absent in the crude ethanolic extract of *Lannea coromandelica* bark and present in the leaf
Anthraquinones		Anthraquinones are absent in the crude ethanolic extract of *Lannea coromandelica* bark and leaf..
Cardiac glycosides	5 ml crude extract was mixed with 2 ml glacial acetic acid & 1 drop of ferric chloride. 1 ml conc. H_2SO_4 was then added. A brown ring at the interface indicates the deoxysugar characteristics of cardenolides and the presence of cardiac glycosides.	Cardiac glycosides are present in the crude ethanolic extract of *Lannea coromandelica* bark and leaf
Saponins		Saponins are present in the crude ethanolic extract of *Lannea coromandelica* bark

but not in leaf.

3.4. Solvent-Solvent Partition Of Crude Extract

3.4.1. *Preparation of Mother Solution*

5 gm of ethanolic extract was triturated with 90 ml of ethanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution, which was partitioned off successively by three solvents of different polarity. In subsequent stages each of the fractions was analyzed separately for the detection of any biological properties.

3.4.2. *Partitioning with n-Hexane*

The mother solution was taken in a separating funnel. 100 ml of the *n*-hexane was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice; *n*-hexane fractions were collected together evaporated in Rota evaporator.

3.4.3. *Partitioning with Dichloromethane*

To the mother solution left after washing with *n*-hexane, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with Dichloromethane (100 ml X 3). The Dichloromethane fractions were collected together and evaporated. The aqueous fraction was preserved for the next step.

3.4.4. *Partitioning with Ethyl acetate*

To the mother solution that left after washing with *n*-hexane and Dichloromethane, 16 ml of distilled water was added and mixed uniformly. The mother solution was then taken in a separating funnel and extracted with Ethyl acetate (100 ml X 3). The $CHCl_3$ soluble fractions were collected together and evaporated. The aqueous ethanolic fraction was preserved as aqueous fraction.

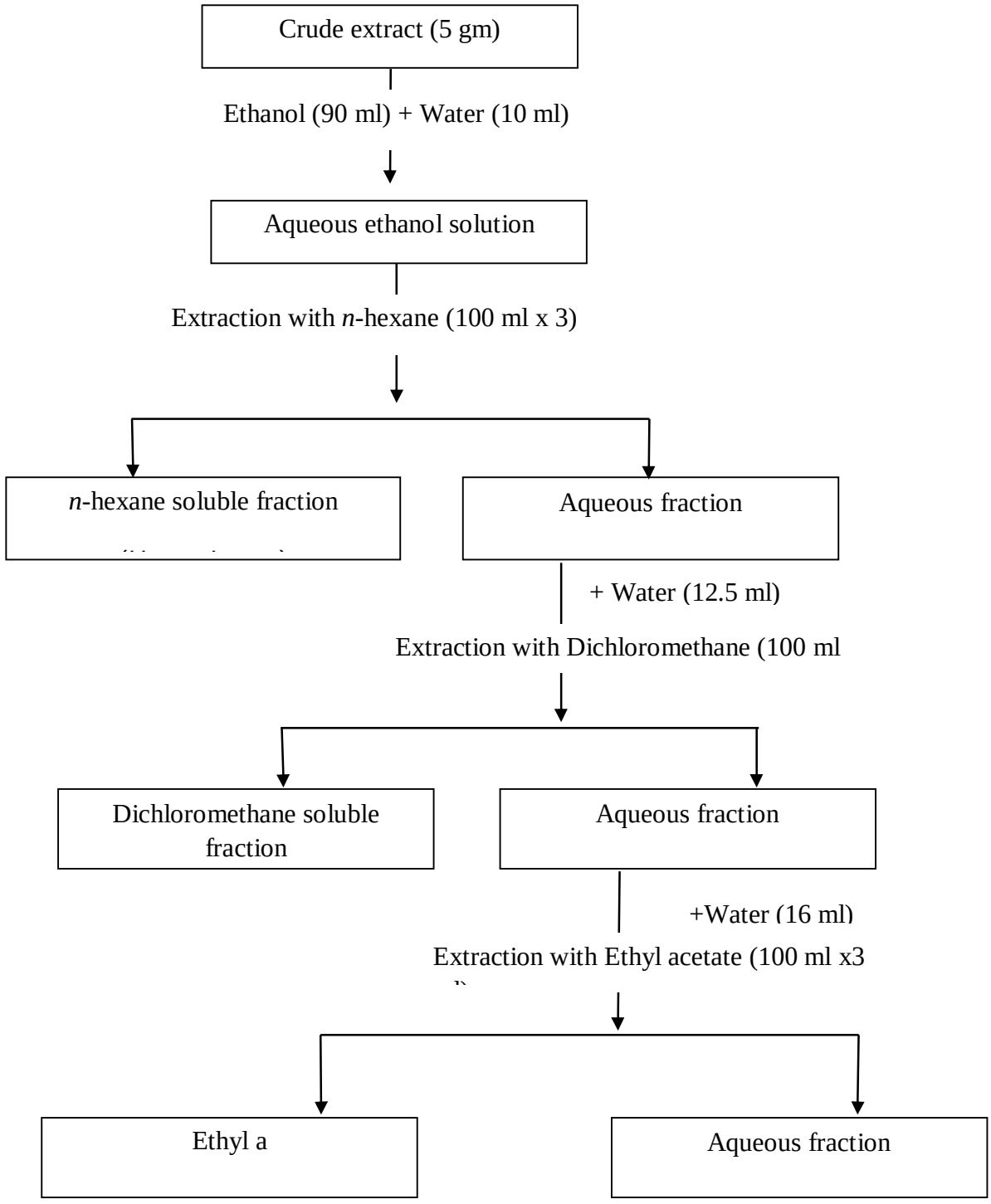


Figure 3.3: Schematic representation of the liquid-liquid extraction of ethanolic crude extract of *Lannea coromandelica*

Figure 3.4: Rotary Evaporator (IKA ®RV05 Basic, Biometra)

After evaporation the weight of the different fractions obtained are as follows:

Table 3.2: Weight of the different fractions

Plant	**Part**	**Fraction**	**Weight**
Lannea coromandelica	Leaf	n-hexane soluble fraction	500 mg
		dichloromethane soluble fraction	350 mg
		aqueous fraction	700 mg
Lannea coromandelica	Leaf	n-hexane soluble fraction	450 mg
		dichloromethane soluble fraction	340 mg
		Aqueous fraction	600 mg

4. DESIGN OF BIOLOGICAL INVESTIGATIONS

4.1. General Approaches To Drug Discovery From Natural Sources

There are three approaches of discovering new drugs and they are traditional, empirical and molecular approaches.

The traditional approach: It makes use of drug that has been found by trial and error over many years in different cultures and systems of medicine. Examples include drugs like morphine, quinine and ephedrine that have been in widespread use for a long time, and more recently adopted compounds such as the antimalarial artemisinin.

The empirical approach: This approach builds on an understanding of a relevant physiological process and often develops a therapeutic agent from a naturally occurring lead molecule. Examples include tubocurarine and other muscle relaxants, propranolol and other β-adrenoceptor antagonists, and cimetidine and other H2 receptor blockers.

The molecular approach: This approach is based on the availability or understanding of a molecular target for the medicinal agent. With the development of molecular biological techniques and the advances in genomics, the majority of drug discovery is currently based on the molecular approach.

The major advantage of natural products for random screening is the structural diversity. Bioactive natural products often occur as a part of a family of related molecules so that it is possible to isolate a number of homologues and obtain structure-activity relationship. Of course, lead compounds found from screening of natural products can be optimized by traditional medicinal chemistry or by application of combinatorial approaches. Overall, when faced with molecular targets in screening assays for which there is no information about low molecular weight leads, use of a natural products library seems more likely to provide the chemical diversity to yield a hit than a library of similar numbers of compounds made by combinatorial synthesis. Since only a small fraction of the world's biodiversity has been tested for biological activity, it can be assumed that natural products will continue to offer novel leads for novel therapeutic agents.

4.2. Experimental Design

4.2.1. *Phytochemical Screening*

The crude extract of the plant was tested to determine the presence of carbohydrates, tannins , steroids, saponins, alkaloids, flavonoids, reducing sugars.

4.2.2. *Microbiological Investigations*

The *in vitro* antimicrobial study was designed to investigate the antibacterial as well as antifungal spectrum of the crude extracts and fractions by observing the growth response. The rationale for these experiments is based on the fact that bacteria and fungi are responsible for many infectious diseases, and if the test materials inhibit bacterial or fungal growth then they may be used in those particular diseases. However, a number of factors viz. the extraction method (Nadir *et al.*, 1986), inocula volume, culture medium composition (Bauer *et al.*, 1966), pH (Levan *et al.*, 1979), and incubation temperature (Lorian, 1991) can influence the results.
Antimicrobial activity was observed by using two methods. The methods are-

a. Kirby- Bauer disk diffusion method.
b. Determination of Minimum Inhibitory Conentration (MIC) & Minimum Bactericidal Concentration (MBC).

4.2.3. *Antioxidant test*

Antioxidant test was done to determine the antioxidant capacity of the various fraction of the *L. coromandelica* skin. The traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. Experiment evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a high number of diseases. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity. Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as byproducts.

Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer,

diabetes, aging, and other degenerative diseases in humans. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant.

Antioxidant property of the various fraction of the plant was determined by following methods-

1.Determination of total phenolic content
2.Determination of DPPH radical scavenging assay
3.Determination of reducing power ability
4.Phosphomolbdate method
5.Determination of total flavonoid content

4.2.4. *Thrombolytic activity test*

One of the major causes of blood circulation problem is the formation of blood clots. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area. A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA). Fribrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by to restore blood supply to ischaemic myocardium, to limit necrosis and to improve prognosis

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic altreplase in most infarct patients while having the advantages of being much less expensive. Tissue-type Plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants

of these drugs. The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity like thrombolytic potentials. Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations have been reported.

5. ANTIOXIDANT TEST

5.1 Rational And Objective

There is considerable recent evidence that free radical induce oxidative damage to biomolecules. This damage causes cancer, aging, neurodegenerative diseases, atherosclerosis, malaria and several other pathological events in living organisms (Halliwell *et al.,* 1992). Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases (Steinmetz and Potter, 1996; Aruoma, 1998; Bandoniene *et al*., 2000; Pieroni *et al.,* 2002; Couladis *et al.,* 2003). A number of reports on the isolation and testing of plant derived antioxidants have been described during the past decade. Natural antioxidants constitute a broad range of substances including phenolic or nitrogen containing compounds and carotenoids (Shahidi *et al.,* 1992; Velioglu *et al.,* 1998; Pietta *et al.,* 1998)

Lipid peroxidation is one of the main reasons for deterioration of food products during processing and storage. Synthetic antioxidant such as *tert*-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) are widely used as food additives to increase self-life, especially lipid and lipid containing products by retarding the process of lipid peroxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects and humans (Ito *et al*., 1986; Wichi,1988), but abnormal effects on enzyme systems (Inatani *et al*., 1983). Therefore, the interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (Jayaprakasha & Jaganmohan Rao, 2000)

5.2 Methods Of Evaluating Antioxidant Activity

Antioxidant property of the various fraction of the plant was determined by following methods-

1. Determination of total phenolic content
2. Determination of total phenolic content Determination of DPPH radical scavenging assay (Quantitative analysis)
3. Determination of reducing power ability
4. Determination of total flavonoids content

5.2.1. *Determination of total phenolic content*

5.2.1.1. Principle

The total phenolic concentration of the extract of *Lannea coromandelica* bark and leaf was determined by the modified Folin-Ciocalteu method (Wolfe *et al.*, 2003). The process of measuring total phenolic content of the crude extract involves the use of Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It measures the amount of substance being tested needed to inhibit the oxidation of the Folin-Ciocalteu reagent. The reagent does not contain phenol. Rather, the reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically. The generated chromogens give a strong absorption maximum at 760 nm.

5.2.1.2. Materials & Reagents

Test tube	Analytical balance
Pipette	UV- visible spectrophotometer
Spatula	Vortex mixer
Folin-Ciocalteu reagent	Distilled water
Sodium carbonate (Na_2CO_3)	Methanol
Gallic acid	Aluminium foil

5.2.1.3. Methods

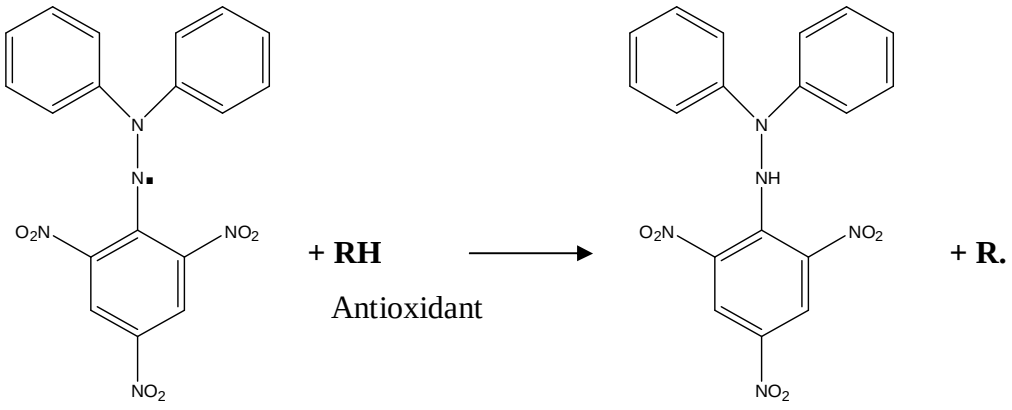
- 0.5ml of a methanol solution of the crude extract of concentration of 1mg/ml was mixed with 5ml Folin ciocalteu reagent (1:10 v/v distilled water) and 4 ml (75g/L) of Sodium carbonate.
- The mixture was vortexed for 15 second and allowed to stand for 30min at room temperature in dark place for color development and the absorbance was measured at 760 nm against methanol as blank by using a UV- visible spetrophotometer.

- The total phenolics was expressed as gm of GAE (gallic acid equivalent)/100gm of the dried extract using the following equation obtained from a standard Gallic acid calibration curve:
  $y = .0162x + 0.0215$, $R^2 = 0.9985$.

5.2.2. *Determination of DPPH radical scavenging assay (Quantitative analysis)*

5.2.2.1. Principle

A rapid, simple and convenient method to measure free radical scavenging capacity of antioxidants involves the use of the free radical, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. DPPH is a stable nitrogen centered free radical with purple color and the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm. When the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H, then the color turns from purple to yellow as the molar absorptivity of the DPPH radical reduces from 9660 to 1640 at 517 nm. Scavenging of DPPH free radicals by antioxidants decreases the absorbance. The lower the absorbance at 517 nm, the greater the free radical scavenging capacity of the crude extracts.



DPPH (oxidized form) DPPH (reduced form)

Diphenyl picrylhydrazyl

5.2.

517 nm

ı,ı-ɑıpnenyı-ƨ-pıcryınydrazyl UV- visible spectrophotometer

L-Ascorbic acid Beaker (100 & 200ml)

Distilled water	Test tube
Methanol	Aluminium foil
Pipette (5ml)	Spatula

5.2.2.3. Methods

- 2.0 ml of a methanol solution of the extract at different concentration (2, 4, 6, 8, 10µg/ml) were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml).
- After 30 min reaction period at room temperature in dark place the absorbance was measured against at 517 nm against methanol as blank by using a UV- visible spetrophotometer.
- Inhibition free radical DPPH in percent (I%) was calculated as follows:

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

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

- Extract concentration providing 50% inhibition *(IC_{50})* was calculated from the graph plotted inhibition percentage against extract concentration.
- L-Ascorbic acid was used as positive control.
- Tests carried out in triplicate and average value was taken.

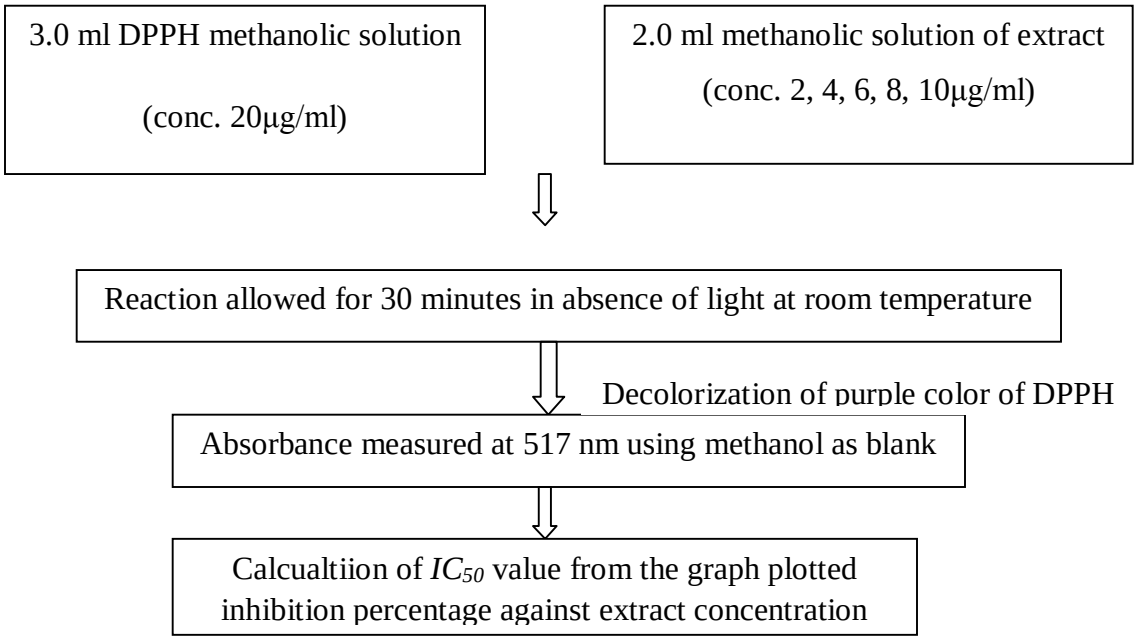


Figure: 5.1.Schematic representation of the method of assaying free radical scavenging activity

5.2.3. *Determination of reducing power assay*

5.2.3.1. Principle

The reducing power assay of the extract of *Spondias pinnata* fruit was determined according to the method previously described by (Oyaizu *et.al.*, 1986). Reducing power assay is based on the principle that substances which have reduction potential react with potassium ferricyanide [$K_3Fe(CN)_6$] to form potassium ferrocyanide [$K_4Fe(CN)_6$], which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard.

5.2.3.2. Materials & Reagents

Test tube	Analytical balance
Pipette	UV- visible spectrophotometer
Spatula	Hot air oven
Vortex mixer	Centrifuge machine
0.2 M Phosphate buffer	1 % Potassium ferricyanide [$K_3Fe(CN)_6$]
10 % Trichloroacetic acid (TCA)	Distilled water
Ferric chloride **($FeCl_3$)**	L-ascorbic acid

5.2.3.3. Methods

- 1 ml of the methanol solution of the crude extract of different concentrations (1, 5, 10, 50, 100µg/mL) was mixed with 2.5 ml phosphate buffer and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] (1%).
- The mixture was incubated at 50°C for 20min.
- 2.5mL of trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000rpm for 10min.

- The upper layer of the solution was separated and mixed with 2.5 ml distilled water and 0.5ml $FeCl_3$.
- The absorbance was measured against a blank at 700nm.
- All the tests were carried out in triplicate and average absorption was noted for each time.
- L-Ascorbic acid was used as positive control.
- Percentage (%) increase in reducing power was calculated as follows:

  % Increase in reducing power = $(A_{Test} / A_{Blank}) -1 X 100$

  Where A_{Blank} is absorbance of blank (containing all reagents except the test material)

  A_{Test} is absorbance of test solution.

5.2.4 *Total Flavonoids Concentration*

5.2.4.1 Principle

Aluminium chloride ($AlCl_3$) (Chang C *et al.,* 2002) colorimetric method is incorporated to determine the total flavonoid contents of the crude plant extract. The amount of flavonoid in the crude extract can be quantified by measuring the absorbance of reaction mixture at 415 nm using a UV-visible spectrophotometer against a blank containing all reagents except the extracts. Quercetin at various concentrations was used as standard.

5.2.4.2 Materials & Reagents

Methanol	UV- visible spectrophotometer
10% aluminum chloride ($AlCl_3$)	Test tube
1M potassium acetate (CH_3COOK)	Aluminium foil
Distilled water	Spatula

Pipette (5ml) Analytical balance

5.2.4.3 Methods (Chang C *et al.*, 2002)

- 0.5 ml of a methanol solution of the extract of concentration of 10 mg/ml was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Sample blank was prepared in similar way by replacing aluminium chloride with distilled water.
- Both sample and blank solution were filtered through double rings filter paper before measuring the absorbance
- Both sample and blank solution were allowed to stand for 30 min at room temperature.
- After 30 minute incubation period, the absorbance of the reaction mixture was measured at 415 nm against a blank by using a UV- visible spectrophotometer.
- All the tests were carried out in triplicate and average absorption was noted for each time.
- The total flavonoids was expressed as mg of Quercetin equivalent per gram of dried extract by using the equation obtained from a standard Quercetin calibration curve

$y = 0.002x + 0.0318$; $R^2 = 0.9989$.

5.3.5 Determination of total antioxidant capacity by phosphomolybdenum method

5.3.5.1 Principle

Phosphomolybdenum assay method is a spectroscopic method for the quantitative determination of antioxidant capacity of crude plant extract. The antioxidant capacity of the crude extract can be quantified by measuring the absorbance of reaction mixture at 695 nm after cooling to room temperature by using a UV-visible spectrophotometer against a blank containing all reagents except the extracts. Ascorbic acid at various concentrations was used as standard. The higher absorbance value indicated higher antioxidant activity.

5.3.5.2 Materials & Reagents

Ethanol UV- visible spectrophotometer

0.6 M Sulfuric acid (H_2SO_4)	Test tube
28mM Sodium Phosphate (Na_2HPO_4)	Aluminium foil
4mM Ammonium molybdate [$(NH_4)_6 Mo_7O_{24}$]	Spatula
Pipette (5ml)	Analytical balance
Hot air oven	L-Ascorbic acid

5.3.5.3 Methods (Prieto *et al.*, 1999)

- 0.3 ml of ethanolic solution of the extract of concentration of 2 mg/ml was mixed with 3 ml of reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate) (all of them were taken in equal volume). Sample blank was prepared in similar way by replacing sample extract with ethanol.
- The reaction mixture was incubated at 95°C for 90 min.
- After that, the samples were allowed to cool to room temperature.
- The absorbance of the reaction mixture was measured at 695 nm against a blank by using a UV- visible spectrophotometer.
- All the tests were carried out in triplicate and average absorption was noted for each time.
- The total antioxidant activity of the crude extract was expressed as the number of grams of ascorbic acid equivalents per gram of dried extract by using the equation obtained from a standard Ascorbic acid calibration curve

$y = 0.113x - 0.146$; $R^2 = 0.9916$.

6. ANTIMICROBIAL SCREENING

6.1 Introduction

Worldwide, infectious disease is one of main causes of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States. Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992; an increase of 58% .It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US (Pinner *et al*., 1996). This is alarming given that it was once believed that we would eliminate infectious disease by the end of the millenium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increases are occurring in the 25–44 year old age group (Pinner *et al*., 1996).

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998).

The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* fungal and bacterial growth. This ability may be estimated by disc diffusion method.

But there is no standardized method for expressing the results of antimicrobial screening (Ayafor *et al*., 1982). Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculum volume, culture medium composition (Bayer *et al*., 1966), p^H, and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion (Bayer *et al*., 1966) is a widely accepted in vitro investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity

or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method (Roland R, 1982).

6.2 Principle of Disc Diffusion Method

In this classical method, antibiotics diffuse from a confined source through the nutrient agar gel and create a concentration gradient. Dried and sterilized filter paper discs (6 mm diameter) containing the test samples of known amounts are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs are used as positive and negative control. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion of the test materials to the surrounding media (Barry, 1976). The plates are then inverted and incubated at 37°C for 24 hours for optimum growth of the organisms. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The antimicrobial activity of the test agent is then determined by measuring the diameter of zone of inhibition expressed in millimeter (Barry, 1976; Bayer *et al.*, 1966.)

In the present study the crude extracts, fractions as well as some pure compounds were tested for antimicrobial activity by disc diffusion method. The experiment is carried out more than once and the mean of the readings is required (Bayer *et al.,* 1966).

6.3. Experimental

6.3.1. *Apparatus and Reagents*

1. Filter paper discs
2. Autoclave
3. Nutrient Agar Medium
4. Laminar air flow hood
5. Petridishes
6. Spirit burner
7. Sterile cotton
8. Refrigerator
9. Micropipette
10. Incubator
11. Inoculating loop
12. Chloroform
13. Sterile forceps
14. Ethanol

15. Screw cap test tubes **16.** Nosemask and Hand gloves

6.3.2. *Test Materials of L. coromandelica*

1. *n*-hexane soluble fraction of etanolic extract
2. Di chloro methane soluble fraction of etanolic extract
3. Aqueous soluble fraction of the etanolic extract

6.3.3. *Test Organisms*

The bacterial and fungal strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 6.1.

Table 6.1: List of micro-organisms used

Gram positive Bacteria	**Gram negative Bacteria**	**Fungi**
Bacillus cereus	*Escherichia coli*	*Candida albicans*
Bacillus subtilis	*Salmonella paratyphi*	*Sacharomyces cerevacae*
Sarcina lutea	*Salmonella typhi*	
Staphylococcus aureus	*Shigella boydii*	
	Shigella dysenteriae	
	Pseudomonas aeruginosa	
	Vibrio mimicus	

6.3.4. *Culture Medium and their Composition*

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

a. Nutrient agar medium

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
p^H	7.2 + 0.1 at 25^0C

Agar medium having this composition was directly brought from the market.

6.3.5. *Preparation of the Medium*

To prepare required volume of this medium, calculated amount of agar medium was taken in a bottle with a cap and distilled water was added to it to make the required volume. The contents were then autoclaved to make a clear solution.

6.3.6. *Sterilization Procedure*

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petridishes and other glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

6.3.7. *Preparation of Subculture*

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The

inoculated strains were then incubated for 24 hours at 37^0C for their optimum growth. These fresh cultures were used for the sensitivity test.

6.3.8. ***Preparation of the Test Plate***

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium. The bacterial and fungal suspension was taken by a loop a mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial/ fungal suspension. Then the bacterial/fungal sample is applied to the petridish with the help of this cotton bud.

6.3.9. ***Preparation of Discs***

Three types of discs were used for antimicrobial screening.

6.3.10. ***Standard Discs***

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Cephradin (30μg/disc) standard disc was used as the reference.

6.3.11. ***Blank Discs***

These were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

6.3.12. ***Preparation of Sample Discs with Test Sample***

Measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

6.3.13. *Preparation of Sample Discs with Test Sample*

Measured amount of each test sample (specified in table) was dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

Table 6.2: Preparation of Sample Discs

Sample	**Dose** (μg/disc)	**Required amount for 20 disc** (mg)
Hexane soluble fraction of ethanolic extract	400	8.0
Hexane soluble fraction of ethanolic extract	800	16
dichloromethane soluble fraction of ethanolic extract	400	8.0
dichloromethane soluble fraction of ethanolic extract	800	16
Aqueous soluble fraction of ethanolic extract	400	8.0
Aqueous soluble fraction of ethanolic extract	800	16

6.3.14. Application of the Test Samples

Standard Cephradin (30 μg/disc) discs were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of produced by the test sample. Blank discs were used as negative controls which ensure that the residual solvents (left over the discs even after air-drying) and the filter paper were not active themselves.

6.3.15. *Diffusion and Incubation*

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria and fungi. The plates were then kept in a refrigerator at 4^0C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^0C for 24 hours.

6.3.16. *Determination of Antimicrobial Activity by Measuring the Zone of Inhibition*

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the Antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

Figure 6.1*:* Zone of inhibition

6.4 Minimum Inhibitory Concentration

6.4 1 *Introduction*

The aim of this study was to determine the minimum inhibitory concentration (MIC) of the fraction ethanolic extract required to kill *S. aureus, S. typhi, S. paratyphi and S. boydi*. In the experiment, medicaments were added to bacterial species into eppendrof tube, in 10 different concentrations. The MIC was the lowest concentration of the drug at which bacterial growth could not be observed.

6.4.2 *Principle of MIC*

The disc diffusion method, which is a 'semi-quantitative' method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. Next to the agar dilution method, the broth dilution method is one of the most frequently used methods to determine MIC's. The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the extract for which the MIC is determined. The first

concentration in the dilution series at which no visual growth can be determined is then considered as the MIC.

6.4.3. *Experimental*

6.4.3.1. Apparatus and reagents

Nutrient Agar Medium	Autoclave
Nutrient Broth Medium	Eppendrof tube
Petridishes	Laminar air flow hood
Sterile cotton	Spirit burner
Micropipette	Refrigerator
Inoculating loop	Incubator
Sterile forceps	Ethanol
Screw cap test tubes	Nosemask and Hand gloves

6.4 3.2. Test Materials of *Lannea coromandelica*

- Dichloromethane fraction of *Lannea coromandelica* leaf of ethanolic extract. This fraction was tested for MIC because we got a potent antimicrobial result in that fraction.

6.4.3.3. Test Organisms

The bacterial and fungal strains used for the experiment were collected as pure cultures from the East West University microbiology lab. Both gram positive and gram-negative organisms were taken for the test and they are listed in the Table 6.3.

Table 6.3: List of micro-organisms used

Gram positive bacteria	**Gram negative bacteria**
Staphylococcus aureus	*Salmonella paratyphi*

	Salmonella typhi *Shigella boydii*

6.4.3.4. Culture Medium and their Composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

b. Nutrient agar medium

Ingredients	**Amount**
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s.	100 ml
P^H	7.2 + 0.1 at 25^0C

Agar medium having this composition was directly brought from the market.

c. Nutrient broth medium

Ingredients	**Amount**
Bacto beef extract	0.3 gm
Bacto peptone	0.5 gm
Distilled water q.s.	100 ml
P^H	7.2 ±0.1 at 25^0C

6.4.4 *Method description* (Huys, 2002)

I. Bacterial cultivation and material preparations (DAY 1-2)

• The organism to be tested should be sub cultured using a suitable medium under optimal incubation conditions to obtain a fresh overnight grown culture. As standard conditions, growth in Agar and Broth at 28 °C under aerobic atmosphere is recommended. For sub culturing, remove one bead from the cryovial and streak out onto an ISA plate. Incubate at 28°C overnight (or longer until clear visible growth is observed).

• After overnight incubation, the streak cultures are checked for purity. A number of pure colonies (app. 5 or more if the isolate is a suspected fastidious organism) are introduced into a glass culture tube containing 10 mL broth and incubated at 28°C overnight (or longer until clear visible growth is observed). Ideally, the culture tubes should fit into a portable spectrophotometer. In this way, the overnight grown culture can be easily adjusted to a standardized cell density by dilution with sterile broth

• Control cultures should be included during each series of MIC determinations.

• A bottle with the necessary volume of double-distilled water should be prepared.

II. Preparation and inoculation of the dilution series (DAY 3)

• The optical density of the overnight culture of the strain is determined spectrophotometrically at 590 nm and is standardized at 0.1 + 0.02 (i.e. app. 10E8 CFU/mL) by diluting with sterile broth.

• For each batch of 4 strains, two 6 mL sterile stock solutions of extract should be prepared in the suitable solvent. However, subsequent dilutions of these stock solutions can be made up in sterile water. The use of two stock solutions is recommended to minimize quantitative errors in the low-concentration range of the serial dilution series.

• Extract concentrations 40, 20, 10, 5, 2.5, and 1.25, 0.625, 0.3125, 0.15625, 0.078125 mg/ml are obtained by making serial dilutions from stock solution. It is absolutely crucial to thoroughly mix every freshly prepared antibiotic dilution prior to using it to prepare the next dilution. It should

be noted that each antibiotic dilution undergoes a final 1:2 dilution when the broth culture is added. If required, the tested MIC range can be extended with additional concentrations.

• Following the preparation of the serial dilutions of extract, 2 mL of freshly standardized broth culture of the strain is inoculated in each tube of the dilution series. In this regard, it is important to note that the standardized cultures should be processed within the hour after preparation. Dilutions and broth cultures should be well homogenized prior to mixing.

• For each batch of MIC determinations, a blank tube (i.e. 2 mL non-inoculated broth mixed with 2 mL water) should be included. In addition, a positive control should be included for each strain. The positive control is made up by mixing 2 mL adjusted broth culture with 2 mL sterile double distilled water.

• All MIC tubes and control tubes of the test isolates and the control strain as well as the blanc are incubated aerobically at 28°C for 24h.

• Each isolate should also be checked for purity by plating a drop of the adjusted culture onto agar medium, and this plate should be incubated under the same conditions as the MIC test itself.
III. Reading of the MIC (DAY 4)

• The purity of the broth culture is checked on agar on the basis of uniform colonial morphology. If contamination is noted than all data generated from the involved strain should be rejected.

• Following a 24 h incubation (or longer until clear visual growth can be determined in the positive control tubes), growth is determined visually among the different tubes of the serial dilution by comparing with the positive control and with the blank. Any series where discontinuity in growth is observed (e.g. growth in tubes 5 and 7 but not in tube 6) should be discarded. The end-point is defined as the lowest antibiotic concentration for which there is no visual growth. This concentration should be reported as the MIC of that antibiotic for that

particular strain. If trailing end-points are observed, this should be reported as a remark and a 80% reduction in growth should be reported as end-point.

7. THROMBOLYTIC ACTIVITY STUDY

7.1 Introduction

Thrombolytic therapy reduces mortality and preserves left ventricular function in patients with myocardial infarction. Streptokinase is widely used fibrinolytic drug that was used in this study as standard. All thrombolytic agents work by activating the enzyme plasminogen that clears the cross-linked fibrin mesh. We test our sample for its thrombolytic activity. All thrombolytic agents activate the enzyme plasminogen that clears the cross linked fibrin mesh. Fribrinolytic drugs can dissolve thrombi in acutely occluded coronary arteries thereby can restore blood supply to ischaemic myocardium and can limit necrosis (Laurence DR, 1992).

7.2 Principle

The in vitro thrombolytic activity of the crude ethanolic extract *Spondias pinnata* (fruit) was determined according to the method reported earlier by Prasad, S. *et al.* (2006). Whole blood drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy was incubated for 45 minutes at 37°C to form clot. After clot formation, the serum was completely removed without disturbing the clot and the weight of the formed clot was taken. Addition of crude extracts may result in the lysis of some of the clot. For clot lysis, the tubes were incubated for 90 minutes at 37°C. Thereby the weight of the clot will decrease. The study was implemented on two volunteers and the average value of weight loss (in %) was calculated to examine the variation of two volunteer. Difference obtained in weight taken before and after clot lysis was expressed as percentage (%) of clot lysis as shown below: **(**Prasad, S. *et al.* 2006)

% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

7.3 Preparation Of Test Sample **(**Prasad, S. *et al.* 2006)

100 mg of ethanolic extract of *Spondias pinnata* (fruit) was suspended in 10 ml distilled water and shaken vigorously on a vortex mixer. Then the suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through filter paper. The solution was then ready for in vitro evaluation of clot lysis activity.

7.4 Methods **(**Prasad, S. *et al.* 2006)

- 10 sterile eppendorf tubes were taken and weights (W_1) were measured.

- 10 ml venous blood samples were drawn from two healthy human volunteers (5 ml from each) without a history of oral contraceptive or anticoagulant therapy.
- The blood samples were then equally distributed into 10 separate pre weighed (W_1) eppendorf tubes (each contains 1 ml) and incubated at 37°C for 45 minutes for clot formation.
- After clot formation, serum was completely decanted and removed without disturbing the clot formed.
- Each eppendorf tube having clot was again weighed to determine the clot weight.

  Clot weight (ΔW) = (weight of clot containing tube, W_2 – weight of tube alone, W_1)

- Each eppendorf tube containing clot was properly labeled and 100 µl of plant extract was added to six eppendorf tubes.
- Streptokinase (100 µl) was added to two eppendorf tubes. (Positive control)
- Distilled water (100 µl) was added to two eppendorf tubes. (Negative control)
- All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis.
- After incubation, fluid obtained was removed and eppendorf tubes were again weighed (W_3)
- The weight of released clot was then calculated.

  Weight of released clot = (weight of clot containing tube, W_2 – weight of tube after after clot disruption, W_3)

- Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

  % clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

  $$= [(W_2 - W_3) / (W_2 - W_1)] \times 100$$

8. RESULTS AND DISCUSSION OF THE TEST SAMPLES OF LANNEA COROMANDELICA

The aim of this chapter is to illustrate the results and discussions of crude ethanolic extract of *Lannea coromandelica* **(**leaf and bark). This chapter will include the following results and discussions of the test samples of *Lannea coromandelica* **(** leaf and bark):

1. In vitro antioxidant activity

    1.1. Total phenolic content

    1.2. DPPH radical scavenging assay (Quantitative analysis) and IC_{50}

    1.3. Reducing power assay

    1.4. Total antioxidant capacity by phosphomolybdenum method

    1.5. Total flavonoids content

2. In vitro antimicrobial screening

3. Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC)

4. In vitro thrombolytic activity

Besides the results of various tests will be represented via suitable graphical representation method.

8.1 In Vitro Antioxidant Activity

8.1.1. *Total phenolic content*

The crude ethanolic extract of *Lannea coromandelica* (leaf and bark*)* was subjected to total phenolic content determination. Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid (table 8.3) equivalents, result of the colorimetric analysis of the total phenolics are given in table 8.4. Total phenolic content of the samples are expressed as mg of GAE (gallic acid equivalent) per gm of dried extract. The phenolic content found in the crude ethanolic extract of *Lannea coromandelica* (leaf and bark*)* was 329.88 mg of gallic acid (GAE) per gm of dried extract.

Table 8.1 Standard curve preparation by using gallic acid

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	100	1.620	y = 0.0162x + 0.0215	0.9985
2	50	0.866		
3	25	0.450		
4	12.5	0.253		
5	6.25	0.120		
6	3.125	0.059		
8	1.5625	0.034		
8	0.88125	0.022		
9	0.3906	0.020		
10	0	0.011		

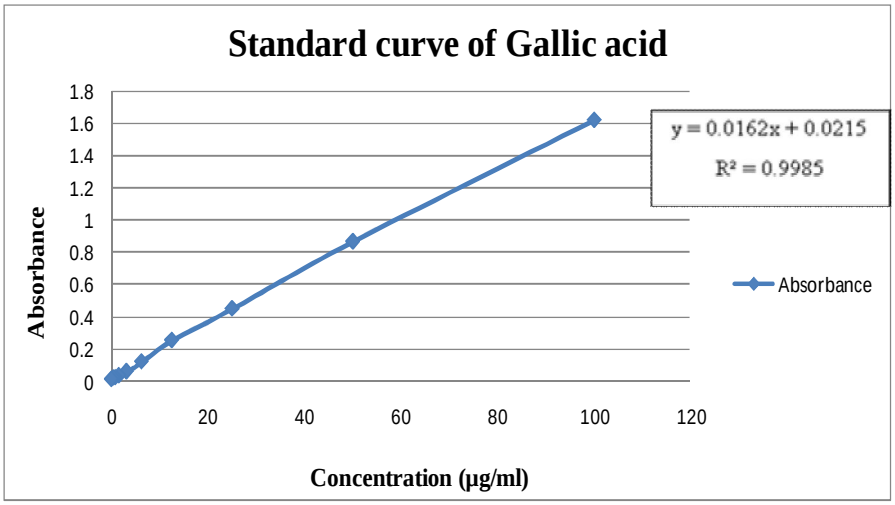


Figure 8.1 Standard curve for gallic acid

Table 8.2 Total phenolic content assay

Sample	**Conc (mg/ml)**	**Absorbance**	**mg of Gallic acid equivalent (GAE) per gm of dried extract)**
Lannea coromandelica bark	1mg/ml	.419	462.10

Lannea coromandelica leaf	1mg/ml	.408	450.08

8.1.2 *Determination of DPPH radical scavenging assay*

Table 8.3: DPPH radical scavenging assay for sample

Name of Extract	**Concentrations** µg /ml	**Absorbance**	**% inhibition of free radical DPPH = (1 – A_{Sample}/A_{Blank}) X 100**	**IC_{50}**
LCBD 1	2	0.035	81.08	3.8 µg /ml
LCBD 2	4	0.031	84.38	
LCBD 3	6	0.026	88.51	
LCBD 4	8	0.022	81.82	
LCBD 5	10	0.016	86.88	
LCBN 1	2	0.12	0.83	8 µg /ml
LCBN 2	4	0.1	18.36	
LCBN 3	6	0.085	38.02	
LCBN 4	8	0.033	82.83	
LCBN 5	10	0.025	89.34	
LCL C 1	2	0.098	16.338	6 µg /ml
LCL C 2	4	0.089	23.282	
LCL C 3	6	0.058	50.862	
LCL C 4	8	0.041	64.665	
LCL C 5	10	0.028	86.828	

N.B:

Absorbance of control, $A_{control}$ = 0.121

LCBN= *Lannea coromandelica* bark(n-Hexane)

LCBD = *Lannea coromandelica* bark (DCM)

LCL C = *Lannea coromandelica* leaf (crude)

Different partitionates of ethanolic extract of *S. pinnata* were subjected to free radical scavenging activity by the method of Brand-Williams *et al.*, 1995. Here, tert-butyl-1-hydroxytoluene (BHT) was used as reference standard.

Table8.4: DPPH radical scavenging assay for standard

SL No	Absorbance of Blank	Concentration (µg/ml)	Absorbance of Extract	%Inhibition	IC_{50}(µg/ml)
1	0.388	500.000	0.020	94.809	**24.00**
2	0.388	250.000	0.024	93.651	
3	0.388	125.000	0.035	90.841	
4	0.388	62.500	0.099	83.810	
5	0.388	31.250	0.149	60.582	
6	0.388	15.625	0.232	38.624	
8	0.388	8.813	0.264	30.159	
8	0.388	3.906	0.291	23.016	
9	0.388	1.953	0.303	19.841	
10	0.388	0.988	0.308	18.519	

In this investigation, the dichloromethane soluble partitionate of ***Lannea coromandelica*** bark showed the highest free radical scavenging activity with IC_{50} value 3.8 µg/ml.that means it shows hiher activity than the standard BHT in lower concentration.

Table8.5: *Lannea coromandelica* bark dichloromethane fraction

Name of Extract	**Concentrations** µg /ml	**Absorbance**	**% Inhibition**	**IC $_{50}$**
LCBD 1	2	0.035	45.08	3.8 µg /ml
LCBD 2	4	0.031	58.38	
LCBD 3	6	0.026	88.51	
LCBD 4	8	0.022	81.82	
LCBD 5	10	0.016	86.88	

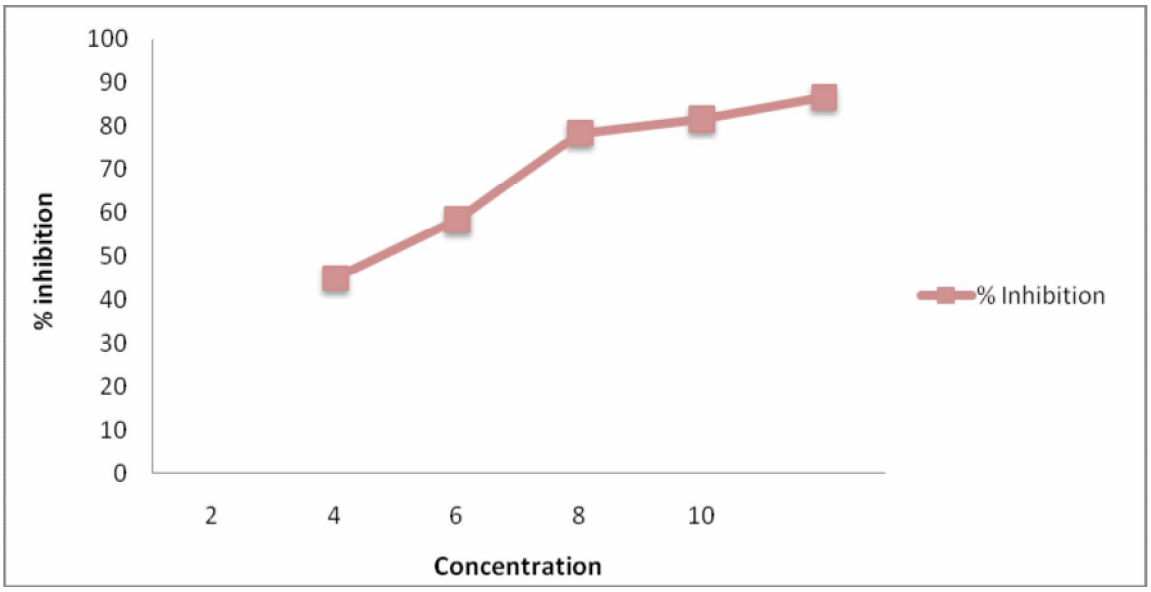


Figure 8.2: IC_{50} value calculation for *Lannea coromandelica* bark dichloromethane fraction

At the same time the n-hexane soluble partitionate also exhibited strong antioxidant potential having IC_{50} value 8 µg/ml .

Table8.6: Lannea coromandelica bark N-hexane fraction

Name of Extract	**Concentrations** µg /ml	**Absorbance**	**% Inhibition**	**IC $_{50}$**
LCBN 1	2	0.120	0.83	8 µg /ml
LCBN 2	4	0.100	18.36	
LCBN 3	6	0.085	38.02	
LCBN 4	8	0.033	82.83	
LCBN 5	10	0.025	89.34	

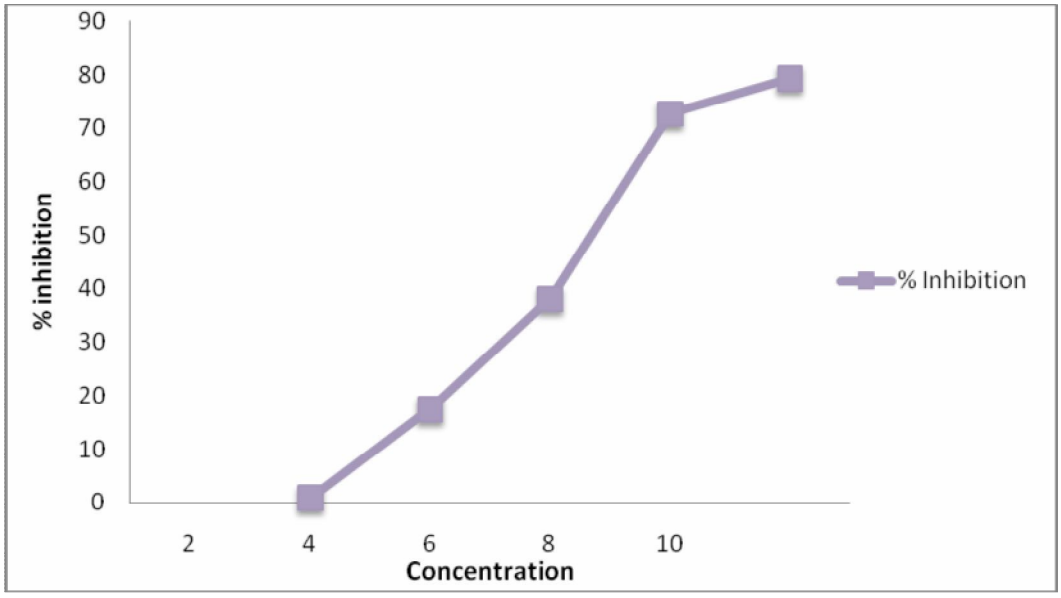


Figure 8.3: IC_{50} value calculation for *Lannea coromandelica* bark n-hexane fraction

Lannea coromandelica leaf crude ethanolic extract also revealed potent scavenging activity IC_{50} 6 µg/ml .

Table 8.7*: Lannea coromandelica* leaf crude

Name of Extract	**Concentrations** µg /ml	**Absorbance**	**% Inhibition**	**IC $_{50}$**
LCL C 1	2	0.098	16.338	6 µg /ml
LCL C 2	4	0.089	23.282	
LCL C 3	6	0.058	50.862	
LCL C 4	8	0.041	64.665	
LCL C 5	10	0.028	86.828	

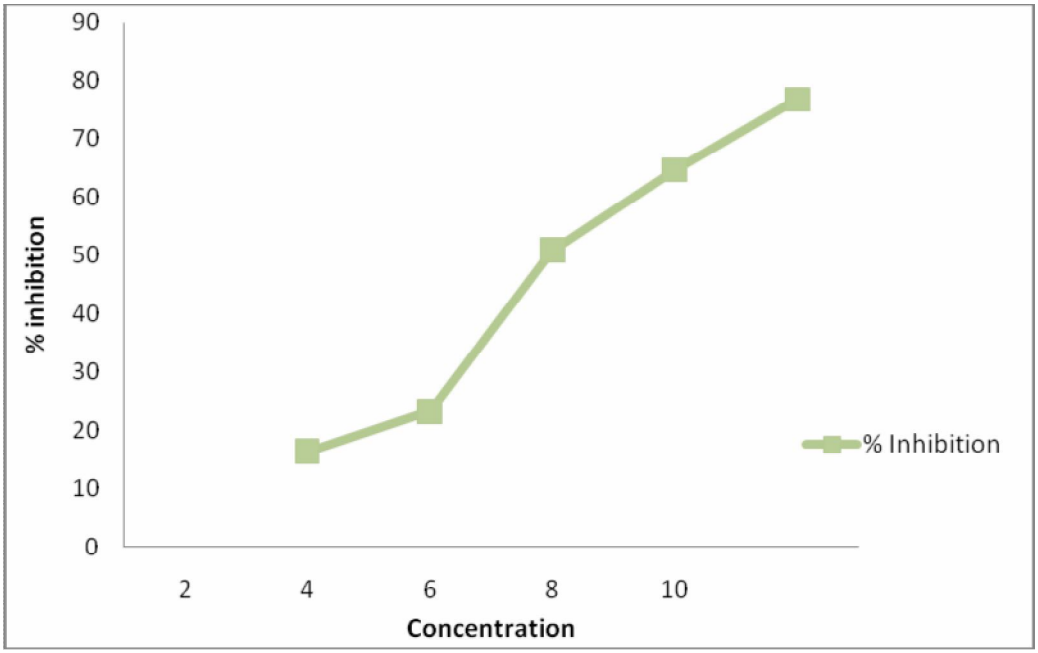


Figure 8.4: IC_{50} value calculation for *Lannea coromandelica* leaf crude ethanolic fraction

8.1.3 *Determination of reducing power assay*

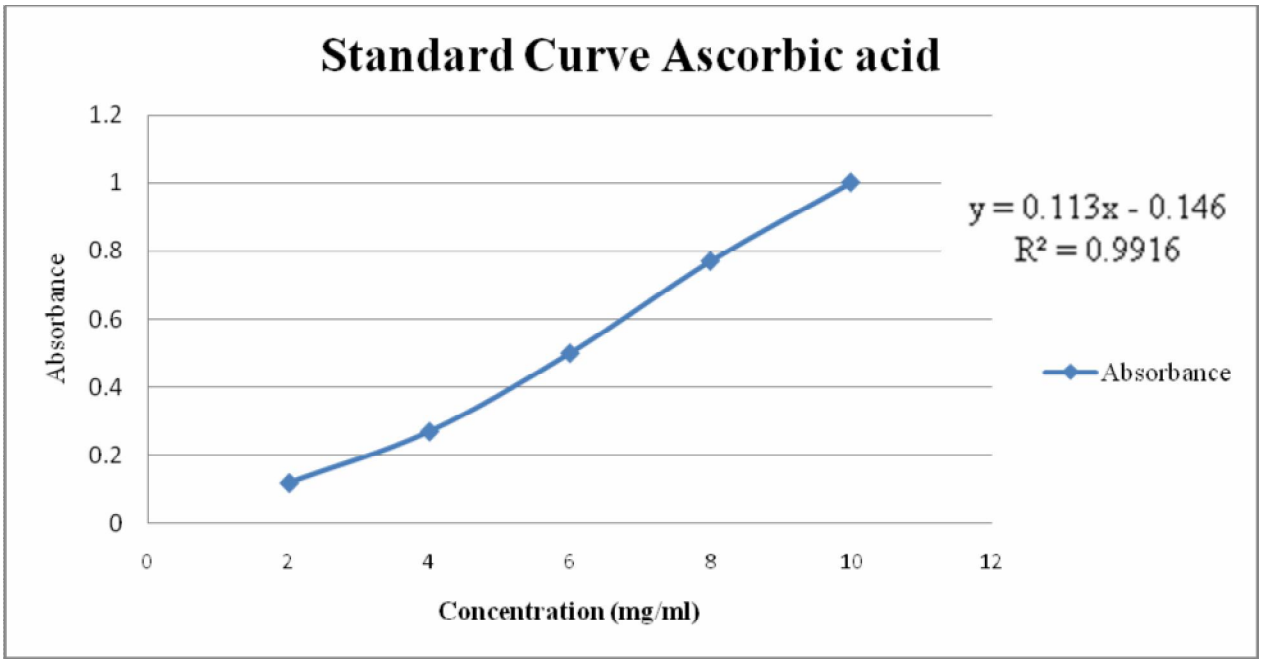


Figure 8.5: Reducing power for ascorbic acid

Table 8.8: Result of ***Lannea coromandelica*** reducing power test

	SL no	Absorbance of blank, A_{Blank}	Concentration (µg/ml)	Absorbance of test, A_{Test}	% increase in reducing power $= (A_{Test} / A_{Blank}) -1 X 100$
LCLN	1		1	0.108	21.34831461
	2		5	0.121	35.95505618
	3		10	0.136	52.80898876
	4		50	0.151	69.66292135

	5	0.089	100	0.163	83.14606742
LCLD	1		1	0.116	30.33707865
	2		5	0.122	37.07865169
	3		10	0.139	56.17977528
	4		50	0.145	62.92134831
	5		100	0.156	75.28089888
LCLAQ	1		1	0.095	6.741573034
	2		5	0.111	24.71910112
	3		10	0.125	40.4494382
	4		50	0.129	44.94382022
	5		100	0.137	53.93258427
LCBD	1		1	0.111	24.71910112
	2		5	0.119	33.70786517
	3		10	0.129	44.94382022
	4		50	0.135	51.68539326
	5		100	0.161	80.8988764
LCBN	1		1	0.105	17.97752809
	2		5	0.126	41.57303371
	3		10	0.138	55.05617978
	4		50	0.146	64.04494382

	5		100	0.151	69.66292135

Here, the entire fraction produces good reducing power capability. Higher absorbance indicates formation of high amount of ferrous complex due to reduction of potassium ferricyanide into potassium ferrocyanide.

Lannea coromandelica **leaf:**

- **Dichloromethane fraction:** among oter fractions DCM produces highest absorbance .156 at 100 µg/ml concentration and %increae in reducing power is75.28.
- **Aqueous fraction** : it produces highest absorbance .137 at 100 µg/ml concentration and %increae in reducing power is 75.28.
- **n-hexane fraction** : produces highest absorbance.151 at 100 µg/ml concentration and %increae in reducing power is 69.66

Lannea coromandelica **bark:**

- **n-hexane fraction** : produces highest absorbance .151 at 100 µg/ml concentration.that is %increae in reducing power is69.668.
- **Dichloromethane fraction:** it produces highest absorbance .161 at 100 µg/ml concentration.and %increae in reducing power is 80.88That is poor than leaf extract of DCM fraction.

8.1.4 *Total flavonoids content*

To determine the total flavonoids content of crude ethanolic extract of *Spondias pinnat*a (fruit) using Chang et al method, a standard curve is needed which is obtained from a series of different quercetin concentrations (table 7.9). The total flavonoids content of the sample is expressed as mg of quercetin per gm of dried extract in table 7.10 by using the standard curve equation of quercetin ($y = 0.002x + 0.0318$, $R^2 = 0.9989$). Where y is absorbance at 415 nm and x is flavonoid content of crude plant extract. The total flavonoids content found in the crude ethanolic extract of *Spondias pinnata* (fruit) was 225.60 mg of quercetin per gm of dried extract.

Table 8.9 Standard curve preparation by using quercetin

SL. No.	Concentration (µg /ml)	Absorbance	Regression line	R^2
1	2.5	0.0365	y = 0.002x + 0.0318	0.9989
2	5	0.0417		
3	10	0.0521		
4	20	0.0735		
5	30	0.0906		
6	40	0.1127		

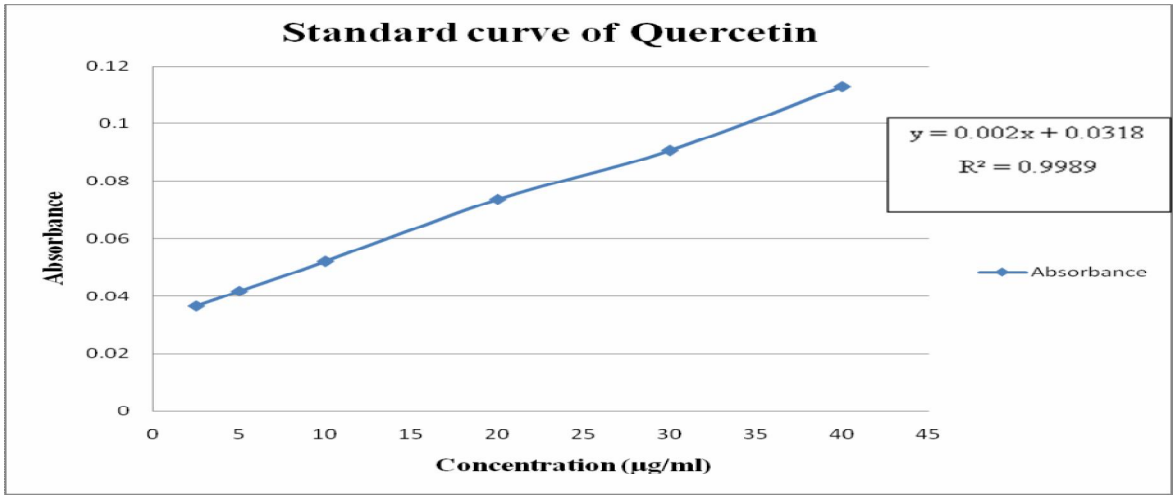


Figure 8.7: Standard curve for quercetin

Table 8.10 Total flavonoids content assay

Sample	Conc (mg/ml)	Absorbance	mg of quercetin equivalent per gm of dried extract)
LCBN	10 mg/ml	0.329	148.6
LCBD	10 mg/ml	0.413	190.6
LCLN	10 mg/ml	0.291	129.2
LCL AQ	10 mg/ml	0.223	95.6
LCLD	10 mg/ml	0.396	182.1

8.2. In Vitro Antimicrobial Screening

Table8.11: In vitro antimicrobial screening

Microorganisms	**LCBDCM800**	**LCBDCM400**	**LCBNH800**	**LCBNH400**	**LCBAQ800**	**LCBAQ400**	**LCLDCM800**	**LCLDCM400**	**LCLAQ800**	**LCLAQ400**	**LCLNH800**	**LCLNH400**	**Control**
Shigella boydii	12	0	0	0	0	0	14	13	0	0	0	0	23
Bacillus subtilis	12	10	10	8	0	0	11	10	0	0	0	0	26
Candida albicans	0	0	0	0	0	0	15	12	0	0	0	0	28
Saccharomyces cerevisiae	8	0	0	0	0	0	10	8	0	0	0	0	20

Vibrio mimicus	10	0	0	0	0	0	19	18	0	0	0	0	22
Shigella dysentery	12	10	8	8	9	10	16	10	11	10	14	9	22
Escherichia coli	10	0	0	0	0	0	16	10	0	0	10	8	28
Bacillus cereus	8	0	0	0	0	0	19	18	0	0	11	8	25
Salmonella typhi	12	0	0	0	0	0	16	15	0	0	0	0	20
Salmonella paratyphi	11	0	0	0	0	0	16	11	0	0	0	0	25
Pseudomonas aeruginosa	12	11	12	8	10	8	16	11	11	8	10	0	25
S.lutea	8	0	0	0	0	0	16	15	0	0	0	0	21
Staphyloccus aureus	9	0	0	0	0	0	14	12	0	0	0	0	15

The antimicrobial activities of extracts were examined in the present study. The results are given in table. The zones of inhibition produced by the *n*-hexane, dichloromethane, ethyl acetate and aqueous soluble partitionates of the ehanolic extract were ranged from 0-15 mm, 8-14 mm, 0-18 mm and 0-12 mm respectively at a concentration of 400µg/disc.

The zones of inhibition produced by the *n*-hexane, dichloromethane, and aqueous soluble partitionates of the ehanolic extract were ranged from 0-13 mm, 10-19 mm, 0-18 mm and 0-16 mm respectively at a concentration of 800µg/disc.

The dichloro metahne partitionate of the ehanolic extract of *L. coromandelica* bark (800 µg) showed moderate activity against *Shigella boydii, Bacillus subtilis, Shigella dysentery, Salmonella typh (*zone of inhibition 12mm). The growth of *B. cereus* (8mm) and *S. lutea* (8 mm each), *B. subtilis* and *S. aureus* (9mm each) were poorly inhibited. *E. coli* (10 mm), *Salmonella* (11mm) were poorly inhibited .

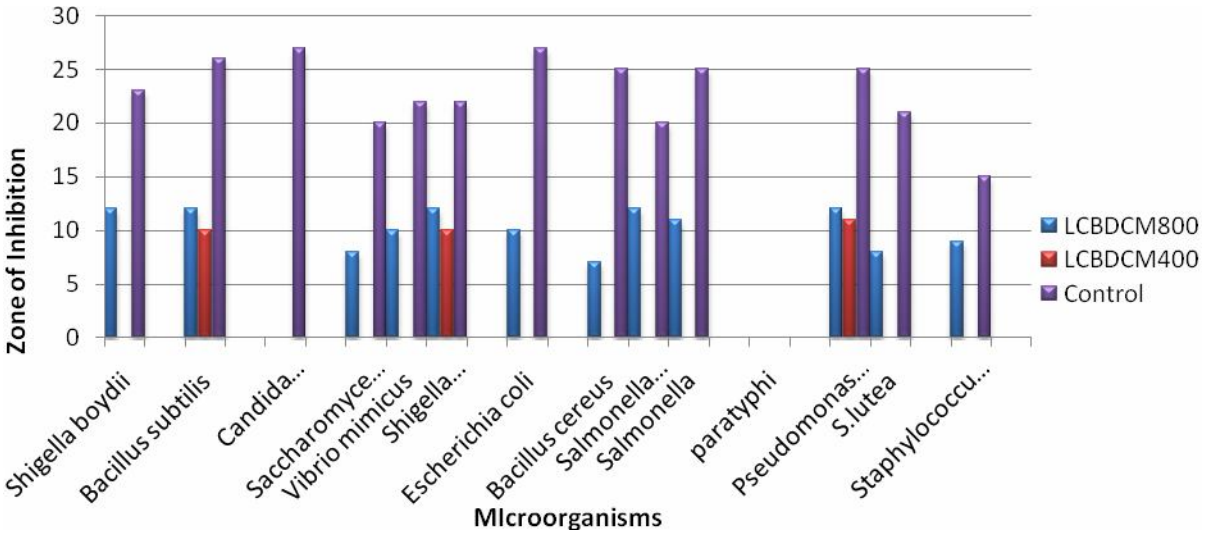


Figure 8.8: Comparison of the zone of inhibition of DCM soluble fraction with the control.

But The dichloro metahne partitionate of the ehanolic extract of *L. coromandelica* leaf (800 µg) showed excellent result against *Shigella boydii* (14mm), *Bacillus subtilis*, *Vibrio mimicus(*19mm), *Shigella dysentery*(16mm), *Escherichia coli*(16mm), *Bacillus cereus*(19mm), *Salmonella typhi*(16mm), *Salmonella paratyphi*(16mm), *Pseudomonas aeruginosa*(16mm), *S.lutea*(16mm).but shows moderate effect on *Staphylococcus aureus*(14mm), *Bacillus subtilis* (11mm). *L. coromandelica* leaf (400 µg) shows high activity against *Vibrio mimicus*(18mm)), *Bacillus cereus*(19mm), *Salmonella typhi*(15mm), *S.lutea*(15mm).but it shows moderate effect on the growth of other bacterias.

The n-hexane partitionate of the ehanolic extract of L. coromandelica bark showed the moderate activity against most of the bacteria.*Bacillus subtilis* having the zone of inhibition of 10 mm (400 µg) and 8 mm (800 µg)..

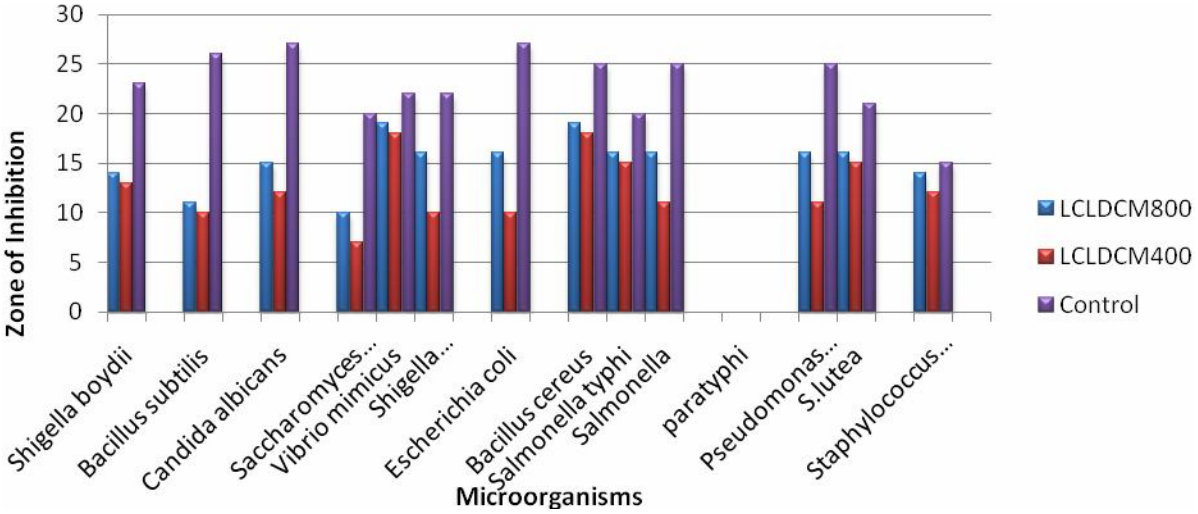


Figure 8.9: Comparison of the zone of inhibition of DCM soluble fraction with the control

Poor activity was found against *S. typhi, S. paratyphi, S. lutea and E.coli.* Among the fungi, the *C. albicans* was weakly inhibited having zone size 0 mm amd 0 mm for 400µg and 800 µg respectively.

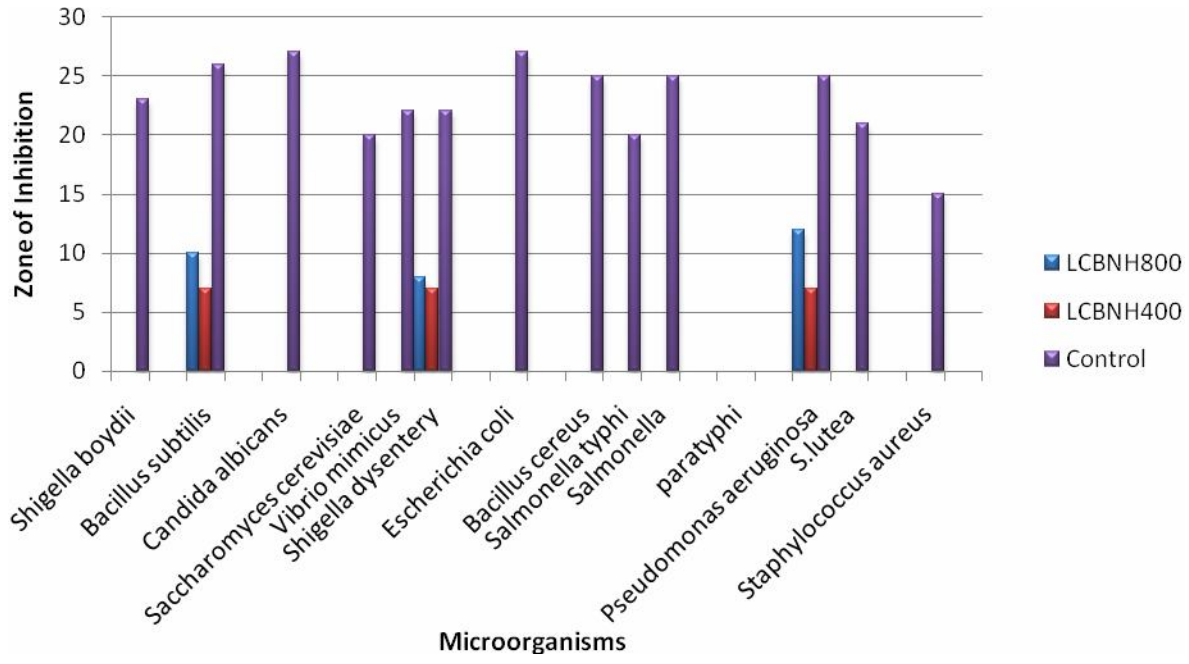


Figure 8.10: Comparison of the zone of inhibition of N-haxane soluble fraction with the control

The n-hexane partitionate of the ehanolic extract *of L. coromandelica* Leaf showed the moderate activity against some of the bacteria. *Shigella dysentery* having the zone of inhibition of 9 mm (400µg) and 14 mm (800 µg). *Bacillus cereus* 11 mm (800µg) and 8 mm (400µg) . *E.coli* 10 mm (800µg) and 8 mm (400µ**g**) poor activity was found against *S. typhi, S. paratyphi, S. lutea* and Among the fungi, the *C. albicans* was weakly inhibited having zone size 0 mm amd 0mm for 400µg and 800 µg respectively.

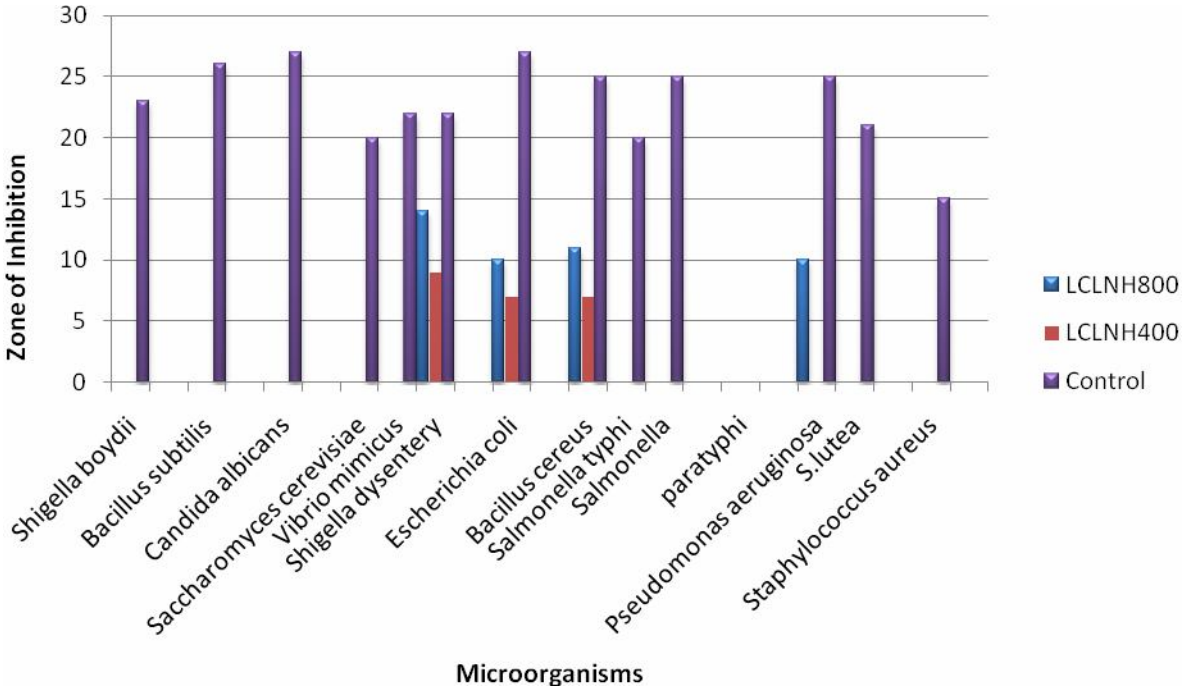


Figure 8.11: Comparison of the zone of inhibition of N-hexane soluble fraction with the control

The aqueous partitionate of the ehanolic extract of *L. coromandelica* bark showed moderate activity against the some microorganisms. *Pseudomonas aeruginosa* 10mm(800 µg) and 8mm (400µg), *Shigella dysentery* 9mm(800 µg) and 8mm (400µg).this part shows zero zone of inhibition for other tested microorganisms.

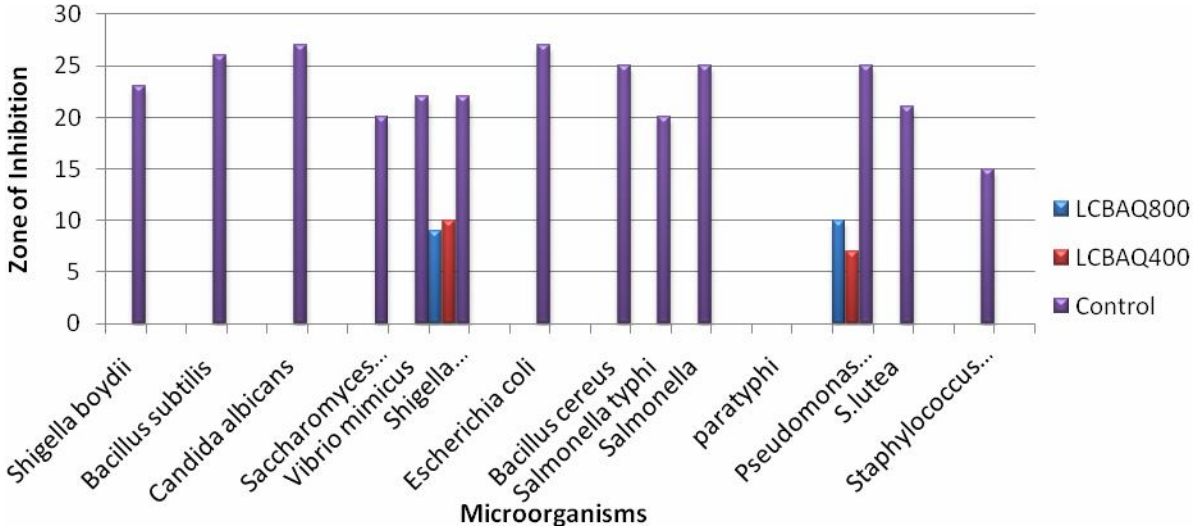


Figure 8.12: Comparison of the zone of inhibition of aquous soluble fraction with the control

The aqueous partitionate of the ehanolic extract of *L. coromandelica* Leaf showed moderate activity against the some microorganisms. *Pseudomonas aeruginosa* 11mm(800 µg) and 8mm (400µg), *Shigella dysentery* 11mm(800 µg) and 8mm (400µg).this part shows zero zone of inhibition for other tested miroorganisms.

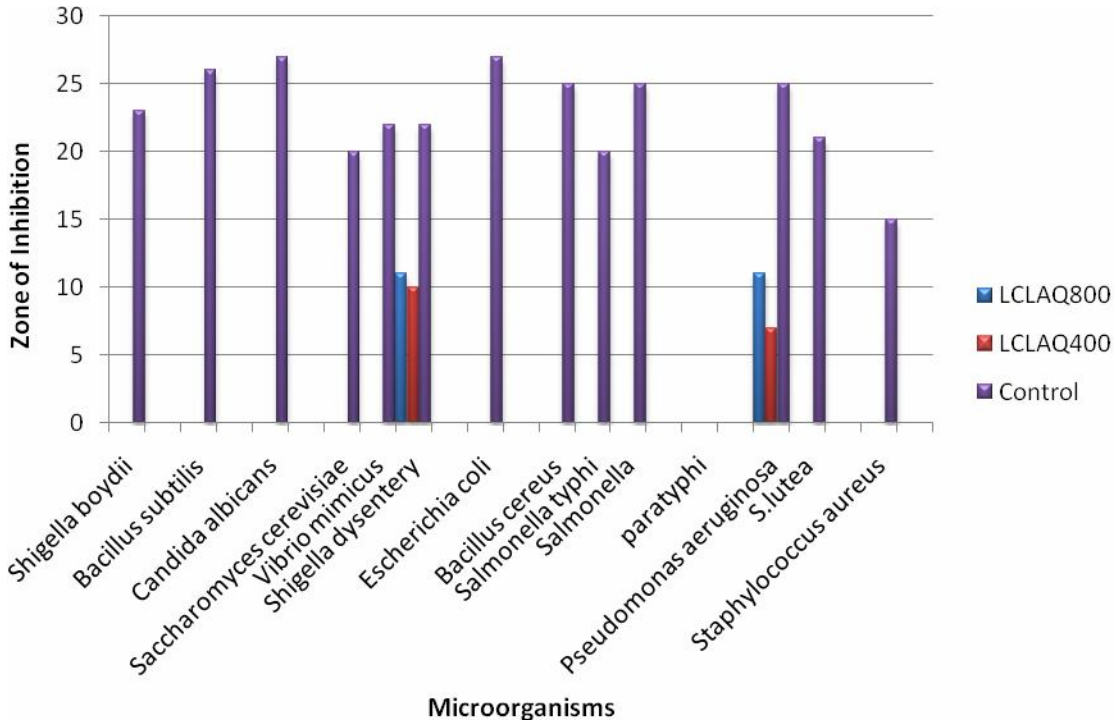


Figure 8.13: Comparison of the zone of inhibition of aquous soluble fraction with the control

Out of all the samples, dichloromethane soluble partitionates of the ehanolic extract were appeared very potent in terms of both zone of inhibition & spectrum of activity. Bioactivity guided isolation can be carried out to separate there bioactive metabolites

8.3 Minimum Inhibitory Concentration

8.3.1 *Result and Discussion*

The zone of inhibition of above antimicrobial study shows that DCM fraction of leaf shows potent activity. Due to the lack of time,we are unable to test MIC for all the fraction and we only choose DCM fration of leaf to determine MIC for some bacteria.

Table8.12: MIC for dichloromethane fraction of *Lannea coromandelica* leaf Of ethanolic extract

Extract	**Bacteria**				
	S. aureus	***S.paratyphi***	***S. typhi***	***B.subtilis***	***S.boydii***
Dichloromethane fraction of *Lannea coromandelica* leaf Of ethanolic extract	20mg/ml	10 mg/ml	5 mg/ml	-	-

So,we have found that DCM fraction *Lannea coromandelica* leaf Of ethanolic extract shows minimum inhibitory concentration of 20mg/ml for *S. aureus*, 10 mg/ml for *S.paratyphi and* 5 mg/ml for *S. typhi*. But shows no result for *B.subtilis and S.boydii*. I think the MIC for this microorganisms are greater than the concentration we use here.

8.4. Thrombolytic Activity Test

Here is the data we have collected after thrombolytic activity test.

Table 8.13: Thrombolytic activity test

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**
Blank	0.881	1.168	1.162	1.8482
StreptokinaseV1	0.892	1.129	0.995	56.5401
StreptokinaseV2	0.865	1.118	0.992	49.8024
LCBDV 1	0.896	1.245	1.032	61.03
LCBDV 1	0.886	1.142	0.98	64.66
LCBDV 1	0.881	1.232	1.068	64.43
LCBDV 2	0.892	1.336	1.181	58.16
LCBDV 2	0.884	1.222	1.202	58.85
LCBDV 2	0.908	1.183	1.104	58.04
LCBNV 1	0.881	1.121	1.055	26.4
LCBNV 1	0.886	1.109	1.042	28.86
LCBNV 1	0.869	1.122	1.052	28.68
LCBNV 2	0.892	1.142	1.086	22.4

LCBN V2	0.885	1.152	1.089	23.6
LCBNV 2	0.908	1.1	1.058	22.4
LCLDV1	0.888	1.208	1.108	31.56
LCLDV 1	0.886	1.206	1.098	32.83
LCLDV1	0.885	1.255	1.138	30.89
LCLDV 2	0.884	1.234	1.125	48.14
LCLDV 2	0.892	1.242	1.054	53.81
LCLDV 2	0.891	1.231	1.058	51.18

N.B

LCBDV = *Lannea coromandelica* bark Dichloromethane fraction volunteer,

LCBNV= *Lannea coromandelica* bark n-hexane fraction volunteer.

LCLDV = *Lannea coromandelica* leaf Dichloromethane fraction volunteer

Here as standard streptokinase is used. The average clot lysis by streptokinase is 55.35849 in volunteer 1 and 50.85403 for volunteer 2.

The n-hexane fraction of the ethanolic extract of *Lannea coromandelica* bark produces poor activity among other extracts. The average clot lysis activity of the fraction is found to be 28.6 1 for volunteer 1 and 22.80 for volunteer 2.

Table 8.14: Thrombolytic activity test n-hexane fraction

Name of Extract	Sample	Wt of empty Eppendrof (W_1) gm	Wt of Eppendrof with clot (W_2) gm	Wt of Eppendrof after clot lysis (W_3) gm	% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)	Average
LCBNV 1	1	0.881	1.121	1.055	26.40	28.61
LCBNV 1	2	0.886	1.109	1.042	28.86	
LCBNV 1	3	0.869	1.122	1.052	28.68	
LCBNV 2	1	0.892	1.142	1.086	22.40	22.80
LCBN V2	2	0.885	1.152	1.089	23.60	
LCBNV 2	3	0.908	1.100	1.058	22.40	

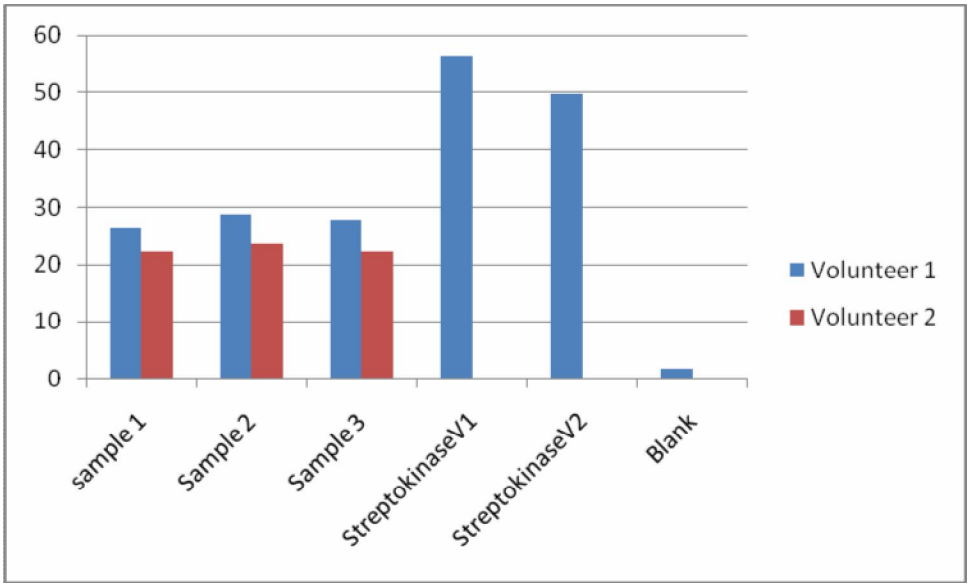


Figure 8.14.Thrombolytic activity test n-hexane fraction

Dichloromethane partitionate of *Lannea coromandelica* bark also produces highest result having average values of clot lysis are 63.383 for volunteer 1 and 58.65 for volunteer 2.

Table 8.15: Thrombolytic activity test dichloromethane fraction

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**	**Average**
LCBDV 1	0.896	1.245	1.032	61.03	63.383
LCBDV 1	0.886	1.142	0.980	64.66	
LCBDV 1	0.881	1.232	1.068	64.43	
LCBDV 2	0.892	1.336	1.181	58.16	58.65
LCBDV 2	0.884	1.222	1.202	58.85	
LCBDV 2	0.908	1.183	1.104	58.04	

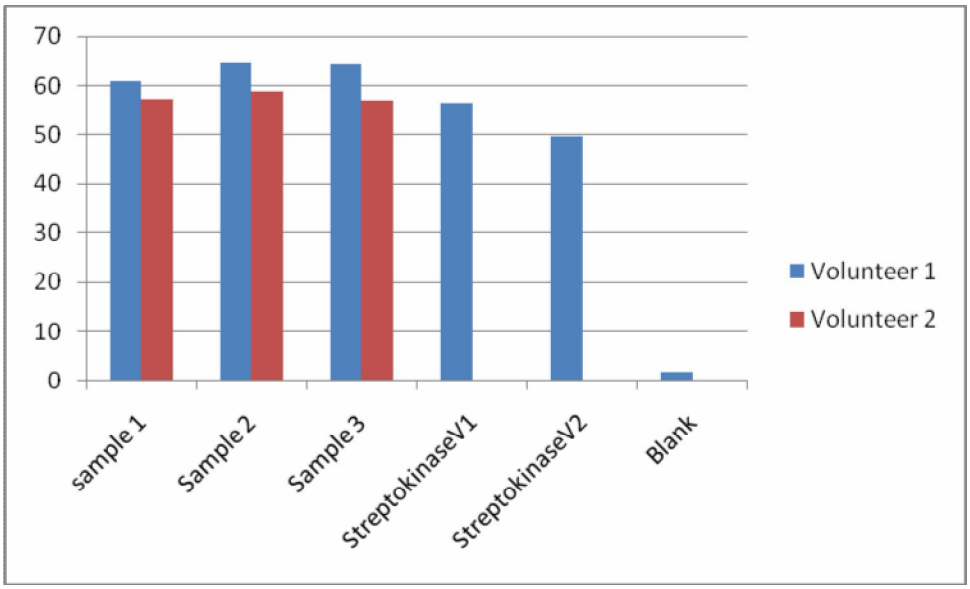


Figure 8.15:Thrombolytic activity test dichloromethane fraction

But. The average clot lysis of Dichloromethane partition of *Lannea coromandelica* leaf fraction is found to be 31.6633 for volunteer 1 and 51.0111 for volunteer 2.

Table8.16: Thrombolytic activity test dichloromethane fraction

Name of Extract	**Wt of empty Eppendrof (W_1) gm**	**Wt of Eppendrof with clot (W_2) gm**	**Wt of Eppendrof after clot lysis (W_3) gm**	**% of Clot Lysis = 100 * (W_2-W_3) / (W_2-W_1)**	**Average**
LCLDV1	0.888	1.208	1.108	31.56	31.6633
LCLDV 1	0.886	1.206	1.098	32.83	
LCLDV1	0.885	1.255	1.138	30.89	
LCLDV 2	0.884	1.234	1.125	48.14	51.0111
LCLDV 2	0.892	1.242	1.054	53.81	
LCLDV 2	0.891	1.231	1.058	51.18	

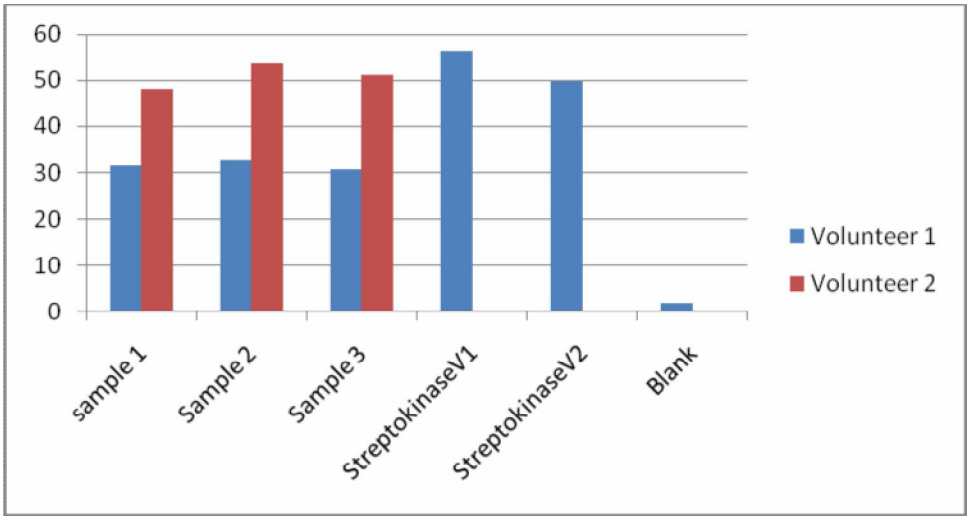


Figure 8.16: Thrombolytic activity test dichloromethane fraction

9. CONCLUSION

The crude ethanolic extract along with *n*-hexane, DCM and aqueous soluble fractions of *Lannea coromandelica* showed significant antimicrobial and antioxidant activities and thrombolytic activity which supports the traditional use of this plant in various diseases.

The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

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