Biological Investigation of the Ethanolic Extract of *Polygonum Hydropiper (L), Coccinia Grandis (L) and Litsea Glutinosa (Lour.)*

A thesis Report submitted to
The department of Pharmacy, East West University
In conformity with the requirements for the
Degree of Bachelor of Pharmacy

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This research paper is dedicated to my parents,

Dr. Mozaffar Hossain

&

Dr. Tahmina Ferdous

CERTIFICATE

This research paper is submitted to the Department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy (B. Pharm) was carried out by Md. Tyeb Mozaffar (Id# 2008-1-70-016).

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This is to certify that the thesis "Biological Investigations of The Ethanolic Extracts of ***Polygonum Hydropiper (L), Coccinia Grandis(L)*** **and** ***Litsea Glutinosa (Lour.)*****."is submitted to the Department Of Pharmacy, East West University, Mohakhali, Dhaka in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B.Pharm) was carried out by Md. Tyeb Mozaffar (ID# 2008-1-70-016) under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed of this connection is duly acknowledged.**

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Table of Contents

	Topics	Pages
	Abstract	1
Chapter 1: Introduction		
1.1	General Introduction	3
1.2	Rationale and objective of the work	4
1.3	Medicinal Plant	6
1.4	Contribution of medicinal plants	7-9
1.5	Medicinal plant users	9
1.6	Reasons behind popularity of plant medicine	9
1.6.1	Historic use	9
1.6.2	Extrapolation	10
1.6.3	Phytomedicinal prospecting	10
1.6.4	Current Legal Status in the US	10
1.6.5	Current Industry Problems	12
1.7	A Few examples which might be useful	12-15
A.1.1	*Litsea Glutinosa*	15
A.1.2	Taxonomy	16
A.1.3	Generic description	18
A.1.4	Common Names	18
A.1. 5	Chemical composition	15
B.1.1	*Coccinia grandis*	19
B.1.2	Folk Mdicine	20
B.1.3	Taxonomy	21
B.1.4	Common Names	22
B.1.5	Botany of *C. grandis*	22
C.1.1	*Polygonum hydropiper*	22
C.1.2	Active Ingredient	22
C.1.3	Taxonomy	23
C.1.5	Hazards	24
C.1.5	Botanical Description	24
C.1.6	Major Constituents	25

Chapter 2: Literature Review		
2.1	Pharmacological Studies of *Litsea Glutinosa*	27-29
2.2	Pharmacological Studies of *Polygonum hydropiper*	29-31
2.3	Pharmacological Studies of *Coccinia grandis*	32-35

Chapter 3 : Method and Materials		
3.1	Selection of plants	37
3.1.1	Collection of the plant part	37
3.1.2	Drying of the plant part	37
3.1.3	Storage and Preservation of plant part	37
3.1.4	Grinding of the plant parts	38
3.1.5	Extraction of the plant material	38
3.1.6	Drying of extract	38
3.2	Antimicrobial Test	
3.2.1	In Vitro Antimicrobial screening	40
3.2.2	Principle of diffusion method	41
3.2.3	Test Materials	43
3.2.4	Test Organism	43
3.2.5	Apparatus and Reagents	44
3.2.6	Sterilization Procedure	44
3.2.7	Culture Media	45
3.2.8	Preparation of Medium	46
3.2.9	Preparation of Subculture	47
3.2.10	Preparation of Test Plates	47
3.2.11	Preparation of Discs	47
3.2.12	Preparation of Discs Containing Sample	48
3.2.13	Placement of Disc, Diffusion and Incubation	48
3.2.14	Procedure	49
3.3	Cytotoxicity Test	51
3.3.1	Brine shrimp lethality bioassay	51
3.3.2	Principle	52
3.3.3	Materials	53
3.3.4	Hatching of brine shrimps	53
3.3.5	Preparation of sea water	54
3.3.6	Preparation of sample	54
3.3.7	Preparation of controls	55
3.3.8	Application of the sample & Brine Shrimp naupli to the vials	55
3.3.9	Counting of Nauplii and Analysis of Data	55
3.3.10	Procedure	56
3.4	*In vitro* Thrombolytic effect	57
3.4.1	Principle	57
3.4.2	Materials	57
3.4.3	Preparation of sample	58
3.4.4	Application of test sample	58
3.4.5	Procedure	59
3.5	Evaluation of scavenging activity	60
3.5.1	Rational and objectine	60
3.5.2	Methods of evaluating of anti oxidant activity	61
3.5.3	Determination of DPPH radical scavenging assay (Quantitative analysis)	61
3.5.3.1	Principle	61
3.5.3.2	Materials and reagents	63
3.5.3.3	Methods	63
3.6	Evaluation of analgesic property	65

3.6.1	Mechanism of pain induction in Acetic acid induced writhing method	66
3.6.2	Materials and Methods	68
3.6.2.1	Drugs and Chemicals	68
3.6.2.2	Animal	68
3.6.2.3	Experimental design	69
3.6.2.4	Method of identification of animal	69
3.6.2.5	Preparation of test material	69
3.6.2.6	Procedure	70
3.6.2.7	Counting of writhing	70

Chapter 4 : Result and Discussion		
4.1	Thrombolytic effect	73
4.1.1	Result	74
4.1.2	Discussion	74
4.2	Antimicrobial Activity	75
4.2.1	Result and Discussion	77
4.2.2	Result and Discussion	79
4.2.3	Result and Discussion	83
4.3	Results of Brine shrimp lethality Bioassay	84
4.3.1	Result	85
4.3.2	Discussion	85
4.4	Results of Analgesic activity by acetic acid induced writhing method	86
4.4.1	Dicussion	89
4.5.1	Result of scavenging activity *Polygonum hydropiper*	91
4.5.2	Discussion	91
4.5.3	Result of scavenging activity of *Litsea glutinosa*	92
4.5.4	Discussion	92
4.5.5	Result of scavenging activity of *Coccinia grandi*s	93
4.5.6	Discussion	93

Chapter 5 : Conclusion		
	Conclusion	93
Chapter 6 : References		
	Refernces	93

List of Tables

	Contents	Pages
Table 1.1	The list of the medicinal substance derived from plants	
Table3.1	List of pathogenic bacterial strains	
Table3.2	Composition of nutrient agar media	
Table4.1	Thrombolytic data for *P. hydropiper* extract	
Table4.2	Data of zone of inhibition of microbial growth for *P. hydropiper*	

Table 4.3	Data of zone of inhibition of microbial growth for *C. grandis*	
Table 4.4	Data of zone of inhibition of microbial growth for *L. glutinosa*	
Table 4.5	Data representation of brine shrimp lethality bioassay	
Table 4.6	Data representation of analgesic activity of *C. grandis*	
Table 4.7	Data representation of analgesic activity of *L. glutinosa*	

List of Figures

	Contents	Pages
Fig.1.1	Lead compound search	
Fig 1.2	*L.glutinosa*	
Fig 1.3	*C. grandis*	
Fig 1.4	*P. hydropiper*	
Fig 3.1	Rotary evaporator	
Fig 3.2	Schematic diagram of Antimicrobial test	
Fig 3.3	Brine shrimp hatchery	
Fig 3.4	Schematic diagram of cytotoxic Bioassay	
Fig 3.5	Schematic diagram of Thrombolytic Bioassay	
Fig 3.4	Reaction of DPPH with reactive free radical	
Fig 3.5	Schematic representation of the method of assaying free radical scavenging activity	
Fig3.6	Schematic diagram of pain induction	
Fig3.7	Swiss albino mice	
Fig3.8	Identification of test animal for analgesic screening	
Fig3.9	Schematic representation of procedure for screening of analgesic property on mice by acetic acid induced method	
Fig4.1	Graphical presentation of Thrombolytic effect of *P. hydropiper* compare to Satandard	
Fig4.2	Zone of inhibition for *P. hydropiper*	
Fig4.3	Zone of inhibition for *C. grandis*	
Fig4.4	Zone of inhibition for *L.glutinosa*	
Fig4.5	Graphical presentation of LC_{50} of *P. hydropiper*	
Fig4.6	Graphical representation of % of writhing of *Coccinia grandis*	
Fig4.7	Graphical representation of % of writhing inhibition *Coccinia grandis*	
Fig4.8	Graphical representation of % of writhing *Litsea glutinosa*	
Fig4.9	Graphical representation of % of writhing inhibition *Litsea glutinosa*	
Fig4.10	Graphical presentation of IC_{50} of *Polygonum hydropiper*	
Fig4.11	Graphical presentation of IC_{50} of *Litsea glutinosa*	
Gig4.12	Graphical presentation of IC_{50} of *Coccinia grandis*	

ABSTRACT

Polygonum hydropiper, Coccinia grandis and *Litsea glutinosa* are common plants in Bangladesh. It has been reported to *Coccinia grandis* have medicinal activities like hypoglycemic, antibacterial, antifungal and *Litsea glutinosa* used for used as demulcent for ulcer; also for dysentery. The leaf of *Polygonum hydropiper* plant has shown cytotoxic and thrombolytic activity. In this study, extracted mixture from *Polygonum hydropiper, Litsea glutinosa* and *Coccinia grandis*, were studied for antibacterial, thrombolytic, cytotoxic and scavenging activity by using *in vitro* techniques. Analgesic activity was tested using *in vivo* techniques. Ethanol extract of the plants were tested for Antibacterial activity using disc diffusion method. Ethanolic extract of the plants were tested for brine shrimp lethality bioassay using brine shrimp nauplii. The LC_{50} of ethanol extract of *Polygonum hydropiper* extract was found to be 6.02µg/ml indicates that extracts have good pharmacological action. The ethanol extract of the extracts were also tested for *in vitro* thrombolytic activity. The extract showed reasonable thrombolytic activity. *Litsea glutinosa* showed very good analgesic activity having percent inhibition of writhing about 76.65. *Coccinea grandis* has extremely good scavenging activity having the IC_{50} of 1.67µg/ml. Further investigation on the plant is required to confirm their pharmacological activity and thereby utilizing them as useful medicinal plant.

Keywords: *Polygonum hydropiper, Litsea glutinosa, Coccinea grandis,* Cytotoxicity, Thrombolytic, Antibacterial, Anti-oxidant, Analgesic.

Chapter 1: Introduction

1.1 General Introduction

The importance of medicinal plants and traditional health systems in solving the health care problems of the world is gaining increasing attention. Because of this resurgence of interest, the research on plants of medicinal importance is growing phenomenally at the international level, often to the detriment of natural habitats and mother populations in the countries of origin. Most of the developing countries have adopted traditional medical practice as an integral part of their culture. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials or in the refined form of crude extracts, mixtures, etc. Recent estimates suggest that several thousands of plants have been known with medicinal applications in various. (Farnsworth N. R. and Fabricant D. S. 2001)

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people. Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. The potential of medicinal plants can be assessed by finding new chemical entities of wide structural diversity. These new chemical substances can also serve as templates for producing more effective drugs

through semi-synthetic and total synthetic procedure. According to World Health Organization (WHO), about 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses (Mukeshwar Pandey, Mousumi Debnath, 2011).

In Bangladesh, ninety percent of the medicinal plants are wild sourced. Out of approximately 5,000 species of indigenous and naturalized phanerogamic and pteridophytic plants growing in the country, more than a thousand of them, including many food, vegetable, beverage, spice & ornamental plants, contain medicinally useful chemical substances (Mia, 1990). Growing in the forests, jungles, wastelands, and along roadsides the types of medicinal plants in Bangladesh are varied. A total of 546 medicinal plants that occur in the country have been counted so far. However, this list is not exhaustive since it is believed that many other medicinal plants also grow there, but have not yet been discovered or identified (Yusuf *et al*., 1994).

The word drug itself comes from the Dutch word "droog" (via the French word Drogue), which means 'dry' because most drugs (medicines) came from dried plants. Some examples are quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove.

Medicinal plants are used as a source of drugs for the treatment of various human and livestock health disorder all over the world from ancient time to the

present day. A total of 25,000 species of flowering plants are referred to as medicinal plants. The World health Organization (WHO) enlisted some 21,000 plants species (Penso *et al*., 1980). The present global herbal market is worth about US$ 62 billion per annum. The annual growth of herbal market is about 15% and the global herbal market by 2050 is expected to be about US$ 5 trillion (WHO *et al.*, 2002).

In Bangladesh, about 500 plant species have been identified as medicinal plant because of their therapeutic properties. In the mean time, a large number of industries (400 herbal factories) have been established in the country for producing Ayurvedic and Unani medicines. It has been estimated that Bangladesh has a market of about 100core taka worth herbal products annually.

Bangladeshi people have traditional medical practice as an integral part of their culture. A lot of medicinal plants are available for their treatment of various diseases. However, scientific studies have been conducted only to a limited extent with few medicinal plants. (Ghani, A. 2003)

1.2 Rationale and objective of the work

Medicinal components from plants play many important roles in traditional medicine. It is estimated that there are about 2,500,000 species of higher plants and the majority of these have not yet been investigated in detail for their pharmacological activities (Ram et al., 2003). In developing countries, about

80% of the population relies on traditional medicine for their primary health care (Matu and Staden *et al.*, 2003).

Since Bangladesh has a vast resource of medical plants and majority of our population has to rely upon indigenous system of medication from economic point of view. The high cost of imported conventional drugs and inaccessibility to western health care facility, imply that traditional mode of health care is the only form of health care that is affordable and available to the rural people. On the other hand, even when western health facilities are available, traditional medicine is viewed as an efficient and an acceptable system from a cultural perspective and as a result, traditional medicine usually exists side by side with western forms of health care. (Munguti, 1997)

Identification and isolation of the active constituents from traditionally used phyto-therapy can ensure the health care of the poor people. In addition, herbal drugs could be scientifically modified for better pharmacological activity and to establish safe and effective drugs and the rationality of the present study lies in meeting the challenge of developing herbal medicines, which needs a systematic research on indigenous medicinal plants for the welfare of the humanity.

There are several familiar approaches for lead searching from the plants (Fig. 1.1) and the isolated bioactive compounds are utilized in three basic ways (Cox, P.A *et al.*, 1994). These can be stated as-

- Unmodified natural plant products where ethno medial uses suggested clinical efficacy, e.g., digitalis.
- Unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use, e.g. vincristine.
- Modified natural or synthetic substances based on a natural product used in folk medicine, e.g., aspirin.

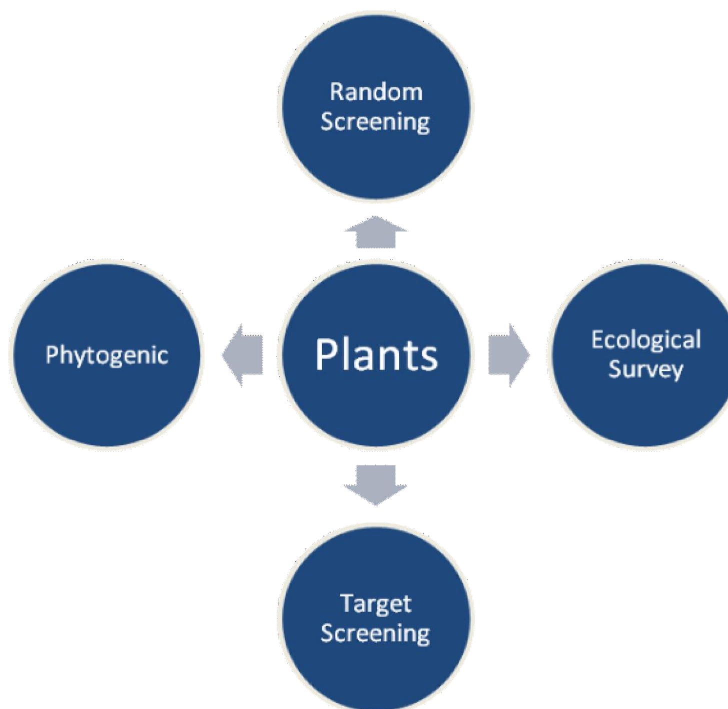


Fig. 1.1 Lead compound search

1.3 Medicinal Plant

"Medicinal Plants *are plants that provide people with medicines - to prevent disease, maintain health or cure ailments." In one form or another, they benefit virtually everyone on Earth. No exact definition of a Medicinal Plant is*

possible. There are related issues, such as for nutrition, toiletry, bodily care, incense and ritual healing.

1.4 Contribution of medicinal plants

All culture from ancient times to the present day have used plants as sources of medicine. Today the majority of the world's population continues to rely on medicinal plants for the health care needs. Cultural global demand for herbal product is vast and growing. The habitants that supports medicinal plant are being rapidly degraded or destroyed for instance by over grazing or conversion to agricultural land.

Many plants synthesize substance that is useful to the maintenance of health in human and other animals. These include aromatic substance, most of which are phenols or their oxygen substituted derivatives such as tannins. Many are secondary metabolites .of which at least 12000 have been isolated –a number estimated to be less than 10% of the total. In many cases, substances such as alkaloids serve as plant defense mechanism against predation by microorganism, insects, and herbivores. Many of the herbs and species used by humans to season food yield useful medicinal compounds. (G Falco et al., 2003)

Plants up regulate and down regulate their biochemical paths in response to the local mix of herbivores, pollinators and microorganisms. The chemical profile of a single plant may vary over time as it reacts to changing conditions. It is the

secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs.

Some examples are given below:-

Table 1.1: The list of the medicinal substance derived from plants.

Drugs /chemical Name	Action /Clinical use	Plant source
Atropine	Antichlolinergic	*Atropa belladonna*
Arecoline	antihelmintic	*Areca catechu*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubifacient	Cinnamonum acemphore
Codeine	Analgesic, Sedative	*Papaver somniferum* linn.
Digitoxin,Digoxin	Cardiotonic	Digitalis purpurea Lin. Digitailis lanata
Ergotametrine,Ergotamine, Ergotoxin	Vasoconstrictor,ergotamine,	Claviceps purpurea. Tul
Hyosine Hyosinamine	Parasympatholytic,Mydriatic,anti spasmodic	Datura , hyocyamus, scopolia ,

		Duboisia
Morphine	Sedative, Narcotic analgesic	*Papaver somaniferum* Linn.
Papaverin	Smooth muscle relaxant	*Paper somaniferum*
Vinflunine	Anticancer agent	*Catheranthus roseus*

1.5 Medicinal plant users

It is estimated by the World Health Organization that approximately 75-80% of the world's population uses plant medicines either in part or entirely. For many this is out of necessity, since many cannot afford the high costs of pharmaceutical drugs. Growing numbers of American health care consumers are turning to plant medicines for many reasons - low cost and seeking natural alternatives with fewer side effects are commonly cited.

1.6 Reasons behind popularity of plant medicine

1.6.1 Historic use:

Based upon the traditions of Europeans, Chinese, Egyptians, American Indians, and other cultures, Information on plant medicines handed down over the

centuries. Currently anthropologists and other scientists are investigating newly-discovered cultures (visits with shamans in Amazonia and Belize, for example), to determine what plants are being used - this "ethnobotany" has introduced a number of new compounds into pharmaceutical research (P. P. Joy et al., 1998)

1.6.2 Extrapolation

Extrapolation can be referred to the process by which a biological test is conducted thinking that some activity in vitro (in the test tube or in the lab) implies major activity in humans. The reasoning involved in predicting a conclusion or making a logical judgment on the basis of circumstantial evidence and prior conclusions rather than on the basis of direct observation.

1.6.3. Phytomedicinal prospecting

Screening plants for biological activity. Huge projects are currently underway by such organizations as INBio, Costa Rica's National Institute of Biodiversity. INBio is cataloging all species of plants and animals in the country - estimated to be around 500,000! To complete this ambitious task they train "community taxonomists" to identify plants and animals (Christopher P. Baker et al., 2008).

1.6.4. Current Legal Status in the US

US currently have an "open market" for anything labeled as a "nutritional supplement". This is both good and bad - it means "caveat emptor" - let the

buyer beware! Unfortunately the buyer may occasionally be gambling with their life if they are not making knowledgeable choices. Since "nutritional supplements" are not sold as a treatment for a specific problem, they buyer must be informed. The bad aspect of this open market approach is that even products with proven health benefits may not be marketed for that use unless the producer has gone through the FDA protocols for determining safety and efficacy.

The best example, perhaps, of this restriction is that it would be absolutely illegal to sell prune juice as a treatment for constipation. While no one would argue that it is safe and effective, no one has gone through the rigorous FDA approval process. A current controversy surrounds the sale of products containing natural sources of ephedrine. Ephedrine is an adrenaline-like compound found in some plants, especially ephedra species such as Ma Huang and Mormon Tea.

This stimulant is found in "thermogenic" weight loss products and also sold as a "legal amphetamine" under such names as "Herbal Ecstasy". A number of people who have taken excessive amounts of this drug have died. The FDA is currently mulling over the idea of taking it off the market. Manufacturers are hurrying to change the labels and provide warnings in an effort to prevent FDA action.

1.6.5. Current Industry Problems

Is the "natural" product you are buying really what it says it is??? This problem has long plagued the industry. When some dried root or leaf products sell for $80 per pound, the temptation to add a little extra something along the way goes from the harvesters to the middlemen to the retailers - all of whom are paid by the pound.

There have been studies of retail ginseng, for example, which show numerous purchased products with no ginseng content whatsoever! Perhaps these products rely on the power of suggestion! Looking at this more innocently, a peasant in the Amazon may not know one plant from a similar-looking cousin. The industry is establishing standards in an effort to improve its credibility - some products, such as ginkgo, are now standardized and offer some uniformity.

1.7. A Few examples which might be useful

1.7.1. Garlic:

Garlic has been shown to reduce cholesterol about 12% and to reduce platelet adhesiveness (the reason an aspirin a day is recommended to reduce the risk of stroke and heart attack). Huge controversy exists over what forms (other than fresh) provide sufficient allicin (the active ingredient) activity. Fresh garlic tends to cause heartburn and social consequences. Garlic preparations in oil have been found to be relatively useless in some studies. Coated tablets are

probably good - the "odorless" kind. Some experts, however, say "if it doesn’t stink, it doesn’t work". The equivalent of 4 to 5 cloves per day is recommended. A 1992 German study of 18 commercial preparations showed significant allicin activity in only five.

1.7.2. Ginkgo:

Flavone glycosides ("ginkgolides") have been shown to reduce capillary fragility and decrease blood cell transit from inflamed capillaries. They have been shown to reduce "PAF" - platelet activating factor". These glycosides are also potent anti-oxidants. Ginkgo preparations are used in Germany to treat "reduced intellectual capacity and vigilance" associated with aging, as well as to treat peripheral vascular disease. Available in Germany in liquid, tablet, and injectable forms, in 1988 over 5.2 million prescriptions were written - even though it is also available over-the-counter. Germany's Commission E, established to study plant medicines, declared ginkgo as safe and effective with very few side-effects (occasional headache). It should be taken three times daily with meals. HYPE ALERT: Ginkgo is being widely advertised as a product which will improve mental functioning of anyone who takes it - this is NOT TRUE! Studies in the elderly have shown some benefit, whereas studies in middle-aged and younger people have not shown any significant benefit.

1.7.3. Cranberry Juice or Extract:

This and related *Uva Ursi* extract has been shown to inhibit the activity of "adhesin", which is needed for the pili, or tiny hairs, on the *E coli* bacteria to stick to the cells lining the urinary tract. They may therefore be useful in the treatment and prevention of bladder infections with those bacteria. (M. A. H. Mollik, 2010)

1.7.4. Valerian:

The roots and rhizomes of *Valeriana officianalis* have been used as a sleep aid for perhaps over one thousand years. Its' effectiveness as a minor sedative or calmative continues to be confirmed. It is safe and effective and a good recommendation for those with minor difficulties or in whom an addictive alternative might be risky. It is frequently combined with hops and/or passionflower, two other safe and effective sedating herbs. Historical note: when the Latin name of a plant has the term *officianalis* in it, it means that it was grown in the monks' garden and kept in the office for medicinal uses.

1.7.5. Others of Interest:

Hawthorne - has shown some beneficial effects on the heart in heart failure. *Melissa officianalis* - lemon balm - has been shown to have some anti-herpes effects. St. John's Wort has been shown to have some anti-depressant activity. A chemical constituent, hesperidin, is being studied for possibly anti-retroviral

(HIV) activity. This plant may cause photo-toxic reactions. Milk thistle is used frequently in Germany to protect the liver from toxins such as poison mushrooms. It may be effective for other toxins such as alcohol.

A.1.1 *Litsea Glutinosa*

Litsea (Lauraceae) is a genus of about 200 species mainly growing in tropical and subtropical Asia, some distributed in Australia and from North America to subtropical South America. In China, this genus is represented by 72 species and mostly distributed in the south and southwestern parts of the country, where many of them are known for their edible fruits and medicinal properties. Previous phytochemical studies have indicated that *Litsea* species contain structurally diverse and biologically active aporphine alkaloids, butanolides and sesquiterpenes. L. glutinosa is found in mixed primary and secondary forest and thickets.

The seeds of *Litsea glutinosa* (Lour.) contain aromatic oil which has been used to make candles and soap. The roots yield fibers used in Thailand for rope manufacture and for paper pulp. The fruits have a sweet creamy edible pulp. The young leaves are eaten by livestock. The pounded seeds are also applied medicinally against boils. The leaves and the mucilage in the gum from the bark have been used for poultices. The bark also acts as a demulcent and mild astringent in diarrhea and dysentery.

Litsea glutinosa (Lour.) is an evergreen tree known in China as "Chan Gao Shu". The leaves and twigs of *L. glutinosa* (Lour.) have been used as a demulcent and mild astringent for diarrhea and dysentery, whereas the roots are used in local folk medicine to poultice sprains and bruises. Earlier phytochemical studies on this plant have been reported, and these include the isolation of an arabinoxylan, an abscisic acid derivative and lignans, aporphine alkaloids and a flavone glycoside. (Botanic Decription, 1995)

A.1.2 Taxonomy

Kingdom: Plantae

(Cladus): Angiosperms

(Cladus): Magnoliids

Order: Laurales

Family: Lauraceae

Genus: *Litsea*

Species: *Litsea glutinosa* (Taxonomy, 2006)

A.1.3 Generic description

A small to medium-sized tree up to 20 m tall, bole straight or curved, up to 60 cm in diameter, not buttressed, bark surface greyish-brown, inner bark yellowish. Leaves arranged spirally, 10-30 cm x 3-13.5 cm, blunt or rounded, hairy on main veins above, yellowish hairy below, midrib raised or flattened above, with 6-11 pairs of secondary veins which are not sunken above, tertiary

venation prominent below, petiole 1-3.5 cm long. Flowers in umbellules arranged in racemes on a 0.7-2.5 cm long peduncle, with 0-3 tepals and 9-15 stamens. Fruit depressed globose or globose, 1-2.5 cm across. Several varieties have been distinguished, but their status is uncertain.

Germination is not very rapid; approximately 85% germination is achieved in 15-45 days. Average annual diameter increments for *L. glutinosa* reported in Luzon are 0.8 cm and 1.9 cm for the 0-5 cm and 5-10 cm diameter classes. *L. glutinosa* is an evergreen or deciduous tree, Altitude is up to 1300 m. Young branchlets are gray-yellow velvety. Alternately arranged leaves are carried on 1-2.6 cm long gray-yellow velvety stalks. Leaves are mostly elliptic but variable, 7-15 x 3-7 cm; velvety on both surfaces when young, lateral veins 5-12 pairs. Leaf base is wedge-shaped blunt or rounded, tip blunt or shortly tapering. Flowers are borne in solitary or several, few-flowered umbels on short branches. Stalks carrying the umbels are 1-1.5 cm. Male flowers have petals imperfect or missing. Fertile stamens are often 15 or more. Fruit is round, 5-7 mm in diameter; fruit-stalk is 3-6 mm, slightly thickened at the top. Flowering usually takes place during May-June.

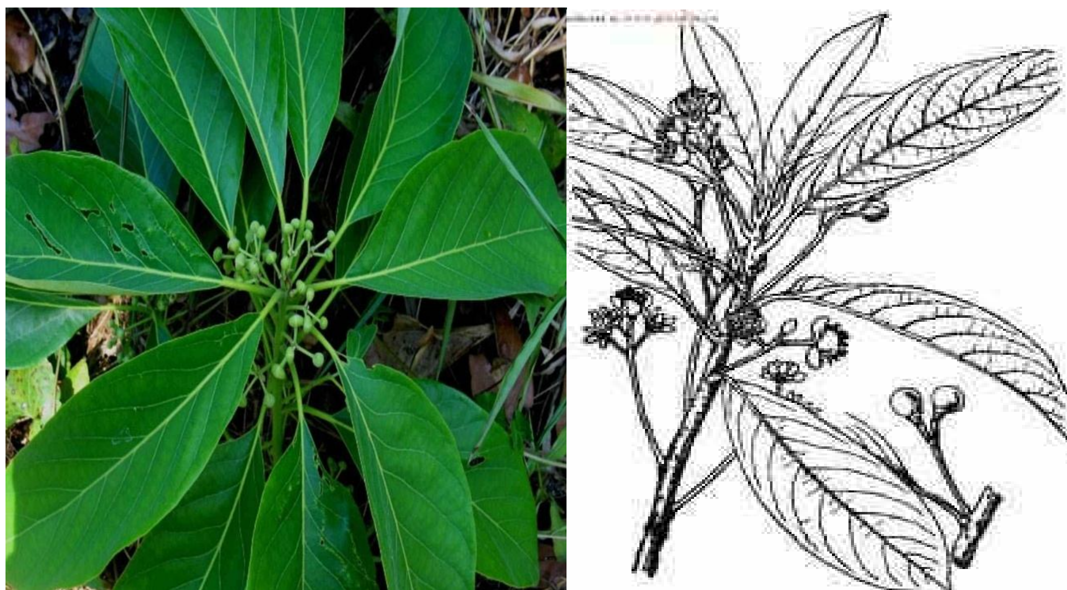


Figure1.2: *L.glutinosa*

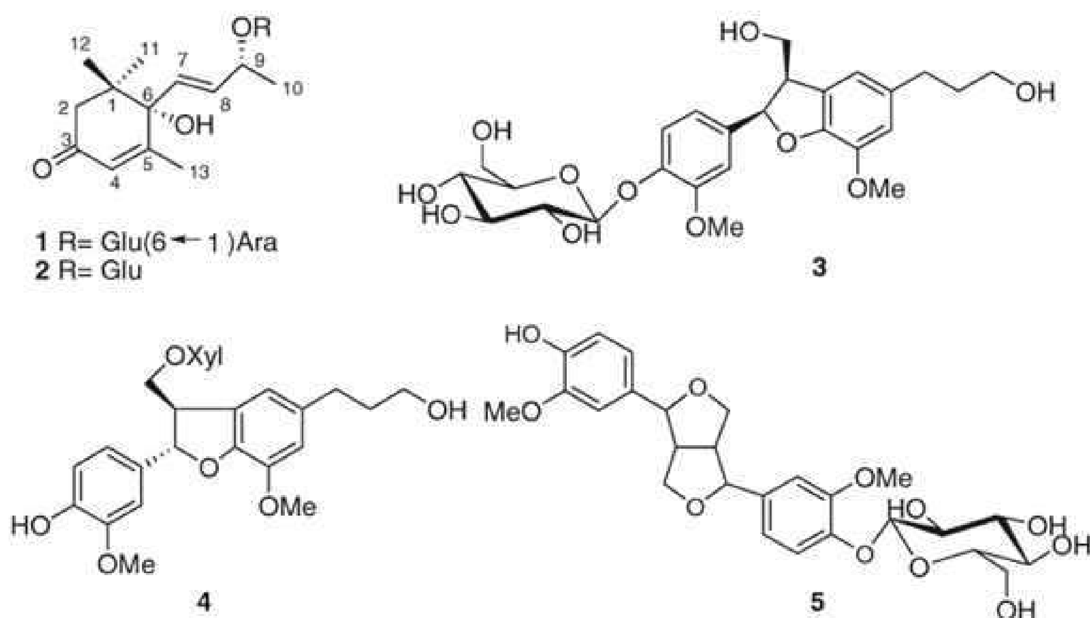
A.1.4 Common Names

Indian Laurel, Kharajora(Bangla), Medh, Chandna, Maidalakri(Hindi), Maidalakdi, Ranamba, Maida Lakadee(Marathi), Gajphala, Gatcho(Oriya), Medasaka(Sanskrit) , Muchaippeyetti, Uralli, Elumburukki(Tamil), Kanugunalike, Meda, Nara-Nalike(Telugu), Litsea laurifolia, Sebifera glutinosa, Tetranthera laurifolia(Synonym). **(**Botanic Decription, 1995**)**

A.1.5 Chemical composition

The present study resulted in the characterization of a new megastigmane diglycoside (1), along with known compounds (6*S*, 7*E*, 9*R*)-roseoside (2), (7'*R*, 8'*R*)- 3, 5'-dimethoxy-9, 9'-dihydroxy-4, 7'-epoxylignan 4'-β-D-glucopyranoside (3), (7'*R*, 8'*S*)-dihydro-dehydrodiconifenyl alcohol 9'-*O*-β-D-xylopyranoside (4), and pinoresinol 3-*O*-β-D-glucopyranoside (5), from the EtOH extract of *L. glutinosa* leaves and twigs. Compound 1 was evaluated for cytotoxic activities

against five tumor cell lines, for which it was proved to be inactive.Following components are the isolated compounds.



B.1.1 *Coccinia grandis*

Coccinia grandis native range extends from Africa to Asia, including India, the Philippines, China, Indonesia, Malaysia, Thailand, Vietnam, eastern Papua New Guinea, and the Northern Territories, Australia. Its documented introduced range includes the Federated States of Micronesia, Fiji, Guam, Saipan, Hawaii, the Marshall Islands, Samoa, Tonga, and Vanuatu.

Seeds or fragments of the vine can be relocated and lead to viable offspring. This can occur when humans transport organic debris or equipment containing *C. grandis*. Once the ivy gourd is established, it is presumably spread by birds,

rats, and other mammals. In Hawaii, it has been suggested that the fruit may be dispersed by pigs. Long-distance dispersal is most commonly carried out by humans due to its culinary uses or by mistake.

B.1.2. Folk Medicine

In traditional medicine, fruits have been used to treat leprosy, fever, asthma, bronchitis and jaundice. The fruit possesses mast cell stabilizing, anti-anaphylactic and antihistaminic potential.

C. grandis is rich source of beta-karotin. The juice of the roots and leaves is used in the treatment of diabetes. The leaves are used as a poultice in treating skin eruptions. The plant is used a laxative. It is used internally in the treatment of gonohhrea. Aqueous and ethanolic extracts of the plants have shown hypoglycemic principles. It helps to regulate blood sugar levels. Compounds in the plant inhibit the enzyme glucose-6-phosphatase which supports the body's on regulatory system and promotes a more balanced and healthy body and therefore it is recommended for diabetic patients.

Coccinia grandis extracts and other forms of the plant can be purchased online and in health food stores. It is claimed that these products help regulate blood sugar levels. There is some research to support that compounds in the plant inhibit the enzyme glucose-6-phosphatase. Glucose-6-phosphatase is one of the key liver enzymes involved in regulating sugar metabolism. Therefore, ivy

gourd is sometimes recommended for diabetic patients. Although these claims have not been supported, there currently is a fair amount of research focused on the medicinal properties of this plant focusing on its use as an antioxidant, anti-hypoglycemic agent, immune system modulator, etc. Some countries in Asia like Thailand prepare traditional tonic like drinks for medicinal purposes.

Figure1.3: *C. grandis*

B.1.3. Taxonomy:

Kingdom: Plantae

Order: Cucurbitales

Family: Cucurbitalaceae

Genus: Coccinia

Species*: C. grandis*

B.1.4. Common name:

Ivy gourd, Scarlet fruited gourd

B.1.5. Botany of *C. grandis*:

From botanical description of C. grandis, it is a dioecious perennial herbaceous vine. Stems are mostly glabrous, produced annually from a tuberous rootstock, tendrils simple, and auxiliary. Leaves are alternate, simple, blade broadly ovate, 5-lobed, acute and mucronate at the apex, corodate with a broad sinus at the base. Surfaces are glabrousor scaly. Stamens are 3; present as staminodes in female flowers.Overy is inferior. Fruits are a smooth, smooth, bright red, ovoid to ellipsoid. (Tamilselvan N, 2011)

C.1.1 *Polygonum hydropiper:*

Water-pepper or Water pepper (*Persicaria hydropiper*, syn. *Polygonum hydropiper*) is a plant of the family Polygonaceae. It grows in damp places and shallow water. It is a cosmopolitan plant, found in Australia, New Zealand, temperate Asia, Europe, and North America. It has some use as a spice because of its pungent flavor.

C.1.2. Active ingredients:

The water-pepper has several active ingredients. Two bicyclic sesquiterpenoids are present, polygodial (tadeonal, an unsaturated dialdehyde with a drimane

backbone) and waburganal, which has been found responsible for the pungent taste (hence its edibility). The plant also contains rutin, a source of the bitter taste impression.

The plant contains an essential oil (0.5%) which consists of monoterpenoids and sesquiterpenoids: α-pinene, β-pinene, 1,4-cineol, fenchone, α-humulene, β-caryophyllene, trans-β-bergamotene. Carboxylic acids (cinnamic, valeric and caproic acid) and their esters were present in traces. The composition depends strongly on genetic factors.

C.1.3. Taxonomy:

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Caryophyllidae

Order: Polygonales

Family: Polygonaceae

Genus: Polygonum L.

Species: *Polygonum hydropiper* L.

C.1.4. Hazards:

Although no specific mention has been made for this species, there have been reports that some members of this genus can cause photosensitivity in susceptible people. Many species also contain oxalic acid (the distinctive lemony flavor of sorrel) whilst not toxic this substance can bind up other minerals making them unavailable to the body and leading to mineral deficiency. Having said that, a number of common foods such as sorrel and rhubarb contain oxalic acid and the leaves of most members of this genus are nutritious and beneficial to eat in moderate quantities. Cooking the leaves will reduce their content of oxalic acid. People with a tendency to rheumatism, arthritis, gout, kidney stones or hyperacidity should take especial caution if including this plant in their diet since it can aggravate their condition.

C.1.5. Botanical description:

Buding is a smooth, rather robust annual, with tufted or shortly creeping roots. Stems are erect, while the branches are ascending, rather stout and leafy, 30 to 45 cm high, often glandular. Nodes are often swollen. Leaves are lanceolate or oblong-lanceolate, up to 7.5 cm long. Racemes are flexuous, leafy at the base, threadlike, decurved and interrupted. Flowers are pinkish. Nuts are usually three-sided.

Figure 1.4: *Polygonum hydropiper*

C.1.6. Major Constituents:

A bicyclic sesquiterpenoid, polygodial (tadeonal, an unsaturated dialdehyd with a drimane backbone) has been found responsible for the pungent taste; rutin (see rue) is the source of the bitter taste impression. Polygodial also appears in an exotic Australian spice, Tasmanian pepper, and, in small quantity, in the Brazil paracress. The plant contains an essential oil (0.5%) which is mainly made up of monoterpenoids and sesquiterpenoids: α-pinene, β-pinene, 1,4-cineol, fenchone and α-humulene, β-caryophyllene, *trans*-β-bergamotene. Carboxylic acids (cinnamic, valeric, capronic acid) and their esters were present in traces. The composition depends strongly on genetic factors.

Chapter 2: Literature review

2.1.1 Analgesic activity of *Litsea glutinosa*

Litsea glutinosa (L.) a medicinal plant is popularly used as herbal remedy for various ailments. But the scientific basis for its medicinal use especially in pain and inflammation remains unknown. Therefore, the present study was designed to evaluate analgesic potential of the ethanolic extract of the bark of the plant. The analgesic activity of standard tramadol HCl was determined for its central pharmacological actions using hotplate method in mice. Simultaneously the ethanolic extract was also investigated for its analgesic action. (Pattari Lohitha 2010)

2.1.2. Antimirobial activity of *Litsea glutinosa*

The powdered material of *Litsea glutinosa* bark was extracted separately by continuous hot extraction process using soxhlet apparatus with different solvents in increasing order of polarity from Petroleum ether, Ethanol, to finally water. After extraction the extracts were subjected to Lyophilization to get dry extract & preserved in aseptic condition. The dried extracts were subjected to various phytochemical analysis to detect the presence of different phytoconstituents like alkaloids, glycosides, flavonoids, saponins, tannins, phenolic compounds. The petroleum ether extract, ethanolic extract and aqueous extracts of the Litsea glutinosa bark have the antibacterial & antifungal activity against Gram positive Staphylococcus aureus bacteria using reference standard like Procain pencillin,Gram negative like Pseudomonas aeruginosa, Salmonella

typhi, E-coli bacteria using reference standard like Streptomycine sulphate. And fungal species like Aspergillus fumigatus and Candida albicans using reference standard like Griseofulvin. The bark of Litsea glutinosa, is one of the most popular of native drugs, is considered to be capable of relieving pain, arousing sexual power & good for stomach are considered to be mildly astringent, include treatment of diarrohea & dysentery. Litsea glutinosa is widely used as a demulcent & as an emollient. Petroleum ether extract & Ethanolic extracts showed good activity against Pseudomonas aeruginosa. Aqueous extract & Petroleum ether extracts showed less activity as that of Ethanolic extract against Salmonella typhi. Then Ethanolic extract showed more effective activity against E-coli. Ethanolic extract had very high activity against Staphylococcus aureus. Likewise Petroleum ether extract of Litsea glutinosa effective in inhibiting the growth of Aspergillus fumigatus & Candida albicans. The phytochemical constituents of bark of Litsea glutinosa showed the effective antibacterial & antifungal activity. (Poornima .V. Hosamath 2011)

2.1.3. Antioxidant, anti-inflammatory and wound healing activity of extracts of *Litsea glutinosa*

Present work was undergone to investigate antioxidant and anti inflammatory and wound healing effects of Litsea glutinosa in rats. The aqueous extract of Litsea glutinosa (250 and 500 mg/kg body weight) was studied for anti inflammatory in animal models. The activity was studied in some acute models

Viz carragenan, histamine and dextrin induced rats paw edema against indomethacin as standard, and it showed significant anti inflammatory activity in all the three models. The preliminary phytochemical analysis was carried out for different extracts. It was found that flavone glycosides, reducing sugars, aminoacids and tannins. Ethanolic extract of *Litsea glutinosa* showed significant response in wound tested when compared with the control group. Nitrofurazone ointment was used as a standard drug. Antioxidant activity was determined by two in vitro methods- DPPH and radical scavenging. The extract having significant antioxidant activity compared to control, BHT and Ascorbic acid were used as reference standard for antioxidant activity. On the basis of the results obtained suggest marked that extracts have significant antioxidant, anti inflammatory and wound healing activity of Litsea glutinosa. The results supported the traditional use of this plant in some painful and inflammatory conditions. (Devi P., Meera R., 2010)

2.2.1. The determination of antibacterial and antifungal Activities of *Polygonum hydropiper* (L.) root Extract:

A study was conducted to determine the antibacterial and antifungal activities of *Polygonum hydropiper* (L.) root extract on chloroform against both bacteria and fungi using the disc diffusion method. The extract showed significant antibacterial activities against four gram-positive (*Bacillus subtilis, Bacillus megaterium*, *Stapphylococcus aureus* and *Enterobacter aerogenes*) and four

gram negative (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Shigella sonnei*) bacteria. The minimum inhibitory concentration (MIC) values against these bacteria ranged from 16 - 64 µg/ml. The antifungal activities were found strong against six fungi (A*spergillus fumigatus, Aspergillus niger, Aspergillus flavus, Candida albicans*, *Rizopus oryzae* and *Tricophyton rubrum*). It can be used in the folk medicine at different parts of the world to treat many diseases including bacterial and fungal infections. (M. F. Hasan, 2009)

2.2.2. In vitro and in vivo anti-inflammatory activities of Polygonum hydropiper methanol extract

Polygonum hydropiper L. (Polygonaceae) has been traditionally used to treat various inflammatory diseases such as rheumatoid arthritis. However, no systematic studies on the anti-inflammatory actions of Polygonum hydropiper and its inhibitory mechanisms have been reported. This study is therefore aimed at exploring the anti-inflammatory effects of 99% methanol extracts (Ph-ME) of this plant. The effects of Ph-ME on the production of inflammatory mediators in RAW264.7 cells and peritoneal macrophages were investigated. Molecular mechanisms underlying the effects, especially inhibitory effects, were elucidated by analyzing the activation of transcription factors and their upstream signalling, and by evaluating the kinase activities of target enzymes. Additionally, a dextran sulphate sodium (DSS)-induced colitis model was

employed to see whether this extract can be used as an orally available drug. (Yang Y, 2012)

2.2.3. Screening of antibacterial, antifungal and cytotoxic activities of Polygonum hydropiper L. stem extracts

The aim of the study was to investigate the antibacterial, antifungal and cytotoxic activities of ethanol extract of *Polygonum hydropiper* stem. Disc diffusion method measuring minimum inhibitory concentration (MIC) was used to demonstrate antibacterial and antifungal activities. Stem extract showed significant antibacterial activities against three gram-positive (*Bacillus subtilis*, *Bacillus megaterium* and *Staphylococcus aureus*) and four gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella sonnei*) bacteria resulting 16 to 64 µg/ml MIC values against these bacteria. Further, the antifungal activities of stem extract were not highly remarked but still to be considered as inhibitory to tested fungi. LC50 of the extract against brine shrimp nauplii was 35.46µg/ml and indicates the cytotoxic potentiality of *Polygonum hydropiper* stem. Results obtained in this study suggest that *Polygonum hydropiper* stem can also be used as a source of antimicrobial and cytotoxic substances for possible for plant protections. (M. F. Hasan, 2011)

2.3.1. Anti-hyperglycemic and insulin releasing effect of *Coccinia grandis* (L.) leaves in Normal and Alloxan Diabetic Rats:

Coccinia grandis (L.) occurs throughout the world and has intensive popular use in the treatment of infections. The main aim of the present work was to investigate the antidiabetic effects of aqueous extracts of leaves of C. grandis obtained by Decoction method. Graded doses of the aqueous extract were administered to normal and experimental diabetic rats for 10 days. Significant ($p < 0.05$) reduction in fasting blood glucose levels were observed in the normal as well as in the treated diabetic animals. Serum insulin levels were not stimulated in the animals treated with the extract. The changes in body weight, serum lipid profiles, liver glycogen levels were assessed in the extract treated diabetic rats and compared with diabetic control and normal animals. (A. Doss, 2008)

2.3.2. A study on antioxidant activity of fruit extracts of *coccinia grandis* l.voigt:

The aim of the present study was investigating the antioxidant activity of the methanolic extract of the fruit of *Coccinia grandis* L. Voigt. (Cucurbitaceae). The antioxidant activity of the fruit has been evaluated by using three in vitro assays and was compared to standard antioxidant, Butylated hydroxyanisole (BHA). All the fractions showed effective H-donor activity, reducing power, free radical scavenging activity. The antioxidant property depends upon

concentration and increased with increasing amount of the fractions. The free radical scavenging and antioxidant activities may be attributed to the presence of flavonoids present in the fractions. The results obtained in the present study indicate that the fruit of *Coccinia grandis* is a potential source of natural antioxidant. (Deshpande S.V. *et al,*2011)

2.3.3. Anti-ulcer effect of *Coccinia grandis*(linn.) On pylorus ligated (albino) rats:

The objective is to evaluate the anti–ulcer activity of ethanolic, aqueous, total aqueous extracts of leaves of *Coccinia grandis* (Linn.) , Anti-ulcer activity of the three extracts was studied in rats by using pylorus ligated ulcer model and it was subjected to preliminary phytochemical studies for the identification of phytoconstituents and also studied for color, consistency and percentage yield of various extracts. Omeprazole was used as the standard drug for comparison. The animals were sacrificed after 19 hrs after the ligation. Stomach was dissected out and contents were drained into tubes and were centrifuged at 1000 rpm for 10min and volume was noted. The PH of gastric juice was recorded using a PH meter. The contents were subjected for analysis of free and total acidity and Na+, k+ ion concentration. The numbers of ulcers per stomach was noted and severity of ulcers scored. Then the blood samples were collected and subjected to estimation of serum calcium and alkaline phosphatase level. The expected result is to get an anti-ulcer activity of the leaf extracts of *Coccinia*

grandis should owing to the presence of one or more phytoconstituents, which may reduce the acidity of the gastric juice and also prevents the mucosal damage and ulcer formation. The Ethanolic Extract 400mg/kg expected to show comparable anti ulcer-activity as that of standard Omeprazole. (P. Manoharan, 2001)

2.3.4. In vivo antitussive activity of Coccinia grandis against irritant aerosol and sulfur dioxide-induced cough model in rodents

Coccinia grandis (Cucurbitaceae) has extensively used to get relief from asthma and cough by the indigenous people of India. The antitussive effect of aerosols of two different concentrations (2.5%, 5% w/v) of methanol extract of *C. grandis* fruits were tested by counting the numbers of coughs produced due to aerosols of citric acid, 10 min after exposing the male guinea pigs to aerosols of test solutions for 7 min. In another set of experiment methanol extract was investigated for its therapeutic efficacy on a cough model induced by sulfur dioxide gas in mice. The results showed significant reduction of cough number obtained in the presence of both concentrations of methanol extract as that of the prototype antitussive agent codeine phosphate. Also, methanol extract exhibited significant antitussive effect at 100, 200 and 400 mg/kg, per orally by inhibiting the cough by 20.57, 33.73 and 56.71% within 90 min of performing the experiment respectively. (S. P. Pattanayak, 2009)

2.3.5. Antibacterial Activity of the Leaves of Coccinia indica (W. and A) Wof India:

The aim of the present research was focused on investigating the antibacterial and preliminary phytochemical properties of *Coccinia indica* (W.A.) via in vitro approach. The aqueous and organic solvent (Petroleum ether, chloroform and ethanol) extracts from the leaves of *Coccinia indica* (Cucurbitaceae) were tested against *Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus epidermidis, Bacillus subtilis* and *Salmonella typhimurium* by agar well diffusion method and broth dilution method. Results showed promising antibacterial activity against the bacteria tested. Among these, ethanol and aqueous extracts were found to have a more potent inhibitory effect comparing with the other extracts, which prove the potentiality of the plant extracts for the treatment of various skin and gastrointestinal infections. (A. Hussain, 2010)

Chapter 3: Materials & Methods

3.1 Selection of plants

Fresh Leaves of plant *Polygonum Hydropiper, Coccina Grandis* and *Litsea Glutinosa* were selected for biological investigation.

3.1.1 Collection of the plant part

For this present investigation the leaves of *Polygonum Hydropiper* and *coccinia Grandis* was collected from Gouripur, Mymensing and the leaves of *Litsea Glutinosa* was collected from Netrokona, Mymensing in August 2011. It was identified at the Bangladesh National Herbarium, Mirpur, Dhaka. These are familiar plant and widely distributed in all over Bangladesh.

3.1.2 Drying of the plant part

The collected leaves of all the plants were washed and unwanted materials were discarded. Collected leaves were sun dried for 15 days.

3.1.3 Storage and Preservation of plant part

Most plant parts from desired plants have undergone a period of storage before they were finally used for research purpose in the laboratory. During this period many undesirable changes may occur in the plant parts if they were not properly stored and preserved against the reabsorption of moisture, oxidation, excessive heat, humidity, direct sunlight, growth of moulds and bacteria and infestation by insects and rodents. Proper storage and preservation of plant parts are thus are very important factors in maintaining a high degree of quality in them. All

efforts towards proper storage should be geared to protect the drugs from all the above deteriorating factors and agents.

3.1.4 Grinding of the plant parts

The dried small pieces of plant parts were grinded into small fine particles by a grinder machine from Department of Pharmacy, East West University. The powder was stored in an air tight container and kept in a cool dark and place until analysis commenced.

3.1.5 Extraction of the plant material

The dried leaves of the *Polygonum Hydropiper*(200mg) was extracted by cold extraction process with ethanol(600ml), the leaves of the *Coccinia Grandis* (300mg) was extracted by cold extraction process with ethanol(900ml) and the leaves of *Litsea Glutinosa*(300 mg) was extracted with ethanol(900ml) in a flat bottom glass container at 1:3 ratio, through occasional shaking and stirring for 10 days. The extract was then filtered through filter paper.

3.1.6 Drying of extract

Using rotary evaporator, the ethanolic extract of all three plants was evaporated at 55-60 degree Celsius temperature and a rotation speed of 160-180 rpm. After 30 minutes of drying process, a slurry concentration were obtained, which were kept in small 50 ml beakers for further drying. During the transfer to the beaker the extracts were ringed by ethyl acetate.

Figure 3.1: Rotary evaporator

3.2 Antimicrobial Test

3.2.1 *In Vitro* Antimicrobial screening

Any chemical substance or biological agent that destroys or suppresses the growth of microorganism is called antimicrobial agent. Antimicrobial screening of a crude extract or pure compound isolated from natural sources is essential to ascertain its activity against various types of pathogenic organisms.

Antimicrobial activity of any plant can be detected by observing the growth response of several of various microorganisms to the plant extract, which is placed in contact with them. In general Antimicrobial screening is undertaken in two phages: a primary qualitative assay to detect the presence or absence of activity and a secondary assay which quantaties the relative potency, expressed as Minimum Inhibitory Concentration (MIC) value, of a pure compound, an important method in the further development of a new Antimicrobial compound.

The primary assay can be done in three ways as -

- Diffusion method
- Dilution method
- Bioautographic method

Among these methods, the disc Diffusion method is widely acceptable for the preliminary evaluation of Antimicrobial activity. Disc Diffusion is essentially a

qualitative or semi- qualitative test indicating the sensitivity or resistance of microorganisms to the test materials. However no distinction between bacteriostatic or bacteriosidal activity can be made by this method.

3.2.2 Principle of diffusion method

Diffusion is based on the ability of a drug tobdiffuse from a confined source through the nutrient agar medium and creates concentration gradient. If agar is seeded with a sensitive organism, a zone of inhibition will result where the concentration exceeds the minimum concentration (MIC) for that particular organism.

In this method, measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentration (µg/ml). Then sterile filter paper discs (5 mm diameters) are impregnated with known amounts of the test substances and dried. The dried discs are places on plates (Petri dishes, 120mm diameters) containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4ºC) for 24 hours to allow maximum diffusion.

A number of events take place simultaneously which include-

- The dried discs absorb water from the agar medium and the material under test is dissolved.

- The test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel.
- There is a gradual change of test material concentration on the agar surrounding each disc.

The plates are then kept in an incubator (37ºC) for 12-18 hours to allow the growth of microorganism. If the test material has antimicrobial activity, it will inhibit the growth of the microorganism, giving a clear, distinct zone called "Zone of Inhibition". The antimicrobial activity of the test agent is determined in term of millimeter by measuring the diameter of the zone of inhibition, the bigger the zone of inhibition of the sample, the greater the activity.

The principles factors which determine the size of the zone of inhibition are

- Intensive antimicrobial susceptibility of the test sample.
- Growth rate of the test organism.
- Diffusion rate of the test sample which is related to its water solubility.
- Concentration of the test organisms incubated in the medium.
- Concentration of the test sample per disc.
- Thickness of the test medium in the Petri discs.

3.2.3 Test Materials

In our present study, the antibacterial activity of ethanolic extracts of *Polygonum Hydropiper, Coccinia Grandis* and *Litsea Glutinosa* was investigated in comparison with standard kanamycin (30 µg/disc) antibiotic agent a number of pathogenic Gram-positive and Gram-negative bacteria, fungus and yeast.

3.2.4 Test Organism

Both Gram-positive and Gram-negative strains of bacteria, fungus and yeast were used as the test organism to observe the anti-bacterial activity of the isolated compounds. The bacterial strains used for this investigation are listed in the Table. These organisms were collected from the Microbiology research laboratory, Department of Pharmacy, East West University. The pure of which was previously collected from the Microbiology Department of Dhaka University.

Table3.1: List of pathogenic bacterial strains.

Microorganism	Gram-negative
Staphylococus aereus Bacillus subtilis Sarcina lutea	Salmonella paratyphi Escherichia coli Shigella dysenteriae

Bacillus circus	Vibrio minicus Salmonella typhi Vibrio parahemolyticus

3.2.5 Apparatus and Reagent

- Filter paper discs (5mm in diameter)
- Petri dishes
- Refrigerator
- Test tubes
- Sterile forceps
- Sterile cotton
- Incubating loop
- Bunsen burner
- Micropipette(10-100µl) & (100-1000µl)
- Laminar air flow unit
- Autoclave
- Incubator
- Nutrient agar media

- Ethanol
- Vials
- Standard disc(Kanamycin 30 µg/disc)

3.2.6 Sterilization Procedure

Petri dishes and other glass wares were sterilized by autoclaving at a temperature 126ºC and a pressure of 20 lbs/sq inches for 45 minutes. Blank discs first kept in a covered Petridis and then subjected to dry heat sterilization at 180ºC for 1 hour. Later they were kept in laminar hood under UV light for 30 minutes. UV light was switched on before one hour working in laminar hood to avoid any accidental contamination.

3.2.7 Culture Media

The main requirement for the growth of bacteria were as follows-

- Source of energy such as carbohydrate, protein and nuclic acid.
- Essential trace elements e.g. Mg, Mn, Fe, and Co
- Optimum pH of media and
- Optimum temperature for incubation

A number of culture media are available to demonstrate the antibacterial activity. This are-

- Nutrient agar media
- Nutrient broth media
- Mueller-Hinton agar media
- Tryptic soy broth(TSB)

Among these, nutrient agar media is most frequently used and its composition is shown in below.

Table3.2: Composition of nutrient agar media

Ingredient	Amount(g/l)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15.0
Distilled water	100 ml
pH	7.5 ±1.0 at 25°C

3.2.8 Preparation of Medium

The instant nutrient agar media was accurately weighted and then reconstituted with distilled water in a conical flask according to specification (2.8% w/v). It was autoclaved to dissolve the agar and make it micro organism free. A transparent solution was obtained.

The prepared media was then transferred in 9ml and 5ml in a number of clean test tubes, respectively to prepare plates and slants. The slants were used for making sub-culture of microorganism, which in turn use for sensitivity tests.

The test tubes were then plugged with cotton and sterilized in an autoclave at temperature of 126ºc and pressure of Ib/sq inch for 15 minutes.

3.2.9 Preparation of Subculture

With the help of a inoculating lop, the test organisms were transferred from the pure culture to the agar containing Petri dishes in a laminar airflow unit. The incubated satins were then incubated at 37º for 18-24 hours to ensure the growth of test organisms. This culture was used for sensitivity test.

3.2.10 Preparation of Test Plates

The test organism was transferred from the subculture to the petri dishes containing 9ml autoclaved medium with the help of an incubating loop in aseptic area. The test tube was shaken by rotation to get a uniform suspension of the organism. The bacterial suspensions were immediately transferred to the sterile perishes in an aseptic area and were rotated several times, first clockwise

and anticlockwise to ensure homogeneous dispersion of the organism into the medium. The depth of media into each Petri dish was approximately 4mm. After plates were cooled to room temperature, it stored in a refrigerator at 4ºC.

3.2.11 Preparation of Discs

Three types of discs were used for antibacterial screening. These were-

- Sample discs
- Standard discs
- Blank/control discs

✓ Sample discs:

Sterilized filters discs (5 mm in diameter) were taken in a blank Petri dish. Sample solution of the desired concentration was applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of solvent.

✓ Standard discs:

These were used to compare to the antibacterial activity of test material. In our investigation Kanamycin (30 g/disc) was used as a reference.

✓ Blank discs:

Only solvent was applied to the disc to determine the antibacterial effects of the solvent used.

3.2.12 Preparation of Discs Containing Sample

Test sample contained *Polygonum Hydropiper, Coccinia Grandis* and *Litsea Glutinosa*. Dried ethanolic extract (8mg) was dissolved in 400µl methanol to prepare a concentration of 400µg/µl.

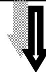
3.2.13 Placement of Disc, Diffusion and Incubation

The sample disc and standard antibiotic disc were placed gently on the solidified agar plates freshly seeded with the organism with the help of a sterile forceps to ensure complete contact with medium surface. The arrangement of the disc was such that the discs were no closer than 15 mm to the plate to prevent overlapping the zone of inhibition.

The plates were then inverted and kept in a refrigerator for about 24 hours at 4ºC. This was sufficient time for the material to diffuse to a considerable area of the medium. Finally, the plates were incubated at 37ºC for 12-18 hours.

3.2.14 Procedure

Bacterial culture was transferred from the subculture to the petri dishes containing autoclaved medium using sterilized loop



Sterilized filters discs were taken and sample solution of the desired concentration was applied on the discs with the help of a micropipette in an aseptic condition

3 Discs containing plant samples, standard and blank control was placed with separate sterilized forceps

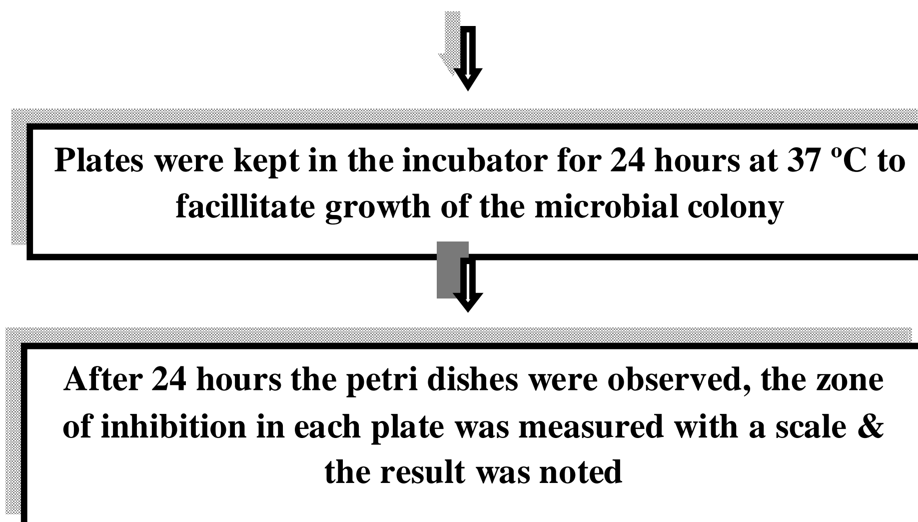


Figure 3.2: Schematic diagram of antmicrobial test

3.3. Cytotoxicity Test

Cytotoxicity test is the quality of being toxic to cells. Examples of toxic agents are a chemical substance, an immune cell or some types of venom (e.g. from the puff adder or brown recluse spider).

3.3.1 Brine shrimp lethality bioassay

The brine shrimp lethality bioassay is considered a useful tool for preliminary assessment of toxicity. It has also been suggested for screening pharmacological activities in plant extracts. However, we think that it is necessary to evaluate the suitability of the brine shrimp methods before they are used as a general bioassay to test natural marine products for pharmacological activity. Pharmacology is simply toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Bioactive compounds are almost always toxic in

high doses. The *in vivo* lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products. Focused on *Artemia salina* as a test organism and developed a protocol for Brine shrimp lethality bioassay to monitor cytotoxicty of a compound.

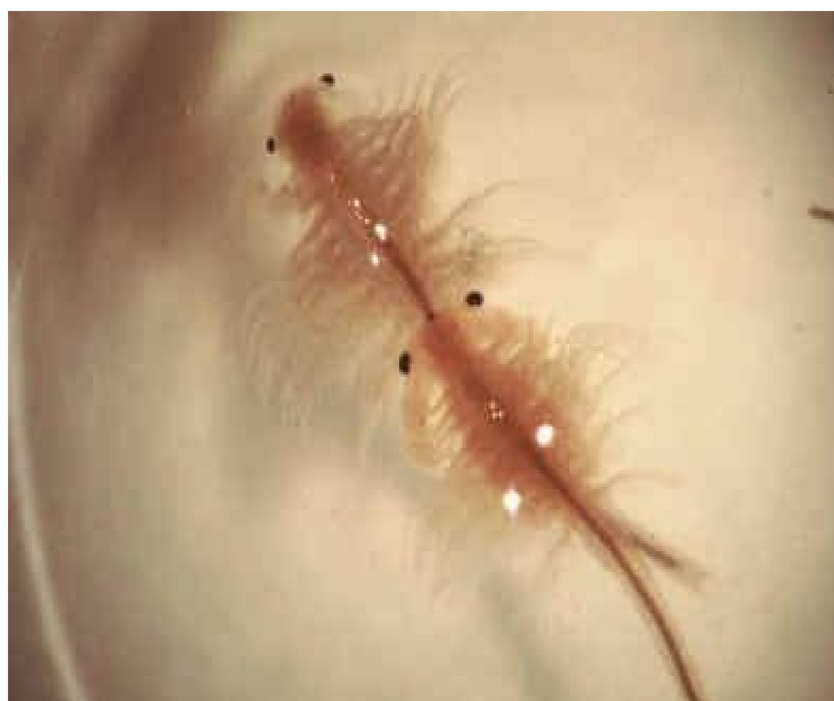


Figure: *Artemia salina*

3.3.2 Principle

Brine shrimp eggs are hatched in simulated seawater to get nauplii. Sample solutions are prepared by dissolving the test materials in pre-calculated amount of DMSO. Ten nauplii are taken in vials containing 5 ml of simulated seawater. The samples of different concentrations are added to the premarked vials with a micropipette. The assay is performed using three replicates. Survivors are

counted after 24 hours. These data are processed in a simple program for probity analysis to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies. (Karmakar *et al*., 2011)

3.3.3 Materials

- Artemia Salina leaches (Brine Shrimp eggs)
- Sea salt (NaCl)
- Small tank with electric air bubbler
- Lamp to attract Shrimp
- Pipettes
- Micropipette
- Glass vials
- Magnifying glass
- Test samples of experimental plant
- Test tube
- 3.3.4 Hatching of brine shrimps

3.3.4. Hatching of brine shrimp:

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and with the help of a Pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of seawater.

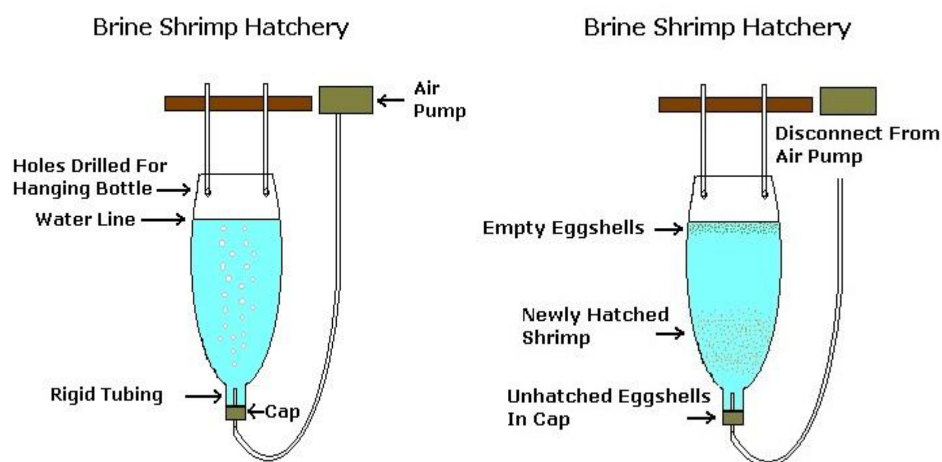


Fig3.3: Brine Shrimp hatchery

3.3.5 Preparation of sea water

38 gm sea salt (pure NaCl) was weighed, dissolved in one liter of distilled water and filtered out to get clear solution.

3.3.6 Preparation of sample

Measured amount (1.00 mg) of each sample was dissolved in saline water (0.9% NaCl) with the help of 1 drop of DMSO. Total 5ml solution was made in a 5ml

cap test tubes. A series of solutions of lower concentrations were prepared by serial dilution with saline water. From each of these test solutions 250 ml were added to premarked glass test tubes containing 5 ml of seawater and 10 shrimp nauplii. So, the final concentration of samples in the test tubes were 400µg/ml, 200 µg/ml,100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.567 µg/ml, 0.781µg/ml .

3.3.7 Preparation of controls

Vincristine sulphate served as the positive control. 0.2mg of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions were made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. The control groups containing 10 living brine shrimp nauplii in 5 ml simulated sea water received the positive control solutions.

As for negative control, 30 µl of DMSO was added to each of three premarked glass vials containing 5 ml of simulated seawater and 10 shrimp nauplii. The test was considered invalid if the negative control showed a rapid mortality rate (Karmakar *et al*., 2011)

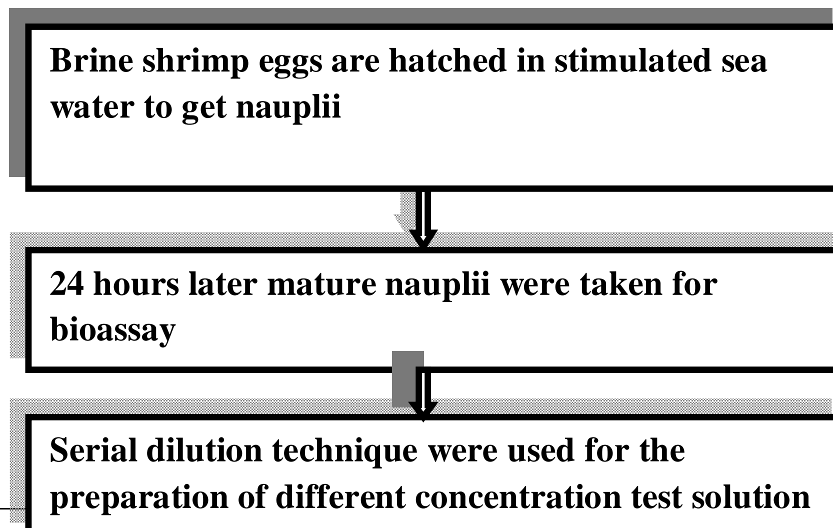
3.3.8 Application of the sample & Brine Shrimp naupli to the vials

Well cleaned vials were taken for 10 different concentrations. Sea water containing a number of Brine Shrimp nauplii were taken in each of the vials. With the help of micropipette specific volumes of the sample were transferred the vials .For each of vials contain these volumes of samples & 5ml of Brine solution contain 10 naupili.

3.3.9 Counting of Nauplii and Analysis of Data

After 24 hours, the vials were inspected using a magnifying glass and the number of survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration-mortality data were analyzed statistically by using probity analysis and linear regression using a simple PC program. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

3.3.10 Procedure



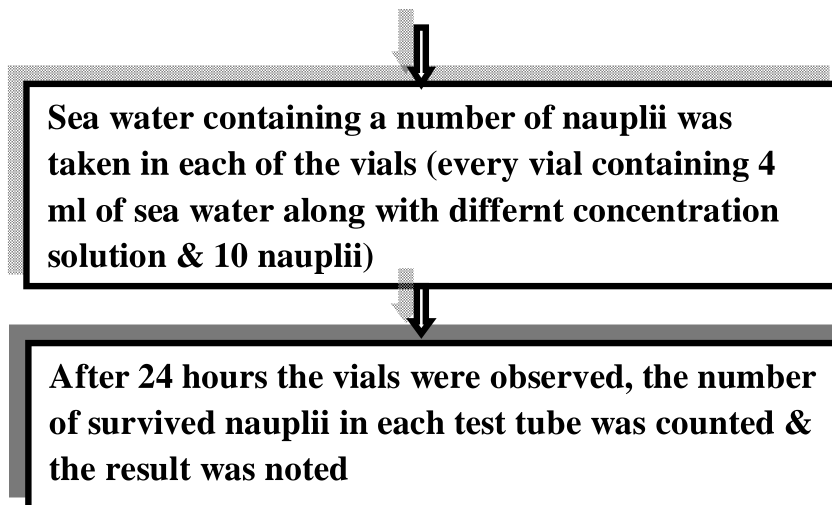


Figure 3.4: Schematic diagram of cytotoxic Bioassay

3.4 *In vitro* Thrombolytic effect

3.4.1 Principle

Atherothrombotic diseases occur as serious impacts of the thrombus formed in blood vessels. Various thrombolytic agents are used to dissolve the clots that have already formed in the blood vessels; but these drugs are not above limitations and can lead to serious and sometimes fatal consequences.

The present study was carried out to investigate the thrombolytic activity of the leaf extracts of *Polygonum Hydropiper, Coccinia Grandis* and *Litsea Glutinosa*. An *in vitro* thrombolytic method was used to investigate the thrombolytic activity of plant extracts in blood sample from healthy human volunteers, along with streptokinase as a positive control. SK, a known thrombolytic drug is used

as a positive control. The comparison of positive control with sample clearly demonstrated about clot dissolution when sample was added to the clot.

3.4.2 Materials

- Sterile syringes (10ml)
- Cotton
- Ethanol
- Micropipette
- Eppendorf tubes (1.5ml)
- Incubator
- Tube holding stand
- Electronic & digital balance
- Sonicator
- Saline water (0.9% NaCl)
- DMSO

3.4.3 Preparation of sample

In glass vials 1mg of N. sativa and 1mg of N. nouchali was weighed using electronic balance. Then 1ml of pyrogen free normal saline (0.9% NaCl) was

added in both vials using a pipette. For enhancing proper dissolution, 1 drop of DMSO was added and sonicated with the help of a “Sonicator”.

3.4.4 Application of test sample

In brief, 8 ml venous blood drawn from healthy volunteers was distributed in eight different pre weighed sterile eppendorf tube (1 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed ,aspirated out without disturbing the clot formed and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube –weight of tube alone).

Each eppendrof tubes containing clot was properly labeled. To each microcentrifuge tube containing pre-weighed clot, 100 µl of ethanolic extract (100 mg/ml) of *Polygonum Hydropiper, Coccinia Grandis* and *Litsea Glutinosa* was added into each eight tubes separately using micropipette. As a positive control, 100 µl of streptokinase was used. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. (Prasad S. 2006)

3.4.5 Procedure

1ml blood was taken into eight separate preweighed sterilized eppendorf tubes and incubated at 37°C for 45 minutes

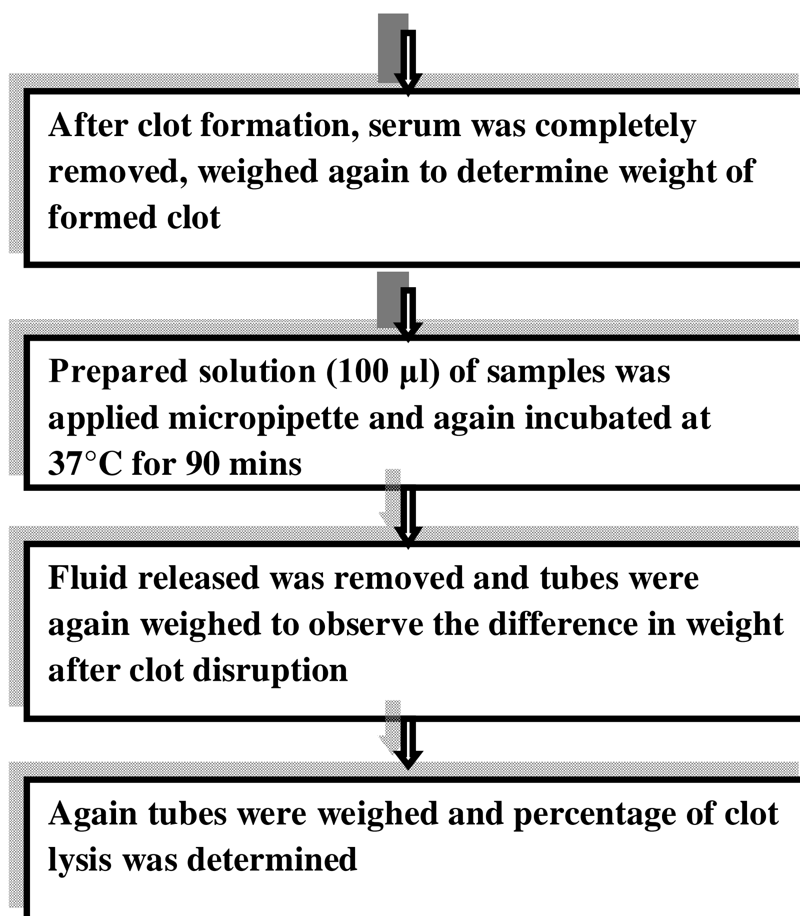


Fig3.5: Schematic diagram of Thrombolytic Bioassay

3.5 Evaluation of scavenging activity

3.5.1 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 posses 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)

3.5.2 Methods of evaluating antioxidant activity

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

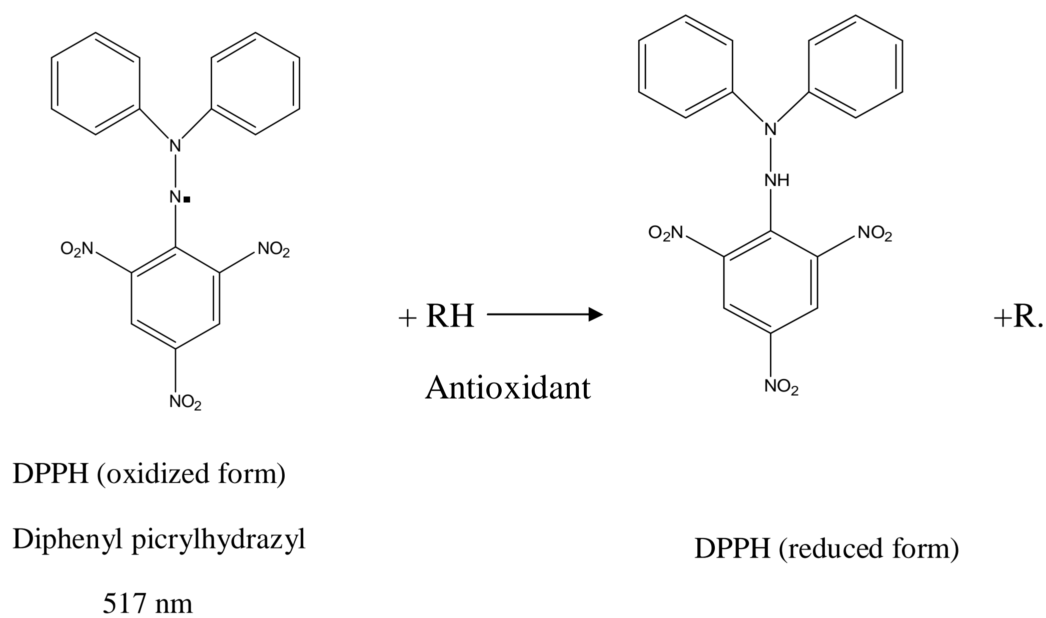
- Determination of DPPH radical scavenging assay (Quantitative analysis)
- Determination of total phenolic content
- Determination of reducing power ability
- Determination of total antioxidant capacity by phosphomolybdenum method
- Determination of total flavonoids content

3.5.3 Determination of DPPH radical scavenging assay (Quantitative analysis)

3.5.3.1 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.



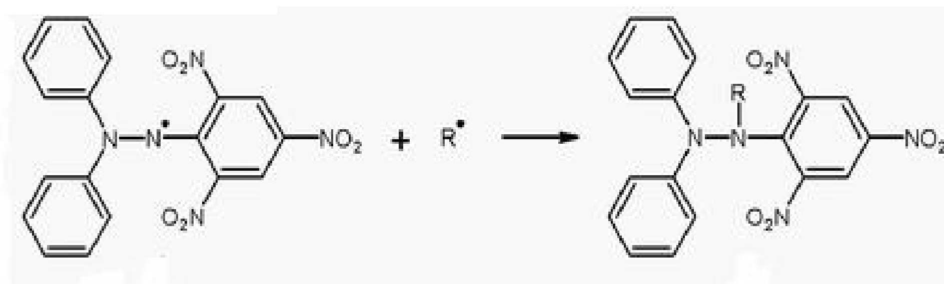


Figure 3.4: reaction of DPPH with reactive free radical

3.5.3.2. Materials & Reagents

- 1,1-diphenyl-2-picrylhydrazyl
- L-Ascorbic acid
- Distilled water
- Methanol

- Pipette (5ml)
- Analytical balance
- UV- visible spectrophotometer
- Beaker (100 & 200ml)
- Test tube
- Aluminium foil
- Spatula

3.5.3.3. 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:

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

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

- L-Ascorbic acid was used as positive control.

- Tests carried out in triplicate and average value was taken.

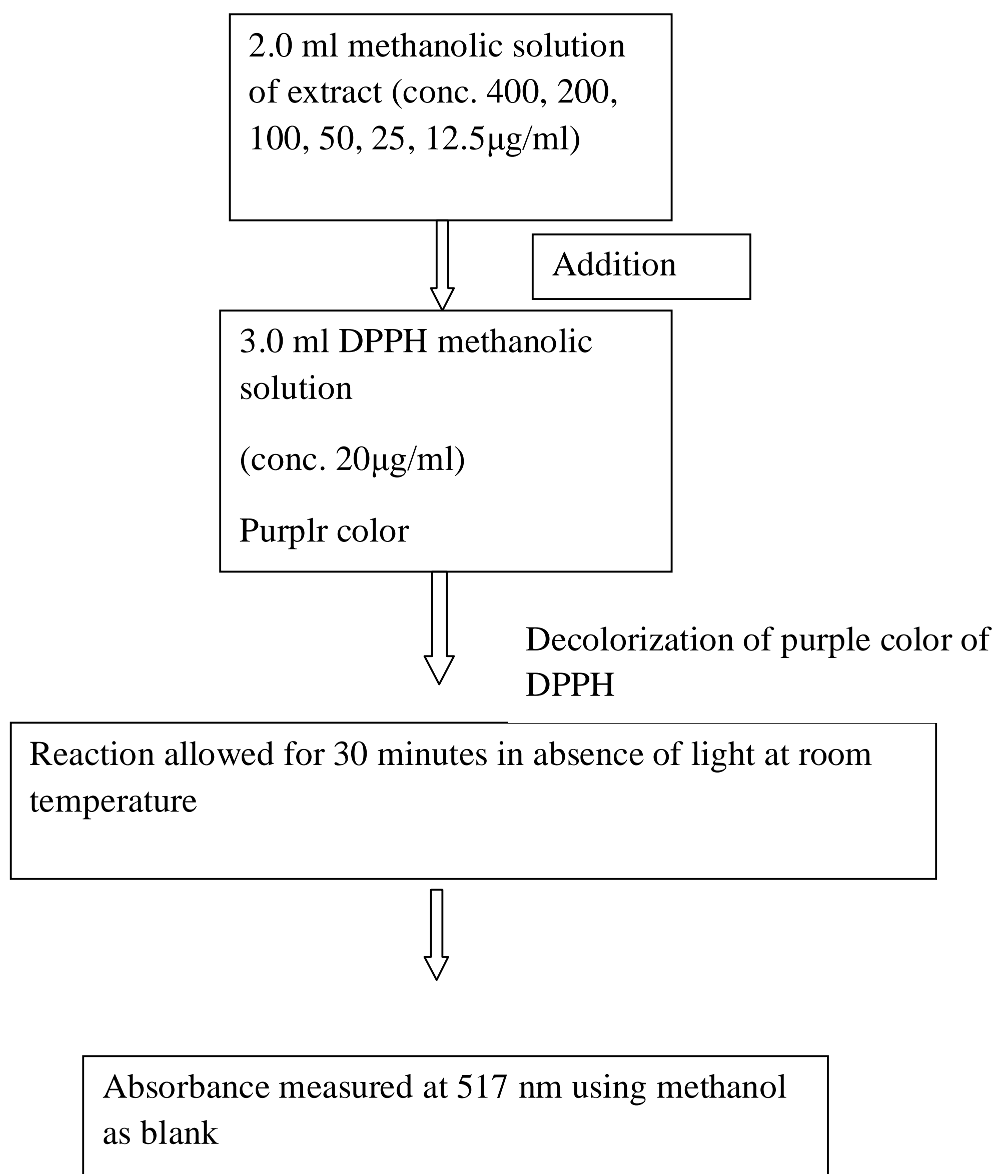


Figure: 3.5.Schematic representation of the method of assaying free radical scavenging activity

3.6. Evaluation of analgesic property

Pain is probably the most prevalent symptom in clinical practice, and characterization of pain is of major importance in the diagnosis and choice of treatment (Thumshirn *et al*., 1999). In the treatment of diseases associated with

pain, the clinical effects typically guide the selection of the analgesics and titration of the dose. However, in practice, the different symptoms of the underlying diseases confound the characterization of pain. These confounders may include complaints relating to psychological, cognitive and social aspects of the illness, as well as systemic reactions such as fever and general malaise (Drewes *et al*., 2003).

Furthermore, treatment with analgesics often causes sedation and other side effects. This may bias the clinical evaluation, as the patients tend to interpret other effects of the medication– such as an effect on the anxiety and depression relating to the disease – as a relief of pain (Le Bars *et al*., 2001).

Because of these confounding factors, *experimental pain models* are often advantageous in preclinical investigations of analgesics. With these models, the investigator can control the experimentally induced pain (including the nature, localization, intensity, frequency and duration of the stimulus), and provide quantitative measures of the psycho-physical, behavioral or the neurophysiological responses (Drewes *et al*., 2003).

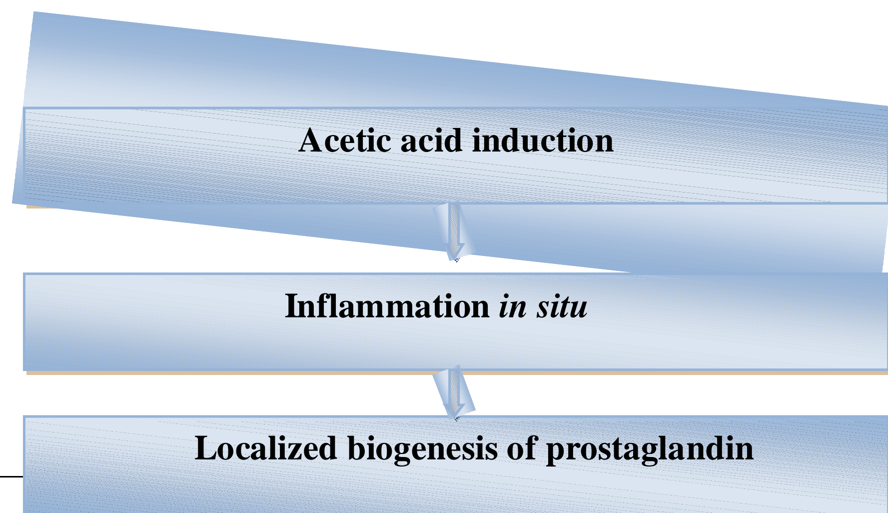
Experimental pain models have been used in *animal studies.* In these experiments, the neuronal nociceptive activity can be recorded or behaviour can be assessed (Sengupta & Gebhart 1994).

However neuronal recordings or reactions do not reveal all aspects of pain, since pain is the net effect of complex multidimensional mechanisms that involve most parts of the central nervous system (Le Bars *et al*., 2001).

Nociceptive reflexes or electrophysiological recordings from selected pathways in the animal nervous system are important in basic research and screening of analgesics. However, animal experiments typically suppress central pain mechanisms and associated complex reactions seen in man. Furthermore, the neurobiology of nociceptive systems differs between species, and this limits the extrapolation of findings from animal studies to man even further (Le Bars *et al*., 2001).

3.6.1. Mechanism of pain induction in Acetic acid induced writhing method

Intraperitonial administration of acetic acid causes localized inflammation in mice. Following inflammation, there is biogenesis of prostaglandins (cyclooxygenase pathway) & leukotrienes (lipooxigenase pathway). The released prostaglandin, mainly prostacyclin (PGI_2) & prostaglandin E have been reported responsible for pain sensation (Le Bars *et al*. 2001).



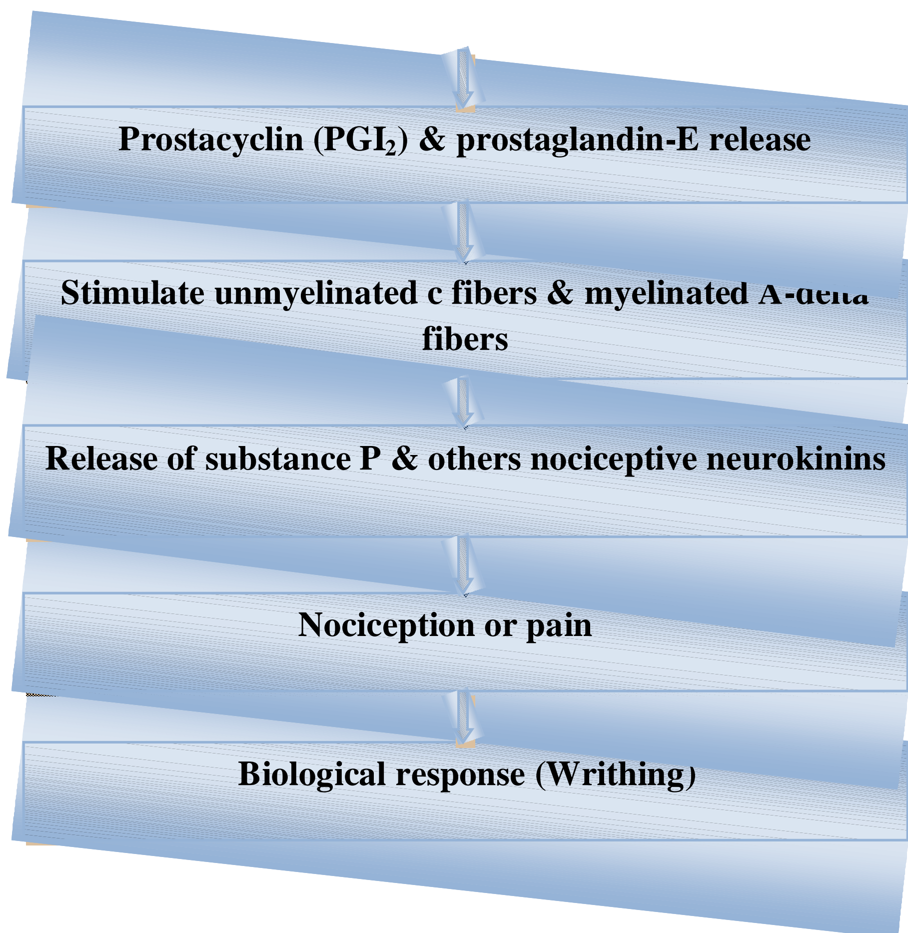


Figure 3.6: Schematic diagram of pain induction

3.6.2 Materials and Methods

3.6.2.1 Drugs and chemicals

Acetic acid was obtained from Merck, Germany. Tween-80 was obtained from BDH Chemicals, UK. Normal saline solution was purchased from Beximco

Infusion Ltd., Bangladesh. Diclofenac was obtained from Square Pharmaceuticals Ltd., Bangladesh.

6.4.2.2 Animal

For the experiment male Swiss albino mice of 3 - 4 weeks of age, weighing between 20 - 25 gm, were collected from the animal research branch of the international center for diarrheal disease & research, Bangladesh (ICDDRB). Animals were maintained under standard environmental conditions and had free access to feed and water which is ICDDRB formulated. The animals were acclimatized to laboratory condition for one week prior to experiments.

Figure 3.7: Swiss albino Mice

6.4.2.3 Experimental design

Twenty experimental animals were taken randomly selected and divided into three groups denoted as experimental group 1, experimental group 2, positive control group & negative control group. Each group mouse was weighed

properly & dose of the test sample & control materials was adjusted accordingly.

6.4.2.4 Method of identification of animals

Each group consists of five animals. It was difficult to observe the biological response of five mice at a time receiving same treatment. It is quite necessary to identify individual animal of groups during treatment.

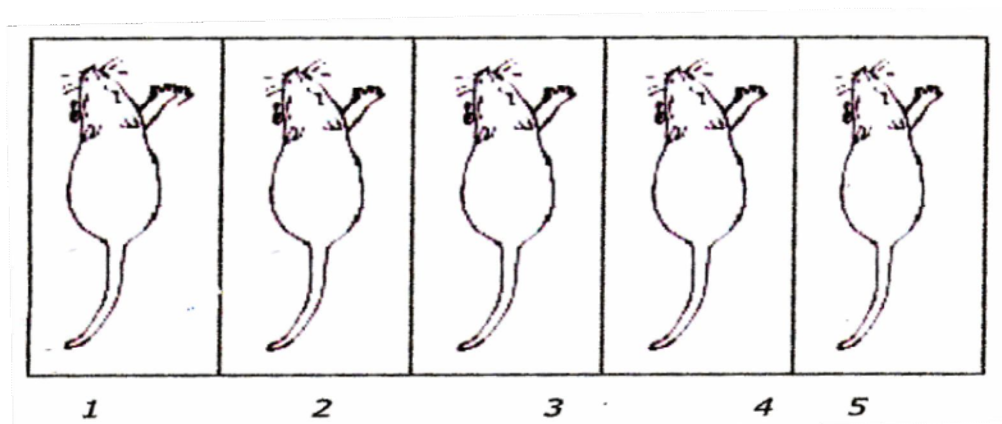


Figure 3.8: Identification of test animals for analgesic property screening

6.4.2.5 Preparation of test material

In order to administer the crude extract at dose 200 & 400 mg/kg body weight of mice. 150 mg of the extract was measured & was triturated unidirectional way by the addition of 5 ml of distilled water. After proper mixing, small amount of suspending agent Tween-80 was slowly added. The final volume of the suspension was made 5.1 ml. To stabilize the suspension it was stirred well. For the preparation of positive control group (10 mg/kg) 30 mg Diclofenac is taken & a suspension of 3 ml is made.

6.4.2.6 P[illegible]

Figure 3.9: Schematic representation of procedu[illegible] analgesic property on mice by acetic acid induced method.

6.4.2.7 Counting of writhing

Each mouse of all groups were observed individually for counting the number of writhing they made in 15 minutes commencing just 5 minutes after the intraperitoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half writhing. Accordingly two half was writhing, I taken as one full writhing.

Chapter 4: Results & Discussion

4.1 Thrombolytic effect

Table 4.1: Thrombolytic data of *Polygonum hydropiper*

No	Wt of clot	Wt of clot (After Applied Sample)	Wt. loss after administration	% Clot lysis	Average of % clot lysis	Standard Deviations
1	0.9954	0.4683	0.29	38.24	37.592	5.8194
2	0.9981	0.4131	0.3657	46.95		
3	0.9965	0.6386	0.3226	33.58		
4	0.9952	0.4599	0.2521	35.407		
5	1.0136	0.6212	0.3258	34.403		
6	0.9942	0.419	0.2809	40.13		
7	0.9975	0.3968	0.3032	43.314		
8	0.9966	0.5065	0.204	28.712		

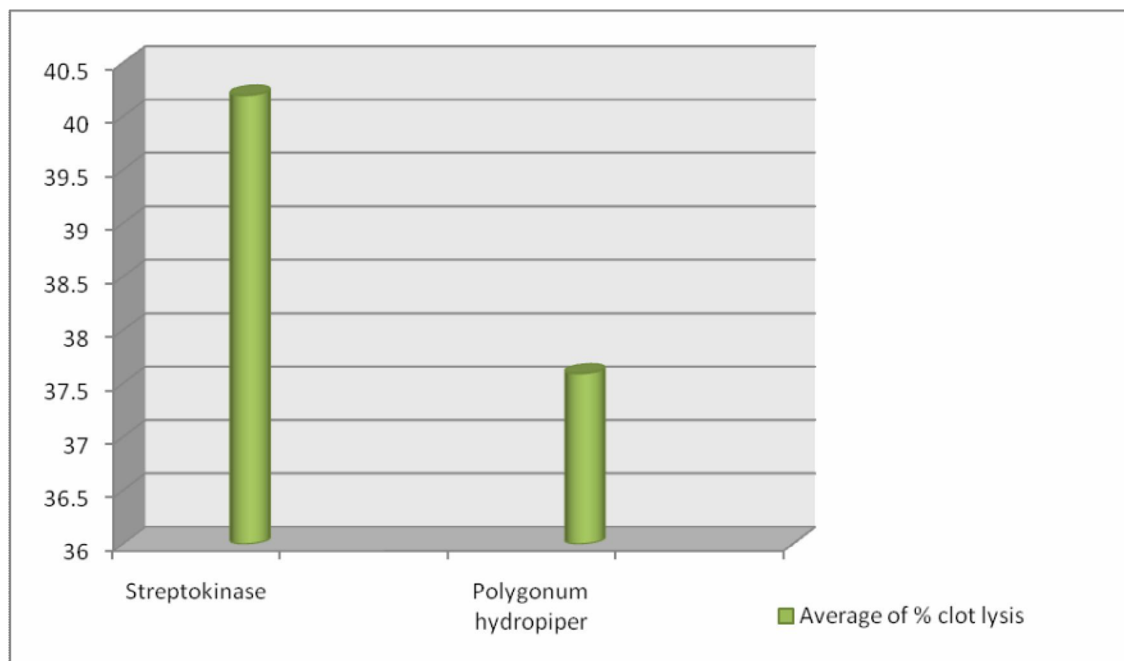


Figure 4.1: Graphical presentation of Thrombolytic effect of *Polygonum hydropiper* compare to Satandard

4.1.1. Results:

The plant extract of *Polygonum hydropiper* showed activity when apllied for clot lysis. *Polygonum hydropiper* showed (37.592±5.8194) percent clot lysis in average.

4.1.2. Discussion:

Figure 4.1 shows the effect of the *Polygonum hydropiper* extract on clot lysis activity. The percentage (%) clot lysis was statistically significant when compared with standard control. The plant extract showed moderate clot lysis activity (37.592±5.8194%) for whereas standard streptokinase showed 40.2±5.37% clot lysis activity.

4.2 Antimicrobial Activity

	Sample (*Polygonum hydropiper*)			Standard (Kanamycin)		
Name of organisms	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**
Gram Negative Bacterias						
1. ***Vibrio mimicus***	6	7.0	7±1.73	31	28.0	28.0±2.64
	9			26		
	6			27		
2. ***Salmonella paratyphi***	7	7.33	7.33 ±5.77	22	25.67	25.67±3.51
	8			26		
	11			29		
3. ***E. coli***	8	8.66	8.66±2.08	23	25.0	25.0±2.0
	11			27		
	7			25		
4. ***Shigella dysenteriae***	-	8.5	8.5±2.12	21	25.0	25.0±3.60
	7			26		
	10			28		
5. ***Vibrio parahemolyticus***	-	5.33	5.33±4.72	29	26.0	26.0±3.60
	7			22		
	9			27		
6. ***Shigella boydii***	-	4.0	4.0±6.92	20	20.0	20.0±1.0
	12			19		
	-			21		
7. ***Salmonella typhi***	11	11.17	11.17±.577	22	23.67	23.67±1.52
	11			24		
	12			25		
Gram Positive Bacterias						
8.***Staphyllococcus aureous***	6	4.66	4.66±4.16	26	23.67	23.67±2.51
	-			24		

	8			21		
9. ***Bacillus cereus***	-	2.0	2.0±3.46	23	21.0	21.0±2.0
	6			21		
	-			19		
10. ***Sarcina lutea***	-	2.33	2.33±4.04	24	23.67	23.67±2.51
	7			26		
	-			21		
11. ***Bacillus subtilis***	-	-	-	22	22.67	22.67±4.04
	-			19		
	-			27		

Table 4.2: Data of zone of inhibition of microbial growth for *Polygonum hydropiper*

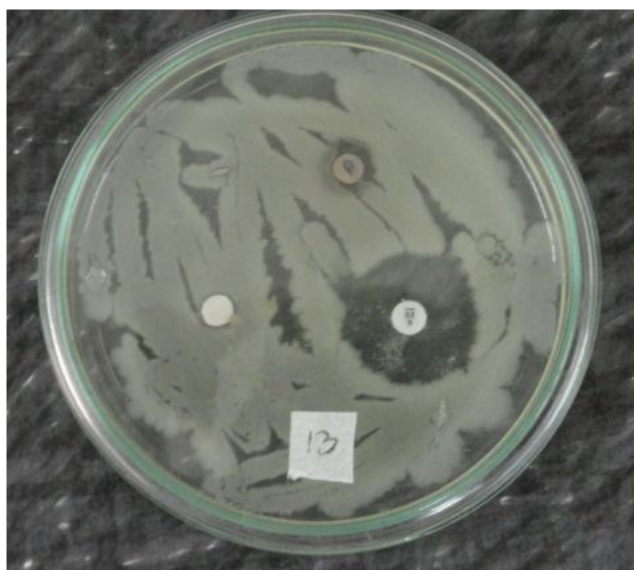


Fig 4.2: Zone of inhibition for *Polygonum hydropiper*

4.2.1. Results & Discussion:

The ethanol extract of was screened against total 11 microorganisms. Among those 7 microorganisms were gram negative bacterias, 4 were gram positive bacterias. Kanamycin disc was used as standard. Kanamycin showed anti microbial activity with average zone of inhibition ranging from 20-28 mm in diameter. The maximum showed zone of inhibition was against *Vibrio mimicus* (gram negative) 28.0 mm.

Ethanolic extract of *Polygonum hydropiper* showed poor anti-microbial activity with average zone of inhibition ranging from 6-9 mm in diameter. The maximum activity was against *Salmonella typhi* (gram negative) 11.11 mm in diameter.

	Sample (*Coccinia grandis*)			Standard (Kanamycin)		
Name of organisms	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**
Gram Negative Bacterias						
1. ***Vibrio mimicus***	-	-	-	31	26.66	26.66±4.04
	-			26		
	-			23		
2. ***Salmonella paratyphi***	-	-	-	22	25.33	25.33±3.05
	-			26		
	-			28		
3. ***E. coli***	8	9.66	9.66±1,52	25	25.66	25.66±1.15
	11			27		
	10			25		
4. ***Shigella dysenteriae***	9	8.66	8.66±1.52	26	26.66	26.66±1.15
	7			26		
	10			28		
5. ***Vibrio parahemolyticus***	11	9.0	9.0±2.0	29	27.33	27.33±1.52
	7			26		
	9			27		
6. ***Shigella boydii***	9	9.33	9.33±2.51	20	21.33	21.33±1.52
	12			23		
	7			21		
7. ***Salmonella typhi***	11	11.17	11.17±.577	22	23.67	23.67±1.52
	11			24		
	12			25		
Gram Positive Bacterias						
8.***Staphyllococcus aureous***	14	13.66	13.66±2.51	26	23.67	23.67±2.51
	16			24		
	11			21		

9. ***Bacillus cereus***	13	12.33	12.33±1.15	23	21.0	21.0±2.0
	11			21		
	13			19		
10. ***Sarcina lutea***	7	7.66	7.66±1.15	24	25.33	25.33±1.54
	7			26		
	9			26		
11. ***Bacillus subtilis***	-	-	-	22	24.33	24.33±2.51
	-			24		
	-			27		

Table 4.3: Data of zone of inhibition of microbial growth for *Coccinia grandis*

Fig 4.3: Zone of inhibition for *Coccinia grandis*

4.2.2. Results & Discussion:

The ethanol extract of *Coccinia grandis* was screened against total 11 microorganisms. Among those 7 microorganisms were gram negative bacterias, 4 were gram positive bacterias.

Kanamycin disc was used as standard. Kanamycin showed anti microbial activity with average zone of inhibition ranging from 21-26 mm in diameter. The maximum showed zone of inhibition was against *Vibrio parahemolyticus* (gram negative) 27.33mm.

Ethanolic extract of *Coccinia grandis showed* moderately good anti microbial activity with average zone of inhibition ranging from 7-10 mm in diameter. The maximum activity was against *Staphyllococcus aureous* (gram positivetive) 13.33 mm in diameter.

	Sample (*Litsea glutinosa*)			Standard (Kanamycin)		
Name of organisms	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**	**Invidual Value(mm)**	**Avg Value(mm)**	**Standard Deviation**
Gram Negative Bacterias						
1. ***Vibrio mimicus***	13	15	15±2.64	31	28.66	28.66±2.51
	14			26		
	18			29		
2. ***Salmonella paratyphi***	13	12.33	12.33 ±1.54	22	23.66	23.66±2.08
	13			26		
	11			23		
3. ***E. coli***	13	11	11±2	23	23.33	23.33±1.52
	11			22		
	9			25		
4. ***Shigella dysenteriae***	8	8.33	8.33±1.52	21	23.66	23.66±2.51
	7			26		
	10			24		
5. ***Vibrio parahemolyticus***	13	9.66	9.66±3.05	29	26.66	26.66±2.51
	7			24		
	9			27		
6. ***Shigella boydii***	9	9.33	9.33±2.51	20	21.33	21.33±1.52
	12			23		
	7			21		
7. ***Salmonella typhi***	-	8	8±1.41	22	23.67	23.67±1.52
	7			24		
	9			25		
Gram Positive Bacterias						
8.***Staphyllococcus aureous***	6	7.66	7.66±1.52	26	23.67	23.67±2.51
	9			24		
	8			21		
9. ***Bacillus cereus***	-			23		

	6	7.5	7.5±2.52	21	21.0	21.0±2.0
	9			19		
10. ***Sarcina lutea***	6	8	8±2.64	24	26.33	26.33±2.51
	7			26		
	11			29		
11. ***Bacillus subtilis***	14	12.33	7.5±.707	22	25.66	25.66±3.21
	11			28		
	12			27		

Table 4.4: Data of zone of inhibition of microbial growth for *litsea glutinosa*

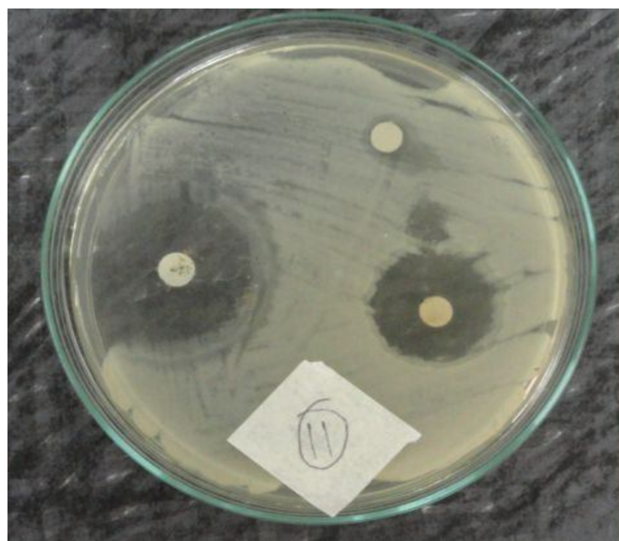


Fig 4.4: Zone of inhibition for *litsea glutinosa*

4.2.3. Results & Discussion

The ethanol extract of *litsea glutinosa* was screened against total 11 microorganisms. Among those 7 microorganisms were gram negative bacterias, 4 were gram positive bacterias.

Kanamycin disc was used as standard. Kanamycin showed anti microbial activity with average zone of inhibition ranging from 22-27 mm in diameter. The maximum showed zone of inhibition was against *Vibrio mimicus* (gram negative) 28.66 mm.

Ethanolic extract of *litsea glutinosa showed* moderate anti-microbial activity with average zone of inhibition ranging from 7-14 mm in diameter. The maximum activity was against *Vibrio mimicus* (gram negative) 15 mm in diameter.

4.3 Result of Brine Shrimp Lethality Bioassay:

Table 4.5: Data representation for Brine Shrimp Lethality Bioassay

Concentration µg/ml	LOG C	Total Population	Alive	Dead	% Mortality	LC_{50} µg/ml (sample)	LC_{50} µg/ml (vincristin sulfate)
400	2.602059	12	0	12	100	6.05	2-4
200	2.301029	11	0	11	100		
100	2	10	0	10	100		
50	1.6989	11	1	10	90.9		
25	1.3979	11	2	9	81.8		
12.5	1.0969	10	2	8	80		
6.25	0.7958	10	3	7	70		
3.125	0.4948	11	5	6	54.5		
1.562	0.1936	13	13	0	0		
0.78	-0.1079	12	12	0	0		

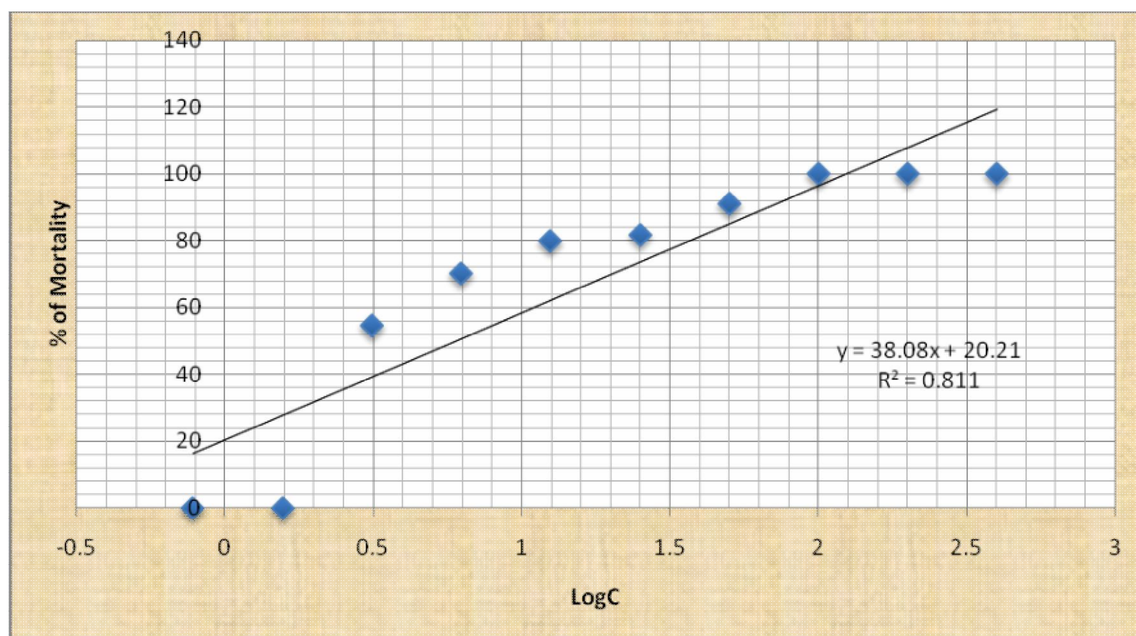


Figure 4.5: Graphical presentation of LC_{50} of *Polygonum hydropiper*

4.3.1. Results:

The toxic potentiality of the plant crude extracts of *Polygonum hydropiper* was evaluated using Brine Shrimp lethality bioassay method whereas doses of 10 different concntration were used (0.78-400 µg/ml). Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA, were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment.

Sea water was used as a solvent and also as a negative control. The median lethal concentration LC of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration.

Maximum 100% test objects found dead after 24hours in case of 400, 200 and 100 µg/ml and the LC_{50} = 6.05 µg/ml for N. sativa.

4.3.2. Discussion:

The plant extract could be said to have extremely good cytotoxic activity against brine shrimp nauplii.

Brine shrimp lethality bioassay was employed to explore a number of noble antitumor, antibacterial and pesticide of natural origin. In this cytotoxic activity study, mortality of the nauplii was observed in all experimental groups. Control group nauplii remained unchanged (no lethality/mortality), is indicative of the cytotoxicity of the extract. The rate of mortality of the nauplii found to be increased with increased concentration of the sample. However, further investigations using carcinoma cell line are necessary to isolate the active compound(s) responsible for the activity. The positive response obtained in this assay suggests that the extracts may have bioactive compounds.

4.4. Results of Analgesic activity by acetic acid induced writhing method

Table 4.6: Data representation on analgesic activity of *Coccinia grandis* by writhing method

Analgesic activities of *Cocccinia grandis*								
Animal Group	**Writhing count**					**Mean**	**% of writhing**	**% of inhibition**
	M1	**M2**	**M3**	**M4**	**M5**			
Control	**14**	**16**	**20**	**19**	**20**	**27.4**	**100**	**0**
Standard	**10**	**7**	**6**	**5**	**8**	**7.2**	**26.27**	**73.73**
Sample 100mg/l	**16**	**4**	**10**	**11**	**17**	**11.6**	**42.33**	**57.67**

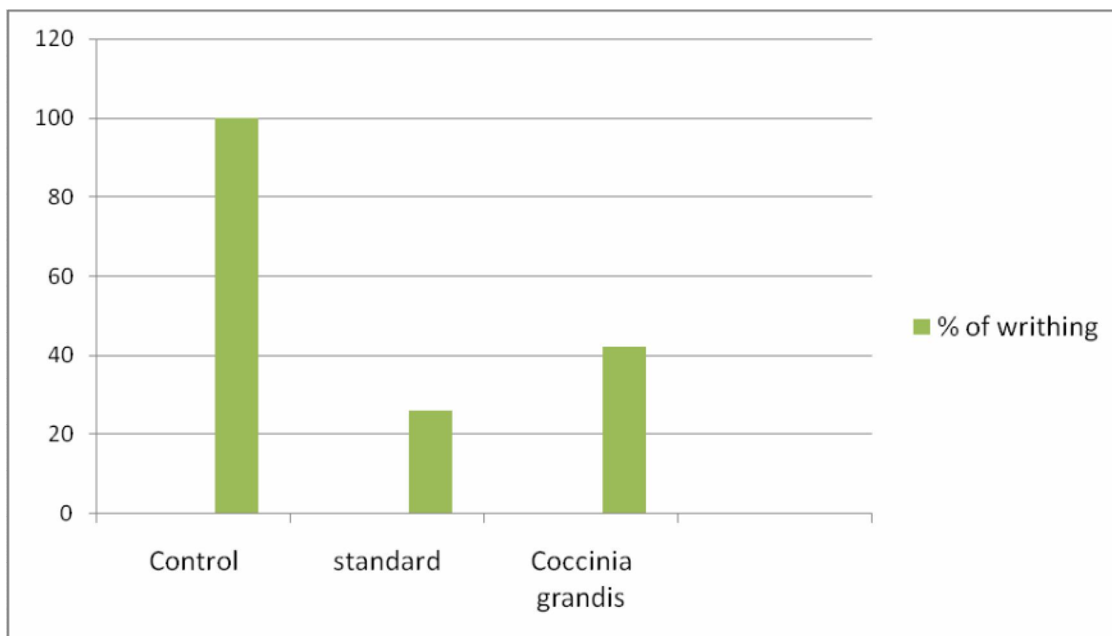


Figure 4.6: Graphical representation of % of writhing of *Coccinia grandis*

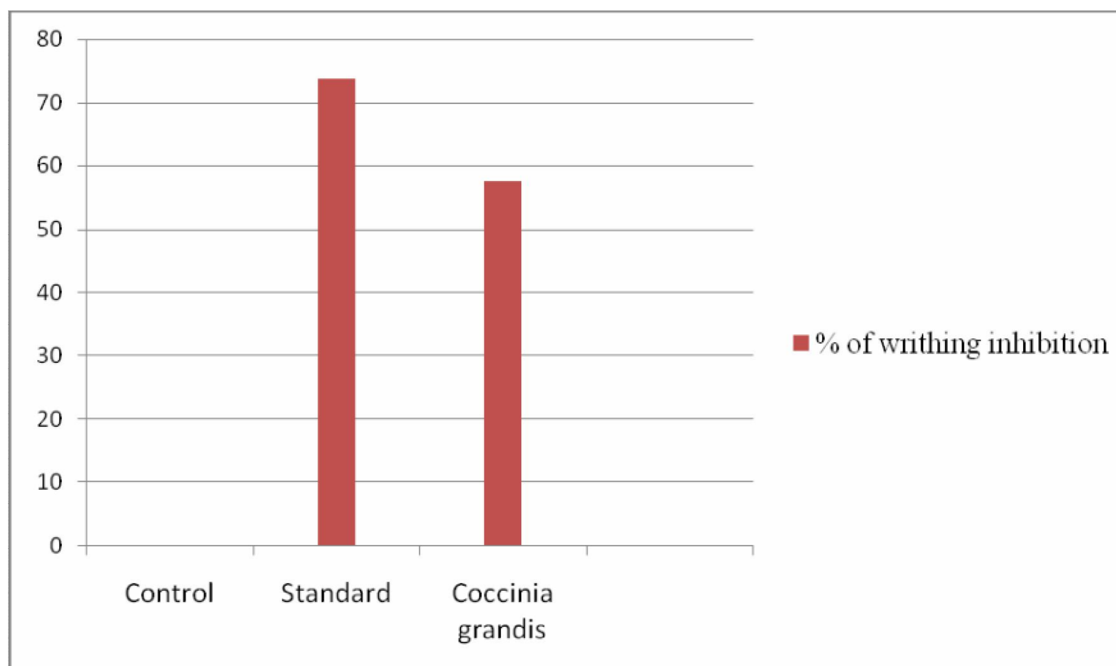


Figure 4.7: Graphical representation of % of writhing inhibition *Coccinia grandis* Table

Table4.7: Data representation on analgesic activity of *Litsea glutinosa* by writhing method

Analgesic activities of *litsea glutinosa*								
Animal Group	**Writhing count**					**Mean**	**% of writhing**	**% of inhibition**
	M1	**M2**	**M3**	**M4**	**M5**			
Control	**14**	**16**	**20**	**19**	**20**	**27.4**	**100**	**0**
Standard	**10**	**7**	**6**	**5**	**8**	**7.2**	**26.27**	**73.73**
Sample 100mg/l	**7**	**6**	**6**	**5**	**8**	**6.4**	**23.35**	**76.65**

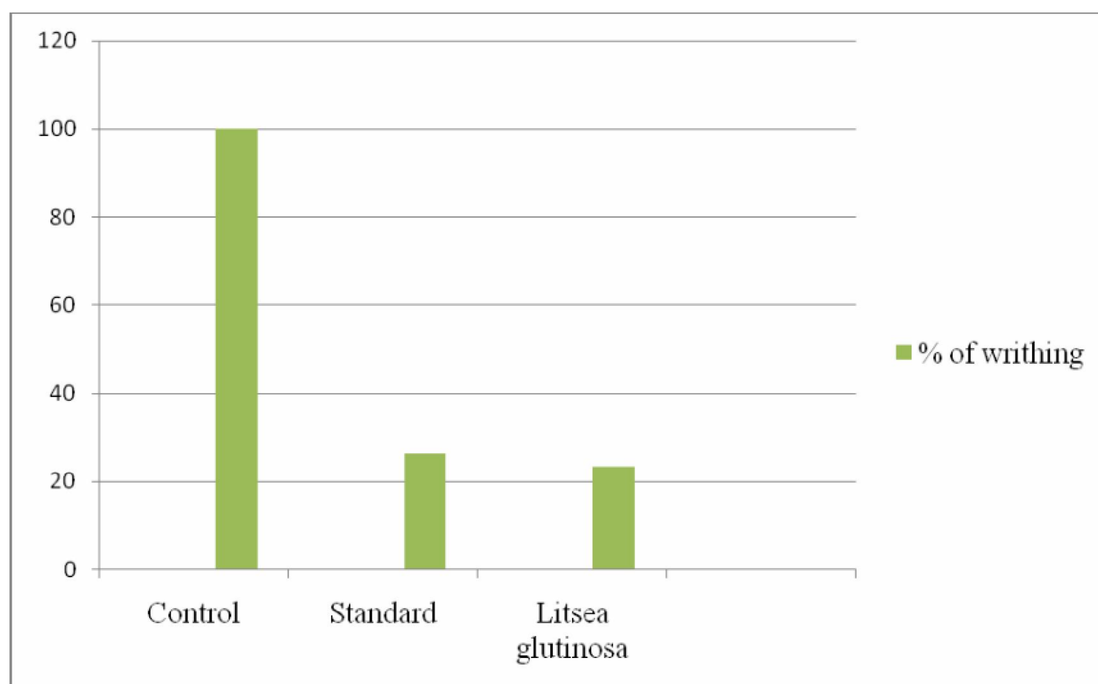


Figure 4.8: Graphical representation of % of writhing *Litsea glutinosa*

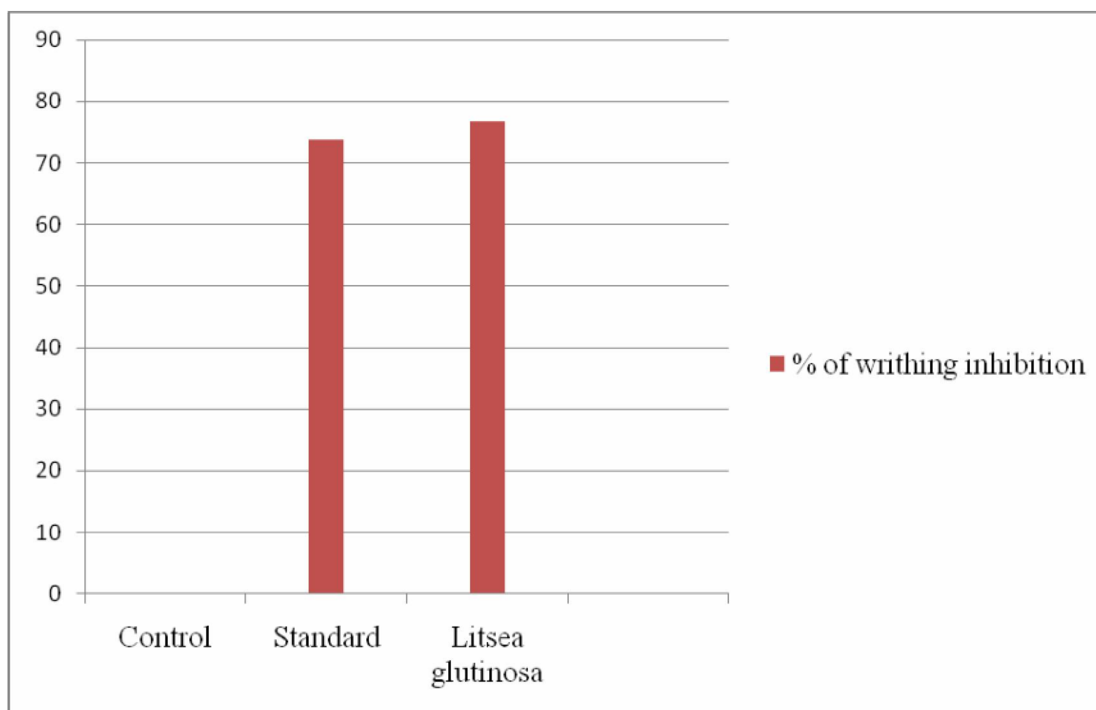


Figure 4.9: Graphical representation of % of writhing inhibition *Litsea glutinosa*

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	404.9333	2	202.4667	49.78689	1.5478E-06	3.885294
Within Groups	48.8	12	4.066667			
Total	453.7333	14				

Discussion:

The % of writhing inhibition of *Coccinia grandis* was 57.67 whereas the % of writhing of standard was 73.73. This represents that the writhing was reduced when standard was administered in comparison with the sample.

The % writhing inhibition of *Litsea gltinosa* is greater than the control. This represents that *Litsea gltinosa* has good analgesic activity compared to Diclofenac Na.

The ANOVA test provide f value = 3.88 with $p<0.001$. Therefore the test is highly significant. Hence there is strong evidence to conclude that the groups are significantly different.

The result indicates that both plant extracts showed good analgesic activity which is statistically significant. So no further study is needed to identify the significant result of *Coccinia grandis* & *Litsea gltinosa* on mice.

4.5. Result of scavenging activity of *polygonum hydropipe, litsea glutinosa* and *Coccinia grandis*

Table 4.7: IC_{50} calculation for *Polygonum hydropiper*

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH= $(1- A_{Sample} / A_{Blank}) \times 100$	IC_{50} (µg/ml)
1		0.78125	0.412	1.670644391	
2		1.5625	0.41	2.14797136	
3		3.125	0.402	4.057279236	
4		6.25	0.277	33.8902148	
5	.419	12.5	0.127	69.68973747	16.97
6		25	0.122	70.88305489	
7		50	0.096	77.08830549	
8		100	0.079	81.14558473	
9		200	0.073	82.57756563	
10		400	0.069	83.53221957	

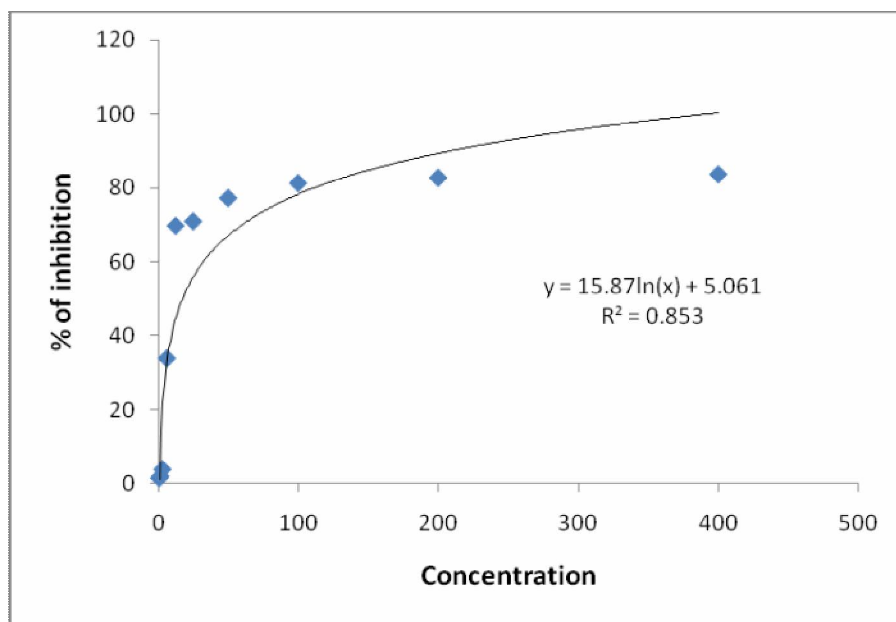


Figure 4.10: Graphical presentation of IC_{50} of *Polygonum hydropiper*

Table 4.7: IC_{50} calculation for *Litsea glutinosa*

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH= (1- A_{Sample} / A_{Blank})×100	IC_{50} (µg/ml)
1		0.78125	0.317	0.626959248	
2		1.5625	0.225	29.46708464	
3		3.125	0.165	48.27586207	
4		6.25	0.115	63.94984326	
5	.419	12.5	0.093	70.84639498	6.70
6		25	0.072	77.42946708	

7		50	0.069	78.36990596	
8		100	0.067	78.9968652	
9		200	0.064	79.93730408	
10		400	0.062	80.56426332	

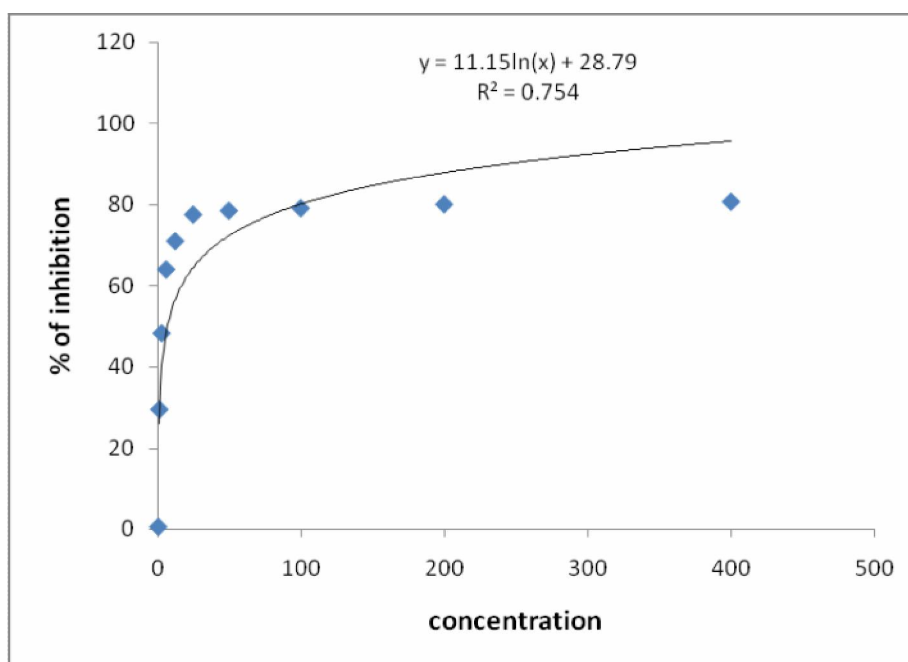


Figure 4.11: Graphical presentation of IC_{50} of *Litsea glutinosa*

Table 4.7: IC_{50} calculation for *Coccinia grandis*

Serial No.	A_{Blank}	Concentration (µg/ml)	A_{Sample}	% inhibition of free radical DPPH= $(1- A_{Sample} / A_{Blank})\times 100$	IC_{50} (µg/ml)
1		0.78125	0.297	6.896551724	
2		1.5625	0.148	53.60501567	
3		3.125	0.091	71.47335423	
4		6.25	0.082	74.29467085	
5	.419	12.5	0.072	77.42946708	1.79
6		25	0.069	78.36990596	
7		50	0.067	78.9968652	
8		100	0.066	79.31034483	
9		200	0.064	79.93730408	
10		400	0.06	81.19122257	

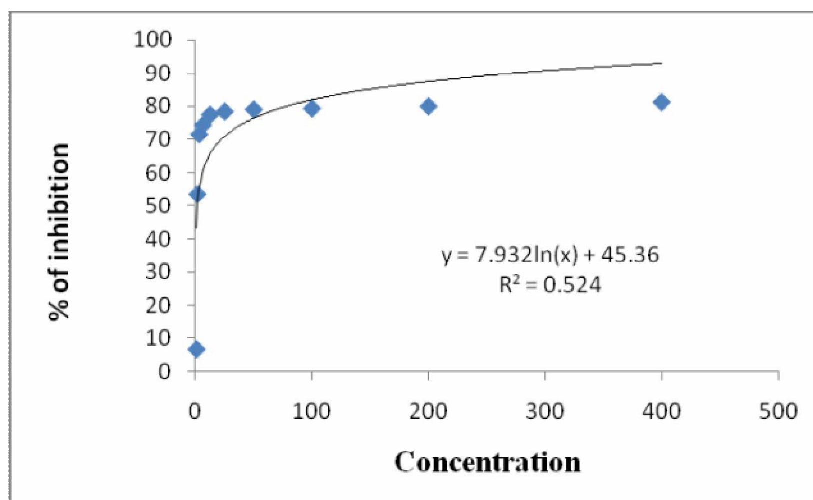


Figure 4.5.12: Graphical presentation of IC_{50} of *Coccinia grandis*

Discussion:

The ethanolic extract of *Polygonum hydropyper* showed good scavenging activity having IC_{50} 16.97. No literature was found on this research. No records or traditional use of *Polygonum hydropiper* as an anti-oxidative species was found. The IC_{50} of *Polygonum hydropiper* (16.97) represents that it has good scavenging activity in comparison with the control.

The plant extract *Litsea glutinosa* showed good scavenging activity. Similar type of research was conducted by Devi P. and Meera R. back in 2010. During that research ascorbic acid and acitic acid both were used as control. In that research work methanolic extract of *Litsea glutinosa* was used. The IC_{50} value that was obtained project was from 8-12 µg/ml. The IC_{50} value of *Litsea glutinosa* obtained in this research is 6.70µg/ml. The possible reason

responsible for the variation could be the solvent that is used for extraction. In this project ethanolic extract of *Litsea glutinosa* was used.

Coccinia grandis showed good scavenging activity. Similar research was conducted by Deshpande S. V. back in 2011. During that research ethanolic extract of *Coccinia grandis* was used. The IC_{50} of *Coccinia grandis,* which was obtained from that research, was 2.70µg/ml. The IC_{50} that is obtained from this research is 1.79µg/ml. The results are similar; the reason behind this similarity between the results could be the methodological similarity.

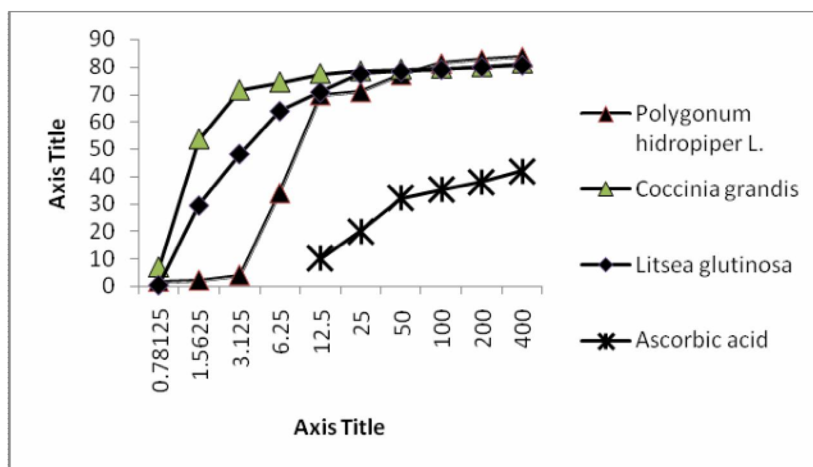


Figure 4.5.13: Comparison of antioxidant activity of *Polygonum hidropiper, Coccinia grandis, Litsea glutinosa* and ascorbic acid.

In the figure 4.5.13, a comparative evaluation of antioxidant activity of test extracts is shown. The IC_{50} value of ascorbic acid which is obtained in this experiment was 6.50µg/ml. The IC_{50} value of *Polygonum hidropiper, Coccinia grandis, Litsea glutinosa* are 16.97 µg/ml, 1.79 µg/ml and 6.70 µg/ml respectively. So it can be said that *Coccinia grandis* and *Litsea glutinosa* has an extensive use as anti-oxidative species as said in the introductory part.

Chapter 5: Conclusion

Conclusion:

In summary, pharmacological evaluation of *Polygonum hydrpiper, Coccinia grandis* and *Litsea glutinosa* extracts showed promosing activities. Cytotoxic and thrombolytic activity of *Polygonum hydropiper* came out very impressive I comparison with the standard. Analgesic activity of both *Litsea glutinosa* and *Coccinia grandis* came out very significant. All three plants have moderate antimicrobial and scavenging activity.

The percent clot lysis of *Polygonum hydropiper* was 37.592, while the streptokinase has the percent clot lysis value of 41.2. *Polygonum hydropiper* showed a poor antimicrobial activity having highest zone of inhibition of 11.11 mm in diameter against *Salmonella typhi* (gram negative). The LC_{50} that was determined by Brian Shrimp lethality bioassay was 6.02µg/ml while the standard (vincristin) value was 2-4µg/ml.

Litsea glutinosa showed poor antimicrobial activity having highest zone of inhibition of 15 mm against *Vibrio mimicus. Litsea glutinosa* showed very good analgesic activity having percent inhibition of writhing about 76.65 while, diclofenac sodium have 73.73. *Litsea glutinosa* has good scavenging activity having IC_{50} of 6.7.

Coccinea grandis showed poor antimicrobial activity having highest zone of inhibition of 13.33 mm against *Staphylococcus aureus. Coccinea grandis* has extremely good scavenging activity having the IC_{50} of 1.67 µg/ml. *Coccinea*

grandis also showed moderate analgesic activity having percent inhibition of writhing 57.67.

However, further studies are necessary to elucidate the mechanism behind these effects. This report may serve as a footstep to use this plant as a new source of medication.

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