

PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF
Terminalia chebula (Family-Combretaceae)



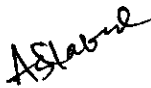
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KHULNA, BANGLADESH.
AUGUST, 2010

DECLARATION

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PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF
***Terminalia chebula* (Family-Combretaceae)**

A thesis

By

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Examination Roll No. MS-070718

Session: 2006-2007

 08.04.2012

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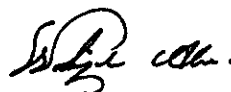
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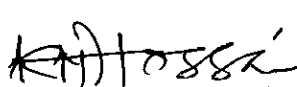
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Submitted to

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, in partial fulfillment of the requirements for the degree of 1 year MS in Biotechnology and Genetic Engineering



Biotechnology and Genetic Engineering Discipline
Life Science School, Khulna University
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August, 2010

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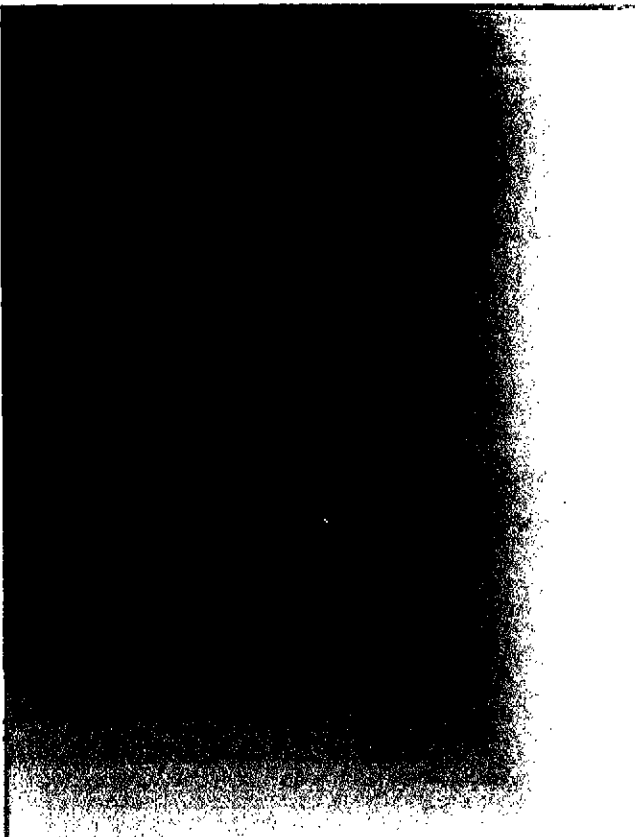
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August, 2010

The Author



**DEDICATED
TO
MY BELOVED PARENTS**

ABSTRACT

Phytochemical analysis of the extract of *Terminalia chebula* (Family- *Combretaceae*) indicated the presence of Alkaloids, Tannin, Steroid and absence of Flavonoids, Gums and Saponins types of compounds. The pharmacological interest of these compounds, coupled with the use of this plant in traditional medicine prompted us to check *T. chebula* for possible analgesic, antioxidant and antidiarrhoeal activities in a dose dependent manner. These activities were investigated on animal model like swiss albino mice. The dried fruits of the plant were subjected to successive extraction with ethanol and the extract was used to investigate the activities. Ethanolic extract of *T. chebula* 500 mg/kg and 250 mg/kg exhibited highly significant ($p < 0.001$) inhibition of writhing reflex by 67.65% and 55.55%, respectively while the standard drug diclofenac- Na inhibition was found to be 80.29% at a dose of 25 mg/kg body weight. The ethanolic extract of the fruits of *T. chebula* showed a prominent ant-oxidant activity ($IC_{50}=3.5 \mu\text{g/ml}$) while the standard ascorbic acid found to be 0.2 $\mu\text{g/ml}$ lower ($IC_{50}=3.3 \mu\text{g/ml}$). For the test of anti-diarrhoeal activity, at the doses of 500 mg/kg body weight, the extract of fruits of *T. chebula* compared to the control group, offered about 2.6 hr. of the mean latent period for diarrhoeal episode and the result was significant ($P < 0.001$). Here standard anti-diarrhoeal agent, loperamide produced means latent period 3.7 hr. The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.



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LIST OF ABBREVIATIONS

NAPRALERT = Natural product Alert Database

eg. = Exempli Gratia (for example)

ie. = Id est (that is)

et al = et alii (and others)

QSAR= Quantitative Structure Activity Relationships

DMSO = Dimethylsulfoxide

UNIDO = United Nations Industrial Development Organization

CVD = Cardio Vascular Disease

gm. = Gram

gr. = Group

HETE = Hydroxy Eicosatetraenoic Acids

HPETE = Hydroperoxyeicosatetraenoic acids

Etc. = Etcetera

hr. = Hour

i.p. = Intraperitoneal

ICDDRDB= International Centre for Diarrhoeal Disease and Research, Bangladesh

kg. = Kilogram

LAF = Laminar Air Flow

MIC = Minimum Inhibitory Concentration

min. = Minute

ml. = Milliliter

mm. = Millimeter

NCI = National Cancer Institute

NSAID = Non-steroidal Anti-inflammatory Drug

PAF = Platelets Activating Factor

PGE = Prostaglandin-E

PGI₂ = Prostacyclines

SEM = Standard Error of Mean

TLC = Thin-layer Chromatography

UV = Ultra-violet

WHO = World Health Organization

wt. = Weight



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Chapter-1
INTRODUCTION

Chapter-1

INTRODUCTION

Medicine is the wonder of the world and blessings for mankind. From the very ancient time, men used various plant parts as their wound healing. Medical and pharmaceutical biotechnology, the science and practice of medicine and its primary source plays an important role in identifying the new molecule of drug through both synthetically and from that of natural origin. The history of drug from natural sources is very significant and well known. By trial and error, primitive men must have acquired knowledge that was useful in determining which plants and animals possessed food value and which were to be avoided because they were unpalatable, poisonous, or dangerous. Their observations were handed down from one generation to another and were added to by his progeny. The healing powers of certain herbs, roots, and juices were undoubtedly discovered by accident; but once these attributes were learned, they were too important to be forgotten. The Babylonians were aware of the medicinal effects of a number of plants. It is a well-known fact that the ancient Egyptians had knowledge of the medicinal usages of many plants and animals, according to *Papyrus Ebers*. As far as medicinal plants are concerned, a single plant is used for each remedy (B. Menale *et al.*, 2006).

Dioscorides, a Greek physician who wrote his "De Materia Medica" in 78 A.D. in which he described about 600 plants that were known to have medicinal properties. Of these, a surprisingly large number is still important in modern medicine. Aloe, belladonna, colchicum, ergot, hyoscyamus, and opium are a few that were used then in much the same manner as they are used today (Tyler and Brady, 1981).

Today, based on the historical information and traditional use, extensive work on finding the actual constituents which is responsible for the medicinal properties of the plant is performed. Isolation of different constituents from the different plants and the rationale of the use of this plant in traditional medicine have been checked. In Ayurvedic system of herbal treatment, pharmaceutical industries are being commercially exploited for the extraction of various ingredients (Mahmood *et al.*, 2003).

Since disease, decay and death have always co-existed with life, the early man had to think about disease and its treatment at the dawn of human intellect. Thus the human race started using plants as a means of treatment of diseases and injuries from the early days of civilization on earth and its long journey from ancient time to modern age the human race

has successfully used plants and plant products as effective therapeutic tools for combating against diseases and various other health hazards.

The information, discussed above, inspired me to search the medicinal properties of a plant of our sub-continent. There are a number of medicinal plants here. Among them, I selected the highly used medicinal plant *Terminalia chebula* (Horitoki). *T. Chebula* is called the "king of medicines" because of its extraordinary powers of healing. In Ayurveda, it is considered to destroy all diseases and eliminate all waste from the body. At the same time, it is known to promote tissue growth and health. *T. chebula* is used to treat digestive diseases, urinary diseases, diabetes, skin diseases, parasitic infections, heart diseases, irregular fevers, flatulence, constipation, ulcers, vomiting, colic pain and hemorrhoids. If taken after meals it prevents imbalance of any of the humors due to bad food or drink. It promotes wisdom, intellect and eyesight. Modern science has found that *Terminalia chebula* as a strong effect against the herpes simplex virus HSV, has antibacterial activity, and exhibits strong cardio tonic properties. *T. chebula* also has antioxidant components, which indicates it can increase the life of tissues. Yet another study shows the anti-tumor activity of *T. chebula* and another study shows that it has considerable effect in inhibiting the HIV virus which ultimately results in AIDS (Barthakur *et al.*, 1991). These influenced me to choose this plant for phytochemical and pharmacological screening.

1.1 Aims and Objectives of the Study

Plants have been used for mankind as remedies from the very beginning of civilization. Still now they play an important role in health care for about 80% of the world's population. The presence of diverse bioactive metabolites like alkaloid, glycosides, flavonoids, tannins etc. in plants has formed the therapeutic basis of herbal medication. Thus emphasis is given on the biological screening of medicinal plants for further exploration of their active constituents.

Consulting with herbal practitioner and potent local people it is found that *Terminalia chebula* (Wild) has been used as a local medicinal plant. Upon significant literature survey it has been found that only a little research work has been performed on other species but not on *T. chebula* and no work has performed to judge their local medicinal use in our locality.

As a student of biotechnology and genetic engineering, I am fascinated on the investigation of the secret behind the traditional use of plant. So from my fascination to search for the mystery of plant that help in the healing of disease this project work was performed on the plant *T. chebula*, traditionally used as an anti-irritant in case of fish sting.

The traditional uses of this plant lead us to investigate the following pharmacological action followed by phytochemical testing. To perform the investigation, I arranged the study as given below:

Part A: Phytochemical study

Identification of different chemical groups present in the active plant parts

Part B: Pharmacological screening

- Analgesic activity
- Antioxidative activity
- Antidiarrhoeal activity

Chapter-2

LITERATURE REVIEW

Chapter-2

LITERATURE REVIEW

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal plants". The exploration of medicinal properties of plants throughout the ages was accomplished principally through careful observation, trial and error, and accidental discovery. Observation of animals' instinctive discrimination between toxic and palatable plants might also have helped primitive man in choosing those plants which are beneficial from nutritive and medicinal standpoints. And in this process, the human race, over the countries, has created a vast heritage of knowledge and experience on medicinal plants in different cultures and civilizations (S.A. Dahanukar, 2000). Plants have contributed and are still contributing to the development of modern synthetic drugs and medicine in a number of ways as stated below:

1. Novel structures of biologically active chemical compounds, isolated from plant sources, often prompt the chemist to synthesis similar or better semi-synthetic compounds.
2. Synthetic drugs with similar or more potent therapeutic activity are often prepared by structural modification of the plant-derived compounds with known biological activity.
3. Various analogues and derivatives of plant constituents with similar or better pharmacological actions and therapeutic properties are often prepared by chemists for use as potent drugs.

Homatropine (a synthetic tropane alkaloid similar to atropine), syrosingopine (a synthetic derivative of reserpine), chloroquine (a synthetic derivative of quinine), dihydromorphinone, methyldihydromorphinone, oxymorphone, ethyl morphine and N-allylnormorphine (synthetic derivatives of morphine) are some of the example of such synthetic drugs, which plants have contributed indirectly. Even in this age of synthetic drugs, there are some naturally occurring drugs, such as the Digitalis glycosides used in cardiac complications and the Catharanthus alkaloids used in cancers, which have no synthetic alternatives. In such cases, plants continue to remain as their principal and only sources (Ghani, 1998).

Phytotherapy, the treatment of disease by the use of plants, was the beginning of pharmacotherapy or treatment of disease by means of drugs (Ghani, 1998). Therapeutic uses of plants had effects, stored at the very beginning of human life on earth when the primitive man, out of necessity and by intuition, resorted to using plants to alleviate his sufferings from injuries and diseases. The medicinal plants have been used in traditional medicine for hundreds of years with reputation as efficacious remedies although there may not sufficient scientific data to substantiate their efficacy. Of these, surprisingly large number are still of importance in modern medicine (Claus, 1993). In this way, phytotherapy laid the foundation stone of all forms of medical treatment that are practiced today. With the development of human civilization, the implementation of phytotherapy exhibits a stepwise development, which can be enumerated as –

1st stage

Crude drugs were employed, prepared in the roughest manner, such as powdered willow bark in the management of pain.

2nd stage

These were converted into more active and manageable forms, such as extracts or solutions, watery or alcoholic.

3rd stage

The pure active principles separated from the crude drug were employed, e.g. salicylic acid.

4th stage

Attempt to synthesize the active drug in the laboratory and indeed structural modification, e.g. aspirin, the wonder drug.

2.1 Herbal Drug Research: Past and Present Perspective

It's really unimaginable how far the modern science has advanced the human civilization. Revolutionary changes have been and are being brought about in day-to-day life. Apart from information technology, most miraculous happenings and advancement have taken place in the biomedical research sector. The complete human genome has been unveiled and this is sure to inspire the exploration of newer facets of biomedical research in near future. More and more insights and emphases are being paid to the molecular bases of diseases. A good number of drugs, either rationally designed or genetically engineered, are coming out each and every year. Gene based therapy along with recombinant DNA

technology has come into the focus of modern medical research. Progress in science is providing crucial insights into the underlying mechanisms of disease, opening up a rich field of potential targets for pharmacological investigation. Day to day creation of new innovative diagnostics and exploration of exciting molecular targets for new drugs has revolutionized and is still enriching the human arsenal against maladies (Samudralwar, 1996).

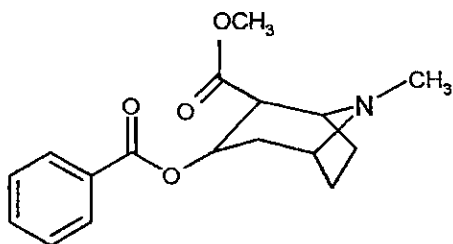
When such is the scenario of present day research, it wouldn't be unnatural for anyone if he turns a bit skeptical about the utility and significance of natural product study. Actually by dint of modern science, our dependence on nature might have lessened to a considerable extent but our concern about nature and its components has enhanced manifold. Surprisingly enough a significant portion of the world population still relies largely on natural products and day by day more interest and importance are being converged to naturopathy, aromatherapy, alternative medicine etc. This is partly due to dissatisfaction with certain aspects of orthodox medicine and partly due to a desire to decrease dependency on 'drugs', seeking a more 'natural' alternative (Eldin and Dunford 1999).

There is no denying the fact that synthetic chemistry has given birth to a lot of present day pharmaceuticals but a significant number of these synthetic compounds do have their lead molecules from plant source. According to a study, it was found that of the 520 new drugs approved by FDA from 1983 to 1994, 30 were natural products and 173 were either semi-synthetic based on a natural product core or modeled on a natural pharmacophore (Cragg *et al.*, 1997).

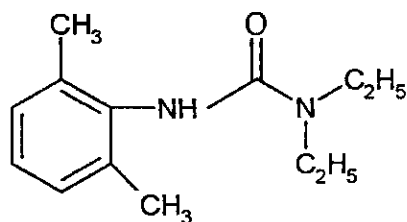
Structural modification and optimization through the QSAR study of many plant derived lead molecules resulted in a good number of wonder drugs. For example the chemical structure of cocaine isolated from coca leaves paves the way for synthesizing modern drugs like procaine, lidocaine, tetracaine and dibucaine (belonging to the local anaesthetic group) in the laboratory. Also the synthesis of life saving antibiotics- penicillin needs a natural precursor 6-aminopenicillanic acid (6-APA) derived from *Penicillium notatum*.

Plants can be envisaged as natural chemical factory (Went, 1939) because numerous chemicals and secondary metabolites are continually being synthesized within the plants in ambient temperature and pressure of nature. The laboratory synthesis of the anti-

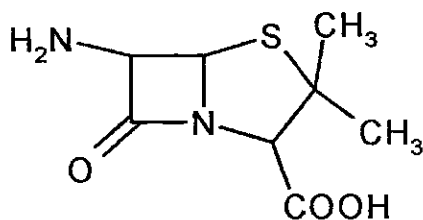
malarial drug – quinine demands an intensive work extending over about half a century but cinchona plant does it every day without much difficulty.



Cocaine from *Erythroxylon coca*

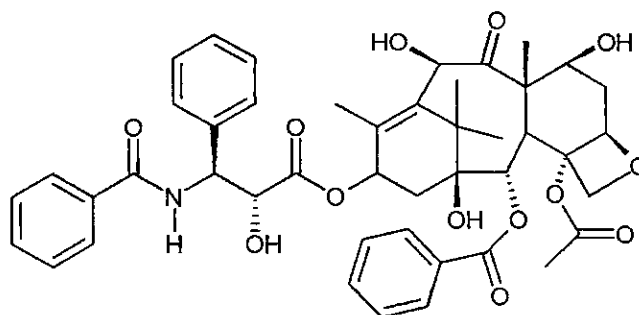


Lidocaine- cocaine analog, local anesthetic

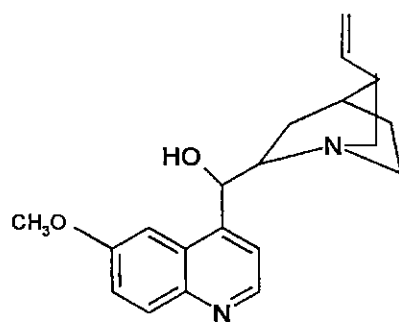


6-APA from *Penicillium notatum*

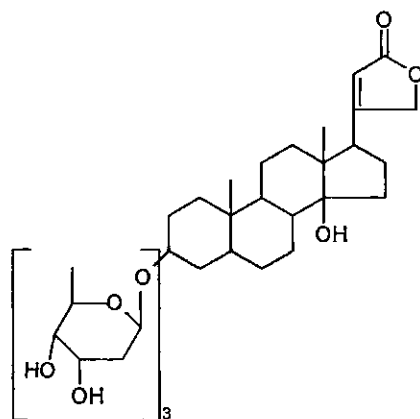
Till to date, extensive phytochemical analysis of plants yielded multifarious chemical compounds of different classes such as steroids, terpenoids, flavonoids, alkaloids, chalcones, glycosides etc. Clinically used plant metabolites such as taxol isolated from *Taxus brevifolia*; vincristine and vinblastine obtained from *Vinca rosea* Linn. ; Morphine & other opoids from poppy plant (*Papaver somniferum*) and digoxin derived from *Digitalis purpurea* are only a few of the many striking examples of wonder drugs from plant sources.



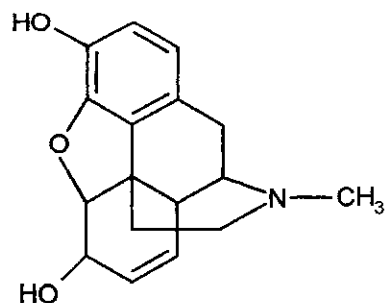
Taxol- from *Taxus brevifolia* (Taxaceae), a potent antineoplastic agent



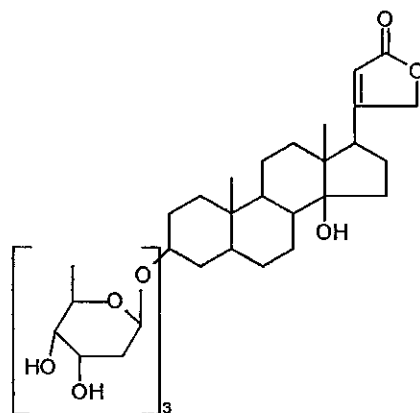
Quinine – from Cinchona bark, a potent antimalarial agent



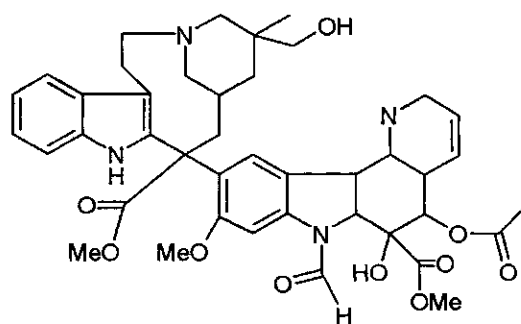
Digitoxin – from *Digitalis purpurea*, a potent cardiotonic drug



Morphine- the prototype opoid analgesic from *Papaver somniferum*



Digitoxin – from *Digitalis purpurea*, a potent cardiotonic drug



Vincristine from *Vinca rosea*, a potent antineoplastic agent

So the value and importance of plants in curing diseases and developing new drugs has not yet tarnished in this modern high-tech world. Obviously there has been no exhaustion of what the plants can offer us to combat ailments. Robert Mendelsohn and M. J. Balick (1995) have researched out that there are about 375 total drugs of pharmaceutical significance in the rain forests of the world, of which only one-eighth have been discovered⁹. Such findings optimistically lend us scope to surmise that plethora of magic molecules are still there in the plant kingdom, awaiting to be explored by mankind. That is why Darwin rightly said:

"We only see how little has been made out in comparison with what remains unexplained and unknown."

2.1.1 Herbal Drug Development: The Never Ending Chronicle

Medicine is a progressive science. In every department attempts are being made to replace empiricism by rationalism and nowhere is this more evident than in the science of pharmacology and therapeutics. Thus when it is said that a drug like *Saraca indica* (Asoka) is useful in menorrhagia or *Boerhavia diffusa* (Punarnava) in dropsy, the profession will not accept these assertions, as these are symptoms and signs but not diseases; what is wanted to know is their particular value in these various conditions and how they help to restore the homeostasis within. The scientific mind is not satisfied by mere statements, no matter from what source they originate, unless corroborated by clinical and experimental evidences (Chopra *et al.*, 1982).

Herbal medicines have certain indications and side effects. Certainly, it has been suggested that several herbs can be involved in immunoallergic reactions³⁵, and patients with atopic conditions such as eczema or with a history of liver disease might be particularly vulnerable to such reactions (Huxtable RJ, 2005). The correct use of herbs requires a clear understanding of how they work. Although most herbs are safe but they

can cause problems. Ancient people knew that certain plants had indisputable healing power. But they could not explain how the plant's medicinal power worked, how much of the leaves or roots or the selected plant part should be used, how long after harvesting should they wait before using the plants or how long should they boil the plant parts for making a decoction. Selection of medicinal plants by early man, without any prior knowledge about them, was largely based on intuition, guesswork or trial and error. Curiosity and search for food had contributed considerably to his knowledge about the plants and their beneficial role. Superficial resemblance between a specific plant part and the affected organ or some symptoms of the ailment had also played some guiding role in his plant selection for curing medicinal purpose. Observation of the other lower animal's instinctive discrimination between toxic and palatable, innocuous plants might have played important role in this respect (Samudralwar, 1996).

Paracellus (Dwyer J. 1990) was the pioneer to consider about the posology- the science about the dose of medicine. Pointing to the plant's inherent chemical composition he argued that herbal drug, if used in crude form might kill a lot for curing a little. Actually the scientific curiosity awakened during Renaissance. But the pace of medicinal discovery had a quantum leap in the 19th century. With the invention of microscope, the germ theory of disease, the use of technology brought a turn to the understanding of how the human body functions, what disease is and how it is cured. The chemists also learned to isolate the active substances from plants. Since then herbal medicine entered into a new era of massive experimentation. Plant extracts, which were once used as traditional beliefs started to get a scientific basis.

Since plant extract may contain a number of ingredients, each of them may have some effects on the living body. On the other hand, a plant extract may contain a chemical component in such a low concentration that may not serve the intended therapeutic interest. Again the crude extract may have a number of chemical constituents performing the same therapeutic role. Ingestion of such an extract may cause severe side effects due to the synergistic action of the constituents. So the use of herbal drugs in crude form may be ineffective or even it may cause toxicity. A proper example is the traditional use of periwinkle plant (*Vinca rosea*) which was intended for treating diabetes. Phytochemical and biological investigation of the plant indicated an insignificant yield of an alkaloid having antidiabetic property. Rather, the presence of anticancer alkaloids such as vincristine, vinblastine, vinrosedine and vineleurosine made this plant an important

source for developing anticancer drug vincristine in large scale. However, the work of plant analysis and research is still on the run. The story of medicinal use of plants is continued to unfold, though in a quiet pace but as an independent way of scientific research.

2.1.2 Revival of Herbalism: The Global Scenario

"A desire to take medicine", wrote the Canadian physician Sir William Osler in 1891 *"is perhaps the great feature which distinguishes man from other animals."* If Osler's wry observation was true in his time, it is more strikingly true today. People take medicines, and take them, and take them – in staggering amount.

In the early phase of human civilization, plants were perhaps the only resort of man seeking cure. But as time passed by, robust advancement in science and technology uplifted allopathic medicine to an insurmountable status and dependence on nature for medicine got a dwindling trend. But recently there has been a worldwide revival of interest in traditional medicine especially those of plant origin. The World Health Organization once estimated that approximately 80 percent of the world population use herbal medicine for primary health care (Fransworth *et al*, 1985) and interestingly, the percentage is unaltered still now (Cordell, 2000).

The WHO has given recognition to these medicines in a resolution adopted by the 30th World Health Assembly in 1977. It defines herbal medicines as any *"finished labeled medicinal parts of plants, or other plant materials or combinations thereof, whether in the crude state or as plant preparations"*.

The WHO has played an active role in identifying and promoting safe and effective herbal medicines for use in primary health care, and has strongly supported the use of herbal medicines to promote the health of communities. In 1978 the *Alma-Ata Conference* on Primary Health Care recommended Governments to give priority to the promotion and development of traditional medicines. To further improve the status of herbal medicine and maintain high standards, the leading professional bodies of France, Belgium, the United Kingdom, Germany and Switzerland came together to form the European Societies Co-operative of Phytotherapy (ESCOP). At the centre of ESCOP's activities is the formation of a set of scientific monographs for the medicinal plants used in phytotherapy (Marilou, 2010).

2.1.3 Rationale for Herbal Drug Research in Bangladesh

The number of plants with medicinal properties included in the Materia Medica of traditional medicine in this subcontinent as present stands at about 2000. More than 500 of such medicinal plants have so far been established as growing in Bangladesh. This number of the indigenous medicinal plants is in the increase with the discovery and introduction of newer plants everyday (Ghani, 1998). Almost all these indigenous medicinal plants are extensively used in the preparation of unani, ayurvedic and homeopathic medicines in Bangladesh.

But the fact is that almost all of them are lacking scientific evaluation of their therapeutic uses. So standard pharmacological and phytochemical methods should be applied in order to:

- Establish scientific evidence of their pharmacological use.
- Isolate the active constituent. This may offer a local natural source of a commonly used drug or a novel therapeutic agent.

2.2 Medicinal Plants Research and Development

In order to fully convert the potential of our medicinal plants into economic wealth, a very active R&D programme is essential. The R & D has to cover all aspects relating to the species from collection to utilization. For convenience we may categorize medicinal species into: (a) those which are of proven medicinal value as per scientific parameters, (b) those on which sufficient leads are available, and (c) those on which much work is required to be done.

Research investigations need to concentrate on the first two categories on the following aspects:-

- Evolving and optimizing the most appropriate technologies for conservation, especially for endangered or endemic species and molecular methods for characterization.
- Detailed studies on life cycle and breeding behavior, taxonomy, seed biology
- Population and habitat viability studies
- Optimizing appropriate methods for post-harvest handling, processing and storage.

- Investigation on quality control standardization and shelf life of raw materials and finished products.

The search for new drugs of plant origin has yielded fruitful result in the past. Today it is possible to use molecular techniques for detection of genetic variability and tagging desired traits as well as culling out duplicates in accessions. The availability of high throughput screen has made the possibility of converting 'hits' into lead compounds in comparatively short time. Drug development from plant sources using gene/molecular techniques is becoming increasingly important (Dahanukar, 1997).

2.2.1 Drug Development from New Molecules/Genes

In terms of a modern research endeavour, drug development from plants must necessarily imply a multi-disciplinary approach. Recent computerized studies of ethnomedical information, albeit in specialized areas of biological activity, have nevertheless confirmed that plants are a reservoir of chemical agents with therapeutic potential. What has been hitherto missed is the collation of data gathered in the trial-and-error is a prolonged clinical trial (Kelly, 2001).

This information includes:

1. Ethnomedical particulars on cultivation, gathering and preparation of plant material for dosage.
2. The role of accompanying plants frequently used in multi-component traditional preparations.
3. Recommended dosage regimens and contra-indications of certain foods.

In addition, it would be wise, in any research effort, to investigate the theories and concepts of the major systems of traditional medicine, as these cannot always be exactly interpreted in terms of modern concepts. In general, natural products that have come into modern medicine are the result of an approach to drug development adopted over the past fifty years or more. The goal has been to find new chemical structures that have a novel biological activity. The alternative approach of finding plant derived therapeutic agents as extracts that could be standardized and formulated, has not received attention. The emphasis of R&D should be on the following:

- Development of technology for bulk production of medicinal products;
- Development of quality control standards for the starting materials as well as for the finished products;

- Development of new formulations and dosage forms specially suited to the prevailing climatic conditions and adapted to locally available raw materials;
- Assimilation of acquired technology and its continuous improvement to make the products competitive;
- Bioequivalence, bioavailability and pharmacokinetic studies on the dosage forms developed;
- Search of new plant sources for known drug and for new drugs from locally available plants.

Given the situation, production of standardized plant fraction should have priority over that of pure active substance, because of the simple technology needed and hence lower cost of the product, provided, of course, the technological testing indicates that the product is safe. It would be advisable to find out the chemical composition of the composite fraction and pharmacological action of each constituent to ensure that they are safe and compatible with each other (Jean Paul, 2002).

In view of the high capital and recurring costs of R & D, collaboration among developing countries and between developed and developing countries are advisable. Keeping in view the national priority the target disease is selected and the plants on the formulation are evaluated clinically using current protocols/criteria. Studies on analytical methods, details chemistry, and pharmacology along with toxicity and safety aspects are required (Senn, 1997).

A multidisciplinary approach to drug development from medicinal plants, used in traditional medicine was tried out in several technical assistance programmed by UNIDO beginning in the mid-1970s.

- Economic mapping of the spontaneous flora.
- Selection and authentication of plant species.
- Collation of ethnomedical and ethnobotanical data.
- Trial propagation to develop high yielding varieties.
- Medium scale plant propagation.
- Chemical studies on plant constituents
- Analytical studies on quantitative assessment of major constituents.
- Pharmacological and toxicological studies
- Pilot plant scale processing of plant extracts.

- Standardization of extracts.
- Formulation studies on extracts into dosage forms.
- Toxicity studies on formulations
- Analytical studies on formulated products

UNIDO's technical Assistance Programme envisaged that if a successful candidate plant was identified, then the large-scale plant cultivation was to be implemented with FAO and the clinical assessment of the drug be conducted with WHO participation. Success was recorded in a number of instances and the programme introduced the concept of goal-oriented research.

2.2.2 Ethnobotanical Approach to Drug Discovery

Many ethnobotanical studies have been carried out in Italian areas, most of them devoted to medicinal plants (Guarrera, 2005). For pharmaceuticals ranging from digitalis to vincristine the ethnobotanical approach to drug discovery has proved successful. The advent of high-throughput, mechanism-based in vitro bioassays coupled with candidate plants derived from painstaking ethnopharmacological research has resulted in the discovery of new pharmaceuticals such as prostratin, a drug candidate for treatment of human immunodeficiency virus, as well as a variety of novel anti-inflammatory compounds. Gastrointestinal maladies, inflammation, skin infections and certain viral diseases are likely to be of high saliency to indigenous healers, whereas diseases such as cancer and cardiovascular illness are unlikely to be easily diagnosed by indigenous peoples. Yet indigenous remedies may indicate pharmacological activity for maladies such as schizophrenia, for which the biochemical mechanisms have yet to be discovered (Rolan, 1997). Ethnopharmacological information can be used to provide three levels of resolution in the search for new drugs:

1. as a general indicator of non-specific bioactivity suitable for a panel of broad screens.
2. as an indicator of specific bioactivity suitable for particular high-resolution bioassays.
3. as an indicator of pharmacological activity for which mechanism-based bioassays have yet to be developed.

Historically, ethnobotanical leads have resulted in three different types of drug discovery:

1. unmodified natural plant products where ethnomedical use suggested clinical efficacy (e.g. digitalis);

2. unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use (e.g. vincristine); and
3. modified natural or synthetic substances based on a natural product used in folk medicine (e.g. aspirin).

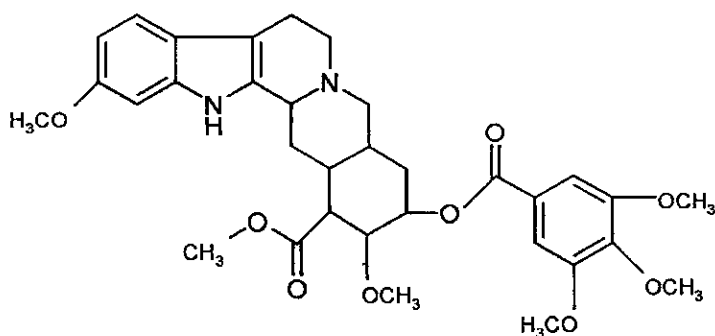
The NAPRALERT database developed by Farnsworth is especially thorough, with systematic searches dating back to 1900, on natural products in the areas of cancer (chemotherapy, chemoprevention, carcinogenesis), fertility regulation (both male and female), diabetes, malaria and other parasitic diseases, viral diseases, sugar substitutes, molluscicides and anti-HIV agents. As an exercise to demonstrate the power of computer analysis, selected 10 diseases searched NAPRALERT for species that had both ethnomedical information and in vitro and/or in vivo experimental data supporting the ethnomedical claims.

2.2.3 Bioactivity Guided Approach

Once the plant medication was provided to the ancient people in the crude form, often exhibited many unwanted effects due to the presence of some toxic components beyond the active constituent. Extensive Phytochemical investigation and isolation of active component(s) in the pure form thus become necessary to avoid untoward effects and to ensure safe use of herbal medicines. With technological advancement, phytochemical studies of medicinal plants got a rapid pace and the presence of many chemical compounds comes in light. These plant-derived compounds often played an important role in directing laboratory synthesis of many new classes of drug molecules. In some cases, the plant components became the starting material in the synthetic process of industrial production of many drug molecules. For example, the use of sterol diosgenin isolated from Mexican Yam for laboratory synthesis of oral contraceptive progesterone reduced the cost of progesterone from a value of \$80 per gram to \$1.75 per gram (Goldstein *et al.*, 1974).

Sometimes Phytochemical analysis of many plants yielded such chemical constituents having no remarkable therapeutic interest. The crude drug containing several constituents was found to be ineffective in case of therapy for which it was used. The phytochemical investigation of Periwinkle plant (Goldstein *et al.*, 1974) (*Vinca rosea*), once used traditionally as an anti-diabetic drug, was found to contain alkaloidal constituents having hypoglycemic potency in minute quantities, whereas late it was found to contain anticancer principles vinca alkaloids in a high yield. The dried seeds of plant *Amni*

visanaga were found to act as a diuretic and antispasmodic in renal colic in the eastern Mediterranean countries and in Arabia, but the research carried out by G.V. Anrep and coworkers (Anrep, 1946) resulted in the isolation of khelin, a component having vasodilatory activity. Khelin appeared as an anti-anginal drug after subsequent clinical trial. The research on *Rauwolfia serpentina* which was traditionally used as an antidote for snakebite, revealed the presence of an antihypertensive and tranquilizing agent reserpine. Thus systemic research with medicinal plants might open the door of many unknown therapeutic choices.



Reserpine- an antihypertensive and tranquilizer from *Rauwolfia serpentina*

From the above discussion it is evident that the search of plant constituents having therapeutic interest requires bioactivity studies with the crude extracts prior to phytochemical investigation. Only the bioactive extracts or fractions would be of interest for next phytochemical analysis. Without prior biological studies, the phytochemical analysis alone can provide the chemical composition of the plant, which may or may not have therapeutic interest. That is why Dr. Kurt Hosttetmann (Irvine, 1995) of university of Lausanne, Switzerland gave emphasis on the biological and pharmacological screening of medicinal plants. Thus in conducting research on medicinal plants, bioactivity guided phytochemical analysis might be a rational approach.

The way of developing drugs from plants thorough the bioactivity-guided approach involves several stages, which include:

1. Selection and taxonomic identification of proper plant(s).
2. Primary processing (cutting, drying, grinding etc) of the collected plant materials.
3. Extraction of the processed/ unprocessed plant materials with suitable solvent or solvent mixture.
4. Primary bioactivity screening of the crude extract.

5. Fractionation of crude extract using the most appropriate chromatographic procedures, biologic evaluation of all fractions and separation of the active fractions and rapid discard of inactive ones (may be subjected to phytochemical investigation later on).
6. Repeated fractionation and purification of active fractions to isolate pure compound(s)
7. Elucidation of the structure of isolated compound(s) by spectroscopic technique.
8. Evaluation of biological activity of the pure compound(s).
9. Toxicity study of the chemically characterized, pure, bioactive compound(s).

2.2.4 Bioengineering of Medicinal Plants

Pharmaceutical importance/therapeutic value of medicinal plants are due to specific constituents/comibination of secondary metabolites present in them. Changes in the proportion of secondary metabolites are often required for the improvement of therapeutic values of medicinal plants. For example, increase in the artemisinin content of *Artemisia annua* in proportion to its immediate precursors and other terpenes is required to make the production of antimalarials from this plant more economically. The biosynthetic pathways for the biologically active chemical compounds in medicinal plants are usually complex and high branches. Tissue and cell cultures such as callus and cell suspension cultural systems of *E. ulmoides* have already been established (Huang *et al.*, 2003; Wang *et al.*, 2003). Genetic manipulations can help increase/decrease the contents of specific compounds in medicinal plants. Detailed understanding of these pathways will be a prerequisite for the identification, cloning and genetic engineering of the concerned structural and regulatory genes. These genetic techniques will also help develop designer medicinal plants. For example it will be possible to produce medicinal plants that will help raise the immunity towards infectious diseases in the use of such plants.

2.2.5 Choosing Priorities

It is necessary to give priority the thrust areas to obtain the output of research efforts and other resources. Several factors help in determining the priorities. These include the distribution of flora, national or regional disease pattern, availability of modern health care etc. In addition we have to keep in mind the global priorities in developing new drugs so as to get a good financial return. The disease pattern and the priorities have

(b) The minimizing of toxicity tests needed to introduce the drug into a health care system. This is particularly necessary when the drug has been in long human use, toxic manifestations could be assessed by studying its long term effect on patients already undergoing treatment in the traditional milieu and the mode of industrial processing does not significantly vary from the ethnomedical methods. Product comparisons by modern instrumental parameters can also be made as between processed product and ethnomedical preparation.

(c) Clinical trials conducted under the supervision of competent authorities (e.g WHO) must be a necessary prerequisite.

(d) Stimulation of traditional processing methods, as well as adherence to ethnomedical regiments will be most helpful in not missing the activity present in an ethnomedical preparation. This will also stimulate examination of ethnomedical theories of disease with a view to interpretation of these, if at all possible, within modern concepts. (The idea particularly refers to long standing and well documented systems such as Ayurveda, Unani and the Chinese systems).

(e) The Selection of the appropriate dosage form and mode of administration should be recently based on economic parameters as well as shelf-life potential in the situations prevailing in the developing world.

(f) There are some concrete evidence that pure compound need not necessarily be the best drugs. But on economic ground as well as on the therapeutic grounds it will serve all interests well if the most appropriate processing methodology of a plant or combination of plants is examined in this light. This would also give rise to interesting researches on the synergistic and/or detoxificant effects of other constituents in the medicinal plants or non medicinal plants that are found often added to polyprescriptions used in traditional systems.

2.3 *T. chebula* Perspective

Owen and Johns (2002) stated that methanolic extracts of *T. chebula*, *Syzygium aromaticum*, *Aquilaria agallocha*, *Santalum album*, *Amomum subulatum*, *Justicia adhatoda* and *Myristica fragrans* contained fractions that displayed significant antioxidant activity.

Khanna *et al.* (1993) postulated that the fruits of *T. chebula* have a long history of use in the treatment of heart diseases and for their laxative, diuretic and cardiogenic properties.

Frawley *et al.* (1985) stated that the fruit of *T. chebula* is also used for pregnancy, dehydration, emaciation, pitta-kopa.

Sharma *et al.* (1988) postulated that the fruit of *T. chebula* is contraindicated in weak digestion, fatigue due to excessive sexual activity, with alcoholic drinks and in hunger, thirst and heart stroke.

Murillo *et al.* (2000) proved that while populations of tropical countries are less likely to have a tradition of wine drinking, and do not often cultivate grapes and blueberries they do consume a tremendous variety of fruit, both whole and as juice. 40 types of tropical fruits were studied and discovered that many demonstrate high levels of antioxidant activity.



Fig 2.1 Mature Fruits (dried and green) and tree of *T. chebula*

Azeem *et al.* (1992) reported that the fruits of *T. chebula* are used to treat heart diseases, coughs, asthma, urinary disorders and constipation.

Saguna *et al.* (2002) reported that *T. chebula* treated wounds healed much faster as indicated by improved rates of contraction and a decreased period of epithelialization.

Hossain (2003) stated that *T. chebula* should not be used for by child. It is harmful for child. But in case of ten years old child it can be applied without risk.

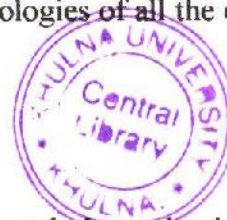
Owen *et al.* (2002) postulated that *T. chebula* may provide a therapeutic benefit towards Cardio Vascular Disease (CVD) prevention and treatment, and reinforces the merits of traditional Tibetan medicine.

Chapter-3
MATERIALS AND METHODS

Chapter-3

MATERIALS AND METHODS

A medicinal plant is any plant which, in one or more of its organs contains substances that can be used for therapeutic purposes or which, is a precursor for synthesis of useful drugs. And the inner objective of this study is to search some of the medicinal characteristics in the fruits of *Terminalia chebula*. To achieve the goal, I conducted some phytochemical group tests, test for analgesic, antioxidative and anidiarrhoeal activity with the methanolic extract of the fruits of the plant. The methodologies of all the experiments are discussed here separately.



3.1 Methodology for Phytochemical Screening

Phytochemistry is related to the identification, isolation and determination of plant constituents with the aid of suitable method. It starts the discovery of new molecule from unknown species.

As a result of modern extraction, isolation techniques and pharmacological testing procedure, new plant drugs usually find their way into medicine as purified substances rather than in the form of galenical preparations.

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted. There are two type of procedure for obtaining organic constituents-

- a. Cold extraction and
- b. Hot extraction.

The extract obtained is then concentrated and constituents are separated by different methods such as chromatography.

3.1.1 Collection and identification

The fruits of *Terminalia chebula* were collected from Dakbangla, Khulna, Bangladesh, during the month of March, 2010 on the day time.

3.1.2 Drying and Grinding

The collected fruits were washed with water, separated from undesirable materials or plants or plant parts. They were dried by shed drying for about one week after cutting it into small pieces. The fruits were ground into a coarse powder (200gm) with the help of a

suitable blender. The powder was stored in an airtight container and kept in a cool and dry place until analysis commenced.

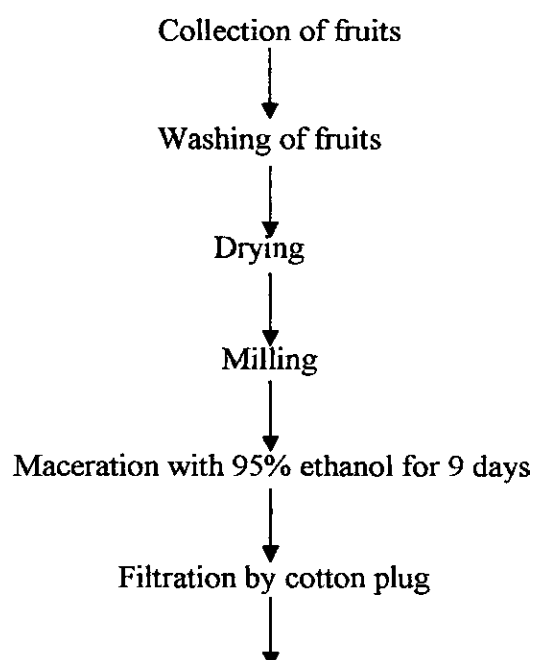
3.1.3 Cold Extraction (Ethanol Extraction) and Filtration

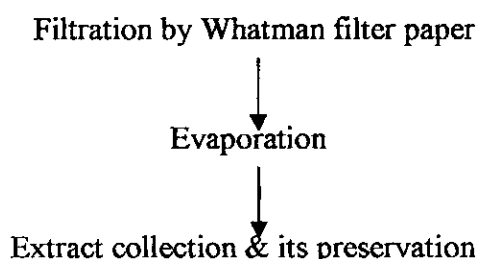
About 100 gm of powdered material was taken in a clean, flat-bottomed glass container and soaked in 700 ml of ethanol up to 3 inches height above the sample surface as it can sufficiently cover the sample surface. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper. After filtration the remaining portion of the plant extract was given for re-extraction for 7 days with another 150 ml of ethanol. Then the mixture was again filtered by the same way as previous

3.1.4 Evaporation of the Solvent

The filtrate (ethanol extract) obtained was evaporated by air supplied from a continuously moving electric fan. It rendered a reddish-black type residue of 28 (yield 28%) gm. The reddish-black type concentrate residue was designated as crude Ethanolic extract of *Terminalia chebula* (Wild) fruits. The extract was then transferred to a small clean beaker with the spatula. The evaporation of re-extract was performed by the same technique as like as 1st extract and the re-extract found one gram and stored in a clean bottle at a cool and dry place.

3.1.5 Extraction at a glance





3.2 Chemical Group Tests

Test of different chemical groups present in the extract represent the preliminary phytochemical studies. In each test 10% (w/v) solution of extract in ethanol was taken unless otherwise mentioned in individual test (Charles *et al.* 1989).

3.2.1 Reagents Used For the Different Chemical Group Test

The following reagents were used for the different chemical group tests (Evans *et al.*, 1989; Ghani, 1998; Dev, 2002; Harborne, 1984).

i) Mayer's reagent

1.36 gm mercuric iodide in 60 ml of water was mixed with a solution contains 5 gm of potassium iodide in 20 ml of water.

ii) Dragendorff's Reagent

1.7 gm basic bismuth nitrate and 20 gm tartaric acid were dissolved in 80 ml water. This solution was mixed with a solution contains 16 gm potassium iodide and 40 ml water.

v) Benedicts Reagent

1.73 gm cupric sulphate, 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

vi) Molisch Reagent

2.5 gm of pure α -naphthol was dissolved in 25 ml of ethanol.

vii) Libermann-Burchard Reagent

5 ml acetic anhydride was carefully mixed under cooling with 5ml concentrated sulphuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling.

viii) 5% ferric chloride solution

ix) 10% potassium dichromate solution.

x) 10% lead acetate solution

xi) Conc. HCl.

- xii) Aqueous extract of plant extract.
- xiii) Ethanolic extract of plant extract.
- xiv) Chloroform extract of plant extract.
- xv) Sulphuric acid.

3.2.2 Tests Performed For Identifying Different Chemical Groups

The following tests were performed for identifying different chemical groups.

3.2.2.1 Tests for tannins

• Ferric Chloride Test

5 ml solution of different extracts was taken in a test tube. Then 1 ml of 5% Ferric chloride solution was added. Greenish black precipitate was formed and indicated the presence of tannins.

• Potassium dichromate test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed. It indicates the presence of tannins.

• Lead Acetate Test

1 ml. of 10% Lead acetate solution was added to 5 ml. of extract solutions. A yellow precipitate was formed. It indicates the presence of tannins.

3.2.2.2 Test for Flavonoids

Added a few drops of concentrated hydrochloric acid to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour indicates the presence of Flavonoids.

3.2.2.3 Test for Saponins

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimetre layer of foam indicates the presence of Saponins.

3.2.2.4 Test for gums

5 ml solution of the extract was taken and then molisch reagent and sulphuric acid were added. Red violet ring produced at the junction of two liquids indicate the presence of gums and carbohydrate

3.2.2.5 Test for Steroids

• Sulphuric acid test

1ml. solution of chloroform extract was taken and then 1ml. Sulphuric acid was added. Chloroform layer showed reddish brown colour and acid layer showed green fluorescence. It indicates the presence of steroids.

3.2.2.6 Test for alkaloids

• Mayer's test

2 ml. solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Mayer's reagent was added. Yellow colour precipitate was formed and that indicates the presence of alkaloids.

• Dragendroff's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. Orange brown precipitate was formed and it indicates the presence of alkaloids.

3.3 Methodology for Analgesic Activity Test

An analgesic selectively relieves pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness. They are used when the noxious stimulus (evoking the pain) cannot be removed or as adjuncts to more etiological approach to pain. Analgesics are divided into two groups, viz.

1. Opioid/narcotic/morphine like analgesics: These are strong analgesics. They depress CNS.
2. Non-opioid/non-narcotic/aspirin like/antipyretic or anti-inflammatory analgesics: These are weaker analgesics. They do not depress CNS, do not produce physical dependence and have no abuse liability. They act primarily on peripheral pain mechanisms, also in CNS to arise pain threshold.

There are several methods to assess the analgesic activity at different steps of pharmacological investigation. The methods are-

1. Acetic acid induced writhing method
2. Radiant heat method

The method, which we used to assess the analgesic activity at different steps of pharmacological investigation, was "*Acetic acid induced writhing method*".

3.3.1 Principle

The acetic acid induced writhing method is an analgesic behavioral observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.7% acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as 'writhing'. A comparison of writhing was made between positive control (Diclofenac sodium), control and test sample. Control, positive control (Diclofenac sodium) and test sample was given orally. Positive control and test sample was given orally 30 minutes prior to acetic acid injection. If the sample possesses analgesic activity, the animal that received the sample will give lower number of writhing than the control, i.e. the sample having analgesic activity will inhibit writhing. Diclofenac sodium was used as reference standard drug. It has analgesic, anti-inflammatory and antipyretic actions. It is one of the important members of non-steroidal anti-inflammatory drugs. It acts by inhibiting cyclooxygenase.

3.3.2. Mechanism of pain induction in acetic acid induced writhing method

Acetic acid is a pain stimulus. Intraperitoneal administration of acetic acid (0.7%) causes localized inflammation. Such pain stimulus causes the release of free arachidonic acid from tissue phospholipid by the action of phospholipase A₂ and other acyl hydrolases. There are three major pathways in the synthesis of the eicosanoids from arachidonic acid. All the eicosanoids with ring structures that is the prostaglandins, thromboxanes and prostacyclins are synthesized via the cyclooxygenase pathway. The leucotrienes, HETE (hydroxy eicosatetraenoic acids) and HPETE (hydroperoxy eicosatetraenoic acids) are hydroxylated derivatives of straight-chain fatty acids and are synthesized via the lipoxygenase pathway.

The released prostaglandins, mainly prostacyclin (PGI₂) and prostaglandin-E have been reported to be responsible for pain sensation by exciting the A δ -fibres. Activity in the A δ -fibres causes a sensation of sharp well localized pain.

Diclofenac sodium was used as the positive control in this method acts by inhibition of prostaglandin synthesis. Any agent that lowers the number of writhing will demonstrate analgesia by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition.

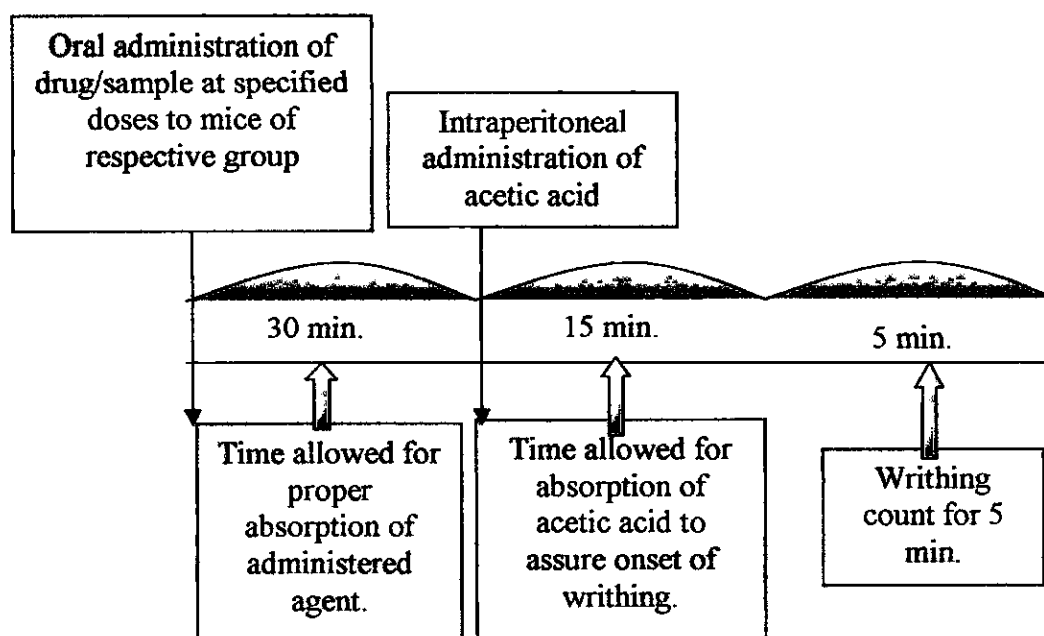


Fig 3.1 Schematic representation of acetic acid induced writhing of mice for investigation of analgesic activity

3.3.3 Experimental animal

Young Swiss-albino mice aged 4-5 weeks, average weight 20-30 gm were used for the experiment. The mice were purchased from the Animal Department ICDDR, Dhaka. They were kept in standard environmental condition for one week for adaptation after their purchase and fed ICDDR formulated rodent food and water *ad libitum*.

3.3.4. Study design

Experimental animals were randomly selected and divided into three groups denoted as group-I, group-II, and group-III (A & B) consisting of 5 mice in each group. Each group received a particular treatment i.e. control, positive control and the two doses of the extract. Each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly.

Table: 3.1 Experimental profile to assess the effect of fruits of *Terminalia chebula* crude ethanolic extract on acetic acid induced writhing of mice

Animal Group	Treatment	No. of animals	Dose (/Kg body weight)	Route of administration
I (Control)	1% Tween 80 in distilled water	05	10 ml	Oral
II (Positive control)	Diclofenac sodium	05	25 mg	Oral
III (A) (Test group)	Ethanolic extract of <i>Terminalia chebula</i>	05	500 mg	Oral
IV (B) (Test group)	Ethanolic extract of <i>Terminalia chebula</i>	05	250 mg	Oral

3.3.5 Preparation of Sample Suspension

To prepare suspension of the test samples at the doses of 500 and 250 mg/kg per body weight, 500mg and 250 mg of samples were measured respectively. The extract was triturated in unidirectional manner by the addition of small amount of water to make a volume about 10 ml. For proper mixing of extract small amount of tween was added to stabilize the suspension.

For the preparation of diclofenac sodium at the dose of 25 mg/kg-body weights, 2 ml ampule of diclofenac sodium containing 75mg was diluted to 3 ml and 1 ml from this lot was diluted to 10 ml.

3.3.6 Working Procedure

Test samples, control and diclofenac sodium were given orally by means of a feeding needle. A thirty minutes interval was given to ensure proper absorption of the administered substances. Then the writhing inducing chemical, acetic acid solution (0.7%) was administered intraperitoneally to each of the animals of a group. After an interval of 15 minutes, which was given for absorption of acetic acid, number of squirms (writhing) was counted for 5 minutes.

3.4 Methodology for Antioxidative Test

The antioxidant activity of the ethanolic extracts was determined on the basis of their scavenging activity of the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. 1ml of each solution of different concentrations (1-500µg/ml) of the extracts was added to 3 ml of 0.004% ethanolic DPPH free radical solution. After 30 minutes the absorbance of the preparations were taken at 517 nm by a UV spectrophotometer which was compared with the corresponding absorbance of standard ascorbic acid concentrations (1-500 µg/ml). The method described by Hatano *et al.* (1988) was used to measure the absorbance with some modifications. Then the % inhibition was calculated by the following equation:

$$\% \text{ radical scavenging activity} = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{(\text{absorbance of blank})} \times 100$$

From calibration curves, obtained from different concentrations of the extracts, the IC₅₀ (Inhibitory concentration 50%) was determined. IC₅₀ value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals (Gupta *et al.*, 2003).

3.4.1 Apparatus used

- Test tubes
- Beakers
- Magnetic stirrer
- Thermometer
- Pipette
- UV spectrophotometer (single beam)
- Electronic balance

Reagents used

- Ethanol
- 0.004% DPPH (Aldrich, USA).
- Ascorbic acid (Loba, India)

3.4.2 Working Procedure

1. At first, 6 test tubes were taken to make aliquots of 6 conc.(1, 5, 10, 50, 100 and 500 μ g/ml) with the samples.
2. Fruit extracts and ascorbic acid were weighed accurately and dissolved in ethanol to make the required concentrations by dilution technique. Here ascorbic acid was taken as standard.
3. DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution. To dissolve homogeneously magnetic stirrer was used.
4. After making the desired concentrations 3ml of 0.004% DPPH solution was applied on each test tube by pipette.
5. The room temperature was recorded and kept the test tubes for 30 mins in light to complete the reactions.
6. DPPH was also applied on the blank test tubes at the same time where only ethanol was taken as blank.
7. After 30 minutes, the absorbances of each test tube were taken by a UV spectrophotometer.
8. IC₅₀'s were measured from % Inhibition vs. Conc. graphs.

3.5 Methodology for Antidiarrhoeal Activity Test

Diarrhoea is too frequent, often too passage of poorly formed stools. In pathological term, it occurs due to passage of excess of water in faeces (Tripathi, 2001). In the ileum and colon active Na⁺K⁺ATPase mediated salt absorption occur, water follows isoosmotically. In addition glucose facilitated Na⁺ absorption take place in the ileum. This mechanism remains intact even in severe diarrhoeas. Diarrhoea associated with carcinoid (secreting 5-HT) and medullary carcinoma of thyroid (secreting calcitonon) is mediated by cAMP. Excess of bile acids also causes diarrhoea by activating adenyl cyclase (Tripathi, 2001).

3.5.1. Principle

Diarrhoea was defined by the presence of stool or any fluid material that stained the absorbent paper placed beneath the cage. Time taken before the first defecation is the 'Latent period'. The total count stool and latent period of test group are comparing with positive control group. Antidiarrhoeal agent increase latent period and decrease total stool count.

3.5.2 Mechanism of diarrheal action of castor oil

Upon oral administration, castor oil mixes with bile and pancreatic enzymes and liberates ricinoleic acid from the tryglyceride. A small amount of ricinoleic acid is absorbed from the gastrointestinal tract and metabolized like any other fatty acid but most remains in the intestine where it produces its anti absorptive or secretory effect. The ricinoleic acid thus liberated readily forms ricinoleate salts with sodium and potassium in the lumen of the intestine. The ricinoleate salt formed as such behaves like a soap or surfactant within the gut and at the mucosal surface. The precise with serotonin (5-HT) have been termed as "diarrhoeogenic hormones mechanism of how ricinoleate salts induce diarrhoea is yet to be known. But most agreed view is that it stimulates the intestinal epithelial cell's adenyl cyclase, release prostaglandins and specially prostaglandins of the E series along" (Tripathi, 2001).

3.5.3 Preparation of Sample

- To prepare suspension of the test samples at the doses of 500mg/kg per body weight and 250mg/kg body weight of samples were measured respectively.
- The extract was triturated in unidirectional manner by the addition of small amount of tween-80.
- After proper mixing of extract and tween-80, the distilled water was slowly added. The final volume of the suspension was made 5ml.
- To prepare suspension of Loperamide at the doses of 3mg/kg body weight, 1.5mg of Loperamide was measured.
- Then distilled water was slowly added with it and the final volume of the suspension was 5 ml.

3.5.4 Study Design and Methodology

- The mice were all screened initially by giving 0.3 ml of castor oil and only those showing diarrhoea were selected for the final experiment.
- The test animals were randomly chosen and divided into four groups having five mice in each.
- Of the experimental groups, group-I or the control received only distilled water containing 1% Tween-80. Group-II or the positive control received standard antimotility drug, Loperamide at a dose of 3mg/kg body weight as oral suspension.
- One test group was treated with suspension of fruits extract of *Terminalia chebula* (Linn.) at the oral dose of 500mg/kg body weight and another test group was treated with the same extract at the oral dose of 250mg/kg body weight.
- The mice were fed with the samples 30 minutes prior to the oral administration of castor oil at a dose of 0.3ml per mouse.
- Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhea every hour in five hours study after the castor oil administration.
- Number of stools or any fluid material that stained the adsorbent paper were counted at each successive hour during the 5 hour period and were noted for each mouse.
- The latent period of each mouse also counted. At the beginning of each hour new papers were placed for the old ones.

Table 3.2 Experimental profile to observe the effect of fruits of *Terminalia chebula* (Linn.) on castor oil induced diarrhoea in mice.

Animal Group	Treatment	Dose (/kg body wt.)	Route Of admin.
I Control (n=5)	Water containing 1% tween 80	30ml	Oral
II Positive control (n=5)	Loperamide	3 mg	Oral
III (A) Test sample (n=5)	Extract	500mg	Oral
IV (B) Test sample (n=5)	Extract	250mg	Oral

Chapter-4

RESULTS AND DISCUSSIONS

Chapter-4

RESULTS AND DISCUSSIONS

The objective has been to explore and evaluate the analgesic, anti-oxidant and antidiarrhoeal activities to provide a suitable lead, which may be utilized in future to peruse a new line of investigation, based on the combined approach of both exploitation and exploration. For this, the investigations were carried out on phytochemistry, pharmacognosy and pharmacology. Results of all the experiments performed are discussed here.

4.1 Result and Discussion of Phytochemical Screening

The crude extract was subjected for chemical group tests and found the results stated in table 4.1.

Table 4.1: Results of different chemical group tests of *T. chebula* fruits

Sample	Test solution	Observation	Inference
<u>Test for Alkaloids:</u> ✓ 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid	0.1 ml of Mayer's reagent.	Yellowish buff colored precipitate was obtained.	Presence of alkaloid. +
✓ 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid.	0.1 ml of Dragendroff's reagent.	Orange brown precipitate was observed.	Presence of alkaloid. +
<u>Test for Steroids:</u> ✓ 1 ml solution of chloroform extract	1 ml sulphuric acid	Greenish color (not red) was observed	Absence of steroid. -
<u>Tests for Flavonoids:</u> ✓ 1 ml solution of ethanolic extract.	Few drops of conc. HCl was added to the extract	Immediate red color was not formed.	Absence of Flavonoids. -
<u>Tests for Saponins:</u>	Shaken in a	One centimeter	Presence of

✓ 1 ml solution of the extract was diluted with distilled water to 20 ml.	graduated cylinder for 15 minutes.	layer of foam was formed.	saponins. +
<u>Tests for Tannins:</u> ✓ 5 ml solution of extract.	1 ml of 5% Ferric chloride solution.	Red colour (not greenish black) precipitate was formed.	Absence of tannins. -
<u>Tests for Gums :</u> ✓ 5 ml solution of extract.	Molisch reagent and sulfuric acid were added.	Red –violet ring was produced at the junction of two liquids.	Presence of gums. +
<u>Test for Carbohydrate:</u> ✓ 5 ml solution of extract	5 ml Fehling solution boiled for 5	Brick red colourd precipitate was obtained	Presence of carbohydrate +
<u>Test for Glycoside:</u> ✓ 1ml of extract with water	A few drops of aqueous NaOH were added	Yellow colour was found	Presence of glycosides. +

We detected alkaloids, saponins, gums, carbohydrates (reducing sugars) and glycosides in the ethanolic extract of fruits of *Terminalia chebula*. In 2008, M. Gupta *et al.* reported that the ethanolic extract of *T. chebula* contains tannins, triterpenes, glycosides and chebulinic acid (M. Gupta, 2008). But we didn't found tannins in it. It may be due to some limitations in our lab. More investigations should be conducted to discover more constituents in it.

4.2 Result and Discussion of Analgesic Activity Test

4.2.1 Tabulation of writhing

Each mouse of all groups was observed carefully to count the number of writhing that they had elicited in 5 minutes.

Table 4.2: Statistical evaluation of the results

Adminis-tered (Dose)	Group	Numbering of mice	Weight (gm)	Dose (ml)	Acetic acid (ml)	Total writhing
Control (1% tween 80 in water, 10 ml/kg)	I	1	20	0.20	0.20	33
		2	24	0.24	0.24	39
		3	25	0.25	0.25	35
		4	21	0.21	0.21	33
		5	22	0.22	0.22	30
Diclo-fenac-Na (25 mg/kg)	II	1	25	0.25	0.25	6
		2	25	0.25	0.25	7.5
		3	21	0.21	0.21	6.5
		4	22	0.22	0.22	7
		5	23	0.23	0.23	6.5
Extract (500 mg/kg)	III (A)	1	18	0.18	0.18	10.5
		2	23	0.23	0.23	10
		3	18	0.18	0.18	11
		4	22	0.22	0.22	11.5
		5	18	0.18	0.18	12
Extract (250 mg/kg)	IV (B)	1	23	0.23	0.23	15
		2	20	0.20	0.20	17
		3	23	0.23	0.23	14
		4	19	0.19	0.19	14
		5	20	0.20	0.20	15

4.2.2 Statistical analysis

Table 4.3: Statistical evaluation of the results of analgesic activity of the Ethanolic extract of *Terminalia chebula*.

Animal Group	Mean Writhing	SD	SE	% Writhing	% of inhibition	t-test (value of p)
Control (10ml/kg)	34	3.88	1.94	100	0	-
Diclofenac sodium (25mg/kg)	6.7	2.33	1.167	19.71	80.29	5.257 P<0.001
Ethanolic extract (500 mg/kg)	11	3.211	1.433	32.35	67.65	4.592 P<0.001
Ethanolic extract (250 mg/kg)	15	4.498	2	44.12	55.88	4.301 P<0.001

SD- Standard deviation, SE- Standard error, n = Number of animals

4.2.3 Result of statistical analysis

Significance

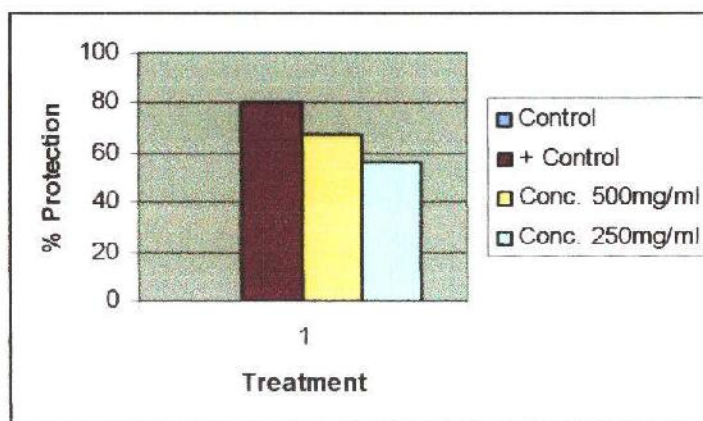
Control Vs Diclofenac sodium: Significant (P< 0.001).

Control Vs Extract (250 mg/kg): Significant (P< 0.001).

Control Vs Extract (500mg/kg): Significant (P<0.001).

% Protection

The ethanol extract of entire parts of *Terminalia chebula* produced 67.65 % and 55.55% protection or writhing inhibition in mice at oral doses of 500mg/kg and 250mg/kg body weights of mice respectively.



Graph 4.1 % protection Vs Treatment curve

4.2.4 Discussion

Terminalia chebula has also been reported for wound healing (analgesic) property (Suguna *et al.*, 2002). The evaluation of analgesic activity of PD-10 (ten medicinal plants, including *T. chebula*) using acetic acid induced writhing model hot plate method and tail immersion methods in mice revealed very significant ($p < 0.001$) analgesic activity (M. Gupta, 2008). Whereas, our detected significant is $p < 0.001$, in case of only *T. chebula*. However, further researches are necessary to dig out in-depth information on the analgesic activity of the active principles.

4.3 Result and Discussion of Antioxidative Activity Test

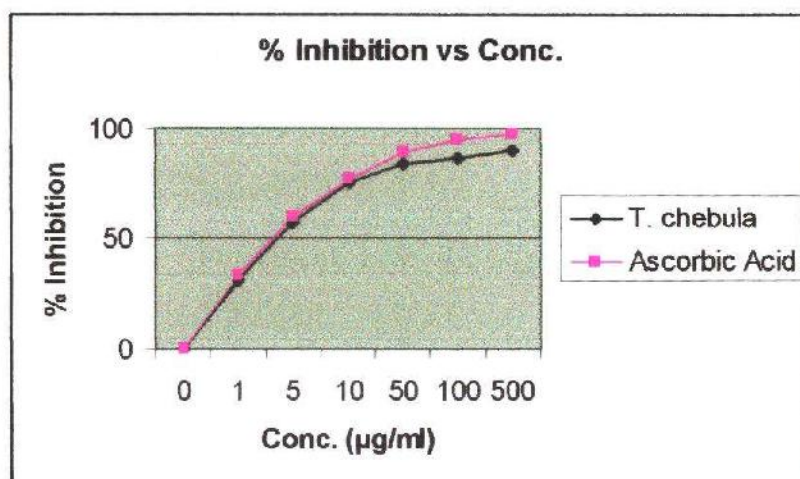
DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or a plant extract. In the present study, ethanolic extract of the fruits of *Terminalia chebula* (Black myrobalan) showed potential free-radical scavenging activity. The antioxidant activities of the individual compounds, present in the extracts may depend on structural factors, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features (Patt *et al.*, 1990).

Table 4.4 Absorbance and evaluation of % inhibition of the fruit extract of *Terminalia chebula* (Black myrobalan).

Conc. of <i>Terminalia</i> <i>chebula</i> (µg/ml)	Absorbance (nm)			Absorbance of blank solution (nm)			% inhibition
	1	2	Average	1	2	Average	
1	0.297	0.296	0.297	0.428	0.438	0.433	31.41
5	0.193	0.183	0.188				56.58
10	0.100	0.114	0.107				75.29
50	0.059	0.077	0.068				84.29
100	0.060	0.055	0.057				86.84
500	0.040	0.047	0.044				89.84

Table 4.5 Absorbance and evaluation of % inhibition of the fruit extract of ascorbic acid.

Conc. of ascorbic acid (µg/ml)	Absorbance (nm)			Absorbance of blank solution (nm)			% inhibition
	1	2	Average	1	2	Average	
1	0.298	0.32	0.299	0.428	0.438	0.433	33.25
5	0.211	0.205	0.208				59.15
10	0.102	0.104	0.103				77.45
50	0.082	0.081	0.081				88.75
100	0.019	0.026	0.022				94.69
500	0.009	0.013	0.011				97.46



Graph 4.2 Evaluation of IC_{50} of the fruit extract of *Terminalia chebula* and standard ascorbic acid.

4.3.1 Discussion

From the graph, it is found that IC_{50} of the fruit extract of *Terminalia chebula* is $3.5 \mu\text{g/ml}$ which indicates the potential antioxidant activity of the extract. On the other hand, the IC_{50} of standard ascorbic acid found $3.3 \mu\text{g/ml}$. In 2003, the same research was conducted by Hua-Yew Cheng *et al.* with MeOH extract and they reported that the IC_{50} of *T. chebula* is $5 \mu\text{g/ml}$ (0.005mg/ml) (Hua-Yew Cheng *et al.*, 2003). Although, the extracts were collected using different solvents, the results were almost same. The antioxidant activity may be due to the presence of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups and other structural features (Patt *et al.*, 1990).

4.4 Result and Discussion of Antidiarrhoeal Activity Test

Prior to castor oil induction, loperamide and solution of extract were induced and the results observed are stated in table 4.6.

Table 4.6 Effect of fruits of *T. chebula* on castor oil induced diarrhoea in mice

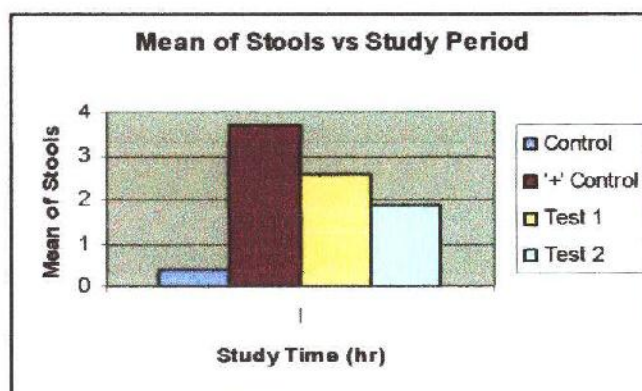
Groups	Period of study (hr)	Mean latent Period $\pm$ S.E.	Mean no. of stools	SD	SE	t-test (P-value)
I Control	1	0.39 $\pm$ 0.059	6.4	2.07	1.036	—
	2		5.2	1.3	0.65	—
	3		7.4	1.95	0.975	—
	4		4.6	1.51	0.75	—
	5		3	1.58	0.79	—
II Positive Control (Loperamide)	1	3.7 $\pm$ 0.424	1.4	1.949	0.9745	3.5156 ^a
	2		4.2	1.483	0.7415	1.014
	3		5.8	3.42	1.71	0.832
	4		3.2	2.786	1.393	0.8855
	5		1.4	2.19	1.095	1.185
III (A) Extract 500mg/kg	1	2.6 $\pm$ 0.438	0.6	0.89	0.445	5.17 ^b
	2		1.8	1.78	0.89	3.09 ^c
	3		3.4	1.95	0.975	2.92 ^c
	4		1.4	1.94	0.97	2.62 ^d
	5		0.6	0.89	0.445	2.649 ^d
IV (B) Extract 250mg/kg	1	1.85 $\pm$ 0.356	1	1	0.5	4.695 ^a
	2		4.3	3.692	1.846	0.459
	3		4.6	3.714	1.857	1.335
	4		4.4	4.23	2.11	0.089
	5		2.2	1.78	0.89	0.67

Values of t-test. ^d p<0.05, ^c p< 0.02, ^b p< 0.001, ^a p< 0.01 vs. control, Student's t-test.

Table 4.7 Effect of fruits of *T. chebula* on the latent period of castor oil induced Diarrhoeal episode in mice.

Group (Dose)	Number Of Mice	Mean latent period (hr)	Standard Deviation (SD)	Standard Error (SE)	t-test (P-value)
I Control	5	0.39	0.1187	0.059	-
II Loperamide (3mg/kg)	5	3.7	0.847	0.424	4.649 (P<.001)
III (A) Extract 500mg/kg)	5	2.6	0.876	0.438	4.977 (P<.001)
IV (B) Extract 250mg/kg)	5	1.85	0.712	0.356	3.882 (P<0.001)

Values of t-test. (n=5); p< 0.001 vs. control, Student's t-test.



Graph 4.3 Effect of crude extract of fruits of *T. chebula* on castor oil induced diarrhoea of mice. Each bar represents mean latent period.

6.4.1 Discussion

At the doses of 500 mg/kg body weight, the fruits extract of *Terminalia chebula* compared to the control group, offered about 3.7 hr of the mean latent period for diarrhoeal episode to ensure and the result was significant ($P < 0.001$).

The mean numbers of stools at the 1st, 2nd, 3rd, 4th and 5th hour of study of loperamide were 1.4, 4.2, 5.8, 3.2, and 1.4 respectively. Whereas the mean number of stools at the 1st, 2nd, 3rd, 4th and 5th hour of study of test-1 were 0.6, 1.8, 3.4, 1.4, 0.6 respectively.

Fruits of *T. chebula* are astringent, digestive, antiseptic, alterative, laxative, diuretic and carminative (Grahame Dixie, 2003). In the present study, *Terminalia chebula* (fruits) showed an antidiarrhoeal activity in castor oil induced test in mice at the doses of 500mg/kg body weight as compared to the standard antidiarrhoeal agent loperamide (dose: mg/kg body weight). *Terminalia chebula* caused an increase in latent period i.e. delayed the onset of diarrhoeal episode and decreased the frequency of defecation.

The decreased frequency of defecation and increase mean latent period of test group than control group and comparing with positive control group, we claimed that *Terminalia chebula* possess antidiarrhoeal activity.

Chapter-5

CONCLUSION

Chapter-5

CONCLUSION

Bangladesh is a very poor country and her 47% people live from hand to mouth. It is impossible for them to take expensive drugs or to eat the costly fruits like apple, grape fruits, orange, litchi, mango, pineapple and so on. In the present study, a cheap local fruit of Bangladesh was used that can easily be taken by the poor people. The investigation showed that the fat free residues of *T. chebula* fruits, possesses considerable analgesic, antioxidative and antidiarrhoeal properties. In short, the findings of the present study are as follows-

- ❑ The chemical groups of alkaloids, saponins, gums, carbohydrates (reducing sugars) and glycosides are present in the ethanolic extract of fruits of *Terminalia chebula*.
- ❑ The ethanolic extract of fruits of *Terminalia chebula* produced 67.65 % and 55.55% protection or writhing inhibition in mice at oral doses of 500mg/kg and 250mg/kg body weights of mice respectively.
- ❑ Mean latent period of control and positive control were 0.39 and 3.7 hours respectively whereas the latent period for 500 and 250 mg/kg concentrated solutions of extracts were 2.6 and 1.85 hours respectively.
- ❑ IC₅₀ of the fruit extract of *Terminalia chebula* is 3.5µg/ml. It means, 3.5µg/ml of sample required to scavenge 50% of the DPPH free radicals.

So, these fruits can be used by the poor people as biological drugs. But, the investigation showed only the intensity of analgesic, antioxidative and antidiarrhoeal compounds present in the extracts. But the study gave no concrete indication of the names of the compounds or exact amounts of them. More work should be done to characterize individual compounds of these fruits in order to assign certain fruit(s) for using as the cheapest source of natural drug to the poor people of Bangladesh as well as the world.

Chapter-6

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Chapter-6

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