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Effects of Selected Indeginous Plant's Extract on Diarrhoeal Diseases in Bangladesh

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University of Rajshahi

Ph.D.
Thesis

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A Thesis

Submitted to the Institute of Environmental Science (IES)
University of Rajshahi
for the Degree of
DOCTOR OF PHILOSOPHY
IN
ENVIRONMENTAL SCIENCE

By

Mrityunjoy Das

Institute of Environmental Science
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Rajshahi-6205
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Declaration

I do hereby declare that the thesis entitled, **"Effects of Selected Indeginous Plant's Extract on Diarrhoeal Diseases in Bangladesh."** Submitted to the Institute of Environmental Science (IES), University of Rajshahi for the award of Doctor of Philosophy (Ph.D) in Environmental Science is the result of my own investigation and research work under the supervision of Dr. Md. Belal Uddin, Professor, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh.

I further declare that this thesis or any part of it has not been submitted to any other University for any degree or diploma or for other similar purposes.

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Certificate

This is to certify that the thesis entitled, "**Effects of Selected Indeginous Plant's Extract on Diarrhoeal Diseases in Bangladesh."** submitted by Mrityunjoy Das in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Science has been carried out by him independently under my direct supervision. I believe that Mr. Mrityunjoy Das has made some distinct contributions in the field of Environmental Science. The thesis or thereof the part of it has not been submitted to any other university for any degree.

I also certify and recommend that the thesis is found satisfactory for submission to the Institute of Environmental Science (IES), University of Rajshahi, Bangladesh in fulfillment for the degree of Doctor of Philosophy in Environmental Science.

Rajshahi.

Dated:

Dr. Md. Belal Uddin

Research Supervisor

Dedicated

To

- My mother to whom give me a lot of support.
- My brothers and sisters who were the sources of my inspiration and energy all-time.
- My supervisor for his guidance.

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In the end, I pray to God that this investigation of bioactive compound may ultimately find its use for the relief of the human ailments in near future.

Mrityunjoy Das

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A Checklist of the Tables Provided

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Abbreviation of the special words used in the text

% = percentage

&=And

α = Alpha

β = beta

(-) = negative

(+) = positive

χ2 = chi-square

[K_2HPO_4+KH_2PO_4] = Phosphate buffer

[$K_3Fe(CN)_6$] = potassium ferricyanide

£ = pound

< =greater than

> =less than

± = positive/ negative

µ = micron

µg = microgram

µl = microlitre

A. indica = Azadirachta indica

A.Juss = Adrien-Henri de Jussieu

A. salina = Artemia salina

AIDS = Acquired Immune Deficiency Syndrome

$AlCl_3$ = Aluminium chloride

Aza.A = Azadirachtin-A

AZAP = Arid Zone Afforestation Project.

B.C. = Before Christ

BBS = Bangladesh bureau of Statistics.

BCSIR = Bangladesh Council of Scientific and Industrial Research

BHT = Butylated hudroxy toluene

C_2H_5OH =ethanol

Cat.E = Cathecin equivalent

CH_3OH = methanol

$CHCl_3$ =chloroform

Cl = Chlorine

Cl_3C-COOH (TCA) = trichloro acetic acid

CLOs = Campytobacter Like Organisms

cm = centimeter

df = degree of freedom

DMSO = Dimethyl sulfoxide

DPPH = 1,1-diphenyl-2-picrylhydrazyl

E. coli. = Escherichia coli

e.g. = Exempli gratia (For example)

ED_{50} = Effective dose 50

et.al. = et alii/alia (and others)

Etc = et cetera

Eto.Ac = ethyl acetate

FAD = Food Drug Administration

FCR = Folin- Ciocalteu Reagent

$FeCl_3$ = Ferric chloride

Fig. = Figure

g = gram

GA.E = Gallic acid equivalent

h = hour

H_2SO_4 = sulphuric acid

HNO3 = Nitric acid

HUS = Hemolytic Uremic Syndrome

i.e = id est (that is)

IC_{50} = Concentration providing 50% inhibition

ICDDR,B = International Centre for Diarrhoeal Diseases Research, Bangladesh.

IR = Infra Red

K = Potassium

LC_{50} = Lethal concentration

LD_{50} = Lethal dose 50

Linn/L. = Linnaeus

m = meter

mg = milligram

MIC = Minimum Inhibition Concentration

ml = mililitre

mM = milli mole

mm = millimeter

mmol/L = milli mole per litre

Na = Sodium

Na_2CO_3 = sodium carbonate

Na_3PO_4 = sodium phosphate

$NaNO_2$ = sodium nitrate

NaOH = sodium hydroxide

A. nilotica = Acacia nilotica

nm = nanometer

NMR = Nuclear Magnetic Resonance.

OCC = Opem Column Chromatography

P.guajava = Psidium guajava

Pet.E = petroleum ether

P^H = Anti logarithms of hydrogen ion concentration.

PO_4 = Phosphate

ppm = parts per million

PTLC = Prepared Thin Layer Chromatography

R_f = Retention factor

rpm = revolutions per minute

S.boydii = Shigella boydii

S.dysenteriae = Shigella dysenteriae

S. flexneri = Shigella flexneri

S. shiga = Shigella shiga

S. sonnei = Shigella sonnei

sp. = species

STD = standard deviation

TLC = Thin Layer Chromatography

UV = Ultra Violet

V. cholerae = Vibrio cholerae

viz = namely

WHO = World Health Organization

Willd = Carl Ludwig Willdenow

Abstract

Babla *(Acacia nilotica),* Neem *(Azadirachta indica)* and Peyara (*Psidium guajava)* leaves have been using as a well-known remedy for the treatment of various types of disorders in the ayurvedic, homoeopathic and traditional system of medicine in Bangladesh, India and many other countries. Considering the medicinal importance and availability in Bangladesh, the plant species*, A nilotica, A.indica* and *P.guajava* leaves were collected from Rajshahi University campus and extracts were prepared using ethanol, methanol, petroleum ether, chloroform and ethyl acetate as solvents. Seven diarrhoeal bacteria were collected from the Department of Pharmacy and Institute of Biological Science of University of Rajshahi, and ICDDR, B. Dhaka.

In this study, we have investigated antibacterial and cytotoxic activities of *A.nilotica, A.indica* and *P.guajava* leaves extracts. Antibacterial activity of the different extracts were evaluated at concentrations (100µg/disc) against *Escherichia coli, Shigella boydii, Shigella dysenteriae, Shigella flexneri, Shigella shiga, Shigella sonnei* and *Vibrio cholerae* bacteria. Ethanol, methanol, petroleum ether, ethyl acetate and chloroform extracts of *A.nilotica* leaves exhibited 10.67, 10.00, 9.33, 9.67 & 9.33 mm clear zone of inhibition against *E.coli & V.cholerae; E.coli; V.cholerae; S.boydii* and *V.cholerae,* with respect to standard ciprofloxacin disc (10 µg/disc). The same solvent extracts of *A.indica* leaves demonstrated 10.33, 9.67, 10.00, 9.67 and 9.33 mm clear zone against *S.boydi; S.flexneri; S.shiga; V.cholerae* and *E.coli*. Similarly, the extracts of *P. guajava* leaves indicated 9.33, 9.67, 9.33, 9.67 and 933 mm, the highest clear zone of inhibition against *S.shiga; V.cholerae; S.shiga; S.sonnei and S.dysenteriae,* respectively. Antibacterial activity was tested on seven diarrhoeal bacteria by the purified ethanol extract of *A.nilotica* leaves (compound E001). The compound E001 (100 µg/disc) was effective against all types of diarrhoeal bacteria.

Extracts of *A.nilotica, A.indica* and *P.guajava* leaves showed detectable MIC against diarrhoeal bacteria. The lowest MIC of *A.nilotica* leaves of ethanol, methanol, petroleum ether, chloroform and ethyl acetate extracts were 16, 16, 32, 16 & 32 µg/ml against *S.sonnei; S.flexneri*; *S.dysenteriae, S.boydii & S.flexneri; S.boydii* and *E.coli & S.sonnei,* respectively. Similarly, the lowest MIC values of the extracts of *A. indica* leaves demonstrated 32, 32, 16, 32 & 16 µg/ml against *S. dysenteriae, S. shiga*

& S. sonnei; S.shiga & S.sonnei; S.dysenteriae; *S.shiga & V.cholerae* and *S.shiga*, respectively. And the lowest MIC of the same solvent extracts of *P. guajava* leaves 32, 16, 32, 32 & 16 µg/ml against *S. shiga, S. sonnei, & Vibrio cholera; S.dysenteriae*; *E.coli, S.shiga, S.sonnei & V.cholerae*; *S.dysenteriae & S.shiga* and *E.coli,* respectively. It was observed that ethanolic extract of *A.nilotica* leaves showed the potent activity than other extracts.

Cytotoxicity test was also performed with various extractives of *A.nilotica, A.indica* and *P.guajava* leaves through brine shrimp (*Artemia salina*) lethality bioassay. LC_{50} values of *A.nilotica* leaves of ethanol, methanol, chloroform, ethyl acetate and petroleum ether extracts were found to be 395.581, 603.472, 830.236, 702.830 and 651.289 ppm, respectively. Similarly, LC_{50} values of *A. indica* leaves extracts were 830.236, 651.289, 682.959, 830.236 and 774.694 ppm. and *P. guajava* leaves extracts were 651.289, 682.959, 830.236, 682.959 and 651.289 ppm. Among the tested extracts, ethanolic extract of *A.nilotica* leaves showed the highest cytotoxicity against *Artemia salina.*

As ethanolic extract of *A.nilotica* leaves showed highest antibacterial and cytotoxicity activities. So, it was purified by PTLC and OCC methods. The purities and bioactivities of single purified compound E001 were reconfirmed by NMR study and further experiment on diarrhoeal bacteria. The isolated compound E001 was a steroid analogue and might be the derivatives of sitosterol.

The phytochemical properties of different solvent extracts of *A.nilotica* leaves were investigated. The extracts showed the presence of saponins, tannins, glycosides, steroids, alkaloids, phenolic compounds, flavonoids and many other bioactive compounds. It has significant reducing power capacity, antioxidant activity and DPPH radical scavenging activity.

The main targets of this research have traced out the isolated bioactive potentials from the ethanolic extract of *A.nilotica* leaves and significant response was found against diarrhoeal bacteria. This investigation of bioactive compound may ultimately find its use for the relief of the human diarrhoea in near future and thereby contribute a lot to the rural poor people of developing countries like Bangladesh.

Chapter One:

Introduction

1.1 General introduction:

Plants have been used as medicine for thousands of years. The ancient people were much more dependent on plants to get rid of any kind of illness. They always tried to use plants as a supreme source of remedy. At the same time, they also tried to find out new and more efficacious one that of the previous one. Only a tiny percentage of plant species are directly used by human for food, shelter, fiber and drugs. Throughout the ages, people have turned for healing to herbal medicine. There is a growing focus on the importance of medicinal plants and traditional health systems in solving the health care problems of the world. Because of this awareness, the international trade of plants for medical importance is growing phenomenally. Most developing countries have viewed traditional medical practice as an integral part of their culture. Therefore, the use of medicinal plants could serve as a basis for the maintenance of good health (Edward, 2001).

Researchers are also trying to explore the precious assets of medicinal plants to help the suffering of humanity. All cultures have folk medicine traditions that include the use of plants & plant products. Many licensed drugs that are used today are originated from the herbal traditions of various cultures. For instance, digitalis derived from foxglove is used for heart disease. In the United States, herbal products are marketed only as food supplements. Since they are not regulated by The Food Drug Administration (FDA), there is no guarantee of their purity or safety. In the recent world, a mentionable percentage of the pharmaceutical preparations are based on plants (Shinwari and Khan, 1998). The World Health Organization (WHO) estimates that 4 billion people (80%) of the world,s population use herbal medicine for some aspect of primary health care (WHO Report, 1993).

1.2 Scope & importance of medicinal plants :

The tradition of using medicinal plant as a remedial system started from the ancient civilization. Over three-quarters of the world population relies mainly on plants and

plant extracts for health care. Of the 2,50,000 higher plant species in the earth, more than 80,000 are medicinal. India is one of the world's biodiversity centres with the presence of over 45,000 different plant species. It has been estimated that in developed countries, such as United States, plant-based drugs are used as much as 25% of the total drugs; whereas in fast developing countries, such as China and India, the contribution is as much as 80%. Some important chemical intermediates (eg. Diosgenin and solasodine) needed for manufacturing the modern drugs are also obtained from plants. Currently, more than 120 pharmaceutical drugs in the market contain extracts from medicinal plants.

1.3 Historical documentation:

Medicinal plants have been used as far back as 5,000 years, and are still widely used today. The earliest record of using medicinal plant was found in China. Among ancient civilizations, India has been known to be rich repository of medicinal plants. The forest in India is the principal repository of large number medicinal and aromatic plants, which are largely collected as raw materials for manufacturing of drugs and perfumery products. In the *Ayurvedic* system of medicine, the *Rigveda* (5,000 BC) has recorded 67 species, whereas *Yajurveda, Atharvaveda* (4,500-2,500 BC), *Charak Samhita* (700 BC) and *Sushrut Samhita* (200 BC) had described properties and uses of 81 species, 290 species, 1100 and 1270 species, respectively. *Ayurveda, Siddha, Unani* and Folk (tribal) medicines are the major systems of indigenous medicines. Among these systems, Ayurveda is the most developed and is widely practiced in India. Ayurveda dating back to 1500-800 BC has been an integral part of Indian culture. Unfortunately, much of the ancient knowledge and many valuable plants are being lost at an alarming rate. The Red Data Book of India has 427 entries of endangered species. Of them, 28 are considered extinct, 124 are endangered, 81 are vulnerable, 100 are rare and 34 are insufficiently known species (Thomas, 1997). Bangladesh possesses a rich flora of medicinal plants. Out of 5000 species of higher plants, more than a thousand has been claimed to possess medicinal and poisonous properties, of which about 550 species have recently been enumerated with their medicinal and therapeutic values. In addition to having various other medicinal properties, about 250 of these medicinal plants have been identified as efficacious

remedies for diarrhoeal diseases (most common ailments in Bangladesh) and 47 for diabetes (Pushpangadan P *et al*., 2001). In Bangladesh most of the rural people are keen to take folk remedy. Maximum people in the hill tracks of Bangladesh, includes three districts, viz., Bandarban, Khagrachari and Rangamati are habituated to use plant-based remedy. *Ayurvedic* and *Unani* are widely practised systems of medicine in Bangladesh. Homoeopathy is also quite popular. There was a time when *Ayurvedic, Unani* or *Hakimi* treatments were the only remedies for both the rich and the poor people used to cure their illness. A majority of the people still use herbal medicines manufactured by indigenous institutions using various plants as raw materials. People in remote areas rely on *Ayurvedic* and *Unani* medicines mainly because of the lack of modern health care services. The traditional medicine practitioners are called Kabiraj or Hakims generally treat everything from temporary illness to chronic diseases.

1.4 Pharmaceutical era:

Pharmaceuticals become popular in Europe during the 19th century, when improvement in chemical analysis techniques allowed scientists to isolate and extract beneficial plant compound. Pharmaceutical industries are using around 120 different compounds derived from plants as drugs. For examples, quinine derived from cinchona tree is being used to treat some strains of malaria. In addition to quinine, salicylic acid and taxol derived from willow bark and yew tree, respectively are now being used to treat heart and cancer diseases. In contrast, traditional use of medicinal plants involves utilizing an entire portion of the plant like root or leaf, rather than extracting a single compound. Pharmaceuticals also go through extensive testing before being available to the public, although scientists report more side effects with pharmaceuticals than medicinal plant. Due to having less scientific evidence exist for the effectiveness of medicinal plants, research interest and investment on plants is increasing day by day.

1.5 Accessibility and cost:

Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of existing drugs in the market and development of resistance to currently used

drugs for infectious diseases have led to emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Global estimates indicate that 80% of about 4 billion population cannot afford the products of the western pharmaceutical industry and have to rely upon the use of traditional medicines which are mainly derived from plant materials. This fact is well documented in the inventory of medicinal plants, listing over 20,000 species. In spite of the overwhelming influences and our dependence on modern medicine and tremendous advances in synthetic drugs, a large segment of the world population still likes drugs from plants. In many of the developing countries, the use of plant-based drugs is increasing, because modern life saving drugs are beyond the reach of three quarters of the third world's population although many such countries spend 40-50% of their total wealth on drugs and health care. As a part of the strategy to reduce the financial burden on developing countries, it is obvious that an increased use of plant-based drugs will be followed in the future.

1.6 Therapeutic use:

Medicinal plants used in herbal remedy provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and/or reduced toxicity. Useful constituents responsible to maintain normal physiology of plant, including vitamins, tannins, saponins, lignins, carbohydrates, proteins, essential oil, polyphenols, flavonoids and so on. Each of these constituents not only has specific activity in the plant physiology but also in the animal physiology. The presence or absence of these constituents varies depending on the parts of the tree. Some of the useful plant-based drugs, including vinblasstine, vincristine, taxol, podophyllotoxin, camptothecin,digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicine, curcumin, artemesinin and ephedrine, have been used for different illness. Not only the phytochemicals, but also the extracts of different parts of plant are used for different purposes. The seeds of the Jatropha plant are used to treat parasitic worms, while the leaves of the some plants are used for wound healing. The root of the Papaya tree treats bronchial asthma, and the leaves are used to treat bloody diarrhoea.

1.7 Other uses:

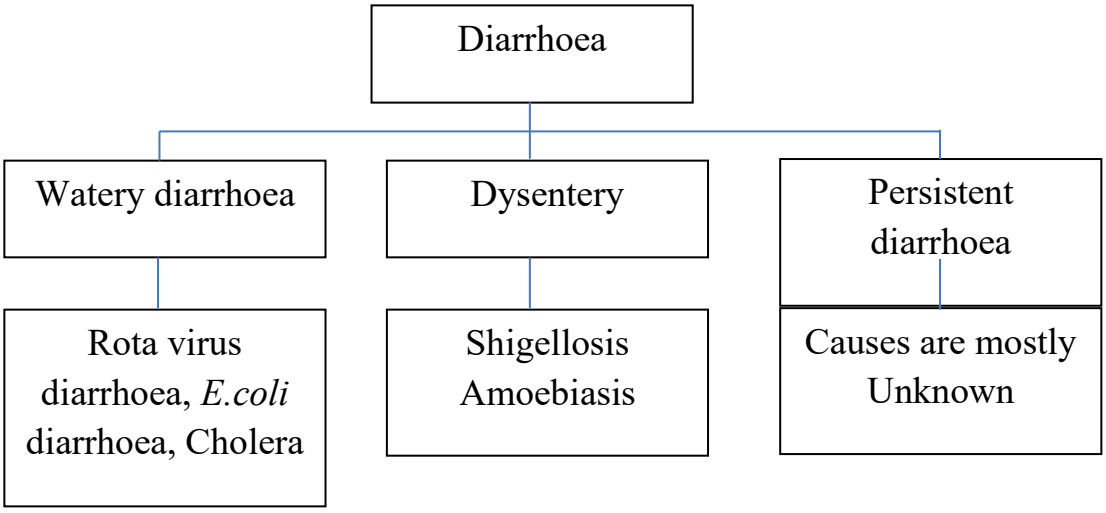
Apart from treating diseases, plants are used in cosmetics for maintaining healthy hair and skin. Commercially available oils, creams and shampoo contain plant extracts like lavender, aloe and mint that provide added benefits. Plants like basil, thyme and rosemary enhance the flavor and nutritional value of foods. Eucalyptus and citronella freshen the air and repel insects. Roots of many plants are edible and contain considerable quantities of food materials, particularly starch. Plants can be considered as one of the most important and interesting subject that should be explored for the discovery and development of newer and safer drug candidates. However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics.

1.8 Diarrhoeal disease

Diarrhoea disease is a common symptom, patient with diarrhoea disease defecates more frequently than in normal time, and stool is loose and there is more water, the quantity of defecation is more than 200 g, or the quantity of defecation is lower than 200 g but the defecation is more than 3 times associated with mucus, bloody pus or undigested food. Generally diarrhoea associated with the symptom including defecation urgency, anus malaise and incontinence. The diagnosis denomination of infant diarrhoea had been altered for several times in domestic, which was customarily called “dyspepsia” from 1950s to 1960s, if complicated with desiccation, acidosis and electrolyte disturbance, which was diagnosed as “toxic dyspepsia”, Diarrhoea caused by bacteria, virus, parasite, fungi and some uncertain pathogens were all called “infantile enteritis”. Broad diarrhoea includes infectious diarrhoea and noninfectious diarrhoea. The former is defined a group of intestines infectious disease caused by pathogen (bacteria, virus, parasite and so on), the clinic characteristic of which is diarrhoea, also denominated diarrhoea disease. The harm of infectious diarrhoea to people embodies in the rapid spread, broad infected area and high incidence rate, death will occur if not be treated in time or reasonably (China CDC, 2005).

1.9 Types of diarrhoeal diseases

According to Mitra (2009), diarrhoea can be classified in following three types.



1.10 Causes of diarrhoeal diseases

A hundred or more different diseases can be associated with diarrhoea. Diarrhoea is a symptom of infection caused by a host of bacterial, viral and parasitic organisms most of which can be spread by contaminated water. Diarrhoea can spread from person to person, aggravated by poor personal hygiene. Food is another major cause of diarrhoea when it is prepared or stored in unhygienic conditions. According to National Digestive Diseases information Clearinghouse (Diarrhoea, 2007) and Parekh (2006) a few of the more common causes of diarrhoea include the following: bacterial infections, viral infections, food intolerances, parasite, reaction to medicines, intestinal diseases, functional bowel disorders, laxatives and chemicals, excessive consumption of alcohol, stomach surgery or removal of the gallbladder.

In many cases, the cause of diarrhoea cannot be found. Recent evidence suggests that genetic factors may also be involved in the developmental response to repeated diarrhoea (Oria *et al.,* 2005). Several studies have found that risk of diarrhoeal disease is associated with environmental variables (Emch and Ali, 2001)

1.11 Symptomps of diarrhoeal diseases

Signs and symptoms associated with diarrhoea may include:

1. Frequent loose, watery stools,
2. Abdominal cramps,
3. Abdominal pain,
4. Fever,
5. Bleeding,
6. Lightheadedness or dizziness form dehydration.

Diarrhoea caused by a viral infection, such as a stomach virus, or bacterial infection also may cause vomiting. In addition, blood and mucus in the stools may appear with diarrhoea caused by bacterial infections.

1.12 Clinical features and complication of diarrhoeal diseases

Watery diarrhoea

Dehydration, electrolyte imbalances, tetany, convulsions, hypoglycemia, renal failure.

Dysentery

Electrolyte imbalances, convulsions, hemolytic uremic syndorme (HUS), leukemoid reaction, toxic megacolon, protein losing enteropathy, arthritis and preformation.

Table 1. Amount of salt loss during diarrhoea

Diarrhoea	Salt (mmol/L)			
	Na	K	Cl	HCO_3
Cholera (child)	88	30	86	32
Cholera (adult)	135	15	100	45
E.coli	53	37	24	18
Rota virus	37	38	22	6

1.13 Causal organisms of diarrhoeal diseases

The past decade has witnessed a proliferation or reports on newly recognized causes of infectious diarrhoea .Table 2. Many bacterial, viral and protozoal agents have been isolated form immune competent patients with diarrhoea. (Mata *et al.,* 1984; Ashkenazi and Pickering, 1991).

Table 2. Newly recognized organisms associated with infectious diarrhoea

Class	Organism
Bacteria	Enterohemorrhagic *Escherichia coli*, Enteroadherent *Escherichia coli,* Aeromonas hydrophila, Plesiornonas shigelloides, Campylobacter-like organisms, (CLOs)*, Mycobaterium aviurnintracellulare*, *Salmonella enteritidis, Escherichia coli, Shigella* species, *Campylobacter* species, *Vibrio* species, *Staphylococcus aureus, Clostridium difficile, Yersinia species*.
Viruses	Rotaviruses of groups B and C, Andenovirus types 40 and 41, Caliciviruses, Astroviruses, Norwalk-like viruses, Coronaviruses, Pesitviruses, Parvoviruses,
Protozoa	*Cryptosporidium, Microsporum*, Isospora betli*, Blastocystis hominis, Giardia lamblia, Entamoeba histolytica*.

* Occur predominantly in immunocompromised hosts such as those with AIDS

1.14 Present situation of diarrhoeal diseases in the world

Diarrhoeal diseases are major contributors to the morbidity and mortality among infants and young children in the developing world. Diarrhoeal diseases kill 2,500,000 annually worldwide (Cholera, 2008). Most deaths are in children under 5 years of age. Diarrhoea occurs worldwide and causes 4% of all deaths and 5% of

health loss to disability. Cholera and dysentery cause severe, sometimes life threatening forms of diarrhoea. Worldwide around 1.1 billion people lack access to improved water sources and 2.4 billion have no basic sanitation. In Southeast Asia and Africa, diarrhoea is responsible for as much as 8.5% and 7.7% all deaths respectively.

1.15 Present situation of diarrhoeal diseases in Bangladesh

Diarrhoea disease is one of the major public health problems in Bangladesh, around 70% of which are food and water borne. The diarrhoea situation in Bangladesh has worsened day by day with the temperature increases. One third of the total child death burden is due to diarrhoea (Victora *et al.,* 1993). Every year, a rural child suffers on average from 4.6 episodes of diarrhoea, form which about 230,000 children die (BBS, 1996; Mitra *et al.,* 1994). Most of the diarrhoea patients are from the lower-income group who work very hard in the high temperature. They have no access to safe water and healthy food and (easily become dehydrated) in the hot weather (Health, 2008). Shigellosis is endemic in Bangladesh and accounts for 20% of deaths related to diarrhoea among children (Victora *et al.,* 1993). According to government statistics, 393 people died of diarrhoea out of over 2.2 million people affected last year.

1.16 General treatment of diarrhoea diseases in world wide

National diarrhoeal disease control efforts have focused mainly on improving the effectiveness of case management. There have been parallel efforts to address factors which prevent the development of diarrhoea, such as the provision of safe water and sanitation, and the promotion of breastfeeding.

Key measures to reduce the number of cases of diarrhoea include:-

1. Access to safe drinking water.
2. Improved sanitation.
3. Good personal and food hygiene.
4. Health education about how infections spread.

Key measures to treat diarrhoea include:-

1. Giving more fluids than usual, including oral rehydration salts solution, to prevent dehydration.
2. Continue feeding.
3. Consulting a health worker if there are signs of dehydration or other problems.

It is very important that the patient should consult a physician as soon as possible and should avoid taking any of the medicine that is not prescribed by physician. The treatment for diarrhoea depends on the condition of the patient and cause of diarrhoea (symptomatic treatment).

Table 3. Antimicrobial agents used

Type of diarrhoea	Antimicrobial agent(s)
Cholera	Tetracycline, Doxacycline, Ciprofloxacin.
Shigellosis	Pivmencillinam (Selexid), Nalidixic acid, Ciprofloxacin, Ceftraxone, Metronidazole.
Amoebiasis	Metronidazole.

1.17 Why necessary to study diarrhoeal diseases

Diarrhoeal diseases caused several million of deaths in the world annually (Field, 2003). In developing countries they are the most common causes of morbidity and mortality (Amstrong and cohen, 1999). Many risks factors have been analyzed, most of them have been done retrospectively, and only few of them have been able to associate the risk factors with subsequent incidence of diarrhoea. In view of this problem, the World Health Organization has initiated the Diarrhoea Disease Control Program, which includes studies of traditional medical practices together with the evaluation of health education and prevention approaches (Pulok *et al.,* 1995). Most of the drugs produce undesirable side effects in man. Finally, a brief review on the actual knowledge of the anti-diarrhoeal potentials in some species of medicinal flora will be performed. Antimicrobial agent resistance has been recognized as an emerging worldwide problem in both human and veterinary medicine, and antimicrobial agent

use is considered the most important factor for the emergence, selection, and dissemination of antimicrobial agent-resistant bacteria. Every time a person takes antibiotics, sensitive bacteria are killed, but resistant germs may be left to grow and multiply. Repeated and improper uses of antibiotics are primary causes of the increase in drug-resistant bacteria. Children are also having the highest rate of infections caused by antibiotic-resistant pathogens. When antibiotics fail to work, the consequences are longer-lasting illnesses; more doctor visits or extended hospital stays; and the need for more expensive and toxic medications. Some resistant infections can cause death. For these reasons, it is necessary to study diarrhoea diseases and continuous research should be undertaken to establish new antibacterial agents to control them effectively and safely.

Plants used in this investigation

1.18 *Acacia nilotica* Linn.

1.18.1 Scientific name: *Acacia nilotica*

1.18.2 Bengali name: Babla

1.18.3 English name : Arabic gumtree; babul acacia; blackthorn.

1.18.4 Scientific classification

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Fabales

Family: Fabaceae

Subfamily: Mimosoideae

Genus*: Acacia*

Species: *Acacia nilotica*

1.18.5 Introduction

Over three quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species at one time or other was used for medicinal purposes. In India drugs of herbal origin have been used in traditional system of medicine such as Unani, Ayurveda since ancient times. The Ayurveda system of medicine uses about 700 species, Unani 700, Siddha 600, Amchi 600 and modern medicine around 30 species. The plant-based traditional medicine system continuously plays an essential role in health care.

Acacia is a genus of shrubs and trees belonging to the subfamily Mimosoidae of the family Fabaceae or Leguminosae first described by the Swedish botanist Carl Linnaeus (1773). They are pod-bearing, with sap and leaves typically bearing large

amounts of tannins and condensed tannins that historically in many species found use as pharmaceuticals and preservatives. This name derives from the Greek word for its characteristic thorns, (akis, thorn). The generic name derives from (akakia), the name given by early Greek botanist-physician Pedanius Dioscorides (ca. 40-90) to the medicinal tree *A. nilotica* in his book Materia Medica. The species name *nilotica* was given by Linnaeus from this tree's best-known range along the Nile river. The genus *Acacia* previously contained roughly 1300 species, about 960 of them native to Australia, with the remainder spread around the tropical to warm temperate regions of both hemispheres, including Europe, Africa, southern Asia, and the America. The extract of pods or legumes is known as Aqaqia in Unani system of medicine. When it is obtained from unripe fruit then called as *Qurz.* It is Native to Egypt, seen throughout the greater part of India, Ceylon, Baluchistan, Waziristan, Arabia, Egypt and tropical Africa. The synonyms are *Acacia arabica* (Lam.) Willd, *Acacia scorpioides* W.Wight, *Mimosa arabica* Lam., *Mimosa nilotica* L., and *Mimosa scorpioides* L.

A. nilotica know as babul is the most important tree of the dried parts of India. Almost all its parts are used in medication including root, bark, leaves, flower, gum, pods etc.

1.18.6 Botanical description

Acacia nilotica (family Leguminosae, subfamily Mimosoideae) grows to 15-18 m in height and 2-3 m in diameter. The bark is generally slaty green in young trees or nearly black in mature trees with deep longitudinal fissures exposing the inner grey-pinkish slash, exuding a reddish low quality gum. The leaves are bipinnate, pinnae 3-10 pairs, 1.3- 3.8 cm long, leaflets 10-20 pairs, and 2-5 mm long. Thin, straight light grey spines present in axillary pairs, usually 3-12 pairs, 5-7.5 cm long in young trees, and mature trees commonly without thorns. Flowers in globulous heads, 1.2-1.5 cm in diameter of a bright golden yellow colour, born either axillary or whorly on peduncles 2-3 cm long located at the end of branches. Pods 7-15 cm long, green and tomentose when immature and greenish black when mature, indehiscent, deeply constricted between the seed giving a necklace appearance. Seeds 8-12 per pod, compressed, ovoid, dark brown shining with hard testa.

1.18.7 Distribution:

A. nilotica is naturally widespread in the drier areas of Africa, from Senegal to Egypt and down to South Africa, and in Asia from Arabia eastward to India, Bangladesh, Burma and Sri Lanka. The largest tracts are found in Sind. It is distributed throughout the greater part of India in forest areas, roadsides, farmlands, tank foreshores, agricultural fields, village grazing lands, wastelands, bunds, along the national highways and railway lines. Mostly it occurs as an isolated tree and rarely found in patches to a limited extent in forests. It has been widely planted on farms throughout the plains of the Indian subcontinent. It is a species of Southern Tropical dry deciduous forests and Southern Tropical thorn forests as distinguished by Champion and Seth (1968).

1.18.8 Chemical constituents:

Leaves and fruits contain tannin 32% and flowers contain stearic acid, kaempferol-3-glucoside, isoquercetin, leucocyanidin. The bark contains 20% of tannin. There is polyphenolic compounds have been reported and those identified are (+) dicatechin, quercetin, gallic acid and ∞ - amyrin, ß-sitosterol. The bark contains sucrose also. Pods of babul also contain tannin, 12-19% in the whole pod and 18-27% after removal of seeds. Several polyphenols are present in pods and those identified are gallic acid, m-digallic acid, (+) catechin, and robidandiol, chlorogenic acid, m-digallic acid, galloylated flavan-3-4-diol. The gum contains 1.8% moisture, galactose, L-arabinose. L-rhamnose and four aldobiouronic acids. It also contains arabinose, 3-O-ß-L-arabinopyranosyl, L-arabinose. Further it contains calcium, polysaccharides, magnesium salts, potassium, sugar, moisture, ash and malic acid and oxidative enzymes. Wood contain chlorides. Analysis of the seeds gave the following values (dry basis). Moisture 8.83, crude protein 26.5, fat 3.3, N-free extract 62.9, crude fibre 2.7, and total ash 4.7, calcium 673.0, phosphorous 420.0, iron 4.95, niacin 3.17, ascorbic acid 4.51, and thiamine 0.24 mg/100 ml. The essential amino acid composition of the seed protein is histidine 3.7, lysine 4.3, methionine 0.4, cystine 0.49, leucine 8.8, valine 4.2, and threonine 3.39/10 g. The seeds on extraction with petroleum ether yield brownish oil (6.7%). The oil contains; saturated acids 27.4%, oleic 30.5% and linoleic acid 42.1% . Both contain 32% tannin and fruit also contain

gallic acid. Root contains hentriacontane, lapachol, n-hentriacontanol, sitosterol, octacosanol, betulin, ß-amyrin. Flower provides stearic acid, kaempferol-3 glucoside, isoquercetin, leucocyanidin.

1.18.9 Pharmacological actions in traditional and ethno medicine

The pharmacological actions described in the traditional system of medicines are concisely summarized and abound of them are pharmacologically proven on modern scientific parameter.

1.18.10 Action mentioned in Unani medicine

Qabiz-Astringent; Muqawwi meda wa kabid-Strengthen the stomach and intestine Habis khoon-Haemostatic;Mujaffif-Desiccant; Radae-Divergent; Mugharri Stimulant; Muqawwi asab-Nervine tonic; Muqawwi badan-General tonic; Mullattif-Demulcent; MuhallilAnti-inflammatory; Dafiae tashannuj Antispasmodic;Mullayin- Laxative; Muqawwi basr-Eye tonic.

1.18 11 Medicinal uses mentioned in Unani medicine

The bark skin, leaves, seeds, pods and the gum of *Acacia nilotica* are used for medicinal purpose. The plant is used internally as well as externally.

Extract of pods (Aqaqia):

The extract of pods is useful to prevent leucorrhoea and drives back the uterus and anus when they come out. It is also useful in istarkha rehm (laxity of vagina). It tightens the perineum if used locally. It is also useful to divert the morbid matter coming towards the organ. It prevents haemorrhagic diarrhoea. It prevents infantile diarrhoea if used along with roghan gul (rose oil). It causes dryness if applied externally on the body. It prevents the foul smelling of sweat, if applied externally on skin along with gulab (rose). It causes constipation when used orally or as enema. It is useful for intestinal abrasions and hemorrhagic diarrhoea. It prevents flabbiness of the joints. It can be used externally in inflammation of anus along with aas and gulab. It promotes and strengthens the vision and cure eye diseases. It gives relief in conjunctivitis, erysipelas and also included in medicine meant for pterygium . It blackens the hair and prevents hair falling. It gives clear complexion and useful for

fissures caused by cold. It is suitable for persons suffering from diarrhoea and dysentery. It is useful in all bleeding disease. It reduces the excessive sweating, when used as tila with gulab and aas. It is useful for whitlow (paronychia), fire burns, pimples and hot swellings when applied with white of egg. It is useful for ulcer of mouth and bleeding gums in the form of zaroor (powder). It is useful in spermatorrhoea and premature ejaculation. It stops bilious diarrhoea. The powdered pods used in impotency and effective in urogenital disorders.

Flowers:

It use as a tonic in diarrhoea and dysentery and good cure for insanity. The flowers are reported to reduce body temperature. Powdered form of flowers and bark useful in premature ejaculation and seminorrhoea.

Leaves:

The decoction of the leaves used for astringent to the bowels, cure bronchitis, heal fracture, good for eye diseases. Bruised leaves applied to sore eyes in children. Paste of burnt leaves effective ointment in itch. Tender leaves beaten into a pulp are used as a gargle in spongy gums, sore throat and as wash in haemorrhagic ulcers and wound.

Leaves extract is an astringent and injected to allay irritation in acute gonorrhoea and leucorrhoea. Further, bruised leaves formed into a poultice and applied to ulcer act as a stimulant and astringent. The tender leaves growing tops rubbed into a paste with sugar and water and given morning and evening act as a demulcent useful in coughs. The tender leaves beaten into a pulp are given in diarrhoea as astringent.

Bark:

The bark contains a large quantity of tannin and is a powerful astringent; its decoction is largely used as a gargle and mouth wash in cancerous and syphilitic affections. Infusion of bark (1½ ounces to one pint of water) is given in chronic diarrhoea and diabetes mellitus in doses of 1½ to 2 ounces twice a day. The juice of the bark mixed with milk is dropped into the eye in conjunctivitis. Decoction of bark is largely used as an astringent douch in gonorrhoea, cystitis, vaginitis, leucorrhoea and prolapse of uterus .

Gum:

The powdered gum mixed with quinine is useful in fever cases complicated with diarrhoea and dysentery. It stops bleeding and urinary and vaginal discharges, also useful in diabetes. Powdered gum mixed with white of egg it is applied to burns and scalds .

Fruits:

The fruits are prescribed in dysentery and opthalmia**.**

1.18.12 Other traditional uses of *A.nilotica* as medicine in different countries:

The African zulu people used bark for cough and also in West Africa, the bark or gum is used to treat cancers/or tumours (of eye, ear or testicles) and indurations of liver and spleen, condylomas and excess flesh. **Lebanon:** The resin is mixed with orange flower infusions for typhoid convalescence. **Tonga**: The root is used to treat tuberculosis. **Egypt**: The Egyptian Nubians believed that diabetics may eat unlimited carbohydrates as long as they also consume powdered pods**. Italian Africa**: The wood is used to treat smallpox**. Ethiopia**: Certain parts of the tree are used as a lactogogue. **Senegal**: Sap or bark, leaves and young pods are strongly astringent due to tannin and are chewed as an antiscorbutic. Dosage mentioned in Unani literature: Extract of pods (Aqaqia): -1½ g ; 2¾ -3½ g; 3½ g.

Substitute: Sandal sufaid (white sandal wood) ; Masoor ki daal (gram); Rasaut (wild turmeric)(Rasaut is the best substitute for Aqaqia); Dammul Akhwain (Dragon blood).

Formulations (Unani medicine): Hab awaz kashk; Hab ral; Hab surkh chashm; Qurs deedan; Qurs ziabitus; Qurs salajit; Qurs sailan; Qurs sailan jadid; Qurs kabid naushadri; Qurs kahruba; Qurs gulnar; Sunun poast mughilan; Majoon bawasir; Lauq sapistan.

1.18.13 Medicinal uses in ethno medicine

Leaves: Tender leaves beaten into a pulp are used as a gargle in spongy gums, sore throat and as wash in hemorrhagic ulcers and wound. Bruised tender leaves formed into a poultice and applied to ulcers act as stimulant and astringent. The leaves are tonic to brain and liver. Also promotes and strengthens the vision and cure eye disease. The leaves are astringent, tonic to the liver and the brain, antipyretic, enriches the blood. The tender leaves infusion used as an astringent and remedy for diarrhoea and dysentery.

Bark: Decoction of bark is largely used as an astringent douche in gonorrhoea, cystitis, vaginitis, leucorrhoea, prolapse of the uterus and piles. The decoction largely used as a gargle and mouth wash. The juice of bark mixed with milk is dropped into the eye for conjunctivitis. The burnt bark and burnt almond shell both pulverized and mixed with salt to make a good tooth-powder. The Italian Africa uses the bark concoction in treating small pox. In Ethiopia, *Acacia nilotica* (booni) is used as a lactogogue (increase milk supply). In Australia*, Acacia nilotica* bark is believed to be an astringent with high tannic acid contents that help to check bleeding, discharge and excess mucus. The extract from this highly astringent herb may block the body's pain triggers.

Gum: Gum administered in the form of mucilage in diarrhoea, dysentery and diabetes mellitus. Fried in ghee, the gum is useful as a nutritive tonic and aphrodisiac in cases of sexual debility. Powdered gum mixed with the white of an egg is applied on burns and scalds. The gum is expectorant, antipyretic, cure lung troubles.The gum is said to be very useful in diabetes mellitus.

Pods: Decoction is beneficial in urogenital diseases and prevents premature ejaculation. It is an astringent and injected to allay irritation in acute gonorrhoea and leucorrhoea. The pods are use for impotency, urino-genital disorder and in dry cough. The seeds and leaves extracts are used for general body vigour.

The fresh pods of *A. nilotica* (booni) tree are effective in treating sexual disorders such as spermatorrhoea, loss of viscidity of semen, frequent night discharges and premature ejaculation. The pods of *Acacia nilotica* are reported helpful in removing

catarrhal matter and phlegm from bronchial tubes; African zulu take bark of *Acacia nilotica* for cough treatment.

Fruits: The fruits are found to be useful in diarrhoea, dysentery and diabetes.

Seeds: Extract of seeds found to be general body vigour.

Root: Powder of root is useful in leucorrhoea, useful in wound healing and useful in burning sensation. Various plants parts used in hair-fall, ear-ache, syphilis, cholera, dysentery and leprosy.

The tender growing tops rubbed into a paste with sugar and water and given morning and evening act as demulcent, useful in caugh.

1.19 *Azadirachta indica* (A.Juss)

1.19.1 Scientific Name: *Azadirachta indica*

1.19.2 Bengali: Nim, Nimgachh, Neem.

1.19.3 English: Indian Lilac, Margosa tree, Neem tree,

1.19.4 Scientific classification

Kingdom : Plantae

Order: Rutales

Suborder: Rutinae

Family: Meliaceae

Subfamily : Melioideae

Tribe : Melieae

Genus *: Azadirachta*

Species: *Azadirachta indica,*

1.19.5 Introduction

The neem tree, botanically referred to as *Azadirachta indica* (A. Juss) is a fast growing hardy and evergreen tropical and sub-tropical plant belonging to the same family as mahogany, Meliaceae. They thrive in climates with annual rainfall of 400–800 mm and an extended dry season, even with poor soils (Schmutterer 1990; Ajayi 2002). It is believed to be native to the whole Indo-Pakistan subcontinent, especially southeast Asia (India, Bangladesh, Burma/Myanmar, Sri Lanka, Thailand, Malaysia and Indonesia), from where it was introduced to Nigeria in 1928 through Ghana by a man named 'Dogon Yaro' and hence its local name in northern Nigeria (Sara and Folorunso 2002)

The neem tree gained prominence in northern Nigeria due to its adoption as a desertification control plant, and the subsequent planting of 14 million neem seedlings in 1978 by the Federal Government of Nigeria under the Arid Zone Afforestation Project(AZAP). According to Sara and Folorunso (2002), the population of neem

trees in the landscape of the dry lands of Nigeria was further boosted in 1986 when 70% of the 1 billion tree seedlings planted under the World Bank-Assisted Forestry II project was neem.

Azadirachta indica is also commonly found in other African countries like Ghana, Togo, Niger, Chad, Cameroon, Ethiopia, Sudan, Somalia, Kenya, Tanzania, Mozambique, Burkina Faso, and Cote' Devoire.

In addition to its use in afforestation programs, authors from different countries have referred to it as ''miracle tree'', ''multipurpose crop'', ''village dispensary'' and ''living pharmacy'' because of its multiple uses. In fact, in its Asian countries of origin, every part of the neem tree has been extensively used in Ayurveda, Unani and Homeopathic medicine as household remedy against various human ailments from antiquity, leading Biswas *et al.* (2002) to conclude that it is a cynosure of modern medicine.

1.19.6 Distribution:

A native to east India and Burma, it grows in much of south East Asia and West Africa, and more recently Caribbean and south and Central America. In India it occurs naturally in Siwalik Hills, dry forests of Andhra Pradesh, Tamil Nadu and Karnataka to an altitude of approximately 700 m. It is cultivated and frequently naturalized throughout the drier regions of tropical and subtropical India, Pakistan, Sri Lanka, Thailand and Indonesia. It is also grown and often naturalized in Peninsular Malaysia, Singapore, Philippines, Australia, Saudi Arabia, Tropical Africa, the Caribbean, Central and South America

1.19.7 Botanical description:

It is a tree 40-50 feet or higher, with a straight trunk and long spreading branches forming a broad round crown; it has rough dark brown bark with wide longitudinal fissures separated by flat ridges. The leaves are compound, imparipinnate, each comprising 5-15 leaflets. The compound leaves are themselves alternating with one another. It bears many flowered panicles, mostly in the leaf axils. The selel are ovate and about 1 cm long with sweet scented white oblanciolate petals. It produces yellow drupes that are ellipsoid and glabrous, 12-20 mm long. Fruits are green, turning

yellow on ripening, aromatic with garlic like odour. Fresh leaves and flowers come in March-April. Fruits mature between April and August depending upon locality.

1.19.8 Medicinal use:

All parts of the tree have been used medicinally for centuries. It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties. The earliest Sanskrit medical writings refer to the benefits of Neem's fruits, seeds, oil, leaves, roots and bark. Each has been used in the Indian Ayurvedic and Unani medicine, and is now being used in pharmaceutical and cosmetics industries.

1.19.9 Advantages of various Neem parts

Neem oil: useful for pest control, cosmetics, medicines, etc.

Neem seed cake: Natural fertilizer and insecticide.

Neem leaves: useful for chickenpox, increase immunity of the body, reduce fever caused by malaria, treating various foot fungi, useful against termites, used in curing neuromuscular pains.

Neem bark and roots: control fleas and ticks on pets, fights against skin infections such as acne, psoriasis, scabies, eczema, etc, treats diabetes, AIDS, cancer, heart disease, herpes, allergies, ulcers, hepatitis and several other diseases.

1.19.10 Health and personal care products :

Neem personal care products derived from seed, oil and leaf include; Skin care - including eczema cream, antiseptic cream, and nail care; hair care - shampoo, and hair oils; oral hygiene - toothpaste and neem twigs; therapeutic - loose Neem leaves – tea, vegetarian capsules, powders; household products - soaps, insect repellent (spray and lotion), and candles.

1.19.11 Therapeutic uses:

Hot water extract of the bark is taken orally by the adult female as a tonic and emmenagouge. Anthraquinone fraction of dried flower, fruit and leaf is taken orally for leprosy. Hot water extract of the flower and leaf is taken orally as an anti-hysteric remedy, and used externally to treat wound. The dried flower is taken orally for

diabetes. Hot water extract of dried fruit is used for piles and externally for skin disease and ulcers. Hot water extract of the entire plant is used as anthelmintic, an insecticide and purgative. Juices of bark of *Andrographic puniculata, Azardiracta indica, Tinospora cardifolia*, are taken orally as a treatment for filariasis. The hot water extract is also taken for fever, diabetes, and as a tonic, refrigerant, anthelmintic. Fruit leaf and root, ground and mixed with dried ginger and 'Triphala" is taken orally with lukewarm water to treat common fever. Leaves due to insecticidal properties are kept with woollen and other cloths for long time. Leaf juice is given in gonorrhoea and leucorrhoea. Leaves applied as poultice to relieve boils, their infusion is used as antiseptic wash to promote the healing of wound and ulcers. A paste of leaves is used to treat wounds, ring worms, eczema and ulcers. Bathing with Neem leaves is beneficial for itching and other skin diseases. Leaf juice is used as nasal drop to treat worm infestation in nose. Steam inhalation of bark is useful in inflammation of throat. Decoction can cure intermittent fever, general debility convalescent, and loss of appetite after fever. Infusion of flower is given in dyspepsia and general debility. The tender twigs of the tree are used as tooth brush which is believed to keep the body system healthy, the breath and mouth clean and sweet. Seed oil is used in leprosy, syphilis, eczema, chronic ulcer.

1.19.12 Chemical constituents:

In India, pioneering work on the isolation and identifications of *A.indica* constituents was initiated in 1942 and has continued in various parts of the world. Leaves have been shown to contain crude fibre (11-24%), carbohydrates (48-58%), crude protein (14-18%), fat (2.3-6.9%), ash (7.7-8.5%), calcium (0.8-2.4%) and phosphorus (0.13-0.24%), as well as a number of amino acids. Recently, a two dimensional TLC method has revealed the presence of carotenoids and other constituents in the leaves of *A.indica*. Its oil is rich in fatty acids and cake (the solid residue following explusion of the oil) has a high sulfur content relative to other oil cakes. A number of sugars and polysaccharides have been identified in the gum and bark of *A.indica*. In addition to these general types of constituents, a number of novel compounds have isolated from most parts of the tree.

All parts of the *A.indica* tree possess insecticidal activity but seed karnel is the most effective. It has a multitude of pesticidal active ingredients which are together called "triterpene" more specifically "limnoids". The four best limnoids compounds are: Azadirachtin, Salannin, Meliantriol, and Nimbin. Azadirachtin (C35H44O16) itself is a group of compounds such as Azadirachtin A,B,C,D,E,F,G etc. Of these, azadirachtin-A (Aza A) is the most plentiful and biologically active one which has shown repellent, antifeedent and insecticidal activity against a number of insect pests and it is generally Aza A that is used for commercial insecticides.

1.20 *Psidium guajava* L

1.20.1 Scientific Name: *Psidium guajava* L.

1.20.2 Bengali name: goaachhi, piyara, peyara

1.20.3 English name: common guava, guava

1.20.4 Scientific classification

Kingdom: Plantae

Subkingdom: Viridiplantae

Infrakingdom: Streptophyta

Superdivision: Embryophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Class: Magnoliopsida

Superorder: Rosanae

Order: Myrtales

Family: Myrtaceae

Genus: *Psidium* L.

Species: *Psidium guajava* L.

1.20.5 Introduction:

Psidium guajava, which is considered a native to Mexico (Rios *et al*.,1977) extends throughout the South America, European, Africa and Asia. Based on archaeological evidence. It has been used widely and known in Peru since pre-Columbian times. It grows in all the tropical and subtropical areas of the world, adapts to different climatic conditions but prefers dry climates (Stone, 1970). The main traditional use known is as an anti-diarrhoeal. Other reported uses include gastroenteritis, dysentery, stomach, antibacterial colic pathogenic germs of the intestine.

Its medicinal usage has been reported in indigenous system of medicines in America more than elsewhere. *Psidium guajava* Linn. (family Myrtaceae), is commonly called guave, goyave or goyavier in French; guave, Guavenbaum, Guayave in German; banjiro in Japanese; goiaba, goiabeiro in Portugal; arac¸a-goiaba, ´arac¸a-guac ´ ¸u, guaiaba in Brazil; guayaba, guayabo in Espa ´ nol ˜and guava in English (Killion, 2000).

1.20.6 Botanic description:

Psidium guajava is a large dicotyledonous shrub, or small evergreen tree, generally 3-10 m high, many branches; stems crooked, bark light to reddish brown, thin, smooth, continuously flaking; root system generally superficial and very extensive, frequently extending well beyond the canopy, there are some deep roots but no distinct taproot.

Leaves opposite, simple; stipules absent, petiole short, 3-10 mm long; blade oblong to elliptic, 5-15.4-6 cm, apex obtuse to bluntly acuminate, base rounded to subcuneate , margins entire, somewhat thick and leathery, dull grey to yellow-green above, slightly downy below, veins prominent, gland dotted.

Inflorescence, axillary, 1- to 3-flowered, pedicles about 2 cm long, bracts 2, linear. Calyx splitting irregularly into 2-4 lobes, whitish and sparsely hairy within; petals 4-5, white, linear-ovate 2 cm long, delicate; stamens numerous, filaments pale white, about 12 mm long, erect or spreading, anther straw coloured, ovary inferior, ovules numerous, style about 10 cm long, stigma green, capitate.

Fruit an ovoid or pear-shaped berry, 4-12 cm long, weighing up to 500 g; skin yellow when ripe, sometimes flushed with red; pulp juicy, creamy-white or creamy-yellow to pink or red; mesocarp thick, edible, the soft pulp enveloping numerous, cream to brown, kidney-shaped or flattened seeds. The exterior of the fruit is fleshy, and the centre consists of a seedy pulp.

From the Greek psidion (pomegranate), due to a fancied resemblance between the two fruits.

1.20.7 Distribution:

Native: Colombia, Mexico, Peru, United States of America

Exotic: Australia, Bangladesh, Brunei, Cambodia, Cameroon, China, Costa Rica, Cote d'Ivoire, Cuba, Dominican Republic, Ecuador, Eritrea, Ethiopia, Fiji, Gabon, Gambia, Greece, Guyana, Haiti, India, Indonesia, Israel, Kenya, Laos, Malawi, Malaysia, Myanmar, Nigeria, Pakistan, Panama, Philippines, Puerto Rico, Samoa, Senegal, South Africa, Sri Lanka, Sudan, Tanzania, Thailand, Togo, Uganda, Venezuela, Vietnam

1. 20.8 Use in traditional medicine

More recent ethnopharmacological studies show that *Psidium guajava* is used in many parts of the world for the treatment of a number of diseases, e.g. as an anti-inflammatory, for diabetes, hypertension, caries, wounds, pain relief and reducing fever . Some of the countries with a long history of traditional medicinal use of guava include Mexico and other Central American countries including the Caribbean, Africa and Asia. Some of these uses will be outlined here.

Medicinal plants are an important element of the indigenous medical systems in Mexico (Lara and Marquez, 1996). These resources are part of their traditional knowledge. The Popoluca Indians of Veracruz rely on medicinal plants for their health care. They appear to have developed a system whereby they select and continue to use plants that they find the most effective for health care purposes. The folk use of guava has been documented in the indigenous groups of Mexican Indians, Maya, Nahuatl, Zapotec and Popoluca. A decoction of the leaves is used to cure cough. According to communities of Nahuatl and Maya origin and Popoluca of the region of the Tuxtlas, Veracruz, they use a guava leaf decoction to treat digestive suffering associated with severe diarrhoea. This is a frequent disease in rainy weather (Heinrich *et al.,* 1998).

P. guajava (Myrtaceae) is widely used in Mexico to treat gastrointestinal and respiratory disturbances and is used as an anti-inflammatory medicine (Aguilar *et al.,* 1994). Commonly roots, bark, leaves and immature fruits, are used in the treatment of gastroenteritis, diarrhoea and dysentery. Leaves are applied on wounds, ulcers and for rheumatic pain, while they are chewed to relieve toothache (Heinrich *et al.,* 1998). A decoction of the new shoots is taken as a febrifuge. A combined decoction of leaves and bark is given to expel the placenta after childbirth (Martinez and Barajas, 1991).

A water leaf extract is used to reduce blood glucose level in diabetics. This hot tea was very common among the local people of Veracruz (Aguilar *et al.,* 1994).

The leaf of *Psidium guajava* is used traditionally in South African folk medicine to manage, control, and/or treat a plethora of human ailments, including diabetes mellitus and hypertension (Ojewole, 2005; Oh *et al.,* 2005).

Guava has been used widely in the traditional medicine of Latin America and the Caribbean in the treatment of diarrhoea and stomach-aches due to indigestion (Mejia and Rengifo, 2000; Mitchell and Ahmad, 2006a,b). Treatment usually involves a decoction of the leaves, roots, and bark of the plant. It also has been used for dysentery in Panama and as an astringent in Venezuela. A decoction of the bark and leaves is also reported to be used as a bath to treat skin ailments. In Uruguay, a decoction of the leaves is used as a vaginal and uterine wash, especially in leucorrhoea (Conway, 2002). In Costa Rica, a decoction of the flower buds is considered an effective anti-inflammatory remedy (Pardo,1999).

In Peru, it is used for gastroenteritis, dysentery, stomach pain (by acting on the pathogenic microorganisms of the intestine), indigestion, inflammations of the mouth and throat in the form of gargles (Cabieses, 1993). In some tribes of the forest (Tipis), the tender leaves are chewed to control toothaches by their weak sedative effect. Tikuna Indians use the decocted leaves or bark of guava for diseases of the gastrointestinal tract. It is also employed by the Indians of the Amazons for dysentery, sore throats, vomiting, stomach upsets, vertigo, and to regulate menstrual periods, mouth sores, bleeding gums, or used as a douche for vaginal discharge and to tighten and tone vaginal walls after childbirth. Flowers are also mashed and applied to painful eye conditions such as sun strain, conjunctivitis or eye injuries (Smith and Nigel, 1992). Guava jelly is tonic to the heart and constipation (Conway, 2002).

In the Philippines the astringent unripe fruit, the leaves, the cortex of the bark and the roots are used for washing ulcers and wounds, as an astringent, vulnerary, and for diarrhoea. Leaves and shoots are used by West Indians in febrifuge and antispasmodic baths; the dust of the leaves is used in the treatment of rheumatism, epilepsy and cholera; and guava leaves tincture is given to children suffering from convulsions (Morton, 1987).

In Latin America, Central and West Africa, and Southeast Asia, guava is considered an astringent, drying agent and a diuretic. A decoction is also recommended as a gargle for sore throats, laryngitis and swelling of the mouth, and it is used externally for skin ulcers, vaginal irritation and discharge (Mejia and Rengifo, 2000). In Mozambique, the decoction of leaves is mixed with the leaves of *Abacateira cajueiro*, to alleviate the flu, cough and pressed chest. In Mozambique, Argentina, Mexico and Nicaragua, guava leaves are applied externally for inflammatory diseases (Jansen and Mendez, 1990).

The use of medicinal plants by the general Chinese population is an old and still widespread practice. *Psidium guajava* leaves are example of the plant commonly used as popular medicine for diarrhoea which is also used as an antiseptic (Teixeira *et al.,* 2003).

In Brazil the fruit and leaves are considered for anorexia, cholera, diarrhoea, digestive problems, dysentery, gastric insufficiency, inflamed mucous membranes, laryngitis, mouth (swelling), skin problems, sore throat, ulcers, vaginal discharge (Holetz *et al.,* 2002). In USA guava leaf extracts that are used in various herbal formulas for a myriad of purposes; from herbal antibiotic and diarrhoea formulas to bowel health and weight loss formulas (Smith and Nigel, 1992).

Besides the medicinal uses *Psidium guayava* is employed as food, in carpentry, in construction of houses and in the manufacture of toys.

1.20.9 Chemical constituents:

Guava contains broad spectrum of phytochemicals including minerals, enzymes, proteins, sesquiterpenoid alcohols and triterpenoid acids, alkaloids, glycosides, steroids, flavanoids, tannins, saponins. Guava is very rich in antioxidants and vitamins and also high in lutein, zeaxanthine and lycopene. The guava leaves contain several chemical constituents such as α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene and curcumene, mallic acids, nerolidiol, β-sitosterol, ursolic, crategolic, and guayavolic acids, cineol, quercetin, 3-L-4-4-arabinofuranoside (avicularin) and its 3-L-4-pyranoside (essential oil), resin, tannin, eugenol, caryophyllene (1a α -, 4a α -,7 α -, 7a β -, 7b α -)]-

decahydro-1H-cycloprop[e] azulene, Guajavolide (2 α -,3 β -,6 β -,23-tetrahydroxyurs-12-en-28,20 β -olide; 1) and guavenoic acid (2 α -,3 β -,6 β -,23-tetrahydroxyurs-12,20(30)-dien-28-oic acid, triterpene oleanolic acid, triterpenoids, flavinone-2 2'-ene, prenol, dihydrobenzophenanthridine and cryptonine. Guavas contain carotenoids and polyphenols, the major classes of antioxidant pigments giving them relatively high potential antioxidant value among plant foods. As these pigments produce the fruit skin and flesh color, guavas that are red-orange have more pigment content as polyphenol, carotenoid and pro-vitamin A, retinoid sources than yellow-green ones.

Guavas contain both carotenoids and polyphenols like (+) - gallocatechin, guaijaverin, leucocynidin and amritoside. It was reported that the leaves of *P. guajava* contain an essential oil rich in cineol, tannins and triterpenes. In addition three flavonoids (quercetin, avicularin, and guaijaverin) have been isolated from the leaves. The leaves of guava are rich in flavonoids, particularly quercetin. The bark of guava tree contains considerable amounts of tannins (11-27%), and hence is used for tanning and dyeing purposes.

Leucocyanidin, luectic acid, ellagic acid and amritoside have been isolated from the stem bark. Five constituents, including one new pentacyclic triterpenoid: guajanoic acid and four known compounds beta-sitosterol, uvaol, oleanolic acid and ursolic acid, have been recently isolated from the leaves of *P. guajava* by Begum *et al*.. The essential oil contains alpha pinene, caryophyllene, cineol, D-limonene, eugenol, and myrcene. The major constituents of the volatile acids include (E) - cinnamic acid and (Z)-3-hexenoic acid. The guava fruit has high water content with lesser amounts of carbohydrates, proteins and fats. The fruit also contains iron, vitamins A and C, thiamine, riboflavin, niacin and manganese. The characteristic fruit odor is attributed to carbonyl compounds. Unripe fruits are high in tannins. The major constituent of the fruit skin is ascorbic acid, largely destroyed by canning and processing.

Active factors of *P. guajava* fruits involve ursolic acid, oleanolic acid, arjunolic acid and glucuronic acid. In comparison, huge amounts of β -sitosterol glucoside, brahmic acid, and polyphenolics including gallic acid, ferulic acid, and quercetin and

triterpenoids, exist in guava leaves. Thus, it is clear that *P. guajava* contains many components reported to display efficacy against various diseases.

1.21 Aim of this research work

To isolate an effective bio-active compounds from Bangladeshi plants which can control the diarrhoeal diseases in Bangladesh.

1.22 Objectives of this research work

1. To identify the plants extracts having the anti-bacterial and antimicrobial properties.

2. To isolate and purify the botanicals those can inhibit the growth of diarrhoeal bacteria.

3. To identify the minimum inhibition concentration (MIC) of extracts for control bacterial growth in lab condition.

4. To study the cytotoxicity of antibacterial compounds.

1.23 Review of Literature

Review of literature is an important section that links previous knowledge with that of objectives of the work and find out the gap. Some important works relevant to the present research work are reviewed and briefly cited.
Shaikh J. Uddin *et al.,* (2008) have discussed cytotoxic effects of Bangladeshi Medicinal plant extracts.

Mohammad shaphiullah *et al*., (2003) have stated that antidiarrhoeal activity of the methanol extract of *Ludwing hyssopifolla* Linn.

Md. Mynol Islam Vhuiyan *et al.,* (2008) have reported that anti-diarrhoeal and CNS Depressant activity of methanolic extract of *Saccharum spontaneum* Linn.

Hemayet Hossain *et al.,* (2012) have studied evaluation of analgesic, antidiarrhoeal and cytotoxic activities of ethanolic extract of *Bacopa monnieri* (L)

Pradeep Parihar *et al.,* (2006) have reported antibacterial activity of extracts of *Pinus roxburghii* sarg.

Rahman *et al.,* (2012) have discussed antibacterial activity of natural spices on multiple drug resistant *E, coil* isolated from drinking water, Bangladesh.

MTH Molla *et al.,* (2010) have studied antibacterial activity in the leaves of seven bitter medicinal plants of Bangladesh.

Md. Afjalus Siraj *et al.,* have reported effect of antidiarrhoeal, analgesic and antibacterial activity of ethanolic extract of *Ecbolium linneanum* (Acanthacea) Leaves.

Md. Aliar Rahman and Md. Safiul Islam have studied antioxidant, antibacterial and cytotoxic effects of the phytochemicals of whole *Leucas aspera* extract.

Ray A. *et al.,* (2011) have reported phytochemical screening, cytotoxicity and antibacterial activities of two Bangladeshi medicinal plants.

A.Akter *et al.,* (2010) have reported screening of elhanol, petroleum ether and chloroform extracts of medicinal plants, *Lawsonia inermis* L. and *Mimosa pudina* L. for antibacterial activity.

Mahmood Ayesha *et al.,* (2011) have observed cytotoxicty and antibacterial activity of *Andrographis peniculta, Euphorbia hirta* and *Urginia indica.*

M. Mashiar Rhaman *et al.,* (2009) tested antibacterial activity of leaf juice and Extracts of *Moringa oleifera* Lam. against some human pathogenic bacteria.

M. A. Rahman *et al.,* (2009) tested antioxidant, antibacterial and cytotioxic activity of the methanol extract of *Urtica crenulata*

M.M. Hossaim *et al.,* (2011) studied on the antidiarrhoeal, antimicrobial and cytotoxic activities of ethanol extracted leaves of yellow oleander (*Thevetia peruviana)*

Md. Amran Howlader *et al.,* (2011) tested anitimicrobial, antioxidant and cytotoxic effects of methanolic extracts of leaves and stems of *Glycosmis pentophylla* (Rete).

M.A. Hossain *et al.,* (2010) have reported antibacterial properties of essential oils and methanol extracts of sweet basil *Ocimum basilicum* occuring in Bangladesh.

M. Abeer *et al.,* (2007) tested antimicrobial activity of *Acacia nilotica* extracts against some bacteria isolated from clinical specimens.

Hatil Hashim *et al.,* (2009) observed Evaluation of antibacterial activity of some medicinal plants used in sudanese traditional medicine for treatment of wound infections.

Kurhekar Jaya Vikans and Bodhankar M.G. (2013) tested in vitro antibacterial activity of few medicinal plants against *E. coli.*

Saba Riaz *et al.,* (2011) researched on antibacterial and cytotoxic activities of *Acacia nilotica* Lam (Mimosaceae) methanol extracts against extended spectrum BetaLactamase producing *E. coil and Klebsiella* species.

Jayshere D. Patel *et al*., (2008) tested screening of plant extracts used in traditional antidiarrhoeal medicines aginst pathogenic *E. coli.*

Hossain Md. Lokman *et al*., (2013) have discussed that phytochemical screening and the evaluation of the antioxidant, total phenolic content and analgesic properties of the plant *Pandanus foetidus*.

Deshpande S.N. (2013) has reported that preliminary phytochemical analysis and *in vitro* investigation of antibacterial activity of *Acacia nilotica* against clinical isolates.

Sapna Malviya *et al.,* (2011) have pointed out that medicinal attributes of *Acacia nilotica* Linn-A comprehensive review on ethnopharmacological claims.

Satish A. Bhalerao and Tushar S. Kelkar (2012) have focused on *Acacia nilotica* Linn: A comprehensive review on morphology, ethnobotany, phytochemistry and pharmacological evaluation.

Kavitha p.A. *et al.,* (2013) have stated that methanolic extract of *Acacia nilotica* and antibacterial activity against hospital isolates of Bengaluru district.

Md. Sariful Islam Howlader *et al*., (2012) have investigated that evaluation of antinociceptive, antimicrobial and cytotoxic activities of ethanolic extract of *Acacia nilotica* leaves from Bangladesh.

This is a result of vast research but compound isolation is absent here.

M.P. Raghavendra *et al.,* (2006) have pointed out that *In vitro* evaluation of antibacterial spectrum and phytochemical analysis of *Acacia nilotica*.

G.O.Solomon-Wisdom and G.A. Shittu (2010) have identified that *In vitro* antimicrobial and phytochemical activities of *Acacia nilotica* leavf extract.

Sarkiyayi S and Abdul Rasheed K (2013) have investigated that properties of *Acacia nilotica* leaf extract: A preliminary investigation on anti typhoid.

Sarangi Rashmi Ranjan *et al*., (2011) have discussed that evaluation of antidiarrhoeal activity of *Sida rhombifolia* Linn. root.

Julius E. Oben *et al*., (2006) have investigated that effect of *Eremomastax speciosa* on experimental diarrhoea.

Muniappan Ayyanar (2012) have explained that Indian medicinal plants as a source of therapeutic agents: A review.

Sunday F *et al*., (2009) have discussed that *Azadirachta indica*(neem): a plant of multiple biological and pharmacological activities.

Imam Hashmat *et al.,* (2012) have pointed out that neem (*Azadirachta indica* A. Juss)- A nature's drugstore: an overview.

M.U.Z.N. *et al*., (2014) have identified that a review of ethnomedicine, phytochemical and pharmacological activities of *Acacia nilotica* (Linn) wild.

Kiran Bargali and S.S.Bargali (2009) have focused that *Acacia nilotica*: a multipurpose leguminous plant.

Raha Saud Orfali (2005) have explained that phytochemical and biological study of *Tanarix nilotica* growing in Saudi Arabia.

Mohammad Mahmudul Hasan *et al.,* (2011) have focused that anti-diarrhoeal, anti-microbial and cytotoxic effect of ethanol extracted guava (*Psidium guajava*) leaves.

M. Ansaruzzaman *et al.,* (2007) have discussed that characterization of enterotoxigenic *Escherichia coli* from diarrhoeal patients in Bangladesh using phenotyping and genetic profiling.

Chapter Two:

Experiment, results and discussion

Materials and method

2.1 Selection of plant materials:

In order to arrive at useful compounds in the shortest possible time, careful selection of plant material is obviously very important. Random collection is one method but it is more judicious to base the selection on certain criteria. By way of illustration, plants used in traditional medicine are more likely to provide pharmacologically active compounds. Similarly, folk use of toxic plants could be taken with desirable output.

In this investigation leaves parts *of Acacia nilotica, Azadirachta indica & Psidium guajava* have been collected for the presence of toxic, as well as, bio-active constituents since the plant is well known as a medicinal plant and also considered to contain toxic constituents. In case of very small plants, such as herbs, shurbs, grass, etc. normally the whole plant is subjected for extraction, because the distribution of constituents generally not vary too much. Being a large timber plant, the distribution of compounds in different parts of this plant is obviously different. The presence of constituents in the heartwood may disappear in the leaves; similarly constituents in the roots may not be the same that present there in the fruits.

2.2 Collection of plants

The mature fresh, disease free *Acacia nilotica*, *Azadirachta indica* & *Psidium guajava* leaves sample were collected from Rajshahi University campus. The collected samples were carefully stored in sterile polythene bags without lightening and used for present study.

2.3 Preparation of leaves extract

Fresh leaves parts of the plant materials were washed under running tap water and air dried for about one week and then they were homogenized to fine powder and were

stored in airtight bottle. The powdered of leaf material (100 gm) was extracted with 100 ml ethanol ,methanol, chloroform, petroleum ether and ethyl acetate using conical flask in a shaking incubator at 28°C for two days. The extracts were filtered and evaporated until dryness. The extracts were stored at 4°C until further use.

2.4 Flow chart of extracts

FLOW CHART

Chemical extract

Plant leaves

↓

Air dry for 7 days

↓

Grinding & Cutting

↓

Dissolve in Methanal/ ethanol/chloroform/ethyl acetate/petroleum ether

↓

Filtration

↓

Rotary evaporation

↓

Botanical extracts

↓

For further use

2.5 Antibacterial assay

2.5.1 Introduction

There are four worldwide accepted methods for the antibacterial assay: Bioautographic method; Serial dilution method; Disc diffusion method; and Streaking methods. Among them the disc diffusion method using different concentrations of the agents absorbed on sterile filter paper discs is widely acceptable in investigation for preliminary screening of compound. So in my work the antibacterial activity of the compounds were studied by using the "Disc diffusion technique".

2.5.2 Materials and methods

2.5.2.1 Test organisms used for the study

The test organisms will obviously depend greatly on the purpose of the investigation. The pure cultures were collected from the Institute of Biological Science, Department of Pharmacy. University of Rajshahi, and Environmental Microbiology Laboratory, ICDDRB, Mahakhali, Dhaka, Bangladesh. The following pathogenic bacteria were used for the study of antibacterial activity.

List of the test pathogenic bacteria

Sl. No.	Name of the test organisms
1.	*Escherichia coli*
2.	*Shigella boydii*
3.	*S. dysenteriae*
4.	*S.flexneri*
5.	*S. shiga*
6.	*S. sonnei*
7.	*Vibrio cholerae*

2.5.2.2 Conditions necessary for growth of pathogen

For optimum growth of bacteria we must have the required nutrients, the permissive temperature, enough moisture in the medium, the proper gaseous atmosphere, proper salt concentration, appropriate P^H and there must be no growth-inhibiting factor.

2.5.2.3 Apparatus, reagents & test materials used for the antibacterial assay

Apparatus

1. Blank sterilized filter paper discs (diameter 5 mm)
2. Petridishes (diameter 120 mm)
3. Test tubes
4. Inoculating loop
5. Bunsen burner
6. Sterile forceps
7. Sterile cotton
8. Laminar air flow unit (Biocraft & Scientific Industries, India)
9. Micropipette (10 µl-100 µl)
10. Autoclave (ALP co. Ltd. KT-30L, Japan)
11. Incubator (OSK-9639A, Japan)
12. Refrigerator (Ariston, Italy)
13. Punch machine
14. P^H meter
15. Beaker
16. Vial

Solvent and Reagents

1. Petroleum Ether
2. $CHCl_3$
3. CH_3OH
4. C_2H_5OH
5. Ethyl Acetate

Test materials

1. Petroleum Ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl Acetate extracts of leaf of *A. indica, A. nilotica* and *P. guajava*
2. Standard antibiotics (Ciprofloxacin)
3. Nutrient agar medium
4. Broth culture medium.

2.5.2.4 Preparation of fresh culture of the pathogenic bacteria

The nutrient agar medium was used to conduct experiments in this investigation and its compositions were used followed by Pramanik (2007).

Table 4. Composition of nutrient agar media used for the investigation

Ingredients	Quantity
Bactopeptone	0.5 gm
Sodium chloride	0.5 gm
Bactoyeast extract	1.0 gm
Bactoagar	2.0 gm
Distilled water	100 ml
P^H (controlled)	7.2±0.1 at 25ºC

The instant nutrient agar medium was weighted and then reconstituted with distilled water in a conical flask according to specification measurement (2.3% W/V). It was then heated in a water bath to dissolve the agar until a transparent solution was obtained. Prepared nutrient agar medium was dispersed in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in an autoclave at 121ºC and 15 Ibs/sq. inch pressure for 15 minutes. After sterilization, the test tubes were kept in an inclined position for solidification. These were then incubated at 37.5ºC to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. Burning the loop after each transfer of microorganism was done to avoid contamination very carefully. The inoculated slants were then incubated at 37.5°C for 24h to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

2.5.2.5 Sterilization procedures

The antibacterial screening was carried out in a laminar air flow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petridishes and other glass wares were sterilized in the autoclave at 121°C temperatures and a pressure of 15 Ib/sq. inch for 15 minutes. Micropipette tips, culture media, cotton, forceps, blank discs etc were also sterilized.

2.5.2.6 Principle of disc diffusion method

Solution of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the sample in definite volume of solvents. Dried and sterilized filter paper discs (5 mm diameter) soaked with known amount of test agents were placed on the nutrient agar media solidified in Petridishes (120 mm diameter) and inoculated with test organisms. These plates were then kept at low temperature (4ºC) for 24h to allow maximum diffusion. During this time the following events took place simultaneously.

- The dried discs absorbed water from the surrounding agar medium and the test samples/ materials are dissolved.
- The test material diffuses from the discs to the surrounding medium. The diffusion took place according to the physical law that controlled the diffusion of molecules through agar gel. As a result, there was a gradual change of test materials concentration in the agar media surrounding each disc.
- The plates were then kept in an incubator at 37.5ºC for 24h to allow maximum growth of the organisms. If the test materials would have any antibacterial activity, they would inhibit the growth of microorganism and moderate zones of inhibition would be observed surrounding the discs.

2.5.2.7 Characteristics of the ingredients used in media

Table 5. Characteristics of the ingredients used in media

Raw materials	Characteristics	Nutritional value
Peptone	The product resulting from the digestion of proteinaceous materials, e.g.; meat, casein and gelatin, digestion of the proteins materials is accomplished with acids or enzymes, many different peptones (depending upon the protein used and the method of digestion) are available for the use in bacteriological media, and peptones differ in their ability to support growth of bacteria.	Principal source of organic nitrogen may also contain some vitamins and sometimes carbohydrates depending upon the kind of proteinaceous materials digested.
Agar	A complex carbohydrate obtained from certain marine algae: Processed to remove extraneous substances.	Used as a solidification agent for media, agar dissolved in aqueous solutions, gels when the temperature is reduced below 45ºC: Agar is not considered as a source of nutrient to the bacteria.
Yeast extract	An aqueous extract of yeast cells, commercially available as powder.	A very rich source of the vitamin B complex; also contains organic nitrogen and carbon compounds.

2.5.2.8 Subculture preparation

The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in aseptic condition. Special care was taken to avoid contamination of microorganism. The inoculated slants were then incubated at 37.5ºC for 24h to assure the growth of test organisms.

2.5.2.9 Preparation of test sample

About 20 mg of petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetate extracts of leaf of *A. indica, A. nilotica* and *P. guajava* were dissolved in 1ml $CHCI_3$ in separate glass vials. Thus the concentrations were 20 µg/µl for each extracts.

2.5.2.10 Preparation of the test plates

The test plates were prepared according to the following procedure:

1) About 10 ml in quantity of distilled water was poured in some cleaned test tubes and plugged with cotton.
2) The test tubes, a number of petridishes, glass rods, a piece of cotton and the medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.
3) About 6 ml of the medium was poured carefully in medium sized petridishes. The petridishes were rotated several times, first clockwise and then anticlockwise to assure homogenous thickness of the medium and allowed to cool and solidify at about 30ºC.
4) The test tubes containing distilled water were inoculated with fresh culture of the test bacteria and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process.
5) A piece of cotton was immerged in the test tubes with the help of individual glass rod and then gently rubbed the medium and the cotton was discarded.
6) Finally, the plates were stored in a refrigerator (4ºC) for overnight.

2.5.2.11 Preparation of the discs treated with the test sample

For the preparation of the discs containing the extracts the following procedures were utilized. Three types of discs were prepared for antibacterial screening. These are as follows:

2.5.2.11.1 Sample discs: Prepared by the selected *A. indica, A. nilotica,* & *P. guajava* leaves extracts using sterilized filter paper discs having 5 mm in diameter (BBL, Cocksville, USA). Sterilized filter paper discs (5 mm in diameter) were taken by the forceps in the plates. Sample solutions of desired concentrations were 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 the solvent.

2.5.2.11.2 Standard discs: These are used to compare the antibacterial activity of the test materials. In the present study, disc containing (10 µg/disc) of the antibiotic

Ciprofloxacin were used as standard discs for the comparison with the extract treated ones.

2.5.2.11.3 Control/blank discs:

These were used as negative controls to ensure that the residual solvents on the filter paper were not active themselves. These were prepared in the previous manner applying only solvent to the discs and were used to examine the effect of the solvents used. For the preparation of discs containing samples, following procedure was utilized.

2.5.2.12 Principle of agar discs diffusion method:

In the discs diffusion assay, the surface of a nutrient agar medium contained in a petri dish was uniformly inoculated with the test bacterial culture. Test sample solution was applied on filter paper disc with the help of a micropipette and dried in room temperature. The filter paper discs were then placed on each of the petridishes previously inoculated.

2.5.2.13 Procedure to determine antibacterial activity:

Antibacterial activity was determined keeping the petridishes in room temperature for 24h. This method was developed by Bondi and standardized by Bauer *et al*. in 1996 for susceptibility test.

2.5.2.14 Placement of the discs for incubation

The following procedure was adopted for the placement of the discs:

1) The dried crude extract containing discs and the standard discs were placed gently on the solidified agar plates seeded with the test organisms with the help of a pair of sterile forceps.
2) Then the plates were kept in a refrigerator at 4ºC for 24h in order to provide sufficient time to diffuse the antibiotics into the medium.
3) Finally, the plates were incubated at 37.5ºC for 24h in an incubator.

2.5.2.15 Precaution

The discs were placed at least 20 mm apart from each other and 15 mm far from the edge of the plate to prevent overlapping the zones of inhibition.

2.5.2.16 Screening through *in vitro* antibacterial activity test

Ethanol, methanol, chloroform, petroleum ether and ethyl acetate extracts were used in this investigation for the detection of antibacterial potentials. The extracts were dissolved in their solvent of extraction and all the extracts were subjected to screen against bacteria. In this antibacterial screening the extracts were used at concentration of 100 µg/disc and a control experiment was set along with ciprofloxacin (15 µg/ml). Considering the size of the clear zone in millimeter (mm) the efficacy of the extracts against the test bacteria was recorded. Paper disc treated with 100 µg of these samples were used to detect biological activity of the extracts.

2.5.2.17 Measurement of the zones of inhibition

After placement of the disc on the petridishes the plates were kept in an incubator (37.5°C) for 24h to allow the growth of the organisms. Developing the test material having antibacterial activity inhibited the growth of microorganisms developing a clear distinct zone called 'Zone of Inhibition'. Effects of the extracts on bacterial growth were quantified by measuring the diameter of the zones of inhibition in term of mm with a transparent scale. The antibacterial activity of the extracts were isolated through measuring the zones of inhibition on petridish comparing with the standard antibiotic (Ciprofloxacin 15 µg/disc) disc.

2.6 Determination of Minimum Inhibitory Concentration (MIC) of the extract

2.6.1 Introduction

Minimum inhibitory concentration (MIC) may be defined as the lowest concentration of antimicrobial drug to inhibit the growth of the pathogenic organism. The data derived from the test tube can be corrected with the knowledge of expected or measured extract level *in vivo* to predict the efficacy of extract. There are two methods for determining the MIC. They are as follows:

1) Serial tube dilution technique or turbidimetric assay.
2) Paper disc plate technique or agar diffusion assay.

Here, serial tube dilution technique was followed using nutrient broth medium to determine the MIC value of extract against the pathogenic bacteria.

2.6.2 Principle of serial tube dilution technique

The tubes of broth medium containing graded doses of extracts are inoculated with the test organisms. After suitable incubation, growth will occur in those inhibitory tubes where the concentration of extract is below the inhibitory level and the culture will become turbid (cloudy). Therefore, growth will not occur above the inhibitory level and the tube will remain clear though the large number of microorganisms present in the tubes.

2.6.3 Principle of inoculums

Fresh cultures of the test organisms were grown at 37.5°C for overnight on nutrient agar medium. Bacterial suspensions were then prepared in sterile nutrient broth medium in such a manner so that the suspension contains 10^6 cells/ml. This suspension was used as inoculums.

2.6.4 Principle of sample solution

The stock solution was prepared by dissolving 2.048 mg of antibiotic in 2 ml of methanol. Thus solutions with a concentration of 1.024 mg/ml were obtained.

2.6.5 Procedure of serial tube dilution technique

1. Twelve test tubes were taken, nine of which were marked as 1, 2, 3, 4, 5, 6, 7, 8, 9 and rest three were assigned as Cm (Medium), Cs (Medium+ Compound) and C1 (Medium+ Inoculums).
2. 1 ml of nutrient broth medium was added to each of the 12 test tubes.
3. These test tubes were cotton plugged and sterilized in an autoclave at 121°C temperature and 15 Ib/sq inch pressure.
4. After cooling, 1 ml of the sample solution was added to the 1st test tube, mixed well and then 1 ml of this content was transferred to the 2nd test tube.
5. The content of the 2nd test tube was mixed well and again 1 ml of this mixture was transferred to 3rd test tube. This process of serial dilution was continued up to the 9th test tube.
6. 10 µl of properly diluted inoculums was added to each of the nine test tubes and mixed well.

7. To the control test tube, 1 ml of the sample solution was added; mixed well and 1 ml of this mixed content was discarded. This is to check the clearity of the medium in presence of diluted solution of the compound.
8. 10 µl of the inoculums was added to the control test tube C1, to observe the growth of the organism in the medium used.
9. The control test tube Cm, containing medium only was used to confirm the sterility of the medium.
10. All the test tubes were incubated at 37.5°C for 24h.

The minimum inhibitory concentration (MIC) was determined against *E. coli, S. boydii, S. dysenteriae, S. flexneri, S. shiga, S. sonnei & V. cholera*

The MIC is the lowest concentration at which there is no growth of the organism.

2.7 Cytotoxicity assay study

2.7.1 Principle

The brine shrimp (*Artemia salina*) assay was proposed by A.S. Michael *et al.,* (1956) and later developed by P. Vanhaecke *et al.,* (1981); Sleet and Brendel, (1983). The brine shrimp assay is very useful tool for the isolation of bioactive compounds from plant extracts (T.W Sam, 1993). The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the microwell scale. In the present work, we report the cytotoxicity studies on various solvent extracts of *A. indica, A. nilotica* and *P. guajava* leaves and evaluating the selected plant's efficiency and safety in light of being used in modern science.

2.7.2 Brine shrimp (*A. salina*) nauplii

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity, as well as, a wide range of pharmacological activities (e.g. anticancer, antiviral, pesticidal, anti-AIDS, etc.) of the

Systemic position:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Branchiopoda
Order: Anostraca
Family: Artemiidae
Genus: *Artemia*
Species: *A. salina* L. (1758)

Fig. 1: *A.salina* (Brine shrimp) nauplius

bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method.

Here *in vivo* lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening a fractionation in the discovery of new bioactive natural products. Generally, the median effective dose (ED_{50}) values for cytotoxicity are one tenth (1/10) of median lethal dose (LD_{50}) values in the brine shrimp test. The *A.salina* belongs to a genus of very primordial crustacean (crawfish - crayfish) the *Anostraca* (Fairy shrimp). Crawfish of this genus just have a divided exoskeleton made of chitin enhanced protein, no usual crust of chitin (escutcheon) as other crawfish have. There are many species within the genus of *Anostraca*, but the *Artemia salina* is very nice to grow, since the rate of successful hatches is very high. To carry on toxicity tests of certain materials these nauplii are very easy to grow from its marketed cysts and to set experiments thereby.

2.7.3 Culture of *A. salina*

In the laboratory condition *A. salina* are very nice to grow, since the rate of successful hatches is very high. To conduct cytotoxicity test the brine shrimp nauplii were used because of its easy hatching and use in the experiment. The cysts were collected from aquarium shops. For their easy hatching and use the requirements were as follows:

- Salt water: 1.5 - 3 tablespoons of marine salt every liter of water;
- Temperature: 26-28°C (80-82°F);
- Light: The beaker was placed near a window with sunlight before hatching;
- Aeration: Picking up some water carefully with a spoon and let it drop back into the beaker once a day (but a small aquarium pump with a little air-stone is better).
- Helpful Hint: Brine shrimp egg is sometimes very buoyant. Swirl the water to knock down eggs;

The cysts absorb water and if the sun is shining (a signal for growing algae and other plankton) they hatch after 24 to 48h, depending on their environment. Freshly hatched *A. salina* called nauplii and have a size of just 0.25 mm (0.01 inch). They molt like any other crawfish when they grow to adult they molt about 17 times. If the breeding temperature is about 26-28°C (80-82°F), a nauplius hatches within 24-48h, gets pubescent in 8-14 days and lives- depending on the concentration of salt-up to 4-5

weeks. The more salt, the less the life expectancy. Freshly hatched nauplii were used in this experiment.

2.7.4 Experimental design for the lethality test

Brine shrimp cysts were hatched in simulated seawater to get nauplii. Test samples were prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii were counted by visual inspection and were taken in test-tubes containing 5 ml of simulated seawater. Then samples of different concentrations were added to the premarked test-tubes through pipette. The test-tubes were left for 24h and then the nauplii were counted again to find out the cytotoxicity of the test agents and compared to the results with positive control.

2.7.5 Test materials:

- Brine Shrimp (*A. salina*) cysts
- Iodine-free salt
- Small tank/ beaker to hatch the shrimp
- Lamp to attract the nauplii
- Pasteur pipette (1 ml and 5 ml)
- Micropipette (10-200 µl adjustable)
- Test tubes (20 ml)
- Magnifying glass

2.7.6 Preparation of simulated seawater (brine water)

Since the lethality test involves the culture of brine shrimp nauplii that is, the nauplii should be grown in the seawater. Seawater contains 3.8% of NaCl. Accordingly, 3.8% sodium chloride solution was made by dissolving sodium chloride (38 g) in normal pond water (1000 ml) and was filtered off. The P^H of the brine water thus prepared was maintained between 8 and 9 $NaHCO_3$.

2.7.7 Hatching of brine shrimp nauplii

Brine water was taken in a small tank and *A. salina* cysts (1.5 g/L) were added to one side of the perforated divided tank with constant oxygen supply. Constant temperature

(37˚C) and sufficient light were maintained to give the sufficient aeration. After 24h, matured shrimp as nauplii was collected and used for the experiment.

2.7.8 Experimentation of lethality test

Petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetate extracts of leaves of *A. indica*, *A. nilotica* and *P. guajava* were applied against brine shrimp nauplii. For each samples, an adhoc test was done before final experimentation. 2 mg extract sample was weighted and taken in a small glass vial, then 1-2 drops of pure Dimethyl sulfoxide (DMSO) added to dissolve initially. 1 ml of pond water was taken into the vial to mix up the sample extract with water to prepare dose. When it mixed up completely added to the test-tube (10 ml marked) for conducting tests. This process was also maintained during final experiment. Separate vials were taken for each dose.

2.7.9 Dose selected for the final experiments

Petroleum ether extracts of *A.nilotica* leaves: 800,600,400,200,100ppm

$CHCI_3$ extracts of *A.nilotica* leaves: 800,600,400,200,100ppm

CH_3OH. extracts of *A.nilotica* leaves: 800,600,400,200,100ppm

C_2H_5OH. extracts of *A.nilotica* leaves: 800,600,400,200,100ppm

Ethyl acetate extracts of *A.nilotica* leaves: 800,600,400,200,100ppm

Petroleum ether extracts of *A.indica* leaves: 800,600,400,200,100ppm

$CHCI_3$extracts of *A.indica* leaves: 800,600,400,200,100ppm

CH_3OH. extracts of *A.indica* leaves: 800,600,400,200,100ppm

C_2H_5OH extracts of *A.indica* leaves: 800,600,400,200,100ppm

Ethyl acetate extracts of *A.indica* leaves: 800,600,400,200,100ppm

Petroleum ether extracts of *P.guajava* leaves: 800,600,400,200,100ppm

$CHCI_3$ extracts of *P.guajava* leaves: 800,600,400,200,100ppm

CH_3OHextracts of *P.guajava* leaves: 800,600,400,200,100ppm

C_2H_5OH extracts of *P.guajava* leaves: 800,600,400,200,100ppm

Ethyl acetate extracts of *P.guajava* leaves: 800,600,400,200,100ppm

2.7.10 Analysis of data

The mortality records of the brine shrimp lethality experiments done against *A.salina* nauplii were corrected by the Abbott's (1925) formula:

$$Pr = \frac{P_0 - P_c}{100 - P_c} \times 100$$

Where,

Pr= Corrected mortality (%)

P_0= Observed mortality (%)

P_c= Control mortality (%), sometimes called natural mortality(%).

Then mortality percentages were subjected to statistical analysis according to Finney (1947) and Busvine (1971) by using 'computer software'. The dose-mortality relationship was expressed as a median lethal concentration (LC_{50}).

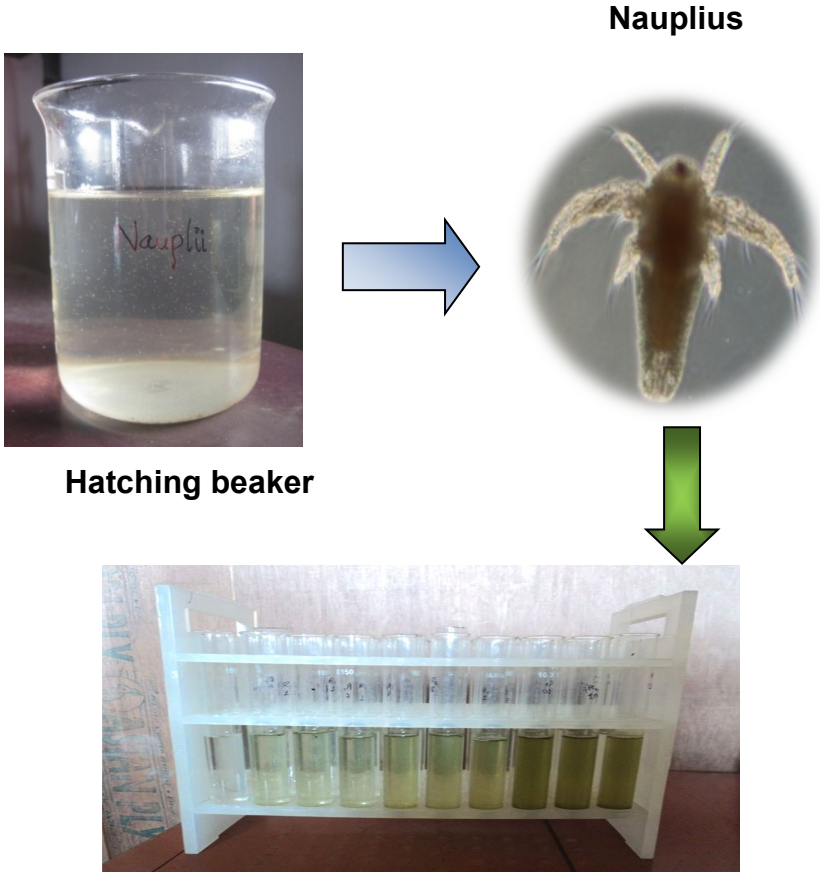


Fig. 2: Bioassay with plant extracts on *A. salina* nauplii by brine shrimp lethality test

2.8 Compound isolation

2.8.1 Introduction

Plants are important source in the field of natural medicine. The ethanolic extract of *Acacia nilotica* leaves were showed potent activity than other extracts. Biological activities consideration is one of the major ideas for the determination of pure compound. Accordingly, an attempt was taken to isolate, purify and characterize the bioactive compounds there in the ethanolic extract of *Acacia nilotica* leaves.

2.8.2 Materials and methods

In this study for isolation, purification and characterization of bioactive constituents some well established techniques have been followed to achieve the research goal. The research approach adopted to obtain an exploitable pure plant constituent involves interdisciplinary work in different research laboratories such as environmental microbiology laboratory at the Institute of Environmental Science, Crops Protection laboratory, Department of Zoology, University of Rajshahi, a number of bacteria were selected to carry out further efficiency tests of the extractives and following basic equipments were used in purpose of sample preparation for the bioactive compound determination. For separation of pure bioactive compounds open column chromatographic methods were applied. The most important preparative separation techniques employed in the isolation and purification of plant constituents, thin layer chromatography (TLC) was used to select the slurry or the solvent system for the successful run of the open column chromatography. Aluminum pre-coated preparative Thin layer chromatographic (TLC) plates (20x20) cm with silica gel GF_{254} with 0.5 mm thickness and active in the usual manner (Merck, Germany) were used in this purpose. Spray reagent was used to detect compound spots following Godin (1954).

2.8.3 Apparatus and reagents

Basic materials and equipment used for TLC separation procedure are presenting in Table

Table 6. Material, equipment and chemicals for the TLC Method.

TLC silica gel plates/ pre-coated TLC (20x20 cm) aluminium oxide- Merck, Germany) and (20x20 cm) cellulose plate.	Sample and standard vials test tubes and rack
TLC Development chamber for 20x20 cm plates.	Measuring cylinder of different size
Chromatography chambers for TLC	Hand gloves
Capillary tubes for TLC spoting	Safety goggles
TLC plate holder 20x20 cm	Oven
TLC guide spotting plate	UV-Lamp
Foil, ruler	M HCl
Tweezers	Solvent (chloroform, methanol, ethanol, ethyl acetate and petroleum ether)
Hair dryer	1% solution of sample (prepared from the ethanolic extract of *A.nilotica* leaves).
Seissors	Spray reagent.
Bera pipets	

NB. Safety precaution was assured on wearing an apron and goggles in the laboratory and washing hand after the completion of the experimental work every time.

2.8.4 Purification through open column chromatography (OCC)

Column chromatography method was applied for the separation of the extract. The stationary phase for the open column chromatography was silica gel Si60 (230-400 mesh) (Merck) and glass column of different sizes (32x2.5 cm, 25x2 cm, 25x1.5 cm, *etc*). Cotton pads washed with acetone, chloroform and methanol were used at the bed of the gel column. A similar cotton cloud was used at the top of the column (after application of the sample and the solvent) to protect destruction of the sample layer of the column. Selected solvent systems were used as eluents and the elution rate was 1ml/min. Glass made cylindrical columns were made by pouring down the slurry of the silica gel (230-400 mesh) in the suitable solvent and allowing the silica gel to settle down. The pouring of slurry, previously selected by thin layer chromatography was continued until the column of desire height was obtained. The solvent layer

always kept above the absorbent bed to avoid cracking of the column. At the end of preparation of the column a little amount of the slurry kept on the upper surface of the gel matrix for the conveniences in application of the extract in dissolved state.

2.8.4.1 Gel filtration

Sephadex LH-20 (Pharmacia) gel was used in the purpose of gel filtration through the open column chromatography method. The separation of the ethanolic extracts by using CH_3OH and a little amount of $CHCI_3$ (not more than 50%). The eluent collected by following about 0.5 ml/min.

2.8.4.2 Targeted compounds for isolation

In this study, isolation of the pure compounds from ethanol extracts of *A.nilotica* leaves were done mainly by open column chromatography (OCC), while thin layer chromatography (TLC) was used as a supporting tool. The selection of the test extracts for the isolation was done depending on their biological activities.

2.8.4.3 Detection of the purified compound on TLC by Godin revelation

2.8.4.3.1 Visual detection: The developed chromatogram was examined visually to detect the presence of coloured compound(s).

2.8.4.3.2 UV light (254 and 366nm): After development and drying the chromatogram was examined under UV light to detect fluorescent compound and the glowing spots, which were then marked.

2.8.4.3.3 Godin reagent spray: Equal volume of 1% ethanolic solution of vanillin and 3% aqueous solution of perchloric acid was mixed and sprayed on to the prepared chromatogram and 10% ethanolic solution of H_2SO_4 was also sprayed afterwards and allowed the plate to dry out at 100°C by using a hair dryer. Revelation was observed in different colours for different compounds according to Godin (1954)

2.8.4.3.4 Measurement of R_f values: The R_f values of the separated

compounds were calculated on a developed chromatogram using the pre-established solvent system.

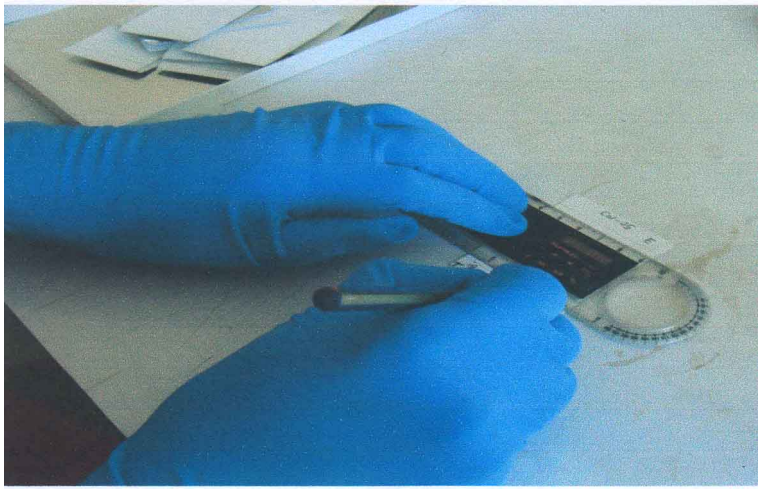


Fig. 3: Measurement of Rf values on TLC plate by a transparent scale

The samples was applied on the TLC plates with the help of a gradient micropipette as a narrow band at 1 cm above the lower edge of the plate to make sure that the sample was not washed away when the plates were placed inside the TLC chamber with the solvent system. The plates were then developed in the usual manner. According to Pramanik (2007) a concentration of 10 mg/ml of the sample in the solvent of extraction offered 100 µl for each of the samples spotted. The chromatograms then developed within a conventional chamber (*Camag*) using the following solvent systems for column chromatographic separation.

2.8.5 Isolation of the compounds from extract:

For the identification and separation of bioactive compound from the ethanol extract of *A.nilotica* leaves firstly 1 mg extract used for the fractionation. In this processes Sephadex gel LH_{20} (Pharmacia) was used as the stationary phase and $CHCl_3$: Cyclohexane (10:1) was the eluent on a glass column of 2.5x32 cm for 1 gm extract. Elution time was adjusted to vials 1 ml/min. It gave 82 test tubes, which were then spotted on TLC to run and reveal the compounds by reagent spray. Six fractions were made for 1st column Fr i T(1-10), Fr ii T(11-15), Fr iii T(16-29), Fr iv T(30-41), Fr v T(42-52) and Fr vi T(53-82). Biological assay with bacteria indicated Fr iii for the presence of bioactive components there in and it was then subjected to fractionation.

Selecting a solvent system by TLC, a slurry of $CHCl_3$: Cyclohexane (10:1) was applied on a glass column of 2x25 cm which was packed with silica gel (230-400 mess, 38 gm) Sigma. The element was kept similar to that of the previous one. This fractionation yielded only 12 test tube and TLC was made for all of them to get 5 fractionate, on silica gel by the end of 2nd column in SFr iii T(4) further subdivided in to each two fraction in 43 test tubes and the fractionate were SSFr i T(1-23) and SSFr ii T(24-43). Biological assay of the SSFr ii T (24-43) fraction against the test bacteria indicated for bioactive compound, which was a pure compound named as compound E001.

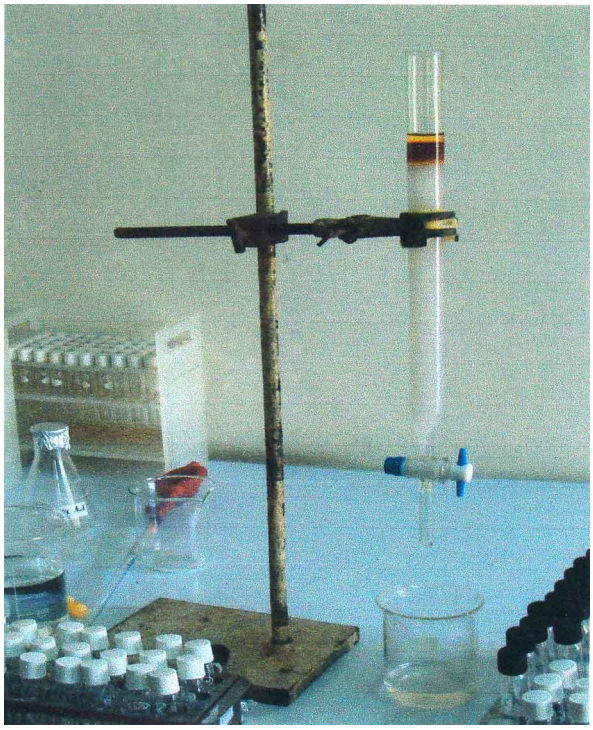


Fig. 4: Collections of slurry solvent for the column separation

2.8.6 Isolation pathway of ethanol extract of *A.nilotica* leaves.

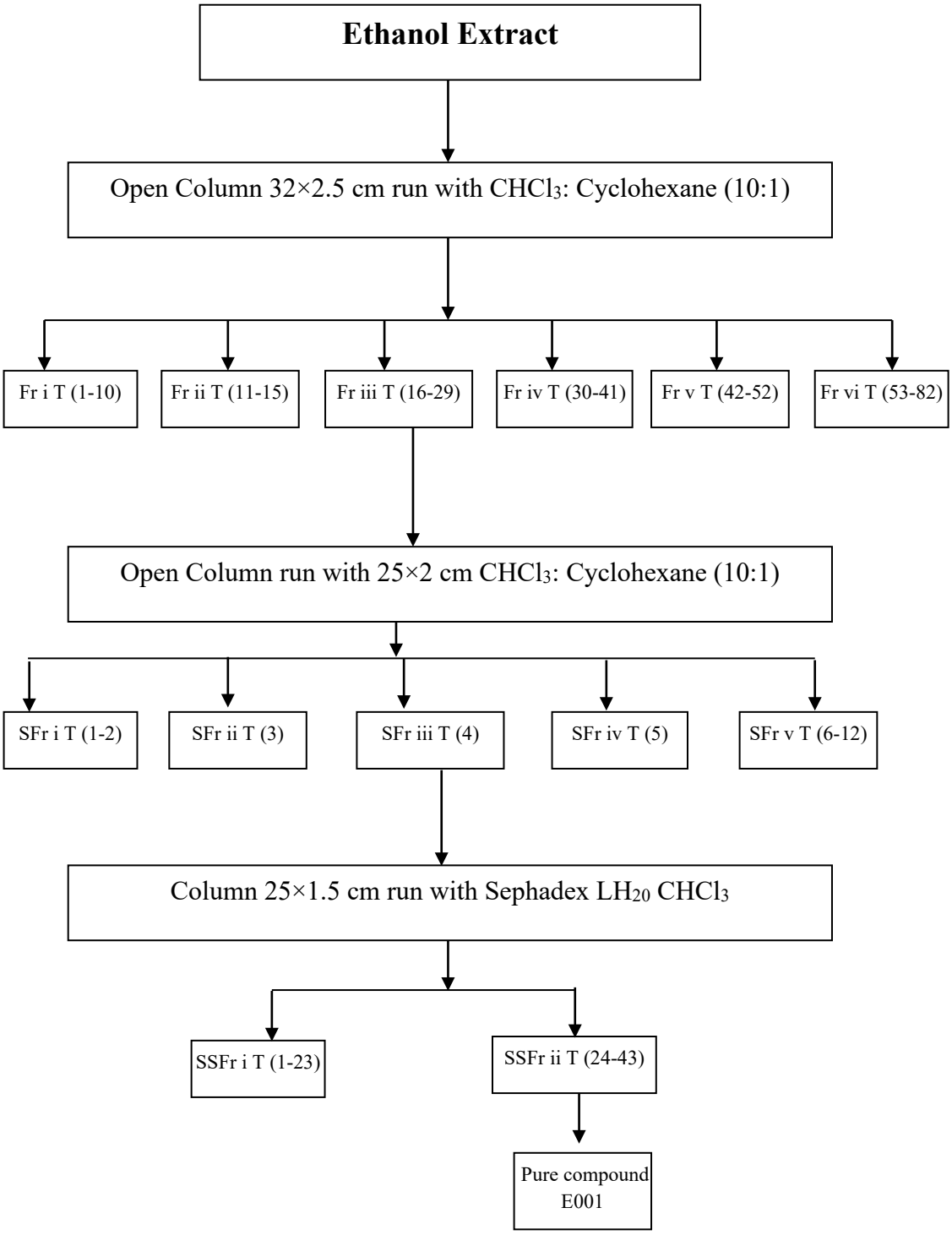


Fig. 5: Isolation pathway of compound E 001

2.9 Antibacterial assay

2.9.1 Results

Antibacterial activity of different solvent extracts of *A.indica, A.nilotica* and *P. guajava* leaves were verified against specific pathogenic bacteria experimentally *in vivo* and the results are presented below:

Table 7. The inhibition zones developed by ethanol, methanol, petroleum ether, ethyl acetate and chloroform extracts (100 µg/disc) of *A.nilotica* leaves and standard Ciprofloxacin disc (10 µg/disc) against test bacteria

Test organisms	Diameter of zone of inhibition(in mm)/ each disc (=6 mm)						
	Ethanol extract	Methanol extract	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Control	Standard Ciprofloxacin
E.coli	10.67	10.00	8.67	8.33	8.33	_	14
S.dysenteriae	9.33	8.67	9.00	8.33	8.00	_	14
S.shiga	9.00	8.33	8.00	8.00	8.33	_	12
S.sonnei	8.33	9.33	8.33	8.00	9.00	_	13
S.boydii	8.67	9.00	8.67	9.67	8.33	_	15
S.flexneri	9.67	8.67	8.33	8.67	8.00	_	13
V.cholerae	10.67	8.33	9.33	8.33	9.33	_	14

Table 8. The inhibition zones developed by ethanol, methanol, petroleum ether, ethyl acetate and chloroform extracts (100 µg/disc) of *A. indica* leaves and standard Ciprofloxacin disc (10 µg/disc) against test bacteria

Test organisms	Diameter of zone of inhibition(in mm)/ each disc (=6 mm)						
	Ethanol extract	Methanol extract	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Control	Standard Ciprofloxacin
E.coli	8.00	8.67	8.67	8.33	9.33	_	15
S.dysenteriae	8.67	9.33	8.00	8.33	8.33	_	14
S.shiga	8.33	8.33	10.00	8.67	8.67	_	14
S.sonnei	9.67	8.00	9.67	8.00	8.00	_	13
S boudii	10.33	8.33	8.67	9.33	9.00	_	15
S.flexneri	8.00	9.67	8.33	8.00	8.67	_	14
V.cholerae	8.67	8.67	9.33	9.67	8.33	_	14

Table 9. The inhibition zones developed by ethanol, methanol, petroleum ether, ethyl acetate and chloroform extracts (100 µg/disc) of *P.guajava* leaves and standard Ciprofloxacin disc (10 µg/disc) against test bacteria

Test organisms	Diameter of zone of inhibition(in mm)/ each disc (=6 mm)						
	Ethanol extract	Methanol extract	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Control	Standard Ciprofloxacin
E.coli	8.33	8.67	8.33	8.00	9.00	_	14
S.dysenteriae	8.33	8.00	8.67	7.67	9.33	_	13
S.shiga	9.33	8.33	9.33	8.00	8.67	_	15
S.sonnei	8.67	9.33	8.67	9.67	8.33	_	14
S.boydii	8.00	8.00	7.67	8.67	8.00	_	15
S.flexneri	9.00	9.00	8.33	8.67	9.00	_	15
V.cholerae	8.67	9.67	8.00	9.00	8.67	_	14

Antibacterial activity of the purified compound E001 from ethanol extract of *A.nilotica* leaves and standard Ciprofloxacin (10 µg/disc)

Table 10. Antibacterial activity of the purified compound E001 (100 µg/disc) and standard Ciprofloxacin (10 µg/disc)

Test organisms	Diameter of zone of inhibition (in mm)/each disc(=6mm)	
	Compound E001 (100 µg/disc	Standard Ciprofloxacin (10 µg/disc)
E.coli	8.00	15
S. dysenteriae	9.67	13
S.shiga	8.33	16
S. sonnei	9.33	14
S. boydii	9.00	15
S. flexneri	8.33	18
V.cholerae	7.67	16

Fig.6: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *S. dysenterae*

Fig.7: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *S. sonnei*

Fig.8: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *S. boydii*

Fig.9: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *S. Flexneri*

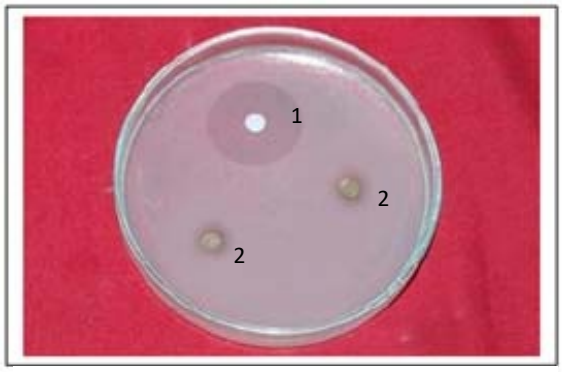


Fig. 10:Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *S. Shiga*

Fig.11: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *E. coli*

Fig.12: Antibacterial test of compound E 001 and Ciprofloxacin (standard) against *V. cholerae*

Key: 1 = Ciprofloxacin, 2 = Compound E 001

2.9.2 Discussion

From the table-7, different extracts (100 µg/disc) of *A.nilotica* leaves represented clear zone of inhibition against bacteria. Among the highest zones of inhibition was 10.67 mm in diameter (in ethanol extract) against *E. coli* & *V. cholerae*. On the other hand methanol, petroleum ether, ethyl acetate. & chloroform extracts showed clear zone of inhibition against bacteria. Among the bacteria the highest zones was 10.00 mm in diameter (in methanol extract) against *E. coli*, 9.33 mm in diameter (in petroleum ether extract) against *V. cholera*, 9.67 mm in diameter (in ethyl acetate extract) against *S. boydii* & 9.33 mm in diameter (in chloroform extract) against *V. cholera*,while the standard antibiotic zones were 14,14, 14,15 and14 mm in diameter respectively. No bacterial growth was observed in control.

From the table-8, different extracts (100 µg/disc) of *A. indica* leaves represented clear zone of inhibition against bacteria. Among the bacteria the highest zone of inhibition was 10.33 mm in diameter (in ethanol extract) against *S. boydii*, 9.67 mm in diameter (in methanol extract) against *S. flexneri*, 10.00 mm in diameter (in petroleum ether. extract) against *S. shiga*, 9.67 mm in diameter (in ethyl acetate extract) against *V. cholera* & 9.33 mm in diameter (in chloroform extract) against *E. coli*, while the standard antibiotic zones were 15, 14,14, 14 and 15 mm in diameter respectively. No protected bacterial growth in control dose.

From the table-9, the ethanol, methanol, petroleum ether, ethyl acetate and chloroform extracts of leaves (100 µg/disc) of *P. guajava* leaves represented clear zones of inhibition against bacteria. The highest zone of inhibition was 9.33 mm in diameter (in ethanol extract) against *S. shiga*, 9.67 mm in diameter (in methanol extract) against *V. cholere*, 9.33 mm in diameter (in petroleum ether extract) against *S. shiga*, 9.67 mm in diameter (in ethyl acetate extract) against *S. sonnei* and 933 mm in diameter (in chloroform extract) against *S. dysenteriae*, while the antibiotic zones were 15, 14, 15, 14 and 12 mm in diameter respectively. No protected bacterial growth in control dose.

From the table 10: Antibacterial activity was tested on the bacteria by the compound E001. The compound E001 was effective against all types of bacteria and the highest zone was measured 9.67 mm in diameter against *S. dysenteriae* while the standard zone of Ciprofloxacin 13 mm in diameter, respectively. Compound E001 was less effective for *V.cholerae* and the minimum zone was measured 7.67 mm in diameter while the standard antibiotic zone of inhibition was 16 mm in diameter, respectively.

2.10 Minimum Inhibitory Concentration (MIC)

2.10.1 Results

Table 11. The MIC of ethanolic extract of *A. nilotica* leaves against bacteria

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	+	-	-	+
5	1	32	10	-	+	-	+	-	+	+
6	1	16	10	+	+	+	+	-	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				32	64	32	128	16	64	128
Notes: + indicates growths, - indicates no growths										

Table 12. The MIC of methanolic extract of *A. nilotica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	+	+	-	-	-
5	1	32	10	+	+	+	+	-	-	+
6	1	16	10	+	+	+	+	+	-	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				64	64	128	128	32	16	64
Notes: + indicates growths, - indicates no growths										

Table 13. The MIC of petroleum ether extract of *A. nilotica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	+	-	-	-	-	-	-
5	1	32	10	+	-	+	-	+	-	+
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				128	32	64	32	64	32	64
Notes: + indicates growths, - indicates no growths										

Table 14. The MIC of chloroform extract of *A. nilotica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	-	-	+	-
5	1	32	10	+	-	+	-	-	+	+
6	1	16	10	+	+	+	-	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				64	32	64	16	32	128	64
Notes: + indicates growths, - indicates no growths										

Table 15. The MIC of ethyl acetate extract of *A. nilotica* leaves against bacteria

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	+	-	-	-	+	-
5	1	32	10	-	+	+	+	-	+	+
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				32	128	64	64	32	128	64
Notes: + indicates growths, - indicates no growths										

Table 16. The MIC of ethanolic extract of *A. indica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	+	-	-	-	-	+	+
5	1	32	10	+	-	-	+	-	+	+
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				128	32	32	64	32	128	128
Notes: + indicates growths, - indicates no growths										

Table 17. The MIC of methanolic extract of *A. indica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	+	-	-	+
5	1	32	10	+	+	-	+	-	+	+
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				64	64	32	128	32	64	128
Notes: + indicates growths, - indicates no growths										

Table 18. The MIC of petroleum ether extract of *A. indica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	+	+	-	-
5	1	32	10	-	-	-	+	+	+	-
6	1	16	10	+	-	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				32	16	32	128	128	64	32
Notes: + indicates growths, - indicates no growths										

Table 19. The MIC of chloroform extract of *A. indica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	+	-	-	+	-	-	-
5	1	32	10	+	+	-	+	+	+	-
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				128	64	32	128	64	64	32
Notes: + indicates growths, - indicates no growths										

Table 20. The MIC of ethyl acetate extract of *A. indica* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	+	-	-	+	-	-
5	1	32	10	+	+	-	-	+	+	-
6	1	16	10	+	+	-	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				64	128	16	32	128	64	32
Notes: + indicates growths, - indicates no growths										

Table 21. The MIC of ethanolic extract of *P. guajava* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	+	-	-	+	-	-	-
5	1	32	10	+	+	-	+	-	+	-
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				128	64	32	128	32	64	32
Notes: + indicates growths, - indicates no growths										

Table 22. The MIC of methanolic extract of *P. guajava* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	+	-	-	+
5	1	32	10	-	-	+	+	-	+	+
6	1	16	10	+	-	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				32	16	64	128	32	64	128
Notes: + indicates growths, - indicates no growths										

Table 23. The MIC of petroleum ether extract of *P. guajava* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	+	-	+	-	-	-
5	1	32	10	-	+	-	+	-	+	-
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				32	128	32	128	32	64	32
Notes: + indicates growths, - indicates no growths										

Table 24. The MIC of chloroform extract of *P. guajava* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	-	+	-	-
5	1	32	10	+	-	-	+	+	+	+
6	1	16	10	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				64	32	32	64	128	64	64
Notes: + indicates growths, - indicates no growths										

Table 25. The MIC of ethyl acetate extract of *P. guajava* leaves against bacteria.

Test tube No.	Nutrient broth medium added (ml)	Diluted solution of extract (µg/ml)	Inoculums added (µl)	Bacteria						
				E. coli	*S. dysenteriae*	*S. shiga*	*S. boydii*	*S. sonnei*	*S. flexneri*	*V. cholerae*
1	1	512	10	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-
4	1	64	10	-	-	-	+	-	-	+
5	1	32	10	-	+	-	+	+	+	+
6	1	16	10	-	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+
Cm	1	0	0	-	-	-	-	-	-	-
Cs	1	512	0	-	-	-	-	-	-	-
C1	1	0	10	+	+	+	+	+	+	+
Results of MIC (µg/ml)				16	64	32	128	64	64	128
Notes: + indicates growths, - indicates no growths										

2.10.2 Discussion

From the table11-15, the first sign of inhibition was seen in the test tube containing C_2H_5OH extract of *A. nilotica* leaves as follows:

32 µg/ml in case of *E. coli,* 128 µg/ml in case of *S.boydii,* 64 µg/ml in case of *S. dysenteriae,* 64 µg/ml in case of *S. flexneri,* 32 µg/ml in case of *S. shiga,* 16 µg/ml in case of *S. sonnei,* 128 µg/ml in case of *V. cholerae*.

No inhibition was recorded in the test tubes containing extract lower than the above concentration. Growth of the organism was observed in the test tube C1 and the content of Cm and Cs was clear. Similarly, the MIC of methanolic extract of *A. nilotica* leaves against *E. coli, S. dysenteriae*, *S. shiga, S. boydii, S. sonnei, S. flexneri* & *V. cholera*e were 64, 64, 128, 128, 32, 16 & 64 µg/ml respectively and those of Petroleum ether, $CHCl_3$ & Ethyl acetate extract of *A. nilotica* were 128, 32 ,64, 32, 64, 32, 64 µg/ml; 64, 32, 64, 16, 32, 128, 64 µg/ml and 32,128, 64, 64, 32, 128, 64 µg/ml respectively.

From the table 16-20, the MIC values of the C_2H_5OH extract of *A. indica* leaves against *E. coli*, *S. dysenteriae, S. shiga, S. boydii, S. sonnei, S. flexneri* & *V. cholerae* showed 128, 32, 32, 64, 32, 128 & 128 µg/ml respectively. Similarly, the MIC of CH_3OH, Petroleum ether, $CHCl_3$ & Ethyl acetate extract of *A. indica* leaves were 64, 64, 32, ,128, 32, 64,128 µg/ml; 32, 16, 32, 128, 128, 64, 32 µg/ml; 128, 64, 32, 128, 64, 64, 32 µg/ml; and 64, 128, 16, 32, 128, 64, 32 µg/ml respectively.

From the table 21-25, the MIC values of the C_2H_5OH extract of *P. guajava* leaves against *E. coli, S. dysenteriae, S. shiga, S. boydii, S. sonnei, S. flexneri* & *Vibrio cholerae* showed 128, 64, 32, 128, 32, 64, 32 µg/ml respectively. Similarly, the MIC of CH_3OH, Petroleum ether, $CHCl_3$ & Ethyl acetate extract of *P. guajava* leaves were 32, 16, 64, 128, 32, 64, 128 µg/ml,;32, 128, 32, 128, 32, 64, 32 µg/ml; 64, 32, 32, 64, 128, 64, 64 µg/ml and 16, 64, 32, 128, 64, 64, 128 µg/ml, respectively.

2.11 Cytotoxicity assay

2.11.1 Results

Lethal effect of the petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetate.extracts of leaves of *A. indica, A. nilotica* and *P. guajava* against the one day aged *A. salina* nauplii were carried out. Doses were selected for the final experiment ranged between 800 to 100 ppm. Observation of mortality was made after 24h. The data was then subjected to probit analysis and the results have been presented in the Appendix Tables 1-15 and Table 26-28.

Table 26. LC_{50} values, 95% confidence limits, regression equations and χ^2 values (along with their df) of the petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetate extracts of *A. nilotica* leaves against *A. salina* nauplii

Extract	Solvents	Exposure (h)	LC_{50} (ppm)	95% confidence limits		Regression equations	χ^2 values (df)
				Lower	Upper		
***A. nilotica* leaves**	C_2H_5OH	24	395.581	238.693	655.589	Y=0.601+1.706X	0.988 (3)
	CH_3OH	24	603.472	273.079	1333.603	Y=1.371+1.305X	0.407 (3)
	$CHCl_3$	24	830.236	256.202	2690.424	Y=1.793+1.099X	0.638 (3)
	Eto.Ac.	24	702.830	268.122	1842.334	Y=1.614+1.189X	0.718 (3)
	Pet.E.	24	651.289	247.806	1711.737	Y=1.830+1.127X	0.661 (3)

Table 27. LC_{50} values, 95% confidence limits, regression equations and χ^2 values (along with their df) of the petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetate extracts of *A. indica* leaves against *A. salina* nauplii

Extract	Solvents	Exposure (h)	LC_{50} (ppm)	95% confidence limits		Regression equations	χ^2 values (df)
				Lower	Upper		
***A. indica* leaves**	C_2H_5OH	24	830.236	256.202	2690.424	Y=1.793+1.099X	0.638 (3)
	CH_3OH	24	651.289	247.806	1711.737	Y=1.830+1.127X	0.661 (3)
	$CHCl_3$	24	682.959	228.649	2039.947	Y=2.093+1.026X	0.475 (3)
	Eto.Ac.	24	830.236	256.202	2690.424	Y=1.793+1.099X	0.638 (3)
	Pet.E.	24	774.694	229.683	2612.949	Y=2.087+1.008X	0.836 (3)

Table 28. LC_{50} values, 95% confidence limits, regression equations and χ^2 values (along with their df) of the petroleum ether, $CHCl_3$, CH_3OH, C_2H_5OH and ethyl acetat extracts of *P. guajava* leaves against *A. salina* nauplii

Extract	Solvents	Exposure (h)	LC_{50} (ppm)	95% confidence limits		Regression equations	χ^2 values (df)
				Lower	Upper		
***P. guajava* leaves**	C_2H_5OH	24	651.289	247.806	1711.737	Y=1.830+1.127X	0.661 (3)
	CH_3OH	24	682.959	228.649	2039.947	Y=2.093+1.026X	0.475 (3)
	$CHCl_3$	24	830.236	256.202	2690.424	Y=1.793+1.099X	0.638 (3)
	Eto.Ac.	24	682.959	228.649	2039.947	Y=2.093+1.026X	0.475 (3)
	Pet.E.	24	651.289	247.806	1711.737	Y=1.830+1.127X	0.661 (3)

2.11.2 Discussion

From the table26-28, the highest lethality has been observed for the C_2H_5OH extract of *A. nilotica* leaves (LC_{50} 395.581 ppm); followed by the CH_3OH extract of *A. nilotica* leaves (LC_{50} 603.472 ppm); followed by the petroleum ether .extract of *A. nilotica* & *P. guajava*; CH_3OH extract of *A. indica* leaves and C_2H_5OH extract of *P. guajava* leaves (LC_{50} 651.289 ppm) and also followed by the $CHCl_3$ extract of *A. nilotica*; CH_3OH and ethyl acetate .extracts of *P. guajava* leaves (LC_{50} 682.959 ppm); followed by the ethyl acetate extracts of *A. nilotica* leaves (LC_{50} 702.830 ppm) and followed by the petroleum ether extract of *A. nilotica* leaves (LC_{50} 774.694 ppm), after 24h of exposure against the nauplii. The lowest lethality has been observed for the $CHCl_3$ extracts of *A. nilotica* and *P. guajava* leaves and C_2H_5OH & ethyl acetate extracts of *A. indica* leaves (LC_{50} 830.236 ppm).

2.12 Compound isolation

Result and discussion

2.12.1 Bioactivity of the Isolated Compound

Biological activities of the purified compound was assessed through antimicrobial activity tests. Doing bioassay with the purified compound has been a major target in this investigation. The purified compound (E001) represented good antimicrobial activities against the pathogenic bacteria, while the strength of activity was elaborately mentioned in the relevant tables included in this chapter.

2.12.2 Characterization of the Compound through TLC

Purity analysis of compound (E001)

The purities of compound E001 was detected by two dimensional TLC method. Physically the compound was granular radish brown colour by open eye examinations in glass vial.

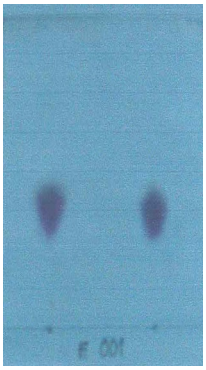


Fig. 13: Single spot of the compound E001.

The results of the final TLC plate (Fig. 13) indicated compound showed only one radish-violet colour spot along the two dimensional chromatography plate. The Retention factor (R_f) was 0.36 against the compound after spraying Godin reagent on TLC plate. That is to say, compound should be a relatively pure compound.

2.12.3 Physical remarks of the purified compound

Physical characteristics of the isolated compound from the ethanolic extract of *A.nilotica* leaves have been presented in (Table 29). The compound was subjected to NMR analysis, as well as its biological activities have been tested against some specific microorganisms *in vivo*.

Table 29. Physical nature, appearance on TLC plate and Retention Factors of the isolated compound from. *A nilotica* leaves.

Solvent of extraction	Compound	Retention factor (R_f)	Physical nature	Coloration after Godin reagent spray on TLC
Ethanol	E001	0.36	Radish brown and granular	Radish Violet spot.

2.12.4 Purity analysis of the isolated compound through TLC method

Purity of the compound (E001) from *A.nilotica* leaves has been confirmed through Thin Layer Chromatography (TLC) method. The purity of compound E001 was detected by two dimensional TLC method. Physically the compound was granular radish brown colour but after spraying Godin reagent compound showed only one radish-violet colour spot along the two dimensional chromatography plate. The Retention factor (R_f) was 0.36 against the compound E001.

2.12.5 NMR Spectroscopic results of the isolated compound

The compound E001 was isolated and successfully separated by using different organic solvents in Open Column Chromatography method. After PTLC evaluation the purities of the compound was confirmed by the NMR spectroscopy and others test analysis and the NMR Spectroscopy are as follows.

2.12.5.1 A. NMR Analysis report of Compound E001

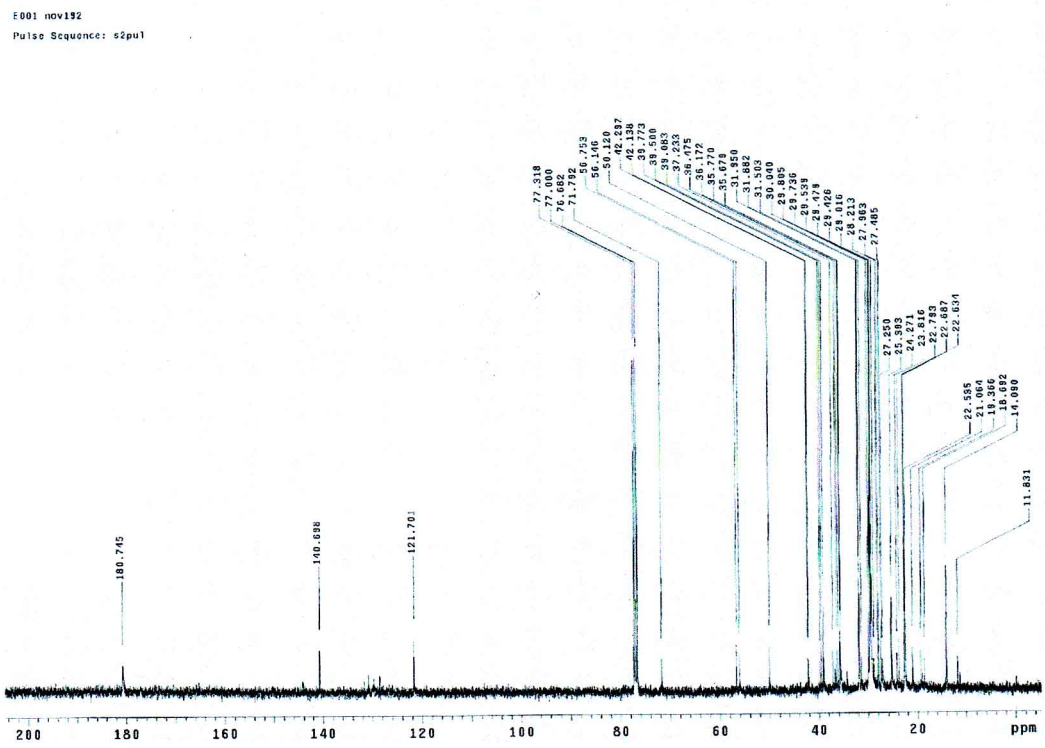


Fig.14: NMR Analysis report of Compound E001(1)

2.12.5.1 B. NMR Analysis report of Compound E001

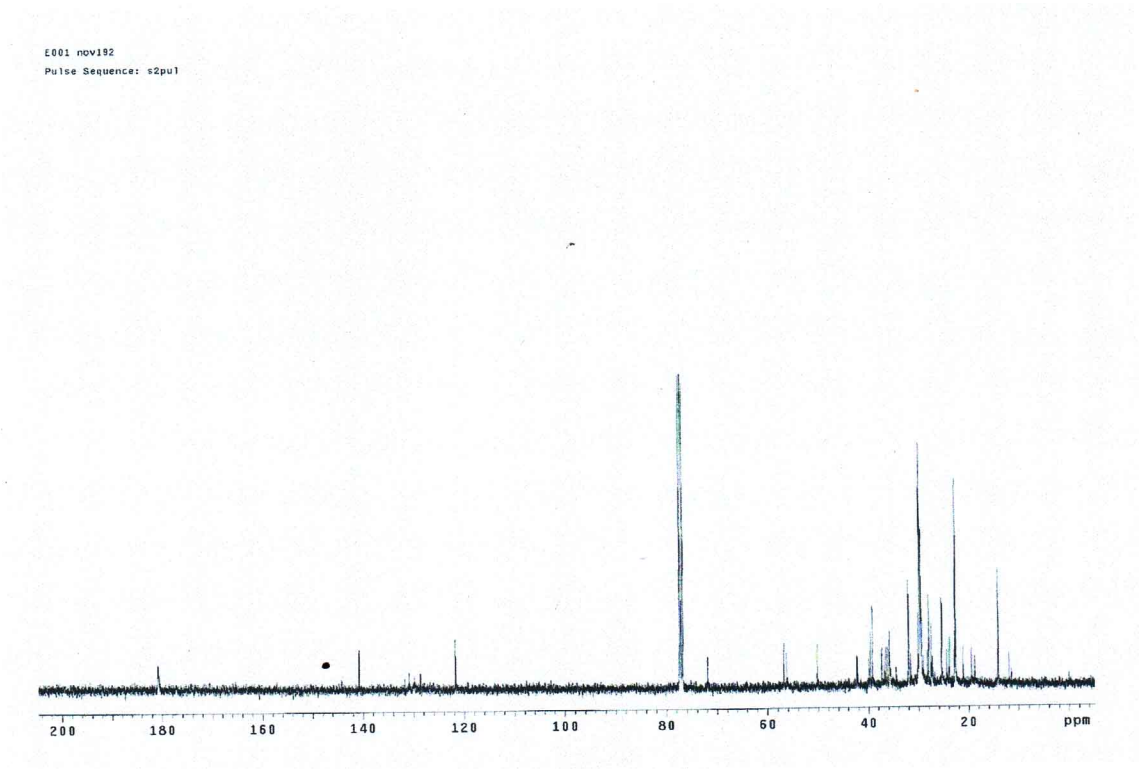


Fig.15: NMR Analysis report of Compound E001 (2)

2.12.5.1 C. NMR Analysis report of Compound E001

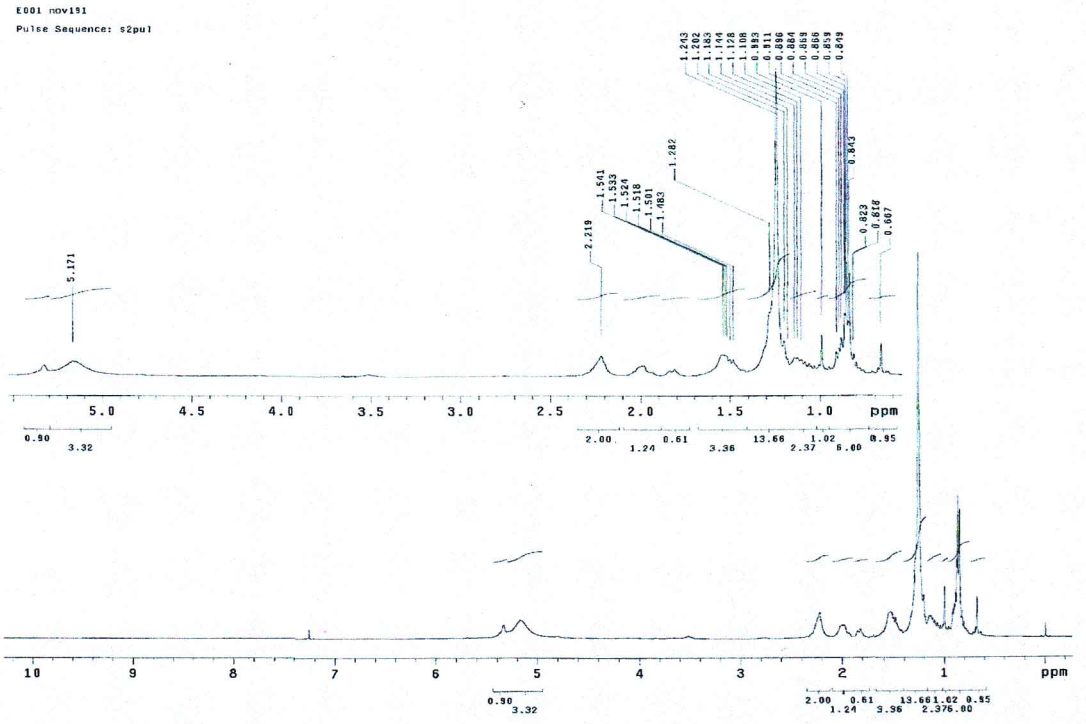


Fig.16: NMR Analysis report of Compound E001(3)

2.12.6 Structure of the purified compound

In case of Compound E001:

In the NMR spectroscopic analysis compound E001 represented the data by which evaluating that the sample E001 of which was isolated from the ethanol extract suspected for the steroid analogues because the similarities NMR spectroscopic data (were recorded from NMR spectra on a Bruker AM 400-400 MHz) with the steroid compound was alike with steroid analogue. But judging from the H and C spectra compound (E001) could be steroid analogues: It has one double bond in the six-membered ring (CH=C, 140 ppm and 121 ppm); one carboxylic acid group (C, 181 ppm), one alcoholic group (-OH, the carbon attached to OH is 71 ppm). But it could also be a triterpenoic acid (less possible, because triterpenoid will have more methyl groups (usually more than 5), which give characteristic methyl singles or doubles in HNMR). So the compound E001 (represented the data from fig. 14-16) is calculated mostly similar with common steroids sitosterols. The main skeleton has one double bond in the six-membered ring (CH=C, 140 ppm and 121 ppm); one carboxylic acid group (C, 181 ppm), one alcoholic group (-OH, the carbon attached to OH is 71 ppm).

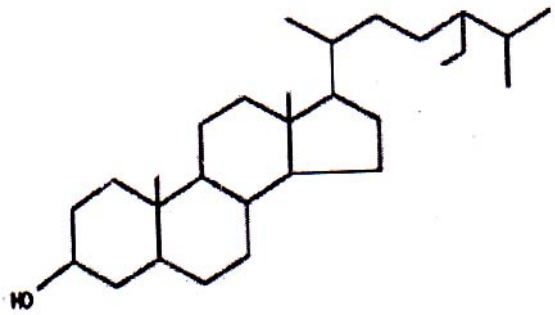


Fig.17: Molecular structure of Sitosterols (Represent for Compound E001)

2.13 Phytochemical test of different extracts of *A.nilotica* leaves

2.13.1 Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, steroids with Libermann-Burchard reagent and reducing sugars with Benedict's reagent.

2.13.2 Chemical group tests of the extract

Testing different chemical groups present in the extract are performed the preliminary phytochemical studies. The chemical group test that was performed along with the results obtained is as follows. In each test 10% (w/v) solution of extract was taken unless otherwise mentioned in individual test.

2.13.3 Reagents used for the different chemical group test

The following reagents were used for the different chemical group test.

i) Mayer's reagent

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

ii) Dragendroff'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 containing 16 gm potassium iodide and 40 ml water.

iii) Fehling's solution A

34.64 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulphuric acid and sufficient water to produce 500 ml.

iv) Fehling's solution B

176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in water to produce 500 ml. Equal volume of above solution were mixed at the time of use.

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 5 ml concentrated sulphuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling.

2.13.4 Tests performed for identifying different chemical groups

The following tests were performed for identifying different chemical groups.

2.13.5 Test for Gums

5 ml solution of the extract was taken and then Molisch reagent and sulphuric acid were added.

2.13.6 Test for Carbohydrates

Molisch's test

A few drops of molisch reagent was added to a little quantity of extract in a test tube and a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube to form a violet layer at the interface indicated the presence of carbohydrates (Trease and Evans, 1983)

Fehlings Test

2 ml extract was taken in a test tube and 5 ml Fehling's reagent was added and the mixture boiled for 8-10 minutes. A brick-red precipitate indicated the presence of free reducing sugar (Trease and Evans, 1983).

2.13.7 Test for Free Anthraquinones (Borntrager‘s test)

Small portion of the extract was mixed with 10 ml benzene and filtered. Then 5 ml of 10% of ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet color indicated the presence of free anthraquinones

2.13.8 Test for Combined Anthraquinones

Sample was boiled with 5 ml of 10% hydrochloric acid for 3 minutes. This would hydrolyze the glycosides to yield glycones which are soluble in hot water only. The solution was filtered hot. The filtrate was cooled and extracted with 5 ml of benzene. The benzene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose- pink or a cherry red colour indicated combined anthracene by presence of free anthraquinones

2.13.9 Test for Cardiac Glycosides

Kella-Killiani Test

Extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45º and 1 ml of concentrated sulphuric acid was added down the side. Purple ring color at the interface indicated cardiac glycosides (Trease and Evans, 1983)

2.13.10 Test for Saponins

Frothing test

Small quantity of the extract was dissolved in 10 ml of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb foam formed for more than 30 minutes indicated the presence of saponins.

2.13.11 Test for Steroid and Triterpenes

Lieberman-Burchards test

Equal volume of acetic anhydride was added to the extract. 1 ml of concentrated sulphuric acid was added down the side of the tube. The colour change was observed immediately and later. Red, pink or purple colour indicated the presence of triterpenes, while blue or blue-green indicated steroids (Trease and Evans, 1983).

2.13.12 Test for Flavonoids

Shinoda Test

About 0.5 g of extract was dissolved in 2 ml of 50% methanol in the tube. Metallic magnesium and 4-5 drops of concentrated hydrochloric acid was added. A red or orange colour indicates the presences of flavanoic aglycones.

2.13.13 Test for Tannins

Lead sub-acetate test

3 drops of lead-sub acetate solution were added to a solution of the extract. A colored precipitate indicated that tannins are present.

Ferric chloride test

About 0.5 ml of extract was dissolved in 10 ml of distilled water, and then filtered. A few drops of ferric chloride solution were added to the filtrate. Formation of a blue-black precipitate indicated the presence of hydrolysable tannins and green precipitate indicated that of condensed tannin.

2.13.14 Test for Alkaloids

Meyer's Test

A few drops of the Meyer's reagent was added to an aliquot of the extract in a test tube. Cream precipitate indicated the presence of alkaloids.

Dragendoffs test

A few drops of this reagent was added to the extract. A rose red precipitate indicated the presence of alkaloids.

Wagners Test

Drops of this reagent was added to a small amount of the extract. A whitish precipitate indicated the presence of alkaloids.

Picric acid test

A few drops of 1% picric acid solution was added to the extract and a yellow colored solution indicated the presence of alkaloids.

2.13.15 Test for Phenol

The extract was dissolved by using 70% ethanol. Resulting solutions was taken as much 1 ml then added 2 drops of $FeCl_3$. The formation of green or blue-green color indicates the presence of phenolic compound

2.13.16 Detection of Amino acids and Proteins

The extract (100 mg) was dissolved in 10 ml distilled water and filtered through Whatman no.1 filter paper and the filtrate was subjected to test for proteins and amino acids.

Biuret test: 2 ml of filtrate was treated with 1 drop of 2% copper sulphate solution. To this 1 ml. of ethanol was added followed by excess of potassium hydroxide pellets. Pink color in the ethanol layer indicates presence of proteins.

Ninhydrin test: 2 drops of ninhydrin solution was added to 2 ml. of aqueous filtrate. A characteristic purple color indicates the presence of amino acids

2.13.17 Detection of Fixed oils and Fats

Spot test: A small quantity of extract was pressed between two filter papers. Oilstain on the paper indicates the presence of fixed oils.

2.13.18 Test for Terpenoids

Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

2.13.19 Results of phytochemical test

Table 30. Phytochemical screening of different extracts of *A. nilotica* leaves.

Test for	Petroleum ether	Ethyl acetate	$CHCl_3$	CH_3OH	C_2H_5OH
Alkaloids	-	-	-	-	+
Carbohydrate	+	+	+	+	+
Anthraquinones (Free state)	+	+	+	+	+
Anthraquinones (Combined state)	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+
Saponins	+	+	+	+	+
Steroid & Triterpenes	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins	+	+	+	+	+
Amino acid & protein	-	+	+	+	+
Terpenoid	+	+	+	+	+
Fixed oil & fat	-	-	-	-	-
Gum	+	+	+	+	+

Key: + = Present; - = Absent

2.13.20 Discussion

The phytochemical tests indicated the presence of carbohydrate, anthrquinones (free & combined state), cardiac glycosides, saponins, steroid & triterpenes, flavonoids, tannins, terpenoid and gum in different extracts. But alkaloids was present in ethanol extract and amino acid & protein was absent in petroleum ether extract. Fixed oil & fat were absent in different extracts**.**

Chapter Three

Evaluation of Antioxidant activity

Materials and method

3.1 Determination of total phenolic content:

Plant polyphenols, a diverse group of phenolic compounds possess an ideal structural chemistry for free redical scavenging activity. Total phenolic content of extract of *A. nilotica* leaves is determined employing the method as described by Singleton *et.al* (1965) involving Folin-Ciocalteu Reagent (FCR) as oxidizing agent and gallic acid as standard.

3.1.1 Principle:

The content of total phenolic compounds of ethanolic extracts of *A. nilotica* leaves was determined by Folin-Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The extract chemical nature of the FCR is not known, but it is belived to contain heteropolyphosphotungstates molybdates. Sequences of reversible one or two electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})_4$. In essence, it is believed that the molybdenum is easier to be reduced in the complex & electron-transfer reaction occurs between reductants and Mo (VI)

$$Mo\ (VI) + e^{-} \rightarrow Mo\ (V).$$

3.1.2 Materials and apparatus:

A) Folin-Ciocalteu Reagent (FCR) (Sigma-Aldrich, India).

B) Sodium carbonate (Na_2CO_3) (Sigma Chemical company,USA).

C) Methanol (Sigma Chemical company,USA).

D) Gallic acid (Wako pure chemicals Ltd, Japan).

e) Micropipette (10-100 µl).

f) Pipette (1-10 ml).

g) UV-spectrophotometer (Shimadzu, USA).

3.1.3 Experimental procedure:

1) 0.5 ml of extract solution was taken in a test tube.

2) 2.5 ml of folin-ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube.

3) 2.0 ml of sodium carbonate (Na_2CO_3) 7.5% solution was added in to the test tube.

4) The test tube was incubated for 20 minutes at 25°C to complete the reaction.

5) Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank.

6) A typical blank solution contained reagent except plant extract or standard solution.

7) The total content of phenolic compounds in plant ethanolic extracts in Gallic acid equivalents (GAE) was calculated by the following formula

$C=cxV/m$

where,

C= Total content of phenolic compounds, mg/g plant extracts in GAE.

c = The concentration of gallic acid in mg/ml established from the calibration curve.

V= The volume of extract in ml.

m= The weight of plant extract in gm.

3.2 Determination of total flavonoid content:

Total flavonoid content of ethanolic extract of *A.nilotica* leaves was determined by aluminium chloride colorimetric method. Catechin was used as standard and the flavonoid content of the extract was expressed as mg of catechin equivalent/gm of dried extract.

3.2.1 Principle:

The content of total flavonoids in ethanolic extract of *A.nilotica* leaves was determined by the well known aluminium chloride colorimetric method. In this method, aluminium chloride forms complex with hydroxyl groups of flavonoids present in the samples. This complex has the maximum absorbance at 510 nm.

3.2.2 Materials and apparatus:

a) Aluminium chloride, $AlCl_3$ (Sigma chemical company,USA)

b) Potassium acetate (Merch, Germany).

c) Methanol (Sigma chemical company,USA).

d) Catechin (Reagent grade).

e) Micropipette (10-100 µl).

f) Pipette (1-10 ml).

g) UV-spectrophotometer (Shimadzu,USA).

3.2.3 Experimental procedure:

1) 0.5 ml of extract solution was taken in a test tube.

2) 1.5 ml of methanol was added into the test tube.

3) 2.5 ml of distilled water was added into the test tube.

4) 1.5 ml of 5% $NaNO_2$ solution was added into test tube.

After another 5 minutes, 1 ml of 0.001% NaOH was added.

Then add 0.55 ml distilled water and solution is mixed well.

5) The test tube was then incubated at room temperature for 30 minutes to complete the reaction.

6) Then the absorbance of the solution was measured at 510 nm using a spectrophotometer against blank.

7) A typical blank solution contained reagent except plant extract or standard solution.

8) The total content of flavonoid compounds in plant extract in catechin equivalents (Cat.E) was calculated by the following equation

C= c x V/m

where,

C= Total content of flavonoid compounds mg/g plant extract in Catechin equivalent (Cat.E).

c= The concentration of Catechin in mg/ ml established from the calibration curve.

V= The volume of extract in ml.

m= The weight of plant extract in gm.

3.3 Determination of total antioxidant activity:

Total antioxidant capacity of ethanollic extract of *A. nilotica* leaves was determined by the method reported by Prieto *et.al*,.(1999) with some modifications.

3.3.1 Principle: The phosphomolybdenum method usually detects antioxidants, such as ascorbic acid, some phenollics,∝-tocopherol and carotenoids. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate (PO_4)/M0 (V) complex at acidic P^H. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI) and the formation of a green phosphate(PO_4)/Mo(V) complex with a maximal absorption at 695 nm.

$$\text{Mo (VI)} + e \rightarrow \text{Mo (V)}.$$

3.3.2 Materials and aparatus

a) Concentrated Sulphuric ,H_2SO_4 (98%) (Merck Germany).

b) Sodium phosphate, Na_3PO_4 (Sigma chemical company, USA).

c) Ammonium molybdate (Sigma chemical company, USA).

d) Cateching (Analytical or Reagent grade).

e) Methanol (Sigma chemical company, USA).

f) Water bath.

g) Micropipette (100-1000 µl).

h) Pipette (1-10 ml).

i) UV-spectrophotometer (Shimadzu, USA).

3.3.3 Experimental procedure:

1) 0.5 ml of plant extract was taken in a test tube.

2) 3 ml of reaction mixture containing 0.6 M H_2SO_4 acid. 28 mM sodium phosphate and 1% ammonium molybdate was added into the test tube.

3) The test tube was incubated at 95°C for 10 minutes to complete the reaction.

4) Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling at room temperature.

5) A typical blank solution contained 3 ml of reaction mixture and the appropriate volume (300 µl) of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples solution.

3.4 Determination of reducing power capacity

The reducing power of ethanolic extracts of *A. nilotica* leaves was evaluated by the method of Oyaizu (1986).

3.4.1 Principle:

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants, such as antioxidant substances in the samples causes the reduction of the Fe^{3+} - Ferricyanide complex to the ferrous from by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

$$Fe^{3+}\text{-ferricyanide} + e^{-} \rightarrow Fe^{2+}\text{-ferricyanide.}$$

3.4.2 Materials and aparatus:

a) Potassium ferricyanide, $[K_3Fe(CN)_6]$ (Merck, Germany).

b) Trichloro acetic acid (TCA),Cl_3C-COOH) (Merck, Germany).

c) Ferric chloride, $FeCl_3$ (Sigma chemical company, USA).

d) Phosphate buffer $[K_2HPO_4+KH_2PO_4]$ (Sigma-Aldrich, USA).

e) Ascorbic acid (Sigma chemical company, USA).

f) Water bath.

g) Centrifuge machine.

h) Pipette (1-10 ml).

i) UV- spectrophotometer (Shimadzu, USA).

3.4.3 Experimental procedure:

1) 1.0 ml of extract solution or positive control was taken in each test tube at different concentration.

2) 2.5 ml of phosphate buffer (0.2M) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution were added into the test tube.

3) The reaction mixture was incubated for 20 minutes at 50°C to complete the reaction.

4) 2.5 ml of TCL, 10% solution was added into the test tube.

5) The total mixture was centrifuged at 3000 rpm for 5 minutes.

6) 2.5 ml supermatant solution was withdrawn from the mixture and mix 2.5 ml of distilled water.

7) 0.5 ml of 0.1% ferric chloride ($FeCl_3$) solution was added to the diluted reaction mixture.

8) Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank.

9) A typical blank solution contained the same solution mixture without plant extract or standard and it was incubated under the same conditions as the rest of the samples solution.

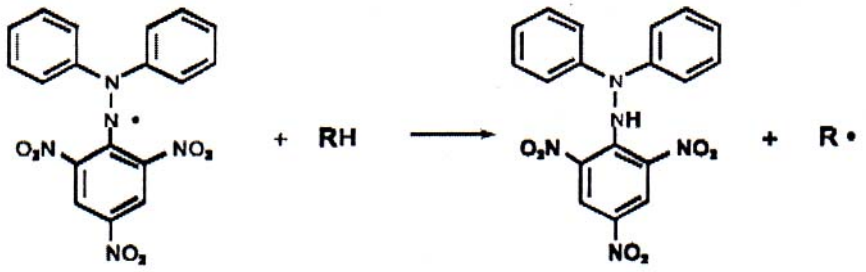
10) Also the absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation. Increased absorbance of the reaction mixture indicated increase reducing power.

3.5 Determination of DPPH radical scavenging activity

DPPH was used to evaluate the free radical scavenging activity of various compounds and medicinal plants (Choi *et.al.,* 2000; Desmarchelier *et.al.,* 1997)

3.5.1 Principle:

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method, it is possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow, the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.



DPPH
517 nm

3.5.2 Materials and aparatus:

a) DPPH (Sigma chemical company,USA).

b) Methanol (Sigma chemical company,USA).

c) Ascorbic acid as standard (SD Fine chem.Ltd. Biosar, India).

d) Pipette (1-10 ml).

e) Micropipette (10-100 µl).

f) UV-spectrophotometer (Shimadzu,USA).

3.5.3 Experimental procedure:

1) 0.1 ml of extract at various concentration (10, 50, 100 and 500 µg/ml) are added to 3 ml of a 0.004% methanol solution of DPPH.

2) After 30 minutes, absorbance of the resulting solution is measured against a blank at 517 nm.

3) The percentage of DPPH radical scavenging activities (%SCV) are calculated by comparing the results of the test with the control (not treated with extract) using following formula:

$\%\ SCV = (A_o - A_1/A_o)\ 100$

where,

SCV= Radical scavenging activity.

A_o = Absorbance of the control and

A_1 = Absorbance of the test (extracts /standard).

4) Extract concentration providing 50 % inhibition (IC_{50}) is calculated from the graph plotted %SCV versus concentration curve.

5) The carried out in duplicate and ascorbic acid is used as standard.

Result & discussion

3.6 Determination of total phenolic content

3.6.1 Result

Table 31. Absorbance of gallic acid at different concentrations.

Sample	Concentration (µg/ml)	Absorbance			Abs. mean ± STD
		a	b	c	
Gallic acid	12.5	0.236	0.307	0.183	0.242±0.062217
	25	0.506	0.430	0.475	0.470333±0.038214
	50	0.823	0.688	0.693	0.734667±0.07654
	100	1.353	1.371	1.121	1.281667±0.139432
	200	2.638	2.323	2.274	2.411667±0.197536

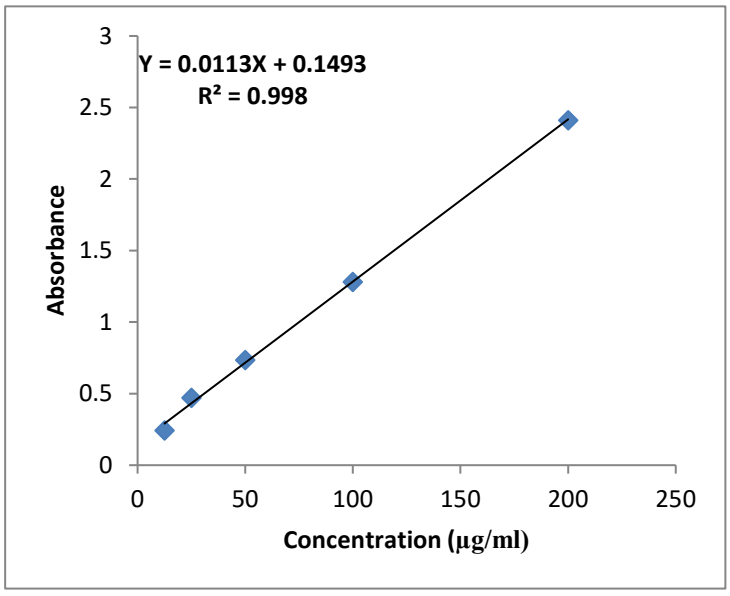


Fig.18: Calibration curve for gallic acid

The concentration of gallic acid in samples are determined by using an equation that is obtained from standard gallic acid curve. The equation is given below

$$Y=0.0113X+0.1493.$$

where,

Y = absorbance

X = gallic acid concentration µg/ml

m = slope = 0.0113

c = Intersection = 0.1493

Now,

The concentration of total phenolic compound in sample was determined as milligram of gallic acid equivalent by using the following equation.

$$A=(c\times v)/m$$

where,

A = Total phenolic content (mg/gm gallic acid equivalent)

C = x/1000= concentration of gallic acid (mg/ml)

v = Volume of extract

m = Mass of the extract (gm)

Table 32. Determination of total phenolic content of ethanol extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
Ethanol extract	1	800	3.784	0.0008	0.322	201.25	193.9583 ±7.864411
	2	800	3.685	0.0008	0.312	195.00	
	3	800	3.505	0.0008	0.297	185.625	
	4	400	2.638	0.0004	0.2202	275.25	258.0417 ±17.3774
	5	400	2.323	0.0004	0.1924	240.50	
	6	400	2.485	0.0004	0.2067	258.375	
	7	200	1.536	0.0002	0.1227	306.75	296.6667 ±10.1376
	8	200	1.486	0.0002	0.1183	295.75	
	9	200	1.444	0.0002	0.1146	286.50	

Total phenolic content of ethanolic extract of *A. nilotica* leaves at 800 µg/ml, 400 µg/ml & 200 µg/ml is 193.9583±7.864411, 258.0417±17.3774 and 296.6667±10.1376 mg/g, GAE.

Table 33. Determination of total phenolic content of ethyl acetate extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
Ethyl acetate extract	1	800	1.229	0.0008	0.0955	59.6875	50.25±8.392472
	2	800	1.008	0.0008	0.0759	47.4375	
	3	800	0.939	0.0008	0.0698	43.625	
	4	400	0.658	0.0004	0.0450	56.25	46.25±8.917153
	5	400	0.541	0.0004	0.0347	43.375	
	6	400	0.503	0.0004	0.0313	39.125	

Total phenolic content of ethyl acetate extract of *A. nilotica* leaves at 800 µg/ml & 400 µg/ml is 50.25±8.392472 and 46.25±8.917153 mg/g, GAE.

Table 34. Determination of total phenolic content of methanol extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
CH_3OH extract	1	800	1.034	0.0008	0.07829	48.93125	48.95125 ±6.442523
	2	800	0.918	0.0008	0.06803	42.51875	
	3	800	1.151	0.0008	0.088646	55.40375	
	4	400	1.318	0.0004	0.103425	129.28125	129.31875 ±37.11251
	5	400	0.983	0.0004	0.07378	92.225	
	6	400	1.654	0.0004	0.13316	166.45	
	7	200	1.214	0.0002	0.09422	235.55	235.483 ±34.85005
	8	200	1.056	0.0002	0.08024	200.60	
	9	200	1.371	0.0002	0.10812	270.30	

Total phenolic content of methanolic extract of *A. nilotica* leaves at 800 µg/ml, 400 µg/ml & 200 µg/ml is 48.95125±6.442523, 129.31875±37.11251 and 235.483±34.85005 mg/g, GAE.

Table 35. Determination of total phenolic content of chloroform extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
$CHCl_3$ extract	1	800	0.586	0.0008	0.03865	24.15625	17.15 ±6.496673
	2	800	0.438	0.0008	0.02555	15.96875	
	3	800	0.354	0.0008	0.01812	11.325	
	4	400	0.259	0.0004	0.0097	12.125	6.8208 ±4.659505
	5	400	0.194	0.0004	0.00396	4.95	
	6	400	0.180	0.0004	0.00271	3.3875	

Total phenolic content of chloroform extract of *A. nilotica* leaves at 800 µg/ml, 400 µg/ml is 17.15±6.496673 and 6.8208±4.659505 mg/g, GAE.

Table 36. Determination of total phenolic content of petroleum ether extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
Petroleum ether extract	1	800	1.793	0.0008	0.14546	90.9125	90.3031 ±0.861786
	2	800	1.771	0.0008	0.14351	89.69375	
	3	400	1.051	0.0004	0.07979	99.7375	89.7875 ±14.07142
	4	400	0.871	0.0004	0.06387	79.8375	
	5	200	0.546	0.0002	0.03511	87.775	78.925 ±12.51579
	6	200	0.466	0.0002	0.02803	70.075	

Total phenolic content of petroleum ether extract of *A. nilotica* leaves at 800 µg/ml, 400 µg/ml and 200 µg/ml is 90.3031±0.861786, 89.7875±14.07142 & 78.925±12.51579 mg/g, GAE.

Table 37. Comparison of total phenolic content of ethanol, ethyl acetate, methanol, chloroform & petroleum ether extracts of *A. nilotica* leaves at 800 µg/ml concentration.

Samples	Total phenolic content (mg/g, GAE)
Ethanol extract	193.9583±7.864411
Ethyl acetate extract	50.25±8.392472
Methanol extract	48.95125±6.442523
Chloroform extract	17.15±6.496673
Petroleum ether extract	90.3031±0.861786

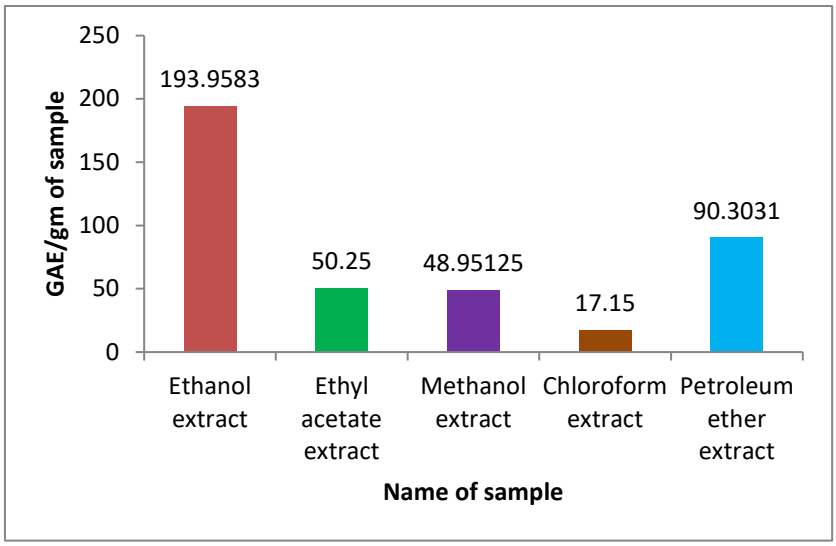


Fig.19: Total phenolic content(mg/gm, gallic acid equivalents) of different extracts.

3.6.2 Discussion

The total phenolic content of ethanol, methanol, ethyl acetate, petroleum ether & chloroform extract of *A. nilotica* leaves is determined using Folin-Ciocalteu reagent. Phenolic content of the samples are calculated on the basis of the standard curve for gallic acid as shown in Table no.31 and in Fig.no. 18. The results are expressed as mg of gallic acid equivalent (GAE/gm) of extract. Ethanol extract possesses the highest phenolic content among all the extracts, at 200 µg/ml, it is found to be 296.6667%, equivalent to gallic acid. Other extracts show no significant response at this concentration except methanol extract. But at 800 µg/ml, all the extracts contain phenolic compound. Phenols are the most important plant constituents because of their scavenging ability due to their hydroxyl group. Phenolic content in extract varies according to the polarity of the solvents used in this study. Therefore, highest phenolic components present in ethanol extract may contribute directly to its highest antioxidant activity.

3.7 Determination of total flavonoids content

3.7.1 Result

Table 38. Absorbance of catechin at different concentrations for quantitative determination of total flavonoids content.

Sample	Concentration(µg/ml)	Absorbance		Abs. mean±STD
		a	b	
Catechin	31.25	0.208	0.22	0.214±0.008485
	62.5	0.397	0.362	0.3795±0.024749
	125	0.461	0.415	0.438±0.032527
	250	0.898	0.853	0.8755±0.03182
	500	2.156	2.466	2.311±0.219203

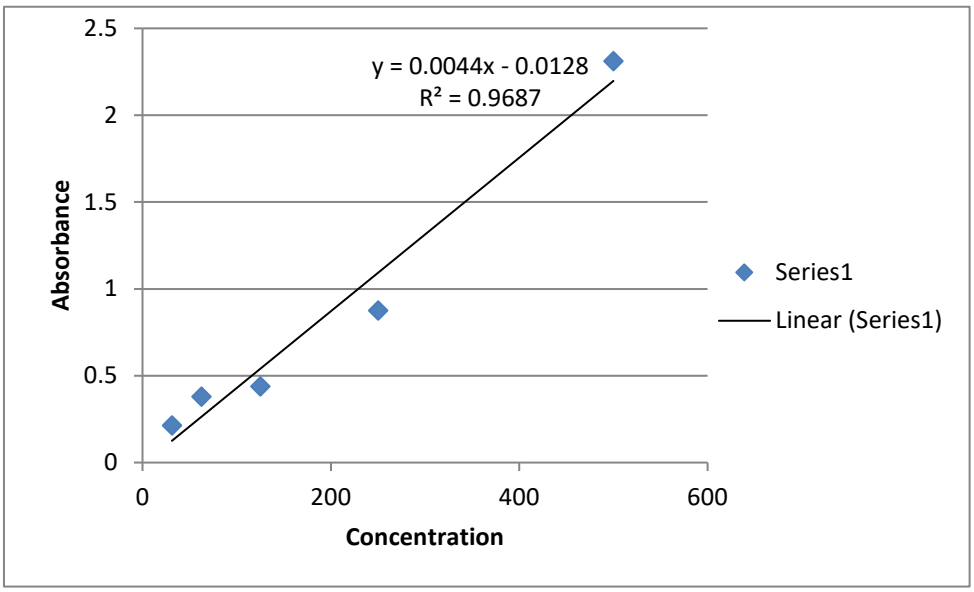


Fig.20: Calibration curve for catechin.

The concentration of catechin in samples was determined by using an equation that was obtained from standard catechin graph. The equation is given below

$$y=0.0044x+0.0128$$

where,

y = absorbance

x = ascorbic acid concentration µg/ml.

m = slope= 0.0044

c = intersection = 0.0128

The total flavonoid content in samples is determined as milligram of quercetin equivalent by using the following equation-

$$A=(c \times v)/m$$

where,

A = Total flaonoid content (mg/gm quercetin equivalent)

c = x/1000= concentration of quercetin in mg/ml

v = Volume of extract

m = Mass of the extract (gm)

The total flavonoid content of the samples with necessary data is shown in the following table.

Table 39. Determination of total flavonoids content of methanol extract of *A.nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(gm)	C(mg/ml)	A= (c×v)/m	A. mean ±STD
Methanol extract	1	800	0.806	0.0008	0.18609	116.30625	106.5542± 9.162535
	2	800	0.678	0.0008	0.157	98.125	
	3	800	0.728	0.0008	0.16837	105.23125	

Total flavonoids content of methanol extract of *A.nilotica* leaves at 800 µg/ml is 106.5542±9.162535 mg/g, (Cat.E).

Table 40. Determination of total flavonoids content of petroleum ether extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(mg)	C(mg/ml)	A= (c×v)/m	A. mean ±STD
Petroleum ether extract	1	800	0.439	0.0008	0.10268	64.175	75.53958± 11.15619
	2	800	0.522	0.0008	0.12155	75.96875	
	3	800	0.596	0.0008	0.13836	86.475	

Total flavonoids content of petroleum ether extract of *A.nilotica* leaves at 800 µg/ml is 75.53958±11.15619 mg/g, (Cat.E)

Table 41.Determination of total flavonoids content of ethanol extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(mg)	C(mg/ml)	A=(c×v)/m	A. mean ±STD
Ethanol extract	1	800	0.937	0.0008	0.21586	134.9125	114.5542±22.1149
	2	800	0.816	0.0008	0.18836	117.725	
	3	800	0.628	0.0008	0.14564	91.025	

Total flavonoids content of ethanol extract of *A.nilotica* leaves at 800 µg/ml is 114.5542±22.1149 mg/g, (Cat.E).

Table 42. Determination of total flavonoids content of chloroform extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(mg)	C(mg/ml)	A=(c×v)/m	A. mean ±STD
Chloroform extract	1	800	0.278	0.0008	0.06609	41.30625	52.90625±10.04814
	2	800	0.402	0.0008	0.09427	58.91875	
	3	800	0.399	0.0008	0.09359	58.49375	

Total flavonoids content of chlorof orm extract of *A.nilotica* leaves at 800 µg/ml is 52.90625±10.04814 mg/g, (Cat.E).

Table 43. Determination of total flavonoids content of ethyl acetate extract of *A. nilotica* leaves.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	m(mg)	C(mg/ml)	A=(c×v)/m	A. mean ±STD
Ethyl acetate extract	1	800	0.303	0.0008	0.07177	44.85625	38.60625±5.530893
	2	800	0.245	0.0008	0.05859	36.61875	
	3	800	0.229	0.0008	0.05495	34.34375	

Total flavonoids content of ethyl acetate extract at 800 ug/ml is 38.60625±5.530893 mg/g, (Cat.E).

Table 44. Comparison of total flavonoids content of methanol, petroleum ether, ethanol, chloroform & ethyl acetate extract of *A.nilotica* leaves at 800 µg/ml

Samples	Total flavonoids content (mg/g, Cat.E)
Methanol	106.5542±9.162535
Petroleum ether	75.53958±11.15619
Ethanol	114.5542±22.1149
Chloroform	52.90625±10.04814
Ethyl acetate	38.60625±5.530893

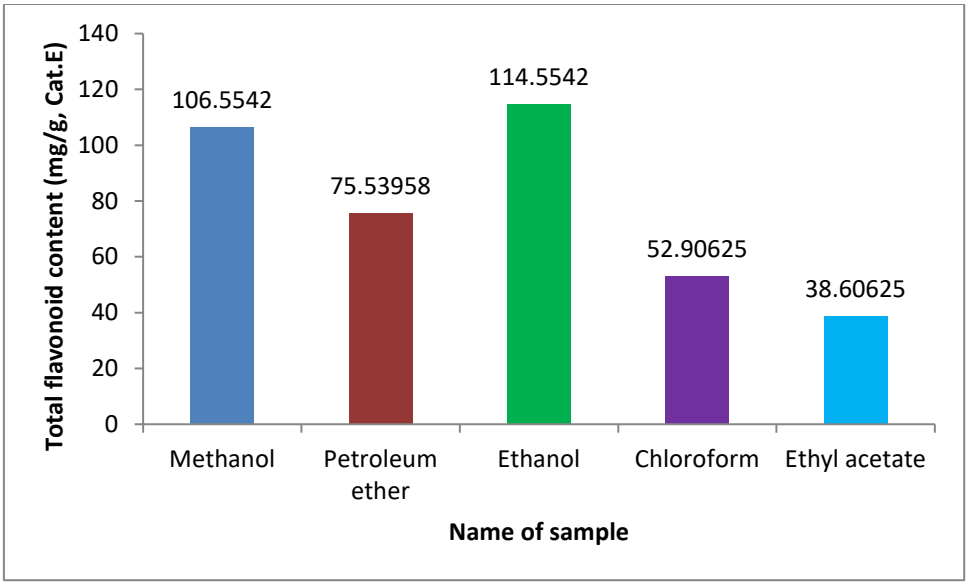


Fig.21: Total flavonoid content (mg/g, Cat, E) of different extracts.

3.7.2 Discussion

Flavonoids are large class of benzo-pyrone derivatves, ubiquitous in plants exhibit antioxidant activities. Total flavonoids content of ethanol, methanol, petroleum ether, ethyl acetate & chloroform extract are determined using much known aluminum chloride colorimetric method. Flavonoid content of the samples is calculated on the basis of the standard curve for catechin as shown in table no. 38 and in fig.no 20. The results are expressed as mg of catechin equivalent (Cat.E)/gm of extracts.

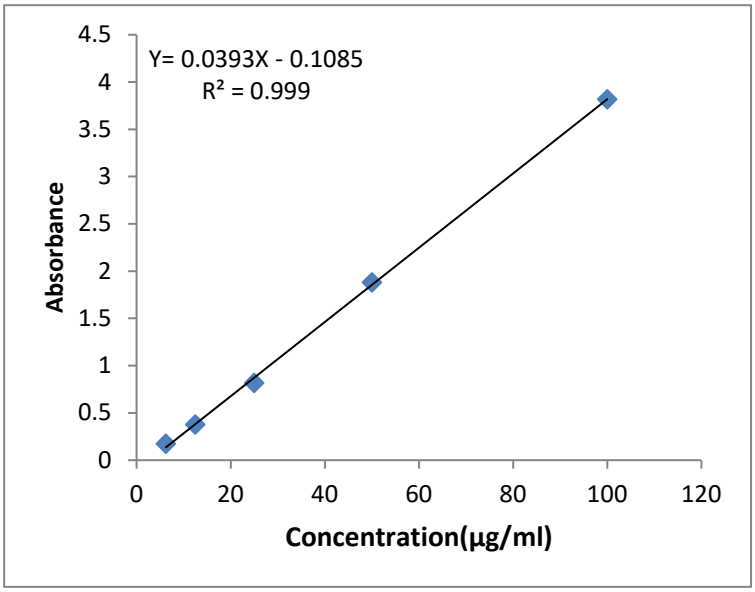
The total flavonoid content in methanol, petroleum ether, ethanol, chloroform & ethyl acetate ia 106.5542±9.162535; 75.53958±11.15619; 114.5542±22.1149; 52.90625±10.04814 & 38.60625±5.530893 mg/gm of catechin, respectively. The result represents that ethanol& methanol extract contain higher amount of flavonoids among all the extracts. The antioxidant property of flavonoids is directed mostly toward hydroxyl superoxide as well as peroxyl and alkoxyl redicals.

3.8 Determination of total antioxidant capacity

3.8.1 Result

Table 45. Absorbance of ascorbic acid at different concentrations for quantitative determination of total antioxidant activity.

Sample	Concentration (µg/ml)	Absorbance		Abs (mean±STD)
		a	b	
Ascorbic acid	6.25	0.167	0.184	0.1755±0.012021
	12.5	0.362	0.390	0.376±0.019799
	25	0.78	0.852	0.816±0.050912
	50	1.769	1.991	1.88±0.156978
	100	3.841	3.797	3.819±0.031113



Concentration (µg/ml)

Fig. 22: Calibration curve for ascorbic acid.

The concentration of ascorbic acid is samples were determined by using an equation that was obtained from standard ascorbic acid graph. The equation is given below:

$$Y = 0.0393X - 0.1085$$

where,

y = absorbance

x = ascorbic acid concentration (µg/ml)

m = slope=0.0393

c = intersection=0.1085

Now,

The total antioxidant capacity in samples is determined as milligram of ascorbic acid equivalent by using the following equation:

$$A = (cxv)/m$$

Where,

A = Total antioxidant capacity (mg/g, ascorbic acid equivalent)

c = x/1000= concentration of ascorbic acid (mg/ml)

v = Volume of extract

m = Mass of the extract (gm)

The total antioxidant capacity of the samples with necessary data is shown in the following Tables.

Table 46. Determination of total antioxidant capacity of ethanolic extract of *A.nilotica* leaves.

Sample (extract)	No. of sample	Concentration(µg/ml)	Absorbance	m(gm)	C (mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
C_2H_5OH extracr	1	100	0.426	0.0001	0.01360	68	63.475±6.399316
	2	100	0.355	0.0001	0.01179	58.95	
	3	50	0.220	0.00005	0.00836	83.6	82.95±0.919239
	4	50	0.215	0.00005	0.00823	82.3	
	5	25	0.085	0.000025	0.00492	98.4	107.3±12.5865
	6	25	0.120	0.000025	0.00581	116.2	
	7	12.5	0.035	0.0000125	0.00365	146	165.4±27.43574
	8	12.5	0.073	0.0000125	0.00462	184.8	
	9	6.25	0.023	0.00000625	0.00335	268	295.2±38.46661
	10	6.25	0.050	0.00000625	0.00403	322.4	

Total antioxidant capacity of ethanolic extract of *A. nilotica* leaves at 100, 50, 25, 12.5 and 6.25 µg/ml is 63.475±6.399316, 82.95±0.919239, 107.3±12.5865, 165.4±27.43574 & 295.2±38.46661 mg/g, ascorbic acid equivalents.

Table 47. Determination of total antioxidant capacity of petroleum ether extract of *A. nilotica* leaves.

Sample (extract)	No. of sample	Concentration(µg/ml)	Absorbance	m (gm)	C (mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
Petroleum ether extract	1	100	0.326	0.0001	0.01106	55.3	54.60±0.989949
	2	100	0.315	0.0001	0.01078	53.9	
	3	50	0.201	0.00005	0.00787	78.7	78.3±0.565685
	4	50	0.198	0.00005	0.00779	77.9	
	5	25	0.126	0.000025	0.00597	159.4	129.7±42.00214
	6	25	0.088	0.000025	0.005	100	
	7	12.5	0.061	0.0000125	0.00431	172.5	181.25±12.37437
	8	12.5	0.078	0.0000125	0.00475	190	
	9	6.25	0.032	0.00000625	0.00358	286.4	274.8±16.40488
	10	6.25	0.021	0.00000625	0.00329	263.2	

Total antioxidant capacity of petroleum ether extract of *A. nilotica* leaves at 100, 50, 25,12.5 and 6.25 µg/ml is 54.60±0.989949, 78.3±0.565685, 129.7±42.00214, 181.25±12.37437 & 274.8±16.40488 mg/g, ascorbic acid equivalents.

Table 48. Determination of total antioxidant capacity of ethyl acetate extract of *A. nilotica* leaves.

Sample (extract)	No. of sample	Concentration(µg/ml)	Absorbance	m (gm)	C (mg/ml)	A=(c×v)/ m	Absorbance (mean±STD)
Ethyl acetate extract	1	100	0.289	0.0001	0.01010	50.5	54.15±5.16188
	2	100	0.346	0.0001	0.01156	57.8	
	3	50	0.179	0.00005	0.00732	73.2	78.8±7.919595
	4	50	0.223	0.00005	0.00844	84.4	
	5	25	0.131	0.000025	0.00609	121.8	134.1±17.39483
	6	25	0.179	0.000025	0.00732	146.4	
	7	12.5	0.117	0.0000125	0.00574	229.6	188.8±57.69991
	8	12.5	0.037	0.0000125	0.00370	148.0	
	9	6.25	0.061	0.00000625	0.00431	344.8	280.7±90.65109
	10	6.25	0.02	0.00000625	0.00327	216.6	

Total antioxidant capacity of ethyl acetate extract of *A. nilotica* leaves at 100, 50, 25,12.5 and 6.25 µg/ml is 54.15±5.16188, 78.8±7.919595, 134.1±17.39483, 188.8±57.69991 and 280.7±90.65109 mg/g, ascorbic acid equivalents.

Table 49. Determination of total antioxidant capacity of CH_3OH extract of *A.nilotica* leaves.

Sample (extract)	No. of sample	Concentration(µg/ml)	Absorbance	m (gm)	C (mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
CH_3OH extract	1	100	0.338	0.0001	0.01136	56.8	54.075±3.853732
	2	100	0.295	0.0001	0.01027	51.35	
	3	50	0.226	0.00005	0.0085	85	74.6±14.70782
	4	50	0.144	0.00005	0.00642	64.2	
	5	25	0.144	0.000025	0.00642	128.4	114.2±20.08183
	6	25	0.088	0.000025	0.005	100	
	7	12.5	0.106	0.0000125	0.00546	218.4	195±33.0926
	8	12.5	0.06	0.0000125	0.00429	171.6	
	9	6.25	0.081	0.00000625	0.0048	384	332.8±72.40773
	10	6.25	0.03	0.00000625	0.00352	281.6	

Total antioxidant capacity of CH_3OH extract of *A. nilotica* leaves at 100, 50, 25, 12.5 and 6.25 µg/ml is 54.075±3.853732, 74.6±14.70782, 114.2±20.08183, 195±33.0926 & 332.8±72.40773 mg/g, ascorbic acid equivalents.

Table 50. Determination of total antioxidant capacity of $CHCl_3$ extract of *A. nilotica* leaves.

Sample (extract)	No. of sample	Concentration(µg/ml)	Absorbance	m (gm)	C (mg/ml)	A=(c×v)/m	Absorbance (mean±STD)
$CHCl_3$ extract	1	100	0.126	0.0001	0.00597	29.85	36.575±9.510586
	2	100	0.232	0.0001	0.00866	43.3	
	3	50	0.055	0.00005	0.00416	41.6	50.75±12.94005
	4	50	0.127	0.00005	0.00599	59.9	
	5	25	0.04	0.000025	0.00378	75.6	83.2±10.74802
	6	25	0.07	0.000025	0.00454	90.8	
	7	12.5	0.035	0.0000125	0.00365	146	149±4.242641
	8	12.5	0.041	0.0000125	0.0038	152	
	9	6.25	0.021	0.00000625	0.00329	263.2	270.4±10.18234
	10	6.25	0.028	0.00000625	0.00347	277.6	

Total antioxidant capacity of $CHCl_3$ extract of *A. nilotica* leaves at 100, 50, 25, 12.5 and 6.25 µg/ml is 36.575±9.510586, 50.75±12.94005, 83.2±10.74802, 149±4.242641 & 270.4±10.18234 mg/g, ascorbic acid equivalents.

Table 51. Comparison of total antioxidant capacity of C_2H_5OH, petroleum ether, ethyl acetate, CH_3OH and $CHCl_3$ extracts of *A. nilotica* leaves at 100 µg/ml concentration.

Samples	Total antioxidant capacity (mg/g, ascorbic acid equivalents)
C_2H_5OH extract	63.475±6.399316
Petroleum ether extract	54.60±0.9899490
Ethyl acetate extract	54.15±5.1618800
CH_3OH extract	54.075±3.853732
$CHCl_3$ extract	36.575±9.510586

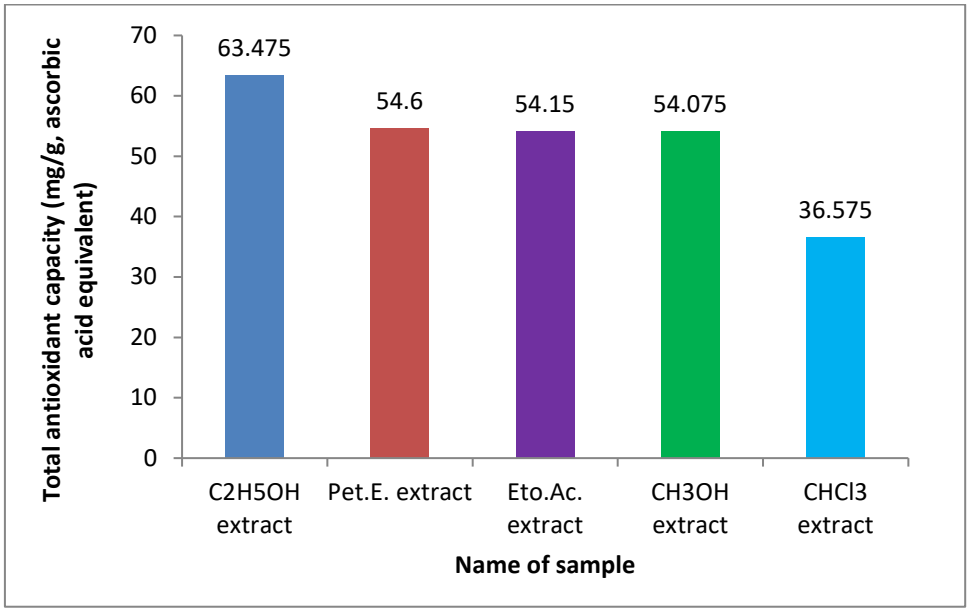


Fig.23: Total antioxidant capacity of different extracts at 100 µg/ml concentration.

Table 52. Comparison of total antioxidant capacity of C_2H_5OH, petroleum ether, ethyl acetate, CH_3OH and $CHCl_3$ extracts of *A. nilotica* leaves at 6.25 µg/ml concentration.

Samples	Total antioxidant capacity (mg/g, ascorbic acid equivalents)
C_2H_5OH extract	295.2±38.46661
Petroleum ether extract	274.8±16.40488
Ethyl acetate extract	280.7±90.65109
CH_3OH extract	332.8±72.40773
$CHCl_3$ extract	270.4±10.18234

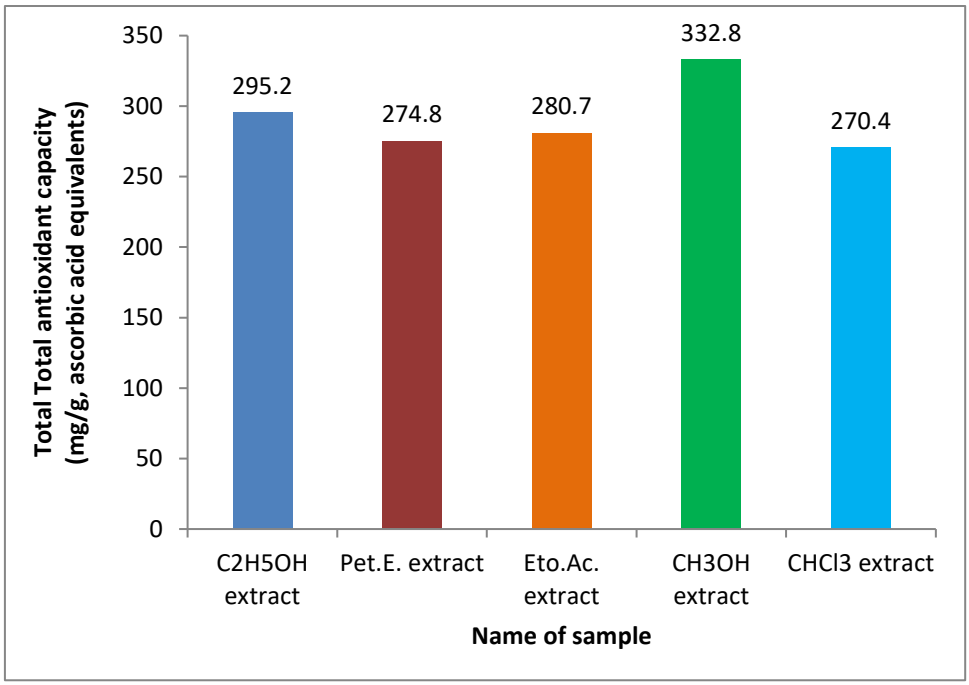


Fig.24: Total antioxidant capacity of different extracts at 6.25 µg/ml concentration.

3.8.2 Discussion

The antioxidant effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free redicals, quenching single and triplet oxygen, or decomposing peroxides.

The phosphomolybdenum method is used on the reduction of Mo (VI) - Mo (V) by the antioxidant compound and the fraction of green phosphate (PO4)/Mo (V) complex with a maximum absorption at 695 nm. Total antioxidant capacity of the plant extracts expressed as the number of mg/g equivalent of ascorbic acid are shown in table no.(46-50) and in fig.22. Total antioxidant capacity of ethanol, petroleum ether, ethyl acetate, methanol & chloroform extract of *A.nilotica* leaves at 100 µg/ml is 63.475±6.399316, 54.60±0.989949, 54.15±5.16188, 54.075±3.853732 & 36.575±9.510586 mg/g, ascorbic acid equivalents, respectively. So, ethanolic extract has highest total antioxidant capacity among all the tested samples.

3.9 Determination of reducing power capacity

3.9.1 Result

Table 53. Reducing power of ascorbic acid (standard) at different concentrations.

Sample	Concentration (µg/ml)	Absorbance			Abs. mean±STD
		a	b	c	
Ascorbic acid	6.25	0.641	0.648	0.6532	0.6474±0.006122
	12.5	1.07	1.04	1.05	1.053333±0.015275
	25	1.65	1.542	1.492	1.561333±0.080755
	50	2.121	2.224	2.17	2.171667±0.05152
	100	3.01	3.04	3.001	3.017±0.020421

Values are the average of duplicate experiments and represented as mean ± standard deviation.

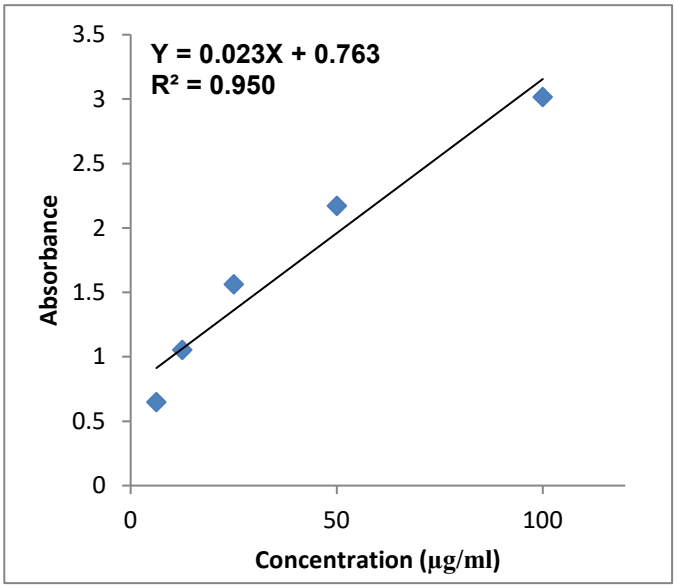


Fig.25: Reducing power of ascorbic acid (Standard) at different concentrations with curve.

Table 54. Reducing power of CH_3OH extract of *A. nilotica* leaves at different concentrations.

Sample	Concentration (µg/ml)	Absorbance			Abs. mean±STD
		a	b	c	
CH_3OH extract	6.25	0.148	0.103	0.124	0.125±0.022517
	12.5	0.349	0.194	0.375	0.306±0.097862
	25	0.621	0.328	0.526	0.491667±0.149487
	50	1.143	0.606	0.743	0.830667±0.279027
	100	2.045	1.130	1.262	1.479±0.494594

Values are the average of duplicate experiments & represented as mean ± standard deviation.

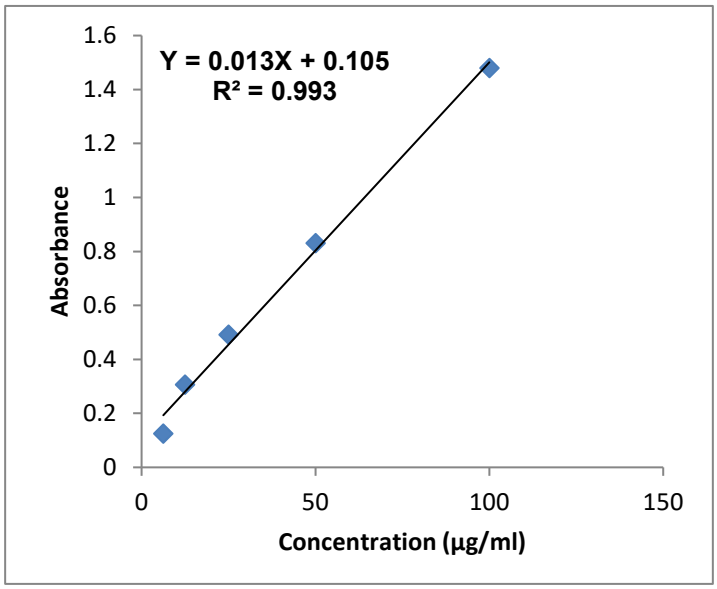


Fig.26: Reducing power of CH_3OH extract of *A. nilotica* leaves at different concentrations with curve .

Table 55. Reducing power of C_2H_5OH extract of *A. nilotica* leaves at different concentrations.

Sample	Concentration (µg/ml)	Absorbance			Abs. mean ± STD
		a	b	c	
C_2H_5OH extract	6.25	0.53	0.55	0.52	0.533333±0.015275
	12.5	1.208	0.537	0.755	0.833333±0.34229
	25	1.121	0.93	1.603	1.218±0.346827
	50	2.018	1.453	1.67	1.713667±0.28502
	100	4.00	2.51	2.336	2.948667±0.914629

Values are the average of duplicate experiments & represented as mean ± standard deviation.

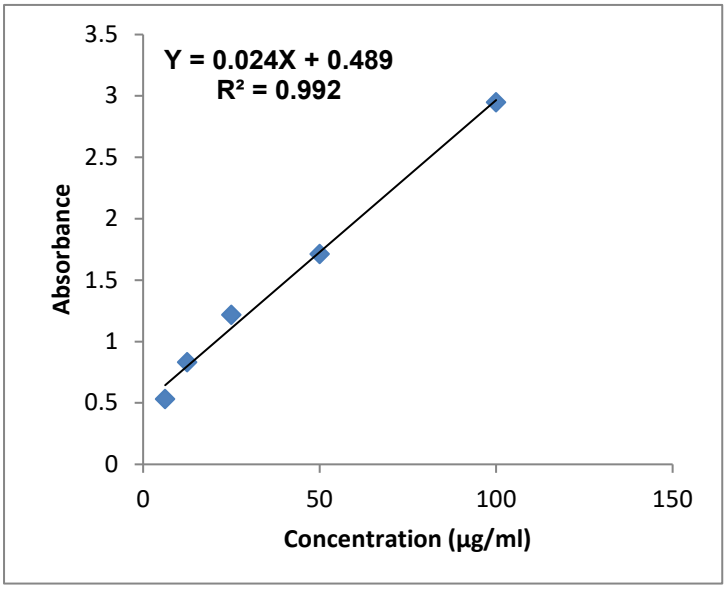


Fig.27: Reducing power of C_2H_5OH extract of *A. nilotica* leaves at different concentrations with curve .

Table 56. Reducing power of $CHCl_3$ extract of *A.nilotica* leaves at different concentrations.

Sample	Concentration (µg/ml)	Absorbance		Abs. mean±STD
		a	b	
$CHCl_3$ extract	6.25	0.045	0.086	0.0655±0.028991
	12.5	0.078	0.105	0.0915±0.019092
	25	0.163	0.201	0.182±0.02687
	50	0.298	0.278	0.2925±0.007778
	100	0.495	0.503	0.499±0.005657

Values are the average of duplicate experiments & represented as mean± standard deviation.

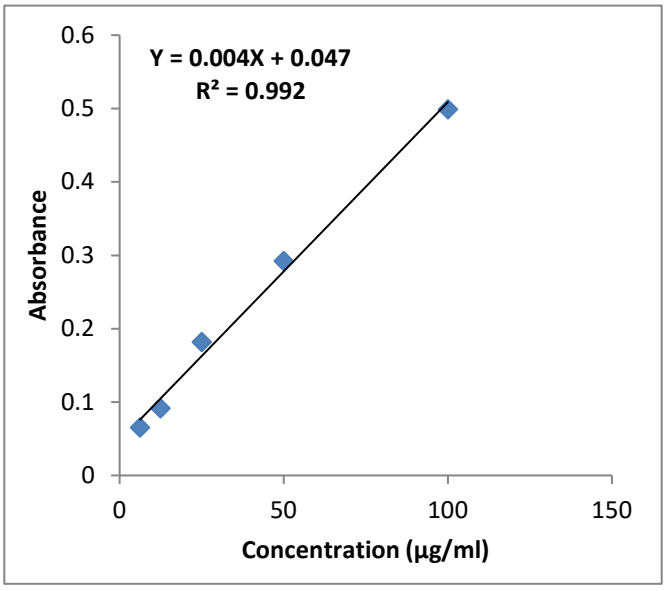


Fig.28: Reducing power of $CHCl_3$ extract of *A. nilotica* leaves at different concentrations with curve .

Table 57. Reducing power of ethyl acetate extract of *A. nilotica* leaves at different concentrations.

Sample	Concentration (µg/ml)	Absorbance		Abs. mean±STD
		a	b	
Ethyl acetate extract	6.25	0.23	0.189	0.2095±0.028991
	12.5	0.32	0.311	0.3155±0.006364
	25	0.552	0.69	0.621±0.097581
	50	0.880	1.084	0.982±0.14425
	100	1.525	1.749	1.637±0.158392

Values are the average of duplicate experiments & represented as mean± standard deviation.

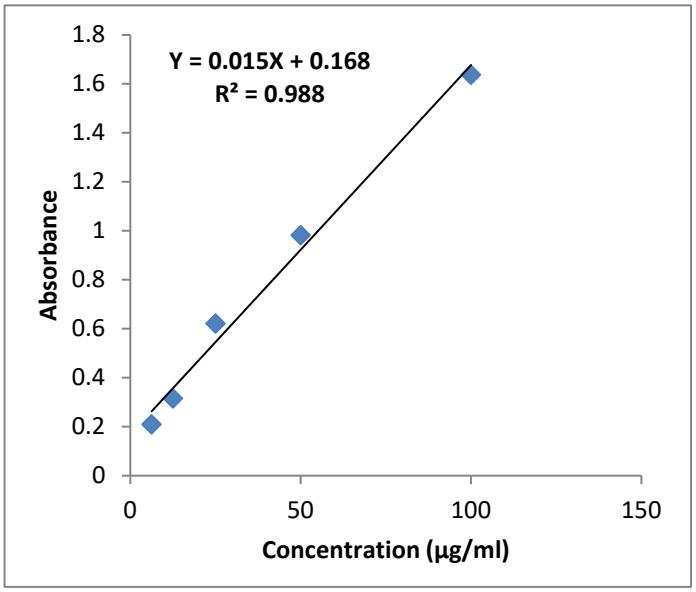


Fig.29: Reducing power of ethyl acetate extract of *A. nilotica* leaves at different concentrations with curve .

Table 58. Reducing power of petroleum ether extract of *A.nilotica* leaves at different concentrations.

Sample	Concentration (µg/ml)	Absorbance		Abs. mean±STD
		a	b	
Petroleum ether extract	6.25	0.068	0.053	0.0605±0.010607
	12.5	0.133	0.112	0.1225±0.014849
	25	0.269	0.256	0.2625±0.009192
	50	0.483	0.555	0.519±0.050912
	100	0.960	1.000	0.98±0.028284

Values are the average of duplicate experiments & represented as mean± standard deviation.

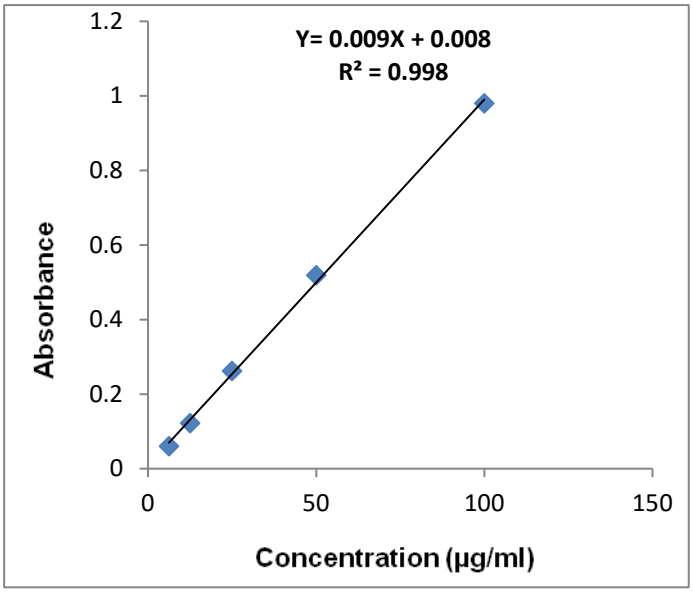


Fig.30: Reducing power of petroleum ether extract of *A. nilotica* leaves at different concentrations with curve.

Table 59. Comparison of reducing power capacity of ascorbic acid and different extracts of *A. nilotica* leaves at 100 µg/ml concentration.

Standard / samples	Iron reducing power capacity (mg/g)
Ascorbic acid	3. 017±0.020421
Ethanol extract	2. 948667±0.914629
Methanol extract	1.479±0.494594
Petroleum ether extract	0.98±0.028284
Chloroform extract	0.499±0.005657
Ethyl acetate extract	1.637±0.158392

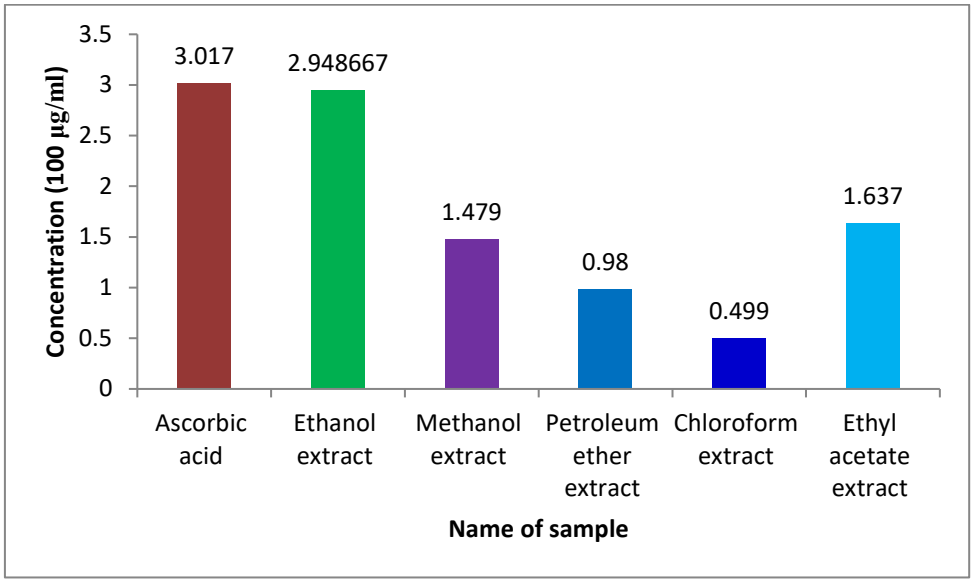


Fig.31:Reducing power capacity (mg/g) of different extracts with ascorbic acid at 100 µg/ml concentration.

3.9.2 Discussion

The Fe^{3+} reducing power of *A. nilotica* leaves extracts is determined by Qyaizu method (1986) with slight modification. The reducing properties are generally associated with the presence of reductants, which have shown to exert antioxidant

action by breaking the free radical chain by donating a hydrogen atom. The reductive capabilities of the extract are shown in Table no.54-58. & in Fig.26-30.. At 6.25 µg/ml, the absorbance of ethanol extract and ascorbic acid is 0.533333 mg/g and 0.6474 mg/g, respectively. At 100 µg/ml, absorbance of ethanol extract and ascorbic acid is 2.948667 mg/g and 3.017 mg/g respectively. The other extracts also show significant reducing power activities as compared to ascorbic acid. It is found that the reducing power increased with concentration of each sample. High absorbance indicates high reducing power. The ranking order of reducing power is ethanol > ethyl acetate >methanol > petroleum ether >chloroform extract. Significantly higher reducing power is evident in Ethanol extract.

3.10 Determination of DPPH radical scavenging activity

3.10.1 Result

Table 60. DPPH radical scavenging activity of ascorbic acid (standard).

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
Ascorbic acid	3.125	0.547	0.450	0.502	11.060	26.89	18.37	18.75333±7.891985	7.9
	6.25	0.411	0.269	0.595	33.170	56.26	3.25	30.89333±26.57823	
	12.5	0.023	0.022	0.107	96.260	96.42	82.60	91.76000±7.933196	
	25	0.017	0.022	0.078	97.240	96.42	87.32	93.66000±5.505888	

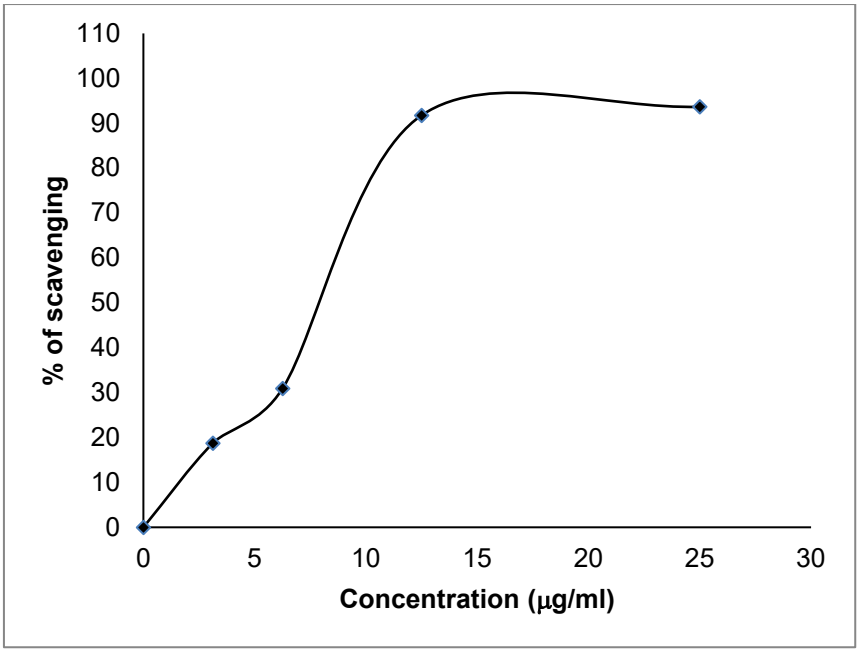


Fig.32: IC_{50} for DPPH radical scavenging activity of ascorbic acid (standard)

Table 61. DPPH radical scavenging activity of ethanolic extract of *A. nilotica* leaves.

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
Ethanol extract of *A.nilotica*	3.125	0.526	0.539	0.513	14.47	12.36	16.59	14.47333±2.115002	8.5
	6.25	0.403	0.354	0.377	34.47	42.44	38.70	38.53667±3.98751	
	12.5	0.261	0.166	0.242	57.56	73.01	60.65	63.74±8.175372	
	25	0.051	0.033	0.100	91.71	94.63	83.74	90.02667±5.636775	

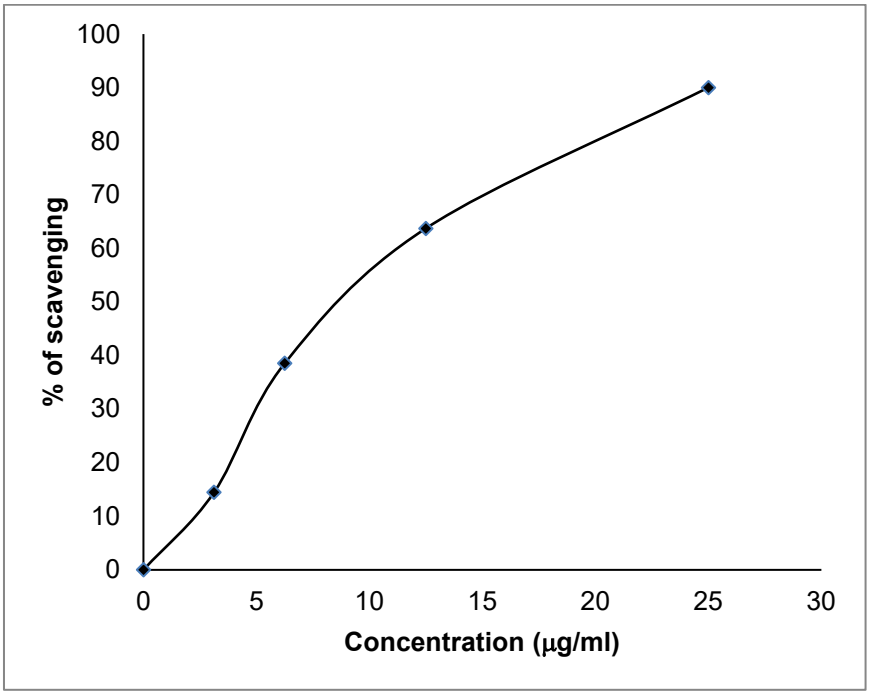


Fig.33: IC_{50} for DPPH radical scavenging activity of ethanol extract of *A. nilotica* leaves

Table 62. DPPH radical scavenging activity of petroleum ether extract of *A. nilotica* leaves.

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
Pet.E. extract of *A. nilotica*	3.125	0.559	0.535	0.577	9.11	13.01	6.18	9.433333±3.426461	24.25
	6.25	0.520	0.539	0.501	15.45	12.36	18.54	15.45±3.09	
	12.5	0.457	0.436	0.477	25.69	29.11	22.44	25.74667±3.335361	
	25	0.262	0.337	0.299	57.40	45.20	51.38	51.32667±6.100175	

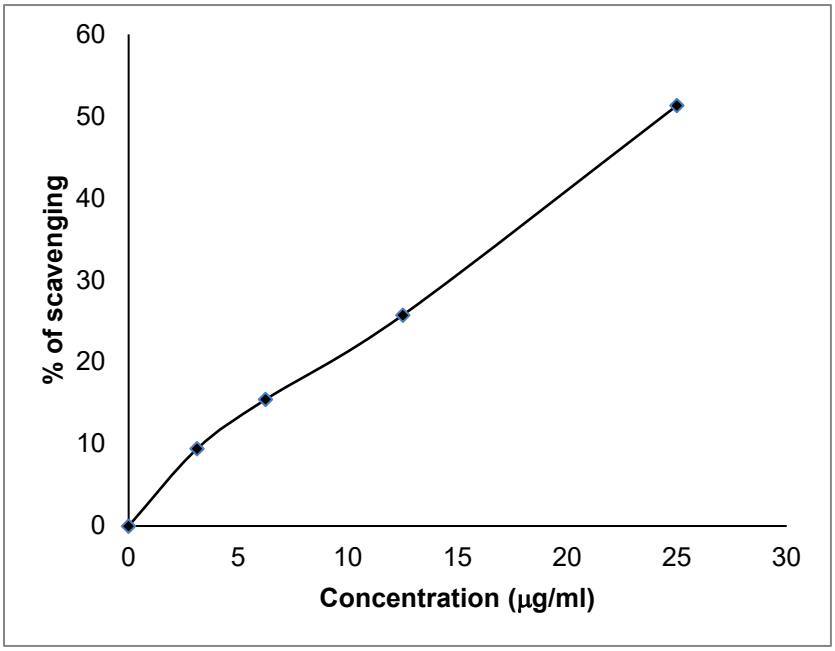


Fig.34: IC_{50} for DPPH radical scavenging activity of petroleum ether extract of *A. nilotica* leaves

Table 63. DPPH radical scavenging activity of CH_3OH extract of *A. nilotica* leaves.

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
CH_3OH extract of *A. nilotica*	3.125	0.563	0.556	0.570	8.46	9.59	7.32	8.456667±1.135004	24.50
	6.25	0.512	0.350	0.431	16.75	43.09	29.92	29.92±13.17	
	12.5	0.552	0.307	0.429	10.24	50.08	30.24	30.18667±19.92005	
	25	0.415	0.198	0.306	32.52	67.80	50.24	50.18667±17.64006	

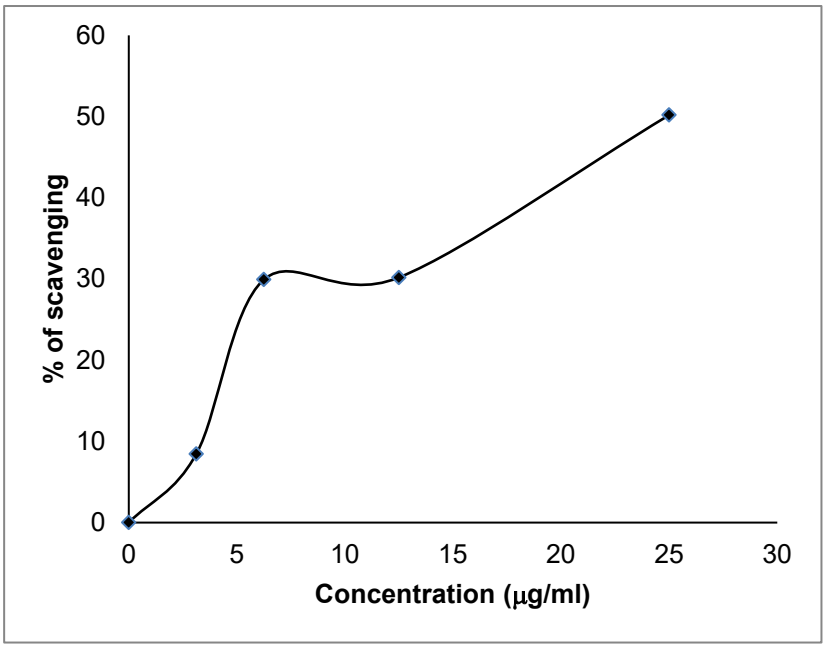


Fig.35: IC_{50} for DPPH radical scavenging activity of CH_3OH extract of *A. nilotica* leaves

Table 64. DPPH radical scavenging activity of $CHCl_3$ extract of *A. nilotica* leaves.

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
$CHCl_3$ extract of *A. nilotica*	3.125	0.54	0.536	0.538	12.20	12.85	12.52	12.52333±0.325013	27.8
	6.25	0.482	0.492	0.487	21.63	20.00	20.81	20.81333±0.815005	
	12.5	0.41	0.339	0.374	33.33	44.88	39.19	39.13333±5.775209	
	25	0.355	0.311	0.313	42.28	49.43	49.11	46.94±4.038849	
	50	0.228	0.106	0.167	62.93	82.76	72.85	72.84667±9.915	
	100	0.099	0.045	0.072	83.90	92.68	88.29	88.29±4.39	

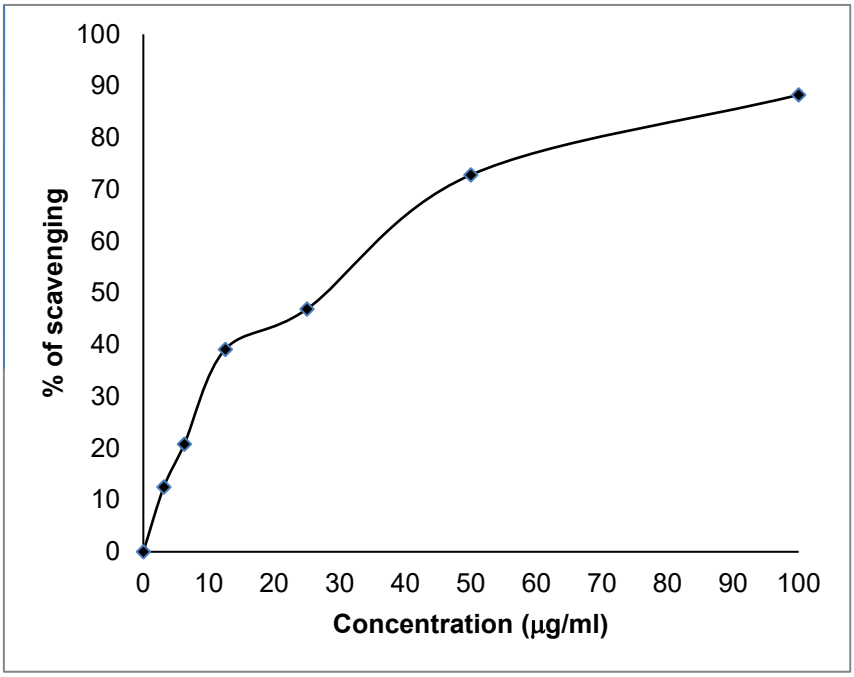


Fig.36: IC_{50} for DPPH radical scavenging activity of $CHCl_3$ extract of *A. nilotica* leaves

Table 65. DPPH radical scavenging activity of ethyl acetate extract of *A.nilotica* leaves.

Sample	Concentration (µg/ml)	Absorbance			% of scavenging			% of scavenging mean±STD	IC_{50} (µg/ml)
		a	b	c	a	b	c		
Eto.Ac. extract of *A. nilotica*	3.125	0.604	0.605	0.602	1.79	1.63	2.11	1.843333±0.244404	48
	6.25	0.556	0.499	0.528	9.59	18.86	14.15	14.20±4.635202	
	12.5	0.546	0.475	0.510	11.22	22.76	17.07	17.01667±5.770185	
	25	0.492	0.411	0.464	20.00	33.17	24.55	25.90667±6.688993	
	50	0.351	0.288	0.259	42.92	53.17	57.89	51.32667±7.653341	
	100	0.164	0.133	0.158	73.33	78.37	74.31	75.33667±2.672252	

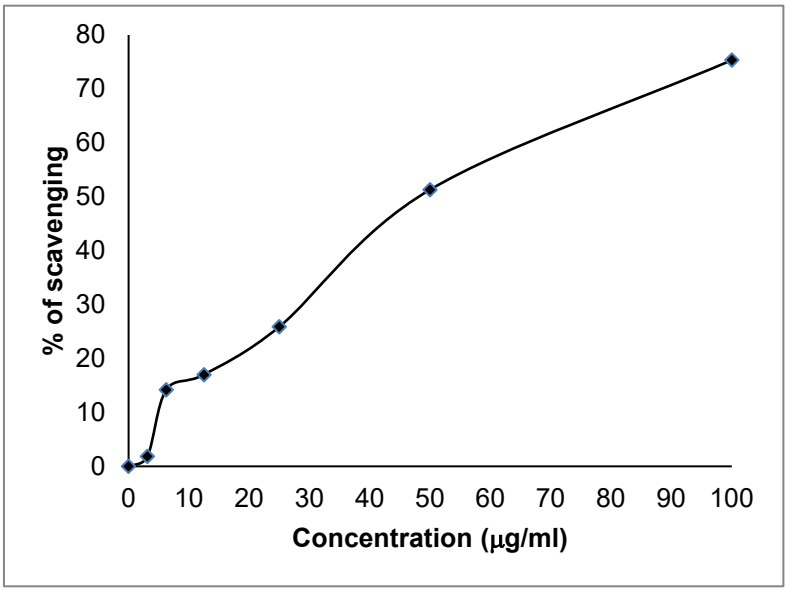


Fig.37: IC_{50} for DPPH radical scavenging activity of ethyl acetate extract of *A. nilotica* leaves

Table 66. Comparative of IC_{50} values of ascorbic acid (standard) & different extracts of *A. nilotica* leaves.

Standard/sample	IC_{50} (µg/ml)
Ascorbic acid	7.9
Ethanol extract	8.5
Petroleum ether extract	24.25
Chloroform	27.8
Ethyl acetate extract	48
Methanol extract	24.50

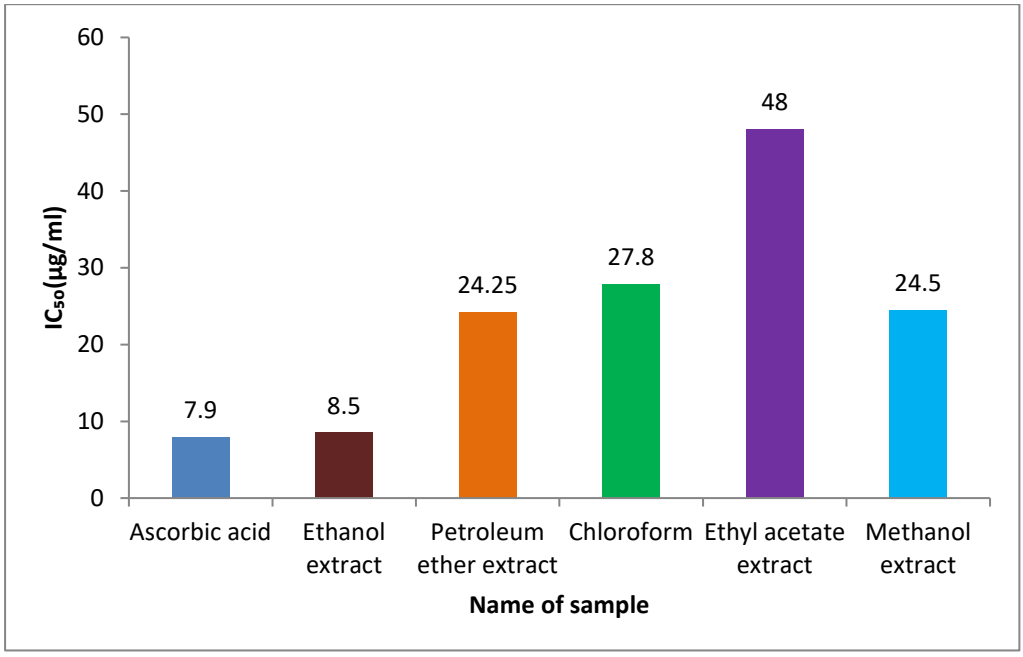


Fig.38: Comparison of IC_{50} (µg/ml) values of different extracts of *A.nilotica* leaves for free radical scavenging activity by DPPH method.

3.10.2 Discussion

DPPH antioxidant assay is based on the ability 1-1 diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and % of scavenging activity is calculated. The activity is increased by increasing the concentration of the sample extract. Concentration providing 50% inhibition (IC_{50}) is calculated from the graph plotted inhibition percentage versus concentration by linear regression analysis

The results of DPPH radical scavenging assays on extract and standard (ascorbic acid) are shown in table (60-65) and IC_{50} value of the extracts are presented in fig (32-37). The IC_{50} values of C_2H_5OH, Pet.E, $CHCl_3$, Eto.Ac. and CH_3OH extracts of *A. nilotica* leaves are 8.5, 24.25, 27.8, 48 & 24.50 µg/ml, respectively. The IC_{50} values of ethanol extract is 8.5 µg/ml, respectively. The IC_{50} value of standard (ascorbic acid) is 7.9 µg/ml. So, among all the extracts ethanol extract shows highest radical scavenging activity (8.5 µg/ml) which is also very close to standard (ascorbic acid). The smaller is the IC_{50} values, the higher is the antioxidant activity of the plant extract.

Conclusion:

The present study demonstrates that the extracts of dried leaves of *A.nilotica, A.indica and P.guajava* contains pharmacologically active substances, possessing significant antibacterial activities against *E.coli, S.boydii, S.dysenteriae, S.flexneri, S.shiga, S.sonne &, V.cholerae* and cytotoxic activities on *A.salina*. Among all the extracts, ethanolic extract of *A.nilotica* leaves showed highest antibacterial and cytotoxic activities, and it was purified by PTLC & OCC methods. The purities and bioactivities of single purified compound was reconfirmed by NMR study and further experiment on the same diarrhoeal bacteria. It was found that the compound was effective against all types of diarrhoeal bacteria. The isolated bioactive purified compound was a steroid analogue and might be the derivatives of sitosterol. The present data provided a scientific support for the traditional use of this plant as drug. However, more detailed phytochemical analysis will be necessary to isolate and characterize the active compounds which are responsible for the antibacterial activities and to understand exact mechanisms of action of these activities. This would be helpful to creates awareness among people for taking control measures based on herbal plants against diarrhoea, dysentery and other infectious diseases and thereby contribute a lot to the rural poor people of developing countries like Bangladesh.

References

Abatan, M.O., Makinde M.J., (1986). Screening *Azadirachta indica* and *Pisum sativum* for possible antimalarial activities. *J Ethnopharmacol* **17** (1):85–93.

Abbott, W. S.,(1925). A method of computing the effectiveness of an insecticide, *J.Econ. entomol* **18**:265-267.

Abdel-Shafy, S., Zayed , A.A., (2002). *In vitro* acaricidal effect of plant extract of neem seed oil (*Azadirachta indica*) on egg, immature, and adult stages of Hyalomma anatolicum excavatum (Ixodoidea: Ixodidae). *Vet Parasitol* **106**(1): 89–96.

Agrawal, S., Kulkarni, GT., Sharma, VN. A Comparative Study on the Antioxidant Activity of Methanol Extracts of *Acacia nilotica* and *Berberis chitria*. *Adv in Nat Appl Sci* **4**(1):78-84.

Aguilar, A., Argueta, A., Cano, L., (1994). Flora Medicinal Indigena de Mexico. Instituto Nacional Indigenista, Mexico.

Agunua, A., Yusuf, S., Andrewa, GO., Zezi, AU., (2005). Abdurahmana EM. Evaluation of five medicinal plants used in diarrhoea treatment in Nigeria. *Journal of Ethnopharmacology*. **101**:27–30.

Ajayi, F.A., (2002). Formulation and promotion of neem—seed derived Biopesticides as alternative to persistent organic pollutants (POPs) for the control of vegetable crop pests. In: Ukwe CN, Folorunso AO, Ibe AC, Z Z, N.E.S NES, Sieghart L (eds) Sustainable industrial utilization of neem tree (*Azadirachta indica*) in Nigeria. UNIDO Regional Development Centre, Lagos, 83–90.

Aladakatti, R.H., Ahamed, R.N., (1999). Effect of *Azadirachta indica* leaves on rat spermatozoa. *Indian J Exp Biol* **37**(12):1251–1254.

Aladakatti, R.H., Nazeer Ahamed, R., Ahmed, M., Ghosesawar, M.G., (2001). Sperm parameters changes induced by *Azadirachta indica* in albino rats. *J Basic Clin Physiol Pharmacol* **12**(1):69–76.

Ali, B.H., (1987). The toxicity of *Azadirachta indica* leaves in goats and guinea pigs. *Vet Hum Toxicol* **29**(1):16–19.

Alli, L.A., Adesokan, A.A., Salawu, O.A., Akanji, M.A., Tijani, A.Y.,(2011). Anti-plasmodial activity of aqueous root extract of *Acacia nilotica, African Journal of Biochemistry Research* **5**(7):214-219.

Almas, K., (1999). The antimicrobial effects of extracts of *Azadirachta indica* (Neem) and Salvadora persica (Arak) chewing sticks. *Indian J Dent Res* **10**(1):23–26.

Al-Rajhy, D.H., Alahmed, A.M., Hussein, H.I., Kheir, S.M., (2003). Acaricidal effects of cardiac glycosides, azadirachtin and neem oil against the camel tick, Hyalomma dromedarii (Acari: Ixodidae). *Pest Manag Sci* **59**(11):1250–1254.

Alzoreky, N.S., Nakahara, K., (2003). Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *Int J Food Microbiol* **15**;80(3):223–230.

Ameh, J.S. *et al*.,(2010). Pods and roots of *Acacia nilotica* have demulcent, styptic and astringent properties. The stem bark can be ingested for leucorrhoea, diarrhea, dysentery, cough and diabetes mellitus type II. *Tropical Journal of Pharmaceutical Research* (**2**):119-125.

Amos, S., Akah, C.J., Odukwe, K.S., Wambede, C.,(1999). The Pharmacological Effects of an Aqueous Extract from *Acacia nilotica* Seeds. *Phytothe Res* **13**:683–685.

Amstrong, D. and Cohen, J., (1999). Infectious Diseases. 2000; £250(two volumes). London: Mosby.

Anonymous.(2003). The Wealth of India, Raw materials. Vol 9. New Delhi CSIR , 37-41.

Antaki, A.D., Tazkiradu Ulil Albab,Ed. 1.,(1998). Beirut: Dar Alkutub Al Ilmiyah 1-3.

Anwar, H.G., Qaiser, J. (2005). Atispasmodie effect of *Acacia Arabica* are mediate through K+ channel activation. *J Ethnopharmacol* , **100**: 347-52.

Arima, H., Danno, G., (2002). Isolation of antimicrobial compounds from guava (*Psidium guajava* L.). *Bioscience, Biotechnology and Biochemistry* **66**, 727–1730.

Arivazhagan, S., Balasenthil, S., Nagini, S., (2000). Garlic and neem leaf extracts enhance hepatic glutathione and glutathione dependent enzymes during N-methyl-N0-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in rats. *Phytother Res* **14**(4):291–293.

Arivazhagan, S., Nagini, S., Santhiya, S.T., Ramesh, A., (2003). N-methyl-N0-nitro-N-nitrosoguanidine (MNNG)-induced in vivo clastogenicity: protective effects of aqueous neem leaf extract. *Pharmazie* **58**(10):750–752.

Arivazhagan, S., Velmurugan, B., Bhuvaneswari, V., Nagini, S., (2004). Effects of aqueous extracts of garlic (*Allium sativum*) and neem (*Azadirachta indica*) leaf on hepatic and blood oxidant–antioxidant status during experimental gastric carcinogenesis. *J Med Food* **7**(3):334–339.

Arora. S., Kaur, K., Kaur, S. (2003). Indian medicinal plants as a reservoir of Protective phytochemicals. Teratogenesis Carcinog Mutagen Suppl. **1**: 295-300.

Asad, M., Aslam, M., Munir, T.A., Nadeem, A.,(2011). Effect of *Acacia nilotica* Leaves Extract on Hyperglycaemia, Lipid profile and Platelet Aggregation in Streptozotocin Induced Diabetic Rats. *J Ayub Med Coll Abbottabad* .**23**(2).

Ashkenazi,S. and Pickering, L.K.,(1991). New causes of infectious diarrhoea. Eur.*J. Clin. Microbiol. Infect. Dis*. **10**(1): 1-3.

Asolkar, L.V., Kakkar, K.K., Chakre, O.J.,(2005). Glossary of Indian medicinal plants with active principles. Part I (A-K). New, Delhi NISCAIR and CSIR : 10-11.

Awasthy, K.S., (2001). Genotoxicity of a crude leaf extract of neem in male germ cells of mice. *Cytobios.* **106**(Suppl 2):151–164.

Awasthy, K.S., Chaurasia, O.P., Sinha, S.P., (1999). Prolonged murine genotoxic effects of crude extracted from neem. *Phytother Res* **13**(1):81–83.

Ayub HM, Tarjuma Aqsarai. Vol 1. Lucknow: Munshi Naval Kishore 1877, 403, 751.

Babu, P.S., Prince, P.S.M., (2004). Antihyperglycaemic and antioxidant effect of hyponidd, an ayurvedic herbomineral formulation in streptozotocin-induced diabetic rats. *J Pharm Pharmacol* **56**(11):1435–1442.

Badam, L., Joshi, S.P., Bedekar, S.S., (1999). '*In vitro'* antiviral activity of neem (*Azadirachta indica* A. Juss) leaf extract against group B coxsackieviruses. *J Commun Dis* **31**(2):79–90.

Baitar I. Al Jameul Mufredat Al Advia wa Al Aghziya. (Urdu translation). Vol 2, New Delhi CCRUM, 1999, 226-27.

Balasenthil, S., Arivazhagan, S., Ramachandran, C.R., Ramachandran, V., Nagini, S., (1999). Chemopreventive potential of neem (*Azadirachta indica*) on 7,12-dimethylbenz[a]- anthracene (DMBA) induced hamster buccal pouch carcinogenesis. *J Ethnopharmacol* **1**;67(2):189–295.

Bandyopadhyay, U., Biswas, K., Sengupta, A., Moitra, P., Dutta, P., Sarkar, D., Debnath, P., Ganguly, C.K., Banerjee, R.K., (2004). Clinical studies on the effect of Neem (*Azadirachta indica*) bark extract on gastric secretion and gastroduodenal ulcer. *Life Sci* **29**;75(24):2867–2878.

Bangladesh Bureau of statistics(BBS) and UNICEF(1996) Progotir Pathey: Achieving the Mid Decade Goals for Children in Bangladesh.

Banso, A.,(2009). Phytochemical and antibacterial investigation of bark extracts of *Acacia nilotica. Journal of Medicinal Plants Research*. **3**(2):082-085.

Baral, R., Chattopadhyay, U., (2004). Neem (*Azadirachta indica*) leaf mediated immune activation causes prophylactic growth inhibition of murine Ehrlich carcinoma and B16 melanoma. *Int Immunopharmacol* **4**(3):355–366.

Bardhan, J., Riar, S.S., Sawhney, R.C., Kain, A.K., Thomas, P., Ilavazhagan, G., (1991). Neem oi a fertility controlling agent in rhesus monkey. *Indian J Physiol Pharmacol* **35**(4): 278–280.

Bargali, S.S., Singh, S.P. and Pandya, K.S. ,(2004). Effect of *Acacia nilotica* on gram crop in a traditional agroforestry system of Chhattisgarh plains. *Int. J. Eco. Environ.Sci*. **30**,363-368.

Bauer,A,W., Kirby, W.M.M., Sherris, J.C. and Turck M.,(1966). Antibiotic susceptibility testing by a standardized single disc method. *Am.J.Clin. Pahol.,* **45**, 403-496.

Begum, S., Hassan, S.I., Siddiqui, B.S., (2002a). Two new triterpenoids from the fresh leaves of *Psidium guajava*. *Planta Medical* **68**, 1149–1152.

Begum, S., Hassan, S.I., Siddiqui, B.S., (2004). Chemical constituents from the leaves of *Psidium guajava*. *Natural Product Research* **18**, 135–140.

Begum, S., Hassan, S.I., Siddiqui, B.S., Shaheen, F., Ghayur, M.N., Gilani, A.H., (2002b). Triterpenoids from the leaves of *Psidium guajava*. *Phytochemistry* **61**, 399–403.

Beniwal, R.S., Toky, O.P.and Sharma, P.K.,(1992). Effect of VA mycorrhizal fungi and phosphorus on growth and nodulation of *Acacia nilotica* (L.) Willd ex Del. *Crop Res*. **5**, 172-176.

Bhatt,B.P., Panwar, M.S. (1990). Plants with fertility regulating potential of Garhlwal Hiimalayas. *J Econ Phytochem* **1**: 33-34.

Bhattacharyya ,N., Chutia, M., Sarma, S., (2007). Neem (*Azadirachta indica* A. Juss), a potent biopesticide and medicinal plant: a review. *J Plant Sci* 2(3):251–259.

Bhonde, S.B., Deshpande, S.G., Sharma, R.N., (1999). In vitro evaluation on inhibitory nature of some neem formulations against plant pathogenic fungi. *Hindustan Antibiot Bull* **41**(1–4):22–24.

Biswas, K., Chattopadhyay, R.K., Banerjee, U., Bandyopadhyay, U., (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*). *Cur Sci* **82**: 1336–1345.

Boeke, S.J., Boersma, M.G., Alink, G.M., Van Loon, J.J., Van Huis, A., Dicke, M., Rietjens, I.M., (2004). Safety evaluation of neem (*Azadirachta indica*) derived pesticides. *J Ethnopharmacol* **94**(1):25–41.

Boral, D., Chatterjee, S., Bhattacharya, K., (2004). The occurrence and allergising potential of airborne pollen in West Bengal. *India Ann Agric Environ Med* **11**(1):45–52.

Bowen, G.D.,(1973). Mineral nutrition in ectomycorrhizae. In: *Ectomycorrhizae: their ecology and physiology* (eds Marks G.C. and Kozlowaski T.T.). Academic Press, New York. 161-205.

Brahmachari, G., (2004). Neem—an omnipotent plant: a retrospection. *Chem BioChem* **5**:408–421.

Busvine, J.R.,(1971). A critical review of the techniques for testing insecticides: Commonwealth Agricultural Buereux:345.

Cabieses, M.F., (1993). Apuntes de la Medicina Tradicional: la racionalidad de lo irracional. II. Lima Per´u, 123–128.

Carcinog Mutagen Suppl (2003); 1:295–300.

Champion, H.G. and Seth, S.K.,(1968). *A revised survey of the forest types of India*. The manager Govt of India press Nasik.

Chandel, B.S., Shah, N.M., Tripathi, R.M..(1992). *In vitro* antibacterial activity of *Acacia Arabica* bark. *Indian J Indigenous Med* **9**:77-79.

Chandra, P., Khuda-Bukhsh, A.R., (2004). Genotoxic effects of cadmium chloride and azadirachtin treated singly and in combination in fish. *Ecotoxicol Environ Saf* **58**(2): 194–201.

Charles,W.W., Philip, E.S., Carl,W.C., (2006). Determination of organic acids and sugars in *guajava* L. cultivars by high-performance liquid chromatography. *Journal of the Food and Agriculture* **33**, 777–780.

Chatterjee, A., Pakrashi, S.C.,(2000). The Treatise on Indian Medicinal Plants. Vol 2, New Delhi NISCAIR Press, 51-3.

Chattopadhyay, I., Nandi, B., Chatterjee, R., Biswas, K., Bandyopadhyay, U., Banerjee, R.K., (2004). Mechanism of antiulcer effect of Neem (*Azadirachta indica*) leaf extract: effect on H?-K?-ATPase oxidative damage and apoptosis. *Inflammopharmacol* 12(2):153–176.

Chattopadhyay, R.R., (1996). Possible mechanism of antihyperglycemic effect of *Azadirachta indica* leaf extract. Part IV. *Gen Pharmacol* **27**(3):431–434.

Chattopadhyay, R.R., (1998). Possible biochemical mode of anti-inflammatory action of *Azadirachta indica* A. Juss. in rats. *Indian J Exp Biol* **36**(4):418–420.

Chattopadhyay, R.R., (2003.) Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: part II. *J Ethnopharmacol* **89**(2–3):217–219.

Chinnasamy, N., Harishankar, N., Kumar, P.U., Rukmini, C., (1993). Toxicological studies on debitterized neem oil (*Azadirachta indica*). *Food Chem Toxicol* **31**(4): 297–301.

Choi, H.Y., Jhun, E.J. and B.O.,(2000). Application of flow injection chemilumineacence to the study of radical scavenging activity in plants. *Phytotheraoy Research*, **14**: 250-253.

Cholera(2008) Principles of Infectious Disease Epidemiology. Principles of Infectious Disease Epidemiology/ EPI 220 UCLA School of Public Health/ Department of Epidemiology.

Chopra, R.N., Nayar, S.L,, Chopra, I.C.,(2002). Glossary of Indian Medicinal Plants. Ed 6, New Delhi NISCIR 2002 , 2.

Choudhary, D.N., Singh, J.N., Verma, S.K., Singh, B.P., (1990). Antifertility effects of leaf extracts of some plants in male rats. *Indian J Exp Biol* **28**(8):714–716.

Complimentary and Alternative Medicine (2008); **5**(4):345.

Conde, G.E.A., Nascimento, V.T., Santiago, S.A.B., (2003). Inotropic effects of extracts of *Psidium guajava* L. (guava) leaves on the guinea pig atrium. *Brazilian Journal of Medical and Biological Research* **36**, 661– 668.

Conway, P., (2002). Tree Medicine: A Comprehensive Guide to the Healing Power of Over 170 Trees. 2001. Judy Piatkus (Publishers) Ltd, 2173– 2177.

Damayanti, M., Susheela, K., Sharma, G.J., (1996). Effect of plant extracts and systemic fungicide on the pineapple fruitrotting fungus, Ceratocystis paradoxa. *Cytobios.* **86**(346): 155–165.

Das, B.K., Mukherjee, S.C., Sahu, B.B., Murjani, G., (1999b) Neem (*Azadirachta indica*) extract as an antibacterial agent against fish pathogenic bacteria. *Indian J Exp Biol* **37**(11):1097–1100.

Das, N.G., Nath, D.R., Baruah, I., Talukdar, P.K., Das, S.C., (1999a) Field evaluation of herbal mosquito repellents. *J Commun Dis* **31**(4):241–245.

Dasgupta ,T., Banerjee, S., Yadava, P.K., Rao, A.R., (2004). Chemopreventive potential of *Azadirachta indica* (neem) leaf extract in murine carcinogenesis model systems. *J Ethnopharmacol* **92**(1):23–36.

De Azambuja, P., Garcia, E.S., (1992). Effects of azadirachtin on Rhodnius prolixus: immunity and Trypanosoma interaction. *Mem Inst Oswaldo Cruz* **87**(Suppl 5):69–72.

Deshpande, S.N.,(2013). Preliminary Phytochemical Analysis and In Vitro Investigation of Antibacterial Activity of *Acacia nilotica* against Clinical Isolates. *Journal of Pharmacognosy and Phytochemistry* **1**(5):23-27.

Deshpande,V.Y., Mendulkar, K.N., Sadre, N.L., (1980). Male antifertility activity of *Azadirachta Indica* in mice. *J Postgrad Med* **26**(3):167–170.

Desmarcheliar, C., Repetto, M., Coussio, J. and Liesuy, S.,(1997). Antioxidant and prooxidant activities in aquesous extract of Argentine plants. *International Journal of Pharmacognocy* **35**: 116-120.

Dhar, R., Dawar, H., Garg, S., Basir, S.F., Talwar, G.P., (1996). Effect of volatiles from neem and other natural products on gonotrophic cycle and oviposition of Anopheles stephensi and A. culicifacies (Diptera: Culicidae). *J Med Entomol* **33**(2):195–201.

Dreyfus, B.L., and Y.R. Dommergues.,(1981). Nodulation of *Acacia* species by fast- and slow-growing tropical strains of *Rhizobium*. *Applied and Environmental Microbiology* **41**, 97-99.

Duhan, J.S., and Lakshinarayana, K.,(1995). Allelopathic effect of *Acacia nilotica* on cereal and legume crops grown in field. *All. J.* **21**: 93-98.

Dunkel, F.V., Serugendo, A., Breene,W.M., Sriharan, S., (1995), Influence of insecticidal plant materials used during storage on sensory attributes and instrumental hardness of dry edible beans (Phaseolus vulgaris L.). *Plant Foods Hum Nutr* **48**(1):1–16.

Dweck, A.C., (2001). A review of *Psidium guajava*. *Malayan Journal of Medical Science* **8**, 27–30.

Dymock, W., Warden,C.J.H., Hooper, D. (2005). Pharmacographica India. *A History of the principal drugs of vegetable origin.* Vol **1**. New Delhi Shrishti book distributors. 556.

Ebenso, I.E., (2004). Molluscicidal effects of neem (*Azadirachta indica*) extracts on edible tropical land snails. *Pest Manag Sci* **60**(2):178–182.

Edward ,J., Sethuramani, A., Devi, P. and Meera, R.,(2010). Anti-diabetic, hypolipidemic and antioxidant activities of the plant extracts of *Morus alba* Linn. *International Journal of Phytopharmacy Research*.**1**:25-28.

Eline, M.B., Ouedraogo, Z.L., Heide, D.,(2004). Effect of aqueous extract of *Acacia nilotica* ssp *adansonii* on milk production and prolactin release in the rat. *J Endocrin* **182**:257–266.

El-Khawas, S.A., (2004). Physiological and biochemical adaptation of *Triticum vulgaris* 1. to PH stress by hormonal application. *Pak. J. Biol. Sci*. **7**, 852-860.

El-Khawas,S.A. and Shehata, M.M.,(2005).The allelopathic potentialities of *Acacia nilotica* and *Eucalyptus rostrata* on monocot (*Zea mays*) and dicot (*Phaseolus vulgaris*)Plants. *Biotechnology* **4**, 23-34.

El-Shazly, M.M., El-Sharnoubi, E.D., (2000). Toxicity of a neem (*Azadirachta indica*) insecticide to certain aquatic organisms. *J Egypt Soc Parasitol* **30**(1):221–231.

Emch, M. and Ali, M.,(2001). Spatial and temporal patterns of diarrhea disease in Matlab. Bangladesh. EPA. **33**(2): 339-350.

Fabry, W., Okemo, P., Ansorg, R. (1996). Fungistatic and fungicidal activity of east African medicinal plants. *Mycoses* **39**(1–2):67–70.

Fabry, W., Okemo, P.O., Ansorg, R., (1998). Antibacterial activity of East African medicinal plants. *J Ethnopharmacol* **60**(1):79–84.

Fag, C. and Stewart, J. L.,(1994). The value of *Acacia* and *Prosopis* in arid and semi-arid environments. *J. Arid Environ*. **27**, 3-25.

FAO/WHO (2004) Regional conference on food safety for Asia and Pacific. Bangladesh Country Paper, Seremban, Malaysia.

Farnsworth, N.R., Waller, D.P., (1982). Current status of plant products reported to inhibit sperm. *Res Front Fert Regul* **2**(1):1–16.

Farzana, MUZN., Shameem, I., Sultana, A,(2012).. Efficacy of Acacia Arabica in Improving Woman's Quality of Life in Uterine Prolapse: A Randomized Controlled Trial. *Sri Lanka Journal of Indigenous Medicine* **2**(1):101-106.

Field, M., (2003). Intestinal ion transport and the pathophysiology of diarrhea. *J. Clin. Invest*. **111**(7): 931-943.

Finney,D.J.,(1947). Probit analysis: A statistical treatment of sigmoid response curve. Cambridge University Press, London,333.

Finney,D.J.,(1971). Probit analysis. 333. Cambridge University Press, London.

Fosberg,F.R., Dasanayakke,M.D. (1980). *Flora of Ceylon*. Vol **1**.New Delhi: Oxford and IBH publishing company, 488.

Gandhi, M., Lal, R., Sankaranarayanan, A., Banerjee, C.K., Sharma, P.L., (1988). Acute toxicity study of the oil from *Azadirachta indica* seed (neem oil). *Ethnopharmacol* **23**(1):39–51.

Garg, S., Taluja, V., Upadhyay, S.N., Talwar, G.P.,(1993). Studies on the contraceptive efficacy of Praneem polyherbal cream. *Contraception* **48**(6):591–596.

Garg, S., Talwar, G.P., Upadhyay, S.N., (1994). Comparison of extraction procedures on the immunocontraceptive activity of neem seed extracts. *J Ethnopharmacol* **44**(2):87–92.

Garg, S., Talwar, G.P., Upadhyay, S.N., (1998). Immunocontraceptive activity guided fractionation and characterization of active constituents of neem (*Azadirachta indica*) seed extracts. *J Ethnopharmacol* **60**(3):235–246.

Ghani, M.N. Khazainul Advia. New Delhi Idarae Kitabul Shifa YNM, 254.

Ghodesawar, M.G., Ahamed, R.N., Ahmed,A.W., Aladakatti, R.H., (2004). Ultrastructural changes in cauda epididymidal epithelial cell types of *Azadirachta indica* leaf treated rats. *Indian J Exp Biol* **42**(11):1091–1095.

Gholap, S., Kar, A., (2004). Hypoglycaemic effects of some plant extracts are possibly mediated through inhibition in corticosteroid concentration. *Pharmazie* **59**(11):876–878.

Ghosesawar, M.G., Ahamed, R.N., Ahmed, M., Aladakatti, R.H., (2003). *Azadirachta indica* adversely affects sperm parameters and fructose levels in vas deferens fluid of albino rats. *J Basic Clin Physiol Pharmacol* **14**(4):387–395.

Gilani, A.H., Shaheen, F., Zaman, M., Janbaz, K.H., Shah, B.H., Akhtar, M.S.,(1999). Studies on Antihypertensive and Antispasmodic activities of Methanol Extract of *Acacia nilotica* pods. *Phytotheraphy Research* B 665-69.

Girish, K., Shankara ,B.S., (2008). Neem—a green treasure. *Electronic J Biol* **4**(3):102–111.

Githiori, J.B., Hoglund, J., Waller, P.J., Baker, R.L., (2004). Evaluation of anthelmintic properties of some plants used as livestock dewormers against Haemonchus contortus infections in sheep. *Parasitology* **129**(Pt 2):245–253.

Githiori, J.B., Hoglund, J., Waller, P.J., Leyden Baker, R., (2003). Evaluation of anthelmintic properties of extracts from some plants used as livestock dewormers by pastoralist and smallholder farmers in Kenya against Heligmosomoides polygyrus infections in mice. *Vet Parasitol* **118** (3–4):215–226.

Godin, P., (1954). *A New Spray Reagent for Paper Chromatography of Polyols and Cetoses Nature*: **174**:4420, 134.

Govindachari, T.R., Suresh, G., Gopalakrishnan, G., Masilamani, S., Banumathi, B., (2000). Antifungal activity of some tetranortriterpenoids.

Grover, I.S., Bala, S., (1993). Studies on antimutagenic effect of guava (*Psidium guajava*) in *Salmonella typhimurium*. *Mutatation Research* **300**, 1–3.

Gulco, P.,(2001). Medicinal Plants in Folklores of Bihar and Orissa. CCRUM New Delhi, 25.

Gupta, R.K.,(1970) Resource survey of gummiferous acacias in western Rajasthan. *Tropical Ecology* **11**, 148-161.

Gupta, S., Kataria, M., Gupta, PK., Murganandan, S., Yashroy, RC., (2004). Protective role of extracts of neem seeds in diabetes caused by streptozotocin in rats. *J Ethnopharmacol* **90**(2–3):185–189.

Hakim, A.,(2002). Bustanul Mufredat. Part I. New Delhi: Idarae Kitabul Shifa; 64, 65.

Halim ,EM., (2003). Lowering of blood sugar by water extract of *Azadirachta indica* and Abroma augusta in diabetes rats. *Indian J Exp Biol* **41**(6):636–640.

Hanachi, P., Fauziah, O., Peng, LT., Wei, LC., Nam, LL., Tian, TS., (2004). The effect of *Azadirachta indica* on distribution of antioxidant elements and glutathione S-transferase activity in the liver of rats during hepatocarcinogenesis. *Asia Pac J Clin Nutr* **13**(Suppl):S170.

Health (2008). Diarrhea situation worsens in Bangladesh as temperature goes up http://news.xinhuanet.com/english/2008-04/18/content 8005020.htm. Cited 21 Feb 2009.

Heinrich, M., (1998). Plants as antidiarrhoeals in medicine and diet. In: Proceedings from a Joint Meeting of the Society for Economic Botany and the International Society London, Royal Botanic Gardens, Kew, UK, July 1–6, 1996, 17–30.

Heinrich, M., Ankli, A., Frei, B., Weimann, C., Sticher, O., (1998). Medicinal plants in Mexico: healers consensus and cultural importance. *Social Science and Medicine* **47**, 1859–1871.

Holetz, F.B., Pessini, G.L., Sanches, N.R., Cortez, D.A., Nakamura, C.V., Filho, B.P., (2002). Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem´orias do Instituto Oswaldo Cruz **97**, 1027–1031.

Holm, L.G., Pancho, J.V., Herberger, J.P., and Plucknett, D.L.,(1979). *A Geographical Atlas of World Weeds.* Wiley, New York.

Hordegen, P., Hertzberg, H., Heilmann, J., Langhans ,W., Maurer, V., (2003). The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. *Vet Parasitol* **117**(1–2):51–60.

Hsieh, C.L., Yang, M.H., Chyau, C.C., Chiu, C.H.,Wang, H.E., Lin, Y.C., Chiu, W.T., Peng, R.Y., (2007). Kinetic analysis on the sensitivity of glucose- or glyoxal-induced LDL glycation to the inhibitory effect of *Psidium guajava* extract in a physiomimic system. *Biosystems* **88**, 92–100.

http://en.wikipedia.org/wiki/Acacia nilotica accessed on 21 Oct, 2009.

http://en.wikipedia.org/wiki/*Acacia*. 16 January, 2014 at 9.30 pm.

Hummel, E., Kleeberg, H., (2002). First results of the application of a new Neemazal powder formulation in hydroponics against different pest insects. Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet **67**(3):631–639.

Ibrahim, IA., Khalid, SA., Omer, SA., Adam, SE., (1994). On the toxicology of *Azadirachta indica* leaves. *J Ethnopharmacol* **42**(1):71–72.

Ibrahim, IA., Omer, SA., Ibrahim, FH., Khalid, SA., Adam, SE., (1992). Experimental *Azadirachta indica* toxicosis in chicks. *Vet Hum Toxicol* **34**(3):221–224.

Iwu, M.M., (1993). Handbook of African Medicinal Plants. CRC Press, 786–789.

Jaiarj, P., Khoohaswan, P., Wongkrajang, Y., Peungvicha, P., Suriyawong, P., Saraya, M.L., Ruangsomboon, O., (1999). Anticough and antimicrobial activities of *Psidium guajava* Linn. leaf extract. *Journal of Ethopharmacology* **67**, 203–212.

Jain, A., Basal, E., (2003). Inhibition of Propionibacterium acnesinduced mediators of inflammation by Indian herbs. *Phytomedicine* **10**(1):34–38.

Jain, N., Dhawan, K., Malhotra, S., Siddiqui, S., Singh, R., (2003). Biochemistry of fruit ripening of guava (*Psidium guajava* L.). *Compositional and Enzymatic Changes* **58**, 309–315.

Jansen, P.C.M., M´endez, O., (1990). Plantas Medicinales: Seu Uso Tradicional em Moc¸ambique, 4 tomos. Ed. Gabinete de Estudos de Medicina Tradicional. (GEMT). Ministerio da Saude. Maputo, Mozambique 298–301.

Jeelani, G.,(1994). Makhzane Hikmat. Vol 2, New Delhi: Oriental publishers, 809-810.

Jensen, JT., (2002). Male contraception. Curr Women Health Rep **2**(5):338–345.

Jimenez, E.A., Rincon, M., Pulido, R., Saura, C.F., (2001). Guava fruit (*Psidium guajava*) as a new source of antioxidant dietary fiber. *Journal of Agricultural and Food Chemistry* **49**, 5489–5493.

Jordan, M.J., Margaria, C.A., Shaw, P.E., Goodner, K.L., (2003). Volatile components and aroma active compounds in aqueous essence and fresh pink guava fruit puree (*Psidium guajava* L.) by GC–MS and multidimensional GC/GC-O. *Journal of Agriculture and Food Chemistry* **51**, 1421–1426.

Joshi, AR., Ahamed, RN., Pathan,KM., Manivannan,B.,(1996). Effect of *Azadirachta indica* leaves on testis and its recovery in albino rats. *Indian J Exp Biol* **34**(11):1091–1094.

Joy, PP., Thomas, J., Mathew, S., Skaria, P.,(1998). Medicinal plants, Kerala Agricultural University Publications; Kerala, India, 3.

Jurjani, I., Zakheerae Khawarzam Shahi (Urdu trans By Khan AH). Vol 2, Lucknow Munshi Nawal Kishore 1903; 632-634

Kabiruddin Md. Makhzanul Mufredat. New Delhi Ejaz Publishing House YNM, 82-83.

Kar, A., Choudhary, BK., Bandyopadhyay, NG.,(2003). Comparative evaluation of hypoglycaemic activity of some Indian medicinal plants in alloxan diabetic rats. *J Ethnopharmacol* **84**(1):105–108.

Karmakar,PR., Chatterjee, BP., (1994). Isolation and characterization of two IgE-reactive proteins from *Azadirachta indica* pollen. *Mol Cell Biochem* **131**(1):87–96.

Kasturi, M., Ahamed, RN., Pathan, KM., Manivannan, B., Aladakatti, RH., (2002). Ultrastructural changes induced by leaves of *Azadirachta indica* (neem) in the testis of albino rats. *J Basic Clin Physiol Pharmacol* **13**(4):311–328.

Kaur, G., Sarwar Alam, M., Athar, M., (2004). Nimbidin suppresses functions of macrophages and neutrophils: relevance to its antiinflammatory mechanisms. *Phytother Res* **18**(5): 419–424.

Kaushik, J.C. and Mandal, B.S.,(2005). The role of mycorrhiza in stree management for seedling growth of Dalbergia sissoo and *Acacia nilotica*. *Bull NIE*, **15**, 133-137.

Kavimani, S., Karpagam, R.I., Jaykar, B., (1997). Anti-inflammatory activity of volatile oil of *Psidium guajava*. *Indian Journal of Pharmaceutical Sciences* **59**, 142–144.

Kenneth, S., Brekke, L., Johon, E., Donald, S., (1970). Volatile constituents in guava. *Journal of Agriculture and Food Chemistry* **18**, 598–599.

Khan A. Muheete Azam. Vol I. Kanpur Nizami press 1313(AH):181-183.

Khan MM. Tohfatul Momineen. Luknow Matba Hasani 1272, 32.

Khan, R., (2009). Antimicrobial Activity of Five Herbal Extracts Against Multi Drug Resistant (MDR) Strains of Bacteria and Fungus of Clinical Origin. Molecules; **14**(2):586-597.

Khillare, B., Shrivastav, TG., (2003). Spermicidal activity of *Azadirachta indica* (neem) leaf extract. *Contraception* **68**(3):225–259.

Khosla, P., Bhanwra, S., Singh, J., Seth, S., Srivastava, RK., (2000). A study of hypoglycaemic effects of *Azadirachta indica* (neem) in normal and alloxan diabetic rabbits *Indian. J Physiol Pharmacol* **44**(1):69–74.

Killion, K.H., (2000). The Review of Natural Products, third ed. Facts and Comparison, USA, 250–251.

Knols, BG., (2002). Traditional use of mosquito-repellent plants in western Kenya and their evaluation in semi-field experimental huts against Anopheles gambiae: ethnobotanicalIts potentiation effects with some non-azadirachtin limonoids in neem against lepidopteran larvae. *J Agric Food Chem* **51**(10):2937–2942.

Kokate, CK., Purohit, AP., Gokhate, SB..(2009). Pharmacognosy. India Nirali Prakashan Pune, Ed 43, 7, 12.

Koul, O., (2004). Biological activity of volatile di-n-propyl disulfide from seeds of neem, Azadirachta indica (Meliaceae), to two species of stored grain pests, Sitophilus oryzae (L.) and Tribolium castaneum (Herbst). *J Econ Entomol* **97**(3):1142–1147.

Koul, O., Multan,i JS., Singh, G., Daniewski, WM., Berlozecki, S., (2003). 6beta-hydroxygedunin from *Azadirachta indica*. Its potentiation effects with some non-azadirachtin limonoids in neem against lepidopteran larvae. *J Agric Food Chem* **51**(10):2937–2942.

Kritikar, KR., Basu, BD.,(2003). Indian Medicinal plants with illustrations. Vol 4, Ed 2, Uttaranchal Oriental Press 1289-92.

Kumar ,A., Rao, AR., Kimura, H., (2002). Radiosensitizing effects of neem (*Azadirachta indica*) oil. *Phytother Res* **16**(1): 74–77.

Labadie, RP., van der Nat, JM., Simons, JM., Kroes, BH., Kosasi, S., van den Berg, AJ., t' Hart LA., Van der Sluis WG., Abeysekera, A., Bamunuarachchi, A., (1989). An ethnopharma- cognostic approach to the search for immunomodulators of plant origin. *Planta Med* **55**(4):339–348.

Lale, NE., Mustapha, A., (2000). Potential of combining neem (*Azadirachta indica* A. Juss) seed oil with varietal resistance for the management of the cowpea bruchid, Callosobruchus maculatus (F.). *J Stored Prod Res* **36**(3): 215–222.

Lara, O.F., Marquez, A.C., (1996). Plantas Medicinales deM´exico. Composici´on. Usos y Actividad Biol´ogica. UNAM, M´exico, D.F, 137–139.

Ley, SV., (1994). Synthesis and chemistry of the insect antifeedant azadirachtin. *Pure Appl Chem* **66**(10/11):2099–2102.

Li, J., Chen, F., Luo, J., (1999). GC–MS analysis of essential oil from the leaves of *Psidium guajava*. Zhong Yao Cai **22**, 78–80.

Li., S.Y. and Wang, Y.F., (1998). Allelopathic potential of *Acacia confuse* and related species in Taiwan. *J. Chem. Ecol* **24**, 2131-2150.

Liu ,F.,(2000). Antioxidative and free radical scavenging activities of selected medicinal herbs. *Life Science*, **66**: 725-735.

Lozoya, X., Reyes, M.H., Chavez, S.M.A., Martinez, G.M., Soto, G.Y., Doubova, S.V., (2002). Intestinal anti-spasmodic effect of a phytodrug of *Psidium guajava* folia in the treatment of acute diarrheic disease. *Journal of Ethnopharmacology* **83**, 19–24.

Lucas, E., Olorunnisola, A., Adewole, N., (2006). Preliminary evaluation of guava (*Psiduim guajava*) tree truss fabrication in Nigeria. *Agriculture Engineering International* **3**, 10–20.

Lutterodt, G.D., (1989). Inhibition of gastrointestinal release of acetylcholine by quercetin as a possible mode of action of *Psidium guajava* leaf extracts in the treatment of acute diarrhoeal disease. *Journal of Ethnopharmacology* **25**, 235–247.

Lutterodt, G.D., (1992). Inhibition of Microlax-induced experimental diarrhea with narcotic-like extracts of *Psidium guajava* leaf in rats. *Journal of Ethnopharmacology* **37**, 51–157.

Mahesh, B., Satish, S., (2008). Antimicrobial Activity of Some Important Medicinal Plant against Plant and Human Pathogens. *World Journal of Agricultural Sciences* **4**(S):839-843.

Mahgoub, S., (1979). On the subspecies of *Acacia nilotica* in the Sudan. *Sudan Silva.* **4**, 57-62.

Majumdar, R., Bag, BG., Maity, N.,(2013). *Acacia nilotica* (Babool) leaf extract mediated size-controlled rapid synthesis of gold nanoparticles and study of its catalytic activity. International Nano Letters **3**:53.

Marjorie ,MC.,(1999). Plant products as antimicrobial agents. *Clin. Microbial. Rev.* **12**(4): 564-582.

Mart´ınez, G.M., Barajas, B., (1991). Estudio etnobot´anico de las plantas medicinales en el mercado Libertad del ´area metropolitana de Guadalajara, Jal. Tesis Licenciatura. Facultad de Agronom´ıa. Universidad de Guadalajara. 100.

Mashram, N.,(2009). Antimicrobial activity of methanol extracts of medicinal plants against bacterial species. *Int Res J I* (**3&4**):147-150.

Masuda, T., Inaba, Y., Maekawa, T., Takeda, Y., Yamaguchi, H., Nakamoto, K., Kuninaga, H., Nishizato, S., Nonaka, A., (2003). Simple detection method of powerful antirradical compounds in the raw extract of plants and its application for the identification of antiradical plants constituents. *Journal of Agriculture and Food Chemistry* **51**, 1831–1838.

Mata, L., Urrutia, JJ. and Simbhon, A.,(1984). Infectious agents in acute and chronic diarrhea of childhood. E. Lebenthal.(ed) Chronic diarrhea in children, nestle, Vevey/Raven press. New York. 237-252.

Meckes, M., Calzada, F., Tortoriello, J., Gonz´alez, J.L., Martinez, M., (1996). Terpenoids isolated from *Psidium guajava* with depressant activity on central nervous system. *Phytotherapy Research* **10**, 600–603.

Medina, M.L., Pagano, F.G., (2003). Characterization of *Psidium guajava* pulp "criolla roja". Revista de la Facultad de Agronom´ıa de La Universidad del Zulia, (LUZ) **20**, 72–76.

Mej´ıa, K., Rengifo, E., (2000). Plantas Medicinales de Uso Popular en la Amazon ´ıa Peruana, 2*a* ed. Tarea Asociaci´on Gr´afica Educativa, Lima Per´u. Mercadante, A.Z., Steck, A., Pfander, H., (1999). Carotenoids from guava (*Psidium guajava*.): isolation and structure elucidation. *Journal of Agricultural and Food Chemistry* **47**, 145–151.

Michael, A.S., Thompson,C.G., Abramovitz M.,(1956). *Artemia salina* as a test organism for a bioassay, *Science* **123**: 464.

Michael, H.N., Salib, J.Y., Isaac, M.S., (2002). Acylated flavonol glycoside from *Psidium gauijava* L. seeds. *Pharmazie* **57**, 859–860.

Miller, JA., Chamberlain, WF., (1989). Azadirachtin as a larvicide against the horn fly, stable fly, and house fly (Diptera: Muscidae). *J Econ Entomol* **82**(5):1375–1378.

Mishra ,AK., Singh, N., Sharma ,VP., (1995). Use of neem oil as a mosquito repellent in tribal villages of Mandla district, Madhya Pradesh. *Indian J Malariol* **32**(3):99–103.

Misra, K., Seshadri, T.R., (1968). Chemical components of the fruits of *Psidium guava*. *Phytochemistry* **7**, 641–645.

Mitchell, MJ., Smith, SL., Johnson, S., Morgan, ED., (1997). Effects of the neem tree compounds azadirachtin, salannin, nimbin, and 6-desacetylnimbin on ecdysone 20-monooxygenase activity. *Arch Insect Biochem Physiol* **35**(1–2):199–209.

Mitchell, PL., Gupta, R., Singh ,AK., Kumar, P., (2004). Behavioral and developmental effects of neem extracts on Clavigralla scutellaris (Hemiptera: Heteroptera: Coreidae) and its egg parasitoid, Gryon fulviventre (Hymenoptera: Scelionidae). *J Econ Entomol* **97**(3):916–923.

Mitchell, S.A., Ahmad, M.H., (2006a). A review of medicinal plant research at the University of theWest Indies, Jamaica, 1948–2001.*West Indies Medical Journal* **55**, 243–269.

Mitchell, S.A., Ahmad, M.H., (2006b). Protecting our medicinal plant heritage: the making of a new national treasure. Jamaica Journal, Institute of Jamaica, Kingston **29**, 28–33.

Mitra ,SN., Ali,MN., Islam,S., Cross,AR. and Saha,T.,(1994). Bangladesh demographic and health survey 1993-1994. Calverton, Maryland and Dhaka, Bangladesh: National Institute of Population Research and Training (NIPORT). Mitra and Associates, and Macro International Inc.

Mitra,A.,(2009). Epidemiology and management of diarrhea diseases. http://www.bibalex.org/supercourse/SupercoursePPT/0011-1001/0931.ppt. Cited 20th Feb 2009.

Mohanty, R.V., Padhy, S.N., Das, S.K.,((1996). Traditional phytotherapy for diarrhoeal disease in Ganjan and phulbani District of South Orissa, *India Ethanobotany* **8**:60-65.

Monzon, R.B., Alvior, J.P., Luczon, L.L., Morales, A.S., Mutuc, F.E., (1994). Larvicidal potential of five Philippine plants against Aedes aegypti (Linnaeus) and Culex quinquefasciatus (Say). *Southeast Asian J Trop Med Public Health* **25**(4):755–759.

Morton, J.F., (1987). Fruits of Warm Climates. Julia F. Morton, Miami, FL, 356–363.

Mossini, S.A., de Oliveira, K.P., Kemmelmeier, C., (2004). Inhibition of patulin production by Penicillium expansum cultured with neem (*Azadirachta indica*) leaf extracts. *J Basic Microbiol* **44**(2):106–113.

Mukherjee, S., Garg, S., Talwar, G.P., (1999). Early post implantation contraceptive effects of a purified fraction of neem (*Azadirachta indica*) seeds, given orally in rats: possible mechanisms involved. *J Ethnopharmacol* **67**(3):287–296.

Mukherjee, S., Lohiya, N.K., Pal, R., Sharma, M.G., Talwar, G.P., (1996). Purified neem (*Azadirachta indica*) seed extracts (Praneem) abrogate pregnancy in primates. *Contraception* **53**(6):375–378.

Mukherjee, S., Talwar, G.P., (1996). Termination of pregnancy in rodents by oral administration of praneem, a purified neem seed extract. *Am J Reprod Immunol* **35**(1):51–56.

Mukhtar, H.M., Ansari, S.H., Bhat, Z.A., Naved, T., Singh, P., (2006). Antidiabetic activity of an ethanol extract obtained from the stem bark of *Psidium guajava* (Myrtaceae). *Pharmazie* **61**, 725–727.

Muruganandan, S., Srinivasan, K., Tandan, S.K., Jawahar, L., Suresh, C., Raviprakash, V., (2001). Anti-inflammatory and analgesic activities of some medicinal plants. *Journal of Medicinal and Aromatic Plant Sciences* **22/23**, 4A/1A, 56–58.

Nadkarni, K.M., (2005). The Indian Plants and Drugs. New Delhi: Shrishti Book Distributors, 4, 5.

Nadkarni, K.M., Nadkarni, A.K., (1999). Indian Materia Medica with Ayurvedic, Unani-Tibbi, Siddha, allophatic, homeopathic, naturopathic and home remedies, vol. **1**. Popular Prakashan Private Ltd., Bombay, India, 142–149.

Nagar, P.K., Rao, T.R., (1981). Studies on endogenous cytokinins in guava (*Psidium guajava* L.). *Annals of Botany* **48**, 845–852.

Nagpal, B.N., Srivastava, A., Sharma, V.P., (1995). Control of mosquito breeding using wood scrapings treated with neem oil. *Indian J Malariol* **32**(2):64–69.

Nair, P.K.R., (1993). *An introduction to agroforestry*. Kluwer cademic Publishers, Dordrecht, The Netherlands,499.

Nakahara, K., Roy, M.K., Ono, H., Maeda, I., Ohnishi-Kameyama, M., Yoshida, M., Trakoontivakorn, G., (2003). Prenylated flavanones isolated from flowers of *Azadirachta indica* (the neem tree) as antimutagenic constituents against studies and application by thermal expulsion and direct burning. *Trans R Soc Trop Med Hyg* **96**(3):225–231.

Nakahara, K., Roy, M.K., Ono, H., Maeda, I., Ohnishi-Kameyama, M., Yoshida, M., Trakoontivakorn, G., (2003). Prenylated flavanones isolated from flowers of *Azadirachta indica* (the neem tree) as antimutagenic constituents againsheterocyclic amines. *J Agric Food Chem* **1**(22): 6456–6460.

Nanduri, S., Thunuguntla, SS., Nyavanandi, VK., Kasu, S., Kumar, PM., Ram, PS., Rajagopal, S., Kumar, RA., Deevi, DS., Rajagopalan, R., Venkateswarlu, A., (2003). Biological investigation and structure–activity relationship studies on azadirone from *Azadirachta indica* A. Juss. *Bioorg Med Chem* Lett **13**(22):4111–4115.

Narayan, D.P., Kumar, U.,(2005). Agro's Dictionary of Medicinal Plants. India (Jodhpur): *Updesh Purohit for Agrobios* **3**.

Narayan, D.P., Purohit, S.S.,(2004). Agro's colour atlas of medicinal plants. India *Agrobios* **3**.

Nasroun, T.H., (1979). Pulp and paper making properties of some tropical hardwood species grown in the Sudan. *Sudan Silva*, **4**, 22-32.

Natarajan, V., Pushkala, S., Karuppiah, V.P., Prasad, P.V., (2002). Anti dermatophytic activity of *Azardirachta indica* (neem) by invitro study. *Indian J Pathol Microbiol* **45**(3):311–313.

Nath, D., Sethi, N., Singh, R.K., Jain, A.K. (1992). Commonly used Indian abortifacient plants with special reference to their teratologic effects in rats. *Journal of Ethnopharmacology* **36**: 147-154.

National Academy of Sciences. *Firewood crops; shrubs and tree species for energy production* (Vol. I & II). National Academy Press, Washington, D.C. 1980, 237.

New, T.R., *A Biology of Acacias.* Oxford University Press, Melbourne, 1984,153.

Njiro, S.M., Kofi-Tsekpo, M.W., (1999). Effect of an aqueous extract of *Azadirachta indica* on the immune response in mice Onderstepoort. *J Vet Res* **66**(1):59–62.

Nwosu, MO., Okafor, JI., (1995). Preliminary against Basidiobolus and some other pathogenic fungi. *Mycoses* **38**(5–6): 191–195.

Ofulla, A.V., Chege, G.M., Rukunga, G.M., Kiarie, F.K., Githure, J.I., Kofi-Tsekpo, M.W., (1995). In vitro antimalarial activity of extracts of Albizia gummifera, Aspilia mossambicensis, Melia azedarach and Azadirachta indica against Plasmodium falciparum. *Afr J Health Sci* **2**(2):309–311.

Oh,W.K., Lee, C.H., Lee, M.S., Bae, E.Y., Sohn, C.B., Oh, H., Kim, B.Y., Ahn, J.S., (2005). Antidiabetic effects of extracts from *Psidium guajava*. *Journal of Ethnopharmacology* **96**, 411–415.

Ojan, H., Nihorimbere, V., (2004). Antioxidant power of phytochemicals from *Psidium guajava*. *Journal of Zhejiang University of Science* **5**, 676–683.

Ojewole, J.A., (2005). Hypoglycemic and hypotensive effects of *Psidium guajava* Linn.. (Myrtaceae) leaf aqueous extract. Methods and Findings in Experimental and Clinical Pharmacology **27**, 689–695.

Ojewole, J.A., (2006). Antiinflamatory and analgesic effects of *Psidium guajava* Linn (Myrtaceae) leaf aqueous extract in rats and mice. Methods and Findings in Experimental and Clinical Pharmacology **28**, 441–446.

Oliver, B.B., (1986). Medicinal Plants in tropical West Africa. Cambridge University Press, Cambridge, 457–461.

Omar, S., Zhang, J., MacKinnon, S., Leaman, D., Durst, T., Philogene, B.J., Arnason, J.T., Sanchez-Vindas, P.E., Poveda, L., Tamez, P.A., Pezzuto, J.M., (2003). Traditionally-used antimalarials from the Meliaceae. Curr Top Med Chem **3**(2):133–139.

Oria ,R.B., Patrick, P.D., Zhang, H., Lomtz, B., Costa, C.M.C., Brito, G.A., Barrett, L.J., Lima, A.A., and Guerrant, R.L.,(2005). APOE4 protects cognitive development in children with heavy diarrhea burdens in Northeast Brazil. *Pediatr. Res*. **57**(2):310-6.

Oyaizu, M., (1986). Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition;* 307-315.

Pai, M.R., Acharya, L.D., Udupa, N., (2004). Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel–a 6-week clinical study. *J Ethnopharmacol* **90**(1): 99–103.

Palm,C.A., (1995). Contribution of agroforestry trees to nutrient requirements of intercropped plants. *Agroforestry Systems* **30**, 105-124.

Palsson, K., Jaenson, T.G., (1999). Plant products used as mosquito repellents in Guinea Bissau. *West Africa Acta Trop* **72**(1):39–52.

Pandey, C. B., Pandya, K. S., Pandey, D., and Sharma, R.B.,(1999). Growth and productivity of rice (*Oryza sativa*) as affected by *Acacia nilotica* in a traditional agroforestry system. *Trop Ecol*, **40**, 109-117.

Pandey, C. B., Singh, A.K., and Sharma, D.K.,(2000). Soil properties under *Acacia nilotica* trees in a traditional agroforestry syatem in Central India. *Agroforestry Systems* **49**, 53-61.

Pandey, C.B., and Sharma.D.K.,(2005). Ecology of *Acacia nilotica* based traditional agroforestry system in Central India. *Bull. NIE,* **15**: 109-116.

Paniandy, J.C., Chane, M.J., Pieribattesti, J.C., (2000). Chemical composition of the essential oil and headspace solid-phase microextraction of the guava fruit (*Psidium guajava* L.). *Journal of Essential Oil Research* **12**, 153–158.

Pardo, O., (1999). Estudio comparativo de ocho especies Americanas de uso medicinal en Mozambique. Revista Chilena de Flora y Vegetaci´on **1**, 323–328.

Parekh, J. and Chands, S.,(2006). *In vitro* antimicrobial activities of extracts of *Launaea procumbens* Roxb.(Labiateae), *Vitis vinifera* L (Vitaceae) and *Cyperus rotundus* L. (Cyperaceae). *Afr.J. Biomed. Res*.**9**: 89-93.

Parida ,M M., Upadhyay, C., Pandya, G., Jana, A .M., (2002). Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on dengue virus type-2 replication. *J Ethnopharmacol* **79**(2):273–278.

Parshad, O., Singh, P., Gardner, M., Fletcher, C., Rickards, E., Choo- Kang, E., (1994). Effect of aqueous neem (*Azadirachta indica*) extract on testosterone and other blood constituents in male rats: a pilot study. *West Indian Med J* **43**(3):71–74.

Pontikis, C., (1996). *Psidium guajava* L. Guava. In: Bajaj,Y. (Ed.), *Biotechnology in Agriculture and Forestry,* vol. **35**, 309–319.

Pradeep, A.R., Happy, D., Garg, G., (2010). Short-term clinical effects of commercially available gel containing *Acacia arabica*: a randomized controlled clinical trial. *Australian Dental Journal* **55**:65–69.

Prakash, A.O., Tewari, R.K., Mathur, R., (1988). Non-hormonal postcoital contraceptive action of neem oil in rats. *J Ethnopharmacol* **23**(1):53–59.

Pramanik, (2007).”Bioactive Properties of the toothbrush plant, *Glycomis pentaphylla* (retz) with Special Reference to stored Product Pests” Unpublished *Ph,D Thesis, University of Rajshahi*. Rajshahi-6205.

Pranee, J., Paranee, K., Yuwadee,W., Penchom, P., Potjanee, S., Saraya, M.L.S., Orawan, R., (1999). Anticough and antimicrobial activities of *Psidium guajava* Linn. leaf extract. *Journal of Ethnopharmacology* **67**, 203–212.

Prieto, P., Pineda ,M., and Aguilar, M.,(1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical of Clinical Biochemistry*, **269**: 337-341.

Pulok, K., Mukherjee., Das, J., Balasubramanian, R., Saha, K., Pal, M. and Saha, B.P.,(1995) .Anti-diarrhoeal evaluation of *nelumbo nucifera* rhizome extract. *Indian J. Pharmacol*. **27**(4): 262-264.

Puri, D.N. and Khybri, M.L., (1975). Economics of Chambal ravine afforestation. *Indian Forester* **101**, 448-451.

Pushpangadan , P., Nair, K.N. and Ahmad, M.R., (2001). Biodiversity and Medicinal Plant Wealth of South Asian Countries, UNESCO sponsored "Regional Training Programme on Biodiversity Systematics: Evaluation and Monitoning with emphasis on Medicinal Plants" held at NBRI, Lucknow from 3rd to 13th September, 2001.

Qadan, F., Thewaini, A.J., Ali, D.A., Afifi, R., Elkhawad, A., Matalka, K.Z., (2005). The antimicrobial activities of *Psidium guajava* and *Juglans regia* leaf extracts to acne-developing organisms. *American Chinese Medical* **33**, 197–204.

Qian, H., Nihorimbere, V., (2004). Antioxidant power of phytochemicals from *Psidium guajava* leaf. *Journal of Science* **5**, 676–683.

Rahman, M.F., Siddiqui, M.K., (2004). Biochemical effects of vepacide (from *Azadirachta indica*) on Wistar rats during subchronic exposure Ecotoxicol. *Environ Saf* **59**(3): 332–339

Rahman, M.F., Siddiqui, M.K., Jamil, K., (2001). Effects of Vepacide (*Azadirachta indica*) on aspartate and alanine aminotransferase profiles in a sub-chronic study with rats. *Hum Exp Toxicol* **20**(5):243–249.

Rahmat, A., Abu, M.F., Faezah, N., Hambali, Z., (2004). The effects of consumption of guava (*Psidium guajava*) or *papaya* (*Carica papaya*) on total antioxidant and lipid profile in normal male youth. *Asia Pacific Clinical Nutrition* **13**, S106.

Rai, M.K., (1996). In vitro evaluation of medicinal plant extracts against Pestalotiopsis mangiferae. *Hindustan Antibiot Bull* **38**(1–4):53–56.

Raji, Y., Ogunwande, I.A., Osadebe, C.A., John, G., (2004). Effects of *Azadirachta indica* extract on gastric ulceration and acid tsecretion in rats. *J Ethnopharmacol* **90**(1):167–170.

Rao, A.D., Devi, K.N., Thyagaraju, K., (1998). Isolation of antioxidant principle from *Azadirachta* seed kernels: determination of its role on plant lipoxygenases. *J Enzyme Inhib* **14**(1):85–96.

Rao, D.R., Reuben, R., Nagasampagi, B.A., (1995). Development of combined use of neem (*Azadirachta indica*) and water management for the control of culicine mosquitoes in rice fields. *Med Vet Entomol* **9**(1):25–33.

Rao, Ghosh, Santhanam.(1967). Folklore practices in Orissa. *Health science* **14**, 161.

Rao, I.G., Singh, A., Singh, V.K., Singh, D.K., (2003). Effect of single and binary combinations of plant-derived molluscicides on different enzyme activities in the nervous tissue of *Achatina fulica*. *J Appl Toxicol* **23**(1):19–22.

Rao, I.G., Singh, D.K., (2001). Combinations of *Azadirachta indica* and Cedrus deodara oil with piperonyl butoxide, MGK- 264 and Embelia ribes against Lymnaea acuminate. *Chemosphere* **44**(8):1691–1695.

Rastogi, R.P., Mehrotra, B.N.(2001). Compendium of Indian Medicinal Plants. Vol **3,** New Delhi NISC 4-7.

Rastogi,R.P.,Mehrotra,B.N. (2001). *Compendium of Indian Medicinal Plants*. Vol **3**, New Delhi NISC. 4-7.

Ray, A., Banerjee ,B.D., Sen, P., (1996). Modulation of humoral and cell-mediated immune responses by Azadirachta indica (neem) in mice. *Indian J Exp Biol* **34(**7):698–701.

Rios, C.D., Salazar, C.R., Cardona, C., Victoria, K., Torres M., (1977). Guayaba. En: Instituto Colombiano Agropecuario. Bogot´a (Colombia), second ed. Frutales. Manual de Asistencia T´ecnica No**. 4**, 221–248.

Romanos, M.T., Santos, N.S., Wigg, M.D., (2005). *In vitro* anti-rotavirus activity of some medicinal plants used in Brazil against diarrhea. *Journal of Ethnopharmacology* **99**, 403–407.

Roy, C.K., Kamath, J.V., Asad, M., (2006). Hepatoprotective activity of *Psidium guajava* Linn. *Indian Journal of Experimental Biology* **44**, 305–311.

Rushd, I. (1987) . Kitabul Kulliyat. Vol **2**, New Delhi: CRUM, 56, 114-117, 210-222, 259.

Sagar,S.K., Sehgal,S.S., (1996). Effects of aqueous extract of deoiled neem (*Azadirachta indica* A. juss) seed kernel and karanja (Pongamia Glabra vent) seed kernel against Culex quinquefasciatus. *J Commun Dis* **28**(4):260–269.

Said, H.M.,(1997). Hamdard Pharmacopeia of Eastern Medicine. Ed 2, New Delhi Sri Satguru publications, 353.

Saini, M.L., (2008). Comparative Pharmacognostical and antimicrobial studies of *Acacia* species (Mimosaceae). *Journal of Medicinal Plants Research* **2**(12):378-386.

SaiRam ,M., Ilavazhagan, G., Sharma, S.K., Dhanraj, S.A., Suresh, B., Parida, M.M., Jana, A.M., Devendra, K., Selvamurthy, W., (2000). Anti-microbial activity of a new vaginal contraceptive NIM-76 from neem oil (*Azadirachta indica*). *J Ethnopharmacol 71*(3):377–382.

Salib, J.Y., Michael, H.N., (2004). Cytotoxic phenylethanol glycosides from *Psidium guaijava* seeds. *Phytochemistry* **65**, 2091–2093.

Sam,T.W.,(1993). Toxicity testing using the brine shrimp: *Artemia salina*. In Colegate SM, Molyneux RJ. (Eds.), bioactive Natural Products Detection, Isolation, and Structural Determination: CRC Press, Boca Raton, FL, 442-456.

Santos, F.A., Rao, V.S.N., Silveira, E.R., (1998). Investigations on the antinociceptive effect of *Psidium guajava* leaf essential oil and its major constituents. *Phytotherapy Research* **12**, 24–27.

Sara, S.B., Folorunso, O.A., (2002.) Potentials of utilizing neem tree for desertification control in Nigeria. In: Ukwe CN, Folorunso AO, Ibe AC, Z Z, Lale NES, Sieghart L (eds) Sustainable industrial utilization of neem tree (*Azadirachta indica*) in Nigeria. UNIDO Regional Dev Centre, Lagos, 45–51.

Schmutterer, H., (1990). Properties and potentials of natural pesticides from neem tree. *Annual review of Entomol* **35**:271–298.

Scott, I.M., Kaushik, N.K., (1998). The toxicity of margosan-O, a product of neem seeds, to selected target and non-target aquatic invertebrates. *Arch Environ Contam Toxicol* **35**(3):426–431.

Sen, P., Mediratta, P.K., Ray, A., (1992. Effects of *Azadirachta indica* A Juss on some biochemical, immunological and visceral parameters in normal and stressed rats. *Indian J Exp Biol* **30**(12):1170–1175.

Seyoum, A., Kabiru, E.W., Lwande, W., Killeen, G.F., Hassanali, A., Knols, B.G., (2002). Repellency of live potted plants against Anopheles gambiae from human baits in semi-field experimental huts. *Am J Trop Med Hyg* **67**(2):191–195.

Sharma, V., Walia, S., Kumar, J., Nair,M.G., Parmar, B.S., (2003). An efficient method for the purification and characterization of nematicidal azadirachtins A, B, and H, using MPLC and ESIMS. *J Agric Food Chem* **51**(14):3966–3972.

Sharma, V.P., Ansari, M.A., (1994). Personal protection from mosquitoes (Diptera: Culicidae) by burning neem oil in kerosene. *J Med Entomol* **31**(3):505–507.

Sharma, V.P., Ansari, M.A., Razdan, R.K., (1993). Mosquito repellent action of neem (*Azadirachta indica*) oil. *J Am Mosq Control Assoc* **9**(3):359–360.

Shetty, K.A.B., (1977). Social forestry in Tamil Nadu. *Indian Farming,* **26**, 82.

Shinwari, M.I. and Khan, M.A. (1998). Indigenous use of medicinal trees and shrubs of Margalla Hills National Park, Islamabad. *Pakistan Journal of Forestry* **48**: 63-90.

Showler, A.T., Greenberg, S.M., Arnason, J.T., (2004). Deterrent effects of four neem-based formulations on gravid female boll weevil (Coleoptera: Curculionidae) feeding and oviposition on cotton squares. *J Econ Entomol* **97**(2): 414–421.

Siddiqui, B., Imran, S., Hassan, S., Begum, S., (2002). Triterpenoids from *Psidium guajava* leaves. Natural Product Letters **16**, 173–175.

Siddiqui, B.S., Afshan, F., Faizi, S., Naeem-Ul-Hassan Naqvi, S., Tariq, R.M., (2002). Two new triterpenoids from *Azadirachta indica* and their insecticidal activity. *J Nat Prod* **65**(8):1216–1218.

Siddiqui, B.S., Afshan, F., Ghiasuddin FaiziS., Naqvi, S.N., Tariq, R.M., (2000). Two insecticidal tetranortriterpenoids from *Azadirachta indica*. *Phytochemistry* **53**(3):371–376.

Siddiqui, B.S., Rasheed, M., Ilyas, F., Gulzar, T., Tariq, R.M., Naqvi, S.N., (2004). Analysis of insecticidal *Azadirachta indica* A. Juss. fractions. Z Naturforsch [C] **59**(1–2):104–112.

Siddiqui, S., Faizi, S., Siddiqui, B.S., Ghiasuddin, S.M., (1992). Constituents of *Azadirachta indica*: isolation and structure elucidation of a new antibacterial tetranortriterpenoid, mahmoodin, and a new protolimonoid, naheedin. *J Nat Prod* **55**(3):303–310.

Simmonds, M.S., Jarvis, A.P., Johnson ,S., Jones, G.R., Morgan, E.D., (2004). Comparison of anti-feedant and insecticidal activity of nimbin and salannin photo-oxidation products with neem (*Azadirachta indica*) limonoids. *Pest Manag Sci* **60**(5):459–464.

Sina, I., (2007). Al Qanoon Fil Tib. Vol 1, New Delhi: Idarae Kitabul Shifa, 33, 34.

Sina, I.,(1993). Al Qanoon Fil Tib. (Eng translation). Book II. New Delhi: Jamia Hamdard, 70, 71, 433.

Singh, A., Singh, D.K., (2001). Molluscicidal activity of Lawsonia inermis and its binary and tertiary combinations with other plant derived molluscicides. *Indian J Exp Biol* **39**(3):263–268.

Singh, A., Singh, D.K., (2004). Effect of herbal molluscicides and their combinations on the reproduction of the snail Lymnaea acuminate. *Arch Environ Contam Toxicol* **46**(4):470–477.

Singh, R., Singh, B., Singh, S., Kumar, N., Kumar, S., Arora, S., (2010). Umbelliferone – An antioxidant isolated from *Acacia nilotica* (L.) Willd. Ex. Del. *Food Chemistry* **120**:825–830.

Singh, R., Singh, R., (1972). Screening of some plant extract for antiviral properties. Technology (Sindri) **9**:415-416.

Singh, R.B., Rastogi, S.S., Singh, N.K., Ghosh, S., Gupta, S., Niaz, M.A., (1993). Can guava fruit intake decrease blood pressure and blood lipids. *Journal of Human Hypertension* **7**, 33–38.

Singh, R.B., Rastogi, S.S., Singh, R., Ghosh, S., Niaz, M.A., (1992). Effects of guava intake on serum total high-density lipoprotein cholesterol levels and on systemic blood pressure. *American Journal of Cardiology* **70**, 1287–1291.

Singleton ,V.L., and Rossi, J.A.,(1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture*. **16**: 144-158.

Sleet,R.B. and Brendel, K.,(1983). Improved methods for harvesting and counting synchronous populations of Artemia nauplii for use in developmental toxicology. *Ecotoxicology and Environmental Safety*, **7**: 435-446.

Smith, J., Nigel, H., (1992). Tropical Forests and their Crops. Cornell University Press, London.

Sonibare, M.A., Gbile, Z.O. (2008). *Acacia nilotica* is good for the treatment of asthma. *African Journal of Traditional*, *Complimentary and Alternative Medicine*, **5(4):** 345.

Stalikas, C.D., (2007).Extraction, separation and detection methods for phenolic acids and flavonoids. *Journal of Separation Science*, **30**: 3268-3295.

Stone, B., (1970). The flora of Guam. *Micronesica* **6**, 454–455.

Stratmann, J.W. and Ryan, C. A., (1997). Myelin basic protein kinase in tomato leaves is induced systematically by wounding and increases in response to systemin and oligosaccharide elicitors. *Proc. Nat. Acad. Sci.,* **94**: 11085-11089.

Subapriya, K., Nagini, S., (2005). Medicinal properties of neem leave: a review. *Curr Med Anti-Cancer Agents* **5**:149–156.

Subapriya, R., Kumaraguruparan, R., Abraham ,S.K., Nagini, S., (2004). Protective effects of ethanolic neem leaf extract on N-methyl-N0-nitro-N-nitrosoguanidine-induced genotoxicity and oxidative stress in mice. *Drug Chem Toxicol* **27**(1):15–26.

Subapriya, R., Kumaraguruparan, R., Chandramohan, K.V., Nagini, S., (2003). Chemoprotective effects of ethanolic extract of neem leaf against MNNG-induced oxidative stress. *Pharmazie* **58**(7):512–517.

Subapriya, R., Nagini, S., (2003). Ethanolic neem leaf extract protects against N-methyl-N0-nitro-N-nitrosoguanidineinduced gastric carcinogenesis in Wistar rats. *Asian Pac J Cancer Prev* **4**(3):167–168.

Sultana, B., Anwar, F., Przybylski, R., (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica, and Eugenia jambolana* Lam. Trees. *Food Chemistry* **104**(3): 1106–1114.

Svetlana, V.D., Reyes, M.H., Flores, H.S., Mart´ınez, G.M.C., Gonz´alez de Coss´ıo, O.M., Ch´avez, S.M.A., Rivera, A.E., Lozoya, X., (2007). Effect of a *Psidii guajavae folium* extract in the treatment of primary dysmenorrhea: a randomized clinical trial. *Journal of Ethnopharmacology* **110**, 305– 310.

Talwar, G.P., Raghuvanshi, P., Misra, R., Mukherjee, S., Shah, S., (1997b). Plant immunomodulators for termination of unwanted pregnancy and for contraception and reproductive health. *Immunol Cell Biol* **75**(2):190–192.

Talwar, G.P., Shah, S., Mukherjee, S., Chabra, R., (1997a). Induced termination of pregnancy by purified extracts of *Azadirachta Indica* (Neem): mechanisms involved. *Am J Reprod Immunol* **37**(6):485–491.

Teixeira, R.S., Camparoto, M.L., Mantovani, M.S., Vicentini, V.E.P., (2003).Ssessment of two medicinal plants, *Psidium guajava* L. and *Achillea millefolium* L., *in vitro* and *in vivo* assays. *Genetics and Molecular Biology* **26**, 234–239.

Tepsuwan ,A., Kupradinun, P., Kusamran, W.R., (2002). Chemopreventive potential of neem flowers on carcinogeninduced rat mammary and liver carcinogenesis. *Asian Pac J Cancer Prev* **3**(3):231–238.

Thaipong, K., Boonprakob, U., Cisneros-Zevallos, L., Byrne, D.H., (2005). Hydrophilic and lipophilic antioxidant activities of guava fruits. *Southeast Asian Journal of Tropical Medicine Public Health* **36**, 254–257.

Thomas, J., (1997). Medicinal and aromatic plants research in India. UNDP.

Toky, O.P. and Bisht, R.P., (1992). Observations on the rooting pattern of some agroforestry trees in an arid region of north-western India. *Agroforestry systs.* **15**, 41- 59.

Toky, O.P., Beniwal, R.S. and Sharma, P.K., (1994). Interaction between Rhizobium inoculation and nitrogen fertilizer application on growth and nodulation of *Acacia nilotica* subsp. i*ndica. J. Arid Environ.* **27**, 49-54.

Tona, L., Kambu, K., Mesia, K., Cimanga, K., Apers, S., de Bruyne, T., Pieters, L., Totte, J., Vlietinck, A.J., (1999). Biological screening of traditional preparations from some medicinal plants used as antidiarrhoeal in Kinshasa, Congo. *Phytomedicine* **6**, 59–66.

Trease, G.E. and Evans, W. C., (1983). Pharcognosy, 12th Ed. Baillieere Tindal, London.

UNICEF/WHO (2009) Diarrhoea: Why children are still dying and what can be done.

Upadhyay, S.N., Dhawan, S., Garg, S., Talwar, G.P., (1992). Immunomodulatory effects of neem (*Azadirachta indica*) oil. *Int J Immunopharmacol* **14**(7):1187–1193.

Upadhyay, S.N., Dhawan, S., Talwar, G.P., (1993). Antifertility effects of neem (*Azadirachta indica*) oil in male rats by single intra-vas administration: an alternate approach to vasectomy. *J Androl* **14**(4):275–281.

Upadhyay, S.N., Kaushic, C., Talwar, G.P., (1990). Antifertility effects of neem (*Azadirachta indica*) oil by single intrauterine administration: a novel method for contraception. *Proc R Soc Lond B Biol Sci* **242**(1305):175–179.

van der Nat JM, Klerx JP, van Dijk H, de Silva KT, Labadie RP (1987) Immunomodulatory activity of an aqueous extract of *Azadirachta indica* stem bark. *J Ethnopharmacol* **19**(2):125–131.

Vanhaecke, P., Persoone, G., Claus. C., (1981). Proposal for a short term toxicity test with Artemia nauplii, *Ecotoxicology and Environmental Safety* **5**: 382-387.

Vanka , A., Tandon, S., Rao, S.R., Udupa, N., Ramkumar, P., (2001). The effect of indigenous Neem Azadirachta indica [correction of (*Azadirachta indica*)] mouth wash on Streptococcus mutans and lactobacilli growth. *Indian J Dent Res* **12**(3):133–144.

Vargas, A.D., Soto, H.M., Gonzalez, H.V.A., Engleman, E.M., Martinez, G.A., (2006). Kinetics of accumulation and distribution of flavonoids in guava (*Psiduim guajava*). Agrociencia **40**, 109–115.

Velu, G., Srinivasan, P.S., Ali, A.M. and Narwal, S.S., (1999). Phytotoxic effect of tree crops on germination and radical extension of legumes. *Intl. Cont. Allel.* **1**, 299-302.

Verma,V.S., (1974). Chemical compounds from *Azadirachta indica* as inhibitors of potato virus X. Acta Microbiol Pol B **6**(1):9–13.

Victora, C.G., Huttly, S.R., Fuchs, S.C., Barros, F.C., Garenne, M., Leroy, O., Fontaine, O., Beau, J.P., Fauveau, V. and Chowdhury, H.R. ,(1993). International differences in clinical patterns of diarrhoeal deaths: a comparison of children from Brazil, Senegal, Bangladesh and India. *J Diiarrhoeal Dis. Res*. **11**(1): 25-9.

Wadood, A., Wadood, N., Shah, S.A., (1989). Effects of *Acacia arabica* and arallum edulis on blood glucose levels of normal and Alloxan diabetic rabbits. *J Pak Med Assoc* **39**(1):33-38.

Wandscheer,C.B., Duque. J.E., da Silva, M.A., Fukuyama, Y., Wohlke, J.L., Adelmann, J., Fontana, J.D., (2004.) Larvicidal action of ethanolic extracts from fruit endocarps of Melia azedarach and *Azadirachta indica* against the dengue mosquito Aedes aegypti. *Toxicon* **44**(8):829–835.

Wang, B., Liu, H.C., Ju, C.Y., (2005). Study on the hypoglycemic activity of different extracts of wild *Psidium guajava* leaves in Panzhihua area. Sichuan Da Xue Xue Bao Yi Xue Ban **36**, 858–861.

Wang, W.X., Vinocur, B., Shoseyov, O. and Altman, A.,(2004). Role of plant heat shock proteins and molecular chaperones in the abiotic stree response. *Trends Plant Sci* **9**, 244-252.

Wei, L., Li, Z., Chen, B., (2000). Clinical study on treatment of infantile rotaviral enteritis with *Psidium guajava* L. Zhongguo Zhong Xi Yi Jie He Za Zhi **20**, 893–895.

WHO(2011) Diarrhoea: http://www.who.int/topics/diarrhoea/en. Cited 10 April 2011.

WHO, (1993). Regional office for Western Pacific Research Guidelines for evaluating safety and efficacy of herbal medicines, Manila.

Wolinsky ,L.E., Mania, S., Nachnani, S., Ling, S., (1996). The inhibiting effect of aqueous *Azadirachta indica* (Neem) extract upon bacterial properties influencing in vitro plaque formation. *J Dent Res* **75**(2):816–822.

Word Health Organization, (1998). Medicinal plants in the South Pacific. WHO Regional Publications. *Western Pacific Series num*. **19**. 163.

Yamashiro, S., Noguchi, K., Matsuzaki, T., Miyagi, K., Nakasone, J., Sakanashi, M., Kukita, I., Aniya, Y., Sakanashi, M., (2003). Cardioprotective effects of extracts from *Psidium guajava* L. and *Limonium wrightii*, Okinawan medicinal plants, against ischemia-reperfusion injury in perfused rat hearts. *Pharmacology* **67**, 128–135.

Yanes, A., Finol, H.J., Hasegawa, M., (2004). Effects of *Azadirachta indica* and Melia azedarach (Meliaceae) extracts from leaves on Trypanosoma cruzi growth and ultrastructure. *J Submicrosc Cytol Pathol* **36**(2):149–154.

Yanpallewar, S.U., Sen, S., Tapas, S., Kumar, M., Raju, S,S., Acharya, S.B., (2003). Effect of *Azadirachta indica* on paracetamolinduced hepatic damage in albino rats. *Phytomedicine* **10**(5):391–396.

Yusof, R.M., Said, M., (2004). Effect of high fibre fruit (Guava-*Psidium guajava* L.) on the serum glucose level in induced diabetic mice. *Asia Pacific Journal Clinical Nutrition* **13**, S135.

Zakaria, M., Mohd, M.A., (1994). Traditional Malay medicinal plants. Penerbit Fajar Bakti Sudan Berhard, 129–132.

Zhang, W.J., Chen, B.T., Wang, C.Y., Zhu, Q.H., Mo, Z., (2003). Mechanism of quercetin as an antidiarrheal agent. Di Yi Jun Yi Xue Xue Bao **23**, 1029–1031.

Zhu, M., Phillipson, J.D., Greengrass, P.M., Bowery, N.E., Cai, Y., (1997). Plant polyphenols: biologically active compounds or non-selective binders to proteins. *Phytochemistry* **44**, 441–447.

Appendix table 1. Toxicity of ethanol extract of *A. nilotica* leaves against *A. salina* naupii after 24h.

Estimate 1
Y = 0.6035461 + 1.706288 X
Estimate 2
Y = 0.6012402 + 1.693632 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2. 903	10	8	80.000	80	5.85	5.557	5.780	5.81	5.518
600.000	2.778	10	6	60.000	60	5.25	5.344	5. 240	6.16	5.306
400.000	2 . 602	10	4	40.000	40	4.75	5.043	4.750	6.37	5.008
200.000	2.301	10	3	30.000	30	4.48	4.530	4.460	5.81	4.498
100.000	2 . 000	10	2	20.000	20	4.16	4.016	4.160	4.39	3.988

CHI=SQUARED IS 0.9880209 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.597236
LD-50 IS 395.5811
95% CONF LIMITS ARE 238.6928 TO 655.5889

Appendix table 2. Toxicity of methanol extract of *A. nilotica* leaves against *A. salina* naupii after 24h.

Estimate 1
Y = 1.435269 + 1.282851 X
Estimate 2
Y = 1.371269 + 1.30499 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	6	60.000	60	5. 25	5.159	5.240	6. 34	5.160
600.000	2.778	10	5	50.000	50	5. 00	4 .999	4.990	6. 34	4.997
400.000	2 . 602	10	4	40.000	40	4. 75	4 .773	4.740	6.16	4.767
200.000	2.301	10	2	20.000	20	4. 16	4.387	4.170	5.32	4.374
100.000	2.000	10	2	20.000	20	4. 16	4.001	4.160	4.39	3.981

CHI-SQUARED IS .4074383 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.780657
LD-50 IS 603.4723
95% CONF LIMITS ARE 273.0789 TO 1333.603

Appendix table 3. Toxicity of chloroform extract of *A. nilotica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.890129 + 1.060803 X
ESTIMATE 2
Y = 1.792453 + 1.098775 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	5	50.000	50	5.00	4.970	4.990	6.34	4.982
600.000	2.778	10	5	50. 000	50	5.00	4.837	5.020	6.27	4.845
400.000	2.602	10	3	30.000	30	4.48	4.650	4.470	6.01	4.652
200.000	2.301	10	2	20.000	20	4.16	4.331	4.170	5.32	4.321
100.000	2.000	10	2	20.000	20	4.16	4.012	4.160	4.39	3.990

CHI-SQUARED IS .6381671 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.919202
LD-50 IS 830.2363
95% CONF LIMITS ARE 256.2023 TO 2690.424

Appendix table 4. Toxicity of ethyl acetate extract of *A. nilotica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.690581 + 1.161543 X
ESTIMATE 2
Y = 1.614375 + 1.189253 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	6	60.000	60	5.25	5 . 063	5.250	6. 37	5.067
600.000	2 . 778	10	4	40.000	40	4.75	4 . 917	4.740	6.34	4.918
400.000	2 . 602	10	4	40.000	40	4.75	4.713	4.740	6.16	4.709
200.000	2.301	10	2	20.000	20	4.16	4.363	4.170	5.32	4.351
100.000	2.000	10	2	20.000	20	4.16	4.014	4.160	4 . 39	3.993

CHI-SQUARED IS .717839 WITH 3 Degrees freedom
NO SIG HETEROGENEITY
LOG LD-50 IS 2.846851
LD-50 IS 702.8301
95% CONF LIMITS ARE 268.122 TO 1842.334

Appendix table 5. Toxicity of petroleum ether extract of *A. nilotica* leaves of against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.875634 + 1.111857 X
ESTIMATE 2
Y = 1.830191 + 1.126533 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	6	60.000	60	5.25	5.103	5.240	6.34	5.101
600.000	2.778	10	5	50.000	50	5.00	4.965	4.990	6.34	4.960
400.000	2.602	10	3	30.000	30	4.48	4 .769	4.480	6.16	4.761
200.000	2.301	10	3	30.000	30	4.48	4 . 434	4.480	5.58	4.422
100.000	2.000	10	2	20.000	20	4.16	4.099	4.160	4.39	4.083

CHI-SQUARED IS .661408 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2 . 813774
LD-50 IS 651.2893
95% CONF LIMITS ARE 247.8056 TO 1711.737

Appendix table 6. Toxicity of ethanol extract of *A.indica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.890129 + 1.060803 X
ESTIMATE 2
Y = 1.792453 + 1.098775 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	5	50.000	50	5.00	4 . 970	4.990	6. 34	4.982
600.000	2 . 778	10	5	50.000	50	5.00	4 . 837	5.020	6. 27	4.845
400.000	2 . 602	10	.3	30.000	30	4.48	4 . 650	4.470	6. 01	4.652
200.000	2.301	10	2	20.000	20	4.16	4.331	4.170	5.32	4.321
100.000	2 . 000	10	2	20.000	20	4.16	4.012	4.160	4.39	3.990

CHI-SQUARED IS .6381671 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.919202
LD-50 IS 830.2363
95% CONF LIMITS ARE 256.2023 TO 2690.424

Appendix table 7. Toxicity of methanol extract of *A.indica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.875634 + 1.111857 X
ESTIMATE 2
Y = 1.830191 + 1.126533 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	6	60.000	60	5.25	5.103	5.240	6. 34	5.101
600.000	2.778	10	5	50.000	50	5.00	4 .965	4.990	6. 34	4.960
400.000	2 . 602	10	3	30.000	30	4.48	4 .769	4.480	6. 16	4.761
200.000	2.301	10	3	30.000	30	4.48	4.434	4.480	5.58	4.422
100.000	2 . 000	10	2	20.000	20	4.16	4 .099	4.160	4.39	4.083

CHI-SQUARED IS .661408 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2 . 813774
LD-50 IS 651.2893
95% CONF LIMITS ARE 247.8056 TO 1711.737

Appendix table 8. Toxicity of chloroform extract of *A.indica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 2.07744 + 1.033264 X
ESTIMATE 2
Y = 2.093083 + 1.025587 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	6	60.000	60	5.25	5.077	5.250	6. 37	5.070
600.000	2.778	10	4	40.000	40	4.75	4.948	4.740	6.34	4.942
400.000	2 . 602	10	4	40.000	40	4.75	4 .766	4.740	6.16	4.762
200.000	2 . 301	10	3	30.000	30	4.48	4 . 455	4.480	5 . 58	4.453
100.000	2 . 000	10	2	20.000	20	4.16	4 .144	4.170	4 . 71	4.144

CHI-SQUARED IS .4750226 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.834395
LD-50 IS 682.9595
95% CONF LIMITS ARE 228.6499 TO 2039.947

Appendix table 9. Toxicity of ethyl acetate extract of *A.indica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.890129 + 1.060803 X
ESTIMATE 2
Y = 1.792453 + 1.098775 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	5	50 . 000	50	5.00	4 .970	4.990	6.34	4.982
600.000	2.778	10	5	50.000	50	5.00	4.837	5.020	6.27	4.845
400.000	2 . 602	10	3	30.000	30	4.48	4 . 650	4.470	6. 01	4.652
200.000	2.301	10	2	20.000	20	4.16	4 .331	4.170	5.32	4.321
100.000	2.000	10	2	20.000	20	4.16	4.012	4.160	4.39	3.990

CHI-SQUARED IS .6381671 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.919202
LD-50 IS 830.2363
95% CONF LIMITS ARE 256.2023 TO 2690.424

Appendix table 10. Toxicity of petroleum ether extract of *A.indica* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 2.130947 + .9905488 X
ESTIMATE 2
Y = 2.087114 + 1.008223 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	6	60.000	60	5.25	5.007	5.250	6. 37	5.014
600.000	2.778	10	4	40.000	40	4.75	4 . 883	4.760	6.27	4.888
400.000	2 . 602	10	3	30.000	30	4.48	4 .708	4.480	6.16	4.711
200.000	2 . 301	10	3	30.000	30	4.48	4 .410	4.480	5 . 58	4.407
100.000	2 . 000	10	2	20.000	20	4.16	4 .112	4.170	4 . 71	4.104

CHI-SQUARED IS .8354511 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.88913
LD-50 IS 774.694
95% CONF LIMITS ARE 229.6833 TO 2612.949

Appendix table 11. Toxicity of ethanol extract of *P.guajava* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.875634 + 1.111857 X
ESTIMATE 2
Y = 1.830191 + 1.126533 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	6	60.000	60	5.25	5.103	5.240	6.34	5.101
600.000	2 . 778	10	5	50.000	50	5.00	4.965	4.990	6.34	4.960
400.000	2 . 602	10	3	30.000	30	4.48	4.769	4.480	6.16	4.761
200.000	2.301	10	3	30.000	30	4.48	4.434	4.480	5.58	4.422
100.000	2.000	10	2	20.000	20	4.16	4 .099	4.160	4.39	4.083

CHI-SQUARED IS .661408 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.813774
LD-50 IS 651.2893
95% CONF LIMITS ARE 247.8056 TO 1711.737

Appendix table 12. Toxicity of methanol extract of *P.guajava* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 2.07744 + 1.033264 X
ESTIMATE 2
Y = 2.093083 + 1.025587 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2 . 903	10	6	60.000	60	5.25	5.077	5.250	6.37	5.070
600.000	2 . 778	10	4	40.000	40	4.75	4 .948	4.740	6.34	4.942
400.000	2.602	10	4	40.000	40	4.75	4 .766	4.740	6.16	4.762
200.000	2 . 301	10	3	30.000	30	4.48	4 . 455	4.480	5. 58	4.453
100.000	2.000	10	2	20.000	20	4.16	4 .144	4.170	4 . 71	4.144

CHI-SQUARED IS .4750226 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.834395
LD-50 IS 682.9595
95% CONF LIMITS ARE 228.6499 TO 2039.947

Appendix table 13. Toxicity of chloroform extract of *P. guajava* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.890129 + 1.060803 X
ESTIMATE 2
Y = 1.792453 + 1.098775 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	5	50.000	50	5.**00**	4.970	4.990	6. 34	4.982
600.000	2.778	10	5	50.000	50	5.**00**	4 .837	5.020	6.27	4.845
400.000	2.602	10	3	30.000	30	4.48	4 . 650	4.470	6. 01	4.652
200.000	2.301	10	2	20.000	20	4.16	4.331	4.170	5.32	4.321
100.000	2.000	10	2	20.000	20	4.16	4.012	4..160	4.39	3.990

CHI-SQUARED IS .6381671 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.919202
LD-50 IS 830.2363
95% CONF LIMITS ARE 256.2023 TO 2690.424

Appendix table 14. Toxicity of ethyl acetate extract of *P.guajava* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 2.07744 + 1.033264 X
ESTIMATE 2
Y = 2.093083 + 1.025587 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800.000	2.903	10	6	60.000	60	5.25	5.077	5.250	6.37	5.070
600.000	2.778	10	4	40.000	40	4.75	4 . 948	4.740	6.34	4..942
400.000	2.602	10	4	40.000	40	4.75	4.766	4.740	6.16	4.,762
200.000	2.301	10	3	30.000	30	4.48	4 .455	4.480	5. 58	4.453
100.000	2.000	10	2	20.000	20	4.16	4 .144	4.170	4.71	4.144

CHI-SQUARED IS .4750226 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.834395
LD-50 IS 682.9595
95% CONF LIMITS ARE 228.6499 TO 2039.947

Appendix table 15. Toxicity of petroleum ether extract of *P.guajava* leaves against *A. salina* naupii after 24h.

ESTIMATE 1
Y = 1.875634 + 1.111857 X
ESTIMATE 2 Y = 1.830191 + 1.126533 X

Dose	Ldos	#U	Kl	%Kill	Cr%	E Pr	Ex Pr	Wk Pro	Weght	F Pro
800 . 000	2 . 903	10	6	60.000	60	5. .25	5.103	5.240	6. 34	5.101
600.000	2 . 778	10	5	50.000	50	5.00	4.965	4.990	6.34	4.960
400.000	2 . 602	10	3	30.000	30	4.48	4 .769	4.480	6.16	4.761
200.000	2 . 301	10	3	30.000	30	4.48	4 .434	4480	5.58	4.422
100.000	2 . 000	10	2	20.000	20	4.16	4.099	4.160	4.39	4.083

CHI-SQUARED IS .661408 WITH 3 DEGREES OF FREEDOM
NO SIG HETEROGENEITY
LOG LD-50 IS 2.813774
LD-50 IS 651.2893
95% CONF LIMITS ARE 247.8056 TO 1711.737