BIOLOGICAL AND PHYTOCHEMICAL STUDIES ON HEARTWOOD OF *Acacia catechu* OF NEPAL

A DISSERTATION

SUBMITTED TO THE CENTRAL DEPARTMENT OF CHEMISTRY

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M. SC. DEGREE IN CHEMISTRY

BY

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CENTRAL DEPARTMENT OF CHEMISTRY

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BIOLOGICAL AND PHYTOCHEMICAL STUDIES ON HEARTWOOD OF *Acacia catechu* OF NEPAL

Submitted

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Yagya Prasad Subedi

Has been accepted as partial fulfillment for the requirement of the

M.Sc. degree in

Chemistry

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RECOMMENDATION LETTER

This is to certify that Mr. Yagya Prasad Subedi has completed this dissertation work entitled BIOLOGICAL AND PHYTOCHEMICAL STUDIES ON HEARTWOOD OF *Acacia catechu* OF NEPAL as a partial fulfillment for the requirement of M. Sc. Degree in Chemistry under my supervision. To my knowledge, this dissertation work had not been submitted to any other degree.

…….

Supervisor

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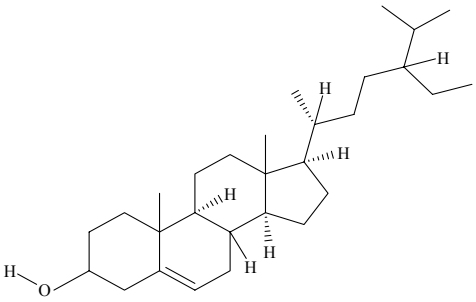
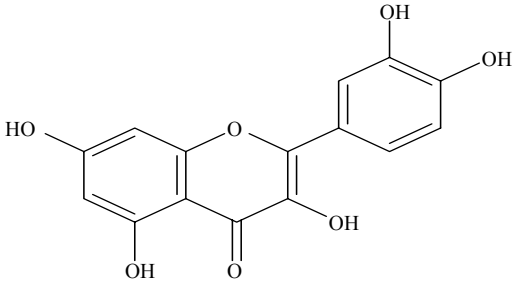
2011

ABSTRACT

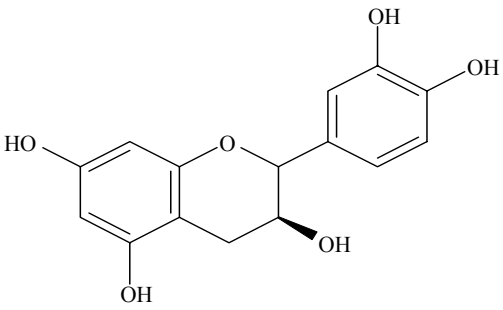
Biological and Phytochemical screening of heartwood of *Acacia catechu* was studied. Phytocostituents found were Sterols, Flavones Aglycones, Tannins and Polyphenols. The column chromatography of diethyl ether fractions result in the isolation of β-Sitosterol (Ac1), Quercetin (Ac4), (+) Catechin (Ac6). Other four compounds Ac2, Ac3, Ac5, Ac7 whose work is on progress, were also isolated. Antibacterial screening, antifungal screening and brine shrimp bioassay of different fractions showed that hexane, chloroform, diethyl ether, ethyl acetate and methanol fraction were biologically active. In brine shrimp bioassay only diethyl ether, ethyl acetate, and methanol fraction showed activity.



β-Sitosterol



Quercetin



(+) Catechin

TABLE OF CONTENTS

CHAPTER- III

CHAPTER- IV

CHAPTER-V

ACRONYMS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
δ	:	Delta
^0C	:	Degree Celsius
DMSO	:	Dimethyl Sulfoxide
gm	:	Gram
IR	:	Infra Red
LC_{50}	:	Lethal Concentration for 50% Mortality
m.p.	:	Melting Point
m	:	Meter
mg	:	Milligram
ml	:	Milliliter
M^+	:	Molecular ion
m/z	:	Mass/Electron
NMR	:	Nuclear Magnetic Resonance
Nm	:	Nanometer
λ_m	:	Maximum Wavelength
ppm	:	Parts per Million
R_f	:	Retension Factor
TLC	:	Thin Layer Chromatography
UV	:	Ultra Violet
Vis	:	Visible
Mg	:	Microgram
Mm	:	Micrometer

µl	:	Micro liter
ZOI	:	Zone of Inhibition
MIC	:	Minimum Inhibitory Concentration
MBC	:	Minimum Bacterial Concentration
NA	:	Nutrient Agar
MHA	:	Molar Hinton Agar
NB	:	Nutrient Broth
EtOAc	:	Ethyl Acetate
MeOH	:	Methanol
$CHCl_3$	:	Chloroform
Hex	:	Hexane
Acet	:	Acetone
Benz	:	Benzene
Lit	:	Liter
PDA	:	Potato Dextrose Agar
PDB	:	Potato Dextrose Broth
Long.	:	Longitudinal

CHAPTER I

1 INRODUCTION

1.1 Background

By directional development in the field of medicinal science had reached to its zenith and thus earned utmost popularity throughout the world. One of these two directions has been led by gene manipulation or by using gene related technologies or genetic engineering and the other by preventing from or curing the diseases by the use of medicinal plants[1].

Nature has been the source of medicinal source for thousands of years and impressive number of modern drugs has been isolated from natural sources, many based on their uses on traditional medicine[2]. Plant produce diverge range of bio-active molecules making them rich source of different medicine. These bio-active molecules are organic compound of very unique and complex structure called secondary metabolites. Actually these are the byproduct of primary metabolites and their role in metabolism of host is not obvious[3]. The most important of these bio-active compounds of plant are Alkaloids, Flavonoids, Tannins and Phenolic compounds. The phytochemical research based on ethno-pharmacological information is generally considered as an effective approach in the discovery of new anti-infective agents from higher plants[4]. The chemical constituent present in plant which are therapeutically active are called active constituent and inactive once are called inert chemical constituents[5].

The history of medicine and medicinal plants in Nepal can be traced back to the Vedic period, where Nepal Himalaya was mentioned as a sacred heaven of potent medicinal and aromatic plants[6]. Good information on the ethno-botanical and medicinal uses of the Nepalese plants can be found in the “Chandra Nighantu”, an herbal pharmacopoeia of medicinal value of plants in 19th century. The earliest mention of the medicinal use of plant

in Hindu culture is found in "Rig-Veda", which has said to have been written between 4500 B.C. and 1600 B.C. is supposed to be the oldest repository of human knowledge[7].

Nepal is situated in the latitude of 26°22'N to 30°27'N and longitudes of 80°04'E to 88°12'E at the central third of the massive Himalaya chain 2500km long with elevation range from 90m to 8848m. Nepal with more or less rectangular shape, stretches lengthwise about 885km east-west and has an average width of 193km north-south. Vegetation of Nepal was classified on the basis of its bioclimatic variations both vertical and horizontal. Accordingly, Nepal is divided in to three main regions: West (Long. 80°40E-83°0'E) Central (Long. 83°0'E-86°30'E) and east (Long. 80°30'-88°12')[1,8].

In Nepal it is estimated that various communities uses approximately 1000 species of wild plants in traditional medicinal practices[9].Nepal has natural gift of over 7000 species of vascular plants among them 1463 species of medicinal plants have been reported, representing about 20% of total flora[10].

1.2 Description of Plant

Acacia belongs to family Fabaceae (Leguminosae). It is estimated that there are roughly 1380 species of *Acacia* worldwide. About two-third of them are native to Australia and rest of spread around tropical and subtropical region of the world[11-13]. In Nepal eight species of *Acacia* are reported[14]. We are here concerning with *Acacia catechu*. Other botanical names of *Acacia catechu* are *Acacia catechoides* (Roxb.)Benth, *Mimosa catechu* L., *Mimosa catechuoides* Roxb. . English name are Black catechu, Cutch tree, Catechu tree, etc. In Sanskrit it is called Khadira, Raktasar, Yagya. Commonly in Nepal it is called by Khayar, where as its trade name are Khair and Cutch[15]. In Nepal, it is commercially threatened[16,17] and banned for transportation, export and felling[18].

Acacia catechu is a deciduous tree from 5-10m, shoot unarmed of with paired stipular spines. Leaves bipinnate, 15-20cm, rachis with a large (2-3mm) rounded gland at base of

lowest, pair of pinnae and 2-4 smaller glands at base of upper ones, pinnae 20-25 pairs, 3-5cm, leaflets numerous, linear-oblong, crowded, (20-)30-45 pairs, base rounded or truncate, ± glabrous. Flowers bisexual in spikes or globose heads. Spikes solitary, axillary, narrowly cylindrical, 8-10cm, densely flowered. Calyx ca. 1.5mm, 4-5 lobed, shortly toothed. Petals as many as calyx lobes, oblong, 2.5-3 mm, connate in lower half. Stamens numerous (more than 20), exserted, nearly 4mm, yellow. Pods oblong, 5-7 x 1-1.5(-2)cm, compressed, leathery; seeds 6-8, suborbicular, nearly 5mm diameter[19]. Chromosome numbers 2n = 26. Flowering in April - June and fruiting in September – December[1].

Acacia catechu is generally distributed in Nepal, India, Thailand, S. China. In Nepal it is distributed in West, Central, East region from tropical to subtropical region with altitude variation 200-1400[19].

1.3 Importance of Plant

In Ayurveda bark of *Acacia catechu* is used as astringent, anti inflammatory, anti helmintic, and useful in toothache, scabies, bronchitis, anorexia, wound, leucoderma, indigestion, blood diseases, leprosy, and cough[20]. It is also used as antidysenteric and antipyretic, and is considered to cure sore throat, ulcers, boils, anemia and piles[21]. It is also considered to use in skin disease, urinary disease and mucous defect[22].

In folk medicine heartwood of *Acacia catechu* is used as anti helmintic, antiseptic, antidysenteric, anti-inflammatory, antipyretic, haemostatic, haematinic[23,24], astringent, depurative, appetizer, tonic, and considered to treat catarrh, cough, leprosy, pruritus, leucoderma, skin disease, anorexia, foul ulcers, wounds, haemoptysis, haematemesis, intermittent fever, inflammation, odontopathy, splenomegaly and pharyngodynia[24,25]. It is considered to be useful in chronic ulceration, anemia and diabetes[26]. Catechu obtained from the heartwood is considered to use in chronic diarrhea, leucorrhea, gingivitis, stomatitis[27], hemorrhoids, and uterine hemorrhage; infusion of catechu is valued in treating nosebleeds

(epistaxis), skin eruption, and sore nipples; the heartwood is boiled in water and applied to relieve backache and other pains caused by arduous work, cough, cold and toothache[28] and colic pain, phthisis, and bronchitis [29,30]. It is a valuable agent for removing cynanche tonsillaris, elongation of uvula and relaxation and congestion of the mucous membrane of fauces, especially of the kind to which public singers are subjected. It is useful in congestion, tenderness and sponginess of the gums[31]. Cutch is considered to cure itching sore throat, indigestion, psoriasis, and urinary discharges[32.]

In folk medicine bark is used as anti helmintic, anti dysenteric and antipyretic which is considered to be useful in melancholia, conjunctivitis, haemoptysis and skin diseases[30]; diarrhea and leprosy[33,34]. Its decoction is considered to treat cough and sprain[35,36]and skin diseases[37,38]**.** And decoction of root is applied in swelling caused by pain or injury[28]. The resinous extract is beneficial in cough and diarrhoea[39]. Root powder is given for drying wounds and it is also used to kill worms in cattles[1,37,38]. The product called kheersal is found within some of older tree in the form of white crystalline deposit of Catechin is used for sore throat and cough[40]**.**

Traditionally Acacia catechu is used in dyeing industries and in Bittle (Paan). Aqueous extract of *Acacia catechu* is used in Indian traditional system of medicine for cancer treatments[41].

1.4 Objective of Study

Acacia catechu has wide range of medicinal value in local application and Ayurveda. But its chemical analysis has not been studied yet properly in Nepal. Being plant banned for transportation and export and present in large number in Nepal with high medicinal importance, it was chosen for dissertation. At present the objective of this work are-

i) Phytochemical screening of different extract of heartwood of *Acacia catechu*.

ii) Biological screening of different extract of heartwood of *Acacia catechu*.

iii) Isolation and characterization of chemical constituents from different extract obtained from heartwood of *Acacia catechu*.

CHAPTER II

2 LITERATURE REVIEW

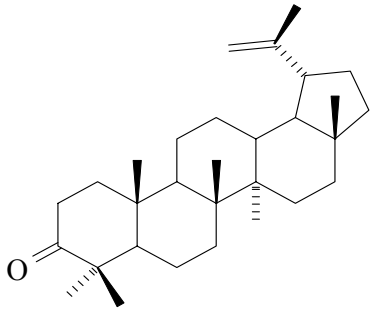
Journals, Paper, Patents etc reveals that a number of chemical constituent have been isolated from heartwood of *Acacia catechu*. Many of the chemical constituent from heartwood of *Acacia catechu* show wide range of biological activity. Different constituents isolated are Triterpenes, Sterols, Alkaloids, Carbohydrates, Tannins, Flavonoids, and Fatty acids.

2.1 Chemical Constituents of *Acacia catechu*

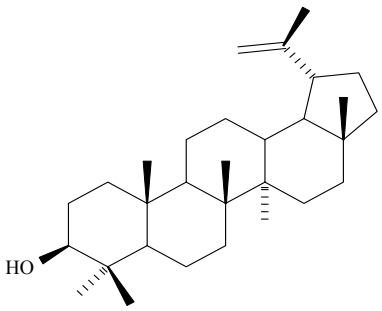
2.1.1 Triterpenes

Triterpenes are hydrocarbon or its oxygenated analog containing 30 carbon atoms and composed of six isoprene units. Resins and Saps contain Triterpenes in the free state as well as in the form of Esters and Glycosides. Biogenetically Triterpenes arise by the cyclization of Squalene. Most Triterpenes are either tetracyclic or pentacyclic compounds[42,43].

Triterpene reported in *Acacia catechu* are Lupenone, Lupeol[44].



Lupenone

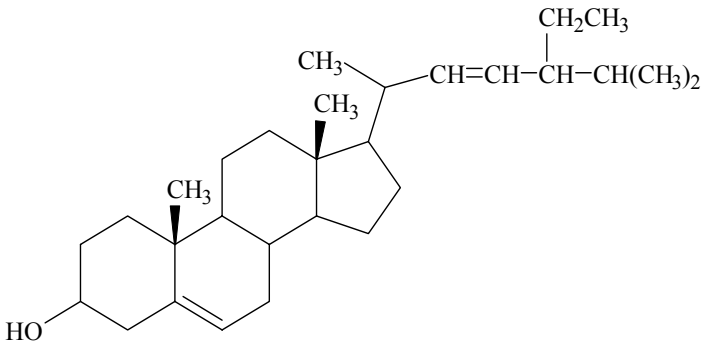


Lupeol

2.1.2 Sterols

Sterols occur as fats and oils in animal and plant respectively. They are crystalline compounds, containing alcoholic functional group in free state as well as esters of higher

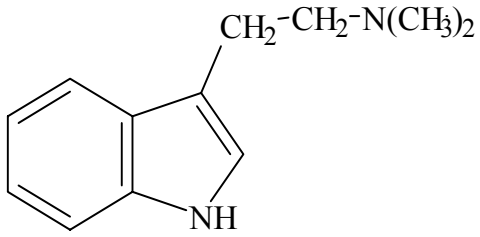
Fatty acids. It is mainly isolated from the unsaponificable portion of oils and fats. The Sterols obtained from the plants sources is Phytosterols and has 1, 2-cyclopentanoperhydro - phenanthrane unit. Ergosterol and Stigmasterols are principal Sterols with 29-30 carbons atoms which are used for the synthesis of Steroids[43,45]. Sterols from *Acacia catechu* are Poriferasterol, Poriferasterol acyl glucosides, Poriferasterol-3-β-glucosides[44].



Poriferasterol

2.1.3 Alkaloids

Alkaloids are a group of naturally occurring chemical compounds which mostly contain basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. Also some synthetic compounds of similar structure are attributed to Alkaloids. Besides carbon, hydrogen and nitrogen, molecules of alkaloids may contain sulfur and rarely chlorine, bromine or phosphorus[43,46]. Alkaloid found in *Acacia catechu* is N,N-dimethyltryptamine[36].

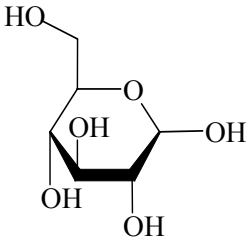


N,N-dimethyltryptamine

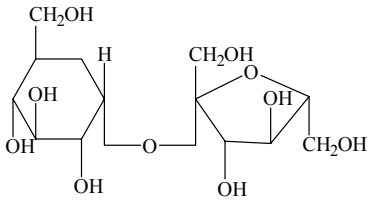
2.1.4 Carbohydrades

Carbohydrate is an organic compound with the general formula $C_m(H_2O)_n$, that is, consists only of carbon, hydrogen and oxygen, with the last two in the 2:1 atom ratio. Carbohydrates can be viewed as hydrates of carbon, hence their name. Structurally however, it is more accurate to view them as polyhydroxy aldehydes and ketones. Carbohydrade are divided into four types: Monosaccharides, Disaccharides, Oligosaccharides, and Polysaccharides[47].

The study of Carbohydrates is one of the most exciting fields of organic chemistry. It deals with understanding the tremendously complicated problems of photosynthesis to the equally difficult problems involved in the unraveling tangle steps during the enzyme catalyzed reconversion of (+) –Glucose into carbondioxide and water[43]. The Carbohydrates found in this plant are D-Glucose and Sucrose[1,32,48].



D-Glucose



Sucrose

2.1.5 Tannins

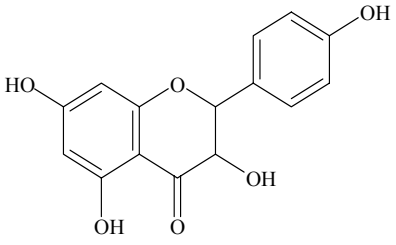
Tannins are colorless non-crystalline substance which forms colloidal solutions in water. The name Tannins is derived from their ability to tan leather. Tannins precipitate proteins from solution, and they from a bluish-black color with ferric salt[43].

There are three major classes of Tannins viz. Hydrolysable Tannins, Non-hydrolysable Tannins or Condensed Tannins & Pseudotannis. Hydrolysable Tannins on heating with HCl or H_2SO_4 yield Gallic or Elagic acids. Non-Hydrolysable Tannins on heating with HCl yield

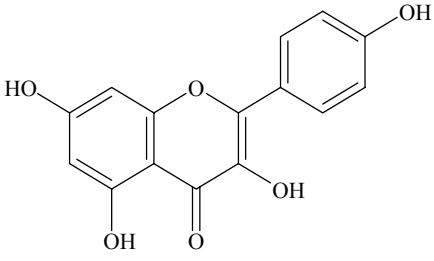
Phlobaphenes like Phloroglucinol[49]. Tannins present in *Acacia catechu* are Tannin, Gallatanin, Phloratannin[50], Catechutannic acids[30].

2.1.6 Flavonoids

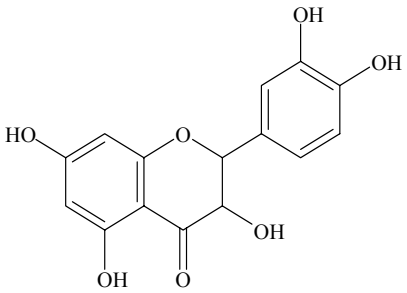
Flavonoids are Polyphenolic compound, which are Ubiquitous in nature. Basic skeleton of Flavonoids contains 15 carbons atoms in which three carbon atoms is present in between two phenyl rings. In most flavonoids three carbons in between two rings are cyclise with oxygen. Flavonoids are categorized according to their chemical structure as Flavone, Flavonal, Flavanone, Flavanonol, Isoflavone, Catechin, Anthocyanidine and Chalcone. Flavonoids are also collectively known as Vitamin P and Citrin. Flavonoids are most popular for their antioxidant activities[3,51]. Flavonoids perform two important functions, they strengthen our body's immune response to attacks from viruses, allergens, and carcinogens and they act as powerful super-antioxidants, protecting our body against free-radical damage, and oxidative stress that can lead to many neurological, cardiovascular, and diabetic diseases**.** Most of the chemical constituents in *Acacia catechu* are Flavonoids. Flavonoids found in *Acacia catechu* are Dihydrokaempferol, Kaempferol, Dihydroquercetin, Quercetin, Isorhamnetin, (+) Afzelchin, (-) Epicatechin,[48,52], Quercetagetin,[32,48] 3 Methyl Quercetin, Gossypetin, (+) Catechin, Dicatechin, Gallochin, Fisetin,[34] 5,7,3',4'-tetrahydroxy-3-methoxyflavone-7-*O*-β-D-galactopyranosyl-(1-4)-*O*-β-D-glucopyranoside[36], 5,7-dihydroxy-3-6-dimethoxyflavone-5-O-α-1-arabinosyl-(1-6)-O-β-D-glucopyranoside(I)[53].



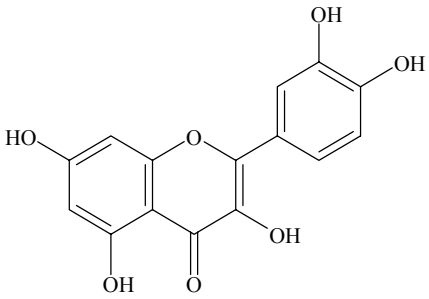
Dihydrokaempferol



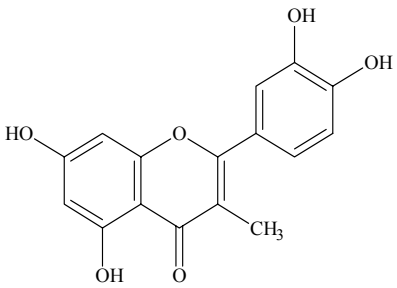
Kaempferol



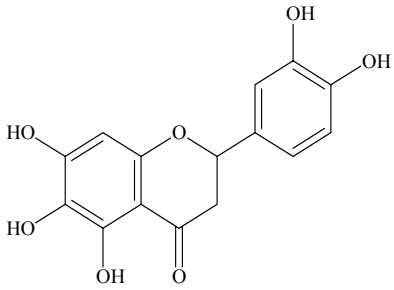
Dihydroquercetin



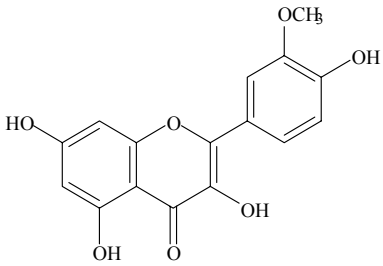
Quercetin



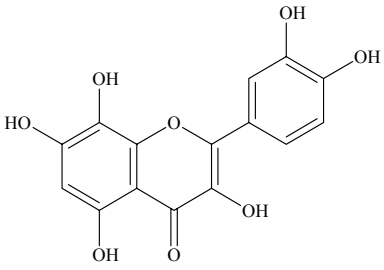
3-Methyl Quercetin



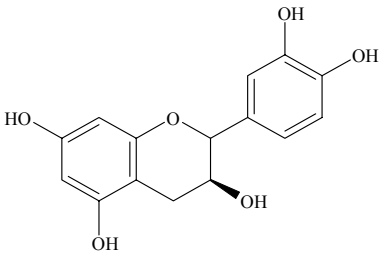
Quercetagetin



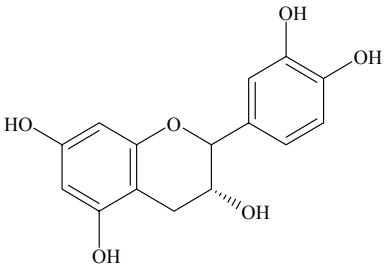
Isorhamnetin



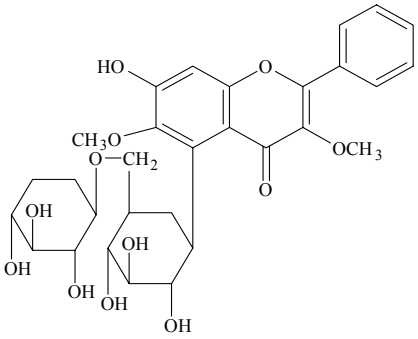
Gossypetin



(+) Catechin



(-) Epicatechin

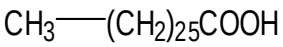


5,7- dihydroxy-3-6-dimethoxyflavone-5-O-α-1- arabinosyl-

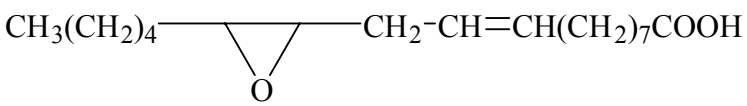
(1-6)-O-β-D -glucopyranoside(I)

2.1.7 Fatty acids

Fatty acids are carboxylic acid with a long unbranched aliphatic chain, derived from, or contained in esterified form in animal or vegetable fat, oil or wax. Naturally fatty acids generally have a chain of 4 to 28 carbons, which may be saturated or unsaturated. Fatty acids reported in *Acacia catechu* are Octacosanoic acids, Urasolic acid[44], Vernolic acid.[36]

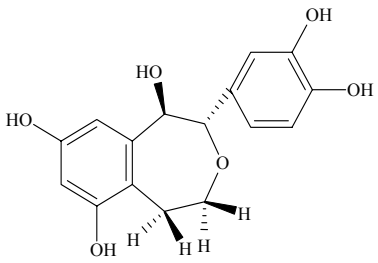


Octacosanoic acid

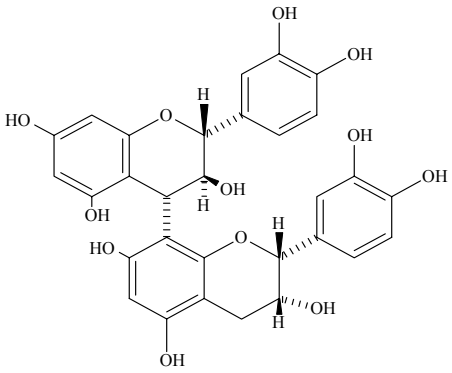


Vernolic acid

In spite of these, other important chemical constituents present in *Acacia catechu* are Hexacosanoic acid ester[44], Cyanidanol, Procyanidin(AC)[52], (+)-homo-iso-catechin[54].



(+) Homo-iso-catechin



Procyanidin AC

2.2 Biological Activities of *Acacia catechu*

In Ayurveda and in folk medicine *Acacia catechu* extract has been extensively used. Many systematic researches conducted on *Acacia catechu* proved that it has many medicinal properties. Some of them are

It was reported that *Acacia catechu* had hypoglycemic activity[55] and Catechin present in it had hepatoprotective, antipyretic and digestive properties[56,57]. Ethyl acetate extract of *Acacia catechu* had significant antipyretic, hypoglycemic and hepatoprotective activities[58]. Its extract had free radical scavenging activity. So its extract was used as a protective measure in cancer patients undergoing radiation therapy, since it had been demonstrated that radiation therapy causes mutations and cancer due to increased production of free radical[41]. It was also reported that *Acacia catechu* had antifungal[59] and antibacterial[60] activities.

Acacia catechu extract had significant inhibitory effect on enzymes cyclooxygenase-2 and 5-lipoxygenase[61]. Condensed tannin from catechu potentially inhibited the animal fatty acid

synthase (FAS). Furthermore, low concentration of condensed tannins were found to suppress the growth of MCF-7 breast cancer cells, and the effect was related to their activity of FAS inhibition[62].

2.3 Some Health Beneficial Compounds Isolated from *Acacia catechu*

Medicinally very important compounds have been isolated from *Acacia catechu.* Some of them with their health benefits are listed below-

2.2.1 Kaempferol

Kaempferol is a polyphenol that is a type of Flavonoid, known as a *flavonol.* Kaempferol supplements are commercially available. It is yellow crystalline solid having melting point 276-278^{o}C and is slightly soluble in water.

Kaempferol was a strong antioxidant and helps to prevent oxidative damage of our cells, lipids and DNA. Kaempferol seemed to prevent arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood. Evidences showed that Kaempferol also prevent atherogenesis through suppressing macrophage uptake of oxLDL[63]. Kaempferol showed anti-cancer activities against pancreatic cancer cell lines[64]. Kaempferol had anti-inflammatory activities and it may help to certain types of brain damage such as ischemic stroke[65]. Specific effect of Kaempferol than other flavonoids was its consumption in broccoli and tea has been correlated with a decrease in ovarian cancer in post-menopausal women.

2.2.2 Dihydroquercetin

Dihydroquercetin also known as Taxifolin is yellow crystalline solid and is slightly soluble in water. Dihydroquercetin had anti-diabetes effect[66], anti-viral effect[67]. It was shown to be highly effective in inhibiting the reverse transcriptase, an important factor of carcinogenesis[68]. Dihydroquercetin had anti oxidative and vasoprotective properties, combined by anti-inflammatory, gastric and hepatoprotective, hypolipidemic and diuretic

effects[69]. The total liver cholesterol concentration was significantly decreased by feeding with taxifolin[70]. It was also responsible for antimutagenic effect, Radio protective effect[71].

2.2.3 Quercetin

Quercetin is yellow crystalline solid, on standing it converted in to red color. It has melting point of 309-311^{o}C. Quercetin prevented the formation of some inflammatory mediators hence, reduced pain in many instances. Quercetin was widely used by those who suffer from constant allergies (such as dust) and seasonal allergies (such as hay fever). It reduced risk of heart disease by lowering the formation of plaque-building substances, specifically oxidized low density lipoprotein (LDL). It was useful in treating diabetic complication. It was believed that Quercetin was useful for prevention or treatment of benign prostatic hyperplasia or enlarged prostate. Quercetin naturally protect flowers from microbes, insects, viruses, fungus and bacteria, as well as oxidative stress and damage from sunlight[72,73].

2.2.4 Isorhamnetin

Isorhamnetin is also a member of Flavonoids. It has melting point of 307^{o}C. Many health benefits of Isorhamnetin were similar to the common health benefits of Flavonoids. Isorhamnetin repressed adipogenesis in 3T3-L1 cells. Isorhamnetin was good anti-oxdidant[74,75].

2.2.5 Epicatechin

Epicatechin is white crystalline solid, soluble in hot water. It is the member of flavanol, Flavonoids. It improved the blood flow and had potential for cardiac health[76]. Epicatechin reduced the risk of four major health problems: stroke, heart failure, cancer and diabetes[77].

2.2.6 Catechin

Catechin is transparent crystal having melting point 176^{o}C. It is the member of flavanol and sparingly soluble in cold water and soluble in hot water. On standing it first converted to

yellow and then to red color and finally to black. In *Acacia catechu* it was major constituents after Tannins. Catechin, when combined with habitual exercise, had delayed some forms of aging[78]. Catechin had also possessed antibiotic properties[79], and anti-carcinogenic effects[80]. Catechin had significant antioxidant and antimicrobial effects[81].Catechin had cyclooxygenase-2 and 5-lipoxygenase enzyme inhibitory effect which were ultimately responsible for rheumatoid arthritis, Osteoarthritis, Alzheimer's diseases and certain type of cancer[61].

CHAPTER III

3 EXPERIMENTAL

3.1 Collection of plant material

Heartwood of *Acacia catechu* is the plant material chosen for the study. Plant material is collected from Chitwan National Park, Pithauli-7, Nawalparasi and is identified by R.P. Chaudhary of Central Department of Botany, Tribhuwan University. Heartwood of *Acacia catechu* was air dried in shade and dismembered with the help of stain free knife. This dismembered heartwood was grinded to powder.

3.2 Chemical and Equipments

All reagent and solvent used were laboratory reagent grade from E. Merck, S.D. Fine, Qualigens and Ranbaxy chemical companies of India.

Isolation and separation of chemical constituents were carried out with the help of column chromatography using silica of 60-120 mesh. Purification was done by re-crystallization. Precoated TLC aluminium plates (silica gel E-Merk 60-F_{254}) with thickness of 0.2 mm were used to check purity of the compounds. The plates are developed in different solvent and visualize under UV light, in iodine chamber and by charring with conc. H_2SO_4. The melting points of compounds were recorded on a hot plate and uncorrected.

3.3 Phytochemical Screening

By this we can aware about the main group of natural product present in plant material. The method employed for phytochemical screening was based on the procedure put forward by Pro. I. Ciulei[82]. This method involves the selective and successive extraction of the plant material by using solvent of increasing polarity. The procedure is given in detail in Annex I.

3.4 Biological Screening

Medicinal plant show different medicinal properties. This is due to different biological properties of the constituents present on the plant material. In biological screening the

effect of the crude plant extract or fraction at arbitrarily fixed dose level in species of organism was studied. In this dissertation work screening of each fraction of extract for their antibacterial, antifungal and brine-shrimp toxicity was performed.

3.4.1 Antibacterial Screening

Agar well diffuse method and two fold dilution method were used in the study for screening and evaluation of antibacterial activity of the crude plant extracts.

Inhibition of bacterial growth was tested by agar well diffused method given by Dingel et al.[83] In agar well diffuse method, the diameter of zone of inhibition (ZOI) produced by plant extract or fraction or compound on particular pathogenic bacteria was measured for the estimation of antibacterial activity of the plant extract or fraction or compound.

3.4.1.1 Preparation of Working Solution

5% of working solution was made by transferring 5mg of each fraction to sterile vial aseptically containing 1ml DMSO solvent. After making working solution, the test tubes were capped sealed and stored in refrigerator until use.

3.4.1.2 Collection of Test Organism

The microbial strains employed were identified strains that were obtained from Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal. The studied strains include 11 different types of bacteria as shown below:

Gram positive bacteria: *Staphylococcus aureus and Bacillus subtilis.*

Gram negative bacteria: *Salmonella paratyphi, Escherichia coli, Pseudomonas, Enterobacter, Salmonella typhi, Shigella, Acenetobacter, Proteus mirabilis, Klebsiella pneumonia.*

Pathogenicity of these bacteria is given in Annex-II

3.4.1.3 Preparation of Media

I. **Nutrient Agar:** Nutrient agar was prepared by adding distills water to 28 gm of nutrient agar to make final volume 1000 ml in conical flask. It was sterilized by autoclaving the media at 15 lb pressure and 121^{o}C for 15 minutes. It was then allowed to cool to 50^{o}C. They were distributed in the sterilized Petri-plates of 90 mm diameter in the ratio of 25 ml per plate aseptically and labeled properly.

II. **Nutrient Broth:** 13 gm of powder weighed and dissolved in distilled water to make final volume of 1000 ml. It was sterilized by autoclaving at 15 lb pressure and 121^{o}C for 15 minutes. It was cooled and 10 ml of it was poured inside screwed capped bottle and again sterilized.

III. **Muller Hinton Agar (MHA):** 3.8 gm of media was suspended 100 ml of distill, boiled to dissolve and sterilized by autoclaving at 15 lb pressure and 121^{o}C for 15 minutes. It was then allowed to cool about 50^{o}C and poured to Petri-plates in 25 ml/plate quantities. The plates were left as such for solidification.

3.4.1.4 Preparation of Standard Culture Inoculums

It was prepared from primary culture plates as described below

- The isolated colony was subculture on nutrient agar plates with the inoculating loops aseptically.
- From this plate a loop of inoculate was transferred in to a tube containing sterile nutrient broth and incubated for 4 hours at 37^{o}C.

3.4.1.5 Screening and Evaluation of Antibacterial Activity

Already prepared Sterile Muller-Hinton Agar (MHA) plates were dried to remove excess of moisture from the surface of the media. Sterile cotton swab was dipped into the prepared inoculums and the excess of inoculums were removed by pressing and rotating against the upper inside side wall of the tube above the liquid level and then swabbed carefully all over

the plates. The plate was rotated through an angle of 60^{o} after each swabbing. Finally the swab was passed round the edges of the agar surface. The inoculated plates were left to dry for few minutes at room temperature with the lid closed[84].

The wells were made in the incubated media plates with the help of sterile cork borer (6 mm) and labeled properly. Then 50 µl of the working solution of the plant extract were loaded into the respective wells with the help of micropipette. The solvent itself was tested for its activity as a control at the same time in the separate well. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37^{o}C[85]. The plates were then observed for zone of inhibition (ZOI) produced by the anti-bacterial activity of different fraction of heartwood of *Acacia catechu*. At the same time ZOI of different organism by different fractions were measured with the help of the ruler for the estimation of potency of anti-bacterial substance.

3.4.1.6 Minimum Bactericidal Concentration (MBC)

Fractions of plant extract which showed antibacterial activity were subjected to two fold serial dilution method to determine MBC[86].

In this method, a set of screw capped 12 test tubes containing 2 ml NB for each bacterium was prepared. The test tubes were labeled as positive control, no. 1 to 10 and negative growth control. In case of negative growth control the nutrient growth was discarded. Then 2 ml of particular fractions were added aseptically to each tube labeled as negative growth control and no. 1 labeled test tube. Now no. 1 tubes contain 2 ml NB and 2 ml of fraction. After complete homogenization 2 ml of its content was transferred aseptically to no. 2 test tubes. In the same way after complete homogenization of the content in the no. 2 test tubes 2 ml of it was transferred aseptically to no. 3 test tubes. In the same way two fold dilutions were prepared up to no. 10 test tubes. From the tenth test tubes, 2 ml of the content was discarded. Hence all the tubes from negative control to no. 10 contained equal volume i.e.

2 ml with gradually decreasing concentration. No fraction was added to the tubes labeled as positive control. Now with the help of micropipette 20 µl of inoculums was added to all the tubes except the negative labeled. The tubes were incubated at 37^{o}C for 24 hours.

After 24 hours a series of test tubes containing the serially diluted fraction and the test organism, the results were compared with positive and negative control tubes. The result was interpreted on the basis of the fact that growth occurs on the positive control and any other tubes in which the concentration of the extract is not sufficient to inhibit the growth. The lowest concentration of anti-microbial agent that inhibits the growth of organism as detected by lack of visible turbidity or growth is the MIC. For colored fraction it was not possible to determine exactly MIC. So all the tubes were subcultured on MHA plates containing no fraction with proper label and incubated at 37^{o}C for 24 hours. Then the plates were examined for the growth of the organism. Tube with minimum concentration of extract in which the growth was completely checked was notified. Then MBC was determined. The minimum concentration of the antimicrobial agent that completely checks out the growth of the bacterial organism is MBC.

3.4.2 Antifungal Screening

Agar well diffuse method was used for screening of antifungal activity of different fraction of plant extract. In this method, the diameter of zone of inhibition (ZOI) produced by plant extract or fraction or compound on particular phytopathogenic fungi was measured for the estimation of antifungal activity of the plant extract or fraction or compound.

3.4.2.1. Preparation of working solution

4 % of working solution was prepared as prepared for screening of antibacterial activity.

3.4.2.2. Collection of Test organism

The fungal spores employed were identified strains that were obtained from Nepal Academy of Science and Technology (NAST), Kathmandu, Nepal. This study included four types of fungal organism, that were- *Fusarium oxysporium, Fusarium moniliformi, Fusarium proliferatum* and *Exherlium turticum*

3.4.2.3. Preparation of Media

I. Potato Dextrose Agar (PDA):

27 gm of medium was dissolved in 1000 ml of distill water and autoclaved at 121^{o}C for 15 minutes.

II. Potato Dextrose Broth

200 gm of peeled potato was weighed and cut into small pieces. It was boiled for few minutes and squeezed as much of pulp as possible through a layer of muslin cheese cloth. 20 gm dextrose and distill water was added to make the final volume 1000 ml. It was then autoclaved at 121^{o}C for 15 minutes.

3.4.2.4. Preparation of Inoculums

For antifungal screening, the standard inoculums of the fungal organism was prepared[87]. A loop of isolated fungal organism were placed in PDB and incubated at 27^{o}C for seven days. The inoculums size was adjusted to range of $1x10^{6}$-$5x10^{6}$ spores/ml by microscopic enumeration with a cell counting Haemocytometer.

3.4.2.5. Screening and Evaluation of Antifungal Activity

Sterile PDA plates were prepared. Before using the plates, they were dried in hot air oven at 40^{o}C for 5 minutes to remove excess of moisture from the surface of the media. Sterile cotton swab was dipped into the prepared inoculums and the excess of inoculums were removed by pressing and rotating against the upper inside side wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated through an

angle of 60^{o} after each swabbing. Finally the swab was passed round the edges of the agar surface. The inoculated plates were left to dry for few minutes at room temperature with the lid closed[84].

The wells were made in the incubated media plates with the help of sterile cork borer (6 mm) and labeled properly. Then 50 µl of the working solution of the plant extract were loaded in to the respective wells with the help of micropipette. The solvent itself was tested for its activity as a control at the same time in the separate well. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated for seven days at 27^{o}C[85]. The plates were then observed for zone of inhibition (ZOI) produced by the anti-fungal activity of different fraction of heartwood of *Acacia catechu*. At the same time ZOI of different organism by different fractions were measure with the help of the ruler for the estimation of potency of anti-fungal substance.

3.4.3 Brine Shrimp Bioassay

Brine shrimp bioassay is a simple and advantageous from the point of view of expense, time and complexity. The brine shrimp toxicity assay was carried out according to Meyer et.al. with Artemia soliana as test organism.

3.4.3.1 Method

The recent trend in phytochemical research is to isolate and characterize biologically active compounds. This method consists of exposing newly hatched brine shrimp nauplii to plant extracts or fraction or isolated compounds. The biological activities are evaluated on the basis of toxicity towards these nauplii. This method determines the LC_{50} value (µg/ml) for the plant extract or fraction or isolated compounds. Plant extract or fraction or isolated compounds having LC_{50} value less than 1000 are supposed to be pharmacologically active[88].

3.4.3.2 Required Material

a. Eggs of Brine-Shrimps

b. Beaker for hatching

c. Test tubes

d. Disposable pipette

e. Artificial sea water

f. Micro pipette

3.4.3.3 General Procedure

I. Sterilization of Apparatus

Plant extract or fraction or isolated compounds

II. Preparation of Artificial Sea Water

The entire bioassay must be carried out in freshly prepared artificial seawater. It was prepared by dissolving some chemicals in distilled water. The composition of sea water. The composition of seawater is given in the following table.

Table No. 1: Composition of artificial sea water

S.N.	Composition	Amount gm/1000ml
1.	NaCl	23.50
2.	Na_2SO_4	4.00
3.	KCl	0.68
4.	H_3BO_4	0.026
5.	$MgCl_2.2H2O$	10.70
7.	$CaCl_2.2H2O$	1.47
8.	$NaHCO_3$	0.196
9.	Na_2EDTA	0.0003

III. Hatching of Shrimps

For hatching of shrimps, the brine-shrimp eggs (about 10 mg) were sprinkled on the 1000 ml beaker half filled with artificial sea water and illuminated with a table lamp (100 w) for 48 hours with temperature adjusted at about 28^{o}C.

IV. Preparation of Samples

20 mg of the samples to be tested were dissolved in 2 ml respective solvent of extraction and they were hexane, chloroform, Ether, Ethyl acetate and methanol. This solution was called stock solution. From the stock solution 500 µl (eqv.1000 ppm), 50 µl (eqv. 100 ppm) and 5 µl (10 ppm) solutions were transferred to total nine sterilized test tubes, three tubes for each doses level. Then the solvent was evaporated by standing overnight.

V. Bioassay

After complete evaporation of solvent, ten matured shrimps and 5 ml seawater were transferred to all test tubes, including three control test tubes taken one for each dose level. After 24 hours, the survivors were counted with the help of disposable pipette.

VI. Data Analysis

LC_{50} value is the lethal concentration dose required to kill 50 % of the shrimps. It can be determined as follows.

If 'n' is the number of replicates (here three), 'x' is the log of constituents in mg/ml (log 10, log 100, log 1000 for three dose level respectively) and 'y is the probit for average survivor of all replicates.

We have,

$$\alpha = 1/n[\sum y - \beta \sum x] \qquad \text{..... (1)}$$

$$\beta = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \qquad \text{..... (2)}$$

Where,

From probit regression

$$Y = \alpha + \beta X \quad \text{……. (3)}$$

$$X = (Y - \alpha) / \beta \quad \text{…….. (4)}$$

Where,

Y is constant having value 5 for calculating LC_{50} value.

Thus,

$$LC_{50} = \text{Antilog } X \quad \text{……. (5)}$$

In the present work, brine–shrimp bioassay of different fractions of plant such as Hexane, Chloroform, Diethyl Ether, Ethyl acetate, Methanol was carried.

3.5 Extraction

2.4 kg of powder of heartwood of *Acacia catechu* was extracted with methanol five times for a two week interval at room temperature. The solvent was evaporated in rotary evaporator to get methanol extract. Thus obtained methanol extract was later successively extracted with Hexane, Chloroform, Diethyl ether, Ethyl acetate. The flow chart of extraction and fractionation is given below.

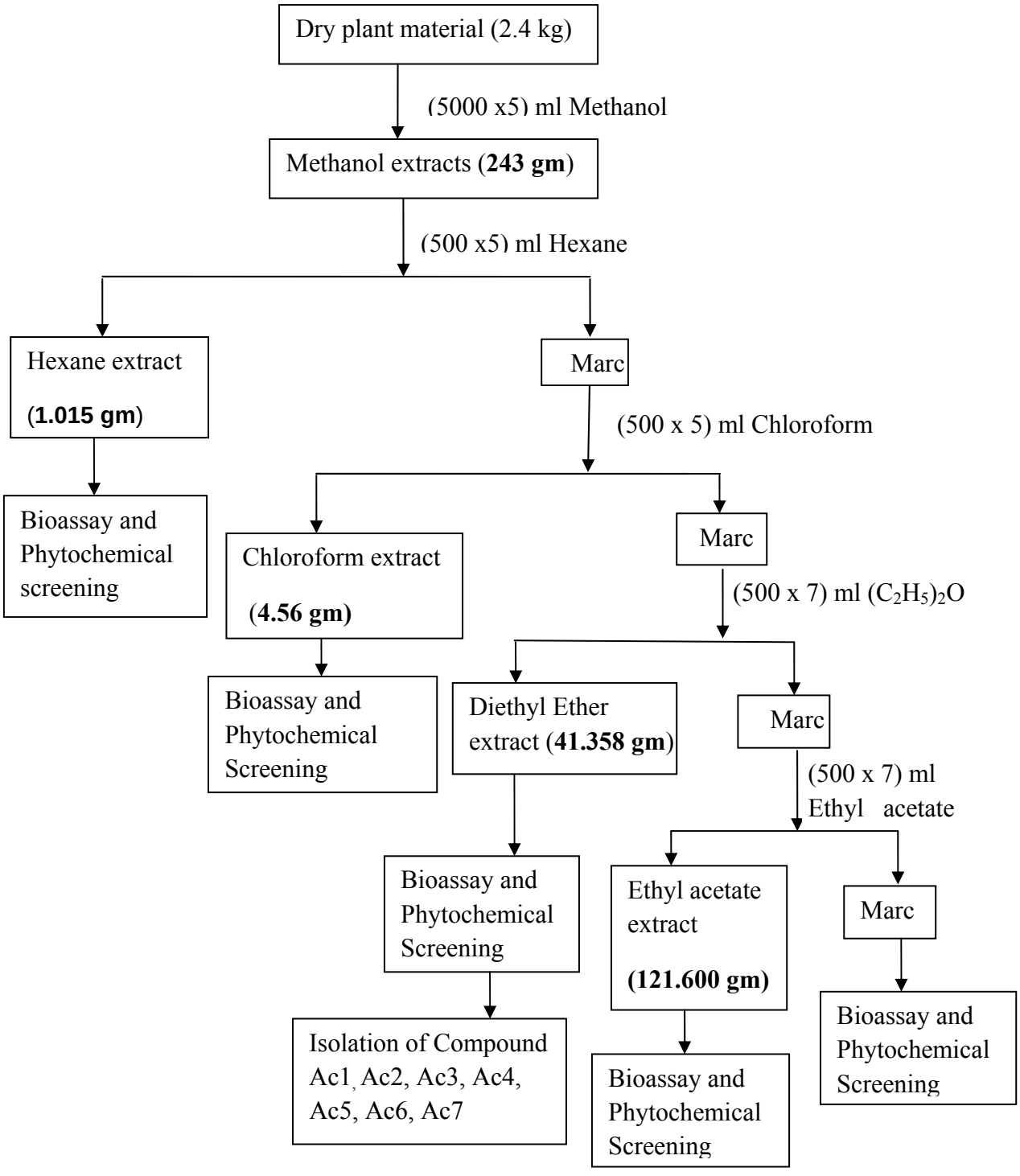


Fig 1: The flow chart of extraction and isolation of compounds

3.6 Separation of Compound from Diethyl ether Fraction by Column Chromatography

31.254 gm of Ether fraction was adsorbed on 40gm of silica gel and loaded in column of internal diameter 7 cm packed with silica gel (285 gm, Qualigens 60-120 mesh) having adsorbent height 38 cm. The column is eluted with gradients of Hexane, Ethyl acetate and Methanol to obtain number of fraction.

Table No. 2: Elution and TLC of different fraction of Diethyl ether extract

S. N.	Eluent	Fraction No.	Volume of eluent	Solvent system for TLC	TLC report	Amount (gm)
1	100% Hexane	1-12	1200 ml	1% EtOAc in Hex	Multi spot	-
2	1% EtOAc in Hex	13-25	1300 ml	5% EtOAc in Hex	No spot	-
3	5% EtOAc in Hex	26-35	1000 ml	15% EtOAc in Hex	No spot	-
4	10% EtOAc in Hex	36-56	2100 ml	30% EtOAc in Hex	2 spot (1 major)	0.043
5	15% EtOAc in Hex	57-65	900 ml	30% EtOAc in Hex	Two spot (1 major)	0.015
6	25% EtOAc in Hex	67-74	900 ml	25% Acet in Benz	2 spot (1 major)	0.057
7	25% EtOAc in Hex	75-110	3600 ml	25% Acet in Benz	3 spot (with tail)	2.932
8	40 % EtOAc in Hex	111-187	7700 ml	25% Acet in Benz	4 spot (with tail)	3.895
9	50% EtOAc in Hex	188-250	6300 ml	45% Acet in Benz	Two spot	4.361
10	70% EtOAc in Hex	251-300	5000 ml	50% Acet in Benz	Two spot (1 major)	6.452
11	100% methanol	301-320	2000 ml	60% Acet in Benz	Tailing	8.321

3.6.1 Isolation and Characterization of Compound Ac1

On eluting the column with 10% EtOAc in Hexane, fraction 40-52 showed two spot with minor impurities. It was re-crystallized from 100% EtOAc and the transparent crystals were obtained. Thus obtained crystals were again re-crystallized from 80% EtOAc in Hexane. Then it gave single spot on TLC plate developed in 30% EtOAc in Hexane. It was soluble in Chloroform, EtOAc, and Acetone. Its melting point was found 133^{o}C.

3.6.2 Isolation and Characterization of Compound Ac2

On eluting column with 25% EtOAc in hexane, fraction 66-74 were obtained which showed two spot with one major on TLC in 25% Acetone in Benzene. It was dissolved in EtOAc and hexane was added drop wise until turbidity appeared, then it was left for few hour in low temperature. After few hours white precipitate was appeared and separated from mother liquor. Then it gave single spot on TLC in 25:74:1 (Acetone:Benzenea:Acetic acid). It was white in color and soluble in Chloroform, EtOAc, and Acetone.

3.6.3 Recolumn of 75-187 Fraction of Ether Extract

4gm of this fraction was adsorbed in equal amount of silica gel (Qualigens 60-120 mesh) and loaded in column of internal diameter 4 cm packed with 90 gm of silica gel (adsorbent height 42 cm). The column was eluted with Benzene, Acetone and Acetic acid to obtain number of fractions.

Table No. 3: Elution and TLC of different fractions of 75-187 fractions of Ether extract

S. N.	Eluent	Fraction No.	Volume of eluent	Solvent system for TLC	TLC report	Amount (gm)
1	100% Benz	E_1-E_{10}	1000ml	5% Acet in Benz	No spot	-
2	1% Acet in Benz	E_{11}-E_{16}	600 ml	5% Acet in Benz	No spot	-
3	5:94:1 (Acet: Benz: AcOH)	E_{17}-E_{21}	400 ml	25:74:1(Acet: Benz: AcOH)	2 spot (1 major)	0.251

4	5:94:1 (Acet: Benz: AcOH)	E_{22}-E_{42}	2000 ml	25:74:1(Acet: Benz: AcOH)	2 spot	0.845
5	5:94:1 (Acet: Benz: AcOH)	E_{43}-E_{51}	900 ml	25:74:1(Acet: Benz: AcOH)	2 spot (1 major)	0.325
6	8:90:2 (Acet: Benz: AcOH)	E_{52}-E_{61}	1000 ml	30:69:1(Acet: Benz: AcOH)	2 spot	0.215
7	8:90:2 (Acet: Benz: AcOH)	E_{62}-E_{71}	2000 ml	30:69:1(Acet: Benz: AcOH)	2 Spot (1 major)	0.156
8	10:88:2 (Acet: Benz: AcOH)	E_{72}-E_{82}	1100 ml	30:69:1(Acet: Benz: AcOH)	2 spot (tailing)	0.056
9	20:78:2 (Acet: Benz: AcOH)	E_{83}-E_{100}	1800 ml	35:64:1(Acet: Benz: AcOH)	1 spot	Negligible
10	30:68:2 (Acet: Benz: AcOH)	E_{101}-E_{110}	1000 ml	45:54:1(Acet: Benz: AcOH)	1 spot (tailing)	Negligible
11	100% Methanol	E_{111}-E_{120}	1000 ml	-	No spot	-

Acet = Acetone Benz = Benzene

3.6.3.1 Isolation and Characterization of Compound Ac3

On eluting the column with 5:89:1 (Acetone: Benzene: Acetic acid) fractions E_{17}-E_{21} were obtained. It showed 2 spot with one major on TLC in 25:74:1 (Acetone: Benzene: Acetic acid). It was re-crystallized from 5% Methanol in Chloroform. Then it gives single spot on TLC in 25:74:1 (Acetone: Benzene: Acetic acid). It was soluble in Ether, Acetone and Methanol.

3.6.4 Recolumn of 188-300 Fraction of Ether Extract

3.5 gm of this fraction was adsorbed in equal amount of silica gel (Qualigens 60-120 mesh) and loaded in column of internal diameter 4 cm packed with 95 gm of silica gel (adsorbent height 43 cm). The column was eluted with Benzene and Acetone.

Table No. 4: Elution and TLC of different fractions of 188-300 fractions of Ether extract

S. N.	Eluent	Fraction No.	Volume of eluent	Solvent system for TLC	TLC report	Amount (gm)
1	100% Benz	A_1-A_5	500ml	5% Acet in Benz	2 spot	Negligible
2	5% Acet in Benz	A_6-A_{35}	3000 ml	25:74:1 (Acet: Benz: AcOH)	2 spot	Negligible
3	10% Acet in Benz	A_{36}-A_{65}	3000 ml	25:74:1 (Acet: Benz: AcOH)	2 spot	Negligible
4	20% Acet in Benz	A_{66}-A_{75}	1000 ml	35:64:1 (Acet: Benz: AcOH)	2 spot	Negligible
5	30% Acet in Benz	A_{76}-A_{85}	1000 ml	45:54:1 (Acet: Benz: AcOH)	2 spot	0.005
6	30% Acet in Benz	A_{86}-A_{104}	1900 ml	45:54:1 (Acet: Benz: AcOH)	1 spot	0.653
7	35% Acet in Benz	A_{105}-A_{120}	1700 ml	7:5:2 (Benz:EtOAc:AcOH)	2 spot (1 major)	1.954
8	40% Acet in Benz	A_{121}-A_{140}	2000 ml	50:49:1 (Acet: Benz: AcOH)	2 spot	Negligible
9	50% Acet in Benz	A_{141}-A_{150}	1000 ml	50:49:1 (Acet: Benz: AcOH)	No spot	-
10	100% Methanol	A_{151}-A_{160}	1000 ml	50:49:1(Acet: Benz: AcOH)	Tailing	Negligible

Acet = Acetone Benz = Benzene

3.6.4.1 Isolation and characterization of compound Ac4

Column on elution with 30% Acetone in Benzene fraction A_{86}-A_{104} were obtained which showed single spot on TLC in 45:54:1 (Acetone: Benzene: Acetic acid). It was re-crystallized with Ether and Methanol (1:1). It was soluble in Ether, Acetone and Methanol.

3.6.4.2 Isolation and Characterization of Compound Ac5

Column on eluting with 35% Acetone in Benzene, A_{105}-A_{120} fraction were obtained. It showed 2 spot with one major. It was re-crystallized with Diethyl ether. Then it gave single spot in 7:5:2 (Benz:EtOAc:AcOH). It was soluble in Ether, Acetone and Methanol.

3.6.5 Recolumn of 301-320 Fractions of Ether Extract

4gm of this fraction was adsorbed in equal amount of silica gel (60-120 mesh) and loaded in column of internal diameter 4 cm packed with 85 gm of silica gel (adsorbent height 39 cm). The column was eluted with Benzene and Acetone.

Table No. 5: Elution and TLC of different fractions of 301-320 fractions of Ether extract

S.N.	Eluent	Fraction No.	Volume of eluent	Solvent system for TLC	TLC report	Amount (gm)
1	100% benz	M_1-M_5	500 ml	5% Acet in Benz	No spot	-
2	10% Acet in Benz	M_6-M_{10}	500 ml	25:74:1 (Acet: Benz: AcOH)	No spot	-
3	20% Acet in Benz	M_{11}-M_{15}	500 ml	35:64:1 (Acet: Benz: AcOH)	Tailing	Negligible
4	30% Acet in Benz	M_{16}-M_{20}	500 ml	45:54:1 (Acet: Benz: AcOH)	2 spot (with tail)	Negligible
5	35% Acet in Benz	M_{21}-M_{41}	2000 ml	7:5:2 (Benz:EtOAc:AcOH)	1 spot	1.663
6	35% Acet in Benz	M_{42}-M_{50}	900 ml	50:49:1 (Acet: Benz: AcOH)	3 spot	0.065
7	35% Acet in Benz	M_{51}-M_{65}	1500 ml	50:49:1 (Acet: Benz: AcOH)	1 spot (with tail)	1.893

8	40% Acet in Benz	M_{66}-M_{75}	1000 ml	50:49:1 (Acet: Benz: AcOH)	Tailing	0.421
9	100% Methanol	M_{76}-M_{85}	1000 ml	50:49:1 (Acet: Benz: AcOH)	Tailing	Negligible

Acet = Acetone Benz = Benzene

3.6.5.1 Isolation and Characterization of Compound Ac5 and Ac6

On elution column with 35% Acetone in Benzene fraction M_{21}-M_{41} were obtained which gave single spot on TLC in 7:5:2 (Benz:EtOAc:AcOH) . It was crystallized from Methanol. This on crystallization from Methanol gave two types of crystal, one was transparent and another was white. Both of them were separated by mechanical separation. Both of these were soluble in Ethyl acetate, Acetone and Methanol. The transparent crystal was re-crystallized from water, dried in fold of Whattman filter paper and melting point of transparent crystal was recorded 177^{o}c. The white crystals show the same properties with compound Ac5.

3.6.5.2 Isolation and Characterization of Compound Ac7

Fractions M_{51}-M_{65} obtained on elution of column with 35% Acetone in Benzene gave single spot with tailing. It was purified by re-crystallization from Chloroform. It was soluble in Ether, Acetone, and Methanol.

CHAPTER-IV

4 RESULT AND DISCUSSIONS

4.1 Phytochemical Screening of heartwood of *Acacia catechu*

Phytochemical screening of heartwood of *Acacia catechu* was performed on the basis of procedure put forward by Prof. I. Ciulei. The results obtained from phytochemical screening are tabulated below.

Table No. 6: Results obtained from phytochemical screening of different fraction of heartwood of *Acacia catechu*

S. N.	Group of compound	Results				
		Hexane	Chloroform	Ether	Ethyl acetate	Methanol
1	Volatile oil	-				
2	Fatty acids	-	-			
3	Basic alkaloids	-	-			
4	Carotenoids	-	-			
5	Sterols	+	+	+		
6	Terpenes	-	-	-		
7	Coumarins	-	-	-		
8	Flavones aglycones	+	+	+		
9	Emodins	-	-	-		
10	Saponins		-	-		
11	Tannins		-	+	+	+
12	Polyoses		-	-	-	-
13	Polyphenol		+	+	+	+

14	Reducing compounds		-	-	-	-
15	Glycosides		-	-	-	-
16	Quinones				-	-

- = Absent + = present

4.2 Biological Screening of *Acacia catechu*

In this dissertation work antibacterial screening, antifungal screening and brine-shrimp bioassay were studied.

4.2.1 Antibacterial Screening

Results obtained from antibacterial screening of heartwood of *Acacia catechu* are given below:

Table No. 7: Results of antibacterial screening of heartwood of *Acacia catechu*

Well diameter= 6 mm Concentration of loaded extract= 5%

S. N.	Plant Fractions	Organisms	Zone of Inhibition (mm)	
			Control	Fractions
1	Hexane	*Staphylococus aureus*	6	6
		Bacillus subtilis	6	7
		Salmonella paratyphi	6	6
		Escherichia coli	6	6
		Pseudomonas sp.	6	6
		Enterobacter sp.	6	9
		Salmonella typhi	6	6
		Shigella sp.	6	9
		Acenetobacter sp.	6	6
		Proteus mirabilis	6	6
		Klebsiella pneumoniae	6	6

2	Chloroform	*Staphylococus aureus*	6	8
		Bacillus subtilis	6	9
		Salmonella paratyphi	6	6
		Escherichia coli	6	6
		Pseudomonas sp.	6	6
		Enterobacter sp.	6	6
		Salmonella typhi	6	6
		Shigella sp.	6	8
		Acenetobacter sp.	6	7
		Proteus mirabilis	6	6
		Klebsiella pneumoniae	6	6
3	Diethyl ether	*Staphylococus aureus*	6	10
		Bacillus subtilis	6	13
		Salmonella paratyphi	6	6
		Escherichia coli	6	6
		Pseudomonas sp.	6	15
		Enterobacter sp.	6	8
		Salmonella typhi	6	6
		Shigella sp.	6	13
		Acenetobacter sp.	6	8
		Proteus mirabilis	6	6
		Klebsiella pneumoniae	6	8
4	Ethyl acetate	*Staphylococus aureus*	6	12
		Bacillus subtilis	6	14
		Salmonella paratyphi	6	6

		Escherichia coli	6	6
		Pseudomonas sp.	6	10
		Enterobacter sp.	6	8
		Salmonella typhi	6	6
		Shigella sp.	6	14
		Acenetobacter sp	6	12
		Proteus mirabilis	6	6
		Klebsiella pneumoniae	6	13
5	Methanol	*Staphylococus aureus*	6	11
		Bacillus subtilis	6	12
		Salmonella paratyphi	6	6
		Escherichia coli	6	6
		Pseudomonas sp.	6	9
		Enterobacter sp.	6	8
		Salmonella typhi	6	6
		Shigella sp.	6	12
		Acenetobacter sp.	6	6
		Proteus mirabilis	6	6
		Klebsiella pneumoniae	6	12

For the estimation of potency of plant fraction on particular bacteria the diameter of Zone of Inhibition (ZOI) was measured. No fractions showed activity against *Salmonella typhy, Salmonella paratyphy, Escherichia coli and Proteus mirabilis.* Hexane and Chloroform showed moderate inhibition while Ethyl acetate, Ether and Methanol showed very good inhibition. Highest inhibition was measure against *Pseudomonas sp.* by Ether fraction.

4.2.1.1 Minimum Bactericidal Concentration (MBC)

The lowest concentration of the antibacterial agents that completely check out the growth of the bacteria is the MBC. Here the MBC of ethyl acetate fraction was studied on Bacillus subtilis, Klebsiella pneumonia, Staphylococus aureus, Shigella sps.

Table No. 8: MBC value of Ethyl acetate fraction

Fraction	Organisms	MBC (mg/ml)
Ethyl acetate	*Bacillus subtilis,*	50
	Klebsiella pneumonia,	100
	Staphylococus aureus,	100
	Shigella sps.	50

These antibacterial screening showed that Ethyl acetate fraction was most antibacterial agent and then Diethyl ether and Methanol. Hexane and Chloroform fractions were poor antibacterial agent.

4.2.2 Antifungal Screening

Results obtained from Antifungal screening of heartwood of *Acacia catechu* are shown below:

Table No. 9: Results of antifungal screening of heartwood of *Acacia catechu*

Well diameter= 6 mm Concentration of loaded extract= 5%

S.N.	Plant fractions	Organisms	Zone of Inhibition (mm)	
			Control	Fractions
1	Hexane	*Fusarium oxysporium*	6	6
		Fusarium moniliformi	6	8
		Fusarium proliferatum	6	6
		Exherlium turticum	6	6

2	Chloroform	*Fusarium oxysporium*	6	9
		Fusarium moniliformi	6	9
		Fusarium proliferatum	6	6
		Exherlium turticum	6	10
3	Diethyl ether	*Fusarium oxysporium*	6	10
		Fusarium moniliformi	6	11
		Fusarium proliferatum	6	10
		Exherlium turticum	6	11
4	Ethyl acetate	*Fusarium oxysporium*	6	17
		Fusarium moniliformi	6	9
		Fusarium proliferatum	6	9
		Exherlium turticum	6	14
5	Methanol	*Fusarium oxysporium*	6	10
		Fusarium moniliformi	6	6
		Fusarium proliferatum	6	7
		Exherlium turticum	6	8

For the estimation of potency of plant fraction on particular fungal organism the diameter of Zone of Inhibition (ZOI) was measured. Highest ZOI was shown by Ethyl acetate fraction against *Fusarium oxysporium*. Here Hexane fraction showed activity only against *Fusarium moniliformi.* Hexane showed poor activity, Chloroform & Methanol showed moderate activity and Diethyl ether & Ethyl acetate showed good activity against fungal organism.

4.2.3 Brine-Shrimp Bioassay

Here LC_{50} values (µgm/ml) for different fractions were determined and those having LC_{50} values less than 1000(10^3) are supposed to be pharmacologically active. The results obtained by exposing newly hatched nauplii are given below:

Table No 9: Cytotoxicity calculation of different fraction of Heartwood of *Acacia*

S. N.	PE_S	z	x	y	xy	x^2	∑x	∑y	∑xy	$∑x^2$	β	α	X	LC50
1	H	10	1	8.33	8.33	1	6	24.33	48.33	14	-0.16	8.44	20.8	$7.05x10^{20}$
		100	2	8	16	4								
		1000	3	8	24	9								
2	C	10	1	8	8	1	6	21.66	41.65	14	-0.83	8.89	4.66	45570.4
		100	2	7.33	14.66	4								
		1000	3	6.33	18.99	9								
3	E	10	1	7	7	1	6	17.66	32.65	14	-1.34	8.56	2.66	461.498
		100	2	6.33	12.66	4								
		1000	3	4.33	12.99	9								
4	EA	10	1	6	6	1	6	12.33	20.99	14	-1.84	7.78	1.51	32.733
		100	2	4	8	4								
		1000	3	2.33	6.99	9								
5	M	10	1	8	8	1	6	19.33	35.66	14	-1.5	9.44	2.96	916.689
		100	2	6.33	12.66	4								
		1000	3	5	15	9								

Where,

PE_s = Plant Extracts

X = log Z

Y = Total Survivor

Z = Concentration

LC_{50} values = Antilog X

H = Hexane

C = Chloroform

E = Ether

EA= Ethyl acetate

M = Methanol

We have,

$$\alpha = 1/n\ [\sum y - \beta \sum x] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (1)$$

$$\beta = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (2)$$

Where,

From probit regression

$$Y = \alpha + \beta X \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (3)$$

$$X = (Y - \alpha)/\beta \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (4)$$

Where Y is constant having value 5 for calculating LC_{50} value.

Thus,

LC_{50} = Antilog X

The brine shrimp bioassay showed that the Diethyl ether fraction with the LC_{50} value 461.498, Ethyl acetate fraction with LC_{50} value 32.733, and Methanol fraction with LC_{50} Value 916.689 were bioactive.

Among the fractions Ethyl acetate showed highest activity and then Diethyl ether and then Methanol. Both Chloroform and Hexane fractions had LC_{50} value more 1000 so they were inactive against brine-shrimp bioassay.

4.3 Analysis and Identification of Isolated Chemical Constituents

Compound Ac1

Compound Ac1 was a crystalline compound, with melting point 133^0C. The compound was found to be single spotted on TLC with R_f 0.51 (30% ethyl acetate/hexane). It gave positive Libermann-Burchard test with greenish red color indicating the compound to be sterol. The mass spectrum showed M^+ at m/z at 414 corresponding to the molecular formula ($C_{29}H_{50}O$).

The IR Spectrum showed presence of –OH group (3434 cm^{-1}). The absorption bands at 2961 cm^{-1} and 1383 cm^{-1} were due to the –C-H stretching and bending respectively. A peak at 1063 cm^{-1} showed the presence of –C-O group.Two singlets at δ 1.00 and at 0.67 are methyl group of C-19 and C-18 respectively. The doublets at δ 0.92 (d, J = 6.1 Hz); 0.81(d, J = 6.9 Hz); 0.83 (d, J = 6.9 Hz) and 0.84 (t, J = 7.3 Hz) were accounted for methyl group at C-21, C-25, C-27 and C-29. A doublet at δ 5.35 (ddd, J = 20, 5 Hz) in ^{1}H-NMR can be accounted for an olefinic proton at C-6. Other multiplet at δ 3.52 (ddd, J = 13.2, 16.0, 16.0 Hz) equivalent to a single proton was assigned for the proton of C-3. The low field signal may be due to the attachment of *β*-OH group at C-3 carbon. Thus, the assignment of hydroxyl group at C-3 and the double bond at C-5 were assigned accordingly. Three multiplets equivalent to two protons each appeared at δ 1.83, 2.00 and 2.27 were assigned for three CH_2 groups. The remaining protons have appeared as multiplets at δ 1.05-1.65.

The respective fragments were assigned by the mass spectrum with m/z 399, 396,381, 329, 273 and 255. These mass fragmentation patterns were given below in Fig. 2.

The compound was further conformed by Co- TLC with authentic sample. From all the spectral analysis, the compound was identified as β-Sitosterol.

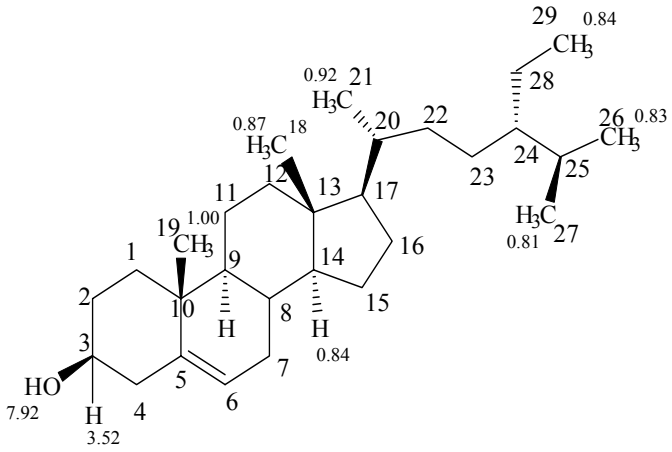
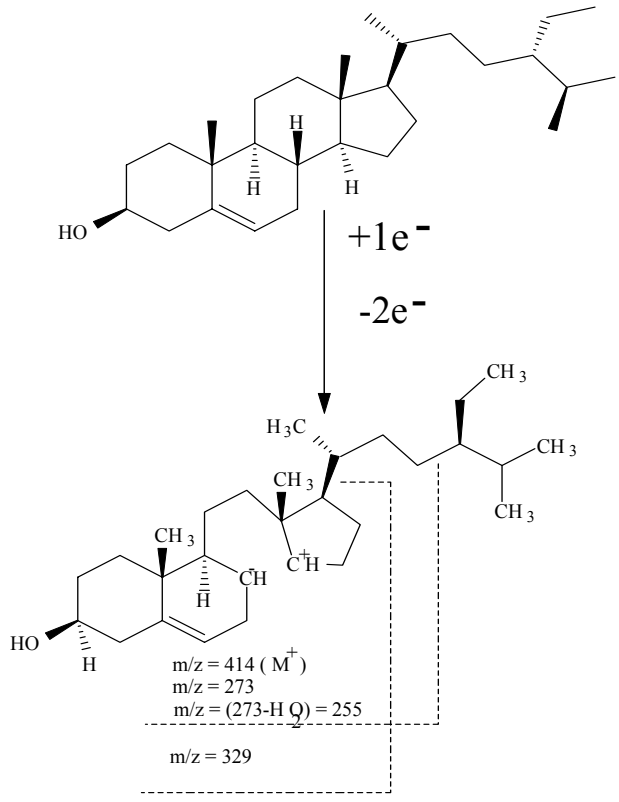


Fig 1: ^{1}H-NMR of Ac1 (*β*-sitosterol)



$M^{+} = 414$

M^{+}-CH_3 (m/z) = 399

M^{+} - H_2O (m/z) = 396

M^{+} - H_2O – CH_3 (m/z) = 381

Fig 2: Mass Fragmentations of Ac1 (β-Sitosterol)

Compound Ac2

Compound Ac2 was white crystalline solid. The compound was found to be single spotted on TLC with R_f value 0.42 (25% Acetone in Benzene). The compound gave positive Flavonoid test. This compound is on the process of work.

Compound Ac3

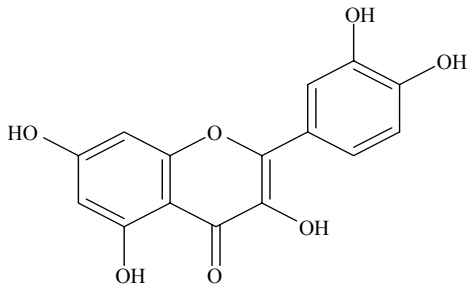
Compound Ac3 was yellow crystalline solid with melting point 336^{o}c. It was found to be single spotted on TLC with R_f value 0.37(29% Acetone in Benzene with 1% acetic acid). This compound gave positive Shibata test. This compound is in the process of work.

Compound Ac4

This compound was yellow crystalline solid having melting point 310^{o}c. It was found to be single spotted in TLC with R_f value 0.34 (49% Acetone in Benzene with 1% acetic acid). This compound gave same R_f value on Co-TLC with authentic sample of Quercetin.

The IR spectra were recorded in the range from 4000 to 600 cm $^{-1}$ and showed absorption bands with maxima at the following frequencies (ν_{max}): 3397.96, 2919.70, 1621.84, 1462.74, 1378.65 cm^{-1}. The peaks at 3397 and 2919 cm^{-1} were characteristic of the aromatic ring structure. The absorption maxima at 1621 cm^{-1} and 1462 cm^{-1} indicate the presence of a quinoid structure and –C=C– bonds, respectively. The absorption maximum at 1378 cm^{-1} was due to Phenolic OH groups. UV-VIS spectra of Quercetin showed absorption maxima at 257nm (Band 1, 0.779) and 356nm (Band 2, 0.619).

A mixture of Ac4 and Quercetin also melted in the range from 309 to 311°C without any depression.



Compound Ac4 (Quercetin)

Compound Ac5

This compound was white crystalline solid. It was found to be single spot in TLC with R_f value 0.47 in 7:5:2 (Benz:EtOAc:AcOH)). This compound gave positive Flavonoids test. This compound is on the process of work.

Compound Ac6

This compound was needle crystal when re-crystallized from water and melting point was found to be 177^{o}c. It had R_f value 0.46 (7:5:2 (Benz:EtOAc:AcOH)). This compound gave positive Flavonoid test.

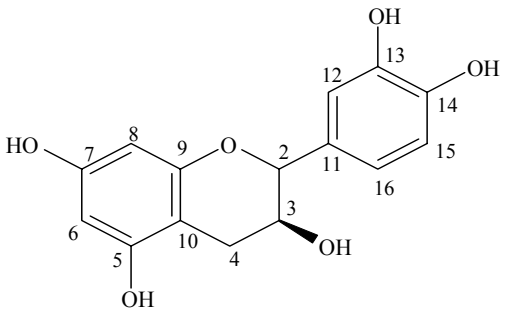
IR Spectra [ν_{max} (KBr)] of this compound showed absorption bands with maxima at the following frequencies: 3400-2600 (broad), 1620, 1520, 1470, 1380, 1280, 1240, 1150, 1120, 1080, 1020, 820 cm^{-1}.

The broad band at 3400-2600 was due to presence of phenolic group. The peak at 1470 indicate the presence of -C=C – bond. Absorption maxima at 1380 was due to phenolic -OH group.

^{13}C-NMR of this compound was comparable with those of authentic (+) Catechin as given in table below.

Carbon Number	δ value of authentic (+) Catechin (DMSO)	δ value of Compound Ac6 (CD_3OD)
C-2	80.96	82.822
C-3	66.27	67.462
C-4	27.84	28.490
C-5	99.00	100.839
C-6	155.31	156.891
C-7	95.05	96.322
C-8	156.41	157.623
C-9	93.78	95.533
C-10	156.12	157.524
C-11	130.55	131.201
C-12	114.48	115.270
C-13	144.63	146.079
C-14	144.79	146.179
C-15	115.01	115.903
C-16	118.36	119.400

This compound was further conformed by Co-TLC with authentic sample.



Compound Ac6 [(+) Catechin]

Compound Ac7

This compound was orange red colored crystalline solid. This compound was found to be single spot on TLC with R_f value0.30 (59% Acetone in Benzene with 1% acetic acid). This compound gave positive Tannin test. This compound is on the process of work.

CHAPTER-V

5 SUMMARY AND CONCLUSION

- *Acacia catechu* is an indigenously important plant due to its medicinal value.
- Phytoconstituents present in heartwood of Acacia catechu were Sterol, Flavonoids, Tannins, Polyphenols.
- Brine shrimp bioassay showed Diethyl ether, Ethyl acetate and Methanol fraction were active.
- Antimicrobial screening showed Ethyl acetate was most bioactive and then Diethyl ether, Methanol, Choloroform and Hexane.
- Compounds β- Sitosterol, Quercetin, (+) Catechin and other four compounds were also isolated from heartwood of *Acacia catechu*.

GLOSSARY

Alzheimer's: A serious disease, especially affecting older people that prevents the brain from functioning normally and causes loss of memory, loss of ability to speak.

Appetizer: A small amount, especially of food or drink to stimulate the appetite.

Anemia: A medical condition in which somebody has too few red cells in their blood making them looks pale and feels weak.

Anorexia: An emotional disorder, especially affecting young women, in which there is an abnormal fear of being fat, causing person to stop eating, leading to dangerous weight loss.

Anti-dysenteric: A drug used to reduce an infection of the bowels that causes severe diarrhea with loss of blood.

Anti-inflammatory: A drug used to inflammation.

Anti-pyretic: Preventing or reducing fever

Antiseptic: A substance that helps to prevent infection in wounds by bacteria.

Astringent: (of liquid or cream) able to make the skin feel less oily or to stop the loss of blood from a cut.

Catarrh: Inflammation of mucous membrane of nose, air passage etc.

Congestion: Abnormal accumulation or obstruction of blood or mucus in part of body.

Conjunctivitis: Inflammation of the conjunctiva.

Depurative: Capable of purifying.

Diuretic: A substance that causes an increase in flow of urine.

Epistaxis: It is relatively common occurrence of hemorrhage from the nose usually noticed when the blood drains out through the nostrils.

Gingivitis: Inflammation of the gums.

Haematemesis: Vomiting of blood.

Haematinic: An agent that improves the quality of blood by increasing the number of erythrocytes and the hemoglobin concentration.

Haemoptysis: Splitting of coughing up of blood streaked mucus as in tuberculosis.

Haemostatic: Capable of stopping hemorrhage or bleeding.

Hemorrhodis: piles

Leprosy: Contagious diseases that damages the skin and nerves.

Leucoderma: The disease where in acquired patches develops is referred as leucoderma.

Leucorrhea: Flow of whitish, yellowish or greenish discharge from the vagina of female.

Melancholia: Depression and anxiety.

Odontopathy: Disease of teeth.

Piles: Hemorrhoids; painful swollen veins at or near the anus.

Pharyngodynia: Pain in the Pharynx.

Pruritus: An itch or sensation that makes a person wants to scratch.

Psoriasis: A skin disease that causes rough red areas where the skin comes off in small pieces.

Scabies: Contagious skin disease causing itching.

Splenomegaly: An enlargement of spleen.

Sprain: Causing pain or swelling; wretch.

Tonic: Invigorating medicine.

ANNEX - I

Phytochemical Screening

General Procedure

1. **Extraction and Fractionation**

In general, three solvent systems were chosen for the extraction of plant material selected to phytochemical screening. These three solvent systems included non-polar lipophilic solvents (e.g. hexane), polar solvent with intermediate polarity (e.g. chloroform) and strong hydrophilic solvents (e.g. alcohol) followed by water.

About 65 gm of air-dried plant material was extracted as alcoholic extract with cold percolation. This extract was first treated with no-polar solvent (Hexane), then by solvent of intermediate polarity (Chloroform, Ether and Ethyl acetate) and finally by solvent of high polarity (Methanol), 200 ml of each, with the help of fractionation.

a) The non-polar fraction.

b) The intermediate polar fraction.

c) The alcohol fraction.

Each fraction was concentrated to reduce its volume up to 50 ml.

2. **Tests carried out in non-polar fraction**

I. Test for Volatile Oils

Two-milliliter solutions were evaporated to get residue and it was mixed with 0.5 ml alcohol. The solution was filtered and the filtrate was put on a filter paper by means of a capillary tube. The formation of spot on filter paper was disappeared indicating the presence of volatile oil.

Saponification of Fraction

The fraction was again extracted by 50% KOH (5 ml × 3 times) in a separating funnel to get aqueous alkaline solution and organic solution. The aqueous alkaline solution was acidified

with conc. HCl using litmus paper as indicator, then it was again extracted by either (5 ml× 3 times) to get etheric extract. The volume of etheric extract was reduced to half and it was reserved for test II, III, IV, V and VI.

II. Test for Fatty Acids

The concentrated fraction was spotted in the filter paper. The persistence of spot even after evaporation of solvent confirmed the presence of fatty acids.

III. Test for Coumarins

The fraction was dissolved in hot water, cooled and the solution was divided into test tubes. The first test tube was taken as standard and the second tube was made alkaline by adding 10% NH_4OH drop by drop. When these two solutions (first and second) were observed in UV light, the occurrence of an intense florescence in second solution (in comparison with standard) indicated the presence of coumarins.

IV. Test for Flavon Aglycones (Shibata's reaction)

The fraction was dissolved with methanol to which a small piece of magnesium and 4-5 drops of conc. HCl were added. A red or orange color indicated the presence of flavones.

V. Test for Alkaloids

The fraction was dissolved in 2 ml, 20% HCl, shake vigorously and filtered, 10% NH_4OH solution was added in filtrate to make it alkaline and it was extracted with $CHCl_3$ (2 ml × 3 times). The chloroform extract was again dissolved in 20% HCl and it was divided into two parts. The first was taken as standard and second was treated with 2-3 drops of Maeyer's reagent. The appearance of white precipitate in second test tube indicated the presence of alkaloids.

VI). Test for Emodins (Borntiager's Test)

The fraction was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for a few minutes to separate two

layers. Neither the upper layer was decolorized nor the lower alkali layer gained red color indicating the absence of emodins.

VII) Test for Sterols and Triterpenes (Libermann-Burchard's Test)

The organic portion was concentrated to yield a residue, which was dissolved in acetic anhydride (1 ml). To this solution, conc. sulphuric acid (2 ml) was added from the side of the test tube without disturbing the solution. A violet ring at the junction of two liquids was observed and the upper was green in color indicating the presence of sterols. The violet superior layer indicated the presence of triterpinnes.

3. Tests carried out in Intermediate-polar and Polar Fraction

a) Test for Polyphenols (Ferric Chloride Test)

The Intermediate-polar/polar fraction (1 ml) was mixed with water (1 ml). To this solution, 1% ferric chlorides solution (3 drops) was added. A greenish blue color was developed indicating the presence of polyphenols.

b) Test of Reducing Compounds (Fehling's test)

The Intermediate-polar/polar fraction (1 ml) was mixed with water (1 ml). To this solution, 1 ml Fehling's reagent (1:1 mixture of Fehling's reagent A and B) was added and then the mixture was warmed over a water bath for 30 minutes. The brick red precipitate was produced indicating the presence of reducing compounds.

C) Test for Alkaloids

The Intermediate-polar/polar fraction (10 ml) was concentrated to yield a residue. To this residue, 2% (v/v) hydrochloric acid (4 ml) was added and shaken vigorously, then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxide solution until pH 8. The solution was then extracted thrice with chloroform (10 ml). The upper alkaline layer was discarded. The lower chloroform layer was concentrated over a water bath. To this

concentrated solution, 2% (v/v) HCl (5 ml) was added. This obtained solution was divided into two parts:

1. The first was treated with Maeyer's reagent (3 drops). No white precipitate was obtained indicating the absence of the alkaloids salts.

2. The second was treated with Dragendorff's reagent (3 drops). No white precipitate was obtained indicating the absence of the alkaloids salts.

Hydrolysis of Intermediate-polar/polar Fraction

The remaining Intermediate-polar/polar fraction was hydrolyzed by refluxing with equal volume of 10% (v/v) HCl acid for 30 minutes. After cooling, the hydrolyzed extract was re-extracted thrice with solvent hexane (10 ml). The lower acidic layer was used for the screening test of anthocyanosides. The upper combined hexane layer was dried over anhydrous sodium sulphate and filtered. The filtrate (hexane solution) was used for screening test's "e" and "f".

d) Test for Anthocyanosides

The red acidic layer was treated with sodium carbonate until basic of the litmus paper. A godrej special gray color was deceloped gradually but not green or blue color indicating the absence of anthocyanosides.

e) Test for Anthracenosides

The hexane solution (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for a few minutes to separate two layers. A greenish yellow color in the lower alkaline layer was observed but no red color indicationg the absence of anthracenosides.

f) Test of Coumarins derivative

The hexane solution (4 ml) was concentrated to yield a residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided in two test tubes. The first test tube was

used as control. To the second tube 10% (v/v) ammonium hydroxide solution was added drop by drop until pH 8 and was then absorbed under ultra violet light. Yellow fluorescence in the control and greenish yellow fluorescence in the senond test tube were observed indicating the presence of coumarin derivatives.

g) Test for Flavonic Glycosides

The intermediate-polar/polar fraction solution (10 ml) was concentrated to yield a residue whice was dissolved in methanol. To this solution, a few pieces of magnesium and concentrated HCl (5 drops) were added. An orange yellow color was developed indicating the presence of flavonic glycosides.

h) Test for Polyoses

The intermediate-polar/polar solution (4 ml) was concentrated in porcelain basin to yield a residue. To this residue, concentrated sulphuric acid (3 drops) was added and allowed to stand for 5 minutes. It was then treated with saturated methanolic solution of thymol (5 ml).

i) Test for Lactone

The intermediate-polar/polar solution (4 ml) was concentrated and treated with 1 drop of 90% alcohol and two drops of 3, 5-dinitrobenzoic acid. This solution was made alkaline by adding NaOH solution. The purple color showed the presence of aglycone containing the unsaturated lactone.

j) Tests for Saponins

The intermediate-polar/polar solution (4 ml) was concentrated and treated with hot water followed by shaking for 15 seconds. The formation of foam showed the presence of saponins.

ANNEX - II

Pathogenicity of Bacteria Involved in the present studies

1. ***Staphylococcus aureus***

   It causes localized infection when enter through break in skin. It causes pyrogenic infection including folliculitis, impetigo, furuncles, caruncles, breast abscess, post-operative wound infections, cellulites, pyomyositis, osteomyelitis, septic arthritis, bronchopneumonia, lungs abscess etc. It also causes boils, secondary infection, septicemia, pneumonia, meningitis, acute endocarditis, conjunctivitis, toxic shock syndrome and more commonly food poisoning[89].

2. ***Bacillus subtilis***

   It is less commonly found opportunistic pathogen. It sometimes causes food poisoning[89].

3. ***Escherichia coli***

   Pathogenic strains of *E. coli* are responsible for three types of infection in humans: urinary tract infection (UTI), neonatal meningitis and intestinal disease (gastrointestinal). The disease caused (or not caused) by particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesions, invasions, toxins and abilities to withstands host defenses[90]. On the basis of their pathogenicity, they are divided in to four groups viz. enteterotoxigenic *E. coli* (ETEC) strains causes an acute watery diarrhea, enteroinvasion strains of *E. coli* (EIEC) can cause *shigella* like blood and mucus in stool, verocytotoxin producing, also termed enterohaemorrhagic *E. coli* (VTEC/EHEC) cause hemorrhagic clitis and enteropathogenic E. coli (EPEC) which is of minor importance[89].

4. ***Pseudomonas sp.***

   It is opportunist pathogens, meaning that it exploits some break in the host defenses to initiate infection. It may cause ear infection and is the major causes melignantotitis media[89].

It also causes urinary tract infections, respiratory system infections, dermatis, soft tissue infections, bacteremia, bone and joints infections, gastrointestinal infections and a variety of systematic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunesupressed[90].

5. ***Enterobactor sp.***

   Several strains of the these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts and in those who are on mechanical ventilation[91]. *Enterobactor* is responsible for various infections, including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections. *Enterobacter* species can also cause various community-acquired infections, including UTIs, skin and soft-tissue infections, and wound infections, among others[92].

6. ***Salmonella typhy/paratyphy***

   Strains of *salmonella* are mostly responsible for enteric fever which includes typhoid fever. Other *Salmonella* causes gastrointestinal tract infections, osteomyelitis in children with sickle cell disease[93].

7. ***Shigella sp.***

   Shigella bacteria produce toxins that can attack the lining of the large intestine, causing swelling, ulcers on the intestinal wall, and bloody diarrhea. The severity of the diarrhea sets shigellosis apart from regular diarrhea. In kids with shigellosis, the first bowel movement is often large and watery. Later bowel movements may be smaller, but the diarrhea may have blood and mucus in it. Other symptoms of shigellosis are abdominal cramps, high fever, loss of appetite, nausea and vomiting, painful bowel movements. In very severe cases of shigellosis, a person may have convulsions (seizures), a stiff neck, a headache, extreme

tiredness, and confusion. Shigellosis can also lead to dehydration and in rare cases, other complications, like arthritis, skin rashes, and kidney failure[94].

8. ***Acenetobacter sp.***

   Acenetobacter is a Gram-negative genus of Bacteria belonging to the Gammaproteo bacteria. *Acenetobacter* are a key source of infection in debilitated patients in the hospital, particularly the species *Acinetobacter baumannii*[95].

9. ***Proteus mirabilis***

   They are opportunistic pathogens. Proteus mirabilis causes 90% of *Proteus* infections and can be considered a community-acquired infection[96].

10. ***Klebsiella Pneumonia***

    Members of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). Both of these antigens contribute to pathogenicity. In recent years, *klebsiellae* have become important pathogens in nosocomial infections[97].

ANNEX - III

Composition of Some Media Used in Study

1. Nutrient Agar (NA)

Composition	gm/lit
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00
Final p^H (at 25^oc)	7.4±0.2

2. Nutrient Broth (NB)

Composition	gm/lit
Peptone	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Final p^H (at 25^oc)	7.4±0.2

3. Mueller Hinton Agar (MHA)

Composition	gm/lit
Beef infusion broth	300.00
Casein Acid Hydrolysate	17.00
Starch	1.00
Agar	17.00
Final p^H (at 25^oc)	7.4±0.2

4. **Potato Dextrose Agar (PDA)**

Composition	**gm/lit**
Potato	200.00
Dextrose	20.00
Agar	20.00

5. **Potato Dextrose Broth**

Composition	**gm/lit**
Potato	200.00
Dextrose	20.00

PREPARATION OF REAGENTS

Mayer's Reagent

Mercuric chloride (0.679 g) was weighed in a 50 ml volumetric flask and dissolved in distilled water. To this solution, potassium iodide (2.5 gm) was added. The scarlet red precipitate was dissolved by shaking and then diluted with distilled water upto the mark of volumetric flask.

Dragendorff's Reagent

Bismuth nitrate (4 gm) was dissolved in 5N nitric acid (10 ml) to make solution A. Next, potassium iodide (13.5 gm) was dissolved in distilled water (20 ml) to make solution B. These solutions A and B were mixed together in a 50 ml volumetric flask.

Sodium Picrate Solution:

Picric acid (0.25 gm) was dissolved in distilled water (50 ml) to make aqueous picric acid solution. The solution was neutralized with sodium bicarbonate.

A strip of whatman no. 1 filter paper was dipped in the prepared solution. The paper was dried completely and protected from external contamination. Thus prepared sodium picrate paper was used for cyanogenic glycoside detection.

Molisch's Reagent:

α- napthol (5 gm) was dissolved in methanol (50 ml).

Neutral Ferric Chloride Solution:

Ferric chloride (1 mg) was dissolved in distilled water (100 ml). To this aqueous ferric chloride solution, sodium carbonate was added little by little with stirring until the slight turbidity was persistent. The mixture was filtered and the colourless filtered was used as neutral ferric chloride solution.

1M HCl

Concentrated HCl (11.7 M) (8.5 ml) was diluted with distilled water up to the mark in 100ml volumetric flask to make 1 M HCl.

28% Ammonia

Liquor ammonia (28 ml) was diluted up to the mark in 100 ml volumetric flask to make 28% liquid ammonia.

REFERENCES

(1) Joshi, K. K.; Joshi, S. D. *Genetic Heritage of Medicinal and Aromatic Plant of Nepal Himalayas*; Buddha Academic Publishers & Distributers Pvt. Ltd., Kathmandu, Nepal, 2001.

(2) Nair, R. I.; Kalaria, T.; Chand, S. *Turk. J. Biol.* **2005**, *29*, 41-47.

(3) Herbert, R. B. *Biosynthesis of Secondary Metabolites*; 2nd ed.; London Newyork, 1989.

(4) Duraipandiyan, V.; Ignacimuthu, M. *BMC compd. and Alt. Med.* **2006**, *6*, 35-41.

(5) Iyengar, M. A. *Study of Crude Drugs* Manipal Power Press, Manipal, India, 1995.

(6) Baral, S. R.; Kurmi, P. P. *A compendium of medicinal plants in Nepal*; Mass Printing Press,Chauni, Kathmandu, Nepal, 2006.

(7) Malla, S. B. *An Overview of Plant Resources in Nepal*; The Green energy Mission,Nepal, 1999.

(8) GoN *NPC's Nepal in Figures* Central Bureau of Statics,Thapathali, kathmandu, Nepal, 1998.

(9) Chaudhary, R. P. *Biodiversity in Nepal*; :Status and Conservation. S. Devi Saharanpur, India & Craftsman Presss, Bangkok, Thailand, 1998.

(10) Tiwari, N. N. *Wild relative of cultivated medicinal and Aromatic plants in Nepal, Proceeding of National Conference on Wild relatives of cultivated wild in Nepal*; Green Energy Mission Nepal, 1999.

(11) Orchard, A. E.; Maslin, B. R. *Australian Systematic Botany* **2003**, *16(1)*, 1-18.

(12) Maslin, B. R.; Miller, J. T.; Seigler, D. S. *Taxon* **2003**, *52(2)*, 362-363.

(13) Saini, M. L.; Saini, R.; Roy, S.; Kumar, A. *Journal of medicinal plants research* **2008**, *2(12)*.

(14) Press, J. R.; Shrestha, K. K.; Sutton, D. A. *Annotated Checklist of the Flowering Plant of Nepal*; The Natural History Museum, London, UK and Central Department of Botany, Tribhuvan University, Kathmandu, Nepal, 2000.

(15) *Medicinal and Aromatic Plant Network*; Nepal.

(16) IUCN *The IUCN Red list of threatened species:2001.Categories and criteria (version 3)*; Kathmandu: IUCN, 2001.

(17) Shrestha, T. B.; Joshi, R. M. *Endemica and Endangered Plants of Nepal*; WWF Nepal Program, 1996.

(18) GoN *Forest Regulation: Third Amendment September 2005*; Ministry of Forest and Soil Conservation, Kathmandu, Nepal, 2005; Vol. 55(37).

(19) Grerson, A.; Long, D. *Flora of Bhutan,vol-1,part 3*; Royal Botanical garden Edinburg, UK, 1984.

(20) Mishra, B. *Bhavprakash Nigantu*; Chaukhumba Bharati Academy, Gokul Bhawan,Varanasi, India, 1993.

(21) Kirtikar, K. R.; Basu, B. D. *Indian Medicinal Plants*; International Book Distributers, India; 195; Vol. IV.

(22) Bajracharya, M. B. *Ayurvedic Medicinal Plants and General Treatment*; Piyusavarsi Ausadhalaya Mahaboudha, Kathmandu, Nepal, 1979.

(23) Babu, S. S.; Madhavi, M. *Green Remedies. Healing power of herbs* Pustak Mahal, Delhi, 2006.

(24) Warrier, P. K.; Nambiar, V. P. K.; Ramankutty, C. *Indian Medicinal Plants: A Compendium of 500 species* Orient Longman Publishers, Kottakkal, India, 1994; Vol. 2.

(25) Adhikari, S. *Sthaniya Jadibuti Dwara Swasthya Raksha(Health Care by Local Herbal Resources)* Himalayan Ayurved Research institute,kathmandu Nepal, 1997.

(26) Oommen, S.; Ved, D. K.; Krishna, R. *Tropical Indian Medicinal Plants: Propagation Methods* Foundation for Revitalization for Local Health Tradition, India, 2000.

(27) Hoffmann, D. *Complete Herbal*; A safe and practical guide to making and using herbal remedies. Element Books Limited, UK, 1996.

(28) Manandhar, N. P. *Plants and People of Nepal* Timber Press, Portland, Oregon, USA, 2002.

(29) Asolkar, L. V.; Kakkar, K. K.; Chakre, O. J. *Second Supplement to Glossary of Indian Medicinal Plants with Active Principles* Publication & Information Directorate, Council of Scientific and Industrial Research, New Delhi, 1965-1981.

(30) Joshi, S. G. *Medicial Plants*; Oxford & IBH Publishing. New Delhi, India, 2006.

(31) Felter, H. W.; Lloyd, J. U. *An extract prepared from the heart wood of Acacia catechu (Linne fillius)*; King's American Dispensatory, 1898.

(32) GoN *Medicinal Plants of Nepal* Bulletin of the Department of Plant Resources No. 28. Ministry of Forest and Soil Conservation, Thapathali, Kathmandu, Nepal, 2007

(33) LISP. *Bikaska Pailaharu: Jadibunti Parbarddan Bisheshanka* Local Initiatives Support Programme, Palpa, 2004.

(34) Prajapati, N. D.; Purohit, S. S.; Sharma, A. K.; Kumar, T. *A Handbook of Medicinal Plants: A Complement Source Book* Agrobios India, 2006.

(35) GoN; Jadibuti Sankalan, Samrakshayan, Sambardan Bidhi. *Jadibuti Parichaya Mala*-1,2,3 Ministry of Forest and Soil Conservation, Thapathali, Kathmndu, 2006.

(36) Watanabe, T.; Rajbhandari, K. R.; Malla, K. J.; Yahara, S. *A Hand Book of Medicinal Plants of Nepal* Ayur Seed Life Environmental Institute (Ayurseed L.E.I.), Japan 2005.

(37) Nepal, I. U. C. N. *National Register of Medicinal and Aromatic Plants* (Revised & updated). The World Conservation Union, Nepal, 2004.

(38) Devkota, K. *Nepali Nighantu*(Medicinal Plants of Nepal) (in Nepali). Royal Nepal Academy, Kathmandu, Nepal, 1968.

(39) Rajbhandari, T. K.; Joshi, R.; Shrestha, T.; Joshi, S. K. G.; Acharya, B. *Medicinal Plants of Nepal for Ayurvedic Drugs* Government of Nepal, Department of Plant Resources, Thapathali, Kathmandu, 1995.

(40) Ambasta, S. P.; Ramchandran, K.; Kashyapa, K.; Chand, R. *The Useful Plants of India* Council of Science and Industrial Research (CSIR), New Delhi, 1992.

(41) Patils, S.; Jolly, C. I.; Narayanan, S. *Indian drugs* **2003**, *40(6)*, 328-332.

(42) In *http://en.wikipedia.org/wiki/Triterpene*.

(43) Finar, I. L. *Organic Chemistry*; 5th ed., 2000; Vol. 2.

(44) Sharma, P.; Dayal, R.; Ayyar, K. S. *J. med. Aromt.Plant sci.* **1999**, *211(4)*, 1002-1005.

(45) In *http://en.wikipedia.org/wiki/sterol*.

(46) In *http://en.encyclopedia.org/wiki/Alkaloids*.

(47) In *http://en.encyclopedia/wiki/carbohydrate*.

(48) Hussain, A.; Virmani, O. P.; Popali, S. P.; Mishra, L. N.; Gupta, M. M.; Srivastava, G. N.; Abraham, Z.; Singh, A. K. *Dictionary of Indian Medicinal Plants* Central Institute of Medicinal and Aromatic Plants. Lucknow, India, 1992.

(49) In *http://en.wikipedia/wiki/Tannins*.

(50) Attar-ur-Rahaman; Said, H. M.; Ahmad, V. *pakistan Encyclopaedia Planta Medica* Hamdard, Pakistan, 1986; Vol. 1.

(51) In *http://en.wikipedia/wiki/Flavonoids*.

(52) Deshpande, V. H.; Patil, A. D. *Indian J. CHEM.* **1980**, *20B*, 628.

(53) Yadav, R. N. *Journal of institute of chemists (India)* **2001**, *73(3)*, 104-108.

(54) Ajad, A. K.; Ogiyama, K.; Sassa, T. *J Wood Sci* **2001**, *47*, 406-409.

(55) Singh, K. N.; et.al. *Indian J Med Res* **1976.**, *64*, 754-757.

(56) Jayashekher, P.; et.al. *Indian Journal of Pharmacology* **1997**, *29*, 426-428.

(57) Chopra, R. N.; et.al. *Acacia catechu Wild.*; Glossary of Indian Medicinal plants. New Delhi, Council of scientific and Industrial research, 1954.

(58) Ray, D.; et.al. *Indian journal of Pharmacology* **2006**, *38(6)*, 408-413.

(59) Nagaraja, T. G.; Sarang, S. V.; Jambhale, D. C. *Journal of Biopesticides* **2008**, *1(2)*, 197-198.

(60) Saini, M. L.; Saini, R.; Roy, S.; Kumar, A. *Journal of Medicinal Plants Research* **2008**, *2(12)*, 378/-386.

(61) Jia, Q.; Nichols, T. C.; Rhoden, E.; Waite, S. United State Patent, Patent No. 7108868, 2006.

(62) Zhang, S.; Zheng, C.; Yan, X.; Tian, W. *Biochemical and Biophysical Reserch Communications* **2008**, *371*, 654-658.

(63) Yu, T. *J Agric Food Chem* **2007** *55(24)*, 9969-9976.

(64) Zang, Y.; et.al. *J Surg Res.* **2008**, *148(1)*, 17-23.

(65) Lopez-Sanchez, C.; et.al *Brain Res.* **2007**, *1182*, 123-137.

(66) Haraguchi, H.; Ohmi, I.; Fukuda, A.; Tamura, Y.; Mizutani, K.; Tanaka, O.; Chou, W. H. *Biosci Biotechnol Biochem* **1997**, *61(4)*, 651-654.

(67) Biziagos, E.; Crance, J. M.; Passagot, J.; Deloince, R. *J Med Virol* **1987**, *22(1)*, 55-66.

(68) Chu, S. C.; Hsieh, Y. S.; Lin, J. Y. *J Nat Prod* **1992**, *55(2)*, 179-183.

(69) Kolhir, V. K.; Tyukavkina, N. A.; V.A.Bykov; et.al. *Chem.-Farm. Jornal* **1995**, 61, 61.

(70) Igarashi, K.; Uchida, Y.; Murakami, N.; Mizutani, K.; Masuda, H. *Biotechnol Biochem* **1996**, *60(3)*, 513-515.

(71) Iluchenok, T. Y.; Homenko, A. I.; Frigidova, L. M.; et.al. *Pharmacology and toxicology* **1975**, *38(5)*, 607.

(72) In *http://ezinearticles.com/?expert=Mila_Sabido*.

(73) In *http://ezinearticles.com/?expert=Dan_Ho*.

(74) Sichuan Da Xue Xue Bao Yi Xue Ban.. Division of Peptides Related with Human Diseases, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China, 2007 Nov.

(75) *Obesity (Silver Spring)*; Biospectrum Life Science Institute, Gunpo City, Gyunggi Do, Republic of Korea, Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul, Republic of Korea, 2009 Feb.

(76) Lee, K. W.; Kim, Y. J.; Lee, H. J.; Lee, C. Y. *J. Agric. Food Chem.* **2003**, *51 (25)*, 7292-7295.

(77) In *Science Daily* March 12,2007.

(78) Murase, T.; Haramizu, S.; Ota, N.; Hase, T. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2008**, *295 (1)*281–289.

(79) Gradisar, H.; Pristovsek, P.; Plaper, A.; Jerala, R. *J. Med. Chem.* **2007**, *50 (2)*, 264-71.

(80) *Fruits, vegetables, teas may protect smokers from lung cancer*; UCLA news 2008.

(81) Naik, G. H.; et.al. *Phytochemistry* **2003**, *63(1)*, 97-104.

(82) Culie, I.; et.al. *Journal of Science, Food and Agriculture* **1953**, *4*, 149-153.

(83) Dingel, J.; Red, W. W.; Solomons, G. L. *J. of science,food and agriculture* **1953**, *40*, 149-153.

(84) WHO. *basic laboratory procedure in clinical bacteriology* World Health Organization, Geneva, 1991.

(85) Parekh, J.; Chanda, S. *African Journal of Microbial Research* **2007**, *1(6)*, 92-99.

(86) Baron, E. J.; Peterson, L. R.; finegold, S. M. *Bailey and Scott's diagnostic Microbiology*; 9th ed.; Mosby tear book, Inc. USA, 1994.

(87) Aberkene, A.; Cuenca-Estrella, M.; Petrikkou, E.; Mellado, E.; Monzon, A.; Rodriguez-Tudela, J. L.; et.al *J. of antimicrobial chemotherarapy* **2002**, *50*, 719-22.

(88) Manandhar, M. D. *C.D. Journal* **2002**, *XXI(35)*.

(89) Collee, J. G.; Fraser, A. G.; Marmion, B. P.; Simmon, A. *Mackie and McCartney Practical Medicinal Microbiology*; Churchill Living Stone, 1996.

(90) Todar, K. *Todar's Online Textbook of Bacteriology*, 2008.

(91) In *http://en.encyclopedia/wiki/Enterobactor*

(92) Fraser, S. L.; Arnett, M. *Enterobacter Infection*, 2010.

(93) Cheesbourg, M. *Mecinal Laboratory Mannual for Tropical Countries* .Microbiology. Llinacre house,Jordan Hill Oxford, 1993.

(94) In *http://kidshealth.org/general/sick/shigellainfection*.

(95) Gerischer, U. *Acinetobacter Molecular Biology*; 1st ed.; Caister Academic Press, 2008.

(96) Struble, K.; Bronze, M. S.; Jackson, R. L.; Gonzalez, G. *Proteus Infection*, 2009.

(97) Umeh, O.; Berkowitz, L. B. *Klebsiella Infection*, 2009.

PHOTOGRAPHS

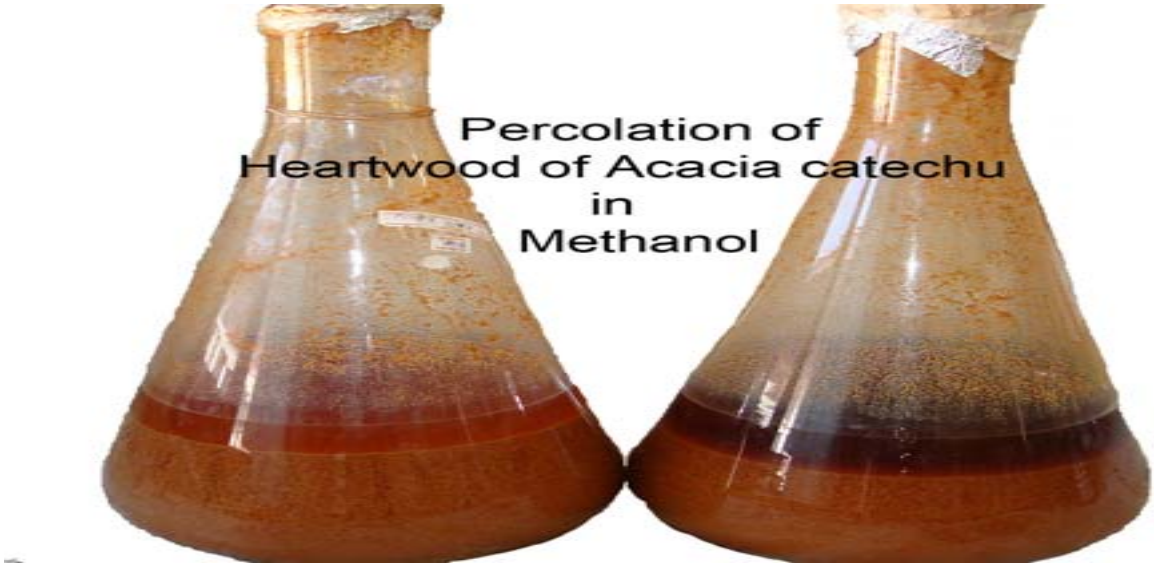


Fig: Percolation of heartwood of *Acacia catechu* in Methanol

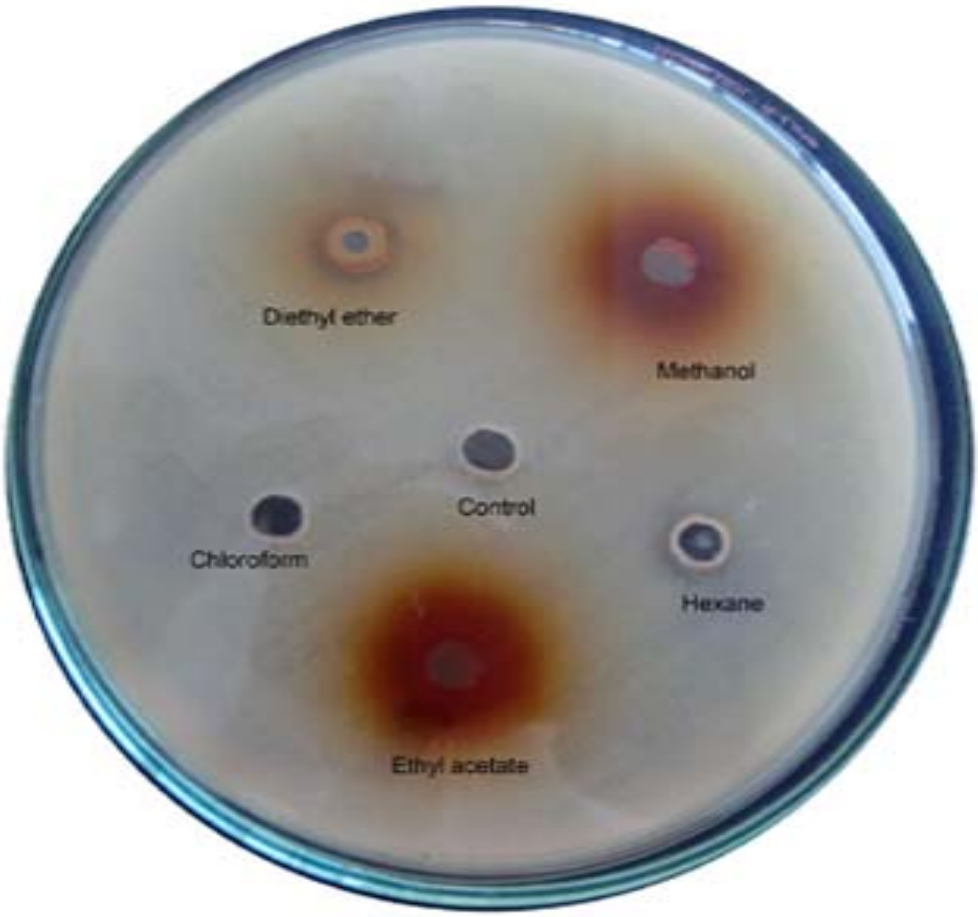


Fig: Zone of Inhibition produced by different fraction of Heartwood of *Acacia catechu* against *Klebsiella pneumonia* bacteria

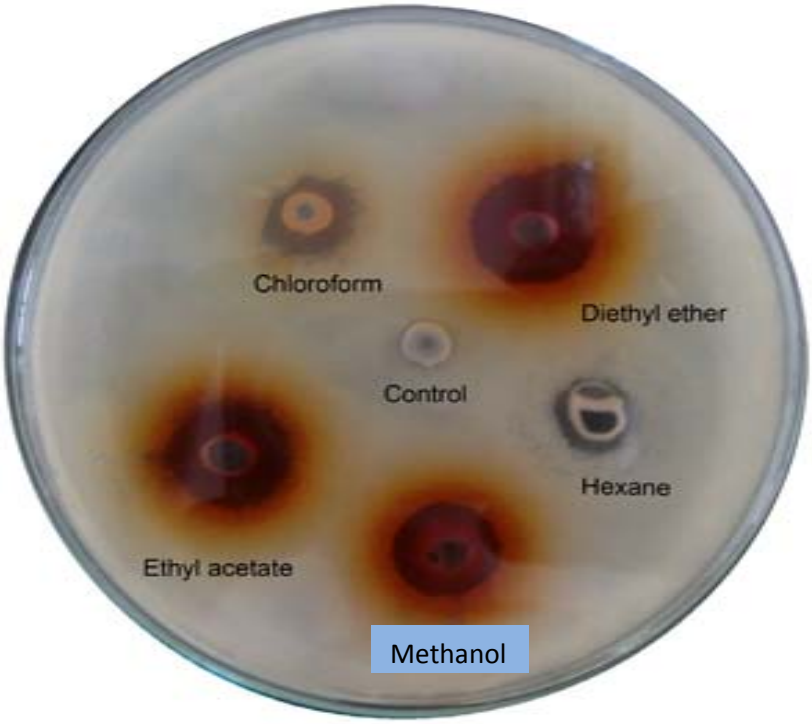


Fig: Zone of Inhibition produced by different fraction of heartwood of *Acacia catechu* against *Bacillus subtilis* bacteria

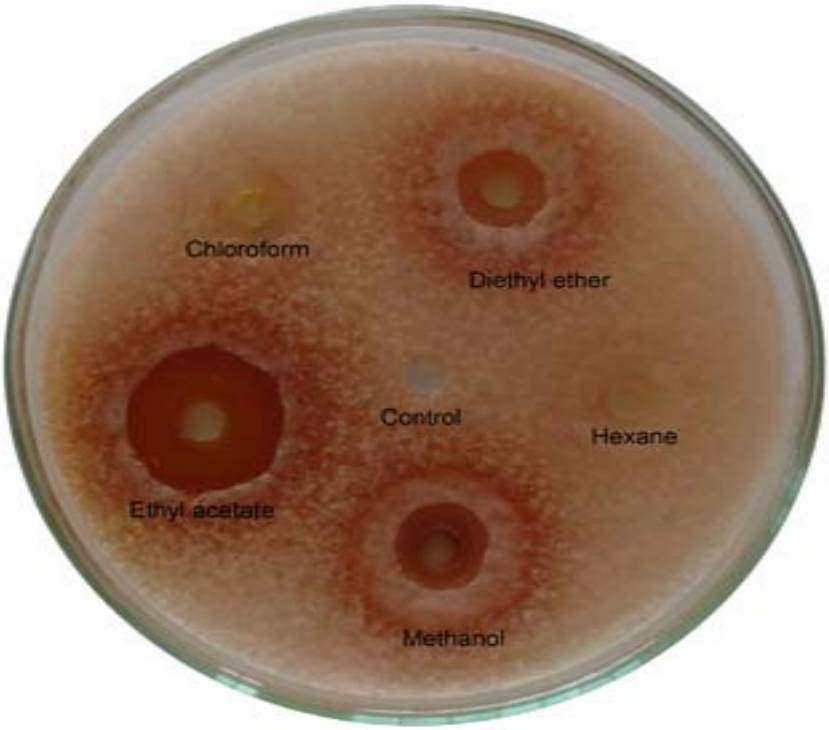


Fig: Zone of Inhibition produced by different fraction of heartwood of *Acacia catechu* against *Fusarium oxysporium* fungi

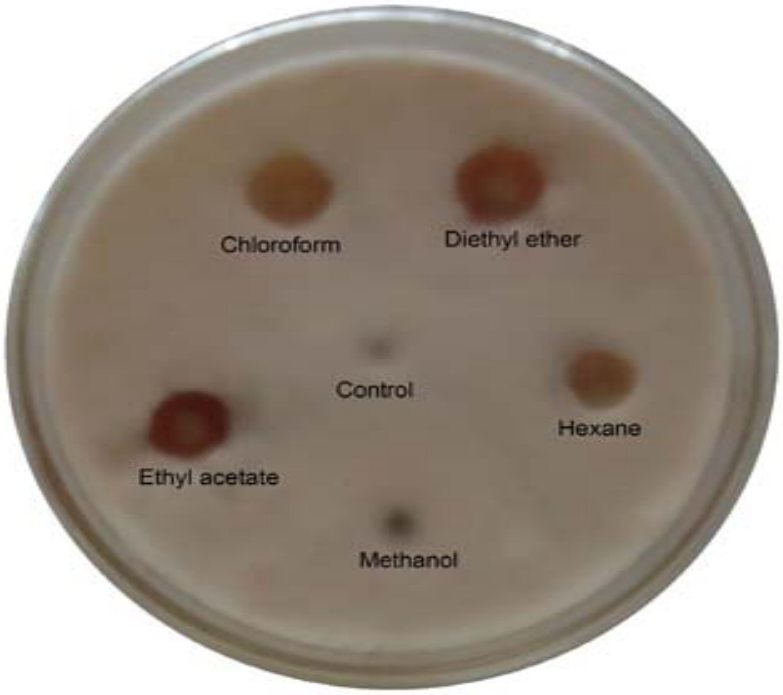


Fig: Zone of Inhibition produced by different fraction of heartwood of *Acacia catechu* against *Fusarium Moniliformi* fungi

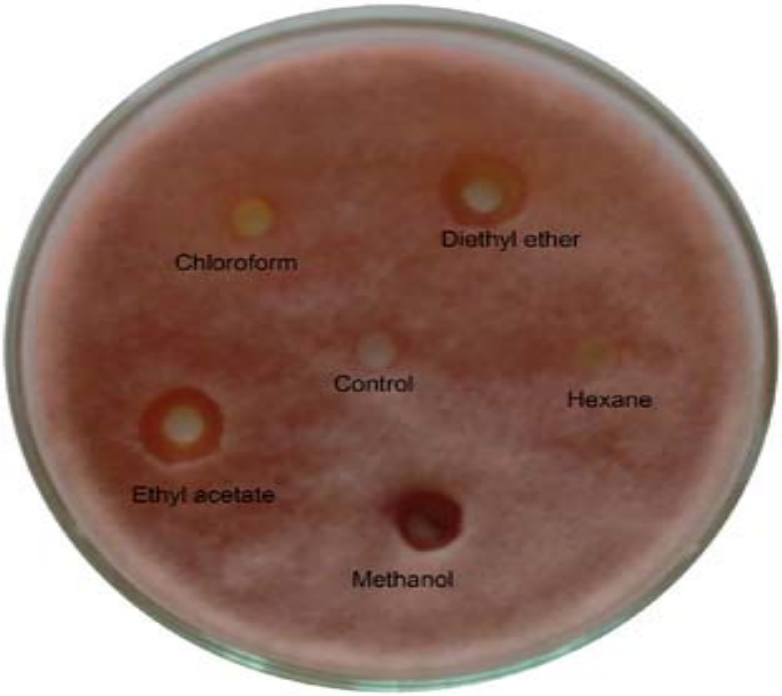
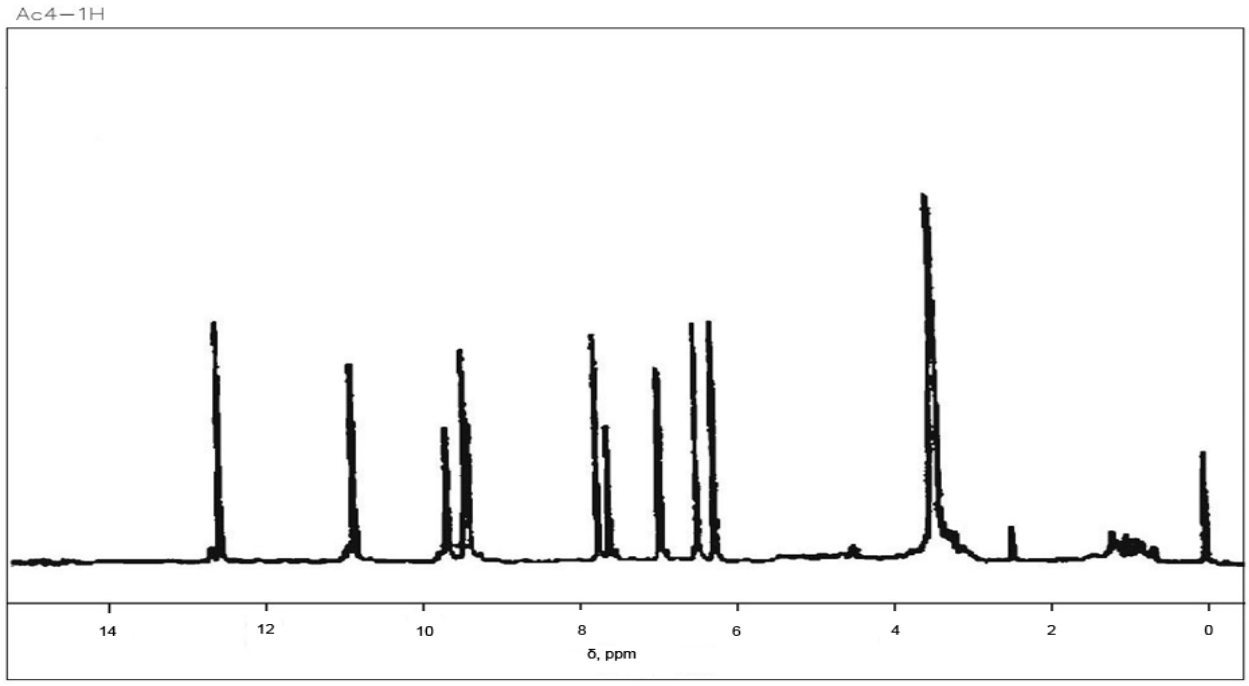
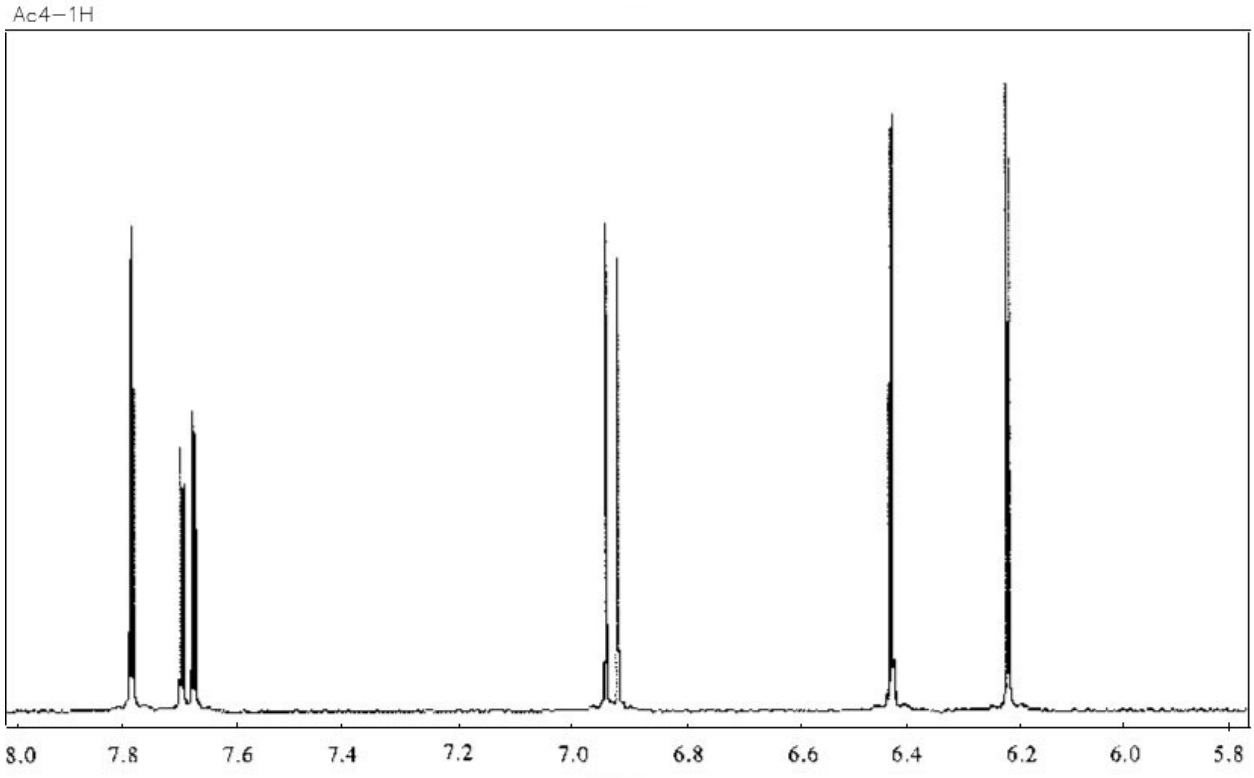


Fig: Zone of Inhibition produced by different fraction of heartwood of *Acacia catechu* against *Fusarium proliferatum* fungi

SPECTRA



^{1}H-NMR Spectra of Compound Ac4 (Quercetin)



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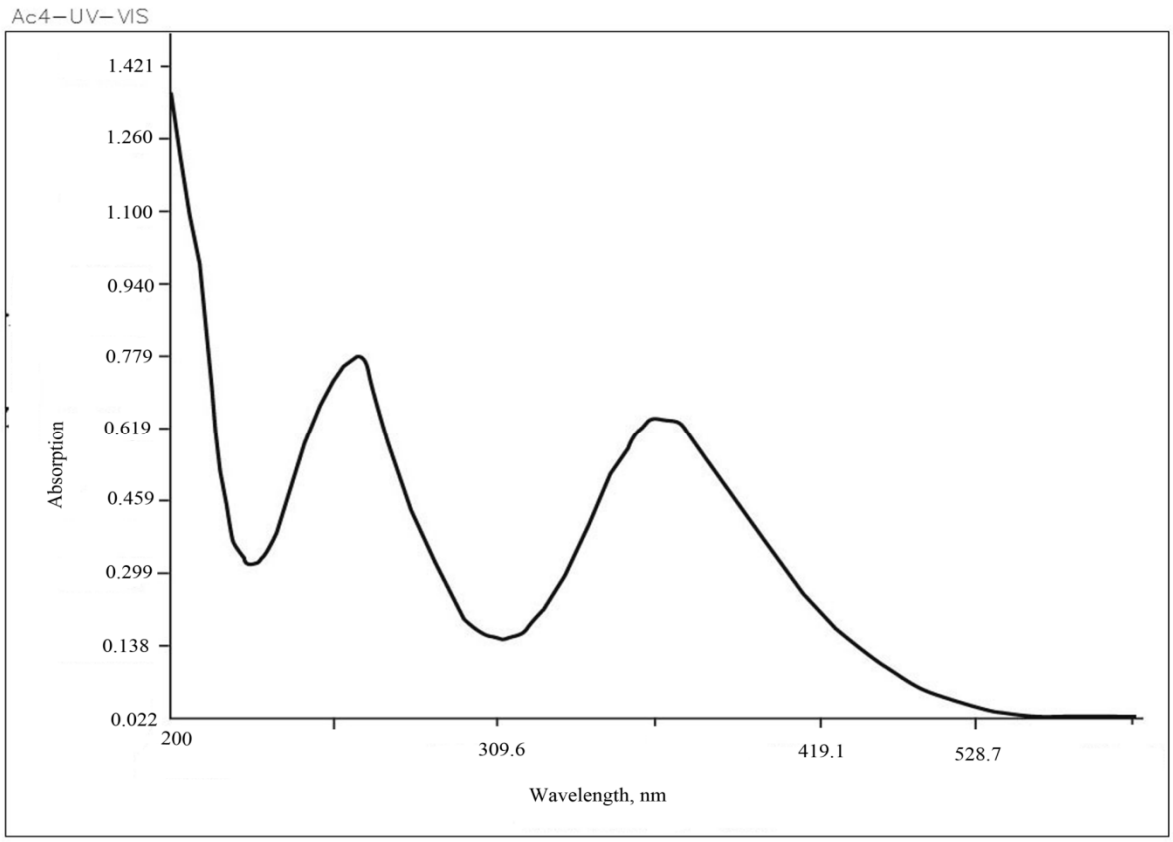
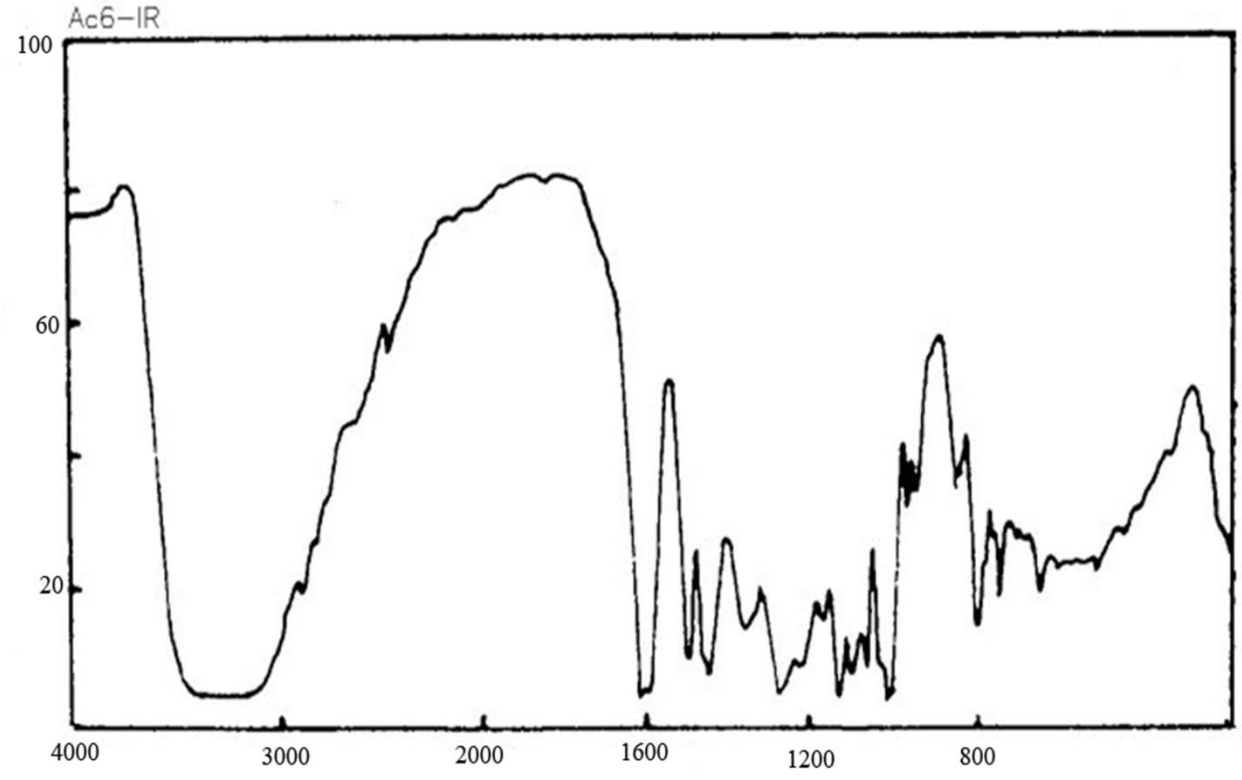
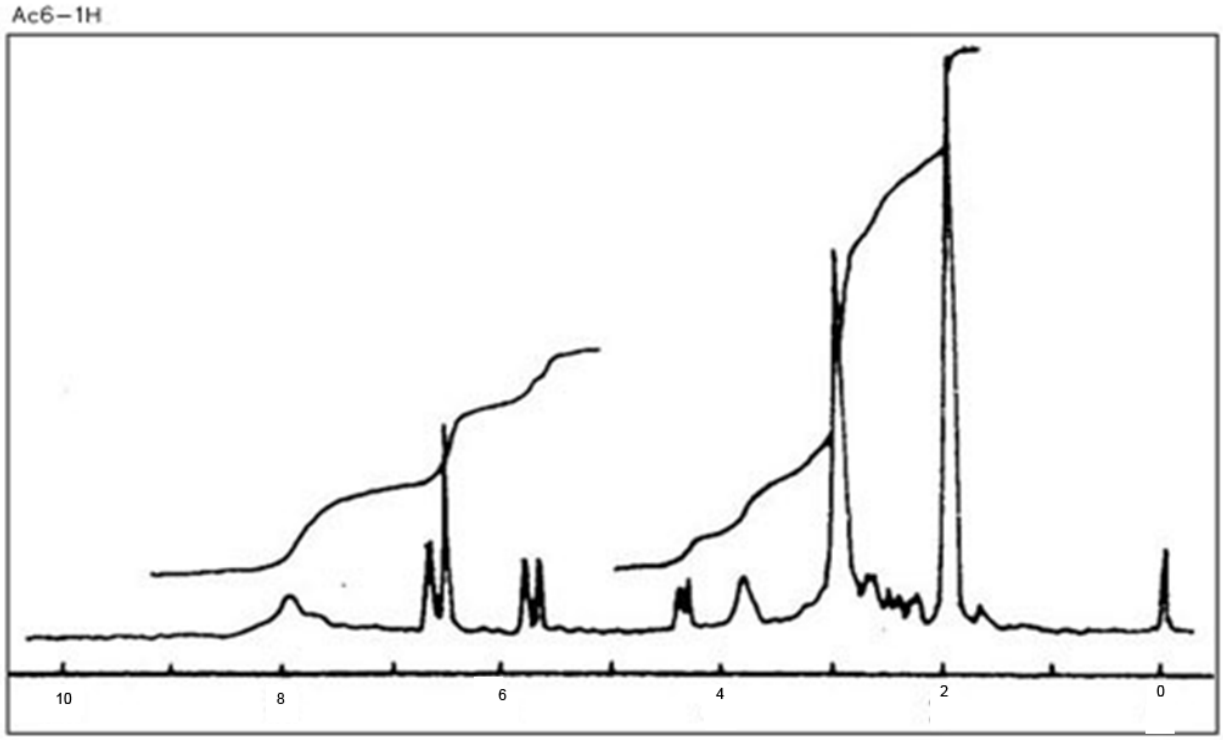


UV-VIS Spectra of compound Ac4 (Quercetin)



IR Spectra of Compound Ac6 [(+) Catechin]



^{1}H-NMR Spectra of Compound Ac6 [(+) Catechin]