# Phytochemical investigation of *Citrullus lanatus* (Watermelon) rind

A thesis report, submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy.


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# Declaration by Research Candidate

I, Bushra Rahman (Student ID: 2010-1-70-017), hereby declare that the thesis report entitled "Phytochemical investigation of *Citrullus lanatus* (Watermelon) rind*"* submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of degree of Bachelor of Pharmacy (Honors) is a genuine and authentic record of original research work carried out by me during Fall 2013 under the supervision and guidance of Dr. Repon Kumer Saha, Associate Professor, Department of Pharmacy, East West University and it has not formed the basis for the award of any other Degree/ Diploma/Fellowship or other similar title to any candidate of any University.

_____

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# CERTIFICATE

This is to certify that this thesis report on "Phytochemical investigation of *Citrullus lanatus* (Watermelon) rind" submitted to the department of pharmacy, East West University, Aftabnagar, Dhaka, in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (B. PHRM) was carried out by Bushra Rahman (Student ID: 2010-1-70-017) under our guidance and supervision and that no part of the thesis has been submitted for any other degree. We further certify that all the sources of information and laboratory facilities availed in this connection is duly acknowledged.


**Dr. Repon Kumer Saha**

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## Objective And Rationale Of The Work

The objective of the work is to trace out important chemicals present in the rind of citrullus lanatus. This process is a part of phytochemical screening which is very important as a priliminary research work. Based on the results of phytochemical screening further research works are conducted.

The medical value of plants are due to some important chemical substances (generally secondary metabolites), which produce a definite physiological action on the human body. Some of these bioactive compounds are alkaloids, flavanoids, caffeine, tannins, phenolics.[1] Many food trees have multipurpose uses and their plant chemical based products satisfy various non-food purposes ranging from timber to forest.[2] There fore, research is focusing on the search for new types of natural chemotherapeutic agents derived from plants, which are proving to be excellent sources of potential new compounds, also called lead compounds.

The hard green rind of the juicy watermelon is discarded by most people. The rind has some nutritional benefit as well as some pharmacological effect. This friut is available in Bangladesh during the summer season. The rind part was selected for phytochemical investigation because it contains an important compound citrullin. If any other important compound could be found from this discarded part than it may become a source of potential medicinal plant and the plant part will also become usefull.

## Abstract

In our study effort was made to find out the constituents present in the rind of *Citrullus lanatus*. Caffiene isolation and catechin isolation process were followed to bring out the specific constituents. Different procedures like TLC, DPPH test and Phenolic assay were done to confirm that the compounds were isolated. The concentration of the isolated sample was also measured by the graph of standard curve.


**Key words:** *Citrullus lanatus*, Caffiene, Catechin, TLC, DPPH test, Phenolic assay, UV screening.

# CHAPTER 1

# Introduction

## 1.1 Introduction

Tracing of different plant constituents by phytochemical screening is a process of fininding lead compound. Lead compound is any compound that has potential pharmacolological activity. The physical, chemical and pharmacological properties are studied to discover any medicinal benefit.

Plants can synthesize a wide variety of chemical compounds that are used to perform important biological functions. Many of these phytochemicals have longer helpfull effects on human health, and can treat human diseases effectively. At least 12,000 lead compounds have been identified and isolated so far, this number estimated to be less than 10% of the total number.[3,4]

The study of traditional human uses of plants is called Ethnobotany. It is an recognised way to discover new effective medicines for future. In ancient Greece, plants were classified and descriptions of them were given by scholars. It aid the identification process. Researchers identified in 2001 that 122 compounds that are used in modern medicine, were isolated and identified from "ethnomedical" plant sources. The current use of the active elements of the plants are 80% similar to those of ethnomedical use.[5] Many of the drugs that are used in pharmaceuticals and currently available to market have a long history of use as herbal remedies, such as quinine, aspirin, digitalis, and opium.

The therapeutic treatment of disease with the use of herbs began long ago.[6] Methods of folk healing throughout the world commonly used herbs as part of their tradition.The practice of using herbs to treat diseases is very common among many non developed societies. It is sometimes more easier to get than purchasing expensive modern pharmaceuticals. The World Health Organization (WHO) estimates that 80 percent of the

Asian and African countries population recently use herbal medicine for some aspect of primary health care. Recent studies done in the United States and Europe have found that their use has scientific evidence of the effectiveness of the medicinal plants. For example salicylic acid from the bark of willow tree has been used for millennia as an effective analgesic and antipyretic. From this aspirin was derived. Slicylic acid is the active metabolite of aspirin. [5]

Fig 1: Aspirin from willow tree

Because "over 50% of prescription drugs are derived from chemicals first identified in plants,"[7] the search for new drugs greatly depend on plants. It is the challenge of the researchers to discover potent compound. In the research developed countries, high-throughput screening tests are used in vitro and in vivo. They are used for bioassay-guided fractionation leading to the isolation of active constituent which may be developed into medicinal agents either as a synthetic chemical or a synthesised analogue with enhanced therapeutic activity or reduced toxic effects.[8]

### 1.2 Medicinal plants of Bangladesh

There are traditional practice of treating diseases from generation to generation in the country. This practices are influnced by cultural habits, religious beleifs and superstitions.

### 1.2.1 Traditional medicinal plants

The earliest mention of traditional medicine is found in Rigveda, the oldest respiratory of knowledge in this subcontinent. Later Ayurveda developed from the Vedic concept of life, became the important source of all medicinal sceinces. With time passing on it became a part of culture and heritage of the people of the Indian subcontinent.[9]

**Table 1**: Crude drugs used as medicines in bangladesh[9,10]

| Common name | Botanical name | Uses |
|---|---|---|
| Ashok | *Saraca asoca* | Menstrual pain, uterine disorder, Diabetes |
| Chirata | *Swertia chiraita* | Skin disease, burning censation, fever |
| Kalmegh | *Andrographis paniculata* | Fever, weakness, gastrointestinal disorder. |
| Gritkumari | *Aloe verra* | Laxative, wound healing, skin burn and care, ulcer |
| Neem | *Azadirachta indica* | Sedative, anlgesic, epilepsy, hypertensive |
| Tulsi | *Ocimum sanclum* | Cough, cold, carminative |

### 1.2.2 Tribal medicinal plants

In different localities of Rangamati and Bandarban Districts of Bangladesh a survey was carried out between 2001 and 2002 to document medicinal plants. A total of 69 medicinal plants under 40 families were documented during this work, which the tribal use to treat about 50 diseases.[11] Some examples are given below-

**Table 2**: Tribal medicinal plants with their use.[11]

| Sceintific name, family name | Tribal name | Locality | Disease |
|---|---|---|---|
| *Leea indica* (Leeacea) | Chakma Haskura | Toolaban Marissa | Sore, leprosy, eczema, itching, bone fracture. |
| *Kalanchoe pinnata* (Crassulaceae) | Tanchongya-Rockkia pangpo | Naramuk Rajasthali | Cough and asthma of children |
| *Eupatorium odoratum* (Asteraceae) | Tonchongya *Demrapata gach* | Naramuk, Rajsthali | Bleeding |
| *Croton caudatus* Geisel. (Euphorbiaceae) | Chakma - Sholokjara | Toolaban, Marissa | Arthritis, paralysis |
| *Annona mouricata* L. (Annonaceae) | Marma - Penchi | Hangshamapara, Bandarban | Pain in hand & leg |

### 1.2.3 The tropical fruits

The tropical fruits of Bangladesh are excellent source of antioxidant vitamins like vitamin C, beta-carotene and antioxidant minerals such as zinc, copper, manganese, iron. Some of these fruits are good source of fiber but poor source of protein and fat. Carbohydrate and brix content are also found in some of the tropical fruits. The major findings of a study include some tropical fruits like star gooseberry, monkey jack, pineapple and golden apple. These are very rich in antioxidant vitamins and minerals. Mango, blackberry, jackfruit and carambola also contains antioxident property but melon and java apple are poor source of antioxidant vitamins and minerals.[12] The antioxidant and beta-carotene amount of the avobe mentioned fruits are given below with their sceintific names.

**Table 3:** Tropical fruits with Vitamin C and Beta-carotene content in mg and ug amount.[12]

| Name of fruit | Local name | Sceintific name | Vitamin C mg | Beta-carotene ug |
|---|---|---|---|---|
| **Blackberry** | Kalojam | *Syzygium Cumini* | 151.51 | 1112.38 |
| **Java apple** | Jamrul | *Syzygium Samarangense* | 40.22 | 1715.51 |
| **Jack fruit** | kanthal | *Artocarpus Heterophyllus* | 35.32 | 4401.82 |
| **Pineapple** | Anarash | *Ananas Comosus* | 104.81 | 1858.73 |
| **Carambola (Star fruit)** | Kamranga | *Averrhoa Carambola* | 65.00 | 1340.87 |
| **Golden apple** | Amra | *Spondius mangifera* | 128.78 | 2377.06 |
| **Mango** | Aam (Fazli) | *Mangifera Indica* | 55.56 | 3504.61 |
| **Melon** | Bangi (Phuti) | *Cucumis melo* | 33.93 | 1850.00 |
| **Monkey jack** | Deophal (Deua) | *Artocarpus lakaocha* | 171.07 | 3718.16 |

# Chapter 2

## Plant review

## 2.1 Plant selection

The targeted plant for this study was *Citrullus lanatus* (watermelon). Watermelon (*Citrullus lanatus*) of family Cucurbitaceae is a flowering plant. It is mainly originated from southern Africa. Its fruit is called pepo by botanist. It is also known as watermelon. Pepo is a berry like fruit. The exocarp is the thick rind, the outer surface of the fruit and mesocarp and endocarp are the freshly coloured center of the fruit.[13]

## 2.2 Plant review

### 2.2.1 Physical Characteristics

Water melon plant

Water melon plant is a spreading, hairy, tendril-bearing annual vine reaching a length of several meters. Leaves are long-stalked, oblong-ovate, 8 to 20 centimeters long, deeply 3- to 7-lobed, pinnatifid with usually narrowed segments. Watermelon leaves are dark green with prominent veins. The plant is self-fertile.[13,14]

Fig 2: *Citrullus lanatus* (watermelon) plants and Leaves

Flowers

Flowers are monoecious, yellow, and about 2 centimeters in diameter, occurring singly in axils of the leaves.[15] It is in flower from Jul to August.[16]

Fig 3: *Citrullus lanatus* (watermelon) flower and fruit.

Fruit

Fruit is very large, smooth, ellipsoid to oblong, light green with irregular dark green-mottled stripes, sometimes covered with a white, waxy bloom, about 30 centimeters long. The flesh is white, yellowish, pink or red; crisp, soft and juicy.

Seeds

Seeds are compressed, sometimes red, usually black. [13] watermelon seeds size:10-12pcs/10cm moisture:13%

Fig 4: *Citrullus lanatus* (watermelon) rind (white part) and seeds

### 2.2.2 Classification

Scientific Classification

Domain- Eukarya

Kingdom- Plantae

Phylum- Embryophyta

Class- Dicotyledoneae

Order: Cucurbitales

Family: Cucurbitaceae

Genus: *Citrullus*

Species: *Citrullus lanatus*

### 2.2.3 Local Names of watermelon

The C. lanatus produces a fruit that is about 93% water, making it the majority of it water, hence the name "water" melon. The "melon" part came from the fact that the fruit is large and round and has a sweet, pulpy flesh. [17]

**Table 4**: Local name of watermelon in different countries[13]

| Name of country | Local name |
|---|---|
| Africa | Waatlemoen |
| Nepal | Tarabuujaa (Tarbuja). |
| China | Han koa, Hia koa. |

| | |
|---|---|
| Egypt | Betteakh |
| India | Tarbnj and Jamaika |
| Germany | Wassemelone |
| Morocco | Dillah |
| Bangladesh | Taramuj, Tormuj |

**2.2.4 Breif description of the classified groups**

Domain: Eukarya[13]

The domain Eukarya contains cells with true nuclei, as well as membrane bound organelles. These factors qualify it as a eukaryotic organism. This domain covers a gigantic variety of organisms ranging from uni-cellular protists to large animals such as the chimpanzee.

Kingdom: Plantae

Kingdom Plantae consist of multi-cellular organisms that have cells with cell walls made of cellulose. Cells also have chloroplasts which allows them to photosynthesize. They receive nutrition by producing energy from sunlight and water. Plantae is a large kingdom that includes huge variety of organisms as well. Another organism within this kingdom is much larger than the Citrullus lanatus and goes by the common name, the white willow.[13,15]

Phylum: Embryophyta

The Citrullus lanatus is under the phylum Embryophyta because it is a land plant, located terrestrially. In addition, it contains seeds, also known as embryos. Another common organism from this phylum includes the cabbage palm.[16]

Class: Dicotyledoneae

The class Dicotyledoneae has all vascular plants; and more specifically dicots, meaning their seeds typically has two outer shell coverings.

Order: Cucurbitales

Cucurbitales is the order containing flowering plants unisexual flowers.

Family: Cucurbitaceae

The Cucurbitaceae family ranks among the highest of plant families for number and percentage of species used as human food.[18] The Cucurbitaceae consist of approximately 125 genera and 960 species, mainly in regions tropical and subtropical. The family Cucurbitaceae consist of plants with sprawling herbaceous vines and melons. A melon is a large fruit with a fleshy and bulky inside with thick,hard, protective skin. This family is also known as the gourd family. Other members within this family include the cantaloupe, squash, and pumpkin. [14]

Genus: *Citrullus*

The Citrullus lanatus is under the genus Citrullus because it is a desert vine and native to Eurasia and Africa.[14] The flowers are yellow and the seeds are somewhat flattened. This genus contains a total of 4 species including -

- *C. colocynthis*,
- *C. ecirrhosus*,

- *C. lanatus*, and
- *C. rehmii*.

Fig 5: Yellow flower of *Citrullus* genus

Species: *C. lanatus*

The watermelon is given the name lanatus because of its pink/red or yellow flesh and black seeds, along with all of the above characteristics.

Fig 6: Citrullus lanatus leaves and fruit.

Sceintific name

The scientific name of the watermelon *Citrullus lanatus* is derived from both Greek and Latin roots. The Citrullus part comes from a Greek word “citrus” which is a reference to the fruit. The lanatus part is Latin. Lanatus has the meaning of being wooly, referring to the small hairs on the stems and leaves of the plant. [14]

## 2.2.5 Origin

Horticulturalists believe that watermelons originated in South Africa, and their name refers not only to the fruit but also to the specific plant, *Citrullus lanatus*.[16]

Since watermelon thrives in warm conditions, it's not surprising that cultivation of the plant spread to places like Egypt, where it is believed to have been cultivated about 2000 BCE. China soon became an avid cultivator of Citrullus lunatus, by at least the 10th century CE, though there are some that contend that the first Asian country to cultivate the watermelon was Viet Nam. When the Moors invaded China, or established trade, watermelon cultivation spread across Asia, the Persian Gulf, and thence to Europe, and Early American settlers were growing the fruit by the 17th century, though some suggest explorers of the New World introduced the plant to Native Americans in the early 16th century. [16]

## 2.2.6:Nutritional Value

2.2.6.1 Fruit - Figures in grams (g) or miligrams (mg) per 100g of food.

**Table 5:** Fruit (Fresh) in grams (g) or miligrams (mg) per 100g [19]

| **Principle** | **Nutrient value** | **Percentage of RDA (Recommended daily allowance** |
|---|---|---|
| Energy | 30 Kcal | 1.5% |
| Carbohydrates | 7.6 g | 6% |
| Protein | 0.6 g | 1% |
| Total Fat | 0.15 g | 0.5% |
| Cholesterol | 0 mg | 0% |
| Dietary Fiber | 0.4 g | 1% |
| **Vitamins** | | |
| Folates | 3 µg | 1% |

| | | |
|---|---|---|
| Niacin | 0.178 mg | 1% |
| Pantothenic acid | 0.221 mg | 4.5% |
| Pyridoxine | 0.045 mg | 3.5% |
| Thiamin | 0.033 mg | 3% |
| Vitamin A | 569 IU | 19% |
| Vitamin C | 8.1 mg | 13.5% |
| Vitamin E | 0.05 mg | 0.5% |
| **Electrolytes** | | |
| Sodium | 1 mg | 0% |
| Potassium | 112 mg | 2.5% |
| Calcium | 7 mg | 0.7% |
| Copper | 42 µg | 4.5% |
| Iron | 0.24 mg | 3% |
| Magnesium | 10 mg | 2.5% |
| Manganese | 0.038 mg | 1.5% |
| Zinc | 0.10 mg | 1% |
| **Phyto-nutrients** | | |
| Carotene-alpha | 303 µg | -- |
| Crypto-xanthin-beta | 78 µg | -- |
| Lutein-zeaxanthin | 8 µg | -- |
| Lycopene | 4532 µg | -- |

# Chapter 3

# Literature review

## 3.1 Phytochemical review

### 3.1.1Chemical constituents

The fruit-

The watermelon fruit contains about 6% sugar and 91% water by weight. Watermelon as with many other fruits, is a source of vitamin C. The amino-acid citrulline was first extracted from watermelon and analyzed. The flesh of a watermelon contains about 250 millligrams of citrulline per cup.Watermelons contain a significant amount of citrulline and after consumption of several kilograms, an elevated concentration is measured in the blood plasma.[20] The fruit is a rich source of pectin. Alongside of tomatoes, watermelon has moved up to the front of the line in recent research studies on high-lycopene foods. Lycopene is a carotenoid phytonutrient that's especially important for our cardiovascular health and bone. Other constituents present are-

- Cucurbitacin E
- vitamin-B6 (pyridoxine),
- thiamin (vitamin B-1),
- vitamin-C,
- manganese.
- Potassium
- carotenoid pigment
- lycosides,
- flavonoids,
- tannins and
- polyphenols.

Flesh of fruit - Flesh of fruit contains[13]-

- saccharose,
- dextrose, levulose,
- invert sugar,
- citrullin,
- lycopin,
- carotin,etc.

The exocarp- The skin contains-

- impressive concentrations of phenolic antioxidants
- a fixed oil,
- arachidic acid, and
- traces of copper.[13]

The seeds- The seeds contain-

- oil, 15 to 45%, made up of glycerides of linoleic acid,
- oleic acid
- palmitic and stearic acids.
- A study suggests the active principle in the seed is a glucoside-saponin named cucurbocitrin.
- Unsaturated fatty acid content of an ether extract in water melon seeds was reported at 76.1%, mainly linoleic acid. [13]

The oil- The oil contains a small amount of phytosterol.

### 3.1.2 Citrulline properties

All parts of the watermelon - rind, flesh, and seeds - contain citrulline, a non-essential amino acid, which converts to L-arginine when eaten.[17]

The organic compound citrulline is an α-amino acid. Its name is derived from citrullus, the Latin word for watermelon, from which it was first isolated in 1914 by Koga & Odake. It was finally identified by Wada in 1930. It is a key intermediate in the urea cycle, the pathway by which mammals excrete ammonia.

Fig 7: Watermelon rind

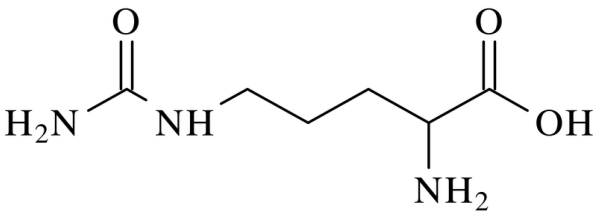



Fig 8: Structure of citrulline

Citrulline is used in the nitric oxide system in humans and has potential antioxidant and vasodilatation roles. Citrulline content ranged from 3.9 to 28.5 mg/g dry weight (dwt) and was similar between seeded and seedless types (16.6 and 20.3 mg/g dwt, respectively). Red flesh watermelons had slightly less citrulline than the yellow or orange flesh watermelons (7.4, 28.5 and 14.2 mg/g dwt, respectively). Rind contained more citrulline than flesh on a dry weight basis (24.7 and 16.7 mg/g dwt, respectively) but a little less on a fresh weight (fwt) basis (1.3 and 1.9 mg/g fwt, respectively). These results indicate that watermelon rind, an underutilized agricultural waste, offers a source of natural citrulline.[17]

## 3.2: Pharmacological Properties

### 3.2.1 Seeds :

- Seeds are considered as cooling agent, demulcent, diuretic, vermifuge, nutritive, pectoral and pectic.[13]
- Seeds are oily which are sometimes used as substitute for peanuts.
- Seeds used to alleviate symptoms of acute cystitis.
- The crude extract of seeds believed to have a lowering blood pressure effect.
- 100 seeds contain 1-2 mg of zinc and iron which are 80-90% bioavailable.[21]

### 3.2.2 The roots:

- The juice of the roots used for hemorrhage after abortion.[13]

### 3.2.3 The fruit:

- High amount of lycopene is present in the fruit which is beneficial for cardiovascular health and for bone. The concentration is greater than that of lycopene in tomatoes.
- In watermelon phenolic compounds including flavonoids, carotenoids, and triterpenoids has anti-inflammatory and antioxidant properties which make the fruit beneficial to health.[22,23]

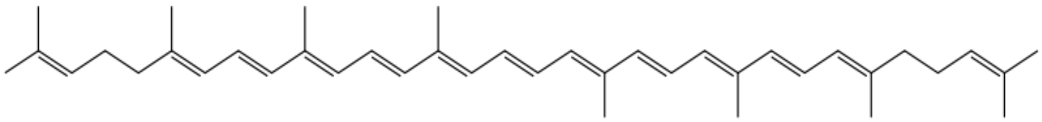


Fig 9: Structure of lycopene

- The flesh of a watermelon contains about 250 millligrams of citrulline per cup. When citrulline is absorbsed, it is converted into arginine.[24,25]

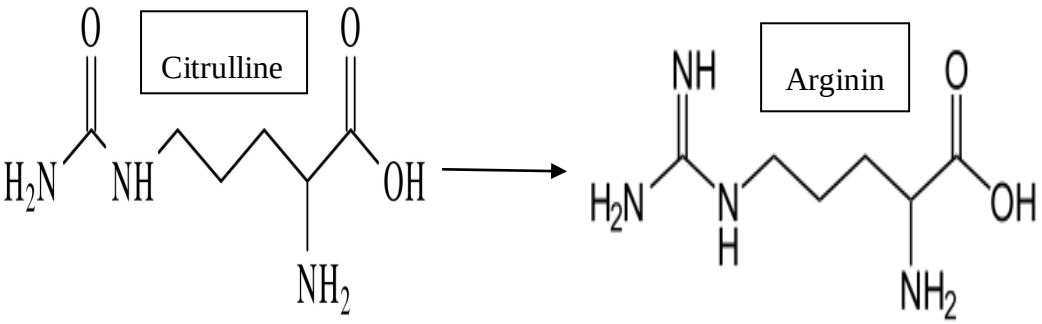



Fig 10: Conversion of Citrulline to Arginine

Specially if a human body can not make enough arginine, higher levels of arginine helps to improve blood flow and thus helps our cardiovascular health.[24] It improves the blood flow through relaxation of blood vessels or causes vasodilation and thus lowers blood pressure. High amount of citrulline content also improves erectile dysfunction. There are certain preliminary evidence from

animal studies that greater conversion of citrulline into arginine results in the formation of polyarginine peptides. It helps to prevent excess accumulation of fat in adipose tissues by blocking the activity of an enzyme called tissue-nonspecific alkaline phosphatase, or TNAP. When TNAP activity is inhibited, then adipocytes increase less fat.[26]

- Cucurbitacin E is an unique anti-inflammatory agent. This phytonutrient is also called a tripterpenoid which is found in watermelon. Cucurbitacin E has been shown to block activity of cyclo-oxygenase enzymes and neutralize reactive nitrogen-containing molecules. This anti-inflammatory agent does not neutralize activity of reactive oxygen species (ROS) but neutralizes activity of reactive nitrogen species (RNS) .[27]

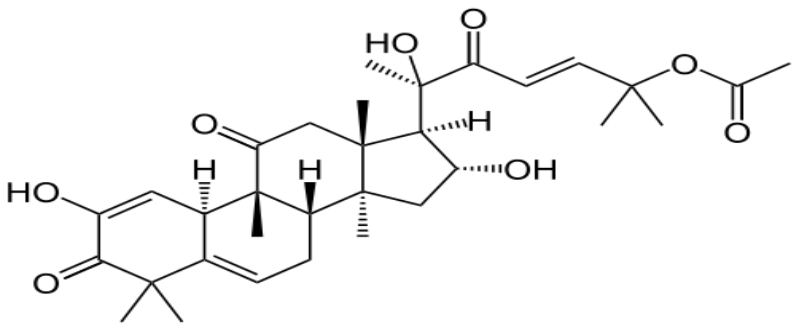



Fig 11: Structure of Cucurbitacin E

- Juice of fruit is used as antiseptic in typhus fever.
- The part eaten is the mature fruit with high nutritional value and rich in Vitamin B1, B2, A, C, beta-carotene, biotin and minerals: S, Mg and also calcium, iron.
- Vitamin C and beta- carotene are usefull antioxidents and watermelon is a good source of them.

- The fruit is used for medicinal purposes like kidneys and urinary tract cleansing.
- In traditional Chinese medicine, it is used to relieve scanty urination, excessive thirst, for treating hepatitis and urinary tract infections.[13]
- Pulp is used as a drastic purgative.[13]

### 3.2.4 The rind:

- The rind contains impressive concentrations of most nutrients like phenolic antioxidants, flavonoids and lycopene.
- In China, rind of the fruit is powdered after drying and after incineration is used for aphthous mouth sores.[13]

# Chapter 4

## Identification and preparation of sample extract

## Section 1- Identification and collection of *Citrullus lanatus* (watermelon rind)

### 4.1.1 *Citrullus lanatus(*watermelon rind) collection:

Watermelons are available through out the country during the summer season. The watermelons were collected from Pullpar, Mymensingh, Bangladesh. 10kg of the rind of water melon *(Citrullus lanatus)* was collected from the fruits during June, 2013.

### 4.1.2 *Citrullus lanatus* (watermelon rind) Identification:

It is advisable to attempt field identification of sample collected. To aid taxonomoic experts in confirming the field identification of sample collected. To aid taxonomic experts in confirming the field identification and to get a permanent scientific record (accession number) a voucher specimen was prepared on 03 November, 2013. In the voucher specimen the dried thick skin with the rind of watermelon fruit and dried seeds were attached and some information like local name, medicinal use, location of the sample plant were also written on that voucher specimen. Finally, from BNH (Bangladesh National Herbarium) I got the identification or accession number of collected sample on 03 November, 2013, and the accession number is 38639 with *Citrullus lanatus* and Cucurbitaceae scientific name and family name of the plant respectively.

### 4.1.3 *Citrullus lanatus* (watermelon rind) preservation

- The rinds were washed with fresh water sothat any dust particles are not attached to the sample.
- Then the rinds were packed and separate packets were made each of 1 kg. All the samples were preserved in deep fridge in $-18^0$ c.

## Section 2- Processing of samples

### 4.2.1 Peeling and maceration of rinds

1.The outer most skin of the exocarp of samples were peeled with a peeler.

2.The rinds were pieced into small pieces.

Fig 12: pieces of the collected rind of *Citrullus lanatus* and maceration with blender

3.Then the rind pieces were macerated with a blender.

4.The liquids were collected in a beaker. It is the crude extract.

### 4.2.2 Filtration of the collected liquid

After the maceration process the liquid was filtered with sterilized cotton filter. The cotton was folded four times and fitted in a funnel. The filtrate was collected in a beaker. Then again it was filtered and this time Whatman's filter (Double Rings Filter Paper, Source- China) was used for getting more clear extract.


Fig 13: Whatman's filter paper

## Section 3- Preparation of Diethyl ether, Chloroform and Hexane extracts

### 4.3.1 Principle of liquid-liquid extraction

Liquid–liquid extraction is also known as solvent extraction and partitioning. It is a method to separate compounds from two different immiscible liquids based on their relative solubilities. The liquids are usually water and an organic solvent. It is an extraction of a substance from one liquid into another liquid phase. Liquid–liquid extraction is a basic technique in chemical laboratories, where it is performed using a separatory funnel.[28]

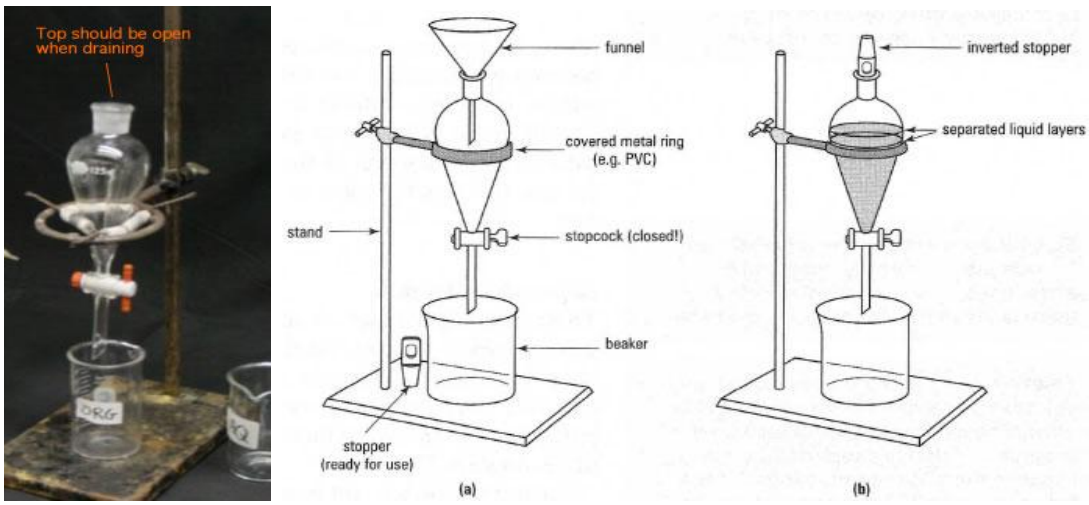



Fig 14: Separatory funnel (a) ready to use and (b) in use.

The term partitioning is the chemical and physical processes that is involved in liquid–liquid extraction. A popular aphorism used for solubility is "like dissolves like". This phrase indicates that a substant will dissolve best in a solvent which has chemical structures that is similar to the solvent. The solvation power of a solvent depends primarily on its polarity. For example, urea which is a very polar (hydrophilic) solute is very soluble in water which is highly polar, less soluble in methanol which is fairly polar, and practically insoluble in solvents such as benzene which is non-polar. In

contrast, a non-polar or lipophilic solute such as naphthalene is insoluble in water, fairly soluble in methanol, and highly soluble in non-polar benzene. The solubility of a substance fundamentally depends on the physical and chemical properties of the solute and solvent as well as on temperature, pressure and the pH of the solution.[28]

### 4.3.2 Preparation of Diethyl ether extract

- 760 ml of crude rind extract was taken in a 1000 ml beaker.
- 300 ml of Diethyl ether (Diethyl ether, source- Germany) was added to the beaker.
- Another 1000 ml beaker was used for vigorous mixing by pouring from one beaker to another for about 30 minutes under the laminar flow hood.
- A separation funnel was set in a stand and some mixture was poured. After pouring the mixture into the separating funnel, the funnel was inverted back and forth ten times stopping every three times to allow gas to escape.
- It was kept stand still for 5 minutes for separation to occur. The organic layer in the funnel was released into a 2$^{nd}$ beaker.
- The process of separation was repeated untill all the mixture was separated. Each time the mixture was shaked well prior to pour into the funnel. Each time the organic layer was released into the 2$^{nd}$ beaker and collected.
- The beaker was labelled properly where sample extract was collected.

### 4.3.3 Preparation of Chloroform extract

- 800 ml of crude rind extract was taken in a 1000 ml beaker.
- 300 ml of Chloroform (EMSURE $^{R}$, source- Germany) was added to the beaker.
- Another 1000 ml beaker was used for vigorous mixing by pouring from one beaker to another for about 30 minutes under the laminar flow hood.

- A separation funnel was set in a stand and some mixture was poured. After pouring the mixture into the separating funnel, the funnel was inverted back and forth ten times stopping every three times to allow gas to escape.
- It was kept stand still for 5 minutes for separation to occur. The organic layer in the funnel was released into a 2nd beaker.
- The process of separation was repeated untill all the mixture was separated. Each time the mixture was shaked well prior to pour into the funnel. Each time the organic layer was released into the 2nd beaker and collected.
- The beaker was labelled properly where sample extract was collected.

### 4.3.4 Preparation of n-Hexane extract

- 770 ml of crude rind extract was taken in a 1000 ml beaker.
- 300 ml of n-Hexane (n-Hexane, source- Germany) was added to the beaker.
- Another 1000 ml beaker was used for vigorous mixing by pouring from one beaker to another for about 30 minutes under the laminar flow hood.
- A separation funnel was set in a stand and some mixture was poured. After pouring the mixture into the separating funnel, the funnel was inverted back and forth ten times stopping every three times to allow gas to escape.
- It was kept stand still for 5 minutes for separation to occur. The organic layer in the funnel was released into a 2nd beaker.
- The process of separation was repeated untill all the mixture was separated. Each time the mixture was shaked well prior to pour into the funnel. Each time the organic layer was released into the 2nd beaker and collected.
- The beaker was labelled properly where sample extract was collected.

## Section 4- Extraction of caffeine

### 4.4.1 Principle of caffeine extraction:

Extraction is a method used for the separation of organic compound from a mixture of compound. This technique selectively dissolves one or more compounds into an appropriate solvent. The solution of these dissolved compounds is referred to as the extract. Caffeine is extracted by using a polar-non polar solvent extraction method. In the case of Caffeine extraction, the solubility of caffeine in water is 22mg/ml at 25°C, 180mg/ml at 80°C, and 670mg/ml at 100°C. Here the organic solvent chloroform is used to extract caffeine from aqueous extract of tea powder because caffeine is more soluble in chloroform (140mg/ml) than it is in water (22mg/ml).The chloroform- caffeine mixture can then be separated on the basis of the different densities of chloroform and water because chloroform is much denser than water and insoluble in it. Residual water is separated from chloroform by drain out the dichloromethane through separating funnel, thus chloroform passed through the funnel while polar solvents such as water is still remains in the funnel. Water and dichloromethane is slightly soluble in each other. So, after separating the solvents, residual water will remain in the organic layer. Sodium hydroxide is used for the removal of water from organic layer. [29]

### 4.4.2 Procedure of isolation of caffeine:

- 700 ml of crude extract was measured and added with 300 ml of chloroform was added in a 1000 ml of glass beaker.

- Under the laminar flow hood the crude extact and the chloroform was mixed by pouring from one beaker to another beaker. Both the beakers were of 1000 ml of volume. The mixing was continued for half an hour.

- A separating funnel on a stand was used for separation. After pouring the mixture into the separating funnel, the funnel was inverted back and forth ten times stopping every three times to allow gas to escape.

- It was kept stand still for 5 minutes for separation to occur. The organic layer in the funnel was released into a $2^{nd}$ beaker.

- The process of separation was repeated untill all the mixture was separated. Each time the mixture was shaked well prior to pour into the funnel. Each time the organic layer was released into the $2^{nd}$ beaker and collected.

- The contents of the separating funnel was discarded and the contents of the $2^{nd}$ beaker were placed into the separating funnel.

- Two washings with 20 ml 6M NaOH were done, followed by one washing with 20 ml NaOH

- During washing with NaOH the organic layer separated with a yellowish color and a middle layer of white foam was formed.Both layers were collected separately.

- The organic layer was the poured into a pre-weighed beaker and evaporated on hot plate at 55ºc-60ºc.

- When all the liquid the liquid had evaporated, the beaker was massed again and mass of caffeine was obtained (2.35gm).

- The extract was then stored at 4ºc for further use.

## Section 5- Isolation of Catechin

### 4.5.1 Principle of Catechin isolation

Catechin is a flavan-3-ol. It is a type of natural phenol and it has antioxidant properties. It is a plant secondary metabolite. It belongs to the chemical family of flavonoids. From the literature survey it is proved that the catechin is a biologically important polyphenolic compound. For this reason to isolate it from natural origins for the benefit of human being will be a great success. The conventional method for the isolation of catechin from catechu is labourious, costly and low yielding (14.2 - 17.2%).[30]

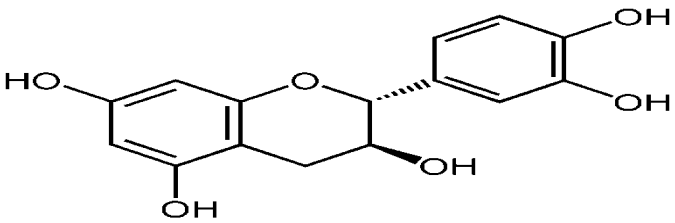



Fig 15: Structure of Catechin

Phenolic compounds classified as phenolic acids and their derivatives, are most numerous and widely distributed groups of natural products in the plant kingdom. In the recent years, phenolic compounds have received growing interest in many edible plant products due to their influence on nutritional, biochemical and physiological functions as well as pharmacological actions. inhibiting reverse transcriptase of human immunodeficiency virus (HIV) by catechins.[31] Phenolic compounds were extracted from beach pea (Lathyrus maritimus L.) seeds using methanol-water, ethanol-water and acetone-water solvent system. Both (+)-Catechin and (-)-Epicatechin were identified in the extraction solution. Acetone-water system extracted considerably higher amounts of phenolic compounds and condensed tannins than the ethanol-water or methanol-water systems.[32]

**4.5.2 Procedure of isolation of catechin:**

- A separation funnel was set in a stand and 300 ml of crude rind extract was taken in a separating funnel.
- 100 ml of dichloromethane (DCM) was added in the funnel.
- After pouring the DCM into the separating funnel, the funnel was inverted back and forth to mix the sample liquid and the DCM ten times stopping every three times to allow gas to escape.
- It was kept stand still for 5 minutes for separation to occur. The organic layer in the funnel was released into a $2^{nd}$ beaker.
- Then in the upper aqous layer 100 ml of acetone was added.
- After that the funnel was inverted back and forth to mix the sample liquid and the acetone for ten times stopping every three times to allow gas to escape.
- It was kept stand still for 25 minutes for separation to occur. The organic layer in the funnel was released into a $3^{rd}$ beaker which is the lower layer.
- The aquous layer was also collected in a $4^{th}$ beaker and it is the upper layer.
- The process was repeated for another 300 ml.

# Chapter 5

# MATERIALS AND METHOD

## Section 1-Thin Layer Chromatography

### 5.1.1 Thin Layer Chromatography:

Chromatography is the separation of two or more compounds or ions by the distribution between two phases, one which is moving and the other which is stationary. These two phases can be solid-liquid, liquid-liquid or gas-liquid.

Thin-layer chromatography or TLC, is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC is a quick, inexpensive microscale technique that can be used to:

- determine the number of components in a mixture
- verify a substance's identity
- monitor the progress of a reaction
- determine appropriate conditions for column chromatography
- analyze the fractions obtained from column chromatography

### 5.1.2 Principle of Thin Layer Chromatography:

In paper chromatography, the stationary phase is a specially manufactured porous paper. The samples are added to one end of the sheet of paper and dipped into the liquid or mobile phase. The solvent is drawn through the paper by capillary action and the molecules are distributed by partition between the mobile and stationary phase. The partition coefficient, k, similar to the distribution coefficient for extraction, is the equilibrium constant for the distribution of molecules between the mobile phase and the stationary phase. It is this equilibrium that separates the components. Different inks and dyes, depending on their molecular structures and interactions with the paper and mobile phase, will adhere to the paper more or less than the other compounds allowing a quick and efficient separation.[33]

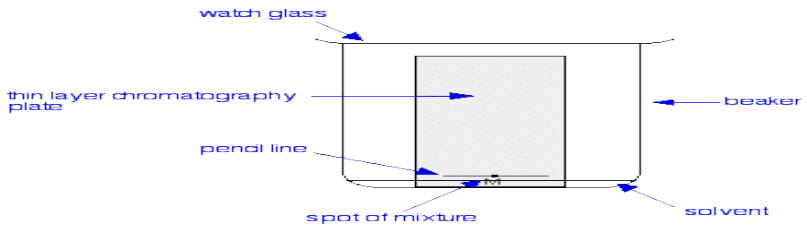



Fig 16: Thin layer chromatography instruments

TLC works on the same principles. In thin-layer chromatography, the stationary phase is a polar absorbent, usually finely ground alumina or silica particles. This absorbent is coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase. Almost all mixtures of solvents can be used as the mobile phase. By manipulating the mobile phase, organic compounds can be separated.[33]

### 5.1.3 Solvent Systems of Thin Layer Chromatography

The extracts of the plant were analyzed by Thin Layer Chromatography process to trace the elements present in the sample. Three solvent systems are there for perfoming TLC. Among them two of the solvent systems were used. The polarity of the solvent systems were increased gradually . It helps the compounds of various polarity to elute according to the solvent system. Nonpolar solvent system is used to help run the nonpolar compounds like benzene. Again Intermediate polar solvent and polar solvent were used to separate polar compounds. There are some common basic compounds like Alkaloid present in nature. So basic solvent is used to help them run on TLC plate.[34]

**Table 6**: Solvent Systems of TLC

| Nonpolar basic solvent | Intermediate polar solvent | Polar Basic solvent |
|---|---|---|
| Benzene 9 ml | Chloroform 5 ml | Ethyl acetate 8 ml |
| Ethanol 1 ml | Ethyl acetate 4 ml | Ethanol 1.2 ml |
| Ammonium hydroxide 0.1 ml | Formic acid 1 ml | Water 0.8 ml |

## 5.1.4 Apparatus used for Thin Layer Chromatography

**Table 7** : List of apparatus

| | |
|---|---|
| 1. TLC tank | 2. Watch glass |
| 3. TLC plate | 4. Spray bottle |
| 5. Pencil and ruller | 6. Hot plate |
| 7. Scale | 8. Uv lamp |
| 9. Pipette | 10. Capillary tube |
| 11. Pumper | 12. Twizzer |


Fig 17: TLC tank (1), TLC Plate (2), Spray bottle (3), Hot plate (4)

## 5.1.5 Chemicals Needed for Thin Layer Chromatography:

**Table 8**: List of chemicals for TLC

| |
|---|
| • Caffeine extracted from *Citrullus lanatus(*watermelon) rind |
| • Caffeine standard |
| • TLC solvent: (solvent system-2: intermediate polar) |
| - Chloroform: Ethyl acetate: Formic acid (5:4:1) |

**5.1.6 Procedure for Thin Layer Chromatography:**

- A spatula tip amount of extracted caffeine was put in a small, labeled test tube. 2.0 ml of $CHCl_3$ was added to dissolve.
- 3 TLC plate were obtained. The plates were marked as plate-1, plate-2 and plate-3. A pencil was used to lightly mark a straight line about 0.5 cm from both end of the plates.
- With a capillary micropipette a small spot of the Caffeine Standard was spotted on the plates. With another capillary micropipette a spot with the Extracted Caffeine from *S. indicum* seed was spotted carefully. To avoid confusion, the spots were lightly labeled below the pencil line.
- The TLC plates were developed by placing them in the TLC chamber that has been filled with the developing solvent. The solvent was allowed to migrate up the TLC plates until it reached the marked top end line.
- The plates were then removed immediately and the solvent was allowed to evaporate. After that it was visualized under UV light and all the spots were marked.
- The distance that the solvent moved was measured for the distances of all spots.

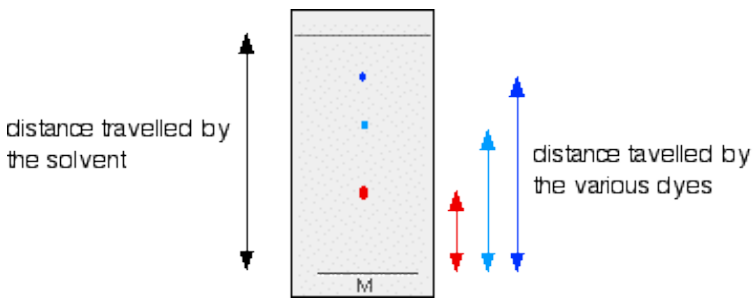



Fig18: Distance of spot for Rf value

- The $R_f$ value was then calculated. It can be calculated as:

$R_f$ = Distance spot travels / Distance solvent travels

**5.1.7 Charing with $H_2SO_4$:** Then charring of plate-1 with 10% $H_2SO_4$ solution was carried out for further confirmation.

**5.1.8 Staining with DPPH (2,2-diphenyl-1-picrylhydrazyl):** 0.4% DPPH solution was prepared with methanol as solvent and labeled as stock solution. From the stock solution 10% DPPH solution was taken in a petri dish and the TLC plate-2 was dipped in it. The plate was visually observed for a color change after sometime. All the processes were carried out in dark place. The antioxidant active regions became yellow in color.

**5.1.9 Staining with FC (Folin-Ciocalteu) reagent:** Staining with FC reagent was carried out as the same procedure above with plate-3. 10% FC solution was prepared with water as the solvent. The antioxidant active regions became yellow/ white in color.

## Section 2-Chemical analysis by UV spectroscopy

### 5.2.1 Principle of UV spectroscopy:

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and near-UV and near-infrared (NIR) ranges.

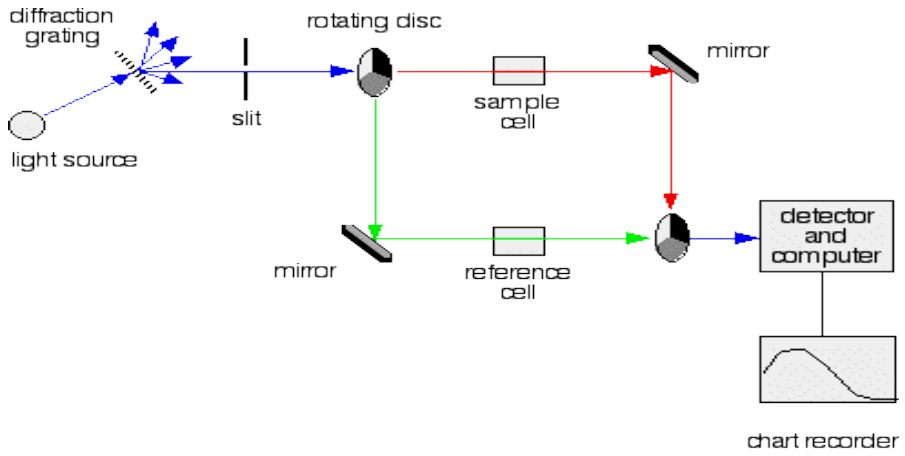



Fig 19: Instrumentation of a UV-Visible spectrometer

Each monochromatic (single wavelength) beam in turn is split into two equal intensity beams by a half-mirrored device. One beam, the sample beam (colored magenta), passes through a small transparent container (cuvette) containing a solution of the compound being studied in a transparent solvent. The other beam, the reference (colored blue), passes through an identical cuvette containing only the solvent. The intensities of these light beams are then measured by electronic detectors and compared. The intensity of the reference beam, which should have suffered little or no light absorption, is defined as $\mathbf{I_0}$. The intensity of the sample beam is defined as **I**. Over a short period of time, the spectrometer automatically scans all the component wavelengths in the manner

described. The ultraviolet (UV) region scanned is normally from 200 to 400 nm, and the visible portion is from 400 to 800 nm.[35] The method is most often used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

$$A = \log_{10}(I_0/I) = \epsilon \cdot c \cdot L,$$

where A is the measured absorbance, in Absorbance Units (AU),

Io is the intensity of the incident light at a given wavelength,

I is the transmitted intensity,

L the pathlength through the sample, and

c the concentration of the absorbing species.

For each species and wavelength, ε is a constant known as the molar absorptivity or extinction coefficient.

**5.2.2 Procedure of UV spectroscopy:**

To find λ max, the wavelength was set on the Lambda UV spectrometer (Shimadzu, Japan) to 400 nm and the maximum and zero absorbance was calibrated using the reference solution (Pure caffeine).The sample solution was inserted and its absorbance at this wavelength was recorded. Then wavelength of 400 to 200 nm was passed. The wavelength at which the absorbance was maximum was recorded. A plot of absorbance versus wavelength shows λ max.

## Section 3- Determination of caffeine in *C. lanatus* by UV-VISIBLE spectroscopy:

### 5. 3.1 Principle

Ultraviolet-visible spectroscopy, or UV-vis, is a method of detection using visible light and its adjacent light wavelengths. A UV-vis spectrometer is an instrument that uses a light source to pass through a sample chamber and detects metal ions and organic compounds. The UV-vis spectrometer is a powerful tool used in many laboratories.

A light source shutter controls the amount of light from a specialized lamp that passes through the sample. The shutter is the only moving component of a UV-vis spectrometer. The advantage of this system lies in the simplistic design of the instrument. However, sample analysis using UV-vis is a very quick process compared to other methods of sample detection, such as HPLC. This rapid analysis is achieved only through proper calibration. The UV-vis technique is non-destructive to the sample and has a high sensitivity for detecting organic compound.

### 5.3.2 Preparation of standard stock solution of Pure caffeine:

Pure caffeine (1.563mg) was accurately weighed and transferred to a test tube containing 5 ml of distilled water. It was boiled at 100 ºC to make a clear caffeine solution of concentration of 312.5 µg/ml. This solution was used as working standard solution. From this solution, by suitably dilution, (156.25,78,39,19.5,9.75) µg/ml concentrations were obtained respectively and used as standard solution.

### 5.3.3 Measurement of absorbance and calibration curve:

The absorbance of the solutions containing pure caffeine at (5-25) µg/ml concentrations are determined in the UV range 260 nm using an appropriate blank. From the standard curve the concentration of caffeine in *C. lanatus* sample were measured.

## Section 4- Determination of antioxident property by DPPH test

### 5.4.1 Principle of DPPH Test (1, 1-diphenyl-2-picrylhydrazyl radical)

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.[36]

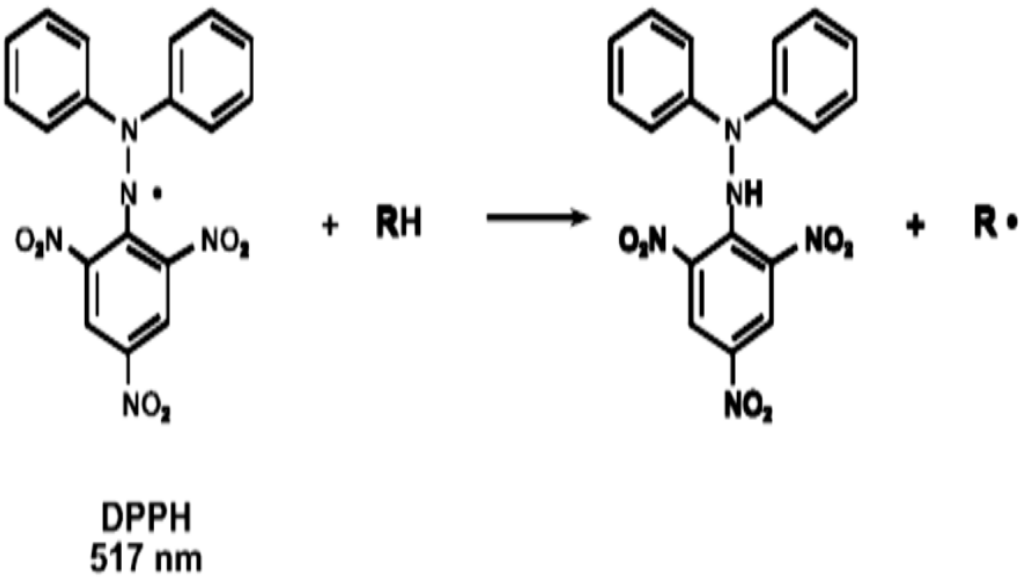



Fig 20: 1, 1-diphenyl-2-picrylhydrazyl radical

**5.4.2 Procedure**

a) The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. Different concentrations of the plant extract (2, 4, 6, 8 & 10 µg/ml, in methanol) were added at an equal volume (10ml) to methanol solution of DPPH (400 µg /ml, 100 µg/m l).

b) Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant.

c) After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the equation, DPPH antiradical scavenging capacity (%) = [1 – (Ab. of sample – Ab. of blank)/Ab. of control] × 100.

d) Different concentrations of plant extract solution was used as a blank, while DPPH solution plus methanol was used as a control .[37]

e) The IC50 values were calculated by the sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity. IC50values denote the concentration of the sample required to scavenge 50% of DPPH radicals.[37]

**5.4.3 Standard Preparation:**

1) 250 mg Ceevit Tablet (Square) was crushed in mortar and pestle.

2) 20 ml distilled water was added and the solution was filtered.

3) It was then diluted by 10 times (2 ml of the filtered solution was taken and 18 ml water added).

4) The solution was taken in 5 test tubes to prepare 5 different concentrations.

5) 1ml, 2ml, 2ml, 4ml and 5ml solution were taken in 5 different test tubes and the volume adjusted to 5 ml with water in all the test tubes.

**5.4.4 Sample Preparation:**

1) 10gm of sample from catechin isolated sample was taken in beaker and water added upto 50 ml. It is the stock solution.

2) Then it was filtered and extract sample was collected.

3) The solutions were taken in 5 test tubes to prepare 5 different concentrations.

4) 1ml, 2ml, 3ml, 4ml, 5ml solution were taken in 5 different test tubes and the volume adjusted to 5 ml with water in all the test tubes.

**5.4.5 Blank Preparation:**

Blank was prepared by adding 5 ml water in a test tube. In all the 16 test tubes 100 μL DPPH solution was added in dark and left for 30 minutes. After that UV absorbance was measured in UV machine at 517 nm.

## Section 5- Determination of total phenolic content

The total phenolic content of isolated caffeine and pure caffeine was determined using Folin-Ciocalteu method using salicylic acid as standard.

### 5.5.1 Principle of total phenolic content

The Folin–Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric in vitro assay of phenolic and polyphenolic antioxidants. The reagent does not only measure phenols, and will react with any reducing substance. It therefore measures the total reducing capacity of a sample, not just phenolic compounds.[38]

### 5.5.2 Procedure for determining total phenolic content

- 1 mg of isolated caffeine and pure caffeine was dissolved in 100µl of chloroform (100µg/µl). Different concentrations of standard salicylic acid were also prepared in test tubes.
- 5 ml of FC reagent diluted up to 10 fold and 5 ml sodium carbonate were added in the test tubes.The test tubes were incubated for 30 minutes in dark place to complete the reaction.
- Then the absorbances of the solutions were measured at 765 nm using a UV spectrometer against blank. A calibration curve of salicylic acid was prepared.
- The total phenolic content was calculated as salicylic acid equivalentby the following equation: T = C xV /M .Where, T is the total phenolic content in $mg \cdot g^{-1}$ of the extracts as SAE, C is the concentration of salicylic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract in g.

## Section 6- Antimicrobial assay

### 5.6.1 Principle of Disc diffusion method:

When a filter paper disc impregnated with a chemical is placed on agar the chemical will diffuse from the disc into the agar. This diffusion will place the chemical in the agar only around the disc. The solubility of the chemical and its molecular size will determine the size of the area of chemical infiltration around the disc. If an organism is placed on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a "zone of inhibition".

### 5.6.2 Procedure of Disc diffusion method:

- Two different bacterial strains of gram positive, two different strains of gram negative bacteria and two different strains of fungi were used to carry out this assay.
- Nutrient agar was used as the culture media. Stocks of these bacterial solutions were revived in nutrient agar by incubating at $37^0$C for 24 hrs.
- A single disk diffusion method was used to assess the presence of antimicrobial activities of the isolated caffeine and pure caffeine.
- Whatman's filter paper was punched, and 6 mm disks were collected in a beaker. The beaker with petri dishes, forceps, tips, cotton bar were covered with foil paper and autoclaved.
- 10 µl of isolated caffeine (600µg/µl) and pure caffeine (1000µg/µl) were loaded per disc with the help of micropippete.

Fig 21: Micropippete, pippete tube

- The revived test organisms were spread onto nutrient agar plates by cotton bar.
- The disc were then placed all plates. Standard disc of Vancomycin (30 µg/disc) was used as positive control.

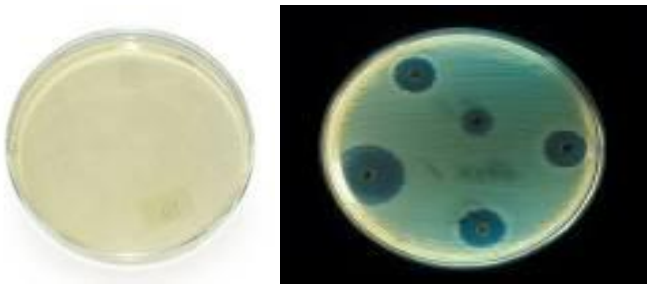


Fig 22: Agar media and disc placed in plate.

- After incubation at $37^0$C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

Fig 23: Incubator of microbiology lab and laminar air flow.

## Section 7- Brine shrimp lethality test

### 5.7.1 Principle of Brine shrimp lethality test

The brine shrimp (Artemia salina Leach) is a simple zoologic organism (an arthropod). The use of brine shrimp test (BST) as a tool to measure general bioactivity in plant extracts was initiated in 1982 and then modified in 1991 as a simple, rapid, in-house, bench-top, and low cost prescreen for cytotoxicity and pesticidal activities. The brine shrimp bioassay has been implemented as a test for the last 20 years and has led to the discovery of the cytotoxic effects of a wide range of plants and bioactive compounds so diverse in their chemical structure. This method is now widely used all over the world with a great success.

### 5.7.2 Procedure of Brine shrimp lethality test:

5.7.2.1 Collection of cysts: The eggs were purchased.

Fig 24: Brine shrimp cyst, Culture medium, Nauplii

5.7.2.2 Culture Medium: The artificial seawater (ASW) was used in our study. Although natural seawater (about 38 g of salts per liter) is the medium of choice, it has been shown the hatching rate and the viability of the nauplii in natural seawater (NSW) are similar to those in the artificial seawater (ASW).

5.7.2.3 Hatching the shrimp: Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial seawater. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened (covered side of the divided tank), while the smaller compartment was illuminated. After 48 h the phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from their shells. Lamp positioned above the uncovered side attracts hatched shrimp. We allowed 2 days (48h) for the eggs to hatch and mature as nauplii. The lamp provides direct light and warmth (about 25° C) throughout embryogenesis.

Fig 25: Hatching of Brine shrimp eggs under light and nauplii after 48 hour.

**5.7.3 Preparation of test samples for isolated caffeine:**

Samples were prepared by dissolving 20 mg of the isolated caffeine in 2 mL of a hot water.

Standard was prepared by dissolving 20 mg pure caffeine in 2 mL of a hot water. Dilution of this stock solution gives the series of concentrations required for testing.

**5.7.4 Preparation of test samples for isolated catechin:**

Samples were prepared both for upper and lower layers of the acetone treatment.

Both the samples were prepared by dissolving 20 mg of the isolated catechin in weight balance in 2 mL of distilled water. The concentration was 10 mg/ml. Dilution of this stock solution gives the series of concentrations required for testing.

**5.7.5 Bioassay:**

6 vials for each sample were taken. The no.6 vial was used as control (nauplii without sample). To each sample vial ten shrimps were transferred using a Pasteur pipette, and artificial seawater (brine) was added to make a total volume of 5 mL. The nauplii were counted against a lighted background. Counting for the chronic LC 50 began 24 hour after initiation of tests. Nauplii were considered dead if they were lying immobile at the bottom of the vials and the percentage of deaths at each dose and at the control were determined.

Fig 26: Pasteur pipette

## Section 8 –Name of the Reagents/ Instrument used with their Brand name and Source

**Table 9 :** Name of the Reagents/ Instrument used with their Brand name and Source

| Name of Chemical or Instrument | Brand Name | Source of Reagent or Instrument |
|---|---|---|
| Formic acid | Unichem | China |
| Dichloromethane (DCM) | G.R-GM4DE | Bangladesh |
| Chloroform | Emsure | Germany |
| Diethyl ether | Diethyl ether | Germany |
| n-Hexane | n-Hexane | Germany |
| Ethyl acetate | Ethyl acetate | Germany |
| Benzene | Benzene | China |
| Laminar air flow | ESCO Laminar flow cabinet | US Fedaral Standard |
| Incubator | EHRET | Bangkok |
| Hot Air Oven | NUVE | Bangkaok |
| Autoclave machinet | HIRAYAMA, HICLAVE | Japan |
| Filter paper | Double rings filter paper | China |

# Chapter 6

# Result and discussion

## Section 1- Result of Crude sample of *Citrullus lanatus* rind

### 6.1.1 Thin Layer Chromatography (TLC) of Crude sample of *Citrullus lanatus* rind

The result obtained after TLC of the Crude sample of *Citrullus lanatus* rind is given below-

(1) (2) (3)

Fig 27: TLC plate in naked eye view (1) ,TLC plate Under UV light (2), TLC plate after charing with $H_2SO_4$ (3)

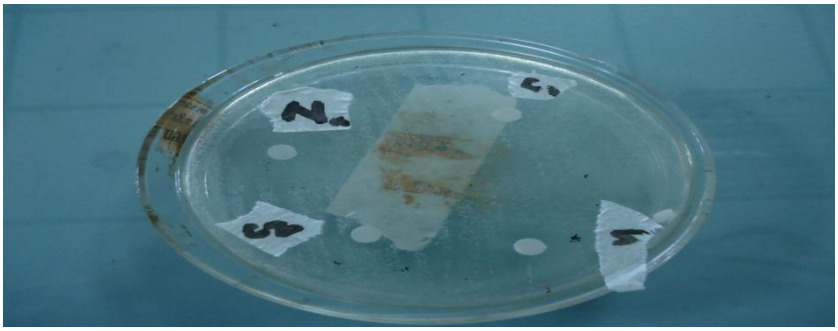
#### 6.1.1.1 Observation

1) Under UV light: TLC was done in solvent system 1 which consist of Benzene 9ml, Ethanol 1ml, Ammonium hydroxide 0.1 ml. It is nonpolar basic solvent. Under UV which is shown in the plate 2, it showed some green flouresence which indicate the presence of compound in crude extract.
2) After charing: After charing of the TLC plate with sulfuric acid was showed (plate 3). In the crude extract layer one spot was observed. No other characteristic spot was visible.

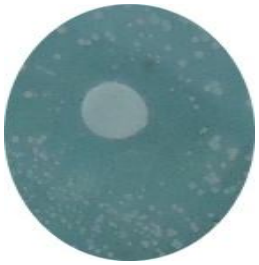
## 6.1.2 Antimicrobial assay of Crude sample

**Table 10:** Data of antimicrobial test for crude sample

| Type | Sceintific name | **Crudee extract(S),(10µl)** Zone of inhibition mm |
|---|---|---|
| Gram (+) | *Streptococcus pyrogen* | 13 |
| | *Staphylococcus aureus* | 13 |
| Gram (-) | *Salmonella typhi* | - |
| | *E.coli* | 12 |
| Fungi | *Candida albicans* | 14 |



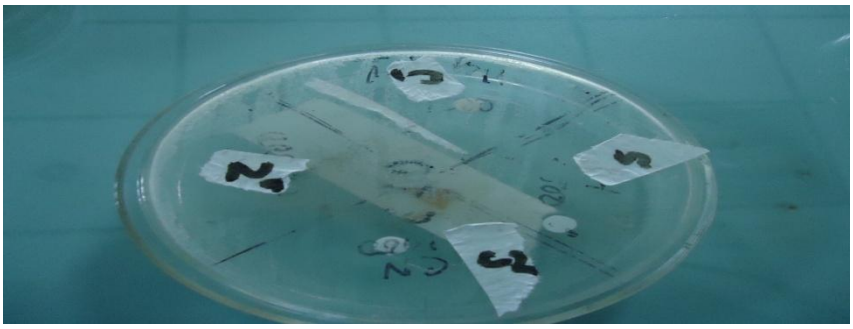
***Streptococcus pyrogen***



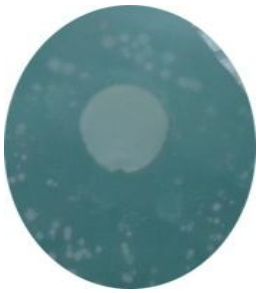
Crude Extract

***Staphylococcus aureus***

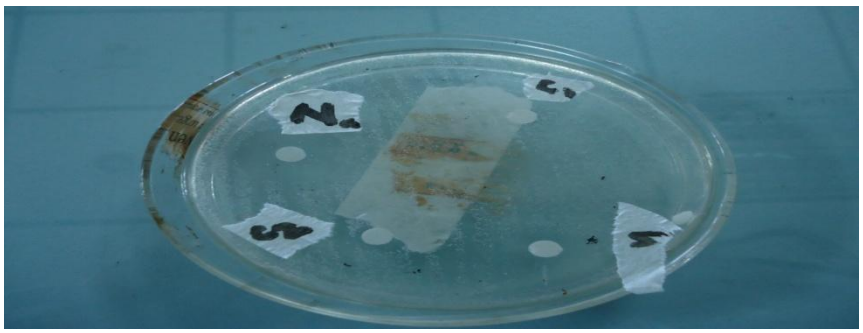
Crude Extract



***Salmonella typhi***

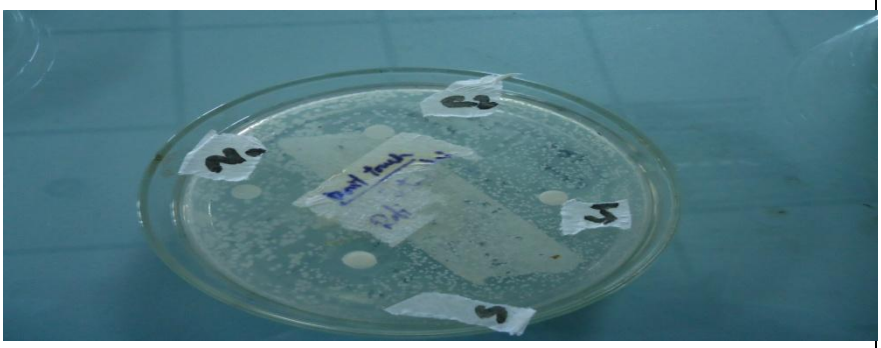


Crude Extract



***E. Coli***

CrudeExtract



***Candida albicans***

Crude Extract

Fig 28: Antimicrobial screening of Crude sample

### 6.1.3 Discussion

From the results of Thin layer chromatograpy and antimicrobial screening it can be assumed that there is presence of compound which has mild antimicrobial property. As there may be mixture of compounds, further experiments need to be done for single compound isolation.

## Section 2- Result of Chloroform, Diethyl ether and n-Hexane phases

### 6.2.1 Thin Layer Chromatography (TLC) of Liquid-liquid extraction solvents

The results obtained after TLC of the liquid-liquid extractions of the *Citrullus lanatus rind* of Chloroform, Diethyl ether and n-Hexane phases are given below-

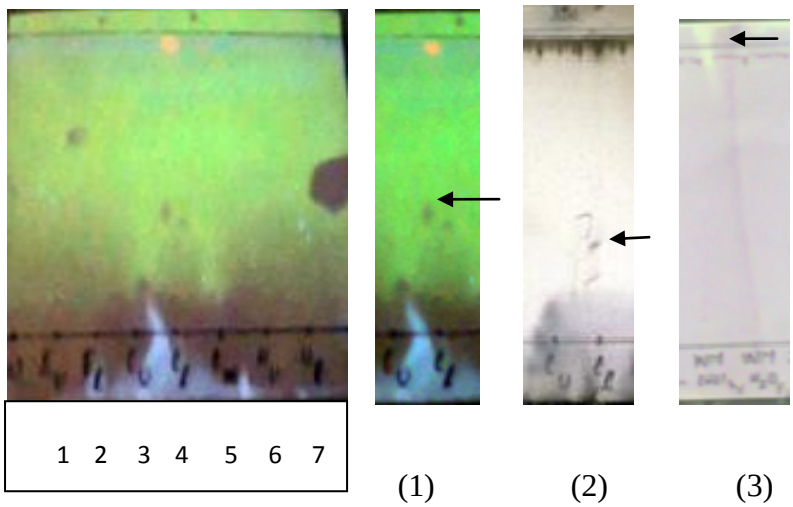



Fig 29: TLC plate under UV light (1), after charing with $H_2SO_4$ (2), after DPPH Staining(3).

Here the spots given are-

1.Ether upper

2. Ether Lower

3.Chloroform upper,

4.Chloroform lower,

5. n- Hexane upper,

6. n- Hexane lower,

7. Pure caffeine

#### 6.2.1.1 Observation

1) Under UV-light: TLC was done in solvent system 2 which consist of chloroform 5ml, ethyl acetate 4ml and formic acid 1ml. Then the plate was observed under UV light which is shown in the plate. It showed some spots which indicate the presence of compounds only in the chloroform layer of the sample.
2) After charing: After charing of the TLC plate with sulfuric acid was showed (plate 2). In the chloroform lower layer of the separation one spot was observed. No other characteristic spot was visible.
3) After DPPH staining: After DPPH staining of the TLC plate was shown in plate-3. Light yellow color at the sides and at the top were visible.

### 6.2.2 Discussion

From the above results of different solvent systems only in the chloroform layer a spot was visible under the ultra-violet light and after charing with 10% $H_2SO_4$ a brown spot was clearly visible. The compound present here may be soluble in chloroform and so the compound has dissolved from the mixture of compounds present in the crude sample of *Citrullus lanatus* rind. For this reason further experiment was done with the solvent Chloroform.

## Section 3- Result of caffeine isolation

### 6.3.1 Thin Layer Chromatography (TLC) after Caffeine isolation

The results obtained after TLC of the caffeine extractions of the *Citrullus lanatus rind* of Chloroform layer are given below-

First isolation-

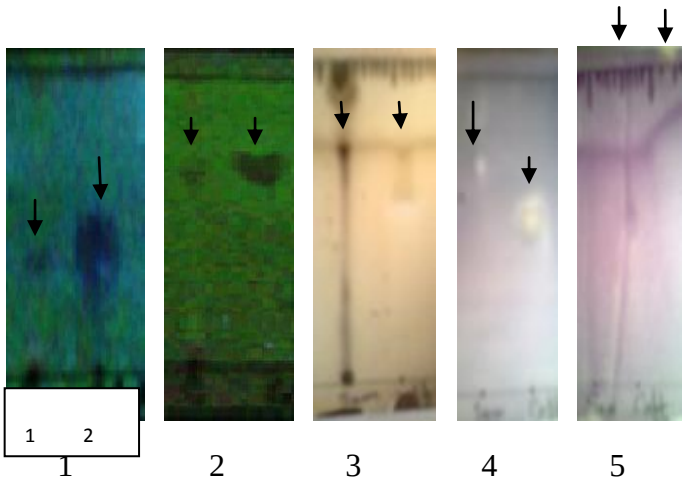
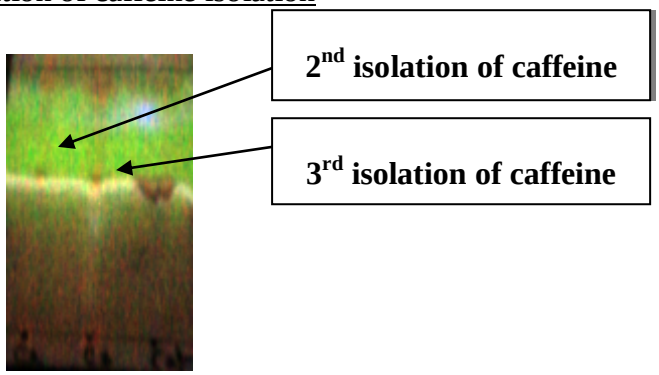



Fig 30: TLC plate under UV light for isolated caffeine -Solvent system 1 (1), Solvent system 2 (2), after Charing with $H_2SO_4$ (3), after staining with FC reagent (4), after staining with DPPH reagent(5), Here spot 1 is for isolated caffeine and spot 2 is for pure caffeine for all the plates.

$$R_f = \frac{\text{Distance spot travels}}{\text{Distance solvent travels}}$$

- Rf value for isolated sample= (2.2/3.2) cm
  - = 0.6875
- Rf value for pure caffeine = (2.2/3.2) cm
  - = 0.6875

Second and third isolation- conformation of caffeine isolation




3

Fig 26: TLC of 2nd and 3rd isolated caffeines with standard pure caffeine.

- Rf value for second isolated sample= (1.85/3.5) cm
  - = 0.528
- Rf value for third isolated sample= (1.84/3.5) cm
  - = 0.526
- Rf value for pure caffeine = (1.84/3.5) cm
  - = 0.526

**6.3.1.1 Observation**

1) Under UV-light: TLC was done in solvent system 1 and solvent system 2. In both systems a peak in the same level of the pure caffeine was visible. The peak was obtained from the chloroform- lower layer of the liquid liquid extraction of the caffeine isolation method. The compounds ran better in solvent system 2. So Rf value was measured in solvent system 2.Rf=0.6875 for both sample and standard. For second and third isolated samples the Rf value measured was 0.528 and 0.526 and standard was 0.526.
2) After charing: After charing of the TLC plate with sulfuric acid was shown in the fig 25. (plate 3). In the plate after amplying heat one clear black spot was visible with the black spot of sample and pure caffeine.

3) After FC reagent staining: After staining with FC reagent yellow spot was visible for pure caffiene and isolated sample.
4) After DPPH staining: After staining with DPPH reagent yellow spot was visible at the top of the plate.

### 6.3.2 Thin Layer Chromatography (TLC) of NaOH layer after Caffeine isolation

The results obtained after TLC of the caffeine extractions of the *Citrullus lanatus rind* of Sodium Hydroxide layer are given below-

1 2 3

Fig 31: TLC plate of NaOH in naked eye view (1) ,TLC plate Under UV light (2), TLC plate after charing with $H_2SO_4$ (3)

#### 6.3.2.1 Observation

1) Under UV-light: TLC was done in solvent system 2 which consist of chloroform 5ml, ethyl acetate 4ml and formic acid 1ml. Then the plate was observed under UV which is shown in the plate 2. It showed one spot which indicate the presence of pure caffeine used as standard.
2) After charing: After charing of the TLC plate with sulfuric acid was showed (plate 2). No characteristic spot was visible.

### 6.3.3 Determination of λ max by UV spectroscopy:

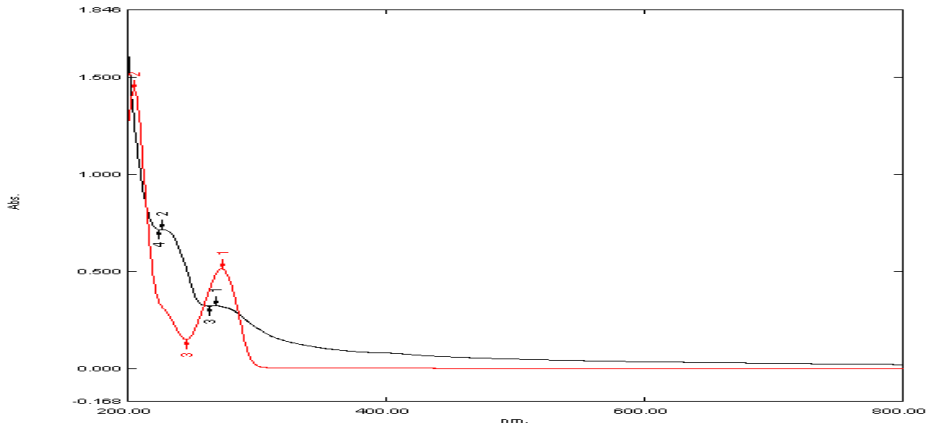



Fig 32: UV scanning of Pure and isolated caffeine

### 6.3.3.1 Observation

The above fig shows the λmax for pure caffeine (black line) and for isolated caffeine (red line). Isolated caffeine gives maximum absorbance at 273 nm which is near the pure caffeine λmax value.

### 6.3.4 Determination of caffeine concentration from standard curve:

**Table 11:** Assay values of standard solution of pure caffeine

| Serial no. | Concentration (µg/ml) | Absorbance (nm) at 260 nm |
|---|---|---|
| 01 | 156.25 | 3.72 |
| 02 | 78 | 1.98 |
| 03 | 39 | 1.23 |
| 04 | 19.5 | 0.744 |
| 05 | 9.75 | 0.434 |
| 06 | 4.87 | 0.234 |

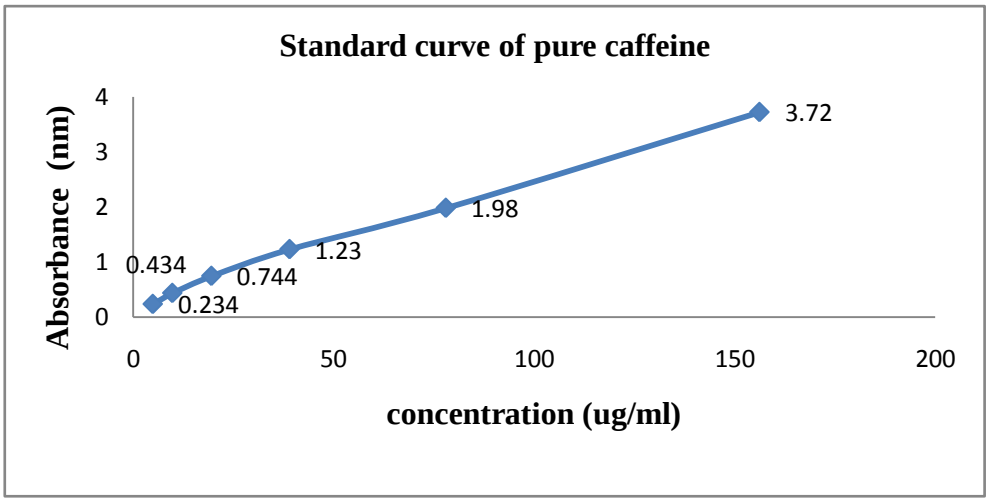



Fig 33: Standard curve of pure caffeine

**Table 12: Assay of isolated caffeine by UV absorbance method**

| Serial no. | Absorbance at 260 nm | Concentration (µg/ml) [x= y-0. 241/0.022] | Amount (mg) |
|---|---|---|---|
| **1.** | 0.349 | x= 4.9 | 0.0049 |

The amount of caffeine was measured from a standard curve. 0.025mg of the obtained extract contains caffeine 0.0049 mg.

## 6.3.5 DPPH test

### 6.3.5.1Standard (ascorbic acid)

**Table 13:**Determination of free radical scavenging capacity for the standard (ascorbic acid)

| Concentration (ug/ml) | Absorbance | % Scavenging |
|---|---|---|
| 10 | 0.086 | 77.48 |
| 20 | 0.084 | 78.01 |
| 30 | 0.082 | 78.53 |
| 40 | 0.080 | 79.05 |
| 50 | 0.075 | 80.36 |
| Controll (Blank) | 0.382 | 0 |

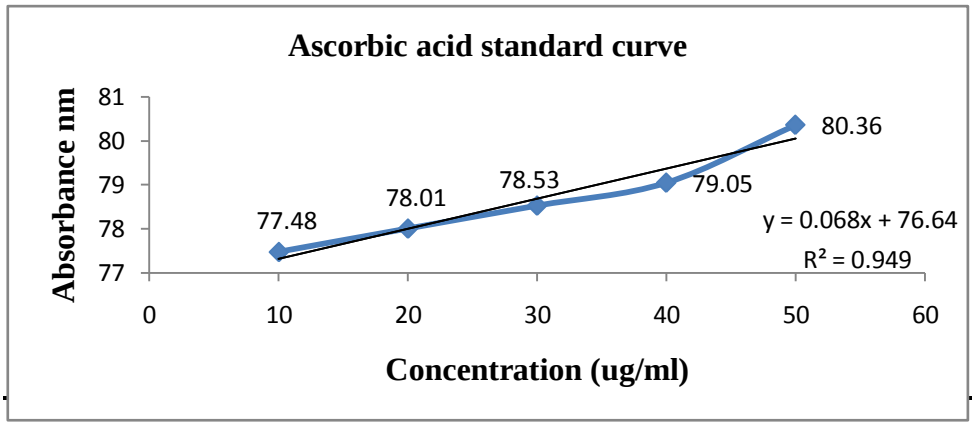



Fig 34: % Scavenging of Standard ascorbic acid

### 6.3.5.2 Determination of free radical scavenging capacity for the pure caffeine and isolated caffeine

**Table 14:** Free radical scavenging capacity for the pure caffeine and isolated caffeine

| Concentration (ug/ml) | Absorbance of pure caffeine | % Scavenging pure caffeine | Absorbance of Isolated caffeine | % Scavenging Isolated caffeine |
|---|---|---|---|---|
| 10 | 0.168 | 56.02 | 0.222 | 41.88 |
| 20 | 0.163 | 57.33 | 0.190 | 50.26 |
| 30 | 0.154 | 59.68 | 0.181 | 52.62 |
| 40 | 0.143 | 62.56 | 0.182 | 52.36 |
| 50 | 0.135 | 64.66 | 0.178 | 53.40 |
| Controll (Blank) | 0.382 | 0 | 0.382 | 0 |

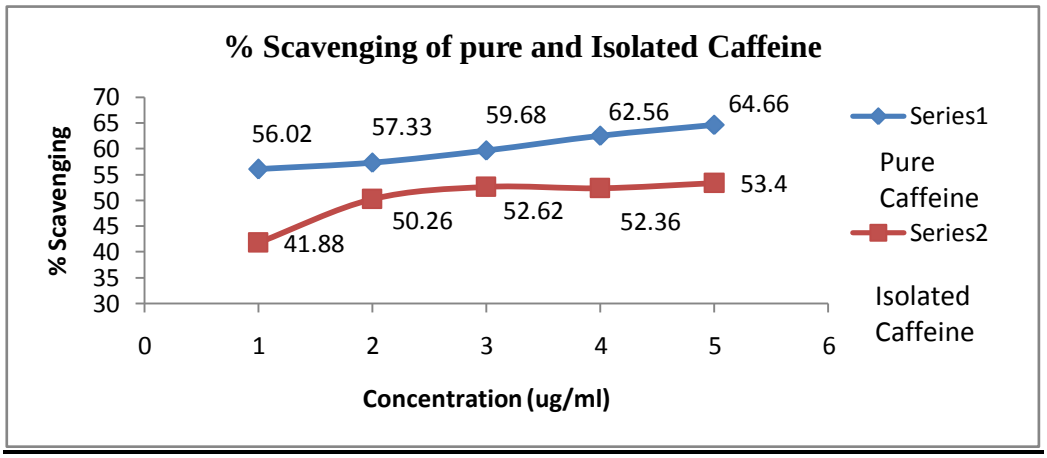



Fig 35: % Scavenging graph of pure and isolated caffeine

## 6.3.6 Determination of total phenolic content

**Table 15:** Assay values of standard solution of salicylic acid

| Serial no. | Concentration (mg/ml) | Absorbance (nm) at 765 nm |
|---|---|---|
| 01 | 5 | 0.162 |
| 02 | 2.5 | 0.135 |
| 03 | 1.25 | 0.119 |
| 04 | 0.625 | 0.112 |
| 05 | 0.3125 | 0.11 |

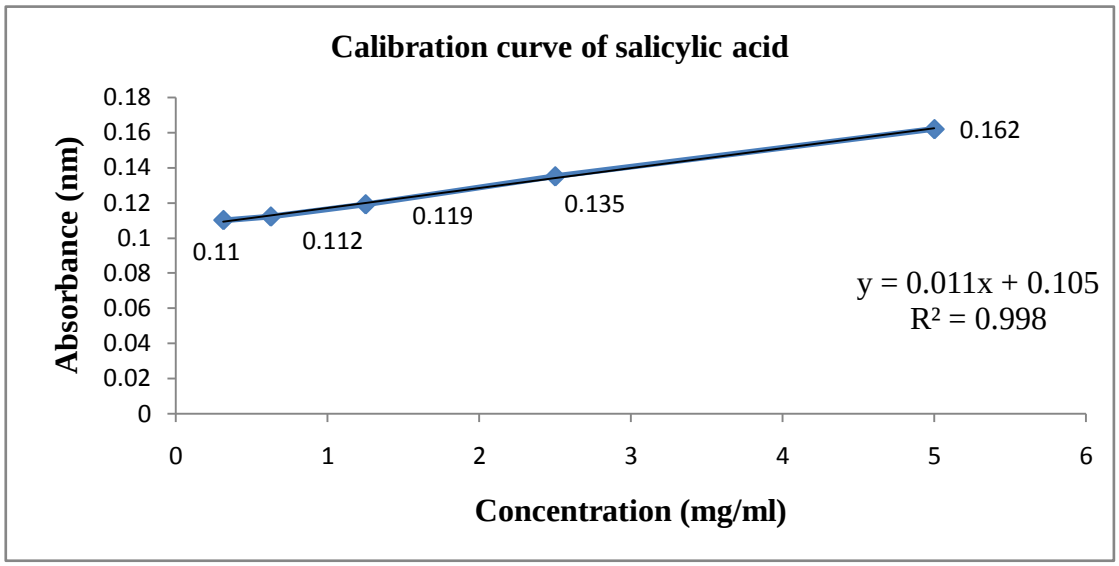



Fig 36: Standard curve of salicylic acid

**Table 16**: TPC of pure caffeine & isolated caffeine

| Sample name | Absorbance at 765 nm | Concentration (mg/ml) [x= y-0.105/0.011] | TPC (mg/g) [T= CxV/M) | Average with SD |
|---|---|---|---|---|
| **Pure** | **0.151** | **4.2** | **42** | **38.8±3.41** |

| | | | | |
|---|---|---|---|---|
| **caffeine** | **0.144** | **3.54** | **35.4** | |
| | **0.148** | **3.90** | **39** | |
| **Isolated caffeine** | **0.147** | **3.82** | **38.2** | **35.63±3.03** |
| | **0.145** | **3.6** | **36** | |
| | **0.141** | **3.27** | **32.7** | |

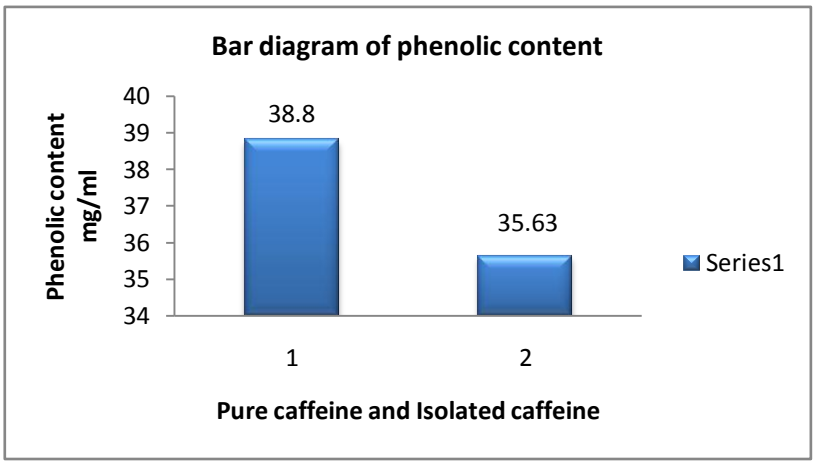



Fig 37: Graph of TPC of pure caffeine & isolated caffeine

**6.3.6.1 Observation**- From the standard curve, the total phenolic compounds as Salicylic acid equivalent (SAE) of the pure caffeine and isolated caffeine was 38.8 mg/g and 35.63 mg/g respectively.

## 6.3.7 Antimicrobial assay

| | | |
|---|---|---|
|  *Streptococcus pyrogen* |  Isolated caffeine |  NaOH |

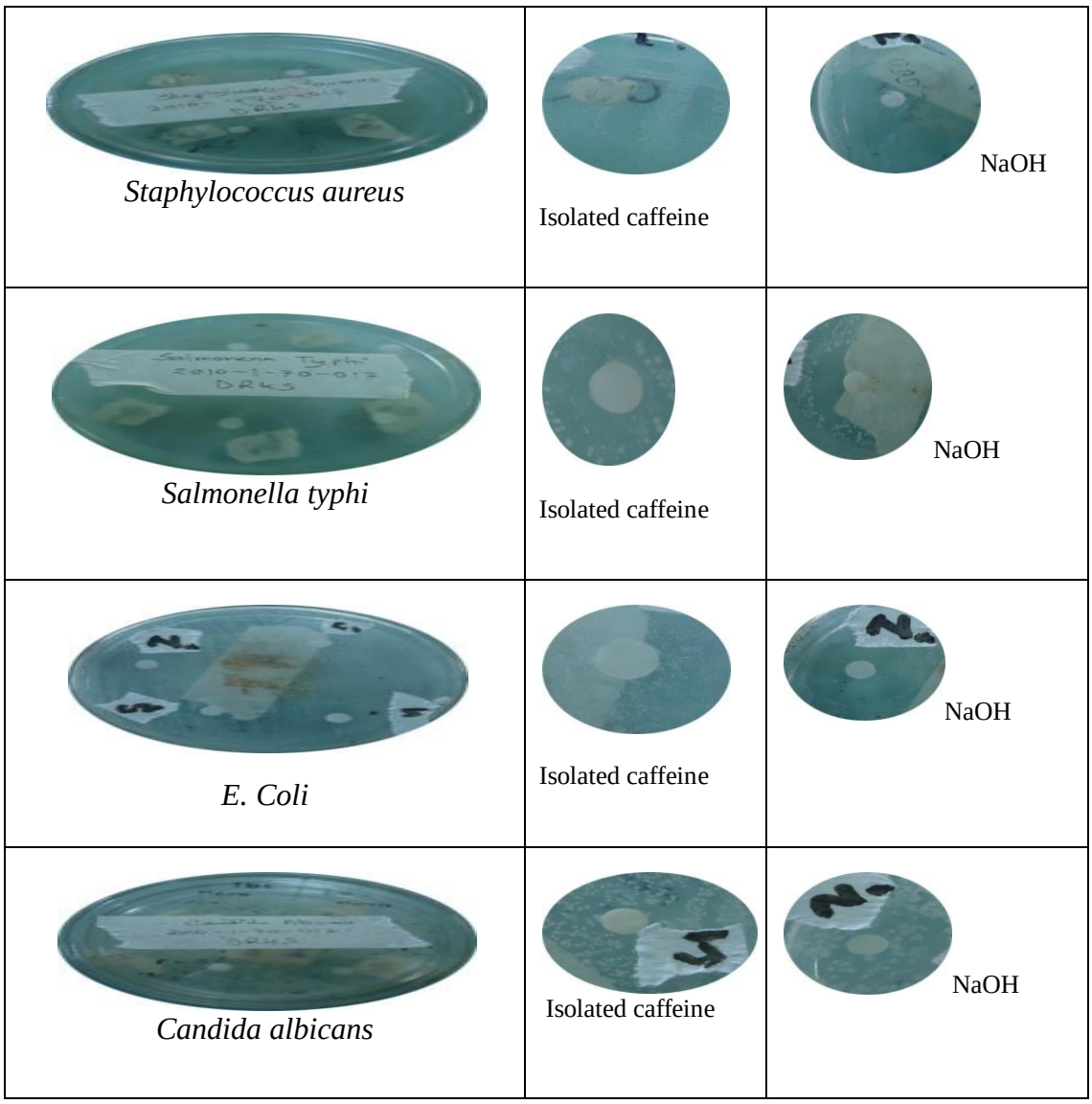



Fig 38: Antimicrobial screening of Isolated caffeine

**Table 17**: Antimicrobial screening for isolated caffeine and NaOH

| Type | **Species** | **Isolated caffeine Chloroform lower layer (C1),(10µl)** | **NaOH layer(Na), (10µl)** | **Negative control Pure NaOH** |
|---|---|---|---|---|
| Gram (+) | *Streptococcus pyrogen* | - | 20 | 23 |
| | *Staphylococcus aureus* | - | 30 | 27 |

| | | | | |
|---|---|---|---|---|
| Gram (-) | *Salmonella typhi* | - | 20 | 25 |
| | *E.coli* | - | 20 | 22 |
| Fungi | *Candida albicans* | - | - | 17 |

**6.3.7.1 Observation:**

The chloroform layer which may contain caffeine has no antimicrobial activity. The crude extract has little antimicrobial activity. The NaOH layer contain no activity. The result found for NaOH is due to the property of NaOH itself as it is very basic. The negative control of NaOH gives similar relust. The PH of NaOH layer was 9.6 and PH of crude extract was 7.4.

**6.3.8 Brine shrimp lethality test**

**Table 18** : Percentage of nauplii alive for isolated and pure caffeine

| Sample | Concentration (mg/ml) | No. of nauplii alive **(Out of 10 nauplii)** | % alive |
|---|---|---|---|
| Pure caffeine | 5 | 0 | 0 |
| | 2.5 | 3 | 33.3 |
| | 1.25 | 4 | 44.4 |
| | 0.625 | 6 | 66.6 |
| | 0.3125 | 7 | 77.7 |
| | Control | 9 | 100 |
| Isolated caffeine | 5 | 1 | 11.1 |
| | 2.5 | 3 | 33.3 |
| | 1.25 | 4 | 44.4 |

| | | | |
|---|---|---|---|
| | 0.625 | 5 | 55.5 |
| | 0.3125 | 8 | 88.8 |
| | Control | 9 | 100 |

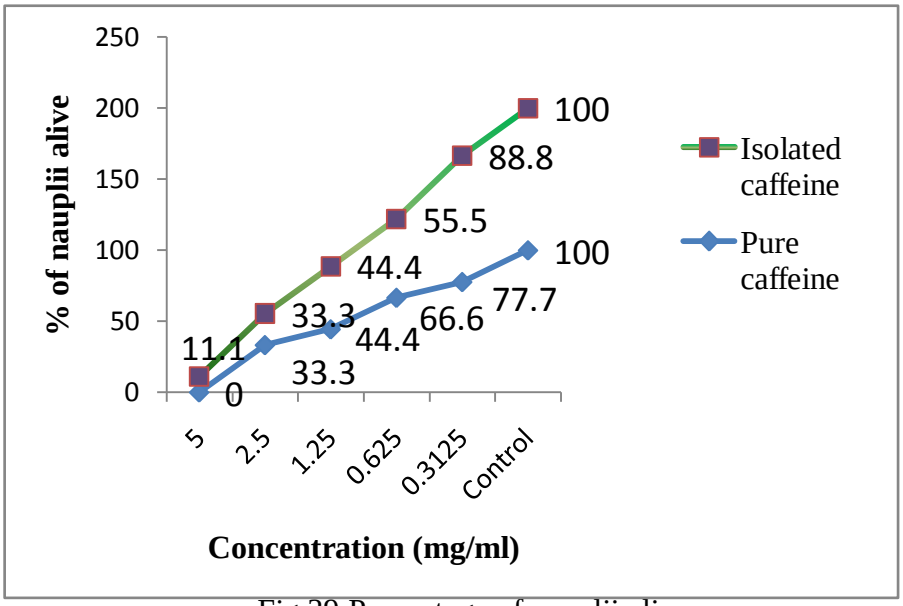



Fig 39:Percentage of nauplii alive

**Table 19** : Percentage of lethality for pure and isolated caffeine

| Sample | Concentration (mg/ml) | No. of nauplii dead**(Out of 10 nauplii)** | % of lethality | $LD_{50}$ (mg/ml) |
|---|---|---|---|---|
| Pure caffeine | 5 | 10 | 100 | 0.9375 |
| | 2.5 | 7 | 70 | |
| | 1.25 | 6 | 60 | |
| | 0.625 | 4 | 40 | |
| | 0.3125 | 3 | 30 | |
| | Control | 1 | 10 | |
| Isolated caffeine | 5 | 9 | 90 | 0.625 |
| | 2.5 | 7 | 70 | |

| | | |
|---|---|---|
| 1.25 | 6 | 60 |
| 0.625 | 5 | 50 |
| 0.3125 | 2 | 20 |
| Control | 1 | 10 |

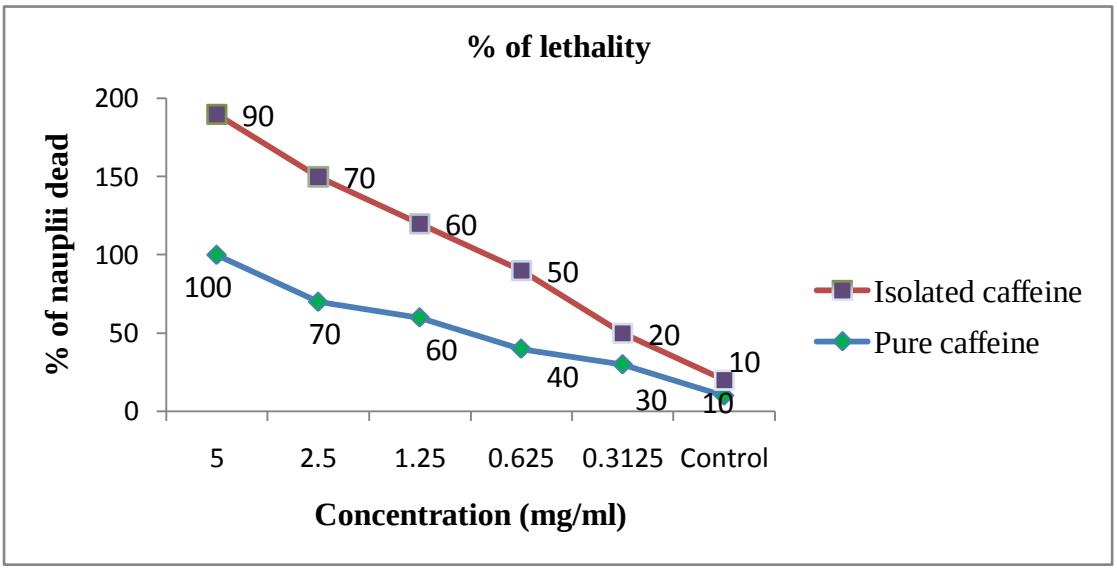



Fig 40: % of lethality for pure and isolated caffeine

**6.3.8.1 Observation**

Lethal Dose, 50% or median lethal dose is the amount of the substance required to kill 50% of the test population. The $LD_{50}$ calculated for isolated caffeine was 0.625 where as for pure caffeine the value was 0.9375. The test population was Brine shrimp nauplii.

**6.3.9 Discussion**

From the observation of TLC plate Rf value was measured in solvent system 2. Rf=0.6875 for both sample and standard. As the Rf value are same the sample and the standard may be same compound. After charing of the TLC plate with sulfuric acid was shown in the fig. (plate 3). In the plate after amplying heat one clear black spot was visible with the black spot of sample and pure caffeine.After staining with FC reagent yellow spot was visible for pure caffiene and isolated sample. The result of DPPH test

for both pure caffine and the isolated sample were similar. The % scavenging from the graph were similar. All these indicate the sample may be caffeine or a derivative of the structure of caffiene.

Caffeine is a white crystalline xanthine alkaloid which is found in varying quantities in the seeds, leaves, and fruit of some plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants, as well as enhancing the reward memory of pollinators. Caffeic acid is an organic compound that is classified as hydroxycinnamic acid. This yellow solid consists of both phenolic and acrylic functional groups. It is found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal sources of biomass.

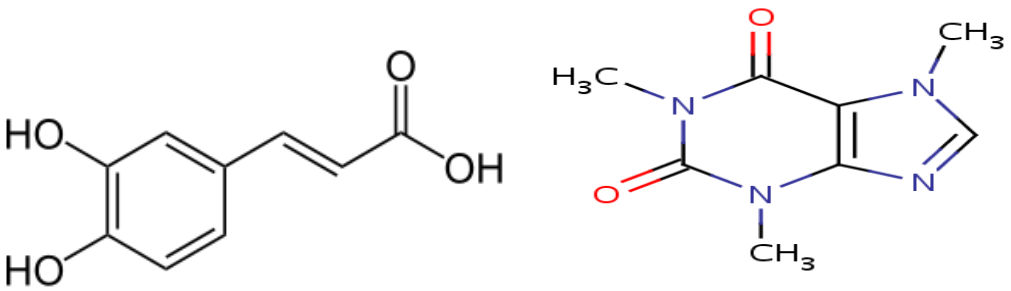



Fig 41: Structure of caffeic acid and caffeine

Caffeic acid has phenolic group which makes it a phenolic acid, where as caffeine has N-based ring. They have less similarity in structure. Again caffeic acid has conjugated double bond, so it will give peak at longer lavelength then caffeine. Pure caffeine show dual peak one at 205 nm and other at 275 nm. Where as caffeic acid show lamda max at 327 nm and a shoulder at c. 295 nm in acidified methanol. The Isolated sample gives lamda max at 273 nm which is near the caffeine value. So the sample might be caffeine.

## Section 4- Isolation of Catechin from *Citrullus lanatus* rind

### 6.4.1 Determination of λ max by UV spectroscopy:

UV screening of the isolated samples of catechine layers were done for confomation of the presence of catechine from the λmax. The graphs showing peak of sample are given below-

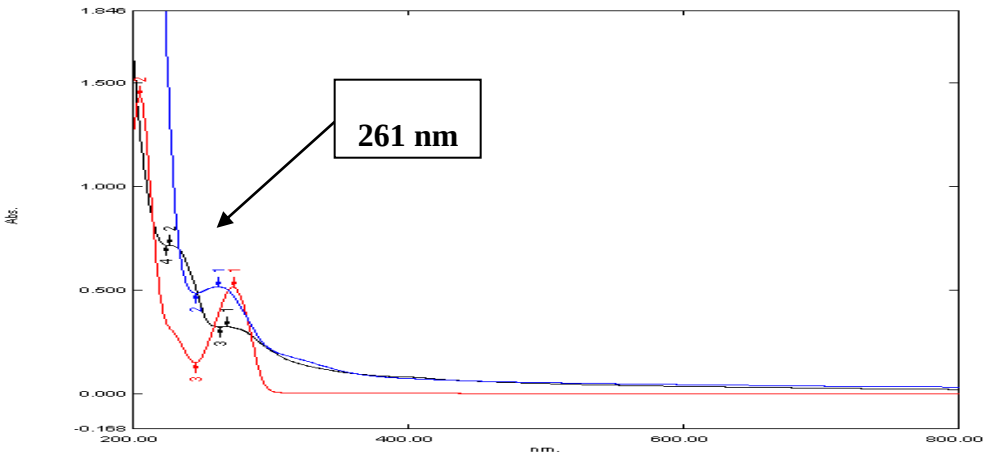



Fig 42:UV scanning of isolated Catechine from the lower phase of separation.(Blue line)

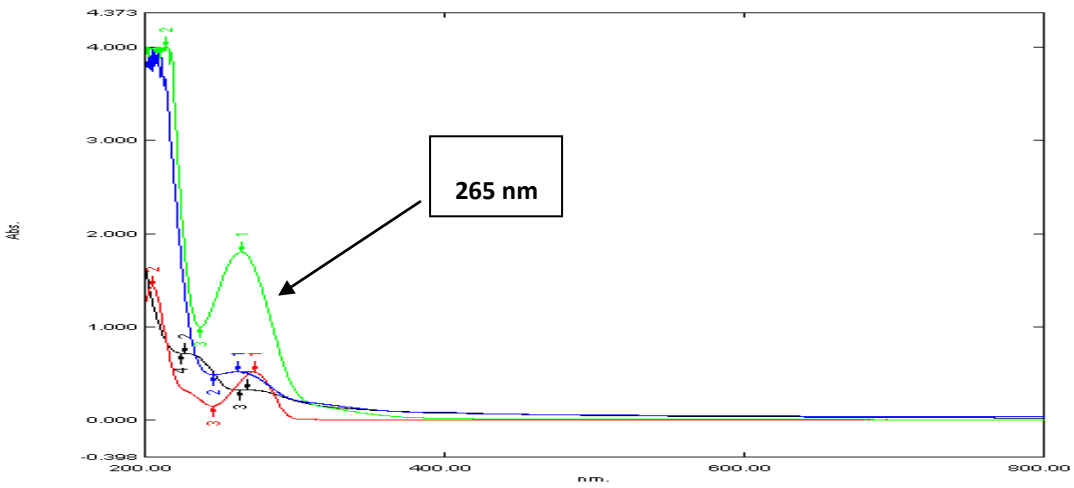



Fig43:UV scanning of isolated Catechine from the upper phase of separation.(Greenline)

### 6.4.1.1 Observation

The above figures show the λmax for isolated catechine from the lower phase (blue line) and the upper phase (green line) of separation when treated with acetone. Isolated

catechine gives maximum absorbance at 261 nm from the lower phase (blue line) and at 265 nm for the upper phase (green line) .

### 6.4.2 TLC of Catechin

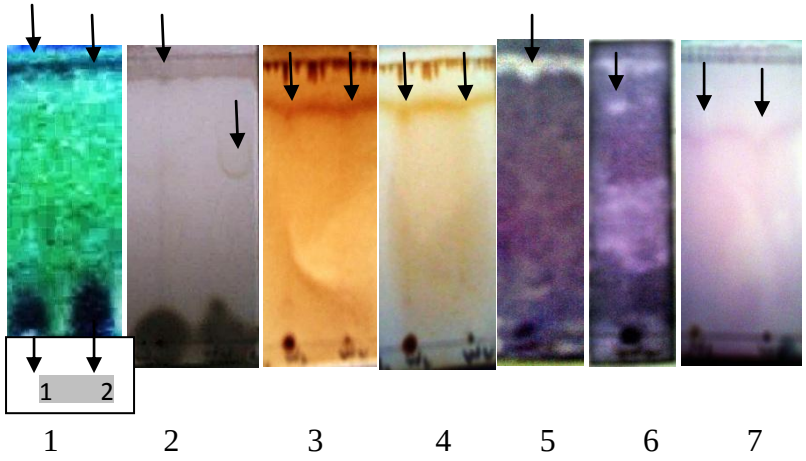



Fig 44: TLC plate of catechin Under UV light(1) , TLC plate after charing with $H_2SO_4$(2),TLC plate after incubation in iodine chamber (3), and Heating after dipping into iodine solution (4), acetone lower layer stained with FC reagent (5), Acetone upper layer stained with Fc reagent (6), DPPH staining of both layers (7)

Here, 1 is the spot of lower layer of acetone and 2 is the spot of upper layer of acetone treated extract for all the plates.

#### 6.4.2.1 Observation

1) Under UV-light: After running the TLC plate in solvent system 2, the plate was observed under the UV light. At the top of the plate two black spots were visible which is shown in plate 1.
2) After charing: After charing with $H_2SO_4$ a small black spot was visible for the loer layer of acetone. A (U) shape curve was visible for the upper layer.
3) Iodine staining: After staining with iodine solution and incubation for 15 minutes in dark chamber the plate was brought to light and a deep orange line was visible. In the line two spots for the upper and lower layer of acetone was more concentrated. After heating the iodine stained plate the concentrated spots were visible.

4) After FC reagent staining: After stainig and incubation for 30 minutes in dark chamber when plate was brought to light white spot was visible for both upper and lower layer of acetone treated extract.

5) After DPPH staining: After stainig and incubation for 30 minutes in dark chamber when plate was brought to light a deep violet line was visible but no yellow color spot was found. Two spots above the violet line were bright indicating the spots.

### 6.4.3 DPPH Test for catechin

#### 6.4.3.1 Standard (ascorbic acid)

**Table 20:** Determination of free radical scavenging capacity for the standard (ascorbic acid)

| **Concentration (ug/ml)** | Absorbance | **% Scavenging** | **IC 50 ug/ml** |
|---|---|---|---|
| 40 | 0.114 | 45.72 | |
| 80 | 0.108 | 48.57 | |
| 120 | 0.103 | 50.95 | 117.76 |
| 160 | 0.100 | 52.38 | |
| 200 | 0.098 | 53.33 | |
| Controll (Blank) | 0.210 | 0 | |

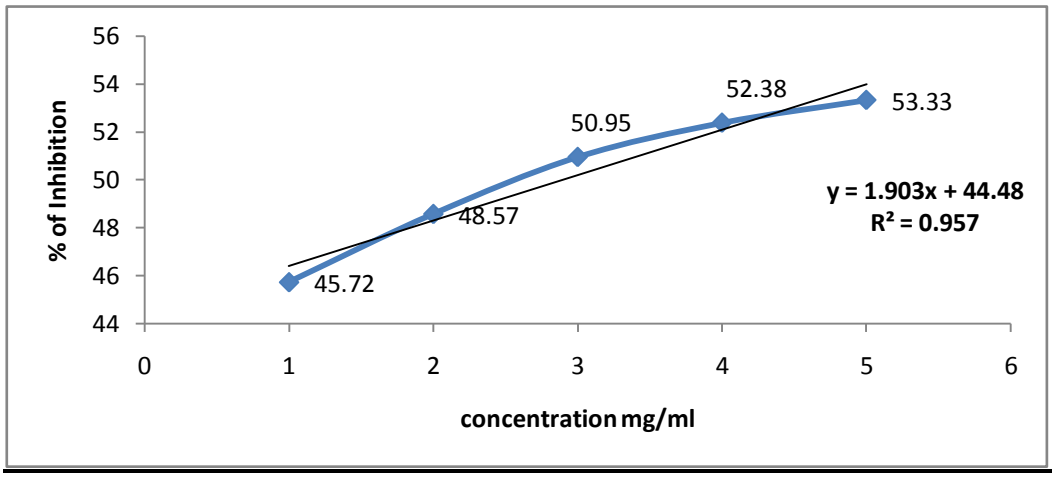



Fig 45: % Scavenging of Standard ascorbic acid

### 6.4.3.2 Determination of free radical scavenging capacity for the isolated catechin upper layer

**Table 21:** Determination of % scavenging capacity for isolated catechin upper layer

| Concentration (ug/ml) | Absorbance | % Scavenging | IC 50 ug/ml |
|---|---|---|---|
| 80 | 0.196 | 6.666 | |
| 160 | 0.188 | 10.476 | |
| 240 | 0.185 | 11.905 | 1135.13 |
| 320 | 0.178 | 15.238 | |
| 400 | 0.173 | 17.619 | |
| Controll (Blank) | 0.210 | 0 | |

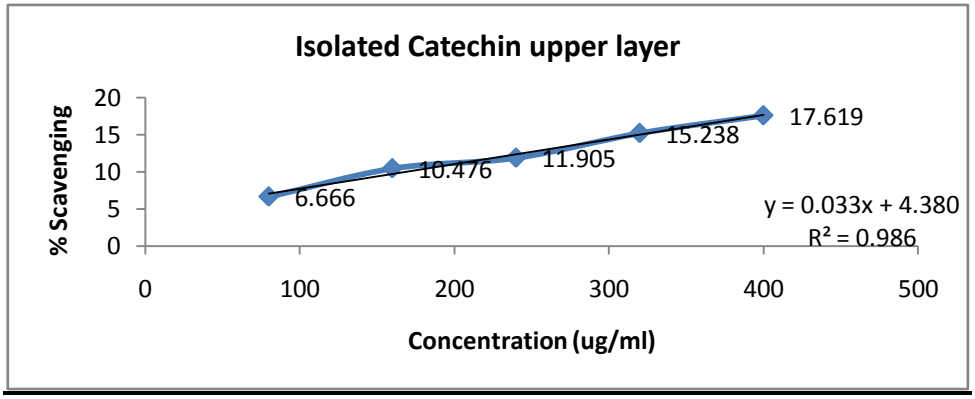



Fig 46: % Scavenging of isolated catechin upper layer

### 6.4.3.3 Determination of free radical scavenging capacity for the isolated catechin lower layer

**Table 22:** Determination of % scavenging capacity for isolated catechin lower layer

| Concentration (ug/ml) | Absorbance | % Scavenging | IC 50 ug/ml |
|---|---|---|---|
| 80 | 0.526 | -169.524 | |
| 160 | 0.447 | -112.857 | |
| 240 | 0.205 | 2.381 | 954.56 |
| 320 | 0.180 | 14.286 | |
| 400 | 0.166 | 20.952 | |
| Controll (Blank) | 0.210 | 0 | |

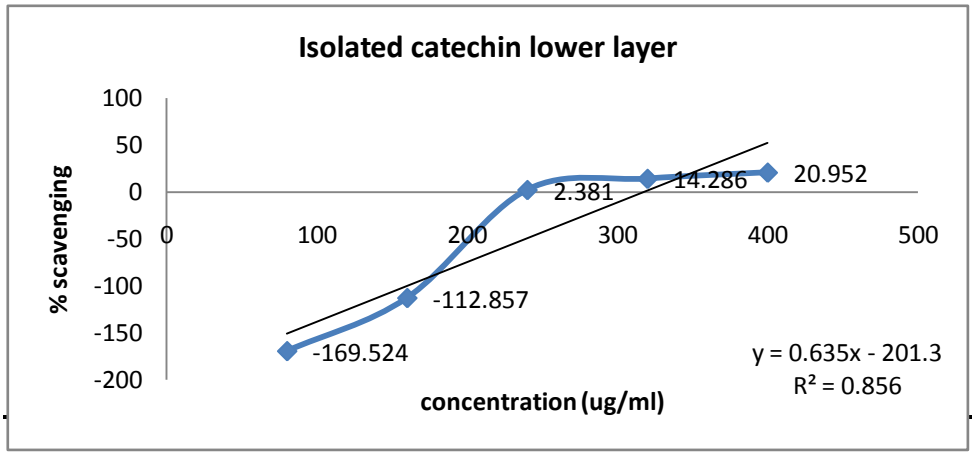



Fig 47: % Scavenging of isolated catechin lower layer

### 6.4.3.4 Observation

DPPH test for both the layers were done to determine the % scavenging capacity. From the above graphs it is visible that the upper layer and the lower layer of acetone treated C. Lanatus extract shows scavenging property. from the upper layer all the values were positive. From the lower layer three values were found positive. Both the results show scavenging capacity near 20% with increasing concentration compared with the standard ascorbic acid. The IC 50 value for lower layer is 1135.13 ug/ml and for lower layer is 954.56 ug/ml. Ascorbic acid has IC 50 value 117. 76 ug/ml.

### 6.4.4 Determination of total phenolic content for catechin

**Table 23:** Assay values of standard solution of salicylic acid

| Serial no. | Concentration (mg/ml) | Absorbance (nm) at 765 nm |
|---|---|---|
| 01 | 5 | 0.653 |
| 02 | 2.5 | 0.467 |
| 03 | 1.25 | 0.277 |
| 04 | 0.625 | 0.121 |
| 05 | 0.3125 | 0.117 |

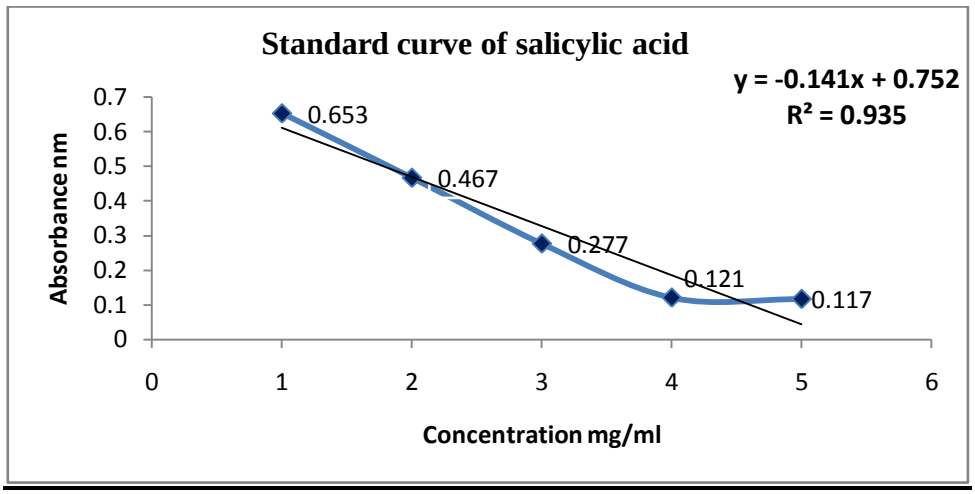



Fig 48: Standard curve of salicylic acid for phenolic test of catechin

**Table 24 :** TPC of isolated catechin

| Sample name | Absorbance at 765 nm | Concentration (mg/ml) [x= y-0.752/-0.141] | TPC (mg/g) [T= CxV/M) | Average with SD |
|---|---|---|---|---|
| **Isolated catechine Lower phase** | 0.271 | 3.411 | 34.11 | 34.296 ± 0.21 |
| | 0.269 | 3.425 | 34.25 | |
| | 0.265 | 3.453 | 34.53 | |
| **Isolated catechine Upper phase** | 0.270 | 3.418 | 34.18 | 34.44 ± 0.25 |
| | 0.266 | 3.446 | 34.46 | |
| | 0.263 | 3.468 | 34.68 | |

**6.4.4.1 Observation**

From the standard curve, the total phenolic compounds as Salicylic acid equivalent (SAE) of the pure caffeine and isolated catechin was 34.296 mg/g for lower phase and 34.44 mg/g for the upper phase.

## 6.4.5 Brine shrimp lethality test

**Table 25**: Percentage of nauplii alive for isolated catechin

| Sample | Concentration (mg/ml) | No. of nauplii alive (Out of 10 nauplii) | % alive |
|---|---|---|---|
| **Isolated Catechine Upper layer** | 10 | 2 | 22.2 |
| | 5 | 3 | 33.3 |
| | 2.5 | 3 | 33.3 |
| | 1.25 | 4 | 44.4 |
| | 0.625 | 4 | 44.4 |
| | Control | 9 | 100 |
| **Isolated Catechine lower layer** | 10 | 2 | 22.2 |
| | 5 | 3 | 33.3 |
| | 2.5 | 3 | 33.3 |
| | 1.25 | 4 | 44.4 |
| | 0.625 | 5 | 55.5 |
| | Control | 9 | 100 |

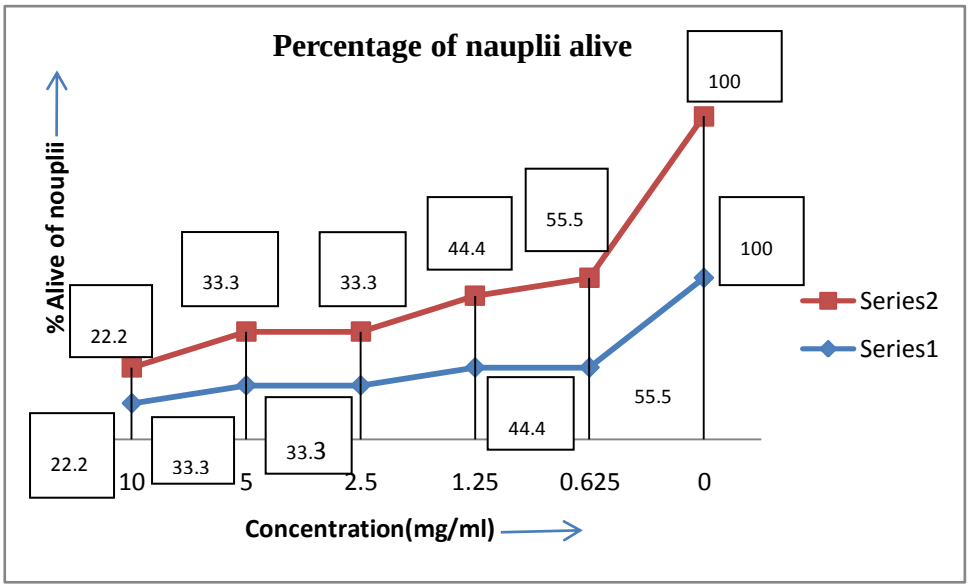



Fig 49:Percentage of nauplii alive

**Table 26**: Percentage of lethality for isolated catechin

| Sample | Concentration (mg/ml) | No. of nauplii dead(Out of 10 nauplii) | % of lethality | $LD_{50}$ (mg/ml) |
|---|---|---|---|---|
| Isolated Catechine | 10 | 8 | 100 | Less than 0.625 |
| Upper layer | 5 | 7 | 87.5 | |
| | 2.5 | 7 | 87.5 | |
| | 1.25 | 6 | 75 | |
| | 0.625 | 6 | 75 | |
| | Control | 1 | 12.5 | |
| Isolated Catechine lower layer | 10 | 8 | 100 | Less than 0.625 |
| | 5 | 7 | 87.5 | |
| | 2.5 | 7 | 87.5 | |
| | 1.25 | 6 | 75 | |
| | 0.625 | 5 | 62.5 | |
| | Control | 1 | 12.5 | |

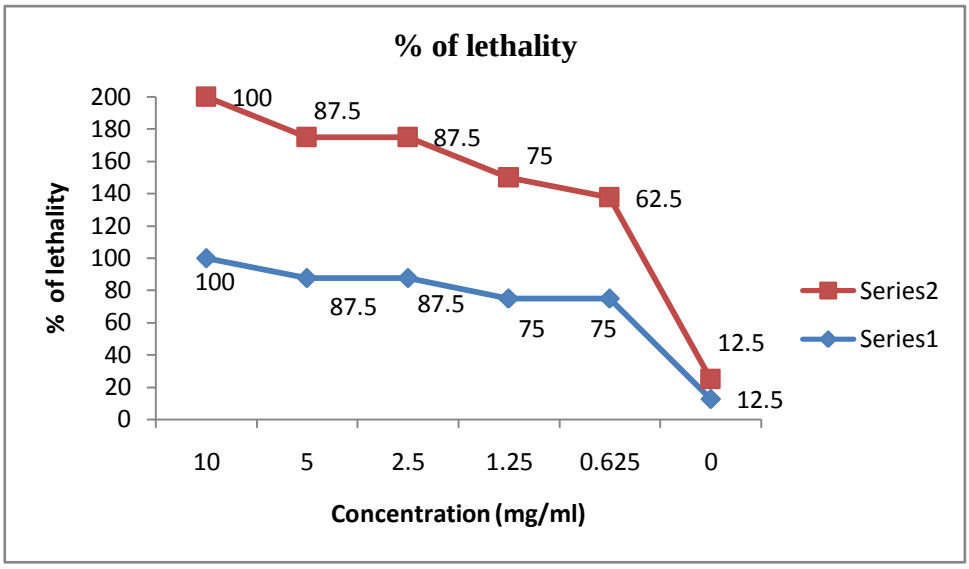



Fig 50: % of lethality (lower phase red line, upper phase blue line)

### 6.4.5.1 Observation

Lethal Dose, 50% or median lethal dose is the amount of the substance required to kill 50% of the test population. The test population was Brine shrimp nauplii. In the above test $LD_{50}$ value could not be determined as no value was observed less or equal to 50%. If more concentrations were taken the value could be obtained. Hence, we can predict that the $LD_{50}$ would be less than 0.625 from the above data.

### 6.4.6 Discussion

Phytochemical compounds like catechin are found in high concentration in a variety of plant based foods and commonly used bevarrages.[39] Based on their structures catechin can be grouped in the class of flavonoids. Some of the compounds of these class are : Catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallaocatechin gallate. Catechins are found in high concentrations in red wine, broad beans, black grapes, apricots and strawberries. Apple, blackberry, broad beans, cherries, black beans,

raspberries are rich in epicatechin. All the compounds are present in black and green tea.[40]

Catechin possesses two benzene rings (called the A- and B-rings) and a dihydropyran heterocycle (the C-ring) with a hydroxyl group on carbon 3. The A ring is similar to a resorcinol moiety while the B ring is similar to a catechol moiety. There are two chiral centers on the molecule on carbons 2 and 3. Therefore, it has four diastereoisomers. Two of the isomers are in trans configuration and are called catechin and the other two are in cis configuration and are called epicatechin.[41]

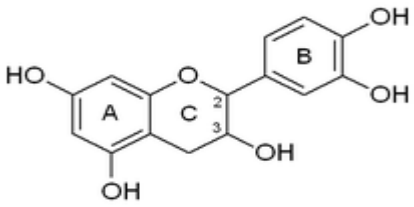



Fig 51: Structure of catechin.

The lamda max of pure catechin is 276 nm.

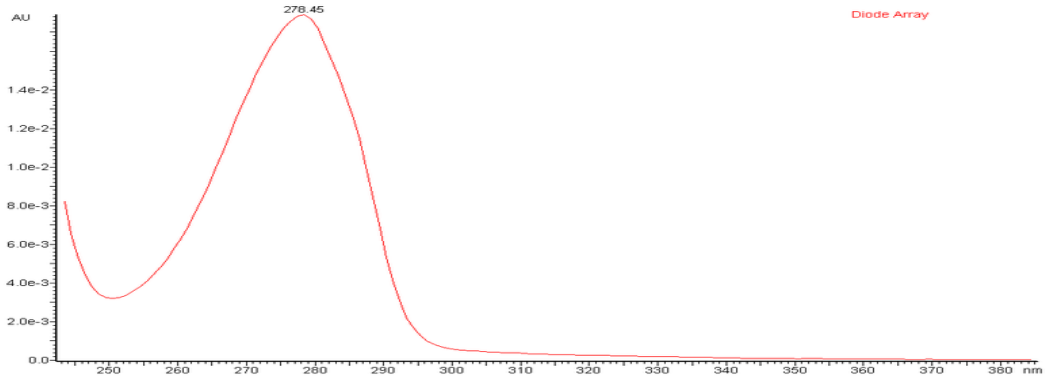



Fig 52: UV-Visible spectra of pure catechin

The most common catechin isomer is the (+)-catechin. Catechin exists in the form of a glycoside.[42] Antioxidant properties can also be provided using a catechin associated with

a sugar. In 1975-76, a group of scientists of Kaz ssr discovered first the catechin rhamnoside using the plants of Filipendula which grow in that region.[43] Rhamnose (Rham) is a naturally occurring deoxy sugar. It can be classified as either a methyl-pentose or a 6-deoxy-hexose. Rhamnose is generally bound to other sugars in nature. It is a common glycone component of glycosides from many plants.

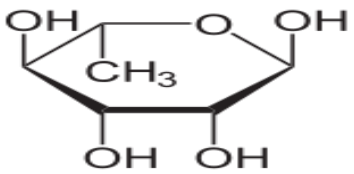



Fig 53: Structure of Ramnose

Epigallocatechin gallate (EGCG) is also named as epigallocatechin-3-gallate, is the ester of epigallocatechin and gallic acid, and is a type of catechin. EGCG is the most abundant catechin in tea and is a potent antioxidant that may have therapeutic applications in the treatment of many disorders like cancer.

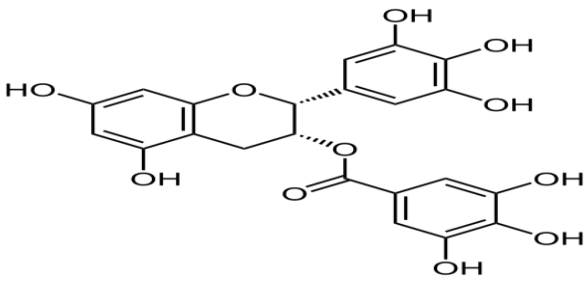



Fig 54: Structure of epigallocatechin

Catechol, also known as pyrocatechol or 1,2-dihydroxybenzene, is an organic compound with the molecular formula C6H4(OH)2. It is the ortho isomer of the three isomeric benzenediols. This colorless compound occurs naturally in trace amounts. It was first discovered by destructive distillation of the plant extract catechin. About 20 million kg

are now synthetically produced annually as a basic organic chemical, mainly as a precursor to pesticides, flavors, and fragrances.

Catechol occurs as feathery white crystals that are very rapidly soluble in water.

(The name "catechol" has also been used as a chemical class name, where it refers generally to the catechins.)

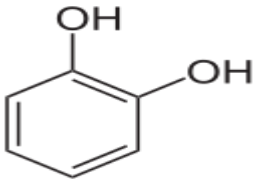



Fig 55: Structure of Brenzcatechin

The compound that is isolated from Citrullus lanatus might be a catechin or a structural derivative.

# Chapter 7

# Conclusion and References

## Conclusion

“In All things there is a poison, and there is nothing without a poison. It depends on only upon the dose whether a poison is a poisonor not”---------Paracelsus (1493-1541,Switzerland). Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons. The building blocks of plants are explored by researchers which enables to identify elements which can be used in beneficial ways. Plant compounds are playing an important role in many medicines as being the major active compound. Taxol, derived from the yew, has been used to treat breast cancer. Now it is being used to treat many other forms of cancer also. The Madagascar periwinkle is the source of vincristine and vinblastine which are being used to treat leukaemia. More humble plants also have their role to play: for example wild relatives of rice contain anti-cancer compounds which have been lost in the highly refined milling process. Many medicinal and recreational drugs, such as tetrahydrocannabinol (active ingredient in marijuana), caffeine, morphine and nicotine come directly from plants.

The phytochemical investigation of *Citrullus lanatus* ( watermelon) rind indicates there is presence of caffeine and catechin in the rind. The crude extract also has mild antibacterial activity. There are also few established research reports on the chemical properties of *Citrullus lanatus*. Still there are plenty of scopes to establish a variety of properties which are significantly beneficial to mankind. Further investigation is needed for the proper identification and isolation of these bioactive compounds.

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