Analysis of the antibacterial and thrombolytic activity of *Citrus sinensis* peel extracts

A project submitted
by
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My inspiration, my mother
Mentor and my sister
&
To my father for being the man he is

Certification Statement

I hereby declare that the research work embodying the results reported in this thesis entitled **'Analysis of the antibacterial and thrombolytic activity of *Citrus sinensis* peel extracts'** submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Najneen Ahmed, Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another. I should add that Dr. Md Mesbah Uddin Talukder finally checked my thesis since my original supervisor Najneen Ahmed is on study leave. Dr. Talukder kindly agreed to sign the thesis on behalf of my supervisor.

Signed,

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Countersigned by the supervisor

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Acknowledgement

Undertaking a project of this stature would not have been possible without the strength and blessings of Allah and the help and support of my family.

I would like to express my wholehearted pleasure and honor to work with the very dedicated teacher of the department, my supervisor Najneen Ahmed, Lecturer, Department of Pharmacy, BRAC University for the continuous support, kind guidance and patience. I am sincerely thankful to her for adding insightful new direction throughout the entire period of my research work and constantly inspiring me to do more.

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Abstract

Citrus sinensis (*C. sinensis*) has been traditionally used for the diagnosis of many diseases which includes- asthma, hypertension, microbial infections, diabetes, tuberculosis, etc. It contains many types of phytochemicals such as flavonoids, saponinss, tannins, glycosides, limonene, citral, neohesperidin, naringin, rutin, rhamnose, eriocitrin, and vitamin C. These phytonutrients may go about as cancer prevention agents, animate the invulnerable frameworks; prompt defensive proteins in the liver or square the harm of the hereditary materials.

C. sinensis showed antimicrobial activity against many micro-organisms which can be taken into account for the treatment of infectious diseases. In this research some of the bacteria which are responsible for gastrointestinal order have been taken into consideration and the antibacterial activity of *C. sinensis* was studied with them. The Studied bacterial strains are *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella sp.* The different concentrations of the sample have been used to evaluate the antimicrobial activity of methanolic peel extracts of *C. sinensis*. It provides antimicrobial activity against these micro-organisms with the increase of sample's concentration. There was no research done for the thrombolytic activity of the *C. sinensis* peel extracts before. Thrombolysis is also referred as clot bursting. Thrombolysis causes disruption in normal blood flow. Methanolic peel extracts of *C. sinensis* show weak thrombolytic activity, but the data can be used for further study. Researchers might use other techniques to reveal thrombolytic activity which can be used for the diagnosis of patients and as it is an herbal drug it would have negligible side effects, adding a preferable treatment for the patients.

List of Content

List of Figures

List of Tables

Abbreviations

C. sinensis- Citrus sinensis

WHO- World Health Organization

NaCl- Sodium Chloride

mg/mL- milligram per milliliter

rpm-Rotaion per minute

DMSO- dimethyl sulfoxide

mm- millimeter

g- gram

mg- milligram

µL- microliter

NA- Nutrient agar

H. pylori- Helicobacter pylori

Chapter One: Introduction

1.1 Brief on herbal Drug:

Herbal medicines refer to plants or parts of plants which can be used to prevent or cure diseases. Most people feel safer in terms of using herbal medicines because it has minimal or negligible side effects (Hassan, 2012).

Plant's many parts such as its flowers, seeds, fruits, peel of fruits, stem are often used to diagnose diseases. Herbal medicines have been used for long, as traditional medicines for health recovery. The use of herbal medicines is becoming more important and more reliable nowadays as it holds the hands of the advance technology, clinical researches, and analytical tools along with quality control assurance (Altschuler et al. 2007).

In past 30 years people are using more herbal supplements and their uses have been increasing drastically day by day. The era of using natural medicines such as the use of plants for medicinal purpose has shown a new direction for the healing purpose of human beings. These eliminate the side effects of the allopathic medicines, adding more advantages to health. According to the World Health Organization (WHO), it has been estimated that more than three fourth of the population finds ease to use herbal medicines and find it more reliable and cheaper. Alternatively herbal medicines can replace the conventional medicines for medical purposes as it has found to have therapeutic effect on our body. Additionally, herbal medicine can also be termed as plant pharmaceutical or phytomedicine as it can be used for the treatment in various diseases. Active ingredients of plants which have therapeutic effects are synthesized in pharmaceuticals for making drugs. Active ingredients of plants become less useful or lose its effect when they get separated from plants (Abeloff et al. 2008).

Nowadays plant-derived substances have become of much importance due to their various applications. Medicinal plants are the high bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Rahmatullah, 2016).

Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use (Prashant Tiwari, 2011).

1.2 Background of herbal medicines:

Although the introduction of scientific study on herbal medicines is new but the use of herbal medicines has been gifted as a blessing to the mankind for its fewer side effects. In history plants have been used for medicinal purposes prevent when all these advanced technology were not introduced. In the early 3000 BC ancient Chinese and Egyptian papyrus used herbal medicines for the betterment of health. Different cultures used herbs in different aspects of treatment and diagnosis. Many herbal plants are used for health beneficial in different region of world (Birks et al. 2007).

As per the WHO, 80% of people in the world have been found to depend on herbal medicines for the treatment and recovery of their health. People in Germany often use plant based medicines and 600-700 plants are used for making drugs and cure health related problems and are prescribed by 70% of doctors. Because of the increasing expenses related to health problems and medicines, enthusiasm for coming back to common or natural cures, has prompted an increment in herbal drugs utilization. Herbal medicines have been widely used in ancient period. Herbal medicines are also found to carry potency and hence, they should be used with precautions. In fact, many pharmaceutical drugs are based on the synthesized versions of naturally occurring compounds found in plants. For instance, digitalis was derived from the herb foxglove and used for the treatment of heart diseases. Nowadays use of herbal medicine has been dramatically touching the sky, so more scientific interest on medicinal plants has come forward. Researchers had many findings about the plants and parts of plants that can be used in treating health problems and boosting health without much side effects and non-toxicity (Damery et al. 2011).

1.3 Usefulness of herbal medicines:

Herbal medicines are widely used because of its safety, efficacy, therapeutic effect and availability. Alternatively, these medicines are found to be more economic and are less expensive than the other allopathic medicines. Their accessibility is more as herbal medicines plants, can also be planted in our gardens. Herbal medicines have been used for many health recovery purposes which includes cough, inflammation, to enhance immune system, problems related to alimentary canal. Improper digestion, constipation, peptic ulcers are also gets recovered by herbal medicines. Patients who are allergic to allopathic medicines can switch to herbal medicines. These herbal plants are also used in the field of cancer research system to bring out active ingredients for diagnosis of cancer (Sissay et al. 2006).

1.4 Traditional uses of *C. sinensis* peel:

Conventional Chinese home grown prescription uses a few citrus peels for particular wellbeing bolster, including those of mandarin orange (*C.reticulata* 'Blanco'), sweet orange (*C. sinensis*) and sharp orange (*C. aurantium*).

For a long time, cultivators prepared in Traditional Chinese Medicine (TCM) have utilized develop sweet orange peel, known as chen pi or ju pi in Chinese pharmaceutical, to enhance assimilation, calm intestinal gas and bloating, and purpose mucus. These peel demonstrations essentially on the digestive and respiratory frameworks. We apply it in conditions including a feeling of distension and totality in the midsection and upper center guts consolidated with loss of hankering, retching or the runs, or hacks with bountiful mucus.

Sweet orange peel, in Chinese medication, acts essentially on the liver and stomach to advance assimilation, ease nourishment maintenance and stomach distension, and advance great liver capacity. Professionals of Chinese herbology utilize this herb when the feeling of distension and distress lies basically under the rib confine as opposed to the focal mid-region.

C. sinensis peel also used as a treatment of anorexia, colds, coughs etc. An essential oil from the peel is used as a food flavouring agent and also in perfumery and medicines.Terpenes extracted from peel are used to paint the ships and boats (Wiesman et al.2005).

1.5 The studied plant (*C. sinensis)*:

C. sinensis is also referred as sweet orange (Fig 1.1). The family of this fruit is Rutaceae (Table 1.3) which is found in tropical and subtropical areas in Southeast Asia. It is mainly originated in South East Asia but it is found worldwide. It is a great source of vitamin C. *C. sinensis* contains various bioactive compounds like acridone alkaloids, flavonoids, vitamin C, carotinoids, limonoids, essential oils, minerals and vitamin B complex (Table 1.1). Sweet orange contains phytochemical nutrients which are important to our health. These fruits also contain abundant of phytochemical compounds like flavanones, polyphenols, anthocyanins and hydroxycinnamic acids which are used mainly in pathological conditions like inflammation, high cholesterol related diabetes and cancer etc. (Milind et al. 2012).

Figure 1.1 *C. sinensis*

1.6 Description of *C. sinensis* as a plant:

C. sinensis is an orange fruit basically and its shape is round and its tree has a length of 9-10 m. Leaves of these trees are in oval shape their barks appear to be green or brown in color which is quite smooth. Leaves have a size of 4-10 cm if its length is taken in to account. The leaves of this tree are green. Leaves have smooth texture with a smell resemblance to the sweet orange. The flower of this tree consists of mainly five petals which smell same as saccharine (Webber et al. 1903).

C. sinensis has seeds in between the parts where juices are present. The seeds are green or cream in color. The fruit's flesh is mostly made of the orange sweet juicy part. The peel has orange color (Fig 1.2) (J.valiant et al.2004).

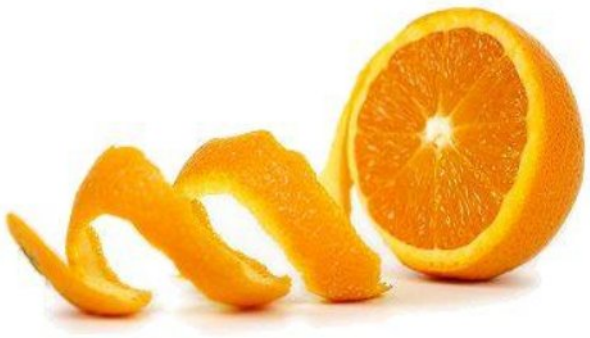


Figure 1.2 Peel of *C. sinensis*

Table 1.1 Nutrient compositions of *C. sinensis* [USDA Nutrient Database (2014)]:

Composition	Amount
Energy	197 kJ (47 kcal)
Sugars	9.35 g
Dietary fibre	2.4 g
Fat	0.12 g
Protein	0.94 g
Water	86.75 g
Vitamin A equiv.	11 µg (1%)
Thiamine (vit. B1)	0.087 mg (8%)
Riboflavin (vit. B2)	0.04 mg (3%)
Niacin (vit. B3)	0.282 mg (2%)
Pantothenic acid (B5)	0.25 mg (5%)
Vitamin B6	0.06 mg (5%)
Folate (vit. B9)	30 µg (8%)
Choline	8.4 mg (2%)
Vitamin C	53.2 mg (64%)
Vitamin E	0.18 mg (1%)
Calcium	40 mg (4%)
Iron	0.1 mg (1%)
Magnesium	10 mg (3%)
Manganese	0.025 mg (1%)
Phosphorus	14 mg (2%)
Potassium	181 mg (4%)

Table 1.2 International synonyms of *C. sinensis* [P.milind et al. (2012)]:

Country	Name
Germany	Apfelsine, orangenbaum
Japan	Orenji, orenzi
China	Tian, cheng
Italy	Arancia, aranciodolce
France	Oranger, orangedouce
Spain	Naranja, naranjodulce
India	Mosambi, narangi, santra
UK	Narineh, narindz, narinjh

Table 1.3 Taxonomy of *C. sinensis* [P. Milind et al. (2012)]:

1.7 Purpose of the study:

From the literature review no thrombolytic activity was found to be done using methanolic extract of *C. sinensis* peel. So this present study is focusing on the evaluation of the thrombolytic effect of *C. sinensis* methanolic peel extracts. Thrombolysis is also referred as clot bursting. Thrombolysis causes disruption in normal blood flow. It mainly causes blood clot and blocks the blood flow causing necrosis or infarction.

Antibacterial was done before on the *C. sinensis* (Table 1.1). The most done research of antimicrobial activity using extract of *C. sinensis* includes dental caries. *C. sinensis* peel has various properties to cure diseases and is widely used against various ailments, such as colic, upset stomach, cancer, diuretic, cormunative, immune-enhancing, stomachic, tonic to digestive system, immune system and skin. It is also used to treat and prevent colds, cough, vitamin deficiencies and scurvy and fight against bacterial and viral infections.

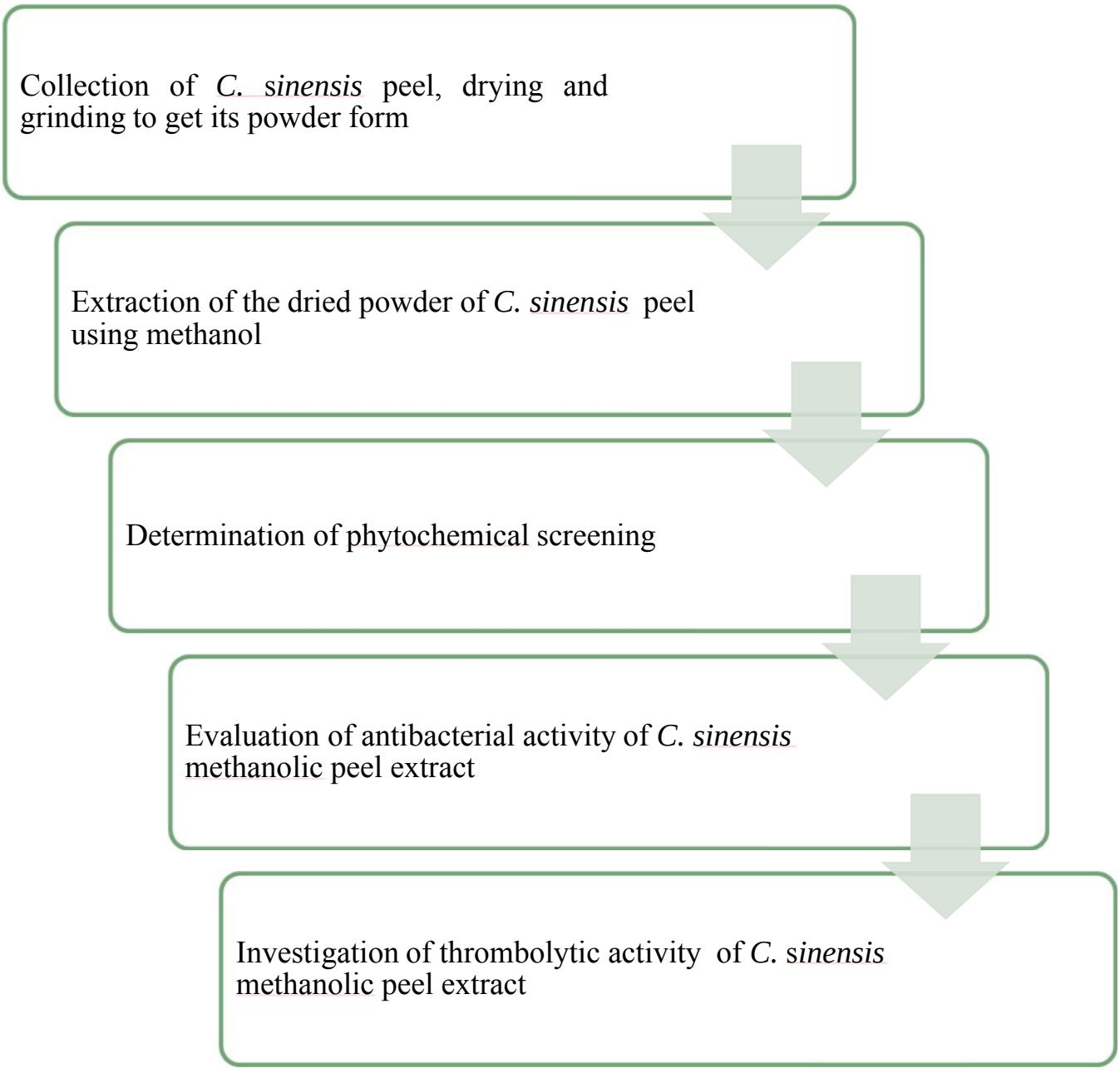
Table 1.4 Antimicrobial activities of *C. sinensis*:

NAME OF SAMPLE	TYPES OF MICRO-ORGANISMS	METHOD OF EXTRACTION
C. sinensis, *Citrus aurentium*	*Colletotrichum capsici, Bacillus cereus, Shigella flexneri, Klebsiella pneumoniae*	Methanol
Citrus limon, Citrus aurantifolia	*Shigella Spp, E.coli, Salmonella Spp*	Chloroform
Citrus peel essential oils	*Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shigella species and Candida albicans*	Hydro distillation
C. sinensis	*Streptococcus mutans and Lactobacillus acidophilus*	Aqueous and ethanol (cold and hot)
C. sinensis	*Staphylococcus auricularis,Staphylococcus aureus, Escherichia coli, Streptococcus mitis, Streptococcus salivarius, Klebseilla pneumoniae, Streptococcus pneumoniae*	Aqueous and ethanol (cold and hot)
C. sinensis	*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae*	Aqueous and ethanol
C. sinensis	*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Pre-votellaintermedia*	Aqueous and ethanol (cold and hot)

Citrus aurentium	*Pseudomonas aeruginosa, Salmonella typhimurium, Micrococcus aureus*	Ethanol, Acetone, Methanol
Citrus peel essential oils	*Aspergillusniger*	Ethanol
Citrus peel essential oils	*Listeria monocytogenesn, Corynebacterium minutissimum, Escherichia coli, Yersinia sp, Klebsiella planticola, Aspergillus flavus, A. fumigates* and *A. niger*	Hydro distillation

In this research, some of the bacteria which are responsible for gastrointestinal order have taken in account and the antibacterial activity of *C. sinensis* was studied on them. The Studied bacterial strains- *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella sp* are found to disturb our normal function of gastrointestinal tract and causes infection. Though there are many allopathic medicines are present for the treatment of gastrointestinal disorder but patients nowadays are tend to use herbal medicines more because of the less side effects and less harmful to health.

1.8 Protocol of the study



Chapter Two: Review of literature

2.1 Activity of *C. sinensis*:

2.1.1 Effect on cardiovascular diseases:

Orange natural product contains vitamin C, carotenoids and flavonoids, which are cardio defensive. As per WHO's late report, citrus natural products offer assurance against cardiovascular illnesses by diminishing levels of homocysteine (Guarnieri et al. 2007).

2.1.2 Effect on kidney stones:

A study has been showed that juices of *C. sinensis* reduce kidney stones which were done over British women (Tanaka et al.1997).

2.1.3 Effect on cancer:

C. sinensis contains limonene which is known to diminish the tumor at the colon, lung, mouth, skin and bosom. The other constituentof sweet orange is hesperidin which showed anti-cancer property in many studies. Most part relies on upon cell reinforcement properties of the atoms causing anti-cancer activity, and also their capacity to regulate the action of detoxifying hepatic chemicals. The polymethoxylated flavones have demonstrated solid hostile to proliferative activity against malignancy cells and antigen initiated T-lymphocytes. β-cryptoxanthin (an orange-red carotenoid) is available in most astounding sums in oranges. It might essentially bring down one's danger of creating lung malignancy (Kurowska et al. 2004).

2.1.4 Effect on micro-organisms:

Oranges are eaten to cure fever. The cooked squash is prepared as a poultice for skin ailments. The fresh peel is rubbed on skin break out. A decoction of the dried leaves and blooms is taken in Italy and France as an antispasmodic, cardio-protective and antagonistic to emetic ailments in China. Orange peel oil creates lethal effect on bugs, fire ants, and houseflies as a result of its 90-95% limonene. Orange peel is used as herbal drugs against living beings (Honow et al. 2003).

2.1.5 Effect on inflammation:

Physical injuries which tend to open skin or ruptures tissues of the skin due to any physical injuries are called wounds. The healing property of orange depends on upon wide blend of phytonutrients, for instance, citrus flavones (hesperidin and naringenin), anthocyanins, hydroxycinnamic acids, and a blended pack of polyphenols. There are reports on the biological activity of *C. sinensis* as antioxidants (Tripoli et al. 2007).

2.1.6 Effect on fungi:

Antifungal constituents of *C. sinensis* are limonene (84.2%), linalol (4.4%) and myrcene (4.1%) which inhibits growth of fungi (Julius et al.2009).

2.1.7 Effect on ulcers:

There are studies which have shown the effect of *C. sinensis* peel on the ulcers. The ulcer diminishes due to decreased occurrence of *Helicobacter pylori* (*H. pylori*) (Sharma et al. 2008).

2.1.8 Effect on diabetes:

Against diabetic kineticism of orange is a result of bioflavonoids, for instance, hesperidin and naringin present in citrus natural item peels. These peels play against diabetic role in C57BL/Ks J-db/db mice by betokens of regulation of glucose managerial impetuses. They lessen the activity of glucose-6-phosphatase and phosphoenol pyruvate. The anti-diabetic capacity of orange peel and juice have one of the reserves of being intervened by betokens of against peroxidation, check of α-amylase impetus kineticism that is responsible for the change of involute starches to glucose, extended hepatic glycogen content, actuation of insulin release, and restoration of secretory disfigurements of pancreatic β-cell (Faturi et al. 2010).

Chapter 3: Methodology

3.1 Preparation of plant extract:

3.1.1 Collection and identification:

The peel of *C. sinensis* was chosen to see the antimicrobial and thrombolytic activity. During the month of March, 2016 the peels of *C. sinensis* were collected from the Dhaka, Bangladesh. Taxonomist of the National Herbarium of Bangladesh in Mirpur Dhaka has given recognition to the sample selected for the experiment. Moreover the voucher specimen was deposited in the National Herbarium for future references.

3.1.2 Preparation of the peel extract:

- The peels which were collected were initially cleaned with distilled water. The peels were taken off from the flesh of the fruits carefully so that no flesh comes out along with the peels.
- The peels were sundried for 7-8 days so that all the moistures are dried away well and the surrounding temperature was about 27-35^{o}C.
- The peels after drying completely were crushed proportionally to fine powders using mortar and pastel and further into a grinding machine.
- The powder weighed 400 gm after crushing and stored in an air-tight container. The container was kept in a dry, cool place before the further investigation.
- The powder sample was soaked in 1 liter of methanol in a glass container and sealed tightly with the help of aluminium foil paper wrapped nicely around the opening of the jar and then with the lead of the jar, so that the soaked material would not get spilled off while shaking and stirring.
- Next the soaked sample was left for 7 days and the soaked powder were daily shaken for thrice at least, so that it gets soaked thoroughly.
- On the eighth day the soaked sample were filtered. Initially the mixture was filtered using cleaned cotton cloth which was sterilized well before use. Afterwards the filtrate collected was further filtered through Whatman filter paper (England).

- Later the filtrate was evaporated in rotary vacuum evaporator (Model Hei-VapAdv Rotary Valve Tech Gwalior, India) at 40^{o}C in a speed of 110 rpm for 1 hour 30 minutes (Fig 3.1). Then it was further dried in water bath.
- The mixture was transferred into a beaker and kept inside the fume cupboard for more evaporation. The beaker was sealed and covered using aluminium foil paper.
- The extract was oily and sticky in texture. The beaker of the crude extract was marked with a marker written "Methanolic peel extract of *C. sinensis"* and placed in a dry, cool place until the next use.
- These crude extract was further used for the experimental test to see the antimicrobial and thrombolytic activity of the methanolic peel extract of *C. sinensis*.

Figure 3.1 Rotary vacuum evaporator

3.2 Phytochemicals:

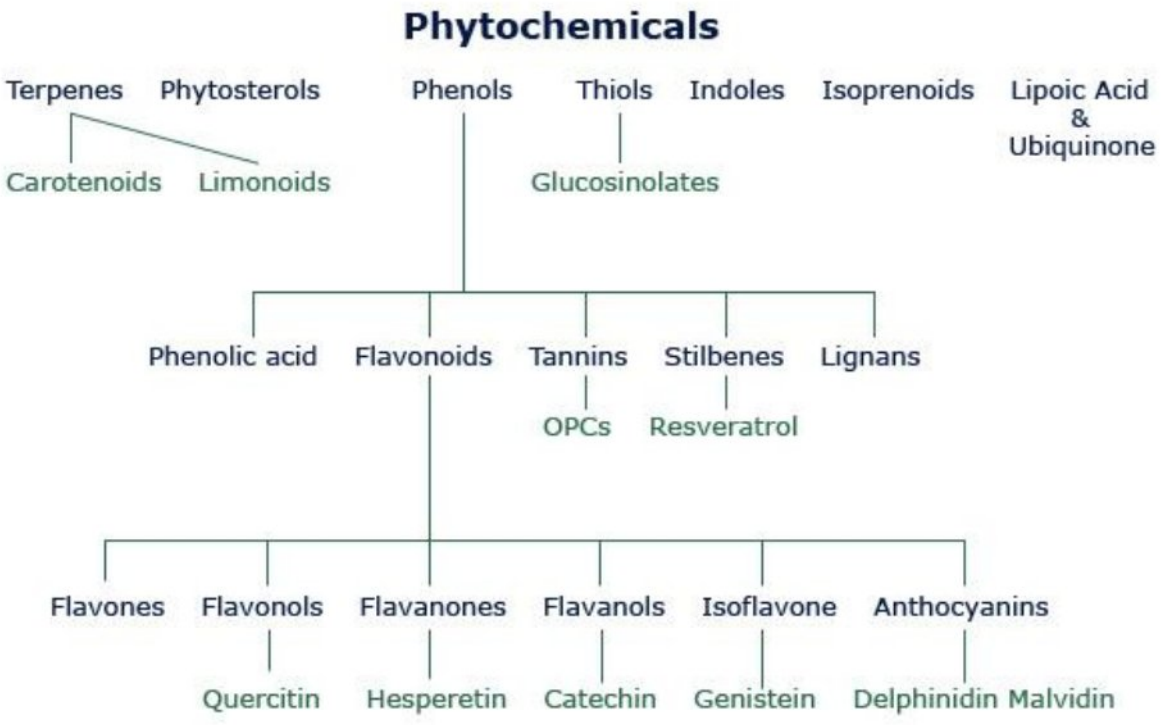


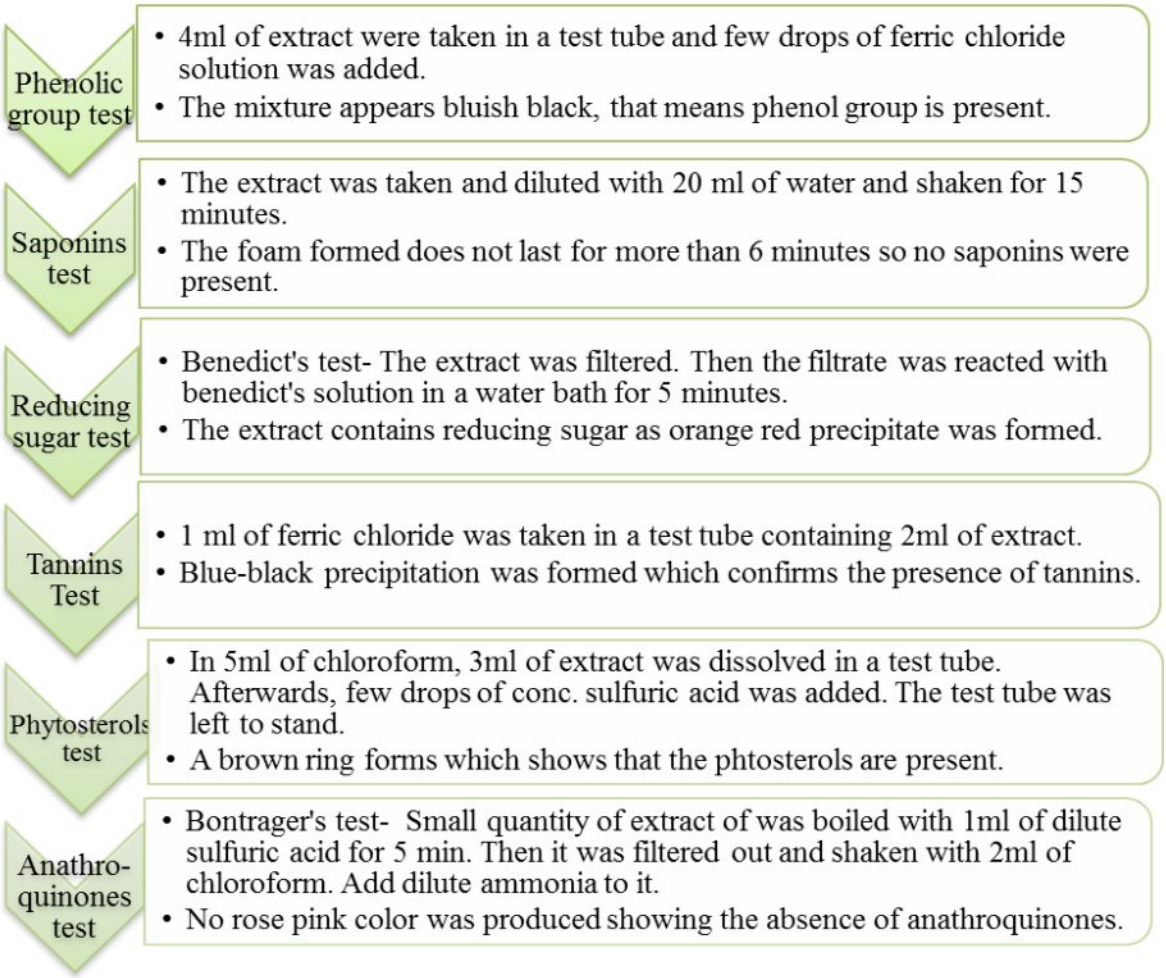
Figure 3.2 Phytochemicals in plants

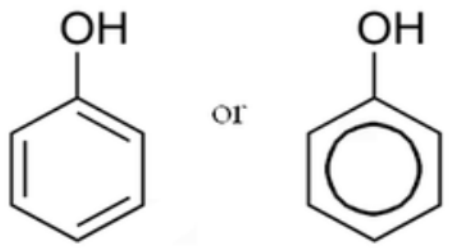
Phytochemicals actually mean "plant chemicals." Researchers have distinguished thousands of diverse phytochemicals, found in vegetables, organic products, beans, entirety, grains, nuts and seeds (Fig 3.2). Eating parts of plants that are rich in phytochemicals may help to counteract at minimum one in each five cases of growth, as well as other genuine illnesses such as heart diseases.

They are actually occurring naturally and found in plants. They predominantly secure plants. They additionally have huge part in assurance of human health. Dietary phytochemicals are found in organic products, vegetables, vegetables, entire grains, nuts, seeds, parasites, herbs and spices. Common sources are broccoli, cabbage, carrots, onions, garlic, entire wheat bread, tomatoes, grapes, fruits, strawberries, raspberries, beans, vegetables, and soy. The definite arrangement of phytochemicals could have not been performed in this way, in light of the wide assortment of them. In detest year Phytochemicals are delegated essential or optional

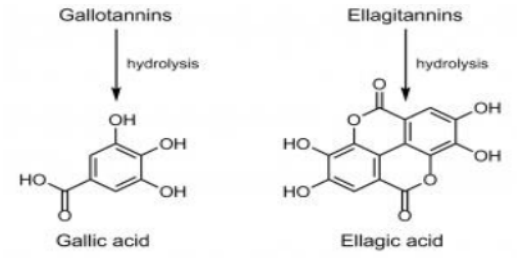
constituents, contingent upon their part in plant digestion system. Essential constituents incorporate the regular sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's and so on. Optional constituents are the rest of the plant chemicals, for example, alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides (Fig 3.3). The phytochemicals present in plants are used for avoiding sickness and advancing wellbeing have been concentrated broadly to build up their adequacy and to comprehend the fundamental component of their activity. Such studies have included identification and seclusion of the concoction parts, foundation of their organic strength both by in vitro also, in vivo concentrates on in exploratory creatures and through epidemiological and clinical-case control concentrates on in man (Saxena, 2013).

3.2.1 Phytochemicals screening of *C. sinensis* [Oikeh et al. (2013)]:

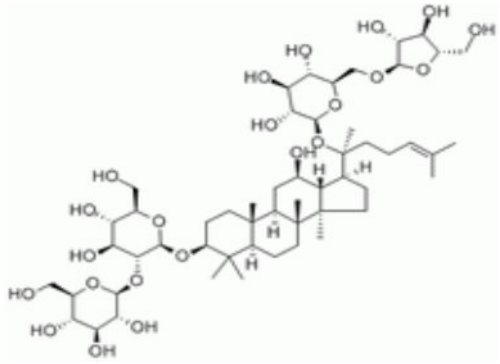




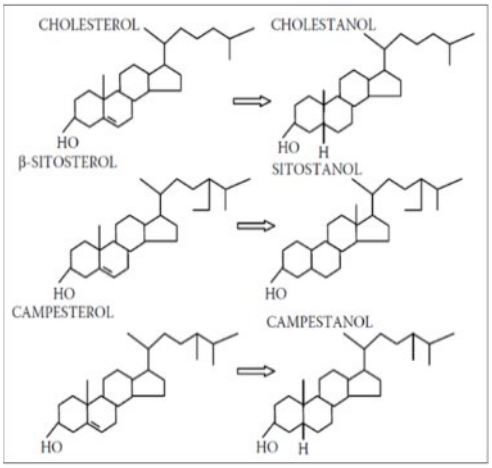
Phenol Group



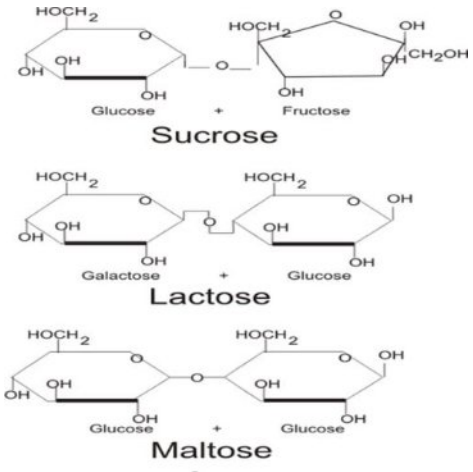
Tannin Group



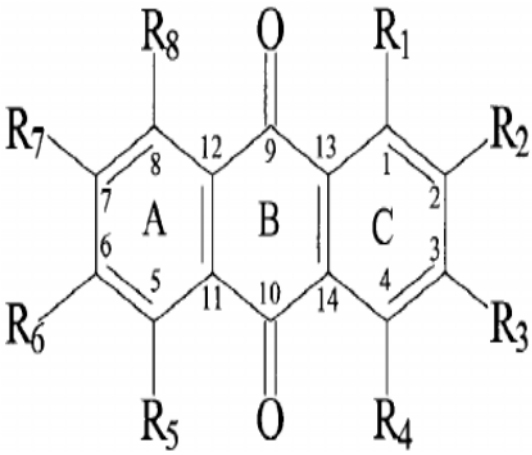
Saponin Group



Phytosterol Group



Reducing Sugar Group



Anathraquinones Group

Figure 3.3 Structures of different phytochemicals.

3.3 Antibacterial activity of methanolic extracts of *C. sinensis*:

An antimicrobial is an agent that kills microorganisms or inhibits their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. The antimicrobial activity of *C. sinensis* was done using the Agar-Well diffusion method. The targeted microbes were assessed to identify their susceptibility for the effect of methanolic peel extract of *C. sinensis*. The three organisms selected were- *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella sp.* The assessment was repeated three times for each organism for better accuracy and validation of the experiment.

3.3.1 Aseptic conditions:

Every time the laminar air flow chamber was cleaned with ethanol to maintain better aseptic condition.

3.3.2 Test organism:

In this experiment the pathogens used were obtained from clinical isolates of icddrb,b that was preserved in the Biotechnology Laboratory, Department of Mathematics And Natural Sciences, BRAC University**.** Three bacterial strains- *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella sp.* were taken for the study.

3.3.3 Maintenance and preparation of inoculums:

Nutrient agar was the culture media for each organism and only fresh cultures were used. In this study only Nutrient Agar media was used. The culture media of nutrient agar was prepared by adding 7.2 mg of NA in a conical flask containing 160 mL of distilled water. Then the mixture was heated in a burner for dissolving completely. Culture media was autoclaved at 121^{o}C for 1 hour. Then the media was poured in the autoclaved petri dishes. The plates are taken and bacteria were sub cultured in these plates. On the day of testing, fresh cell suspensions were prepared for more accurate results. Test organisms were taken in the loop from freshly sub cultured bacteria that was incubated 24 hours preceding the test and disintegrated in individual autoclaved test tubes, each containing 10 mL of 0.9% NaCl to make the separate bacterial cell suspensions. The test tubes were enthusiastically blended utilizing a vortex to guarantee complete disintegration of

the bacterial cells inside the saline. The cell suspensions were of equivalent focus to ensure that there are an equivalent number of cells in each cell plate. The concentration of cell suspensions were checked until it has same or more turbidity than of a 0.5 McFarland Standard. In accordance to that of the 0.5 McFarland the acquired turbidity of the suspensions are adjusted by comparing. Then the cotton swab was dipped into this saline containing each organism

3.3.4 Preparation of extract solution for antibacterial activity test:

The methanol peel extract of *C. sinensis* were dissolved in 0.25% (v/v) autoclaved dimethyl sulphoxide (DMSO) to make five different concentrations of extract solutions for the antibacterial activity tests which were 5 mg/mL, 10 mg/mL, 15 mg/mL, 20mg/mL and 25 mg/mL.

3.3.5 Inoculation of media:

Antimicrobial activity was observed by preparing lawn culture after turbidity of bacterial suspension that was adjusted to equivalent of 0.5 McFarland on nutrient agar plates. The lid was left slightly ajar to allow inoculums to be absorbed before carrying out agar-well diffusion method.

3.3.6 Agar-Well diffusion and application of disks for bioassay of different concentrations of extracts:

The discs of about 0.6mm diameter were aseptically cut out from the inoculated plates using the back of sterile micropipette tips allowing 30mm between adjacent wells and the edge of the petri dishes. 16 µL of each extract were then added into the wells. This process was repeated for the extract three times. Antimicrobial discs were applied as positive control and for comparison. Bend-forceps were sterilized in flame and used to impregnate the discs on the agar by tapping them gently to ensure complete contact with the agar surface.

Care was taken not to relocate the disks once it has come in contact with the agar surface as the drug diffuses almost instantaneously. Plates were kept in an upright position in an incubator to allow the extracts to diffuse into the agar. The plates were incubated for 24 hours in the incubator at 37^0C and observed for zone of inhibition (mm) around the wells.

3.4 Thrombolytic test:

Thrombolysis causes disruption in normal blood flow. It mainly causes blood clot and blocks the bood flow causing necrosis or infarction. Thrombolytic agents act by converting the proenzyme, plasminogen to plasmin, the active enzyme. Plasminogen activators that preferentially activate fibrin-bound plasminogen are fibrin-specific. In contrast, nonspecific plasminogen activators do not discriminate between fibrin-bound and circulating plasminogen. Activation of circulating plasminogen results in the generation of unopposed plasmin that can trigger the systemic lytic state (Fig 3.4).

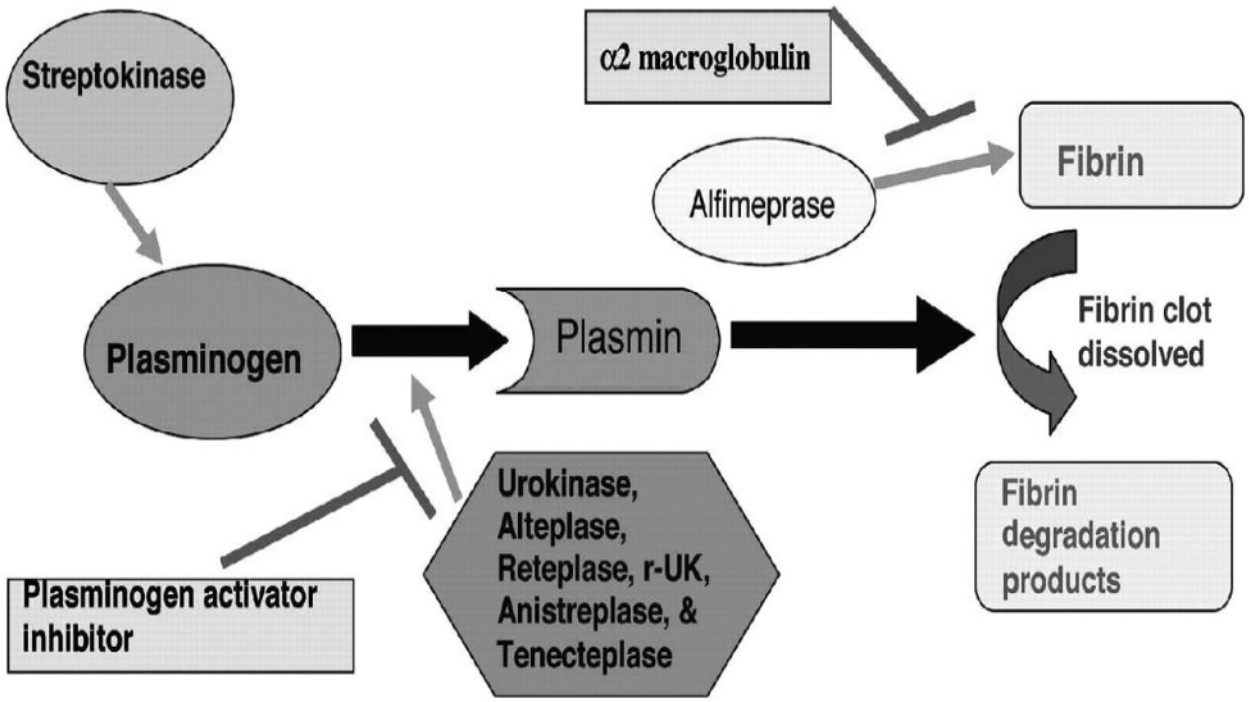


Figure 3.4 Thrombolytic activity in blood. [Jacquelyn L. Baskin, (2012)]:

3.4.1 Collection of blood sample:

For the blood specimens venous blood of two healthy volunteers (male) were taken (age 20-28 years) who has no recent history of oral contraceptive and anticoagulant therapy. Each pre-microcentrifuge tube were weighed and about 5 mL of blood from each volunteers were taken into microcentrifuge tube to form clots, and these were separated from each other by assigning a distinct number to each- A, B, C. The ethical committee of pharmacy department, BRAC University approved the study protocol. Each volunteer has provided the written consent for collecting blood (Hassan, 2012).

3.4.2 Streptokinase:

The commercially available streptokinase (15,00,000 IU) vial (STK, Incepta Pharmaceuticals Ltd., Bangladesh) was collected and 5 mL of methanol was added and mixed properly. The concentration of the streptokinase was adjusted to be 30,000 IU and 100 µL of this suspension was used for in vitro thrombolysis. This was used as the reference standard for thrombolytic activity because it is commonly used as a thrombolytic drug (Fahad Hussain, 2014).

3.4.3 Sample preparation:

Initially 100 mg of plant extract was taken and dissolved in 10 mL of methanol. This was left overnight. Then the mixture was filtered (Fahad Hussain, 2014).

3.4.4 Thrombolytic activity test:

The blood samples were allowed to incubate for 45 minutes at 37^{o}C. After clot formation, serum was completely removed (clot should not be disturbed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube − weight of tube alone). Each microcentrifuge tube containing clot was properly labeled, and 100 µL of plant extract, 100 µL of methanol (as a negative control), 100 µL of 30,000 IU reference streptokinase (as a positive control) were added to tubes with clots. All the tubes were incubated at 37°C for 90 minutes. The fluid left was then carefully removed, and the tubes were weighed again. The difference in weight before and after clot lysis was expressed as percentage of clot lysis (Md. Shahrear Biozid, 2015).

3.4.5 Statistical analysis:

The significance of percentage clot lysis between plants extracts and water by means of the weight difference was tested by the Dunnett*t*-test analysis. Significance was set at both $P< 0.001$ and $P< 0.05$ levels. Data are expressed as mean ± standard error mean. SPSS is a statistical analysis program developed by IBM Corporation;USA was used to for this purpose.

Percentage clot lysis = (weight of the clot after lysis by sample and removal of serum/weight of the clot before lysis by sample) ×100 (Fahad Hussain, 2014).

Chapter 4: Results and discussion

4.1 Antibacterial activity of different concentrations ofmethanolic extract of *C. sinensis*:

In vitro antimicrobial screening of methanolic extracts of *C. Sinensis* were carried out using different concentrations of 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL to have the extent of antimicrobial activity. Ciprofloxacin was used as positive control for this study. The results are shown in Table 4.1.

Bacillus cereus shows the greatest zone of inhibition which increases with the concentration. *Pseudomonas aeruginosa* shows zone of inhibition which increases with the concentration but in the concentration of 20 mg/mL and 25 mg/mL it has same zone of inhibition. *Klebsiella sp* shows the poor zone of inhibition and it has no zone of inhibition in the concentration of 5 mg/mL of *C. sinensis*' methanolic peel extract (Fig 4.1).

Table 4.1 Zone of inhibition of the sample and positive control:

Methanolic extract of*C. sinensis*' peel	Zone of inhibition (mm)			
Concentration (mg/mL)	Positive control (Ciprofloxacin)	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Klebsiella sp*.
5	18.5	7	10	_
10	19.3	9	12	3
15	20.9	11	12	7
20	21	12	13	9
25	22.5	12	14	9.9

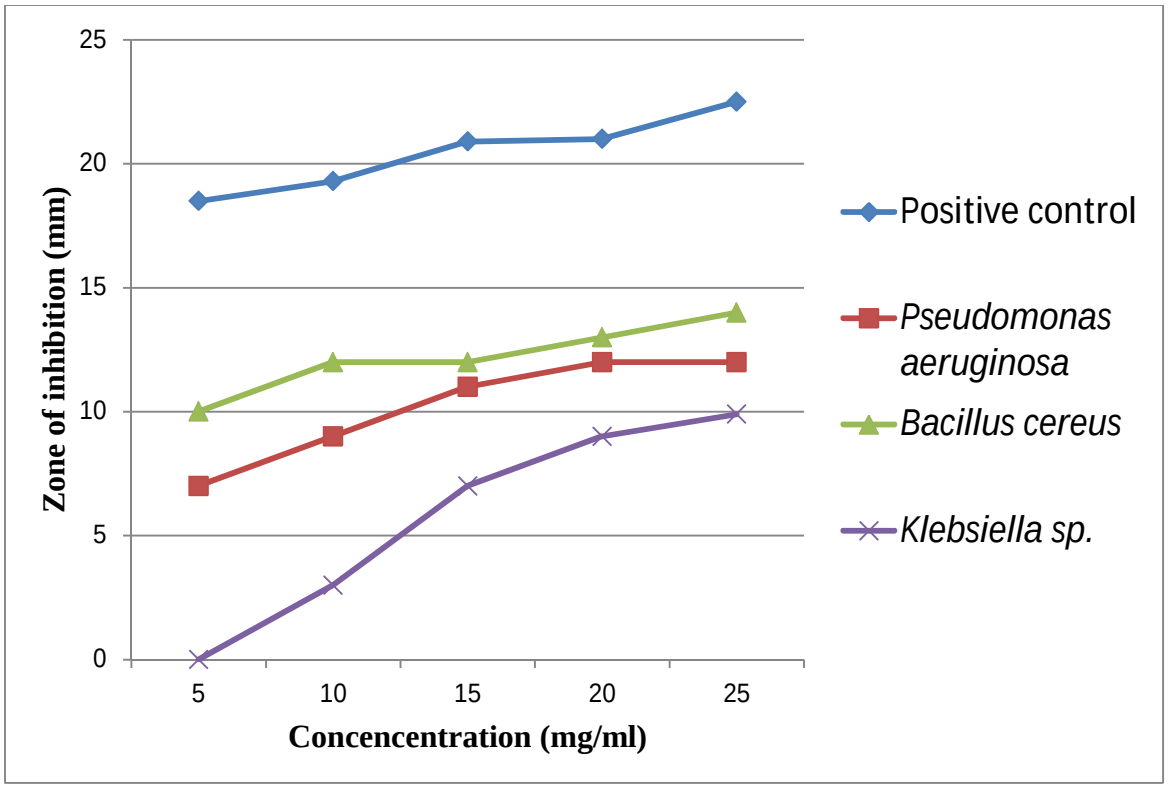


Figure 4.1 Graphical relation for antimicrobial test

4.2 Results of the thrombolytic test:

The percentage of clot lysis of positive control was 66.12%. The sample methanolic extract of *C. sinensis* peel shows weak percentage of clot lysis- 26.96% (Fig 4.2).

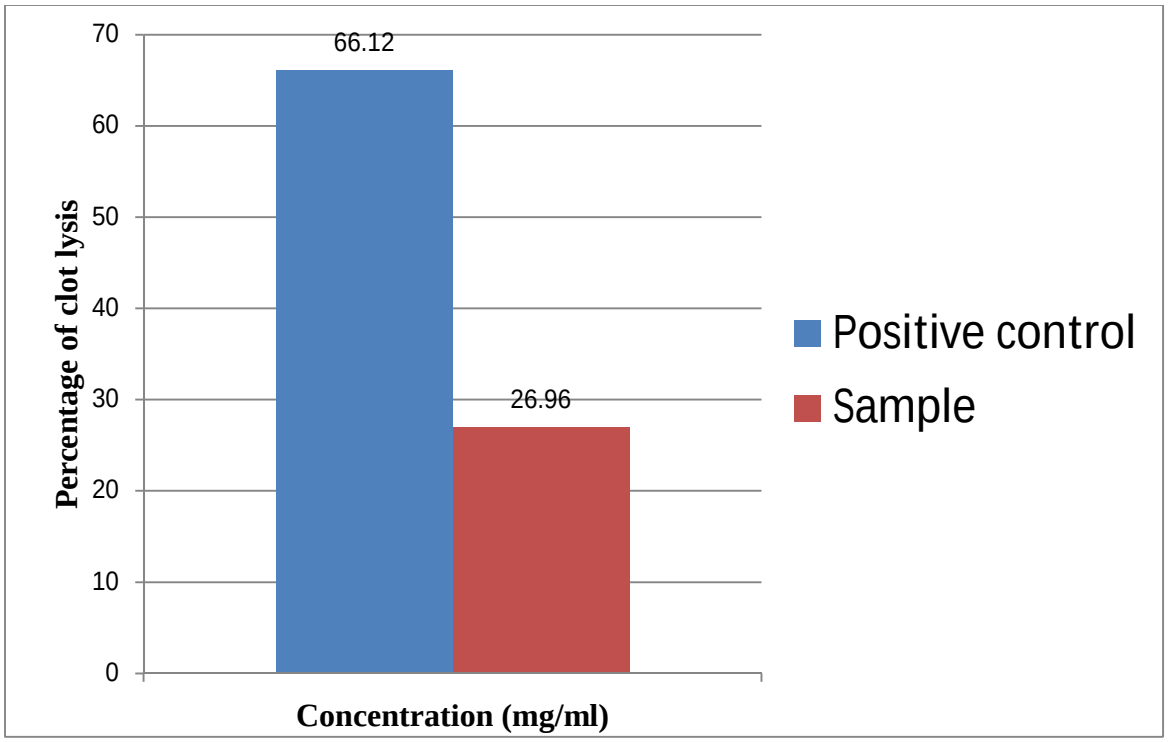


Figure 4.2 Graphical representation of thrombolytic test

Chapter 5: Conclusion

Herbal medicines are produced from different parts of particular plants, for example- seeds, roots, stems, barks, leaves, berries etc. The components determined by the phytochemical screening of the methanolic peel extract of *C. sinensis* showed the presence of various valuable chemicals like- reducing sugars, tannins, phytosterols etc. The result of the present study revealed that the methanolic extract of the *C. sinesis* peel have antimicrobial activity. It can be used as a therapeutic agent in the treatment of gastrointestinal infection. Further detailing study is necessary to observe the mechanism of impacts closely and to draw extractive conclusion.

Thrombolytic study of *C. sinensis* shows that it has thrombolytic activity which was weak but using different isolation techniques the constituents could be separated and further investigation on thrombolytic activity can be done. Therefore it could be used for the diagnosis of patients and as it is an herbal drug it would have fewer side effects which could be a better choice for mankind. The glorious environment of Bangladesh is great for citrus generation which gave a proper chance for agronomic practices and which are taken after for gaining splendid citrus organic product. Wise use of symptoms from plant sources can moreover be valuable for most great utilization of normal sustenance's and meanwhile help with environment protection.

If all together things are taken, an amazing number of settled data of affirmation has avowed that *C. sinensis* peel show a critical scope of viable common activities. Impressive outcome of *C. sinensis* to have great bioavailability which along these lines pulls in authorities to perform sensible studies for intense ailment balancing activity. Moreover, treatment besides it has demonstrated that it has no deadly impact.

References

Abeloff: Abeloff's Clinical Oncology, 4th ed. Philadelphia, PA: Churchill Livingstone, An Imprint of Elsevier. 2008.

Altschuler, J.A, Casella S.J, MacKenzie T.A, Curtis K.M. (2007). The effect of cinnamon on A1C among adolescents with type 1 diabetes. *Diabetes Care*. 30(4), 813-6.

Ashok, K.(2011). Antimicrobial activity and phytochemical analysis of citrus fruit peels - utilization of fruit waste. *International Journal Engineering Science and Technology*, 3(6), 5414 – 5421.

Birks, J. (2007). Ginkgo biloba for cognitive impairment and dementia. *CochraneDatabaseSyst Rev*, (2)

Bright, J.J. (2007). Curcumin and autoimmune disease. *AdvExp Med Biol*, (595), 425-51.

Botanical-online. (1999-2016). Retrieved from www.botanical-online.com: http://www.botanical-online.com/english/historyofmedicinalplants.htm

Damery, S.Gratus, C. and Grieve R. (2011). The use of herbal medicines by people with cancer: a cross-sectional survey. *Br J Cancer*, 104(6):927-33.

Don, M. (2010). "Discover the tradition of healing with citrus peel—the zesty, invigorating flavor can help support your health."

Essen Bioscience. (2016). Retrieved from www.essenbioscience.com: http://www.essenbioscience.com/applications/cytotoxicity/

FahadHussain, M. A. (2014). In vitro thrombolytic potential of root extracts of four medicinal plants available in Bangladesh. *NCBI* , 162-164.

Faturi, C.B., Leite, J.R., Alves, P.B., Canton, A.C. and Teixeira silva F. (2010). Anxiolytic effect of sweet orange aroma in wistar rat. *E pub*. 34, 605-609

Faturi, C.B., Leite, J.R., Alves, P.B., Canton, A.C. and Teixeira silva F. (2010). Anxiolytic effect of sweet orange aroma in wistar rat. *E pub*. 34, 15-19

Food and agriculture organization of United Nations. (2016). Retrieved from www.fao.org: http://www.fao.org/docrep/010/ai387e/AI387E08.htm

Ghani, A. (1990). Present state-of-the art of traditional medicine practice in Bangladesh.

Guarnieri, S., Riso, P. and Porrini, M. (2007). Orange juice vs vitamin C: effect on hydrogen peroxide-induced DNA damage in mononuclear blood cells. *Br j. Nutr*. 97, 639-643.

Hamid, A. U. (2010, June 25). Antioxidants: Its medicinal and pharmacological applications. *African Journal of Pure and Applied Chemistry, 4(8)*, 142-151.

Hassan, B. A. (2012). Medicinal plants (Importance and uses). *PharmaceuticaAnalyticaActa*, 3(10).

Jacquelyn, L. (2012). Thrombolytic therapy for central venous catheter occlusion. *Haemotologia*, 641-652.

Julius, O., Ebangha, E., Shil, K., Walter, C., Robert, G. and Deanne, D. (2009). Citrus extracts benefit joint health in osteoarthritis patients: a pilot, double-blind, placebo-controlled Study.*Biomed central*. 1, 8-38.

Kurowska, E.M. et al. (2004). Hypolipidemic effects and absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia. *J Agric Food Chem*, (19), 2879-2886.

Md. ShahrearBiozid, M. N. (2015). A comparative study of thrombolytic effects of methanolic. *The pharmainnovation* , 05-07.

Milind, P. (2012). Orange range of benefits.*IRJP*,3(7), 61.

Parmar, H.S. (2008). Medicinal values of fruit peels from *C. sinensis*, *Punicagranatum* and *Musa paradisiacal* with respect to alterations in tissue lipid peroxidation and serum concentration of glucose, insulin and thyroid hormones. *J Med Food*, (11), 376-381.

Rahmatullah, M. A. (2016). A survey of medicinal plants used by folk medicinal practitioners in two villages of Tangail district, Bangladesh. *American-Eurasian journal of sustainable agriculture,* 556-566.

Reference. (2016). Retrieved from www.reference.com: https://www.reference.com/science/phytochemical-screening-6c0234abce82bf22#

Saxena, S. N. (2013). Phytochemistry of Medicinal Plants. *Journal of pharmacognosy and phytochemistry*, 1(6), 168-182.

Sharma, N. (2008). Effects of C. sinensis (L.) Osbeckepicarp essential oil on growth and morphogenesis of Aspergillusniger (L.). *Microbiological research*, (163) 337-344.

Sissay, B.M. (2006).*C. sinensis* disease survey: knowledge, attitude and management practices in Ethiopia.*Tropical research journal*, (4), 1-9

Strange, R.R. (1993). An antifungal compound produced by grapefruit and valencia orange after wounding of the peel. Journal of Natural Products, (56), 1627-1629.

Tanaka, Y., Makita H, Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Fukutani, K., Tanaka, T. and Ogawa, H. (1997). Modulation of N- methyl-N-nitrosamine-induced rat oesophagealtumorigenesis by dietary feeding of diosmin and hesperidin, both alone and in combination. *Carcinogenesis, Agricultural and food chemistry.*18, 761-769.

Tanaka, Y., Makita H, Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Fukutani, K., Tanaka, T. and Ogawa, H. (1997). Modulation of N- methyl-N-nitrosamine-induced rat oesophagealtumorigenesis by dietary feeding of diosmin and hesperidin, both alone and in combination. *Carcinogenesis, Agricultural and food chemistry.*18, 957-965.

Tiwari, P. K. (2011). Phytochemical screening and extraction: A review. *International Pharrmaceutica Sciencia,* 1(1), 98-106.

Vivek, K.R. (2010). Anti typhoid activity of aqueous extract of fruit peel *C. sinensis. IJPRD*, (2), 217-219.

Wiesman, Z. and Chapagain, B.P. (2005).Larvicidal of aqueous extracts of Balanitesaegyptiaca (desert date) against the larvae of culexpipiens mosquitoes. *Afr. Biotechnoln*. 4, 1351-1354.

(2010). Antioxidants.american dietetic association .

Healing wonders of Philippine medicinal plants. (2015). Retrieved from Filipino Herbs Healing Wonders: http://www.filipinoherbshealingwonders.filipinovegetarianrecipe.com/pandan.htm

(2016). Retrieved from https://www.reference.com/science/phytochemical-screening-6c0234abce82bf22#