Phytochemical screening and evaluation of hypoglycemic activity of *Citrus sinensis* peel extract

A project

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Certification Statement

This is to certify that this project titled ‘ Phytochemical screening and evaluation of hypoglycemic activity of *Citrus sinensis* peel extract ’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.

Signed,

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Countersigned by the supervisor

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Acknowledgement

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Dedicated to my parents, who sacrificed their every desire since my birth to make me a real man and inspire me in every steps of my life.

Abstract

Orange has been traditionally used as an adjuvant in different diseases including diabetes, tuberculosis, asthma and hypertension. Phytochemically, entire plant contains flavonoids, saponinss, tannins, glycosides, limonene, citral, neohesperidin, naringin, rutin, rhamnose, eriocitrin, and vitamin-C.

An examination on the impacts of convergences of peel extract from *Citrus sinensis* in hyperglycemic Albino mice uncovered the glucose lowering activity of *Citrus sinensis* . In this test, the methanolic extract of the fruit peel was assessed in two dosage regimen- 200mg/kg and 400mg/kg, regarding the regulation of glucose incited diabetes mellitusand both the doses were find to lower the serum glucose level , uncovering the antihyperglycemic activity of the methanolic extract of *Citrus sinensis* peel. In the Brine Shrimp Lethality Toxicology study, the bioactivity of the extract was found sound and the phytochemical screening of the methanolic extract of the peel showed presence of different constituents like flavonoids, alkaloids etc.

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List of Abbreviations

BSL: Brine Shrimp Lethality

ME: Methyl extract

CLT: Controlled group

STD: Standard group

PMFs: Polymethoxylated flavonoids

TCM: Traditional Chinese Medicine

DMSO: Dimethyl sulfoxide

Chapter One: Introduction

1.1 Herbal Drug:

The word *Herbal* comes from the word *herb* and it includes the description of plant parts which is used for medicinal purposes. The study of herbal drug includes the use of medicinal herbs to prevent and treat diseases and to promote health and healing. Alternatively it can be said that, a drug or preparation of drug made from a plant or plants parts which are useful for human health and also has no or minimal side effects are known as herbal drug. (Abeloff et al., 2008).

Additionally, herbal medicine can also be termed as plant pharmaceutical or phytomedicine-alludes which utilizes plant seeds, roots, fruits, leaves, bark, or blooms for therapeutic purposes. Herbalism has been applied to treat illness from ancient times. Investigation on medicinal plants and quality control of herbal medicine has been turning out to be more standard and developed with time and significant advancement has been seen in clinical studies of herbal medications in treatment of various diseases. (Altschuler et al., 2007).

1.1.1 History of herbal medicine:

Plants had been utilized for restorative purposes much sooner than written history. Therapeutic uses for plants were reported in 3,000 BC as found from the depictions of the Old Chinese and Egyptian papyrus compositions. African, Native American and other indigenous societies have utilized herbs as a part of their recuperating ceremonies, while others created therapeutic customary frameworks, (for example, Ayurveda and Traditional Chinese Medicine) in which home grown treatments were utilized. Scientists found that in different regions of the world there was a tendency to utilize the similar or comparative plants for the identical purposes.
(Birks et al., 2007).
Around 1950, when experiments on different substances first got to be accessible, researchers began to do researches on plant extracts. (Bright et al., 2007).

Later, the World Health Organization or WHO evaluated that 80% of individuals around the world rely on home grown plant medicine or herbal medicine. In Germany, almost 70% of German doctors prescribe herbal medicine where around 600 - 700 plant based pharmaceuticals are accessible. 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 home grown drug utilization. (Damery et al., 2011).

1.1.2 Advantages of Herbal Drug:

There are abundant benefits of herbal drug like all other alternative medicines. It is known that Allopathic medicines are very expensive in comparison with herbal medicines which are of very low cost. Some herbal medicine plant can even be planted at home. Herbal medicines have been proven to be effective to the body for the process of natural detoxification. Herbal medicines might be used to clear the colon, to improve digestion and food intake. Herbal medicines are also helpful for immune system as they are very effective in healing many types of intestinal disorders like colitis, indigestion, peptic ulcers and irregular bowel movement. This kind of medicines is necessary for patients who are allergic to many types of allopathic drug. They can also be used to remove weight by regular appetite.

Chapter Two: Review of literature

2.1 *Citrus sinensis*:

In general language *Citrus sinensis* is also called sweet orange. The family of this fruit is Rutaceae which is found in tropical and subtropical areas in Southeast Asia. The fruits of Citrus are plentiful sources of bioactive compounds which have health giving effect on human body like vitamin-C, carotinoids, flavonoids, limonoids, essential oils, acridone alkaloids, minerals and vitamine-B complex.

Citrus sinensis fruits also contain phytochemical compounds like flavanones, polyphenols, anthocyanins and hydroxycinnamic acids which are useful mainly in pathological conditions like inflammation, high cholesterol related diabetes and cancer etc.

In this world more than 130 countries are farming *C.sinensis* Countries like Spain, UK, USA, Germany, Holland, Brazil, China, France, and India are mainly cultivating this fruit for their medicinal and economical purposes.

C.sinensis is available mainly in winter season. (Milind et al., 2012).

2.2 History of *Citrus sinensis*:

Sweet orange (*C.sinensis*) is mostly cropped and uncommonly found in the forests. Southern China and North-eastern India first planted this fruit. In European history, the first recording of citrus fruits was found to be in 350 BC by Theophrastus and the fruit was further introduced by Alexander the Great. In the early European history, many writers described the Persian sweet orange which had a beautiful, amazing smell and they thought this fruit to be toxic. In Europe *Citrus* was first known to be a citron. Later on , the edibility of sweet orange was reported . Alexander the Great was very fond of this fruit and it's smell. 11th century the Parisan orange was introduced in Italy and after that Southern Europe broadly planted this fruit which was bitter in taste and that was basically planted for medicinal purposes. After 1450 AD, Italian traders introduced it in Mediterian area. In 1493 AD Christopher Columbus brought the seed of sweet orange to Haiti and Caribbean region on the very second journey of sea in his travelling life.

Portuguese navigators have also been credited for taking *C.sinensis* orange tree to Mediterranean region around 1500 AD. Spaniards introduced the sweet orange into South America and Mexico in the mid-1500s. In Europe, orange was well introduced as an eatable fruit in 1646. Orange trees had survived on American soil, since the independence of the country was declared, and was harvested in Florida (in 1820s) and California (in 1870s) as economical plant. In normal diet sweet oranges were not included by the Americans until around 1880s. When refrigerators were introduced and heavy transport system was advanced, sweet oranges were distributed country wide. (Sissay et al., 2006).

2.3. Botanical Description of *Citrus sinensis*:

On the planet the greater part of the trees hues are green however *Citrus sinensis* is orange in most dark green blooming tree. Orange tree is fundamentally 9-10m long (in spite of the fact that which examples are extremely old those may be ache for 15m). The length of leaves of these trees is 4-10 cm is arranged alternatively. These leaves are in oval shape and have crenulated margins. These trees barks look like gray brown to greenish in color and those barks are very smooth. (Webber et al., 1903).

Table 1: Botanical relegation of Sweet Orange

Kingdom	Plantae
Division	Magnoliophyta
Class	Dicotylidones
Subclass	Sapindales
Order	Rosidae
Family	Rutaceae
Subfamily	Aurantoideae
Genera	Citrus
Subgenera	Papeda
Species	Sinensis

[Source: Milind et al., 2012].

Fruit:

The *C.sinensis* natural product's similar to a hesperidium, it can be characterize as a classification of organic product like berry that is extensively extends inside and out, color, figure and juice highlight. *Citrus sinensis* are globosely to dodge fit as a fiddle. In common orange organic product has an unruffled external surface shin and whole is loaded with petiole wings. The petioles of sweet oranges leaves are much littler than that of harsh orange.

Table 2: International synonyms of *Citrus sinensis*

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

[Source: Milind et al., 2012].

Seeds:

Citrus sinensis or saccharine orange has withal seeds in their juice part. Seeds are green to pastel white in color, smooth and sharp cornered. Rudimentally the seed is immature. The immature seeds are either “zygotic” or “nuclear”. (Milind et al., 2012).

Flowers:

Maximum flowers of sweet orange have saccharine smell. This flower is fundamentally represented with five petals. This flower additionally constitutes some oil glands. The stamen

number extent from give or take 20-40. The ovary of sub circular is superior which having 8-18 spaces of fruit (cavities) having 4-8 spore per spaces in two rows. Flowers sizes are diminutive and those colors are waxy greenish white. (Valiant et al., 2004).

Leaves:

Leaves surface are smooth, orange leaves shape are ovoid or egg shaped. Colors are tenebrous green and effulgent carrying a special perfume often same to the fruit of orange. The leaves of petioles are fundamentally winged. The leaf of sweet orange of incipient branch is green and those are intersections in vexed area and these have auxiliary single spine, whether the leaf of first-born branches and division are not single spine and those are round in vexed area.

2.4. Phytoconstituents of *Citrus sinensis*:

Citrus sinensis fruit constituent is essential oil which is about 1.5%. The most important phytoconstituents existing in C.sinensis fruits are D-limonene (amount: 90%), citral, citronellal, nootkaton, sinesal, n-nonanal, n- decanal, n-dodecanal, linalyl acetate, geranyl acetate, citronelyl acetate and anthranil acid methyl ester. Lipophilic flavonoids and furanocumarines are presented in squeeze oils. There is some approval that active ingredients of *C.sinensis* stimulate the secretion of gastric juice. *C.sinensis* also contains several bitter flavone glycosides likes neohesperidin and naringine, whose neohesperidose is a sugar component and rutinose is also a sugar component of rutin. It is sayed that both of two sugars are disaccharide of glucose and rhamnose (6-desoxymannose). (Ihrig et al., 1995).

Table 3: Phytoconstituents of *Citrus sinensis*

Srl No.	Plant Part	Phytoconstituents
1	Leaves	Terpenoids; Linalool, β-elemene
2	Flowers	Triterpenes; Limonene
3	Fruits	Minerals: Calcium, Iron, Magnesium, Zinc, Phosphorus, Potassium Vitamins: B1, B2, B3, B5, B6, and Vitamin C
4	Fruit Peel	Triterpene; Limonene, citrol Pigment; Anthocyanin, β-cryptoxanthin, Crytpoxanthin, Zeaxanthin, Flavone glycosides; Neohesperidin, Naringin, Hesperidin, Narirutin, and Rutin, Eriocitrin, Homocysteine, Polymethoxylated flavones; Tangeretin and Nobiletin Flavonoids; Citacridone, Citbrasine and Noradrenaline

[Source: Milind et al., 2012].

2.5. Bioactivity of *Citrus sinensis*:

2.5.1. Antioxidant property:

Many plants contain several active photochemical compounds including vitamins, flavonoids, topenoids, carotenoids, cumarins, lignin, saponin, plant sterol etc which have antioxidant property. *Citrus sinensis* fruits and juices are vital source of bioactive compounds including antioxidant such as ascorbic acid, flavonoids, phenolic compounds and pectins that are necessary for health nutrition. (Lucia et al., 2008; Fernandez-L et al., 2005; Ebrahimzadeh et al., 2008).

Hesperidin methyl Chalcone is also an active compound and it is a ketone as well as a polyol and used as an antioxidant . It is known as bioflavonoid which origin is *Citrus*. *sinensis* outer surface of skin and specifically in the most outer surface of sweet oranges. (Jayaprakasha et al., 2008).

C. sinensis is a plentiful source of vitamin C, flavonoids, phenolic compounds and pectins. Amongst these compounds, flavanoids are main compound which get going on citrus species; those are hesperidine, narirutine, naringin and eriocitrin. (Kumar et al., 2011). But one citrus species orange provides 116% of the regular necessity of vitamin C. Vitamin C is an essential water soluble anti-cancer agent. Vitamin C is also very essential to improve immune system. For reducing cold cough and recurrent ear infection, vitamin C is very effective.

2.5.2. Protection against Cardiovascular Diseases:

As per World Health Organization's latest report, citrus natural products offer assurance against cardiovascular illnesses by diminishing levels of homocysteine. *Citrus sinensis* contains vitamin C, carotenoids and flavonoids, which are cardio defensive. Cholesterol bringing down impact of orange is created by Limonene. Polymethoxylated flavones (PMFs) are available in *Citrus sinensis* peel, which can lower cholesterol more successfully than some professionally prescribed medications, without demonstrating any side effects. In spite of the fact that, citrus natural products contains PMFs, the most generally perceived PMFs are tangeretin and nobiletin, which are found in the peels of oranges. PMFs work like statin prescriptions.

2.5.3. Anti-carcinogenic property:

Limonene, one of the primary constituents of orange, diminishes the danger of mouth, skin, lung, diseases, and stomach and colon tumor. Another constituent of orange is hesperidin, has additionally displayed effectiveness against cancer-causing elements in different in vivo studies. The polymethoxylated flavonoids have demonstrated effect against malignant 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 having lung cancer. (Kurowska et al., 2004).

2.5.4. Reduced risk of kidney stones:

In a study of the British Journal of Nutrition, it was found that when ladies drank 1/2 liter of squeezed orange every day, their urinary pH esteem and citrus extract discharge expanded consequently lessening the danger of shaping calcium oxalate stones essentially. (Tanaka et al., 1997).

2.5.5. Anti-bacterial activity:

Oranges are eaten to assuage 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. (Honow et al., 2003).

2.5.6. Anti-inflammatory, Healing and Anti-arthritic activity:

Wounds are generally described as physical injuries that result in an opening or breaking of the skin. The healing property of orange depends on upon wide blend of phytonutrients, for instance, citrus flavonoids (hesperidin and naringenin), anthocyanins, hydroxycinnamic acids, and a blended pack of polyphenols. The most important flavone in orange is hesperidin that has been shown to abatement hypertension and also cholesterol in animal studies. (Haiqing et al., 2004).

Carotenoids, zeaxanthin and β-cryptoxanthin, are the phytonutrients, which lessen amazingly the pain of rheumatoid joint. Those persons consuming high amount of zeaxanthin and cryptoxanthin have exhibited 52% less dangers of making rheumatoid joint pain. *Citrus sinensis* (orange) peel extracts contain bio-flavonoids, including polymethoxylated flavones (PMFs), which have cooling, tumor counteractive action and hypolipidemic effect, cell reinforcement specialists and so on. (Sandhya et al., 2011).

2.5.7. Anti-fungal activity:

Citrus sinensis peel oil is an effective inhibitor of fungal infection. The antifungal constituents of orange are limonene (84.2%), linalol (4.4%) and myrcene (4.1%). (Julius et al., 2009).

2.5.8. Anti-ulcer property:

Eating orange decreases the disease occurrence with *Helicobacter pylori* and in this way turning away advancement of ulcers. (Sharma et al., 2008).

2.5.9. Anti-diabetic activity:

Against diabetes, orange shows effect because of bio-flavonoids, for instance, hesperidin and naringin present in citrus peels. These peels play an important role against diabetes. They lessen the activity of glucose-6-phosphatase and phosphoenol pyruvate and thus are responsible for decreasing serum glucose level.(Faturi et al., 2010; Leite et al., 2010).

2.6. Traditional Uses of *Citrus sinensis* Peel :

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

For a long time, Traditional Chinese Medicine (TCM) have utilized sweet orange peel, known as chen pi or ju pi in Chinese pharmaceutical, to enhance assimilation, to calm intestinal gas and bloating, and to remove mucus. This peel is useful in the diseases of digestive and respiratory systems.

Citrus sinensis peel has also been 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).

2.7. Medicinal uses of *Citrus sinensis:*

Sweet oranges are useful in the administration of:

- Arthritis
- Asthma
- Alzheimer's Disease
- Parkinson's Disease
- Macular degeneration
- Diabetes mellitus
- Gallstones
- Multiple sclerosis
- Cholera
- Gingivitis
- Optimal lung capacity
- Cataracts
- Ulcerative colitis
- Crohn's Disease

[Source: Don et al., 2010].

2.8. Nutritional Value:

A solitary orange gives 12.5% of the everyday requisite for fiber, which has been demonstrated to decrease elevated cholesterol levels in this manner accommodating to evade atherosclerosis, in holding glucose levels under control, which may justify why oranges can be an used as effective medicine in diabetes. The fiber in oranges can get disease bringing on chemicals and keep them far from cells of the colon, giving yet a different line of security from colon tumor. (Milind et al., 2012).

Chapter Three: Preparation and phytochemical screening of plant extracts

3.1. Collection and identification:

The entire plant was gathered from Dhaka, Bangladesh and recognized by the taxonomist of the national herbarium of Bangladesh, Mirpur, Dhaka. The voucher examples of the plants have been store in the herbarium for further references.

Figure 1: Herbarium sheet of *Citrus sinensis*

3.2. Plant material preparation

Crisp *Citrus sinensis* fruit peels were amassed from in the neighborhood market in the month of June 2015. The sweet orange were washed well using distilled water .The peel is divided , then the mash was separated by cutting them into scintilla then it was shed sun dried for a time of 6-7 days, at a surrounding temperature of 30˚C. The dried specimens were crushed harmoniously using a mortar and pestle and later using a processor, to acquire the powdered structure. The powder of the peels and the pulps were put away discretely in sealed shut containers.

3.3. Preparation of Extract:

Extraction procedure was as follows-

1. 850g *Citrus sinensis* peel was soaked in 1450 ml methanol in a jar.
2. The jar was closed and kept for seven days.
3. The mixture was stirred well every day.
4. Due to some solvent loss by evaporation, about 200 ml methanol was added in each jar and the jars were sealed with foil paper.
5. After seven days, the soaked peel was filtered by cloth, cotton and Whatman filter paper.
6. The filter was dried in rotary evaporator at 50˚C for 40 minutes where the rotation speed was 100 rpm.
7. Then it was poured in a beaker and kept in the fume hood for further evaporation of the solvent.
8. After a week, sticky extract was obtained which was kept in a dry place in normal temperature.
9. The crude extract was used for photochemical and pharmacological evaporation.

3.4. Phytochemical screening:

Phytochemical examinations of the extracts were performed as per the protocol depicted by Sofowora (1994) also, Harborne and Harborne (1998).

Table 4: Phytochemical screening test and result

Sr No	Name of the test	Procedure	Observation	Result
1	Tannin Test	1ml ferric chloride was taken in a test tube and 2ml extract was added there.	Blue-black colored precipitate was formed.	Tannins were present.
2	Flavonoid test / Lead acetate test	1 ml of lead acetate was treated to 3 ml of extract.	Yellow colored precipitate was formed.	Flavonoids were present.
3	Alkaloid test / Wagner's test	1 ml of extract was treated with few drops of Wagner's reagent.	Orange brown precipitate was formed.	Alkaloids were present.
4	Saponins test	Around 2 ml of extract was treated with 5 ml of distilled water and the solution was shaken vigorously for 20 second.	Foam was not formed that lasted for more than 10 minutes.	Saponins were not present.
5	Steroid test	1ml extract was treated with 5 drops of concentrated sulfuric acid.	A red colored Indication was given.	Steroids were present.
6	Terpenoid test	2ml chloroform, 3ml concentrated sulfuric acid and 5ml extract was taken in a test tube.	Brown colored indication was given.	Terpenoids were present.
7	Glycoside test	5ml extract, 2ml glacial acetic acid, 1 drop of ferric chloride and 1ml of concentrated sulfuric acid was taken in a test tube.	Brown colored ring was formed.	Glycosides were present.

[Source: Oikeh et al., 2013]

Chapter Four: Evaluation of Brine Shrimp Lethality Bioassay

4.1. Principle:

For quick and far reaching bioassay of the plant extracts and synthesized compounds, Brine Shrimp Lethality Bioassay is a well known test. By performing this technique, bioactivity of plant extracts can be predicted. In this system, In vivo lethality in Brine shrimp nauplii is used as a useful screen for screening of the bioactive items.

Bioactive compounds are frequently lethal to living body when higher concentrations are administered. Brine shrimp lethality bioassay helps to assess the toxicological part of the bioactive preparations.

As it is a quick process (circadian), reasonable and obliges no unique gear or aseptic system, Brine Shrimp Lethality Bioassay is considered as a better experiment than other cytotoxicity testing techniques. It can be performed for measurable acceptance in cytotoxicity study and generally small amount of test sample is required (2-20 mg or less). This bioassay can also be used in antimicrobial, antiviral, pesticidal and anticancer studies. (Meyer et al., 1982; McLaughlin et al., 1988).

In this study, the cytotoxicity test was performed on brine shrimp nauplii as indicated by Mayer system (Hossain et al., 2004). Brine shrimp naupli can be hatched by incubating the shrimp eggs in simulated seawater. To dissolve the test examples, Dimethyl sulfoxide (DMSO) was used. The naupli were checked by visual assessment and were taken in test tubes containing 5 ml of simulated sea water where test samples were dissolved. The test tubes were left for 24 hours. After that, the number of alive nauplii was counted in each test tubes and these information were used in calculating LC_{50} values. (Meyer et al., 1982)

4.2. Apparatus:

- Little tank with punctured separating dam to incubate the shrimps
- Lamp
- 20 Test tubes.
- Test tube holder
- Pipettes, micropipette (50-100 μl)
- Glass vials (10ml)
- Magnifying glass
- Dropper
- Test samples containing experimenting compounds

4.3. Chemical Ingredients:

- *Artemia salina* leach (brine shrimp eggs)
- Sea salt - Sodium Chloride, NaCl

4.4. Preparation of Artificial Sea water:

38 gm salt (NaCl) was measured and dissolved in one liter of distilled water and cotton filtered for two times to remove any impurities or darts.

4.5. Hatching of Brine shrimp:

Brine shrimp eggs were gotten from the BRAC University Laboratory in Mohakhali, as a blessing specimen for the exploration work. Separated, simulated seawater was made by dissolving 38 g of ocean salt in 1 liter of distilled water for hatching the shrimp eggs. The seawater was placed in a little glass compartment (incubating chamber) with a parcel for dull (secured) and light zones. Shrimp eggs were joined into the dull side of the chamber while the light over the other side (light) will polarize the brought forth shrimp. Two days ,after the shrimp eggs were hatched forth and became adult as naupli (hatchling). Following two days, when the

shrimp eggs hatchings was done, 5 mL of the artificial seawater was incorporated to every test tube and 10 brine shrimp naupli were brought into each tube. In this manner, there was a sum of 10 shrimps for each weakening. At that point the volume was balanced with simulated seawater up to 5 mL for each test tube. The test tubes were left uncovered under the light. The quantity of surviving shrimps were tallied and recorded following 24 hours. Using probit investigation, the lethality fixation (LC_{50}) was evaluated at 95% certainty interims. This is to discover that the mortality of the naupli against different concentration of extract.

4.6. Preparation of test samples:

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of each of the test sample and ten test tubes were taken for standard drug vincristine sulphate and for negative control test ,dimethyl sulfoxide was taken. 4 mg of test sample was taken and dissolved in 100 µl of pure dimethyl sulfoxide (DMSO) in glass vials to get stock solutions. Then 50 µl of solutions was taken in test tube each containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus the final concentration of the prepared solution in the first test tube was 400 µg/ml. Then a series of solutions having varying concentrations were prepared from the stock solution by serial dilution method. In each case 50 µl of sample was added to test tube and fresh 50 µl DMSO was added to vial. Thus the concentrations of the obtained solutions in each test tube were as Table.

Table 5: Preparation of test samples

Test Tube No	Concentration
1	400
2	200
3	100
4	50
5	25
6	12.5
7	6.25
8	3.125
9	1.56
10	0.781

[Source: Oikeh et al., 2013]

4.8. Preparation of the positive control group

In the present study, Vincristine Sulfate was utilized as the positive control. Measured amount of the Vincristine Sulfate was dissolved in DMSO to get an initial concentration of 20 µg/ml and serial dilution was done utilizing DMSO to get 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.039 µg/ml. The standard solution of different concentrations was added to test tubes containing ten living naupli in 5ml of simulated salt water.

4.9. Preparation of the negative control group

50 µl of DMSO was added to each of three pre-marked test tubes containing 5 ml of simulated sea water and 10 brine shrimp naupli. These test tubes were used as control groups. If the brine shrimps in these test tubes show a rapid mortality rate, then the test is considered as invalid because the naupli died due to some reason other than cytotoxicity of the compounds. It may be died due to the toxic action of the solvent or the prepared simulated seawater.

4.10. Counting of nauplii

After 24 hours, the test tubes were inspected accurately using a magnifying glass and the number of survivors of shrimp nauplii were counted. The percentage (%) of mortality was calculated for each dilution of concentration.

Table 6: Effect of vincristine sulphate (Positive Control) on shrimp nauplii

Sl No	Conc(µg/ml)	Log C	% mortality of Vincristine sulphate	LC_{50} (µg/ml)
1	40	1.60206	100	1.283
2	20	1.30103	90	
3	10	1	90	
4	5	0.69897	90	
5	2.5	0.39794	80	
6	1.25	0.09691	70	
7	0.625	-0.20412	40	
8	0.3125	-0.50515	0	
9	0.156	-0.80688	0	
10	0.078	-1.10791	0	

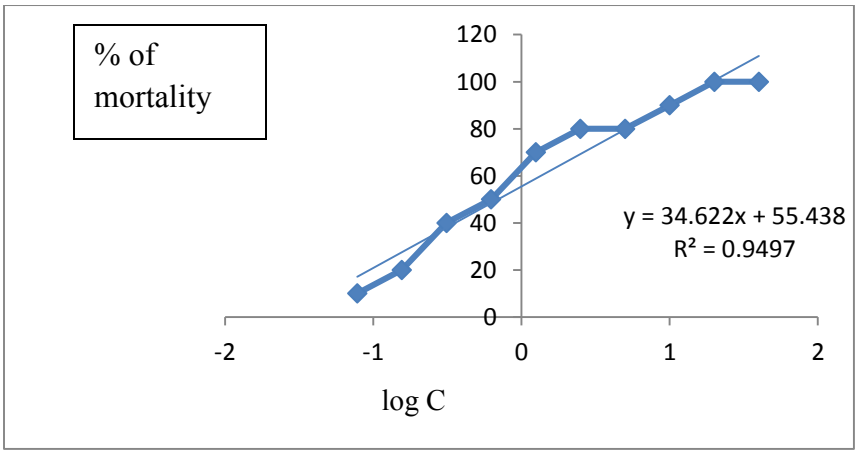

Figure 2: Percentage of mortality of Vincristine sulphate

Table 7: Effect of methanolic extract of *Citrus sinensis* peel on shrimp nauplii.

Srl No	Conc (µg/ml)	Log C	% of Mortality	LC_{50} (µg/ml)
1	400	2.60206	50	
2	200	2.30103	30	
3	100	2	20	
4	50	1.69897	10	
5	25	1.39794	10	17.885
6	12.5	1.09691	0	
7	6.25	0.79588	0	
8	3.125	0.49487	0	
9	1.56	0.193125	0	
10	0.78	-0.10735	0	

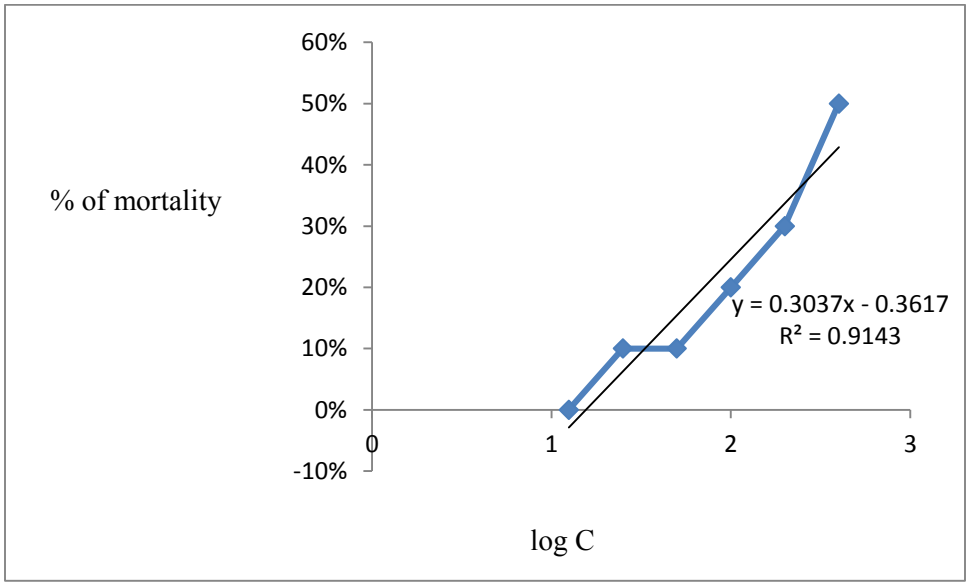

Figure 3: Percentage of Mortality of *Citrus sinensis*

4.11. Result and Discussion:

Brine shrimp lethality is the simple bioassay useful for screening large number of extracts in the drug discovery process from the *Citrus sinensis* peels. The procedure (Mayer et al, 1982) was adopted to determine the lethality of plant extracts to brine shrimp. The method allows the use of smaller quantity of the extracts and permits larger number of samples and dilutions within shorter time than using the original test vials (Sam et al., 1993). The LC_{50} values of the brine shrimp obtained for extracts of these medicinal peels.

According to the aforementioned data, showed no significant differences in percentage mortalities between different concentrations within extract and negative control which indicating that no brine shrimp lethality, compared to that of control.

The synthesized chemical compounds were subjected to Brine Shrimp Lethality Bioassay following the procedure of Meyer. The median lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by plot of percentage of the shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best fit line was obtained from the curve data by means of regression analysis.

Chapter Five: Evaluation of Hypoglycemic Activity

5.1. Principle:

Diabetes mellitus is an endocrine issue everyday in both male and female. Earlier it was believed that it was an illness that has touched base from the west. Be that as it may, with the modernizing and urbanizing of the populace in our nation, it has turned into an endemic infection.

A standout amongst the most worthy systems for assessing the hypoglycemic action is glucose resistance test (GTT). It is a therapeutic test where glucose is given and blood tests and after that amassed a short time later to decide how quickly it is cleared from the blood. The test is routinely used to test for diabetes, insulin resistance, receptive hypoglycemia and more rare issue of starch digestion system. This test has been performed throughout the years for sundry purposes with diverse standard measurements of glucose, distinctive courses of organization, distinctive interims and terms of examining, and sundry substances evaluated in additament to blood glucose.

In this study, hypoglycemic impact of methanolic concentrate of the peel of *Citrus sinensis* at 200 mg/kg and 400 mg/kg measurements were analyzed & contrasted with relative with that of control and standard gathering.

5.2. Experimental Animals

Swiss-albino mice of either sex, weighed 25gm in average were obtained from the animal house of State University of Bangladesh located in Dhanmondi, Dhaka, Bangladesh. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 2ºC; relative humidity 60-70%). Total six mice were used to perform this experiment.

Figure 4: Swiss albino mice

Figure 5: Oral administration glucose

5.3. Experimental Design

Six experimental animals were randomly selected and divided into two groups denoted as group-I, group-II consisting of 3 mice in each group. Each group received different doses of extract. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. As it was difficult to observe the biologic response of three mice at a time receiving same treatment, it was necessary to identify individual animal of a group during the treatment. So, the animals were marked as 1=Mice 1, 2=Mice 2, 3=Mice 3, 4= mice-4, 5= mice-5 and 6= mice-6.

5.4. Preparation of extract containing dosage:

- For 200mg dose, 24mg extract was taken in a 5ml vial with 0.8 ml distilled dihydrogen monoxide was integrated there.
- For 400mg dose, 48mg extract was taken in a 5ml vial with 0.8ml distilled dihydrogen monoxide.
- 2drops of Tween-80 was integrated in each vial to ascertain felicitous coalescence of the extract.
- Then the extracts were dissolved in the vial by utilizing vortex machine.

5.5. Preparation of glucose solution:

10g glucose was disintegrated in 100 ml water which was utilized to build the blood glucose level of the mice.

5.6. Procedure:

- The tail of each mouse was pierced and blood was taken into the strip of diabetes measuring machine. Current blood glucose level of every mouse was recorded.
- 1 ml glucose solution was taken in a syringe and administered orally.
- After 20 minutes, glucose level was checked again and the data was recorded.
- Then 0.2ml extract was given orally. (200mg dose to mouse1-3 and 400mg dose to mouse 4-6).
- Blood glucose level was checked after 30minutes, 90 minutes, 150 minutes. The data was recorded in a chart.

5.7. Results and Discussion

The effects of methanolic extract of peel of *Citrus sinensis* at 200 and 400 mg/kg dose to lower blood glucose level were observed as follows to evaluate their hypoglycemic activity.

Table 8: Test materials used in the evaluation of hypoglycemic activity of crude extract of peel of *Citrus sinensis*

Code no.	Test Samples	Group	Identification	Dose (mg/kg)
CTL	1% Tween-80 & DMSO in normal saline	I	Control Group	0.1 ml/10 g of body wt
STD	Glibenclamide	II	Standard Group	10
ME 1	Methanolic extract of peel of *Citrus sinensis*	III A	Test Sample	200
ME 2	Methanolic extract of peel of *Citrus sinensis*	III B	Test Sample	400

Table 9: Plasma level of glucose (mmol/L) of mice at different time

Code No	0 minute		30 minute		90 minute		150 minute	
	Data	Mean	Data	Mean	Data	Mean	Data	Mean
CTL	5.8		10.1		7.6		5.7	
	5.8		10.9	10.67	7.2	7.33	5.8	5.60
	5.5	5.70	11		7.2		5.3	
STD	4.1		3.6		3.3		3.6	
	4.2		3.7	3.73	3.6	3.53	3.2	3.30
	4.2	4.17	3.9		3.7		3.1	
ME 1	3.8		4.2		3.1		2.4	
	5.7		7.9		6.6		4.2	
	5.7	5.06	6.9	6.3	3.6	4.43	3.7	3.43
ME 2	3.4		4.3		2.2		2.3	
	4.3		9.1		2.9		5.0	
	4.9	4.2	7.0	6.8	2.5	2.53	Low	2.43

Table 10: Percentage reduction of plasma glucose level by test materials

Code no.	Percentage reduction		
	After 30 minutes	After 90 minutes	After 150 minutes
STD	10.55	15.35	20.86
ME 1	(-)24.50	12.45	32.21
ME 2	(-)61.90	39.76	42.14

From the above table it is clear that ME 1 and ME 2 have greater percentage reduction values than STD after 30, 90 and 150 minutes of administration.

Table11: Hypoglycemic activity of crude extract of peel of *Citrus sinensis*

Code no.	Plasma level of glucose (Mean)			
	0 minute	30 minute	90 minute	150 minute
CTL	5.70	10.67	7.33	5.60
STD	4.17	3.73	3.53	3.30
ME 1	5.06	6.3	4.43	3.43
ME 2	4.2	6.8	2.53	2.43

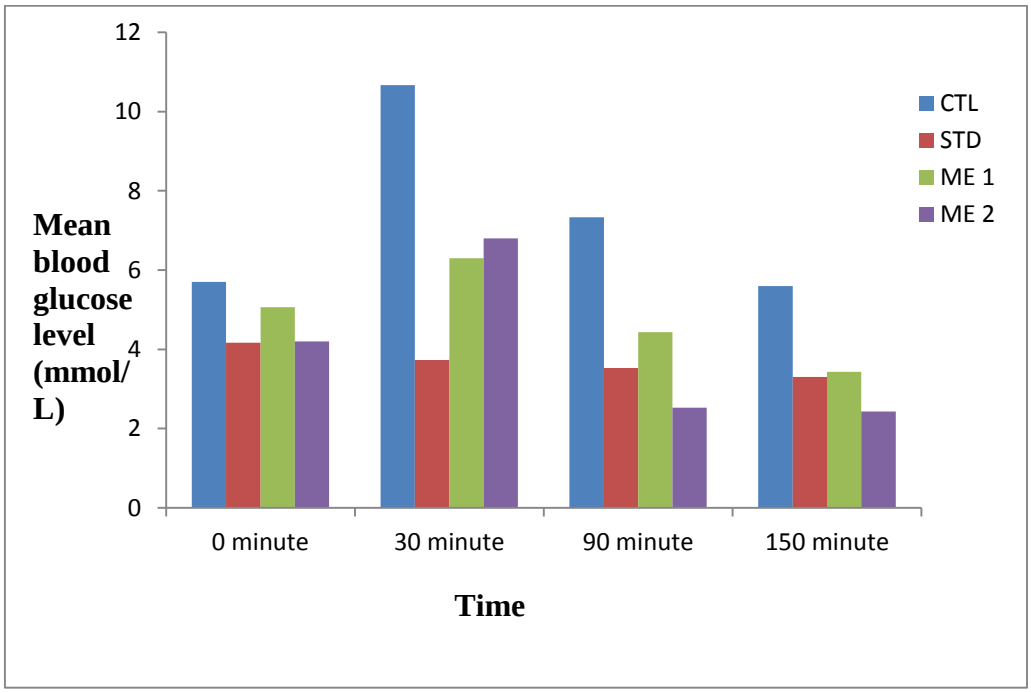

Figure 4: Mean blood glucose level

5.8. Statistical data evaluation

The standard t-Test was carried out for the test samples in comparison with the positive control and the statistical significance of the data was calculated.

Table 12: Statistical evaluation of the data

Code No	t-Test value	Degree of Fredrom	P value
STD	3.0412	6	0.0228
ME 1	1.8988	6	0.1064
ME 2	2. 1339	6	0.0768

[Significant at 5% level]

5.9. Result and Discussion

Both the two dosages 200mg/kg and 400mg/kg of methanolic extract showed reduction in mean blood glucose level. Although the reduction of mean blood glucose level of ME 1 and ME 2 were not quite statistically significant which could be because of the increased plasma glucose level in 30 minutes, reduction in mean blood glucose level in 60, 90 and 120 minutes was found. After administration of glucose, it was quite obvious that blood glucose increment would take place.

Chapter Six: Concluding remarks

Herbal medicines are produced from different parts of particular plants, for example, seeds, roots, stems, barks, leaves, berries or containers or pills. (D. E. Okwi, 2006).
The result of the present study revealed that the methanolic extract of the *citrus sinesis* peel have promising hypoglycemic activity and it's a bioactive compound. It can be used as a therapeutic agent in the treatment of hyperglycemia .

The regal environment of Bangladesh is favorable for citrus generation gave that fitting agronomic practices are taken after for acquiring brilliant citrus organic product. Judicious utilization of side effects from plant sources can likewise be useful for most extreme usage of common sustenances and in the meantime help with environment insurance.

Taken all together, an impressive number of settled information of confirmation has affirmed that *Citrus sinensis* peel display a noteworthy range of effective natural exercises. Fabulous consequence of citrus sinensis to have awesome bioavailability which thusly pulls in specialists to perform logical studies for powerful illness counteractive action furthermore, treatment furthermore it has proved that it has no lethal effect. There are more adjusted flavonoids in citrus peel being explored, which could offer assistance to make strides dosage impact relationship enormously.

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