INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

PHYTOCHEMICAL SCREENING, CYTOTOXIC AND HYPOGLYCEMIC ACTIVITY OF METHANOLIC EXTRACT OF *CITRUS SINENSIS* PEEL

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Article Received on: 10/01/16 Revised on: 04/02/16 Approved for publication: 10/02/16

DOI: 10.7897/2230-8407.07328

ABSTRACT

Orange has been traditionally used as an adjuvant in different diseases including diabetes, tuberculosis, asthma and hypertension. An examination of the impacts of peel extract from *Citrus sinensis* in hyperglycemic Albino mice uncovered the glucose lowering activity of *Citrus sinensis*. In this study, 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 mellitus. Both doses were being found to reduce the serum glucose level significantly (P value<0.0001) by 32% and 42%, respectively after 120 minutes of administration; revealing the anti hyperglycemic activity of the methanolic extract of *Citrus sinensis* peel. In the Brine Shrimp Lethality Toxicology study, the LC_{50} value of the extract was found 17.885µg/ml which indicated minimum lethal effect compared to that of standard (vincristine sulfate) which was 1.283µg/ml and the phytochemical screening of the methanolic extract of the peel showed presence of different bioactive compounds like flavonoids, saponins, tannins, alkaloids which are health promoting secondary metabolites.

Keywords: *Citrus sinensis*, methanolic extract, anti hyperglycemic activity, mice

INTRODUCTION

Herbal drug is a drug or preparation of drug made from a plant or plant parts which are useful for human health and also have no or minimal side effect[1].Additionally, herbal medicine can also be termed as plant pharmaceutical or phytomedicine- alludes which utilize 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[2].

Plants have been utilized for restorative purposes much sooner than written history. Therapeutic uses of 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 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[3]. Around 1950, when experiments on different substances first got to be accessible, researchers began to conduct researches on plant extracts[4].

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 and juice of Citrus are plentiful sources of bioactive compounds like vitamin-C, carotenoid, flavonoids, limonoids, essential oils, pectins, minerals and vitamin-B complex which are necessary for health nutrition[5,6,7].

Citrus sinensis fruits contain phytochemical compounds like polyphenols, anthocyanins and hydroxycinnamic acids which are useful mainly in pathological conditions like inflammation, high cholesterol related diabetes and cancer etc. *C. sinensis* also contains several bitter flavones glycosides likes neohesperidin and naringin, whose neohesperidose is a sugar component and rutinose is also a sugar component of rutin. It is said that both of two sugars are disaccharide of glucose and rhamnose (6-desoxymannose)[8,9]. Limonene, one of the primary constituents of orange, diminishes the danger of mouth, skin, lung diseases, stomach and colon tumor. Another constituent of orange is hesperidin, has additionally displayed effectiveness against cancer-causing elements in different in vivo studies. Effect has been found against malignant cells and antigen initiated T-lymphocytes by polymethoxylated flavonoids and B-cryptoxanthin (orange-red carotenoid) respectively in oranges. It brings down one's danger of having lung cancer[10].Peel of *Citrus sinensis* is used as a treatment of anorexia, colds, coughs etc**.** An essential oil from the peel functions as a food flavoring agent and also in perfumery and medicines**.** Terpenes extracted from peel are used to paint ships and boats[11]. Orange shows effect against diabetes because of bio-flavonoids, for instance, hesperidin and naringin present in citrus peels**.** They lessen the activity of glucose-6-phosphatase and phosphoenol pyruvate and thus are responsible for decreasing serum glucose level.

A standout amongst the mostworthy systems for assessing the hypoglycemic action is glucose tolerance test (GTT). It is a therapeutic test where blood glucose is measured. The test is routinely used to diagnose diabetes, insulin resistance, and hypoglycemia.

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, cytotoxic activity of plant extracts can be predicted. In this system, in vivo lethality of Brine shrimp nauplii is used as a useful screen for screening of the bioactive items. In this study, the cytotoxicity test was performed on brine shrimp nauplii by Meyer system[12] after phytochemical screening of *Citrus sinensis* peel extract. Also, hypoglycemic impact of methanolic concentrate of the peel of *Citrus sinensis* at 200 mg/kg and 400 mg/kg measurements were analyzed and contrasted with that of control and standard collection.

MATERIALS AND METHODS

Collection and identification of the plant

The entire plant was collected and identified by the taxonomist of the national herbarium of Bangladesh in Mirpur, Dhaka.

Plant material preparation

The collected sweet oranges (*Citrus sinensis*) were washed well using distilled water. The peel was divided; the mash was separated by cutting them into scintilla and sun dried for a time of 6-7 days, at 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 containers.

Preparation of Extract

850 g *Citrus sinensis* peel was soaked in 1450 ml methanol in a closed container for 7 days with occasional stirring. Then the soaked peel was filtered by Whatman filter paper followed by drying in rotary evaporator at 50˚C for 40 minutes at 100 rpm rotational speed. Then concentrated extract was poured in a beaker and was left covered by cloth in open air. After a week, sticky extract was obtained and kept in a dry place in normal temperature. The crude extract was used for photochemical and pharmacological evaporation.

Phytochemical screening

Phytochemical examinations of the extracts were performed as per the protocol depicted by Harborne[13], Sofowora[14].

Preparation of artificial sea water and hatching of Brine shrimp

Brine shrimp eggs were collected from the BRAC University Laboratory in Mohakhali. Artificial seawater was made by dissolving 38 g ocean salt in 1 liter 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) would polarize the brought forth shrimp. Two days later, shrimp eggs were hatched forth and became adult as nauplii (hatchling). After 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 nauplii were brought into each tube. The test tubes were left uncovered under the light. The quantity of surviving shrimps were tallied and recorded following 24 hours. Using the number of alive nauplii, lethal concentration for 50% mortality (LC_{50}) was evaluated in order to determine the mortality of the nauplii against different concentrations of the vincristine sulfate and methanolic extract.

Preparation of the test samples

Samples and the positive control-drug vincristine sulfate were taken in ten different test tubes. Pure dimethyl sulfoxide solution was used as a negative control. 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 400µg/ml. The other concentrations were 200, 100, 50, 25, 12.5, 6.25 etc µg/ml. Then a series of solutions having varying concentrations were prepared from the stock solution by serial dilution method.

Preparation of the positive control group

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 nauplii in 5ml of simulated salt water.

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 nauplii. These test tubes were used as control groups.

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 2.1-2.2).

Collection of experimental animal for evaluating hypoglycemic activity

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%).

Experimental Design

Six experimental mice were marked as 1 to 6 , 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 orally. Prior to any treatment, each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly.

Preparation of extract containing dosage

For 200 mg and 400 mg dose, 24 mg and 48 mg extract was taken respectively in a 5ml vial with 0.8 ml distilled dihydrogen monoxide. Two drops of Tween-80 was poured in each vial to ascertain felicitous coalescence of the extract. The extracts were dissolved by utilizing vortex machine. Three types of test materials were used in the evaluation of hypoglycemic activity of crude extract of *Citrus sinensis* peel (Table 3).

Measurement of blood glucose

10 g glucose was dissolved in 100 ml water which was utilized to build the blood glucose level of the mice. 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 0.2 ml extract was given orally (200 mg dose to mouse 1-3 and 400 mg dose to mouse 4-6). Blood glucose level was checked after 30 minutes, 90 minutes, 120 minutes.

RESULT

Phytochemical screening of *Citrus sinensis* peel methanolic extract showed precipitates of different colors (Table 1). The peel showed positive result for tannin, steroid, lead acetate, flavonoid, alkaloid, terpenoid and glycoside. In Brine shrimp lethality bioassay, consuming even lower concentration of vincristine sulfate caused higher mortality rate of the shrimp than high dose of methanolic extract of the sweet orange peel (Table 2). No significant difference in percentage of mortalities was observed between different concentrations of extract and negative control; which indicated that no brine shrimp lethality occurred comparing to negative control.

Table 1: Phytochemical screening test and result

SL 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.

Table 2.1: Effect of vincristine sulfate (positive control) on Shrimp nauplii

Serial No	Concentration (µg/ml)	Log C [a]	% mortality using Vincristine Sulphate	LC_{50} [b] (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	

Log C [a], Log value of the concentration; LC_{50} [b], lethal concentration

Table 2.2: Effect of methanolic extract of *Citrus sinensis* peel on Shrimp nauplii

Serial No	Concentration (µg/ml)	Log C [a]	% Mortality	LC_{50} [b] (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	

Log C [a], Log value of the concentration; LC_{50} [b], lethal concentration

Table 3: 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 4: Plasma level of glucose (mmol/l) of mice at different time

Code No	0 minute		30 minute		90 minute		120 minute	
	Data	Mean	Data	Mean	Data	Mean	Data	Mean
CTL	5.8	5.70	10.1	10.67	7.6	7.33	5.7	5.60
	5.8		10.9		7.2		5.8	
	5.5		11		7.2		5.3	
STD	4.1	4.17	3.6	3.73	3.3	3.53	3.6	3.30
	4.2		3.7		3.6		3.2	
	4.2		3.9		3.7		3.1	
ME 1	3.8	5.06	4.2	6.3	3.1	4.43	2.4	3.43
	5.7		7.9		6.6		4.2	
	5.7		6.9		3.6		3.7	
ME 2	3.4	4.2	4.3	6.8	2.2	2.53	2.3	2.43
	4.3		9.1		2.9		5.0	
	4.9		7.0		2.5		LOW	

Table 5: Hypoglycemic activity of crude extract of *Citrus sinensis* peel

	Plasma level of glucose (Mean) ± SEM (mmol/L)				
	0 minute	30 minute	60 minute	90 minute	120 minute
CTL	5.70±0.52	7.3±0.145	10.6±0.065	6.3±0.112	5.60±0.097
STD	4.17±0.21	3.5±0.048	3.7±0.06	3.6±0.106	3.30±0.073
ME 1	5.06±0.380	6.3±0.643	5.96±0.700	4.43±0.522	3.43±0.336
ME 2	4.2±0.253	6.8±0.933	5.3±0.744	2.53±0.141	2.43±0.649

Table 6: Percent reduction of plasma glucose level by test materials

Code no.	Percent reduction (%) (mmol/L)			
	30 minutes	60 minutes	90 minutes	120 minutes
STD	10.55	15.35	15.35	20.86
ME 1	(-)24.50	5.39	12.45	32.21
ME 2	(-)61.90	28.30	39.76	42.14

Minus (-) indicated the increase in blood pressure

Table 7: Statistical data evaluation

Code No	t-Test value	Degree of Freedom	P value	Level of significance
STD	17.94	10	< 0.0001	****
ME 1	6.595	10	<0.0001	****
ME 2	7.036	10	<0.0001	****

Here, **** → extremely statistically significant at 999% confidence interval; N= No. of samples = 6.

The effect of methanolic extracts of the peel of *Citrus sinensis* at 200 and 400 mg/kg dose to lower blood glucose level was observed to evaluate their hypoglycemic activity (Table 4 and 5). After methanolic extract consumption, mean plasma glucose was lowered (3.4 mmol/L and 2.43 mmol/L) compared to control (CTL: 5.6 mmol/L). Methanolic extracts (ME 1 and ME 2) have greater percent reduction values (32.21 % and 42.14 %) than standard (STD) vincristine sulfate (20.86 %) after 120 minutes of administration (Table 6). All the test samples showed highly significant results (P value <0.0001) in comparison with the positive control (Table 7) found by the Graph Pad software.

DISCUSSION

Citrus sinensis peel is the resource of the bioactive compounds-tannin, steroid, lead acetate, flavonoid, alkaloid, terpenoid and glycoside which provide nutritional effect to health. Tannin is an antimicrobial agent and antioxidant[15]. Steroid and terpenoid lower blood cholesterol[16].

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 measuring 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, pesticides and anticancer studies[12, 17]. The LC_{50} values of the brine shrimp obtained for extracts of these medicinal peels. Increased dose of methanolic extract of the sweet orange peel does not cause notable growth of mortality in shrimp.

The synthesized chemical compounds were subjected to Brine Shrimp Lethality Bioassay following the procedure of Meyer. Even high median lethal concentration (LC_{50}) value caused less percent mortality of shrimp-which indicated that consumption of methanolic extract did not exert much toxicity on Brine shrimp with respect to vincristine sulfate. In spite of this variation, it can be ascertained that vincristine sulfate and the methanolic extract of *Citrus sinensis* peel have cytotoxic activity. Further bioactivity guided investigation was performed by checking the hypoglycemic activity of the *C. sinensis* peel extract. Both two dosages 200mg/kg and 400mg/kg of methanolic extract showed reduction in mean blood glucose level. The reduction of mean blood glucose level of methanolic extracts, ME 1 and ME 2 were extremely statistically significant. Although an increased plasma glucose level was found in 30 minutes after the administration of ME 1 and ME 2, reduction in mean blood glucose level in 60, 90 and 120 minutes were found. After administration of glucose, it was quite obvious that blood glucose elevation would take place.

CONCLUSION

Methanolic extract of *Citrus sinensis* peel possesses bioactive compound and can reduce hyperglycemia of hyperglycemic mice**.** It can be practiced clinically to evaluate its impact on diabetic patients with acute hyperglycemia**.** Besides, the moderate cytotoxic activity of the methanolic extract indicates that the glucose lowering activity does not involve any extensive cell death within the body**.**

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Cite this article as:

Najneen Ahmed, Tasnim Ahmed, Nusrat Akbar and Sharmin Ahmed. Phytochemical screening, cytotoxic and hypoglycemic activity of methanolic extract of *Citrus sinensis* peel. Int. Res. J. Pharm. 2016;7(3):44-48 http://dx.doi.org/10.7897/2230-8407.07328

Source of support: Nil, Conflict of interest: None Declared

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