International Journal of Sciences & Applied Research

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Analysis of the antibacterial and thrombolytic activity of the methanolic extract of citrus sinensis peel

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Abstract
Citrus sinensis (C. sinensis), a member of the Rutaceae (citrus family) has been traditionally used for various ailments such as asthma, hypertension, microbial infections, diabetes, tuberculosis, etc. It is rich in many phytochemicals which include flavonoids, saponins, tannins, glycosides, limonene, citral, neohesperidin, naringin, rutin, rhamnose, eriocitrin, and vitamin C. In this study the antibacterial activity of the methanolic extract of *C. sinensis* was investigated on some bacterial strains like *Pseudomonas aeruginosa*, *Bacillus cereus* and *Klebsiella* sp that are responsible for gastrointestinal disorder. The different concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20mg/mL and 25 mg/mL) of the sample have been used to evaluate the antimicrobial activity and increment in the antimicrobial activity was found with the increasing concentration of the sample. In the investigation of the thrombolytic activity of the *C. sinensis* methanolic peel extract, weak thrombolytic activity (26%) was found.

Keywords: *Citrus sinensis*, methanolic extracts, gastrointestinal bacteria, antimicrobial activity, thrombolytic activity

Introduction

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). Nowadays plant-derived substances have become of much importance due to their various applications. 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).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).

Citrus sinensis, also referred to as sweet orange belong to the family of Rutaceae (Milind, 2012). It is mainly originated in the

tropical and subtropical areas of South East Asia but can be found worldwide. Apart from being 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 (USDA Nutrient Database, 2014). These fruits also contain abundant 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, 2012).

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. Essential oil from the peel is also used as a food-flavouring agent, in perfumery and medicines (Wiesman and Chapagain, 2005).

C. sinensis has been used for ages for its healing properties traditionally (Don, 2010). C. sinensis peel has various properties to cure diseases and is widely used against various ailments, such as colic,upset stomach, cancer, diuretic, carminative, immune-enhancing, stomachic, tonic to digestivesystem, immune system and skin (Faturi et al., 2010a and b; Guarnieri et al., 2007; Honow et al., 2003; Kurowska, 2004; Parmar, 2008;Sharma, 2008; Tanaka et al., 1997a and b; and Tripoli et al., 2007). It is also used to treat and prevent colds, cough, vitamindeficiencies and scurvy and fight against bacterial, fungal and viral infections (Ashok, 2011;Honow et al., 2003; Julius et al., 2009;Strange, 1993; Vivek, 2010 and Wiesmanand Chapagain, 2005).

In this study, the common bacteria which are responsible for gastrointestinal infections were taken into account and the antibacterial activity of *C. sinensis* was studied on them. The Studied bacterial strains were *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella* sp are found to disrupt our normal function of gastrointestinal tract causing infection.

This project also investigated the thrombolytic activity of the methanolic extract of *C. sinensis* peel. Thrombolysis is also referred as blood clot bursting and thrombolysis disrupts normal blood flow through formation of blood clot and blocks the blood flow causing necrosis or infarction (Jacquelyn, 2012).

Materials and methods

Drugs, Chemicals, Reagents and Instruments: Streptokinase (15,00,000 IU) vial was bought from local drugstore (STK, Incepta Pharmaceuticals Ltd., Bangladesh).Chemicals and reagents used for this study were Methanol, Nutrient Agar, Sodium Chloride (NaCl), Dimethyl Sulphoxide (DMSO) were purchased from the local vendor of Sigma Aldrich (USA). Evaporation of the peel extract was performed using rotary vacuum evaporator (Model Hei-Vap Adv Rotary Valve Tech Gwalior, India).

Test organisms: The microbes used in this study were the clinical isolates collected from the icddr,b that was preserved in the Biotechnology Laboratory of BRAC University. Three bacterial strains- *Pseudomonas aeruginosa, Bacillus cereus* and *Klebsiella* sp. were subjected for the study.

Preparation of plant material: Fresh peels which were to be discarded as fruit wastage were collected from local juice shops, cleaned with distilled water and cut into smaller pieces. Later, the peels were sundried for 7-8 days at about 27-35^{0}C which were then grinded into fine powders using processor.

Preparation of the peel extract: The process of peel extract preparation was

adapted from another study of the researchers (Najneen Ahmed et al., 2016). The powder of *C. sinensis* peel was soaked for 7 days in methanol in a glass container, sealed tightly and occasionally stirred. Afterwards the sample was filtered using sterilized cotton cloth followed by whatman filter paper, the filtrate was evaporated in rotary vacuum evaporator at 40^0C in a speed of 110 rpm for 1 hour 30 minutes and 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 with aluminium foil paper. The crude extract was oily and sticky in texture and placed in a dry, cool place.

Antibacterial activity test:

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 accuracy and validation of the experiment.

Maintenance and preparation of inoculums: The culture media of nutrient agar was prepared by dissolving 7.2 mg of NA in a conical flask containing 160 mL of distilled water, autoclaved at 121^0C for 1 hour and poured into the autoclaved petri dishes in which bacteria were sub cultured. Test organisms were taken in the loop from freshly sub cultured bacteria (24 hours preceding the test), disintegrated in individual autoclaved test tubes containing 10 mL of 0.9% NaCl to make separate bacterial cell suspensions. The cell suspensions were of equivalent focus to ensure that there are an equivalent number of cells in each cell plate. The concentrations of cell suspensions were checked until it has equal or more turbidity than of a 0.5 McFarland Standard. Then the cotton swab was dipped into this saline containing each organism.

Antibacterial activity of methanolic extracts of *C. sinensis*: The methanol peel extract of *C. sinensis* were dissolved in 0.25% (v/v) autoclaved dimethylsulphoxide (DMSO) to make five different concentrations which were 5 mg/mL, 10 mg/mL, 15 mg/mL, 20mg/mL and 25mg/mL. Turbidity of bacterial suspension was adjusted to equivalent of 0.5 McFarland on nutrient agar plates. The discs of about 0.6mm diameter were aseptically cut out from the inoculated plates allowing 30mm between adjacent wells and the edge of the petridishes. 16 µL of each extract were added into the wells and the process was repeated three times. Antimicrobial discs were applied as positive control and for comparison. 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.

Thrombolytic test

Collection of blood sample: The ethical committee of the Department of Pharmacy, BRAC University approved the study protocol. Each volunteer has provided the written consent for collecting blood (Hassan, 2012). Proper safety protocol was maintained and a medical officer was in charge during the collection of the blood samples.

Reagent and sample preparation: The commercially available streptokinase (15,00,000 IU) was diluted to 5 mL of methanol to make 30,000 IU and 100 µL of this suspension was used as the reference standard for thrombolyticactivity because it is commonly used as a thrombolytic drug (Fahad, 2014).100 mg of plant extract was

dissolved in 10 mL of methanol, left overnight and filtered (Fahad, 2014).

Thrombolytic activity of methanolic extracts of C. sinensis: Venous blood was collected from five healthy volunteers (male, age 20-28 years) who have no recent history of oral contraceptive and anticoagulant therapy. The blood samples were allowed to incubate for 45 minutes at 37^0C. After clot formation, serum was completely removed and each tube having clot was weighed. 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 carefully removed, and the tubes were weighed. Thedifference in weight before and after clot lysis was expressed as percentage of clot lysis (Md. Shahrear, 2015,)

Statistical analysis

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

Percentage of clot = (weight of the clot after lysis by sample and removal of serum ⁄ weight of the clot before lysis by sample) X 100% (Fahad, 2014)

Results

Antibacterial activity of different concentrations of methanolic extract of *C. sinensis*: In vitro antimicrobial screening of methanolic extracts of C. Sinensis were carried out using 5 different concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL). Ciprofloxacin was used as positive control for this study [Table 1].

Bacillus cereus showed the greatest zone of inhibition among all as the concentration increased. *Pseudomonas aeruginosa* also showed zone of inhibition with an increase in concentration but it had same zone of inhibition in 20 mg/mL and 25 mg/mL. *Klebsiella sp* showed poor zone of inhibition with no zone of inhibition in the concentration of 5 mg/mL of *C. sinensis*' methanolic peel extract [Fig 1].

Table 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

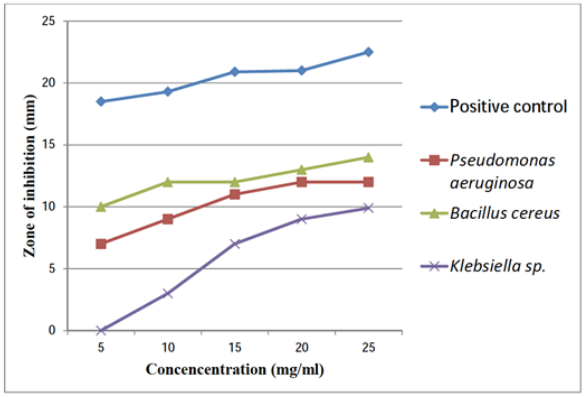

Figure 1: Graphical presentation of the antimicrobial test.

Thrombolytic activity of methanolic extracts of C. sinensis: 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% as shown in the figure [Fig 2].

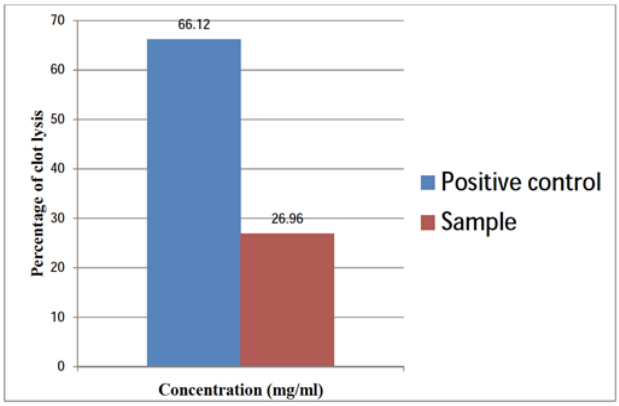

Figure 2: Graphical representation of thrombolytic test.

Discussion

The present study was a continuation of a previous study (Najneen Ahmed et al., 2016) and the antibacterial activity of *C. sinensis* methanolic peel extract against common bacterial strains responsible for GI disorders and infections was explored. Antimicrobial resistance has been a burning issue worldwide (Ventola,C. L. ,2015) and different strategies have been adopted to combat this problem. Extensive research for the development of new antibiotic and the possible solutions to this problem could be development of synthetic and semi synthetic antibiotics or use of herbal medicine as a potential alternative (Piddock LJ., 2012). *C. sinensis* methanolic peel extract when tested against the three of the common bacterial strains such as *Bacillus cereus, Klebsiella sp and Pseudomonas aeruginosa which are* responsible for the GI infection. From the experiments, it was found that *C sinensis* peel extract was effective in reducing the growth of all the studied bacterial strains in a concentration dependent manner. *Bacillus cereus* was found to be more sensitive amongst the studied bacterial strains. But in comparison to Ciprofloxacin which was the positive control for this study, methanolic peel extract of *C. sinensis* was found less effective (Fantin, B. et al., 2009)

In addition to the antimicrobial activity, thrombolytic activity of the *C.sinensis* peel extract was also investigated. Clot or thrombus formation in blood vessel creates blockage in blood flow and may lead to cardiovascular diseases like hypertension, stroke to to the heart etc (Bentzon, J. F. et al., 2014). According to the present study, *C.sinensis* methanolic peel extract was not found to be effective as a thrombolytic agent . The positive control used in this study was streptokinase which shown to be dissolving around 66% of the formed clot whereas the *C.sinensis* methanolic peel extract caused around 26% lysis of the formed clot.

Conclusion

Herbal medicines are produced from different parts of particular plants such as- 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. This study revealed that the methanolic extract of the *C. sinesis* peel have potent

antimicrobial activity. It can be used as a therapeutic agent in the treatment of gastrointestinal infection. Further investigation is necessary to observe the mechanism of impacts closely and to draw extractive conclusion. Thrombolytic study of *C. sinensis* showed weak thrombolytic activity but different isolation techniques to separate the constituents more effectively and in depth investigation on thrombolytic activity could be done. Therefore it could be used for the diagnosis of patients and as being an herbal drug it possesses fewer side effects and makes an excellent choice of treatment. The glorious environment of Bangladesh is great for citrus generation providing a proper chance for agronomic practices. 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.

Acknowledgments

Dr. Raushanara Akter, Assistant Professor in the Department of Pharmacy of BRAC University helped the authors in the preparation of the article.

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