Annals of Plant Sciences
ISSN: 2287-688X
OPEN ACCESS
www.annalsofplantsciences.com

Original Research Article

Study of *in vitro* antibacterial activity of *leave extract of Citrus maxima*

Amiya Kumar Prusty* and Saroja Kumar Patro
Institute of Pharmacy and Technology, Salipur, Cuttack, Odisha–754 202, India

Received for publication: November 10, 2014; **Accepted:** November 21, 2014.

Abstract: The objective of the study was to evaluate the antibacterial activity of the ethanolic extracts of leave of *Citrus maxima (Burm.) Merr.* on *Staphylococus aureus, Bacillus subtilis, Escherichia hermanii and Pseudomonas aeruginoss.* The ethanolic leave extract of *Citrus maxima (Burm) Merr.* was prepared by percolation method followed by antimicrobial susceptibility test using disc diffusion method. The whatman-1 filter paper discs of 6mm sizes impregnated with the extract were placed on agar plates seeded with bacterial cultures of 0.5 McFarland standards. The antibacterial activities were assessed by the presence or absence of inhibition zones after incubating the plates at 37^0C for 24 hours. Minimum inhibitory concentration (MIC) of ethanolic extract for the selected pathogens were determined by broth macro dilution method. Maximum zone of inhibition in antibacterial susceptibility test was shown by *Staphylococus aureus*. MIC value of the extract for *Staphylococus aureus* and *Bacillus subtilis* (20µg/ml) was found to be lower than *Escherichia hermanii* (30µg/ml) *and Pseudomonas aeruginoss* (40µg/ml). The Leave extract of *Citrus maxima* showed significant antibacterial activity against *Escherichia hermanii, staphylococcus aureus,* Bacillus *subtilis and Pseudomonas aeruginoss.*

Key words: *Citrus Maxima*, Ethanol extract, Antimicrobial activity, MIC.

Introduction

Infectious diseases remain one of the major threats to human health. Although a number of natural and synthetic antimicrobial agents have been isolated or developed to kill pathogenic microorganisms effectively, global antimicrobial resistance is an increasing public health problem. Therefore, novel antimicrobial agents from different biological sources are continuously sought. India is rich in its biodiversity. A number of plants have been documented for their medicinal potential, which is in use by the traditional healers, herbal folklorists and in Indian system of medicine namely Siddha, Ayurveda and Unani (Ahmedulla and Nayar, 1999). Plants have the capacity to produce a large number of organic chemical called as phytochemicals. The accumulation of phytochemical in the plant cell cultures had been studied for more than thirty years and the generated knowledge had helped in realization of using cell culture for the production of desired phytochemicals (Castello *et al.,* 2002).The plants also act as a source of inspiration for development of novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (kumar *et al.,* 2011). According to the World Health Organization (WHO), almost 80% of the world's population relies on traditional medicines for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body (Pawar *et al.,* 2011). The plant, *Citrus maxima (Burm.) Merr.* (Rutaceae), is commonly known as shaddock or pomelo. The plant is indigenous to tropical parts of Asia. The fruit and pulp are cited as antitoxic, appetizer, cardiac stimulant and stomach tonic in ancient and medieval literature. Recently, leaves are found to exhibit antitumour activity (Sen *et al.,* 2011). Alcoholic extracts of the fruit also shows antidiabetic and antihyperlipidaemic potential (Bhandurge *et al.,* 2010). The essential oil of the fruit shows in vitro activity against *Staphylococcus aureus* and *Escherichia coli* (Oydepo *et al.,* 2012). Antibacterial activity of the leave of *Citrus maxima (Burm.) Merr*. has not been evaluated so far. In this study, an attempt has been made to evaluate the antibacterial activity of the leaves of *Citrus maxima (Burm.) Merr*. against different microorganisms.

Materials and Methods

Microbial Strains Used

Following four microbial strains were used for the antimicrobial activity determination.

***Corresponding Author:**
Dr. Amiya Kumar Prusty
Institute of Pharmacy and Technology,
Salipur, Cuttack,
Odisha –754 202, India.

1. *Staphylococcus aureus* MTCC 9886
2. *Bacillus Subtilis* MTCC 1789
3. *Escherichia heranii* MTCC 9144
4. *pseudomonas aeruginoss* MTCC 10070

[MTCC: Microbial type culture collection] that were obtained from the culture collection of the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and cultured and preserved in Institute of Pharmacy and Technology, Salipur microbiology laboratory.

Collection of Leave

The Leave of Citrus maxima were collected from the nearby village of Salipur, Odisha during the month of January. The leave were authenticated by the taxonomist of department of Botany, Salipur autonomous college, Salipur and a specimen sample was preserved in the museum of Institute of Pharmacy and Technology, Salipur, Cuttack. The collected leave were properly washed in sterile water to remove any dirt or other unwanted materials adhere to it, shade dried for fifteen days followed by oven dry at 50ºC for 24hours and preserved in a dry container for future use (Prusty *et al.,* 2008).

Preparation of extract

Ethanolic extract was prepared using 90% ethanol by percolation method. This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The dried leave were cut into small pieces and ground to powder by a mechanical grinder. The powdered materials (20 gm) were moistened with 50ml of ethanol and allowed to stand for approximately 4 hrs in a well closed container, after which the mass was packed and the top of the percolator was closed. Additional 25ml of ethanol was added to form a shallow layer above the mass, and the mixture was allowed to macerate in the closed percolator for 24 hrs. The outlet of the percolator was then opened and the liquid contained therein was allowed to drip slowly. Additional menstruum was added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc was pressed and the expressed liquid was added to the percolate. Sufficient menstruum was added to produce the final volume of 200ml, and the mixed liquid was clarified by filtration. The extract was evaporated to dryness.

Phytochemical Analysis

The dried extract was subjected to qualitative phytochemical analysis for the presence of flavonoids, alkaloids, tannins, saponins, sterols and terpenoids by following standard procedures.

Tests for Alkaloids

Wagner's Test: With alkaloid it shows reddish brown precipitate. It was prepared by dissolving 1.27 gm of Iodine and 2 gm of Potassium Iodide in 5ml of water and the final volume was made up to 200 ml.

Mayer's Test: It is another method of detecting alkaloids. To prepare the reagent, 1.36gm of mercuric chloride was dissolved in distilled water. In another part 5gm of potassium iodide was dissolved in 60 ml of distilled water. Then both parts were mixed and the volume was adjusted to 200ml. With alkaloids it produces white to buff precipitate.

Dragendroff's Test: With alkaloids this reagent gives orange-brown colored precipitate. To prepare this reagent, 14 gm of sodium iodide was boiled with 5.2 gm of bismuth carbonate in 50 ml glacial acetic acid for few minutes. Then it was allowed to stand for overnight and the precipitate of sodium acetate was filtered out. To 40 ml of filtrate 160 ml of acetate and 1 ml of water was added. The stock solution was stored in amber-colored bottle. During experiment; to 10 ml of stock solution 20 ml of acetic acid was added and the final volume was made up to 100 ml with water.

Hager's Test: This reagent shows characteristic crystalline precipitate with alkaloids. In this case a saturated aqueous picric acid was used for detection of alkaloids.

Tests for Tannins and Phenolic Compounds

Test with Lead Acetate: Tannins get precipitate with lead acetate.

Test with Ferric Chloride: Generally phenols were precipitated with 5% w/v solution of ferric chloride in 90% alcohol and thus phenols were detected.

Test with Gelatin Solution: To a solution of tannins (0.5 - 1%) aqueous

solution of gelatin (1%) and sodium chloride (10%) were added. A white buff precipitate confirms tannis.

Tests for Saponins

Foam Test: About 1 ml of alcoholic and aqueous extract was diluted separately with distilled water to make the volume up to 10 ml, and shaken in a graduated cylinder for 15 minutes and kept aside. 1 cm layer of foam after standing for 30 minutes indicates the presence of saponins.

Tests for Flavonoids

Test with NaOH: For the detection of flavonoids, the extract was first dissolved with water. It was filtered and the filtrate was treated with sodium hydroxide. A yellow color confirms the presence of flavonoids.

Shinoda test: A small quantity of extract was taken in a test tube and dissolved in methanol (1 ml). A pinch of magnesium powder was added followed by concentrated. HCl. Appearance of pink color indicates the presence of flavonoids, bioflavonoid.

Tests for Steroids and Sterols

Salkowski's Test: To 5ml of the solution of the extract in chloroform was taken in a dry test tube, equal volume of conc. H_2SO_4 was added along the side of the test tube. The presence of steroids and sterols were confirmed by the upper chloroform layer showing a play of colors first from bluish red to gradually violet and lower acid layer showing yellow color with green fluorescence.

Libermann Burchard Reagent Test: In this method of detection, about 2 ml of the solution of extract in chloroform was placed in a dry test tube. Then 2 ml of acetic anhydride and 2-3 drops of conc. H_2SO_4 was added to it and allowed to stand for few minutes. An emerald green color develops if steroid or sterols are present.

Tests for Triterpenoids

Test with Tin and Thionyl Chloride: For detection of triterpenoids the extract was dissolved in chloroform. A piece of metallic tin and 1 drop of thionyl chloride was added to it. Pink color confirms the result.

Determination of Minimum Inhibitory Concentration

An agar dilution assay was used for determining the minimum inhibitory concentration (MIC) of the extract. This method was used as a modification of Parish and Davidson (1993). Tubes of 15 ml molten agar were prepared and maintained at 50ºC. A single concentration of antimicrobial was added to the agar in each test tube to obtain a range of final concentrations of 10, 20, 30, 40 and 50µg/ml. After addition of the different concentration of extract to the agar, plates were poured and agar allowed solidifying. The test microorganisms were diluted in 0.1% (w/v) peptone water to 10^6 CFU/ml. After each dilution the samples were taken for optical density determination using a spectrophotometer. When OD becomes 04 it indicates the microbial concentration is the required concentration. The test microorganism was then added to the plates and spreaded by a spreader. A control plate, without added antimicrobial, was prepared and inoculated to ensure adequate growth of the test microorganism. The plates were incubated at 37ºC for overnight. The MIC was defined as the lowest concentration that completely inhibited growth up to 24 hr (Ahmed *et al.,* 2007).

Study of Antibacterial activity

For antibacterial activities, the samples were evaluated following a modified filter paper disc method (Norrel *et al.,* 1997). The samples were diluted to 50µg/ml with sterilized distilled water. The sterile filter paper (Whatman filter paper) discs of 6mm diameter were soaked with 50µl of the solution and placed on bacteria seeded plate (10^6 CFU/ml) of solid nutrient agar. For positive control, gentamycin sulphate in same concentration was used as similar manner. The plates were first incubated at 4ºC for 12 hours to allow proper diffusion of the extract into the medium and then re-incubated at 37ºC for 24 hours (Praven *et al.,* 2012). After incubation period, the inhibition zone was observed and measured after marking at six different positions.

Statistical Analysis

The mean zone of inhibition and standard deviation was calculated by using Smith's statistical package (SSP) software. The data were put in the first variable column. Then by going to statistical inference followed by one simple test of mean, the

value of mean and standard deviation was determined. Six mean reading were used to calculate the standard deviation (Prusty *et al.,* 2014).

Results and Discussions

Percentage yield of extract

After complete extraction and drying, the dried mass of the ethanolic extract of *Citrus maxima* was weighed in a digital balance. A net yield of 5.2 g (26% w/w) was obtained by percolating 20g of dry powder of the leave of *Citrus maxima*.

Preliminary phytochemical screening of extract

The results of different phytochemical tests of the crude ethanol extract of *Citrus maxima* showed presence of components like flavonoids, saponins, alkaloids and tannins. The detail results were represented in table no.1.

Table 1: Preliminary phytochemical tests for identification of phytoconstituents in *Citrus maxima*.

CONSTITUENTS	NAME OF THE TEST	RESULT
Alkaloids	Mayer's test	+
	Wagner's test	+
	Hager's test	+
	Dragendorff's test	+
Saponins	Foam test	+
	Bromine water test	+
Steroids	Salkowaski test	-
	Liebermann Burchard test	-
Terpenoids/Volatile oils	Sudan-III test	-
	Tincture alkane test	-
Flavonoids	Shinoda test	+
	Lead acetate test	+
	Ferric-chloride test	+
Tannins	Gelatin test	+
	Potassium dichromate test	-

Table 2: Antimicrobial activity of different concentration of ethanolic leave extract and antibiotic against microorganism.

Microorganisms	Mean zone of inhibition in mm+/- SD			
	Extracts			Antibiotic
	1mg/ml	0.5 mg/ml	0.25 mg/ml	0.25 mg/ml
Staphylococcus aureus	17.08+/-0.51	13.87+/-0.2	10.98+/-0.32	21.82+/-0.44
Bacillus subtilis	16.17+/-0.56	13.08+/-0.40	10.47+/-0.36	20.7+/-0.46
Escherichia heranii	14.63+/-0.64	11.08+/-0.29	8.7+/-0.32	18.66+/-0.21
Pseudomonas aeruginoss	11.95+/-0.43	10.43+/-0.28	8.4+/-0.93	16.53+/-0.51

Table 3: Minimal Inhibitory Concentration of extract and antibiotic against different bacteria.

Organism	Ethanol extract (µg /ml)					Antibiotics (µg /ml)				
	10	20	30	40	50	10	20	30	40	50
S. aureus (MTCC 9886)	+	+	-	-	-	+	-	-	-	-
*B. subtilis (*MTCC 1789*)*	+	+	-	-	-	+	-	-	-	-
E.heranii (MTCC 9144)	+	+	+	-	-	+	+	-	-	-
P. aeruginoss (MTCC 10070)	+	+	+	+	-	+	+	-	-	-

[+ ve sign indicates growth, and – ve sign indicates no growth]

Antimicrobial activity study

From the Zone of inhibition data for Gentamycin sulphate it was observed that *Staphylococcus aureus* was having maximum zone of inhibition of 21.82+/-0.44, followed by *Bacillus subtilis* with 20.7+/-0.46, *Escherichia heranii* with 18.66+/-0.21 and *Pseudomonas aeruginoss* with 16.53+/-0.51. In addition from the Zone of inhibition data for 0.25% ethanolic extract it was observed that *Staphylococus aureus was having* maximum zone of inhibition of 10.98+/-0.32 followed by *Bacillus subtilis with* 10.47+/-0.36, *Escherichia heranii with* 8.7+/-0.32, followed by *Pseudomonas aeruginoss* with 8.4+/-0.93. The Zone of inhibition data for 0.5% ethanolic extract it was observed that *Staphylococus aureus was having* maximum zone of inhibition of 13.87+/-0.2 followed by *Bacillus subtilis with* 13.08+/-0.40, *Escherichia heranii* with 11.08+/-0.29, followed by *Pseudomonas aeruginoss* with 10.43+/-0.28. The Zone of inhibition data for 1% ethanolic extract it was observed that *Staphylococus aureus was having* maximum zone of inhibition of 17.08+/-0.51 followed by *Bacillus subtilis* with 16.17+/-0.56, *Escherichia heranii* with 14.63+/-0.64, followed by *Pseudomonas aeruginoss* with 11.95+/-0.43.

The observations of antimicrobial study were more likely to be due to the fact that Gram negative bacteria possess an outer lipid membrane that acts as a barrier to many environmental substances including antibiotics [Prusty *et al.,* 2008]. Therefore it may be a reason that the leave extracts were

more active against gram-positive microorganisms than gram-negative microorganisms [Jigna *et al.,* 2005].

The minimum inhibitory concentration of different dilution of extract against different microorganism was shown in Table 3. The Ethanolic extract had shown lowest MIC against both *Staphylococcus aureous* and *Bacillus subtilis* i.e. 20µg/ml and had shown maximum MIC against *Pseudomonas aeregonoss* i.e. 40µg/ml followed by *Escherichia heranii* with 30µg/ml. The antibiotic had shown lowest MIC against *staphylococcus aureous* and *Bacillus subtilis* whereas maximum MIC against *Pseudomonas aeregonoss* and *Escherichia heranii*.

In the present investigation, the results of detail activity of different concentration of ethanolic extract of the leave of Citrus maxima along with the activity profile with standard commercial antibiotics (gentamycin) was presented in the observation. In this investigation, the leave extract of Citrus maxima showed significant in-vitro antimicrobial effect.

The antibacterial activity of the plant extract can be attributed to the different phytochemicals present in the leave of Citrus maxima (Burm.) Merr. There was growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. Phytochemicals were non-nutritive plant chemicals that may have protective or disease preventive antimicrobial activities. Because of their structural differences from those of the more studied antimicrobial sources, their mode of action may too differ. Flavonoids, alkaloids and saponins were found to be associated with antimicrobial effects in various studies using plant extracts. These phytochemicals had been found to be present in the leave of Citrus maxima (Burm.) Merr. Flavonoids had been found to exhibit antimicrobial activity through various mechanisms like inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism. The mode of action of antibacterial effects of saponins seems to involve membranolytic properties. Saponins possess detergent like activity and might increase the permeability of bacterial cell membrane without destroying them. In theory, this activity might facilitate antibiotic influx through the bacterial cell wall membrane. The mechanism of action of highly aromatic quaternary alkaloids is attributed to their ability to intercalate with DNA. These may explain the probable mechanism of antibacterial activity of the plant. In recent years, ethnobotanical and traditional uses of natural compounds, especially of plant origin received much attention as they were well tested for their efficacy and generally believed to be safe for human use. They obviously deserve scrutiny on modern scientific lines such as phytochemical investigation, biological evaluation on experimental animal models, toxicity studies, investigation of molecular mechanism of actions of isolated phytoprinciples and their clinical trials [Swarnamoni *et al.,* 2013].

Conclusion

Results obtained from the antimicrobial study by paper disc method in the present study revealed that the ethanolic leave extract of *Citrus maxima* showed inhibitory effect against all the test microbial strains but showing more activity against gram positive as compared to gram negative strains. The zone of inhibition study also suggests that the extracts possess antimicrobial activity in a concentration dependent manner against the test microorganisms and was comparable with the standard drugs. From previous studies in addition to the current it could be concluded that Leaves of *Citrus maxima* contain a potential metabolite and can be further utilized for its antimicrobial applications in agriculture, medicine and other area of interest.

References

1. Ahmed I, Chandana J, Geon WL,Tae SL, Antibacterial and Antifungal Activities of Stereum ostrea, an Inedible Wild Mushroom. Mycobiology, 2007, 35(4), 210-214.

2. Ahmedulla M and Nayar MP, Red data book of Indian plants, 4, Calcutta: Botanical survey of India, 1999.

3. Bhandurge P, Rajarajeshwari N, Alagawadi KR, Agrawal S, Antidiabetic and hyperlipaemic effects of Citrus Maxima Linn fruits on alloxan-induced diabetic rats. Int J Drug Dev & Res, 2010, 2(2), 273-278.

4. Castello M, Phatak A, Chandra N and Sharon M, Antimicrobial activity of crude extracts from plant parts and corresponding calli of FBixaorellana L. Indian J Exp Biol, 2002, 40,

1378-1381.

5. Jigna P, Rathish N, Sumitra C, Preliminary screening of some folklore medicinal plants from Western India for potential antimicrobial activity. Indian J Pharmacol, 2005; 37: 408-409.

6. Kumar S, Choudhary HS, Seniya C, In vitro antibacterial study of aqueous and methanolic extracts of some selected medicinal plants. J Chem Pharm Res, 2011, 3(4), 854-860.

7. Norrel S A and Messley K E, Microbiology Laboratory Manual Principles and Applications. Prentice Hall, Upper Saddle River, New Jersey, 1997.

8. Oyedepo T A, Antioxidant potential of Citrus maxima fruit juice in rats. Glo Adv Res J Med Med Sci, 2012, 1(5), 122-126.

9. Pawar NK and Arumugam N, Leaf extract of Centratherumpunctatum exhibits antimicrobial, antioxidant and anti proliferative properties. Asian J Pharm Clin Res, 2011, 4(3), 71-76.

10. Praveen K, Usha KY, Naveen MB, Rajasekhar R, Antibacterial Activity of a Mushroom - Stereum ostrea, J Biology Agri Healthcare, 2012, 2(1), 1-5.

11. Prusty AK, Ghosh T, Sahu S K, Anthelmintic, antimicrobial and antipyretic activity of various extracts of Clerodendrum infortunatum linn. Leaves Ori Pharm Exper Med, 2008, 8(4), 374-379.

12. Prusty AK, Samad L, Rout A, Patra A, In-Vitro Antibacterial Activity of Stereum Ostrea a Wood Decaying Macro Fungi. Journal of Microbiology Research and Reviews, 2014, 2(2), 12-18.

13. Sen SK, Haldar PK, Gupta M, Mazumder UK, Saha P, Bala A, Antitumor activity of citrus maxima (Burm.)Merr.leaves in Ehrlich's ascites carcinoma cell-treated mice. ISRN Endocrinology, 2011, 1-7.

14. Swarnamoni D, Mukundam B, Shagufa A, Antibacterial activity of the ethanolic extract of leaves of citrus maxima (burm.) merr. On escherichia coli and pseudomonas aeruginoss. Asian J Pharm Clin Res, 2013, 6(4), 136-139.

15. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H, Phytochemical screening and extraction: A Review. Inter Pharmaceutica Sciencia 2011, 1(1), 98-106.

Source of support: Nil
Conflict of interest: None Declared