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Isolation of Citric Acid from *Citrus maxima* Fruit Juice, Phytochemical Analysis and *In-Vitro* Antifungal Activity of Ethanolic Extract of *Citrus maxima*

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ABSTRACT

In the traditional system of Ayurveda, there are several kinds of medicinal formulations and preparations which are used in the treatment of various acute and chronic infirmities by employing specific organised and unorganised parts of medicinal plants like leaves, root, fruits, seeds, stems, barks etc. The purpose of this study is to describe the specific segment of phytochemical analysis and to find out *in-vitro* antifungal activity of ethanolic extract of the leaves of Citrus maxima and isolation of citric acid from *Citrus maxima* fruit juice. The principal objective of this project is to investigate Citrus maxima (leaves) phytochemically and pharmacognostically, to isolate citric acid from the fruit extract of *Citrus maxima* and to evaluate the i*n-vitro* anti-fungal potentials of the dried leaves of Citrus maxima on pathogenic fungal stain via Disc-diffusion method. The antifungal activity was detected by paper disc-diffusion method. The ethanolic extract of *Citrus maxima* leaves, inhibited growth of different fungal strains like Rhizopus, Fusarium and Penicillium at various concentrations from 100 mg to 400 mg, in which test result found out the 400 mg concentration, shows the maximum zone of inhibition with respect to the standard drug.

INTRODUCTION

A genus of Citrus (Linn) of Rutaceae an evergreen aromatic shrub and small trees occupies an important place in the medicine and also in the fruit economy of India. Scientifically it is also known as *Aurantium maximum* Burm. Ex Rumph, *Citrus aurantium* L. *Var grandis* L., *Citrus Decumana* L, *Citrus grandis* Osbeck & *Citrus pamplemos. Citrus grandis* (Linn) Osbeck is a crop plant of India, China, Indonesia, America, Thailand etc. The pummelo tree is normally about16 to 50 ft tall. Pomelo is native plant of Malayu island and East of India. It is wide spread in China, Japan, Philipines, Indonesia, USA and Thailand (1, 2).

Nowadays there are various antifungal drugs employed to treat Fungal infections. In the one hand few infections show good response against superficial treatment of fungal infections and in another hand *Tinea unguium* and *Tinea capitis* and other chronic infections requires Systemic and prolong therapy. Sometime in few cases when we are using prolong therapy, failure can occur due to the antifungal resistance caused by pathogen of fungi. That's why it is mandatory to evaluate the antifungal using a standardized, easy and reproducible *in-vitro* assay to know the antifungal activity of drugs against isolates. (Esteban A *et al*, 2005).

Figure No. 1: Leaves of *Citrus maxima*

MATERIAL AND METHODS:

Collection, Identification and authentication

The plant Citrus maxima are collected from the locality of Dehradun and the collected plant material were subjected to preparation of Herbarium file and sent for the identification and authentication.

Herbarium of Citrus maxima plant:

- ✓ Kingdom: Plantae
- ✓ Division: Magnoliophyta
- ✓ Class: Magnoliopsida
- ✓ Order: Sapindales
- ✓ Genus: Citrus
- ✓ Species: Citrus maxima
- ✓ Family: Rutaceae
- ✓ Locality: Bhauwala, Dehradun
- ✓ Habit: Evergreen tree
- ✓ Remark: Tree
- ✓ Collected by: Harendra Bisht
- ✓ Identified by: Dr. Praveen Kumar Verma

The herbarium of the plant specimen has been deposited to Systemic Botany Discipline, Forest Research Institute, Dehradun and the voucher specimen No. 126/Dis./2019/Syst.Bot./Rev.Gen./4-5.

Material and Methodology

Plant material: The plant of Citrus maxima (White) fruit and leaf sample were collected from Bhauwala, Dehradun, Uttarakhand. Leaves sample collected during the month of March to April 2019. The fresh fruit sample were detached from the peel.

Preparation of Extract:

Extract from Dried powder:

1. The leaves of the plant are ground into uniform powder with electronic blender.
2. 25gm of Citrus maxima dried leaves powder was taken into the Soxhlet assembly.
3. Add 175ml of ethanol in round bottom flask for Soxhlation (10hours).

Standardization of plant Material:

Determination of extractive value: The confirmation of water soluble or alcohol soluble extractive is used as a mean of evaluating the constituents of which are not readily estimated by another method. As suitability and availability, the extractive tests are no longer required as pharmacopoeial standards.

Determination of water-Soluble Extractive value:

Method: 5gm of fine powdered drug was macerated with 100ml of 0.01% v/v chloroform: water of the describe strength in a closed flask for 24 hours. The flask was shaken frequently during the starting 6 hours and then stand for 18 hours. After that filtration was done and precaution was taken to prevent loss of water and tared flat-bottomed shallow dish died at 105ºC & weighted. The percent of the water- soluble extractive value was calculated. (Kharjil A. *et al*, 2012)

Determination of alcohol-soluble extractive value:

Method: 5gm of air-dried fine powdered drug was macerated with 100 ml of ethanol with the specified manner in a closed container for 24 hours and allow standing 18 hours.

To avoid the loss of ethanol precaution was taken, dried in and tared flat-bottomed shallow dish died at 105ºC & weighted. The percent of the ethanolic extractive value was calculated.

Determination of Total Ash value:

In the estimation of total ash value, the carbon content must be removed at low temperature 450ºC as possible because the presence of alkali chloride, which maybe volatile at high temperature.

If carbon content is yet present after heating at moderate temperature, the water-soluble ash may be separated, and residue again ignited.

Procedure: Take 2gm of crude drug was weighted in a tared silica crucible dish and incinerated at a temperature 450ºC until free form carbon. After that silica dish allow to stand for cooling than weighed. The percentage of ash value was calculated with standard to air-dried crude drug. (Kharjil A. *et al*, 2012)

Determination of Acid soluble and Acid insoluble Ash value:

Method: The total ash of air-dried crude drug was boiled with 25ml of 2M hydrochloric acid for 5min. than filter with ashless filter paper. After that ignited and cooled in a desiccator and weighed. The percentage of acid soluble and acid-insoluble ash was calculated.

Determination of loss of drying: Although the loss on weight, in the sample so tested, principally is due to water, smell amounts of other volatile content also involve in weight loss. For volatile material direct drying (100-105ºC) to constant weight can be employed. (Moisture balance= Drying process + weight recording)

Procedure: Weigh a glass stoppered, shallow weighing bottle that was dried at 105ºC. Transfer 2gm of sample crude drug powder in bottle and weighed. Calculated the LOD. (Kharjil A. *et al*, 2012)

Phytochemical Screening tests for ethanol extract:

The phytochemical analysis has carried out on the crude of the ethanol extract (0.2gm) using standard phytochemical procedures for the determination of alkaloids, carbohydrates, Glycosides, proteins, tannins, amino acids and flavonoids etc. (Kathirkamanathan S. *et al*, 2017)

Procedure for detecting phytochemical screening

Table No. 1: Test for Alkaloids

Sr. No.	Test	Reagent	Observation
1	Mayer's test	Potassium mercuric iodide	Cream or white colour precipitate
2	Dragendroff's test	Potassium bismuth iodide	Orange/reddish brown precipitate
3	Wagner's test	Potassium Iodide	Reddish-brown precipitate
4	Hager test	Saturated solution of picric acid	Yellow precipitate

Test for Glycoside:

Baljet Test: 2ml of crude drug extract and sodium picrate solution, it gives yellow to orange colour.

Lagal's test: Take 2ml of dried extract and 1ml pyridine solution and 1 ml sodium nitroprusside, it shows blood red colour.

Kedder's Test: Take 2ml of extract add 1 drop of 90% alcohol and 2 drops of 3,5 dinitro benzoic acid in 90% alcohol, make this solution alkaline with 20% NaOH solution. It produces purple colour.

Borntrager's test: Take a 2 ml of test extract add 1 ml of sulphuric acid, boil & filter than allow to stand for cooling. Shake with equal volume of chloromethane, separate the lower layer and shake it with half its volume of dilute ammonia. It produces rose pink to red colour. (Patel P. *et al*, 2014)

Test for Steroids:

Salkowski Reaction: Take 2 ml of crude drug extract add 2ml chloroform and concentrate sulphuric acid then shake well. The acid layer shows green/yellow fluorescence and chloroform layer shows red colour.

Liebermann-Burchard reaction: Take 2 ml of crude drug extract in chloroform, add 1 ml of acetic anhydride and 2 drops of concentrate sulphuric acid.

Liebermann's reaction: Mix 3ml of crude drug extract with 3 ml of acetic anhydride and boil than allow for Colling and add few drops of concentrate sulphuric acid. Blue colour appears. (Patel P. *et al*, 2014)

Test for Proteins:

Biuret test: Take 3ml of crude drug extract, 4% sodium hydroxide and add few ml of 1% copper sulphate solution. It gives Violet or pink colour.

Xanthoprotein test: Take 2ml of crude drug extract, add 1% concentrate sulphuric acid. White precipitate formed which on boiling change to yellow. By the addition of ammonium hydroxide change to yellow.

Million's Test: Take 2ml of crude drug extract and add few drops of million's reagent. It gives white precipitate at the junction of two liquids. (Ghongade R. *et al*, 2013)

Test for Carbohydrate:

Molish test: Mix 2 ml of crude drug extract with Molish reagent. It produces violet ring at the junction of liquids.

Fehling's solution test: Mix 1 ml Fehling solution A and B (Boil for 1 min), add 2 ml of crude drug extract and heat for 5-10 min. It shows the brick-red colour.

Benedict's test: Take 2 ml of crude drug extract and add few drops of Benedict's reagent. It shows green, red, yellow colour.

Barfoed's test: Take equal quantity of crude drug extract with Barford's reagent. Red colour precipitate was observed.

Iodine test: Take few ml of crude drug extract add iodine solution it gives blue colour. (Ghongade R. *et al*, 2013, Mathew BB. *et al*.2012)

Test for flavonoids:

Shinoda test: Take few ml of crude drug extract add 5ml of 95% ethanol and add few drops of concentrate hydrochloric acid along with magnesium turning. It produces pink colour.

Alkaline reagent test: Mix equal quantity of sodium hydroxide and crude drug extract. Yellow colour form and warm it, then add dilute acid. It shows colourless solution.

Zinc Hydroxide test: Take few ml of crude drug extract and add zinc dust with hydrochloric acid. On standing it gives red colour. (Ghongade R. *et al*, 2013)

Isolation of citric acid from citrus fruit:

There are following steps of isolation of citric acid from the fruits of Citrus maxima.

Source of citric acid: The Citrus maxima fruit is rich source of citric acid. So, by the normal fruit extracts from using a homogeniser or domestic lemon squeezer.

Recovery of citric acid: There were several stages in this method for the isolation of citric acid via application of filtration.

Conversion of citric acid (soluble) to trisodium citrate: In the first step addition of sodium hydroxide which result in the formation of a precipitate trisodium citrate.

$$2C_6H_8O_7+3\ NaOH= (C_6H_5O_7)_2.Na_3.4H_2O+ 2H_2O$$

After the addition of sodium hydroxide, adjust the pH 8-9 and stirrer continuously. When complete neutralization was done go for the filtration.

Conversion of trisodium citrate (insoluble) to Tricalcium citrate: The addition of cloudy solution of calcium chloride produces white precipitated on stirring and boiling. By using vacuum filtration, the white precipitate is collected and dried.

Conversion of tricalcium citrate to citric acid (soluble): The addition of sulphuric acid to the tricalcium citrate resulted in the precipitation of Tricalcium citrate, leaving citric acid in solution.

Recovery of citric acid crystal: The precipitate is washed out via hot water and the filtrate used to evaporate by boiling at 70ºC temperature and the citric acid crystal was formed. (Tariq V-N *et al*, 1995)

***In-vitro* antifungal analysis by disc diffusion method:**

Micro-organisms (Fungi)

The test organism or isolated fungi were Rhizopus nigricans, Fusarium and Penicillium. Stock culture were maintained on Potato-Dextrose Agar (PDA) and Sabourauds-Dextrose Agar (SDA) slants are subcultures at 37°C prior each antifungal test.

Evaluation of antifungal activity:

Culture media: Potato-Dextrose agar (PDA) and sabouradus-Dextrose agar (SDA) media was prepared for culturing and incubation the microbial cell culture.

Table No. 2: Composition of sabouraud-dextrose agar media:

Sr. No.	INGREDIENTS	In gm/1000ml
1	Dextrose (Glucose)	40gm
2	Peptone	10gm
3	Agar	15gm
4	Distilled water	Up to1000ml
5	pH	5.6 ± 0.2
6	Temperature	25°C

Table No. 3: Composition of potato-dextrose agar media:

Sr. No.	Ingredients	In gm/1000ml
1	Potato infusion	100gm
2	Dextrose	10gm
3	Agar	10gm
4	Distilled water	Up to1000ml
5	pH	5.6 ± 0.2
6	Temperature	25°C

Chemicals for antifungal assay: Itraconazole or Fluconazole was used positive reference for standard as for all the stains of fungi. The normal distilled water was applied as solvent for tested samples.

***In-vitro* antifungal assay method:** The whole experiment was done under sterilized environment and laminar airflow cabinet. In this, we have used disc diffusion method for

analysis. The 5mm uniform size disc is prepared by Whatman filter paper. These discs were impregnated in standard drug solution as well as in crude drug extract solution (different concentration). (Dubey N. K. *et al*, 2010)

The previously incubated culture media were poured into sterilized Petri dishes and after solidified disc, the impregnated disc containing drug extract or standard drug were placed in the petri dishes consist of fungi. The plates were kept into the incubator at 37°C for 24 hours. At the completion of incubation time, the zone of inhibition was measured in the Petri dishes.

RESULTS

Table No. 4: Determination of extractive value (%w/w) of the leaf with different solvents

Sr. No.	Extractive	Extractive Value (%w/w)
1	Water soluble extractive	17.8
2	Alcohol soluble extractive	6.2

Table No. 5: Determination of total ash value

Sr. No.	Reading	I	II	III
1	Weight of crucible(gm)	29.35	29.14	29.31
2	Weight of air-dried drug(gm)	2	2	2
3	Weight of crucible + air-dried drug(gm)	31.35	31.14	31.31
4	Weight of crucible + ash(gm)	29.44	29.24	29.40
5	Total ash(gm)	0.07	0.11	0.08
6	Percentage of total ash (%w/w)	4.5	5.0	4.5

Table No. 6: Determination of acid-insoluble ash

Sr. No.	Reading	I	II	III
1	Weight of silica crucible(gm)	29.352	29.352	29.352
2	Weight of silica crucible (gm) +gm	29.359	29.357	29.359
3	Total acid insoluble ash(gm)	0.007	0.005	0.007
4	Percentage total acid insoluble ash (%w/w)	0.35	0.25	0.35

Table No. 7: Determination of acid-soluble ash

Sr. No.	Reading	I	II	III
1	Weight of silica crucible(gm)	29.491	29.359	29.482
2	Weight of silica crucible (gm) +gm	29.583	29.455	29.571
3	Total acid soluble ash(gm)	0.092	0.096	0.089
4	Percentage total acid insoluble ash (%w/w)	4.6	4.8	4.5

Figure No. 2: Chemical test for alkaloids and glycosides

Table No. 8: Phytochemical analysis

S. No.	Phytochemical	Interference
1	Alkaloids	+ve
2	Glycosides	+ve
3	Steroids	-ve
4	Proteins	+ve
5	Carbohydrates	-ve
6	Flavonoids	+ve
7	Tannins	+ve

Isolation of citric acid from citrus fruit:

Table No. 9: Acid content of Citrus maxima fruit extract

Sr. No.	Source	Concentration of acid g/l #
1	Citrus maxima	60±4 (13)

Assumed that acid content equivalent to only citric acid

Mean ± SD (n)

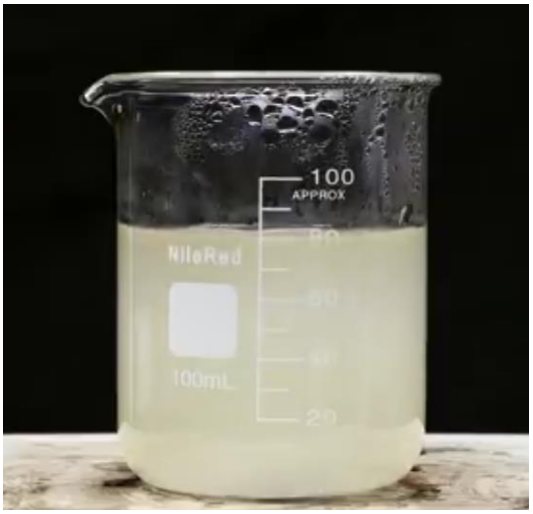

Figure No. 3: Evaporation of tricalcium citrate and development of citric acid crystal

Figure No. 4: Formation of citric acid crystal

Table No. 10: *In-vitro* antifungal evaluation [zone of inhibition (mm)]

Extract	Concentration (mg)	Disc size (mm)	Zone of inhibition (mm)			
			Disc 1	Disc 2	Disc 3	Disc 4
Standard drug	**150**	**5**	**9**	**8**	**10**	**14**
Test 1	100	5	5	5	6	5
Test 2	200	5	5	5	5	6
Test 3	300	5	6	6	6	6
Test 4	400	5	7	6	6	7

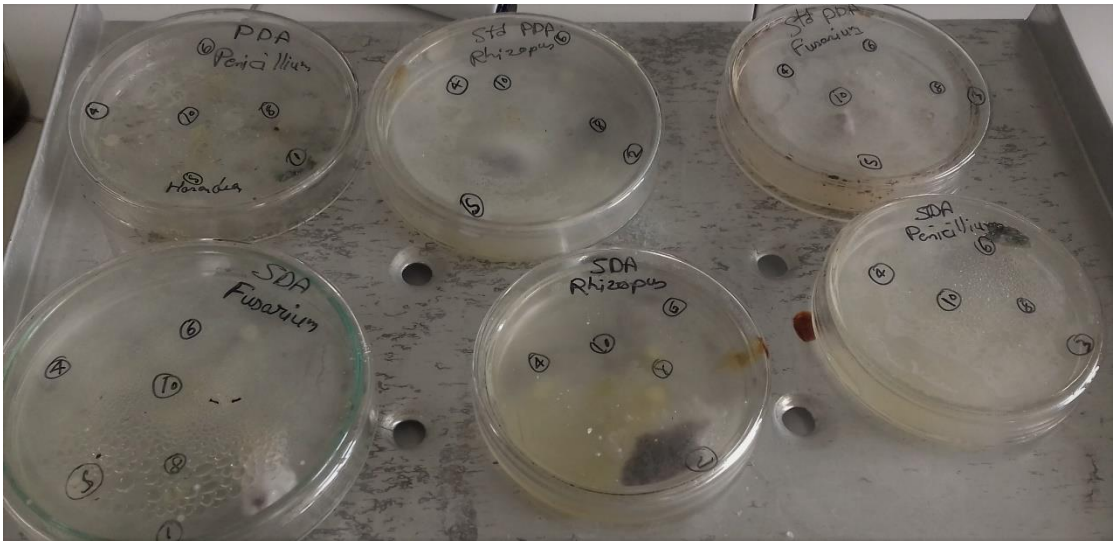

Figure No. 5: Crude drug extract for *in-vitro* evaluation of antifungal activity

DISCUSSION

Citrus maxima belong to the family of Rutaceae. It is rich source of citric acid. The inner fruit part having higher amount of citric acid and the leaves having various phytochemicals that has been investigated. Bioactive or phytochemical properties of *Citrus maxima* leaves had been determined. Now further study is needed to modify the techniques and quantitatively and structurally find out the different active principles.

In our study, Itraconazole shows inhibition zone around the discs which concludes the crude citrus extract having a potency of antifungal activity.

This study proved that ethanolic extract of *Citrus maxima* leaves having various phytochemical like alkaloids, glycosides, saponins, protein, tannins, carbohydrate etc. which are essential for their pharmacological response. During antifungal assay, as we increased the concentration of crude drug extract the activity also increased. So, we can say that antifungal activity of extract is dose dependent. The findings of present investigation revealed the *Citrus maxima* leaf extract having antimycotic activity. Such a variation in chemical composition of ethanolic extract would alter their biological activity. Hence determination of chemical profile is essential before performing antifungal activity.

The major chemical of the *Citrus maxima* fruit is vitamin-C (Ascorbic acid). Due to presence of various phytochemical, it can be used as potent natural antifungal agent, antioxidant additive in food product and as well as a dietary supplement.

In-vitro antifungal activity was determined by various procedures and it has been observed that ethanolic extract of *Citrus maxima* leaves possessed antifungal activity. Thus, it can be said that citrus fruit are useful for consumption and are beneficial for health. This study may lead to the formulation of an antifungal drug which can be used for human health.

CONCLUSION

At present study of medicinal plant Citrus maxima belongs to the family Rutaceae was investigated for the phytochemical screening and the Antifungal activity. The plant leave was screen out with the various chemical constituents like alkaloids, glycosides, protein, flavonoid and tannins. Further estimation involves the isolation of citric acid from the fruit juice of plant *Citrus maxima*. Antifungal activity was detected by paper disc-diffusion method. The ethanolic extract of *Citrus maxima* leaves, inhibited growth of different fungal strains like, Rhizopus, Fusarium and Penicillium at various concentrations from 100 mg to 400 mg, in which test result found out the 400 mg concentration, shows the maximum zone of inhibition. The isolated compound citric acid is useful for its Pharmaceutical properties such as buffering agent, chelating agent, emulsifying agent and acidulant in topical products.

In conclusion, the investigation deals with the potential constituent of *Citrus maxima* for its antifungal activity and pharmaceutical aid properties in area of Biocentres, Pharmaceutical industries, cosmeceuticals and other area of interest. The previous findings have shown that it is a plant of quality which is cost effective and non-hazardous in nature for living being health. Hence, such good antifungal efficacy plant used in future for developing the eco-friendly, safer and effective herbal formulations.

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CONFLICT OF INTEREST: NIL

SOURCE OF SUPPORT: NIL

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