Phytochemical Screening and Antimicrobial Properties of *Citrus Sinensis* leaf extract

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Abstract

Resistant bacteria represent a challenge in the treatments of various well-known infections and necessitate the need to find new substances with antimicrobial properties to be used against these microorganisms. Plants have anchored to the mother earth long before man can set his feet on earth. Plants of citrus sinensis leaf were used widely for medicinal purposes in the olden days, and in the modern times, the use of medicinal herbs and shrubs for the treatment of diseases are a breakthrough in pharmacognosy. The study investigated the phytochemicals and anti-microbial properties of the leaf extract of Citrus sinensis commonly known as sweet orange. Phytochemical screening of the leaf extract of Citrus sinensis commonly known as sweet orange showed the presence of saponin, alkaloid and tannin in both chloroform and ethanolic extract. Flavonoids, alkaloids, tannins and steroids were present in methanol extract, while alkaloid and steroid were observed in aqueous extract, and tannin in citrus sinensis residua, Antimicrobial property of aqueous extract of Citrus sinensis against Staphylococcus aureus and Escherichia coli was higher than other solvent extracted leaf extract of Citrus sinensis with an average zones inhibition of about 22 and 18 respectively, followed by that of ethanol and methanol extracts which have showed a greater antimicrobial activity against Escherichia coli and Staphylococcus aureus with an average equal zones of inhibition of about 10 and 9 respectively, n-Hexane extract shows the least antimicrobial activity against Staphylococcus aureus and Escherichia coli with an average zone of inhibition of 9 and 8 respectively. Hence, the presence of secondary metabolites in the leaves of Citrus sinensis shows that the plant can be effective against many ailments. Based on the result of this study, the water-soluble extract (F003) showed a greater antimicrobial (antibacterial) activity on Escherichia coli and Staphylococcus aureus. The n-Hexane soluble extract (F005) showed the least activity among other soluble extracts. Alkaloids, Tannins, Steroids and Flavonoids were present in leaf of Citrus sinensis.

Keywords: Citrus sinensis, Escherichia coli, Staphylococcus aureus, antimicrobial activity.

Criblage Phytochimique Et Propriétés Antimicrobiennes De L'extrait De Feuilles De Citrus Sinensis

Résumé

Les bactéries résistantes représentent un défi dans le traitement de diverses infections bien connues et nécessitent la nécessité de trouver de nouvelles substances dotées de propriétés antimicrobiennes à utiliser contre ces micro-organismes. Les plantes se sont ancrées à la terre mère bien avant que l'homme puisse poser les pieds sur terre. Les plantes à feuilles de citrus sinensis étaient autrefois

largement utilisées à des fins médicinales, et dans les temps modernes, l'utilisation d'herbes et d'arbustes médicinaux pour le traitement des maladies constitue une percée dans la pharmacognosie. L'étude a examiné les propriétés phytochimiques et antimicrobiennes de l'extrait de feuille de Citrus sinensis, communément appelé orange douce. L'analyse phytochimique de l'extrait de feuille de Citrus sinensis, communément appelé orange douce, a montré la présence de saponine, d'alcaloïde et de tanin dans l'extrait chloroformique et éthanolique. Des flavonoïdes, des alcaloïdes, des tanins et des stéroïdes étaient présents dans l'extrait au méthanol, tandis que des alcaloïdes et des stéroïdes ont été observés dans l'extrait aqueux, et des tanins dans les résidus de Citrus sinensis. La propriété antimicrobienne de l'extrait aqueux de Citrus sinensis contre Staphylococcus aureus et Escherichia coli était supérieure à celle des autres solvants extraits. extrait de feuille de Citrus sinensis avec une zone d'inhibition moyenne d'environ 22 et 18 respectivement, suivi de celui des extraits éthanoliques et méthanoliques qui ont montré une plus grande activité antimicrobienne contre Escherichia coli et Staphylococcus aureus avec une zone d'inhibition égale moyenne d'environ 10 et 9 respectivement, l'extrait de n-Hexane présente la moindre activité antimicrobienne contre Staphylococcus aureus et Escherichia coli avec une zone d'inhibition moyenne de 9 et 8 respectivement. Ainsi, la présence de métabolites secondaires dans les feuilles de Citrus sinensis montre que la plante peut être efficace contre de nombreuses affections. Sur la base des résultats de cette étude, l'extrait hydrosoluble (F003) a montré une plus grande activité antimicrobienne (antibactérienne) sur Escherichia coli et Staphylococcus aureus. L'extrait soluble au n-hexane (F005) a montré le moins d'activité parmi les autres extraits solubles. Des alcaloïdes, des tanins, des stéroïdes et des flavonoïdes étaient présents dans les feuilles de Citrus sinensis.

Mots-clés : Citrus sinensis, Escherichia coli, Staphylococcus aureus, activité antimicrobienne.

تمثل البكتيريا المقاومة تحديًا في علاجات مختلف الإصابات المعروفة وتقتضي الحاجة إلى إيجاد مواد جديدة ذات خصائص مضادة للميكروبات لاستخدامها ضد هذه الكائنات الحية الدقيقة لقد رست النباتات على الأرض الأم قبل وقت طويل من أن يضع الإنسان قدميه على الأرض. تم استخدام نباتات أوراق الحمضيات على نطاق واسع للأغراض الطبية في الأيام القديمة وفي العصر الحديث يمثل استخدام الأعشاب والشجيرات الطبية لعلاج الأمراض إنجازا كبيرا في العقاقير بحثت الدراسة في المواد الكيميائية النباتية والخصائص المضادة للميكروبات لمستخلص الأوراق من سينينسيس الحمضيات المعروف باسم البرتقال الحلو أظهر وجود الصابونين والقلويد والتانين في كل من مستخلص الكلوروفورم والإيثانوليك كانت الفلافونويد والقلويدات والعفص والمنشطات موجودة في مستخلص الميثانول بينما لوحظ وجود القلويد والستيرويد في المستخلص المائي والتانين في الحمضيات خاصية مضادات الميكروبات للمستخلص المائي للحمضيات ضد المكورات العنقودية الذهبية وكان الإشريكية القولونية أعلى من مستخلص أوراق المذيب المستخرج من الحمضيات بمتوسط تثبيط للمناطق يبلغ حوالي 22 و 18 على التوالي تليها مستخلصات الإيثانول والميثانول التي أظهرت زيادة في مضادات الميكروبات... ضد الإشريكية القولونية والمكورات العنقودية الذهبية بمتوسط مناطق تثبيط متساوية يبلغ حوالي 10 و 9 أقل نشاط مضاد للميكروبات ضد المكورات العنقودية الذهبية و الإشريكية القولونية بمتوسط منطقة تثبيط يبلغ 9 و 8 n-Hexane على التوالي يُظهر مستخلص على التوالي وبالتالي، فإن وجود مستقلبات ثانوية في أوراق سيتروس سينينسيس يظهر أن يمكن أن يكون النبات فعالاً ضد العديد من الأمراض بناءً على النتيجة، القابل n-Hexane أظهر المستخلص القابل للذوبان في الماء نشاطًا أكبر ضد الميكروبات على الإشريكية القولونية والمكورات العنقودية الذهبية أظهر مستخلص أقل نشاط من بين المستخلصات الأخرى القابلة للذوبان. القلويدات والعفص والمنشطات والفلافونويد كانت موجودة في أوراق الحمضيات (F005) للذوبان

Introduction

The orange peels are usually considered as waste materials, which may create environmental problems for local communities because of the presence of biomaterials in orange peel. Every ton of food waste means 4.5 ton of CO2 emissions. (Adams *et al*., 2006). There is a great need for development of new

and environmentally friendly design processing techniques which could be turned into an asset. Also, the development of drug resistance by some microbial strain to commercial antibiotics has posed concerns to scientist which is a serious problem. The problem of environmental pollution can also be reduced. Citrus sinensis is an orange with a round shape and tree has a length of 9-10 m. Leaves of these trees are in oval shape, their barks appear to be green or brown in color which is quite smooth. Leaves have a size of 4-10 cm (if it is length is taken in to account). Leaves have smooth texture with a smell resemblance to the sweet orange. The flower of this tree consists of mainly five petals which smell same as saccharine (Webber *et al*., 2003). Citrus sinensis has seeds in between the parts where juices are present. The seeds are green or cream in color. The fruit's flesh is mostly made of the orange sweet juicy part. The peel has orange color. The endocarp is the palatable portion, partitioned into 10-14 sections segregated by thin septa, containing up to 8 seeds/septa, but it was appeared regularly with one. Citrus fruit extracts and citrus flavonoids exhibit a wide range of promising biological properties due to their phenolic profile and antioxidant properties (Addi, M., et al., 2021). They have been seen to have wide range of antimicrobial activity on pathogenic microorganism. Citrus is the largest fruit crop in the world (100 million cubic tons per year) and the orange account for 60% (Odubanjo, O. *et al*., 2002). The remaining orange-peel account for approximately 45% of the total bulk (Yeoh et al., 2008). Consequently, significant amounts of orange peel are available as a by-product. The orange peel, if treated as waste materials, may create environmental problems, particularly water pollution, due to the presence of biomaterials such as essential oil (Ferhat, 2008), pectin (Yeoh et al., 2008; Berna et al., 2000) and sugar. This problem could be turned into an asset, if potentially marketable active principles such as essential oil could be extracted from the peel. After extraction, the peel could be a high protein stock feed in dry form, increasing the potential return for the orange juice industry and reducing the pollution (Yeoh et al., 2008).

The orange is a small tree with irregular branches armed with thick spines, stiff and sharps leaves of 5 to 7.5 cm long, flowers of 2 cm long. Fruit is round with 3.0 to 6.0 cm, greenish yellow with plenty of acid pulp, small white seeds with shaped oval, in local applications, it eases rheumatic aches and is very effective for acute attack of lumbago (Gulsen, O. *et al* 2011)

Materials and method

Ethanol, methanol, chloroform, distilled water, n-hexane, mercuric chloride, potassium iodide , ferric chloride, concentrated sulphuric acid, magnesium fuming, concentrated hydrochloric acid, olive oil, Fehling's solution, Dimethyl Sulphoxide (DMSO), Mueller Hinton Agar and Gentamacin were used as received.

Sample collection

Fresh samples of sweet orange leaves were collected from Unguwar Fulani Quarters, Wudil Local Government Area of Kano State. The leaves were chopped into pieces, washed with distilled water and allowed to dry under the shade. The dried leaf sample were ground into powder using a cleaned pestle and mortar.

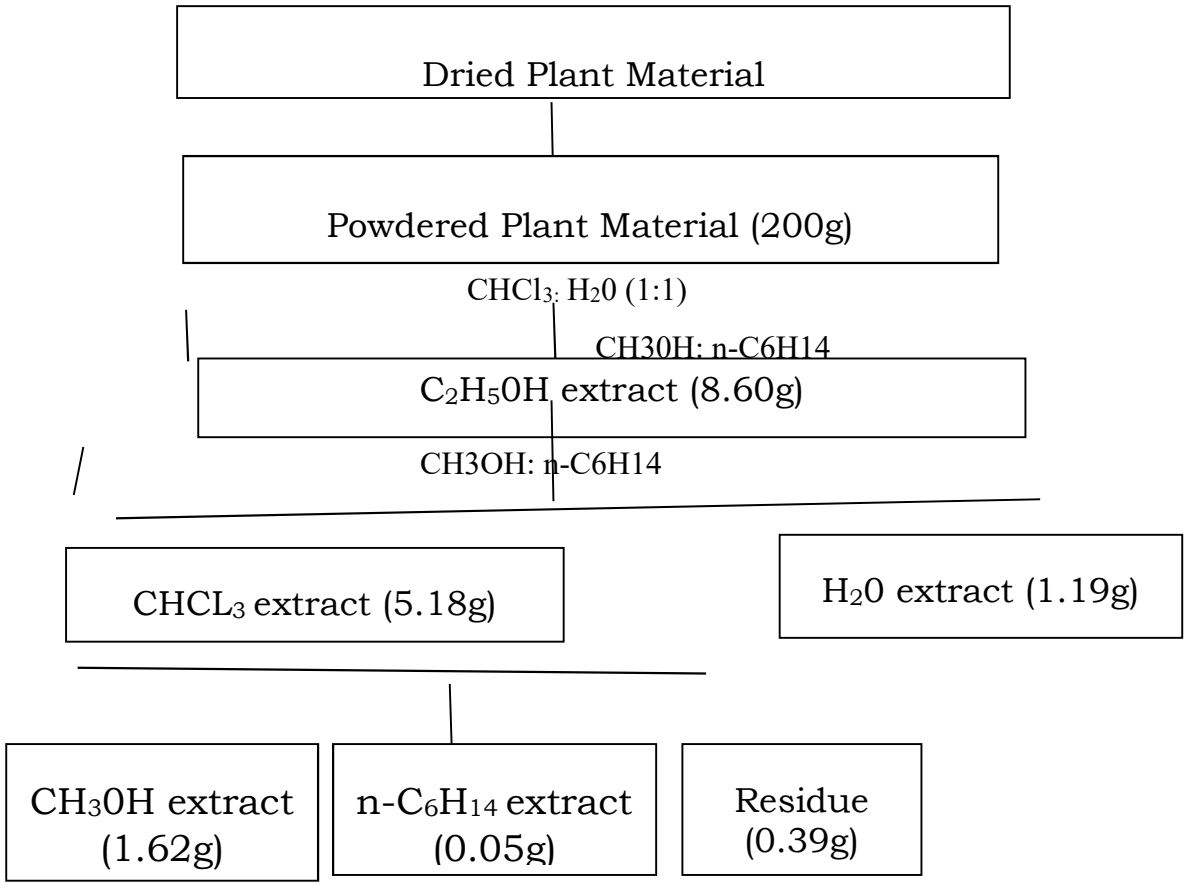
Percolation of Citrus Sinensis

200 g of the ground *Citrus sinensis* leaf sample was dissolved in 800 mL of ethanol at room temperature for 48 hours with a regular shaking. The mixture s decanted, filtered and concentrated using a rotary evaporator at 50 ℃. The crude ethanol extract obtained after drying was weighed (8.60 g) and labelled as (F_{001}).

Extraction

Fractionation with chloroform and distilled water

Seven grams (7.0 g) of the dried ethanol extract (F_{001}) was fractionated with chloroform and distilled water. Thirty milliliters (30 mL) each of chloroform and distilled water were added to the crude extract and diluted completely. The mixtures were transferred into a separating funnel (250 mL), and gently shaken with a regular opening of the tap to reduce the pressure until it became completely saturated. The mixture was allowed to separate for 24 hours. The separated portions were collected separately into a clean, dry, pre-weighed and labelled beakers. 3.00 g of dried chloroform extract (F_{002}) were fractionated with methanol and n-Hexane to obtain the methanol and n-Hexane fractionated fractions. Methanol (30 mL) and n-Hexane (30 mL) were added to the dried crude extract and diluted until it dissolved completely. It was then transfered into separating funnel (250 mL) and shaken gently with regular opening of the tap to reduce the internal prssure until it became completely diluted. It was allowed to separate on a retort stand for 24 hours duration. Each portion was separately collected into a clean; dry weighed and labelled two different beakers. Both extracts were allowed to evaporate to give the methanol and the *n-Hexane* dried fractionated fractions, labelled as (F_{004}) and (F_{005}), respectively, and weighed (1.62 g) for methanol and (0.05 g) for the *n-Hexane* fractions after it has completely dried. The filtered residue was also dried on the filter paper and labelled (F_{006}) and weighed as (0.39 g). Fractionation



Scheme 1: flow chart of extraction process

Preparation of Meyer's reagent

1.30 g of mercuric chloride were dissolved in 50 mL of distilled water, 5.00 g of potassium iodide was dissolved in 50 mL distilled water. The two solutions were combined to make 100 mL of Meyer's reagent.

Ferric chloride solution

Ferric chloride solution was prepared by dissolving 5.00 g of Ferric chloride in 100 mL distilled water. The flask was shaken to ensure homogeneity.

Test for alkaloids

Small portion of each dried test extract was mixed with 15 mL of methanol and stirred with a glass rod until it became completely dissolved. One milliliters (1 mL) of the test extracts were taken with sterile micro syringe and transferred to a clean dry tube. One milliliters (1 mL) of Mayer's reagent was added to the mixture. A creamy colour precipitate indicates the presence of alkaloids.

Test for tannins

Small portion of each dried extract was mixed with 15 mL of methanol and stirred with a glass rod until it became completely dissolved. One milliliters (1 mL) of the test extract was taken with a sterile micro syringe and transferred into a clean and dry test tube, and then about 2 drops of the ferric chloride solution ($FeCl_3$) were added to the mixture. A bluish-black or greenish-black precipitate indicates the presence of tannins.

Test for steroids

A small portion of each dried test extract was mixed with 15 mL of methanol and stirred with a glass rod until it became completely dissolved. One milliliters (1 mL) of the test extract was taken with a sterile micro syringe and transferred to a clean and dry test tube, then 1 mL of concentrated sulphuric acid (H_2SO_4) was added to the mixture for 40 minutes duration. A red coloration indicates the presence of steroids.

Flavonoid test

About 0.4 g of the extract was dissolved in 1 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of three (3) drops of concentrated HCL. The occurrence of a red or orange colouration was indicative of the flavonoids.

Test for saponin

Precisely 0.5g /ml of each sample was stirred with water in a test-tube Frothingpersiston warming was taken as evidence for the presence of Saponin.

Antimicrobial analysis test

Overnight cultures of the Gram-positive strains S. aureus, and the Gram-negative strains E. coli were prepared on nutrient brothplates. All bacterial isolates were suspended in saline to a turbidity equivalent to 0.5 McFarland (1.5 x 108 CFU/ml) and a sterile swab stick was dipped into the test tube containing the organisms and it was used to seed the organism on the solidified Nutrient agar (MHA, pH 7.3 ± 0.1, Difco). Then 6 mm wells were prepared. In these wells 3.125ug, 6.125 ug, 12.50 ug, 25, 50 ug and 100 ug of the solvent extract of Citrus sinensis peel was added and allowed to stand for 30 minutes for pre-diffusion. The plate was incubated overnight at 37ºC for 24 hours. After incubation the zones of inhibition were measured and recorded (Bonev, 2008; Yagoub, 2005).

Test organisms

Staphylococcus aureus and *Escherichia coli* were isolated, and obtained from the microbiology laboratory, department of microbiology Kano University of Science and Technology, Wudil, Kano State.

Preparation of stock solution

0.2 g of extract was dissolved in 5 mL of dimethyl sulphoxide (DMSO) . Different concentrations were prepared according to Kabiru *et al*. (2023).

Preparation of sensitivity discs

Discs of about 6 mm in diameter were made from Whitman's No. 1 filter paper using a puncher. About 50 discs were transferred into each bijou bottle (15 bijour bottles) and sterilized at 121℃ for 15 minutes.

Preparation of concentrations

Organic solvent extracts were dissolved in 1 mL Dimethyl Sulphoixide (DMSO) while aqueous extract were dissolved in 1mL sterile distilled water that is to say: 0.06 g of each extract was dissolved in 1mL of the solvent. 0.5 g of the sterile discs respectively in bijour bottles to make 60μg/disc concentration. Half (0.5ml) of DMSO was added into the remaining stock solution making 1ml, 0.5ml was taken and placed into another bottle containing 50 filter paper discs and labeled 30μg/disc, 0.5ml of DMSO was added, another 0.5ml was taken and placed into another 50-filter paper disc and labeled 15μg/disc. With each disc, was capable of absorbing 0.01ml of the solution, the procedure was employed to prepare 15, 30 and 60 μg/disc doubling dilution as explained above was employed in the preparation of organic solvent extracts for disc and Mic preparation (Kabiru *et al.,* 2023).

Preparation of culture media (Mueller Hinton agar)

Thirty-five grams (35 g) of Muiler Hinton agar was dissolved in 100 mL of distilled water and autoclaved at 131℃ for15 minutes. Nutrient broth was equally prepared by dissolving 14 g into 100 mL of distilled water. The solution was dispensed into test tubes and autoclaved at 121℃ for 15 minutes (Siraj and Ado, 2018).

Preparation of control solution/concentration

1 mL of Dimethyl Sulphoxide (DMSO) was poured into a bijour bottle containing 50 filter paper discs. Then, 1 mL of gentamycin was added to make up the control solution (Siraj and Ado, (2018).

Bioassay procedure

Standard inocula of the isolates were swabbed on the surface of prepared and solidified Mueller Hinton agar in separate Petri dishes. The discs of the extracts (60 μg/disc, 30 μg/disc and 15 μg/disc) and the standard antibiotic disc (gentamycin) were placed on the surface of the inoculated media at intervals. The Petri dishes were incubated at a temperature of 37℃ for 24 hours. Thereafter, zones of inhibition was measured as described by Siraj and Ado (2018).

Results

Table 1: Phytochemical screening of ethanol extract

S/N	Phytochemicals	Test Performed	Ethanol Extract
1	Carbohydrates	Fehling's test	-
2	Flavonoids	Test for flavonoids	-
3	Saponins	Froth with water test	+
4	Alkaloids	Mayer's test	+
5	Tannins	Ferric chloride test	+

Table 2: Phytochemical screening of chloroform extract

S/N	Phytochemicals	Test Performed	Chloroform Extract
1	Carbohydrates	Fehling's test	
2	Flavonoids	Test for flavonoids	
3	Saponins	Froth with water test	+
4	Alkaloids	Mayer's test	+
5	Tannins	Ferric chloride test	+

Table 3: Phytochemical screening of aqueous extract

S/N	Phytochemicals	Test Performed	Water Extract
1	Carbohydrates	Fehling's test	-
2	Flavonoids	Test for flavonoids	-
3	Saponins	Froth with water test	-
4	Alkaloids	Mayer's test	+
5	Tannins	Ferric chloride test	-

Table 4: Phytochemical screening of methanol extract

S/N	Phytochemicals	Test Performed	Methanol Extract
1	Carbohydrates	Fehling's test	-
2	Flavonoids	Test for flavonoids	+
3	Saponins	Froth with water test	-
4	Alkaloids	Mayer's test	+
5	Tannins	Ferric chloride test	+

Table 5: Phytochemical screening of residue extract

S/N	Phytochemicals	Test Performed	Residue Extract
1	Carbohydrates	Fehling's test	-
2	Flavonoids	Test for flavonoids	-
3	Saponins	Froth with water test	-
4	Alkaloids	Mayer's test	-
5	Tannins	Ferric chloride test	+

Key: (+) sign represent present and (-) represents absent

Table 6: Antimicrobial activities of n-hexane, chloroform, ethanol, aqueous and methanol respectively

n-Hexane extract			
Test organism	Zones of inhibition (mm)		
Bacteria	60 μg/mL	30 μg/mL	15 μg/mL
Escherichia coli	9	8	7
Staphylococcus aureus	12	9	7

Chloroform extract			
Test organism	Zone of inhibition (mm)		
Bacterial	60 μg/ml	30 μg/ml	15 μg/ml
Escherichia coli	13	8	7
Staphylococcus aureus	13	9	8

Ethanol extract			
Test organism	Zone of inhibition (mm)		
Bacterial	60μg/ml	30μg/ml	15μg/ml
Escherichia coli	12	10	8
Staphylococcus aureus	12	9	8

Aqueous extract			
Test organism	Zones of inhibition (mm)		
Bacteria	60 μg/ml	30 μg/ml	15 μg/ml
Escherichia coli	24	20	12
Staphylococcus aureus	26	21	18

Methanol extract			
Test organism	Zone of inhibition (mm)		
Bacteria	60 μg/mL	30 μg/mL	15 μg/mL
Escherichia coli	13	10	7
Staphylococcus aureus	12	9	8

Discussion

The bioactivity of this plant extracts is attributed to its phytochemical constituents. The leaf extract contains Alkaloids, Tannins, some Saponins and Steroids, with a very less Flavonoids. The presence of Saponins supports the fact that orange leaf has cytotoxic effects such as permealization of the intestine as Saponins are cytotoxic, presence of Alkaloids in the leaves of orange shows that the

plant can be effective anti-malaria as an antioxidant the plant can be used in herbal medicine for the treatment of common cold and other diseases. Also, this study has shown the antimicrobial activity of the citrus sinensis plant extracts against the tested organisms as described by Hussain, K. A *et al*., (2015)

Conclusion

Based on the result of this study, the water-soluble extract (F_{003}) showed a greater antimicrobial (antibacterial) activity on *Escherichia coli* and *Staphylococcus aureus.* The *n-Hexane* soluble extract (F_{005}) showed the least activity among other soluble extracts. Alkaloids, Tannins, Steroids and Flavonoids were present in leaf of *Citrus sinensis.*

Recommendations

Research on Isolation and characterization of the bioactive components in *Citrus sinensis* leaf are encouraged.

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