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Preliminary Phytochemical Analysis of Lemon (*Citrus limon*) Leaf Nano Powders

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Abstract

For thousands of years, people have utilized plants as medicine in ancient cultures. Extracts from medicinal plants are said to possess health-promoting qualities since they include secondary metabolites such flavonoids, alkaloids, amino acids, carotenoids, and steroids, among others. The leaves of certain plants are used to treat multiple leaves, convulsive cough, and epilepsy. A review of the literature reveals a number of health advantages as well as pharmacological characteristics, including anti-diabetic, anti-inflammatory, and anti-oxidative effects, among many others. This study is to characterize the powdered citrus limon leaves using Xrd and Edax and to analyze the powdered citrus limon leaves using two extracts, methanol and aqueous, for both qualitative and quantitative analysis. The nano powder of citrus limon leaves, according to a preliminary qualitative and quantitative study, included flavonoids, alkaloids, limonoids, carotenoids, phenolic compounds, coumarins, and essential oils. The phenolic chemicals, limonoids, and alkaloids found in citrus limon leaf nano powder make it a potent bioactive substance. It can be used to cure some illnesses and is an excellent source of phytochemicals.

***Key words*:** Citrus limon leaves, Xrd, Edax, Phytochemical analysis, Aqueous, Methanol extracts

Lemon citrus L. leaf is a common plant that is native to Asia and from to the Rutaceae family. Following a preliminary phytochemical assessment, the leaf extract of Citrus limon L has been identified to contain alkaloids, tannins, phenolic compounds, carbohydrates, proteins, oils, lipids, and flavonoids. Three varied concentrations of anthelmintic activity, such as 5 mg/ml, 10 mg/ml, and 20 mg/ml, were investigated. It is these phytochemicals that give plants their biological action. This investigation examined the antibacterial properties of leaf extracts in light of their diverse biological activities. C. lemon leaves have been shown to be useful in the development of new antibiotics and in the study of their method of action on the cells of pathogenic microorganisms [1]. *C. lemon* leaves have been shown to be useful in the development of new antibiotics and in the study of their method of action on the cells of pathogenic microorganisms. The potential of *C. lemon* leaf oil as a new antibiotic against harmful bacteria, as well as its impact on the creation of proteins, lipids, DNA, and RNA in *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells, should be estimated [2]. The Rutaceae family includes lemons (*Citrus limon* L.). After orange and mandarin trees, lemons are the third most popular citrus species grown worldwide. Geranial, neryl acetate, limonene, cis-α-bergamotene, and β-bisabolene were the most prevalent chemicals. Spraying AgNPs at 5, 7.5, and 10 mg/L increased the leaf chemical composition of N, P, K, Fe, Zn, and Mn significantly as compared to the control over the two experimental seasons [3]. Using ethanol, acetone, and distilled water as extraction solvents, the phytochemical and antioxidant properties of *Citrus limon* L. Osbeck leaf extracts were investigated. The present work provides support for the use of *C. limon* leaf extracts in folk medicine by demonstrating their high phytochemical content and antioxidant activity [4]. To determine the methanol extract's phytochemistry and antioxidant properties from *Citrus limon* (L.) Osbeck (CLM) leaves. Numerous flavonoids (rutin and apigenin) and phenolics (gallic acid, catechin, vanillic acid, coumaric acid, and resveratrol) were detected by HPLC tests. Phenolics (302.91 µg EAG/mg), tannins (36.86 ± 0.71 µg ECT/mg), flavonoids (19.77 ± 0.06 µg EQ/mg), and coumarins were detected by qualitative analysis. *Citrus limon* contains antioxidant properties and is a rich source of bioactive phytochemicals such as tannins, flavonoids, and phenolic acids. These findings offer verifiable proof of the health advantages of conventional plants and present a promising profile of naturally occurring antioxidant phytochemicals [5]. In 2010, lemons ranked among the world's most significant crops, with an estimated 123 million tons produced annually. After orange and mandarin, lemon (*Citrus limon* L.) is the third most significant citrus species in the world with 4.200.000 metric tons of output [6]. One of the key components of the Tunisian economy is the lemon (*Citrus limon* L). As a matter of fact, in 2005, the production of lemons and limes approached 27,000 tons. Several significant species are found in the genus Citrus worldwide: 56% of the species are oranges, 17% are tangerines and clementines, 11% are lemons and limes, and 6% are

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grapefruit [7]. Lemon is very rich in important natural compounds, including citric acid, ascorbic acid, minerals, flavonoids, and essential oils [8].

MATERIALS AND METHODS

Citrus limon leaves were collected locally from Tuticorin District, Tamil Nadu, India. The Plant identities were verified and authenticated by the Botanist at Sadakathullah Appa college in Tirunelveli District. The herbarium specimen was preserved in Department of Botany for future references. The part which was collected was washed with distilled water it is then shadow dried for 7-10 days. The dried leaves were grinded using Mortal and pestal for three hours and then it is sieved. The synthesized powder was again grinded for another two hours and then is allowed to II sieve for the reduction in to nano-size powder. It is then stored in sterile air tight container.

Characterization

The synthesized leaves (*Citrus limon*) nano powder was characterized using XRD and EDAX for conformation of the sample size and identification of elements [9-11]. To find out the quantitative and qualitative phytochemical analysis were done by using two extracts Aqueous and methanol.

Phytochemicals screening test

Test for tannins

10 ml of bromine water was added to the sample. Decoloration of bromine water showed the presence of tannins.

Tests for flavonoids

Alkaline reagent test: 2 ml of 2.0% NaOH mixture was mixed with the sample; concentrated yellow color was produced, which became colorless when we added 2 drops of diluted acid to mixture. This result showed the presence of flavonoids.

Tests for glycosides

Liebermann's test: Added 2.0 ml of acetic acid and 2 ml of chloroform with the sample. The mixture was then cooled and we added H_2SO_4 concentrated. Green color showed the entity of aglycone, steroidal part of glycosides.

Test for terpenoids

2.0 ml of chloroform was added with the sample and evaporated on the water path and then boiled with 3 ml of H_2SO_4 concentrated. A grey color formed which showed the entity of terpenoids.

Test for alkaloids

Sample is dissolved in dilute Hydrochloric acid and filtered. Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow-coloured precipitate indicates the presence of alkaloids.

Test for phenolic compounds

2 ml of distilled water followed by few drops of 10% ferric chloride was added to the sample. Formation of blue or green color indicates presence of Phenolic compounds.

Test for steroid

Analytical method used is according to Ejikeme *et al.* [11]. Each sample (0.30 g) weighed into a beaker was mixed with 20 cm 3 of ethanol; the component was extracted for 2 hours. To the ethanolic extract of each sample (5 cm 3) was added 2 cm 3 acetic anhydride followed with 2 cm 3 of concentrated tetraoxosulphate (VI) acid. A violet to blue or green colour change in sample(s) indicates the presence of steroids.

Test for saponins

5.0 ml of distilled water was mixed with sample in a test tube and it was mixed vigorously. The frothing was mixed with few drops of olive oil and mixed vigorously and the foam appearance showed the presence of saponins.

Estimation of Kaempferol/Quentin/Hesperia and Naringin

Flavonoid compounds were measured by the aluminium chloride colorimetric assay

The reaction mixture consists of 1 mg of sample and 4 ml of distilled water was taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5% sodium nitrite was treated and after 5 minutes, 0.3 ml of 10% aluminium chloride was mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water. Reference standard solutions of Kaempferol/Quentin/Hesperia and Naringin (20, 40, 60, 80 and 100 µg/ml) were prepared separately in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The above compounds were expressed separately for each standard.

Estimation of β-carotene

β-carotene was estimated following approved method as described below:

Sample was taken in 150 ml glass stoppered Erlenmeyer flask and 40 ml water saturated butanol (WSB) was added. The contents of the flasks were mixed vigorously for 1 minute and kept overnight (16-18 hours) at room temperature under dark for complete extraction of β-carotene. Next day, the contents were shaken again and filtered completely through the Whatman No. 1 filter paper into a 100 ml volumetric flask. The optical density of the clear filtrate was measured at 440 nm using spectrophotometer.

Pure WSB was used as blank. The β-carotene content was calculated from calibration curve from known amount of β-carotene as discussed below and expressed as parts per million (ppm). Standard solution of β-carotene (Sigma) was prepared in water saturated butanol (WSB) at the concentration of 5 µg/ml. WSB is prepared by mixing n-butanol with distilled water in 8:2 ratios. Calibration curve is made from known amounts of pure β-carotene from 0.25 µg/ml to 1.5 µg/ml which are prepared after suitable dilutions of original stock with WSB in calibrated 10 ml volumetric flasks (from 0.5 ml to 3 ml of standard solution in 10 ml). Absorbance of each dilution is measured and a calibration curve is established. β-carotene content of unknown samples is calculated from standard curve. Ref: Biochemical methods/AOAC.

Essential oils

Extraction of essential oils according to the distillation technique by means of a calvinger device. The essential oils were extracted by hydro-distillation method using a Clevenger equipment, by placing a quantity of sample in a 100 ml glass flask, to which a quantity of distilled water (60 ml) was added until the entire sample was immersed. The extraction process continued for 18 hours, after which the essential oil was separated from the aqueous extract by a 100 ml separating funnel using chloroform solvent in several batches. Evaporater at °C 40 to a volume of 3ml and then leave the solution until complete evaporation of the solvent. The oils were measured and calculated.

Determination of alkaloid (Rutin)

The sample was dissolved in dimethyl sulphoxide (DMSO), added 1ml of 2 N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of Rutin (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The Rutin content was expressed as mg/g of extract.

Determination of coumarins content

The concentration of coumarins in sample was determined using spectrophotometric method. The reaction mixture consists of 1 ml of ethanol extract sample and 9 ml of distilled water was taken in a volumetric flask (25 ml). One millilitre of Folin-Ciocalteu phenol reagent was treated to the mixture and shaken well. After 5 minutes, 10 ml of 7% Sodium carbonate (Na_2CO_3) solution was treated to the mixture. The volume was made up to 25 ml. A set of standard solutions of Coumarins (20, 40, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV) /Visible spectrophotometer. Coumarins content was expressed as mg/gm.

Determination of phenolic compounds: Phenolic acid, caffeic acid, cholorogenic acid

The concentration of phenolics in sample extract was determined using spectrophotometric method. Folin-Ciocalteu assay method was used for the determination of the Phenolic compounds. The reaction mixture consists of 1 ml of alcoholic extract and 9 ml of distilled water was taken in a volumetric flask (25 ml). One millilitre of Folin-Ciocalteu phenol reagent was treated to the mixture and shaken well. After 5 minutes, 10 ml of 7% sodium carbonate (Na_2CO_3) solution was treated to the mixture. The volume was made up to 25 ml. A set of standard solutions of phenolic acid, caffeic acid, cholorogenic acid (20, 40, 40, 60, 80 and 100 µg/ml) were prepared separately in the same manner as described earlier. Incubated for 90 min at room temperature and the absorbance for test and standard solutions were determined against the reagent blank at 550 nm with an Ultraviolet (UV) /Visible spectrophotometer. Phenolic acid, caffeic acid, cholorogenic acid were calculated separately and expressed as mg/g.

Limonoids determination: (Limonin and Nomilin)
Preparation of the reference solution

Limonin and Nomilin, reference substance (10mg) was accurately weight separately, and added in a 10ml volumetric flask, diluted with ethyl acetate to the marked line to afford a concentration of 1.0mg/ml standard solution.

Preparation of the test solution: The sample was precisely measured and placed in a 10ml volumetric flask, diluted with ethyl acetate to the marked line.

Chromogenic method: The color developing agent applied on this experiment was prepared by the procedure as follows, 5% vanillin-acetic acid solution plus 2mL of perchloric acid were heated at 65°C for 20min, then cooled in ice water and warmed up to room temperature after being shaken. Vanillin (500mg) was dissolved in acetic acid (10ml) to prepare the vanillin solution. The standard curve 0.0, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0 ml Limonin and Nomilin standard solution were precisely measured, placed in a 10 ml flask with ethyl acetate to volume marked line, The sample solution and standard mixture was then shaken, colored according to the chromogenic method. The absorbance (A) of each solution was measured at 210nm wavelength, a blank solution as the control reference. The Limonin and Nomilin content was calculated separately and expressed as mg/g.

RESULTS AND DISCUSSION

XRD analysis

The XRD patterns were recorded for the citrus lemon leaf nano powders (Fig 1). In this pattern the 3 most prominent peaks are at 2θ = 24.3973, 14.8907 and 21.6334. All the diffraction patterned in studied samples have good agreement with standard lines as given in the below file. Thus, the comparison confirms the presence of Hexagonal Wurtzite crystal structures of synthesized nanoparticles. The 'd' values for all the lines have been calculated and the patterns have been indexed in (Table 1). The values of crystallite size have been calculated by the Scherrer method considering the FWHM of the most prominent diffraction lines. The results show that the values of the average crystallite size are 30.91 nm for citrus lemon leaf nano powders [12].

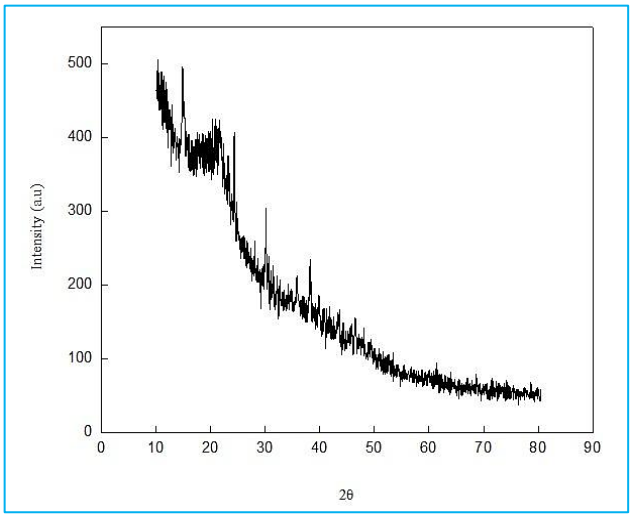

Fig 1 XRD pattern of citrus lemon leaf nano powders

Table 1 XRD parameters of citrus lemon leaf nano powders

2θ	FWHM (degree)	d-spacing (Å)	Crystallite size (nm)
24.3973	0.1574	3.64851	50.65
14.8907	0.2362	5.94948	33.66
21.6334	0.9446	4.10798	8.42
Average size of citrus lemon leaf nano powders			30.91

EDX analysis

EdX patterns were recorded for the citrus lemon leaf nano powders (Fig 2). The patterns have been indexed in (Table 2). Potassium, calcium, carbon, oxygen, magnesium, copper, phosphorus, boron, and zinc are present in the EDX spectra of citrus lemon leaf nano powders. The components composition, which is in good agreement with the initial composition, was determined to be 0.21%, 0.35%, 20.89%, 12.22%, 0.11%,

0.12%, 0.084%, 4.21%, and 0.10% respectively. Potassium, magnesium, phosphorus, zinc, and other elements are present in good amounts in processed samples that are devoid of contaminants [13].

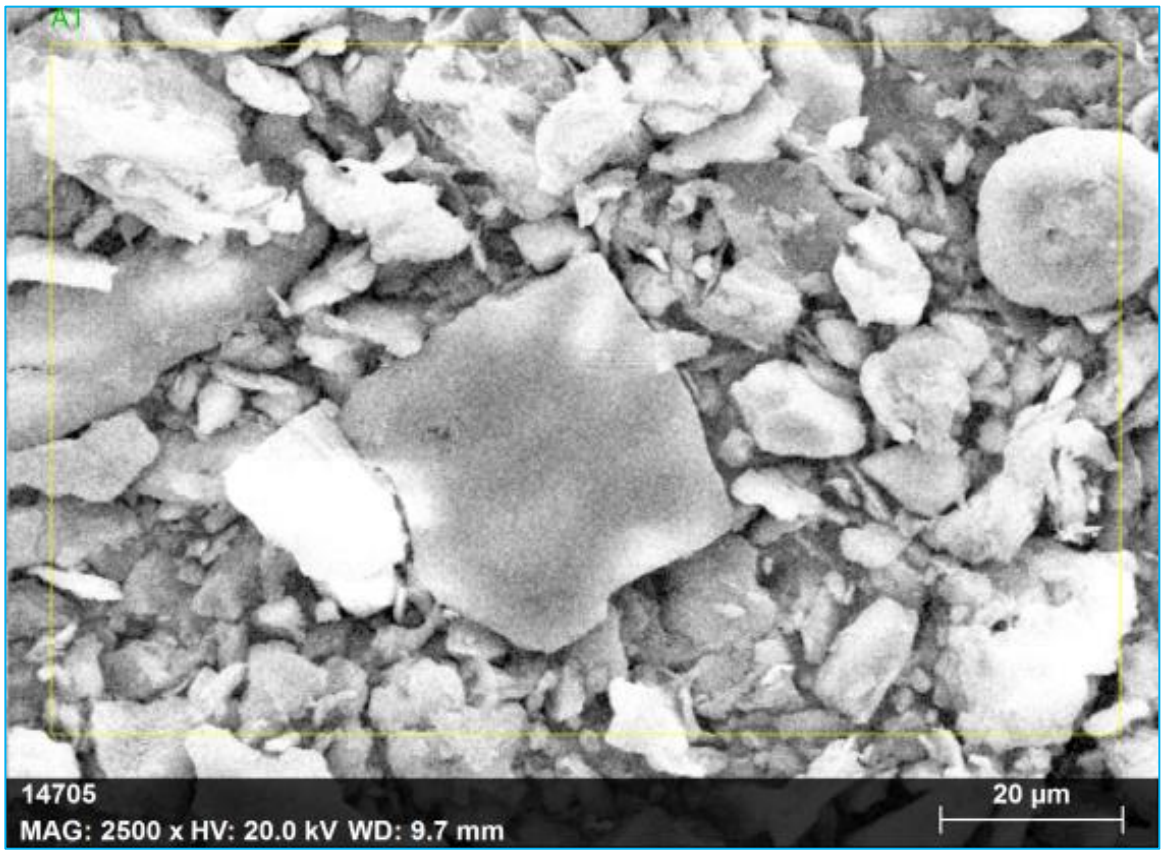

Fig 2 EDX images of citrus lemon leaf nano powders

Table 2 EDX spectrum of citrus lemon leaf nano powders

Element	Series	unn. C [wt.%]	norm [wt.%]	Atom. C Error [at.%]	3 sigma [wt]
Potassium	K-series	1.37	1.37	0.47	0.21
calcium	K-series	3.02	3.02	1.01	0.35
carbon	K-series	58.88	58.88	65.95	20.89
Oxygen	K-series	31.44	31.44	26.43	12.22
Magnesium	K-series	0.18	0.18	0.10	0.11
Copper	K-series	0.25	0.25	0.05	0.12
Phosphorus	K-series	0.02	0.02	0.01	0.084
Boron	K-series	4.79	4.79	5.96	4.21
Zinc	K-series	0.05	0.05	0.01	0.10

Table 3 Phytochemical screening of *Citrus limon* leaves nano – size powder

Phytochemical test	Aqueous	Methanol
Tannin	+	+
Alkaloids	+	+
Phenolic compounds	+	+
Flavonoids	+	+
Glycosides	+	+
Terpenoids	+	+
Saponins	+	+
Limonoids	+	+
Carotenoids	+	+
Coumarins	+	+
Essential oils	+	+

From the above (Table 3) shows the qualitative phytochemical screening of citrus limon leaf nano powders using two extracts.as follows tannin, alkaloids, phenolic compounds, flavonids, glycosides, terpenoids, saponins, limonoids, carotenoids, coumarins essential oils respectively. The presence of these compounds in citrus limon leaf nano powders suggest their potential utility in various applications such as medicine, food, or cosmetics, depending on their concentrations and bioactivities [14].

From the above (Table 4) shows the mean and standard deviation of the citrus limon leaf powder using two extracts aqueous and methanol as follows flavonoid kaempferol, Quentin, Hesperia, naringin, rutin, limonin, nomilin, beta carotene, phenolic acids, caffeic acid, chlorogenic, coumarins, essential oils respectively [15].

CONCLUSION

Citrus limon leaves nano powder can be used to cure some illnesses, infections and it play an important role in pharmacological industry and *Citrus limon* is an excellent

source of phytochemicals. *Citrus limon* leaves contain various phytochemicals such as flavonoids, alkaloids, phenolic compounds, terpenoids, and essential oils. These compounds have antioxidant, anti-inflammatory, antimicrobial, and other bioactive properties, which contribute to their medicinal value. Citrus limon leaves and their nano powder hold promise as natural remedies for a range of health issues and as valuable resources for the pharmaceutical and nutraceutical industries.

Table 4 Mean and standard deviation of *Citrus limon* leaves nano powder using aqueous and methanol extracts

Parameters	*Citrus limon* nano leaf powder	*Citrus limon* nano leaf powder
	Flavnoids	
Kaempferol	27.1 ± 0.1	45.7 ± 0.1
Quentin	13.05 ± 0.05	22.05 ± 0.05
Hesperia	4.25 ± 0.05	14.45 ± 0.05
Naringin	10.15 ± 0.05	18.01 ± 0.1
	Alkaloids	
Rutin	118 ± 1	243.5 ± 1.5
	Limonoids	
Limonin	19.25 ± 0.05	35.7 ± 0.1
Nomilin	6.1 ± 0.1	10.45 ± 0.05
	Carotenoids	
Beta carotene	90.5 ± 0.5	102.5 ± 0.05
	Phenolic compounds	
Phenolic acid	141 ± 0.1	116 ± 1
Caffeic acid	115.5 ± 0.5	80.5 ± 0.1
Chlorogenic acid	95.4 ± 0.1	63.65 ± 0.05
Coumarins	102.5 ± 0.5	134 ± 1
Essential oils	1.1 ± 0.1	30.65 ± 0.15

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