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Preliminary phytochemical screening with the evaluation of antioxidant and antimicrobial efficacy *Citrus limon* and C*itrus aurantifolia* leaf hydro-alcoholic extract: A Comparative Study

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Abstract

In recent years, focus on plant-oriented research has been increasing as they are potential sources of therapeutic agents comprising secondary metabolites, free antioxidant radicals, and many other well-known phytochemicals that have a high salutary value and massive impact on the healthcare system. Our present research study mainly aims at the comparative investigation of quantitative phytochemical estimation, evaluating the antioxidant and antimicrobial potential of *Citrus limon* (*C.limon*) and *Citrus aurantifolia* (*C.aurantifolia*) leaf hydro-alcoholic extracts. It has been manifested that the presence of antioxidant free radicals and phytoconstituents enhances the antimicrobial activity of the plant. The result revealed the presence of various phytochemicals, including carbohydrate, protein, steroid, phenol, alkaloid, flavonoid, tannin, cardiac glycosides, terpenoid, and quinones in both the leaves extracts. It has also been highlighted that *C.limon* hydro-alcoholic extract have the highest extractive value (18.89±3.84%) as well as the maximum polyphenols content (63.28±0.22 mg GAE/g of dry tissue), flavonoids content (26.46±0.30 mg QE/g of dry tissue) that support their antioxidant capacity, i.e. % DPPH (56.33±0.56%) radical scavenging potential along with antimicrobial property with respect to the *C.aurantifolia*. The overall result highlighted that the leaves of these two Citrus species possess the good amount of phytochemicals that can be used to treat many oxidative stress-related disorders and may have some beneficial antibacterial properties that can be further used in controlling the microbial infection.

Keywords: *Citrus limon*, *Citrus aurantifolia*, Hydro-alcoholic extracts, Phytochemicals, Anti-oxidant, Anti-microbial.

1. Introduction

Medicinal plants have been treated as a source of therapeutic agents since ancient time. Most of these plants contain certain phytochemicals referring to various compounds that occur naturally in plants which play an essential role in drug discovery. Although most people in developing countries rely on herbal medicines and their derived bioactive compounds to develop new antimicrobial compounds from alternative sources such as medicinal plants [1, 2]. Plants belonging to the family Rutaceae under the genus- *Citrus* are rich in these secondary bioactive metabolites [3]. Among many other citrus species plants, our study deals with two important species, *Citrus aurantifolia*, commonly known as lime, and the other *Citrus limon*, natively known as a lemon. *Citrus limon (C.limon)* is a spiny, evergreen shrub or small tree native to tropical and subtropical Southeast Asia [4]. Generally, a lemon tree can grow not more than 10 meters and smaller in size. The leaves are green, elliptical-acuminate and shiny. The flowers with mature citrus are 1.5-3 cm long, with a pedicel and a strong fragrance. Lemon flowers are full and flawless and have the same overall properties as other commercial citrus species [5, 6, 7]. Lemon is more precisely well known for its distinctive features and importance related to food or nutrition. Nevertheless, the most important point is it has great medicinal and nutritional importance [8]. The plant was found to acquire effective analgesic, anti-inflammatory, antioxidant, anthelmintic, antibacterial, antifungal, and hypolipidemic properties. They are found to possess a significant amount of antihyperglycemic, antidiabetic, as well as hypoglycemic activity. Some other medicinal benefits are Blood Sugar Balance, Brain and Nerve Food, Rheumatism, Arthritis and Bone-Related Diseases, Treat Throat Infections, Insomnia and many more [9, 10]. There were distinct elements to the essential oil of the leaves and peel of the *Citrus limon*. In both essential oils, limonene is the primary element. Lemon leaf oil was recognized with β-pine, myrcene, neryl acetate and β-caryophyllene. Caffeine is present in flowers and lemon tree leaves. Lemons contain various phytochemical substances, including polyphenols and terpenes [11,12]. The intake of lemon juice and honey is one of the key health benefits associated with Weight loss, also used in case of UTI (Urinary Tract Infection) and people with high uric acid level. The concoction of lemon pulp with olive oil helps to cure gall bladder stones and kidney stones. Lemon juice is also used as a liver stimulant, controls nausea and relieves heartburns and irritable bowel syndrome [13]. Culinary uses include lemon juice, peel in a wide variety of foods and drinks, making marmalade, lemon curd, lemon liqueur, and garnishing the food items using lemon zest the grated outer rind of the peel. The lemon tree-leaves are generally used to make tea and to prepare cooked meats and seafood [14]. *Citrus aurantifolia* (*C. aurantifolia*) is a small, densely and irregularly branched tree with short, sharp spines, characterized by alternate; elliptical to oblong-ovate (4-8cm×2-5cm) shaped leaves and has a crenulated

margin, principally cultivated in hot subtropical or tropical regions such as Southern Florida, India, Mexico, Egypt, and the West Indies [15]. The fruits are globose to ovoid berry of about 3 - 6 cm in diameter and sometimes have apical papilla, generally are yellow when ripe but usually picked green commercially [16]. The importance of *C. aurantifolia* in both domestic and ethnomedicinal use cannot be overemphasized. It is valued for its nutritional qualities as well as numerous health benefits. The plant is traditionally used as a folk medicine for its role as antiseptic, antiviral, antifungal, anthelmintic, astringent, diuretic, and mosquito repellent for the treatment of stomach ailments, constipation, headache, arthritis, colds, coughs, sore throats and used as an appetite stimulant [17, 18]. This health-related prosperity of *C. aurantifolia* is correlated with its elevated amounts of photochemical and bioactive compounds such as flavonoids, limonoids, phenols, carotenoids, minerals and vitamins. A total of 46 compounds were identified from the *Citrus aurantifolia* leaf essential oil; most of these were terpenes, which were found in greater amounts than aldehydes, ketones, phenols, and free acids [19]. Approximately one-third of total citrus production is utilized for processing [20, 21]. In the kitchen, it is used for cooking, to add flavour to food, cakes, to garnish salad and to add flavourings to drinking water [22]. The health benefits of both these plant leaves are due to the presence of bioactive compounds, such as phenolics (e.g. flavanone, glycosides, hydroxycinnamic acid) and carotenoids, flavonoids, alkaloids, terpenoids and tannins, which are responsible for antimicrobial activities [23, 24]. Plant-based anti-microbials represent a vast untapped source. The adoption of plant extract for medicinal treatment has become prominent, especially now when people are starting to realize that the competent life span of anti-microbials is limited and over-prescription and misuse cause microbial resistance [25]. Based on the above scientific findings, our present course of study aims to compare phytochemical screening with subsequent analysis of the antioxidant and antimicrobial activity of *C. limon* and *C. aurantifolia* leaves extract. The overall workflow is being represented through a graphical abstract in Figure 1.

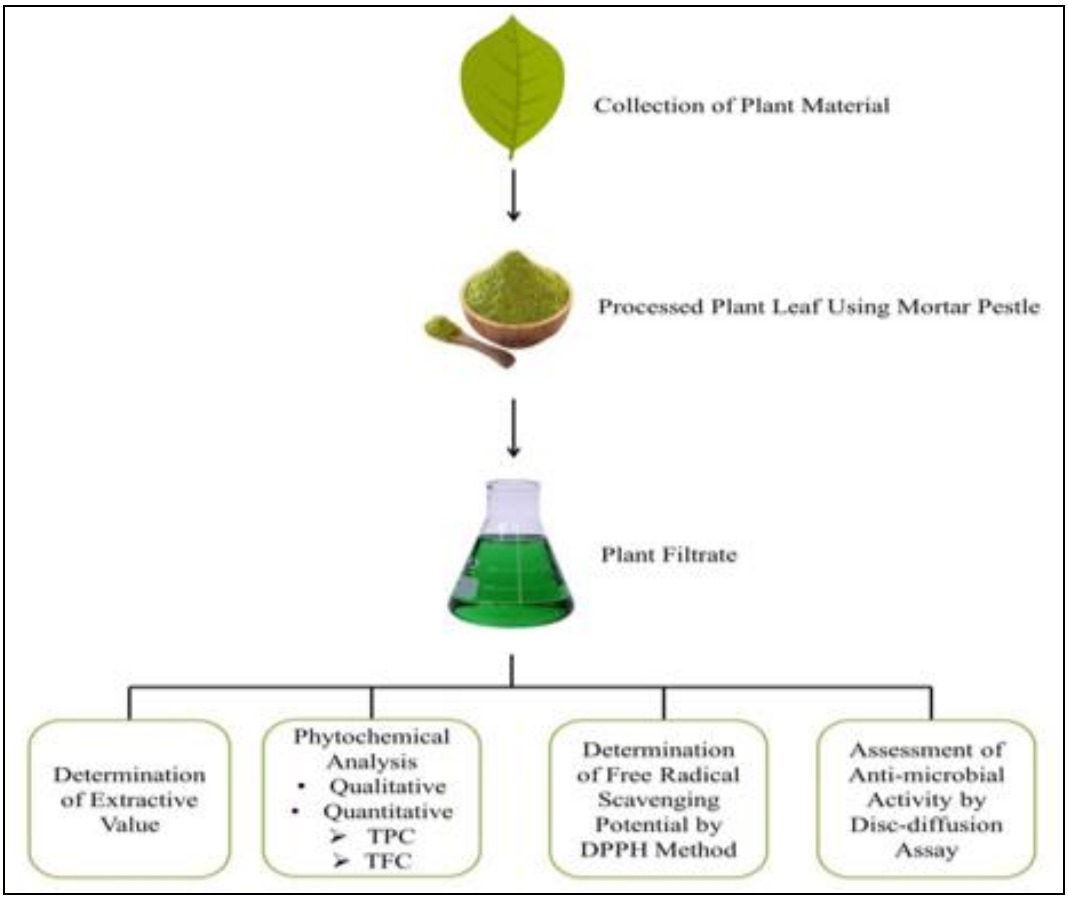

Fig 1: Graphical abstract

Materials

Chemicals

All the chemicals that were used in the experiments were of analytical grade. Folin-ciocalteu, aluminium chloride and ascorbic acid were obtained from Merck Life Science, Mumbai.

Gallic acid, sodium hydroxide was supplied by SD Fine-Chem Limited, Mumbai. Quercetin, Sodium nitrite, sodium carbonate were purchased from SRL Pvt. Ltd. DPPH was obtained from Sisco Research Laboratories Pvt. Ltd., Maharashtra. Besides these, both the nutrient agar and nutrient broth were obtained from Himedia Laboratories Pvt. Ltd., Mumbai.

Methods

Sample collection and preparation

The leaves of both plant species *Citrus limon* and *Citrus aurantifolia* (Figure 2a and 2b), were collected from North 24 Parganas, West Bengal, in the month of August-2019. After collecting all the samples, the leaves were carefully separated, cleaned, washed well to remove all the dirt and are shade dried separately until all the water molecules were

evaporated; next, each leaf sample was mechanically ground and coarsely powdered. The powder was subjected to solvent extraction with 70% ethanol for their respective purposes.

Fig 2: [A] Plant of *citrus limon*; [B] Plant of *citrus aurantifolia*

Extraction technique

1gm of each leaf powder was added to 50 ml of solvent (70% ethanol) in a conical flask. They were kept in a shaker for 24 hours at room temperature. All the extracts were filtered by using Whatman No.1 filter paper. The filtered solution of extract was stored at 4°C and diluted for further studies according to the need for the specific assay.

Extractive value

The extractive value was determined by the standard method with slight alteration using 70% ethanol as solvent [26].

Preliminary Qualitative Phytochemical Assays

To detect the presence of carbohydrate (Molisch's test) [27], protein (Biuret test) [28], reducing sugars (Benedict test) [29], steroids (Liebermann-Burchard test) [30], alkaloid (Wagner test) [31], phenol ($FeCl_3$ method) [32], flavonoids (Alkaline reagent test) [33], tannin ($FeCl_3$ test) [34], cardiac glycosides (Keller-Kelliani's test) [35], quinones [36], terpenoids (Salkowski's test) [37], leucoanthocyanin [38] standard methods were used with little modifications.

Quantitative phytochemical assays

Quantification of total phenolic content (TPC)

The total polyphenol contents were determined by using the Folin-Ciocalteu method with adjustments. The Gallic acid was used for preparation of standard curve. The absorbance was read at 765 nm. The results were expressed as mg Gallic acid equivalents/g of dry tissue [39].

Quantification of total flavonoid content (TFC)

Aluminium chloride colourimetric assay with adjustments was used for determination of flavonoids. The absorbance was read at 510 nm. Quercetin was used for prparation of the standard curve. The results were expressed as mg Quercetin equivalents/g of dry tissue [40].

Determination of antioxidant activity

DPPH radical scavenging assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was evaluated using the standard protocol with slight adjustments. The standard curve was made using ascorbic acid. Absorbance was read at 517 nm. The DPPH radical scavenging capacity was expressed in terms of Ascorbic Acid Equivalent, as the percentage of inhibition of the assay was calculated by the following formula [41].

% Inhibition of DPPH= OD of control-OD of sample/ OD of control*100

Determination of antimicrobial property by Kirby-Bauer Disk Diffusion method (Biological assay)

The four bacterial strains used in the present study were obtained from the Department of Microbiology, Calcutta University.

Gram-positive bacteria

Staphylococcus aureus and *Bacillus subtilis*

Gram-negative bacteria

Klebsiella pneumonia and *Pseudomonas aeruginosa*

An antimicrobial activity study was carried out by using a disc diffusion assay. Agar media was weighed and dissolved in sterile distilled water. After sterilization, the media was poured into sterile plates and allowed to solidify. The sample extracts for this assay were filtered through a 0.2 µm Whatman Filter paper before use for this purpose. In 5ml of sterile Nutrient Broth, 100 µl of each microbe was subcultured. 20µl of test bacteria from the log phase of freshly subcultured tubes were spread and seeded onto pre-warmed sterile Agar plates. Sterile paper discs were placed onto the surface of inoculated agar plates with the help of sterile forceps. 30µl aliquots of sample extracts were then pipetted out onto the paper discs embedded on the agar surface. The plates were allowed to dry for some minutes and then incubated at 37ºC for 24hrs. The antibacterial property was expressed as the diameter of the zone of inhibition (mm) produced by the extracts around the disc. All tests were carried out in triplicates [42].

Statistical analysis

All the experimental measurements were performed in triplicate and expressed as the average ± standard deviations. The magnitude of the means, standard curve, standard errors, standard deviations, one way ANOVA was calculated by using Microsoft Excel 2010 Software. $P<0.05$ is accepted as statistical significance.

Results

Extractive value

The present course of study has shown that the extractive value of *C. limon* hydro-alcoholic extract was highest, i.e. 18.89±3.84%, whereas *C. aurantifolia* hydro-alcoholic extract shows 11.11±2.77%, which is the lowest. Measurements of extractive value that determine the amount of the bioactive constituents in a given amount of plant material when extracted with the solvent are represented in Table 1 [26].

Table 1: Extractive Value %

Sample	Colour	Extractive Value % (Mean ± Sd)
C.limon	Deep green	18.89±3.84
C.aurantifolia	Brownish green	11.11±2.77

Qualitative assay

Results obtained from the qualitative study of both *Citrus* species leaf extracts are represented in Table 2. A series of twelve tests were performed in order to find out different

phytomolecules. Among them, ten tests gave a positive result for both the extracts of *C. limon* and *C. aurantifolia.* These are carbohydrate, protein, steroids, alkaloid, phenol, flavonoid, tannin, cardiac glycosides, quinones and terpenoids. Only two phytochemicals, i.e. reducing sugars and leucoanthocyanin, were found to be absent in all of the cases. The results indicate that the experimental plants harvours therapeutically, pharmaceutically and nutritionally critical bioactive compounds. Therefore, the estimation of these phytomolecules becomes essential [43].

Table 2: Results of Qualitative Assays

SL No.	Test Name	*C. limon*	*C. aurantifolia*
1	Carbohydrate	+	+
2	Protein	+	+
3	Reducing sugars	-	-
4	Steroids	+	+
5	Alkaloid	+	+
6	Phenol	+	+
7	Flavonoid	+	+
8	Tannin	+	+
9	Cardiac glycosides	+	+
10	Quinones	+	+
11	Terpenoids	+	+
12	Leucoanthocyanin	-	-

[Where, "+" present, "-" absent]

Table 3: Standard Curve Equation and Their Respective R^2 Value

Sl no.	Name of quantitative assay	Standard curve equation	R2 value
1	Polyphenol	4.504X+0.073	0.999
2	Flavonoid	6.652X+0.090	0.994
3	DPPH	183.8X+3.256	0.996

Quantitative assay

The present course of study aims to determine the antioxidant and antimicrobial potential of two *Citrus* species leaf extracts. Since it is well established that the antioxidant property of plants largely depends on the total polyphenols (TPC) and flavonoids (TFC) content, that we quantify their concentration using standard protocols and correlate them with their free radical scavenging activity.

Standard curve equation and R^2 value for TPC, TFC and DPPH free radical scavenging assay are given in Table 3, which highlighted the strength and accuracy of further quantitative estimation assays. The total polyphenol content (TPC) of both *C. limon* and *C. aurantifoia* hydro-alcoholic extracts were quantified. The result showed that *C. limon* leaf extract contained the highest amount of polyphenol content, i.e. 63.28±0.22 mg GAE/g of dry tissue, wherein the case of *C. aurantifolia* leaf, it was found to be 56.51±0.29 mg GAE/g of dry tissue (Figure 3A). A significant difference ($p<0.05$) in polyphenols concentration between the hydro-alcoholic extracts of both the *Citrus* species is evident from the study. Polyphenol compounds are reactive species towards oxidation and regulate bio-physiological activity.

The total flavonoid content (TFC) of both *C. limon* and *C. aurantifoia* hydro-alcoholic extracts were estimated. The maximum amount of flavonoid content was found to be present in *C.limon* leaf extract, i.e. 26.46±0.30 mg QE/g of dry tissue, and the lowest amount, i.e. 22.05±0.39 mg QE/g of dry tissue, present in *C.aurantifolia* extract (Figure 3B). The p-value <0.05, showed the significant difference in TFC between the hydro-alcoholic extracts of both the *Citrus* species.

Depending on their specific structure, flavonoids compounds can inhibit all possible reactive oxygen species.

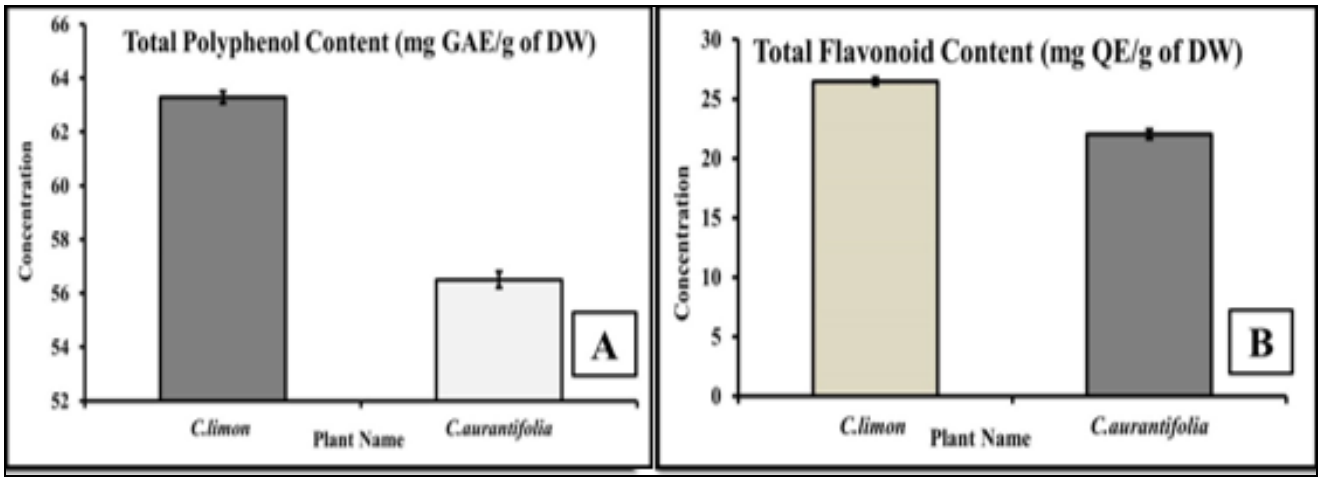

Fig 3: [A] Total polyphenol content (mg GAE/G OF DW); [B] Total flavonoid content (mg QE/g of DW)

Inhibition of free Radical scavenging assay

Bioactive compounds can act as an antioxidant by scavenging the free radicals. DPPH is a stable free radical, which is used to investigate the free radical scavenging capacity of an antioxidant. The inhibition percentage of DPPH radical scavenging assay was found to be the highest in *C. limon* hydro-alcoholic extract, i.e. 56.33±0.56%, on the other hand, *C. aurantifolia* hydro-alcoholic extract showed the lowest inhibition percentage, i.e.53.73±0.46% (Figure 4A and 4B). A significant difference ($p<0.05$), in %DPPH radical scavenging capacity between the hydro-alcoholic extracts of both *Citrus* species is present.

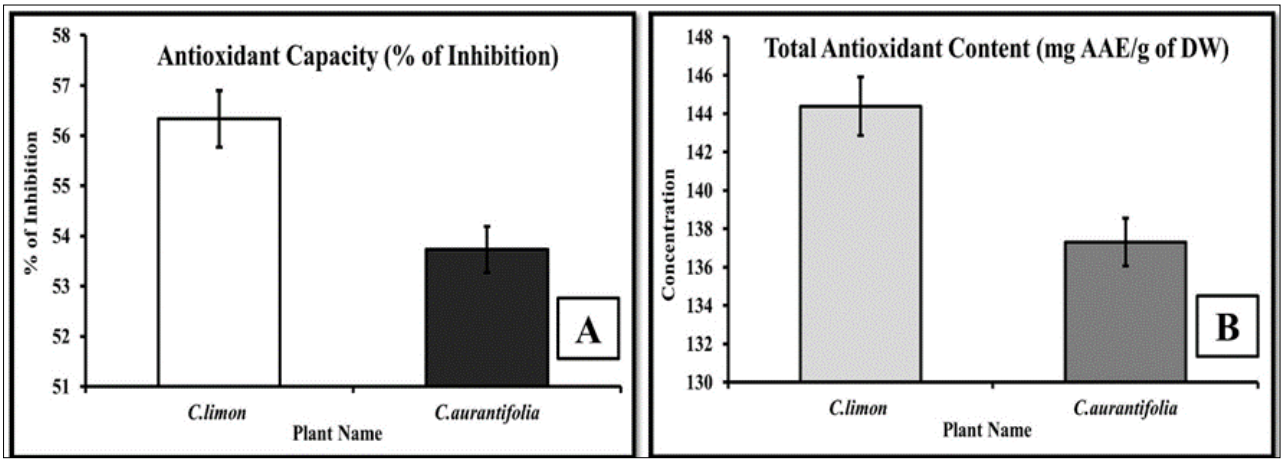

Fig 4: [A] DPPH free radical scavenging percentage; [B] Total antioxidant concentration (mg AAE/G OF DW)

Determination of antimicrobial property by kirby bauer Disk diffusion method

We further evaluated and compared the antimicrobial properties of hydro-alcoholic leaf extracts of both *Citrus* species [45]. The results obtained were represented in Table 4, 5 and Figure 5A, 5B, 6A and 6B.

The antimicrobial potential of both the experimental plant species was evaluated according to their zone of inhibition against two gram-positive and two gram-negative bacteria, and the results were compared, which highlighted that in the case of *C.limon* extract, it showed the maximum zone of inhibition, i.e. net zone (7 mm.) against *K. pneumonia,* i.e. a gram-negative, whereas *C.aurantifolia* extract showed the highest zone of inhibition, i.e. net zone (6.7 mm.) against *S.aureus*, i.e. gram-positive.

Table 4: Comparison of antimicrobial activity of *C.limon* Leaf extract by measuring zone of inhibition (MM.) against four different bacteria

Organism	Zone oF inhibition(MM.) mean±sd	
	CONTROL	EXTRACT
S.aureus	5.6±0.1	11.6±0.1
B.subtilis	5±1	11±1
K.pneumoniae	5.3±0.20	12.3±0.1
P.aeruginosa	6±1	11.3±0.2

Table 5: Comparison of antimicrobial activity of *C.aurantifolia* leaf extract by measuring zone of inhibition (MM.) against four different bacteria

Organism	Zone of inhibition(MM.) mean±sd	
	CONTROL	EXTRACT
S.aureus	5.3±0.1	12±0.5
B.subtilis	6.7±0.1	12.7±0.1
K.pneumoniae	5.6±0.1	11±1
P.aeruginosa	6±2	10.7±0.2

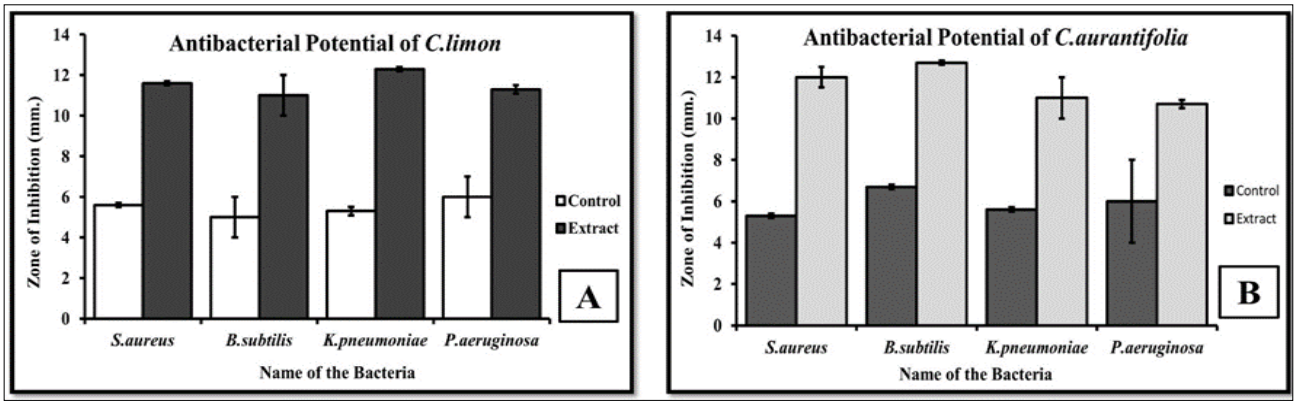

Fig 5: [A] Graph showing comparative vision of antibacterial potential between control and *C. limon* extract against different bacterial strains; [B] Graph showing comparative vision of antibacterial potential between control and *C. aurantifolia* extract against different bacterial strains

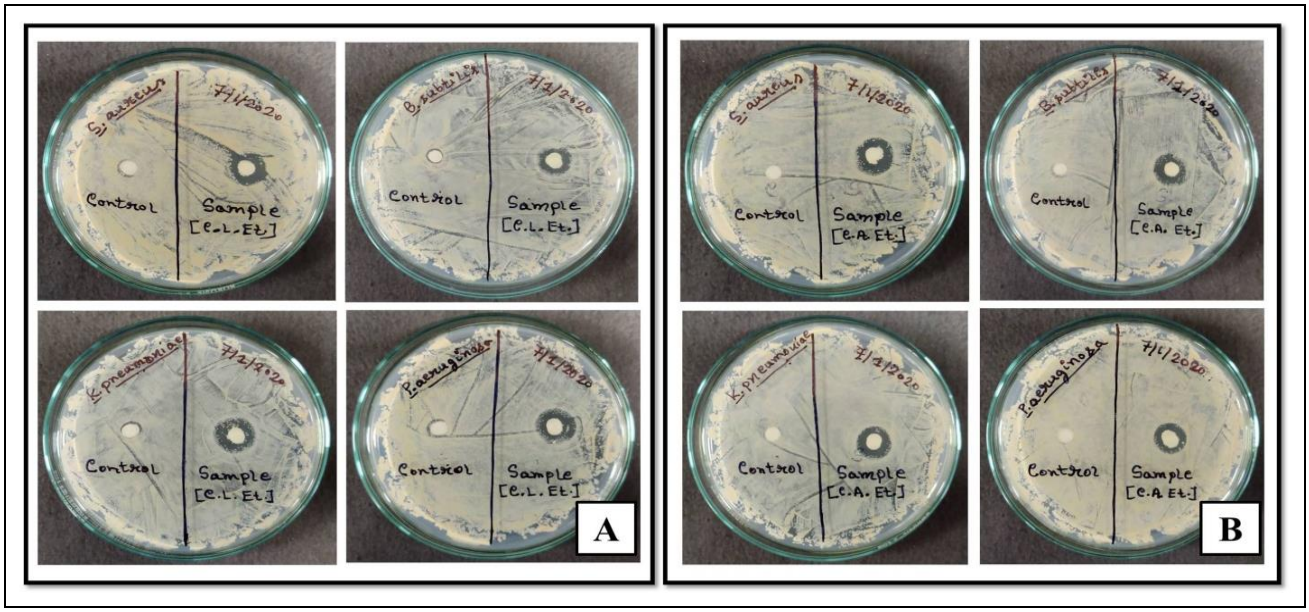

Fig 6: [a] Agar plates showing zone of inhibition against *C. limon* extract with respect to control (70%hydro-alcoholic solvent), [B] agar plates showing zone of inhibition against *C. auarantifolia* extract with respect to control (70%hydro-alcoholic solvent)

Discussion

The remedial nature of plants is perhaps due to the presence of various secondary metabolites such as alkaloids, phenols, flavonoids, tannins, quinones, and many other phytonutrients, which are rich in showing antioxidant activity [44]. The successive extraction leaves of both *Citrus* species in hydro-alcoholic solvent revealed the presence of carbohydrates, proteins, flavonoids, tannins, quinones, cardiac glycosides, terpenoids, phenols, alkaloids (Table 2). Thus the preliminary phytochemical screening may be helpful in detecting bioactive principles and subsequently may lead to drug discovery and development [45]. On the other hand, quantitative analysis showed the presence of the maximum amount of polyphenols and flavonoids that enhanced their higher antioxidant activity DPPH radical scavenging assay. The findings of the study support the fact that traditionally used medicinal plants are the primary source of therapeutically used antioxidants [46]. Significant antimicrobial activity was exhibited by both plant extract comparable to each other. Based on the results obtained, it can be concluded that *the Citrus* species leaf is a rich source of natural antioxidants and various phenolic and flavonoid compounds that increase their nutritional and medicinal aspects [47, 48].

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Abbreviation

GAE= Gallic Acid Equivalent; QE= Quercetin Equivalent; AAE= Ascorbic Acid Equivalent; DPPH= 1, 1-diphenyl-2-picrylhydrazyl; OD= Optical Density.

Conflict of interest

The author declares no conflict of interest.

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