Research Article

Supercritical CO_2 extraction, physicochemical and phytochemical screening of *Citrus* leaves extract and study of microbial activity

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Abstract

Objective: The main theme of the present study is that, to compare the other method of extraction from supercritical fluid extraction. **Material and methods:** The leaves of *citrus* are used for the investigation of inorganic compounds in the form of total ash. The leaves of *citrus* are also used for the latest extraction technique such as supercritical fluid extraction and the phytochemicals are present in it are compared with other method of extraction. The leaves and its extracts were used for fluorescent test, ash analysis, extraction, phytochemicals analysis, spectral analysis and antibacterial investigation. **Results and Conclusion:** The florescent test gives different color in different solvents. Ash analysis was gives about 15% of total ash. The samples were extracted using three different methods out of which SFE is found to be more effective. Phytochemical such as alkaloids, carbohydrate, phenolic compounds and tannins were present. All the extracts of *citrus* leave possess less antibacterial activity as compare with standard. Out of which SFE extract is moderately active against gram +ve bacteria. While all the extracts are showing less antibacterial activity against gram –ve bacteria. Conventional extract is inactive against all bacteria. This may be due to the degradation of flavonoids due to continue reflux, which possess antibacterial activity.

***Keywords*:** Supercritical fluid extraction, Citrus leaves, phytochemicals, physicochemical, antibacterial activity

Introduction

Extraction is the initial and most important step in the recovery and purification of bioactive compounds from plant material (Mostapha et al., 2014). The convectional extraction techniques and solvent extraction have many disadvantages such as they are time consuming and more amount of organic solvents are required. There may be possibility of degradation of thermal labile components (Paula et al., 2013; Marelo et al., 2014). There is increasing number of research related to the extraction of bioactive compounds, including new technologies (Andre et al., 2013). The factors affecting on the process of SFE for solid substrate is selected extraction time, which also affects extraction yield depending on particles size, CO_2 temperature, pressure and supercritical velocity (Valle et al., 2014). The addition of modifier or co-solvents to CO_2 improves the efficiency of the extraction for increasing the solubility of the solute and the extraction yields (Marsni et al., 2013; Maksimovic et al., 2013). Supercritical extraction is one of the most desirable techniques which is used in last decade (Basa'ar et al., 2017).

Plant produces phytochemical which can be used by humans for healthy life (Samreen et al., 2018). Plants, animals and micro-organisms represent of natural sources. Plants contain several species which still used as remedies for several diseases throughout the world (Brusotti et al., 2014). According to world health organization (WHO) near about 80% of the world population of developed country uses herbal medicine (Samreen et al., 208). The citrus fruit belongs to the *Rutaceae* family (Okwu et al., 2006). Literature survey shows that citrus fruit contain flavonoids (Nogata et al, 2006), Carbohydrate (Bean et al., 1961), Vitamin C (Rekha et al., 2012), phenols (Ghasemi et al., 2007). It also possesses antioxidant property (Tanizawa et al., 1992; Goulas et al., 2012).

Materials and methods

The leaves of *Citrus* leaves were collected from local area of

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Aurangabad city in the month of December. The leaves were washed and kept for drying under at room temperature under shade for one week.

Fluorescent test

0.5 g of samples was added in different solvents and fluorescent behavior was observed. Normal light florescent behavior was different in different solvent (Samreen et al., 2018).

Ash analysis

Accurately weighted 10 gm of sample was taken in thoroughly cleaned silica crucible and ignited for 4 hrs with gradually increasing in temperature up to 300°C. After ignition of leaves of plant, the residue was remain is designated as ash. The residue was again ignited with the interval of 10 min, till to get the constant weight. This ash was used to determine the three parameters called as total ash, acid insoluble ash and water soluble ash.

Acid insoluble ash

Acid insoluble ash is the ash which is insoluble in dilute HCl. 1 gm of total ash was dissolved in 2N hydrochloric acid. Stirred well for the digestion of ash and filtered through wattman filter paper no. 41. The residue remained after filtration is ignited in clean silica crucible by gradually increase in temperature up to 300°C. The residue was cooled and weighted and again kept for ignition till to get the constant weight. The residue is remains after ignition is acid insoluble ash. The percentage was calculated for the acid insoluble ash.

Water soluble ash

Take 1 g of total ash was boiled with20ml of double distilled water. The residue was collected by filtration through wattman filter paper no 41. Residue was washed with hot water and kept for ignition not more than 400°C. The weight of residue was subtracted from total ash. This difference between residue and total ash represent the water soluble ash. The percentage was calculated.

Extraction procedure

The sample was extracted by three extraction method for getting aqueous extract, methanol extract and by supercritical fluid extraction.

Aqueous Extraction

Accurately weight 30 g of sample was introduced into the 500 ml round bottom flask (which was first clean by dilute hydrochloric acid and then distilled water) with 300 ml double distilled water. The pourciline pieces were add to avoid bumping of the sample. The condenser was fitted with circulation of water. The sample was refluxed on flame for six hrs. The sample was cooled and filtered by the suction pump. The excessive water was evaporated for the preservation of the sample and it was kept at 4°C for 12 hrs. The percentage of the extract was calculated.

Methanol extraction

Accurately weight 30 g of sample was introduced into the 500 ml round bottom flask (which was first clean by acetone and then HPLC grade methanol) with 300 ml methanol. The round bottom flask was closely thigh and kept for 6 hrs at room temperature.

Supercritical fluid extraction

The sample was kept in the sample holder. The critical temperature for carbon dioxide was -7°C with that co-solvent was used as methanol. The temperature and pressure was kept as 35°C and 100 Mpa.

Physicochemical test evaluation

The physical parameters like relative density, surface tension, viscosity and refractive index were measured (Basa'ar et al., 2016).

Relative density

Clean and dry empty density bottle with stopper weighted accurately .The density bottle was filled with double distilled water up to it fall from the bottle and stopper was fitted and the bottle was cleaned from outside. The bottle was weighted.

The procedure was repeated for the samples. The density was measured by taking difference between bottle with sample and empty bottle. Relative density was calculated by the formula

Relative density = ρ_2 / ρ_1

Where ρ_1 is density of the water and ρ_2 is the density of the sample.

Viscosity

The Ostwald's viscometer was cleaned by NaOH to remove greasy impurities than with chromic acid and finally with the distilled water. The 10 ml of double distilled water was inserted in viscometer from large diameter tube. And the sample was sucked through second tube of the same viscometer till it rises with 2-3 cm above the mark. By keeping stop watch ready the liquid was allowed to decent down the time required to flow of the liquid between two points was noted. The same procedure was repeated for the samples which have to study.

Viscosity was measured by formula:

$$\eta_2 = \frac{t_2}{t_1}\frac{\rho_2}{\rho_1}\eta_1$$

Surface tension

The stalagmometer was cleaned by NaOH to remove greasy

impurities then with chromic acid and finally with the distilled water. The rubber tubing with the with a screw clip was attached to the top of the stalgmometer. The flat end of the stalagmometer was dipped into the standard solution (double distilled water) suck through the water tubing until the water level rises above the mark. The screw was adjust the pressure until the rate of the drop was 10 to 15 per minute. The number of drops were counted for double distilled water when passes from upper mark to the lower mark.

The stalagmometer was removed and rinse with alcohol and dried. Stalagmometer was filled with the test sample and number of drops was determined. Same procedure was repeated for every concentration three times and mean was taken.

The surface tension was then calculated by the formula:

$$\gamma_2 = \frac{n_1}{n_2}\frac{\rho_2}{\rho_1}\gamma_1$$

Where γ_1 and γ_2 are the surface tension of the double distilled water and the sample respectively,

and n_1, ρ_1 and n_2, ρ_2 are the number of drops and relative densities of the double distilled water and samples under study respectively.

Refractive index

Refractive index depends upon temperature and concentration. However the specific refraction is independent of temperature and it is characteristic property of the liquid. The refractive index was measured by Abbe's refractometer. The refractometer was placed on the table near to the window so that sufficient light should reached to the prism. The prism box was opened b turning the lock nut and both the phases of the prism was cleaned with the help of cotton wool by the acetone and prism box was closed after drying. Few drops of the liquid were pumped through the small hole on the prism box with the help of the dropper. The crosswire of the telescope was focus by rotating the eye piece and the mirror was adjust for the reflection of maximum light towards the prism box. The prism box was moved forward and backward until the clear boundary between the light and dark region was appear. The scale reading was noted down and refractive index were calculated by using the formula:

$$\text{Specific refraction (r)} = \frac{n^2 - 1}{n^2 + 2} \times \frac{1}{\rho}$$

Where ρ is the density of the samples

Qualitative Phytochemical Screening

The different qualitative chemical tests were performed for establishing profile of given extract for its chemical composition. Qualitative test for alkaloids, carbohydrate, proteins and tannins, saponins etc were analyzed (Samreen et al., 2018).

Detection of alkaloids

Solvent free extract, 50 mg was stirred with 5 mL of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows.

I Mayer's test: To a few mL of filtrate, two drop of Mayer's reagent added by the side of test tube. A white or creamy precipitate indicated the test as positive.

Mayer's reagent: Mercuric chloride (1.36 g) was dissolved in 60 mL of water and potassium iodide (5.0 g) was dissolved in 10 mL of water. The two solutions were mixed and made up to 100 mL with water.

ii) Wagner's test: To a few ml of filtrate, few drops of Wager's reagents were added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

Wagner's reagent: Iodine (1.27 g) and potassium iodide (2 g) was dissolved in 5 ml of water and made up to the 100 ml with distilled water**.**

iii) Hager's test: To a few ml of filtrate 1 or 2 of Hager's reagent (saturated aqueous solution of picric acid) were added. A prominent yellow precipitate indicated the test as positive.

Detection of Carbohydrate

The extract 100 mg was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

I Molisch's tests: To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of test tube and allwed to stand. A violet ring indicates the presence of carbohydrates.

ii) Fehling's test: One ml of filtrate was boiled on water bath with 1 ml each Fehling's solutions A and B. Red precipitate indicates the presence of suger.

Fehling's solution: Solution A; copper sulphate (34.66 g) was dissolved in distilled water made up to 500 ml with distilled water.

Solution B: potassium sodium tartarate (173 g) and sodium hydroxide (50 g) was dissolved in water and made up to 500 ml.

iii) Benedict's test: To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristics colored precipitate indicates the presence of sugar.

Benedict's reagent: Sodium citrate (173 g) and sodium carbonate (100 g) were dissolved in 800 ml distilled water and boiled to make it clear solution. Copper sulphate (17.3 g) dissolved in 100 ml distilled water.

iv) **Barfoed's test:** To 1 ml of filtrate, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2

min. red precipitate indicates the presence of suger.

Barfoed's reagents: copper acetate 30.5 g was dissolved in 1.8 ml of glacial acetic acid.

Detection of Saponins by Foam test

The 50 mg was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 15 min. A two cm layer of foam indicated the presence of saponins.

Detection of proteins and Amino acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatman filter paper no. 41 and filtrate was subjected to test of proteins and amino acids.

I Millon's test: To 2 mL of filtrate, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins.

Millon's reagent: Mercury (1 g) was dissolved in 9 mL of fuming nitric acid. When the reaction was completed, equal volume of distilled water was added.

ii) Biuret test: An aliqot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. To this 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink color in the ethanolic layer indicates the presence of proteins.

iii) Ninhydrine test: two drops of ninhydrine solution (10 mg of ninhydrine in 200 ml of acetone) were added to two ml of aqueous filtrate. A characteristic purple color indicates the presence of amino acids.

iv) Nitric acid test: The aqueous extract of plant leaves were added in 3ml of nitric acid. Appearance of yellow color indicates the presence of proteins

Detection of phenolic compounds and tannins

i) Ferric chloride test: The extract (50 mg) was dissolved in 5 ml distilled water. To this few drops of neutral 5% ferric chloride solutions were added. A dark green color indicates the presence of phenolic compounds.

ii) Gelatin test: The extract (50 mg) was dissolved in 5 ml of distilled water and 2 ml of 1% solutions of gelatin containing 10% sodium chloride was added to it. White precipitate indicates the presence of tannins.

iii) Lead acetate tests: The extract (50 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of flavonoids compounds.

iv) Alkaline reagent test: an aqueous solution of the extract was treated with the 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

Spectral analysis: UV-Visible: The powder of extract was dried and dissolved in distilled water to prepared solution of approximately 50 ppm. The spectra was recorded in the range from 190 to 800 nm by using double beam spectrophotometer of Model Elico-159 and λmax is determine from the software Spectra treat**.**

IR Spectra: The FTIR instrument IRT3000, JASCO, having serial No. B051061016, and the spectra were recorded using spectra manager. IR instrument is calibrated by using polystyrene. Spectra were recorded by using potassium bromide (KBr) of IR grade manufactured by Marck life sciences. KBr was kept in hot oven at 50°C for half hour to free it from moisture. Spectra of that dry KBr is measured within IR range. The samples of leave extract were crushed to make it fine powder and mixed with dry KBr, and spectra of the samples were measured.

Anti-microbial study: antibacterial activity is investigated by cup plate method in which 70 µL of standard test solution were added in each cups or wells and these cups were prepared by using sterile metal borer. The media used was sterile nutrient agar and sterilization was performed in autoclave at 121^{0}C for 20 min. the bacterial culture which were used are the standard is as streptomycin. The physical parameters like relative density, surface tension, viscosity and refractive index were measured (Basa'ar et al., 2016).

Results

Fluorescent test

The powdered samples were treated with different chemicals and observed with naked eyes. The results were summarized in table 1. Different colors were observed in different solvents due to fluorescent phenomenon. The constituents of plant powder when treated with acid or base may undergo chemical changes and hence sometime fluorescent light is emitted by them. Hence, they emit light, which can be even seen by necked eyes. This can be

Table1. Florescent test for leave powder

Sr. No.	Solutions	Observation
1	Powder as such (P)	Dark green
2	P + chloroform	Light green
3	P + Conc. HCl	Dark green
4	P + Conc. HNO_3	Dark orange
5	P + Conc. H_2SO_4	Dark brown
6	P + Chloroform	Light green
7	P + 2N acetic acid	Yellowish white
8	P + Glacial acetic acid	Yellowish green
9	P + 2N HCl	Whitish cream
10	P +2N NaOH	Light green
11	P + 2N H_2SO_4	Whitish green
12	P + Ammonia	Fluorescent yellow

ascertained as significant qualitative test for plant powder. The powder is green and remains almost green in acid and strong base media as well as in chloroform. It shows fluorescent yellow colour in ammonia only.

Ash value

Inorganic compounds present in the leaves of *citrus* may be calculated by the measuring of total ash. The results are shown in table 2. The powder contained 15% of total ash. It represents inorganic residue i.e. metal oxides. Out of which 28% is water soluble indicate the oxides of alkali metals like sodium and potassium. The acid insoluble ash represents heavy metal oxides including silica.

Extraction

The sample was extracted using three different methods. Out of which for methanol extract less amount of extract and supercritical fluid extraction gives more amount of extract. In compare with supercritical fluid extraction and conventional extraction supercritical extraction is more superior because there may be degradation of compounds in conventional extraction due to continues heating. Since the polarity of constituent are different and solubility of constituents is different for different solvent system. Hence the yield is different.

Physicochemical properties

Physicochemical parameters of the extract such as pH, Conductance, relative density, viscosity, surface tension and refractive index were calculated as described in the literature (Samreen et al., 2018). It is observed that all the parameters of all three extract were different. This indicates that pH, Conductance, relative density, viscosity, surface tension are changes due to change in extraction method (Table 4).

Phytochemical test

Phytochemicals are secondary metabolize produced by plant. Qualitative test for the phytochemicals were performed. The results are described in table 5. Out of all three extract near about all test are having similar result. The only difference is not in the conventional extract. The test for saponins and phenolic compounds was found to be positive. This results in the high viscosity of aqueous extract compounds.

FTIR spectrum

All the extracts were examined for Fourier transform infrared spectroscopy. All the extracts are having near about same

Table 2. Ash analysis and densities of leave powder

Ash	Results
Total Ash	15%
Acid Insoluble	28.8%
Water Soluble	22.8%

Table 3. Extractive value of *Citrus leaves* plant leaves.

Solvent	Percentage
Supercritical fluid extraction	20.03
Conventional Hydro Extraction	19.94
Methanol extract	5.26

Table 4. Physicochemical parameters of the extracts

Parameters	SFE	CE	ME
pH	9.14	9.66	9.45
Conductance	0.396	0.429	0.211
Relative density	1.004	1.035	0.984
Viscosity	0.9843	1.2713	0.7945
Surface tension	66.67	74.47	74.84
Refractive index	0.848	0.844	0.889

SFE: Supercritical Fluid Extract; **CE:** Conventional extract; **ME:** Methanol extract

Table 5. Qualitative test for *Citrus* fruit leaves extract

Sr. No.	Reagent	SFE	CE	ME
1.	**Detection of Alkaloids**			
A.	Mayer's test	-ve	-ve	-ve
B.	Wagner's test	+ve	+ve	+ve
C.	Hager's test	+ve	+ve	+ve
2.	**Detection of carbohydrate**			
A.	Molish test	+ve	+ve	+ve
B.	Fehling's test	+ve	+ve	+ve
C.	Benedic test	+ve	+ve	+ve
D.	Barfoad's test	-ve	-ve	-ve
4.	**Test for saponins**	-ve	+ve	-ve
5.	**Detection of proteins and amino acid**			
A.	Millon's test	**-ve**	-ve	-ve
B.	Nitric acid test	-ve	-ve	-ve
C.	Biuret test	-ve	-ve	-ve
D.	Ninhydrine test	-ve	-ve	-ve
6.	**Detection of phenolic compound and tannins**			
A.	Ferric chloride test	-ve	+ve	-ve
B.	Gelatin test	-ve	+ve	-ve
C.	Lead acetate test	-ve	+ve	-ve
D.	Alkaline reagent test	+ve	+ve	+ve

FTIR spectra. The peaks at 3829, 3775, 3664, 3644, 3444, 3367, 3262, 3079 $cm^{-1}$are observed are responsible for different functional groups. Such as NH_2 stretching, -OH stretching, C-H stretching etc. FTIR spectra can be used as monograph for the identification of plant leaves only.

Antibacterial study

The anti-bacterial properties vary with part of plants, solvent used for the extraction etc. antibacterial activity is investigated by cup plate method in which 70 µL of standard test solution were added in each cups or wells and these cups were prepared by using sterile metal borer. The media used was sterile nutrient agar and sterilization was performed in autoclave at 121^0C for 20 min. the bacterial culture which were used are the standard is as streptomycin. All the extracts of *citrus* leave possess less antibacterial activity as compare with standard. Out of which SFE extract is moderately active against gram +ve bacteria. While all the

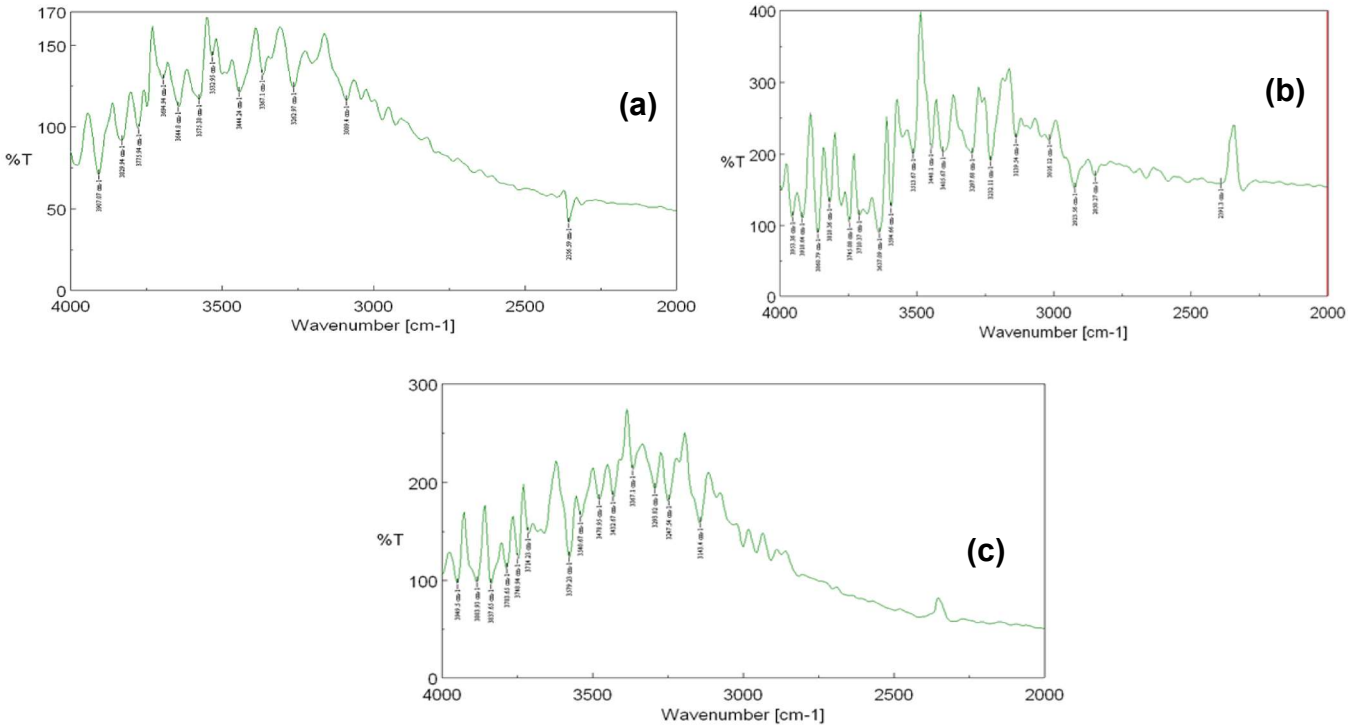

Figure 1. IR spectra of (a) conventional extract (b) methanol extract (c) Supercritical fluid extract

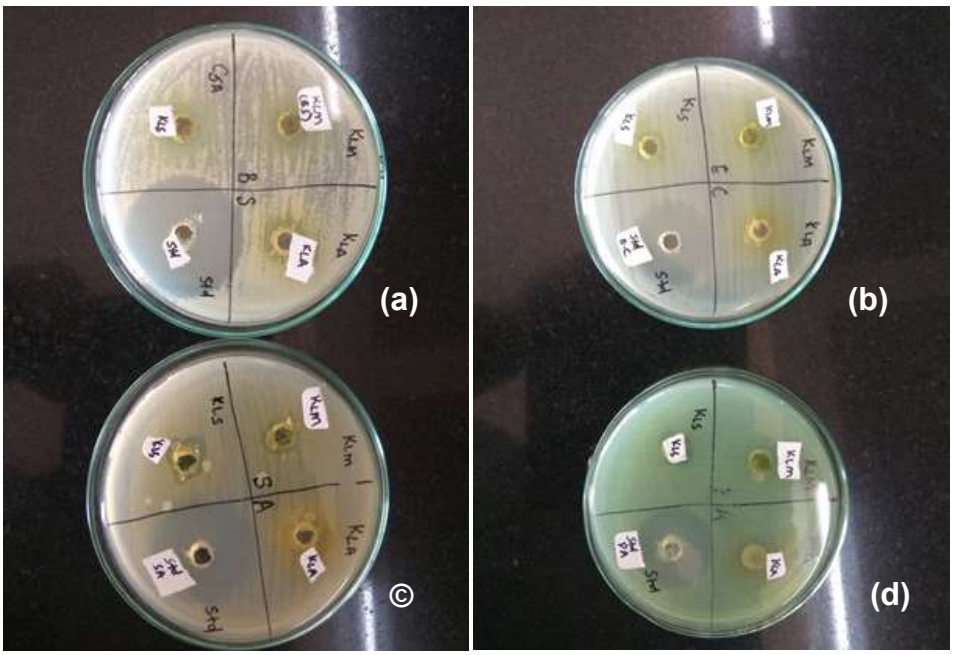

Figure 2. Antibacterial activity of different extracts (a) BS (b) EC (c) SA (d) PA

Table 6. Antibacterial properties of leaves extract

Extract sample		Gram +ve		Gram –ve	
	Std	***Bacillus subtilis***	***Staphylococcus Aureus***	***E. coli***	***Psedomonas aeroginosa***
SFE	1.2cm	0.4 cm	0.6 cm	0.2 cm	0.1 cm
CE	1.2 cm	0.1 cm	0.1 cm	0.05 cm	0.01 cm
ME	1.2 cm	0.2 cm	0.4 cm	0.1 cm	Nil

extracts are showing less antibacterial activity against gram –ve bacteria. Conventional extract is inactive against all bacteria. This may be due to the degradation of flavonoids due to continue reflux, which possess antibacterial activity. The standard is used as streptomycin. Results are given in table 6.

Conclusion

A qualitative aspect of *Citrus* plant leave powder has been investigated with characteristic physico-chemical properties, FTIR spectrum as identification mark. The powder extract has less potential as antimicrobial agent compound to streptomycin. Phytochemicals such as alkaloids, carbohydrate, phenolic compounds and tannins were present. FTIR spectra show various peaks for the functional group present in the extracts. This also confirms the presence of alkaloids and phenolic compound.

Conflict of interest: Authors have no conflict of interest.

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