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Antimicrobial Efficacy of *Citrus limon* Leaf and Peel Extracts on Indeterminate Tomato Plants to Achieve Sustainable Agriculture

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ABSTRACT

Background: The present study was carried out with the aim to extract and evaluate antimicrobial efficiency of lemon peel against bacteria (*Ralstonia solanacearum*) and fungi *(Fusarium oxysporum*). In the current scenario various microorganisms are developing resistance against all biotics.

Methods: In this experiment, volatile compounds from lemon peel were extracted by hydro-distillation process. Extract contains D-limonene (70-90%), flavonoids and several polymethoxylated flavones and showed many therapeutic properties like anticancer, antiviral, antitumor, anti-inflammatory activities including many bioactive compounds such as carotenoids, minerals, flavonoids and vitamins.

Result: It may be concluded that maximum zone of inhibition against the bacteria and fungus was around 40% when methanol is used as solvent. On the other hand, T_3 (Lemon peel extract with 40% methanol) also showed very good results concerning vegetative and reproductive parameters of tomato along with very less disease incidence and plant mortality rate.

Key words: Antibacterial sustainable agriculture, Antifungal, Extract, Lemon peel, Methanol.

INTRODUCTION

Citrus is a fruit of lucrative category and commercially produced in almost all subtropical and tropical regions. Besides table fruit, it contains many phenolic compounds including flavonoids, which are considered to exhibit antiviral, antioxidant, anti-allergenic, anti-carcinogenic, anti-inflammatory, antimicrobial activity. Use of naturally occurring antimicrobial materials is becoming more important in plant chemotherapy. Moreover, several alkaloids are present in various plant parts like stem, leaves, juice, flower and peel which are very effective against different bacterial strains (Kawaii *et al*., 2000). Production of mandarin (6 million metric tons) was the highest and followed by sweet oranges (4.25 MT) during 2022 in India (Statista, 2022). Phenolic compounds, Alkaloids, sesquiterpenes, glycosides, resins, saponins flavonoids, lipids, oleoresins and oils are only a few of the chemical components found in medicinal plants. Secondary metabolites of various aromatic plants can be accessed through the seeds/ leaves/peel of the fruit. Tawfik *et al.* (2010) also revealed that citrus fruits contain numerous antifungal and antibacterial properties. Alpha-pinene, sabinene, m-cymene, linalool, 4-terpineol, B-myrcene and d-limonene are among the other substances which may be extracted from citrus oil. Citrus peel contains all above-mentioned phytochemicals. Fruit (citrus) shows a high level of antioxidants and antimicrobial chemicals compounds which are responsible for antiviral, antibacterial, antidiabetic, anticancer and antifungal properties. Citrus peel is made up from limonene (90%) and citral (5%). Limonene, a biologically active compound re-sponsible for antimicrobial property is present in the essential oil of the citrus peel which has potential application in food systems as natural preservative (Han *et al*., 2021). Different authors have mentioned the antimicrobial effect of aqueous extract of juice and peel against eight gram-negative and six gram-positive bacteria, such as *Enterobacter aerogen Enterococcus faecalis, Staphylococcus pyogenes, Staphylococcus agalactiae Staphylococcus pneumonia, pseudo coccus aeruginosa* and *S. aureus*. It was revealed that antibacterial activity of the *Citrus limon* fruit peel's methanolic extract and the phytochemical analysis. Abundant phytochemical tests assessed saponin, alkaloids, citric acids, sugars, flavonoids glycosides and tannins in plant biomass (Ali *et al*., 2017). Antibacterial activity and phytochemical components of volatile *Citrus lemon* oil extracted from pruning leaves obtained and were accessed by Asker *et al*. (2020) against gram-negative and gram-positive bacteria. Aruna *et al*. (2022) proved in an experiment that lemon peel oil may be used as a preserving substance in the food system as it displayed several antifungal and antibacterial activities.

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Worldwide, waste from citrus peel production amounts to over 54 billion tons (Teigiserova *et al*., 2021) and they comprised pruning materials and juice producing wastes (El-gengaihi *et al*., 2020).

MATERIALS AND METHODS

Designed study was carried out in post-harvest lab of department of Horticulture in Lovely Professional University, Phagwara during 2021-2022. Lemon (*Citrus limon* var. PAU baramasi lemon 1) leaves and fruits were collected from the Centre of Excellence for Fruits Khanaura (Kapurthala) Punjab, washed with distilled water and peeled. Both components (leaf and peel) were dried in tray dryer (35°C) for 72 hours and converted into fine powder using a waring blender.

Ethyl alcohol extract

Ethanol (C_2H_6O) is used frequently in making antiseptic hand sanitizer liquid against bacteria including a wide range of industries. It plays a major role in extracting antimicrobial compounds from plants/parts of citrus species. El-Desoukey *et al*. (2018) also assessed antimicrobial properties in similar way and evaluated them. Extraction components were used effectively against various bacterial strains. Same compound having anti-bacterial properties were detected by Pandey *et al*. (2011). Ahmad and Ahmad (2016) also found antimicrobial properties in case of citrus limon through extraction process.

Methyl alcohol extract

Methanol (CH_3OH) can also serve as a significant solvent for the extraction of citrus species.

Aqueous extract

Different scientists used aqueous methods of extraction for purposes. Following this method, El-Desoukey *et al*., (2018) added dried citrus peel to the boiling distilled water and maintained its temperature at 4°C. But Amengialue *et al*., (2016) used distilled water along with 10 g of peel powder and covered it with aluminum foil and filtered it prior to use. Similarly, Ahmad and Ahmad (2016) adopted this extraction technique for evaluating the antibacterial activity of citrus species by drying powder with hot distilled water and boiling whole matter for 30 minutes.

Extract of acetone

Acetone (also known as propanone) is a highly volatile, flammable, or colorless liquid having a pungent odor which may be used for antimicrobial extraction purpose. Yashaswini and Arvind (2018) also employed dried orange powder (kept at 4°C) and extracted with 20 ml of acetone. They stir the mixture for 36 hours at 30°C while extracting *Citrus reticulata* var. kinnow. Pathogens like *Staphylococcus aureus* and *Klebsiella pneumoniae* were supposed to be controlled.

Preparation of lemon leaf extracts

Plant leaves were collected, washed and kept under shade for 1-2 hours. After weighing, placed in a tray dryer at temperature of 40°C and then stored at 4°C. 50 grams of each dried leaf powder were dissolved in 500 ml of distilled water (1:10) to make aqueous extract (Plate 1). It was centrifugated at 3000 rpm to enable the diffusion of active ingredients. Later filtrate was evaporated to powder by a water bath steam at 100°C. Same procedure was further carried out with ethanol, methanol and acetone.

Preparation of lemon peel extracts

Fruits were washed and kept in 100°C boiling water for blanching process. Peel of the lemon was removed and kept in tray dryer at 40°C for 72 hours. After drying, peel was grinded into a fine powdered form. This procedure was repeated for methanol extract: B_1 (10% concentration) and acetone extract: B_4 (10% concentration).

Solvents used during study

Methanol (10%, 20% and 40% concentration).
Acetone (10%, 20% and 40% concentration).

Factor details

Factor	Names and notations
Factor-1	Fruit peel (A_1), Plant leaf (A_2)
Factor-2	Solvent type and concentration-10% methanol-notated as B_1, 20% methanol as B_2, 40% methanol-B_3, 10% acetone-B_4, acetone 20% as B_5 and 40% acetone as B_6.
Factor-3	Tomato plants used in experiment were notated as P_1.

There were 13 treatments (including control) with replications 3 and number of plants per treatment was 6. In this experiment total 78 treatments and their combinations were studied and various observations like plant height, number of branches, length of internode, stem girth, number of flower cluster per plant, no. of flower per cluster, number of fruit per cluster, fruit length, fruit diameter, average fruit weight (grams), number of fruits/ plant, plant mortality and number of plants affected by the disease incidence, were recorded. Recorded data was analyzed in Factorial RBD.

Preparation of lemon peel and leaf extract

Fresh collected lemon leaf samples were washed out to remove the contamination. Then peel was separated and cut it into small pieces (Plate 1). Both were kept in a tray dryer at 30-35°C for 72 hours (Plate 2).

Grinding of peel and leaf

Dried lemon peel and leaf (Plate 3) were placed in a grinder mixer to make a fine powdered material and sieved (Plate 4). Prepared powder was stored in an airtight jar at temperature 4°C.

Maceration

This method involves shaking of grounded material, using an orbital shaker incubator at 250 rpm overnight with a solvent followed by filtration and evaporation of the solvent. A weighted sample of lemon peel and leaf powder (50 grams, each) and 500 ml of solvent is mixed. Made solution was

kept in an orbital shaking incubator at 37°C and filtered and evaporated to dryness using a water bath. Finally, solidified mass of the extract, was obtained (Plate 5).

Investigation of antibacterial traits of lemon leaf and peel

Diffusion technique was employed to evaluate the antibacterial properties of lemon peel and leaf. The experiment was carried out under controlled conditions in a laminar air flow chamber.

Preparation of nutrient agar

300 grams of nutrient agar (comprised of 5 g of peptone, 5 g dextrose, 5 g sodium chloride, 3 g beef extract and 20 g agar) was suspended in distilled water (one liter) in a conical flask and mixed well. For complete dissolution, solution was also sterilized by autoclaving at 15 lbs pressure at 121°C for 15-20 minutes. The prepared mixture was cooled and poured into sterile petri plates and permit it to harden under a laminar air flow condition.

Preparation of nutrient broth

In a conical flask, 300 gms of broth (comprising 3 g of beef extract, 5 g of peptone, 5 g of dextrose and 5 g sodium chloride) were mixed with distilled water (one liter) and agitated for homogenization. The solution was autoclaved at 15 lbs pressure, 121°C for 20 minutes. The prepared medium was cooled and kept at a temperature of 25°C.

Agar well diffusion method

Dey *et al*. (2010) also evaluated antibacterial and antifungal properties of the lemon leaf and peel extract by using modified agar well diffusion method. *Ralstonia solanacearum* was added to the broth and stirred for 24 hours. Using spreader, 0.1 ml of inoculum from broth was dispersed uniformly over nutrient agar plates. Plates were incubated for a period.

After growth of the plates, a uniform well was created with the help of sterile cork borer of 5 mm diameter and extract (lemon peel) was poured in the well and kept for 48 hours. Zone of clearance around the well was observed to determine the antibacterial activity. The antibacterial activity was assessed by measuring the diameter of the inhibition zone formed around the discs. These studies were accomplished in triplicate.

Preparation of potato dextrose agar from powdered material

39 g of commercialized powder was added to 1 liter of distilled water. It was boiled and sterilized media by autoclaving at 121°C for 15 minutes. The prepared mixture was cooled, poured into sterile petri plates and allowed to solidify in a laminar air flow. After growth of the plates, a uniform well was formed with the help of sterile cork borer of 5 mm diameter and lemon peel extract was poured in the well and plates were then kept for 48 hours. Zone of clearance around the well was observed to determine the antibacterial activity. Extent of antibacterial activity was assessed by measuring the periphery of the inhibition zone formed around the discs.

Optimization of extraction time

The yield obtained by the extraction process of lemon peel and leaf at different time periods and concentration (150 g/l) revealed that after 12 hours, 24 hours and 36 hours yield was 2.09 g, 21.00 g and 29.40 g, respectively.

Thus, the yield percentage of lemon peel and leaf extract was found to be highest with 19.60% with extraction timeframe of 36 hours. The yield percentage improved with increase in the time *i.e*., 8.6% for 12 hours, 14% for 24 hours and 19.6% for 36 hours (Table 1). It was concluded that the optimum time for maximum yield of lemon peels and leaves extract was 36 hours and therefore, recommended as optimum extraction time for the lemon peels/leaves extraction.

RESULTS AND DISCUSSION

In has been observed that zone of inhibition was different for the various solvents. Required amount of solvent increased with the extent of the zone of inhibition. Methanol had the largest zone of inhibition, trailed by acetone. In this study different concentrations of methanol peel extract (%) were used like 10%, 20% and 40%. Against *fusarium oxysporum* and *ralstonia solanacearum*, inhibition zone was noted in various concentrations (10%, 20%, 40%) as shown in Table 2. Amengialue *et al.* (2016) also showed that methanol peel exhibits a greater zone of inhibition as compared to ethanol. They revealed that in this case the zone of inhibition was 15.2 mg/ml. Methanol and ethanol seed extract had a minimum inhibitory concentration having range of 3.12 mg/ml -50 mg/ml against all tested bacteria.

Zone of inhibition (%, for acetone peel extract)

Acetone as a solvent found to be working at concentrations 20%, 40% and exhibited a higher zone of inhibition as Table 3. Yashaswini and Arvind (2018) determined the antimicrobial

Plate 1: Citrus Limon leaves.

Plate 2: Tray dryer.

properties and phenolic content of the kinnow against pathogenic bacteria. The maximum zone of inhibition in case of acetone was 7.93+0.06 mm for *Klebsiella pneumoniae* and 7.75+0.06 mm in case of *E. coli*. It was observed that acetone was proved less effective than methanol.

Zone of inhibition of methanol leaf extract

Methanol leaf extraction showed action at concentrations @ 20% and 40%. But maximum zone of inhibition was recorded at 40% (Table 4). Ibrahim and Kabede (2020) also claimed that methanol extracts of various plant leaves displayed improved activity as compared to other extracts. Amit *et al.* (2015) revealed that Methanolic extract of lemon peel exhibited higher antimicrobial activity when against tested many microorganisms like *Trichophyton rubrum, E. coli, Candida albicans* and *S. aureus*.

Zone of inhibition of acetone leaf extract

Leaf extract of acetone exhibited maximum zone of inhibition against fungus and bacteria at the 40% concentration (Table 5).

Peel and fruit leaf extract (methanol, acetone) was sprayed on tomato crop for recorded different growth and disease parameters.

A) Vegetative parameters

Plant height

Data concerning plant height was recorded at an interval of 20 days. Maximum height was noted in T_3 (peel + 40% conc. of methanol + tomato) followed by T_9 (leaf + 40% conc. of methanol + tomato), T_2 (peel + 20% conc. of methanol + tomato), T_8 (leaf + 20% conc. of methanol + tomato) treatments as shown in Table 6, Fig 1 and Fig 2 Methanol peel and leaf responded positively concerning plant height. In T_3 height was 93.67 cm while in T_9 and T_2, the height was 89.33 cm, 86.67 cm, respectively. Above all, control treatment (T_{13}) showed lowest results with height 66.67 cm.

Number of branches

Effect of peel and leaf extracts on height of tomato plants was recorded manually at an interval of 20 days. Maximum number of branches was noted in the treatment T_3 (peel + 40% conc. of methanol + tomato). Above all, methanol peel extract at 40% conc. showed very encouraging results as compared to the other treatments (Table 6 and Fig 1 and Fig 2). In this treatment (T_3) number of branches were 7.5 while in control it was only 4.73.

Internodal length

Maximum internodal distance was measured in T_3 (peel + 40% conc. of methanol + tomato) *i.e.*, 8.37 and followed by T_9 (Leaf + 40% conc. of methanol + tomato) and T_2 (peel + 20% conc. of methanol + tomato) as shown in Table 7 and Fig 1 and Fig 2. Above all, methanol exhibited a decent zone of inhibition contrary to the bacteria (*Ralstonia solanacearum*) and fungus (*Fusarium oxysporum sp. lycopersici*) and shown in Plate 1.

Plate 3: Dried peel and leaf.

Plate 4: Peel powder.

Plate 5: *Citrus limon* peel and leaf extract.

Table 1: Optimization of extraction time for lemon peels/leaves.

Weight of the dry extract (g)	Weight of the air-dry extract (g)	Time period (hours)	Yield (%)
12.90	150	12	8.06
21.00	150	24	14.00
29.40	150	36	19.06

Table 2: Zone of inhibition of acetone peel against certain organisms.

Test against organisms	10%	20%	40%
Fusarium oxysporum	+	+	+
Ralstonia solanacearum	+	+	+

Table 3: Zone of inhibition of acetone peel against certain organisms.

Test against organisms	10%	20%	40%
Fusarium oxysporum	-	+	+
Ralstonia solanacearum	-	+	+

Table 4: Zone of inhibition (%, for methanol leaf extract).

Test against organisms	10%	20%	40%
Fusarium oxysporum	-	+	+
Ralstonia solanacearum	-	+	+

Stem girth

Highest stem girth was noted in case of T_3 (Peel + 40% conc. of methanol + tomato) *i.e.*, 8 cm followed by the treatment T_2 (Peel + 20% conc. of methanol + tomato) and T_1 (Peel + 10% conc. of methanol + tomato) as shown in Table 7 and Fig 1. Moreover, it was also seen that stem girth occurred in the methanol solvent followed by acetone.

Table 5: Zone of inhibition of acetone leaf extract against some organisms.

Test against organisms	10%	20%	40%
Fusarium oxysporum	-	-	+
Ralstonia solanacearum	-	-	+

B) Flowering and fruiting parameter

Number of clusters per plant

Table 6 reveals that maximum number of clusters per plant were noted in T_3 (peel + 40% conc. of methanol + tomato) *i.e.*, 24.67 followed by T_2 (peel + 20% conc. of methanol +

Table 6: Effect of lemon peel and leaf extract on plant height, fruit weight, no. of fruit per plant and no. of flower cluster in tomato.

Treatment	Plant height (cm)	Average fruit weight (g)	No. of fruit per plant	No. of flower cluster
T_1	81.67	78.33	23.67	21.67
T_2	86.67	80.33	24.33	23.33
T_3	93.67	83.67	26.00	24.67
T_4	72.00	74.67	19.67	18.00
T_5	74.00	76.67	20.67	19.67
T_6	76.67	78.33	21.67	21.00
T_7	79.33	77.33	22.33	19.33
T_8	82.67	79.33	23.67	20.33
T_9	89.33	81.33	24.33	21.00
T_{10}	71.67	75.33	17.33	16.33
T_{11}	73.33	75.00	18.33	18.00
T_{12}	75.33	77.00	19.33	19.33
T_{13}	66.67	69.67	15.67	15.00
C.D.	5.07	5.37	1.43	1.12

Table 7: Effect of lemon peel and leaf extract on plant height, fruit weight, no. of fruit per plant and no. of flower cluster in tomato.

Treatment	Fruit length (cm)	No. of branches	Length of internode	Girth of stem	No. of flowers/cluster	Fruit diameter
T_1	3.67	5.67	8.00	7.30	6.00	3.67
T_2	4.33	6.50	8.13	7.80	7.00	4.33
T_3	5.33	7.50	8.37	8.00	8.00	5.67
T_4	3.00	4.50	7.33	5.87	4.33	3.00
T_5	3.67	5.17	7.53	6.88	5.33	3.33
T_6	3.67	5.93	7.63	6.97	6.33	3.67
T_7	3.00	5.87	7.77	6.17	5.00	2.67
T_8	3.67	6.80	7.85	6.65	5.67	3.67
T_9	4.67	7.13	8.17	7.07	6.33	4.33
T_{10}	2.67	4.60	6.97	5.42	3.67	2.33
T_{11}	3.00	4.80	7.13	5.47	4.67	2.67
T_{12}	3.00	4.97	7.33	5.77	5.00	3.00
T_{13}	2.00	4.73	6.73	4.97	2.67	2.00
C.D.	0.75	1.19	0.21	0.80	1.07	1.29

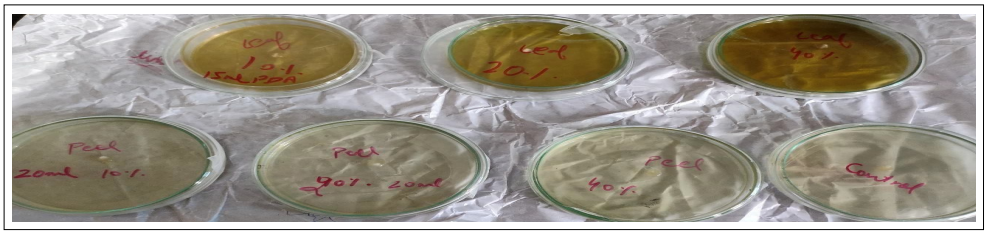

Fig 1: Checking zone of inhibition of peel and leaf extracts against the fungus (*Fusarium oxysporum* sp. lycopersica).

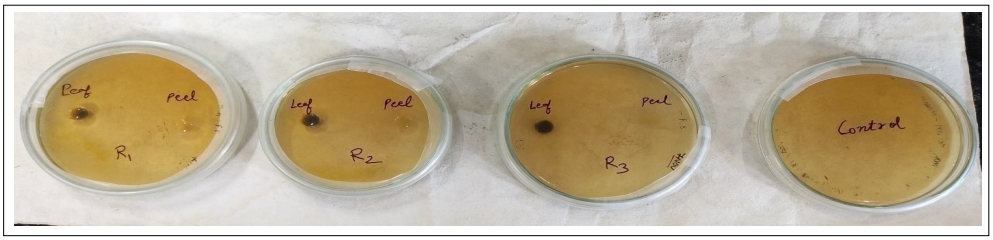

Fig 2: Checking zone of inhibition of the extract against the bacteria (*Ralstonia solanacearum*).

Table 8: Effect of lemon peel and leaf extract on plant height, fruit weight, no. of fruit per plant and no. of flower cluster in tomato.

Treatment	Plant mortality	No. of affected plants by diseases	No. of fruits per cluster
T_1	0.67	1.00	3.33
T_2	0.00	0.00	4.67
T_3	0.00	0.00	5.67
T_4	1.67	2.00	2.33
T_5	1.33	2.00	3.67
T_6	1.00	1.33	4.67
T_7	0.67	1.00	3.00
T_8	0.00	0.67	3.33
T_9	0.00	0.33	3.67
T_{10}	2.00	2.00	2.67
T_{11}	1.67	2.00	3.33
T_{12}	1.33	2.00	4.00
T_{13}	2.00	2.00	1.33
C.D.	0.55	0.43	0.94

tomato) *i.e.*, 23.33 along with T_1, T_9 and T_6. On the contrary, minimum number of the flower cluster was found in the control treatment T_{13} *i.e.*, 15. This data was recorded at an interval of 20 days after the flower emergence.

Flowers per cluster

Data concerning flowers per cluster is exhibited in Table 7. Maximum flowers per cluster was 8, in case of methanol peel solvent at 40% concentration in T_3 (peel + 40% conc. of methanol + tomato) followed by T_2, T_9 and T_1. On the other hand, in control (T_{13}) the no. of flowers per cluster was very less (2.67).

Fruits per cluster

Maximum number fruits per cluster was noted in T_3 (Peel + 40% conc. of methanol + tomato) to the tune of 5.67. The lowest value was displayed in T_{13} *i.e.*, 1.33 (Table 7).

Fruit length

Maximum length of the fruits was noted in T_3 (5.33 cm) followed by T_9 and T_2 (4.67 and 4.33 cm, respectively). Lowest value was observed in control (T_{13}) (Table 7).

Fruit diameter

Table number 7 reveals that maximum diameter was recorded in case of T_3 (Peel + 40% conc. of methanol + tomato) *i.e.*, 5.67 cm. It was noted that methanol @ 40% concentration acts as a good solvent in case of peel extract. On the other hand, the minimum diameter (out of treated fruits) was observed in T_2 (4.33 cm) as shown in Table 7. While lowest value was found in T_{13} (control) to the extent of 2 cm.

Fruit weight

Table 6 exhibited regarding maximum fruit weight existed in case of T_3(Peel + 40% conc. of methanol + tomato) with a value of 83.67 gm followed by T_9 and T_2. Above all, the lowest weight was seen in T_{13} (69.67 gm).

Number of fruits per plant

Maximum no. of fruits was found in T_3 (Peel + 40% conc. of methanol + tomato) *i.e.*, 26 but other treatments like T_2, T_9 and T_8 also showed encouraging results. On the contrary minimum number of fruit per plant were recorded in control (T_{13}) *i.e.*, 15.67 as compared to other treatments as shown in Table 6.

C) Disease incidence parameters

Plant mortality

Minimum mortality rates were found in T_3 (Peel + 40% conc. of methanol + tomato) followed by T_2, T_8 and T_9. On the other hand, highest mortality rates were found in the treatment T_{13} *i.e.*, in control. It is interesting to note that out of various treatments highest plant mortality was 1.67 found in T_{11} and T_4 (Table 8).

Number of plants affected by diseases

Analysis of observed data (Table 8) reveals that maximum plants were affected in T_4 (peel + 10% conc. of acetone + tomato), T_5 (Peel + 20% conc. of acetone + tomato), T_{11}, T_{12} and T_{13}. On the contrary, minimum no. of affected plants were seen in T_3 and T_4.

CONCLUSION

Maximum zone of inhibition against the bacteria and fungus shown by the solvent methanol (both lemon peel and leaf extracts) at concentration is around 40%. Lemon peel + 40% conc. of methanol showed very good results in the case of vegetative and reproductive parameters of tomato. Moreover, in this treatment disease incidence and plant mortality rate were also very low. It may be concluded that methanol peel solvent showed very optimistic results concerning antifungal and antibacterial properties and may be recommended.

Conflict of interest: None.

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