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Antibacterial activities of green tea extracts on five plant pathogenic bacteria

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Abstract

Tea is one of the medicinal plants that is used all over the world. In the preparation of green tea, the leaves are dried after harvesting and then used without fermentation. Tea extract has antimicrobial properties, but there is little information about this. The leaves of three clones of 100, seed hybrid and Assam were collected from tea gardens, and then the collected leaves were dried. Three solvents; aqueous, methanolic and ethanolic, were used for extraction. The effect of these extracts on the proliferation of five bacteria *Clavibacter michiganensis, Xanthomonas arboricola*, *Pseudomonas syringae, Dikeya zeae* and *Pectobacterium carotovorum* was investigated by the paper disc method by measuring the non-growth halo. The results showed that the highest dilutions of aqueous, ethanolic and methanolic extracts (50%) were the most effective dilutions on the investigated bacteria. All extracts had the ability to control bacteria. The extracts obtained from clone 100 showed the highest inhibition rate on the studied bacteria. Among the extracts and clones, the highest amount of inhibition on Gram-positive bacteria C. michiganensis was by water extract. On average, aqueous extract showed the most effect and ethanol extract showed the least effect on bacteria. *D. zeae* showed the highest resistance against the extracts. It seems that different factors such as type of solvent, concentration, types of microorganisms are effective in the inhibition rate of green tea extract.

Key words: Solvent, Green tea, Antibacterial, Aqueous extract, Alcoholic extract, Biological control

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Introduction

Tea (Camellia sinensis) is among the most common and important soft drinks consumed in the world, especially in Iran. The composition of green tea leaves differ and vary with climate, season and the type of tea itself. The beneficial of green tea extracts are attributed due to the presence of polyphenolic compounds, polysaccharides, Vitamin B, C, E and amino acids (Sarwa et al., 2013). Antibiotics provide the mainstay of treatment for microbial infections. Recently, antibiotic resistance is one of the biggest problems in the antibiotic industry in the world, various resistant strains have emerged after the discovery of antibiotics more than 50 years ago (Chatterjee et al., 2009; Adil et al., 2018; Adil et al., 2019). Excessive use of antibiotics has led to the emergence of multidrug resistance of several microorganisms. Therefore, in this increase of antibiotic-resistant microbes, there is a great demand to find new antimicrobial agents. The use of herbal medicines has increased worldwide (Pandey et al., 2016). The medicinal value of plants is due to some chemical compounds. Some of these biologically active components in plants include flavonoids, alkaloids, phenolic compounds and tannins (Gupta et al., 2014; Pérez-Burillo et al., 2021). Green tea leaves contain polyphenolic compounds, such as theaflavin and thearubigin, with antimicrobial effects (Nataro, 2006). The amount of polyphenol oxidase enzyme activity in different clones is different from each other. This value for clones varied between 16 and 46 units. Research has shown that the inhibitory effect of tea is directly related to its antioxidant power, and tea polyphenols act as prooxidant in certain conditions by producing hydrogen peroxide, and in this way, exert their inhibitory effect on the growth of bacteria (Sugita et al., 1999). Among the tested catechins, EGCG (*Epigallocatechin gallate*) and EPCG (export promotion capital goods) showed the highest inhibitory activity (Graham, 1992). Most of the methods adopted in recent years have not been based on sustainable agriculture. The most common method to control plant diseases is the use of chemical pesticides. However, the side effects of pesticides make it necessary to find alternative methods to control plant diseases, including the use of beneficial microorganisms, extracts and plant products (Rahanandeh et al., 2014). In this research, the extracts of three tea clones were extracted with three solvents and their effects on five types of plant pathogenic bacteria were investigated. The main purpose of this research was to investigate the antibacterial effects of green tea extract on important plant pathogenic bacteria.

Materials and Methods

Collecting leaves of various green plants and tea flowers

Green tea leaves were collected from clone 100, seed hybrid and Assam plants in spring. In order to deactivate the polyphenol oxidase enzyme, the collected leaves were immediately placed in the oven at 105 °C for 24 hours. The dried green leaves were ground for more uniformity. Tea flowers were also collected from tea bushes in autumn and dried by the leaf method.

Extraction

Preparation of ethanol extract of green tea leaves

The percolation method with 70% ethanol was used. First, 10 grams of each tea sample was transferred to different Erlenmeyer along with 200 ml of 70% ethanol. After 48 hours of incubation at 60 °C, the resulting mixture was passed through Whatman number two filter paper. Then, 200 ml of 70% ethanol was added to the pressed pulp and the previous steps were repeated. After re-purification, the obtained extracts were concentrated with the rotary evaporation until the volume of each of them reached 2 ml (Chou & Chung, 1999).

Preparation of aqueous extract of green tea leaves

Ten grams of each tea sample was added to different Erlenmeyer flasks containing 100 ml of sterile distilled water and heated on the flame for 30 minutes. Then, using Whatman filter paper No. 2, the tea grounds were separated from each sample. The liquid obtained from different tea samples was transferred to the ceramic crucibles that had been sterilized in the oven for 24 hours and placed on steam for one hour to dry, then

transferred to the oven at 103 °C and left for 24 hours. After this time, the ceramic crucibles were removed from the oven and the dried extract was shaved and weighed with a spatula.

Preparation of methanol extract of green tea leaves

Ten grams of the ground tissue was placed on a shaker in 100 ml of 80% methanol for 24 hours at a temperature of 20 °C, after this period, 75 ml of the solution was removed and 25 ml of sterile distilled water was added. Then its volume increased to 100 ml, and the equivalent of hexane added. This mixture was placed on the shaker for 2 hours, then the different parts were separated and the methanol part placed under the hood for evaporation and extract extraction.

Preparation of tea flower aqueous extract

In order to extraction of aqueous extract from tea flowers, 10 grams of dried flowers were added to different Erlenmeyer flasks containing 100 ml of sterile distilled water and heated on a flame for 30 minutes. Then, it was filtered using Whatman filter paper No. 2 and the procedure was similar to the preparation of aqueous green tea leaf extract.

Dilution of extracts

For the dilution of ethanolic, methanolic, aqueous green tea leaf and tea flower extracts, 2 ml of the concentrated extracts of each tea sample were completely dried by incubation at 50 °C and then scraped with a spatula and ground in a mortar. Four sterile test tubes were selected and 0.5 ml of sterile distilled water was added to each. Then 0.5 ml of tea sample extract was added to the first test tube. Then serial dilution was prepared from this solution. Thus, for each tea sample, dilutions of 0.5 (50%), 0.25 (25%), 0.125 (12.5%), 0.0625 (6.25%), and 0.03125 (3.125%) ml extracts in ml water were prepared separately, the resulting solution was poured into capped vials and stored at 4 °C.

Cultivation condition

In this study, nutrient agar medium was used for culturing and maintaining bacteria, bacterial suspension and putting discs soaked in ethanolic, methanolic and aqueous extracts in disc-diffusion method.

Preparation of bacterial strains

The bacteria used in this research were pure strains of plant pathogenic bacteria, all of which were obtained from the Iranian Research Institute of Plant Protection (IRIPP) and included; Xanthomonas arboricola sp. Juglandis, Pseudomonas syringae, Dickeya zeae, Pectobacterium carotovorum sub sp. carotovorum, Clavibacter michiganensis sub sp.michiganensis.

Preparation of discs containing different extracts by disc-diffusion method

Plank discs manufactured by Padtan Teb Iran Company were inoculated with 25 µl of different dilutions of 0.5 (50%), 0.25 (25%), 0.125 (12.5%), 0.0625 (6.25%), and 0.03125 (3.125%) of tea samples by using sampler. Then, they were placed in an incubator at a 37 °C to dry for one hour to prepare for disking. Discs of sterile distilled water and antibiotics; ciprofloxacin, tetracycline, cefotaxime, and ampicillin were used as controls. The prepared discs were placed on the passaged plates and the plates were placed in an incubator at 27 °C for 24 hours in order to create favorable conditions for the bacteria. The minimum inhibitory concentration (MIC) was determined as the lowest dilution capable of inhibiting any visible growth by measuring the diameter of the halo of non-growth of bacteria around the disks containing the extract of tea samples (BBL manual of products & laboratory procedures, 1973).

Data analysis

These experiments were performed with a completely randomized design with three replications. The analysis of variance was calculated with SAS (9.2) and the comparison of means was done by LSD with a significance threshold of 5%.

Results

In this research, the effect of aqueous, ethanolic and methanolic extracts of three tea clones on 5 types of plant pathogenic bacteria was investigated.

The results of the inhibitory effect of aqueous extract of tea clones on bacterial growth: The results of the evaluation of aqueous tea extract on the investigated bacteria showed that they have inhibitory ability on all bacteria at least at a di-

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Table 1. Comparison of the average interaction effect of aqueous extract in tea clones against pathogenic bacteria in laboratory tests

Treatment	Inhibition rate (mm)				
	P. carotovorum	*D. zeae*	*P. syringae*	*X. arboricola*	*C. michiganensis*
Clone 100 extract with a dilution of 50%	3 ^{b}	2 ^{cd}	2.3 ^{a}	10 ^{a}	19.3 ^{a}
Clone 100 extract with a dilution of 25%	1.3 ^{c}	3 ^{c}	1.3 ^{b}	4.3 ^{bc}	17.6 ^{a}
Clone 100 extract with a dilution of 12.5%	3 ^{cd}	0 ^{d}	0 ^{c}	0 ^{d}	10.6 ^{b}
Clone 100 extract with a dilution of 6.25%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	9.8 ^{bc}
Clone 100 extract with a dilution of 3.125%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	6.1 ^{cd}
\]Assam clone extract with a dilution of 50%	4.2 ^{a}	4.3 ^{b}	2 ^{a}	4.6 ^{b}	4.5 ^{de}
Assam clone extract with a dilution of 25%	1.3 ^{c}	1.3 ^{cd}	1 ^{b}	1.3 ^{cd}	1.5 ^{ef}
Assam clone extract with a dilution of 12.5%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Assam clone extract with a dilution of 6.25%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Assam clone extract with a dilution of 3.125%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 50%	0 ^{d}	8.8 ^{a}	0 ^{c}	6.6 ^{b}	3.6 ^{def}
Seed hybrid clone extract with a dilution of 25%	0 ^{d}	5 ^{b}	0 ^{c}	1.3 ^{cd}	1.3 ^{ef}
Seed hybrid clone extract with a dilution of 12.5%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 6.25%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 3.125%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}
Control	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{d}	0 ^{f}

According to Tukey's test, means with similar letters do not have a significant difference at the 1% probability level

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Table 2- Comparison of the average interaction effect of once filtered ethanolic extract in tea clones against pathogenic bacteria in laboratory tests.

Treatment	Inhibition rate (mm)				
	P. carotovorum	*D. zeae*	*P. syringae*	*X. arboricola*	*C. michiganensis*
Clone 100 extract with a dilution of 50%	9.8 ^{a}	5.1 ^{a}	1.8 ^{b}	7.5 ^{a}	5 ^{ab}
Clone 100 extract with a dilution of 25%	9.5 ^{a}	4.5 ^{a}	1.3 ^{b}	5.5 ^{abc}	3.3 ^{bc}
Clone 100 extract with a dilution of 12.5%	5 ^{b}	2 ^{bc}	1.3 ^{b}	4.6 ^{bc}	1 ^{de}
Clone 100 extract with a dilution of 6.25%	1.6 ^{cd}	1 ^{cd}	0 ^{c}	0 ^{e}	1 ^{de}
ml/ml	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	0 ^{e}
Assam clone extract with a dilution of 50%	1.3 ^{cd}	2.6 ^{b}	5.3 ^{a}	5.3 ^{abc}	2 ^{cde}
Assam clone extract with a dilution of 25%	0 ^{d}	1 ^{cd}	1.8 ^{b}	3.8 ^{bc}	1 ^{de}
Assam clone extract with a dilution of 12.5%	0 ^{d}	0 ^{d}	1.3 ^{b}	1.1 ^{de}	0 ^{e}
Assam clone extract with a dilution of 6.25%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	0 ^{e}
Assam clone extract with a dilution of 3.125%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	0 ^{e}
Seed hybrid clone extract with a dilution of 50%	5.3 ^{b}	5 ^{a}	5 ^{a}	6.1 ^{ab}	5.6 ^{a}
Seed hybrid clone extract with a dilution of 25%	2.6 ^{c}	5 ^{a}	1 ^{bc}	3.5 ^{cd}	5.3 ^{ab}
Seed hybrid clone extract with a dilution of 12.5%	0 ^{d}	1.6 ^{bc}	0 ^{c}	0 ^{e}	2.3 ^{cd}
Seed hybrid clone extract with a dilution of 6.25%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	3 ^{de}
Seed hybrid clone extract with a dilution of 3.125%	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	0 ^{e}
Control	0 ^{d}	0 ^{d}	0 ^{c}	0 ^{e}	0 ^{e}

According to Tukey's test, means with similar letters do not have a significant difference at the 1% probability level

lution of 0.5 (50 %) ml/ml. Among tea clones examined in aqueous extract, clone 100 had the most effect on three bacteria *C. michiganensis*, X. arboricola and *P. syringae* at a dilution of 50%. The Assam clone showed the highest inhibitory effect on *P. syringae* and P. carotovorum at the first dilution. The seed hybrid clone showed the greatest inhibitory effect on *D. zeae* bacteria at the first dilution (Table 1).

The results of the inhibitory effect of ethanol extract of tea clones on bacterial growth. The evaluation results of tea ethanol extract on the investigated bacteria showed that they have inhibitory ability on all bacteria at least at a dilution of 0.5 (its unit). Among the tea clones examined in the ethanolic extract, clone 100 on three bacteria, *C. michiganensis*, X. arboricola, *D. zeae* and P. carotovorum had the greatest effect at a dilution of 0.5 ml in one ml. The seed hybrid clone has the greatest inhibitory effect on C. michiganensis, X. arboricola, *P. syringae* and *D. zeae* showed in the first dilution. Ethanol extract of Assam clone only in *P. syringae* species and at a dilution of 50%, its effect was placed in group A (Table 2).

The results of the inhibitory effect of the methanol extract of tea clones on bacterial growth

The results of the evaluation of the methanol extract of tea on the investigated bacteria showed that they have inhibitory ability on all bacteria at least at a dilution of 0.5. Among the investigated tea clones, the methanolic extract of clone 100 had the greatest effect on three bacteria C. michiganensis, *D. zeae* and *P. syringae* at a dilution of 50%. The methanolic extract of hybrid seed clone did not have a good effect on bacteria and it was only on *D. zeae* bacteria in the first dilution with two other clones in the same group (a). The methanolic extract of Assam clone at a dilution of 50% had the most inhibitory effect on X. arboricola, *D. zeae* and P. carotovorum species.

According to Tukey's test, means with the same letters do not have a significant difference at the 1% probability level.

The results of the inhibitory effect of tea flower extract on bacterial growth

The results showed that tea flower extract had inhibitory power on four other bacterial species except *Pseudomonas syringae* only at a dilution of 50%.

Discussion

Tea is known as a medicinal plant (anti-inflammatory, antimicrobial, anti-oxidative, anti-aging and anti-tumor) (Sharma et al., 2012). In the preparation of green tea, the leaves are first dried and then used without fermentation. Green tea contains 30 to 40 types of polyphenols, while black tea contains 3 to 10 types of polyphenols. (Archana & Abraham 2011). Various factors such as: geographical area where tea is grown, soil type, extraction method, type of solvent and inhibition measurement method have an effect on the inhibitory power of green tea extract (Sartini et al., 2015). Tea polyphenols are effective on gram positive and negative bacteria (Reygaert, 2014). In this study, it was shown that green tea extract with different solvents have different killing abilities on 5 types of plant pathogenic bacteria. Green tea contains polyphenols and other compounds that have different solubility in different solvents; therefore, their inhibitory ability is different (Sartini et al., 2015). In the present study, it was observed that aqueous extract was more effective than methanolic and ethanolic extracts in controlling bacteria. In a study conducted by Nihal on extracts of green tea with three solvents, water, ethanol and methanol, the aqueous extract had the highest inhibitory power on bacteria (Sartini et al., 2009). Based on the statistical results, methanolic extract was more effective than ethanolic extract, which is consistent with past research (Cumar et al., 2012; Fakoori et al., 2020). Catechins in green tea extract prevent the pathogenicity of Xanthomonas campestris pv. citri, pv. vesicatoria and *Pseudomonas syringae* pv. Tomato have been effective on citrus fruits and tomatoes (Kodama et al., 1991). Amlording to the research of Fukai et al., tea extract was effective on different strains of plant pathogenic bacteria such as Erwinia, Pseudomonas, Clavibacter, Agarobacterium and Xanthomonas. These bacteria were pathogenic on different products such as lettuce, tomato, eggplant, carrot, Irish potato, onion and grape. Different dilutions

Table 3- Comparison of the average interaction effect of methanol extract in tea clones against pathogenic bacteria in laboratory tests.

Treatment	Inhibition rate (mm)				
	P. carotovorum	*D. zeae*	*P. syringae*	*X. arboricola*	*C. michiganensis*
Clone 100 extract with a dilution of 50%	7.3 ^{b}	1 ^{a}	10.5 ^{a}	6.1 ^{b}	7.3 ^{a}
Clone 100 extract with a dilution of 25%	5.6 ^{bc}	0 ^{b}	6 ^{b}	5 ^{bc}	5.6 ^{b}
Clone 100 extract with a dilution of 12.5%	4.3 ^{bcd}	0 ^{b}	3.6 ^{c}	1 ^{cd}	4.3 ^{bc}
Clone 100 extract with a dilution of 6.25%	2.3 ^{cde}	0 ^{b}	2.3 ^{d}	0 ^{d}	2.3 ^{de}
Clone 100 extract with a dilution of 3.125%	0 ^{e}	0 ^{b}	1 ^{ef}	0 ^{d}	0 ^{f}
Assam clone extract with a dilution of 50%	11.3 ^{a}	1.3 ^{a}	2 ^{de}	11.6 ^{a}	3 ^{cd}
Assam clone extract with a dilution of 25%	7 ^{b}	0 ^{b}	0 ^{f}	5.3 ^{b}	1 ^{ef}
Assam clone extract with a dilution of 12.5%	3.5 ^{bcde}	0 ^{b}	0 ^{f}	2.3 ^{bcd}	0 ^{f}
Assam clone extract with a dilution of 6.25%	2.3 ^{cde}	0 ^{b}	0 ^{f}	1 ^{cd}	0 ^{f}
Assam clone extract with a dilution of 3.125%	1 ^{de}	0 ^{b}	0 ^{f}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 50%	0 ^{e}	1 ^{a}	1.3 ^{de}	2.3 ^{bcd}	1.3 ^{ef}
Seed hybrid clone extract with a dilution of 25%	0 ^{e}	0 ^{b}	0 ^{f}	1 ^{cd}	6 ^{f}
Seed hybrid clone extract with a dilution of 12.5%	0 ^{e}	0 ^{b}	0 ^{f}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 6.25%	0 ^{e}	0 ^{b}	0 ^{f}	0 ^{d}	0 ^{f}
Seed hybrid clone extract with a dilution of 3.125%	0 ^{e}	0 ^{b}	0 ^{f}	0 ^{d}	0 ^{f}
Control	0 ^{e}	0 ^{b}	0 ^{f}	0 ^{d}	0 ^{f}

According to Tukey's test, means with the same letters do not have a significant difference at the 1% probability level.

Table 4. Comparison of the average effect of tea flower extract against pathogenic bacteria in laboratory tests.

Treatment	Inhibition rate (mm)				
	P. carotovorum	***D. zeae***	***P. syringae***	***X. arboricola***	***C. michiganensis***
Tea flower extract with a dilution of 50%	6.6 ^{a}	7.6 ^{a}	0 ^{a}	4 ^{a}	3.6 ^{a}
Tea flower extract with a dilution of 25%	0 ^{b}	0 ^{b}	0 ^{a}	0 ^{b}	0 ^{b}
Tea flower extract with a dilution of 12.5%	0 ^{b}	0 ^{b}	0 ^{a}	0 ^{b}	0 ^{b}
Tea flower extract with a dilution of 6.25%	0 ^{b}	0 ^{b}	0 ^{a}	0 ^{b}	0 ^{b}
Tea flower extract with a dilution of 3.125%	0 ^{b}	0 ^{b}	0 ^{a}	0 ^{b}	0 ^{b}

Amlording to Duncan's test, means with similar letters do not have a significant difference at the probability level of one percent

of the extract had different effects so that its effectiveness increased with increasing concentration (Fukai et al., 1991). In another study, it was observed that tea extract had an inhibitory effect on different species of Pseudomonas (*P. syringae* pv. pisi race 1, *P. syringae* pv. pisi race 2 and *P. syringae* pv. phaseolicola) (Alstrom, 1992). Polyphenol extract of tea has the ability to reduce the growth of bacteria *P. syringae* pv.lacrymans, X. campestris pv. citri, X. c. pv. vesicatoria and R. solanasearum (Kodama et al., 1991). The results of the present study were completely consistent with previous studies. There are many materials related to the effect of green tea extract on human pathogenic bacteria, but there is not much material on the effect on plant bacteria and the mechanism of effect (Yang & Zhang, 2019)). Despite the many researches that have been done on the effect of tea extracts on bacteria (Sartini et al., 2009), this is the first research on the effect of green tea extract on 5 genera of plant pathogenic bacteria with three solvents: water, ethanol and methanol.

The authors declare that there is no conflict of interest.

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