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Original Article

Nicotiana plumbaginifolia Leaf Extracts as an Efficient Antibacterial and a Biocontrol Agent Versus Tomato Pathogenic Bacteria

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Abstract

Antibacterial agents derived from plants are known as suitable alternatives for synthetic pesticides because of their health and the lack of any side effects on plants. This study aims to assess *in vitro* effect of water, ethanol and methanol extracts of tobacco (*Nicotiana plumbaginifolia* Viv.) leaves on plant pathogenic bacteria and their effect on the control of bacterial infection in tomato plant in the farm conditions. Also, effective compounds of the selected extract were characterized. Water, ethanol and methanol extracts were prepared from the leaves of *N. plumbaginifolia* by soaking method. Antibacterial effects of three extracts were determined by agar well diffusion method in 100, 400 and 800 mg/ml concentrations on plant pathogenic bacteria including *Xantomonas campestris* PTCC1473, *Pseudomonas aeruginosa* PTCC1181, *Pseudomonas syringae* PTCC1290, *Enterobacter aerogenes* PTCC1221, *Clavibacter michiganensis* PTCC1399, *Ralstonia solanacearum* PTCC1600 and *Gluconacetobacter* PTCC1734. Then the effect of the extract was analyzed on the protection of infection and some characteristics of the tomato fruit in farm conditions. For this purpose, the tomato bushes were inoculated with the bacterium that were highly sensitive to the extracts and in order to evaluate the effect of the extract on the tomato fruit characteristics, factors including pH, ascorbic acid content, lycopene content, texture stiffness and antioxidant activity of tomatoes were assessed post treatment. Effective compounds of the extract with the maximum antibacterial effect were measured using GC/MS method. Water, ethanol and methanol extracts of *N. plumbaginifolia* in the minimum concentration (MIC) of 400 mg/ml were effective on the most of studied bacteria and had the most effect on *P. aeruginosa* PTCC1181 with the growth inhibition zone of 22.51 mm (water extract), 19.39 mm (ethanol extract) and 20.94 mm (methanol extract). The concentration of 800 mg/ml of the extracts was detected as minimum bactericidal concentration (MBC). The results showed that the methanol extract of *N. plumbaginifolia* had the most effect on the studied bacteria without changes in plant growth indicators. GC/MS analysis of the extract approved the existence of several organic acids and antimicrobial compounds including benzoic acid, hydroxymethylfurfural, nicotine, nonanoic acid, neophytadiene and plant esters. The methanol extract of *N. plumbaginifolia* leaves had the greatest impact on *P. aeruginosa* PTCC1181 as one of the pathogens of tomato, and this extract also controlled the growth of bacteria on the tomato plant in farm condition and this plant extract is recommended as an antimicrobial agent to control the bacterial disease of tomato.

Keywords: *Nicotiana plumbaginifolia*, Antibacterial, Biocontrol agent, Plant pathogenic bacteria, Effective compounds

Introduction

In the sixteenth century tobacco seeds were brought to France by Jean Nicot. After it, the genus of this plant was named as *Nicotiana* [1]. Species of this genus are primarily belonging to the Neotropics and Australia [2]. *Nicotiana plumbaginifolia* Viv. belongs to the monophyletic group section *Alatae* [3]. The use of natural herbal antimicrobial compounds is today widely used to control pathogenic bacteria and fungi [4]. Extracts and essential oils have long been known as natural antimicrobial compounds in the fields of pharmacy, medical microbiology, herbal pathology and food preservation and unlike antibiotics do not cause drug resistance to pathogens [5]. Most extracts of essential oils

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and extracts have antifungal, antiparasitic, antiviral and antibacterial properties. These compounds affect bacteria by different mechanisms and inhibit their growth and proliferation. Most of the essential oils and extracts are actually produced as inactive precursors stored in plant tissue and released in response to environmental stresses. Antibacterial activity of essential oils and plant extracts is mainly due to the presence of terpenoids and phenolic compounds including thymol, carvacrol and eugenol [6]. The tobacco leaves contain several types of pyridine alkaloids that the main of them is nicotine. Other compounds include organic acids, glycosides, caffeic acid, amino acids, isoprenoids, terpenoids, volatile oilsand inorganic compounds such as metal compounds [7]. Analyses of the tobaccos harvested from different geographical areas have been provided an overview of major differences in the composition and the amounts of compounds in different tobaccos [8]. This study aimed to estimate *in vitro* effect of water, ethanol and methanol extracts of tobacco (*N. plumbaginifolia*) leaves on some tomato plant pathogenic bacteria and their effect on the control of growth and infection induction of bacteria inoculated onto tomato plant in the farm conditions.

Material and Methods

Plant Seeds

Tomato seeds with the herbarium number 106/012/001 and tomato seeds with the herbarium number Acc: l00134 were purchased from Isfahan Pakan Bazr Co in February 2017.

Preparation of Tobacco Leaves Extracts

For preparation of water extract, the tobacco leaves were dried in room temperature and powdered. Then every 50 g of tobacco powder was added to 100 ml of sterile distilled water, 100 ml ethanol 70% and 100 ml absolute methanol respectively and was shacked for 48 to 72 hrs at room temperature. Finally, the extracts were refined by sterile gas and filtered by filter paper No. 4. The filtrates were dried up for 24-48 hours at the temperature of 45 ºC and stored in 4 ºC [9].

Antibacterial Activity Assessment

Pathogenic or saprophytic bacteria including *Xantomonas campestris* PTCC1473, *Pseudomonas aeruginosa* PTCC1181, *Pseudomonas syringae* PTCC1290, *Enterobacter aerogenes* PTCC1221, *Clavibacter michiganensis* PTCC1399, *Ralstonia solanacearum* PTCC1600 and *Gluconacetobacter* PTCC1734 were purchased from Tehran Research Institute of Technology. Antibacterial activity of extracts was assessed by agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). A dilution series of 100, 400 and 800 mg/ml of each extract was prepared in DMSO (10%). Inoculums with the turbidity as McFarland standard No. 0.5 of each bacterial suspension in nutrient broth was spread on Muller Hinton agar (MHA) plates with a sterile swab. Subsequently, bottom sealed wells with 8 mm diameter were punched in MHA medium and each well was filled with 100 µl of one of the plant extract concentrations and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 37° for 24 h. Wells containing the same volume of DMSO (10%), distilled water, ethanol and methanol served as negative controls while standard antibiotic disc of gentamycin (10 µg) was used as the positive controls. After incubation time, the diameters of the growth inhibition zones were measured. Three replicates of experiments were carried out for each extract against each bacterium.

Assessment of Plant Infection Control

First 100g of garden soil was sterilized with autoclave and then, was mixed with the number of 1.5×10^8 CFU/ml cells of pathogen bacterium that showed the most sensitivity to the extracts. Then tomato seeds were dispersed on the soil and covered with a thin layer of soil. The seeds were watered every 3 days to germinate. Then the best extract was sprayed on tomato leaves 2 times with 2 weeks, interval in field conditions. The leaves of infected plants had tubular form in contrast to flat leaves of healthy leaves (Fig. 1). In order to evaluate the effect of the extracts on tomato plant occurrence of the infection in addition to physicochemical characteristics of tomato fruits were detected. For this purpose, factors including pH, ascorbic acid production (N-bromosuccinimide titration), lycopene production (dissolving in hexane and acetone and color metering), texture stiffness (texture meter apparatus), and antioxidant activity (DPPH reduction method) of tomatoes were assessed after treatment with the extracts.

Phytochemical Analysis of Tobacco Leaves

Gas chromatography (GC) system (Agilent 6890, column with 30 meters, length, 0.25 mm inner diameter and 0.25 µm layer thickness) was used for analysis of the composition of the most effective extract. Dried powdered leaves of tobacco were dissolved in N-hexane and injected to the system. The temperature program was adjusted as 5 min at 50 °C, rising to 240 °C, decreasing to 15 °C with a stop for 5 min and then increase up to 300 °C with a slope of 15 ºC every min and a stop in the last temperature for 3 min. The temperature was adjusted to 290 °C as split of 1 to 20. Helium gas with the flow rate of 0.5 ml/min was used as the carrier gas. Mass spectrophotometer (MS) system (Agilent 5973) with the ionization method of EI (ionization voltage of 70 e.V and source temperature of 220 °C) was used. The range of the masses was set from 40 to 500 nm. The Commissions software was used for compounds detection.

The compounds were identified by their retention time according to the retention indexes using standard mass media and information available in the computer library.

Fig. 1 The leaves of healthy tomato plant (A) in comparison to the leaves that had been infected by *P. aeruginosa* (B).

Statistical Analysis

Data were expressed as mean±standard deviation. Determination of significant differences between groups was done by Kruskal-Wallis H test with the significance level is 0.05.

Discussion

One of the benefits of volatile compounds extracted from plant and vegetables as an alternative to chemical pesticides is that these compounds have wide range antimicrobial activity. These compounds which include different alcohols, aldehydes, terpenoids and other contributing compounds can widely use as natural substitutes for treating variety of infectious diseases and as food preservatives [10-13]. The findings of this study showed that the effect of water, ethanol and methanol extracts of *N. plumbaginifolia* leaves on plant pathogenic bacteria including *X. campestris*, *P. aeruginosa*, *P. syringae*, *E. aerogenes*, *C. michiganensis*, *R. solanacearum* and *Gluconacetobacter* showed that although the concentration of 800 mg/ml of all 3 extracts had the most effectiveness against all plant pathogenic bacteria, the MIC for the extract was 400 mg/ml on all studied bacteria except of methanol extract against *X. campestris* and *E. aerogenes* which its MIC was 100 mg/ml. The tobacco extract has previously mentioned as a biological defense against human pathogenic bacteria. Singh *et al*. (2010) showed that the highest antibacterial activity of aqueous and methanol leaf extract of *N. plumbaginifolia* was in the concentration of 600 mg/ml and least activity was observed by a lower concentration (200 mg/ml) on Gram-positive *Bacillus* spp [14]. However, the Gram-negative bacterium (*Salmonella typhimurium*) showed the inhibition zone with lower diameter to the concentration of 200 mg/ml of methanol extract. Okorondu *et al*. (2015) investigated the antimicrobial effects of *N. tabacum* leaves using ethanol and water extracts and showed that the extract inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* with the MIC of 100 mg/ml [15]. The compounds identified in the methanol extract of tobacco plant in the present study included some antimicrobial agents with proven properties. One of these compounds was benzoic acid. Benzoic acid is used in medicine as an ointment to treat skin diseases with fungal origin such as dermatophytosis and the leg wound of athletes [16]. It is also used in the early 20th century as a topical disinfectant and inhalation drug to open the nasal passages [17]. Hydroxymethylfurfurole was also one of the effective compounds identified in tobacco extract in the study. It is found in sweet natural nutrients such as honey, and it is produced by dehydration of certain sugars during heating or baking, and it is added to food as an additive flavor. Also the antioxidant effect of this material is proved [18]. It also has antimicrobial effect. Nafea *et al*. (2011) added hydroxymethyl furfurole with different ratios to honey and studied the antibacterial effect of honey on bacteria. The results showed that the addition of 24 mg/kg of this material to honey by 15 to 20 percent had inhibited the growth of *Escherichia coli* in laboratory conditions [19]. Nanoic acid was another ingredient in tobacco methanol extract. The esters and salts of nanoic acid, commonly called nanoates, are found in many plant extracts and are used in agriculture to control weeds [20]. These compounds have also antimicrobial effects. Inouye *et al*. (2001) found the compound in 14 plant essential oils. The extracts had antibacterial effects based on the types of pathogenic bacteria such as *Haemophilus influenza*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. Nicotine was also detected in tobacco methanol extract. It is an organic compound that is found mostly in plants such as tobacco and in lower amounts in tomatoes, potatoes, eggplant and green pepper [21]. The rate of 3 to 5 percent nicotine exists in dried tobacco plant, which is an effective material on the nervous system and is used in many insecticides [12]. It has an antimicrobial effect on different types of bacteria and fungi. Pavia *et al*. (2000) found antimicrobial effect of the material at a concentration of 250 µg/ml. Neophytadiene was also observed among compounds in the extract [22]. It also was found in the study of Alagić *et al*. (2002) as 23% in tobacco plants [23]. Finally, sterol compounds such as stigmasterol, which are found in the extract of many plants and are found in the extract of the tobacco plant, have known antimicrobial effects against a variety of Gram-positive and Gram-negative bacteria, and fungi [13].

Results

Water Extract Effect

The results based on the mean values of inhibition zones (mm) created by the concentrations of 100, 400 and 800 mg/ml of tobacco leaves water extract on the plant

pathogen bacteria is illustrated in Figure 2. The extract showed no effect on *P. syringae* and the most effectiveness against *X. campestris*, *Ralstonia solanacearum*, *Gluconacetobacter*, *C. michiganensis* and *E. aerogenes* in the concentration of 800 mg/ml. In all six bacteria there was a significant difference between growth inhibition zone diameters in different concentrations of the extract. Kruskal-Wallis H test results showed that the inhibition zone diameters on *X. campestris*, *Ralstonia solanacearum*, *Gluconacetobacter*, *C. michiganensis* and *E. aerogenes* had significant differences between every two concentrations and had more effects with significant differences by concentration increasing but there were no differences between the effectiveness of 400 and 800 mg/ml concentrations on *P. aeruginosa* and *C. michiganensis* but the inhibition zone by the concentration of 800 mg/ml on *P. aeruginosa* was significantly greater than *X. campestris*, *Ralstonia solanacearum*, *Gluconacetobacter* and *E. aerogenes*. Also there were no significant differences between the inhibition zones on the last 4 bacteria. *P. aeruginosa* was the most sensitive bacterium. The minimum inhibitory concentration (MIC) was detected as 400 mg/ml in all studied bacteria.

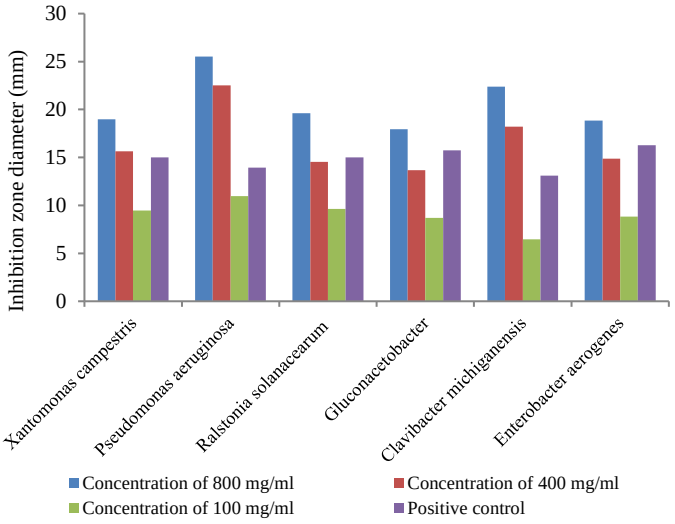

Fig. 2 The results from the effects of different concentrations of tobacco water extract on plant pathogen bacteria.

Ethanol Extract Effect

Figure 3 illustrates the mean values of inhibition zones (mm) created by the concentrations of 100, 400 and 800 mg/ml of tobacco leaves water extract on the plant pathogenic bacteria. The extract showed the most effectiveness against *P. aeruginosa*, *X. campestris*, *Ralstonia solanacearum*, *Gluconacetobacter*, *C. michiganensis*, *E. aerogenes* and *P. syringae* in the concentration of 800 mg/ml.The results of Kruskal-Wallis H test showed that the inhibition zone diameters were significantly different by all concentrations of the extracts as well as each two different concentrations. Also the effect was significantly increased by concentration increasing. By the concentration of 800 mg/ml, the inhibition zones on *C. michiganensis*, *E. aerogenes* and *P. aeruginosa* was significantly greater than *X. campestris* and *P. syringae*, and the inhibition zone on *Ralstonia solanacearum* and *Gluconacetobacter* was significantly greater than *P. syringae*. *P. aeruginosa* was the most sensitive bacterium. The minimum inhibitory concentration (MIC) was detected as 400 mg/ml in all studied bacteria.

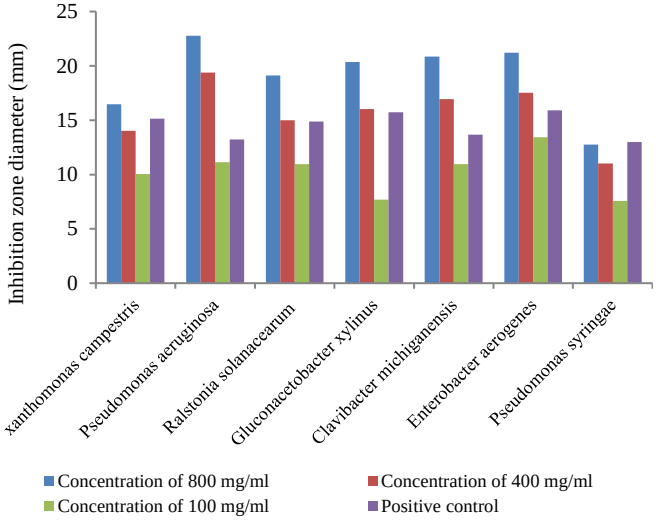

Fig. 3 The results from the effects of different concentrations of tobacco ethanol extract on plant pathogenic bacteria.

Methanol Extract Effect

The results of the mean values of inhibition zones (mm) created by the concentrations of 100, 400 and 800 mg/ml of tobacco leaves methanol extract on the plant pathogenic bacteria is illustrated in Figure 4. The most effectiveness of the extract was shown in the concentration of 800 mg/ml against all plant pathogenic bacteria.

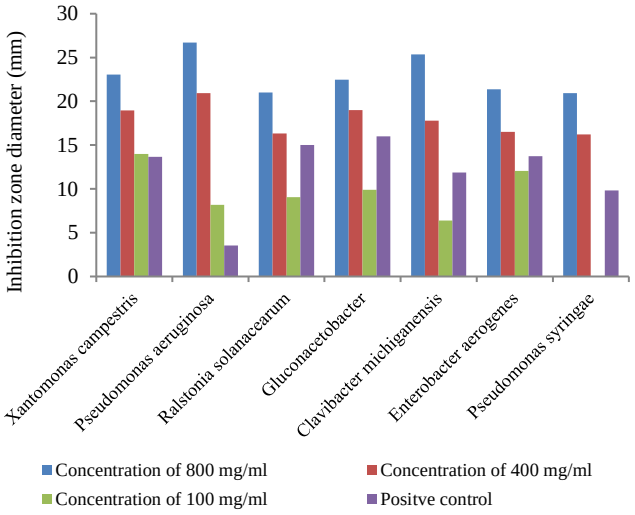

Fig. 4 The results from the effects of different concentrations of tobacco methanol extract on plant pathogenic bacteria.

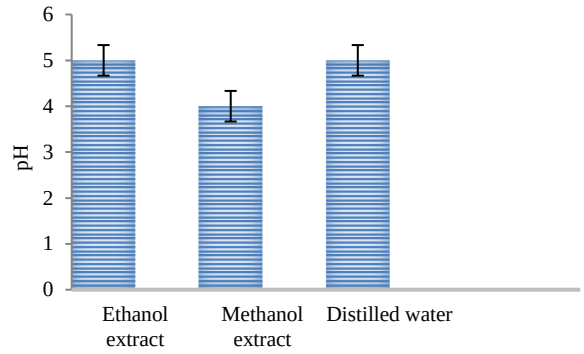

Fig. 5 The results from the effects of 800 mg/ml concentrations of ethanol and methanol extracts of tobacco leaves on pH values of the fruits of contaminated tomatoes with *P. aeruginosa* PTCC1181

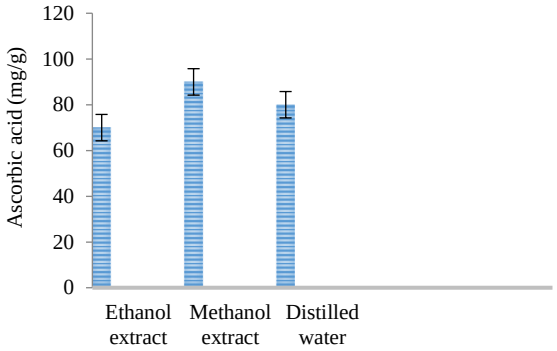

Fig. 6 The results from the effects of 800 mg/ml concentrations of ethanol and methanol extracts of tobacco leaves on ascorbic acid amounts in the fruits of contaminated tomatoes with *P. aeruginosa* PTCC1181

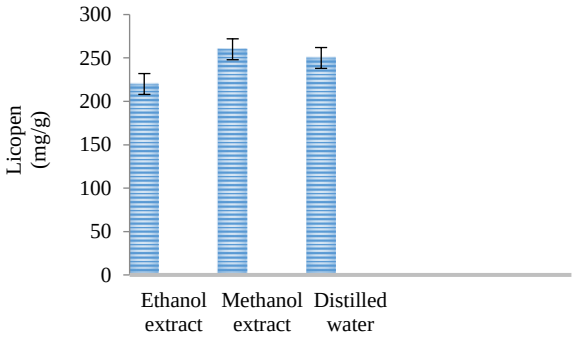

Fig. 7 The results from the effects of 800 mg/ml concentrations of ethanol and methanol extracts of tobacco leaves on lycopene

Kruskal-Wallis H test results showed that the inhibition zone diameters on *X. campestris*, *P. aeruginosa*, *E. aerogenes*, *C. michiganensis*, *R. solanacearum* and *Gluconacetobacter* had significant differences between different concentrations. Also every two concentrations and had more effect with significant differences by concentration increasing. In the case of *P. syringae*, 800 mg/ml methanol extract was significantly more effective than 400 mg/ml but there was no significant difference between the concentrations of 400 and 100 mg/ml. There were no significant differences between the effects of 800 mg/ml concentration between all plant pathogenic bacteria except between *X. campestris* and *P. aeruginosa*. *P. aeruginosa* was the most sensitive bacterium. The minimum inhibitory concentration (MIC) was detected as 400 mg/ml in all studied bacteria except of *X. campestris* and *E. aerogenes* which was 100mg/ml.

Phytochemical Analysis of Tomato Fruits

The results of mean values of pH, and the amounts of ascorbic acid and lycopene in fruits of tomatoes contaminated with methanol extract of tobacco are observed in Figures 5 to 9. The treatment did not have a significant effect on plant characteristics ($p>0.05$), while controlling the plant infection.

Methanol extract also showed strong ability for the control of *P. aeruginosa* on the tomato plant. As tomato is from the most important economic plant products in the world and Iran [23], this extract is recommended as an antimicrobial agent in agricultural application for this plant.

The Results from GC/MS Analysis of the Methanol Extract

Name and characteristics of the compounds which were detected in the methanol extract of *N. plumbaginifolia* Viv. leaves are shown in table 1.amounts in the fruits of contaminated tomatoes with *Pseudomonas aeruginosa* PTCC1181

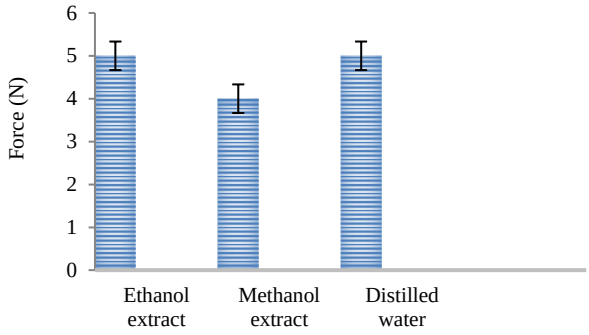

Fig. 8 The results from the effects of 800 mg/ml concentrations of ethanol and methanol extracts of tobacco leaves on texture stiffness of the contaminated tomatoes with *P. aeruginosa* PTCC1181

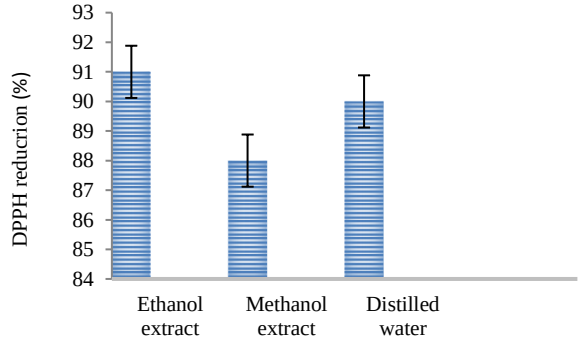

Fig. 9 The results from the effects of 800 mg/ml concentrations of ethanol and methanol extracts of tobacco leaves on antioxidant activity of the contaminated tomatoes with *P. aeruginosa* PTCC1181

Table 1 The results obtained from GC/MS analysis of the *N. plumbaginifolia* Viv. leaves

Retention time (min)	Area (%)	Name	Quality	CAS Number
9.916	2.68	Alpha-ethythexanoic acid	95	000149-57-5
10.077	0.40	4H-Pyran-4-one,2,3-dihydro-3,5-Dihydroxy-6-methyl	59	028564-83-2
11.328	9.47	Benzoic acid	94	000065-85-0
11.888	1.21	Hydroxymethy lfurfurole	86	000067-47-0
12.931	0.51	Nonanoic acid	93	000112-05-0
13.444	0.40	Dodecamethy lcyclohexasiloxane	91	000540-97-6
14.145	72.00	Nicotine	95	000054-11-5
16.599	0.44	Beta-Nicotyrine	92	000487-19-4
17.486	0.42	4,5,7,7a-Tetrahydro-4,4,7a-Trimethyl- 2(6H)benzofuranon	53	015356-74-8
20.356	0.87	Cotinine	96	000486-56-6
21.087	0.58	Myristic acid	98	000544-63-8
21.57	0.55	N(b)-formylnornicotine	80	003000-81-5
22.208	0.57	Neophytadiene	99	000000-00-0
24.05	4.99	Palmitic acid	99	000057-10-3
26.395	0.87	2-Methyl-Z,Z-3,13-octadecadienol	95	000000-00-0
26.675	0.92	Stearic acid	99	000057-11-4
40.737	0.45	Cholest-2-ene-2-methanol,(5,alpha)	80	053287-18-6
41.473	0.95	Stigmasterol	97	000083-48-7
42.916	0.75	Gamma-Sitosrerol	96	000083-47-6

Conclusion

Present study concludes that *N. plumbaginifolia* leaves are rich source of various phytochemicals and have strong antimicrobial properties. Methanol extract of the leaves showed comparatively more antibacterial activity on plant pathogenic bacteria than the water and ethanol extracts. The methanol extract of *N. plumbaginifolia* leaves had the greatest effect on *P. aeruginosa* as one of the pathogens of tomato, and this extract also controlled the growth of bacteria on the tomato plant in farm condition. Thus, this plant extract is recommended as an antibacterial agent to control the bacterial disease of tomato.

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