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Effectiveness of Novel Herbal Extracts and Antibiotics in Controlling the Bacterial Soft-Rot Disease in Potatoes and Carrots

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Abstract

Potatoes and carrots are one of the major tuber crops in the world with high exposure to various pests and diseases, especially soft rot disease caused by Pectobacterium carotovorum subsp. carotovorum. In recent years, several reports relating to the spread of this disease in different parts of Northwest Iran have been published, but so far, no effective solution has been found. Due to the high prevalence of this disease in the region, the use of chemical pesticides, new antibiotics and safe antimicrobial compounds such as herbal extracts and essential oils appears to be necessary. The current research was conducted to assess the antibacterial effects of the three antibiotics Oxytetracycline, Dihydrostreptomycin and Chloramphenicol, in addition to thyme and mint essential oils, as well as neem aqueous and alcoholic extracts on the causal agent of the soft rot disease. Copper oxychloride pesticide was used as the control to compare the efficiencies of the compounds and extracts. The antibacterial effects on soft rot-inducing bacteria were analyzed using the agar gel diffusion and disc diffusion methods in three replicates by applying completely randomized design, and the results were analyzed in the SPSS software. The results showed that among the antibiotics the highest inhibition effect belonged to oxytetracycline. This antibiotic with its inhibitory halo diameter of ٤۳ mm and percentage inhibition of ٤۷,۷۷٪ showed better performance than all other treatments, whereas copper oxychloride pesticide even at the highest concentration (٥۰۰۰mg/L), the inhibitory halo diameter was ۱۳ mm and the inhibitory effect was ۱٤,۷۷٪. Among the essential oils and extracts, the thyme essential oil with the inhibitory halo diameter of ۲۷ mm and percentage inhibition of ۳۰٪ was the highest among the natural compounds.

Keywords: soft rot, bacteria, Pectobacterium carotovorum, extract, antibiotic, pesticides

۱. Introduction

Potato (Solanum tuberosum L.) and carrot (Daucus carota L.) are one of the most important tuber crops in Iran and the world being economically and nutritionally important. Various pests and diseases expose the cultivation and production of these two crops to risk. One of the most important diseases in these two plants is bacterial soft rot caused by Pectobacterium carotovorum subsp. carotovorum (PCC) (Davis & Raid, ۲۰۰۲; Shattock, ۲۰۰۲). This bacterium causes considerable damage to the tuber crop of these two plants in both storage houses and fields (Agrios, ۲۰۰٥). Various types of agricultural, physical, and biological methods have been introduced to control the damage incited by this bacterium, most of which are either ineffective or expensive and applicable at a limited level. However, one of the most common, effective and cheapest control methods to control bacterial diseases in plants is to use various chemicals, especially copper pesticides and antibiotics (McManus et al., ۲۰۰۲). Nowadays, various pesticides and antibiotics have become conventional to control the population of plant pathogenic bacteria, but there is little information on the effect of such antibiotics against soft rot-inducing bacteria. Besides, plant extracts and essential oils, with their antimicrobial properties, are environmentally friendly and have long been used widely to control human diseases (Sarawaneeyaruk et al., ۲۰۱٥); however, their effect on plant pathogenic bacteria especially soft rot-inducing bacteria is not well-known. To overcome the disease with a new safe and cheap measure, the present study was conducted to find antibiotics and herbal substances that act against the disease.

۲. Materials and Methods

۲,۱. Preparing bacterial samples

The bacterial isolate of Pectobacterium caratovorum subsp. carotovorum was prepared from the Bacteriology Laboratory, Plant Protection Department, Faculty of Agriculture, University of Tabriz. This isolate had been accurately identified earlier using conventional laboratory methods (biochemical and molecular tests) and their pathogenicity had been proven in inducing soft rot diseases (Ansari, ٢٠١٥).

٢,٢. Preparing plant extracts and essential oils

Leaves from three plant species including thyme (*Thymus vulgaris* L.), mint (*Mentha spicata* L.), and neem (*Azadirachta indica* L.) were used for extracting the essential oil after removing the veins and petioles. Thyme and mint samples were collected from fields and pastures in West Azerbaijan Province in the northwest of Iran at the flowering stage, but neem was collected from Hormozgan Province in the south of Iran at different growth stages. After transferring to the laboratory and rinsing, the plant samples were dried at the ambient temperature (~٢٥ ˚C) in the shade. Then, the dried plants were powdered by an electric grinder and stored in the freezer (-٢٠˚C). Essential oils were extracted from the collected plant samples via the water distillation method by the micro-Clevenger apparatus using Jaymand and Rezaei's (٢٠٠٧) method. Accordingly, almost ١٠٠ g of the powdered parts of each plant was poured into a balloon and ٥٠٠ ml of water was added. After heating the distillation vessel by a heating source for ٤ h, water vapour and distilled volatile oils were collected as liquid droplets. This was repeated several times to prepare adequate amounts of essential oil from each plant. Then, the aqueous and oily phases of the essential oils were collected separated and stored in dark-coloured vessels to prevent oxidation and stored in the refrigerator at ٤ °C. Sodium sulfate powder was used to dehydrate the extracted essential oil samples. In the case of neem, no enough essential oil was extracted (due to the very low available samples); thus, only the alcoholic and aqueous extracts were provided for the study. To prepare aqueous and alcoholic extracts, the neem plant was used by the method developed by Alisi et al. (٢٠٠٨) with some modifications. For water extraction, the first ١٠٠ g of lower aerial parts of plants were mixed with ٧٥٠ ml of sterile distilled water, properly agitated for ٢٤ h in a shaker, and kept in the refrigerator at ٤ °C for ٢٤ h. The samples were filtered twice by Whatman filter paper and placed in Bain-marie at ٧٥ °C for ٤٨ h for water evaporation. After passing through a special Whatman biological filter (٠,٢ µm), the extract was stored in opaque and closed vessels away from light in the refrigerator to prevent oxidation. In the ethanol extraction method, ١٠٠ g of the milled plant sample was mixed with ٢٥٠ ml ethanol and placed in a shaker at ٢٠ °C for ٢٤ h; and the rest of the extraction steps were performed in accordance with the previous method. The neem leaf aqueous and alcoholic extracts were diluted with distilled water at the ratio of ١:٥, ١:١٠, ١:٢٠, and ١:٤٠.

٢,٣. Preparing antibiotics and pesticides

To investigate the effect of antibiotics on the causal agent of soft rot disease, three new, inexpensive, and commercially available antibiotics called Dihydrostreptomycin, Oxytetracycline Hydrochloride, and Chloramphenicol were used. These antibiotics were prepared by the Faculty of Pharmacy, Tabriz University of Medical Sciences. Concentrations of ٥٠, ١٠٠, ٢٥٠, ٥٠٠, ١٠٠٠, ٢٥٠٠, and ٥٠٠٠ mg/L were prepared for each of these antibiotics. For comparison, copper oxychloride, a common copper-based bactericide for controlling most bacterial diseases, was prepared by the Tabriz Department of Agriculture at concentrations of ٥٠, ١٠٠, ٢٥٠, ٥٠٠, ١٠٠٠, ٢٥٠٠, and ٥٠٠٠ mg/L and tested similar to that of antibiotics.

٢,٤. Examining the antibacterial effect by agar diffusion method

A suspension at the concentration of $١٠^{٧}$-$١٠^{٩}$ cells/ml was prepared from ٢٤-h bacterial culture (٣٠˚C). About ٢٥٠ µl of the prepared bacterial suspension was spread on a Petri dish containing Nutrient Agar medium. After complete absorption of the suspension to the solid medium in the Petri dishes, four wells each ٦ mm in diameter were made in the medium and about ٧٥ µl aliquot from each essential oil, antibiotics, or copper oxychloride was poured into each well. For each concentration, four replicative wells were devoted and sterile distilled water was used as the control. All the inoculated agars were kept in an incubator at ٢٥ °C. After ٧٢ h, the agars were examined for inhibitory halos around the wells (Bauer et al., ١٩٦٦). The inhibition percentage (IP) was calculated by using the formula IP (%) = [(R١ – R٢)/R١] × ١٠٠ wherein R١ is the radial diameter of the control halo and R٢ is the radial diameter of the treatment halo. The experiments were repeated three times.

Evaluating the antibacterial effect of essential oils and neem extracts with disc diffusion

Whatman filter paper was used to make the raw paper discs. The discs were prepared at the diameter of ٥ mm and sterilized using an autoclave. An hour before the cultivation, paper discs were impregnated with the antibacterial solutions or essential oils as defined above. Sterile distilled water was used to prepare control discs. The modified Bauer et al.'s method was used to determine the antibacterial activity (Bauer et al., ١٩٦٦). The experiment was performed as a completely randomized design. About ٠,٢٥ mL of ٢٤-h bacterial suspension (at concentration of $١٠^{٧}$-$١٠^{٩}$ cells/ml) was spread on the solid nutrient agar. Shortly after the surface of the Petri dish dried, ٤ discs impregnated with solutions and a control disc related to each dilution were placed over the medium. Each dilution was tested in three replications. After the incubation period, the diameter of growth inhibitory halos around the discs was measured and the average inhibitory halo was recorded for each Petri dish. The inhibition percentage (IP) was calculated using the same formula as used in the agar diffusion method. The data obtained from three replications were analyzed as completely randomized design experiments by SPSS software.

٣. Results & Discussion

٣,١. Antibacterial effect of essential oils and plant extracts

In the extraction process of the essential oils, the greatest quantity of essential oil was obtained from thyme, followed by the mint. In the case of the neem plants, since insufficient pure essential oil was obtained after ٤ h of oil extraction, only aqueous and alcoholic extracts were examined as antibacterial agents instead of essential oil. By comparing the two methods, agar- and disc- diffusion in all treatments (antibiotics and herbal extractions), the agar diffusion method gave better results than the disc diffusion method with a higher percentage of inhibition. According to the results from the agar diffusion method (Fig. ١) (Table ١), both mint and thyme essential oils had significant antibacterial effects on Pectobacterium carotovorum. Accordingly, thyme essential oil with an inhibitory halo diameter of ٢٧ mm and inhibition percentage of ٣٠٪ showed a higher antibacterial effect than the mint essential oil which appeared to have a growth inhibition diameter of ١٥ mm and ١٦,٦٪ as the inhibition percentage. However, the aqueous and alcoholic extracts of neem did not affect inhibiting the bacterial growth. In the method involving paper discs impregnated with the potential antibacterial agents, only thyme essential oil showed growth inhibition with an inhibitory halo of ٢٣ mm, but there was no significant inhibition by the use of mint essential oil, and aqueous and alcoholic extract of neem (Fig. ٢).

Table ١: Statical analysis of the inhibitory Inhibition Percentage (IP) of thyme, mint, and neem plant extracts on Pectobacterium carotovorum subsp. carotovorum

Variability Sources	P-Value	Average of square	Degree of freedom	Sum of squares
Treatment	≤ ٠,٠١	١١٨,٨٥٧*	٣	٣٥٦,٥٧١
error		.٤٢٢	٨	٣,٣٧٥
Sum			١١	٣٥٩,٩٤٦

Figure ١. The effect of neem extract (right), essential oil of mint (middle), and thyme (left) on *Pectobacterium carotovorum subsp. carotovorum*

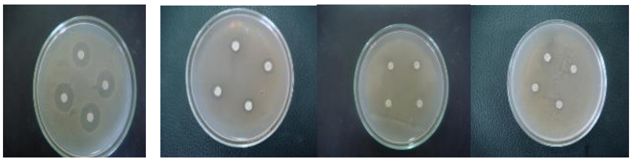

Figure ٢. Inhibitory halo of paper disc impregnated with thyme, mint and neem extracts, and copperoxychloride on *Pectobacterium carotovorum* subsp. *carotovorum* (left to right)

٣,٢. *Effect of antibiotics and copper pesticide*

The results at the probability level of ١٪ error showed that oxytetracycline hydrochloride with the highest inhibition level was significantly different from the other two antibiotics (Fig. ٣). Comparison of the effects of the three antibiotics on the bacterial population showed that oxytetracycline hydrochloride at the concentration of ٥,٠٠٠ mg/l had the inhibitory halo diameter of ٤٣ mm and inhibition percentage of ٤٧,٧٧٪. This antibiotic showed inhibitory effect in all the replications and at all concentrations, so even at the lowest concentration of ٥٠ mg/l, the inhibition rate was ٢٤,٤٤٪ (Fig. ٣&٤). The other antibiotic, dihydrostreptomycin at the concentration of ٥٠ mg/l showed no inhibitory effect on the growth of the disease-inducing bacteria, but slight inhibition was observed at higher concentrations. Chloramphenicol, like oxytetracycline, showed inhibitory effect at all the concentrations, but less inhibition was recorded in comparison to the latter. This antibiotic had the inhibition rate of ٢٨٪ and inhibitory halo diameter of ٢٦ mm, even at the highest concentration (٥٠٠٠ mg/l), which was ٦٠٪ less than that of oxytetracycline hydrochloride. According to the results of this study, there was a significant difference between oxytetracycline hydrochloride and the two other antibiotics that were observed in all the replications, suggesting a lack of bacterial resistance to this antibiotic. In this research, examining the effect of copper oxychloride showed that it was not highly effective in controlling the causal agent of soft rot disease in vitro and it was less than ٥٠٪ of the effect of selected antibiotics. However, previous studies have reported copper-based pesticides to have enough efficiency to control most of the plant pathogenic bacteria; Even at the highest dose (٥٠٠٠ mg/l), the bacterial inhibitory effect of this compound was ١٤,٤٤٪, which was one-third that of oxytetracycline hydrochloride and half of thyme essential oil.

Figure ٣. Effect of ٥٠٠٠ mg/l concentration of oxytetracycline (left), dihydrostreptomycin(middle), and chloramphenicol (right) on Pectobacterium carotovorum subsp. carotovorum growth

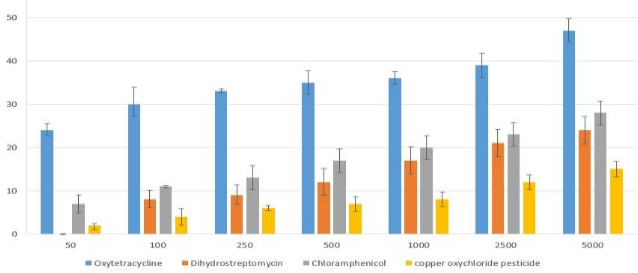

Figure ٤. Comparison of the inhibition percentage of oxytetracycline, dihydrostreptomycin, and chloramphenicol, plus copper oxychloride pesticide in different concentrations (ppm) on Pectobacterium carotovorum subsp. carotovorum

٤. Discussion

In this research, the effect of essential oils and extracts from three plants, i.e., thyme, mint and neem as well as the effect of three inexpensive and available antibiotics along with a common pesticide on soft rot-inducing bacteria of carrots and potatoes were tested. Numerous studies have shown the antibacterial effect of herbs such as thyme, mint and neem. Mahmoudi et al. (٢٠٠٨) examined the antibacterial effect of ١٤ essential oils and ٤ aqueous extracts on canker-inducing bacteria and bacterial leaf spot of nutrient fruits (*Pseudomonas syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *proni*). The results showed that in Pseudomonas bacteria, thyme and ajwain essential oils had the highest inhibitory effect whereas common sage and asafetida essential oils did not affect the bacterial inhibition. These results are similar to the results of the present study and show that the highest percentage of inhibition belongs to thyme essential oil. In another similar study, Kokoskova and Paula (٢٠٠٦) examined the effect of several plants essential oils on *Erwinia chrysanthemum* and *Pseudomonas marginalis* and reported that thyme essential oil had the highest percentage inhibition on the growth of these bacteria. Earlier studies have shown that essential oils extracted from the aerial parts of thyme contain compounds such as thymol and carvacrol (Pauli and Knoblauch, ١٩٨٧). Thymol is a non-polar compound with high solubility in organic solvents with the general formula $C_{١٠}H_{١٤}$. (Galambosi et al., ٢٠٠٢). Carvacrol which is a phenolic compound with the general formula $C_{٦}H_{٣}CH_{٣}(OH)(C_{٣}H_{٧})$ and the odour similar to that of oregano causes damage to the outer membrane of Gram-negative bacteria, increases the permeability of the cell membrane, and causes ATP to leave the bacterial cell (Galambosi et al., ٢٠٠٢). Accordingly, carvacrol has an inhibitory effect on ATPase pump activity. In addition, carvacrol is an antibacterial agent with a synergistic effect with thyme oil (Bart et al., ٢٠٠٧) and its function depends on its concentration and time of contact with microorganisms. Carvacrol reacts with cell membranes by altering the permeability of H+/K+ canals. According to the results of current research, thyme essential oil is widely suggested as a natural substance to control plant diseases, especially bacterial diseases, due to the ease of preparation and the possibility of growing thyme in many parts of Iran. In this research, the antibacterial effect of thyme was higher than that of mint while Mohasaneh et al. (١٩٩٦) who worked on the antibacterial properties of several plant species, *Erwinia amylovora*, *Pseudomonas savastanoi*, *Pseudomonas syringae* pv *syringae*, and *Xanthomonas vesicatoria* showed that essential oils from mint species caused higher inhibitory effect than that from thyme. This disagreement could be because various bacteria have different susceptibility against a given essential oil and accordingly the susceptibility of soft rot bacteria to thyme essential oil is probably higher than mint oil. Moreover, the amount and even type of metabolites in these plants may be somehow different under distinct geographical and climatic conditions (Yazdani et al., ٢٠٠٢). Therefore, plant essential oils extracted in various areas of their growth habitat may have different results and could be the reason for this difference. In the case of neem extract, its antimicrobial effect had already been reported on many plant pathogens; thus, it was expected to be effective against the soft rot bacterium. However, no significant effect was observed in any of the treatments. Likewise, our results, in earlier studies the effect of neem on pectolytic bacteria has been reported to be small (Sarawaneeyaruk et al.,٢٠١٥). They showed that Pectobacterium bacteria are resistant to the effect of neem tree extract and are less affected by this extract than other terrestrial bacteria. Rashid et al. (٢٠١٢) also showed that neem extracts slightly affected the *Pectobacterium atrocepticum*. So, it seems that the soft rot bacterium and the Pectobacterium react similarly against neem extract. In the case of using antibiotics to control plant diseases, only a handful of antibiotics such as streptomycin have received further attention. Chiu and Jones (١٩٩٣) and Norley et al. (٢٠٠٣) have demonstrated that the use of streptomycin is severely restricted owing to the emergence of resistant bacterial strains and the risk of horizontal transmission of antibiotic-resistant genes to animal and other plant pathogens bacteria; thus, looking for new antibiotics or new forms of existing antibiotics is necessary.

In this research, the effect of three antibiotics, i.e., Dihydrostreptomycin, Oxytetracycline Hydrochloride, and Chloramphenicol, was tested on a soft rot-inducing bacterium. Dihydrostreptomycin is one of the most common forms of streptomycin antibiotic, which is widely used in medicine and veterinary medicine with very low toxicity to humans and pets (LD٥٠=>٩٠٠٠mg/kg/day). The effect of this antibiotic on some plant bacterial diseases has been previously shown, but its effect on Enterobacteriaceae has been reported to be less. In this research, this antibiotic indicated a very little effect on soft rot bacterium which belongs to the Enterobacteriaceae family; thus, these results demonstrate that this antibiotic is not appropriate for widespread and commercial use to control the soft rot-inducing bacteria. Contrary to streptomycin, oxytetracycline hydrochloride showed very good inhibitory effects. After the streptomycin group, this antibiotic is the most widely used antibiotic in agriculture to control plant diseases (Al-Rimawi et al., ٢٠١٩).

Conclusions

The results of the present study showed that the antibiotics examined in this research could be effective even at much lower concentrations if used in more suitable formulations for farm or storage conditions. Oxytetracycline hydrochloride can be used to control the disease even better than common copper-based pesticides such as copper oxychloride; though, the tetracycline group is not used in agriculture for the first time and has previously been reported to control phytoplasma and some bacterial diseases. According to the results of the current study, as well as the low price and availability of this antibiotic, tetracycline can be recommended as an alternative for copper-based pesticides and other antibacterial agents in agriculture.

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