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ANTIBACTERIAL ACTIVITY OF SOME ESSENTIAL OILS ON BACTERIAL SPOT DISEASE OF TOMATO PLANT CAUSED BY *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA*

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A B S T R A C T

Bacterial spot disease caused by *Xanthomonas axonopodis* pv. *vesicatoria* is considered one of the major diseases of tomato crop worldwide. The objective of this paper was to study the effect of certain essential oils (EOs), lemongrass, oleum and thyme, on *X. axonopodis* pv. *vesicatoria* (PHYX14) for controlling bacterial spot disease in tomato plants. The tested three essential oils (EOs) showed antibacterial activity *in vitro* test at 1:10 concentration against the PHYX14.Thyme oil exhibited the highest inhibition against PHYX14 followed by lemongrass and finally oleum. Under greenhouse conditions, the effect of EOs on the bacterial spot of tomato was evaluated on tomato seedlings. Thyme oil exhibited the highest reducing of tomato bacterial spot followed oleum and then lemongrass. Results indicated that the application of the tested (EOs) to tomato plants two days after the infection caused the highest reduction of disease severity. While the application of oleum oil exhibited the highest induction of the oxidative enzymes, peroxidase (PO) and polyphenol enzyme (PPO). Also increased total phenolic contents of tomato leaves followed lemongrass and then thyme oil as compared by control. The application of EOs two days before the infection caused the highest induction of PO, PPO enzymes and total phenolic contents in tomato leaves than two days after the inoculation.

Keywords: Bacterial spot, essential oils, enzyme oxidase, fresh and dry weight, tomato.

INTRODUCTION

Bacterial diseases of tomato plants (*Solanum lycopersicum* L.) are responsible for significant yield losses. Bacterial spot disease caused by a number of species of the genus *Xanthomonas* (Dowson) Jones *et al.* (2000). Such disease is among the most important bacterial disease among other diseases. Bacterial diseases caused by the pathogens of genus *Xanthomonas* have distressed different plants, leading to considerable losses in yield and quality of the harvests (Ji *et al.*, 2008). Quick and easily applicable management of plant diseases and microbial contamination is the use of chemical compounds (Burr, 2001). At present, only a small assortment of chemical compounds is available for the farmers. The compounds on the market are almost absolutely based on copper or different antibiotic preparations (Janse, 2005). Because of the general toxicity and the development of resistant strains, spraying with copper-based compounds exert a negative impact on the environment and on the yield. In many countries, antibiotics are prohibited due to their high and acute toxicity, food chain accumulation and lengthy periods of degradation. Members of the genus *Xanthomonas* are reported to have developed resistance to several antibiotics such as streptomycin, ampicillin, kanamycin and penicillin (Rodríguez *et al.*, 1997). As an alternative strategy for plant protection and the spread of the diseases of different plant compounds have been tested for their antimicrobial activity. In the past few years, studies have been conducted providing evidence that some aromatic plants might be a potential source of natural pesticides (Tripathi and Kumar, 2007; Patharakorn *et al.*, 2010).

Essential oils, as odorous and volatile products of the secondary metabolism of aromatic plants, normally formed in special cells or groups of cells could be used as

antimicrobial agents in the control of the disease caused by *Xanthomonas* species (Nguefack *et al.*, 2005; Bajpai *et al.*, 2011). Due to their more systematic and easily biodegradable nature, essential oils were proved better substitutes for synthetic antibiotics (Claflin, 2003). The antimicrobial activity of the essential oils is assigned to small terpenoids and phenolic compounds that exhibit antimicrobial activity when tested separately (Horváth *et al.*, 2004). This growing interest in finding bio-pesticides in order to control the diseases is emphasized by the fact that the diseases caused by the plant pathogenic bacteria of *Xanthomonas* species are still a major problem even in the developed countries (McManus *et al.*, 2002). Recently, a wide range of essential oils (EOs) have been extensively studied for their activity against many phytopathogenic bacteria (Gormez *et al.*, 2015; Gakuubi *et al.*, 2016; Todorović *et al.*, 2016; Popović *et al.*, 2017), usually using a direct-contact antimicrobial assay (Bajpai *et al.*, 2011).

Polyphenol oxidases (PPOs) are a group of Cu-containing enzymes that catalyse the oxidation of several phenols to o-quinones (Oliveira *et al.*, 2011; van Gelder *et al.*, 1997). In turn, o-quinones are highly reactive molecules that can undergo non-enzymatic secondary reactions to form brown complex polymers known as melanins and cross-linked polymers with protein functional groups (Golan-Goldhirsh *et al.*, 1984; Wong, 1989; Rolff *et al.*, 2011; Friedman, 1996).

The aim of this study was to examine the antibacterial activity of three essential oils and several of their components against the causative agents of bacterial spot of tomato *Xanthomonas axonopodis* pv. *vesicatoria in vitro* and under greenhouse conditions. The fresh and dry weight were determined, and dry weight was recorded. As well as the plant pathways triggered in response to peroxidase (PO), polyphenol oxidase (PPO) enzymes as well as total phenolic compounds contents were investigated.

MATERIALS AND METHODS

Bacterial culture and inoculum production: *X. axonopodis* pv. *vesicatoria* isolate (PHYX14) was isolated and identified according to their morphological, cultural and physiological characteristics as recommended by Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology 9th edition (Holt *et al.*, 1994) it was isolated by Abo-Elyousr and El-Hendawy (2008). Their pathogenicity was proved on tomato leaves. The bacterium was streaked onto nutrient sucrose agar (NSA) medium and grown at 28 ºC for 24 h. The inoculum of the pathogen was prepared by soaking the bacterial colonies in terms of mL of distilled water, scraping it, and the concentration was adjusting to A620 = ($5x10^8$ CFU mL^{-1}). Plants tomato at the age of 4 weeks were inoculated by spraying it with the bacterial suspension until runoff.

Effect of essential oils *in vitro:* Three essential oils namely lemongrass, oleum and thyme were screened for their antibacterial activity against *X. axonopodis* pv. *vesicatoria* PHYX14. They were obtained from local markets in Assiut governorate. The activity of the essential oils was tested using the well diffusion plate method against PHYX14 (Kalemba and Kunicka, 2003). Different dilutions of essential oils were tested for their activity to inhibit the growth of the pathogen PHYX14 *in vitro*. A single colony of the isolate was selected from NA medium streaked with PHYX14 and grown in 250 ml Erlenmeyer flasks containing 100 ml of nutrient agar (NA) and incubated at 27±2°C for 48 h on a rotary shaker at 150 rpm. Bacterial cell suspension was centrifuged (8 min. at 10,000 g), the cells resuspended in tap water and cell density adjusted to be $5x10^8$ CFU ml^{-1} using a spectrophotometer at a wavelength of 620 nm (McGuire and Kelman, 1984). Then bacterial inoculum was prepared by pouring 200 µL of the bacterial suspension ($5x10^8$ CFU mL^{-1}) on NA plates and spreading uniformly. The applied inoculum was spread on the surface of the medium by sterile glass spreader. One well of 10 mm diameter was made in the middle of the plate carried out using sterilized cork-borer. One ml of each essential oil was dissolved in 100 of distilled water and one µl of Tween 80 at (1:10 concentration) and sterilized by filtration through a 0.20 µm Seitz filter. 100 µl of each oil was individually pipetted into the well. The incubation of these dishes was done at 28°C for 48 h. Three Petri dishes for each tested oil were maintained as replicates. Petri dishes containing wells supplemented with sterile Tween 80 were deemed as a control. The inhibition zones (mm) were measured from the edge of the zone to the edge of the well.

Plant material: Tomato cultivar Super Marmande was used for all of the following experiments. Plants were grown in 30 cm pots in a soil mix containing sand-clay soil (5 kg/pots) and plants watered and fertilized with 1% NPK (12:4:6) when necessary.

Effect of essential oils under greenhouse condition: Mello disease assessment key was used for evaluations of severity (Lobo *et al.*, 2005), and the efficiency of tested essential oils was conducted in the greenhouse to study their effect in reducing the severity bacterial spot disease of tomato plants. The experiment was conducted in a

randomized complete block design, with four replicates and three plants per pot. Thirty ml of each essential oil was kept in 100 ml conical flask (1.5 ml essential oil + 30 ml of distilled water + 1 µl of Tween 80) and keep at shaker for 24 h for continuous agitation at 150 rev/min for thorough mixing and complete elucidation of active materials to dissolve in the respective solvent. The plants were divided into two groups. The first group was inoculated with the pathogen PHYX14 (5×10^8 CFU mL^{-1}), two days after application with essential oils (30ml of each essential oil). Plants in the second group were inoculated with spraying pathogen PHYX14 two days before application of essential oils and control plants sprayed with water). Disease severity was recorded after four weeks from application. The experiment was repeated twice.

Determination of fresh and dry weight: Plants were removed from different treatments, washed with distilled water, blotted with tissue paper, and determined the fresh weight. Plants were then dried for 72 h at 60°C and recorded dry weight. Four replicates were used and the experiments were repeated twice.

Preparation of samples for determining enzyme activities: Leaf tissue samples (1 g fresh weight) were harvested after 15 days from the application of essential oils and immersed in liquid nitrogen for enzyme extraction. The frozen leaf segments were homogenized in a mortar using 50 mM potassium phosphate buffer (pH 7·0) containing 1 mNaCl, 1% polyvinylpyrrolidone, 1 mM EDTA and 10 mM B-mercaptoethanol. The homogenates were centrifuged at 17 000 g for 20 min at 4°C and, finally, the supernatant (crude enzyme extract) was collected and divided into 1.5 ml portions. Protein concentrations were determined using bovine serum albumin (BSA) as a standard according to Bradford (1976) using Bradford reagent the extract was then used to determine the activities of PO and PPO. Estimation of peroxidase (PO) was determined according to Urbanek *et al.* (1991) and the methods of Gauillard *et al.* (1993) for polyphenol oxidase (PPO) activity.

Extraction of total phenolic compounds: For determining the concentration of total phenolic compounds, leaf extracts were prepared according to the method of Malamy *et al.* (1992) with some modifications and the methods of Malik and Singh (1980) was used for determination of total phenolic contents.

Statistical analysis: The data were evaluated by analysis of variance, using MSTAT-C. Differences between treatment means were determined using the least significant difference (LSD) test ($P < 0.05$) (Freeman *et al.*, 1985).

RESULTS AND DISCUSSION

Effect of certain essential oil treatments on growth of pathogen PHYX14 *in vitro:* The effect of three essential oils lemongrass, oleum and thyme on the growth of *X. axonopodis* pv. *vesicatoria* (PHYX14) was tested *in vitro*. Results in Figure 1 indicated that thyme oil exhibited the highest inhibition zone against the PHYX14 producing 3 cm inhibition zone followed by lemongrass oil in the second rank resulting 2 cm inhibition zone and then oleum oil showed low fungicidal effects causing inhibition zones less than 1.8 cm. White *et al.* (2002) reported that many plant pathogenic bacteria have acquired resistance to synthetic pesticides. For instance, pathovars of *Xanthomonas axonopodis* have developed resistance to some antibiotics such as kanamycin, ampicillin, penicillin and streptomycin (Rodríguez *et al.*, 1997). Also, Huang and Lakshman (2010) mentioned that clove oil has antibacterial activity against many phytopathogenic bacteria e.g. *Rhodococcus fascians*, *Erwinia carotovora* pv. *carotovora*, *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *syringae*, *X. campestris* pv. *pelargonii*, and *Streptomyces* spp. Both Gram-negative and Gram-positive bacteria were sensitive to clove essential oil at a concentration (0.1 and 0.5%). Vancheva *et al.* (2015) reported that various essential oils have the potential for use in alternative strategies for plant pathogen control. Also, Abdel-Rahim and Abo-Elyousr (2017) mentioned that thyme oil showed a great inhibitory impact on *B. cinerea* at MIC value 0.2 µL/mL and it reduced the disease by 78.6.

Effect of certain oil treatments on diseases severity of *X. axonopodis* pv. *vesicatoria* (PHYX14) under greenhouse conditions: The effect of certain oil treatments on diseases severity of *X. axonopodis* pv. *vesicatoria* (PHYX14) under greenhouse conditions was shown in Table 1. Data also revealed that thyme oil exhibited the highest disease severity followed by oleum oil and then lemongrass oil. Results also showed that inoculation plants two days after the inoculation resulted in the highest reduction of disease index than inoculation of two days before the inoculation. Also, Lucas *et al.* (2012) reported that 0.1% of EOs have reduced the severity of tomato bacterial spot under greenhouse conditions. da Silva *et al.* (2019) demonstrated that *Lippia gracilis* EOs have antimicrobial activity and have a potential to be used in the control of black rot caused by *X. campestris* pv. *campestris*.

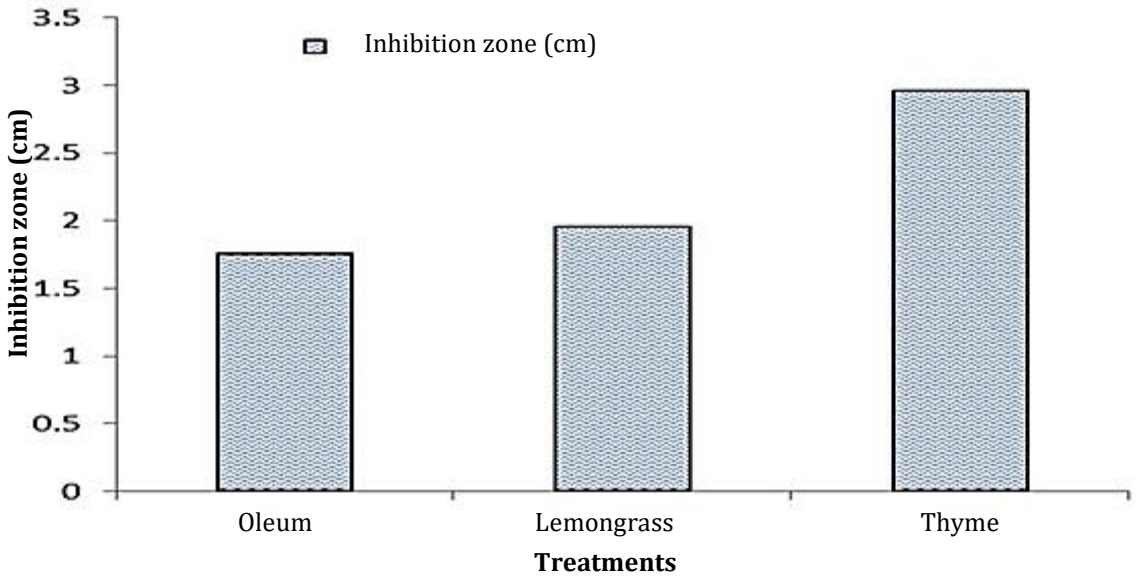

Figure 1. Effect of certain essential oils on the growth of pathogen PHYX14. Means of standard deviation for eight plants per treatment are shown.

Table 1. Effect of certain oil treatments on diseases severity of *X. axonopodis* pv. *vesicatoria* (PHYX14) under greenhouse conditions.

Treatment	Two days before	Two days after	Mean
Thyme oil	6 d	6 d	6 d
Lemongrass oil	24 b	24 b	24 b
Oleum oil	18 c	12 c	15 c
Infected control	78 a	70 a	74 a
Healthy control	0 e	0 e	0 e
Mean	31.5	28	

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05.

Determination of fresh and dry weight before and after treatments: Results in Tables 2 indicated that lemongrass oil exhibited the highest fresh and dry weight of tomato followed by thyme oil as a comparison with healthy control. Oleum oil exhibited the lowest fresh and dry weight of tomato as a comparison with infected control. Abreu (2006) mentioned that treated tomato plant with the essential oil, of cinnamon, caused reduction in the incidence of tomato black spot and the same trend was observed under the field conditions. The reductions were 12.5 and 32.8%, at the concentrations of 3,000and 5,000 µL L-1 (0.3 and 0.5%). Under greenhouse conditions, the EOs of citronella, tea tree, clove and lemongrass reduced disease severity and caused damage to the bacterial cell wall.

Results in Table 3 showed the effect of certain oil treatments on fresh and dry weights of tomato plants under greenhouse conditions two days before application with essential oils of lemongrass, oleum and thyme. Tomato harvested after 3 months of planting and the fresh weight was determined. Thyme oil exhibited the highest fresh and dry weights of tomato followed by lemongrass oil as compared with healthy control. Oleum oil exhibited the lowest fresh and dry weights of tomato as compared with infected control. Our results are agreed with those reported by Pereira *et al.* (2012). They mentioned that the essential oils, eucalyptus, citronella and tea tree promoted to control of the disease similar to that of the fungicide. Also, Pereira *et al.* (2012) reported that protection was 54.3% when using clove oil (0.1%) against coffee plant rust.

Effect of certain oil treatments of PO, PPO and phenol activities in tomato leaves: To explore the potential of

lemongrass, oleum and thyme oils in inducing plant defense system when applied in a more preserved way, peroxidase (PO), polyphenol oxidase (PPO) activities and total phenolic compounds contents were investigated in tomato leaves. Data in Table 3 (Figure 2) revealed that the activity of peroxidase was decreased in leaves of tomato with oleum, lemongrass and then thyme oils. Data also revealed that oleum oil exhibited the highest PO activity in tomato leaves followed lemongrass oil and then thyme oil as compared with healthy control, whereas two days before the infection resulted in the highest induction of PO activity in tomato leaves than two days after the infection.

Table 2. Effect of certain oil treatments on fresh and dry weights of tomato plants under greenhouse conditions after two days from treatment.

Treatment	Fresh weight (g/plant)	Dry weight (g/plant)	Mean
Thyme oil	25.3 b	8.4 d	16.96 b
Lemongrass oil	25.93 b	9.2 d	17.56 b
Oleum oil	8.8 d	6.83 d	7.8 d
Infected control	17.25 c	6.95 d	12.1 c
Healthy control	39.2 a	8.9 d	24.1 a
Mean	23.33 a	8.1 b	

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05.

Table 3. Effect of certain oil treatments on fresh and dry weights of tomato plants under greenhouse conditions before two days from treatment.

Treatment	Fresh weight (g/plant)	Dry weight (g/plant)	Mean
Thyme oil	25.93 b	9.2 d	17.56 b
Lemongrass oil	25.53 b	8.4 d	16.96 b
Oleum oil	8.78 d	2.68 d	7.8 d
Control infected	17.25 c	6.95 d	12.10 c
Control healthy	39.15 a	8.9 d	24.03 a
Mean	23.33 a	8.01	

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05.

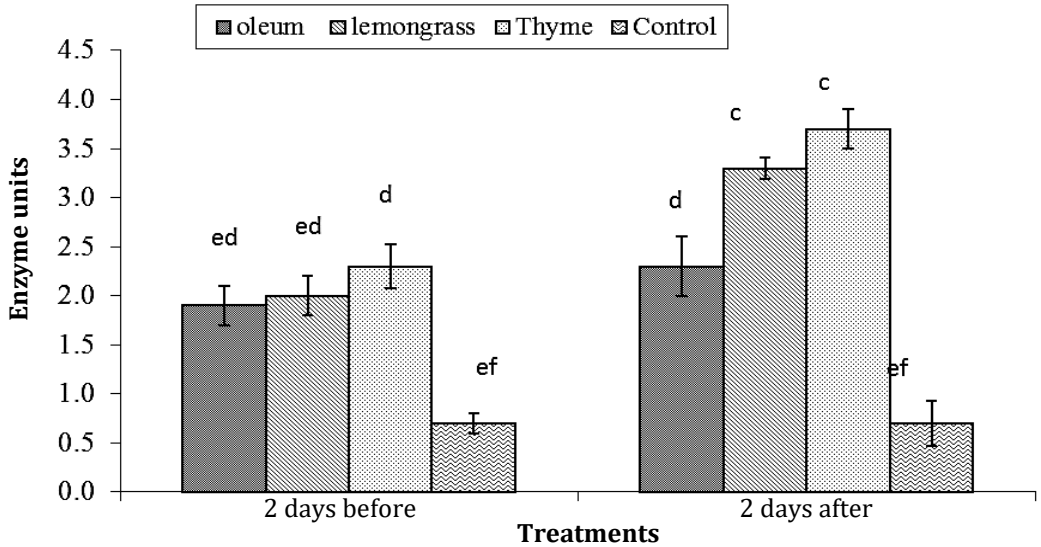

Figure 2. Effect of certain oil treatments on the induction of PO activity in tomato leaves. Means of standard deviation for eight plants per treatment are shown.

Data declared that the activity of polyphenol oxidase (PPO) compounds was decreased in leaves of tomato plants treated with oleum, lemongrass and then thyme oils (Figure 3). Data also revealed that oleum oil exhibited the highest induction of PPO activity in tomato leaves followed lemongrass oil and then thyme oil as compared with healthy control, whereas two days before the infection resulted in the highest induction of PO activity in tomato leaves than two days after the infection. Data showed that the level of total phenolic compounds (Figure 4) was decreased in leaves of tomato plants treated with oleum, lemongrass and then thyme oils. Data also revealed that oleum oil exhibited the highest induction of total phenolic compounds contents in tomato leaves followed lemongrass oil and then thyme oil as compared with control healthy, whereas two days before the infection resulted in the highest induction of PO activity in tomato leaves than two days after the infection.

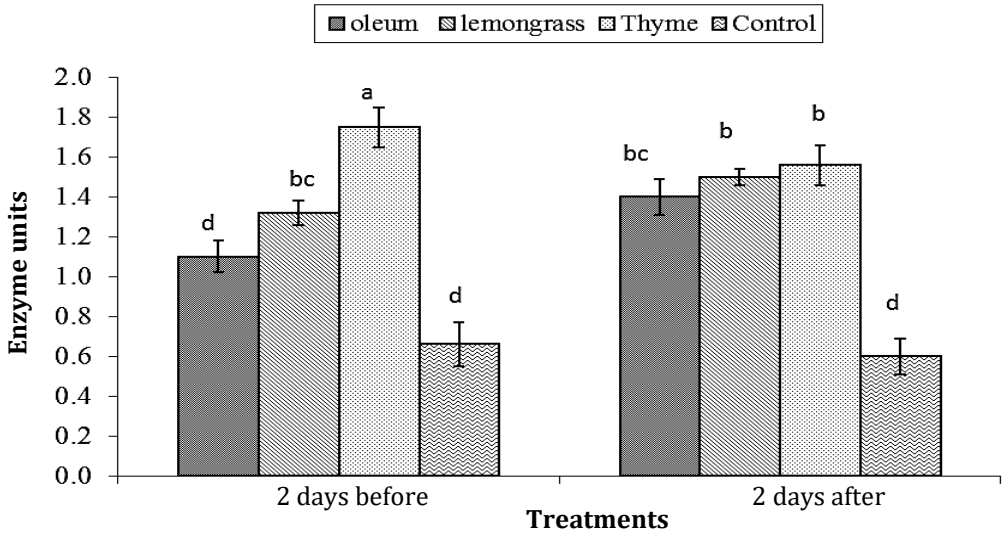

Figure 3. Effect of certain oil treatments on the induction of PPO activity in tomato leaves. Means of standard deviation for eight plants per treatment are shown.

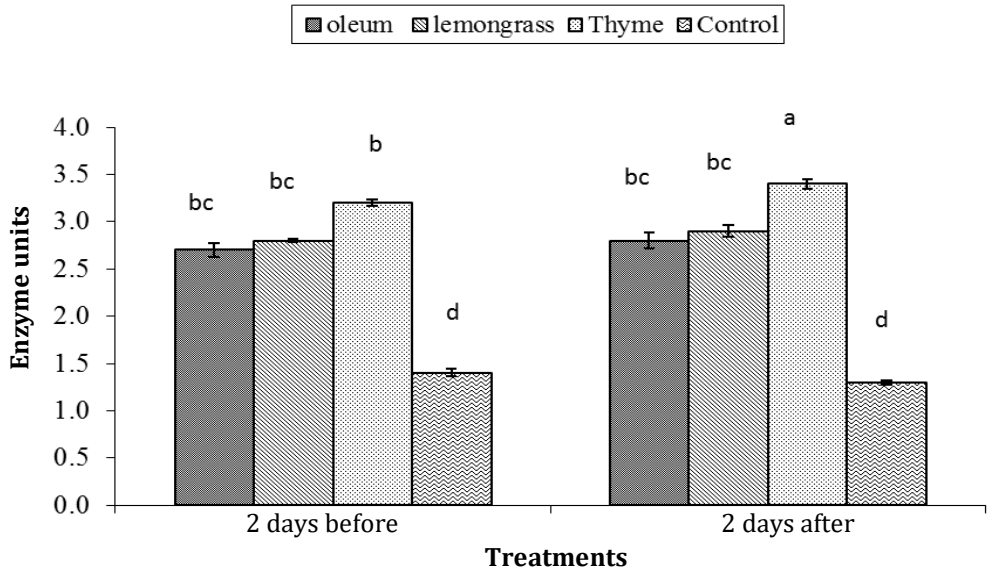

Figure 4. Effect of certain oil treatments on the induction of phenol content in tomato leaves. Means of standard deviation for eight plants per treatment are shown.

To explore the potential of lemongrass, oleum and thyme oils in inducing plant defense system when applied in a more preserved way, peroxidase (PO), polyphenol oxidase (PPO) activities and total phenolic compounds contents were investigated in tomato leaves. Data revealed that the level of peroxidase and polyphenol oxidase activities and total phenolic compounds contents were decreased in leaves of tomato treated with oleum, lemongrass and then thyme oils. Data also revealed that oleum oil exhibited the highest induction of PO, PPO activities and total phenolic compounds contents in tomato leaves followed lemongrass oil and then thyme oil as compared with healthy control. Two days before the infection resulted in the highest induction of PO activity in tomato leaves than two days after the infection. EL-Fiki and El-Habbak (2016) using the tested bacterio toxicants in program form increased the total phenol content, activities of oxidative enzymes (peroxidase, polyphenoloxidase and chitinase) in tomato leaves comparing to treated-copper control (prog-4) and untreated control treatment. Successful disease management and control practices greatly are determined by a comprehension of the ecology of the pathogenic organism in the environmental surroundings (Pradhanang *et al.*, 2000). Also, SAR mechanisms might be as a result of induces plant resistance (de Meyer *et al.*, 1998).

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