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Preliminary Studies on Antibacterial and Antiviral Activities of Five Medicinal Plants

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Abstract

It is of interest to inhibit the pathogenic bacteria and virus by medicinal plants. This method is safe and cheap. The antimicrobial activities of aqueous infusion and decoction of five medicinal plants: *Nigella sativa* (NS), *Zingiber officinale, Thymus vulgaris, Syzygium aromaticum, Mentha piperita* were investigated. The screening of antibacterial activity was against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Listeria monocytogenes, Lactococcus lactis* and *Bacillus cereus*. The aqueous infusion of ginger exhibited maximum activity against *E. coli* with 15.00 mm inhibition zone (IZ). Also, the infusion of black seeds showed highest antibacterial activity against *Lactococcus lactis, P. aeruginosa, L. monocytogenes,* and *Staphylococcus aureus* with 22.30, 9.60, 9.50 and 9.00 mm IZ, respectively. The decoction of peppermint exhibited significant inhibition against *E. coli* and *Lactococcus lactis* (20 and 19.5 mm, respectively), while decoction of black seeds showed maximum inhibition against *P. aeruginosa* and *B. cereus* with 9.50 and 9.3 mm. The decoction of clove inhibited *L. monocytogenes* and *Staphylococcus aureus* significantly compared to other plants. Further antiviral screening was done. The effect of NS extracts as inhibitors against Zucchini yellow mosaic virus (ZYMV) infectivity *in-vitro* and *in-vivo* was studied. Aqueous decoction and infusion of NS inhibit the production of ZYMV symptoms on squash plants by 85% and 80%, respectively, *in vitro* treatment. Upon using of NS infusion and decoction, the phenolic compounds, the total protein as well as peroxidase and polyphenol oxidases were increased, comparing to viral controls. The infusion and decoction of medicinal plants were effective against tested virus and bacteria.

Keywords: Medicinal plants; Pathogenic bacteria; ZYMV

Introduction

Pathogenic bacteria can invade in the body through inhalation into nose and lungs, ingestion in food or through sexual contact. General symptoms of bacterial diseases include fever, chills, headache, nausea and vomiting. Commonly occurring pathogenic bacteria are *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, *Listeria monocytogene* [1]. Commonly occurring pathogenic bacteria are *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Listeria monocytogene.*

The use of medicinal plants is very important to our health. All drugs of the past were extracted from medicinal plants [2]. The medicinal plants have been screened for their antimicrobial activities. Medicinal plants were used in traditional medicines to treat infectious diseases. It contains new bioactive secondary metabolites [3]. The phenolic compounds showed antibacterial and antiphytoviral activities [4]. The increased quantity of phenolic in Chilli may be attributed to resistance to viral infection [5]. Phenolics inhibit diseases development through inhibition of exteracellular enzymes and antioxidant activity in plant tissue [6].

This study describes the antibacterial and antiviral properties of five medicinal plants. *Nigella sativa* L., (Black seed) plant is an annual herb of the Ranunculaceae family. The seeds contain volatile and fixed oil which are used in pharmaceutical industry. The extracts of the seeds have been shown to possess antioxidant, anticancer and antimicrobial activities [7]. Broad bean mosaic virus was inhibited by the extracts of *Nigella sativa* and Zizyphus plants [8].

Syzygium aromaticum (Clove) is the aromatic dried flower buds of a tree in the family *Myrtaceae*. Cloves are antimutagenic, anti-inflammatory, antioxidant, antiulcerogenic and antiparasitic [9]. Several studies have demonstrated potent antifungal, antiviral and antibacterial effects of clove [10,11].

Thymus vulgaris (Thyme), locally known as zaatar, belonging to family *Lamiaceae*; The aromatic and medicinal properties of the genus *Thymus* have made it one of the most popular plants all over the world. Thymol oil derived from thyme has demonstrated biological properties such as antimicrobial, antioxidant and antiseptic activities [12]. It has high activity on inhibition of respiratory tract pathogenic bacteria [13]. Generally, thyme species are commonly used as herbal tea, flavoring agent and medicinal plants [14].

Zingiber officinale (Ginger), belonging to the family *Zingiberacae*, is a perennial herb with thick tuberous rhizomes. The ginerols (an essential oil) have antibacterial properties [15]. Malu et al. [16] showed that ginger extracts have medicinal properties, antibacterial activity. Ginger inhibits the growth of *Staphylococcus aureus* and *Streptococcus pyogenes* [17].

Mentha piperita (Peppermint) belonging to family *Labiate*, and is of huge importance medicinally [18]. Peppermint leaves contain about 0.5-4% volatile oil. This oil is composed of 50-78% free methanol, monoterpene, menthofurane and traces of jasmine (0.15%) to improve the quality [19]. Peppermint has antibacterial, antiviral and fungicidal activity [20].

Therefore, the objective of this study was to assess antibacterial

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activity of some medicinal plants on pathogenic bacteria, and investigate the antiviral activity of black seed against zucchini yellow mosaic virus, *in vitro* and *in vivo* on squash. Also, to evaluate the effect of the medicinal plants on morphological and physiological identity of squash mechanically infected with ZYMV.

Materials and Methods

Medicinal plants

Five medicinal plants, ginger, black seed, thyme, clove and peppermint were purchased from local market of Zagazig, Egypt.

Preparation of infusion and decoction

The aqueous infusion was prepared by taking 10 g of tested medicinal plants in 100 ml distilled water and left for 24 hours at room temperature with occasional shaking, and filtered to obtain clear infusion. The aqueous decoction was prepared by boiling 10 g of tested medicinal plants in 100 ml distilled water in a flask for 20 minutes. The flask was removed from heat and allowed to cool. The content of flask was filtered to obtain clear decoction [21].

Tested microorganisms

Bacterial species: Six bacterial species belonging to Gram-negative and Gram-positive were tested. *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* were obtained from Faculty of Medicine, Zagazig University, while *Listeria monocytogenes, Lactococcus lactis* and *Bacillus cereus* were obtained from Faculty of Science, Zagazig University.

Sources of antibacterials

Infusion and decoction of ginger, black seed, thyme, clove and mentha.

Screening of antibacterial activity: Screening of antibacterial activity was performed by standard disc diffusion method [22]. Fifty sterilized discs of filter paper (6 mm diameter) were soaked in 1 ml of infusion or decoction, separately, for 2 minutes and then used for screening. The potency of each disc was 10 µl (each 50 discs of filter paper absorbed 0.5 ml). Nutrient agar was used as base medium. The inoculated plates were incubated at 37°C for 24 hours. After incubation, inhibition zone diameters of 4 discs for each treatment were measured to the nearest millimeter (mm).

Zucchini Yellow Mosaic Virus (ZYMV) isolated from naturally infected zucchini fruit: This virus was identified by Abdel-Shafi [23], by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA), as described by Clark and Adams [24]. The virus was propagated and maintained in squash plants (*Cucurbita pepo* L.), according to Faccioli and Capponi [25], 5 g of mechanically infected squash leaves were ground in sterile morter with a pestle in 10 ml of 0.01 M phosphate buffer solution of pH 7.2, then filtered by cotton piece. The volume was made up to 20 ml with phosphate buffer, then 100 µl of viral sap were mechanically inoculated into coteledonary leaves and first leaf of squash dusted by carborundum (600 mesh-Prolab). Then, wash the inoculated leaves by distilled water according to Yarwood [26]. After 21 days, the symptoms were recorded and the infected leaves were frozen and used as inocula in further experiments.

Squash seeds (Host plant): Squash seeds (*Cucurbita pepo* L.), were obtained from Agriculture Research Institute, Giza, Egypt, were cultivated in plastic pots (1000 cm^3) one cm depth below the soil surface and kept under the natural day light in greenhouse of Faculty of Science, Zagazig University.

Sources of antivirals

Infusion and decoction of *Nigella sativa* seeds.

Screening of antiviral activity

***In vitro* studies:** (2 ml of sap containing virus+2 ml of NS seeds extraction mixed in test tubes for 15 min.). Equal volumes of infusion or decoction and ZYMV inoculums were mixed together, then 100 µl of the mixture after dusting the leaves with carborundum (600 meshes, Prolab), inoculated directly into cotyledonary leaves and first leaf of *Cucurbita pepo* L., then the inoculated leaves were washed with distilled water. The numbers of plants which have symptoms were counted after 21 days and the mean of 20 plants/treatment was calculated. General control plants (healthy plants) were inoculated with buffer only. Viral control plants were inoculated by ZYMV only. The percentage of inhibition calculated according to the equation: % of viral inhibition=number of symptomatic plants in viral control–number of symptomatic plants in treatment/number of symptomatic plants in viral control×100. Plants were harvested, and then number of leaves, shoot length and fresh weight were determined.

In vivo studies

Post inoculation experiment (NS seed extraction treatment after 24 hrs from viral infection): After 24 hrs of virus inoculation, the leaves were treated with NS seeds infusion and decoction 100 ul/leaf. The developing symptoms were recorded after 21 days (20 plants for each treatment), and the percentages of inhibition were calculated. The viral control (squash plants inoculated with ZYMV only) and the general control (healthy plants treated with phosphate buffer) were done. Plants were harvested, and then number of leaves, shoot length and fresh weight were determined.

Pre-inoculation experiment (NS extraction treatment before 24 hrs from viral infection): Before 24 hrs. of virus inoculation, the leaves were treated with NS seeds infusion and decoction 100 ul/leaf. The symptoms were recorded after 21 days (20 plants/treatment), and the percentages of inhibition were calculated.

Seed treatment experiment: The squash seeds were soaked in NS extracts for 6, 12 and 24 hrs, then the seeds germinated in the pots. After germination, the cotyledonary leaves and first leaf which were dusted by carborundum inoculated with ZYMV inoculums (100 µl/leaf), then the inoculated leaves were rinsed with distilled water. The viral control is squash seeds soaked in distilled water for 6, 12 and 24 hrs, then germinated and mechanically inoculated with virus inoculum only. Plants were harvested, and then the number of leaves, shoot length and fresh weight were determined.

Methods used for antioxidant investigation

Estimation of phenolic compounds: Extraction of free and bound cell wall phenols: Free phenols were extracted from plant material of both control and treated plants according to the method of Campbell and Ellis [27]. Phenolic acids esterified to the cell wall by ester linkages were saponified, according to the method of Funk and Brodelius [28]. The mixtures were neutralized with 2 M HCl, centrifuged, and the supernatant also was used for Folin-Ciocalteu assay.

Determination of phenols by Folin-Ciocalteu assay: Phenolic content of the methanolic and NaOH extracts, described above, was determined by the method of Julkunen-Tiitto [29].

Estimation of antioxidant enzymes (polyphenol oxidase and peroxidase): Extraction: A known fresh weight of plant material was homogenized in 0.05 M cold phosphate buffer (pH 6.5) and centrifuged at 10.000 rpm for 10 min. The supernatant was completed to total known volume and used as enzyme source [30].

Assay of polyphenol oxidase activity (PPO): The optical density of the produced color was measured at 430 nm using spectrophotometer (WP 0803006), and the enzyme activity was expressed as the change in the optical density/mg protein/min.

Assay of peroxidase activity (POX): Five ml of assay mixture comprising 300 µM of phosphate buffer (pH 6.8), 50 µM catechol, 50 µM H_2O_2 and 1 ml crude enzyme were prepared. After incubation at 25°C for 5 min, the reaction was stopped by the addition of 1 ml 10% (v/v) H_2SO_4. The optical density of the produced color was measured at 430 nm using spectrophotometer (WP 0803006), and the enzyme activity was expressed as the change in the optical density/mg protein/min.

Determination of total soluble protein content: The total soluble protein content was determined according to the method of Lowry et al. [31].

Statistical analysis: Data of the experiment were statistically analyzed using the General Linear Model Program of SAS [32]. Significant differences between treatment means were tested by Duncan's Multiple Range Test [33].

Results

The current work was designed to evaluate the effect of five medicinal plants on pathogenic bacteria, and on ZYMV infectivity *in vitro* and *in vivo*.

The effect of aqueous infusion and decoction of five medicinal plants on pathogenic bacteria

Results obtained in the present study revealed that the tested five medicinal plants extracts have potential antibacterial activity against all tested bacteria, except thyme against *Staphylococcus aureus*. Table 1 presents the results of antibacterial activity of aqueous infusion of five medicinal plants.

The aqueous infusion of ginger exhibited maximum activity against *E. coli* with 15.00 mm mean diameter of inhibition zone (IZ), compared to other medicinal plants. Also, the infusion of black cumin seeds showed highest antibacterial activity against *Lactococcus lactis, Pseudomonas aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus*, with 22.30, 9.60, 9.50 and 9.00 mm, respectively. The results in Table 2 showed that decoction of peppermint exhibited significant inhibition against *E. coli* and *Lactococcus lactis* (20 and 19.5 mm, respectively), while decoction of black seeds showed maximum inhibition against *P. aeruginosa* and *B. cereus* with 9.50 and 9.3 mm compared to other medicinal plants. The decoction of clove inhibited *Listeria monocytogenes* and *Staphylococcus aureus* significantly compared to other plants. This means the most potent medicinal plants was black seeds extract; therefore, further antiviral screening was done.

Tested bacteria	Mean zone of inhibition (mm) ± SD				
	Ginger	Black cumin	Thyme	Clove	peppermint
Gram-negative:					
E. coli	15.00 ± .78^a	2.00 ± 0.14^d	8.00 ± .14^b	2.00 ± 0.12^d	5.00 ± .19^c
Pseudomonas aeruginosa	4.00 ± 0.18^c	9.60 ± 0.15^a	9.00 ± .12^a	6.60 ± 0.12^b	4.00 ± 0.15^c
Gram-postive:					
Bacillus cereus	11.60 ± 0.43^a	5.00 ± 0.14^e	8.30 ± 0.22^c	9.30 ± 0.22^b	7.00 ± .10^d
Lactococcus lactis	11.00 ± 0.22^c	22.30 ± 0.50^a	7.30 ± .15^d	7.60 ± 0.19^d	16.00 ± 28^b
Listeria monocytogens	9.00 ± 0.18^b	9.50 ± 0.22^a	9.00 ± 0.23^b	8.30 ± 0.21^c	6.30 ± .18^d
Staphylococcus aureus	5.70 ± 0.20^c	9.00 ± 0.1^a	0.00 ± 0.0^d	0.00 ± 0.0^d	6.33 ± 0.15^b

a,b,c,d means in the same row with different superscript differ significantly ($p<0.05$)

Table 1: Antibacterial activities of infusion of medicinal plants.

Tested bacteria	Mean zone of inhibition (mm) ± SD				
	Ginger	Black cumin	Thyme	Clove	peppermint
Gram-negative:					
E. coli	7.30 ± 0.11^d	5.30 ± 0.11^e	11.30 ± 0.55^b	9.30 ± 0.15^c	20.00 ± 0.26^a
Pseudomonas aeruginosa	8.00 ± 0.15^c	9.50 ± 0.41^a	7.30 ± 0.17^d	3.00 ± 0.14^e	8.70 ± 0.20^b
Gram-postive:					
Bacillus cereus	4.30 ± 0.11^e	9.30 ± 0.15^a	8.30 ± 0.22^c	5.00 ± 0.20^d	7.00 ± 0.10^b
Lactococcus lactis	13.00 ± 0.19^b	8.30 ± 0.35^c	7.30 ± .15^d	5.00 ± 0.16^e	19.50 ± 1.00^a
Listeria monocytogens	12.30 ± 0.22^c	7.00 ± 0.11^d	9.00 ± 0.23^b	14.30 ± 0.22^a	13.00 ± 0.30^b
Staphylococcus aureus	0.00 ± 0.0^b	0.00 ± 0.0^b	0.00 ± 0.0^d	4.00 ± 0.18^a	0.00 ± 0.0^b

a,b,c,d means in the same row with different superscript differ significantly ($p<0.05$)

Table 2: Antibacterial activities of decoction of medicinal plants.

In vitro and *in vivo* screening of antiviral activities of *Nigella sativa* on ZYMV

The effect of aqueous infusion and decoction of *Nigella sativa* seeds on inhibition of ZYMV symptoms is present in Figure 1. The results of *in vitro* exp. revealed that decoction and infusion of NS seeds inhibit the ZYMV symptoms on squash plants by 85% and 80%, respectively. In post experiment decoction and infusion gave 70 and 65% ZYMV inhibition, respectively (Figure 1). The observed symptoms on mechanically inoculated squash plants, including malformation, dark green blisters, thread-like leaf, mosaic, leaf roll, abnormal leaf shape and chlorosis (Figure 2). Also, the application of infusion and decoction of black seeds not only inhibit the virus symptoms, but also, increase the leaf size (Figure 3), and the growth parameter of squash plants (Table 3) in comparison with mechanically infected viral control and healthy control plants.

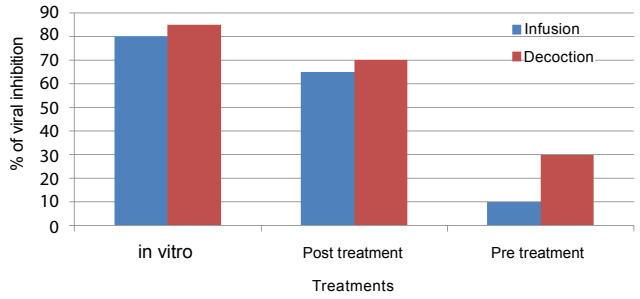

Figure 1: Antiviral activity of Infusion and Decoction of black seed extraction on the infectivity of ZYMV on squash plants *in-vitro* and *in-vivo*. % of inhibition=mean of three reading, Healthy control=untreated plants, viral control=0% inhibition (all plants are diseased).

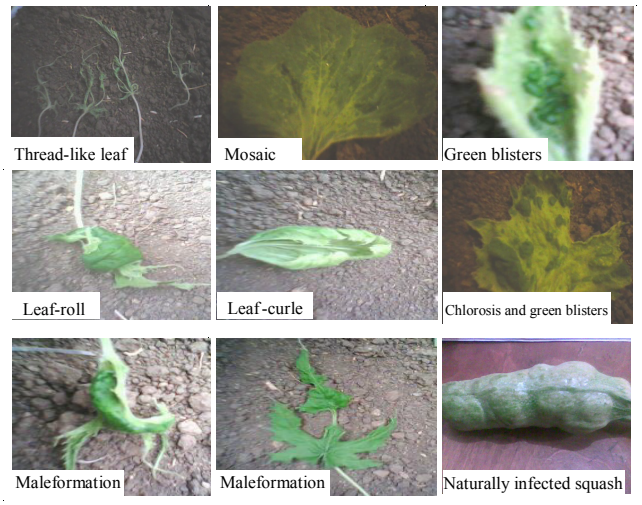

Figure 2: ZYMV Symptoms on mechanically infected squash leaves and on naturally infected squash (Unmarketable).

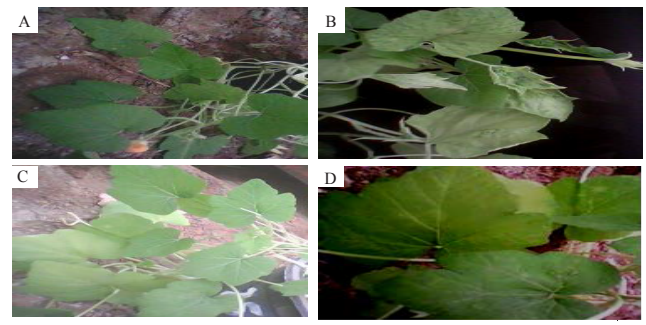

Figure 3: A-Healthy squash plants control, B-Viral control, C and D, Decoction a infusion of black seeds (*in vitro* exp.), respectively, the treatment inhibit virus symptoms and increase the plant growth.

Soaking of squash seeds in decoction and infusion of black seeds

The results in Figure 4 showed that the soaking of squash seeds in decoction exhibited higher activity against ZYMV than infusion. The decoction showed maximum percentage of viral inhibition (95%) after

Treatments	Healthy control	Viral control	Decoction of black seed	Infusion of black seed
In vitro				
No. of leaves	7.01 ± 0.74^{b}	6.20 ± 0.63^{b}	8.60 ± 1.70^{a}	8.2 ± 1.40^{a}
Shoot length (cm)	30.00 ± 3.09^{b}	29.6 ± 3.69^{b}	42.6 ± 8.37^{a}	39.6 ± 5.52^{a}
Fresh weight (g)	10.13 ± 2.51^{b}	7.92 ± 1.95^{b}	11.63 ± 2.02^{a}	12.53 ± 2.60^{a}
Pre inoculation treatment				
No. of leaves	5.5 ± 0.53 ^{bc}	5.00 ± 0.82^{c}	8.7± 0.48^{a}	6.00 ± 0.82^{b}
Shoot length (cm)	35.00 ± 4. 11^{a}	26.6 ± 3.06	35.00 ± 0.84^{a}	30.00 ± 5.23^{b}
Fresh weight (g)	10.77 ± 1.81^{a}	5.86 ± 1.71^{c}	12.00 ± 5.23^{a}	11.92 ± 1.22^{a}
Post inoculation treatment				
No. of leaves	5.5 ± 0.53^{bc}	5.00 ± 0.82^{c}	7.40 ± 0.52^{a}	5.70 ± 0.48^{b}
Shoot length (cm)	35.00 ± 4. 11^{a}	26.6 ± 3.06^{c}	31.40 ± 3.37^{b}	32.8 ± 4.24^{ab}
Fresh weight (g)	10.77 ± 1.81^{a}	5.86 ± 1.71^{d}	8.72 ± 0.58^{b}	7.20 ± 0.98^{c}

Healthy control=twenty plants normal (without any treatments) all are healthy. Viral control=squash seedling mechanically inoculated with ZYMV on cotyledonary leaves and first stage leaves.
a,b,c,d means in the same row with different superscript differ significantly ($p<0.05$)

Table 3: Effect of black seed extraction *in vitro* and *in vivo* on growth parameters of mechanically infected squash plants by ZYMV. (Each value is the mean twenty reading ± SD).

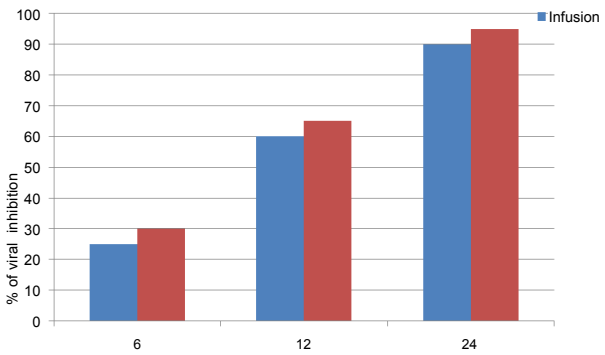

Figure 4: Effect of soaking of squash seeds in Decoction and Infusion of black seed extraction on the infectivity of ZYMV on squash plants at different time intervals.

% of inhibition=mean of three reading, Healthy control=untreated plants, viral control=0% inhibition (all plants are diseased).

Treatment \ Time	6 hrs			12 hrs			24 hrs		
	No. of leaves	Shoot length (cm)	Fresh weight (g)	No. of leaves	Shoot length (cm)	Fresh weight (g)	No. of leaves	Shoot length (cm)	Fresh weight (g)
Healthy control	6.20 ± 0.79^{c}	36.7 ± 2.79^{a}	10.4 ± 1.64^{a}	6.20 ± 0.79^{c}	36.7 ± 2.79^{a}	10.37 ± 1.64^{a}	6.20 ± 0.79^{b}	36.7 ± 2.79^{a}	10.37 ± 1.64^{a}
Viral control	4.70 ± 0.67^{d}	26.6 ± 3.06^{b}	4.89 ± 0.64^{c}	4.70 ± 0.67^{d}	26.60 ±3.06^{c}	4.89 ± 0.64^{c}	4.70 ± 0.67^{c}	26.60 ± 3.06^{c}	4.89 ± 0.64^{c}
Infusion of black seeds	7.70 ± 0.48^{b}	34.10 ± 3.73^{a}	6.76 ± 0.96^{b}	7.20 ± 1.03^{b}	31.9 ± 2.73^{b}	7.25 ± 1.18^{b}	7.1 ± 1.20^{a}	32.3 ± 4.00^{b}	8.95 ± 1.12^{b}
Decoction of black seeds	8.50 ± 0.97^{b}	33.70 ± 2.98^{a}	7.39 ± 1.02^{b}	8.00 ± 0.94^{a}	31.40 ± 2.76^{b}	7.49 ± 1.66^{b}	7.8 ± 0.92^{a}	35.20 ± 3.08^{ab}	9.66 ± 1.14^{ab}

a,b,c,d means in the same column with different superscript differ significantly ($p<0.05$)

Table 4: Effect of squash seeds soaking in black seed Infusion and Decoction extraction on growth parameters of treated plants with ZYMV at different time intervals.

Treatments	Free phenolic compounds	Cell bound phenolic compounds	Total phenols	Total protein	Polyphenol oxidase	Peroxidase
***In vitro* experiment**						
Viral control	21.67	8.21	29.88	9.60	0.0493	0.0410
Healthy control	26.25	7.23	33.48	8.80	0.0330	0.0323
Infusion	27.50	9.13	36.63	10.50	0.0500	0.0501
Decoction	31.30	9.50	40.80	11.10	0.0551	0.0600
Pre-inoculation experiment						
Viral control	19.33	11.23	30.56	9.50	0.0482	0.0400
Healthy control	24.50	8.49	32.99	8.49	0.0312	0.0301
Infusion	25.39	8.23	34.67	9.76	0.0481	0.0419
Decoction	29.78	8.97	35.99	10.88	0.0523	0.0557
Post-inoculation experiment						
Viral control	20.35	9.22	29.57	9.30	0.0490	0.0422
Healthy control	23.45	7.99	31.44	8.70	0.0340	0.0313
Infusion	26.91	8.99	35.26	9.87	0.0477	0.0450
Decoction	30.21	9.30	37.22	10.97	0.0548	0.0569

Viral control: Mechanically infected squash plants with ZYMV. Healthy plants: squash plants without any treatments

Table 5: The influence of *Nigella sativa* infusion and decoction on phenolic compounds (mg/g), total protein, polyphenol oxidase and peroxidase of ZYMV infected squash plants *in vitro* and *in vivo*.

soaking for 24 hrs, while infusion gave 90% inhibition after soaking for 24 hrs. Moreover, results of the growth parameters of plants in seed soaking experiments in Table 4 revealed that number of leaves, shoot length and fresh weight increased significantly ($p<0.05$) more than the viral control at all times. The decoction increases the growth parameters more than infusion. Soaking in decoction for 24 hrs, give number of leaves, shoot length and fresh weight 7.8, 35.2, 9.66 compared to 4.7, 26.6, and 4.89 of viral control.

The physiological responses of squash plants infected with ZYMV and treated by aqueous infusion and decoction of *Nigella sativa* seeds

Data shown in Table 5 and 6 showed that *in vitro* application of infusion and decoction of *Nigella sativa* seeds led to an increase in total phenols contents of squash plants compared to viral or healthy control plants. The application of NS decoction increased the total phenolic compounds in all experiments compared to viral control. The total soluble intracellular protein significantly increased upon treatment with infusion and decoction of *Nigella sativa* seeds comparing to viral control where it was 10.50,11.10 compared to 8.8, respectively. As well as, the activity of polyphenol oxidase and peroxidase increased after NS extracts treatment, comparing to viral and healthy control plants. The infusion treatment for 24 hrs leads to total phenol 30.73; total protein 9.01; polyphenol oxidase 0.0388 and peroxidase 0.0391. The decoction treatment for 24 hrs leads to total phenol 32.17; total protein 10.1; polyphenol oxidase 0.0533 and peroxidase 0.0490.

Discussion

It is important to find safe and cheap effective antimicrobial agents to inhibit virus and pathogenic bacteria. The virus and bacteria cannot mutate with the medicinal plants because of their chemical constituents. One essential oil of these plants can have from 200-800 chemical constituents. Due to increasing antibiotic resistance in microorganisms and side effects of synthetic antibiotics, medicinal plants are now gaining popularity in treatment of bacterial infections [34]. Medicinal plants are a rich source of antimicrobial agents due to the secondary metabolites such as alkaloids, flavonoides, tannins and terpenoids that are present in these plants [35,36].

Plants are important source of potentially useful structures for new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay [37]. In the present study, the results for the antibacterial screening have shown that the infusion of ginger significantly ($p<0.05$) inhibit *E. coli* and Bacillus cereus compared to other medicinal plants. This result agrees with that obtained by Ekweny and Elegalam [38], who reported that ginger inhibit *E. coli*. Also, Malu et al. [16] reported that ginger extracts possesses antibacterial properties and could be used for the treatment of bacterial infections.

This study showed that the infusion and decoction of black

Treatments	Free phenolic compounds	Cell bound phenolic compounds	Total phenols	Total protein	Polyphenol oxidase	Peroxidase
Viral control	20.22	11.19	30.56	10.20	0.0499	0.0402
Healthy control	20.66	9.76	30.42	9.30	0.0410	0.0403
Soaking in Infusion for						
6 hrs	19.25	10.72	29.97	9.60	0.0371	0.0400
12 hrs	9.70	11.00	20.70	9.23	0.0383	0.0392
24 hrs	19.9	10.83	30.73	9.01	0.0388	0.0391
Soaking in decoction for						
6 hrs	19.98	10.87	30.85	9.99	0.0500	0.0452
12 hrs	20.15	11.66	31.81	10.00	0.0510	0.0477
24 hrs	20.50	11.67	32.17	10.10	0.0533	0.0490

Viral control: Mechanically infected squash plants with ZYMV. Healthy plants: Squash plants without any treatments

Table 6: Effect of soaking in *Nigella sativa* infusion and decoction on phenolic compounds (mg/g), total protein, polyphenol oxidase and peroxidase of ZYMV infected squash plants.

seed strangely inhibited *Lactococcus lactis, P. aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus* with 22.30, 9.60, 9.50 and 9.00 mm, respectively. These results agree with that reported by Mashhadian and Rakhshandeh [39]. Black seed extracts have weak antibacterial activity on *E. coli*. Similar results obtained by Zuridah et al. [40]. Aqueous decoction and infusion of black seed inhibit the production of ZYMV symptoms on squash plants by 85% and 80%, respectively, *in vitro* treatment. Soaking in NS decoction and infusion for 24 h inhibited ZYMV by 95% and 90%, respectively. These results are similar to previous studies. Mohamed [8] showed that the extract of NS inhibited the infectivity of Broad bean mosic virus (BBMV) *in vitro* and *in vivo*. NS infusion and decoction not only inhibit ZYMV symptoms, but also increase the growth parameters. The application of NS extract improved antioxidant response of squash plant in terms of phenolic compounds, total soluble proteins, polyphenol oxidase and peroxidase, which were summarized in Tables 5 and 6. The accumulation of the phenolic compounds and their derivatives may be considered as a defense mechanism or as a hypersensitive reaction. The disease resistance response correlates with changes in cell biochemistry and physiology [41]. Many studies showed that induced resistance through the accumulation of various phenolic compounds and activation of oxidative and key enzymes in phenylpropanoid and isoflavonoid pathways [42].

In this study, it was found that the peroxidase enzyme and polyphenol-oxidase enzyme activities showed an increase in the infected plants. Moreover, the activity of such enzymes was higher in the plants treated with infusion and decoction of black seed. These results could give an explanation for the increase in phenolic compounds, since the oxidative enzymes play an important role in oxidation of free phenols, which are accumulated as a result of viral infection, and these newly synthesized polyphenols and their oxidation products may limit the viral activity in resistance tissues. Similarly, many studies observed an increase activity in peroxidase, catalase and polyphenoloxidase in the infected plants [43,44]. Accumulation of such compounds and the high activity of some enzymes involved in the metabolism of ROS, such as peroxidase (POX) and polyphenol oxidase explain the reduction in viral disease symptoms, and the increase in growth and metabolic activities in squash plant treated with NS infusion and decoction, which activate the biochemical and structural defense systems that help word off the spread of pathogen, and consequently, increase squash growth if compared to the ZYMV infected ones. These results agree with that obtained by Ghosal et al. [45] and Devanathan et al. [46].

Recently, it was found that a common feature of abiotic and biotic stress factors is the generation of ROS, such as H_2O_2 in plant cell ROS formation also accompanies normal metabolic processes. To ameliorate the danger posed by the presence of cellular oxidants, plant cells have evolved complex defense mechanisms [47]. Plants possess several mechanisms that detoxify O^-_2 and H_2O_2 called antioxidant systems. The primary components of antioxidant systems include non-enzymatic antioxidants (carotenoids, ascorbate, glutathione and tocopherols), and enzymes such as catalase, glutathione peroxidase and those involved in the ascorbate glutathione cycle; ascorbate peroxidase, dehydroascorbate reductase, monodhydroascorbate reductase and glutathione reductase. The components of these antioxidant defense systems can be found in different sub cellular compartments [48,49]. Virus infection has also been shown to increase peroxides [50]. (Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency [51]. The free and total phenols were more concentrated in leaves of squash plants after black seed treatments compared to viral control, and this was in contrast with bound phenols. These results were in agreement with Kofalvi and Nassuth [52], who reported a significant increase in phenols accumulation in wheat plants infected with the wheat streak mosaic potyvirus (WSMV) compared to the healthy controls. Phenolic compounds produced by plants are formed through phenylpropanoid metabolism. However, since free phenols can be cytotoxic in the cytoplasm, plants sequester these compounds in the vacuole or deposit them in or on the cell wall. Once the phenolic acids or cinnamyl alcohols reach the cell wall, they may be either esterified or linked to the cell wall polysaccharides or hemicelluloses, or be polymerized into lignin [53].

Squash plants infected with zucchini yellow mosaic virus (ZYMV) show high content of total protein compared to healthy plants. However, there was a progressive increase in protein contents in plants treated with mixture of NS and ZYMV. This result agreed with that obtained by Cheema et al. [54], who showed that protein content in two soybean varieties increased with infection with soybean yellow mosaic virus. Haque et al. [55] reported that ZYMV infection increased the protein content of pumpkin leaves compared to healthy ones. Muqit et al. [56] showed that total protein increased in the infected leaves of *Benincasa hispida* due to Papaya ring spot virus. Rao et al. [57] concluded that the increased protein content in virus infected plants was due to increased activity of RNA synthetase or RNA polymerase. The treated plants also show high protein content compared to viral control. This may be due to the formation of new antiviral protein. This agrees with that obtained by Abdel-Shafi [23].

The infusion and decoction of thyme inhibited all tested bacteria, except *Staphylococcus aureus*. Stahl-Biskup and Saez [14] reported that the extracts of Thymus species have strong antibacterial, antiviral, antifungal and antioxidant activities. This is similar with that obtained by Seden et al. [58], who reported that thyme inhibited the pathogenic

bacteria. Thymol oil derived from thyme (*Thymus vulgaris*), has demonstrated biological properties such as antimicrobial, antioxidant and antiseptic activities [12]. It has high activity on inhibition of respiratory tract pathogenic bacteria [13].

The infusion of clove inhibited all tested bacteria, except *Stapylococcus aureus*. This disagrees with that obtained by Saeed and Tariq [21]. The decoction of clove inhibited *Listeria monocytogenes* and *Staphylococcus aureus* significantly compared to other plants. The infusion of peppermint inhibited all tested bacteria, while the decoction exhibited significant inhibition against *E. coli* and *Lactococcus lactis* (20 and 19.5 mm, respectively), but did not inhibit *Staph aureus*. Similar results were obtained by Irshad et al. [2], who reported that that plant peppermint inhibited 7 bacterial strains (*E. coli, B. subtilis, S. typhi. P. aureus, K. pneumonia* and *S. epidermitus*). Also, Saeed and Tariq [59] reported that the juice of peppermint leaves exhibited highest antibacterial activity.

Further work for quantitative analysis of virus and electron microscope will be done.

Conclusion

This study has shown that ginger, black seed, clove, thyme and peppermint extracts have antibacterial activity. Due to the increase of antibiotic resistant bacteria and bad effect of synthetic antibiotic, the decoction and infusion of medicinal plants above could be used as alternative to antibiotics in treatments of bacterial infections. Moreover, the infusion and decoction of black seeds showed high antiviral activity against ZYMV on squash plants. Black seed induce systemic resistance and defense response in squash against ZYMV infection, and increase the plant growth parameters. The enzymes involved in the defense against reactive oxygen species (ROS) showed an increase of POX, phenolic, polyphenol oxidase activities, compared to the control, suggesting a significant ROS decrease.

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