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RESEARCH ARTICLE

Possible sustainable management of onion *Botrytis* brown stain disease by some plants aqueous extract

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Abstract

This study was conducted to survey the fungal microorganisms that cause common diseases and reduce the quality of onion bulbs produced in Assiut and Sohag governorates in Egypt, particularly Botrytis Brown Stain (BBS) disease on onion bulbs. *Aspergulls niger*, *A. flavus*, *Penicillium oxalicum*, *Botrytis allii* and *B. cinerea* strains were isolated from onion. *B. cinerea* was the strain that clearly showed brown spot symptoms while other tested fungal strains did not show significant symptoms. The highest severity of 52.00% was found for *B. cinerea*. Eucalyptus (*Eucalyptus chamadulonsis*), Bitter apple (*Citrullus colocynthis*), and Neem (*Azadiratcha indica*) aqueous plant extracts were used and their ability to reduce the growth of the fungal pathogen. The extract of Bitter apple (*Citrullus colocynthis*) at concentrations of 5 and 10% resulted in the highest growth inhibition by 13.33 and 8.89%, respectively. The plant extracts ability to reduce the disease severity on onion bulbs under storage condition were evaluated. The *C. colocynthis* plant extract exhibited significantly increased of the total phenolic and salicylic acid contents in onion plants compared to untreated plants. The results also indicated that *C. colocynthis* plant extract was the most effective and comparable with the recommended fungicide. Therefore, the natural plant extracts can be recommended as alternative to highly toxic and expensive fungicides.

Keywords: *Allium cepa*; *Botrytis cinerea*; *Citrullus colocynthis;* Induced resistance; salicylic acid.

1. Introduction

Onion is native Asian then moved to Egyptian trade road (Raghavan, 2006). Onion (*Allium cepa* L.) cultivated area in Egypt is 87948 ha; Egypt's productivity of onions is close to three million tons. Egypt is one of the ten most productive countries of onions in the world. The exports of Egyptian onions, at end of 2019 recorded 550,000 tons, compared to 310,000 tons in 2018 (Anonymous, 2020). Onion damaged by many diseases; neck rot, blast and downy mildew, purple blotch, rust, leaf spot, white rot, basal rot, and pink rot (Hill and Waller, 1988). Onion was facing post-harvest fungal diseases that decreased the quality and the quantity of yield. Botrytis brown stain is one of these diseases (Clark, and Lorbeer, 1973). Storage fungal diseases Botrytis spp. was found on diseased onion bulbs (lee *et al.*, 2001). Noticed lose in onion storage has been recorded for Botrytis spp. (Ibrahim, 2005).

Mohana *et al.* (2006) reported that many screenings conducted using plant extracts of fruit and seeds as sources of bioactive, compounds that shown antifungal and antibacterial properties in vivo. *Eucalyptus chamadulonsis*, *C. colocynthis* and *Azadiratcha*

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indica, Researchers have successfully employed Datura stramonium, *E. globules*, *Salix* spp., *Ocimum* spp., *Cydonia oblonga*, *Foeniculum vulgare*, and *Rosmarinus officinalis*, to manage plant pathogens (Abdel-Monaim *et al.*, 2011; Nashwa and Abo-Elyousr, 2013). Induction of resistance in peroxidase, polyphenol oxidase was recorded for many plants against plant bacterial and fungal pathogens (Zahid *et al.*, 2012; Hassan *et al.*, 2008).

Plant responses to biotic and abiotic stress are largely regulated by phytohormones. Many phytohormones are being investigated for their function in abiotic stress responses, including salicylic acid, jasmonic acid, auxin, ABA, ethylene, and gibberellic acid (Santner *et al.*, 2009; Peleg and Blumwald, 2011; Kohli *et al.*, 2013)

The objective of this research is to study the efficacy of aqueous plant extracts on reducing the disease severity of brown stain disease on onion bulbs.

2. Materials and Methods

2.1.Samples Collection

The samples collected from different areas in Assiut and Sohag during July-January 2019, samples were fully mature bulbs then deposited for storage. Twenty-four samples of Giza 6 onion cultivar were collected,.

2.2.Isolation and identification the causal pathogen

Onion Samples kept in 30x17.5 cm paper bags, labeled and stored for 30 days under room condition (25±2 C and 70% humidity), diseased parts were cut in small pieces and put on PDA medium for 10 days till growth of fungal and for 3 days for *Aspergillus spp.* pathogens appears after that the fungal hyphal tip was reinoculated on new petri dishes included PDA medium to purify the fungal growth. Fungal species was identified according the observed features and descriptions of Chilvers and Dutoit (2006), and confirmed in Assiut Mycological Center. Strains preserved under 4^{o}C for further experiment.

2.3.Pathogenicity Tests

Three replicates of healthy onion Giza 6 cultivar bulbs used, surface sterilization were made using ethanol 70% spray followed by scratches on each bulb. bulbs were scratches with the fungal inoculum (10^4 CFU/ml). Tissues of onion sealed with parafilm tape, labeled and incubated at 28^{o}C for 10 days. Constant observation was made and then re-isolated and compared with the original isolates. Controls were prepared by making the wounds aseptically on the fresh onion without inoculation.

Symptoms in Figure 1 were employed to calculate the disease severity as the followed equation that used to calculate disease severity percentage, where: Number of bulbs in each category on infection =n, Values numerical categories of infections=V, Total number of bulbs examined=N, Constant of highest numerical value=4, The value scales from 0 - 4 used, No infection=0, 25% bulbs area infected=1, 50% bulb area infected=2,100% bulb area infected=3, Disease severity percentage DS%= [(n x V) / 4 x N] x 100.

Figure 1. Disease symptoms on onion bulbs, healthy for highest disease severity from right to left

2.4. Plants Extraction

According to Taiga (2011), Eucalyptus (*Eucalyptus chamadulonsis*), Bitter apple (*Citrullus colocynthis*), and Neem (*Azadiratcha indica*) leaves were properly cleaned with water, air-dried, and crushed into a fine powder using a hammer mill. The fine powder of each plant extract was mixed in a 250 ml Erlenmeyer flask with distilled water up to 100 ml to generate a cold water extraction for all concentrations. The crude aqueous extracts of 1, 5, and 10% were then filtered for 48 hours through four layers of sterile cheese cloth.

2.5. Effect of plant extract on pathogen radial growth in vitro

Ten milliliters of different concentration of each plant extract placed into 9 cm Petri dishes with solidified potato dextrose agar and drained. Then, using a sterile 5 mm cork borer, three days old colony was sliced and aseptically inserted upside down in the centre of the PDA in the plates, where it was cultured for 7 days at 28^{o} C. Regular observation was made, where radial growth diameter measured to evaluate inhibition of the causal pathogen by following equation, R=(C–T/C)x100. Whereas, growth percentage of reduction=R, control hyphal growth diameter=R and treated hyphal growth diameter=T (Hussein *et al*., 2007).

2.6. Effect of plant extracts on pathogen growth during storage

The plants were sprayed with different plant extracts Eucalyptus (*Eucalyptus chamadulonsis*), Bitter apple (*Citrullus colocynthis*), and Neem (*Azadiratcha indica*) at three concentrations (1, 5, 10 percent) five days before harvest time, and the infection occurred at the same time in the treatment before harvest. Post-harvest treatment and infection were done in the same way just after the onion bulbs were harvested (Hussein *et al*., 2007). Ten days later, the results were obtained.

2.7. Salicylic acid contents and total phenolic contents determination

2.7.1. Samples Preparation

One gram of onion bulb and liquid nitrogen were used to ground it and homogenized in ten milliliters of 80% methanol, then centrifuged at 4°C, 10,000 g for 30 minutes. ascorbic acid (0.02 $g \cdot mL^{-1}$) add, the pellet was discarded. At 65°C, the supernatant was evaporated using a rotary evaporator, procedure was done three times, each for five minutes. In 5 mL of 80 percent methanol, the remains were dissolved. Four replicates were utilized for each treatment, 48 hours after treatment each sample were analyzed (Abbasi *et al.*, 2002).

2.7.2. Total Phenolics

Gallic acid was used as a standard, total phenolic contents were assessed 48 hours of treatment using a spectrophotometer at 767 nm model Spectronic 20 Genesys, company Schutt Labor Technik, of Cambridge UK. The amount of total phenol in each gram of plant material was measured in milligrams of gallic acid (Sahin *et al*, 2004)

2.7.3. salicylic acid Content

The salicylic acid (SA) contents were estimated 48 hours of treatment using adapted method of Dat *et al.*, (1998). Five hundred µL leaf homogenate was combined with HCl 10-N (250 µL) and methanol (1 mL). For 2 hours, 80°C water bath used to incubate the samples. 4–5 drops of 1-M NaHCO3 was neutralized each sample after adding one mL of methanol to the mixture. At a wavelength of 254 nm, the OD optical density measured. SA contents were computed and represented as gram of SA per gram of plant material.

2.7.4. Enzymatic Activities

One g fresh tissue was homogenized in liquid nitrogen with 10 mL of pH 5.2 sodium acetate buffer 0.1-M and for 30 minutes 4°C centrifuged at 1000 g to evaluate polyphenol oxidase (PPO) activity. Each treatment was used 4 replicates. Supernatant of total protein content was measured using Bradford reagent

as determined by Dat *et al.* (1998).

2.7.5. Activity of Polyphenol Oxidase

Described method by Batra and Kuhn (1975) was used to determine the activity of PPO polyphenol oxidase. Two mL 50-mM Sorensen phosphate pH 6.5, substrate pyrocatechol 0.5 mL with 0.5 mL of supernatant were the reaction mixture contents, and reaction mixture was incubated in water bath for 2 h at 37 ˚C and measured at 410 nm. Activity of PPO on OD at 410 nm was calculated and expressed as OD· mg protein^{-1}.

2.8. Electrophoresis and protein analysis

Protein analysis and electrophoresis were performed under denaturing conditions using 12 percent polyacrylamide and 1 percent SDS (w/v) according to Laemmli's (1970) technique. Coomassie Blue R was used to stain the gels for protein bands. The gels were then destined by repeatedly immersing them in a solution of methanol, acetic acid, and water (1:1:8, by volume). Using the GS 365 electrophoresis data system application version 3.01, the molecular weight of protein bands was measured by comparing them to standard protein markers.

2.9. Experimental Design and Statistical Analysis

The experiment design CRD Completely Randomized Design was used as described by Gomez and Gomez (1984). Results were analyzed using ANOVA and means compared using Least Significant Difference (LSD) at p = 0.05.

3. Results and Discussion

3.1. Isolation and Identification of the causal pathogens

Results in Table (1) showed that the survey of associated fungi with the onions brown stains disease in different regions in Assiut and Sohag, seven isolates were obtained and identified as *Botrytis cinerea*, three isolates identified as *Botrytis allii*, two isolates as *Aspergillus niger*, two isolates *Aspergillus flavus and one isolate Penicillium oxalicum*. These results match with several researchers such (Clark and Lorbeer, 1973; Clark and Lorbeer, 1975; Kritzman and Netzer, 1978).

3.2. Pathogenicity Tests

Results in Figure (2) isolated strains were used to conduct pathogenicity tests on Giza 6 cultivar, *Botrytis cinerea.* The highest disease severity by 52 %, followed by *Botrytis cinerea 2* and *Botrytis cinerea* 4 by 36 and 35 % respectably. While isolates of *Aspergillus niger*, *A. flavus* and *Penicillium oxalicum* didn't have any symptoms. The results supported *Botrytis* spp. ability to be a pathogen on onion bulbs mentioned many times by (Steentjes, *et al.*, 2021; Hussein, *et al.*, 2014; Chilvers, and du Toit, *et al.*, 2006). The pathogenicity test revealed that *Botrytis cinerae* was the most virulent when compared with the other tested organisms that not match what mentioned by Lee, *et al.*, (2001) and Ibrahim, *et al.*, (2014) whom reported that that *Aspergillus spp.* were the most virulent and prevalent in onion bulbs rot.

3.3. Effect of Plant Extracts on Pathogen Growth In Vitro

Three plant extracts of *Eucalyptus chamadulonsis*, *Citrullus colocynthis* and *Azadiratcha indica* with different concentrations (1, 5, 10 %), were tested *in vitro* against *B. cinerea* No. 1 the casual pathogen of Botrytis Brown Stain (BBS).

Data presented in Figure (3) indicates that *Citrullus colocynthis* 1, 5, 10 % and *Azadiratcha indica* 5, 10 % plant extracts were able to decrease growth of mycelium of the causal pathogens with variation in their growth inhibition. The highest inhibition of *B. cinerea* (13.33 %), by *Citrullus colocynthis* at the concentration 10%. Data also indicate that other plants extract of *Eucalyptus chamadulonsis* 1, 5, 10 % didn't showed mycelial inhibition. According to the previous results, 10% concentration *Citrullus colocynthis* was selected to carry out the *in vivo* studies. Tebuconazole 125 µg/l Folicur 430 SC Bayer gave no growth

in the positive control. Results match with results showed by Abo-Elyousr and Asran (2009) they reported that garlic extract gave high impact on fungal pathogen. Previous study reported that (GC-MS) analysis of *C. colocynthis* compounds including imidazole that have effect as antibacterial and antifungal and another thirty-seven compounds (Rahman and Gray, 2005; Hussein *et al.*, 2018).

Table 1. Sources of the potential causal pathogen.

No.	Isolates	Source of isolate
1	*Botrytis cinerea* 1	Manfalout- Assiut
2	*Botrytis cinerea* 2	Dairout- Assiut
3	*Botrytis cinerea* 3	Abnoub- Assiut
4	*Botrytis cinerea* 4	Reefa- Assiut
5	*Botrytis cinerea* 5	Abnoub- Assiut
6	*Botrytis cinerea* 6	Assiut
7	*Botrytis cinerea* 7	Sohag
8	*Botrytis allii*1	Mousha- Assiut
9	*Botrytis allii* 2	Sohag
10	*Botrytis allii***3**	Elzawia- Assiut
11	*Aspergillus niger 1*	Drunka- Assiut
12	*Aspergillus niger2*	Sohag
13	*Aspergillus flavus 1*	Drunka- Assiut
14	*Aspergillus flavus2*	Assiut
15	*Penicillium oxalicum*	Assiut

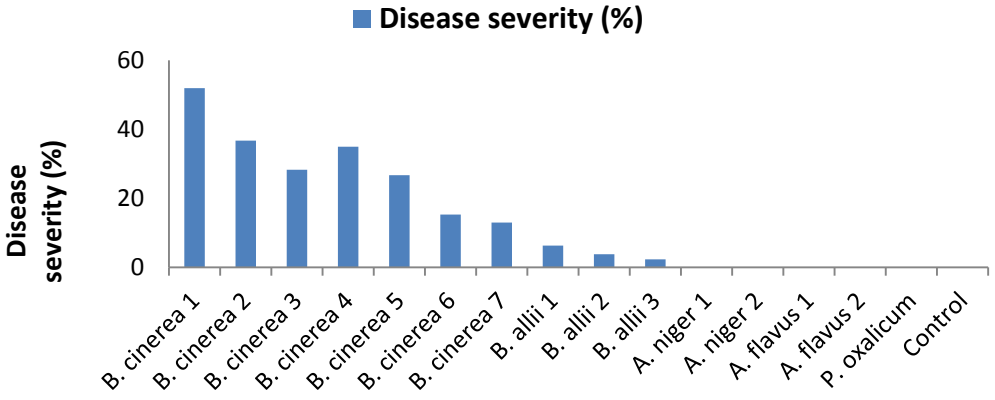

Figure 2. Pathogenicity tests of certain pathogenic fungi to incite BBS on Giza 6 onion cultivar, Least significant difference L.S.D. at 0.05 ≥ 3.02.

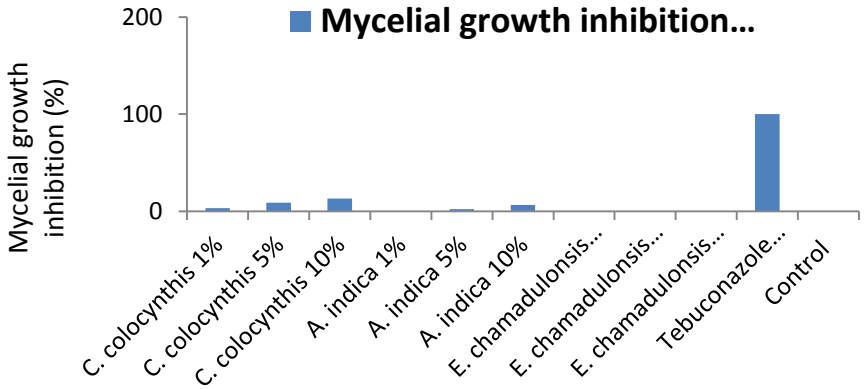

Figure 3. Antifungal activity of certain plant extracts to inhibit the mycelia growth of *B. cinerea in vitro*, L.S.D. at 0.05

≥ 6.07.

3.4. *Effect of Plant Extracts on Disease Severity in vivo pre harvest treatments*

Data presented in Figure (4) indicates that *Citrullus colocynthis*, *Azadiratcha indica* and *Eucalyptus chamadulonsis* 10% resulted in 14.33, 15.07 and 10.66 respectively, all tested plant extracts were able to affect the disease severity with slight significant difference between different concentrations.

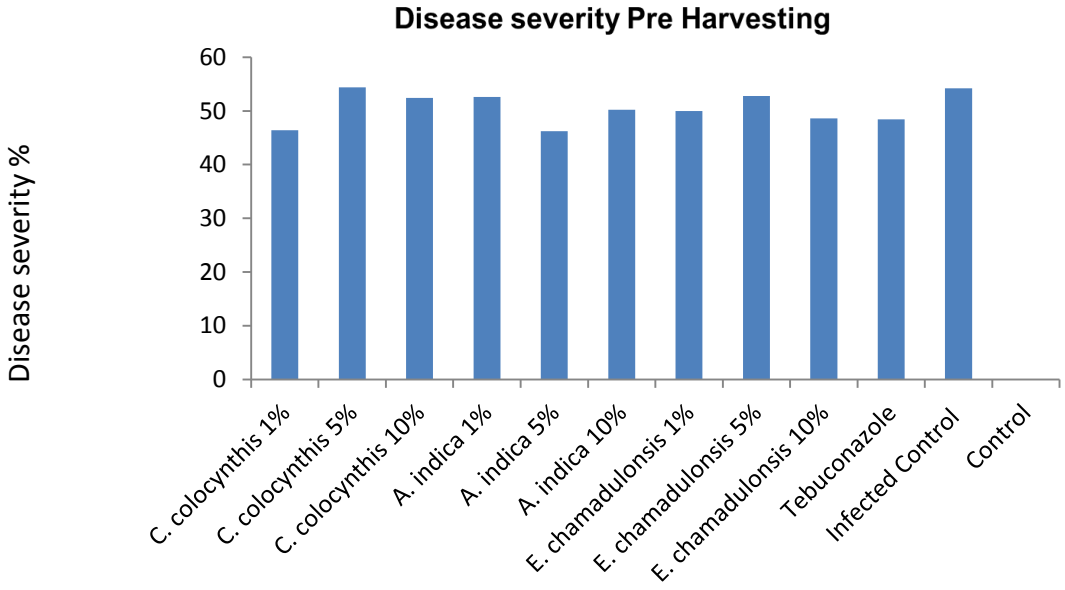

Figure 4. Effect of Pre-harvest treatment with certain plant extracts on severity of BBS diseases *in vivo*, L.S.D. at 0.05 disease severity ≥ 8.20 and disease reduction ≥ 9.86.

3.5. *Effect of Plant Extracts on Disease Severity in vivo post-harvest treatment*

Data presented in Figure (5) indicates that all tested plant extracts were able to affect the disease severity with variation in their toxicity. The highest reduction of *B. cinerea* (70.49, 54.71 and 33.20 %) on Giza 6 onions caused by *Citrullus colocynthis* at the concentration 10, 5, 1% respectively. Data also indicate that other plants extract of *Azadiratcha indica* 28.69, 25.82 and 12.70% for 10, 5, 1% respectively. *Eucalyptus chamadulonsis* showed the lowest mycelial inhibition percentage of growth by 3.28%. According to the previous results. Tebuconazole 0.12-0.24 ml/l Folicur 430 SC Bayer gave no infection with Botrytis brown stains.

Previous studies showed that bioagents can cause high impact on *Botrytis spp.* Such Wahab *et al.* (2020) indicated that organisms were isolated, i.e: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium expansum* and *Botrytis aclada* these organisms were frequently encountered decreased during the experiments. Abdulsalam *et al.* (2015) and Hussein *et al.* (2018) noted that *Botrytis spp.* effect decreased when treated with bio-agents, and the disease incidence also decreased.

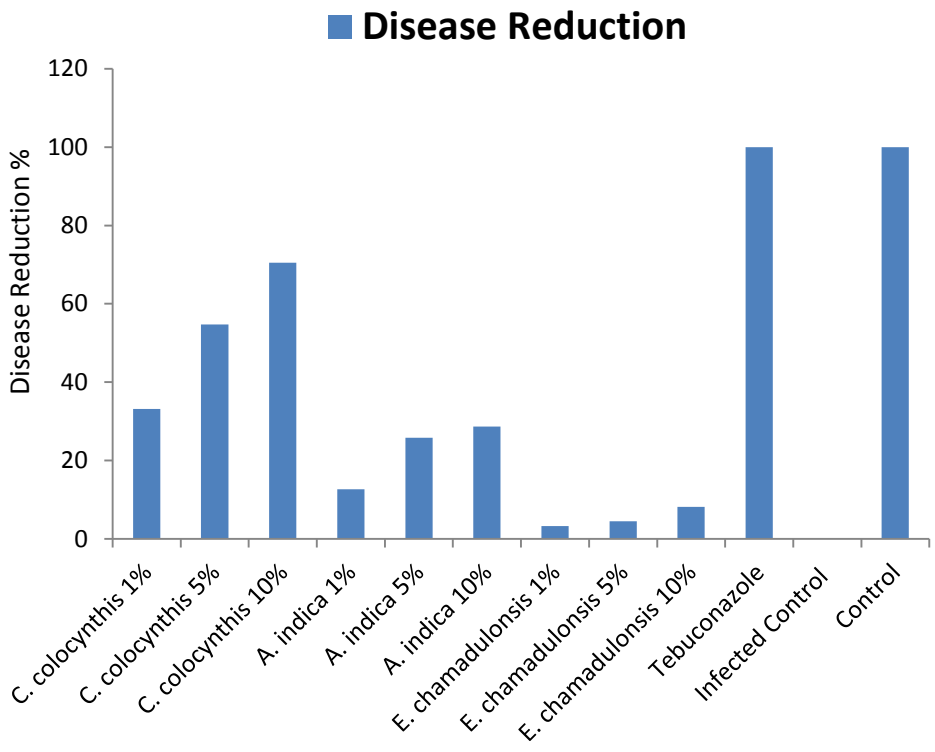

Figure 5. Effect of Post-harvest treatments of certain plant extracts to reduce severity of BBS diseases *in vivo*, L.S.D. at 0.05 ≥ 11.30.

This study results do not match with previous study with plant extract Neem leaf extract (*Azadiratcha indica* L.). In other related studies Suleiman and Falaiye (2013) reported that Neem leaf extract was most effective in suppressing of *A. niger* causing biodeterioration in sweet potato while it was less effective in present study.

John *et al.* (2016) reported that an aqueous extract of 80 % *V. amygdalina* gave inhibition effect against *Fusarium oxysporum Geotricum*, *Candida* and *Aspergillus niger* with more than 60 % aqueous concentration of the test plant extract.

3.6. Plant Extracts Effect on total phenolic contents

Data presented in Figure (6) indicates that all tested plant extracts were able to increase total phenol contents. The highest total phenol contents on Giza 6 onion cultivar caused by *Azadiratcha indica* at the concentration 5, 1% by 6.04 for both, followed by plants extract of *Eucalyptus chamadulonsis* at the concentration 5, 1% 5.40 and 5.12 respectively and *C. colocynthis* 10% by 1.16.

Data presented in Figure (7) indicates that all tested plant extracts were able to increase salicylic acid in Giza 6 onion cultivar. The highest salicylic acid on Giza 6 onion cultivar caused by the standard fungicide by 6.42. while among the treatments *C. colocynthis* 1 0 % showed the highest salicylic acid by 4.88 at the concentration 10%. While the lowest salicylic acid recorded by healthy control

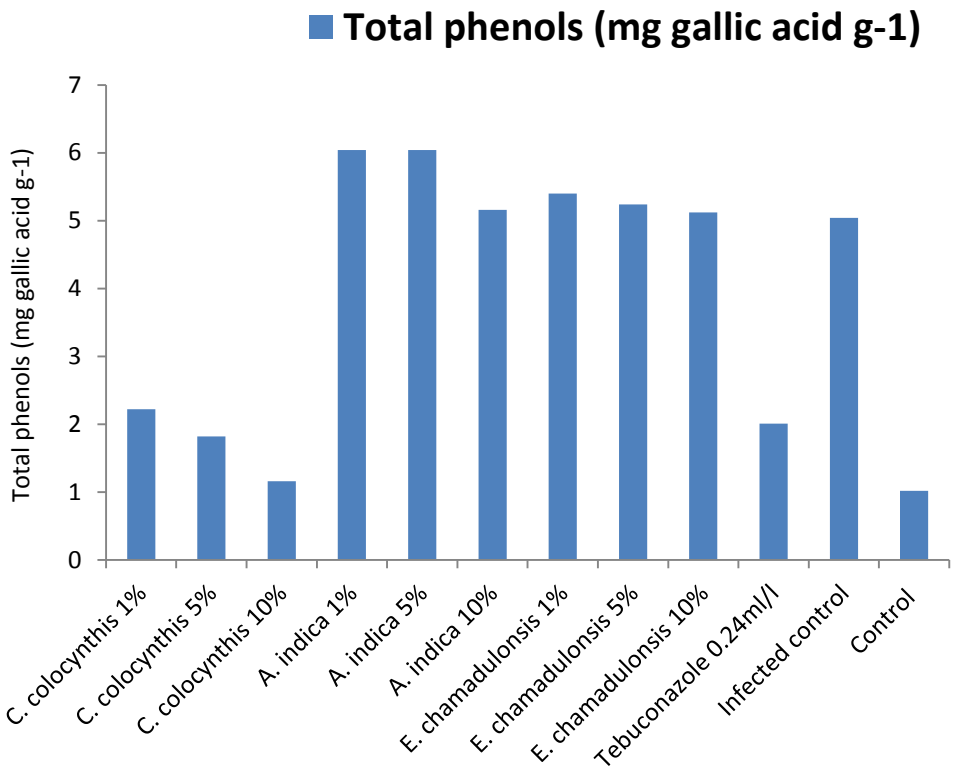

Figure 6. Effects of plant extracts treatments on total phenolic contents, LSD at 0.05 ≥0.96.

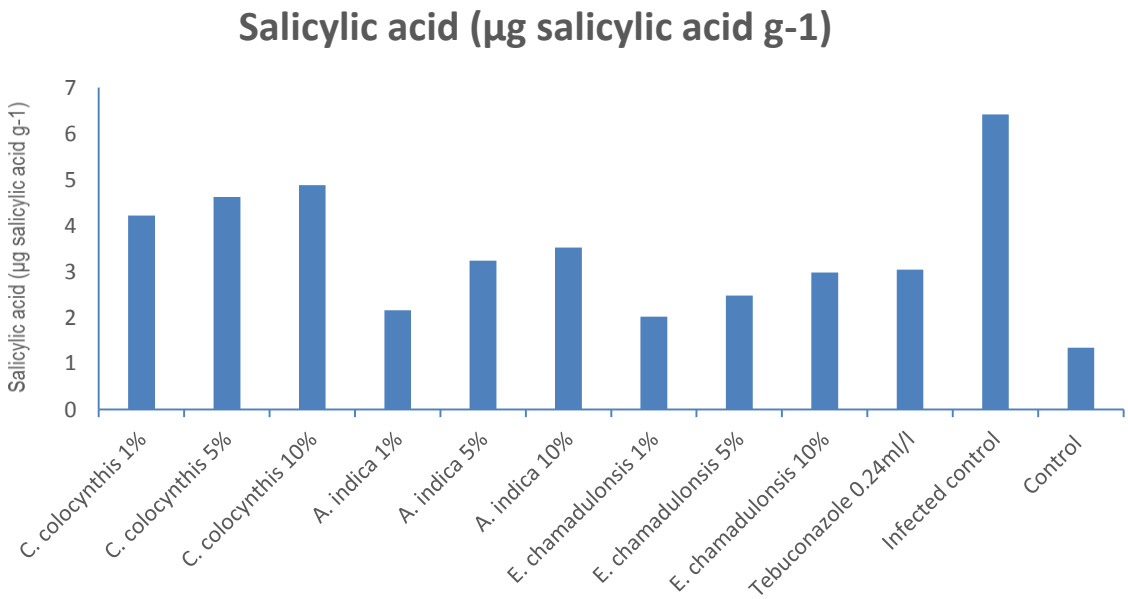

Figure 7. Effect of treatment with certain plant extracts on Salicylic Acid Contents, LSD at 0.05 ≥ 1.10

Except for *C. colocynthis* extract, total phenolic contents were considerably greater in treated plants compared to infected or healthy control plants. Plant extracts treatment elevated the phenolic contents accumulation in response to infection. The amount of phenol in samples ranged from 1.02 to 6.04 mg gallic acid per gramme. Because phenolic compounds were phytotoxic pathogenic, their accumulation at the

infection site was related to pathogen suppression. Increased phenolic acid compounds in the plant cell cytoplasm may help to increase resistance by preventing pathogen development by raising the pH of the plant cell cytoplasm. (Sagar *et al.*, 2020).

With the application of *C. colocynthis* extract, the content of salicylic acid (SA) increased considerably by (4.88 µg salicylic acid g−1) 7 days for 10% concentration after treatment, Despite the fact that there was no significant difference between other concentrations of C. colocynthis (Figure 7).

In comparison to the control group, all treatments demonstrated statistically significant increases in SA levels. However, extracts exhibited levels of SA in all treatments, and extract of *C. colocynthis* generated the greatest SA increases of all the treatments. (Abd-Rabboh and El Shennawy, 2016; Dempsey *et al*., 1999) SA accumulation has been hypothesized to be critical for the activation of numerous modes of plant disease resistance. In addition, in response to pathogen infection, SA accumulates in both infected and distant leaves, mediating plant defense against pathogens. These findings could potentially suggest that plant extracts elicit pathogen resistance in plants by activating a signaling pathway that is dependent on SA or a novel subsequent decrease that is not depending on jasmonic, ethylene or SA signaling.

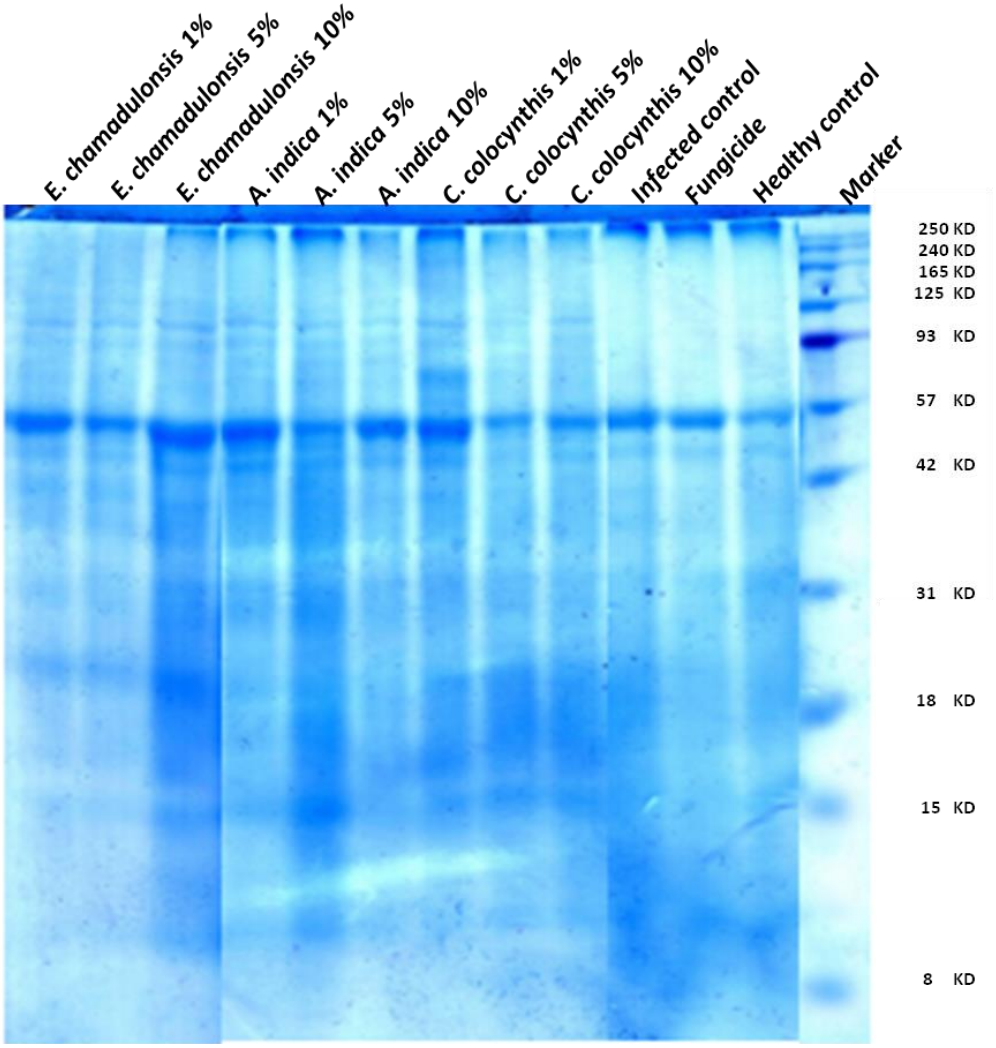

Figure 8. Protein patterns (SDS-PAGE) for treated onion with different concentrations of three plant extract.

3.7. Protein profiling of tested onion treatments

Protein profiling was done to determine whether some new proteins was associated with resistance in onion different treated plants in compare with control. SDS-PAGE is used for finding the banding pattern of proteins existance or disappeared. It has been found that the banding patterns of protein of different treatments as shown in Figure (7). The number of protein bands presents ranged from 3 to 10. The highest numbers of bands were found in onion treated with *E. chamadulonsis* concentration 10% followed by *A. indica* concentration 1 and 5% and *C. colocynthius* concentration 1% while *C. colocynthius* concentration 5 and 10% resulted in the least number of bands and it was comparable to healthy control lane.

Data, also reveal that the protein patterns which are present in control of onions treated plants corresponding to the molecular weight in KDs (260, 110, 100, 93, 70, 54.50, 40, 38, 31, 20, 13,10 and 8), the plant extract treatments contains one two or three more protein band than the healthy control at 110, 100 and 93KD. The detection or absence of bands is may be due to the treatments on onion this is can reinforce the view of ability of Plant extracts to enhance the resistance system in plant (Gurudeeban *et al*., 2010; Hussain *et al*., 2013; Hussein *et al*., 2018).

4. Conclusions

The aqueous extracts of *Eucalyptus chamadulonsis*, *Citrullus colocynthis* and *Azadiratcha indica* plants exhibited promising antifungal action against the pathogen that causes Onion Botrytis brown stains and could be used as integrated management of the disease.

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Institutional Review Board Statement
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Data presented in this study are available on fair request from the respective author.
Ethics Approval and Consent to Participate
This work carried out at Plant Pathology department and followed all the department instructions.
Consent for Publication
Not applicable.
Conflicts of Interest
The authors declare no conflict of interest

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