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EFFECT OF SOME ESSENTIAL OILS ON THE GROWTH AND DEVELOPMENT OF FRUIT ROT OF LEMON CAUSED BY *ASPERGILLUS NIGER* UNDER *IN-VITRO* CONDITIONS

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A B S T R A C T

It is common observations that during storage, transportation and marketing fruits and vegetables suffer from many postharvest diseases. Generally the diseases are controlled by applying different chemicals in the form of fungicides. On the other hand, these fungicides are also hazardous for health and disturb the equilibrium of the natural environment. In this regard, researchers have found that certain plant essential oils not only keep away the insects but also show fungicidal actions against some plant pathogens. The aim of current investigation is to find out some suitable and effective essential oils against the growth fruit rot of lemon caused by *Aspergillus niger* under *in-vitro* conditions. For this purpose, pathogenicity test against *A. niger* was performed. The antifungal components of some essential oils like clove, tarpin, neem, castor, chamomile, and rose oil were carried out at different doses i.e. 50 µl and 100µl to find out their effectiveness against *A. niger.* The results showed that, high severity of disease was determined through the cut method of inoculation as compared to the injection method. Minimum colony growth (1.11% and 0.55%) was examined when plates treated with clove oil at the dosage of 50µl and 100µl followed by tarpin oil (42.22% and 38.89%), neem oil (42.96% and 41.11%), castor oil (52.96% and 50.00%) and chamomile oil (71.85% and 67.77%) respectively. In comparison to control (100%), maximum growth (91.85% and 89.62%) was observed when treated with rose oil at the dosage of 50µl and 100µl. When the inoculated lemon fruits were treated with aqueous solution of essential oils, it was observed that minimum rotting (12.53%) was recorded for clove oil followed by neem oil (13.30%), tarpin oil (13.42%), rose oil (14.27%), castor oil (15.16%) and chamomile oil (17.25%), respectively as compared to control (23.37%). On the basis of present investigation it was concluded that clove oil is highly effective against linear colony growth of *A. niger* under *in-vitro* conditions.

Keywords: Essential oils, *Aspergillus niger*, Pathogenicity, Cut and Injection Methods

INTRODUCTION

Across 100 countries of the world, lemon fruit a crop of winter season has grown for its commercial purposes (Terol *et al.,* 2014).The cross between citron (*Citrus medica* L.) and sour orange thought to produce a hybrid variety of fruit known as lemon. Lemon fruits are available in round, oblong, or elongated shapes. China, U.S.A, Brazil, Mexico, Spain and India are the world's largest producing countries contributing approximately two-third of global production of citrus fruit (Sabulal *et al.,* 2006). Worldwide, Pakistan being an agricultural country, ranked 10th in producing citrus fruit (Sabir *et al.,* 2010). Lemon is a rich source of vitamin 'C' and plays an important role in health promotion. Lemon fruits contain less proportion of proteins and fats, providing sucrose, glucose and fructose as carbohydrates (Spina *et al.,* 2008). Lemon fruits are also a good source of some dietary fiber which preventing gastrointestinal disease and lowering the cholesterol level in the blood.

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Most important plant pathogens are fungi, bacteria, phytoplasmas and viruses. Among such pathogens, fungi can cause severe losses in fruits as well as in vegetables. Lemon due to low pH (less than 4) and high moisture contents make them susceptible to various fungi (Moss, 2012). The fruit rot of lemon caused by *Aspergillus niger* is one of the major postharvest disease which may cause considerable losses to the lemon growers. The disease caused by *A. niger* is more sever in rainy season in lemon fruits. In Pakistan, storage, inappropriate method of packing, transportation and marketing become the cause of nearly 40% loss to the lemon fruits (Samuel *et al.,* 2017). Most post-harvest pathogens (*Penicillium, Alternaria, Aspergillus, Diplodia, Phomopsis*) are quite selective in their host and pH. Most of the fungi attack lemon fruits due to weak defense mechanism in term of low pH which is less than 4 (Ladanyia and Ladaniya, 2010).

Over many decades, attempts and efforts have been made so as to prevent and control the plants from such heavy loss (Inouye *et al.,* 2006). In this regard, scientists have developed synthetic fungicides to check the growth of such pathogens in host plants. Obviously, these pesticides are able and highly effective in controlling a variety of pre and post harvest diseases of vegetables and fruits. On the other hand, the continuous and repeated use of various fungicides may disturb the equilibrium of ecosystems and produces high threats for health than insecticides and herbicides (Gomi *et al*., 2003). Susceptibility of lemon fruits to rotting has been associated with ripeness and over ripeness of the fruits and long storage periods.

Therefore, the challenges have been emerged to develop alternative method which is safer and eco-friendly and causes less risk to the health of human lives and nature. In this regard, researchers have shown an interest for using non-toxic alternatives instead of synthetic fungicides. There are about 2600 species of plants out of which 700 are noted for their use as medicinal herbs. Different essential oils and its use have been found to be more effective and an alternative method against pathological breakdown of certain pathogens of lemon fruits as compare to other methods (Klieber *et al*., 2002; Ahmed *et al*., 2007).The natural and complex compound properties of plant essential oils make them able to act as an antioxidant and antimicrobial against certain pathogens (Bakkali *et al*., 2008). The biodegradability of EOs compounds has shown an effective control against post-harvest diseases like; green mould, blue mould and sour rot of lemon fruits (Arras and Usai, 2001). No doubt, postharvest fungal disease of lemon fruit causes significant economic losses to the growers. Yet satisfactory attention has not been given on this group of diseases. Due attention must be given to reduce the risk of chemicals methods hence the current study was conducted to isolate and identified and afterwards find the potential of some EOs clove, tarpin, neem, castor, chamomile and rose oil for the control of fruit rot of lemon with the following objectives and techniques. First of all the fungus was isolated from the diseased lemon fruits and the identification was made using microscopic technique. The identified fungus was then purified for further use. The pathogenicity test against the *Aspergillus niger* was performed. The antifungal potential of different essential oils was checked on the linear colony growth of most predominant fungus. Then, further the efficacy of some aqueous solution of essential oils against the disease development can be checked and evaluated on the disease development of fruit rot of lemon.

MATERIALS AND METHODS

Survey and sampling: Survey and sampling of infected lemon fruits were done at the local market of Tando Jam and Hyderabad city.

Disease incidence: The disease incidence of rotted lemon fruits can be calculated through the following formula.

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased fruits}}{\text{Total number of fruits}} \times 100$$

Isolation, identification and multiplication of the causal fungus: The infected portion of lemon fruits was cut into small pieces of 3 to 4 mm, surface sterilized with 5% commercial bleach for 2 minutes. The sterilized pieces were washed twice with sterilized water and shifted over the surface of sterilized filter paper for drying and then sterilized portions were placed in Petri dishes containing PDA medium. All Petri plates were kept in incubator at 25±2°C temperature for 7 days to observe sporulation of the fungi. The fungus was identified through microscope with different magnifications, the pure culture of fungus was made, maintained and multiplied for future use. The following formula gives information on the frequency of isolated fungi.

$$\text{Frequency (\%)} = \frac{\text{Number of pieces colonized by the fungus}}{\text{Total no of pieces cultured in plates}} \times 100$$

Pathogenicity test of the most predominant fungus: Pathogenicity test of predominant fungus (*A. niger*) was conducted on six [6] healthy, mature and fresh lemon fruits through two different methods i.e. wound and injection method. The pathogen disc from 8-10 days old culture plates was taken and inserted into [03] fresh lemon fruits through cut method and [03] lemon fruits were inoculated by the solution of pathogen [1ml of pathogen solution] through injection method. The inoculated portion of lemon fruits were covered with paraffin film and then placed in plastic bags. Lemon fruits were monitored daily for disease development.

To evaluate the efficacy of various essential oils on the linear colony growth of the casual fungus: The efficacy of different essential oils such as clove, neem, tarpin, castor, chamomile and rose oil was examined under *in-vitro* conditions against *A. niger.* The essential oils were used, with two different doses i.e. 50µl and 100µl. Pathogens disc approximately (5mm) from 8-10 days old reserved pure culture were taken and placed in the middle of Petri dishes containing PDA and then mounted in an incubator at 28°C. Petri dishes without EOs were served as control. Day wise radial colony growth of *A. niger* was recorded till the Petri dishes will completely fill with the fungus in any treatment.

Effect of some essential oils on the growth and development of fruit rot of lemon under *in-vitro* conditions: Similarly, effects of EOs were checked against the disease development and growth by the method introduced by (Lassois *et al.,* 2008) with little modification. In this method, the collected infected samples of lemon fruits were first washed with tap water. After that the lemon fruits were sterilized with 5% commercial bleach for 2 minutes. The fresh and healthy specimens of lemon fruits were inoculated with 8-10 days old culture of *A. niger.* The selected essential oils were added in distilled water at the rate of (1ml/300ml) with a drop of tween-20 as to mix the oil in water. After a day the inoculated lemon fruits were immersed for about 5 minutes in the aqueous solution of selected essential oils. The lemon fruits specimen treated with different EOs were put separately on the tissue papers at 20ºC with humidity of 80%. The treated samples were then observed daily for the growth of fungal pathogen. The decrease in disease development showed the effect of EOs. The rotting (%) in the inoculated lemon fruits in aqueous solution of EOs can be calculated through following formula.

$$\text{Rotting (\%)} = \frac{\text{Rotted portion of the fruit}}{\text{Total fresh area of the fruit}} \times 100$$

RESULTS

This study was carried out to find the effect of some essential oils on the growth and development of fruit rot of lemon caused by *Aspergillus niger* under *in-vitro* conditions. The pathogenicity test against *A. niger* was performed. The antifungal potential of different essential oils like clove, tarpin, neem, castor, chamomile and rose oil was carried out at different doses i.e. 50µl and 100µl to find out the effective and suitable oil for the growth inhibition of *A. niger.* The result indicates that high severity of disease was observed through the cut method of inoculation as compared to injection method (Figure 1). The results regarding the linear colony growth of *A. niger* under different essential oils were presented in (Table 1). The data clarified that minimum colony growth of *A. niger* (1.00 and 0.5 mm) was examined under clove oil at the dosage of 50µl and 100µl followed by tarpin oil (38.33 and 33.83 mm), neem oil (39.16 and 37.00 mm), castor oil (46.50 and 44.50 mm) and chamomile oil (64.16 and 60.33 mm), respectively. Maximum colony growth of *A. niger* (82.66 and 80.33 mm) was observed under rose oil at the dosage of 50µl and 100µl. Under control the *A. niger* showed (90 mm) colony growth. On the basis of means clove oil ranked 1st, tarpin oil ranked 2nd, neem oil ranked 3rd, castor oil ranked 4th, chamomile oil ranked 5th, rose oil ranked 6th for controlling colony growth of *A.niger* under *in-vitro* conditions presented in (Figure 2). Statistical analysis reveals that there was significant difference in the linear colony growth of *A. niger* among the essential oils at different dozes. The results showed that mean minimum rotting (12.53%) was recorded for clove oil followed by neem oil (13.30%), tarpin oil (13.42%), rose oil (14.27%), castor oil (15.16%) and chamomile oil (17.25%), respectively (Table 2). Maximum rotting (23.37%) was recorded for under control shown in (Figure 3). The analysis of obtained data revealed that there was significant difference in rotting percentage between the essential oils on different days.

Table 1. Effect of different essential oils on the linear colony growth of *A. niger*

Sr. No.	Essential oils	Doses		Mean
		50µl	100µl	
1.	Clove oil	1.00 h	0.50 h	0.75 f
2.	Tarpin oil	38.33 f	33.83 g	36.08 e
3.	Neem oil	39.16 f	37.00 f	38.08 e
4.	Castor oil	46.50 e	44.50 e	45.50 d
5.	Chamomile oil	64.16 c	60.33 d	62.25 c
6.	Rose oil	82.66 b	80.33 b	81.50 b
	Control	90.00 a	90.00 a	90.00 a

Table 2. Evaluation of rotting (%) of lemon fruits through direct contact with aqueous solution of essential oils

Sr. No.	Essential oils	Days							Mean
		1st	2nd	3rd	4th	5th	6th	7th	
1.	Clove oil	1.79	4.63	7.35	9.46	16.16	21.24	27.11	12.53 b
2.	Tarpin oil	1.59	3.53	6.68	10.90	18.16	22.90	30.22	13.42 b
3.	Neem oil	2.05	4.21	7.42	11.83	17.60	22.23	27.76	13.30 b
4.	Castor oil	1.64	3.53	6.18	11.37	21.32	26.92	35.20	15.16 b
5.	Chamomile oil	1.63	3.46	8.56	14.43	23.74	30.82	38.12	17.25 b
6.	Rose oil	1.70	3.22	6.85	12.15	18.69	25.02	32.30	14.27 b
	Control	4.32	8.02	14.23	19.93	29.14	44.14	46.49	23.37 a

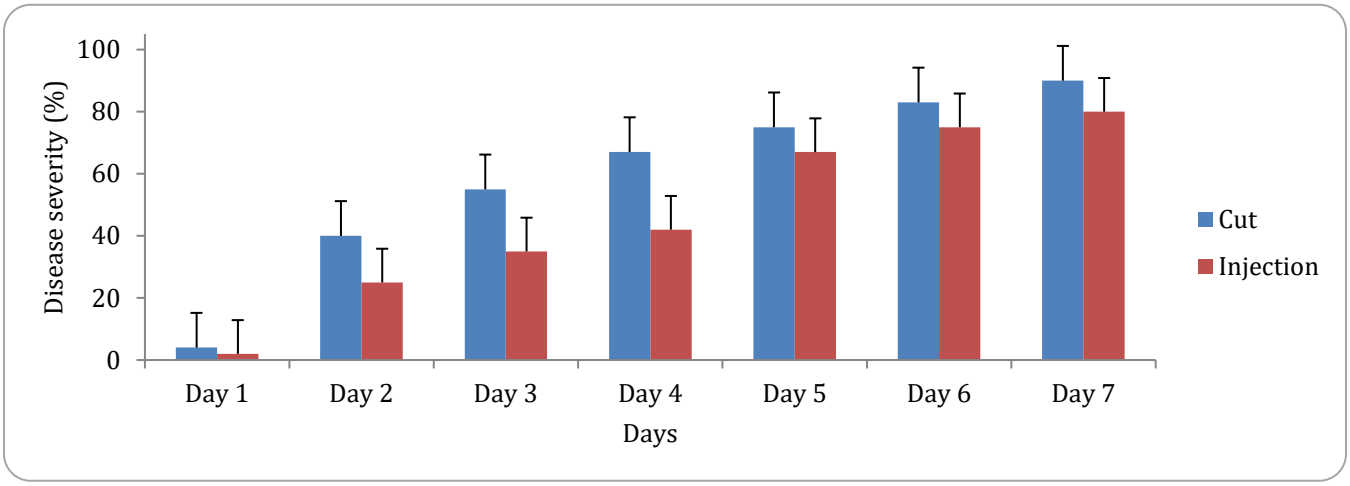

Figure 1. Pathogenicity test against *Aspergillus niger*

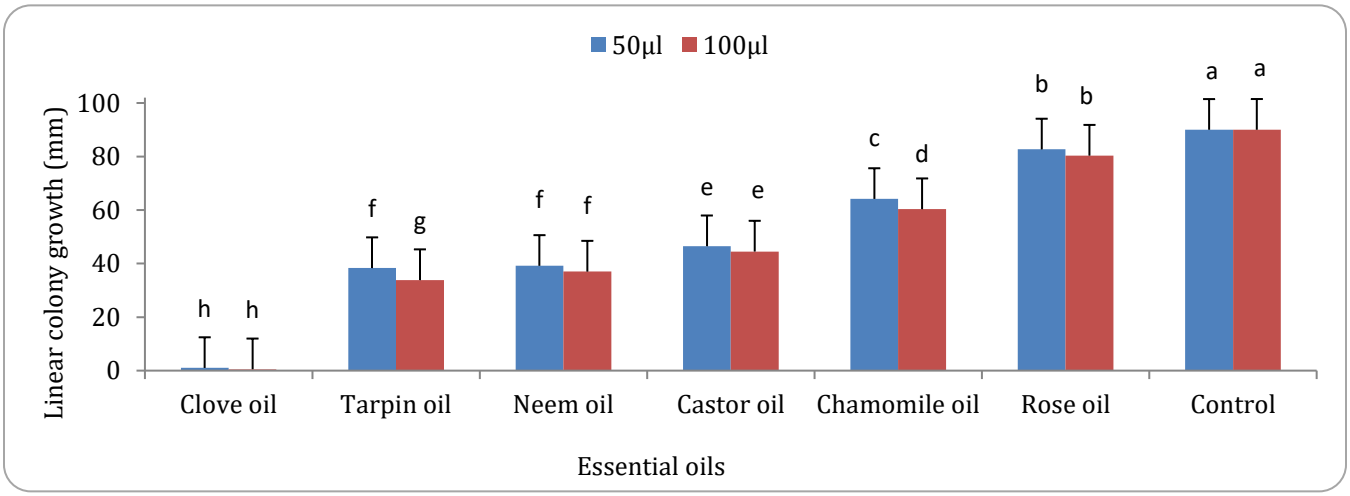

Figure 2. Effect of different essential oils on the linear colony growth of *Aspergillus niger*

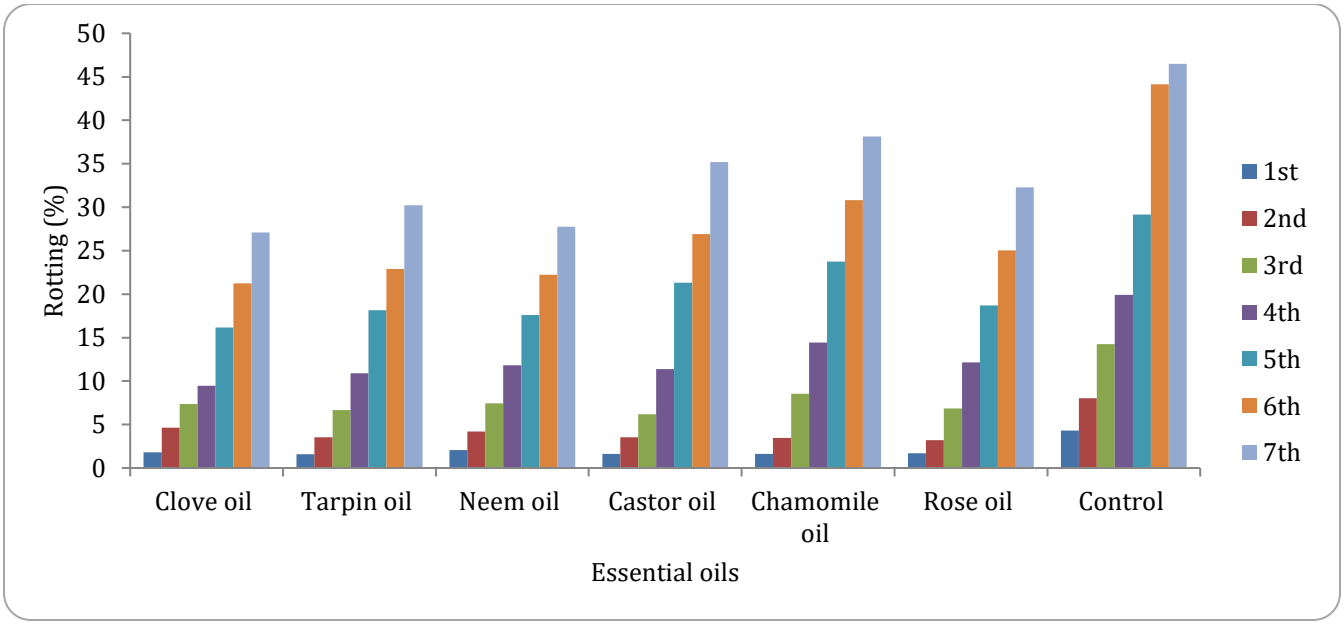

Figure 3. Evaluation of rotting (%) of lemon fruits through direct contact with aqueous solution of essential oils.

DISCUSSIONS

Lemon fruits of the local market of Hyderabad and Tando Jam found with varying incidence of different types of post-harvest rots. Among the rots, fruit rot of lemon found to be the most severe disease throughout the survey districts. In order to reduce the side effect of chemicals, an alternative method is introduced through this study for controlling the fruit rot of lemon. In this regards, some essential oils found to have promising history for the antimicrobial activity against a number of plant diseases. In present study six different oils such as clove, tarpin, neem, castor, chamomile and rose oil are used against fruit rot of lemon caused by *A. niger* under *in-vitro* conditions. *In-vitro* test against *A. niger* by the effect of volatile produced by essential oils or by the amendment of oils in the media revealed a great variation of fungal growth diameter among different treatments.Volatile effect of few essential oils are highly effective-completely inhibited the growth of *A. niger,* few were moderately effective-brought some reduction in the growth, while few were almost ineffective and failed to check the growth of the test pathogen.

Direct mixing of clove oil with media shows almost 100% inhibition of *A. niger* at all the used doses. The second most effective oil remains tarpin. The antifungal property of clove oil is determined by numerous researchers (Baratta *et al.,* 1998). The mycelial growth of post-harvest fungal pathogens isolated from Embul banana can be reduced by the use of clove oil (Ranasinghe *et al.,* 2002). Alrajhi, (2014) evaluated the effects essential oils extracted from Cinnamon , tea-tree and lemon grass against fungal pathogens of cinnamon *viz*. *Curvularia, Helminthosporium, Pestalotiopsis, Aspergillus, Rhizopus, Cladosporium, Nigrospora* and *Trichoderma*. On the other hand all the other used oil viz., castor, chamomile and rose oil also reduced the growth of the targeted pathogen but in most of the cases at higher used concentrations. Sitara *et al.* (2008) evaluated antifungal activity of neem seed EO against *Aspergillus niger*, *A. flavus, Fusarium oxysporum, F. moniliforme, F. nivale, F. semitectum, Drechsleraha wiinesis* and *Alternaria alternata*.

A number of fresh and healthy lemon fruits were inoculated with *Aspergillus niger* using cut and injection method of inoculations. In cut method, some mature lemon fruits were inoculated from 8-10 days old pure culture disc of *A. niger* in the cut made in the lemon fruits using sterilized cutter. While in injection method, the lemon fruits were inoculated with the aqueous solution of same pathogen (1ml of pathogen solution) using syringe needle. The inoculated portions of the lemon fruits were covered with the paraffin film as to check the invasion of other pathogens. Both the inoculated lemon samples were observed for 07 days. It is found that high severity of disease was determined through cut method of inoculation as compared to injection method. Clove oil ranked 1st, tarpin oil ranked 2nd, neem oil ranked 3rd, castor oil ranked 4th, chamomile

oil ranked 5th, rose oil ranked 6th for controlling the linear colony growth of *A. niger* under *in-vitro* conditions. The antifungal ability of EO found to be challenging to combat *A.niger* and is actually in accordance with the findings of Pawar and Thaker (2006). For further study of effectiveness of essential oils, the inoculated lemon fruits were immersed in aqueous solution of essential oils. Minimum rotting (12.53%) was recorded for clove oil followed by neem oil (13.30%), tarpin oil (13.42%), rose oil (14.27%), castor oil (15.16%) and chamomile oil (17.25%), respectively. Findings published by Guynot *et al.*, (2003) and Viuda-Martos *et al.*, (2007) are in agreement with the results obtained in our experiment. The method described in this study may be used on a broader level for obtaining a qualitative result and it will be no more than an idea about the volatile fraction of EOs.

Attack of different biotic and abiotic factors, can affect the production of lemon fruit and cause severe problems to the growers (Timmer and Graham, 1992). In 2014 survey, lemon fruit displayed wrinkled dark brown lesion in citrus production at Islamabad, Taxila, Rawalpindi and Wah districts, Pakistan. The severity of disease was higher (36.6%) in Islamabad followed by Rawalpindi (23%), Taxila (23%) and Wah (20%), correspondingly. Around 20% vegetable and fruits spoilage are associated with the damages caused by a variety of fungi. A study has been carried out to identify the fungi causing spoilt in fruits sold in the market of Gwagwalada, Abuja. According to their findings *Aspergillus niger* was one of the most prominent fungi which causing spoilage in lemon, oranges, pawpaw, pineapple, watermelon with a frequency (38%) followed by *Fusarium avenaceum* (31%), *Penicillium digitatum* and *Rhizopus stolonifer* (4%), *Saccharomyces* species (10%), *Fusarium solani* (8%), *Aspergillus flavus* (5%), respectively. *A. niger* were highly prevalent in orange (70%) followed by *F. avenaceum* (65%) in pawpaw. *Saccharomyces* species (40%) in watermelon, *P. digitatum* (20%) in tomato and *R. stolonifer* (5%) in orange, respectively (Samuel *et al.,* 2017).

CONCLUSION

On the basis of present investigation it is concluded that pathogenicity test against *A. niger* through cut method is more severe than that of injection method. Similarly, for controlling the linear colony growth of *A. niger* under *in vitro* conditions clove oil ranked 1st, tarpin oil ranked 2nd, neem oil ranked 3rd, castor oil ranked 4th, chamomile oil ranked 5th, and rose oil ranked 6th. The effect of EOs can also be shown by the numerous rotting percentages in lemon through direct contact of inoculated fruit samples with the aqueous solution of different essential oils.

RECOMMENDATIONS

As per conclusion it is suggested that clove oil may be used for controlling *A. niger* causing fruit rot of lemon. Furthermore, studies should be conducted to find the efficiency of clove oil against other fungal species on various fruits and vegetables.

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