Management of Post-Harvest Fruit Spoilage Fungi by Some Potential Spice Extracts

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Abstract: The experiment was carried out to evaluate the antifungal potential of aqueous and ethanolic extracts of four spices (*Allium sativum*, *Zingiber officinale*, *Cinnamomum zeylanicum* and *Capsicum annuum*) against the important post-harvest spoilage fungi isolated from diseased fruits. In total, 276 isolates of post-harvest spoilage fungi were isolated from four different fruit types (*Persea americana, Musa acuminate, Citrus sinensis* and *Lycopersicom esculentum*), out of which 183 isolates were identified while 93 isolates were remain unidentified. The most dominant post-harvest spoilage fungus was *Rhizopus* sp., (26.45%), followed by *Penicillium* sp., (19.93%), *Aspergillus* sp., (10.86%) and *Fusarium* sp., (9.06%). Results of disc diffusion assay showed that ethanolic extract of *C. zeylanicum* was found to be most effective against *Penicillium* sp., followed by aqueous extracts of *A. sativum*. The ethanolic extract of *C. zeylanicum* in agar amended assay and minimal inhibitory concentrations was found to be very efficient (100% inhibition) against all the tested fungi. Result of *in-vivo* study showed that pre-inoculated *C. zeylanicum* ethanolic extract and *A. sativum* aqueous extract were found effective in reducing the disease severity (6.24-13.67%) and (7.91-13.15%) against the *Penicillium*, *Rhizopus*, *Aspergillus* and *Fusarium* spp.

Keywords: Aqueous extract, Diseased fruits, Disease severity, Ethanolic extract, Inhibition.

INTRODUCTION

Fruits play a vital role in the human nutrition and are the major source of nutrients, dietary fibers and vitamins C, thiamin, riboflavin, niacin, folate, A and E [1], beside this it also contains antioxidant compounds protect it against the oxidative damages caused by free radicals [2]. It may also reduce the risk of cardiovascular, cancers and neurological diseases [3]. Due to highly-diversified agro-ecological conditions the fruits were cultivated only in the central rift valley and eastern part of the country. The commonly cultivated fruits are avocado, banana, orange, papaya and guava. These fruits have the short shelf-life and were usually deteriorated with some fruit spoilage fungi.

Post-harvest fruit losses were more severe due to inadequate storage and transportation facilities in developing countries [4, 5]. It has been reported that about 50% post-harvest loss was due to poor storage conditions [6], which may permit the spoilage fungi to infect the fruits. The common post-harvest spoilage fungi are *Alternaria* sp., *Aspergillus* sp., *Botrytis cinerea*, *Penicillium* sp., *Monilinia lax* and *Rhizopus stolonifer* [7]. However, Ibrahim and Rahma [8] found that *Rhizopus*, *Mucor*, *Torula*, *Penicillium*, *Aspergillus* and *Alternaria* sp. were the dominant fungal pathogens associated with date fruits. Droby [9] stated that about 20-25% of the harvested fruits were deteriorate by spoilage fungi during post-harvest handling. However, Serrano *et al*. [10] found that *Penicillium*, *Fusarium* and *Aspergillus* sp. were common post-harvest spoilage fungi caused 10-30% loss in fruit yield. Similarly, Palumbo *et al.* [11] reported that *A. flavus* is responsible fungi for the deterioration of fruits under stored condition.

Synthetic fungicides, such as thiabendazole, imazalil and sodium ortho-phenyl phonate have been traditionally used to control the diseases caused by post-harvest spoilage fungi [12]. Use of synthetic fungicides is expensive, having negative impact on the human as well as environmental conditions [13]. In this regards, spice extracts provide an alternative way to control postharvest spoilage fungi because of bioactive compounds, volatile essential and antifungal property [14-16]. Application of plant extracts is now gaining popularity due to its biodegradable nature and safeness towards health against the fungal diseases [17]. Earlier studies clearly indicated that use of plant extracts had the great potential against the fungal pathogens [18, 19]. Gupta *et al.* [15] reported that *Cinnamon* plant extract caused the inhibitory effect against *Penicillum* sp., while *Cinnamon* oil extract was effective against *Alternaria* sp., *Aspergillus fumigates*, *Aspergillus niger*, *Aspergillus* sp., *Penicillum* sp., *Rhizopus* sp. and *Rhizomucor* sp. Similarly, aqueous, methanolic and ethanolic extracts of *A. sativum* have high potency against the pathogenic fungi on sweet potato and yam [20], and the *C. zeylanicum* ethanolic extract was found to be effective against *Penicillium* sp. [21].

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The objective of this study was i) to isolate and characterize some fruit spoilage fungi from selected fruits ii) to evaluate the effectiveness of spice extracts in inhibiting the growth of important post-harvest fruit spoilage fungi iii) to determine the MIC (minimum inhibitory concentration) of the spice extracts iv) to evaluate the effectiveness of the spice extracts in extending the shelf life of some fruits samples challenged with spoilage fungi.

MATERIALS AND METHODS

Description of the Study Area

The study was conducted at Department of Biology, College of Natural Sciences, Jimma University, Jimma, located at 353 km in south-west of Addis Ababa. This town is geographical coordinated between 7°41' N latitude and 36°50' E longitude with an average altitude of about 1,780 m above sea level. The annual temperature ranges from 14-30°C and the rainfall ranges from 1138-1690 mm.

Collection of Spice Samples

The spice samples were selected because on the traditional practices of these spices by the local peoples as preservatives, food flavoring and seasoning agents. The spice samples (Garlic (bulb), Ginger (root and rhizome), Cinnamon (bark) and Chilli (fruit) were purchased from the local market of Jimma town.

Preparation of Spice Extracts

Each spice samples were washed thrice with sterile distilled water to remove the adhering the soil particles and dried at room temperature. After drying each samples were macerated in Waring blender and the macerated materials were extraction purposes.

Preparation of Crude Ethanolic Extracts

A 90% ethanolic extract was prepared by the method of Alade and Irobi [22] with minor modification. The modification was made by increasing the concentration of the solvent (ethanol) used from 70% to 90% to enhance the inhibitory effects of the spice extracts. About 100 g of each dried powder spice was soaked in 500 ml of 90% ethanol for 48 hrs. After soaking the mixtures were filtered using cheese cloth followed by Whatman filter paper No.1. The organic solvents were evaporated using Rota vapor under reduced temperature and about 3 g extract of each samples were taken and diluted by adding 10 ml of sterile distilled water to obtain a concentration of 30% of the extract. The stock extracts were transferred to per-labeled sterile screw capped bottles enclosed with clothes and stored at 4°C for further used.

Preparation of Crude Aqueous Extracts

Aqueous extracts were prepared in 100 g/500 ml (w/v) of sterilized distilled water and shaken at 130 rpm continuously for 1 hr using a rotary shaker following the method of Onwuliri and Wonang [23]. The mixtures were allowed to stand for 48 hrs and filtered using cheese cloth followed by Whatman filter paper No. 1 and stored at about 4^{o}C in the refrigerator until used.

Isolation and Characterization of Spoilage Fungi

A total of 120 samples comprising of 30 samples of each fruit (*Persea Americana* (Avacado), *Musa acuminate* (Banana), *Citrus sinensis* (Orange) and *Lycopersicon esculentum* (Tomato)) showing the post-harvest disease symptoms were collected randomly in sterile polyethylene bag from the local market of Jimma town.

The post-harvest spoilage fungi were isolated from diseased fruits by the method of Al-Hindi *et al*. [5] on Potato Dextrose Agar (PDA) medium having the pH 5.5. Chloramphenicol (100 mg per 1000 ml of sterilized medium) was added to suppress bacterial contamination. Small infected portions of the fruit were taken by sterilized scalpel and placed on the pre-prepared Petri plates PDA. The inoculated Petri plates were incubated at 25 ± 2^{o}C for 5 days. The colonies of each isolates were identified using taxonomic and morphological keys [24] and the pure culture of each isolate was maintained separately and stored in refrigerator 4^{o}C for further used. The fungal isolates were designated as JUA_n (Jimma University avocado isolates), JUB_n (Jimma University banana isolates), JUO_n (Jimma University orange isolates) and JUT_n (Jimma University tomato isolates).

Antifungal Analysis

The extent of antifungal activity of the crude extracts was evaluated against the four post-harvest fruit spoilage fungi. *In-vitro* antifungal activity of crude extracts was carried out by disk diffusion and amended agar methods. Determination of the minimal inhibitory concentrations (MIC) by agar dilution method and *in-vivo* antifungal activity that involves evaluation of the effectiveness of the spice extracts in extending the shelf life of fresh tomato fruits by artificial inoculation.

In-vitro Antifungal Activity of Crude Extracts by Disc Diffusion Method

The isolated fruit spoilage fungi were grown on PDA (Hi-Media, Pvt. Ltd. Mumbai, India) medium and the spore suspension (1×10^4 spore/ml) was prepared. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were prepared and aseptically placed on each Petri plates containing PDA, pre-inoculated with respective spoilage fungi. A 30 µl of each crude extracts was impregnated on-to the disk by sterile micropipette tips and the same amount of sterile distilled water was added as negative control. The Petri plates were left for 30 min at room temperature to allow the extract diffusion, and were incubated at 25°C for 5 days. At the end of the incubation period, antifungal activity was evaluated by measuring zone of inhibition against the tested spoilage fungi using a ruler. All treatments were replicated thrice.

In-vitro Antifungal Activity of Crude Extracts by Amended Agar Method

The potato dextrose agar (PDA) medium with pH 3.5 was prepared. To prevent the bacterial growth, chloramphenicol 0.25% was also added. One ml of each tested crude extracts was added to 20 ml acidified PDA medium. The content was mixed well and poured into Petri-dishes and allowed to solidify at room temperature for 30 min. After solidification of the plates, mycelia discs (5 mm in diameter) were collected from the periphery of actively growing fungal cultures using sterile cork borer and placed at the centre of the amended PDA Petri plates. Petri plates with PDA without spice extract were used as control. Inoculated plates were incubated at 25°C for 5 days. All treatments were replicated thrice. Percentage radial growth inhibition of the spice extracts was calculated by the formula of Hernandez-Castillo *et al.* [25].

$$\% \text{ Inhibition} = \frac{\text{Growth of fungus in control} - \text{Growth of fungus in extract}}{\text{Control}} \times 100$$

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the crude extracts was conducted by agar dilution method [26]. Different concentrations of the extracts (50, 100, 150, 200, 250, and 300 mg/ml (w/v)) were prepared. One ml from each concentration of the extract was added to 20 ml of PDA medium and vortexes for 30s. The content was poured into Petri-plates and allowed to solidify at room temperature for 30 min. After solidification of the plates, mycelia discs (5 mm in diameter) were collected from the periphery of actively growing fungal cultures using sterile cork borer and placed at the centre of the amended PDA plates. The Petri plates without any extract were used as control. After inoculation, the plates were incubated at 25°C for 5 days. At the end of the incubation period, the plates were evaluated for the presence or absence of fungal growth. The Minimum Inhibitory Concentration (MIC) was determined as the least concentration of the spice extract showed an inhibitory effect against the mycelial growth of the tested spoilage fungi against the control by the radial growth method [27]. All treatments were replicated thrice.

In-vivo Antifungal Activity of Crude Extracts

To confirm the effectiveness of the crude extracts against the disease causing spoilage fungi healthy fresh tomato fruits of almost similar shape, size were collected. These fruits were surface sterilized with 1% Sodium hypochlorite (NaOCl) solution for 2 min, rinsed thrice with sterile distilled water, and air-dried before treatment. *In-vivo* antifungal activities of the spice extracts against the spoilage fungi were estimated by method of Sharma *et al.* [18].

Inoculums Preparation and Standardization

The tested fungi were grown on PDA medium for sporulation on Petri plates for 5 days and the fresh stock suspension (1×10^4 spore/ml) of each spoilage fungi was prepared.

Inoculations Procedure

The aqueous and ethanol extracts of spices at a concentration of 30µl were pre-inoculated (12 hrs before fungal spore inoculation); simultaneous and post-inoculated (12 hrs after fungal spore inoculation) to tomato fruits.

Experimental Design

The experiment was carried out in a randomized block design with following treatment 1) Fruit wound + aqueous *C. zeylanicum* extract + spoilage fungi; 2) Fruit wound + ethanolic *C. zeylanicum* extract + spoilage fungi; 3) Fruit wound + aqueous *A. sativum* + spoilage fungi; 4) Fruit wound + ethanolic *A. sativum* extract + spoilage fungi; 5) Fruit wound + Sodium benzoate (0.1%) + spoilage fungi (positive control); 6) Fruit wound + spoilage fungi (negative control). All the treatments were replicated thrice. The disease severity

(percentage of spoilage) was computed by measuring the radius of the rotted symptom (lesion) from edge of the inoculums wound on the fruit surface [28].

Data Analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD. The collected data were statistically analyzed using one-way analysis of variance (ANOVA) by SPSS ver. 16.0 followed by Tukey's post hoc multiple comparison test at p = 0.05.

RESULTS

Isolation and Characterization of Spoilage Fungi Associated with Fruits

A total of 276 fruit spoilage fungi were isolated from 120 samples of four types of diseased fruits namely Avocado (*Persea americana)*, Banana *(Musa acuminata)*, Orange (*Citrus sinensis*) and Tomato (*Lycopersicom esculentum*). The microscopic study also revealed that a mass of brownish, necrotic patches were present on the surface of diseased fruits. The mycelial colonies of each isolates were identified (Table **1**) using taxonomic and morphological keys of Cheesbrough [24].

Prevalence of Spoilage Fungi Isolated from Diseased Fruits

In total 276 isolates of post-harvest spoilage fungi were isolated from four fruit types (*P. americana, M. acuminate, C. sinensis* and *L. esculentum*), out of which 183 (66.30%) isolates were identified while 93 (33.70%) isolates were remain unidentified (Table **2**). The highest number of spoilage fungi were isolated from tomato (31.88%) followed by banana (27.17%), orange (25.00%) and avocado (15.94%). The most dominant post-harvest spoilage fungus was *Rhizopus* sp. (26.45%), followed by *Penicillium* sp. (19.93%), *Aspergillus* sp. (10.86%) and *Fusarium* sp. (9.06%). *Rhizopus* and *Aspergillus* sp. were wide spread among all examined fruits, while, *Fusarium* and *Penicillium* sp. were isolated from orange and tomato (Table **2**).

Antifungal Analysis

In-vitro Antifungal Activity of Crude Extracts by Disk Diffusion Assay

The crude ethanolic and aqueous extracts of all the spices (*C. zeylanicum*, *C. annuum*, *A. sativum* and *Z. officinale*) showed different degrees of mycelial growth inhibition on the different tested spoilage fungi (Table **3**). The ethanolic extracts of all spices showed

Table 1: Colonial and Morphological Characteristics of Fungi Associated with the Spoilage of Fruits

Isolates No.	Characteristics of colony	Structural Morphology	Identified genera
JUA000 JUB001-007 JUO001-004 JUT001-014	Cottony, pink, purple, brown colonies.	Extensive septet mycelium, conidiophores simple or branched with ovoid to elongated conidia of variable sizes. Conidia are septate, fusiform, slightly curved and pointed at both ends.	*Fusarium* sp.
JUA001-018 JUO005-029 JUT015-026	Greenish or blue green colonies.	Conidia in long chains on branched conidiophores resembles brush like head (Penicllus). Conidiophores are smooth, relatively short. Penicillia arranged very irregular and asymmetrical with branches of various lengths.	*Penicillium* sp.
JUA019-024 JUB008-021 JUO030-034 JUT027-031	Colonies with loose white to yellow mycelium rapidly becoming dark brown to black on the development of conidia. Colonies light green-yellow.	Black, brownish black or purple brown conidiophores. Conidia are yellow to green with dark sclerotia. Conidiophores arising from a foot-cell, catenate (basipetal) conidia on phialides (1or 2 series) on vesicle.	*Aspergillus* sp.
JUA025-039 JUB022-038 JUO035-055 JUT021-051	White to dark grey colonies, fast growing with dense cottony mycelium, producing mass of sporangia.	Non-septate mycelium with root like rhizoids; black columellate, sporangiophores, in clusters and dark sporangia containing dark to pale spores.	*Rhizopus* sp.
JUA040-044 JUB039-075 JUO056-069 JUT052-088	–	–	Unidentified

A= Avocado; B= Banana; O= Orange; T= Tomato; n= Code no. of isolates.

Table 2: Prevalence of Spoilage Fungi Isolated from Deteriorating Fruits Sold in Jimma Town

Fruits	No. of samples	No. of isolates	*Aspergillus* sp.	*Fusarium* sp.	*Penicillium* sp.	*Rhizopus* sp.	Unidentified
Avocado	30	44 (15.94)	6 (13.63)	-	18 (40.90)	15 (34.09)	5 (11.36)
Banana	30	75 (27.17)	14 (18.66)	7 (9.33)	-	17 (22.66)	37(49.33)
Orange	30	69 (25.00)	5 (7.24)	4 (5.79)	25 (36.23)	21 (30.43)	14 (20.28)
Tomato	30	88 (31.88)	5 (5.68)	14 (15.9)	12 (13.63)	20 (22.72)	37 (42.04)
Total	120	276 (100)	30 (10.86)	25 (9.06)	55 (19.93)	73 (26.45)	93 (33.69)

Note: Values in parenthesis are percentages of the isolated fungi.

significant antifungal activity (p = 0.05) compared to control (untreated one) (Table **3**).

The ethanolic extracts of cinnamon had the highest antifungal activity (16.00-24.33mm) against all the tested fungi followed by *A. sativum* (10.33-13.33mm), *C. annum* (9.66-11.66mm) and *Z. officinale* (6.00-10.66mm), respectively. However, there is no significant difference (p = 0.05) in the antifungal activity of the *A. sativum* and *C. annuum* ethanolic extracts against all the tested spoilage fungi. Of all ethanolic extracts tested, the *C. zeylanicum* extract showed the highest antifungal activity against *Penicillium* sp. (24.33mm), and *Fusarium* sp. (16.66mm). However, ethanolic extracts of *A. sativum* showed the maximin inhibition zone (13.33mm) against *Penicillium* sp., while, *Z. officinale* and *C. annuum* showed a maximum inhibition zone of 10.66mm and 11.66mm against *Aspergillus* sp. Aqueous extracts of all the spices had a lesser inhibitory effect against all the tested fungi compared to ethanolic extracts. Aqueous extracts from *A. sativum* and *C. annuum* were more effective compared to others tested extracts. *A. sativum* showed the maximum inhibition zone (14.60mm) against *Aspergillus* sp., followed by *C. zeylanicum* (11.30mm) against *Fusarium* species (Table **3**). However, *Z. officinale* and *C. annuum* exhibit the maximum inhibitory zone (11.30mm and 10.00mm) against *Penicillium*, respectively. There was no significant difference in the antifungal activity of *C. zeylanicum*, *C. annuum* and *Z. officinale* aqueous extracts against *Aspergillus, Penicillium* and *Fusarium* isolates.

In-vitro Antifungal Activity of Crude Extracts by Amended Agar Method

In-vitro antifungal activity of aqueous and ethanolic extracts of all spices against the tested spoilage fungi was measurement by the radial growth of respective fungi. The crude extracts of all spices showed different degrees of inhibition in the mycelial growth against the tested spoilage fungi compared to control (Table **4**). The inhibitory effects of all the spices were significant (p = 0.05) against all the tested fungi. The inhibitory effects of ethanolic extracts of all species ranged from 68.34-100% (Figure **1**). However, the ethanolic extracts of *A. sativum* inhibited the growth of all the tested fungi in the range of 76.13-93.40% against *Penicillium* and

Table 3: *In-vitro* Antifungal Activity of Four Crude Extracts of Spices Against the Tested Fungi Using Disk Diffusion Assay

Solvents	Spices	Inhibition zone diameter in mm (Mean ± SD)			
		Aspergillus sp.	*Fusarium* sp.	*Penicillium* sp.	*Rhizopus* sp.
DDW	Control	—	—	—	—
Ethanol	*C. zeylanicum*	16.00±1.00^{a}	16.66±2.08^{a}	24.33±2.51^{a}	16.00±0.86^{a}
	C. annuum	11.66±1.15^{bc}	10.66±0.58^{b}	10.00±1.00^{b}	9.66±1.53^{bc}
	A. sativum	11.00±1.00^{cd}	10.33±0.58^{b}	13.33±0.58^{b}	11.00±1.00^{b}
	Z. officinale	10.66±2.08^{cd}	9.33±1.15^{b}	9.66±0.58^{b}	6.00±1.00^{c}
Aqueous	*C. zeylanicum*	10.00±1.00^{cd}	11.33±1.52^{b}	10.66±1.15^{b}	9.00±1.00^{bc}
	C. annuum	9.66±0.57^{cd}	10.00±1.00^{b}	10.00±1.00^{b}	7.0±2.00b^{c}
	A. sativum	14.66±1.15^{ab}	11.00±1.00^{b}	12.60±1.53^{b}	8.66±1.15^{bc}
	Z. officinale	8.00±1.00^{d}	8.00±2.00^{c}	11.33±1.15^{b}	8.33±1.52^{bc}

Mean values followed by same superscripts within a column are not significantly different using Post Hoc multiple Comparison test ($P > 0.05$).

Table 4: ***In-vitro*** **Antifungal Activity of Four Crude Spice Extracts Against the Tested Fungi Using Amended Agar Method**

Solvents	Spices	Mean radial growth (mm) and Inhibition (%) of tested fungi							
		***Aspergillus* sp.**		***Fusarium* sp.**		***Penicillium* sp.**		***Rhizopus* sp.**	
		R.G	**I (%)**	**R.G**	**I (%)**	**R.G**	**I (%)**	**R.G**	**I (%)**
DDW	Control	30.33^{a}	0.00^{e}	28.66^{a}	0.00^{e}	29.33^{a}	0.00^{e}	32.00^{a}	0.00^{e}
Ethanol	*C. zeylanicum*	0.00^{e}	100.00^{a}	0.00^{e}	100.00^{a}	0.00^{e}	100.00^{a}	0.00^{e}	100.00^{a}
	C. annuum	4.66^{d}	84.63^{b}	5.33^{c}	81.40^{c}	5.00^{cd}	82.95^{bc}	3.00^{d}	90.62^{b}
	A. sativum	2.00^{d}	93.40^{b}	6.66^{c}	76.76^{c}	7.00^{c}	76.13^{c}	2.36^{d}	92.62^{b}
	Z. officinale	3.00^{d}	90.10^{b}	3.36^{d}	88.27^{b}	5.33^{c}	81.82^{c}	3.33^{d}	89.59^{b}
Aqueous	*C. zeylanicum*	9.66^{c}	68.34^{c}	7.00^{c}	75.52^{c}	6.00^{c}	79.54^{c}	9.33^{c}	70.84^{c}
	C. annuum	12.30^{b}	59.40^{d}	10.60^{b}	63.01^{d}	11.66^{b}	60.45^{d}	12.33^{b}	61.46^{d}
	A. sativum	2.00^{d}	93.40^{b}	3.36^{d}	88.27^{b}	4.66^{d}	84.11^{b}	8.66^{c}	72.93^{c}
	Z. officinale	12.66^{b}	58.25^{d}	11.33^{b}	60.46^{d}	11.66^{b}	60.24^{d}	13.00^{b}	59.37^{d}

Mean values followed by same letters within a column are not significantly different using Post Hoc multiple Comparisons test ($P > 0.05$); RG= Radial Growth, I= Inhibition.

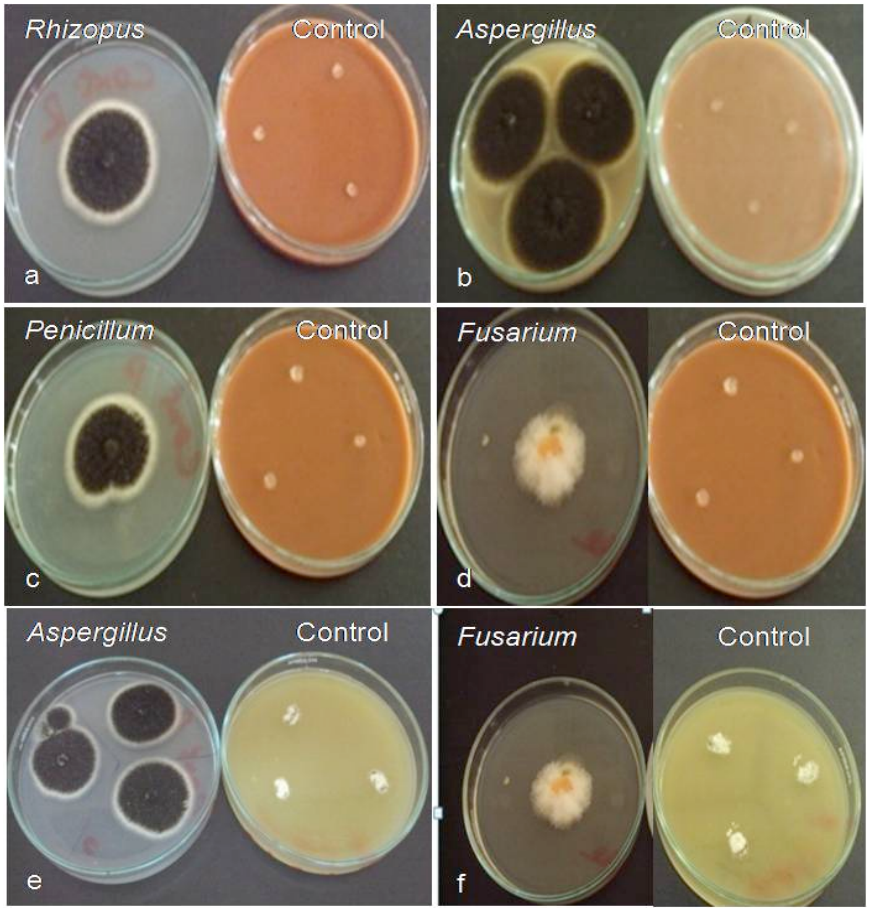

Figure 1: *In-vitro* antifungal activity of the most effective spice extracts against the tested fungal pathogens (**a**) *C. zeylanicum* ethanolic extract against *Rhizopus* (**b**) *C. zeylanicum* ethanolic extract against *Aspergillus* (**c**) *C. zeylanicum* ethanolic extract against *Penicillium* and (**d**) *C. zeylanicum* ethanolic extract against *Fusarium* spp. along with their respective controls; (**e**) *A. sativum* aqueous extract against *Aspergillus* (**f**) *A. sativum* aqueous extract against *Fusarium* spp. along with their controls.

Aspergillus spp. *C. annuum* and *Z. officinale* ethanolic extracts were also active against all of the tested fungal spp., (Table **4**). The inhibitory effect of aqueous extracts of tested spices ranged from 58.25-93.40% with the highest inhibition (93.40%) by *A. sativum* against *Aspergillus* spp. (Figure **1**) followed by *C.*

zeylanicum (79.54%) against *Penicillium* sp., *C. annuum* (63.01%) against *Fusarium* sp. and ginger (60.46%) against *Fusarium* sp. (Table **4**).

In-vivo Antifungal Activity of Crude Extracts

In-vivo antifungal activity of crude ethanolic and aqueous extracts of *C. zeylanicum* and *A. sativum* showed significant reduction in disease severity (Figures **2**, **3** and **4**) compared to the control (untreated) when applied 12 h before, after and at the same time with the pathogen. Pre-inoculated ethanolic extracts showed significant disease severity reduction ranging from 6.24% against *Penicillium* to 13.88% against *Aspergillus* (Figure **2**). *C. zeylanicum* ethanolic extract recorded a disease severity reduction of 6.24%, 10.18%, 12.77% and 13.67% against *Penicillium, Rhizopus, Aspergillus* and *Fusarium*, respectively where as ethanolic extracts of *A. sativum* recorded 8.5%, 12.22%, 13.23% and 13.88% against *Penicillium, Rhizopus, Fusarium* and *Aspergillus* isolates respectively (Figure **2**). However, the two ethanolic extracts showed no significant (p = 0.05) difference in disease severity reduction against all the tested fungal isolates. The two extracts also showed significant relationship in disease severity reduction with that of sodium benzoate (positive control) against *Penicillium* only.

Similarly, Pre-inoculated aqueous extracts of the two spices also showed significant disease severity reduction ranging from 7.9% to 40.20% against the tested fungi (Figure **2**). *A. sativum* aqueous extract showed the highest percentage of disease severity reduction against *Penicillium* (7.91%) followed by *Aspergillus* (9.55%), *Rhizopus* (11.19%) and *Fusarium* (13.15%). In addition, the two aqueous extracts showed significant (p = 0.05) difference in disease severity reduction against all the tested fungi. *A. sativum* aqueous extract showed significant relationship in disease severity reduction with that of sodium benzoate (positive control) against *Penicillium* only (Figure **2**).

Simultaneous inoculation, ethanolic extracts showed significant disease severity reduction ranging from 12.33% against *Aspergillus* to 15.46% against *Rhizopus* (Figure **3**). *A. sativum* ethanolic extract recorded a disease severity reduction of 12.33%, 14.01%, 15.41%, 15.46% against *Aspergillus, Fusarium, Penicillium, Rhizopus*, respectively where as *C. zeylanicum* ethanolic extract scored 15.22%, 13.23%, 14.29% and 15.29% respectively against the tested fungi in the same order (Figure **3**). Nevertheless, the two ethanolic extracts have no significant (p = 0.05) difference in disease severity reduction against all the tested fungi. Aqueous extracts applied with the pathogen at the same time were also showed disease severity reduction ranging from 27.91% against *Fusarium* to 61.03% against *Penicillium*. A reduction of disease severity (27.91%) against *Aspergillus* was the highest by *A. sativum* aqueous extract, followed by *C. zeylanicum* which recorded 27.99% disease severity reduction against *Aspergillus*. In addition, the two aqueous extracts showed significant difference (p = 0.05) in disease severity reduction against *Aspergillus* and *Penicillium* (Figure **3**).

Post-inoculated ethanolic extracts showed a reduction in disease severity which ranged from

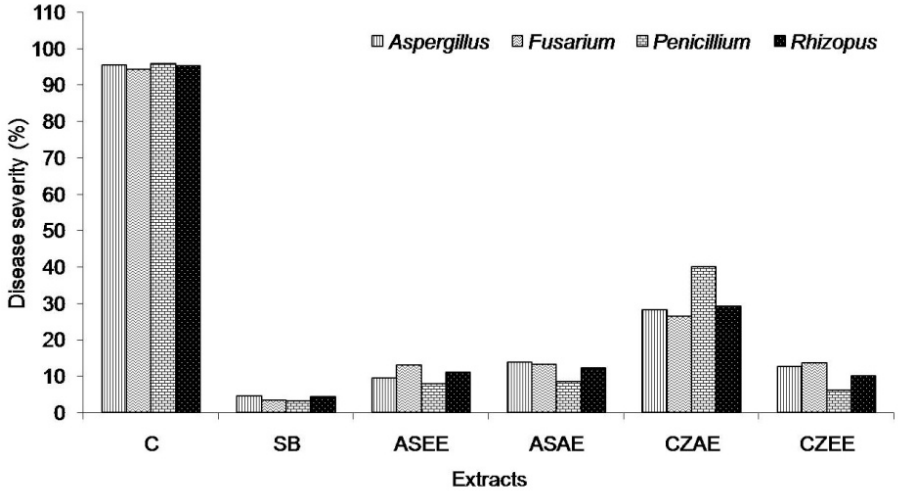

Figure 2: *In-vivo* antifungal activity of pre-inoculated spice extracts on tomato against the fruit spoilage fungi. SB= Sodium Benzoate, ASEE= *A. sativum* ethanolic extract, ASAE= *A. sativum* aqueous extract, CZEE= *C. zeylanicum* ethanolic extract CZAE= *C. zeylanicum* aqueous extract.

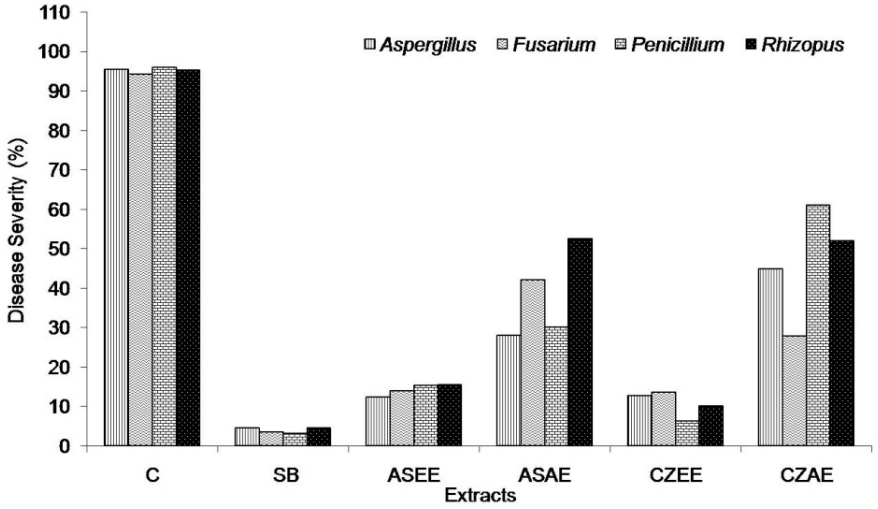

Figure 3: *In-vivo* antifungal activity of simultaneously inoculated spice extracts on tomato against the fruit spoilage fungi. SB= Sodium Benzoate, ASEE= *A. sativum* ethanolic extract, ASAE= *A. sativum* aqueous extract, CZEE= *C. zeylanicum* ethanolic extract CZAE= *C. zeylanicum* aqueous extract.

23.54% against *Penicillium* to 71.66% against *Rhizopus* which was lower than that of pre and simultaneous inoculation (Figure **4**). *C. zeylanicum* recorded a disease severity reduction of 51.66%, 53.23%, 23.54% and 37.28 against *Aspergillus, Fusarium, Penicillium* and *Rhizopus,* respectively whereas *A. sativum* reduced disease severity by 57.22%, 47.06%, 65.2% and 71.66% against the tested fungi in the same order (Figure **4**). However, the two ethanolic extracts have no significant (p = 0.05) difference in disease severity reduction against *Aspergillus* and *Fusarium.* Of the post-inoculated aqueous crude extracts, *A. sativum* recorded the highest disease severity reduction against *Penicillium* (9.20%) and *Rhizopus* (16.42%). On the other hand, *C. zeylanicum* showed a reduction in disease severity ranging from 46.88% against *Rhizopus* to 68.11% against *Fusarium*. In addition, the two aqueous extracts showed significant (p = 0.05) difference in disease severity reduction against all the tested fungal isolates.

Determination of the Minimal Inhibitory Concentrations (MIC)

All the crude extracts showed a significant reduction in mycelial growth at a concentration of less than 30% against the tested fungi compared to control. *C. zeylanicum* ethanolic extract had the potential to inhibit the growth of spoilage fungi at a very low concentration compared to the others (Table **5**). The lowest concentration of *C. zeylanicum* ethanolic extract inhibited the growth of *Penicillium* spp. and *Fusarium* spp. was 20 %, while in case of *Aspergillus* sp. and

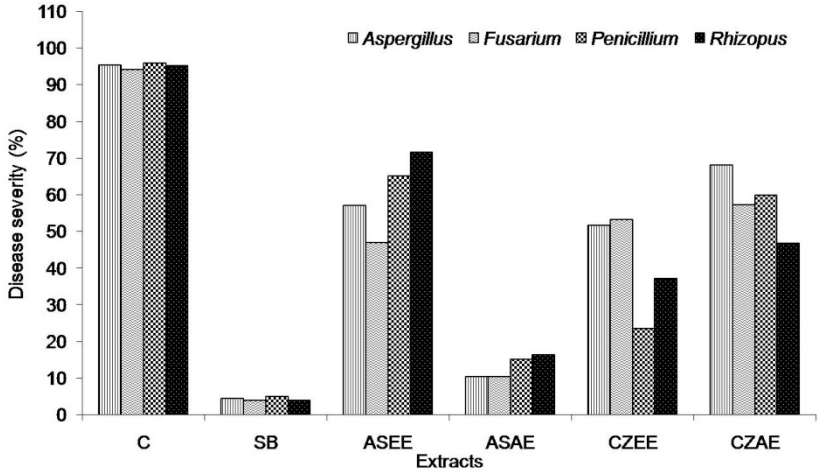

Figure 4: *In-vivo* antifungal activity of post-inoculated spice extracts on tomato against the fruits spoilage fungi. SB= Sodium Benzoate, ASEE= *A. sativum* ethanolic extract, ASAE= *A. sativum* aqueous extract, CZEE= *C. zeylanicum* ethanolic extract CZAE= *C. zeylanicum* aqueous extract.

Table 5: Minimum Inhibitory Concentrations (in %) of Crude Extracts of *C. zeylanicum* and *A. sativum* Extracts (Ethanolic and Aqueous) Against Mycelial Extension (mm) of the Tested Fungi

Extracts	Conc. (%)	Mycelial growth (mm)			
		***Aspergillus* sp.**	***Fusarium* sp.**	***Penicillium* sp.**	***Rhizopus* sp.**
Control	0	30.33±0.57^{a}	28.66±0.57^{a}	29.33±0.57^{a}	32.00±1.00^{a}
CZEE	5	15.33±0.58^{e}	15.00±0.00^{f}	13.50±0.86^{c}	15.50±0.50^{f}
	10	11.00±1.00^{g}	11.50±0.50^{g}	9.50±0.50^{d}	11.66±0.28^{g}
	15	9.66±0.57^{g}	7.83±0.76^{h}	8.00±1.00^{d}	9.16±0.28^{h}
	20	7.33±0.57^{h}	0.00±0.00^{i}	0.00±0.00^{f}	7.00±1.00^{h}
	25	3.16±0.28^{j}	0.00±0.00^{i}	0.00±0.00^{f}	3.00±0.05^{i}
	30	0.00±0.00^{k}	0.00±0.00^{i}	0.00±0.00^{f}	0.00±0.00^{j}
CZAE	5	27.83±0.76^{b}	26.00±2.00^{b}	25.66±0.57^{b}	28.00±2.00^{b}
	10	25.16±0.28^{c}	23.33±0.28^{c}	22.00±0.28^{c}	24.33±0.57^{c}
	15	20.33±0.57^{d}	19.00±1.00^{e}	17.60±0.57^{c}	20.00±1.00^{d}
	20	15.50±0.50^{e}	14.66±0.28^{f}	12.00±0.50^{cd}	16.33±0.57^{ef}
	25	12.66±0.50^{f}	10.33±0.57^{g}	8.33±0.57^{d}	12.00±0.00^{g}
	30	9.66±0.57^{g}	7.00±0.50^{h}	6.00±0.28^{e}	9.33±0.57^{h}
ASEE	5	16.00±0.00^{e}	16.00±0.50^{f}	16.33±0.57^{c}	15.66±0.28f
	10	12.33±0.57^{f}	13.33±0.28^{f}	13.16±0.28^{cd}	12.66±0.57^{g}
	15	10.50±0.86^{g}	11.83±0.76^{g}	11.66±0.57^{cd}	10.50±0.50^{gh}
	20	8.00±0.00^{gh}	9.83±0.76^{g}	9.33±0.57^{d}	7.66±0.76^{h}
	25	5.33±0.28^{i}	7.66±0.62^{h}	8.00±1.00^{d}	4.33±0.57^{i}
	30	2.00±0.57^{j}	6.63±0.57^{h}	4.00±0.50^{ef}	2.33±0.57^{i}
ASAE	5	27.66±0.28^{b}	25.33±0.28^{b}	26.00±0.50^{b}	27.33±0.57^{b}
	10	24.33±0.57^{c}	21.66±0.28^{d}	22.33±0.58^{c}	23.00±0.50^{c}
	15	18.50±0.86^{de}	16.33±0.57^{f}	17.00±1.00^{c}	17.33±0.28^{e}
	20	14.33±0.57^{e}	12.66±0.28^{fg}	13.33±0.57^{cd}	14.66±0.28^{f}
	25	8.00±1.00^{gh}	9.00±0.50^{g}	8.62±0.28^{d}	11.00±1.00^{g}
	30	2.00±0.28^{j}	3.36±0.57^{i}	4.66±0.28^{ef}	8.66±1.15^{h}

Mean values followed by same superscripts within a column are not significantly different using Post Hoc multiple Comparison test ($P > 0.05$). CZEE= *C. zeylanicum* ethanolic extract; CZAE= *C. zeylanicum* aqueous extract; ASEE= *A. sativum* ethanolic extract; ASAE= *A. sativum* aqueous extract.

Rhizopus sp. it was 30%. The results showed a significant reduction in the mycelial growth of the tested spoilage fungi, as concentration of the extract increases by 5% (Table **5**). However, there was no significant (p =0.05) difference between 10% and 15% against *Aspergillus* and *Penicillium*, among concentrations of 20%, 25%, and 30% against *Fusarium* and *Penicillium,* between 15% and 20% against *Rhizopus*.

In *C. zeylanicum* ethanolic extract, *Penicillium* sp. showed a mycelial growth ranging from an average of 29.3 mm, 13.5 mm, 9.5 mm, 8.0 mm, 0.0 mm, 0.0 mm and 0.0 mm at concentrations of 0%, 5%, 10%, 15%, 20%, 25%, and 30%, respectively. *Fusarium* sp. recorded mycelial growth of 28.7 mm, 15.0 mm, 11.5 mm, 7.8 mm, 0.0 mm, 0.0 mm and 0.0 mm at similar spice extract concentrations, respectively. The radial growth observed for *Aspergillus* spp. was 30.3 mm, 15.3 mm, 11.0 mm, 9.7 mm, 7.3 mm, 3.2 mm and 0.0 mm respectively at concentrations in the ascending order. Similarly, *Rhizopus* sp. showed a mycelial growth ranging from an average of 32.0 mm, 15.5 mm, 11.7 mm, 9.2 mm, 7.0 mm, 3.0 mm and 0.0 mm, respectively. The control treatments showed maximum mycelial growth without inhibition (Table **5**).

A. sativum ethanolic extract is the second most mycelia growth inhibitor (Table **5**). It has the lowest MIC value of 30% with 2 mm mycelial growth

(*Aspergillus* sp.), 6.66 mm (*Fusarium* sp.), 4mm (*Penicillium* sp.) and 3.33 mm (*Rhizopus* sp.). Results also showed that the test organisms are sensitive to all ethanolic extracts and the concentration at which they are inhibited to grow under the test condition varied. It is also observed that the 30% showed the best result in inhibiting the mycelial growth in all the four fungi studied though there was no total inhibition observed by extracts of *A. sativum* (aqueous and ethanolic) and *C. zeylanicum* (aqueous).

The aqueous extracts of *C. zeylanicum* and *A. sativum* also inhibited the mycelial growth of the tested fungi though not as effective as that of the ethanolic extract (Table **5**). Results showed that the aqueous extracts of the two spices have more or less similar antifungal properties against the test fungi. All the extracts also had similar MIC (Table **5**). There was no significant ($p = 0.05$) difference in the mycelial growth of all the test fungi at 30% concentration of *C. zeylanicum* and *A. sativum* aqueous extracts.

DISCUSSION

It is obvious from our results that four post-harvest spoilage fungi (*Aspergillus, Fusarium*, *Penicillium* and *Rhizopus* spp.) were associated with diseased fruits. Al-Hindi *et al*. [5] found that *A. niger*, *P. digitatum* and *R. stolonifer* were associated with the spoilage of citrus, while *Fusarium* sp. were also the promising spoilage fungi for citrus fruits [29]. Similarly, Mukhtar and Adamu [30] found that *Aspergillus, Penicillium, Mucor, Rhizopus,* and *Fusarium* spp., were able to caused the infection in healthy the *Citrus sinensis.* However, Akinmusire [31] reported that *Fusarium oxysporium, Fusarium moniliform, Aspergillus niger* and *Rhizopus stolonifer* caused the infection on tomato fruits. Our results showed that *Aspergillus* sp. was the most prevailing spoilage fungi isolated from all types of fruits. Our result is in agreement with the finding of previous researchers [32-34].

The prevalence of spoilage fungi may depend on various factors associated with the handling process to consumption of fruits. This may also influence by physiological conditions of the produce and associated extrinsic factors [35]. The damage inflicted on produce at harvesting time is the major cause behind the infection. The spoilage fungi invade to produce through the damaged tissues and extent of deterioration is influenced by the depth of the wound. Akinmusire [31] stated that contamination of fruits caused by spoilage fungi could be the result of poor handling and marketing practices, storage conditions and transportation. Our survey result revealed that the poor hygienic conditions of the vending house or store, utensils of venders and mixing of deteriorating fruits with the healthier were the main cause behind the prevalence of fruit spoilage fungi. In addition, the climatic conditions of studied area especially the high moisture content favor the growth of spoilage fungi on fruits. Andrews and Harris [36] reported that the contamination might be arisen from fungal spores derived from the atmosphere before harvest in the field or during harvest, storage, transport and marketing.

Results of *in-vitro* antifungal activity showed that the aqueous and ethanolic extracts of *C. zeylanicum*, *A. sativum*, *C. annum* and *Z. officinale* exhibited various degree of antifungal activity against tested fungi. The reason behind this is the presence of various phytochemicals associated with different spices as reported by earlier workers [20, 23]. Results of present study showed that *C. zeylanicum* ethanolic extract completely inhibit the growth of all tested fungal isolates, however, *Penicillium* spp. were susceptible to all isolates. Similar results were also obtained by Al-Fatimi *et al*. [21] and reported that *C. zeylanicum* is the most effective in inhibiting the growth of *Penicillium*. Gupta *et al*. [15] reported that *C. zeylanicum* extract showed the widest inhibition zone only against *Penicillium* sp. amongst all the tested fungi but our result showed that *C. zeylanicum* ethanolic extract had inhibitory effect against both *Rhizopus* and *Aspergillus* spp. The main reason behind this result is the differences in the contents of the tested plants might be directly influenced active ingredient of the plants under different agro-ecological and climatic conditions. The amount of rainfall, photoperiod, edaphic and harvesting period also influenced the chemical constituents of the plants [37].

In general the crude extracts of spices extracted during or immediately after flowering possess the strongest antimicrobial activity. Moreover, the methods of extraction, bioassay used and differences in test strains can be attributed to different results [38, 39]. Therefore, the high efficacy of *C. zeylanicum* extract may be related to cinnamaldehyde, eugenol, cinnamic acid as well as to various organic acids [40]. The fungicidal effect of *C. zeylanicum* spice/oil extract is due to the inhibitory action of these natural products. Cytoplasm granulation, membrane rupture, inactivation/ inhibition of intracellular and extracellular enzymes were the various responsible mechanisms behind this cause [41].

A. sativum showed the second highest antifungal activity against all the tested fungi, but the potency of aqueous extracts was low compared to ethanolic extracts. Udo *et al*. [20] reported that methanolic and ethanolic *A. sativum* extracts have high potency for the control of pathogenic fungi on potato and yam tubers, while, Abdel-Hafez and El-Said [42] observed that *A. sativum* inhibit the growth of *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. terreus, Penicillium chrysogenum, P. puberulum, P. citrinum, P. corylophilum, Rhizopus stolonifer, Stachybotrys chartarum, Eurotium chevalieri* and *Emericella nidulans*. Similarly, Anjorin *et al.* [43] found the significant inhibition of *Fusarium* spp. by *A. sativum* extract.

Our results of minimum inhibitory concentration showed that crude extracts of *C. zeylanicum* and *A. sativum* poses strong *in-vitro* antifungal activity against all the tested fungi. *Penicillium* and *Fusarium* spp. were completely inhibited by *C. zeylanicum* ethanolic extract at (20%) while, it was recorded 30% in *Rhizopus* and *Aspergillus* spp. The increase in the antifungal activity of the extracts was enhanced by increase in the concentration of the extracts. Mann *et al.* [44] found that high concentration of plant extract had higher antifungal activity. The values of minimum inhibitory concentration of plant extracts against the tested organisms showed variation antifungal activity. Similarly, Prescott *et al*. [45] reported that antimicrobial agents with low activity against an organism have high minimum inhibitory concentration while a highly antimicrobial agent has a low minimum inhibitory concentration of the extracts. However, Banso and Adeyemo [46] stated that the antifungal substances found in the extracts behave as fungi static at lower concentrations while it become fungicidal at higher concentrations. The differences in the susceptibility of the test organisms to crude extracts could be attributed to variation in the rate of crude extract constituent's penetration through the cell wall and cell membrane structures, the site where their antimicrobial action is suggested to be expressed [47].

Results of *in-vivo* antifungal activity showed that aqueous and ethanolic extracts of both *C. zeylanicum* and *A. sativum* significantly reduced severity of diseases on tomato fruits compared to the untreated control. The aqueous extracts of *A. sativum* showed significant reduction in disease severity in pre or post inoculated spoilage fungi on tomato. However, no significant reduction in disease severity was recorded in simultaneous inoculation. Our results showed that the pre inoculation of aqueous *C. zeylanicum* extract reduced disease severity, while, ethanolic extracts significantly reduced the severity in post and simultaneous inoculation against the tested fruit spoilage fungi. However, the disease severity on the control fruits and sodium benzoate treated fruits were not significantly affected by the time of inoculation. The antifungal property of spice is due to their phytochemical contents and this seems to have inhibitory effect against the fungal infection and the inhibitory potency of the spices may depend on their types, mode of extraction and the concentration of the extracts used. Thus, the spices extracts can use as alternative to chemical preservatives to preserve the agricultural products. Considering their attribute and broad-spectrum activities, successful development of such compounds as antifungal would not only provide a potent tool for control of spoilage fungi of fruits, but also could promise success in multipurpose bio-rational alternatives for the integrated management of other plant diseases. The usage of spice plants as extract for shelf life extension of fruit and vegetable might be considered as safe, cheep and easily applied method for controlling post-harvest spoilage fungi of fruits taking in consideration the avoidance of environmental pollution and side effects of fungicide application.

CONCLUSION

It is obvious from our results that spice extracts have the ability to inhibit the growth of fruit spoilage fungi. Amongst all the tested spice extracts *C. zeylanicum* and *A. sativum* have proved their potential to be used as candidates extracts because of their wide antifungal activity against the tested fruit spoilage fungi. *In-vivo* assay showed that *A. sativum* and *C. zeylanicum* extracts have the strong potential to inhibit disease severity against the spoilage fungi compared to other tested extracts. The broad antifungal spectrum exhibited by these spice extracts could be used in food preservation. Thus it would be advisable that people can used spices to inhibiting growth of spoilage fungi on some agricultural commodities such as fruits and also prolonged their shelf life.

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