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Original Research

Growth Inhibition of Grain Spoilage Fungi by Selected Herbs and Spices Essential Oils

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Abstract

Natural plant extracts are promising alternatives for chemical food additives and synthetic pesticides. In this study, essential oils of selected herbs and spices were tested for their antimicrobial activities against *Aspergillus flavus* and *Aspergillus niger*, two of the most common food spoilage microorganisms. Agar disk diffusion assay was used for screening of the most effective essential oils, agar dilution assay was used to determine Minimum Inhibitory Concentration (MIC) of the essential oils and broth dilution assay was employed to the spore germination inhibition assay. Tests were also conducted to examine the effects of the essential oils for sorghum kernel protection against the tested fungi, and the optimal protective dosages on the sorghum grains were also determined. From the preliminary tests, essential oils of *Cinnamomum zeylanicum* (Cinnamon) and *Thymus schimperi* (thymus) were found to be the most effective. However piper nigrum (black pepper) had no effect on the test organisms. In MIC, spore germination inhibition and grain protection assay, cinnamon essential oil was found to be superior where its MIC on the isolates was found to be 0.0156% and its optimum protective dosage on the sorghum grain was 5%. It inhibited spore germination at a concentration of 3µL/mL. The effect of thymus oil was also very much comparable to these results (no significant difference at $P>0.05$). Finally, it was concluded that essential oil extracts of cinnamon and thymus can be a useful source of antifungal agents for protection of grain spoilage by fungi.

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INTRODUCTION

Contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and sub-tropics, where climatic conditions, agricultural and storage practices are conducive to fungal growth and toxin production (Kumar *et al*., 2008). Fungal contamination of grains can occur in the field or in store with the extent of contamination largely determining the rate of deterioration of stored grains (Samapundo *et al*., 2007; Amiri *et al*., 2008; Rasoolia *et al*., 2008). The most important species of field fungi are from the genera *Alternaria*, *Cladosporium*, *Fusarium* and *Drechslera* (Amare, 2002). *Fusaria* and *Aspergilli* are the most commonly isolated contaminants of corn worldwide, with the most important species being *F. verticillioides*, *F. proliferatum*, *A. flavus* and *A. paraciticus* (Velluti *et al.,* 2004; Samapundo *et al*., 2007; Amiri *et al*., 2008). Estimated losses of grains, especially staple food grains in store, caused from insects and pathogens vary widely. They may amount to 10% worldwide but can reach 50% in tropical regions (Velluti *et al*., 2004; Zhang *et al*., 2009). Agricultural commodities such as peanuts, corn, maize, sorghum and others are the highly affected crops by fungi and ultimately end up unfit for consumption on long time storage (Rasoolia *et al*., 2008). Contamination of cereal commodities by moulds and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain (Paster *et al*., 1994; Ngueface *et al*., 2004).

Generally, the presence of toxigenic fungi and mycotoxins in foods and grains stored for long periods of time presents a potential hazard to human and animal health (Omidbeygi *et al*., 2007). Therefore, the contamination of foods and feeds by mycotoxins should be minimized by designing a series of measures of prevention and control.

There is evidence that spices or herbal essential oils contain strong antimicrobial constituents which are derivatives of phenolic compounds and they are generally regarded as safe (GRAS) by the FDA, at least at concentrations commonly found in foods (Ngueface *et al*., 2004; Shan *et al*., 2007; Tatsadjieu *et al*., 2009). Phenolic compounds in olive oil (oleuropein) and tea-tree oil (terpenes),which are not classified as either spices or

herbs, also show antimicrobial activity (Holley and patel,2005). Spice and herbs such as *Cinnamomum zeylanicum* (Cinnamon), *Thymus schimperi* (Thymus), *Zingiber officinalis (*Ginger), *Allium sativum* (Garlic), *Laggera tomentosa* (Keskese), *Piper nigrum* (Black pepper) and *Citrus limon* (Lemon) are essential oil plants commonly grown in various parts of Ethiopia. Thus in the present study, the antifungal activities of these plants essential oil were investigated against *A. flavus and A. niger*, two most common grain spoilage fungi. Their potential in fungal growth inhibition in sorghum grains was also evaluated.

MATERIALS AND METHODS

Plant Sample Collection and Extraction of Essential Oils

Different parts of herb and spice plants were collected from highlands and low lands areas of Ethiopia (Table 1). The selection was based on the traditional practices that local people use these plants as food preservatives, food flavoring and seasoning agents. The collected plant materials were dried at room temperature and ground using a grinder (NIMA-8300 Burman, Germany) and extraction of the essential oils was conducted through the process of hydro-distillation (Clevenger 77-550 type apparatus) following the procedure outlined by Hettiarachichi (2008) at Ethiopian Health and Nutrition Research Institute (EHNRI). 500g of these materials were packed in a distillation flask with approximately four times water (w/w) of the test materials. The distillation chamber was heated to 50^0c and allowed to boil until extraction completed (4-5 hrs). The distillate was collected in the separating funnel in which the aqueous portion was separated from the volatile oil. The water (lower) layer was slowly drowned off until the oil layer remained. Colored oils, with pleasant odors, were obtained. Finally the oils were collected in sterile container, dried over minimum amount of anhydrous sodium sulfate to remove traces of moisture and preserved in refrigerator until it was used for further experiments.

Grain Sample Collection

Visibly healthy sorghum grains were purchased from the local market to carry out the grain protection assay. The grains that are not damaged by insects, which do not have any broken parts and other physical injury, were visually inspected seriously for the decision to buy them.

Table 1: Plant samples used

Species	Parts of the plants used	Sampling area
Allium sativum (garlic)	Bulb	Debre zeit
Cinnamomum zeylanicum (cinnamon)	Bark	Jima/Tepi
Citrus limon (lemon)	peel	Addis Ababa
Laggera tomentosa (Keskese)	Seed	Chancho
Piper nigrum (black pepper)	Seed	Jima/Tepi
Thymus schimperi(thymus)	Leaf	Chancho
Zingiber officinale (ginger)	Root	Jima/Tepi

Fungi Sample Collection and Maintenance

For antimicrobial testing, the test fungi *Aspergillus flavus* (ATCC 13697) and *Aspergillus niger* (ATCC 10535) standards were kindly supplied by Ethiopian Health and Nutrition Institute (EHNRI), while local isolates of *Aspergillus flavus* and *A. niger* were obtained from Addis Ababa University Department of Biology, Mycology Lab. The fungi were maintained on potato dextrose agar (PDA; Himedia Ltd., India) slant at refrigerator temperature and periodic transfers were done to keep the microorganism viable.

Inoculums Preparation and Standardization

The fungal culture was grown on PDA (Himedia Ltd., India) medium for sporulation on petri dishes for 5-7 days. Fresh stock suspension of the organisms were prepared in 10 ml of sterile normal saline solution and were adjusted to 0.8-1 OD range with 6051-type colorimeter at 625 nm wave length to obtain a concentration of 10^4 spores/mL and was utilized the same day.

Disk Diffusion Assay

Screening of essential oils for antifungal activity was carried out by disc diffusion method, which is normally used as a preliminary screening of efficient essential oils (Burt, 2004) following the procedure approved by NCCLS. Filter paper discs (Whatman no.1, 6 mm in diameter) were prepared and sterilized. 5µL of each essential oil extracts were impregnated on to the disk by sterile micropipette tips. Using an ethanol dipped, flamed and cooled forceps; these disks were aseptically placed soon individually over the middle of freshly prepared PDA plates already seeded with the respective test microorganisms and gently pressed down on to the agar. Sterile distilled water was added on the discs to provide negative control. The plates were left for 30 minutes at room temperature to allow the oil diffusion, turned upside down and were incubated at 27^0C for 3 days. At the end of the incubation period, antifungal activity was evaluated by measuring zone of complete inhibition (including diameter of the disk) against the test fungi using a ruler. All treatments consisted of three replicates, and the averages values were determined.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of all the essential oils was determined by agar dilution method (Hammer *et al*., 1999, Cao *et al*., 2009; Rusenova and Parvanov, 2009). Stock solutions of the oils were prepared in 2% Tween-80 and different concentration of the oils from the stock ranging from 2% (v/v) to 0.0078% (v/v) was prepared in sabroud-dextrose agar (SDA; Oxoid, England) and vortexed for 30 seconds. The oils extracted were added to the culture medium at a temperature of 30-40^0C. The content was poured into Petri-dishes and allowed to solidify at room temperature for 30 minutes. After the medium was solidified, a loop full of the fungi suspension containing 10^4 cfu/mL was spot inoculated using sterile loop. The SDA plate with 2% Tween-80 but no oil was used as a positive growth control. All plates were incubated for 72 hours at 28^0C. At the end of the

incubation period, the plates were evaluated for the presence or absence of microbial growth. The MIC was determined as the lowest concentration of oil inhibiting the visible growth of each organism on the plates (Tullio *et al*., 2006; Cao *et al*., 2009; Goni *et al.,* 2009).

Spore Germination Inhibition Assay

Different concentrations of the most effective essential oils were tested for spore germination inhibition of the assay. The tested fungi were cultured on PDA medium for sporulation on Petri-dishes for 5-7 days and the suspension was prepared in 10 ml sterile distilled water containing 0.1% (v/v) Tween 80 for better spore separation (Tzortzakis and Economakis, 2007) by aseptically dislodging the spores with a sterile inoculating loop. The spore suspensions were aseptically filtered off the mycelia in a funnel containing sterile cotton wool and adjusted with sterile water to give a final spore concentration of approximately 10^4 spore/mL. Various concentrations of the oils (20 µL, 15 µL, 10 µL and 5 µL) were added to 5 mL of nutrient broth in a small flat bottom flask and 1ml of the spore suspension was added to each flask in tripilicate. The flasks were allowed to germinate at 25°C for 24 h on a rotary shaker (121 rpm). Germinated spores were observed using a light microscope at 400 x magnification for the presence or absence of germ tubes. The nutrient broth without the essential oils was served as positive control. Results were expressed in terms of the percentage of spores germinated as compared to the control.

Grain Protection Assay

The grain protection assay was carried out using visibly healthy sorghum grains following the methods described by Montes-Belmont and Carvajal, 1998; Juglal *et al*., 2002; Atanda *et al*., 2007. 120 seeds of sorghum grains were immersed in the essential oils (5% each) for 30 min, dried for another 30 min at room temperature and distributed in three petri-dishes with sterile wet cotton wool. They were then inoculated (sprayed) with 10^4 spores per mL fungal spore suspension of *A.flavus* and *A.niger* and incubated at a temperature of 27^0C for seven days. Binocular microscope (40 x magnifications) was used to see growth of the tested fungi on the surface of the grain.

To examine proliferation of the fungi into the kernel, the grain was surface sterilized using 1% commercial sodium hypochlorite solution (Amare, 2002; Dikbas *et al*., 2008) and subsequently rinsed three times in sterile distilled water, dried for an hour over sterile filter paper and placed on freshly prepared PDA plates (30 grains each) using ethanol dipped and flamed forceps. The plates were then incubated for 5 days and the effects of each oil were observed for fungal growth from the grains. The percentage of contaminated grains was obtained from three replications.

Optimum Protective Dosage of the Essential Oils

Optimum protective dosage test was carried with different concentrations of the most effective essential oils (cinnamon and thymus) at a concentration of 2, 3, 4, 5, 6, 7, and 8 % prepared using hexane as a solvent as described by Atanda *et al*. (2007).

Statistical Analysis

All the measurements were replicated for each assay and the results are presented as mean±SD. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Post Hoc Multiple Comparison Tests using statistical software (SPSS) package version 15.0 for windows and *P* values < 0.05 were considered as significant.

RESULTS

Screening for Antifungal Activity using Disk Diffusion Assay

All essential oils extracted from the common herb and spice plants have shown antifungal activity on the test organisms, except *Piper nigrum* that didn't show growth inhibit of the tested fungi (Table 2). Essential oil extracts from *Thymus schimperi* (thymus) and *Cinnamomum zeylanicum* (cinnamon) were found to be the most effective as compared to the others revealed by the broader zone of inhibition followed by *Allium sativum* (garlic). *Thymus schimperi* has shown the greatest inhibition zone diameter of 62.3 mm against *A.niger* standard which is the largest zone diameter recorded in this study. Result of ANOVA showed that there is no significant difference between the three essential oils against *A. flavus* standard and *A. niger* standard. All the tests were conducted in parallel to the control which didn't show any fungal activity.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the essential oils was determined by agar dilution method. All the essential oils inhibited the mycelia growth at a concentration less than 2% except *piper nigrum.* Cinnamon EOs has exhibited the lowest MIC value and it was determined to be 0.0156% (v/v) for the tested fungi. Thymus EOs was with the second lowest MIC value and it was determined to be 0.0315% (v/v) and 0.0625% (v/v) against *A. flavus* and *A.niger* respectively (Table 3).

Spore Germination Inhibition Assay

Spore germination inhibition potential of the two most effective essential oils, thymus and cinnamon oils was conducted by broth dilution assay. Cinnamon EOs inhibited spore germination of both *A. flavus* and *A. niger* at the concentration of 3µL/mL while thymus EOs inhibited spore germination of both *A. flavus* and *A. niger* at the concentration of 4µL/mL (Table 4).

Table 2: Determination of antifungal properties of oil extracts by disk diffusion assay

Tested fungi	Inhibition Zone Diameter in mm (Mean ± SD)						
	Cinnamon	Thymus	Ginger	Keskese	Lemon peel	Garlic	Piper nigrum
A. flavus (local isolates)	35.57±0.60	58.33±0.42	11.0±1.00	11.5±1.32	12.5±0.50	20.33±0.58	0.00± 0.00
A. flavus (standard)	42.33± 0.58	61.00±1.00	12.5± 0.50	13.50± 0.5	13.33±0.58	23.0±1.00	0.00± 0.00
A.niger (local isolates)	35.47± 0.70	61.00±1.00	11.0±1.00	7.8±0.280	13.17±0.29	20.0±1.00	0.00±0. 00
A. niger (standard)	38.33± 0.58	62.33±1.52	14.5±0.50	12.5±0.50	15.33±0.58	26.0±1.00	0.00± 0.00

Table 3: Determination of Minimum Inhibitory Concentration (MIC) of the oil extracts

EO extracts	Tested fungi and its corresponding MIC (%)			
	***A. flavus* (Local isolates)**	***A. flavus* (Standard)**	***A. niger* (Local isolates)**	***A.niger* (Standard)**
Cinnamon	0.0156	0.0156	0.0156	0.0156
Thymus	0.0625	0.0315	0.0625	0.0625
Ginger	0.5	0.125	0.25	0.25
Keskese	1	0.25	0.5	0.5
Lemon peel	1	0.5	1	1
Garlic	0.25	0.0625	0.125	0.125
Piper nigrum	>2	1	>2	1

Table 4: Determination of antifungal property by spore germination assay

Tested fungi	Thymus oil (µL/mL)				Cinnamon oil (µL/mL)			
	1	2	3	4	1	2	3	4
A. flavus	51.98+*0.*46	20.76+0.88	5.71+0.27	0.00	34.04+0.66	9.62+0.40	0.00	0.00
A.niger	39.76+0.46	19.05+1.01	0.00	0.00	28.07+0.37	5.06 +0.11	0.00	0.00

Grain Protection Assay

Cinnamon and thymus EOs completely inhibited growth of the tested fungi on sorghum grains; garlic, ginger and lemon EOs had minimal effect while black pepper and Keskese EOs had shown no effect (Table 5).

Optimum Protective Dosage

The optimal protection dosage of the two most effective essential oils were studied at concentrations of 4, 5,and 6% (v/v) (Table 6). Both of the tested fungi were sensitive to 5% cinnamon essential whereas they were sensitive to 6% thymus essential oil (Table 6).

Table 5: Effects of oil extracts on sorghum grain protection assay

Treatments at 5% concentration	*A. niger*		*A. flavus*	
	Contamination	Reduction of Contamination	Contamination	Reduction of Contamination
Cinnamon	0 + 0.00	100	0 + 0.00	100
Thymus	0 + 0.00	100	1.66+0.835	98.34
Ginger	29.72+ 4.59	40.83	31.39+ 2.09	48.33
Keskese	25.00+1.67	45.83	25.56+ 3.47	54.16
Lemon peel	68.33+ 2.21	2.5	80+ 3.33	0
Garlic	62.56+3.41	8.27	74.72+ 6.02	5.28
Piper nigrum	44.77+ 9.61	25.83	48.61+ 7.74	31.66
Conntrol	70.55+ 2.68	-	80 + 3.33	-

Table 6: Optimum sorghum grain protective dosage by the most effective essential oils (result expressed in (Mean+SD)

Treatments	Contaminated kernels (%) at:						
	Oil concentration (%)						
	2	3	4	5	6	7	8
CAF	45±5.00	21.67+3.81	2.5+0.86	0	0	0	0
CAN	33.67+3.21	18.33+4.41	0	0	0	0	0
TAF	53.61+5.55	40+1.67	23.89+2.55	3.33+0.83	0	0	0
TAN	46.94+3.93	33.61+1.27	14.45+2.09	0	0	0	0

(CAF=Cinnamon EO against *A.flavus*, CAN = Cinnamon EO against *A.niger*, TAF=Thymus EO against *A.flavus*, TAN=Thymus EO against *A.niger*)

DISCUSSION

In this study, the susceptibility of the test organisms to the essential oils has shown wide variation. This could be attributed to the difference in the rate of essential oil constituent's penetration through the cell wall and cell membrane structures, the site where their antimicrobial action is suggested to be expressed (Tatsadjieu *et al*., 2009). Furthermore, the composition, structure as well as functional groups of the essential oils which play an important role in determining the antimicrobial activity of essential oils (Holley and Patel, 2005) is one of the major factor for difference in action of the essential oils. Usually compounds containing phenolic functional groups are the most effective (Carmo *et al*., 2008). Thymus and cinnamon EOs showed the highest zone of inhibition measured as 62.33mm and 38.33mm against *A.niger* while 61.00mm and 42.33mm against *A.flavus* respectively. This supports the work of earlier researchers, Dikbas *et al*. (2008) conducted an antifungal activity of *Satureja hortensis* oil on *A. flavus* and reported an inhibition zone of the oil to be 61mm by disk diffusion assay. Juglal *et al*. (2002) reported that clove oil (eugenol) was the most inhibitory oil against growth of *F.moniliforme* and *A.paraciticus* followed by cinnamon, oregano, mace, nutmeg turmeric and aniseed oils. Ginger EO exhibited inhibition zone diameter of 12.5mm and 14.5mm against *A. flavus* and *A.niger* respectively. These findings were in the contrary to past works. Singh *et al*. (2008) tested the efficacy of ginger essential oils and reported a higher inhibition zone diameter of 44.4 mm and 27.9 mm against *A. flavus* on *A. niger* respectively. The variation could be attributed to EO contents due to local climate and

environmental conditions. Burt *et al*. (2005); Clausen and Yang (2008) out lined that the geographical area of production and weather conditions during the growing season and particularly at harvest can have a significant effect on the content of active ingredients found in plant.

In the MIC determination, cinnamon essential oil was found to be the strongest mycelia growth inhibitor followed by thymus essential oil. The MIC value of cinnamon was 0.0156% against the tested fungi and that of thymus oil was 0.0315% (v/v) against *A. flavus*. This result was in accordance with the work of Hammer *et al*. (1999) who reported that the MIC value *Thymus vulgaris* showed 0.03% against *Candida albicans.* Rusenova and Parvanov (2009) reported that cinnamon, oregano and lemongrass has exhibited MICs values between 0.03-0.06% (v/v) against *C. albicans* and *Malassezia pachydermatis*.

The spore germination inhibition assay showed that fungal spore germination was suppressed by cinnamon and thymus EOs. The inhibitory effect of the oils increased in proportion to their concentrations. It was observed that total inhibition (zero spore germination) for cinnamon EO was at 3µL/mL and that of thymus EO was at 4µL/mL against both the tested fungi. The cinnamon essential oil inhibited the spore germination of the tested fungi at a concentration less than that of thymus which showed that cinnamon oil was more potent than thymus. This could be attributed to the dispersion and persistence of the oil in the nutrient broth (Ashenafi, 2007). Chalfoun *et al*., (2003) analyzed the inhibitory effect of ten powdered spices on mycelial growth, sporulation and production of aflatoxins by toxinogenic fungi at the concentrations of 1, 2, 3 and 4%, and reported that clove and cinnamon powders promoted total inhibition of *A. niger* mycelia development in all doses tested. They also reported that level of inhibition was always proportional to the concentration used.

A similar result with that of disk diffusion assay was observed during grain protection assay. Cinnamon and thymus essential oils were found to be the most active in reducing contamination of the grain. Ginger and garlic essential oils showed similar properties while black pepper showed poor effect against both the tested fungi. Cinnamon and thymus essential caused 100% growth reduction at 5% concentartion, garlic showed 40.83% and 48.33% against *A,niger* and *A.flavus* respectively and ginger showed 45.83 and 54.16% against *A,niger* and *A.flavus* respectively. Montes-Belmont and Carvajal (1998) and Juglal *et al*. (2002) reported a similar result by using *Cinnamomum zeylanicum, Thymus volgaris* and *piper nigrum EOs* against *Aspergillus parasiticus* and *Fusarium moniliforme* on contaminated maize grains. The variation in the result of the grain protection could be related to the volatility of the respective active compounds of the oils. For example, onion and garlic sulfides may not be retained for more than 24 h and their effect is only partial and if they don't kill all the spores, the remaining spores germinate and mycelia develop (Montes-Belmont and Carvajal, 1998). Moreover the effectiveness of essential oils was influenced by changes in their concentrations, substrate water availability, and time of incubation (Bluma and Etcheverry, 2008).

The result of optimum protective dosage on sorghum grains showed that cinnamon essential oil prevented contamination of the grain by *A.niger* and *A.flavus* at concentration of 4% and 5% (v/v). On the other hand, thymus essential oil had a protective dosage of 6% (v/v) against *A.flavus* and 5% (v/v) against *A.niger*. This finding was supported by past works such as Atanda *et al*. (2007) who reported the optimum protective dosage or fungistatic concentration of sweet basil (*Ocimum basilicum*), cassia (*Cinnamomum cassia*) against *A. parasiticus* CFR 223 was 5% (v/v) on sorghum grains protection assay.

CONCLUSION

In addition to boosting flavor, herbs and spices are known for their preservative, antioxidative, antimicrobial and various other medicinal values (Singh *et al*., 2008). With this background, out of the seven different plants studied, c*innamomum zeylanicum* and *Thymus schimperi* essential oils were determined as the most effective antifungal agents against *A. flavus* and *A. niger,* the two most common food spoilage microorganisms. The effectiveness of these EOs was further confirmed by spore germination and grain protection assay in which both the EOs of the two plants showed greater efficacy against the tested fungi. Hence, it was concluded that essential oil extracts of cinnamon and thymus can be a useful source of antifungal agents. These findings pointed out that there is an alternative source than chemical fungicides in inhibiting growth of spoilage molds on some stored agricultural commodities such as sorghum grains.

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