Mycopath (2015) 13(2): 97-104 **Research Article**

Exploiting antifungal potential of ginger for the management of *Alternaria alternata,* the cause of leaf spot disease of spinach

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Abstract

Present study was conducted to explore the antifungal activity of ginger (*Zingiber officinale* Roscoe), a medicinal plant of family Zingiberaceae, for the management of *Alternaria alternata,* the cause of leaf spot disease of spinach (*Spinacia oleracea* L.). Dry powder of ginger was extracted in ethanol and tested for antifungal activity at 0.5%, 1.5%, ..., 5.5% concentrations *in vitro*. Poisoned food technique and spore germination assay were used to evaluate the antifungal efficacy of extracts of ethanolic. All the concentrations showed inhibitory effect on the growth of test pathogen. The highest concentration (5.5%) had 90.4% inhibition on mycelia growth. Spore germination was completely inhibited at both 4.5% and 5.5% concentrations. Ethanolic extract was further partitioned using petroleum ether, chloroform, ethyl acetate, *n*-butanol and water. Antifungal activity of different concentrations (2.5 to 320 mg mL^{-1}) of each of the five fractions of ethanolic extract was investigated against the target fungal pathogen using potato dextrose broth as growth medium. All the concentrations of ethyl acetate fraction significantly reduced the biomass of the target fungal pathogen by 85-100%. Similarly, chloroform fraction was equally effective and suppressed the fungal biomass. In contrast, all the concentrations of *n*-butanol and aqueous fraction of ethanol extract had negligible inhibitory effect on the fungal growth.

Keywords: *Alternaria alternata*, ginger, leaf spot, natural fungicides, spinach.

Introduction

Worldwide, plants suffer huge amount of yield losses due to fungi. The genus *Alternaria* is an important plant pathogen infecting crops in fields, causing aerial diseases and is also responsible for spoilage of post harvest crops and many plant products (Thomma, 2003). Total losses caused by this genus, rank amongst the highest caused by any plant pathogen (Agrios, 2005). In recent past, a new leaf spot disease on spinach caused by *Alternaria alternata* was reported in kingdom of Saudi Arabia (Marraiki *et al*., 2012). Mortality due to the disease was found to be 20-80%**.** Besides causing crop loss, there is also doubt that sensitivity to *Alternaria* is an important factor in the induction of allergic rhinitis and asthma on immunodepressed patients, especially in children (Kuna *et al*., 2011). Over the past few years there is a growing concern on the toxic effects of the secondary metabolites produced by *Alternaria* spp., which are harmful to both human and animal health. Application of fungicides is one of the most extensively used practices in foliar disease management. Although intensive use helps in controlling the pathogens, it results in accumulation of toxic compounds which are potential environmental pollutants and hazardous to mankind (Reshu and Khan, 2012). Their wide spread use has also resulted in creating resistance in the pathogen (Heydari and Pessarakli, 2010). As a consequence, several researches have been conducted in the last decades on plant extracts to find safer and effective alternatives. Enormous studies have revealed the potential of extracts from different parts of plant as an excellent biocontrol method in controlling phytopathogenic fungi (Shafique *et al.,* 2011). Hence biological control methods which are eco- friendly besides being less hazardous is gaining increased attention all over the world.

Zingiber officinale is well known for its ethanomedicinal and nutritional values. Ginger, which is a rhizome, has antimicrobial and antimycotoxigenic effects (Tatsadjieu *et al.,* 2009).Traditionally, it is used as a cure for for a wide array of ailments that include pains, muscular aches, arthritis, rheumatism, sore throats, nausea, vomiting, hypertension, constipation, indigestion and infectious diseases. Hence our present study deals with investigating the antifungal property of ginger extracts against *Alternaria alternata*, the cause of leaf spots of spinach.

Materials and Methods

Isolation of target fungal species

Alternaria alternata was isolated from spinach grown in Riyadh, Saudi Arabia. Leaves with infected portions were cut into small pieces and surface sterilized with 1% sodium hypochlorite solution, then rinsed thrice with sterile water and cultured on potato dextrose agar with incubation at 25±1 °C for a week. Thereafter, pure culture was stored in the refrigerator at 4 °C.

Plant material and Preparation of extracts

Ginger used in this study was purchased from local market in Riyadh. Fresh rhizomes were thoroughly washed with running tap water followed by sterilized distilled water and then air dried. The dried rhizomes were powdered and subjected to various extraction procedures.

Extract preparation

Powdered ginger (2 kg) was soaked in the eight litre of ethanol for one week and then filtered with Whatmans filter paper. The filtrate was evaporated with the help of a rotary evaporator at 45 °C under reduced pressure to obtain crude ethanolic extract. This extract was divided into two parts. One part was used to test its antifungal activity while the other portion was subjected to fractionation by different solvents.

Antifungal activity of ethanolic extract

Potato dextrose broth was used for the bioassay. Stock solutions were prepared by dissolving 16 g of crude ethanolic extract in 5 mL DMSO and raising the volume to 20 mL by adding sterilized distilled water. Control solution was prepared by adding 5 mL DMSO to 15 mL sterilized distilled water minus the extract. Various concentrations (0.5, 1.5, 2.5, 3.5, 4.5 and 5.5%) were prepared by adding appropriate quantities of stock and control solution to each flask, containing 76 mL of sterilized medium to make the total volume 80 mL. Corresponding control treatment received 4 mL of control solution alone without the plant extract. Mycelial disc of 5 mm diameter was removed from the periphery of 9 days old actively growing culture of *Alternaria alternata* using a sterilized cork borer and transferred to the each flask aseptically. Flasks were incubated for one week in an incubator at 25±2 °C. Each treatment was replicated five times. Fungal biomass in each flask was filtered on pre weighed filter papers, dried to constant weight at 60±1 °C and weighed.

Effect of Ethanolic Extract on Radial Growth

Various concentrations of ethanolic extract (0.5, 1.5, 2.5, 3.5, 4.5 and 5.5%) were added to sterilized Petri dishes followed by pouring selected amount of PDA aseptically. The Petri dishes were shaken gently to allow the extract to disperse evenly in the medium. After the medium solidified, a mycelial plug of 5 mm diameter was removed from the periphery of 9 day old actively growing colony and placed in the centre of the plate. Five Petri plates were prepared for each concentration. Culture plates were incubated for a week at 25±2 °C and radial growth was measured and recorded. Control treatment was without the extract.

Effect of ethanol extract on sporulation

Spore germination assay was done by harvesting spores aseptically from 9 day old culture maintained on PDA, using sterilized distilled water. Antifungal effect of ethanol extract was tested using different concentrations by spore germination method using cavity slides (Maji *et al*., 2005). A 50 µL spore suspension and 50 µL of different concentrations of ethanol extracts were taken on separate cavity slides .These slides were incubated at 25±2 °C in moist chambers for 24 h. Each slide was then observed under the microscope for spore germination. The spores that generated germ tubes were enumerated and percentage of spore germination was calculated. One cavity slide was maintained as control without adding any extract

Fractionation of crude extracts

The second portion of crude ethanol extract was dissolved in distilled water (500 mL) and shaken until the crude extract dissolved. The solution was transferred into a separating funnel and extracted successively and separately with petroleum ether, chloroform, ethyl acetae and butanol, respectively. The lower aqueous phase at the end of the process was used as aqueous fraction. At the end of the extraction process all the different solvents were evaporated in a rotary evaporator resulting in five extracts i.e. petroleum ether fraction (5.8 g), Chloroform fraction (3.37 g), ethyl acetate fraction (6.0 g), butanol fraction (4.60 g) and aqueous fraction (10.6 g). All the fractions were tested for their antifungal activity against *Alternaria alternata* by the method of Iqbal and Javaid (2012) with slight modifications. Stock solutions were made by dissolving 2.4 g of each of the fraction mentioned above by dissolving them in 1ml DMSO followed by addition of 5 ml

potato dextrose broth to prepare a concentration of 400 mg mL^{-1}. Appropriate quantity of this stock was serially double diluted by adding potato dextrose broth to prepare lower concentrations of 320, 160, …, 2.5 mg mL^{-1}. Similarly, various control treatments were prepared corresponding to each extract treatment by adding 1mL DMSO to 5ml of potato dextrose broth followed by serial double dilution. This was done in order to avoid the inhibitory effect of DMSO on fungal growth based on previous studies. Test tubes (10ml) were used for the assay. To each tube, 1 ml medium was added and inoculated with one drop of spore suspension. Test tubes were incubated for one week at 25±2 °C. Fungal biomass in each test tube was filtered on pre weighed filter papers, dried to constant weight at 60±1 °C and weighed. Each treatment was replicated three times.

Statistical analysis

Completely randomized design (CRD) was applied and comparison of means (lettering) was done using LSD test at 5% level of significance. All the data for ethanolic fractions were analyzed by analysis of variance followed by Tukey's HSD test at 5% level of significancs.

Results

Effect of ethanol extract on the biomass of *Alternaria alternata*

In the present study, all the concentration of ethanol used, inhibited the mycelial biomass of test pathogen. Inhibitory activity of ethanol extracts of various concentrations on growth of *Alternaria alternata* is presented (Fig. 1). There was a gradual decline in the mycelial biomass as the concentration of the extract increased. Significant loss in mycelial biomass was (76%) was observed from 3.5% concentration onwards reaching a maximum of 90.47% at the highest concentration of the extract (5.5%).

Effect of ethanol extract on radial growth of the pathogen

Various concentrations of extracts had a moderate to high inhibitory effect on the radial growth of test pathogen**.** Data in Table 1 indicate that higher concentrations of the extract could significantly reduce the growth of fungi. Pronounced radial growth reduction of 85%, 97% and 98% at 3.5, 4.5 and 5.5% concentrations was noted

Antifungal effect of ethanol extract on spore germination of the pathogen

The results of antifungal effect of *Z. officinale* extract on the spore germination of the test pathogen are presented in Table 2. Spore germination responded remarkably to the extract treatment by completely inhibiting the germination at 4.5% and 5.5% followed by 3.5% concentration which caused 95% inhibition.

Inhibitory effect of different fractions of ethanol extracts on test fungi

The different fractions of ethanol extracts, exhibited varying levels of antifungal activity. The control treatment which comprised of different concentrations of DMSO, inhibited the growth of *Alternaria alternata*. As the concentration of DMSO increased the mycelia biomass decreased gradually (Table 3). Different fraction of ethanol possessed varying levels of inhibitory effects on the growth of test pathogen. It was observed that the ethyl acetate fraction of the extract showed excellent antifungal activity amongst all the other fractions tested followed by chloroform and petroleum ether. Ethyl acetate fraction had pronounced antifungal effect on the fungi by reducing the fungal biomass completely at 320 and 160 mg mL^{-1} and inhibiting the fungi by 85% even at the lowest concentration of 2.5 mg mL^{-1}. ^{C}hloroform fraction also exhibited good antifungal effect on test pathogen by completely inhibiting the growth of fungi at higher concentration of 320 and 160 mg mL^{-1} . Lower concentrations of 2.5 mg mL^{-1} also suppressed the growth by 70%. However, n-butanol and aqueous extract responded poorly on the growth of the fungi, n butanol showed 50-73% reduction at higher concentration of 80 mg mL^{-1}, but lower fractions did not show significant reduction. Aqueous fraction of extracts exhibited negligible antifungal effects at 320 mg mL^{-1} while the concentration below 160 mg mL^{-1} had no effect on the fungal growth. In fact it stimulated the growth partially over control.

Discussion

Present findings clearly demonstrate that ethanol extract of *Zingiber officinale* had considerable inhibitory effect on the mycelia growth and spore germination of *Alternaria alternata*. Shirzadian *et al.* (2009) used ethanol, petroleum ether and aqueous extracts of some plant and found ethanolic extracts to be most effective in controlling the growth of some pathogenic fungi including *Alternaria alternata*. Manoharan *et al.* (2010) compared the ethanolic

extracts of both Malaysian and Thailand gingers and reported both of them to posses showed good to excellent activities on *Aspergillus niger* and *Candida albicans.* Similarly, a comparative study on ginger, garlic and lime with ethanol base extraction medium showed that all the extracts inhibited the fungal growth with ginger exhibiting the highest antimicrobial activity (Tagoe *et al*., 2010). In another study Tagoe *et al*. (2011*)* screened different fungal test pathogens with ethanolic extracts of onion ,ginger and garlic and reported ginger extracts to inhibit the test fungi with a mean diameter of 1.40cms over 1.80 cm by onion, hence possessing highest antimicrobial activity. These findings further support our results stating that ethanol as a solvent is very effective in extracting antifungal bioactive compounds (Zaker and Mosallannajed, 2010). Our results also point at the inhibitory effects of DMSO in control treatments, such inhibitory effects were also observed earlier on *A.alternata*, *Ascochyta rabiei* and *Macrophomina phaseolina* (Javaid and Samad, 2012; Javaid and Munir, 2012; Naqvi *et al*., 2012). Amongst the ethanolic fractions screened, ethyl acetate fraction exhibited maximum antifungal effect on the *A. alternata* followed by chloroform and petroleum ether, while n-butanol and aqueous extract showing minimum to nil inhibitory effects. Several researchers have reported the strong antifungal activity of ginger. They attribute this strong inhibition potential to over 400 different compounds a mixture of both volatile and non-volatile chemical constituents such as zingerone, shogaols, ingerols, sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene) and a small monoterpenoid fraction (β-phelladrene,cineol and citral) (Chrubasik *et al*., 2005; Grzanna *et al*., 2005). Significant antifungal efficiency of ethyl acetate fraction in comparison to other ethanolic fractions of ginger was also observed by Ficker *et al.* (2003a). Furthermore they used this fraction to isolate Gingerol and gingerdiols. Both the compounds inhibited the mycelial growth of *A. alternata* along with several other filamentous fungi. In another study, Ficker *et al.* (2003b) screened 29 plant extracts, of which ginger extract had the broadest range of anti-fungal activity, measured either by the fungal inhibition or as the average diameter of the zones of inhibition. Similarly ethanol and ethyl acetate extracts of some plants were very effective in inhibiting *A. solani* and *F. oxysporum*, while aqueous extracts had the least effect (Hassanein *et al*., 2008). Mostafa *et al*. (2011) screened five plant extracts for their antifungal activity including *Z. officinale*. They isolated and reported phenol compounds, gingerol, cedrene and zingiberene as the most effective antimicrobial component in *Z. officinale* extract. Growth inhibition of phytopathogenic fungi was attributed to the presence of phenolic compounds such as gingerol, cedrene, zingiberene and α-curcumene isolated from Z. *officinale* extract (Rahmah *et al*., 2013). The variations in fungitoxicity of different ethanoilc fractions of ginger towards the test pathogen could be due the fact that different compounds dissolve in different organic solvents due to their different polarities.Chemical variations leads to difference in their antimicrobial activity. (Manoranjitham *et al*., 2001; Singh *et al*., 2008 ; Nadia *et al*., 2014).

Present study shows ethanol extracts of ginger as excellent antifungal agent as it inhibited the mycelia growth as well as spore germination.The same applies to its ethanolic fractions, particularly with ethyl acetate. Hence *Z.officinale* could serve as a safe,ecofriendly and inexpensive bio control agent and an excellent alternative to chemical fungicides. Especially in an era where safety of human health and environment is a burning issue due to concerns on accumulation of fungicides and its residual toxicity in soil and vegetables. Moreover several clinical trials have proved *Z. officinale* root and root extracts were found to be safe (Mowrey and Clayson, 1982). Hence it is a promising alternative to chemical fungicides.

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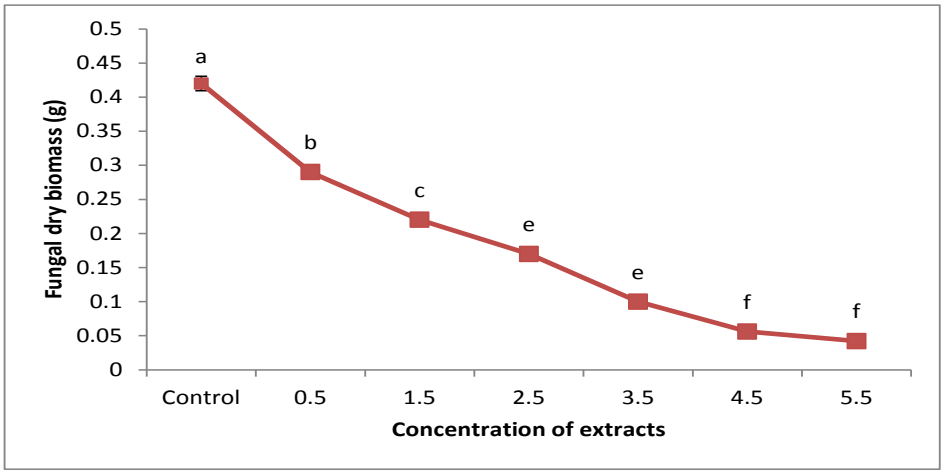

Fig. 1: Effect of different concentrations of ethanolic extarct of Zingiber officinale on the biomass of *Alternaria alternate*. Comparison of means (lettering) was done using LSD test at 5% level of significance.

Table 1: Effect of ethanolic extracts on the mycelia growth of *Alternaria alternate.*

Concentrations of extract (%)	Mean radial growth	Percent inhibition
Control	90.00	-
0.5	70.80	21.33±0.29 e
1.5	54.50	39.44±0.68 d
2.5	28.26	68.60±1.80 c
3.5	13.33	85.19±1.97 b
4.5	2.46	97.27±1.54 a
5.5	1.03	98.86±1.90 a

Comparison of means (lettering) was done using LSD test at 5% level of significance.

Table 2: Effect of ethanol extract of *Zingiber officinale* on spore germination of *Alternaria alternate.*

Concentrations of extract (%)	Spore's density	Percent Inhibition
0.5	92	16.36±0.25 e
1.5	62	43.63±0.24 d
2.5	46	58.18±0.12 c
3.5	5	95.45±2.24 ab
4.5	0	100.00±3.15 a
5.5	0	100.00±3.15 a

Comparison of means (lettering) was done using LSD test at 5% level of significance.

Table 3: Antifungal activity of various fractions of ethanolic extracts of *Zingiber officinale o*n the growth *of Alternaria alternate.*

Ethanolic fractions	DMSO conc (mL mL^{-1})	Concentration of Extract (mg mL^{-1})	Fungal biomass (mg)
Control	0.2665	0	0.86 opq
	0.1332	0	1.00 mno
	0.0666	0	1.56 k
	0.0333	0	1.90 j
	0.0166	0	2.16 i
	0.0083	0	3.62 f
	0.0041	0	4.53 d
	0.0020	0	5.00 b
Petroleum ether	0.2665	320	0.20 wxy
	0.1332	160	0.31 uvw
	0.0666	80	0.62 rs
	0.0333	40	0.86 opq
	0.0166	20	1.10 m
	0.0083	10	1.90 j
	0.0041	5	2.40 h
	0.0020	2.5	2.90 g
Chloroform	0.2665	320	0.00 z
	0.1332	160	0.00 z
	0.0666	80	0.16 xy
	0.0333	40	0.37 tuv
	0.0166	20	0.51 st
	0.0083	10	0.88 nop
	0.0041	5	1.25 l
	0.0020	2.5	1.50 k
Ethyl acetate	0.2665	320	0.00 z
	0.1332	160	0.00 z
	0.0666	80	0.12 yz
	0.0333	40	0.17 wxy
	0.0166	20	0.28 u-x
	0.0083	10	0.48 st
	0.0041	5	0.62 rs
	0.0020	2.5	0.73 qr
n-Butanol	0.2665	320	0.23 v-y
	0.1332	160	0.40 tu
	0.0666	80	0.78 pq
	0.0333	40	1.01 mn
	0.0166	20	1.42 k
	0.0083	10	2.94 g
	0.0041	5	4.41 d
	0.0020	2.5	4.88 b
Aqueous	0.2665	320	0.74 pqr
	0.1332	160	0.93 no
	0.0666	80	1.50 k
	0.0333	40	1.90 j
	0.0166	20	2.16 i
	0.0083	10	4.01 e
	0.0041	5	4.72 c
	0.0020	2.5	5.50 a

In vertical columns, values with different letters show significant difference ($P \leq 0.05$) as determined by Tukey's HSD test.

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