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Comparative antifungal study of cadaghi gum (*Eucalyptus torelliana* F) flowering buds, leaves and bark extracts against foliar blight pathogen in control conditions

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Key words: *Eucalyptus torelliana* F. Muell.; Aqueous extract; Organic solvent extracts; Spot blotch disease of wheat.

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Abstract

The present study objective was to evaluate and compare the utmost antifungal activity by screening different parts of *Eucalyptus torelliana* F. Muell. (Cadaghi) have been successively extracted in a variety of solvents against most hostile isolate of *Bipolaris sorokiniana* Sacc. In-vitro bioassay conducted with crude extracts at different concentrations (1%, 05% & 10%) for radial mycelial growth, inhibition zone and biomass production of *B. sorokiniana*. Inhibition of pathogen was check by food poison technique and well diffusion assay in seeded agar plates. Colony growth inhibition due to leaves extracted in ethanol (76%) and methanol components of flower buds (75%) had been found to get higher than both ethanol and methanol extracts of bark (58%). Likewise for aqueous ingredients bark and leaf proved higher inhibition (52% & 50%) compare to flower buds. The inhibition zone observed for flowering buds were 29.15±0.88; 27.40±1.25; 26.15±1.03 and 0.00±0.00mm at highest concentration (5.3mg/100µl) for methanol, ethanol, aqueous and control treatments respectively against *B. sorokiniana*. The highest decrease found in hyphae fresh and dry weight (0.026 & 0.02g) treated with flower bud methanol extract in contrast of aqueous extracts (2.28 & 2.23g). Length/width of extract treated conidia (30±2.88/20±1.91µm) and conidiophores (111±16.42/5.50±0.56µm) have been significantly decreased with respect of control treated conidia 77±0.54/25±0.15µm and conidiophores 141±1.69/7.33±0.44µm. The average numbers of septa within treated conidia were basically 2-6 in control 2-7 had been observed. Really small variations were seen in colony color, margin, texture and hyphae thickness in extract treated and control treatments.

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Introduction

Wheat (*Triticum aestivum* L.) is affected by different fungal disease e.g Stem rust (*Puccinia graminis* f. sp. *Tritici*), Stripe rust (*Puccinia striiformis* f. sp. *Tritici*), Leaf rust (*Puccinia triticina*), Powdery mildew (*Blumaria graminis* f. sp. *Tritici*) Loose smut (*Ustilago tritici*), Downy mildew (*Sclerophthora macrospora*), *Septoria tritici* blotch (*Septoria tritici*) and *Fusarium* head blight (*Fusarium graminearum*). Among these diseases, Spot blotch caused by *Bipolaris sorokiniana* is of severe concern all around the world particularly to south Asia and south America due to its wide spread, occurrence and rising severity (Joshi *et al*., 2002). It causes seedling blight, root rot and spot blotch of wheat. An initially very small, dark brown lesion without chlorotic margin appears. After that, these lesions enlarge in oval to elongated blotches up to several centimeters, light brown to dark brown in colour and resulting death of the leaf. Fruiting bodies are generally observed on old lesions. Shriveled grain and black pointed seed results if infection reached to spikelet (Duveiller and Dubin 2002).

The ideal condition for the development of spot blotch are 85-100% humidity and 20-30^{0}C temperature for a long duration of 12-24 hours specially when host leaves are wet either by rainfall, irrigation or dew. If conditions are best germination of conidia completed within four hours on host leaf surface and infects new host plant within 24 hours. Sources of inoculum for this disease are infected seeds; air, crop residues and soil also contain conidia that survive when temperature and humidity are appropriate. The host range of *B. sorokiniana are mostly* small grain cereals, like *Triticum aestivum, hordeum vulgare, Avena sativa, Sorghum bicolor* and a large number of wild grasses. Several plant species other than monocotyledons including *Brassica compestris, Glycine max, Lens culinaris, Vigna radiata, Sesamum indicum, Vigna mungo and Pennisetum amaricanum* are identified as the host of *B. sorokiniana*. Iftikhar *et al.* (2012) stated spot blotch of wheat to be of economic importance. This disease causes yield losses of 10-30% and these yield losses dependent on heat, late sowing and low fertilizers uses. Significant losses due to spot blotch disease in wheat crops were reported by Rattu *et al.* (2011) in Pakistan. According to them spot blotch disease prevalence on five commercial varieties of wheat i.e. Bhakkar-2001, Inqilab -91, Faisalabad-08, Lasani-08 and Seher-2006, was 100%, 14%, 10%, 5% and 3% respectively.

Spot blotch is controlled generally by the application of agrochemicals. But, in recent years, there are rising concern associated with farming methods that are feasible both environmentally and economically. Natural plant products are important sources of new agrochemicals for the control of plant diseases (Kagale *et al.,* 2004). Studies revealed that plants (oil & extracts) contain natural ingredients that are efficient against disease managements (Goussous *et al.,* 2010). This disease management can be achieved by direct toxic effects of active ingredients. These natural substances can inhibit the mycelial growth or slow down the spore germination and generate the resistance induced by physiological changes in the plant, such as stimulation of pathogenesis-related enzymes, lignifications and phytoalexins (Schwan-Estrada & Stangarlin, 2005). Garlic extract reduce germination of spores more than 50% and induced modifications in the morphology of hyphae and conidia of *B. sorokiniana* Perello *et al.* (2012). Therefore the objective of present study was to investigate the efficiency of *Eucalyptus torelliana* leaf, bark and flowering buds extracts against the management of spot blotch of wheat under *in-vitro* conditions.

Material and methods

Test pathogen of spot blotch disease

Bipolaris sorokiniana was isolated from diseased wheat plants grown in National Agricultural Research Center (NARC) Islamabad, research fields and after confirmation of Koch's postulates pathogen was maintained on growth medium until used.

Extraction procedure

Samples of different parts of *Eucalyptus torelliana*

Were collect from different localities of Islamabad, Pakistan. Shade dried sterilized plant material were powder in electric grinder. Powdered material was successively extracted according to Jeyaseelan *et al.* (2012) with water, methanol and ethanol. Plant material of 20g were suspended in 100 ml sterilized water and shake for 3 days on a rotary shaker. It was first filtered with eight fold muslin cloth and then through Whatman filters paper (No.01) and completely dry on water bath at 45^{0}C. The consequential residue were used for further extraction with methanol and followed by ethanol just like the procedure that carried out for the water extraction. Thereafter both extracts were dry separately under reduced pressure on Rota evaporator at 45^{0}C. After full drying, weight the yield of the each extract separately and use for potential antifungal activities.

Antifungal bioassay

Treatments of different concentrations were tested against the test fungi by agar well diffusion and food poison technique. To form stock solution 1.6g dry extracts were dissolve in 30ml sterile water. Then 100, 300 and 600µl stock solution that containing (5.3, 16 & 32mg) dry extract were dissolve separately in 2ml sterile water and 5% ethanol, methanol to form (1, 5 & 10%) concentrations. An aliquot (2ml) of each concentration was then added to 90ml autoclaved growth medium (potato dextrose agar) and mixed thoroughly. Thirty ml was poured in each of three Petri plates of 9cm diameter. Thereafter, mycelial discs of approximately 6mm diameter, cut from the periphery of a 10-day old culture were inoculated in the centre of each Petri plate. Methanol ethanol and distilled water instead of extracts served as the control. For each treatment three replicates were maintained in a completely randomized design. These Petri plates were then incubated at 28±2°C and observations were recorded from the 3rd day until completed growth in control plates (Sasode *et al.*, 2012).

Potato dextrose agar growth medium was prepared in distilled water, pH was adjusted to 6.5, autoclaved at 121^{0}C and 15 psi for 20 minutes and then allowed to cool up to 30°C, add pathogen suspension in growth medium and mix uniformly. Petri plates (90mm) were poured with 30ml of seeded PDA and allowed to solidify. Wells were made in the center of agar plates with the help of sterile cork borer of 8mm diameter. About 100µL of the crude extract at 50mg/ml concentration were added into each well for about 2h leave it at room temperature, for controls treatments the wells were filled with water, methanol and ethanol and incubated at 28 °C. After 48h zones of inhibition were measured and treatments were compared with control.

For the determination of dry weight of the test fungi, approximately 1ml of each treatment contains 50 mg/ml concentration were added to 20ml of sterilized PD broth in 100ml flask and inoculated with a 5mm disc of test fungi. The flasks containing medium with 1ml of methanol, ethanol and distilled water served as control. After 10 days of incubation on rotary shaker dry weights of mycelia (*Bipolaris sorokiniana*) were determined (Ramezani *et al.*, 2002).

The morphological affects caused by different treatments on conidia and conidiophores were determined from the comparative structures analysis that was observed in the control and in each of the treatments under an optical microscope at (10X & 40X).

Statistical analysis

The entire experiments were independently repeated three times. Three-factor factorial under Completely Randomized Design with 95% confidence level was used for statistical analysis of the results by STATISTIX 8.1. The results of antifungal bioassays were presented as mean value ± standard deviation.

Results

Leaves, flowering buds and bark of *Eucalyptus torelliana* F. Mueller were evaluated for fungicidal activity against *B. sorokiniana* causes spot blotch in wheat. Samples were extracted in water, ethanol and methanol and antifungal activity was checked against

mycelial growth, spore germination, sporulation, biomass production, hyphae and conidial morphological characterization. Leaf and flowering buds extracts of *E. torelliana* showed very significant antifungal activity while the bark extracts also having antifungal activity but not significant against pathogen. The ethno botanical data of *E. torelliana* are shown in Table 2.

Table 1. Analysis of Variance Table for Radial mycelial growth.

Source	DF	SS	MS	F	P
Parts	2	383.9	192.0	3.21	0.0410*
Solvents	2	2756.1	1378.0	23.05	0.0000**
Concentration	2	21779.0	10889.5	182.18	0.0000**
Parts*Solvents	4	1086.0	271.5	4.54	0.0013**
Parts*Concentration	4	547.2	136.8	2.29	0.0586
Solvents*Concentration	4	78.4	19.6	0.33	0.8593
Parts*Solvents*Concentration	8	1916.0	239.5	4.01	0.0001**
Error	621	37119.1	59.8		
Total	647	65665.7			

P Value < 0.05= significant result.**

Table 2. Ethno-botanical data of collected sample of *Eucalyptus torelliana* F. Muell.

Botanical name	Common name	Local name	Family	Part of plant used	Place collection
Eucalyptus torelliana F. Muell	Cadaghi gum	Lachi, Sufaida	Myrtaceae	Leaves, Bark, Flowering Buds	Pakistan forest Institute, Peshawar

Redial mycelia growth inhibition caused by different treatments

In present study the inhibitory effect of *E. torelliana* different parts aqueous and organic extracts were evaluated using different treatments combination against *B. sorokiniana*. The highest mycelial growth was observed in control treatments (water) followed by methanol and ethanol as shown in Fig 1, 2 and 6.

Table 3. Conidia characters of *Bipolaris sorokiniana* subjected to different concentrations of *Eucalyptus torelliana* F. Muell extracts.

Treatments			Color		Size (Mean±S.Error)				No of septa		Shape of conidia
					Length (µ)		Width (µ)		Conidio phore	Conidia	
B. extracts	E. solvent	Part used	Conidiophore	Conidia	Conidiophore	Conidia	Conidiophore	Conidia			
E. torelliana F. Muell	Ethanol	Leaf	Light brown	Olivaceous brown	115±16.30	51±3.17	6.91±0.52	23±2.23	4-9	2-6	Elliptical or oval
		Flower Buds	Light brown	Olivaceous brown	114±15.00	52±6.29	5.33±0.66	21±3.05	2-7	1-4	Oval to nearly round
		Bark	Light brown	Olivaceous brown	119±20.51	67±4.04	7.07±0.36	25±1.76	3-9	2-6	Elliptical or oval
	Methanol	Leaf	Light brown	Olivaceous brown	122±20.21	52±11.8	7.09±0.36	36±4.16	4-9	2-6	Oval with round ends
		Flower Buds	Light brown	Olivaceous brown	111±16.42	30±2.88	5.50±0.56	25±1.46	2-7	1-5	Oval to nearly round
		Bark	Light brown	Olivaceous brown	122±16.44	52±6.40	5.96±0.38	22±2.18	4-9	1-6	Oval with round ends

Water	Leaf	Light brown	Olivaceous brown	117±16.91	49±5.02	6.33±0.49	23±2.34	4-8	2-7	Oval slightly curved
	Flower Buds	Light brown	Olivaceous brown	112±15.81	50±6.75	6.50±0.42	25±1.83	2-9	2-6	Elliptical or oval
	Bark	Light brown	Olivaceous brown	121±14.86	62±3.33	6.00±0.51	20±1.91	2-9	1-7	Oval with round ends
Control	Ethanol	Light brown	Olivaceous brown	127±1.79	75±0.76	6.87±0.30	27±0.18	2-7	2-7	Oval with round ends
	Methanol	Light brown	Olivaceous brown	118±1.97	75±1.11	6.84±0.44	20±0.20	2-8	1-6	Oval with round ends
	Water	Light brown	Olivaceous brown	141±1.69	77±0.54	7.33±0.44	25±0.15	2-9	2-7	Oval with round ends

Table 4. Sporulation rate and conidial germination of *Bipolaris sorokiniana* affected by *Eucalyptus* torelliana extracts.

Botanical	Extraction solvent	Part used	*Eucalyptus torelliana* F. Muell		
			Conidial recount (Mean±S.Error)		Conidial germination
			Sporulation	No of spores/10µl	Germination pattern
E. torelliana F. Muell Flowering buds	Ethanol	Leaf	+++^{c}	173±16.83	Mostly unipolar sometime bipolar
		Flower Buds	++ ^{b}	130±9.53	Mostly unipolar sometime bipolar
		Bark	+++^{c}	211±19.45	Mostly unipolar sometime bipolar
	Methanol	Leaf	+++^{c}	136±12.51	Mostly unipolar sometime bipolar
		Flower Buds	++^{b}	157±7.49	Mostly unipolar sometime bipolar
		Bark	+++^{c}	203±12.86	Mostly unipolar sometime bipolar
	Water	Leaf	+++^{c}	208±15.91	Mostly unipolar sometime bipolar
		Flower Buds	+++^{c}	199±10.33	Mostly unipolar sometime bipolar
		Bark	+++^{c}	258±15.50	Mostly unipolar sometime bipolar
	Control	Ethanol	+++ ^{c}	146±8.29	Mostly unipolar sometime bipolar
		Methanol	+++ ^{c}	124±7.62	Mostly unipolar sometime bipolar
		Water	++++ ^{d}	181±12.36	Mostly unipolar sometime bipolar

=No Sporulation, +^{a} =Sporulation rate less than 40% compared to control, ++^{b} = Sporulation rate >40% and <70%, +++^{c} = Sporulation rate > 80% and <100%, ++++^{d} = 100% Sporulation.

Combinations of Eucalyptus torelliana (Extract in ethanol, methanol, and water)

Inhibition zone made by different treatments of Eucalyptus extracts

The *E. torelliana* flowering buds extract showed the highest inhibition zone of 29.51±0.71 compared to inhibition zone made by leaf extract and bark extract. Minimum antifungal activities regarding to inhibition zone observed was (9.20±1.86mm**)** for bark aqueous extract as shown in Fig. 3 and 6.

Biomass production of B. sorokiniana affected by Eucalyptus extracts

In present study different treatments of *E. torelliana* crude extract against biomass production of *B. sorokiniana* were observed.

Treatments of flowering buds extracts showed very good results in term of fresh and dry biomass weight as presented.

The maximum fresh weight (2.281g) for biomass of *B. sorokiniana* was found with *E. torelliana* bark aqueous extract and the maximum dry weight (0.266g) of biomass was found with *E. torelliana* flowering buds ethanol extract see Fig. 5.

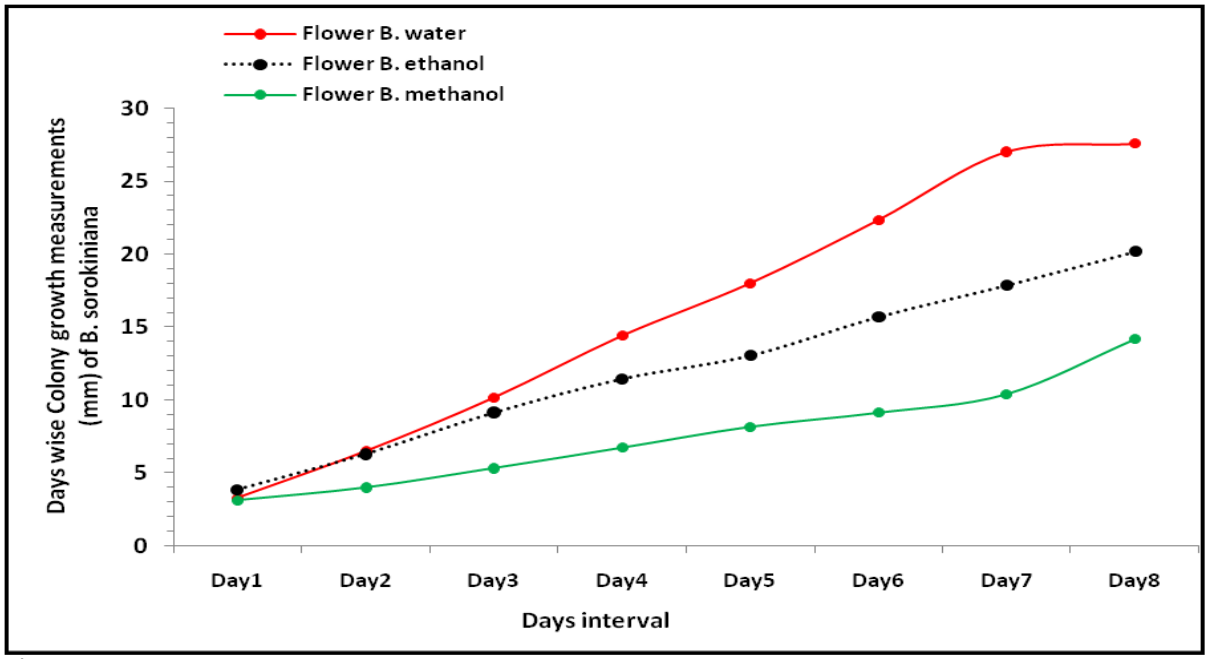

Fig. 1. Daily observation for colony growth of *Bipolaris sorokiniana* effectted by lower buds treatments of *Eucalyptus torelliana*.

Discussion

Effect of Eucalyptus torelliana F. extracts on mycelial growth inhibition and inhibition Zone of B. sorokiniana at different treatments combination

In present study the inhibitory effects of different treatments combinations of ethanol, methanol and aqueous extracts of *Eucalyptus torelliana* different parts were evaluated against Spot blotch causing pathogen of wheat crop. The antifungal activities of ethanol methanol and water extracts of *E. torelliana* in response to *B. sorokiniana* are prsented in Fig. 2, 3 and 6 as mycelial growth inhibition and zone of inhibition. The radial mycelia growth of *B. sorokiniana* was significantly decline by fruit and leaves extracts treatments and bark extracts were also found to causes the inhibition of fungal growth.

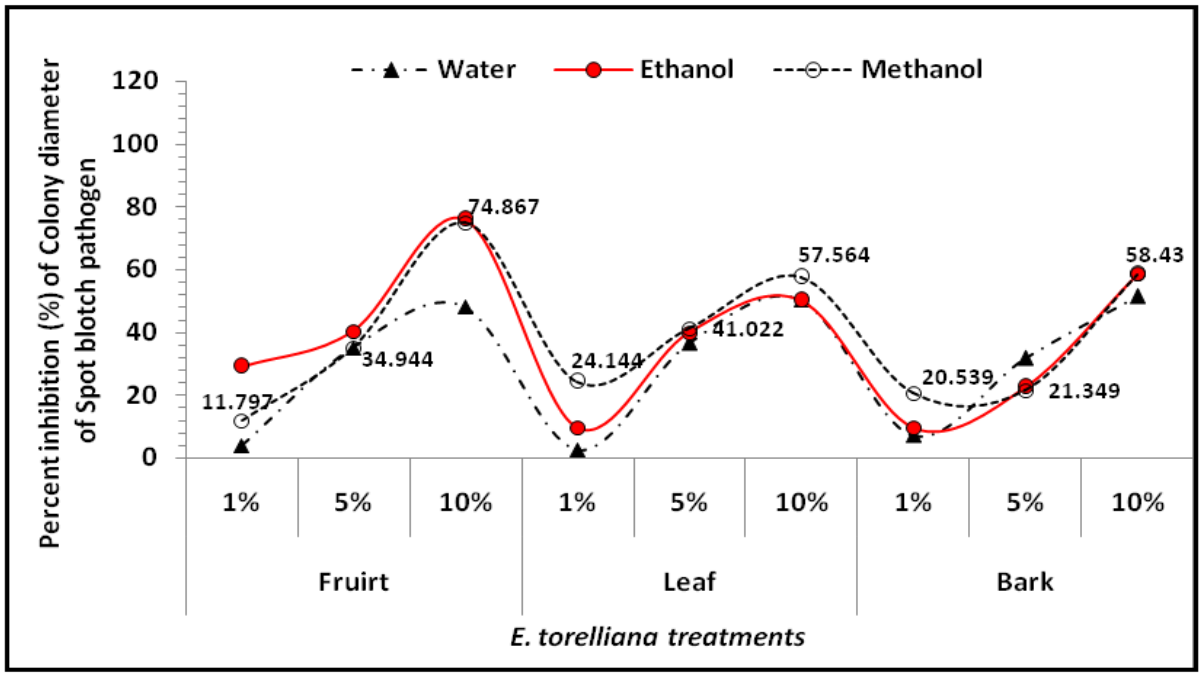

Fig. 2. Percent inhibition of *Bipolaris sorokiniana* colony growth with respect to different treatments.

The highest growth was observed in water control followed by methanol and ethanol control. The antifungal activities were compared among different concentration of *E. torelliana* extracts and it was found that treatments of 10% concentration gives best results of inhibition against *B. sorokiniana*. For instance, 10% leaves ethanol extract and 10% flowering buds methanol extract showed lowest mycelial growth (7.196±0.737mm & 7.633±0.713mm) and highest mycelia growth inhibition (75.9% & 74.9%) respectively as given in Fig. 5 and 6. The lowest mycelial growth inhibition observed in whole study was for leaf water extract at 1% concentration (2.6%). Similar to Hasan *et al.* (2012) the inhibition percentage of hyphae growth increases with the increases of treatments concentrations.

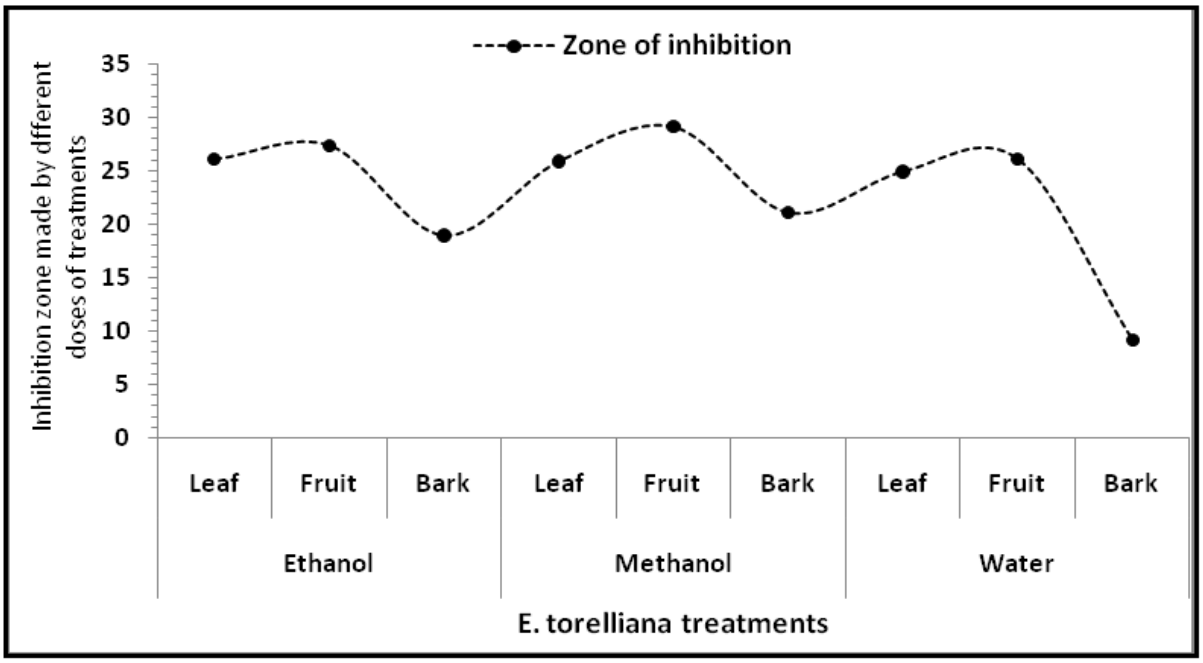

Fig. 3. Inhibition zone made by different treatments combinations of *Eucalyptus torelliana* against *Bipolaris sorokiniana* (Extract in ethanol, methanol, and water).

Maximum antifungal activities and minimum antifungal activities regarding to zone of inhibition were recorded for flowering bud methanol extract (29.15±0.88mm) and bark water extract (9.20±1.86 mm)**.**

They have statistically no match with other different treatment combination. Results of bark and leaf treatments were also supported by Jain *et al.* (2010) that the *E. treticornis* bark methanol extract more significantly inhibiting *Candida albicans* compared to leaf methanol extract with inhibition zone ranging between 17-27mm and 18-24mm respectively. Water extract of *Eucalyptus* leaf also causes reduction in spot blotch disease severity caused by *B. sorokiniana* (Yadav *et al.*, 2015). A lot of studies are reported regarding to antifungal efficacy of different plant extracts and their E. oil against various dermatophytes and filamentous pathogens (Tyagi and Malik, 2010).

Relationship between colony growth inhibition and concentration of most active extracts

The regression equation between concentrations of *Eucalyptus* flowering buds ethanol extract and mycelial growth inhibition indicates that for each unit increase in concentration the percent inhibition increases 529.0 units. The R^2=0.949 means that more than 94% variability's in mycelial growth inhibition was explained by changes in treatment concentrations. The straight line evidences that the rate of increasing trend of mycelial growth inhibition due to gradual increase of concentration of treatments (Fig. 4).

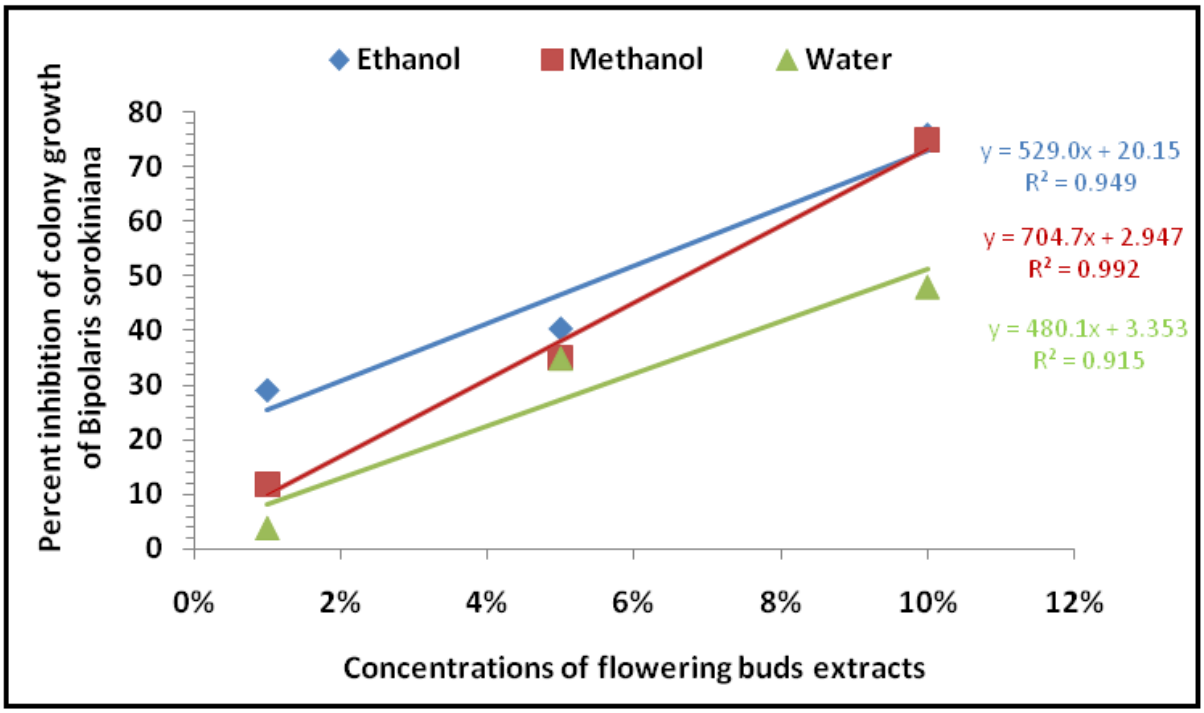

Fig. 4. Functional relationship between concentration and colony growth inhibition of *Eucalyptus* extracts (Extract in ethanol, methanol, and water).

Effect of Eucalyptus torelliana extracts on Conidia characteristics, fresh and dry weight of B. sorokiniana at different treatment combination

Flowering buds treatments showed very good results in term of fresh and dry weight are presented in Fig. 5. Like 0.026g fresh weight and 0.02g dry weight was observed with 10% flowering buds methanol treatment. Similarly Hasan *et al.* (2012) also reported the lowest fresh weight 1.6g with 5% Neem extract and dry weight 0.052g with 15% Onion extract.

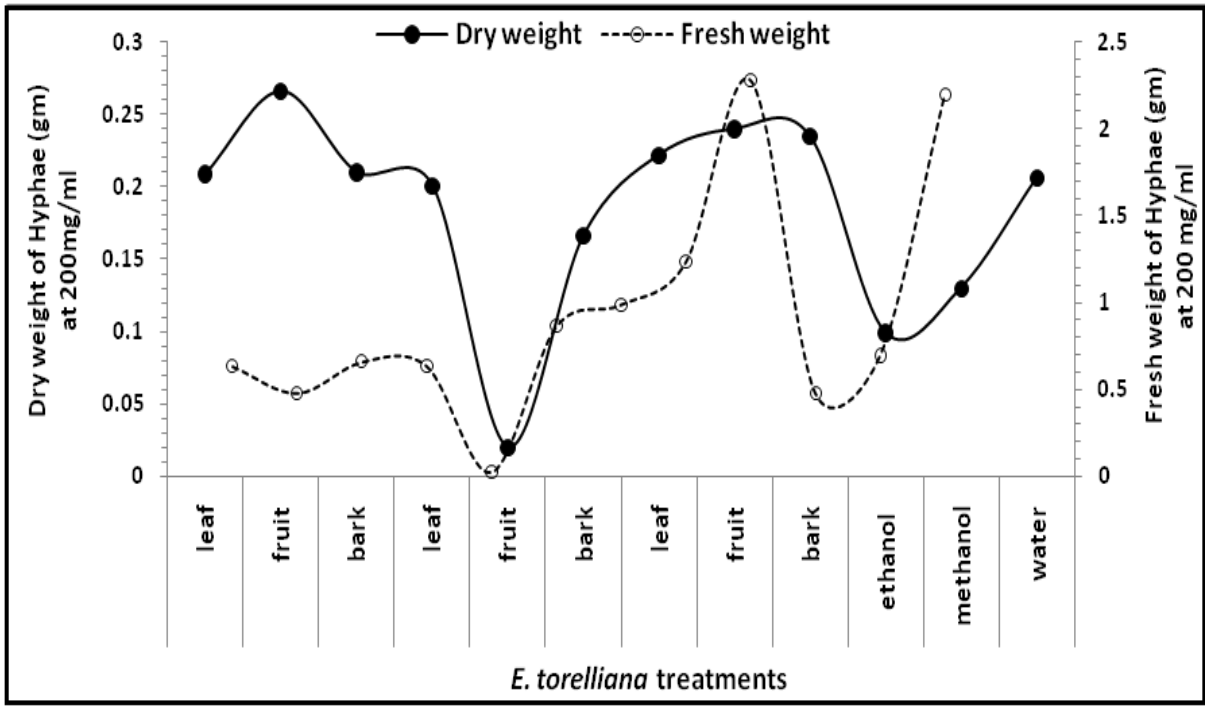

Fig. 5. Relationship between different *Eucalyptus* treatments combinations, colony growth and percent inhibition of *Bipolaris sorokiniana*.

Different variety of other plants species that are effective against *Bipolaris sorokiniana* were reported by several authors, such as *Eucalyptus camaldulensis* essential oil against *Bipolaris sorokiniana* (Katooli, *et al.,* 2014), Garlic extract (Perello *et al*., 2012; Hasan *et al.,* 2012), Neem leaf and cake aqueous extract (Yadav *et al*., 2015; Al-Hazmi, 2013), Bauhinia extracts ((Elisabeth Bach *et al*., 2012), *Adhatoda vasica* (leaf) and *Zingiber Officinale* (rhizome) extracts (Akhter *et al*., 2006).

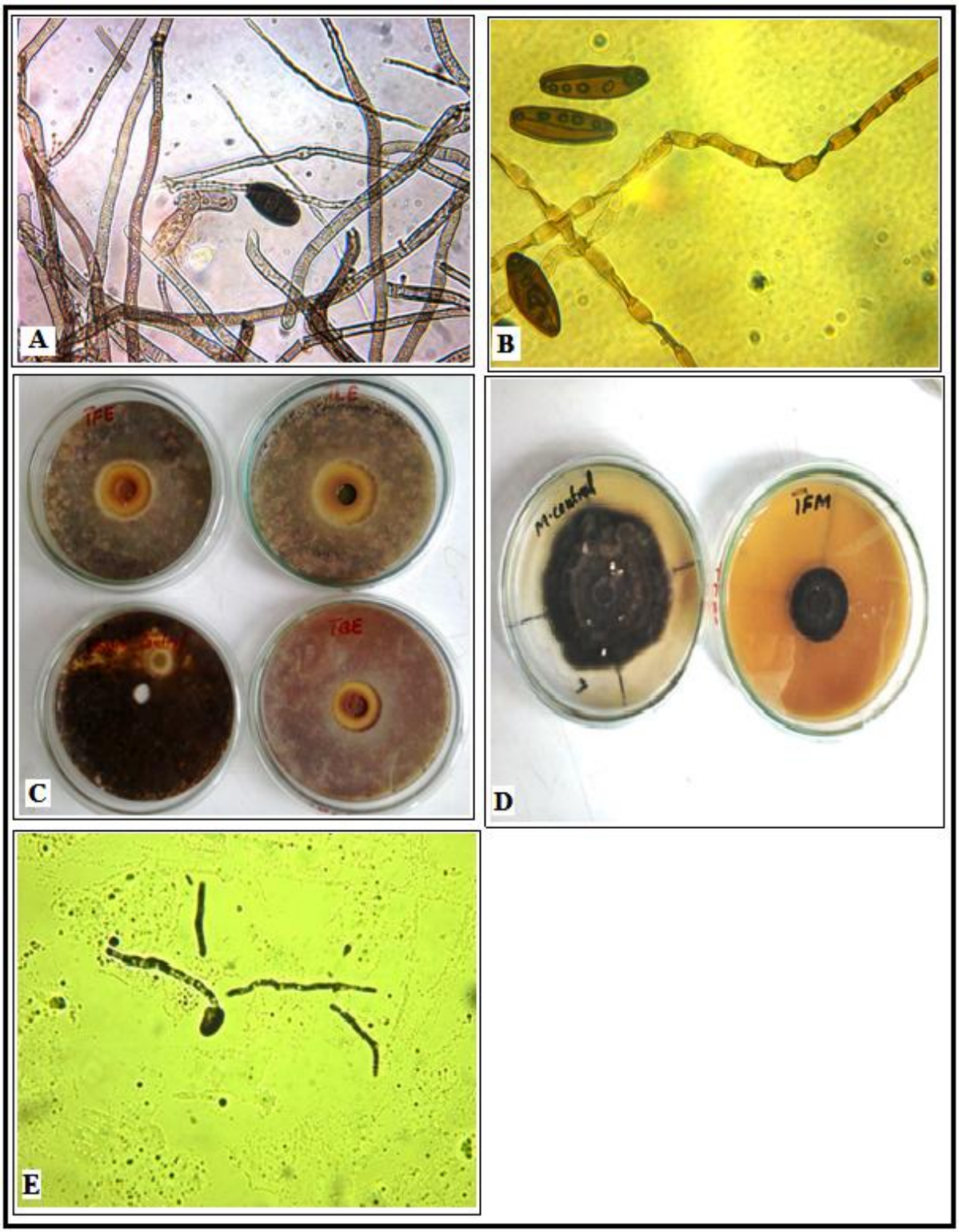

Fig. 6. A: extract treated spore; B: spore in control treatment; C: Zone of inhibition made by leaf, F.bud, bark & control treatment; D: colony growth effects by F.bud treatment; E: unipolar germination of conidia.

Significant results were obtained in regarding to morphological changes occurred in conidia and conidiophores after treatments applications as shown in Table 3. Generally the light brown colour conidiophores and olive brown colour conidia were observed. Mostly the shapes of conidia were oval to nearly round but in some treatments oval to curved and elliptical conidia were also founded. Chowdhury *et al.* (2013) also reported the elliptical shaped conidia with 5-9 septa.

The minimum and maximum length of conidiophores observed after application of different treatments combinations was 111±16.42 to 122±20.21µm compared to control treatments 141±1.69µm with 2-9 septa whereas length of conidia range from 51±3.17 to 67±4.04µm having 1-7 septa. Similar results for conidia size of control treatments was reported by Muchovej *et al.* (1988). They observed that *B. sorokiniana* presented conidia more than 75 micron long and less than 25 micron wide.

The highest percentage of sporulaion was observed in case of ethanol and methanol treatments at 10% concentration. The minimum number of spores 130±9.53 counted in 10µl suspension. The germination pattern mostly found was unipolar. Sometime bipolar germination was also founded.

From the above results it is evident that *Eucalyptus* extracts exert antifungal effects against different kind of pathogens such as *E. citriodora against Macropomena phaseolene* Javaid and Rahman (2011); *E. citriodora* against *A. rabiei* Jabeen and Javaid (2008); *E. citridora* against *A. alternata and F. solani* Shafique *et al.* (2007); *Eucalyptus camaldulensis* against *Candida albicans Uz*ama *et al.* (2011); *E. globules* E.oil against dermatophytes Elaissi *et al.* (2012). *Eucalyptus* methanol extract against *Alternaria alternate* Zaker & Mosallanejad (2010). Through these findings, it is clear that *Eucalyptus* definitely contains some active ingredients and because of that, uses of crude eucalyptus extracts resulted in reducing disease severity in crops including spot blotch in wheat caused by *Bipolaris sorokiniana*.

Conclusion

This is concluded from the results of whole study that both the leaves and flowering buds of *Eucalyptus torelliana* F. Muell have more efficacy as antifungal agents against *Bipolaris sorokiniana* with respect to bark. Methanol and ethanol extract are more effective inhibitor against test organisms compared to water extracts. These investigations defend all those claims which made by different researchers about *Eucalyptus* species uses in conventional medication to treat various infectious diseases. These results will definitely provide the strong basis to select different plant species and explore the new discovery of natural active ingredients. However more research require for isolation, purification and structural elucidation of the targeted active compounds present in the crude extracts mixture has been initiated that not only justify these inhibitory claims but also once the structure of most potent natural compounds are identified that can be used as a lead for the synthesis of synthetic pesticides.

Acknowledgement

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Ethical statement

This material has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content. No animal or human studies were carried out by the authors.

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