INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 10, No. 5, p. 138-147, 2017
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Antifungal potential of Gymnospermic cones against Panama wilt fungus (*Fusarium oxysporum* f. sp. *cubense*)

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Article published on May 29, 2017

Key words: Panama disease, *Fusarium oxysporum* f. sp. *cubense*, Food poisoning technique, Percent inhibition, Gymnospermic botanicals.

Abstract

Yield of commercial banana plantations across the world is tremendously affected by vascular wilt (Panama disease) of banana, caused by a soil-borne hyphomycete *Fusarium oxysporum* f. sp. *cubense*. Six Gymnospermic cones extract (*Pinus roxburghii, Pinus wallachiana, Cedrus deodara, Thuja orientalis, Cupressus sempervirens* and *Picea smithiana*) prepared in selected organic solvents (methanol, ethanol and n-hexane) were *in vitro* screened against *Fusarium oxysporum* f. sp. *cubense* using food poisoning technique. Percent inhibition results from preliminary screening clearly indicated that 10% cone extracts were more effective compared to 5%. Extracts showing ≥ 60% inhibition were further evaluated using 50% extract concentration. Methanolic extract of *Picea smithiana* cone, ethanolic extract of *Thuja orientalis* cone, *Pinus wallachiana* and *Pinus roxburghii* cone extract in n-hexane exhibited ≥ 70% inhibition against tested fungus. Results revealed that these Gymnospermic botanicals are potential source of antifungal phytochemicals, therefore extensive research is required to extract their active compounds thus providing a substitute of fungicide and an alternative approach to current management practices of Panama wilt disease.

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Introduction

Yield of cultivated plants is greatly affected by plant pathogenic fungi that raises continuous threat to global food security (Savary *et al*., 2006; Montesinos, 2007). Vascular wilt (Panama disease) of banana, caused by a hyphomycete *Fusarium oxysporum* f. sp. *cubense*, is one such devastating disease that is reason for destruction of commercial banana plantations across the world (Ploetz, 2005; Ploetz, 2006; Pereira and Gasparoto, 2006; Grim, 2008).

Three out of total four different races of this soil-borne fungus are found pathogenic to particular varieties of banana (Ploetz and Pegg, 1997). Carribean and Central American banana plantations relying on 'Gros Michel' variety were destroyed by FOC race 1 during the mid-twentieth century (Daly and Walduck, 2006; Ploetz and Pegg, 1997). Shift of banana plantation from susceptible varieties to a resistant Cavendish group overcome the issue but emergence of FOC race 4 has again raised an alarming situation worldwide as this particular virulent race affects Cavendish group as well (Huang *et al*., 2012).

Fungus entry point is the banana roots from where it spreads to the corm. It finally invades xylem tissues that results in external symptoms appearance that includes gradual wilting, progressive leaf chlorosis and necrosis and eventually leaves collapse at the petiole, pseudostem splitting. Internal symptoms include discoloration of vascular tissues. Continuous progression of disease leads to corm and pseudostem collapse that ends up in plant death (Nelson, 1981; Ploetz, 2000; Yin *et al*, 2011).

Flood fallowing (Stover, 1962), crop rotation and intercropping (Zhang *et al*., 2013), addition of organic amendments (Kidane and Laing, 2010), planting resistant cultivars (Smith *et al*., 2006), molecular techniques for production of resistant banana varieties (Yip *et al*., 2011; Paul *et al*., 2011), soil fumigation (Herbert and Marx, 1990; Ploetz, 2000), chemical control (Nel *et al*., 2007) and biocontrol (Cao *et al*., 2005; Lian *et al*., 2009; Akila *et al*., 2011; Thangavelu and Mustaffa, 2012) are all management strategies. Though giving promising results under laboratory and green house conditions, little or short-term success has been observed under field conditions in all mentioned control options (Thangavelu and Mustaffa, 2012; Zhang *et al*., 2013; Gnanasekaran *et al*., 2015).

Widespread use of synthetic pesticides to combat diseases has developed microbial resistance against such commercial antimicrobial products (Brent and Hollomon, 1998; Mukherjee *et al*., 2002). It's Cost, health hazards, environmental pollution, bioaccumulation are all additional drawbacks. Public awareness has elevated interest in discovering safer alternate protectants to substitute synthetic chemical pesticides (Paster and Bullerman, 1988; Daoubi *et al*., 2005). For thousands of years plants have been used as medicines (Samuelsson, 2004). Since ancient era, plants have been documented for their ethnopharmacological properties. 50 percent of modern drugs are derived from natural sources and plants (Gurib-Fakim, 2006; Dias *et al*., 2012).

Medicinal plants are continuously exploited to discover novel antimicrobial agents by scientists. Higher plants, including angiosperms and gymnosperms, are more frequently been investigated for their bioactivity. Angiosperms are more often explored than gymnosperms (Joshi and sati, 2008). This group of naked-seeded vascular plants, forming occasionally dominant trees of forest areas, have also been used as folk medicines for centuries (Kirtikar and Bassu, 1935).Such plants are used as antimicrobial (Appendino, 1993), antioxidant, anti-inflammatory and even anticancer (Erdemoglu and Sener, 2001). Alkaloids, phenols, terpenoids, tannins, ligans, steroids, glycosides and sugar derivatives have been reported in gymnosperms as secondary metabolites (Niemann, 1988; Stermitz *et al*., 1999; Harborne and Baxter, 2001).

Therefore, in this present study, six Gymnospermic plants were *in vitro* screened for their antimicrobial potential against vascular wilt fungus of banana.

Material and methods

Study area

In vitro study for evaluation of cone extracts was done in fungal pathology laboratory of Plant and environment protection (PEP) department, National Agriculture Research Center (NARC).

Maintenance of Plant Pathogen

Fusarium oxysporum f. sp. *cubense* race 4 was provided by plant and environment protection (PEP) department, NARC. Fungus culture was maintained in Potato Dextrose Agar (PDA) media. Ten days old culture plates were used for *in vitro* experiments.

Plant material

Pinus roxburghii, Pinus wallachiana, Cedrus deodara, Thuja orientalis, Cupressus sempervirens and *Picea smithiana* cones were selected for the test of their antifungal efficacy against *Fusarium oxysporum* f. sp. *cubense*. Fresh Cones of these plants were collected, washed with tap water, disinfected with 5% Clorox for 5 minutes to remove surface microorganisms and again cones were washed three times with water to remove Clorox. Cones were shade dried for 10 days to remove moisture. Moisture dried cones were packed in paper bags and placed in air circulating oven at 35˚C for 48 hours. Oven dried cones were powdered and placed in air tight containers for further use.

Plant extracts preparation

Methanol, ethanol and n-hexane were used as extraction solvents. 5 and 10 percent extracts were prepared using 5 and 10 grams of plant sample respectively. Powdered cones were separately mixed with each 100ml solvent placed in 250ml Erlenmeyer flasks. Flasks were shaken at 60rpm for two days on mechanical shaker and then filtered. Solvent from each filtrate was dried using rotavapor. Extracts thus formed were placed in glass vials and labeled. Before use extracts were reconstituted in 5% respective solvents.

Antifungal efficacy test of extracts

Extracts were used by food poisoning method (Nene and Thapilyal, 2000). 5 and 10 percent concentrations of each plant cones, prepared separately in the three solvents, were individually mixed with autoclaved PDA and poured into sterilized petri plates and labelled. Disks of *Fusarium oxysporum* f. sp. *cubense* were taken from 10 days old culture plate with the help of cork borer and were aseptically placed at the center of petri dishes (one disk per plate) containing poisoned medium at different concentration. Plates having PDA mixed with simple 5% solvent, without plant extract, serve as the control. There were 5 replicates for each treatment. Plates were incubated at 25±2 ˚C. Radial growth of fungus was measured from third day of inoculation of plate till that day when *Fusarium oxysporum* f. sp. *cubense* completely covers the PDA control plate.

Percent Inhibition =(C-T)/C × 100

Where,

T = radius of the mycelia growth for each treatment

C = radius of the mycelia growth in Control plate

Cone extract showing ≥ 60% inhibition at 10 percent concentration were further selected for another test to clearly distinguish performance of each extract. For this purpose 50g of each cone powder was used in 500ml of solvent. Flasks were placed on shaker at 60 rpm for 48 hours so that solvent can easily extract the phytochemicals from plant cone's powder. Using Whatmann no 1 filter paper extracts were filtered. Filtered extracts were dried in rota vapor and placed in autoclaved glass vials. Extracts were reconstituted in 5% respective solvents and were incorporated into PDA using the same food poisoning technique. 5% solvents without plant extract serve as solvent controls. Propiconazole fungicide was used as positive control and used in the same manner as that of cone extracts. PDA Plates without any extract and solvent but having only FOC disk in them served as simple control. Five replicates for each treatment were taken. Radial mycelial growth was measured from 3rd till 7th day when control plates were filled up with fungal mycelialmass. Using aforementioned formula percentage inhibition was calculated.

Statistical analysis

Using Statistix 8.1 software, ANOVA was applied on the experimental data. Completely randomized design of experiment was used.

Result and discussion

In 5% concentration test, percentage inhibition ranged between 1.55%-56.54%. Ethanolic extracts of *Pinus roxburghhi* showed least activity i.e. 1.55% while n-hexane extract of *Picea smithiana* and *Pinus wallachiana* cone had maximum inhibition i.e. 56.54 and 56.30 respectively. Majority of the solvent cone extracts had above 40% inhibition percentages (Table 1a). ANOVA applied on the experiment data demonstrated significant difference among treatments (Table 1b).

Table 1a. Percent inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *cubense* using plant cone extract at 5 % concentration. Presentation of data as Mean±SE.

Treatment	Percent Inhibition by methanolic cone extract	Percent Inhibition by ethanolic cone extract	Percent Inhibition by n-hexane cone extract
T_1 Control	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000
T_2 *Pinusroxburghi*	46.308 ± 0.8698	1.5480 ± 1.1227	52.138 ± 1.8010
T_3 *Pinus wallachiana*	33.928 ± 0.3268	18.808 ± 1.4901	56.306 ± 1.1697
T_4 *Cedrus deodara*	46.308 ± 0.4374	48.810 ± 0.6264	58.808 ± 0.4755
T_5 *Thuja orientalis*	44.166 ± 0.5123	50.238 ± 0.9716	55.952 ± 1.6733
T_6 *Cupressus sempervirens*	41.186 ± 0.5778	28.926 ± 0.7681	32.260 ± 2.1417
T_7 *Picea smithiana*	51.874 ± 0.4591	42.020 ± 0.9703	56.546 ± 0.9420

($p< 0.05$)

Table 1b. Analysis of Variance for percent Inhibition of *Fusarium oxysporum* f. sp. *cubense*by cone extracts at 5%.

Source	DF	SS	MS	F	P
Replicate	4	3.0	0.74		
Treatment	6	29084.3	4847.38	915.72	0.0000
solvents	2	5361.6	2680.80	506.43	0.0000
Treatment*solvents	12	7595.4	632.95	119.57	0.0000
Error	80	423.5	5.29		
Total	104	42467.8			

Grand Mean 36.482 CV 6.31

In 10% concentration test, percent inhibition ranged between 17.02%-66.664%. Minimum inhibition was noted in case of ethanolic extract of *Pinus roxburghii* cone, while rest of all the extracts had above 34% inhibition. Methanolic cone extract of *Picea smithiana*, Ethanolic cone extract of *Thuja orientalis* and *Cedrus deodara*, n-hexane cone extracts of *Pinus wallachiana, Picea smithiana, Cedrus deodara, Pinus roxburghii* and *Thuja orientalis* had ≥60% inhibition (Table 2a). ANOVA applied on the experiment data provided significant difference among treatments (Table 2b). Hexane cone extracts followed by ethanol and then methanol cone extracts were found effective against *Fusarium oxysporum* f. sp. *cubense* (Fig. 1a-1c).

Table 2a. Percent inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *cubense* using plant cone extract at 10 % concentration. Presentation of data as Mean ± SE.

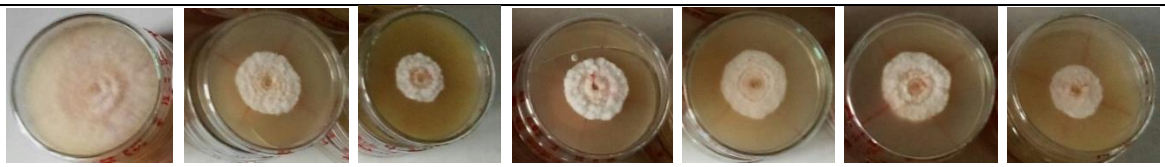
Treatment	Percent Inhibition by methanolic cone extract	Percent Inhibition by ethanolic cone extract	Percent Inhibition by n-hexane cone extract
T_1 Control	0.0000 ± 0.0000 ^{K}	0.0000 ± 0.0000 ^{K}	0.0000 ± 0.0000 ^{K}
T_2 *Pinusroxburghi*	56.902 ± 1.0400 ^{EF}	17.022 ± 1.3651 ^{J}	62.974 ± 0.9287 ^{BC}
T_3 *Pinus wallachiana*	44.642 ± 0.8622 ^{H}	34.764 ± 1.5612 ^{I}	66.664 ± 1.2483 ^{A}
T_4 *Cedrus deodara*	57.736 ± 0.6513 ^{DE}	60.474 ± 0.5525 ^{CD}	66.074 ± 1.5403 ^{AB}
T_5 *Thuja orientalis*	56.068 ± 1.1814 ^{EF}	65.832 ± 0.3553 ^{AB}	60.592 ± 1.7906 ^{CD}
T_6 *Cupressus sempervirens*	54.172 ± 0.9027 ^{FG}	43.094 ± 2.1999 ^{H}	51.428 ± 0.6140 ^{G}
T_7 *Picea smithiana*	65.474 ± 0.3755 ^{AB}	57.738 ± 1.1139 ^{DE}	66.070 ± 2.3209 ^{AB}

($p< 0.05$)

Table 2b. Analysis of Variance for percent Inhibition of *Fusarium oxysporum* f. sp. *cubense*by cone extracts at 10%.

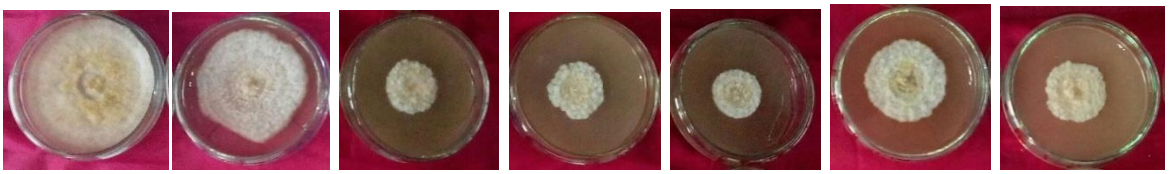
Source	DF	SS	MS	F	P
Replicate	4	15.4	3.86		
Treatment	6	43181.5	7196.92	1013.45	0.0000
Solvents	2	3250.4	1625.21	228.86	0.0000
Treatment*Solvents	12	6616.2	551.35	77.64	0.0000
Error	80	568.1	7.10		
Total	104	53631.7			

Grand Mean 47.034 CV 5.67



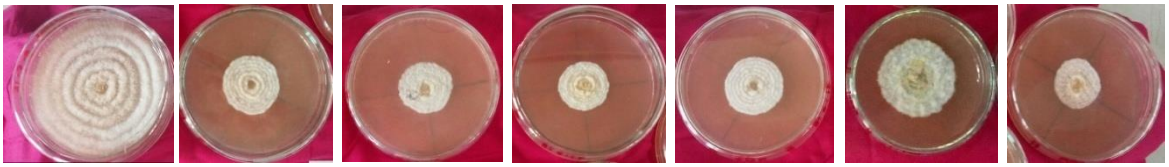
T_1 T_2 T_3 T_4 T_5 T_6 T_7

Fig. 1a. Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* on 10% methanolic cone extracts.



T_1 T_2 T_3 T_4 T_5 T_6 T_7

Fig. 1b. Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* on 10% ethanolic cone extracts.



T_1 T_2 T_3 T_4 T_5 T_6 T_7

Fig. 1c. Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* on 10% n-Hexane cone extracts.

Fig. 1a-1c. Where, T_1: Respective solvent control, T_2: Cone extract of *Pinus roxburghi*, T_3 Cone extract of *Pinus wallachiana*, T_4 : Cone extract of *Cedrus deodara*, T_5: Cone extract of *Thuja orientalis*, T_6 : Cone extract of *Cupressus sempervirens*, T_7: Cone extract of *Picea smithiana*.

Methanolic cone extract of *Picea smithiana*, Ethanolic cone extract of *Thuja orientalis* and *Cedrus deodara*, n-hexane cone extracts of *Pinus wallachiana, Picea smithiana, Cedrus deodara, Pinus roxburghii* and *Thuja orientalis* had ≥60% inhibition. There was a mixed kind of results in case of 10% concentration therefore in order to clearly differentiate antifungal potential of these cone extracts against *Fusarium oxysporum* f. sp. *cubense*, 50% concentration test was performed using same food poisoning technique.

Percent inhibition at 50% concentration ranged between 55.9%-77.37%. Methanolic extract of *Picea smithiana* cone, ethanolic extract of *Thuja orientalis* cone, *Pinus wallachiana* and *Pinus roxburghii* cone extract in n-hexane exhibited ≥ 70% inhibition.

Minimum inhibition was observed in case of n-hexane extract of *Picea smithiana* and maximum inhibition was exhibited by methanolic cone extract of *Picea smithiana* (Table 3a). ANOVA result clearly shows that there was significant difference among treatments (Table 3b). Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* was effectively inhibited using these cone extracts (Fig. 2).

Gymnospermic botanicals have been evaluated for their antimicrobial efficacy during recent time. Results from such investigations provide ample evidence that these botanicals had the potential to inhibit the microbial growth and thus are sources from which antimicrobial products could be formed.

Table 3a. Percent inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *cubense* using selected plant cone extract at 50% concentration. Presentation of data as Mean ± SE.

Treatment	Percent Inhibition using 50% concentration
T_1 Simple Control	0.0000 ± 0.0000 ^{J}
T_2 Hexane control at 5%	0.0000 ± 0.0000 ^{J}
T_3 Ethanolic control at 5%	0.0000 ± 0.0000 ^{J}
T_4 Methanolic control at 5%	0.0000 ± 0.0000 ^{J}
T_5 Fungicide control	100.00 ± 0.0000 ^{A}
T_6 Hexane extract of *Pinus wallachiana*cone	75.950 ± 1.4040 ^{C}
T_7 Hexane extract of *Cedrus deodara* cone	66.664 ± 0.6793 ^{F}
T_8 Hexane extract of *Picea smithiana* cone	55.952 ± 1.1617 ^{I}
T_9 Ethanolic extract of *Thuja orientalis*cone	74.760 ± 1.8673 ^{D}
T_{10} Methanolic extract of *Picea smithiana* cone	77.378 ± 0.9034 ^{B}
T_{11} Hexane extract of *Pinus roxburghi*cone	70.834 ± 0.8418 ^{E}
T_{12} Hexane extract of *Thuja orientalis*cone	63.216 ± 0.8714 ^{G}
T_{13} Ethanolic extract of *Cedrus deodara* cone	62.978 ± 1.8955 ^{H}

($p < 0.05$)

Table 3b. Analysis of Variance for percent inhibition of *Fusarium oxysporum* f. sp. *cubense* by selected cone extract at 50%.

Source	DF	SS	MS	F	P
Treatment	12	78129.4	6510.78	1288	0.0000
Error	52	262.9	5.06		
Total	64	78392.3			

Grand Mean 49.826 CV 4.51

Aqueous and alcoholic extracts from leaves, bark, stem and cones of *Pinus roxburghii* were tested against bacterial human and plant pathogen. Maximum inhibition was observed in case of aqueous and alcoholic female cone extract against *Salmonella typhi* (24 and 22mm) followed by *Escherichia coli* (20 and 18mm) (Parihar *et al*., 2006).

Aqueous, petroleum ether, chloroform and ethanol extracts, from the ariel parts of *Pinus roxburghii* and *Thuja orientalis*, were tested using disc diffusion method. Petroleum ether extracts were most effective and needle extract of *Pinus roxburghii* displayed maximum inhibition (20mm) against *Klebsiella pneumonia* (Bissa *et al*., 2008).

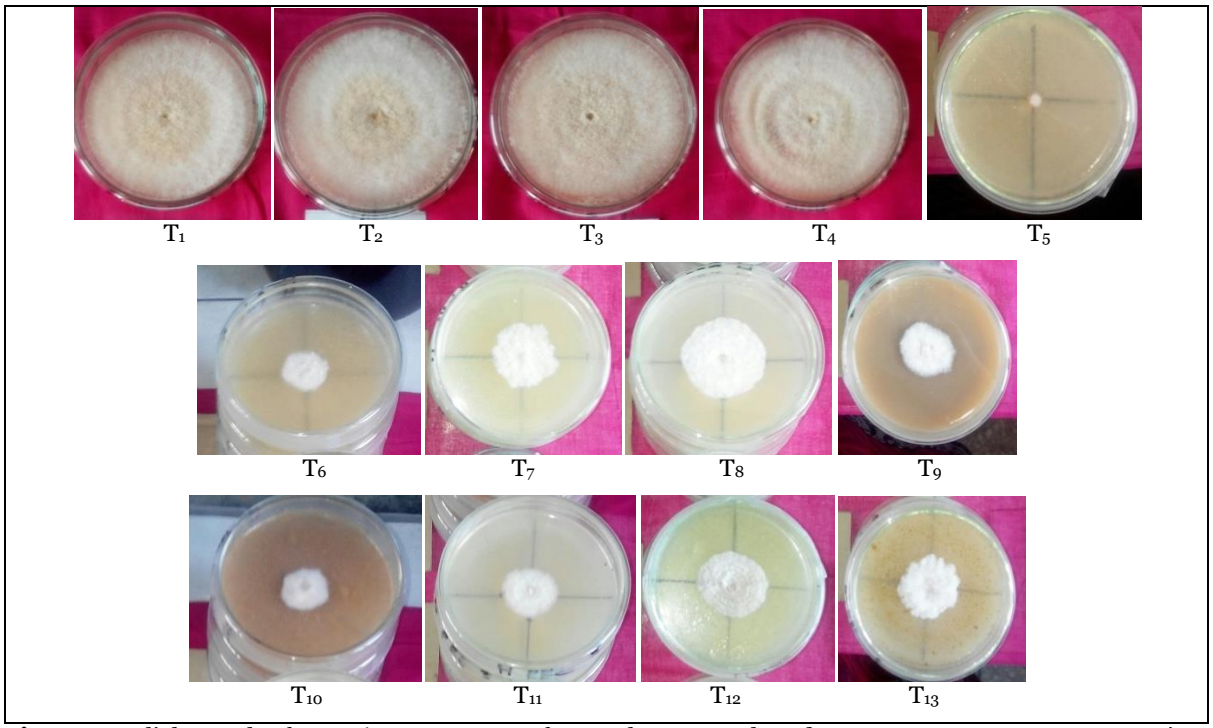


Fig. 2. Mycelial growth of *Fusarium oxysporum* f. sp. *cubense* on selected cone extracts at 50% concentration. where, T_1: Simple Control, T_2: Hexanic control at 5%, T_3: Ethanolic control at 5%, T_4 Methanolic control at 5%, T_5: Fungicide control, T_6 : Hexanic extract of *Pinus wallachiana*cone, T_7 : Hexanic extract of *Cedrus deodara* cone, T_8: Hexanic extract of *Picea smithiana* cone, T_9: Ethanolic extract of *Thuja orientalis*cone, T_{10}: Methanolic extract of *Picea smithiana* cone, T_{11}: Hexanic extract of *Pinus roxburghi*cone, T_{12} : Hexanic extract of *Thuja orientalis*cone, T_{13}: Ethanolic extract of *Cedrus deodara* cone.

Essential oil from fresh leaves of *Thuja orientalis* L. was analysed by GC/MS and its antifungal activity against *Alternaria alternata* was done in a direct bioautography assay. Bioactive compounds mainly b_1 and b_2 produced 5 and 10mm inhibition zone using 30.5 and 4.5µg respectively. b_1 was determined as α-cedrol (Guleria *et al*., 2008).

Cedrus deodara root oil and its compounds were tested for their antifungal activity against *Aspergillus fumigatus* and *Candida albicans*. 150µg/disc concentration of *C. deodara* oil showed zone of inhibition (7mm) against *A. fumigatus*. Himachalol (compound of root oil) showed zone of inhibition (10m) against *A. fumigatus* at 150 µg/disc concentration (Parveen *et al*., 2010).

Good antibacterial activity of ethanolic extract of *Cedrus deodara* wood was observed against *Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis* using 600 µg/well concentration. Highest zone of inhibition (33mm) at 600 µg/well was observed in case of *S. aureus* (Devmurari, 2010).

Hexane extract of *Thuja orientalis* cones has the highest inhibitory effect on cell viability of *Listeria monocytogenes*. Damaging effect of the hexane extract on the morphology of *L. monocytogenes* was clearly demonstrated with SEM observation (Yang *et al*., 2011).

Aspergillus flavus and *A. niger* were found to be sensitive towards the methanol extract of *Thuja orientalis* L. leaf with inhibition zones of 15.50 and 16.00 mm, respectively compared to fungicides (e.g. fluconozole and ketoconazole) (Duhan *et al*., 2013).

Thuja orientalis L. extracts, at three different concentrations i.e. 2, 4 and 6 g L^{-1}, were applied under in vitro culture conditions to *Citrullus lanatus* hypocotyls explants that were infected with Watermelon mosaic potyvirus (WMV). 6 g L^{-1} followed by 4 g L^{-1} of *Thuja* extract concentration was more effective to reduce the infection of WMV compared with 2g L^{-1} or control treatment either in healthy or infected hypocotyl explants (Elbeshehy *et al*., 2015).

Ethanol and methanol extracts of *Picea smithiana* bark were tested using disc diffusion method. The lowest value of MIC and MBC were recorded against *Agrobacterium tumefaciens* in methanol extract (31.25 µg/ml and 62.5 µg/ml respectively). Methanol extract exhibited highest inhibitory activity against *A. tumefaciens* (ZOI, 19 mm) while ethanol extract showed its highest activity *for Erwinia chrysanthemi* (ZOI, 12 mm) (Sati *et al*., 2015).

Antibacterial effects of *Cedrus deodara* oil were determined against 5 Pathogenic Strains. Antimicrobial activities of *C. deodara* oil was evaluated by well and disc diffusion methods. *C. deodara* oil exhibited excellent inhibitory effects against tested pathogens (Majid *et al*., 2015).

Leaf extracts of eleven Gymnospermic plants including *Pinus wallichiana, Picea smithiana* and *Cedrus deodara* were tested using disc diffusion method. At 1000 µg/ml concentration, studied gymnospermic leaves extract showed effective antibacterial activity. Methanol extract were found most effective (54% to 81%) followed by ethanol extract (45-72%), hexane extract (18-27%), while in chloroform extract it was 9-27%. *Ginkgo biloba* displayed maximum Antibacterial Value Index (ABVI) i.e. 90%, followed by *P. wallichiana* and *Araucaria cunninghamii* (ABVI, 55% each) (Joshi *et al*., 2016).

Ethyl acetate, n-hexane, chloroform, water and residue extract fractions of *Picea smithiana* leaves were used for antimicrobial investigation. Effective minimum inhibitory concentrations were observed in case of ethyl acetate (20 µg/mL against *Microsporum canis*), chloroform (10 µg/mL against *Microsporum canis*) and water fraction (30 µg/mL against *Fusarium solani*) (Taj Ur Rahman *et al*., 2016).

Female cones and needles of *Pinus roxburghii* were extracted in different solvents (methanol, petroleum ether, Chloroform and distilled water) and

antimicrobial tests were performed using disc diffusion method. Chloroform extract of female cone showed maximum inhibition zone against *A. alternata* (22±2) followed by cone's methanolic extract against *F. solani* (20±2) (Khalid *et al*., 2016).

Conclusion

Research, conducted in recent decades, on Gymnospermic botanicals expresses their antimicrobial potential. More focus has been given to evaluate their efficacy against pathogenic bacteria either human or plant pathogen. Present work provides antifungal potential of these botanicals against Panama wilt fungus which is a hot topic in current scenario as far as fungal diseases of banana are concerned. It is evident from this current research that majority of the cone extracts in their crude form effectively inhibited the mycelia growth of *Fusarium oxysporum* f. sp. *cubense*. Results depict that Gymnospermic cone extracts are good source of novel phytochemicals that can serve as an excellent antifungal products. Exploitation of such natural sources of phytochemicals would definitely yield better antifungal products that will be environment friendly and biodegradable.

Acknowledgement

Author is thankful to Higher Education Commission, for their financial support. Special thanks to PEP department, NARC for providing laboratory facilities for this research.

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