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Bioactive Botanics against Pathogenic and Mycotoxigenic Fungi Isolated from Rice

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Abstract

Cereal fungal contamination causes both economic and human health problems. In the present study, the chemical composition of commercial bay leaf, cinnamon, clove and oregano essential oils, and antifungal activity against three pathogenic fungi isolated from rice, were investigated. Essential oils presented a high percentage of oxygenated components: 78.8% in bay leaf (eucalyptol 52%); 90.5% in clove (eugenol 90%); 92% in cinnamon (eugenol 60% and eugenyl acetate 18.5%); 72% in oregano (carvacrol 50% and thymol 20%). Monoterpenes and sesquiterpenes were: 18% in bay leaf, 9% in clove, 5% in cinnamon, 25% in oregano. Cinnamon, and clove essential oils reduced fungal growth by 70%, 80% and almost 90%. Moreover, there was total inhibition using oregano until the seventeenth day. Oregano, clove and cinnamon oils could provide an alternative for controlling *Bipolaris spicifer*, *Fusarium culmorum* and *Fusarium sambucinum* in stored grains and seeds, so extending their shelf life.

Keywords: Antifungal activity; Essential oils; Bipolaris; Fusarium

Introduction

New methods to control spoilage fungi in food are being investigated because the application of synthetic fungicides has led to several environmental and health problems. The antimicrobial properties of different natural products from plants have been recognized and used in medicine and food preservation since ancient times. Among these different groups of natural plant products, essential oils are especially recommended as one of the most promising for formulating safer antifungal agents [1-2]. Cereal fungal contamination causes both economic and human health problems. Plant Pathogenic fungi cause some of the most devasting universal crop diseases [3], Fusarium and Bipolaris, together with Alternaria, are the most economically important genera of phytopathogenic fungi in the family Poaceae. Several Fusarium species can infect cereal grains, and the predominant species can vary according to crop, geographic region and environmental conditions [4]. *Fusarium* toxins are secondary metabolites produced by toxigenic fungi that naturally contaminate cereals and are a source of grave concern in cereals and cereal-based products [5]. The commonest *Fusarium* mycotoxin groups are trichothecenes, zearalenones, and fumonisins. However, other mycotoxins as enniantins, moniliformin, beavericin and fusaproliferin can be presented in combination with them [6-7]. The aim of this work was to determine the chemical composition of bay leaf, cinnamon, clove and oregano essential oils, and to evaluate their antifungal activity against *Bipolaris* spicifer, *Fusarium culmorum* and *Fusarium sambucinum*, isolated from rice.

Experimental Details

Essential oils

Commercial samples of clover leaf (*Syzygium aromaticum* (L.) Merr. & Perry), batch 9449600032, essential oil purchased from Guinama (Valencia, Spain), cinnamon leaf (*Cinnamomum verum J.Presl*), batch 405, bay leaf (*Laurus nobilis L.*), batch 216, and oregano (*Origanum vulgare L.*), batch 624, essential oils supplied by Plantis Artesania Agrícola S.A., were stored at 4°C until chemical analysis and antifungal studies were done. Other materials and chemicals used were of analytical grade and purchased from local suppliers.

Gas chromatography (GC/FID)

GC was performed using a Perkin-Elmer Clarus 500GC apparatus equipped with a flame ionization detector (FID), and a Hewlett-Packard HP-1 (cross-linked methyl silicone) capillary column (30 m long and 0.2 mm i.d., with 0.33 µm film thickness). The column temperature program was 60°C during 5 min, with 3°C/min increases to 180°C, then 20°C/min increases to 280°C, which was

maintained for 10 min. The carrier gas was helium at a flow rate of 1 mL/min. Both the FID detector and injector port temperature were maintained at 250 and 220°C, respectively.

Gas chromatography and mass spectrometry (GC-MS)

GC-MS analysis was carried out with a Varian Saturn 2000 equipped with a Varian C.S VA-5MS capillary column (30m long and 0.25 mm i.d. with 0.25 µm film thickness). The same working conditions used for GC and split mode injection (ratio 1:25) were employed. Mass spectra were taken over the m/z 28–400 range with an ionizing voltage of 70eV. Kovat's retention index was calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by MS and their identity was confirmed by comparison of their RIs, relative to C8-C32 n-alkanes, and by comparing their mass spectra and retention times with those of authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature [8].

Fungal species

Three phytopathogenic fungi, *Bipolaris spicifer* (CECT 27761), *Fusarium culmorum* (CECT 209241) and *Fusarium sambucinum* (CECT 209252) were isolated in the Botany Laboratory of the Department of Agroforest Ecosystems from rice samples collected in "La Albufera" rice-producing Mediterranean area in Valencia (Spain). Fungal species were morphological and moleculary identified and then deposited in the Spanish Type Culture Collection (CECT).

Antifungal activity

The bioassay was performed in Petri dish (90×15mm and 150×20mm), dissolving 300 µg/mL (Tween 20, 0.1%) of commercial essential oils in previously sterilized Potato Dextrose Agar (PDA) growth medium flasks at 45-50°C while the medium was still in a liquid form and distributed into Petri dishes. Petri dishes were inoculated with an 8mm diameter disk of 7-day old colony on PDA of each tested fungi. Plates were incubated in the dark at 25°C during 7, 14 and 21 days. Petri dish control contained equal amounts of sterilized water/Tween 20 (0.1%) on PDA was employed. Fungal growth was evaluated by measuring daily the diameter of the colony in two perpendicular directions and speed of growth was calculated. For each essential oil and fungi, six replicate dishes were used. The Petri plate control contained only PDA.

Statistical analysis

The fungal growth results were submitted to variance analysis (ANOVA) using Fisher test of least significant difference (LSD) with significant values at $P<0.05$. Data analysis was performed using Stat Graphics Plus 5.0 software (Stat Point, Inc., Herndon, Virginia, USA).

Results and Discussion

Chemical composition of commercial essential oils

Table 1: Identified compounds in commercial chemical composition of comercial essential oils of bay leaf (Bl) *(Laurus nobilis)*, cinnamon (C) *(Cinnamomum verum)*, clove (Cl) *(Syzygium aromaticum)* and oregano (O) *(Origanum vulgare)*.

RI	Compound	Peak Area (%) Bl	Peak Area (%) C	Peak Area (%) Cl	Peak Area (%) O
853	3-hexen-1-ol	0.06	-	-	-
918	butanoic acid,2-methylpropyl ester	0.06	-	-	-
932	α-thujene	0.17	0.08	-	0.32
939	α-pinene	3.95	0.83	-	0.39
954	camphene	-	0.36	-	0.08
979	sabinene	7,48	-	-	-
980	β-pinene	3.6	0.27	-	0.08
983	1-octen-3-ol	-	-	-	0.12
994	β-myrcene	0.65	0.08	-	0.16
995	3-octanone	-	-	-	0.81
1005	phellandrene	-	0.53	-	-
1013	δ-3-carene	-	-	-	0.06
1020	α-terpinene	0.47	0.16	-	1.35
1029	p-cymene	-	0.71	-	11.5
1033	limonene	-	1	-	0.33
1035	1,8-cineole (eucaliptol)	51.8	0.07	-	-
1063	γ- terpinene	1.2	-	-	9.22
1071	sabinene hydrate	0.12	-	-	0.12

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1091	terpinolene	-	-	-	0.2
1001	linalool	3.66	0.41	-	1.08
1168	acetic acid,phenylmethylester	-	0.13	-	-
1168	borneol	0.12	-	-	0.1
1180	terpinen-4-ol	2.17	-	-	0.44
1192	linalyl propionate	-	0.11	-	-
1193	α-terpineol	2.301	-	-	0.19
1199	estragole	0.38	-	-	
1201	dihydrocarvone	-	-	-	0.07
1232	nerol	0.3	-	-	-
1261	linalyl acetate	0.25	-	-	-
1273	2-propenal, 3-phenyl-	-	1.99	-	-
1288	bornyl acetate	0.55	-	-	-
1290	thymol	-	-	-	20.18
1296	2-undecanone	0.14	-	-	-
1298	carvacrol	-	-	-	50.02
1328	myrtenyl acetate	0.06	-	-	-
1357	*α*-terpinenyl acetate	13.1	-	-	-
1362	eugenol	1.4	59.8	90.01	-
1380	copaene	-	0.36	-	-
1409	methyl eugenol	3.8	-	-	-
1420	*β*-caryophyllene	0.18	1.9	6.59	1.59
1447	cinnamyl acetate	0.09	4.78	-	-
1453	*α*-caryophyllene	-	0.5	1.99	0.09
1519	δ-cadinene	0.08	-	-	0.1
1538	eugenylacetate	-	18.48	-	-
1582	caryophyllene oxide	0.1	0.71	0.4	0.15
1731	4-hydroxy-2-methoxycinnamaldehyde	-	-	0.08	-

The identified components are shown in Table 1 with their relative percentages. In bay leaf, the oxygenated components amounted to 78.8% of their entire composition, of which the majority were epoxide 1.8-cineole (52%) and ester α-terpinenyl acetate (13%), followed by esters bornyl acetate (0.5%) and linalyl acetate (0.25%). Hydrocarbons (monoterpenes and sesquiterpenes) accounted for 18% of their composition. Clove essential oil presented 90.5% of oxygenated compounds, and eugenol had the highest proportion (90%). Hydrocarbons (monoterpenes and sesquiterpenes) accounted for 9%; of these, 6.6% were β -caryophyllene and 2% β-caryophyllene. In cinnamon essential oil, the percentage of oxygenated compounds was 92%, eugenol was approximately 60%, followed by esters eugenyl acetate (18.5%) and cinnamyl acetate (5%), while hydrocarbons (monoterpenes and sesquiterpenes) represented only 5%, β -caryophyllene (1.9%), followed by limonene (1%), α-pinene (0.8%), el p-cymene (0.7) and α-caryophyllene (0.5%). Eugenol was the main volatile component of both extracts and was more abundant in clove than in cinnamon. Oregano essential oil contained 72% of oxygenated compounds, characterized by its high content of phenols carvacrol (50%) and thymol (20%). Monoterpenes and sesquiterpenes accounted for 25%, the most outstanding of which were p-cymene (11.5%), γ-terpinene (9.2%), β-caryophyllene (1.6%), α-terpinene (1.4%) and α-pinene (0.4%). The high content of carvacrol and thymol, and their precursors γ-terpinene and p-cymene, has been previously reported for their antimicrobial activity [2].

Antifungal activity

The responses of the tested fungal species differed but displayed the same behavior pattern when faced with the distinct essential oils under study. Growth and sporulation of fungi were totally inhibited until day 17, and even then, speed of growth was very low (2-3mmd-1) when oregano essential oil was used. Oregano essential oil at 300μg/mL displayed the most antifungal potential (Table 2, Figure 1) against all the tested phytopathogenic fungi. In vitro the most susceptible fungi against clove and cinna-

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mon essential oils were *B. spicifer* and *F. culmorum* (which reduced speed of fungal growth by 83-87%), followed by F. sambucinum (reduced fungal growth exceeded 70%). The effect as powerful inhibitors of fungi of clove and cinnamon oils was similar. In our study bay leaf oil had no inhibitory effect on the tested fungi. Cinnamon, clove and oregano essential oils significantly reduced the growth of *Bipolaris spicifer, Fusarium sambucinum* and *Fusarium culmorum* (P<0.05) (Figure 2). The chemical composition of bay leaf oil, which had no inhibitory effect on the fungi tested in our study, was 52% of 1,8 cineole (eucalyptol), but with only a small proportion of cinnamon and oregano (0.07% and 0.04%, respectively), which were not detected at all in clove; the 1.8-cineole compound has been described for its antifungal activity by various authors [9-10]. Several authors have studied the effect of cinnamon and clove oils on the growth of the *Fusarium* species isolated from plants, and their application in disinfecting seeds [11-12]. The antifungal activity of a gelatin film containing clove, oregano and cinnamon EOs has been demonstrated against *Colletotrichum gloesporoides*, *Alternaria alternata*, *Fusarium oxysporum* and *F. proliferatum* [13-15]. The main compound in clove and cinnamon essential oils was a natural phenolic compound, eugenol, characterized by its antifungal activity. It has been recently approved (Reg. (EU) No 546/2013) by the European Food Safety Authority (EFSA) as a fungicide (Dossier completed 2011/266/EU). The use of essential oils rich in eugenol and cinnamaldehyde is interesting because it has been recently found that their antifungal activity was even more effective in an encapsulation state than in a pure state [16].

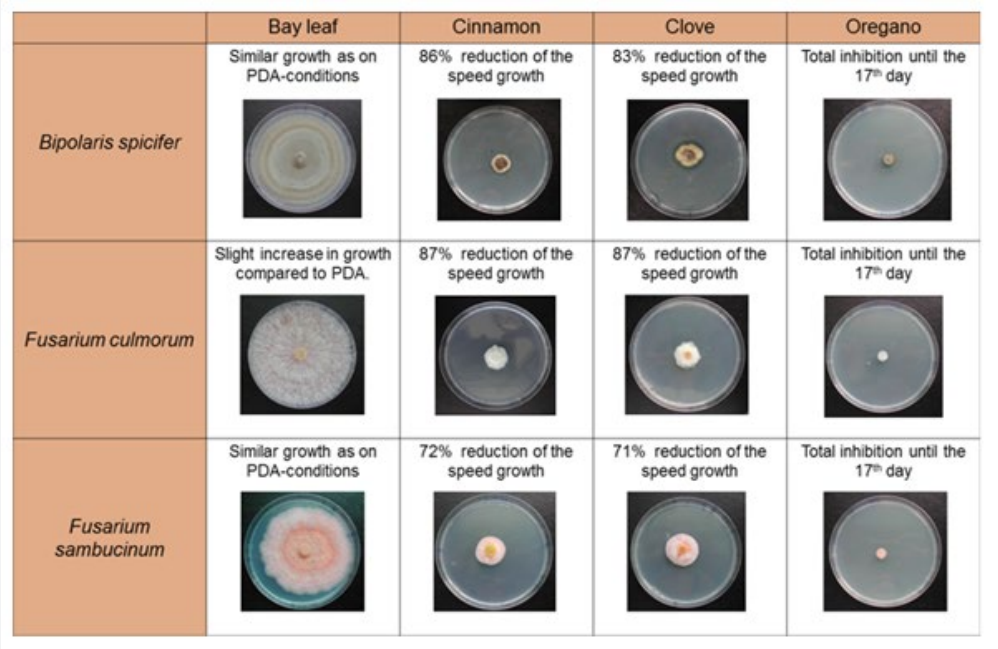

Figure 1: Inhibition growth of *Bipolaris spicifer*, *Fusarium culmorum* and *Fusarium sambucinum* on PDA-Bay Leaf, PDA-Cinnamon, PDA-Clove and PDA-Oregano. Photographs of 14th day and 17th for oregano.

Table 2: Effects of bay leaf, cinnamon, clove and oregano essential oils (300µg/mL) on radial growth and growth rates of B. spicifer, *F. culmorum* and F. sambucinum. Confidence intervals with probability of 0.95.s.

Species-Treatment	Mean	Lower limit	Upper limit	GR
B. spicifer-PDA	23.52 ± 1.46	20.64	26.39	6,83 (0.99)
B. spicifer-BL	24.32 ± 1.22	21.92	26.73	6.63 (0.99)
B. spicifer -C	4.24 ± 0.99	2.27	6.2	0.92 (0.89)
B. spicifer-CL	3.57 ± 1.33	0.96	6.18	1.16 (0.89)
B. oryzae-O	9.74 ± 1.69	6.42	13.05	3.00 (0.94)
F. culmorum-PDA	32.62 ± 2.24	24.23	35.01	11.80 (0.99)
F. culmorum-BL	36.25 ± 2.24	26.86	37.64	11.95 (0.99)
F. culmorum-C	4.01 ± 1.22	1.6	6.42	1.55 (0.89)
F. culmorum-CL	2.87 ± 1.46	0	5.75	1.50 (0.92)

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F. culmorum-O	5.02 ± 1.73	1.72	8.43	2.11 (0.94)
F. sambucimun-PDA	22.82 ± 1.22	20.42	25.23	6.59 (0.98)
F. sambucinum-BL	20.88 ± 1.12	18.69	23.08	6.00.(0.98)
F. sambucinum-C	11.99 ± 0.82	10.36	13.61	1.84 (0.87)
F. sambucinum-CL	12.45 ± 0.84	10.79	14.11	1.90 (0.96)
F. sambucunum-O	3.83 ± 3.16	1.23	10.04	2.50 (0.98)

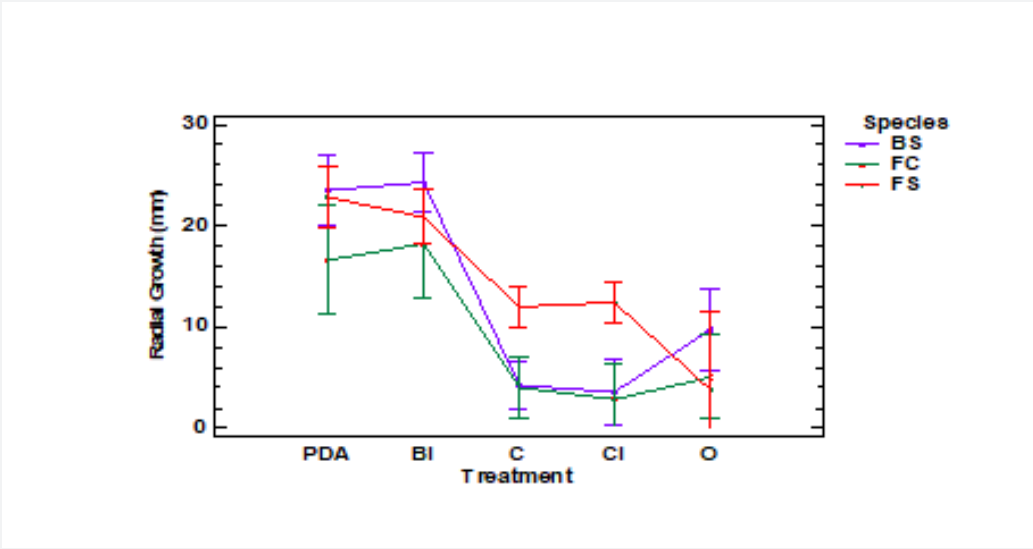

Figure 2: Graphical representation HSD Tukey Interval according to species and treatment. BS: *Bipolaris spicifer*, FC: *Fusarium culmorum* and FS: *Fusarium sambucinum* on PDA, PDA-Bay Leaf, PDA-Cinnamon, PDA-Clove and PDA-Oregano.

The studied oregano essential oil presented highly inhibited fungal growth. These results are in concordance with other authors, who have indicated that compounds carvacrol and thymol present high antifungal activity against food spoilage fungi, including *Rhizoctonia*, *Colletotrichum* and *Fusarium* [12, 15,17]. Oregano essential oil contains large amounts of phenolic compounds, thymol and carvacrol, and their biogenetic precursors p-cymene and γ-terpinene. Thymol has been recently approved (Reg. (EU) No 568/2013) by EFSA as a fungicide active substance (Dossier completed 2011/266/EU).

Conclusion

The results obtained in this work proved highly satisfactory against *Bipolaris spicifer*, *Fusarium culmorum* and *Fusarium sambucinum* with reductions in their growth of around 70%, 80% and almost 90% when cinnamon and clove essential oils were used. Furthermore, their growth and sporulation were totally inhibited up to almost 20 trial days when oregano oil was used. Addition of oregano, clove and cinnamon oils could provide an alternative for controlling *Bipolaris spicifer*, *Fusarium culmorum* and Fusarium sambucinum in stored products, and could thus extend their shelf life. As they are biodegradable, they could be used as preservatives and additives in foodstuffs and be applied in the storage of grain and seeds.

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