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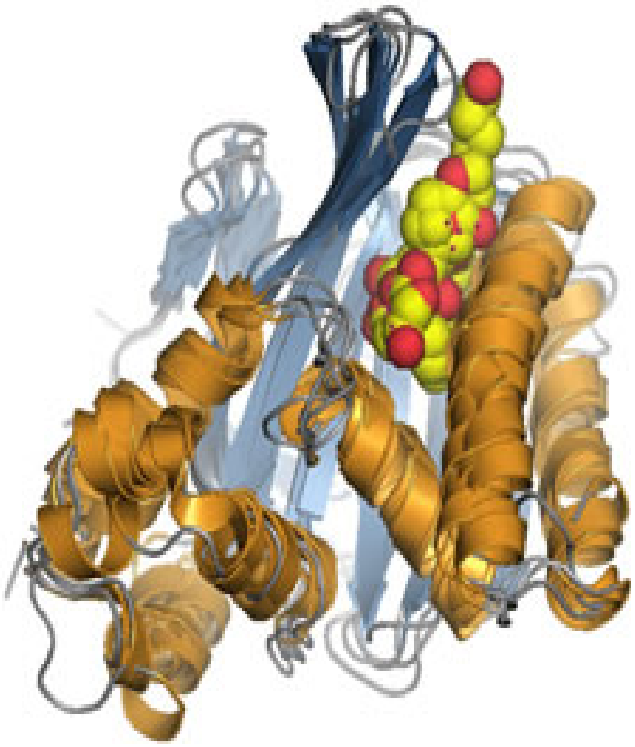

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Direct Bioautography for Antifungal Activity Measurement "Case of Bayoud Disease"

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Abstract. *Fusarium oxysporum f. sp. albedinis (Foa) is the causal agent of a lethal disease of date palm "Phoenix dactylifera L." called Bayoud. The antifungal test against Foa was evaluated using direct bioautography for extracts from four medicinal and/or poisonous plants (Acacia raddiana, Asteriscus graveolens (Forsk.), Citrullus colocynhis (L.) Schrad, Pergularia tomentosa). The choice of extracts was based on activity against Foa using disc diffusion techniques and relative virulence. The most effective extracts (detected inhibition and relative virulence decreased below 50%) were chosen for direct bioautography test.*
Direct bioautography has a great importance based on effectiveness. Extracts from Acacia raddiana showed no effect on chromatograms. The best results were represented by ethyl acetate extract of Citrullus colocynthis fruits (7.75 ±1.06 mm) and ethyle acetate extract of Asteriscus graveolens stems (7.00±1.41 mm).
The efficiency of some species especially Citrullus colocynthis indicates the presence of highly sensitive targets in foa, which can be exploited for the development of efficient treatment against Bayoud.

***Key Words*:** Date palm, *Fusarium oxysporum* f. sp. *albedinis*, antifungal, Medicinal plants, Poisonous plants, direct bioautography

1. Introduction

Palm trees (*Phoenix dactylifera* L.) represent for the saharian populations the trees which maintain the ecological and socio-economic equilibrium (Ben Abdallah, 1990; Djerbi, 1991). Many *Fusarium* species are serious plant pathogens (Herrmann *et al.*, 1996). *Fusarium oxysporum* f. sp. *albedinis* (Killian & Maire) Malençon, is the causal agent of Bayoud disease, the most dangerous disease of "*Phoenix dactylifera* L." (Bounaga and Djerbi, 1990). There is no effective treatment to date.
The studies realized by our research group (Boulenouar *et al.*, 2009; Boulenouar *et al.*, 2012) on nine plants prompted us to conduct a study on the extracts that showed the most important effect on Foa using antifungal evaluation by direct bioautography (Boulenouar *et al.*, 2011).

2. Materials and Methods

Plant material

On the basis of results obtained from antifungal and virulence tests of four medicinal and/or poisonous plants extracts (*Acacia raddiana, Asteriscus graveolens* (Forsk.), *Citrullus colocynhis* (L.) Schrad, *Pergularia tomentosa*) (Boulenouar *et al.*, 2009; Boulenouar *et al.*, 2012). The heat reflux extraction was realized using four solvents (hexane, dichloromethane, ethyle acetate, methanol). The extracts showed an inhibition effect and/or diminished the virulence below 50% had been chosen for direct bioautography test. Under these conditions, fifteen medicinal plants extracts and fourteen poisonous plants had been selected.

Antifungal activity using direct bioautography

The process of direct bioautography can be divided into three steps: (1) Culture of microorganism to be tested; (2) Chromatographic separation; (3) Post chromatographic detection (Horvath *et al.*, 2004a; b).

Culture of Foa

The culture of Foa was realized on PDA medium from spores. Then, the fungus was subcultured on SNA medium to get sufficient spores concentration (10^6 spores/ml).

Chromatographic separation

In this step, two chromatograms were realized, one for secondary metabolites revelation, the other for antifungal test (post-chromatographic detection). The separation was realized on silica gel 60 F254 aluminium sheets using ethyl acetate/butanol (75/25) as mobile phase. The quantity of extract used for each chromatographic separation was 400 µg.

Post-chromatographic separation

The 2nd chromatogram had undergone the following teatments:

- Immersion of chromatograms in PDB containing Foa spores (10^6 spores/mL) for 10s.
- Incubation at 21 °C for 4 days (in humide environnement).
- Immersion of chromatograms in solution of iodonitrotetrazolium chloride (INT) (2 mg/mL) (incubation for 24 h at 21 °C).
- Immersion in ethanol (70 %) for 10 s to stop growth.

The inhibition zones were visible as clear zones on a red background. The inhibition degree is represented by the diameter. In addition, the retardation factors (Rf) were calculated. The controls were represented by chromatograms passing all the steps except for some without extracts, and for others without Foa.

All experiments were repeated twice. The results were expressed as mean ± standard error (SE).

3. Results and discussion

Some extracts have demonstrated an antifungal effect by disc diffusion technique but not with bioautography. This could be related to the synergistic effect of the compounds present in the crude extract. Contrary, the chosen extracts for their effect on relative virulence (RV<50%) but have shown an inhibition on the chromatogram, can be explained by the phenomenon of antagonism in the crude extracts, then this phenomenon disappear after separation. Cowan (1999) has shown that sometimes the antimicrobial effect is related to the mixture of compounds (synergy).

Table1: Antifungal activity of plants extracts on *Fusarium oxysporum* f. sp. *albedinis* using direct bioautography.

			Direct Bioautography	
Species	**Part used**	**Extraction solvent**	**∅ (mm)**	**Rf**
Acacia raddiana	Leaves	Methanol	ND	ND
		Hexane	ND	ND
	Bark	Methanol	ND	ND
		Ethyl acetate	ND	ND
		Hexane	ND	ND
Asteriscus graveolens	Leaves	Ethyl acetate	5.50±2.12	0.00±0.00
			4.75±0.35	0.85±0.04
		Dichloromethane	ND	ND
		Hexane	3.00±0.00	0.00±0.00
	Stems	Ethyl acetate	7.00±1.41	0.50±0.10
		Dichloromethane	ND	ND
Citrullus colocynthis	Leaves and Stems	Methanol	ND	ND
		Hexane	ND	ND
		Ethyl Acetate	ND	ND
		Dichloromethane	3.23±0.35	0.00±0.00
	Fruits	Methanol	ND	ND
		Ethyl Acetate	7.75±1.06	0.82±0.08
Pergularia tomentosa	Leaves	Methanol	3.50±0.71	0.82±0.06
	Stems	Methanol	ND	ND
		Hexane	ND	ND

∅: Inhibition zones diameter; **Rf:** Retention factor; **ND:** Not detected.

The bioautography showed different retention factor (Rf) values for inhibition zones, a result related to the presence (in plants) of anti-Foa compounds with different polarities. Moreover, this result confirms the presence of different targets in the Foa and thus inhibition is not necessarily tied to a single mechanism. In addition, the efficacy varies from one plant to another and from one part to another (Shinde *et al.*, 2009). The realization of bioautography has allowed us to separate the constituents present in a given extract and evaluate their antifungal effect. For the Rf of inhibition zones, the higher the value is close to (1.00) over the polar character decreases (when the stationary phase is polar, silica gel for example) (Mdee *et al.*, 2009). According to our results, the antifungal compounds present in these plants are different in efficacy (inhibition zone), polarity and chemical nature.

Any plant part may contain antimicrobial compounds (Cowan, 1999). This information is true and general, but these different results can be noticed by the plant source, the protocol of extraction and evaluation, and the host (pathogen). Our results have demonstrated that leaves are the best representative as antifungal source. Concerning the efficacy, the fruits of *Citrullus colocynthis* are the most important. The roots extracts have not inhibited the Foa by bioautography. Among 19 extracts chosen in this study, only 6 (31.58%) had shown at least one inhibition zone (Table 1).

As extraction solvent, ethyl acetate presented the best effect (3 out of 6 extracts present inhibition effect). The ethyl acetate extracts are the most effective on Foa, which is probably related to the nature of compounds in this solvent extracts and to the presence of their targets in Foa. The efficacy of ethyl acetate extracts reflects that antifungal compounds are of medium polarity. The ethyl acetate is known for its efficacy to extract active biological

compounds which play a role in plants defense such us phenolic compounds (Flavonoids, tannins, coumarins,…). Many studies have demonstrated the presence of antifungal compounds in ethyl acetate extracts (Chang *et al.*, 1999; Deepa *et al.*, 2004; Mouokeu *et al.*, 2011; Velmurugan *et al.*, 2012). Tung *et al.* (2007) have shown that soluble fraction in ethyl acetate (from bark of *Acacia confusa*) is the richest in phenolic compounds.
Asteriscus graveolens is the most efficient plant as inhibiton zones number (3 spots). *Citrullus colocynthis* and *Asteriscus graveolens* take the leader place as inhibition zone diameters (7.75 ± 1.06 mm; 7.00 ± 1.41 mm, respectively). The fact that *Citrullus colocynthis* and *Asteriscus graveolens* extracts are the most active against Foa indicates the presence of potential antifungal compounds in these plants and presence of their targets in Foa. Phytochemical studies have showed the richness of *Citrullus colocynthis* in phenolic compounds and flavonoids (Maatooq *et al.*, 1997; Kumar *et al.*, 2008), well known for their antimicrobial action (Pengelly, 2004; Marica *et al.*, 2008). The remarkable effect of *Citrullus colocynthis* is probably due to phenolic compounds. The flavonoids, for example, can play a role of Foa enzymes inhibiton. Waksmundzka-Hajnos and Miroslaw (2008) have signaled that coumarins play a protection role in plants and some of them have antifungal effect. The difference in diameter is possible related to the concentration and/or effectiveness of the antifungal compound in each extract.

4. Conclusions

The effectiveness of some species especially *Citrullus colocynthis* and *Asteriscus graveolens* extracts indicates the presence of highly sensitive targets in Foa, which can be exploited for the development of efficient treatment against Bayoud disease.

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