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Keywords: Immediately after the abstract, provide a maximum of 7 keywords, avoiding general and plural terms and multiple concepts (Times New Roman; Size-12; Italic).

Introduction: Should clearly establish the objectives of the work and its relationship with other works in the same field

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Bioautographic test of *Punica granatum* extracts on *Fusarium oxysporum.f.sp.albidinis*

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Abstract *Fusarium oxysporum f. sp. albidinis*, is among the most aggressive soil fungi causing wilt and rot of date palm. Bayoud disease, caused by *Fusarium oxysporum f. sp. albidinis* (Foa), is a major limiting factor of the date palm cultivation. In order to look for other alternatives to fight against this fungus, in this work we study the antifungal potency of substances extracted from the peel of *Punica granatum*. The analysis by the method of direct bioautography allows the detection of 17 bioactive substances, Three are indicated in the ethyl acetate extract, two on the level of hexane extract, two in the extract of dichlorométhane, one in the butanol extract and nine on the level of the chloroformic extract.

Key Words: *Fusarium. oxysporum f. sp. Albidinis*, *Punica granatum*, bioactive substances, bioautography.

1. Introduction

Plants have been known to be a reservoir of secondary metabolites which are being exploited as source of bioactive substance for various pharmacological purposes [1,2]. The fact that some of these plants have been used traditionally for centuries and modern scientific studies have shown the existence of good correlation between the traditional or folkloric application of some of these plants further strengthens the search for pharmacologically active compounds from plants [2]. One of such plants with wide ethnomedicinal use is *Punica granatum*

The pomegranate, *Punica granatum* L., is one of the oldest known edible fruits. This fruit is mentioned in the Koran and Bible and is often associated with fertility [3]. The pomegranate tree, *Punica granatum* possesses a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal value [4], and it is an important source of bioactive compounds [5]. The pomegranate tree can be divided into several anatomical

compartments like seed, peel, juice, flower, leaf, bark, roots, etc. each of which has interesting pharmacologic activities [4].

From the literature review, it was noticed that the peels of *Punica granatum* possess various pharmacological activities and also considering the use of fruit in indigenous medicines [5, 6]. In an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. In this regard, several bioassays were developed for screening purposes [7]. Once the technique has been mastered, bioautography is a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore facilitates the target-directed isolation of the active constituents [7]. Among the all bioautographic methods, the most widely applied is direct bioautography [8]. Bayoud disease of date palm caused by *Fusarium oxysporum f.sp.albidinis* [9-12] part constraints of date palms against which it is difficult to fight. In this regard we proposed this study to look for other alternatives to fight against this fungus, studying the antifungal potency of some extracts of *punica granatum* peel by direct bioautography.

2. Material and method

2.1. Solvant and reagent

All solvents used in this study are from SIGMA-ALDRICH (Hexane) ;Biochem pharma (n-Heptane, Acetone); PROLABO VWR (Dichloromethane, Chlorofom, Ethyle acetate)and Cheminova (Butanol) .Reageant Iodonitrotetrazolium chloride (p-Iodonitrotetrazolium Violet, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, INT) is used from Fulka Analytical.

2.2. Plant materials

Pomegranate peels were obtained from local markets. The peels were manually removed, sun-dried and powdered.

2.3. Fungal Strain

The fungal strain used in this study is *Fusarium oxysporum f. sp. albedinis*, the causal agent of Bayoud disease that affects the date palm trees (*Phoenix dactylifera* L). The strain used in this study was obtained from The Technical Institute for Saharian Agronomy (TISA), Adrar, Algeria.

2.4. Preparation of plant extracts

The *Punica granatum* peels was shade dried and pulverized to fine powder in a mechanical grinder. The powder (150g) was extracted using heat reflux extraction with 300 milliliter of hexane, chloroform, dichloromethane, Ethyle acetate, methanol, butanol and water as solvent successively for 2h. The extracts were concentrated under reduced pressure in a rotary evaporator. The crude extract was collected, partially dried and stored in a cool place until use.

2.5. Separation of extracts by TLC

The thin layer chromatography (TLC) has established itself as one of the basic techniques in research on natural substances, due to its simplicity of implementation and its ability to separation and analysis complex mixtures that are crude extracts of natural origin [13,14].

The crude extracts obtained as described above was analyzed by TLC on Silica Gel plates (7 x 1.5 cm) using solvent system: AcOEt/Heptane (5/15:V/V) for Hexane, Chloroform and Dichlorometan extracts ; AcOEt/Heptane (15/5:V/V) for Ethyl acetate extracts . TLC plates

were run in duplicates and one set was used as the reference chromatogram. Spots were visualized by UV irradiation (254 and 366 nm). The other set was used for bioautography.

2.6. Method of screening by bioautography

2.6.1. Bioautography

Bioautography is a very convenient way of testing plant extracts and pure phytochemical compounds for their effect on both human pathogenic and plant pathogenic microorganisms [15]. A number of bioautographic assays have been developed, which can be divided into three groups. These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate [7, 16]. Among the all bioautographic methods, the most widely applied is direct bioautography [4]. The beginnings of coupling microbiological assay with planar chromatography date back to 1946, when Goodall and Levi combined paper chromatography method (PC) with contact bioautography detection for the determination of the different penicillins [4, 17]. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture [7].

2.6.2. Antifungal screening by direct bioautography (Thin-layer chromatography-direct bioautography)

A bioautography method based on the direct immersed and growing of a fungal spore suspension on a developed TLC chromatogram was used to screen for and identify compounds with antifungal activity present in the plant extracts. Amount of 400 µg/5 µl of each extract were applied to the TLC plate of silica gel 60 F254 (7 × 1.5 cm). After developing the chromatogram using a suitable eluent for efficient separation and location of the UV absorbing spot, chromatograms are then moved directly into petri dishes containing 20 ml of spores for 10 seconds, where the concentration is $2 \cdot 10^7$ spores/ml. Bioautograms were examined for fungal growth at regular intervals until TLC plates were completely covered with a lawn of mycelial growth. Then, they are added in other petri dishes sterile containing cotton wet with distilled water to provide moisture. These are incubated for 4 days at 21 °C. The plates were immersed in 2 mg/ml solution of p-iodonitrotetrazolium violet (INT) and further incubated for 1 hour at 21 °C. Rf zones on the plates were compared to that of reference plates to find the Rf of the active compound.

3. Results and discussion

Bioautography allows easy localization of activity even in complex matrix as that derived from natural products. Metabolites with antimicrobial activity were identified as clear inhibition zones on a red-colored background that indicated microbial growth.

The crude extracts of *Punica granatum* peels was analyzed by TLC on silica gel. Plates A (1-4) (Figure 1-4) shows the chromatogram used as the reference one. Plates B(1-4) (Figure 1-4) shows part of the chromatogram after treatment with spores suspension, indicating the fungal inhibition zone. The zones of inhibition (clear zones on plate) for organisms of *Fusarium oxysporum f.sp.albidinis* were measured in mm and Rf of the inhibition zones on the plate (plates B(1-4)) was compared with the Rf of the reference chromatogram (plates A(1-4)) to determine the Rf of the active compound (Table.1).

For visualization of fungal growth, tetrazolium salts (INT) are used, which are converted by the dehydrogenases of living microorganisms to intensely colored formazan. Clear white zones against a purple background on the TLC Plate indicate antifungal activity of the extracts. The same method is used in deferent study such as K. Das and all, 2010 for evaluation of medicinal plant products as antimicrobial agents; L. Lindy andall , 2006 and C. Valgas and all ,2010.

In the present study, The analysis by the direct bioautography detects the presence of 17 antifungal substances on the level of *Punica granatum* peels , Three are indicated in the ethyl acetate extract, two on the level of hexane extract, two in the extract of dichlorométhane, one in the butanol extract and nine on the level of the chloroformic extract.

In addition, we are showed that the extract of chloroform is the mixture richest with antifungal substances because all his constituents are indicated by INT as a clear color in plate. The same are observed for the dichloromethane extract.

Also, the ethyl acetate extract showed good activity against the fungus *Fusarium*, the presence of this activity was observed by three well-defined zones of inhibition ($R_f=1.2, R_f=0.45$ and $R_f=0.78$). The compound of $R_f = 0.78$ is the most active compound, their inhibition zone equals 1 cm. This activity may be due either to the amount of this compound in the complex mixture or to the right of this activity on the *Foa*.

Table.1: The results of direct bioautography test.

<i>Extract</i>	<i>N^{ber} of spots</i>	<i>R_f</i>	<i>Diameter (D) in mm</i>
Hexane extract	2 spots	0.43	3
		0.52	3
Chloroform extract	9 spots	0	3
		0.1	3
		0.21	2
		0.36	4
		0.48	5.5
		0.5	5
		0.54	4
		0.56	6.5
		0.66	7
Dichloromethane extract	2 spots	0	4
		0.45	5
Ethyl acetate extract	3 spots	0.12	4
		0.446	6
		0.784	10
Butanol extract	1 spot	0.58	

According to a study carried out by G. Prakash Yoganandam et al., 2010 on the phytochemical screening of *Punica granatum* peel extracts; the ethyl acetate extract is a rich

mixture flavonoïds and tannins, poor in alkaloids, glycoside and saponins. Therefore, we can deduce that the antifungal activity from the components of the extract acetate of ethyl due to the presence of these compounds.

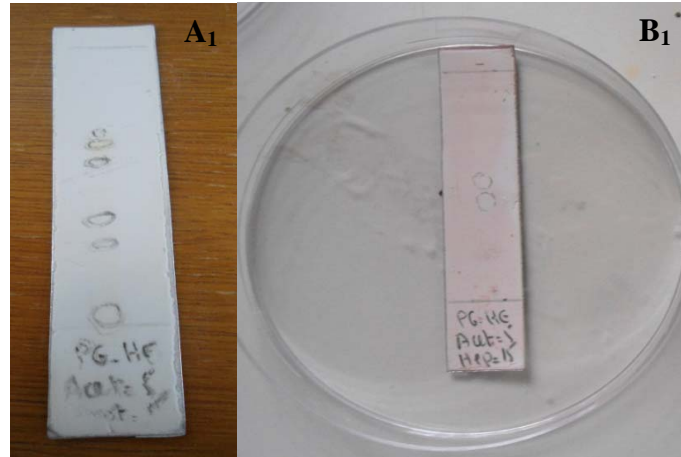


Fig.1: Test bioautography of hexane extract.

Eluent: AcOEt/Heptane (5/15)(V/V), A₁ reference chromatogram, B₁ chromatogram for bioautography

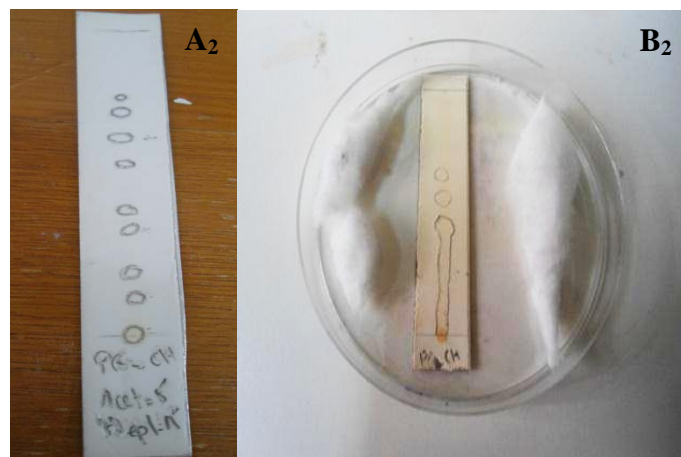


Fig.2: Test bioautography of chloroform extract.

Eluent: AcOEt/Heptane (5/15)(V/V), A₂ reference chromatogram, B₂ chromatogram for bioautography

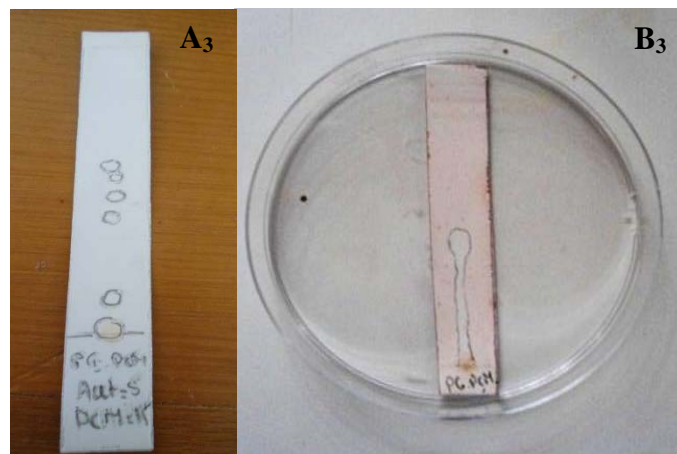


Fig.3: Test bioautography of dichloromethane extract.

Eluent: AcOEt/Heptane (5/15)(V/V), A₃ reference chromatogram, B₃ chromatogram for bioautography

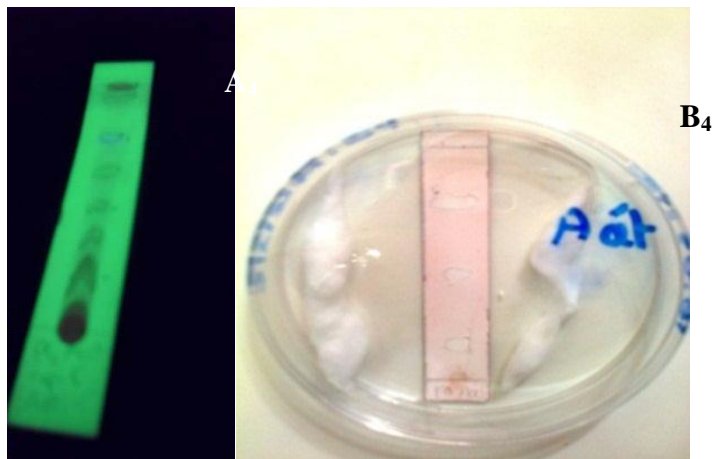


Fig.4: Test bioautography of Ethyl acetate extract.

Eluent: AcOEt/Heptane (15/5)(V/V), A₄ reference chromatogram, B₄ chromatogram for bioautography

4. Conclusion

TLC–bioautography, a convenient and simple mean of testing plant extracts and pure substances for their effects on pathogenic microorganisms, enables easy detection of active fractions. The direct bioautography assay, as an identify and localize compounds with antifungal activity in crude extracts of *punica granatum*. This assay has the advantage of being quick, easy to perform and relatively cheap, requiring no sophisticated infrastructure, only requiring a small amount of the tested compounds, with high sample throughout and the results are easy to interpret.

According to this assay we concluded that, *Punica granatum* is a rich plant with antifungal substances, especially antifusarium substances. It can be used as a solution to fight against the bayoud disease.

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