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Antifungal activity of *Matricaria pubescens .Desf et Juniperus oxycedrus* and control of mycotoxin-producing molds.

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Abstract

This work is part of the development of two medicinal plants (*Matricaria pubescens (Desf) and Juniperus oxycedrus)*, growing spontaneously in the region of Bechar and Ain Sefra respectively, in order to know their phytochemical compositions and their properties. antifungal and anti-mycotoxin control *in vitro.*

Phytochemical screening revealed the presence of saponosides, tannins, flavonoids, steroids, fatty acids, volatile oils and triterpenes in both plants. The moisture content of *J. oxycedrus* contains slightly higher than *M. pubescens* (Desf) with rates of the order of 32.66% and 30.80% respectively.

The antifungal activity of the extracts (aqueous, ethanolic and methanolic) of the aerial parts of the two plants was tested on five fungal strains: *Aspergillus niger; Aspergillus flavus; Fusarium oxysporum* f.s albedinis (FOA1 and FOA2) and *Penicellium purperogenum* by the method of evaluation of radial growth on solid medium as well as the antifungal effect was obtained by the development of biomass in liquid medium.

With the exception of the aqueous extract of *J. oxycedrus* where a reduction in radial growth of the FOA2 strain was recorded, the other tests involving the aqueous extracts showed increases as a function of the concentrations.

Based on the measurements of diameters at the end of the cultures compared to the controls, very important antifungal indices were noted for the five strains according to the concentration of the extracts, with percent inhibition of 100% on FOA with the metholic and ethanolic extract of *M. pubescens (Desf)* at concentrations of 1.86 and 1.57 mg / ml respectively.

With regard to the liquid media, almost complete inhibition was observed for all the strains tested with concentrations of the order of 1.57 and 2.7 mg / ml relative to the methanolic and ethanolic extracts added to the two plants.

The most effective inhibitory potency is that of the methanolic, ethanolic extract of *M. pubescens*, and the aqueous extract of *J.oxycedrus* at concentrations of 0.046 and 0.059 mg / ml, respectively.

Key words: *Matricaria pubescens*, *Juniperus oxycedrus*, antifungal activity, anti-mycotoxin, methanolic extract, ethanolic extract, aqueous extract.

1.Introduction

Nowadays, contamination of foods with fungi and their mycotoxins is one of the most dangerous with regard to food safety and pose serious health risk. Mycotoxins are molecules produced as secondary metabolites by filamentous fungi, are toxic compounds found in many feedstuffs. They contaminates cereals, oil seed products, worldwide due to infestations by funguous species .

Mycotoxins metabolites (Aflatoxin, Zearalenone , Trichothecene,Ochratoxin A , Citrinin, Patulin, Penicillic acid, Tenuazonic acid, Cytochalasins, Deoxynivalenol, Fumonisins, Fusarin C, fusaric acid…) produced by specific fungi are introduced into the food chain by pre-harvest and post-harvest contamination of foods and livestock feed .

Mycotoxins are intermediates metabolites or products, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism". The term was later applied to other toxic fungal natural products (Bennett and Klich, 2003).

These metabolites primarily affect the seed quality, germination, viability, seedling vigour, growth of root and cleoptile. Additionally, since the fungi responsible for the production of these mycotoxins are often endophytes that infect and colonize living plant tissues, accumulation of mycotoxins in the plant tissues may at times be associated with development of plant disease symptoms. (Ismaiel and Papenbrock ,2015)

Climate changes seem to be another important factor affecting mycotoxin contamination of foods and feedstuffs .Depending on the geographical and climate conditions, different fungal species can invade foods and feedstuffs. *Aspergillus*, *Penicillium*, and *Fusarium* species are the most important mycotoxin

producers. *Penicillium* and *Aspergillus* spe cies can grow at higher temperature and lower a_w than *Fusarium. Fusarium* species grow well at higher a_w and lower temperature. *Aspergillus* species can be found on nuts, cereals, palm kernels, cocoa, and coffee beans(Afsah-Hejri *et al.*,2013).

The proper storage facilities for moisture and temperature control and aeration provide protection from mycotoxigenic fungal growth. Numerous natural and chemical agents have also been used to prevent the fungal growth and mycotoxin formation..

Essential oils and extracts from aromatic plants have long been used for a wide variety of medicinal and domestic purposes. Antimicrobial properties of essential oils obtained from aerial parts and seeds of aromatic plants against food-related microorganisms as well as their applications in food system have been investigated and reviewed(Makhloufi *et al.*,2014).

2. Materials and Methods

2.1. Plant material

Matricaria pubescens (Desf.) Schultz (Asteraceae) (figure 01) is a small, pleasant-scented annual plant endemic to North Africa (Makhloufi *et al.*,2015). *Juniperus oxycedrus* L (Figure 02) is a shrub belonging to *Cupressaceae* family, in the mediterranean area, including Algeria which lies on the arid slopes and hills It is more frequent in the Saharan Atlas of Algeria.

M.pubescens (Desf.) and *J. oxycedrus* L specimens were collected from Bechar and Ain Safra region respectively , south-west of Algeria, during January and February 2012. These biomasses were dried for fifteen days in the dark at ambient laboratory temperature (20-28°C).

Figure01 : : *Matricaria pubescens(Desf)*

Figure02 : : *Juniperus oxycedrus L*

2.2. Phytochemical tests

Phyto-chemical screening of the *J. oxycedreus L* was performed using the methods described by Akermi et al (2017), detection of steroids, alkaloids, flavonoids, saponins and tannis.

2.3. Antifungal Activity (*In vitro)*

2.3.1 Preparation of ethanolic and methanolic extract

Fresh plants were dried in the shade at room temperature and ground in a coffee bean grinder. 15g of plant material powder is macerated in 100 ml of ethanol or methanol in a balloon protected from light for 24 hours, then the mixture is filtered.
The filtrate obtained is evaporated using a Rotavapor. The dry extract is kept cold in hermetically closed sterile vials. Solutions at different concentrations are prepared from this dry extract (Abbassi *et al.,* 2005, Kassi *et al.,* 2008, Makhloufi *et al.,* 2012).

2.3.2.Preparation of aqueous extract

Aqueous extract of *M. pubescens(Desf.)* and *J. oxycedrus* were prepared by boiling 25g in 500 ml sterile distilled water for 15 to 20 min. The flasks were then plugged and removed from heat and allowed to cool. After cooling, the contents of flasks were filtered (Sqalli *et al.,* 2007).This was repeated and the filtrates were combined and concentrated in a rotary evaporator to obtain the crude extract (Kassi *et al.,* 2008)

2.3.3 Fungal Strains

The antifungal activity was evaluated by dilution methods against five selected fungi of *Fusarium oxysporum f. sp. Albedinis(*1) FOA(1), *Fusarium oxysporum f. sp. Albedinis*(2) FOA(2), isolated from Tabelbala and Kerzaz region respectively, *Aspergillus niger Aspergillus flavus and Penicillium purpurogenum* ,were isolated from infected date.

Fungal spores were prepared by growing mold on Potato Dextrose Agar (PDA) at 27°C for 7 days, and spores were suspended in sterile 1% tween-80. Spore count was performed by using hemacytometer, and adjusted to obtain 10^5 to 10^6 spores/ml (Makhloufi et *al.,* 2011).

2.3.4. Determination of minimal inhibition concentration (MIC)

Known volumes of the extract were added to the sterile PDA medium to obtain different concentrations. Petri dishes containing 15 ml of PDA medium without extract are used as controls for each fungal strain (Mishra and Dubey 1994; Khallil, 2001). The seeding is done by the method of single spore using a platinum loop calibrated to take the same volume of inoculum. After incubation at 25 ° C for 7 days taking into account control growth (Leroux , 1978). The MIC is defined as the lowest concentration of extract for which no growth is visible compared to the

control without extract (Motiejūnaité and Peiculyté, 2004)

The antifungal index or percentage of inhibition (PI) which is determined by the formula is calculated (Leroux , 1978)

PI= [(DC- D) / DC] ×100 %

D: The diameter of the growing area of the test

DC: The diameter of the growth zone of the control.

2.3.5. Assessment of biomass in a liquid medium:

In flasks containing 45 ml of Yes or Czapeck liquid glucose medium (with a few drops of lactic acid), different concentrations of each extract are added. After stirring, a disc of the inoculum harvested from a 7-day culture is added. After 7 to 22 days of incubation at 25 ° C., this solution is filtered on filter paper and then dried in an oven at 105 ° C. for 3 hours and the weight of filter paper is taken after drying.

The weight of the biomass formed is determined by the following formula:

W = W1-W0

W_0: Weight of the filter paper in grams (g).

W_1: Weight of the filter paper with the biomass of the mushrooms after drying in gram (g).

2.3.6.Mycotoxicological analyzes:

2.3.6.1.Seeding on Yeast Extract Sucrose medium(YES)

Aspergillus flavus Strains are reseeded on yeast Extract Sucrose medium, this medium promotes secondary metabolism and induces anabolic reactions. The plates were then Incubated at 25 ± 2C for 7 to 22 days.

2.3.6.2.Seeding on Czapek Dox Glucose medium(CZG)

The ***Czapek Dox Glucose*** medium used for the growth and identification of fungi belonging to the genera *Fusarium.* In addition, this medium also used in the aflatoxin production test, and for the conservation of molds (André , 2007).

2.3.6.3.Extraction of mycotoxins

After 14 days of incubation, the biomass formed is filtered off by filtering the YES or CZG medium through filter paper. 45 ml of the filtrate obtained are added to 162 ml of chloroform, the whole is vigorously shaken for 30 minutes, the homogenized solution is deducted (using a light bulb to count down). The chloroform phase is concentrated by evaporation under vacuum until 2 to 3ml of volume using a rotavapor. The filtrate is kept to 4 ° C in haemolysis tubes well closed to undergo on thin-layer chromatographic separation.(Abdellah,2004)

2.3.6.4.Chromatographic analysis with TLC:

Thin layer chromatography (TLC) is a good step for screening samples (detection

of positive samples), but quantification is often difficult (Cahagnier *et al.*, 1998).

In the starting line, the spots of each extract to be analyzed are deposited. The plate is then placed in a chromatographic tank and quenched in an elution solvent consisting of toluene, acetaldehyde and formic acid of volume (50/40/10 ml) respectively. After migration and evaporation of the dry elution solvent using a drier, the plate is examined under UV at 365 nm. The presence of mycotoxins results in characteristic fluorescences.

3. Results And Discussion

3.1. Qualitative phytochemical screening

The preliminary phytochemical screening of bioactive constituents of ethanolic,methanolic and aqueous leaves extracts of *M. pubescens(Desf.)* and *J. oxycedrus* was carried out flavonoids, sterols , triterpenes, fatty acids , Tannins, volatile oils, and steroids were found in all samples with remarkable quantity (table01). However, Alkaloids, Emodols and Quinone were not found.

Table 01 : Result of the phytochemical screening of aerial part of studied plants.

researched Compounds	***M.pubescens* (Desf.)**	***J.oxycedrus***
Strach	+	-
Saponosid	+	+
Tanins	+	+
Alkaloids	-	-
fatty acids	+	+
Flavonoïds	+	+
Stérols	+	-
stéroïdes	+	+
Emodols	-	-
volatile oils	+	+
Triterpen	+	+
Quinone	-	-

These results are consistent with those of the work of (Azari,2009) that proved that *J.oxycedrus* is very rich in tannins, Saponosid and Flavonoïd**s.** For *M.pubescens* (Desf.)These results confirmed the substantiation of previous studies which have reported that *M.pubescens* (Desf.) has a wide variety of secondary metabolites and different biosynthetic processes according to the type of chemical compounds: coumarins, flavonoids, , terpenes, glycosides,

sesquiterpenes lactones Saponosid(Gherboudj, 2014 ;Dehimat,2014).

3.2.Antifungal activity of extracts :

3.2.1. Results of evaluation of the radial growth on solid medium

From the results shown in Figures 03, 04, 05, and 06. It is noted that there is a decrease in radial growth of all fungal strains with methanolic and ethanolic extracts. On the other hand, for the aqueous extrac,t the radial growth is inversely proportional to the concentration of this extract, except for FOA 2. This increase can be due probably, that the strains use the aqueous extract as carbon and nitrogen source , or there is the existence of a stimulator for the growth of these fungi. This hypothesis is also used by other authors, who noticed the same phenomenon. (Holetz *et al.*, 2002, Nahal, 2008).

By comparison, it can be seen that the compounds of the alcoholic extracts are more active than the aqueous extracts. Noting that this maceration indicates the presence of flavonoids, tannins, steroids and terpenes, these compounds are endowed with antimicrobial activity (Nakatsu and Shu 1998).

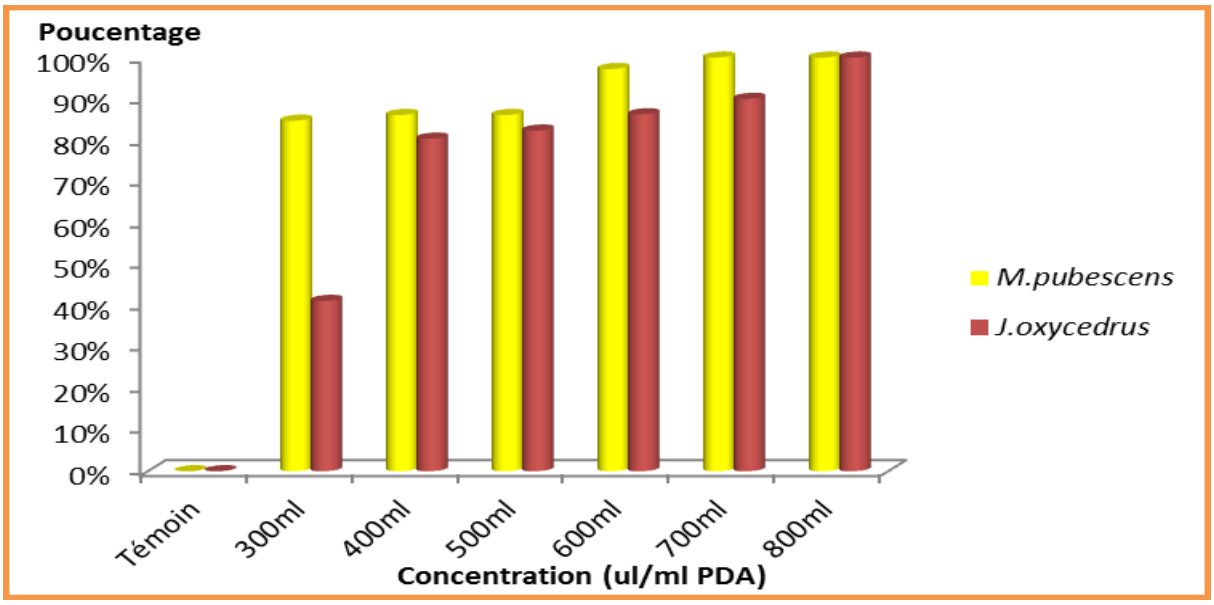

Figure 3: Antifungal index of the methanolic extract on *A.niger*

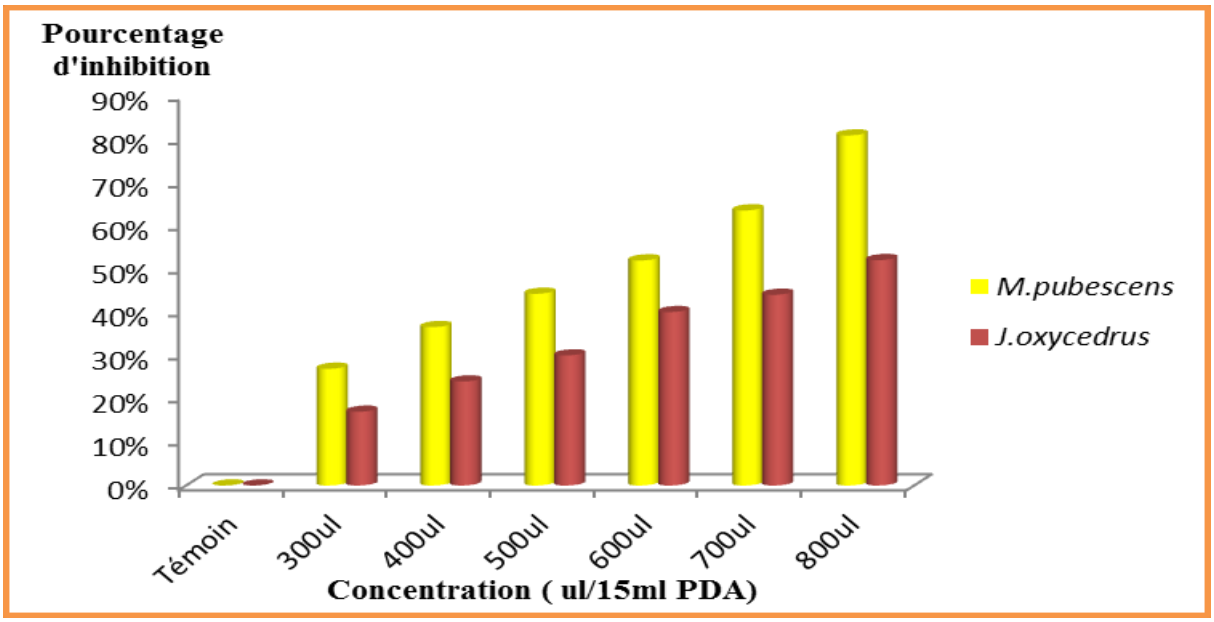

Figure 4: Antifungal index of the ethanolic extract on *A.niger*

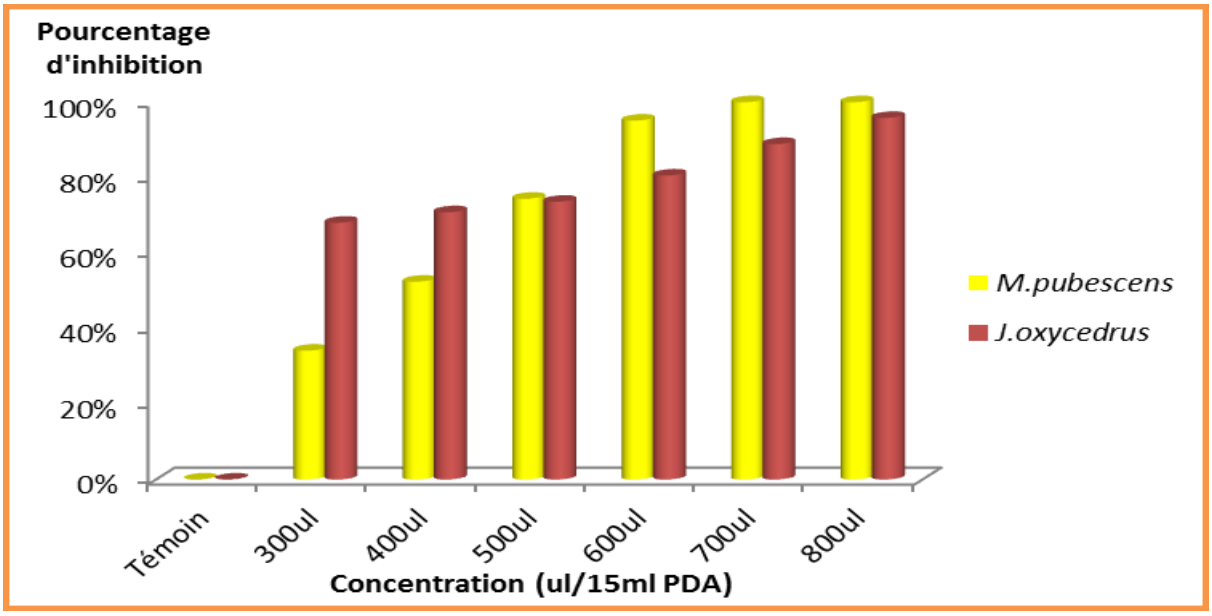

Figure 5: Antifungal index of the methanolic extract on *FOA1*

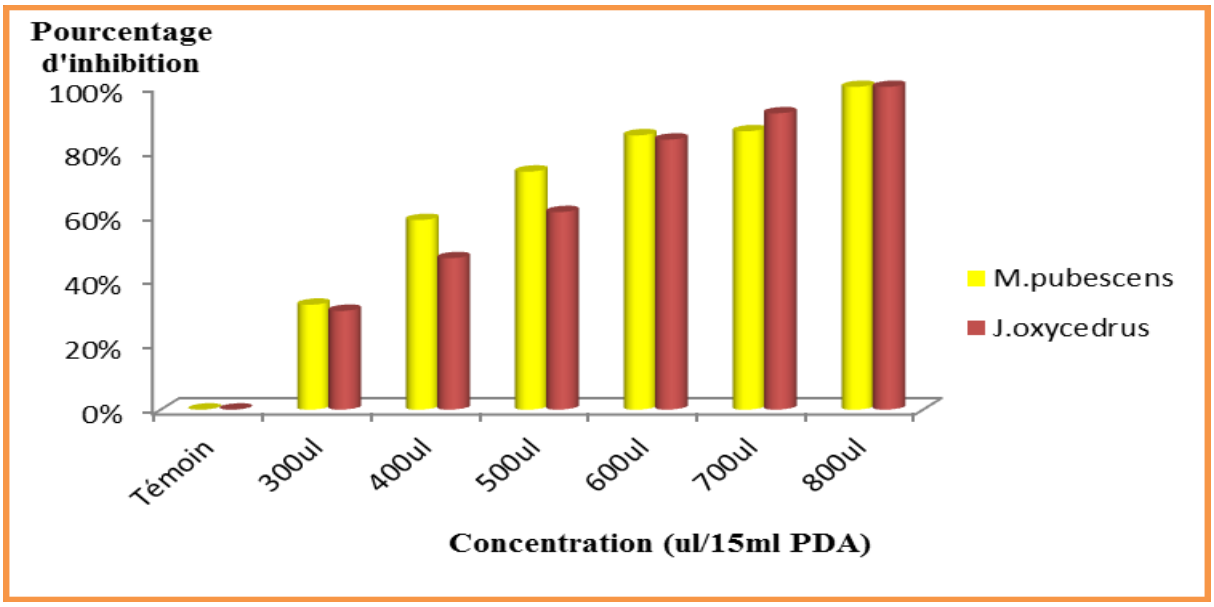

Figure 6: Antifungal index of the ethanolic extract on *FOA1*

3.2.2.Determination of the MIC

The methanolic and ethanolic extracts of *J.oxycedrus* reacted positively on all fungal strains tested (Table 02 and 03); with MICs of 2.38 g / l for *A.niger*, 2.51 g / l for *A.flavus* and FOA2 and 2.64 g / l for *P. purpurogenum*, and 2.25 g / l for FOA1.

Table 02: Result of the MIC Determination of the Methanolic Extract of *J. oxycedrus*. (g / l) on the fungal strains.

Concentrations (g/l) / Strains	**1.85**	**1.98**	**2.25**	**2.38**	**2.51**	**2.64**	**2.78**
A.niger	+	+	+	**CMI**	-	-	-
A.flavus	+	+	+	+	**CMI**	-	-
P. purpurogenum	+	+	+	+	+	**CMI**	-
FOA1	+	+	**CMI**	-	-	-	-
FOA2	+	+	+	+	**CMI**	-	-

(-): Inhibition;(+) Growth

Table 03: Result of the MIC Determination of the ethanolic Extract of *J. oxycedrus*. (g / l) on the fungal strains.

Concentrations (g/l) / Strains	**1.85**	**1.90**	**2.19**	**2.32**	**2.45**	**2.58**	**2.70**
A.niger	+	+	+	-	-	**CMI**	-

A.flavus	+	+	+	+	+	+	**CMI**
P. purpurogenum	+	+	+	+	+	**CMI**	-
FOA1	+	+	+	**CMI**	-	-	-
FOA2	+	+	+	+	+	**CMI**	-

The methanolic and ethanolic extracts of *M. pubescens Desf* reacted positively on all fungal strains tested (Table 04 and 05); with MICs of 2.38 g / l for *A.niger*, 2.51 g / l for *A.flavus* and FOA2 and 2.64 g / l for *P. purpurogenum*, and 2.25 g / l for FOA1.

Table 04: Result of the MIC Determination of the Methanolic Extract of *M. pubescens Desf*. (g / l) on the fungal strains.

Concentrations (g/l) / Strains	**1.23**	**1.5**	**1.86**	**2**	**2.45**	**2.58**	**2.70**
A.niger	+	+	+	-	-	**CMI**	-
A.flavus	+	+	+	+	+	+	**CMI**
P. purpurogenum	+	+	+	+	+	**CMI**	-
FOA1	+	+	**CMI**	-	-	-	-
FOA2	+	+	+	+	+	**CMI**	-

Table 05: Result of the MIC Determination of the Ethanolic Extract of *M. pubescens Desf*. (g / l) on the fungal strains

Concentrations (g/l) / Strains	**1.38**	**1.57**	**1.61**	**1.76**	**1.85**	**1.94**	**2.03**
A.niger	+	+	+	+	**CMI**	-	-
A.flavus	+	+	+	+	+	**CMI**	-
P. purpurogenum	+	+	**CMI**	-	-	-	-
FOA1	+	**CMI**	-	-	-	-	-
FOA2	+	+	**CMI**	-	-	-	-

All strains are inhibited between 1.57 and 2.7 g / l, we found that both extracts have almost similar activity on the strains tested, this is clear by comparing the inhibitory concentrations. The extracts of *M.pubescens Desf*. Are very active comparatively with *J.oxycedrus* extract, whose MIC was of 1.94 g / l of

M.pubescens Desf. And 2.25 to 2.7 g / l *J. oxycedrus*.

For *M.pubescens Desf*, The results obtained show that FOA 1 is the most sensitive strain with a MIC of 1.85 g / l for the methanolic extract 1.57 g / l for the ethanolic extract, for the extracts of *J. oxycedrus*, the MIC is of the order of 2.25 g / l for methanolic extract, 2.32 g / l for the ethanolic extract .

3.3. Results of Mycotoxicological analyzes

In order to test the effect of the extracts of both plants on the inhibition of mycotoxin production, the results obtained are shown in the figure 07 and 08

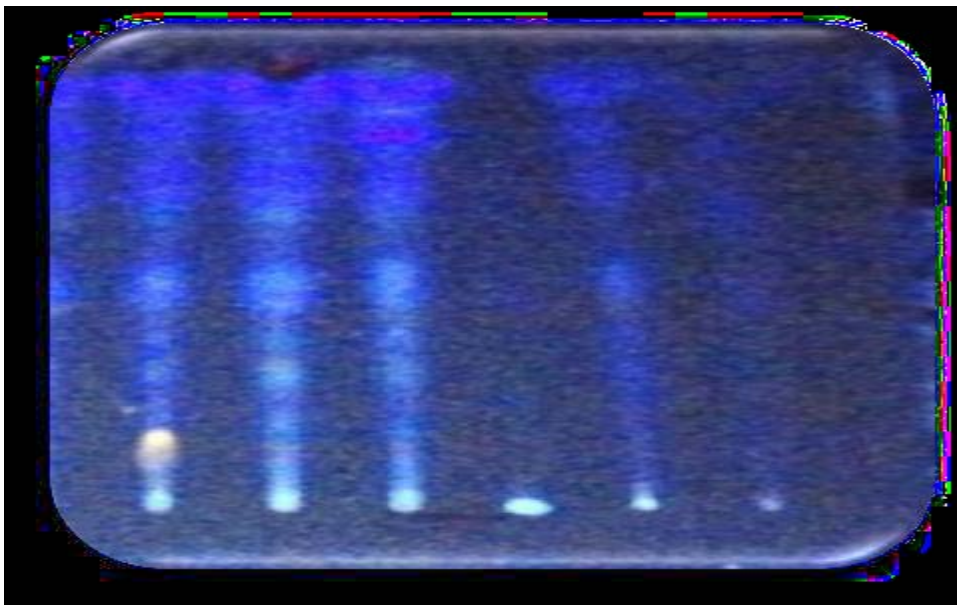

Figure 07 : the effect of Methanolic extract of *M.pubescens Desf* on the production of mycotoxins on TLC

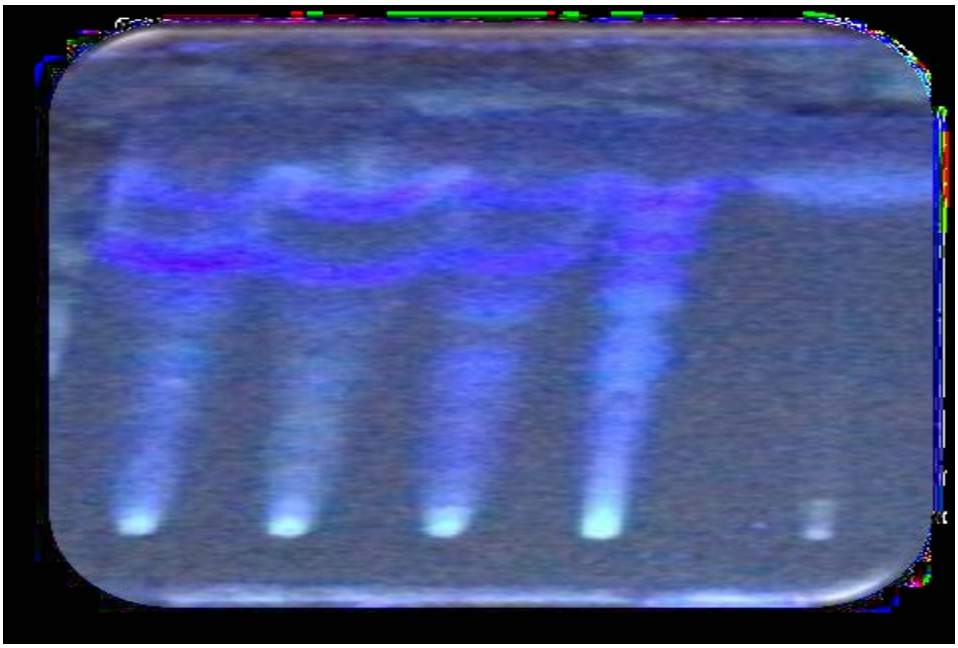

Figure 08 : the effect of Ethanolic extract of *J. oxycedrus* on the production of mycotoxins on TLC

It is noted that there is an inhibitory effect of some studied extracts on the secretion of mycotoxins by *A. flavus* and FOA1.

According to the results of anti-mycotoxicological analyzes, there are three phenomena:

- Activation of the synthesis of certain toxins by the extracts used;
- There is no effect of the extracts on inhibition of mycotoxin synthesis;
-Total inhibition of mycotoxin secretion.

The most effective inhibitory potency is that of the methanolic, ethanolic extract of *M. pubescens*, and the aqueous extract of *J.oxycedrus* at concentrations of 0.046 and 0.059 g / l, respectively. This effect is probably due to the different chemical families existing in the mentioned extracts, which have an inhibitory effect on the synthesis of these secondary metabolites.

It is also noted that there is a proportional increase as a function of different concentrations, this phenomenon can be explained by the stimulating effect of the chemical families exist extracts that promote the secretion of mycotoxins in the culture media used.

Conclusion

The antifungal activity of extracts isolated from the arial parts of *M. pubescens Desf*, and *J.oxycedrus* has been evaluated using spore germination assay. They has the potential to be considered as a new natural drug for the treatment of some fungal infections, and reducing mycotoxin production by different fungal.

The extracts exhibited variable efficacy against different isolates strains and 100% inhibition of spore germination of all the fungi tested was observed at 500 ppm. However, the most resistant fungus,

Fusarium udum was inhibited 80% at 400 ppm. Kinetic studies showed concentration as well as time dependent complex inhibition of spore germination by the essential oil.

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