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In vitro antifungal effect of aqueous extracts of some Moroccan plants

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Abstract

The antifungal activity of aqueous extracts of nine spontaneous Moroccan aromatic and medicinal plants was examined against four phytopathogenic fungi. The primary secondary metabolites were qualitatively determined in the extracts, which showed inhibitory activity against at least one of the targeted fungal species. The poisoned food approach was used to evaluate antifungal activity in vitro. The mycelial growth of phytopathogenic fungi was greatly inhibited by phyto-extracts from five different plants. The inhibitory effect of Ptychotis verticillata extract was 47.40 on Fusarium oxysporum and 78.81% on Fusarium culmorum. For Botrytis cinerea, mycelial growth was reduced by over 50% by aqueous extracts of Cistus monspeliensis and Saxifraga granulata, but was stimulated by three other extracts, including Quercus suber, which had a significant positive effect 31% higher than the control. The growth of Verticillium dahliae was inhibited by extracts of Cistus monspeliensis, C. salviifolius and C. ladaniferus, and the greatest effect was caused by the extract of C. ladaniferus with a percentage inhibition of 94%. Qualitative phytochemical analysis of the five active extracts showed the presence of phenolic compounds, tannins and flavonoids. In addition, extracts from the three rockroses were rich in saponins and alkaloids. The antifungal activities revealed in the phyto-extracts depended on the source plant and the phytopathogenic fungi targeted. The plants P. verticillata, C. salviifolius, C. ladaniferus, C. monspeliensis and S. granulata could be exploited in phytopharmacy for the treatment of diseases caused by F. oxysporum, F. culmorum, V. dahliae and B. cinerea.

Keywords: Plant extract, Secondary metabolites, Antifungal activity, Phytopathogenic fungi, Poisoned food technique.

Full length article*Corresponding Author*, e-mail: <soukaina.aithaddou@uit.ac.ma>, <atmane.rochdi@uit.ac.ma>

1. Introduction

Over 40% of agricultural productivity losses worldwide are attributed to phytopathogenic fungi [1], of which the species most damaging to horticulture and agriculture belong to the genus Fusarium [2]. *Fusarium oxysporum*, for instance, is ranked fifth among plant diseases that are significant to the plant crops [3]. It causes yellowing and wilting in more than 100 plant species [4]. However, one of the diseases most commonly linked to fusarium head blight in wheat, which results in yield losses of 30–70% globally, is *Fusarium culmorum* [5]. Additionally, hundreds of dicotyledonous species, such as cotton, tomato, lettuce, potato, strawberry, and olive, are susceptible to vascular wilt disease due to *Verticillium dahliae*, a widespread fungal pathogen that damages soil [6]. Intravenous leaf necrosis and wilting are the disease's primary symptoms, which ultimately lead the affected plants to die [7]. Moreover, *Botrytis cinerea* is one of the phytopathogenic fungi that cause the largest agricultural losses both before and after harvest, as it is the cause of gray mold in over 1400 plant species [8]. Unfortunately, industrial pesticides that are used to manage pests and plant diseases have detrimental impacts on both human health and the environment [9]. Additionally, a lot of plant diseases have become resistant to current pesticides, necessitating the use of ecological alternatives in order to successfully battle agents detrimental to agriculture without posing a risk to public health or the environment. Plant secondary metabolites are chemicals produced by plants to guard against a range of environmental threats, such as disease invasions, drought, and other stress [10]. According to Sabitha et al.2018, these substances -also referred to as phytochemicals- are commonly divided into three categories: terpenes, phenols, and nitrogen compounds. [11]. Given their greater host specificity, phytochemicals offer advantages over industrial pesticides in terms of efficacy, durability, and

lack of toxicity [12]. In this context, four phytopathogenic fungi (*Fusarium oxysporum*, *Fusarium culmorum*, *Verticillium dahliae*, and *Botrytis cinerea*) were used to assess the antifungal effect of nine plants from the Moroccan spontaneous flora.

2. Materials and methods

2.1 Biological material

2.1.1. Isolation and identification of phytopathogenic fungi

Parts of tomato, strawberry and wheat plants showing symptoms of cryptogamic infection (lesions, rot, wilting, etc.) were cut into small pieces, disinfected and then rinsed 3 times with sterile distilled water, then placed in containers. Sterile petri dishes containing rounds of filter paper moistened with sterile distilled water and incubated in the dark at a temperature of 25°C. After 5 days, the presence of fungi was examined under a binocular microscope and the conidia were taken and cultured in petri dishes containing PSA (Potato Sucrose Agar) medium under sterile conditions and incubated in the dark at 25°C. After 7 days, if the sectors of the mushrooms were not homogeneous, then a purification step was carried out and which consisted of repeating the transplanting several times until obtaining a homogeneous petri dish presenting a single type of mushroom. Finally, the identification was made on the basis of the determination keys and four fungi were selected for the experiment because of their significant damage to agriculture: *Fusarium oxysporum* and *Verticillium dahliae* isolated from tomato leaves, *Botrytis cinerea* isolated from strawberry fruit and *Fusarium culmorum* isolated from wheat roots.

2.1.2. Plant material

The aerial parts of nine species (the Moroccan fir: *Abies marocana*, the Atlas cedar: *Cedrus atlantica*, the gum cistus: *Cistus ladaniferus*, the Montpellier cistus: *Cistus monspeliensis*, the sage-leaved cistus: *Cistus salviifolius*, the False fluet ammi: *Ptychotis verticillata*, the Cork oak: *Quercus suber*, the granulated Saxifrage: *Saxifraga granulata* and the Sinuated Mullein: *Verbascum sinuatum*) of the Moroccan medicinal flora were collected between June and August then left to dry naturally in the shade at room temperature of 25 to 30°C for 3 to 4 weeks. The dry plant material was collected and ground into a fine powder, then sieved and packed into glass jars which were stored in the dark and at laboratory temperature until use.

2.2 Preparation of aqueous plant extracts

The preparation of the plant extracts was done using the method of infusion in hot water. 10g of the powder of each plant is infused for 30 minutes with magnetic stirring in 100ml of sterile distilled water previously heated to boiling. The solution obtained is filtered successively through muslin cloth then filter paper. The filtrate was then sterilized by passing through Millipore filters of 0.22 µm pore diameter and stored in the refrigerator at 4°C until use within 7 days.

2.3 In vitro test of the antifungal effect of aqueous plant extracts

The effect of plant extracts on mycelial growth was tested at a concentration of 2.5% by the poisoned food method described by [14]. Fungal fragments 6 mm in diameter were taken by a punch from the periphery of a mycelial mat from a young culture of seven days. These fragments were seeded by depositing in the center of the petri dishes containing a mixture of 7.5 ml of PSA medium and 2.5 ml of the extract (test) or sterile distilled water (controls). To minimize experimental error, each test is repeated five times. Incubation was done in the dark for seven days at 25°C and the diameter of the mycelial growth was then measured from two perpendicular axes drawn on the back of the petri dish. The evaluation of the antifungal effect of the plant extracts consisted of calculating the percentage of inhibition (PI) relative to the control by the following formula:

$$PI = ((DC-DE)/DC) \times 100$$

Where DC is the average diameter of mycelial growth in the 5 control boxes; DE is the average diameter of mycelial growth in the 5 boxes containing a plant extract.

2.4 Phytochemical tests of active phyto-extracts

Aqueous extracts that showed significant antifungal activity were subjected to standard qualitative phytochemical analysis with some modifications described by [15,16].

2.4.1. Phenols

❖ ***Phenolic compounds test***

A few drops of a 5% neutral ferric chloride ($FeCl_3$) solution were added to 5 ml of each plant extract. Dark green or blackish blue indicates the presence of phenolic compounds.

❖ ***Tannin test***

1 ml of a 10% aqueous solution of potassium dichromate was used to treat 5 ml of each plant extract. A yellowish-brown precipitation suggests the presence of tannins.

❖ ***Flavonoid test***

1ml of hydrochloric acid and 1ml of iso-amyl alcohol were used to treat 1ml of each extract then some magnesium shavings were added. The release of heat then the appearance of a pink-orange or purplish color indicates the presence of flavonoids.

2.4.2. Terpenes

❖ ***Saponin test***

In a test tube, 5 ml of distilled water and 5 ml of extract were mixed and stirred for 15 minutes. A stable and persistent layer of foam indicates the presence of saponins.

2.4.3. Nitrogen compounds

❖ ***Alkaloid Test***

Four drops of Mayer's reagent were added to 2 ml of plant extract. A creamy white precipitation indicates the presence of alkaloids.

2.5 Statistical analyzes

The results of the mycelial diameters of the fungal strains in the presence and absence of plant extracts were expressed as mean ± the standard error of the five repetitions. Significant differences were assessed by 1-way analysis of variance (ANOVA) using SPSS software.

3. Results and Discussions

Analysis of variance showed a significant difference between the inhibition exerted by the aqueous extracts for each fungal isolate according to the Tukey HSD test at the threshold of $p<0.05$. One-way analysis for *F. oxysporum* showed that there is no significant variation between the growth in the controls and the growth in the presence of the extract of *Q.suber* ($p=0.507$) and that of S. granulate ($p=0.153$). For *F. culmorum*, growth was significantly different from the control only in the presence of three extracts: *V. sinuatum*, *P. verticillate* and *C. atlantica*. For *B. Cinerea* and *V. Dahliae*, one of the nine treatments was not significantly different from the controls *C. salviifolius* and *V.sinuatum* respectively (Table 2). The sensitivity of the fungus *B. cinerea* was only observed towards extracts of two plants *S. granulata* and *C. monspeliensis* with rates of inhibition of mycelial growth of 55 to 56% (Figure 1, A). The relatively highest level of sensitivity was noted in *V. dahliae*, particularly under the effect of cistus extracts (Figure 1, B) which caused very high inhibition rates (94, 73 and 60% respectively with *C. ladaniferus*, *C. salviifolius* and *C.monspeliensis*) on the mycelial growth of this fungus. Very generally, *F.culmorum* showed very significant resistance in comparison with the three other strains of mushrooms tested and against the majority of phyto-extracts. However, the species *P. verticillate* exerted a very high inhibitory effect on the growth of *F. culmorum* (79%) (Figure 1, D), as well as on the growth of *F. oxysporum* (74%) (Figure 1, C). Furthermore, contrary to our expectations, some extracts seem to have biostimulatory effects of 22 to 32% on the mycelial growth of some phytopathogenic fungi. These include *V.sinuatum* on *F.oxysporum* and *Q.suber* on *B.cinerea*.

The results shown in Table 3 show a difference between species in the presence or absence of secondary metabolites, with the exception of a similarity in phenolic compounds, tannins and flavonoids with a positive reaction in all the species tested. Furthermore, alkaloids are only found in the three cistuses (*C. salviifolius*, *C. ladaniferus* and *C. monspeliensis*). Finally, saponins are absent in *P. verticillata*. The aqueous extract of *Ptychotis verticillata* showed strong antifungal activity (PI>74%) against both Fusarium (*F. culmorum* and *F. oxysporum*) and the phytochemical test revealed the presence of phenolic compounds, tannins and flavonoids. In a study carried out by Taybi et al. (2023) *P.verticillata* essential oil contains mainly 45.54% phenolic compounds and it has been shown to be effective as an antifungal agent to inhibit the growth of various fungi [17]. Concerning the cistaceae, the extract of *C. salviifolius* showed a strong antifungal effect on *V. dahliae* (PI = 73%) which may be due either to the richness of the extract in phenolic compounds, tannins, flavonoids, saponins and alkaloids whose presence we revealed through phytochemical tests. These bioactive molecules can act as a natural antifungal agent [18,19]. Furthermore, the *Cistus monspeliensis* extract contains all the secondary metabolites tested (phenolic compounds, tannins, flavonoids, saponins and alkaloids) and it showed a very high inhibitory power against *B. cinerea* and *V. dahliae* with 56% and 60% respectively of inhibition. Rebaya et al. (2016) also demonstrated that *C.salviifolius* and *C.monspeliensis* have antifungal and antibacterial activity against all the species targeted in their work [20]. The strongest inhibitory activity of the aqueous extracts of the three cistaceae tested was caused by *Cistus ladaniferus* which inhibited more than 94% of the mycelial growth of *V. dahliae*. In addition, this extract contains phenolic compounds, tannins, flavonoids, saponins and alkaloids. Numerous studies have evaluated the phytochemical composition of extracts from different cistus and have all shown the richness of these plants in polyphenolic compounds, flavonoids, terpenes and several other molecules characterized by their antimicrobial potential [21–22–23].

Regarding the extract from Saxifraga granulate exerted a remarkable inhibitory effect on the mycelial growth of *B.cinerea* with an inhibition percentage which exceeded 55% despite the fact that this fungus is renowned for its ability to develop resistance to fungicides [24]. The aqueous extract of *Q. suber* had no effect on the mycelial growth of *F. culmorum*, however it stimulated that of *F. oxysporum*. Our results differ from those reported by Subhashini et al. (2016), according to which *Q.suber* extract inhibited the mycelial growth of *F.oxysporum* [25]. However, in our study a higher stimulatory effect of *Q. suber* was noted on *Botrytis cinerea* (PI = -32%). It turns out that infusion in hot water does not always extract the antifungal biomolecules found in *Q. suber*. in fact, other researchers who have used the hydrodistillation extraction technique have noted that the essential oils of this same species have antimicrobial activities against all the fungal and bacterial species tested in their work [26–28]. *Verbascum sinuatum* extract showed weak antifungal activity against phytopathogenic fungi and even a stimulatory effect on *F. oxysporum*. On the other hand, in other works, a strong antimicrobial activity of *V. sinuatum* against several bacteria and yeasts was noted by methanol and ethanol extracts [27,28]. Moreover, [29] showed that the antibacterial activity of secondary metabolites of *V.sinuatum* is due to their ability to increase the permeability of bacterial membranes. Antifungal activity has been reported in other Verbascum species such as *V.arianthum* and *V.thapsus* [30,31]. The aqueous extracts of the two pinaceae *Cedrus atlantica* and *Abies marocana* were ineffective against the four targeted fungi. On the other hand, in other works, other extracts such as the essential oil of *C.atlantica* exerted antifungal activity against *Aspergillus niger*, *Thielavia hyalocarpa* and four other fungi of the Penicillium genus [32], as well as against *Aspergillus flavus* [33]. *C.atlantica* hydrodistillate also has antimicrobial activity: it inhibited the growth of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus sp* and *Salmonella sp* [34]. In general, there is little mycelial growth of mushrooms in the presence of plant extracts compared with the controls. We can therefore assume that increasing the concentration used (2.5%) will increase the antifungal effect, because several studies have shown that the antimicrobial activity of plant extracts requires in the majority of cases strong (10%) concentrations [35,36].

Table 1: Zones of inhibition exerted by phyto-extracts on mycelial growth of the four phytopathogenic fungi

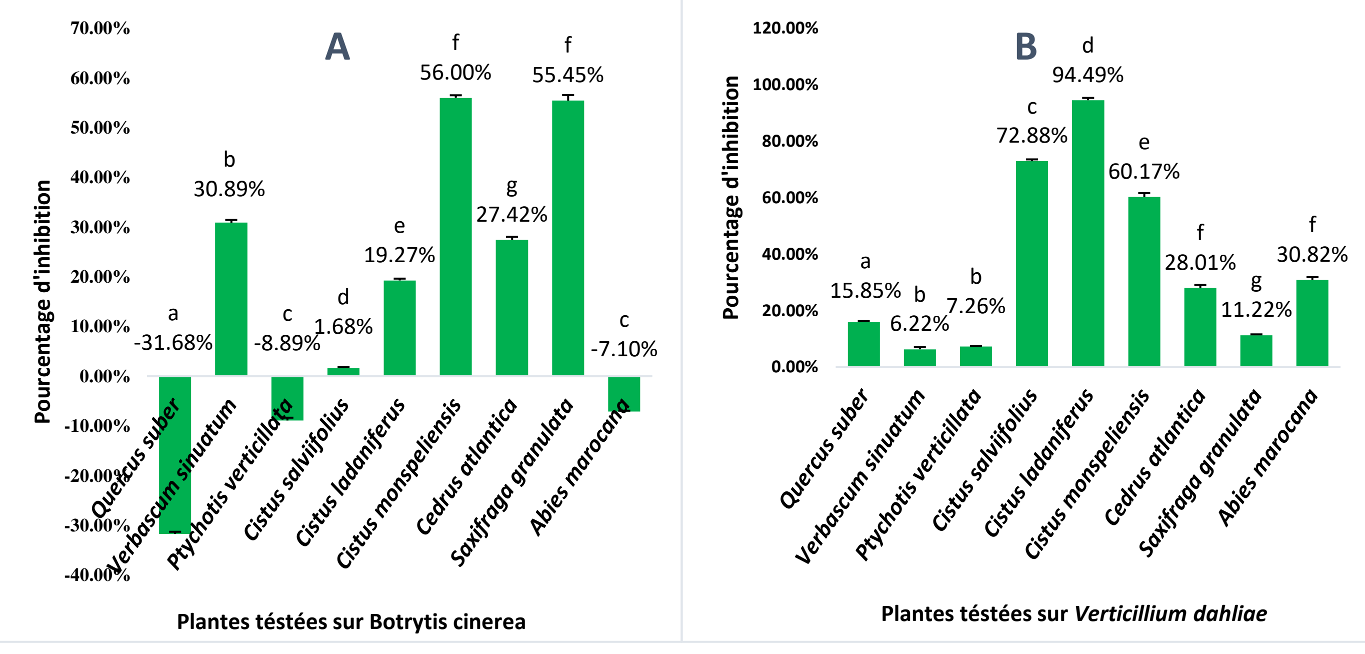
Plant extract	Phytopathogenic fungi			
	F. Oxysporum	*F. Culmorum*	*B. Cinerea*	*V. Dahliae*
Abies morocana	20.00 ± 2.00*	0.00 ± 0.00	-3.40 ± 0.40*	22.40 ± 1.29
Cedrus atlantica	14.80 ± 0.58*	7.80 ± 0.37*	19.40 ± 0.93*	20.20 ± 0.58
Cistus salviifolius	14.6 ± 1.08*	0.00 ± 0.00	1.20 ± 0.20	26.80 ± 4.89
Cistus ladaniferus	19.40 ± 0.81*	0.00 ± 0.00	13.60 ± 0.24*	68.20 ± 0.37
Cistus monspeliensis	10.20 ± 0.20*	0.00 ± 0.00	39.60 ± 2.73*	43.40 ± 0.60
Ptychotis verticillata	37.20 ± 6.89*	66.20 ± 0.80*	-5.20 ± 0.20*	5.80 ± 0.20
Quercus suber	-2.20 ± 0.20	0.00 ± 0.00	-15.40 ± 0.24*	8.40 ± 0.24
Saxifraga granulata	4.80 ± 0.37	0.00 ± 0.00	26.80 ± 1.98*	8.20 ± 0.58
Verbascum sinuatum	-9.80 ± 0.49*	29.40 ± 0.24*	18.60 ± 0.40*	2.00 ± 0.00*

Values are means of five replicates ± SD. Within a column, mean values with a star * indicate significant differences with controls (Tukey HSD test ; <0.05)

Table 2: The presence of secondary metabolites in aqueous extracts

Molecules	Phenolic compounds		Tannins		Flavonoids		Saponins		Alkaloids	
Extrait	Pictures	Result	Pictures	Result	Pictures	Result	Pictures	Result	Pictures	Result
Ptychotis verticillata		+		+		+		–		–
Cistus salviifolius		+		+		+		+		+
Cistus ladaniferus		+		+		+		+		+
Cistus monspeliensis		+		+		+		+		+
Saxifraga granulata		+		+		+		+		–

+ means positive result; - means negative result.



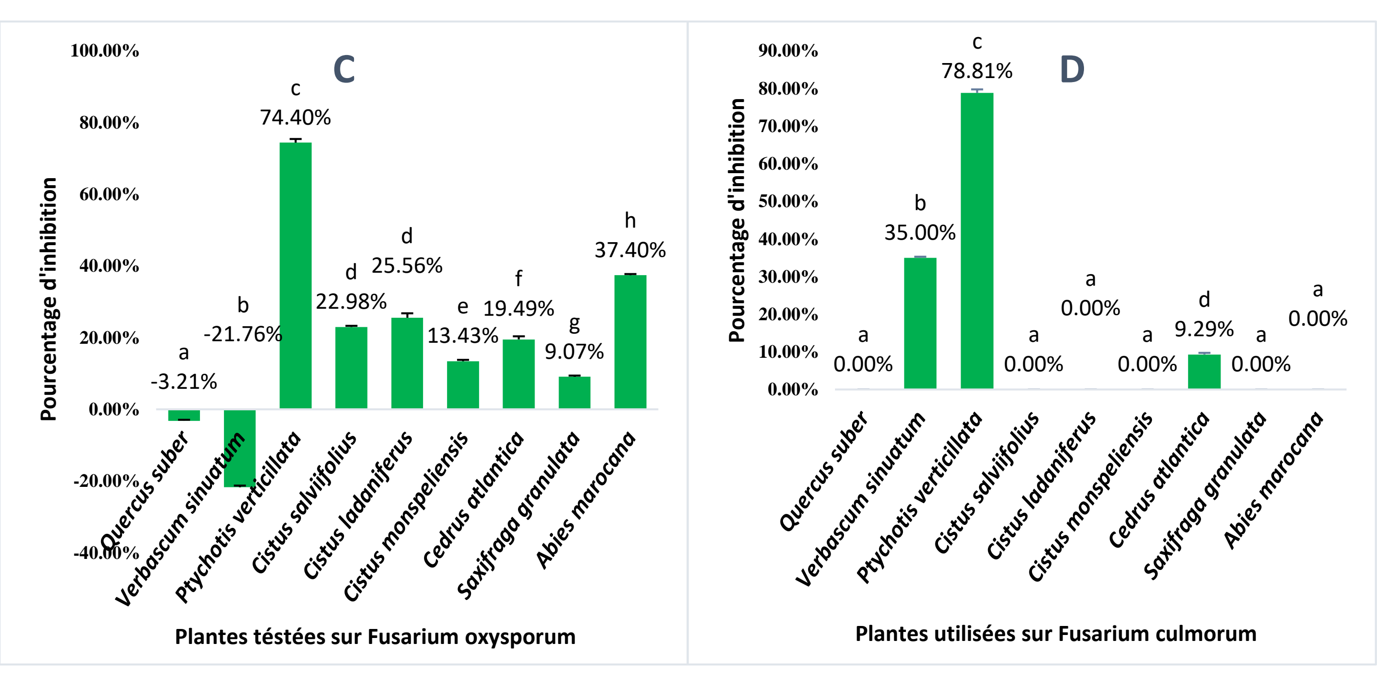


Figure 1: The percentage of inhibition of mycelial growth of: A (B. cinerea) B (V. Dahliae) C (F. Oxysporum) D (F. Culmorum); at a concentration of 2.5% of aqueous plant extracts. Bars with the same letter do not differ significantly according to the Tukey HSD test, at ($p<0.05$)

4. Conclusions

This study demonstrated the possibility of controlling fungal diseases caused by *Fusarium oxysporum*, *Fusarium culmorum*, *Verticillium dahliae* and *Botrytis cinerea*, by aqueous extracts of *Ptychotis verticillata*, *Cistus salviifolius*, *Cistus ladaniferus*, *Cistus monspeliensis* and *Saxifraga granulata*. This antifungal activity is influenced by the phytochemical profile of the source plants. It offers a new opportunity to improve the biological fight against phytopathogens which cause undeniable losses in crop yields without endangering the environment and the health of consumers.

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