

***In Vitro* Evaluation of Antifungal Activity of *Daphne Gnidium* Extracts against Six Human Pathogenic Fungi**

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ABSTRACT

There is ample scientific evidence today on the existence of a heightened and growing resistance of fungal agents to antifungal molecules. The search for natural bioactive substances that present lower risks to health has become a must. Scientific studies are on the rise, thus increasingly proving the therapeutic efficacy of plants.

In the present work, we sought to study the antifungal activity of the aqueous and methanolic extracts of *Daphne Gnidium* leaves, a wild species used in traditional Moroccan medicine. The plants' antifungal activity was evaluated *In Vitro* through the method of incorporation solid medium, against six pathogenic isolates: *Candida albicans* (AL62), *Candida albicans* (AL73), *Candida tropicalis* (TR6), *Candida glabrata* (GL1), *Trichophyton violaceum* (VI11) and *Scopulariopsis brevicaulis* (BR1), all of which are pathogenic fungi that occur in humans.

The results obtained show that the two types of extracts, aqueous and methanolic, have a positive but different antifungal impact depending on the pathogenic strains tested, with the exception of the two isolates of *C. albicans* (AL62 and AL73) and the isolate of *C. tropicalis* (TR6) which proved to be resistant to the aqueous extract of *D. gnidium*. This plant extract remarkably inhibited the *Candida glabrata* strain with a percentage of inhibition of diametrical growth -P.I.D.G- of 79.82% at the maximum concentration used which was 64.7mg. The aqueous extract also inhibited mycelial growth of *Trichophyton violaceum* and *Scopulariopsis brevicaulis*. The inhibition was total for *Trichophyton violaceum* at a minimal inhibitory concentration (MIC_{100%}) of 2.588 mg/ml. For *Scopulariopsis brevicaulis*, the inhibition of diametrical growth was 77.84% at a 64.7mg/ml concentration.

The methanolic extract was much more effective than the aqueous one. Indeed, we found that this extract had a significantly inhibitory effect on the isolates tested at lower concentrations than those of the aqueous extract: the P.I.D.G of *Candida glabrata* was 70% at a concentration of 8 mg/ml, while inhibition was total for *Trichophyton violaceum* and *Scopulariopsis brevicaulis* at a MIC_{100%} of 0.8 mg/ml and 6 mg/ml, respectively. The effectiveness of *D. gnidium* extracts against these pathogenic fungi opens new prospects for a natural fight against fungal agents.

KEY WORDS: *Daphne Gnidium*, antifungal activity, *Candida albicans*, *C. glabrata*; *C. tropicalis*, *Scopulariopsis brevicaulis*, *Trichophyton violaceum*.

1. INTRODUCTION

Antifungal therapy must be safe, effective and inexpensive. Yet, these qualities are almost absent in the products marketed due to side effects, toxicity and lengthy treatments that could last for months, and result in the development of resistance to these active ingredients. Thus, researchers have turned their attention to natural resources, including medicinal plants.

From ancient times, these plants have played a very important role for humanity. They are able to synthesize complex organic molecules that can treat certain diseases by a simple therapeutic procedure.

Morocco is a Mediterranean country where traditional medicine has always occupied an important place in the medical tradition. Herbal remedies are still a common traditional practice. Surveys in some provinces of the Kingdom have shown that over 60% of patients have a spontaneous recourse to herbal medicine. Phytotherapy, or how to treat some diseases with herbs, is a long-standing and well-entrenched phenomenon in Morocco. Besides, Moroccan traditional medicine is one of the richest and most versatile in the world. Thus, many plants are considered beneficial in the fight against microbial infections.

Having scientifically established the role of microorganisms in local or systemic infections, we explored many plant compounds in search of antimicrobial agents. Several studies have brought to light the different biological activities of aromatic and medicinal plants, especially their antifungal and antibacterial effectiveness.

Among the plants used, the *Daphne* a plant has been the subject of numerous studies. Indeed, in recent decades, a wide range of bioactive compounds was identified in this plant, particularly diterpenoids, coumarins, flavonoids and bi-flavonoids. Some *Daphne* species with antimicrobial powers include, for example: *D. mucronata*, *D. malyana* and *D. cneorum*.

The objective of this study is to test *In Vitro* the antifungal activity of aqueous and methanolic extracts of *D. gnidium*, a Moroccan indigenous plant, against five pathogenic fungi that occur in humans. We tested two isolates of *C. albicans*, one isolate of *C. tropicalis*, one isolate of *C. glabrata*, one isolate of *S. brevicaulis* and one isolate of *T. violaceum*. All these species affect human health.

2. EXPERIMENTAL SECTION

Plant material: The *Daphne Gnidium* leaves used in this study were harvested from the *Maamora forestin* of the Rabat region in March-April 2015 on randomly chosen plants. The samples selected for this experiment were dried in the shade for 15 days.

Extraction of natural substances: The previously dried leaves were first crushed in an electric grinder (such as Krups, Power X). The extraction of natural substances from the plant powder was performed with a Soxhlet, using water or methanol. The quantity of the plant material placed in the cartridge was 100g.

The final extracts were obtained after concentration and removal of the solvent and water by rotary evaporation. The extractions were repeated at least three times.

Total yield is expressed by the following equation:

$$R (\%) = (\text{mass of extract/mass of plant powder}) \times 100$$

Bioassays:

Fungal material: The microorganisms tested were five pathogenic fungi that affect humans: *Candida albicans* (AL62), *Candida albicans* (AL73), *Candida glabrata* (GL1), *Candida tropicalis* (TR6), *Trichophyton violaceum* (VI11) and *Scopulariopsis brevicaulis* (BR1).

Evaluation of the antifungal activity of the extracts: The antifungal activity of natural extracts was investigated *In Vitro* in a PDA medium (200g of potato starch, 20 g of glucose, 15 g of agar, and 1000ml of distilled water). The minimum inhibitory concentrations were determined by macro-dilution. The dilution solution was 0.2% agar.

Fungus subcultures were produced in the presence of different concentrations of both *D. gnidium* extracts. The same operation was performed on the culture medium alone to serve as control sample. Incubation was carried out at 25°C, in the dark. Antifungal activity tests of the two extracts were conducted on an agar culture medium by comparing their action on the fungi's diametrical growth at different concentrations.

Reading the findings: The test carried out is both qualitative and quantitative. Firstly, it allowed us to have an idea about the inhibiting power of the natural substance tested (presence or absence of inhibition), and then to calculate the diametrical growth inhibition rate of the fungal plant species, using the following formula:

$$P.I.D.G = (\text{Øt} - \text{Øe}/\text{Øt}) \times 100.$$

P.I.D.G = percentage of inhibition of diametrical growth.

Øt = average diameter of the control sample.

Øe = average diameter of the plants exposed to relevant plant extracts.

Three petri dishes were used per condition and at least three repetitions were carried out at different times.

The antifungal activity of the extracts studied was evaluated according to the following criteria:

- 30-40% inhibition = low activity
- 50-60% inhibition = moderate activity
- Above 70% inhibition = significant activity.

3. RESULTS

Evaluation of *D. gnidium* aqueous extract: The yield of the aqueous extract was:

$$R (\%) = (32.35/100) \times 100 = 32.35.$$

According to the results, the *D. gnidium* aqueous extract acted on three of the five tested fungal species. The effectiveness of the aqueous extract was positive but different on three strains of *C. glabrata* (GL1), *S. brevicaulis* (BR1) and *T. violaceum* (VI11) (Table.1). In the case of *GL1* and *BR1*, we found that the growth inhibition of both fungi increased with the extract concentration. At the concentration of 12,34mg, the diametrical growth inhibition was 40.2% for *GL1* and 40.2% for *BR1*, whereas at 64.7 mg/ml, the effect of the plant extract was remarkably positive with a diametrical growth inhibition of 79.82% and 77.84%, respectively for *GL1* and *BR1* (Table.1).

As for the *VIII* isolate, the diametrical growth was remarkably delayed under low concentrations, with an inhibition percentage higher than 50% at a 0,647mg/ml concentration (Table 1). The inhibition was complete for this isolate with a minimum inhibitory concentration (MIC_{100%}) of 2,588mg/ml (Table 2). Completely inhibited, *T. violaceum* (*VIII*) was transferred onto the plain PDA medium. After a stop phase under the effect of the plant extract, this pathogen came back to life. Therefore, the aqueous extract of *D. gnidium* has a fungistatic effect on *VIII* at this concentration.

Furthermore, we found that other species of *C. albicans* (AL62), *C. albicans* (AL73) and *C. tropicalis* (TR6) displayed insensitivity to the different concentrations used in our experiment. The aqueous extract had no effect on these pathogenic strains (Table 1).

Table.1. Effect of *Daphne Gnidium* aqueous extract on five pathogenic fungi, estimated by percentage of inhibition of diametrical growth (P.I.D.G).

Species tested	Concentrations of <i>Daphne Gnidium</i> aqueous extract in mg								
	12.94	19.41	25.88	32.35	38.82	45.29	51.76	58.23	64.7
<i>AL62</i>	-	-	-	-	-	-	-	-	-
<i>AL73</i>	-	-	-	-	-	-	-	-	-
<i>TR6</i>	-	-	-	-	-	-	-	-	-
<i>GL1</i>	40.2	40.87	41.89	43.23	57.76	59.12	69.13	73.24	79.82
<i>BR1</i>	40.12	46.33	50.1	52.13	56.23	57.15	59.15	69.53	77.84
<i>VIII</i>	100	100	100	100	100	100	100	100	100

Table.2. Search for the MIC of *Trichophyton violaceum* (VIII)

Species tested	Concentrations of <i>Daphne gnidium</i> aqueous extract in mg					
	0.647	1.294	1.941	2.588 (MIC)	3.235	6.47
<i>VIII</i>	52.6	61.88	73.33	100	100	100

Evaluation of *D. gnidium* methanolic extract: The yield of the methanolic extract was: $R (\%) = (38/100) \times 100 = 38$.

For this plant extract, similarity was noted in terms of the inhibition reactions of *GL1*, *BR1* and *VIII* in comparison with those of the aqueous extract. Indeed, the inhibitory effect of the methanolic extract on these three fungi rises with concentration. However, the methanolic extract is effective even at lower concentrations. The diametrical growth of the *T. violaceum* (*VIII*) isolate was remarkably inhibited under the action of the lowest concentration at 0.4 mg/ml with a 45.13% inhibition percentage, and this inhibition was full at the MIC_{100%} of 0.8mg/ml. As for *S. brevicaulis* (*BR1*) at 2.4mg/ml, inhibition of the diametrical growth reached 60.2% and became total at a MIC_{100%} of 6mg/ml (Table 3). The transfer of *VIII* and *BR1* cuttings on the plain PDA medium showed that *VIII* and *BR1* came back to life after a stop phase in the methanolic extract of *D. gnidium* (Table.4). Therefore, this plant extract has a fungistatic effect on both pathogenic strains.

In the case of *C. glabrata* (*GL1*), we also found that the inhibition of fungal growth increases with the concentration of the methanolic extract. Indeed, at 3.6 mg/ml, the diametrical growth of *GL1* was reduced by 52.8% and the extract was significantly active at the maximum concentration used of 8 mg/ml with a P.I.D.G of 69.44% (table.3).

Table.3. Effect of the methanolic extract of *Daphne Gnidium* on five pathogenic fungi, estimated by percentage of inhibition of diameter growth (P.I.D.G)

Species tested	Concentrations of <i>D. gnidium</i> methanolic extract in mg						
	0.4	0.8 MIC	2.4	3.6	4.8	6 MIC	8
<i>AL62</i>	-	-	-	-	-	-	-
<i>AL73</i>	-	-	-	-	-	-	-
<i>TR6</i>	-	-	-	-	-	-	-
<i>GL1</i>	29.5	41.65	50.87	52.8	55.7	62.63	69.44
<i>BR1</i>	38.6	47.77	60.2	67.3	73.296	100	100
<i>VIII</i>	45.13	100	100	100	100	100	100

∴ absence of inhibition

Table.4. Radial growth of *VIII* and *BR1* transferred to the plain PDA medium after exposure to *Daphne Gnidium* methanolic extract.

Fungal strains exposed to methanolic extract	<i>VIII</i>	<i>BR1</i>
Diameters of plants in mm, after one week in plain PDA	5.33	12.27
Nature of methanolic extract action	fungistatic	fungistatic

It appears from these results that the methanolic extract of *D. gnidium* has a remarkable and action on *T. violaceum* (*VIII*), followed by *S. brevicaulis* (*BR1*) and finally *C. glabrata* (*GL1*) which turned out to be much less vulnerable to this plant extract.

The results obtained for the two types of *D. gnidium* extracts showed that the aqueous extract is not active on the three strains of *Candida*, *C. albicans* *AL62*, *C. albicans* *AL73* and *Candida tropicalis* *TR6*. It can be concluded that these fungal strains are resistant to these treatments.

In the case of *T. violaceum*, we found that the aqueous and methanolic extracts have resulted in complete inhibition of the fungal species' diametrical growth. Indeed, it was noted that the methanolic extract's MIC_{100%} was much lower (0.8 mg/ml) compared to that of the aqueous extract which was 2.588mg/ml. As for *S. brevicaulis*, inhibition was complete with the methanolic extract with a MIC_{100%} of 6mg/ml, whereas this inhibition did not exceed 78% at the 64.7mg/ml concentration of the aqueous extract. As for *C. glabrata*, both aqueous and methanol extracts of *D. gnidium* had a relatively interesting effect with an inhibition rate, which did not exceed 80%.

4. DISCUSSION AND CONCLUSION

During the past two decades, the search for new biologically active substances and the use of herbal remedies have become a major scientific concern. Extracts of medicinal plants are endowed with antimicrobial power, which varies according to their chemical composition, and also with the nature of the extraction solvents. Plant extracts have a very broad spectrum of action and also inhibit the growth of bacteria as well as fungi, such as the extracts of *Lawsoniainermis* which both antifungal and antibacterial properties.

A number of studies have thus been conducted to investigate the secrets that medicinal plants hold such as the present study, dedicated to exploring the potential antifungal effects of aqueous and methanolic extracts of *D. gnidium* leaves.

Bioassays performed under this study showed that the growth of some of the tested fungal species was inhibited in accordance with the concentration of the *D. gnidium* extracts. Indeed, results obtained showed that the two types of extracts proved to be active though different on the mycelial growth of three of the five fungi tested and that the inhibition rate differed from one fungal species to the other.

The antifungal power of the methanolic extract was found to be higher compared to that of the aqueous extract. Indeed, the latter required higher doses to inhibit the tested fungi as opposed to the methanolic extract. These findings are consistent with those of Bougandoura & Bendimerad who showed in 2012 that the aqueous extract of *Satureja Calamintha* had the lowest antimicrobial effect. This can be explained by the low levels or absence of active ingredients contained in the aqueous extract.

According to the results obtained with the *D. gnidium* aqueous extract on the five pathogenic fungi, among the three species of *Candida* (*C. albicans* AL62, *C. albicans* AL73, *C. tropicalis* TR6 and *C. glabrata* GL1), this extract had a significant effect on *C. glabrata* with an inhibition rate of 79.82% at a concentration degree of 64,7mg/ml. The other *Candida* species have proven completely resistant. This confirms what had previously been reported by Cottiglia (2001).

The *Daphne Gnidium* aqueous extract tested in our experimental conditions on *S. brevicaulis* BR1 has also proven to be quite effective but required relatively high concentrations.

For *T. violaceum* VIII, the aqueous extract totally inhibited this isolate at the MIC_{100%} of 2,588mg. As a result, *T. violaceum* appears to be the most sensitive fungal pathogen.

The evaluation of the impact of *D. gnidium* methanolic extract on five pathogenic fungi showed that the activity of this plant extract was quite remarkable at lower concentrations compared to those needed with the aqueous extract on three of the five fungi tested: *T. violaceum* (VIII) *S. brevicaulis* (BR1) and *C. glabrata* (GL1). This shows that the methanolic extract had a very potent antifungal activity. The inhibition rate was variable depending on the species. The diametrical growth of the tested *T. violaceum* VII1 and *S.brevicaulis*BR1 was inhibited at 100%, while for the *C. glabrata* GL1, this inhibition did not exceed 70% at the highest concentration tested, which was 8 mg/ml. The methanolic extract of *D. gnidium* was found to be remarkably active on *S. brevicaulis* BR1 and resulted in its total inhibition. This outcome was very encouraging considering the resistance this fungus had shown to other antifungal agents used in the treatment of fungal infections.

Similar to the aqueous extract, the methanolic extract was very effective against *T.violaceum*VIII at a MIC_{100%} of 8mg/ml. This result is in agreement with other studies that found the aqueous extracts of eleven tested plants to be highly effective (with a 90-100% inhibition rate) against *T. violaceum*.

The great antifungal power of *D. gnidium* against the tested strains can be attributed to the presence of a greater variety of secondary metabolites, most of which are flavonoids and coumarins, known for their antimicrobial action.

The results thus obtained can be attributed to one of the compounds or a combination of substances: flavonoids and coumarins, or a family of compounds present in the *D. gnidium* extracts.

Other studies by Mohammadi (2013), confirmed the presence of coumarins and flavonoids of which the antifungal power has been proven with other fungal species. In fact, these studies have shown that the natural substances present in *D. gnidium* are likely to significantly reduce the fungal population of the *flavus* *Aspergillus* species. Moreover, the work of Kouassi (2012), showed that the *Capsicum annuum* and *Capsicum frutescens* varieties contain, amongst others, alkaloids, flavonoids, tannins, polyphenols and steroids that have antifungal properties against *Alternaria* sp, *Fusarium* sp, *Penicillium* sp and *Aspergillus flavus*.

The action of medicinal plants is often reduced to the activities of their major compounds, or those likely to be active. However, minor compounds could also act synergistically. Many studies have shown a qualitatively similar antimicrobial activity between plants and their chemical compounds tested in isolation. However, some quantitative differences exist. Indeed, it was shown that the antimicrobial effect of the plant is higher than that of its key compounds when these are tested separately. According to Lambert (2001), the association of major active compounds would synergistically act by enhancing the antimicrobial action of the tested plant.

Furthermore, other researchers have proven the antioxidant action of *D. gnidium* by using methanolic extracts of the leaves. Indeed, results of the phytochemical analysis of methanolic extracts revealed the presence of four new coumarins and flavonoids. Other studies have shown that *D. gnidium* leaves contained 131.9 ± 0.0072 mg (GAE)/g, the flavonoid content was 112.4 ± 0.02 mg (EQ)/g for these leaves.

The antifungal power of *D. gnidium* extracts is promising and suggests possibilities of drugs derived from these natural products.

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