academicJournals

Vol. 7(46), pp. 2947-2952, 15 December, 2013
DOI: 10.5897/AJPP2013.3627
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Anvillea radiata as a source of natural antifungal compounds

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Accepted 3 December, 2013

Recently, natural products have been evaluated as sources of antimicrobial agents with efficacies against a variety of microorganisms. This study describes the phytochemical screening and the antifungal activity of *Anvillea radiata.* Phytochemical analysis revealed the presence of some chemical groups such as volatile oils, fatty acids, tannins, flavonoids, anthracenosides, emodols, saponins, free quinones, anthraquinones, alkaloids, sterols and triterpenes. The antifungal activity of flavonoid extracts and cell-wall polysaccharide extracts derived from the flower and leaves of *A. radiata* were tested against plant pathogenic fungi *Fusarium oxysporum* f. sp. *albedinis* (Foa) (causing vascular wilt of date palm) by agar well diffusion method. The results indicate that flavonoid extracts had the strongest inhibitory effects on spore germination and on soil population density of Foa. Highly methylated pectins (HMP) from flowers produced the greatest inhibitory effect on mycelial growth. The sporulation was strongly inhibited using cellulose-based agar of leaves. Thus, it can be concluded that the use of *A. radiata* extracts could be considered as an antifungal available to develop novel types of natural fungicides and to control several plant pathogenic fungi.

Key words: Antifungal activity, *Anvillea radiate,* flavonoids extracts, *Fusarium oxysporum* f. sp. *albedinis,* phytochemical screening, polysaccharide extracts.

INTRODUCTION

Since ancient times, people have been exploring nature particularly plants in search of new drugs (Verpoorte, 1998; Dabai et al., 2012). Medicinal plants represent a rich source of antimicrobial agents and they are widely used in human therapy, veterinary, agriculture, scientific research and countless other areas (Vasu et al., 2009; Hussain et al., 2012). Drugs from plants can be derived from barks, leaves, flowers, roots, fruits, seeds (Criagg and David, 2001). In addition, knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances (Parekh and Chanda, 2007). Although hundreds of plant species have been tested for antimicrobial properties, a vast majority have not been adequately evaluated (Balandrin et al., 1985). In this context, *Anvillea radiate*, a plant belonging to the Asteraceae family that grows in Northern Africa, particularly in the two Maghreb countries of Morocco and Algeria. It is widely used in traditional medicine for the treatment of dysentery, gastric-intestinal disorders (Bellakhdar, 1997), chest cold, and has been reported to have hypoglycemic activity (Maizak et al., 1993). Although so far, there has been no study on

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its antimicrobial effects and phytochemical constituents. Hence, the main goal of this report is to study the qualitative phytochemical constituents of *Anvillea radiate* in the South-western region of Algeria. Also of interest is the antifungal effects of its polysaccharide extracts and flavonoid extracts against Foa, the causal agent of vascular wilt of date palm called "Bayoud disease" which represents a major limiting factor of date palm culture in Morocco and constitutes a serious threat to date palm plantations in Algeria and all other countries.

MATERIALS AND METHODS

Plant materials

At flowering stage, fresh parts (leaves and flowers) of *A. radiata* were collected from the Bechar area in Southwest, Algeria during the period January to May 2012. The plant was authenticated at the Sciences Department, University of Bechar, Algeria and it was deposited in the herbarium of this department. The harvested parts were shade dried for 15 days. After drying, the plant materials were ground well using mechanical blender into fine powder and transferred into airtight containers for future use.

Plant pathogenic fungi

One isolate of Foa, was used in the study. The fungus was received from the Laboratory of Valorization of Vegetal Resources and Food Security in Semi-arid Area, South-west Algeria, University of Bechar, Algeria. It was isolated from the rachis of infected date palms in an infested palm grove in the region. This fungal species was maintained on a potato dextrose agar acidified (PDAa) and stored at 5°C for further study.

Phytochemical analysis

Phytochemical tests were performed on different extracts prepared from the dried plant materials and ground, using four solvents of different polarities: water, ethanol, diethyl ether and petroleum ether. Phytochemical analysis for major phytoconstituents of the plant extracts was undertaken using standard qualitative methods as described by various authors (Bruneton, 1999; Paris and Moyse, 1969).

Preparation of flavonoids extract

A large number of flavonoid extraction methods have been developed in the past few years (Gao and Liu, 2005). In this study, flavonoid extracts from leaves and flowers of the plant studied were prepared as described by Lee et al. (1995) using a series of solvents (mixture of distilled water/ethanol, then n-butanol and finally a mixture of distilled water/ethyl acetate). The final organic phase (represents flavonoids extract) was dried before being weighed and recovered in 1% ethanol for biological tests.

Preparation of the cell-wall polysaccharides extracts

According to Harche et al. (1991) the extraction of cell-wall polysaccharides requires a preliminary operation which consists of preparation of the parietal residue. For this, each of the powdered plant materials (30 g) was put in an Erlenmeyer flask containing a mixture of methanol and chloroform (1:1, v/v) and was stirred for 14 h under a fume hood in order to remove the lipid soluble, tannins and other cytoplasmic constituents. The operation was repeated twice. After filtration, the residue was placed in 95% ethanol for 2 h while stirring to remove traces of chloroform and then was incubated in boiling 95% ethanol for 2 h to get better elimination of chloroform traces. The residue was then dried in an oven at 60°C for 48 h (Redgwell and Selvendran, 1986).

Extraction of cellulose

Cellulose was obtained by putting 5 g of parietal residue in an Erlenmeyer flask containing 100 ml of 4% NaOH and was stirred for 14 h. After filtration, the residue was rinsed with distilled water and then with acetone after which the residue was dried in an oven at 60°C for 14 h before being weighed. The resulting part represents the cellulosic fraction (Chanda et al., 1950).

Extraction of hemicelluloses

The two filtrates obtained in the previous step were neutralized by pure acetic acid and then precipitated in ethanol (1:3, v/v) for 14 h. After centrifugation at 3600 *g* at room temperature for 30 min, the pellet was washed with distilled water and then with acetone. Next, it was dried in an oven at 60°C for 14 h and finally weighed. This part represents the hemicellulosic fraction (Chanda et al., 1950).

Extraction of HMP

HMP extracts were prepared by putting 5 g of parietal residue in an Erlenmeyer flask containing 100 ml of distilled water, and stirred for 14 h at room temperature, and were put to boiling under reflux twice for two hours. After filtration, the filtrate was concentrated in a rotary evaporator. Then it was precipitated in cold acetone (1:2, v/v) for 14 h. After centrifugation at 3600 g at room temperature for 30 min, the pellet was rinsed with distilled water and alcohol after which the residue was dried in an oven at 50°C and was weighed. This part represents the HMP (Thibault, 1980).

Antifungal activity assay

Before being added to the PDAa medium, hemicelluloses, HMP and flavonoids were diluted in NaCl solution (5 mM), sterile distilled water and in 1% ethanol, respectively. A range of concentrations (0.25; 0.5; 1; 2 and 4 mg/ml of culture medium) of the extract were used. However , the cellulose extracts were intended to reconstruct cellulose-based media (with the following composition : $NaNO_3$ 2 g; K_2HPO_4 1 g; $MgSO_4,7H_2O$ 1 g; KCl 0.5 g; $ZnSO_4,7H_2O$ 0.005 g; $CuSO_4,5H_2O$ 0.001 g; $MnCl_2$ 0.001 g; Cellulose 2.5 ; agar 16 g; distilled water 1000 ml; pH = 5. (Lekchiri et al., 2006) on which development of *Foa* was studied.

Spore germination assay

Spore germination assay was performed as described by Maouni et al. (2001): 0.1 ml of a spore suspension (10^5 spores/ml) of *Foa,* prepared in sterile distilled water (counting using a Malassez cell) was spread on the Petri dishes containing PDAa media incorporated with flavonoids, hemicellulose and HMP extracts (separately) at different concentrations and on the cellulose-based media. The Petri dishes were incubated at 25 °C for 24 h. The counting of spores germinated or ungerminated was determined under a microscope on a total of 200 spores. A spore is considered germinated if

the germ tube length is greater than its diameter.

Mycelial growth assay

Petri dishes containing PDAa media with extracts (hemicelluloses, HMP and flavonoids in separated experiments) at different concentrations were inoculated with a mycelial disc (6 mm diameter, by using the reverse tip of a Pasteur pipette) obtained from a pure culture of *Foa*. The Petri dishes containing cellulose-based medium were inoculated in the same way. The Petri dishes were incubated at 25°C for 7 days. The diameter of the *Foa* colony was obtained by calculating the average of two perpendicular diameters (Hassikou et al., 2002).

Sporulation assay

All colonies used to evaluate mycelial growth and incubated for 10 days at 25 °C were used to study the effect of the extracts on sporulation. Sporulation assay was performed as described by Maouni et al. (2001).

Control tests were performed under the same conditions in the absence of extracts. The percentage of inhibition was calculated using the formula of Amadioha (2003), as shown below:

Inhibition (%) = (C - T). 1OO / C.
Where C and T represent *Foa* germination (radial growth or sporulation) in control and treated plates, respectively.

Fusarium soil population assay

Foa was incorporated into soil (previously sifted and sterilized) at an inoculum density of 10^6 spores/g of soil. Aliquots (10 g) of the infested soil were introduced aseptically into sterilized tubes and treated by 1, 5 and 10% (w/w) of cellulose, hemicellulose, HMP and flavonoids in separated experiments. Population density of *Foa* was determined using dilution plate techniques at 0 (before soil treatment), 1, 3, 7, 14, and 21 days after soil treatment (Bowers and Locke, 2000). 0.5 g of soil (sample unit) from each tube (replication) was placed in 5 ml of distilled water. 0.1 ml from the appropriate dilution (depending on the treatment) was pipetted onto the surface of Petri plates containing PDAa media. Plates were then incubated at 25°C for 24 to 48 h. Colony forming units (CFU) were counted in Petri dishes and then reported per gram of soil. CFU were then transformed to log 10 (CFU/g of soil) (Si Moussa et al., 2010). Control tests (infested soil with no medicinal plant treatment) were performed under the same conditions.

RESULTS

Phytochemical analysis

The phytochemical screening of Anvillea *radiata* showed the presence of medically active compounds in this plant (Table 1). Table shows that volatile oils, fatty acids, tannins, flavonoids, anthracénosides, emodols, saponins, free quinones are present in both organs studied. Anthraquinones, sterols and triterpenes were absent in the leaves. Alkaloids were absent only from the flowers. However, starch and reducing compounds were absent in the leaves and also in the flowers.

The extractions revealed the presence of 4.25% and 3.52% (w/w) flavonoids in leaves and flowers respectively, 22.83% and 25.66% for cellulose compounds. Hemicellulose content was found more in leaves (13.85%) and flowers contained 6.05% of hemicellulose but the content of HMP was low in the leaves (2.18%) compared to the flowers (7.2%).

Antifungal activity

On spore germination, the evaluation of the effectiveness of the extracts tested was based on the calculated percentages of spore germination (200 spores counted represent a percentage of 100%). The percentage of spore germination in control treatments was equal to 83.44% (Table 2). Compared with the control test, spore germination was stimulated by the polysaccharide extracts at all the tested concentrations (with spore germination percentages ranging from 99.66% to 100%). By contrast, flavonoid extracts inhibited spore germination at all dosages. The strongest inhibitory activity was observed with floral flavonoid (48.66%) at high concentration. Cellulose-based media were also capable of inhibiting the spore germination (the inhibition rate has reached 16% and 46% on cellulose-based medium of leaves and flowers respectively).

For mycelial growth fungal strain colony radius in control treatments was equal to 45.9 mm (Table 3). From the concentration of 0.5 mg/ml all the incorporated extracts (hemicellulose, HMP and flavonoid) showed inhibitory effect with varying degrees of inhibition rate. An increase in antifungal activity was observed with increase in concentration. Compared with the other extracts, HMP from flowers exhibited the most interesting inhibition on mycelial growth (30.71% at the highest concentration). On cellulose-based agar the mycelial hyphae elongation of *Foa* has occurred in a similar way as on PDAa without addings. But the significant difference is that the cellulose-based media have developed a very weakly dense mycelium compared to the PDAa with no addings. Inhibitory but also stimulatory effect of conidial production by the tested compounds was also found (Table 4). In particular, flavonoid and hemicellulose from both organs inhibited sporulation of *Foa* at all dosages (the dose increase is not necessarily accompanied by better efficiency) while sporulation of the pathogen was only inhibited at the intermediate and high dosages of HMP from both organs. The highest sporulation inhibition was obtained with hemicellulose of flowers (39.70% at 4 mg/ml). In addition, cellulose-based media also greatly inhibited production of conidia of *Foa* (inhibition percentage reached 80 and 77% on the cellulose-based agar of leaves and flowers respectively).

Twenty-one days after soil treatment with 1%, flavonoid from both organs reduced population densities of *Foa* by 22.80%, compared with the non-treated control soil (Table 5). However, the treatment with the other extracts has no significant effect. No significant change in the case

Table 1. Phytochemical constituent of *Anvillea radiata.*

Plant part	Starch	Reducing compounds	Volatile oils	Fatty acids	Alkaloids	Tannins	Flavonoids	Anthracénosides	Anthocyanosides	Emodols	Saponins	Sterols and triterpenes	Quinones free	Anthraquinones
Leaves	-	-	+	+	+	+	+	+	-	+	+	-	+	-
Flowers	-	-	+	+	-	+	+	+	-	+	+	+	+	+

Table 2. Effect of *A. radiata* extracts on spore germination of *Foa*.

Plant organ	Extract	Concentration (mg/ml)					Control	Percentage of spore germination (%)
		0.25	0.5	1	2	4		
Leaves	Flavonoid	70	69.66	59.66	61.66	45.83	83.44	
	Hemicellulose	99.83	99.83	99.33	98.16	96.16	83.44	
	HMP	100	99.16	100	100	99.66	83.44	
Flowers	Flavonoid	64.16	61.5	49.33	55.83	42.83	83.44	
	Hemicellulose	97.33	100	98.33	100	99.83	83.44	
	HMP	98.6	99.83	100	99.66	97.5	83.44	

Table 3. Effect of *A. radiata* extracts on mycelial growth of *Foa*.

Plant organ	Extract	Concentration (mg/ml)					Control	Colony diameter (cm)
		0.25	0.5	1	2	4		
Leaves	Flavonoid	4.95	4.58	4.51	4.35	3.88	4.59	
	hemicellulose	4.45	4.37	4.05	4.07	3.6	4.59	
	HMP	4.63	4.56	4.4	4.03	3.77	4.59	
Flowers	Flavonoid	4.63	4.56	4.4	4.03	3.77	4.59	
	hemicellulose	3.75	4.52	3.9	3.82	3.47	4.59	
	HMP	3.8	3.6	3.81	3.65	3.18	4.59	

case of the treatment with 5% of *A. radiata* extracts. The population of *Foa* in the soil treated with 10% of medicinal plant powders was not significantly less than in the untreated soil after 21 days.

DISCUSSION

Phytochemical screening conducted on the *A. radiata* extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities (Parekh and Chanda, 2008). Analysis of the plant extracts revealed the presence of phytochemicals such as tannins, flavonoids, saponins, volatile oils, sterols, anthracénosides, emodols, triterpenes, and alkaloids. The presence of these bioactive compounds indicates the medicinal value of this plant. Natural phytochemicals derived from fruits, vegetables and herbs have been reported to possess a wide range of biological effects, including antioxidant, antimicrobial and anti-inflammatory actions (Brunet et al., 2009).

Various extracts of medicinal plants have shown inhibitory effects against phytopathogenic fungi *in vitro* (Shalini and Srivastava, 2009). In this study, the biological tests showed that flavonoids exhibited appreciable inhibitory effect against *Foa* in all tests. Previous studies

Table 4. Effect of *A. radiata* extracts on sporulation of *Foa*.

Plant organ	Extract	Concentration (mg/ml)					Sporulation inhibition rate (%)
		0.25	0.5	1	2	4	
Leaves	Flavonoid	02.43	08.26	08.57	16.55	19.91	
	hemicellulose	36.94	15.77	18.53	18.99	39.24	
	HMP	-11.67	00.90	03.35	24.37	31.59	
Flowers	Flavonoid	36.80	01.21	03.35	24.22	31.59	
	hemicellulose	17.15	13.80	01.21	26.82	39.70	
	HMP	-07.14	-05.53	-11.90	11.63	28.20	

Table 5. Soil population densities of *Foa* 21 days after soil treatment with *A. radiata* extracts.

Plant organ	Extract	Rate treatment			Untreated	$\log_{10}$ (CFU/g soil)
		1%	5%	10%		
Leaves	Flavonoid	4.3	4.47	5.44	5.57	
	Hemicellulose	5.43	6.12	5.14	5.57	
	HMP	5.34	5.36	6.31	5.57	
	Cellulose	5.53	4.3	5.61	5.57	
Flowers	Flavonoid	4.3	4.47	5.44	5.57	
	Hemicellulose	5.44	5.07	4.9	5.57	
	HMP	5.71	5.2	5.38	5.57	
	Cellulose	5.71	5.76	5.71	5.57	

indicate that flavonoids of several plant extracts showed antifungal activities (Cushnie and Lamb, 2005; Pavithra et al., 2009; Akroum et al., 2009). The other roles attributed to them were to promote physiological survival of the plant by protecting them from fungal pathogens (Galeotti et al., 2008). According to Huang and Chung (2003) phenolic compounds caused swelling of hyphal tips, plasma seeping around hyphae, leaking of plasma, cell wall distortion, abnormal branching or fusion of hyphae and consequently wrinkling of hyphae surface.

Our results showed that polysaccharide extracts have presented an inhibitory effect both on mycelial growth and sporulation neatly superior to that on the spore germination. The antifungal activity of polysaccharide extracts was also confirmed experimentally by Ballance et al. (2007). The antimicrobial properties of natural polysaccharides are based on their chemical structure where the presence of a highly reactive carbonyl group was detected. The carbonyl group is able to bond primary amines to produce a stable combination of polysaccharides with proteins (glycolconjugates). Bonding of exoenzymes of saprogenic microorganisms by reactive polysaccharides is likely the reason for their antimicrobial activity (Painter, 1991).

Conclusion

The results revealed the presence of medicinally important constituents in the plant studied. In this study, the antifungal activity of cell-wall polysaccharide extracts and flavonoid extracts of *A. radiata* against *Foa* was determined. Flavonoid extracts showed the strongest inhibitory effects on spore germination and on soil population density of *Foa.* HMP from flowers had the greatest inhibitory effect on mycelial growth. The sporulation was strongly inhibited using cellulose-based agar of leaves. Based on these results, these extracts may have important role in biologically based management strategies for the control of fungal diseases in plants.

ACKNOWLEDGEMENTS

We are highly thankful to the members of LP2VM, USTOMB, Oran, Algeria, for their encouragement during the course of this study and the members of the Laboratory of valorization of vegetal resources and food security in semi arid area, south west of Algeria, University of Bechar, Algeria, in providing the fungal strain.

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