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Exploring the potential of using bioactive plant products in the management of *Fusarium oxysporum* f.sp. *albedinis*: the causal agent of Bayoud disease on date palm (*Phoenix dactylifera* L.)

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Abstract

Background: "Bayoud" disease caused by *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) poses a serious threat to date palm (*Phoenix dactylifera* L.) in Morocco. However, research studies performed to discover biological methods to control this disease remain limited. The present study has set objectives to determine antifungal activity of five plants extracts (*Acacia cyanophylla*, *Cupressus atlantica*, *Eucalyptus torquata*, *Nerium oleander*, and *Schinus molle*) against *Foa* and link this effect to their content in polyphenols and flavonoids as well as their antioxidant properties.

Results: Plant extracts showed significant differences ($p < 0.05$) regarding their antifungal activity. The extracts of *E. torquata* and *C. atlantica* showed the strongest antifungal effect resulting in the inhibition of mycelial growth, sporulation, and spore germination in a dose-dependent manner. In addition, there were significant differences among the examined plant extracts in respect to their total polyphenols (1.536–7.348 g GAE/100 g DW), flavonoids (0.986–5.759 g RE/100 g DW), and antioxidant properties measured by Trolox equivalent antioxidant capacity (TEAC) (7.47–38.97 mmol TE/100 g DW) and ferric-reducing antioxidant power (FRAP) assay (8.95–47.36 mmol TE/100 g DW). Moreover, the antifungal potential of plant extracts was found to be moderately to strongly correlated with their polyphenol and flavonoid contents as well as their antioxidant activity, implying that the effective inhibitory activity of these plant extracts is partly due to their richness in antioxidative secondary metabolites.

Conclusion: Our findings shed further light on plants as a-yet-untapped resource of bioactive compounds and constructed the foundation for the development of new biological approaches to best manage Bayoud disease.

Keywords: Plant extract, Antifungal compound, Bayoud, *Phoenix dactylifera* L

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1 Background

Date palm (*Phoenix dactylifera* L.) has long been a major fruit crop in the semi-arid regions of Southern Morocco [1]. In these areas, date production is an important source of food and income for local populations and plays key economic and environmental roles by creating a suitable microclimate for growing other food crops and protecting oases from silting and desertification [2]. Date palm in Morocco is grown over 48,000 ha of traditional and genetically diverse oases, and 20,000 ha of modern plantations cultivating commercial varieties [3]. While the practice of agroforestry-based production system in the former offers opportunities for sustainability, intensive production coupled with cultivar genetic uniformity increase vulnerability to diseases caused by plant pathogens.

"Bayoud" disease is the principal enemy of palm trees that puts at risk the future of the date industry in Morocco [4]. Bayoud is a tracheomycosis caused by the soil-borne fungal pathogen *Foa*. The parasite attacks palms through the roots and ends up colonizing the entire vascular system, leading to wilt and ultimately to date palm death [5]. Since its first appearance in Morocco in the late nineteenth century, the disease has spread throughout all date-producing areas in Morocco and part of Algeria, killing respectively more than 10 and 3 million palm trees in these countries [6]. In addition, this epidemic has wiped out a wide range of cultivars that were highly appreciated by growers, leading to an impoverishment in palm genetic diversity and a decrease in farmer's income [6]. Currently, genetic resistance is the only effective method to control the disease [6, 7]. However, although many Bayoud-resistant varieties have been selected, most of commercial varieties, such as "Mejhool" and "Bouffegous", are highly sensitive and require intensive management against the pathogen. These varieties are principally cultivated in modern farms established not far from Bayoud-infested areas, which constitute a constant risk of its introduction into pathogen-free locations [8]. Thus, there is an urgent need for developing appropriate anticipative management strategies to best manage the disease.

Plants produce a broad spectrum of secondary metabolites, such as alkaloids, phenols, quinones, flavonoids, glycosides, saponins, tannins, and terpenoids [9]. The roles of these bioactive compounds include inducing plant defence system against pathogen attacks during which their concentration in plants depends on pathosystem and environmental conditions [10]. Previous studies have reported that plant-derived products from members of the genera *Acacia*, *Cupressus*, *Eucalyptus*, *Nerium*, and *Schinus* possess strong antifungal activities against a broad spectrum of plant pathogenic fungi [10–14]. Species of these botanical groups are widely distributed worldwide, including date palm-growing areas in Morocco, which present a great potential for their use in the management of Bayoud disease. This could be possible by recycling plant pruning waste of bioactive species as a soil amendment material in palm plantations. The breakdown of plant debris in the soil may result in the release of antimicrobial compounds that would confer protection against infection of date palm roots by *Foa*.

In the perspective of developing bioactive soil amendments using selected plants, the aim of this study is to (i) investigate antifungal effects of aqueous extracts of 5 plant species (*Acacia cyanophylla*, *Cupressus atlantica*, *Eucalyptus torquata*, *Nerium oleander*, and *Schinus molle*) against *Foa*, (ii) to determine their richness in secondary metabolites (polyphenols and flavonoids), and (iii) to measure their antioxidant properties. This work contributes to constructing a more comprehensive understanding of the mechanisms by which plant compounds may affect *Foa* survival and development.

2 Materials and methods

2.1 Plant material

Plant pruning wastes (leaves, branches, twigs etc.) of *Acacia cyanophylla* (ID CRRAE 06/2020), *Cupressus atlantica* (ID CRRAE 07/2020), *Eucalyptus torquata* (ID CRRAE 08/2020), *Nerium oleander* (ID CRRAE 09/2020), and *Schinus molle* (ID CRRAE 10/2020) generated following green urban space management in Errachidia City (South-Eastern Morocco) were collected during October 2019 from public parks and gardens in the former city. The plant species were identified by Dr. Homrani Bakkali, a botanist in National Institute of Agricultural Research and the herbarium specimens for each plant species were prepared and deposited at the same institute. The collected plant material was air-dried under shade, ground to a powder using an electric grinder and stored at room temperature (25 °C) in dark for further use.

2.2 Preparation of polyphenol rich extracts

Stock solutions (10%, w/v) of polyphenol rich extracts were prepared for each plant species using a modified method reported by Hmidani et al. [15]. Briefly, 30 g of plant fine powder were mixed with 300 mL of water at 50 °C using an orbital shaker-incubator during 6 h. The mixture's filtrates were used to assess the antifungal activity, phenolic, and flavonoid contents, as well as antioxidant properties.

2.3 Antifungal activity of plant extracts

2.3.1 Spore germination inhibition assay

The ability of plant extracts to inhibit spore germination of *Foa* was examined using the method described by

Bammou et al. [16]. Fractions of each extract's stock solution (10%) were diluted down to 1%, 2% and 3%. The resulting dilutions were used in the preparation of Potato dextrose agar (PDA) medium. For each plant extract and concentration, an aliquot of 100 μL of *Foa* spore suspension adjusted to 10^5 spores/mL of sterile distilled water (using Malassez's hemocytometer) was spread onto the plates of the supplemented PDA medium. The number of spores germinating out of 50 counted was determined after 24 h of incubation at 25 ± 2 °C. A spore was considered germinating when the length of its germ tube was greater than its smallest diameter. Each treatment included five replicates and a negative control containing un-supplemented media. Using these counts, the percentage of spore germination inhibition by each plant extract was calculated:

$$\text{Inhibition of spore germination } (\%) = \frac{(\text{Nc} - \text{Nt})}{\text{Nc}} \text{x } 100$$

Where
Nc: number of germinating spores in the control
Nt: number of germinating spores in the treatment

2.3.2 Mycelial growth inhibitory assay

Mycelial growth inhibition of *Foa* by plant extracts was assessed using the method described by Sellam et al. [17] with slight modification. Five millimeters of mycelial discs from 7-day-old culture of *Foa* was transferred onto Petri dishes containing PDA medium supplemented with plant extracts as indicated above. Thereafter, the plates were incubated at 25 ± 2 °C for 7 days. Mycelial growth was determined as the average diameter of the colony measured at two right angles. Each treatment included five replicates and a negative control containing unsupplemented media. *Foa*'s mycelial growth inhibition was calculated using the following formula:

$$\text{Growth inhibition } (\%) = \frac{(\text{Dc} - \text{Dt})}{\text{Dc}} * 100$$

Where
Dc: Diameter of colony in the control (mm)
Dt: Diameter of colony in the treatment (mm)

2.3.3 Sporulation inhibition assay

The plates used to perform the mycelial growth inhibition assay were kept in incubation for 10 days under the same conditions to assess the effect of plant extracts on *Foa* sporulation according to the method described by Islam et al. [18]. For each colony, four discs (5 mm) taken along a diameter were transferred into a 1 mL tube of sterile distilled water. The tube was vortexed for 30 s, and the spore concentration of the resulting suspension was determined using a Malassez Counting Chamber. Five counts per suspension were made.

2.4 Antioxidant activity of plant extracts

2.4.1 Measurement of total phenolic compounds

The total phenolic compounds (TPC) in each plant extract were quantified based on the method described by Bouhlali et al. [2]. Briefly, 100 μL of plant filtrate was mixed with 500 μL of 1/10 water-diluted Folin–Ciocalteau's reagent. Subsequently, 400 μL of sodium carbonate solution (7.5% w/v) was added. The mixture was left at room temperature for 60 min, and its absorbance was measured at 765 nm. Gallic acid at various concentrations (0 to 500 mg/L) was used to construct the calibration curve and the results (means of four measurements) were expressed in mg of Gallic acid equivalents per 100 g dry weight (DW) of plant material (mg GAE/100 g DW).

2.4.2 Measurement of flavonoid content

The total flavonoid compounds (TFC) of plant material were measured according to the method previously described by Bouhlali et al. [19]. A sample of 500 μL of plant extract was filled up to 2500 μL using distilled water, mixed with 150 μL of 5% sodium nitrite and 10% aluminum chloride, and incubated for 5 min at room temperature before adding 1 mL of 1 M sodium hydroxide. The final volume of the mixture was made up to 5000 μL using distilled water. A standard curve was constructed using rutin at various concentrations ranging from 0 to 800 mg/L. The absorbance of the resulting solution was measured at 510 nm after homogenization. The results (means of four measurements) were expressed in milligram of rutin equivalents per 100 g DW of plant material (mg RE/100 g DW).

2.4.3 Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay was performed using the method of Re et al. [20]. Aqueous solutions of both ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) (7 mM) and potassium persulphate (2.45 mM) had been mixed and kept in dark at room temperature for 12–16 h to generate ABTS radical cations ($ABTS^+$). Subsequently, the absorbance of the mixture at 734 nm was adjusted to 0.700 ± 0.005 by adding distilled water. Finally, 30 μL of diluted plant filtrate was added to 3 mL of the diluted ABTS radical solution. The mixture was allowed to stand for 6 min at room temperature before the absorbance was measured at 734 nm. Total antioxidant activity (mean of four measurements) was expressed in mmol of Trolox equivalents per 100 g DW (mmol TE/100 g DW) using an aqueous solution of Trolox as a standard curve.

2.4.4 Ferric-reducing antioxidant power assay (FRAP)

Ferric-reducing ability of plant extracts was evaluated according to the method previously described by Benzie and Strain [21]. Briefly, 5 mL of TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM TPTZ in 40 mM HCl), 50 mL of acetate buffer (300 mM, pH 3.6), and 5 mL of $FeCl_3$ (20 mM in water solution) were mixed to prepare the FRAP reagent. For each sample, 10 μL of plant filtrate was added to 2 mL of the FRAP reagent. The resulting solution was incubated at room temperature for 30 min before the absorbance at 593 nm was measured against a blank solution. Total antioxidant activity (mean of four measurements) was expressed in mmol of Trolox equivalents per 100 g DW (mmol TE/100 g DW) using an aqueous solution of Trolox as a standard curve.

2.5 Statistical analysis

Statistical analysis was performed using StatView 5.0 software. One-way analysis of variance (ANOVA) and post hoc Bonferroni tests were carried out to determine significant differences between plant extracts in respect to their antifungal activity, phenolic and flavonoid contents, and antioxidant property with $p < 0.05$ as the significance level. In addition, Pearson's square correlation coefficient (R^2) was calculated to measure pairwise associations among variables.

3 Results

3.1 Evaluation of antifungal activity

The plant extracts affected significantly ($p < 0.05$) and dose-dependently the pathogen's mycelial growth, spore germination, and sporulation. At every concentration, *E. torquata* had the greatest inhibitory effect on fungal mycelial growth followed by *C. atlantica*, *N. oleander*, *S. molle*, and *A. cyanophylla* (Fig. 1). In addition, higher plant extract's concentration induced more potent inhibition of the pathogen's growth, regardless of plant species, though the effect of *A. cyanophylla* extract at the lowest concentration (1%) was not significant.

Moreover, for all plant extracts, except *A. cyanophylla* and *S. mole* at the lowest concentration, increased extract doses resulted in more intense inhibition of *Foa*'s spore germination (Fig. 2). At the dose of 4%, *E. torquata* extract induced the strongest spore inhibition (79.21%), followed by *C. atlantica* (67.97%) and *N. oleander* (50.56%). The other extracts exhibited a weaker anti-germinative effect, ranging between 43.82% for *S. molle* and 16.85% for *A. cyanophylla*. However, *A. cyanophylla* extract at the concentrations of 1% and 2% had no significant effect on *Foa*'s spore germination.

Finally, the same pattern of inhibition was observed in *Foa*'s sporulation with the extracts of *E. torquata* and *C. atlantica*, causing the greatest sporulation reductions

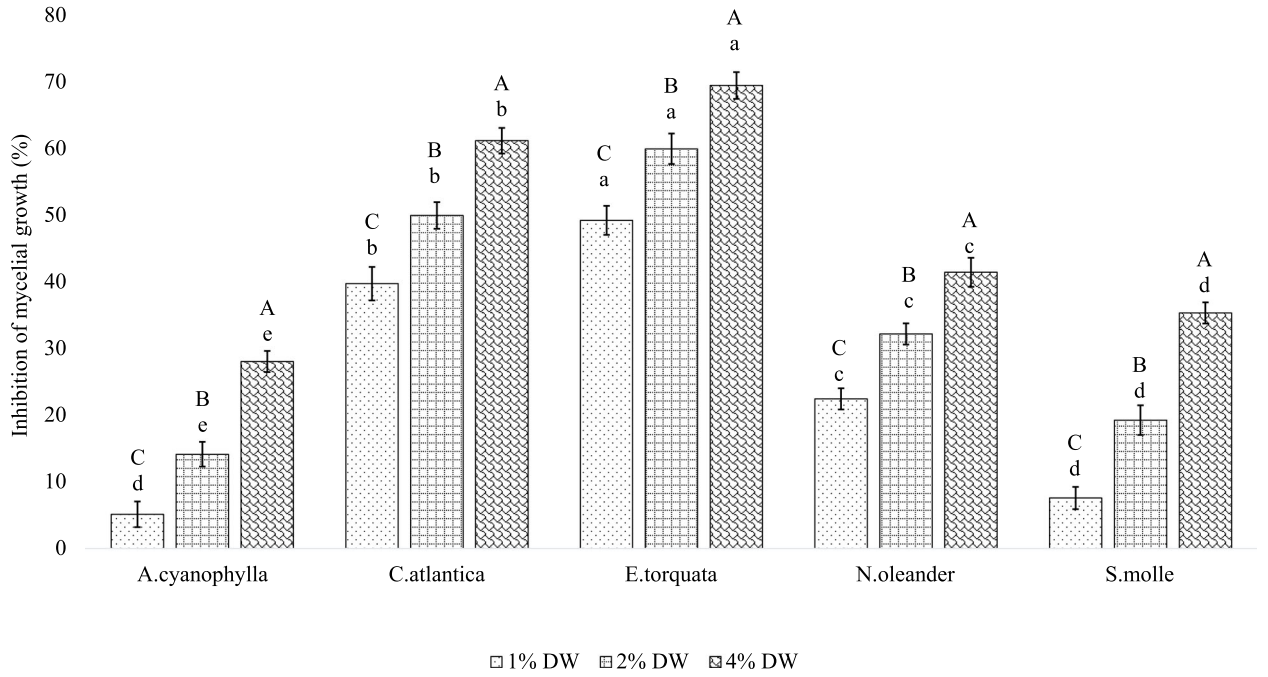

Fig. 1 Effect of different plant DW percentage on *Foa* mycelial growth. Plant species and extract concentration had significant effects on the percentage of radial growth inhibition (one-way ANOVA, $p < 0.05$). Each bar represents the mean of five replicate plates per concentration. Within each plant species, bars followed by the same uppercase letters indicate no significant differences among extract concentrations, while within each concentration; bars followed by the same lowercase letters indicate no significant differences among plant species (Bonferroni tests, $p < 0.05$). Error bars represent standard errors

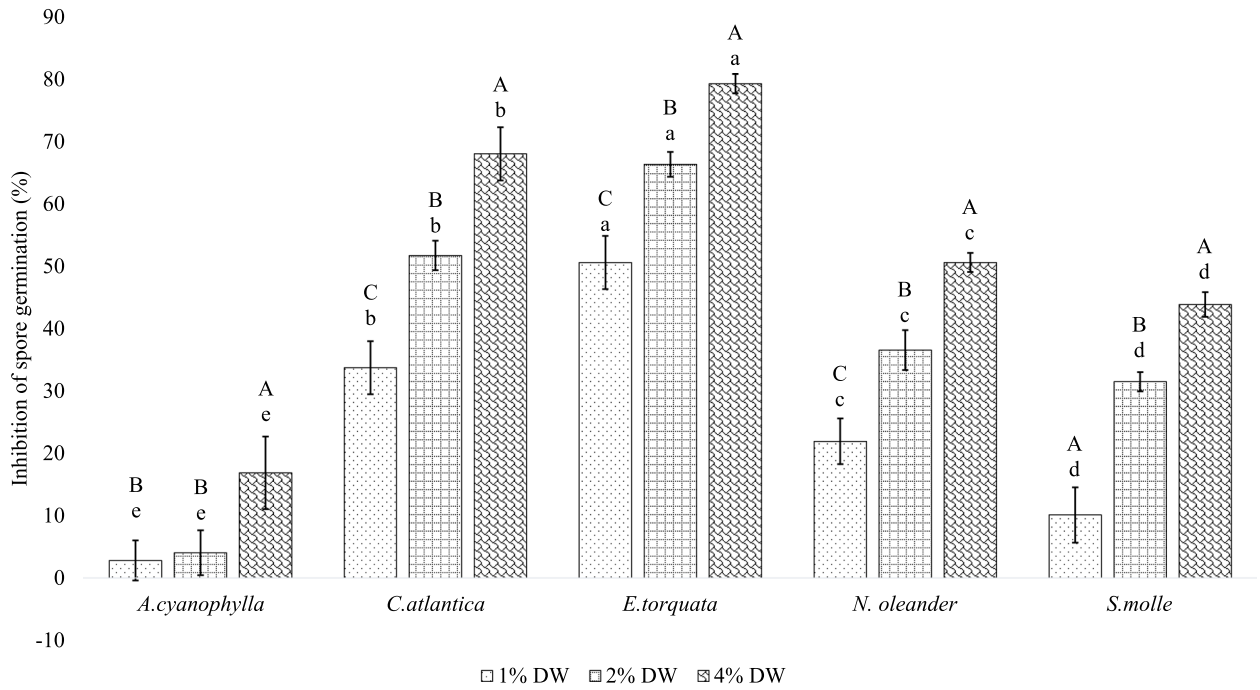

Fig. 2 Effect of different plant DW percentage on *Foa* spore germination. Plant species and extract concentration had significant effects on the percentage of inhibition of spore germination (one-way ANOVA, $p < 0.05$). Each bar represents the mean of five replicate plates per concentration. Within each plant species, bars followed by the same uppercase letters, indicate no significant differences among extract concentrations, while within each concentration; bars followed by the same lowercase letters indicate no significant differences among plant species (Bonferroni tests, $p < 0.05$). Error bars represent standard errors

(44.97% and 37.32% respectively at the concentration of 4%), followed by the extract of *N. oleander* that lowered *Foa* sporulation by 23.92% and 14.35% at the doses of 2% and 4% respectively (Fig. 3). In contrast, the extracts of *A. cyanophylla* and *S. molle* at the lowest concentration (1%) fostered *Foa* sporulation respectively by 30.62% and 10.52%. The extracts of *N. oleander* (at 1%), *A. cyanophylla* (at 4%), and *S. molle* (at 4%) showed no significant effect on *Foa* sporulation.

3.2 Total phenolic and flavonoid contents

The plant extracts displayed significant differences ($p < 0.05$) concerning their total polyphenols and flavonoids level (Fig. 4). Specifically, total phenolic content ranged between 7.348 g GAE/100 g DW in *E. torquata* and 1.536 g GAE/100 g DW in *A. cyanophylla*. Similarly, *E. torquata* had the greatest total flavonoids content (5.759 g RE/100 g DW), followed by *C. atlantica*, *S. molle*, *A. cyanophylla*, and *N. oleander* which showed the lowest concentration of flavonoids (0.986 g RE/100 g DW).

3.3 Evaluation of antioxidant activity

The antioxidant property of the studied plant extracts, as measured by both TEAC and FRAP assays, showed significant differences ($p < 0.05$) (Fig. 5) and strong correlations ($R^2 \geq 0.974$, $p < 0.01$) with total phenolic and flavonoids contents (Table 1). TEAC values ranged between 7.47 and 38.97 mmol TE/100 g DW and were strongly correlated ($R^2 = 0.999$, $p < 0.01$) with those of FRAP which varied between 8.95 and 36.43 mmol TE/100 g DW (Table 1). Finally, phenolics and flavonoids were found to be correlated with antifungal activity, especially mycelial growth inhibition ($R^2 \geq 0.669$, $p < 0.01$) and spore germination inhibition of *Foa* ($R^2 \geq 0.54$, $p < 0.01$) (Table 1).

4 Discussion

Organic soil amendment contributes greatly to fostering plant biomass and health [22]. For example, the use of compost improves physico-chemical properties and structure of soil, enhances the diversity of microbial communities, and enriches potentially the taxa which play major role in suppressing various fungal and bacterial plant diseases [23]. In addition, the use of organic waste with biocidal effects in composting process can offer an additional way of increasing compost's impact on plant pathogens. Our work provides strong evidence that the extracts of some plants (trees and bushes) possess potent inhibitory effect on *Foa*. These plants produce a great deal of pruning biomass that can be utilized in the production of bioactive compost as a soil amendment for sustainable control of Bayoud in date palm groves.

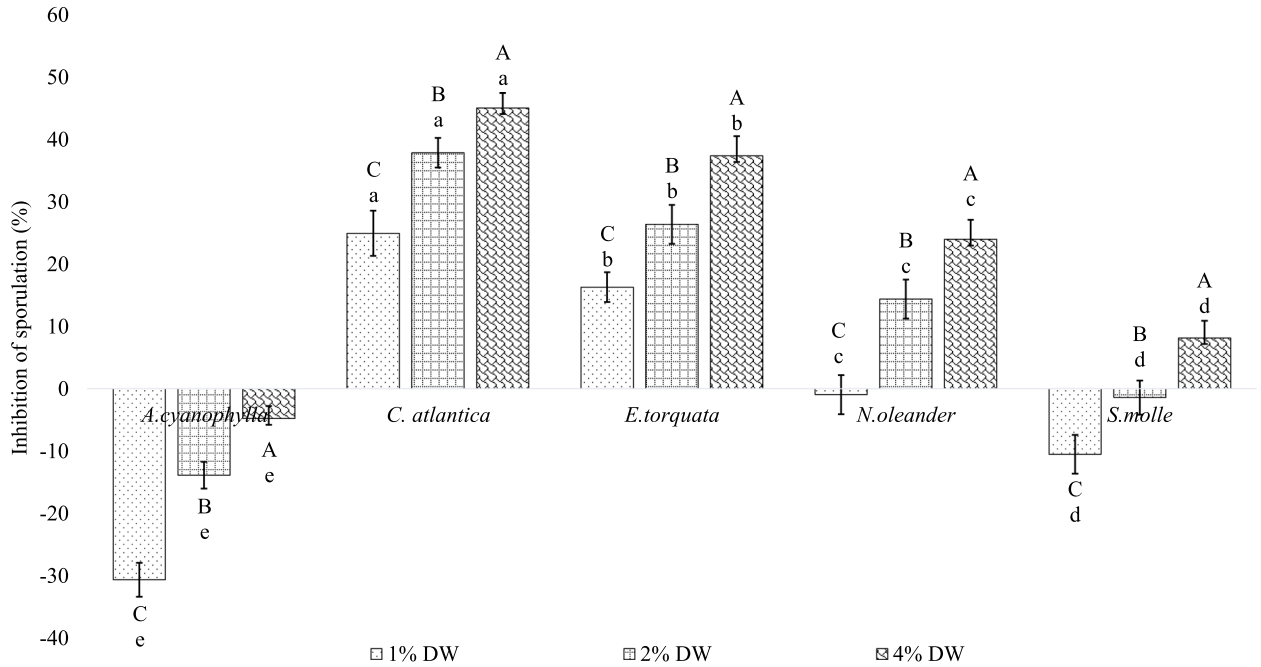

Fig. 3 Effect of different percentage of plant DW on *Foa* sporulation. Plant species and extract concentration had significant effects on the percentage of sporulation inhibition (one-way ANOVA, $p < 0.05$). Each bar represents the mean of five replicate plates per concentration. Within each plant species, bars followed by the same uppercase letters indicate no significant differences among extract concentrations while within each concentration, bars followed by the same lowercase letters indicate no significant differences among plant species (Bonferroni tests, $p < 0.05$). Error bars represent standard errors

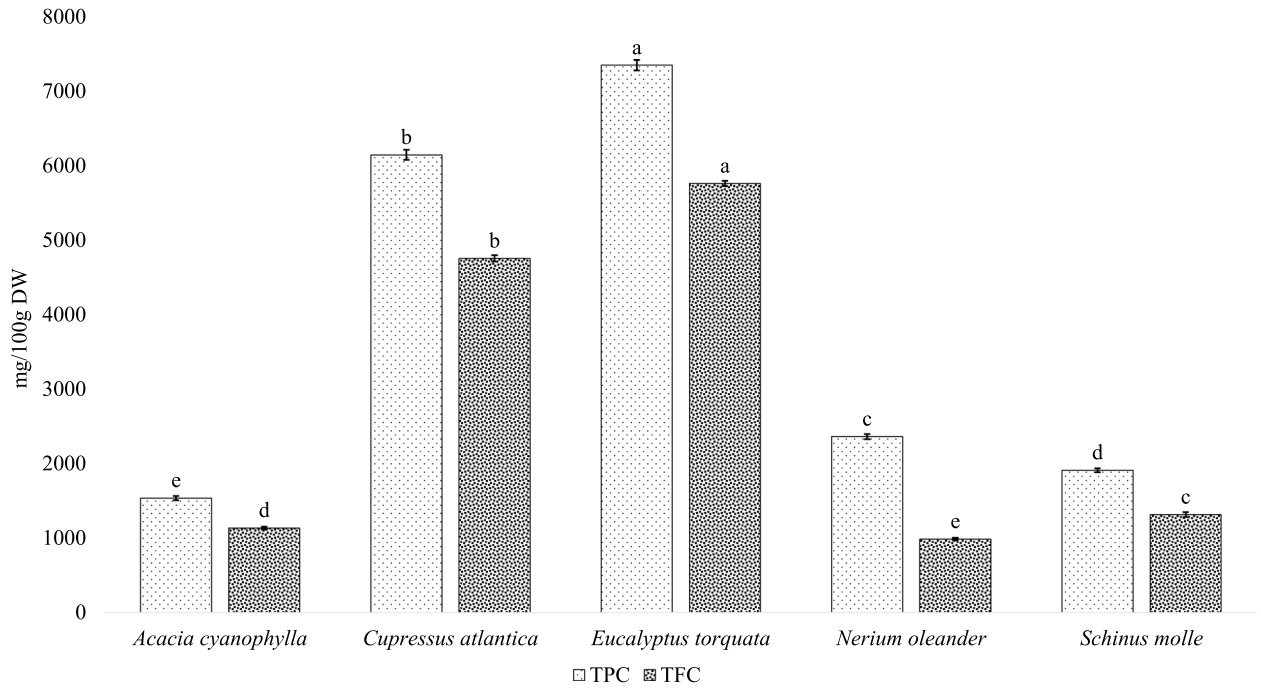

Fig. 4 Total polyphenols (TPC) and flavonoids (TFC) content in plant extracts. Plant species had significant effects on TPC and TFC (one-way ANOVA, $p < 0.05$). Each bar represents the mean of four replicate measurements. Within each secondary metabolite group (TPC or TFC), different letters above bars indicate significant differences among plant species (Bonferroni tests, $p < 0.05$). Error bars represent standard errors

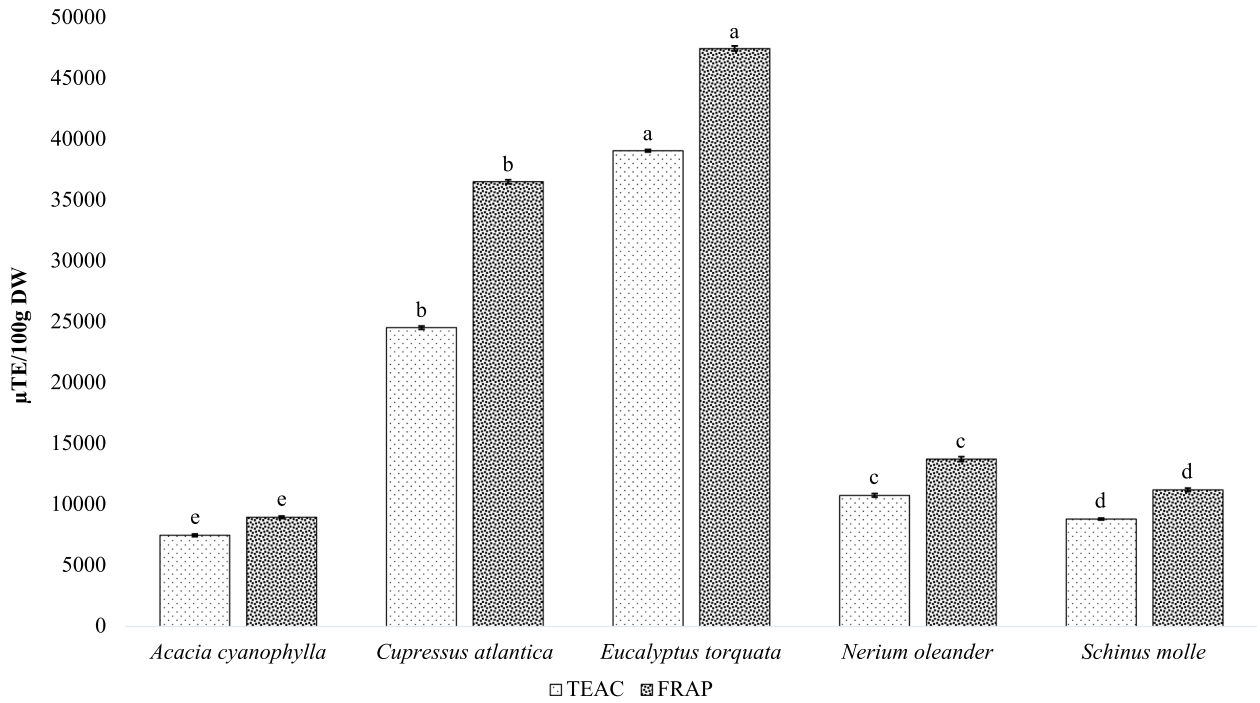

Fig. 5 Antioxidant activity of plant extracts based on TEAC and FRAP assays. Plant species had significant effects on TEAC and FRAP (one-way ANOVA, $p < 0.05$). Each bar represents the mean of four replicate measurements. Within each antioxidant activity indicator (TEAC or FRAP), different letters above bars indicate significant differences among plant species (Bonferroni tests, $p < 0.05$). Error bars represent standard errors

Bayoud is an incurable disease of date palm trees caused by *Foa* [24]. Its control is based on the combination of many approaches, including prophylactic measures, cultural practices, and intensive management of the pathogen's populations in order to prevent its growth and establishment. The use of natural biocidal products can reinforce this strategy. In this study, we demonstrate that the plants *E. torquata* and *C. atlantica* have strong antifungal activities, suggesting that their incorporation in compost may contribute to the management of *Foa*'s community in soil. Specifically, the capacity of these plant extracts to decrease sporulation and inhibit spore germination in the pathogen population can be detrimental to the onset of the disease and the spreading of fungal spores which are considered the primary inoculum for the infection of date palm by *Foa*. Additionally, the finding that *E. torquata* and *C. atlantica* filtrates affect strongly the pathogen's mycelial growth suggests that the use of these two bioactive plant species in compost can lead to a reduced saprophytic development of *Foa* and thus to a decreased soil infectious potential. In overall, our work laid the foundation for developing novel strategies to the control of Bayoud disease in palm groves based on the use of bioactive plant wastes.

Table 1 Correlation between polyphenols, flavonoids, antioxidant, and antifungal activities assays

	TPC	TFC	FRAP	TEAC	PRGI	PISG	PSI
TPC	1						
TFC	0.978**	1					
FRAP	1**	0.978**	1				
TEAC	0.999**	0.974**	0.999**	1			
PRGI	0.719**	0.669**	0.731**	0.724**	1		
PISG	0.606**	0.54**	0.622**	0.616**	0.925**	1	
PSI	0.568**	0.527**	0.579**	0.552**	0.864**	0.835**	1

TPC total phenolic content, *TFC* total flavonoid content, *FRAP* ferric-reducing antioxidant power, *TEAC* Trolox equivalent antioxidant capacity, *PRGI* percentage of radial growth inhibition, *PISG* percentage of inhibition of spore germination, *PSI* percentage of sporulation inhibition, **$p < 0.01$

A large body of work has reported that the essential oils and extracts drawn from some Eucalyptus species showed a dose dependent antifungal activity against seven *Fusarium* species, namely, *F. oxysporum*, *F. solani*, *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, *F. graminearum*, and *F. sporotrichioides* [12, 25–27]. In addition, different species of the genus *Cupressus* had a strong antifungal effect against the mycelial growth of *F. oxysporum*, *F. solani*, *F. culmorum*, *F. equisiti*, *F. verticillioides*, *F. nygamai*, and *F. subglutinans* that was positively correlated with the applied dose [11, 28, 29]. There is a general consensus that the antifungal effect of plants might be associated with their content of secondary metabolites. Our results are in line with previous

findings as plant antifungal activity was moderately to strongly correlated with phenolic and flavonoid contents ($R^2 \geq 0.527$) [30, 31]. In fact, *E. torquata* and *C. atlantica* that showed the greatest concentrations of polyphenols and flavonoids induced the strongest inhibition effects. In contrast, the relatively weaker inhibitory effects exhibited by the other plants may be attributed to low amounts and/or bio inactive composition of their secondary metabolites. These findings corroborate also with those reported by Daayf et al. [32] who found a significant increase in the synthesis of phenolic compounds by date palm-resistant and susceptible cultivars following infection by *Foa*, indicating a possible role of phenolic compounds in the inactivation of the pathogen upon infection.

Diverse mechanisms can underlie fungal toxicity of plant secondary metabolites including the inhibition of enzymes by oxidized compounds, probably by reactions with sulfhydryl groups or by less specific interactions with proteins, interference with cell wall synthesis, alteration of cell permeability, interference with electron transport, absorption of nutrients, adenosine triphosphatase, and other cell metabolic pathways [33–35]. Bioactive compounds found in plant extracts may act simultaneously or separately, their modes of action can be similar or different, and their targets include metabolic pathways involved in mycelial growth, sporulation, and spore germination, which result in effective antifungal activity [36].

Moderate to strong correlations ($R^2 \geq 0.552$, $p < 0.01$) were also found between antioxidant activity and the inhibition of fungal growth, spore germination, and sporulation. As a matter of fact, antioxidants have been reported to play a major role in increasing treatment effectiveness against plant fungal pathogens when combined as adjuvants with fungicides [37]. Their effect may be due to increased fungal cell permeability that subsequently allows greater fungicide diffusion into cells or to reduced intracellular fungicide oxidation that results in stronger fungitoxicity [38, 39]. In agreement with these investigations, our results suggest that the antifungal properties of plant extracts coupled with their antioxidant activity are responsible for potent inhibitory effect against the causal agent of the Bayoud disease (*Foa*).

Regardless of their mechanisms of action, the present work demonstrated that extracts from *E. torquata* and *C. atlantica* impede the mycelial growth, spore production, and spore germination of *Foa* under controlled laboratory conditions. However, because of the lack of field data, the use of these plants in an integrated strategy for Bayoud management requires further research. Specifically, related future investigations must address the effect of raw plant material in compost on the pathogen, microbial community, and structure, especially beneficial taxa, physical, and chemical properties of soil, as well as date palm growth and development.

5 Conclusion

Taken together, our data showed that extracts from *E. torquata* and *C. atlantica* waste produced during trimming of green urban spaces have a strong antifungal activity against *Foa*. The use of these plants as composted material may offer a better alternative to costly and environmentally unfriendly fungicides. Studies are underway with the objective of developing and implementing a compost-based biological approach to control Bayoud disease in Moroccan date palm groves. Further studies are recommended to evaluate the stability of antifungal effect retained by these plant species after composting process.

Abbreviations

TEAC: Trolox equivalent antioxidant capacity; FRAP: Ferric-reducing antioxidant power; TPTZ: 2,4,6-tripyridyl-s-triazine; TE: Trolox equivalent; DW: Dry weight; GAE: Gallic acid equivalent; RE: Rutin equivalent; *Foa*: *Fusarium oxysporum* f. sp. *Albedinis*; TPC: Total phenolic content; TFC: Total flavonoids content; PRGI: Percentage of radial growth inhibition; PISG: Percentage of inhibition of spore germination; PSI: Percentage of sporulation inhibition

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Authors' contributions

E.D.T.B., A.E., and R.M. conceived the presented idea. A. E and R. M verified the analytical methods and supervised the findings of this work. E.D.T.B., M.D., and H.B. carried out the experiment. E.D.T.B, M.D., and A.E. wrote the manuscript after discussion of the results with all authors. All authors have read and approved the manuscript for submission.

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Availability of data and materials

The available data is included in the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

None

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