Full Length Research Paper

High-performance liquid chromatography (HPLC) Identification of five new phenolic compounds involved in the olive tree (*Olea europea* var. Sigoise) resistance to *Verticillium dahliae*

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Verticillium wilt is a vascular disease caused by *Verticillium dahliae* which represents a serious threat for olive growing in Algeria. Many studies have shown the potential involvement of phenolic compounds in the reaction of plants to pathogens. Our study shows that the presence of Verticillium wilt induces a high production of polyphenols in infected olive trees compared to uninfected ones. The presence of high concentrations of flavonoids (3.45%) and alkaloids (0.44%) in the infected trees suggests that flavonoids and alkaloids may play a role in the olive tree resistance to verticillium wilt. The high performance liquid chromatography (HPLC) analysis showed the presence of five phenolic compounds: oleuropeine, luteonine, catechin, and for the first time verbascoside, apigenine-7-glycoside and some derivatives hydroxycinnamic compounds. These substances are good resistance markers and should help to make efficient strategies for the biocontrol of verticillium wilt.

Key words: *Olea europea* var. Sigoise, verticillium wilt, *Verticillium dahliae*, phenolic compounds, resistance, HPLC.

INTRODUCTION

The olive tree (*Olea europea)* is in full expansion in many countries. This tree, grown for its fruit, is sensitive to a great number of diseases such as Verticillium wilt which causes considerable losses in productivity. In recent years, deteriorations of the olive tree due to Verticillium wilt have worsened. This disease can propagate very quickly due to the large dissemination of the disease-causing agent and its long survival in the ground (Serrhini and Zeroual, 1995). Plants have complex mechanisms to protect themselves against pathogens. Phenolic secondary metabolites, which are considered as involved in the special organoleptic properties of oil, have been shown to

play a role in the resistance of some olive (*Olea* europea L.) varieties to oil autoxidation (Botia et al., 2001). In addition, some reports (Ruiz-Barba et al., 1991; Marsilio and Lanza, 1998) have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and fungi like *Phytophthora* (Del Rio et al., 2003). Similarly, the phenolic metabolism of the olive tree is considered as a plant-response to the infection by *Verticillium dahliae* (Daayf, 1993). Thus, increasing the endogenous levels of these secondary metabolites can improve the resistance properties of the plant and be

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used as a natural alternative for preventing plant diseases.

Methods for detecting and recognizing phenolic compounds rely mainly on chromatographic separation, using the HPLC analysis (El Modafar et al., 1993; El Modafar and El Boustani, 2001) which allows their successful identification.

In Algeria, little is known about the resistance of *Olea europea* var. Sigoise. The aim of this work was to detect the presence of phenolic compounds potentially involved in the resistance of *Olea europea* var. Sigoise to verticillium wilt and determine the chemical nature of these compounds using HPLC method.

MATERIALS AND METHODS

Isolation of the pathogen

To isolate the pathogen, sections from the stem of infected olive tree were superficially disinfected with 95% ethyl alcohol for 30 s, rinsed three times with sterile distilled water, dried on sterile filter paper and plated on Potato Dextrose Agar (PDA) medium amended with streptomycin (100 p.p.m.) and incubated at 25°C in the dark, for two weeks (Tsror et al., 1998). The isolates were identified as *V. dahliae*, based on the description of Hawksworth and Talboys (1970).

Plant material

The study was carried out with 4 years old infected and uninfected olive trees (*Olea europea* var. Sigoise) grown in a commercial plantation, located in Tlemcen, Algeria. The stems were washed, dried with paper towel, cut into approximately 1 cm squares, dried in an oven at 60°C for at least 24 h, crushed and degreased in a Soxhlet, before use. All analyses were conducted in triplicate, and the results were based on dry weight per 100 grams of sample.

Yields extraction

Tannins extraction

Powdered material (100 g) was extracted at 4°C using 500 ml of a mixture of acetone-water (25/45, v/v) for 4 days (Bruneton, 1999). The extracts were filtered under vacuum through filter paper and the acetone was evaporated under reduced pressure. Subsequently, dichloromethane (2 × 25 ml) was used for the extraction of lipids and pigments from the aqueous extracts using a separating funnel. Afterward, the aqueous phase was extracted with 25 ml of ethyl acetate. This process was repeated four times. After filtration, the organic phases (ethyl acetate) containing tannins were recovered and concentrated to dryness under vacuum, using a rotary evaporator. The residue obtained after evaporation was kept at 4°C and used for further investigation.

Flavonoids extraction

A quantity of 10 g of dried material was extracted with 100 ml of methanol and 5 g of calcium carbonate by boiling for 1 h (Danguet and Foucher, 1982). After filtration, through Whatman filter paper,

the methanol was evaporated under reduced pressure to eventually give an aqueous extract. Subsequently, the dry extract was recovered with 50 ml of boiling water. The aqueous extract was filtered and subjected to solvent fractionation, firstly with diethyl ether, then ethyl acetate and finally n-butanol, using separating funnel (pyrex). All fractions were concentrated, dried to constant weight in an oven at 45°C and kept at 4°C.

Extraction of alkaloids

An amount of 10 g of dried sample was mixed with 250 ml of HCl 2% and 110 ml of ethyl acetate. After cold soaking (4 °C) for 10 h, the mixture was filtered and basified with NH_4OH. The basic aqueous phase was extracted twice with ethyl acetate until no alkaloid was detected in the aqueous phase. The alkaloid residue was obtained by decantation and evaporation of the organic phase (Bruneton, 1999).

Crude extraction

The dried powder of olive stem (10.0 g) was extracted in triplicate, using EtOH (96% v/v) at room temperature, under stirring. The aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic et al., 2008).

Total phenol content analysis

The total phenolic content (TPC) was determined using Folin Ciocalteu reagent (Singleton and Rossi, 1965). Briefly, 5 µl of the crude extract was added to 1.70 ml of distilled water and 300 µl of Folin Ciocalteu reagent (previously diluted 3-fold in distilled water). The mixture was allowed to stand for 3 min, then 0.5 ml of Na_2CO_3 (20%, w/v) was added to the mixture. After 1 h in the dark at room temperature, the absorbance was measured at 760 nm. The results were expressed as gallic acid equivalents (mg of gallic acid/mg dry weight extract).

HPLC analysis

HPLC analyses of polyphenols were performed by RP-HPLC coupled with diode array detection DAD using a Symmetry C18 column (5 µm, 100 × 4.6 mm) (Waters ref. WAT186002616) equipped with a membrane degasser, a Rheodyne injector 7725i (La Jolla, the USA) and a binary pump 1525 (flow rate, 0.75 mL min^{-1}; T = 30 °C; volume injected: 10 µl). The mobile phase was made up of two solvents: methanol (A) and acidified distilled water with acetic acid 1% (v/v) (B). The gradient was performed in three steps: step 1, from 5 to 45 % of A in B for 25 min; step 2, from 45 to 100 % of A in B for 3 min; step 3, isocratic to 100 % of A for 2 min. The total time of elution was 35 min. Each polyphenol was identified by its retention time and UV-vis spectrum after comparison with standards. Empower software was used to control the device.

RESULTS

Identification of *Verticillium dahliae*

The symptoms from infected plants were very similar to those described for verticillium wilt of olive tree, including foliar chlorosis and necrosis, stunting and vascular
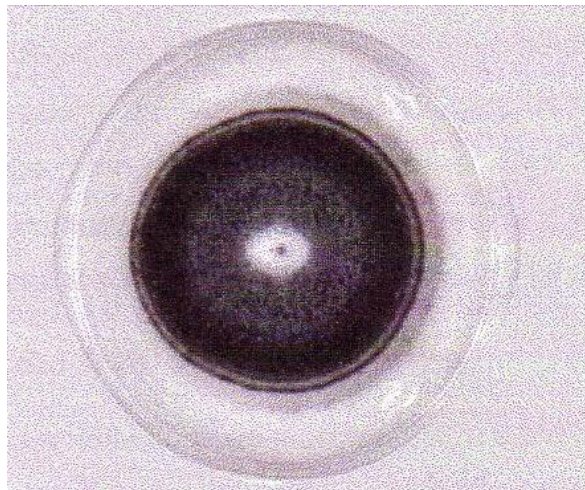
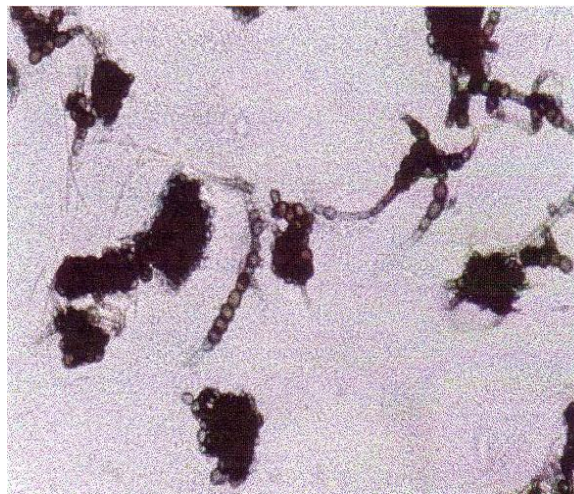**Figure 1.** Colony type of *Verticillium dahlia.*

Figure 2. Microsclerotia of *V. dahliae* on PDA medium (40 ×).
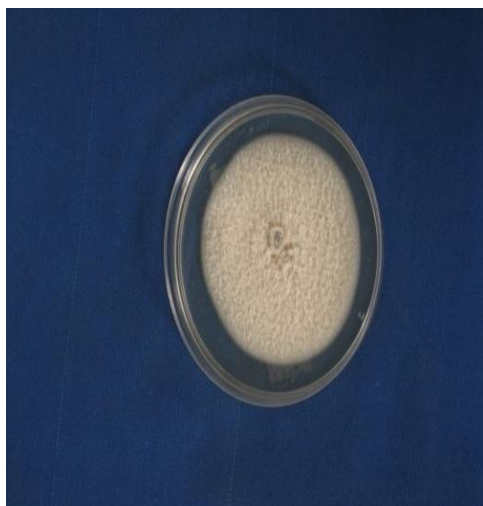

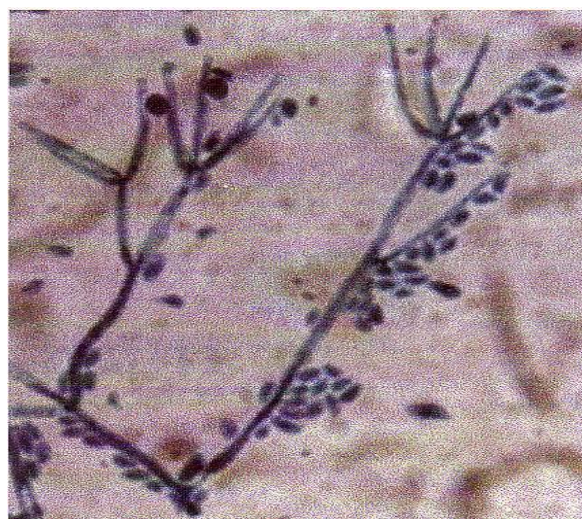**Figure 3.** Hyaline colony of *V. dahlia.*

Figure 4. Mycelium and conidiophores of *V. dahliae (40 ×)*

discoloration.

Fungal colonies were black (Figure 1) and produced microsclerotia characteristic of *V. dahliae* (Figure 2). The color of the colony change from black to white after many transfers (Figure 3). The presence of single-celled oval conidia and distinct verticillate conidiophores confirmed the isolate of *V. dahliae* (Figure 4).

Total phenol content

Figure 5 shows the total phenol content in a whole stem from uninfected and infected olive plants. The total phenol contents in the whole stems of the infected plants (780 mg/g) was practically double that measured in the uninfected plants (440 mg/g).

Yields extraction

The yields of tannins, flavonoids and alkaloids are presented in Figure 6. The yield of tannins in whole stem from infected and uninfected olive plants was 0.1 and 0.3 %, respectively.

The yield of flavonoïds and alkaloids was higher in infected plants: (3.45 % and 2 %) for flavonoids and (0.44 % and 0.04 %) for alkaloids content in uninfected and infected plants respectively.

Identification of phenolic compounds by HPLC
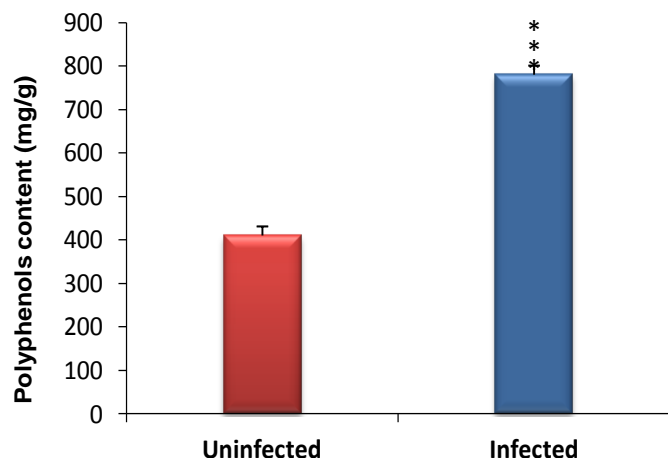
The data (retention time, λ max in the visible region, and

Figure 5. Polyphenols content of uninfected and infected olive-tree.

Figure 6. Tannins, flavonoids and alkaloids contents of uninfected and infected olive-tree.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax) and tentative identification of phenolic compounds in whole stem and leaf of olive-tree.

Peak	Rt (min)	Λmax (nm)	Tentative identification
1	10,364	288	Flavonoid (N.D.)
2	14,541	288	N.D.
3	15,084	327	N.D.
4	15,680	322	Hydroxycinammic derivative
5	15,710	331	Verbascoside
6	16,748	349	Luteoline-7-glucoside
7	17,117	288	Flavonoid (N.D.)
8	17,196	322	N.D.
9	18,598	280	Oleuropein
10	18,710	337	Apigenine-7-glucoside
11	20,000	332	N.D.
12	20,495	281	Catechin
13	21,000	330	N.D.
14	21,696	287	Flavonoid (N.D.)
15	22,000	333	N.D.

tentative identification) obtained for the phenolic compound peaks in the HPLC-DAD analyses are presented in Table 1 and Figure 7a. HPLC studies point to five phenolic compounds determined in olive stem extracts: oleuropein (t_R = 18.59 min, maximum absorbance at 280 nm), luteolin-7-glucoside (t_R = 16.74 min, maximum absorbance at 349 nm), verbascoside (t_R = 15.71 min, maximum absorbance at 290 nm), apigenine-7-glucoside (t_R = 18.71 min, maximum absorbance at 263 and 337 nm) and catechin (t_R = 20.49 min, maximum absorbance at 281 nm) (Figure 7b). The retention time and absorption spectrum (obtained by means of a UV/vis DAD) were identical to those obtained for the corresponding standards.
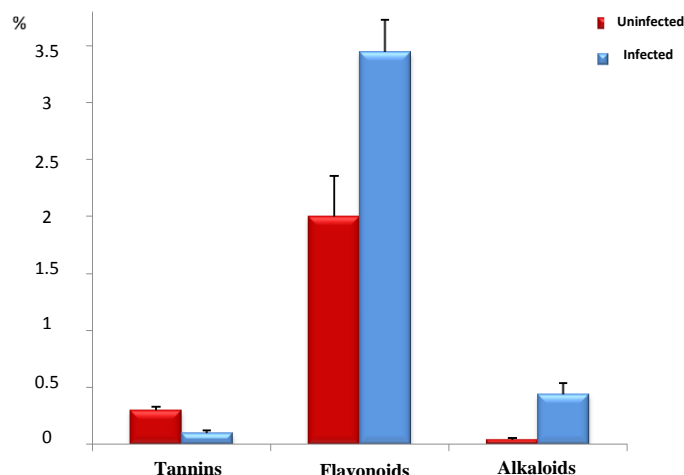
DISCUSSION

V. dahliae was isolated from the whole stem and leaf, presenting the typical symptoms of Verticillium wilt, according to Vigouroux (1975). These include xylem browning and leaf rolling to their inner face, with color changing from yellow to brown. Then, the dry leaves became brittle and fell.

V. dahliae isolate produced black microsclerotia as resting structures and was identified according to the published descriptions of Hawksworth and Talboy (1970) and Goud et al. (2003). The color of colonies mainly changed from black to white on PDA medium. The hyaline isolates lost their ability to produce microsclerotia, most probably due to many transfers and long maintenance in several laboratories under different conditions (Smith, 1965).

The obtained results show that total polyphenols were present in infected olive trees at higher levels than in uninfected olive trees; and the difference was statistically significant ($p < 0.001$). However, the difference in tannin and flavonoid yields was significant ($p < 0.05$).

This is certainly due to the type of analysis which shows that the total polyphenols synthesis was better after *V. dahliae* infection. The TPP content obtained confirms this idea because the TPP analysis gives a quantitative result whereas the yield gives a qualitative one.

In a previous work, El Boustani et al. (1998, 2nd International Electric Conference of Synthetic Organic Chemistry, personal communication), showed that inoculation of the olive twigs by a conidial suspension of *V. dahliae* resulted in important modifications in flavone and phenol levels. These findings suggest that the first step of the response mechanism to infection in olive plants is a rapid accumulation of phenols at the infection site, thus reducing or slowing the pathogen growth, as
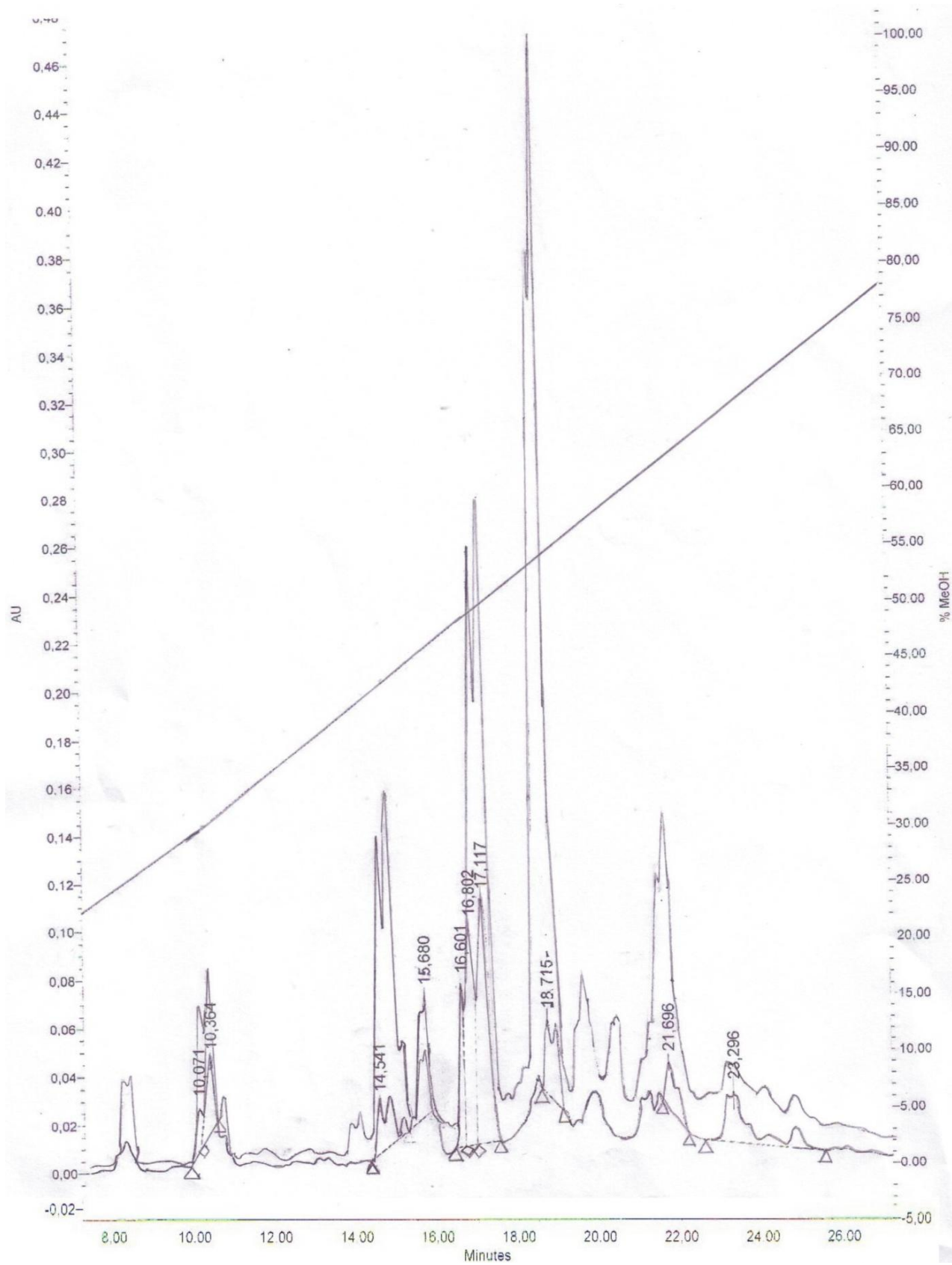
Figure 7a. Chromatogram (zoom) recorded at 350 nm showing the phenolic compounds profiles identified and not identified of olive-tree stem (*Olea europea* var. Sigoise).
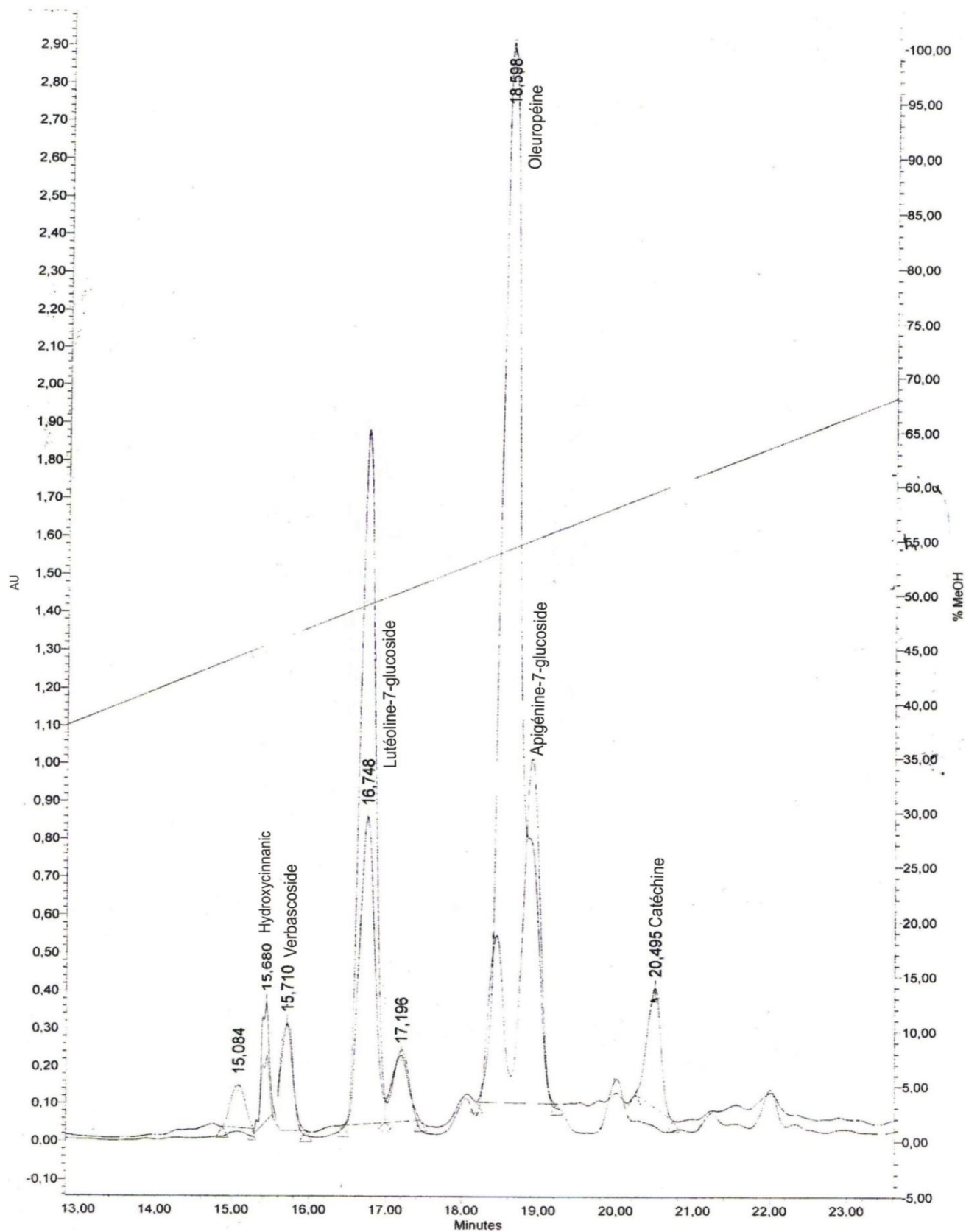
Figure 7b. Chromatogram (zoom) recorded at 350 nm showing the phenolic compounds profiles identified of olive-tree stem (*Olea europea* var. Sigoise).

reported for other vegetal materials (Matern and Kneusel, 1988; Del Rio et al., 2004). Therefore, in contrast to flavonoids and alkaloids, the tannin content of the uninfected sample was higher than that of the infected one. This suggests that tannins, which are constitutive substances, mainly present in the bark, were synthesized and used initially by the olive plant in its defense against pathogens before transforming into flavonoids. Tannins were found in tropical plants at high concentrations, by Makkar and Becker (1998), because their synthesis is promoted by light, whereas flavonoids and alkaloids are inducible compounds, since they are not produced directly during the photosynthesis, but result from further chemical reactions. Our results are in agreement with those of Corbaz (1990), whose study results show that the young leaves at the cotton plant are often resistant to *V. dahliae* and become sensitive as they grow older. This phenomenon might be ascribed to the inhibition of mycelium growth in young tissues, which contain higher concentrations of substances such as tannins than those in the old leaves.

In selective extractions, that concentrations of alkaloids in infected olive plants were higher than in uninfected ones also suggest that alkaloids may have a role in the response mechanism of olive plants to Verticillium wilt. Williams and Charles (2006) showed that alkaloids have antibiotic properties; these nitrogenous substances, synthesized from amino acids, salt out the cyanhydric acid when the plants are damaged (Hopkins, 2003). Our results show that the concentration of flavonoids was higher in infected olive trees compared to the uninfected ones. Similar results were found in potato plants inoculated with *V. dahliae*, which induced a production of flavonol glycosides two to three times higher than in the uninoculated plant (El Hadrami et al., 2011). Likewise, Daayf (1993) showed that infection of the cotton plant by *V. dahliae* stimulated the accumulation of phenolic compounds (flavones and flavonols). All these results highlighted that tannins, as constitutive substances, should be first used by the olive tree in their reaction to pathogen attacks; whereas flavonoids are inducible molecules, since they are obtained from tannin degradation.

Our main findings were that the HPLC analysis revealed the presence of three new phenolic compounds in infected olive stems, namely verbascoside, apigenine-7-glycoside and hydroxycinnamic derivatives which have, to our knowledge, never been isolated before, from this variety of olive tree.

Conclusion

This study strongly suggests that some of the secondary metabolites, especially flavonoids and alkaloids, present in olive plants act as phytoanticipins and/or phytoalexins in the plant's natural defense mechanism, as it has been established for other phenolic secondary metabolites in different plant materials infected by pathogenic fungi. The HPLC analysis revealed the presence of three new phenolic compounds, namely verbascoside, apigenine-7-glycoside and hydroxycinnamic derivatives.

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