Journal of Research in Ecology

An International Scientific Research Journal

ISSN No: Print: 2319 –1546; Online: 2319– 1554

Original Research

In vitro evaluation of the anti-fungal activity of the crude *Rosmarinus eriocalyx* Jord. & Fourr. extracts in batch mode

Authors:
Meraghni M[1], Bordjiba O[1] and Rahmouni N[2]

Institution:
1. Plant Biology and Environment Laboratory, 1: Badji Mokhtar Annaba University, P.O. Box 12, Annaba Algeria

2. Unité de Recherche et Valorisation des Ressources Naturelles, Molécules Bioactives et Analyse Physico chimique et Biologiques, Université des Frères Mentouri Constantine 1, Algérie

Corresponding author:
Meraghni M

ABSTRACT:

Four pathogenic fungal species of *Alternaria* isolated from tomato plants were subjected to the effects of foliar and methanolic extract of *Rosmarinus eriocalyx* Jord. & Fourr. Batch mode (discontinuous growth) for 120 h, was used to assess the growth of two fungal species according to three parameters *viz*., pH, dry weight of inoculum and optical density. The kinetics of the parameters analyzed showed lower development of most fungal strains and a low microbial biomass in the presence of the plant extract compared with those of the controls, or even with those obtained in the presence of synthetic chemical pesticide. The most promising results were obtained with *Alternaria alternata* and *Alternaria arborescens*; these two species seem the most sensitive to the leaf and floral extracts of the tested plant. This reductive effect of the natural extract on the biomass of fungal strains and its influence on decreasing the pH during the growth phase corresponds with the composition of the rosemary leaves extracts. A major peak, representing rosmarinic acid, was revealed by ESI LC-MS /MS and is compatible with the pH decline. The foliar and floral extracts of *R. eriocalyx* indicated significant antifungal properties. The results warrants further investigations and the testing of other phytopathogenic fungal species responsible for serious damage to crops. Identifying the natural bioactive substances in plant extracts should provide alternatives to the use of synthetic pesticides.

Keywords:

Antifungal activity, *Alternaria*, Foliar and floral extract, *Rosmarinus eriocalyx*, Batch mode.

Article Citation:
Meraghni M, Bordjiba O and Rahmouni N
In vitro evaluation of the anti-fungal activity of the crude *Rosmarinus eriocalyx* Jord. & Fourr. extracts in batch mode
Journal of Research in Ecology (2018) 6(1): 1481-1491

Dates:
Received: 26 Feb 2018 **Accepted:** 08 Mar 2018 **Published:** 24 Mar 2018

Web Address:
http://ecologyresearch.info/documents/EC0532.pdf

Journal of Research in Ecology

INTRODUCTION

Cryptogamic diseases affect plant production and cause considerable losses in the economy (Fletcher *et al*., 2006; Abdel-kader *et al*., 2012). *Alternaria* sp are post-harvest pathogens, grow at low temperatures, giving them the power to degrade foods during refrigerated storage (Ostry, 2008). However most pesticides do not specifically target the pathogen or parasite and so often affect non-target plants and animals. Repeated application results in loss of biodiversity. The majority of pesticides are not biodegradable and accumulate in soils, groundwater and surface water causing wide spread environmental contamination. Broad-spectrum insecticides such as carbamates, organophosphorus and pyrethroids can diminish beneficial insect populations such as bees, spiders and beetles. For example, the number of earthworms were 1.3 to 3.2 times higher in biological plots compared to the conventionally treated plots (Mäder *et al*., 2002).

Broad spectrum pesticides will also affect those species that play an important role in the food web or are natural enemies of the pests (Kaki, 2004). Increased awareness of the environmental problems associated with pesticides have led to the search for unconventional chemicals of biological origin to fight against fungal diseases (Neela *et al*., 2014). Natural pesticides (biopesticides) are easily accessible and relatively inexpensive and are amenable to sustainable production methods. Among the natural substances already used, essential oils extracted from aromatic plants can serve as effective bio-pesticides (Koul *et al*., 2008). In North America, various studies have shown the fungicidal efficacy of plant extracts, some of which have been effective against potato late blight. In the latter case the extract was based on the commercial composted tea (Jolly Farmer) and kelp (100% algae) (Moreno *et al*., 2006). One plant extract of particular interest in North Africa is *Rosmarinus eriocalyx* (Lamiaceae), a plant common in the semi-arid region of North-Eastern Algeria which is rich in medicinal and aromatic plant species. This rosemary is known for its richness in terpene and phenolic compounds (rosmarinic acid), caffeic acid, ferulic acid, chlorogenic acid, lutein, apigenin, genkwanin, quercitrin, rutin, epicatechin, catechin (Farhat *et al*., 2009; Castro-Vazquez *et al*., 2009). Several interesting biological activities have been identified in rosemary, including antimicrobial activity (Jordán *et al*., 2013) and antioxidant activity (Yan *et al*., 2015) of its terpenoids. Rosemary essential oil has been used as an antibacterial and antiseptic agent. They have been the subject of numerous studies (Pintore *et al*., 2002, Daferera *et al*., 2003). *Rosmarinus eriocalyx* is common in Mediterranean areas with *Rosmarinus officinalis*. It is common in the arid and semi-arid regions of Algeria.

This study is based on the evaluation of the biopesticide power and its raw methanolic extracts of leaves and flowers of *R. eriocalyx* on dieback diseases caused by the phytopathogenic agents of *Alternaria* during and after the harvest of tomato, *Lycopersicum esculentum*, an economically intensive crop in eastern Algeria.

MATERIALS AND METHODS

Biopesticide

Rosmarinus eriocalyx was harvested in March 2015 at the flowering stage in the region of Souk Ahras (northeastern Algeria), located on the high slopes of the Tellian Atlas. The climate is characterized by a hot and dry summer (25 - 35°C in July-August), and a cold and wet winter (1 to 15°C in January). Flowers and leaves from the harvested plants are dried in a ventilated place, protected from sun and dust at ambient temperature conditions and then ground at moderate speed in an electric mill. The powder obtained was used for the preparation of the extract by extracting 100g of macerate in 1000ml of pure methanol with gentle shaking for 24h in the dark at ambient temperature. The mixture is filtered three times using sterile doubled gauze. The

Table 1. Identification of major phenolic compounds in methanolic extract of *Rosmarinus* by using ESI LC-MS /MS

S.No	RT (min)	[M-H]⁻(m/z)	Main fragment ESI⁻ MS^n	Identification	References
1	1.38	191	MS^2 (191) : 127, 173, 85,	Quinic acid	Cirlini *et al.* (2016)
2	3.63	431	MS^2 [431]: 281, 251, 179, 279	Apigenine-7-*O*-glugoside	Linares *et al.* (2011)
3	4 .41	325	MS^2 [325] : 163	Coumaric acide-*O*-hexoside	Vallverdú-Queralt *et al.*(2014)
4	5.33	305	MS^2 [305] : 225, 97	Gallocatechin	Hossain *et al.* (2010)
5	8.09	355	MS^2 [355] :327, 337	6-formyl-Isoophiopogonanone A	Ye *et al.* (2015)
6	8.58	477	MS^2 [477] : 301	Quercetinuronic acid	Plazonić *et al.* (2009)
7	10.00	597	MS^2 [597]: 311,329, 579]	NI	
8	10,49	555	MS^2 [555] : 538,512, 392, 259, 193	Isoaloeresin D	Sayed *et al.* (2016)
9	12.13	477	MS^2 [477] :315, MS^3 :300, 153	Isorhamnetin-3-O-hexoside	. Kontogianni *et al.* (2013)
10	12.53	571	MS^2 [571] : 527, 553, MS^3 :311, 169	NI	
11	14.25	461	MS^2 [461] : 285	Luteolin-3'-glucuronide	Hossain *et al.* (2010)
12	**14.50**	**359**	**MS^2 [359] : 161, 179, 197**	**Rosmarinic acid**	Kontogianni *et al.* (2013)
13	16.99	637	MS^2 [637] : 537, 538, 555, MS^3 :493, 255	NI	
14	17.35	285	MS^2 [285] : 267	Luteolin	Hossain et *al.* (2010)
15	17,69	503	MS^2 [503] : 443, 285, 461, --MS^3 285, 381	Luteolin 3'-*O*-(*O*-acetyl)-β-D-glucuronide Isomer II	Borrás-Linares *et al.* (2014)

NI= not identify

recovered filtrate is evaporated under vacuum at 40°C. The dry extract obtained is taken up in 5ml of absolute methanol (Falleh *et al*., 2008).

Identification of the plant extract by ESI LC-MS / MS

The identification was carried out at the level of Department of Chemistry, CICECO-Aveiro Institute of Materials. The foliar and floral extract was analyzed by ESI LC-MS /MS. A concentration of 10mg/ml of the filtered methanolic extract using a 0.2ml membrane (Whatman). Three analyses were conducted separately for confirmation. A Thermo Scientific Ultimate 3000RSLC (Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer is used in this technique. A thermo-scientific gold and hypersil column (1000mm x 20mm) with a part size of 1.9µm width at 30°C was maintained.

The mobile phase consists of (A) acetonitrile and (B) 0.1% formic acid (v/v), degassing and filtering done before injection the methanolic extract. The flow rate was 0.2ml / min. At the beginning of the analysis, a gradient of 5% for solvent B for 14 min. followed by 40% of solvent B for 2 min, of 100% over 7 min and finally of 5% over 10 min with a volume of 2µl of injection. The UV-visible spectrum data are collected in a range of 250 to 500nm and the chromatographic profiles are made at 280nm. The mass spectrometer used is an LTQ XL linear ion trap equipped with an orthogonal Electrospray Ion Source (ESI). The equipment operated in negative ion mode with a 5.00 kV electrospray ionization source and an ESI capillary temperature of 275°C. The complete scan covered a mass range of 50 to 2000m / z. MS / MS and MSn collision-induced dissociation experiments were acquired simultaneously for precursor ions.

Synthetic chemical pesticide

Hymexazole is the active substance in a commercial systemic seed fungicide from Golden Union Agrochemical, marketed in Algeria as tachigazol. It

belongs to the chemical family Triazines.

- Raw chemical formula: $C_4H_5NO_2$.
- Structural formula: 5-methyl-3(2H)-isoxazolone. (Hadjeres, 2015)
- Chemical structure shown in Figure 1:

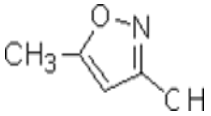

Figure 1. Chemical structure of hymexazole

Isolation, purification and identification of *Alternaria* strains responsible for the damage on the tomato crop

Collected from different greenhouses, the tomato leaves are carefully washed three times with running water and twice with sterile distilled water and then soaked for 5 min in 2% sodium hypochlorite (NaOCl). Then, they are rinsed several times with distilled water. The infected portions of the aseptically cut fruits are deposited in sterile petri dishes containing the Potato-Dextrose Agar (PDA) medium. The dishes are incubated at a temperature of 37°C for 5-7 days. The isolated fungal strains are then transferred to Kzapeck medium supplemented with antibiotic (streptomycin) to prevent bacterial growth. This operation is repeated several times until the pure strains are obtained. The pure strains are sub-cultured on Potato Carrot Agar (PCA) medium (Simmons, 2002). The isolates obtained are identified on the basis of the macroscopic morphological characteristics of the colonies and the microscopic characteristics of spores and mycelium based on identification keys of the fungus (Botton *et al*., 1990). Four *Alternaria* species were identified *viz*: *Alternaria alternata* 1, *A. alternata* 2, *A. tenuissima* and *A. arborescens*.

Principle of the method

To determine the antifungal power property, or inhibition effect, the crude extract was tested on *Alternaria* stem growth capacity. The susceptibility or resistance to the treatment was determined the medium (Methanolic Extract=ME or Pesticide=Pst). Growth potential of the tested *Alternaria* strains were evaluated through growth kinetics using three parameters: pH, OD and dry weight of the inoculum extracts. This method relies on the kinetic evaluation of the antimicrobial effect of the extract on fungal growth based on three indicators: pH, opacity, or fungal biomass concentration. The pre-cultures and cultures are carried out in a batch mode on Galzy and improved Solnimski (GS) synthetic

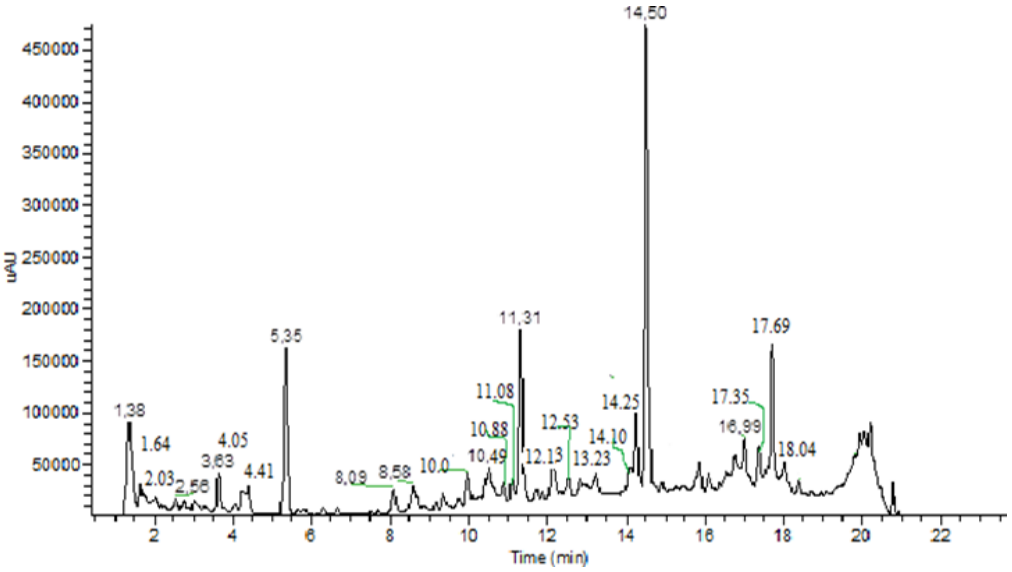

Figure 2. Chromatogram of methanolic extract of *Rosmarinus eriocalyx* by ESI LC- MS/MS in the negative ionization mode

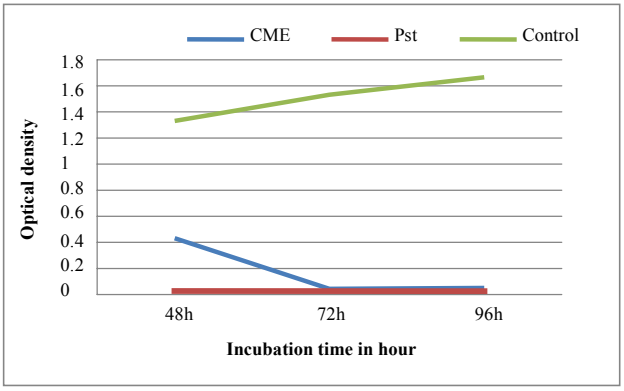

Figure 3. Growth kinetics of *Alternaria alternata* 1 (Under the effect of CME and Pst)

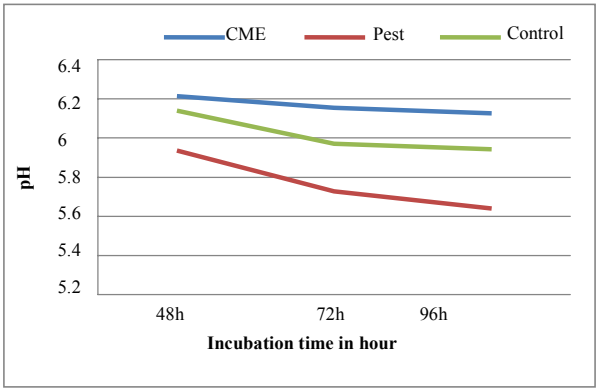

Figure 4. pH kinetics of *Alternaria alternata* 1 (Under the effect of CME and Pst)

medium, (Bordjiba, 2003) supplemented with glucose (5 g/l) at pH 4.5, the medium is distributed in 250ml Erlenmeyer flasks with 100ml per vial, the whole is then sterilized under saturated vapor pressure (20 min at 120°C). The inoculum (mycelium plus spores or fungal colonies) is removed from the petri dishes and aseptically introduced into vials. Each test is performed in triplicate (three repetitions for each strain. The incubation is done at a temperature of 27°C under 1200 Lux (12h / 24 h) illumination and at agitated conditions (on an orbital plate at a rotation speed of 180rpm). After 48h (T_0), the dry extract obtained is taken up in DMSO and added to the cultures aseptically to a final concentration of 1mg / ml. The fungicide molecules are then prepared in a mixture of DMSO (50/50, v/v) and sterilized by filtration through a 0.22µm Millipore membrane (Bedford, MA) and before adding to the cultures in an aseptic manner at a final concentration of 100mg/l. Control trials (medium + inoculum without extract and without pesticides) are included in the trials. The experiments lasted for 5 days (120h). The parameters studied are pH, the optical density or turbidity and the dry weight of the inoculum. Measurements are taken from the medium at T_0 (moment of addition of the extract or the pesticide) and then at every 24h. The measured values allow the kinetics of the pH, optical density and dry weights of the inoculum of the strains to be calculated and plotted. (Bordjiba, 2003).

pH measurement

The growth and maintenance of microbial survival are influenced by the pH of the medium. The fungal suspensions, submitted under the effect of the methanol extract and the pesticide is measured daily using a pH meter type HI 2211 Hanna (Bordjiba, 2003).

Determination of optical density

Turbidimetry is used to determine the growth of fungal strains in the presence of the extract or pesticide used as a carbon source at room temperature for 5 days. The optical density is determined during the incubation period from T_0 at 24h time intervals. It is measured at a wavelength of 550nm on a WPA biowave spectrophotometer. The determination of the concentration of the fungi studied is carried out on the basis of the calibration curves for the growth of non-treated strains (Bordjiba, 2003).

Calculation of the dry weight of the inoculum

After 120h, the contents of the Erlenmeyer flasks are recovered and filtered through a millipore 0.45 µm membrane. The contents of the membrane are allowed to dry at 105°C for 24h and then weighed to determine the weight in mg / 100ml of the dry inoculum of the strain tested. The dry weight is calculated according to the following equation:

$Pis = (M-M_0)$

where, M: weight of the membrane after drying; M_0: weight of the empty membrane (Bordjiba, 2003). All

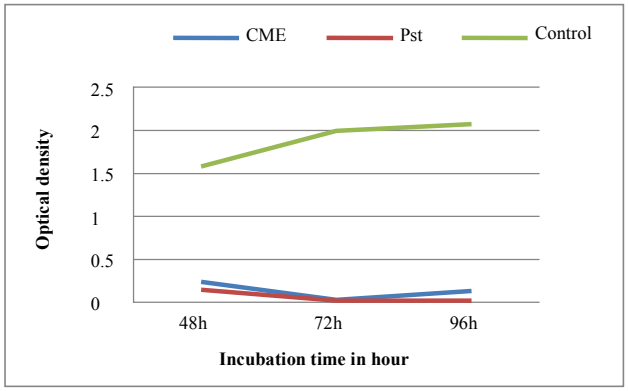

Figure 5. Growth kinetics of *Alternaria alternata* 2 (Under the effect of CME and Pst)

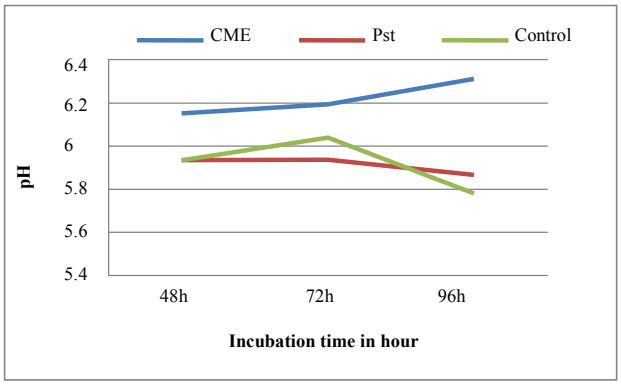

Figure 6. pH kinetics of *Alternaria alternata* 2 (Under the effect of CME and Pst)

results obtained are validated by 2 statistical tests (Tukey, Anova with Minitab Inc, 2016).

RESULTS AND DISCUSSION

Following identification by SEI LC MS/MS, it appears in Table 1 that the extract contains a mixture of major compounds. Among these components, the most abundant major compound is rosmarinic acid at 14.5min (Figure 2).

Optical density and pH and dry weight of inoculum biomass

The results obtained showed that the antifungal activity of the extract tested varies according to the nature of the strain. The strain *Alternaria alternata* 1 seems most sensitive to the methanolic extract. At the beginning and up to 48h, growth is not affected, the optical density is of the order of 0.431. After 72h , inhibition is important since the calculated optical density is only of the order of 0.046 (Figure 3). The tested extract has an effect quite similar to that of the chemical pesticide (D.O Pesticide = 0.022). Significant differences are detected ($p<0.000$) using statistical tests. As for 't', (Figure 4) there is an increase in the (pH) in this case of the medium inoculated by the plant extract and a decrease in the pH in the case of the medium inoculated with the synthetic pesticide. The Turkey's test also revealed significant differences in pH results between the two treatments. The calculation of the dry weight of the inoculum obtained at the end of the experiment after 120h (Figure 11) showed a non-negligible decrease in the total biomass when the strain is exposed to the effect of the natural extract. However, this weight (p = 0.36mg / 100ml) is reduced compared with the control's weight and this indicated that extract has a pesticide effect against this strain.

For the second strain of *A. alternata* 2, it can be

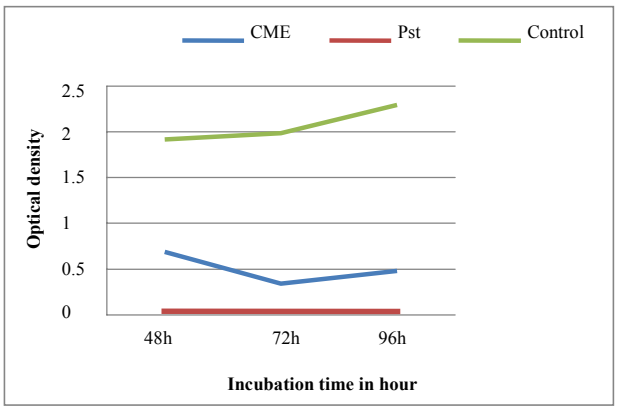

Figure 7. Growth kinetics of *Alternariatenuissima* (Under the effect of CME and Pst)

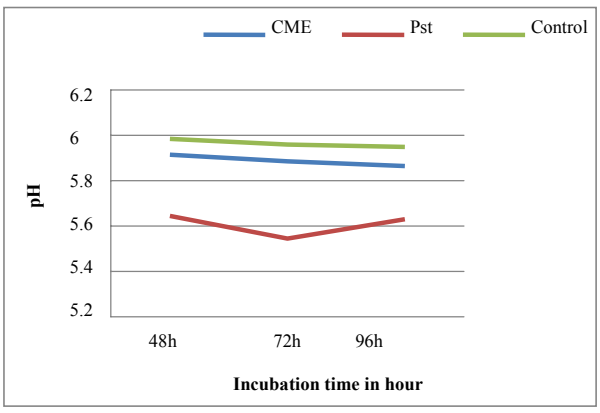

Figure 8. pH kinetics of *Alternariatenuissima* (Under the effect of CME and Pst)

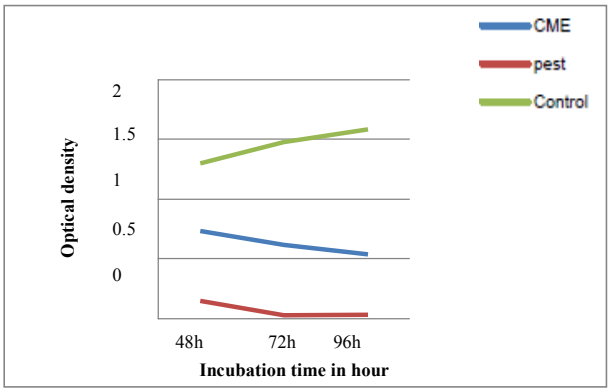

Figure 9. Growth kinetics of *Alternaria arborescens* (Under the effect of CME and Pst)

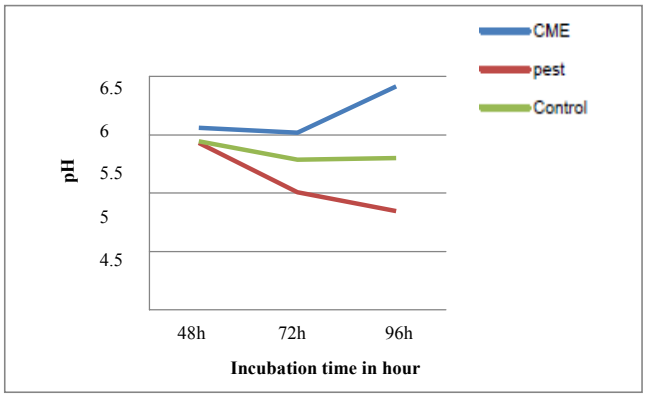

Figure 10. pH kinetics of *Alternaria arborescens* (Under the effect of CME and Pst)

seen from the data shown in the (Figure 5) that the efficiency of the optical density decreases to the lowest level (OD = 0.031) contrary to the value recorded by the untreated sample (1.996). As for the optical density (Figure 5) displayed by the medium supplemented with the pesticide, it is close to that of the sample treated with the plant extract (OD = 0.015). After 72h of incubation, the growth of *A. alternata* 2 rises slightly for both the control (2.069) and the methanolic plant extract (OD = 0.135). The acidity of the media treated with the plant extract (Figure 6) and that of the control is quite similar (pH of the plant extract is 6.19, pH of the untreated control is 6.04. From 72h, the pH of the medium injected with the plant extract rises whereas that recorded for the control drops. The pH of the solution with the plant extract is greater than those of the other samples analyzed. A decrease in weights for both treatments means inhibition of strain development but this antifungal activity is more pronounced for the chemical or pesticide. A decrease in *Alternaria tenuissima* growth is revealed by low optical density values between 0.482 for the extract and 0.027 for the pesticide (Figure 7). This decrease is observed also for the pH (Figure 8). However, this decrease is greater for the pesticide (pH = 5.6). The weight taken indicated that the Methanolic Extract (ME) induced a reduction on the growth of the strain with a weight of the order of 131.67mg compared to that of the control which is 323.33mg / 100ml.

The activity of ME on *Alternaria arborescens* is very powerful almost of the same order as that of the pesticide, according to OD (Figure 9), which dropped after 72h from 0.736 to 0.053. This followed a marked increase in pH (Figure 10) due to different reactions caused by the action of ME. The differences are very significant pH ($p<0.05$) for different treatments that demonstrated that the strain does not have the same sensitivity to different treatments. The weight recorded strongly matches the OD obtained; ME reduced the weight of 89.67mg quite similar to that of the pesticide 75.67mg / 100ml. The optical density values (indicating the fungal concentration) confirm the weight values. Low density indicated low growth and therefore low weight.

The analyzes of the three parameters, pH, OD and dry weight of inoculums showed that there is a relatively low development of biomass shown by all the strains tested in control without treatment. Whereas quite significant decreases occur in the presence of the crude methanolic extracts of *Rosmarinus eriocalyx*. This decrease is more pronounced for *Alternaria alternata* 1 and *Alternaria tenuissima*. The toxic effect caused by the natural extract on most of the tested strains seems quite similar if not more to that caused by the conventional pesticide. This reduction in the weight of the inoculum indicated good antifungal activity of the tested ethanolic extract which shows the sensitivity of fungal

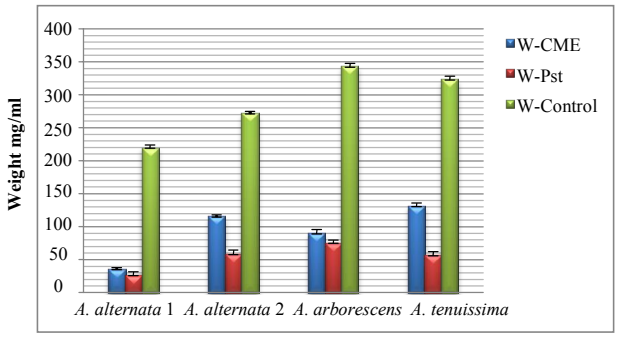

Figure 11. Weight of *Alternaria* sp. strains of batch culture

strains to the plant components. The qualitative identification of the extract revealed flavonoids and diterpene lactones. These active compounds identified in the methanolic extracts seem to be toxic to the different species of strains of *Alternaria* under the conditions of our experimentation. Indeed rosmarinic acid, caffeic acid, luteolin are well known for their antimicrobial power (Moreno *et al*., 2006; Widmer and Laurent, 2006; Su *et al*., 2014). Modified medium pH was observed in all cases. Low or high pH values are recorded after 120h of incubation in media treated with the extract of the plant. This acidification or alkalinization of the medium is greater or less important than that recorded in the culture media with pesticide added as well as that of the controls. This pH decrease is particularly strong with *Alternaria alternata* 1 and *Alternaria tenuissima* and *Alternaria arborescens* at 72h. Acidification of the medium can be caused by the inhibition process following the different cellular metabolic reactions.

Our results are fully consistent with those of Hirasawa and Takada (2004) who reported a change in pH of the medium during the microbial growth slowing process. It can be deduced that the change in pH during the experiment probably affects weight reduction since pH is an important factor in fungal culture. In our present study, methanolic extract infected the growth of all *Alternaria* strains. Similar results were obtained by Bomfilm *et al*. (2015), who showed a strong reduction in the mycelial weight of *Fusarium verticillioides* by applying the essential oil of *Rosmarinus officinalis* to the fungal culture. These same authors assert that in addition to growth inhibition, variations in the morphology of microconidia were observed. Our results on four *Alternaria* strains subjected to the effect of the methanolic extract of *Rosmarinus eriocalyx* however differ in that an increase in the biomass coincided with a rise in pH. Nevertheless, clearly pH has an indirect influence on the development of these microorganisms by disturbing the fungal enzyme system and reducing germination of the fungus (Amiri *et al*., 2011) Another effect on, medium pH concerns the effectiveness of the compounds in the extract used. In this context, Hirasawa and Takada, (2004) demonstrated that catechins exert a strong antifungal activity on *Candida albicans* but only in an acid pH environment. This is explained by the fact that the active compounds in the plant extract exert a strong capacity for toxicity on the development of these strains despite their development during the first growth phases (after 48h of culture). Hence, strains after 120h of extract exposure are completely unable to survive the conditions of the test medium. This type of behaviour could be explained by a change in cellular content and the fungal cell membrane together with the genetic apparatus necessary to maintain metabolic multiplication reactions; consequently, they cannot control and sustain their metabolic functions (Raybaudi-Massilia *et al*., 2006; Oussalah *et al*., 2007). Notably, metabolism is also under the influence of the environment the metabolic activity and in particular the enzymatic activities of the microbial strains cultivated in the presence of a source of organic substrate (energy source) depend on this source of energy (carbon, nitrogen), pH and temperature (Bordjiba, 2003).

CONCLUSION

The results obtained indicated that *Alternaria alternata* 1 and *Alternaria arborescens* are most sensitive to the active compounds in the foliar and floral extract of *Rosmarinus eriocalyx*. This efficiency is some-

times greater than that of hymexasole, the synthetic fungicide. The tested crude methanolic extract seems to have an interesting antifungal power which is probably due to its composition of secondary metabolites and in particular rosmarinic acid appears to be responsible for inhibition of fungal growth. Further experiments on the isolation and the qualitative and quantitative identification of the relevant polyphenolic molecules for inhibitory activity on different strains of *Alternaria* are needed. More natural phytopathogenic agents are urgently required for bio -control as bio-pesticides to reduce side effects on non-pathogenic organisms and help eliminate serious diseases and reduce agricultural losses.

ACKNOWLEDGEMENTS

I thank all the staff of the Department of Chemistry, CICECO-Aveiro Institute of Materials University of AveiroAveiroPortugal for their help

REFERENCES

Abdel-Kader MM, El-Mougy NS, El-Gamal NG, El-Mohamdy RS and Fatouh YO. 2012. *In vitro* assay of some plant resistance inducers, essential oils and plant extracts on antagonistic ability of fungal bio-agents. *Journal of Applied Sciences Research,* 8(3): 1383-1391.

Ait Kaki A. 2004. Research of new potentialities of bacteria of the genus *Bacillus* for the agriculture and agribusiness Ph. D thesis. Mentouri University Constantine 1, Algeria,, 127p.

Amiri A, Chai W and Schnabel G. 2011. Effect of nutrient status, pH, temperature and water potential on germination and growth of Rhizopus *Stolonifer* and *Gilbertella Persicaria*. *Journal of Plant Pathology,* 93 (3): 603-612.

Ben Farhat M, Jordan MJ, Chaouech-Hamada Rym, Landoulsi A and Sotomayor JA. 2009. Variations in essential oil, phenolic compounds, and antioxidant activity of tunisian cultivated *Salvia officinalis* L. *Journal of Agricultural and Food Chemistry,* 57(21): 10349-10356.

Bordjiba O. 2003. Effect of pesticides on the fungal microflora of the soil biodegradation of herbicides by isolated strains. PhD thesis, University Joseph Fourier Grenoble, France 185p.

Borrás Linares I, Arráez-Román D, Herrero M, Ibáñez E, Segura-Carretero A and Fernández-Gutiérrez A. 2011. Comparison of different extraction procedures for the comprehensive characterization of bioactive phenolic compounds in *Rosmarinus officinalis* by reversed-phase high-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight mass spectrometry. *Journal of Chromatography A,* 1218(42): 7682– 7690.

Borrás-Linares Isabel, Stojanovic Zorica, Quirantes-Piné Rosa, Arráez-Román David, Švarc-Gajić Jaroslava, Fernández-Gutiérrez Alberto and Segura-Carretero Antonio. 2014. *Rosmarinus officinalis* leaves as a natural source of bioactive compounds. *International Journal of Molecular Sciences,* 15(11): 20585-20606.

Botton B, Breton A, Fèvre M, Gauthier S, Guy P, Larpent JP, Reymond P, Sanglier JJ, Vayssier Y and Veau P. 1990. Useful and harmful moulds, industrial importance Masson, Paris, 512 p.

Castro-Vazquez Lucia, Diaz-Maroto M Consuelo Hidalgo, Gonzalez-Vinas Manuel Angel and Perez-Coello M Soledad. 2009. Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys based on volatile composition and sensory descriptive analysis. *Food Chemistry,* 112(4): 1022 -1030.

Cirlini M, Mena P, Tassotti M,. Herrlinger KA, Nie-

man KM, Dall'Asta Chiara and Del Rio Daniel. 2016. Phenolic and volatile composition of a dry spearmint (*Mentha spicata* L.) extract. *Molecules*, 21(8): 1007.

Daferera DJ, Ziogas BN and Polissiou MG. 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea, Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis. Crop Protection,* 22(1): 9-44.

Da Silva Bomfilm N, Nakassugi LP, Oliveira FP, Kohiyama CY, Mossini SA, Grespan R, Nerilo SP, Mallmann CA, Alves AFB and Machinski M Jr. 2015. Antifungal activity and inhibition of fumonisin production by *Rosmarinus officinalis* L. essential oil in *Fusarium verticillioides*. (Sacc.) Nirenberg. *Food Chemistry,* 166: 330-336.

El Sayed AM, Ezzat SM, El Naggar MM and El Hawary SS. 2016. *In vivo* diabetic wound healing effect and HPLC–DAD–ESI–MS/MS profiling of the methanol extracts of eight *Aloe* species. *Revista Brasileira de Farmacognosia,* 26(3): 352–36.

Falleh H, Ksouri R, Chaieb K, Karray-Bouraoui N, Trabelsi N, Boulaaba M and Abdelly C. 2008. Phenolic composition of *Cynara cardunculus* L. organs, and their biological activities. *Compte Rendus de Biologie,* 331(5): 372-379.

Fletcher J, Bender C, Budowle B, Cobb WT, Gold SE, Ishimaru CA, Luster D, Melcher U, Murch R, Scherm H, Seem RC, Sherwood JL, Sobral BW and Tolin SA. 2006. Plant pathogen forensics: capabilities, needs, and recommendations. *Microbiology and Molecular Biology Reviews,* 70(2): 450–471.

Hadjeres N. 2015. Index des produits phytosanitaires à l'usage agricoles. 217p. Available from: www.minagri.dz/pdf/Dpvct/ INDEX_PRODUITS_PHYTO_2015.pd

Hirasawa M and Takada K. 2004. Multiple effects of green tea catechin on the antifungal activiti of antimycotics against *Candida albicans*. *Journal of Antimicrobial Chemotherapy,* 53(2): 225-229.

Hossain MB, Rai DK, Brunton NP, Martin-Diana AB and Barry-Ryan C. 2010. Characterization of phenolics composition in Lamiaceae spices by LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry*, 58 (19): 10576-10581.

Jordán MJ, Lax V, Rota MC, Lorán S and Sotomayor JA. 2013. Effect of the phenological stage on the chemical composition, and antimicrobial and antioxidant properties of *Rosmarinus officinalis* L essential oil and its polyphenolic extract. *Industrial Crops and Products,* 48: 144-152.

Kontogianni VG, Tomic G, Nikolic I, Nerantzaki AA, Sayyad N, Stosic-Grujicic S, Stojanovic I, Gerothanassis IP and Tzakos AG. 2013. Phytochemical profile of *Rosmarinus officinalis* and *Salvia officinalis* extracts and correlation to their antioxidant and anti-proliferative activity. *Food Chemistry*, 136(1): 120–129.

Koul O, Walia S and Dhaliwal GS. 2008. Essential oils as green pesticides: potential and constraints. *Biopesticides International,* 4(1): 63–84.

Mäder P, Fliessbach A, Dubois D, Gunst L, Fried P and Niggli U. 2002. Soil fertility and biodiversity in organic farming. *Science*, 296(5573): 1694-1697.

Minitab Inc, 2016. Statistical software. Computer software. State college, PA: Minitab, INC (www.minitab.com). Available from: http:// fr.softwaresea.com/software-download/minitab-16

Moreno S, Scheyer T, Romano CS, Vojnov AA. 2006. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition.

Free Radical Research, 40(2): 223–231.

Neela FA, Sonia IA, Shamsi S. 2014. Antifungal activity of selected medicinal plant extract on *Fusarium oxysporum* schlechtthe causal agent of *Fusarium* wilt disease in tomato. *American Journal of Plant Sciences,* 5(18): 2665–2671.

Ostry V. 2008. *Alternaria mycotoxins* an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal,* 1(2): 175-188.

Oussalah M, Caillet S, Saucier L and Lacroix M. 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Control,* 18 (5): 414- 420

Pintore G, Usai M, Bradesi P, Juliano C, Boatto G, Tomi F, Chessa M, Cerri R and Casanova J. 2002. Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from sardinia and corsica. *Flavor Fragrance Journal,* 17(1): 15-19.

Plazonić A, Bucar F, Maleš Z, Mornar A, Nigović B and Kujundţić N. 2009. Identification and quantification of flavonoids and phenolic acids in burr parsley (*Caucalis platycarpos* L.), using high - performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. *Molecules,* 14(7): 2466-2490.

Raybaudi-Massilia RM, Mosqueda-Melgar Jonathan, Martín-Belloso O. 2006. Antimicrobial activity of essential oils on *Salmonella enteritidis*, *Escherichia coli*, and *Listeria innocua* in fruit juices. *Journal of Food Protection,* 69(7): 1579-1586.

Simmons EG. 2002. *Alternaria* themes and variations (305-309) Lewia/ *Alternaria* revisited. *Mycotaxon,* 83: 127-145.

Su Y, Ma L, Wen Y, Wang H and Zhang S. 2014. Studies of the in vitro antibacterial activities of several polyphenols against clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Molecules,* 19: 12630-12639.

Widmer TL and Laurent N. 2006. Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora* spp. pathogenic to *Theobroma cacao*. *European Journal of plant pathology,* 115(4): 377.

Vallverdú-Queralt Anna, Regueiro Jorge, Martínez-Huélamo Miriam, Alvarenga José Fernando Rinaldi, Leal Leonel N and Lamuela-Raventos Rosa M. 2014. A comprehensive study on the phenolic profile of widely used culinary herbs and spices: rosemary, thyme, oregano, cinnamon, cumin and bay. *Food Chemistry,* 154: 299–307.

Yan M, Li G, Petiwala SM, Householter E, Johnson JJ. 2015. Standardized rosemary (*Rosmarinus officinalis*) extract induces Nrf2/sestrin-2 pathway in colon cancer cells. *Journal of Functional Foods,* 13: 137-47.

Ye M, Guo D, Ye G and Huang C. 2005. Analysis of homoisoflavonoids in Ophiopogon japonicus by HPLC-DAD-ESI-MSn1. *Journal of the American Society for Mass Spectrometry,* 16(2): 234-243.