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Antioxidant Activities and Antifungal Screening of *Salviaofficinalis* Flavonoids

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ABSTRACT

Medicinal plants constitute an immense source of bioactive molecules, endowed with numerous biological activities.
This study aimed at performing antioxidant and antifungal screening of flavonoids extract from *Salvia officinalis* leaves, this plant with therapeutic properties serving to the curative needs of certain peoples
An antioxidant and antifungal test were performed after the extraction of flavonoids, which are in the value of 6.96%.
Antioxidant activity of *Salvia officinalis* flavonoids was evaluation by the DPPH test revealed a fairly high anti-radical effect with results ranging from 75.67% to 90.27% and an effective concentration to reduce 50% of free radicals of 10.60 µg / ml.
The evaluation of antifungal activity was fined by radial growth technique gave the antifungal indices according to the order of efficacy of the flavonoids; on the following recorded antifungal registers; *Aspergillusflavus* 24.16%, *A.ochraceus* 21.61%, *A. niger* 20.16% and *P.expansum* 16.95%.
KEY WORDS: Medicinal plant, *Salvia officinalis*, flavonoids, antioxidant, antifungal.

INTRODUCTION

Algeria has a climatic diversity: Mediterranean, Saharan and tropical, which influences the richness of its flora, where there are more than 3000 species of several botanical families, of which 15% are endemic [7]. The molecules responsible for the therapeutic effect of so-called natural plants are considered a very important source of drugs; knowing that more than 120 herbal compounds are now used in modern medicine and nearly 75% of them are applied according to their traditional use.
During recent decades, different plant-derived extracts and phytochimicals have been ascribed a variety of potentially health-promoting biological activities [27].
Therapeutically, the antioxidant potential of medicinal plants has a very important role in reducing the damage caused by free radicals [35].
Salvia officinalis is distinguished by the effectiveness of its essential oils and bioactive substances [37]. Sage is a perennial round shrubin the family of labiatae/ Lamiaceae; Salvia is the largest genus of this family and includes near 900species. Plants of this genus grow all over the world and the species of *Salvia officinalis* is native to Middle East and Mediterranean areas [20]. *Salvia officinalis* is known for its wide range of therapeutic activities including antibacterial, antiviral, antifungal and antioxidant effects [31, 5 and18]
In the present study, flavonoids extracted from *Salvia officinalis* were tested, the effect of scavenging free radicals and the antifungal effect were tested on fungal strains isolated from durum wheat stored.

MATERIALS AND METHODS

1. Plant samples collected

Salvia officinalis used in this study is collected from Hassi 20 located north of the town of Bechar at 20 km, during the month of September 2015.Wilaya of Bechar is located in the southwest of Algeria, 1600Km from the capital Algiers. The leaves of the collected plant were dried to amber, at room temperature, for 25 days, the dried leaves are stored in closed containers and milled in an electric mill on the day of extraction.

1. Flavonoids extraction protocol

Following the extraction protocol was described by Lee *et al.,* (1995), modified and used by Bouchelta *et al*., (2005); 50g of these plant leaves are milled and added to a mixture compounded with 250ml of distilled water and 250ml absolute ethanol, putted under the 90°C of temperature. In an assembly of reflux for 4 hours, the extract is filtered through a filter paper. The hydro-ethanolic phase was evaporated to remove the ethanol, after that it extracted with 100 ml of n-butanol. This step is followed by a decantation and then acidification with HCl

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(10%) until a pH=3, the n-butanolic phase is up to the dried up evaporation. The dry residue was collected in three times with 200 ml of the distilled water / ethyl acetate (v / v) for one hour, the organic phase was basified with NaOH until pH = 9, after 15 minutes of resting; organic phase (flavonoids) was evaporate dry.
The yield of flavonoids is calculated by the following formula:

$$\text{Yields} = \frac{\text{Balloon mass after drying} \;\; \text{Empty balloon mass}}{\text{Mass of plant material}} \text{x } 100$$

The dry residue was dissolved in 1% ethanol for biological tests

2. **Antioxidative activity by DPPH technique**

the technique used to test the antioxidant activity of flavonoids extracted from *Salvia officinalis* leaves is that of radical scavenging, using the stable radical DPPH [9]. After, 100µl of methnolic solution of flavonoids at different concentrations (4.37, 8.75, 17.5, 26.25; 35) µg/ml was prepared and placed in cuvettes, a volume of 2.9 ml of a methanolic solution of DPPH (0.004%) is added to each cuvette.
After incubation for 30 minutes in the dark, and at room temperature, the absorbance of each sample was measured by a spectrophotometer at a wave length 517 nm.
For the calibration of the apparatus methanol was used; Ascorbic acid is used as a positive reference
The inhibitory percentage of DPPH by the flavonoids extract was calculated according to the following equation:

$$\text{Scavengin efect}(\%) = \frac{\text{Ac} - \text{As}}{\text{Ac}} \text{x } 100$$

Where Ac is the absorbance of the DPPH solution, and As is the absorbance of DPPH solution after the addition of the samples.

3. Antifungal activity

3.1. Fungal strains

The fungal strains, *A. flavus*; *A. ochracceus*; *A. niger* and *P. Expansum* were obtained from durum wheat during our laboratory work.
The genus Aspergillus and Penicillium were identified by the micro-culture technique described by Haris (1989) & Barnette (1972).
Furthermore, for species identification, the single spore technique is performed using three culture media: Malt Extract Agar (M.E.A) at 25°C; Glycerol Nitrate Agar (G25N) at 25°C and Czapek Yeast Agar (C.Y.A) at 5°C and 37°C. The key of pitt (1973) is used for the identification of *Aspergillus flavus, Aspergillus niger* and *Aspergillus ochraceus*. The key of Ramirez (1982) is used for the identification of Penicillium species.
The observation was made after 14 days of incubation. The fungal strains were stored in tubes of PDA acidified at 4 ° C.

3.2. Growth radial technique

The following volumes: 20, 40, 60 et 80 µl of leaves flavonoids of *Salvia officinalis* were added to 15 ml of PDA_{ac} (Potatos Dextrose Agar acidified), in order to have respectively, the concentrations (46, 93, 140, 180) µg/ml, the latter were transferred into a Petri plats, after ensemensing of fangal strains with single spore method, these Petri plats were incubated during 7 days at 25±2°C.A Petri plats containing 15 ml of PDA_{ac} medium without extract is inoculated to serve as growth controls for each strain and each series of tests.
The diameter of the fungal mycelium is measured from the third day of incubation [40, 24].
The seeding of Petri plats for radial growth evaluation was approved out starting from a previously prepared sporulation suspension, by counting the number of spores in order to obtain the concentration of 10^5 spores/ml [38].
The inhibition percentage of mycelial growth of each extracts was calculated using the formula of Singh *et al*., (2009):

$$\text{IP} = \frac{\text{Dt} - \text{D}}{\text{Dl}} \text{x} 100$$

Dt: Mean diameter of mycelial growth control
D: Mean diameter of mycelial growth in treatment
All tests were performed in triplicate for confirmation of results.

4. Statistical analysis

The difference between the control and the various tests, is determined by analysis of variance by one-way ANOVA followed by the Tukey HSD test for multiple comparisons of means and the determination of the significance rates. Value of $p \leq 0.05$ is considered statistically significant.
The computer programs used are:

- Microsoft Office Excel 2007.Ink.
- R version 3.2.2 Copyright (C) 2015 The R Foundation for Statistical Computing (ANOVA).

RESULTS AND DISCUSSION

1. Flavonoids extraction

The dried leaves of *Salvia officinalis* gave a yield of 6.96% of flavonoids; this richness of S*alvia officinalis* in bioactive photochemical compounds is proved by several studies, Yinrong & Yeap, (2002) confirmed the presence of flavonoids in the genus Salvia, Lima *et al.,* (2007) recorded that the most abundant phenolic compounds present in the water extracts were rosmarinic acid and luteolin-7-glucoside.

This richness of bioactive and phenolic compounds especially is related to several parameters as, the origin of plant material [17]; the variety, the season of culture, the season of harvest, the climatic and environmental conditions, the geographical location, the different diseases which can affect the plant, the maturity of the plant [33]; the shelf life[30]; and the species and the type of the plant material, the solvents and the extraction technique have an influence on the yield of flavonoids [42].

2. Antioxidative activity

DPPH is a dark purple color radical, his coloring to yellow indicates the degree of reduction of the solution to be tested [6, 43]. DPPH, this stable radical is very used in the assessment of free radical scavenging capacity and antioxidant properties [17, 9, and 44]. The results of scavenging of DPPH by *Salvia officinalis* flavonoids and ascorbic acid are respectively illustrated in figure 1 and 2, in these study flavonoids from *Salvia officinalis* leaves are trapped between 75% and 90.27% of DPPH radical with over a range of flavonoids concentrations of 4.375 to 35 µg/ml. In order to trap 50% of free radicals of *Salvia officinalis* flavonoids, the effective concentration should be equal to 10.60 µg/ml, so that the IC50 concentration of ascorbic acid is 62.6µg/ml.

The lower the IC50 value, the higher the antiradical activity [21]. The flavonoids of *Salvia officinalis* have an IC50 of 10.60 µg / ml, which shows an antioxidant activity greater than that of ascorbic acid. This activity is due to the phytochemical composition of the leaves of *Salvia officinalis* which is influenced by the difference in terms of culture conditions, geographical location, climatic conditions, mode and extraction time, and techniques used [23, 29].

A strong anti-oxidant activity of *Salvia officinalis,* equivalent or superior than that of synthetic antioxidants were shown by Dauksas *et al.,* 2001; many studies have established relationships between the chemical structures of flavonoids and their antioxidant capacity [12]

The species *Salvia officinalis* was featured by apigenin, luteolin and 6-OH-lut-6-OMe, while apigenin and luteolin are flavons [42].

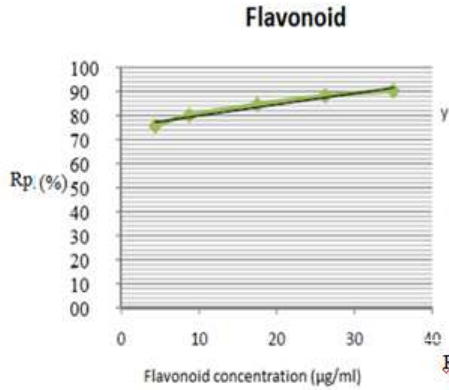
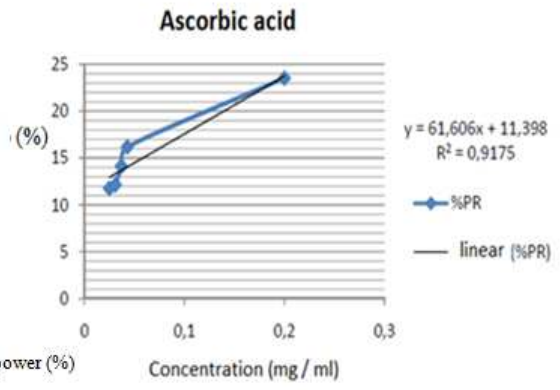


Figure 1. DPPH scavenging effect of Flavonoids of *Salvia officinalis*



Rp: Reduction power (%)

Figure 2. DPPH scavenging effect of Ascorbic acid

According to Cosio *et al., (*2006) and Dimitrios & Vassiliki (2006) the flavonoids of *Salvia officinalis* identified are ranked in descending order according to their antioxidant powers as follows: Quercetin, luteolin, apigenin, and their derivatives.

The antioxidant activity of flavonoids can take many forms in the regulation of oxidative stress. These components can intervene by directly capturing the free radicals or by inhibiting the generators of the reactive species of oxygen, or by capturing the metal cations [3, 28].

3. Antifungal activity with growth radial technique

Different concentrations of flavonoids extracted of *Salvia officinalis* leaves were tested for their efficacy by growth radial technique against fungi of food stored. The histograms (figure3) and pictures (figure4) show that varying concentrations of flavonoids have diminished the diameters of all the fungal strains, because of inhibition of radial growth of *Aspergillus flavus*; *Aspergillus ochraceus*; *Aspergillus niger* and *Penicillium expansum*. The best inhibition recorded is for *Aspergillus flavus* with a decrease of a diameter of 49.66mm to 37.66mm for the control and to 180µl / ml respectively.

The antifungal indexes of fungal strains tested are calculated at the concentration of 180µg/ml, *Aspergillus flavus 24.16%, Aspergillus ochraceus 21.61%, Aspergillus niger 20.16*, and *Penicillium expansum 16.95%.*

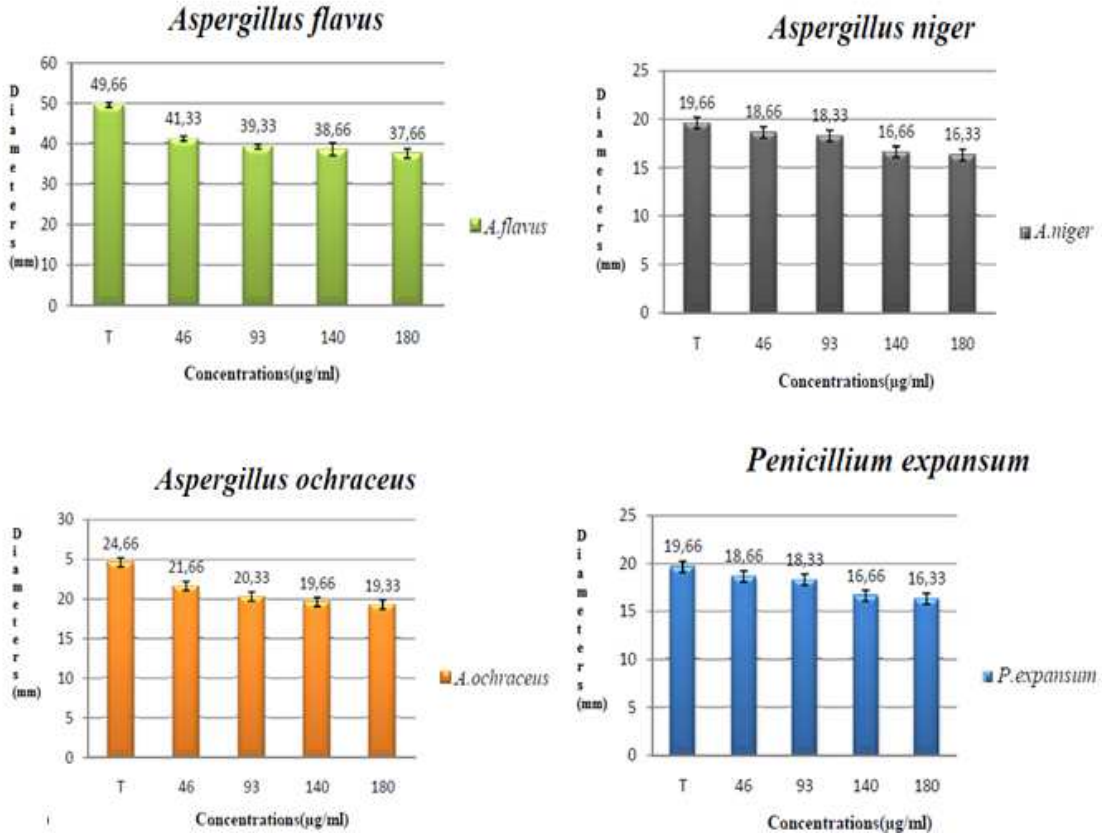


Figure 3. Results of Antifungal activity of *Salvia officinalis* leaves Flavonoids with Growth radial technique.

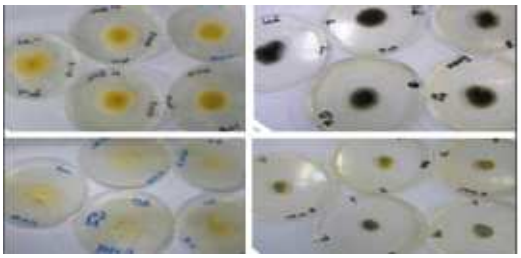


Figure 4. Pictures of Antifungal activity results of *Salvia officinalis* Flavonoids with Growth radial technique

Statistical analysis

The comparison of the mycelial growth between the control and the different concentrations of the flavonoids was carried out by the ANOVA statistical test (one way) followed by the Tukey HSD test illustrated in figure 5under, which shows a significant difference between the diameters of the control and all tested concentrations; which reflects a decrease in growth as a function of the concentration of flavonoids.

In relation to the results obtained by the statistical test (ANOVA) a significant difference characterizes the effects of the different doses of flavonoids on the four fungal strains. The genus Aspergillus is similar in

appearance with antifungal index values. It's the case of *A. flavus, A.ochraceus*, and *A.niger* strains. Penicillium has a lower and rather different inhibition.

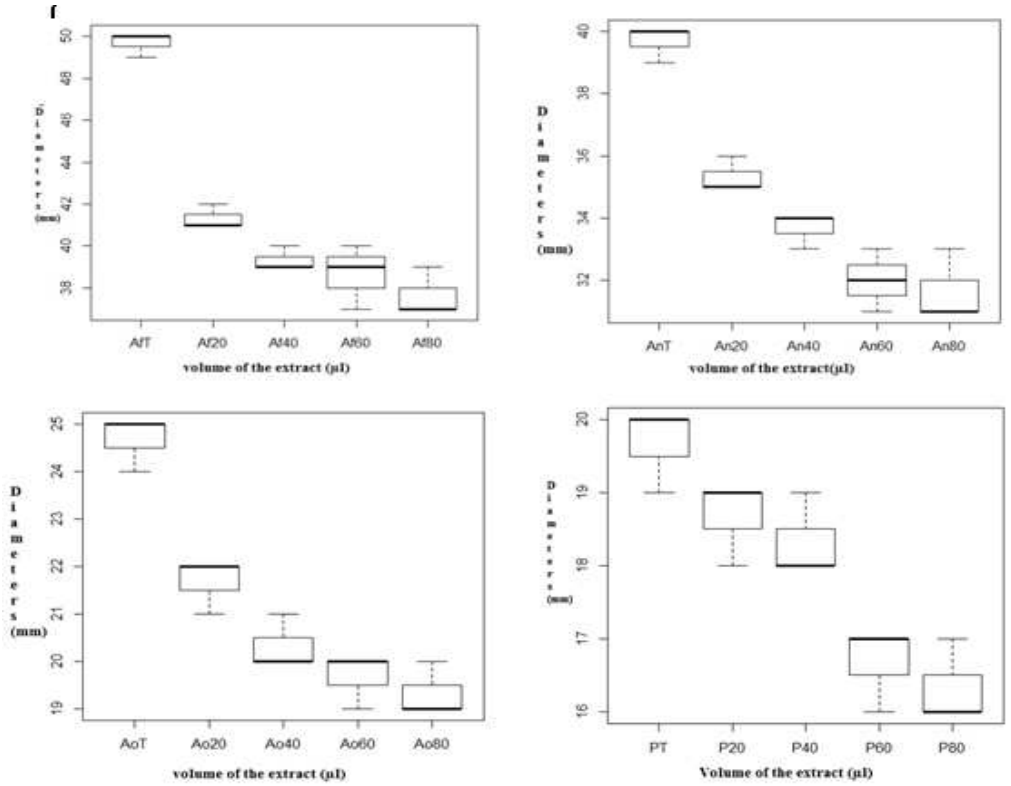


Figure 5. Statistical analysis.

In case of *Penicillium expansum*, the concentrations 46-93 µg/ml exhibit similar behaviours, this is the case of concentrations 140-180 µg/ml. This same result was found by Alilou *et al.,* (2016)

When talking about antifungal activity, there are two kinds of effects: lethal or fungicidal activity and inhibition of growth or fungistatic activity [12].

According to Beigi Boroujeni & Gholami (2017); Ulanowska *et al.,* (2006), the sage has an effect fungistatic, this activity is mainly due to the ability of flavonoids to inhibit the expression of DNA and the synthesis of certain enzymes and membrane proteins of microorganisms.

The inhibitory effect of flavonoids about some enzymes and their antioxidant activity, classified him among the best substances with therapeutic effects. Flavonoids are recommended for use as antibacterial, antifungal, antiviral and anti-inflammatory agents, which are proved by Velikhovic *et al*., (2007).

This activity is probably related to their ability to form complexes with extracellular soluble proteins or with bacterial cell walls [13].

Carolina *et al.,* (2011) indicate that the antifungal action of substances has a predictive target for the cytoplasmic membrane. Nahal & *al., (*2016) assumed that this membrane is the microbial target for flavonoids with chemical affinity on membrane lipids; this hypothesis is more plausible than that of Cowen (1999), lipophilic compounds which disturb the structure of this membrane; flavonoids are lipophilic agents that can disrupt this target.

Phenolic compounds cause damage to the outer membrane of bacteria, resulting in increased membrane permeability to protons and potassium ions, reduced intracellular ATP stores, disruption of proton motive force, and denaturation of cellular proteins [2, 10 and 3].

Chabot *et al*., (1992) affirmed that flavonoids, characterized by the absence of the hydroxyl group on ring B, have higher antimicrobial activity than those belonging to a -OH group, these have at least one hydroxyl group on cycle B. The number and sites of the hydroxyl groups; are the two factors responsible for toxicity to microorganisms.

It is very likely that each constituent has its own mechanism of action, which could explain this divergence of results.

Exploration of the antifungal activity of flavonoids could be done by twinning the mechanism of action with the target action and the chemical structure of each bioactive compound.

By phytochemical screening we can identify the secondary metabolites accumulated in an organ of the plant, these metabolites are used by the plant to protect against herbivores and pests [34]; flavonoids are known to be synthesized by plants in response to microbial infection [19].
Several authors Grayer & Harborne (1994); have reported that secondary metabolites of medicinal plants have antifungal properties and are capable to inhibit mycotoxin synthesis.
It is necessary to explore the chemical structure of flavonoids to try to understand their mode and site of action within the cell, or studies have explored the relationship between the chemical structure of flavonoids and their antimicrobial potency.

CONCLUSION

The richness of *Salvia officinalis* in phytochemical constituents is identified by their quality and quantity.
Flavonoids of sage have a very good power antioxidant where IC 50 is three times higher than that of acide ascorbique
As a result, it was represented that *Salvia officinalis* flavonoids shown antifungal effect on *A. flavus, A. ochraceus, A. niger and P.expansum*.
The biological activities studied could be an alternative solution to the problem of post-harvest deterioration related to molds as well as a likely path for biomedical research including a solution to antimicrobial resistance with bioactive substances.

Conflict of interests
None.

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