Comparative study on the antifungal and antioxydant properties of natural and colored *Juglans regia* L. barks: A high activity against vaginal *Candida* strains

Emira Noumi[1,2*], Mejdi Snoussi[3], Inès Noumi[4], Eulogio Valentin[3], Mahjoub Aouni[1] and Abdulbasit Al-sieni[5]

[1]Laboratoire des Maladies Transmissibles et des Substances Biologiquement Actives, Faculté de Pharmacie, Université de Monastir, Tunisie.
[2]Departamento de Microbiologia y Ecologia, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain.
[3] Laboratoire de Traitement des Eaux Usées, Centre de Recherches et des Technologies des Eaux (CERTE), Technopole de Borj-Cédria, BP 273- Soliman 8020, Tunisie.
[4] Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Tunisie.
[5] Department of Biochemistry,Faculty of Science, King Abdul Aziz University, Jeddah, Kingdom of Saudi Arabia.
*Corresponding author: emira_noumi@yahoo.fr

Abstract: We report in this work the antifungal and antioxydant activities of *Juglans regia* L. natural and treated barks on several vaginal *Candida* isolates and *Candida* type strains. Water, methanol, ethyl acetate and diluted acetone extracts of natural and colored *J. regia* barks were screened for *in vitro* activity against vaginal *Candida* strains. These plants were selected due to their traditional use for the treatment of fungal infections. Plant preparations were screened for antifungal activity using a standard agar disc diffusion assay. Following study of the antifungal activity of plant extracts, their minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values were determined using broth microdilution assay. Several antioxydant properties of this plant were tested such as total phenolic content and determination of reducing power. Among the four *J. regia* L. extracts (for natural and colored bark), methanolic extract of natural bark had the best antifungal activity against all vaginal *Candida* strains. The most important MICs and MFCs values of the methanol extract were obtained for the majority of the vaginal *Candida* isolates (MIC = 0.012 mg/ ml and MFC = 0.024 mg/ml) as compared to amphotericin B (MIC = 0.097 mg / ml and MFC: 0.78 mg/ml). This study reveals that the antioxidant activity of "Swak" extract was more important for natural bark than the treated bark. This plant can contain compounds with therapeutic potential against vaginal *Candida* strains and, hence, their possible use as therapeutic agents.
[Emira Noumi, Mejdi Snoussi, Inès Noumi, Eulogio Valentin, Mahjoub Aouni and Abdulbasit Al-sieni. **Comparative study on the antifungal and antioxydant properties of natural and colored *Juglans regia* L. barks: A high activity against vaginal *Candida* strains.** *Life Sci J* 2014;11(8):327-335]. (ISSN:1097-8135). http://www.lifesciencesite.com. 44

Keywords: Vaginal *Candida*, *J. regia*, natural bark, colored bark, antifungal, antioxydant activities.

1. Introduction:

Candida strains are opportunistic pathogens associated with local and systemic infections in predisposed persons (Chong, 2007). The development of *Candida* vaginitis involves some breakdown of a balance between an aggressive fungal traits and host active immune surveillance that allows *Candida* growth and mucosal colonization (Amouri et al., 2010). It is now thought that women who experience recurrent vulvovaginal *Candida* do so because of persistent infection, rather than re-infection. The aim of treatment in this situation is therefore to avoid the overgrowth of *Candida* that leads to symptoms, rather than necessarily being able to achieve complete eradication or cure.

The difficulties associated with the management of *Candida* infections necessitate the discovery of new antifungal agents in order to widen the spectrum of activity against *Candida*. Plant-derived natural products may offer potential leads to new compounds which could act on these fungi (Lachoria, 1999). In fact, medicinal plants have been used for several years in developing countries as alternative treatments to health problems. Many plant extracts and essential oils isolated from plants have been shown to exert biological activity, which develop a new research opportunity and characterization of antifungal activity of these plants (Chaieb, 2007; Clark, 1990; Darout, 2000; Hajlaoui, 2008; Runyoro, 2006).

The *Juglans* genus (family Juglandaceae) comprises several species and is widely distributed throughout the world. *J. regia* L., or walnut, constitutes an important species of trees. Walnut leaves are considered to be a source of healthcare compounds, and have been intensively used in traditional medicine for the treatment of venous insufficiency and for its antidiarrheic properties (Bruneton, 1993; Darout, 2000; Hajlaoui, 2008; Okemo, 2001; Runyoro, 2006). Antifungal activity of this plant has also been described (Girzu, 1998; Noumi et al., 2010; Valnet, 1992). Some

studies have demonstrated the antimicrobial activity of walnut products, particularly of bark and the specific compound juglone (Alkhawajah,1997; Amaral et al., 2005). In Tunisia, this tree is widely known as the "Swak" tree and its fresh leaves and dried bark used are by women to clean the teeth and to maintain a good oral hygiene. The bark of this tree are collected and treated with yellow dye and dried before use (Noumi et al., 2010). In fact, walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional medicine to treat venous insufficiency and due to its antidiarrheic, antifungal, antibacterial and antioxidant properties (Amaral et al., 2003; Miraliakbari and Shahidi, 2008; Pereira, 2007).

These benefits are usually attributed to the presence of essential fatty acids and tocopherols and especially linoleic, oleic, linolenic, palmitic, and stearic acids Juglone (Amaral et al., 2003) (5-Hydroxy 1,4 naphtoquinone) is one of the most important flavonoides of walnuts green husk (Amaral et al., 2003). Walnut leaf and shell have some medicinal effects, as walnut green husk has antioxidant (Pereira, 2007), antifungal (Noumi et al., 2010) astringent, wart liquidator effects and uses for skin diseases and anemia cures. In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fiber, melatonin (Reiter, 2005), plant sterols (Amaral et al., 2005), folate, tannins, and polyphenols. Plant derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest.

Despite the wide use of *J. regia* L. (walnut) and especially the bark and the leaves in Tunisia, this plant has not received much attention and have not been intensively studied. Therefore, the present work is the first study with aims to evaluate the antifungal and antioxidant properties of natural and colored *J. regia* barks against vaginal *Candida* strains.

2. Materials and methods

2.1. Plant material

The root bark's of Tunisian *J. regia* L. was tested for its antifungal activity against vaginal *Candida* strains. The plant material was purchased from a local supermarket from the region of Menzel Bouzalfa, Nabeul (Tunisia). Harvesting was carried out in December 2010. Two types of bark were tested: natural untreated bark and treated bark with a food yellow dye that also was tested for its anti-*Candida* activity (Figure 1).

A **B**

Figure 1. Plant material: *Juglans regia* L. (A): natural bark; (B): colored bark.

2.2. Plant extracts

The extraction of the various components from (natural and colored *J. regia* bark) was made with four different solvents: water, diluted acetone, ethyl acetate, and methanol. The extracts were prepared by adding 10 g of small-particles of plant material to 100 ml of solvent. The mixtures were kept overnight at room temperature, after which the supernatants were filtered. The solvent was removed under reduced pressure below 45°C to give a crude extract. The crude extract was further dried in a vacuum dessicator over anhydrous copper sulfate to give a dry solid of the extract for bioassay.

2.3. Fungal isolates

A total of 34 *Candida* strains including four species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. parapsilosis*) were used in this study. Twenty eight vaginal *Candida* strains were collected from the vaginal cavity by using a swabbing method. A sterile cotton swab (Nippon Menbo, Tokyo, Japan) was immediately cultured on Sabouraud chloramphenicol agar (Bio-rad, France) and on chromogenic medium *Candida* ID2 for 24 to 48 h at 35°C.

Five type strains (*C. albicans* SC 5314, *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258)

and one *C. krusei* strain (code "Roma") from Italy were also tested.

2.4. Antifungal activity

2.4.1. Disc diffusion method

The anti-*Candida* spp. activity was achieved by the agar–well diffusion method. All *Candida* strains were inoculated onto Sabouraud dextrose agar and incubated for 18 h at 37°C. The yeast cultures were harvested and then suspended in sterile saline (0.8% NaCl) and the cell density was adjusted to 10^7 cells/ml (OD_{540nm}= 0.5).

For the antifungal activity of the plant extracts used in this study, three sterile 6-mm paper discs (Whatman paper no. 3), impregnated with 30 mg of extract (10 µl/disc) at a final concentration of 300 mg/ml were placed on the inoculated surface. The plates were then incubated at 37°C for 18-24 h. The ATCC strains were used as quality control strains. The diameter of the zones of inhibition around each disc were examined after 24 h, measured and recorded as the mean diameter (mm) of complete growth inhibition. As a positive control, 10 µg of amphotericin B (Fungizone, BioBasic, Inc., Toronto, Canada) was used. Tests were done in triplicate and the results are given as the mean average.

2.4.2. Microdilution method for the determination of minimal inhibition concentration (MIC) and minimal fungicidal concentration (MFC) values

Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values were determined for all *Candida* strains. The fungi strains inocula were prepared from 12 h broth cultures and suspensions and were adjusted to 0.5 McFarland standard turbidity. Each extract was dissolved in 10% dimethyl sulfoxide (DMSO) and subjected to a series of dilutions from an initial concentration. The 96 well plates were prepared by dispensing into each well 95 µl of fresh Sabouraud dextrose broth and 5 µl of *Candida* inoculum. *J. regia* barks extracts (100 µl) were added into wells. The last well containing 195 µl of nutrient broth without extracts and 5 µl of the inoculum on each strip was used as negative control. The final volume in each well is 200 µl. The plates were incubated at 37°C for 18 to 24 h and after incubation at 37°C for 24 h, the plates were passed to the visual reading of MIC and MFC. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of the fungi. The MFC values were interpreted as the highest concentration of the sample which showed clear fluid with no development of turbidity and without visible growth.

Amphotericin B (12.5-0.003 mg/ml) was used as a positive control. All of the experiments were conducted in triplicate and average values were calculated using the SPSS v13.0 statistical software package for Windows.

3. Antioxidant properties

3.1. Evaluation of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH according to the protocol described by Prieto et al. (1999). An aliquot (0.1 ml) of plant extract was combined to 1 ml ofreagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95° C for 90 min. After, the mixture had cooled to room temperature; the absorbance of each solution was measured at 695 nm (Anthelie Advanced 2, SECOMAN) against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0–500 µg/ml. All samples were analyzed in triplicate.

3.2. Total phenolic content

Phenolic content was assayed using the Folin-Ciocalteu reagent, following Singleton's method slightly modified (Dewanto et al., 2002). An aliquot (0.125 ml) of appropriately diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin-Ciocalteu reagent. After 3 min, 1.25 ml of Na2CO3 solution (7g/100 ml) were added and the final volume was made up to 3 ml with distilled water. The absorbance was measured at 760 115 nm, after incubation for 90 min at 23°C in dark. Total phenolic content of leaves was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE.g-1 DW) through the calibration curve with gallic acid. The calibration curve range was 0-400 µg.ml-1. Triplicate measurements were taken for all samples.

3.3. Total condensed tannins

Proanthocyanidins were measured using the modified vanillin assay described by Sun et al. (1998).To 50 µl of suitably diluted sample were added 3 ml of methanol vanillin solution and 1.5 ml H_2SO_4 respectively. The mixture was allowed to stand for 15 min at room temperature, and the absorption was measured at 500 nm against solvent as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin g-1 DW.

3.4. Antioxidant activities

3.4.1. DPPH radical–scavenging activity

The antioxidant activity of different solvent extracts was measured in term of hydrogen donating or radical scavenging ability using the stable DPPH method according to the method described by Hanato et al. (1988). The sample was diluted in pure solvent of extraction at different concentrations (10, 20, 100 and 200 µg.ml-1), then 1 ml of each diluted plant extract was added to 0.25 ml of a 0.2 mmol/l DPPH. methanolic solution. The mixture of different extract concentration and DPPH were placed in the dark at

room temperature for 30 min. The absorbance of the resulting solution was then read at 517 nm. The antiradical activity was expressed as IC50 (µg.ml-1). The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0]*100$ (1)

Where A_0 is the absorbance of the control at 30 min, and A1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

3.4.2. Determination of reducing power

The ability of the extracts to reduce Fe3+ was assayed by the method of Oyaizu (1986). Briefly, 1 ml of *S. persica* extract was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1g/100 ml). After incubation at 50 °C for 25 min, 2.5 ml of trichloroacetic acid (10g/100ml) was added and the mixture was centrifuged at 650x*g* for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of aqueous $FeCl_3$ (0.1g/100 ml). The absorbance was measured at 700 nm. The mean of absorbance values were plotted against concentration and a linear regression analysis was carried out. Increased absorbance of the reaction mixture indicated increased reducing power. EC50 value (mg.ml-1) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as positive control.

3.4.3. β-carotene-linoleic acid model system (β -CLAMS)

The β-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (Koleva et al., 2002). In this study the β -CLAMS was modified for the 96-well micro-plate reader. In brief, the B-carotene was dissolved in 2 ml of $CHCl_3$, to which 20 mg of linoleic acid and 200 mg of tween 40 were added. $CHCl_3$ was removed using rotary evaporator. Oxygenated water (100 ml) was added, and the flask was shaken vigorously until all material dissolved. This test mixture was prepared fresh and using immediately. To each well, 250 µl of the reagent mixture and 35 µl sample or standard solution were added. The plate was incubated at 45°C. Readings were taken at 490 nm using visible/UV microplate kinetics reader (EL x 808, Bio-Tek instruments) All samples were prepared and analyzed in triplicate.

4. Results

In this study, we tried to determine the antioxydant activity of *J. regia* extracts and anti-*Candida* of natural and colored *J. regia* bark extracts *in vitro* by the disk diffusion method (Table 1) based on the determination of inhibition on solid medium (agar Sabouraud- Chloramphenicol) and determination of MIC and MFC (Table 2) on liquid medium (Sabouraud broth). The results of the antifungal activity were illustrated in Tables 1 and 2.

In the present study, the three extraction solvents (methanol, ethyl acetate and diluted acetone) used for *J. regia* L. were active against all *Candida* strains, but their effectiveness varied between the strains. Aqueous extract had no antifungal activity against all vaginal *Candida* isolates and also against type strains. The some result has been observed for the tested alimentary yellow dye that showed the absence of anti-*Candida* activity. Vaginal *C. albicans*was the most sensitive one. The zones of inhibition ranged from 7-12.33 mm (diluted acetone extract) to 7.66–14 mm (methanol extract) and from 9 to 18.66 mm for ethyl acetate extracts.

The colored bark with yellow dye showed no activity against vaginal *Candida* strains except methanol extract that presented antifungal activity against some strains (example: IZ = 11.66 mm for vaginal *C. albicans* strain "24"). We noted that the aqueous extract had no activity for natural and colored bark and against all *Candida* isolates (e.g. IZ = 7 mm for vaginal *C. albicans* strain "19") as shown in Figure 2 and table 1.

Vaginal *C. tropicalis* (strain 1) and *C. krusei* (strain 3') were sensitive for all extracts (water, methanol, ethyl acetate and diluted acetone) of natural and colored *J. regia* barks. These results were demonstrated by the diameter of inhibition zone on solid medium (e.g IZ= 7 mm for vaginal *C. tropicalis* strain with methanolic extract of natural *J. regia* bark and IZ= 6 mm for vaginal *C. krusei* strain with methanolic extract of colored *J. regia* bark).

According to the results obtained on solid medium (disk diffusion method), we chose to determine the MIC and MFC values to the most active extract against the majority of *Candida* isolates: the methanol extract of the *J. regia* natural bark by the turbidity test. The most important of MICs and MFCs values of methanol extract were obtained for almost of vaginal *Candida* isolates (MIC = 0.012 mg/ ml and MFC = 0.024 mg/ml) compared to amphotericin B (MIC = 0.097 mg / ml and MFC: 0.78 mg/ml). All these results were summarized in Table 2. Our study showed that low concentrations of the methanol extract of natural *J. regia* bark could partially (MIC) and completely (MFC) inhibit growth of vaginal *Candida* species (Table 2).

This is the first time that the antioxidant activities and the identification of polyphenols in the natural and treated bark of *J. regia* were evaluated. In fact, special attention is accorded to nuts due to their regular consumption and their benefits. In Tunisia, *J. regia* barks were treated with a yellow dye and dried before its use to clean the mouth.

Table 1. Antifungal activity of aqueous, methanol, ethyl acetate, and diluted acetone extracts of natural and colored *Juglans regia* L. barks

Strains	Inhibition zone as diameter in mm (mean ± SD) around the discs impregnated with 10µl (300µg/ disc) of plant extract and antifungal drug										
	Natural bark				Colored bark				C		AmB
	1	2	3	4	1	2	3	4	(30 mg/ml)		(10 µg/ml)
C. albicans											
ATCC 90028	12±0	10±0	9.33±0.57	7±0	7±0	9±0	10±0	6±0	7±0		7±0
3	11.66±0.57	10±0	10±0	7±0	7±0	11±0	8±0	6±0	7±0		8.66±0.57
4	11±0	10.66±0.57	12±0	7±0	8±0	9±0	8.66±0.57	7±0	9±0		11±0
6	12.66±0.57	11±0	12.33±0.57	8±0	8±0	10.33±0.57	11.33±0.57	6±0	7±0		9±0
7	11.33±0.57	10.33±0.57	11.33±0.57	7±0	8±0	10.66±0.57	10.33±0.57	6±0	7±0		8±0
10	8±0	9±0	11±0	6±0	7±0	10±0	8.33±0.57	6±0	6±0		12±0
14	11±1	8±0	9±0	7±0	7±0	8±0	8±0	6±0	6±0		8±0
15	12.33±0.57	10.33±0.57	11±0	6±0	7±0	9±0	11.33±0.57	6±0	6±0		7±0
16	12±0	10±0	13±0	7±0	8±0	9±0	10.66±0.57	7±0	7±0		7±0
18	13.66±0.57	11±0	11.33±0.57	7±0	8±0	11±0	10.66±0.57	6±0	8±0		9.33±0.57
19	8±0	8±0	8±0	7±0	6±0	7±0	9.33±0.57	7±0	7±0		8±0
20	12.66±1.15	10±0	12±0	7±0	7±0	10±0	10±0	6±0	7±0		7±0
22	12.66±0.57	12±0	10.33±0.57	7±0	10±0	10±0	8.33±0.57	6±0	7±0		8±0
23	10.66±0.57	9.33±0.57	10±0	6±0	7±0	8±0	11.66±0.57	6±0	6±0		8±0
24	11±1	9.33±0.57	12.66±0.57	6±0	7±0	8.66±0	11.66±0.57	7±0	6±0		8±0
SC 5314	9.66±0.57	9±0	10.66±1.15	7±0	8±0	9±0	8.66±0.57	6±0	6±0		7.33±0.57
C. glabrata											
ATCC 90030	14±0	7±0	9±0	7±0	7±0	7±0	9±0	6±0	6±0		7±0
2	8±0	9.66±0.57	12±0	7±0	7±0	7±0	10±0	7±0	7±0		11±0
5	8±0	7±0	8.33±0.57	7±0	8±0	7±0	7.66±0.57	7±0	7±0		9±0
8	14.33±0.57	10±0	11.66±0.57	9.66±0.57	9±0	10±0	11.33±0.57	6±0	7±0		9.66±0.57
9	11.66±0.57	12±0	9.33±1.15	12±0	10±0	9±0	11.33±0.57	7±0	7±0		10±0
11	8±0	7±0	8±0	7±0	7±0	7±0	8±0	6±0	6±0		7±0
12	8.33±0.57	8±0	11.66±0.57	7±0	7±0	7±0	9±0	7±0	7±0		10±0
13	8.33±0,57	8±0	9±0	7±0	7±0	9±0	8±0	6±0	6±0		9±0
14’	9,66±0.57	7±0	9±0	7±0	7±0	7±0	8±0	6±0	7±0		9±0
15’	8±0	10.66±0.57	11±0	7±0	7±0	7±0	9±0	7±0	7±0		9±0
17	14.66±0.57	12±0	13.66±0.57	8±0	9.33±0.57	11.66±0.57	11.33±0.57	6±0	7±0		9.66±0.57
21	12.33±0.57	11±0	12±0	7±0	7±0	10.33±0.57	9.66±0.57	6±0	8.66±0.57		8±0
25	8±0	8±0	8±0	8±0	7±0	8±0	7±0	8±0	7±0		6±0
C. parapsilosis											
ATCC 22019	12.66±0.57	8±0	9.66±0.57	6±0	7±0	7±0	8.66±0.57	6±0	7.33±0.57		8±0
C. tropicalis											
1	7±0	11±0	7±0	6±0	7±0	9±0	8.33±0.57	6±0	6±0		9±0
C. krusei											
3’	10.33±0.57	8±0	12.33±0.57	7±0	8±0	8±0	10±0	6±0	7±0		7±0
ATCC 6258	11.33±0.57	9±0	9±0	7±0	9±0	9.66±0.57	9.33±0.57	7±0	6±0		7±0
Roma	11.33±0.57	9±0	18.33±0.57	6±0	7±0	7±0	8±0	6±0	7±0		7±0

1: ethyl acetate extract**; 2:** diluted acetone extract**; 3:** methanol extract; **4:** water extract; **C :** yellow dye; **AmB:** amphotericin B.

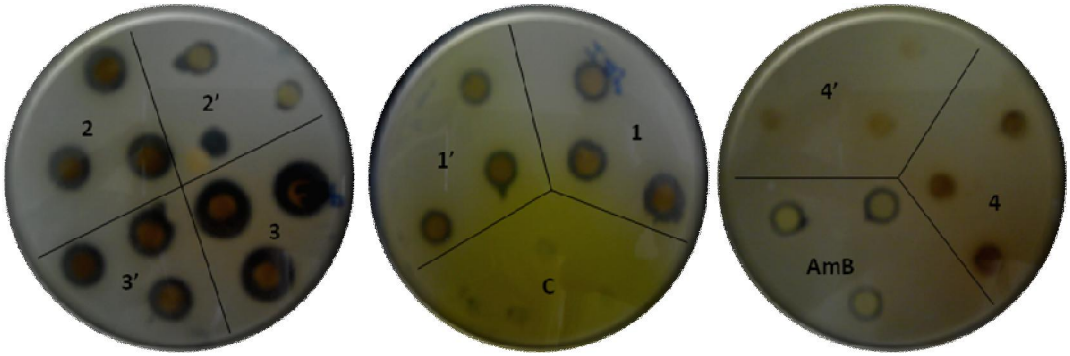

Figure 2. Antifungal activity of *J. regia* bark extracts against *Candida* strains. 1: methanol extract; 2: acetone 80; 3: ethyl acetate extract; 4: water extract; C: yellow dye; AmB: Amphotericin B; figures with (') are the different extracts from the colored bark.

Table 2. MIC and MFC values of *J. regia* L. natural bark methanolic extract tested against vaginal *Candida* strains.					
trains	***J. regia* L. natural bark methanolic extract (mg/ml)**			**Amphotericin B (mg/ml)**	
	MIC	**MFC**		**MIC**	**MFC**
C. albicans					
ATCC 90028	0.0122	0.048		0,097	1.562
3	0.0122	0.195		0,195	3.125
4	0.0122	0.097		0.097	0.39
6	0.0122	0.097		0.097	0.78
7	0.0122	0.097		0.097	0.78
10	0.0122	0.024		0.097	1.562
14	0.0122	0.195		0.097	0.78
15	0.0122	0.097		0.097	0.39
16	0.0122	0.097		0.097	0.78
18	0.0122	0.195		0.097	0.78
19	0.0122	0.097		0.097	0.39
20	0.0122	0.195		0.097	0.78
22	0.0122	0.097		0.097	0.39
23	0.0122	0.097		0.097	0.78
24	0.0122	0.097		0.097	0.78
SC 5314	0.0122	0.097		0.097	0.78
C. glabrata					
ATCC 90030	0.0122	0.048		0.097	0.78
2	0,024	0.048		0.195	1.562
5	0.0122	0.097		0.097	0.78
8	0.0122	0.097		0.39	1.562
9	0.0122	0.097		0.097	1.562
11	0.0122	0.097		0.097	0.78
12	0.0122	0.048		0.097	1.562
13	0.0122	0.195		0.097	1.562
14’	0.0122	0.195		0.195	3.125
15’	0.0122	0.097		0.097	0.78
17	0.0122	0.048		0.097	0.39
21	0.0122	0.39		0.097	0.78
25	0.0122	0.048		0.195	1.562
C. parapsilosis					
ATCC 22019	0.0122	0.097		0.39	0.78
C. tropicalis					
1	0.0122	0.097		0.097	0.78
C. krusei					
3’	0.0122	0.097		0.097	0.78
ATCC 6258	0.0122	0.097		0.097	0.78
Roma	0.0122	0.097		0.195	1.562
MIC: Minimal inhibitrice concentration; MFC: Minimal fungicidal concentration.					

Table 3. Antioxidant activities of extracts of *J. regia* natural and treated barks.											
Antioxidant activities	**Untreated barks**					**Colored barks**				**C**	**BHT**
	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**		
TAA: (mg GAE.g^{-1} DW)	194.66	420.66	334	173.33		160	370	254	372.66	342.66	-
Total Polyphenols: (mg GAE.g^{-1} DW)	138.5	311.5	172.5	182		197	247.5	160.5	249.5	-	-
Tannins : (mg EC.g^{-1} DW)	16	38.5	11.03	7.86		36	34.33	15.16	15.16	-	-
DPPH: IC_{50} (µg.ml^{-1})	36	4.8	13.6	14		11.2	5.8	14.4	11	-	11.5
β- carotenes: IC_{50} (µg.ml^{-1})	1105.3	790	730	3800		600	470	1150	620	-	75
RP: EC_{50} (µg.ml^{-1})	700	220	560	913.8		600	230	590	320	-	75
TAA: Total Antioxidant Activity is expressed as mg gallic acid equivalents per gram of dry weight; **Total Polyphenols** is expressed as mg gallic acid equivalents per gram of dry weight; **Tannins** is expressed as mg (+)-catechin/g DW; **DPPH** radical–scavenging activity is expressed as IC_{50} values (µg/ml); **β-Carotens** bleaching test is expressed as IC50 values (µg.ml-1); **RP**: reducing power was expressed as EC_{50} values (µg/ml). **1:** ethyl acetate extract**; 2:** diluted acetone extract**; 3:** methanol extract; **4:** water extract; **C:** yellow dye.											

The results of the antioxidant properties of *J. regia* barks were summarized in table 3. The study reveals that the antioxidant activity of "Swak" extract was more important for natural bark than treated bark and for the majority of activities. In fact, the TAA was 194.66 mg GAE.g-^{1} DW for natural *J. regia* bark and only 160 mg GAE.g-^{1} DW for treated bark and the total phenolic content was 33,833 mg GAE.g-1 DW. Similarly, β- carotenes of ethyl acetate extract was estimated at IC_{50}= 1105.3 µg.ml^{-1} for natural bark and only 600 µg.ml^{-1} for colored bark. For the methanolic extract, all the antioxydant activities tested (TAA, total polyphenols, tannins, DPPH, β- carotenes and RP) were more important for treated bark than natural bark (Table 3). Indeed, the concentration of β- carotenes was estimated at IC_{50}= 1150 µg.ml^{-1} for treated bark and only 730 µg.ml^{-1} for colored bark. TAA, total polyphenols, tannins and DPPH activities were more important for treated *J. regia* bark for the four solvent. In the other side, the antioxidant activity measured by the bleaching of β-carotenes and the reducing power assay showed important results for natural plant. The total antioxydant activity (TAA) of the alimentary yellow dye was 342.66 mg GAE.g-^{1} DW and important than those of both natural and colored barks. Our results showed that the values of DPPH, β-carotenes and RP were about IC_{50}= 11.5 µg.ml^{-1}, IC_{50}= 75 µg.ml^{-1} and EC_{50}= 75 µg. ml^{-1}respectively for the standard BHT and less important than the plant extracts as shown in table 3.

In conclusion for these antioxydant activities, the TAA was more important for diluted acetone natural bark extract (420.66 mg GAE.g-^{1} DW), also for total polyphenol (311.5 mg GAE.g-^{1} DW) and tannins (38.5 mg EC.g^{-1} DW). Whereas, the antioxidant activity measured by the bleaching of β-carotenes and DPPH assay showed important results for in ethyl acetate natural bark (IC50= 11.05 µg.ml^{-1} and IC50= 36 µg.ml^{-1} respectively). For the last tested antioxydant activity (the RP), the EC50 was maximum for natural bark aqueous extract and equal to 913.8 µg.ml^{-1}.

5. Discussion

The activity of plant extracts against bacteria has been studied for many years. In this idea, several Chinese, African, and Asian plant extracts have been evaluated for their antimicrobial and antifungal activities (Al Bagieh, 1997). The present study was conducted for the first time in order to investigate the antifungal and antioxydant activities of natural and treated *J. regia* L. barks extracts against vaginal *Candida* isolates.

Our study showed that the three extraction solvents (except water) of *J. regia* L. were active against all *Candida* strains, but their effectiveness varied. Methanolic extract of natural bark was the more effective extraction solvent and *C. albicans* was the most sensitive species. Some studies have demonstrated the antimicrobial activity of walnut products, particularly of bark (Alkhawajah, 1997), and the specific compound juglone (Amaral et al., 2005). Pereira et al. (2007) proved that the bark of walnut were not active on *C. albicans* strains. This plant was well characterized for its antioxidant activities but not for its antifungal properties, especially against *Candida* species. The results of the present work, and for its inexpensive cost, show that the use of *J. regia* L. can help developing countries limit oral infections, especially oral candidiasis.

In fact, walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional medicine to treat venous insufficiency and due to its antidiarrheic, antifungal, antibacterial and antioxidant properties (Amaral et al., 2003). These benefits are usually attributed to the presence of essential fatty acids and tocopherols and especially linoleic, oleic, linolenic, palmitic, and stearic acids (Amaral et al., 2008). Juglone (5-Hydroxy 1,4naphtoquinone) is one of the most important flavonoides of walnuts green husk (Cosmulescu et al., 2009; Jaimand et al., 2004). Walnut leaf and shell have some medicinal effects, as walnut green husk has antioxidant (Pereira, 2007), antifungal (Noumi et al., 2010), astringent, wart liquidator effects and uses for skin diseases and anemia cures. In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fiber, melatonin (Reiter, 2005), plant sterols (Amaral et al., 2005), folate, tannins, and polyphenols. Plant derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest.

And, additionally to the known molecules with benefic interest for Human health described in the different organs of *J. regia* tree, the identified polyphenols in the present work contribute to highlight and reinforce the large scale of biological properties of this plant and therefore suggested that *J. regia* can be used as an inexpensive and easily accessible source of effective natural antioxidants and chemo-preventive agents.

Indeed, we have demonstrated in a last work (Noumi et al., 2012) that the extract of "Swak" contains 24 compounds which the percentage varies from 1.37 to 15.02% among them there are: caffeic acid (15,02%), rutin trihydrate (12.71%), syringic acid (3.44%), gallic acid (2.58%) and 55 compounds with a percentage varying from 0,02 to 0,93% among them there are: chlorogenic acid (0.8%), resorcinol (0.78%), vanillic acid (0.77%), naphtho-resorcinol (0.68%), quercetin dihydrate (0.57%), *p*-coumaric acid (0.56%), trans-cinnamic acid (0.53%) and catechine hydrae (0.18%). Our results showed the presence of several

compounds with known antioxidant properties and we noted that caffeic acid, rutin trihydrate, syringic acid and gallic acids were the predominant compounds. In fact, walnuts contain several phenolic compounds which are thought to contribute to their biological activities (Table 4). The identified polyphenols and flavonoids possess anti-inflammatory, antimutagenic, anticancer, antioxidant, anti-bacterial, anti-viral (an important anti-HIV capacity), anti-allergic, antihypertensive, anti-arthritic and many other biological activities. The tannins (such as condensed tannins) have various therapeutic effects through their antibacterial, antiviral, anti-carcinogenic, anti-inflammatory and anti-allergic (Table 4). In fact, no study was found to report the antioxidant activities of the treated *J. regia* bark either in Tunisia or from the rest of the world.

Table 4. Review of the chemical composition and the biological activities of natural *J. regia* bark.

Organ	Chemical composition/References	Biological activities/References
Bark	*Tannins, polyphenols and flavonoids (Alkhawajah 1997). *Polyphenols in the treated bark: caffeic acid, rutint rihydrate, syringic acid, gallic acid, chlorogenic acid, resorcinol, vanillic acid, naphtho-resorcinol, quercetin dihydrate, *p*-coumaric acid, trans-cinnamic acid and catechine hydrae (This study).	Antibacterial activities (Alkhawajah 1997). Antifungal activities (Noumi et al., 2010) Antioxidant activities (**This study**)

6. Conclusion

In conclusion, the results of this study represent the first comparison between biological properties of natural and treated Tunisian *J. regia* bark. Methanolic extract of natural walnut possess high anti vaginal *Candida* strains. This study reveals that the antioxidant activity of "Swak" extract was more important for natural bark than treated bark and for the majority of activities correlated with a high concentration of tannins and polyphenols. These results suggested that *J. regia*can be used as an inexpensive and easily accessible source of effective natural antifungal and antioxidant agent. Our study demonstrated that *J. regia* can also be used to treat vaginal candidiasis.

References

1. AlBagieh, N.H., Almas, K. 1997. *In vitro* antibacterial effects of aqueous and alcohol extracts of miswak (chewing sticks). Cairo. Dent. J., 13, 221-224.
2. Alkhawajah AM (1997). Studies on the antimicrobial activity of *Juglans regia*. Am. J. Chin. Med., 25:175-180.
3. Amaral JS, Casal S, Pereira J, Seabra R, Oliveira B (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. J. Agric. Food. Chem., 51: 7698-7702.
4. Amaral JS, Casal S, Seabra RM, Oliveira BPP (2005). Development and evaluation of a normal phase liquid chromatographic method for determination of tocopherols and tocotrienols in walnuts. J. Liq. Chromatogr. Relat. Technol., 28: 785-795.
5. Amaral JS, Valentão P, Andrade PB, Martins RC, Seabra RM (2008). Do cultivar, geographical location and crop season influence phenolic profile of walnut leaves? Molecules., 13: 1321-1332.
6. Amouri I, Abbes S, Sellami H., Makni F, Sellami A and Ayadi A (2010).Vulvovaginal candidiasis: A review. J. Mycol. Méd., 20: 108-115.
7. Bruneton J (1993). Pharmacogosie, phytochimie, plantes médicinales. Technique & Documentation, Lavoisier, Paris, p 348
8. Chaieb K, Zmantar T, Ksouri R (2007). Antioxidant properties of the essential oil of *Eugenia caryophyllata* and its antifungal activity against a large number of clinical *Candida* species. Mycoses., 50:403-406.
9. Chong PP, Abdul Hadi SR, Lee YL (2007). Genotyping and drug resistance profile of *Candida* spp. in recurrent and one-off vaginitis, and high association of non-albicans species with nonpregnant status. Infect. Genet. Evol., 7:449-456
10. Clark AM, Jurgens TM, Hufford CD (1990). Antimicrobial activity of juglone. Phytother. Res., 4:11-14.
11. Cosmulescu SA, Baciu G, Achim M, Trandafir I (2009). Mineral composition of fruits in different walnut (*Juglans regia* L.) cultivars. Not. Bot. Hort. Agro. Cluj-Napoca., 37(2):156-160.
12. Darout IA, Christy AA, Skaug N (2000). Identification and quantification of some potentially antimicrobial anionic components in miswak extract. Ind. J. Pharmacol., 32:11-14.
13. Dewanto V, Wu X, Adom KK, Liu RH (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food. Chem., 50: 3010-3014.
14. Girzu M, Carnat A, Privat AM (1998). Sedative effect of walnut leaf extract and juglone, an isolated constituent. Pharm. Biol., 36:280-286.

15. Hajlaoui H, Snoussi M, Ben Jannet H (2008). Comparison of chemical composition and antimicrobial activities of *Mentha longifolia* L. ssp. longifolia essential oil from two Tunisian localities (Gabes and Sidi Bouzid). Ann. Microbiol., 58(3):513–520.
16. Hanato T, Kagawa H, Yasuhara T, Okuda T (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem. Pharm. Bull., 36: 2090-2097.
17. Jaimand K, Baghai P, Rezaee MB, Sajadipoor SA, Nasrabadi M (2004). Determination of Juglone from Leaves and fresh peels of *Juglans regia* L. by High Performance Liquid Chromatography. Iran. J. Med. Aromat. Plan. Res. 20: 323-331
18. Koleva II, Teris AB, Jozef PH, Linssen AG, Lyuba NE (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal., 13: 8-17.
19. Lachoria R, Jain PC, Agrawal SC (1999). Activity of some plant extracts against dermatophytes. Hindustan Antibiot. Bull., 41:17-21
20. Miraliakbari H, Shahidi F (2008). Oxidative Stability of tree Nut Oils. J. Agric. Food Chem., 56: 4751-4759.
21. Noumi E, Hajlaoui H, Trabelsi N, Ksouri R, Bakhrouf A, Snoussi M (2012). Antioxidant activities and RP-HPLC identification of polyphenols in the ethyl acetate extract of Tunisian *Juglans regia* L treated barks. JMPR., 6(8):1468-1475.
22. Noumi E, Snoussi M, Hajlaoui H, Valentin E, Bakhrouf A (2010).Antifungal properties of *Salvadora persica* and *Juglans regia* L. extracts against oral *Candida* strains. Eur. J. Microbiol. Infect. Dis., 29: 81-88.
23. Okemo PO, Mwatha WE, Chhabra SC (2001).The kill kinetics of *Azadirachta indica* A. Juss. (Meliaceae) extracts on *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Afr. J. Sci. Technol., 2:113-118.
24. Oyaizu M (1986). Studies on products of browning reaction: Antioxidative activity of products of browning reaction. Jpn. J. Nutr., 44: 307-315.Pereira JA, Oliveira I, Sousa A (2007). Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. Food. Chem. Toxicol., 45:2287-2295.
25. Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.
26. Reiter RJ, Manchester LC, Dun-Xian Tan (2005). Melatonin in walnuts: influence on levels of melatonin and total antioxidant capacity of blood. Nutrition., 21: 920-924.
27. Runyoro DKB, Matee MIN, Ngassapa OD (2006). Screening of Tanzanian medicinal plants for anti-Candida activity. BMC Complement Altern. Med., 6:11.
28. Sun B, Richardo-Da-Silvia JM, Spranger I (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food. Chem., 46: 4267-4274.
29. Valnet J (1992). La phytothérapie: traitement des maladies par les plantes. Maloine, Paris, pp 476-478.

5/6/2014