J. Mater. Environ. Sci. 5 (3) (2014) 823-830
ISSN : 2028-2508
CODEN: JMESCN

Chemical Composition and Antifungal Activity of *Vitex agnus-castus l.* Seeds Oil Growing in Morocco

A. Asdadi[1*], L.M. Idrissi Hassani[1], B. Chebli[2], R. Moutaj[3], S. Gharby[4], H. Harhar[4], R. Salghi[2], M. EL Hadek[5]

[1]*Laboratoire de Biotechnologies Végétales, Equipe Planta Sud, Faculté des Sciences d'Agadir, Université Ibn Zohr, B.P 28/S, Agadir, Maroc.*
[2]*Laboratoire de Mécanique de Procédé de l'Energie et de l'Environnement, Ecole Nationale des Sciences Appliquées, Université Ibn Zohr, BP 1136, Agadir, Morocco.*
[3] *Service de Parasitologie et Mycologie Hôpital Militaire Avicenne, CHU Med VI. Marrakech 40 000. Maroc.*
[4]*Laboratoire de Chimie des Plantes et de Synthèse Organique et Bioorganique, Faculté des Sciences, Université Mohammed V-Agdal, BP 1014- Rabat, Morocco.*
[5]*Laboratoire de Génie des Procédés, Faculté des Sciences d'Agadir, Université Ibn Zohr, B.P 28/S, Agadir, Maroc.*

Received 28 Dec 2013, Revised 31 Jan 2014, Accepted 31 Jan 2014.
[*]Corresponding Author. E-mail: asdadiali@gmail.com; Tel: (+212661093000)

Abstract
The present study describes the chemical composition and the antifungal activity of *Vitex agnus-castus L.* oil extracted from seeds using Soxhlet apparatus. The *Vitex* seeds oil contains significant quantity of fixed oil (7.9 ± 0.75 %). Unsaturated fatty acids are the major component of *Vitex* oil. Oleic and linoleic acids constitute 87% of the fatty acids. While, very low percentage of linolenic acid was found in *Vitex* seeds oil (<0.4%). β-sitosterol and stigmasterol were among the major components, together constituting about 69 % of total sterols. In *Vitex* seeds oil, cholesterol was found at about 0.1% of the total sterols. *Vitex* oil has a strong antifungal activity compared with the conventional fungicides. The MIC_{50}, MIC_{90} and the Minimal Fungicidal Concentration (MFC) were respectively 1.75, 7 and 7 (mg/ml) against all tested *Candida* species isolated from nosocomial infections in hospitals. Our findings demonstrate that *Vitex* oil possesses antifungal activity that might be a natural potential source of antifungal used in food, in cosmetics and in pharmaceuticals products.

Keywords: Antifungal activity, *Candida* species, Fatty acids, *Vitex agnus-castus L.*, Vitex oil.

1. Introduction
The incidence of fungal infections has dramatically increased during the past two decades due to several factors, such as increased numbers of immunocompromised patients and the widespread use of broad-spectrum antibiotics [1-7]. Systemic candidiasis is fourth among nosocomial infections with a percentage of 10 to 15% [8, 9]. At this time *Candida albicans* is the most frequently isolated yeast pathogen, but *C. glabrata* is rapidly emerging as a common cause of nosocomial yeast infection. Other pathogenic yeast species isolated from clinical samples include *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. famata*, *C. lusitaniae*, *C. guilliermondii*, *C. norvegensis*, etc though these are isolated less frequently in comparison to *C. albicans* and *C. glabrata* [4, 8, 10-14]. It should also be mentioned that *C. krusei* whose emergence is attributed to it primary resistance to fluconazole [4, 14-17], A new species of Candida with similar phenotypic characteristics to those of *C. albicans* was identified in 1995 and was named *C. dubliniensis* that less sensitive than *Candida albicans* to azole antifungals especially fluconazole [14, 18, 19].
Emerging fungal infections are therefore a major challenge for health professionals, a poor prognosis [20, 21], and resistance to conventional antifungal used in current hospital practices [15, 17]. Despite therapeutic advances marked by the emergence of new antifungal molecules to high cost of taken into load [4], the mortality rate is still around 50% [8, 22-24]. Fluconazole and amphotericin B are still the antifungal agents of choice commonly used in infections related to Candida species; however they are known to have side effects and high

toxicity, in addition to emerging resistance among clinical isolates of *Candida albicans*. Therefore, it is necessary to isolate new antifungal agents, mainly from plant extracts. In recent years, some researchers have focused on the use of components made from extracts of plants that exhibit biological activity *in vitro* and *in vivo*, thereby justifying based research on traditional medicine for their characterization activity looking for molecules that are both highly effective antifungal, with fewer side effects, very tolerable by the human body and less costly [25, 26].

Therefore increasing the resistance to conventional antifungal, toxicity and the costs involved justified the search for new therapeutic approaches. Among these new approaches, fixed oils are one of the groups of promising natural compounds for use in the prevention and treatment of fungal infections. In this study, we attempted to evaluate the antifungal activity of the fixed oil extracted from natural fruits of *Vitex agnus-castus* 'Rosea' against Candida species isolated from patients with nosocomial candidiasis.

Vitex agnus-castus L., common name Vitex and local vernacular name Anguerf, formerly classified in the family of *Verbenaceae*, but now phylogenetic classification situated the plant within the *Lamiaceae* family [27], *V. Agnus-castus* is a small tree or shrub, it has been used medicinally for at least two thousand years ago, Hippocrates recommended the use of chaste tree for injuries and inflammation [27, 28]. This plant is widely distributed in the Middle East and the Southern Europe [29, 30], in the Mediterranean area and common also in Morocco [31]. It has been proven that it has many biological activities. The fruit of Vitex has been reported in the literature not only be used to relieve uterine cramps and to regulate menstruation in women but was also used as a lactogen [30], in the treatment of hyperprolactinaemia and mastalgia [32], as diuretic, as anorectic, hypnotic, for dyspepsia [33], for the treatment of acne [34, 35], antifungal and also against anxiety and early birth [29, 35], locally this plant is widely used for its sedative, antispasmodic, anaphrodisiac as an infusion of fruits and flowering tops [36]. Secondary effects are rare and may include rash, gastrointestinal disorders, headache and increased menstrual flow [30]. In our knowledge, this study is the first report on the chemical composition and antifungal activity of the Moroccan seeds oil of *Vitex agnus-castus* against *Candida* species isolated from nosocomial infections in hospitals.

2. Material and Methods

2.1. Plant materials

Fully ripened seeds of *Vitex agnus-castus* were harvested in July 2012 from the region of Imouzzer Idaouatanane Southwest of Morocco. After harvest, the seeds were stored at 4 °C until extraction. The plant and seeds was identified by Pr. Fouad Msanda, and specimens were deposited in the herbarium of Laboratory of Biotechnology, Planta Sud unity. Faculty of Sciences, Ibnou Zohr University, Agadir, Morocco.

2.2. Oils extraction

All the reagents were of analytical or HPLC grade. Oil extraction was performed according to the AOCS Official method Am 2-93[37]. About 20 g of ground seeds were extracted for 4 h using a Soxhlet apparatus and n-hexane (100 mL) as extraction solvent. The organic phase was then concentrated under vacuum and dried for 5 min in an oven at 103 ± 2 °C. Oil obtained was weighed and stored at 4 °C in a sealed brown vial until use. The yield obtained was 7, 9 %.

2.3. Determination of oil chemical composition

2.3.1. Fatty acid composition of the oil

Fatty acids (FAs) were converted to fatty acid methyl esters (FAMEs) before analysis by shaking a solution of 60 mg oil and 3 mL of hexane with 0.3 mL of 2 N methanolic potassium hydroxide. They were analyzed by gas chromatograph (Varian CP-3800, Varian Inc.) equipped with a FID. The column used was a CP- Wax 52CB column (30 m×0.25 mm i.d.; Varian Inc., Middelburg, The Netherlands).The carrier gas was helium, and the total gas flow rate was 1 mL/min. The initial column temperature was 170 °C, the final temperature 230 °C, and the temperature was increased by steps of 4 °C/min. The injector and detector temperature was 230 °C. Data were processed using Varian Star Workstation v 6.30 (Varian Inc., Walnut Creek, CA, USA). The results were expressed as the relative percentage of each individual fatty acid (FA) presents in the sample.

Iodine values (IV) were calculated from fatty acid percentages [38] using the formula: **Eq.1**

$$\mathbf{IV} = (\%\mathbf{Palmitoleic} \times \mathbf{1.001}) + (\%\mathbf{Oleic} \times \mathbf{0.899}) + (\%\mathbf{Linoleic} \times \mathbf{1.814}) + (\%\mathbf{Linolenic} \times \mathbf{2.737})$$

2.3.2. Sterols composition of the oil
Sterol composition was determined after trimethylsilylation of the crude sterol fraction using a Varian 3800 instrument equipped with a VF-1 ms column (30 m 9 0.25 mm i.d.) and helium (flow rate 1.6 mL/mn) as carrier gas, column temperature was isothermal at 270 °C, injector and detector temperature was 300 °C. Injected quantity was 1 µL for each analysis. Data were processed using Varian Star Workstation v 6.30 (Varian Inc., Walnut Creek, CA, USA).

2.3.3. Physical and chemical parameters of the oil
Acidity index, peroxide value (PV), extinction coefficients (K232 & K270) and refractive index were determined according to AOCS recommended practices Cd 1c-85, Cd 3a-94, Cc7-25, Ca 6a-40 and Ca 5a-40, respectively[37]. Acidity was expressed as the amount of oleic acid as %. PV was expressed as milliequivalents of active oxygen per kilogram of oil (Meq O2/kg oil), and extinction coefficient (K232 & K270) was expressed as the specific extinctions of a 1% (w/v) solution of oil in cyclohexane in 1 cm cell path length, using a CARY 100 Varian UV spectrometer.

2.4. Isolation and identification of microorganisms
The 6 isolates of *Candida* studied in this work include *C. albicans* (n = 12), *C. dubliniensis* (n = 1), *C. glabrata* (n = 7) and *C. krusei* (n = 5). All *Candida* species were clinically isolated from infected patients in the laboratory of parasitology-mycology and bacteriology Avicenna military hospital –Marrakech, MOROCCO. On Sabouraud chloramphenicol agar plates and identified by the germ tube test and an API 20 C AUX (Bio-merieux, marcy-l'etoile, France) according to the Manufacturer's recommendations and chromogenic medium CandiSelect 4 (Bio-RAD, Marnes-la-Coquette, France).For this experiment, all isolated strains were tested and yeast cells were harvested from Sabouraud dextrose agar, supplemented with chloramphenicole, and then counted and adjusted density in serum glucose sterile 5% of $2.16X10^5$ Cells/ml to $5.22X10^5$ Cells/ml with haemocytometer for each repetition.

2.5. Drugs used as controls
The used drugs usually eradicate fungal infections such as those belonging to the family of imidazole agents like Fluconazole and those belonging to the family of polyenes, agents such as Amphotericin B. These antifungal agents are dissolved in 2 ml DMSO 10% to give the following stock solutions: Fluconazole 75 mg/ml and Amphotericine B 33 mg/ml.

2.6. Preparation of fungal suspension
A fresh overnight culture, in log phase, of the tested yeasts was used to prepare the cell suspension by inoculating 5 ml of serum glucose 5% broth with an appropriate yeast strain and incubating for 24 hour at 37°C to ensure that yeast cells were actively dividing, then adjusted between: 2.16×10^5 Cells/ml to 5.22×10^5 Cells/ml for fungal strains with counting with haemocytometer for each repetition.

2.7. Antifungal activity
Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)
The broth macro dilution method was used to determine the minimum inhibitory concentration (MIC) according to NCCLS M38P [42] for yeasts. The fungal suspension was then used to inoculate the tubes in the test group. The test groups were prepared with 1 ml of medium containing 10 µl of dimethyl sulfoxide (DMSO) 10%, the final concentration never exceeded 2%, and 200 µl of each strain of yeast suspension previously adjusted. 10 µl of *Vitex agnus-castus* seeds oil (700 mg / ml), 10 µl of fluconazole (75 mg / ml) and 10µl of amphotericin B (33 mg / ml) were respectively added to tubes containing culture medium in the test groups, then adding a sufficient amount of glucose 5% to a final volume of 1 ml and serial dilutions and concentration gradient is established as follows: *Vitex agnus castus* L. seeds oil of 7 mg / ml to 0.875 mg / ml, Fluconazole 0.75 mg / ml to 0.09375 mg / ml and Amphotericine B of 0.33 mg / ml to 0.04125 mg /mL . The test tubes were incubated at 37 ° C on a shaker ELISA plates for 48 hours, and the MICs were determined. The MIC was defined as the lowest concentration of the oil at which the microorganism did not demonstrate visible growth. The growth of micro-organisms is evaluated counting with haemocytometer for each repetition.
To determine the minimum fungicidal concentration (MFC), aliquots (20 µl) of broth were taken from each negative after reading MIC tube, and cultured in the Sabouraud agar plates (SDA) and incubated at 37 ◦C for 48 h. The MFC was defined as the lowest concentration of the essential oil at which the microorganism was

incubated completely killed. Each test was performed in triplicate. Fluconazole and amphotericin B were used as positive controls antifungal.
For each strain tested, the growth conditions and the sterility of the medium were checked in two control tubes. The safety of DMSO was also checked at the highest tested concentration. All experiments were performed in triplicate and repeated if the results are different.

2.8. Statistical analysis
Statistical analysis was performed using a statistical package, SPSS windows version 19, by applying mean values using one-way ANOVA with post-hoc Newman-Keuls. A P value of less than 0.05 was considered significant.

3. Results and Discussion

3.1. Physicochemical characterization of Vitex agnus-castus L. seeds oil
Vitex seeds oil contains significant quantity of fixed oil that needs further investigation to be explored as a source of edible oil. The oil content was found to be 7.9 ± 0.75 % (Table 1). Physicochemical properties of the Vitex seeds oil are shown in Table 1. Physical properties of lipids derive directly from their chemical structures and functional groups and greatly influence the functions of lipids in foods and the methods required for their manipulation and processing. They can also be used to assess the purity or quality of lipid material in reference to known standards or preferred characteristics [43]. The Vitex seeds oil was dark greenish brown peppery taste. It was liquid at room temperature and in the refrigerator heavy viscous. Refractive index is used by most processors to measure the change in unsaturation as the fat or oil is hydrogenated. The refractive index of oils depends on their molecular weight, fatty acid chain length and degree of unsaturation [43]. The Vitex seeds oil showed a refractive index of 1.467 ± 0.003. Pure oils have marked ranges of refractive index and density; thus, the degree of variation of typical oil from its true values may indicate its relative purity.
Spectrophotometric measurements are widely used in quality assessments. The K232 is usually considered as an indicator of the oil autoxidation and has been well correlated with peroxide value, but the K270 is a more useful quantity that measures the presence of conjugated dienes and trienes [44] . Furthermore, both measurements have been used to determine the addition of oil to pure ones [45]. As can be seen in Table 1, the K232 and K270 of vitex seed oil were 1.88 ± 0.05 and 1.37 ± 0.05 respectively. Markovic and Bastic found that oils with the same peroxide values show different specific extinctions and the Vitex seeds oil had specific extinctions considerably slightly higher than those of other vegetable oils at both wavelengths [46]. Considering the content of free fatty acids (2.73 ± 0.01 % as oleic acid), acid value (5.46 ± 0.01 mg KOH/g oil) and peroxide value (5.5 ± 0.5 meq O_2/kg oil) (Table 2), the extracted Vitex seeds oil had an acceptable initial quality. The Codex Alimentarius Commission expressed the permitted maximum acid values of 10 and 4 mg KOH/g oil for all vegetable oil [47]. On the other hand, according to the Codex Alimentarius Commission, the peroxide value for virgin olive oil may be maximum 20 meq/kg oil [48] and virgin argan oil 15 meq/kg oil [44, 49]. Therefore, considering that the oil studied was unrefined and its initial quality indicators were within the reported limits, the Vitex seeds oil can be regarded as edible oil with high quality. The Vitex seeds oil had an iodine value of 142.23 ± 0.0 (Table 2), indicating a high degree of unsaturation. This value is comparable with that of other seeds oils such as argan oil (102.5), olive oil (90.2), soybean seed oil (134.5 ± 0.5) and Sunflower oil (130 ± 0.5) [50].

Table 1. Physicochemical characterization of the Moroccan *Vitex agnus-castus L.* seeds oil.

Oil yield (%)	7.9 ± 0.75
Free fatty acids (%)	2.73
PV (Meq/kg)	5.5
E232	1.885
E270	1.37
Iodine value (g of I_2/100 g of oil)	142.23
Refractive index at 20 °C	1.4671 ± 0.003

3.2. Fatty acid composition of Vitex agnus-castus L. seeds oil

Fatty acid composition determination is an important characteristic for vegetable oils [51]. Data regarding fatty acid composition of Vitex oil is presented in Table 2. Unsaturated fatty acids are the major component of Vitex oil. Values are listed in Table 2. Together oleic and linoleic acids constitute 87% of the fatty acids. Linolenic acid was detected at a low level in Vitex seeds oil (<0.4%) but another survey can be found in literature [52], where linolenic acid is described as one of the major compounds of the fatty acids. The major unsaturated fatty acid detected was linoleic acid followed by oleic acid (Table-2). Vitex seed oil constitutes an important source of polyunsaturated fatty acid (PUFA) (70.5%), while olive oil presents a high content in monounsaturated fatty acids (MUFA) (over (61-80%). Vitex seeds and sunflower oil present a similar level of linoleic acid [50]. In this study, saturated fatty acids accounted for 17 % of total fatty acids. Among them, the main saturated normal chain fatty acids were palmitic (6.18%), stearic (4.23%) and arachidic (0.5%). The dietetic value of the oil is high as the total unsaturated fatty acids/total saturated fatty acids ratio is approximately 7.52, which is similar to that of olive oil. It is worth mentioning that the high amount of linoleic acid makes Vitex seed oil specifically prone to oxidation. Yet, this fatty acid may have favorable nutritional implications and beneficial physiological effects in the prevention of both coronary heart disease and cancer [53].

Table 2. Fatty acid composition of the Moroccan *Vitex agnus-castus L.* seeds oil.

Fatty Acid (%)	% of total fatty acids	Fatty Acid (%)	% of total fatty acids
Myristic Acid (C14:0)	0.1 ± 0.1	Linolenic Acid (C18:3)	0.35 ± 0.1
Palmitic Acid (C16:0)	6.18 ± 0.1	Arachidic Acid (C20:0)	0.5 ± 0.1
Palmitolic Acid (C16:1)	0.85 ± 0.1	Gadoleic Acid (C20:1)	ND
Stearic Acid (C18:0)	4.23 ± 0.1	Behenic Acid (C22:0)	0.6 ± 0.1
Oleic Acid (C18:1)	16.41 ± 0.1	Total saturated fatty acids (TSFA)	11.61 ± 0.5
Linoleic Acid (C18:2)	69.75 ± 0.1	Total unsaturated fatty acids (TUFA)	87.36 ± 0.5

Values are given as means of three replicates ± SD.

3.3. Sterol Composition

Sterols constitute a sizeable proportion of the unsaponifiable matter in vegetable oil. Investigated sterol profile of seeds oil of Vitex oil is given in Table 3. In Vitex seeds oil, β-sitosterol and Stigmasterol were among the major components, together constituting about 69 % of total sterols. In the studied Vitex seeds oil, the sterol marker was β-sitosterol, which constituted 59.28% of the total sterol content. β-sitosterol is the sterol marker in extra virgin olive oil and ranges from 75 to 87% of total sterols [54]. High β-sitosterol content was also found in the majority of vegetable oil such as olive oil, soybean seed oil, groundnut oil and sunflower oil, in which the mean relative contents were 84.3, 69.2, 62.3 and 61.9% of total sterols, respectively [49]. The next major component is the Stigmasterol, constituted 10.37% of total sterols in Vitex seeds oil. Existing differences between sterol compositions make them the most suitable for determining the botanical origin of oils and hence detecting adulteration among vegetable oils [55, 56]. Cholesterol is known as being specific to animal lipids. However, it is possible to detect a high cholesterol level in some vegetable oils such as tomato seeds in which cholesterol reaches about 20% of total sterols [57]. In Vitex seeds oil, cholesterol was found at about 0.1% of the total sterols. This value is lower than other vegetable oils as olive, soybean oils (~0.40%) palm oil (2.30%), argan oil (~0.40%) and cactus oil (~0.90%) [50, 57]. Sterols from vegetable oils have been shown to lower total and LDL cholesterol levels in humans by inhibiting cholesterol absorption from the intestine [58, 59]. These finding may lead to the use of Vitex seeds oil sterols as new therapeutic agents for treatment of hypercholesterolemia.

Table 3. Sterol composition of the Moroccan *Vitex agnus-castus L.* seed oil

Sterols	% of total sterols	Sterols	% of total sterols
Cholesterol	0.1± 0.1	β-Sitosterol	59.28± 1.5
Brassicasterol	Nd	Δ-5avenasterol	7.67± 0.5
Campesterol	6.56± 0.2	Δ-7 stigmasterol	0.82± 0.1
Stigmasterol	10.37± 0.5	Δ-7 avenasterol	0.59± 0.1

Values are given as means of three replicates ± SD.

3.4. Antifungal activity

Determination of minimum inhibitory and fungicidal concentration

The results presented in the table 4 show that the Vitex oil has a strong antifungal activity compared with the conventional fungicide. The MIC_{50}, MIC_{90} and the Minimal Fungicidal Concentration (MFC) were respectively 1.75, 7 and 7 (mg/ml) against all tested *Candida* species isolated from nosocomial infections in hospitals (Table 5). We note a very important antifungal activity especially against strains non *Candida albicans* species, this activity extends even to species resistant to conventional antifungal used as control. The antifungal activity of Vitex seeds oil may be attributed to the presence of β-sitosterol and oleic acid as the main components in the oil composition of *Vitex agnus-castus L.*, several studies have shown that long chain fatty acid has a fungistatic activity against a few strains of *Candida* [60, 61]. Also another study showed that different components such as β-sitosterol and stigmasterol has antifungal activity against pathogenic fungi to humans, *Aspergillus flavus*, *A. Niger*, *Geotrichum candidum*, *Candida tropicalis* and *C. albicans* [62]. A fraction composed of other β-sitosterol, isolated from the hexane extract of *Lantana hispida* showed significant antimycobacterial even among strains of *Mycobacterium tuberculosis* multidrug-resistant activity [63] and as antibacterial [64]. Also, previous studies showed that β-sitosterol have antifungal activity, in agreement with the current results [65].

Table 4. Minimum Inhibitory concentrations (MIC_{50} and MIC_{90} [mg/ml]) and Minimum Fungicidal Concentration MFC ([mg/ml]) of *Vitex agnus-castus* seeds oil against *Candida* strains.

MIC and MFC (mg/ml) at 48 h					
Species (Number of isolates)	Vitex oil & Antifungal agent	Range	MIC_{50}	MIC_{90}	MFC
Candida albicans (n=12)	Vitex Oil	7 - 0.875	1.75	7	7
	FLC	0.75-0.09375	0.01172	0.0234375	0.0234375
	AMB	0.33-0.04125	0.0825	0.165	0.165
Candida dubliniensis (n=1)	Vitex Oil	7 - 0.875	3.5	7	7
	FLC	0.75-0.09375	0.75	>0.75	>0.75
	AMB	0.33-0.04125	0.00516	0.01031	0.01031
Candida glabrata (n=7)	Vitex Oil	7 - 0.875	1.75	7	7
	FLC	0.75-0.09375	0.75	>0.75	>0.75
	AMB	0.33-0.04125	0.165	0.33	0.33
Candida krusei (n=5)	Vitex Oil	7 - 0.875	1.75	7	7
	FLC	0.75-0.09375	>0.75	>0.75	>0.75
	AMB	0.33-0.04125	0.165	0.33	0.33

Fluconazole (FLC); Amphotericine B (AmB); *Vitex agnus-castus L.* Seeds oil (Vitex oil).

4. Conclusion

The antifungal activity of the *Vitex agnus-castus L.* seeds oil has been demonstrated even in *Candida* species responsible of nosocomial infections resistant to certain antifungal synthesis , which suggests its use in the future as adjuvant to conventional antifungal therapy. However, this oil should be tested to evaluate its

effectiveness in different environments and cropping systems and also assess its toxicity and safety in clinical and pharmacological trials. This study demonstrates the composition and the antifungal activity of the *Vitex agnus-castus* seeds oil which could be a new source of edible oil and consolidate the idea of using *Vitex agnus-castus L*. oil in foods, in cosmetics and in pharmaceuticals products.

References:

1. Ascioglu S.,Rex J.H., De Pauw B., Bennett J.E., Bille J., Crokaert F., Denning D.W., Donnelly J.P., Edwards J.E., Erjavec Z., Fiere D., Lortholary O., Maertens J., Meis J.F., Patterson T.F., RitterJ., Selleslag D., Shah P.M., Stevens D.A., Walsh T.J. *Clin. Infect. Dis*. 34 (2002) 7.
2. Robert R. Tests rapides pour le diagnostic des parasitoses et des mycoses. *Immuno-analyse & Biologie Spécialisée*. 12 (1997) 232.
3. Chabasse D., Pihet M., Bouchara J.P. *revue francophone des laboratoires*.416 (2009) 71.
4. Toubas D. *Revue Francophone des Laboratoires*. 450 (2013) 27.
5. Marcia G., Nucci M. *Ann Nestlé ed fr .67(* 2009) 135.
6. Silva F., Ferreira S., Duarte A., Mendonça D.I., Domingues F.C . *Phytomedicine* . 19(2011) 42.
7. Buitron-Garcia-Figueroa R.,Araiza-Santibanez J.,Basurto-Kuba E., Bonifaz-Trujillo-A. *Cir.Ciruj*. 77(2009) 423.
8. Eggimann Ph., Pittet D. Schweiz Med Wochenschr. *Candidoses .130(*2000)1525.
9. Yera H., Sendid B., Francois N., Camus D. *Eur. J. Clin. Microbiol. Infect. Dis.*20(2001) 864.
10. Hanna S., Blancard A., De La Roziere J.C., Dumon H., Manelli J.C. *j. mycol. méd.* 13(2003) 61.
11. Rimek D., Singh J., Kappe R. *j. clin. microbiol.* 41 (2003) 3395.
12. Anane S., Kallel K., Kaouech E., Belhaj S., Chaker E. *Ann. Biol. Clin*. 65(2007)13.
13. Bouchoux J. Candidoses: apparition d'espèces émergentes et lute antifongique. Epidémiologie, traitement et résistance croisée entre 5-Fluorocytosine et Fluconazole chez Candida lusitaniae. *Ed. Univ. eur*.(2012) 72.
14. Vazquez J.A., Sobel J.D. *Essentials. Clin. Mycol*., Springer, New York. (2011),
15. Develoux M., Bretagne S. *EMC - Maladies Infectieuses*. 2(2005) 119.
16. Sleth J.C., *Ann. Fr. Anesth.Rèanim.* 26 (2007) 266.
17. Dannaoui É. *revue francophone des laboratoires.*450(2013) 71.
18. Sullivan D.J., Westerneng T.J., Haynes K.A., Bennett D.E., Coleman D.C. *Microbiology*. 141(1995)1507.
19. Perfect J.R., Schell W.A. *Clin Infect Dis*. 22(1996) S112.
20. Anane S., Khalfallah F. *Pathol.Biol*.55(2007) 262.
21. Pihet M., Marot A. *revue francophone des laboratoires*.450(2013) 47.
22. Poulain D. *Lett.Infectiologue 2000*.15(2000)182.
23. GRANIER F. *La Presse médicale*.29(2000) 2051.
24. Mishra B.B., Singh D.D., Kishore N., Tiwari V.K., Tripathi V. *Phytochemistry.* 71(2010) 230.
25. Kumar A., Thakur S., Thakur V.C.,Kumar A., Patil S., Vohra M.P. *JKIMSU*. 1(2012) 61.
26. Agarwal V., Lal P., Pruthi V. *J. Microbiol. Immunol. Infect*. 43 (2010) 447.
27. Chantaranothai P. *Tropical Natural History*.11(2011) 91.
28. Meier B., Allemann C., Kreuter M. H. *Vitex agnus-castus*. (1994)
29. Baytop T. *Istanbul University Pub*.3255(1984).
30. Saglam H., Pabuçcuoglu A., Kivçak B. *Phytotherapy research*. 21(2007) 1059.
31. Bellakhdar J. *Thèse de doctorat.Univ. Metz. (1997).*
32. *Focus on Alternative and Complementary Therapies*.9 (2004) 281.
33. Blumenthal M. Herbal medicine. *American Botanical Council*. (2000)435.
34. Pearlstein T., Steiner M. *J. Psychiastry. Neurosci*.33 (2008) 291.
35. Chhabra G.S.,Kulkarni K.S. *j. Nat. Remedies*.11(2011) 90.
36. Sijelmassi A. *Les plantes médicinales du Maroc*. Casablanca. Le Fennec éd.(1991).
37. AOCS. *Official Methods and Recommended Practices of the AOCS, 5th ed. AOCS Press, Champaign, USA.(*1998).
38. Maestri D.M., Labuckas D.O., Meriles J.M., Lamarque A.L., Zygadlo J.A., Guzman C.A. *j. Sci. food Agric.* 77(1998) 494.
39. Chen C.N., Weng M.S., Wu C.L., Lin J.K. *Evid. Based. Complement. Alternat. Med.J.* 1(2004)175.
40. Leitao G.G., Leitao S.G.,Vilegas W., *Z. Naturforsch. J*. 57 (2002)1051.

41. Wang S.V., Wu J.H., Shyur L.F., Kuoy H., Chang S.T. *Holzforschung*. 56(2002)487.
42. NCCLS. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard. M27-A3*. 2nd ed. Wayne, Pa: NCCLS.(2002).
43. Nichols D.S., Sanderson K. *Chemical and Functional Properties of Food Lipids*. CRC Press. Zdzislaw E Sikorski and Anna Kolakowska ed. Florida.USA. (2002).
44. Gharby S., Harhar H., Guillaume D., Haddad A., Matthäus B., Charrouf Z. *LWT-Food Science and Technology*. 44(2011)1.
45. Ogutcu M., Mendes M.,Yilmaz E. *J. Am. Oil Chem. Soc*.85(2008) 441.
46. Markovic V. V., Bastic L.V. *J. Am. Oil Chem. Soc*. 53(1975) 42.
47. CODEX. *CODEX Alimentarius Commission.* Stan 33, 1981(revised 2). (2003).
48. COI, *COI/NCn°3/Rev.4, Norme commercial applicable aux huiles d'olive et aux huiles de grignon d'olive.* (2009).
49. Gharby S., Harhar H., El Monfalouti H., Kartah B., Maata N., Guillaume D., Charrouf Z. *Med.j.nutr. metab*.44(2011)1.
50. Zine S., Gharby S., El hadek M. *biosci. biotech. res. asia* .10(2013)1.
51. Gharby S., Harhar H., Kartaha B., Hilali M., Guillaume D., Charrouf Z. *Nat. Prod. Commun*.8(2013)25.
52. Berger D. *Universität Basel ed*. *Inauguraldissertation*.(1998).
53. Oomah D.B., Ladet S., Godfrey D.V., Liang J., Girard B., *Food Chem*. 69(2000) 187.
54. Cercaci L., Rodriguez-Estrada M.T., Lercker G. *j chrom A*.985(2003) 211.
55. Hilali M., Charrouf Z., El Soulhi A., Hachimi L., Guillaume. D. *J. Am. Oil Chem. Soc*.84(2007)761.
56. Aparicio R., Aparicio-Ruiz R. *J. Chromatogr. A*.881(2000) 91.
57. Feinberg M., Favier C., Ripert J. *Répertoire Général des Aliments. Tome 1. Technique et Documentation. Ed Lavoisier. Paris*. (1987).
58. Jones P., Racini-sarjaz M., Ntanios F., Feng J., Parson W. *J. Lipid Res*.41(2000)697.
59. Tapiero H., Townsend D.M., Tew K.D. *Biomed. Pharmacother*. 57(2003) 321.
60. Ouraïni D., Agoumi A., Ismaili-Alaoui M., Alaoui K., Cherrah Y., Alaoui M.A.,Belabbas M.A. *Phytothérapie*.5(2007) 6.
61. Kabara J.J. *J. Am. Soc. Oil. Chem*. 56(1979) 757.
62. Gupta S., Satishkumar M.N., Duraiswamy B., Das S., Chhajed M. *World Journal of Pharmaceutical research*.1(2012)1.
63. Jimenez-Arellanes A., Meckes M., Torres J., Luna-Herrera J. *J. Ethnopharmacol*. 111 (2007) 202.
64. Sen A., Dhavan P., Shukla K.K., Singh S., Tejovathi G. *Science Secure Journal of Biotechnology*.1(2012) 9.
65. Moshi M., Joseph C., Innocent E., Nkunya M. *Pharm Biol*. 42 (2004) 269.