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Research Article

COMPARATIVE STUDY OF THE ANTIFUNGAL EFFECT OF OILS AND THEIR UNSAPONIFIABLE FRACTIONS EXTRACTED FROM SEED COAT AND BARKS OF ALGERIAN *CITRULLUS COLOCYNTHIS* SEEDS

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Abstract

The main objective of the study was to assess the *in vitro* antifungal potency of the unsaponifiable fraction extracted from coat and bark seeds oils of *Citrullus colocynthis* L against pathogenic fungal strains namely *Aspergillus flavus, Aspergillus ochraceus, Penicillium expansum* and *Fusarium oxysporum*. In terms of the physico-chemical characterization, oils under study showed evidence of quality standards relating to vegetable oils. Unsaponifiable matter yield recorded was approximately 0.93% and 1.03%, for the seed coat oil and bark seed oil respectively. Antifungal activity carried out by radial growth on solid medium (Potatoes Dextrose Agar acidified) revealed that the oils and the corresponding unsaponifiable fractions exhibited complete inhibition of fungal growth. Maximal antifungal index inhibition (IAF=100%) were recorded at 5% and 2.5% dilutions of each fraction tested. The results provided evidence that the unsaponifiable oils fractions might indeed be potential sources of natural antifungal agents and deserve further studies to characterize the biological compounds included in these fractions.

Keywords: *Citrullus colocynthis seeds*, seed coat, bark, oils, unsaponifiable fraction, antifungal activity.

Introduction

Regularly placed under the headline news, food security has become the main concern of the food industry and health professions considering the food both as source and vector of toxic (FAO, 2003).

Many natural and industrial xenobiotics are likely to be found in food for human and animal populations causing foodborne diseases, these diseases are widespread and represent a serious threat to health, both in developing countries than in developed ones, affecting most severely children, pregnant women and the elderly. (Castegnaro et al., 2002) The existence of these infections has multiple consequences can range from mild poisoning, through serious pathophysiological manifestations, until the fatal outcome. “Cancerous tumors”. Statistics of International Cancer Centre (IARC) report that a significant number of human cancers caused by environmental factors and more particularly to dietary factors (Canet, 2009).

Fungal contamination is one of the main causes of deterioration of food, especially after the discovery of mycotoxins produced by these microorganisms has compounded the problems of farmers, industrialists, scientists and ordinary consumer (Tabuc, 2007).

A real subtle opponent, fungi inoculums is a quiet, invisible, sleeping and yet capable of producing very harmful effects see catastrophic when conditions become favorable. More subtle when visible damage to materials were offset by processing methods, making disappear perceptible signs of mold activity without eliminating it toxic principles, often very stable, which may have been formed earlier and preventing opponents at the same time any research of correlation between the presence of mold and mycotoxin risk (Castegnaro et al., 2002 ; Pfohl-Leszkowicz, 1999).

With the growing problem of fungal contamination, mycotoxin production and the emergence of a sense of insecurity, we are witnessing more and more to the installation of new preventive strategies to limit fungal contamination, reduce or prevent mycotoxin production (Nguyen, 2007), (Pineiro et al., 2005).

Facing this situation, many researchers have turned to alternative reductions or fight against this contamination, such as natural control based on the use of different metabolites of medicinal plants in order to contribute to their recovery, because of their efficiencies, and their lower cost compared to other methods of reductions (Krishna et al. 2009).

The search for new natural substances in antifungal activity is an important component of that sought compete several laboratories of very high levels (kitondo et al. 2013). The antifungal activities of these plant metabolites depend essentially on the presence of various bioactive agents belonging to different chemical classes, namely fixed oils characterized by their wide antimicrobial spectra (Kubmarawa et al. 2007).

Many studies have confirmed the ability of a wide range of plants and their extracts to reduce see inhibit the growth of fungal strains including toxigenic ones. In this continuum topic, this work falls within the evaluation of the antifungal effect of the insaponifiable fractions of the almond oil and bark seed oil extracted from *Citrullus colocynthis* L. seeds against four toxinogenic fungi namely *Fusarium oxysporum, Penicillium expansum Aspergillus flavus and Aspergillus ochraceus*.

Materials and Methods

Collection of plant material and extraction of oil

The *C. colocynthis* seeds used for the present study were collected in December 2013 at Kenadessa area, (Bechar, Algeria). The seeds were shade dried at room temperature. The seed coat are separated from the bark seeds. The dried part were milled to a fine powder in an electrical mill and stored in the dark at room temperature in recipients until required. The finely powdered were extracted with organic solvent petrolium ether using soxhlet apparatus for 6 hours. The different extracts obtained were subsequently concentrated under reduced pressure to get their corresponding oils. The physicochemical parameters of the oils such as density, refractive index, saponification value, iodine value, insaponifiable matter, acid value, acidity and peroxide value were performed according to standard methods by AFNOR (1988).

Saponification of fatty acids

A mixture of 3.4 g of oiliness residue, ethanol (15 mL), water (15 mL) and sodium hydroxide 3 g was refluxed for about 45 min. After evaporating of the ethanol under reduced pressure, the solution obtained was filtered using whattman filter paper to separate the fatty acids of the unsaponifiable fractions.

Fungal materials and confirmation of testing strain

Four pure fungal strains such as *A. flavus* MTTC 2799, A*. ochraceus* CECT 2092, *Penicillium expansum* and *Fusarium oxysporum* were procured from biology laboratory of Tahri Mohamed University- Bechar. The fungal culture were further maintained by sub culturing regularly on PDA acidified and stored at 4°C until the time of use.

Determination of percent mycelial inhibition by growth radial technique on solid medium

Technique consists in placing simultaneously in test tubes different volumes of insaponifiable fractions tested for their antifungal potency. Range of final concentrations 5, 2.5, 1, 0.5, 0.25, 0.125 and 0.0625% obtained by dilution were tested. The tubes are completed to 20 mL by solid medium PDAa (Potatoes Dextrose Agar acidified). After agitation their contents are poured into Petri dishes which were inoculated by the respective spore solution of each tested fungal strain (Agar solution at 0.2%+5% Tween 80 and spores of fungal strains). After 7 days of incubation at 25 ± 2 °C Hibar et al (2006), mycelial radial growth was measured from the third day of incubation (Soro et al, 2010) (Kra et al, 2009). The inhibition percentage of mycelial growth was calculated using the following formula: (PIg= ((DT-D)/DT)*100 where DT is mean diameter of mycelial growth in control and D is mean diameter of mycelial growth in treatment (Singh et al, 2009).

Statistical analysis

All statistical analysis were performed using analysis of variance (MS excel 2007). All values are expressed as Mean ± standard deviation.

Results

Physicochemical analysis of oils

In terms of physicochemical characterization results depicted in table 1, oils subject of this investigation have provided variable yields: 18, 14.45 and 09.86%, respectively for *C. colocynthis seeds,* seed coat and bark seeds oils. In terms of density value, the values recorded ranged between [0.9080 – 0.9102] the bark oil seeds provided the highest density index. The refractive index is recorded for our oil is spread over a range of 1.4705-1.4743.

The values recorded for the saponification are consistent with the values indicated by the *Codex Alimentarius*, which sets values between 170-265 mg KOH /g oil for vegetable oils. The iodine value generally allows appreciating the oil degree of unsaturation. The average values indicate that tested oils provided iodine value in the range [171 – 219] which imply that the number of unsaturation on the carbon chain of these oils is very important.

Results of the mean values for determining the rate of insaponifiable matter shows that tested oils had a richness insaponifiable matter. Findings are confirmed by the determination of the quality indices or alteration of the studied oils which are in agreement with the values of the *codex Alimentarius* on vegetable oils (Olle, 2002).

Table 1. Physico-chemical characterization of oils.

Oils	*C. colocynthis* seed oil	Seed coat oil	Seed Bark oil
Extraction yields (%)	**18**	**14.45**	**09.86**
Color	Light yellow	Light yellow	yellow
Aspect	Clear	Cloudy	Clear
Physical characterization			
Density	0.9080	0.9095	0.9102
Refractive index (20°C)	1.4743	1.4705	1.4713
Chemical characterization			
Saponification value (mg KOH/g oil)	219	196.35	171.28
Iodine value	86	58.86	52.02
Insaponifiable matter (%)	2.26	0.93	1.03
Characteristics of alteration			
Acid value	3.64	5.7	6.34
Acidity (%)	1.83	3.91	4.15
Peroxide value (Meq O_2/kg oil)	1.17	4.5	6.63

Antifungal activity

Reading of graphic supports giving the effect of the tested oils and their corresponding insaponifiable fractions determinated by the radial growth on solid medium to prevent the mycelial growth of the fungal organisms known for their harmful to human beings and their production of mycotoxins showed clearly that the extract under study exhibited a very pronounced antifungal activity. This inhibition was variable on tested strains; the effectiveness antifungal action was more marked on *A. ochraceus, Penicillium expansum, Fusarium oxysporum* and finally *A. flavus*. Indeed, the antifungal potency recorded increased proportionally to the concentrations of tested fractions particularly in case of the insaponifiable fractions. As illustrated on figures bellow, total inhibition index ($IAF_{[0.5\%], [1\%], [2.5\%], [5\%]}$ =100%) were detected on 0.5, 1, 2.5 and 5% concentrations of almonds and bark insaponifiable fractions oils. In contrast, the reading of Figure 5 revealed less antifungal effect of insaponifiable fraction extracted from the whole seed at the same concentrations for all the tested strains ($IAF_{[0.5\%]}$ = 53.71 %, $IAF_{[1\%]}$ =52.30 %, $IAF_{[2.5\%]}$ = 48.71% and $IAF_{[5\%]}$ = 47.94 % for *A. ochraceus*).

Discussion

The use of herbs in herbal medicine has received great interest in biomedical research and is as important as chemotherapy. This renewed interest is that herbal remedies are an inexhaustible source of bioactive natural compounds and other parts of the need for research to better medication by a more gentle therapy without side effects (Kitondo et al. 2013). A broad spectrum of organic substances extracted from medicinal plants, including oils were tested to substitute certain means of struggle against xenobiotic including those fungal (Kubmarawa et al. 2007). In this part, several authors have confirmed the effectiveness of the oils on toxigenic fungi.

The problem of food deterioration by fungi microscopic (mold) is attracting increasing interest in recent decades (Nguyen, 2007). In view of the requirements of food safety and in order to overcome the problems of fungal contamination, many researchers have turned to alternative reductions or struggles against this contamination, such as natural control based on the use of various extracts medicinal plants in order to contribute to their development, because of their effectiveness, safety evidence and their lower cost compared to other methods of reductions.

Vegetable oils consist essentially of triglycerides represented by the fatty acids which are combined to non-glyceride components also called minor constituents (Ndeye, 2001). Furthermore, the quality of oil depends mainly on its chemical composition. However, depending on the conditions of extraction and storage, the various components of the oil are subject to more or less significant changes could adversely affect their quality (Akroun, 2010). The determination of the physical characteristics of the oils (relative density, and the refractive index) depends on the composition of the fatty acids and of the temperature.

In terms of the physico-chemical characterization, results showed that oil yield level of almonds was higher (14.45%) compared to the bark (09.86%). This difference can be assigned to the fact that the fatty acids is concentrated in the almond seed. For instance, it is difficult to compare the results with those of the bibliography. Performance is relative and depends on the method and conditions under which the extraction was performed (Lee et al, 2003). It's important to underline that the whole seed has provided an oil yield of 18%. These findings are in agreement with those obtained by Daoudi et al, 2014; Sebbagh et al. (2009), Abu Naser and Potts (1953), and Schafferman et al., (1998)] who reported the same yield.

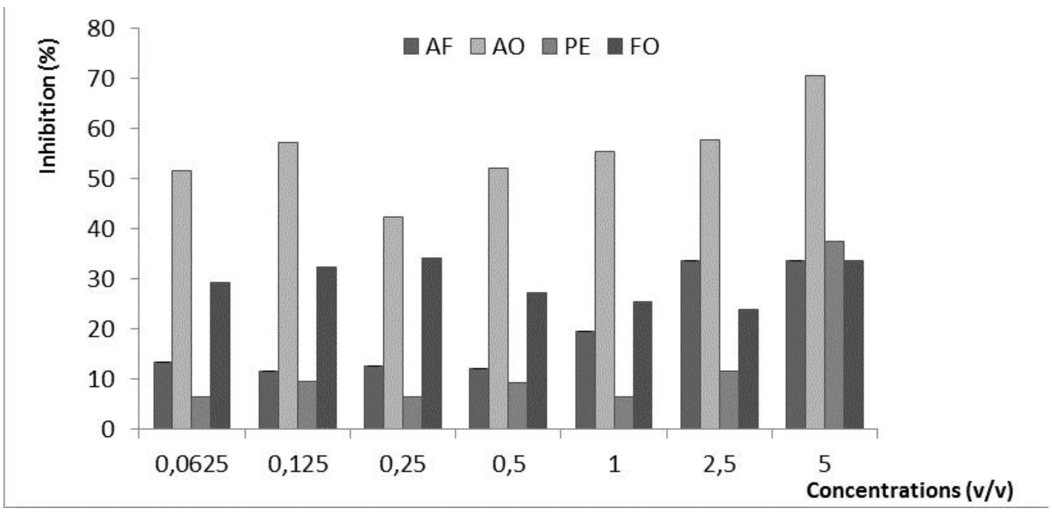

Figure 1. Antifungal index of seed coat oil of *Citrullus colocynthis* seeds (Growth radial technique on solid medium)

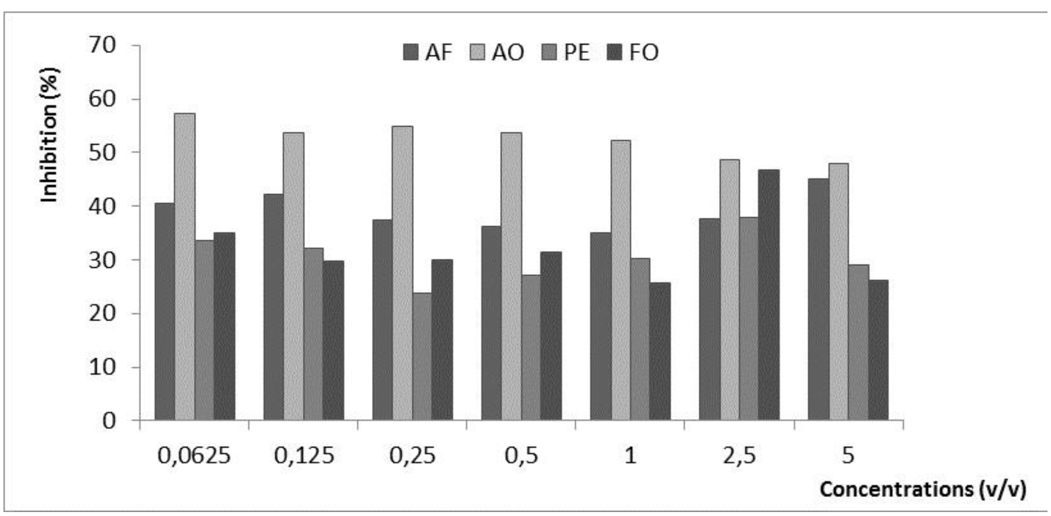

Figure 2. Antifungal index of bark oil of *Citrullus colocynthis* seeds (Growth radial technique on solid medium)

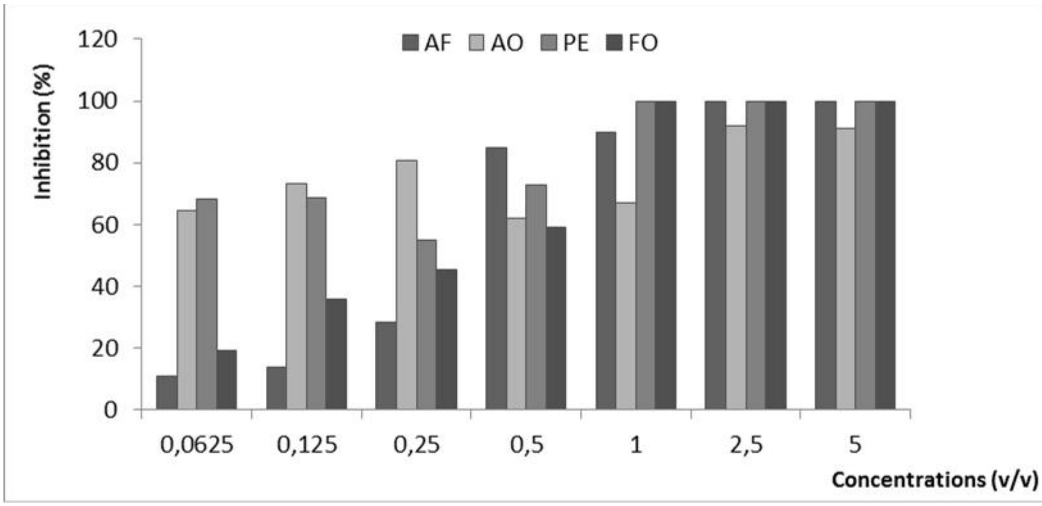

Figure 3. Antifungal index of seed coat oil of *Citrullus colocynthis* seeds (Growth radial technique on solid medium)

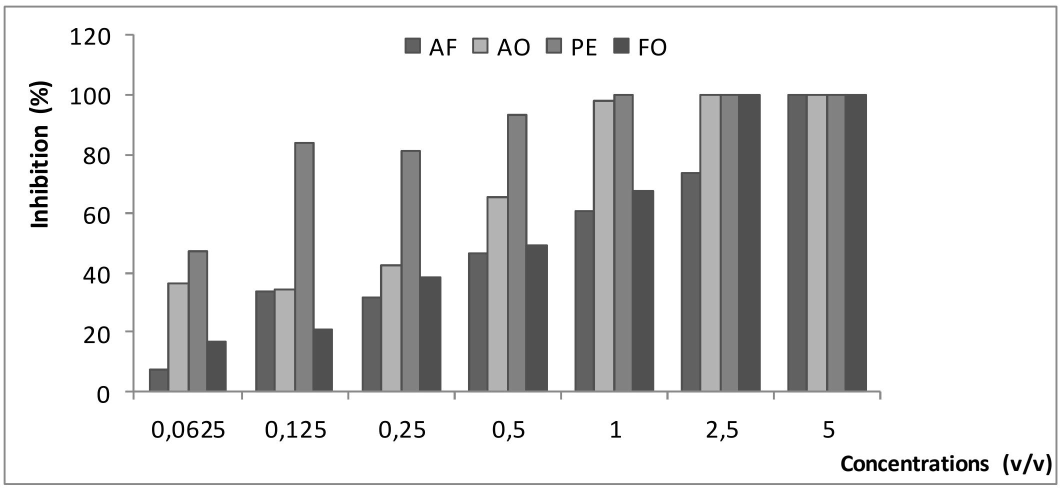

Figure 4. Antifungal index of insaponifiable fraction extracted from bark oil of *Citrullus colocynthis* seeds (Growth radial technique on solid medium)

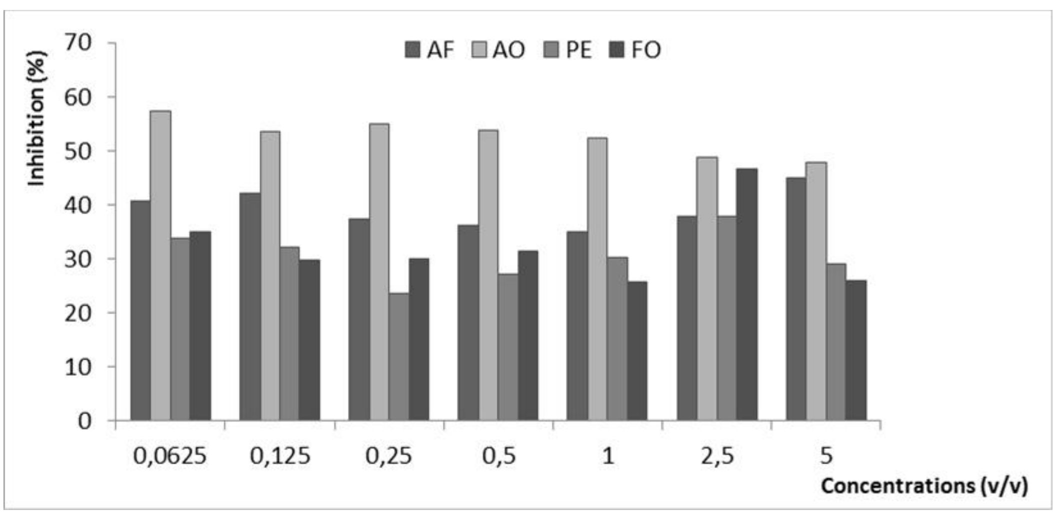

Figure 5. Antifungal index of insaponifiable fraction extracted from the whole *Citrullus colocynthis* seeds (Growth radial technique on solid medium)

In terms of the physico-chemical characterization, results showed that oil yield level of almonds was higher (14.45%) compared to the bark (09.86%). This difference can be assigned to the fact that the fatty acids is concentrated in the almond seed. For instance, it is difficult to compare the results with those of the bibliography. Performance is relative and depends on the method and conditions under which the extraction was performed (Lee et al, 2003). It's important to underline that the whole seed has provided an oil yield of 18%. These findings are in agreement with those obtained by Daoudi et al, 2014; Sebbagh et al. (2009), Abu naser and Potts (1953), and Schafferman et al., (1998)] who reported the same yield.

Analysis of the results of the relative density and refractive index shows that the values obtained are variable, but remain within the range of the standards required by the *Codex Alimentarius* which establish values [0919-0925] for the density and on [1.4677 - 1.4771] for the refraction index. The values obtained for the relative density of the *C. colocynthis* oil [0.9087] are consistent with those found by (Sebbagh et al. 2009) and those of Akpambang et al (2008).

Analysis of the results of the refractive index shows that it has been around [1.4743, 1.4705 and 1.4713], respectively for *C. colocynthis* oil., almond seeds and bark seeds oils, these values are close to those established by the *Codex Alimentarius* and those described in the literature. These results agree with those found by Akpambang et al (2008) with a refractive index of 1.47 ± 0.02 for the *C. colocynthis* oil and the work of Hassimi et al (2007) describe a refractive index in the range size [1.4607 -1.4620].

It's important to underline that our findings agree with those of Adrian et al (1998) and Olle (2002) who reported that the refractive index and the density depends on the composition of the fatty acids and the temperature, and the refractive index increase is due to the appearance of other high molecular weight compounds. Thus, several authors have adopted classifications of fatty acids. As Adrian et al (1998), who suggest that plant lauric fats have refractive indices between [1.448 <R <1.458] and vegetable fats have indices between [1.468 <R <1.490]. Olle (2002) proposed a classification of oils based on their fatty acid compositions include oleic acid, linoleic acid, linolenic acid and α refractive index acid. According to this classification oils whose refractive index is between [1.468 <R <1.472], are oils rich in oleic acid case of oils under.

The qualitative analytical control of oil is based on the determination of chemical characteristic indices (peroxide index, acid value and acidity). The determination of acidity is the first step providing information about the degree of mainly triglycerides present in the fat body after a chemical or enzymatic hydrolysis. It also provides information on the amount of free fatty acids, which are responsible for much of the alteration of the oil, in particular oxidation (Adrian et al., 1998).

Results of determination of the acid value of our oils showed that the seed coat oil [5.7%] and bark seed oil [6.34%] are greater to the standard edible oils set by the *Codex Alimentarius* (4%). However, Akpambang et al (2008) reported that oil of *C. colocynthis* outcome of collected seeds from Nigeria presented an acid value of [8.02 ± 0.07 mg KOH / g]. This is probably due to its content of free fatty acids resulting from the action of lipases on triglycerides; moreover, this enzyme is very abundant in the seeds of *C.colocynthis*. This difference may be allocated according to some environmental studies at geographical conditions even varietal differences of the plant.

The peroxide provides an assessment on the quality of peroxides present in fat. Furthermore, this index provides whether our oils have been the area of oxidations more or less important. Index values of peroxides obtained for the three oils studied are consistent with the standards of *Codex Alimentarius*, which sets a value less than [10 Meq O_2 active / kg of body fat] for fresh oils which have undergone no oxidation.

Findings for the three oils ie oil *C. colocynthis* [1.17 Meq O_2 active / kg of fat], almond seed oil [4.5 Meq oil L. O_2 active / kg fat] and bark seed oil [6.63 Meq O_2 active / kg of fat], allows us to see that our oils have not undergone oxidation to the formation of peroxides and hydroperoxides responsible of alteration oils. Bark seed oil had elevations of the peroxide probably due to the extraction conditions and conservation. Outcomes were confirmed by the work of Li et al (2013), who obtained very high peroxide [65.76 Meq O_2 active/kg of body fat] for *C. colocynthis* oil. Appelbaum (1989) had assumed that other substances present in the oils, such as carotenoids, vitamins A and E, which are prone to oxidation and therefore induce a strong indication peroxide, could also cause the oxidation of oils.

The saponification informs about the wealth of oil fatty acids with long chain triglycerides for a given weight, the quality and quantity of carboxylic groups linked or complexed present in substances capable to form soap after hydrolysis with a base. This index depends on the molecular weight of the fatty acids used in the formation of triglycerides mixture. In addition, the number of carbon atoms in acid molecules is high; less the saponification is important (Karlesking, 1992). The amount of potassium hydroxide necessary for saponifying therefore increases with decreasing chain length of the fatty acids.

The *Codex Alimentarius* fixed for the saponification of an interval [170-265 mg KOH / g oil] for crude oils. In this study, the three inspected oils showed mean values of [171.28, 196.35 and 219 mg KOH / g oil] respectively for bark oil seeds, almond oil seeds and oil *C. colocynthis*. This finding are similar with good quality of these oils. These results are in line with those of the saponification of oils *C. colocynthis* studied in Sudan and Malaysia 206 and 204.44 mg KOH / g of oil, respectively. These values are also in the same order of magnitude as those found by (Obeid, 1996; Ziyada 2008; Giwa et al, 2010.).

According AFNOR (1988), the unsaponifiable fraction, which represents 0.2 to 2%, is formed of non-glyceride components of natural fat, extractable by organic solvent (hexane, ether, etc..), Such as sterols, higher aliphatic alcohols , pigments, hydrocarbons, liposoluble vitamins, as well as non-volatile organic substances foreign to 103 ° C optionally they can contain. Some of them can be very useful and more involved in the stability of oil against oxidation, such as the case of tocopherols and carotenoids. Other unsaponifiable matter can have negative impact on the quality of the oil.

Analysis of the results of the unsaponifiable index shows that it was of the order of [0.93, 1.03 and 2.26%], respectively for almond oil seeds, bark oil seeds, and *C. colocynthis* oil. These values are slightly higher than the standard required by AFNOR particularly for oil *C. colocynthis*. In fact, some researchers (Abu Nasr, 1953 Obeid, 1996) found that *C. colocynthis* oil contained only 1.01% to 1.72% unsaponifiable. This difference may be attributed to the variety of the *C.colocynthis* seeds species studied.

Exploitation of results of the antifungal activity carried out by the effect of the unsaponifiable fractions extracted from different parts of the seed oils *Citrullus colocynthis*, shows a significant inhibition of unsaponifiable fractions of the fungal strains tested. This inhibition was proportional to the concentration added to the culture medium. Higher and complete inhibition (IAF =100%) was recorded at high concentrations [0.5%], [1%], [2.5%], [5%] for the unsaponifiable fractions extracted from *Citrullus colocynthis* almonds and bark seeds oil. These findings correlate with the studies of several authors such as Belsem et al, 2009; Marzouk et al., 2009; Sebbagh et al, 2009; Amrouche et al., 2013 and Gacem et al., 2013 who attested that the unsaponifiable fractions extract from *Cucurbitaceae* are provided with particular biological properties for instance antidiabetic, antiobesity, antimicrobial, drastic and tonic laxative properties. The effect was very noticeable on all strains tested compared to the effect of the unsaponifiable fraction extracted from the whole seed having provided a moderate inhibition.

The higher activity of unsaponifiable extracted from *Citrullus colocynthis* seed coat and bark seeds oil compared to complete *Citrullus colocynthis* oils is significant. Less effects of the unsaponifiable extract from the whole seed is a paradoxe and could be due to antagonistic effect of the bioactive compounds.

Conclusion

The present study reveals that unsaponifiable fractions under study displayed strong antifungal activity and can be used for the treatment of various fungal deseases. Evidence of antifungal activities of the unsaponifiable extract from *Citrullus colocynthis* almonds and bark seeds oil suggest that further studies are need to characterize and elucidate of the bioactive compounds of this unsaponifiable fractions.

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