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Full Length Research Paper

Viscosimetric study and synergistic antifungal activity of oils extract from three Algerian medicinal plants against toxigenic *Aspergillus*

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Oils extracted from seeds of three medicinal plants used in Algerian folk medicine (*Citrullus colocynthis, Nigella sativa* and *Linum usitassimum*), were tested for their antifungal synergy against one strain of *Aspergillus flavus* toxin (*A. flavus* MTTC 2799) by the method of radial growth on solid medium Potateos Dextrose Agar (PDA). Antifungal results revealed that all oils tested at the concentration 1/5000 inhibited the growth of the studied fungus, where the best antifungal potency was observed for the *L. usitassimum* seed oil. The values of antifungal indices (55.65, 48.65, 40.54) were recorded, respectively for *L. usitassimum* oil, *C. colocynthis* oil and *N. sativa* oil at concentrations of (1/5000, 1/500 and 1/2000). The results of the synergistic antifungal activity obtained by oils combination revealed that *L.* oil/*C.* oil showed the best antifungal effect (54.19%) before the *L.* oil/*C.*oil/*N.* oil system with 48.76% of inhibition. In order to explain the synergistic effect and the best behavior of oils, especially determination of optimal concentrations C* (C star) needed for antifungal synergistic activity, viscosimetric investigation was carried out at room temperature and at several concentrations.

Key words: Viscosity, oil carbon chain, synergistic antifungal activity, oils, *Aspergillus flavus*.

INTRODUCTION

Fungal contamination is one of the main causes of deterioration of food, especially after the discovery of mycotoxins produced by these micro-organisms that only accentuated the concerns of farmers, industrialists, scientists and simple consumer (Tahani and Elamrani, 2008). Real adversaries are subtle; mold inoculum is quiet, hidden and dormant and yet capable of producing very harmful and even catastrophic effects when conditions become favorable. Opponents are even more subtle when the visible damage to raw materials have been erased by the processes of transformation, by removing the visible signs of mold activity without eliminating the toxic principles provided, and often very stable, which

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may have been formed before and at the same time preventing any search for correlation between the presence of mold and mycotoxin risk (Bejaoui, 2005). Indeed, faced with these food hazards and the emergence of a sense of insecurity, consumers are increasingly demanding healthier products whose quality and food security are guaranteed (Canadas, 2006).

Today, the use of medicinal plants in phytotherapy received a great interest in biomedical research and is as important as chemotherapy. This renewed interest is that medicinal plants are an inexhaustible source of natural compounds and other bioactive, and share the need to search for better medications for milder therapy without side effects (Yakhlef et al., 2011). The antimicrobial properties of medicinal plants depend on the presence of various bioactive agents belonging to different chemical classes (Bouzouitta, 2008).

Recently, numerous studies have been focused on antifungal activity of the oils extracted from medicinal plants because the scientific evidence derived from experimental studies confirm the amplitude spectrum of antifungal activity confined to these class of compounds (Ziyada et al., 2008; Yingying et al., 2008; Dale et al., 2004; Marzouk et al., 2009). In the same route, the results collected from our previous study revealed that the oils of *C. colocynthis, L. usitassimum* and *N. sativa* reduced the growth of *A. flavus* MTTC 2799, producing aflatoxin at various concentrations_(Amrouche et al., 2011). That is why we focused our attention on the search of eventual synergistic effects from different combinations of the *L. usitassimum*, *C. colocynthis* and *N. sativa* oils against *A. flavus* strain and to introduce the viscosity parameter in order to explain the synergistic potency of these oils.

MATERIALS AND METHODS

Collection of plants material

The *C. Colocynthis* seeds used for the present study were collected in December 2010 in Beni Abbes area (Bechar). Whereas, the *L. usitatissimum* and *N. sativa* seeds were purchased from local market of Bechar Department, Algeria. The seeds were shade dried at room temperature for 10 days. The dried seeds were milled to a fine powder in an electrical mill and stored in the dark at room temperature in recipients until required.

Extraction of the oils

The finely powdered seeds materials (15 g) of each plant were submitted to oils extraction with petrolium-ether (180 mL) using soxhlet apparatus for 6 h. The different extracts obtained were subsequently concentrated under reduced pressure to give the corresponded oils. The corresponding physicochemical parameters of the oils were performed according to AFNOR (1988).

Viscosity measurement

Viscosity was measured with a capillary viscosimeter ubbelohde-schott Gerat AVS400 (Bock et al., 1988). The temperature is kept constant by a thermostat (25 ± 0.1)°C. Dilution of oil solutions was performed manually. The viscosity measurement is based on the determination of the flow time of a volume of the solution through a capillary length of "1" and diamèter "a".

Fungal material and confirming of testing strain

The seeds oil extracts were assayed for antifungal activity against the fungal strain *A. flavus* MTTC 2799 obtained from biology laboratory at Bechar University. Confirmation of *Aspergillus* genera was performed by micro-culture method described by Haris (1989) and Barnett (1972). Furthermore, confirmation of *A. flavus* species was carried out by Single Spore method using three cultures media: Malt extract agar (MEA) at 25°C, Glycerol Nitrate Agar (G25N) at 25°C and Czapek yeast agar (CYA) at 5 and 37°C. Using the identification keys of Pitt (1973), observation has been made after the first and second week. Confirmation of *A. flavus* strains was carried out by inoculation at 25°C in Attenuated familial adenomatous polyposis (AFAP) medium which gave oranges Revers plate. The fungal strain is regularly maintained by sub-culturing on PDA medium. Conservation of the strain was carried out on tube of acidified PDA at 4°C.

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

The final culture of fungal strain was carried out on potato dextrose agar medium (PDA) supplemented oils in different proportions. To obtain a homogeneous distribution of oil and to maximize the contact germ/compound, oils subject of this investigation were used as emulsions to be able to handle as solutions. The agar (0.2%) was chosen as the emulsifying agent because it is devoid of any interaction on the activity of the used oils (Remmal et al., 1993). Using the protocol_described in Remmal's report, the different dilutions were prepared in a solution of agar in sterile distilled water for different concentrations of oil, and then they were added to the culture medium acidified potato dextrose agar (PDA acidified) contained in the tubes (Remmal et al., 1993). The mother solution of oil must be at 1/10; it was prepared in a solution of 0.2% agar.

In order to test the synergistic antifungal effect, mother solution of studied oils was prepared from 1.5 ml of each oils and 13.5 ml of potato dextrose agar medium, then different oils system were prepared in various concentrations (1/100, 1/250, 1/500, 1/1000, 1/2000, 1/3000, 1/5000). The tubes were agitated in vortex mixer and the contents are poured into Petri dishes. Note that for each dilution, five dishes are prepared. The inoculation was made from a spore suspension adjusted to 10^5 to 10^6 spores by counting on a Malassez slide. Aseptically, a drop was deposited on the surface of the culture medium. Finally, incubation was performed at 28 ± 4°C for 3 to 14 days. After incubation and taking into account the growth of the witnesses, the diameters of the mycelia formed were measured to calculate the antifungal index using the formula ID = (1

Table 1. Physico-chemical caracterization of oils.

Oils	*C. colocynthis*	*L. usitatissimum*	*N. sativa*
Extraction yield (%)	18	39.96	33.71
Color	Light yellow	Yellow straw	Brown green
Physical caracterization			
Density	0.9087	0.9319	0.921
Refractive index (20°C)	1.474	1.4595	1.4720
Freezing point (°C)	-6	-4	-7
Chemical caracterization			
Saponification value (mg KOH/g)	219	191	195
Iodine values	86	192	126
Insaponifiable matter (%)	2.26	1.7	3.47
Characteristics of alteration			
Acide value (%)	3.64	3.08	5.42
Acidity (%)	1.83	2.03	3.81
Peroxide value (meq/kg)	1.17	4.74	6.02

Table 2. Antifungal index values of the different tested oils.

Tested oils	1/100	1/250	1/500	1/1000	1/2000	1/3000	1/5000
Colocynthis oil (C. oil)	42,12	45,27	48,65*	37,61	39,05	38,51	41,22
Lin oil (L. oil)	45,97	46,41	50,53	48,17	53,47	50,24	55,65*
Nigela oil (N. oil)	38,73	34,68	32,57	34,04	40,54*	35,59	38,75
C.oil/L.oil	32,43	26,32	28,38	28,38	40,54	54,19*	41,75
C.oil/N.oil	33,78	36,49*	25,68	25,68	26,35	27,03	26,35
N.oil/L.oil	22,97	21,62	22,63	23,65*	20	21,62	23,51
C.oil/L.oil/N.oil	38,16	47,97	40,95	47,97	44,35	45,27	48,76*

(*): Values of the best antifungal index.

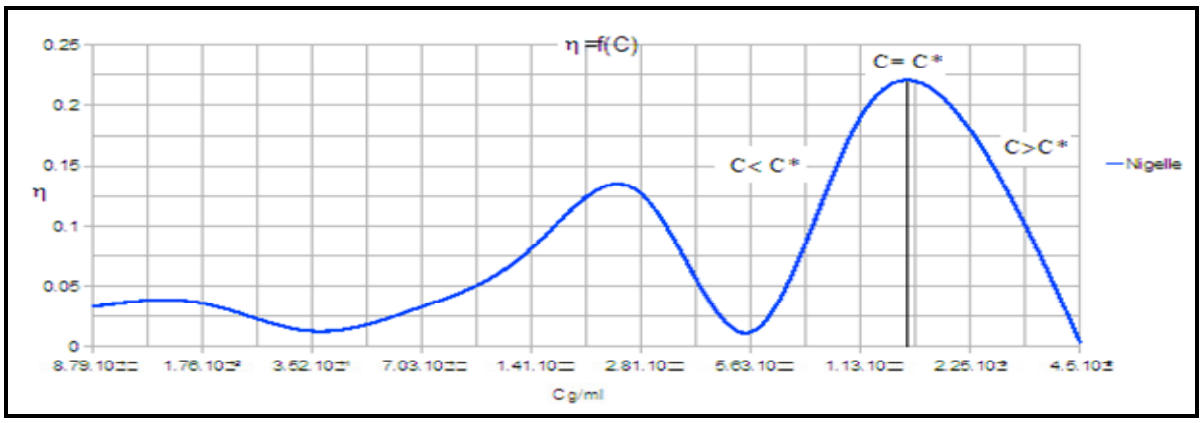
- Da / Db) × 100. Where Da: diameter of the growth area of the test, Db: diameter of the growth area of the samples (Singh et al., 2009).

RESULTS

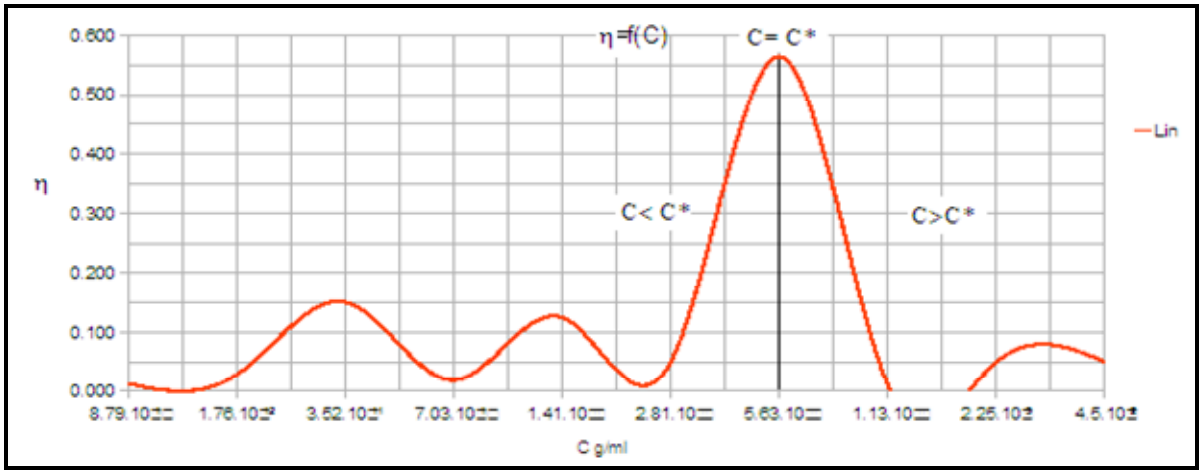
Physico-chemical characterization of oils covered the determination of quality and the alteration criteria. The values obtained were compared to those given by the Codex Alimentarius (1996). The results depicted in Table 1 present the profile of physicochemical oils tested. The exploitation of these results shows variability in the extraction yield (23, 39.96 and 33.71%), respectively for *C. Colocynthis, L. usitatissimum* and *N. sativa* oils. On the other hand, from Table 2, we observed high iodine values (86, 192, 126), respectively for *C. Colocynthis, L. usitatissimum* and *N. sativa* oils. These physico-chemical data confirmed well the best behaviour of the oils chain carbon and their ability to bind the iodine molecules.

As outlined in the curves a, b and c, the viscosimetric investigation of the studied oil allowed us to find the optimal concentration of each oil necessary to produce the synergistic antifungal activity. The values of the oils concentrations were found $C_1^* = 1.5.10^{-3}$ g/ml, $C_2^* = 5.63.10^{-6}$g/ml and $C_3^* = 1.41.10^{-8}$ g/ml, respectively for *N. sativa*, *L. usitatissimum* and *C. Colocynthis*. Furthermore, curves d, e, f and g showed the profile of the oils behaviour during the synergistic effect (Figure 1).

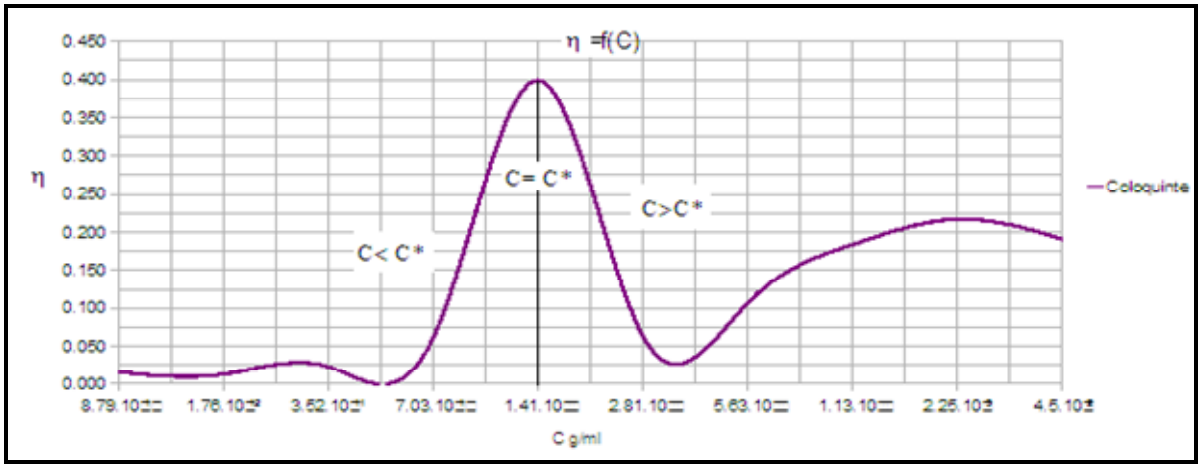
All oils evaluated in this work exhibited more or less antifungal potency against toxigenic strain *A. flavus*. The results obtained by the method of Remmal et al. (1993) pronouced the antifungal activity of these oils. Indeed, growth retardation of the strain was generally perceived


η =f(C)
C= C*
C< C*
C>C*
Nigelle
η
C g/ml

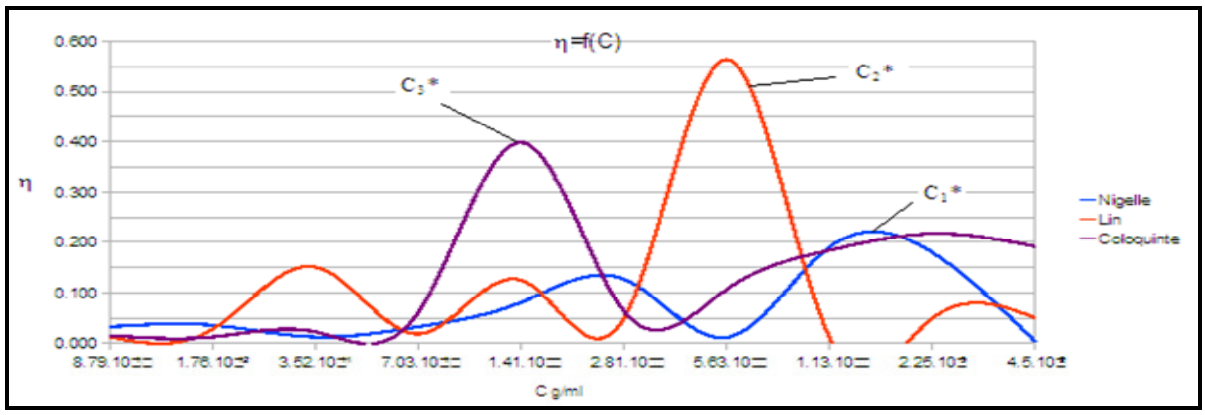
(a)


η =f(C)
C= C*
C< C*
C>C*
Lin
η
C g/ml

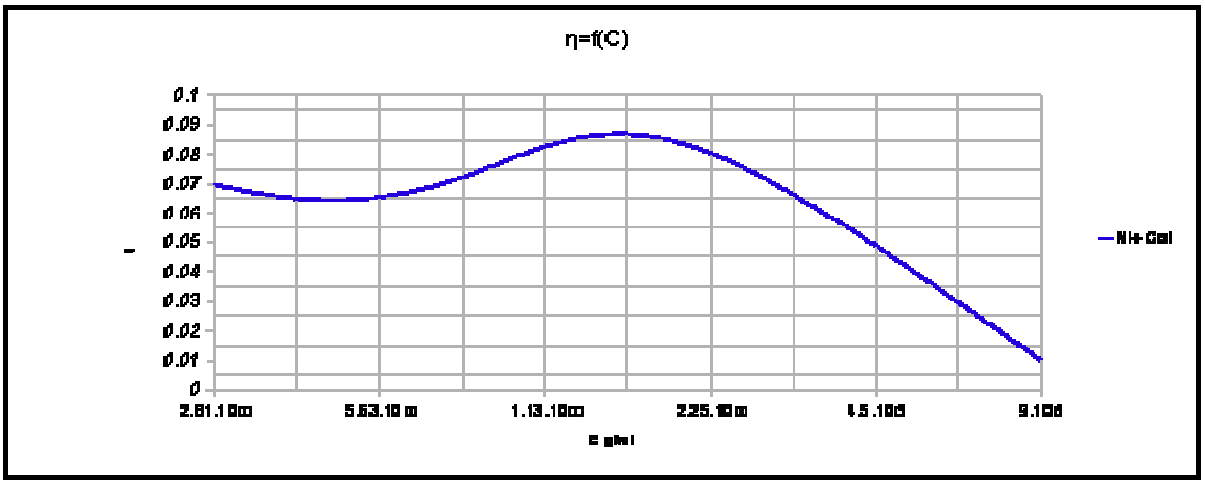
(b)


η =f(C)
C= C*
C< C*
C>C*
Coloquinte
η
C g/ml

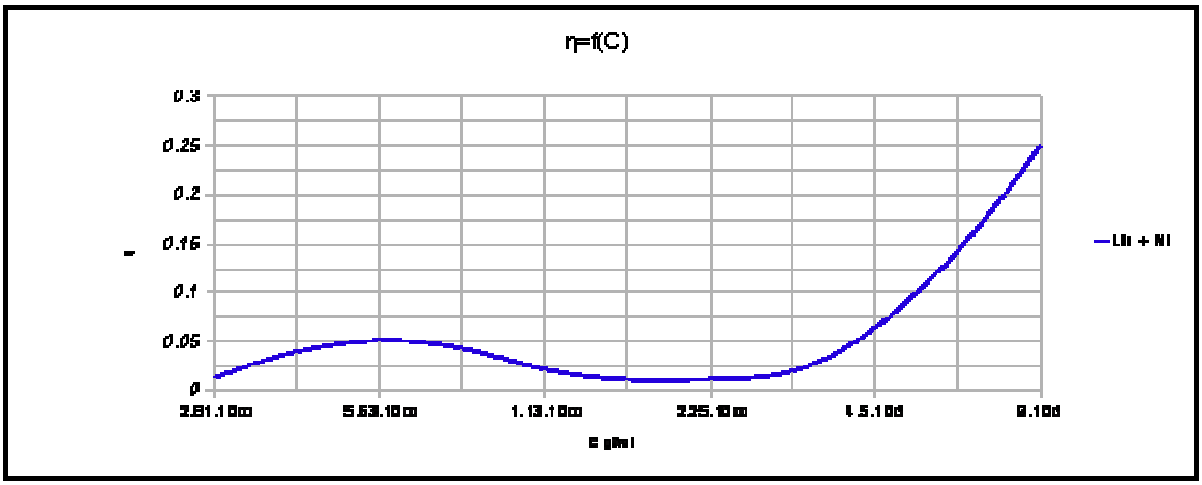
(c)


η=f(C)
C1*
C2*
C3*
η
0.600
0.500
0.400
0.300
0.200
0.100
0.000
C g/ml
Nigelle
Lin
Coloquinte

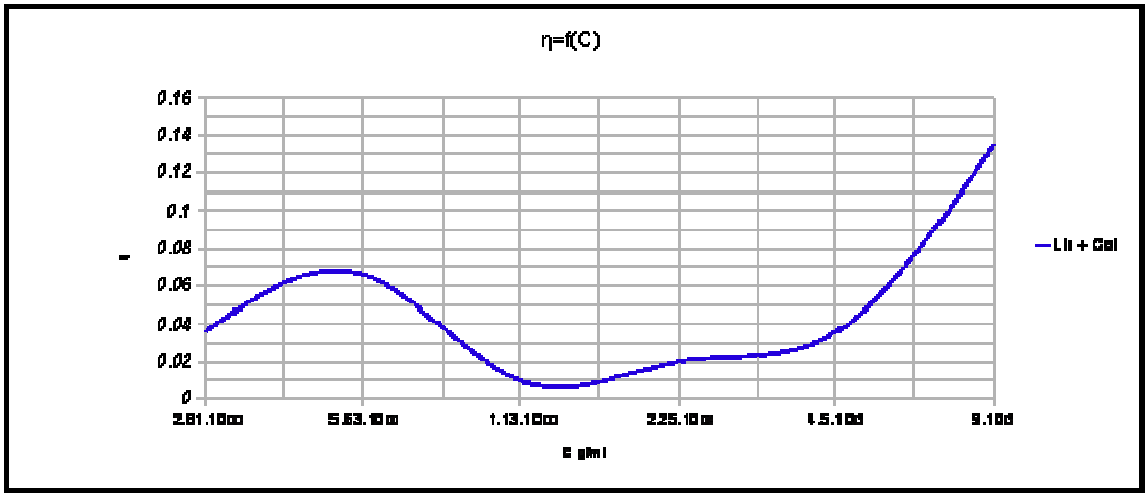
(d)


η=f(C)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Nig+Gel

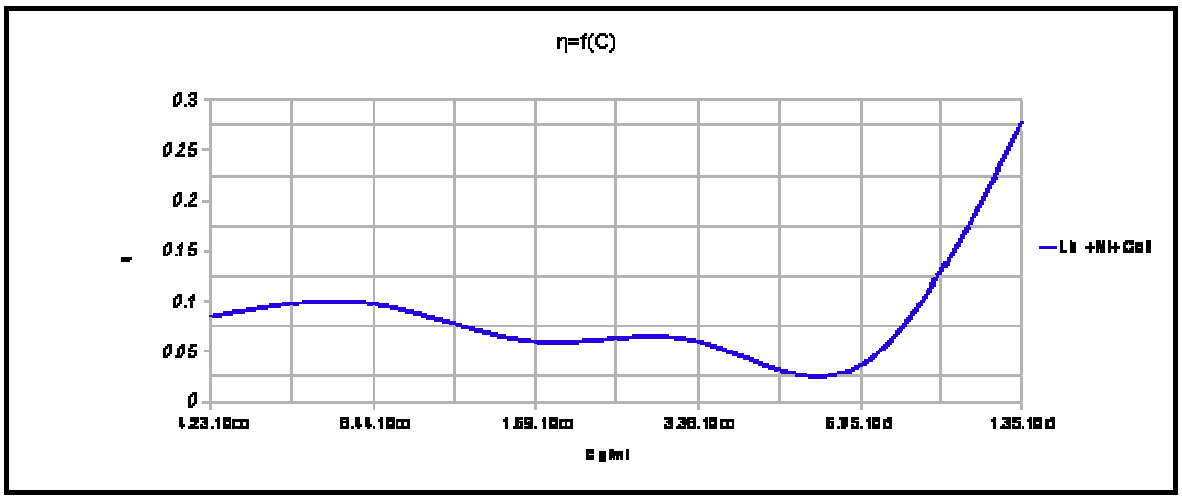
(e)


η=f(C)
0.3
0.25
0.2
0.15
0.1
0.05
0
Lin + Nl

(f)



(g)



(h)

Figure 1. Variation of specific viscosity with concentration (η_{sp} = f(C)) for different oils systems. (a) η_{sp} = f(C) (g/ml) (C_1^* = 1.5.10^{-3} g/ml); (b) η_{sp} = f(C) (g/ml) (C_2^* = 5.63.10^{-6} g/ml); (c) η_{sp} = f(C) (g/ml) (C_3^* = 1.41.10^{-8} g/ml); (d) η_{sp} = f(C) (g/ml) for the three oils; (e) η_{sp} = f(C) (g/ml) synergy (*N.* oil - *C.* oil); (f) η_{sp} = f(C) (g/ml) synergy (*N.* oil - *L.* oil); (g) η_{sp} = f(C) (g/ml) synergy (*L.* oil - *C.* oil); (h) η_{sp} = f(C) (g/ml) synergy for the three oils.

to *A. flavus* merger (1/2000 and 1/5000) at 6th day of incubation, with an optimum concentration of 1/5000 for all oils. As seen in Table 2, we observed that the *L. usitatissimum oil* was shown to be the most potent against the fungus, with inhibition of 55.65%.

The oil of *C. colocynthis* with indices displayed antifungal means (37.61 to 48.65) at concentration (1/500) during the 4th day of incubation. For *N. sativa*, antifungal indices varied between of (40.54 to 32.57) at the concentration (1/2000) during the 5th and 6th day of incubation and was found to be less potent than the other oils.

The evaluation tests of the antifungal effect by synergistic action performed by different oils combination formulas, showed at least a more efficacy of these oils. The combination of oils in systems (*C.* oil - *L.* oil) and high and less potency values of about 54.19 and 23.65% after the 6th and 5th days of incubation. Besides, the

comparison of antifungal indices collected from the different formulas of synergy showed that (*C.* oil - *L.* oil) system had the best antifungal effect against *A. flavus* strain with value of about 54.19% at concentration of (1/3000) after the 9th days of incubation. Indeed, the combinations of *L.* oil and *N.* oil and *C.* oil - *N.* oil showed synergistic antifungal effect with value of 23.65 and 36.49%. As observed in Table 2, the combining formula of the three oils (*C.* oil - *N.* oil - *L.* oil), showed a decrease of the antifungal effect with value of 48.76% at the concentration of (1/5000).

DISCUSSION

Preventing contamination of food by mold and mycotoxins is a major challenge for stakeholders in the food chain (Duris, 2000). Various strategies may exist to try to limit fungal growth and mycotoxin production in the field, at harvest and during storage (Bejaoui, 2005). In spite of the precautions taken to prevent the presence of fungal flora and their toxins from food, accidents still occur because there is no food safety to guarantee zero risk (Canadas, 2006).

Plants are currently a priority source for searching for new biologically active substances, their contents and the chemical nature of their constituents confers great application prospects. A wide spectrum of biological substances extracted from medicinal plants, including oils, was tested to replace some ways to fight against xenobiotics, including fungal. In this section, numerous investigations have confirmed the effectiveness of oils on toxigenic strains of fungi (Ziyada et al., 2008; Yingying et al., 2008; Dale et al., 2004; Marzouk et al., 2009).

In continuation of work done on assessing the antifungal activity of oils extracted from seeds of *C. colocynthis, L. usitatissimum* and *N. sativa*, we aimed to test the eventual synergy between these oils, to assess the ability to inhibit or remove the toxigenic strain *A. flavus*.

The analytical control of oils based on determination of chemical indices (values given in Table 1) showed that the values are in agreement with the codex norms'. In fact, the refraction index values of *C.* oil, *L.* oil and *N.* oil were found to be 1.474, 1.480 and 1.472. According to Ollé (2002) data, at intervals (1.472 to 1.480), the oils are rich in fatty acids such as oleic and linoleic acids. Besides, Schafferman et al. (1998) and Dale et al. (2004) confirmed that *C. colocynthis* oil with refraction indice (IR= 1.474) is rich in linoleic acid.

The saponification values of oils (219, 191 and 195) mg_{KOH}/g, respectively for *C.* oil, *L.* oil and *N.* oil, were superior to the codex standards. This result may be an indication of the presence of short carbon chain of fatty acids (Ollé, 2002). From our finding, iodine values of our samples exceeded the standard requested by codex norms; this may be an index on chains moderately rich in unsaturated fatty acids (karleskind, 1992). As seen in Table 1, we observed high values of iodine number which confirmed well the best behaviour of the oils chain carbon and their ability to bind the iodine molecules. This finding correlated well with the viscosimetric data described. From Table 1, we observed also that the peroxide value of oils was less than the standard of 10 meq O_2/kg oil.

The evaluation of the antifungal potency of oils tested separately showed a satisfactory antifungal effect. The oils efficacy was ordered as follows: *L. usitatissimum*, *C. colocynthis* and *N. sativa*. Determination of antifungal index for *L.* oil, *C.* oil and *N.* oil, respectively gave values (55.65, 48.65 and 40.54). These results are in agreement with findings of Yingying et al. (2008) who demonstrated that the seeds powder of *L. usitatissimum* (6%) inhibit completely the mycelial growth of *A. flavus*. Dale et al. (2004) believed that the antifungal effect could be due to the action of α-linolenic acid present in this oil.

The oil of *C. colocynthis* produced also antifungal efficacy but with less index than *L. usitatissimum*. The obtained results confirmed the reports of Marzouk et al. (2009) and Belsem et al. (2009) who reported the antifungal activity of this oil, which was believe to be due to unsaturated fatty acids. Finally, *N. sativa* seed oil gave slight antifungal index (16.44), where several workers confirmed the effectiveness of some *A. Niger* strains which inhibit completely this fungi (Al Jabre et al., 2005). This action was attributed to thymoquinones present in this oil.

In order to improve the antifungal effect, combination of the three oils in various systems; (*L.* oil -*C.* oil), (*L.* oil - *N.* oil), (*C.* oil - *N.* oil) and (*L.* oil - *C.* oil - *N.* oil) were carried out. Unfortunately, the different formulas have not pronounced the desired results. The association of oils (*L.* oil - *C.* oil), (*L.* oil - *N.* oil) and (*C.* oil - *N.* oil) gave antifungal index (54.19, 23.65, 36.49), respectively, besides, the association of three oils (*L.* oil - *C.* oil - *N.* oil) enhanced the antifungal indice (48.76). These indices are the same as those obtained by the use of each oil separately.

Employing viscosity investigation helped us to explain the oils behaviour used separately or in several synergistic formulas. The concentrations required for antifungal effect corresponded well to optimal concentration (C*). At these concentrations, the oils carbon chain covered the space of solution (Flory, 1971). According to the results of Benmansour et al. (2003), the chains are impenetrable and form a temporary micelles at high concentrations (C > C*). Figure 2 showed the behaviour of oils in different solution systems (Doi and Edwards, 1986). The viscosimetric behaviour design of the studied oils allowed

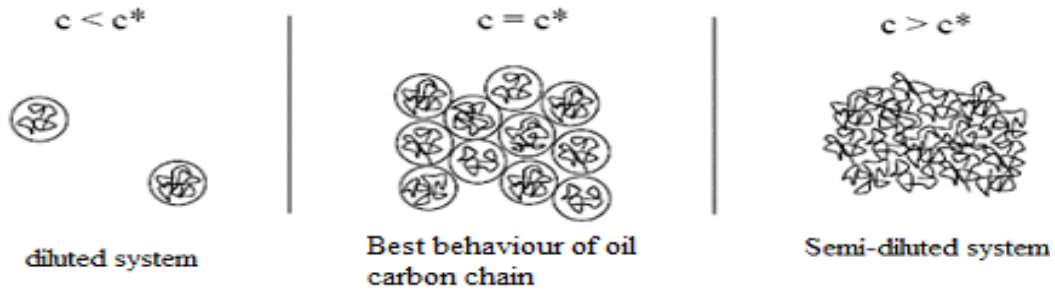


Figure 2. Behaviour of oils carbon chain at different concentrations.

us to highlight the area of ideal concentration for optimal synergistic antifungal activity and therefore understand the behavior of these oils together to advocate the critical micelle concentrations for a possible synergistic antifungal activity. In this field and as observed in Figure 2, the discussion must be in the area of (C = C*), in this area, the oil carbon chain is more spread and flexible, and act in better manner than other ranges. These findings confirmed well the reports of Regalado et al. (1999) and Benmansour et al. (2003) who showed that the effect of longer chains increased the viscosity in an exponential manner (Allouche, 2003). This result showed clearly the importance of the viscosity parameter which informed us about the variations of conformations of macromolecules in solution.

Indeed, this technique provided the presence of intra- or intermolecular associations between oils chains. For our best knowledge, it is important to underline that the study of the synergistic effect of the oils was not carried out through employment of the best behavioural oil carbon chain at C*. This study open the way for further investigations to test the synergistic effect of the studied oils using the obtained C* values, identify, purify the active molecules, determine the antifungal activity and elucidate the mechanism of antifungal purified molecules.

Conclusion

The results obtained are only the first step of the synergistic antifungal activity of the studied oils, further studies should be performed to confirm or refute the effectiveness of these oils on the reduction of fungal flora. From the viscosimetric behaviour investigation, the optimal synergistic antifungal activity of the oils used must be and will be tested at concentration of C*. At the end, fine analytical study on the composition of these oils is more than necessary to search on the possible proportions of these oils for synergistic antifungal potency.

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