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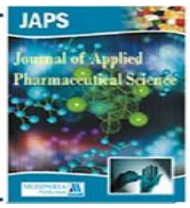

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Evaluation of antifungal activity of some oils from Algerian medicinal plants against *Aspergillus flavus* strain produced aflatoxins

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ABSTRACT

Antifungal activity of the oils extracts of *Citrullus colocynthis* L., *Linum usitatissimum* L., *Nigella sativa* L. collected from Bechar Department in the region of (Algeria) has been evaluated in this present study. Two methods were used in this investigation: radial growth on solid medium and biomass on liquid medium. The oils extracts were obtained by Soxhlet extraction of the seeds part. The values of physicochemical indices of our oils such as acid, acidity and peroxide were also determined. The results of the antifungal potency revealed that the seeds oils exhibited different degrees of inhibition against *Aspergillus flavus* MTTC 2799. However, evaluation of radial growth on *Potatoes Dextrose Agar* (PDA) solid medium showed slight mycelial growth proportional to oil concentration added to the medium. Antifungal indices investigation allowed as to put our oils in the order of effectiveness: *L. usitatissimum* (29%) > *C. colocynthis* (26.5 %) > *N. sativa* (18.75 %). Evaluation of *Aspergillus flavus* biomass production on *Potatoes Dextrose Broth* (PDB) on liquid medium revealed that the studied oils produced a variable weight reduction (*C. colocynthis* and *L. usitatissimum* at concentration superior to (80µl/ml). On the contrary, *N. sativa* oil reduced *Aspergillus flavus* biomass at concentrations of 80µl/ml and 100µl/ml.

Keywords: Antifungul activity, oils, *Citrullus colocynthis* L., *Linum usitatissimum* L., *Nigella sativa* L., *Aspergillus flavus*

INTRODUCTION

Fungi are significant destroyers of foodstuffs and grains during storage, rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins (Marin *et al*., 1999; Janardhana *et al*., 1998). A significant portion of the agricultural produce in the country and the world over become unfit for human consumption due to mycotoxins contamination of grains, especially those produced by species of *Aspergillus* (Janardhana *et al.*, 1999; Chandra R. and Sarbhoy A.K., 1997; Devi *et al.*, 2001; Lopez *et al.*, 2010; Pitt *et al.*, 2000). More than 25% of the world cereals are contaminated with known mycotoxins and more than 300 fungal metabolites are reported to be toxic to man and animals (Matheron *et al*..; 2005). Mycotoxins especially aflatoxins produced by *A. flavus* species are reported to be toxic. Besides, they are thermicaly stable and resistant at high pH which they make detoxication skills are not efficacy (Bourais and Amine; 2006). Unfortunately, remove completely the mycotoxins still an impossible mission. Thus, there is a need to search for alternative approaches to store

grains/cereals for human consumption without toxicity problems that are ecofriendly and not capital intensive. Plant extracts of many higher plants have been reported to exhibit antifungal properties under laboratory trails (Okigbo and Ogbonnaya, 2006; Bouamama *et al.*, 2006; Mohana and Raveesha, 2006; Ouraïni *et al*., 2005). Plant metabolites and plant based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers. This led the authors to screen *in vitro* oils extracts of *C. colocynthis*, *L. usitatissimum*, *N. sativa*. for antifungal activity against *A. flavus* specie with the ultimate aim of developing plant based formulations for plant disease management and safe storage of grains.

MATERIALS AND METHODS

Plant material and extracts preparation

The *C. Colocynthis* plant used for the present study was collected in February 2010 in ain Essdar, Kenadsa area, Bechar Department, Algeria. The seeds were separated from fruits and shade dried at room temperature for 10 days. In contrast, the *L. usitatissimum* and *N. sativa* seeds were purchased from Bechar Market in the same date. The dried seeds part plants 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 seeds materials were extracted with organic solvent chloroform using soxhlet apparatus for 6 hours. The different extracts obtained were subsequently concentrated under reduced pressure to get their corresponding oils extracts residues (AFNOR, 1988).

Fungal material and confirmation of testing strain

The seeds oil extracts were assayed for antifungal activity against the fungal strain *A. flavus* MTTC 2799 obtained from *Arachis hypogaea* in biology laboratory at Bechar University. Confirmation of *Aspergillus* genera was realized by micro-culture method described by Harris (1989) and Barnett (1972). Furthermore, confirmation of *A.flavus* species was carried out by Single Spore method using three cultures media: Malt Extract Agar (M.E.A) at 25°C, Glycerol Nitrate Agar (G25N) at 25°C and Czapek Yeast Agar (C.Y.A) at 5°C and 37°C. Using the identification keys of Pitt and Hocking (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 AFAP medium which give oranges Revers plate. This fungus was stored in tubes of PDA acidified at 4^{o}C.

Aflatoxinogenic Test

A. flavus was sowed on Y.E.S (Yeast Extract Sucrose) medium rich on B vitamin complex. After two weeks of incubation at 30 ± 2 °C, the biomass was removed by filtration of Y.E.S. The filtrate was added then to 180 ml of chloroform and stirred for 30min. After decantation, the organic layer was concentrated to 2ml. Aflatoxins were determined in each extract by spotting samples onto thin layer chromatography plate. Plate was developed with a toluene/Ethyl acetate/ Formic acid (50:40:10, v/v/v) solvent system. Aflatoxin standard was spotted on the same plate as reference and aflatoxin spots were identified using 365 nm UV (Asso. of off. Anal. Chem., 1975). The presence of Aflatoxins was provided by appearance of blue fluorescence for AFB which has the same R_f as control.

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

Selected concentrations of 50 µl/ml ,100 µl/ml ,150 µl/ml ,200 µl/ml ,250 µl/ml, 275 µl/ml and 300 µl/ml of seeds oil extracts of *C. colocynthis*, *L. usitatissimum* and *N. sativa* were prepared using 5% Tween 80 and 20% Agar added then to 20 ml of PDAa solid medium. After agitation, the selected solutions were transferred into a Petri plates which were incubated for 7days at 25 ± 2°C. Mycelial radial growth was measured from the third day of incubation (Soro, 2010; Kra, 2009). The inhibition percentage of mycelial growth of each oil was calculated using the following formula (PI_g = ((DT –D)/DT) x 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).

Determination of percent mycelial inhibition by biomass technique on liquid medium

Evaluation of biomass liquid medium was achieved by counting of spores' number using Malassez hematimeter in order to obtain the concentration of 10^5spores/ml (Serghat *et al.*.; 2004). This technique consists to put different volumes of oil in flasks and completed them with 50 ml of PDBa (Potato Dextrose Broth acidified) in order to obtain the following concentrations: 20 µl/ml ,40 µl/ml ,60 µl/ml ,80 µl/ml ,100 µl/ml, 110 µl/ml and 120 µl/ml. These liquid cultures were sowed with 30µl of sporal suspension. The flasks were incubated for 14 days at 25 ± 2°C (Tubajika, 2006; Hibar *et al.*, 2006). After filtration, the filter paper was dried at 60°C during 24hours (Dhandhukia P.C. and Thakkar; 2007). Biomass weight formed (P) was determined using the following formula of Imtiaj and Lee (2007) ($P = P_1 – P_0$) where P_0 is the filter paper weight and P_1 is the filter paper and fungal biomass weight after dryness.

RESULTS

Extraction carried out on seeds part plants has given the following yields of oils of *L.usitatissimum* (39.96%), *N.sativa* (33.71%) and *C.colocynthis* (17.63%). In order to determine the quality of our oils, physico-chemical indices were measured and illustrated in table 1. On the other hand, our results obtained by identification methods provided that our strain corresponded well to *A. flavus* group. Besides, as seen in figure 1, aflatoxinogenic test revealed that our strain produced AFB1 (blue fluorescence).

The antifungal activity of oils extracts was tested to prevent the mycelial growth of *A.flavus* fungal organism that is harmful to human beings. Different concentrations of oils of seeds tested (Figure 2). Oil extract of *L.usitatissimum* significantly arrested the mycelial growth of *Aspergillus flavus* MTTC 2799 and showed moderate effect on sporulation (figure 2A). Figure 2B

Table 1. Physico-chemical results analysis.

	C. colocynthis oil	*L. usitatissimum* oil	*N. sativa* oil
Refraction indice	1.4754	1.4595	1.4700
Relative density	0.895	0.987	1.030
Acid indice (KOH/g)	5.3	1.59	52.9
Acidity (%)	2.66	0.80	26.58
Peroxide indice (meq.O_2/kg)	7.35	5.68	30.01

shows the activity of oil residue of *C. colocynthis* with complete sporulation and means efficacies on mycelial growth of *A.flavus.* In contrast, *N.sativa* oil appears more efficacies on sporulation and shows less inhibition on mycelial growth of *A.flavus* (Figure 2C).

As shown in figure 3, the results of biomass technique on liquid medium revealed that the biomass weight formed under action of our oils were variable and superior to controls. Results analysis of fungal biomass produced by different concentrations of *L.usitatissimum* oil showed that the biomass weight (0.2190g and 1.1530 g) were more or less superior to controls (0.2584 g) (Figure 3A). Whereas, biomass weights at concentrations of 20, 40 and 60 µl/m were proportional to concentration of oil added in the medium. In contrast, biomass formed under effect of the rest concentrations were inversely proportional. Same results were obtained for *C. colocynthis* oil (figure 3B). As given in Figure 3(C), we observed a fluctuation in mean biomass weight under action of the different concentrations of *N. sativa* oil. So, at concentrations of 20, 40, and 60 µl/ml the weights are nearly constant (0.6400g), then decrease in concentrations of 80 and 100µl/ml (0.5047 g - 0.3390 g). We observed also, elevation of biomass weight by increase of the oil concentrations (110 and 120 µl/ml) where the weight value was found to be 0.6000 g.

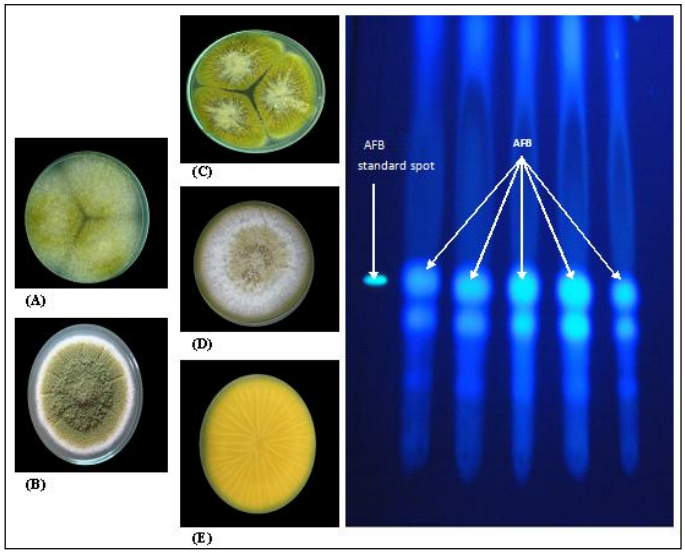


Fig 1. *A. flavus* strain by *« Single Spore »* method: A: G25N at 25°C; B: MEA medium at 25°C ; C : CYA medium at 37°C; D : AFAP medium at 25°C ; E: AFAP revers strain and TLC of aflatoxinogenic test.

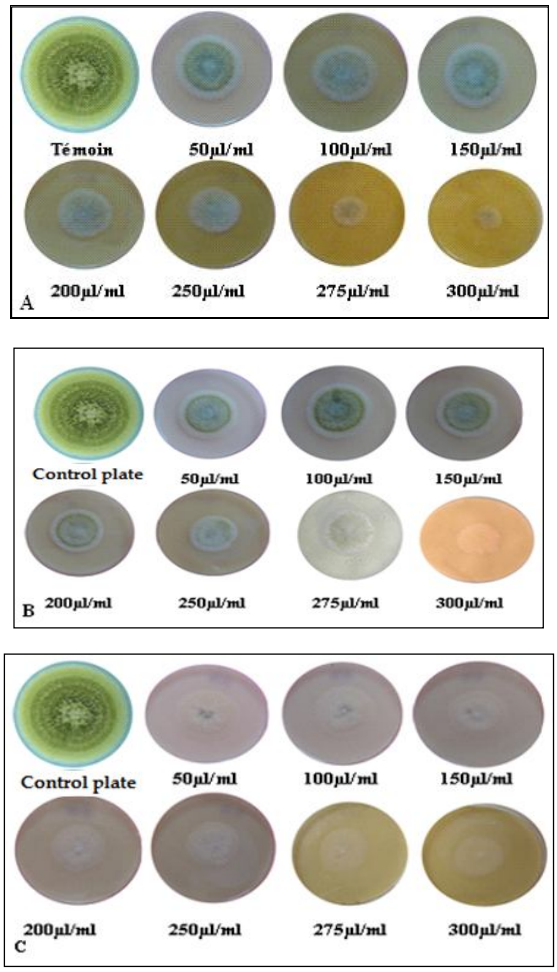
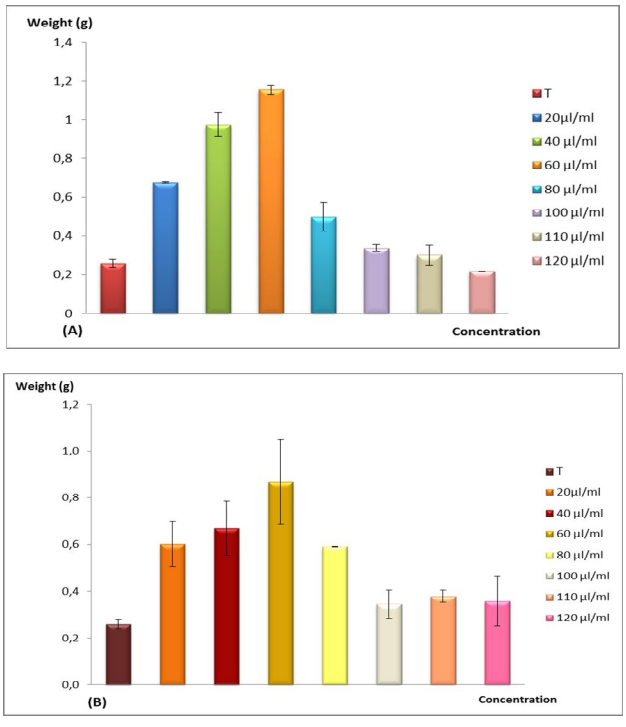


Fig 2. Specimens of radial growth strain under effect of different concentrations of oils: *L. usitatissimum* (A); *C. colocynthis* (B) and *N. sativa* (C).



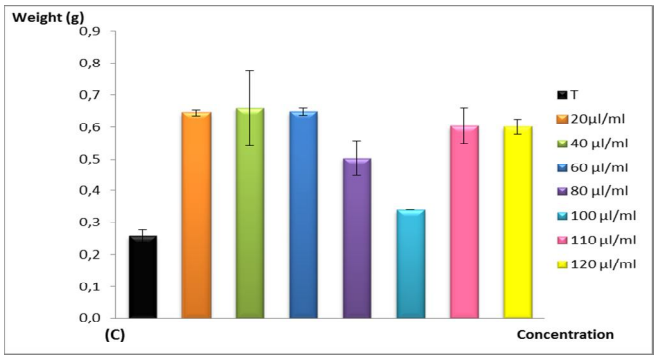


Fig 3. Biomass weight formed in the presence of different concentrations of oils: *L. usitatissimum* (A); *C. colocynthis* (B) and *N. sativa* (C)

DISCUSSION

Aflatoxins are the most notorious of the mycotoxins causing acute and chronic toxicoses in foodstuff (CAST, 2003). *A.flavus* is one of the species of *Aspergillus* that produce aflatoxins. This fungus can grow on a wide range of agricultural commodities. Aflatoxins can be produced in preharvest as well as in stored products as cereals (Wilson and Payne; 1994). Plant-produced compounds are becoming of interest as a source of safer and more effective substances than synthetically produced antimicrobial agents. Many oils obtained from plant parts have been reported as effective inhibitors of fungal growth and aflatoxin production and have also been shown to contain antitoxigenic properties (Bhatnagar and McCormick, 1988; Ansari and Shrivastava , 1991). The present study was undertaken in order to observe the effect of various concentrations of seeds oils of *C.colocynthis* L., *L.usitatissimum* L. and *N. sativa* L on the growth of *A. flavus* and aflatoxin production.

In the first experiment for determination of oils extraction yields, the values varied between 17 and 39%. For *N. sativa* oil, the result of extraction has found to be 33.71% which is in agreement with findings of Meral *et al.*. (2004), AL-Beitawi *et al.*. (2009), and Rouhou-Cheikh *et al.*. (2007) who reported similar oil yield varying between 33.71% and 40.35%. This variation of weight residue may be due to collect period, environment conditions, soil, extraction and storage conditions. For *C. colocynthis*, the yield of extraction oil was found to be (17.63%) which is supported by some reports of Sebbagh *et al.*. (2009), Abu naser and Potts (1953) and Schafferman *et al.*. (1998). In addition, from *L. usitatissimum*, yield of 39.90% of oil was obtained from seeds part plant. This value corresponded well to Quebec research Center (35 to 45 %) and Diedrichsen findings (26 to 45%).

In the second experiment, the analytical control of oils was based on determination of chemical indices (peroxide, acid and acidity). The values of peroxide indice of *C. colocynthis* (7.35 meq. of O_2/kg) and *L. usitatissimum* (5.68 meq. of O_2/kg) are in agreement with codex normes (inferior to 10 meq. of O_2/kg of oil). These results confirm that our oils are not oxidized, in contrast to *N.sativa* with high value of peroxide indice (30.01 meq. of O_2/kg). This oxidation may be due to extraction and storage conditions that produced oxidation reaction of unsaturated fatty acids, carotenoids or vitamins A and B (Ghedira, 2006; Appelbaum *et al.*, 1989). For acid indice, the data showed that *L. usitatissimum* has as value 1.59 mg KOH/g conformed to codex normes (4 mg KOH/g). It is to note that *C. colocynthis* presented a slight elevation (5.3 mg KOH/g) compared to codex normes. However, Akpambang and Amoo (2008) reported that *C. colocynthis* oil obtained from Nigerian country has as acid indice value 8.02 ± 0.07 mg KOH/g. From our findings, we observed well that *N.sativa* oil presented high value which may be attributable to free fatty acids high present in the seeds. In contrast to our study, Tauseef (2009) showed that *N.sativa* oil possess indice acid value of 0.34 mg KOH/g inferior to our oil (52.9 mg KOH/g). In addition to these indices, other parameters such as refraction indice and relative density were also determined. As seen in table 1, our results of refraction indice (1.4595, 1.4700 and 1.4754 respectively for *L. usitatissimum*, *N. sativa* and *C. colocynthis*) are nearly similar to codex norms (1.4677-1.4707) and Akpambang report. Furthermore, the relative density values of *C. colocynthis* reported by Akpambang *et al.* (2008) (1.51 ± 0.02) is higher than our oil 0.8950. Besides, Tauseef (2009) demonstrated that *N. sativa* presented relative density of 0.923 ± 0.001 inferior to our obtained data (1.0300).

In the third experiment, and in order to complete the previous works on antimicrobial plants, we have attempted to evaluate antifungal potency of seeds oils of *N.sativa*, *L.usitatissimum* and *C.colocynthis* against *A. Flavus* strain by growth radial on solid medium and biomass evaluation on liquid medium. The preliminary results of growth radial technique revealed that all the oils tested exhibited different degrees of antifungal activity against *Aspergillus flavus*. The percent of inhibition of *L.usitatissimum* was found to be 29% compared to *C.colocynthis* (26.5%) and *N.sativa* (18.75%). Yingying *et al.*. (2008) reported about the antifungal activity of seeds oil of *L.usitatissimum* (seeds powder at 6% concentration inhibit completely (100%) the development of *A. flavus)*. The present study confirms this investigation where activity may be due to fatty acids more frequent in the seeds especially linoleic acid (Schafferman *et al.*, 1998; Dale *et al.*, 2004). For *C.colocynthis*, Belsem *et al.*. (2009) reported the antifungal activity of the fruits parts. Moreover, Schafferman *et al.*. (1998) suggested that the reduction of the growth strain may be correlated to linoleic acid more present in *C.colocynthis* seeds oil.

For *N.sativa*, numerous works have been made to provide the antifungal activity of this plant against various strains. Agrawal *et al.*. (1979) found that oil extract has been proved to possess excellent activity against *A.niger*, besides Nazrul *et al.*. (1989) confirmed the studied effect of *N.sativa* seeds on *A.flavus* strain. Furthermore, Singh *et al.*. (2005) showed that 6 µl/ml of oil concentration exhibited inhibition activity with 73.3%. Thymoquinone was believed to be the responsible constituent to this effect. This assumption was confirmed by Al-Jabre *et al.*. (2005) who reported that thymoquinone at concentration of 2 mg/ml after one week of incubation has antifungal activity via inhibition of 100% against *A.niger* strain.

Results obtained from biomass weight on liquid medium showed different fungal weights which are superior to controls. These data may be due to inoculum quantity added on culture medium or strain using oil as carbon source. Comparing biomass weight to selected concentrations, *L.usitatissimum* and *C.colocynthis* oils reduced biomass weights at concentration of 80µl/ml. This reduction effect may be due to linolenic and linoleic acids abundant in these oils. This assumption has been confirmed by Dale *et al.*. (2004) report (reduction of biomass weight at 100 µl/ml concentration).

Finally, for *N.sativa*, fluctuation of biomass weights was observed beginning by diminution at 80µl and 100 µl/ml concentrations of oil. Same observations have been reported by Al-Jabre *et al.*. (2005) and Akhtar *et al.*. (2007) who suggested that biomass variation is believed to be due to thymoquinone.

CONCLUSION

The oil extracts of the plants part used showed prominent antifungal activity against *A. flavus*. This potency may be attributable to fatty acids especially linoleic and linolenic acids or synergic effect of their constituents. Further investigations on their mechanisms will be able to provide a great potential for the control of AFB production.

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