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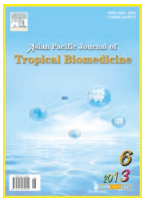

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Evaluation of antifungal activity of free fatty acids methyl esters fraction isolated from Algerian *Linum usitatissimum* L. seeds against toxigenic *Aspergillus*

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Comments

This contribution can be a remedy for the gravity and the abundance of the contamination of the foodstuffs. Indeed, FAMEs of seeds of *L. usutatissimum*, exit of the Bechar (Algeria) area, showed an appreciable activity about (54.19% and 40.48%) successively on *Aspergillus F* and *A. oracles*. This antifungal capacity can be due to the abundance of the linoleic and linolenic acids in the linseed oil which seems promising to treat the fungi infections, the mushrooms of storage and deterioration of food in the field of food industry.

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ABSTRACT

Objective: The aim of this study was to evaluate the antifungal activity of the major fraction of fatty acids methyl esters (FAMEs) isolated from *Linum usitatissimum* L. seeds oil collected from Bechar department (Algeria). **Methods:** The assessment of antifungal activity was carried out in terms of percentage of radial growth on solid medium (*potatoes dextrose agar* PDA) and biomass growth inhibition on liquid medium (potatoes dextrose broth PDB) against two fungi. **Results:** The FAMEs was found to be effective in inhibiting the radial mycelial growth of *Aspergillus flavus* more than *Aspergillus ochraceus* on all tested concentrations. The highest antifungal index was found to be (54.19%) compared to *Aspergillus ochraceus* (40.48%). The results of the antifungal activity of the FAMEs inhibition of biomass on liquid medium gave no discounted results, but this does not exclude the antifungal activity. **Conclusions:** We can assume that the observed antifungal potency may be due to the abundance of linoleic and α-linolenic acids in linseed oil which appears to be promising to treat fungal infections, storage fungi and food spoilage in food industry field.

KEYWORDS

Antifungal activity, FAMEs, *Linum usitatissimum* L., Extraction, *Aspergillus*

1. Introduction

Fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life[1]. In addition, in some cases fungi are indirectly

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responsible for allergic or toxic disorders among consumers because of the production of mycotoxins or allergens[2].

Fungi are generally controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment[3]. The increased risk of high-level toxic residues in the products and the emergence of pathogens resistant to the products employed, justifies the search for novel actives molecules and new control strategies. Thus, there are a growing interest on the research of possible use of the plant extracts for control of the pest and diseases in agriculture which is less harmful to the health and environment[4,5]. Several works have demonstrated in laboratory trials that plants tissues, such as roots, leaves, seeds and flowers posses inhibitory properties against bacteria, fungi and insects[6,7].

Facing these health problems, the use of medicinal plants in herbal medicine has received great interest in biomedical research and is as important as chemotherapy. This because herbs are an inexhaustible source of bioactive natural compounds and other parts of the need for research to better medication therapy by softer without side effects[8]. Medicinal plants are now an endless source of interesting molecules for scientists and industry. Molecules from these plants have similar active ingredients which have specific properties giving them an intrinsic behaviour[9]. A wide spectrum of biological substances extracted from medicinal plants, including oils were tested to replace some of the ways to fight against xenobiotics including fungi. In this section several authors have confirmed the effectiveness of the oils on toxigenic fungi[10-12].

This study was carried out with an objective to investigate *in vitro* the antifungal potency of free fatty acids methyl esters fraction (FAMEs) extracts from the seeds oil of *Linum usitatissimum* L. (*L. usutatissimum*). In this context and in a first time, FAMEs was separated from the linseed oil, in a second one the antifungal effect of these esters has been investigated against two fungal strains producing aflatoxins and ochratoxin A among other *Aspergillus flavus* (*A. flavus*) and *Aspergillus ochraceus* (*A. ochraceus*).

2. Materials and methods

2.1. Plant materiel and extract preparation

The *L. usutatissimum* seeds used for the present study were collected in February 2012 in Kenadsa area, Bechar Department, Algeria. The seeds were shade and dried at room temperature for 10 d. 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. Samples (100 g) of dried powdered form seed were taken into the soxhlet apparatus. A piece of cotton is placed at the top and bottom of the apparatus to evenly distribute the solvent as it drops on the sample during extraction. Extraction was carried out with Petroleum ether for 6 h without interruption by heating around 40 to 60°C. After the extraction, solvent was evaporated on the Buchi-Rotavapor until no odour of solvent remains and finally oil (6.8 g) was collected in a separate beaker for further analysis[12].

2.2. Isolation of ester from fatty acids extract

2.2.1. Saponification of fatty acids

Into a 250 mL round-bottomed flask, fitted with a reflux condenser, place 3.4 g of oiliness residue, ethanol (15 mL), water (15 mL) and sodium hydroxide 3 g. Reflux the mixture for about 45 min. Distill off the ethanol under reduced pressure using a rotary evaporator. Extract the solution with 15 mL of diethyl ether. Acidulate the organic layer with concentrate acid (HCl) then extract it with 20 mL of diethyl ether. Distill off the solvent. Yield: 3.24 g of fatty acids residue[13].

2.2.2. Methylic esterification of fatty acids

Into a 250 mL three-necked flask equipped with a drooping funnel, a sealed stirrer unit a double surface condenser, place 3 g of fatty acids and 25 mL of methanol. Add slowly through the dropping funnel and with vigorous stirring a solution of concentrate sulfuric acid (1 mL). Reflux the mixture for about 2 h. Allow the reaction mixture to reach room temperature and to stand for 2 h, cool the mixture and pour onto 300 g of crushed ice. The aqueous layer was then extracted with chloroform. The organic layer was dried on anhydrous sodium sulfate. The solvent was distilled off. Yield: 1.12 g of methyl esters residue[15,16].

2.2.3. Column separation of methyl ester fraction

The methyl esters of extract (1.12 g) was purified by column chromatography 1150 mm long and 35 mm in diameter, filled with 250 g of silica gel S, subsequently eluted with a mixture of petroleum ether-chloroform (9:1). A total of 10 fractions was collected and combined into two fractions (Frac.1 (0.9 g) and Frac.2 (0.3 g)). Analysis via thin layer chromatography showed that the fraction 1 is pure in comparison with the fraction 2.

2.3. Fungal materials and confirmation of testing strain

The seeds oil extracts were assayed for antifungal activity against the fungal strain *A. flavus* MTTC 2799 and *A. ochraceus* CECT 2092 obtained from biology laboratory at Bechar University. Confirmation of *Aspergillus* genera was realized by micro-culture method described by Wheelis and Dugan[17,18]. Furthermore, confirmation of *A. flavus* species was carried out by Single Spore method using three cultures media: malt extract agar at 25 °C, glycerol nitrate agar (G25N) at 25 °C and Czapek yeast agar at 5 °C and 37 °C. Using the identification keys of Pitt and Hocking[19], 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. The fungal strains are regularly maintained by subculturing on PDA medium. This fungus was stored in tubes of PDA acidified at 4 °C.

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

Selected volumes of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μL of FAMEs isolated from *L. usitatissimum* seeds oil are placed in test tubes and fill up to 15 mL by solid medium PDAa (*Potatoes Dextrose Agar*) to obtain the following concentrations: 0.7, 1.3, 2.0, 2.7, 3.3, 4.0, 4.7, 5.3, 6.0 and 6.7 μL/mL after agitation, the selected solutions were transferred into a petri plates which were inoculated by the respective spore solution of each fungal strain tested (Agar solution at 0.2%+5% tween 80 and spores of fungal strains). The plates were incubated for 7 d at 25±2 °C. Mycelial radial growth was measured from the third day of incubation[20,21]. 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[22].

2.5. 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 in order to obtain the concentration of 10^6 spores/mL[23]. This technique consists to put different volumes of esters 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μL in flasks and completed them with 50 mL of PDBa (Potato Dextrose Broth acidified) in order to obtain the following concentrations: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 μL/mL. These liquid cultures were sowed with 30 μL of sporal suspension. The flasks were incubated for 14 d at 25±2 °C[24,25]. After filtration, the filter paper was dried at 60 °C during 24 h[26]. Biomass weight formed (P) was determined using the following formula of Swagatica (2012) ($P=P_1-P_o$, where P_o is the filter paper weight and P_1 is the filter paper and fungal biomass weight after dryness[27].

2.6. Statistical analysis

All experiments were repeated at least five times. The experimental data were analyzed statistically by the analysis of variance (ANOVA) under excel 2007.

3. Results

Through the results depicted in Table 1, it appears that the seeds of *L. usitatissimum* gave a good yield of oil (39.96%). On the other hand, our results obtained by isolation methods from fatty acids showed a yield of 37.44% of crude esters this extraction yield is quite similar to the first one. Contrary to these results, the crude ester gave a great efficiency of major esters (80%). On the other hand, the results obtained by identification methods provided that ours trains corresponded well to *A. flavus* and *A. ochraceus*.

Table 1

Table 1

Mean yields of isolated esters.

	Oil	Crude esters	Major fraction of esters after Column separation
Weight (g)	6.80	1.12	0.90
Yields (%)	–	37.44	80.00

The antifungal activity recorded in the present study by the inhibition of radial growth on solid medium, revealed that the tested FAMEs possess potentiel antifungal activity against *A. flavus* and *A. ochraceus*. The effect of different concentrations of the FAMEs is summarized in Table 2. Indeed, the tested proportions inhibited the growth of fungus even at low concentrations. The potency of the *L. usitatissimum* FAMEs was optimal on *A. flavus* more than the second fungi on all tested concentrations. The highest antifungal index was found to be (54.19%) compared to *A. ochraceus* (40.48%) and the least activity recorded on *A. flavus* (46.11%) was observed at 70 μL. In contrast, less antifungal activity was revealed against *A. ochraceus* on almost tested dilutions. Statistical analysis of variance ($P<0.05$) revealed significant results.

The results obtained from biomass weight on liquid medium showed different and variable fungal weights. These last were almost superior to controls as shown in Table 2. Results analysis of fungal biomass (*A. flavus* − *A. ochraceus*) product under action of our FAMEs showed that the biomass were more important than control weights. However, some exception were recorded at certain concentrations (0.4 μL/mL, 1 μL/mL, 1.6 μL/mL) on which inhibition of biomass was perceived ($IAF_{0.4\ \mu L/mL}$=18.25% ; $IAF_{1\ \mu L/mL}$=12.15% and $IAF_{1.6\ \mu L/mL}$ =17.89%). Same findings were obtained for *A. ochraceus*. The

Table 2

Antifungal index of FAMEs isolated from *L. usutatissimum* tested against *A. flavus* and *A. ochraceus*.

		10 μL	20 μL	30 μL	40 μL	50 μL	60 μL	70 μL	80 μL	90 μL	100 μL
A. flavus	Solid medium	54.19±0.85	53.16±0.91	48.22±0.47	48.62±0.40	51.69±0.40	50.73±0.12	46.11±0.06	49.25±0.33	47.83±0.26	48.88±0.73
	Liquid medium	−10.43±0.13	18.25±0.59	−25.49±0.51	−0.82±0.22	12.15±0.61	−5.20±0.31	−13.95±0.34	17.89±0.14	−16.03±0.31	−11.37±0.63
A. ochraceus	Solid medium	39.17±0.79	29.56±0.47	38.24± 0.83	40.44± 0.14	37.18± 0.55	35.87± 0.45	38.47± 0.78	30.07± 0.83	40.48± 0.12	30.60± 0.64
	Liquid medium	2.90±0.46	−13.23±0.70	−6.45± 0.97	−33.23±0.16	−11.29±0.02	21.61± 0.04	−12.90±0.28	10.87± 0.72	−42.58±0.37	−12.58±0.32

(Growth radial technique on solid medium and Biomass technique on liquid medium). ($P<0.05$)

weight of biomass decrease in dilutions of 1.2 μL/mL and 1.6 μL/mL) ($IAF_{1.2\ \mu L/mL}$=21.61% ; $IAF_{1.6\ \mu L/mL}$=10.87%). Statistical analysis showed that there is no statistically significant difference but *A. ochraceus* was found to be more or less sensitive.

4. Discussion

Fungi are ubiquitous in the environnement, and infection due to fungal pathogens has become more common. The genus *Aspergillus* is widely distribued in nature and its species are among the most common destroyers of foodstuffs and grains during storage. It includes species that may damage crops in the field or cause post harvest decay[28]. In addition, the genus products mycotoxins and studies in the last decade have emphasized its toxicogenic properties. Indeed, the palette adverse effects of mycotoxins on the human and animal health is very extensive and sometimes unknown[29,30]. Besides acute toxic effects or chronic hemorrhagic, immunotoxic, hepatotoxic, nephrotoxic, neurotoxic, teratogenic, or oestrogenic, certianes mycotoxins have shown mutagenic and carcinogenic effects in laboratory animals and humans[31].

Over the last decades, concerns were expressed about the increasing prevalence of pathogenic fungi that are resistant and more precisely those producing mycotoxins. But, the problem posed by the high cost and the increased toxic side effects of some synthetic substances coupled with their failure to treat cannot be underestimated. For this reason, this last decade we witness an increased intensive studies of extracts and biologically active compounds isolated from natural plants[32,33]. Facing this situation, the aim of this work was to evaluate *in vitro* the potentiel antifungal of FAMEs extract from seeds oil of *L. usitatissimum* against toxigenic fungi producing mycotoxins such as *A. flavus* and *A. ochraceus* in order to check possible inhibition activity.

Results for determination of oils extraction yields, showed that yield of 39.90% of oil was obtained from seeds part of plant *L. usutatissimum* this value correspond well to Quebec research center (35%-45%) and (Diedrichsen *et al*, 2008) findings (26%-65%)[34]. Preliminary analysis of the results of the isolation of esters from *L. usutatissimum* oil revealed a yield of 37.44% of crude esters which have provided a considerable rate of FAMEs (82.80%). These results are in analogy with literature data which confirm the richeness of this oil with fatty acids and esters of fatty acids[35].

The obtained antimicrobial results showed a pronounced antifungal activity of FAMEs isolated from *L. usutatissimum* seeds oil. These results are in harmony with the work of Lima (2011) and Canales (2011) who showed that the methyl esters of fatty acids are endowed with antibacterial and antifungal capacity[36,37]. Exploitation of the evaluation of antifungal solid medium of FAMEs finding, revealed their inhibitory effect against the two fungal strains on all concentrations tested. Furthermore, we found that the antifungal effect of the FAMEs was more pronounced on *A. flavus* than *A. ochraceus* ($IAF_{10\ \mu L}$=54.00%, $IAF_{20\ \mu L}$=53.16%, $IAF_{50\ \mu L}$=51.69%). These results correlate with the work of Yingying[11], who reported that *linum*seeds powder at 6% concentration inhibit completely the development of *A. flavus*. Previous reports have shown that the FAMEs provided antibacterial and antifungal activities[36-38]. Chandrasekaran showed that fatty acid methyl esters extracted from halophyte plants exhibited strong antimicrobial activity against micro-organisms pathogenic to human as *A. fumigatus* and *A. Niger*[39]. Nehdi *et al.* and Choi *et al.* have emitted the hypothesis that the antifungal effect was probably due to the predominantly linoleic acid present in linseed oil[40,11].

Furthermore, the biomass in liquid medium technique showed that FAMEs have no inhibitory except on some concentrations ($IAF_{1.2\ \mu L/mL}$=21.61% for *A. ochraceus* and $IAF_{0.4\ \mu L/mL}$=18.25%) for *A. flavus*. Same findings (fluctuation of biomass weights) has been reported by Ozdemir *et al.* and Elsemra *et al*[42,43]. At this stage of the analysis, we have not yet a clear explanation for the perceived difference in the two methods of evaluation of the antifungal activity of our FAMEs, but it is sure that this difference does not exclude the antifungal activity. The assumption is that the fractions present in the ester that may be responsible for the antifungal activity by reducing mycelial growth in solid media do not react by the same way in the liquid medium. Otherwise, incubation under agitation might be given a better homogenizing culture medium and an increased chance of contact between the fungus and the bioactive fraction. We believe also that antifungal effect may be due to the abundance of linoleic and linolenic acids in linseed oil[44]. This assumption has been confirmed by Choi *et al*[12].

The results of the present work indicate that the *Linum* FAMEs assayed possess antifungal properties which could be used on various diseases whose symptoms might involve fungal infections and storage fungi and food spoilage in food industry field. Further, phytochemical research is needed to identify the active fatty acid responsible for the antifungal effect.

Conflict of interest statement

We declare that we have no conflict of interest.

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useful suggestions and information's for this study.

Comments

Background

A. flavus and *A. ochraceus* are able to deteriorate food, inducing organoleptic and chemical deteriorations with serious risks of intoxications for the consumers. Produced *A. flavus* of aflatoxine group's substances are toxic and carcinogenic (disease X of turkeys and other livestock). *A. ochraceus* produced of the ochratoxine, this mycotoxin is the cause of human renal disease, and easily contaminates the cereal grains stored in the mould.

Research frontiers

The means of fight against the moulds are numerous: A rigorous hygiene, physical agents like the temperature or radiations but cannot be used in a universal way, certain chemicals (fungicidal) can also be used, but harmful for the man, very recently lactic Bacillus and other bacteria can play a determining role. This work is interested has the action of the FAME extradited of the plant: *L. usutatissimum*. Indeed, several work showed that certain parts of the plants has inhibiting properties with respect to the bacteria, mushrooms and insects without side effects.

Related reports

The objective of this study is to evaluate the antiphonic activity of the fatty acids (FAME) of seeds of *L. usutatissimum* Exit of the Bechar (Algérie) area. Methodology is well described, the means implemented make it possible to control an experimental work well on the living organisms: Identification of the moulds and quantification of the activity, the latter was evaluated in terms of percentage of the radial growth on solid medium and by the inhibition of the growth on liquid medium, with respect to two moulds: *Aspergillus F* was shown more sensitive with a rate of 54.19% compared to *A. okraceus* (40.48%), but the results on the biomass seems to be can convincing.

Innovations and breakthroughs

Fatty acids from the plant have antifungal activity may be quite large and the natural products label food preservation, but deeper studies should be conducted to confirm that they do not present a danger to the living; it would be interesting to see how they are working on these foods (spray coating ...).

Applications

The presence of producing toxin moulds must be taken with the serious one, they are carcinogenic molecules even has low dose since, not or with difficulty metabolize and thus accumulates in the organization, the prevention is essential in the silos and other places of storages which must be refractory mediums with the development of mushrooms. This plant and well of others of the area must undergo a screening for their metabolites have fine to replace: Chemicals and other radiations for the conservation of food.

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This contribution can be a remedy for the gravity and the abundance of the contamination of the foodstuffs. Indeed, FAMEs of seeds of *L. usutatissimum*, exit of the Bechar (Algeria) area, showed an appreciable activity about (54.19% and 40.48%) successively on *Aspergillus F* and *A. oracles*. This antifungal capacity can be due to the abundance of the linoleic and linolenic acids in the linseed oil which seems promising to treat the fungi infections, the mushrooms of storage and deterioration of food in the field of food industry.

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