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Full Length Research Paper

Evaluation of antifungal effect of organic extracts of Algerian *Citrullus colocynthis* seeds against four strains of *Aspergillus* isolate from wheat stored

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Anti-mycotic activity of the methanolic and aqueous extracts from Algerian Colocynth *(Citrullus colocynthis* L. Schrad) seed parts were screened *in vitro* against four important plant pathogenic fungi, namely, *Aspergillus flavus, Aspergillus ochraceus, Aspergillus fumigatus* and *Aspergillus niger,* using micro-dilution technique in the broth medium after their phytochemical analysis. The qualitative phytochemical screening of organic extracts revealed the presence of biochemical substances such as alkaloid, flavonoid, steroid and saponins liable for biological activity. Extracts inhibited the mycelial growth of almost all strains of *Aspergillus* and the results unveiled that the methanol extract was more of antifungal effect than the aqueous extract with minimum fungicidal concentration (MFC) of 15 mg/ml against *A. ochraceus,* 17.5 mg/ml against *A. niger* and 25 mg/ml against *A. fumigatus*. *A. flavus* proved to be the most resistant strain to the two types of extracts and no activity was recorded. These results showed that the extracts could be considered suitable alternatives to chemical additives for the control of fungal diseases in plants.

Key words: *Citrullus colocynthis,* methanolic extract, aqueous extract, phytochemical screening, antifungal activity.

INTRODUCTION

Some foodstuffs are most susceptible to fungal infection such as vegetable products, including fruits, green vegetables and cereal grains. During their growth, harvesting and storage, the microbiology of cereals is highly dominated by fungi, being *Aspergillus* and *Fusarium*, the most commonly isolated genera. These genera produce most of the damage to cereals by their presence itself, but due to their capacity to produce and accumulate mycotoxins (Del Castillo, 2007), it represents a threat to the wholesomeness of the food and thus constitutes an important health risk for consumers (Desjardins et al., 2000; Thompson and Henke, 2000; Bennett and Klich, 2003).

Currently, about 300 fungal toxins have been identified, being aflatoxins, fumonisins, ochratoxins, and deoxynivalenol, the most frequently found (Dutta and Das, 2001; Bennett and Klich, 2003). Mycotoxins have an immediate toxic effect, as well as immunosuppressive, mutagenic, teratogenic and cancerogenic properties (Del Castillo, 2007).

The decontamination of fungi and their mycotoxins in food may be carried out by means of physical, chemical or biological methods. These methods should be efficient, economic and should not significantly modify the nutriational value of the food. In addition, treatment by these methods should not leave residuals that could adversely affect animal or human health (Murphy et al., 2006). The use of the physical and chemical methods available for the detoxification of mycotoxin contaminated agricultural products is restricted due to health security issues and the

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issues and the possible decrease in the nutritional quality of the food. These methods are also of limited efficiency and involve expensive procedures, which has led to the search for alternative strategies such as biological control (Kabak et al., 2006).

The increase in the demand for natural and increasingly safe food products has led to the use of methods that biologically monitor microorganisms that contaminate foodstuffs. Among these is the addition of natural products with antimicrobial properties which ensure the quality of the food (Kabak et al., 2006). Among these natural products, bioactive substances of aromatic plants with known antiseptic and antimicrobial properties and mainly used traditional medicine have been studied for use in the control of fungi (Bluma et al., 2008; Kumar et al., 2008). The objective of this study was to know the phytochemicals families and to assess the antifungal effect of organic extracts of *Citrullus colocynthis* seeds, an aromatic and medicinal plant known by their antimicrobial activity, against *Aspergillus fumigatus, Aspergillus ochraceus, Aspergillus flavus* and *Aspergillus niger* isolated from wheat stored, taken from the Algerian Interprofessional Cereals Office (AICO).

MATERIALS AND METHODS

Plant

C. colocynthis Schrad. fruits were collected in December near Ouargla, Algeria in the area of Oued N'sa. The identification was performed according to the flora of Tunisia (Pottier-Alapetite, 1981) and the botanists of Faculty of Biology of Saida University (Algeria).

Extraction protocol

The extractions were performed on the seeds of *C. colocynthis*. Plant materials were washed with tap water, disinfected by immersion in 2% sodium hypochlorite solution for 30 min, rinsed with sterile distilled water to eliminate residual hypochlorite. Afterwards, the seeds are ready for extraction (Jasso de Rodriguez et al., 2005).

Methanol extract

Twenty grams of seeds were ground with a mixer and added to 100 ml of methanol. After 3 h of maceration with continuous stirring at 200 rev/ min, the mixture was then filtered using filter paper (Whatman No 1). This operation is repeated four times after each filtration with renewal of the solvent in order to exhaust the marc and increase the yield. At the end of extraction, the fractions obtained were collected in a vial and then were evaporated by rotavapor at a specific temperature to the solvent (Senhaji et al., 2005).

Aqueous extract

The aqueous extract is prepared by soaking 20 g of the ground seeds in 100 ml of cold distilled water for 3 h with continuous agitation. The mixture was then centrifuged at 3600 g for 30 min. The supernatant was recovered and then filtered through Whatman No. 1 filter paper. This operation was repeated four times after each filtration with renewal of the solvent. At the end of extraction, the fractions obtained were collected in a vial and lyophilized, yielding the lyophilized aqueous extract (Senhaji et al., 2005).

Qualitative phytochemical screening

Saponins

Ten milliliters of extract was placed in a test tube shaken for 15 s and then deposited for 15 min. A persistent foam height greater than 1 cm indicates the presence of saponins (Koffi et al., 2009).

Steroids

After addition of 5 ml of acetic anhydride to 5 ml of hot extract, the mixture was added to 0.5 ml of concentrated sulfuric acid. After stirring, the appearance of a purple or violet ring turning blue to green indicates the presence of steroids (Bruneton, 1999).

Flavonoids

A mixture of a few drops of magnesium ion and drops of concentrated HCl were placed in a tube and 2 ml of extract was added to it. The appearance of pink coloration, orange or red indicates the presence of flavonoids (Malec and Pamelio, 2003).

Alkaloids

Alkaloids have been characterized using reagents of Mayer. 10 milliliters of the extract were evaporated until a volume of 0.2 ml was obtained on which 1.5 ml of HCl (2%) was added. After stirring the acid solution, 1 to 2 drops of reagent were added, and the appearance of a yellowish white precipitate indicates the presence of alkaloids (Mojab et al., 2003).

Antifungal activity of plant extracts

Fungal isolation

Dilution plating was used as isolation technique (Pitt and Hocking, 2009). Ten grams of the sample were added to 90 ml of 0.1% peptone water. This mixture was then shaken on a rotary shaker for approximately 15 min and diluted 10^{-2}, 10^{-3} and 10^{-4} fold. Aliquots composing of 0.1 ml of each dilution were spread (in triplicate) on the surface of the Dichloran Rose-Bengal Chloramphenicol Agar (DRBC medium), Czapek Dextrose Agar (CDA) and Potatoes Dextrose Agar (PDA). All plates were incubated for 5 to 7 days at 28°C in the dark and under normal atmosphere.

The identification of fungal strain is realized on the basis of morphological characteristics under the microscope (Raper and Fennell, 1965; Barnett and Hunter, 1972; Pitt, 1973; Pitt and Hocking, 2009) and single spore method by colony characteristics after their culture on three different culture media: Malt Extract Agar (MEA) at 25°C, Glycerol Nitrate Agar (G25N) at 25°C, CDA at 25°C and Czapek Yeast Agar (CYA) at two different temperatures: 5 and 37°C.

Antifungal screening test of plant extracts

The antifungal activity of the prepared extracts was determined by

Table 1. Extraction yields (%) and phytochemical screening of *C. colocynthis* seeds.

Extract	Extraction yields (%)	Phytochemical screening of *C. colocynthis* seeds			
		Alkaloids	Flavonoids	Saponins	Steroids
Aqueous extract	2.72	+	+	-	+
Methanol extract	4.89	+	+	+	+

+: Presence; –: Absence.

using micro dilution method. The plant extract residues were re-dissolved in 5 ml of Czapek Dox Broth (CDB), sterilized in disposable Millipore filter (0.22 µm pores) and mixed with 45 ml of sterile Czapek broth in 150 ml Erlenmeyer flasks to obtain final concentrations ranging from 1 up to 25 mg/ml of each plant extract. The control set was kept in parallel to the treatment sets without plant extracts (Al-Rahmah et al., 2011). The flasks were inoculated with discs of 6 mm diameter of *A. flavus, A. ochraceus, A. fumigatus* and *A. niger* isolate and incubated at 25 ± 2°C for 7 days. After incubation, content of each flask was filtered (Whatman No. 1) and biomass of the filtered mycelium was determined after drying at 70°C for 4 days till their weights remains constant. The percentage of mycelial inhibition was calculated (Table 3) using the following formula.

Percentage of mycelial inhibition = [C – T / C] × 100

where, C and T are the mycelial dry weight (mg) in control and treatment respectively.

RESULTS

Phytochemical screening and extraction yield

Methanol extract of *C. colocynthis* seeds have significantly much higher extraction than aqueous extract, but both types of extraction gave a yield greater than 1% (Table 1).

The results (Table 1) reveal that the aqueous extract contained alkaloids, flavonoids and steroids. Saponins were not detected in this extract. The methanol extract reacted positively with all families of the phytochemicals tests.

Identification of fungal strains

The different strains of *Aspergillus* isolated from wheat stored and their aspects on different medium of identification by single spore method are shown in Table 2. Fungal strains appear with different colors on culture media, which facilitates their identification by referring to the identification key.

The colonies of *A. flavus* and *A. fumigatus* are absent on the CYA and the colors of colonies of *A. niger* are indeterminate on this medium. The confirmation of *A. flavus* species is made on attenuated familial adenomatous polyposis (AFAP) medium; the latter gives an orange reverse after growth.

Antifungal activity of methanol and aqueous extracts

Minimum inhibitory concentration and minimum fungicidal concentration (MIC and MFC) were employed by poisoned food technique to assess fungistatic and fungicidal properties of the plant extract. As illustrated in Figures 1 and 2, the inhibitory plant extracts showed various capabilities to suppress *A. ochraceus, A. niger* and *A. fumigatus* grown on broth medium.

Although, the inhibitory effect of the plant extracts increased in proportion to their concentrations and reached to a maximum in the final concentration of 25 mg/ml. The methanolic extract of *C. colocynthis* showed completely inhibited mycelial growth of *A. ochraceus* at concentration of 15 mg/ml followed by *A. niger* and *A. fumigatus* with a concentration 17.5 and 15 mg/ml, respectively, while *A. flavus* appears more resistant to the concentration range achieved and need more than 25 mg/ml to suppress fungal mycelial growth.

Methanolic extract of *C. colocynthis* were strongly active at MIC of 12.5 mg/ml with 76.90% inhibition and at MFC of 15 mg/ml against *A. ochraceus*. For *A. niger*, the MIC is 15 mg/ml with 86.91% inhibition and MFC is 17.5 mg/ml and finally mycelial growth of *A. fumigatus* was inhibited at 22.5 mg/ml with 90.42% inhibition and MFC is 25 mg/ml. The aqueous extract of *C. colocynthis* has no inhibitory effect against *A. flavus* by concentrations; this extract completely inhibits the mycelial growth of *A. ochraceus* and *A. niger* at a MFC of 22.5 and 25 mg/ml, respectively. The last two fungal strains present with aqueous extract the MIC of 20 mg/ml with 85.55% inhibition and MIC of 22.5 mg/ml with 89.66% inhibition, respectively. For *A. fumigatus*, inhibition was only 10.47% with 25 mg/ml of aqueous extract; these strains need more than 25 mg/ml to suppress fungal mycelial growth. The impact of plant extracts on the biomass of mycelial growth of fungal strains is shown in Table 3. All biomass of mycelial growth decreases with augmentation of plant extracts.

DISCUSSION

Medicinal plants have been used for ages in the treatment of diseases. In recent years, herbal medicines have increasingly been used to treat infections difficult to manage, but their use as food preservative was rarely

Table 2. Identification of the species *A. flavus, A. fumigatus, A. niger and A. ochraceus* by single spore method.

Genera species	Medium	Reading	
		Color	Diameter (mm)
A. flavus	MEA 25°C	Pistachio green	58
	CYA 37°C	Dark brown	52
	CYA 5°C	Absent	Absent
	G25N 25°C	Greenish yellow	50
	AFAP	White + Orange back	48
A. fumigatus	MEA 25°C	White	45
	CYA 37°C	Pale yellow to yellow	50
	CYA 5°C	Absent	Absent
	G25N 25°C	Yellow white	51
A. niger	MEA 25°C	Black	45
	CYA 37°C	Gray to black	56
	CYA 5°C	ID*	30
	G25N 25°C	ID*	Micro colony
A. ochraceus	MEA 25°C	Yellow gold	70
	CYA 37°C	Yellow	53
	CYA 5°C	Beige	20
	G25N 25°C	Yellow	55

ID*: indeterminate.

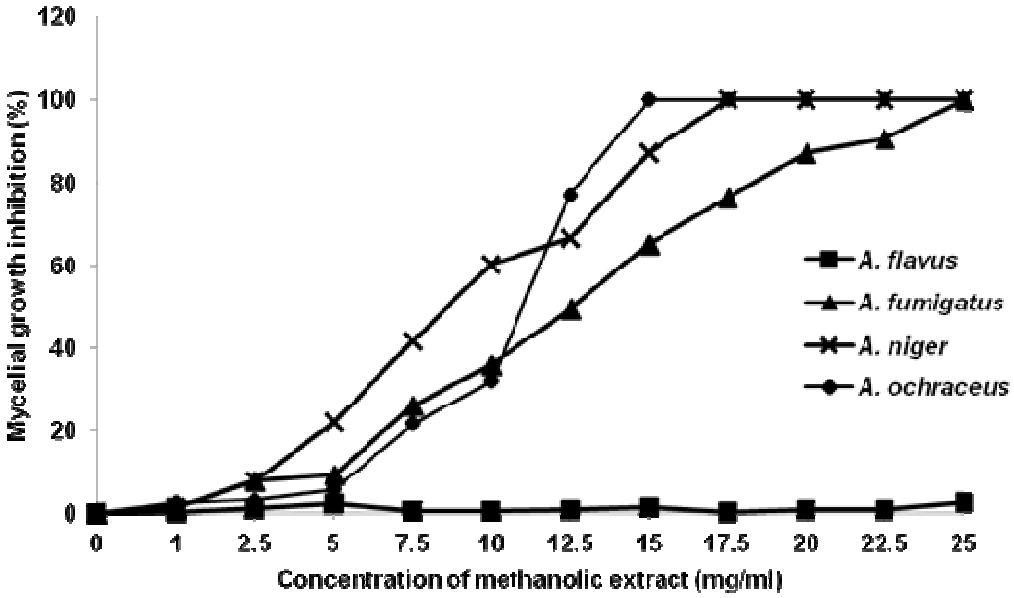

Figure 1. Percentage of mycelial growth inhibition of the toxigenic *A. flavus, A. ochraceus, A. niger* and *A. fumigatus* by various concentrations of methanolic extract.

studied. Although, the antibacterial and the anticandidal activities of all organs of *C. colocynthis* has been investigated (Marzouk et al., 2009; Marzouk et al., 2011; Najafi et al., 2010), but the screening of the seed organic

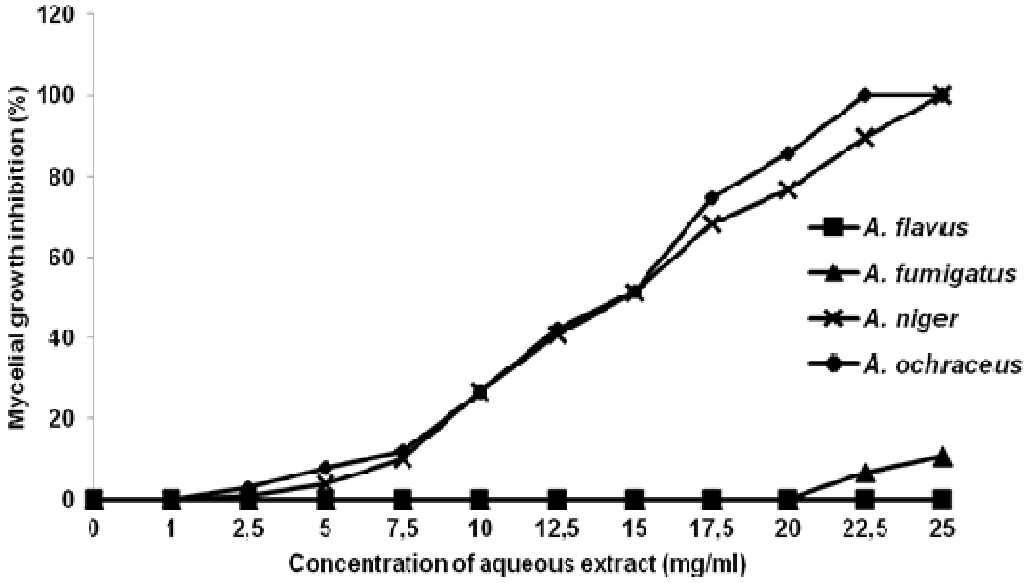

Figure 2. Percentage of mycelial growth inhibition of the toxigenic *A. flavus, A. ochraceus, A. niger* and *A. fumigatus* by various concentrations of aqueous extract.

extracts against fungal strains responsible for alteration of wheat has not been elucidated. This investigation has provided multifaceted results as made obvious by the extraction yields, phytochemical screening and antifungal activities of organic extracts of *C. colocynthis* seeds.

The phytochemical screening reported in this paper revealed that the seeds of *C. colocynthis* are rich in bioactive substances which are responsible for antifungal activity which is in agreement with the study of Benariba et al. (2013). Among these substances, flavonoids and alkaloids are also shown to inhibit microbes (Fogliani et al., 2005; Yan et al., 2008). Saponins and steroids are active antifungal agents (Sadipo et al., 1991; Nawaz Khan et al., 2007). The efficiency of the antifungal activity of each extract depends on the type of plant extract which is generally a crude mixture of non-active and active compounds. The antibacterial activity depended on the tested strain (the plant organ and the nature of the extract); for a given micro-organism, the most effective plant organ can also change according to the organic extract type. The studies of Gurudeeban et al. (2010) showed that the methanolic extract of leaf of *C. colocynthis* have a good antifungal activity against *A. fumigatus*, but the aqueous extract did not present any activity against *A. flavus*.

The studies of Marzouk et al. (2011) confirm that the organic extract of *C. colocynthis* have a good antimicrobial activity. Other results published by Gurudeeban et al. (2011) showed that the ethanolic extract of *C. colocynthis* have a good antifungal activity against *A. fumigatus* and *A. flavus* and even the results of Amrouche et al. (2011) revealed that the oil of *C. colocynthis* has a very good capacity to inhibit the growth of *A. flavus*.

Several studies have been conducted to understand the mechanism of action of plant extracts; however, it is still unclear. Several researchers attributed this function to the phenolic compounds: the amphipathicity of these compounds can explain their interactions with bio-membrane and thus the antimicrobial activity (Veldhuizen et al., 2006). Possible action mechanisms by which mycelial growth may be reduced or totally inhibited have been proposed. It is commonly accepted that it is the toxic effects of components of extracts on the functionality and structure of the cell membrane that is responsible for the aforesaid activity (Sikkema et al., 1995). Omidbeygi et al. (2007), Mshvildadze et al. (2000) and Abdel-Ghani et al. (2008) suggested that components of extracts cross the cell membrane, interacting with the enzymes and proteins of the membrane, so producing a flux of protons towards the cell exterior which induces changes in the cells and, ultimately, their death. Cristani et al. (2007) and Lucini et al. (2006) reported that the antimicrobial activity is related to the ability of terpenes to affect not only permeability, but also other functions of cell membranes. These compounds might cross the cell membranes, thus penetrating into the interior of the cell and interacting with critical intracellular sites. These components would increase the concentration of lipidic peroxides such as hydroxyl, alkoxyl and alkoperoxyl radicals and so bring about cell death. For Sharma and Tripathi (2006), components of the extracts would act on the hyphae of the mycelium, provoking exit of components from the cytoplasm, the loss of rigidity and integrity of the hypha cell wall, resulting in its collapse and death of the mycelium.

With all these wide spectrum antifungal properties, *C.*

Table 3. Impact of plant extracts on the biomass of mycelial growth of *A. flavus, A. fumigatus, A. niger* and *A. ochraceus* isolate.

Extract	Concentration (mg/ml)	Biomass of mycelial inhibition			
		A. flavus	***A. fumigatus***	***A. niger***	***A. ochraceus***
Methanol	0	0.708 ± 0.006	0.960 ± 0.006	0.833 ± 0.007	0.417 ± 0.006
	1	0.707 ± 0.006	0.946 ± 0.003	0.821 ± 0.011	0.407 ± 0.012
	2.5	0.701 ± 0.006	0.886 ± 0.004	0.768 ± 0.020	0.404 ± 0.016
	5	0.692 ± 0.005	0.872 ± 0.003	0.651 ± 0.006	0.393 ± 0.006
	7.5	0.705 ± 0.006	0.710 ± 0.009	0.486 ± 0.012	0.327 ± 0.006
	10	0.705 ± 0.007	0.616 ± 0.006	0.334 ± 0.015	0.284 ± 0.002
	12.5	0.703 ± 0.011	0.487 ± 0.004	0.280 ± 0.013	0.096 ± 0.003
	15	0.699 ± 0.010	0.337 ± 0.004	0.109 ± 0.020	0.000 ± 0.000
	17.5	0.709 ± 0.003	0.227 ± 0.003	0.000 ± 0.000	0.000 ± 0.000
	20	0.702 ± 0.007	0.124 ± 0.004	0.000 ± 0.000	0.000 ± 0.000
	22.5	0.702 ± 0.011	0.092 ± 0.003	0.000 ± 0.000	0.000 ± 0.000
	25	0.690 ± 0.010	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Aqueous	0	0.721 ± 0.008	0.993 ± 0.011	0.822 ± 0,018	0.422 ± 0,010
	1	0.721 ± 0.010	0.999 ± 0.002	0.859 ± 0,014	0.423 ± 0,001
	2.5	0.725 ± 0.011	0.996 ± 0.006	0.815 ± 0,007	0.410 ± 0,009
	5	0.730 ± 0.007	0.999 ± 0.003	0.793 ± 0,014	0.389 ± 0,010
	7.5	0.722 ± 0.011	1.032 ± 0.059	0.740 ± 0,018	0.372 ± 0,003
	10	0.728 ± 0.013	1.000 ± 0.001	0.605 ± 0,006	0.310 ± 0,010
	12.5	0.724 ± 0.009	1.001 ± 0.003	0.488 ± 0,013	0.245 ± 0,012
	15	0.722 ± 0.012	0.998 ± 0.002	0.403 ± 0,018	0.205 ± 0,008
	17.5	0.719 ± 0.004	1.001 ± 0.002	0.263 ± 0,046	0.107 ± 0,007
	20	0.729 ± 0.,007	0.999 ± 0.002	0.192 ± 0,006	0.061 ± 0,043
	22.5	0.733 ± 0.006	0.928 ± 0.024	0.085 ± 0,006	0.000 ± 0,000
	25	0.735 ± 0.007	0.889 ± 0.009	0.000 ± 0,000	0.000 ± 0,000

colocynthis Schrad. can be considered an effective antimicrobial agent to treat infectious diseases. This plant, namely its seeds extracts demonstrated activity against some fungi prevalent in dermatology, gynaecological and pulmonary infections. Also, it can be added as a food preservative. The study supported scientifically the ethnopharmacological use of the plant as an antifungal agent and could account for some of the variations observed in the ethnopharmaceutical preparation methods. Therefore, the use of this plant as antimicrobial agent is validated by the results obtained in this work. Further studies are ongoing to identify the chemical compounds of these antimicrobial extracts and even their toxicity.

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