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Aqueous and organic extracts of *Trigonella foenum-graecum* L. inhibit the mycelia growth of fungi

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Abstract

Aqueous extracts from various plant parts of fenugreek (3%) (aerial parts: leaves and stems (LS), roots (R), ground seeds (GS) and not ground seeds (NGS)) and petroleum ether, ethyl acetate and methanolic fractions of the aerial parts were assayed to determine their antifungal potential against *Botrytis cinerea*, *Fusarium graminearum*, *Alternaria* sp., *Pythium aphanidermatum*, and *Rhizoctinia solani*. All fenugreek plant parts showed antifungal potential and the magnitude of their inhibitory effects was species and plant parts dependent. R extract was shown less toxic (30.38%), whereas NGS extract expressed the strongest inhibition, with an average of 71.44%, followed by GS (58.56%) and LS (57.1%). Screening indicated that *P. aphanidermatum* was the most resistant species, with an average inhibition of 34.5%. *F. graminearum*, *Alternaria* sp. and *R. solani* were the most sensitive species, and were similarly inhibited (63.5%). The stability test indicated that the aqueous extracts of all plant parts lost approximately 50% of their relative activity after one month of storage at 4°C, whilst they lost 60%–90% of their activity when stored at ambient temperature for one month. The antifungal activity resided mainly in the methanol fraction and the minimum inhibitory concentration (MIC) of methanol fraction witch caused total inhibition of *R. solani* and *Alternaria* sp. was 60 μg/ml. Results of current study suggested that the constituents of *Trigonella foenum-graecum* have potential against harmful pathogenic fungi. Therefore, fenugreek could be an important source of biologically active compounds useful for developing better new antifungal drugs.

Key words: antifungal potential; aqueous extracts; fungi; methanol fraction; minimum inhibitory concentration (MIC); *Trigonella foenum-graecum* L.

Introduction

Use of fungicides in agricultural crops causes huge economic loses to farmers in two ways: first, it reduces crop quality, and secondly it increases cost of labour to control pathogenic fungi. Since fungi can cause disasters on the crops, the metabolites of many fungi may have adverse or stimulatory effects on plants (Rice, 1995), such as suppression of seed germination, malformation, and retardation of seedling growth (Lynch and Clark, 1984). Many crop seeds are infected by fungi before harvest or during storage (Neergaard, 1979). If conditions are not favourable, the situation is more serious (Kozakiewicz, 1996). Some fungi on the surface of seeds may produce mycotoxins that affect food quality (Betina, 1984), and some may produce phytotoxins that affect seed germination and seedling growth (Neergaard, 1979). The fungi also could cause other various symptoms such as vascular wilt, yellows, corm rot, root rot, and damping-off. Thus, the presence of these microorganisms in crops, vegetables, and fruits has significant impact on the quality of foods and related products. Furthermore, the increasing use of fungicides has resulted in a dramatic increase in the fungal resistance (Ritchie, 1985; Bertrand and Padgett, 1997; Horton *et al*., 2004). To reduce heavy reliance on herbicides and fungicides, there is a need to move to low-input sustainable agriculture as a component of integrated weed and fungi management. Indeed, the search of natural compounds and management methods alternatives (or complements) to classical pesticides and fungicides has become an intense and productive research field. In this regard, greater attention is towards the use of allelopatic plants and their products for managing the fungi in a sustainable manner (Chin, 1987; Han *et al*., 2005). A number of plants have been demonstrated to control pathogenic fungi (Singh *et al*., 1998; Zygadlo and Grosso, 1995; Dubey *et al*., 2000; Manohar *et al*., 2001). Therefore, it is worthwhile to explore the plants as sources of biological active compounds.

Trigonella foenum-graecum L. (fenugreek) has been of medicinal use within the traditional system of many countries for centuries. It is a plant easily cultivated with

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a significant production of biomass and its allelopathic potential has been reported in a previous work (Haouala *et al*., 2008). However, no information is available about its antifungal activity.

In the present work, the effects of aqueous extracts of *T. foenum-graecum*, plant parts as well as three organic fractions of aerial parts on the growth of five pathogenic fungi (*Botrytis cinerea*, *Fusarium graminearum*, *Pythium aphanidermatum*, *Alternaria* sp., and *Rhizoctinia solani*) were studied. These fungi species are known to have very serious effects on the crops.

1 Materials and methods

1.1 Plant materials

Trigonella foenum-graecum plants were collected at maturity from the south of Tunisia (North Africa). The leaves and stems (LS) and the roots (R) of plants were separated, well washed with water, disinfected by immersion in a 2% sodium hypochlorite solution for 30 min, and rinsed with distilled water to eliminate residual hypochlorite (Jasso *et al*., 2005). The fresh material was directly oven-dried at 60°C for 2 d, then ground into powder and stored at 2°C until use.

1.2 Preparation of aqueous and organic extracts

Three grams of dried materials were extracted by soaking in 100 ml distilled water at ambient temperature for 24 h in a shaker to give a concentration of 3% dry tissue. For fenugreek seeds, two types of aqueous extracts were tested: 3 g of ground seeds (GS) and 3 g of not ground seeds (NGS) were macerated in the same conditions as S and R. Extracts were then filtered in vacuum. First, through a Whatmman #3 disk and then, re-filtered through a nitro-cellulose paper (Ø= 0.45 μm) to reduce the risk of interference by micro-organisms. Antifungal activity was evaluated on target fungi at aqueous extract concentrations of 3%, 1.5%, 0.75%, 0.375%, and 0.18%, respectively. Aqueous extracts pH was measured, but only minor differences were registered (from pH 5.8 to pH 6.1). Sterilised deionise water was used as control.

The powdered material of fenugreek shoots (LS) was subjected to Soxhlet extraction successively with petroleum ether, ethyl acetate and methanol to obtain respective fractions (Harborne, 1998). Solvents were evaporated under reduced pressure using Rotavapour R-114 (Buchi, France) and dry fractions were stored at 4°C until use. The three organic fractions were also examined for their antifungal activity.

Two further complementary control series were used. The first group of control plates contained only PDA (Potato-Dextrose-Agar), but the pH was not manipulated because this group was employed as a control for statistical purposes. The second group of control plates also contained only PDA but its pH was adjusted to 5.9. To monitor possible interferences by bacteria through the experiment, another series of growth media containing PDA, Streptomycine (75 mg/L) and Cloranfenicol (10 mg/L) was prepared and used as control.

1.3 Antifungal activity assay

The antifungal activity was tested on five phytopathogenic species fungi: *B. cinerea*, *F. graminearum*, *P. aphanidermatum*, *Alternaria* sp. and *R. solani*. The extracts were put in dug wells (5 mm broad and 20 mm length) in the culture medium PDA, in sterilized Petri dishes. Fungal plugs (0.4 mm in diameter) were placed opposite the well with 1 cm of the edge limps. Limps control consists in putting water distilled in the well. After an incubation of 72 h at 24±2°C, we observed the mycelium development of pathogenic fungi in each Petri dish and noted the phytotoxic effect of fenugreek extracts with measuring the distance covered by the mycelium. The inhibition percentage of growth (I, %) was calculated following Eq.(1) (Hmouni *et al*., 1996).

$$I = (1 - d_t/d_c) \times 100\% \quad (1)$$

where, d_c is the fungal colony diameter measured in control sets, d_t is the fungal colony diameter measured in treatment sets after 72 h of incubation. The antifungal effect was measured under a totally random design with three replications.

1.4 Stability testing

Stability tests were carried out on the crude extracts of all fenugreek plant parts. The extracts were divided into two parts, and stored at 4 and 25°C (ambient temperature), respectively, for one month. The extracts were then assayed for the antifungal activity.

1.5 Statistical analysis

The triplicate data were subjected to an analysis of variance for a completely random design using SPSS 13.0 for Windows program. Comparison of means was analyzed by Duncan's multiple range test and differences were considered significant when $P < 0.05$.

2 Results and discussion

2.1 Effect of fenugreek aqueous extracts on fungi growth

The inhibition of each plant part was determined from the average of inhibition percentage of leaves and stems (LS), roots (R), ground seeds (GS), and not ground seeds (NGS) on the mycelium growth of the target fungi. The variance analysis showed that fenugreek extracts had a highly significant effect on fungi growth ($P < 0.001$). Moreover, the variation of the pH does not have effect on the mycelium growth. Limps, which contain the bacteria, also did not show significant variation with those that did not contain. This allowed discarding the effects of possible contaminations by bacteria.

Roots extract was shown less toxic, which caused the strongest inhibition of *F. graminearum* mycelium followed by *Alternaria* sp., with an average inhibition of 53.8% and 44.3%, respectively. For the other recipient species, the inhibition average was 34.1% for *B. cinerea* and only 11.2% and 8.5% for *P. aphanidermatum* and *R. solani*,

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respectively (Fig.1). For LS extract, the strongly inhibition was shown in the growth of *R. solani* and *Alternaria* sp. (71% inhibition average). *F. graminearum* and *B. cinerea* showed similar inhibition (an average of 61.25%) and *P. aphanidermatum* was the most resistant fungi against this extract, with an average inhibition of only 10.6% (Fig.1). Mycelium growth inhibition percentages induced by the extracts GS and NGS showed the highest values for the five target fungi, and NGS extract was more inhibitor than GS extract. Indeed, GS extract suppressed *R. solani* growth by a percentage of 72.1%, and an average of 61.7% for *Alternaria* sp., *P. aphanidermatum* and *F. graminearum*. The lowest percentage of the inhibition was recorded at *B. cinerea* (35.5% in average). In the presence of NGS extract, percentage of inhibition varied between 58.5% and 87.21% for the five fungi species. Nevertheless, *B. cinerea* showed the lowest inhibition percentage (58.5%) again (Fig.1).

Data revealed that all fenugreek plant parts showed antifungal potential and the magnitude of their inhibitory effects was species dependent and varied among plant parts. NGS extract expressed the strongest inhibition, with an average of 71.44%, followed by GS (58.56%), LS (57.1%) and R (30.38%). Screening indicated that *P. aphanidermatum* was the most resistant species, with 34.5% as average of inhibition. *F. graminearum*, *Alternaria* sp. and *R. solani* were the most sensitive species, and showed a similar comportment with a total inhibition of 63.5%. *B. cinerea* had presented a middle resistance.

Many researchers (Ouf *et al*., 1994, Rhajaoui *et al*., 2003, Jbilou *et al*., 2006) have reported antifungal potential and selective effects of plants crude extracts. Antifungal activity of *T. foenum-graecum* could be attributed to their phenol, alkaloid, tannin, etc., compounds. Phenolic acids are often mentioned as putative allelochemicals (Lattanzio *et al*., 2001) and have an important effect on rhizospheric microbial populations (Lin *et al*., 2007). The mechanisms as how natural compounds in herbs exert their function have been previously discussed by Brul and Coote (1999).

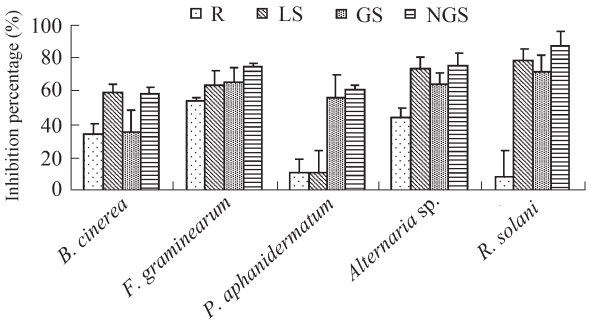

Fig. 1 Effects of aqueous extracts (3%) of different plant parts of fenugreek on mycelium growth of *B. cinerea*, *F.* graminearum, *P. aphanidermatum*, *Alternaria* sp. and *R. solani*, after 72 h incubation. The bars on each column show standard error. R: roots; LS: leaves and stems; GS: ground seeds; NGS: not ground seeds.

2.2 Dose-response effect of fenugreek aqueous extracts

Plant parts extracts "dose-response effect" of fenugreek is reported in Fig.2. The results show that the effectiveness of the extracts decreases with the importance of dilution. However, exceptions must be noted. Thus, for the root extract we recorded significant variation only at 0.18% for *B. cinerea* and from concentration 0.75% for *F. graminearum*. Insignificant variation was recorded with concentration variation for the other fungi species. In the same way, the dilution of GS extract did not show significant variation of its effect on *B. cinerea*. Nevertheless, for the other fungi, the effect of this extract was most efficient with concentrations of 1.5%. Below this level the inhibiting effect of GS extract had decreased proportionally with dilution when experimented with all the target species. Concerning LS extract, it proved very inhibiting fungi growth and its effectiveness remains detectable until more or less weak concentrations, depending on the fungi species. We registered an inhibition for about 50% with *R. solani* and 63% with *Alternaria* sp. in presence of LS extract at 0.375% and 0.18% concentrations, respectively. For the other species, a concentration of 0.75% was enough to

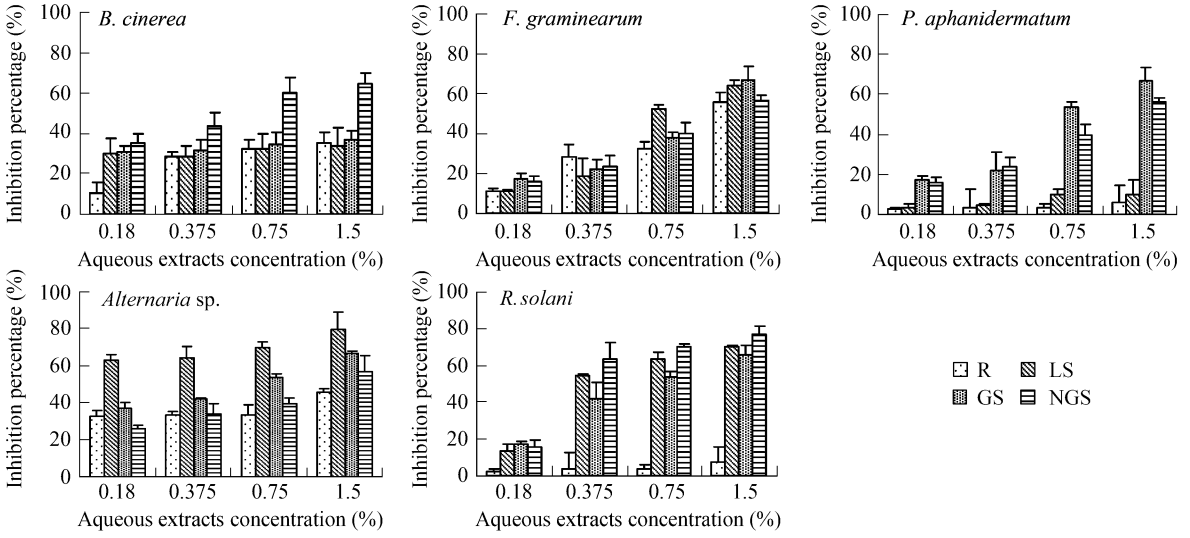

Fig. 2 Effects of aqueous extracts concentrations of different plant parts of fenugreek on mycelium growth of *B. cinerea*, *F. graminearum*, *P. aphanidermatum*, *Alternaria* sp. and *R. solani*, after 72 h of incubation. The bars on each column show standard error.

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induce an inhibition superior to 50% of the growth fungi mycelium. NGS acted similarly to extract LS, except with *Alternaria* sp. where inhibition became higher than 50% only at 1.5% concentration. However, *P. aphanidermatum* was shown resistant against this extract (9.9% of inhibition at a concentration of 1.5%) (Fig.2).

Thus, the minimum inhibitory concentration (MIC) values registered in this study were ranged between 0.18% and 0.75% depending on the extract origin and the target fungi. Lee *et al*. (2007) had registered MIC values (4.4%) required to inhibit the growth of fungi higher than those mentioned above. Our findings show that even only 1 g of the plant parts dried powder was sufficient to show an inhibitory effect against fungi growth.

2.3 Stability of fenugreek aqueous extracts antifungal activity

The stability test was carried out to examine whether the activity of the plant extracts could be maintained after one month of storage at 4 and 25°C. The results indicate that the aqueous extracts of all plant parts lost approximately 50% of their relative activity after one month of storage at 4°C, whilst they lost 60%–90% of their activity when stored at ambient temperature (25°C) for one month (Fig.3). The results indicate that fresh extracts have to be stored at lower temperature to preserve their antifungal activity. The antifungal substances that presented in the plant extracts may be unstable at higher temperatures. Similar results were obtained by Singh *et al*. (2001), Motsei *et al*. (2003), and Lee *et al*. (2007).

2.4 Effect of organic extracts of fenugreek

The screening of plant parts for antifungal activity showed that all parts of plant fenugreek had potential against the five phytopathogenic target species. Therefore, powdered aerial parts of *T. foenum-graecum* were subjected to stepwise extraction with organic solvents to obtain petroleum ether, ethyl acetate and methanol fractions. Dry powder of fenugreek yielded 0.84%, 1.41%, and 1.74% of petroleum ether, ethyl acetate and methanolic fractions, respectively.

All three fractions of fenugreek aerial parts were evaluated at 60 mg/L for their antifungal activity using in addition 1/2 and 1/4 dilutions, against *B. cinerea*, *F. graminearum*, *P. aphanidermatum*, *Alternaria* sp. and *R. solani*. It was observed that the antifungal activity resided mainly in the methanol fraction of leaves and stems of fenugreek. The wells containing a concentration of 60 mg/L of the methanol fraction caused a total suppression of *R. solani* and *Alternaria* sp. growth. The hexane and ethyl acetate fractions were found to be inactive. The percentage growth inhibition increased with the increase of fraction concentration (Fig.4). The MIC of methanol fraction against *R. solani* and *Alternaria* sp. was found to be 60 μg/ml. This concentration of methanol fraction inhibited 100% and 98.67% growth of the two species respectively. *B. cinerea* and *F. graminearum* showed similar behaviour with an average percentage inhibition of 69.38% at the same concentration. However, *P. aphanidermatum* was the most resistant vis-a-vis the metanolic extract with a growth inhibition percentage of only 9.45% at 60 μg/ml concentration.

In the current study, the methanol fraction of fenugreek showed activity against *B. cinerea*, *R. solany*, *F. graminearum*, and *Alternaria* sp. The MIC was 60 μg/ml (Fig.4). The activity of fenugreek methanol fraction appears to be very quite. Indeed, there are reports where the crude extracts of the plants having MIC 380.0 mg/ml against *A. fumigatus* were considered to be of activity significance (Sadyojatha and Valdya, 1996), and the MIC of chloroform

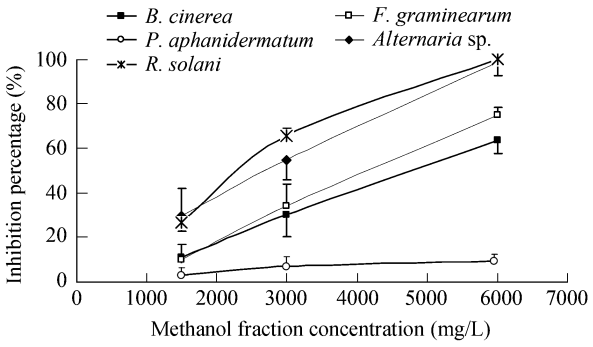

Fig. 4 Effect of methanol fraction concentration of fenugreek aerial parts on the inhibition percentage of mycelium growth of five fungi species. Values represent the average of three measurements ± standard error of the mean (SEM).

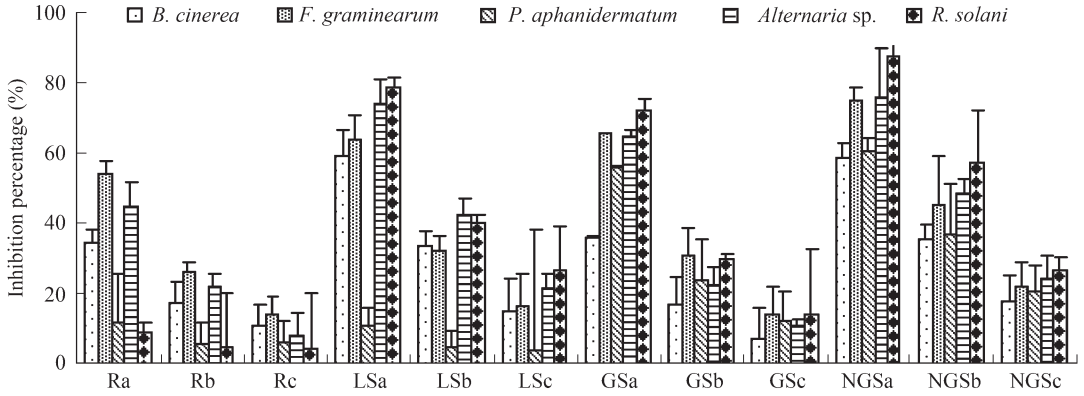

Fig. 3 Effect of storage of aqueous plant extracts on the inhibition percentage of mycelium growth of five fungi species. Plant extracts were freshly prepared (a) or stored for one month at 25°C (b) and 4°C (c) from different plant parts of fenugreek. Values represent the average of three measurements ± standard error of the mean (SEM).

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fraction of *Datura metel* was 625 μg/ml and was considered to be significantly important (Rajesh and Sharma, 2002).

3 Conclusions

In this study, the antifungal action of aqueous crude extracts of different plant parts of fenugreek were examined and indicated, using bioassay methods, that all fenugreek plant parts extracts are potentially natural sources of antifungal agents. Also, the methanol fraction of fenugreek aerial parts showed activity against four fungi species and the MIC was 60 μg/ml. Fenugreek extracts may be an attractive alternative for the use of a natural product for control of fungi that attack industrial crops, avoiding chemical fungicides application. The indicated antifungal activity of fenugreek could be exploited and accomplished with future studies focusing on the identification and isolation of the allelochemicals, as long as these substances should certainly serve to integrated weed management as a model for future fungicides if environmental compatibility is a required feature.

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