ISSN (Online) = 2522-6754 ISSN (Print) = 2522-6746
WORLD JOURNAL OF BIOLOGY AND BIOTECHNOLOGY
Research Manuscript www.sciplatform.com

Antifungal and antioxidant properties of chloroform soluble compounds of fennel seeds

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Authors' Contribution | **Akhtar, N., A. Shoaib** and **S. Ashraf** conducted the experiments, analyzed the data and wrote the manuscript.

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Digital Object Identifier (DOI) Number: **https://dx.doi.org/10.33865/wjb.007.02.0609**

ABSTRACT

Bio-pesticides are replacing chemicals being their potential alternatives. The present study was conducted to evaluate the antifungal effect of chloroform soluble fractions of *Foeniculum vulgare* seeds against seed rotting caused by *Aspergillus flavus*, *A. welwitschia*, *A. minisclerotigenes,* and *A. oryzae*. The effect was determined in terms of inhibition in fungal biomass production, the total content of cellular proteins, and the enzymatic rate of catalase and peroxidase. The maximum growth inhibition was recorded for *A. flavus* where the extract of *F. vulgare* reduced 97.29% fungal dry weight without significantly affecting the total cell protein content as well as the catalase and peroxidase enzymatic activities. Seed extract caused 92% inhibition in the mycelial growth of *A. minisclerotigenes* over the control treatment, reducing the cell protein content to 1.25 from 1.57 mg/g fresh weight. However, catalytic and peroxidase activities increased about three and two times, respectively. Under the stress of seed extract, *A. welwitschia* mycelial weight suppression was almost 72%, total protein content increased by two folds, and catalase and peroxidase activities decreased significantly. The effect of chloroform seed extract of *F. vulgare* was found to be insignificant on growth and selected physiological attributes of *A. oryzae*.

Keywords: Antioxidant, *Aspergillus*, growth inhibition, pathogen, pesticide.

INTRODUCTION: Fungal pathogens are responsible for contaminating grain crops at pre or post-harvest stages hence producing mycotoxins, which reduces seed market value by deteriorating grain quality, seed health, and vigor (Goko *et al.*, 2021). Furthermore, along with the mycotoxins, seed-borne pathogens also produce various types of metabolites that have adverse effects on seed metabolism (Hussain *et al.*, 2013). Mycotoxins associated drawbacks to human and animal life are well reported, while losses in livestock and poultry production from mycotoxin-contaminated feeds have been documented to cause suppression in the immune system, and reduced growth rates (Hussain *et al.*, 2013; Goko *et al.*, 2021). Ergot alkaloids are one of the adverse effects of mycotoxins by seed-borne pathogens, which have been affecting both man and animals for centuries. Opportunistic storage molds or *Aspergillus* is a widely distributed genus established in 1729 and comprises 339 known species that show great diversity in preferred substrate/host and mycotoxins production. The toxins of agricultural pests threaten food safety worldwide (Abo Nouh *et al.*, 2020; Navale *et al.*, 2021).

Practically the possible ways to manage fungal pathogens are proper quarantine measures, seed testing, and seed treatment. Apart from chemical treatments, various other non-chemical treatments are also used also for protection against agricultural pests (Amodu and Aku, 2015). Several herbs and spices have been used for centuries for account of their antimicrobial potential along with their medicinal properties. Many of these spices and herbs usually produce flavonoids, alkaloids, tannins, and many phenolic compounds which are the secondary metabolites and are definitive sources of drugs, pesticides and many microbicides (Mahesh and Satish, 2008). *Foeniculum vulgare* Mill. (family Apiaceae) is a biennial medicinal and aromatic herb with yellow flowers and feathery leaves indigenous to central Europe and Mediterranean regions but now cultivated in almost every country (Muckensturm *et al.*, 1997). It contains protein, lipids, and carbohydrates along with minerals (Ca, Fe, Mg, Zn, P, K, and Na), dietary fiber, and vitamins (C and thiamin B1), relative to human needs. On account of its aroma, it is extensively utilized for culinary and medicinal purposes with diverse pharmacological (Rather *et al.*, 2016). Essential oil and seeds extract of *F. vulgare* have been screened for their anti-fungal potential and found to be equally good with broad-spectrum antibiotics against *Aspergillus niger, A. flavus*, *Fusarium graminearum* and *F. minliforme* (Singh *et al.*, 2006; Upadhyay, 2015). Likewise, aqueous and alcoholic seed extracts of *F. vulgare* inhibited the growth of *Alternaria alternata*, *Mucor rouxii*, and *A. flavus* (Thakur *et al.*, 2013), and in some cases, antifungal activity of aqueous seed extract was greater compared to reference fungicidal agent ((Taie *et al.*, 2013). Khan (2017) observed the substantial potential of methanolic and aqueous seed extract of *F. vulgare* against different species of *Candida*. Aamir *et al.* (2018) results indicated the significant antifungal potential of the aqueous, alcoholic, acetone, ethyl acetate, and chloroform extracts of fresh seeds of *F. vulgare* against *A. niger.* Natural agents displaying innovative chemistry and mode f action are always momentum as an alternative antimicrobial agent particularly on account of increasing resistance in the pathogen. *F. vulgare* may be a potential source for new antifungal agents against Aspergillus spp.

OBJECTIVES: The purpose of this research work was to evaluate the *in vitro* antifungal potency of chloroform seed extract of *F. vulgare* against seed-borne Aspergilli.

MATERIALS AND METHODS: Fungal cultures: Four different *Aspergillus* species [*A. flavus* (FCBP0051), *A. welwitschia* (FCBP0087), *A. oryzae* (FCBP1213) and *A. minisclerotigenes* (FCBP1353)] were procured from the First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore, Pakistan

and maintained on Malt Extract Agar; MEA (Dhingra and Sinclair, 2017).

Biological control assays: Growth assays were conducted in 250 mL conical flasks each containing 50 mL of malt extract broth (pH 6.5) amended with the respective concentration of chloroform extract of *F. vulgare* seeds. About 50 g of dry powdered *F. vulgare* seeds were soaked with 500 mL of methanol in airtight glass jars. After 7 days, undissolved material was removed by filtration through double-fold muslin cloth followed by Whatman filter paper. The crude methanolic soluble fraction of seeds was obtained by evaporation of filtrate in a hot air oven at 45 °C. The paste obtained was partitioned using chloroform in a separating funnel. The solvent was evaporated on a rotary evaporator and the weight of the solvent-soluble fraction was measured and then stored in pre-weighing sterilized glass bottles.

Experimental setup for fungal growth assays: About 2 g crude chloroform extracts of seed were dissolved in 10 mL dimethyl sulfoxide (DMSO) to make a sticky solution. About 4 concentrations (w/v), 0, 0.02, 0.04, 0.08, and 0.12 % were prepared by dissoloving 0, 10, 20, 40, and 60 mg of stock solution per 50 mL of malt extract medium. Each experiment was replicated twice. Each treatment was inoculated with 6 mm agar plugs from actively growing 7 days old pure fungal culture and incubated at 25±2 °C for 10 days. Fungal dry weight was determined after overnight drying the filtered mycelia in a hot air oven at 40 °C. The effect of different concentrations of chloroform fractions of fennel seeds was determined in terms of percent increase/decrease in average fungal dry weight.

$$\text{Percentage increase/decrease in biomass} = \frac{\text{Biomass in control} - \text{Biomass in treatment}}{\text{Biomass in control}} \times 100$$

Effect of plant extracts on fungal physiology: Changes in total protein content and rate of peroxidase and catalase enzymes activities were determined and compared in extract-treated and non-treated strains. Fungal cells were grown and collected as described for growth assays. To remove extra moisture, filtered mycelia were blotted by pressing in folds of filter papers and used for physiological assays. Total cell protein content (mg g^{-1} fresh weight, FW) was calculated using the method of Lowry (1951). Peroxidase activity (mmol/min/mg protein) was determined by following the protocol of Kunwar and Khan (1982). Finally, catalytic activity (mmole H_2O_2 utilized/min/mg protein) in treated or non-treated fungal cells was assessed according to the method described by Glick (2009).

RESULTS AND DISCUSSION: Effect on growth and antioxidant properties of *A. oryzae*: Seeds of *F. vulgare* have been used as a traditional medicine to cure many microbial diseases (Dua *et al.*, 2013). The production of fungal biomass of *A. oryzae* was inhibited with the increase in the concentration of chloroform soluble compounds of *F. vulgare* seeds (figure 1A-E). In the control treatment, the dry weight of *A. oryzae* was 4.39 g which increased to 6.33 g in 10 mg treatment. However, under the effect of 20 and 40 mg extract concentration, an insignificant difference was observed in the dried fungal matt of *A. oryzae* markedly declined to 3.17 g when the fungal strain was grown in the medium amended with 60 mg extract concentration (figure 1A). The higher concentration of extract inhibit the fungal biomass production probably due to the increase in quantity of bioactive metabolites (e.g. oxygenated

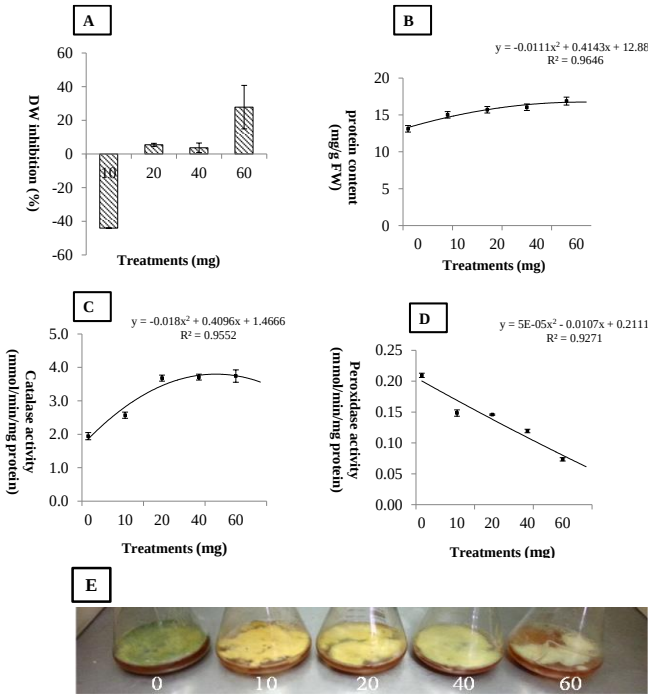

Figure 1: Effect of different concentration of chloroform soluble fraction (0.02-0.08%) of *Foeniculum vulgare* seed on growth, total protein content, and antioxidant properties of *Aspergillus oryzae*. (About 10, 20, 40, and 60 mg of stock solution of chloroform fraction per 50 mL of growth medium is equivalent to 0.02, 0.06 and 0.08% concentration, respectively).

monoterpenes) in the seed extract (Badgujar *et al.*, 2014). The antifungal compounds might cause denaturation of the enzymes responsible for growth and reproduction in the fungi. Proteins play a major role in fungal growth metabolism. A concentration-dependent increase in *A. oryzae* total cell protein content was recorded (figure 1B) in the fungal cells is directly correlated with nitrogen metabolism (Akhtar *et al.*, 2022). Proteolysis or denaturation is usually the main reason for lower cellular protein content (Lubaina and Murugan, 2013). Activities of antioxidant enzymes can be used as indicators of stress physiology in cells. Enhanced activity of antioxidant enzymes activity helps the organisms to grow under stress and is a good sign of stress tolerance which in the present case is the chloroform extract of *F. vulgare* seeds. Catalytic activity in *A. oryzae* cells rose with the addition of *F. vulgare* seed extract. In the control treatment, the rate of catalytic activity was 1.94 mmol/min/mg protein that enhanced to 2.57, 3.67, 3.70, and 3.74 mmol/min/mg protein due to the extract concentrations 10, 20, 40, and 60 mg (figure 1C). However, *A. oryzae* peroxidase enzyme activity declined to 0.07 mmol/min/mg protein from 0.20 mmol/min/mg protein (control treatment) as the extract concentration increased to 60 mg (figure 1D).

Effect on growth and antioxidant properties of *A. flavus*: Increasing concentration of *F. vulgare* seed extract caused significant growth inhibition of *A. flavus* (figure2A-E). The antifungal potential of *Cymbopogon nervatus* has been reported for its inhibitory effect against *Aspergillus flavus* for aflatoxins production. Similarly, during the present study, antifungal potential of *F. vulgare* has been proven very effective against *A. flavus* biomass production. The weight of the mycelium fungal cells grown in a non-treated growth medium was 6.16 g which was reduced to 0.16 g when cells were grown in 60 mg extract

treatment (figure 2A). Arshad and Hafiza (2011) found a 90% reduction in fungal biomass production by chloroform extract of medicinal plants.

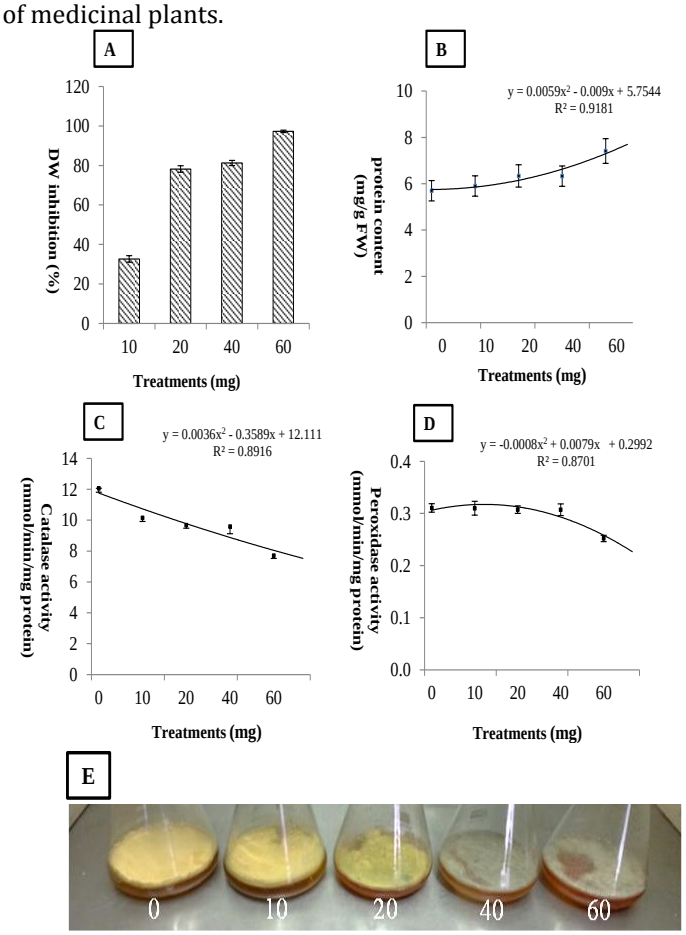

Figure 2: Effect of different concentration of chloroform soluble fraction (0.02-0.08%) of *Foeniculum vulgare* seed on growth, total protein content, and antioxidant properties of *Aspergillus flavus.* (About 10, 20, 40, and 60 mg of stock solution of chloroform fraction per 50 mL of growth medium is equivalent to 0.02, 0.06 and 0.08% concentration, respectively).

The transcriptome profile of any organism under stress is needed to adjust continuously. That may result in the over-expression of some genes (Gupta *et al.,* 2013). A similar pattern of protein content was observed in cells of *A. flavus* that improved with a rising concentration of extract (figure 2B). Catalase and peroxidase activities showed decreasing pattern with the addition of *F. vulgare* chloroform extract (figure 2C & D). Cellular response to a particular stress is highly hooked on the tolerance or susceptibility level which is generally under the effect of genes or proteins expression (Nouri *et al.,* 2015)

Effect on growth and antioxidant properties of *A. minisclerotigenes*: Results of present assays showed that chloroform extract of *F. vulgare* seeds significantly arrested the growth of *A. minisclerotigenes* fungus even at the lowest concentration (figure 3A & E). Almost complete cessation in the growth of *A. minisclerotigenes* was achieved which might be due to the impact of antifungal compound in extract on mitosis and cell division (McCarroll *et al.,* 2002).

In contrast to *A. oryzae* and *A. flavus*, the total cell protein content of *A. minisclerotigenes* which is an important parameter to study the changes in cellular metabolism (Shoaib *et al.*, 2022) decreased with the increase in the concentration of seed extract (figure 3B).

Catalase and peroxidase activities of fungal cells increased with the increasing concentration of *F. vulgare* extract (figure 3C & D). Undergrowth stress conditions, reactive oxygen species (ROS) are produced which are generated as the result of oxygen metabolism.

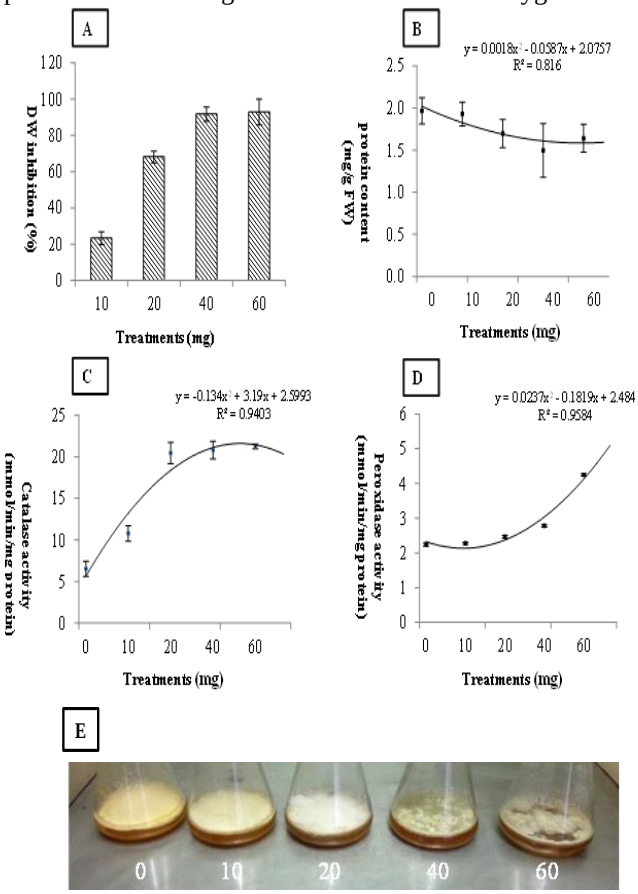

Figure 3: Effect of different concentration of chloroform soluble fraction (0.02-0.08%) of *Foeniculum vulgare* seed on growth, total protein content, and antioxidant properties of *Aspergillus minisclerotigenes.* (About 10, 20, 40, and 60 mg of stock solution of chloroform fraction per 50 mL of growth medium is equivalent to 0.02, 0.06 and 0.08% concentration, respectively).

Catalase and peroxidase are antioxidant enzymes that help the cell to maintain the hydrogen peroxide to a harmless level and save the cells from damage by these ROS (Mustapha *et al.*, 2009).

Effect on growth and antioxidant properties of *A. welwitschia*: An even decline in *A. welwitschia* biomass production was seen with the addition of *F. vulgare* seed extract in the growth medium. In the highest concentration, approximately 75% reduction was observed (figure 4A & E). These results suggested that this extract can be an alternative to synthetic fungicides for the treatment of *A. welwitschia* infections. Results depicted an increase in *A. welwitschia* total cell protein content with the rising concentration of seed extract and at the highest concentration of plant extract cell proteins became almost double (figure 4B). The increasing concentration of chloroform extract exhibited an inhibitory effect on both catalase and peroxidase enzymatic rates as compared to the control (figure 4C). Peroxidase enzyme lost its activity from 0.56 to 0.14 mmol/min/mg protein at 60 mg concentrations (figure 4D). The decline in antioxidant enzyme rate is an indication that due to the *F. vulgare* extract, the transcript level of these enzyme coding genes is suppressed. It has been widely accepted that response of each organism to a particular stress is different for other organisms (Caverzan *et al.*, 2012).

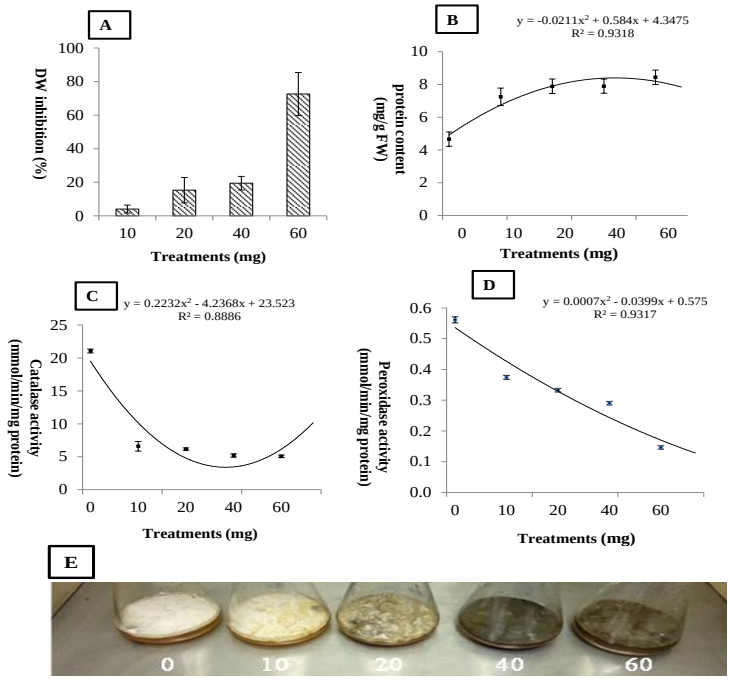

Figure 4: Effect of different concentration of chloroform soluble fraction (0.02% to 0.08%) of *Foeniculum vulgare* seed on growth, total protein content, and antioxidant properties of *Aspergillus welwitschia*. About 10, 20, 40, and 60 mg of stock solution of chloroform fraction per 50 mL of growth medium is equivalent to 0.02, 0.06 and 0.08% concentration, respectively).

It is also established that fungicidal compounds interfere with the biosynthetic pathways of amino acids and proteins ultimately affecting all other cellular functions of organisms differentially under chemical stress (Shoaib *et al*., 2021).

CONCLUSIONS: The chloroform seed extract of *F. vulgare* inhibited growth and physiological responses in the species of Aspergilli, which suggested that this extract can be an alternative to synthetic fungicides against Aspergilli species.

ACKNOWLEDGMENTS: The authors are grateful to the First Fungal Culture Bank of Pakistan for procurement of the fungal culture. The research was carried out within the Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The authors are grateful University of the Punjab, Lahore, Pakistan to provide facilities to carry this research work.

CONFLICT OF INTEREST: There is no declaration of interest by the authors concerning the publication of this paper

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