COMPARATIVE EFFICACY OF ORGANIC SOLVENT FRACTIONS OF LEAF EXTRACT OF HEMP AGAINST *Aspergillus versicolor*

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Abstract

The fungus *Aspergillus versicolor* is generally found on food products and produces sterigmatocystin, a carcinogenic and hepatotoxic mycotoxin. This study reports the usefulness of polar and non-polar fractions of methanolic extract of hemp (*Cannabis sativa* L.) leaves against *A. versicolor.* Dried leaves of hemp were soaked in methanol for two weeks. The material was filtered and the methanol was evaporated on a rotary evaporator. Thereafter, water was added to the residues and the aqueous mixture was successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. Different concentrations of each fraction were prepared which ranged from 1.562 to 200 mg mL^{-1}. Antifungal activity was carried out in malt extract broth medium. In general, all the concentrations of the four organic solvent fractions significantly controlled the growth of *A. versicolor.* There were 71–100%, 59–100%, 65–100% and 69–100% decline in the biomass of *A. versicolor* due to *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions, respectively. It is concluded that different fractions of leaf extract of *C. sativa* have remarkable potential in controlling growth of *A. versicolor.*

Keywords: *Aspergillus versicolor, Cannabis sativa, leaf extract, organic fractions.*

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INTRODUCTION

Aspergillus versicolor is among the most significant food-rotting and toxicant fungi. It has been derived from many types of foodstuffs. It is reported to produce sterigmatocystin, a cancer-causing agent i.e. a precursor of aflatoxins (Stanojević-Nikolić *et al.,* 2020). Various *Aspergillus* species synthesize sterigmatocystin that has been derived from various foods and feeds. Due to its toxicogenic properties, this compound has been categorized as a B2 substance, which has been blacklisted as potential carcinogen. *A. versicolor* has mesophilic growth with temperature range of 9 °C to 39 °C. It has an optimum growth at 27 °C (Houbraken *et al.,* 2020). Its growth requires pH range of 3.1 to 10.2. Current concept of controlling fungal contaminations generally involves the use of natural food preservatives, to avoid adverse effects due to fungal toxins on the consumer's health (Campêlo *et al.,* 2019).

Weed plants denote an imperative affluence and cost-effective factor of biodiversity. Selected wild herbs and weeds are used in conventional medicines throughout the globe for curative purpose (Hassan, 2020). Numerous weed plants are also known for their antimicrobial properties (Javaid *et al.,* 2018; Naqvi *et al.,* 2019; Khan *et al.,* 2021). Use of natural products including botanical extracts has proved an excellent alternative methodology to manage mycological infections of plants (Javaid and Saddique, 2011; Akhtar *et al.,* 2020).

Hemp (*Cannabis sativa* L.) is among primogenital recognized medicinal plants and has been designated in more or less all the ancient handbooks related to phyto-medicines. It is generally known as a potent psychoactive constituent (Booth and Bohlmann*,* 2019). It grows as a wild plant in various parts of Punjab. It is also cultivated in some countries largely for its fibers, which are raw materials for manufacturing of strings, fabrics and vessel sails. This plant has been used for its analgesic, antispasmodic, narcotic, sedative and anti-inflammatory properties (Singh *et al.,* 2018). However, studies regarding its antifungal activities are very rare. Therefore, this study aimed to examine the performance of different organic solvent fractions of *C. sativa* leaf extracts against *A. versicolor.*

MATERIALS AND METHODS

Extraction

Leaves of *C. sativa* were collected in 2018 from barren lands of Gujrat, Pakistan. Freshly collected foliage was rinsed thoroughly with tap water. The collected leaves were washed repeatedly in sterilized water. Cleaned leaves were dried under shade, pulverized to fine powder and preserved properly for future use.

About 3 kg of dried powdered leaves of *C. sativa* were soaked in 7 L of methanol for 14 days at room temperature (32–37 ºC). The soaked leaves were kept in an air tight container. On 14^{th} day, the extract was strained using cheese cloth followed by double layered filter papers. The filtrate was evaporated on a rotary evaporator at 45 °C followed by evaporation in an electic oven at 40 °C to get 198 g residues. This viscous methanolic extract was mixed with 200 mL of double distilled water. The mixture was partitioned with *n*-hexane (4 × 500 mL), followed by chloroform (400 mL), ethyl acetate (500 mL) and *n*-butanol (300 mL) by fractionating funnel. Initially, the solvents were removed on a rotary evaporator. Complete evaporation at 40 °C oven-drying resulted in 18.4, 11.7, 9.5 and 10.3 g of *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions.

Antifungal bioassays

An amount of 1.2 g of each fraction was weighed and dissolved in 1 mL of dimethyl sulfoxide (DMSO). To this solution, 5 mL of 2% (w/v) malt extract broth was added to make a 200 mg mL^{-1} concentration growth medium. It was used to prepare a serial dilutions of 100, 50, 25,…., 1.562 mg mL^{-1}. A control series was also prepared similarly by excluding the extracts and was used as reference for each extract concentration in antifungal studies.

Mature colony of *A. versicolor* was scratched in double distilled water for inoculum preparation. Fungal suspension (20 µL) was inoculated in culture tubes under aseptic conditions. Tubes were incubated at 28 ºC for 7 days. *A. versicolor* mats were strained through pre-weighed filter papers, dried at 70 ºC and weighed (Khan and Javaid, 2020b).

Statistical analysis

Experiment was done in completely randomized design using three replicates of each treatment. Data is presented as means ± standard errors. Data were analyzed by ANOVA followed by separation of treatment means by LSD method at $P \leq 0.05$. Software Statistix 8.1 was used for analysis.

RESULTS AND DISCUSSION

The comparative antifungal efficacy of different fractions of *C. sativa* against *A. versicolor* is shown in Fig. 1 and 2. Although both the organic solvents and the aqueous fractions significantly ($P \leq 0.05$) controlled growth of the target fungus, however, the aqueous fraction was less effective than the organic solvent fractions. Organic fractions reduce the biomass of *A. versicolor* from 59–100% as compared to 43–100% decline in fungal growth due to aqueous fraction. The *n*-hexane fraction surpassed the others with 71–100% suppression in fungal biomass (Fig. 1A). Efficacy of *n*-butanol fraction was very close to the efficacy of *n*-hexane fraction with 69–100% decline in biomass of *A. versicolor* (Fig. 1D). Chloroform and ethyl acetate fractions showed a little lesser antifungal activity than the other two organic solvent fractions resulting in 59–100% and 65–100% reduction in biomass of *A. versicolor* (Fig. 1 B and C). Previously, there are just a few reports regarding antifungal properties of *C. sativa* against other fungal species which showed moderate to low fungicidal effect of different type of extracts of this plant (Glodowska and Lyszcz, 2016). Khan and Sabir (2016) reported 18.96% and 18.10% reduction in growth of *Aspergillus flavous* and *A. niger,* respectively, due to extracts of *C. sativa* in disc diffusion bioassays. Likewise, Ali *et al.* (2012) reported low activity of whole plant methanolic extract of *C. sativa* against *Candida albicans*. However, Wasim *et al.* (1995) reported that ethanolic and petroleum leaf extracts of *C. sativa* growing in Pakistan had pronounced activities against *A. niger* and *Candida albicans.* Findings of a recent study carried out by Khan and Javaid (2020a) strengthen the earlier reports of these works. In their study, they found 38–82% reduction in growth of *Aspergillus flavipes* due to organic fractions of leaf extract of *C. sativa.* Chemical analysis of *C. sativa* showed that it contains over 480 compounds including a number of terpenes and about 180 cannabinoids (Fischedick *et al.,* 2010). Most of the cannabinoids showed pharmaceutical properties (Aizpurua-Olaizola, 2016). However, these compounds are more effective against bacteria than against fungi (Karas *et al.,* 2020).

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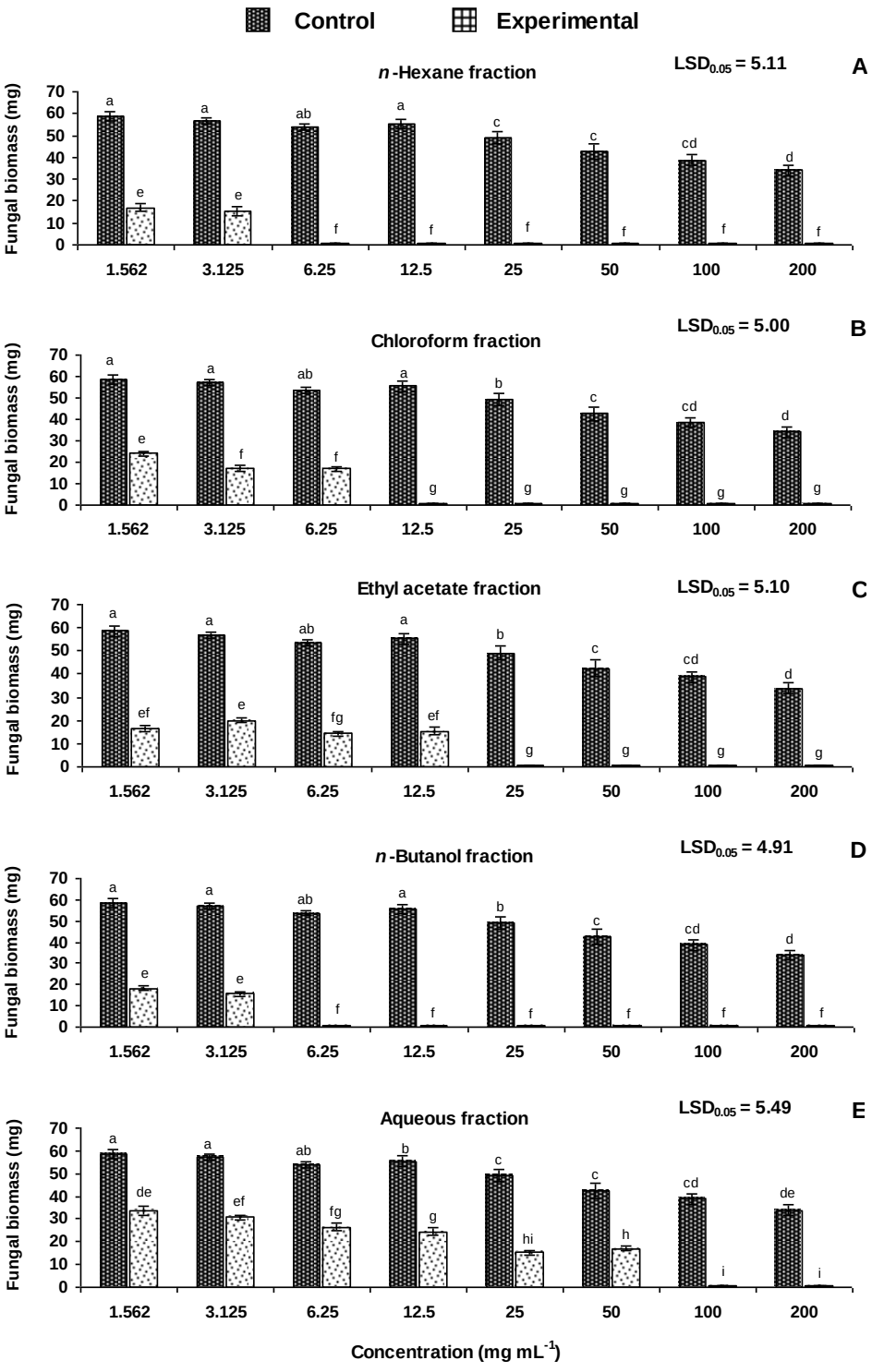

Fig. 1 (A-E): Effect of different concentrations of fractions of methanolic leaf extract of *Cannabis sativa* on growth of *Aspergillus versicolor*. Vertical bars show standard errors of means of three replicates. Values with different letters show significant difference as determined by LSD Test at $P \leq 0.05$.

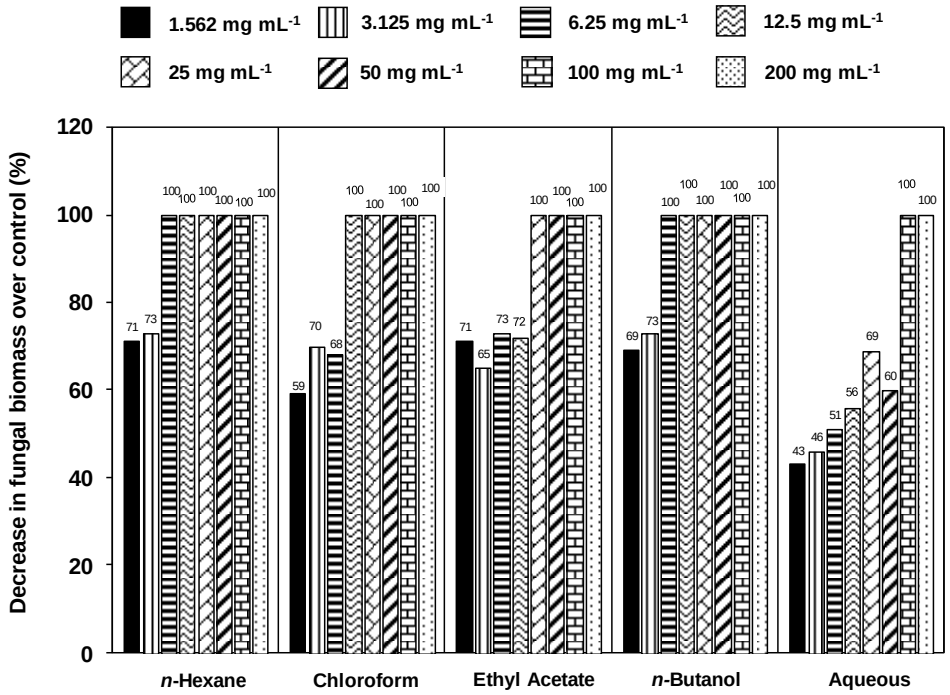

Fig. 2: Percentage decrease in biomass of *Aspergillus versicolor* due to different fractions of leaf extract of *C. sativa* over control.

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Initially the leaves of hemp were extracted in methanol. Although traditional users generally use water as a solvent for extraction, however, it has been found that methanol is better solvent than water for antimicrobial studies (Kaur *et al.,* 2015), owing to better solubility of components in organic solvents as compared to water (Salama and Marraiki, 2010). Moreover, Kaur *et al.* (2015) also demonstrated that methanolic extracts of different weed species showed higher antifungal activities than acetone and ethanolic extracts. The methanolic extract was further partitioned using four organic solvents of different polarity natures. There was much variation in antifungal activities between organic solvent fractions and the aqueous fraction. Various earlier studies have also revealed lesser antifungal activity of aqueous fraction as compared to organic solvent fractions (Aftab *et al.,* 2019; Khan and Javaid, 2020a; Banaras *et al.,* 2021). Even there are reports where aqueous fractions of *Tribulus terrestris* and *Sisymbrium irio* methanolic extracts stimulated the fungal growth (Javaid *et al.,* 2017, 2019). In the present study, there was also a variation in activities among the organic solvent fractions. The *n*-hexane and *n*-butanol fractions were more inhibitory to *A. versicolor* as compared to the ethyl acetate and chloroform fractions. Although in the present study, there were small differences in antifungal efficacy of the four organic solvent fractions, however, in various similar earlier studies a much higher differences in antifungal activities among the organic solvent fractions have been reported. As for example, recently Banaras *et al.* (2021) reported that chloroform fraction was highly effective against *Macrophomina phaseolina* causing up to 93% reduction as compared to *n*-hexane fraction that reduced the fungal biomass up to 70%. Likewise, Banaras *et al.* (2020) demonstrated that chloroform and *n*-hexane fractions of *Sonchus oleraceous* extract reduced *M. phaseolina* growth by 60–90% and 15–66%, respectively, while ethyl acetate and *n*-butanol showed an insignificant affect on fungal growth. Khan and Javaid (2020b) showed that *n*-butanol fraction of *C. sativa* was more antifungal than *n*-hexane and ethyl acetate fractions against *Aspergillus flavipes*. The difference in antifungal activities of fractions was due to different polarity natures of the four solvents. Compounds of different polarity natures present in leaf extract of *C. sativa* were grouped in different solvent fractions according to the polarity natures of these solvents, and thus showed variable antifungal activities against *A. versicolor.*

Conclusion

n-Hexane and *n*-butanol fractions were highly antifungal against *A. versicolor* and can be used for isolation of purified antifungal constituents for the control of this and possibly other related species, and preparation of natural products based fungicides having lesser or no harmful effects on the human health and the surrounding environment.

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