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EVALUATION OF FUNGICIDAL POTENTIAL OF *OCIMUM SANCTUM* AND *NICOTIANA TABACUM ASPERGILLUS FLAVUS* AND *A. NIGER*

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Abstract

The current study aimed to assess the antifungal potential of two medicinal plants (*Nicotiana tabacum* L. and *Ocimum sanctum* L. belonging to the Solanaceae and Lamiaceae families respectively against *Aspergillus niger* van Tieghem and *Aspergillus flavus* Link. Both plants were selected on the basis of their ethnobotanical uses. Dried test plants were dipped in methanol as the extraction solvent, and after getting filtrated by means of maceration procedure, their crude extracts were prepared. Methanolic crude extracts were then subjected to bioassay-guided fractionation to make various solvent fractions viz. n-hexane, dichloromethane, ethyl acetate and n-butanol. Phytochemical analysis was also carried out afterwards to confirm the absence or presence of secondary metabolites in test plants. Two concentrations i.e 0.1% and 0.3% of each fraction of *O. sanctum* and *N. tabacum* were prepared to conduct the antifungal assay. Results of antifungal test revealed that *n*-butanol fraction of *N. tabacum* had more potential in controlling the growth of *A. flavus* at 0.1 % in comparison to *A. niger*. On the other hand, *O. sanctum* proved to be efficient in the retardation of *A. niger* with a fraction of *n*-hexane (100%) at 0.3% concentration. Hence it is depicted by results that both of tested plants have vast potential to be used as antifungal agents.

Key words: Antifungal, Phytochemical analysis, Bioassay guided fractionation, *Ocimum sanctum*, *Nicotiana tabacum*.

Introduction

The genus *Aspergillus* includes those fungal pathogens which are incredibly poisonous to a greater extent and cause several diseases to plant species such as black mold. *A. niger* is one of these fungi affecting various fruits, vegetables and peanuts (Pitt and Hocking, 1997; Perfect *et al*., 2001; Perrone *et al*., 2007). Though this fungus has been considered as harmless by US drug administration but it is still responsible for triggering several disease symptoms in plants and human (Gautam *et al*., 2011). *A. flavus* is another member of this genus which is saprophytic in nature due to the occurrence of aflatoxins in it. Aflatoxins are lethal and prevailing hepatocarcinogenic natural composites until now categorized responsible for several certain disorders (Hedayati *et al*., 2007).

Herbalism is known for preparing natural suppositories directly from plant source of medicinal importance to possessing several organic components in them (Hassan, 2012). These biologically derived medicines proved to be useful as they are cheap and exhibit fewer side effects, so man has been utilizing plants since ages to make herbal medicines (Mazid *et al*., 2012).

Due to the presence of micro and macronutrients, some plant chemicals are known to exhibit biotic actions and improve human health as well. Such compounds are known as phytochemicals (Hasler & Blumberg, 1999). Plants defence-system also enables plants to fight ecological circumstances like pollution, UV exposure, drought, stress and pathogenic attacks (Gibson *et al.*, 1998; Mathai, 2000). Plants also are known to contain biological entities which proved helpful in suppressing diseases as they are rich in phytochemicals (Arif *et al*., 2011). Such living entities are termed as antifungal agents as they can kill or retard the fungal growth and minimize disease symptoms with minimal side effects (Dixon & Walsh, 1996).

Ocimum sanctum, a member of family Lamiaceae, also known as "Tulsi" is medicinally important and used worldwide to combat fungal infections. This plant possesses analgesic, fever, antiemetic, diaphoretic, anticancer, expectorant, antistress, bronchitis and hepatoprotective properties. *Nicotiana tabacum* is a well-known member of family Solanaceae whose leaves are used in making tobacco and because of this reason it is also known as cultivated tobacco. Depending on the illness to be cured, the plant has gained much importance in making plasters and herbal teas by using leaves alone or in combination with other herbs. Usually, leaves are most significant in healing when crushed and ground with slaked lime to produce toxic oral odour which acts as a shielding source against several diseases (Kevin, 2010). Nicotine is medicinally important phytochemical, which is present in *N. tabacum* and the presence of this chemical accounts for its insecticidal potential (Ware & Whitacare, 2004).

So, this study is designed to investigate the antifungal potential of crude methanolic extracts of *O. sanctum* and *N. tabacum* upon fungal growth of *A. niger* and *A. flavus*.

Materials and Methods

Plant material and preparation: Both plants were purchased in cultivated form from Hassan nursery at Kalma chowk, Lahore. After collection, they were weighed using an electronic balance. The plants were dried to remove moisture content using a sheet of paper under shade. Finally, for natural handling, plants were ground into a fine powder.

Source of fungal pathogens: Pure cultures of *A. niger* accession no. 764 and *A. flavus* no. 994 were collected from First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of Punjab, Lahore and maintained on 2% MEA (Malt Extract Agar).

Extraction and Fractionation

Extraction: The measured amounts of the selected plants were soaked in methanol to get crude extract at room temperature. Extraction was carried out for 15-20 days. Then it was subjected to filtration and to obtain a semi-solid crude extract, the filtrate was condensed by the use of rotary evaporator.

Fractionation: The crude methanolic extract of both plants was then dissolved in 10 mL of distilled water to make it aqueous. The aqueous fraction was successively partitioned with n-hexane, dichloromethane, ethyl acetate, n-butanol and methanol at room temperature by using separating funnel. These organic extracts were thoroughly evaporated on a rotary evaporator at 40℃ to obtain their gummy masses (Sherazi *et al*., 2016).

Phytochemical analysis: Phytochemical analysis of each fraction was carried out to confirm the absence or presence of secondary metabolites such as phenolics, flavonoids, saponins glycosides, steroids, triterpenes and tannins (Sofowora, 1982; Harborne, 1973, 1983; Evans, 1989).

Antifungal activity: Antifungal activity was carried out following many steps mentioned below:

a) Initial step was the culturing of fungal strains using malt extract and agar as media for fungal growth along with distilled water in flasks. Flasks comprising media were autoclaved at 121ºC for 30 min. After this, flasks were then allowed to cool then the media was poured into Petri plates. When media in plates get solidified then culturing was carried out from pure strains obtained from the fungal bank by the use of sterilized cork borer. Discs of 5 mm were taken through cork borer for inoculation.
b) Stock solutions of all the fractions of both test plants were prepared up to 20% for using distilled water. Depending on the availability of plant material, two concentrations were prepared for each plant, and three replicates of each concentration were also made.
c) After developing fungus and plant stock solutions for the experiment, again using malt extract (ME broth) medium was prepared. Two flasks were serving as a control treatment, and all other flasks were used as an experimental treatment. Chloromycetin capsule was added in each flask in order to inhibit bacterial contamination. Then fungus was inoculated in all flasks followed by the application of stock solutions. Stock solution was not added to the control treatment and received only distilled water.
d) In all the flasks (control + experimental) 5 mm discs of both the test fungi were added respectively and incubated at 25ºC for 7 days in order to allow the fungi to grow. After seven days, fungal colonies were filtered using pre-weighed Whatman no. 1 filter papers and dried biomass of colonies were measured (Waheed *et al.*, 2016).
e) The last step of the antifungal assay was to find out percentage growth inhibition of dried fungal mats using the formula mentioned below:

$$\text{Fungal growth inhibition}(\%) = \frac{\text{Growth in treatment} - \text{Growth in control}}{\text{Growth in control}} \text{X } 100$$

Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) was used to analyze the data (Steel *et al*., 1997).

Results

Phytochemical analysis: Qualitative phytochemical investigation revealed the presence of various essential secondary metabolites in both experimental plants. In the case of *O. sanctum*, saponins and phenolics were present all the tested fractions. Saponins were found to be absent in just *n*-butanol fraction of *O. sanctum*, while steroids were completely absent. However, glycosides were observed in only *n*-butanol fraction. Flavonoids were present in methanol, ethyl acetate and *n*-butanol fraction. While tannins and triterpenes were present in methanol, *n*-hexane and ethyl acetate fraction. In the case of *N. tabacum*, saponins were present all tested fraction except *n*-hexane fraction. Phenolics also existed in all fractions except *n*-hexane and aqueous fractions. While, steroids and tannins were found in dichloromethane, *n*-butanol and aqueous fractions (Table 1).

Antifungal bioassay of *O. sanctum*: In current studies, different solvent fractions of *O. sanctum* were used for controlling the *in vitro* growth of pathogenic fungal strains. Two concentrations of crude extract of each fraction were prepared viz. 0.3% and 0.7%. Both of the applied concentrations of all the tested fractions significantly suppressed the growth rate of test fungi.

Results revealed that at 0.3% conc. all the fractions showed considerable antifungal activity against *A. niger* but maximum inhibition (100%) was observed in *n*-hexane fraction. While minimum retardation in the biomass of *A. niger* was showed by ethyl acetate fraction, i.e. 27%. Other fractions also reduced the biomass of *A. niger* as 39%, 61%, 64% & 48% inhibition was recorded in methanol, dichloromethane, *n*-butanol and aqueous fractions respectively in comparison to control (Fig. 1). In the case of *A. flavus* at 0.3% concentration *n*-hexane fraction was found more efficient with maximum 69% growth inhibition. However, dichloromethane and butanol fractions also significantly reduced the biomass of *A. flavus* up to 21%. While other tested fractions were found less inhibitory against *A. flavus* (Fig. 2).

Table 1. Qualitative phytochemical analysis of *O. sanctum* and *N. tabacum*.

Phytochemicals	Extract fractions					
	MeOH	n-hexane	DCM	EtOAc	BuOH	Aqueous
	O. sanctum					
Flavanoids	+++	_	_	+	+	_
Glycosides	_	_	_	_	+++	_
Phenolics	+++	+	++	+++	+++	+
Saponins	+++	+	+	+	_	+
Steroids	_	_	_	_	_	_
Tannins	++	+	_	+	_	+
Triterpene	+	+	_	+	+	_
	N. tabacum					
Flavanoids	++	_	+	+	+	_
Glycosides	+	_	_	+	+	_
Phenolics	+	_	++	++	+++	_
Saponins	+	_	+	+++	+++	++
Steroids	_	_	+++	_	_	+
Tannins	_	_	+	_	+++	+
Triterpene	+	_	+	_	+	+

Positive sign shows that phytochemicals are present and negative sign shows the absence of phytochemicals

The effect of n-hexane, dichloromethane, ethyl acetate, n-butanol, methanol and aqueous fractions at 0.7% concentration was displayed in Fig. 3. Maximum antifungal activity against *A. niger* was observed in *n*-butanol fraction (45%) while minimum suppression in test fungus biomass was showed by methanol fraction i.e. only 9%. Other applied concentrations viz. *n*-hexane, dichloromethane, ethyl acetate and aqueous fractions inhibited *A. niger* germination up to 12%, 33%, 18% and 42% respectively. On the other hand, against *A. flavus* aqueous fraction gives maximum growth inhibition, i.e. 69%. While minimum antifungal activity (24%) was shown by methanol fraction. Other fractionated extracts also exhibit mycotic activity to some extent such as *n*-hexane (41%), dichloromethane (44%), ethyl acetate (27%) and *n*-butanol (52%) (Fig. 4).

Antifungal bioassay of *N. tabacum*: All the applied concentrations (0.1 and 0.3%) of all the test fractions significantly suppressed the *in vitro* growth of *A. niger* and *A. flavus*.

Maximum (79%) inhibition in the biomass of *A.niger* was observed in ethyl acetate fraction at 0.1% extract concentration. Other fractions, i.e. *n*-hexane, dichloromethane, *n*-butanol, methanol and aqueous, caused up to 39% - 70% inhibition in the growth of *A. niger* (Fig. 5). In the case of *A. flavus* at 0.1% concentration maximum inhibitory activity was displayed by butanol fraction, i.e. 96%. However ethyl acetate, *n*-hexane and dichloromethane fractions also significantly suppressed the germination of *A. flavus* by 62%, 30% and 83% respectively as compared to control. While methanol fraction showed at least inhibitory effects (Fig. 6).

Maximum growth inhibition (88%) in *A. niger* was noticed in dichloromethane fraction, and minimum antifungal activity was observed in methanol fraction i.e 33% at 0.3 concentration. *n*-butanol and ethyl acetate fractions gave 64% - 39% inhibition, while *n*-hexane and aqueous fractions caused a 51% reduction in *A. niger* (Fig. 7). At 0.3% concentration maximum retardation (48%) in *A. flavus* was showed by methanol and dichloromethane. Other fractions viz. *n*-hexane, aqueous, *n*-butanol, and ethyl acetate fraction retarded the biomass of *A. flavus* up to 31% - 17% (Fig. 8).

Discussion

Plant essential oils and extracts have been used for thousands of years in food preservation, food supplements and pharmaceuticals. Plant and plant-based foodstuff have been severely affected by fungal infections caused by Aspergillus species, probably *A. flavus* and *A. niger* trailed by *A. parasiticus, A. ochraceus, A. carbonarius*, and *A. alliaceus*. These fungal specimens contaminate agronomic entities during pre and post harvest stages as well as in their processing and handling. Plant-based natural products could be a way to overcome these destructive fungal pathogens. Recently Adil *et al*., (2020) suggested that chloroform and methanolic extracts of *Achillea millefolium* and *Chaerophyllum villosum* effectively suppressed the growth of *A. flavus, Fusarium solanum* and *Penicilium notatum*. Hence to contain the spread of these harmful microbes in food products, *O. sanctum* and *N. tabacum* were used as experimental plants in the current study. They were collected and subjected to extraction process using methanol. The methanolic extract of each of the plant was then subjected to fractionation, and different organic fractions were tested for their antifungal potential. Two concentrations, i.e. 0.1% and 0.3% of *O. sanctum* and *N. tabacum* of all the isolated fractions were checked *in vitro* against test fungi. Phytochemical analysis of individual fractions for both plants was also carried out.

In the present study, various secondary metabolites like phenolics, flavonoids, saponins glycosides, steroids, triterpenes and tannins were identified in both the tested plants *O. sanctum* and *N. tabacum.* The presence of these phytochemicals in test plants proved their antifungal efficacy. The therapeutic effects of the plant-based product are because of the collective response of multiple chemical constituents. Therefore, it is necessary to define as many phytochemicals as possible to recognize and explain the bioactivity.

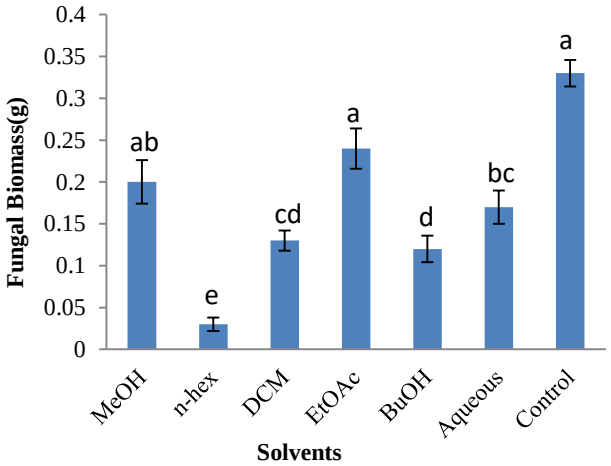

Fig. 1. Assessment of *O. sanctum* solvent fractions with respect to fungal biomass (g) at concentration of 0.3% against *A. niger*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

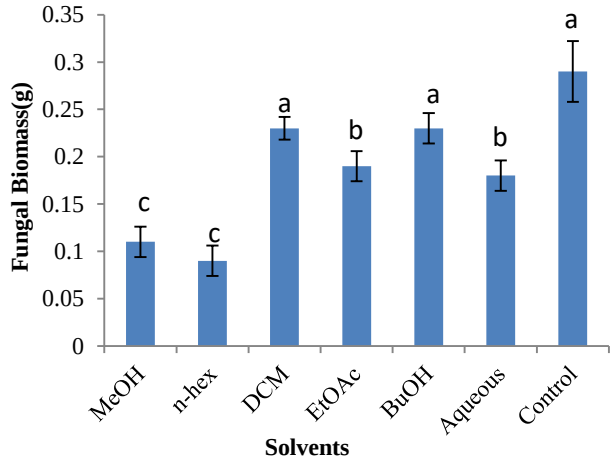

Fig. 2. Assessment of *O. sanctum* solvent fractions with respect to fungal biomass (g) at concentration of 0.3% against *A. flavus*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

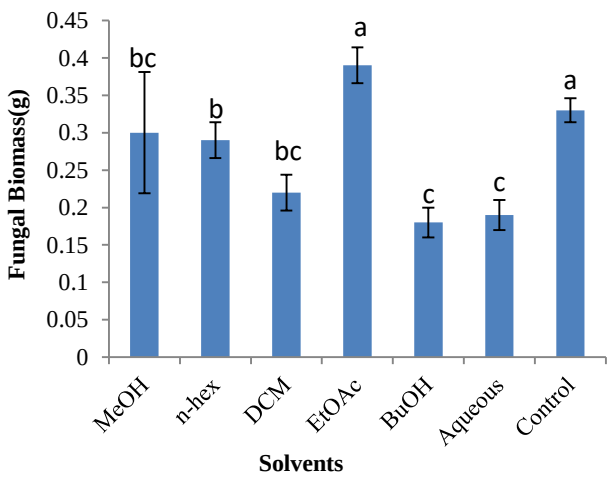

Fig. 3. Assessment of *O. sanctum* solvent fractions with respect to fungal biomass (g) at concentration of 0.7% against *A. niger*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

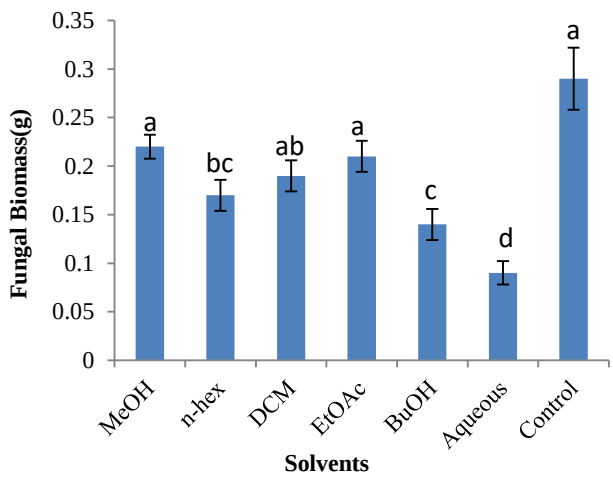

Fig. 4. Assessment of *O. sanctum* solvent fractions with respect to fungal biomass (g) at concentration of 0.7% against *A. flavus*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

In *O. sanctum* as a whole n-hexane fraction was found most effective against *A. niger* as this plant exhibits a wide variety of essential oils. Jaggi *et al.* (2003) investigated the existence of triterpenoids, tannins, saponins and flavonoids in the leaves and stem of *O. sanctum* which contribute towards its medicinal importance (Sethi *et al.,* 2003). Similar results were also reported by (Pachkore *et al.*, 2012; Suleiman, 2011). Due to the presence of these compounds, this plant has been widely used as expectorant, antiemetic, antistress, analgesic and antimicrobial agent. Balakumar *et al*., (2011) reported the antifungal activity of *O. sanctum* against *Microsporum canis, Microsporum gypseum, Epidermophyton floccosum, Trichophyton mentagrophytes* and *Trichophyton rubrum* respectively. *O. sanctum* oil proved to be important in curing mycotic taints caused by Aspergillus species (Soul *et al.*, 2015). The current study also confirmed the existence of various phytochemical compounds that contribute towards its antifungal potential.

As for *N. tabacum*, it possesses many secondary metabolites that account for its antifungal activity, so in this study maximum control for fungal growth had been recorded in butanol fraction. *N. tabacum* displayed broad spectra of antimicrobial activity, and it was efficiently subdued the growth of the pathogens due to the existence of essential secondary metabolites. The leaves of *N. tabaccum* had excellent antihelmintic, antifungal, antibacterial and antimicrobial potential as reported by Rawat & Mali (2013). Owing to the existence of terpenoids, saponins, alkaloids, glycosides, tannins and phenolics, which are potent plant-based secondary metabolites (Saxena *et al.,* 2013). Also, the existence of such a wide range of antimicrobial metabolites has been reported by (Shekins *et al*., 2016) in the methanol extract.

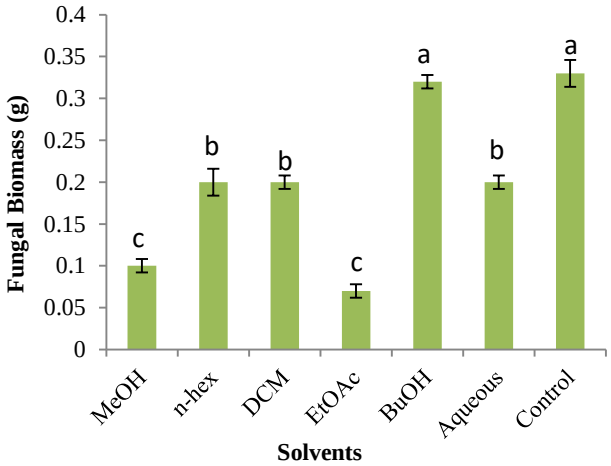

Fig. 5. Assessment of *N. tabacum* solvent fractions with respect to fungal biomass (g) at concentration of 0.1% against *A. niger*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

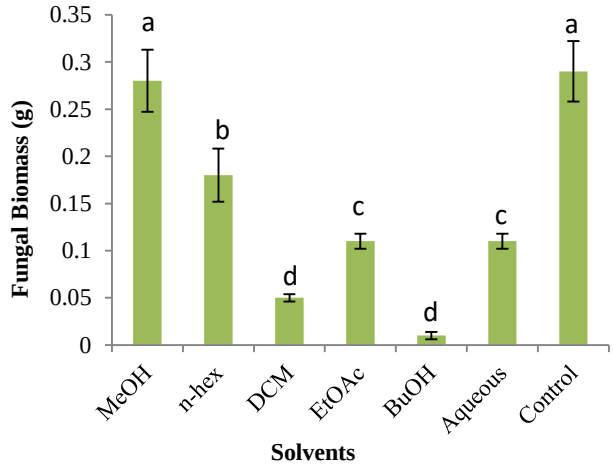

Fig. 6. Assessment of *N. tabacum* solvent fractions with respect to fungal biomass (g) at concentration of 0.1% against *A. flavus*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

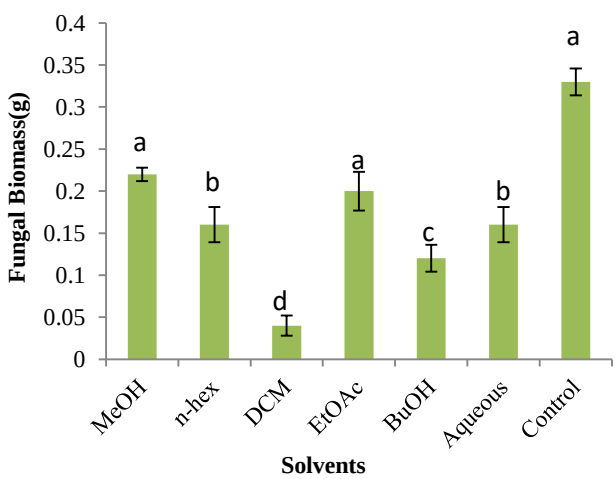

Fig. 7. Assessment of *N. tabacum* solvent fractions with respect to fungal biomass (g) at concentration of 0.3% against *A. niger*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

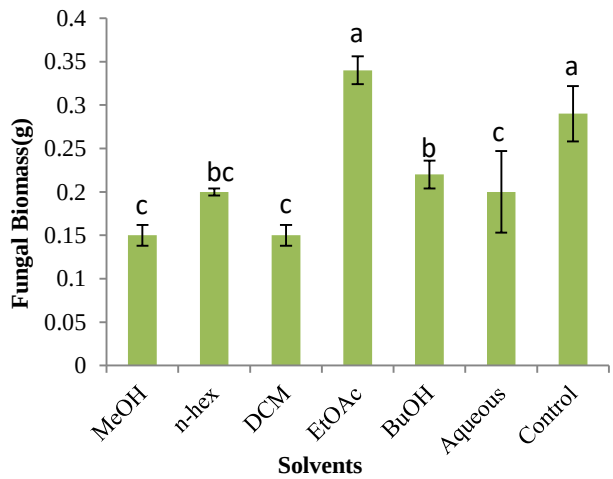

Fig. 8. Assessment of *N. tabacum* solvent fractions with respect to fungal biomass (g) at concentration of 0.3% against *A. flavus*. Significant differences are shown by values with different letters as determined by DMR Test while standard error of means of three replicates is shown by vertical bars.

Conclusion

This study can be concluded that *N. tabacum* proves to be best in the control of *A. flavus* while *O. sanctum* is most suited for retarding *A. niger*. So these plants can be used as disease control natural agents caused by fungal pathogens.

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