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QUALITATIVE AND QUANTITATIVE STUDIES OF REDUCING SUGARS, PROTEIN CONTENTS AND ANTIFUNGAL ACTIVITY OF SECONDARY METABOLITES OF *CLAVICEPS PURPUREA*

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Abstract

Current research work is aimed at qualitative and quantitative investigation of reducing sugars, protein and amino acids in fungal culture filtrate and ethyl acetate extract of *Claviceps purpurea* for antifungal activity. Reducing sugars were quantitatively estimated by DNS method and protein concentration was determined by the Lowry's method. At optimized conditions (Temperature 28°C, pH 7.0, 1% sucrose as carbon source, 1% beef extract as a nitrogen source, 120hr incubation period and 7.5% of inoculum size) *Claviceps purpurea* produces highest reducing sugars and proteins. This fungus culture filtrate contains alkaloids which have pharmacology property. The preliminary phytochemical qualitative analysis of ethyl acetate extract of *Claviceps purpurea* showed the presence of carbohydrates, proteins and amino acids. Antifungal activity was tested against different fungal pathogens. Among all test fungal pathogens, ethyl acetate extract of *Claviceps purpurea* showed a maximum zone of inhibition against *Aspergillus niger* (14.2 ± 0.3mm) compared to standard fluconazole (38.76 ± 0.1mm). It indicates that secondary metabolites of *Claviceps purpurea* has a good antifungal property.

Key words: *Claviceps purpurea*, DNS, Secondary metabolites, Antifungal activity

Introduction

The ergot fungus *Claviceps purpurea* is a phytopathogenic ascomycete which infects the ears of several grasses, replacing the ovary and producing a hibernation structure, the so-called sclerotium, in which the ergot alkaloids are formed. These substances show a high level of structural homology to some neurotransmitters like serotonin and dopamine and can therefore bind to the same receptors in the central nervous system (CNS), which is the basis for the application of ergot alkaloids in a variety of clinical conditions (Lorenz *et al.*, 2010). The sclerotium and the disease are comm only referred to as ergot and ergotism, respectively. Distribution of *C. purpurea* is basically Holarctic, but it has been recorded in Arctic regions and also occurs in southern temperate and subtropical regions (Stefan *et al*., 2011). Most of the methods for determination of carbohydrase activity are based on the analysis of reducing sugars (RSs) formed as a result of the enzymatic scission of the glycosidic bond between two carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Different methods for assaying the RS have been applied in the carbohydrase activity measurements. The Nelson-Somogyi (NS) assay with copper and arsenomolybdate reagents and the 3,5-dinitro salicylic acid (DNS) assay described by Miller are the most popular methods used by many researchers (Gusakov *et al*., 2011). Fungi are remarkable organisms that readily produce a wide range of natural products often called secondary metabolites. In many cases, the benefit these compounds confer on the organism is unknown. However, interest in these compounds is considerable, as many natural products are of medical, industrial and/or agricultural importance. Some natural products are deleterious (*e.g.*, mycotoxins), while others are beneficial (*e.g.*, antibiotics) to humankind (Ana *et al*., 2002). Alkaloids are pharmacologically active basic compounds with nitrogenous heterocyclic rings in their molecular structures. Alkaloidal compounds are sub-divided into subgroups corresponding to the structural moiety of the heterocyclic system they possess. There are various heterocyclic nuclei that have been characterized so far such as indole, ergoline and quinolone and ergosterol

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conjunction-type nuclei (Mohd *et al*., 2016). Secondary metabolite production usually commences late in the growth of the microbe, often upon entering the stationary or resting phase. In early observations it was noted that the environmental conditions required for sporulation and secondary metabolism were often similar and were more stringent than those for pure vegetative growth. Although it was once thought that natural products were essential for sporulation, there are many examples of fungal strains that still sporulate but are deficient in secondary metabolite production. Reducing sugars and proteins are helpful to well growth of hyphae thickness, biomass and pigment production (Barry *et al*., 2015). The interaction of endophytes plants, with these microorganisms can produce several substances of biotechnological interest, including secondary metabolites with pharmaceutical application being used in the production of antimicrobials that inhibit the development of pathogens (Garcia *et al*., 2012). The present work was focused on qualitative and quantitative investigation of biochemicals present in the culture filtrate of fungus *Claviceps purpurea*. Optimum conditions for maximum production of carbohydrates and proteins was determined, then fungal organism was grown at these conditions and culture filtrate was obtained for further analysis.

Materials and Methods

Quantitative estimation of reducing sugars by DNS Method

The reducing sugars were estimated by Dinitro salicylic acid method. The aliquots of extract were pipette out from 0.5 to3ml in test tubes, the volume was equalized to 3ml with distilled water in all the tubes. Then 3ml of DNS reagent was added, mixed and heated for 5 min in a boiling water bath. After the color has developed, 1 ml of 40% Rochelle salt solution was added and mixed. The tubes were cooled under running tap water and the absorption was read at 510 nm. The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose (Naveen *et al*., 2012).

Protein estimation

The protein concentration was determined by the Lowry's method (Lowry *et al*., 1951), by using bovine serum albumin (BSA) as a standard (0.2 mg/mL), absorbance was read at 660 nm using JENWAY-6305, UV-VIS Spectrophotometer and plotted the standard protein calibration curve. The culture filtrates from the culture flasks at different days of incubation were used as crude proteins (Shivakumar *et al*., 2014).

Qualitative Alkaloid analysis.

The alkaloid concentration in the culture filtrate was determined, when added Van Urk reagent formation of blue color indicated presence of alkaloids. (Mantle *et al*., 1976).

Qualitative test for carbohydrates

Molisch test

2-3ml of ethyl acetate extract was added with alpha naphthol solution, shaken well and Conc. H_2SO_4 was added from sides of test tube. Violet ring formation at the junction of two liquids indicates presence of carbohydrates (Syed *et al*., 2015).

Benedict's Test

Few drops of Benedict's reagent were added to the test solution and boiled on water bath. Formation of reddish-brown precipitate indicates the presence of sugars. Depending on the concentration of the reducing sugar, the amount and color of the precipitate produced varied. A positive Benedict's test appears green, yellow, orange, or red (Vimal *et al*., 2014).

Fehling's test

The filtrate was treated with 1 mL of Fehling's A and B, and heated in a boiling water bath for 5-10 min. Appearance of reddish orange precipitate shows the presence of carbohydrates.

Qualitative test for proteins & Amino acids

The fungal extract was dissolved in 10 ml of distilled water and the filtrate was used for the following tests.

Biuret test: To 0.5 mg of extract equal volume of 40% NaOH solution and two drops of one percent copper sulphate solution was added. The appearance of violet color indicates the presence of protein (Santhi *et al*., 2016).

Ninhydrin test: About 0.5 mg of extract was taken and two drops of freshly prepared 0.2% Ninhydrin reagent was added and heated. The appearance of pink or purple color indicates the presence of proteins, peptides or amino acids.

Xanthoproteic test

The fungal extract was treated with few drops of conc. Nitric acid. The formation of yellow color indicates the presence of proteins (Gusthinnadura *et al*., 2017).

Antifungal activity

Microbial strains

The antifungal activity of the ethyl acetate extract was individually tested against a set of eight fungal

pathogenic isolates namely; Alternaria sp, Aspergillus flavus, Aspergillus niger, Candida albicans, Cladosporium sp, Dreschlera sp, Fusarium annulatum and Fusarium oxysporum. These isolates were cultured and incubated at room temperature for 3 to 5 days in laboratory.

Agar well diffusion method

Potato Dextrose Agar was used to culture the fungal organism, the plates were inoculated with culture of respective fungi. With the help of a flamed cork borer, 6 mm wells were cut out and to each of the well 0.1 ml extract (25, 50, 75 and 100 mg/ml) were aseptically added with the help of pipette and were left for an hour to facilitate diffusion of the test solution (the extract). Later the plates were incubated at room temperature. Inhibition was recorded by measuring the diameter of the inhibition zone after 72hr. Fluconazole (10mg/ml) was used as a standard for comparison of antifungal activity (Senthil *et al*., 2014).

Results and Discussion

Fungal pigments are secondary metabolites known as polyketides. Although these come under the supplementary metabolites for growth and reproduction, the developmental stage of fungus greatly influences the production of these pigments, and the developmental stages are influenced by the extrinsic as well as intrinsic factors, including pH, substrate, oxygen, temperature, water activity, and light availability. Like any other fermentative production medium composition, aeration rate, agitation rate, nutritional limitation, status of carbon supply affects the production of secondary metabolites (Gunjan *et al*., 2017).

The 3, 5-dinitrosalicylic acid (DNS) assay for reducing sugars are widely used in measurements of carbohydrase activities against different polysaccharides. In this comparative study, amount of reducing sugars present in culture filtrate was evaluated by DNS method. The cellulase activity of fungal strains was mathematically

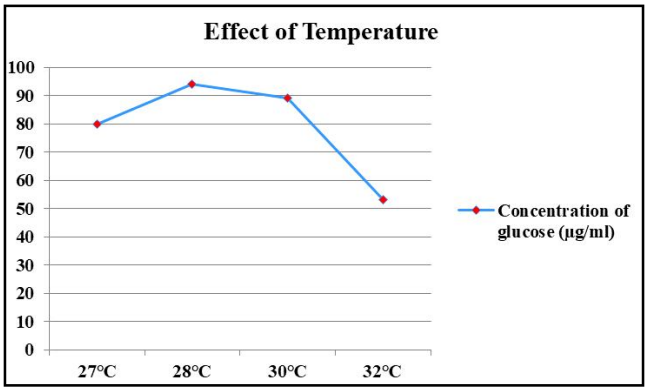

Fig. 1: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different temperature

Table 1: Qualitative test of proteins and amino acids.

Sl. No	Name of test	Result
01	Biuret test	Present
02	Ninhydrin test	Present
03	Xanthoproteic test	Present

Table 2: Qualitative test of carbohydrates.

Sl. No	Name of test	Result
01	Molisch's test	Present
02	Benedict's test	Absent
03	Fehling's test	Present

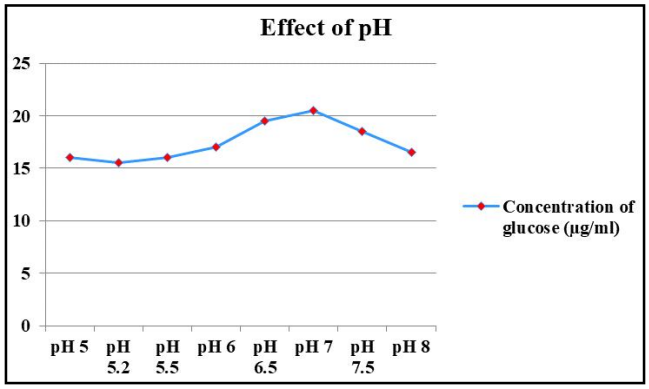

Fig 2: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different pH.

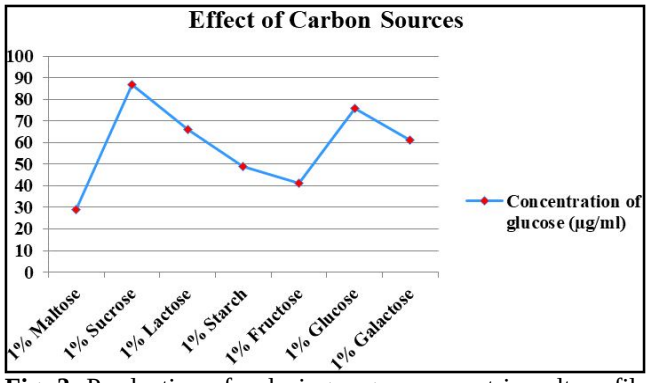

Fig. 3: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different carbon sources.

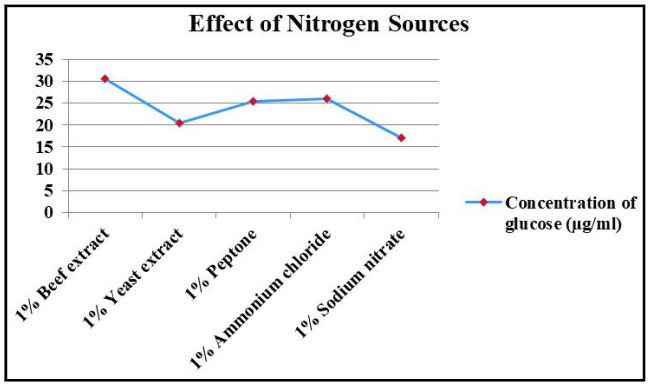

Fig. 4: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different nitrogen sources.

calculated by using the data of protein content (µg/ml) present in the supernatant of isolates which was estimated by Lowry's method. The amount of reducing sugars and protein content present in culture filtrate of *Claviceps purpurea* was estimated in present study.

In the present study culture filtrate of *Claviceps purpurea*, produces more reducing sugars (94 µg /ml) at 28°C compared to the other conditions.

In the present study culture filtrate of *Claviceps purpurea*, produced more reducing sugars (20.5 µg/ml) at pH 7.0 compared to the other conditions. Earlier (Patil *et al*., 2015) stated that pH has an important role for the medium plays in pigmentation. The optimum pH for the biomass and pigment production was found to be 5.

In this study, culture filtrate of *Claviceps purpurea*, produces more reducing sugars (87µg /ml) at concentration of 1% sucrose carbon source compared to the other conditions. Rosemeire *et al*., 2015 reported, the growth rate of the fungus cultured on solid medium containing different carbon sources was similar but the macroscopic characteristics of the colonies were clearly different. Here our present study revealed that *Claviceps purpurea* utilizes carbon sources for growth and pigment production is well on liquid media.

Table 3: Antifungal activity (zone of inhibition, mm) of ethyl acetate extract of Claviceps purpurea against various fungal pathogens.

Test Fungi	*Fluconazole (10mg/ml) Zone of inhibition(mm)*	*DMSO (10%) Zone of inhibition (mm)*	*Concentration of extract (mg/ml)*	*Diameter of zone of inhibition (mm)*
Alternaria sp.	*34.65±0.3*	*00*	*100*	*00*
			75	*00*
			50	*00*
			25	*00*
Aspergillus flavus	*32.26±0.2*	*00*	*100*	*13.1±0.3*
			75	*12.3±0.3*
			50	*10.3±0.1*
			25	*09.1±0.2*
Aspergillu-sniger	*38.76±0.1*	*00*	*100*	*14.2±0.3*
			75	*12.3± 0.2*
			50	*11.5± 0.2*
			25	*10.4± 0.1*
Candida albicans	*28.35±0.4*	*00*	*100*	*10.1±0.2*
			75	*08.2±0.3*
			50	*07.5±0.2*
			25	*06.6±0.1*
Cladosporium sp	*26.12±0.2*	*00*	*100*	*00*
			75	*00*
			50	*00*
			25	*00*
Dreschlera sp	*28.85±0.5*	*00*	*100*	*12.4±0.4*
			75	*11.2±0.2*
			50	*10.4±0.2*
			25	*10.1±0.1*
Fusarium annulatum	*38.88±0.4*	*00*	*100*	*10.5±0.4*
			75	*10.1±0.3*
			50	*10.4±0.3*
			25	*08.2±0.2*
Fusarium oxysporum	*40.10±0.6*	*00*	*100*	*00*
			75	*00*
			50	*00*
			25	*00*

Note: ± Indicates triplicates of Standard error.

In the present study culture filtrate of *Claviceps purpurea*, extract produces more reducing sugars in nitrogen source condition of, 1% beef (30.5µg / ml) compared to the other conditions. In earlier report (Stephanie *et al*., 2019) three nitrogen sources (ammonium chloride, nicotinic acid and yeast extract) were tested for growth and pigment production of fungus *Chlorociboria aeruginascens*. In yeast extract significant fungal growth and pigmentation was detected.

In the present study culture filtrate of *Claviceps purpurea*, produces more reducing sugars (92µg/ml) at 120hr incubation period condition, compared to the other conditions.

In the present study culture filtrate of *Claviceps purpurea*, produces more reducing sugars (96µg/ml) at inoculum size condition of, 7.5% compared to the other conditions (Lone *et al*., 2012).

Same work has completed in relevant to estimation the reducing sugar content per gram of Arecanut husk waste sample- A (4.12mg/gm) was initiated (Naveen *et al*., 2012). Compare to this sample our fungal culture filtrate shows very high concentrations.

Quantitative estimation of proteins

Soluble crude proteins were evaluated by Lowry's method, in different culture conditions. but maximum protein production by *Claviceps purpurea* fungus was at 28°C temperature (98 µg/mL), at pH 5.5 (78µg/ml), in 1% maltose (78µg/ml), in 1% beef extract (82 µg/ml), at incubation period of 120 hr (98 µg/ml) and finally at inoculum size 2.5% (78 µg/ml).

Soluble crude proteins were evaluated by Lowry's method, in different culture conditions. but maximum protein production (98 µg /mL), was at 28°C temperature, at pH 5.5 (78µg /ml), in different

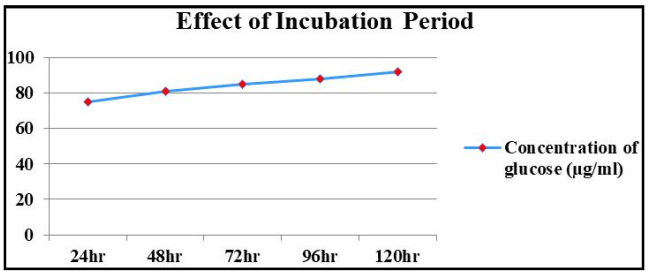

Fig. 5: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different incubation period.

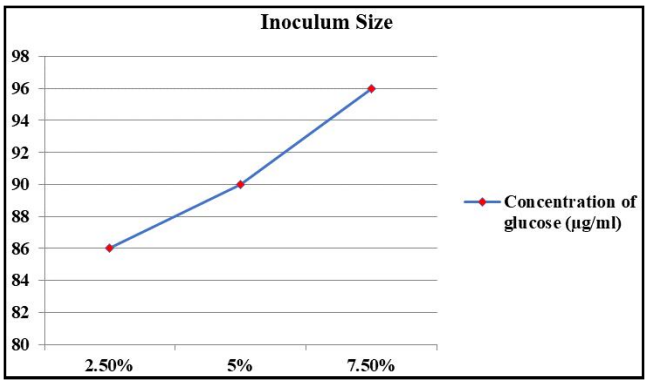

Fig. 6: Production of reducing sugars present in culture filtrate of *Claviceps purpurea* at different inoculum size.

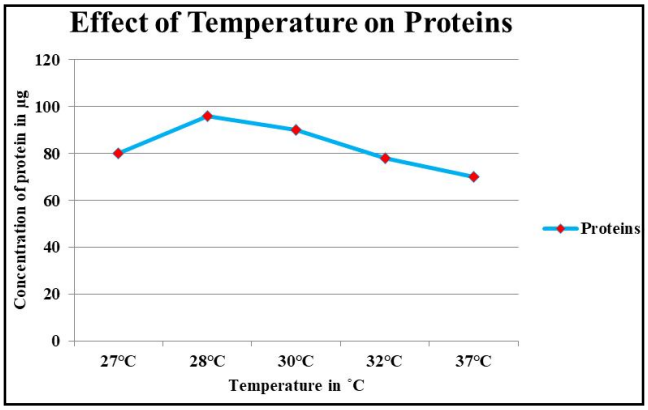

Fig. 7: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at temperature.

carbon sources 1% maltose showed maximum protein production (78µg/ml).

In assessing the true protein content present in culture filtrate of *Claviceps purpurea*, it is important that a method of protein determination is chosen which is accurate, yet suitable enough to use for routine testing. A comparison of the currently used methods was made with protein determination, based on measurement of the quantities of individual protein content as shown in Fig. 7-12 (Christias *et al*., 1975).

Qualitative study of alkaloid analysis

When culture filtrate was mixed with Van Urk reagent formation of blue colors indicates presence of

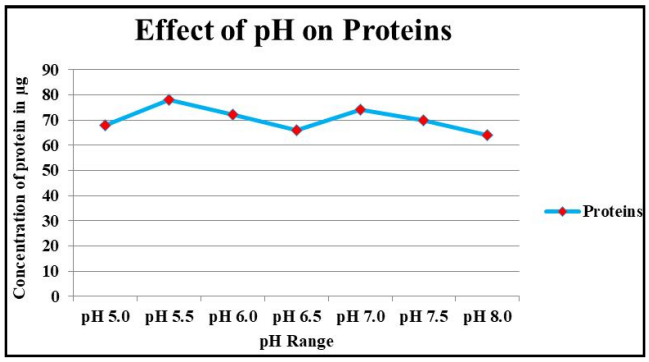

Fig. 8: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at pH.

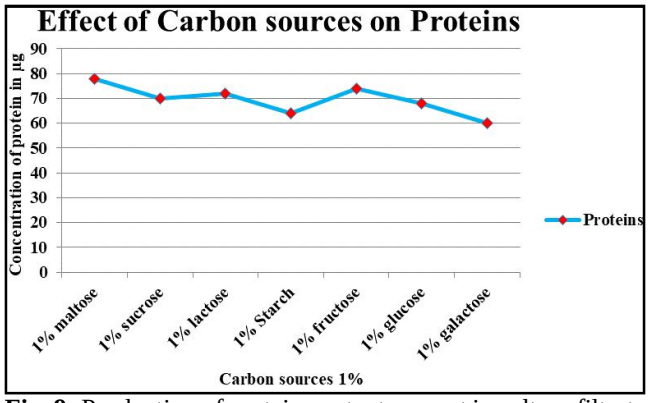

Fig. 9: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at carbon source.

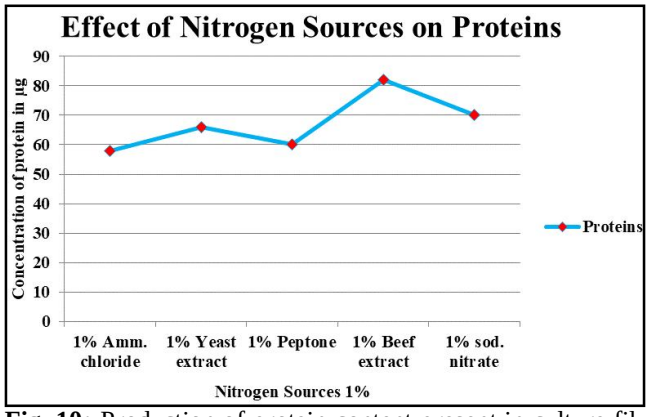

Fig. 10: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at nitrogen source

alkaloids Fig. 13.

Qualitative test of Carbohydrates, Proteins and amino acids

This study revealed the presence of numerous Phyto nutrients as very important and active medicinal constituents of fungi. In carbohydrates estimation, the Molisch's test and Fehling's test showed the positive results and Benedict's test showed negative result. The results are interpreted in table 1. In proteins and amino acids estimation, Biuret test, Ninhydrin test, and Xanthoproteic

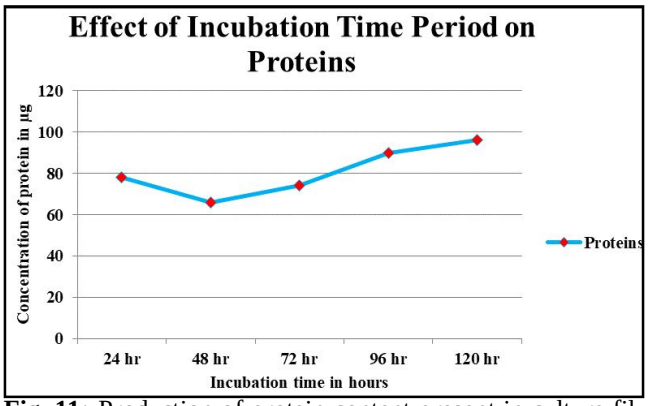

Fig. 11: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at incubation period.

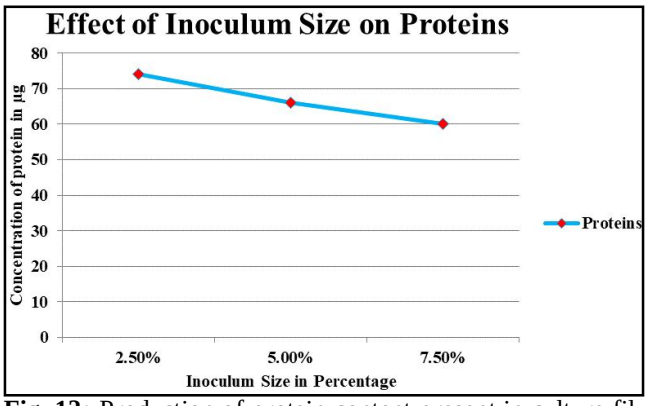

Fig. 12: Production of protein content present in culture filtrate of *Claviceps purpurea* on optimized conditions at inoculum size.

test showed positive results. The results are interpreted in table 2. The above discussion revealed that the culture filtrate of *Claviceps purpurea* showed a richer phytochemicals constituent and they also have many medicinally important properties.

Antifungal activity test

The result indicated that, among the tested fungal organisms, extract exhibited significant antifungal activity against Aspergillus niger, Aspergillus flavus, Dreschlera sp, Fusarium annulatum, Candida albicans compared to standard drug Fluconazole. Table 3 depicts the antifungal screening results of the ethyl acetate extract of *Claviceps purpurea* (Garudachari *et al*., 2012).

Maximum antifungal activity of ethyl acetate extract of *Claviceps purpurea* was against *A. niger* (14.2 ± 0.3mm), followed by *A. flavus* (13.1±0.3mm), Dreschlera sp. (12.4 ± 0.4), *Fusarium annulatum* (10.5 ± 0.4mm) and *Candida albicans* (10.1 ± 0.2). Extract did not show antifungal activity against Alternaria sp., Cladosporium sp., *Fusarium oxysporum.* Fig. 14 (Antara *et al*., 2012).

Conclusion

The particular fungal culture filtrates have a rich

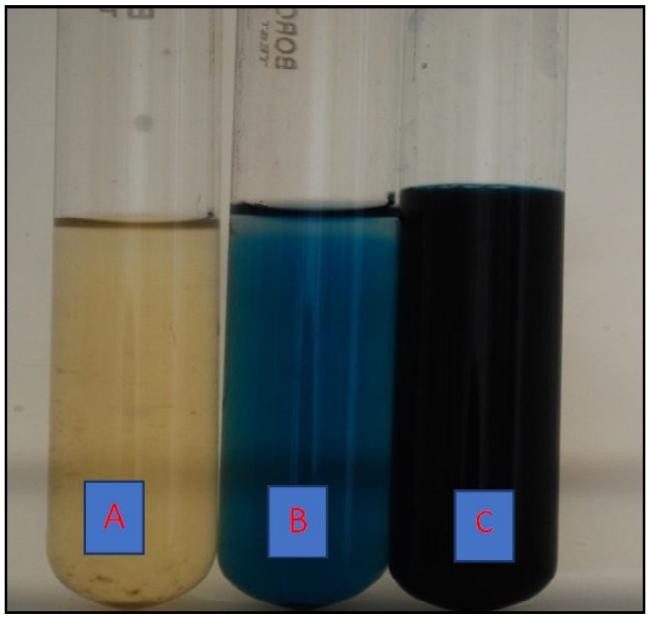

Fig. 13: Qualitative Alkaloid analysis A. Control Culture Filtrate, B and C. Blue color indicates alkaloids present**.**

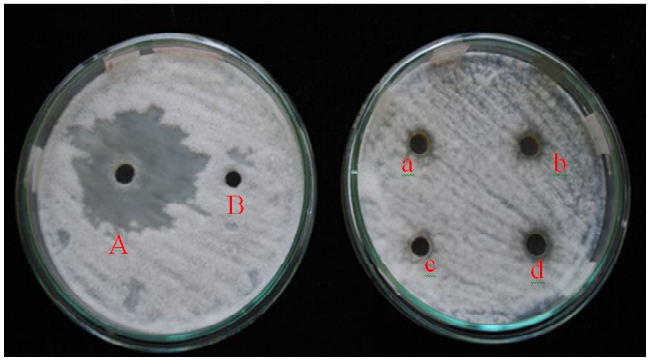

Fig. 14: Ethyl acetate extract of *Claviceps purpurea* against *Fusarium annulatum* A. Positive control B. Negative control. a. 100mg/ml, b. 75mg/ml, c. 50mg/ml, d. 25mg/ml.

source of secondary metabolites *i.e.* reducing sugars, carbohydrates, proteins and amino acids. These are helpful for production of pigments and growth of organism as well. Based on Carbohydrates, protein and amino acid analysis, it appears that, *Claviceps purpurea* is a better organism to produce secondary metabolites.

For antifungal activity result *Aspergillus niger* (14.2 ± 0.3mm) showed a maximum zone of inhibition compare to other fungi. In antifungal activity Fluconazole was used as the positive control and 10% DMSO used for negative control against the test fungi. Fluconazole, standard antifungal showed a higher antifungal activity than the ethyl acetate extract of *Claviceps purpurea* and extract showed a significant value of this activity. These secondary metabolites have pharmacological importance. Finally, secondary metabolites of *Claviceps purpurea* showed a good antifungal property.

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