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RESEARCH ARTICLE

PHYTOCHEMICAL SECREENING OF SECONDARY METABOLITES FROM *ASPERGILLUS NIGER* (MTTC-961) AND THEIR ANTIBACTRIAL ACTIVITY

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ABSTRACT

Fungi are the potential and promising sources for biologically active secondary metabolite production. Secondary metabolites are the chemical compounds that are produced during the stationary phase of the organism. Many years of study revealed that fungi are excellent sources for novel bioactive secondary metabolites. In the present study *Aspergillus niger* extract was screened for the presence of phytochemicals by standard procedures. After phytochemical screening check their antibacterial activity against human pathogens. The present work shows the presence of significant secondary metabolites and show maximum antibacterial activity.

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INTRODUCTION

Secondary metabolites are small molecules that are not directly involved in metabolism and growth of the organism. Both plants and fungi are known for producing a large number of chemically diverse secondary metabolites. While the role of some of these metabolites makes sense biologically as inferring an advantage to the producer, e.g. antibiotics, virulence factors, siderophores and pigments, the benefit of others is less obvious or unknown. The general belief is that the secondary metabolites must contribute to the survival of the producer in its environment where it competes with other organisms (Fox *et al*., 2008). The actual production of secondary metabolites has, in broad terms, been reported to be affected by the developmental stage of the fungus (i.e. conidiation) and intrinsic and extrinsic factors of the environment as substrate, composition, pH, water activity, temperature, light and oxygen availability (Sagaram *et al*., 2006 and Bayram *et al*., 2008). *Aspergillus niger* is a versatile filamentous fungus found in the environment all over the world in soil and on decaying plant material, and it has been reported to grow on a large number of foods and feeds (Sorensen *et al*., 1996). The word "Phyto" is the Greek word for plant. Phytochemicals, which not only are non-nutritive chemicals that have protective or disease preventive properties but also protect humans from a host of diseases (Suleima 2011). Phytochemical studies have shown that fungi with antimicrobial activity contain bioactive constituents such as tannins, flavonoids, alkaloids and saponins (Chukwuka *et al*., 2011) Alkaloids & flavinoids have been used as antiviral, antibacterial, antiamoebial & anticancer agents. Phenolics and polyphenolics are the other group of secondary metabolites (Amit Pandey *et al*., 2011). The aim of this study was analysis of the secondary metabolites and the evaluation of antibacterial activity using *Aspergillus niger*.

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MATERIALS AND METHODS

Culture collection and Maintenance

Pure cultures of *Aspergillus niger* (MTCC No-961) Chandigarh, India and were immediately transferred to sterile agar slants of potato dextrose agar media. The strains were grown in potato dextrose media. The *Asperigillus niger* culture from potato dextrose broth was streaked on a Potato dextrose agar slant and it was incubated at 27°C for 72 hours. It was then sub cultured and was stored in refrigerator for further use.

Extraction of Secondary Metabolites

The pure culture of *Aspergillus niger* inoculate to the sterilized Potato Dextrose Broth and the culture flasks were incubated at 27°C for 15 days. After one week incubation ethyl acetate is added to the broth in 1:1 ratio and kept for incubation in orbital shaker at 170rpm and kept for 5 hr. Minimal shaking is required for dissolving the metabolites into ethyl acetate solvent.

Separation of Metabolites: The metabolites which are now dissolved in ethyl acetate solvent are separated by using separating funnels. In the separating funnel add the media with ethyl acetate. To that add some amount of ethyl acetate, shake well and allow it to settle for few minutes. Later two layers were observed in the separating funnel. The bottom layer is Broth Layer which is discarded and the upper layers of ethyl acetate with metabolites are collected which is called the organic layer. The washes were repeated for three times to extract the complete metabolites. This separated extract was Rota vapored for the collection of crude extract. Crude extract is dissolved in Dimethyl Sulfoxide

Phytochemical Screening

The crude extract was subjected to preliminary phytochemical screening for the detection of various phytochemicals.

Test for Alkaloids

Wagner's test: A fraction of extract was treated with Wagner's test reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish brown colour precipitate.

Test for Flavonoids

NaOH test: A small amount of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour.

Lead acetate test: A small amount of extract was treated with lead acetate and observed for the formation of white precipitate.

Test for Tannins

Braymer's test: Few ml of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution.

Test for Saponins

Foam test: A small amount of extract was shaken with water and observed for the formation of persistent foam.

Test for Carbohydrates

Molisch's test: Few drops of Molisch's reagent were added to each of the portion dissolved in distilled water; this was then followed by addition of 1 ml of conc. H_2SO_4 by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet colour at the interphase of the two layers was a positive test.

Test for Quinones: A small amount of extract was treated with concentrated HCl and observed for the formation of yellow colour precipitate.

Test for Terpenoids

Liebermann – Burchard test: Extract (1ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark green colour.

Test for Sterols

H_2SO_4 test: The fraction of extract was treated with ethanol and H_2SO_4 and observed for the formation of violet blue or green colour.

Test for Phenols

Ferric chloride test: The fraction of extract was treated with 5 % ferric chloride and observed for formation of deep blue or black colour.

Test for Anthraquinones

Borntrager's test: About 50 mg of powdered extract was heated with 10% ferric chloride solution and 1ml concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia; pink or deep red colourations of aqueous layer indicate the presence of anthraquinone.

Test for Proteins

Ninhydrin test (aqueous): The extract was treated with aqueous ninhydrin, purple colour indicates the presence of protein.

Antibacterial Activity (Agar - well diffusion method)

The antibacterial activities of the fungal extract were tested against the selected bacterial strains. The 20ml of sterilized agar medium was poured into each sterile petriplates and allowed to solidify. The test bacterial cultures were evenly spread over the appropriate media by using a sterile cotton swab. Then a well of 0.5cm was made in the medium by using a sterile cork borer, 150µl of each methanol, ethanol and aqueous fungal extracts were transferred into separate wells. After these plates was incubated at 37^{o}C for 24-48 hours. After incubation period, the results were observed and measure the diameter of inhibition zone around the each well.

Microbial Test Organisms

The organisms like ***Staphylococcus aureus*** (MTCC-3160), ***Streptococcus*** (MTCC-2327), ***Ksebsiella pneumonia*** (MTCC-452), ***Escherichia coli (***MTCC-443), was procured from

Microbial Type Culture Collection(MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained freshly prepared potato dextrose agar slants respectively. The organisms were preserved at - 20 ºC in the presence of glycerol (15 %, v/v) for longer periods.

RESULTS

The results of the phytochemical, biochemical screening and antimicrobial activity of fungal extract were showed in Table 1 and 2.

Table 1. Qualitative screening of the phytochemicals in the ethylacetate extract of *Aspergillus niger* (+ = Positive - = Negative)

S.No	Test	Result
1	Alkanoids	+
2	Tannins	-
3	Steroids	+
4	Saponins	-
5	Andhraquinine glycosides	+
6	Terpenoids	+
7	Phlobatannins	-
8	Proteins	-
9	Phenols	+
10	Glycosides	+
11	Quinones	-
12	Gums& Carbohydrates	-
13	Lignins	-
14	Coumarins	+
15	Aldehydes	+
16	Ketones	+
17	Flavanoids	-

Fig. 1. Phytochemical screening of Fungal extract

Table 2. Antibacterial Activity of *A.niger* Extract

S.No.	Organism	Zone of Inhibition(mm)			
		Control (20µg/ml) Gentamycin	20 µg/ml	30 µg/ml	50 µg/ml
1	*Staphylococcus aureus* (MTCC-3160)	25mm	12mm	15m	16mm
2	*Streptococcus (*MTCC-2327),	20mm	18mm	20mm	32mm
3	*Ksebsiella pneumonia (*MTCC-452)	12mm	10mm	15mm	18mm
4	*Escherichia coli* (MTCC-443)	1 5mm	18mm	20mm	21mm

Fig. 1. Antibacterial activity of fungal Extract against *Staphylococcus aureus*

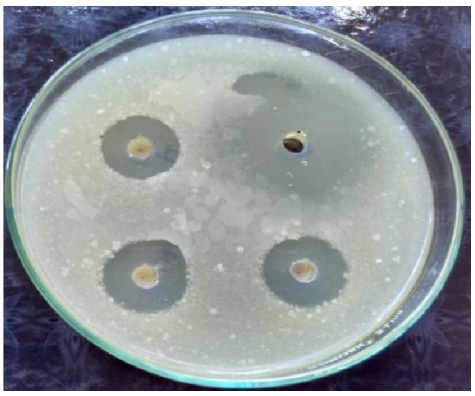

Fig. 2. Antibacterial activity of fungal extract against *Streptococcus*

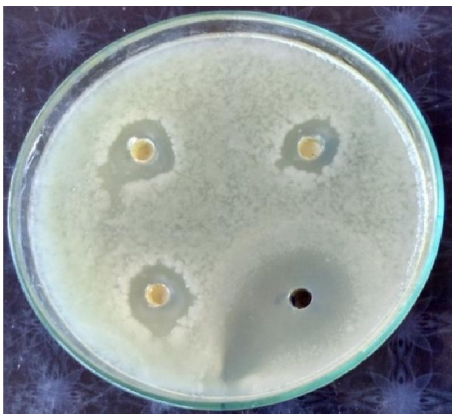

Fig. 3. Antibacterial activity of fungal Extract against *Klebsiella pneumoniae*

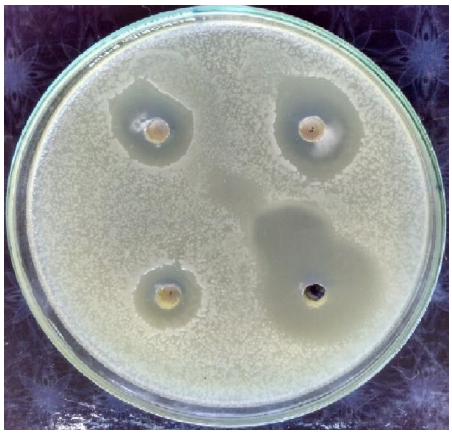

Fig. 4. Antibacterial activity of fungal extract against *E.coli*

DISCUSSION

The crude extract *of Aspergillus niger* was subjected to phytochemical analysis by standard procedures. The crude extract showed the presence of different phytochemicals, phenolic compounds, Steroids, Cardiac glycosides, Tannins, Alkaloids and Flavanoids. Major natural products of secondary metabolism in fungi are phenolic compounds. Phenol and flavonoid compounds have been reported to possess different bioactivies (Huang *et al*., 2010). Flavonoids enhance the effects of Vitamin C and function as antioxidants. They are also known to be biologically active against liver toxins, tumors, viruses and other microbes (Korkina *et al*., 1997). Phytochemical tests of the above extracts were performed through chemical reagents as described by Harbone 1998. Natarajan *et al*. (2010) found that the compound isolated from broth extract of *A.niger* showed a high degree of antibacterial activity against Bacterial organisms. The results of antimicrobial activity assay showed that, the fungal extracts possess antimicrobial activities against different concentration of 20, 30, 50 µg/ml for *Aspergillus niger* (Table 1). The *A.niger* extracts compared with the standard antibiotic Gentamicin. In the agar diffusion assay, the highest zone of inhibition was observed in 50 µg/ml concentration that is 32mm against *Streptococcus*. The lowest zone of inhibition was observed in 20 µg/ml concentration that is 12mm against *Staphylococcus aureus*. The extract of *Aspergillus niger* showed inhibition activity against *Escherichia coli* (21mm). While the extract of *Aspergillus niger* showed the highest inhibition zone against *Escherichia coli* and *Streptococcus* (20mm) followed by *Streptococcus,* and *Ksebsiella pneumomonia* (18mm) respectively. Fawzy *et al*., 2011 studied the antimicrobial activity of extracellular and intracellular extracts of *A.niger* against 10 different bacterial isolates comprising of both Gram negative and Gram positive organisms. They found that the extracellular extracts of fungi were found to posses activity against the Gram-negative bacteria only, while the intracellular extracts didn't do so. The fungal extract showed maximum antibacterial activity against human pathogens.

Conclusion

The present work was attempted to *A.niger* which is capable of producing efficient antibacterial. The presence of phytochemicals as phenolic compounds and flavanoids were present in crude extract. The future scope of this work is to isolate these biologically active compounds to use in pharmaceutical applications.

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