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Antimicrobial activity of *Polyalthia longifolia* extract, fungal chitosan and its combinatorial studies

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ABSTRACT: Medicinal plants play an important role in protective our health and these plants are nature's gift to lead defensive life. India is one of the greatest ethanoculturally varied nations in the world, where the remedial plant area is part of a time privileged practice that is treasured even today. Pharmacologic research on the plant parts displays effective cytotoxic function, hypotensive effect, hypoglycemic activity, antiulcer activity, and antimicrobial activity. In this present study the antimicrobial activity of combinatorial studies of aqueous extract *Polyalthia longifolia* and chitosan (F*Ch*) extracted from *Aspergillus niger*. The extracted FCh characterized by by using UV-visible spectroscopy, FT-IR, and SEM coupled with EDX analysis. Further, the antimicrobial activity of aqueous extract of *P. longifolia* and chitosan (F*Ch*) was checked by using disc diffusion assay. Also the combinatorial antimicrobial activity studies of F*Ch* and *P. longifolia* were studied. The results of FT-IR showed, the characteristic peaks of F*Ch* shows at 3435 cm^{-1}, 2924cm^{-1}, 1645 cm^{-1}, 1412 $cm^{-1}$and 1021 cm^{-1} attributing towards O-H strech, C-H strech, N-H bend C-H bend-C-N stretch. The characteristic peak at 191 nm obtained from spectrophotometric evaluation confirms the presence of Ch. The microscopic view of F*Ch* was also observed through SEM coupled with EDX showed that F*Ch* smooth surface. The antimicrobial action of aqueous extract of *P. longifolia* zone of inhibition was ranges from 10.6±0.1 to 12.6±0.3mm and F*Ch* show 11.6±0.5 to 18.2±0.3 mm at 0.5 to 10mg/ml concentrations. The combinatorial studies F*Ch* and *P. longifolia* showed highest percent mycelial inhibition against *A. flavus* and *F. verticillioides*. The percent mycelial inhibition of *A. flvaus* was 87.6±0.4 % and *F. verticillioides* 97.6±0.5% at 0.5 to 10mg/ml concentrations. The obtained results confirms that the combinatorial studies F*Ch* and *P. longifolia* could be used as alternative green strategy for the control of microbial activity.

Keywords: *Polyalthia longifolia,* chitosan, characterization, antimicrobial activity, mycelial inhibition, combinatorial studies.

INTRODUCTION

In developing countries, infectious diseases are a significant cause of morbidity and mortality among the general population. According to the Centers for Disease Control and Prevention, food-borne pathogens leads to approximately 48 million people illness, 128,000 hospitalizations and 3000 deaths annually (Severino *et al.,* 2014). A greater concern is the emergence of microbial pathogens not formerly associated with processed or raw products, which could further escalate the chances of food-borne illness and outbreaks. Furthermore, due to the overuse of antibiotics, there has been a global emergence of multidrug-resistant microorganisms, which reduces the efficacy of current antibiotic therapy and results in thousands of death.

The drug resistance to human pathogenic bacteria commonly reported worldwide is members of the family *Enterobacteriaceae* (*E. coli*, *Shigella* spp., and *Salmonella* spp.) and other pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Campylobacter*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*. However, this situation is alarming in developing countries due to indiscriminate use of antibiotics. The existence of multidrug-resistant (MDR) bacteria have also creates special problems in treating infections in immuno-compromised persons particularly cancer and AIDS patients (Exner *et al.,* 2017). Although treatment and management of microbial infections has improved by using efficient antibiotics during the last decades, the increase in antimicrobial resistance, the emergence of new pathogens and re-emergence of known pathogens form a major threat for health systems worldwide (Zipfel *et al.,* 2007; Hosseinnejad and Jafari 2016).

Fungal Chitosan (*FCh*) is thought to have a higher

bioactivity than Chitosan (*Ch*) obtained from traditional sources (Jaworska and Konieczna 2001). Whereas fungal derived *Ch* has a medium-low molecular weight of 1-12 × 10^4 Da (Wibowo *et al.,* 2007). Ch is a natural biopolymer obtained by deacetylation of chitin and is a non-toxic polymer (Wang *et al.,* 2011). The Ch extraction of fungi is considered as a safe, there are several mycelium fungi, such as *Mucor rouxii*, *Absidia glauca*, *Aspergillus niger*, *Gongronella butleri*, *Pleurotus sajor-caju*, *Rhizopus oryzae*, *Lentinus edodes*, and *Trichoderma reesei* have been considered as possible sources of chitin and *Ch* due to their presence in the cell walls (Pochanavanich and Suntornsuk, 2002; Suntornsuk *et al.,* 2002). The production of *Ch* from fungi and is recognized as safe and free from allergenic shrimp protein (Dhillon, 2013). The *Ch* has excellent biocompatibility and biodegradability, *Ch* is used as enzyme immobilization, emulsifying, thickening and stabilizing agent, packaging membrane, antioxidant, dietary, antimicrobial agent, drug-delivery systems, blood anticoagulants, gene therapy, occulants and chelating agent for metals (Ahmed *et al.,* 2018).

Polyalthia longifolia (commonly known as Ashoka tree) belongs to family Annonaceae. They consist of various components in different parts of plant such as clerodane, diterpenoids and alkaloids which are known for their medicinal effects (Katkar *et al.,* 2010). *P. longifolia* has been observed to show resistance to pathogenic infections. In indigenous systems of medicine this plant has been used as an antipyretic agent. Traditionally this plant has been used as a medicine to treat various diseases such as fever, diabetes, hypertension, skin diseases and helminthiasis (Malairajan *et al.,* 2008; Dasta and Aliahmadi, 2015). The plant extract and the number of biologically active compounds isolated from this plant have been studied for various biological activities such as cytotoxicity, antifungal and antibacterial (Nematolahi *et al.,* 2015; Tan *et al.,* 2015; Abdelghany *et al.,* 2019; Nguyen *et al.,* 2020; Konappa *et al.,* 2018; 2019; 2020). Using a combination of active ingredients may increase antimicrobial activity (Nair and Chanda, 2005). In this study, an attempt has been made to analyze the antimicrobial activities of *FCh* and plant extract.

MATERIAL AND METHODS

A. Test microorganisms

The four human pathogenic bacteria *viz., Escherichia coli* (NCIM 2065)*, Klebsiella pneumoniae* (NCIM 2957), *Pseudomonas aeruginosa* (NCIM 5031), and *Staphylococcus aureus* (2079) were procured from the National Collection of Industrial Microorganisms (NCIM), Pune (India). All the test bacteria were maintained on MHA (Hi-Media, India) and twenty-four hours old cultures were used for antibacterial activity assay. The seed borne fungi were isolated from maize seed samples *A. flavus* and *F. verticillioides* as field fungi.

Collection of plant material and preparation of aqueous extract. Aqueous extracts of fresh plant materials were prepared following the procedure of Mohana and Raveesha (2006). The plant materials (50g) were separately macerated with 100 ml sterile distilled water in a warring blender, filtered through muslin cloth and then centrifuged at 4000*g* for 30 min. The supernatant was filtered through Whatman No. 1 filter paper and subjected to antifungal activity assay using poisoned food technique by amending SDA medium with 10% aqueous extract (Mohana and Raveesha, 2006).

Extraction and characterization of Chitosan from *Aspergillus niger.* The seven-day old culture of *Aspergillus niger* was inoculated in potato dextrose broth and incubated at 28±0.8 °C for 15 day. Mycelial growth harvested by centrifugation, washed with double distilled water and ho and then homogenized with 2N NaOH at 100 °C for 1 h. The alkali insoluble fraction was separated, washed and neutralized with 2% acetic acid (v/v). Subsequently it was washed with ethanol (95%) and finally with acetone. Lastly it was dried in vacuum oven at 50 °C and weighed (Nadarajah *et al.,* 2001). The obtained samples were subjected for characterization, UV Spectrophometer, FTIR analysis and SEM analysis used for the further antimicrobial assay.

B. Characterization of fungal chitosan

UV-Spectrophometer and Fourier transform infrared (FTIR) spectroscopy. The UV-visible spectrum of obtained sample was analyzed using UV–visible spectroscope (UV-1800, Shimadzu). FTIR analysis (Shimadzu IRAffinity-1S) was used to characterize the bonding characteristics of the fungal chitosan. Samples were mixed uniformly with potassium bromide in 1:10 proportion and spectral scanning was done in the range of 4000–400 cm^{-1}.

Scanning electron microscopy and energy dispersive X-ray analysis (EDX). The obtained F*Ch* was determined using SEM (Hitachi). The lyophilized samples of small aliquots were mounted on metal stubs. The stub was then coated with conductive gold with a sputter coater attached to the instrument. The photographs were taken using a scanning electron microscope (SEM Hitachi SU 3500) under a magnification of 15000X and also analyzed by the elements present in the compounds.

C. Antimicrobial assay

Antibacterial assay. Antibacterial activity of FC and *P. longfolia* were determined by disc diffusion method on the Muller-Hinton agar (MHA) medium following the procedure of National Committee for Clinical Laboratory Standards (NCCLS, 2000). One hundred µL of each bacterial inoculum (10^8 CFU/mL) was spread separately on the MHA plates using sterile moistened swab. Then, disc of 5 mm diameter separately impregnated with F*Ch* and *P. longfolia* (20 µg/ disc) were placed on the pre inoculated MHA plates and incubated at 37 °C for 24 hrs. The same amount of sterilized distilled tween 20 served as a control. The diameters of zone of inhibition (ZOI) around the wells were measured in millimeter (mm). For each treatment three replicates were maintained.

Antifungal assay. Antifungal activity of F*Ch* and *P. longfolia* solution was determined by poisoned food technique. The extracts were separately added to the sterile petri dishes contains PDA medium. After

complete solidification of the medium, 5mm disc of 7 day old culture of the test fungi was placed at the center of agar surface. The experiment was carried out in three replicates. The petri dishes containing medium devoid of the extract served as control. The plates were incubated at 28±2°C for 7 days (Roshan *et al.,* 2021). The fungi toxicity of the extract in terms of percentage inhibition of mycelial growth was calculated using the formula

$$\text{Percent mycelial inhibition (\%)} = \frac{dC - dT}{dC} \times 100$$

Where, dC = average mycelial growth; dT = average mycelial growth in treatment.

RESULTS AND DISCUSSION

Chitosan synthesis involves the various chemical steps such as preparation of chitin from mycelial mat, there are removal of proteins in this shells followed by demineralization for the removal of the carbon and other salts present in the crude form which will be preceded by the deacetylation of the chitin that would result in chitosan. In this present studies the extracted compounds was white in color and its average yield was 0.986g/l after 13 days of incubation (Fig. 1). Similar studies were carried out by Maghsoodi *et al.* (2009) investigated the effect of different nitrogen sources and effect of glucose supplementation on chitosan produced by *A. niger*.

The isolated *Ch* was subjected to identify the compounds using UV-visible spectroscopy, FT-IR, and SEM coupled with EDX analysis. The results showed that the FT-IR, the characteristic peaks of F*Ch* shows at 3435 cm^{-1}, 2924 cm^{-1}, 1645 cm^{-1}, 1412 $cm^{-1}$and 1021 cm^{-1} attributing towards O-H strech, C-H strech, N-H bend C-H bend-C-N strech. Due to some interaction between acetic acid and nitrogen donors of the *Ch* polymer the amine band has shifted to 1566 NH_2-deformation (Kaneko, 1997). These signaling peaks confirm the extracted compound was Chitosan. Further, the characteristic peak at 191 nm obtained from spectrophotometric evaluation confirms the presence of Ch (Fig. 2 and 3). The microscopic view of F*Ch* was also observed through SEM coupled with EDX was showed F*Ch* was smooth surface (Fig. 4a and Fig. 4b). In case of previous data reports also represented that F*Ch* has crystal structure and smooth surface (Johney *et al.,* 2017; Tayel *et al.,* 2010).

Fig. 1. *A. niger* growth and mycelial mat formation on PDB broth.

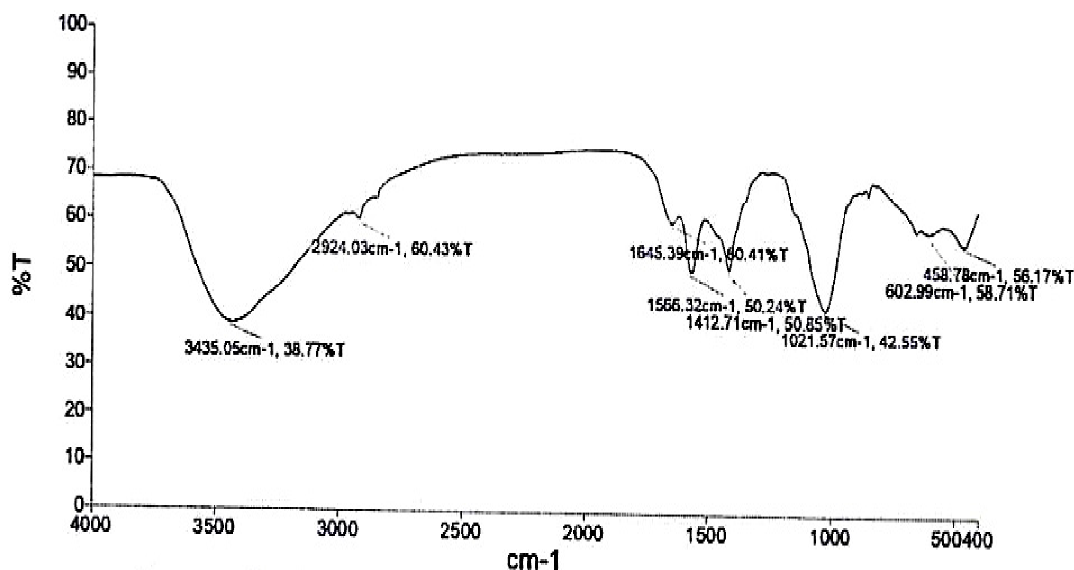

Fig. 2. Fourier transform infrared spectrum of fungal chitosan extracted from *A. niger.*

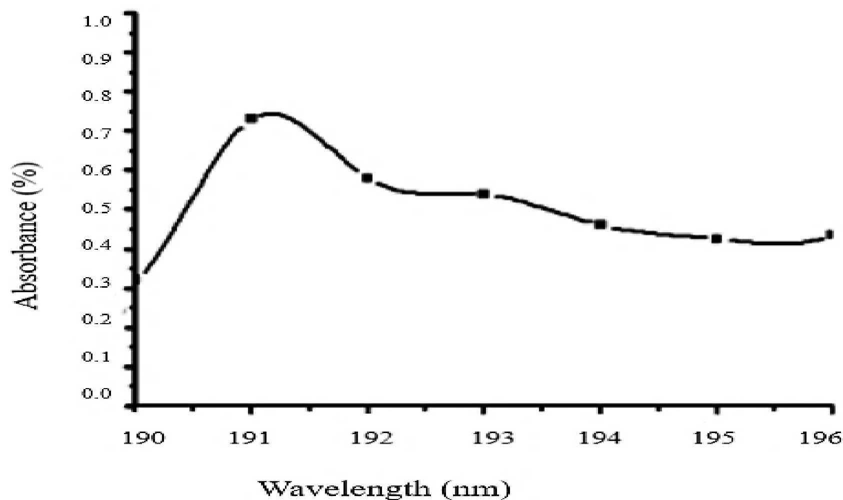

Fig. 3. UV-spectroscopic spectrum of F*Ch* extracted from *A. niger*.

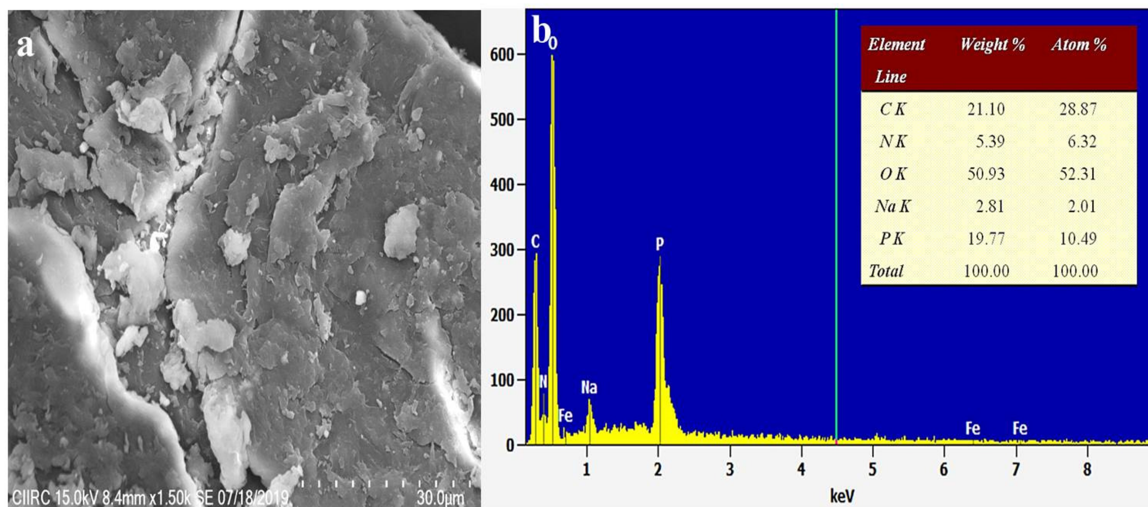

Fig. 4 a. Scanning electron microscopy images of fungal Chitosan. **b.** Energy dispersive X-ray analysis of F*Ch*.

The antimicrobial activity of multidrug resistant bacteria and fungi received more considerable attention in recent years. The interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. The antimicrobial action of aqueous extract of *P. longifolia* zone of inhibition was ranges from 10.6±0.1 to 12.6±0.3mm at 0.5 to 10mg/ml concentrations. Similarly, Thenmozhi and Sivaraj (2010) stated that the antibacterial activity of *P. longifolia.* Further, the antimicrobial studies of F*Ch* show 11.6±0.5 to 18.2±0.3mm (Fig. 5).

In our study the antimicrobial action of combinatorial studies *FCh* and *P. longifolia* encouraging results against bacteria and fungi at 0.5 to 10mg/ml concentrations. The zone of inhibition was ranged between 15.6±0.2 to 21.3±0.4 mm (Table 1). The highest zone of inhibition was observed *P. aeuroginosa, K. pneumonia* when compared with the *E. coli* and *S. aureus* (Johney *et al.,* 2016).

Table 1: Antibacterial activity against some human pathogens.

Test Bacteria	ZOI (mm)		
	P. longifolia	**F*Ch***	***P. longifolia* + F*Ch***
E. coli	10.6±0.1	11.6±0.5	15.6±0.2
S. aureus	11.3±0.1	12.3±0.4	16.3±0.4
K. peunominae	12.0±0.4	15.6±0.5	21.6±0.5
P. aerugenosa	12.6±0.3	18.2±0.3	21.3±0.4

Note: ZOI- Zone of inhibition values are expressed in mg/ml. *P. longifolia*- Aqueous extract of *P. longifolia*. F*Ch*- Fungal chitosan, *P. longifolia* + F*Ch*- Aqueous extract of *Polyalthia longifolia*+ fungal chitosan. Data given are the mean values of three replicates mean ± standard error ($p \leq 0.05$)

The combinatorial studies *FCh* and *P. longifolia* showed highest percent mycelilal inhibition against *A. flavus* and *F. verticillioides*. The percent mycelial inhibition of *A. flvaus* was 87.6±0.4 % and *F. verticillioides* 97.6±0.5% at 0.5 to 10mg/ml concentrations (Table 2; Fig. 6).

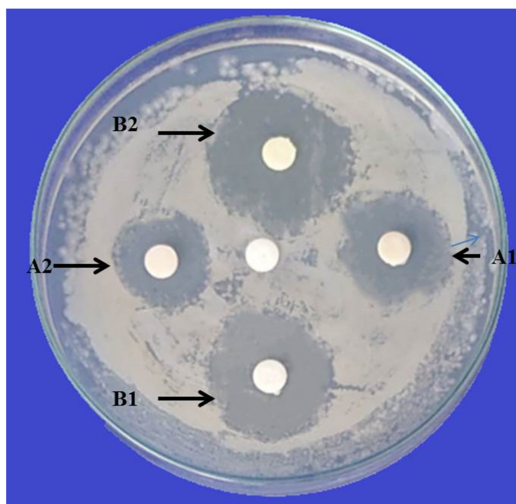

Fig. 5. Antibacterial activity of A1 & A2 represents F*ch* and B1 & B2 *P. longifolia* and F*Ch*.

Table 2: Antifungal activity against *A. flavus* and *F. verticillioides*.

Test fungi	Percent of mycelial inhibition(%MI)		
	P. longifolia	**F*Ch***	***P. longifolia* + FCh**
A. flavus	38.3±0.3	74.3±0.4	87.6±0.4
F. verticillioides	40.3±0.4	80.3±0.4	97.6±0.5

Note: %MI-percent mycelial inhibition values are expressed in mg/ ml. *P. longifolia*- Aqueous extract of *P. longifolia*. F*Ch*- Fungal chitosan, *P. longifolia* + F*Ch*- Aqueous extract of *P. longifolia* + fungal chitosan. Data given are the mean values of three replicates mean ± standard error ($p \leq 0.05$).

Fig. 6 Antifungal activities of *F. verticillioides* (*P. longfolia* + F*Ch*).

CONCLUSION

In this study the chitosan was extracted from *A. niger*. The yield after 13th day of incubation. The UV-visible spectrophotometer, FTIR, SEM coupled with EDX also confirmed that chitosan. The extracted fungal chitosan was subjected to antimicrobial activity, and it was found the fungal chitosan were active against the bacteria and fungi. Our finding suggests that the *A. niger* is the potential candidate to produce eco-friendly chitosan in the development of drugs and food industries. Future prospective of the study may be extended in the following future directions. Apart from aqueous extract of *P. lonifolia,* the major components and its combition of F*Ch* used as antimicrobial agent. Further, the preparation of fungal chitosan and its combination of *P. lonifolia* can be evaluated at nanolevel. The F*Ch* and its combination of other plant extracts, their components can be studied at nanolevel used as antimicrobial agent.

Declaration of competing interest. No conflicts of reports were reported by the authors.
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