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PRODUCTION, OPTIMIZATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUND FROM *ASPERGILLUS* SP

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ABSTRACT

Fungi have been reported to be active producers of secondary metabolites. In this study, a fungal isolate (*Aspergillus* sp) isolated from soil has been evaluated for its antimicrobial activity. The activity was studied under various physio-chemical parameters, such as pH, temperature, incubation period, carbon and nitrogen sources. The best antimicrobial activity was observed in the production medium having pH 5-7, on fifth day of incubation at 25 ºC when grown as static culture. Starch was the most promising carbon source, while yeast extract and soyabean meal acted as best nitrogen sources. Butanolic extract was comparable to standard antibiotics in contrast to aqueous extract. Response surface analysis showed that the antimicrobial activity was enhanced by 1.25 folds in *S.aureus*, 1.28 folds (*S.epidermidis*), 1.6 folds (*K.pneumoniae* 1), 1.37 folds (*C.albicans*), 1.38 folds (*MRSA*). Characterization of the purified compound responsible for antimicrobial activity was carried out by various analytical procedures i.e. TLC, HPLC, NMR and IR. MIC of the butanolic extract ranged from (0.016mg/ml-18mg/ml) while purified compound exhibited lower MIC value of 6µg/ml, 20 µg/ml and 20 µg/ml respectively for *S.epidermidis*, *C.albicans* and *MRSA*. VCC (Viable cell count) studies revealed *E.coli* to be the most sensitive and demonstrated 100% killing at 0 hr. Butanolic extract (crude) and the purified compound were found to be neither cytotoxic nor mutagenic.

Key words: *Aspergillus*, Antimicrobial, Minimum inhibitory concentration, Viable cell count, Bioactive compound, Resistance.

INTRODUCTION

Microbial populations are the most promising sources of natural secondary metabolites. Fungi produce a wide range of such products with therapeutic value as antibiotics, along with many other bioactive compounds of pharmaceutical and agricultural use. Many Aspergilli have been reported to be the active producers of different secondary metabolites with various bioactivities such as antiviral, antimicrobial, tumor suppressing, antihypercholesterolemic and immunosuppressant activities [1]. Soil holds an enormous biodiversity that can be screened for various bioactive metabolites. Number of fungi showing different biological activities have been listed in the literature, still a lot remains untapped from diverse soil habitats. Further, bacterial resistance is spreading throughout the world, revealing the steadily decreasing potencies of prevalent antibiotics [2]. Strains of methicllin resistant *Staphylococcus aureus* have increased to an alarming number leading to many infectious diseases, so there is a need to explore and expand the spectrum of suitable organisms yielding novel antimicrobials. Keeping this in mind, the present study was designed to screen the fungi from soil collected from different areas of Punjab (30˚ 4’ N 75˚ 5’ E) for their antimicrobial activity. One such promising isolate, was perused further for optimization of various physiochemical parameters to enhance the production of antimicrobial agent/s. Minimum inhibitory concentration and viable cell count has been carried out to check the bacteriostatic or bactericidal nature of the butanolic extract of *Aspergillus* sp. Purification

of the compound was carried out by various analytical techniques and toxicity testing was done by AMES mutagencity test and MTT assay.

MATERIALS AND METHODS

Fungal isolation and extract preparation: The organism used for study was isolated from soil and maintained by standard procedures. It was identified by National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India and found close to be *Aspergillus wentii* gr, where it has been deposited with vide accession no (NFCCI 2565). The GenBank accession no of the sequence reported in this paper is KF039712. The isolate was grown in YPDS medium and the filtrate obtained was used for testing antimicrobial activity [3].

Test organisms: The reference strains of bacteria and two yeasts, used for testing their sensitivity to fungal extracts, were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and the clinical isolate methicillin resistant *Staphylococcus aureus* (MRSA) was obtained from Post graduate Institute of Medical Education and Research, (PGIMER), Chandigarh, India. Reference strains included Gram positive bacteria –*Enterococcus faecalis* (MTCC439), *Staphylococcus aureus* (MTCC740), *Staphylococcus epidermidis* (MTCC435). Gram negative bacteria *Escherichia coli* (MTCC119), *Klebsiella pneumoniae* 1 (MTCC109), *Klebsiella pneumoniae* 2 (MTCC530) *Pseudomonas aeruginosa* (MTCC741) , *Salmonella typhimurium*1 (MTCC-98), *Salmonella typhimurium2* (MTCC1251), *Shigella flexneri* (MTCC1457) and two yeast strains viz *Candida albicans* (MTCC227), *Candida tropicalis* (MTCC230) .The bacterial cultures were maintained on nutrient agar slants, except *Enterococcus faecalis* which was maintained on Trypticase soya agar . *Candida albicans* and *Candida tropicalis* were respectively maintained on yeast malt agar and Sabouraud agar.

Inoculum preparation: A loopful of isolated bacterial and yeast colonies were inoculated into 5ml of their respective medium and incubated at 37˚C and 25˚C respectively for 4h. This was used as inoculum after adjusting the turbidity as per Mc Farland turbidity standard. This turbidity is equivalent to approximately 1 to 2×10^8 colony forming units per ml (CFU/ml). The inoculum thus prepared was used further for further testing [3].

Determination of fungal biomass: The dry weight of the fungal mycelium was measured after repeated washing of the mycelium with distilled water and drying overnight at 70˚C to a constant weight.

Screening the fungal extract for antimicrobial activity: The fungal extract was tested against different microbial cultures grown on their respective media by agar well diffusion assay. The plates were incubated at 37˚C and 25˚C for 24h and diameter of resultant zone of inhibition was measured. Experiments were run in duplicate for each combination of extract and microbial strains.

Optimizing the physiochemical parameters and media components: To optimize various physiochemical and nutritional conditions, the aqueous extract obtained after growing the fungi under various physiochemical parameters, were tested for their respective antimicrobial activity by agar well diffusion method along with thermostability studies of the fungal extract [3].

Effect of different carbon and nitrogen sources: To study the effect of different carbon and nitrogen sources ,total carbon sources i.e. dextrose and starch in YPDS medium were replaced by a single carbon source (dextrose, starch, sucrose, lactose or maltose) at a concentration of 1%, Similarly, to work out the effect of nitrogen sources in the YPDS medium, yeast extract and peptone were replaced with one or the other nitrogen rich sources (sodium nitrate, yeast extract, soybean meal, potassium nitrate, ammonium chloride, ammonium sulphate, ammonium dihydrogen phosphate, ammonium nitrate, peptone, malt extract, urea and casein) at a concentration of 1%.

Fractionation of extracts: The fungal extract obtained was extracted with different solvents viz diethyl ether, chloroform, butanol, hexane and ethyl acetate as described previously [3].

Comparison of antimicrobial activity of butanolic extract with some standard antibiotics: In order to compare the effectiveness of the extract, their activity was compared with some standard antibiotics by agar diffusion method.

Statistical optimization of the medium: On the basis of results obtained from screening of different carbon and nitrogen sources through one-factor-at-a-time classical method; starch, dextrose and yeast extract were taken independent variables for the optimization by Response Surface Methodology (RSM) using Box-Behnken design of experiments. Each variable was studied at three levels (-1, 0, +1); for starch and dextrose these were 0, 1% and 2%, while for yeast

extract it was 0, 0.4% and 0.8% **.**The experimental design included 17 flasks with five replicates having all the three variables at their central coded values. The mathematical relationship of response G (for each parameter) and independent variable X (X_1, dextrose; X_2, Starch; X_3, Yeast extract) was calculated by the following quadratic model equation [4].

$$G = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

Where, G is the predicted response; β_0, intercept; β_1, β_2, and β_3, linear coefficients; β_{11}, β_{22} and β_{33}, squared coefficients and β_{12}, β_{13} and β_{23} interaction coefficients. MINITAB statistical software was used to obtain optimal working conditions and generate response surface graphs. Statistical analysis of experimental data was also performed using this software.

Minimum inhibitory concentration **(MIC):** Minimum inhibitory concentration of the butanolic extract was worked out by agar dilution method [5], with final concentrations ranged from (0.016mg-18mg/ml). The experiment was performed in duplicate and repeated three times.

Microbicidal activity: Microbicidal activity of the butanolic extract of *Aspergillus* sp was measured by viable cell count studies [6], with a stock solution of (100mg/ml). The mean number of colonies were determined and compared with that of control in which the butanolic extract was replaced with DMSO.

Extraction and purification of active components from butanolic extract of Aspergillus **sp:** For the extraction and purification of active group/component from *Aspergillus* sp, 3 litres of the culture broth was extracted with equal volume of butanol (1:1) .The organic layer was separated and treated with Na_2SO_4 and then evaporated to dryness in vaccuo and the resulting solids (3g) were subjected to column chromatography using silica gel (100-200 mesh size, column 18mm×300mm) packed and pre-equilibrated with hexane. The column was first eluted with equilibration solvent i.e. hexane (2 bed volumes) followed by linear gradients of Hexane : Butanol (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100) at a flow rate of 1ml/min. A total of 65 fractions were collected and the fraction size kept to 20ml. Each fraction after concentration was subjected to thin layer chromatography and agar disc diffusion assay. Butanol: Hexane (9:1) was used as screening system to develop the chromatograms which were observed under UV light (254 and 365nm) and in iodine chamber. Fractions which showed similar TLC pattern were pooled, concentrated and again loaded to silica gel (100-200 mesh size), column (10mm×300mm) and pre-equilibrated with hexane (2 bed volumes). The column after elution with hexane was eluted with linear gradients of Butanol: Hexane (as above) and fraction size reduced to 5ml. The collected fractions were tested for antimicrobial activity, thin layer chromatography and HPLC.

HPLC Analysis: The purified fraction was subjected to high pressure liquid chromatography (HPLC) using Dionex P680 HPLC. Acetonitrile (75% aqueous solution) was used as mobile phase at a flow rate 0.3ml/min and injection volume was 20µl at a column temperature of 25 ºC. The detections were monitored at different wavelengths (225,250,275 and 300nm). NMR spectroscopy (H^1 and C^{13}) was outsourced from Punjab University, Chandigarh and the data were recorded on 400 MHz Bruker. IR spectrum of the purified compound was recorded on varian 660-IR Fourier Transform Infra-Red Spectrometer. UV spectrum was measured on Shimadzu UV mini 1240 UV-visible spectrophotometer.

Safety evaluation: Butanolic extract of the fungus and the purified compound were subjected to Ames test to evaluate their mutagenicity [7] by using *S.typhimurium* (MTCC 1251, IMTECH, Chandigarh), while their toxicity was determined by MTT (3-(4, 5-di methylthiazole-2yl)-2, 5-di phenyl tetrazolium bromide) assay. The absorbance was measured at 570 nm using an automated microplate reader (Biorad 680-XR_1 Japan). The wells with untreated cells served as control [8].

RESULTS

On the basis of colony characteristics and microscopic morphology, the isolated fungus was identified as *Aspergillus* sp. close to *A. wentii* gr. (HT-113) by Agharkar Research Institute, Pune. The purified amplified internal transcribed spacer (ITS) region was sequenced by single primer analysis (SPA) services (Genei, Bangalore, India). The ITS sequence was deposited with NCBI gene bank with access No (KF039712). The fungus grown on Yeast extract glucose agar medium at 25˚C for 8 days. The aqueous extract of the selected fungal isolate showed antimicrobial activity against four bacteria and one yeast. *MRSA* was the most sensitive organism with a inhibition zone of 27mm followed by *C. albicans* (26mm) *S.epidermidis* (25mm), *S.aureus* (24mm) and *K.pneumoniae* 1 (14mm).

Effect of incubation period and other growth conditions: Maximum biomass (8.34mg/ml) and antimicrobial activity against most of the organisms was found on day 5 which remained more or less stable upto day 9. *MRSA* was the most sensitive organism with inhibition zone of 28mm, followed by *C. albicans*, *S. aureus*, *S. epidermidis* and *K. pneumoniae* 1. Further experiments were carried out for 5 days incubation period. The antimicrobial activity was better expressed under stationary conditions as compared to shake flask cultures. With increase in revolutions per minute (RPM), fungus showed a reasonable decline in its biomass as well as antimicrobial activity against all the five microorganisms. Thus, further experimentation was carried out under static conditions only. Maximum antimicrobial activity and fungal biomass was recorded at 25˚C while the fungus was not able to grow above 30˚C. The antimicrobial activity was completely lost on heating the aqueous extract at 100˚C for 1hr whereas up to 60˚C it suffered a moderate loss in activity (4-20 %) and with a further loss in activity at 80˚C. (Fig.1). Relatively low antimicrobial activity was expressed at pH 3- 4 which increased from pH-5 and remained more or less stable upto pH 7 and declined, subsequently indicating the pH optima between pH 5-7.

Effect of carbon and nitrogen sources: Starch was the best carbon source and the order of antimicrobial activity for different carbon sources was starch > dextrose > lactose > maltose>sucrose .Maximum biomass was recorded in the presence of maltose, however, the antimicrobial activity exhibited by both maltose and sucrose was not statistically significant at 5% confidence level. Analysis by fischer's LSD procedure indicated starch containing media to be the best amongst the other the carbon sources tested. (Fig.2). similarly, yeast extract was the best among organic and inorganic nitrogen sources. Soybean meal and peptone were also good sources of nitrogen for bioactivity while fungal biomass was maximum in the former medium. The fungus was not able to grow in the presence of urea. However, other nitrogen sources showed variable biomass without supporting any antimicrobial activity.

Effect of media components and their concentration

In experimental setup I, the maximum antimicrobial activity was observed at 1% dextrose which declined appreciably at 4% and showed no activity at all at 6% against any bacteria except the yeast strain which showed only a moderate decline up to 10% dextrose. Similarly, in set up II, maximum biomass was obtained at the highest concentration of starch i.e. 10% while the best antimicrobial activity was observed at 1% starch which declined with further increase in the concentration. In the third set of experiment, 1% peptone gave the best antimicrobial activity which was more or less stable upto 2%. Similarly, when the fungus, was grown in setup (IV), the antimicrobial activity was maximum at 1% yeast extract and decreased with increase in its concentration. Biomass of the fungus increased with increase in concentration of nitrogen sources. Thus, to work out the combined effect of carbon and nitrogen sources, these were respectively tested at 1% concentration.

Box-Behnken design for statistical optimization of carbon and nitrogen sources

Fitting the model

The data obtained from quadratic model equation was found to be significant. It was verified by F value and the analysis of variance (ANOVA) by fitting the data of all independent observations in response surface quadratic model. The results for model *F*-value implies that the model is significant which indicate it to be suitable to represent adequately the real relationship among the parameters used. R^2 value for all the responses ranged between 90 -95.1%, which showed suitable fitting of the model in the designed experiments. The final predictive equations for each response *S.aureus* (G_1), *S.epidermidis* (G_2), *K. pneumoniae*1 (G_3), *C. albicans* (G_4), *MRSA* (G_5) obtained are as follows

(G_1)

$25+2.75X_1+6.125X_2+0.125X_3-2X_1^2-1.25X_2^2-6.75X_3^2-0.25X_1X_2+0.25X_1X_3-1X_2X_3$

(G_2)

$26+2.75X_1+6.125X_2+0.125X_3-1.80X_1^2-1.05X_2^2-6.55X_3^2-0.25X_1X_2+0.25X_1X_3-1X_2X_3$

(G_3)

$16.6+1X_1+4.375X_2+0.875X_3-0.05X_1^2+1.20X_2^2-2.80X_3^2+0.25X_1X_2-0.25X_1X_3-1.5X_2X_3$

(G_4)

$30+2.75X_1+7.25X_2-0.25X_3-0.75X_1^2-1.75X_2^2-7.75X_3^2-0.50X_1X_2-0.0X_1X_3-0.50X_2X_3$

(G_5)

$29.2+2.625X_1+7.75X_2-0.375X_3-0.60X_1^2-1.35X_2^2-7.1X_3^2-0.75X_1X_2-0.0X_1X_3-0.75X_2X_3$

The optimized values for factors were validated by repeating the experiment in triplicates

1) (G_1) Linear effect of starch (X_2) and squared effect of yeast extract(X_3^2) was highly significant with P value $\leq$ 0.005 similarly linear (X_1) and squared effect(X_1^2) of dextrose was significant with P value $\leq$ 0.05 and $\leq$ 0.5 respectively. The response surface graph showed highest activity at Dextrose 1%-2%, Starch 2% and Yeast extract 0.4% (Fig.3a).

2) (G_2) Similarly linear effect of starch (X_2) and squared effect of yeast extract (X_3^2) was highly significant with P value ≤ 0.005. Linear effect of dextrose (X_1) and squared effect of starch (X_2^2) was significant with P value ≤ 0.05 and ≤ 0.5 respectively. Highest activity was found with Dextrose 1%-2%, Starch 2% and yeast extract 0.4% (Fig.3b).
3) (G_3) Linear effect of starch (X_2) was highly significant with P value ≤ 0.005.Squred effect of yeast extract (X_3^2) and starch (X_2^2) was also significant with P value ≤ 0.05 and ≤0.5 respectively. Linear effect of dextrose (X_1) and yeast extract (X_3) significant with and P value ≤0.5 respectively. The response surface graph showed highest activity at 2% Starch, 2% Dextrose and Yeast extract 0.4% (Fig.3c).
4) (G_4) Linear effect of starch (X_2) and squared effect of yeast extract (X_3^2) was highly significant with P value ≤ 0.005. Linear effect of dextrose (X_1) and squared effect of starch (X_2^2) was significant with P value ≤ 0.05 and ≤0.5 respectively. The response surface graph of (G_4) showed highest activity at Starch 2%, Dextrose 2%, Yeast extract 0.4% (Fig.3d).
5) (G_5)Linear effect of starch (X_2) and squared effect of yeast extract (X_3^2) was highly significant with P value ≤ 0.005.Similarly linear effect of dextrose (X_1) and squared effect of starch (X_2^2) was significant with P value ≤ 0.05 and ≤ 0.5, respectively. Thus the highest activity was expressed at Starch 2%, Dextrose 2% and Yeast extract 0.4%. (Fig.3e).

Validation of Results

Thus from the overall assessment 2% Dextrose, 2% starch and 0.4% yeast extract and (1%) peptone in YPDS medium may be regarded as the optimized conditions for antimicrobial activity The F value and R^2 value showed that the model correlated well with measured data and was statistically significant. To confirm the adequacy of the model the verification experiments using optimum medium composition as described above were carried out in triplicates. The results showed the antimicrobial activity was enhanced by 1.25 folds in *S.aureus*, 1.28 folds (*S.epidermidis*), 1.6 folds (*K.pneumoniae* 1), 1.37 folds (*C.albicans*), 1.38 folds (*MRSA*).

Solvent extraction and antibacterial activity: Antimicrobial activity of different solvents indicated that chloroform, ethyl acetate, petroleum ether, hexane and aqueous extract were not significantly different at 5% confidence level with an inhibition zone range of 11mm to 30mm. Butanol demonstrated to be the best extractant with inhibition zone range of 14mm to 43mm for all test organisms (Fig 4). DMSO was used as negative control and it, did not show any activity. *S.aureus*, *S.epidermidis* and *K. pneumoniae*1 were the most sensitive and the inhibitory zone ranged from 24 to 43mm. Similarly, methicillin resistant. *S aureus* gave a maximum zone of inhibition (29mm). Hexane extract was least effective and inhibited only three microorganisms i.e. *K.pneumoniae* 2, *MRSA* and *C. albicans*. *K.pneumoniae* 2 which was found resistant to aqueous extract was found to be sensitive to chloroform , butanol, petroleum ether and hexane extract. As, butanol was found to be the best organic solvent responsible for antimicrobial activity in terms of zone size as well as number of sensitive microorganisms (Fig 4.), so it was used for further studies.

Comparison of antimicrobial activity of butanolic extract of Aspergillus sp with some standard antibiotics: Butanolic extract of *Aspergillus* sp exhibited better activity against *S.aureus*, *K. pneumoniae* 1, *MRSA* and *C. albicans*, better than even standard antibiotics. *E. faecalis* which was resistant to most of the antibiotics was sensitive to butanolic extract with an inhibition zone of 27mm. *E.coli* resistant to co-trimaxazole and ampicillin gave 22mm zone of inhibition. *MRSA* was resistant to many antibiotics but was sensitive to the butanolic extract. (Table 1)

MIC of butanolic extract of Aspergillus sp: The MIC values were strain dependent. MIC of butanolic extract ranged from 0.016mg-18mg/ml. *K.pneumoniae* 1 and *S.epidermidis* were found to be most sensitive and inhibited at (0.016mg/ml) followed by *S aureus* (0.1mg/ml), *C.albicans* (0.5mg/ml), *E. faecalis* and *MRSA* (1mg/ml) *S.typhimurium* 2 and *E.coli* (5mg/ml), *Sh.flexneri* (6mg/ml) while *P.aeruginosa* and *S.typhimurium* 1 gave the highest MIC values (18mg/ml).

Viable cell count: On the basis of MIC of butanolic extract obtained for different organisms they were subjected to viable cell count studies. Complete killing of *E.coli* was observed at 0 hr. *S.epidermidis*, *S.typhimurium* 2 and *MRSA* were killed at 4hrs while *S.aureus* took 6hrs for complete killing. *S.typhimurium* 1 took the longest time and showed 99.5% killing at 12 hr while the residual cells restarted their growth after 12 hrs (Fig.5).

Purification of the active compound: All the fractions obtained from column chromatography were subjected to antimicrobial testing by agar diffusion assay. Sixty such fractions were subjected

to TLC and the active fractions (fraction no 40-50) were effective against *S.epidermidis* ,*K.pneumoniae*1, *MRSA* , *C.albicans*, *S.typhimurium* 2 with inhibition zone ranging from 18 -25mm . The fractions having the same R_f values (0.8 cm) were pooled and again loaded on to column. In the second column with total 55 fractions, fraction no 20 to 29, having same R_f values (0.7cm) showed antimicrobial activity (18 to 20mm) were again pooled and concentrated for further checking their activity and TLC. One single band was observed on TLC under UV-225 and iodine chamber with R_f value (0.7cm) (Fig.6a, 6b). The active fraction was subjected to HPLC analysis and the single peak of the compound indicates its purity (Fig.7). The retention time for the compound was 8.924 min which were further analyzed for NMR, and IR. MIC of the compound obtained was found to be 6 µg/ml with *S.epidermidis*, 20 µg/ml with *C.albicans* and 20 µg/ml with *MRSA*. The UV spectrum of the fraction demonstrated maximum absorbance at 210nm (Fig.8). ^{1}H NMR of the isolated compound showed peaks in the range of 7.9-6.8ppm that correspond to aromatic groups. ^{1}H NMR showed some resonance at alkenic region indicating the presence of some alkenic proton in unknown compound. In addition aliphatic region was also observed from 2.3-1.5ppm. The presence of –OH group is indicated from the broad peak observed at 8.6ppm. (Fig. 9). ^{13}C NMR also showed the peak at δ 170.9 which may be due to (C=O) group which was further confirmed by its IR spectrum that showed carbonyl stretching at 1668 cm^{-1} (Fig.10). ^{13}C NMR of the compound showed the presence of both aliphatic and aromatic region which ranged from 68.05-21.44 and 165.2-127.5 respectively. The IR spectrum of the compound showed band at 3238.06 cm^{-1} indicating the presence of –OH group. The bands corresponding to C=O, C-O and C=C were observed at 1668.9, 1437.8 and 2928.2 cm^{-1} respectively. Similarly the bands ranging from 1325.2- 964.3 cm^{-1}, confirmed the presence of methyl or methylene group (Fig.11).

Toxicity testing: The numbers of revert ant colonies in the positive control were numerous, whereas the bacteria incubated with the butanolic extract and purified compound isolated from *Aspergillus* sp did not show any revert ant colonies. The glucose minimal agar media plates layered with top agar containing excess of biotin and no histidine also served as control as no colonies were observed. Since, reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) can only occur in metabolically active cells, where MTT is converted to insoluble formazan crystals that are dissolved in DMSO and the absorbance of purple colored solutions directly represents the viability of the cells. In the present studies, 95.4% and 96% of the viable cells were observed in butanolic extract and purified compound respectively showing the extracts to be noncytotoxic.

DISCUSSION

Natural products isolated from different fungi play an important role in human medicine. Each fungal species make its unique set of secondary metabolites, to complement its own biological traits, and a large number of compounds await discovery in *Aspergillus* as a whole. In the present study, *Aspergillus* sp was explored for its potential antimicrobial activity. Optimization studies revealed 5 days incubation period to be optimal for maximum production of antimicrobial activity which is in consonance with earlier studies [9]. The subsequent decline in the antimicrobial activity may be attributed to depletion of nutrients in the medium required for the growth. However, the time course for production of antimicrobial agent differs according to the strain and cultivation conditions; for instance, the maximum antimicrobial agent production was achieved after 4 days of incubation of *Cladosporium* sp [10]. Maximum biomass and antimicrobial activity were best expressed under static culture conditions in consonance with earlier studies [11-13]. Fungal growth at different temperatures revealed 25˚C to be the optimum for growth and metabolite production similar to earlier observations [14-16]. Low temperature may slow down the metabolic activity and high temperature may kill the organism and inactivate the responsible antimicrobial compound [17]. The antimicrobial activity and biomass production was optimally best between pH 6-9. This may be due to better metabolite production under such pH conditions. The pH is related to permeability characteristics of the cell wall and membrane and thus has got effect on either ion uptake or loss to the nutrient medium and may also be governing the extracellular release of requisite metabolites [17]. These observations corroborate the previous studies on *Aspergillus fumigatus* and *Varicosporina ramulosa* [18-19]. Rubini et al. [20] reported the growth and antimicrobial agent production at neutral pH. One percent starch was optimum for antibiotic production which decreased with increase in its concentration while the biomass increased. This might be due to its suppressive effect on production of secondary metabolites and is in consonance with other studies [21]. Other carbon sources that can readily serve as growth substrates, often repress secondary metabolites. During formulation of fermentation medium, polysaccharides or

oligosaccharides are often found to be better than monosaccharide for antibiotic production [22]. In consonance with earlier studies [17, 23-25] yeast extract (2%) was found to be the best nitrogen source for antimicrobial as well as biomass production. Thermostability studies revealed the compound responsible for antimicrobial activity to be quite stable at 50˚C. The application of statistical experiment designs in fermentation process development can result in improvement of product yield and reduce process variability. Moreover, it is an efficient way to generate useful information with limited experimentation, thereby cutting the process development time and cost. Response surface methodology resulted in enhancement in antimicrobial activity ranging from 1.25 to 1.6 folds. A good agreement between the predicted and experimental results verified the validity of the model and the improvement of antimicrobial activity indicated the RSM to be a powerful tool for determining the exact optimal values of the individual factors and the maximum response value. These results are comparable with the antimicrobial activity of other microorganisms such as bacteria, actinomycetes and fungi [26]. Butanolic extract demonstrated the maximum antimicrobial activity including methicillin resistant *S.aureus* and also including those resistant to aqueous extract. Our observations with butanol extract are in consonance with earlier studies [16]. MIC of butanolic extract ranged from (0.016mg/ml- 18mg/ml) and also supported the data obtained by agar well diffusion assay. *S.epidermidis* and *K.pneumoniae* 1(0.016mg/ml) were found to be the most sensitive while *S.typhimurium* 1 and *P. aeruginosa* were least sensitive with highest MIC values of 18mg/ml. It got credence from viable cell count studies in which *S.typhimurium* 1 took the longest time of 12hrs for 99.5% killing and again started growing after 12hrs of incubation. *E.coli* was found to be killed instantly at 0hr. The importance of the study was further highlighted where the butanolic extract showed higher or comparable activity than standard antibiotics. Resistant strains like *MRSA*, found to be resistant to some of the antibiotics was sensitive to butanolic extract of *Aspergillus* sp. This might be due to effective elution of broad spectrum antimicrobial compound. Purification of the compound resulted in one single band as observed on TLC, with HPLC, single peak was detected at retention time 8.924 min. UV –Vis spectra also showed one peak with maximum absorbance at 210nm and purified compound when tested by disc diffusion method, showed a good zone of inhibition against different microorganisms with a MIC value of 6µg/ml, 20 µg/ml and 20 µg/ml respectively for *S.epidermidis*, *C.albicans* and *MRSA* respectively. These results are also comparable with earlier MIC studies [27-28]. This also proved the purity of the compound as MIC of the butanolic extract was higher than the purified compound. The present study get further importance as the purified compound did not show any cytotoxicity or mutagenicity thus validating it as potential antimicrobial agent. The above study suggests *Aspergillus* sp as a potential candidate offering a better scope for the production and purification of broad spectrum antimicrobial compound. These findings will facilitate the further studies to gain better understanding and production of bioactive metabolites by fungi and will be helpful in their biotechnological mass production in near future.

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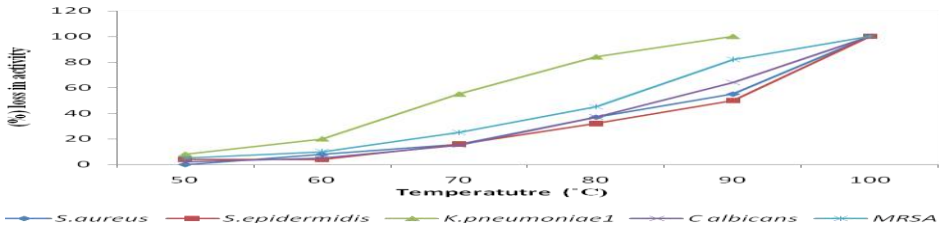


Figure 1. Thermostability of aqueous extract of *Aspergillus* sp

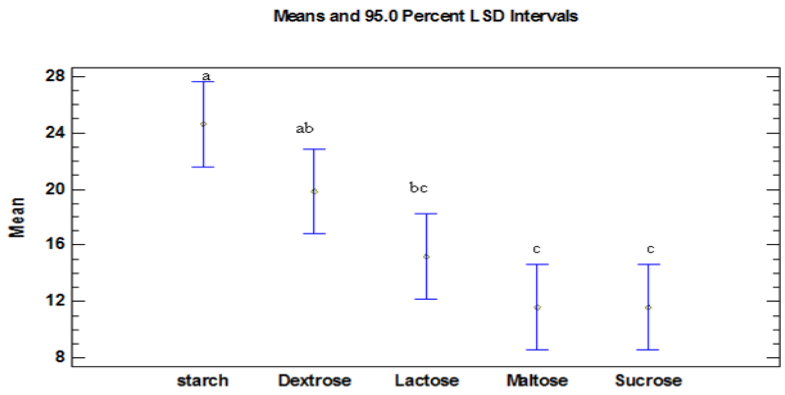
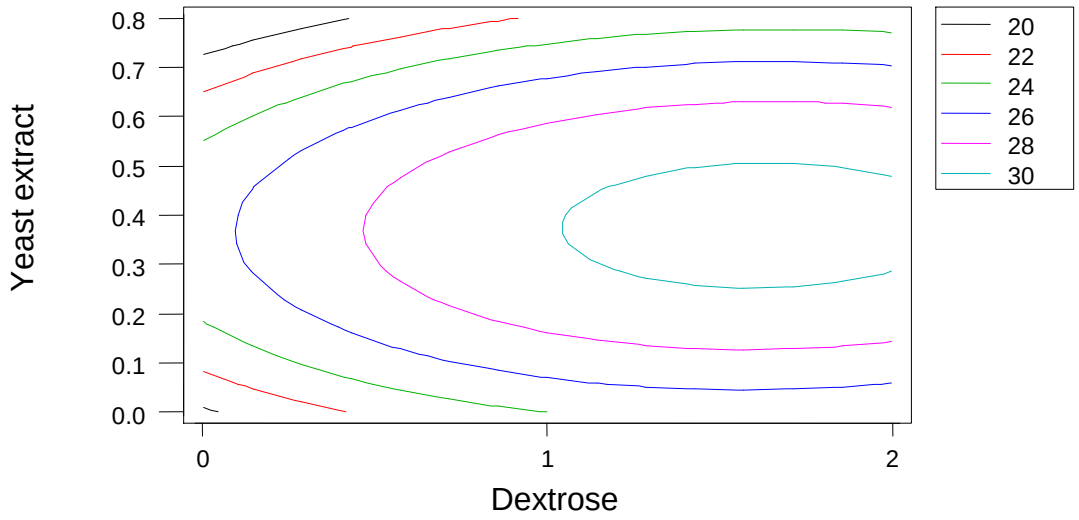
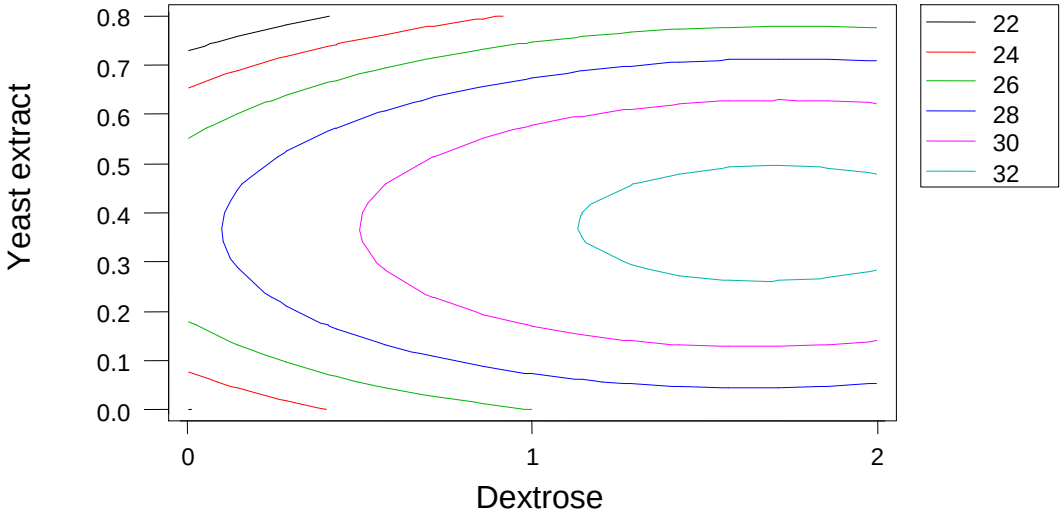


Figure 2. Effect of different carbon sources on antimicrobial activity.



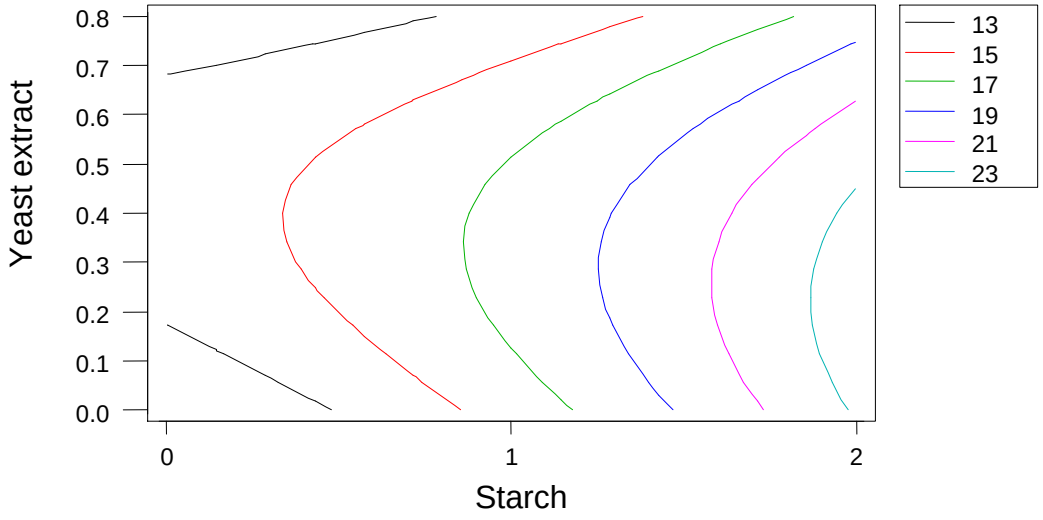
Hold values: starch: 2.0

Figure 3a. Contour Plot of *Staphylococcus aureus*



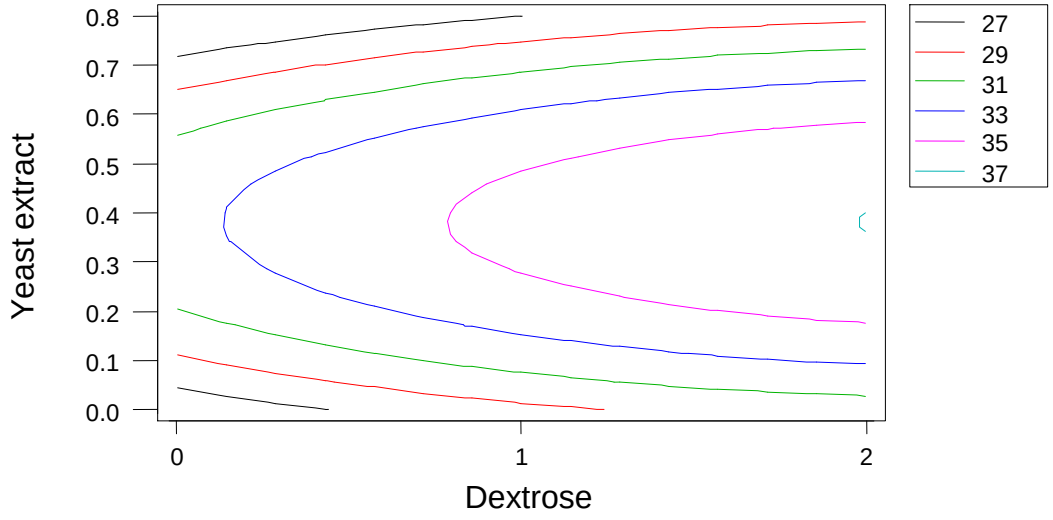
Hold values: starch: 2.0

Figure 3b. Contour Plot of *Staphylococcus epidermidis*



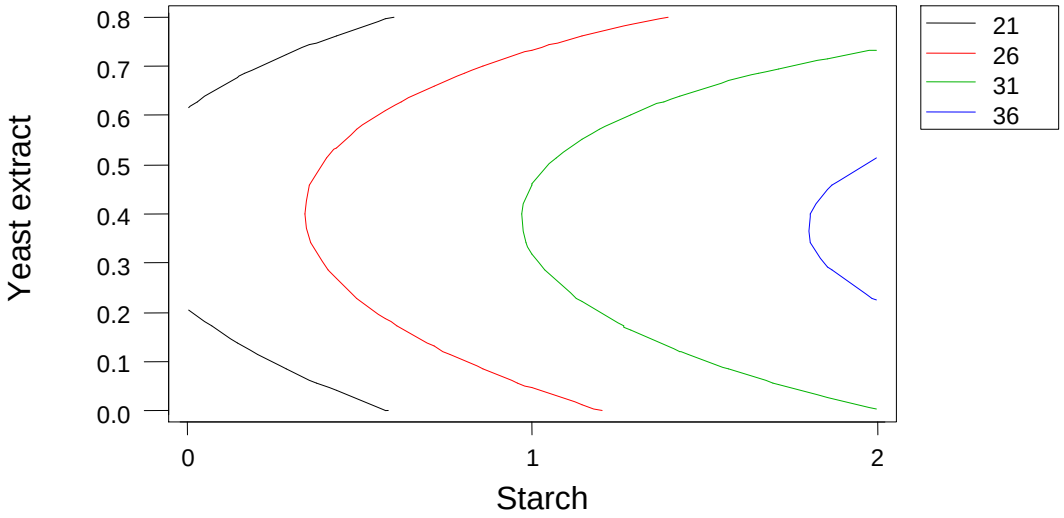
Hold values: Dextrose: 2.0

Figure 3c. Contour Plot of *Klebsiella pneumoniae* 1



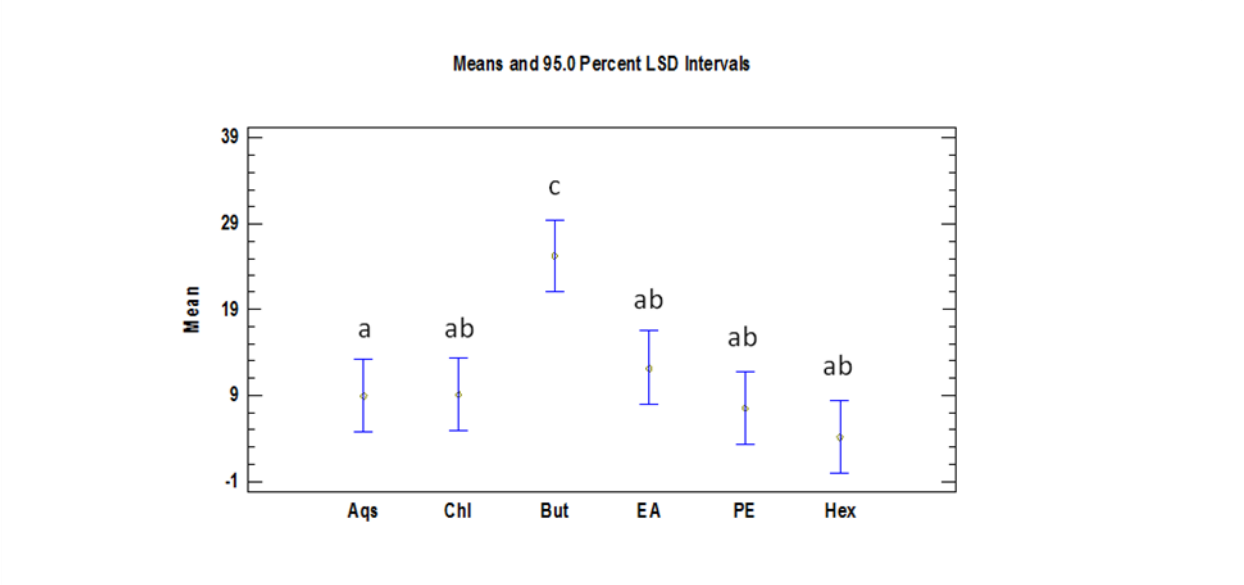
Hold values: starch: 2.0

Figure 3d. Contour Plot of *Candida albicans*



Hold values: Dextrose: 2.0

Figure 3e. Contour Plot of *MRSA*



Aqs-Aqueous extract, Chl-Chloroform extract, But- Butanol extract, EA-Ethyl acetate extract, PE-Petroleum ether extract, Hex-Hexane extract.

Figure 4. Effect of different solvent extracts on antimicrobial activity of *Aspergillus* sp.

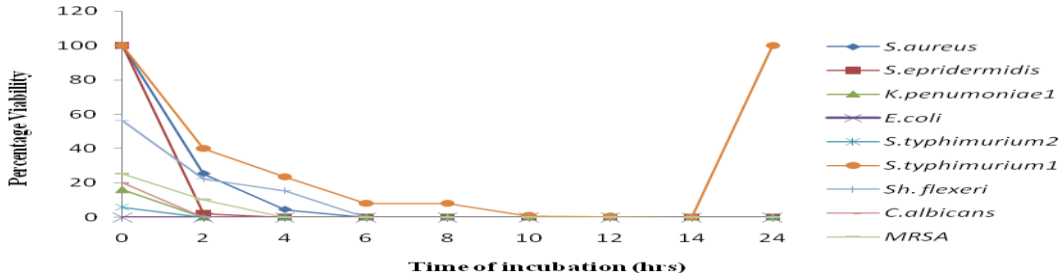


Figure 5. Viable cell count studies on butanolic extract of *Aspergillus* sp

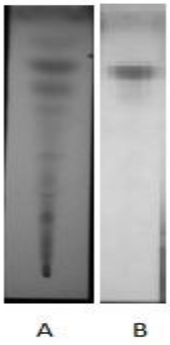


Figure 6 (a). TLC showing different bands of butanolic extract of *Aspegillus* sp
Figure 6 (b). TLC showing single active band after purification by column chromatography

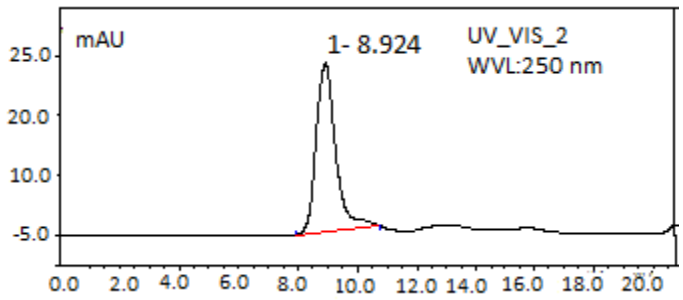


Figure 7. HPLC showing single peak of active band after purification from column chromatography

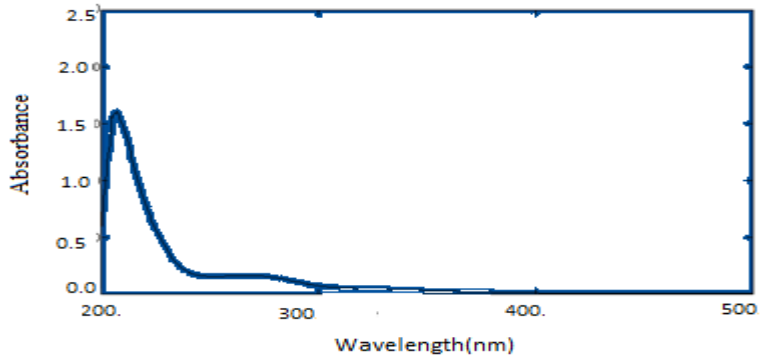


Figure 8. UV spectrum of the purified compound.

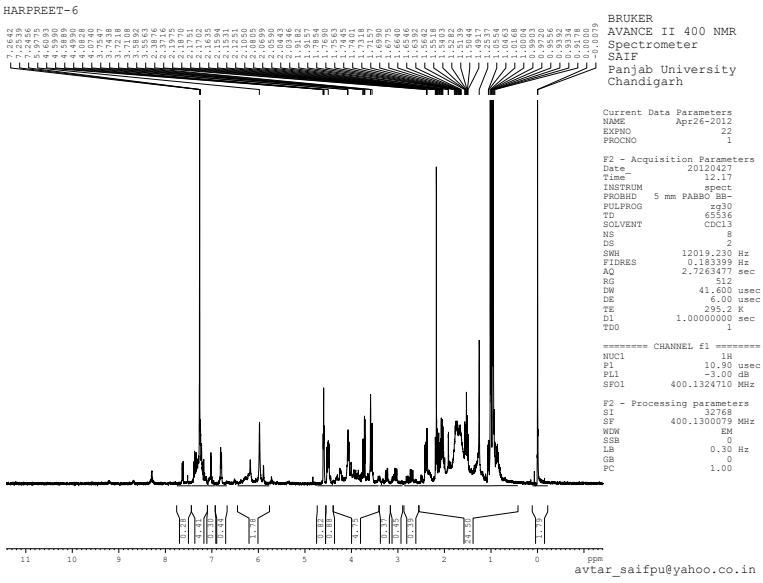


Figure 9. ^{1}H NMR spectrum of the purified compound.

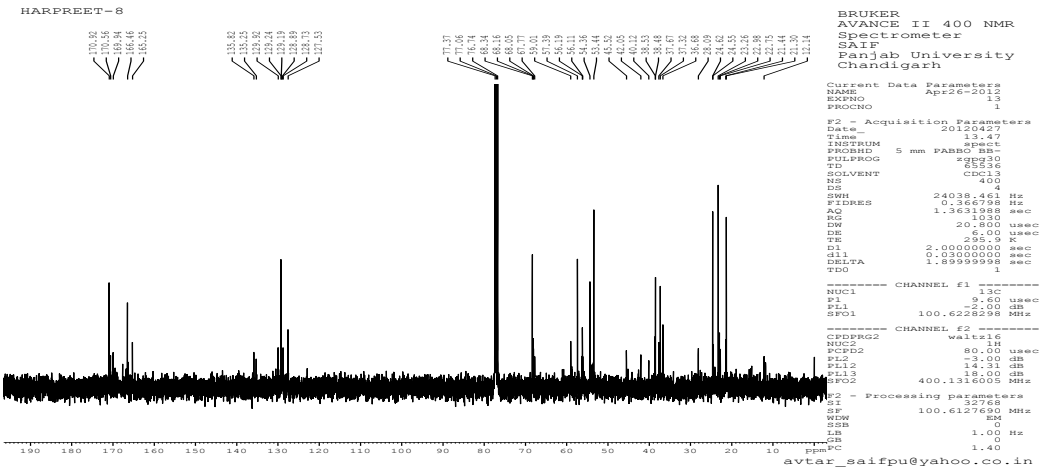


Figure.10. ^{13}C NMR spectrum of the purified compound

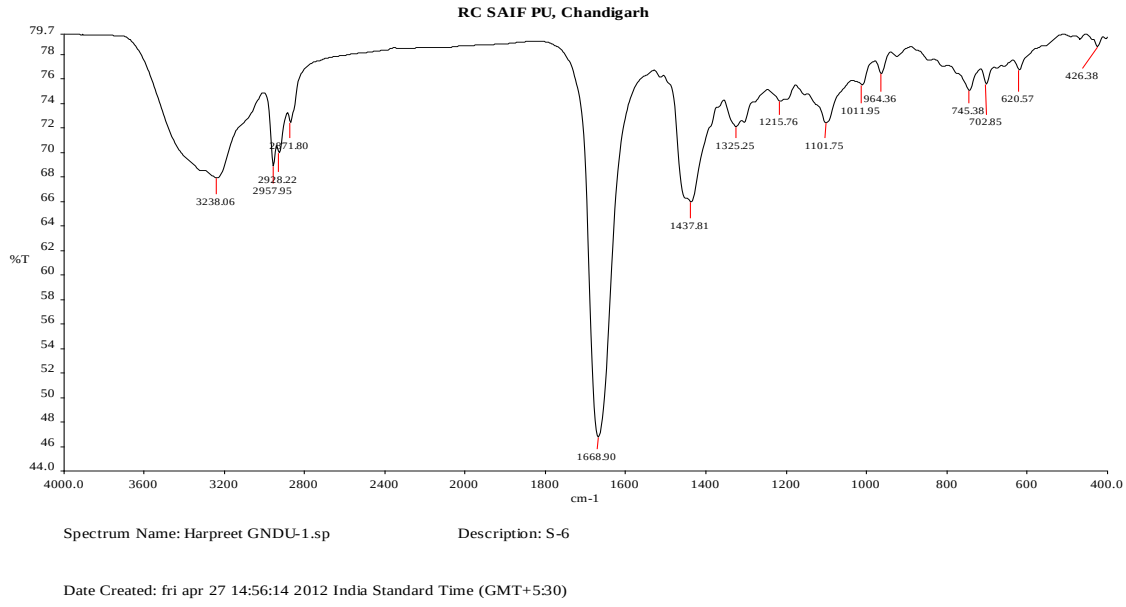


Figure 11. IR spectrum of the purified compound

Table 1 Comparison of antimicrobial activity of butanolic extract of *Aspergillus* sp with some standard antibiotics

		Standard antibiotics[a]										
b	**c**	M	Cfx	PN	K	G	Co	A	Imp	Pt	C	T
		Zone of inhibition (mm)										
SA	42±0.57	26±1	20±0	28±0.57	25±0.57	25±0.57	21±0	30±0	15±1	31±0	24±0.57	19±1
SE	39±0.57	35±1.5	19±0	32±0.57	22±0.57	16±0.57	19±0.57	20±0	18±0	27±0	20±0.57	17±1
SF	20±1	19±1.5	25±0	-	17±0	20±0	25±0.57	-	24±0	21±0	30±0.57	23±1
ST1	14±0	-	23±0	-	20±0	20±0	21±1	19±0.57	22±0.57	26±0.57	22±0	20±0
ST2	20±1.15	18±0.57	20±0.57	-	25±0.57	27±1	29±1	-	22±0.57	24±0.57	34±0.57	20±0.57
EC	22±0.57	17±0.57	21±0	-	21±0.57	23±1	-	-	21±1	20±0.57	34±0.57	13±1
KP 1	46±1	30±1.5	-	-	33±1	32±0.57	35±0.57	-	23±1	25±0.57	31±0.57	24±1.5
PA	16±0	-	-	-	-	17±0.57	-	-	29±1	17±1.5	-	-
EF	27±1	20±1	-	-	-	-	25±0.57	-	-	15±1.5	20±0.57	13±0.57
CA	30±0.57	-	18±0.57	-	20±0	18±0.57	-	-	16±0	24±1	21±0.57	-
MRSA	28±0	-	-	11±0	17±0.57	-	-	12±0.57	14±0	10±0.57	24±0.57	18±1.5

[a] T-30µg-Tetracycline; M-5 µg- Methicillin; K-30 µg-Kenamycin; P-10 µg-Penicillin; Co-25 µg Co-Trimaxazole; A-10 µg Ampicillin; Imp-10 µg Imipenem; Pt-10 µg Piperacillin/Tazobactam; C- 30 µg Chloramphenicol; G-10 µg Gentamycin; Cfx-30 µg Cefixime

[b] Organisms: SA (*Staphylococcus aureus*); SE (*Staphylococcus epidermidis*); SF(*Shigella flexneri*); ST1(*Salmonella typhimurium* 1); ST2 (*Salmonella typhimurium* 2); EC (*E.coli*); KP1(*Klebsiella pneumoniae* 1); PA (*Pseudomonas aeruginosa*); EF(*Enetrococcus faecalis*); CA (*Candida albicans***)**; MRSA(Methicillin resistant *Staphylococcus aureus*) [c] Butanolic extract

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