Role of growth media and chemical enhancers in secondary metabolites production from *Aspergillus carbonarius* (NRL-369) and their pharmaceutical potentials

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Abstract: The present study investigates the effect of different growth media and chemical enhancer on silent genes in *Aspergillus carbonarius* (NRL-369) for secondary metabolites production and its *in vitro* biological activities. Results revealed that *Aspergillus carbonarius* (NRL-369) grown in Czapeak yeast extract broth medium produced more metabolites compared with other media. Chemical epigenetic modifiers (suberoyl-anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) at concentration of 15mM were effective for the expression of silent genes resulting in increased secondary metabolites production. Secondary metabolites extracted in ethyl acetate and fractionized in *n*-Hexane showed variable degree of growth inhibitions of the tested microorganisms. Similarly, these samples were also active against brine shrimps and *Lemna.*

Keywords: *Aspergillus carbonarius*, silient genes, chemical enhancers, growth media, pharmaceutical activities

INTRODUCTION

About half of all deaths in the developing countries are due to various infectious diseases caused by *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus.* Though therapeutic agents of natural or synthetic origin are available to treat these diseases, however, the misuse of these therapeutic agents has increased resistance in pathogens (Mathias *et al*., 2000; Valverde *et al*., 2008). Therefore search for new, safe and more potent biologically active metabolites is very much needed in the developing countries (Hadi *et al*., 2008; Daoud *et al*., 2011). Since long, nature has been contributing considerably to drug discovery for human beings by providing remedial treatments (Stuffness *et al*., 1982; Baker *et al*., 1995; Aida *et al*., 2001). Natural products research still has a huge unexploited potential, and the significant advantages and disadvantages of natural product-derived molecules as drug candidates have been reported elsewhere in the literature (Rogers, 2004; Bakht *et al*., 2011 a, b, c, d; 2012; 2013 a, b; 2014a,b, c; 2015; Nasir *et al*., 2015; Ullah *et al*., 2015; Bilal and Bakht, 2016; Wajid *et al*., 2016). About three quarter of the world population depends on indigenous plants and their materials for treating different kinds of ailments (WHO, 2002).

Even though fungi are good producer of biologically active metabolites, however, fungi cannot be used directly for domestic health care because of their toxic nature. Many human and animal therapeutic medicines have been produced by fungi through fermentation, or by modification of the product (Montenegro *et al*., 2004).

Filamentous fungi produce a number of metabolites which can be used as antibiotics, cytotoxins, pesticides etc. (Siddhardha *et al*., 2009). The increasing number of fungal genome sequences has demonstrated that their biosynthetic potential is far from being exploited. In fungi, the genes required for the biosynthesis of a secondary metabolite are clustered and many of these newly discovered gene clusters are silent under standard laboratory conditions. Genetic approaches could be helpful for estimating the biosynthetic potentials of microorganisms. This approach has remained successfully for the gene(s) involved in the synthesis of polyketides and histone deacetyalse inhibitor (Bode and Muller, 2005; Pelzer *et al*., 2005; Fisch *et al*., 2009; Cherblanc *et al*., 2013). Micro-organisms like fungi can produce a wide range of diverse metabolites depending on the culturing media and conditions (Knight *et al*., 2003; Bode *et al*., 2002). The objectives of this study were (i) to investigate the role of culture media and epigenetic modifiers on secondary metabolites production by *A. carbonarius* (NRL-369) and (ii) to study the *in vitro* pharmaceutical activities of secondary metabolites produced by *A. carbonarius* (NRL-369).

MATERIALS AND METHODS

Fungal strain growth condition

About 106 spores/ ml were inoculated in different culture media i.e. Czapeak-dox Broth (CB), Czapeak Yeast-

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extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapeak-dox (supplemented with glucose and starch) Broth (CGSB). The culture was incubated in shaking incubator (28^oC) at 200 rpm for 12 days. Two epigenetic modifier suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) were used in different concentration i.e. 1, 5, 10, 15 and 20mM/100ml in Czapeak yeast broth (CYB) media to activate the silence gene (s) for the production of metabolite (Fisch *et al*., 2009).

Extraction of metabolites from liquid culture and fractionation

After the incubation, 200 to 500μl of concentrated HCl were added and culture was grinded with blender and filtered with Whatman filter paper using vacuum pump. Equal volume of ethyl acetate was added and mixed thoroughly for half an hour. The organic layer were recovered and washed with 2M brine solution. Anhydrous sodium sulphate (Na_2SO_4) was used to remove the aqueous components. The metabolites were concentrated by rotary evaporator at 45^oC. The crude metabolites were recovered and dried under liquid nitrogen. The metabolites (400mg) were suspended in 200ml distilled water and defatted with *n*-Hexane and about 180mg crude metabolites were reserved for biological screening. Equal volumes of the solvent i.e. 100ml per flask were added for the extraction.

In vitro antibacterial activity

Different bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*) were incubated in 10 ml x 6 of nutrient broth (different additive) at 37^oC for 24 hours for the production of fresh culture. Then 20ml x 6 of the nutrient agar were taken in sterile petri plates and allowed to cool and 0.2ml of each experimental organism was taken from broth culture and poured on the agar media. Stock solutions were prepared in sterile di-methyl sulfoxide (DMSO) with 1, 10, 20, 50, 100, 250, 500 μg/ml concentrations and was poured in each well. Carbenicillin was used as standard drug at concentration of 100μg/ml. The plates were left at room temperature for 2-3 hours for the diffusion of the samples and transferred to incubator for 24 hours at 37^oC (Benkeblia, 2004; Gulluce *et al*., 2003). These experiments were conducted in triplicate and the zone of inhibitions was determined by the following formula.

$$\text{inhibition }\% = \frac{\text{Zone of sample}}{\text{Zone of control}} \times 100$$

Antifungal activity

Different fungi (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*) were cultured in 25ml x 6 of potato dextrose agar (PDA-different additive) at 28^0C for 7 days for the production of fresh culture. Then 10ml x 6 of the potato dextrose agar were taken in sterile test tubes and the test samples were added at concentrations of 10, 20, 50, 100, 250, 500 and 1000μg/ml from stock solution prepared in sterile di-methyl sulfoxide (DMSO) allowed in slanted position to cool and a small piece of about 4 mm in diameter was detached from the old culture (7 days old) of fungi and implanted. Meconazol was used as standard drug ath concentration of 100μg/ml. The test tubes were transferred to incubator for 7 days at 28^oC (Gulluce, 2003). These experiments were conducted in triplicate and inhibition percentage was determined by the following formula.

$$\text{inhibition }\% = \frac{\text{Growth of control}}{\text{Growth of sample}} \times 100$$

Brine shrimp lethality assay

One milligram (1mg) of brine-shrimp eggs were transferred to the artificial hatching media (3.8gm of sea salt in 1000ml of de-ionized water) in to a small tank and left for 24 hours at 25^oC for hatching. The test samples were transferred to sea water at concentration of 1000, 100 and 10μl/ml. Ten (10) shrimps were transferred to each vial and left for 24 hours, the surviving shrimps were recorded and value of LD_{50} were calculated as described by Meyer *et al.* (1982).

$$\text{Mortality }\% = \frac{\text{Shrimps in sample}}{\text{Shrimps in - ve control}} \times 100$$

Phyto-toxic activity

The test samples were transferred to E-medium at concentration of 1000, 100 and 10μl/ml. The solution was allowed for 24 hours for the evaporation of excessive solvent under aseptic condition. After 24 hours, 20 ml of the medium with slightly basic pH was added to sterilized flasks having ten healthy plants of *Lemna acquinoctialis* with three fronds each and kept in growth cabinet/ chamber for seven days (30^oC; light intensity of 9000 lux and 60% humidity). On eighth day the fronds were measured and parquet was used as positive control (De Almeida *et al.*, 2010).

$$\text{Growith inhibition }\% = \frac{\text{Fronds in Sample}}{\text{Fronds in - ve control}} \times 100$$

STATISTICAL ANALYSIS

The experiment was repeated in triplicate and MSTAT computer software was used for the analysis of the data. Least Significant Difference (LSD) test was employed upon obtaining significant difference at $p<0.05$ (Steel *et al*., 1997).

RESULTS

Our results revealed that metabolites production in Czapeak-dox (supplemented with glucose and starch) Broth (CGSB) started at day 3 (6mg) with abrupt increase and peak production of 85mg was noted at day 8.

Similarly, Czapeak Yeast Extract Broth started its production at day 2 (9mg) with a gradual increase and maximum production was achieved at day 9 (62mg). Czapeak-dox Broth, Yeast Extract Sucrose, Potato Dextrose Broth on the other hand was not effective to induce maximum production of metabolite as evident from their low yield (29mg at day 8) (fig. 1). The data indicated that medium containing 10mM concentration of SAHA showed an increase of 17mg by swapping the production from 25 to 42mg on day 4. The highest production of 105mg was observed on day 8 with an increase of 20 mg and afterward a gradual decrease was noted in metabolite production. These results suggested that both the lower and higher concentrations of SAHA (1, 5, 10 and 20*μ*M/100 ml) were ineffective to induce salient genes for significant production of metabolites in the optimized media. In case of 5-AZA, addition of 15 *μ*M/100 ml resulted in maximum production (30mg by swapping the production from 25 to 55mg) of metabolites on day 4. After days 4, a rapid increase of 28, 58 and 28 mg was observed by swapping the production from 40 to 68, 52 to 110 and 72 to 100mg on day 5, 6 and 7 respectively. Highest production of 115mg was observed on day 8 with an increase of 30mg and then a gradual decrease was observed (fig. 2).

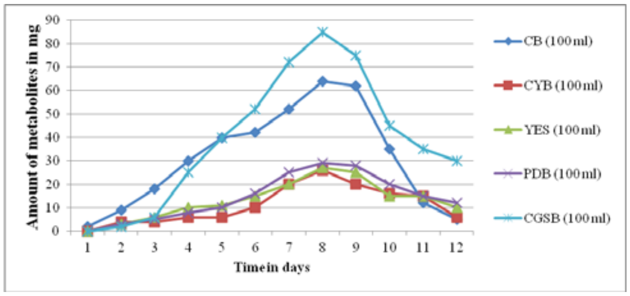

Fig. 1: Optimization of media for production of secondary metabolites from *Aspergillus carbonarius*.

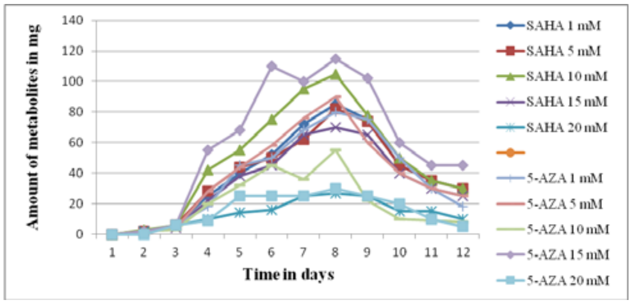

Fig. 2: Effect of epigenetic modifier (suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) on the expression of silent genes of *Aspergillus carbonarius* for their metabolites production.

Tables 1 and 2 shows antibacterial activities of the crude secondary metabolites obtained from *Aspergillus carbonarius* (NRL-369) against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella flexneri* and *Staphylococcus aureus*. Different concentrations of metabolites extracted with ethyl acetate revealed variable inhibition of growth in the tested micro-organisms. The data suggested that slight growth reduction was noted against *B. subtilis* (7.5%) and *S. aureus* (13%) at 50*μ*g/ml concentrations. The growth of *S. flexneri* and *S. aureus* was reduced by 26% and 25% respectively at 100*μ*g/ml concentrations. Similarly, at 250*μ*g/ml concentration, growth of *B. subtilis* and *S. aureus* was inhibited by 36% and 31% respectively. The same concentration reduced the growth of *E. coli*, *K. pneumonia*, *S. flexneri* and S. *typhi* by 25%, 25%, 27% and 15% respectively. When concentration was increased to 500*μ*g/ml, highiest activity was noted against *B. subtilis*, *S. flexneri* and *K. pneumoniae* (64.5%, 59.5% and 51% respectively). Similarly, the same concentration reduced the growth of *E. coli*, *S. typhi* and *S. aureus* by 35%, 38.5% and 36% respectively. The first four concentrations of *n*-Hexane fraction (1 to 50*μ*g/ml) did not inhibit the growth of tested strains. However, our results suggested that 100, 250 and 500*μ*g/ml concentration of the same fraction showed 10%, 32% and 57.5% growth inhibition respectively against *E. coli*.

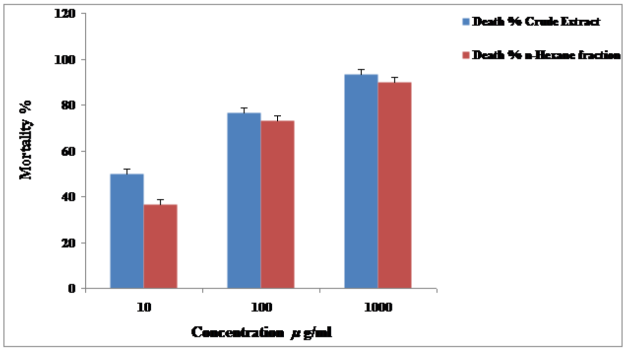

Fig. 3: Cytotoxic activities of ethyl acetate extract & n-Hexane fraction of *A. carbonarius* (NRL-369). Bars show ± LSD at p<0.05.

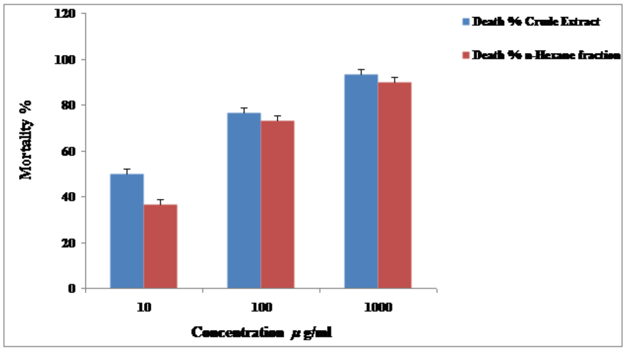

Fig. 4: Phytotoxic activities of ethyl acetate extract & *n*-Hexane fraction of *A. carbonarius* (NRL-369). Bars show ± LSD at p<0.05.

Antifungal activities of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against *Aspergillus flavus*, *Candida albicans*, *Candida glabrata*, *Fusarium solani*, *Microsporum canis* and *Trichophyton longifusus* are shown in tables 3 and 4. Different concentrations of metabolites extracted with ethyl acetate have shown variable inhibition. All the pathogenic fungi survived and no zone of inhibition was observed at 10, 20 and 50μg/ml concentration of metabolites in ethyl acetate fraction. Low linear inhibitions were recorded against all the tested fungi except *M. canis* using 100 μg/ml concentrations. Similarly, low activities were recorded against *F. solani, A. flavus, C. albicans, C. glabrata,* and *T. longifusus* (6%, 10%, 15.5%, 14.5% and 16% respectively) and no activity against *M. canis* at 250μg/ml concentrations. The data further suggested the growth of *C. Albicans, C. glabrata,* and *T. Longifusus, A. Flavus, F. solani and M. canis* was 35 %, 28.5%, 24.5%, 15%, 13.5% and 5.5% at 500μg/ml concentrations. When concentration was increased to 1000 μg/ml concentration, good activity was observed against *C. Albicans* (72%), *C. glabrata* and *T. Longifusus* (58.5% 57%) and again low activity was against *M. canis* (10%) at the same concentration. The first five concentrations of *n*-Hexane fraction (10 to 250μg/ml) did not show any inhibition activity against any of the pathogenic fungi. At 500μg/ml concentration, low activity was noted against *M. canis* (13%). The results also indicated that 1000μg/ml concentration reduced the growth of *C. Albicans* by 14% and *M. canis* by 35.5% .

Fig. 3 presents cytotoxic activities of three different concentrations (10, 100 and 1000μg/ml) of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against the test organism (b*rine shrimps*). The ethyl acetate extract showed 50%, 77% and 94% mortality at 10, 100 and 1000μg/ml concentration respectively, whereas the same concentrations of n-hexane fraction showed 37%, 73% and 90 % mortality respectively of the same organism. The data also indicated that ethyl acetate and n-hexane displayed very low LD_{50} value of 84.75 and 126.94 respectively (table 5). Phytotoxic activities of three concentrations (10, 100 and 1000μg/ml) of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against the test organism (*Lemna*) were measured (fig. 8). Our results suggested that ethyl acetate extract resulted in 40%, 64% and 90% mortality at 10, 100 and 1000μg/ml respectively, whereas the same concentrations of n-hexane fraction revealed 54%, 80% and 94% mortality respectively with very low LD_{50} value of 159.25 and 43.37 respectively (table 6).

DISCUSSION

Optimization of media is an important factor for the growth of any microbe and subsequent metabolites production. The present paper describes the results of five different media for the growth and maximum production of secondary metabolites. The data showed that metabolites production in Czapeak-dox (supplemented with glucose and starch) Broth (CGSB) started at day 3 with abrupt increase and peak production was observed at day 8. The data showed that production of secondary metabolites started at day 2 in Czapeak Yeast Extract Broth with a gradual increase and maximum production was obtained at day 9. Czapeak-dox Broth, Yeast Extract Sucrose and Potato Dextrose Broth were ineffective to induce maximum production of metabolite as evident from their low yield. Similar results are also reported by Sunesson *et al*. (1995), Hestbjerg *et al*. (2002) and Bragulat *et al*. (2011). Two epigenetic modifiers i.e. suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) in different concentrations (1 to 20 μM/100 ml) were tested in Czapeak Yeast Extract Broth to study their role in secondary metabolite production. Our results revealed that medium containing 10mM concentration of SAHA showed an increase of secondary metabolite at day 4. Maximum production was observed on day 8 and afterward a gradual decrease in metabolite production was noted. These results suggested that both the lower and higher concentrations of SAHA were ineffective to induce salient genes for significant production of metabolites in the optimized media. In case of 5-AZA, addition of 15μM/100ml resulted in maximum production of metabolites on day 4, and afterward a rapid increase was observed on day 5, 6 and 7. Highest production observed on day 8 and a gradual decrease afterward was observed. These results agree with Fisch *et al.* (2009).

The antibacterial activities of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi*, *Shigella flexneri* and *Staphylococcus aureus*) were also investigated using different extracts of the secondary metabolites. Different concentrations of metabolites extracted with ethyl acetate revealed variable growth inhibition of the tested micro-organisms. Our results indicated that slight reduction in growth was measured for *B. subtilis* and *S. aureus* at 50 μg/ml concentrations. The growth of *S. flexneri* and *S. aureus* was inhibited reduced at 100μg/ml concentrations. Similarly, at 250μg/ml concentration, growth of *B. subtilis* and *S. aureus, E. coli, K. pneumonia, S. flexneri* and S. *typhi* was also inhibited. When concentration was increased to 500μg/ml, highest activity was noted against *B. subtilis, S. flexneri* and *K. pneumoniae*. Similarly, the same concentration reduced the growth of *E. coli*, *S. typhi* and *S. aureus.* The first four concentrations of *n*-Hexane fraction (1 to 50μg/ml) did not reduce the growth of the tested strains. However, our results suggested that 100, 250 and 500 μg/ml concentration of the same fraction were effective against *E. coli*. Our results are supported by Christophersen *et al*. (1998) and Rabteb and Rainer (2011). Similarly, Nquyen *et al*. (2007) reported that the fungal broth of a marine *Aspergillus* species yielded a new polyoxygenated decalin derivative, dehydroxychlorofusarielin B (30), which was found to exhibit mild antibacterial activity against *S. aureus*,

Table 1: Antibacterial activities (% zone of inhibition) of ethyl acetate extracted samples of *A. carbonarius* (NRL-369).

Ethyl acetate concentration (µg/ml)	*B. subtilis*	*E. coli*	*K. pneumonae*	*S. typhi*	*S. flexeneri*	*S. aureus*
1	0	0	0	0	0	0
10	0	0	0	0	0	0
20	2.0	0	2.0	2.5	18.0	5.0
50	7.5	4.5	5.0	4.0	23.5	13.0
100	15.0	14.0	9.5	11.0	25.5	25.0
250	36.0	24.5	25.0	15.0	26.5	31.0
500	64.5	35.0	51.0	38.5	59.5	36.0
Control (Carbenicillin)	100	100	100	100	100	100

Table 2: Antibacterial activities (% zone of inhibition) of n-hexane extracted samples of *A. carbonarius* (NRL-369).

Ethyl acetate concentration (µg/ml)	*B. subtilis*	*E. coli*	*K. pneumonae*	*S. typhi*	*S. flexeneri*	*S. aureus*
1	0	0	0	0	0	0
10	0	0	0	0	0	0
20	0	0	0	0	0	0
50	0	0	0	0	0	0
100	0	9.5	0	0	0	0
250	0	32.0	0	0	0	0
500	0	56.5	0	0	0	22.5
Control (Carbenicillin)	100	100	100	100	100	100

Table 3: Antifungal activities (% zone of inhibition) of ethyl acetate extracted samples of *A. carbonarius* (NRL-369).

Ethyl acetate concentration (µg/ml)	*A. flavus*	*C. albicans*	*C. glaberata*	*F. solani*	*M. canis*	*T. longifusus*
10	0	0	0	0	0	0
20	0	0	0	0	0	0
50	0	0	1.0	0	0	0
100	4.0	5.0	5.0	1.0	0	4.0
250	9.5	15.5	14.5	6.0	0	16.0
500	15.0	35	28.5	13.5	5.5	24.5
1000	28.0	71.5	58.5	30.0	10.0	57.0
Control (Meconazol)	100	100	100	100	100	100

Table 4: Antifungal activities (% zone of inhibition) of n-hexane extracted samples of *A. carbonarius* (NRL-369).

Ethyl acetate concentration (µg/ml)	*A. flavus*	*C. albicans*	*C. glaberata*	*F. solani*	*M. canis*	*T. longifusus*
10	0	0	0	0	0	0
20	0	0	0	0	0	0
50	0	0	0	0	0	0
100	0	0	0	0	0	0
250	0	0	0	0	2.0	0
500	0	0	0	0	13.0	0
1000	0	14.0	0	0	35.5	0
Control (Meconazol)	100	100	100	100	100	100

methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* with MIC values of 142.36µM for all strains.

Antifungal activities of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against *Aspergillus flavus*, *Candida albicans*, *Candida glabrata*, *Fusarium solani*, *Microsporum canis* and *Trichophyton longifusus* were also screened. All the pathogenic fungi survived and no zone of inhibition was observed at 10, 20 and 50*µ*g/ml concentration of metabolites in ethyl acetate fraction. Low linear growth reduction was measured in all the tested fungi at 100*µ*g/ml concentrations except *M. canis*. Similarly, low activities were recorded against *F. solani, A. flavus, C. albicans, C. glabrata*, and *T. longifusus* and

Table 5: Cytotoxic activities of crude and n-Hexane of *Aspergillus carbonarius* showing low LD_{50}

Extract	Dose, ug/ml	No of Shrimps	Shrimps Survived	Shrimps Dead	Death Ratio	log10 (Dose)	% Probability		
Crude	10	30	24	6	0.200	1	2.122	Intercept	1.917
Crude	100	30	8	22	0.733	2	2.559	Slope	0.263
Crude	1000	30	2	28	0.933	3	2.648	R-Square	0.872
LD_{50}									84.75
n-Hexane	10	30	26	4	0.133	1	1.996	Intercept	1.753
n-Hexane	100	30	9	21	0.700	2	2.543	Slope	0.319
n-Hexane	1000	30	3	27	0.900	3	2.634	R-Square	0.855
LD_{50}									126.94

Table 6: Phytotoxic activities of crude and n-Hexane of *Aspergillus carbonarius* showing deq4e./low LD_{50}

Extract	Dose, ug/ml	No of Fronds	Fronds Survived	Fronds Dead	Death Ratio	log10 (Dose)	% Probability		
Crude	10	30	27	3	0.100	1	1.910		1.641
Crude	100	30	10	20	0.667	2	2.525	Slope	0.355
Crude	1000	30	4	26	0.867	3	2.620	R-Square	0.848
LD_{50}									159.81
n-Hexane	10	30	21	9	0.300	1	2.252	Intercept	2.101
n-Hexane	100	30	6	24	0.800	2	2.591	Slope	0.198

no activity against *M. canis* at 250*µ*g/ml concentrations. The data further revealed the growth of *C. Albicans, C. glabrata,* and *T. Longifusus, A. Flavus, F. solani and M. canis* was also reduced at 500*µ*g/ml concentrations. Good activity was observed against *C. albicans*, *C. glabrata* and *T. Longifusus* at 1000*µ*g/ml concentrations and again low activity was noted against *M. canis* at the same concentration. The first five concentrations of *n*-Hexane fraction (10 to 250*µ*g/ml) did not show any inhibition activity against any of the pathogenic fungi. At 500 *µ*g/ml concentration, low activity was noted against *M. canis*. The results also indicated that 1000*µ*g/ml concentration reduced the growth of *C. Albicans* and *M. canis.*· These results agree with Hansen (1998).

Cytotoxic activities of three different concentrations (10, 100 and 1000*µ*g/ml) of the crude metabolites of *Aspergillus carbonarius* (NRL-369) against the test organism (b*rine shrimps*) showed that ethyl acetate and n-hexane fractions revealed maximum mortality 1000*µ*g/ml concentration. The data also suggested that ethyl acetate and n-hexane displayed very low LD_{50} value. Our findings are in accordance with Wu *et al*. (2012). Greve *et al*. (2008) reported that the extract of the marine fungus *Curvularia* sp. (strain no. 768), isolated from the red alga *Acanthophora spicifera* was active towards a panel of human tumour cell lines. Phytotoxic activities of three concentrations (10, 100 and 1000*µ*g/ml) of the crude metabolites against the test organism (*Lemna*) were measured. Our results suggested that maximum mortality as achieved at highest concentration of ethyl acetate and n-hexane fractions of the secondary metabolites with very low LD_{50} value . Similar results are also revealed by Jiao *et al.* (2004).

ACKNOWLEDGMENTS

The authors are highly thankful to the Higher Education Commission (HEC) of Pakistan for financial support and ARS culture collection USA for providing the fungi.

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