Synthesis of secondary metabolites by *Cladosporium resinae* (NRL-6437) under different growth media and chemical inducers and their pharmaceutical activity

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Abstract: The role of different growth media and chemical enhancer on synthesis of secondary metabolites *Cladosporium resinae* (NRL-6437) was investigated for their *in vitro* biological activities. *Cladosporium resinae* (NRL-6437) were grown in various nutrient media (Czapeak-dox Broth (CB), Czapeak Yeast-extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapeak-dox (supplemented with glucose and starch) Broth (CGSB) for the production of metabolites. Two chemical epigenetic modifiers (suberoyl-anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) were also used for the expression of silent genes for secondary metabolite production. Our results indicated that among different media, Czapeak yeast extract broth produced more secondary metabolites. Application of 15mM of both modifiers was effective for the expressions of silent genes resulting in an increased metabolites production. Secondary metabolites extracted in ethyl acetate and fractionized in *n*-Hexane were also tested for their biological activity. The secondary metabolites revealed varying degrees of growth inhibitions of the tested organisms. Similarly, these metabolites were also active against brine shrimps and *Lemna.*

Keywords: *Cladosporium resinae*, silient genes, chemical enhancers, growth media, pharmaceutical activity.

INTRODUCTION

About 50% of all deaths in the developing countries are caused by different microbial infections. Though therapeutic agents of both natural and synthetic origins are available to cure different microbial infections, however, the injudicious use of these therapeutic drugs has increased resistance in different microbes (Valverde *et al*., 2008). Therefore, searching for new, safe and more potentially biologically active metabolite is an urgent need of the developing countries (Hadi *et al*., 2008). About three quarter population of the world depends on indigenous plants and their materials for treating different kinds of health related problems and about 56% of lower income population of the world use plants medicine for their major health care (Pirzada *et al*., 2010). A renewed curiosity has occurred in the last two decades to investigate the phytochemicals and antimicrobial activities of native and naturalized plants for pharmaceutical and nutritional purposes (Bakht *et al*., 2011 a, b, c and d; 2012; 2013 a,b; 2014 a, b,c; 2015; Nasir *et al.*, 2015; Ullah *et al*., 2015; Zakir *et al*., 2015; Chaun *et al*., 2015; Parveen and Bakht, 2015; Bilal *et al*., 2016; Wajid *et al*., 2016 a, b; Amjad *et al*., 2016; Anwar *et al*., 2016; Anwar *et al*., 2017). However, fungi cannot be used directly for domestic health care due to its toxic nature, even though fungi are good producer of biologically active metabolites. Majority of the human and animal therapeutic agents have been produced and isolated from fungi through fermentation, or by modification of the product (Montenegro *et al*., 2004).

Secondary metabolites are low-molecular-weight compounds with often potent physiological activities. Digitalis, morphine and quinine are plant based secondary metabolites, whereas penicillin, cephalosporin, ergotrate and the statins are also well known important fungal secondary metabolites (Keller *et al*., 2005). Although chemically diverse, all secondary metabolites are produced by a few common biosynthetic pathways, often in conjunction with morphological development. Fungi are remarkable organisms that readily produce a wide range of natural products often called secondary metabolites (Calvo *et al*., 2002; Abid *et al*., 2016). The benefits of these secondary metabolites are some times not known for the producing organism (Bu'Lock, 1961). However, these compounds are of considerable interest as many natural products are of medical, industrial and/or agricultural importance. Some natural products are deleterious (e.g., mycotoxins), while others are beneficial (e.g., antibiotics) to humankind (Dorn, 1970). It is well known that natural products are generally related to cell differentiation or development, and in fact most secondary metabolites are produced by organisms that reveal filamentous growth and have a relatively complex

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morphology. Fungi produce variety of metabolites with high therapeutic agent such as antibiotics, cyto-toxins, pesticides etc (Siddhardha *et al*., 2009). Genetic approaches are useful techniques for estimating the biosynthetic potential of microorganisms. This approach has been 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). Fungi may produce broad spectrum of diverse metabolites depending on the culturing conditions as well as on additives or chemical modifiers (Knight *et al*., 2003; Bode *et al*., 2002).

MATERIALS AND METHODS

Fungal strain and growth condition

The spore suspensions (106 spores/ml) of *Cladosporium resinae* were transferred to different culture media i.e. Czapeak-dox Broth (CB), Czapeak Yeast-extract Broth (CYB), Yeast Extract Sucrose (YES), Potato Dextrose Broth (PDB) and Czapeak-dox (supplemented with glucose and starch) Broth (CGSB). The culture was then incubated at 28^0C in shaking incubator at 200rpm for 12 days.

Use of epigenetic modifiers

Two epigenetic modifier suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) were tested in different concentrations of 1, 5, 10, 15 and 20 mM/100 ml 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 45^0C. 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.

In vitro antibacterial activity

The antibacterial activity was carried by disc diffusion assay as described in Bauer *et al*. (1966) Six bacteria (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexneri* and *Staphylococcus aureus*) were incubated in 10ml x 6 of nutrient broth (different additive) at 37^0C 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 0.2 ml 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, 500μg/ml concentrations and was poured in each well. Carbenicillin was used as standard drug with 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 incubation at 37 ^{0}C[14-15] (Benkeblia, 2004; Gulluce *et al*., 2003). The 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

Antifungal activity was carried out as describes in Ramdas *et al*. (1998). 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 4mm in diameter was detached from the old culture (7 days old) of fungi and implanted. Meconazol was used as standard drug with concentration of 100μg/ml. The test tubes were transferred to incubator for 7 days and 28^0C[15]. The experiments were conducted in triplicate and inhibition percentage was determined by the following formula.

$$\text{Inhibition \%} = \frac{\text{Growth of controle}}{\text{Growth of sample}} \times 100$$

Brine shrimp lethality assay

Artificial hatching media or sea water was made by dissolving water and then filtered. 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 24hours at 25^oC for hatching. The test samples were transferred to Sea water with 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 with concentration of 1000, 100 and 10μl/ ml. The solution was allowed for 24hours to evaporate the excessive solvent under aseptic condition. After 24hours 20ml 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 (at 30 ^{0}C; light intensity of 9000 lux and 60% humidity). On eighth day the fronds was measured and parquet was used as positive control (De Almeida *et al.*, 2010).

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

STATISTICAL ANALYSIS

Data are presented as mean values of three replicates. MSTATC computer software was used to carry out statistical analysis (Russel and Eisensmith, 1983).

RESULTS

Optimization of growth media

Optimization of media is an important factor for the growth of any microbe and metabolites production. For this purpose five different media were used for the growth and maximum production of metabolites (fig. 1). Metabolites production in Czapeak Yeast Extract Broth started at day 2 (9mg) with gradual increase and maximum production was achieved at day 9 (62mg). Czapeak-dox Broth, Yeast Extract Sucrose, Potato Dextrose Broth Czapeak-dox (supplemented with glucose and starch) Broth were not effective to induce maximum production of metabolite as evident from low yield by these media (29mg at day 8).

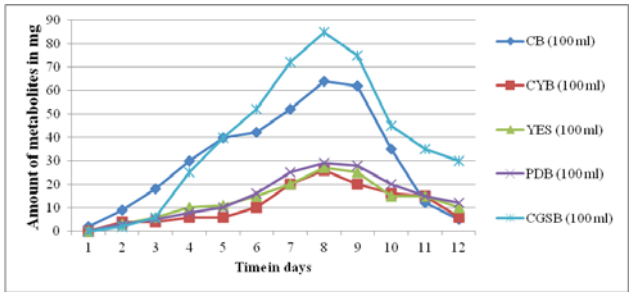

Fig. 1: Optimization of media for production of secondary metabolites, it is clear from the graph that Czapeak-dox (supplemented with glucose & starch) broth is the best for the production of secondary metabolites.

Effect of epigenetic modifiers on metabolites

Two epigenetic modifiers i.e. suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) in different concentrations (1 to 20μM/100ml) were tested in Czapeak Yeast Extract Broth to investigate their role in secondary metabolite production (fig. 2). The medium containing 10mM concentration of SAHA showed an increase of 17 mg 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 20mg 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 μM/100ml) 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 (30mg by swapping the production from 25 to 55mg) of metabolites on day 4. After days 4, a rapid increase of 28, 58 and 28mg by swapping the production from 40 to 68, 52 to 110 and 72 to 100mg on day 5, 6 and 7 respectively was observed. Highest production of 115mg was observed on day 8 with an increase of 30mg and then a gradual decrease was observed.

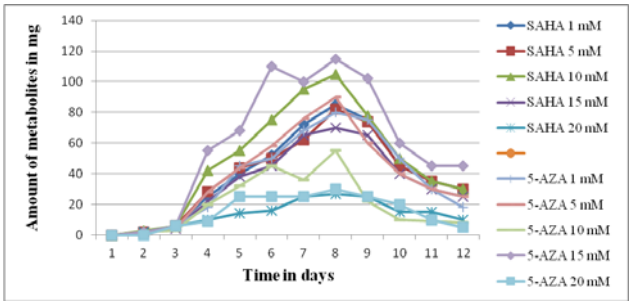

Fig. 2: Epigenetic modifier (suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA)) were used in (CGSB) media for the expression of the silent genes for their metabolites production. The graph shows that 10mM of SAHA and 15mM of 5-AZA is effective for activation of the genes.

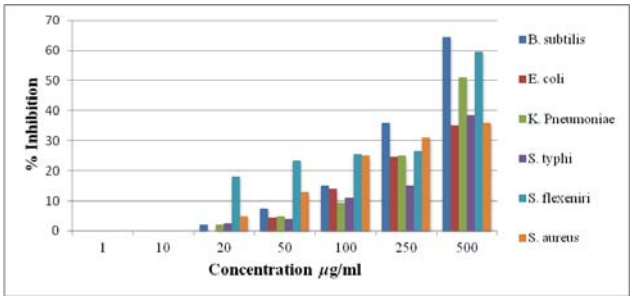

Fig. 3: Antibacterial activities of ethyl acetate extract of *C. resinae* (NRL-6437), the graph shows that 500μg/ml concentrations of the metabolites in ethyl acetate are active against all the pathogenic bacteria.

Antibacterial activity

Figs. 3 and 4 show antibacterial activities of the crude metabolites of *Cladosporium resinae* (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 the test micro-organisms. The data suggested that slight growth inhibition was noted against *B. subtilis* (7.5%) and *S. aureus* (13%) at 50μg/ml concentrations. The growth of *S. flexneri* (26%) and *S. aureus* (25%) was reduced by 26% and 25% respectively at 100μg/ml concentrations. Similarly, at 250μg/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 up to 500µg/ml, high activity was noted against *B. subtilis, S. flexneri* and *K. pneumoniae* (64.5%, 59.5%, 51% respectively. Similarly, the same concentration reduced the growth of *E. coli, S. typhi* and *S.s aureus* by (35% 38.5% and 36% respectively. The first four concentrations of *n*-Hexane fraction (1 to 50µg/ml) showed no inhibition activity against any pathogenic bacteria. The results suggested that hundred and 250µg/ml concentration of the same fraction showed 10% and 32% growth inhibition respectively against *E. coli*. Good activity was observed for *E. coli* (57.5%) at 500µg/ml concentration.

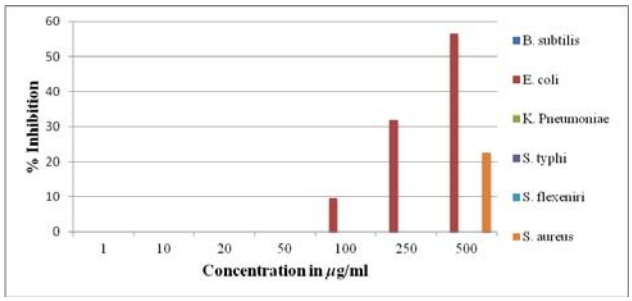

Fig. 4: Antibacterial activities of n-Hexane fraction of *C. resinae* (NRL-6437), the graph shows that 500µg/ml concentrations of the metabolites in *n*-Hexane were active against *E. coli* and *S. aureus*.

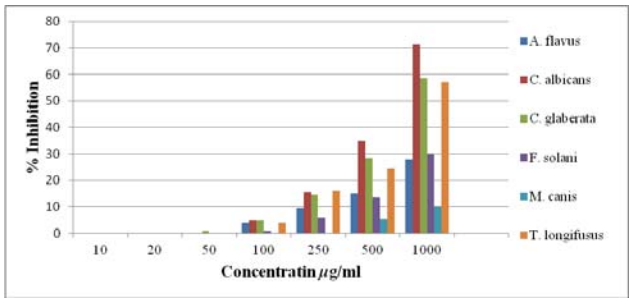

Fig. 5: Antifungal activities of ethyl acetate extract of *C. resinae* (NRL-6437), the graph shows that 1000µg/ml concentrations of the metabolites in ethyl acetate are active against all the pathogenic fungi.

Antifungal activities

Antifungal activities of the crude metabolites of Aspergillus carbonarius (NRL-369) against the test organisms (*Aspergillus flavus, Candida albicans, Candida glabrata, Fusarium solani, Microsporum canis* and *Trichophyton longifusus*) are shown in fig. 5 and 6. 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 & 50µg/ml concentration of metabolites in ethyl acetate fraction. Low linear inhibitions were recorded against all the tested fungi except *M. canis* using 100µg/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 1000µg/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% .

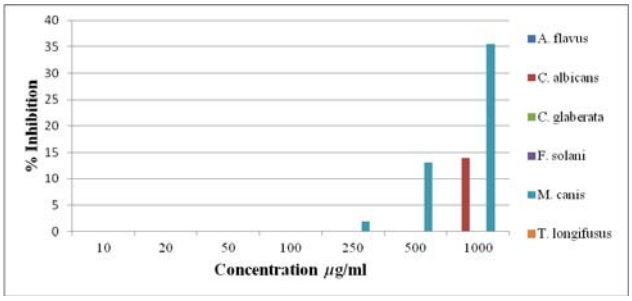

Fig. 6: Antifungal activities of n-Hexane fraction of *C. resinae* (NRL-6437), the graph shows that 1000µg/ml concentrations of the metabolites in *n*-Hexane were active against *C. albicans* and *A. flavus*.

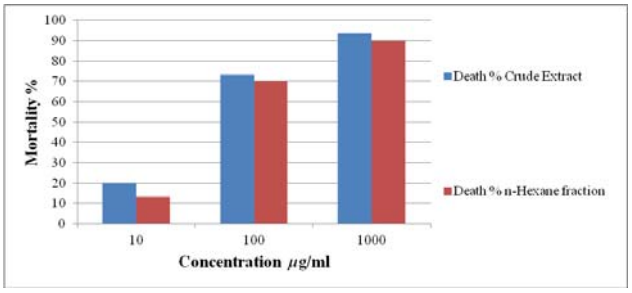

Fig. 7: Cytotoxic activities of ethyl acetate extract & n-Hexane fraction of *C. resinae* (NRL-6437), the graph shows that 1000µg/ml concentrations of the metabolites are highly active against the brine shrimps**.**

Cytotoxic activity

Fig. 7 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 1000 µg/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

Table 1: Cytotoxic activities of crude and n-Hexane of *C. resinae* (NRL-6437) 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 2: Phytotoxic activities of crude and n-Hexane of *C. resinae* (NRL-6437) 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

and n-hexane displayed very low LD_{50} value of 61.18 and 136.29 respectively (table 1).

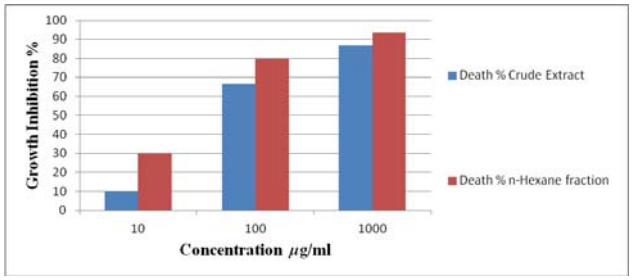

Fig. 8: Phytotoxic activities of ethyl acetate extract & *n*-Hexane fraction of *C. resinae* (NRL-6437) (NRL-639), the graph shows that 1000µg/ml concentrations of the metabolites are highly active against lemna.

Phytotoxic activity

Phytotoxic activities of three concentrations (10, 100 and 1000 µg/ml) of the crude metabolites of *Cladosporium resinae* (NRL-6437) 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 2).

DISCUSSION

The present study investigates the effect of growth media and epigenetic modifers on the growth and secondary metabolites production by *Cladosporium resinae* (NRL-6437). Our results revealed that metabolites production started at day 2 in Czapeak Yeast Extract Broth with gradual increase and maximum production was achieved at day 9. The other growth media under study were not effective to induce maximum production of metabolite Similar results are also reported by Sunesson *et al.* (1995), Hestbjerg *et al.* (2002) and Bragulat *et al.* (2011). To assess the effect of epigenetic modifiers on metabolite production, suberoyl anilide hydroxamic acid (SAHA) and 5-azacytidine (5-AZA) in different concentrations were used in Czapeak Yeast Extract Broth. The data indicated that 10mM of SAHA produced maximum metabolites on day 8 after which a gradual decrease was noted. These results suggested that both the lower and higher concentrations of SAHA were did not induce salient genes for significant production of metabolites in the optimized media. These results agree with Fisch *et al.* (2009).

The antibacterial activities of the crude metabolites of *C. resinae* (NRL-6437) against *B. subtilis*, *E. coli*, *K. pneumoniae*, *S. typhi*, *S. flexneri* and *S. aureus*) were also investigated Different concentrations of metabolites extracted with ethyl acetate measured variable zone of inhibition against the studied micro-organisms. The data suggested that when concentration was increased up to 500 µg/ml, high activity was noted against *B. subtilis*, *S. flexneri* and *K. pneumoniae*. Similarly, the same concentration inhibited the activity 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 any pathogenic bacteria. However, good activity was observed for *E. coli* at the highest concentration. Our results are supported by Christophersen *et al*. (1998) and Rabteb and Rainer (2011). Our results regarding the antifungal activity of the crude metabolites of *C. resinae* (NRL-6437) against the test organisms (*A. flavus*, *C. albicans*, *C. glabrata*, *F. solani*, *M. canis* and *Trichophyton longifusus*) revealed that no activity was recorded upto 50 µg/ml concentration of metabolites in ethyl acetate

extracted fraction. Low linear inhibitions were recorded against all the tested fungi except *M. canis* at 100µg/ml concentrations. Similarly, low activities were recorded against *F. solani, A. flavus, C. albicans, C. glabrata,* and *T. longifusus* and no activity against *M. canis* at 250µg/ml concentrations. The data further suggested at 1000µg/ml concentration, good activity was observed against *C. clbicans*, *C. glabrata* and *T. Longifusus* and low activity was against *M. canis*. The first five concentrations of *n*-Hexane fraction were ineffective to control the growth of any pathogenic fungi, however, highest concentration of the same extract reduced the growth of *C. albicans* and. These results agree with Hansen (1998).

The cytotoxic activity of the ethyl acetate and n-hexane extracted crude metabolites of *Cladosporium resinae* (NRL-6437) against the test organism (*brine shrimps*) revealed high mortality at 1000µg/ml concentration. The data also showed that ethyl acetate and n-hexane displayed very low LD_{50} value of 61.18 and 136.29 respectively. Our findings are in accordance with Wu *et al*. (2012). Ethyl acetate and n-hexane extracted metabolites were also screened for their phytotoxic activity at three concentrations against the test organism (*Lemna*). Our results showed that ethyl acetate and n-hexane extract samples measured good phytotoxic activity at highest concentration with very low LD_{50} value. Similar results are also revealed by Jiao *et al.* (2004).

CONCLUSION

It can be concluded from these results that Czapeak yeast extract broth was good medium for the production of secondary metabolites compared with other media. Application of 15mM of both modifiers was effective to express silent genes for maximum metabolites production. The secondary metabolites revealed different levels of growth inhibitions of the tested organisms and were active against brine shrimps and *Lemna.*

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