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Antimicrobial and cytotoxic activities of endophytic fungus *Colletotrichum gloeosporioides* isolated from endemic tree *Cinnamomum malabatrum*

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Abstract

In a survey of endophytic fungi associated with endemic plant *Cinnamomum malabatrum* leaves harbored a bioactive endophytic isolate CMS 3 was identified as *Colletotrichum gloeosporioides* through morphological and phylogenetic analysis based on ITS-rDNA. The ethyl acetate extract of fermentation broth of *Colletotrichum gloeosporioides* CMS 3 displayed antimicrobial activity against gram positive and gram negative bacteria as well as the fungal pathogen, *Candida albicans*. The ethyl acetate crude extract showed in *vitro* cytotoxicity against the HeLa, MCF-7 and MG63 cancer cell lines with the IC_{50} values of 94.2µg/ml, 84.3µg/ml and 162µg/ml respectively. Gas chromatography and Mass Spectrophotometry (GC-MS) analysis of crude extract confirmed that CMS 3 was a prolific producer of secondary metabolites, in which nearly 74% of the metabolites not listed in the NIST database. Major compounds were phenol 3, 5-dimethoxy acetate (11.82 %), 4'-isopropylidene-bis-(2-cyclohexyl) phenol, N-Didehydrohexacarboxyl-2, 4, 5-trimethylpiperazine and 1, 2, 4-Triazolium ylide. These metabolites may be responsible for its antimicrobial and cytotoxic activities.

Key words – Antimicrobial potential – *Colletotrichum gloeosporioides* – Cytotoxic activity, Endemic tree – Endophytic fungus – GC-MS

Introduction

Globally, there is an ascending trend in life threatening diseases and emergence of drug resistant- bacterial pathogens which has become a serious health concern (WHO 2008). Therefore, there is an urgent need to search for newer and effective novel compounds from various biota. One such unexplored treasure trove is endophytic fungi, which reside in the internal tissues of plants, without causing apparent disease symptom (Tan & Zou 2001). Plant endophytic fungi have the ability to produce array of bioactive compounds such as antibiotics, antiviral compounds,

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anticancer agents, antioxidants, antidiabetic agents, immunosuppressive compounds and biocontrol agents (Strobel et al. 2004, Kusari et al. 2014). Hence, there is a significant interest in screening of endophytic fungi for the discovery of novel metabolites to treat life threatening diseases. Areas with high endemicity possess specific endophytes that may have evolved with endemic plant species and could be a source of novel molecules heralding drug discovery (Strobel 2002).

In India, the Western Ghats is one of the most important hotspots of biodiversity and endemism, which harbors ~45% of endemic plants (Myers et al. 2012). In this paper, we present the endophytic fungi associated with endemic tree *Cinnamomum malabatrum* (Lauraceae) and its bioactivities. *Cinnamomum* species are widely used in herbal therapy in treating colds, sinusitis, bronchitis and fungal infection (Kharwar et al. 2012). It also possesses diverse pharmacological effects such as anti-inflammatory (Annegowda et al. 2012), hepatoprotective (Maridass 2009), antioxidant (Harikumar et al. 2010), anticancer (Agarwal et al. 2013). Endophytes from species of *Cinnamomum* have been studied elsewhere (Strobel et al. 2001, Suwannarach et al. 2010, Santiago et al. 2012). However, there is no study to date on fungal endophytes and their bioactivities from endemic tree *Cinnamomum malabatrum.*

Materials & Methods

Sample collection, fungal isolation and purification

Cinnamomum malabatrum leaves were collected during September 2013 from Uppangala village in the central Western Ghats of the Karnataka state, India (12^0 32' N latitude and 75^0 39' E) and stored in sterile plastic bag and transported to lab in a igloo icy box within 48 hours. The identification of plant species was done at French Institute of Pondicherry, Puducherry.

During the process of endophytic fungal isolation, healthy leaf samples were thoroughly washed in running tap water, then surface sterilized by submerging them in 70% ethanol for 2 minutes, followed by 5% sodium hypochlorite (NaOCl) solution for 5 min, 70% ethanol for 30 seconds and washed with sterile distilled water. Leaves were cut into 1 cm segments and placed on the Petri dish with potato dextrose agar (PDA) amended with streptomycin (250 mg/L) to avert bacterial growth and incubated at 30^o C till the fungal mycelia started growing on the samples. Hyphal tips emerging out from the leaf segments were picked and grown on potato dextrose agar in pure culture and periodically checked for purity. Each pure isolate was stored on PDA slants at room temperature for further investigations.

Identification of endophytic fungus

Endophytic fungi were preliminarily grouped into genus or species level based on their microscopic characteristics (Ellis 1971, Muthumary 2013). Potential bioactive endophytic fungal strain was subjected to molecular identification amplifying by ITS-rDNA sequence based on preliminarily antimicrobial activity. Fungal genomic DNA was extracted using fungal DNA kit (InstaGene TM Matrix). Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for amplification of ITS-rDNA sequence. The PCR products were purified, sequenced and the sequence data were analyzed using BLAST software (BLASTN) at NCBI and submitted to Genbank (accession no. KT795465**)**. The sequence obtained and some from known species were used to construct a neighbor-joining (NJ) phylogenetic tree by using MEGA 6.0 (Tamura et al. 2013) for identification of endophytic fungus.

Fermentation and metabolite extraction

Endophytic fungus was cultured in Potato dextrose broth at room temperature for 21 days under stationary conditions. Fermentation broth was separated from mycelium by filtration. The culture filtrate was extracted thrice with ethyl acetate (EtOAc). The ethyl acetate fraction was evaporated to dryness under reduced pressure at 45 - 55º C using rotary evaporator to obtain a crude extract. The concentrated crude extract was tested for the bioactivities.

Antimicrobial activity

Antimicrobial activity of crude extracts was evaluated by means of agar-well diffusion assay (Basha et al. 2012) with some modifications. At various concentrations, the crude extract of fungi was tested against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus faecalis* (Gram positive bacteria), *Escherichia coli*, *Salmonella typhimurium*, *Shigella boydii* (Gram negative bacteria) on Muller-Hinton agar medium. The antifungal activity was tested against *Candida albicans* using potato dextrose agar medium. Experiments were done in triplicates and antimicrobial activity was expressed as the average inhibition zone diameter produced by the fungal extract.

Cytotoxicity assay

The cytotoxicity of the crude extract was evaluated against human cancer cell lines such as HeLa, MCF-7 and MG63. The cell lines were obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37 ° C, 5% CO_2, 95% air and 100% relative humidity. Cytotoxicity was assessed using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, hundred microlitres of 1×10^4 cells were seeded in each well of 96 well plate, and allowed to grow for 24-h at 37 ° C and 5% CO_2 conditions. After 24-h of incubation, the cells were treated with serial concentration of crude extract (18.75 µg, 37.5 µg, 75 µg, 150 µg, 300 µg). An equal concentration of DMSO was used as control. After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ^{0} C for 4-h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then the absorbance at 570 nm was measured using micro plate reader. The % growth inhibition was determined using the following formula and the IC_{50} was determined using Graphpad Prism software.

% Growth Inhibition = 100- Abs (Test)/Abs (control) x100.

GC-MS Analysis

The fungal extract was subjected to GC-MS analysis to identify the bioactive compound. The sample was analysed in Thermo GC- Trace Ultra Ver. 5.0 (DB 5- MS Capillary Standard Non-polar Column with diameter 0.25 mm, length 30 m, film thickness 0.25 µm) interfaced with mass detector Thermo MS DSQ II (Ion source Single quad EI+), programmed at temperature 70 - 260° C with a rate of 6 ° C/min. Injector flow rate was 220 ° C; carrier gas was ultra pure He, column flow rate 1.0 ml/min., injection mode- spilitless. The compounds produced by CMS 3 were tentatively identified through library comparison with NIST database. Compounds listed were tentatively identified strictly on the basis of their quality match (50% or better) with the NIST database compounds.

Results

Fungal isolation and identification

In the present study, 28 isolates of fungal endophytes were obtained from the leaves of endemic tree *Cinnamomum malabatrum*. Of these, 13 isolates were sporulated and grouped into five species, namely, *Colletotrichum* sp., *Aspergillus* sp., *Alternaria* sp., *Fusarium* sp. and *Curvularia* sp. Among the isolates, species of *Colletotrichum* was found to be predominant (~60%). Preliminary antimicrobial activity of all the isolates revealed that one of the isolates of *Colletotrichum*, CMS 3, showed significant activity and went through further analysis. The colony morphology of CMS 3 on PDA medium was dense aerial hyphae, initially white gray and later becoming dark gray. Conidia were hyaline, aseptate and cylindrical with obtuse apices, size of the conidia was 10 to 15 µm in length and width 2 - 3 µm. Based on the morphological character the species was assigned to *Colletotrichum* sp. (Fig. 1a-b).

Precise identification was done by amplifying ITS region using ITS1 and ITS4 primers and the sequence was determined. Sequence similarity search was done with NCBI BLAST resulted in several closely related sequences along with them phylogenic analysis was conducted, which resulted in CMS 3 fell into the group of *Colletotrichum gloeosporioides* (Fig. 2).

Antimicrobial and Cytotoxic activities

Biological activities of the crude extract were evaluated using antimicrobial activity and cytotoxicity against three cell lines. Ethyl acetate extract of *Colletotrichum. gloeosporioides* fermentation broth was tested at various concentration (50, 100, 200, 300, 400 µg/ml) against Gram- positive, Gram- negative bacteria and fungi, *Candida albicans*. At low concentration, crude extract (50 µg/ml) did not show the zone of inhibition against the tested organisms, whereas 100 µg of crude extract inhibited the growth of all the test organisms (Table 1). The zone of inhibition was measured and this concentration was considered as the minimum inhibitory concentration (MIC). Maximum inhibition was reported at higher concentration (400 µg/ml) which was more or less equal to the standard antibiotics.

The evaluation of cytotoxicity of *Colletotrichum gloeosporioides,* CMS 3, extract was performed using three cell lines (HeLa, MCF-7 and MG63), which showed reasonable activity against cell lines (Fig. 3). MCF-7 has more sensitivity to crude extract than HeLa and MG63 cell lines. Over all, the extract did not show significant cell inhibition at lower concentrations (18.75 and 37.5 µg /ml), moderate inhibition (> 25% and 38%) at 75µg/ml and > 80% cell inhibition at 150 µg /ml. At high concentration (300 µg /ml) crude extract had showed 100% cell inhibition. The IC_{50} values for MCF-7, HeLa and MG63 cell lines were respectively 84.2, 94.2, and 162 µg /ml.

GC-MS analysis

The extract of *Colletotrichum gloeosporioides,* CMS 3 was analyzed by GC-MS. Twenty three components were detected in the crude extract and six of them were identified by comparison with MS Data of NIST (Table 2). Seventeen compounds remained unidentified because the spectral data could not match with any compound in the database. Among the listed compounds, phenol 3, 5- dimethoxy acetate was the most abundant with the relative area of 11.82%. The other notable compounds are 4'-isopropylidene-bis-(2-cyclohexyl) phenol, N-Didehydrohexacarboxy l- 2, 4, 5-trimethylpiperazine and 1, 2, 4- Triazolium ylide. GC-MS analysis of crude extract of *Colletotrichum gloeosporioides,* CMS 3 by and large produced phenolic derivatives (Table 2).

Discussion

Plants growing in biodiversity rich areas may contain novel endophytic microorganism (Strobel 2003) and are the lest-explored organisms for biologically active small molecules (Chowdhary et al. 2012, Radic & Strukelj 2012). *Cinnamomum* species are widely used in herbal medicine because of its therapeautic potential. Over the last decades, fungal endophytes have been inventoried from various species of *Cinnamomum* (Strobel et al. 2001, Ezra et al. 2004, Suwannarach et al. 2010, Wang et al. 2011, Han et al. 2012, Kharwar et al. 2012, Santiago et al.2012, Saxena et al. 2014) to harness its potential. In this study, *Colletotrichum* isolates were found to be predominant fungal isolate from *Cinnamomum malabatrum,* in which one of isolates CMS 3 showed significant biological activity. The isolate was identified as *Colletotrichum gloeosporioides* both by morphological and molecular characterization based on ITS rDNA sequences. *Colletotrichum gloeosporioides* is found to be the most common endophyte in various host plants in the tropics (Zou et al. 2000, Kumar & Hyde 2004, Gangadevi & Muthumary 2008, Wang et al. 2008, Arivudainambi et al. 2011, Suwannarach et al. 2012, Zhang et al. 2012, Senthilkumar et al. 2013).

Colletotrichum gloeosporioides crude extract showed significant antimicrobial activity against Gram –Positive and negative bacteria and fungi (Table 1). This findings corroborated with the early study that reported that *Colletotrichum gloeosporioides* was the prolific producer of

plethora of antimicrobial compounds, such as colletotric acid and colletoic acid (Zou et al. 2000), diketopiperazines (Trigos et al. 1997), artemisinin (Wang et al. 2006), phillyrin compounds (Zhang et al. 2012) and piperine (Chithra et al. 2014).

Many plant derived natural products like camptothecin, taxol, vinblastine, vincristine are of immense value to combat malignancies (Srivastava et al. 2005). Many of the cytotoxic compounds reported from plants are also produced by endophytic fungi (Schulz et al. 2002; Strobel et al. 2004; Kharwar et al. 2011, Deshmukh &Verekar 2014). The cytotoxicity evaluation of *Colletotrichum gloeosporioides* CMS 3, was moderate (Fig. 3) when compared to the National Institute Criteria for Cytotoxicity ($IC_{50} < 20$ µg/ml) for screening of crude extracts. About 80% of the screened crude extracts of endophytic fungus isolated form *Stryphnodindron adstringens* showed significant cytotoxicity ($IC_{50} < 20$ µg/ml) against MCF-7 cell line (Carvalho et al. 2012). However, extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia* showed moderate activity ($IC_{50} > 20$ µg/ml) against JURKAT cell line (Guimaraes et al. 2008).

The bioactivity of the extract possibly as a result of innate compounds in the extract including N-Didehydrohexacarboxy l- 2, 4, 5-tri methyl piperazine and other phenol derivatives, identified following GC-MS fragmentation. Piperazine derivatives have potent biological activities such as antibacterial activity (Graz et al. 1999), cytotoxicity (Wang et al. 2012) and antiviral activity (Wang et al. 2013).

In the recent past, few studies have reported dwindling production of plant secondary metabolites by endophytic fungi in successive culturing (Gurudatt et al. 2010, Kusari & Spiteller 2010, Kumara et al. 2012, Zhao et al. 2013). It may be due to loss of genes or silencing of genes (Kumara et al. 2014). In our case, we have sub cultured *Colletotrichum gloeosporioides* CMS 3 more than seven times before conducting the biological activity, so it may be the probable reason for moderate bioactivity. Recently, various methodologies were employed to overcome the silencing of genes and to induce the production of bioactive compounds through epigenetic modulations (Gacek & Strauss 2012, Chen et al. 2013), microbial co-cultivation (Netzker et al. 2015), addition of polysaccharide elicitors (Simic et al. 2014) and in some cases addition of various carbon sources (methanol and ethanol) in microbial fermentation (Zhao et al. 2013).

Even after successive sub culturing of *Colletotrichum gloeosporioides,* CMS 3, endophytic fungi showed moderate biological activity, which confirm that CMS 3, have great promise as a sustainable resource of biologically active novel secondary metabolites. We have initiated the fermentation of endophytes with elicitors for the discovery and structural elucidation of bioactive compounds from the endophyte *Colletotrichum gloeosporioides,* CMS 3.

Table 1 Antimicrobial activity of endophytic fungus *Colletotrichum gloeosporioides* CMS 3, isolated from endemic tree *Cinnamomum malabatrum*

Test organisms	Diameter of inhibition zone (mm) mean ± S.D.				
	100µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	Standard antibiotic 25 µg/ml
Staphylococcus aureus	15.0 ± 2.0[a]	17.0 ± 1.7[a]	17.0 ± 1.7[a]	24.0 ± 2.0[a]	28.3 ± 0.6[s]
Bacillus subtilis	14.3 ± 2.1[a]	16.7 ± 1.5[a]	17.3 ± 3.5[a]	24.0 ± 1.7[a]	29.3 ± 0.6[s]
Streptococcus faecalis	14.0 ± 1.0[a]	15.3 ± 0.6[a]	15.7 ± 0.6[a]	23.0 ± 2.6[a]	30.0 ± 0.0[s]
Escherichia coli	12.3 ± 2.1[a]	15.7 ± 2.1[a]	17.3 ± 1.5[a]	20.7 ± 1.2[a]	29.0 ± 1.0[s]
Salmonella typhimurium	14.3 ± 1.2[a]	14.3 ± 1.2[a]	16.3 ± 1.5[a]	22.7 ± 1.5[a]	29.7 ± 0.6[s]
Shigella boydii	14.3 ± 2.1[a]	15.7 ± 1.5[a]	16.7 ± 2.3[a]	24.3 ± 1.2	24.7 ± 1.5[s]
Candida albicans	14.0 ± 1.7[a]	15.3 ± 2.1[a]	16.0 ± 2.6[a]	20.3 ± 0.6[a]	27.0 ± 1.0[f]

Data are mean ± S.D. of triplicate reading of the inhibition zone diameter.
[a]Significantly different from the control at $P < 0.05$ using one way ANOVA
[s]Standard antibiotic Stretomycin for bacterial pathogens.
[f]Standard antibiotic fluconazole for fungal pathogens.

Table 2 GC-MS Analysis of the compounds presents in Ethyl acetate extract of *Colletotrichum gloeosporioides* CMS 3

Retention Time	Relative area (%)	Compound Name	Quality	Molecular weight	Molecular formula
23.37	7.09	N-Didehydrohexacarboxyl-2,4,5-tri methyl piperazine	96.17	222	$C_{13}H_{22}N_{2O}$
27.26	1.19	6-(t-Butyl imino)-8-(3'-trifluoromethylphenyl)-3,4-dihydro-2H, 6H-pyrimido[2,1-b][1,3]thiazine-7-carbonitrile	61.17	392	$C_{19}H_{19}F_3N_4S$
30.76	5.25	1,2,4-Triazolium ylide	79.36	244	$C_{12}H_{12}N_4O_2$
31.07	7.99	4,4'-isopropylidene-bis-(2-cyclohexyl phenol)	57.00	392	$C_{27}H_{36}O_2$
33.69	2.27	Fluoro bis [3-fluorodimethylsilyl-2,2,4,4,6,6-hexamethyl-1,3,5-triaza-2,4,6-trisilacyclohexyl]borane	98.22	618	$C_{16}H_{50}BF_3N_6Si_8$
38.39	11.82	Phenol, 3,5-dimethoxy- acetate	58.29	196	$C_{10}H_{12}O_4$

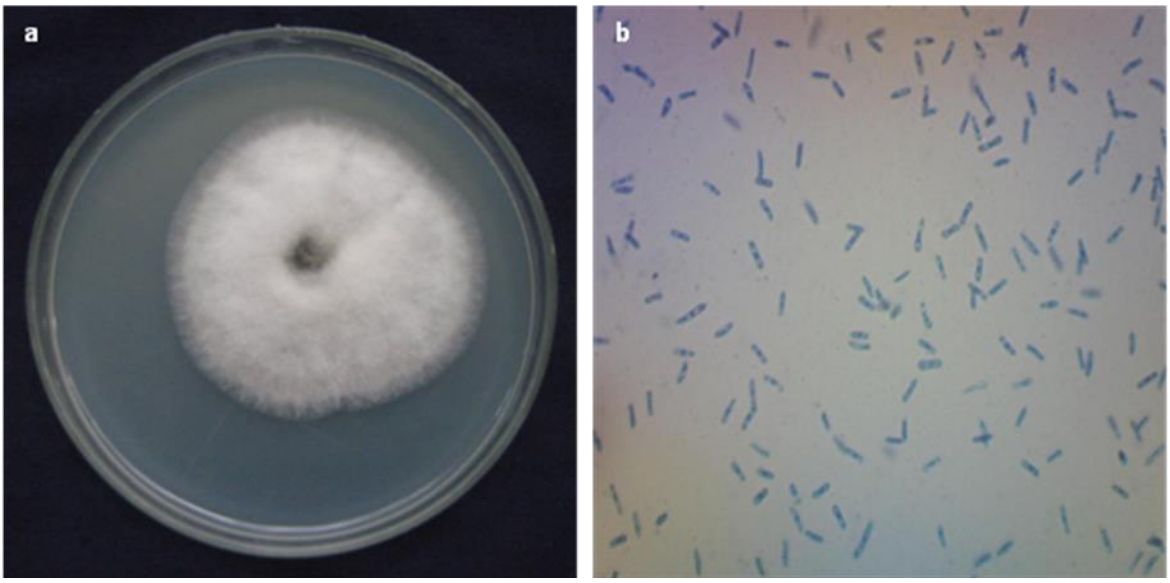

Fig. 1 – Colony morphology and conidia of *Colletotrichum* isolate, CMS 3 on PDA medium. (a) Colony of CMS 3 after 5 days, (b) Conidia of CMS 3

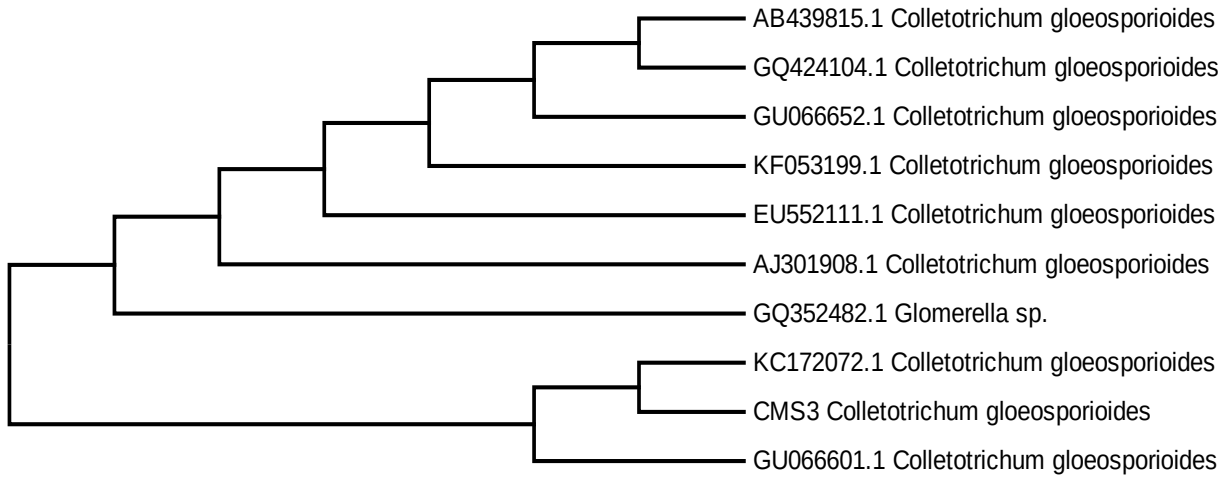

Fig. 2 – Phylogeny NJ tree inferred from ITS rDNA sequences of *Colletotrichum gloeosporioides*, CMS 3

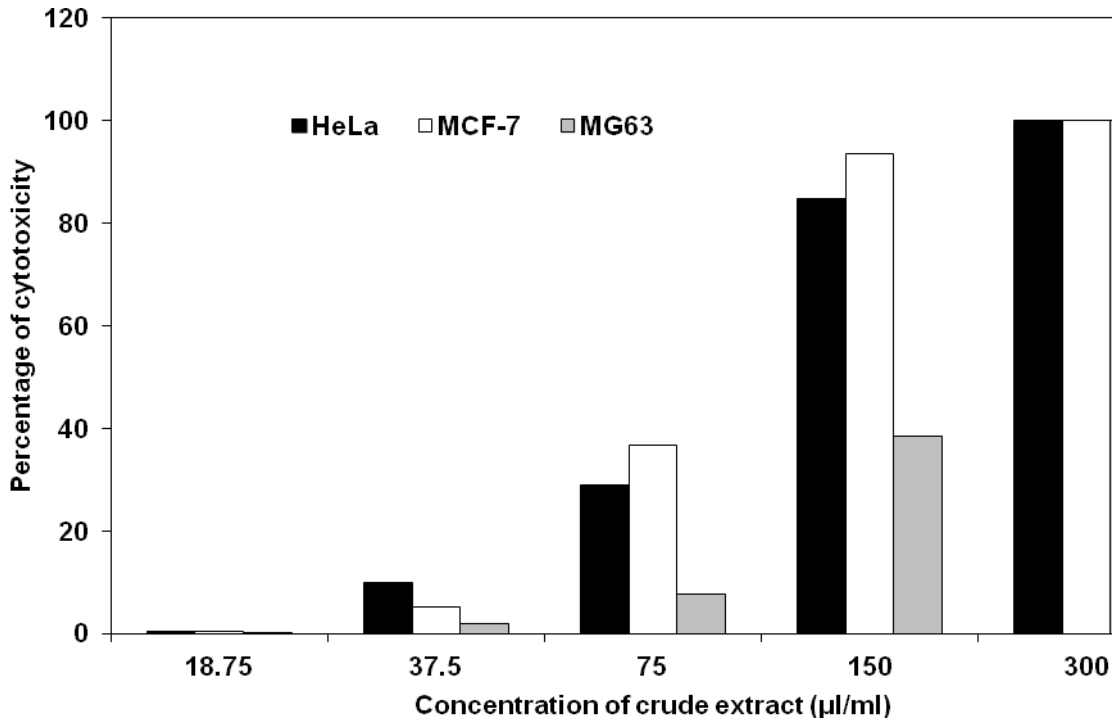

Fig. 3 – *In vitro* cytotoxic activity of ethylacetate extract of *Colletotrichum gloeosporioides* against HeLa, MCF-7 and MG63 cancer cell lines

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