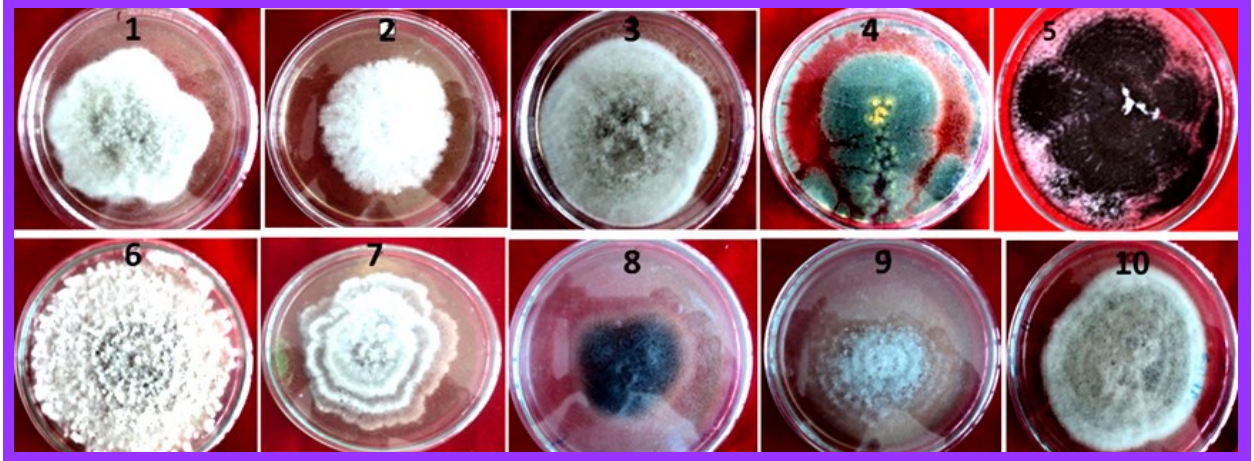

BJP

Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
Bangladesh J Pharmacol 2014; 9: 129-139
ISSN: 1991-0088

First report of anti-cancer agent, lapachol producing endophyte, *Aspergillus niger* of *Tabebuia argentea* and its *in vitro* cytotoxicity assays

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Article Info

Received: 7 August 2013
Accepted: 7 January 2014
Available Online: 17 March 2014

DOI: 10.3329/bjp.v9i1.15622

Cite this article:
Channabasava, Govindappa M. First report of anticancer agent, lapachol producing endophyte, *Aspergillus niger* of *Tabebuia argentea* and its *in vitro* cytotoxicity assays. Bangladesh J Pharmacol. 2014; 9: 129-39.

Abstract

All parts of *Tabebuia argentia* were used for isolation and identified the lapachol producing endophytes were used for evaluation of *in vitro* cytotoxicity (antimitotic, anti-proliferative, determination of cell viability, DNA fragmentation). Five endophytes (leaf endophytes, *Alternaria alternata*, *Alternaria* sp., *Aspegerillus niger*, *Penicillium* sp. and the bark endophyte, *A. alternata*) are able to produce potent anticancer agent lapachol. The 3rd and 4th fractions of endophytic extracts (*A. niger* and *Penicillium* sp.) exhibited the pure lapachol. The 3rd fraction of *A. niger* lapachol strongly inhibited the *Allium cepa* root actively growing cells at various stages of cell cycle in antimitotic assay and the index was 22.5 mg/mL, the yeast cells were died due to toxicity and possessed the cell necrosis and they exhibited the DNA fragmentation. Present promised endophytes can be used for production of lapachol using endophyte *A. niger* for large scale production of lapachol within short period of time.

Introduction

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals (Baker et al., 2000). Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. Only a few these plants have ever been completely studied relative to their endophytic biology (Strobel and Daisy, 2003). Novel antibiotics, antimycotics, immunosuppressants, and anti-cancer compounds are only a few examples of what has been found after the isolation, culture, purification, and characterization of some choice endophytes in the recent past. The potential prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great (Strobel and Daisy, 2003). Many endophytes are producing antibiotics, antiviral compounds, anti-cancer agents, anti-oxidant molecules, insecticidal compounds, anti-diabetic compounds, immunosuppressive compounds etc.

Tabebuia argentea (Bignoniaceae) is a large and yellow flowering tree and have proven to be a rich source of many organic compounds, especially, of phenolic and poliophenolic nature. The plant able to produce anti-cancer agent, lapachol it is ability to interfere with the bioactivities of enzymes known as, topoisomerases, a group of enzymes that are critical for DNA replication in cells (Wuerzberger et al., 1998). The antitumor activity of lapachol may be due to its interaction with nucleic

acids and the interaction of the naphthoquinone moiety between base pairs of the DNA helix occurs with subsequent inhibition of DNA replication and RNA synthesis (Murray and Pizzorno, 1998). Other biological activities of lapachol are antimetastatic activity (Balassiano et al., 2005), anti-microbial and antifungal activity (Da Silva et al., 2003), antiviral activity (Breger et al., 2007), anti-inflammatory (Almeida et al., 1990), antiparasitic activity (Murray and Pizzorno, 1998), leish -manicidal activity (Teixeira et al., 2001) and molluscicidal activity (Silva et al., 2005). Only one report is avaliable on identification of lapachol producing endophytes of *Tabebuia argentea* from our lab only (Sadananda et al., 2011). Present investigation was aimed to isolate and identification of lapachol producing different endophytes and evaluation of endophytic lapachol for antimitotic, anti-proliferative and DNA fragmentation assays in *in vitro*. This is the first report of endophytic lapachol and its *in vitro* antimitotic, anti-proliferative and DNA fragmentation assays nationally and internationally.

Materials and Methods

Plant material collection

T. argentea collected in the month of September 2012 from Shridevi Institute of Engineering and Technology campus, Tumkur, Karnataka, India and it is authenticated by Dr. P. Sharanappa, Department of Studies in Biosciences, University of Mysore, Hemagangothri, Hassan, Karnataka.

Isolation and identification of endophytic fungi

The protocol for isolation follows methods used in other endophyte studies (Theantana et al., 2009) but adjusted for the specific plant tissues used here following pilot experiments. The plant parts were washed in running tap water for one hour. Fifty segments of leaves from each plant were cut into 5 mm 2 pieces, including a vein (25 samples) and intervein (25 samples). 25 segments of branches were then cut randomly to a length of 5 mm. Endophytic fungi were isolated from the different parts of the plant (25 segments). Twenty five segments (5 mm long) were cut from the stems and the roots. The total 150 segments of plant material were treated by triple surface sterilization techniques (Bussaban et al., 2001). Each piece is then placed on malt extract agar (malt extract (20 g/L), rose Bengal (0.033 g/L), chloromphenicol (50 mg/L, agar (15 g/L). All plates were incubated at 26 ± 2°C until mycelium grew out hyphal tips were cut and transferred to potato dextrose agar (PDA). Half strength PDA is used for subculture and stock culture. Identification was made based on colony and hyphal morphology of the fungal cultures, characteristics of the spores (Barnett and Hunter, 1972).

Fungal cultivation and extraction of metabolite

The fungal endophytes were cultivated on potato dextrose broth (Himedia) by placing agar blocks of actively growing pure culture (3 mm diameter) in 250 mL Erlenmeyer flasks containing 100 mL of the medium. The flasks were incubated at 26 ± 2°C for 1 week with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats.

The fungal metabolites from different endophytic mycelial mats were extracted by using methanol. Equal volume of the filtrate and solvents were taken in a separating funnel and shaken vigorously for 10 min. The solution were then allowed to stand, where the cell mass got separated and the solvent so obtained was collected. All solvents were evaporated and the resultant compound was dried in vacuum evaporator using $MgSO_4$ to yield the crude extracts (Raviraja et al., 2006).

Test for lapachol identification

Dried endophytic extract and plant flower extract of *T. argentea* was extracted with ethyl acetate. 1 g of the endophytic and flower extract was re-crystallized in petroleum ether and benzene (80:60) and heated at 139 to 140°C for 5 min. 2 mL of ferric chloride solution was added and observed for the color change (Thomson, 1987).

Test for anthraquinones (Borntrager test)

2 mL of endophytic test sample (of plant and endophytic extracts) was shaken with 4 mL of hexane. The lyophilic layer was separated and adds 4 mL of dilute ammonia was added and color change was observed.

Thin layer chromatography

A thin mark is made at the bottom of the plate with a pencil to apply the sample spots. Then samples solutions are applied on the spots marked on the line at equal distances.

The mobile phase (chloroform:methanol, 19:1) (Houghton et al., 1994) is poured into the TLC chamber to a level few centimeters above the chamber bottom. Then the plate prepared with sample spotting is placed in TLC chamber such that the side of the plate with sample line is towards the mobile phase. Then the chamber is closed with a lid.

The plate is immersed such that sample spots are well above the level of mobile phase but not immersed in the solvent for development. Allow sufficient time for development of spots. Then the plates were removed and allowed to dry. The sample spots were visualized in UV light chamber for the sample.

Column chromatography

Column chromatography is a type of adsorption chromatography techniques. Here stationary phase is a solid material packed in a vertical column.

The column chromatography requires a vertical glass column with a knob at the bottom end. Silica gel used as stationary phase for the column. Hexane used as the mobile phase of chromatography for the separation. Cotton wool has plugged before to hold stationary phase and let allow only the solvent and sample. The stationary phase material (silica) is suitably moistened with mobile phase (hexane) and packed sufficiently in the column with a cotton pad at the bottom. The extract sample to be separated is placed on the top of packed stationary phase. The mobile phase is poured into the column over the sample. A collecting beaker is placed at the bottom of column near the end to collect the elute. Totally 12 endophytes were used, six fractions were collected (for each endophytes) in 10 mL intermetant gap.

Figure 1: *Tabebuia argentea* plant during flowering

Antimitotic activity

Method adopted by Shweta et al. (2012) was used for determination of antimitotic activity using *Allium cepa* root with slight modification. *A. cepa* were collected from Tumkur vegetable market. *A. cepa* bulbs were sprouted in water for 24 hours at room temperature. The uniform root tips of *A. cepa* were selected for the study. These roots were dipped in the extract (10 and 5 mg/mL) for 48 hours. Water was used for dilution and lapachol was used as a standard for study. After 48 hours, the root tips were fixed in the fixing solution of acetic acid and alcohol (1:3). Squash preparation was made by staining with acetocarmine stain. Morphology and the number of the cells were observed under microscope (40x). In all 350-400 cells were counted and cells manifesting different stages of mitosis i.e., interphase and prophase (P), metaphase (M), anaphase (A) and telophase (T) were recorded. The mitotic index was calculated using the following formula (Shweta et al., 2012; Subhadradevi et al., 2011).

$$\text{Mitotic index} = \frac{P + M + A + T}{\text{Total cells}} \times 100$$

Anti-proliferative activity

Evaluation of anti-proliferative activity of plant extract was done by yeast *Saccharomyces cerevisiae* model according to Shwetha et al. (2012).

Yeast inoculum preparation

The yeast was inoculated with sterilized potato dextrose broth and incubated at 37°C for 24 hours and it was referred as seeded broth.

Determination of cell viability

Cell viability assay was performed with 2.5 mL of potato dextrose broth and 0.5 mL of yeast inoculums in four separate test tubes. In the first test tube distilled water, in second test tube quercetin (Sigma-Aldrich) as standard (1 mg/mL), in third and fourth test tubes plant extract (10 and 5 mg/mL respectively) were added. All tubes were incubated at 37°C for 24 hours. In the above cell suspension, 0.1%methylene blue dye was added in all tubes and they were observed under low power microscope. The number of viable cells, those does not stain and look transparent with oval shape while dead cells get stained and appeared blue in color were counted in 16 chambers of hemocytometer and the average number of cell was calculated. The percentage of cell viability was calculated using the formula (Sehgal et al., 2006).

%Cytotoxicity = No. of dead cells/No. of viable cell + No. of dead cells X 100

DNA fragmentation assay

DNA fragmentation assay was performed by the method of Bicas et al. (2011). Briefly, 0.1 mL of extract mixed with 2.5 mL potato dextrose broth and 0.5 mL of yeast inoculums. Cell suspension was incubated for 24 hours at 37°C. DNA was isolated from the treated cell suspension with Tris EDTA buffer and DNA was electrophoresed (Shwetha et al., 2012).

Results

Figure 1 showing flowering period of the *T. argentea*. Totally 12 endophytes were isolated and identified from three parts (bark, leaf and stem) of *T. artentea. A. alternata* was present in all the parts. The leaf part alomost all the endophytes except unidentified fungi. Leaf yielded *A. alternata, Alternaria* sp., *A. niger, Fusarium oxysporum, Fusarium* sp., *Penicillium* sp., *Rhizopus*

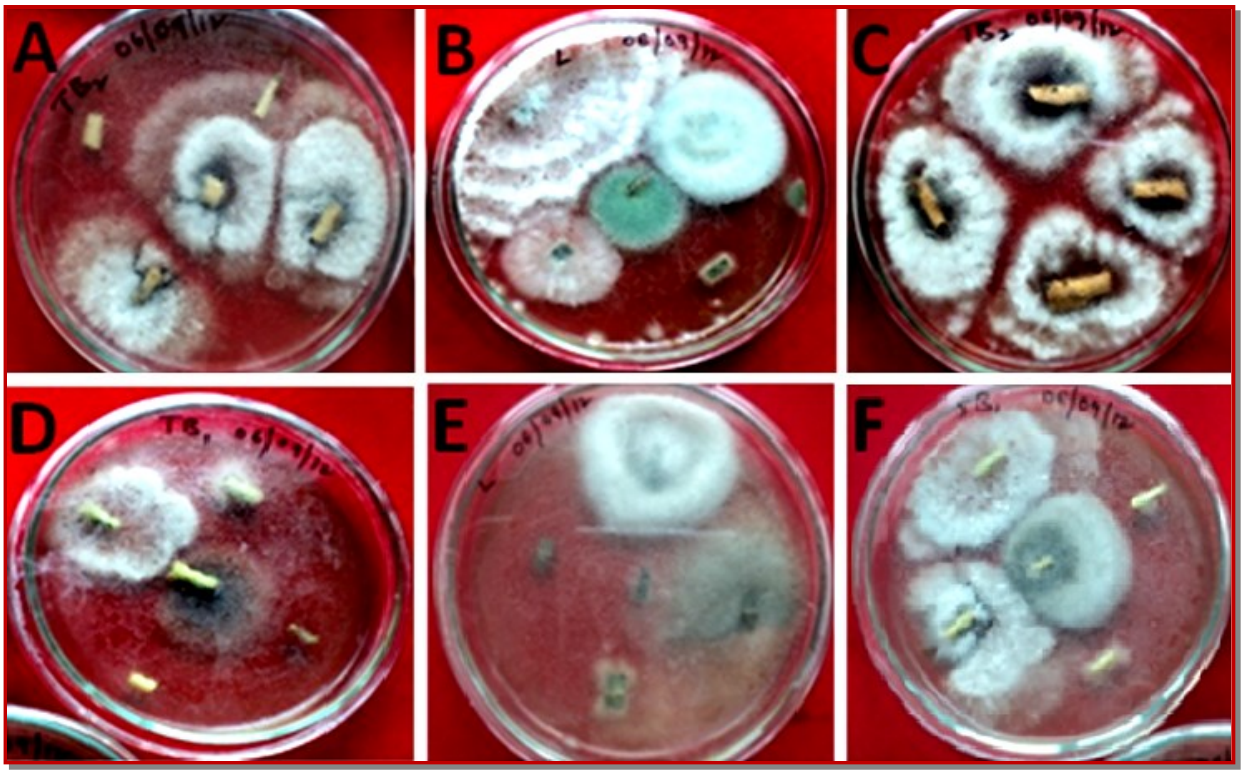

Figure 2: Incubated plant parts showing different endophytes

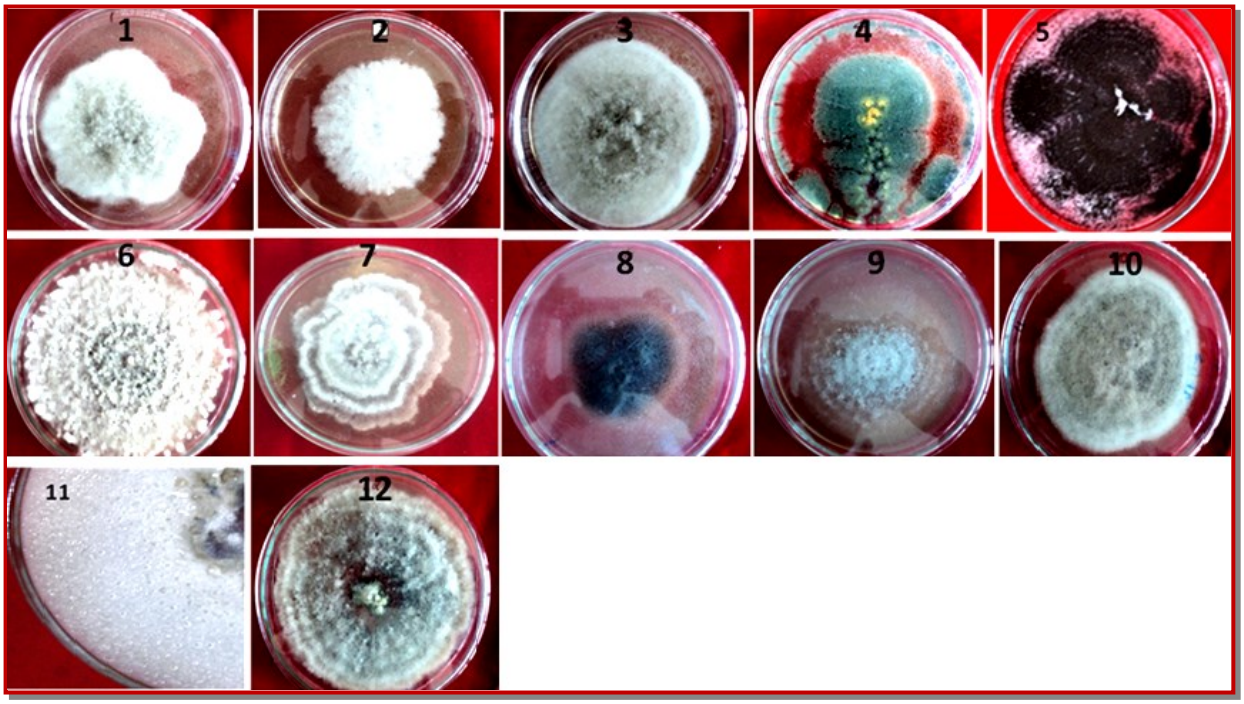

Figure 3: Mass production of different endophytes in Petri plates, 1: Leaf *Fusarium oxyporum*, 2: Bark *Fusarium oxysporum*, 3: Leaf *Alternaria alternata*, 4: Leaf *Penicillium* sp., 5: Leaf *Aspergillus niger*, 6: Leaf *Alternaria* sp., 7: Stem *Alternaria* sp., 8: Bark unidentified fungi, 9: leaf *Fusarium* sp., 10: Bark *Alternaria alternata*, 11: Leaf *Rhizopus* sp., 12: Stem *Alternaria alternata*

sp., the bark yileded *A. anlternata*, *F. oxysporum* and unidentified fungi whereas, *A. alternata* and *Alternaria* sp (Table I; Figure 2). Each fungal species were identified based on morphology and conidial structures using different standard fungal manuals. The identified endophytes were mass cultured on potato dextrose agar (Figure 3) and potato dextrose broth.

Yellow color confirmed the presence of quinoned compounds (Naphthoquinone). The leaf endophytes, *A. alternata*, *Alternaria* sp., *A. niger*, *Penicillium* sp., the bark endophytes, *A. alternata* has showed the presence of lapachol compared with standard lapachol and plant parts. Naphthoquinone was identified in leaf endophytes, *A. alternata*, *Alternaria* sp., *A. niger*, *Penicillium* sp., the bark endophytes, *A. alternata* and in plant extract. Other endophytes did not show the presence of naphthoquinone presence. The five endophytes have shown the presence of napthoquinone and lapachol (Table II), whereas the Figure 4 showing only positive reports of *A. niger* and *Penicillium* sp.

The leaf endophytes, *A. alternata*, *Alternaria* sp., *A. niger*, *Penicillium* sp., and the bark endophyte, *A. alternata* has showed confirmed the presence of lapachol. TLC was performed for the extract of endophyte in comparison with standard (Figure 5). The developed bands were observed under UV light. TLC for the endophytic extract was compared with standard lapachol.

Column chromatography was performed to partially purify the lapachol from the endophytic extract using silica gel as the stationary phase and the hexane used as the mobile phase solvent. Only two leaf endophytes (*A. niger* and *Penicillium* sp.) were used for this experiment. The column fractions of about 20 mL for every 20 min was collected, 6 fractions were collected.

TLC was performed for the collected fractions in comparison with standard lapachol; at fraction 3 and 4 exhibited the clear band of lapachol (Figure 6). The both endophytes exhibited presence of lapachol clearly. Most of the lpachol is yielded at 3 and 4 fractions of endophytic extracts. The 3rd fraction of leaf endophyte, *A. niger* extract was used for *in vitro* antimitotic, antiproliferative and DNA fragmentation assay.

The antimitotic assay revealed that the lapachol fraction of *A. niger* extract showed good inhibition of meristamatic cell in different stages of cell cycle due various process. We have found that *A. niger* lapachol is induced mitotic activity at various levels of cell cycle viz., shrinked interphase cells, abnormal chromosomal distribution after metaphase, chromosomal bridges with vagrant chromosomes at anaphase, chromosomal stickiness and clumping in telophase, chromosomal bridge and clumping, chromosomal degeneration, large nucleolus, binucleolar and nucleolar burst and mega cell and chromosomal clumping in small cells was observed was compared with normal mitotic phases of interphase, metaphase and anaphase (Figure 7 and 8). The value decrease dose dependently with lapachol from standard lapachol, *A. niger* and *Penicillium* sp. lapachol and the mitotic index was found to be 12.2, 22.5, 28.7 mg/mL respectively whereas the untreated control showed 91.6 mg/mL (Figure 9).

The *A. niger* lapachol (3rd fraction) was evaluated using *Saccharomyces cerevisiae* in anti-proliferative activity and it showed potent inhibition of yeast cell growth. The number of dead cells was calculated using above mentioned formula. The *A. niger* lapachol inhibited the yeast cell growth above 70% whereas the standard showed more than 80%. This result confirms the purity of the lapachol and also similar activity (Figure 10). The *A. niger* lapachol leads to death of yeast by showing dead cells with debris (Figure 11). We sequentially observed the yeast cells treatment of *A. niger* lapachol after 24 hours. This results clearly idicates that how our endophytic lapachol acts on yeast cells. The antiproliferative activity of standard lapachol, endophytes, *A. niger* and *Penicillium* sp was noticed at different range was 86.5, 72.8 and 68.2 respectively (Figure 12).

DNA fragmentation assay confirmed the anti-proliferative activity of *A. niger* lapachol. After 48 hours treatment with *A. niger* and *Penicillium* sp. lapachol is breakdown the DNA of yeast it is one of the method of inhibition of DNA replication in cancer therapy. The DNA fragmentation is may be due to inhibtion of topoisomerase, are the key enzymes in DNA replication (Figure 13).

Discussion

Many of the natural anti-cancer agents are exploring by using of endophytes. Taxol is a diterpenoids it can kill tumor cells by enhancing the assembly of microtubules and inhibiting their depolymerisation, which was first extracted from endophyte, *Taxomyces andreanae* (Stierle et al., 1993), *Pestalotiopsis microspora* (Li et al., 1996), *A. alternata, Periconia* sp., *Pithomyces* sp., *Chaetomella raphigera*, *Monochaetia* sp. and *Seimatoantlerium nepalense* (Visalakchi and Muthumary, 2010). Torreyanic acid, a selectively cytotoxic quinone dimer (anti-cancer agent), was isolated from a *P. microspora* strain (Lee et al., 1996). The lapachol ability to interfere with the bioactiviries of enzymes, topoisomerases are group of enzymes are critical for DNA replication in cells (Wuerzberger et al., 1998). Sadananda et al. (2011) have primarily reported the lapachol producing endophytic fungi (*A. niger* and *A. alternata*). In curent study, we have identified four endophytic which are capable to produce lapachol. The pure endophytic lapachol was isolated at 3rd and 4th fraction in column chromatography and it was proved in TLC comparision with the standard. The 3rd fraction

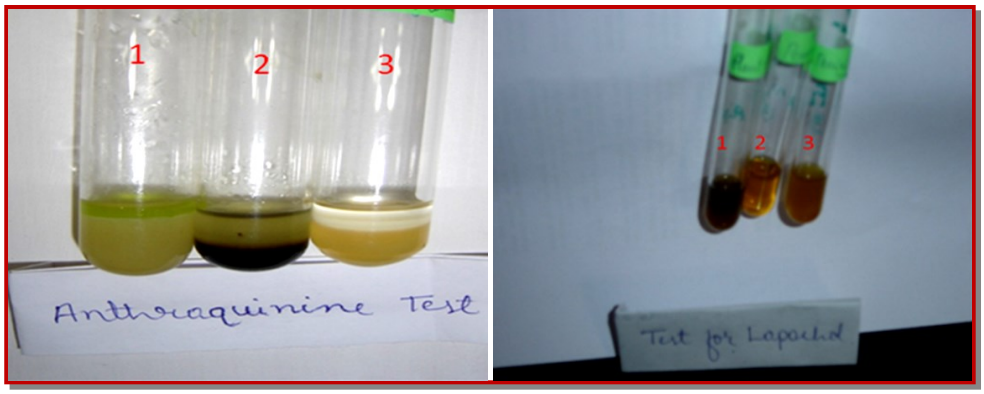

Figure 4: Anthraquinone and lapachol test for endophytic extracts, 1: Plant extract, 2: *Aspegerillus niger* extract, 3: *Penicillium* sp. extract

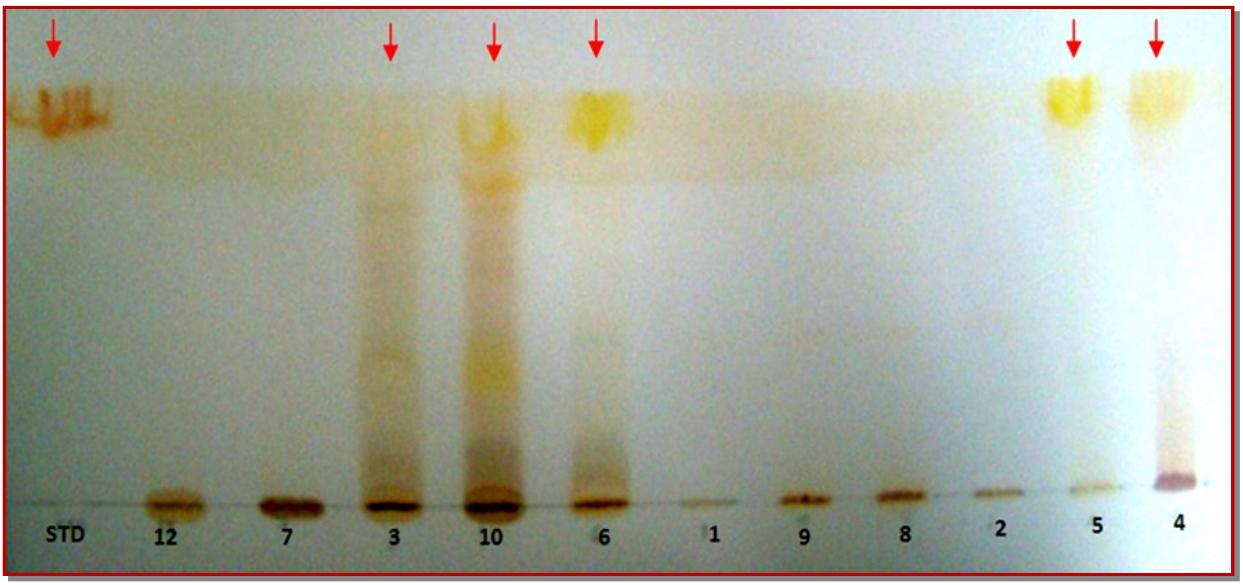

Figure 5: TLC plate showing presence of lapachol from different endophytes (3, 10, 6, 5 & 4) compared with the standard

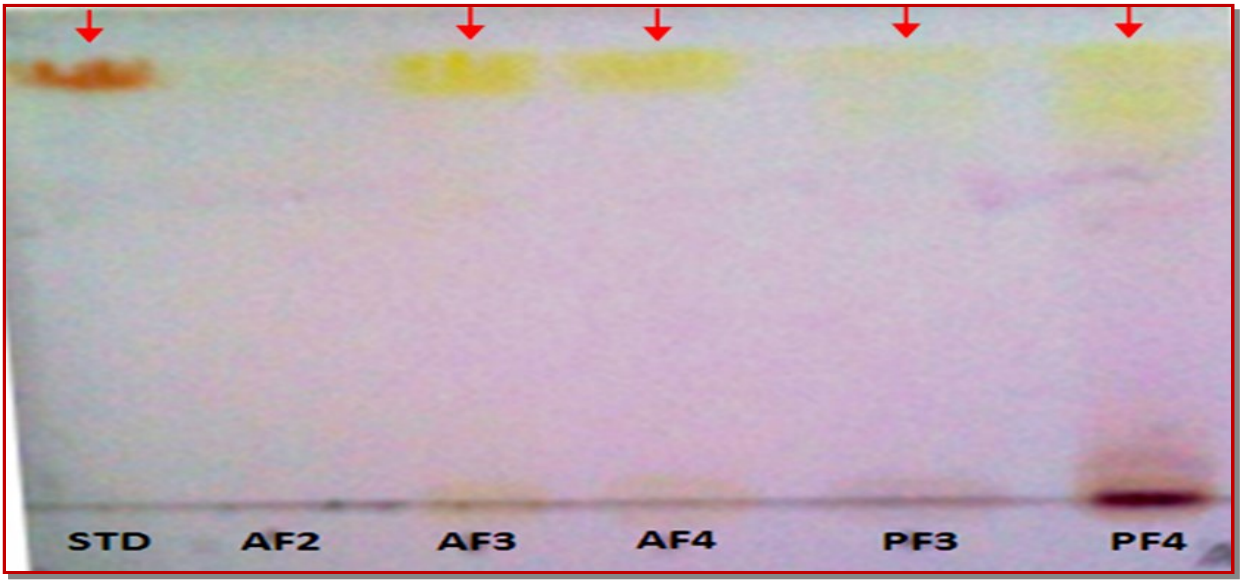

Figure 6: The 3rd and 4th fractions of *Aspergillus niger* and *Penicillium* sp. showing pure lapachol when compared with standard lapachol

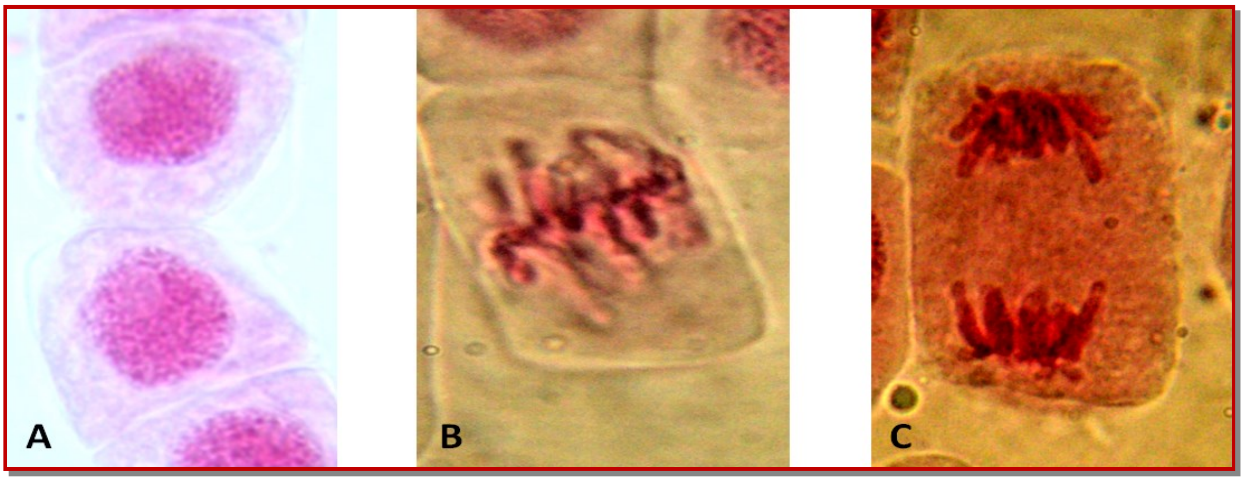

Figure 7: Normal mitotic phases, A) Interphase, B) Metaphase and C) Anaphase

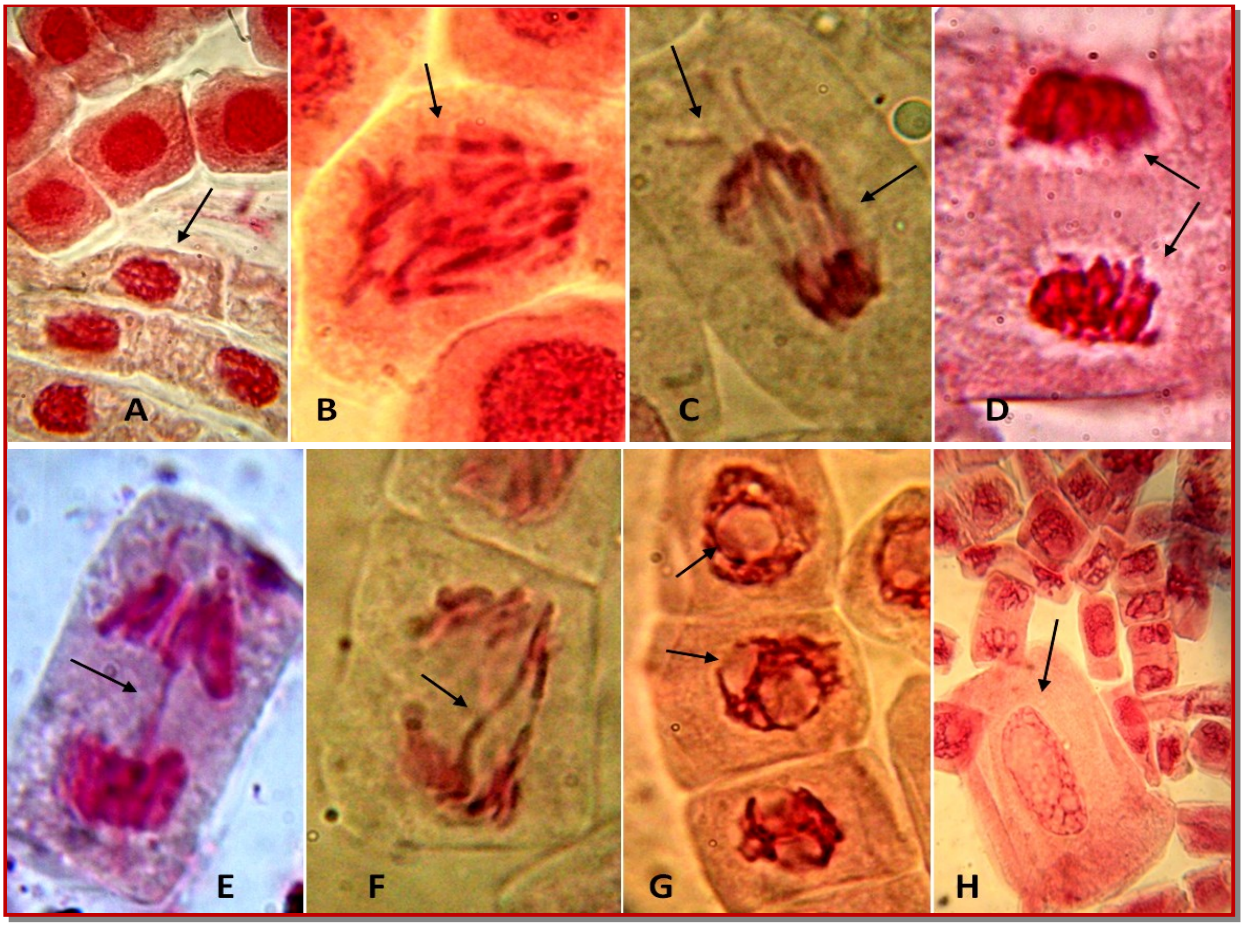

Figure 8: Chromosomal, nucleolar and cellular abnormalities, A) Shrinked interphase cells along with normal ones, B) Abnormal chromosomal distribution after metaphase, C) Chromosomal bridges with vagrant chromosomes at anaphase, D) Chromosomal stickiness and clumping at telophase, E) Chromosomal bridge and clumping F) Chromosomal bridges and chromosome degeneration, G) Large nucleolus, binuncleolar and nucleolar burst, H) Mega cell and chromosomal clumping in small cells.

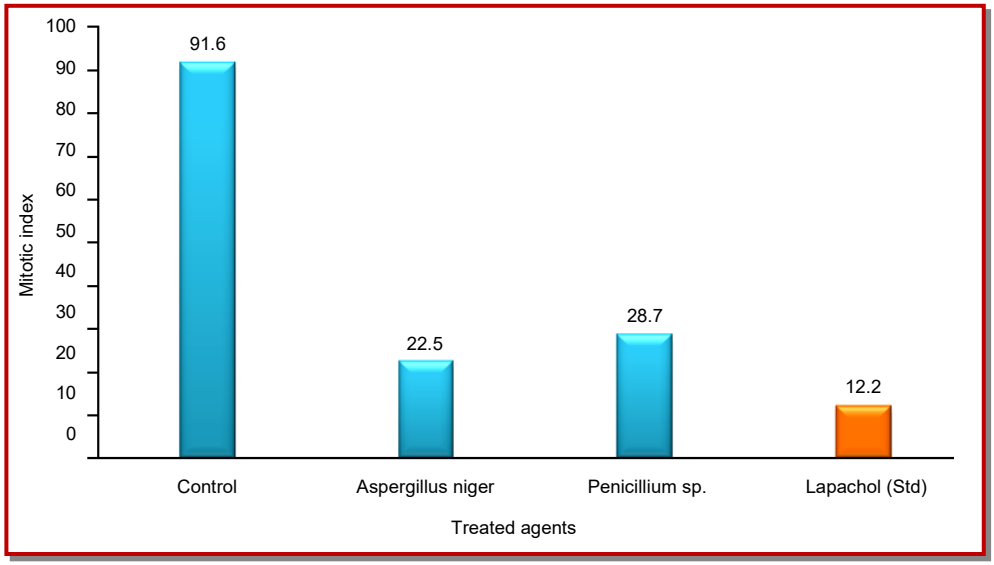

Figure 9: Mitotic index in *Allium cepa* root tips for endophytic extracts and standard

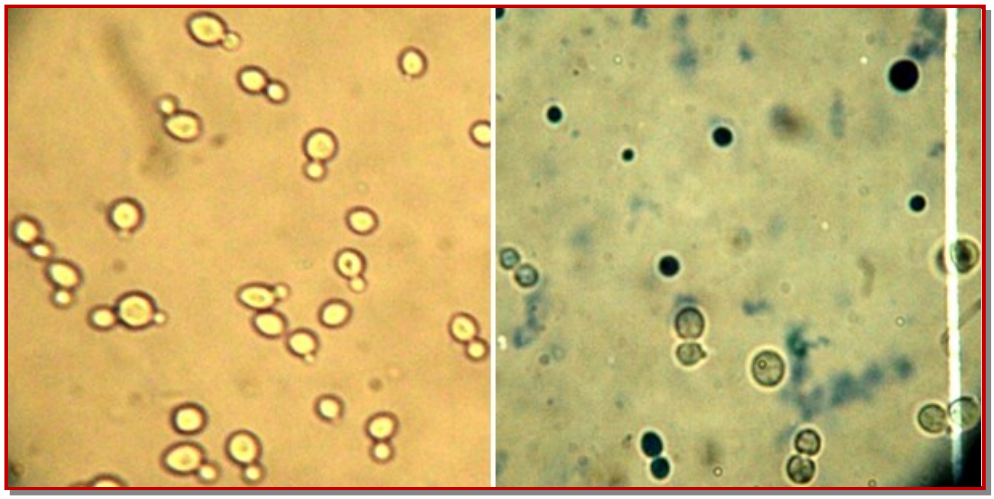

Figure 10: Endophytic lapchol treated yeast cells. A. control, B. *Aspergillus niger* extract treated yeast (stained cells are dead cells with cell debris

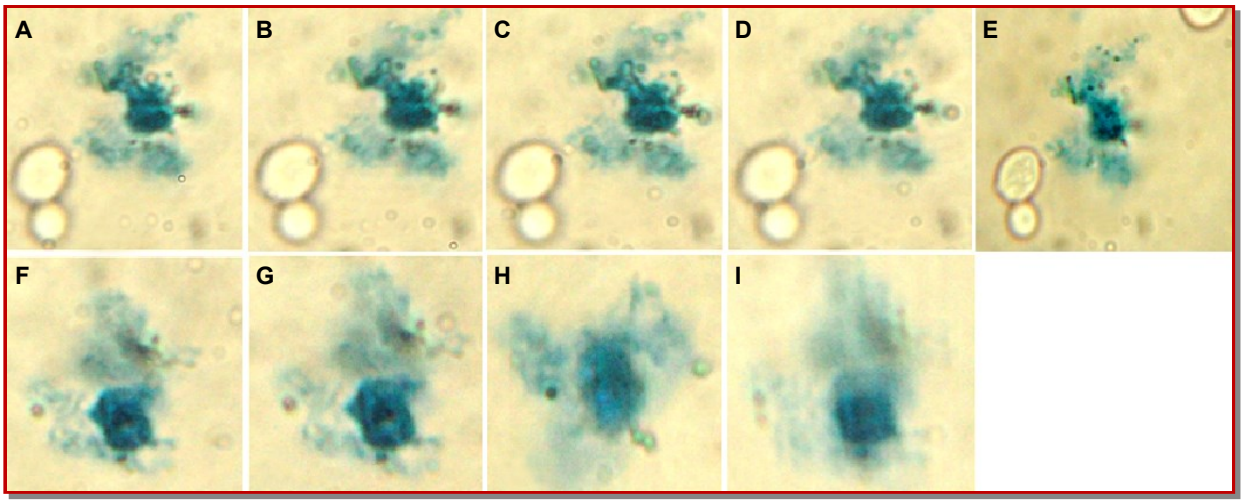

Figure 11: Sequential process of cell necrosis in yeast cells due to effect of *Aspergillus niger* lapachol

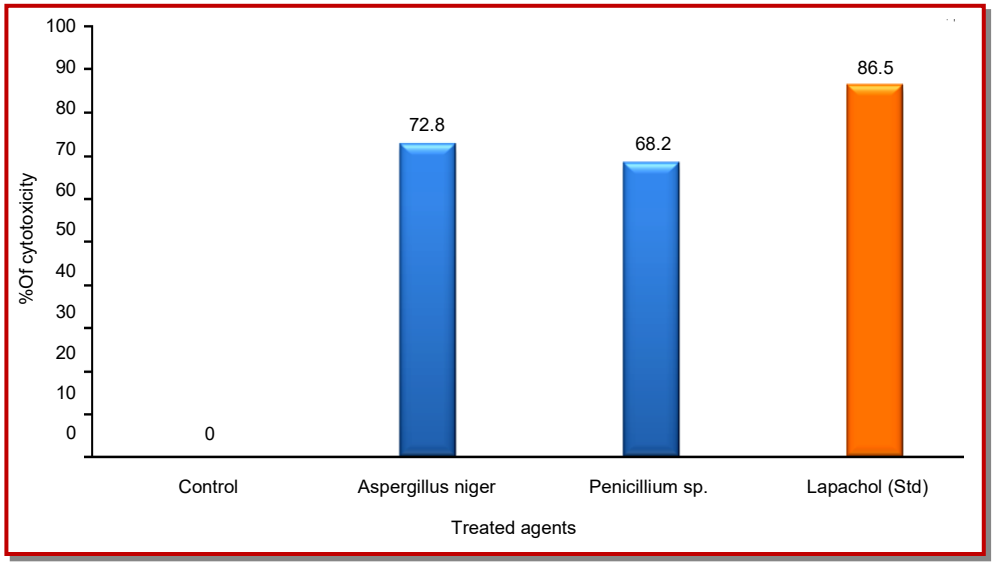

Figure 12: *In vitro* antiproliferative activity of endophytic extracts and standard

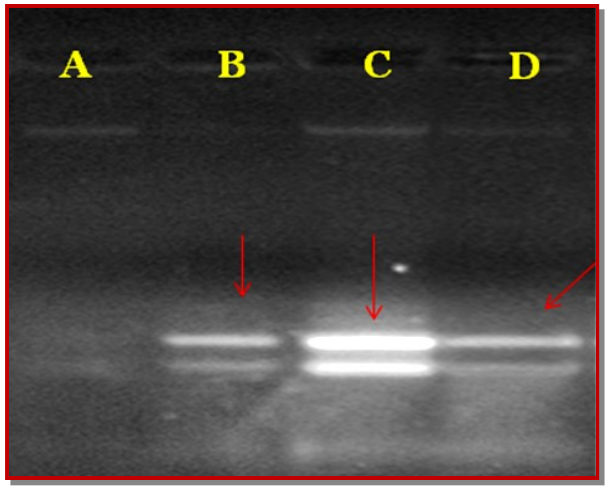

Figure 13: DNA fragmentation due to potent activity of endophytic lapachol on yeast cells

A: Distilled water, B: standard lapachol, C: *Aspergillus niger* lapachol, D: *Penicillium* sp. lapachol, arrow denotes the DNA fragmentation

of *A. niger* and *Penicillium* sp. lapachol induced the antimitotic, anti-proliferative and DNA fragmentation

Table II

Presence of naphthoquinone and lapachol in different endophytes

Endophytes	Napthoquinone	Lapachol
Alternaria alternata [10]	+	+
Alternaria alternata [3]	+	+
Alternaria alternata [12]	-	-
Alternaria sp. [6]	+	+
Alternaria sp. [7]	-	-
Aspergillus niger [5]	+	+
Fusarium oxysporum [2]	-	-
Fusarium oxysporum [1]	-	-
Fusarium sp. [9]	-	-
Penicillium sp. [4]	+	+
Rhizopus sp. [11]	-	-
Unidentified fungi [8]	-	-
Bark	+	+
Leaf	+	+
Stem	+	+

(+)= present, (-)= not present, each experiment was repeated for thrice for each

Table I

List of endophytes isolated from different parts of *Tabebuia argentea*

Endophytes	Parts used	Parts used	Parts used
Endophytes	Bark	Leaf	Stem
Alternaria alternata	+ [10]	+ [3]	+ [12]
Alternaria sp.	-	+ [6]	+ [7]
Aspergillus niger	-	+ [5]	-
Fusarium oxysporum	+ [2]	+ [1]	-
Fusarium sp.	-	+ [9]	-
Penicillium sp.	-	+ [4]	-
Rhizopus sp.	-	+ [11]	-
Unidentified fungi	+ [8]	-	-

(+)= present, each experiment was repeated for thrice for each. Parenthesis given numbers are given for identification of endophytes from different parts origin

activity and by this results clearly indicating that it has potential role in cancer management like plant lapachol. The antimitotic activity, the endophytic lapachol arrest the cell growth in cell cycle as reduce the rate of cell division by preventing the entry of cell into the other different stages. This result is confirmation with the reports of others (Shwetha et al., 2012; Bhujbal et al., 2011; Jangala et al., 2012; Ping et al., 2012; Sehgal et al., 2006).

Now-a-days, yeast is using as a model system for anti-cancer drug discovery in *in vitro* assays against enzyme inhibition or selective toxicity or in screen using model organism. Use of yeast as model organism for anti-cancer drug discovery is that of the process affected by cancer related genetic and epigenetic alterations are fundamentals to all living organisms; cell cycle progression, DNA replication and segregation, maintenance of genomic integrety and stress responses are highly conserved among eukaryotes, making yeast an attactive model in which to results in premature death of the living cells. It was observed with endophyte, *A. niger* lapachol.

In the present investigation of cell anti-proliferative activity, most of the cells died and cell debris was observed due to the treatment of endophytic lapachol toxicity. An interesting point, we have observed that yeast cell necrosis sequentially due to lapachol toxicity. Similar results were reported by using cancer cell lines using plant extracts (Türk et al., 2011; Yu et al., 2007). Another set was subjected to DNA fragmentation assay concludes the breakdown of yeast DNA due to the effect of endophytic lapachol.

Conclusion

Thus, it can assume the possible mechanism of 50% concentration of lapachol anti-cancer activity it induces strong cytotoxicity, anti-proliferative, DNA fragmentation activity in tested experiments.

Financial Support

Visvesvaraya Technological University, Karnataka, India

Conflict of Interest

Authors declare no conflict of interest

Acknowledgements

We thank Dr. M. R. Hulinaykar, Sri Shridevi Charitable Trust and Dr. K. Sukumaran, Principal, SIET, Tumkur, India for encouragement and Dr. S Lokesh, Applied Botany and Biotechnology, University of Mysore, Mysore, and Dr. P. Sharanappa, Department of Studies in Biosciences, University of Mysore, Hemagangothri, Hassan, India for assistance in identifying endophytic fungi.

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