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Antioxidant and in vivo genoprotective effects of phenolic compounds identified from an endophytic *Cladosporium velox* and their relationship with its host plant *Tinospora cordifolia*

Bahaderjeet Singh [a], Prince Sharma [b], Arun Kumar [c], Pooja Chadha [b], Ramandeep Kaur [c], Amarjeet Kaur [a, *]

[a] *Department of Microbiology, Guru Nanak Dev University, Amritsar 143005, India*
[b] *Department of Zoology, Guru Nanak Dev University, Amritsar 143005, India*
[c] *Department of Biotechnology, Guru Nanak Dev University, Amritsar 143005, India*

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ABSTRACT

Ethnopharmacological relevance

Tinospora cordifolia (Willd. Hook. f. & Thomson; family: Menispermaceae), has a long history of use in various traditional medicinal systems including "Ayurveda". It is reported to possess anticancer, antidiabetic, antimicrobial, antispasmodic, and antiinflammatory activities. *T. cordifolia* has also been well documented for production of various bioactive metabolites and their antioxidant activity, but the microorganisms associated with it have been least explored for the same properties.

Aim of the study

Aim of the present study was to evaluate antioxidant and in vivo genoprotective potential of phenolic compounds produced by an endophytic fungus *Cladosporium velox* TN-9S isolated from *T. cordifolia*.

Materials and methods

The isolate of *C. velox* TN-9S was cultivated in malt extract medium and extracted with ethyl acetate. Total phenol content was determined by Folin Ciocalteu reagent. The antioxidant activity was measured in terms of DPPH and FRAP assay. The in vivo genoprotective activity was assessed using fish *Channa punctatus* as model. Identification of phenolic compounds was carried out using RP-HPLC. The fungal extract was evaluated for biosafety using *Salmonella typhimurium* His^- strain and CHO cell lines for mutagenicity and cytotoxicity, respectively.

Results

The total phenolic content in the ethyl acetate extract of the fungus was determined to be 730 µg gallic acid equivalent/mL. The extract evinced significant antioxidant activity with IC_{50} value of 22.5 µg/mL in DPPH scavenging assay. The phenolic extract showed good in vivo genoprotective activity against the genetic damage induced in fish *C. punctatus* after treatment with a non-ionic surfactant 4-nonylphenol. RP-HPLC analysis revealed the presence of peaks corresponding to various phenolic compounds in the extract. Mutagenicity and cytotoxicity results revealed the extract to be nonmutagenic and non cytotoxic in nature.

Conclusion

The results indicate the potential of an endophytic *C. velox* isolated from *T. cordifolia* as a producer of phenolic compounds with antioxidant and genoprotective activities which could be exploited in pharmaceutical industry. The ability of endophytes to produce similar compounds as the host, is also revealed in the present study.

1. Introduction

Reactive oxygen species (ROS) are a class of highly reactive molecules derived from the metabolism of oxygen. Rapid production of free radicals may lead to oxidative damage to biomolecules and result in degenerative disorders such as cancer, diabetes, neural disorders and ageing (Hyun et al., 2006; Sas et al., 2007). Antioxidants are molecules, which can scavenge free radicals and prevent cellular damage by reducing the oxidative stress and therefore have a beneficial effect on human health. A number of antioxidants are known to provide protection against several diseases. Recently, increasing attention has been focused on the use of natural antioxidants, such as ascorbic acid, tocopherols, phenolic compounds including flavonoids, phenolic acids and volatile compounds for preventing oxidation of biomolecules which can lead to cell injury and death (McCord, 2000). Antioxidants have also been reported for their genoprotective potential as they prevent cellular damage against several mutagenic compounds (Matkowski and Wołniak, 2005).

Many plants have been widely reported for the production of antioxidants (Guleria et al., 2013; Premanath and Lakhmidevi, 2010). *T. cordifolia* known as amrita (guduchi) in Sanskrit, is widely used in folk and ayurvedic systems of medicine (Singh et al., 2003; Saha and Ghosh, 2012). It is reported to possess-osteoporotic, antiinflammatory, immunomodulatory, anticancer, antioxidant and antidiabetic

* Corresponding author.
Email addresses: sidhubahaderjit@yahoo.in (B. Singh); princeranayal@gmail.com (P. Sharma); arunbiochemju@gmail.com (A. Kumar); poojachadha77@yahoo.co.in (P. Chadha); gndu.ramandeep@gmail.com (R. Kaur); amarjeet_b@rediffmail.com (A. Kaur)

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properties. It also used as a remedy for the treatment of rheumatoid arthritis and urinary disorders (Rajalakshmi et al., 2009; Abiramasundari et al., 2012; Aranha et al., 2012; Sharma et al., 2012). Several chemical compounds like N-formylannonain, cordifolioside A, magnoflorine, tinocordiside, syringing, phenols, tannins, flavonoids, saponins and polyphenols have been reported from *T. cordifolia* by various researchers after phytochemical analysis (Yadav and Agarwala, 2011; Upadhyay et al., 2014; Bala et al., 2015). It is possible that the microorganisms residing in the plant could possess similar activities or compounds as the plant. This has been exemplified in the case of paclitaxel which is an anticancer agent found in yew trees (*Taxus* spp.) and the endophytes residing in it (Stierle et al., 1993). Exploration of endophytic microbes is expected to yield important metabolites that are produced by the host plant as a result of genetic exchange and evolution of a long relationship (Tan and Zou, 2001). A microbial source of such bioactive compounds would be easier and economically viable to produce.

An endophytic fungus *Cladosporium velox* TN-9S possessing high phenol content was isolated in our lab from *T. cordifolia*. As phenolics are reported to exhibit antioxidant activities, this culture was selected for in vitro antioxidant & in vivo genoprotective studies.

2. Materials and methods

2.1. Materials

1,1-diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteu reagent, ascorbic acid, aluminium chloride, sodium nitrate, sodium hydroxide were purchased from Himedia (India). Gallic acid (98%, SRL), catechin (≥99%, Sigma), chlorogenic acid (98%, SRL), epicatechin (≥90%, Sigma), caffeic acid (≥98%, Sigma), umbelliferon (99%, Sigma), coumaric acid (≥99%, Sigma), ellagic acid (≥95%, Sigma) and *tert*-butylhydroquinone (97%, Sigma) and all other chemicals used were of analytical grade. Chemicals of Himedia and SRL have been supplied by Namco agencies, Amritsar while Sigma chemicals were supplied by Meenakshi trading company, Amritsar, Punjab, India..

2.2. Microorganism and plant used

Microorganism used in the present study was an endophytic *C. velox* TN-9S (MTCC 12032) isolated from stem of *T. cordifolia* (deposited vide voucher no. 423 dated: June 3, 2016 in Herbarium, Department of Botanical and Environment Sciences, Guru Nanak Dev University, Amritsar, Punjab, India). Isolation was carried out from different parts of visibly healthy plant (stem, leaves and bark) of *T. cordifolia* collected from Guru Nanak Dev University campus, Amritsar (India), a region located in Indo Gangetic plains of North West part of Indian subcontinent (Singh et al., 2015). Stem and leaves of the same plant were used for analysis of phenolic components.

2.3. Production of polyphenols from C. velox

Production of polyphenols was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of liquid production medium (malt extract 20 g/L, dextrose 20 g/L, proteose peptone 1 g/L, pH 5.5) inoculated with one plug (8 mm diameter) taken from the periphery of actively growing culture. The flasks were incubated at 250 rpm on a rotary shaker at 30 °C for 10 d. Thereafter, the culture broth along with the fungal biomass was extracted with 50 mL of ethyl acetate under shaking conditions at 120 rpm and 40 °C for 2 h twice. The upper organic phase thus obtained was separated and concentrated on rotary evaporator (BUCHI). The concentrated samples were then re-suspended in 1 mL of HPLC grade water for further studies (Singh et al., 2012).

2.4. Determination of total phenolic content (TPC)

TPC of the concentrated fungal extract obtained was determined using Folin-Ciocalteu (FC) assay as described by Zhang et al. (2006) with minor modifications. Ten μL of extract and 100 μL FC reagent was incubated for 5 min followed by addition of 80 μL of 7.5% sodium carbonate solution and mixed well. This was kept in dark at room temperature for 30 min. The absorbance was measured at 750 nm on ELISA plate reader. Quantification of phenol content was achieved by using the standard curve generated from the gallic acid standard over a concentration range of 0.10–0.80 mg/mL. TPC was expressed as gallic acid equivalent (GAE) in mg/mL of the extract.

2.5. Antioxidant activity

2.5.1. DPPH free radical scavenging assay

The free radical scavenging activity of fungal extract at different concentrations was measured by the method of Blois (1958) with minor modifications. One mL of 0.1 mM 1,1-diphenyl-2- picrylhydrazyl (DPPH) in methanol and 0.5 mL of test sample at different concentrations were added in the test tubes and the change in absorbance was measured at 517 nm 30 min later. The positive control contained 0.5 mL of methanol instead of test sample. L-ascorbic acid was used as a reference standard. The standard curve generated from the ascorbic acid standard over a concentration range of 0.10–0.80 mg/mL. The percentage (%) of inhibition was calculated by the following formula:

$$\%\ \text{inhibition} = \text{absorbance of control} - \text{absorbance of sample} / \text{absorbance of control} \times 100$$

2.5.2. Ferric (Fe^{3+}) reducing antioxidant power (FRAP) assay

Ten microlitre of fungal extract was mixed with 15 μL of 0.1 M sodium phosphate buffer (pH 6.6) and 15 μL of potassium ferricyanide (1% w/v) and incubated at 50 °C for 20 min (Ak and Gulcin, 2008) with minor modifications. The reaction mixture was acidified with 15 μL of trichloroacetic acid (10%) and mixed well. To this, 55 μL of distilled water and 110 μL of ferric chloride (0.1%w/v) were added and the absorbance was measured at 700 nm on a ELISA reader. Increased absorbance of the reaction mixture indicated increased reducing potential. L-ascorbic acid was taken as a reference standard.

2.5.3. In vivo genoprotective activity

To study the in vivo genoprotective effect of fungal extract the freshwater air breathing fish *C. punctatus* was procured from local market having an average weight and length of 17±3.2 g and 12±1.9 cm, respectively. *C. punctatus* is distributed throughout India and is of high commercial importance due to its food value and availability throughout year. The fish were fed on boiled eggs and fecal matter was removed daily to reduce the ammonia content in water. Three groups of fish were studied. One group was of control fish, 2nd group was treated for 72 h with 0.63 mg/L of 4-nonylphenol (4-NP) which is 1/2 of LC_{50} determined in earlier study (Sharma et al., 2014) and in the 3rd group, the fish were pre-treated with the intramuscular

injection of extract of fungus (1 mL/100 mg of body weight) and then the fish was exposed to the same concentration of 4-NP for 72 h.

Micronuclei assay was carried out according to Palhares and Grisolia (2002). Blood from gill was smeared on to the slides from experimental as well as control group. After air drying slides were fixed in absolute ethanol for 10 min and stained with 10% Giesma. Finally, the slides were analysed for the presence of nuclear abnormalities (notched, binucleated, micro nucleated, blebbed, lobed etc.) under a binocular microscope using a 100× oil immersion lens. In each fish 1000 erythrocytes were counted from experimental and control group.

2.6. Reverse Phase High performance liquid chromatography (RP-HPLC)

2.6.1. Sample preparation of fungal extract for RP-HPLC

Fungal extract was prepared as described above. Ten µL extract was loaded on RP-HPLC from the fungal extract containing total phenolic compounds equivalent to 730 µg/mL of gallic acid.

2.6.2. Sample preparation of stem and leaf of T. cordifolia for RP-HPLC

The stem and leaves of plant *T. cordifolia* were dried at 30–35 °C and ground to fine powder and precisely weighed. Five gm powder of both stem and leaf was dipped in 30 mL of ethyl acetate for overnight, the ethyl acetate extract was collected and distilled under reduced pressure. The procedure was repeated twice after addition of fresh solvent. The combined extract was distilled and subjected to evaluation of total phenol content. The dried extracts were resusupended in water and 10 µL extract containing total phenolic compounds equivalent to 1 mg/mL of gallic acid was loaded on RP-HPLC.

2.6.3. RP-HPLC of fungal and plant extract

The RP-HPLC analysis was performed on Nexera UHPLC (Shimadzu) system. The system was equipped with LC-30 AD quaternary gradient pump, SPD-M20 A diode array detector (DAD), CTO-10 as VP column oven, DGU-10 A5 prominence degasser, and SIL-30 AC Nexera auto sampler. The extracts were filtered through 0.2 µm filter and loaded on Microsorb–MV 100-5 C18 (150×4.6×5 µm particle size) column (Agilent, USA) with mobile phase solution I (0.1% acetic acid pH 3.2) and solution II (methanol) (70:30) at flow rate of 1 mL/min. The column oven temperature was 25 °C and the full loop injection was 10 µL (Kaur et al., 2015).

2.7. Safety evaluation of C. velox extract

2.7.1. Ames test for mutagenecity testing

The mutagenicity of the fungal extract was determined using *Salmonella* histidine point mutation assay using his$^-$ *Salmonella* Typhimurium strain (MTCC-1251, IMTECH, Chandigarh, India) as proposed by Maron and Ames (1983). For toxicity testing, 0.1 mL of bacterial culture and 0.1 mL of phenolic extract was added to 5 mL of top agar and poured onto the minimal agar plates followed by incubation at 37 °C for 48 h. To determine the spontaneous reversion sodium azide (5 µL of 17.2 mg/mL) was used as a positive control while 0.5% DMSO was used as negative control. After incubation for 48 h, the number of revertant His$^+$ bacteria colonies were scored. The mutagenic potential of the extract was determined by comparing the number of colonies with control plates where no test compounds were added.

2.7.2. SRB (sulforhodamine B) assay for cytotoxicity testing

CHO (Chinese Hamster Ovary) cell line was procured from National Centre for Cell Science (NCCS), Pune. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) with 10,000U Penicillin, 10 mg streptomycin and 25 µg amphotericin B/mL in a CO_2 incubator (5% CO_2; 90% RH) at 37 °C. The in vitro cytotoxicity of fungal extract was determined by SRB Assay (Skehan et al., 1990). 3×10^3 cells were added in each well of 96 well plate and incubated at 37 °C, 5% CO_2 for 24 h. Different concentrations of the sample were used (125, 250, 375 and 500 µg/mL) for cytotoxicity assay. The dried sample was dissolved in water, 10 µL of each compound added to 96 well tissue culture plate in triplicates. The cells were fixed with 50 µL of ice cold 50% (w/v) trichloroacetic acid at 4 °C for 1 h. To the washed and air dried plate, 100 µL SRB (0.057% w/v SRB in 1% acetic acid solution) solution was added and incubated at room temperature for 30 min. The unbound SRB solution was removed by washing with 1% acetic acid solution and plates were air dried. 200 µL of 10 mM Tris base solution (pH 10.5) was added in each well and shaken on a gyratory shaker for 5 min. The optical density was measured at 510 nm by microplate reader. Percentage of cell growth inhibition was calculated by using formula below.

% cell viability=(OD of treated cells/ OD of control)×100.

2.8. Statistical analysis

Each experiment was performed in triplicate. To compare difference in means, one way analysis of variance (ANOVA) with Tukey's test at $P\leq0.05$ was performed. SPSS software for windows version 16.0 (SPSS Inc., Chicago) and microsoft office excel 2007 (Microsoft Corp., USA) were used to perform the statistical analysis.

3. Results

3.1. Total phenol content and antioxidant activity of extract obtained from C. velox

The detailed antioxidant potential of fungal extract obtained from *C. velox* was evaluated with various antioxidant assay techniques. The results of Folin-Ciocalteu assay revealed significant amount of total phenolic compounds equivalent to 730 µg/mL of gallic acid in the fungal extract. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The fungal extract significantly reduced the DPPH radicals in a dose dependent manner with an IC_{50} of 22.5 µg/mL using DPPH. Maximum scavenging activity of 80% was obtained at a concentration of 22.5 µg/mL. Reductive ability of the phenolics extract was investigated by ferric ion-ferrous ion transformation in the presence of fungal extract in the ferric reducing antioxidant power (FRAP) assay.

3.2. 3.2 In vivo genoprotective potential of extract

The fungal extract was also evaluated for its in vivo genoprotective potential. The nuclear abnormalities in blood cells from gill tissue among the three groups studied are revealed in Fig. 1..

The % nuclear abnormalities frequency was found to be lowest for control group (4.64±0.053). In the group, which was treated with nonylphenol, the value was found to be significantly higher than the control group (29.35±0.442). While in the third group which was pretreated with extract of fungus + nonylphenol, a decrease in the nuclear abnormalities was observed and the value was determined to be

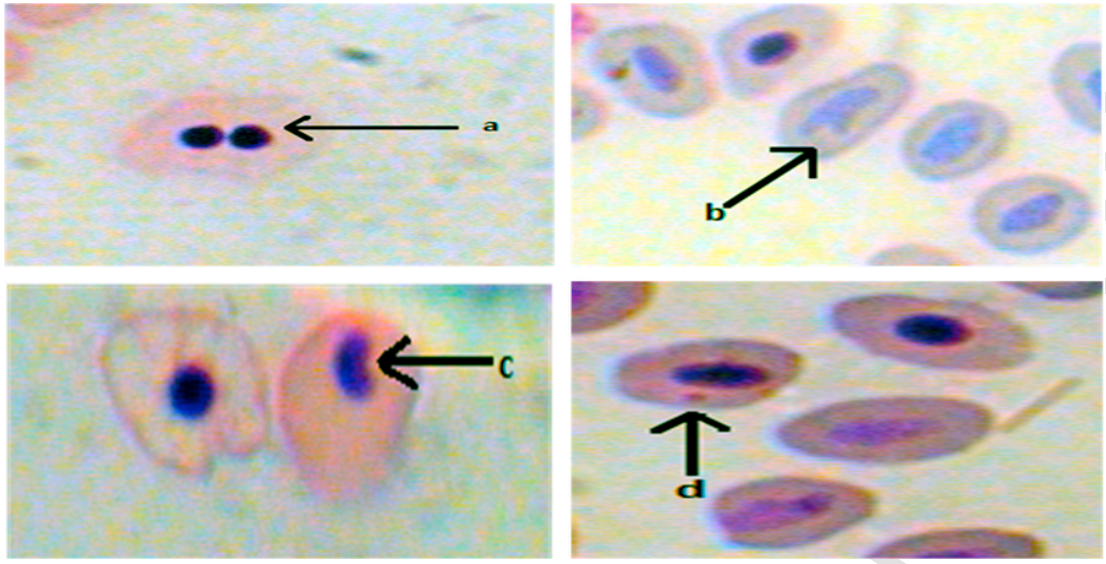

Fig. 1. (a-d): Nuclear abnormalities observed (a) binucleated (b) blebbed (c) notched (d) micronucleated cell.

24.27±0.159. One way analysis of variance was applied to study the effect of nonylphenol and nonylphenol+extract followed by post hoc Tukey's test ($F=2288.712$, $P\leq0.0001$). Tukey's test revealed a significant difference between the three groups. The nuclear abnormalities caused due to 4-NP got significantly reduced in the group pretreated with extract of fungus indicating the genoprotective effect of the fungal extract (Fig. 2)..

3.3. Identification of phenolic compounds in fungal extract using RP-HPLC

Attempts were made to identify the phenolic compounds responsible for antioxidant activity. The fungal extract was subjected to RP-HPLC for analytical analysis. The results obtained in RP-HPLC analysis revealed peaks at retention times of 2.482, 3.837, 4.574, 6.013, 6.796, 9.292, 10.703, 15.719 and 16.077 min, which were comparable to the retention times of gallic acid, catechin, chlorogenic acid, epicatechin, caffiec acid, umbelliferon, coumaric acid, ellagic acid and *tert*-butylhydroquinone, respectively on comparison with standard phenolic compounds (Fig. 3A)..

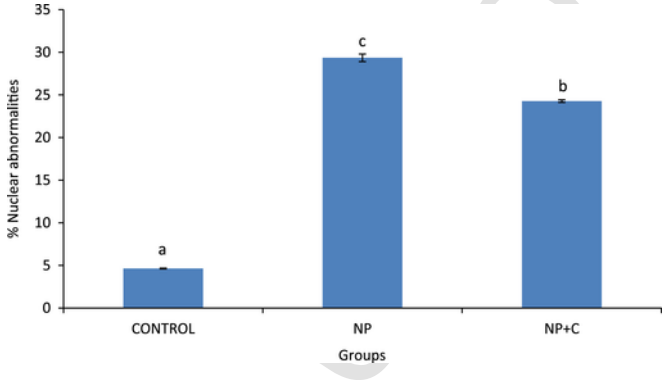

Fig. 2. % Nuclear abnormalities observed in different groups. NP (4-nonylphenol), NP+C (4-nonylphenol+fungal extract). Columns and bars represent the means±SE. Means followed by different superscript letters within a column are significantly different. Tukey's test p≤0.0001.

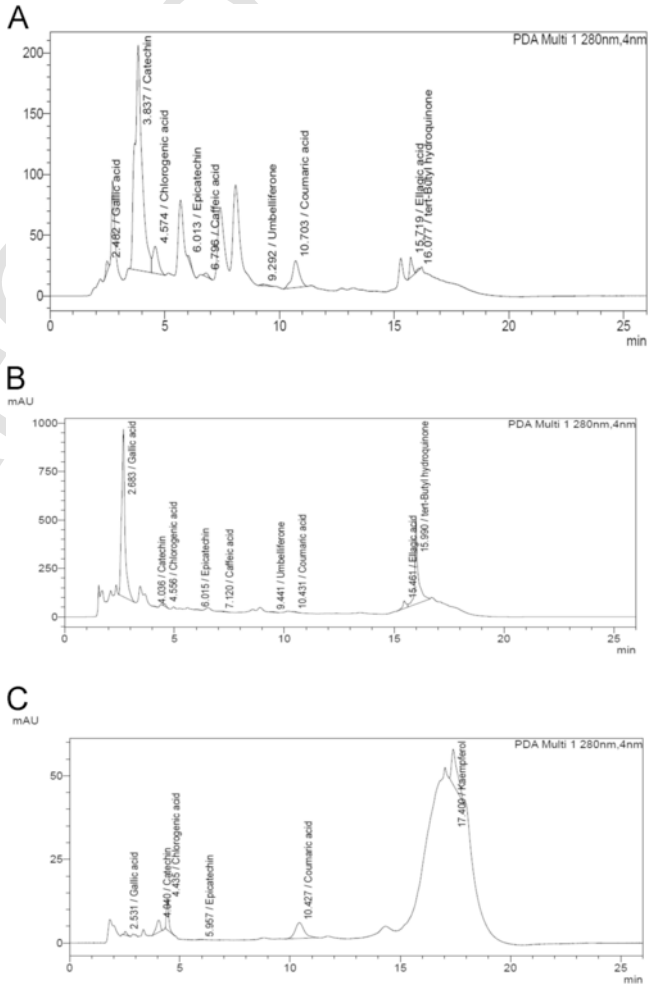

Fig. 3. (A): RP-HPLC chromatogram of the extract obtained from endophytic *C. velox* TN-9S, detected at 280 nm. (B): RP-HPLC chromatogram of extract obtained from stem of *T. cordifolia* detected at 280 nm. (C): RP-HPLC chromatogram of extract obtained from leaf of *T. cordifolia* detected at 280 nm.

3.4. Comparative analysis of extract of T. cordifolia and C. velox using RP-HPLC

Comparative analysis of phenolic profile of *T. cordifolia*., leaf, stem and the endophyte *C. velox* isolated from stem was carried out. RP-HPLC analysis revealed that the endophyte isolated from stem contained similar phenolic compounds as the stem of the host plant. In addition to other phenolics, both the endophyte and stem extract showed the presence of peaks corresponding to ellagic acid whereas kaempferol was absent (Fig. 3B). In case of leaf peak corresponding to kaempferol was present in high amount (Fig. 3C).

3.5. Biosafety evaluation

To determine the biosafety of the compounds, the extract obtained from the endophytic *C. velox* was subjected to Ames mutagenicity test and SRB cytotoxicity test. Number of revertant colonies appeared in the positive control, whereas the bacteria incubated with fungal extract did not show any revertants (Fig. 4)..

SRB cytotoxicity assay revealed mild cytotoxic effect at high concentration of the extract (10% cell death) which was not significant when compared with positive control containing doxorubicin (50%) (Fig. 5)..

4. Discussion

The compounds obtained in the present study from *C. velox* were determined to be phenolic in nature in RP-HPLC analysis. Phenolic antioxidants are usually products of secondary metabolism in plants/ microorganisms and their antioxidant activity is mainly due to their redox properties and chemical structure (Mohamed et al., 2013). The antioxidant activities of extracts are often correlated with their total phenol contents. High phenol content was detected in the selected strain which led to further determination of antioxidant activity. DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants (Naik et al., 2003). The small percentage of remaining DPPH in the presence of extract in dose dependent manner indicated presence of good amount of antioxidants. Strong reducing activity was also observed in the extract after FRAP assay, confirming the antioxidant activity.

Antioxidant activity of the phenolic acids, alkaloids and flavonoids has been reported from several plants and fungi (Guo et al., 2012; Mayur et al., 2010; Jakovljevic et al., 2014). The phenolic compounds produced by the endophyte *C. velox* in the present study have been reported for various biological activities viz. antimicrobial, antioxidant, antidiabetic, antiproliferative, antihypertensive etc. (Narayanan et al., 1999; Seeram et al., 2005; Friedman et al., 2006; Wang et al., 2009; Calderon-Montano et al., 2011; Zhao et al., 2011; Onakpoya et al., 2014). Positive association between the consumption of foods containing polyphenolics and a reduced risk of developing several disorders such as cancer and cardiovascular diseases has been observed (Pandey and Rizvi, 2009). It was observed that both the host plant and the endophyte isolated from it produced similar spectrum of phenolic compounds. A microbial source of such important bioactive compounds could be helpful in their easy, economic and large scale production. Secondly, if the endophytic microorganisms can produce the same rare and important bioactive compounds as their host plants, it would help to preserve the world's ever diminishing biodiversity (Muller, 2015). Previous researchers have also demonstrated the presence similar of activities and compounds in the host plants and the endophyte isolated from them. Azadirachtin, an insecticidal compound exclusive to *Azadirachta indica* was also reported to be produced by an endophytic *Eupenicillium* residing in it (Kusari et al., 2012). Huperzine A is an acetylcholinesterase inhibitor produced by the plant *Huperzia serrata* as well as two endophytic fungi *Shiaria* sp. and *Cladosporium cladosporoides* residing in it (Zhu et al., 2010; Zhang et al., 2011). This strengthens the opinion that a plant can contain several endophytic microbes that can produce biological compounds or secondary metabolites as a result of genetic transfer from the host plant into endophytic microbes.

In vivo genoprotective effect of the fungal extract was also observed in the present study. Polyphenols have been reported to show antioxidant and genoprotective properties (Ramos et al., 2008, Lima et al., 2006). Wilms et al. (2005) reported the protective effects of quercetin against the formation of oxidative DNA damage in human lymphocytes induced by H_2O_2. Quercetin and caffeic acid are also reported to have genoprotective effect against H_2O_2 induced DNA damage (Szeto and Benzie, 2002). In the present study the fungal extract has been found to show genoprotective efficacy against the DNA damage induced after treatment with a non ionic surfactant nonylphenol. Micronuclei assay has been used as index for cytogenetic damage assessment. Several scientists have identified nuclear abnormalities including nuclear bud, fragmented nucleus, lobed nucleus and binucleated cells as an indicator of genotoxicity (Ayllon and Garcia-Vazquez, 2000; Talapatra and Banerjee, 2007). These nuclear abnormalities may arise due to detrimental effect produced by the clastogenic agents. According to Wu et al. (2005) the metabolism of surfactant by organism may produce highly reactive oxygen species (ROS). But pretreatment with fungal extract was found to show genoprotective effect in fish. This genoprotective efficacy

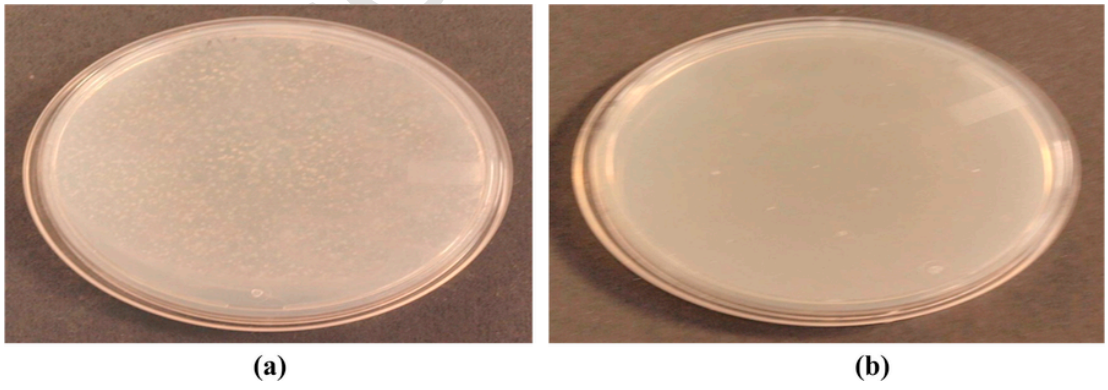

Fig. 4. Visible colony count studies for mutagenicity testing in Ames test. (a) positive control (b) fungal extract from *C. velox* TN-9S.

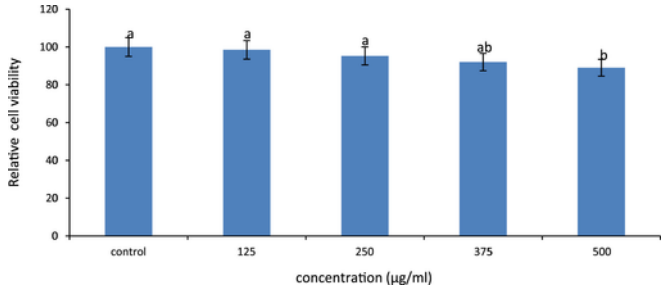

Fig. 5. Cytotoxicity testing of the extract obtained from *C. velox* TN-9S by SRB assay. Columns and bars represent the means±S.E, followed by different superscript letters within a column are significantly different. Tukey's test $p \leq 0.001$.

could be attributed to antioxidant activity of various phenolic compounds present in the extract. Antioxidants are known to prevent cellular damage against several mutagenic compounds (Matkowski and Wolniak, 2005; Kumar et al., 2012). The protective effect of two dietary antioxidants ellagic and chlorogenic acid has been reported by Pavlica and Gebhardt, (2005) against oxidative stress in PC12 cells.

5. Conclusion

The endophytic fungus, *C. velox* isolated from *T. cordifolia* in the present could be used as a source of phenolic compounds with antioxidant and genoprotective potential. The phenolic compounds obtained may be exploited in varied pharmaceutical applications. The nonmutagenic and noncytotoxic nature of these compounds serves to enhance their potential. The present study also emphasizes the fact that endophytes have the ability to synthesize similar compounds as their host plant.

Conflict of interest

The authors do not have any conflict of interest.

Author contribution statement

Amarjeet Kaur was involved in the conception of the idea, designing of experiments, analysis of results and preparation of the manuscript. Pooja Chadha designed the experiments to assess the genoprotective potential and was involved in the preparation of the manuscript. Ramandeep Kaur designed the experiments to evaluate the bisafety. Bahaderjeet Singh, Prince Sharma and Arun Kumar performed the experiments, helped in the analysis of the data and preparation of the manuscript.

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