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ANTIOXIDANT POTENTIALS OF ENDOMYCOPHYTES ISOLATED FROM ADIANTUM SP.

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ABSTRACT

Endomycophytes are fungi residing inside healthy plant tissues without any discernible infectious symptoms. Endophytic strains of many plants produce a plethora of secondary metabolites, most of which are of immense pharmaceutical importance. With the increase in ROS linked cancers emerging owing to the modern lifestyle, an attempt at screening natural sources for free radical scavenging properties is inevitable. In the present study, ten endomycophytes have been isolated from the Adiantum sp. fern collected from the Western ghats and subjected to antioxidant potential screening by DDPH assay and NO assay in Colo320 cell lines. Results showed significant inhibition of DPPH radicals at 67.32% and NO radicals at 72.06% by the A21 extract.

Keywords: DPPH, NO, Colo320.

INTRODUCTION

Natural products have been playing a major role in the search for novel drugs for numerous illnesses including cancer (Butler and Newman 2008; Molinari 2009). The plant extracts have traditionally been screened for such discoveries, however many of the endophytic fungi have also been shown to possess associated plant active principles such as Taxol®, vincristine, podophyllotoxin, camptothecin, etc. (Stierle *et al*.,1993; Eyberger *et al*.,2006; Kusari *et al*., 2009; Kumar *et al*., 2013). Moreover, these endophytes have been shown to be a versatile source of many new biologically active chemical structures (Aly *et al*., 2010). Many endophytic fungi are added every year to this ever growing list of biologically relevant strains producing valuable drug candidates which is a testimony to the fact that only a tiny fraction of an estimated one million endophytic fungal species has been cultured and screened for their bioactivities (Suryanarayanan *et al*., 2009).

There is increasing evidence indicating that reactive oxygen species (ROS) and free radical-meditated reactions can cause oxidative damage to biomolecules, eventually contributing to, for example, aging, cancer, atherosclerosis, coronary heart ailment, diabetes, Alzheimer's disease, and other neurodegenerative disorders (Finkel and Holbrook 2000). Antioxidants are thought to be highly effective in the management of ROS-mediated tissue impairments. Naturally derived antioxidants have received much attention in recent years (Hu and Kitts 2000; Schulz *et al*. 2002).

The present study involves the analysis of the secondary metabolites for their efficacy against ROS free radicals by DPPH Assay and NO assay in Colo320 cell lines.

MATERIALS AND METHODS

Chemicals and Reagents

Rosewell Park Memorial Institute 1640 (RPMI 1640) medium, DPPH (2,2-diphenyl-1-picrylhydrazyl), Trypsin Phosphate Versene Glucose (TPVG) and Fetal Bovine Serum (FBS) were purchased from HiMedia, India. Dimethyl sulfoxide (DMSO), Penicillin and Streptomycin was bought from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture plastic wares were from Nunc Co. (Denmark). All other chemicals and solvents were of laboratory grade.

Human Colon carcinoma cell lines Colo320 were purchased from the National Centre for Cell Sciences (Pune, India). The cells were cultured in RPMI 1640 containing L- Glutamine and 25 mM 4- [2- hydroxyethyl] –1– piperazineethane sulfonic acid (HEPES), Penicillin (100µg/mL), Streptomycin (50µg/mL) and 10% foetal bovine serum. Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO2.

Sample Preparation

Fresh, disease free aerial parts of the *Adiantum* sp., was collected during the months of June 2015 from the Pangode village, Quilon district, Kerala and transferred to the laboratory within 24 hours. The leaves were subjected to regular washing, surface sterilization (Johannes *et al*., 2006) and aseptically dissected to expose cortex region and placed on to Potato dextrose agar plates, supplemented with Streptomycin 250 mg/L. Aliquots of 1.0 mL of the last wash were also inoculated in PDA to evaluate the effectiveness of the disinfection process. The plates were incubated for 12 to 15 days at 28˚C dark condition. After incubation period the mycelium originating from the tear of the sample was purified and cultured under the same conditions for further bioactivity screening.

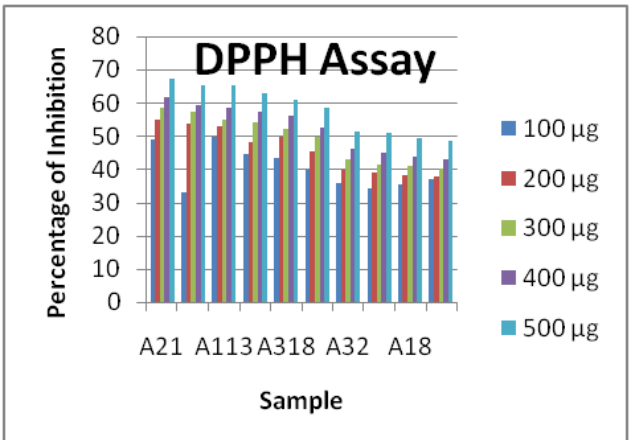

Fig.1. Graphical representation of DPPH assay of secondary metabolites from Endomycophytes of Adiantum sp.

The endophytic fungi were grown in 500 ml conical flasks containing 200 ml PDB. Three mycelial agar plugs (0.5 cm) were used as inoculum and the organism was grown at 25 ± 2 °C statically for 21 days.

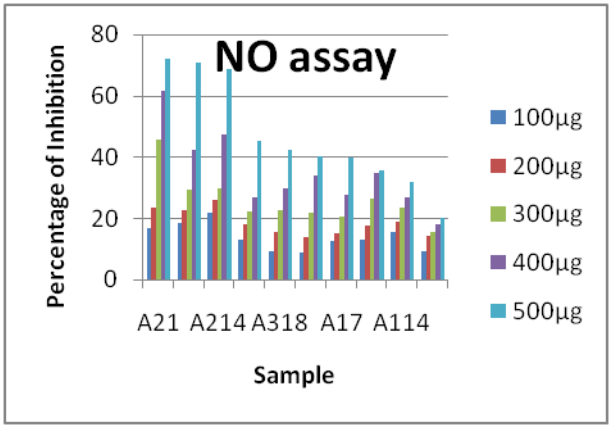

Fig.2. Graphical representation of NO assay of secondary metabolites from Endomycophytes of Adiantum sp.

After incubation, the crude bioactive metabolite was extracted by using separatory funnel with equal volume of chloroform: methanol (3:1). The solvent layer was collected and then evaporated in a rotary evaporator under vacuum.

DPPH assay

DPPH quenching ability of chloroform: methanol extract of endophytes was measured (Blois 1958). Methanol DPPH solution (0.15 %) was mixed with serial dilutions (200–1,000 µg/ml) of the extracts and after 10 min, the absorbance was read at 515 nm. The antiradical activity was expressed as IC_{50} (µg/ml), (the antiradical dose required to cause a 50 % inhibition). Vitamin C was used as standard. The ability to scavenge the DPPH radical was calculated by the following formula:

DPPH radical scavenging activity % $=A_0 - A_1/A_0 \times 100$

Where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate

NO Assay

The nitric oxide assay was performed as described previously with slight modification (Yang *et al*., 2009). After preincubation of Colo320 cells (1.5 × 105 cells/mL) with LPS (1µg/ml) for 24h, the extracts (50µg/ml, 100 µg/ml, 200 µg/ml) were added and incubated for 48h. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was measured using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µl of cell culture medium was mixed with 100 µl of Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader (Tecan, Switzerland). Fresh culture medium was used as a blank in every experiment.

RESULTS AND DISCUSSION

Secondary metabolite extracts of the ten endomycophytes from *Adiantum* sp. were tested for their free radical scavenging properties using DPPH assay and NO assay in Colo320 cell lines.

Figure 1 demonstrates DPPH scavenging activity, expressed in percentage of inhibition, caused by different concentrations of the microbial extracts. DPPH is a purple colour dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple colour fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The weakest radical scavenging activity (34.27%) was exhibited by the A114 extract of 100µg, whereas the strongest activity (67.32%) was exhibited by the A21 extract at a concentration of 500 µg (Figure 1).

The Griess reaction, a spectrophotometric determination for nitrite, was carried out to establish the nitrite levels in conditioned medium of Colo320 cells treated with the extracts. Metabolite extract of A21 showed the highest NO inhibition activity at 72.06%, as shown in Fig.2.

As reviewed by Schulz *et al*. (2002), 51% of the biologically active substances isolated from endophytes were previously unknown. Although a number of bio-pharmacological compounds with antimicrobial, antitumor, antiinflammatory, and antiviral activities have been previously isolated from endophytes (Aly *et al*., 2008; Souza *et al*., 2008; Liu *et al*., 2008), information related to their antioxidant activities is very scanty (Strobel *et al*., 2002). The results of this study are in concurrence with the earlier findings of antioxidant potentials from endomycophytes.

CONCLUSION

In response to the growing consumer demand for food supplements that are free of synthetic antioxidants with carcinogenic potential, such as butylated hydroxytoluene (BHT) (Baardseth, 1989), there is an overwhelming trend to search for naturally occurring antioxidants in the past decades (Gould, 1995; Reische *et al*., 1998).

Since utilizing the plant sources for obtaining these valuable molecules called secondary metabolites present many

formidable challenges towards their sustained supply, e.g. seasonal variations and large-scale deforestation concerns, their endo-microbial sources have generated substantial interest among academicians and commercial entities in the recent times. While plants and plant extracts have been playing an important role in traditional medicine for thousands of years, the discovery of and investigations on fungal and bacterial natural products had only been made possible with the development of natural sciences during the 20th century. The secondary metabolites of microbial origin are well known as valuable supply of lead molecules in the quest for drug candidates against infectious diseases, cancer and many other illnesses (Tejeswi *et al*., 2009).

The current study is an attempt on similar lines to elucidate antioxidant drug candidates from the endomycophytes from ferns, which are abundantly present in the lush green mountains of the western ghats.

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