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Antioxidant and anticancer potential of endophytic fungus *Alternaria tenuissima* PE2 isolated from the leaves of *Psidium guajava L.*

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For reducing pathological conditions generated by free radicals, it is essential to intake of sufficient amount of antioxidant in the body. Although a large number of plants have been studied for novel source of natural antioxidants but endophytic microorganisms as antioxidant potential were not studied sufficiently. In the present endeavor, potent endophytic fungal strain PE2 was isolated from the healthy and mature leaves of *Psidium guajava* L. and was identified as *Alternaria tenuissima* by 28S rDNA sequence homology. EA extract of PE2 was subjected for antioxidant studies following different methods. EA extract displayed very good antioxidant activities through DPPH, ABTS as well as superoxide radical scavenging properties with IC50 values of 63.01± 2.12 µg/ml, 11.21± 2.12ìg/ml and 138.29 ± 3.10ìg/ml respectively. Apart from this, EA extract found effective to inhibit the proliferation of breast cancer cell line MDA-MB-231. Survivability of peripheral blood mononuclear cells in the presence EA extract was also observed. Based on the present study, PE2 are considered as a probable source of bioactive compounds for the development of new anticancer drugs.

Keywords: *Alternaria tenuissima*, antioxidant potential, bioactive compounds, breast cancer cell line, endophytic microorganisms

INTRODUCTION

In recent years, the free radical chemistry becomes an interested area and achieves more attention by scientists as well as the relation of oxidative stress with human disorders is considered as one of important research fields. Free radicals are molecules or part of molecules with an unpaired electron in their atomic or molecular orbital and are produced naturally by various physiological activities in the body.

These are reactive oxygen (ROS) or reactive nitrogen (RNS) species which include superoxide anion (O2•-), hydroxyl (HO•), peroxyl (ROO•) radicals, nitric oxide (NO•) radical, peroxynitrite anion (ONOO-) etc. Although free radicals produced naturally inside the body for performing important role in a number of cellular functions, their uncontrolled production stimulates cellular and molecular damages which eventually cause health disorders. If the body is not able to remove these excess free radicals efficiently, they can cause a wide array of degenerative human diseases like cancer, neurodegenerative disorders, cardiovascular diseases, inflammatory diseases, Alzheimer's disease, Parkinson's disease and others (Finkel and Holbrook, 2000; Gülçin, 2007). Antioxidants are those substances which play important roles to protect the health by providing an electron to the free radical and stop the chain reaction, therefore slow down cellular disruption.The human body possess variety of enzyme systems for scavenging the free radicals. Besides this, various micronutrients viz. vitamin C, vitamin E, beta-carotene are also considered as major antioxidants. These nutrients

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must be supplied in the body through regular diet as our body is unable to produce these (Ramassamy, 2006). Although medicinal plants, fresh vegetables and fruits are the main sources of natural antioxidants, several bioactive compounds obtained from endophytic fungi are also found to have antioxidant properties (Zhao *et al*. 2012; Khiralla *et al*. 2015).

On the other hand, different types of cancers are the leading cause of death worldwide. About 7.6 millions death happened in over the world due to cancer and it is predicted that this number will increase to 13.1 millions in 2030 (WHO, 2013). The majority of the existing anticancer drugs are toxic to normal cells; therefore exhibiting adverse side effects as well as they are less effective against several kinds of cancer (Remesh, 2017). To overcome these, there is a basic need for searching of bioactive metabolites from natural sources. The medicinal plants therefore considered as an alternative source of anticancer drugs due to their intrinsic potential without side effects, as well as low levels of cytotoxicity (Mbaveng*et al*. 2011; Debbie *et al*. 2012). Several plant derived compounds, for example, taxol, vinblastine, vincristine, camptothecin, podophyllotoxins played an important role in the development of potent anticancer drugs (Cragg and Newman, 2004; Srivastava *et al*. 2005). It was already reported that some of the endophytic fungi have the ability to produce similar or identical secondary metabolites as their host plants which revealed promising biological potential (Tan and Zou, 2001; Zhao *et al.* 2011;Jia *et al.* 2016).

Therefore, they have been considered as probable source of novel anticancer drugs (Kharwar*et al.*2011; Schulz *et al.*2002; Newman and Cragg, 2007). The world's first billion dollar anticancer drug taxol was initially discovered from the gymnosperm *Taxus brevifolia*. Later on the taxol producing endophytic fungi *Taxomyces andreanae* was isolated from the plant *Taxus brevifolia* (Strobel, 2003), which significantly reduced the destruction of *Taxus* plant.

Psidium guajava L. (Family Myrtaceae), commonly known as 'guava' is a popular fruit plant as well as a very common home garden tree in India. Different parts of this plant, including leaves, barks, roots and fruits have several medicinal importance likeanti-microbial, anti-oxidant, anti-inflammatory, anti-cancer, analgesic,anti-malarial, anti-diabetic and others (Guti´errez*et al*. 2008).In traditional medicines,it is widely used for a number of illnesses. From various parts of *P.guajava*,numbers of chemical constituents have been reported which includedphenolic compounds,isoflavonoids, carotenoids, catechin, epicathechin, gallic acid, rutin, kaempferol, ascorbic acid, naringenin etc. (Barbalho *et al*. 2012).

In our previous study, we have isolated an endophytic fungus *Alternaria tenuissima* PE2 with bioactive potential from the leaves of *Psidium guajava* L. grown in the lateritic belt of Santiniketan, West Bengal, India. (Chatterjee *et al*. 2022). The EA extract of PE2 was found very effectivefor controlling the growth of pathogenic as well as spoilage bacterial and fungal strains. Apart from this, it was also exhibited anticandidal activity against human pathogenic yeast *Candida albicans* (Chatterjee *et al*. 2022).

The present study was aimed to evaluate the antioxidant activity of the ethyl acetate (EA) extract of potent endophytic isolate *Alternaria tenuissima* PE2 by DPPH, ABTS and superoxide free radical scavenging assays. Besides this, anticancer properties of PE2 were also determined by evaluating its effects on human breast cancer cell line MDA-MB-231. The survivability of normal cells in the present of EA extract were also checked using human peripheral blood mononuclear cells (HPBMCs).

MATERIALS AND METHODS

Collection of plant materials and identification of PE2

The endophytic fungus PE2 was isolated from fresh and mature leaves of *Psidium guajava* L. were collected randomly from plants grown in Santiniketan (23.6816660 °N, 87.6714825 °E), West Bengal, India, during the month of September. (Chatterjee *et al*. 2022).The leaves were collected when the plants were blossomed. The plant was identified by Professor Subrata Mondal, Plant Biosystematics Laboratory,

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The potent isolate PE2 was identified based on the morphological characters as well as by amplification followed by sequencing of D1/D2 region of large sub unit 28S rDNA (Chatterjee *et al.* 2022)

Solvent extraction of PE2

Initially different solvents were used but based on ability of the ethyl acetate (EA) for extracting the bioactive compounds was chosen as the most suitable one. PE2 was grown in 1 l of ME broth at 28 ºC for 21 days. The cell free supernatant (CFS) was collected by filtration with muslin cloth followed by centrifugation at 10,000 rpm for 20 min. Each 100 ml of CFS was mixed with 40 ml of EA, shaken vigorously and the upper EA fraction was collected by separating funnel. The EA portion was evaporated to dryness using rotary vacuum evaporator at room temperature (26 ± 2 °C) and stored at 4 °C for future experimentations.

Study of Antioxidant activity of EA extract of PE2

To evaluate the antioxidant potential of EA extract of PE2, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2' – azino- bis (3- ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radicals and superoxide radical assay were performed.

Determination of antioxidant activity by DPPH free radical scavenging assay

DPPH free radical scavenging assay was carried out following the method described by Lee *et al*. (1998). The dried EA extract was dissolved in methanol at a concentration of 10 mg/ml. 100 ìl of methanolic solutions containing different concentrations of extract of PE2 were mixed with 2900 ìl of DPPH solution and incubated for 30 minutes at room temperature in dark condition. In control set, only 100 ìl of methanol was added to the DPPH solution. The absorbance was recorded at 517 nm after the incubation period and the percentage of inhibition (%I) of free radicals was calculated as: %I = [(A blank-A sample)/A blank]×100, where, A blank: absorbance of control, A sample: absorbance of methanolic solution and DPPH. IC50 value was calculated by plotting straight line equation. The well known antioxidant ascorbic acid was considered as positive control during this assay and its free radical scavenging potential also determined following the same process.

Determination of antioxidant activity by ABTS free radical scavenging assay

Free radical scavenging activities of EA extract of PE2 was also checked using 2, 2'– azino- bis (3- ethylbenzothiazoline- 6- sulphonic acid) (ABTS) radical cation decolorization assay (Re *et al*. 1999). The reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1) produced ABTS·+ cation radical. After 12-16 hours incubation at room temperature in the dark, ABTS·+ solution was diluted with methanol for getting an absorbance of 0.700 ± 0.002 at 734 nm. 5 ìl of sample solutions containing different concentrations of EA extracts were added to 3.995 ml of diluted ABTS·+ solution. After 30 minutes of the initial mixing, the absorbance was measured at 734 nm. Percentage of inhibition was calculated using the formula-

ABTS·+ scavenging effect (%) = [(AB–AA)/AB)]×100 , where,

AB is absorbance of ABTS radical + methanol; AA is absorbance of ABTS radical + sample extract or standard. Trolox was used as a standard in this assay.

Determination of antioxidant activity by superoxide radical scavenging assay

Superoxide radical scavenging activities of the EA extract was determined by using phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system (Fontana et al., 2001). One ml of reaction mixture was prepared by adding sodium phosphate buffer (20 mM, pH 7.4), NADH (73 ìM), nitro blue tetrazolium or NBT (50 ìM), PMS (15 ìM) and EA extract of PE2 (0-250 ìg/ml). Absorbance was recorded at 562 nm in the presence of appropriate blank after five minutes of incubation at room temperature. Quercetin was taken as positive control during this study.

Percentage of inhibition (%I) of each radical was calculated as: %I = [(A blank-A sample)/ A blank] ×100, where, A blank: Absorbance of control (without EA extract of PE2), A sample: Absorbance of sample (with EA extract of PE2). IC50 value was calculated from straight line equation.

Study of anticancer activity by MTT assay

Anticancer study was carried out using human breast cancer cell line MDA-MB-231 after treating them with different concentrations (20- 300 µg/ml) of EA extract of PE2. Cell culture plates were incubated at 37°C in a 5% CO_2 incubator (Thermo Scientific, USA). After 48 hours, cell proliferations were estimated by MTT assay (Mosmann, 1983) where absorbance was taken at 595 nm. Cell survival percentages were calculated considering 100% survival in control set where only equal amount of DMSO was added without any EA extract. 50 mM mitomycin C was used as positive control. Survivability of normal cells in the presence of EA extract were also checked using human peripheral blood mononuclear cells (HPBMCs) using the same process.

Statistical analysis

All the data represented the means of at least three replicates. Means and standard deviation were calculated with the help of Microsoft Excel program version 2007.

RESULTS AND DISCUSSION

Isolation and identification of PE2

The potent strain PE2 with antioxidant and anticancer potential which was isolated from fresh and mature leaves of *Psidium guajava*, were identified as *Alternaria tenuissima* on the basis of its morphological characteristics as observed under light microscope as well as based on D1/D2 region of LSU (Large subunit 28S rDNA) gene sequences (Chatterjee *et al*. 2022).

Antioxidant activities of EA extract of PE2

DPPH free radical scavenging potential

Antioxidants are obtained naturally from different plants, fruits and vegetables, few reports suggested that endophytic organisms also have very good antioxidant potential (Khiralla *et al.* 2015). By considering EA as most suitable solvent for extracting bioactive compounds from endophytic fungi, EA extract of PE2 was subjected for antioxidant studies following different methods. During DPPH free radical scavenging assay in the presence of different concentrations of EA extract of PE2, the pink color DPPH solution turned into yellow along with increased concentration of the extracts which suggested its strong antioxidant property. In the presence of 150 ìg/ml concentration of EA extract of PE2, 91.38% inhibitions of free radicals were recorded. From the standard curve of percentages of inhibition, IC50 value revealed as 63.01 µg/ml for EA extract of (Table 1). On the other hand, ascorbic acid, which was used as positive control in this study, exhibited an IC50 value of 20.23 µg/ml (Table1). Endophytic fungi *Chaetomium* sp. and *Aspergillus* sp. isolated from *Eugenia jambolana* Lam. also exhibited 80% inhibition of free radicals during DPPH free-radical scavenging assay (Yadav *et al*. 2014).

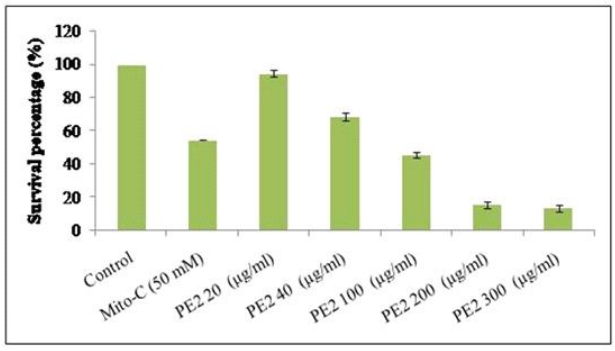

Fig. 1: Survival percentage of Cancer cell lines against different concentrations of EA extracts of endophyte strain PE2

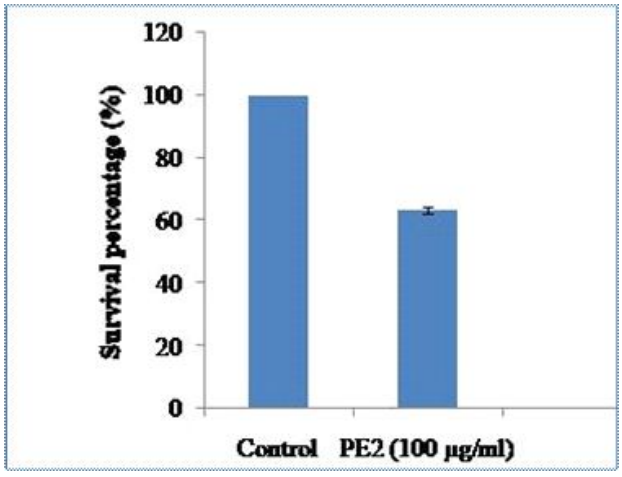

Fig. 2: Survivability of cells using human peripheral blood mononuclear cells (HPBMCs) in the presence of EA extract of PE2

Table 1: DPPH free radical scavenging activities of EA extract of PE2

	Percentage (%) of inhibition	
Concentration (µg/ml)	Ethyl acetate extract of PE2	Ascorbic acid (positive control)
5	5.94 ± 1.88	23.76 ± 1.67
10	11.18 ± 1.93	31.72 ± 2.88
20	20.82 ± 1.87	49.43 ± 2.22
30	24.0 ± 1.93	79.23 ± 3.12
40	35.07 ± 1.95	88.98 ± 2.82
50	43.38 ± 2.11	97.27 ± 1.70
60	48.61 ± 2.26	97.77 ± 1.51
80	67.07 ± 2.82	98.25 ± 1.20
100	75.38 ± 2.54	98.42 ± 0.41
150	91.38 ± 2.11	98.51± 0.22
IC_{50}	63.01 ± 2.12	20.23 ± 1.83

Table 2 : ABTS free radical scavenging activities of EA extract of PE2.

	Percentage (%) of inhibition	
Concentration (µg/ml)	Ethyl acetate extract of PE2	Trolox (positive control)
5	25.37 ± 1.87	9.95 ± 1.26
10	44.60 ± 2.23	19.84 ± 2.12
20	76..81 ± 1.85	42.18 ± 2.67
30	90.79 ± 1.32	65.63 ± 1.68
40	96.10 ± 1.89	72.42 ± 2.41
50	97.52 ± 1.10	87.54 ± 1.82
100	98.05 ± 1.04	96.23 ± 1.54
IC_{50}	11.21 ± 2.12	23.21 ± 1.32

Table 3: Superoxide radical scavenging activities of EA extract of PE2.

	Percentage (%) of inhibition	
Concentration (µg/ml)	Ethyl acetate extract of PE2	Quercetin (positive control)
10	14.93 ± 1.41	35.92 ± 1.78
20	29.87 ± 1.76	41.63 ± 2.11
30	31.65 ± 1.14	59.18 ± 2.34
40	38.15 ± 2.34	93.22 ± 2.72
50	54.23 ± 3.12	98.65 ± 1.02
100	69.25 ± 3.00	98.88 ± 1.08
IC_{50}	138.29 ± 3.10	42.31 ± 1.80

ABTS free radical scavenging potential

A prominent decoloration of ABTS solution from greenish blue color was noticed along with increased concentrations of the EA extract of PE2. ABTS is most commonly used substance by the various food industry and researchers for estimating the antioxidant properties of different foods or other natural products. EA extract was able to inhibit more than 90% free radicals at a concentration of 30 μg/ml. IC50 value of EA extract of PE2 was calculated as 11.21μg/ml (Table 2), which was much lower than the IC50 value (23.21μg/ml) of the standard substance trolox. The EA extract of *Cytospora rhizophorae* and *Seiridium ceratosporum* isolated from *Rhizophora stylosa*, found most effective scavengers of ABTS radicals with IC50 values of 0.50 mg/mL and 0.37 mg/ml respectively (Zhou *et al*. 2018).

Superoxide radical scavenging assay

It has been reported previously that the presence of flavonoids in the plant or their endophytic extracts is responsible for scavenging the superoxide free radicals which are highly reactive molecules. In addition to DPPH and ABTS free radical scavenging properties, the EA extract of PE2 also exhibited substantial superoxide free radical scavenging potential. Along with increased concentration of EA extract, prominent reduction of the purple color solution was noticed. EA extract of PE2 showed an IC50 value of 138.29 ìg/ml. In comparison, the IC50 value of the well-known standard quercetin (positive control) was calculated as 42.31μg/ml (Table 3). EA fraction of *Alternaria alternata* AE1 exhibited superoxide free radical scavenging activity with IC50 value of 11.38 ìg/ml (Chatterjee *et al*.2019).

Anticancer activity of EA extract of PE2

Now a day, searching of novel anticancer drug from natural sources becomes one of the important fields of research. EA extract of PE2 was also found effective to inhibit the proliferation of breast cancer cell line MDA-MB-231 when tested at different concentrations (20- 300 μg/ml). 45.70 % cell survival was found in the presence of 100 ìg/ml which decreased to 15.72 % in the presence of 200 μg/ml EA extract (Fig. 1). On the other hand, in the presence of 50 mM mitomycin C which was used as positive control, 54.56 % cell survivals was recorded during MTT assay. On the other hand, when the survivability of normal cells were checked using human peripheral blood mononuclear cells (HPBMCs) in the presence of EA extract, 63.21 % cells remain survived in the presence of 100 μg/ml of EA extract PE2 (Fig. 2). The inhibition of proliferation of human breast cancer cell line suggested strong anticancer potentiality of the EA extract of PE2. Anticancer properties of bioactive compounds derived from endophytic fungi have also been reported earlier (Fernandes *et al*. 2009; Arivudainambi *et al*. 2014).

CONCLUSION.

From the above study, DPPH and ABTS free radical as well as superoxide scavenging assays suggested very good antioxidant activity of EA extract of endophytic isolate PE2. In addition, EA extract also found effective to inhibit the proliferation of breast cancer cell line MDA-MB-231. Therefore it was considered as a most promising source of bioactive compounds for the development of new anticancer drugs.Survivability of peripheral blood mononuclear cells in the presence EA extract also suggested its specific activities to cancer cells and may open new window for cancer chemotherapy.

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DECLARATIONS

Conflict of Interest. Authors declare no conflict of interest.

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