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Bioactivities of phenolics-rich fraction from *Diaporthe arengae* TATW2, an endophytic fungus from *Terminalia arjuna* (Roxb.)

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ABSTRACT

The study demonstrated the *in vitro* and *in vivo* anti-hyperchoeterolemic activity of purified fraction of fermentation extract of endophytic fungus *Diaporthe arengae* TATW2 isolated from *Terminalia arjuna* Roxb. (Combretaceae). *In vitro* anti-hypercholesterolemic activity was carried out using human red blood cells (hRBCs) of healthy and hypercholesterolemic donors. Bioactive fraction of the crude extract was obtained using activity guided fractionation followed by Thin-Layer Chromatography (TLC). *In vivo* efficacy studies of this isolated compound were carried out using albino Wistar rats. After 24 h incubation of whole blood as well as isolated erythrocytes from hypercholesterolemic donors with 100 μg/mL of extract of TATW 2, the concentration of RBC membrane cholesterol was found to be reduced significantly ($P < 0.05$). Bioactivity guided fractionation of crude extract followed by TLC afforded a colorless semisolid phenolic compound. *In vivo* efficacy studies of this isolated compound were carried out using albino Wistar rats revealed significant alteration in the serum and tissue lipid profile of the animals. Co-administration of the isolated compound at 50 mg kg^{-1} body weight resulted in a considerable decline in the levels of serum total cholesterol (TC), triglycerides (TG), very low density lipoproteins (VLDL) and low density lipoprotein (LDL) cholesterol in the compound treated group (group C) *vis-a-vis* animals fed with high fat diet (group B) and the results were comparable to the animals receiving high fat diet plus 10 mg kg^{-1} body weight of atorvastatin (group D). Chemical characterization of the fractionated compound using GC-HRMS chromatogram revealed one major peak at a retention time of 39.67 as well as three minor peaks. The spectra revealed three phenolic compounds identified as Benzene propionic acid, 3, 5–bis (1, 1-dimethylethyl)-4-hydroxy methyl ester; Pterin-6-carboxylic acid and 2, 6-ditert-butyl-4- phenol. Endophytic *Diaporthe* produces a variety of secondary metabolites, but, till date, no reports on the production of anti-hypercholesterolemic phenolic compounds. Thus, *Diaporthe arengae* could be a potential source of phenolic compounds with potential anti-hypercholesterolemic activity.

1. Introduction

Annual mortality from cardiovascular disease (CVD) is projected to increase from 17.5 million in 2012 to 22.2 million in 2030 (Patel et al., 2011). Clinical studies indicated that hypercholesterolemia is the most important contributing factor in the development of CVDs and it remains a leading cause of mortality despite extensive therapeutic progress due to the widespread use of statins, an inhibitor of HMG-CoA reductase, the key enzyme in cholesterol biosynthesis. Anti-hypercholesterolemic activity of statins is attributed to a decrease in plasma low-density lipoprotein (LDL) cholesterol concentrations. However, it has been proved that treatment with statins alone can achieve only about 30% reduction in the incidence of cardiovascular events (Baigent et al., 2005, 2010). Although statins are the first choice for treatment of hypercholesterolemia (Lefer, 2002; Wang et al., 2010), there are associated side effects with the statin use as well as many patients do not show regression in atherosclerosis. Moreover, statins intolerance is frequently encountered during their clinical use (Cooney et al., 2010; Arca et al., 2012). Moreover, elevated level of plasma LDL, especially oxidized LDL, is the major factor which promotes monocyte adhesion ultimately leading to atherosclerosis. Therefore, preventing LDL oxidization is an important target for the prevention of atherosclerotic diseases.

"Endophytes are the microbes that colonize the living internal tissues of the plant without causing any negative effect to it"(Bacon and White, 2000). Every plant species on the earth is host to one or more endophytic microorganism. They are increasingly recognized as a novel source of bioactive metabolites with therapeutic potential.

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Terminalia arjuna is a celebrated plant for its cardioprotective role and has been reported as a valuable source of phenolic compounds (Bajpai et al., 2005). It is believed that endophytes mimic the chemistry of their host plant because of horizontal gene transfer during the course of evolution (Strobel et al., 2004). The phenolics of natural origin act as excellent antioxidants mainly due to their redox properties and their ability to quench reactive oxygen. Polyphenols also display excellent antioxidant, antibacterial, anti-atherogenic and cytotoxic activities (Husain et al., 1987; Rice-Evans et al., 1997; Lee et al., 2013). Despite of their significant anti-atherogenic properties, the bioactive phenolic compounds from natural sources such as endophytic fungi are relatively less explored.

This works reports endophytic fungus from Indian medicinal plant, *Terminalia arjuna,* which is capable to produce a number of phenolic compounds. The therapeutically important metabolites were isolated by bioactivity guided fractionation. Chemical characterization was done by spectroscopic and chromatographic techniques and the anti-hypercholesterolemic activity was established using animal models.

2. Materials

The test fungus used in this study, endophytic *Diaporthe arengae* TATW2 was isolated from the twig tissue of the healthy, symptomless plant, *T. arjuna* and identified based on morphology and ITS gene sequencing (Maheshwari et al., 2014). The chemicals and reagents used in the study were of analytical grade.

2.1. Methods

2.1.1. Laboratory cultivation of endophytic fungi and preparation of crude extract

The isolated fungal endophyte TATW 2 was allowed to sporulate in a sporulation medium. After development of sufficient number of spores, a suspension at concentration 10^6 spores /mL was inoculated in the inoculum development medium (g/L) comprising of sucrose, 30; sodium nitrate, 2; K_2HPO_4, 1; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01 (pH 6.5). Inoculated medium was incubated at 28 °C on a rotary shaker (Steelmate Novatech, India) to develop biomass. After development of sufficient biomass, 10% of inoculum was transferred aseptically into 500 mL Erlenmeyer flask containing 120 mL production medium having same composition as that of the inoculum development medium in multiple sets. All fermentations were carried out at 28 °C for 20 days on a rotary shaker at 120 rpm. At the end of stationary phase of the culture, the fermented broth was subjected to extraction. It was filtered using three layered muslin cloth in order to remove fungal mycelia and the culture filtrate was extracted with equal volume of the ethyl acetate. To remove the salts and polar constituents, ethyl acetate phase was washed twice with water and concentrated using a rotary vacuum evaporator under reduced pressure (Equitron, India).

2.1.2. In-vitro anti-hypercholesterolemic activity of crude extract of TATW 2

The crude extract of TATW2 was evaluated for *in vitro* anti-hypercholesterolemic activity as described by Piotr et al. (2012) with slight modifications. Blood samples from hypercholesterolemic and healthy donors were collected after taking their written consent, in sterile tube containing anticoagulant (23 mmol/L citric acid, 45.1 mmol/L trisodium citrate, 45 mmol/L glucose). Erythrocytes were washed three times in phosphate buffered saline (0.9% NaCl; pH 7.4) and centrifuged at 3000 rpm for 15 min. The isolated erythrocytes were suspended in the incubation medium containing (140 mM NaCl, 10 mM KCL, 1.5 mmol/L $MgCl_2$, 10 mmol/L glucose, 100 μg/mL streptomycin, 5 mmol/L Tris-HCl buffer, pH 7.4) at a hematocrit of 5%. Similarly, whole blood and hematocrit were incubated with and without crude extracts, standard quercetin (1, 10, 100 μg/mL concentration) for 24 h at 37 °C. Cholesterol concentration was determined (Allain et al., 1974) using standard cholesterol estimation kits (Nicholas Piramal, India).

2.1.3. Peroxidation of lipids

The lipids from erythrocytes membrane were extracted by organic solvent extraction method (Rodríguez-Vico et al., 1991). Peroxidation of lipids from erythrocyte membrane was measured by thiobarbituric acid (TBARS) method with slight modifications (Rice-Evans et al., 1991; Stocks and Dormandy, 1971). Absorbance was recorded at 535 nm and the malondialdehyde concentration in the sample was calculated by an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ using formula $A = \Sigma CL$, where A is absorption, Σ is molar extinction coefficient, C is concentration and L is path length (Onoja et al., 2014). The results were expressed as μmol/mg protein.

2.1.4. Characterization of bioactive compound

The phenolic content of crude extract of TATW 2 was determined by Folin Cio-Calteau reagent method (Lister and Wilson, 2001). Different concentrations of gallic acid equivalent were prepared in 95% ethanol and standard curve was plotted. The absorbance was measured at 760 nm and the results were expressed as Gallic acid Equivalents (mg GAE /mL of the crude extract) (Qiu et al., 2010). Separation of bioactive metabolites from crude extract was performed by thin-layer chromatography (TLC) using 20 × 20 cm TLC plates coated with silica gel 60 F254 (Merck, Germany). Development was carried out in 25 × 25 cm chromatography trough chamber in an ascending manner in (Aetron, Mumbai, India) using chloroform: methanol (9:1, v/v, mobile phase volume 10 mL) as a mobile phase. The mobile phase saturation time was 20 min. After separation, plates were observed under UV (both shorter and longer wavelengths), and R_f values of band were calculated. Same mobile phase was used for elution in silica gel column chromatography for isolation of metabolites. Fractions of 2–5 mL were collected and simultaneously checked for presence of the compounds by TLC and scanned on a UV visible spectrophotometer (Shimadzu 1750, Japan). Fractions showing similar pattern in TLC were pooled together. The Fourier Transform-Infra Red (FT-IR) spectra of the purified fractions were recorded using KBr as a matrix (Shimadzu 8202 PC, Japan 4000–400 cm^{-1}). HPLC analysis of bioactive fraction was performed using C18G 120 (250 × 4.60 mm, i.d. 5 μm) column on the liquid chromatography system equipped with Shimadzu SPD- M20A PDA detector (Shimadzu, Japan). Sample volume was 20 μL and the eluction was carried out using the mobile phase comprised of mixture of acetonitrile: double distilled water (40:60 v/v). The flow rate was 0.5 mL/min; the detection wavelength was 350 nm. and the HPLC run time was 20 min. The GC-HRMS analysis of isolated compound was carried out at SAIF, IIT Bombay, Mumbai, (India) using Agilent 7890-GC fitted with hp 5 fused silica capillary column (30 m × 0.20 mm, phase thickness 0.25 μm) coupled with JeolAccu TOF GCV mass spectrometer with FID detector. Structure elucidation was done using GC-MS spectral analysis and comparison with National Institute of standards and Technology (NIST) database.

2.1.5. Acute oral toxicity testing

Acute toxicity studies of the crude extract were performed according to the current guidelines set by Organization for Economic Co-operation and Development (OECD, guideline no 423) and approved by the institutional Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, approval no. SPTM-IAEC/Dec-14/07/17). Animals were fasted for 3–4 h and crude extract at doses of 300, 1000 and 2000 mg kg^{-1} and fractionated compound at 10, 25 and 50 mg kg^{-1} body weight per day were given orally. Animals were observed for clinical signs of toxicity at 0–0.5, 0.5–1, 1–2, 2–4, 24–48 h post dosing with special attention during first 4 h.

2.1.6. Bioefficacy studies

Six weeks old healthy female Wistar albino rats weighing 150–170 g were purchased from National Institute of Bio Sciences, Pune, MS,