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Review Article

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COMPARATIVE STUDY OF ENDOPHYTIC FUNGAL METABOLITE ISOLATED FROM BLACK TURMERIC (*CURCUMA CAESIA* ROXB.) IN ROS ASSOCIATED *CAENORHABDITIS ELEGANS* MODEL SYSTEM

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ABSTRACT

Free radicals containing oxygen, known as reactive oxygen species (ROS) is one of the important factors of aging-associated diseases. Excess production of ROS and insufficient cellular antioxidant reverse compromise cell repair and metabolic homeostasis, which serves a mechanical switch for a variety of aging-related pathways. Black turmeric (*Curcuma caesia* Roxb.) is a perennial herb, native to North-East and Central India, with bluish-black rhizome of high economical importance because of its medicinal values. It was reported that curcumin from this rhizome has anti-inflammatory and *in vitro* antioxidant activity. In this review, we discuss the possible role of major endophytic fungi present inside the healthy tissue of rhizome of black turmeric coupled with analysis of *in vivo* antioxidant and antiaging potential of the fungal metabolite by employing a popular experimental model-*Caenorhabditis elegans*. Because endophytes are a potential source of novel natural products for exploitation in medicine, agriculture, and industry and the endophytes of black turmeric have yet not explored in comparison to *Curcuma longa.*

KEYWORDS: Black turmeric, ROS, Anti-aging, Curcumin, Endophytic fungi, fungal bioactive compounds.

INTRODUCTION

Endophytes are microorganisms that colonize intercellularly or intracellularly within healthy plant tissues and exhibit a symbiotic relationship drawing nutrition and protecting the host against pathogens, pests, and insects by synthesizing bioactive metabolites.[1,2] In current years, fungal endophytes, especially those from medicinal plants, have drawn worldwide attention as they have not only mimicked the biosynthetic pathways of their host plant but have also revealed potentials to synthesize unrelated bioactive molecules that find wide-ranging applications in the medicinal, agricultural, and industrial sector.

Curcuma caesia Roxb. a member of the family *Zingiberaceae* and commonly known as black turmeric, is a perennial, erect rhizomatous herb with bluish-black rhizome of high economical importance because of its medicinal values.[3] It is native to North-East and Central India.[6] Curcumin is a major compound of the rhizomes is reported to contain anti-inflammatory, *In vitro*, antioxidant activity and the paste of fresh rhizomes are used as a remedy for insect and snake bite by the *Khamti* tribe of Lohit district of eastern Arunachal Pradesh.[3,4]

Free radicals containing oxygen, known as reactive oxygen species (ROS), are the most biologically significant free radicals play a key role in the development of various degenerative diseases, including aging, cancer, inflammation, diabetes, Alzheimer's disease and other neurodegenerative disorders.[3,5] Previous research indicated free-radical scavenging or *In vitro* antioxidant properties of *C. caesia* metabolites, but similar type of activity of the compound from the endophytic fungus of this plant is not yet explored. Moreover, many of the molecules of natural origin with antioxidant properties that scavenge ROS have attained a central stage in anti-aging research.[7]

In the background of this information, the present study is designed to isolate, identify, and chemically characterize the major endophytic fungi of black turmeric coupled with analysis of *in vivo* antioxidant and anti-aging potential of the fungal metabolite by employing a popular experimental model-*Caenorhabditis elegans*, which mimics the age-related physiological processes of mammals, including humans.[8]

OBJECTIVES

1. Isolation, Identification, and characterization of endophytic fungus of black turmeric and extraction of metabolites in response to stress stimuli.
2. Comparative Chromatographic & mass spectrum (MS) analysis of the metabolites of the black turmeric and the endophytic fungus.
3. To evaluate the *In vivo* antioxidant and anti-aging potential of the fungal metabolites in *C. elegan* model system.
4. Molecular docking to predict the molecular targets for these compounds.

METHODOLOGY

Fungal isolation

In vivo-grown black turmeric rhizomes of a single-plant sibling, lines will be collect from Lakhimpur district of Assam, India, and needs to be planted in the green house. After 2 months of acclimatization, the fresh rhizome portions will be aseptically collected. The rhizomes will be washed thoroughly under running tap water and subsequently surface sterilized with 70 % ethanol and 0.1 % HgCl2 for 5 min, followed by 3–4 thorough washing with sterilized distilled water. The sterilized rhizomes will be dried on sterile filter papers, dissected into small (3–5 mm) pieces, and then placed on Potato Dextrose Agar (PDA) medium supplemented with antibiotics (100 µg/ml streptomycin) followed by incubation at 27±2 °C for 3–10 days. The plates will be methodically examined for fungal growth, and the fungal hyphae spreading out from the explants will be isolated as single-fungal strain, which will be subsequently incubated on PDA for 7 days at 27±2 °C.

Fungal Identification

Genomic DNA of the exponentially growing culture of the endophytic fungus will be isolated by using the InstaGeneTM Matrix Genomic DNA Isolation Kit according to the manufacturer's instructions. Fungal 5.8s rDNA-ITS region will be amplified from the extracted genomic DNA by using the fungal domain-specific primers of ITS1 (5-TCCGTAGGTGAACCTGCGG -3) and ITS4 (5-TCCTCCGCTTATTGATATGC -3). The reaction has to be performed in a 20 μL final volume containing 1 μL of genomic DNA, 50 pM of each primer, 1 × *Taq* pol. buffer, 1.5mM MgCl2, 0.2mM dNTPs. The following PCR thermal cycle parameters have to be used: Denaturation: 94℃ for 45 sec, Annealing: 55℃ for 60 sec, Chain Elongation: 72℃for 60 sec. The amplification of ITS region was confirmed by running the amplicons on 1.5 % agarose gel. The PCR-amplified product (100 µl) has to be cleaned using Montage PCR Clean-up kit (Millipore) and sequenced using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq®DNA polymerase (FS enzyme) (Applied Biosystems). The nucleotide sequence has to be subjected to homology search using Blast tool (www.ncbi.nlm.nih.gov/org) and will be submitted to (GenBank, NCBI). The phylogenetic analysis will be performed by neighbor-joining in MEGA X, using 1,000 bootstrap replicates.[3,9]

Fermentation, extraction, and isolation

The fungal culture will be grown in two different potato dextrose broth (PDB) (one will be supplemented with **Histatin** an oxidative stress inducer and another will be normal [fig. 2])[13] at 27±2 °C, will harvest from 4th to 18th day of incubation at 2 days interval and will regularly analyze for compound isolation. After incubation, the culture filtrate from each experimental flask will filter through a muslin cloth to remove mycelia. Filtered medium (250 ml) will be fractionated with organic solvents in triplicates. These fractions will be dehydrated on dried Na_2SO_4 and concentrated in vacuo (Rotavapor). All the extracts will subject to LCMS analysis. The product will be isolated through preparative LCMS. In the meanwhile, the metabolite from the rhizome of black turmeric needs to be extracted and will be performed GCMS analysis.

Anti-aging activity

N2 Bristol (wild-type) strain of *C. elegans* and *E. coli* OP50 need to be procured from the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA). The bacterial culture will be maintained on Luria agar (LA) plates, and the nematode strain will be routinely cultured and maintained at 20 °C on nematode growth medium (NGM) seeded with *E. coli* OP50 bacteria.[3]

The effects of isolated fungal metabolites with respect to that of the commercially available molecule, i.e., 1, 5-pentanediol[3] will be evaluated for different anti-aging parameters of *C. elegans*. For all the assays, worm eggs will be synchronized in different concentrations of test compound. After 3 days of synchronization, the worms (L4, young adults) will be transferred to the treated NGM plates.

Life span assay; Age-synchronized L1 worms will be treated with the test compound of different concentrations till their L4 stage. Hundred (n=100) pretreated (L4) worms will be transferred to fresh NGM plates having test compound supplementation as previously used concentrations. The plates having no supplementation (0 %) will be termed as control plates. To block the progeny development, 5-fluorodeoxyuridine (FUdR; Sigma, St. Louis, MO, USA) will be added to the NGM plates and the worms will be allowed to grow at 20 °C. The worms will be transferred after every alternate day to ensure the proper supplementation of the test compound. The survival of the worms will observe daily under a stereoscopic microscope. Worms (L4) will be scored daily for survival until all nematodes have to die. There will be five replicates for each treatment. The experiment will be repeated thrice independently. Mean and maximum life span values will be analyzed and statistical significance of the survival curves have to demonstrate.

Pharyngeal pumping assay; To examine the effect of fungal metabolites on pharyngeal pumping of the worms, movement of the pharynx terminal bulb will be recorded according to the protocol described previously.[10]

***Intracellular ROS detection assay*;** To determine the intracellular ROS level in treated and control worms will be performed according to the reported protocol with minor modification.[3]

***Stress resistance assay*;** In order to evaluate the effects, fungal metabolite on worms resistance against oxidative stress, an intracellular ROS generator, Paraquat will be used.[3] From the abovesaid synchronized worms, adult day-2 worms will be transferred to 10 mM paraquat and assayed for survival after 72 h of continuous exposure. Stress tolerance of the worms will be determined as illustrated earlier with minor modifications. For thermal stress analysis, adult day-1 worms with and without treatments will be placed at 37 °C and survival of worms will be recorded.

Statistical analysis; Data including differences between the life span will be statistically analyzed by one-way ANOVA using specific statistical assistance software.

Molecular docking; Computational approaches need to be applied to predict the molecular targets for these compounds using a specific docking program.[11]

Significance

1. Since oxidative stress and inflammation are major determinants of the aging process, it was reported that curcumin can have a more general effect that slows down the rate of aging. The hypothetical significance of this particular work will produce novel antioxidants with anti-aging potential.
2. It is evident from the literature that the endophytes of black turmeric have yet not explored in comparison to *Curcuma longa*. It is interesting to note that instead of the plant related metabolites, the present study will report production of a different class of compound by the isolated endophyte of black turmeric for therapeutic applications pertaining to age-related progressions.
3. It was reported that microorganism can release compounds in response to stress stimuli that can confer stress resistance and survival benefits to animals that consume them.[12] In this work, if the isolated fungus release compounds in response to histatin then it will help to do work on aging by using such compounds.
4. Since the source of black turmeric is limited due to non-available in all habitat, the endophytic fungus will be an alternative of it for non-plant-based bioactive compounds.

Figure 1: Rhizome of Curcuma ceasia Roxb. Ref. Curcuma caesia; www.ebay.com.

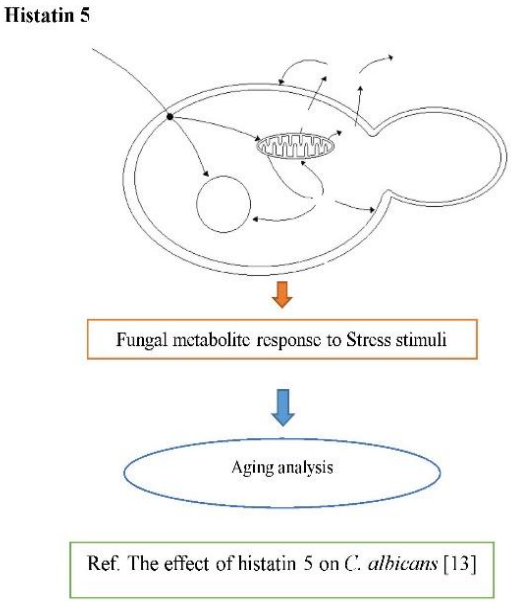

Figure 2: Hypothetical strategy.

Normally Histatin 5 targets the mitochondrion that induces the release of ATP and affects the ability of the cell to respire, subsequently ATP loss may activate a cell death pathway. It also causes the generation of ROS that induces G_1 phase arrest of the nucleus.[13] As they can mimic the biosynthetic potential of their host, if the isolated endosymbiotic fungi can produce metabolite in response to the stress stimuli histatin 5, it will link to do work on aging analysis by using such the fungal metabolite.

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DISCLOSURE STATEMENT

The authors like to make a declaration that there is no conflict of interest to publish the paper.

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