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Endophytic Fungus Isolated From Zingiber zerumbet (L.) Sm. Inhibits Free Radicals And Cyclooxygenase Activity

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Abstract: An endophytic fungus was isolated from rhizomes of *Zingiber zerumbet* (L.) Sm. (*Z.z*) an important medicinal plant of North-East India. Colonial morphological trait, microscopic observation and molecular sequence analysis of ITS region of isolated fungus insinuated 99% similarity with *Fusarium oxysporum*. The isolated and identified fungus was then cultivated in Potato Dextrose Broth for 25 days at 25 ± 1^0 C. Broth and mycelia were separated, fungal broth was extracted with different solvent system; methanol (*Zfe 1*), n-butanol (*Zfe 2*), hexane (*Zfe 3*), and mycelia was extracted with ethyl acetate (*Zfe 4*). The extracts (*Zfe 1, Zfe 2, Zfe 3, Zfe 4*) were subjected to screening and estimation of DPPH free radical scavenging activity, total phenolics content, total flavonoid content, cyclo-oxygenase inhibition assay. Among the tested extract, *Zfe 3* shows highest DPPH radical scavenging activity and COX II inhibition with IC_{50} value of 41.68 µg and 14.27 µg/100 mg respectively. However, phenolic and flavonoid content is highest in *Zfe 4* with concentration of 260 µg/100 mg and 29 µg/ 100 µg.Thus, fungal endophytes inhibit free radicals and Cyclo-oxygenase activity, and could be an alternative natural products of therapeutical importance.
Keywords: Endophytes; *Zingiber zerumbet* (L.) Sm.; ITS, ; *Fusarium oxysporum*; free radicals; cyclo-oxygenase activity.

Introduction

Medicinal plants are reported to harbour endophytes [1-2], which in turn provide protection to their host from infectious agents and also provide adaptability to survive in adverse environmental conditions. Endophytes commonly live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no outward manifestation of their presence and have typically gone unnoticed [3]. Identification and ascertaining taxonomic identity of endophytic fungi using available taxonomic tools at the disposal of fungal taxonomists is often difficult as most of the endophytic fungal isolates tend to present cryptic properties[4]. Diversity and Biological Activities (antimicrobial, reducing power assay, radical scavenging activity, total phenolic content) of Endophytic Fungi of *Emblica officinalis*, was reported. Endophytic fungi produce a number of substances such as antioxidants, novel antibiotics, antimycotics, immunosuppresants and anticancer compounds, and thus rich source of biologically active metabolites that find wide-ranging exploitation in medicine, agriculture, and industry[5-6]. Endophytic fungi from *Amomum siamense* was studied, initiated to investigate the fungal assemblages of the wild ginger, Amomum siamense Criab., at Doi Sutherp-Pui National Park, Thailand, and reported *Fusarium* spp. as one of the endophyte of *A.siamense*[7]. A study shown that Zingibearceous species contain similar endophytic fungal communities as those of other monocotyledons[8-10]. There is report on the

isolation of endophytic fungus from Zingiberaceae plants (*Curcuma amada, C.angustifolia, C.aromatica, Kaempferia angustifolia, Zingiber officinale*)[11].

Studies have reported that rhizomes of *Zingiber zerumbet* has multipotential bioactivies like anti-inflammatory activity, anti-cancer and anti-apoptogenic activity, antinociceptive activity, antimicrobial activity, antiplatelet aggregation activity, antipyretic and cytotoxic activity, antihyperglycaemic activity, chondroprotective activity, LPS (lipopolysaccharide)-induced NO production activity, anti-AD (Alzheimer's disease), chemopreventive activity, anti-oxidant activity, hepatoprotective activity, immuno-modulatory activity, anti-edema, antiepileptic seizures and angiogenic activity, antipancreatitic activity, antiallergic activity, enzyme activation activity, anti-oomycete activity, anti-HIV [12]. Recent studies have demonstrated that plant-associated micro-organisms are prolific producers of novel and pharmacologically active secondary metabolites[13]. Certain endophytic fungi are capable of synthesizing the medicinal products produced in plants[14]. *Fusarium oxysporum* Dzf17 as an endophytic fungus isolated from the rhizomes of *Dioscorea zingiberensis*, a well known traditional Chinese medicinal herb indigenous to the south of China [15-16]. *In vitro* antioxidant activities of polysaccharides from endophytic fungus *Fusarium oxysporum* dzf17 was reported[17]. Phytochemical analysis and anti-oxidant activity of endophytic fungi (*Fusarium, mucor, Aspergillus*) isolated from *Lobelia nicotianifolia* was done and reported the presence of flavonoids and glycosides[18]. The antioxidant capacities of the endophytic fungus cultures were significantly correlated with their total phenolic contents, suggesting that phenolics were also the major constituents of the endophytes [19]. In our present study, an endophytic fungus was isolated from rhizomes of *Z. zerumbet* and its colonial morphological trait and microscopic characters were studied. However, the number of reports on the study of anti-oxidant and COX inhibition activity of endophytic fungal extract is very less.

Materials And Methods

Plant material

The rhizomes of *Zingiber zerumbet* (L.) Sm. were collected from Chavangphai village, Chandel district of Manipur, (N24°18′39.9′, E 94° 18′39.9″, 215 msl). A voucher specimen No.-IBSD/M/1004 was deposited for reference to Plant systematic and conservation Lab, IBSD, Takyelpat, Imphal. Healthy and mature rhizomes of *Zingiber zerumbet.* Smith collected from field were used as host plant for the isolation of endophytes.

Isolation of endophytic fungus

The endophytic fungus from the rhizome was isolated according to Kjer *et al*. 2010. Rhizomes were washed in running tap water, and its scales were removed using a sterile blade. It was then washed with 70% ethanol for 1-3 min, followed by washing in 5% aqueous solution of sodium hypochloride for 3 minute. It was again washed with 70% ethanol for 1-4 min, rinsed with sterile distilled water. It was aseptically cut with sterile blade and inner tissues were excised. The excised tissue pieces were inoculated to potato dextrose agar (PDA) containing 1 mM Gentamicin (to avoid bacterial growth). Inoculated for 6-25 days at 25 ± 1°C. Pure cultures were then transferred to PDA plates free of antibiotics and cultivated for 20 days on PDA plated at 28°C [20].

DNA extraction and sequence analysis

DNA was extracted from the microbial isolate following White *et al*. 1990. Isolated DNA was quantified by NanoDrop 2000 (Thermo Scientific) and amplified by PCR using primers ITS1 (TCCGTAGGTGAA CCTGCGG) & ITS4 (TCCTCCGCTTATTGATATGC). The reaction was performed in a total volume of 50µl PCR mix containing 1X standard PCR incubation buffer, 0.5 µM of each primers, 200 µM of each four deoxyribonucleotide triphosphate, 1.25 U Taq polymerase and 20 ng genomic DNA. Thermal cycling conditions include initial denaturation step for 5 min. at 94^0 C, followed by 30 cycles each for 1 min. denaturation at 94^0 C, 1 min. annealing at 52^0 C and 90 sec elongation at 74^0 C with a final extension of 7 min. at 74^0 C. Amplified PCR product was purified, lyophilized and sent for sequencing to SAP services, GeneI, Bangalore. DNA sequences were then aligned and searched for similarity using BLAST. The sequences were submitted to NCBI GenBank to obtain an accession number[21].

Extraction and isolation of crude extracts from fugal fermentation broth

Each of the pure cultures was re-cultivated on PDA at 28°C for 7-20 days. Three pieces of mycelia agar plugs (0.5 X 0.5 cm^2) were inoculated into 500 ml Erlenmeyer flasks containing 300 ml Potato Dextrose Broth and incubated at room temperature for 4 weeks under static phase/ condition. The broth culture was then filtered to separate the filtrate and mycelia. The filtrate was extracted three times by shaking with an equal volume of ethyl

acetate. The ethyl acetate extract was partitioned with an equal volume of distilled water. The water phase was separated and partitioned with equal volume of n-butanol. EtOAc phase was evaporated and again partitioned in equal ratio 1:1 (v/v) of 90% MeOH and n-Hexane [20]. Methanol (*Zfe* 1), n-butanol (*Zfe 2*), hexane (*Zfe 3*), ethyl acetate extract of mycelia (*Zfe 4*) were dried and concentrated under Vaccum evaporator (Buchi, Switzerland). The extracts were stored at 4 ^{0} C, till further analysis.

Estimation of Total phenolic content

100µl of each sample (*Zfe 1, Zfe 2, Zfe 3, Zfe 4*) was transferred to respective microtube (assaytube, 2ml). 200 µl Folin–Ciocalteu (F–C) reagent (10% v/v) was added and vortex thoroughly. 800 ml of 700 mM Na_2Co_3 was added into each tube. 100 µl gallic acid in various concentration (10– 200 mg/ml), 100 µl methanol (99.98% v/v) were taken as standard and blank respectively. In order to avoid the air-oxidation of phenols (present in test samples or standard gallic acid) by alkali, samples or gallic acid were mixed with methanol and F–C first followed by vortexing, thereafter added alkali (Na_2Co_3). The assay tubes were incubated at 45 ^{0}C for 30 min. Transferred 200 ml samples, standard and blank solution from respective assay tubes to clear 96 well microplate and read the absorbance of each well at 765nm (A_{765}) in Thermo Multis- kan reader. A standard curve was drawn from the blank-corrected A_{765} of the gallic acid standard. The total phenolics contents was calculated as gallic acid equivalents using the regression equation between gallic acid standards and A_{765} [22].

Estimation of Total flavonoid content

Total flavonoid content was estimated using $AlCl_3$ method. 0.5 ml of the extract (*Zfe 1, Zfe 2, Zfe 3, Zfe 4*) was taken, added 1.5 ml of methanol. To the reaction solution 0.1 of 10% AlCl3 was added. 0.1 ml of IM Potassium acetate was added. The volume of the solution was make upto 5 ml with distilled water. The reaction mixture was incubated at room temperature for 30 minute. Absorbance was read at 415 nm at Multiscan reader (Thermo). A curve was generated using Rutin (1-10µg/ml) as standard flavonoids. Total flavonoid content was expressed as Rutin equivalent (µg/100µl) of the extract [23].

DPPH free radical scavenging activity

3 ml of fungal extract was taken at different concentration (10-200µg). To this, 1 ml of DPPH (0.1 mM in ethanol) was added. The reaction mixture was incubated under dark for 30 minutes. Decolourization of DPPH was determined by measuring the decrease in absorbance at 517 nm [23]. DPPH radical scavenging effect was calculated using the equation;

% scavenging rate = $(A_0 – A_1)/A_0 \times 100$, where, A_0 = Absorbance of control, A_1= Absorbance of sample.

Cyclooxygenase (COX) Inhibition Assay

COX-1 and COX-2 assays were performed in separate assay plates (96 flat bottom well plates). COX enzyme activity was determined by using a colorimetric COX inhibitor screening assay kit (Cayman Chemical Company, Ann Arbor, USA) in a free system according to the manufacturer's instructions. The Colorimetric COX Inhibitor Screening Assay measures the peroxidase component of cyclooxygenases. The peroxide activity was assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm. All the samples were dissolved in DMSO. Briefly, 160 µl of assay buffer and 10 µl of heme were added to the background well. 150 µl of assay buffer, 10 µl of heme and 10 µl of COX-I & COX - II enzyme were added to the 100% initial activity well. 10 µl of fungal extracts (Zfe 1, Zfe 2, Zfe 3, Zfe 4; the final concentration is 100 µM and 50 µM) was added to the sample wells and 10 µl of DMSO was added to the background wells. The plate was carefully shaken for a few seconds and incubated for 5 min at 27°C. 20 µl of the colorimetric substrate solution and then 20 µl of arachidonic acid were added to all the wells. The plate was carefully shaken for a few seconds and incubated for 5 min at 27°C. The absorbance at 590 nm was read by using a microplate reader (Thermo Scientific Multiskan Spectrum) and the inhibition ratio on COX-1 & COX – 2 enzymatic activities was calculated according to the manufacturer's instructions.

Results And Discussion

Colonies of the microbial isolate grew rapidly, 4.5 cm in 4 days, white cottony aerial mycelium, becoming purple.Hyaline septate, conidiophores, phialides, macroconidia, and microconidia were observed microscopically. Sickle shaped macroconidia, and short simple conidiophores bearing small, conidia in clusters were observed. Extracted DNA of the fungal isolate was quantified to be 501.3 ng. Based on the ITS1 & ITS4

region sequence data, the isolate show 99 % similarity with *Fusarium oxysporum*, Acc.No. HQ647333.1 from the existing NCBI GenBank database.

In *in-vitro* DPPH radical scavenging assays, the IC_{50} values of the samples *Zfe 1, Zfe 2, Zfe 3, Zfe 4* was calculated as 182.00µg, 180.90µg, 41.68µg and 189.40µg respectively. Total Phenolic Content of the samples *Zfe 1 Zfe 2 Zfe 3 Zfe 4* were found to be 260.00µg, 81.00µg, 5.00µg and 20.00µg respectively per 100 mg each, equivalent to Gallic acid (100mg). Whereas the Total Flavonoid Content of the samples *Zfe 1 Zfe 2 Zfe 3 Zfe 4* were found to be 3µg, 6µg, 1µg and 29µg respectively per 100 µg, equivalent to Rutin (100µg). Details of results are shown in Table 1. In *in-vitro* assays, the samples *Zfe 1 Zfe 2 Zfe 3 Zfe 4* inhibited both COX-1 and COX-2, but their inhibition concentration differs with each others. IC50 value for *Zfe 1 Zfe 2 Zfe 3 Zfe 4* of COX-1 were found to be 134.63µg, 112.34µg, 456.78µg, 334.56µg respectively and IC_{50} of COX-2 were found to be 21.38µg, 26.52µg, 14.27µg, and 25.76µg respectively. Details of results are shown in Table 2.

Tabe 1. IC_{50} of DPPH free radical scavenging activity, total phenolic content, total flavonoid content of endophytic *Fusarium oxysporum* extract.

Fungal extract	DPPH free radical scavenging activity IC50 (µg)	Total Phenolic Content of sample equivalent to Gallic acid (µg/100 mg)	Total flavonoid content Rutin equivalent (µg/100 µg)
*Zfe 1**	180.90± 0.0024	81.00 ± 0.0037	3 ± 0.0017
*Zfe 2***	189.40±0.0031	20.00 ± 0.0005	6 ± 0.0029
*Zfe 3****	41.68±0.0009	5.00 ± 0.0014	1 ± 0.004
*Zfe 4*****	182.00± 0.0047	260.00 ± 0.0061	29 ± 0.0072

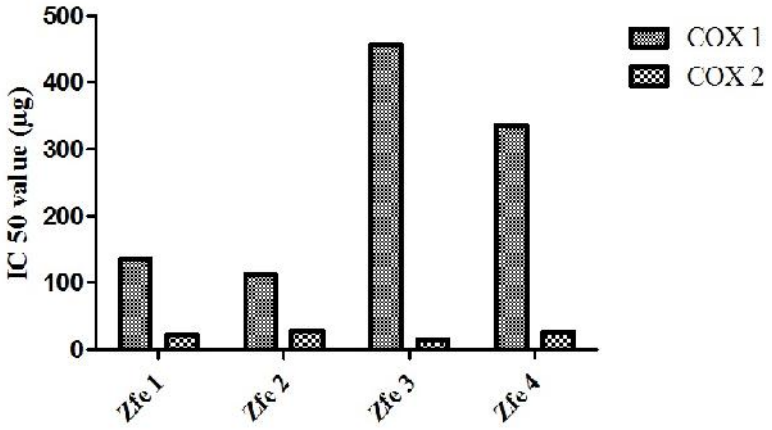
* methanol,** n-butanol, *** hexane,**** ethyl acetate extract.

DPPH radical scavenging activity of the fungal extract range from 41.68 µg- 189.40 µg Among the tested extract, *Zfe 3* shows highest DPPH radical scavenging activity of 41.68 µg. However, phenolic and flavonoid content is highest in *Zfe 4* with concentration of 260 µg/100 mg and 29 µg/ 100 µg.

Table 2. COX inhibition assay of different extract of endophytic *Fusarium oxysporum*

Fungal extract	IC $_{50}$ VALUE (µg)	
	COX – I	COX - II
*Zfe 1**	134.63 ± 0.098	21.38 ± 0.067
*Zfe 2***	112.34 ± 0.032	26.52 ± 0.007
*Zfe 3****	456.78 ±0.040	14.27 ± 0.043
*Zfe 4*****	334.56 ± 0.009	25.76 ± 0.004

* methanol,** n-butanol, *** hexane,**** ethyl acetate extract.
Among the tested extract, *Zfe 3* shows highest COX II inhibition with IC_{50} value of 14.27 µg/100 mg.



Effect of different extracts of *Fusarium oxysporum* on COX inhibition assay

Endophytes provide a wide variety of structurally unique bioactive natural products, such as alkaloids, benzopyranoids, chinones, flavonoids, phenolic acids, quinines, steroids, terpenoids, tetralones, xanthones, and others[14]. Antibiotics, antiviral compounds, anticancer agents, immunosuppressive compounds as well as antioxidants have been reported from endophytic metabolites, and medicinal plants have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance[24-25]. Because they are relatively unstudied, much attention is now being paid to endophytic biodiversity, the chemistry and bioactivity of endophytic metabolites, and the relationships between endophytes and host plants[14, 26]. *Fusarium oxysporum* is an abundant saprophyte in soil and organic matter and occurs worldwide in the rhizosphere of many plant species. Many of the fungus belonging to genus *Fusarium* produce a wide range of biologically active secondary metabolites (e. g. mycotoxins) with extraordinary chemical diversity. Although extensive studies have been performed on the biology of *F. oxysporum*, strains and the colonization process of *F. oxysporum* strains is well explored metabolism [27-31], the role of root exudates, primary signals in fungus–plant interactions in the rhizosphere, still remains mostly unclear. Apart from their role as plant pathogens several strains of *F. oxysporum* are known to control *Fusarium* diseases [31], and are involved in the suppressiveness of soils [32]. An antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f.sp.*ciceris* was induced[33]. The antioxidant activity of *Aspergillus fumigatus* was assayed by different procedures and correlated with its extracellular total phenolic contents[34]. The total anti-oxidant capacity of the endophytic fungal metabolites was significantly correlated with their total phenolic content. The sample which has anti-oxidant capacity is also correlated with the anti-inflamatory activity. Although, there is report that endophytic metabolites can mimic the host metabolites, without controlled experiments, however we cannot conclude with certainty that the same compounds are in fact produced in vivo by the endophytes alone or by both endophytes and the host plant[19].

Conclusion

The present study confirm that the endophytic fungus isolated from *Zingiber zerumbet* has the potential to scavenge the DPPH free radicals, also shows the presence of phenolics and flavonoid content. COX inhibition assay also shows positive result. There is report that the metabolites produced by host plant, can also be produced by endophytes isolated from the host, and it is possible that the medicinal property imparted by the plant is attributed by the endophytes within the host. Further detailed study is needed to investigate the anti-oxidant metabolites and also other bioactive metabolites to take endophytes as an excellent source of natural products.

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