Endophytic fungi of *Tinospora cordifolia* with anti-gout properties

Neha Kapoor^{1,2} · Sanjai Saxena¹

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Abstract

Targeting xanthine oxidase inhibition has been a prime strategy for exploration and development of non-purine based drugs for anti-hyperuricemic therapy. Recent studies from our laboratory have suggested that fungal endophytes from medicinal plants could play a pivotal role in exploration of novel therapeutic moieties. In the present study, 19 fungal endophytes were isolated from the medicinal plant *Tinospora cordifolia* and evaluated for their XO inhibitory activity. In the qualitative assay, Out of 19 fungal endophytes, 7 endophytes exhibited > 30% XO inhibition, of which isolates #1 TCSTITPLM, #53 TCSTITPLM, #105 TCSTITPLM and #83 TCSTITPLM were found to exhibit XO inhibition in the range of 38–45%. Further, ethyl acetate extract of #1TCSTITPLM exhibited 69% XO inhibitory activity followed by chloroform extract of #53 TCSTITPLM with 63% XO activity in the in vitro uric acid estimation assay. In the quantitative NBT titer plate assay, both extracts exhibited 85% of XO inhibition. The present study is the first report of fungal endophytes isolated from *T. cordifolia* exhibiting XO inhibitory activity. Ethyl acetate and chloroform extract of #1 TCSTITPLM and #53 TCSTITPLM demonstrated potent XO inhibitory action. Further studies are warranted for purification and characterization of the XO inhibiting moieties.

Keywords Endophytic fungi · Xanthine oxidase · Hyperuricemia · Enzyme inhibition · Uric acid

Introduction

Nearly all the modern medicines today find their origin from nature. Of these, plants have served as an excellent source of novel medicinal compounds which in due course have been developed into medicines. However, the inherent bottleneck encountered by the pharmaceutical industry is their bulk production as it requires huge quantities of biomass for their extraction which eventually threatens the existence of these plants. Thus, pharmaceutical industry is focusing on alternative methods for production of these phytomedicinals. It has been amply demonstrated by various studies that the endophytic fungi possess the capacity to biosynthesize the host metabolites like podophyllotoxin, camptothecin and huperzine, and therefore are promising alternative source

of secondary metabolites known from plants (Aly et al. 2010; Kaul et al. 2012; Dong et al. 2014; Nisa et al. 2015). Further they may also produce novel congeners of the phytomedicinals which can be exploited in the development of medicines directly or indirectly as they exhibit anti-bacterial, anti-cancer, cytotoxic, anti-oxidant and neuro-protective properties (Nisa et al. 2015; Gouda et al. 2016; Maheshwari et al. 2017).

Tinospora cordifolia, commonly known as Guduchi or Giloy, is a large deciduous climbing shrub belonging to the family Menispermaceae. It is generally found at high altitudes. It is a well-documented plant in Ayurveda and is known for its anti-rheumatic, anti-spasmodic, anti-microbial, anti-osteoporotic, anti-inflammatory, anti-arthritis, anti-allergic, and anti-diabetic properties (Warrier et al. 1996; Tripathi et al. 1997; Nair et al. 2004; Sinha et al. 2004). Thus it is expected that endophytic mycoflora existing in *T. cordifolia* may possess anti-gout or anti-rheumatic properties.

Xanthine oxidase (XO) is a key enzyme responsible for development and progression of various metabolic and oxidative stress related disorders. The over activity of XO results in increase in serum urate levels which in turn leads to development of hyperuricemia, a predisposing factor for gout (Billet et al. 2014). The cornerstone in the prevention

✉ Sanjai Saxena
sanjaibiotech@yahoo.com; ssaxena@thapar.edu

¹ #BC-213, BC Link Block (First Floor) Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab 147004, India

² Present Address: Department of Microbiology, Uttarakhand (PG) College of Biomedical Sciences and Hospital, Dehradun, Uttarakhand 248002, India

and treatment of hyperuricemia involves the exploitation of uricosurics and uricostatic drugs by excessive uric acid excretion or blocking the uric acid production respectively. Till date, allopurinol and febuxostat are commercially available xanthine oxidase inhibitors used in hyperuricemia therapy. Owing to the increasing serious side effects imposed by these drugs, there arise an urgent need for exploration and development of novel non-purine scaffold based xanthine oxidase inhibitors.

However, exploration of XO inhibitors from endophytic fungi is limited (Kapoor and Saxena 2014, 2016). Based on the above assumptions, the present study was undertaken to isolate and screen the endophytic mycoflora of *T. cordifolia* for xanthine oxidase inhibiting properties to aid in screening of new chemical templates which could be developed as anti-gout therapeutics.

Materials and methods

Isolation of endophytic fungi

The plant sample (leaves and stems) of *T. cordifolia* was collected from Palampur, Himachal Pradesh, India. Each sample was properly cleaned and stored in sterile zip pouches at 4 °C. Healthy leaves and stems of the plant were surface sterilized by dipping in 0.1% solution of sodium hypochlorite followed by 70% ethanol for 45 s and subsequently dipping in 30% ethanol for 30 s. The sterilized sample was aseptically cut into 1–3 mm segments and inoculated onto potato dextrose agar plate. The plates were incubated at 26 ± 2 °C, 16 h/8 h light/dark cycles for 8–10 days. The plates were regularly monitored for any fungal growth. Hyphal tips emerging out of the inoculated segments were picked up by sterile loop and aseptically transferred to the fresh PDA plate to obtain pure culture. The pure cultures were then further transferred on to PDA slants containing 10% glycerol for long term preservation (Mitchell et al. 2008). The isolation rate, relative frequency and Menhinick's and Camargo index were calculated (Whittaker 1977; Huang et al. 2008; Chowdhary and Kaushik 2015).

Production of culture filtrate

Each endophytic isolate was subjected to submerged fermentation in potato dextrose broth (PDB) medium by following protocol of Raviraja et al. (2006). Briefly, 5 mm mycelial plug of actively dividing culture was inoculated into 100 ml pre-sterilized PDB medium and incubated at 26 ± 2 °C, 120 rpm for 8–10 days. After the incubation is over, the supernatant was separated from fungal mass by filtration through Whatmann filter paper no. 4 followed by centrifugation at 10,000 rpm at room temperature for 10 min.

Qualitative screening for XO inhibition

This assay was carried out by following the procedure as described by Aggarwal and Banerjee (2009) with slight modifications. Briefly describing, 40 µl of master-mix containing pre-incubated 30 µl of culture filtrate of each endophytic isolate and 0.04 U of xanthine oxidase (Bovine milk, Sigma Aldrich, USA) was dispensed into each well in xanthine—NBT agar plate followed by overnight incubation at 37 °C. The control well consisted of 30 µl of un-inoculated broth and 0.04 U of XO. Allopurinol and Febuxostat (Stock-1 mM) were used as positive controls. Appearance of blue colored halo indicated the XO activity in control well while reduction in diameter of blue colored halo in comparison to control well indicated XO inhibition. All the tests were performed in triplicates. The halo diameter was recorded and data were represented as mean $\pm$ SD.

Liquid–liquid extraction

The cell free supernatants of all the cultures were extracted thrice with chloroform in the ratio of 1:2. The pooled organic layers were clubbed together followed by dehydration with anhydrous sodium sulphate. The residual aqueous layer was then extracted with ethyl acetate. The crude chloroform and ethyl acetate residues were obtained by evaporating the solvent till dryness by Rotatory Evaporator (IKA RV10, Germany) at 25 °C. The residue so obtained was weighed and a stock solution was prepared using methanol/DMSO.

Quantitative estimation of xanthine oxidase inhibition

NBT assay

The crude chloroform and ethyl acetate fractions of cultures were subjected for determination of xanthine oxidase inhibitory activity as described by Aggarwal and Banerjee (2009) with slight modifications. The crude fractions were pre-incubated with bovine milk xanthine oxidase at 37 °C for 1 h prior to assaying the activity. The reaction was started by adding 130 µl of xanthine (10 mM) and 30 µl of NBT to the pre-incubated master-mix. After the incubation at 37 °C for 1 h, the amount of formazan produced was estimated by measuring the absorbance at 575 nm using Biotek Powerwave 340 Plate reader. Allopurinol and Febuxostat were chosen as positive control. A control reaction was set up consisting of everything except any inhibitor. All the reactions were performed in triplicates.

In vitro uric acid estimation assay

This assay was carried out as per the method devised by Chang et al. (1993) with slight modifications to estimate the uric acid produced from xanthine which is dependent on the XO activity. Briefly, the initial reaction mixture was prepared by adding 40 µl of culture filtrate to 960 µl of 200 µM of xanthine buffer solution. The reaction was initiated by adding 15 µl of xanthine oxidase (200 mU/ml) followed by incubation at 37 °C for 25 min. Further, the reaction was stopped by adding 1 N HCl solution. The concentration of uric acid produced was measured by taking absorbance at 290 nm. Allopurinol and Febuxostat served as positive controls. The inhibitory percentage of xanthine oxidase was calculated by the formula:

$$\% \text{ Inhibition} = [(A - B) - (C - D)] / (A - B) \times 100 \%,$$

where *A* is the OD at 290 nm with enzyme but without sample, *B* is the OD at 290 nm without sample and enzyme, *C* is the OD at 290 nm with sample and enzyme, *D* is the OD at 290 nm with sample, but without enzyme.

Results

A total of 19 fungal endophytes belonging to 4 different taxa were isolated from stem internal tissue (vascular tissue) of *T. cordifolia* (Table 1). No endophyte was obtained from

Table 1 Endophytic isolates recovered from internal stem tissue of *Tinospora cordifolia* located in Palampur, Himachal Pradesh, India

S. no	Culture code	Tentative identification
1.	#1TCSTITPLM	<i>Colletotrichum</i> sp.
2.	#4 TCSTITPLM	Unidentified
3.	#11TCSTITPLM	<i>Alternaria</i>
4.	#16TCSTITPLM	<i>Hypoxylon</i> sp.
5.	#21TCSTITPLM	Unidentified
6.	#33 TCSTITPLM	<i>Fusarium</i> sp.
7.	#41 TCSTITPLM	Unidentified
8.	#42 TCSTITPLM	<i>Alternaria</i>
9.	#45 TCSTITPLM	Unidentified
10.	#46 TCSTITPLM	<i>Hypoxylon</i> sp.
11.	#51 TCSTITPLM	<i>Curvularia</i> sp.
12.	#53 TCSTITPLM	<i>Fusarium</i> sp.
13.	#59 TCSTITPLM	<i>Fusarium</i> sp.
14.	#61 TCSTITPLM	Unidentified
15.	#76 TCSTITPLM	<i>Fusarium equiseti</i>
16.	#83 TCSTITPLM	<i>Fusarium</i> sp.
17.	#88 TCSTITPLM	<i>Fusarium</i> sp.
18.	#105 TCSTITPLM	<i>Fusarium</i> sp.
19.	#107 TCSTITPLM	<i>Fusarium</i> sp.

leaves of the plant. Among the taxa identified, the frequency of occurrence of *Fusarium* species was much higher when compared to the *Alternaria*, *Curvularia* and *Colletotrichum* sp. The isolation rate, relative frequency, species richness and Camargo index are listed in Table 2.

In the preliminary XO inhibition screening assay over xanthine-NBT agar plate, the culture filtrate of seven isolates were found to exhibit $\geq 30\%$ in vitro XO inhibitory activity with a zone diameter ranging from 6.5 to 8.0 mm in comparison to negative control (no inhibitor) with 12.5 mm. As per one-way ANOVA analysis [$F(20, 40) = 114.9$, $p < 0.0001$], Febuxostat exhibited highest mean XO inhibition of 60.3% followed by allopurinol with 55.8%. Further, based on Tukey's post-hoc analysis, among 19 cultures tested, #1 TCSTITPLM exhibited highest relative XO inhibition (Fig. 1) of 45.1% followed by #53 TCSTITPLM (42.4%), #105 TCSTITPLM (41.9%) and #83 TCSTITPLM (38.4%). An insignificant difference was observed between the percent XO inhibitory profile of #16 TCSTITPLM, #61 TCSTITPLM and #59 TCSTITPLM (Table 3).

The crude chloroform and ethyl acetate residues of all the isolates were further evaluated on NBT microtiter plate assay and in vitro uric acid estimation assay for XO inhibitory activity. In NBT microtiter plate assay, chloroform residue of #53 TCSTITPLM and ethyl acetate residue of #1 TCSTITPLM exhibited maximum relative XO inhibition of 85% followed by ethyl acetate residue of #83TCSTITPLM (76%) as per one-way ANOVA [$F(21,41) = 533.7$, $F(21,41) = 56.77$, $p < 0.0001$] and Tukey's post hoc analysis (Fig. 2). On further evaluating selected crude extracts of potent endophytes using uric acid estimation assay, maximum relative XO inhibition was observed in EA extract of #1 TCSTITPLM (69%) followed by CHL extract of #53 TCSTITPLM as per one-way ANOVA [$F(6,12) = 1604$, $p < 0.0001$] and Tukey's Post hoc analysis (Fig. 3).

Table 2 Isolation rate, relative frequency and Menhinick's & Camargo index of different fungal endophytes obtained from *Tinospora cordifolia*

Parameter	Result
Total no. of segments inoculated	110
Total no. of isolates obtained from stem internal tissue	19
Isolation rate	0.1727
Relative frequency	
<i>Fusarium</i> sp.	0.421
<i>Hypoxylon</i>	0.105
<i>Alternaria</i>	0.105
<i>Colletotrichum</i>	0.052
<i>Curvularia</i>	0.052
Menhinick's index	1.146
Camargo's index	0.8718

Fig. 1 In vitro XO inhibitory activity of #1TCSTITPLM and Febuxostat over Xanthine-NBT agar plate assay

Table 3 In vitro XO inhibitory activity of 19 fungal endophytes over Xanthine-NBT agar plate assay

S. no	Culture code	Relative XO inhibition (%)
1.	#1 TCSTITPLM	45.14 ^b ± 0.99
2.	#53 TCSTITPLM	42.42 ^{bc} ± 1.36
3.	#105 TCSTITPLM	41.89 ^{bc} ± 2.00
4.	#83 TCSTITPLM	38.37 ^{cd} ± 1.41
5.	#16 TCSTITPLM	34.58 ^{de} ± 1.34
6.	#61 TCSTITPLM	34.58 ^{de} ± 1.34
7.	#59 TCSTITPLM	32.98 ^{de} ± 0.85
8.	#51 TCSTITPLM	28.33 ^{ef} ± 3.51
9.	#88 TCSTITPLM	26.20 ^{fg} ± 1.58
10.	# 107 TCSTITPLM	25.67 ^{fg} ± 2.08
11.	#21 TCSTITPLM	24.83 ^{fg} ± 2.84
12.	#46 TCSTITPLM	24.33 ^{fg} ± 4.04
13.	#4 TCSTITPLM	20.28 ^{gh} ± 0.48
14.	#76 TCSTITPLM	17.56 ^h ± 2.14
15.	#41 TCSTITPLM	16.22 ^{hi} ± 0.38
16.	#11 TCSTITPLM	14.89 ^{hij} ± 2.52
17.	#42 TCSTITPLM	10.78 ^{ij} ± 2.11
18.	#45 TCSTITPLM	9.44 ^j ± 2.21
19.	#33 TCSTITPLM	9.44 ^j ± 2.21
20.	Febuxostat	60.31 ^a ± 0.54
21.	Allopurinol	55.81 ^a ± 1.32
22.	Control	0 ^k

Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Tukey's post-hoc test ($p < 0.05$)

Discussion

Hyperuricemia is considered as a major risk factor for the development of gout which is characterized by overproduction or under excretion of uric acid or combination of both leading to elevated serum urate level beyond 6.8 mg/dl. Higher SUA levels in the body reflect the higher XO activity and a state of oxidative stress. XO plays a major role in various forms of ischemic reperfusion, vascular injuries, acute arthritis, chronic heart failure, diabetes type1 (Desco et al. 2002; Berry and Hare 2004). Currently, only two XO inhibitors, viz. allopurinol and febuxostat are clinically approved for hyperuricemia. However, due to serious medical conditions imposed by the continual use of these drugs, development of novel and safe alternatives is the need of the hour.

Several plants and their phytochemicals are being explored in search of novel XO inhibitors. Methanolic extracts of *Momadica charantia* and *Zingiber officinalis* exhibited 96.5 ± 2.1 and $81.5 \pm 3.7\%$ XO inhibitory activity (Alsultane et al. 2014). The leaf extract of *Carica papaya* exhibited $86.9 \pm 1.9\%$ XO inhibitory activity with an IC_{50} of $4.3 \mu\text{g/ml}$ (Azmi et al. 2012). Geraniin, a tannin purified from 70% water–acetic extract of *Phyllanthus winaria* showed a dose-dependent XO inhibition with IC_{50} of $30.5 \mu\text{M}$ (Lin et al. 2008).

Besides plant based phytochemicals, various varieties of mushrooms viz. Acetonic, methanolic and aqueous extracts of *Pleurotus cornucopiae*, *P. citrinopileatus*, *P. salmoneostramineus*, *P. nebrodensis* were found to exhibit dose-dependent XO inhibitory activity (Alam et al. 2011a, b, c, d). The aqueous extract of *Agaricus brasiliensis* was found to exhibit maximum XO inhibitory activity of 72.9% (Zanabaatar et al. 2010). Further, endophytic fungi

Fig. 2 In vitro XO inhibitory activity of Ethyl acetate and chloroform extracts of 19 fungal endophytes over NBT microtiter plate assay. Data presented are mean $\pm$ standard deviation of three replications. Means with different colored superscript letters are different by Tukey's post-hoc test ($p < 0.05$)

Fig. 3 In vitro XO inhibitory activity of selected crude extracts of potent endophytes by uric acid estimation assay. Data presented are mean $\pm$ standard deviation of three replications. Mean values with different superscript letters are different by Tukey's post-hoc test ($p < 0.05$)

are recognized as relatively unexplored source of novel therapeutic molecules with a greater probability to inhibit XO in anti-hyperuricemic therapy regimen. There exists very limited data on the exploration of endophytic fungi as a source of XO inhibitors (Kapoor and Saxena 2014). A cerebroside named Fusaruside was purified from culture filtrate of endophytic *Fusarium* sp. IFB-121 exhibited

a potent mixed type XO inhibition with IC_{50} value of $43.8 \pm 3.6 \mu M$ (Shu et al. 2004). Chloroform extract of *Lasiodiplodia pseudotheobromae* and *Muscador darjeelingensis* isolated from *Aegle marmelos* and *Cinnamomum camphora* also exhibited potent XO inhibition with an IC_{50} of 0.61 and 0.54 $\mu g/ml$, respectively (Kapoor and Saxena 2016).

The present study, for the first time, reports the potential of fungal endophytes isolated from *T. cordifolia* as potential candidates for exploration of novel XO inhibitors. Two fungal endophytes viz. #1 TCSTITPLM and #53 TCSTITPLM were found to be potent producer of XO inhibitory substances comparable to that of allopurinol. The production of XO inhibitory molecule from endophytic fungi can be correlated with the modified metabolism for the survival and reproduction inside the host plant. The present study further embroils the pivotal role of endophytic fungi as repository for selective xanthine oxidase inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no financial or commercial conflicts of interest.

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