On: 28 October 2014, At: 01:17
Publisher: Taylor & Francis
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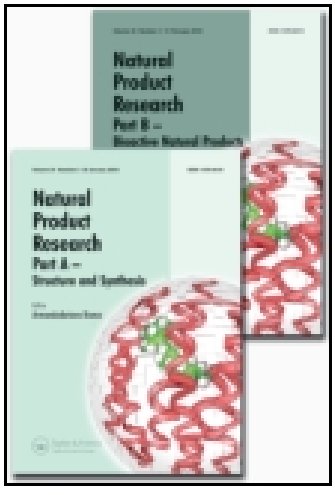

Natural Product Research: Formerly Natural Product Letters

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

Characterisation and bioactivity of oosporein produced by endophytic fungus Cochliobolus kusanoi isolated from Nerium oleander L.

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Published online: 17 Jun 2014.

To cite this article: Ramesha Alurappa, Madhusudhan Reddy Muthukurpalya Bojegowda, Vijith Kumar, Naveen Kumar Mallesh & Srinivas Chowdappa (2014) Characterisation and bioactivity of oosporein produced by endophytic fungus Cochliobolus kusanoi isolated from Nerium oleander L., Natural Product Research: Formerly Natural Product Letters, 28:23, 2217-2220, DOI: 10.1080/14786419.2014.924933

To link to this article: http://dx.doi.org/10.1080/14786419.2014.924933

Natural Product Research, 2014
Vol. 28, No. 23, 2217–2220, http://dx.doi.org/10.1080/14786419.2014.924933

SHORT COMMUNICATION

Characterisation and bioactivity of oosporein produced by endophytic fungus *Cochliobolus kusanoi* isolated from *Nerium oleander* L.

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(*Received 10 February 2014; final version received 13 May 2014*)

Bioactive compounds comprising secondary metabolites produced by endophytic fungi have wide applications in pharmacology and agriculture. Isolation, characterisation and evaluation of biological activities of secondary metabolites were carried out from *Cochliobolus kusanoi* an endophytic fungus of *Nerium oleander* L. The fungus was identified based on 18S rDNA sequence analysis. There are no reports available on the compounds of *C. kusanoi* hence, antimicrobial metabolite produced by this fungus was extracted and purified by fractionation using hexane, diethyl ether, dichloromethane, ethyl acetate and methanol. Out of all the solvent fractions, the methanol fraction exhibited better antimicrobial activity which was further purified and characterised as oosporein. Oosporein from *C. kusanoi* exhibited broad spectrum *in vitro* antimicrobial, antioxidant and cytotoxic activities. The characterisation and antioxidant activity of oosporein from *C. kusanoi* are reported for the first time.

Keywords: *Nerium oleander* L; *Cochliobolus kusanoi*; oosporein; antimicrobial activity; antioxidant activity; anticancer activity

1. Introduction

The *Nerium oleander* L. is a medicinal plant belonging to the Apocynaceae family and is grown worldwide. The leaves and flowers of *N. oleander* L. are traditionally used for the treatment of heart diseases, emesis and scabies; roots are used to treat leprosy and skin diseases (Sheikh et al. 2009). In recent years, endophytic fungi are gaining wide importance for their bioactivity by secondary metabolites (Eduardo et al. 2012). The genus *Cochliobolus* is known to produce quinone compounds such as cochlioquinone A and isocochlioquinone A, which exhibits leishmanicidal and anticancer activities (Campos et al. 2008). In this study, a secondary metabolite exhibiting antimicrobial activity was extracted from the endophytic fungus *Cochliobolus kusanoi*, and its structure was elucidated by using spectroscopic methods. The antimicrobial, antioxidant and cytotoxic activities of the purified compound were determined.

2. Results and discussion

The endophytic fungus isolated from the stem of *N. oleander* L. displayed profuse growth on potato dextrose agar and produced a diffusible red exudate (Figure S1). The identification of

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fungus was carried out by 18S rDNA sequence, and phylogenetic analysis revealed 100% sequence similarity to *C. kusanoi* (Figure S2). The ITS rDNA sequence of the strain has been deposited in the NCBI GenBank database, Maryland, USA (Accession number KF193636). The fungus was grown on potato dextrose broth and the culture filtrate was extracted with ethyl acetate followed by 0.1 N acetic acid (pH 3) to obtain a red colour crude extract. The extract was fractionated using hexane, diethyl ether, dichloromethane, ethyl acetate and methanol. Each fraction was concentrated using a rotary evaporator. The antimicrobial tests were performed using these fractions against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans*. The ethylacetate fraction revealed weak activity against the tested pathogens, whereas methanol fraction exhibited broad-spectrum antimicrobial activity (Table S1). Hence, methanol fraction was further purified using preparative RP-HPLC. A single peak was observed at the retention time of 3.318 min which conferred purity of the compound (Figure S3). Finally, this compound was crystallised using methanol:water system (95:5).

The R_f value of the purified compound was found to be 0.19 on TLC using the solvent system toluene:ethylacetate:formic acid (4:5:1). The melting point of the crystal was found to be at 263–270°C. UV–vis spectrum was recorded in DMSO, and λ_{max} was observed at 281 nm (0.7706 Å). Molecular weight of the compound was determined as *m/z* 329.0278 [M + Na] by HR-ESI-MS (Figure S4), and the percentage of carbon and hydrogen was found to be 55.21% and 3.26%, respectively from CHNS analysis. Based on the above-mentioned analysis, the empirical formula of the purified compound was found to be $C_{14}H_{10}O_8$.

Structural elucidation of the molecule was done by using FT-IR, ^{1}H NMR, ^{13}C NMR, HSQC and HMBC spectral analysis. FT-IR spectrum of the compound found stretching at 3308, 1648 and 1624 cm^{-1} conferring the functional groups O—H, C=O and C=C, respectively (Figure S5). ^{1}H NMR spectrum of the compound revealed broad peak at $\delta = 10.97$ ppm (br) confirmed four hydroxyl protons, at $\delta = 1.78$ ppm singlet peak was observed with two methyl protons (Figure S6). ^{13}C NMR spectrum of the compound revealed $\delta = 168.94, 197.72, 112.84, 107.22, 7.59$ ppm (Figure S7). The HSQC experiment suggested that methyl protons resonating at 1.78 ppm have a correlation with methyl carbon resonating at 7.59 ppm (a) (Figure S8). This proves that there are only two identical sets of protons coupled directly to carbon representing the methyl group. From HMBC experiment, it was concluded that the methyl proton (a) resonating at 1.78 ppm has a multiple bond correlation with ring quaternary carbon resonating at 112.84 ppm (b) (Figure S9, Figure 1). Further, single-crystal X-ray diffraction study of the block-shaped crystals revealed that the crystal structure belonged to the monoclinic centrosymmetric space group *C*2/*c* with $Z = 4$. The two symmetry-related substituted benzoquinone rings are connected to each other. Due to the extensive electron delocalisation in the compound, the benzoquinone ring is almost coplanar [r.m.s. deviations of the ring = 0.0010 Å]. Whereas owing to the steric hindrance from the methyl and hydroxyl groups, both benzoquinone rings are tilted towards each other, enclosing a dihedral angle of 67.68°. The bond length and bond angles are in good agreement with the reported structure

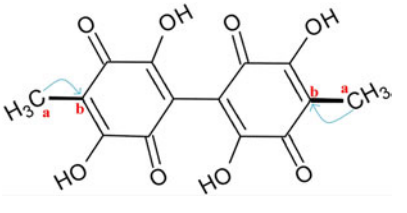

Figure 1. The structure and HMBC correlations of oosporein.

(Kaftory & Weisz 1984; Keegstra et al. 1994; He et al. 2012). Figure S10 shows the ORTEP diagram of the molecule. A summary of the crystallographic data and structural refinement details of the compound is given in Table S2. The intermolecular packing in the compound is mainly due to O—H···O hydrogen-bonded dimers (Figure S11), which is further supported by C—H···O hydrogen bonding and C—H··· π interactions (Figure S12, Table S3). From all the above-mentioned spectral data, the isolated compound from *C. kusanoi* was identified as 3,3′,6,6′-tetrahydroxyl-4,4′-dimethyle-[1,1′-bi(cyclohexane)]-2,2′,5,5′-tetraone (oosporein), and the empirical formula of the purified compound was confirmed to be $C_{14}H_{10}O_8$. The crystal data have been submitted to the Cambridge Crystallographic Data Centre (CCDC number 942418).

Oosporein from *C. kusanoi* exhibited a broad-spectrum antimicrobial activity against all tested pathogens. The minimum inhibitory concentration (MIC) of all the tested pathogens was found to be at 12.5 μM (Table S4), whereas 50% inhibitory concentration (IC_{50}) of oosporein varied with the different pathogens. The IC_{50} was found to be 13, 46, 40, 48, 64 and 35 μM for *S. aureus*, *B. cereus*, *E. coli*, *S. typhimurium*, *P. aeruginosa* and *C. albicans*, respectively. The compound was highly inhibitory to *S. aureus* followed by *E. coli*, *B. cereus*, *S. typhimurium* and *P. aeruginosa*. The compound at 25 μM revealed effective inhibition of *S. aureus* compared with positive control, and activity against *S. typhimurium* was at par with the positive control. The compound exhibited moderate activity against *C. albicans* compared with fluconazole, whereas Taniguchi et al. (1984) reported that oosporein exhibited antimicrobial activity by oxidative metabolism against Gram-positive bacteria *S. aureus* and *B. subtilis* with MIC of 12.5–50 μg/mL and was inefficient against Gram-negative bacteria, yeasts and moulds. In this study, oosporein inhibited Gram-negative bacteria and *C. albicans*. Oosporein revealed strong antioxidant activity that increased in a dose-dependent manner from 0.075 to 1.5 mM (Figure S13). The compound exhibited 50% DPPH-scavenging activity at 0.194 mM, which is very close to the reference standard (ascorbic acid) at 0.189 mM. The large conjugated system and hydroxyl groups in the chemical structure of oosporein may confer it to be having the strong antioxidant activity. This discovery is similar to that of Yuan et al. (2013), who reported methyl β-D-ribofuranoside, 2-deoxyadenosine and 3-methylpiperazine-2,5-dione, which have a large conjugated system and many hydroxyls; hence, these compounds show strong antioxidant activity. Huang et al. (2007) reported that most endophytic fungi isolated from *N. oleander* exhibited antioxidant activity similar to the host plant. The cytotoxicity of the oosporein was evaluated by using MTT assay on human lung carcinoma type II epithelial cells (A549). The cells treated with 5–50 μM oosporein exhibited decreased cell proliferation with increasing concentration of the compound, and the IC_{50} was observed at 21 μM (Figure S14). Mao et al. (2010) reported antitumour activity of oosporein on A549 cell lines at IC_{50} 28.66 μM. Earlier studies reported that oosporein at 100 μg/mL had no effect on *in vitro* cell cultures of hamster tumour cells and baby hamster kidney cells, and at 600 ng/mL had no adverse effect on two different mammalian cell lines (Wainwright et al. 1986; Strasser et al. 2000).

3. Conclusion

From the above-mentioned data, the orange red compound isolated from the endophytic fungus *C. kusanoi* of *N. oleander* L. was oosporein exhibiting antioxidant, antimicrobial and cytotoxic properties.

Supplementary material

The experimental section relating to this article is available online, alongside Figures S1–S14, and Tables S1–S4.

Acknowledgements

The authors are grateful to Prof. T.N. Guru Row, Solid State and Structural Chemistry Unit, Indian Institute of Science, Bangalore for providing single-crystal XRD facility. We also sincerely thank the Society for Innovation and Development and NMR Research Centre, Indian Institute of Science, Bangalore for providing the facility for spectral analysis.

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