SUPPLEMENTARY MATERIAL

Urease inhibition potential of Di-naphthodiospyrol from *Diospyros lotus* roots

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Abstract

The dimeric naphthoquinone 5,8,4'-trihydroxy-1'-methoxy-6, 6'-dimethyl-7,3'-binaphthyl-1,4,5',8'-tetraone (**1**) was isolated from the chloroform fraction of *Diospyros lotus* extract. Compound **1** was screened for its inhibitory effects against four enzymes: urease, phosphodiesterase-I, carbonic anhydrase-II, and α -chymotrypsin. It showed selective activity against urease with an IC₅₀ value of 254.1 ± 3.82 μ M as compared to the standard thiourea (IC₅₀ = 21 ± 0.11 μ M). Further, *in silico* docking study was carried out to explain the molecular mechanism of compound **1** against the target receptor.

Key words: *Diospyros lotus*; Ebenaceae; di-naphthodiospyrol M; urease inhibitory activity; molecular docking.

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Experimental

Plant material

D. lotus roots were collected from Razagram (Khall), Dir, KPK, Pakistan, in May 2009. The specimen was identified and authenticated by senior Prof. Dr. A. Rashid, a taxonomist at the Department of Botany, University of Peshawar (DBUP), Peshawar, Pakistan. A voucher specimen (Bot. 20036 (PUP) was deposited at the herbarium located at the Department of Botany, University of Peshawar, Pakistan.

Extraction and isolation

Shade-dried roots of *D. lotus* (14 kg) were ground with the aid of a local grinder machine and repeatedly extracted with methanol (MeOH) at room temperature. Extracts were combined and concentrated under reduced pressure at a temperature below 55^oC by rotary evaporator, which furnished a dark red residue (202 g). The crude extract was suspended in distilled water and successively partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butyl alcohol subsequently. The residue (30 g) from the chloroform fraction was subjected to column chromatography (CC). The column was eluted with *n*-hexane-ethyl acetate (100:0→0:10→0:100) as solvent system. A total of 105 fractions, PS-1 to PS-105 were obtained based on TLC profiles. Further elution the column with *n*-hexane-ethyl acetate (100:0 → 20:80) a violet red fraction was obtained which showed two compounds based on TLC profile TLC. This fraction was purified by preparative chromatography which resulted in the isolation of an orange red compound identified as 5,8,4'-trihydroxy-1'-methoxy-6,6'-dimethyl-7,3'-binaphtyl-1,4,5',8'-tetraone (**1**). Identification of compound **1** was confirmed by means of various 2D NMR techniques (Rauf et al., 2014).

2.3. Enzymes inhibition assay

Mixtures of 25 μ L of jack bean (*Canavalia ensiformis*) urease, 55 μ L of buffer at pH 6.8, 100 mM of urea, and 5 μ L of various concentrations of test compounds (from 0.5 to 0.00625 mM) were incubated at 30°C for 15 min in 96-well plates. In kinetics experiments, various concentrations of both substrates and test compounds were used. Subsequently, 45 μ L of phenolic reagents (1% w/v phenol and 0.005% w/v sodium nitroprusside), and 70 μ L of alkali reagent (0.5% w/v NaOH and 0.1% w/v NaOCl) were added to each well. Urease activity through indophenols method was measured by the production of ammonia, as described by Weatherburn (Weatherburn, 1967). After 50 min, the increasing absorbance at 630 nm was measured in a microplate reader (SpectraMax M2, Molecular Devices, CA, USA). All reactions were performed in triplicate in a final volume of 200 μ L. Thiourea was employed as the standard inhibitor of urease (Uddin et al., 2013). The phosphodiesterase-I, carbonic anhydrase-II, and chymotrypsin inhibition assays were evaluated according to literature protocols (Ahmad et al., 2003; Arslan, 2001; Cannell, Kellam, Owsianka, & Walker, 1988). Finally, results were processed by software SoftMax Pro (Molecular Devices, CA, USA), MS-Excel and Ez-fit programs. Percentage inhibition was calculated according to the formula given below:

$$\% \text{ Inhibition} = 100 - (\text{OD test} / \text{OD control}) \times 100.$$

2.4. Computational docking

Urease enzyme 3D structure (PDB code 4GY7 from jack bean) was downloaded from protein data bank (PDB). Geometry optimization of the crystal structure was carried out with the aid of a Swiss PdbViewer v4.1.0 program (Guex & Peitsch, 1997). Compound **1** and standard thiourea structures were prepared for docking by using ChemSketch (Li, Wan, Shi, & Ouyang, 2004) and Avogadro softwares (Hanwell et al., 2012). Docking studies were carried out through Autodock

Vina (Trott & Olson, 2010) and i-GEMDOCKv 2.1 softwares (Hsu, Chen, Lin, & Yang, 2011). Initially, method optimizations of the docking softwares were carried out.

PyRx virtual screening tool was connected to Autodock vina (Yellamma, Nagaraju, Peera, & Praveen). Removal of solvent molecules, hydrogen addition and Gasteiger charges calculation was carried out (Chang, Ayeni, Breuer, & Torbett, 2010). Furthermore, all default parameters were used for the docking through Autodock vina (Jacob, et al., 2012; Rauf et al., 2015a). Another docking was also carried out with i-GEMDOCKv2.1 software, implemented with genetic evolutionary algorithm and empirical scoring function. All the default parameters were used for docking simulation (Rauf et al., 2015b). Interaction analysis of docked complexes were performed by LIGPLOT+ version v.1.4.5 (Laskowski & Swindells, 2011) and PyMOL version 1.7.2 (Jacob et al.,2012).

Table S1. Enzymes inhibitory activities of compound **1**

Compounds	Urease		Phosphodiesterase-I	Carbonic anhydrase-II	α -Chymotrypsin
	% Inhibition (0.5 mM)	IC ₅₀ $\pm$ S.E.M. (μ M)	% Inhibition (0.5 mM)	% Inhibition (0.5 mM)	% Inhibition (0.5 mM)
1	72.11	254.1 $\pm$ 3.82	43.2	15.9	46.1
Standard	Thiourea	Thiourea	EDTA	Acetazolamide	Chymostatin
	98.2	21 $\pm$ 0.11	80.1	89	98.6

Table S2. Docking statistics of compound **1** and reference thiourea against the urease enzyme

Compounds	Autodock Vina score (kcal/mol)	i-GEMDOCK score (kcal/mol)			
	B. Affinity	Total Energy	VDW	H-bond	Elec
Compound 1	-7.9	-108	-94	-14	0
Thiourea	-3.4	-44	-24	-20	0

Figure S1. The best docked conformation of compound **1** (sticks green color) along with the standard thiourea (sticks red color), against urease enzyme (cyan color). These binding conformations were produced in the binding site, where metals (black dots representing nickel) are already present in the active site of crystal structure.

Figure S2. The 2D (left) and 3D (right) interaction profile of Compound **1** with the active site of urease enzyme. In the above 2D image half-moon shows the hydrophobic interactions whereas hydrogen bond is represented by the dotted green lines with distance in Angstrom.

Figure S3: Chemical structure of compound **1**

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