Computational Prediction of Molecular Targets responsible for Antioxidant Activity of D-pinitol in *Caenorhabditis elegans*

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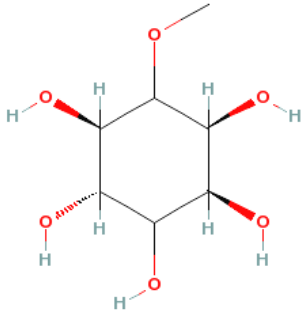
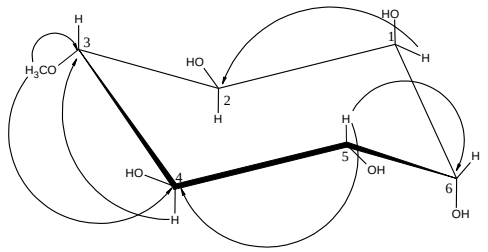
ABSTRACT
D-pinitol (3-O-methyl-D-inositol), a form of vitamin B inositol is a sugar-like molecule used for natural healing purposes for various diabetic-associated conditions. It is found in various plants like legumes, leafy vegetables, and citrus fruits, but is not found in animals and humans. In the present investigation, we have predicted possible biological molecular targets for D-pinitol using reverse docking approaches. In the process, we have identified that D-pinitol have affinity for most of the enzymes directly/indirectly associated with the free radical scavenging processes, indicating that D-pinitol might use as a potential antioxidant. The prediction was further in vivo validated on *C. elegans*, a multicellular model system using chemotaxis, thermo-tolerance and ROS scavenging activities assay. A strong correlation was observed in the computational prediction and *in vivo* antioxidant activities assays of D-pinitol in a dose-dependent manner. The findings broaden our current perspectives in understanding the antioxidative properties of D-pinitol.

Keywords
Caenorhabditis elegans, D-pinitol, Anti-oxidative activity, Reverse docking approach

1. INTRODUCTION
Extensive research on different phytomolecules for their therapeutic applications has led to the need for developing novel herbal drugs. D-pinitol, an alicyclic polyalcohol is a naturally occurring compound and is found in various plants, trees, and foods such as soy. It is a methyl-inositol extract that promotes the transport of glucose and glycogen synthesis. D-pinitol possesses multifunctional properties by exerting insulin-like effects by driving creatine and other nutrients into muscle cells [1], and is also used for the prevention of cardiovascular diseases. D-pinitol was isolated from *Desmodium gangeticum* DC. (Shalparni), a well-recognized medicinal plant from the family Fabaceae, used in the treatment of various ailments. The immense medicinal potential of the herb is mainly due to its anti-oxidative [2], anti-diabetic[3], anti-inflammatory, anti-fertility [4], anti-ulcer [5] and analgesic [6] properties. The active constituents of the plant extract were examined through HPLC and NMR techniques which revealed the novel source for the isolation of D-pinitol (3-O-methyl-D-chiro inositol) from *D. gangeticum* (Fig. 1A, 1B).



(A)



(B)

Figure 1 (A) two dimensional structure of D-pinitol. (B) Most significant HMBC Correlations (NMR). IUPAC: (1R,2S,4S,5S)-6-methoxycyclohexane-1,2,3,4,5-pentol | MW: 194.182460 g/mol | MF: C7H14O6

D-pinitol is an alicyclic polyalcohol, belonging to the group of compounds called 'Cyclitols'. Besides possessing insulin-like effects, via driving creatine and other nutrients into muscle cells [1], it also exert multifunctional properties, including anti-inflammatory activity [7], and has also been implicated in the prevention of cardiovascular diseases [8]. The isolated bioactive molecule is an important stress metabolite in plants and its accumulation may be related to plant tolerance to water deficit stress [9]. The molecular insight of antioxidative property shown by D-pinitol is still not investigated in detail. We used several bioinformatics resources to predict the molecular targets of D-pinitol followed by in vivo validation in *C. elegans* model.

2. MATERIALS AND METHODS

2.1 Identification of molecular targets for D-pinitol

Reverse docking approach was used to predict the molecular targets of D-pinitol using Potential Drug Target Database (PDTD) [10]. PDTD is a web accessible database that currently contains 1207 proteins covering 841 known and potential drug targets with structures in Protein Data Bank [11]. PDTD provides quick screening of potential binding proteins for any drug like molecule using TarFisDock server [12] that works on reverse ligand-protein docking approach [13-16].

TarFisDock server tries to dock the ligand into already known active site of protein using PatchDock algorithm [17] where the surfaces of receptor and ligand are first divided into pathches and then further use of geometric hashing algorithm to indentify maximum surface shape complementary, while minimizing the number of steric clashes. Server generates the target list in the increasing order of interaction energy (E_{inter}), which is collectively expressed with the van der Waals and electrostatic interaction:

$$E_{\text{int}er} = \sum_{i=1}^{lig}\sum_{j=1}^{rec}\left(\frac{A_{ij}}{r_{ij}^{a}} - \frac{B_{ij}}{r_{ij}^{b}} + 332.0\frac{q_i q_j}{Dr_{ij}}\right)$$

Where i and j are the atoms in ligand and receptor, r_{ij} is the distance between the receptor and ligand atom, A_{ij}, B_{ij} and a, b are the parameters and exponent of van der Walls repulsion and attraction, q is the charges on the atoms, D is dielectric function, and conversion factor 332, convert the electrostatic energies in to the kcal/mol.

2.2 Geneontology (GO) analysis of the targeted proteins

In order to predict the molecular processes, biological functions and metabolic pathways associated with the proteins targeted by D-pinitol, we performed gene ontology analysis using PANTHER server (Protein ANalysis THrough Evolutionary Relationship: http://www.pantherdb.org). For this, PDB ids of targeted proteins were converted into UniProt IDs using ID Mapper/ Converter tool available on UniProt server (http://www.uniprot.org/mapping/) and uploaded to Panther Ver. 8.0 database for the assignment of GO terms. All the proteins that are involved in the oxygen and reactive oxygen species metabolic processes were filtered and analyzed for the mechanism of antioxidative activities shown by D-pinitol.

2.3 Identification of protein homologs in *C. elegans*

To validate the antioxidant property of D-pinitol *in vivo*, we used *C. elegans* model system. For all the probable proteins responsible for antioxidant property of D-pinitol, we searched the homologs in *C. elegans* using BLAST/BLAT search tool available on wormbase (www.wormbase.org/tools/blast_blat). The antioxidant potential of D-pinitol was further evaluated using various *in vivo* assays.

2.4 Maintenance of *Caenorhabditis elegans* strains

The wild type *C. elegans* N2 Bristol strain and daf-16 (mgDf50) mutant were used in this experiment. Worms were maintained on nematode growth medium (NGM) and fed with OP-50 *Escherichia coli* [18] at 20^{o}C. The strains used in this experiment were obtained from the Caenorhabditis Genetics Center, University of Minnesota, and Minneapolis, USA. D-pinitol was added directly to the bacterial OP-50 food in order to get the final concentrations of 500µM, 50µM and 5µM. Since D-pinitol toxicity for the above concentrations was not found, so these concentrations were selected for further evaluation.

2.5 Measurement of intracellular oxidative free radicals

To measure the intracellular ROS levels, age-synchronized N2 worms were grown on untreated and treated NGM plates. The assay was done using adult day 2 worms according to the method of Kampkotter et al., 2007 with some modifications [19]. Worms were collected in groups of 50 in sterile eppendorf tubes containing 300 µl of 0.1% PBST. To break the outer cuticle and lyse the cellular contents, the worms were then subjected to equally-timed sonication (Branson Sonifier 250, VWR Scientific, Suwanee, GA). Immediately prior to reading, 15µl of 10mM DCF-DA was added to sonicated samples in a final concentration of 50 µM. Samples were pipetted into 96-well plates. Non-fluorescent DCF-DA is a freely cell permeable dye which interacts with intracellular ROS and is readily converted to fluorescent 2'7'-dichlorofluorescein (DCF). Fluorescent readings were measured using Spectra Max M2 multimode micro plate reader, (Molecular Devices) at 485 nm excitation and 530 nm emission. Observations were recorded after every 20 min for 210 minutes at 37^{o}C.

3. RESULTS AND DISCUSSION

We analyzed top 10% protein (106 proteins) returned by TarFisDock server as potential targets for D-pinitol. We further screened them for their involvement in oxidative stress

related processes by mapping the GO terms with PANTHER server. Figure 2 highlights various biological processes governed by potential enzymes targeted by D-pinitol.

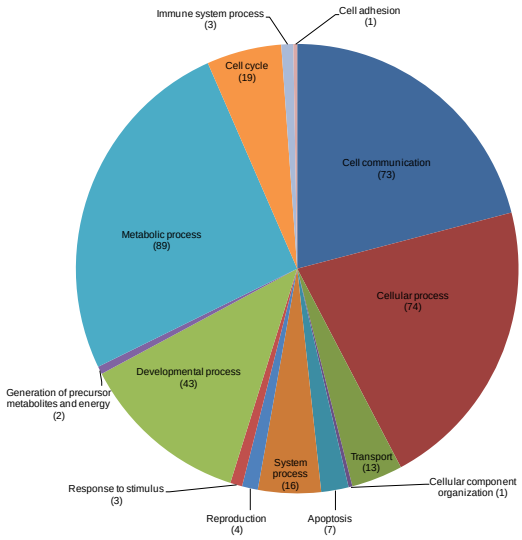


Figure 2 Pie chart showing various biological processes that might get affected by D-pinitol treatment. Number below each biological process represents total number of potential enzymes involved in respective biological processes and targeted by D-pinitol in *C. elegans*.

In total 6 important enzymes were identified as potential targets for D-pinitol and involved in oxidative stress related processes (Table 1).

Table 1 Enzymes involved in oxidative stress related process and targeted by D-pinitol

S. No.	Enzyme targeted	UniProt ID	PDB ID	Class
1	Glutathione S-transferase P	GSTP1_HUMAN	18GS	Transferase
2	Nitric oxide synthase	NOS2_HUMAN	4NOS	Oxidoreductase
3	Glutathione S-transferase A3	GSTA3_HUMAN	1TDI	Transferase
4	Glutathione S-transferase theta-2	GSTT2_HUMAN	3LJR	Transferase
5	Mitogen-activated protein kinase 10	MK10_HUMAN	1JNK	Transferase
6	Signal transducer and activator of transcription 1-alpha/beta	STAT1_HUMAN	1YVL	Signaling protein

For all the 6 enzymes mentioned in the table 1, amino acid sequences were downloaded from UniProt and BLAST/BLAT search was performed using sequence alignment tool available on WormBase. Table 2 highlights all the potential homologs from *C. elegans* for all the enzymes targeted by D-pinitol in Table 1.

Table 2 Blast result from *C. elegans* protein database with the enzymes targeted by D-pinitol

Enzyme targeted by D-pinitol	Homologous *C. elegans* enzyme (WormBase id)	E-value	% identities	% similarity
Glutathione S-transferase P	GST-1 (WP:CE00302)	1e-42	39	58
Nitric oxide synthase	EMB-8 (WP:CE02018)	2e-52	26	43
Glutathione S-transferase A3	GST-37 (WP:CE21504)	2e-18	29	50
Glutathione S-transferase theta-2	GST-31 (WP:CE22421)	1e-3	24	42
Mitogen-activated protein kinase 10	JNK-1, isoform a (WP:CE27574)	1e-157	69	83
Signal transducer and activator of transcription 1-alpha/beta	STA-1, isoform C (WP:CE22342)	2e-35	26	45

The interaction of D-pinitol with GSTP is shown in Figure 3. Total 5 hydrogen interactions can be seen in the figure with SER65, ASP98, GLN51 amino acid residues of GSTP in the binding cavity.

Previous studies have already reported the upregulation of glutathione S-transferase gene in *C. elegans* during oxidative stress conditions [20, 21]. Another protein nitric oxide synthase has similarity with EMB-8 protein i.e. NADPH-cytochrome P450 reductase associated with increased production of ROS. Similarly, D-pinitol also target JNK-1 isoform a in *C. elegans* that promote DAF-16 translocation into the nucleus in the stress condition which in turn activates several genes to increase stress resistance [22, 23]. Oxidative stress also activates STAT1, which is one of the important signaling molecule that transmit signals from the cell surface in response to several cytokines/ growth factors [24]. Thus computational data clearly indicate that D-pinitol will show the antioxidative activities in *C. elegans* model system as most of the stress related enzymes targeted by D-pinitol have homologs in *C. elegans.*

To validate our results of antioxidant activity of D-pinitol, we measure the intracellular ROS levels in control and D-pinitol treated worms. The anti-oxidative potency of D-pinitol in *C.*

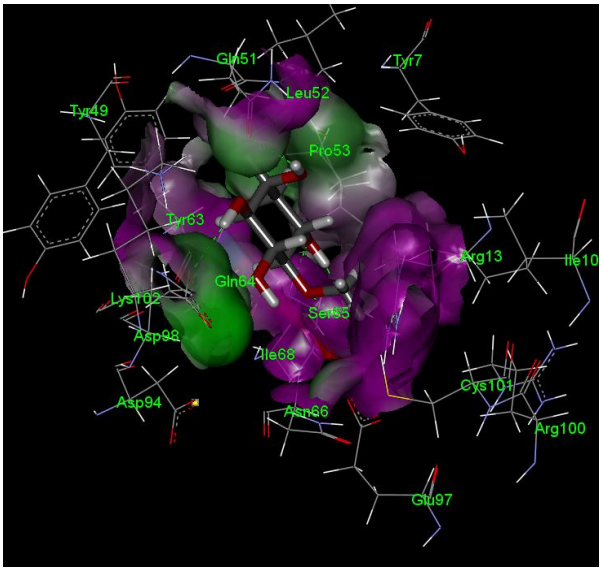


Figure 3 Interaction of D-pinitol with GSTP, generally upregulated in the stress conditions. Amino acid residues in the binding cavity are labeled. Surface is generated around the D-pinitol and colored on the basis of hydrogen bond donor/ acceptor. Donors colored in green and receptor acceptors in cyan. Hydrogen bonds are shown with dotted green color lines.

elegans was determined using a DCF method according to standard protocol. Age-synchronized worms were exposed to different concentrations of D-pinitol. The adult day 2 worms were collected for DCF assay and the levels of H_2O_2 using a fluorescent microplate reader were determined. Dose-dependent diminution of H_2O_2 levels in the wild type worms is shown in Figure 4 . Significant differences in ROS level were observed for different treatment groups when compared with untreated control ($P<0.001$).

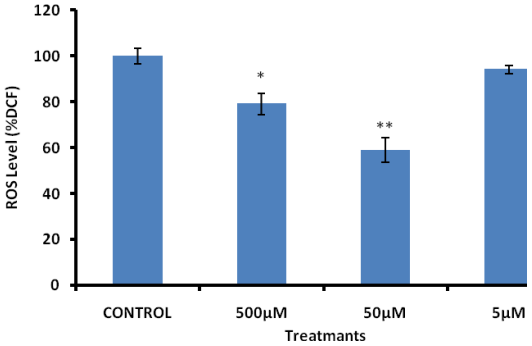


Figure 4 The effect of D-pinitol on ROS accumulation at 37°C. *C. elegans* were grown on solid NGM media at 20°C. Varying concentrations of D-pinitol were added directly to OP50 food source and animals were treated from L1 until experimentation. Following treatment, age synchronized 2 days old nematodes were collected and incubated with DCF-DA. Levels of H_2O_2 were detected at 37°C in a fluorescent microplate reader.

In summary, D-pinitol, a potential alicyclic polyalcohol possesses antioxidant property as it is able to able to decrease free radical generation within the organism in *in vivo* settings.

4. CONCLUSION

Identification of novel compounds with antioxidative property in multiple species are highly desirable, both as a tool for further research along with potential therapeutic avenues for ROS related diseases. The present experimentation evaluates the effect of different pharmacological doses of D-pinitol on the stress tolerance in the nematode *Caenorhabditis elegans*. A variety of properties of D-pinitol such as anti-inflammatory effects, reduction of glucose concentrations, antihyperglycemic effect, maltase dehydrogenase activity, regulation Th1/Th2 balance in ovalbumin-induced asthama, increase in plant tolerance against water deficit stress has already been reported. Owing to these immense properties of D-pinitol, a keen interest is generated to evaluate its anti-oxidant potentials as well. The present study provides computational protocols followed by *in vivo* validation of antioxidant property of D-pinitol. These protocols may be further generalized to screen other therapeutic compounds with antioxidant property.

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