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Antibacterial activity of leaf extract of *Delonix elata* and molecular docking studies of luteolin

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Abstract

In vitro antibacterial activity of leaf extract of *Delonix elata* was screened against both gram negative and gram positive bacteria. The methanol extract of leaf showed significant antibacterial activity against *Klebsiella pneumonia* (18.40 ± 0.80) and *Bacillus subtilis* (17.60 ± 0.55) with minimal inhibitory concentration of 1mg ml^{-1} and exhibited less bactericidal activity against *P. vulgaris* (9.83±0.31). *In silico* docking of Luteolin, a bioactive compound from leaf extract of *D. elata* with a key enzyme of bacterial glucosamine-6-phosphate synthase, showed significant inhibition with minimum docking energy -10.12 $kJmol^{-1}$, binding energy -10.07 kJ mol^{-1} and inhibition constant 4.14e-008. The molecular docking study was comparatively evaluated with the standard drug ciprofloxacin with an inhibition constant 4.2e-007. This investigation supports the medicinal claim of *D. elata.*

Key words: *Delonix elata,* Antibacterial activity, Luteolin, Glucosamine-6-phosphate synthase, Molecular docking.

Introduction

Medicinal plants have been used from centuries to treat infectious diseases as an alternative form of health care. In recent years there has been a rising interest in the discovery of new antimicrobial compounds; due to alarming increase in the rate of infections with multi-drug resistant microorganisms (Bassam et al. 2006). This has lead researchers to investigate the antimicrobial activity of the medicinal plants. The antimicrobial resistance among key microbial pathogens continues to grow at an alarming rate (Kresken and Wiedemann 1988; Bhavani and Ballow 2000). In many parts of the world fluoroquinolone antibiotics like pefloxacin, and ciprofloxacin are recommended for serious infections associated with *Klebsiella*, *Pseudomonas* and *Staphylococcus* species. The increased prevalence of antibiotic-resistance bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control some bacterial diseases (Cowan 1999). Plants produce bioactive molecules that allow them to interact with other organisms in their environment. These bioactive compounds are important in defense mechanism and contribute to the resistance to diseases. Many investigators evaluated the bioactivity of plant extracts and the constituents against the serious infectious organisms (Parekh and Sumithra 2006; Kausik et al. 2002).

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Delonix elata (Linn.) is a tree species belongs to the family Fabaceae (Leguminosae), sub family Caesalpinioideae. The traditional practioners residing in the villages of Chitradurga and Davanagere districts of Karnataka, India have been using for the treatment of bronchitis or pneumonia in infants, fever, abdominal pains and flatulence. Medicinal uses of *D. elata* were reported by Pavithra et al. (2010). Wijayasiriwardena et al. (2009) reported the presence of luteolin from ethyl acetate fraction of methanol extract of leaf of *D. elata*. Luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one), a polyphenolic flavonoid compound widely occurring as glycosylated forms in most of the plant species (Kim et al. 2005; Shimoi et al. 2000). Many investigators reported the antibacterial activity of luteolin against a number of pathogenic bacteria (Basile et al.1999; Mori et al. 1987; Ramesh et al.2002; Bashir et al. 1994; Sato et al.2000; Miski et al.1983).

Bacterial proteins are the ultimate target to inhibit their growth since these are the executors of many cellular functions. The key enzyme L-glutamine: D-fructose-6phosphate amidotransferase, known under the trivial name of glucosamine-6-phosphate synthase (EC 2.6.1.16) is responsible for the synthesis of glucosamine-6-phosphate (GlcN-6-P) from D-fructose-6-phosphate and L-glutamine. This is the key enzyme in the pathway leading to the formation of UDP-N-acetylglucosamine (UDP-GlcNAc), the major intermediate in the biosynthesis of all aminosugar containing macromolecules both in prokaryotic (Bates and Pasternak 1965; Imada et al. 1977) and eukaryotic cells (Cabib et al. 1982; Winzler and Bekesi 1967). In bacteria, this enzyme is concerned to build peptidoglycan of

bacterial cell wall. This is due to the fact that glucosamine-6-phosphate synthase (GlcN-6-P synthase) has been exploited as a target molecule for the authentication of antibacterial drug. In addition, inhibition of this bacterial life sustaining enzyme has some important implications for therapy (Chmara et al. 1984). It has been reported that even a short-time inactivation of GlcN-6-P synthase was lethal to the pathogenic microorganisms by inducing morphological changes, agglutination and lysis. (Bates et al. 1966; Chmara and Borowski 1986; Milewski et al. 1986).

In the present study, we made an attempt to screen the *in vitro* antibacterial activity of leaf extract of *D. elata* against eight different human pathogenic bacterial species and *in silico* docking of Luteolin, a bioactive principle from leaf extract *D. elata* with the GlcN-6-P synthase .

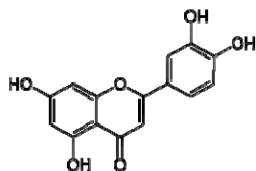

Figure 1: Luteolin structure

Materials and methods

Plant material and extraction

The leaves of *D. elata* were collected from the villages near by Chitradurga district, Karnataka, India. The plant material was identified and authenticated by Dr. Manjunatha B.K., comparing with the voucher specimen deposited at Kuvempu University Herbarium specimen FDD, (Flora of Davanagere District, Karnataka, Manjunatha B.K et al. 2004). The leaves were cleaned with deionized water and were shade dried, grounded porously by using mechanical blender and passes through 40-mesh sieve. About 1 kg of powdered material was loaded into four Soxhlet timbles of 250 g each and extracted using methanol for about 48 h. The extracts were filtered (Whatman No.1 filter paper) and concentrated in vacuum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and then the extract was kept on water bath for complete evaporation of solvent. Finally the dried extract was preserved in air tight container until use.

Bacterial strains and culture media

Various cultures of human pathogenic gram positive bacteria namely, *Bacillus subtilis*-NCIM-2063, *Staphylococcus aureus*-NCIM-2079, and *Pseudomonas aeruginosa*-NCIM-2036 and gram negative bacteria namely, *Escherichia coli*-NCIM-2065, *Proteus vulgaris*-NCIM-2027, *Salmonell typhi*-NCIM- 2501, *Klebsiella pneumoniae*-NCIM- 2957 and *Salmonell paratyphi*-MTCC-735 were obtained from National Chemical Laboratory, Pune and Microbial type culture collection and gene bank, Chandigarh, India and were used for screening of antibacterial activity of Leaf extract of *D. elata*. The microorganisms were repeatedly subcultured on sterile nutrient agar media in order to obtain pure isolates. A loop full test organism was inoculated on nutrient broth and incubated for 24 h at 37 ± 1°C and maintained in sterile condition.

Agar well diffusion assay

Antibacterial activity of leaf extract was screened against eight different bacterial strains by agar diffusion method. The culture plates were prepared with sterile nutrient agar media. 100 µl of bacterial culture (10^5cells/ ml) was inoculated on to the culture plate using sterile L-shaped glass rod to get uniform distribution of bacteria. Wells were created using a stainless steel sterilized cork borer (6.0 mm) under aseptic conditions. 50µl of the plant extract at different concentrations (100, 80, 60, 40, 20, 10 mg/ml) were aseptically loaded into wells. For comparative evaluation, Ciprofloxacin (BioChemika, ≥98.0% (HPLC) (Fluka)) was used as a positive reference standard and sterile distilled water as negative control. Then, the cultured plates were incubated for 24 h at 37^{0}C. After incubation, inhibition of the bacterial growth was measured in mm. The result was statistical analysed using one-way ANOVA and all the values are expressed as Statistical analysis mean ± S.D. (n = 9). A value of $P < 0.05$ was considered as significant.

Minimal inhibitory concentration determination

Minimal inhibitory concentration (MIC) values were determined by broth dilution method. Serial dilutions (final volume of 1 ml) of leaf extract of *D. elata* (2 to 0.25 mg ml^{-1}) were performed with 0.9% saline. Following this, 9 ml of nutrient broth was added. Broths were inoculated with 100 µl of each bacterial suspension (5 × 10^4CFU) and incubated for 24 h at 37^{0}C. Ciprofloxacin was used as a positive control and 0.9% saline as negative control. After 24 h, bacterial growth was assayed by measuring absorbance at 625 nm.

Molecular docking studies

Automated docking was used to determine the orientation of inhibitors bound in the active site of GlcN-6-P synthase. A Lamarckian genetic algorithm method implemented using the program AutoDock 3.0. The ligand molecules, Luteolin, and Ciprofloxacin were designed and the structure was analyzed by using ChemDraw Ultra 6.0 and 3D coordinates were prepared using PRODRG server (Ghose and Crippen 1987). The protein structure file (PDB ID: 1gdo) was taken from PDB (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C- terminal oxygen (Binkowski et al. 2003). For docking calculations, Gasteigere–MarsiliMpartial charges (Gasteiger and Marsili 1980) were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at the following residues of the protein namely, Cys 1, Trp 74, Thr 76, His 77, Gly 99, and Asp 123 were predicted from the Lig Plot and were generated with help of AutoGrid. The Lamarckian genetic algorithm was applied for minimization, using default parameters.

Results and discussion

The Soxhlet extraction of 1000 g of leaves powder of *D. elata* yielded 10% w/v methanolic extract. The preliminary phytochemical screening of the leaf extract gave positive tests for the presence of flavonoids, glycosides, triterpenoids, saponins, and tannins (Table 1).The antibacterial activity of methanol extract of leaf of *D. elata* was examined concomitantly with the standard drug Ciprofloxacin a well-known broad-spectrum antibacterial agent. The results of antibacterial screening of the leaf extract and the standard drug Ciprofloxacin are presented in Table 2. In the agar diffusion method, leaf extract of *D. elata* showed a significant level of bacterial inhibition against *Klebsiella pneumonia* (18.40 ± 0.80) and *Bacillus subtilis* (17.60 ± 0.55). It showed moderate activity against *Escherichia coli* (15.00±0.37), *Staphylococcus aureus* (14.33±0.33), *Salmonella typhi* (14.33±0.74) and the antibacterial activity was very less against *Salmonella paratyphi* (12.17±0.17) and *Proteus vulgaris* (9.83±0.31). The MIC values determined by broth dilution method indicated significant antibacterial activity at

Table 1: Phytochemical screening of methanolic extract of leaf of *D. elata*

Sl. No.	Test	Procedure	Observation	Result
1	Alkaloids	Extract + Dragondroffs reagent	No orange ppt	−
		Extract + Mayer's reagent	No white ppt.	−
		Extract + Hager's reagent	No yellow ppt.	−
2	Sterols	Extract + Liebermann test	No change in color	−
3	Flavonoids	Shinodaw's test	Red color	+
		Zn-HCl acid reduction test	Magneta color	+
4	Glycosides	Extract + Anthrone + H_2SO_4 +Heat	Purple color	+
5	Terpenoids	Extract + chloroform + con. H_2SO_4	Lower layer turns yellow	+
6	Tannins	Extract + lead acetate + water	White ppt.	+
7	Quinones	Extract + conc. H_2SO_4	No red color	−
8	Saponins	Extract + water + Shake well	Formation of stable froth	+

+ = Present, − =Absent

2.0 to 1.0 gml^{-1}(Table 3). Overall leaf extract showed significant antibacterial activity against tested bacterial strains.

Table 2: Antibacterial activity of Leaf extract of *D. elata* against various bacterial strains by agar diffusion method

Sl. No.	Bacterial strains	Gram stain	Ciprofloxacin	Leaf extract of *D. elata*
1	*B.subtilis*- NCIM-2063	+	23.17±0.31	17.60 ± 0.55
2	*E.coli* -NCIM-2065	−	23.67±0.33	15.00±0.37
3	*K.pneumoniae*-NCIM-2957	−	25.00±0.26	18.40 ± 0.80
4	*P. aeruginosa* -NCIM-2027	+	23.00±0.26	12.33±0.21
5	*P.vulgaris*-NCIM-2027	−	21.67±0.42	9.83±0.31
6	*S.aureus*-NCIM-2079	+	23.17±0.17	14.33±0.33
7	*S.typhi*-NCIM- 2501	−	22.50±0.62	14.33±0.74
8	*S.paratyphi*-MTCC-735	−	23.50±0.50	12.17±0.17

Note: Zone of inhibition in mean± SE mm (n=9)
Values*p<0.05 are considered significant compared to standard

Medicinal claim indicated leaf methanol extract of *D. elata* have been used to treat bacterial infections like treatment of bronchitis or pneumonia in infants, fever. The results of this investigation provided additional support for the confirmation of antibacterial activity. This could be the presence of a flavones compound Luteolin. Wijayasiriwardena et al. (2009) isolated luteolin from the methanol extract of leaves of *D. elata*. In addition, Ramesh et al. (2002) also isolated luteolin from leaves of *Begonia malabarica*. Reports also indicated that most of the flavonoid compounds exhibited anti bacterial propereties (Tim et al. 2005) and the antibacterial property of luteolin has been evaluated against human pathogenic bacterial strains(Ramesh et al. 2002). This showed that the significant antibacterial activity of *D. elata* is could be the presence of the bioactive compounds like luteolin. However, the mode of action of the drug ligands on the target molecules has not been evaluated.

Comparative docking of Glutamine amidotransferase domain of bacterial Glucosamine 6-phosphate synthase (PDB ID: 1gdo) with the luteolin and the ciprofloxacin was done to support the *in vitro* antibacterial activity. The docking of luteolin with glutamine

Table 3: MIC values bacterial strains

Sl. No.	Test bacterial strains	Leaf extract (mg/ml)
1	*B.subtilis*	1
2	*E.coli*	2
3	*K.pneumoniae*	1
4	*P.aeruginosa*	2
5	*P.vulgaris*	2
6	*S.aureus*	2
7	*S.typhi*	2
8	*S.paratyphi*	2

Table 4: Molecular docking of ligand molecules with glucosamine-6-phosphatesynthase

Sl. No.	Drug	Docking energy (kJ mol-1)	Ligand efficiency	RMS
1	Luteolin	-10.12	-0.48	0.0
2	ciprofloxacin	-9.36	-0.36	0.0

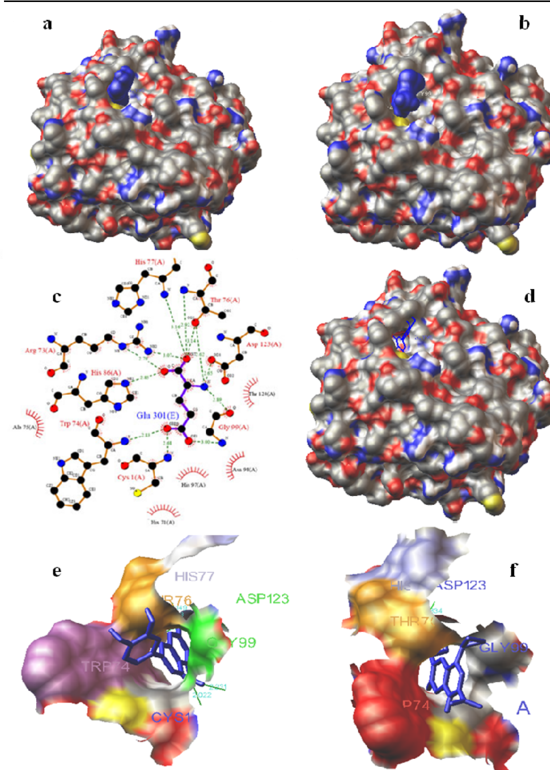

Figure 2: a) The Luteolin molecule (MSMS-Mol) enfolded completely in the active pocket of GlcN-6-P synthase (MSMS-MOL). b) Enfolding of ciprofloxacin (MSMS-MOL) in the active pocket of GlcN-6-P synthase (MSMS-Atom). c) Interacting amino acids as predicted from the ligplot. D. The luteolin (S&B indicated blue) found to sit in the proper orientation complementary to the topology of the active site. E. Orientation of luteolin molecule perpendicular to the grove made by Cys 1, Arg 73, Thr 76, and Gly 99. F. Orientation of ciprofloxacin molecule in active pocket.

amidotransferase domain reveals that, the luteolin exhibiting interaction with one or the other amino acids in the active pocket (Fig. 2). The docking results for luteolin and ciprofloxacin are documented in Table 4. Theoretically, luteolin showed good docking energy -10.68 kJ mol^{-1} with -0.51 of ligand efficiency. The docking energy of the ciprofloxacin was-10.77 kJ mol-1with -0.15 of ligand efficiency. The luteolin was completely enfolded in the entire active pocket of GlcN-6-P synthase (Fig. 2a) as compared to ciprofloxacin (Fig. 2b). The topology of the active site of GlcN-6-P synthase was similar in both luteolin and ciprofloxacin, which is lined by interacting amino acids as predicted from the ligplot

(Fig. 2c). The luteolin (S&B indicated blue) found to sit in the proper orientation complementary to the topology of the active site (Fig. 2d). However, orientation of luteolin molecule was perpendicular to the grove made by Cys 1, Arg 73, Thr 76, and Gly 99 (Fig.2 e&f). The earlier investigator (Isupov et al. 1996) noticed that, the Cys1 is the catalytic nucleophile in glutaminase domain of bacterial glucosamine 6-phosphate synthase, and the nucleophilic character of its thiol group appears to be increased through general base activation by its own alpha-amino group. Cys1 can adopt two conformations, one active and one inactive; luteolin binding locks the residue inactive conformation. Luteolin has been proved to be one of the potent antibacterial agents. By *in silico* analysis, it seems that luteolin is promoting the remarkable antibacterial activity through the inhibition of GlcN-6Psynthase.Theoretical reason behind low potency of luteolin could be due to minimum accessibility to enter through the peptidoglycan layer of bacteria as ciprofloxacin does to inhibit the growth of bacteria, but the ability of luteolin interaction with membrane bound Glucosamine 6-phosphate synthase is more since the active pocket has more hydrophobicity, hence affinity of luteolin is highly significant compared to ciprofloxacin and also we are providing here efficiency of ligands toward enzyme under *in silico* condition in support of our claim.

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