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In vitro and *In Silico* Evaluation of Anti-Microbial Potential of *Leptadenia reticulata* - An Endangered Medicinal Plant

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ABSTRACT: The present study reports the antimicrobial efficacy of bioactive compounds present in *Leptadenia reticulata*. The ethyl acetate extract shows highest antibacterial activity against *E. coli*. with 16.6 mm zone of inhibition. The methanol extract showed moderate activity against bacteria but produced highest zone of inhibition (24.8 mm, 23.6 mm) against *Aspergillus niger* and *Trichoderma viride*. The strong antibacterial activity of ethyl acetate extract against *Staphylococcus aureus, Bacillus sibtilis, klebsiella pneumonia* was also observed. *Trichoderma viride* and *penicillium citrinum* was found to be resistant to aqueous extract. To determine the binding interaction, *in silico* docking analysis was carried out between important phytoconstituents of *L. reticulata*, reference molecule and macromolecular enzymes that involved in key cellular biosynthetic process. Stigmasterol, a bioactive compound from *L. reticulata* exhibited significant docking score of -44.669kcal/mol with Dihydrofolate synthase. Similarly, Diosmetin and beta-sitosterol also exhibited remarkable binding affinity (-43.6159kcal/mol, -42.9215kcal/mol) with Dihydrofolate synthase. Our study further opens new avenues for further exploitation of the bioactive compounds from this important medicinal plant for pharmaceutical application.

Keywords: Jivanti, Molecular Docking, Antibacterial Assay, Antifungal Assay.

INTRODUCTION

Leptadenia reticulata, popularly known as Jivanti a versatile medicinal plant owing to its several medicinal uses. The use of this plant as medicine goes back to Vedic age of 4500 to 1600 BC. Several pharmaceutically important compounds were isolated from *L. reticulata* such as amyrin, ferulic acid, luteolin, diosmetin, rutin, beta-sitosterol, stigmasterol, hentriacontanol, a triterpene alcohol simiarenol, apigenin (Krishna *et al.*, 1975; Subramanian *et al.*, 1977; Sastry *et al.*, 1985; Dhalani and Nariya 2017; Godara *et al.*, 2019). Besides these, novel pregnane glycosides namely reticulin, deniculatin, leptaculatin have also been reported from the same plant (Srivastav *et al*., 1994; Kaushik and Joshi 2013). This valuable medicinal plant has been used for the treatment of various ailments such as hematopoesis, emaciation, cough, dyspnoea, fever, burning sensation, night blindness and dysentery (Sivarajan and Balachandran 1994; Pal *et al.*, 2012). It is regarded as a good cure for tuberculosis and also effectively used for several problems associated with ear and nose (Parabia *et al.*, 2007).

Interest in this plant was further enhanced due to its anti-tumor, anti-carcinogenic and usefulness as antifungal agent (Sathiyanarayanan *et al.*, 2007; Mishra *et al*., 2010; Sulaiman *et al.*, 2021). The bioactive compounds from this species alone or in combination with other compounds have been extensively used in several herbal formulations such as Speman forte (Himalaya drugs), Galactin Vet (Himalaya drugs), Speman forte vet (Himalaya drugs), Speman vet (Himalaya drugs), Chyavanprasha (Himalaya drugs), calshakti (Intas Pharma), Chyavanaprash (Adrenal herbs) and many more. In spite of multifold usages, the mechanism of interaction of the bioactive compounds present in this plant with macromolecular enzymes involved in key cellular biosynthetic process is still not been reported so far. Therefore, the present study is designed to explore the antimicrobial potential of *L. reticulata* using *in vitro* and the molecular interaction study of major bioactive constituents through molecular docking analysis.

MATERIAL AND METHODS

A. Antimicrobial activity

The fresh naturally grown plants were collected from herbal garden of Padmashree Institute of Management and Sciences, Bangalore. The collected samples were shade dried and ground to fine powder. The powder was subjected to aqueous and organic solvents (ethyl acetate, methanol) extraction using a soxhlet extractor. The extracts were evaporated to dryness by vacuum distillation and stored at 4°C.

B. Antimicrobial assay of different solvent extract

The well diffusion method was adopted to assay the antibacterial activity of different extract against the test microorganisms (Kirby-Bauer Methods described by Drago *et al*. (2000).

C. Determination of Minimum Inhibitory Concentration
The extracts that showed significant antimicrobial activity were selected to determine the minimum inhibitory concentration (MIC) as described by De Paiva *et al*., (2003).

D. Molecular docking analysis
The molecular docking studies were carried out to determine the binding interactions between the active phytoconstituents of *L. reticulata* (Alpha amyrin, Beta-amyrin, Ferulic acid, Luteolin, Diosmetin, Rutin, Beta-Sitosterol, Stigmasterol, Quercetin and Coumeric Acid), reference analogs (Benzyl Penicillin, Sulfadiazine, Trimethoprim, Rifampacin, Ciprofloxacin and Ketoconozole) and proteins (2RF4, 3INV, 3M4I, 3NRS and 3OCL). The protein 4KOF was marked as "hold for release". Molecular docking was initiated by drawing 3D structures of the phytoconstituents using chemsketch and converted to pdb file using chimera (https://www.cgl.
ucsf.edu/chimera/). The target proteins were downloaded from PDB (https://www.rcsb.org/). Medusa Dock, a flexible docking software was used to perform docking analysis (https://dokhlab.med.psu.edu/medusa
dock).

RESULTS AND DISCUSSION

A. Antimicrobial activity
Currently demand of plant based antimicrobials has gained more importance because of enormous therapeutic potential with lesser side effect than its synthetic counterpart. The efficacy of different solvent extracts at various concentrations against selected bacteria and fungi were tested. It was observed that ethyl acetate extract showed highest activity with 16.6 mm zone of inhibition against *E. coli* whereas methanol extract with 24.2 mm zone of inhibition against *Aspergillus niger*. The strong antibacterial activity of ethyl acetate extract against *Staphylococcus aureus*, *Bacillus sibtilis*, *klebsiella pneumonia* was also noticed with (12.4 mm, 17.6 mm, 15.9 mm) zone of inhibition (Table 1).
It was observed that the zone of inhibition was increased with increase in the concentration of extract (Fig. 1). It was also evident that the organic solvent extract exhibited potent antimicrobial activity than the aqueous extract. This may be due to the antimicrobial principle of this plant are either polar or non polar in nature and thus extracted to organic solvent medium.
On the whole, result of this experiment highlights that ethyl acetate fraction exhibited significant antimicrobial potential than methanol and water. Aqueous extract was found to be least effective showing minimum zone of inhibition. This result indicates that antimicrobial compounds extracted from *L. reticulata* are more concentrated in ethyl acetate fraction than other solvent. The methanol extract showed moderate activity against bacteria but produced highest zone of inhibition (24.8 mm, 23.6 mm) against *Aspergillus niger* and *Trichoderma viride* respectively (Table 2, Fig. 2).

Table 1: Antibacterial activity of various extracts of *L. reticulata.*

Extract	Conc. of Extract µg/100µl	Zone of inhibition diameter in (mm)				
		S. aureus	***K. pneumonia***	***E. coli***	***B. Subtilis***	***P.fluorescens***
Methanol	200	5.6±0.15	4.8±0.13	NI	3.6±0.14	4.6±0.21
	400	7.4±0.12	5.2±0.28	NI	4.2±0.12	5.2±0.11
	600	9.0±0.16	5.6±0.22	NI	4.8±0.22	5.8±0.18
	800	10.2±0.56	6.2±0.31	NI	5.4±0.31	6.2±0.22
Ethyl acetate	200	6.2±0.41	6.1±0.32	6.4±0.77	6.4±0.17	1.8±0.21
	400	9.6±0.19	8.2±0.56	8.8±0.24	9.6±0.31	2.2±0.30
	600	12.4±0.63	12.4±0.21	12.20.44	12..3±0.18	2.6±0.31
	800	14.6±0.27	15.9±0.14	16.6±0.1	17.6±0.29	3.2±0.11
Water	200	2.4±0.44	NI	NI	2.1±0.08	NI
	400	3.2±0.20	3.6±0.81	NI	2.6±0.24	3.5±0.4
	600	3.8±0.41	3.80±0.43	NI	3.5±0.20	5.6±0.25
	800	4.1±0.26	4.1±0.22	NI	4.2±0.17	5.8
+ve control	Tetracycline	30µg/disc	18.4±0.31	22.6±0.34	26.00±0.11	22.0±0.62

The values within each column represent, Mean ± SE

The variation in activity of different extract may be attributed to the ability of solvents to dissolve specific phytochemicals responsible for inhibition of microbial growth. *Trichoderma viride* and *penicillium citrinum* was found to be resistant to aqueous extract. As methanol extract shows potent antifungal activity further exploitation and characterization of the bioactive molecules present in it need to be focused.

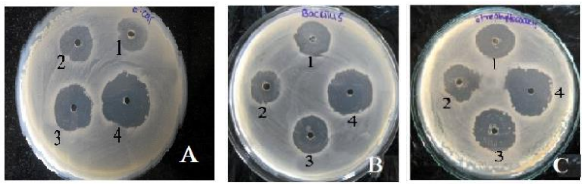

Fig. 1. Antibacterial activities of Ethyl acetate extract of *Leptadenia reticulata*. (A) *E. coli*; (B) *B. subtilis*; (C) *S. aureus.*

Table 2: Antifungal activity of various extract of *L. reticulata.*

Extract	Conc. of Extract µg/100µl	Zone of inhibition diameter in (mm)			
		Aspergillus niger	*Candida albicans*	*Penicillum citrinum*	*Trichoderma viride*
Methanol	200	4.2±0.43	NI	NI	3.2±0.04
	400	9.1±0.26	3.4±0.27	NI	5.6±0.04
	600	14.6±0.15	7.2±0.51	5.1±0.11	11.8±0.07
	800	24.8±0.34	16.8±0.19	9.4±0.21	23.6±0.45
Ethyl acetate	200	2.4±0.26	NI	NI	NI
	400	4.6±0.15	3.4±0.43	4.2±0.11	NI
	600	8.2±0.19	7.7±0.25	5.6±0.11	NI
	800	12.6±0.32	17.2±0.10	9.6±0.09	NI
Water	200	NI	NI	NI	NI
	400	NI	4.6±0.25	NI	NI
	600	NI	7.2±0.10	NI	NI
	800	NI	10.4±0.26	NI	NI
+ve control	Ketoconaz-ole 25µg/disc	30.2±0.43	28.9±0.11	22.00±0.46	30.00±0.26

The values within each column represent, Mean ± SE

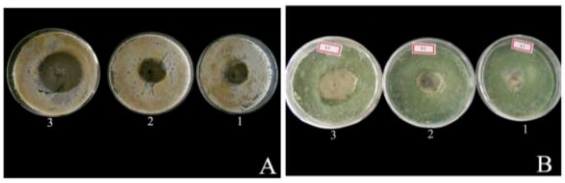

Fig. 2. Antifungal activities of *Leptadenia reticulata.* (A) *Aspergillus niger* (Methanol extract); (B) *Trichoderma viride* (methanol extract).

The zone inhibition diameter of ethyl acetate extract at 800µg against *K. pneumonia, B. subtilis, E. coli* can be comparable to positive control tetracycline at 30µg/disc (Table No. 1) which reveals the potent antimicrobial properties of *Leptadenia reticulata* extract.

Minimum inhibitory concentration (MIC) was determined for those extract which exhibited significant result in the well diffusion assay. The minimum inhibitory concentration of ethyl acetate and methanol extract showed strong antibacterial activity with MIC against *Staphhylococcus aureus* (110µg/l; 140µg/l), *Bacillus subtilis* (150µg/ml; 160µg/ml), *Aspergillus niger* (200µg/ml; 190mg/ml). The above results further indicate the extract having potent antimicrobial activity (Table 3). The positive control ampicilin was found to be most effective than all extract with MIC value ranging from (0.02-0.08mg/ml).

Table 3: Minimum inhibitory concentration (MIC) of different extract of *L. reticulata* on test organisms.

Microorganisms	Methanol extract (µg)	Ethyl acetate extract (µg)	Aqueous extract (µg)
Staphylococcus aureus	140±1.2	110±1.9	200±2.8
Klebsiella pneumonia	190±1.5	160±2.2	350±3.5
Escherichia coli	NI	180±2.5	NI
Bacillus subtilis	160±2.1	150±1.5	200±2.7
Pseudomonas fluorescens	180±2.5	190±2.4	400±3.9
Aspergillus niger	190±2.4	200±2.8	NI
Candida albicans	340±3.3	330±3.3	360±3.6
Penicillium citrinum	560±4.1	340±3.5	NI
Trichoderma viride	170±2.5	NI	NI

The values within each column represent, Mean ± SE

The low MIC value of ethyl acetate and methanol extract confirms that antimicrobial compounds of this plant are concentrated in mid polar fraction. Our previous finding from the preliminary phytochemical screening also showed the presence of various bioactive constituents like phenols, alkaloids, saponins, Flavonoids and glycosides (Sudipta *et al.,* 2011). The presence of these phytochemicals is probably responsible for antimicrobial potential of the plant.

B. Molecular docking

The docking analysis shows dihydrofolate synthase (3NRS) docks with the reference analogs and phytoconstituents Diosmetin, Rutin, BetoSitosterol, Stigmasterol and Apigenin. It was observed that phytocompound Stigmasterol has strong binding affinity with docking score of -44.669kcal/mol with dihydrofolate synthase. Dihydrofolate synthase also observed to dock with a very good docking score of -43.6159kcal/mol with Diosmetin and -42.9215kcal/mol with beta- sitosterol (Table 4). The significant docking score of the phytoconstituents with dihydrofolate synthase indicate its antimicrobial potential as

dihydrofolate synthase is involved in biosynthesis of aminoacid and nucleic acid (Sharma and Chauhan 2012). Overall dihydrofolate synthase was observed to have good binding affinity with all the phyto-compounds tested in the present study. The docking of penicillin binding proteins (3OCL) with Benzyl Penicillin, Trimethoprim, Alpha amyrin and Quercetin was ignored owing to positive docking scores (Table 4).

Table 4: Binding energy of bioactive compounds from *L. reticulata* with key molecular enzymes and reference analogs.

Analogs (Ligands)			Binding energy with target molecules (receptors)				
			2RF4	3INV	3M4I	3NRS	3OCL
Reference Analogs	1	Benzyl Penicillin	-26.0629	-22.1831	-23.7777	-11.318	106.998
	2	Sulfadiazine	-30.7062	-21.2031	-34.2709	-42.6199	-27.389
	3	Trimethoprim	-28.6079	-28.4467	-30.4807	-26.244	3.02417
	4	Rifampacin	-19.7864	29.8057	-25.9191	-39.8805	-35.8839
	5	Ciprofloxacin	-13.5112	-15.9762	-27.1797	-43.6159	-18.6564
	6	Ketoconozole	-27.4171	-18.1512	-27.1797	-42.6199	-26.6725
Analogs from *L. reticulata*	7	Alpha amyrin	-24.2415	-24.9453	-26.2234	-19.0142	8893.38
	8	Beta amyrin	-19.7656	-20.4634	-27.1797	-28.1987	-18.6564
	9	Ferulic acid	-21.8457	-33.3622	-20.0203	-38.2146	-35.8103
	10	Luteolin	-32.7232	-34.6375	-31.5033	-26.244	-20.5528
	11	Diosmetin	-21.5507	-34.6375	-28.1731	-43.6159	-29.3663
	12	Rutin	-24.0715	-28.4467	-29.9765	-38.2146	-21.2371
	13	BetoSitosterol	-24.4196	-27.5268	-17.6955	-42.9215	-30.1408
	14	Stigmasterol	-24.147	-28.6039	-34.2709	-44.669	-29.3663
	15	Quercetin	-24.147	-18.1512	-31.5033	-30.7857	79.4363
	16	Coumeric Acid	-21.8457	-33.3622	-28.1731	-26.213	-35.8103
	17	Apigenin	-24.1366	-29.4204	-26.8997	-37.7069	-26.8044

Again, another phytoconstituent from *L. reticulata*, Stigmasterol also showed potential binding affinity with docking score of -34.2709 kcal/mol with DNA gyrase (3M4I). Detail analysis of docked complex reveals that catalytic residues from dihydrofolate synthase (GLU 149, LYS 63, GLY 64, GLY 62, ASN 61, LYS 191) that interact with beta-sitosterol, diosmetin, stigmasterol which are key factor for its inhibition (Fig. 3- 5).

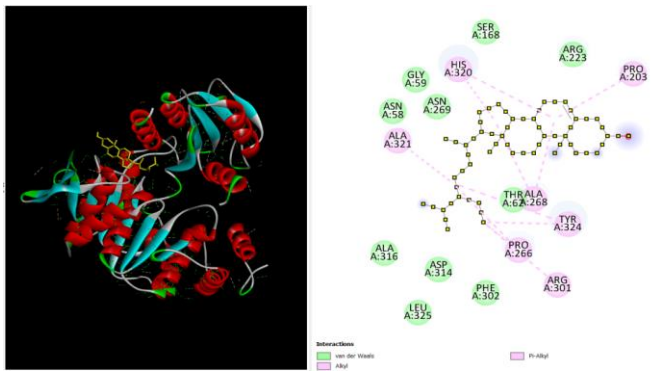

Fig. 3. Docked confirmation with possible interaction of dihydrofolate synthase with phytoconstituents from *L. reticulata* (Beta-sitosterol).

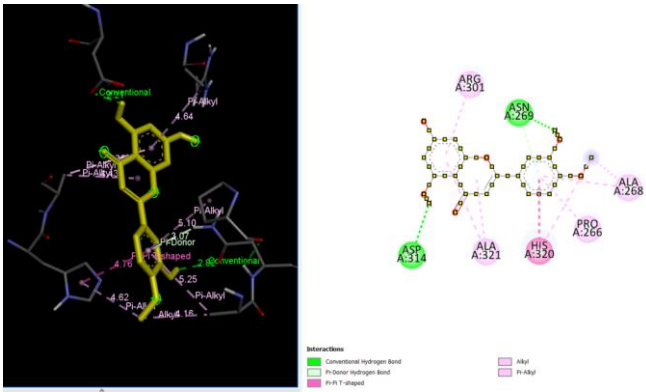

Fig. 4. Docked confirmation with possible interaction of dihydrofolate synthase with phytoconstituents from *L. reticulata* (Diosmetin).

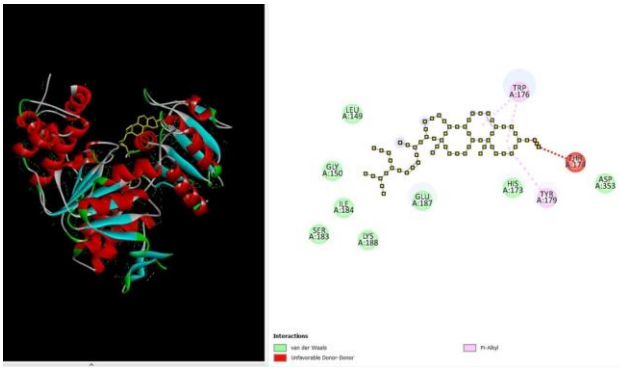

Fig. 5. Docked confirmation with possible interaction of dihydrofolate synthase with phytoconstituents from *L. reticulata* (Stigmasterol)

The above result clearly indicates the antimicrobial potential of *L. reticulata*, as DNA gyrase is a key enzyme in prokaryotes that introduce negative supercoiling in DNA and proven to be an appealing therapeutic target for antibacterial agents (Nagaraja *et al.*, 2017; Mdluli and Ma 2007; Khan *et al.*, 2018). Similarly, Ferulic acid also shows good binding affinity of -33.3622kcal/mol, -38.2146kcal/mol and -35.8103 kcal/mol with dihydrofolatereductase (3INV), dihydrofolate synthase (3NRS) and penicillin binding proteins (3OCL) respectively. From the present biocomputational studies, it is clearly evident that *L. reticulata* contain several valuable biologically active compounds with microbial growth inhibiting properties.

CONCLUSION AND FUTURE SCOPE

The present study validates that *L. reticulata* has potent bioactive phytoconstituents like Stigmasterol, Diosmetin, beta-sitosterol which are capable of inhibiting microbial growth. However, detail characterizations of the other phytocompounds are required to explore their mode of action and to evaluate the clinical and biosafety potential.

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Conflict of Interest. None.

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