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In-silico Antiurolithiatic Screening of Lupeol derived from Bhadra, *Aerva lanata* Linn.

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Abstract— Introduction*: The decoction prepared from the whole plant of Bhadra, Aerva lanata Linn. (Amaranthaceae) is widely using as an antiurolithiatic and diuretic drug by traditional Ayurvedic practitioners of Kerala. Lupeol is an important phytoconstituent present in this drug having antiurolithiatic activity. The qualitative and quantitative estimation of Lupeol in decoction of A. lanata was done by High performance Thin Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC) methods, respectively and antiurolithiatic potential was screened by in-silico method.* ***Materials and methods****: The authentication of the plant A. lanata was done by analyzing its physico-chemical parameters and compared with Ayurveda Pharmacoepia of India (API) standards. The decoction of A. lanata was prepared as per the standard Ayurvedic procedure. Presence of Lupeol in decoction was authenticated by HPTLC method and quantification was done using HPLC. The main target proteins involved in urolithiasis are 2ETE of Oxalate Oxidase, 4wrn of Tamm horsfall protein and 2ckj of Xanthine oxidoreductase. Lupeol is docked with these proteins to check its inhibitory activity using Auto Dock Vina 4.2.6 software.* ***Results and Discussion****: The quantity of Lupeol present in A. lanata decoction was estimated by HPLC as 2.29 ppm. In molecular docking study, Lupeol from the A. lanata showed good inhibitory activity against these proteins with the best binding score against the protein Xanthine oxido reductase supporting the traditional claim for their therapeutic utility in urolithiasis.* ***Conclusion****: The Lupeol estimation helped in developing standardization parameter for A. lanata decoction. The docking study has created good docking scores which reveal its molecular mechanism of action. The study provides scientific data substantiating the traditional claim of A. lanata decoction in urolithiasis.*

Keywords*— Aerva lanata, Lupeol, Anti urolithiatic, in-silico screening.*

I. INTRODUCTION

Aerva lanata Linn. (family: Amaranthaceae) is a popular medicinal plant used by traditional Ayurvedic practitioners of Kerala as an antiurolithiatic and diuretic agent [1,2,3,4.] It is a herbaceous plant growing as a perennial weed, distributed in almost all regions of central India and Southern India, especially in Kerala and Tamil Nadu. The plant is commonly known as *Cherula* in Malayalam and *Bhadra* in Sanskrit[5]. The references for this plant is not available in ancient medical literatures such as *Samhithas* and *Nighantus*. However, this plant is widely mentioned in the text books related to medicinal plants of India *viz*., as Indian Materia Medica(6), Indian Medicinal plants[7], Hendry Van Rheeds Hortus Malabaricus, an ethno medicinal compendium[8] and is listed in popular ancient medical books of Malayalam language such as Chikitsamanajari, Vishajyotsnika, Sahasrayoga and Vaidyamanorama[4,9]. *Gorakshaganja*, *Chaya*, *Cherupulai*, *Cherula* etc are the vernacular names in different languages[6]. Morphologically it is a many branched, hardly erect or prostrate woody herb with short and stout stem, simple alternate leaves and spike inflorescence growing upto a height of 30-60cm[5].

Traditionally, *Aerva lanata* has been used in treating cough, dysuria, hemiplegic migraine and kidney related disorders, urinary problems, different types of fevers, and poisonous bites by reptiles. Besides, it has many scientifically proven pharmacological properties like demulcent, diuretic, ulcer wounds, rheumatism, antimicrobial, anti-urolithiasis, antiulcer, anti-asthmatic, acute kidney injury, anti-diarrheal activity, antioxidant, antihyperglycemic, hypolipidemic and antiulcer activities[10].

The decoction prepared from the whole plant of decoction of *A. Lanata* is a simple and highly utilized pharmaceutical preparation which is commonly used in the management of *muthrasmari* (urolithiasis)[3], which is formation of stone in kidney or urinary tract affecting around 5 to 13% of the world population[11]. However, studies on its molecular mechanism of action and standardization of the formulation is found to be limited. The major phytoconstituents present in *Aerva lanata* are alkaloids (ervine, methylervine, ervoside, aervine, methylaervine, aervoside, ervolanine, and aervolanine), flavonoids (kaempferol, quercetin, isorhamnetin, persinol, and persinosides A and B), β-sitosterol, α-amyrin, betulin, hentriacontane, sitosterylpalmitate, D-glucoside, glycosides, kaempferol-3-galactoside and kaempferol-3-rhamnoglucoside, methyl grevillate, Lupeol, Lupeol acetate benzoic acid, β-sitosteryl acetate, tannic acid, starch and free sugars[12]. Among these, Lupeol is a pentacyclic triterpenoid with an antiurolithiatic and diuretic activity[1].

Standardization of herbal formulations in terms of active ingredients is essential to ensure quality and to prevent adulteration[13]. HPLC and HPTLC are widely used for the identification of chemical constituents and considered as the simple and cost-effective method for standardizing herbal formulations. Preparative and analytical HPTLC and HPLC are used in pharmaceutical industry for isolating and purification of herbal compounds[14]. Lupeol is an important phytoconstituent present in *Bhadra* decoction having

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antiurolithiatic activity. Hence the present study focuses on physico-chemical analysis of *Aerva lanata* and the quantitative estimation of Lupeol in decoction of *A. Lanata* by HPTLC and HPLC method for developing standardization parameters for this formulation.

Moreover, antiurolithiatic potential of Lupeol can be screened by *in-silico* molecular docking method which is a bioinformatics tool and is helpful in the compound isolation and drug discovery from herbal drugs. *In silico* approaches such as virtual screening and network analysis have been extensively utilized to elucidate the pharmacological basis of the functions of traditional medicinal plants[15]. *In-silico* analysis helps to predict and clarify the mechanism of action of various chemical constituents in medicinal plants for the better understanding of their therapeutic effects[16]. The important target proteins involved in Calcium Oxalate crystal formation of urolithiasis are 2ETE of Oxalate Oxidase[17,] 4wrn of Tamm horsfallprotein[18] and 2ckj of Xanthine oxidoreductase[19]. Hence, in-silico screening of urolithiatic activity of Lupeol against these proteins were also studied in this study.

II. Materials and Methods

Plant material

The whole plant of *Bhadra* (*Aerva lanata*) was collected from their natural habitat, Bhutathankettu in Kerala (10.1364^0N, 76.6622^0E). The plant was identified botanically based on macroscopic and microscopic characters. The collected plant materials were washed thoroughly with water and dried in shade at room temperature and grinded to coarse powder.

Solvents and chemicals

Methanol (HPLC grade), ethanol and phosphoric acid (analytical reagent grade), potassium dihydrogenphosphate, Acetonitrile, orthophosphoric acid, purchased from Merck (Darmstadt, Germany) and Himedia. The solvent mixtures were filtered and degassed before use. The standard marker compound, Lupeol was acquired from Himedia laboratories, India.

Physico-chemical analysis of Aerva lanata

The physico-chemical test parameters such as total ash, acid insoluble ash, water soluble ash, the extractive values were evaluated to understand the purity and quality of the drug. The qualitative analysis was done for estimating the presence of secondary metabolites such as alkaloids, flavonoids, steroids, phenols. All the analysis were done as per procedures mentioned in Ayurvedic Pharmacoepia of India (API)[20] and compared with standards mentioned in API[5].

Preparation of Bhadra decoction

The decoction of powdered and dried whole plant *A. lanata* was prepared according to standard procedures mentioned in *Sarngadhara Samhitha*[21]. 10 g coarse powder of whole plant of *Aerva lanata* was taken in an earthen vessel, added with 160ml water, boiled and reduced to $1/4^{th}$ (40ml). The decoction thus obtained was strained through a clean cotton cloth. Further, the decoction was concentrated to $1/4^{th}$ (10ml) by boiling and was stored in an air-tight container for the further analysis.

Qualitative analysis of Bhadra decoction by HPTLC

Preparation of test sample and standard

10ml of concentrated decoction was taken in a beaker and kept on water bath at $70\text{-}80^0$c for 2-3 hour until a dry residue was obtained. The residue was dissolved in 5ml methanol and filtered through Whatman no.1 filter paper. The standard solution of lupeol was prepared by dissolving 10mg of the compound in 10ml of methanol.

HPTLC conditions for the detection of Lupeol in the decoction

HPTLC was done (CAMAG, Switzerland) using 60 F 254 TLC plate, keeping in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase Cycohexane: Toluene: Ethyl acetate at the ratio of 7.5:3:2 upto 70 mm and the developed plate was dried and kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and photo of the chromatogram was taken using the software WinCATS version 1.3.4. The presence of Lupeol in the decoction was confirmed by HPTLC chromatogram.

Quantitative estimation of Lupeol in Bhadra decoction by HPLC

Standard preparation

Standard stock solution of Lupeol was prepared by dissolving 1mg Lupeol in 1ml of mobile phase in a volumetric flask and filtered through 0.2 µm millipore membrane filter. From this stock solution, different concentration viz., 25ppm, 50ppm, and 100 ppm were prepared by serial dilution methods.

Sample preparation for HPLC

10 ml of concentrated decoction was taken in a beaker and kept on a water bath at $70\text{-}\ 80^0$C for 2-3 hours until a dry residue was obtained. 10 mg of this residue was weighed accurately and dissolved in 1 ml of methanol and was filtered through 0.22 µm nylon microfilters prior to use. 20 µl of the filtered sample was directly injected in HPLC sample injector.

Quantitative estimation of Lupeol from *A. lanata* is carried out using HPLC (Agilent 1260 series HPLC system) comprising a quaternary pump (Agilent Technologies 1260), a vacuum degasser, a variable wavelength detector and a 20 µl sample injector, column thermostat. The data were analyzed using open lab CDS ezchrom Workstation VL software [22]

The separation was performed on Zorbax Eclipse plus C18 column. Column specification: 3.5µm, 100 X 4.6mm, Agilent. The reverse phase column with an extended guard column was used as a stationary phase and isocratic elution of Methanol:Acetonitrile (30:70) at 1ml/minute as mobile phase. Chromatograms were recorded at 210nm. The decoction sample was appropriately diluted, filtered through 0.22µm nylon microfilters and injected into HPLC sample injector. The column thermostat was maintained at room temperature.

The graph obtained for the concentration of lupeol in the sample is compared with that of the standard and the peak area was measured, plotted against concentration and concentration

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of Lupeol in the test sample was estimated. The proposed RP-HPLC method was validated according to the international Conference on Harmonization guidelines [23].

Molecular docking studies

Anti urolithiatic activity of bioactive compound, Lupeol isolated from *A. lanata* was studied against the proteins 2ETE of Oxalate Oxidase, 4wrn of Tamm horsfall protein and 2ckj of Xanthine oxidoreductase by *in-silico* method using automated docking software Auto Dock Vina 4.2.6 software. The structure of these proteins which reportedly participate in kidney stone formation were downloaded from pdb (www.rcsb.org/pdb).

III. RESULTS

The physico-chemical parameters analyzed for the *A. lanata* found to be within the limit as per the API standards (Table 1). The qualitative analysis of methanolic extract of *A. lanata* confirmed the presence of alkaloids, flavonoids, phenols, steroids and tannins (Table 2). The HPTLC analyses were done to estimate the presence of Lupeol in the decoction of *A. lanata* HPTLC and comparative fingerprint profiling indicated the presence of Lupeol in the decoction of *A. lanata (*Fig. 1).

Table 1: Results of physico-chemical analysis of *A. lanata*. values represent mean± S.E (n=3)

Sl no	Parameters	Values (%)
1	Foreign matter	Nil
2	Total ash	11.69±1.54
3	Acid Insoluble Ash	0.18 ±0.28
4	Water Insoluble Ash	6.88 ±0.54
5	Moisture Content	6±0.19
6	Volatile oil	Nil
7	Fibre	86.42±2.08
8	Total sugar	0.83 ±0.31
9	Reducing sugar	0.66 ±0.52
10	Non-reducing sugar	0.17± 0.85
11	Alcohol soluble extractive value	14.5±2.15
12	Water soluble extractive values	19.16±1.60

Table.2: Results of qualitative analysis of *A. lanata* methanol extract.

Experiment			Presence (+)/absence (-)
1)	Alkaloids		
	a)	Dragendroff's test	+
	b)	Meyer's test	+
2)	Flavonoids		+
3)	Saponins		-
4)	Carbohydrates		
	a)	Fehling's test	+
	b)	Benedict's test	+
5)	Proteins		+
6)	Phenols		
	a)	Ferric chloride test	+
	b)	Lead acetate test	+
7)	Steroids		+
8)	Tannins		
	a)	Ferric chloride test	+
	b)	Lead acetate test	+

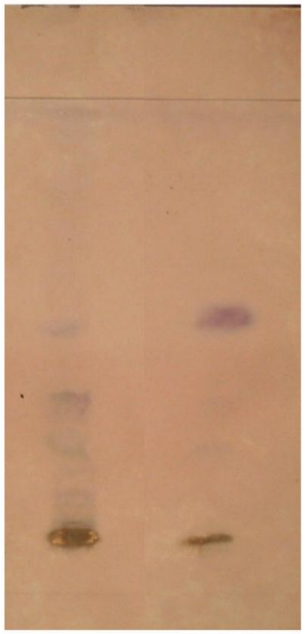

Fig. 1: Chromatogram showing the presence of lupeol in decoction of *A. lanata* (Track 1) on comparison with standard compound, Lupeol (Track 2)

The concentration of lupeol in the decoction of Aerva lanata was found to be 2.29ppm (Fig. 2A and Table 3). Fig 2B shows chromatogram of standard of lupeol. Fig 2C represents the plotted graph with area against concentration. The results of analytical characteristic performance of decoction are shown in figure 2. The retention time (Rt) of of Lupeol in the chromatogram is found to be 12.2min.

Binding affinity of Lupeol

The binding affinity of Lupeol was predicted against the proteins 2ETE (Oxalate Oxidase), 4wrn (Tamm horsfall protein) and 2ckj (Xanthine oxidoreductase) which is shown in the Table 4 and Fig 3, 4 and 5.

Lupeol showed best activity against 2ckj-chainB based on the binding affinities. The docking results has shown that the binding strength of Lupeol in the active site of the enzyme Xanthine oxidoreductase was -8.5kcal/mol. This indicates that lupeol can be a better inhibitor of this enzyme. Hence the best possible molecular target of Lupeol is Xanthineoxidoreductase enzyme. From this, it is evident that Lupeol is a natural inhibitor of these proteins and is a promising drug in prevention and treatment of urolithiasis.

IV. DISCUSSION

Urolithiasis is one of the oldest diseases affecting humans, and have been one among the causes of renal failure[1]. In spite of substantial progress in understanding the pathophysiological mechanisms, treatment options are still limited, and treatment is often expensive. Hence, there is a great interest in herbal remedies for the treatment of urinary stone disease as an alternative or adjunct therapy[24].

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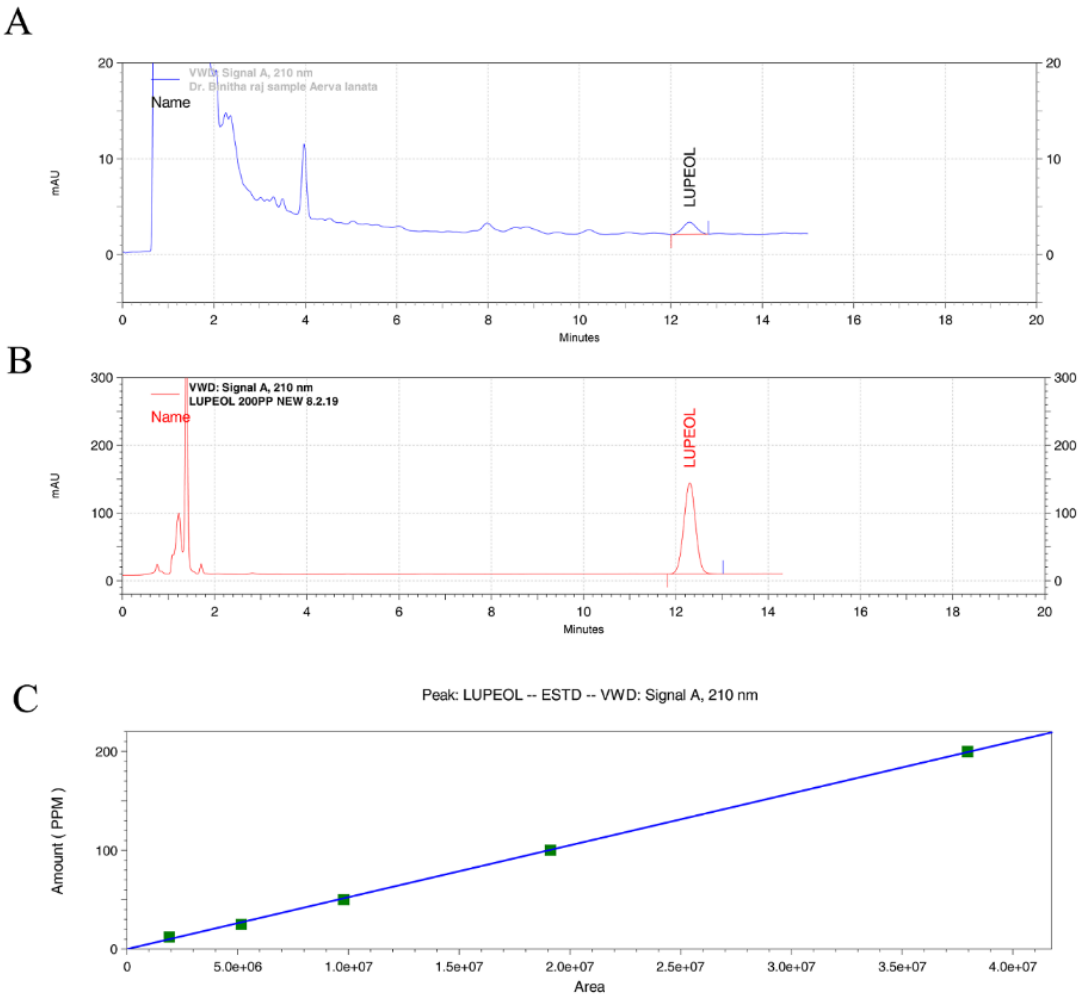

Fig. 2: Analytical characteristic performance of decoction of *A. lanata* and the concentration of marker Lupeol in decoction of *A. lanata*.

Table 3: Concentration of Lupeol in the decoction of *A. lanata*

Name	Retention time	Area	Area %	Height	Concentration (ppm)
Lupeol	12.410	435814	100.00	21113	2.28833

Table 4: Results of binding affinity of lupeol against three proteins

Target proteins	**Binding affinity (kcal/mol)**
2ETE	-6.2
4wrn	-8.2
2ckj-chainB	-8.5

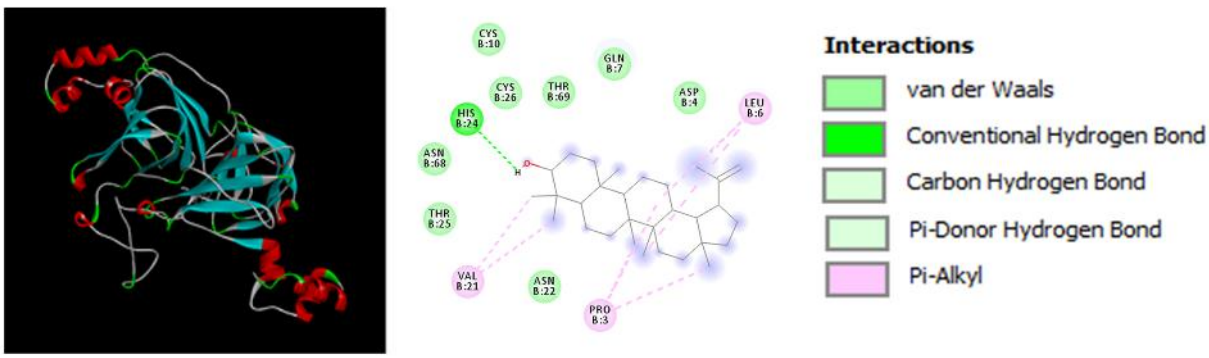

Fig. 3: Predicted binding site of 2ETE with Lupeol

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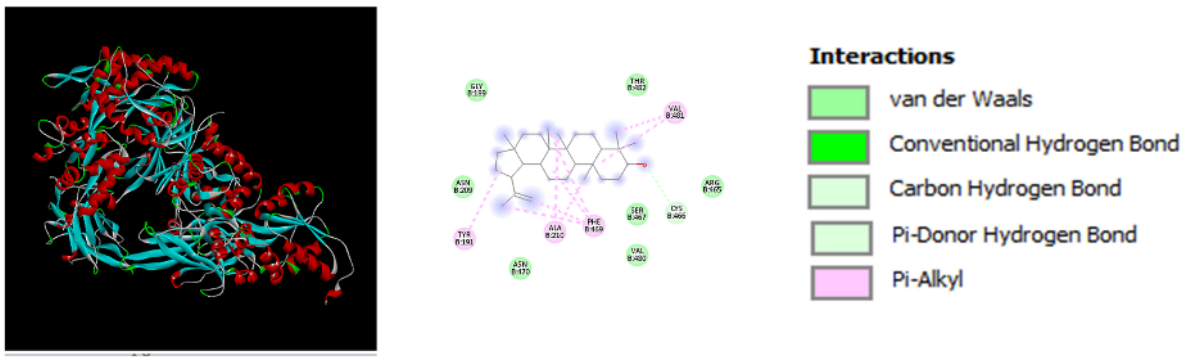

Fig. 4: Predicted binding site of 4wrn with Lupeol

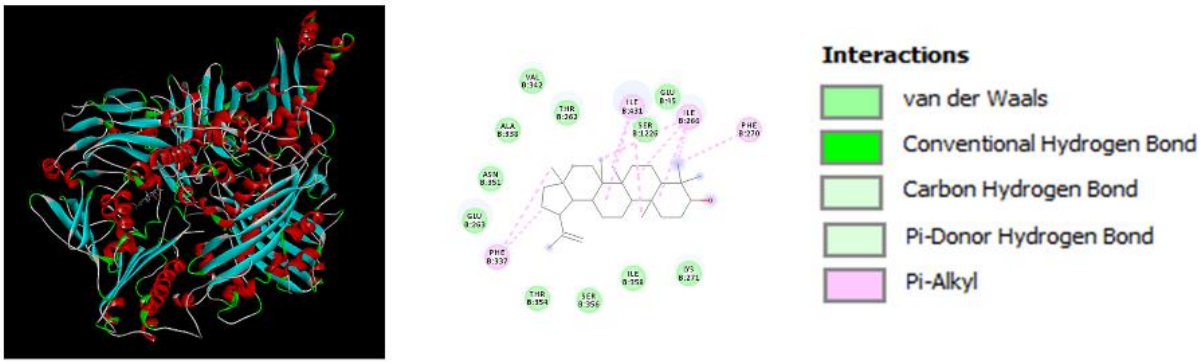

Fig. 5: Predicted binding site of 2ckj-chainB with Lupeol

Decoction, a herbal formulation prepared by boiling the whole plant of *A. lanata* is used effectively by traditional *Ayurvedic* practioners of *Kerala* [3,4]. However, its molecular mechanism of action was not clearly known and standardization of data was not available in the current scientific literatures. Hence, present study is an attempt to standardize the decoction for its main active phytoconstituent, Lupeol by HPLC method and screening the antiurolithiatic activity of *A. lanata* by molecular docking method.

The fresh plant of *A. lanata* was collected and taxonomic confirmation was done based on the macroscopy, microscopy and physicochemical parameters mentioned in API[4]. The quality control parameters such as foreign matter, total ash, acid insoluble ash, water insoluble ash, moisture content, volatile oil, fibre, total sugar, reducing sugar, non-reducing sugar, alcohol extractive value, water soluble extractive value and qualitative assay of major phytoconstituents were carried out and found comparable with API standards[4]. The assay of Lupeol, which is one of the main phytoconstituent present in *A. lanata*, was carried out on the decoction prepared from whole plant of *A. lanata* since this dosage form is specifically indicated in urolithiasis[3,4]. Bioactive markers are used to identify the presence of ingredients in formulation easily. The detection of the presence of bioactive markers and its quantification by HPTLC and HPLC are the best ways to identify and evaluate the quality of the finished formulation for standardizing the protocol for quality control in Ayurvedic formulations[13].

HPTLC and HPLC methods were selected for the quantification of the Lupeol in the decoction of in *A. lanata* since these methods are widely used in pharmaceutical industry for isolating and purification of herbal compounds[14]. Lupeol was proven to be an effective antiurolithiatic agent by preventing the formation of vesical calculi and decreasing the size of preformed stones. In addition, Lupeol's antiurolithiatic mechanism was revealed due to their capacity of minimizing crystal induced renal peroxidative changes and subsequent tissue damage[25].

Some of the major enzymes such as Oxalate Oxidase, Tamm horsfall protein and Xanthine oxidoreductase are considered to be the responsible factor for the urinary stone deposition. Docking study was done to understand the orientation of the ligand with these enzymes which reportedly participate in kidney stone formation in patients. Figure 3, 4and 5also shows the *in-silico* active pocket prediction of amino acids of protein 2 ETE,4wrn, 2ckj-chainB involved in binding with the ligand obtained from PDB. The binding energy of Lupeol against the enyme 2ckj-chainB was found to be high with -8.5 kcal/mol. Hence, Lupeol can be considered as the best inhibitor of Xanthine oxidoreductase. *In-silico* study helps to understand that the Lupeol, present in *A. lanata*, contribute to the antiurolithiatic activity of this drug which support the traditional claim for its usage in the treatment of *Muthrashmari (*urinary stones). Present study is the first attempt to understand the mechanism of action of the drug against the enzyme involved in the Urolithiasis using automated docking software.

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V. CONCLUSION

Ayurvedic practitioners of Kerala are widely using the decoction of *A. lanata* for the treatment of urolithiasis and was found to be very effective. In this study, we confirmed this traditional claim by elucidating the molecular mechanism by *in-silico* technique. The efficacy of Lupeol was studied by *in-silico* technique for its antiurolithiatic activity by downloading the protein compounds 2ETE of Oxalate oxidase, 4wrn of Tamm horsfall protein and 2ckj of Xanthine oxidoreductase from PDB and docking with it. The docking analysis attempted in this study created good docking scores which established the good inhibitory activity against the enzyme responsible for the formation of kidney stone formation. However, our study should be treated as preliminary in nature and further pharmacological experiments are necessary for supporting this finding for developing a promising drug for urolithiasis.

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