How to Cite:
Suvarchala Reddy, N. V. L. V., Ganga Raju, M., Anusha, V., Gaikwad, A., Pulate, C., Mahajan, K., & Tare, H. (2022). Investigation of potential antiurolithiatic activity and in silico docking studies of Karpura shilajit. *International Journal of Health Sciences*, *6*(S4), 8900–8916. https://doi.org/10.53730/ijhs.v6nS4.11875

Investigation of potential antiurolithiatic activity and in silico docking studies of Karpura shilajit

Dr. N V L Suvarchala Reddy V
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally - 500090, Affiliated to Osmania University, Bachupally, Hyderabad 500090, Telangana, India
Corresponding author email: suvarchalakiran@gmail.com

Dr. Ganga Raju M
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally - 500090, Affiliated to Osmania University, Bachupally, Hyderabad 500090, Telangana, India
Email: mgrpharma@gmail.com

Anusha V
Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally - 500090, Affiliated to Osmania University, Bachupally, Hyderabad 500090, Telangana, India
Email: vemulapallyanusha1998@gmail.com

Asmita Gaikwad
Department of Pharmaceutical Chemistry, Sharadchandra Pawar College of Pharmacy, Otur, Tal. Junnar, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India
Email: asmitavgaikwad@gmail.com

Chetan Pulate
Department of Pharmaceutical Chemistry, Sharadchandra Pawar College of Pharmacy, Otur, Tal. Junnar, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India
Email: pulatechetan2222@gmail.com

Dr. Kiran Mahajan
Department of Pharmaceutics, Sharadchandra Pawar College of Pharmacy, Otur, Tal. Junnar, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India
Email: kirancmahajan@gmail.com

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 9 April 2022, Manuscript revised: 18 June 2022, Accepted for publication: 27 July 2022

Dr. Harshal Tare
Department of Pharmacognosy, Sharadchandra Pawar College of Pharmacy, Otur, Tal. Junnar, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India
Email: harshaltare51@gmail.com

***Abstract*---**In this present study, Karpura shilajit, an Ayurvedic herbo-mineral, currently used in the Ayurvedic clinical practice for various ailments, was investigated chemically and pharmacologically.To screen anti-lithiatic activity of Karpura shilajit against sodium oxalate (70 mg/kg, i.p) for 7 days and in vitro activity by nucleation-aggregation assay. Cystone (500 mg/kg, p.o.)used as a standard drug in the present study.Nephrolithiasis may also be associated with nephrocalcinosis, *i.e.*, crystal depositions in tubular lumen and/or interstitium, an entity which suggests specific pathological processes. Preliminary Phytochemical screening resulted in the presence of alkaloids, flavonoids, saponins, carbohydrates, terpenoids and steroids. Karpura shilajit significantly restored creatinine, urea, uric acid, calcium, total protein, sodium and potassium levels insodium oxalate induced urolithiasis model. Histopathological examination further confirmed the induction of a long standing hypercalciuria or hyperoxaluria, conversely to nephrocalcinosis in the sections of kidney treated with sodium oxalate. Karpura shilajit treatment normalized the biochemical and renal stone markers in the experimental rats and showed better activity in vitro and in vivo models. Karpura shilajit had shown significant effect on urine volume, urine pH, urine excretion, sodium and potassium levels. In silico experiments were performed by using Schrodinger in order to establish affinity of compounds in shilajit by APRTase promoters and SOD promoters towards antilithiatic and antioxidant activity. Karpura shilajit treatment restored the antioxidants in experimental rats by its anti-urolithiatic properties.

***Keywords*---**Karpura shilajit, nephrocaliculi, sodium oxalate.

Introduction

Urolithiasis is derived from the Greek words ouron (urine) and lithos (stone) [1]. Kidney stones are mainly lodged in the kidneys. Mankind has been afflicted by urinary stones since centuries dating back to 4000 B.C[2]. It is the most common disease of the urinary tract. The prevention of renal stone recurrence remains to be a serious problem in human health. The prevention of stone recurrence requires better understanding of the mechanisms involved in stone formation [3].Kidney stones have been associated with an increased risk of chronic kidney diseases, end-stage renal failure, cardiovascular diseases, diabetes, and hypertension. It has been suggested that kidney stone may be a systemic disorder linked to the metabolic syndrome. Nephrolithiasis is responsible for 2 to 3% of end-stage renal cases if it is associated with nephrocalcinosis[4]. Kidney stone

formation is a complex process that results from a succession of several physicochemical events including super saturation, nucleation, growth aggregation and retention within the renal tubules. Among the used treatments, there are extracorporeal shock wave lithotripsy (ESWL) and drug treatment which revolutionized urological practice almost become the standard procedure for eliminating kidney stones [5].

Shilajit also known in the north of India as *salajit*, *shilajatu*, *mimie*, or *mummiyo* is a blackish-brown powder or an exudate from high mountain rocks, especially in the Himalayans mountains. *Shilajit* has been known and used for centuries by the *Ayurvedic* medicine, as a rejuvenator and as antiaging compound. There are two important characteristics of a *rasayana* compound in the ancient Indian medicine: that is, to increase physical strength and to promote human health. The health benefits of *shilajit* have been shown to differ from region to region, depending on the place from which it was extracted[6].Shilajit contains 14-20% humidity, 18-20% minerals, 13-17% proteins, 4-4.5% lipids, 3.3-6.5% steroids, 18-20% nitrogen free compounds, 1.5-2.0% carbohydratesand 0.5-0.8% alkaloids, amino acids and other compounds.

Docking is a method of molecular modeling, which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Molecular docking can be defined as an optimization problem, which would describe the “best-fit” orientation of a ligand that binds to a particular protein of interest and is used to predict the structure of the intermolecular complex formed between two or more molecules [7].Molecular docking is an attractive scaffold to understand drug bimolecular interactions for the rational drug design and discovery, as well as in the mechanistic study by placing a molecule (ligand) into the preferred binding site of the target specific region of the DNA/protein (receptor) mainly in a non-covalent fashion to form a stable complex of potential efficacy and more specificity. The information obtained from the docking technique can be used to suggest the binding energy, free energy and stability of complexes.Depending upon binding properties of ligand and target, it predicts the three-dimensional structure of any complex.Molecular docking generates different possible adduct structures that are ranked and grouped together using scoring function in the software.Usually, the receptor is kept rigid or partially rigid while the confirmation of ligand molecules is allowed to change[8].

Materials and Methods

Preliminary Phytochemical Investigation

The extract has shown the presence of not only chemical compounds such as carbohydrates, protein and lipids, but multitude of compounds like alkaloids,volatile oils, flavonoids etc. that exert a physiological and therapeutic effect. Shilajit were screened for the presence of various groups of compounds.

Acute toxicity studies

Studies were carried out in order to check the toxic effects of the extract. The study was performed as per organization for economic cooperation and development (OECD). The method is used to evaluate the acute oral toxicity is up and down procedure (OECD).

Animals

Adult wistar albino rats weighing about 150-200 gm which are inbred are utilized for the current study. They were kept in polypropylene cages at 25 ± 2°C, with relative humidity 45- 55% under 12-hour light and dark cycles. All the animals were acclimatized to the to the laboratory conditions for a week before use. They were fed with standard animal feed and water ad libitum. All the pharmacological experimental protocols were approved by the IAEC. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of experiments on Animals (CPCSEA).

Experimental Protocol

Wistar albino rats weighing approx. 150 to 200 g were procured from Gentox Bioservices, Hyderabad, and this study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India (Reg, No. 1175/PO/ERe/S/08/CPCSEA).

***In vitro* evaluation of antilithiatic activity**

Nucleation and aggregation assay

Newly pre-arranged solution of calcium chloride dihydrate 10 mM and sodium oxalate1.0 mM, containing NaCl and sodium acetate trihydrate200 mM, 10 mM respectively, it was adjusted to pH 5.7. All examinations were conducted at 37°C, utilizing a circulating water bath. For experiments of crystallization, 25 mL of sodium oxalate solution was moved to a beaker and set in the hot plate magnetic stirrer (Model 2MLH, REMI), which was maintained at a temperature of 37°C and continually stirred at 800 rpm. An extra 1 mL of distilled water/standard (Cystone)/extract were added and finally calcium chloride solution (25 mL) was added. At 620 nm in spectrophotometer (UV 1800, Shimadzu Corporation, Japan) the optical density was measured after addition of calcium containing solution, on every 15 s over 5 min and then every 1 min over 10 min. Every one of the trials was performed in triplicate. The final solutions were seen under a light microscope to analyze the density of shaped crystals in the solution (Olympus,USA). Percent inhibition in the presence of Cystone or Shilajit was compared with the control by the following formula. Percent inhibition of nucleation and aggregation assay was measured by simultaneous model described by Sharma.The percentage inhibition was calculated as:

$$1 - \frac{Tsi*}{Tsc} 100$$

Where T sc, the turbidity slope of the control; and Tsi, the turbidity slope in the presence of the inhibitor[9, 10].

In vivo evaluation of antilithiatic activity

Sodium oxalate induced urolithiasis

Sodium oxalate prompted calcium oxalate urolithiasis model was utilized to assess antilithiatic impact of *Karpura shilajit* in Wistar albino rats. This is an acute model of urolithiasis and the treatment period for 7 days. Group I served as control group that receives normal (0.5 %) gum acacia, Group II, III, IV and V getsSodium oxalate (70 mg/kg, bd. wt.,*i.p*) in distilled water for 1-7 days but Group III and IV received additionally *Karpura shilajit* (200 mg/kg, bd. wt., *p.o*) for 1-7 days and Group V received additionally Cystone (500 mg/kg, bd. wt.*, p.o*) for 1-7 days.Every one of the animals in different groups put separately in metabolic cages for a daythey were given complete access to drinking water, analyzed for urine volume and urine pH on 0 th and 7 th day. Conc Hclone drop was added to urine and stored at 40°C. Blood was withdrawn from retro orbital sinus under anaesthesia on 0th and 7th day and sample were centrifuged at 3000 rpm for 15 min. Serum obtained was analyzed for creatinine, BUN, uric acid, calcium, phosphate, oxalate, sodium and potassium [11, 12].

Histopathology of kidney

For the assessments of tissues histopathologically, the kidney tissue from both kidneys of the animal were fixed in 10% formalin for a minimum of 24 h. Then the paraffin sections were kept ready and cut into 5-µm thick segments in a rotary microtome. The areas were stained with Haematoxylin-eosin dye. The histopathological examination of slides was performed under plain and polarized light microscope and photographed by camera. Histopathological changes, aggregation of calcium oxalate crystals and stones in the kidney tissues were recorded[13].

Insilico analysis

a) Molecular docking studies
   Molecular docking continues to hold great promise in the field of computer-based drug design which screens small molecules by orienting and scoring them in the binding site of a protein[14].Structures of proteins are downloaded from PDB. The analysis and interpretation of the binding behavior play a crucial role in rational drug designs and in elucidating fundamentals of biochemical processes.
b) Ramachandran plot
   The Ramachandran plot is a fundamental tool in the analysis of protein structures [15]. Used to access the quality of model.Here, red region indicates favored region, yellow region for allowed and light-yellow shows generously allowed region and white for disallowed region. Phi and Psi angels determine torsion angels[16].
c) Molinspiration
   Lipinski's rule of five is helpful in describing molecular properties of drug compounds required for estimation of important pharmacokinetic

parameters such as absorption, distribution, metabolism, and excretion.There are various physicochemical descriptors and pharmacokinetic relevant properties of the adrenergic agents were evaluated by using the tool Molinspiration Cheminformatics server (http://www.molinspiration.com). Molinspiration Cheminformatics offers broad range of tools supporting molecule manipulation and processing, including SMILES and SDfile conversion, normalization of molecules, generation of tautomers, molecule fragmentation, calculation of various molecular properties needed in QSAR, molecular modelling and drug design, high quality molecule depiction, molecular database tools supporting substructure and similarity searches. This software also supports fragment-based virtual screening, bioactivity prediction and data visualization [17].

Statistical analysis

Values are expressed as Mean ± SEM, (n=6). All the groups were compared with control, disease control and standard. By using Dunnett's test significant values were expressed as p= 0.0001, p< 0.0001, p< 0.0005, p< 0.001.

Results

Preliminary phytochemical analysis

Phytochemical screening of extract revealed the presence of terpenoids, alkaloids, carbohydrates, saponins and steroids.

Table 1:Preliminary phytochemical analysis

Phytochemical constituents	Result
Phenolicacids	+
Flavonoids	+
Essentialoils	+
Thiophenederivatives	+
Triterpenoids	+
Sterols	+
Alkaloids	+
Carbohydrates	+

Acute toxicity studies

The pure resin of *Karpura shilajit* was tested on Swiss albino mice up to adose of 2000 mg/kg bd. wt. The animal did not exhibit any signs of toxicity or mortality upto2000 mg/kg bd. wt. various morphological and behavioral characters were observed during the study. Hence the extract was found to be safe up to 2000 mg/kg bd. wt.

Dose selection

From toxicity studies, a dose of 2000 mg/kg bd. wt. was identified to be safe, and the working dose was considered as 1/20 th i.e., 100 mg/kg, bd. wt. In the

present study pharmacological evaluations were done using 100 mg/kg, bd. wt. and 200 mg/kg, bd. wt.

In vitro evaluation of antiurolithiatic activity

Nucleation and aggregation assay

Table 2:Effect of *Karpura shilajit* and Cystone on turbidity and percentage inhibition in '*in vitro* nucleation and aggregation' assay method

Group	Turbidity	Percentage Inhibition(%)
Blank	0.80	0
Karpura shilajit (100 mg)	0.64	21.52
Karpura shilajit (200 mg)	0.56	32.37
Cystone (500 mg)	0.32	60.49

In vivo evaluation of antilithiatic activity

Sodium oxalate induced lithiasis model

Groups	BUN (mg/dL)		Sodium (mEq/mL)	
	Day 0	Day 7	Day 0	Day 7
Control	35.90±0.85	36.82±0.76	141.05±0.27	141.58±0.55
Disease Control	38.45±0.23	92.10±0.92 *	140.21±0.29	93.28±0.42*
Karpura shilajit (100 mg/kg)	36.62±0.337	73.61±0.80 *aB	141.58±0.41	116.72±0.30 *aB
Karpura shilajit (200 mg/kg)	37.60±0.53	52.10±0.61 *a ns	139.49±0.31	127.50±0.28 *aA
Cystone (500 mg/kg)	37.64±0.45	47.97±0.84 *a	138.39±0.18	129.71±0.17 *a

Table 3: Effect of *Karpura shilajit* on serum Blood Urea Nitrogen and Sodium levels in Sodium oxalate model

Values are expressed as mean ± SEM (n=6) analysis was performed with one-way one way ANOVA followed by Dunnett's multiple comparison test against control (*= p= 0.0001), against disease (a= p= 0.0001) and against Cystone 500 (A = p<0.0005, B= p= 0.0001), ns= non-significant.

Table 4: Effect of *Karpura shilajit* on serum Calcium, Potassium and Total protein levels in Sodium oxalate model

Groups	Calcium (mg/dL)		Potassium (mEq/dL)		Total protein (g/dL)	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Control	8.81±0.56	9.4±0.39	3.9±0.15	4.06±0.18	6.1±0.07	7.10±0.07
Disease Control	8.47±0.19	16.20±0.41*	4.09±0.16	10.50±0.52 *	6.73±0.07	10.23±0.18**
Karpura shilajit (100 mg/kg)	8.19±0.14	13.60±0.18 *aB	3.69±0.08	8.09±0.30 *aC	6.54±0.14	9.08±0.09 **aB
Karpura shilajit	8.38 ± 0.32	12.50±0.10	3.95±0.21	6.81±0.29	6.8±0.07	8.98±0.19*

(200mg/kg)		*bA		*b ns		*aB
Cystone (500mg/kg)	8.75 ± 0.03	10.9±0.21 **a	3.89±0.11	6.722±0.30 *b	7.1±0.05	7.91±0.13 ***c

Values are expressed as mean±SEM (n=6) analysis was performed with one way ANOVA followed by Dunnett's multiple comparison test against control (* = p= 0.0001), (** = p<0.0001, *** = p=0.01), against disease (a = p< 0.0005, b = p= 0.0001, c = p<0.0001) and against Cystone 500 mg/kg (A = p< 0.0005, B= p< 0.0001, C = p<0.001).

Table 5: Effect of *Karpura shilajit* on serum Creatinine and Uric acid levels in Sodium oxalate model

Groups	Creatinine (mg/dL)		Uric acid (mg/dL)	
	Day 0	Day 7	Day 0	Day 7
Control	0.76±0.05	0.78±0.02	0.78±0.07	0.72±0.10
Disease Control	0.73±0.06	2.62±0.09*	0.75±0.07	2.84±0.06*
Karpura shilajit (100 mg/kg)	0.65±0.01	2.14±0.03 *aA	0.8±0.14	1.88±0.10 *aB
Karpura shilajit (200 mg/kg)	0.71±0.04	1.62±0.13 *a ns	0.68±0.07	1.64±0.09 *a ns
Cystone (500 mg/kg)	0.69±0.05	1.47±0.02 *a	0.71±0.05	1.45±0.07 *a

Values are expressed as mean±SEM (n=6) analysis was performed with one way ANOVA followed by Dunnett's multiple comparison test against control (*= p= 0.0001), against disease (a= p= 0.0001), and Cystone 500 mg/kg (A= p= 0.0001, B= p<0.0001), ns= non-significant.

Table 6: Effect of *Karpura shilajit* on following physical parameters in Sodium oxalate model

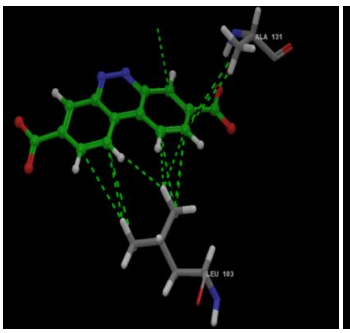
Groups	Percent change in bd.wt (g)	Urine volume (ml)	Wet Kidney wt (g)	Dry Kidney wt (g)	Urine pH
Control	4.83±0.40	8.1±0.10	1.06±0.01	0.45±0.03	6.5±0.22
Disease Control	-4.33± 0.65*	4.6±0.15*	1.73±0.07*	1.10±0.08*	3.33±0.21*
Karpura shilajit (100 mg/kg)	2.23±0.33***a ns	5.3±0.08**bB	1.41±0.02*aA	0.67±0.02**aC	5.5±0.22***aB
Karpura shilajit (200 mg/kg)	3.89±0.90***aC ns	6.4±0.9c ns	1.27±0.01**aC	0.53±0.001a ns	6.83±0.30 a ns
Cystone (500 mg/kg)	4±0.25a ns	6.6±0.16bns	1.13±0.02a ns	0.52±0.001a ns	7.16±0.30a ns

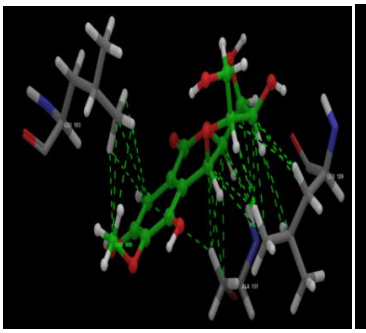
Values are expressed as mean ± SEM (n=6) analysis was performed with one way ANOVA followed by Dunnett's multiple comparison test against control

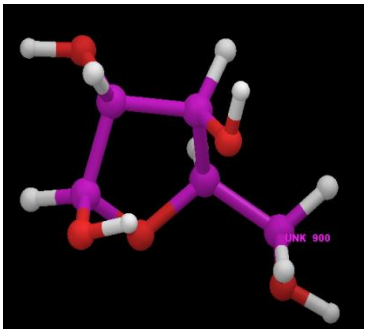
(a=p<0.0001, b=p<0.005,c=p<0.05), against disease (*=p<0.0001,**=p<0.01, ***p<0.05) and against cystone 500 (A=p<0.0001, B=p<0.01,C=p<0.05). ns = Nonsignificant.

***Insilico* analysis**

Molecular docking
PDB ID: 6FD4





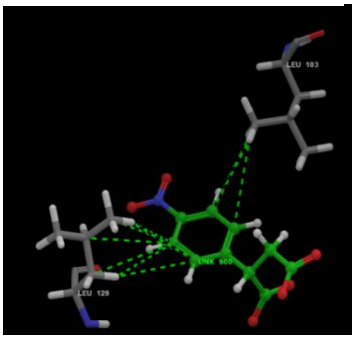


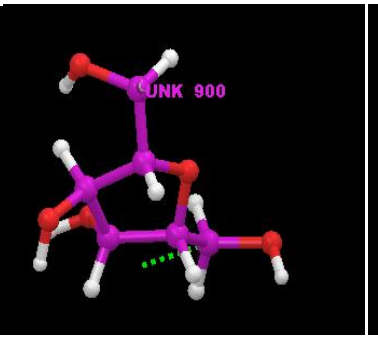
a) Berginin (-9.47)b) Cinnolinedicarboxylic acid (-8.23) c) Xylofuranose (-7.69)

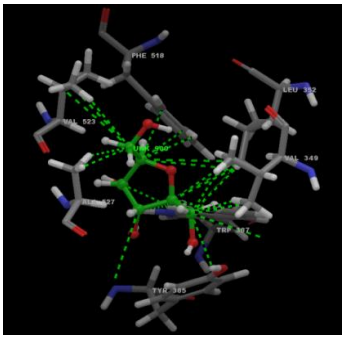
Demonstrated hydrophobicinteractions with LEU 103, LEU 129, ALA

Demonstrated hydrophobicinteractions with LEU 103, ALA 131

Demonstrated No hydrophobicinteractions







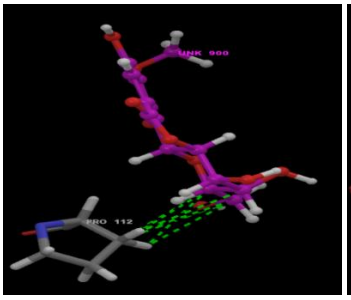
d) Nitrophenyl succinic acid (-7.56)e) Anhydrotalitol (-8.69) f) Bendazol (-8.72)

Demonstrated hydrophobicinteractions with LEU 103, LEU 129

Demonstrated No hydrophobicinteractions

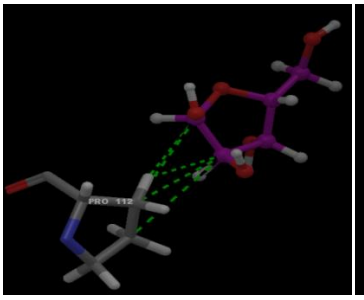
Demonstrated hydrophobicinteractions with LEU 163, LEU 129, AL

PDB ID: 2JLP



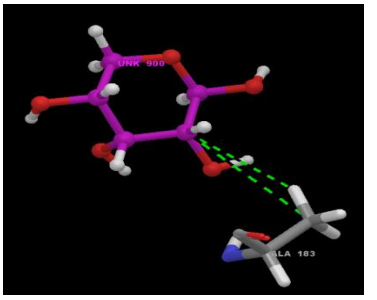
a) Berginin (-7.23)

Demonstrated hydrophobic interactions with PRO 112



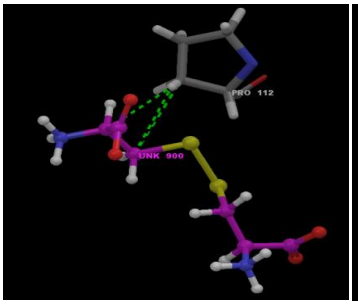
b)Xylofuranose (-7.00)

Demonstrated hydrophobic interactions with PRO 112



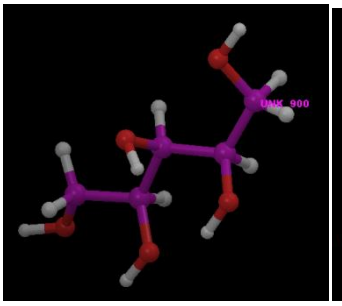
c) Ribopyranoside (-6.70)

Demonstrated hydrophobic interactions with ALA 183



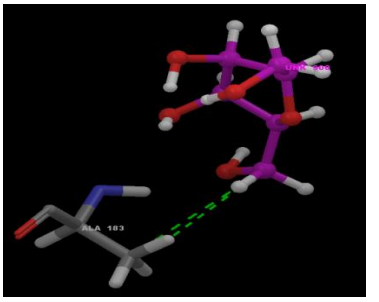
d) Cystine (-6.64)

Demonstrated hydrophobic interactions with PRO 112



e) Ribitol (-5.97)

Demonstrated No hydrophobic interactions



f) Anhydrotalitol (-8.57)

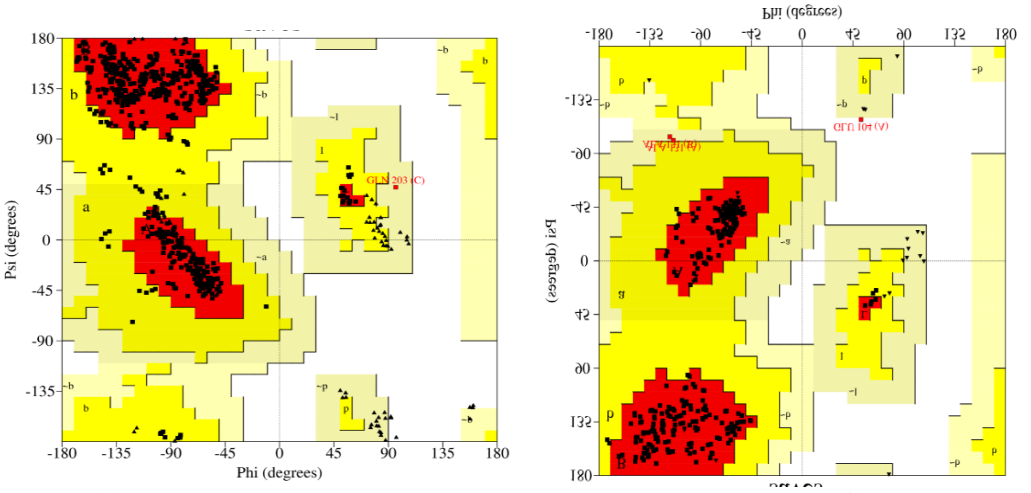
Demonstrated hydrophobic interactions with ALA 183

Figure 1:Protein-ligand interaction analysis

Table7:Glide score of proteins by molecular docking (Schrodinger software)

Constituent	Docking Score(kcal/mol)	
	PDBID:6FD4	PDBID:2JLP
Berginin	-9.47	-7.23
Bendazol	-8.72	-4.01
Anhydrotalitol	-8.69	-8.96
Cinnolinedicarboxylic acid	-8.23	-4.63
Ribitol	-7.45	-5.97
Cystine	-7.51	-6.64
Xylofuranose	-7.69	-7.00
Isocembrol	-5.20	-3.93
Trimethoxychalcoe	-6.43	-2.20
Tartronic acid	-6.40	-4.31
Ferulic acid	-2.74	-4.69

Ramachandran plot



(a) 2JLP (b) 6FD4

Figure 2: Ramachandran plot of Proteins 2JLP and 6FD4

Table8:Ramachandran plot status with protein 6FD4 and 2JLP

Residues	6FD4	2JLP
Most favoured region (%)	96.6	91.7
Additional allowed region (%)	2.4	8.1
Generously allowed region (%)	0.7	0.2
Disallowed region (%)	0.3	0.0

Molinspiration

Table 9: ADME properties of Shilajit compounds by molinspiration

Compound	MW	nON	nOH	nV	nrotb	TPSA	miLogP
Berginin	328.27	9	5	0	2	145.91	0.90
Xylofuranose	150.13	5	4	0	1	20.23	2.41
Trimethoxychalcone	228.4	1	1	0	10	20.23	4.92
Ribitol	280.5	1	1	1	14	20.23	7.21
Cystine	198.3	0	0	1	11	0.00	7.70
Cinnolinedicarboxylic acid	307.2	2	1	1	12	37.30	6.27
Nitrophenyl Succinic acid	73.09	2	2	0	1	43.09	0.08
Tartronic acid	31	5	3	0	6	86.99	4.01
Cycloheptatrien	92.14	0	0	0	0	0	2.00
Anhydrotalitol	164.16	5	4	0	2	90.15	0.51
Ferulic acid	194.19	4	2	0	3	66.76	1.25
Cyclopropane Dicarboxamide	566.60	11	6	3	10	161.89	3.11
Bendazol	208.26	2	1	0	2	28.68	3.50
Thymol	150.22	1	1	0	1	20.23	3.34
Nitroimidazole pyridine	204.19	6	0	0	2	76.54	1.27

MW = Molecular weight, nON = number of hydrogen bond acceptors, nOH = number of hydrogen bond donors, nV = number of violations of Lipinski's rule of five, nrotb = number of rotatable bonds, TPSA = Total Polar Surface Area and miLogP = Octanol-water partition coefficient logP.

Table 10: Bioactivity of Shilajit compounds by molinspiration

Compound	GPCR	IonCM	KI	NRL	PI	EI
Berginin	0.06	-0.09	-0.09	-0.08	-0.14	0.35
Xylofuranose	-0.12	-0.16	-0.84	-1.58	-0.34	0.74
Trimethoxychalcone	-0.07	-0.05	-0.14	0.01	-0.21	0.05
Ribitol	-0.89	-0.26	-1.20	-1.20	-0.97	-0.13
Cystine	0.08	-0.12	-0.88	-0.57	0.03	0.36
Cinnolinedicarboxylic acid	-0.10	0.05	0.02	-0.06	-0.14	0.14
Nitrophenyl Succinic acid	-0.22	0.22	-0.72	0.00	-0.30	-0.04
Tartronic acid	-2.76	-2.49	-3.43	-2.50	-2.67	-2.35
Cycloheptatrien	-3.03	-2.81	-3.46	-3.19	-3.42	-2.85
Anhydrotalitol	-0.24	0.13	-0.60	-1.26	-0.69	0.33
Ferulic acid	-0.47	-0.30	-0.72	-0.14	-0.81	-0.12
Cyclopropane Dicarboxamide	0.34	-0.18	0.50	-0.58	0.20	0.20
Bendazol	-0.10	0.16	-0.02	-0.59	-0.38	0.15
Thymol	-1.05	-0.53	-1.29	-0.78	-1.34	-0.57
Nitroimidazole pyridine	-0.54	-0.28	-0.35	-1.24	-1.37	-0.15

GPCR = G protein coupled receptor, Ion cm = Ion channel modulator, KI = Kinase inhibitor, sNRL = Nuclear receptor ligand, PI = Protease inhibitor, EI = Enzyme inhibitor.

Calculation of molecular properties

Compounds obtained from GC-MS were subjected to docking with protein, were selected and ADME properties, drug likeness (Lipinski's rule of five) which are given in Table 9 (MWT ≤ 500).

Prediction of Bioactivity properties

Bioactivity of compounds was evaluated against six different protein structures. Biological activity is measured by bioactivity score that are categorized under three different ranges (Table 10)

1. If bioactivity score is more than 0.00, having considerable biological activity.
2. If bioactivity score is 0.5 to 0 having moderately activity.
3. If bioactivity score is less than -0.50, having inactivity[18].

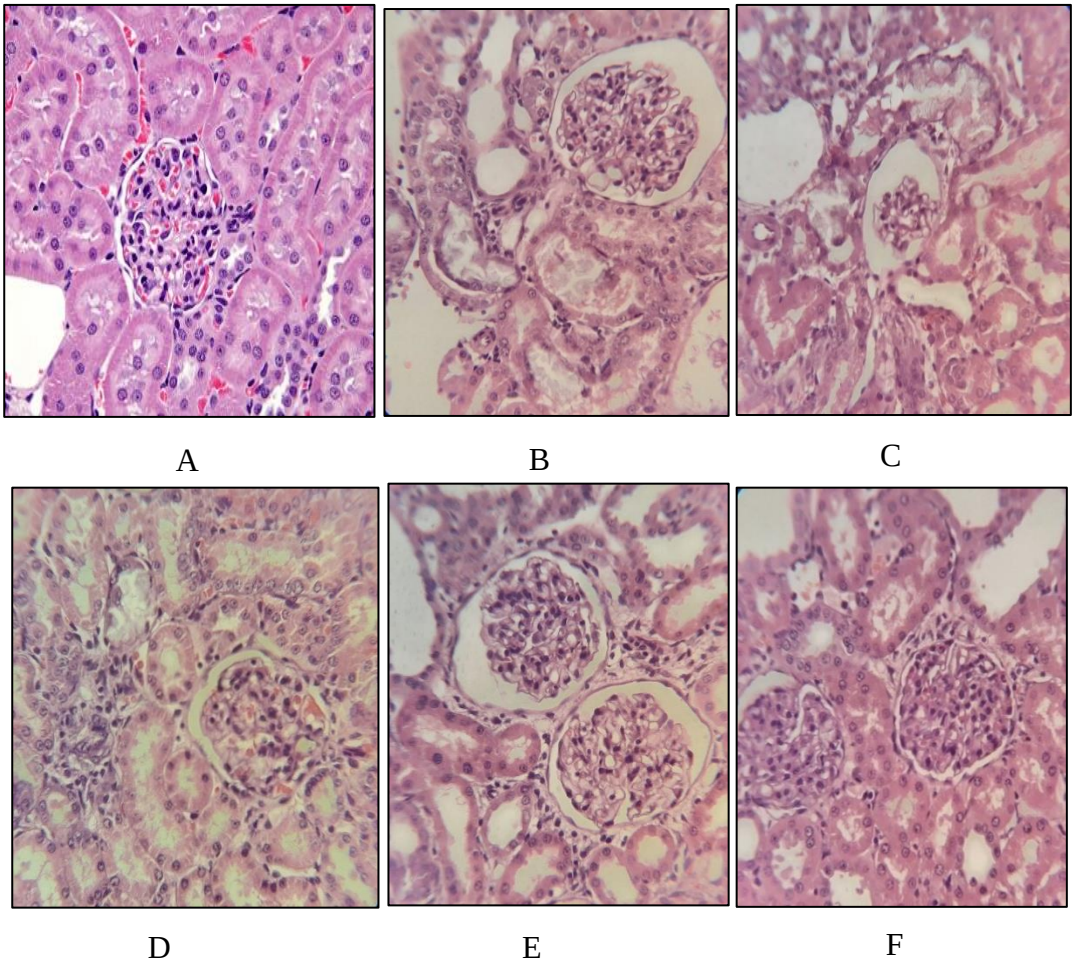


Figure 3: Histopathological Examinations of Kidney sections. (A) Normal Healthy glomeruli and Tubule. (B) Calcium oxalate crystals formation in Disease control group. (C) Degeneration of glomeruli & Acute Nephritis observed in disease control group. (D) Regeneration of glomeruli with RBCs in group treated with Shilajit 100 mg/kg. (E) Healthy tubules observed in group treated with Shilajit 200 mg/kg. (F) Normal glomeruli without any crystal deposition in group treated with standard Cystone 500 mg/kg.

Discussion

Urolithiasis is the condition where urinary calculi are formed or the method of formation of stones within the excretory organ, bladder, and/or ureters (urinary tract). Urine composition factors are important in crystal formation as urine is a metastable liquid containing several coexisting substances that can crystallize to generate renal calculi [19].It is reported that flavonoids, terpenoids, steroids, saponins from different plants showed antilithiatic and diuretic activity [20]. Flavonoids and phenols act as antioxidants through scavenging free radicals, enhancing endogenously produced antioxidant enzymes, chelating the metals, and decreasing membrane lipid oxidation [21].In the crystallization study, the

turbidity increased linearly up to 5 min and then decreased linearly up to 15 min after the addition of calcium chloride dihydrate. Earlier increase in the turbidity was suggestive of the nucleation phenomenon, while the decrease in the later part indicated the aggregation. These two phenomena represented the complete process of in-vitro crystallization [22].Thepresent study revealed anti-urolithiatic activity in '*in vitro* nucleation and aggregation' method and *in vivo* Sodium Oxalate induced lithiasis model. The mechanism of sodium oxalate-induced renal calculi causes hyperoxaluria which is due to the poor solubility of oxalate in urine and its precipitation. Hyperoxaluria damage renal tubules and lead to nucleation, aggregation, and growth of CaOx crystals[23]. During histopathological examination of the kidney sections derived from sodium oxalate model after 7th day study, varying amounts of glomeruli were seen in the kidneys of experimental animal's tissue. Disease group elicited in elongation of tubules with acute nephritis in glomeruli and high infiltration with interstitial inflammation. Shilajit 100 group showed degeneration of glomeruli and elongation of tubule. Co treatment with the Shilajit 200 and Cystone resulted normal glomerulus but Cystone treated group there we observed slight elongation of tubule. Constituents present in Shilajit showed antilithiatic property.In our study *Karpura shilajit* at two dose levels prevented the aggregation of calcium oxalate crystals in *in vitro* nucleation and aggregation method and showed significant reduction in serum creatinine, uric acid, BUN, Total protein and phosphate levels in *in vivo* sodium oxalate model. The chemical composition of Shilajit is a Phytocomplex. The components, humins, humic acids and fulvic acids, are found in all Shilajit along with dilbenzo-α-pyrones which act as carrier of other substances. The humaric substances are the results of degradation of organic matter mainly vegetable substances which is the results of the action of many micro-organisms. A large amount of benzoic acid, benzoates, hippuric acid and their salts as active substances are reported from Shilajit. Chemical investigation of Shilajit furnished six compunds namely, shilajityl acetate, shilajitol, silacatechol, silaxanthone, shilaanthranil and naphsilajitone. The other molecules present in Shilajit are lipids, steroids, carbohydrates, alkaloids, amino acids, free fatty acids, colouring matters such as carotenoids and indigoids, coumarins, organic acids including adipic, succinic, citric, oxalic and tartaric acids, waxes, resins, polyphenols, essential oils, and vitamins like B and B12, elagic acid, latex gums, albumins, triterpenes, sterols, aromatic carbocylic acids, phenolic acids, tannoids and lignins [24, 25, 26].

Molecular docking

Identified compounds were studied by in silico technique using Schrodinger software. A protein sequence of Adenine phosphoribosyltransferase (APRTase) and Super Oxide Dismutase were downloaded from PDB (www.rcsb.org/pdb) which reportedly participate in kidney stone formation and anti-oxidation respectively. Protein sequence of adenine phosphoribosyltransferase (APRTase) and SOD of Homo sapiens and the structure were downloaded in PDB format. The docking analysis of isolated compounds from *Karpura shilajit* and standard drug like Cystone were carried out usingschrodinger software. The various constituents identified in extract are Ribitol, Cystine, Xylofuranose, Isocenbrol, Tartronic acid, Hexenoic acid, Anhydrotalitol, Ferulic acid, Trimethocychalcone and standard drugCystone are subjected to docking against PDB ID: 6FD4,PDBID:2JLP. The

proteins identified were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is clear from the plot that anticipated models have most favorable regions, additionally allowed regions, generally allowed regions and disallowed regions. Percentage distribution of such protein residues determined by Ramachandran plot indicates that the predicted models are of high quality. According to Ramachandran plot a decent quality model would be relied upon to have more than 90% in most favored regions.

The results disclose that Anhydrotalitol, Cyclopropane dicarboxamide, Xylofuranose, Ribitol, Cystine showed highest glide scores when compared to other phytochemical constituents present in the extract against protein models selected. Anhydrotalitol, Cyclopropane Dicarboxamide, Ribitol, Cystine showed highest glide score against PDB,ID:6FD4. Anhydrotalitol, Xylofuranose, Ribopyranoside, Cystine, Ribitol have shown high score against PDBID:2JLP. The glide scores of constituents namely Anhydrotalitol found in the extract has shown higher value than the standard drug Cystone indicating that the compound has more affinity to bind to the proteins. These results clearly indicate that the phytoconstituents might have shown similar mechanism of action to that of the standard drug Cystone in decreasing the crystal growth.

Conclusion

The herbo-mineral extract of *Karpura shilajit* possess anti-urolithiatic and antioxidant activity in rodent models. It significantly reduced the elevated serum biochemical parameters after administration of Sodium oxalate by causing a hyperoxaluric condition leading to rapid formation of calcium oxalate crystals in renal tubules of experimental animals. This high oxalate levels and deposition of calcium oxalate crystals particularly in nephrons destroys the epithelial layer in turn causing nucleation followed by aggregation of these crystals eventually leading to formation of a clinical stone. All compounds showed good glide score when docked against 6FD4 and 2JLP proteins. Lipinski's rule is the basis for determining and calculating the Molinspiration molecular properties, all the compounds that are docked had lower molecular weight so that they are easily absorbed, diffused and transported and its components, values of molecular weight, nON, nOHNH, nrotb and octanol water partition coefficient logP under the acceptable limits.Overall results explained that Shilajit has nephroprotective activity and antioxidant activity by controlling renal stone formation and increasing urine flow. The application of molecular docking studies and *in silico* ADME molinspiration calculations added a practical approach to calculate the molecular properties and predicting the bioactivity scores for the active constituents that are isolated from GC-MS. Further investigations are required to be done to isolate phytochemical constituents of the extract separately and to establish the exact mechanism for its antilithiatic and antioxidant activities.

References

1. Alelign, T., Petros, B. (2018). Kidney Stone Disease: An Update on Current Concepts:Hindawi advances in Urology.1–12.
2. Bawari, S., Sah, A.N., Tewari, D.(2018).Anti- Urolithiatic activity of *Daucus carota*: An *in vitro* study:Pharmacogn J.,10(5): 880-884.

3. Bosco, K. H., Brasseur, R. (2005). The Ramachandran plots of glycine and pre-proline: BMC Part of Springer Nature., 5(14): 1-5.
4. Christina, A.J.M., Muthumani, P.(2013). Phytochemical investigation and anti-lithiatic activity of *Abelmoschusmoschatus*medikus: Int. J. Pharm. Pharm. Sci., 5(1): 108-113.
5. Dinnimath, B.M., Jalalpure, S.S, Patil, U.K.(2017). Antilurolithiatic activity of natural constituents isolated from *Aervalanata*: J. Ayur. Int. Med., 8: 226-232.
6. Harsahay, M.,Pandey, H.K.,Arya, M.C., Ahmed, Z. (2010). Shilajit- A panacea for high-altitude problems:Int. J. Ayur. Res.,1(1): 35-37.
7. Ilhan, M., Ergene, B., Suntar, I., Ozbilgin, S., Citollu, G.S., Demirel, M.A., Kele, G.H., Altun, L.,Akkol, E.K. (2014). Preclinical Evaluation of Antiurolithiatic Activity of Viburnum opulus L. on Sodium Oxalate-Induced Urolithiasis Rat Model: Evidence-Based Complementary Alternative Med.,1-10.
8. Kumar, N., Mishra, S.S., Sharma, C.S., Singh, H.P.(2016). *In silico* ADME, bioactivity and toxicity analysis selected antimalarial agents:International J. Appl. Pharm. Biol. Res., 1(5): 1-8.
9. Lopez, M., Hoppe, B. (2008). History, epidemiology and regional diversities of urolithiasis:Pediatric Nephrology., 25(1): 49–59.
10. Meng,X., Zhang,H. X., Mezei, M., Cui, M.(2012). Molecular Docking- A powerful approach for structure-based drug discovery:Current Computer Aided Drug Des.; 7(2): 146–157.
11. Mishra, H., Rai, K., & Alex, A. (2022). *In vitro* antilithiatic and antioxidant potential of ethanolic extract of *Bauhinia variegata* leaves. Int. J. Health Sci., *6*(S5), 3264–3280. https://doi.org/10.53730/ijhs.v6nS5.9354
12. Mohd,A.K., Pradhan,D. (2011). Antiurolithiatic Activity of *Ageratum Conzoides* Extract in Rats:Pharmacologyonline.,3: 953-958.
13. Onaran, M., Orhan, N., Farahvash, A., Ekin,H.N., Kocabıyık, M., Gonul, I.I., Sen, I., Aslan,M.(2016). Successful treatment of sodium oxalate induced urolithiasis with Helichrysum flowers:J. Ethnopharmacol.,186: 322–328.
14. Pandey, S.P. (2019). Shilajit - A Wonder Drug of Ayurveda: An Overview: Int. J. Pharm. Sci. Rev. Res.,59(1): 140-143.
15. Pawar, A.T., Vyawahare, N.S. (2017). Protective effect of ethyl acetate fraction of *Biophytum sensitivum* extract against sodium oxalate-induced urolithiasis in rats: Journal of Traditional and Complementary Medicine. 7: 476-486.
16. Pawar, A.T., Vyawahare, N.S. (2017). Protective effect of ethyl acetate fraction of *Biophytum sensitivum* extract against sodium oxalate-induced urolithiasis in rats: J. Trad. Complementary Med.,7: 476-486.
17. Rai, K., Mishra, H., & Alex, A. (2022). *In vitro* antilithiatic and antioxidant potential of methanolic extract of *Polyalthia longifolia* leaves. Int. J. Health Sci., *6*(S6), 2582–2598. https://doi.org/10.53730/ijhs.v6nS6.10041
18. Raju, M.G., Goud, P.P., Reddy, N.V.L.S.(2019). Antihypertensive effect of Rutin: Pharmacological and Computational Approach. Asian J. Pharm. Clin. Res., 12(8):87-92.
19. Reddy,N.V.L.S., Raj, G.B.P., Anarthe,S.J., Raju, M.G., Akhila, M.(2019). Molecular docking studies of isolated compounds from *Cassia fistula* on HMG-COA reductase. Asian J. Res. Chem., 12(2):89-93.
20. Reddy,V.N.V.L.S., Raju, M.G., Mamatha,M., Nandini, N. (2021). GC-MS analysis.*In silico* docking studies and diuretic activity of methanolic extract of

Citrus medica l. Leaf in Wistar albino rat model: Bull.Env.Pharmacol. Life Sci., 10(3): 42-50.

21. Sharma, D.,Yadu,D.N.,Sikarwar, I.,Sijoria, R.,Wanjari, M.M.,Jadhav, A.D. (2016). *In vitro* study of aqueous leaf extract of *Chenopodium album* for inhibition of calcium oxalate and brushite crystallization:Egyptian J. Basic Appl. Sci.,1-8.
22. Shehzad, A., Saleem, U., Shah, M.A., Cruz,C.V., Khan, A.H., Ahmad, B. (2020). Antiurolithic evaluation of *Cucurbitapepo* seeds extract against sodium oxalate-induced renal calculi:Pharmacognosy Magazine.,16(68): 174-180.
23. Srinivas,S., Venkanna, B., Mohan, E.M., Mohan, C.K. (2012). Urolithiasis an overview: Int. J. Pharm. Res. Biomed. Sci., 1(1): 20-31.
24. Suvarchala Reddy VNVL, Raju MG, Mamatha M. Antilithiatic activity of leaf extracts of *Citrus medica* on sodium oxalate urolithiais - *in vitro* and *in vivo* evaluation: Int. J. Pharma. Sci. Res.2021; 12(7): 3709-3715.
25. Vijaya, T., Rao, N.V.R., Bab, A.N., Kumar, M.S., Nirojini, P.S., Reddy, B.S., Nadendla, R.(2013). Anti-urolithiatic activity of methanolic extract of dried leaves extract of *Golchidion velutinium* using ethylene glycol induced rats:Int. J. Biol., & Pharm. Res., 4(12): 878-884.
26. Vijayakumari, B.,Venkatachalam, S.,Singanallur, R.,Yadav, H. (2016). *In silico* analysis of aqueous root extract of Rotula aquatica Lour for docking analysis of the compound 3-O-acetyl-11-keto-β-boswellic acid contents:SpringerPlus.,5(1): 1486.