Pharmacological Evaluation of Ganoderma lucidum for the treatment of Kidney Stones

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KEYWORDS

kidney stones, urolithiasis, nephrolithiasis, Ganoderma lucidum, nucleation, aggregation

ABSTRACT

Kidney stones are prevalent and painful urological conditions with limited effective treatment options. This study aimed to investigate the therapeutic potential of Ganoderma lucidum for in vitro and in vivo models for the management of kidney stones.

Method- In-vitro experiments using standardized assays were used to assess the antioxidant, anti-nucleation, and anti-aggregation properties of the mushroom extracts against common calcium-based stones. Also, an in vivo study was carried out using a rat model of calcium oxalate urolithiasis, induced by 0.75% v/v ethylene glycol, animals were divided into 5 groups (n=6), group 1 normal control, group 2 disease control, group 3 treated with Standard drug, group 4 and 5 with mushroom extracts of 250mg/kg and 500mg/kg, for 4 weeks. The biochemical parameters were measured

via blood and 24-hour urine volume. The Calcium Oxalate depositions were evaluated by hematoxylin and eosin staining.

Result- The in vitro findings revealed that the mushroom extracts exhibited significant inhibition of calcium oxalate by nucleation at 48.1±1.42 % and by aggregation at 61 ± 0.46 %. Moreover, in the in vivo study, the administration of these mushroom extracts led to a reduction in stones formed in the rat kidneys. Histopathological examination of kidney tissue from the treated group demonstrated positive results, indicating a reduction in tissue damage and inflammation compared to the disease control group. These effects were accompanied by a decrease in serum creatinine, total protein, urea, uric acid and calcium levels and an increase in serum magnesium levels, indicative of improved renal functions. These findings open avenues for further research into the development of natural remedies for kidney stone prevention and treatment.

Introduction

Deposition of minerals and salts promotes hard substance formation in the kidney, known as kidney stone. The most common type of kidney stone is calcium oxalate, which forms when there is less amount of liquid intake and high levels of calcium and oxalate in the urine. Other types of kidney stones include uric acid, struvite, and cystine stones. [1]The pathophysiological mechanisms for calcium kidney stone formation are complex and diverse and include low urine volume, hypercalciuria, hyperuricosuria, and hyperoxaluria**.**[2]**,**[3]. Calcium oxalate stones are the most common type of kidney stone, accounting for about 70% of all kidney stones. They form when there are high levels of calcium and oxalate in the urine and too little liquid[4] [5] . Calcium oxalate stones are caused by too much oxalate in the urine, which can form crystals that grow and form kidney stones [4]. The risk of developing calcium oxalate stones in a kidney is highest at a pH value of five [6]. Calcium oxalate monohydrate (COM) is thermodynamically more stable with aggregatory and adhesive tendencies. Hence, COM tends to form large crystal aggregates and adheres strongly to renal epithelial tissue, injuring the same. Therefore, of the two polymorphs, COM significantly promotes crystal retention and eventual stone formation.[7]

The treatment of calcium oxalate stones depends on the type, symptoms and severity of hyperoxaluria and how well the patient responds to treatment [8]. To reduce the amount of calcium oxalate crystal formation in the kidneys, the medical practitioner may recommend one

or more of the following treatments: medication to reduce the oxalate, increasing fluid intake, increasing dietary intake of calcium, and restricting the intake of salt, animal protein, and oxalate-rich food [9]. If large kidney stones are causing pain or blocking urine flow, they may need to be removed or broken up so they can pass in the urine [8]**.** To relieve mild pain, doctors may recommend pain relievers. In addition, doctors may give medication to help pass the kidney stone, such as an alpha-blocker, which relaxes the muscles in the ureter, helping the patient pass the kidney stone more quickly and with less pain. Several biomarkers have been studied for kidney stones.

Materials and methods

Mushroom collection

Ganoderma lucidum was procured from Shilpa farm Turpan village, Hyderabad, and was identified and authenticated from NIScPR Delhi. A voucher specimen of *G.lucidum* authentication number **NIScPR/RHMD/Consult/2023/4516-17** Was also deposited in the herbarium of NIScPR Delhi.

Processing and extraction

G. lucidum fruiting bodies were cut into small pieces, dried in shade for some time, and coarsely powdered. The Soxhlet method was used for the extraction of powdered *G. lucidum* with 70% v/v ethanol. *G. lucidum* fruiting bodied extract thus obtained was dried in a rotatory evaporator under reduced temperature and pressure, and then the final residue was evaporated by using a water bath to obtain the concentrated extract.[14 15]

Preliminary Mycochemical screening

Preliminary mycochemical evaluation of *G.lucidum* was carried out for qualitative estimation of mycoconstituents.[16]

Physiochemical parameters

Physiochemical parameters were measured to determine the physical properties of the mushroom. [17 18]

Anti-oxidant assay

DPPH

The free radical scavenging activity of the extract was measured by the 1,1-diphenyl -2-picryl hydrazyl (DPPH)method. Five concentrations (10,20,30,40 and 50 microgram/ml) of extract and ascorbic acid were prepared in ethanol in different volumetric flasks. The volume of all samples was made up to 3 ml using ethanol. Methanolic DPPH (0.1mM) was prepared and 1 ml of this solution was added to each volumetric flask, shaken vigorously, and allowed to stand for 30 min at room temperature. A control was prepared as described above without a test or standard. Absorbance was measured at 517 nm. All tests were performed in triplicates. Low absorbance of the reaction mixture indicates higher free radical scavenging activity. Radical scavenging was expressed in terms of percentage inhibition and was calculated by using the formula[19]

%RSA = (Abs of Control)- (Abs of the sample) / (Abs of control) x100

NO_2

Sodium nitroprusside (5 µl) in standard phosphate buffer solution was incubated with different concentrations of the test extracts dissolved in standard phosphate buffer (0.025M. pH 7.4) and the tubes were incubated at 25°C for 5 hr. After 5 hr 0.5 ml of incubated solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing an equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in water). The absorbance of the chromophore formed was read at 546 nm. distilled water was used for control. Readings were taken in triplicates and compared with ascorbic acid (standard antioxidant). Nitric oxide radical scavenging is calculated by the Formula mentioned above for DPPH [20 21].

Nucleation assay

The impact of calcium oxalate (CaOx) crystal formation was evaluated using a nucleation test. Calcium chloride ($CaCl_2$) (5 mmol/l) and sodium oxalate ($Na_2C_2O_4$) solution (7.5 mmol/l) were prepared In Tris-HCl (0.05 mol/l) and NaCl (0.15 mol/l) buffer (pH 6.5). different dilutions of *G.lucidum* were prepared (100-1000µg/ml) in distilled water. One millilitre of each *G.lucidum* concentration was mixed with 3 ml $CaCl_2$ solution followed by the addition of 3 ml $Na_2C_2O_4$ solution. The final mixture was incubated at 37°C for 30 minutes. At a wavelength of 620 nm, the optical density of the mixture was measured. The percent inhibition of nucleation was then

determined using the formula given below for *G. lucidum* and compared with the standard drug Cystone. [22]

% Inhibition = (1- $OD_{test}/OD_{control}$) × 100

Aggregation Assay

An aggregation assay was used to assess the impact of *G. lucidum* on CaOx crystal aggregation. Solutions of $CaCl_2$ and $Na_2C_2O_4$ (50 mmol/l each) were mixed well, heated to 60 C in a water bath for one hour, and then incubated overnight which helps to form Seed of CaOx crystals. After drying, CaOx crystal solution (0.8 mg/ml) was prepared in a 0.05 mol/l Tris-HCl and 0.15 mol/l NaCl buffer (pH 6.5). One milli litter of aliquots (100–1000 µg/ml) of *G.lucidum* was added to 3 ml CaOx solution, vortexed, and incubated at 37 °C for 30 min. After that, the final combinations' OD was read at 620nm, and % inhibition of aggregation was then calculated as the same formula as the nucleation assay.[22]

CaOx crystal characterization by FT-IR

FT-IR analysis was used for the confirmation of the calcium oxalate crystal formation in aggregation assay [23]

Animals Study

Thirty male Wistar rats were taken from the departmental animal house, and were housed at a temperature of 22 °C ± 3 °C with relative humidity 30-70 % and 12-hr light and 12-hr dark cycle. The animals were allowed to acclimate laboratory condition for one week prior to experimentation. They were fed with pellets diet and provided aqua water *ad libitum* [24]. The protocols and procedures were approved by the IAEC of KUDOPS with protocol number KUDOPS/207.

The animals were divided into five groups, each containing 6 animals. In the previous study, the ethanolic extract was safe up to 5000 mg/kg[26]. so, we have taken 250 and 500 mg/kg dose**.** Group 1 is considered as a normal control group provided normal saline 0.9%. Group 2 is Lithiatic Control given 0.75% ethylene glycol, Group 3 Standard 750 mg/kg (Cystone) orally and 0.75% v/v ethylene glycol, Group 4 *Ganoderma lucidum* low dose {GLL) 250mg/kg, Group 5 *Ganoderma lucidum* high dose (GLH) 500 mg/kg orally. 1% w/v ammonium chloride in drinking water for the first three days was given to group II, III IV and V to induce stable stone[27]**.**

Biochemical Parameter

To measure the levels of Calcium, Magnesium, Uric acid, Urea, Creatinine, Potassium, Sodium, Total Protein, SGPT, SGOT, BUN, the rat urine was collected on day 27th by placing each rat in metabolic cage for 24 hr and blood was collected on 28th day of the experiment. The animals were brought into general anaesthesia by intramuscular injection of a ketamine–xylazine solution (0.2 mL/kg). Blood samples were collected from the eye by retro orbital vein. Blood was immediately transferred in tubes and centrifuged at 5,000 rpm for 15 min at room temperature. Subsequently, the serum was collected and stored at −20 °C to be further analysed. The parameters were evaluated by following the manufacturer's guidelines [28] [20].

Histopathology

Higher dose of anaesthesia (ketamine and Xylazine) is given to animals to sacrifice them, then both kidneys were extracted out by using surgical tools and excess of blood was removed by blotting with filter paper and transferred to container containing 10% formalin and placed them for 24 hours to fix the tissue. [29]

Histopathology analysis

These tissues were sectioned horizontally and vertically and fixed kidney tissues were sectioned (5-micron thickness), embedded in paraffin and stained with Haematoxylin and Eosin (H&E). Light microscopic examination of multiple tissue sections from each were performed. Changes in the experimental histopathologic parameters for kidney tissues were analysed. [30]

Statistical analysis

Quantitative results of all the experiments performed in triplicates were expressed as mean ± standard error of the mean (S.E.M). A mean difference was considered significant when $p<0.05$ Statistical computations were performed on GraphPad Prism 10 software using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

Results

Preliminary phytochemical screening

The percent yield of *G.lucidum* was 23.3%. Qualitative phytoconstituents determination in *G.lucidum* showed the presence of the following chemical compounds by positive sign and the absence is marked by negative sign.

S. no.	Phytochemical screening	Chemical test performed	Result
1	Carbohydrates	Molisch's test Fehling's test Benedict's test	+ + +
2	Flavanoids	Shinoda Test Sulphuric acid test	+ -
3	Steroids	Salkowski's test Libermann –burchard test	+ +
4	Glycosides	Keller killani test	+
5	Phenolic Compound	Lead acetate solution Potassium dichromate	+ -
6	Amino acid	Ninhydrin test	+
7	Saponins	Foam Test	+
8	Acids	Litmus test	-
9	Organic Acid	Calcium chloride test	+

Physiochemical parameters

S.NO	Physiochemical parameter	Powder (%w/w)
1	Loss on drying	20%
2	Total ash	11%
3	Acid insoluble ash	5.25%
4	Water soluble ash	4.2%

Antioxidant assay

DPPH

The DPPH scavenging activity of Ethanolic extract of *Ganoderma lucidum* extract increased with increasing concentration. DPPH scavenging potential of extract at various concentrations was measured. The extract showed a radical scavenging ability at 100 µg/ml of 65 ± 0.40 %. whereas ascorbic acid showed 79 ± 0.18 % at 1000 µg/ml highest and IC_{50} values are 57.36 and 72.59 for test and standard respectively.

NO2

The NO_2 antioxidant activity of Ethanolic extract of *Ganoderma lucidum* extract increased with increasing concentration. NO_2 scavenging potential of extract at various concentrations (100-1000 µg/ml) was measured. The extract shows a radical scavenging ability at 1000 µg/ml of 63.19 ± 0.%. whereas ascorbic acid showed 73 ± 0. % at 1000 µg/ml highest and IC_{50} values are 63 and 57.39 for test and standard respectively.

***In-Vitro* Urolithiasis activity**

Nucleation Assay

Addition of $Na_2C_2O_4$ solution to the mixture consisting of $CaCl_2$, promote the formation of numerous CaOx crystals. Presence of *G.lucidum* (1000 µg/ml) in the reaction mixture produced a % reduction in nucleation of 48.1±1.42% which was significantly higher (P<0.01) than that produced by Cystone (39.67±0.98%).

Aggregation assay

G.lucidum produced a significant reduction in aggregation of performed CaOx crystals. Percent reduction in aggregation produced by *G.lucidum* was found to be 61 ± 0.46 When compared with Cystone 76± 0.09 at 1000 µg/ml.

Characterization of CaOx crystals by FT-IR

FT-IR analysis of CaOx crystals produced ten bands. Spectrum showed an asymmetric C-O vibration at 1613.6 cm^{-1}; a symmetric C-O vibration at 1317.23 cm^{-1}; an O-H stretch at 3428.52 cm^{-1}; one C-C stretch at 884.45 cm^{-1} was observed and one O-H bend at 661.14 cm^{-1}. These values are also been reported in previous studies which indicates CaOx monohydrate form. [23]

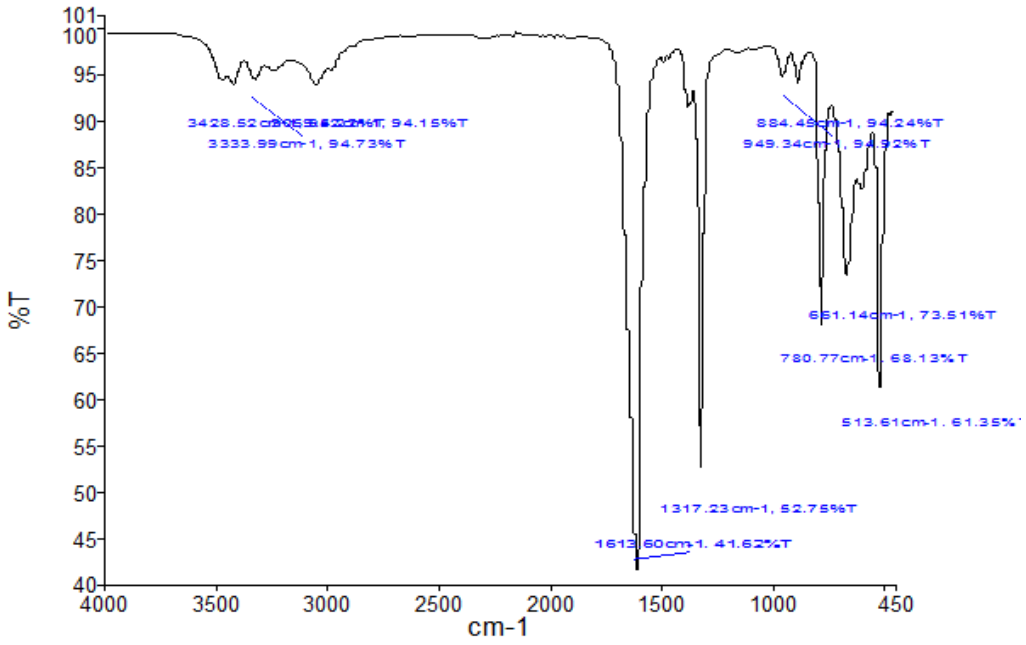
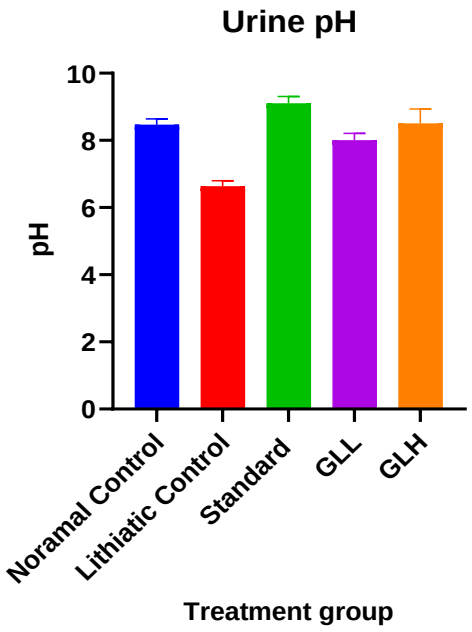


Figure: 1- FT-IR spectra of seed CaOx Crystals.

Urine Biochemical parameters



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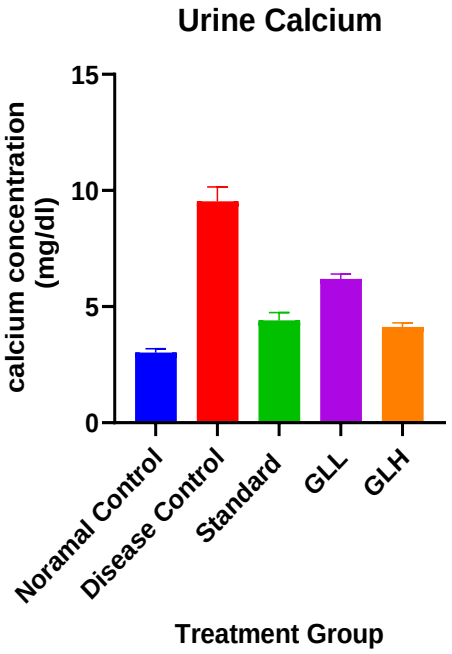

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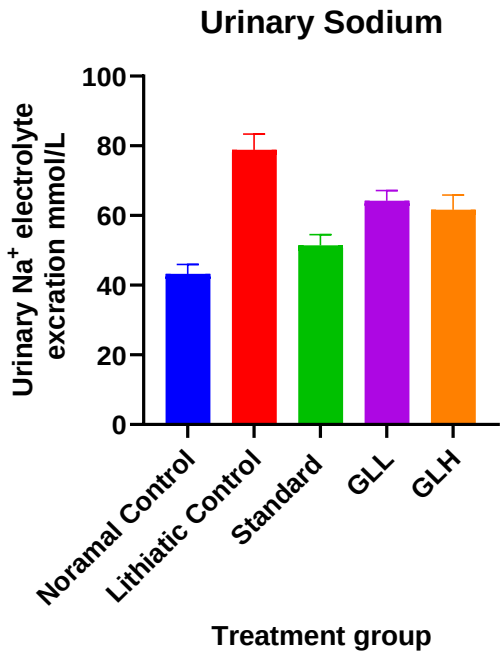

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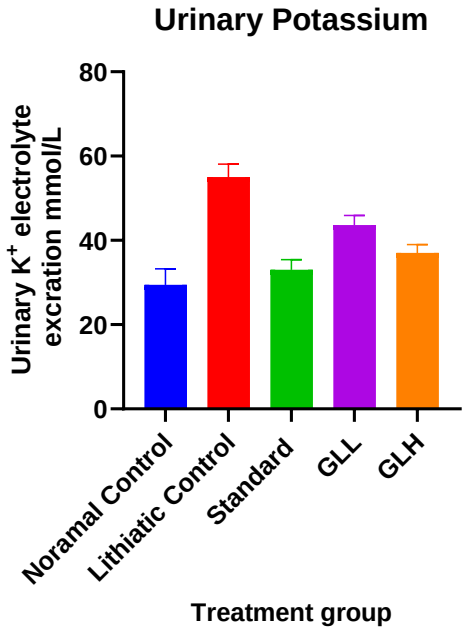
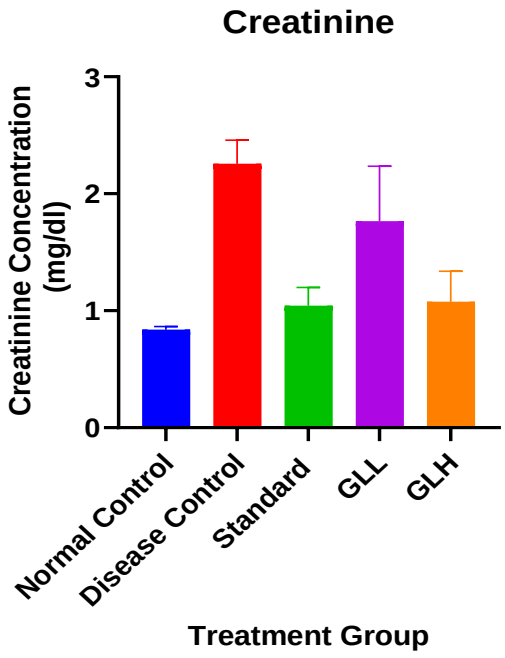


Figure: 6

Serum Biochemical parameters



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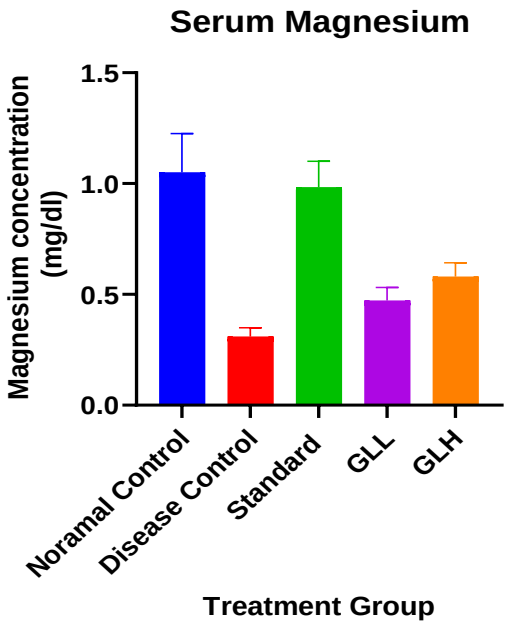

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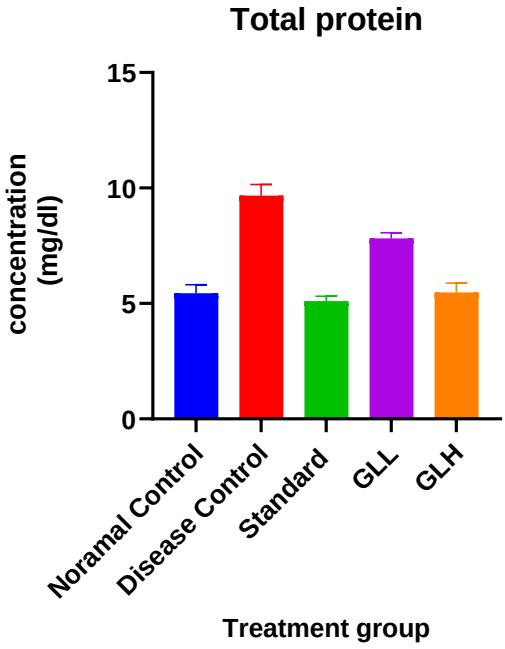

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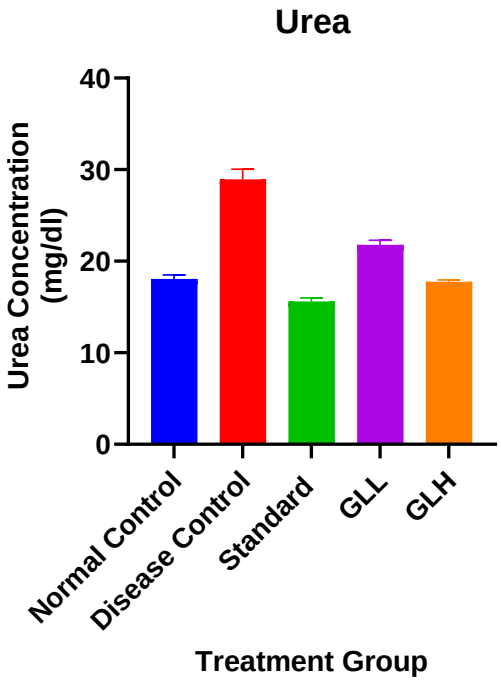

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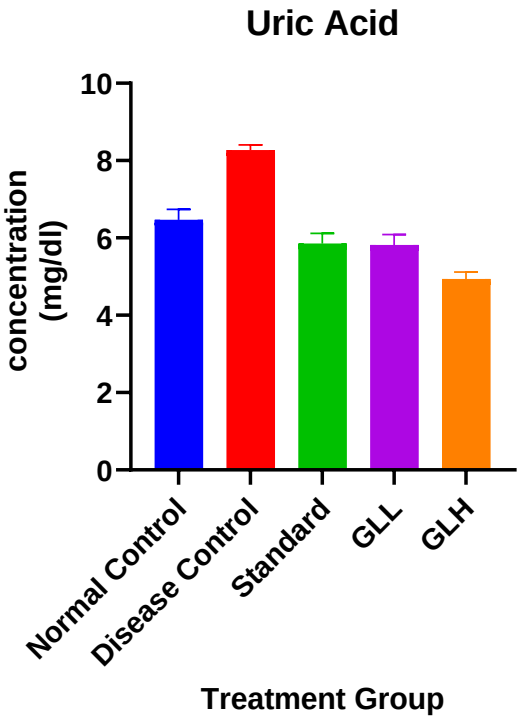

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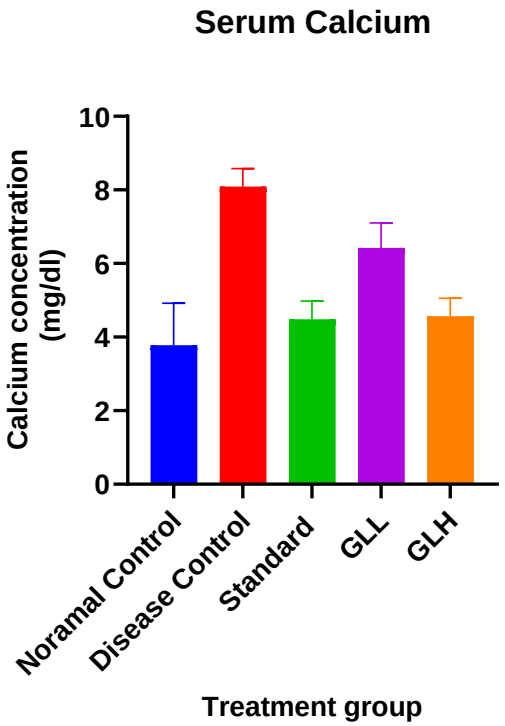

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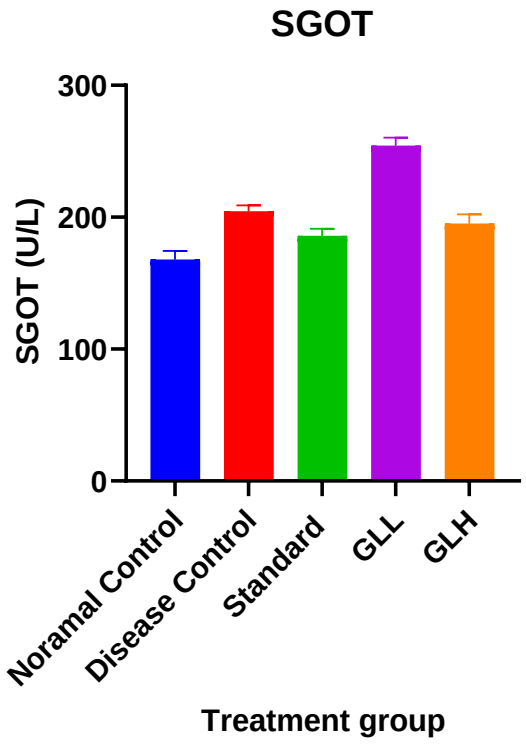

Figure: 13

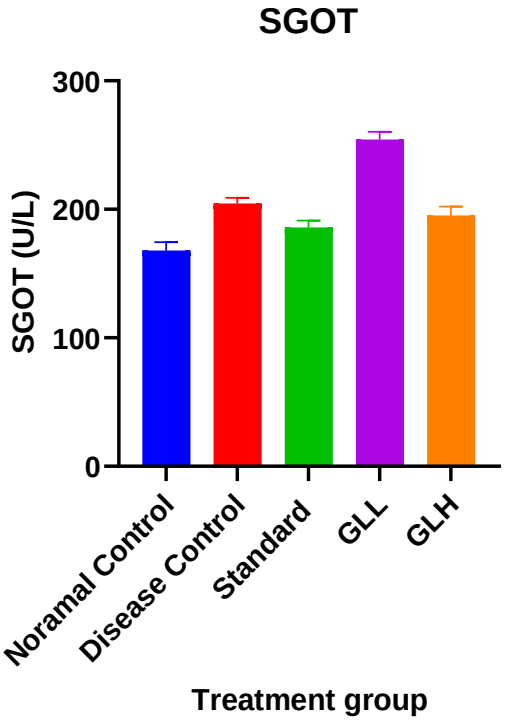

Figure: 14

Serum Biochemical parameters

**P <0.01, ***P<0.001, values are mean ± SEM, n=6, when compared with the disease control using one-way variance analysis (ANOVA) followed by Dunnett's multiple comparisons test.

Serum Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Creatinine	0.83 ± 0.01	2.25 ± 0.08	1.04 ± 0.06***	1.76 ± 0.19***	1.077 ± 0.10***
Total protein	5.43 ± 0.19	9.67 ± 0.19	5.09 ± 0.08***	7.87 ± 0.10***	5.469 ± 0.16***
BUN	18.05 ± 0.18	28.92 ± 0.45	15.58 ± 0.16***	21.78 ± 0.20***	17.75 ± 0.08***
Uric acid	6.40 ± 0.15	8.25 ± 0.06	5.10 ± 0.02***	5.90 ± 0.09***	4.95 ± 0.07***
Serum calcium	3.78 ± 0.46	8.08 ± 0.20	4.48 ± 0.20***	6.41 ± 0.27***	4.56 ± 0.20***
SGPT	11.75 ± 2.34	35.93 ± 1.80	15.78 ± 0.52***	29.07 ± 1.04**	19.73 ± 0.48***
SGOT	167.9 ± 2.64	204.3 ± 1.88	185.9 ± 2.14****	254.2 ± 2.51***	195.1 ± 2.89*

Urine Biochemical parameters

**P <0.01, ***P<0.001, values are mean ± SEM, n=6, when compared with the disease control using one-way variance analysis (ANOVA) followed by Dunnett's multiple comparisons test.

Urine Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Urine calcium	3.07 ± 0.07	9.53 ± 0.25	4.40 ± 0.13***	6.18 ± 0.09***	4.17 ± 0.07***

Urine Na	43.20 ± 1.24	78.80 ± 2.03	51.40 ± 1.40***	64.20 ± 1.31***	61.60 ± 1.91***
Urine K	29.40 ± 1.72	55.00 ± 1.37	33.00 ± 1.09***	43.60 ± 1.03***	37.00 ± 0.89***
Urine Mg	1.05 ± 0.01	0.31 ± 0.01	0.98 ± 0.04***	0.47 ± 0.02*	0.58 ± 0.02***
Urine pH	8.46 ± 0.07	6.63 ± 0.06	9.10 ± 0.08***	8.00± 0.08***	8.50 ± 0.17***

Histopathology –In histopathology study no pathologic changes was observed in the kidneys of the normal group (figure 15)

In lithiatic Group the CaOx crystals damaged the tubular walls; in this group, the kidneys represented a granular view, and in some cases, the medulla was destroyed. The polyuria and very dark blackish-brown colour urine samples was obtained in this group (figure 16).

In standard drug treated group Morphology of tissue was intact and slight inflammation was observed and crystals were least in standard group. (figure 17)

In test drug treated group (250 mg/kg) the crystal accumulation was more marked than all groups, the widening of tubular lumen was seen, haematuria was observed, the kidneys appeared abnormal and the crystal accumulation was less than the group of disease control (figure18). In test group *500 mg/kg treated* group, tubules containing stones were considerably lower than other groups. the integrity of the tissue was more preserved than other groups (figure 19).

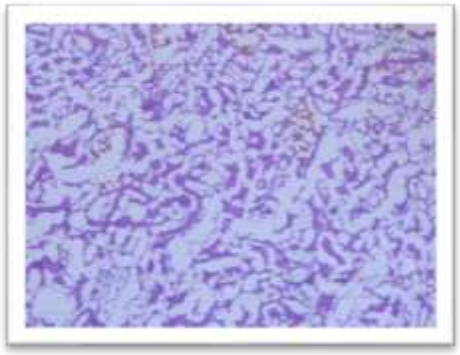

Figure 15

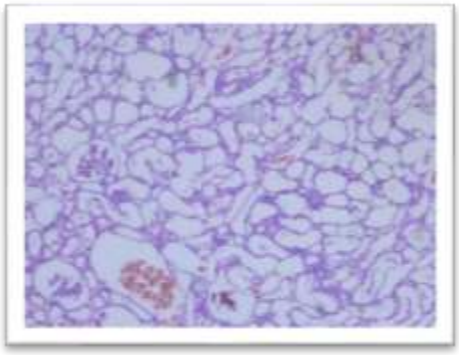

Figure 16

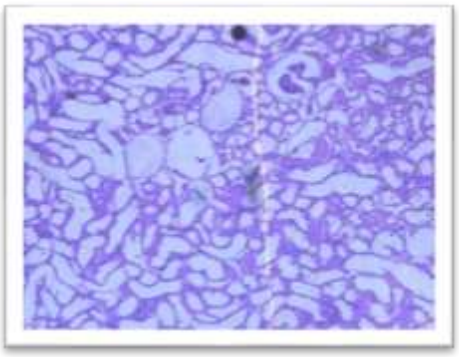

Figure17

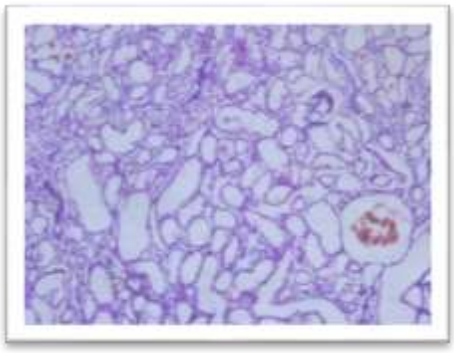

Figure 18

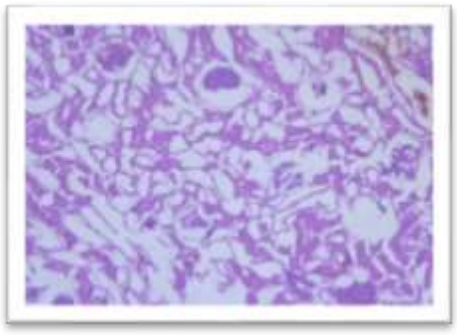

Figure 19

Discussion

The present study was done to evaluate the kidney stone-removing properties of *Ganoderma lucidum.* The results obtained from this research provides valuable insights into the potential therapeutic application of the mushroom extract.

This study demonstrate that the administration of high dose of (500 mg/kg) ethanolic extract of *G.lucidum* extract resulted in a significant reduction in the size of kidney stone in the experimental group. This reduction was observed both in terms of stone size and number of stones and level of serum calcium level, urinary calcium, potassium and sodium levels. Serum and urinary levels of these elements are decreased in treatment (250mg/kg, 500mg/kg) and standard group when compared with lithiatic control (group II). urinary sodium and calcium levels were previously reported as stone promoters. these finding suggested that *G.lucidum* may possess the properties that facilitate the dissolution of the expulsion of kidney stones.

G.lucidum might have a positive effect on kidney stone dissolution due the presence of various phytoconstituents like triterpenoids, polysaccharides, polyphenols. These phytoconstituents are previously reported to reduce kidney stones, reduce oxidative stress and increases urine pH along with lowering SGPT, SGOT levels. Same effect was shown by the *G.lucidum* extract.

Magnesium complexes with oxalate and reduces the supersaturation of calcium oxalate by reducing the saturation of calcium oxalate and as a consequence reduces the growth and nucleation rate of calcium oxalate crystals. Both the doses of 250 and 500 mg/kg/day of extract for 28 days treatment significantly increased the urinary magnesium level and decreased free calcium level in serum when as compared to the lithiatic control group and thus reduced the risk of stone formation.

In urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction to the outflow of urine by stones in the urinary system. Due to this, the waste product particularly nitrogenous substances such as creatinine, urea, BUN and uric acid accumulated in the blood. The serum creatinine, urea, BUN and uric acid were increased in urolithiatic control (group II). The ethanolic extract of *Ganoderma lucidum* of 250 and 500 mg/kg day treatment lowered the elevated serum levels of creatine, urea, BUN and uric acid[62].

Higher level of protein promotes kidney stone formation. Total serum protein level is decreased in Ethanolic Extract of *G.lucidum* of 250 and 500 mg/kg and standard group when compared with lithiatic control(group II).

The potential kidney stone-removing properties of *G.lucidum* present an exciting prospect for the development of alternative, natural treatments for kidney stone sufferers. This could reduce the need for invasive procedures such as lithotripsy or surgery and may offer cost-effective and less invasive solutions

Further research is needed to understand the mechanism by which *G.lucidum* exerts its effect and compare its efficacy with other established treatments.

Conclusion

Our study suggested that *G.lucidum* extract may hold promise as a natural remedy and prophylactic use for kidney stone management. While the results are encouraging, possible mechanism of anticrystallization activity of *G.lucidum* could be its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complex, and acting through various steps it helps to prevent kidney stones. *G.lucidum* could potentially offer a valuable addition to the array of treatment options available to individuals suffering from kidney stones.

References

1. Cui X, Zhao Z, Zhang G, Chen S, Zhao Y, Lu J. Analysis and classification of kidney stones based on Raman spectroscopy. *Biomed Opt Express*. 2018;9(9):4175. doi:10.1364/boe.9.004175
2. Alelign T, Petros B. Kidney Stone Disease: An Update on Current Concepts. *Adv Urol*. 2018;2018. doi:10.1155/2018/3068365
3. U.S Department of Health and Human Services Litwin MS, Saigal CS E. Urinary tract stones. National Institutes of Health. Published 2012. https://www.niddk.nih.gov/health-information/urologic-diseases/kidney-stones/definition-facts
4. Massey LK, Roman-Smith H, Sutton RAL. Effect of dietary oxalate and calcium on urinary oxalate and risk of formation of calcium oxalate kidney stones. *J Am Diet Assoc*. 1993;93(8):901-906. doi:10.1016/0002-8223(93)91530-4
5. Dawson CH. Kidney stone disease : pathophysiology, investigation and medical treatment. *Clin Med (Northfield Il)*. 2012;12(5):467-471.
6. Werner H, Bapat S, Schobesberger M, Segets D, Schwaminger SP. Calcium Oxalate Crystallization: Influence of pH, Energy Input, and Supersaturation Ratio on the Synthesis of Artificial Kidney Stones. *ACS Omega*. 2021;6(40):26566-26574. doi:10.1021/acsomega.1c03938

7. Sheng X, Ward MD, Wesson JA. Crystal surface adhesion explains the pathological activity of calcium oxalate hydrates in kidney stone formation. *J Am Soc Nephrol*. 2005;16(7):1904-1908. doi:10.1681/ASN.2005040400

8. Fontenelle LF, Sarti TD. Kidney stones: Treatment and prevention. *Am Fam Physician*. 2019;99(8):491-496.

9. Vadim A. Finkielstein DSG. Strategies for preventing calcium oxalate stones. *CMAJ*. 2014;174(10):2-7.

10. Golshan A, Hayatdavoudi P, Hadjzadeh MAR, et al. Kidney stone formation and antioxidant effects of Cynodon dactylon decoction in male Wistar rats. *Avicenna J phytomedicine*. 2017;7(2):180-190. http://www.ncbi.nlm.nih.gov/pubmed/28348973%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5355823

11. Shen S, Wei J, Kang W, Wang T. Elucidating shared biomarkers and pathways in kidney stones and diabetes: insights into novel therapeutic targets and the role of resveratrol. *J Transl Med*. 2023;21(1):1-27. doi:10.1186/s12967-023-04356-4

12. Shen X, Chen Y, Chen Y, Liang H, Li G, Hao Z. Is the METS-IR Index a Potential New Biomarker for Kidney Stone Development? *Front Endocrinol (Lausanne)*. 2022;13(July):1-8. doi:10.3389/fendo.2022.914812

13. LYNDA FRASSETTO and INGRID KOHLSTADT. Treatment and Prevention of Kidney Stones: An Update. *Am Acad Fam Physician*. 2011;84(1):10-27. https://medium.com/@arifwicaksanaa/pengertian-use-case-a7e576e1b6bf

14. Luque de Castro MD, Priego-Capote F. Soxhlet extraction: Past and present panacea. *J Chromatogr A*. 2010;1217(16):2383-2389. doi:10.1016/j.chroma.2009.11.027

15. Oludemi T, Barros L, Prieto MA, Heleno SA, Barreiro MF, Ferreira ICFR. Extraction of triterpenoids and phenolic compounds from: Ganoderma lucidum: Optimization study using the response surface methodology. *Food Funct*. 2018;9(1):209-226. doi:10.1039/c7fo01601h

16. Khandelwal k. R. *Practical Pharmacognosy Techniques and Experiments*. 12th ed. Nirali Prakashan; 2010.

17. WHO. Quality control methods for medicinal plant materials. Published 1998. https://www.who.int/docs/default-source/medicines/norms-and-standards/guidelines/quality-control/quality-control-methods-for-medicinal-plant-materials.pdf?sfvrsn=b451e7c6_0

18. Balekundri A, Mannur V. Quality control of the traditional herbs and herbal products: a review. *Futur J Pharm Sci*. 2020;6(1). doi:10.1186/s43094-020-00091-5

19. Dev SK, Choudhury PK, Srivastava R, Sharma M. Phytochemical Characterization and Antioxidant Assessment of Herbal Extracts. *J Drug Deliv Ther*. 2018;8(4):126-133. doi:10.22270/jddt.v8i4.1736

20. Everett SA, Dennis MF, Patel KB, Maddix S, Kundu SC, Willson RL. Scavenging of nitrogen dioxide, thiyl, and sulfonyl free radicals by the nutritional antioxidant β-carotene. *J Biol Chem*. 1996;271(8):3988-3994. doi:10.1074/jbc.271.8.3988

21. Rajnish Srivastava*, Pratim Kumar Choudhury SKD and VR. Molecular Simulation

Studies of Alpha Pinene, an Alkene in Search for Oxidative Stress Targeted Therapeutic Paradigms for the Treatment of Parkinson's Disease: A Computational Approach and Its In-Vitro Antioxidant Validation. *Bentham Sci*. 2021;18(12-2021):1117-1135.

22. Bawari S, Sah AN, Tewari D. Antiurolithiatic Activity of Daucus carota : An in vitro Study. 2018;10(5):880-884.

23. Rampazzi L, Andreotti A, Bonaduce I, Colombini MP, Colombo C, Toniolo L. Analytical investigation of calcium oxalate films on marble monuments. *Talanta*. 2004;63(4):967-977. doi:10.1016/j.talanta.2004.01.005

24. OECD. Test Guideline 425: Acute Oral Toxicity - Up-and-Down Procedure. *Guidel Test Chem*. 2022;(December):26.

25. Yuan S, Ibrahim IAA RR. Anti-urolithiatic Activity of Daidzin in Ethylene Glycol-Induced Urolithiasis in Rats. *Appl Biochem Biotechnol*. Published online 2023:905-198.

26. Bhakshu LM, Ratnam K V., Yadav PR, Saheb CM, Pandita A, Pandita D. Reishi Mushroom (Ganoderma lucidum). *Mushrooms Nutraceuticals Funct Foods*. Published online 2023:170-190. doi:10.1201/9781003322238-11

27. Fan J, Glass MA, Chandhoke PS. Impact of ammonium chloride administration on a rat ethylene glycol urolithiasis model. *Scanning Microsc*. 1999;13(3):299-306.

28. Mark A. Suckow, Steven H. Weisbroth CLF. *The Laboratory Rat*.; 2005. https://shop.elsevier.com/books/the-laboratory-rat/suckow/978-0-12-074903-4

29. Kocoglu H, Ozturk H, Ozturk H, Yilmaz F, Gulcu N. Effect of dexmedetomidine on ischemia-reperfusion injury in rat kidney: A histopathologic study. *Ren Fail*. 2009;31(1):70-74. doi:10.1080/08860220802546487

30. Arsad SS. Histopathologic Changes in Liver and Kidney Tissues from Male Sprague Dawley Rats Treated with Rhaphidophora Decursiva (Roxb.) Schott Extract. *J Cytol Histol*. 2014;s4(01). doi:10.4172/2157-7099.s4-001