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Antiurolithiatic Activity of *Diplazium Esculentum* Plant Extract: An *In-Vitro* Approach

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KEYWORDS

D. esculentum, Antiurolithic, Aggregation assay, nucleation assay, oxalate depletion assay

ABSTRACT:

Introduction: Urolithiasis, a condition of growing clinical significance, is among the most common diseases encountered in urology clinics. The primary types of uroliths found in humans include oxalate, struvite, urate, brushite, and cystine. With its increasing global prevalence and severity, urolithiasis has become a pressing medical concern. Recent studies have highlighted the effectiveness of medicinal herbs and natural substances in its treatment and management. Diplazium esculentum, an edible fern from the Athyriaceae family, has long been consumed as a vegetable and is valued for its economic, cultural, ecological, and health benefits. However, its potential role in urolithiasis prevention or treatment remains unexplored.

Objectives: The intention of this article is to appraise the antiurolithiatic potential of the plant extract *Diplazium esculentum* through an in-vitro study.

Materials and Methods: The in-vitro antiurolithiatic activity was evaluated using nucleation assay, aggregation assay, and oxalate depletion assay.

Results: The IC50 values of the *Diplazium esculentum* extract were quantified as 67.04 µg/mL, 69.33 µg/mL, and 1161.92 µg/mL in the nucleation assay, aggregation assay, and oxalate depletion assay, respectively. *D. esculentum* demonstrated significant suppression of calcium oxalate (CaOx) crystal nucleation, aggregation, and oxalate depletion.

Conclusion:*D. esculentum* has strong antiurolithiatic activity against calcium oxalate (CaOx) urolithiasis in vitro, likely due to the phytochemicals it contains.

1. Introduction

Urolithiasis refers to the development of kidney stones in the urinary system. These kidney stones are made of crystals like calcium oxalate, Ca3(PO4)2, uric acid, or struvite, along with some proteins. They typically develop due to supersaturation of urine with stone-forming compounds, altered pH, urinary stasis, or the existence of inhibitors/promoters of crystallization. Urolithiasis can develop in the kidneys (nephrolithiasis), ureters (ureterolithiasis), or bladder (cystolithiasis), leading to symptoms such as pain (renal colic), hematuria, and urinary obstruction.[1]. It affects approximately 12% of the population, with recurrence rates of 70–80% in males and 47–60% in females[2].The majority (80%) of urinary calculi are made up of calcium, primarily calcium oxalate, while the remaining 20% consist of other types[3].

Five primary set of urinary stones, with calcium oxalate (CaOx) being the most common, accounting for 80% of cases. Other prevalent types include calcium phosphate (5%) and uric acid stones. Urolithiasis, one of the oldest and most painful urologic conditions, affects approximately 5–7 million people. The word "urolithiasis" derives from Greek concept urone (urination) and lithos (stone). Around 50% of Patients face relapse within five years, and currently, no

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universally accepted treatment can completely prevent recurrence. Nephrolith development is an intricate process involving a chain of physicochemical events, including supersaturation, nucleation, growth, aggregation, and retention within the renal tubules[4].Various advanced investigative techniques, including radiological and laboratory methods,have not fully achieved effectiveness in determining the precise causes and mechanisms of stone formation[5]. Although, the current research has thoroughly explored the potential contributing factors. It is believed that kidney calculi develop when urinary fluid becomes excessively concentrated with insoluble substances due to excessive excretion rates, leading to crystal formation, aggregation, and eventual stone development [6]. Urolithiasis requires both preventive and curative approaches. Currently, modern medicine lacks highly effective drugs capable of dissolving stones, leading physicians to explore alternative medical systems for better treatment options[6]. The primary medical management of urolithiasis involves surgical removal of stones through techniques such as Kidney stone shock wave therapy or (ESWL)Extracorporeal Shock Wave Lithotripsy and percutaneous nephrolithotomy (PCNL). However, these procedures do not prevent recurrence and can lead to adverse effects like hemorrhage, hypertension, tubular necrosis, and subsequent kidney fibrosis.Traditional medicine, particularly herbal remedies, has been widely utilized for treating various diseases, including urinary stones. Notably, herbal treatments are often effective, cause less adverse effects than modern drugs, and may help reduce the recurrence of renal stones. The extensive Ayurvedic literature documents numerous botanical species with Possible advantages for urinary stone treatment, though many remain unexplored for their full pharmacological potential[7]. Diplazium esculentum, an edible fern from the Athyriaceae family, is a forest edible plant has been traditionally used as a vegetable for generations. Beyond its role in cooking, this plant offers significant economic, cultural, ecological, and health benefits[8,9]. It is known by various regional names, including ‘Dhekishak’ in Bengali, ‘Paloi’ in Hindi, ‘Dhekia’ in Assamese, and ‘Okang’ in Manipuri. Among the Tripuri community, It is identified as ‘Sikiomamoidu’ or ‘Maikhando[10,11]. Traditionally, D. esculentum has been used by various communities in India and other Countries use it to manage various medical situation, including diabetes, smallpox, asthma, diarrhea, arthritis, dysentery, migraines, fevers, injuries, pain, measles, high blood pressure, and constipation, oligospermia, bone fractures, glandular swellings, and skin diseases[12,13]. Scientific research has emphasized its wide-ranging biological effects, which are due to its abundant phytochemical composition. Some of its notable pharmacological properties include: Antimicrobial [14,15,16], Antioxidant[17,18,19,20], Antidiabetic[21,18], Anti-inflammatory [22], Hepatoprotective[18,23], Antibacterial[24,28], Anthelmintic[25,26], Neuromodulatory activity [27], Anticancer [29,30]. The growing interest in plant-based medicine as an alternative or complement to conventional healthcare has highlighted its capability as an important supply of new drug discoveries. Accordingly, this research sought to assess the in vitro antiurolithic potential of the Alcohol-based extract from the entire Diplazium esculentum plant.

2. Methods

Collection of the fern and its authentication:

The plant Diplazium esculentum was collected from Manaspara, Lakhipur, Goalpara, Assam. For authentication, the above-ground portions of the plant were dried, mounted on herbarium sheets, and sent to Guwahati University in Assam, India. The plant has been assigned the accession number GUBH20435 and the reference number Herb/GUBH/2023/79.

Preparation of plant extract:

The plants Diplazium esculentum were collected ,washed , dried under shade and powdered by electric grinder into coarse powder[31].

Soxhlet extraction method:

The 50g roughly ground plant material was extracted by using Hot continuous Soxhlet extraction method.The roughly ground was extracted with liquid Petroleum ether of 500ml for 48 hours in Soxhlet apparatus.The product is collected and evaporated by using rotary vaccum evaporator at 40°C.The marc were again extracted with solvent ethanol (95% v/v) of 500ml for 48hours and the product is collected and evaporated by using rotary vaccum evaporator at 40°C. The % yields for the liquid petroleum ether and ethanol-based extracts of Diplazium esculentum were Identified as

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approximately 9.33% and 6.66%, respectively. The extracts were kept in the refrigerator for storage.

Preliminary phytochemical screening:

The extracted samples were analyzed for qualitative analysis to identify various plant constituents. Tests for alkaloids, carbohydrates, phenolic compounds, saponins, tannins, steroids, and others were conducted following standard procedures to ascertain the phytoconstituents[8,32,33,34].

In-vitro Antiurolithic Activity:

Nucleation assay [35,36]:

This experiment was performed using Diplazium esculentum and the reference compound cystone at concentrations of 20, 40, 60, 80, and 100 µg/mL. For each test sample, one mL of 0.025 M $CaCl_2$, 2 mL of 0.05 M Tris-buffer, and 1 mL of plant extract or standard at varying concentrations were initially introduced into a test tube. Subsequently, one mL of 0.025 M $Na_2C_2O_4$ was added at 37°C to assess the percentage of inhibition. The protocol was conducted in triplicate for each sample.The nucleation rate was assessed by evaluating the formation of crystals that attained a critical or optically detectable size in the existence of the extract with those in the control group (without extract). After 30 minutes, the absorbance was measured at 620 nm, and the % of inhibition was computed using the formula:

Percentage Inhibition (%) = Abs of control- Abs of test /Abs of control × 100.

Aggregation assay [37]:

CaOx crystals were generated by combining 1 mL of 0.025 M $CaCl_2$ with 1 mL of 0.025 M $Na_2C_2O_4$, followed by stabilization at 60°C in a temperature-controlled bath for 1 hour. The solutions were subsequently left to cool overnight at 37°C. The crystals formed were centrifuged for 5 min and harvested crystals were evaporated for 5 min at 37°C. The crystals were used at concentration of 0.8 mg/mL, buffered with tris hydrochloride 0.05 mol/L, and sodium chloride 0.15 mol/L at pH 6.5. Experiments were conducted at 37°C with 1 mL of Diplazium esculentum and cystone (standard) at various concentrations (20, 40, 60, 80, and 100 µg/mL). The solution was mixed thoroughly, and the aggregation rate was assessed by measuring the turbidity in the presence of DE or Cystone (reference) against that of the control. The Abs at 620 nm was recorded. The experiment was done in triplicate. The aggregation rate or percentage inhibition rate (Ir) was determined using the formula:

Percentage Inhibition (%) = Abs of control- Abs of test /Abs of control × 100

Oxalate Reduction Assay [38,39,2,40]:

The impact of DE on CaOx crystal development was assessed using the oxalate reduction assay. Various strength of DE (100 µg/ml, 500 µg/ml, and 1000 µg/ml) were dispersed in purified H_2O. A CaOx crystal suspension with a strength of 1.5 mg/ml was prepared in a 50 mM sodium acetate buffer (pH 5.7). A 4 mM $CaCl_2$ solution and a 4 mM $Na_2C_2O_4$ solution (1 ml each) were combined with 1.5 ml of Tris-HCl (10 mM) and NaCl (90 mM) buffer (pH 7.4). Subsequently, 30 µl of the CaOx crystal suspension was added. CaOx crystal growth was then evaluated by monitoring the oxalate reduction rate in the solution at a wavelength of 214 nm for 600 seconds. The impact of each DE concentration on crystal growth was assessed by introducing 1 ml of DE (100 µg/ml, 500 µg/ml, and 1000 µg/ml) into the reaction mixture, followed by measuring changes in optical density. The percentage of crystal growth inhibition was then determined as outlined in the nucleation assay.

Crystal growth Inhibitory (%) = (C – S) /C) × 100

Where, C= free oxalate reduction in the absence of extract, and

S= free oxalate reduction in the presence of an inhibitor (plant extract).

3. Results

Primary Phytochemical Analysis:

The Percentage yield for liquid Petroleum ether and Ethanol extract of Diplaziumesculentum,found to be ~ 9.33% and ~ 6.66%.The phytochemical screening of Diplazium esculentum were enriched with alkaloids,glycosides ,steroid,tannins,flavonoids,Fats& Oils ,proteins & amino acid and carbohydrates. The existence of diverse bioactive compounds in the plant extracts, which could contribute to their therapeutic effects.

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Nucleation Assay:

The percentage inhibition of extract on nucleation of CaOx crystals were found to be 19.53-69.45%, whereas with Cystone (standard) it was 34.41-74.57%. IC50 value of the DE was 67.04 µg/mL, compared with 47.34 µg/mL for Cystone. Inhibition on nucleation of CaOx crystal formation increased with rising extract strength and was comparable to Cystone.

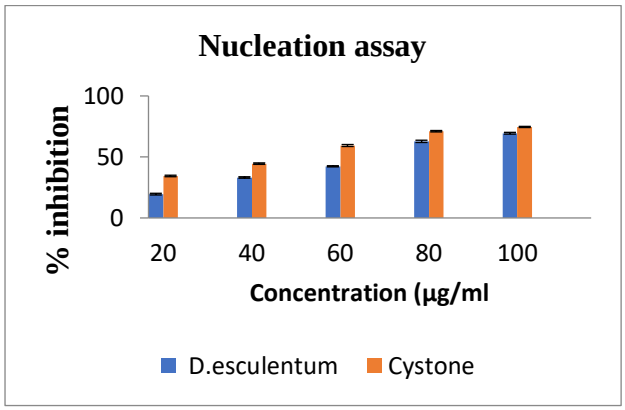

Fig 1: Nucleation assay of D.esculentum and cystone

Aggregation Assay

Figure 2 illustrated that D. esculentum extract exhibited a notable dose-dependent suppression of CaOx crystal aggregation. % inhibition of DE on CaOx aggregation was confirmed as 35.81%–58.91%, whereas with cystone,the most potent, it was 42.79%–70.77%. IC_{50} of D.esculentum was 69.33µg/mL, and for cystone, it was confirmed as 39.12µg/mL. Higher concentrations of DE showed lower turbidity (aggregation). Therefore, this finding suggests that DE contains bioactive compounds that prevent the aggregation of CaOx crystals.

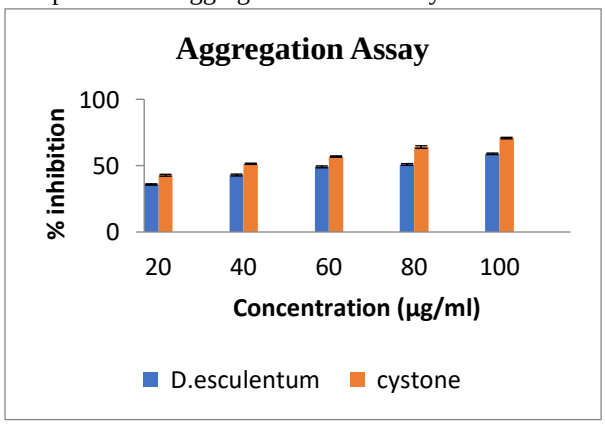

Fig 2: Aggregation Analysis of *D. esculentum* and Cystone

Oxalate depletion assay:

The % decrease in growth with DE was observed at 45.27%, while Cystone demonstrated 48.87% at 1000 µg/ml. The suppression of CaOx crystal growth by DE was markedly less than that of Cystone at lower concentrations but was on par with Cystone at the highest concentration.

lower concentrations but was on par with Cystone at the highest concentration.

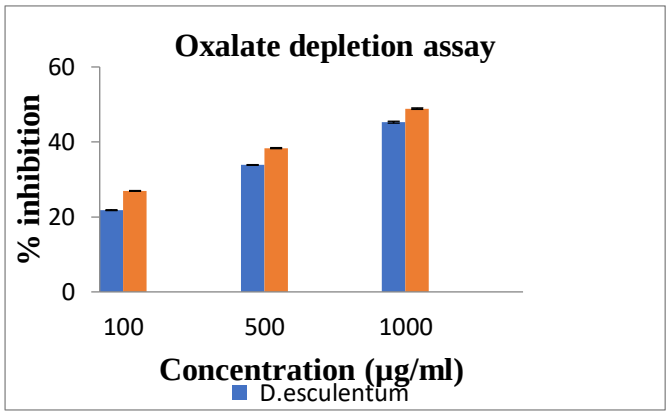

Fig 3: Oxalate Depletion Assay

3. Discussion

The kidneys are among the most vital organs within the human system, carrying out vital roles that sustain life. They continuously filter the blood, regulate fluid balance, and help eliminate waste, toxins, and excess fluids through urine. Additionally, they serve a vital function in red blood cell production, maintaining strong bones, and regulating blood pressure. Therefore, proper kidney function is essential, as its failure can lead to severe, potentially life-threatening consequences[41]. The mineral accumulation in the kidneys or urinary tract is referred to as urolithiasis. The causes of renal calculi is a multi-step process that includes crystal nucleation, growth, and aggregation assay[42]. The in-vitro investigation aimed at analyzing the effectiveness of D. esculentum in preventing CaOx stone formation. Nucleation is an energy-driven process in which liquefied compounds in a oversaturated solution naturally form crystals. [43]. The outcomes of this research demonstrate that the alcohol-based extract of Diplazium esculentum (D. esculentum) effectively

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inhibits the nucleation of calcium oxalate crystallization in a dosage-dependent fashion. The extract inhibited CaOx crystal nucleation by 19.53% to 69.45%, while Cystone, the standard reference, showed an inhibition range of 34.41% to 74.57%. This implies that D. esculentum possesses significant antiurolithiatic potential, although marginally less than Cystone. Furthermore, the IC_{50} value of D. esculentum (67.04 µg/mL) was greater than Cystone (47.34 µg/mL), indicating that a relatively higher strength of the extract is required to achieve 50% inhibition of nucleation, compared with the reference medication. Nevertheless, the growing suppression with rising extract concentration suggests a dose-dependent effect, reinforcing the healing capability of D. esculentum in preventing CaOx stone formation. These results support the utilization of D. esculentum as a naturally derived alternative for managing urolithiasis.

Crystal aggregation is the phenomenon in which multiple crystals in a solution aggregate and bind to form large crystal clusters. Coalescence is a vital factor in crystal retention, as these large agglomerates can obstruct renal tubules as a result, fostering stone formation[44].This study's findings imply that the ethanol extract of Diplazium esculentum (D. esculentum) significantly inhibits the aggregation of calcium oxalate (CaOx) crystals in a dosage-dependent fashion. The extract exhibited an aggregation inhibition of 35.81%–58.91%, whereas Cystone, the standard reference, showed a higher inhibition range of 42.79%–70.77%. This suggests that while D. esculentum is effective in preventing CaOx crystal aggregation, Cystone remains the more potent inhibitor.The IC_{50} value of D. esculentum (69.33 µg/mL) was higher compared to Cystone (39.12 µg/mL), indicating that a greater strength of the extract is required to achieve 50% inhibition of aggregation. However, the observed reduction in turbidity with increasing concentrations of D. esculentum suggests that its phytoconstituents play a role in preventing crystal aggregation.These findings support the potential use of D. esculentum as a natural antiurolithiatic agent, potentially contributing to the prevention of kidney stone formation. Further studies, including in vivo research and phytochemical characterization, are needed to identify the effective constituents accountable for this effect and to fully elucidate the underlying mechanisms. In the oxalate depletion assay, Diplazium esculentum (D. esculentum) exhibited a lower crystal growth inhibition at lower concentrations compared to Cystone, indicating that a higher dose of the extract is required to achieve a similar inhibitory effect. This suggests that the functional constituents found in D. esculentum may share to its antiurolithiatic properties by interfering with crystal growth. The capability of D. esculentum to supress calcium oxalate (CaOx) crystal growth implies its effectiveness in preventing kidney stone formation. While its effect at minimal doses was less potent than Cystone, the dose-dependent trend indicates that increasing extract concentration enhances its inhibitory action. This supports the hypothesis that D. esculentum contains phytoconstituents that might be involved in modulating crystal formation and reducing stone development.

The phytochemical profiling of Diplazium esculentum (D. esculentum) confirmed the occurrence of diverse active constituents, such as alkaloids, glycosides, steroids, tannins, flavonoids, lipids, proteins, amino acids, and carbohydrates.These phytoconstituents are reputed to play a role in the remedial benefits of medicinal plants, suggesting that D. esculentum may possess significant pharmacological activity.

Flavonoids and tannins are widely recognized for their antioxidative and anti-inflammatory effects, which may aid in alleviating oxidative damage—a major contributor to urolithiasis[38]. Alkaloids and glycosides may contribute to diuretic effects, promoting the excretion of urinary crystals and reducing the risk of stone formation. Steroids are associated with anti-inflammatory activity, while proteins, amino acids, and carbohydrates could participate in maintaining cellular functions and metabolic stability. The occurrence of fats and oils could aid in the absorption and bioavailability of these active compounds.

The diverse phytochemical composition of D. esculentum suggests that its antiurolithiatic potential may occur through different pathways, such as antioxidant activity, inhibition of crystal aggregation, and modulation of urinary parameters. However, further quantitative analysis and in-vivo additional exploration is required to establish its efficacy and to identify the particular compounds contributing to its therapeutic effects.

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Conclusion

The discoveries of this investigation suggest that Diplazium esculentum (D. esculentum) possesses significant antiurolithiatic potential by inhibiting key stages of calcium oxalate (CaOx) stone formation, including nucleation, aggregation, and crystal growth. The ethanol extract of D. esculentum demonstrated a dose-dependent inhibitory effect, although its effectiveness was somewhat reduced compared to the standard drug, Cystone.

Phytochemical screening revealed the detection of therapeutic constituents such as flavonoids, tannins, alkaloids, glycosides, steroids, proteins, and carbohydrates, that could be a factor in the extract's therapeutic effects. Flavonoids, in particular, have been linked to CaOx crystal dissolution, further supporting the antiurolithiatic potential of D. esculentum.

Overall, these results support the possibility of D. esculentum as a nature-derived alternative for managing urolithiasis. However, further in vivo studies and detailed phytochemical characterization are necessary to uncover the distinct pharmacologically active components influencing its antiurolithiatic activity and to explore its clinical applications.

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