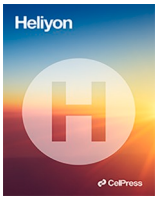
Heliyon 10 (2024) e27330

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Syrupy herbal formulation of green bean pod extract of *Phaseolus vulgaris* L.: Formulation optimization by central composite design, and evaluation for anti-urolithiatic activity

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ARTICLE INFO

Keywords:
Calcium oxalate crystals
Phaseolus vulgaris L.
Anti-urolithiatic activity
Syrupy formulation
Central composite design

ABSTRACT

The green bean pods of *Phaseolus vulgaris* L. are traditionally used as a folk remedy for treating calcium oxalate kidney stones. The current research aimed to develop a syrup formulation containing green bean pod extract for anti-urolithiatic activity. The syrup was prepared using a simple blending method and optimized through a central composite design (CCD) with two independent variables: the ratio of pod juice (PJ) to sugar solution (SS) ranging from 1:0.5 to 1:1.5, and the percentage of CMC from 0.2% to 0.4% w/v. These variables were analyzed for their impact on viscosity (CP) and sedimentation percentage, helping to identify the best formulation out of 13 variants. The finalized formulation (F-opt) underwent assessment for physicochemical characteristics such as organoleptic properties, viscosity, density, sedimentation rate, and stability. Additionally, a microbiological assessment was performed utilizing the spread plate method. Further, it was evaluated for *in vitro*, *ex vivo*, and *in vivo* anti-urolithiatic activity in rat models for 28 days and compared with that of the reference standard (Cystone syrup). Additionally, acute toxicity was assessed in albino Swiss mice. Histopathological evaluations were then conducted on the kidneys of the Wistar rats that had been used for the *in vivo* studies, providing insight into the treatment effects on kidney tissue structure. The optimized formulation (F-opt) was a green, viscous, clear syrup with a pH of 5.8, a viscosity of 256.38 CP, a density of 1.31 g/ml, and a sedimentation rate of 0.69%. The optimized formulation was found to be stable, showing no significant changes in physicochemical and microbiological properties. The results of the *in vitro*, *ex vivo*, and *in vivo* anti-urolithiatic studies indicated that the optimized formulation effectively inhibited the aggregation of calcium oxalate. The acute toxicity studies revealed no mortality or adverse effects for both the optimized formulation and pure bean pod juice at a dose of 2000 mg/kg body weight. Histopathological examination revealed that rats treated with the optimized formulation exhibited a significant reduction in both the number and size of calcium

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https://doi.org/10.1016/j.heliyon.2024.e27330
Received 3 March 2023; Received in revised form 20 January 2024; Accepted 28 February 2024
Available online 29 February 2024

oxalate deposits within various parts of the renal tubules. It can be concluded that the syrupy formulation of *Phaseolus vulgaris* L. green bean pod extract demonstrated significant anti-urolithiatic activity. This activity could be due to its diuretic properties and its ability to inhibit the formation of calcium oxalate crystals. However, limitations of the study included a lack of elucidation of the mechanism and limited generalizability of the findings.

1. Introduction

Urolithiasis, which affects 10–15% of the general population, is a third common metabolic disorder with an elevated risk of renal failure and is thought to be a substantial health concern worldwide [1]. Urolithiasis is a pathological condition of the genitourinary system, which is referred to as calculi (stones) in the urinary tract. Many people are suffering from urinary stones, and it is an important reason for chronic and acute renal failure. Lithiasis, or stone formation, encompasses the development of stones in the kidney as well as in various parts of the urinary system, including the ureter and the bladder. Among these different kinds of stones are calcium oxalate stones, which occur mainly in men, while phosphate stone formation occurs more frequently in women, the pathogenesis of lithiasis seems to be complicated and multifactorial [2]. Urolithiasis is a complex process that results from several physicochemical events including supersaturation, nucleation, growth, aggregation, and retention of urinary stone constituents within tubular epithelial cells [3].

The orientation of calcium and oxalate ions on the surfaces of other crystals, such as uric acid, can contribute to the development of calcium oxalate stones which is the most common type of kidney stone. Supersaturation and heterogeneous nucleation processes specifically contribute to the development of urolithiasis. Surgical procedures, lithotripsy, and laparoscopy are commonly used to remove the calculi [1,4]. However, these procedures carry a risk of causing acute renal injury, which can lead to a decline in renal function. Stone recurrence is frequently observed, with approximate recurrence rates of 10% in the first year, 33% in the fifth year, and 50% in the tenth year. The high cost of the treatment regimen and the complex clinical care of patients emphasize the urgent need for finding appropriate alternative therapies [1].

In current pharmacotherapy, allopathic medicine, commonly referred to as conventional or Western medicine, focuses on diagnosing and treating diseases using pharmaceutical drugs, surgical interventions, and evidence-based medical techniques. It relies on diagnostic tests and emphasizes medical decisions supported by scientific research and clinical trials. There is a growing trend towards the use of alternative medicinal systems, particularly plant extracts or their active ingredients, in the treatment of various diseases. Traditional medicinal plants, which have been used by indigenous or local communities for medicinal purposes, are the subject of intensive research globally. Many plants, such as *Cassia fistula, Argemone maxicana, Aegle marmelose*, and *Phylanthus emblica*, are traditionally used to treat urinary tract infections and kidney stones. They are known to inhibit stone formation, break down calculi, dissolve them, and expel them from the body, providing relief from the associated pain [5].

Many researchers have conducted studies on the anti-urolithiatic effects of various naturally occurring active principles. These include the rhizomes of *Zingiber officinale* (6-Shogaol) [6], the seed extract of *Peganum harmala* [7], the leaf extract of *Duranta erecta* [1], the bark extract of *Cinnamomum zeylanicum* [8,9], the seed extract of *Dolichos biflorus* [10], the fruit extract of *Piper cubeba* [11], the leaf extract of *Bryophyllum pinnatum* [12], and more.

Phaseolus vulgaris L. (Family: Fabaceae), a common green bean, ranks as one of Africa's most important and popular legumes [13]. Raw green beans, also known as string beans, have enclosing pods before the bean seeds inside fully mature. They are commonly sold fresh, canned, and frozen in many countries and can be consumed raw, steamed, boiled, stir-fried, or baked [14]. Phytochemical constituents such as flavonoids, phenolic acids, tannins, and saponins are present in green beans. This versatile plant is utilized in folk medicine to address a range of conditions, including eczema, acne, diarrhea, diuretic properties, dysentery, rheumatism, bladder issues, burns, diabetes, kidney stones, carminative traits, and burns [15,16].

Traditionally, green beans are used for combating many diseases/conditions, such as obesity, diabetes, high cholesterol, UTI infections, lung cancer, and kidney stones [17–20]. Further, it also exhibited antioxidant and anti-inflammatory activity [16,21]. The rate of effectiveness for some of the above-said conditions like kidney stones requires scientific evidence. To date, there is no evidence in modern therapy supporting the anti-urolithiatic activity of any of the reported green bean or bean pod formulations. The absence of evidence regarding the effectiveness of green bean pods in modern therapy for managing renal stone necessitates the development of a bean pod formulation that retains its desired active constituents. Based on the aforementioned facts, the primary aim of this research was to develop and assess a syrup formulation derived from green bean pods for its anti-urolithiatic properties. The syrup formulation was prepared through a simple blending method and optimized using central composite design (CCD), considering key factors such as pod juice to sugar solution ratio (1:0.5–1:1.5) and a percentage of CMC (0.2–0.4% w/v). The effects of these factors on viscosity and sedimentation were observed to choose the optimized formulation from 13 formulations. The optimized formulation (F-opt) was evaluated for organoleptic properties, density, stability, and microbiological study, along with a stability assessment following ICH guidelines. This formulation was further tested for *in-vitro* and *ex-vivo* anti-urolithiatic activities, acute toxicity in albino mice, *in-vivo* anti-urolithiatic efficacy using an ethylene glycol-induced urolithiatic rats' model, and compared with the anti-urolithiatic activity of the reference standard (commercially available Cystone syrup). Finally, histopathological evaluation of rat kidneys was conducted.

2. Materials and methods

2.1. Materials

Green beans were sourced from the local commercial market in Asella, Ethiopia. Certain materials, including sucrose and glycerol from Merck Sigma Aldrich (USA), methylparaben and propylparaben from SRL Pvt Ltd (India), calcium chloride and sodium oxalate from Qualigens, Thermo Fischer Scientific Pvt Ltd (India), Tris from Avantor Pvt Ltd (India), sodium chloride and carboxy methyl cellulose from Qualigens, GSK Pvt Ltd (India), Nutrient agar, Sabouraud dextrose agar, and Ringer's solution from HiMedia Laboratories (India), as well as polyethylene glycol, citric acid, Tween 80, acetone, and ethanol from Sigma-Aldrich (St. Louis, MO, USA) were utilized. Eosin and biochemical estimation kits were obtained from ERBA (USA), while ELISA kits were sourced from BioTek (USA). Edible color and flavor were procured locally from bakers in Addis Ababa, Ethiopia. The study employed Cystone syrup from Himalaya Drug Company Pvt Ltd (India) as a reference standard. Purified water was used during the syrup preparation.

2.2. Methods

2.2.1. Procurement and separation of pods from beans of Phaseolus vulgaris L

Fresh green beans were obtained from the commercial vegetable market in Asella, Oromia region, Ethiopia, and cleaned thoroughly with running water. The seeds were then removed to extract the pods. These fresh, undried pods were specifically used to preserve the vital phytochemicals for the study.

2.2.2. Extraction of juice from fresh pods and determination of its yield (Y)

The juice of fresh *Phaseolus vulgaris* L. pods was extracted using a modified version of the method by Jayamanohar et al. [22]. One kilogram of fresh pods was ground in a mixer with an appropriate amount of purified water (100 ml). The resulting extract was then filtered through a muslin cloth to obtain the filtrate [23,24]. The yield of the juice was determined using equation (1), and the filtered extract was subsequently utilized for the study.

$$Y = \frac{Wj - Ww}{Wp} x100 \ldots\ldots\ldots \quad (1)$$

Where, Y= Yield of Juice, Wj = Wt of juice, Ww = Wt of water added, Wp = Weight of Pods.

2.2.3. Preparation of syrup containing extract of pods of Phaseolus vulgaris L

The extract was mixed with a simple sugar solution (66.67% w/v), followed by the addition of the required quantity of CMC and agitation for 15 min. After this, the necessary amount of glycerol (1% w/w) and citric acid (1% w/w) was added, along with an ethanolic solution of preservatives (0.15% w/v, methylparaben + propylparaben, in an 8:1 ratio). The solution was thoroughly mixed to achieve the desired viscosity. Finally, vanilla flavor was added before the mixture was filtered through a 200-mesh muslin cloth and preserved in an amber-colored glass bottle [23,25,26].

2.2.4. Optimization by central composite design (CCD)

To optimize the syrupy herbal formulation, CCD by Design-Expert® software (version 8.0.7.1, Stat-Ease, USA) was employed [27]. The syrup was prepared using a simple mixing procedure without heating. Initially, a preliminary screening was conducted to identify the key independent factors that influence the formulation parameters significantly. Factors such as pod juice (PJ), syrup solution concentration, glycerol percentage, citric acid, mixing time (minutes), CMC percentage, and pod juice to syrup solution ratio were tested for their impact on various parameters including viscosity, density, taste, texture, sedimentation percentage, turbidity, and pH. Following the preliminary study and referenced literature [28–30], we selected two independent factors—pod juice (PJ) to sugar solution ratio (SS) (1:0.5–1:1.5) and percentage of CMC (0.2–0.4% w/v)—with viscosity (CP) and sedimentation (%) as dependent parameters for optimization through CCD (Table 1). Furthermore, several parameters such as preservative (0.3% w/v, methylparaben and propylparaben, 8:1), ethanol (2.5% w/v), glycerol (1% w/v), citric acid (1%), mixing time (15 min), and vanilla flavor (0.04% w/v) were held constant [31]. The number of experiments required for the CCD was calculated using the following equation [32,33].

$$2^k + 2k + n \ldots \ldots \ldots \ldots \quad (2)$$

Table 1
Factors and their Levels Investigated for Central Composite Design.

Independent factors				Dependent factors	
Name	Levels			Name	Goals
	−1	0	+1	Viscosity (CP)	The formulation should be free-flowing
PJ: SS (ratio)	1:0.5	1:1	1:1.5	Sedimentation (%)	Minimum
CMC (% w/v)	0.2	0.3	0.4		

PJ= Pod Juice, SS= Sugar Solution, CMC= Carboxy Methyl Cellulose.

Where 'n' represents the number of experiments performed at the center points (n = 5), and 'k' is the number of factors (k = 2) to assess experimental repeatability. The primary factorial design included 2^k experiments in the first set, and in the second set, 2k experiments were conducted at the star points—a distance [$\pm\alpha$ ($2^{k/4}$) away from the center]. With $a = \pm 1.41$, the aforementioned CCD model was designed to be rotatable. To minimize experimental bias, a total of 13 experiments were randomly conducted, including five center points.

2.2.4.1. Response surface modeling. To ensure the best-fit model, the responses of each run were fitted to multiple models, including linear, 2F1, and quadratic models. Conclusions were drawn using the resultant polynomial equation (3) after evaluating the magnitude and numerical sign (positive or negative) of the coefficients. Findings from the polynomial regression were presented using 3-D and contour plots. The selection of optimum formulations was primarily based on achieving the ideal viscosity (free-flowing) and the lowest possible sedimentation percentage [34].

$$\text{Response (R)} = \beta0 + \beta1A + \beta2B + \beta12\ AB + \beta11A^2 + \beta22B^2 \ldots\ldots\ldots \quad (3)$$

Where R was the expected response, β0 represented the intercept, β1 and β2 were linear coefficients, β11 and β22 were squared coefficients for quadratic terms, β12 and β13 were interaction coefficients, and A and B were the independent variables selected based on the results of a preliminary study and previous findings. To evaluate the best-fit model, predicted R2 and adjusted R2 values were compared.

2.2.4.2. Selection of optimized formulation and validation of response surface methodology (RSM). The study utilized a point prediction optimization approach, employing the desirability function to select the optimized syrupy formulation based on the desired objectives outlined in Table 1 [35]. A desirability value reaching one indicates the obtained response's proximity to its intended target value (predicted response). The optimized formulation was then developed to verify and validate the RSM. The prediction error was calculated using the following equation [36].

$$\text{Prediction error } (\%) = \frac{\text{Predicted value} - \text{Observed value}}{\text{Observed value}} \text{x}100 \quad (4)$$

In the desirability approach, the desirability function was calculated, and the best formulation—having a desirability value close to one and meeting the maximum response requirements—was selected.

2.3. In-vitro evaluation of optimized syrup formulation (F-opt)

2.3.1. Observation of organoleptic properties

The syrup was tested for color, odor, taste, and appearance.

2.3.2. Crystallization evaluation

As the syrup contains sucrose, it was subjected to a crystallization test by refrigerating it at 2–8 °C for a week. Subsequently, the syrup was evaluated for the presence of any crystallization or precipitation [28,37].

2.3.3. Determination of viscosity

The viscosity of the developed syrup was assessed at room temperature using spindle No. 5 and at 60 rpm with a Brookfield viscometer [28,30].

2.3.4. Determination of density

The density (g/mL) of the syrup was determined using a 10 ml measurement-capacity pycnometer [38].

2.3.5. Determination of sedimentation rate

The syrup (100 g) was centrifuged at 5000 rpm using a centrifuge (Remi, India) to separate the sediment [25]. Subsequently, the sediment was dried in a oven (Remi, India) at approximately 80 °C. After thorough drying, the residue was weighed, and the percent sedimentation was calculated using the following formula:

$$\text{Sedimentation percentage} = \frac{\text{Weight of sediment (g)}}{\text{Weight of syrup (g)}} \text{x}100\ldots\ldots\ldots \quad (5)$$

pH parameters of the optimized formulation were also measured using a pH meter.

2.4. Microbiological quality studies of syrupy pod formulation during storage

Microbial contamination was assessed by applying the formulation on nutrient agar and Sabouraud dextrose agar, followed by incubation for 24–48 h at 37 °C for bacteria and 5 days at 28–30 °C for molds (fungi). Standardized methods were used for purifying and identifying isolates down to the species level.

To determine the level of contamination, 1 ml of the formulation was mixed with 4 ml of sterile Ringer solution containing 0.25%

tween 80. Appropriate dilutions were made in the same dispersing vehicle and 0.5 ml was plated out on the appropriate solid medium using the spread plate method (MacConkey agar is for Gram-negative bacteria- *E.Coli* and mannitol salt agar is selective for Gram-positive bacteria-*Staphylococcus aureus* and xylulose-lysine-deoxycholate agar medium-*S. typhi*) [39]. Emergent colonies were counted after the necessary incubation using a colony counter machine (Fisher Scientific, Mumbai, India). Culture media without samples were kept at the same test conditions during each step of the analysis.

2.5. Stability study

In compliance with ICH Q1C guidelines from the European Medicines Agency [40], a stability study was carried out. The optimized syrupy formulation (F-opt) was packaged in an amber glass vial and placed in a stability chamber (Hicon Instruments, New Delhi, India) maintained at 40 ± 2 °C/75 ± 5% humidity. The sample was withdrawn at specified intervals (0, 1, 3, and 6 months) for evaluation of physical appearance, color, odor, pH, viscosity, and subsequent microbiological analysis.

2.6. Anti-urolithiatic activity

2.6.1. In-vitro anti-urolithiatic activity (synthetic urine – in-vitro aggregation assay)

The ability of the optimized formulation (F-opt) to inhibit oxalate crystal aggregation was assessed and compared to that of the pure bean pod juice extract (which acts as the pure active pharmaceutical ingredient) and the reference standard (Cystone syrup). In a buffer comprised of 0.05 mol/L Tris and 0.15 mol/L NaCl at pH 6.5, solutions of calcium chloride ($CaCl_2$) and sodium oxalate ($Na_2C_2O_4$) were prepared at final concentrations of 6.0 mmol/L and 6.5 mmol/L, respectively. To initiate crystallization, 950 μL of calcium chloride solution was mixed with 100 μL of either the optimized formulation, pure bean pod juice extract, or reference standard at various concentrations (200, 400, and 600 μg/mL), followed by the addition of 950 μL of sodium oxalate solution. For the control experiment, 100 μL of buffer instead was added to the calcium chloride solution. Incubation was conducted for 1 h at a constant temperature of 37 °C. The optical density (OD) of the resulting suspension was measured at 620 nm with a colorimeter (Fisher Scientific, Mumbai, India). The percentage of inhibition was calculated by comparing the turbidity seen with the formulation, juice extract, and commercial herbal formulation against the control, using the following formula [41]:

$$\text{Inhibition } \% = [1\text{-}(OD_{\text{sample}} / OD_{\text{control}})] \text{ x } 100 \ldots \ldots \ldots \quad (6)$$

The expected growth of crystals is explained by the chemical reaction below.

$$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$$

2.6.2. Ex-vivo anti-urolithiatic activity by ex-vivo turbidity assay method using rat plasma

The capability of the optimized formulation (F-opt) to inhibit the formation of oxalate crystals was evaluated using rat blood plasma, which provides a biological environment for the assay. Plasma samples were diluted with equal volumes of 12 mmol/L solutions of calcium chloride and sodium oxalate, respectively. For the nucleation assay, separate plasma solutions containing either sodium oxalate or calcium chloride were prepared. To initiate crystallization, 950 μL of the plasma solution containing 6.0 mmol/L sodium oxalate was mixed with 100 μL of either the optimized formulation or varying concentrations (200 and 400 μg/mL) of the commercial herbal formulations. Then, 950 μL of plasma containing 6.0 mmol/L calcium chloride was added. The solutions were incubated for 1 h at 37 °C. Post-incubation, the optical density (OD) of the suspension was measured at 620 nm, and the potential for crystal growth inhibition was determined by comparison with a control, consisting of 100 μL of buffer. The percentage of inhibition of aggregation was calculated through a comparison of the turbidity seen with the formulation, juice extract, and commercial herbal formulation against the control, employing the same formula used in the in-vitro anti-urolithiatic study [41].

2.6.3. In-vivo animal studies

In the present study, Swiss Albino female mice weighing between 25 and 30 g and Albino Wister Male Rats weighing between 350 and 400 g were used for acute toxicity and anti-urolithiatic activity, respectively. The animals were maintained under the standard light/dark cycle at room temperature with free access to the standard diet and water *ad libitum*. The protocol of the study has been approved by IAEC (Ref: HSKCP/IAEC-2/E, dated: September 07, 2020), for all the animal experimental studies.

2.6.3.1. Acute toxicity studies. The acute toxicity study was conducted in accordance with OECD-421 guidelines using albino mice [42]. Female albino Swiss mice, after an overnight fast, were used for the toxicity study. The animals were divided into two groups: one received the optimized formulation, and the other received pure bean pod juice extract, with six animals in each group. The optimized formulation and pure bean pod juice extract were diluted with water and administered orally to the respective group at a dose of 2000 mg/kg body weight (3.04 ml of optimized formulation and 1.52 ml of pure bean juice extract per kg body weight, respectively).

After dosing, animals were individually examined at least once during the first 30 min (and then periodically during the first 4 h), followed by examinations every 24 h and then daily for up to three days. The animals were assessed for changes in skin, fur, eyes, respiratory rate, mucous membrane (nasal), circulatory system (heart rate and blood pressure), autonomic functions (salivation, perspiration, lacrimation, piloerection, urinary incontinence, and defecation), and central nervous system function (ptosis, drowsiness,

gait, tremors, and convulsions).

2.6.3.2. Evaluation of in-vivo anti-urolithiatic activity in ethylene glycol-induced urolithiatic rats. The Hodgkinson et al. protocol was followed to induce lithiasis in rats [7,43]. In this model, animals were randomly divided into 8 groups, each with six animals (Table 2). Group I served as the normal control, while groups II to VIII were administered ethylene glycol (EG) (0.75%) to induce renal calculi. Group II served as the lithiatic control, receiving EG in drinking water. Group III was treated with a reference standard (750 mg/kg b.w) from the 1st to the 28th day, serving as a reference standard for the preventive regimen to assess the ability to prevent renal stone formation. Groups IV and V were treated with an optimized bean pod syrupy formulation at doses of 500 and 750 mg/kg b.w from the 1st to the 28th day, also as part of the preventive regimen. For the curative regimen to assess the curing ability, separate groups (VI-VIII) were set up. Group VI was treated with the reference standard at 750 mg/kg b.w. from the 15th to the 28th day, serving as the reference standard for the curative regimen. Groups VII and VIII were treated with the optimized bean pod syrupy formulation at doses of 500 and 750 mg/kg b.w from the 15th to the 28th day. The group separation and their respective treatments are shown in Table 2.

On the 28th day, immediately after the administration of the assigned doses, the rats from each group were individually placed in metabolic cages for 24 h for urine collection, while being provided with water and food. Parameters studied included.

a) **Morphological parameters:** Urine volume was measured, and urine pH was checked using narrow range pH paper [44]**.** Kidney weight was also measured after sacrificing the animals.
b) **Biochemical parameters - Serum analysis:** Blood samples were collected (retro-orbital bleeding) from the animals under anesthesia before being sacrificed. The collected blood samples were centrifuged to obtain serum for the analysis of blood urea nitrogen (BUN), creatinine, and uric acid.
c) **Urine parameters:** Urine was analyzed for Calcium, oxalate, and phosphorus.

2.6.3.3. Histopathological studies. Following the sacrifice of the animals on the 29th day, their abdomens were opened to dissect their kidneys [7,41]. The isolated kidneys were thoroughly cleansed before being placed in 10% neutralized formalin. Subsequently, they were processed through a series of progressively stronger alcohol and xylene solutions, and then embedded in paraffin wax. Using a microtome, histological sections, approximately 5 μm thick, were prepared, and stained with hematoxylin-eosin (H&E) dye. These sections were then observed under a light microscope at 10x magnification. Histological examinations were conducted to determine whether changes in the kidneys had occurred, with observations including hemorrhages, congestion, focal tubular swelling, vacuolar changes in the cytoplasm, changes in tubular epithelium, and interstitial fibrosis.

2.7. Statistical analysis

All data were expressed as mean ± standard error mean (SEM). Statistical analyses were conducted using GraphPad® software (version 5, GraphPad, San Diego, CA, USA). Analysis of Variance (ANOVA) followed by Dunnett's Multiple Comparisons Test was performed. A *p-value* less than 0.05 was considered statistically significant.

3. Results

3.1. Extraction of juice and determination of juice yield (Y)

The percentage yield of juice was determined to be 66.37 ± 0.93%. Fresh juice, without the use of any organic solvent or heating, was employed for the preparation of the syrup. In a similar manner, Jayamanohar and colleagues extracted water-extractable polysaccharides from kidney beans and assessed their prebiotic potential [22].

3.2. Preparation and optimization of syrupy formulation

The syrup was prepared through simple mixing without heating. In light of the preliminary study results, two independent factors,

Table 2
Group's separation and their respective treatments.

Preventive Regimen		
Group I	:	Normal Control
Group II	:	EG (0.75%) in drinking water (1st to 28th day)
Group III	:	EG (0.75%) + Reference Standard (750 mg/kg b.w., p.o. 1st to 28th day)
Group IV	:	EG (0.75%) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 1st to 28th day)
Group V	:	EG (0.75%) + Optimized Syrupy Formulation (750 mg/kg b.w., p.o. 1st to 28th day)
Curative Regimen		
Group VI	:	EG (0.75%) (1st to 28th day) + Reference Standard (750 mg/kg b.w., p.o. 15th to 28th day)
Group VII	:	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 15th to 28th day)
Group VIII	:	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (mg/kg b.w., p.o. 15th to 28th day)

[a]EG-ethylene glycol, b.w-body weight, p.o.-per os, in latin (orally).

namely pod juice (PJ) to sugar solution ratio (SS) (ranging from 1:0.5 to 1:1.5), and the percentage of CMC (0.2–0.4% w/v), were chosen, while dependent parameters such as syrup viscosity (CP) and sedimentation (%) were considered. The optimization of the syrupy formulation was conducted using response surface methodology via a central composite design (CCD) generated using Design-Expert® software [23,45].

3.3. CCD for the optimization of syrupy formulation

A total of 13 formulation compositions were obtained from CDD. All formulations were prepared and analyzed for responses, namely viscosity (R1) and sedimentation percentage (R2). Table 3 displays the viscosity and sedimentation percentages of all formulations, ranging from 478.48 to 205.36 CP and 2.12%–0.37%, respectively. As shown in Table 4, the best-fit polynomial model for viscosity (R1) and sedimentation percentage (R2) was found to be quadratic (*p-value* < 0.0001). The difference between the adjusted R2 and predicted R2 values for viscosity and sedimentation percentage was less than 0.2, indicating reasonable agreement. A low coefficient of variance (% CV) for both cases (3.01 and 7.69 for viscosity and sedimentation, respectively) indicated that the model was significantly fitted with adequate signal. The model F-values of 199.52 and 160.42 at a *p-value* < 0.0001 for viscosity and sedimentation percentage, respectively, indicated that the quadratic model in both cases was significant and best fitted. The lack of fit was found to be non-significant, with F-values of 6.27 at a *p-value* of 0.0542 for viscosity and 2.48 at a *p-value* of 0.2004 for sedimentation percentage (Table 5). Similar results were reported by Mohanty and co-workers during the optimization of atomoxetine NLCs for nose-to-brain delivery [46].

3.3.1. Effect of independent variables on viscosity

The viscosity of all formulations ranged from 478.48 to 205.36 CP. Viscosity plays a crucial role in liquid formulations, with higher viscosity often leading to enhanced stability by potentially reducing the required water content for microbial growth, thus constraining microbial proliferation.

Table 3 exhibited both the observed and predicted viscosity values. he polynomial equation provided below illustrates the individual, interaction, and quadratic effects of the formulation factors on viscosity.

$$\text{Viscosity (R1)} = +281.35 + 15.21A + 96.96B + 7.04AB + 7.71A^2 + 34.21B^2 \ldots \ldots \ldots \quad (7)$$

In this case, the quadratic model emerged as the most effective, as indicated in Table 4. To confirm the model's effectiveness, multiple correlation tests (R2) and ANOVA (as shown in Table 5) were conducted. With an R2 value of 0.9930 and a *p-value* of 0.0001, Table 4 solidified the significance of independent variables in response prediction. The model's F-value of 199.52, at a *p-value* of <0.0001, underscored its significance. The model terms A, B, and B^2 were found to be particularly significant. Conversely, the lack of fit demonstrated an F-value of 6.27 and a *p-value* of 0.0542, illustrating the insignificance of these terms. Furthermore, the adjusted and predicted R^2 values, 0.9881 and 0.9572, respectively, indicated a reasonable agreement, affirming the model's fitness. The 3D and contour plots in Fig. 1 capture the impact of different factors on viscosity. In Fig. 1C and Table 3, the experimental (actual) viscosity values closely mirrored those predicted by the Design-Expert software, highlighting the accuracy of the predictions. Similar results were also reported by Kazemalilou and colleagues [25].

3.3.2. Effect of independent variables on sedimentation percentage

The sedimentation should ideally be as low as possible to ensure dose uniformity and promote better stability. The polynomial equation provided below illustrates the individual, interaction, and quadratic effects of formulation factors on sedimentation percentage.

Table 3
Compositions of various batches of syrup containing juice of pods of *Phaseolus vulgaris* L based on independent variables using CCD.

Run (FC)	Standard order	Factor A (PJ: SS ratio)	Factor B (CMC %)	Experiment type	Response R1 (Viscosity, CP)		Response R2 (Sedimentation %)	
					Actual	Predicted	Actual	Predicted
F1	4	1:1.5	0.50	2^2 factorial design	450.41	442.48	0.43	0.37
F2	2	1:1.5	0.20		241.63	234.49	1.55	1.48
F3	1	1:0.5	0.20		225.91	218.15	1.88	1.84
F4	3	1:0.5	0.50		406.52	397.97	0.46	0.43
F5	8	1:1.0	0.56	Stars or axial points	478.48	486.88	0.37	0.41
F6	5	1:0.29	0.35		266.98	275.26	0.88	0.91
F7	7	1:1.0	0.14		205.36	212.65	2.12	2.18
F8	6	1:1.71	0.35		310.88	318.29	0.55	0.62
F9	10	1:1.0	0.35	Center points (n = 5)	278.75	281.35	0.68	0.70
F10	13	1:1.0	0.35		287.13	281.35	0.73	0.70
F11	11	1:1.0	0.35		275.52	281.35	0.69	0.70
F12	12	1:1.0	0.35		278.91	281.35	0.71	0.70
F13	9	1:1.0	0.35		285.06	281.35	0.78	0.70

Run and standard order was obtained from the design expert software, FC= Formulation code.

Table 4
Statistics summary of regression analysis results for responses R1, and R2.

Model	R^2	Adjusted R^2	Predicted R^2	SD	% CV	Remark
Response (R1)						
Linear	0.8953	0.8743	0.8101	30.02		
2FI	0.8976	0.8634	0.7249	31.30		
Quadratic	0.9930	0.9881	0.9572	09.26	3.01	Suggested
Response (R2)						
Linear	0.8277	0.7932	0.6774	0.26		
2FI	0.8335	0.7779	0.5808	0.27		
Quadratic	0.9913	0.9852	0.9553	0.069	7.69	Suggested

SD= Standard deviation, CV= Coefficient of variance.

Table 5
ANOVA Table for the Selected Model of Viscosity (R1) and Sedimentation percentage (R2).

Result of ANOVA	Viscosity (CP) (R1)	Sedimentation (%) (R2)
Regression		
Sum of Squares	85467.04	3.87
Degree of freedom	5	5
Mean squares	17093.41	0.77
F-value	199.52	160.42
P-value	<0.0001	<0.0001
Inference	Significant	Significant
Lack of fit test		
Sum of Squares	494.51	0.022
Degree of freedom	3	3
Mean squares	164.84	7.318e-003
F-value	6.27	2.48
P-value	0.0542	0.2004
Inference	Non-Significant	Non-Significant
Residual		
Sum of Squares	599.71	0.034
Degree of freedom	7	7
Mean squares	85.76	4.882e-003

$$\text{Sedimentation \% (R2)} = +0.70–0.10A - 0.63B + 0.075AB + 0.033A2 + 0.30B2 \ldots \ldots \ldots \quad (8)$$

In Table 3, both the observed and predicted values of sedimentation percentage were presented. To confirm the model's effectiveness, multiple correlation tests (R2) and ANOVA (Table 5) were performed. Notably, Table 4 revealed an R^2 value of 0.9913 and a *p-value* of 0.0001, affirming the significance of independent variables in response prediction**.** The model's F-value of 160.42, at a *p-value* of <0.0001, indicated its significance, with terms A, B, and B2 being particularly noteworthy. Conversely, the lack of fit demonstrated an F-value of 2.48 and a *p-value* of 0.2004, highlighting the insignificance of these terms. Furthermore, the adjusted and predicted R^2 values, 0.9852 and 0.9553, respectively, indicated reasonable agreement, confirming the model's fitness. The 3D and contour plots provided in Fig. 2A and B effectively illustrate the impact of different factors on sedimentation. The experimental (actual) sedimentation values closely mirrored those predicted by the Design-Expert software as depicted in Fig. 2C and Table 3. These results are consistent with previous findings [25]. The sedimentation percentage of all formulations ranged from 0.37% to 2.12%.

3.4. Selection of optimized formulation and validation of RSM

A point prediction approach was used to identify the optimized syrupy formulation [35]. F9 formulation was selected from various compositions obtained from the software and was further modified using point prediction to achieve the optimized formulation. Based on the predicted viscosity (251.94 CP) and sedimentation value (0.7%), a batch with a 1:1 (PJ: SS ratio) and 0.30% CMC was chosen as the optimized formulation (F-opt) (Table 6). The formulation was developed using the selected composition, and the experimental values of viscosity and sedimentation were compared with the predicted values to determine the prediction error, as depicted in Table 6. The prediction error for RSM validation was found to be −1.73% and −1.45% for viscosity and sedimentation value, respectively, affirming the validity of the obtained responses.

For the optimized formulation, the desirability function was noted as 0.98, signifying the robustness of the optimized formulation (Table 6). Upon analyzing the results, it was evident that all the obtained values aligned closely with the predicted values, highlighting the potential of CCD in combination with the desirability function as a promising approach for optimizing the syrupy herbal formulation of *Phaseolus vulgaris* L. Similar findings were reported by Zafar and colleagues using RSM in the development of brain-targeted rotigotine surface-engineered NLCs [35].

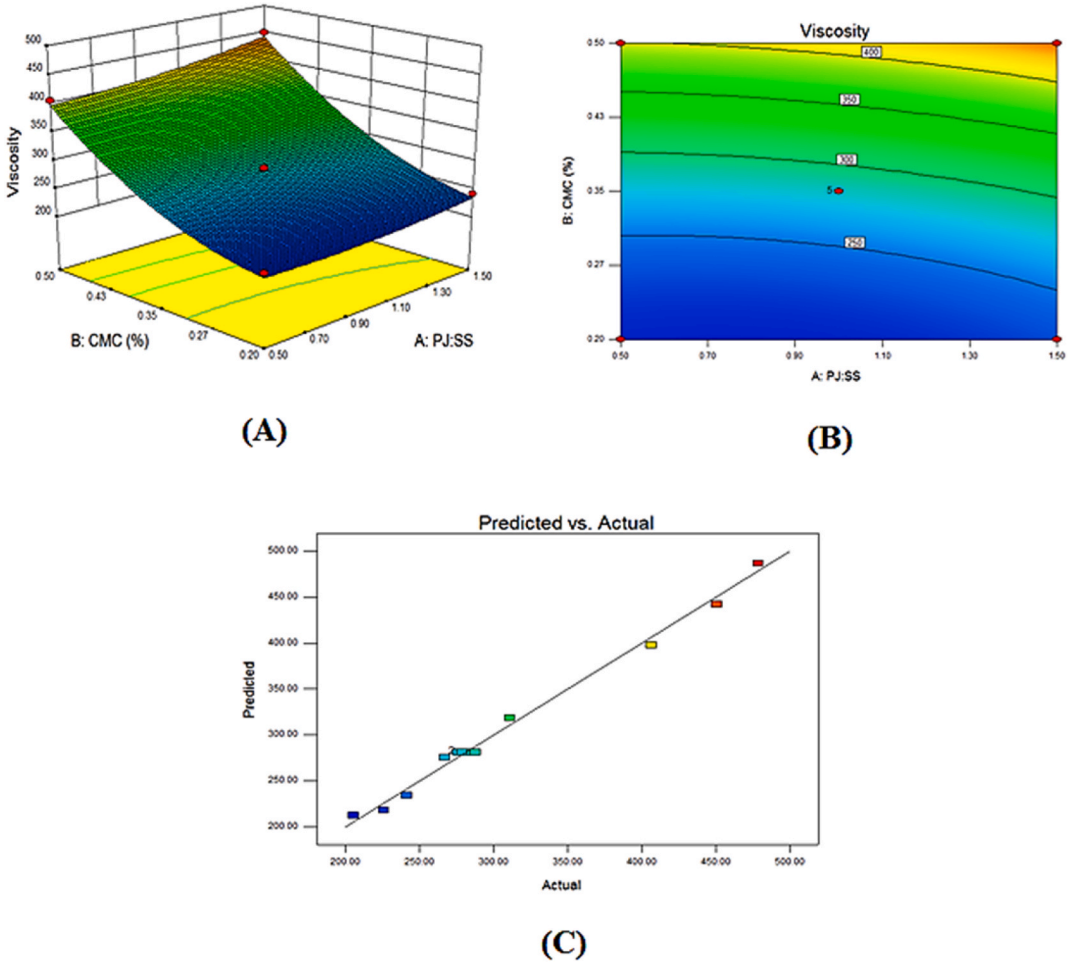

Fig. 1. (A) 3D response surface and (B) contour plots obtained from the design of expert software showing the effect of independent variables on viscosity and (C) The graph between actual and predicted values of viscosity.

3.4.1. Formula of optimized formulation

The complete formula for the optimized syrupy formulation (F-opt) included a 1:1 ratio of bean pod juice to sugar solution and 0.30% CMC, with additional components such as 2.50% w/v ethanol, 0.3% w/v preservative (methylparaben and propylparaben in an 8:1 ratio), 1% w/v glycerol, 1% citric acid, a mixing time of 15 min, and 0.04% w/v vanilla flavor. The optimized syrup was determined to contain 0.5 ml of fresh bean pod juice per milliliter, equivalent to 0.655 g of bean pod extract.

3.5. In-vitro evaluation of optimized syrupy formulation

Optimized formulation (F-opt) was subjected to various *in-vitro* evaluation parameters such as.

3.5.1. Observation of organoleptic properties

The syrupy herbal formulation exhibited a green color, presenting a clear, viscous solution with a pleasant vanilla odor and sweet taste. The pH of the formulation was found to be 5.8. In general, an acidic formulation may not represent the most ideal pH for dissolving kidney stones. However, our mildly acidic formulation, with a pH of 5.8, is within the expected pH range. The formation of kidney stones can be associated with both more acidic urinary pH as well as a more alkaline pH (for different types of stones). Our study indicates that our formulation's mildly acidic nature at a pH of 5.8 indeed prevented crystal aggregation and could potentially have promoted stone dissolution (breakdown). Furthermore, it is essential to note that the pH of a formulation has the potential to change *in vivo*. Several factors can influence the pH of a formulation once it is administered in the body, including its interaction with bodily fluids, the presence of enzymes or other substances that can alter pH, as well as the overall physiological environment [47,48].

3.5.2. Crystallization evaluation

The optimized syrup (F-opt) was subjected to a crystallization study under cool conditions at a temperature of 2–8 °C for a week.

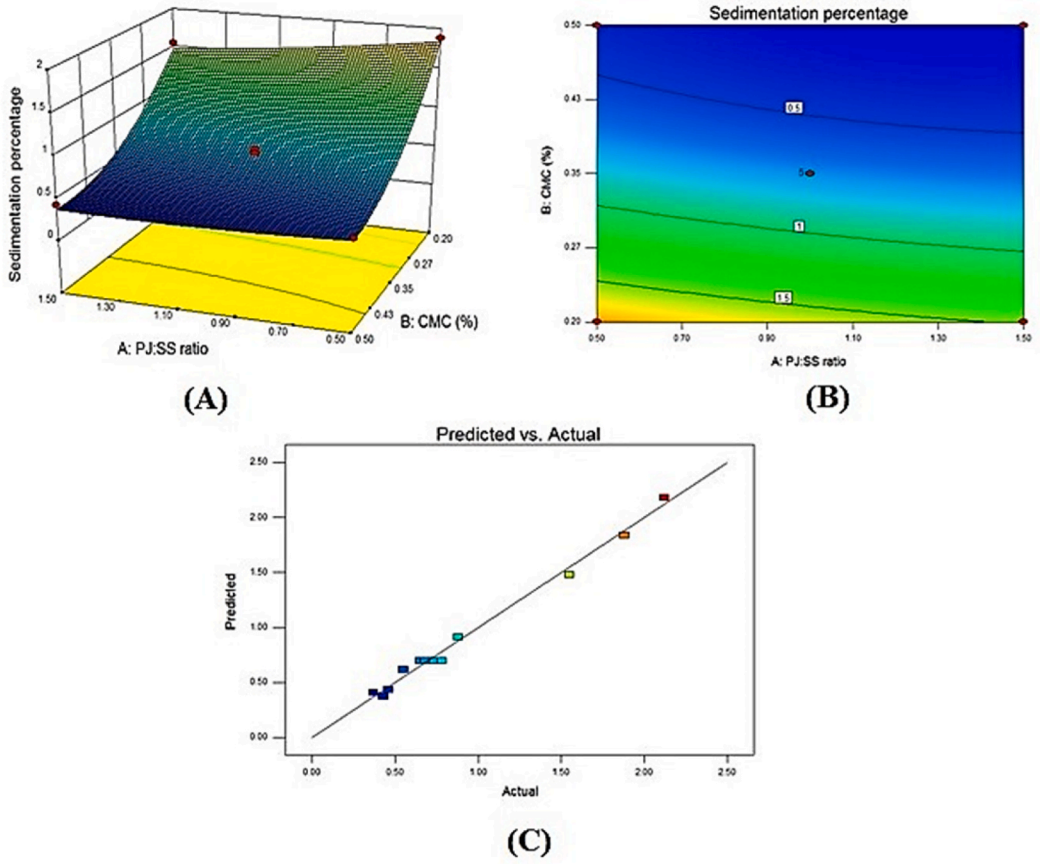

Fig. 2. (A) 3D response surface and (B) contour plots obtained from the design of expert software showing the effect of independent variables on sedimentation percentage and (C) The graph between actual and predicted values of sedimentation rate.

Table 6
Composition, actual, predicted, and desirability values of optimized syrupy herbal (F-opt) formulation.

Independent parameters (unit)	Optimal value	Response	Actual value	Predicted value	Prediction error (%)	Desirability function
PJ: SS ratio	1:1	Viscosity (CP)	256.38	251.94	−1.73	0.98
CMC (%)	0.30	sedimentation value (%)	0.69	0.7	1.45	

PJ: SS ratio = Pod juice (PJ) to Sugar solution ratio, CMC= Carboxymethylcellulose.

The presence of sucrose in pharmaceutical liquid preparations can lead to crystallization and result in cap locking [49]. In the present study, no crystals or signs of cap locking were observed. M.H. Moghadam and colleagues developed syrup of Prunus domestica and evaluated it for crystal development, observing no signs of crystallization, precipitation, or cap locking [28].

3.5.3. Determination of density

The density of the optimized formulation was found to be 1.31 g/ml. Density plays a crucial role in converting volume to weight and vice versa.

Table 7
Microbiological study of optimized syrupy formulation (F-opt).

S.No	Microorganisms	Count
1	Total microbial count (TMC)	100 per gram
2	Total yeast mold count (Fungai)	<100 per gram
3	*E. coli*	Absent
4	*S. aureus*	Absent
5	*S. typhi*	Absent

3.6. Microbiological quality studies of syrupy pod formulation during storage

Microbial loads, as well as the presence of undesirable organisms or pathogens, may be present in herbal products. According to the World Health Organization [50] and the European Pharmacopoeia [51], the total viable aerobic count for herbal medicinal products that aren't boiled before use is restricted to 10^5 bacteria and 10^3 fungi per gram or milliliter [50]. The presence of Gram-negative bacteria such as *E. coli* and *S. typhi*, and Gram-positive bacteria like *S. aureus*, is not acceptable in the formulation. In this study, the total microbial count was approximately 100 per gram, and the total fungal count was found to be less than 100 per gram (Table 7).

3.7. Stability study

According to the ICH (International Council for Harmonization) and WHO technical report series, No. 953, 2009, the stability study was conducted at 40 ± 2 °C/75 ± 5% for 6 months [40]. Several physicochemicals and microbiological evaluations were performed, and the results are shown in Table 8. The formulation was found to be stable with no distinct changes in its physicochemical and microbiological properties.

3.8. Anti-urolithiatic activity

3.8.1. In-vitro anti-urolithiatic activity (synthetic urine – in-vitro aggregation assay)

Calcium oxalate and calcium phosphate are the two major types of crystals found in kidney stones, and they can combine to form small, hard masses called stones [3]. The *in-vitro* inhibitory effect of the optimized syrupy formulation, pure juice extract, and reference standard on various phases of calcium oxalate crystallization was determined by measuring the time course of turbidity in synthetic urine across a range of concentrations [41]. Fig. 3 depicts the graph showing the percentage of inhibition of the crystallization of calcium oxalate at different concentrations of the optimized syrupy formulation, pure juice extract, and reference standard. Turbidance is directly proportional to the aggregation process. When aggregation occurs, particles come together to form larger clusters or aggregates (calcium oxalate crystals). These aggregates can scatter light, leading to an increase in turbidance. The turbidance (OD) of the crystallized suspension in the presence of samples and the standard, in comparison to that obtained in the control (with no aggregation inhibition), was measured, and the resulting percentage inhibition was calculated [26,41].

The optimized syrupy formulation, at concentrations of 200 μg/mL to 600 μg/mL, demonstrated a greater ability to inhibit the aggregation of calcium oxalate crystals (60.15 ± 1.9%, 78.34 ± 2.35%, 98.45 ± 1.56%, respectively) compared to the pure juice extract (43.45 ± 2.82%, 69.46 ± 1.27%, 87.46 ± 1.15%, respectively). The optimized syrupy formulation exhibited better inhibition of the aggregation of calcium oxalate at concentrations of 200 μg/mL to 600 μg/mL compared to the reference standard (56.98 ± 2.72%, 95.28 ± 1.21%, respectively), but less so at 400 μg/mL (80.73 ± 1.53%). Pure juice extract displayed a reduced ability to inhibit the aggregation of calcium oxalate compared to the reference standard at all concentrations (200, 400, and 600 μg/mL). The optimized syrupy formulation's ability to inhibit the aggregation of calcium oxalate appears to be higher than that of the pure juice extract and even the reference standard. This may be attributed to the formulation's properties, such as viscosity, which might enhance its retention at the site of action [52].

3.8.2. Ex-vivo anti-urolithiatic activity (rat plasma – ex-vivo turbidity assay)

The anti-urolithiatic activity of the optimized syrupy formulation and the reference standard was assessed in rat plasma through an ex-vivo assay. The results revealed that both the optimized syrupy formulation and the reference standard were effective in inhibiting the formation of calcium oxalate crystals. As depicted in Fig. 4, the reference standard and the optimized syrupy formulation demonstrated 72.70% and 82.12% inhibition, respectively, of calcium oxalate crystal formation after 30 min of incubation.

Table 8
Stability testing result of the optimized syrupy formulation (F-opt).

Physicochemical evaluation	Duration of sampling (Months)			
	0	1	3	6
Physical appearance	Uniform	Uniform	Uniform	Uniform
Texture	Smooth	Smooth	Smooth	Smooth
Color	Green	Green	Green	Green
odor	Vanilla	Vanilla	Vanilla	Vanilla
Taste	Sweet	Sweet	Sweet	Sweet
Turbidity	No	No	No	No
pH	5.80	5.85	5.86	5.88
Viscosity (CP)	256.38	258.73	259.15	261.63
Sedimentation (%)	0.69	0.71	0.72	0.75
Microbiological evaluation				
Total microbial count (TMC)	Less than 100	Less than 100	Less than 100	Less than 100
Total yeast mold count (Fungi)	Less than 100	Less than 100	Less than 100	Less than 100
E. coli	Absent	Absent	Absent	Absent
S. aureus	Absent	Absent	Absent	Absent
S. typhi	Absent	Absent	Absent	Absent

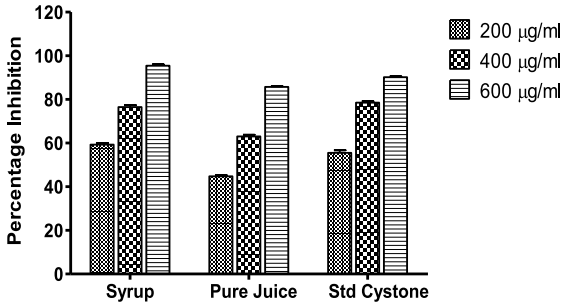

Fig. 3. Effect of different concentrations of syrupy formulation of bean pod juice, pure bean pod juice, and reference standard on CaO_x crystallization in synthetic urine. Comparison was made $p < 0.05$.

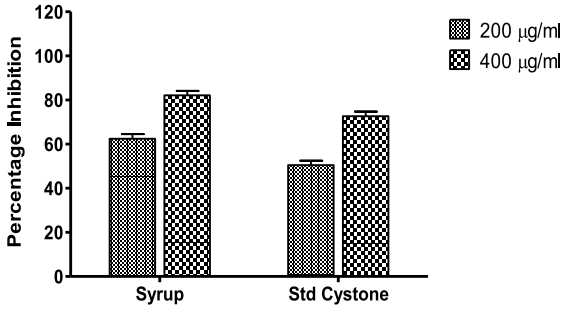

Fig. 4. Effect of different concentrations of syrupy formulation of bean pod juice, pure bean pod juice, and reference standard on CaO_x crystallization in plasma. Comparison was made $p < 0.05$.

3.8.3. In-vivo animal studies

3.8.3.1. Acute toxicity studies. The results of acute toxicity studies revealed no mortality or adverse effects from the optimized formulation and pure bean pod juice at the dosage of 2000 mg/kg body weight (equivalent to 3.04 ml of the optimized formulation and 1.52 ml of the pure bean juice extract per kg body weight) during 24 h of observation and daily monitoring for an additional three days. Both the formulation and juice extract were deemed safe up to 2000 mg/kg body weight. Consequently, we selected 25% and 37.5% from the LD50 cut-off values as screening doses, namely 500 mg/kg and 750 mg/kg body weight doses for evaluating the *in-vivo* anti-urolithiatic activity of the optimized bean pod syrupy formulation.

3.8.3.2. In-vivo anti-urolithiatic activity

3.8.3.2.1. Urine parameters. In this study, the urine output of all groups of rats on the 28th day is detailed in Table 9. The urine volume in the normal group of animals was measured at 7.86 ml. In the E.G. treated group (Gr-II), this volume decreased to 4.61 ml. Conversely, in the reference standard and syrupy formulation treated groups (Gr-III to VIII), the urine volume notably increased. Ethylene glycol (E.G.) is recognized for contributing to the development of urolithiasis, or kidney stones. When E.G. is metabolized in the body, oxalate crystals can form and combine with calcium, leading to the formation of calcium oxalate stones in the kidneys. These stones can obstruct the flow of urine, resulting in reduced urine volume. Moreover, the presence of kidney stones can cause irritation and inflammation in the urinary tract and kidneys, further impacting urine production. The resulting pain and discomfort may also lead to decreased fluid intake, which contributes to a lower urine volume [7]. Comparable findings were noted by Patel and colleagues during their assessment of the urolithiatic effect of *Macrotyloma uniflorum* in E.G.-induced urolithiasis in rats [53]. The urine pH decreased and kidney (wet weight) increased in the lithiasis control group of animals compared to the normal group and the treated groups (Gr-I and III to VIII) of rats.

3.8.3.2.2. Serum parameters. Serum analysis was conducted, and the values are summarized in Table 10. A notable increase in creatinine, uric acid, and BUN was observed in Gr-II (0.75%EG), while Gr-III, IV, V, VI, and VIII, treated with a reference standard (750 mg/kg b.w.) and varying concentrations of the syrupy formulation (500 and 750 mg/kg b.w.), exhibited significant restoration in BUN, creatinine, and uric acid levels. However, Gr-VII (syrupy formulation 500 mg/kg b.w.) did not show a significant restoration of creatinine compared to the lithiasis control group.

3.8.3.2.3. Urine calcium, oxalates, and phosphate. The concentrations of calcium, oxalate, and phosphate ions in the urine fluctuated after treatment with E.G. On the 28th day of the experiment, the control group (0.75% EG) showed a significant increase in

Table 9
Effect of Optimized Syrupy Formulation on Urine pH, Urine Output, and Kidney Weight against Ethylene Glycol-Induced Urolithiasis.

Groups		Urine pH	Urine output (ml)	Kidney weight (gm)
Preventive regimen				
Gr-I	Normal Control	8.06 ± 0.02	7.86 ± 0.11	1.10 ± 0.03
Gr-II	Control (0.75% EG) (1st to 28th day)	4.81 ± 0.23$^{\#}$	4.61 ± 0.38$^{\#}$	3.65 ± 0.22$^{\#}$
Gr-III	EG (0.75%) + Reference Standard (750 mg/kg b.w., p.o. 1st to 28th day)	6.97 ± 0.15***	7.92 ± 0.24***	1.75 ± 0.19***
Gr-IV	EG (0.75%) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 1st to 28th day)	6.10 ± 0.31***	7.01 ± 0.39***	1.98 ± 0.15***
Gr-V	EG (0.75%) + Optimized Syrupy Formulation (750 mg/kg b.w., p.o. 1st to 28th day)	7.13 ± 0.15***	8.23 ± 0.24***	2.17 ± 0.19***
Curative regimen				
Gr-VI	EG (0.75%) (1st to 28th day) + Reference Standard (750 mg/kg b.w., p.o. 15th to 28th day)	6.98 ± 0.45***	7.01 ± 0.32***	1.28 ± 0.42***
Gr-VII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 15th to 28th day)	5.96 ± 0.28*	5.91 ± 0.38***	2.79 ± 0.13**
Gr-VIII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (mg/kg b.w., p.o. 15th to 28th day)	6.60 ± 0.29***	6.98 ± 0.33***	2.15 ± 0.18***

All values are expressed as Mean ± SEM, (n = 6 animals), $^{\#}p < 0.01$ as compared to control and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns-non significant.

Table 10
Effect of optimized syrupy formulation on serum BUN, creatinine and uric acid levels against ethylene glycol induced urolithiasis.

Groups		BUN (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Preventive regimen				
Gr-I	Normal Control	41.20 ± 1.01	0.74 ± 0.09	1.8 ± 0.11
Gr-II	Control (0.75% EG) (1st to 28th day)	56.52 ± 1.69$^{\#}$	1.01 ± 0.068$^{\#}$	3.75 ± 0.16$^{\#}$
Gr-III	EG (0.75%) + Reference Standard (750 mg/kg b.w., p.o. 1st to 28th day)	43.97 ± 2.56***	0.77 ± 0.074**	1.98 ± 0.13***
Gr-IV	EG (0.75%) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 1st to 28th day)	41.21 ± 1.01***	0.80 ± 0.064**	1.79 ± 0.16***
Gr-V	EG (0.75%) + Optimized Syrupy Formulation (750 mg/kg b.w., p.o. 1st to 28th day)	41.44 ± 2.32***	0.74 ± 0.060***	1.88 ± 0.10***
Curative regimen				
Gr-VI	EG (0.75%) (1st to 28th day) + Reference Standard (750 mg/kg b.w., p.o. 15th to 28th day)	43.12 ± 1.27***	0.72 ± 0.060***	1.82 ± 0.12***
Gr-VII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 15th to 28th day)	41.89 ± 1.16^{ns}	0.80 ± 0.06^{ns}	2.67 ± 0.13**
Gr-VIII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (mg/kg b.w., p.o. 15th to 28th day)	42.92 ± 1.06***	0.80 ± 0.064**	1.79 ± 0.16***

All values are expressed as Mean ± SEM, (n = 6 animals), $^{\#}p < 0.01$ as compared to control and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns-non significant.

Table 11
Effect of optimized syrupy formulation on urine calcium, oxalates, and phosphates against ethylene glycol-induced urolithiasis.

Groups		Calcium (mg/dl)	Oxalates (mg/dl)	Phosphates (mg/dl)
Preventive regimen				
Gr-I	Normal Control	1.68 ± 0.18	2.09 ± 0.09	4.5 ± 0.17
Gr-II	Control (0.75% EG) (1st to 28th day)	6.33 ± 0.42$^{\#}$	4.17 ± 0.11$^{\#}$	10.21 ± 0.38$^{\#}$
Gr-III	EG (0.75%) + Reference Standard (750 mg/kg b.w., p.o. 1st to 28th day)	1.68 ± 0.16***	2.00 ± 0.15***	4.57 ± 0.55***
Gr-IV	EG (0.75%) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 1st to 28th day)	3.35 ± 0.14 *	2.10 ± 0.18***	5.32 ± 0.41 **
Gr-V	EG (0.75%) + Optimized Syrupy Formulation (750 mg/kg b.w., p.o. 1st to 28th day)	1.16 ± 0.38***	1.30 ± 0.20***	3.61 ± 0.27 ***
Curative regimen				
Gr-VI	EG (0.75%) (1st to 28th day) Reference Standard (750 mg/kg b.w., p.o. 15th to 28th day)	2.24 ± 0.10***	2.33 ± 0.13***	3.09 ± 0.27***
Gr-VII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (500 mg/kg b.w., p.o. 15th to 28th day)	5.27 ± 0.58 *	3.25 ± 0.19***	7.57 ± 0.31*
Gr-VIII	EG (0.75%) (1st to 28th day) + Optimized Syrupy Formulation (mg/kg b.w., p.o. 15th to 28th day)	1.31 ± 0.13***	2.14 ± 0.33***	3.19 ± 0.34 ***

All values are expressed as Mean ± SEM, (n = 6 animals), $^{\#}p < 0.01$ as compared to control and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns-non significant.

calcium, oxalate, and phosphate levels, and the results are shown in Table 11. The rise in urinary calcium levels may be associated with the mechanism of E.G. toxicity. When metabolized in the liver, E.G. produces glycolic acid and oxalic acid, which can bind to calcium ions in the body, leading to the formation of calcium oxalate crystals. These crystals can deposit in the kidneys, causing urinary tract obstruction and kidney stone formation. Consequently, there might be an increase in urinary calcium levels due to the presence of calcium oxalate crystals being excreted in the urine. E.G. toxicity can also lead to kidney damage, influencing calcium handling and reabsorption in the kidneys, resulting in an altered calcium balance and increased excretion of calcium in the urine. As a result, urinary calcium levels may be elevated in E.G.-induced urolithiasis [54]. Our study findings align with the results reported by Choudhary and colleagues in their investigation of the effect of *Cucumis callosus* fruits on calcium oxalate urolithiasis in an ethylene glycol-induced hyperoxaluric rat model [55]. All groups treated with the reference standard (750 mg/kg b.w) and varying concentrations of the syrupy formulation (500 and 750 mg/kg b.w.) displayed a substantial reduction in the excretion of calcium, oxalate, and phosphate ions. Among these, Gr-III, V, VI, and VIII exhibited highly significant reduction in urine ions, while Gr-IV showed moderate reduction and Gr-VII displayed less significance. All values were compared to the lithiasis control group of rats.

3.8.3.2.4. Histopathological evaluation of kidney. As shown in Fig. 5A, prominent critical tubules and Bowman's capsules were displayed by the Normal Group (Gr-I) rats. When compared to control groups, the Gr-I group kidney has a normal glomerular structure. In contrast, the lithiasis control group (Gr-II) displayed loss of architecture and hypercellularity, with dilated and congested glomerular capillaries in Fig. 5B. Degenerative alterations in tubules, along with congested blood vessels, focal aggregates, microcrystals, and inflammation, were seen. The reference standard group (Gr-III) (Fig. 5C) demonstrated a distinct reduction in renal tubular membrane, hemorrhages, tubular dilatation, and glomerular atrophy compared to the control kidney sections, with no deposition of colored calcium oxalate crystals in the inter-tubular space. Further analysis of the paraffin kidney sections revealed no crystals in any of the treatment groups, contrasting with the untreated groups, which displayed significant necrosis and few basophile deposits. In the preventive regimen, the group treated with the optimized syrupy formulation at 500 mg/kg b.w (Gr-IV) (Fig. 5D) exhibited cloudy changes with normal cellularity and no alterations in tubules. Meanwhile, the group treated with 750 mg/kg b.w (Gr-V) (Fig. 5E) displayed unaltered architecture, normal cellularity of glomeruli, tubules, and blood vessels, showcasing prevention of inflammation and aggregates. In the curative regimen, the reference standard group (Gr-VI) (Fig. 5F) and the 500 mg/kg syrupy formulation group (Gr-VII) (Fig. 5G) displayed normal architecture with tubular dilatation, minimal interstitial inflammation, and occasional calcium oxalate crystal deposits. The 750 mg/kg b.w syrupy formulation group (Gr-VIII) (Fig. 5H) demonstrated normal characteristics similar to the Normal Control Gr-I (Fig. 5A).

4. Discussion

In response to the numerous side effects and the limitations of current urolithiasis treatments in preventing recurrence, there is growing interest in alternative herbal remedies. Despite the traditional use of green beans/pods in combating urolithiasis [56], there

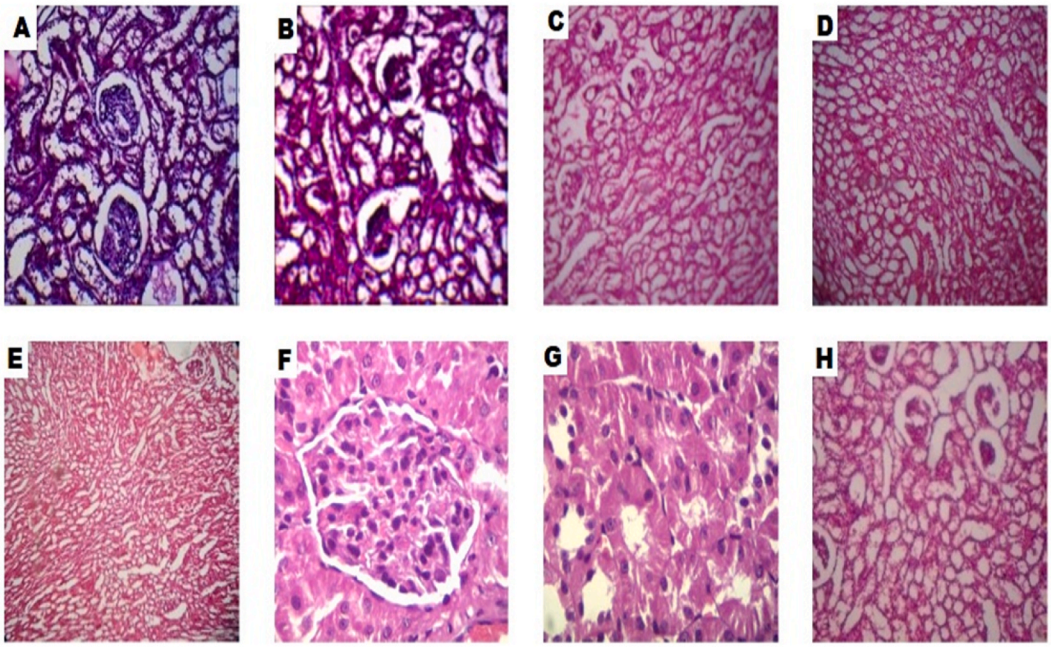

Fig. 5. Microscopic images of kidney sections under a light microscope (10x) after hematoxylin and eosin staining from animals of (A) Normal Control (Gr-I), (B) Control (Gr-II), (C) Reference standard (750 mg/kg) (Gr-III, Preventive regimen), (D) Syrupy Formulation (500 mg/kg) (Gr-IV, Preventive regimen), (E) Syrupy Formulation (750 mg/kg) (Gr-V, Preventive regimen), (F) Reference standard (750 mg/kg) (Gr-VI, Curative regimen), (G) Syrupy Formulation (500 mg/kg) (Gr-VII, Curative regimen), and (H) Syrupy Formulation (750 mg/kg) (Gr-VIII, Curative regimen).

remains a lack of evidence in the literature regarding the anti-urolithiatic activity of green bean/bean pod aqueous extract/formulations.

It is hypothetically believed that the process of drying and/or extracting with organic or aqueous solvents, as well as the application of heat during extraction, may lead to the loss of important phytoconstituents crucial for the traditionally claimed activity. In the context of this hypothesis, the present research attempt was made to use fresh/wet green bean pods instead of drying, and a simple aqueous-based solution was proposed for formulation development. The use of fresh drugs (just collected/wet) to prepare the prescribed formulations was also emphasized & supported by *Sarangadhara* (An eminent Ayurvedic physician, 1400 AD). Avoiding organic solvents for extraction retains the aqueous soluble phytoconstituents and also reduces the burden of post-extraction workup to remove solvents (residual solvents) completely. Moreover, by grinding the whole pods completely, we ensure the presence of non-aqueous phytoconstituents as well. This attempt is expected to lend support to the traditional claim regarding the proposed activity of the plant.

The syrup was prepared by simple mixing without heating. We utilized a Central Composite Design (CCD) for the optimization of the syrupy formulation, developing a total of 13 formulations (including four factorial, four-star/axial, and five center points). The effect of independent variables (PJ: SS ratio and CMC, % w/v) was observed on characteristic parameters such as viscosity and sedimentation percentage. Both the PJ: SS ratio and CMC concentration showed a positive effect on viscosity, but the dominant effect was attributed to CMC concentration, with a higher coefficient (96.96) compared to PJ: SS ratio (15.21) (equation (7)). This can be explained by the fact that CMC, when in contact with water, becomes hydrated, swells, and effectively increases the viscosity of the syrupy formulation [57]. In a related study, B. Dalila and co-workers developed a paracetamol suspension using CMC as a viscosity enhancer to improve the stability of the suspension by reducing the rate of sedimentation of the suspending particles [52].

In the case of the sedimentation percentage, both the PJ: SS ratio and the CMC concentration demonstrated a negative effect. However, the impact was predominantly attributed to the CMC concentration, as it displayed a higher coefficient (−0.63) compared to the PJ: SS ratio (−0.10) (equation (8)). This decrease in sedimentation percentage can be attributed to the viscosity-enhancing property of CMC [57,58].

Based on the results of viscosity and sedimentation percentage, batch F9 was selected for further optimization using the point prediction approach with the desirability function. The prediction error for viscosity and sedimentation values validated the selected quadratic model for the formulation (F-opt). The robustness of the optimized formulation was confirmed by the high value of the desirability function (0.98). Predicted and actual (experimental) values of viscosity and sedimentation aligned with each other (Table 6), demonstrating that the Central Composite Design (CCD) combined with the desirability function is a promising approach for optimizing syrupy herbal formulations of *Phaseolus vulgaris* L.

The optimized formulation was found to be physically stable with acceptable organoleptic properties and without any crystallization. The viscosity of the optimized formulation ensured proper flow, with negligible sedimentation ensuring dose uniformity. Microbial study results were within an acceptable range as per the WHO guidelines [59,60] for herbal products. Notably, microorganisms like *E. coli, S. typhi,* and *S. aureus* were completely absent. Furthermore, stability studies conducted over various time intervals (0 [initial], 1, 3, and 6 months) confirmed that there was no distinct difference in the physicochemical and microbiological properties of the optimized syrup formulation [40,61], indicating no spoilage or deterioration over time. The stability of the formulation may be attributed to the presence of sugar solution, ethanol, methylparaben, and propylparaben. Sucrose, as a vehicle and sweetening agent, can cause osmotic disturbances in the cellular components of microorganisms [62]. Ethanol aids in solubilization and has the capacity to denature proteins, thus inhibiting the growth of microorganisms [63]. Additionally, methyl and propylparaben act as preservatives by interfering with cellular membrane transfer processes and inhibiting the synthesis of DNA, RNA, and enzymes in bacterial cells [62–64].

Both *in-vitro* and *ex-vivo* anti-urolithiatic activity were performed on the optimized formulation. The results showed that the optimized syrupy formulation's ability to inhibit the aggregation of calcium oxalate appears to be higher than that of pure juice extract and even that of the reference standard. This enhanced performance may be attributed to the formulation's properties like viscosity, which might improve the retention of the formulation at the site of action [52]. Hewagama and co-workers observed similar results when they assessed the ability to inhibit the nucleation, growth, and aggregation of calcium oxalate crystals in *Kalanchoe laciniata, Aegle marmelos,* and *Drymoglossum piloselloides* compared to the same reference standard [26]. *Ex-vivo* conditions produced better results than in-vitro conditions, as they were performed in rat plasma. This phenomenon of improved calcium oxalate crystal inhibition may be attributed to the inhibition of glycolate oxidase (GOx) by some of the formulation's active constituents [65].

In-vivo study was conducted to explore the preventive and curative properties of the formulation in ethylene glycol-induced renal calculi in Wistar rats. In this study, ethylene glycol treatment significantly increased the level of oxalate in the urine of urolithiatic rats, which was alleviated by 28 days of treatment with the syrupy formulation. The reduction in oxalate excretion attributed to the syrupy formulation could be due to either the desolvation of calcium oxalate crystals or the suppression of oxalate formation. Additionally, literature suggests that a plant extract high in phenolic compounds, flavonoids, and iso-flavonoids can relax the smooth muscle of the urinary and biliary tract, enabling stones to be expelled from the kidney, and reducing the size of calculi in rats [66]. Beans (*P. vulgaris*) were found to be rich in phytochemicals such as proteins, carbohydrates, flavonoids, phenolic acid, tannins, and saponins [56]. These constituents have the antioxidant property and reduce renal cell injury, decreases calcium oxalate retention in renal epithelial cells. Due to this, calcium oxalate crystals pass through urine and do not participate in kidney to form stone. Hence antioxidants, play an important role in prophylactic management of kidney stones [20].

The current study found that ethylene glycol administration increased calcium levels in urine, serum, and kidney homogenate, leading to the formation of CaOx stones. In urolithiatic rats, the syrupy formulation reduced calcium levels in urine, serum, and kidney homogenate, thereby preventing stone formation and aggregation. The anti-urolithiatic activity of the bean pod syrupy formulation

has been linked to its high phenolic content, particularly flavonoids [67]. Other possible reason behind the anti-urolithiatic potential of the syrupy formulation may be its potential to increase the bioavailability of nitric oxide, which activates cGMP, controlling intracellular calcium levels [66].

In the current study, the urolithiatic rats showed an increase in serum creatinine, urea, and uric acid, which was improved by treatment with a syrupy formulation, leading to enhanced renal functions. In calculi-induced animals, histopathological examination of the kidney revealed an accumulation of calcium oxalate deposits inside the tubules. Additionally, significant changes such as proximal tubule dilation and interstitial inflammation were observed. The syrupy formulation-treated rats exhibited a significant reduction in the number and size of calcium oxalate deposits in various parts of the renal tubules, as well as a reduction in renal tubule damage. The *in-vivo* activity of the formulation includes both a preventive regimen and a curative regimen, as urolithiasis is a condition that may have a risk of future repeated occurrences. Both regimens yielded significant results, indicating the efficacy of the treatment and prevention of urolithiasis in rats.

The overall activity of the bean pod syrupy formulation is believed to rely on its extraction and formulation development. This belief is based on a traditional claim for beans/bean pods, and as of now, there are no reports of conventional extracts claiming the said activity. Therefore, it is strongly believed that the developed extraction protocol, which involves using fresh whole bean pods without drying, heat treatment, or organic solvents, would have preserved the crucial phytochemicals for the claimed activity. From the obtained results, it is evident that the optimized syrupy formulation successfully delivers the active components of the phytochemical drug to the correct part of the body, at the right concentration, and in the right manner. This achievement is facilitated by maintaining various factors such as viscosity, pH, solubility, and stability, resulting in a beneficial medicinal product.

5. Limitations of the study

The study focused on the green bean pods of *Phaseolus vulgaris* L as an herbal remedy for kidney stones using a simple syrupy formulation approach. Although the study compared the formulation with a reference herbal remedy standard (Cystone syrup), we did not compare the results with commonly used standard treatments for kidney stones. Comparative studies with standard treatments would provide a better understanding of the formulation's effectiveness and potential advantages, limiting the generalizability of the findings in our case.

Furthermore, although our study demonstrated the anti-urolithiatic activity of the formulation, we neither investigated the specific mechanisms or compounds responsible for these effects, nor the pharmacokinetic properties of the formulated syrup. Exploring the formulation's specific mode of action would enhance the understanding of its efficacy and facilitate further development.

6. Conclusion

The need for surgery and its associated risks could be avoided if a natural remedy with therapeutic properties could dissolve and remove stones. The syrup formulation was successfully designed, optimized, and evaluated for its physical properties. The formulation was found to be physically and microbiologically stable under the tested conditions. In summary, this study confirms the effective use of whole bean pod syrup in kidney malfunctioning and stone formation. Moreover, the formulation was found to be non-toxic, and no significant changes were observed in the histology of the rats' kidneys after the administration of the formulation. Based on the results of the in-*vivo* study, the present research provides scientific evidence for the use of a syrupy formulation derived from the bean pod of *Phaseolus vulgaris* L as an effective therapy in the prevention and curative management of urolithiasis. Both regimens showed significant results, affirming the efficacy in the treatment and prevention of urolithiasis in rats. Therefore, it is strongly believed that the present study may provide a ray of hope in the management of urolithiasis.

In the future, this study may aid in the discovery of novel phytochemicals from fresh whole bean pods (without – drying or heating or using organic solvents) and may be explored as new phytopharmaceutical drugs. Additionally, beans are considered safe and widely used edible vegetables in almost all parts of the world.

Funding information

The project was approved and funded by the University Research Committee office of the Research Vice President, Arsi University, Asella, Ethiopia with the Project Number: CoHS/R/0063/2019/2020.

The authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

The protocol of the animal study was approved by the Institutional Animal Ethical Committee (IAEC), Hanagal Shri Kumareshwar College of Pharmacy, Bagalkot- 587101 Karnataka, India. The protocol number HSKCP/IAEC-2/E, dated: September 07, 2020, is for all the animal experimental studies. The experimental protocol for animals was performed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Consent to participate was not applicable.

Human and animal rights

No humans were involved in the study. All animal-related experiments were conducted as per the guidelines of CPCSEA

(Committee for the purpose of control and supervision of experiments on animals).

Data availability

All necessary data is included in this article. Access to the raw data will be made available from the corresponding author after a reasonable request.

CRediT authorship contribution statement

K.M. Noorulla: Methodology, Investigation, Conceptualization, Writing – review & editing. **Debesa Doyo Dalecha:** Visualization, Resources. **Misbahu Jemal Haji:** Software, Methodology. **Roshan S:** Supervision, Project administration, Formal analysis. **Manikandan Arumugam:** Writing – review & editing. **Ameeduzzafar Zafar:** Data curation, Conceptualization. **Wondesen Gadisa Gobena:** Visualization, Resources. **Shimelis Mekit:** Project administration, Investigation, Formal analysis. **Hussein Haji Negawo:** Supervision, Project administration, Formal analysis. **Mohammednur Hussein:** Project administration, Formal analysis. **Hailu Fekadu Demessie:** Writing – review & editing, Validation. **Mohd Yasir:** Writing – original draft, Software.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Department of Pharmacy, College of Health Sciences, Arsi University, Asella, Ethiopia for providing the necessary support for the successful completion of the work. We also thank Hanagal Shri Kumareshwar College of Pharmacy, Bagalkot, Karnataka, India for their support in the completion of the animal studies.

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