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PHYTOCHEMICAL STANDARDIZATION, ANTIOXIDANT AND *IN-VITRO* ANTIUROLITHIATIC EVALUATION OF A POLYHERBAL FORMULATION

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ABSTRACT

The present study aimed to develop and scientifically evaluate a polyherbal formulation for phytochemical standardization, antioxidant activity, and *in-vitro* antiurolithiatic activity. Extracts of selected medicinal plants traditionally used for urinary disorders were prepared by the cold percolation method and formulated into three polyherbal formulations (F1, F2, and F3). Organoleptic evaluation, preliminary phytochemical screening, and TLC fingerprinting analysis were performed for phytochemical characterization and standardization. The antiurolithiatic activity was assessed using in-vitro calcium oxalate crystal nucleation and aggregation assays, along with microscopic evaluation of calcium oxalate crystals. Antioxidant activity was evaluated using DPPH free radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) assays. Preliminary phytochemical screening revealed the presence of flavonoids, tannins, glycosides, terpenoids, and saponins in the developed formulations. Phytochemical consistency was confirmed through characteristic TLC fingerprint profiles with specific R_f values. All formulations exhibited concentration-dependent biological activity, with F3 showing the highest inhibition of calcium oxalate crystal nucleation and aggregation. Microscopic evaluation further demonstrated a reduction in crystal size and aggregation in formulation-treated groups. Among all formulations, F3 exhibited the highest antioxidant activity in DPPH, FRAP, and PFRAP assays and showed activity comparable to the standard antioxidant compound. The results of the present investigation indicated that the developed polyherbal formulation possesses significant antioxidant and *in-vitro* antiurolithiatic activities, which may be attributed to the synergistic action of its phytoconstituents. The formulation may serve as a potential therapeutic candidate for the prevention and management of urolithiasis. However, further *in-vivo* studies and clinical investigations are required to establish its safety and therapeutic efficacy.

KEYWORDS: Polyherbal formulation; Urolithiasis; Antiurolithiatic activity; Antioxidant activity; Calcium oxalate; TLC fingerprinting.

1. INTRODUCTION

Urolithiasis, or kidney stones, is a common disorder of the urinary system in most parts of the world. It is a disease in which there are hard, crystalline deposits in the kidneys caused by supersaturation of urates (calcium, oxalate, phosphate) in urine.[1] Calcium oxalate stones are said to be the most common type of urinary stones. In recent years, the incidence of urolithiasis has risen considerably with improper food habits, less water consumption, sedentary lifestyle, metabolic disorders and environmental changes. Recurrent stone formation may cause significant pain and discomfort, and can lead to obstruction of the urinary tract, damage to renal tissues and loss of renal function.[2]

Kidney stone formation is thought to be a multiphasic process of crystal nucleation, crystal growth, crystal aggregation and retention in the renal tubules. Since then, oxidative stress and renal epithelial injury have been shown to be important factors in the onset and development of urolithiasis.[3] High levels of ROS produce cell damage to the renal epithelial cells, and lead to adhesion of calcium oxalate crystals to the tissues of the kidney, thus hastening the stone formation. Thus, anti-oxidant antioxidant and anti-urolithiatic therapeutic agents would be beneficial in prevention and management of renal calculi.[4]

The treatment of urolithiasis according to the rules includes pharmacological treatment, extracorporeal shock wave lithotripsy, surgical interventions and removal of the stones with a ureteroscopic.[5] These treatments are effective for stone removal, but have been linked to various drawbacks, including high cost of treatment, risk of stone recurrence, side effects and failure to prevent stone reformation. The above limitations have prompted researchers to turn to herbal medicine as an alternative treatment which is not only safer but also more cost effective in treating kidney stone disease.[6]

Traditional medicines have utilized medicinal plants for centuries in their treatment of urinary malfunction and kidney stone for centuries.[7] Herbal drugs have been reported to contain a variety of phytoconstituents such as flavonoids, terpenoids, glycosides, tannins and saponins which show antioxidant, diuretic, nephroprotective, anti-inflammatory and antiurolithiatic activity.[8] These phytochemicals can prevent the formation of calcium oxalate crystals and aggregation of crystals, and also help to prevent the damage to renal tissues caused by oxidative stress. Polyherbal formulations are generally found to be very effective due to its synergistic therapeutic action where two or more medicinal plants of complementary action are used together to produce a powerful combined effect as compared to the effect when they are used individually.[9]

In the present study, a polyherbal formulation has been developed using selected medicinal plants such as *Tribulus terrestris, Caesalpinia bonducella, Xanthium strumarium, Moringa oleifera, Bergenia ligulata, Acorus calamus* and other medicinal plants which are traditionally reported as having antiurolithiatic and nephroprotective activity. The preliminary phytochemical screening and TLC fingerprinting analysis were done on the developed formulations to carry out phytochemical standardization. In addition, the antioxidant activity was analysed by DPPH free radical scavenging assay and the *in-vitro* antiurolithiatic activity was analysed by calcium oxalate crystal nucleation and aggregation assay as well as microscopic crystal evaluation.[10]

Hence, the present study was conducted to scientifically evaluate the phytochemical constituents, antioxidant activity and *in-vitro* antiurolithiatic activity of the prepared polyherbal formulation and to find out the best formulation for further therapeutic use in the management of urolithiasis.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Materials

Plants used in the present study were chosen because of their reported traditional application in the treatment of urolithiasis, urinary tract disorders, nephrotoxicity and renal inflammation. Scientific reports of antioxidant, nephroprotective, diuretic, anti-inflammatory, and antiurolithiatic effects of the selected herbs were found, which helped to support the selection. The medicinal plants studied were *Tribulus terrestris*, *Caesalpinia bonducella*, *Xanthium strumarium*, *Phaseolus vulgaris*, *Moringa oleifera*, *Bergenia ligulata*, *Acorus calamus*, *Desmodium gangeticum*, *Daucus carota*, *Piper cubeba*, *Adiantum capillus-veneris*, *Chenopodium album*, and *Plectranthus barbatus*.

All the crude drugs were purchased from the known herbal drug suppliers and local medicinal plant markets. The collected plant materials were thoroughly checked for microbial contamination, fungal infection, insects and other foreign matters. The plant materials were authenticated by a qualified taxonomist with the help of the standard taxonomic keys, morphological features, and vouchers from herbarium. Voucher specimens were prepared and preserved for future use and reproducibility of this study.

2.2 Chemicals and Reagents

Analytical reagent (AR) grade chemicals, solvents and reagents were all used in the investigation. Ethanol, methanol, sodium oxalate, calcium chloride, ferric chloride, DPPH, Folin–Ciocalteu reagent, aluminium chloride, potassium acetate, gallic acid, quercetin and other reagents were purchased from the standard certified laboratory suppliers. Distilled water was used throughout the study for preparation of solutions and reagents. The standard antioxidant reference compound used was ascorbic acid and wherever required, comparative evaluation was made with the standard antiurolithiatic drug.[11]

2.3 Processing of Plant Materials

All the plant materials collected were well washed with distilled water to eliminate dust particles and extraneous materials. The shade dried cleaned materials were dried under controlled room temperature for the period of about 10–14 days for the preservation of thermolabile and photosensitive phytoconstituents. The drying was done in the absence of direct sunlight to prevent possible degradation of phytochemicals.

Plant materials were dried and then coarsely ground in a mechanical grinder and sieved through Sieve No. 40 to get a uniform particle size. Powdered samples were kept in separate light-resistant, airtight containers at room temperature until further extraction procedure.[12]

2.4 Solubility Study and Selection of Extraction Solvent

A preliminary solubility study was conducted to select the most appropriate extraction solvent system for efficient solvent extraction of phytoconstituents from the selected medicinal plants. Various solvents such as distilled water, methanol, ethanol and hydroalcoholic solvent system were tested for their ability to extract phytochemicals and their efficiency in dissolving the phytochemicals.[13]

Powdered plant material (about 1–5 g) was added to a number of different solvents, shaken intermittently for 1-2 h at room temperature. The mixtures were filtered using Whatman filter paper and then the filtrates were taken for

spectrophotometric analysis. The solvent system which gave the highest absorbance and extraction efficiency was chosen for subsequent extraction. Based on the above results, hydroalcoholic solvent system was chosen as the optimized extraction medium to prepare the extracts.[14]

2.5 Preparation of Hydroalcoholic Extracts

The powdered plant materials were extracted using the cold percolation method, which is suitable for extracting heat-sensitive phytoconstituents. About 100g of plant powder was accurately weighed and placed in clean glass containers. About 1000 mL of hydroalcoholic solvent was added to each container maintaining a drug-to-solvent ratio of 1:10. The mixtures were kept at room temperature for 7 days, shaken periodically for maximum extraction of phytoconstituents. The extracts were filtered first through muslin cloth and then Whatman filter paper after extraction to get the clear filtrates.

The collected filtrates were concentrated by using Rotary evaporator under reduced pressure at temperature below 45°C to prevent thermal degradation of the active constituents. These concentrated extracts were then further dried to a constant dry matter, weighed and the percentage extractive yield was calculated and stored in airtight containers at 4°C for further phytochemical and pharmacological investigations.[15]

2.6 Preparation of Polyherbal Formulations

The polyherbal formulations were developed by mixing the dried hydroalcoholic extracts of the selected medicinal plants in the desired proportions. To optimize the phytochemical composition and biological activity, three formulations, F1, F2 and F3, were developed with various proportions of the plant extracts.

The individual extracts were mixed in a uniform manner using the geometric dilution technique to obtain the homogeneity of the formulation. The blended formulations were sized using a sieve (No. 40) to ensure that the particles are not aggregated and that the mixture has a uniform particle size. The ready mixtures were kept in moisture-proof and light-proof containers for further testing.[16]

2.7 Preliminary Phytochemical Screening

Standard Pharmacognostic procedures were adopted for preliminary phytochemical screening of individual extracts and developed polyherbal formulations for detection of major secondary metabolites like alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, steroids, carbohydrates, proteins and amino acids.

Various qualitative tests, such as Dragendorff's test for alkaloids, Shinoda test for flavonoids, Ferric chloride test for tannins, foam test for saponins, Keller-Killiani test for glycosides, Liebermann-Burchard test for steroids and Salkowski test for terpenoids were carried out. Presence of corresponding phytoconstituents was confirmed by the formation of characteristic color reactions or precipitates.[17]

2.8 TLC Fingerprinting Analysis

Phytochemical profiling and standardisation of developed polyherbal formulations was carried out by using Thin Layer Chromatography (TLC) fingerprinting analysis.

Silica gel 60 F254 aluminium TLC plates were used as stationary phase. The sample solutions were dissolved in an appropriate solvent and delivered as single spots on the surface of the plates by capillary tube. Optimized mobile phase

Toluene: Ethyl acetate: Formic acid (5:4:1 v/v/v) was used in a pre-saturated TLC chamber to develop the chromatographic plates.

The plates were developed and then air dried and seen under UV light at 254nm. The separated spots were identified based on their appearance and Rf values. All formulations were fingerprinted using TLC to ensure phytochemical identity and consistency of the developed formulations.[18]

2.9 *In-vitro* Antiurolithiatic Study

2.9.1 Calcium Oxalate Crystal Nucleation Assay

The ability of developed polyherbal formulations to inhibit the calcium oxalate crystal nucleation was analyzed using standard *in-vitro* calcium oxalate crystal nucleation assay. Different concentrations of formulations (100–800 µg/mL) were prepared and added to calcium chloride and sodium oxalate solutions. Formation of calcium oxalate crystals lead to turbidity which was measured spectrophotometrically at 620 nm.[19]

Absorbance of the test sample compared to the absorbance of the control solution was used to determine the percentage inhibition of the crystal nucleation. Antiurolithiatic drugs used as standard were cystone. All the experiments were repeated three times and the data was reported as Mean ± SEM.

2.9.2 Calcium Oxalate Crystal Aggregation Assay

The calcium oxalate crystal aggregation assay was performed to evaluate the ability of the developed formulations to inhibit aggregation of preformed calcium oxalate crystals. Calcium oxalate crystals were prepared by mixing equal volume of calcium chloride and sodium oxalate solutions then incubated with different concentration of the formulations.[20]

The reaction mixture was then incubated at 37°C and spectrophotometric readings were taken at 620 nm. The percentage inhibition of crystal aggregation was determined by comparing to control and standard drug-treated groups.

2.9.3 Microscopic Evaluation of Calcium Oxalate Crystals

The effect of the developed formulations on crystal morphology, size and pattern of aggregation was carried out by microscopic examination. Each sample of crystal obtained from the control and standard and formulation treated was mounted on the clean glass slides and examined at a proper magnification on optical microscope. Calcium oxalate crystals were counted, measured for size and observed for aggregation pattern in various treatment groups. Decrease in crystal size and aggregation was taken as an evidence of antiurolithiatic activity.[21]

2.10 *In-vitro* Antioxidant Study

2.10.1 DPPH Free Radical Scavenging Assay

The developed polyherbal formulations were evaluated for their antioxidant activity by DPPH free radical scavenging assay. The concentrations of the formulations (20–100 µg/mL) were added to the DPPH solution, and incubated in the dark for 30 min at room temperature.[22]

The absorbance was spectrophotometrically determined at 517 nm with blank solution. For standard antioxidant compound ascorbic acid was used. Standard formula was used to calculate the percentage free radical scavenging activity. Each experiment was repeated three times and the results are reported as Mean ± SEM.

2.10.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power of the developed formulations was determined using FRAP assay. Acetate buffer, TPTZ solution and ferric chloride solution were prepared and mixed in suitable proportions to prepare the FRAP reagent. The various concentrations of formulations were mixed with the FRAP reagent and incubated for 30 min at 37°C.[23]

Reduction of Ferric ions to Ferrous ions led to the formation of Blue coloured complex, absorbance was measured at 593 nm in UV–Visible spectrophotometer. The standard antioxidant compound used was ascorbic acid. The more the absorbance value increased, the ferric reducing antioxidant power increased.

2.10.3 Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) Assay

The reducing power activity of the developed formulations was determined by Potassium Ferricyanide reducing antioxidant power assay. The various concentrations of the formulations were combined with phosphate buffer and potassium ferricyanide solution and allowed to incubate for 20 min at 50°C.[24]

After incubation, trichloroacetic acid was added to stop the reaction and centrifuged. The supernatant was mixed with distilled water and ferric chloride solution. Prussian blue type complex formation was used as an indicator of reducing activity, and the absorbance of the complexes was spectrophotometrically measured at 700 nm.[25] The higher absorbance values, the higher reducing antioxidant potential of developed formulations. All experiments were carried out three times and results are expressed as Mean ± SEM.

2.11 Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as Mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism software. Statistical significance was considered at $p < 0.05$.[26]

3. RESULTS

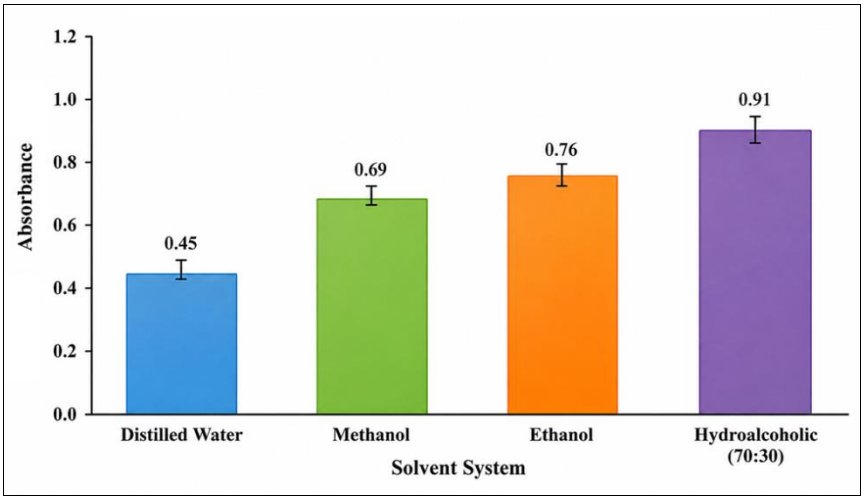
3.1 Solubility Study and Selection of Extraction Solvent

Various solvent systems such as distilled water, methanol, ethanol and hydroalcoholic solvent were tested for suitability as an extraction medium in the selected medicinal plants. The extraction efficiency was successfully analyzed by UV–Visible spectrophotometric analysis.

The highest absorbance value was obtained for the hydroalcoholic solvent system amongst all tested solvent systems, which implied high extraction efficiency for phytoconstituents (Table 1 and Figure 1). The enhancement in extraction could be explained by the balanced polarity of the hydroalcoholic solvent that allows the extraction of both polar and moderately polar bioactive compounds like flavonoids, tannins and glycosides. Thus, it was decided to proceed with hydroalcoholic solvent system for further extraction studies.

Table 1: Solubility Study Results.

S. No.	Solvent System	Absorbance (Mean ± SEM, n = 3)
1	Distilled Water	0.45 ± 0.02
2	Methanol	0.69 ± 0.03
3	Ethanol	0.76 ± 0.02
4	Hydroalcoholic (70:30)	0.91 ± 0.01



Values are expressed as Mean ± SEM (n=3)

Figure 1: Solubility Study of Different Solvent Systems.

3.2 Extractive Yield of Selected Medicinal Plants

The selected medicinal plants were extracted using the hydroalcoholic extraction method and the percent yield of the extracts was computed after the complete drying of the extract. The extraction efficiencies obtained were different for different plant materials, from 10.6% to 15.2% (Figure 2).

Bergenia ligulata, *Moringa oleifera* and *Tribulus terrestris* were the plants with high extractive yield among the selected plants. This difference in extractive yield can be explained by the varying phytochemical composition as well as solubility properties of plant materials. The satisfactory extraction yields showed the suitability of the hydroalcoholic solvent system for extracting bioactive compounds efficiently.

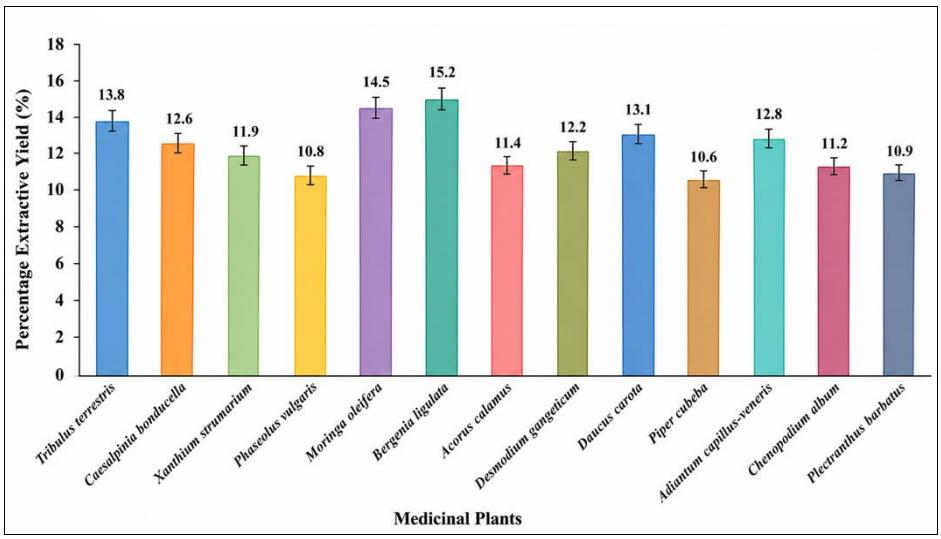


Figure 2: Percentage Extractive Yield of Selected Medicinal Plants.

3.3 Preparation and Organoleptic Evaluation of Polyherbal Formulations

The three polyherbal formulations (F1, F2 and F3) were successfully prepared by mixing the hydroalcoholic extract of the selected medicinal plants in specific ratios by using the geometric dilution method. The formulations prepared were seen as fine, free flowing powders with no evidence of aggregation, clumping or instability.

The organoleptic evaluations showed minor differences in color, smell and feel of the formulations (Table 2, Figure 3). The F1 had a characteristic brown color, with slight aromatic odour and smooth texture, while F2 had dark brown colour and strong aromatic odour and slightly coarse texture. F3 was brownish green in colour, had a distinct herbaceous smell and smooth surface. The differences can be explained due to the difference in phytochemical composition and percentage of extracts of the medicinal plants incorporated. In overall, it is found that all formulations showed good physical stability and compatibility, which are suitable for the next phytochemical and pharmacological investigations.

Table 2: Organoleptic Characteristics of Polyherbal Formulations.

Formulation	Color	Odor	Texture
F1	Brown	Mild aromatic	Smooth
F2	Dark brown	Strong aromatic	Slightly coarse
F3	Brownish green	Characteristic herbal	Smooth

Figure 3: Physical Appearance of Polyherbal Formulations (F1, F2 and F3).

3.4 Preliminary Phytochemical Screening

The phytochemical screening of developed polyherbal formulations showed presence of various important secondary metabolites such as alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, steroids and carbohydrates (Table 3).

In comparison to all formulations, the amount of flavonoids, terpenoids compounds was comparatively highest in F3. These phytoconstituents could be responsible for some of the antioxidant and antiurolithiatic activity of the developed formulations. The findings obtained were in confirmation of successful incorporation of bioactive constituents into the formulated polyherbal formulations.

Table 3: Preliminary Phytochemical Screening of Polyherbal Formulations.

Phytoconstituents	F1	F2	F3
Alkaloids	+	++	++
Flavonoids	++	++	+++
Tannins	+	++	++
Saponins	+	+	++
Glycosides	++	++	+++
Terpenoids	+	++	+++
Steroids	+	+	++
Carbohydrates	++	++	+++

Note: (+) Present, (++) Moderately Present, (+++) Strongly Present

3.5 TLC Fingerprinting Analysis

Polyherbal formulations were developed and to check their phytochemical profile and standardization, TLC fingerprinting analysis was performed using pre-coated silica gel 60 F254 TLC plates. The separation by chromatography gave different R_f values, showing that the formulations contain several phytoconstituents (Table 4 and Figure 4).

All formulations showed typical dark spots under the UV light (254 nm) due to separation of phytochemical constituents. The moderate number of phytoconstituents was revealed by formulation F1 with five well-resolved spots in the range of R_f 0.14 to 0.79. Formulation F2 showed 6 different spots ranging from R_f value 0.17 to 0.83 which indicated comparatively good phytochemical diversity.

From all the formulations, F3 showed six sharp and distinct spots ranging from R_f value 0.19 to 0.89. The more intense and well-resolved spots observed in F3 suggest the presence of more concentrated bioactive phytoconstituents like flavonoids compounds and terpenoids. The improved chromatographic profile of F3 could be correlated with its potency in biological evaluation to prevent formation of urinary stones and its antioxidant activity.

In general, the TLC fingerprint profiles have demonstrated the phytochemical identification, uniformity and incorporation of medicinal plants extracts in the developed polyherbal formulations. The resulting chromatograms can also be used as a reference standard in future studies on quality control and standardization.

Table 4: R_f Values Observed in TLC Fingerprint Analysis of Polyherbal Formulations.

Formulation	Number of Spots	Observed R_f Values
F1	5	0.14, 0.27, 0.41, 0.55, 0.79
F2	6	0.17, 0.30, 0.45, 0.59, 0.72, 0.83
F3	6	0.19, 0.33, 0.48, 0.61, 0.75, 0.89

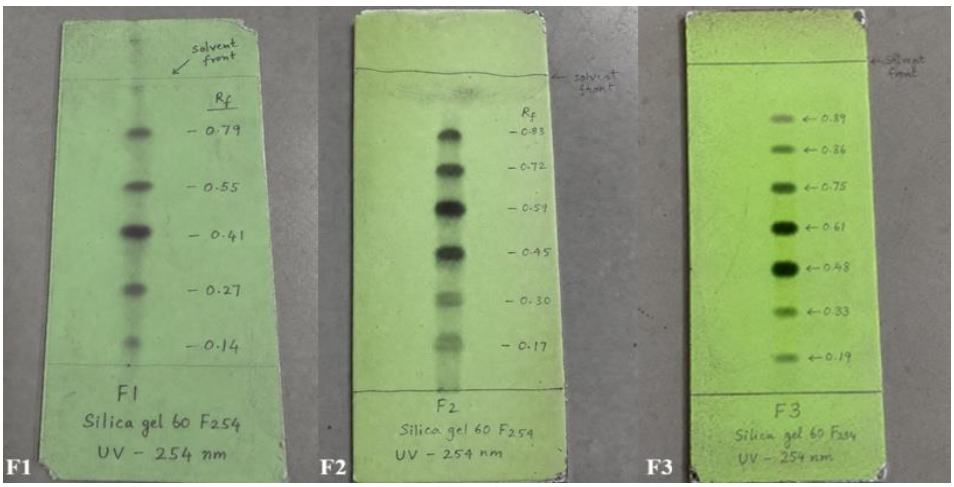


Figure 4: TLC Fingerprint Profile of Polyherbal Formulations (F1, F2 and F3) under UV Light at 254 nm.

3.6 *In-vitro* Antiurolithiatic Activity

3.6.1 Calcium Oxalate Crystal Nucleation Assay

Inhibitory effect of the developed polyherbal formulations on the calcium oxalate crystal nucleation was assessed by a standard *in-vitro* nucleation assay. All formulations inhibited the calcium oxalate crystal growth in a concentration-dependent manner as shown by results (Table 5 & Figure 5).

When all the formulations were compared, the highest inhibitory activity was observed in F3 followed by F2 and F1.

The turbidity value was significantly decreased by effective inhibition of calcium oxalate crystal nucleation at higher concentrations in the case of F3. The increased inhibitory effect of F3 could be explained by the fact that the amount of flavonoids is greater, which shows their ability to inhibit crystal formation and crystal aggregation processes.

The observed antiurolithiatic activity implies that the developed polyherbal formulations have the potential to decrease the supersaturation and inhibit the initial stages of the calcium oxalate crystal formation. The results thus obtained were similar to that of the standard drug treated group.

Table 5: Inhibitory Effect of Polyherbal Formulations on Calcium Oxalate Crystal Nucleation.

Concentration (µg/mL)	Standard (%)	F1 (%)	F2 (%)	F3 (%)
100	37.4 ± 1.5	25.2 ± 1.2	30.8 ± 1.6	34.6 ± 1.4
200	48.9 ± 1.9	32.7 ± 1.7	40.6 ± 1.8	45.3 ± 1.6
400	65.1 ± 2.2	44.1 ± 2.0	53.8 ± 1.9	59.8 ± 2.1
800	77.6 ± 2.4	57.3 ± 2.3	66.4 ± 2.2	73.2 ± 2.5

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

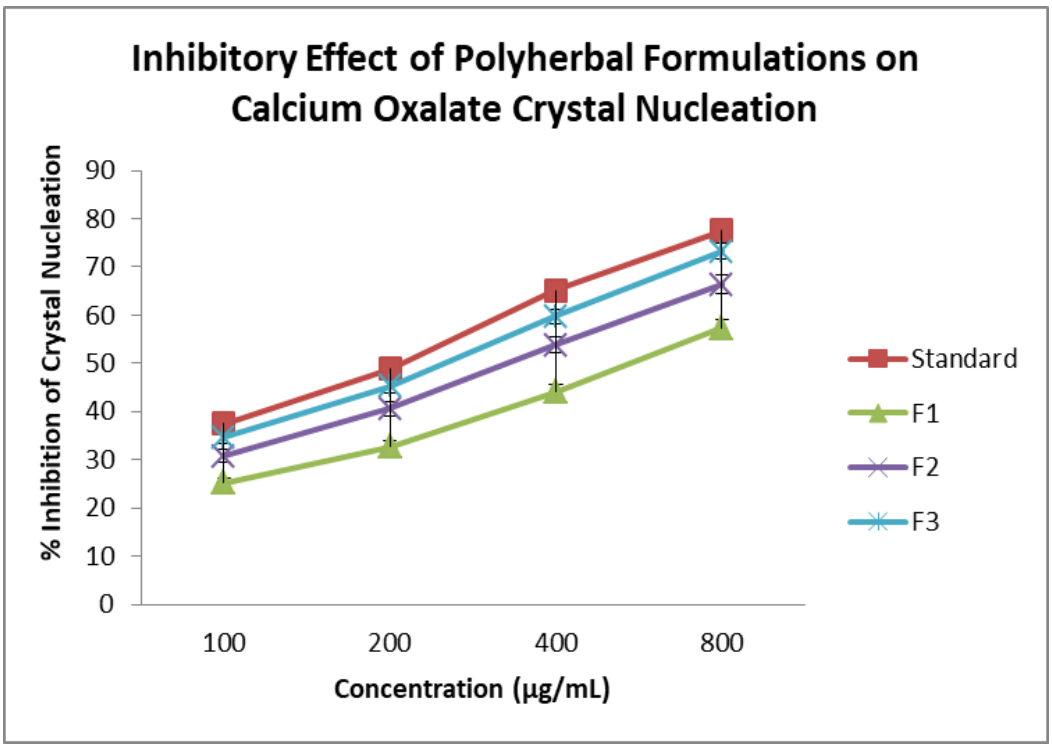


Figure 5: Effect of Polyherbal Formulations on Calcium Oxalate Crystal Nucleation.

3.6.2 Calcium Oxalate Crystal Aggregation Assay

The developed polyherbal formulations were assessed for the inhibition of the preformed calcium oxalate crystall aggregation by conducting the calcium oxalate crystal aggregation assay. The effect of inhibiting the crystal aggregation was also concentration-dependent for all formulations (Table 6 and Figure 6).

Based on the results of the tested formulations, F3 appeared to show the highest inhibitory activity followed by F2 and F1. At higher concentrations F3 showed a marked reduction in crystal aggregation, thus effectively preventing the crystal growth and clumping. The increased inhibitory effect of F3 is due to more flavonoids and terpenoids, which are known to inhibit crystal aggregation processes.

The results obtained indicated that the formulated polyherbal formulations have potential to exert antiurolithiatic effect by inhibiting the development of larger crystal aggregates which form kidney stones. The inhibitory effect of F3 was shown to be similar to that of the standard drug treated group.

Table 6: Inhibitory Effect of Polyherbal Formulations on Calcium Oxalate Crystal Aggregation.

Concentration (µg/mL)	Standard (%)	F1 (%)	F2 (%)	F3 (%)
100	34.6 ± 1.5	22.8 ± 1.3	28.4 ± 1.6	31.2 ± 1.4
200	45.3 ± 1.9	29.7 ± 1.7	37.5 ± 1.8	42.6 ± 1.6
400	61.8 ± 2.4	41.2 ± 2.1	50.9 ± 2.0	57.1 ± 2.3
800	73.4 ± 2.8	54.6 ± 2.5	63.8 ± 2.6	69.3 ± 2.7

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

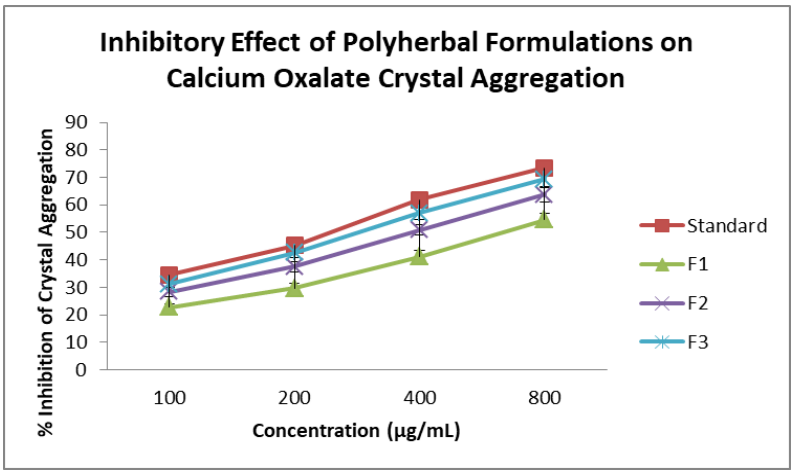


Figure 6: Effect of Polyherbal Formulations on Calcium Oxalate Crystal Aggregation.

3.6.3 Microscopic Evaluation of Calcium Oxalate Crystals

The effect of the developed polyherbal formulations on the calcium oxalate crystal morphology, size, and aggregation pattern was done using microscopic evaluation. Significant differences in both control and formulation treated groups were observed when viewed under a microscope (Figure 7).

The crystals of Ca oxalate of the control group were large, dense and highly aggregated while the crystals treated with polyherbal formulations were noticed to be reduced in size and amount of aggregation. The formulation F3 showed the highest inhibition effect in all formulations, as it gave the smallest and less aggregated crystals than the other two formulations.

The high concentration of flavonoids compounds in F3 may be responsible for the crucial decrease in crystal aggregation, as flavonoids are known to affect the crystal growth and adhesion processes. The results obtained further demonstrated that the developed polyherbal formulations have a promising antiurolithiatic activity.

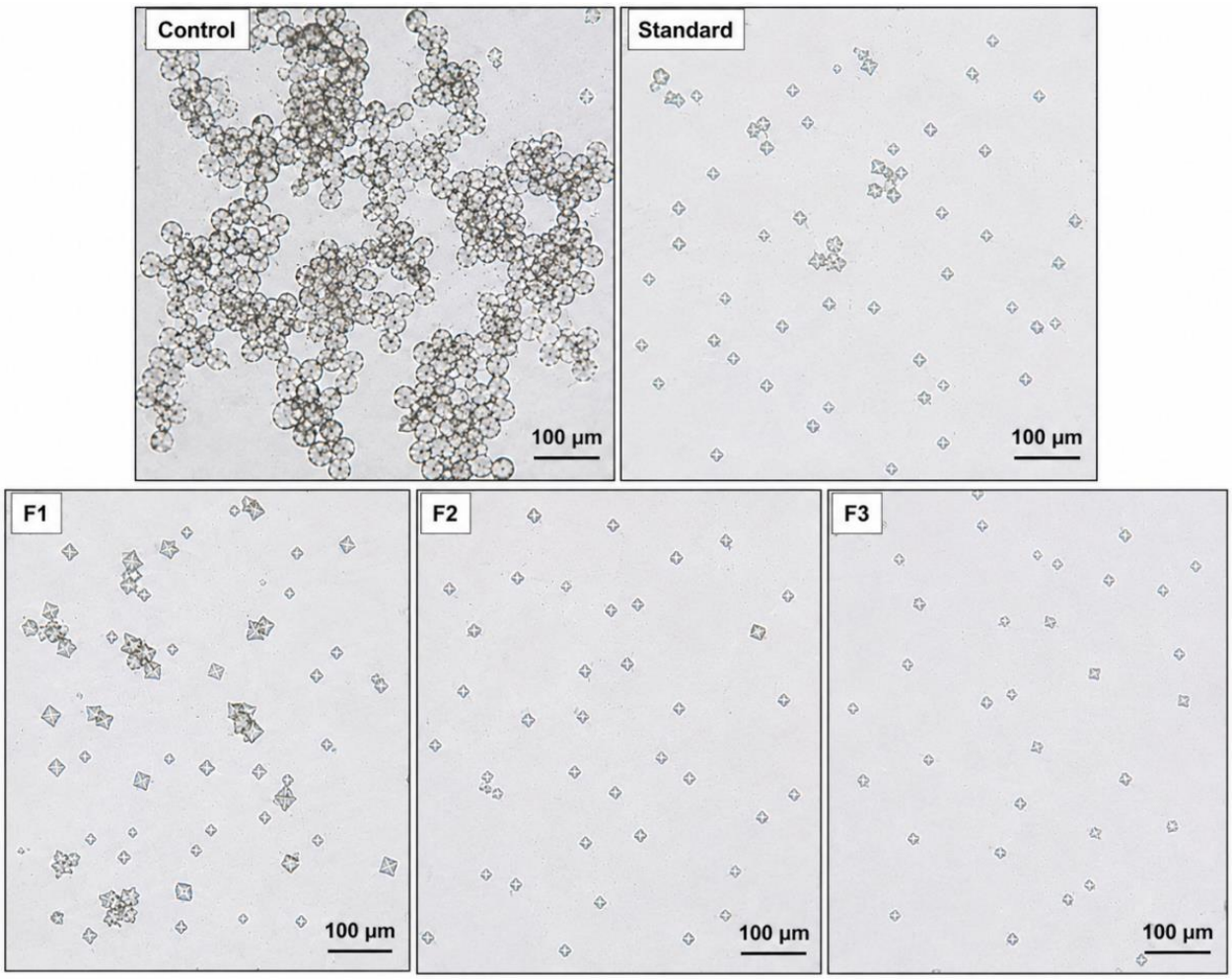


Figure 7: Microscopic Evaluation of Calcium Oxalate Crystals in Control and Formulation-Treated Groups.

3.7 *In-vitro* Antioxidant Activity

3.7.1 DPPH Free Radical Scavenging Assay

The developed polyherbal formulations were assessed for the antioxidant activity by using DPPH Free radical scavenging assay. The scavenging activity of free radicals in all formulations was found to be concentration dependent (Table 7 and Figure 8).

The levels of antioxidant activity of all formulations was highest for F3, followed by F2 and then F1. The DPPH free radical scavenging activity was highly significant for F3 at higher concentrations, similar to the standard antioxidant compound. Increased antioxidant activity of F3 might be due to increased amounts of the phytochemicals, which were flavonoid, observed during phytochemical analysis.

The results obtained indicated that the polyherbal formulation developed possessed effective antioxidant activity which was able to reduce the oxidative stress caused due to the formation of calcium oxalate crystals and damage to renal epithelial cells.

Table 7: DPPH Free Radical Scavenging Activity of Polyherbal Formulations.

Concentration (µg/mL)	Standard (%)	F1 (%)	F2 (%)	F3 (%)
20	31.2 ± 1.3	19.4 ± 0.9	23.7 ± 1.2	27.6 ± 1.1
40	42.8 ± 1.8	26.9 ± 1.3	34.1 ± 1.6	39.5 ± 1.5
60	58.4 ± 2.2	41.8 ± 1.9	49.6 ± 2.0	54.2 ± 2.1
80	71.3 ± 2.9	52.7 ± 2.3	63.8 ± 2.5	67.9 ± 2.6
100	82.1 ± 3.1	65.6 ± 2.8	74.2 ± 2.7	80.3 ± 3.0

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

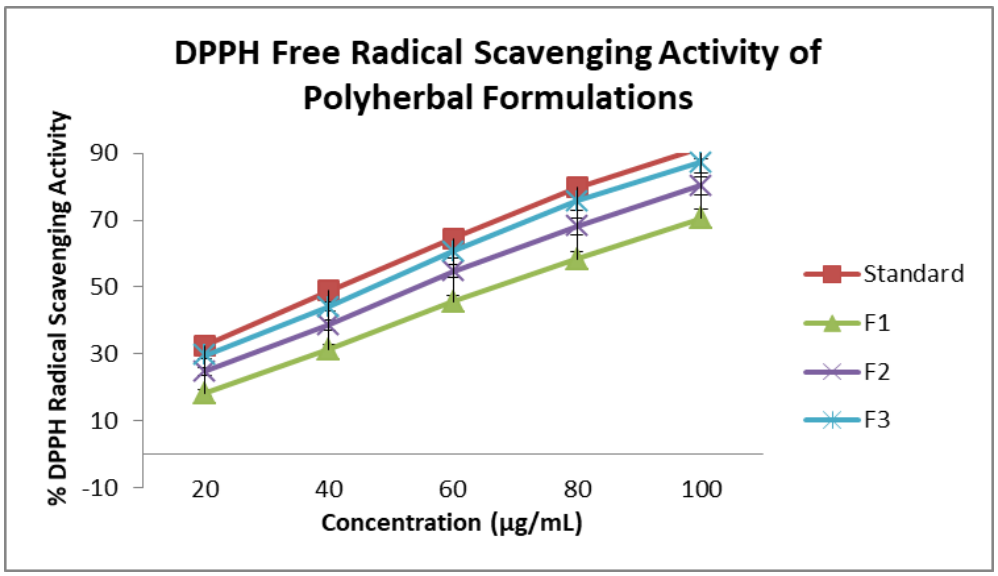


Figure 8: DPPH Free Radical Scavenging Activity of Polyherbal Formulations.

3.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power of developed polyherbal formulations were determined by FRAP assay. The ferric ion reducing activity of all the formulations was concentration dependent (Table 8 and Figure 9). The antioxidant potential increased significantly with increase in concentration of the formulations.

The F3 formulation showed maximum ferric reducing antioxidant activity among all the formulations tested, followed by F2 and F1. This increased reducing potential of F3 might be explained by the amount of flavonoids found in the formulation. The results obtained were indicative of high electron donating potential of the developed formulations that can be beneficial in mitigating oxidative stress and renal epithelial damage seen in urolithiasis.

Table 8: Ferric Reducing Antioxidant Power (FRAP) of Polyherbal Formulations.

Concentration (µg/mL)	Standard (%)	F1 (%)	F2 (%)	F3 (%)
25	29.6 ± 1.1	18.2 ± 0.8	22.9 ± 1.0	26.8 ± 0.9
50	41.8 ± 1.6	27.6 ± 1.2	35.1 ± 1.4	39.7 ± 1.3
100	59.4 ± 2.1	42.3 ± 1.8	50.8 ± 1.9	56.2 ± 2.0
150	73.1 ± 2.7	54.8 ± 2.3	64.2 ± 2.4	69.7 ± 2.6
200	84.7 ± 3.2	67.1 ± 2.8	76.4 ± 2.9	82.3 ± 3.1

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

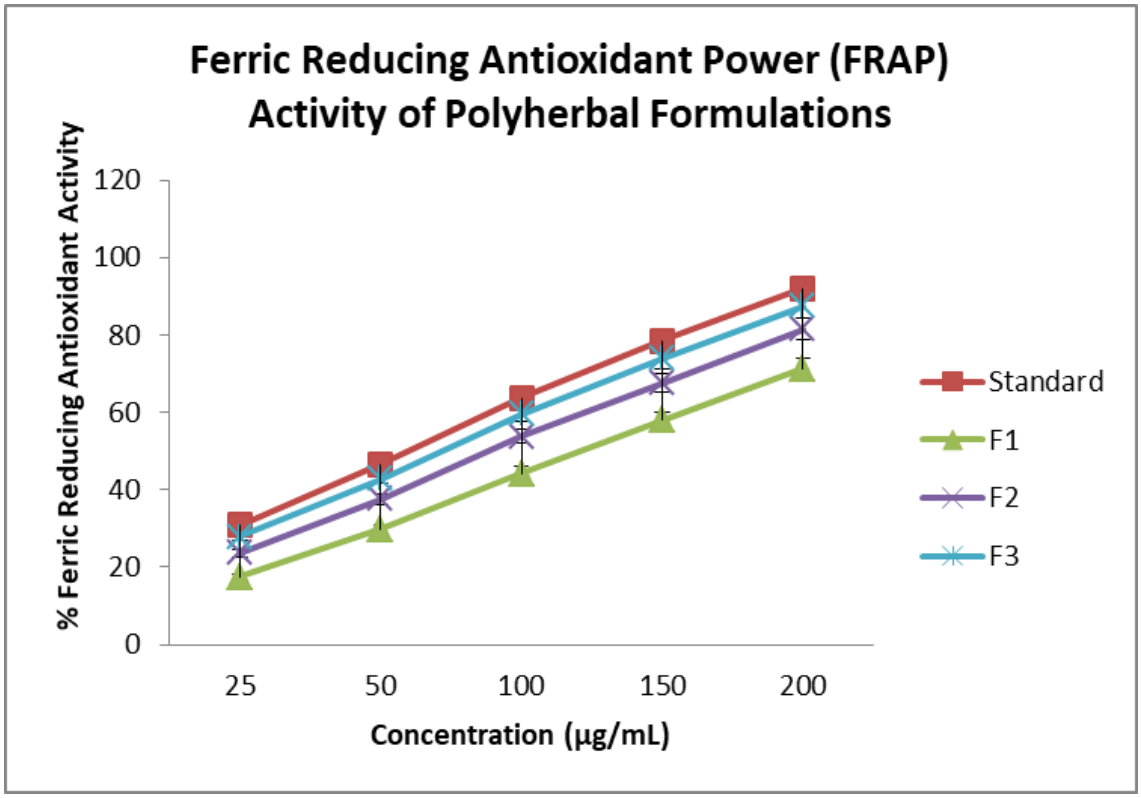


Figure 9: Ferric Reducing Antioxidant Power (FRAP) of Polyherbal Formulations.

3.7.3 Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) Assay

Further the reducing antioxidant potential of the developed polyherbal formulations was evaluated by potassium ferricyanide reducing antioxidant power assay. The absorbance values of all the formulations showed a concentration dependent reducing activity (Table 9 and Figure 10). F3 formulation had the highest reducing antioxidant activity and F2 and F1 had an intermediate reducing antioxidant activity. The absorbance was greater in F3, suggesting a higher electron donating property and higher reducing potential than the other formulations. The increased antioxidant activity of F3 can be explained by the improved amount of flavonoid compounds in the formulation. The results obtained supported the developed polyherbal formulations as having good reducing antioxidant capacity which can help to combat free radicals and reduce the level of oxidative stress caused by renal injury caused by calcium oxalate crystal.

Table 9: Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) of Polyherbal Formulations.

Concentration (µg/mL)	Standard (%)	F1 (%)	F2 (%)	F3 (%)
25	23.8 ± 1.2	17.1 ± 0.9	19.4 ± 1.1	21.9 ± 1.0
50	34.2 ± 1.9	22.4 ± 1.5	30.8 ± 1.6	33.1 ± 1.5
100	52.6 ± 2.8	39.7 ± 2.2	44.8 ± 2.5	49.3 ± 2.6
150	64.1 ± 3.6	47.3 ± 3.0	59.6 ± 3.1	61.8 ± 3.3
200	77.8 ± 4.1	61.9 ± 3.5	69.2 ± 3.8	75.4 ± 3.9

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

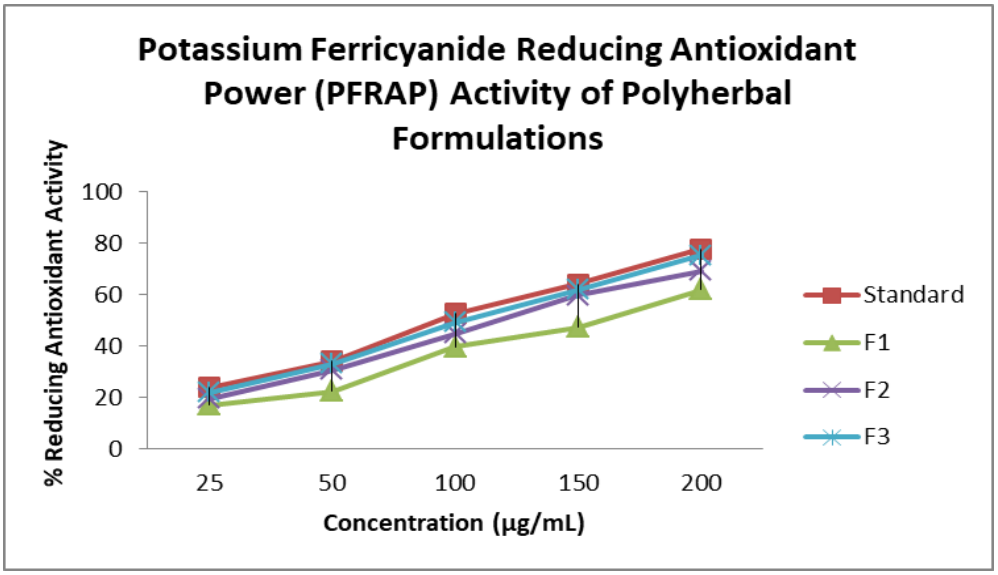


Figure 10: Potassium Ferricyanide Reducing Antioxidant Power (PFRAP) of Polyherbal Formulations.

4. DISCUSSION

The present study was aimed at developing and scientifically testing the phytochemical, antioxidant and *in-vitro* antiurolithiatic potential of polyherbal formulations. The results secured showed that the developed formulations have significant biological activity which may be due to the synergistic effect of various medicinal plant extracts used in the formulations.

Based on the preliminary study of the solvent system, the hydroalcoholic solvent system exhibited the highest extraction efficiency over distilled water, methanol and ethanol (Table 1 and Figure 1). This increased extraction efficiency of hydroalcoholic solvent could be attributed to its medium polarity that aids in the extraction of both polar and moderately polar phytoconstituents such as flavonoids, tannins and glycosides. Previous phytochemical studies indicated that hydroalcoholic solvents resulted in better recovery of bioactive constituents from medicinal plants.

The range of percentage extractive yield of various medicinal plants was 10.6% to 15.2% (Figure 2). The highest extractive yield was observed in *Bergenia ligulata* (15.2%), whereas the lowest extractive yield was recorded in *Phaseolus vulgaris* (10.6%). The differences in extractive yield can be correlated with differences in phytochemical composition, various degree of solvent penetration and solubility of the plant materials. The satisfactory extractive yield also supported the process of hydroalcoholic extraction for recovery of phytoconstituents.

All the formulations developed showed satisfactory organoleptic properties with respect to color, odor, texture and flow properties (Table 2 and Figure 3). There were no signs of aggregation or instability in the formulations, suggesting successful blending and compatibility of the extracts added. A physical stability is necessary for uniformity and reproducibility of herbal formulations.

The preliminary phytochemical screening showed the presence of important secondary metabolites such as alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, steroids and carbohydrates in the developed formulations (Table 3). Various phytochemicals like flavonoids have been reported to have free radical scavenging, nephroprotective and crystal growth inhibitory properties.

TLC fingerprint analysis resulted in unique chromatographic profiles of all the formulations in terms of the R_f values (Table 4 and Figure 4). In the formulation F3, the numbers of well resolved spots were comparatively higher in intensity which was an indication of the presence of more number of phytoconstituents in the formulation. The achieved chromatographic fingerprints verified the phytochemical identity, consistency and effective inclusion of medicinal plant extracts in the formulations. TLC profiling can thus be used as a helpful method for quality control and standardisation of the formulated polyherbal preparations.

The *in-vitro* antiurolithiatic evaluation indicated that the prepared formulations showed a significant anti-calcium oxalate crystal nucleation and anti-crystal aggregation activity (Tables 5 and 6, and Figures 5 and 6). Concentration dependent inhibitory activity was observed with all the formulations, but the most inhibitory formulation was F3. The decrease in crystal nucleation and crystallization may indicate that the formulations can disrupt the supersaturation and crystal growth and crystal adhesion process in kidney stone formation. The observed activity can be due to the synergistic effect of flavonoids and terpenoids found in the formulation.

The developed formulations were also confirmed by microscopic evaluation (Figure 7) which showed antiurolithiatic action. Large and highly aggregated calcium oxalate crystals were observed in the control group while the polyherbal formulations reduced the size of the calcium oxalate crystals and their aggregation. In comparison to all formulations, F3 gave the smaller and less aggregated crystals, which showed effective crystal growth and adhesion inhibition. The results indicated the developed formulations could be useful for preventing formation and retention of kidney stones inside renal tubules.

Antioxidant studies carried out by DPPH, FRAP and PFRAP assays revealed good antioxidant activity of the prepared formulations (Tables 7-9 and Figures 8-10). All the formulations demonstrated the concentration dependent free radical scavenging and reducing activity and the maximum antioxidant potential was found in the formulation F3 comparable with the standard antioxidant compound. The increase in antioxidant activity of F3 may be due to the increase of flavonoid compounds that were detected in the phytochemical screening. The antioxidant activity of the formulations may significantly account for their antiurolithiatic activity, as oxidative stress is known to be one of the important etiological factor in renal epithelial injury and calcium oxalate crystal retention.

The findings obtained overall indicated that the polyherbal formulations produced are very potent in terms of phytochemical content, antioxidant activity, and in-vitro antiurolithiatic activity. In all of the tested formulations, the formulation # 3 showed the highest biological activity and it could be recommended as the most promising formulation for in-vivo pharmacological/clinical studies for treatment of urolithiasis.

5. CONCLUSION

In the present study, the developed polyherbal formulations were successfully phytochemical standardized, antioxidant activity and *in-vitro* antiurolithiatic potential of the same was evaluated. Preliminary phytochemical screening and TLC fingerprinting analysis validated presence of important bioactive phytoconstituents in the developed formulations, such as flavonoids, tannins, glycosides and terpenoids.

The antioxidant and antiurolithiatic activities of the developed formulations were found to be significant in DPPH, FRAP, PFRAP, calcium oxalate crystal nucleation and aggregation assays, in a concentration-dependent manner. The

microscopic examination also revealed effective reduction of crystal size and aggregation after treatment with the formulations. F3 had a relatively high phytochemical profile, antioxidant activity and calcium oxalate crystal and aggregation inhibitory activity among all the tested formulations.

The results obtained showed that the developed polyherbal formulation, especially F3, had a promising therapeutic potential against the prevention and management of urolithiasis. But additional *in-vivo* pharmacologic studies and clinical trials need to be conducted to define its safety, efficacy, and therapeutic potential in future drugs development process.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this research work.

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