FORMULATION & CHARACTERIZATION OF NANO DOSAGE FORM USING *KALANCHOE GASTONIS-BONNIERI* (CRASSULACEAE) AGAINST UROLITHIASIS INCLUDING DNA BARCODING AND HPLC STUDIES

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ABSTRACT

Kalanchoe gastonis-bonnieri is a medicinal herb found in the geography of southern Indian region and the leaf part has been consumed for the management of urolithiasis for expulsion of kidney stone. This research work focuses on genetic authentication of the plant using Ribosomal RNA gene ITS-2 as a molecular marker, quantifying the phytochemicals using marker compounds by HPLC studies, formulating the herbal medicine into nano dosage form and scientifically validating its cytotoxic and anti-urolithiatic activity. Genetic authentication was done using ITS2 marker gene. Two flavonoids Quercetin and Apigenin were quantified by HPLC studies by using marker compounds. o/w Nano emulsion was formulated by high-speed homogenization method using 1% tween 80 as surfactant and physically characterized by particle size analysis, polydispersity index, thermal analysis by DSC and TGA, Raman spectroscopy and Dielectric spectroscopy. For cytotoxic activity, the test drug was investigated in NRK-52E rat kidney cell lines and for anti-urolithiatic activity invitro nucleation assay was carried out. In DNA barcoding studies Kalanchoe gastonis-bonnieri ITS2 partial sequence 5.8s ribosomal RNA gene was sequenced and genetically authenticated. In HPLC studies the amount of Apigenin content was found to be 159.1µg/gm and Quercetin content was found to be 236.1159.1µg/gm quantified in 70% solvent free hydro-alcoholic leaves extract. The herbal medicine is formulated into o/w Nano emulsion and physically characterized. The particle size

was found to be Z average in 160.7d. nm, poly disperse in nature and thermally stable up to 450ºC. From pharmacological studies it is observed that 62.5µg/ml exhibited 88% of cytoprotective effect in NRK-52E rat kidney cell lines and 40µg/ml exhibited 61.38% inhibition of calcium oxalate nucleation.Genetic authentication studies, quantifying the phytometabolites, formulating herbal medicine into nano dosage form and fingerprinting the safety and efficacy of the herbal medicine is the need of the hour. Accordingly, the research work was carried out to scientifically explore the herbal medicines to discover the novel therapeutics in drug discovery process from the potent source of Herbal medicines.

Keywords: Kalanchoe; DNA authentication; HPLC; Nanoemulsion; Physical characterization; cytotoxic activity; anti-urolithiasis

1. Introduction

Urolithiasis is one of the metabolic disorders with recurrence rate of 53% and there is no permanent cure. [1] The treatment in modern system of medicine is either lithotripsy or surgery or only timely management with pain killers.[2] The genus *Kalanchoe* commonly called as Bryophyllum includes 145 species having varied therapeutic uses. [3] *Kalanchoe gastonis-bonnieri* is one among the herbal medicine geographically found in southern Indian origin and also in Ecuador.[4,5] One or two fresh leaves were consumed (dosage regimen) followed by consumption of adequate water in an empty stomach in the morning, for three consecutive days expel the renal calculi. The size of the renal calculi expelled is even up to 8mm. On the other side from the literature review it is reported that, most of the species belonging to the genus *Kalanchoe* exhibits cytotoxic effect due to the presence of Bufadienolides. Cytotoxicity is due to DNA damage associated NF-kB inhibitor gene overexpression, involved in the regulation of gene transcription.

Despite of therapeutic management against renal calculi and the cytotoxic effect exhibited by the genus *Kalanchoe*, the safety and its efficacy for antiurolithiatic effect must be focused for all species belonging to the genus *Kalanchoe*. In this research work *Kalachoe gastonis-bonnieri* is scientifically validated for its antiurolithiatic activity and its safety profile was fingerprinted in NRK-52E rat kidney epithelial cell line by performing cytotoxicity studies to identify the toxic and safe dose.

DNA authentication of herbal medicines is the need of the hour as per regulatory and Pharmacopeial requirement. [6] DNA barcoding studies of *Kalanchoe gastonis-bonnieri* were done by using ITS-2 universal marker gene for DNA authentication of the herb.[7] The phytometabolites Quercetin and Apigenin were quantified by HPLC studies as chemical profiling of the herbal plant.

Further the herbal dosage regimen was formulated into nano dosage form as o/w nanoemulsion and physically characterized by determining the particle size in nano range, identifying the thermal stability of the formulation by thermal analysis – Differential scanning calorimetry and Thermogravimetry analysis, fingerprinting the formulation by Raman spectroscopy and determining the dielectric constant and conductivity of the formulated nano dosage form by

dielectric spectroscopic studies. Thus, the plant is bio scientifically validated, and its chemical and pharmacological profile was fingerprinted by identifying its safe and effective dose. This research work might serve as a platform to discover novel therapeutics against urolithiasis in drug discovery process from potent source of herbal medicine.

2. Materials & Methods

2.1. Collection & Authentication of Plant Material

Fig.1. Habitat of *Kalanchoe gastonis-bonnieri*

Kalanchoe gastonis-bonnieri is a flowering succulent medicinal herb (Figure 1) also have ornamental value, belong to the kingdom: Plantae, subkingdom: Tracheobionta, super division: Spermatophyta, division: Magnoliophyta, class: Magnoliopsida, subclass: Roxidae, order: Saxifragales, family: Crassulaceae, genus: *Kalanchoe*. They are commonly called as Palm Beach bells and Donkey ear plant because of its phenotypic characters.[8] The plant leaves were collected from in and around Puducherry, India. Three plant specimens were collected from three different geographical locations (GPS) of 38 km apart. The samples are taken from Sample 1 (Latitue:12.26627º Longitude: 79.657929º), Sample 2 (Latitude: 11.91972 N 11º55’10.97868” longitude: 79.8089 E 79º48’32.05368”), Sample 3 (Latitude: 11.95678 N 11º57’24.39468” Longitude: 79.80113 E 79º48’4.07304”). The collected plant’s leaves specimens were botanically authenticated by a Taxonomist, Department of Ecology, French Institute of Pondicherry, Puducherry, India and the voucher specimen were deposited at the department of Pharmacognosy, College of Pharmacy, Mother Theresa Post Graduate & Research Institute of Health Sciences, Puducherry, India (Herbarium Accession No: DOPG/2022/003).

3. DNA barcoding – Genetic authentication

3.1. Isolation of DNA

100 mg of leaf samples were taken in a mortar and pestle and ground gently with 1ml of CTAB (Cetyl Methyl Ammonium Bromide) buffer, an anionic surfactant, cleaves cell wall. Polyvinyl pyrrolidine (PVP) was added to the samples to remove the phenolic compounds and polysaccharides by forming hydrogen bonds. The mixture was transferred to 1.5ml centrifuge tube and shaken well. β-Mercaptoethanol 40-70µl (Reducing agent removes polyphenols and tannins, denatures proteins by breaking disulphide bonds) was added to the tubes and inverted three times while mixing. The tubes were incubated at 65ºC for 45 mins and mixing with 15 mins time interval. Incubation causes cell lysis and protein denaturation, suspends lipids and

break celluloid contents result in free DNA. To the cooled mixer 400µl of chloroform (denaturates protein): Isoamyl alcohol (antifoaming agent that precipitate protein) – 24:1 is added and the tubes are vortexed in the direction of 8. The resultant was centrifuged and 1.5ml of the supernatant is collected (persistent of cell debris if observed, the process is repeated). 500µl of ice-cold isopropanol was added, resultng in crystallization of DNA. The tubes are inverted gently for 1-2 times and refrigerated overnight. Again, centrifuged at 10,000rpm after bringing to room temperature. The supernatant was removed carefully without disturbing the DNA pellets. The pellets were washed with 500µl of 70% ethanol twice and centrifuged at 10,000rpm for 2 minutes. The pellets are dried at room temperature for 12 hours and dissolved in 100µl of 1xTE buffer and stored at recommended temperature. [9]

3.2. DNA Amplification

The DNA was subjected to barcodes by PCR amplification using universal primer ITS-2 (Table 1). The reaction mix for PCR consists of 1x buffer includes 1.5nM $MgCl_2$, 0.2µM dNTPs, 5.0pml primers, 1unit Taq DNA polymerase and 20-50ng genomic DNA. The amplification includes an initial denaturation 95ºC for 5mins, 30 cycles for denaturation at 95ºC for 30s, for annealing 55ºC for 30s and extension at 72ºC for 1min, final extension at 72ºC for 5mins and stored at 16ºC. [10]

Table.1. Primer Pairs for PCR used in DNA barcoding of *Kalanchoe gatonis-bonnieri*

Gene	Primer sequences	Used in	Amplified fragment length	Annealing Temperature
ITS-2	F5'GGAAGGAGAAGTCGTAACAAGG3' R5'TCCTCCGCTTATTGATATGC3'	PCR	505 bp	55ºC

3.3. Genotyping and Identifications

Purification of PCR amplicons were done using ez-10 spin column kit (Biobasic, Canada) and sequencing were done using dideoxy method at sequencing facility by 3130x1 Genetic analyser (Life Technology, USA). DNA barcodes sequence quality was determined by software v1.0 Applied biosystems, USA. Full length sequences were assembled using Codon code Aligner version 4.2.4 (Codon code corporation). BLAST (Basic Local Alignment Search Tool) algorithm used for similarity search against non-redundant nucleotide databases at NCBI. The phylogeny tree is assembled using sequence (ITS2) obtained from NCBI with reference sequence with 505bp[11] and the sequenced gene was submitted in NCBI-National centre for Biotechnology Information database (Genebank Accession Number: OK085467.1). Phylogenetic analysis was made to authenticate the taxonomic identification of the samples. The Neighbour Joining (NJ) bootstrap method was applied to construct the dendrogram with using closely related reference sequences with 1000 bootstrap replicates. The tamura 3 parameter model was applied to construct the dendrogram with a default 50 number of threads.

4. Phytochemical Profiling

Bufadienolides and Flavonoids were reported in the genus *Kalanchoe*. Based on the reported molecules in the genus *Kalanchoe*, marker compounds Quercetin, Kaempferol and Apigenin were procured from Yukka enterprises, Mumbai, India. Solvent free 70% hydroalcoholic leaf extract (Soxhlet extraction) along with marker compounds were subjected for Thin layer chromatography using Mobile phase (Trial & error basis) Methanol: Acetic acid: Water (18:1:1). Quercetin and Apigenin were identified in TLC chromatogram. Based on the TLC profile for the confirmation of two flavonoid compounds Quercetin and Apigenin, further HPLC studies were done for quantification of identified flavonoid compounds in 70% hydroalcoholic solvent free leaf extract.

5. HPLC studies for Quantification of Quercetin & Apigenin

5.1. Chromatographic Condition

High Performance Liquid Chromatography studies for quantification was done using Shimadzu LC 2010 HPLC (Japan), equipped with quaternary pump, autosampler and UV detector. The molecules were quantified using C-18 column (150 x 4.6mm internal diameter, 5µm particle size) and mobile phase containing 400ml of Potassium dihydrogen ortho phosphate buffer pH-3 (272 mg KH_2PO_4 in 400 ml of HPLC grade water), 520ml of Methanol (HPLC grade) and 80ml of Acetonitrile (HPLC grade) and the run time is 20mins. Flow rate is 0.7ml/min and the volume injected is 10µl. UV detection was set at 347nm for Quercetin and 310 nm for Apigenin.

5.2. Standard solutions preparation

1.11mg of Quercetin was weighed and added with 5 ml of Methanol (HPLC Grade) and sonicated for 10 mins. Further Methanol was added in 10ml of volumetric flask and maked up to 10ml (111µg/ml concentration). 1.12mg of Apigenin was weighed and added with Methanol 10ml as given above (112 µg/ml concentration). The above solutions were individually filtered in syringe filter (0.22µ pore size) and 10µl was injected for HPLC analysis.

5.3. Sample solution preparation

338.1mg of 70% hydroalcoholic solvent free leaf extract was dissolved with 7ml of Methanol (HPLC grade) by sonication for 10 mins. Further make up into 10ml of Methanol in volumetric flask. The above solution was centrifuged, and the supernatant was taken and filtered in syringe filter (0.22µ pore size). 10µl from the filtered solution was injected for HPLC analysis for quantification.

6. Formulation of o/w Nanoemulsion from *Kalanchoe gastonis-bonnieri*

6.1. Preparation of Extracts

Leaves of *Kalanchoe gastonis-bonnieri* are collected, washed in running tap water, wiped in muslin cloth, chopped into fine pieces and shade dried. The dried finely chopped leaf

drug is coarsely powdered by grinding. The coarsely powdered leaf drug was subjected for extraction process.

The phytoconstituents from the leaves of *Kalanchoe gastonis-bonnieri* was individually loaded into oil phase and aqueous phase. To load the Phytoconstituents into oil phase, 6.5gms of coarsely powdered leaf drug is macerated in 13ml of sesame oil (1:2 ratio) for 3 days with occasional shaking. [11] After maceration, the macerated content is filtered and the filtrate is subjected for Thin layer chromatography to confirm the presence of Phytoconstituents extracted into oil phase by optimizing mobile phase on trial and error method. [12] The macerated sesame oil containing Phytomolecules from *Kalanchoe gastonis-bonnieri* was taken as oil phase to formulate O/W

For loading the phytoconstituent in aqueous phase, 20gms of coarsely powdered leaf drug is extracted with 70% hydroalcoholic solvent by continuous hot percolation method using Soxhlet apparatus. [13]The extract obtained was subjected for solvent evaporation and the crude solvent free hydroalcoholic extract was mixed in distilled water (0.1%w/w concentration) into homogenous mixture. The resultant solution was taken as aqueous phase to formulate O/W Nanoemulsion.

6.2. Formulation of o/w Nanoemulsion

O/W Nanoemulsion was formulated by High-speed homogenization method using Tween 80 as surfactant. [14,15] 50ml of prepared aqueous phase (0.1% hydroalcoholic extract in distilled water) is taken in 250ml beaker. 1ml of Tween 80 (1%v/v concentration) was homogenized with aqueous phase using high speed homogenizer. To the above homogenized solution of aqueous phase with tween 80, 50ml of macerated sesame oil containing phytoconstituents is added dropwise under high-speed homogenization (T18 Ultra Turrex, IKA) using 10,000rpm for 2 hours.[16,17]

Table.2. Formulation of O/W Nanoemulsion from the leaves of *Kalanchoe gastonis-bonnieri [18]*

S.No	Ingredients	Quantity	Phase
1.	0.1%w/w Hydroalcoholic extract in distilled water	50ml	Aqueous Phase
2.	1% v/v Tween 80	1 ml	Homogenised in 0.1%w/w Hydroalcoholic extract in distilled water
3.	Macerated Sesame oil containing Phytoconstituents	50 ml	Oil Phase

7. Characterization of formulated o/w Nanoemulsion

The formulated o/w Nanoemulsion was subjected for Physical characterization such as Colour, odour, density, viscosity, pH, particle size analysis and poly dispersible index (Zeta

Sizer, Malvern, Model: Zeta Sizer Nano S), Thermal analysis (TA Instruments: Q600SDT and Q20DSC) by Differential Scanning Calorimetry (DSC) & Thermogravimetry Analysis (TGA) to identify the thermal stability, Laser Confocal Raman Microscope with Spectrometer (Renishaw Metrological Systems UK, Model: Invia Reflex, Laser Excitation: 514/488nm and 785nm) for fingerprinting as qualitative analysis and Dielectric spectroscopy (Novocontrol Technologies GmbH & Co. Germany, Model: Concept 80) to study the dielectric permittivity and conductivity of the formulated herbal drug. [19-23]

8. Pharmacological studies

For the Pharmacological studies, the coarsely powdered leaves of *Kalanchoe gastonis-bonnieri* was boiled with distilled water. The decoction is filtered, evaporated and the solvent free extract (freshly prepared to mimic the traditional dosage) was subjected for *in-vitro* cytotoxicity and *in-vitro* antiurolithiatic activity by nucleation assay.

8.1. Cytotoxicity Activity

NRK-52E is the rat kidney cell line and was plated separately in 96 well plates with $1x10^4$cells/well concentration in DMEM media along with 1x antibiotic antimycotic solution and 10% fetal bovine serum (Himedia, India) and incubated at 37ºC with 5%CO_2 in CO_2 incubator. [24] Further the cells were washed using 200µl of 1xPhosphate buffer saline. Subsequently the cells were treated with test drug of varied concentration, standard drug Cisplatin and 30%DMSO as positive control and then subjected to incubation for 24 hours. At the end of the treatment period, the medium was aspirated from cells. The cells were incubated at 37ºC for 4hours in CO_2 incubator with MTT (0.5mg/ml in 1xPBS). At the end of incubation, the medium containing MTT was discarded and the cells were washed with 200µl of PBS. The crystals formed were dissolved in 100µl of DMSO and mixed, resulted in development of colour. The intensity of colour (Formazan dye turns to purple colour) was measured at 570nm using Microplate reader. [25] The percentage of cell viability was determined by using the formula

$$\% \text{ of cell viability} = \frac{\text{Test OD/Standard OD}}{\text{Control OD}} \times 100$$

8.2. *Invitro* Anti-urolithiatic activity – Nucleation assay

Anti-urolithiatic activity of *Kalanchoe gastonis-bonnieri* leaf extract on calcium oxalate nucleation formation is determined by adding 1ml of various concentrations of extract and standard drug cystone (10-60µg/ml) with 3ml of 5mM $CaCl_2$ and 3ml of 7.5mM $Na_2C_2O_4$ solution (5mM $CaCl_2$ and 7.5mM $Na_2C_2O_4$ were prepared in 0.5M Tris HCl and 0.15M NaCl buffer 6.5). The above mixture was incubated at 37ºC for 30mins and the optical density of the solutions were measured at 620nm in UV Spectrophotometer. [26] Percentage inhibition of nucleation of calcium oxalate produced by test drug and standard drug was calculated by

$$\% \text{ of Inhibition} \left[1 - \frac{OD_{\text{test/standard}}}{OD_{\text{control}}}\right] \times 100$$

9. Results and Discussion

Kalanchoe gastonis-bonnieri is a succulent herb easily propagated by leaf cuttings and adventitious buds by developing roots at the tip of leaves. The leaves are greenish, succulent in nature, elliptical in shape and curved with crenate margin (Figure 1).

9.1. DNA Barcoding studies

DNA barcoding study is used for identification of species with the aid of DNA sequences from small fragment of genome. The internal transcribed space 2 (ITS-2) is the nuclear ribosomal DNA [27] is one among the phylogenetic marker genes (*psba-trn*H, *mat*K, *rbcl, rpo*C1, *ycf*5, ITS2 and ITS) [28] used for DNA barcodes for plant species accurate and précised authentication because of its availability of conserved regions for designing universal primers. The ITS region consists of two sub-regions: ITS1 and ITS2. These regions are located between the conserved ribosomal RNA genes (18S, 5.8S, and 28S) and vary in sequence among different species, making them useful for species identification.

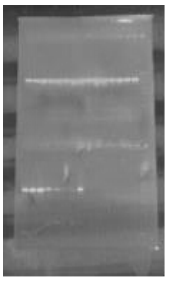


Fig.2. Amplification of ITS2 region (Amplicon length of 505bp) of *kalanchoe gastonis-bonnieri*

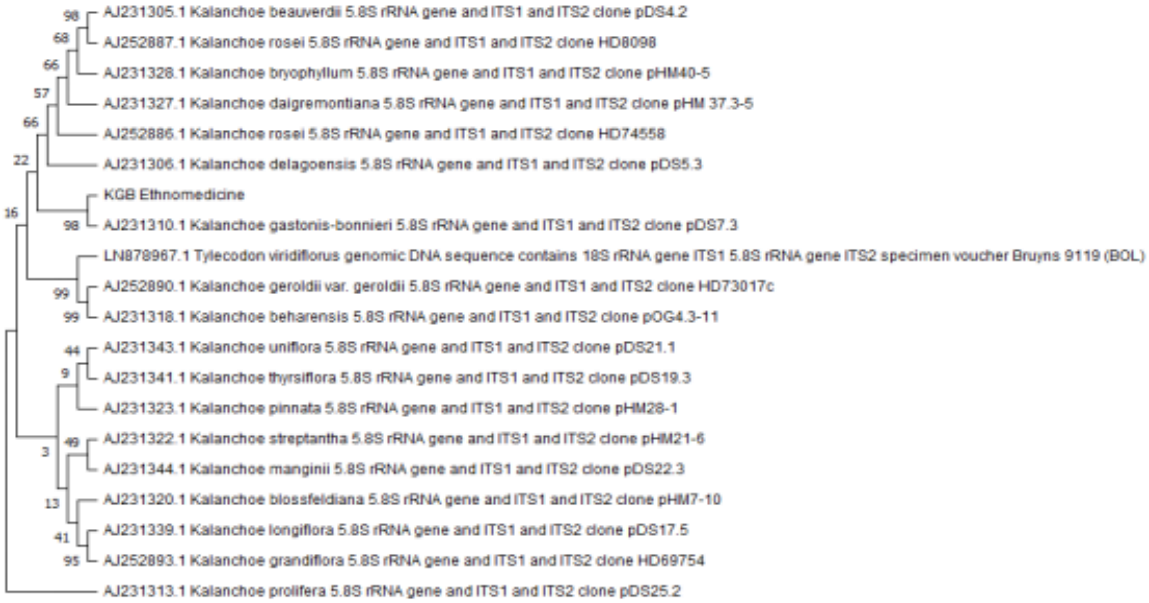


Fig.3. Phylogenetic analysis of *Kalanchoe gastonis-bonnieri* (Crassulaceae) using ITS2 marker (505bp). The Neighbour Joining (NJ) tree was generated using closely related

sequences with 1000 bootstrap replicates. Numbers at the branch nodes are bootstrap values. Codes presiding is the gene accession numbers and the species names

The leaf DNA was isolated by CTAB method and the isolated DNA was subjected to barcodes by PCR amplification using universal primers (Table.1). The PCR amplicons (Figure 2) were purified and sequenced by dideoxy method. The sequenced DNA results were compared with reference sequences of known *Kalanchoe* species available in public databases, such as GenBank or the International Nucleotide Sequence Database Collaboration (INSDC). Used software tools like BLAST (Basic Local Alignment Search Tool) to align and compared the sequences with the reference sequences. Based on the sequence alignment and comparison, closest matches of *Kalanchoe gastonis-bonnieri* was determined.

Figure 3 showed Phylogenetic analysis of *Kalanchoe gastonis-bonnieri* (Crassulaceae) using ITS2 marker (505bp). The Neighbour Joining (NJ) tree was generated using closely related sequences with 1000 bootstrap replicates. 98% of similarity is observed by comparing the sequenced DNA results with the standard sequence retrieved from databases and hence the collected samples are genetically authenticated as *Kalanchoe gastonis bonnieri*. In addition, the sequenced DNA results were deposited at NCBI https://www.ncbi.nlm.nih.gov/nuccore/OK085467.1

9.2. HPLC studies for Quantification of Quercetin and Apigenin

Preliminary Thin Layer Chromatography studies were done for the 70% hydroalcoholic leaf extract along with marker compounds Quercetin, Kaempferol and Apigenin using mobile phase Methanol: Acetic acid: Water (18:1:1) to identify the presence of above three flavonoids, hence the above three phytometabolites are reported in most of the species belonging to the genus *Kalanchoe*. In TLC studies presence of Quercetin and Apigenin were identified with Rf value 0.875 (Quercetin) and 0.928 (Apigenin) similar to the respective standards Rf values. Resoluted spot corresponding to Kaempferol was absent in the TLC chromatogram of leaf extract. Further to quantify the identified compounds HPLC studies were done.

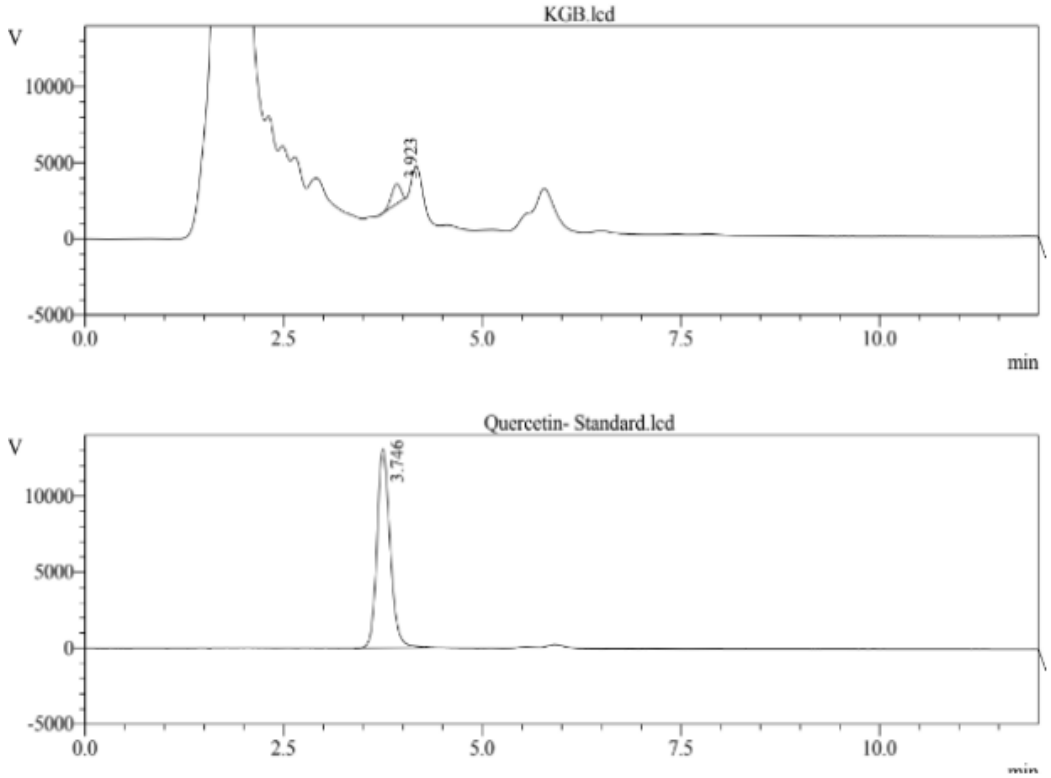


Fig.4. HPLC Chromatogram of Standard Quercetin and Sample solution

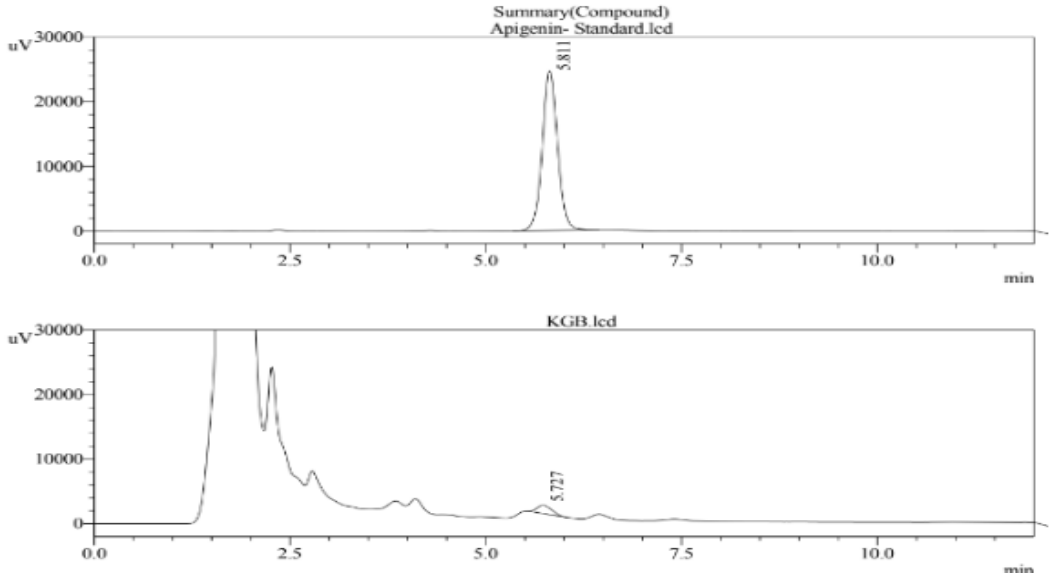


Fig.5. HPLC Chromatogram of Standard Apigenin and Sample solution

HPLC chromatographic analysis (Figure 4 & 5) was done by Shimadzu LC 2010 HPLC equipped with C-18 Column, UV detector, isocratic mobile phase Potassium dihydrogen ortho phosphate buffer pH-3, Methanol and Acetonitrile (400:520:80) chosen by trial and error method. The concentration of Apigenin content was found to be 159.1µg/gm and Quercetin content was found to be 236.1 µg/gm in solvent free 70% hydroalcoholic leaf extract.

9.3. Preparation of aqueous and oil phase for Formulation

The phytoconstituents from the coarsely powdered *Kalanchoe gastonis-bonnieri* was extracted and loaded in two phases. The first phase consists of polar phytomolecules extracted by Soxhlet extraction using 70% hydroalcoholic solvent (70ml of ethanol and 30ml of water). The percentage yield of solvent free hydroalcoholic extract is found to be 0.5%w/w. The hydroalcoholic extract (0.1%) was further homogenized in distilled water and taken as an aqueous phase for formulating nanoemulsion. Second phase consists of nonpolar phytomolecules in oil phase. For oil phase, the coarsely powdered leaf drug was macerated with sesame oil. The macerated sesame oil was subjected for TLC to identify the presence of phytoconstituents. From fig.6, the TLC chromatogram of non-macerated oil showed absence of resoluted spots whereas macerated oil showed the presence of four resoluted uv active phytomolecules with Rf value of 0.038, 0.06, 0.114 and 0.2 using Hexane: Ethylacetate (9:1) as mobile phase. The macerated oil containing UV active phytoconstituent was taken as oil phase to formulate nanoemulsion.

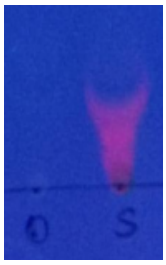


Fig.6. TLC Chromatogram of Non Macerated (O) and Macerated Sesame oil (S)

9.4. Formulation and Characterization of o/w Nanoemulsion

50 ml of aqueous phase was initially homogenized with 1% tween 80 as surfactant (Table 2). Further the macerated oil phase was added dropwise into the above homogenized aqueous phase by high-speed homogenization method using 10,000 rpm for 2 hours (Figure 7). The formulated nanoemulsion was subjected for physical characterization.

Fig.7. Formulation of o/w Nanoemulsion by High-Speed Homogenizer

9.4.1. Physical characterization

The formulated o/w Nanoemulsion was subjected for physical characterization. It is observed as milky white in colour, characteristic odour, acrid in taste, viscous in nature, Density – 0.9519 g/ml, Viscous-0.1797 poise and acidic in pH – 5.44 (Table 3).

Table.3. Physical Characters of Formulated o/w Nano emulsion

S.No	Parameters	Physical Characters
1	Colour	Milky white
2	Odour	Characteristic
3	Taste	Acrid
4	Nature	Viscous
3	Density	0.9519 g/ml
4	Viscosity	0.1797 poise
5	pH	5.44
6	Particle size (Z Average in d.nm)	160.7
7	Poly Dispersity Index (Pdi)	0.432

9.4.2. Particle size analysis and Poly Dispersity Index

Particle size is an important factor that determine the dissolution of a molecule. The smaller the particle size will have greater surface area, that enhance the fastest dissolution rate with quicker onset of action that enhances drug permeability and reduce drug metabolism leading to improved bioavailability. [29] The particle size of phytomolecules in the macerated oil globules (oil phase) was found to be 160.7d.nm (Figure 8) in the formulated phytonanoemulsion that denotes the phytomolecules are formulated in nano range.

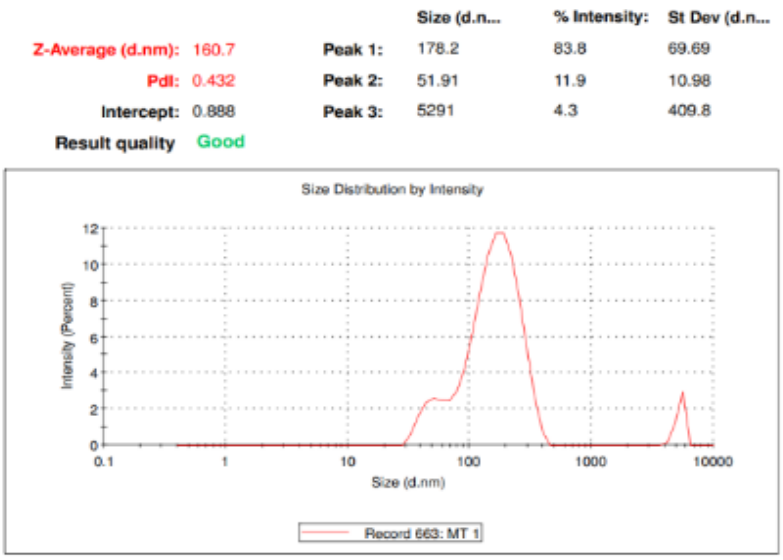


Fig.8. Particle size & Poly Dispersibility Index of formulated o/w Nanoemulsion

Poly Dispersity Index (PDI) is the size distribution of the nanoparticle. It gives the measurement of heterogenicity. PDI values <0.05 indicates monodisperse distribution of particles, <0.7 indicates polydisperse distribution of particles and >0.7 indicates very polydisperse distribution of particles. [30] PDI value of formulated nanoemulsion is found to

be 0.432 (Figure 8) indicates polydisperse distribution of phytomolecular particles in turn denotes varieties of dimensions of Plant molecules present in the formulation.

9.5. Thermal Analysis

Thermal analysis is used to determine the drug decomposition temperature and to predict the drug molecules thermal stability. [31] Differential scanning calorimetry is the measurement of absorbed or released energy by a sample molecule with respect to function of time or temperature needed for phase transition [32] i.e., transformation from one state to another with a wide range of temperature from -90 to 550ºC. Thermogravimetry analysis is the measurement of changes in weight of a sample molecule with respect to temperature.[33] It is used to determine the decomposition temperature in turn gives information about the thermal stability. The formulated phytonanoemulsion was subjected for Thermal analysis by Differential Scanning Calorimetry and Thermo Gravimetry Analysis. From the thermogravimetry graph (Figure 9) the decomposition of phytomolecules occurred at three stages. 5.643% of weight loss occurred upto 80ºC, 5% upto 105ºC and 26.7% upto 140ºC. Interestingly from 140º-320ºC, the phytomolecules are thermally stable. 57% of weight loss observed from 320º-450ºC and at 450ºC complete decomposition of phytomolecules observed. From 320º-400ºC showed 2 peaks of Phase transitions. The above said weight loss with respective to temperatures and the phase transitions are the physical characters of Phytomolecules in the formulated nanoemulsion in turn gives the information that at 450ºC the phytomolecules are completely decomposed and the phyto nanoformulation is thermally stable upto 450ºC.

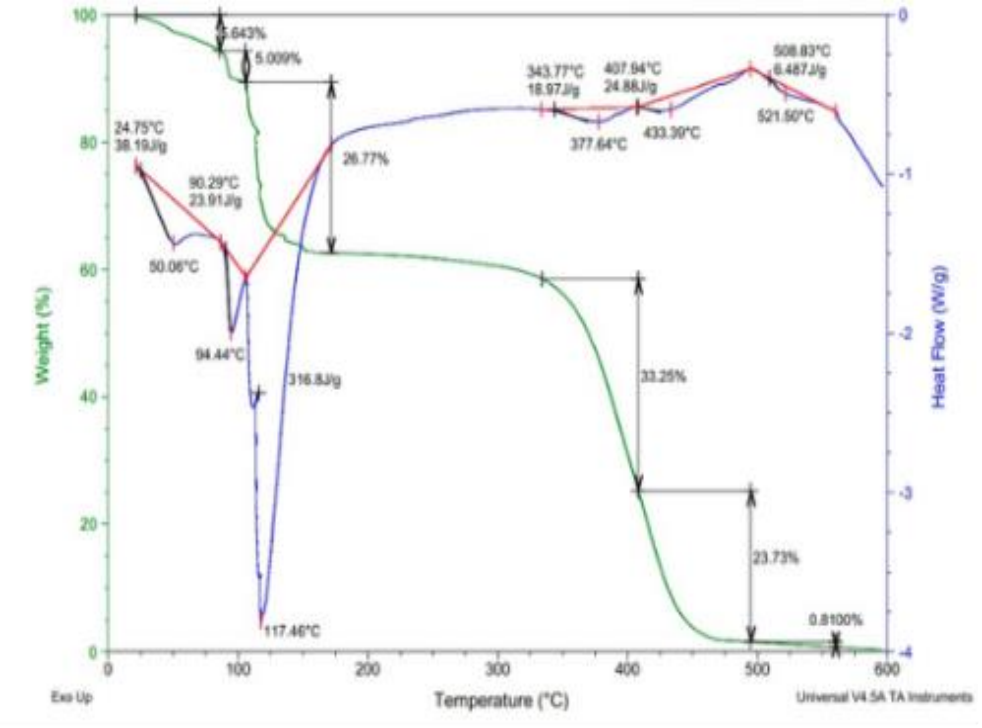
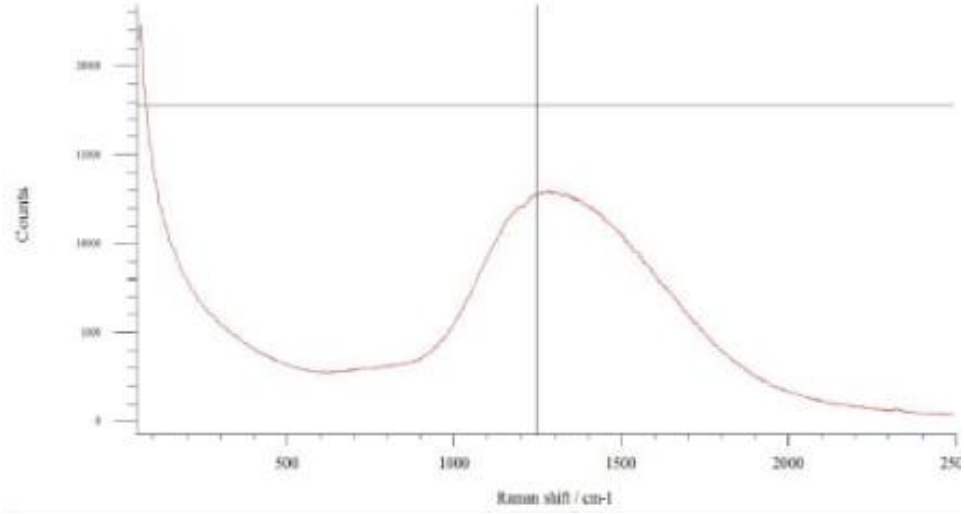


Fig.9. Thermogravimetric graph (DSC & TGA) of formulated o/w Nanoemulsion

9.6. Raman Spectroscopy

Raman spectroscopy is a non-destructive chemical analytical technique. It gives information about crystallinity, structure of molecules, molecular interactions, phase and polymorphism. It is a light scattering technique in the molecular vibrational level. The scattering of light called Raman scattering by molecules at different frequencies depends on the chemical structure of molecules. Raman scattering is complimentary to IR absorption in which both technique gives vibrational spectra in the range of wavenumber upto 4000cm^{-1}. In Raman spectrum, number of peaks with shifted frequencies corresponds to the molecular bond vibrations such as N-O, C-C, C-H, C=C, O-H, etc., [34] Hence the formulated nanoemulsion consist of combination of phytomolecules, the Raman spectrum (Spectrum 1) provides chemical fingerprint which is used for identity as qualitative analytical spectrum[35] for authenticity of the formulated nanoemulsion inorder to identify the adulterants by the presence of additional shifted frequencies. In addition, the excitation of the phytomolecules by laser source (514/488nm and 785nm) is the physical character of the phytomolecules in the formulated nanoemulsion.

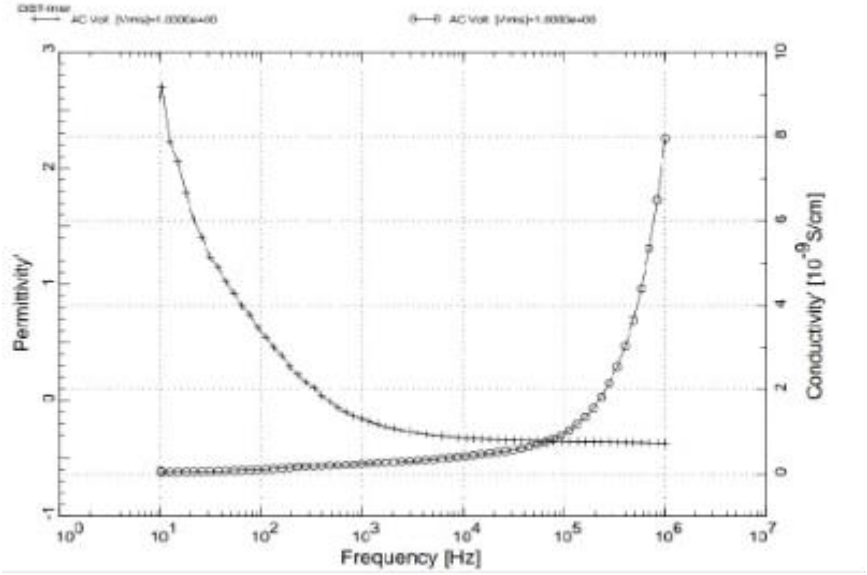


Spectrum.1. Fingerprint of Raman Spectrum of the formulated o/w Nanoemulsion

9.7. Dielectric spectroscopy

Dielectric spectroscopy is used to measure the dielectric property of a sample molecule with respect to frequency. It is the interaction of external field with electric dipole moment of the molecule that expressed by permittivity. Dielectric constant/permitivity is a physicochemical property of a molecules that denotes the polarity which regulates the solubility of the drug. Molecule with higher dielectric constant denotes the solubility in greater polarity of solvent. Conductivity is also the physicochemical property of the drug molecule to conduct electric flux. [36] The dielectric spectrum of formulated nanoemulsion (spectrum 2) showed the permittivity on the left side and conductivity on the right side. The permittivity values showed 0.6 at 1 MHz, 0.1 from 1MHz to 1KHz, 1 at 60 Hz, 2.75 at 1 Hz and the conductivity value observed is 0.1x10^{-9}s/cm at 1 Hz, 0.4 x10^{-9}s/cm at 10 KHz and 8 x10^{-9}s/cm at 1 MHz. From these values it is observed that the phytomolecules have high insulative property with respect to conductivity and with respect to dielectric constant / permittivity value, in higher frequency have higher value and in lower frequency have lower permittivity value that denotes that the

phytomolecules in nanoemulsion are soluble both in polar and non-polar solvents due to loaded phytomolecules in non-polar oil phase and polar aqueous phase while formulating nanoemulsion.



Spectrum.2. Dielectric Spectrum of formulated o/w Nanoemulsion

9.8. Invitro Pharmacological screening

Some of the species belonging to the genus *Kalanchoe* was reported for cytotoxic effect due to presence of bufadienolides. [37] Hence *Kalanchoe gastonis-bonnieri* have ethnic claim against urolithiasis, the plant material is first screened for cytotoxic activity to identify the safe dose and subsequently investigated for therapeutic efficacy of the plant against renal calculi.

9.8.1. Cytotoxic activity in NRK-5E cell lines

Cytotoxic effect of the traditional dosage form, solvent free decoction is subjected for Invitro cytotoxicity studies in NRK-52E rat kidney cell lines. The cell lines were treated with varied concentration of the solvent free decoction of leaf drug and against cytotoxic agent Cisplatin as a standard drug by MTT assay. [38] From the study it is observed that at 15.625µg/ml concentration, the test drug is safe i.e., 100% of cells are viable and at the dose of 62.5µg/ml 88.73% of cells are viable that denotes 88% cytoprotective action exhibited by the test drug *Kalanchoe gastonis-bonnieri* (Table 4, Figure 10, Graph 1 & 2).

Table.4. Cytotoxic activity of *Kalanchoe gastonis-bonnieri* in NRK-5E cell lines

Test Drug (Average of Triplicates)			**Standard Drug Cisplatin (Average of Triplicates)**		
Conc. (µg/ml)	**OD at 570nm**	**% of cell viability**	**Conc. (µg/ml)**	**OD at 570nm**	**% of cell viability**
250	0.196	55.31	20	0.173	48.42
125	0.245	69.20	15	0.195	54.66
62.5	0.315	88.73	10	0.258	72.07

31.25	0.330	92.96	5	0.286	79.89
15.625	0.355	100.00	2.5	0.301	84.26
Negative control	0.355	100.00	Negative control	0.357	99.91
Positive control	0.143	40.28	Positive control	0.178	49.72

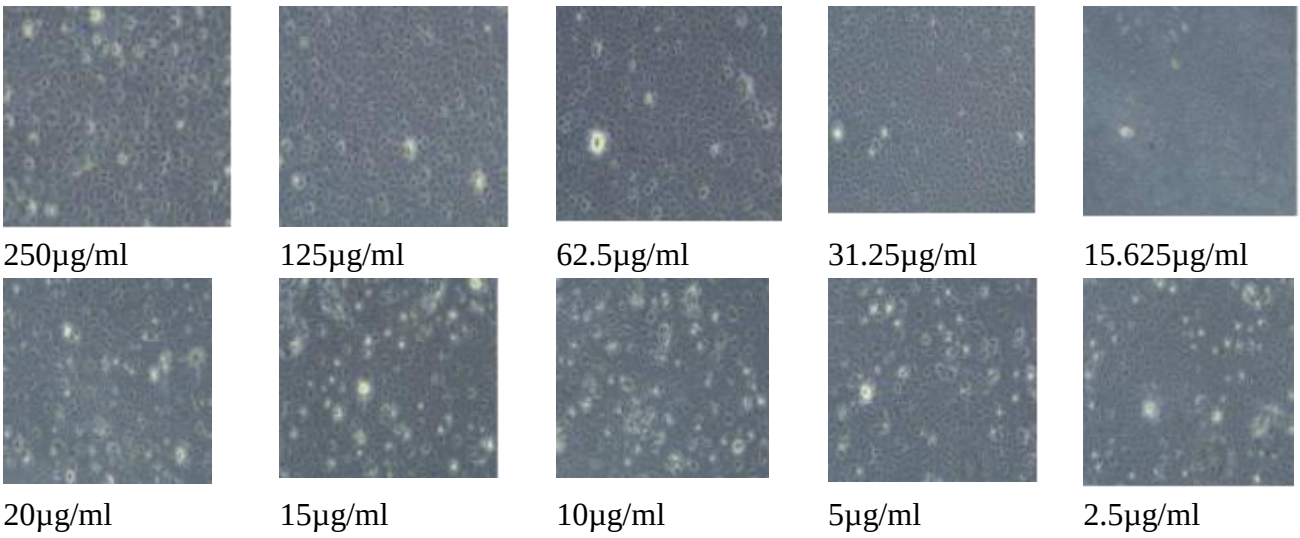
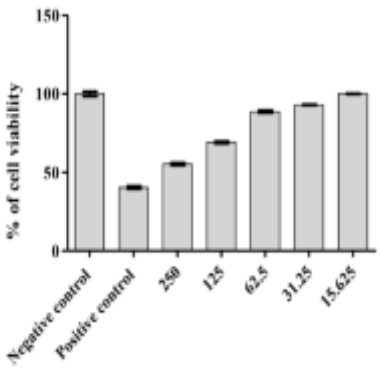
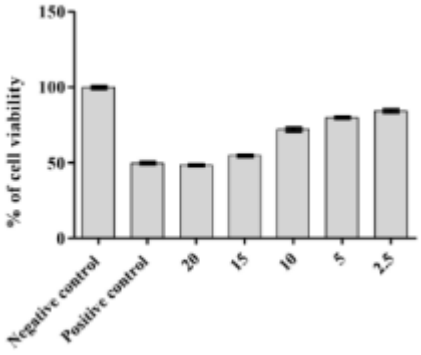


Fig.10. Microscopic images of NRK-52E cells after treated with Test & Standard drug (10x)



Graph.1. Percentage of cell viability after treatment with test drug



Graph.2. Percentage of cell viability after treatment with cisplatin

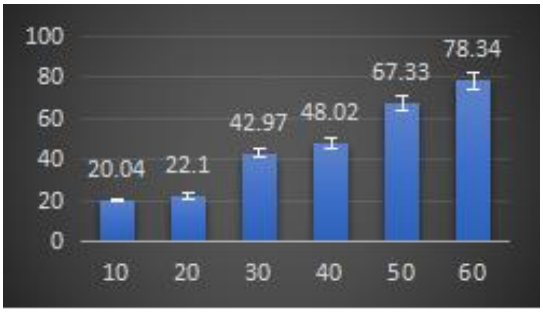
9.8.2. Invitro antiurothiatic activity by Nucleation assay

The dose at 62.5µg/ml exhibited 88% of cytoprotective effect to NRK-52E rat kidney cell line. It is identified that the safe dose selected for antiurolithiatic activity is upto 60µg/ml. The principle involved in the nucleation assay for antiurolithiatic effect is upon addition of calcium

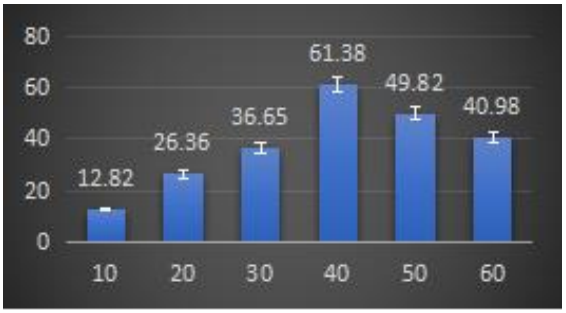
chloride with sodium oxalate results in the formation of calcium oxalate and sodium chloride. The rate of nucleation of calcium oxalate crystals is determined by the comparison of induction of time for nucleation in presence of test drug and standard drug cystone with control. [39] From antiurolithiatic activity, varied concentration of test drug from 10 µg/ml to 60 µg/ml was screened by nucleation assay. It is observed that the standard drug cystone at 60 µg/ml showed higher inhibition of nucleation of calcium oxalate stone formation of 79.34% inhibition whereas the test drug at 10 µg/ml concentration showed 12.82% and at 40 µg/ml showed highest inhibition of 61.38% inhibition in dose independent manner (Table 5, Graph 3 & 4).

Table.5. In-vitro antiurolithiatic activity of *Kalanchoe gastonis-bonnieri* by Nucleation assay

Standard Drug Cystone (Average of Triplicates)			**Test Drug (Average of Triplicates)**		
Conc. (µg/ml)	**OD at 620 nm**	**% of inhibition of nucleation**	**Conc. (µg/ml)**	**OD at 620 nm**	**% of inhibition of nucleation**
10	0.443	20.04	10	0.483	12.82
20	0.431	22.10	20	0.408	26.36
30	0.316	42.97	30	0.351	36.65
40	0.288	48.02	40	0.214	61.38
50	0.181	67.33	50	0.278	49.82
60	0.120	78.34	60	0.327	40.98
Control	0.554		Control	0.554	



Graph.3. Percentage inhibition of nucleation after treatment with Cystone



Graph.4. Percentage inhibition of nucleation after treatment with test drug

10. Conclusion

Kalanchoe gastonis-bonnieri (Crassulaceae) is an herbal medicine for the management of Kidney stone. The herbal medicine was scientifically validated by identifying the safe dose as less than 62.5µg/ml in NRK-52E rat kidney cell line and effective dose in the management of urolithiasis is 40 µg/ml, exhibited 61.38% inhibition of calcium oxalate nucleation. The plant was genetically authenticated using ITS-2 marker gene and the sequenced Nucleotide was deposited in the Gene bank of NCBI. Two flavonoid compounds Apigenin and Quercetin

were quantified in leaf part by HPLC analysis. The herbal medicine was formulated into o/w Nanoemulsion with nanosize, z average in d.nm of 160.7 with poly disperse in nature and upon physical characterization, it was observed that the herbal Nano dosage form is thermally stable up to 450ºC. Raman and Dielectric spectra give the data as qualitative fingerprint for the authenticity of the formulated nanoemulsion. Further pharmacokinetic profiling must be done to identify whether the formulated dosage form complies to meet the regulatory standards for subsequent preclinical and clinical trial investigations in drug discovery process against urolithiasis from potential sources of Herbal Medicines.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

Selvakumari.E: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Validation, Visualization, Writing original draft and Editing, Conceptualization & Data curation. **Muthukumar. R & Rubini.S:** Data curation, Formal Analysis & Investigation, **Subramanian Muthukumaravel**: Resources and Software in Genetic studies.

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