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In Vitro Study of The Effect of *Celosia argentea* and *Boerhavia diffusa* Root Plant Extracts on Calcium Oxalate Crystals

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Abstract

Since ancient times, people have used plants to treat illnesses. Urolithiasis has been a problem for people worldwide for thousands of years. Nature has given our country a huge range of plants that can be used as medicine. Medicinal plants have been used for hundreds of years because they are safe, effective, culturally acceptable, and don't have as many bad side effects as manufactured drugs. Crystals in the urinary stream separate from urine and form kidney stones. These stones are small, hard deposits of minerals and acid salts. Biochemical processes, such as kidney formation and aggregation, cause renal stones to form. Nucleation is the process by which free ions in a fluid join together to form small particles. Crystals that were grouped were defined as having three or more different crystals that were tightly connected. A study is being done to determine how well plant medicines treat calcium oxalate crystals. Nucleation and aggregation tests were used to look at the study *In Vitro*. The current study aims to determine how well *Boerhavia diffusa* and *Celosia argentea* work as antiurolithiatics. Plant extracts good at nucleation and aggregation could be used to make herbal medicines that help fight kidney stones. This study aimed to determine how well an extract from the roots of *Celosia argentea* stopped calcium oxalate (Caox) from crystallizing in a lab setting.

Keywords- Medicinal plant, Kidney stone, Nucleation, Aggregation, Antiurolithiasis

Introduction

India is a land of ancient medicinal healing plants. Over thirty percent of all plant types are used to make medicines (Gupta and Kanwar, 2018). *Celosia argentea* plant belongs to the Asteraceae family, having medicinal value in its vegetative parts such as roots and stem. Ethnobotanical data reveals the root extracts of *C. argentea* can be used against inflammation, make you pee, fight free radicals, and anti-cancer therapy. Micturition percentage in males and females is going to vary with a variable is disorder too. Men have high urinary calcium oxalate saturation in the summer, while women have it in the early winter (Parmar, 2014). Three types of urological disorders include urinary disorders, urinary tract infections, and prostate diseases (Mandal *et al.*, 2017). Many biological processes can lead to kidney stones, including oversaturation, nucleation, growth, aggregation, and retention (Yadav *et al.*, 2011). It has been recorded that over ten to twelve percent of people in developed countries have kidney stones. The chance of getting stones over a person's lifetime is between 5 and 10% on average (Sofia and Walter, 2016). In many cultures, medicinal herbs are a safe way to get natural remedies (Arya *et al.*, 2017). Renal stones are a common problem that costs a lot of money and causes a lot of illness (Aggarwal *et al.*, 2017). Urinary calculi are mostly made up of calcium oxalate (CaOx), especially in developed nations. A lot of attention has been paid to the effect of inhibitors on CaOx

crystallization because studies can be done *In Vitro* to see how different inhibitors affect the total inhibitory activity of urine (Hennequin *et al.*, 1993). Stone formation is a painful urologic disease affecting about 1 in 12 people worldwide. In men, it happens 70–81% of the time, and in women, it happens 47–60% of the time (Mikawlawng *et al.*, 2014). So, these different problems that happen during urinary disorders lead to pain and stomach issues. As a potential remedy, it can be cured with a mix of herbs that will lessen the side effects and help the crystals dissolve quickly in the urinary system. Our data significantly shows the healing power of herbs used during experimental trials and bioassay to reduce the disorder caused by urological infections.

Materials and Methods

Extraction of plant material

Celosia argentea and *Boerhavia diffusa* plants (root) were collected from the Sangli and Kolhapur districts. The extraction process was done by the Soxhlet method by continuous extraction for 12 hr., using ethanol (40 - 45°C boiling range) as a solvent. The extraction of the solvent was stored at 4°C for further analysis. The various concentration of ethanolic extracts was prepared for further analysis.

1) *In Vitro* antiurolithiatic activity assays

a. Crystallization assay:

Assay was done in whole urine. Urine samples (collected over 24 h) from a healthy subject were accumulated in a polypropylene bottle containing sodium azide as an antibacterial agent; the urine sample was refrigerated during collection. Aliquots of 2 mL of urine were transferred to tubes and allowed to warm to 37°C; 50 µL of Test Sample at different concentrations were added to the tubes. Tubes with no sample added were used as controls. Finally, 50 µL of 0.1 mol/L sodium oxalate solution was added, and the tubes incubated at 37°C for 30 min. The solution optical density (OD) was then read at 620 nm, the samples filtered through 0.22 µm membranes, and the filtrate processed for microscopy. In a separate experiment, the test sample solution was used after dialysis against distilled water overnight at 40°C, using membranes with a threshold of 6±8 kDa, and filtration through 0.45 µm membranes.

b. Nucleation assay

A spectrophotometric assay was used to evaluate the extracts' ability to inhibit the nucleation of CaOx crystals (Hennequin *et al.*, 1993). The crystallization process was started by adding calcium chloride (4 mmol/L) and sodium oxalate (50 mmol/L) solutions to artificial urine, which was produced in a buffer comprising Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 and 37°C. This action initiated the crystallization process. Then calculated, the rate of nucleation was calculated using the induction time of the crystals, the period between 0 and 180 minutes when they first appeared and reached a critical size, making them optically detectable in the presence of the extract and that of the control, which had no extract. At 620 nm, using a single-beam UV-vis spectrophotometer the absorbance (optical density, or OD) was measured. The % inhibition was computed as $[\text{OD (experimental)}/\text{OD (control)}] \times 100$.

c. Aggregation assay

The technique of Hess *et al.*, (1989) was used to calculate the rate of aggregation of the CaOx crystals, with a few minor adjustments. The calcium chloride and sodium oxalate solutions were combined at a 50 mmol/L ratio to create the COM crystals. Then, both solutions were allowed to acclimate for one hour at 60 degrees Celsius. After cooling to 37 °C, the solutions evaporated. After that, the COM crystals were dissolved to a final concentration of 1 mg/mL at pH 6.5 using Tris 0.05 mol/L and NaCl 0.15 mol/L. Then measured, the absorbance was measured at 620 nm using a single-beam UV-vis spectrophotometer at 30, 60, 90, and 180 minutes. The rate of aggregation was calculated by comparing the turbidity slope in the extract-containing solution to the one obtained in the control. The formula for calculating the percentage inhibition was $(1 - \text{Si}/\text{Sc}) \times 100$, where Sc is the slope of the control plot (without inhibitor) and Si is the slope of the plot when the inhibitor (extract) is present.

d. Microscopy

Prepared samples of Nucleation/ Aggregation were prepared for the analysis and after eight minutes of reaction, drop of sample dried was observed under 40 resolution and photographed for variation in the crystal size. Olympus Trinocular model Bx53.

2) Bioactive Compound Analysis (GC-MS/MS):

The GC-MS/MS analysis of these extracts was performed using a Shimadzu (Japan) TQ 8050 Plus with QP 2010 model system and Gas chromatograph interfaced to a Mass Spectrometer (GC- MS/MS) equipped with an EliteI, fused silica capillary column (30mmX0.25mm 1D X 1 μ Mdf, composed of 100% Dimethylpolysiloxane). An electron ionization system with an ionizing energy of 70 eV was used for GC-MS detection. The carrier gas utilized in the experiment was 99.999% helium gas, flowing at a steady flow rate of 1 ml/min. An injection volume of 2 μ l (with a split ratio of 10:1) was used; the injector temperature was 250°C, and the ion source temperature was 280°C. The oven temperature was set to start at 110°C (isothermal for 2 minutes), increase by 10°C/min to 200°C, then by 5°C/min to 280°C, and finally end at 280°C for a 9-minute isothermal. Mass spectra were obtained at 70 eV with fragments ranging from 45 to 450 Da and a scan interval of 0.5 seconds. The GC ran for 46 minutes in total. By comparing each component's average peak area to the total areas, the relative percent amount was determined. Turbomass was the software used to handle the mass spectra and chromatograms. The National Institute of Standards and Technology (NIST) Library was used to interpret the mass spectra and identify the compounds (Patil *et al.*, 2020).

3) Fourier Transform Infrared Spectroscopy (FT-IR)

The resulting extracts were concentrated at 40 °C using an evaporator, filtered through Whatman No. 1 filter paper, and any leftover extracts were refrigerated at 4 °C in sterile, small amber glass bottles. performed FTIR analysis using this finely powdered solution of *C. argentea* and *B. diffusa* roots (Patil *et al.*, 2020).

4) Bioactive Compound Analysis (HR)LC-MS/MS:

The alcoholic extract of *C. argentea* and *B. diffusa* roots was studied using a 6200 series TOF/6500 series Q-TOF B.05.01 (B5125.3) LC-MS instrument. Precursor ions were selected in Q1 with a 2D isolation width and fragmented in the collision cell using a collision energy slope of 5-45 eV. Found productions with a 150/400 Vpp collision RF, a transfer time of 70 ms, a pre-pulse storage of 5 ms, a pulse frequency of 10 kHz, and a spectra rate of 1.5 Hz for the collision-induced dissociation (CID) of in-source fragment ions as the in-source CID energy rose from 0 to 100 V. We used nitrogen as a collision gas. obtained accurate mass spectra in the m/z range of 50–1000 at a rate of two spectra per second. Carried out internal calibration in the positive mode, employing signals at m/z 121.0509 (protonated purine) and 922.0098 (protonated hexakis (1H,1H,3H-tetrauoropropoxy) phosphazine). Then, using the Mass Hunter Workstation software, we processed raw HPLC-QTOFMS (Agilent 6540 UHD QTOF LC-MS) full single MS and MS/MS data, mining the data to look for molecular formulas and fragment patterns (qualitative analysis). The algorithm for full single MS data, Molecular feature extraction (MFEs), identified ions with similar elution profiles and m/z values. These ions were different isotopes of the same compound (Narayankar *et al.*, 2021).

5) Determination of Antioxidant Potential:

a) DPPH

The DPPH radical scavenging activity of the extract was estimated as per the method of (Koleva et al., 2002) using DPPH. The 2.5 ml of 0.1 mM DPPH was mixed with 0.1 ml plant extract up to 3 ml volume with the addition of methanol. The absorbance of the reaction mixture read at 0 min and after 30 min at 517nm. The standard BHT was used for calculation. The scavenging effect was expressed as percent inhibition as shown below: % discoloration = $(1 - \text{Abs sample} / \text{Abs control}) \times 100$.

Method

In a 96-well plate, 5 μ l of a different test compound stock was mixed with 0.1 ml of 0.1 mM DPPH. Set the reaction in triplicate and prepared duplicates of the blank using 0.2 ml of DMSO/methanol and 5 μ l of compounds of different concentrations, as specified in the Excel sheet. The plate was incubated for 30 minutes in the dark. At the end of the incubation, the decolorization was measured at 495 nm with a microplate reader (iMark, BioRad). The control mixture consisted of 20 μ l of deionized water. The scavenging activity was expressed as a '% inhibition' for control. The IC₅₀ was calculated using the software Graph Pad Prism 6.

Results:

1) a. Nucleation assay

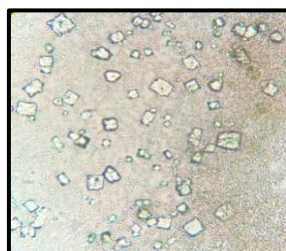


Plate I: Nucleation of Calcium Oxalate Crystals (Untreated)

i. *Celosia argentea*

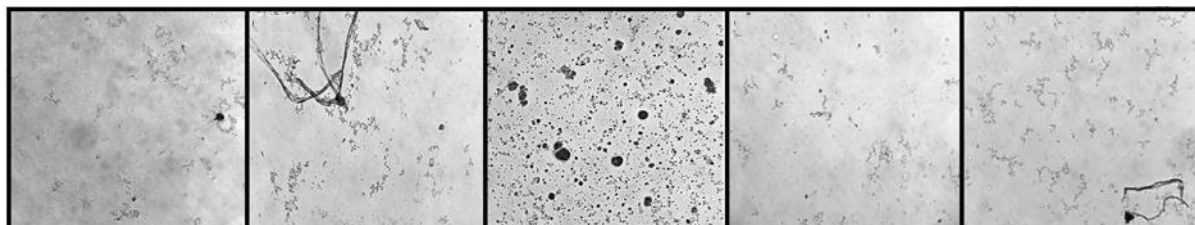


Plate II: Effect of *Celosia argentea* extract on Nucleation Inhibition

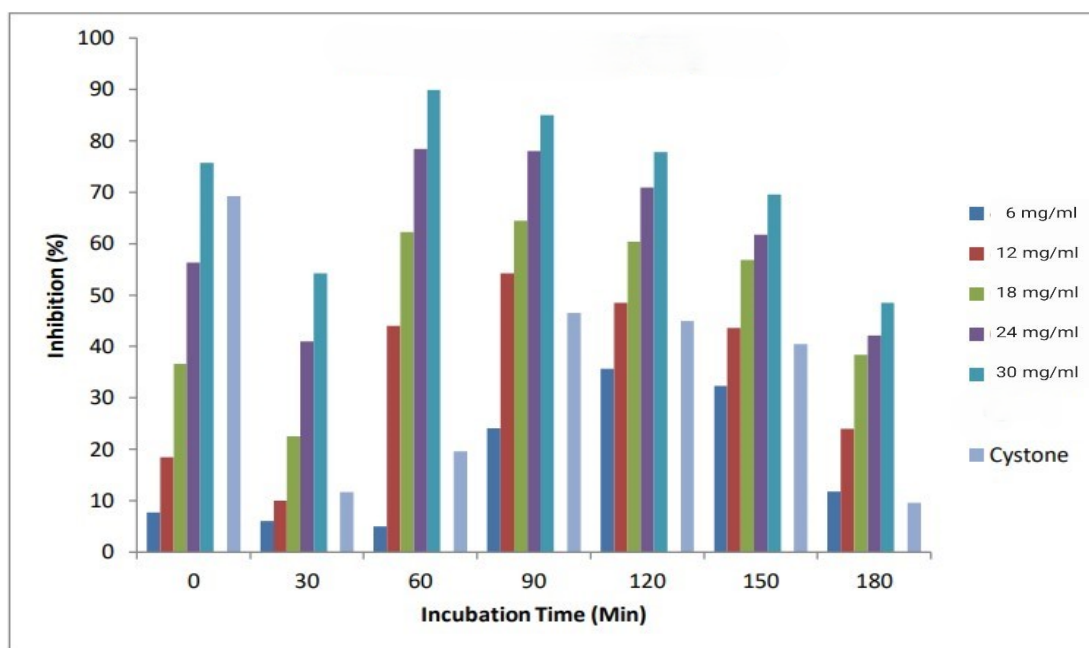


Figure I: Impact of Extracts of Roots of *Celosia argentea* on the nucleation process

ii. *Boerhavia diffusa*

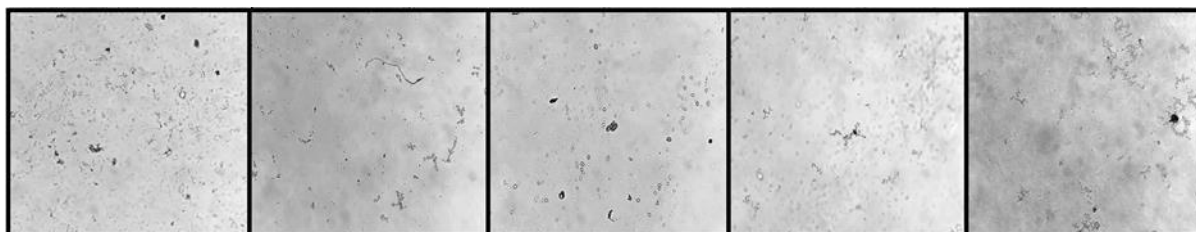


Plate III: Effect of *Boerhavia diffusa* extract on Nucleation Inhibition

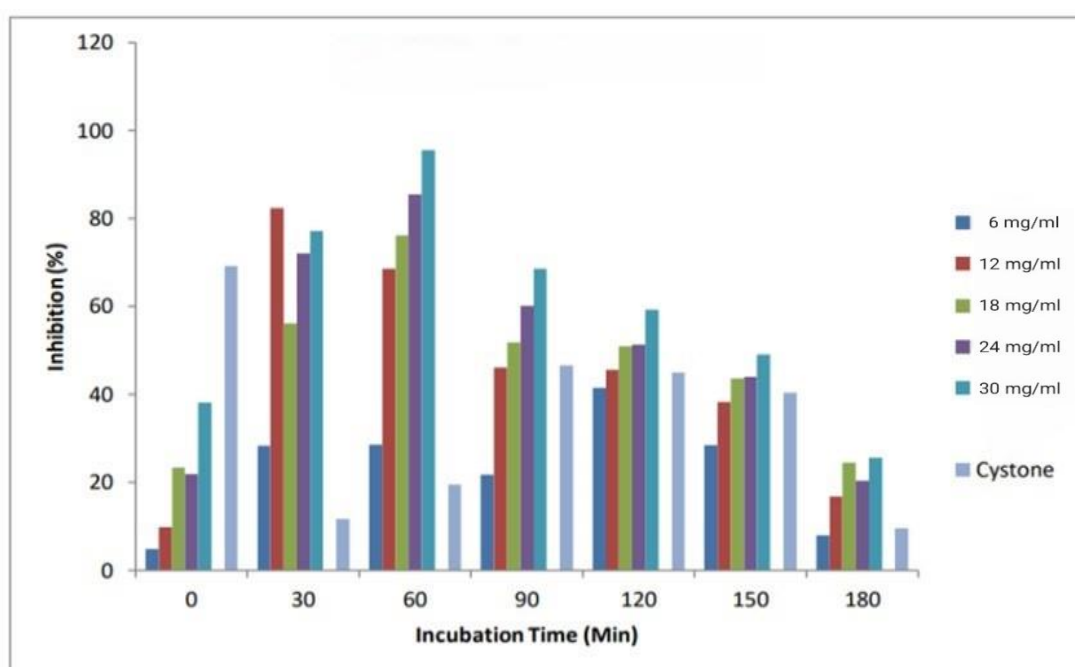


Figure II: Impact of Extracts of Roots of *Boerhavia diffusa* on the nucleation process

1. b. Aggregation assay

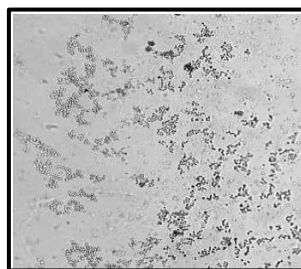


Plate IV: Aggregation of Calcium Oxalate Crystals (Untreated)

i. *Celosia argentea*



Plate V: Effect of *Celosia argentea* extract on Aggregation Inhibition

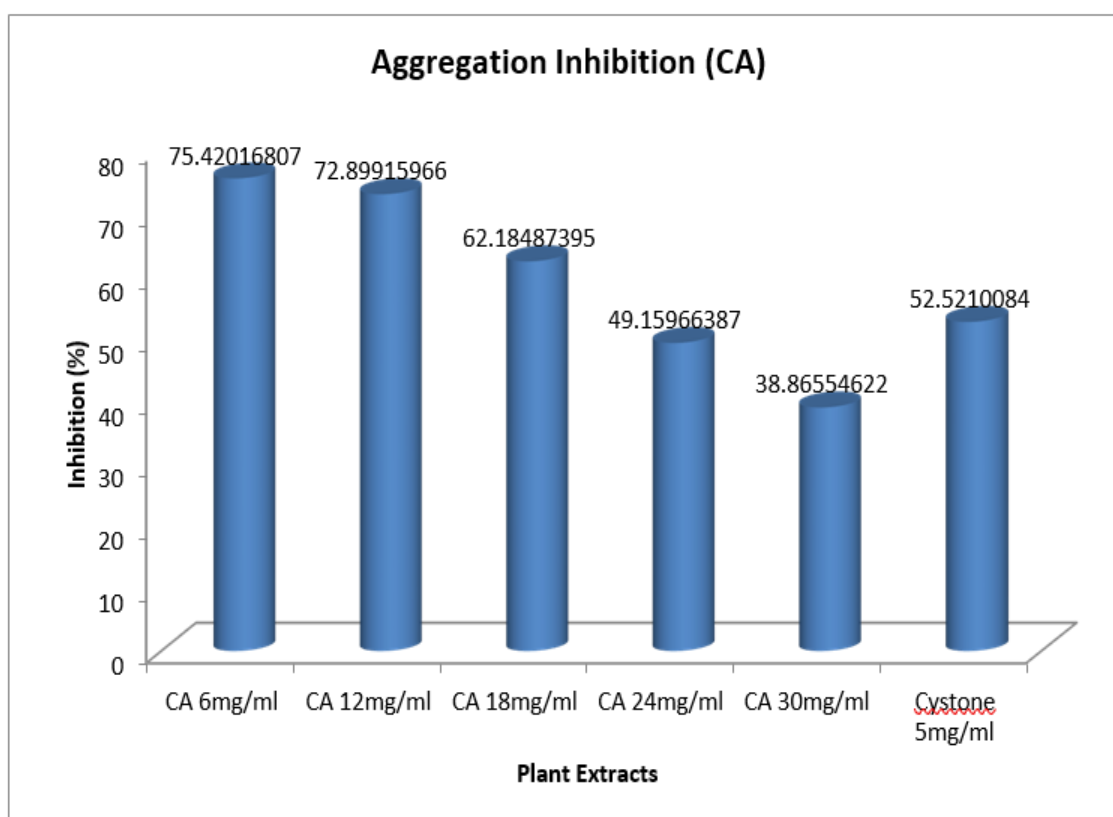


Figure III: Impact of Extracts of Roots of *Celosia argentea* on the aggregation process

ii. *Boerhavia diffusa*

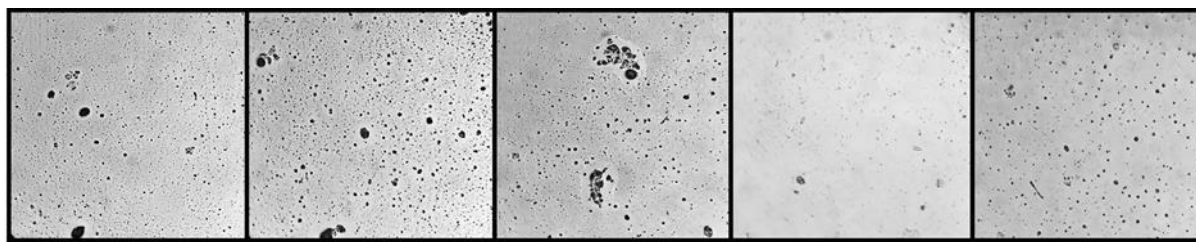


Plate VI: Effect of *Boerhavia diffusa* extract on Aggregation Inhibition

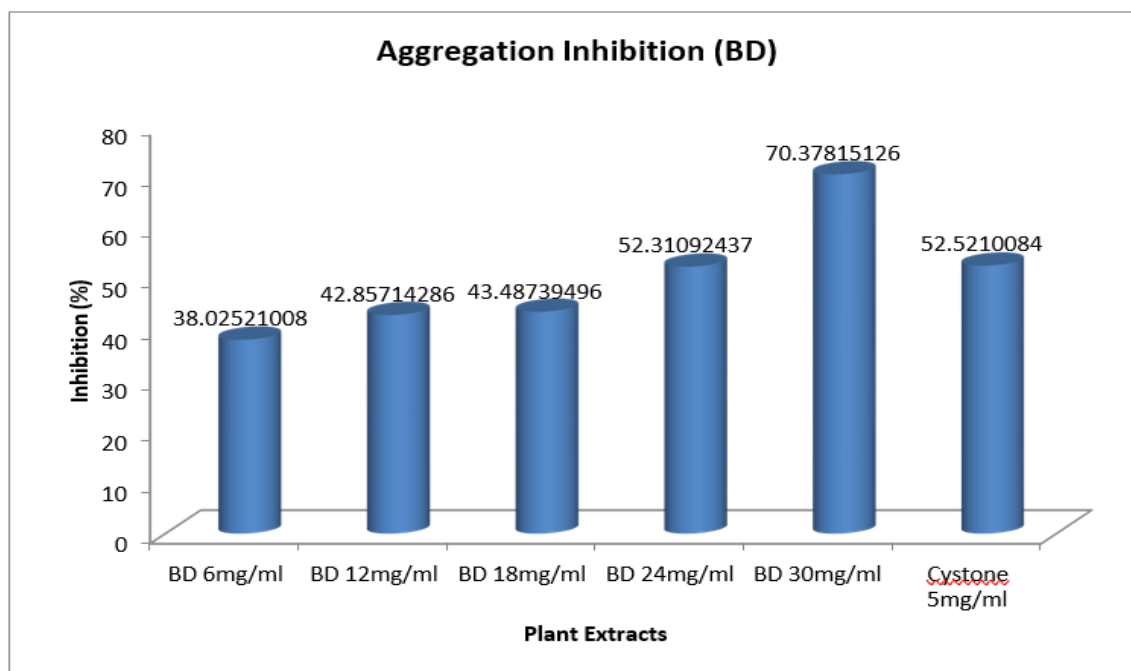


Figure IV: Impact of Extracts of roots of *Boerhavia diffusa* on the aggregation process

2) Gas chromatography-mass spectrometry (GCMS/MS)

i. *Celosia argentea*

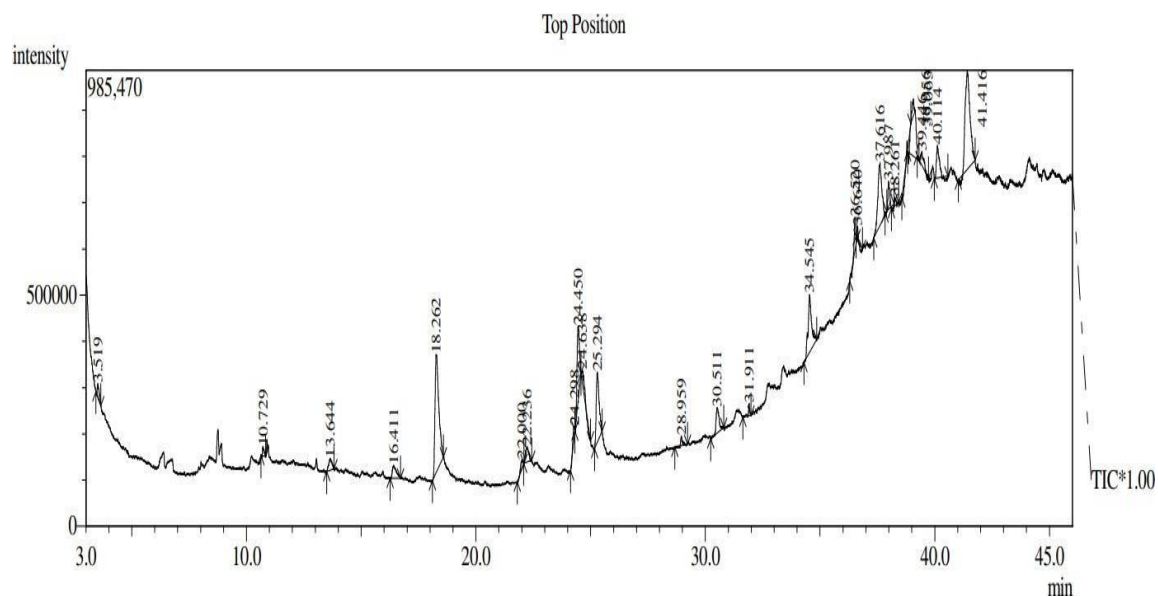


Plate VII: GC-MS/MS Chromatogram of *Celosia argentea* root extract

Table I: GC-MS/MS analysis of Major Compounds in the root of *Celosia argentea*

Sr. No.	Name of compound	Retention time	% peak area	Molecular formula	Mol. Weight g/mol
1)	Propane, 1,1,3-tri ethoxy-	3.519	1.03	C ₉ H ₂₀ O ₃	176.25
2)	Eicosane, 1-iodo-	10.729	0.57	C ₂₀ H ₄₁ I	408.4
3)	Sulfurous acid, pentadecyl 2-propyl ester	13.644	1.39	C ₁₈ H ₃₈ O ₃ S	334.6
4)	Hexadecanoic acid, methyl ester	16.411	1.98	C ₁₇ H ₃₄ O ₂	270.5
5)	Hexadecanoic acid, ethyl ester	18.262	18.10	C ₁₈ H ₃₆ O ₂	284.5
6)	9,12-Octadecadienoic acid, ethyl ester	22.000	0.77	C ₂₀ H ₃₆ O ₂	308.5
7)	9-Octadecenoic acid, methyl ester, (E)-	22.236	1.76	C ₁₉ H ₃₆ O ₂	296.5
8)	Ethyl Oleate	24.638	0.89	C ₂₀ H ₃₈ O ₂	310.5
9)	Cyclononasiloxane, octadecamethyl-	28.959	0.54	C ₁₈ H ₅₄ O ₉ Si ₉	667.4
10)	Ethyl 15-methyl-hexadecanoate	30.511	2.93	C ₁₉ H ₃₈ O ₂	298.5
11)	Octacosanal	36.520	1.74	C ₂₈ H ₅₆ O	408.7
12)	Tetracosamethyl-cyclododecasiloxane	36.640	-0.30	C ₂₄ H ₇₂ O ₁₂ Si ₁₂	889.8
13)	gamma.-Sitosterol	37.616	9.87	C ₂₉ H ₅₂ O ₂	432.7
14)	Ethyl henicosanoate	37.987	1.46	C ₂₃ H ₄₆ O ₂	354.6
15)	Olean-12-en-3-ol, acetate, (3.beta.)-	39.063	9.87	C ₃₂ H ₅₂ O ₂	468.8
16)	Olean-18-ene	39.446	1.23	C ₃₀ H ₅₀	410.7
17)	n-Nonadecanol-1	40.114	3.13	C ₁₉ H ₄₀ O	284.5

ii. *Boerhavia diffusa*

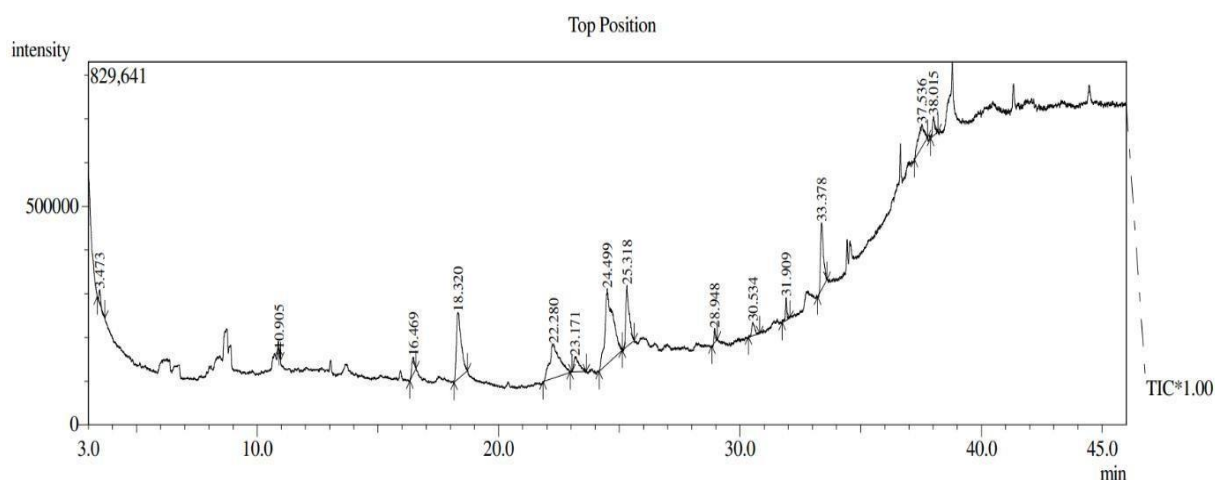
Plate VIII: GC-MS/MS Chromatogram of *Boerhavia diffusa* root extract

Table II: GC-MS/MS analysis of Major Compounds in the root of *Boerhavia diffusa*

Sr. No.	Name of compound	Retention time	% peak area	Molecular formula	Mol. Weight g/mol
1)	Propane, 1,1,3-triethoxy-	3.473	0.80	C ₉ H ₂₀ O ₃	176.25
2)	Hexadecanoic acid, methyl ester	16.469	2.19	C ₁₇ H ₃₄ O ₂	270.5
3)	Hexadecanoic acid, ethyl ester	18.320	15.50	C ₁₈ H ₃₆ O ₂	284.5
4)	6-Octadecenoic acid, methyl ester, (Z)-	22.280	16.43	C ₁₉ H ₃₆ O ₂	296.5
5)	Heptadecanoic acid, 16-methyl-, methyl ester	23.171	3.88	C ₁₉ H ₃₈ O ₂	298.5
6)	Ethyl Oleate	24.499	29.09	C ₂₀ H ₃₈ O ₂	310.5
7)	Octadecanoic acid, ethyl ester	25.318	10.05	C ₂₀ H ₄₀ O ₂	312.5
8)	Cyclononasiloxane, octadecamethyl-	28.948	0.68	C ₁₈ H ₅₄ O ₉ Si ₉	667.4
9)	Methyl 19-methyl-eicosanoate	30.534	1.95	C ₂₂ H ₄₄ O ₂	340.6
10)	Cyclononasiloxane, octadecamethyl-	31.909	1.50	C ₁₈ H ₅₄ O ₉ Si ₉	667.4
11)	Bis(2-ethylhexyl) phthalate	33.378	9.14	C ₂₄ H ₃₈ O ₄	390.6
12)	Chondrillasterol	37.536	6.58	C ₂₉ H ₄₈ O	412.7
13)	Ethyl henicosanoate	38.015	2.21	C ₂₃ H ₄₆ O ₂	354.6

3) Fourier Transform Infrared Spectroscopy (FT-IR)

i. *Celosia argentea*

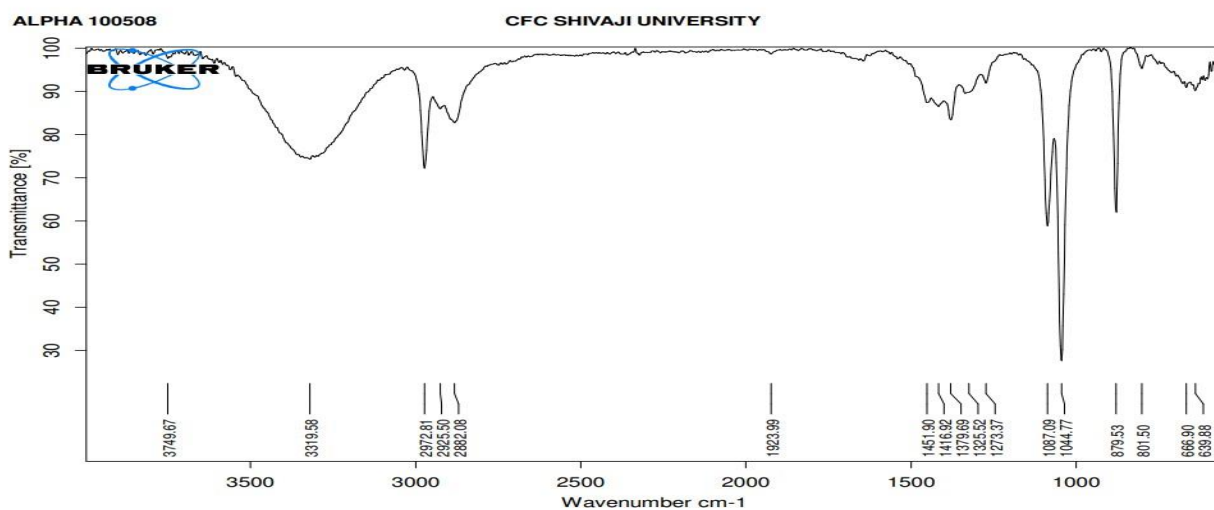
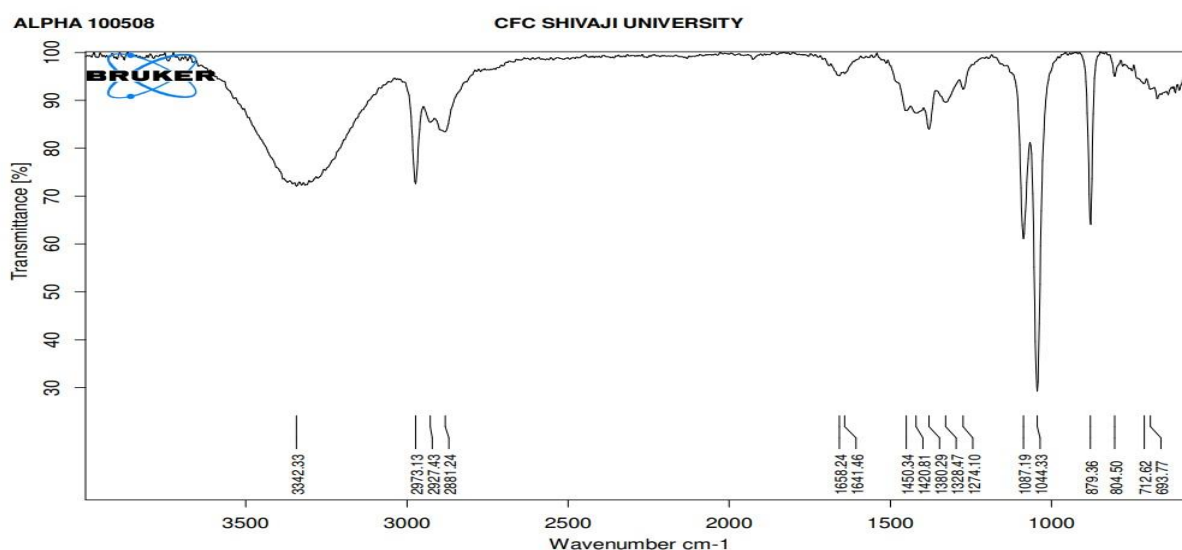
Plate IX: Fourier transform infrared spectroscopy (FTIR) spectral graph of the root of *Celosia argentea*

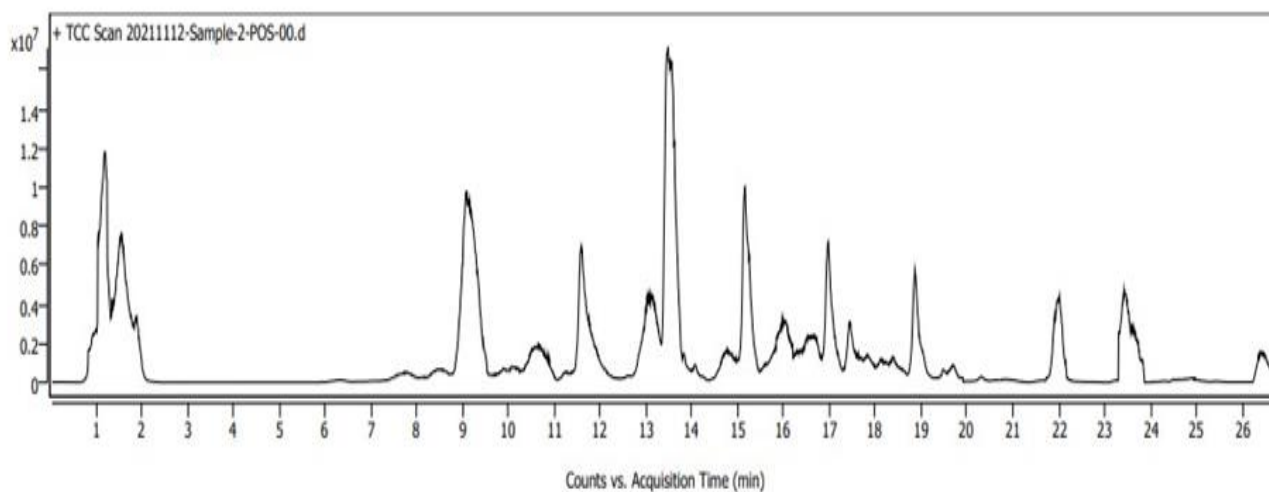
Table III: FTIR peak values of *Celosia aregentea* of root ethanolic extract

Sr. No.	Peak value	Group frequency cm-1	Functional compound class
1.	639.88	C-Cl stretching	halo compound
2.	879.53	C-H bending	1,2,4-trisubstituted
3.	1044.77	C-N stretching	amine
4.	1087.09	C-O stretching	aliphatic ether
5.	1273.37	C-O stretching	aromatic ester
6.	1325.52	O-H bending	phenol
7.	1379.69	O-H bending	alcohol
8.	1416.92	O-H bending	carboxylic acid
9.	1451.90	C-H bending	alkane
10.	2882.08	N-H stretching	amine salt

ii. *Boerhavia diffusa*Plate X: Fourier transform infrared spectroscopy (FTIR) spectral graph of root of *Boerhavia diffusa*Table IV: FTIR peak values of *Boerhavia diffusa* of root ethanolic extract

Sr. No.	Peak value	Group frequency cm-1	Functional compound class
1.	693 .77	C=C bending	aklene
2.	804 .50	C-Cl stretching	halo compound
3.	879 .36	C-H bending	1,2,4-trisubstituted
4.	1044.33	C-F stretching	fluoro compound
5.	1328.47	C-O stretching	alkyl aryl ether
6.	1420.81	O-H bending	carboxylic acid
7.	1450.34	C-H bending	alkane
8.	1641.46	C=N stretching	imine / oxime
9.	1658.24	C=C stretching	alkene
10.	2881.24	O-H stretching	carboxylic acid
11.	3342 .33	O-H stretching	alcohol

4) High Resolution Liquid chromatography-mass spectrometry (HR-LCMS/MS)

i. *Celosia argentea*Plate XI: (HR)LC-MS/MS Spectrum of Roots of *Celosia argentea*Table V: (HR) LC-MS Profiling of Bioactive Compounds from ethanolic extract of root of *Celosia argentea*

Sr. No.	Name	Formula	RT	Mass	Score	Activity	Reference
1	2-Hydroxymyristic Acid	$C_{14}H_{28}O_3$	9.398	244.2045	97.11	Colorectal cancer	Silke <i>et al.</i> , 2016.
2	Sphinganine	$C_{18}H_{39}NO_2$	15.160	301.2981	95.09	Colorectal cancer	Silke <i>et al.</i> , 2016.
3	2-Eicosenoyl glycerol	$C_{23}H_{44}O_4$	17.814	384.3252	90.82	Colorectal cancer	Silke <i>et al.</i> , 2016.

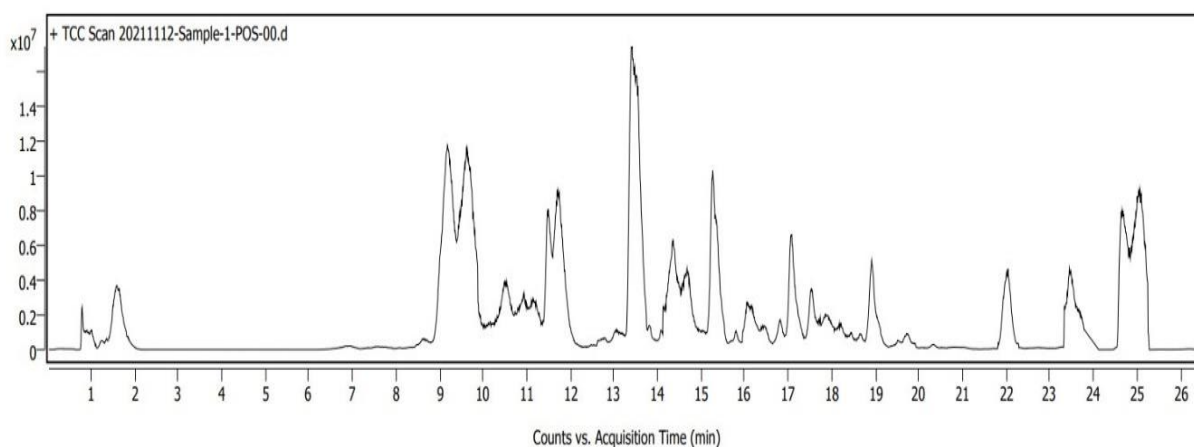
ii. *Boerhavia diffusa*Plate XII: (HR)LC-MS/MS Spectrum of Roots of *Boerhavia diffusa*

Table VI: (HR) LC-MS Profiling of Bioactive Compounds from ethanolic extract of root of *Boerhavia diffusa*

Sr. No.	Name	Formula	RT	Mass	Score	Activity	Reference
1	2-Hydroxymyristic Acid	C ₁₄ H ₂₈ O ₃	9.494	244.2040	98.07	Colorectal cancer	Silke <i>et al.</i> , 2016.
2	13Z,16Z-docosadienoic acid	C ₂₂ H ₄₀ O ₂	16.686	336.3032	95.53	Colorectal cancer	Silke <i>et al.</i> , 2016.
3	Glyceryl erucate	C ₂₅ H ₄₈ O ₄	19.724	19.724	92.91	Colorectal cancer	Silke <i>et al.</i> , 2016.

5) DPPH radical scavenging activity

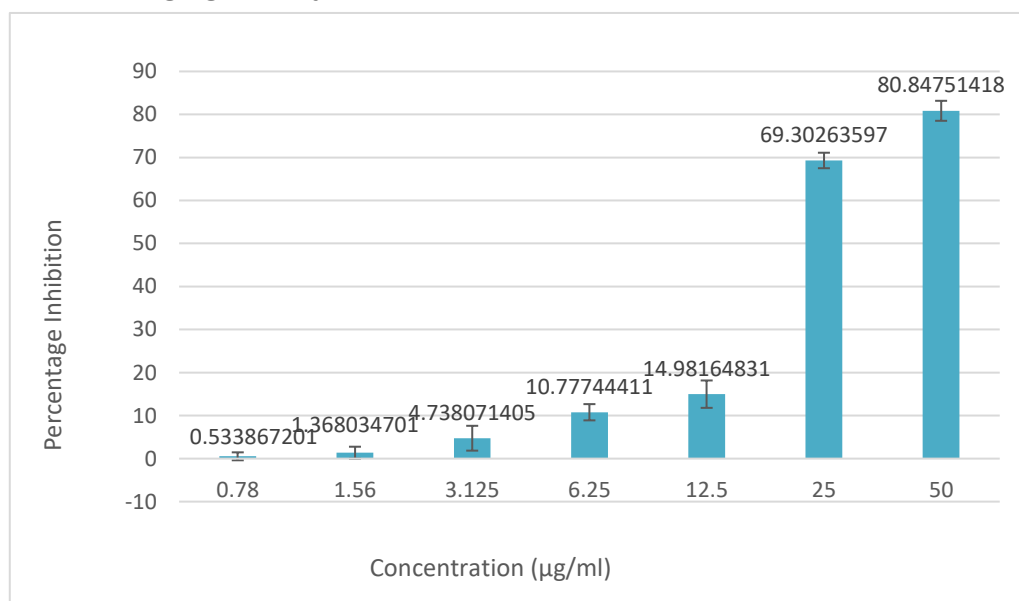
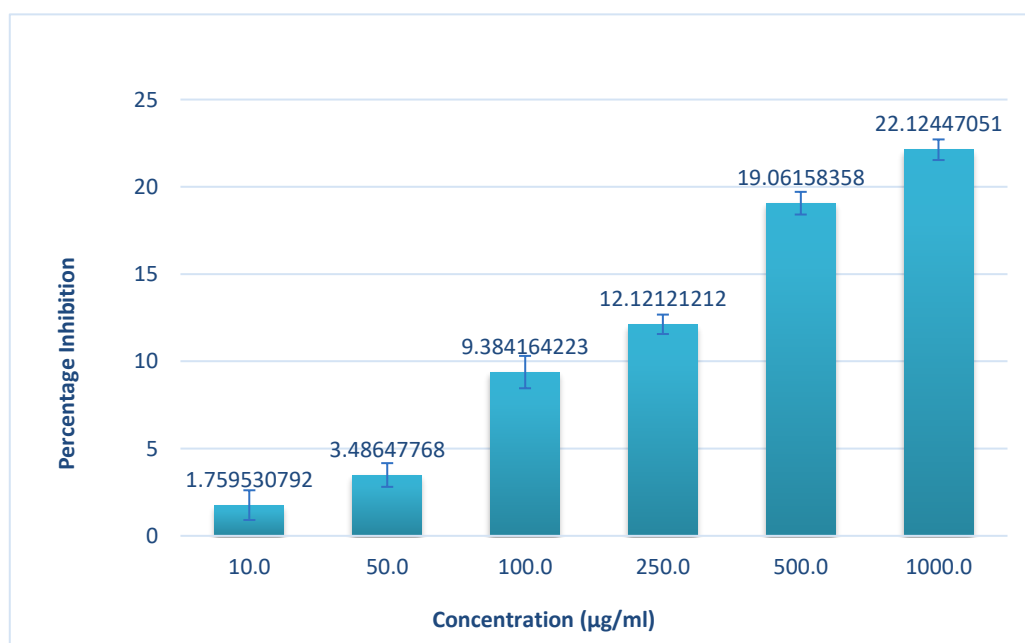


Figure V: DPPH radical scavenging activity of Standard Ascorbic acid

Figure VI: DPPH radical scavenging activity of *Celosia argentea*

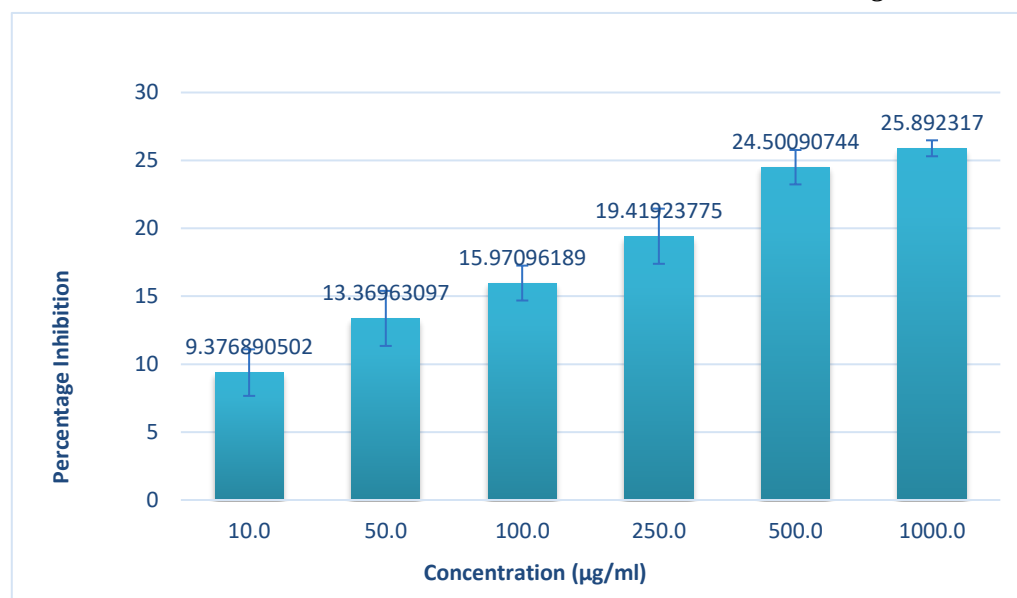


Figure VII: DPPH radical scavenging activity of *Boerhavia diffusa*

Discussion:

The current study on *C. argentea* and *B. diffusa* revealed the existence of many types of phytoconstituents. The existence of numerous secondary metabolites in medicinal plants may account for their healing powers. Thus, the preliminary screening test may be effective in detecting bioactive substances that can dissolve kidney stones. These 2 plant species' consecutive ethanolic extracts revealed the presence of a root plant portion. This suggests that the two *C. argentea* and *B. diffusa* plant species are useful for treating kidney stone illnesses. The herbs *C. argentea* and *B. diffusa* can help with urinary tract stones because they have chemicals in them that stop calcium oxalate crystals from forming and sticking together. Researchers have extensively studied plant extracts for their potential immunomodulatory effects. We previously reported on the ethanolic extract of *C. argentea* and *B. diffusa* root, an Indian traditional medicine plant (Pandeya *et al.*, 2005). Several plants used in the traditional Indian medicine system have immunomodulatory properties. *Boerhavia diffusa* (Punarnava; Family Nyctaginaceae) is a creeping weed found abundantly all over India. In Indian traditional medicine, the roots of this weed are used for the treatment of dyspepsia, jaundice, enlargement of the spleen, abdominal pain, and as an antistress agent (Kirtikar *et al.*, 1996). *Boerhavia diffusa* has several ethnobotanical uses (the leaves are used as a vegetable, and the root juice is used to treat asthma, urinary problems, leucorrhoea, rheumatism, and encephalitis), as well as therapeutic usage in the old Ayurvedic system. Furthermore, the plant is said to have numerous pharmacological, therapeutic, and antibacterial characteristics (Chopra *et al.*, 1996). The morning root decoction is effective in treating kidney stones (Sangeetha *et al.*, 2023).

Conclusion:

Ethanolic plant extracts treat calcium oxalate crystals using nucleation and aggregation assays. The extracts of *C. argentea* and *B. diffusa* (root) have antiurolithiatic properties. According to the test used to measure major nucleation and aggregation, some medicinal herbs are antiurolithiatic and have a strong chance of stopping the process of nucleation and aggregation. Conducting additional phytochemical studies to pinpoint the bioactive component that inhibits and degrades calcium oxalate crystals. We use methods like GC-MS/MS, FTIR, DPPH, and (HR) LC-MS/MS. The ethanolic extract's GC-MS/MS results revealed the presence of several phytochemical components. Several compounds possess antioxidant, antibacterial, and anticancer properties, with some finding application in the treatment of urological issues. The (HR) LC-MS/MS results demonstrate anti-cancer activity. The existence of these phytochemicals in *C. argentea* and *B. diffusa* (root) is an important result of our investigation. Numerous bioactive chemicals have demonstrated their medicinal value. These species demonstrate antioxidant, antibacterial, anti-nutritional, antifungal, antidiabetic, anti-inflammatory, and anticancer actions, implying that they have a variety of therapeutic qualities and high nutritional potential.

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