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In-Vitro Study of The Effect of *Celosia argentea* And *Boerhaiva 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 find out how well *Boerehavia 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 leaves of *Celosia argentea* stopped calcium oxalate (Caox) from crystallizing in a lab setting.

Keywords- Medicinal plant, Kidney stone, Nucleation, Aggregation, Antiurolithiatic activity.

Introduction

India is a land of ancient medicinal healing plants. Over thirty percent of all plant types are used to make medicines. (Kanwar and Gupta 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 (Mikawlrawng *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 – 45ºC boiling range) as a solvent. The extraction of the solvent was stored at – 4ºC for further analysis. The various concentration of ethanolic extracts was prepared for further analysis.

A) Nucleation assay

A spectrophotometric assay was used to evaluate the extracts' ability to inhibit the nucleation of CaOx crystals. (Hennequin *et al*., 1993). Artificial urine was mixed with solutions of calcium chloride (4 mmol/L) and sodium oxalate (50 mmol/L), which were both prepared in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 and 37 °C. This action initiated the crystallization process. We calculated the rate of nucleation 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, the absorbance (optical density, or OD) was measured. The % inhibition was computed as [OD (experimental)/OD (control)].100.

B) 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. We measured the absorbance at 620 nm 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-Si/Sc).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.

C) Gas chromatography-mass spectrometry (GCMS/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 μMdf, 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 μl (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)

D) 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).

E)High-Resolution Liquid chromatography-mass spectrometry (HR-LCMS/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).

F)DPPH

Assay of DPPH radical scavenging activity

The extracts' DPPH free radical scavenging activity was determined using the method described by (Braca *et al*., 2001). 200 µl plant extract (5, 25, 50, 100, 500 µg/ml) was combined with 2 ml of a 0.004% methanol solution of DPPH. After 30 minutes, the absorbance at 517 nm was measured using a Shimadzu UV-1601PC spectrophotometer against a blank. The absorbance of the DPPH solution without extract or standard agent was used as a control. The extracts' percentage scavenging activity was calculated using the formula: % scavenging activity = {(A0 – A1) / A0} × 100, where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard. Determining the reducing power Antioxidants in the sample break down the Fe3+/ferricyanide complex into its ferrous form, which is used for the test (Oyaizu, 1986). In the experiment, extract solutions (5-200 µg/ml) were mixed with 2.5 ml (1%) potassium ferricyanide and incubated at 50 °C for 10 minutes. After adding 2.5 ml of 10% trichloroacetic acid, we centrifuged the mixture at 3000 rpm for 10 minutes. 2.5 mL of the supernatant solution was combined with 2.5 mL of distilled water and 0.5 mL (0.1%) of ferric chloride. The solution's absorbance was measured at 700 nm against a blank. The reaction mixture without extract was used as a blank. The percentage reducing capacity was calculated using the formula: % reducing ability = {(Am – Ab) / Ab} × 100, where Am is the absorbance of the reaction mixture and Ab is the absorbance of the blank. (Imam *et al*., 2011).

Methods

In a 96-well plate, 5 µl 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 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 µl of deionized water. The scavenging activity was expressed as a '% inhibition' for control. The IC-50 was calculated using the software Graph Pad Prism 6.

FRAP Assay

The ferric-reducing antioxidant power (FRAP) assay The FRAP assay was carried out according to Benzie et al. (1996), with minor modifications suggested by Firuzi and Lancana (2005). The FRAP reagent was prepared and stored at 37 °C for 5-10 minutes before use. In a 96-well plate, add 175 µL of FRAP reagent and pre-read at 593 nm. To each well, add 25 µL of sample solution and mix thoroughly with a pipette. We measured the absorbance at 593 nm for 4, 15, 30, and 60 minutes, using methanol as a blank. The change in absorbance at different time intervals was then converted to a FRAP value using a standard Trolox curve. (Rao *et al*., 2013).

Results

1) Nucleation assay

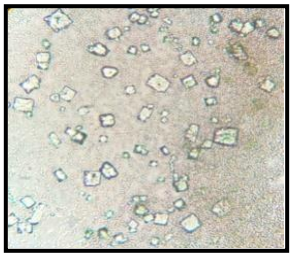

Plate I: Nucleation of Calcium Oxalate Crystals (Untreated)

*i. **Celosia argentea***

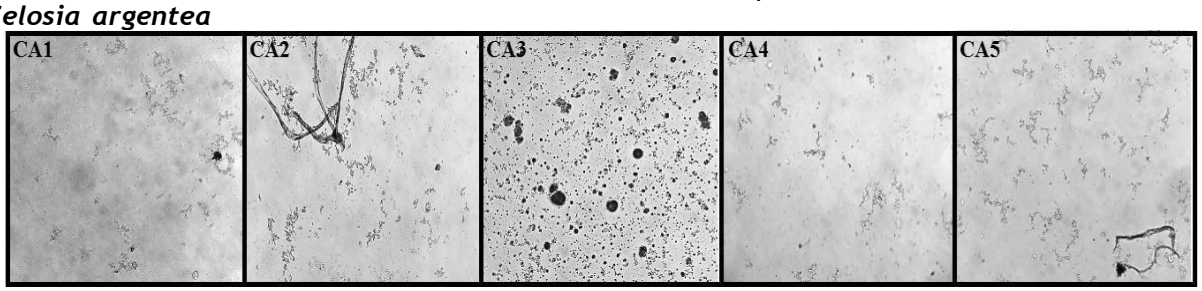

Plate II: Effect of *Celosia argentea* extract on Nucleation Inhibition

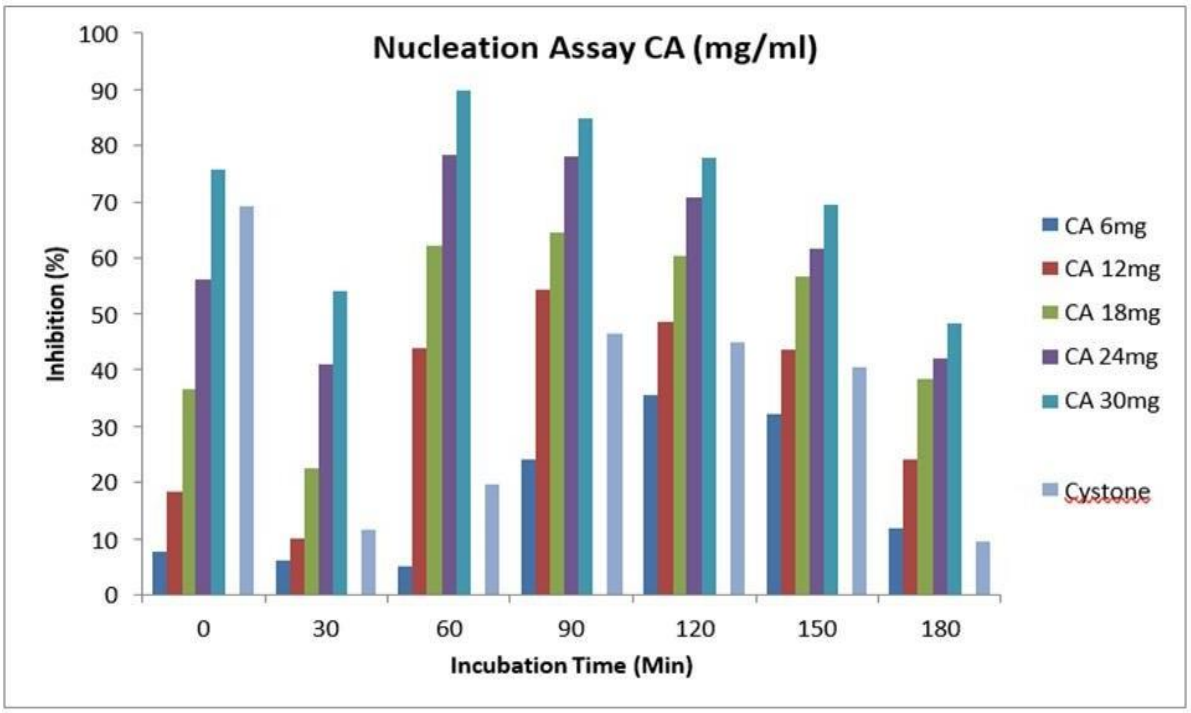

Fig I: Nucleation Assay of Plant Extract (*Celosia argentea*)

*ii. **Boerhavia diffusa***

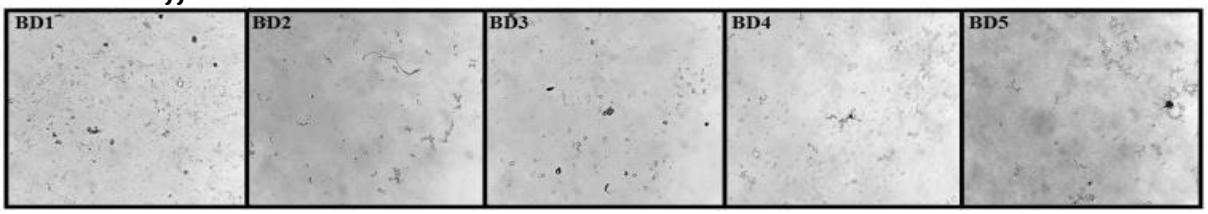

Plate III: Effect of *Boerhavia diffusa* extract on Nucleation Inhibition

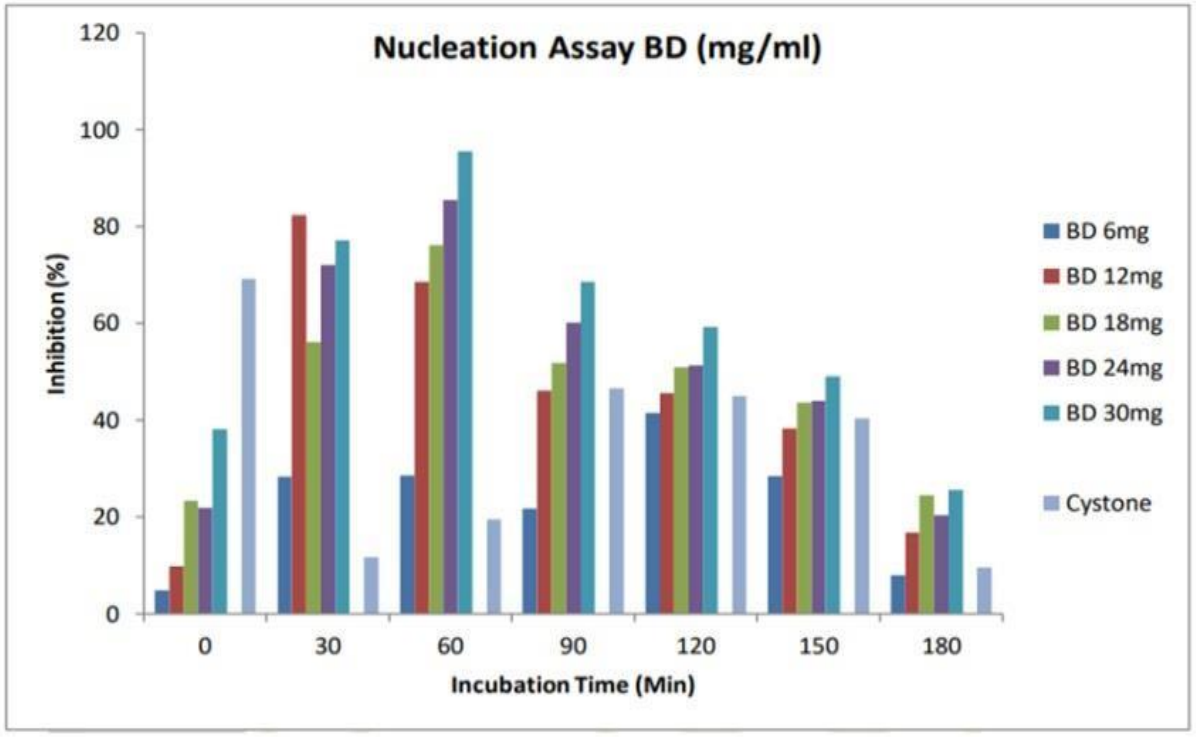

Fig II: Nucleation Assay of Plant Extract (*Boerhavia diffusa*)

2) Aggregation assay

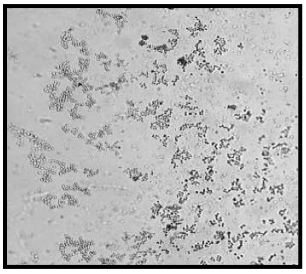

Plate IV: Aggregation of Calcium Oxalate Crystals (Untreated)

i. ***Celosia argentea***

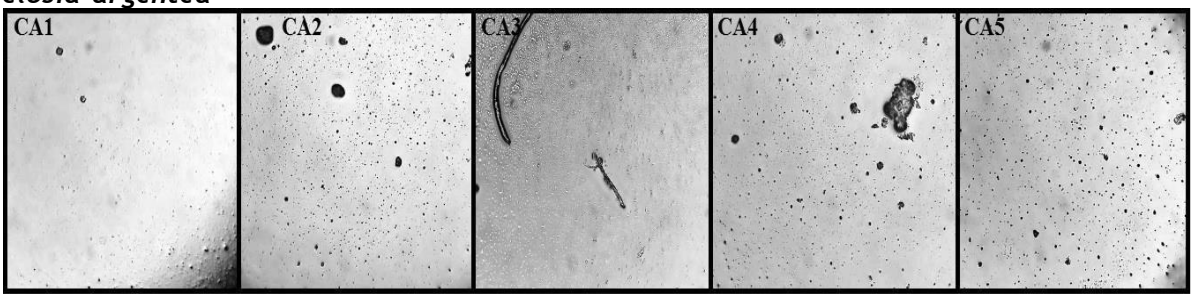

Fig V: Effect of *Celosia argentea* extract on Aggregation Inhibition

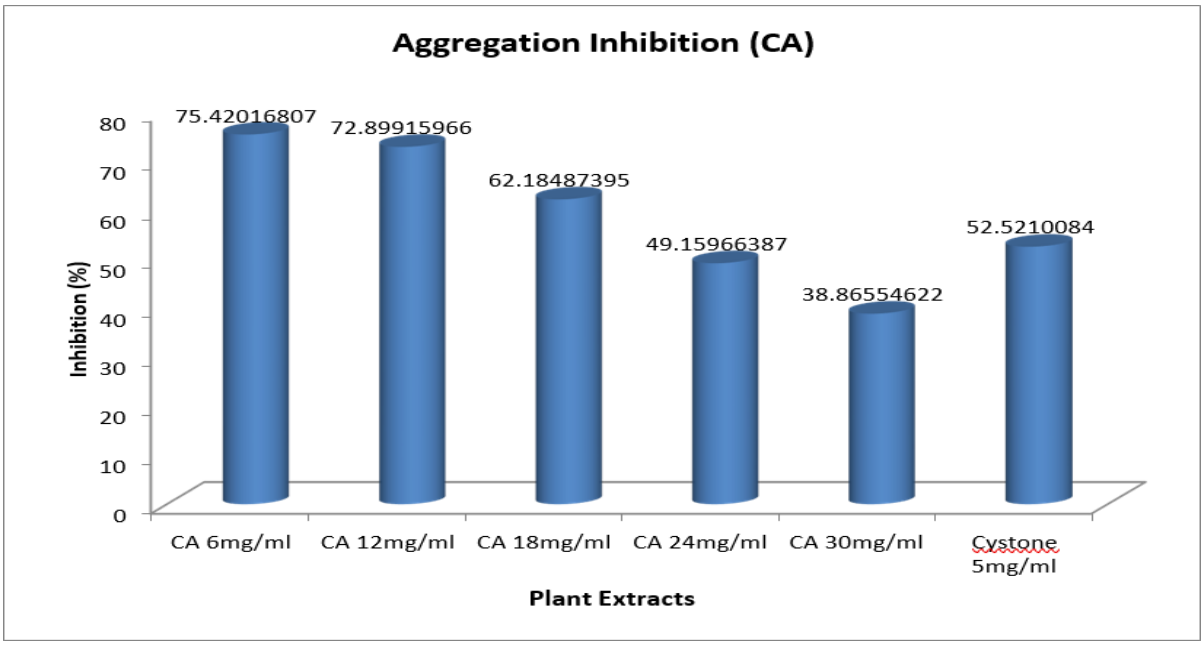

Fig III: Aggregation Assay of Plant Extract (*Celosia argentea*)

ii. Boerhavia diffusa

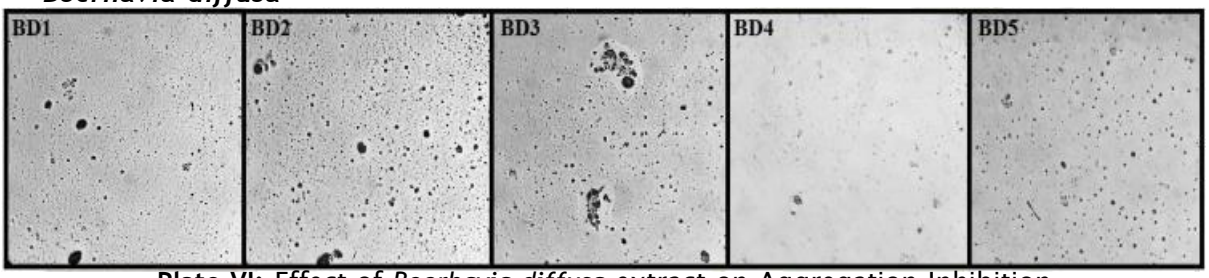

Plate VI: Effect of *Boerhavia diffusa* extract on Aggregation Inhibition

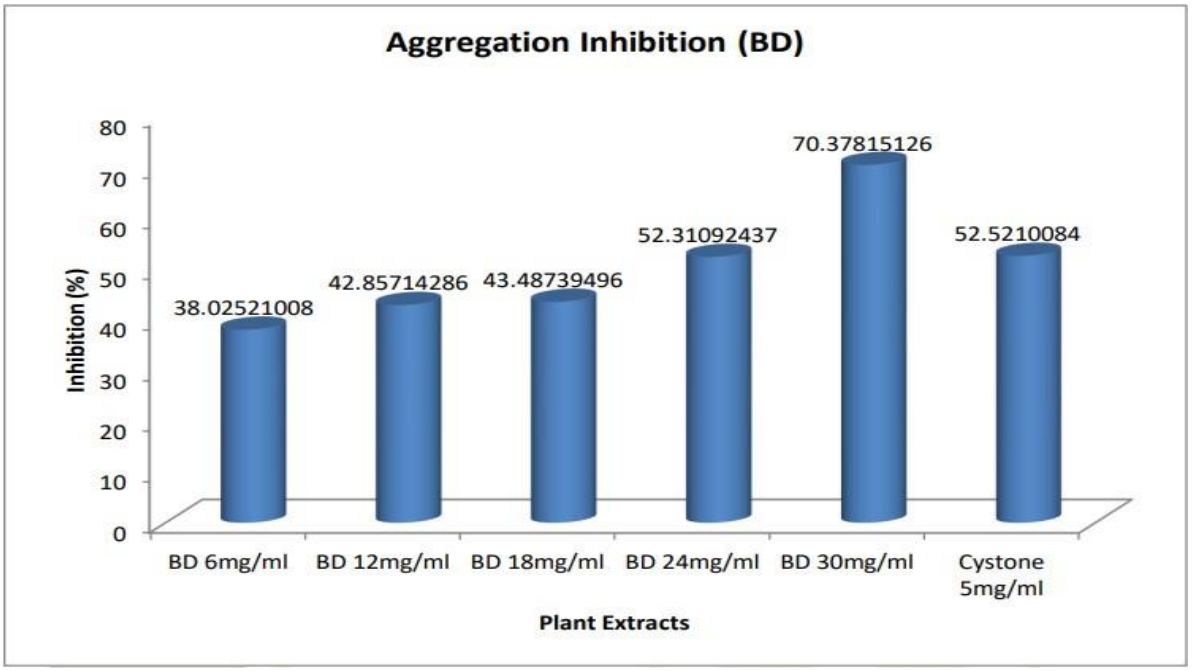

Fig IV: Aggregation Assay of Plant Extract (*Boerhavia diffusa*)

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

i. Celosia argentea

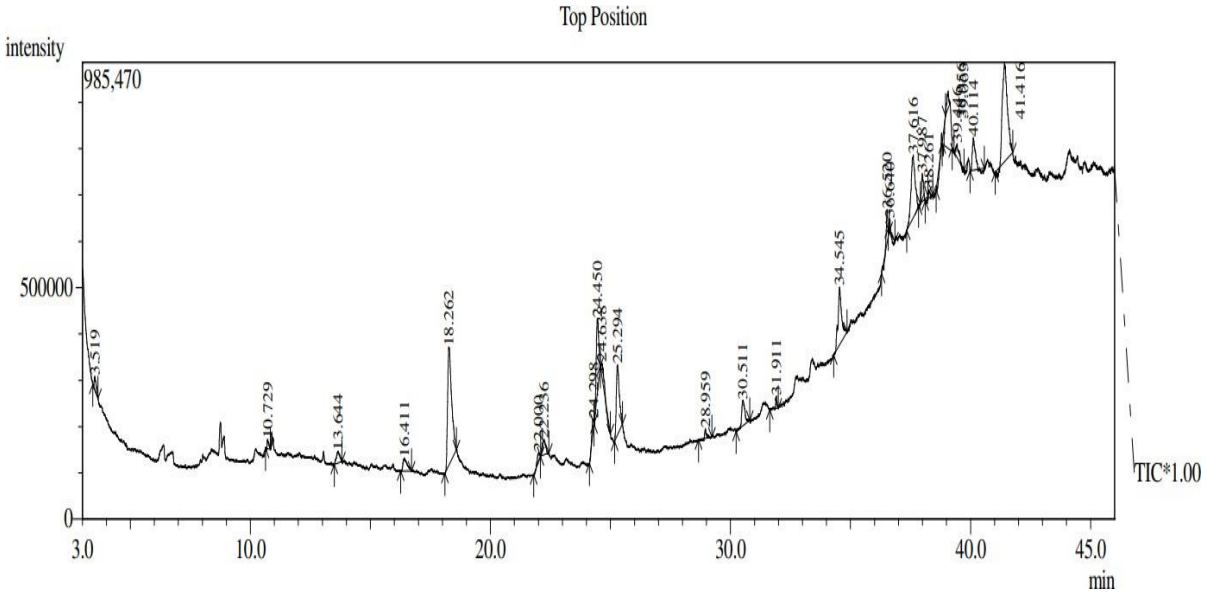

Fig V: GC-MS/MS chromatogram of ethanolic extract of roots of *Celosia argentea*

Table I: GC-MS/MS analysis Major compounds identified an ethanolic extract of *Celosia argentea* with molecular formula and molecular weights.

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	$C_9H_{20}O_3$	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	$C_{18}H_{38}O_3S$	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)	(E)-9-Octadecenoic acid ethyl ester	24.450	7.29	$C_{20}H_{38}O_2$	310.5
9)	Ethyl Oleate	24.638	0.89	$C_{20}H_{38}O_2$	310.5
10)	Octadecanoic acid, ethyl ester	25.294	8.19	$C_{20}H_{38}O_2$	310.5
11)	Cyclononasiloxane, octadecamethyl-	28.959	0.54	$C_{18}H_{54}O_9Si_9$	667.4
12)	Ethyl 15-methyl-hexadecanoic	30.511	2.93	$C_{19}H_{38}O_2$	298.5
13)	Tetracosamethyl-cyclododecasiloxan e	31.911	0.62	$C_{24}H_{72}O_{12}Si_{12}$	889.8
14)	Ethyl 14-methyl-hexadecanoic	34.545	7.95	$C_{19}H_{38}O_2$	298.5
15)	Octacosanal	36.520	1.74	$C_{28}H_{56}O$	408.7

16)	Tetracosamethyl-cyclododecasiloxane	36.640	-0.30	$C_{24}H_{72}O_{12}Si_{12}$	889.8
17)	gamma-Sitosterol	37.616	9.87	$C_{29}H_{52}O_2$	432.7
18)	Ethyl henicosanoate	37.987	1.46	$C_{23}H_{46}O_2$	354.6
19)	Olean-12-en-3-ol, acetate, (3 beta)-	39.063	9.87	$C_{32}H_{52}O_2$	468.8
20)	Olean-18-ene	39.446	1.23	$C_{30}H_{50}$	410.7
21)	n-Nonadecanol-1	40.114	3.13	$C_{19}H_{40}O$	284.5
22)	Lup-20(29)-en-3-ol, acetate, (3 beta.)-	41.416	18.81	$C_{32}H_{52}O_2$	468.8

ii. ***Boerhavia diffusa***

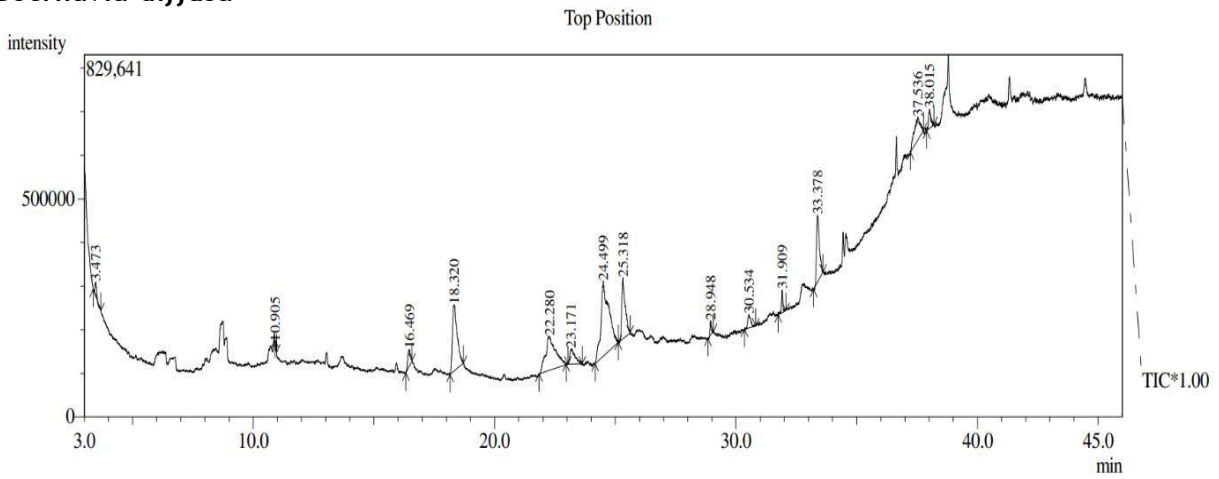

Fig VI: GC-MS/MS chromatogram of ethanolic extract of roots of *Boerhaiva diffuse*

Table II: GC-MS/MS analysis Major compounds identified an ethanolic extract of *Boerhaiva diffusa* with molecular formula and molecular weights.

Sr. No.	Name of compound	Retention time	% peak area	Molecular formula	Mol. Weight g/mol
1)	Propane, 1,1,3-tri ethoxy-	3.473	0.80	$C_9H_{20}O_3$	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	$C_{18}H_{54}O_9Si_9$	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	$C_{18}H_{54}O_9Si_9$	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

The current study was conducted to identify its phytochemical using GC-MS/MS analysis. The current study examines the Gas Chromatography Mass Spectroscopy of Celosia argentera and Boerhaiva diffusa, which have a variety of medicinal properties. A GC-MS/MS analysis was also performed, and it revealed the presence of phytochemicals. As a result, plant extracts with high bioavailability and retention of specific compounds can be recommended for use as a natural medicine for treating infectious diseases.

4) Fourier Transform Infrared Spectroscopy (FT-IR)

The presence of functional groups in the ethanolic extracts was determined through FTIR analysis. An FTIR spectrophotometer was used to identify the functional groups in a subset of plant samples. The standard peak value and the verified current functional groups were compared to the FTIR image locations. FTIR analysis revealed that the ethanolic extracts of C. aregentea and B. diffusa contained a number of different functional groups.

i. Celosia aregentea

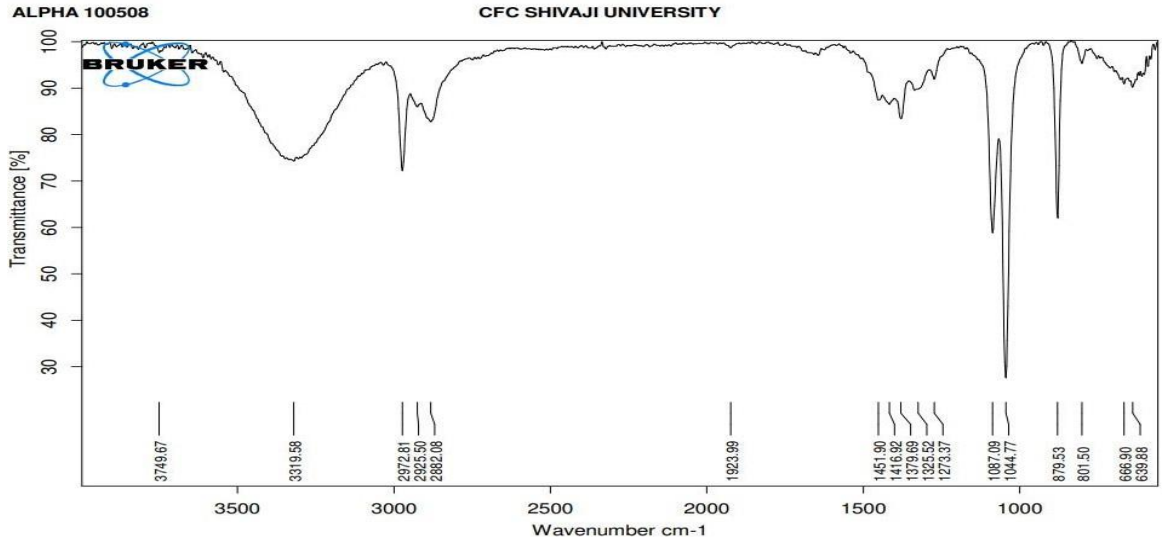

Figure VII: FTIR spectral analysis of an ethanolic extract of *Celosia aregentea*

ii. Boerhaiva diffusa

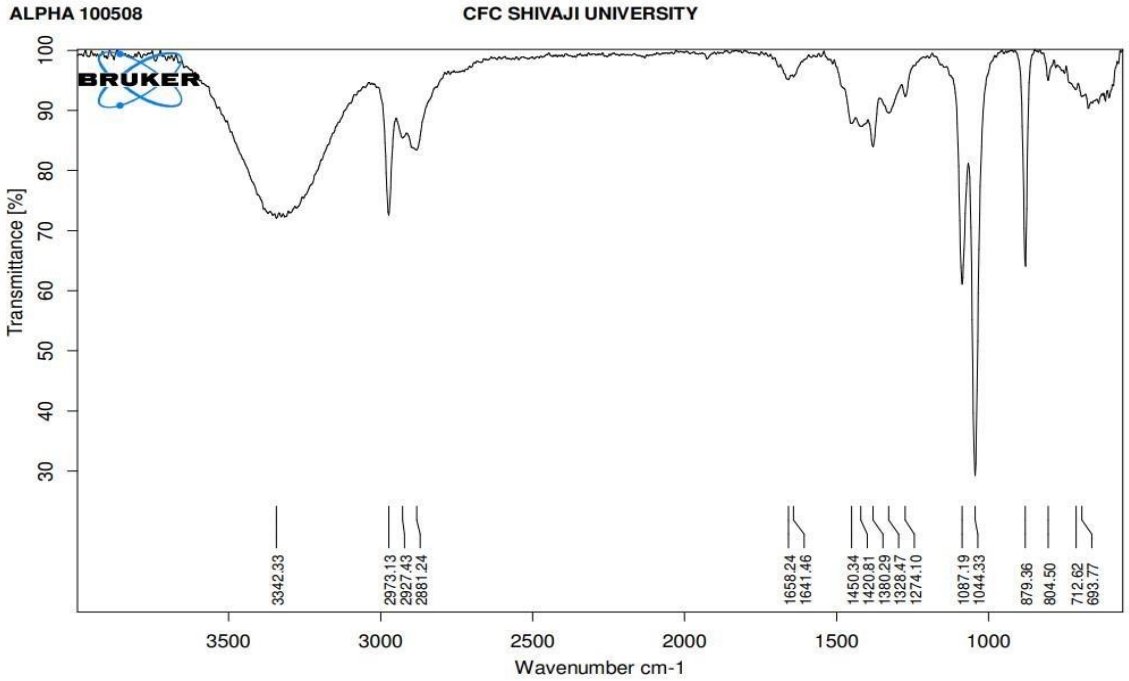

Figure VIII: FTIR spectral analysis of ethanolic extract of *Boerhaiva diffusa*

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

i. Celosia argentea

Figure IX: HRLC-MS/MS chromatogram of ethanolic extract of Roots of *Boerhavia diffusa*

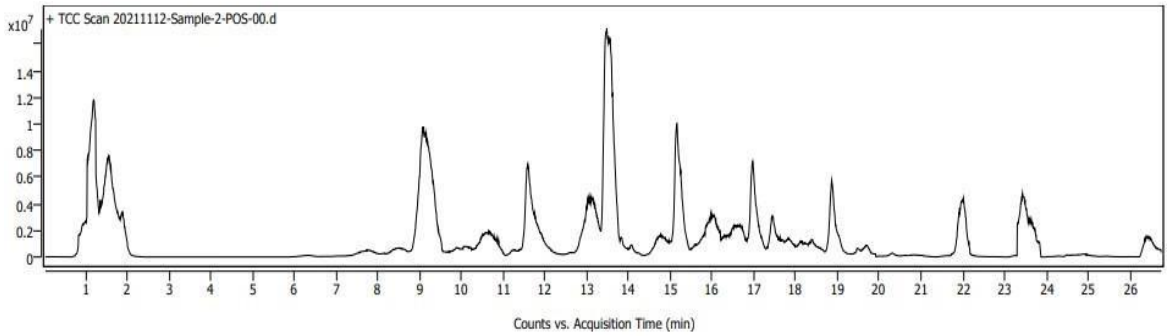

Table No. III. Chemoprofiling of *Celosia aregentea* by HRLC-MS/MS Analysis

Sr.No.	Name	Formula	RT	Mass	Score	Properties	Reference
1	2-Hydroxymyristic Acid	C14 H28 O3	9.398	244.2045	97.11	Colorectal cancer	Silke *et al*., 2016.
2	Sphinganine	C18 H39 N O2	15.160	301.2981	95.09	Colorectal cancer	Silke *et al*., 2016.
3	2- Eicosenoyl -glycerol	C23 H44 O4	17.814	384.3252	90.82	Colorectal cancer	Silke *et al*., 2016.

ii. Boerhavia diffusa

Figure X: HRLC-MS/MS chromatogram of ethanolic extract of Roots of *Boerhavia diffusa*

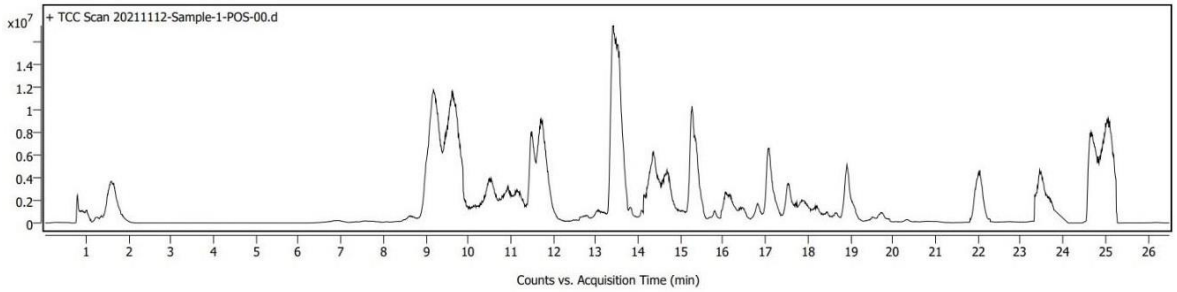

Table No. IV. Chemoprofiling of *Boerhavia diffusa* by HRLC-MS/MS Analysis

Sr.No.	Name	Formula	RT	Mass	Score	Properties	Reference
1	2-Hydroxymyristic Acid	C14 H28 O3	9.494	244.2040	98.07	Colorectal cancer	Silke *et al*., 2016.
2	13Z,16Z-docosadienoic acid	C22 H40 O2	16.686	336.3032	95.53	Colorectal cancer	Silke *et al*., 2016.
3	Glyceryl erucate	C25 H48 O4	19.724	19.724	92.91	Colorectal cancer	Silke *et al*., 2016.

6) DPPH radical scavenging activity

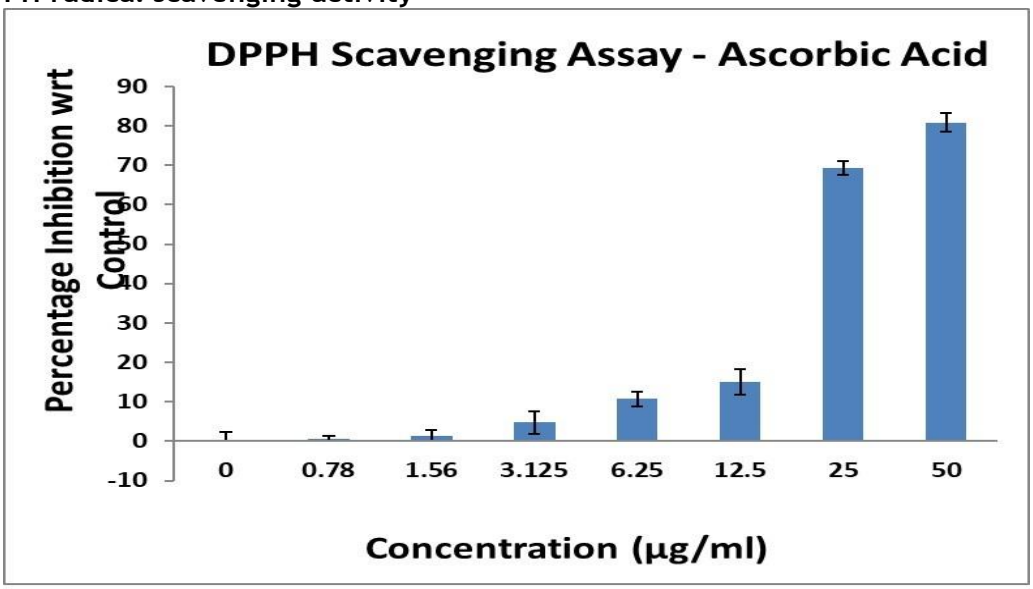

Figure XI: DPPH analysis of Standard Ascorbic Acid

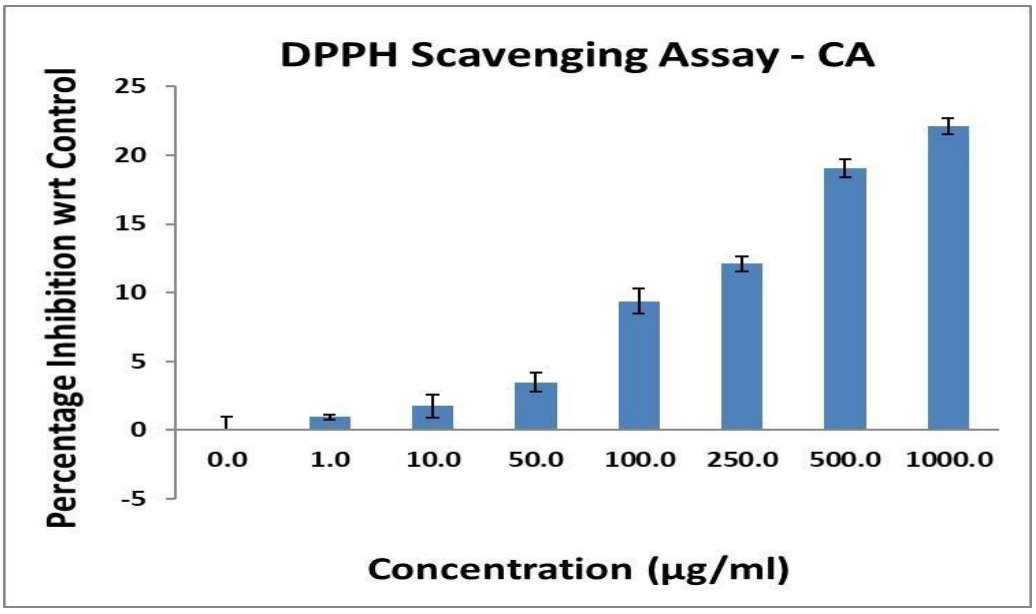

Figure XII: DPPH analysis of ethanolic extract of *Celosia aregentea*

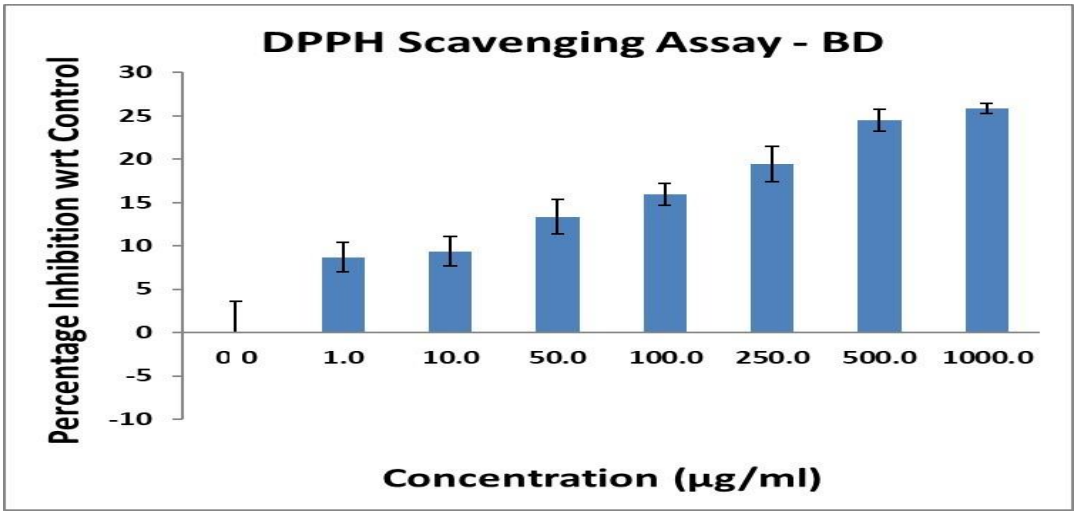

Figure XIII: DPPH analysis of an ethanolic extract of *Boerhaiva diffusa*

7) FRAP

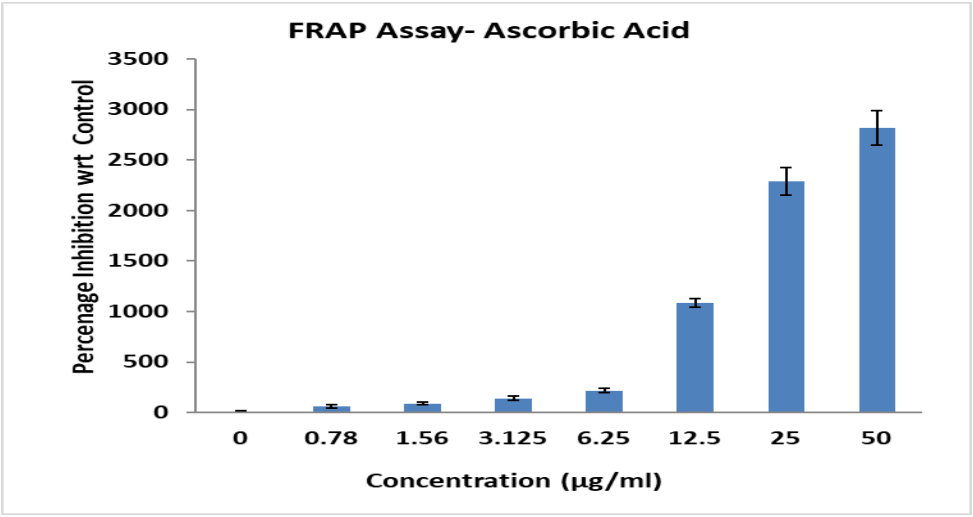

Figure XIV: FRAP analysis of Standard Ascorbic Acid

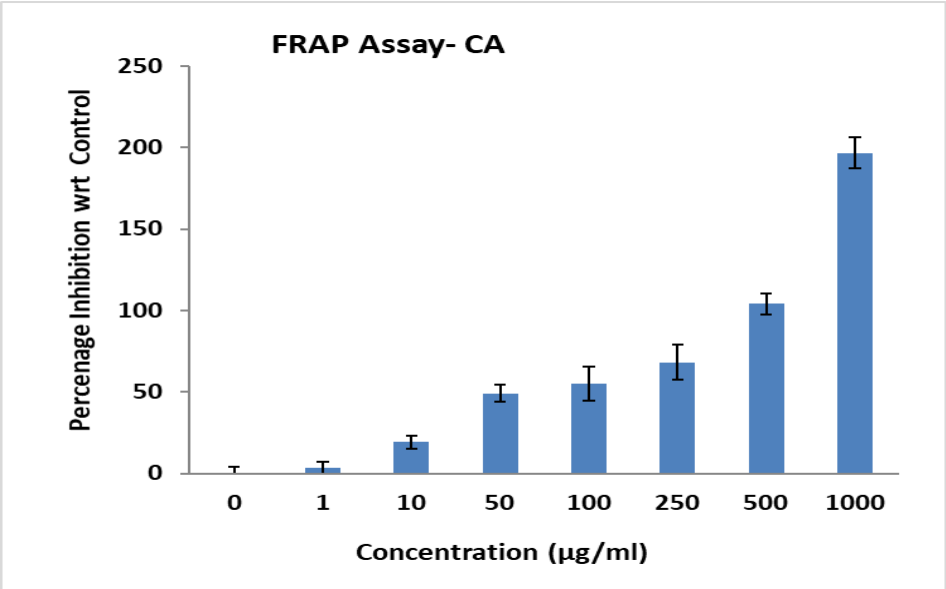

Figure XV: FRAP analysis of an ethanolic extract of *Celosia aregentea*

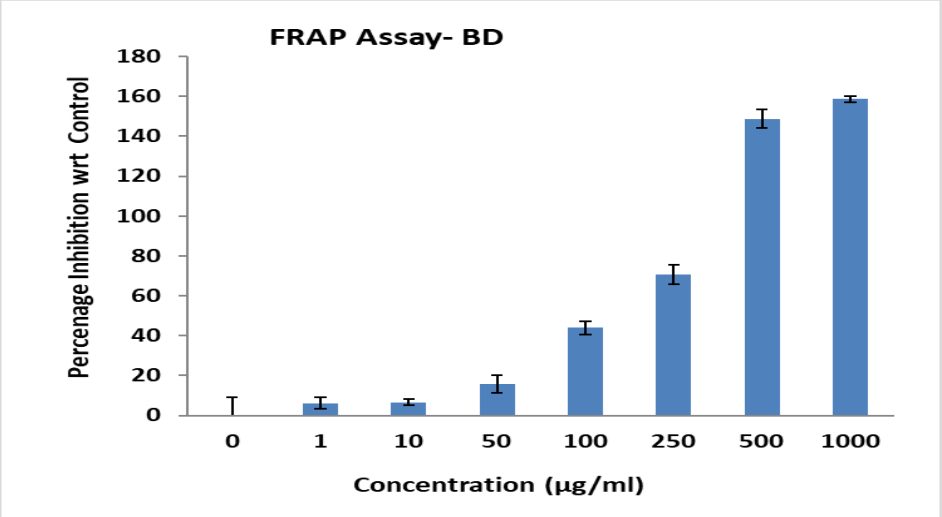

Figure XVI: FRAP analysis of ethanolic extract of *Boerhaiva diffusa*

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 2plant 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 Boerhaavia diffusa root, an Indian traditional medicine plant. (Pandeya *et al.*, 2005). Several plants used in the traditional Indian medicine system have immunomodulatory properties. Boerhaavia 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). Boerhaavia 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 Boerhaavia Diffusa 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 aggregation and nucleation 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. Researchers are 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, FRAP, and LCMS/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 LCMS/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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