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Impact of alcoholic extract of Morus alba leaves on the in vitro crystallization of calcium oxalate

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ABSTRACT

The *in vitro* analysis of nucleation and aggregation assays to evaluate the antiurolithiatic efficacy of *Morus alba* was carried out. The ethanolic extracts of *M. alba* shown antioxidant potential with DPPH radicals Scavenging activity with, increasing concentrations. The GCMS/MS, FT-IR and HRLC-MS/MS profiling of *M. alba* extracts indicates the presence of phytoconstituents and secondary bioactive compounds, which improves the aggregation and nucleation inhibition of calcium oxalate crystals. The extracts of *M. alba* leaves possessing significant nucleation and aggregation properties, may be utilized in the formulation of herbal medications for kidney stones.

Keywords: *Antiurolithiasis, Nucleation, Aggregation, Phytochemical*

1. INTRODUCTION

Urinary calculi have been identified in the graves of North American Indians dating from 1500–1000 BC and in the tombs of Egyptian mummies from 4000 BC. Ancient Sanskrit texts from India, dating between 3000 and 2000 BC, reference stone production. In El-Amara, the pelvic bones of a 7000-year-old adolescent male were found to harbor the earliest known urinary tract calculi (Probert, 2008).

The formation of kidney stones, or nephrolithiasis, is among the most common urinary disorders. Kidney stones adversely affect individuals and society due to their prevalence, high treatment costs, and significant recurrence rate. In recent years, there has been a worldwide rise in concern regarding kidney stones, which can induce severe back pain and may lead to serious complications such as acute renal failure and acute kidney injury. Bioinformatics research will examine ferroptosis-related genes to identify potential indicators of kidney stones. Kidney stones are affected by these genes (Gill *et al*., 1982).

Due to their efficacy, low toxicity, and absence of adverse effects, interest in herbal remedies is increasing. Pifferi *et al*., (1999) indicate that numerous phytotherapeutic agents have been utilized for centuries to address urolithiasis. Litholytic herbs have been employed to treat kidney stones since antiquity, predating the advent of modern therapies. Phytotherapy has proven crucial in diminishing stone recurrence, prompting significant scientific research focus on its Gilhotra and Christina, (2011). Botanical remedies have historically been employed to address urolithiasis. Before the advent of modern medicine, kidney stones were addressed with litholytic herbs (Thenmozhi *et al*., 2016). Furthermore, individuals are reverting to natural remedies for safe medications due to the misuse of synthetic pharmaceuticals, which heightens the risk of adverse drug reactions. Numerous sources assert that the origins can be traced to the 1976 Canberra meeting of the World Health Organization, which promoted the concept of "traditional" medicines for developing countries (Edwin *et al*., 2005).

Mulberry leaves (*Morus alba* Linn., Family: Moraceae) are employed globally as both a vegetable and livestock feed, although they are predominantly used as sustenance for silkworms. Researchers have performed extensive applications and investigations on flora. Residual plant matter is utilized in pharmaceuticals. (Singh *et al*., 2013).

The principal constituents of *M. alba* are flavonols with anti-inflammatory and antioxidant attributes, including rutin, astragalin, quercetin, and isoquercitrin. These compounds are reported to exhibit antioxidant properties. In addition to their antioxidant properties, flavonoids exhibit a diverse array of biological activities. For instance, they can safeguard the liver, dilate blood vessels, combat bacteria, viruses, inflammation, allergies, thrombosis, and cancer (Kim *et al*., 2014). In traditional Turkish medicine, the leaves of *Morus alba* are utilized to safeguard the liver, fortify joints, enhance vision, facilitate urination, and reduce blood pressure (Emniyet *et al*., 2014).

A preliminary chemical profile of *Morus alba* L. plants cultivated in Cuba using various extraction solvents. The majority of these include polyphenolic compounds, enzymes, digestive enzyme inhibitors, flavonoids, anthocyanins, lectins, oligosaccharides, antimicrobial agents, unsaturated fatty acids, and numerous other physiologically active chemicals. Fruits, roots, and stems possess bioactive compounds; however, leaves are the most prolific source (González *et al*., 2022). Hence it is thought worthwhile to evaluate the effect of *M. alba* Leaf extracts on crystallisation of calcium oxalate.

2. MATERIAL AND METHODS

A. Material:

Collection of plant material

The leaves of *Morus alba* Were collected from Shivaji University campus and examined prototypes along with relevant literature to confirm the plant material. Prior to the immediate processing of the plant parts, we carefully collected, labelled, and organized them.

B. Methods:

The gathered plant parts were cleaned by using tap water. The clean material was left to shade at room temperature before being ground into a powder and then filtered through a muslin cloth to create a fine powder. Then, for later use, the powder was stored in inexpensive, airtight packets in a dry, cool place. The powder was used for phytochemical analysis, and Soxhlet extraction.

1. Extract preparation by Soxhlet method

An ethanolic extract was prepared by soxhlet method by Contain were extraction for 12h. with a boilinf range of 40–45 °C (Horwitz, 1980). The extract was preserved at a temperature of -4 °C for subsequent examination. Several different concentrations of ethanolic extracts were prepared for further analysis.

a. Bioactive Compound Analysis (GC-MS/MS):

The ethanolic extract obtained from the leaves of *Morus alba* were injected into GC-MS for the identification of the different bioactive compounds. The GC-MS/MS analysis of the Ethanolic extract was run on 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.

b. 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 *Morus alba* (Leaves) (Patil *et al.,* 2020).

c. Bioactive Compound Analysis (HR)LC-MS/MS):

The alcoholic extracts of the leaves of *Morus alba* were analyzed with the help of 6200 series TOF/6500 series Q-TOF B.05.01 (B5125.3) LC-MS instrument. Precursor ions were selected in Q1 with an isolation width of 2 D and fragmented in the collision cell, applying a slope of collision energies in the range of 5–45 eV. nitrogen is used as Collision gas and product ions were identified with a collision RF of 150/400 Vpp, transfer time of 70 ms, prepulse storage of 5 ms, pulse frequency of 10 kHz, and spectra rate of 1.5 Hz for collision-induced dissociation (CID) of in-source fragment ions, with the in-source CID energy increased from 0 to 100 V. Accurate mass spectra were acquired in the m/z range of 50–1000 at an acquisition rate of 2 spectra per seconds. Internal calibration was carried out using signals at m/z 121.0509 (protonated purine) and 922.0098 (protonated hexakis (1H,1H, 3H-tetrauoropropoxy) phosphazine) in positive mode. Both raw HPLC–QTOFMS (Agilent 6540 UHD QTOF LC-MS) full single MS and MS/MS data, and for data mining based on molecular formulae estimations and fragment patterns was processed with Mass Hunter Workstation software (Qualitative Analysis). Using the algorithm employed for full single MS data, ions with identical elution profiles and related m/z values (representing different isotopes of the same compound) were extracted by molecular features extraction (MFEs).

2. Determination of Antioxidant Potential:

a. Preparation of Extract:

The fresh plant parts (2, 4, 8, 16, 32, 64 mg) leaves of *Morus alba* were dissolved in (98, 96, 92, 84, 68, 36 ml) ethanol and kept for overnight and next day used for different assay.

b. 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 adding methanol. The absorbance of the reaction mixture read at 0 min and after 30 min at 517nm. The standard BHT was 44 used for calculation. The scavenging effect was expressed as percent inhibition as shown below: % discoloration= (1-Abs sample / Abs control) X 100.

3. *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 37^0C; 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 37^0C for 30 min. The solution optical density (OD) was then read at 620 nm; the samples filtered through 0.22 mm membranes and the filtrate processed for microscopy. In a separate experiment, the test sample solution was used after dialysis against distilled water overnight at 4^0C, using membranes with a threshold of 6±8 kDa, and filtration through 0.45 mm membranes.

b. Nucleation assay

A spectrophotometric assay was used to evaluate the extracts' ability to inhibit the nucleation of CaOx crystals, as per the methods of Hennequin *et al*., (1993)., and described by Kolekar and Gaikwad, (2024).

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 Modification as per Kolekar and Gaikwad, (2024).

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.

e. Statistical Analysis of Data and Significance Level

In the present study all the generated data was analysed using appropriate statistical tests with the help of PASW 18.0 software, (formerly known as SPSS 18.0). The descriptive statistic like mean, standard deviation, minimum, maximum, etc. were determined from the raw data. The inferential statistic such as one way ANOVA was used to test the difference in the means obtained as a function of secondary metabolite concentration in the plant extract. The qualitative data was organized in such a way that reasonable interpretation can be made with it. The significance level was chosen to be 0.05 or equivalently, 5% by keeping in view the consequence of such an error. That is, we want to make the significance level as small as possible in order to protect the null hypothesis and to prevent, as far as possible, from inadvertently arriving at false conclusions.

3. RESULTS

1. Analysis of GC-MS/MS compounds

The results of GCMS analyses of in *Morus alba* (Leaves), is given in Table No. 1 and respectively and Plate No. 1 which indicate GCMS experimental data, retention time (RT), mass fragment of compounds for terpenoids and other fatty acid compounds and their applications.

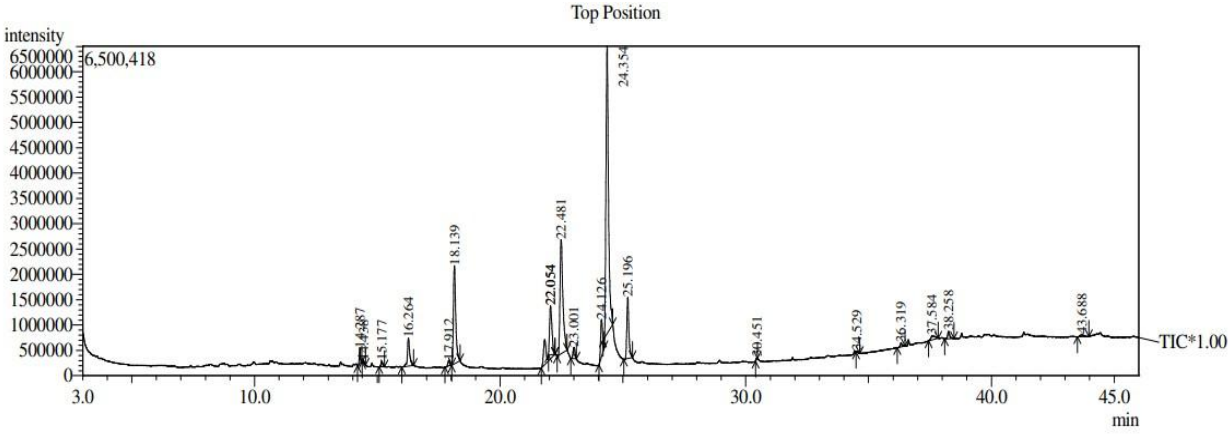


Plate 1: GC-MS/MS Chromatogram of *Morus alba* leaves extract

Table No. 1: GC-MS/MS analysis of Major Compounds in leaves of *Morus alba*

Sr. No.	Name of compound	Retention time	% peak area	Molecular formula	Mol. Weight g/mol
1)	Neophytadiene	14.287	0.9	C20H38	278.5
2)	2-Pentadecanone, 6,10,14-trimethyl-	14.438	0.19	C18H36O	268.5
3)	Hexadecanoic acid, methyl ester	16.264	3.36	C17H34O2	270.5
4)	Ethyl 9-hexadecenoate	17.912	0.4	C18H34O2	282.5
5)	Hexadecanoic acid, ethyl ester	18.139	11.9	C18H36O2	284.5
6)	8,11,14-Docosatrienoic acid, methyl ester	22.054	9.96	C23H40O2	348.6
7)	Phytol	22.481	16.97	C20H40O	296.5
8)	Methyl stearate	23.001	1.15	C19H38O2	298.5
9)	Linoleic acid ethyl ester	24.126	3.64	C20H36O2	308.5
10)	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	24.354	36.36	C27H52O4Si2	496.9
11)	Octadecanoic acid, ethyl ester	25.196	6.29	C20H40O2	312.5
12)	Ethyl henicosanoate	30.451	0.1	C23H46O2	354.6
13)	Ethyl 14-methyl-hexadecanoate	34.529	0.06	C19H38O2	298.5
14)	Tetrapentacontane	36.319	0.35	C54H110	759.4
15)	gamma-Sitosterol	37.584	0.86	C29H52O2	432.7
16)	Squalene	38.258	0.78	C30H50	410.7
17)	gamma-Tocopherol	43.688	0.34	C28H48O2	416.7

2. Analysis of organic compounds by FT Raman spectrum

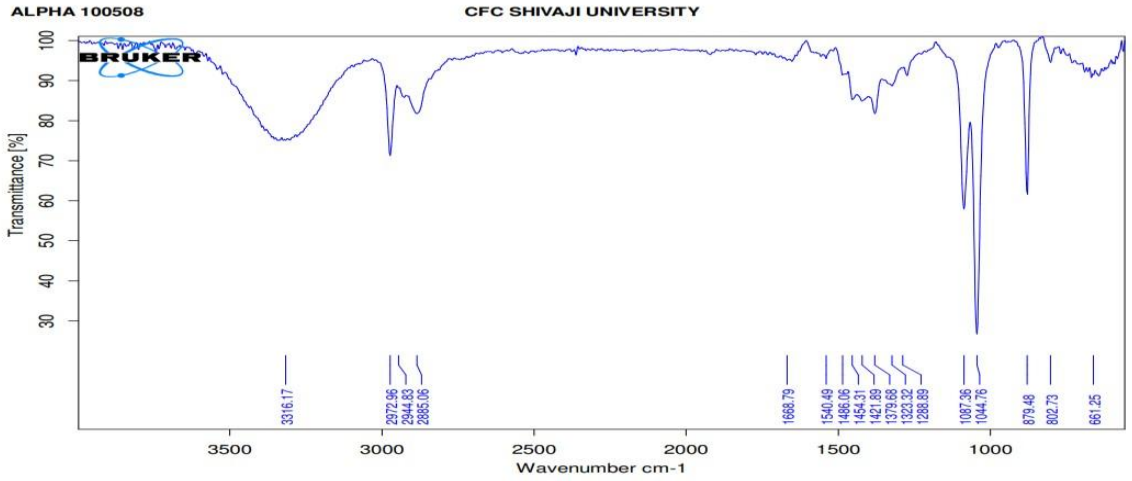


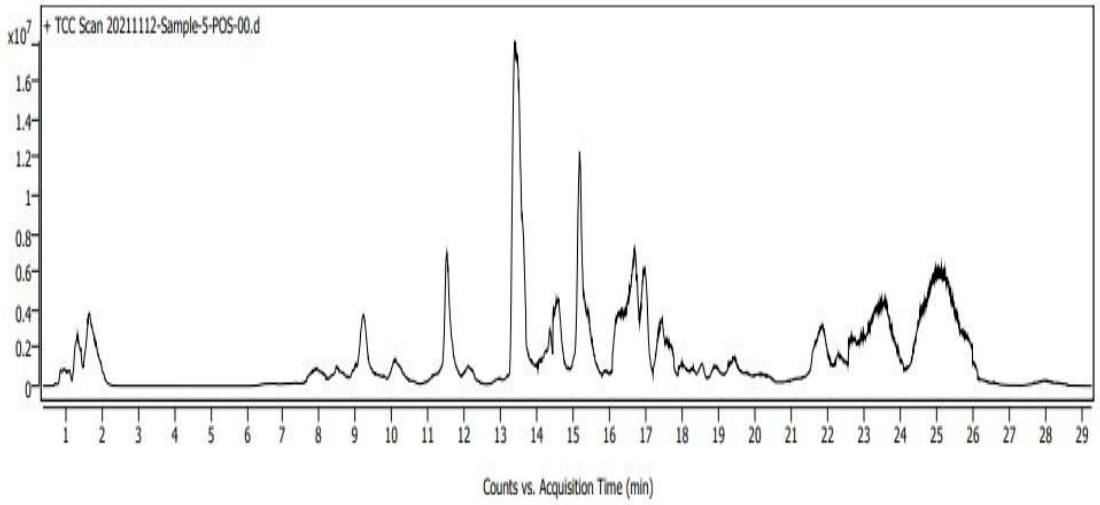
Plate 2. Fourier transform infrared spectroscopy (FTIR) spectral graph of leaves of *Morus alba*

Table No. 2: FTIR peak values of *Morus alba* of leaves ethanolic extract

Sr. No.	Peak value	Group frequency cm-1	Functional compound class
1.	661.25	C-Cl stretching	halo compound
2.	802.73	C-H bending	1,4-disubstituted
3.	1044.76	CO-O-CO stretching	anhydride
4.	1087.36	C-O stretching	secondary alcohol
5.	1288.89	C-N stretching	aromatic amine
6.	1323.32	S=O stretching	sulfone
7.	1421.89	C-H bending	alkane
8.	1540.49	N-O stretching	nitro compound
9.	1668.79	C=O stretching	conjugated ketone
10.	2885.06	N-H stretching	amine salt
11.	3316.17	O-H stretching	alcohol

The spectrum of ethanolic extract of *Morus alba* of leaves, Table No. 2 and Plate. 2 which is characteristic of Wave number 661.25 cm^{-1} C-Cl stretching halo compound, Wave number 802.73 cm^{-1} C-H bending 1,4-disubstituted, wavenumber 879.48 cm^{-1} C-H bending 1,2,4trisubstituted, Wave number 1044.76 cm^{-1} CO-O-CO stretching anhydride, Wave number 1087.36 cm^{-1} C-O stretching secondary alcohol, Wave number 1288.89 cm^{-1} C-N stretching aromatic amine, wavenumber 1323.32 cm^{-1} S=O stretching sulfone, Wave number 1379.68 cm^{-1} C-F stretching fluoro compound, Wave number 1421.89 cm^{-1} C-H bending alkane, Wave number 1540.49 cm^{-1} N-O stretching nitro compound Wave number 1668.79 cm^{-1} C=O stretching conjugated ketone, Wave number 2885.06, 2944.83, 2972.96 cm^{-1} N-H stretching amine salt, Wave number 3316.17 cm^{-1} O-H stretching alcohol.

3. Bioactive compounds (HR)LC-MS/MS analysis



Plates 3. (HR)LC-MS/MS Spectrum of Leaves of *Morus alba*

Table No. 3: (HR) LC-MS Profiling of Bioactive Compounds from ethanolic extract of leaves of *Morus alba*

Sr. No.	Name	Formula	RT	Mass	Score	Activity	Reference
1	2-Hydroxymyristic Acid	C14H28O3	9.522	244.2036	98.90	Colorectal cancer	Matysik *et al*., 2016.
2	Misoprostol	C22H38O5	11.367	382.2723	98.65	Kidney diseases Urinary Bladder Acute kidney injury Stomach Ulcer Peptic ulcer	Wong *et al.,* 1995. Gray *et al*., 1986. Ozer *et al*., 2011. Arakawa *et al*., 1991. Wu *et al*., 2011.
4	Mesoporphyrin IX	C34H38N4O4	24.498	566.2907	95.08	Compositions and their Uses for treating cancers	Keum *et al*., 2003.

The 2-Hydroxymyristic Acid, Misoprostol, Mesoporphyrin IX as bioactive compounds were detected in *M. alba* leaves (Table 3), among these misoprostol exhibits, the activity against kidney and urinary bladder disorder (Wong *et al.,* 1995 and Ozer *et al*., 2011).

4. Antioxidant Potential

a. DPPH radical scavenging activity

DPPH radical scavenging activity in the ethanolic extract of *Morus alba* is depicted in Figure No. 2 Graph shows that the DPPH radical scavenging activity is increases with increasing concentrations of the ethanolic extract of *Morus alba* (Leaves).

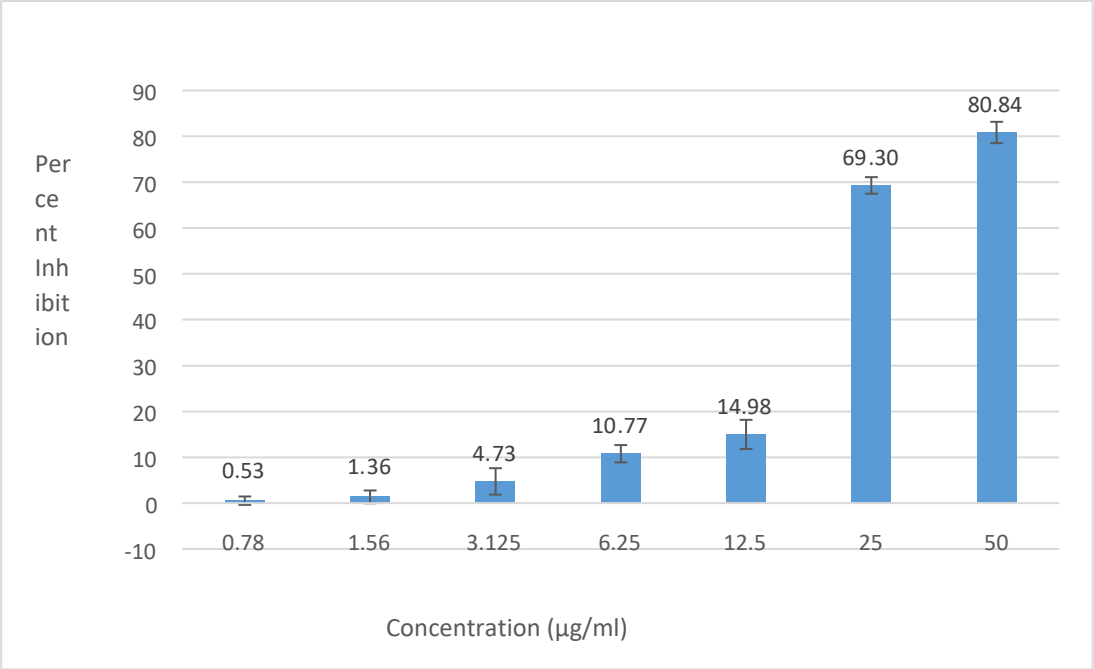


Figure No. 1: DPPH radical scavenging activity of Standard Ascorbic acid

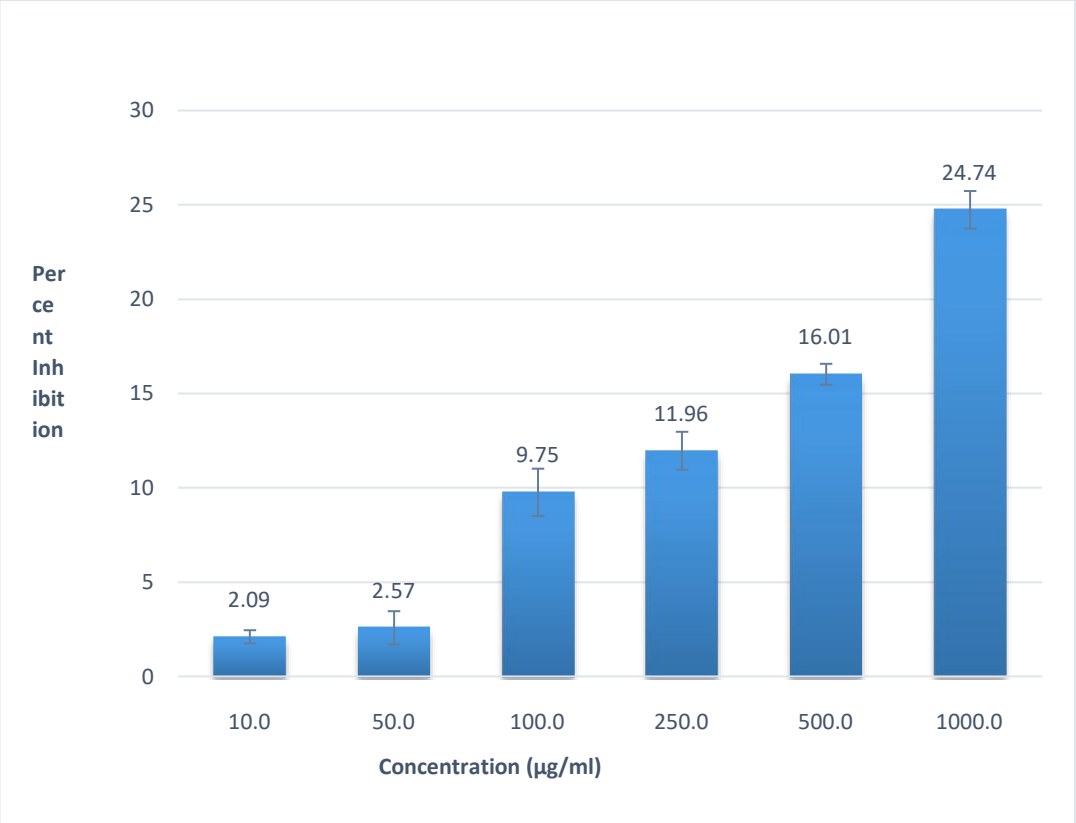


Figure No. 2: DPPH radical scavenging activity of *Morus alba*

DPPH is an electron transfer-based method characterized by a rapid electron transfer from the sample to DPPH (Miguel, 2009). Robak and Marcinkiewicz, (1995) assert that antioxidants mediate their effects by directly interacting with reactive oxygen species (ROS), neutralizing them and/or chelating catalytic metal ions. Shahidi and Wanasundara, (1992) assert that

antioxidants mitigate oxidative damage induced by free radicals by reacting with them, chelating catalytic metals, and functioning as oxygen scavengers.

5. The Crystallization Inhibitory Action of Plant Extracts

a. Nucleation Process

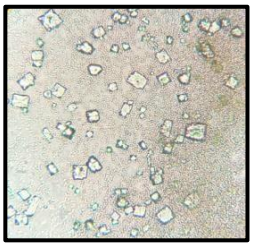


Plate. 4. Nucleation of Calcium Oxalate Crystals (Untreated)

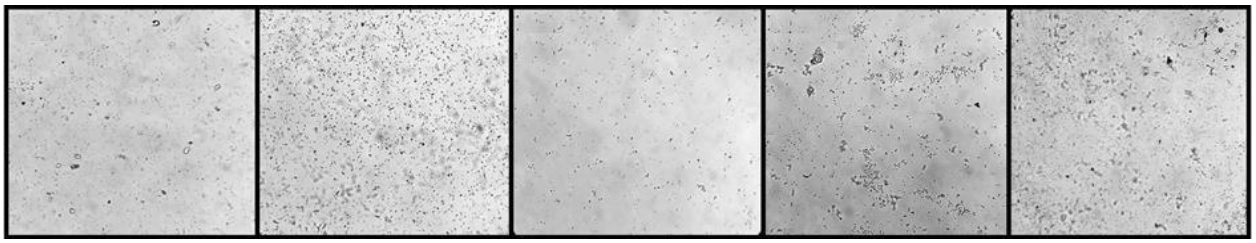


Plate. 5. Effect of *Morus alba* extract on Nucleation Inhibition

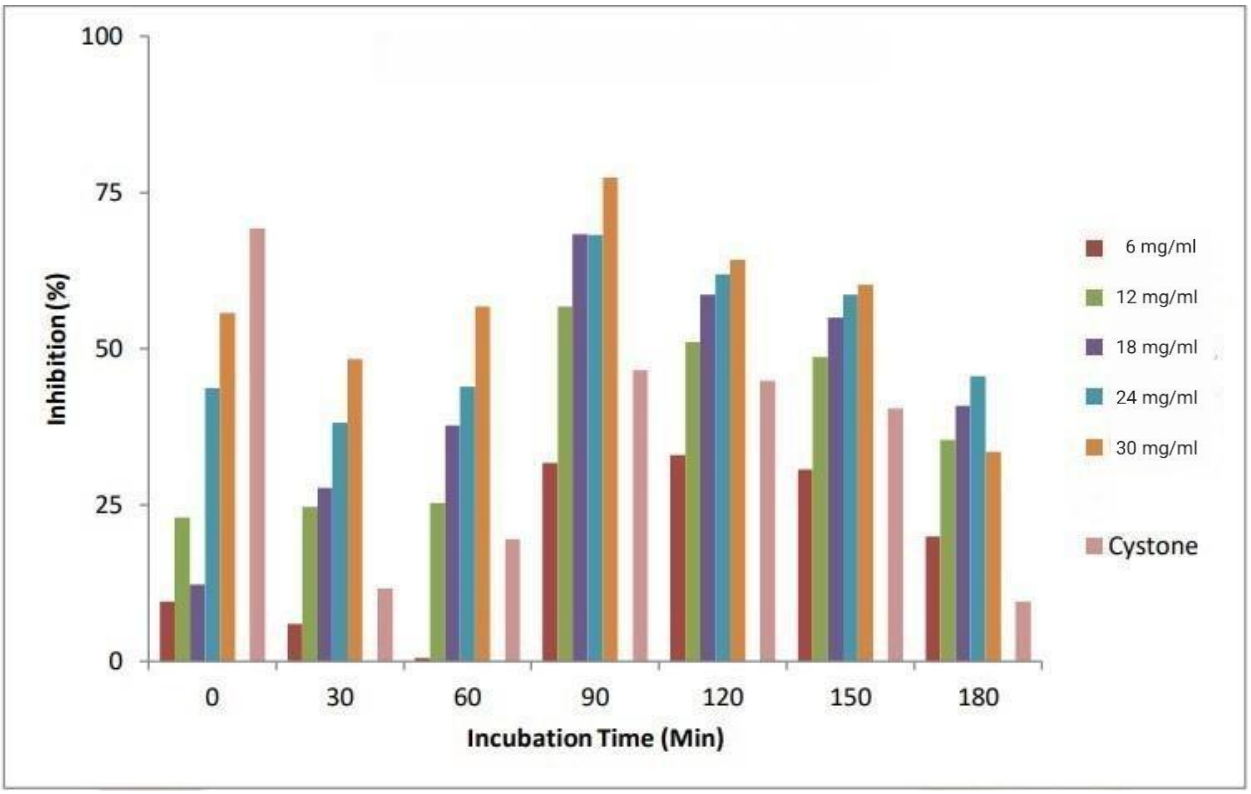


Figure 3. Impact of Extracts of leaves of *Morus alba* on the nucleation process

The control runs were conducted without the use of plant extract before the effect of the extract on the inhibition of the nucleation and aggregation process was evaluated. The microscopic analysis revealed crystal formation. (Plate 4).

The Plate 5 shows the findings of the study conducted to evaluate the effect of *Morus ala* plant extract on the inhibition of

the nucleation process. The varying quantities of plant extract (6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml) were utilized here as well. The results indicate a change in absorbance when the 30 mg/ml plant extract was utilized over the recorded time period of 0 to 180 minutes. Figure. 3. The microscopic evaluation of the reaction mixture provides strong support for this conclusion. The plant extract demonstrates a significant effect on nucleation cessation. Therefore, based on the study results, it is clear that the extract of *Morus alba* plant exhibits an inhibitory effect on the nucleation process of calcium oxalate crystals, even when utilized in smaller quantities.

b. Aggregation Process

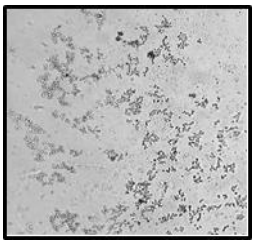


Plate. 6. Aggregation of Calcium Oxalate Crystals (Untreated)

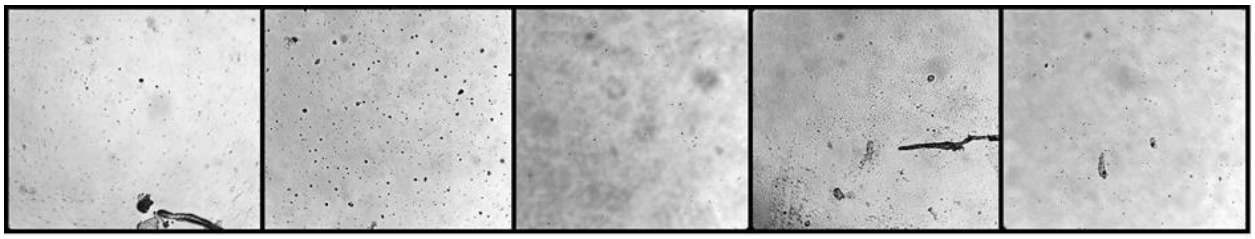


Plate 7. Effect of *Morus alba* extract on Aggregation Inhibition

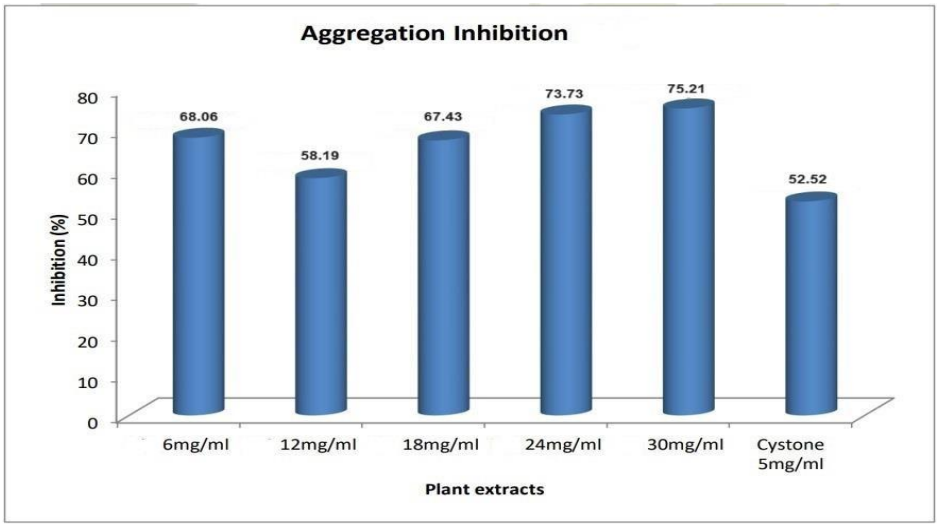


Figure 4. Impact of Extracts of leaves of *Morus alba* on the aggregation process

Comparable to the evaluation of the nucleation process, prior to assessing the effect of plant extract on the inhibition of the aggregation process, control runs were conducted without the application of the plant extract. This was conducted to elucidate the function of plant extract in inhibiting the aggregation process. The results indicate that there was no gradual decline in absorbance in the absence of plant extract (Plate. 7), suggesting that the aggregation process was not inhibited. Furthermore, the microscopic analysis revealed the formation of crystals.

The study was conducted to evaluate how plant extract plays a role in putting the brakes on the aggregation process. (Fig. 4). To get to the bottom of the inhibitory effect, various amounts of plant extract were put to the test: 6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml. The findings show that when 6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml of

plant extract were in the mix, the aggregation hit the brakes at 180 minutes. The microscopic evaluation also showed that the aggregation process came to a halt, pointing to the plant extract's inhibitory effect. Therefore, based on the study results, it's clear as day that the extract of *Morus alba* plant puts the brakes on the aggregation process when used in all amounts, 6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml. (Fig 4).

4. DISCUSSION

The present Investigation of *M. alba* indicates the presence of various types of Phyto constituents. The presence of some secondary metabolites that can dissolve the stone crystals These indicates that the *M. alba* is useful for kidney stone disease.

The principal cause of urolithiasis, characterized by the nucleation, aggregation, and formation of calcium oxalate crystals, is mitigated by the antioxidative properties of specific antiurolithitic nutraceuticals. Epicatechin and catechin, the principal antioxidants found in plant sources like grape seeds and green tea (Mendoza-Wilson and Glossman-Mitnik, 2006), are chiefly recognized for their capacity to chelate metals, scavenge free radicals, and rejuvenate enzymes and transcription factors. These antioxidants offer protection against the formation of kidney stones, oxidative stress associated with renal failure, and renal injuries (Khan *et al*., 2009).

Rutin, present in various plants such as apple peels and black tea, significantly diminished stone formation when administered alone or in combination with curcumin (Pastukhov *et al*., 2007). These phytophenols inhibited the growth and aggregation of COM crystals and altered the filtration rates of the glomeruli (Ghodasara *et al*., 2010).

Quercetin exhibits potential antioxidative activity in rats with induced hyperoxaluria due to its ability to elevate serum PON1 (Paraoxonase) levels. These therapeutic bioflavonoids have demonstrated efficacy in managing renal lithiasis by enhancing the activities of superoxide dismutase (SOD) and catalase, which possess antioxidant properties that protect renal tubular cells from damage, exhibit anti-apoptotic effects, and inhibit the accumulation of oxalate crystals. (Nirumand *et al*., 2018).

Historically, renal ailments and urinary calculi have been addressed using the leaves of *Chenopodium album* Linn. Sharma *et al*., (2016) investigated the effect of an aqueous extract of *C. dalbum* leaves (CAAE) on the *in vitro* crystallization of calcium oxalate and brushite crystals.

Agarwal, (2017) evaluated the efficacy of various extract concentrations on calcium oxalate crystallization *in vitro* using these assays. The assay demonstrated a maximum inhibition of 55.36% at a concentration of 1000 mg/ml, indicating that the percentage inhibition of calcium oxalate crystal formation was directly correlated with the increase in the plant extract's concentration (Atmani *et al*., 1998). *Ageratum conyzoides* L. has been identified as a potent and promising antiurolithiatic agent, consistent with its application in traditional medicine.

5. CONCLUSION

The Ethanolic extracts are treated on calcium oxalate crystals using nucleation and aggregation studies, which indicates that the varying quantities of plant extract (6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml) indicate a change in absorbance when the 30 mg/ml plant extract was utilized over the recorded time period of 0 to 180 minutes, Indicating inhibition of nucleation processes and the extract reduces the aggregation process when used in all Concentrations, 6mg/ml, 12mg/ml, 18mg/ml, 24mg/ml, and 30mg/ml. The presence of several Phyto constituents and bio active compounds that can dissolve kidney stones, thus the ethanolic extracts of *M. alba* leaves are useful for treating the urine stone due to the chemicals in the leaves that inhibits the formation and sticking together of calcium oxalate crystals.

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