Preparation of Superfine Bael Leaf Nanopowder, Physical Properties Measurement and its Anti- Microbial Activities

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Abstract
The recent advent of nanoscience and nanotechnology as a cutting edge science has created oppoertunities for novel and creative uses as herbal nanomedicine. In this present research, high energy Ball milling was used to produce the superfine nanoparticles from shade-dried *Aegle marmelos* leaves (bael). The SEM analysis shows highly agglomeration in the milled sample. The particle size was evaluated using TEM analysis and found to be 19.89 nm. The Zeta potential found to increase with milling hour from -15.56 mV to -22.57 mV, showing the stability of material increases with milling hours. Despite the nanoparticles' size changes as a result of milling time, and crystallinity of prepared bael nanopowder remained unchanged, which were confirmed by XRD measurement. Uv-Visible-NIR measurement indicates the maximal absorbance found almost the same at 400 nm and somewhat lower at 270 nm, showing that the synthesized sample has protein structure. The functional group were remains unchanged during milling, which was analysed by FTIR. Antimicrobial activities of prepared nanoparticles against Gram-negative *E.coli, E.aerogenes, and* Gram-positive *S.aureus, B.cereus* Streptomycin were investigated. The longer the milling times, more superfine behaviour , which enhances the antibacterial action. The result supports the superfine behaviourhave potential against antibacterial action on specific microorganism. The understanding of how particle size affects antibacterial activities that have been demonstrated would aid in maximizing the synthesis of required nanoparticles for various biomedical applications.
Keywords:Bael leaf**,** *nanomaterials , Physical Properties, , Zeta potential, Anti-Microbial.*

1. Introduction

Nature has conferred us meticulously with countless medicinal plants with various functional properties. The use of medicinal plants is still a significant therapeutic tool for treating human illnesses. Early man was well managed to discover his local natural environment in order to utilize numerous minerals, plants and animal products etc. For the synthesis of a variety of medicinal agents in his quest for eternal health and longevity[1]. People were aware of plants' and their phytochemicals' therapeutic properties since ancient times. Modern research has made it possible to create herbal medications with great therapeutic activity and no adverse effects [2]. It is time to find and synthesize novel medicinal chemicals derived from natural sources, as well as to create affordable and potentially effective antibiotics which are resistant to drug-resistant bacteria [3]. In the fields of nanoscience and nanotechnology, which are crucial for producing nanoparticles for various applications, green chemistry plays a significant role [4]. Even now, there is a growing need for additional medications derived from plants as a resurgence of interest in traditional medicine [5].This renewed interest in plant-based medications is largely attributable to the widely held notion that green medicine is safer and highly reliable than pricey synthetic medications, some of which have negative side effects. Traditional medicine made from plant resources are still used by people, a majorportion of the developing countries. The preponderance of therapeutic herbs is produced in India. Herbal remedies have been the cornerstone of traditional Indian medical practises like Ayurveda, Unani, and Siddha for treating and curing a wide range of diseases and physiological abnormalities [6]. Emerging field of science and technology sector, which is expanding rapidly, includes a rising area known as nanotechnology. The science behind nanotechnology aims to produce or synthesise materials with a size of the nanometer or less. These materials are extremely beneficial in all areas of research, including synthetic and biology, chemistry. Nanoparticles, which have not received much attention, display a variety of unique physical, chemical, and biological characteristics that primarily depend on their diverse crystal structure, shape, size, and morphology [1]. In this present research, high energy ball milling machine has been employedto synthesise *aegle marmelos* nanopowder where this is a mechanical process which is frequently used to combine materials and grind powders into uniform particles. It has widespread use in industry across the globe as it is an eco-friendly method [7]. The abundance of phytonutrients in plants has minimized the risk of many diseases that affect humans, including diabetes, cancer, malaria, cardiovascular disease, and neurological disorders. Medicinal herbs and the derivatives of them play important roles in modern medications, as has been well-documented [8]. *Aegle marmelos* L., also known as "Bael," is the sole species belongs to monotypitonus genus *Aegle*in the Ructaceae family. Found in an indigenous to the dry (subtropical regions) vegetation of the hilly and plain regions of South Asian nations such as India, Pakistan, Bangladesh, Thailand, Sri Lanka, India, and Malaysia [9]. This plant has a lot of bioactive substances that are good for health, like vitamins, flavonoids, polyphenols, carotenes, and organic acids. Moreover, it has considerable amounts of vital minerals like potassium, phosphorus, calcium sodium, manganese, copper, and iron. [10] The tree also goes by the name Shivduma since it is considered to be a holy plant and is accordingly used to make prayers to Lord Shiva and the goddess Parvati. Hindus think that the goddess Lakshmi resides among leaves of bael [11]. Charak has described about the *Aegle marmelos* that it is one of most essential medicinal plants since 1500 B.C. of India. Enourmous phytochemical more than 100 have been extracted for various use [12]. The bioactive components (luvangetin, psoralen, marmelosin, aurapten, marmelide, riboflavin, tannin aegeline, carotene, lupeol, eugenol) of *Aegle marmelos* leaves exhibit a variety of biological effects, including anti-helminthic, antibacterial, antiulcer, antispasmodic, artemicide, astringent[13]. Despite being abundant in these beneficial elements and activities, it is nonetheless neglected due to its year-round unavailability. The largest accumulation of bioactive phytochemical substances, present in the leaf which are produced as secondary metabolites, that is why, we have selected this plant leaf, studied their physical properties for its applications [14]. Microscopic and macroscale properties of the treated material, such as its structure, crystallinity,morphology and thermal stability, are significantly influenced by ball milling. Okajima and colleagues have discussed how the ball mill affects the morphological and structural characteristics of cellulose [15]. Ahmad and colleagues also looked into how ball milling affected the cellulose's crystallinity index. [15] They ground crystalline cellulose at 600 rpm for two hours in a planetary ball mill, and they used a variety of methods to examine the differences between the raw material and the finished product, including FT-IR, X-ray diffraction, and SEM. On the basis of understanding and thorough research, the herbal Bael leaf powder at the nanoscale are produced by us. In general, it is understood that the structure and characteristics of nanoparticles depend on the processes/methods used to synthesise them. One top-down method for producing homogenous nanoparticles is ball milling which is used in this present research [16]. The purpose of this work is to produce superfine nanoparticles from powdered herbal Bael leaf using a ball milling approach and to explore its physical properties and antibacterial activity to show biomedical potential.

2. Materials and Methods : Synthesis Process

2.1. Materials

In order to fulfilment of the present research, fresh leaves of *Aegle marmelos* were obtained from nearby plant in the Patna, Bihar, India. The *Aegle marmelos* leaves were cleansed with distilled water. After cleaning, it was allowed to dry for two days in the sun under a shed. The dried leaves were then pulverised in a pristine, common mixer grinder to synthesize the powder. The coarsely powder was stored in an airtight container labelled, called as 0hr sample.

In an E_{max} Retsch high energy ball mill, this prepared powder was milled to a super-fine nano powder labelled with 3hr and prepare it for further characterisation using modern scientific tools and its applications.

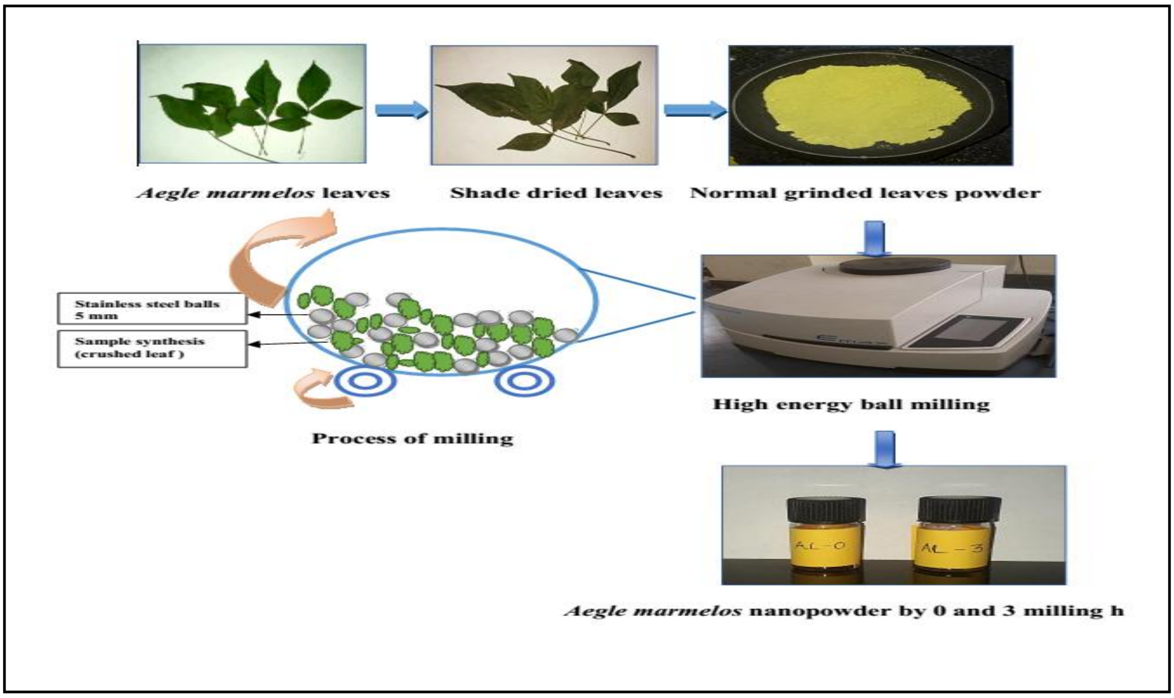


Fig.1. Schematic flow chart of synthesis process.

2.2.Methodology

2.2.1.Synthesis of super fine nanosized powder of *aegle marmelos* Bael leaves

Figure 1 shows the flowchart for the procedure used to create superfine bael leaf nanosize powder. At Nanoscience and Nanotechnology center, Aryabhatta Knowledge University in India, fresh leaf powder was ground to get *aegle marmelos* leaf nanopowder using a high energy ball milling machine (E_{max} Retsch, Germany). High-energy ball milling, which is essentially a planetary ball mill device, uses a stainless steel ball with a 20 mm diameter to superfinely grind the ultrafinely powdered sample inside the stainless steel jar, which has a 50 ml capacity. Some other researchers have reported using a similar synthesis process (Aman et al. 2018). A sample-to-ball weight ratio of 1:20 is kept in the stainless steel jars with a 50 ml capacity. In order to fill the jar to capacity by volume, the sample and the ball together occupy 1/3 of its surface. With a continuous rotational speed of 600 rpm, the grinding was done for 3 hours.
Horizontally revolving inside the jar are 20 mm stainless steel balls. After 30 minutes at intervals of 2 minutes, the ball in the jar rotates in the opposite direction, clockwise or anticlockwise. In order to avoid the high energy ball mill equipment from overheating while milling, the chiller was employed to keep the temperature below 25 ^{0}C. Modern scientific techniques including Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD),Transmission electron microscopy(TEM), and UV-Visible-NIR spectroscopy are used for evaluation of physical properties.

3. Antimicrobial activities:

3.1. Strain of Microorganism:

4 Strains of bacteria were used for the test which include. *E.coli, E.aerogenes, S.aureus, B.cereus*

3.2. Sterile disc preparation:

To perform this experiment 5 mm sized (in diameter) a Whatman's filter paper No.1 was used. The leaf extracts were inoculate in to sterile disc. Each sterile disc was contained discretely with 40 mg/ml, 20 mg/ml, 10 mg/ml and 5 mg/ml. Laminar air flow was used to dry the discs. Disc diffusion technique was used to test the medicinal plants extract's antimicrobial activity.

3.3. Mueller Hinton Broth (Disc Diffusion method):

The test bacterial strain was applied to the surface of the prepared nutrient agar plates with a sterile cotton swab. The nutrient agar plates were covered with the antibiotic disc that had been filled with plant extract. Dimethyl sulfoxide was loaded onto the disc in order to maintain the controls. The plates were then incubated for 12 to 24 hours at temperature 37°C. The size of the inhibitory zone showed how the bacteria responded to the nanomaterial.

3.4.Antibacterial test

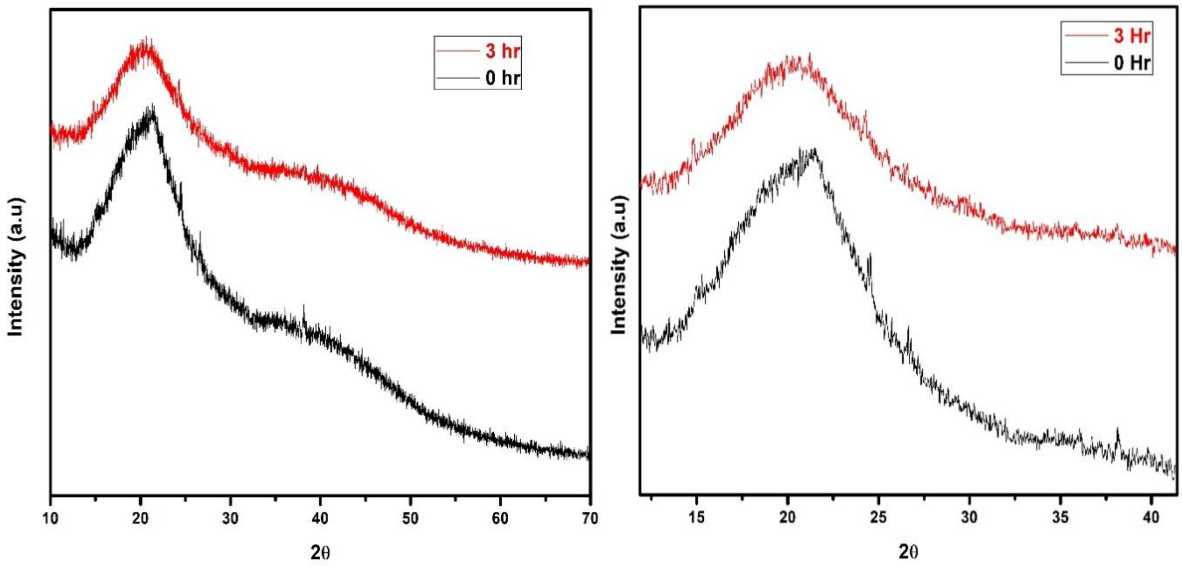
The antibacterial activity of *aegle marmelos* leaf nano powder towards the bacterial strains *E.coli, E.aerogens, S. aureus and B.cereus* were tested. Nutrient broth and nutrient agar were used to grow the bacterium strains. Prior to tests, every medium was autoclave-sterilized. To determine the effects of bacteria, a loop filled with bacterial culture was placed on a medium made of Muller Hinton broth for *aegle marmelos* nano powder. The Zone of inhibition were calculated after 24 hrs of incubation at 37 °C. The size of the inhibitory zone was used to predict how the bacteria would respond to the nanopowder.

4. Results and Discussions

4.1. X-ray Diffraction (XRD) analysis:

XRD analysis is used to determine physical properties of synthesized materials [17]. For the current study, theBruker Germany based D8 Advance X-ray diffractometer (XRD) was used to analyze synthesised superfine grinded *Aegle marmelos* (bael leaf) powder at 3hmilling time for the current study. Voltage, current, and temperature were maintained at 40 kV, 40 mA, and room temperature using CuK radiation (= 1.5406A). At a scan rate of 2°/min, the diffraction angles were measured from 10 to 90°. The XRD data were obtained for *aegle marmelos* (bael leaf) powder processed in a high energy ball mill for 0 hours (B0) [18] and 3 hours (B3). The raw powder was prepared using a mixer grinder (Figure 2). The XRD peak intensity did not significantly alter, suggesting that grinding had little impact on the general structure of cellulose and that the produced material was only visible as nanopowder. Since amorphous materials have irregular or curved surfaces, they do not yield well-resolved X-ray diffraction patterns. According to XRD data, the angular peak positions did not significantly change as the size of *aegle marmelos* was reduced at milling hours (3hr). Through XRD analysis, the crystal structure of superfine *aegle marmelos* bael leaf powder was examined at the atomic and molecular levels.

We have found that there is slight shift in the angular 2-theta position and the peak intensity of the pattern has also decreased confirming the particle size and crystal structure has been considerably changes by milling hours, which were also confirmed further by TEM, SEM and FTIR.



(a) (b)

Fig.2 (a-b) XRD analysis of bael leaves nanomaterials (0h) and (3h)

4.2. Fourier Transform Infrared analysis (FTIR):

The native bael leaves contained functional groups such carboxylic acids, sulphonamides amides, and thioesters, as shown by the FT-IR (Fourier Transform infrared spectroscopy) spectra. In fact, in their research on the therapeutic benefits of bael leaves, Sudha Rameshwari and Radhika found that bael leaves include sterols, carboxylic acids, alkaloids phenols, sand xanthoproteins. [19] The intricate spectra of Fig. 3 shows a number of notable absorption bands. The bands were assigned using information from FT-IR spectra of cell wall constituents [20] and plant leaf tissues [21] as well as information on the structure and chemical composition of the cuticle and cell wall of the plant. The hydroxyl group showed the broad band about 3435cm^{-1} on the stretching vibration. The presence of the band was attributed to the rich hydroxyl groups in the polysaccharide structure of the plant cell walls and the high water content of the plant leaves. The absorption bands at 1631 cm^{-1} and 1565cm^{-1} [22] indicate the fragrant regions of the cuticle and plant leaves. At 1700-1650cm-1, you may find the characteristic amide-I frequencies of protein secondary structures. The absorbance at 1655cm^{-1} is caused by C-O of carboxylic acids [23]. The N-H bending and C-N stretching in protein amide groups that result in the amide-II band are frequently associated with an absorbance of about 1550 cm^{-1}. The thioester group is represented by the band at 672 cm^{-1}. The band at 1382cm^{-1} is designated as the sulphonamide group [24].

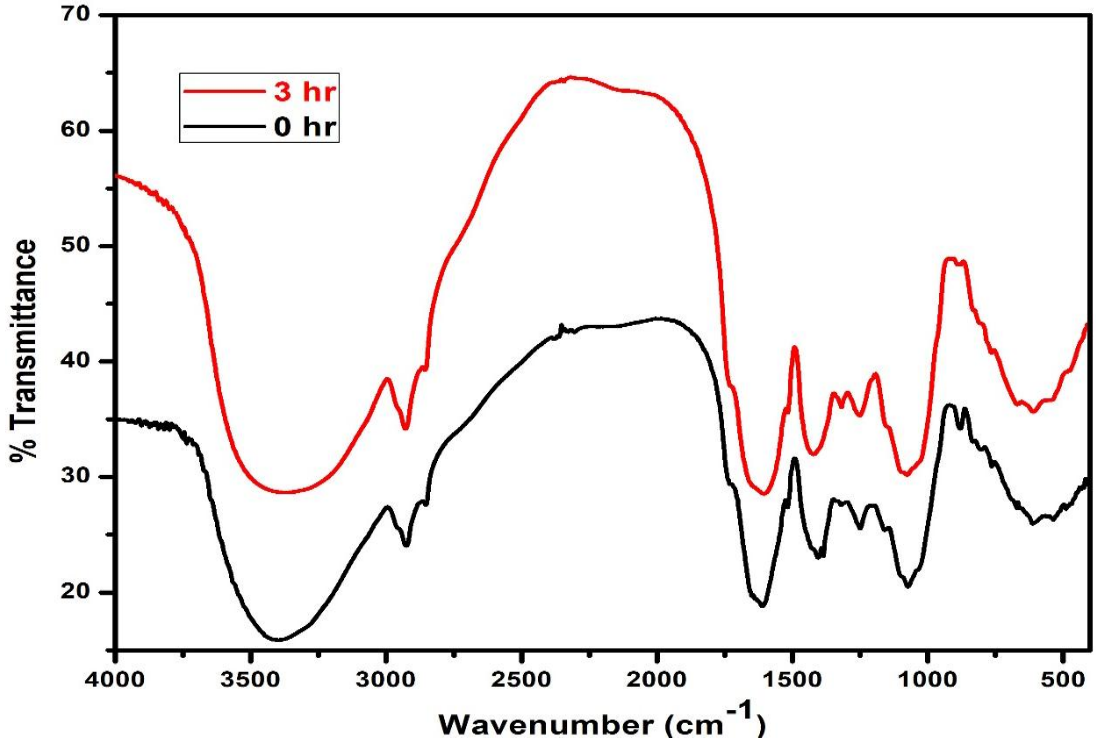


Fig.3. Functional groups showing through FTIR spectra of Bael leaf nanoparticles

4.3. UV-visible NIR Measurement

In Uv-Visible-NIR measurement, shown in figure-4, the maximal absorbance including all samples is almost the same at 400 nm and somewhat lower at 270 nm, showing that the generated samplehas protein structure. The cellulose concentration of *aegle marmelos* (bael leaf) nanoparticles might be the cause of the apparent absorption in the visible zone. Three additional bands in the blue-red region were seen at all concentrations at 508, 541, and 607 nm, indicating the presence of the pheophytin fraction (chlorophyll derivatives and/or breakdown products) in the extract [25]. Considerable changes in absorbance intensity, shows changes in physical properties of superfine powder materials, which are supported by XRD, FTIR and TEM measurement.

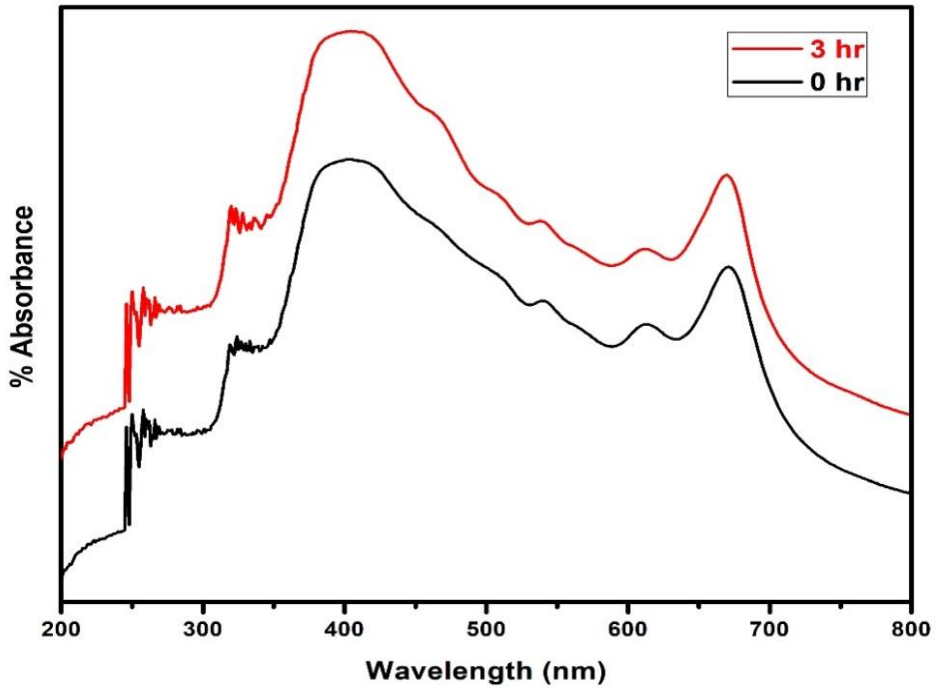
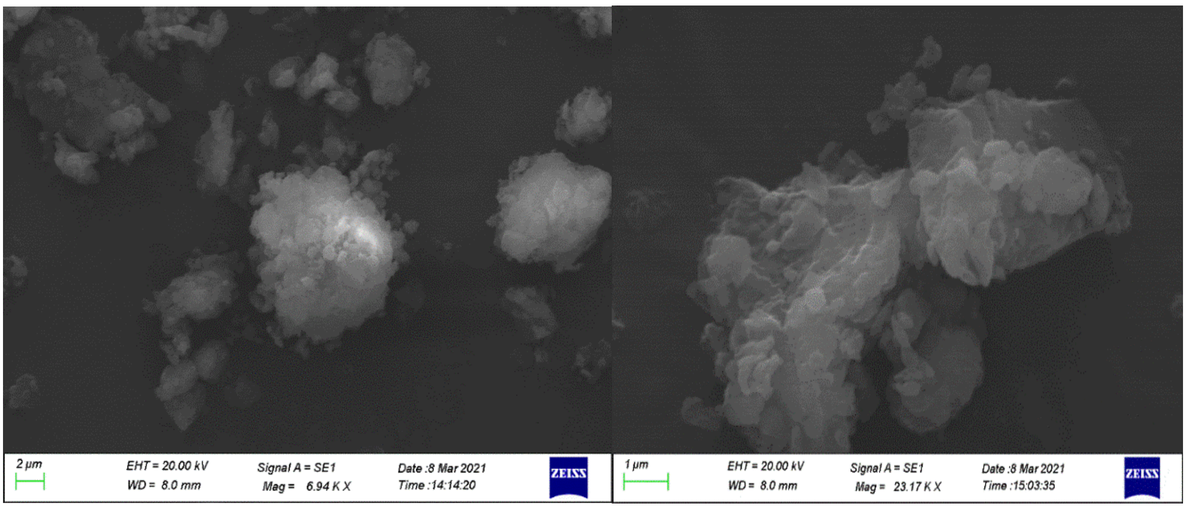


Fig 4. UV-vis spectra of bael nanoparticles

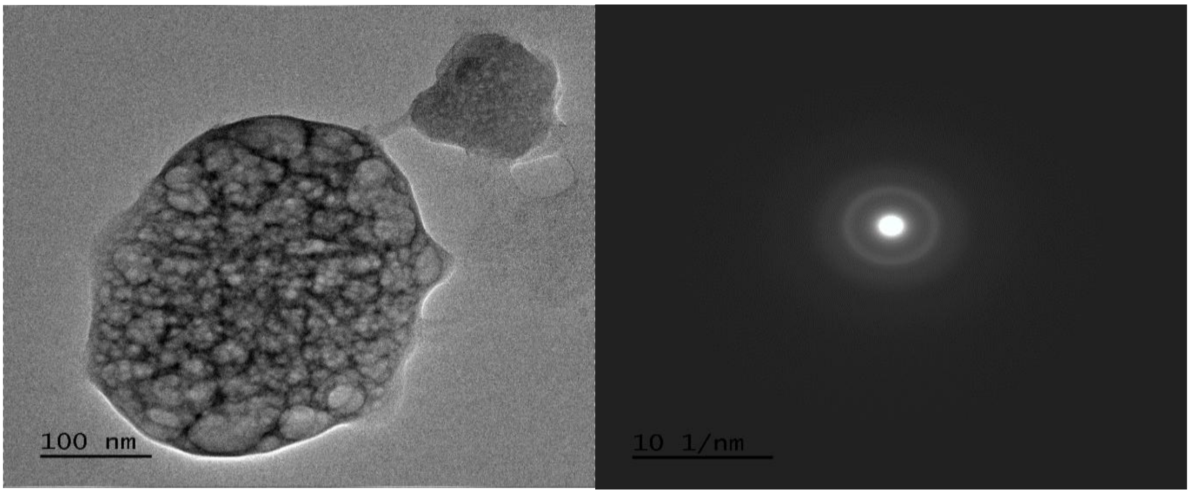
4.5.Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy(TEM) measurement

SEM was used to observe the surface morphology and reactivityand form of the bael leaf power and are shown in figure-5(a-b). It was feasible to observe the change from a typical blocky shape (coarse powder) when the particle size and interparticle interaction changes. Through the breaking of intermolecular connections and the reduction of particle size, bael leaf powder was clearly shown to undergo a structural metamorphosis during ball milling from an ordered structure to a disordered structure. It was noteworthy that it showed greater aggregation as a result of the milling's flattening, aggregation, and fracture effects [26]. Higher magnification made it crystal evident that as particle size decreases; the surface tends to be flat and smooth[27-28].

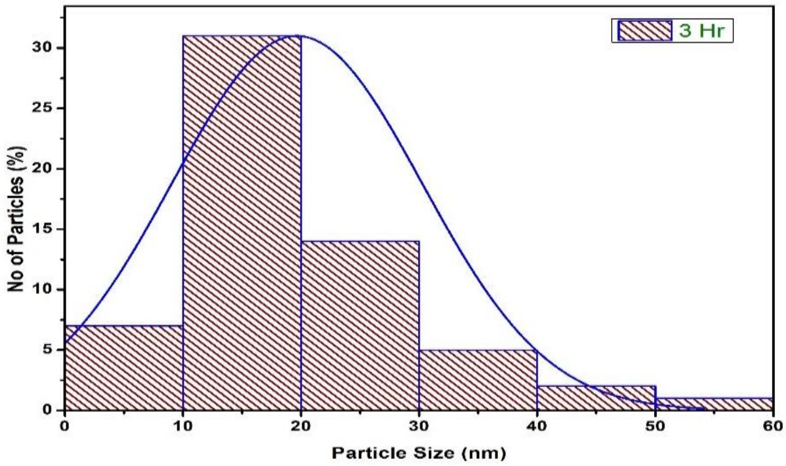


(a) (b)

Fig.5 (a-b) SEM images of bael leaf powder at 0 hr and 3 hr respectively.



(a) (b)



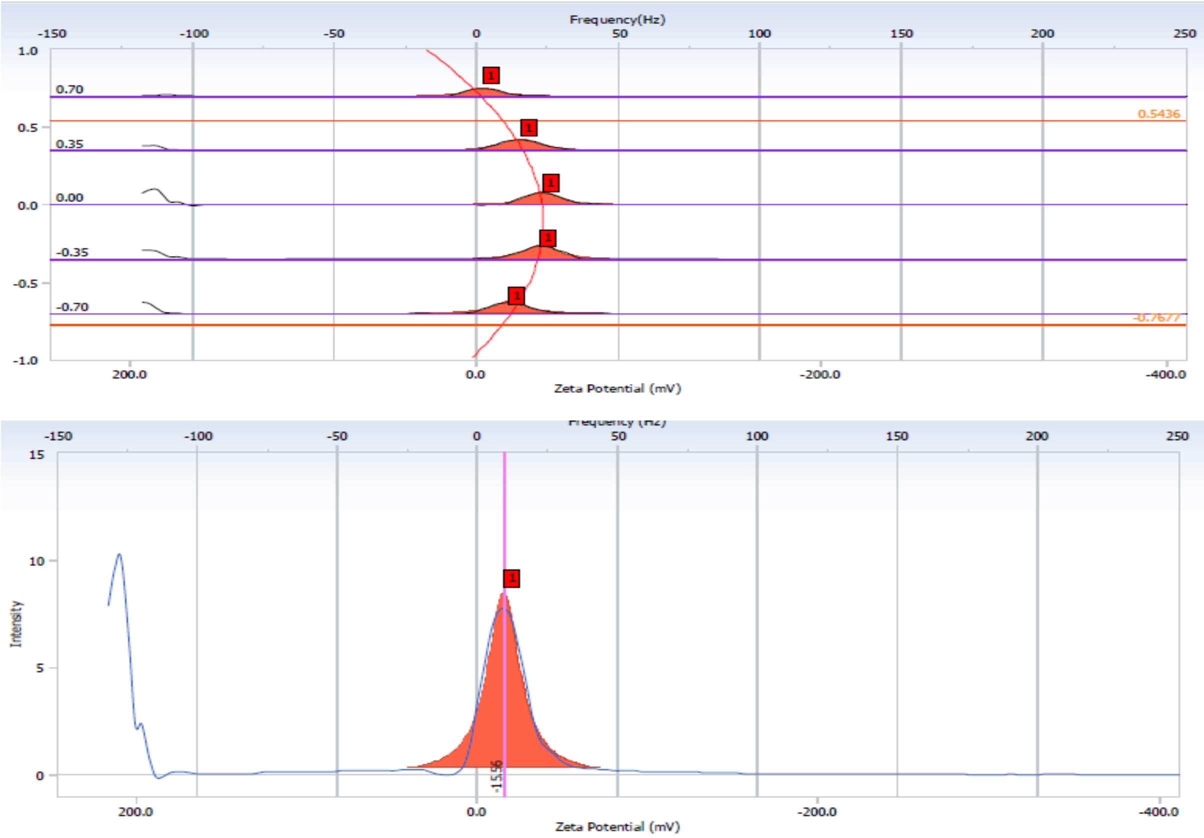
(c)

Fig. 6. HRTEM was used to examine the microstructure of superfine samples of (bael leaf) that had been ground for 3 hours. (a-c).

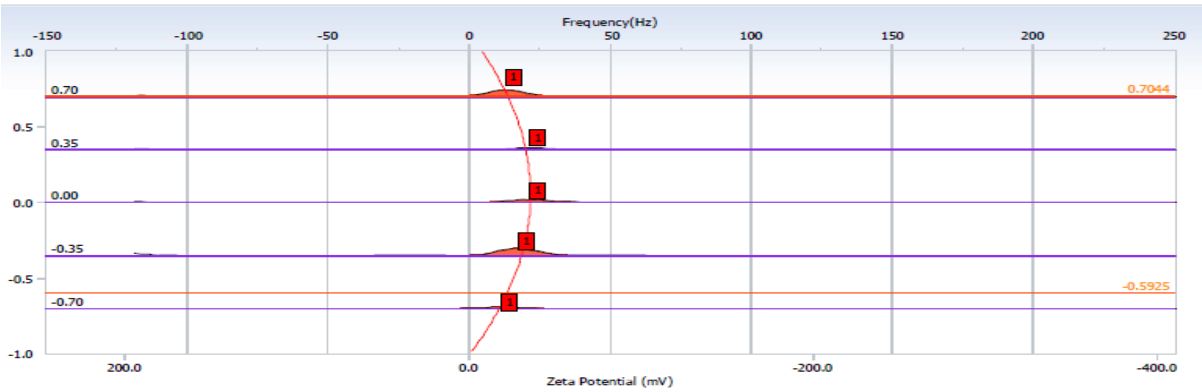
Fig.6 (a) demonstrates the microstructure how tiny nanoparticles develop into an agglomerated structure. In order to determine the average particle size, we created a histogram using the observed size of nanoparticles, as shown in Fig.6 (c). The particle size was calculated via statistical analysis of histogram plots to be around 19.89 nm. Because there are no bright spots or concentric circles in the pattern, as shown by the SAED, the generated nanomaterials are amorphous Figure 6(b) [29]. This measurement indicates that, prepared ball milled bael leaf powder are in nanometric scale. Further as applications and its importance were evaluated by Zeta potential and antimicrobial activities.

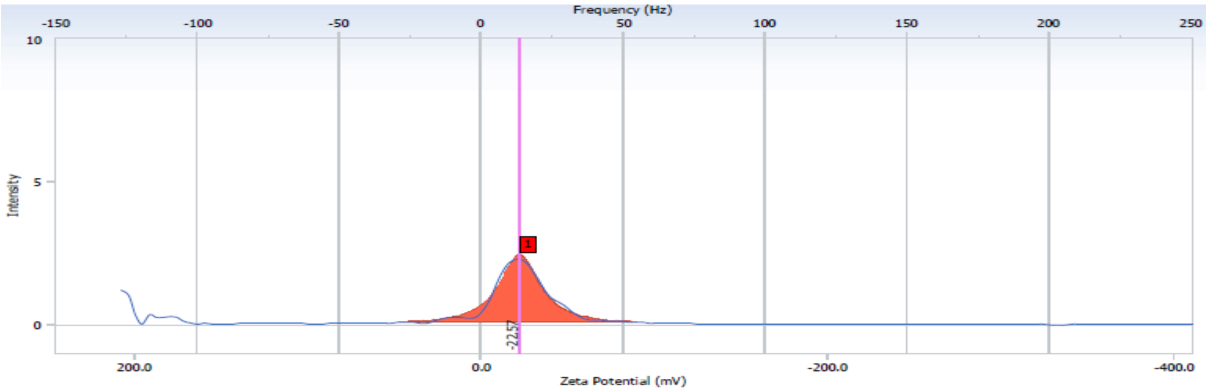
4.6. Zeta Potential Measurement

Zeta potential is a crucial metric because it may be used to assess the stability of colloidal systems and it reflects their potential as carriers of contrast chemicals for ultrasound imaging, which must be inert and simple to detect and remove during in-vivo use. Zeta potential levels greater than +25 mV or lower than -25 mV allows for a largely stable suspension. It is well known that larger absolute values of Zeta potential indicate higher stable states of colloidal systems [30,31]. Moreover, Zeta potential data have been utilized to describe the adsorption mechanisms of chemical and biological ligands on surfaces in addition to how stable colloidal systems of the bael superfine dispersion in water. According to Figure 7(a-b), the fine powders of Aegle marmelos (bael leaf) milled at 0 hours and 3 hours had Zeta potentials of -15.56 mV and -22.57 mV, respectively. Due to the strong electrostatic repelling interactions between the particles, the generated tiny particles diffused in water. Thus, higher milled sample will be more stable having higher value of Zeta potential. Theothers parameters measuredfor the0 hour sample and 3hr samples are - Zeta Potential : 15.56(mV)Doppler shift : 9.48 (Hz) Mobility : -1.214e-004 (cm²/Vs)Base Frequency : 117.8 (Hz) Conductivity : 0.2797 (mS/cm) and Zeta Potential : -22.57 (mV)Doppler shift : 13.76(Hz) Mobility : -1.760e-004 (cm²/Vs) Base Frequency : 118.5 (Hz) Conductivity : 0.2045 (mS/cm) respectively. This shows that milling and pressure grinding changes properties at atomic and molecular level.



(a) 0 hr milled





(b) 3 hr milled

Fig 7. (a-b) . Zeta measurement 0 and 3 h.

5. Antimicrobial Activity

Table.5.1. Zone of inhibition of synthesised bael leaf nanopowder.

SI.No.	Microorganism	40mg/ml	20mg/ml	10mg/ml	5mg/ml
1	*E.aerogenes*	40	38	28	20
2	*E.coli*	42	38	32	26
3	*B.cereus*	10	6	4	0
4	*S.aureus*	8	6	2.5	1

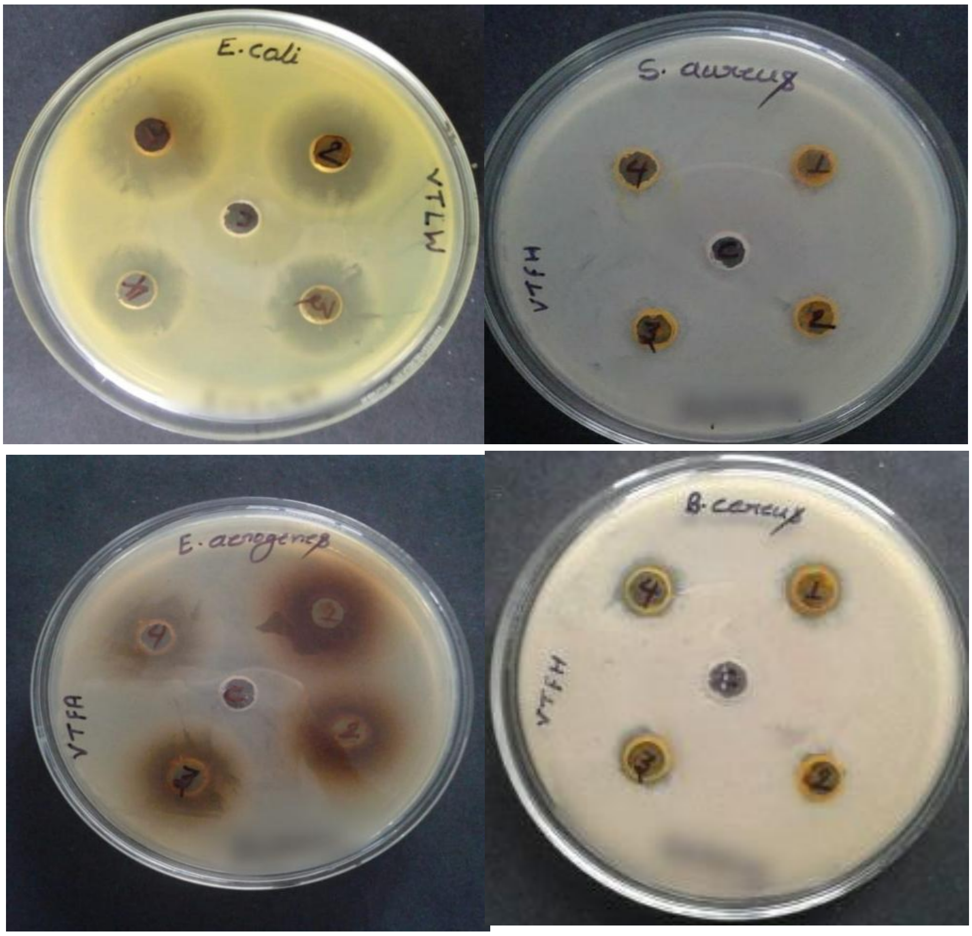


Fig.7 Zone of inhibition of *Aegle marmelos* showingfor different microorganisms.

Values of the inhibition zone were measured for nano size *Aegle marmelos* leaf powder examined against the bacterial strains Gram–negative *E.coli, E.aerogenes* and Gram- positive *S.aureus, B.cereus* Streptomycin is used as standard control. Based on the findings of the experiment, it determined that the nanopowder made from *Aegle marmelos* leaves had substantial in vitro antibacterial activity and maximum zone of inhibition was found 42 for *E coli* .

The details of zone of inhibition for different microorganisms for the same material are shown in table. 5.1. The most potent extracts can be subjected to further isolation, therapeutic antibacterial drug discovery, and pharmacological testing. As per analysis *E.coli, E.aerogenes* gram-negative microorganism shows higher zone of inhibition than *S.aureus* and *B.cereus* gram-positive microorganism results were in the synthesised samples.The nanoparticles may therefore be considered as potent antibiotics. These studies on the antibacterial effect may be helpful for bioremediation research, pharmaceutical formulations, and industrial applications. K.Suresh *et al* and Farina Mujeeb reported the similar antimicrobial effect of plant extract of bael against aegle marmelos leaf and flower [6,12].

6. Conclusions

Using a ball milling process, the superfine nanoparticles were successfully synthesizedfrom the shade-dried bael plant leaves. The size of the prepared powder found 19.87nm, which was confirmed by TEM. Crytallinity changes considerably, which are confirmed by XRD spectrum. The prominent superfine absorption peaks in UV-Visible –NIR measurement in the range of 270, 508, 541, and 607 nm indicates that the sample was milled for three hours having higher absorbance. The functional group were remains unchanged, which was analysed by FTIR. The fine powders of *Aegle marmelos* (bael leaf) milled at 0 hours and 3 hours have been found Zeta potentials of -15.56 mV and -22.57 mV, respectively, which highlights the more stability during longer duration of milling.The zone of inhibition confirms that synthesis of homogenous nanoparticles, which improve the antibacterial activity of bael leaves nanoparticles, is significantly influenced by the milling time periods**.** Maximum zone of inhibition was found 42mm for *E coli.*This study demonstrates that greater particle sizes with smaller surface areas do not generate the greatest zone of inhibition like smaller nanoparticles with higher surface areas do on specific microorganism. Also, this study showed superfine nanoscale powder of bael and their antimicrobial analysis will be more beneficial for biomedical applications as evidence based herbal medicine.

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