Superfine black pepper nanometric food powder synthesis by eco-friendly approach and its characteristic effect on its structural, morphological and toxicity for its applications in Biomedical/ Agriculture sciences as new functional nanomaterials.

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Abstract: The superfine powder of black pepper at nanometeric scale was synthesized using High Energy Ball Milling equipment. Current research study shows alteration in crystal structure and surface morphology due to grinding at different milling time. The Electron microscopy studies revealed that the superfine Nano powder is less than 50 nm in size and surface structure was found to change. The microstructure accessibility clearly specifies tiny nanoparticles development, which is observed as an agglomerated structure. FTIR and UV-vis-NIR spectrum shows that the internal covalent nature of materials did not change due to milling, but surface reactivity was found changed, which was further confirmed by Scanning Electron Microscope. The optical properties were highlighted by the observed changes in the colour which has changed from dark brown to light brown confirming the changes in properties in pretext to the crystal structure of the synthesized black pepper nano powder. The effect of the prepared Nano formulations was tested on the cell health index of the isolated mice splenocytes, which was done by using MTT assay. The result of the assay showcased reduced cell toxicity and increased cell viability due to change in crystal structure of superfine powder. The present research reveals that nanometric food particles can remarkably enhance the physicochemical properties, which are useful for its applications in agriculture, food, Biomedical science as new functional food materials.

Key Words: Black Pepper, nanoparticles, structural, HRTEM, optical, cytotoxicity.

1.0 Introduction

Since ages many medicinal plants have been rigorously discovered which in layman language, we refer them as medicinal herbs are still in continued usage as traditional medicine globally. Enormous chemical compound extracted from the medicinal herbs have exhibited varied functional properties like antibacterial, insecticidal and also predominantly showing defence against many herbivores. Large volumes of phytochemical compound with proven functional and biological activities have been in continuous mode of discovery till date [1]. Medicinal plants have been used since ages for potentially benefiting human life. According to a report cited by W.H.O (World Health Organisation) globally a large population which constitute approximately 80% is still dependent on plant parts or plant extracts to carry out their age-old traditional therapies [2]. The prime objective of this current research is to prepare superfine black pepper nanoscale powder as new functional materials for its various uses.

Piper nigrum- Black pepper is popularly called as Kaali mirch in India. It is a flowering vine falling in the family of Piperaceae. The black pepper vines are mainly cultivated for peppercorn which is basically the fruit of the aforesaid vine. After harvesting the peppercorn, it is dried for further usage. The black pepper corn is widely used globally as a spice, food seasoning and for treating varied ailments. The matured fruit size is about 5 mm (0.20 in) in diameter. The corn contains a single seed which is dark red in colour [3]. Peppercorns have been categorised as per their method of synthesis and time of harvesting precisely as black pepper which is the peppercorn which is cooked and is basically dried unripe fruit, secondly green pepper which is basically the unripe fruit which is dried and third most is the white pepper which is basically ripe fruit [4]. Black pepper is synthesised from the green harvested peppercorns, that are unripe fruit of the aforesaid vine. The harvested peppercorns are initially cooked for a little while in hot water, for cleansing purpose and simultaneously preparing the peppercorn for drying for further ready usage. The intensity of heat while cooking disrupts the cell walls of the black pepper which directly speeds up the process of browning enzymes required for drying purpose. Post the aforesaid process the drupes are dried via two mechanisms firstly by sun drying or secondly taking the aid of drying machine. Both the process of drying is carried for several days until the outer layer of the peppercorn shrinks by drying and finally turning of the shirked layer into dark colour. Post drying due to the acquired black colour the drupes are called as black peppercorns. In certain estates the black pepper is handpicked and sun dried without involving the boiling process [3, 4]. The South Asia is considered to be the primitive native to Black pepper and since 2000 BCE black pepper has been an integral part of the Indian cuisines, therapies and medicines, [5]. As cited by J. Innes Miller it had been noted that the important source of black pepper was from Kerela, India which was earlier a dominant state ruled by the dynasty of the Cheras, while it was extensively cultivated at Southern Thailand and Malaysia [6]. Piperine is the active ingredient of black pepper. Alkaloids, volatile oils and oleoresins have also a marked presences in black pepper. Piperitine, chavicine, piperine and piperidine are the major alkaloids that constitute black pepper [7]). Terpenes, steroids, lignans, flavones, and alkamides form the primary constituents [8]. The peppercorns are superbly rich in vitamins A and K along with dietary fibre. The black pepper is a storehouse of magnesium, calcium, manganese, potassium, β-carotene and phosphorous. Pepper oil, grounded pepper and oleoresins are the well-known product extensively derived from black pepper [9-11].

High antioxidant activity has been exhibited in black pepper. In high efficiency the antioxidant property was observed in oleoresins and black pepper essential oil in comparison to synthetic antioxidants [12]. Black pepper is a storehouse to high amount of natural antioxidants [13]. Due to these amazing range of components black pepper has a effective impact in treating oxidative stress [14]. Vitiligo is a skin disorder which is marked by the presences of depigmentation lesions has been seen to be treated efficiently by black pepper extract as evident by several studies [15]. Restoration value of various spices and other food materials are seen to be enhanced by adding black pepper to meals and varied recipes [16]. Various nerve ailments such as Alzheimer, depression epilepsy etc are seen to be treated by black pepper which acts in a supporting role in the treatment process. Studies in the aforesaid pretext are very low in human subjects. Owing to immense surface area to volume enhancement the nanocrystalline material synthesised show superior and varied properties as compared to their bulk counterparts. Crystal structure are categorized in nanometric scale only when they are in the nanometric range in the scale ranging between 1 nm - 100 nm [17,18]. Varied pressure dependent grinding techniques are widely used to form nano superfine crystal which is achieved by changes brought in size from micron to nano which is due to changes in surface reactivity and internal cohesion force. Effective medicinal benefits can be derived effectively from the natural food material via the recent development brought in the superfine grinding technology which is a powerful tool in synthesizing the super fine powder [19]. Solubility, dispensability, and surface reactivity phenomenon have been exhibited in an enhanced form by the superfine grinding technique as compared to the regular mechanical method used for normal preparation of powder. In varied commercial applications, numerous food stuffs and in biotechnological sector superfine grinding has been used extensively with high end potential [20]. The extensive usage of collision, friction, shear, impingement, or other mechanical actions in Ball milling technology have enormously modified the physiochemical structure the of food powder [21]. To effectively bring about changes in pretext to the relative structure of the food which would directly enhance the food value in terms of availability, solubility and targeted delivery of varied food materials ball milling method is becoming highly popularised. Very few research works have been carried out and there is very less published research papers availability in pretext to imapct of ball milling grinding technique of black pepper superfine nano powder at the nanometric scale and its significance in medicine, agricultural, nutraceuticals and other areas related to it as per the information availed to the best of my knowledge. Current research work aims to, observe and analyse the effect on physical and chemical property changes occurring in black pepper superfine powder with aid of ball milling machine and to study other related properties as per observation. In the current research we have tried to investigate and study the possibilities of the large scale production at industrial level of superfine black pepper superfine powder for various sectors predominantly medicine, biotechnological, agricultural and some others related field. The prime objective of the current research work is to gain deeper studies for the occurring changes in pretext to structural parameter and surface morphology of black pepper super fine powder occurred during the process of superfine grinding and their possible effective applications in various sectors as a new functional food powder. Enhanced changes were observed in bioavailability, density, new surface area, and hydrolysis and pore size and hydrolysis reaction mechanism [22]. The results of the MTT assay also exhibited changes in behaviour, which are explained in details in results and discussion section.

2.0 Materials and Method

2.1 Material

For the fulfillment of the present research, sample of black pepper was procured from the local market of Patna, Bihar, India in form of black pepper corn. To make black pepper powder the black pepper corns were coarsely grinded in a clean mixer grinder. Some part of the coarsely grinded pepper powder was labelled as 0hrs and kept in air tight container for further characterizations and determining applications. Further the coarsely grinded mixture was further subjected to pressure grinding in Emax, Retsch, Germany- high energy ball milling machine [Fig 1] for 2.5 hrs, 5.0 hrs and 7.5 hrs respectively to form superfine black pepper powder. Post high energy ball milling the resultant superfine powders were labelled and stored for further characterization and for determining applications. The equipment and synthesis process of the black pepper superfine powder is depicted in the flow chart are shown in figure-1-2.

2.2 Methodology

2.2.1 Synthesis of Black Pepper *(Piper nigrum)* superfine powder

The methodology adopted for the synthesis of superfine black pepper as flow chart are shown in in Fig.1-2. In High Energy Ball milling which is basically a Planetary Ball Mill instrument the powdered sample is kept in a stainless stell jar of 50ml capacity and the stainless-steel ball of 20mm diameter is used for superfine grinding inside the stainless-steel jar. 1:20 sample to ball ratio by weight is maintained in the stainless-steel jars of 50 ml capacity. 1/3rd space is occupied by sample and the ball as whole in the jar in pretext to capacity by volume. The speed of rotation is maintained at 500rpm for 2.5hrs, 5 hrs and 7.5hrs respectively. The rotation of the 20mm stainless steel balls is horizontal inside the jar. The movement of the ball inside the jar is both clockwise and anticlockwise which changes every 30minutes with a time interval of 2 minutes respectively. The temperature of the instrument was maintained below 25ºC throughout the milling hours. Due to high energy ball milling, there is heat generation so the overall temperature of the instrument is maintained by air-conditioner. Similar methodology has been employed to prepare various superfine powder of different food materials [21]. The samples were then characterized using modern scientific tools like XRD-X-ray diffractometer , SEM-Scanning Electron Microscope, TEM- Transmission Electron Microscopy and FTIR- Fourier Transform Microscope and some others. Optical images of superfine grinded black pepper powder are shown in fig. 2(a – d). Colour changes of different superfine powder states the crystal structure changes, which may be considered as new functional food materials for its varied applications.

2.3 Biomedical application

In-vitro cytotoxicity assay

For the bio application of nanomaterial cell viability and cytotoxicity assay are considered as vital index for cell health evaluation. Cytotoxicity of synthesised nano powder of Black Pepper at different time interval 0hrs, 2.5hrs, 5hrs and 7.5hrs nanoparticles was thoroughly performed against splenocytes which are basically murine derived spleen cells and Human leukemia monocytic cell line -THP-1 cell lines using exclusion assay by Trypan blue dye along with assay done by MTT(3-(4, 5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) by methodology described previously [13,14]. The splenocytes and THP-1 cells were thoroughly taken and thereafter washing was done twice with the help of phosphate buffer saline (PBS), and thereafter the resultant suspension was suspended in RPMI-1640 medium. 10% foetal bovine serum (FBS), 50 μg streptomycin, 50U penicillin, and 25 μg/ml gentamicin was also added to the medium. The cell count was intensively brought to 1×10^6 cells/ml by taking the aid of Neubauer cell counting chamber. Thereafter, cell suspension was inoculated in culture plate and, with concentration of 25, 50, 100, 250, 500, 1000 μg/ml respectively of nanoparticles which were added to assigned wells of the culture plate. The culture plate was incubated in a CO_2 incubator at 37 °C which had 5% CO_2 and 95% humidity for a time period of 48 hrs. After incubating it, some part of cell suspension was taken from all wells respectively and thereafter 10% MTT reagent was thoroughly added to each respective wells which was again further incubated for the time interval of next 4 hrs. MTT solubilizing agent of equal amount was added to each well to thoroughly dissolve the formazan crystal of MTT for unhindered and accurate reading. Finally, the culture was further transferred to a 96 well culture plate (100 μl/well) and was extensively mixed well with the help of a pipette by very gentle mixing. The absorbance was taken at 570 nm on Multiskan EX reader. Control of the experimentation were the cells in the well without any treatment. Further cell viability percentage was meticulously calculated by the formula as stated below.

Cell viability % = (Mean absorbance of treated samples/ Mean absorbance of untreated samples) × 100

On the other hand, exclusion assay was performed by Trypan blue dye to distinguish between viable cells which were the living cells and unviable cells. Approximately 50µl of cell suspension from each well was taken and thoroughly mixed with 0.4% trypan blue dye. Thereafter the percent viability of cells was determined with the aid of microscope.

3.0 Results and Discussion

Physical Property Measurement

3.1 XRD-X-ray Diffraction and SEM- Scanning Electron Microscope Measurements

D8 Advance, Bruker Germany X- ray diffractometer was used to determine the crystalline size and related details of the black pepper powder. 40 kV voltage, 40 mA of current and 25^0C temperature were maintained along with a nickel-filtered Cu Kα radiation (λ = 1.5406 Å). 2 theta (2Θ) diffraction angle was scanned from 10 to 90^0 maintaining a scan rate of 2^0/ minute. The XRD data was recorded for black pepper superfine powder which had been milled for 0 h (BP 0) (powder, prepared by using mixer grinder),2.5hr (BP 2.5), 5 hr (BP 5) and 7.5 hr (BP 7.5) using high energy ball milling machine (Figure 3). Similar XRD pattern was also reported by Mariselvi S et al [26] but he further not reported the effect of milling on structural properties. EVO 18 Research Zeiss, UK scanning electron microscope having accelerating potential of 10KV was used for analysing surface topology of the milled superfine powder of black pepper. With utmost care the sample was mounted on aluminium studs with the aid of double-sided adhesive tape (instrument specific) followed by sputtering process in which the sample is thoroughly coated with 10nm gold film and finally the sample is scanned under scanning electron microscope. The SEM images of the superfine black pepper powder is depicted in the Fig 5 (a-d). SEM has been an excellent tool for analysing surface morphology and interparticle interaction of material. The SEM images obtained states that there is a considerable change in external surface morphology along with visible agglomerations of particles which clearly indicates changes in molecular structure; as irregular and/or disordered shape of different sizes are clearly visible in the images. With gradual increase in the hours of milling it was observed that the agglomerated shapes and structures of the particles has also changed considerably. It can be stated that pressure grinding is an effective technique of altering the original surface structure and intermolecular cohesion of black pepper powder. Different milling hours have changed the surface morphology considerably to a great extent as surface to volume ratio changes the surface reactivity also increases.

3.2.1 TEM and Microstructural analysis:

The microstructural analysis of 7.5hrs milled black pepper powder was done HRTEM depicted in fig 6 (a-c). The microstructure visible in Fig 6 (a), shows clearly tiny nanoparticles agglomerated structure development. Hence, for the calculation of the average particle size, a histogram has been plotted using the measured size of nanoparticles and depicted in Fig. 6 (b). statistical analysis of the histogram plotting calculated the crystallite size of black pepper nano powder approximately 49 nm. Fig 6 (c) depicts SAED Pattern. The SAED pattern shows no bright spots or any concentric rings confirming that the synthesized superfine powder in nano scale range.

3.3 Functional group measurement using FTIR

Composition of functional groups, nature, type and purity present in Black pepper superfine powder was identified using Fourier Transform Infra-red spectroscopy (Frontier, PerkinElmer, UK). To form the samples for FTIR pelletization method was adopted which was done with the aid of pelletizer. Potassium bromide (KBr) was added to sample in the ratio of 1:20. Fig. 7, exhibits the FTIR spectra for sample BP (Black pepper). Tables has been made, depicting the functional group that are present at different wave number in the Black pepper superfine powder at four different milling time durations (a) 0 h (b) 2.5 h (c) 5 h (d) 7.5 h respectively. FTIR spectra of black pepper showed varied range of absorption band. Absorption bands were observed from 400cm^{-1} - 4000cm^{-1} at room temperature. Broad IR absorption bands were observed from 2955-3395 cm^{-1}, 1631 -2150 cm^{-1}, 1190 -1480 cm^{-1} and 510 - 994 cm^{-1}. The intense broad band region 2955 – 3395 cm^{-1} corresponds to O-H, N-H, H=C=H, HO-C=O and C–H, this might be due to aromatic ring stretching of the bioactive in sample. 2148 – 2150 cm^{-1} absorption band corresponds to alkynes in the sample. Absorption band from 1190 -1251 cm^{-1} shows presence of C=O, C=C, vibrational stretching of aldehyde, ketone, ester, nitrile and alkynes. Band spectrum 1190 – 1645 cm^{-1} is analogous to C=C, CH_2, C-O vibrational stretching of the aromatic compounds. Vibrational stretching located at around 514 - 997 cm^{-1} represent CH_2, C-Br. In the fingerprint region absorption bands are observed below 1000 cm^{-1}. Table gives a detailed account on the different functional groups present in Black pepper superfine powder. The result establishes the fact that, no new chemical/ functional group was formed owing to pressure grinding. Few changes in characteristic bonds were observed in pretext to transmittance / wavenumber. Functional groups of Black pepper superfine powder did not show any direct linkage to crystallinity. Minor shifts in the position of spectrum states that superfine grinding did not break any intermolecular hydrogen bond predominantly hence it can be stated that, superfine grinding roughens the amorphous area present on the surface of the ordered amorphous sample. Superfine grinding causes breakage in the intermolecular bonds and reform again to attain a characteristic unique crystalline structural parameters and hence new functional Black pepper superfine powder was synthesized. Changes in porosity, density, internal forces of cohesions, changes in surface area and change in the number of the bond were observed in the FTIR studies. SEM, TEM and XRD data are in accordance to the above findings. Similar processes have been employed by various research groups to elucidate different functional groups present in different food samples [23].

3.4 Optical measurement using UV-VIS -NIR Spectroscopy

Figure 8 shows the Uv-visible spectra of black pepper nanoparticles milled at different hours of milling time. The maximum absorbance for all four synthesised samples is approximately identical at approximately 400 nm and observed slightly lower at 270 nm, indicating that protein structure is present in the different sample of superfine powder. This could be related to the three types of amino acids that make up the protein (namely tryptophan, tyrosine, and cysteine) [24]. FTIR results support the UV-Vis-NIR spectrum.

4.0 Biomedical Applications

Cell Viability Assay

The percentage of cell viability was done by comparing with control is been shown in fig 9. The results of the current study exihibit that as the crystallite size of the nano powder is changing with respect to the increasing time duration 0, 2.5, 5 and 7.5 hrs respectively. There is significant reduction in its cell toxicity with increase in the cell viability. Hence the increase in cell viability with size reduction can further be explored for various other applications in diversified fields, such as in food sector, biomedical Engineering and health. Such behaviour is due to change surface reactivity and crystal structure of black paper using high energy ball mill [25-27].

Conclusions:

Nanocrystalline superfine Black pepper powder with diverse crystal arrangement and morphology were successfully synthesized with the aid modern high energy ball milling machine with significant scientific evidence as a new functional nanomaterial. The TEM analysis confirms the material having average crystallite size of approximately 49 nm. FTIR and UV-vis NIR measurement shows that the internal bond nature cannot change, while SEM measurement reveals those changes in surface morphology and reactivity. The MTT assay result showed reduced cell toxicity with increase in cell viability, which depends on crystal structure and thus present research opens window for production of more functionalised food material as a new functional food material and creates a new window for production of Nano crystalline black pepper food material, which can be used in variety of industrial and biomedical industries and many others.

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Conflicts of interest/Competing interests – There were no conflict of interest among the authors.

List of Figure and Table

Fig. 1- High energy Ball Milling machine and flowchart of synthesis process
(Source:- Water cooled bench top grinding machine (Planetary ball mill type) (Make: Retsch, Germany) – Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

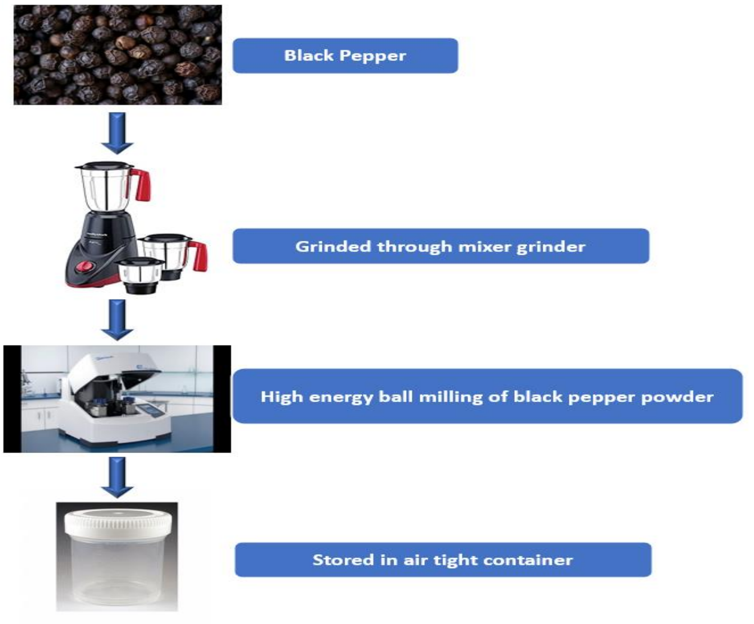


Fig. 2- Pictorial representation of the synthesis process
(Source- Water cooled bench top grinding machine (Planetary ball mill type) (Make: Retsch, Germany) – Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

(a) 0 hours

(b) 2.5 hours

(c) 5 hours

(d) 7.5 hours

Fig 3 (a-d) – Optical images of Black pepper superfine powder after grinding; (Source:- Synthesis Lab of Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

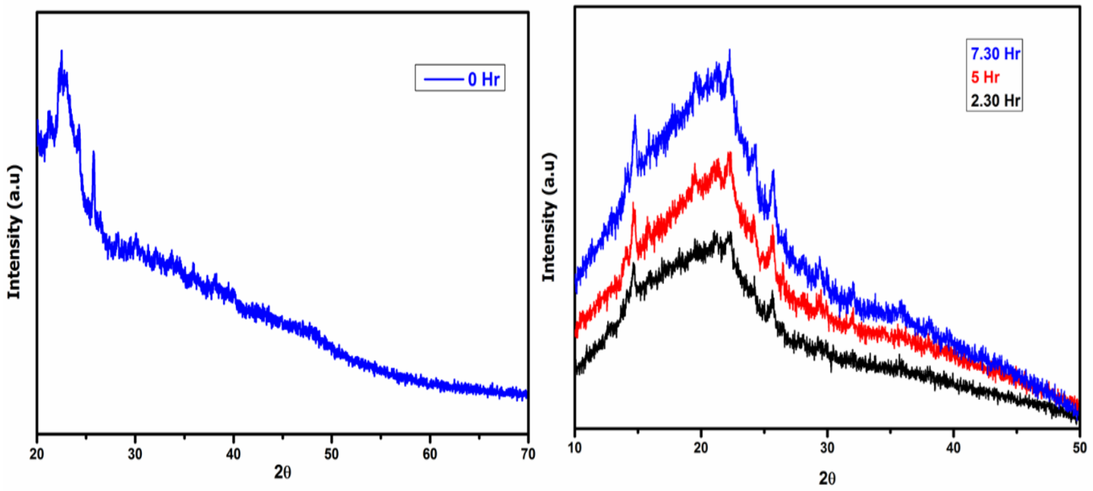
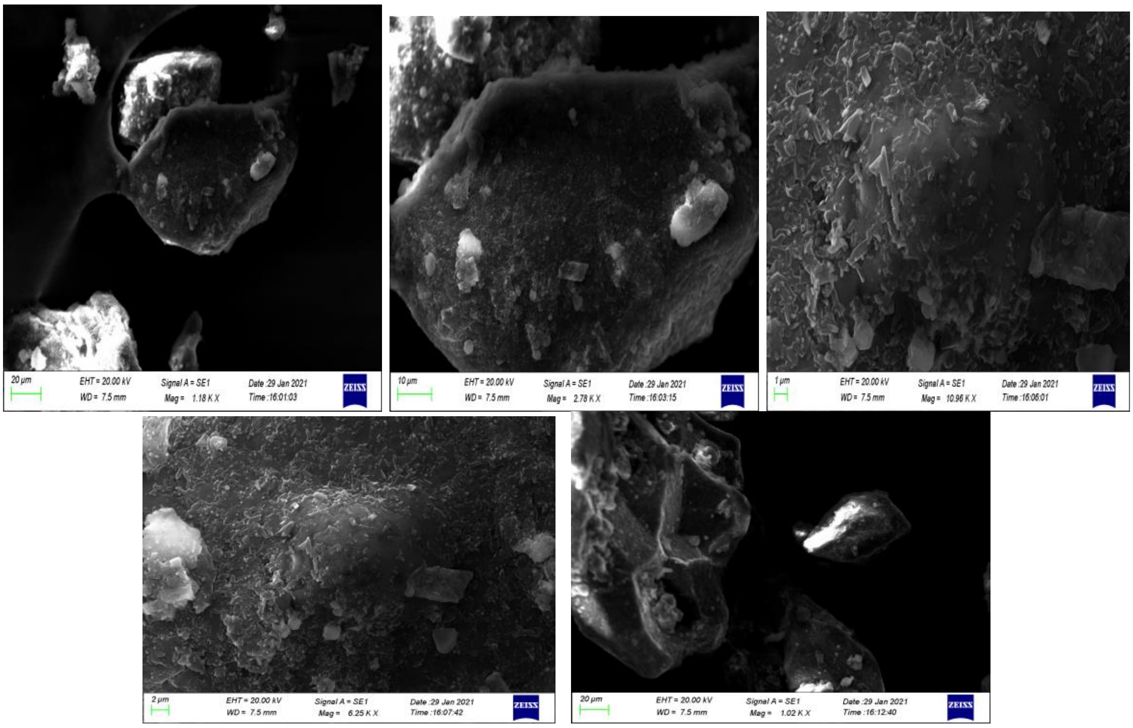


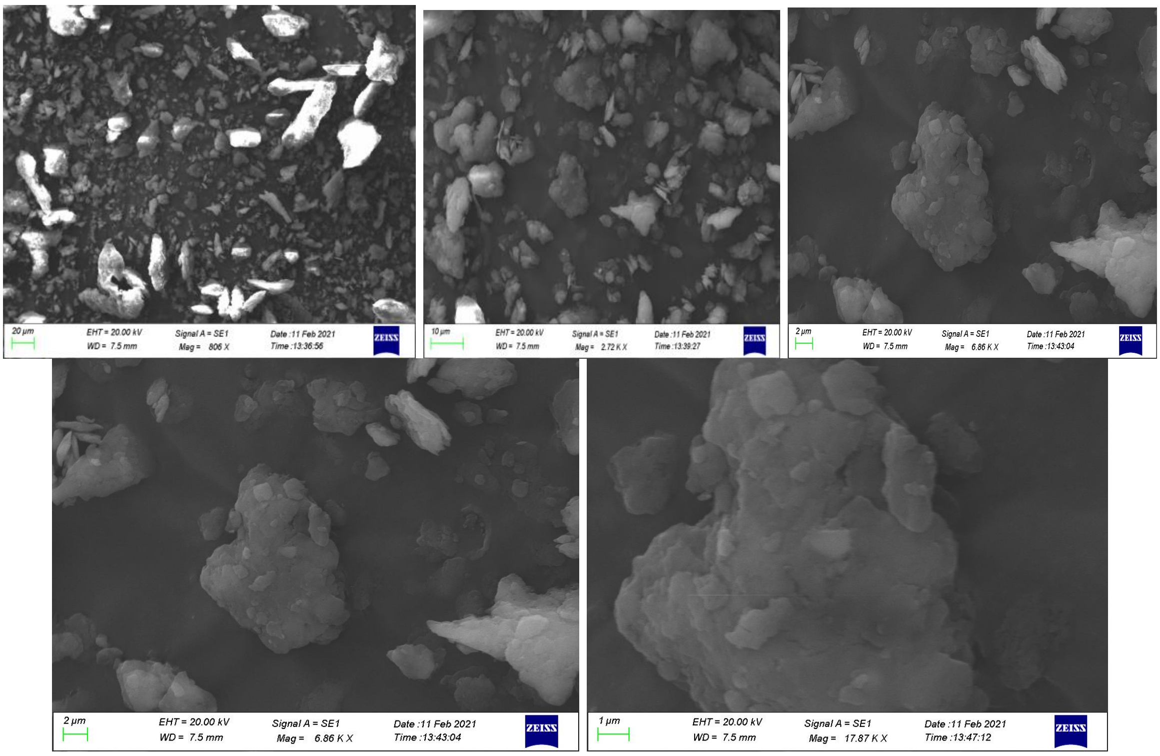
Fig 4. XRD Spectra of Black Pepper at different milling hours.

(Source:- X-Ray Diffractometer with temperature (Iq. N2 – 1200°C) variation facility (Make: Bruker, Germany) and graph by OriginPro 8.5 Software – Characterization Lab of Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

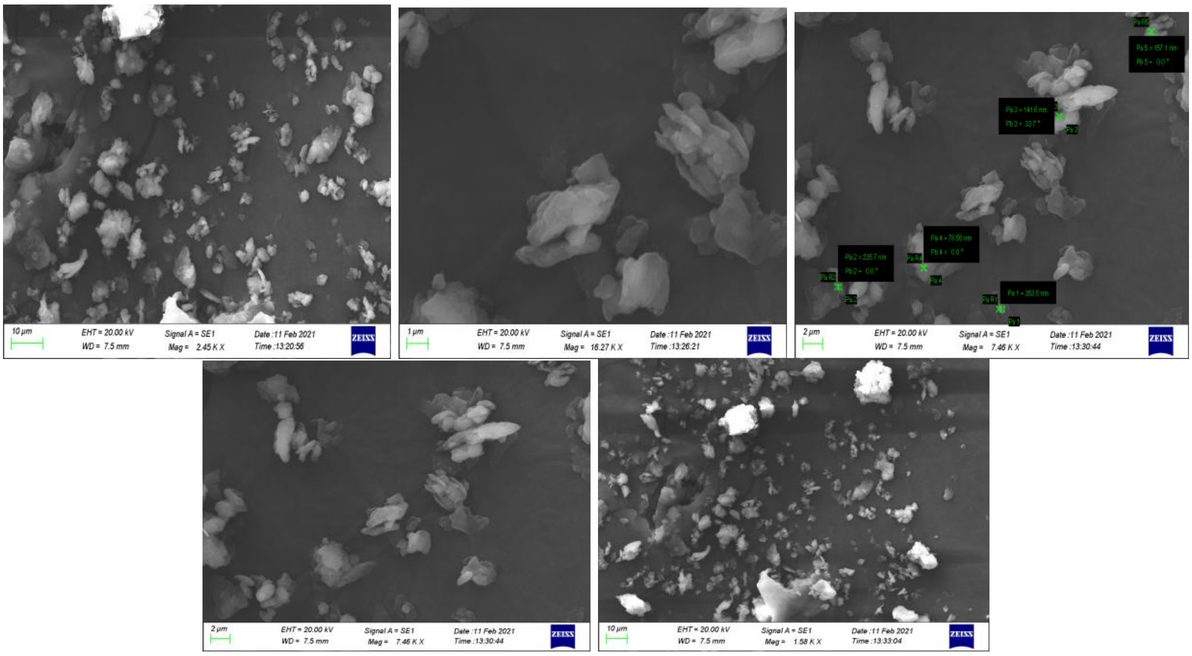
(a) Black pepper SEM images for 0Hrs milled Superfine Powder



(b) Black Pepper SEM images for 2.5Hrs milled superfine powder



(c) Black pepper SEM images for 5hrs milled superfine powder



(d) Black pepper SEM images for 7.5hrs milled superfine powder

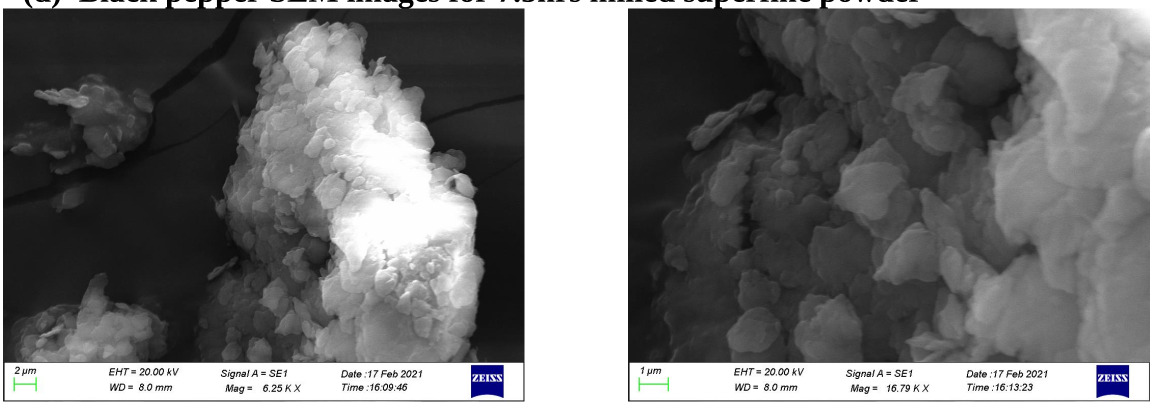
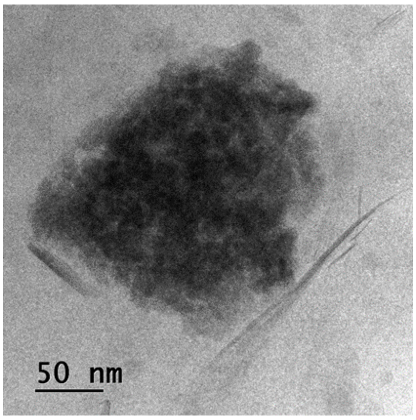
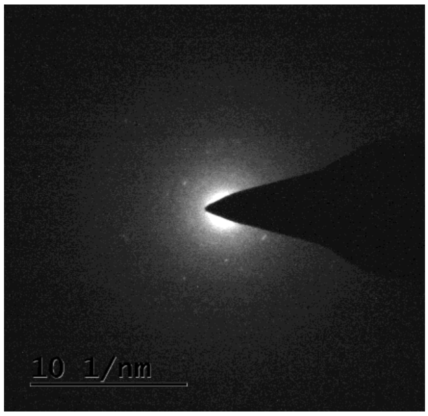


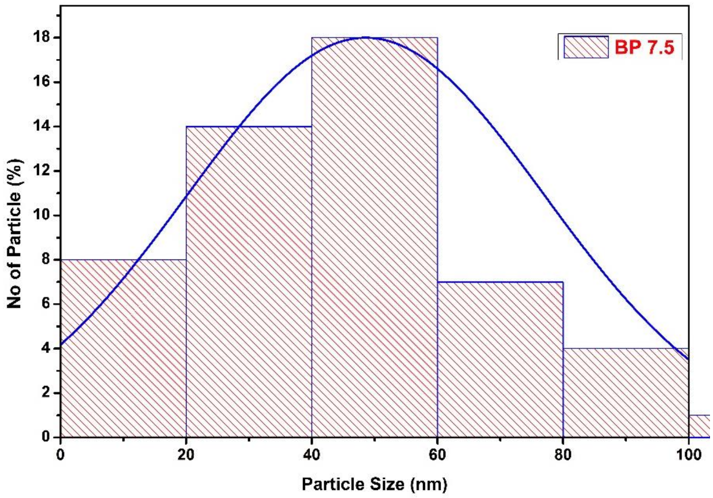
Fig. 5 (a-d)– SEM images of Black pepper superfine powder
(Source: Scanning Electron Microscope (Make: Carl Zeiss Microscopy Ltd., UK - Characterization Lab of Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)



(a)



(b)



(c)

Fig. 6 (a-c) HRTEM images of Black pepper nanoparticles (a) measurement (b) SAED Pattern (c) Histogram

(Source: Transmission Electron Microscope (Make: JEOL Ltd., Japan)

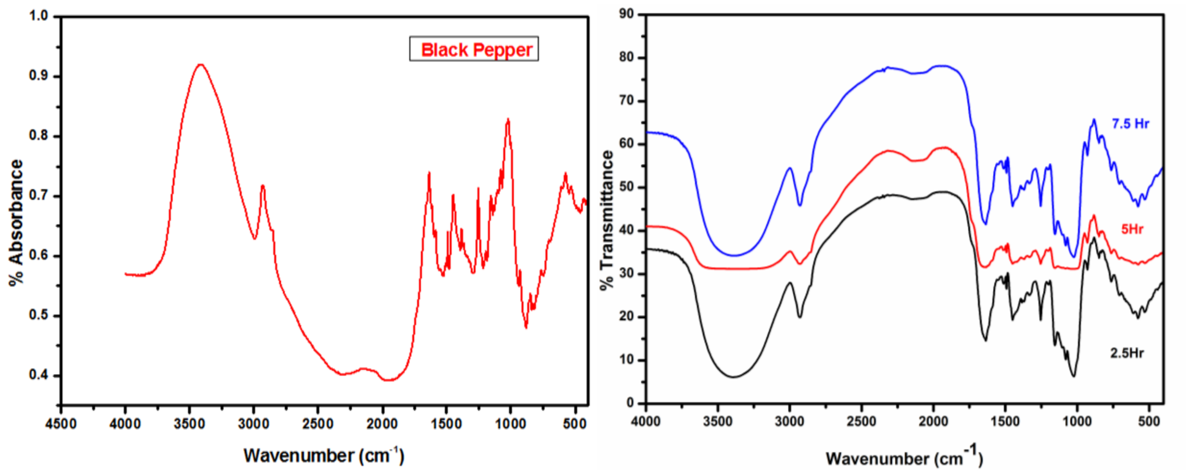


Fig.7 – FTIR spectra and table of black pepper

Table 1 – FTIR Spectra of Black Pepper at 0,2.5,5 & 7.5 Hrs

FTIR spectra of BP-0		FTIR spectra of BP-2.5		FTIR spectra of BP-5		FTIR spectra of BP-7.5	
Wavenumber (Cm^{-1})	Functional Group	Wavenumber (Cm^{-1})	Functional Group	Wavenumber (Cm^{-1})	Functional Group	Wavenumber (Cm^{-1})	Functional Group
3415 Cm^{-1}	O-H	3395	O-H	3387	O-H	3384	O-H
2985 Cm^{-1}	N-H	2965 Cm^{-1}	N-H	2955 Cm^{-1}	N-H	2955 Cm^{-1}	N-H
2155 Cm^{-1}	Alkynes	2150 Cm^{-1}	Alkynes	2148 Cm^{-1}	Alkynes	2148 Cm^{-1}	Alkynes
1639Cm^{-1}	C=C	1632 Cm^{-1}	C=C	1631 Cm^{-1}	C=C	1631 Cm^{-1}	C=C
1482 Cm^{-1}	CH_3	1480 Cm^{-1}	CH_3	1478 Cm^{-1}	CH_3	1478 Cm^{-1}	CH_3
1252 Cm^{-1}	C-O	1251 Cm^{-1}	C-O	1251 Cm^{-1}	C-O	1251 Cm^{-1}	C-O
1194 Cm^{-1}	C-O	1191 Cm^{-1}	C-O	1190 Cm^{-1}	C-O	1190 Cm^{-1}	C-O
996 Cm^{-1}	CH_2	994 Cm^{-1}	CH_2	994 Cm^{-1}	CH_2	994 Cm^{-1}	CH_2
512 Cm^{-1}	C-Br	511 Cm-1	C-Br	510 Cm^{-1}	C-Br	510 Cm^{-1}	C-Br

(Source: FTIR spectrophotometer (Make: PerkinElmer, UK) and graph by OriginPro 8.5 Software - Characterization Lab of Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

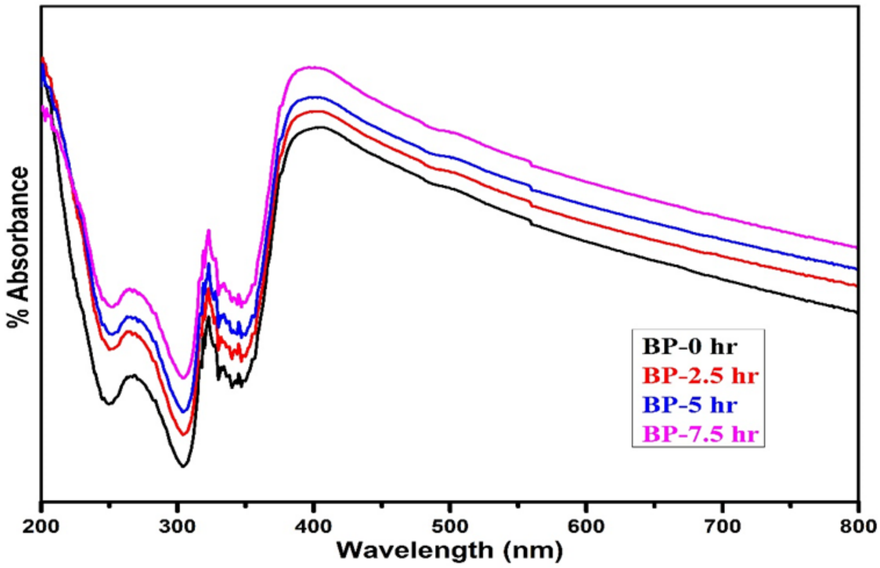


Fig 8. Uv-Visible spectra of black pepper at different milling hours.
(Source: UV-Vis-NIR spectrophotometer with temperature variation facility (Make: PerkinElmer, UK - Characterization Lab of Nanoscience & Nanotechnology Centre, Aryabhatta Knowledge University, Patna)

Black Pepper cytotoxicity MTT assay

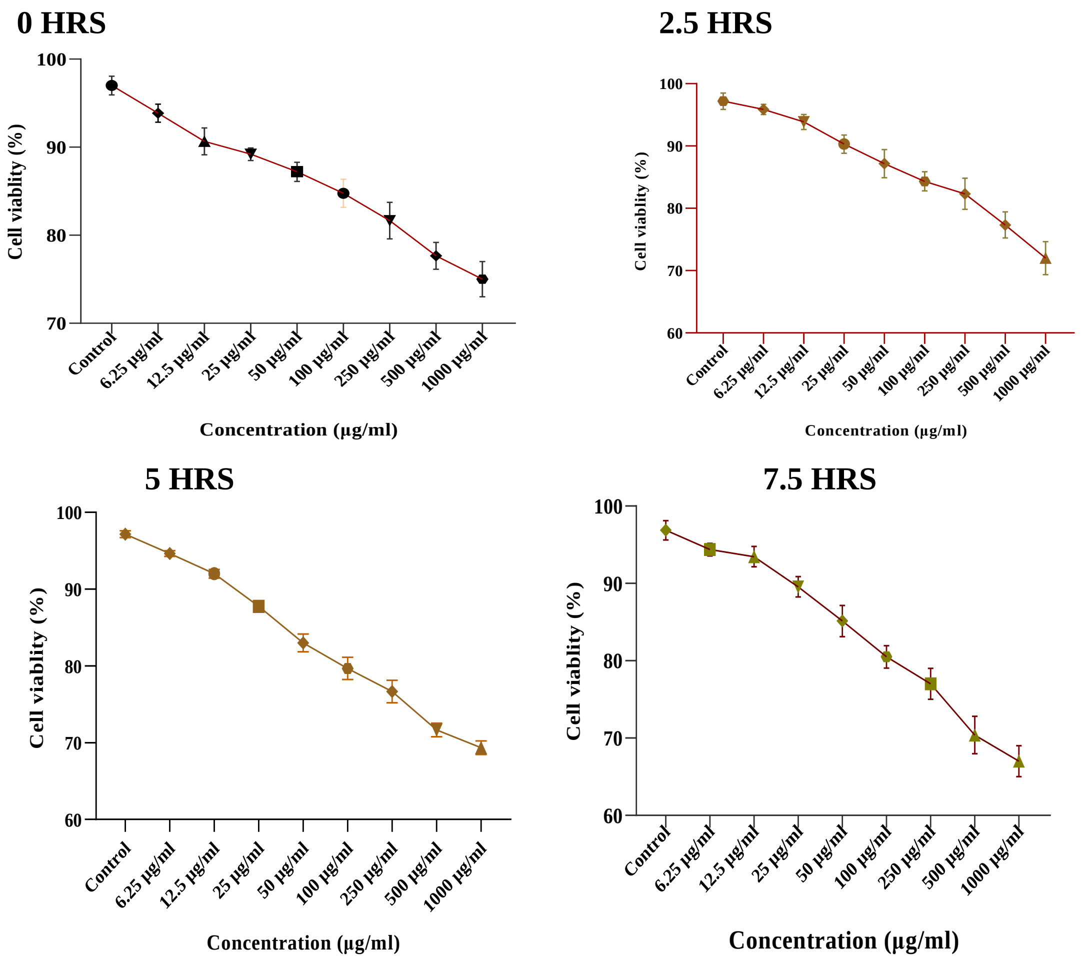


Figure 9. Cytotoxicity assay of black pepper at different milling hours (Source: Microbiology Lab of Rajendra Memorial Research Institute of Medical Sciences (RMRI), Patna)

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