ORIGINAL ARTICLE

Effect of Herbomineral Preparation and their Corresponding Metal Nanoparticle on Enzymatic Activity and Growth Pattern of Baker's Yeast

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Abstract

In modern medicine, heavy metals are thought to be existing toxic and trace amount of metals such as lead and mercury is a regulatory concern in drug development and approval process. However, metals are an integral part of Indian traditional medicine Ayurveda and have a safe history of usage for the past 5000 years. Bhasma, which is a herbomineral preparation containing heavy metals are subjected to heat, pressure and is heated with herbal juice and this process for detoxification of metals as claimed by Ayurveda. The objective of the present investigation is to understand the physiochemical changes in metal due to Sodhana and impact on bio-molecules such as enzyme and eukaryotic cells. Bhasma is compared with nanoparticles of the same metal to study the effect of Sodhana on biocompatibility of metal nanoparticles. The basic aim of this research was to find out the ill effect of such metallic preparation (Mandur bhasma) used in Ayurvedic medicine system and their corresponding iron nanoparticles using Baker's yeast (*Saccharomyces cerevisiae*) and biological enzymes. For the study, initially culture Baker's yeast was prepared in the pre-sterilized yeast extract, peptone, dextrose media. Growth and morphological change in baker's yeast cell was studied in the presence of the marketed ayurvedic formulation and its corresponding metal nanoparticles. At a similar time, standard microbiological assay procedures were also performed to find out the impact of these preparations on growth and morphology of yeast cells. An enzyme blocking study using the enzymes was also performed. Results showed that the iron nanoparticles (in higher concentration) have an inhibitory effect on the growth of yeast cells in comparison to the respective formulation. At the same time, the yeast cells show aggregation behavior and damaging with the abnormal surface in case of metallic nanoparticles. Effect on enzymatic activity was also found significant. On the basis of the present study it could be concluded that metals present in the ayurvedic preparations in Sodhit form do not have any objectionable behavior but there are certain need of pharmacovigilance to follow standard protocol to establish the safety and efficacy of such Ayurvedic preparations, and before coming to any final conclusion, still number of studies will also be needed.

Key words: Ayurvedic preparations, metallic toxicity, microbiological assay, yeast cells, yeast growth curve

INTRODUCTION

Iron is one of the most important and essential low molecular weight element which catalyzes a number of reactions involved in biological processes and sometimes leads to the generation of free radicals, which are supposed to have a deleterious effect on the various biomolecules including lipids, proteins, and nucleic acids.[1] Iron is one of the very much important elements for the biological function, being the integral of blood and necessary on regular basis[2] but still, there is a phrase in Sanskrit that *atya sarvatra varjate* and it has also been reported in various other studies that iron may also its toxic impact if it is given for a longer time.[3] Literature reveals that overload of the iron leads to the deposition in the cytoplasm of the cells of various

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organs including liver, pancreas, heart, endocrine glands, and skin.[4]

While in Ayurveda bhasma prepared by the use of iron as Mandura bhasma is being used from ancient time too, even in higher daily doses for longer time without any reported toxicity and this highly efficacious and safer use of iron-based bhasma may be attributed due to the process of Sodhana as mentioned in literature for the preparation of Bhasma.[5,6]

Ayurveda and different ayurvedic medicine are leaving their footprints worldwide, and in recent few years, the use of ayurvedic herbomineral preparation has been increased tremendously. Indian system has witnessed the golden era of Ayurveda since the ancient time. Charka, Sushruta, Vagbhata Atreya, Bhavamisra, Rusa, etc., are few names who have set a milestone in the development of Ayurveda.[7,8]

Charaka Samhita is one of the most ancient literatures mentioning the use of metals for their use in therapy.[9] Ancient literature reveals that when the metals are heated till red hot condition in the presence of various herbal juices, the physiochemical properties get alter completely and the toxicity associated with such metals get removed and have much safer activity.[10] The change in physiochemical properties and toxicity occurred due to the process as mentioned in literature is known as Sodhana. Sometimes, Sodhana process also involves the use of mercury and abhrak, which may have serious deleterious impact on the human form if it gets not converted in the safer form due to improper Sodhana process.[8,11]

Apart from the above, several metals such as lead and tin may have serious ill effect if used in its raw form even in a smaller amount.[12] While several other metals such as silver, gold, and copper which are regarded as much safer and beneficial, may also have a negative effect in their raw form in comparatively higher dosage. While the ancient and modern, both the literatures revealed the fact that proper validated Sodhana process may overcome such chances of ill effects by changing the properties and toxic impact of metals.[13] However, still it is matter of great concern that how the Sodhana process may be validated, how the quality of such herbomineral preparations may be assured, how to ensure the elimination of toxic impact of the metallic materials used in such preparation, etc.[14]

However, still, there are several questions arisen on the use of such metal-based herbomineral preparations due to lack of very much scientific evidence behind the change of physiochemical properties of metals after Sodhana which are mainly responsible for the safe uses of such preparation. At the similar time, proof of the safety of such metal-based preparation only by the animal-based studies has also set several limitations, and the process is comparatively tedious having much cost behind. Sometimes, results obtained by such studies are lacking with the significant result by which a clear inference can be drawn. Hence, it is much needed to have certain methods for such type of toxicological studies, which are more sensitive, cost effective and which may result to the significant difference between control and test for the better conclusion.[15] In the present work, we have tried to use the yeast growth and inhibition of enzymatic activity to prove and compare the toxicity of such metal-based formulations.

MATERIALS AND METHODS

Mandur bhasma was procured from the local ayurvedic store while iron oxide nanoparticles were prepared in the institute laboratory. Baker's yeast was procured from the local market while Pepsin and Diastase were procured from Central Drug House (P) Ltd.

Preparation of iron oxide microparticles

Iron oxide microparticles were prepared by reaction of ferric chloride and sodium hydroxide followed by drying of the precipitate at 350°C. Initially, 1 molar (M) solution of ferric chloride was prepared in distilled water. Further, 1 M solution of sodium hydroxide was prepared and added to the ferric chloride solution with high-speed agitation (2500 rpm). Resultant system was filtered and set to settle down. Further, it was decanted off to get the ferric hydroxide. After repeated washing, it was dried at 350°C for approximately 4 h. After cooling, it was again dispersed into isopropyl alcohol and sonicated for 1 h.[16]

Characterization of herbomineral preparation and corresponding microparticles

XRD studies

Powder XRD of herbomineral preparation and corresponding bhasma was performed using PANalytical Empyrean XRD X-ray diffractometer with the parameters using CuKα radiation, l = 1.5406 Å over the range 5.203° 80.148°.

IR spectral analysis

The IR analysis was performed with spectra measured over the frequency range 500–4000 cm^{-1} using Bruker's FT-IR.

Particle size analysis

The particle size of both the preparation was found out using Zetasizer (Horiba, Japan). Samples were suspended in the 0.5% Carboxy methylcellulose solution to avoid the settling of the particle during the study. SEM (JEOL-JSM-T330A) studies were performed to find out the size as well as surface behavior of the particles present.

Study of the toxicological impact of bhasma using a standard procedure of pepsin activity (I. P)

The assay procedure for pepsin (Indian Pharmacopoeia, 1996) was modified to study the effect of bhasma and corresponding nanoparticle on the catalytic activity of the enzyme. The assay procedure is based on the digestion of egg albumin by the proteolytic enzyme pepsin. 0.05 g of pepsin was accurately weighed and triturated with 200 mg of sodium chloride with slow addition of acidified water and volume was made up to 200 ml with continuous shaking for 15 min. Then, 3 g of coagulated egg albumin already passed through sieve no. 44 was taken and mixed thoroughly with 10 ml of the acidified water, ensuring that the particles of egg albumin are completely disintegrated. 10 ml of acidified water was further added with or without bhasma or metal nanoparticle at a concentration of 1 mg/ml to it and kept in a water bath at 51°C temperature for 15 min. After that, 4.0 ml of already prepared solution of pepsin was added and the entire material was kept at 51°C for the digestion until 4 h with intermittent shaking at the intervals of 15 min. After completion of digestion, the complete suspension was centrifuged and the supernatant was decanted off. Remaining material was washed into a 10-ml graduated cylinder and allows to stand for 30 min.[17] After digestion, 10 ml of the above system was centrifuged and precipitated volume of undigested protein was measured.

Study of the toxicological impact of bhasma using a standard procedure of α-amylase (diastase) activity (I. P)

1 g of fungal alpha-amylase (diastase) was weighed accurately and triturated with 2 ml of acetate buffer (pH 5) with the further addition of sufficient acetate buffer to produce 10 ml. Further, this solution was diluted up to 100 ml with acetate buffer pH 5 filtered. Further, 5 ml of starch solution was prepared as per the monograph and added into the test-tube without touching the sides of the test-tube and placed in a water-bath maintaining the temperature at 40° ± 0.1°C. 1 ml of already prepared diastase solution was added to the starch solution and again kept in the water bath to maintain the temperature. After heating the above up to 60 min, it has been cooled rapidly in cold water and added 0.05 ml of iodine solution and mixed well. For finding the inhibitory effect of the herbomineral preparation on the diastase activity, standard diastase solution was treated with bhasma for 30 min, and then the capability of change in starch digestion was recorded.[18]

Toxicological study using Baker's yeast

Growth of yeast was obtained using yeast extract, peptone, and dextrose (YPD) media. For the cup plate method, solid agar plate using YPD and for suspension culture liquid YPD media was used. All the ingredients mentioned in Table 1 were dissolved in 1 L of distilled water and sterilized by autoclaving at 121°C and 15 mps for 20 min. Combination of the ingredient 1, 2, and 3 of Table 1 was taken to prepare the liquid YPD media while solid agar plate of YPD media was prepared using all the four ingredient.[19]

Cup-plate method

Baker's yeast was grown in YPD media and inoculated the yeast suspension on YPD agar plate and spread with a spreader incubated the plates for 48 h which led to the development of lawn of yeast. For further studies, the cup was made in the center and seeded with the formulation before incubation. Three different plates were prepared and cup was made for the addition of the material to be checked for the toxic effect.[20,21] Three different plates were prepared:

- Plate 1-cup was seeded with sterile normal saline
- Plate 2-cup was seeded with sterile suspension of bhasma in normal saline
- Plate 3-cup was seeded with sterile suspension of micro/nano-particles in normal saline.

All the plates were incubated and zone of inhibition was observed after 48 h.

By suspension culture

Impact of bhasma and corresponding nanoparticles were studied on the growth and morphology of yeast cell. Yeast suspension was prepared using YPD media[22] aseptically and divided into three different sets:

- Set 1-YPD media with yeast
- Set-2 YPD media with yeast treated with bhasma
- Set 3-YPD media with yeast treated with nano-particle.

Ten vials of each set were taken and incubated in shaker incubator, and optical density (OD_{590}) of the yeast

Table 1: Composition of yeast extract, peptone, dextrose media used for the study (liquid or solid)

S.No.	Reagent	Amount (g)
1	Yeast extract	10
2	Peptone	20
3	Dextrose	20
4	Agar (for plates only)	20

Table 2: Pepsin activity on digestion of protein in different condition

S.No.	Metals+ pepsin	Total volume of test material containing protein	Precipitated volume after centrifugation
1	Standard	10 ml	<0.2 ml
2	Iron bhasma	10 ml	0.6 ml
3	Iron particles	10 ml	1.8

suspension of each set was measured at 590 nm using the corresponding blank. Graph between the time and OD_{590} was plotted to prepare the growth curve. Change in the growth curve pattern and change in the microscopic view of the yeast cell (using trinocular brightfield Microscope) was observed.

RESULTS AND DISCUSSION

The IR spectra analysis reveals the presence of organic matter in the herbomineral preparation characterized by the peaks above 2000-cm as shown in Figure 1a. The presence of IR spectral bands near to 420, 440, 460, 560, 640, and 680 and 990–1010 shows the presence of Hematite, Magnetite, Meghamite, and other iron oxides. IR band close to 1900 and 3650 show the presence of siderite ($FeCO_3$). The shift of band toward 3700 shows the presence of $MgCO_3$.

Peak identification and matching was done by Match (version 3.6.2.121) software, and the major X-ray diffraction peak in case of Bhasma preparation was observed at $2\theta = 24.16$, 32.28, 33.30, 35.74, 40.95, 54.19, 62.75, and 64.22 corresponding to Hematite-proto, $(Na_{3.9}\ Fe_{0.1})$ $(Ti_{1.86}\ Fe_{0.14}\ O_{1.96}\ (O\ H)_{0.04})$ $(Si\ O4)_2$, Magnetite(Fe_3O_4), Maghemite (Fe_2O_3 and Fe_3O_4) Quartz(SiO_2), and Ciderite ($FeCO_3$) as depicted in Figure 2a. Sharp and high-intensity XRD peaks indicate the presence of these materials in crystalline form with a different structure such as trigonal, orthorhombic, cubic, and tetragonal. In case of the iron nanoparticles shown major X-ray diffraction peak seen at 2θ = 9.05, 18.14, 29.04, 33.98, 40.07, 44.10, 44.78, 47.74, 60.38, and 74.81which corresponds to iron oxide (FeO), iron(Fe), magnetite(Fe_3O_4), and iron (III) oxide (Fe_2O_3) as mentioned in Figure 2b. Sharp and high-intensity XRD peaks indicate the presence of crystalline (orthorhombic and cubic) structure. In comparison to the bhasma, iron nanoparticles

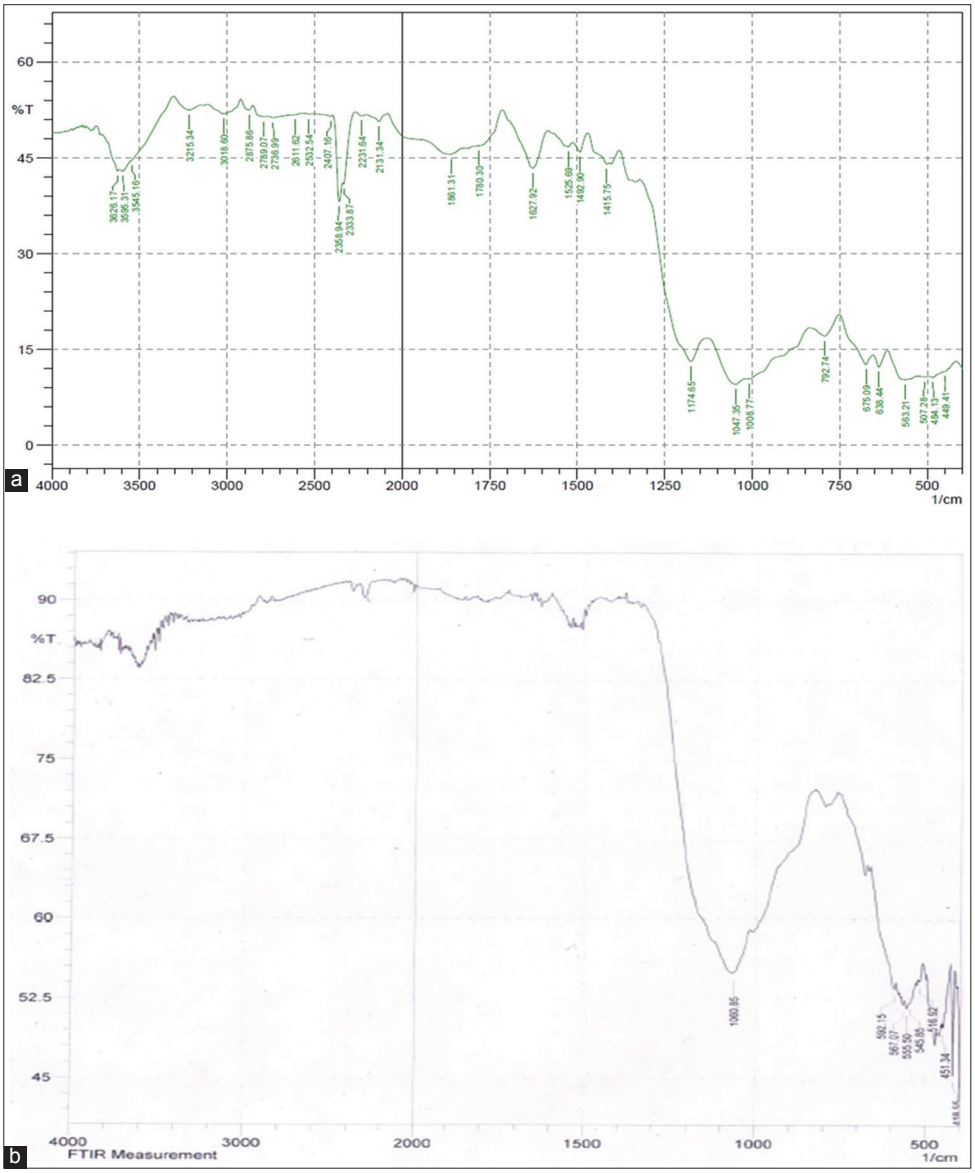

Figure 1: (a) IR spectra of Mandur Bhasma. (b) IR spectra of nanoparticles

shows orthorhombic structure rather than the mixed form, i.e., trigonal, orthorhombic, cubic, and tetragonal. At the same time, Bhasma preparation lack with the free iron particles rather than the nanoparticles.

The formulations were filtered through a 0.45 µm filter to collect the nanosize particles in the formulation which are likely to be absorbed in the intestine. When these formulations were filtered through a 0.45 µm filter, the average size of bhasma was found to be 350 µm with a yield of 37.2%. The average size of herbomineral preparations was found to be 1.45 µm while synthetic iron particles were 1.01 µm. Morphological study of iron nanoparticles was done using SEM shows agglomerates of almost spherical shaped particles [Figure 3a]. A large fraction of observed particles were in the nanosize range, which is also substantiated by the particles size study. The SEM of the herbomineral preparation, however, shows variations in shape with smooth structure with no agglomerates [Figure 3b]. At higher resolution, it can be observed that the bigger particles of herbomineral preparation are also associated with several tiny particles at their surface.

One of the major causes of heavy metal toxicity is due to their effect on the enzyme function and enzyme inhibition. The inhibitory effect of bhasmas on two different enzymes (pepsin and diastase) was studied by a standard assay protocol (IP 1996). It was observed that in comparison to the bhasma, Micro/nano-material, as mentioned in Table 2 and Figure 4a shows an inhibitory effect on the pepsin activity (I. P 1996). Both bhasma and nanomaterial (at higher concentration) demonstrated an inhibitory effect on pepsin. While in the case of diastase activity, it was observed that pre-treatment of diastase with bhasma shows no change in catalytic property in the digestion of starch. Hence, it can be said that the bhasma do not have any major inhibitory impact on the diastase activity while pre-treated diastase with iron nanoparticles shows better activity in digesting the starch. It was observed during the study that pre-treated diastase with nanoparticles shows faster catalysis of the standard starch solution than pure diastase. Result of diastase activity is shown in Figure 4b.

In the toxicological study, the microbiological assay was performed using cup-plate method and it was found that both the samples show inhibitory action in comparison to the control plate loaded with normal saline [Figure 5]. Control plate seeded with 0.9% NaCl showed tremendous growth within 48 h and formed a smear like structure on the entire plate, including the cup. While, a clear zone of inhibition in form of black patches as shown in Figure 5, was observed in both the plates seeded with 10 mg of bhasma and 10 mg of Micro/nanoparticle respectively. However, at similar concentration bhasma shows comparatively larger zone of inhibition. At a higher amount (20 mg), nanoparticles show similar zone of inhibition as compared to 10 mg of bhasma. However, as the remaining area of the plate has well-defined growth of yeast cells, so on the basis of it, a clear conclusion may not be drawn regarding the toxicity of the same as the growth may be checked due to the development of a micro-osmotic area due to presence of several other

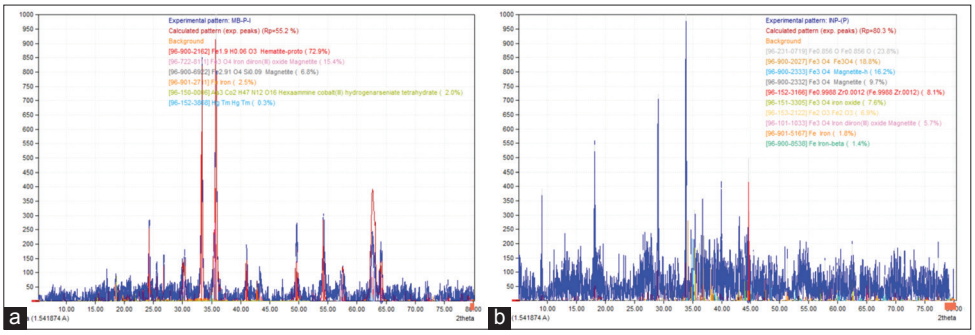

Figure 2: (a) XRD spectra of Mandur Bhasma. (b) XRD spectra of iron nanoparticle

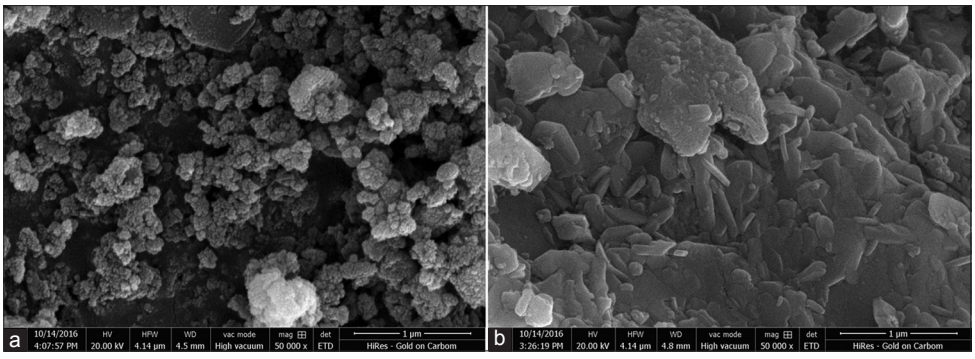

Figure 3:(a) Scanning electron microscope (SEM) photograph of Micro/nano-particle. (b) Scanning electron microscope (SEM) photograph of Bhasma

element such as Pb, Na, Mg, and Si. Hence, in the further study it was planned to find out the cumulative growth of the yeast cell for a certain period of time in YPD media as the micro-osmotic pressure generation due to the several other inorganic materials will be distributed to the larger area and almost it will be negligible as a comparison to the cup-plate method.

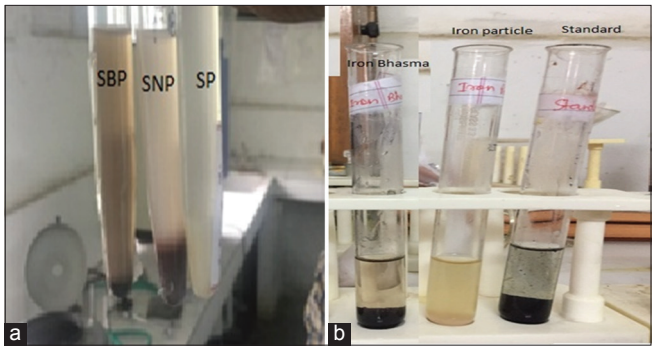

Figure 4: (a) Digestion of egg protein by pepsin (SP) pre-treated with bhasma (SBP) and nanoparticle (SNP). (b) Digestion of starch by different types of diastase i.e., Standard, Diastase pre-treated with Bhasma and iron nanoparticles

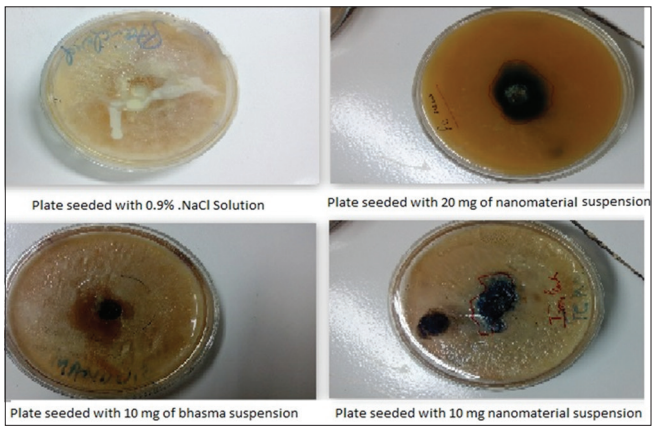

Figure 5: Yeast extract, peptone, dextrose – agar plate seeded with different samples

Yeast was chosen to find out the toxicological impact just because of eukaryotic cells. In comparison to other microorganism its bigger size, non-pathogenic behavior makes it, a better choice. It is easier to find out the surface behavior, dividing plane, and budding process, etc., which also provide the information about the metabolic process of the cells and any hindrances during this process indicates the inhibition of normal cell process due to toxicity. During the present study, growth of the yeast cell was measured after a regular time interval and growth curve plotted using time and OD_{590} of the cell. It has been observed that in all cases, the growth rate of the yeast has been hindered in comparison to the standard yeast suspension. In Figure 6, it can easily be observed that the yeast growth in the presence of bhasma has attained the saturation phase little bit early. It has also been observed that increasing the concentration of bhasma has also decreased the growth rate and attain the plateau phase early in comparison to the lower concentration of bhasma while the nano-material has decreased the yeast growth significantly in comparison to the standard cell culture. Nanomaterial has also exhibit concentration-dependent inhibition of yeast growth. From Figure 6, it can be concluded that the growth of yeast cell has been hindered by all the samples but still as the growth has been observed it cannot be said that the materials are toxic.

Differences in the yeast cell morphology are shown in Figure 7. Normal yeast cells have a more regular and uniform

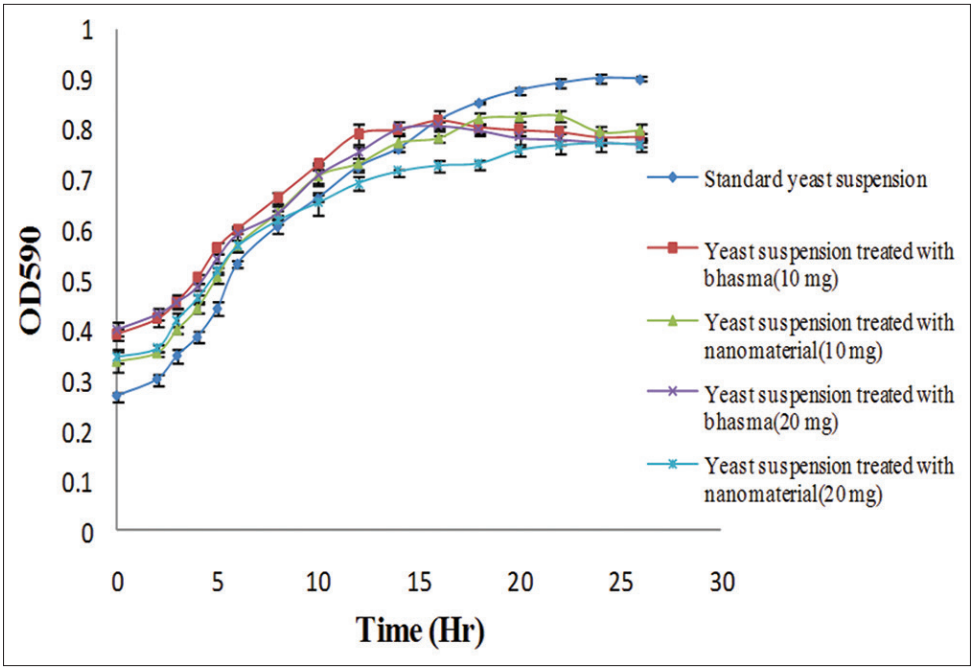

Figure 6: Yeast growth curve in different conditions

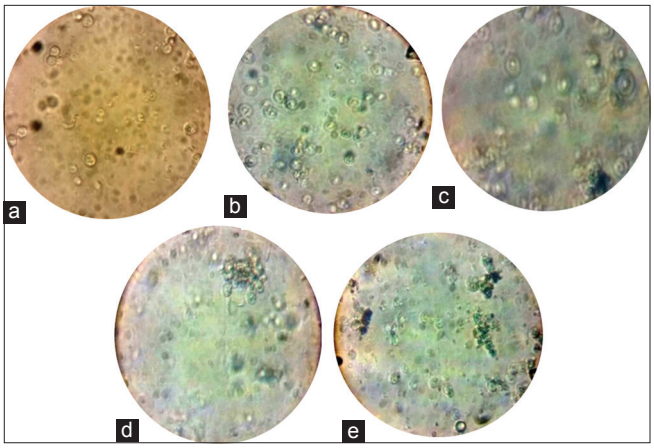

Figure 7: Microscopic photograph at ×600, (a) normal yeast cells (b) yeast cells with normal sample of bhasma (c) yeast cells with nanosized sample of bhasma (d) yeast cells with normal sample of prepared nanoparticles (e) yeast cells with sample of prepared nanoparticles having size <0.45 μ

structure than the pre-treated cell. During the study, it was observed that the yeast cells uptake herbomineral particulates with a change in their size which is clearly visible in Figure 7b. Similarly, it is shown in Figure 7d that the metallic particle has also been taken up by the cell which led to the aggregation of the yeast cell to form a bunch or it may be due to the agglomeration of yeast cell around the particle. Some changes in the cell structure were also observed.

The impact of particle size on the cell morphology and aggregation behavior was also observed. Figure 7c depicts that the herbomineral preparations having the particle size <0.45 μ is up taken by the cell more easily, and it leads to the enlargement of the cells. The aggregation of the yeast cells was increased in case of the metallic preparation with a particle size of 0.45 μ, which decreased the cell size in comparison to the normal samples.

CONCLUSION

In the present time, Ayurvedic medicines are getting wide acceptability in becoming an integral part of the health-care industry. Use of different types of Ayurvedic formulations such as, churna, vati, and bhasma has been increased tremendously in the past 10 years and still increasing day by day. However, at the same time, use of heavy metals during the manufacturing of such preparations is leading to a debate on the safety and efficacy of these preparations. While the ancient literatures reveal that these formulations are being prepared by the Sodhana process, which completely removes the toxicity associated with heavy metals. However, it is the utmost need of the present time to have some specific methods which may be utilized to access the toxicity, if any. In present work, we have tried to prove the utility of the enzymatic assay method and yeast growth pattern to establish the toxicity in spite of animal studies. The results show that these methods can be used to access the toxicity related to the heavy metals which are likely to be present in these preparations.

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