Research Article

Nanostructured silica particles from *Cymbopogon flexuosus* (Nees ex Steud.): extraction, characterization and study of their blend with osteoporotic drugs

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Received: 13 March 2020 / Accepted: 24 November 2020 / Published online: 11 December 2020
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Abstract
Cymbopogon flexuosus (lemongrass) is one of the potential herbaceous sources for the extraction of silica. Silica plays a vital role in formation of bone and its maintenance by improving the quality of the bone matrix and facilitating bone mineralization. This work presents the extraction of silica nanoparticles from different parts of lemongrass such as root, stem and leaf. IR spectra of all the three extracts confirmed the presence of siloxane and silanol linkage. The SEM analysis revealed the SiNPs from root, stem and leaves to be aggregates of tubular, fibrous and spherical structures, respectively. Significant differences were observed in elemental composition of SiNPs as determined by EDAX. Optimization of the acid leaching process further enhanced the yield by around 30% from leaves. EDAX revealed significant differences in the elemental composition. The DLS histogram showed a size distribution of 62.4 nm validating Zetasizer evaluation having PdI of 0.127. The work also reports the screening of drugs through *in silico* approach wherein the drug risedronate showed ideal parameters satisfying the Lipinski's rule of 5. Further, the cytotoxicity of SiNPs and SiNPs with risedronate (drug) was tested on HUVEC and Saos-2 cell lines. The SiNPs showed lesser IC_{50} value in comparison with risedronate and SiNPs with risedronate tested on both the cell lines. LDH assay elaborated prominent cell leakage by risedronate-anchored SiNPs which is found significant in comparison with bare SiNPs. Treatment of the osteoporotic cells with risedronate/silica blend (1:1000) revealed efficient deposition of calcium and drug in the cells as determined by microscopic observations. Drug release studies of SiNPs with risedronate were performed. FTIR indicated the presence of siloxane (Si–O–Si) and silanol (Si–OH) functional groups. In vitro study of different ratios of risedronate with silica on Saos-2 cell lines was performed to determine the mineralization potential of the combinatorial drug. It was found that the cells incubated with 0.6 mg/ml and 0.8 mg/ml of risedronate with silica showed efficient deposition of calcium inside the cells. The wild variety of *Cymbopogon flexuosus* could be a potential source for the extraction of SiNPs, which finds much use in biomedical applications.

Keywords *Cymbopogon flexuosus* · Silica nanoparticles · Characterization · Osteoporosis · Risedronate

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42452-020-03952-z) contains supplementary material, which is available to authorized users.

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1 Introduction

Cymbopogon flexuosus, commonly known as lemongrass, is one of the significant aromatic grasses in the family of Poaceae. *Cymbopogon* sp. is native to the Indian subcontinent, particularly to Southern India. The species are cultivated in Kerala, Assam, Maharashtra, Uttar Pradesh and Gujarat for commercial purposes [1]. The grass has applications in Asian cuisine because of its exclusive aroma. The tea prepared from the leaves of this plant acts as a sedative to the central nervous system. The leaves serve as a source of cellulose for manufacturing paper and cardboard. The lignocellulosic material of the plant is used in the production of ethanol in the USA [2]. *C. flexuosus* is rich in essential oil and yields one of the top ten major essential oils in the world known as "lemongrass oil". The oil is used as one of the prime components in most perfumes, soaps, cosmetics, flavours, and in aromatherapy [3, 4]. The dried spent grass is used as fuel in the distillery industry, as animal feed, like manure, and is planted for controlling soil erosion. The grass has medicinal properties and is used from ancestral time as a remedy for coughs, gingivitis, malaria, pneumonia, and vascular disorders [5].

Silica, the predominant mineral in the ash of perennial grasses, is the second abundant element found in soil [6]. The presence of silica in the grass depends upon the amount of silicic acid absorbed by the roots and the photosynthetic activity. Silica from grasses is obtained by heating to an elevated temperature, removing carbon and other volatile compounds [7]. The species of *Cymbopogon* contains majorly carbohydrates (38.44%), crude fibre (27.72%), ash (23.4–25%), crude protein (15.6%), and fat (1.2%) [8]. The proximate analysis of the grass revealed the presence of minerals such as silica, phosphorus, zinc, and potassium.

The silica has been generally regarded as safe by the Food and Drug Administration (FDA). No adverse effects of silica have been reported by the studies hitherto [9]. The various physicochemical properties of silica-based nanoparticles, such as encapsulation efficiency, surface area, low density, and adsorption capacity, made them a potential inert material in various biomedical applications [10]. Osteoporosis is a condition of low bone mass and micro-architectural disruption that results in fracture with minimal trauma [11]. The complication is known to develop due to the depletion of the hormone estrogen. The sites of a bone fracture include vertebral bodies, distal radius and the proximal femur, leading to generalized skeletal fragility. Besides, the fractures in other areas such as ribs and long bones are common [11]. Bisphosphonates such as alendronate (Fosamax), risedronate (Actonel), ibandronate (Boniva), and zoledronic acid (Reclast) are the most common drugs prescribed for the treatment of osteoporosis. The bisphosphonates are characterized by low intestinal absorption, high selective localization and prolonged storage in targeted sites. The absorption of these drugs by the intestine is deficient and variable (1–10%). Passive diffusion occurs in the stomach and upper small intestine and is reduced if the drug is administered with iron or calcium. Hormones, such as estrogen, play a vital role in preventing osteoporosis and treatment [12]. However, there has been some concern about the potential side effects associated with the use of hormone therapy. The current treatment suggests the use of the lowest dose of hormones for the shortest period. Silica enhances the desired properties of the bone matrix and facilitates bone mineralization. Bone mineral density increases by increasing the intake of bio-available silica [13]. But the commonly used drugs for the treatment of osteoporosis, such as alendronate, which increases the absorption of calcium by the bone, are not administered with silica. Hence, there is a need for complexation of drugs with silica before administration.

In this work, the silica was extracted from different parts of *C. flexuosus*, the extraction process was optimized, and the silica obtained was characterized for composition, structural properties, and cytotoxicity. Further, the study also reports the in vitro and in silico analysis of drug screening and determining the effect of silica/drug blend on osteoporotic cells.

2 Materials and methods

2.1 Sample collection

The wild *C. flexuosus* were collected from Sri Champakadhama Swamy temple located at Bannerghatta, Bengaluru, Karnataka, with latitude and longitude of 12.8138° N and 77.5762° E, respectively. The collected grass was submitted to Regional Ayurveda Research Institute for Metabolic Disorder (RARIMD), Bangalore, for authentication.

2.2 Extraction of silica by acid leaching

The leaf, stem and root of *C. flexuosus* were collected and washed to remove impurities and further dried at 105 °C for 24 h. The samples were soaked separately in 5 M HCl at different temperatures of 40–100 °C for 3 h. The acid-treated samples were rinsed with distilled water thrice and dried in oven at 105 °C for overnight. The dried samples were calcined at 600 °C until it turned to white ash [14].

2.2.1 Optimization of acid leaching process

The silica was obtained from the leaves by modifying the protocol of acid leaching process. Fifty grams of lemongrass powder was soaked in 5 M HCL at 50 °C for 3 h. Treated sample was then rinsed with distilled water and dried overnight at room temperature. It was then calcinated at 600 °C for 5 h until it became white ash. It was further subjected to sonication (500 kHz, 30 °C) for 2 h. The silica ash obtained was then quantified and characterized by SEM, EDAX, FTIR, XRD and DLS.

2.3 Characterization of silica

The calcined samples were subjected to scanning electron microscope (SEM) (JSM 6460 LA, JEOL, USA) analysis using a computer-controlled field emission, and the images were recorded. Further, the preliminary structural features of all the three samples were determined by Fourier transform infrared spectroscopy (FTIR) (Spectrum 3 Tri-Range, PerkinElmer, USA). The spectra were recorded in the range between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}as reported [13]. The elemental composition of extracted silica from all the three parts of the plant was studied through energy-dispersive X-ray analysis (EDAX) (Si(Li) EDS detector, LA, JEOL, USA). The particle size of SiNPs was determined by dynamic light scattering (DLS) measurements followed by size measurements using a Zetasizer Nano ZS (Malvern Instruments, UK) employing a nominal 5-mW HeNe laser operating at a 633 nm wavelength; the scattered light was detected at 173°.

2.4 In silico modelling of drugs

The extracted silica was studied for bone applications in the remedy of osteoporosis. Three drugs, namely ibandronate, risedronate and alendronate (Apollo pharmacy, India) commonly employed in treatment of osteoporosis, were selected. All three drugs were analysed for their affinity towards bone cells using an online tool, SWISS ADME. The different properties were studied against the Lipinski rule of 5 [15].

2.5 Preparation of silica–drug blend

Risedronate was powdered, and stock solutions were prepared for 1 mg/ml and 10 mg/100 ml (35 ml in total) [16]. The stock solutions were equally divided for control and other for adding silica. The solutions were sonicated for 10 min to ensure proper dissolution of drug in water and later centrifuged for 10 min at 10,000 rpm to remove excipients. Supernatant was collected, and O.D was determined using spectrophotometer. Later collected supernatant was filtered using syringe filter to make it sterile. Again, these stock solutions were divided equally, where one acts as a control and another one for adding silica. After that, 0.062 g of sterile silica powder was added to prepare new drug formulation [17]. Then, control and SiNPs with risedronate solutions were kept in shaker for 24 h. After 24 hr, different concentrations of control and SiNPs with risedronate ranging from 0.2 to 1 ml were withdrawn and were made up to 3 ml using distilled water. O.D was taken at 262 nm using water as blank. Amount of the risedronate released was determined from the standard graph drawn by using the obtained readings. Remaining solution was centrifuged again, the supernatant was discarded, and the pellet was stored on cryo-vials. FTIR was done to check the presence of new bonds.

2.6 In-vitro cytotoxicity assay

The primary human umbilical vein endothelial cell (HUVEC) line was purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), and 100 µg/mL penicillin and 100 µg/mL streptomycin were cultured at 37 °C in 5% CO_2 humidified environment. For investigations, the cells were seeded in 6-well plates (except MTT assay using 96-well plates) at a density of 1×10^5 cells/mL and allowed to attach for 24 h and then treated with different concentrations of SiNPs (10, 25, 50, 75 and 100 µg/mL) for another 24 h along with normal (normal saline), negative (Triton *X* 100) and standard control (risedronate alone and cisplatin alone) (Apollo pharmacy, India). The stock suspensions of SiNPs were sonicated for 5 min before the use. Controls were supplied with an equivalent volume of DMEM without SiNPs. For all experiments, each group had five replicate wells. Data are expressed as means ± *SD* from three independent experiments ($*p < 0.05$).

Cell morphology was observed by optical microscope (Olympus IX81, Japan). The above protocol was carried out for MTT assay on human osteosarcoma cell (Saos-2) line purchased from the National Centre for Cell Science (NCCS), Pune, India.

2.6.1 MTT assay

The cell cytotoxicity was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay [18]. MTT assay is most commonly used for the detection of cytotoxicity or cell viability following exposure to toxic substances. MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by

succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes, and therefore, it accumulates in healthy cells. The absorbance of formazan was measured at 492 nm using a microplate reader (ThemoMultiscan MK3, USA).

2.6.2 LDH assay

The lactate dehydrogenase permeability assay (LDH), which is based on the measurement of lactate dehydrogenase activity in the extracellular medium, was determined using a commercial LDH Kit (Jiancheng, China) according to the manufacturer's protocols. The loss of intracellular LDH and its release into the culture medium are an indicator of irreversible cell death due to cell membrane damage. After HUVECs and Saos-2 treated with different concentrations (10, 25, 50, 75 and 100 µg/mL) of SiNPs for 24 h, the supernatants were collected for measurement of LDH content. Hundred microlitres of cell medium was used for determining LDH activity, and the absorbance at 440 nm was measured using UV–visible spectrophotometer (Beckman DU-640B, USA).

2.7 Combinatorial drug analysis on Saos-2 cell lines for calcium uptake

The cells were seeded onto 96-well plates, and the capacity of each well was 70–200 µl. Calcium carbonate solution of concentration 18 mM was prepared to supplement the media. The stock solutions were prepared, i.e. control with 24 mg/ml and test with 32.5 mg/ml (Table 1).

Table 1 Composition of each well for in vitro study

Sl. No	Concentrations	Control	Test
1	0.2 mg/ml	D-0.83 µl M-99.17 µl Ca-19.23 µl	D-0.614 µl M-99.386 µl Ca-19.23 µl
2	0.4 mg/ml	D-1.664 µl M-98.336 µl Ca-19.23 µl	D-1.228 µl M-98.772 µl Ca-19.23 µl
3	0.6 mg/ml	D-2.496 µl M-97.504 µl Ca-19.23 µl	D-1.842 µl M-98.158 µl Ca-19.23 µl
4	0.8 mg/ml	D-3.328 µl M-96.672 µl Ca-19.23 µl	D-2.456 µl M-97.454 µl Ca-19.23 µl
5	1 mg/ml	D-4.16 µl M-95.84 µl Ca-19.23 µl	D-3.07 µl M-96.93 µl Ca-19.23 µl

**The total volume was made up to 130 µl in each of the above wells of the control and test samples*

2.8 Microscopic examination of treated cells

The drug-treated cell suspension was washed with PBS and aspirated without disturbing the cell monolayer and was fixed with ethanol for 60 min. Ethanol was aspirated, and the cells were stained with the Alizarin Red stain (2% in water); cells were incubated at room temperature for 45 min. The cell monolayer was washed four times with distilled water, and PBS was added again. The ability of the cells to uptake the drug was studied by determining penetration of drug/silica blend suspension with the use of inverted microscope. The cells treated with aqueous drug solution were used as control.

3 Results and discussion

3.1 Yield of silica

The plant sample for study was authenticated as *Cymbopogon flexuosus* with the accession number—SMPU/RARIMD/BNG/2019–20/230/RRCBI-mus231. The extracted SiNPs from grass was obtained by heating to elevated temperatures to remove carbon and other volatile compounds. The extraction of silica by acid leaching and gasification methods are well established and are reported [7]. The analysis of silica content by EDAX showed that higher amounts of silica were extracted from leaf with 56.57% followed by 23.49% in root and 21.65% in the stem. The modified acid leaching process resulted in increased yield of 81.03% from leaf samples. The chemical reaction between metals and acid will decrease the metal impurities in the treated lemon grass [19]. The silica precipitates out from different types of bio-waste such as rice hull, rice husk, bagasse and lemon grass. The grasses belonging to family Poaceae contain high silica content and are present predominantly in leaf and leaf sheaths when compared to its root and stem counterparts [20].

3.2 Chemical composition of plant extracts

The present investigation has explored the differences in silica content and its composition in different parts of *C. flexuosus* when subjected to treatment with varying temperatures and final calcination at 600 °C. Enhanced agglomeration of Silica particles was observed. The results obtained revealed the presence of amorphous nature of the silica particles in all the three parts of the plant studied. The higher percentage of silica was found in leaf (56.57), followed by root (23.49) and stem (21.65) (Figs. 1, 2, 3). Earlier reports revealed that the amorphous form of silica is usually pure and has small particle size and high surface area [14]. The present study showed that the treated ash obtained from all the three

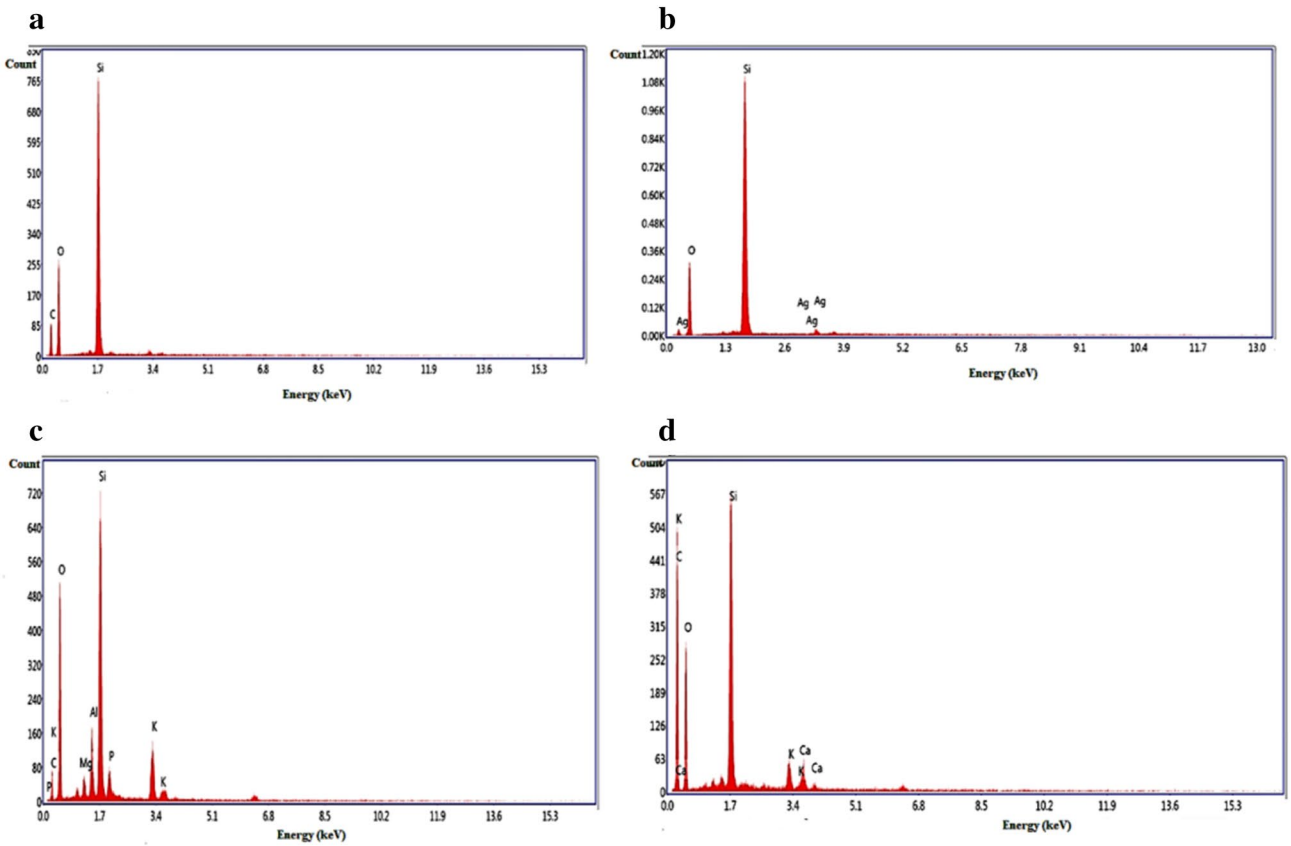

Fig. 1 EDAX analysis of SiNPs from leaf extract of *C. flexuosus* at different temperatures (a: 40 °C, b: 60 °C, c: 80 °C and d: 100 °C)

parts of *C. flexuosus* contained predominantly silica particles and no original compounds were observed. The modified protocol resulted in highest percentage of SiNPs (81.03%) (Fig. 4).

The elemental analysis for different silica samples of *C. flexuosus* was studied using EDAX method. EDAX spectra were measured with a Si (Li) EDS detector having an active area of 10 mm. The leaf sample contained mostly silicon (Si) in addition to aluminium (Al), calcium (Ca), potassium (K), magnesium (Mg) and silver (Ag) which is consistent with the previous reports involving *Cymbopogons* [13]. The silica from stem showed the presence of silicon (Si), magnesium (Mg), aluminium (Al), potassium (K), chlorine (Cl), calcium (Ca), phosphorous and copper (Cu), while the root sample contained elements such as silicon (Si), aluminium (Al), sodium (Na), iron (Fe) and chlorine (Cl). The modified protocol revealed different elemental composition wherein the silica and oxygen were present in more amount of percentage, i.e. 27.01 and 54.02%, respectively, along lesser amounts of carbon (C), potassium (K), and calcium (Ca).

3.3 Structural and morphological properties of SiNPs

3.3.1 FTIR studies

IR spectroscopic studies of all three samples showed the major functional groups which represent silica between the wavenumbers 3300–3200 cm^{-1} present in the acid leached samples (leaf, stem and root). The peaks obtained corresponded to the stretching vibrations of Si–OH or H–OH. The fibres of *C. flexuosus* are hydrophilic in nature. The hydroxyl group present in the cell wall forms hydrogen bond with water molecules [21].

In this study, the strongest broad IR band recorded was between 1200 and 1000 cm^{-1}. The peaks in this range revealed asymmetry between Si–O and Si–O–Si bonds. The peaks ranging from 800 to 700 cm^{-1} are due to the symmetric stretching mode of the Si–O-Si bond, and the peaks from 450 to 600 cm-1 correspond to the Si–O–Si bending vibration (Figs. 5, 6, 7), which is in agreement with the previous report [22].

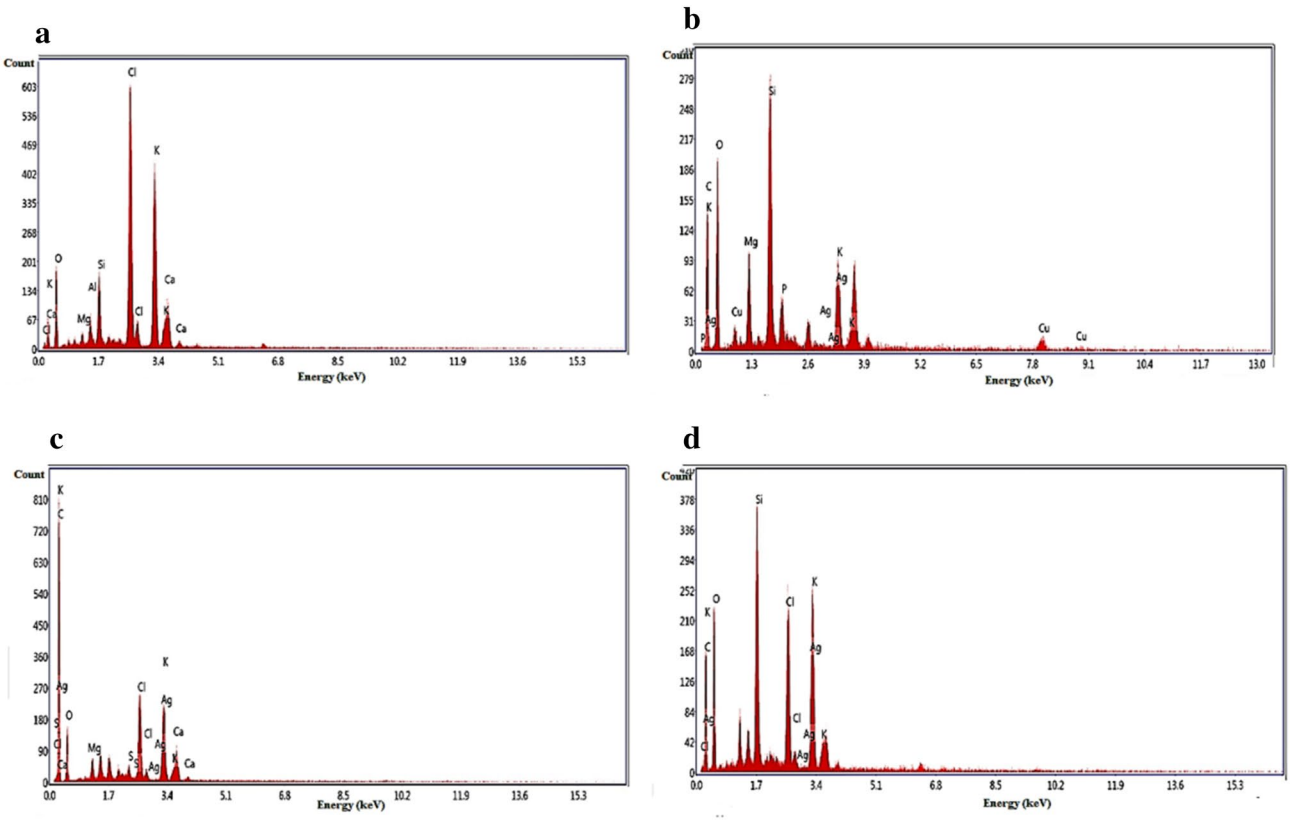

Fig. 2 EDAX analysis of SiNPs from stem extract of *C. flexuosus* at different temperatures (a: 40 °C, b: 60 °C, c: 80 °C and 100 °C)

The SiNPs from leaf extracted through modified protocol showed the sharp peak at wavenumber 1200 cm^{-1}. The peak revealed the asymmetry between Si–O and Si–O–Si bonds. The peaks ranging from 800 to 700 cm^{-1} are due to the symmetric stretching mode of the Si–O–Si bond, and the peaks from 450 to 600 cm^{-1} correspond to the Si–O–Si bending vibration (Fig. 8).

3.3.2 SEM studies

The scanning electron microscopic observations of ash obtained from leaf, stem and root samples of *C. flexuosus* treated at different temperatures are shown in Fig. 9, 10 and 11. Different morphological structures were noted such as tubular aggregates, spherical aggregates and fibrous aggregates corresponding to silica obtained from different parts of plant. At all the corresponding temperatures, the ash obtained from leaf, stem and root samples showed aggregate of spherical, tubular and fibrous particles, respectively. The modified protocol of silica extraction from leaf showed spherical nanoparticles of uniform size ranging from 50 to 60 nm (Fig. 12).

Among the samples investigated, the silica particles from leaf extract had high yield when compared to the other counterparts with spherical aggregates which was further optimized by modifying the extraction method. The optimized process resulted in SiNPs with highest percentage of 81.03% (± 2) with uniform size ranging from 50 to 60 nm and spherical aggregates with uniform distribution. It is well established that the spherical nanoparticle aggregates are ideal structures for efficient encapsulation of bio-active components and their delivery to the targeted sites as well [23]. In addition, the nanoparticles from leaf extract showed significant differences in elemental composition when compared to the particles from other two extracts. Hence, in further investigations, the SiNPs obtained from the leaf extract through the optimized process were used.

3.3.3 Measurement of particle size

The dynamic light scattering (DLS) profile showed more distinct particles size measurement and their distribution. The results demonstrated that at pH 7.4 it offers better and stable particle size between 60 and 75 nm (Fig. 13) due to optimum absorbance of and extraction of silica from the medium. The outcomes of DLS evaluation of synthesized SiNPs confirmed the Zetasizer evaluation of size

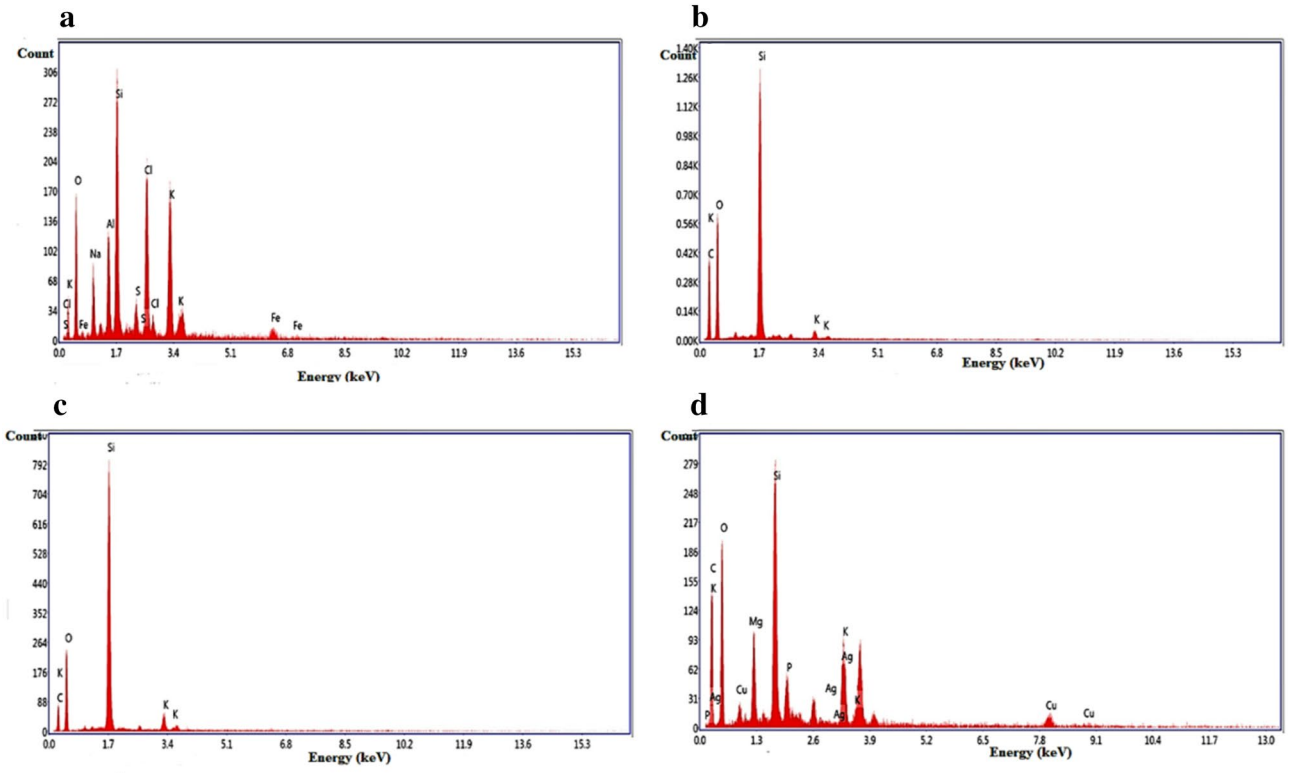

Fig. 3 EDAX analysis of SiNPs from root extract of *C. flexuosus* at different temperatures (a: 40 °C, b: 60 °C, c: 80 °C and d: 100 °C)

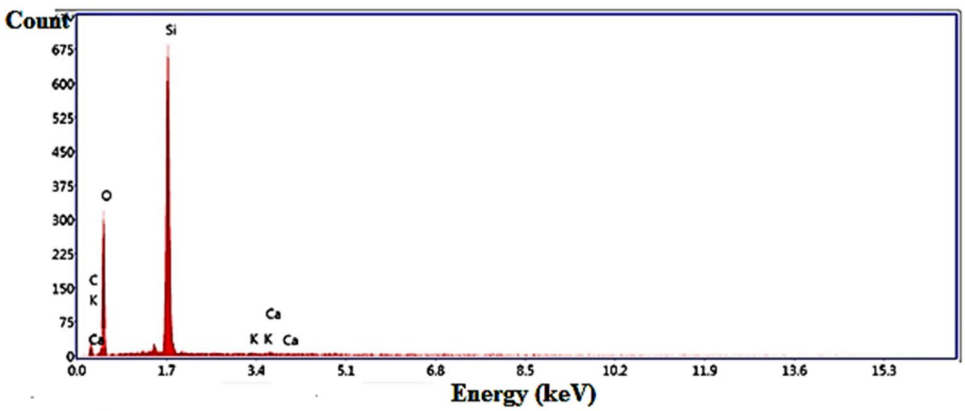

Fig. 4 EDAX analysis of SiNPs (modified protocol) from leaf of *C. flexuosus* at 50 °C

measurement of SiNPs and evaluation of advanced picture of more distinct size distribution pattern of prepared SiNPs on longer exposure of time.

3.4 In-silico modelling

Ibandronate, risedronate and alendronate are the most commonly used drugs for the treatment of osteoporosis. The suitability of the drug for therapeutic purpose is usually determined based on the Lipinski's rule of 5 that considers number of hydrogen bonds, distribution coefficients (clog *P*), in n-octanol to water (O/W), molecular weight, aqueous solubility (log *S*), donors and acceptors of the hydrogen bond, rotatable bonds and membrane permeability [24]. In this work, the properties of three commonly used drugs were studied using SwissADME informatics tool. The major properties investigated include water solubility, log p, topological polar surface area (TPSA), druggability and lead-likeness. Among the drugs studied, risedronate was found to be an ideal drug satisfying criteria of range of molecular weight, water solubility, lead-likeness and drug-likeness (Table 2). Hence, further investigations to determine the role of silica particles in enhancing the drug targeting

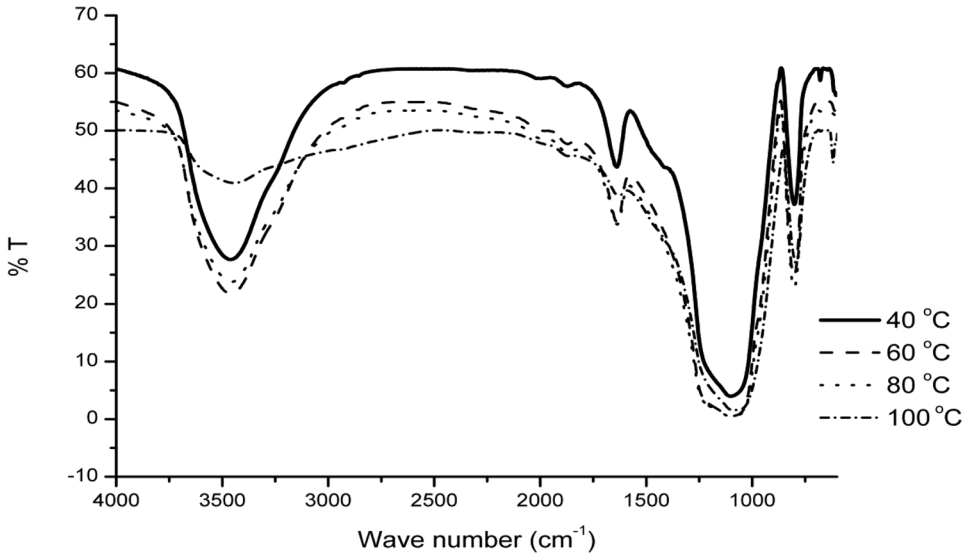

Fig. 5 FTIR spectrum of SiNPs from Leaf extract of *C. flexuosus* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

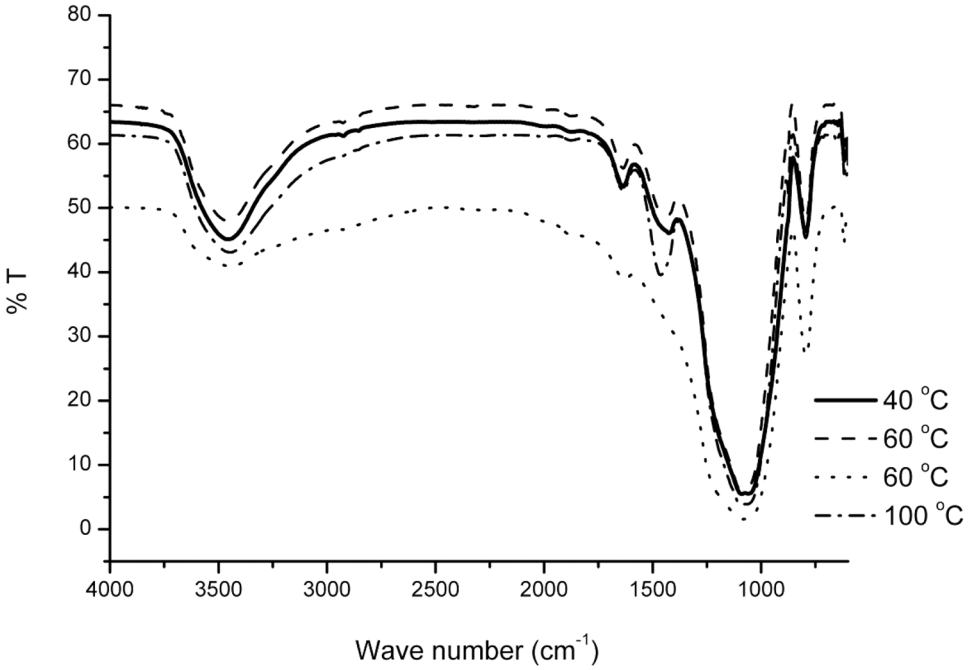

Fig. 6 FTIR spectrum of SiNPs from stem extract of *C. flexuosus* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

and distribution across the tissues were conducted in combination with the drug, risedronate.

The most important criterion for any drug carrier is the targeted and controlled delivery of loaded drug or bioactive components. The porous and nanostructured silica particles are widely used as drug carriers and their sustained release. The porous nature of these particles allows the efficient loading of drugs and their release as well [25]. In this work, the SiNPs extracted from the wild *Cymbopogon* sp. have been complexed with risedronate 6:1 proportion (by wt.) and the effect of silica on drug absorption was determined in the presence and absence of encapsulation by silica particles.

3.5 Drug formulation results

3.5.1 Drug release study

The silica which is bound to risedronate is heavier and will settle as pellet during centrifugation. The pellet was collected and washed to remove the unbound particles of SiNPs and risedronate. The supernatant will contain

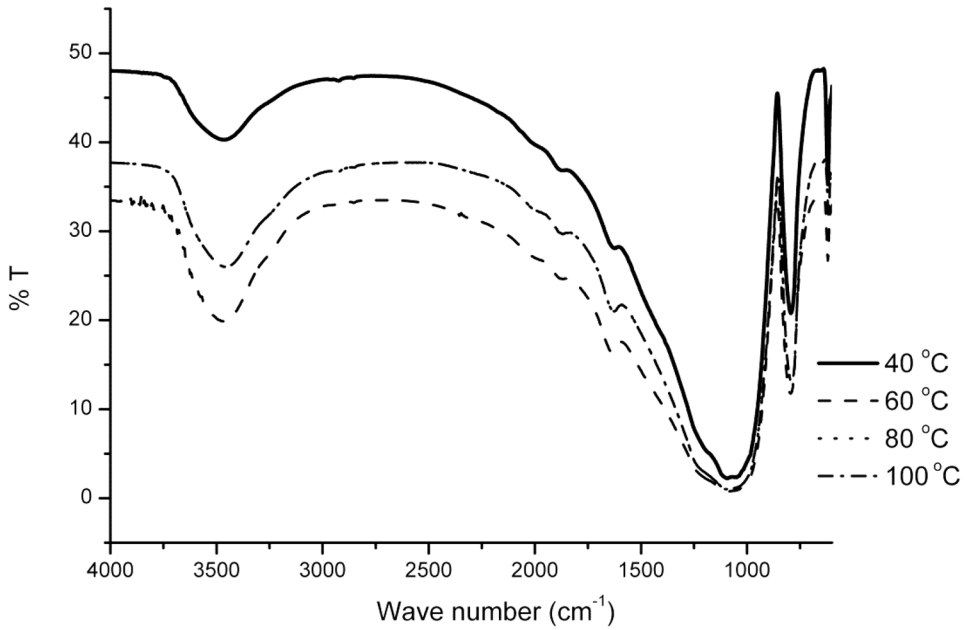

Fig. 7 FTIR spectrum of SiNPs from root extract of *C. flexuous* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

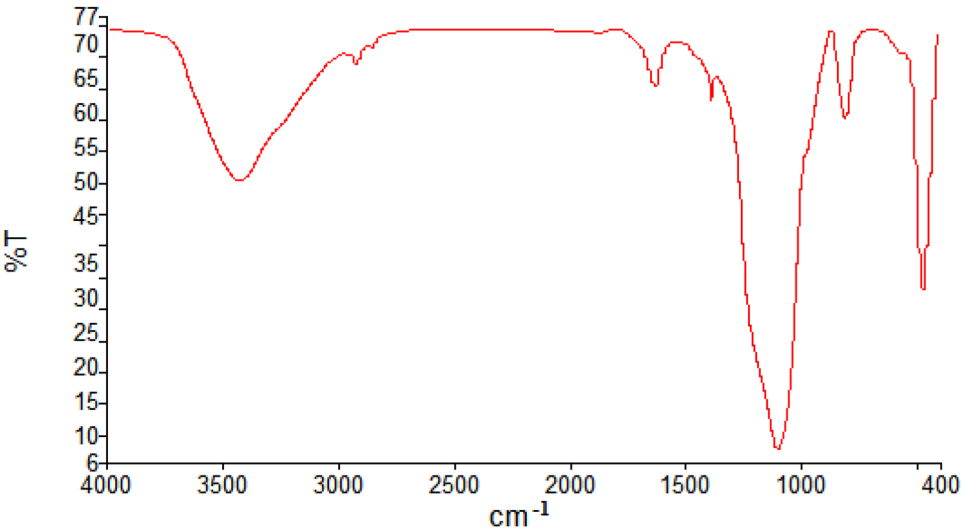

Fig. 8 FTIR spectrum of SiNPs from leaf extract of *C. flexuosus* (modified protocol) at 50 °C

the lighter free particles whose concentrations are measured using UV spectrophotometer.

It can be observed from the graph that the curve of SiNPs with risedronate for 1 mg/ml is nearly equal to the curve of the control 1 mg/ml for the concentrations of 0.0–1 mg/100 ml. There is a small decrease in sample for 0.2–0.4 mg/ml.

It can be observed from the graph (Figs. 14, 15) that the curve of the SiNPs with risedronate for 10 mg/100 ml is lower than the curve for the control 10 mg/ml for the concentrations of 0.2–0.8 mg/100 ml (Tables 3, 4). This indicates that there is more adsorption of silica with risedronate; this is further confirmed by FTIR analysis.

3.6 In-vitro cytotoxicity

In vitro cytotoxicity studies of different ratios of SiNPs and SiNPs with risedronate on HUVEC and Saos-2 cell lines were performed to determine the mineralization potential of the combinatorial drug. The SiNPs showed lesser IC_{50} value in comparison with SiNPs with risedronate tested on both the cell lines. The difference of LDH release was observed between treated groups and control group after HUVECs exposure to SiNPs and SiNPs with risedronate for 24 h (Figs. 16, 17, 18, 19) (Table 5). Our results indicated that SiNPs and SiNPs with risedronate induced cytotoxicity and cell leakage in a dose- and time-dependent manner.

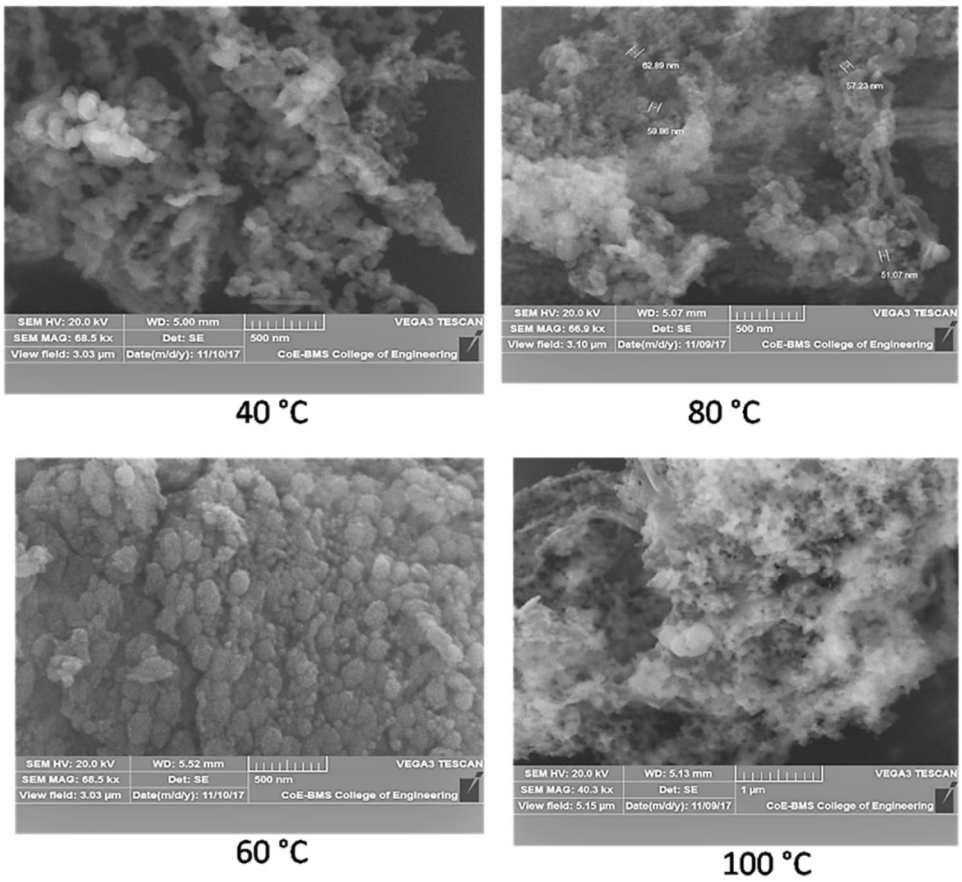

Fig. 9 SEM micrographs of SiNPs from leaf extract of *C. flexuous* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

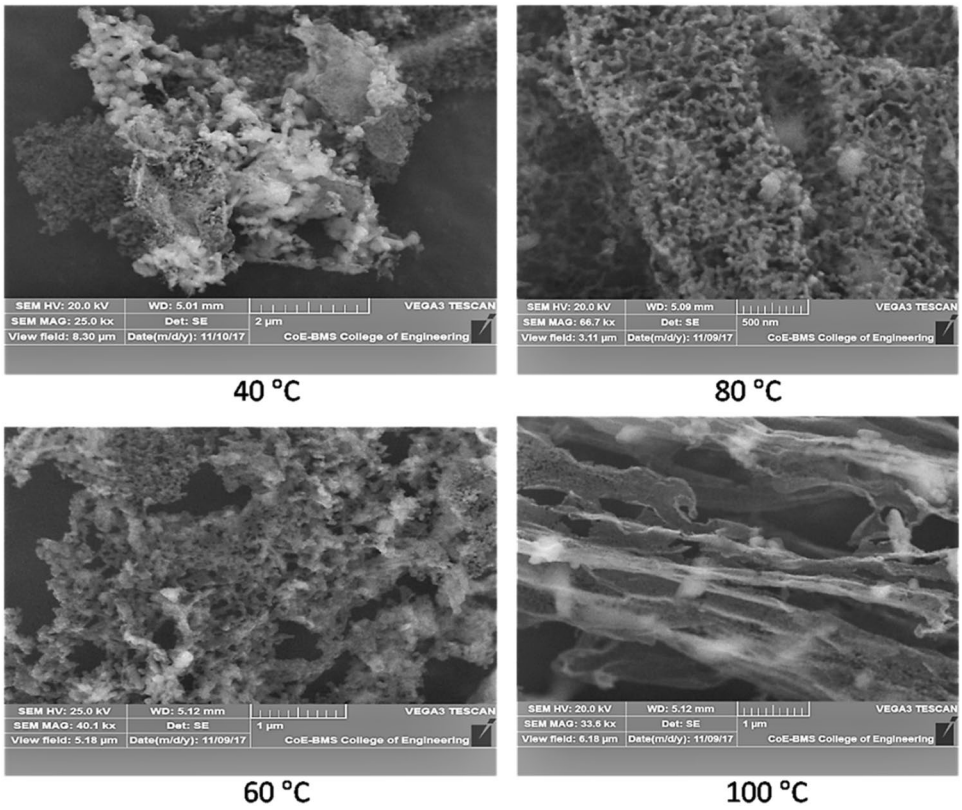

Fig. 10 SEM analysis of SiNPs from stem extract of *C. flexuous* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

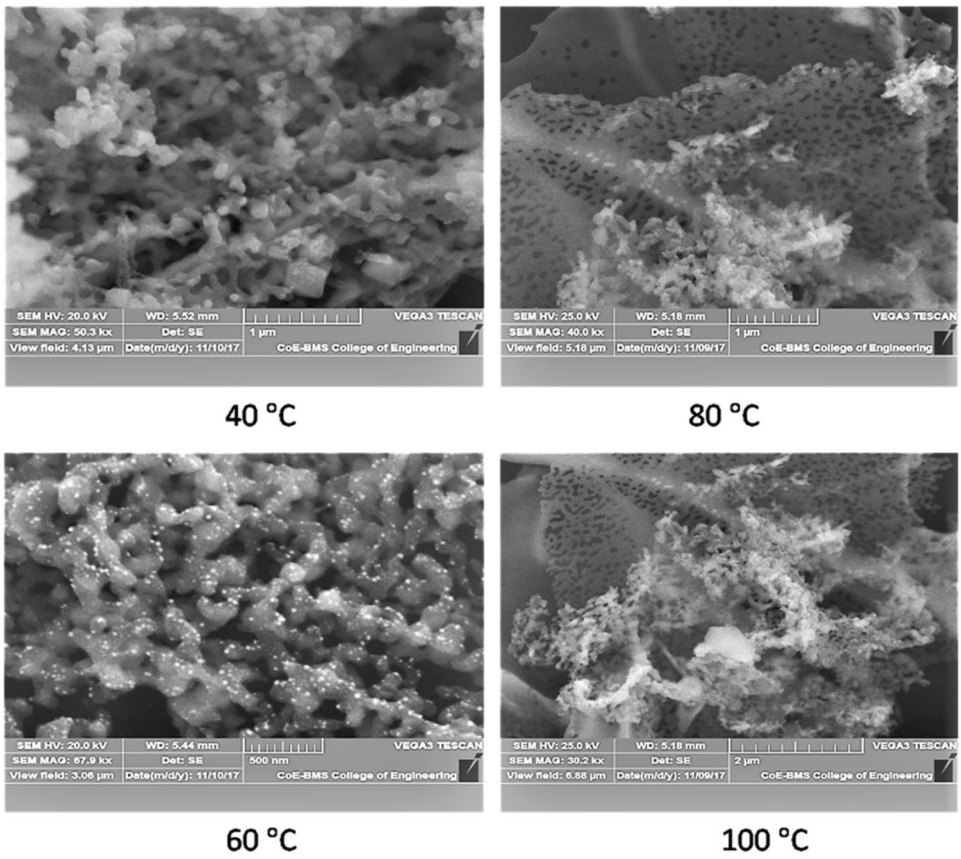

Fig. 11 SEM micrographs of SiNPs from root extract of *C. flexuosus* at different temperatures (40 °C, 60 °C, 80 °C, 100 °C)

The cytotoxic effect is mediated by permeabilization of the cytoplasmic membrane and reduction of the redox potential of the cells, suggesting a decrease of the mitochondria activity.

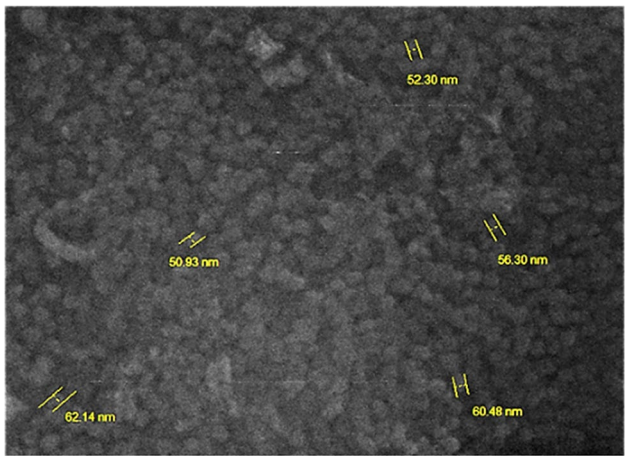

Fig. 12 SEM micrographs of SiNPs extracted by modified acid leaching technique from leaves of *C. flexuosus*

3.7 Microscopic examination of treated cells

Alizarin red is used to identify calcium in tissue sections. Calcium and alizarin red forms a complex. The reaction is identified as birefringence. By applying this principle, we can detect the absorption of calcium by cells. We noticed that for some concentrations, calcium deposits were present outside the cells. This could indicate that the cells need to be incubated for longer or the given concentration was not strong enough(Fig. 20).

For the incubation duration of 72 h, the most suitable concentrations of SiNPs with risedronate were found to be at 1 mg/ml followed by 0.8 mg/ml and 0.6 mg/ml. Uniform deposition of calcium was observed at 1 mg/ml concentration inside the cells. Further analysis could determine the toxicity and long-term effects of the drug combination. (Fig. 21).

To show that alizarin red forms a complex with calcium and the reaction is characterized by a bright red colour; we treated the cells with 1 mg/ml concentration of drug with no calcium. The cells were then fixed and subjected to alizarin red.

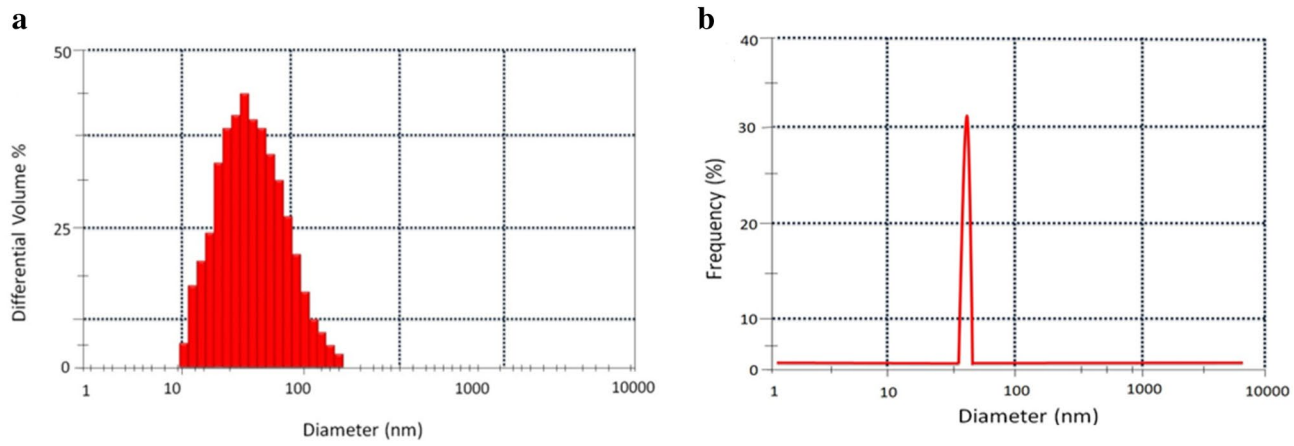

Fig. 13 DLS histogram of SiNPs from *C. flexuosus,* **a** Particle size, **b** Size distribution

Table 2 Physicochemical and biological properties of the drugs used in the work

Parameters	Ibandronate	Risedronate	Alendronate
Log *S*	1.23	0.39	3.07
Molecular weight (g/mol)	341.21	283.11	249.10
Hydrogen bond donor	4	5	6
Hydrogen bond acceptor	8	8	8
Rotatable bonds	9	4	5
TPSA (A2)	160.98	167.80	180.93
Drug-likeness	No, 1 violation	Yes	No, 1 violation

Under observation by inverted microscope, we could not identify any change in colour indicating that alizarin red forms a coloured complex upon reaction with calcium.

4 Conclusion

This work reports the extraction of SiNPs from leaf, stem and root of wild variety, *C. flexuosus* at a calcination temperature of 600 °C, its characterization and blend with osteoporotic drug. The high yield of silica was from leaf extract, followed by root and stem. The modified process of extraction has increased the yield of SiNPs by around 30%. The proximate analysis of the plant extracts

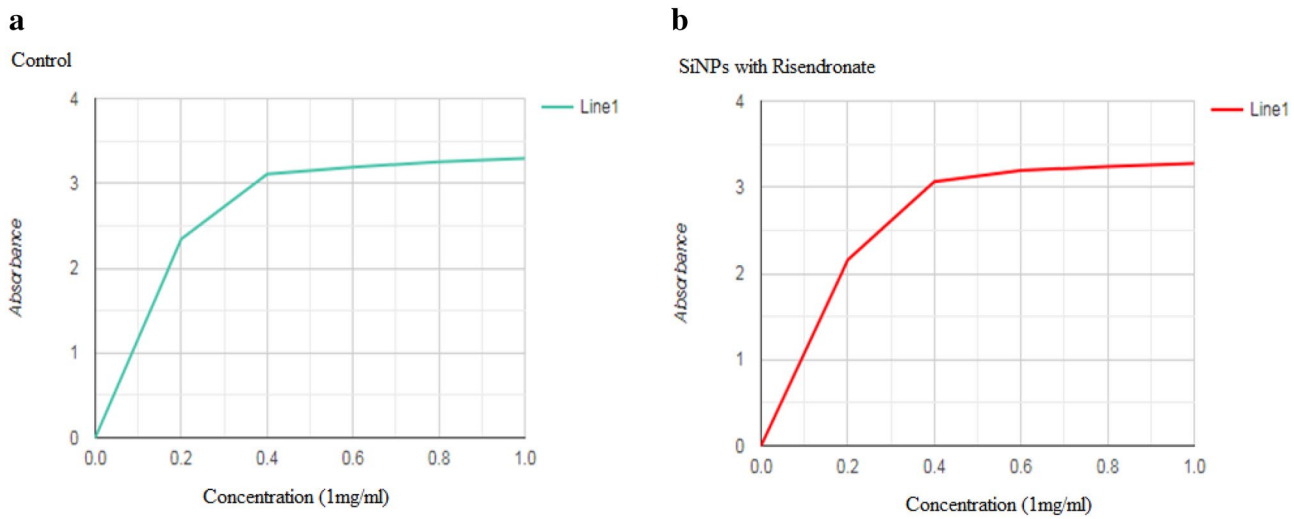

Fig. 14 Spectrophotometric analysis for drug release studies with 1 mg/ml concentration: **a** control, **b** SiNPs with risedronate

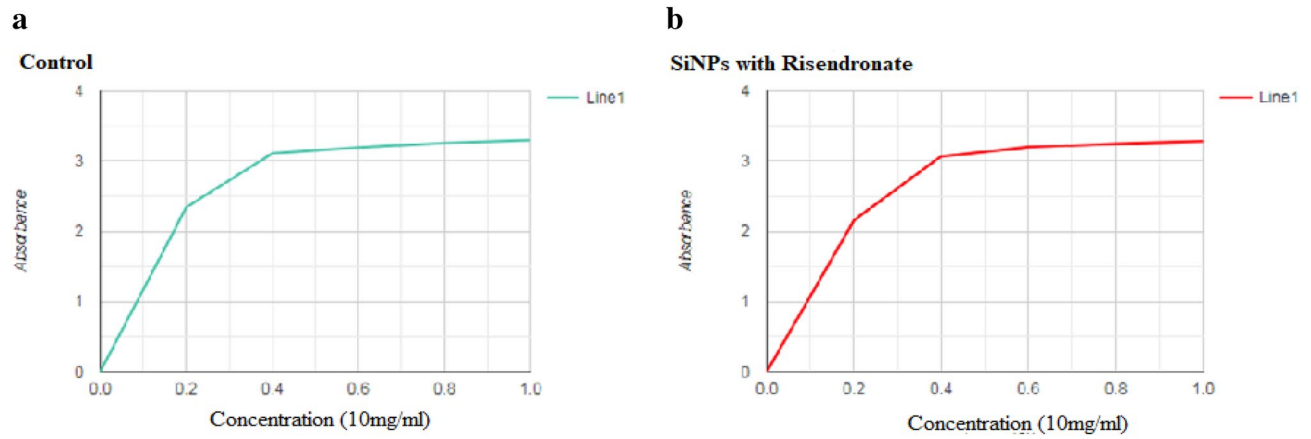

Fig. 15 Spectrophotometric analysis for drug release studies with 10 mg/ml concentration **a** Control **b** SiNPs with Risendronate

Table 3 OD values for control and SiNPs with risedronate (1 mg/ml)

Concentration (mg/ml)	OD	
	Control (1 mg/ml)	SiNPs with risedronate
0	0	0
0.2	2.155	2.339
0.4	3.065	3.107
0.6	3.195	3.192
0.8	3.238	3.254
1	3.276	3.294

Table 4 OD values for control and SiNPs with risedronate (10 mg/ml)

Concentration (mg/ml)	OD	
0	0	0
0.2	0.054	0.042
0.4	0.061	0.101
0.6	0.116	0.139
0.8	0.149	0.165
1	0.211	0.298

revealed significant differences in elemental composition, and SEM analysis showed differences in structural organization of SiNPs. The spherical aggregates of nanoparticles from leaf extract were further selected in the study for biomedical applications. The chemical properties of the drug were analysed in silico approach, and risedronate was chosen as the most suitable in this study. In vitro study of different ratios of SiNPs and SiNPs with risedronate on Saos-2 cell lines was performed to determine the mineralization potential of the combinatorial drug. The difference of LDH release was observed between treated groups and control group after HUVECs exposure to SiNPs and SiNPs with risedronate for 24 h. Our results indicated that SiNPs and SiNPs with risedronate induced cytotoxicity and cell leakage in a dose- and time-dependent manner. Further investigations needed to demonstrate the concentration-dependent effect of silica/drug blends for the efficient treatment of osteoporosis. Microscopic analysis revealed that, when compared to treatment with only risedronate, the SiNPs with risedronate complex were found to be more efficient in the absorption of calcium into the bones. The

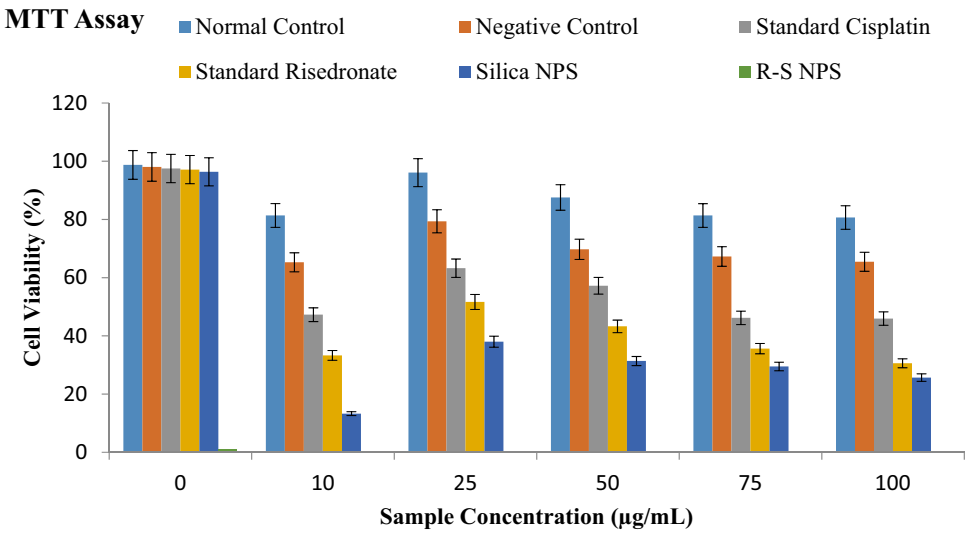

Fig. 16 Cell viability of HUVECs treated with different samples at different concentrations was measured by MTT assay after 24-h exposure

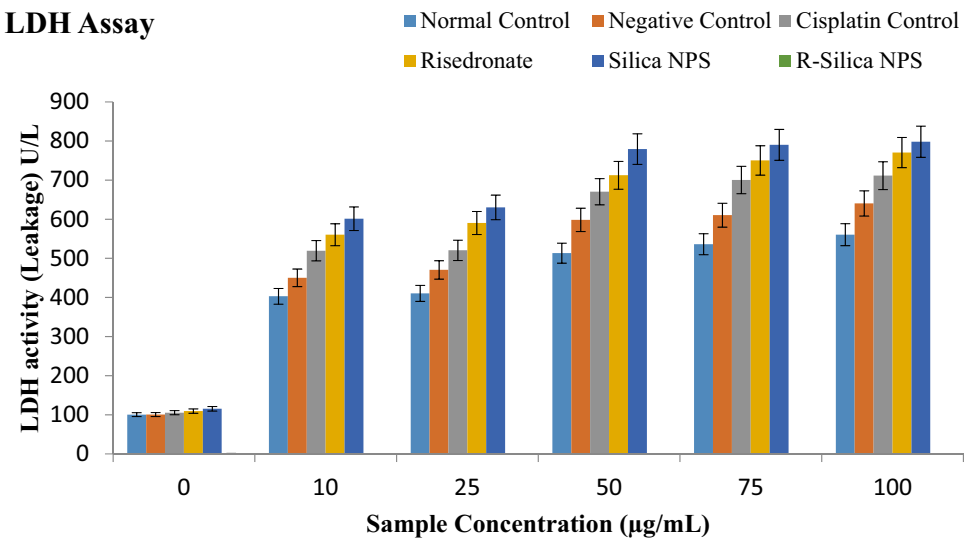

Fig. 17 LDH leakage of HUVECs exposed to various samples at different concentrations for 24 h. The results indicated that SiNPs and risedronate-bound SiNPs induced cytotoxicity in a dose- and time-dependent manner. Data are expressed as means ± S.D. from three independent experiments (*$p < 0.05$)

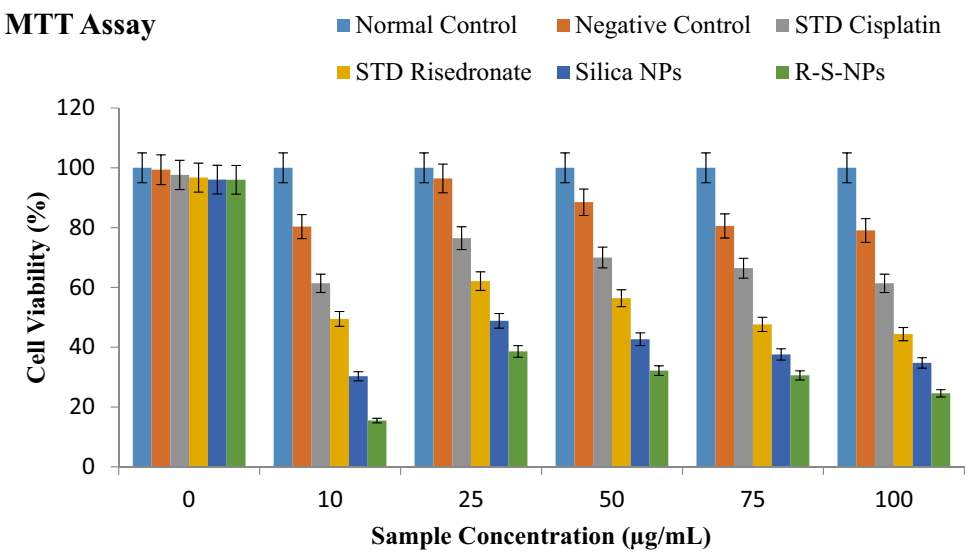

Fig 18 Cell viability of Saos-2 treated with different samples at different concentrations was measured by MTT assay after 24-h exposure

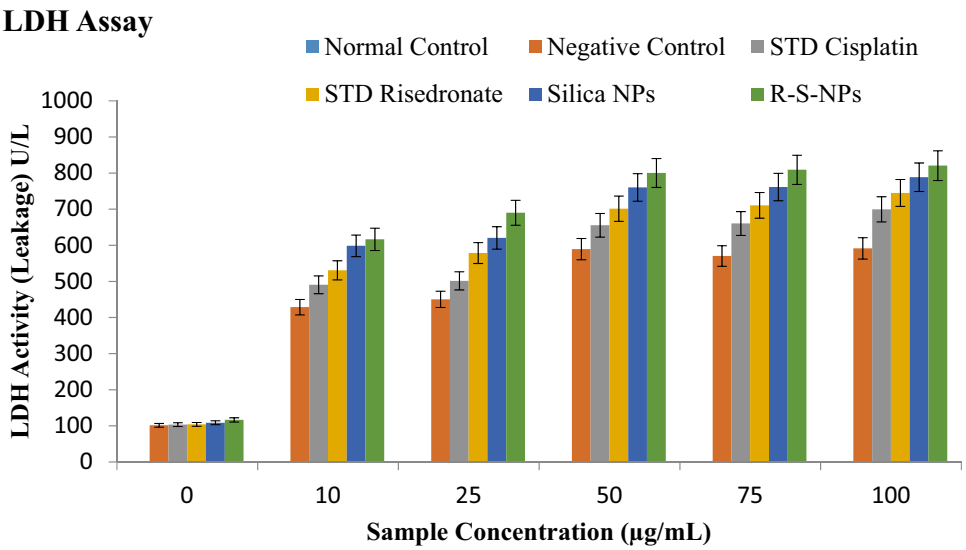

Fig. 19 LDH leakage of Saos-2 exposed to various samples at different concentrations for 24 h. The results indicated that SiNPs and risedronate-bound SiNPs induced cytotoxicity in a dose- and time-dependent manner. Data are expressed as means ± *S.D.* from three independent experiments (*$p < 0.05$)

Table 5 The cytotoxicity effect of standards (cisplatin and risedronate), SiNPs and SiNPs with risedronate tested on HUVEC and Saos-2 cell lines with their IC_{50} values

	IC_{50} (µM)	
Test sample	HUVEC	Saos-2
Std control (cisplatin)	6.338	5.99
Std control (risedronate)	6.173	5.027
SiNPS	5.922	4.987
SiNPs with risedronate	4.99	4.068

The IC_{50} values represent the mean of at least 3 independent experiments (SD)

concentrations that were most effective were 1 mg/ml followed by 0.8 and 0.6 mg/ml and showed uniform uptake of calcium.

By increasing the efficiency of the bisphosphate by addition of silica, we can prevent long-term exposure to the drug. This helps in reducing the risk of side effects such as fever, anaemia, numbness and swelling.

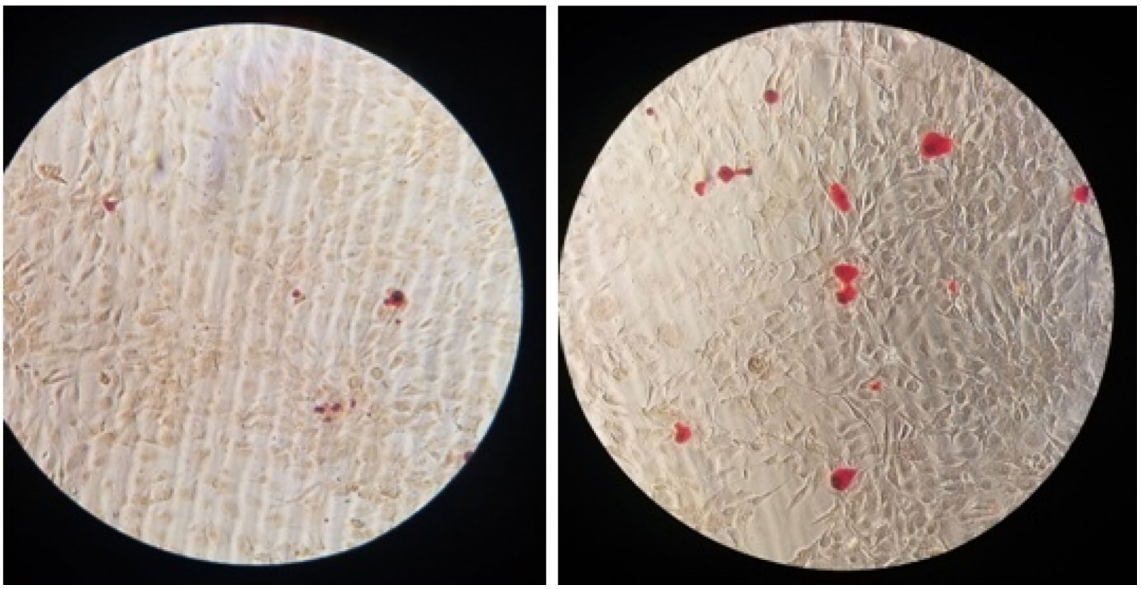

Fig. 20 Alizarin Red stained Saos-2 cell line showing calcium deposition in control and test samples at 1 mg/ml conc

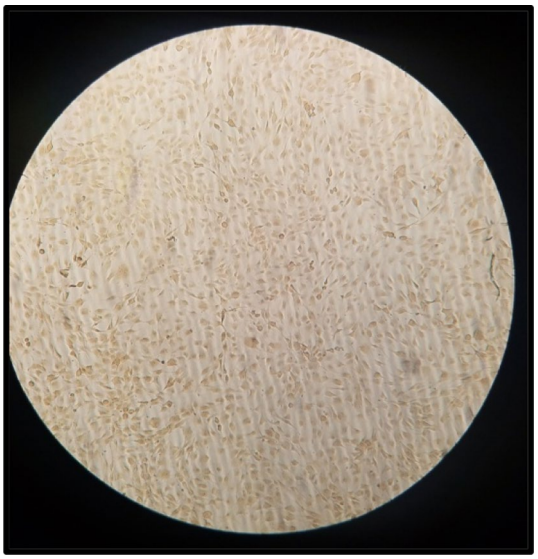

Fig. 21 Cells observed under the microscope without the addition of calcium

Acknowledgement Authors acknowledge Center of Excellence, BMSCE and R&D, Department of Biotechnology, BMSCE, for analytical facilities.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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