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Study on osteopotential activity of *Terminalia arjuna* bark extract incorporated bone substitute

G KRITHIGA[1], T HEMALATHA[1], R DEEPACHITRA[1], KAUSIK GHOSH[2] and T P SASTRY[1,*]

[1]Bioproducts Laboratory, Central Leather Research Institute, Adyar, Chennai 600 020, India
[2]Footwear Design and Development Institute, International Testing Centre, Irungattukottai 602 105, India

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Abstract. **Bark extract of *Terminalia arjuna* (TA) possesses potent medical properties and therefore, holds a reputed position in both Ayurvedic and Unani systems of medicine. Bone substitutes play an inevitable role in traumatic bone damages. Growth factors induce osteoinductivity, but suffer from limitations such as high cost and side effects. This study aims to evaluate the osteoinductive potential of bark extract of TA in bone substitutes. Bone substitutes prepared with TA bark extract were characterized for their physicochemical properties. *In vitro* biomineralization study was carried out using simulated body fluid. Cytotoxicity, alkaline phosphatase activity and mineralization potential were assessed using MG-63 cell lines. Scanning electron microscope revealed apatite formation on the surface after biomineralization. Thermogravimetric analysis showed 15% increase in residual weight by deposition of calcium and phosphate and their presence was identified by energy dispersive analysis. Increased alkaline phosphatase and calcium release was observed in bone substitutes prepared with TA extract compared with control. The functional groups of TA bark extract help in *in vitro* biomineralization. In MG-63 cells, it showed potential influence in cell differentiation. TA extract may be used as low-cost alternative for growth factors for treatment of fractured bones.**

Keywords. ***Terminalia arjuna*; osteoinductivity; bone substitutes; biomineralization.**

1. Introduction

Natural products are recognized as an endless source of medicine and have dominated the human pharmacopoeia for thousands of years (Raskin and Ripoll 2004). Alternative medical therapies have become common trend the world over for the past decade owing to their fewer side effects, low costs and increased efficacy (Bussmann and Sharon 2006). In both developed and developing countries, the use of plant-derived products in health care is very common and this incorporates the use of botanical medicines (either alone or in combination with prescribed medicines). Natural products continue to make an impact in new drug discovery, and in recent years the interest has intensified in the field of orthopaedics. Plant active compounds are tested for their bone formation ability in osteoblast cell lines. India is well established as a huge source of medicinal plants, viz. *Silybum marianum*, *Picrosshiza kursoa*, *Phyllanthus niruri*, *Terminalia arjuna*, etc., in the treatment of bone fracture.

Terminalia arjuna (Roxb.) (TA) is a deciduous tree of combretaceae family widely distributed throughout India. It is well recognized in Ayurveda (Indian traditional medicine) for its various therapeutic values (Dwivedi 2007). The bark of TA is recommended as cardioprotective agent for hypertension and ischaemic heart diseases (Gauthaman *et al* 2005; Hemalatha *et al* 2010). The bark paste of TA is being successfully used for the treatment of fractured bone of animals as well as of humans. It is believed that fractured bones regenerate at a faster rate if the bark paste is used and plastered with the bark itself. In practice, the decoction of the bark is used therapeutically to relieve the pain and inflammation (Patnaik *et al* 2007).

Bone damages or traumatic events lead to the removal of large portions of bone, creating critical size defects and necessitating bone substitutes to help in regeneration. In the past few decades, application of calcium phosphate ceramics as a bone substitute material is becoming inevitable. Bioactivity is its key factor along with biodegradability and biocompatibility (Ogose *et al* 2005). *In vitro* studies proved that only bioactive materials are able to form bone apatite on the surface when placed in simulated body fluids (SBFs). Among the bioactive materials, biphasic-calcium phosphate (BCP) is gaining interest because of its flexibility in changing resorbability by change in ratio of hydroxyapatite and tricalcium phosphate. To impart osteoinductivity to any bone substitute, growth factors like bone morphogenetic proteins are incorporated by different methods. Growth factors are

*Author for correspondence (sastrytp@hotmail.com)

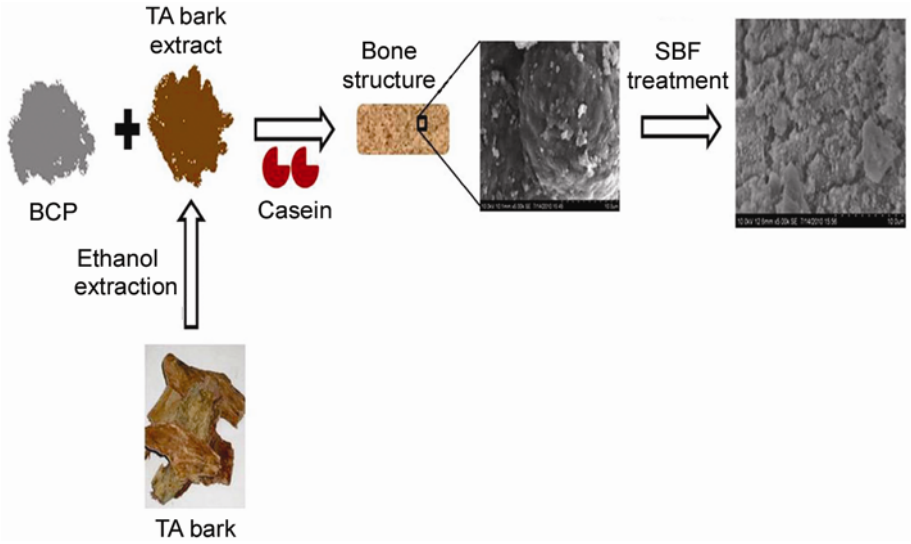

Figure 1. Study on osteopotential activity of *Terminalia arjuna* bark extract incorporated bone substitute.

degraded easily *in vivo* and thus requires high quantity to achieve the intended results; moreover, it will lead to side effects, including cancer and high costs (Helms *et al* 2005). Recent evidence in clinical studies has further indicated that use of recombinant human bone morphogenetic protein-2 (rhBMP-2) is associated with side effects in the central and peripheral nervous systems (Dmitriev *et al* 2011).

Casein is a phosphoprotein which accounts for 20% of the milk protein. It is insoluble in water and thus utilized to prepare organic adhesive. Casein phosphopeptides (CPPs) stabilize the amorphous phase of calcium phosphate (ACP) in solution, and these CPP–ACP complexes have anticariogenic potential (Cross *et al* 2001; 2004; Shen *et al* 2001). Casein hydrolysate fraction, when complexed with calcium compounds, help in remineralization of teeth (Huq *et al* 2003).

The study aims to evaluate the osteoinductive potential of bark extract of TA. Hence, bone implants were prepared using BCP, casein and ethanolic bark extract of TA and its efficacy for inducing new bone formation was analysed *in vitro* (figure 1). The prepared implant was characterized physicochemically and its *in vitro* bioactivity was assessed using MG-63 cell line.

2. Materials and methods

2.1 *Materials*

TA bark powder was obtained from the Indian Medical Practitioners Cooperative Pharmacy and Stores Limited (IMCOPS), Chennai, India. All other reagents were of analytical grade and used as received. β tricalcium phosphate (β TCP) was synthesized by the method of Bow *et al* (2004). Hydroxyapatite was synthesized by modifying the method of Noorjahan and Sastry (2005).

2.2 *Ethanolic extract of TA*

TA bark powder (50 g) was soaked in 95% ethanol for 7 days with intermittent shaking and the solvent was filtered with Whatman no. 1 filter paper. The filtrate was evaporated under vacuum drier and the brown residue obtained was stored at –4 °C for further use. The brown residue weighed 3·5 g and the yield was about 7%.

2.3 *Preparation of BCP*

BCP was prepared by physically mixing hydroxyapatite and β TCP in the ratio of 60 : 40.

2.4 *Preparation of bone implant with BCP and casein (BC)*

Casein (2 g) was soaked in 6 mL of 10% $Ca(OH)_2$ solution. As much as 5 g of BCP was mixed well with the mixture of casein and calcium hydroxide and a dough was made. This dough was extruded through a glass cylinder (1 cm diameter) using a glass rod. The implants were allowed to set at 30 °C for 24 h and then cured at 80 °C.

2.5 *Preparation of bone implant with BCP, casein and TA extract (BCTA)*

Casein (2 g) was soaked in 6 mL of 10% $Ca(OH)_2$ solution. As much as 5 g of BCP along with 5 mg of TA was

mixed well with the mixture of casein and calcium hydroxide and a dough was made. This dough was extruded through a glass cylinder (1 cm diameter) using a glass rod. The implants were allowed to set at 30 °C for 24 h and then cured at 80 °C.

2.6 *Physicochemical characterization*

2.6a *Mechanical properties*: Cylindrical specimens (diameter 1 cm, height 0·8 cm) of BC and BCTA were prepared and allowed to set at 30 °C for 24 h and then cured at 80 °C. To measure the compressive strength, the samples were loaded along the cylindrical axis in between the platens of Instron (4501 model) at a cross-head speed of 1 mm/min. The compressive strength was calculated from the break load and dimensions of the pellets.

2.6b *Fourier transform infrared spectroscopy*: Various functional groups present in the prepared and tested samples were identified by FT–IR (Nicolet Magna IR 560, USA). Here, 1 wt% of the powder was mixed and ground with 99% KBr. Tablets of 10 mm diameter were prepared by pressing the powder mixture at a load of 5 tons for 2 min and the spectrum was taken in the range of 500–4000 cm^{-1} with resolutions of 4 and 128 times scanning.

2.6c *Thermogravimetric analysis*: TGA of samples prepared and tested in SBF were carried out using a Seiko SSC 5200 H in nitrogen atmosphere (80 mL/min) at a heating rate of 10 °C/min. Primary weight loss of these materials as a function of temperature was recorded using this study.

2.6d *Scanning electron microscopy*: Dried samples were coated with an ultra-thin layer of gold using an ion coater (fisons sputter coater) under the following conditions, viz. 0·1 Torr pressure, 200 Ma current and 70 s coating time. Surface structure was visualized by scanning electron microscope (SEM model LEICA stereoscan 440) using 15 kV accelerating voltage.

2.6e *Energy dispersive X-ray analysis*: For EDX analysis, samples were mounted on holders and 6 nm sputter-coated with carbon by means of an MED 020 sputter device (Bal-Tec). In a Philips scanning electron microscope (Philips, Eindhoven, the Netherlands), EDX analyses were performed at 5 and 10 kV.

2.7 *Biomineralization assay*

2.7a *Preparation of simulated body fluid*: To investigate bioactivity of biomaterials, SBF was used as growth medium. In SBF, chemical components are similar to the inorganic mineral ions of human blood plasma and of which ion concentrations are also similar to those of human blood plasma. For SBF preparation, pre-determined amounts of reagent-grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $Na_2SO_4 \cdot 10H_2O$ and $CaCl_2 \cdot 2H_2O$ were weighed and dissolved in deionized (DI) water. The ionic concentrations in SBF were as follows: Na^+ (142 mM), K^+ (5 mM), Mg^{2+} (1·5 mM), Ca^{2+} (2·5 mM), Cl^- (147·8 mM), HCO_3^- (4·2 mM), HPO_2^{4-} (1 mM), SO_4^{2-} (0·5 mM), which are nearly equal to those in human blood plasma at 37 °C. SBF was then buffered at pH 7·4 with *tris*(hydroxymethyl) aminomethane and hydrochloric acid at 37 °C.

2.7b *In vitro bioactivity test*: BC and BCTA implants were well immersed in SBF solution for 21 days. After the specified time intervals, the scaffolds were removed from the SBF solution and washed thrice with DI water to remove adsorbed minerals. BC and BCTA implants tested in SBF were denoted as SBC and SBCTA, respectively. BC, BCTA, SBC and SBCTA were characterized by FT–IR, XRD, TGA, SEM and EDX analyses.

2.8 *Biological assessment*

2.8a *Cell culture*: The *in vitro* assay was performed on osteoblast-like cells (MG-63) obtained from National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's Minimum Eagle's Medium (DMEM) (HiMedia) supplemented with 10% foetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 25 μL cell suspension (5×10^3 cells/well) was seeded in each of the 96 wells and incubated at 37 °C for 48 h in 5% CO_2 for the formation of a confluent monolayer.

2.8b *MTT assay*: Cytotoxicity was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MG-63 cells were exposed to various concentrations (25, 50, 100 and 200 μg) of BC and BCTA. The cell viability was measured after 96 h using MTT assay (Carmichael *et al* 1987). MTT was added at the end of the incubation period and the plates were incubated for 4 h at 37 °C. Following incubation, the medium was aspirated completely and MTT formazan crystals formed were dissolved by adding DMSO and the absorbance was read at 570 nm using a multiwell plate reader (Multiskan Ascent VI.24). Controls were maintained throughout the experiment (untreated wells served as cell control). Data were normalized to control to evaluate the effect of the sample BC and BCTA on cells.

2.8c *Alkaline phosphatase activity*: Alkaline phosphatase (ALP) was measured in culture supernatants as per Pacheco-Pantoja *et al* (2011). The procedure involves a colorimetric assay using *p*-nitrophenyl phosphate as

substrate, and measuring *p*-nitrophenol spectrophotometrically released at 405 nm, with the absorbance being proportional to ALP activity. Cells were seeded onto 24-well plates and cultures were treated with 100 μg/mL concentration of BC and BCTA. After different periods of incubation, cell-free supernatants were collected for ALP assay. The determinations were performed using 3 replicates each time. All the treatments were compared against control wells (without treatment).

2.8d *Calcium release assay*: Alizarin red is used to determine quantitatively the calcium deposition by osteogenic cell lines by colorimetric method. The formation of calcium phosphate by MG-63 osteoblast-like cells was determined as described by using the alizarin red-S assay. The medium was removed, and the cell layers on the matrix were rinsed with phosphate-buffered saline (PBS) three times and fixed in 3·6% (v/v) formaldehyde at room temperature for 15 min. The fixed cells were stained with 2% alizarin red S (pH 4·1–4·3) for 15 min at 25 °C. Then the cell layers were washed with DI water for quantification of staining, 800 mL of 10% (v/v) acetic acid was added to each well and the wells were incubated at room temperature for 30 min with shaking. The cell layers on the wells were lifted and transferred to a 1·5 mL micro centrifuge tube. After vortexing for 30 s, the pellet was overlaid with 50 μg/mL mineral oil, heated to 85 °C for 10 min and transferred to ice for 5 min. The pellet was centrifuged at 20,000 g for 15 min and 50 μg/mL of the supernatant was removed to a new 1·5 mL micro centrifuge tube. Then, 200 μg/mL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. The absorbance of the supernatant was measured at 405 nm (Gregory *et al* 2004). Calcium release assay was done on 1st, 7th and 14th day in triplicates.

2.9 *Statistical analysis*

The data were presented as mean ± standard deviation (SD) of three individual experiments. The comparison between means of different groups was made by one-way analysis of variance followed by Duncan's multiple range analysis using statistical software package SPSS, version 13. Means bearing different superscripts in a column differed significantly at $p < 0{\cdot}05$.

3. Results and discussion

3.1 *Physicochemical characterization*

3.1a *Compression strength*: The bone implants were used to induce new bone formation at the implant site or sometimes implant itself gets ossified thereby filling the gap with new bone. The compression strength of bone implant depends on the particle size, porosity, binding of individual components, nature of the binding material and moisture content present in the sample. The compression strength of BC was found to be 3·04 ± 0·13 MPa, whereas that of BCTA was 4·24 ± 0·18. After immersion of BC and BCTA implants in SBF for 21 days, the compression strength of SBC and SBCTA implants was found to be 3·12 ± 0·15 and 4·31 ± 0·06 MPa, respectively. Compression strength of the prepared implants is low for the application as load-bearing implants (10–80 MPa), but significant increase after the addition of TA extract may be due to the cross-linking effects of phenolic groups in TA extract with the functional groups of casein. These implants may be used as osteoinductive materials in the defects of non-load-bearing bone defects.

3.1b *FT–IR*: IR spectrum of TA (figure 2a) exhibits strong absorption band at 3438 cm^{-1} representing hydroxyl groups. The vibrational band at 2938 cm^{-1} was due to the presence of saturated –C–H bond. Characteristic bands of carboxylic acid (1716 cm^{-1}), amines (1625 and 1593 cm^{-1}) and phenols (1257 and 1171 cm^{-1}) are present. Glycosidic linkage represented the peak at 1051 cm^{-1} (Patnaik *et al* 2007). IR spectrum of BC (figure 2b) has shown characteristic vibration peaks of phosphate (PO_4^{3-}) ν_1 and ν_3 at around 961 and 1038 cm^{-1}. Carbonate peaks are identified at 1431 and 875 cm^{-1}. Absorption band at 1676 and 1525 cm^{-1} represent amides I and II of casein. IR spectrum of BCTA (figure 2c) shows all peaks of BC except amide II peak of casein and amide I peak shifted from 1676 to 1652 cm^{-1}, indicating a reaction between TA extract and functional groups of casein.

3.2 *In vitro bioactivity test*

The biocomposite formed using casein was stable in SBF solution for 21 days. A variety of binding agents like

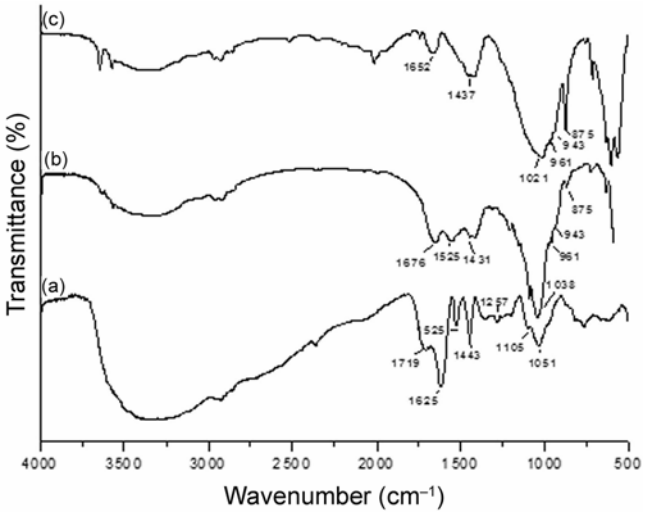

Figure 2. FT–IR spectra of (**a**) *Terminalia arjuna* ethanol extract, (**b**) BC and (**c**) BCTA.

gelatin, chitosan and fibrin are employed for preparing bone substitutes, whereas few attempts have been made with casein. In case of gelatin and others, a chemical cross-linking agent is necessary to give stability over a given period of time. Without cross linking, the gelatin-based biocomposite loses its stability in SBF within a day. In this study, casein, a milk protein, was used to prepare BCP biocomposite. Casein, insoluble protein of milk, is rendered soluble by the alkali and then it forms the binder (Silva *et al* 2003). The stability of the prepared implant is necessary for both *in vitro* and *in vivo* bio-activities as it has to be stable at the defective site till it is ossified. Apart from introducing stability to the prepared biocomposite, casein may also be helpful in bone regeneration (James *et al* 2002).

3.3 *TGA*

Thermogravimetric analysis reveals loss of weight of the sample with increase in the temperature. This loss may be due to decomposition of sample into carbon dioxide (CO_2) carbon monooxide (CO) nitrous oxide (NOX) and water vapour. Normally inorganic mineral is stable up to 800 °C. In the present investigation, BC, when subjected to thermal analysis, showed residual weight percentage of 83·22% at 800 °C. SBC has shown the residual content of 84·77% under similar conditions. There is a negligible increase of 1·5% due to the possible ossification. BCTA has shown the residual content of 62·89%, whereas the sample SBCTA has shown 77·04% residual content at 800 °C. An increase of 15% in the inorganic residue shows that BCTA is a good osteoinductive material. The results are shown in figure 3. The presence of apatite can be confirmed by the increase in residual weight in thermogravimetric analysis for both BC and BCTA samples. The increase was more in BCTA than in BC and this increase may be attributed to the osteoinductive nature of the TA extract.

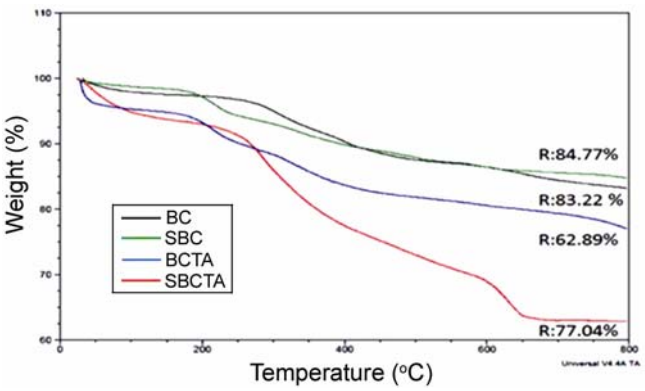

Figure 3. Thermogravimetric analysis of BC, BCTA, SBC and SBCTA.

3.4 *SEM*

Scanning electron micrographs of the samples BC, BCTA, SBC and SBCTA are given in figure 4. In case of BC, crystals are visible in both individual and aggregated forms. In SBC, the surface is modified, the size of individual crystals has increased and it shows possible ossification of the samples. In case of BCTA, crystals appear with a smooth surface, but in SBCTA roughness is observed on the surface of the crystals and there is an increase in size of the individual crystals indicating mineralization. The roughness and increase in size is more prominent in BCTA samples than in BC samples after treatment in SBF. Figure 4(d) clearly depicts the formation of cracks in apatite layers. Ning and Zhou (2002) reported that long periods of immersion would induce formation of cracks in the apatite layers due to drying and shrinkage.

3.5 *EDX*

EDX analysis of the samples is shown in figure 5. In BC and BCTA, calcium level increased up to 2·35- and 4·31-fold, respectively, after treatment in SBF, whereas the level of phosphorous increase was about 4·29- and 1·89-fold. EDX results reveal the enhancement in the levels of calcium and phosphorus, which may be attributed to the synthesis of hydroxyapatite crystals.

3.6 *Biological assessment*

3.6a *MTT assay*: The effect of BC and BCTA on cell viability was assessed using MG-63 in order to evaluate their biocompatible nature (figure 6a). More than 85% viability was observed in all the groups, except at 200 μg of BCTA (which was about 76%). There was no significant difference between BC and BCTA in all concentrations. Both groups showed significant reduction in cell viability at 200 μg concentration. As there was no significant difference between BC and BCTA at 200 μg concentration, the decrease may be attributed to the increased calcium phosphate content rather than TA extract. Cell inhibitions at higher concentrations of calcium phosphate were well documented in previous studies (Kim *et al* 2005). Sivalokanathan *et al* (2006) reported that TA extract inhibited the proliferation of human hepatoma cells (HepG2), osteosarcoma (U2OS), and glioblastoma (U251) cells in a concentration-dependent manner. The study results clearly infer that concentration of TA bark extract used is within the biocompatible range, and it has not inhibited cell proliferation and the observed effect at 200 μg could be due to the levels of calcium phosphate. 100 μg concentration was selected for further studies, viz. alkaline phosphatase assay and calcium release activity assay, since 100 μg shows higher cell viability compared with 200 μg concentration.

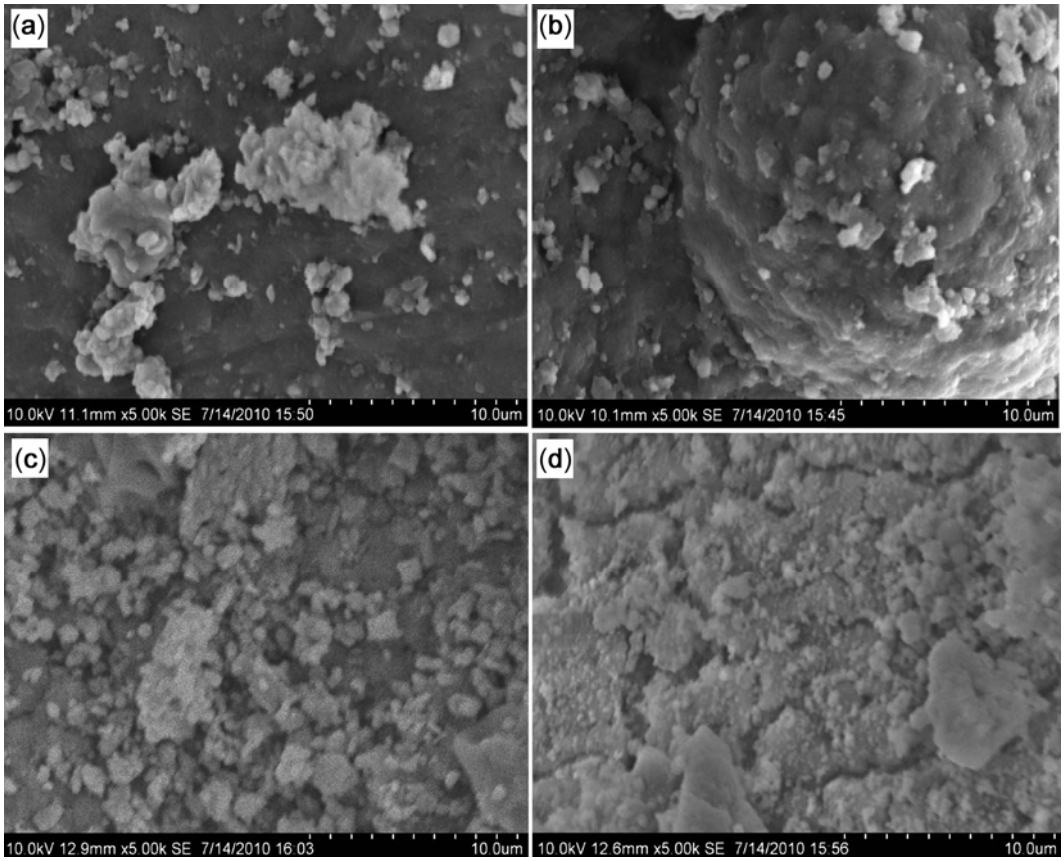

Figure 4. SEM micrograph (magnification 500×) of (**a**) BC, (**b**) BCTA, (**c**) BC after treatment in SBF for 21 days and (**d**) BCTA after treatment in SBF for 21 days.

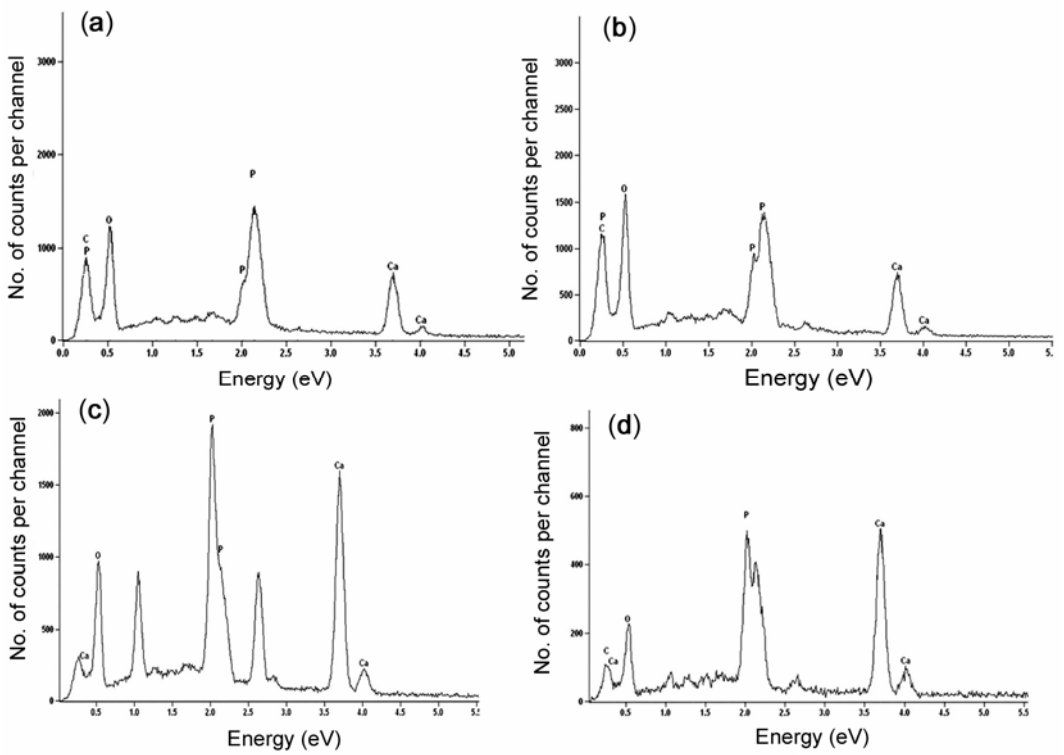

Figure 5. EDX data of (**a**) BC, (**b**) BCTA, (**c**) BC after treatment in SBF for 21 days and (**d**) BCTA after treatment in SBF for 21 days.

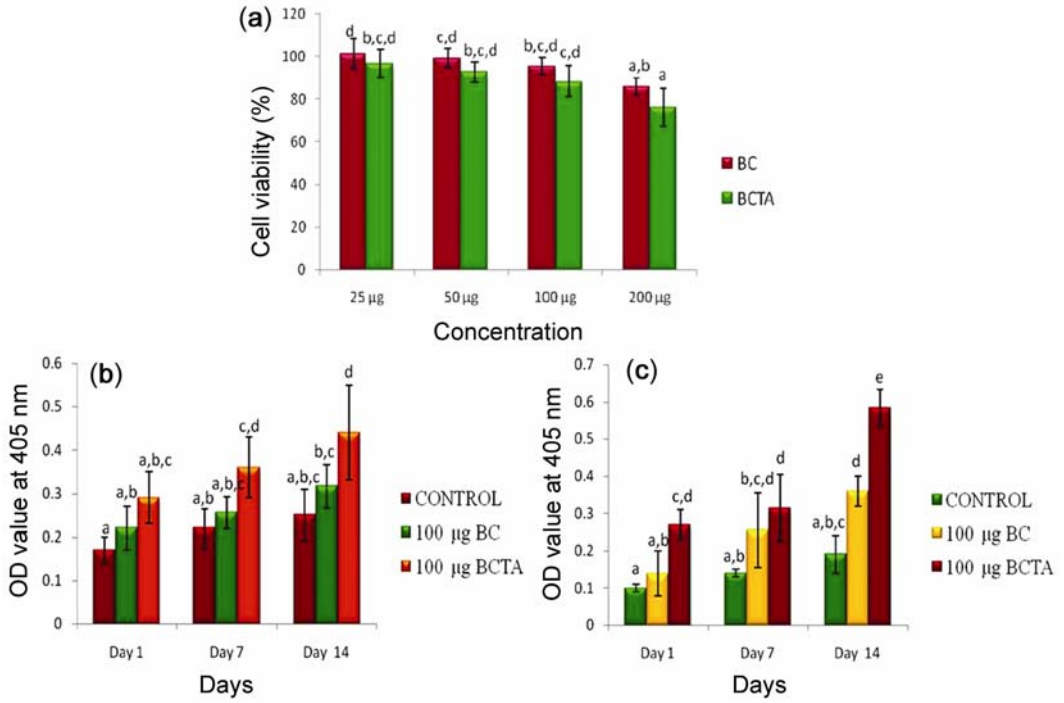

Figure 6. (**a**) MTT assay. The data are presented as mean ± SD of three individual experiments. Data were normalized to control. Means bearing different superscripts differed significantly by Duncan's multiple range analysis at $p < 0{\cdot}05$. (**b**) Alkaline phosphatase activity assay. The data are presented as mean ± SD of three individual experiments. Means bearing different superscripts differed significantly by Duncan's multiple range analysis at $p < 0{\cdot}05$. (**c**) Calcium release assay. The data are presented as mean ± SD of three individual experiments. Means bearing different superscripts differed significantly by Duncan's multiple range analysis at $p < 0{\cdot}05$.

3.6b *Alkaline phosphatase (ALP) activity*: Alkaline phosphatase is an important marker which is expressed during osteoblastic differentiation (Weinreb *et al* 1990). At the end of cell proliferation and maturation, cells express higher level of ALP activity. ALP expression levels are an important factor in determining the functions of osteoblast cells. Figure 6(b) reveals the levels of ALP activity expressed by BC and BCTA at 100 μg concentration on the 1st, 7th and 14th day. On the 1st day there was no significant difference in ALP activity between the groups. On the 7th and 14th days significant increase in ALP activity was observed only in BCTA, but not in BC. This may be due to the influence of TA extract on cell differentiation. Morris (2006) has also reported an increase in ALP activity by soy isoflavones during osteoblastic differentiation in MG-63 cell lines.

3.6c *Calcium release assay*: Mineralization potential of the cells was quantified by alizarin red assay. The calcium released by BC and BCTA at 100 μg concentration on various day intervals are given in figure 6(c). There was no significant increase in calcium content in BC up to 7th day and the significant increase was evident on the 14th day. In case of BCTA there was a significant increase in calcium content compared to control throughout the experiment. On the 14th day BCTA showed significant increase in calcium content compared to BC. The significant increase in calcium levels in BCTA may be due to the osteogenic effect of TA extract on MG-63 cells. The increased calcium level and osteogenic differentiation of MG-63 cells on electrospun collagen and electrospun gelatin was earlier reported by Tsai *et al* (2012).

4. Conclusions

The study validates the potential of TA bark extract. Total TA bark extract was used for the study mainly because of the synergistic effect of the phytochemicals on different pathways leading to better bioactivity. Biocomposite prepared using bark extract of TA (BCTA) possessed stability and compression strength. The functional groups of TA extract help in *in vitro* biomineralization and in MG-63 cells, it showed potential influence on cell differentiation and cell viability. Thus, the prepared biocomposite may be used as potential bone filler in the non-load-bearing areas or as a coating agent in metallic implants to impart bioactivity. With further molecular studies, TA extract might be tried as a possible alternative to BMPs.

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