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SYNTHESIS, CHARACTERIZATION, *IN VITRO* DEGRADABILITY AND BIOACTIVITY OF STRONTIUM SUBSTITUTED RICE HULL ASH SILICA BASED MELT DERIVED 45S5 BIOACTIVE GLASS

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ABSTRACT

The purpose of this study was to synthesize strontium substituted melt derived 45S5 bioactive glasses by use of rice hull ash silica and commercial silica. The degradation behaviour of bioactive glass samples was studied through the weight loss in the tris-(hydroxymethyl)-aminomethane solution. The *in vitro* bioactivity of the bioactive glass samples was evaluated by soaking studies in simulated body fluid. The apatite like layer formation on glasses surface was assessed by X-ray powder diffraction, scanning electron microscope, inductively coupled plasma and Vickers hardness measurement analyses. Also, the effect of bioactive glass samples on osteoblasts *in vitro* and cell mineralisation was investigated. The present investigation revealed that both of the samples show similar bioactivity but 45S5 bioactive glass from rice hull ash silica supported cell activity better than commercial silica. Thus, rice hull ash silica can be used to synthesize strontium substituted 45S5 bioactive glasses and it is also good alternative to commercial silica.
Keywords: Bioactive glass, rice hull ash silica, strontium.

STRONSIYUM KATKILI PİRİNÇ KABUĞU KÜLÜ SİLİKALI ERGİTME YÖNTEMİ İLE 45S5 BİYOAKTİF CAM SENTEZİ, KARAKTERİZASYONU, IN VİTRO BOZUNMA VE BİYOAKTİVİTESİ

ÖZET

Bu çalışmanın amacı, pirinç kabuğu külü silikası ve ticari silika kullanılarak stronsiyum katkılı ergitme türevli 45S5 biyoaktif camların sentezlenmesidir. Biyoaktif cam numunelerinin, bozunma davranışı tris-(hidroksimetil)-aminometan çözeltisi içinde ağırlık kaybı ile çalışıldı. Biyoaktif cam numunelerinin *in vitro* biyoaktiviteleri yapay vücut sıvısında bekletilerek değerlendirildi. Cam yüzeyinde apatit tabakası oluşumu X-ışınları difraksiyonu, taramalı elektron mikroskobu, indüktif eşlenmiş plazma ve Vickers sertlik ölçümü analizleri ile değerlendirildi. Ayrıca, biyoaktif cam numunelerinin osteoblastların üzerindeki *in vitro* ve hücre mineralizasyonuna etkisi araştırıldı. Bu araştırma, her iki örneğin de benzer biyoaktivite gösterdiğini fakat pirinç kabuğu külünden elde edilen 45S5 biyoaktif camların hücre aktivitesini daha iyi desteklediğini göstermiştir. Bu nedenle pirinç kabuğu külü silikasının stronsiyum katkılı 45S5 biyoaktif cam üretiminde kullanılabileceği ve ticari silikaya iyi bir alternatif olduğu görülmektedir.
Anahtar Sözcükler: Biyoaktif cam, pirinç kabuğu külü silikası, stronsiyum.

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1. INTRODUCTION

Bioactive glasses have been widely used in craniofacial, maxillofacial, and periodontal applications to repair hard tissues due to their surface reactivity with living tissue and osteoblast stimulating properties [1,2]. The chemical composition of bioactive glass is a key parameter to enhance a successful interaction with living tissues [3].

Strontium is one of the trace elements in human bone that have attracted considerable attention for the management of osteoporosis as a drug over 75 years due to its bone-seeking property [4]. It is chemically similiar to the calcium, and thus strontium can replace with calcium in metabolic processes of hard tissue [4, 5].Strontium has been regarded as a promising incorporation agent to enhance the replication of preosteoblastic cells and lessen osteoclast activity [1,6]. Due to these roles of strontium, it drew attention in bioactive glass production in recent years [1, 7]. Gorustovich et al. (2010) showed that the strontium doped 45S5 Bioglass® exhibits strong bonding to bone with high biocompatibility [8].

In general, commercial silica sources was used as starting materials for bioactive glass production. Based on the earlier work of Yucel and co-workers, rice hull ash silica can be used as an alternative silica source for preparation of melt derived bioactive glasses [9].

The objective of the present study was to synthesize a rice hull ash silica based melt derived strontium substituted bioactive glass based on SiO_2–Na_2O-CaO–SrO–P_2O_5 system and to compare its bioactivity and cellular properties to that of a commercial silica based bioactive glass using appropriate techniques.

2. EXPERIMENTAL SECTION

2.1. Materials

The rice hull ash sample was obtained from the Yetiş Food Factory (Turkey). All reagents used in the experiments were analytical grade. Calcium chloride ($CaCl_2.2H_2O$), calcium carbonate ($CaCO_3$), hydrochloric acid (HCl), potassium chloride (KCl), magnesium chloride ($MgCl_2.6H_2O$), magnesium sulfate ($MgSO_4.7H_2O$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium hydroxide (NaOH), sodium sulfate (Na_2SO_4), tris-(hydroxymethyl)-aminomethane ($(CH_2OH)_3CNH_2$) were purchased from Merck (Darmstad, Germany). Silica (SiO_2) was purchased from Riedel-deHaën. Alkaline phosphatase (ALP), Dulbecco's Modified Eagle's medium (DMEM), Fetal bovine serum (FBS), β-glycerophosphate, L-ascorbic acid, p-nitrophenylphosphate and strontium oxide (SrO) were purchased from Sigma-Aldrich.

2.2. Preparation of Rice Hull Ash Silica

The rice hull ash was burned in a muffle furnace (Protherm, TURKEY) at 600°C for 5 hours. An acid leaching step was applied to remove impurities. In this step, 40 grams of rice hull ash was mixed with 6M HCl solution (pH 1) and then boiled with stirring for 2 hours. The mixture was filtered under vacuum and the acid leached rice hull ash was dried at 80°C for 20 minutes. The dried ash was boiled in 240 mL of 1M NaOH solution in a covered flask for an hour. The dispersion was filtered and the carbon residues were washed with 400 mL of distilled water. The obtained sodium silicate solution was cooled down to room temperature and then pH of the solution was adjusted to nine with 1 M HCl to obtain silica gel. The silica gel was aged for a day. After aging the gels were gently crushed and centrifuged. Distilled water was added on to the gels after the supernatant solution was poured. The washing and centrifugation steps were repeated for four times. The silica particles were obtained after the drying (80°C for 12 hours) and grinding processes [10].

2.3. Preparation of the Bioactive Glass Samples

Glasses in the SiO_2–Na_2O-CaO–SrO-P_2O_5 system were manufactured using the melting process. The compositions of prepared bioglasses were given in Table 1. Calcium carbonate ($CaCO_3$), sodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), sodium bicarbonate ($NaHCO_3$), strontium nitrate ($Sr(NO_3)_2$) and silicon dioxide (SiO_2) (Sigma-Aldrich, St. Louis, MO, USA) or rice hull silica were mixed and grinded in a mortar. The powdered mixture was transferred to a platinum pot and was heated at 1400°C. An hour later, the molten material was poured into cold distilled water. The bioactive glass samples were dried in an oven at 150°C for 15 minutes, then grinded to powder and were heated 2 hours at 1450°C in a platinum pot. The molten material was poured on a metal vessel and annealed at 550°C for two hours. Finally, the samples were allowed to cool down to room temperature at a rate of 1°C min^{-1}.

Table 1. Chemical compositions of bioactive glass samples

		Chemical Composition of Components (wt. %)				
Symbolic name	**Silica source**	**SiO_2**	**Na_2O**	**CaO**	**P_2O_5**	**SrO**
45S5-SrC	Commercial	45	24.5	14	6	10.5
45S5-SrR	Rice hull ash	45	24.5	14	6	10.5

2.4. *In vitro S*tudies in Simulated Body Fluid

The glass samples were immersed at 37°C for 7, 14, 21 and 28 days in a simulated body fluid (SBF) which was prepared as described by Taş [11] to determine the hydroxyapatite formation on the bioactive glass surfaces. The composition of SBF is given in Table 2.

Table 2. Composition of the simulated body fluid

Ion	**Concentration (mmol/L)**
Na^+	142.0
K^+	5.0
Ca^{2+}	2.5
Mg^{2+}	1.5
Cl^-	125.0
HPO_4^{-2}	1.0
HCO_3^-	27.0
SO_4^{-2}	0.5

2.5. *In vitro* Biodegradation Studies in tris-(hydroxymethyl)-aminomethane Solution

The *in vitro* biodegradation behavior of bioactive glass samples were investigated by immersing them in tris-(hydroxymethyl)-aminomethane (TRIS) buffer solution for 7 days at 37°C. TRIS buffer solution was prepared by dissolving TRIS in distilled water. The solution was bufferred at pH 8 with 1 M HCl solution. The pH variation of TRIS solution was monitored.

2.6. Preparation of Bioactive Glass Containing Medium

In order to prepare the bioactive glass containing medium with a concentration of 20 mg/mL (powder/DMEM- F12), 0.1 g of powdered material was added in 5 mL of serum free DMEM-F12 at 37 ^oC for 24 h. After 24h, the mixture was centrifuged; the supernatant was collected and then passed through a 0.20 µm filter for sterilization. The elemental concentration of ions was

determined by analyzing the filtered extracts using the Inductively Coupled Plasma Optical Emission Spectroscopy.

2.7. Cell Culture, Cell Viability and Cytotoxicity Studies

SAOS-2 osteoblast- like cells were used in cell viability and cytotoxicity studies. Cells were cultured in DMEM- F12 with 10% FBS at 37^{o}C, 5% CO_2. The culture medium was changed once in 2 days. Cell viability was measured by the MTT assay according to manufacturer's protocol (Sigma USA). Cells were plated as 10^3 cells per well in 96 well plates. After 24 h of incubation, bioactive glasses were added at different concentrations. After 7, 14 and 21 days 20 µL MTT solution (5 mg/mL) was added into each well. After additional incubation for 4 h, 100 µL DMSO was added. The absorbance values were measured at 495 nm with a microplate reader (Biotek synergy, USA).

2.8. Differantiation, Mineralization and Alkaline Phosphatase Assay

To perform osteoblast differentiation and mineralization assays, SAOS-2 cells were cultured in osteo-inductive conditioned medium for 4, 7, and 14 days and were then harvested individually. Differentiation and mineralization were induced by the osteoinductive conditioned medium, which was composed of 10% FBS DMEM supplemented with 50 µg/mL L-ascorbic acid and 10 mM β-glycerophosphate. Cells treated with 10% FBS DMEM normal medium were used as controls for all experiments.

2.8.1. Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was determined with p-nitrophenylphosphate as a substrate. SAOS-2 cells were seeded onto 6 well plates at a density of 2 x 10^5 cells per well and treated for the designated time. Cells were then obtained and sonicated for 10 min to completely lyse. Subsequently, sonicates were centrifuged for 10 min. The supernatants were collected to be used as samples for the ALP activity assay. The bicinchoninic protein assay reagent was used to determine the protein concentrations. The relative activity of the sample is reported as the ratio of activity and the corresponding protein concentration (U/mg).

2.8.2. Mineralization Assay

Calcified nodules on the cells were determined by Alizarin red staining. SAOS-2 cells were seeded into 3.5 cm dishes at a density of 2 x 10^5 cells per dish and treated for the designed time. After incubation, the cells were rinsed with PBS (without $Mg^{2+}$and Ca^{2+}) and fixed in ice-cold 95% ethanol for 30 min at -20°C. Afterwards, the matrix and the cells were stained with Alizarin red-S (40 mM, pH 4.2), for an hour at room temperature. The stained nodules that appeared bright red color were identified by light microscopy. To compare the stained region of mineralization, the whole dish was photographed.

Calcification was assessed also by spectrophotometry. The cultures were decalcified with HCl (300 mL, 0.6 N) for a day. The Sigma Diagnostics calcium reagent that contains 2,2'-[1,8-Dihydroxy-3,6-disulfonaphthylene-2,7-bisazo]-bis-benzenearsonic acid (Arsenazo III), which specifically binds to calcium to form a purple coloured complex was used to colorimetrically determine the calcium content of the HCl supernatant. The optical densities were measured at 600 nm. A bicinchoninic acid which was used for the colorimetric detection of the cuprous cation obtained by protein from Pierce, Rockford, IL was used to measure the total protein content. The calcium content of the cell layer has been normalized to protein content.

2.9. Characterisation of Bioactive Glass Samples

The microstructure of the bioactive glass sample surfaces were observed by the use of a SEM (JEOL JSM 5410 LV, Japan; CamScan- Oxford Instruments, France) before and after *in vitro* studies in SBF.

The bioactive glass samples were analyzed with XRD system (X'pert Pro Pan'alytical, Holland). This instrument works with an acceleration voltage of 40 kV and a current of 45 mA. XRD diagrams were recorded with an angular 2θ range of 5^o - 90^o and 0.03° step size. The ionic concentration change of calcium, silica, phosphorus, sodium and strontium in SBF was obtained by the use of ICP-OES system (Perkin Elmer Optical Emission Spectrometer Optima 2100 DV, USA).

The Vickers hardness values of bioactive glass samples were determined with Vickers hardness equipment (BulutMakina HVS 1000, Turkey). The indentations were made within 10 s from the loading (500g) for all specimens at each time point.

3. RESULT AND DISCUSSIONS

3.1. Scanning Electron Microscope

The microstructure of bioactive glass samples is shown in Figure 1. Before soaking in SBF solution the surface of bioactive glass samples were smooth as can be seen from Figure 1a and Figure 1e. Bioactive glass surfaces were coated by apatitic crystals after 7 days (Figure 1 b and Figure 1 f). By the increment in soaking time from 7 to 21 days, the hydroxyapatite layer formation increased proportionally.

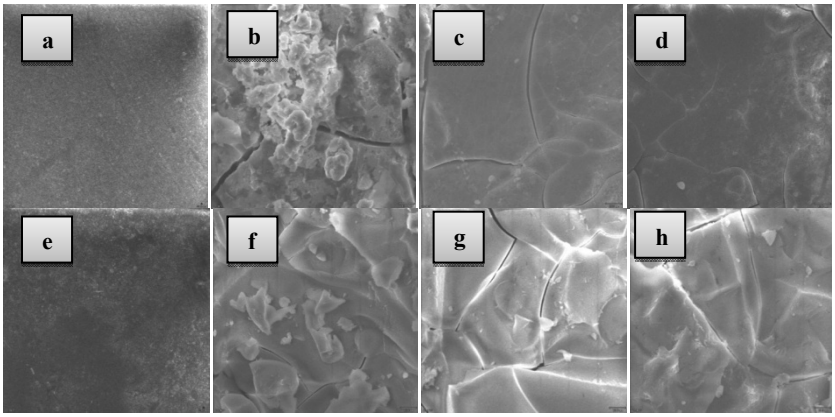

Figure 1. SEM micrographs showing the morphology of the 45S5-SrR sample a) before SBF, b) on 7th day in SBF, c) on 14th day in SBF, d) on 21th day in SBF and 45S5-SrC sample e) before SBF, f) on 7th day in SBF, g) on 14th day in SBF, h) on 21th day in SBF

3.2. X-ray Diffraction

Figure 2 shows XRD patterns of 45S5-SrR sample before and after soaking in SBF solution. The 45S5-SrR sample had both amorphous and crystalline phases before soaked in SBF (Figure 2a). Also, the $(Ca_{10}(PO_4)_6(OH)_2)$ crystal phase formation on 45S5-SrR sample surface can be observed on its 28th day in SBF (Figure 2b).

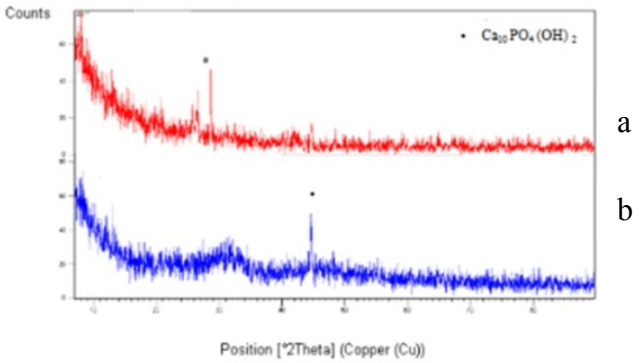

Figure 2. XRD patterns of 45S5-SrR a) before SBF b) on 28th day in SBF

3.3. Inductively Coupled Plasma Optical Emission Spectroscopy

The effect of strontium substituted bioactive glass samples immersion on variations of calcium, phosphor and silicon concentrations in SBF solution on 28th day is given in Figure 3.

Bioactive glass soaking in SBF increases the Ca^{2+} and P^{5+}concentrations of SBF solution. This results in calcium phosphate and hydroxyapatite layer formation, respectively. The P^{5+} ion concentration in 45S5-SrC soaked SBF was found to be higher than 45S5-SrR soaked SBF. This result shows that hydroxyl carbonate apatite layer formation on 45S5-SrR surface is more than the 45S5-SrC surface.

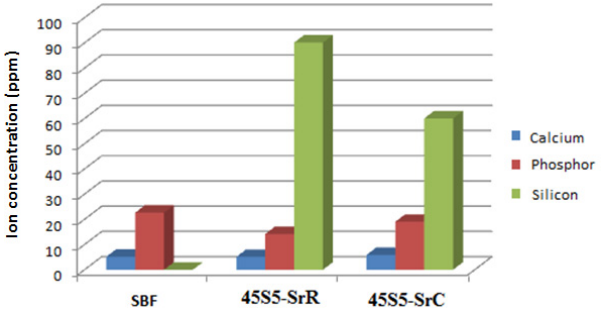

Figure 3. The changes in calcium, phosphor and silicon concentrations of SBF solution with the strontium substituted bioactive glass immersion on 28th day

The release of ions from bioactive glasses into solution is one of the key characteristics of glasses.

The elemental concentrations of calcium, silicon and strontium released from bioactive glass samples into the cell culture media are shown in Table 3. The incubation of strontium substitued bioactive glass samples results in increased strontium concentration in DMEM cell culture media as expected. Also, silicon levels of cell culture media increased which is related to the dissolution of the outer silica layers of the bioactive glass network [12].

Table 3. Ionic concentrations of cell culture media

	Ion Concentration (µg/mL)			
	Si	**Ca**	**Na**	**Sr**
DMEM- F12 neat	0.10	3.00	320.00	0.00
45S5-SrR conditioned medium	12.60	3.49	632.30	0.89
45S5-SrC conditioned medium	18.47	Not determined	600.93	4.88

3.4. Vickers Hardness Analysis of Bioactive Glass Samples

The effect of SBF immersion on mechanical properties of bioactive glass samples was determined with Vickers microhardness test at different time intervals. Microhardness values obtained at different SBF immersion periods give information about bioactivity and degradation behavior of the samples. Microhardness test results obtained were presented in Table 4. Microhardness values of prepaired bioactive glass samples decrease with increasing immersion period as can be seen in the table. Results revealed that degradation and bioactivity behavior of 45S5-SrR and 45S5-SrC was similar.

Table 4. Vickers hardness date for strontium substituted glass samples before and after SBF immersion

Samples	**Before soaked in SBF solution**	**7. Day**	**14. Day**	**21. Day**	**Vickers hardness reduction (%)**		
					7. Day	**14. Day**	**21. Day**
45S5-SrR	534	235	172	145	56	68	73
45S5-SrC	445	222	151.8	126	50	66	71

3.5. Ion Release of Bioactive Glass Samples in TRIS Solution

The bioactive glass loses weight due to the release of Ca, Na, P and Si ions from bioactive glass to TRIS solution. The degradation behavior of glass samples gives information about the bioactivity of glass. As can be seen from Figure 4, the weight loss of glass samples was high at first day. However, at second day there was an increment in the weight of glass samples that was due to precipitation of released ions from TRIS solution to the glass surface. It was observed that the weight loss was continuous with a slow rate from third to seventh day.

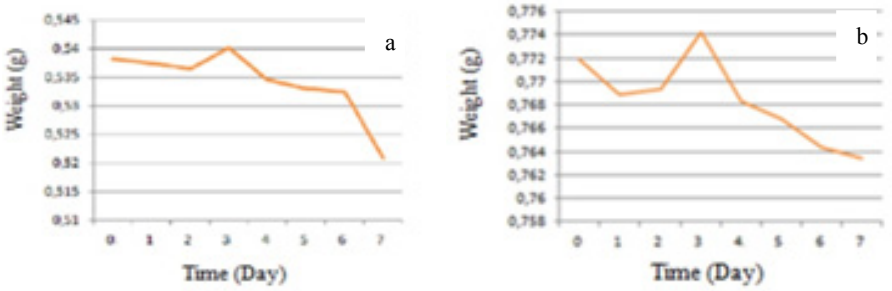

Figure 4. Weight loss of bioactive glass samples in TRIS solution a) 45S5-SrR b) 45S5-SrC

Due to the release of ions from glass surface by the contact of bioactive glass samples with TRIS solution, pH of TRIS solution increases. The pH changes in 45S5-SrR and 45S5-SrC sample soaked TRIS solutions were similar and thus it can be said that both of the glass samples showed similar bioactivity.

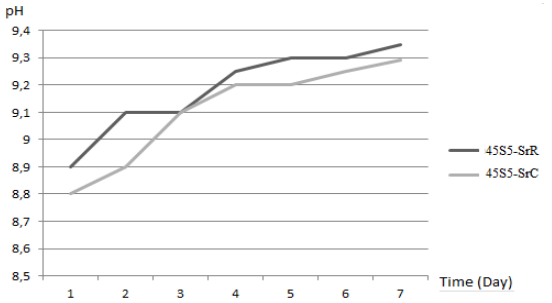

Figure 5. The pH variation of TRIS solution with soaking time for 45S5-SrR and 45S5-SrC sample

3.6. Metabolic Activity of Cells

After 14 days in culture, the MTT activity in cells treated with dissolved ions from all compositions of bioactive glass samples was significantly enhanced compared to controls. After 14 days in culture, MTT activity was significantly greater in SAOS-2 cells treated with dissolved ions. When the results of 45S5-SrR and 45S5-SrC samples were compared 45S5-SrR showed more metabolic activity and viability than 45S5-SrC, as can be seen in Fig. 6.

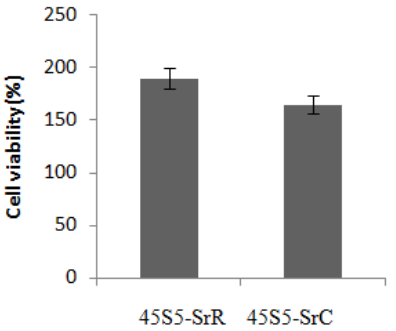

Figure 6. Cell viability under 100 µM bioactive glass solution

3.7. ALP Activity

SAOS-2 cells cultured on bioactive glass discs showed increased total ALP activity with increasing strontium substitution for calcium. As can be seen from Figure 7, cells with bioactive glass 45S5-SrR had significantly higher total ALP activity than 45S5-SrC in 7 days culture.

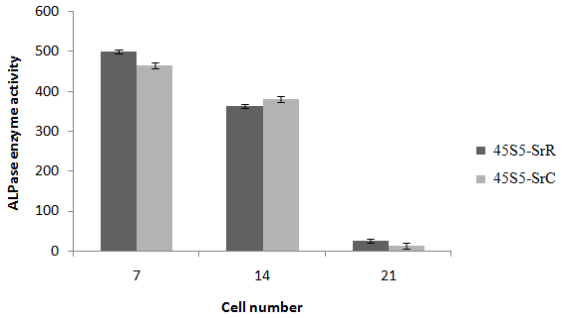

Figure 7. The ALP activity of 45S5-SrR and 45S5-SrC sample at day 7, 14 and 21

Calcium has been reported to act as a network modifier in silicate glass systems such as bioactive glass [13]. Strontium has a similar ionic radius and charge to calcium. Thus, by the substitution of strontium into the glass network, it may act a similar role as calcium. The lower charge to size ratio of the strontium ion compared to the calcium ion (due to the slighter larger ionic radius of strontium), however, should create an expanded and more loosely cross-linked glass network, but not fundamentally alter the glass structure. The release of calcium, phosphorus, silicon and strontium into cell culture medium during incubation with bioactive glass is complicated because of ions already present in cell culture media, which vary among media formulations.

Figure 8 shows that the calcium deposition of 45S5-SrR sample is higher than 45S5-SrC sample.

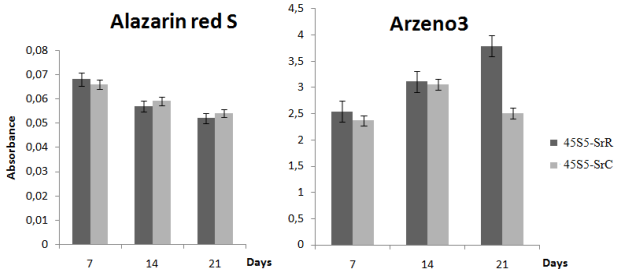

Figure 8. Semi-quantitative Alizarin red S and Arzeno 3 staining intensities on 45S5-SrR and 45S5-SrC samples.

4. CONCLUSION

In this study, 45S5-SrR and 45S5-SrC bioactive glasses were produced by melt derived process and their mechanical and bioactivity properties were evaluated by SEM, XRD, Vickers Hardness analysis and degredation in TRIS solution. Also, the effect of bioactive glass samples on osteoblast cells was determined by MTT and ALP activity methods.

It is concluded from the results that rice hull ash silica is a convenient raw material to prepare melt derived bioactive glass. Bioactivity and mechanical properties of 45S5-SrR and 45S5-SrC bioactive glasses were similar but 45S5-SrR showed better metabolic activity and viability than 45S5-SrC.

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