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Note

Ginseng used for bone tissue scaffold

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The scaffold based tissue engineering materialized for bone tissue therapy. Gelatin-glutaraldehyde cross linked scaffold was prepared by solvent casting -porogen leaching method. It was characterized by FTIR and SEM microphotograph analysis. Absence of peak at waves no. 1625 cm^{-1} in ATR-FTIR indicated formation of cross-linking. FE-SEM micrograph showed honeycomb pad like structure with high porosity. Methanolic extract of *Withania somnifera* (Ashwagandha) root extract induced MC3T3 E1 osteoblast cell adhesion and proliferation on porous gelatin scaffold. GC-MS analysis pointed out presence of 4-amino- 2-ethyl-3-methylquinoline, an active phyto-chemicals having tissue regeneration potential. High anti-oxidant capacity down regulates cell death mechanism by scavenging free radical. The biocompatible gelatin scaffold has RGD moiety that attune the MC3T3 E1 osteoblast cell adhesion. *Withania somnifera* root extract may boost up cell proliferation on scaffold. Therefore treatment with *Withania somnifera* root extract may be the new approaches for designing bone tissue scaffold for bone tissue therapy.

Keyword: Ginseng scaffold, Honeycomb scaffold, MC3T3 E1 cell, Regenarative therapy

The field of tissue engineering has an objective to regenerate damaged and injured tissue on highly porous biomaterial called scaffold. The scaffold provides appropriate environment and biophysical stimulus for cell growth, regeneration and proliferation[1]. Besides, an interconnected pore gives a large surface area, suitable for cell growth. Biocompatible scaffolds slowly degrade and provides construct for cellular structure *in vitro* and *in vivo*[2]. The arginine-glycine-aspartic acid (RGD) moiety of gelatin modulates the cell adhesion. Besides that, it is a natural biopolymer obtained by partial or alkaline hydrolysis of animal collagen from bone and skin[3]. *Withania somnifera* (Indian Ginseng) root extract is the well known ayurvedic herb that promote anti ageing and anti oxidant activity along with revitalizing capacity for cell and tissue[4]. Therefore, our experiment was planned for studying the *Withania somnifera* root extract induced osteoblast bone cell proliferation on porous gelatin scaffold with intention of designing bone tissue scaffold for therapeutic purpose.

Materials and Methods

Preparation of Withania somnifera root extract

The *Withania somnifera* was collected from the Govt. approved Herbal medicinal Plant Nursery placed near to Jadavpur University. It was washed properly and then cut into fine pieces with a scalpel. The extract was prepared by mixing 20 g of the root in methanol with little exposure to warm heat. The concentrated solution was lyophilized and stored in 4°C for future use.

Antioxidant activity by DPPH assay

The antioxidant activity of the methanolic extract was determined by DPPH assay[5]. 0.1 mM DPPH (2,2 diphenyl-1-pierylhydrozyl) solution was prepared in alcohol. Sample solution was prepared by mixing 1 mL of freshly prepared DPPH solution with 3 mL of extract. 4 mL of DPPH solution was kept in another test tube for using as control. And 4 mL of extract was taken as a blank for our experiment. All test tubes were shaken vigorously in vortex and allowed to stand at room temperature (25°C ± 2°C) for 30 min. Five serial dilution of L-ascorbic acid (SRL) in the range of 1-0.625 µg/mL were used to obtain standard curve. All measurements were performed at 517 nm in Agilent Technologies Cary 60 UV-vis Version 2.00 Spectrophotometer. Finally the % of antioxidant activity (AA %) was calculated using the formula 1.

$$AA\% = \left[100 - \left\{\frac{(Abs\ Sample - Abs\ Blank)}{Abs\ Control}\right\}\right] \times 100 \quad \ldots (1)$$

GC-MS analysis

GC-MS analysis of the *Withania somnifera* root extract[5-7] was carried out using Ion trap technology on M/S Thermo Scientific instrument coupled with Mass

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detector on Polaris-Q machine. The instrument was set to initial temperature 70°C and maintained isothermally for 3 min followed by increase at a rate of 10°C/min upto 240°C and finally ending with 9 min isothermal at 280°C. 1 µL sample was injected manually into silica capillary column (DB5MS; Agilent Technologies with specification of 30 m × 0.25 mm ID × 0.25 µM thick, composed of 5% phenyl/95%dimethylpolysiloxane) for employing analysis in 10:1 split ratio when injector temperature was 240°C and ion source temperature 220°C. Helium gas (99.999%) was used as carrier gas at a constant flow of 1 mL/min, operating in electron impact mode at 70 eV. The detector performs scanning from 50-950 *m/z* in total running time starting at 2 min to 40 min. All data were store at data store software (X-caliber). The identifications were based on mass spectral matching with standard compound in NIST library version 2014.

Preparation of porous gelatin Scaffold

Porous gelatin scaffold was prepared by slight modification of conventional solvent casting and porogen leaching method 8.5% gelatin granules (Merck) was mixed with porogen *i.e.* 3% sodium chloride (Merck) solution and with 4% glutaraldehyde solution (SRL). The crosslinked gelatin scaffold was promptly transferred to petriplate for providing shape and support. It was shifted to −20°C and freeze dried. The newly synthesized scaffold was washed in appropriate wash buffer solution , to get rid of most of excess porogen and excess glutaraldehyde. Therefore it was incubated at least 4h at room temperature in 50 mM NH4Cl containing previous washing buffer followed by refluxed extensively in hot water in a Soxhlet extractor 8. It was air dried and ultimately the fabricated gelatin scaffold was obtained.

FE-SEM micrograph analysis of Scaffold

Cross linked gelatin scaffold were cut and the cross-section were coated with gold. It is then observed at 5.0 kV with Hitachi S-4800 (serial no: HI-9155-0005) FE-SEM. Pore size were analyzed by measuring the maximum diameter of the pore and calculated by using the formula 2:

$$\text{Area of pore} = \pi r^2 \quad \ldots (2)$$

Scaffold optimization

MCF-7 and MDA-MB-231 cell line were purchased from NCCS Pune and cultured in a sterilized aseptic condition prescribed as per standard protocol of cell culture[5]. PBMC cell was isolated from the blood of healthy donor after obtaining their legal consent that was prior approved by Institutional Bioethics Committee, Jadavpur University. It was cultured as per standard protocol[7,9]. ATR-FTIR chemical analysis, pH dependent scaffold swelling study, cytotoxicity using PBMC and evaluation of anchorage dependent (MCF-7) and anchorage independent (MDA-MB-231) cell line growth on scaffold were performed for evaluating the scaffold based cell or tissue growth and biocompatibility. Samples were observed at different coming wavelength ranges from 3000 cm^{-1} – 1000 cm^{-1} in FTIR instrument Irprestage-21. The scaffold was cut into three sections weighing about 1.1 g averages and having same dimension (L × W × T). Each section dipped into the test tube containing three buffer medium (acidic, alkaline and neutral). The weight of the each section was measured at specific time interval[8]. Hemo- compatibility of the scaffold was assessed as per the ASTM protocol.

Cell viability, cytotoxicity and cellular proliferation rate measurement through MTT assay

The MC3T3 E-1 cells were cultured in a sterial ascetic environment in complete DMEM media with high glucose (Himedia) add on to 10% FBS (Gibco). The cells reaching confluences were treated with Trypsin-EDTA (Himedia) and harvested. The porous gelatin scaffold was sterilized with 70% (*v/v*) ethanol and washed with PBS. The appropriate number of cells was seeded on porous gelatin scaffold.

The seeded scaffold were transferred to 6 well plate and divided into two groups (24 & 48 h) containing 3 well in each group, designated as blank (containing no seeded scaffold and having no treatment), no treatment and treatment with Aswagandha root extract. The number of cell seeded on scaffold of different groups was measured after 24 h and 48 h MTT assay (as per standard protocol). Aswagandha root extract induced cellular proliferation was measured by comparing the MTT assay results of treatment and no treatment group. % of cell viability/ adhesion was measured against blank[8,9].

Statistical methods

All data was recorded on Ms excel-2007. One way ANOVA was performed using Origin 6.1 software packages. The compiled data (mean value ± SD) was given in figure either by line diagram or by bar

diagram. The significant difference at $P< 0.05$ was marked with alphabet where same letter designated for no significant and different letter denotes significant difference. The letter on line signified for group analysis.

Results and Discussion

Withania somnifera root extract mostly used as herbal drug for arresting cellular aging and to enhance cellular longevity. Routine biochemical analysis of the methanolic extract reveled that it contains 433 ± 40 mg alkaloid, 24.26 ± 1.44 mg flavonoids and 0.28 ± 0.009 mg Polyphenolic compound. DPPH antioxidant assay was performed. DPPH has nitrogen centered free radical that contains an odd electron which gives a strong absorbance at 517 nm. Its colour changes from purple to yellow when odd electron of DPPH accept electron from antioxidant compound and became paired off to form reduced DPPH-H.

The DPPH antioxidant activity of *Withania somnifera* root extract was calculated 74.66% which is equivalent to 0.8 mg/dL L-ascorbic acid concentration. The phytochemical screening of the extract were carried out and identified by GC-MS techniques. The GC-MS analysis result (Fig. 1) reveled total 27 peaks. Among them 9 major peaks stated as retention time (RT) 6.42, 7.57, 8.08, 11.01, 13.42, 14.66, 15.11, 17.9, 25.76 corresponds to 9 different compound named Spiro{3,3]hepta-1,5-diene, 1,3,5- Cycloheptatriene, Acetoxyacetic acid, 2-naphthyl ester, 8-Azabicyclo [3,2,1]octan-3-ol, 8-methyl- acetate[ester], 8-Azabicyclo [3,2,1]octan-3-ol, 8-methyl-acetate[ester], 4-Amino-2-ethyl-3- methylquinoline, 1H-Indole, 2-pyrrolidin-2-yl, Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4- hydroxy,methyl ester, 9,12,15-Octadecatrienal respectively. Among them the most prominent peak at RT_14.66 with relative peak area of 30013.19 units is for the compound of 4-amino-2-ethyl-3- methylquinoline. It may be our compound of interest that induced the cell growth and proliferation.

The cross linked gelatin scaffold under the FE-SEM showed highly porous structure with an open fully connected geometry (Fig. 2A). The honeycomb pad like structure provides least density with high compression properties (Fig. 2B & C). The diameter of the pores ranges from 2-8nm and the area was calculated on an average 20 nm^2.

The ATR-FTIR expressed in (Fig. 3A) showed a peak on wave no 1625 cm^{-1} on non cross linked gelatin scaffold. This peak was absent in cross linked gelatin scaffold. The aldehyde functional group of the glutaraldehyde formed an amide bond with free non-protonated amino group ($-NH_2$) of lysine or hydroxyl lysine. The -COO stretching vibration indicated the

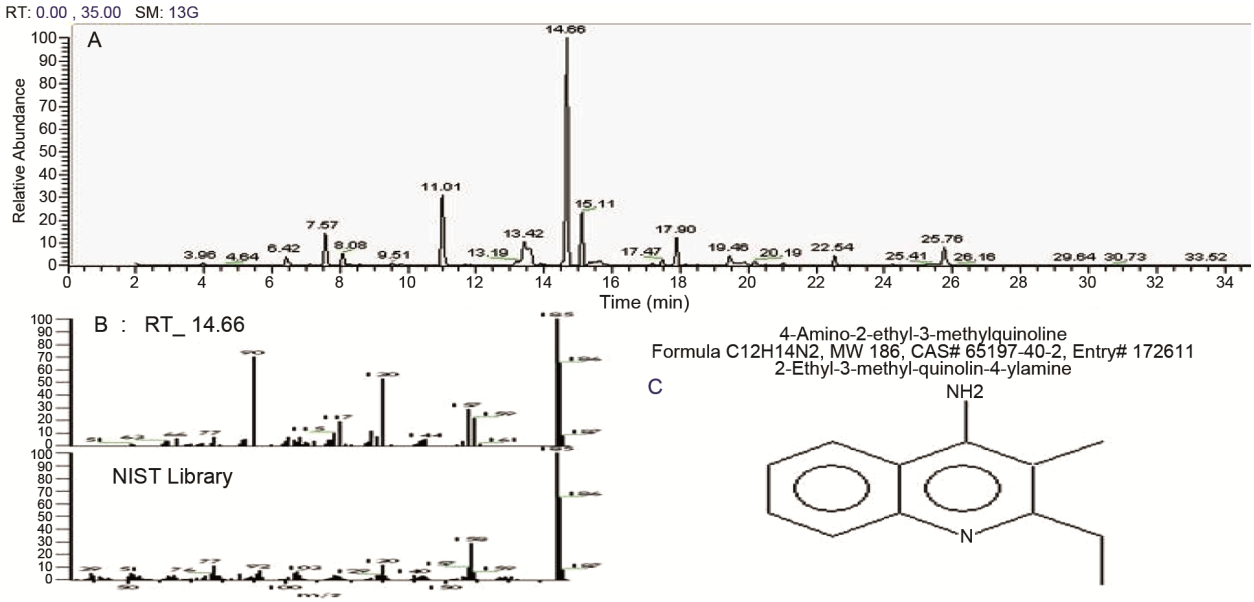

Fig. 1 — GC-MS analysis of *Withania somnifera* (Ashwagandha) root extract

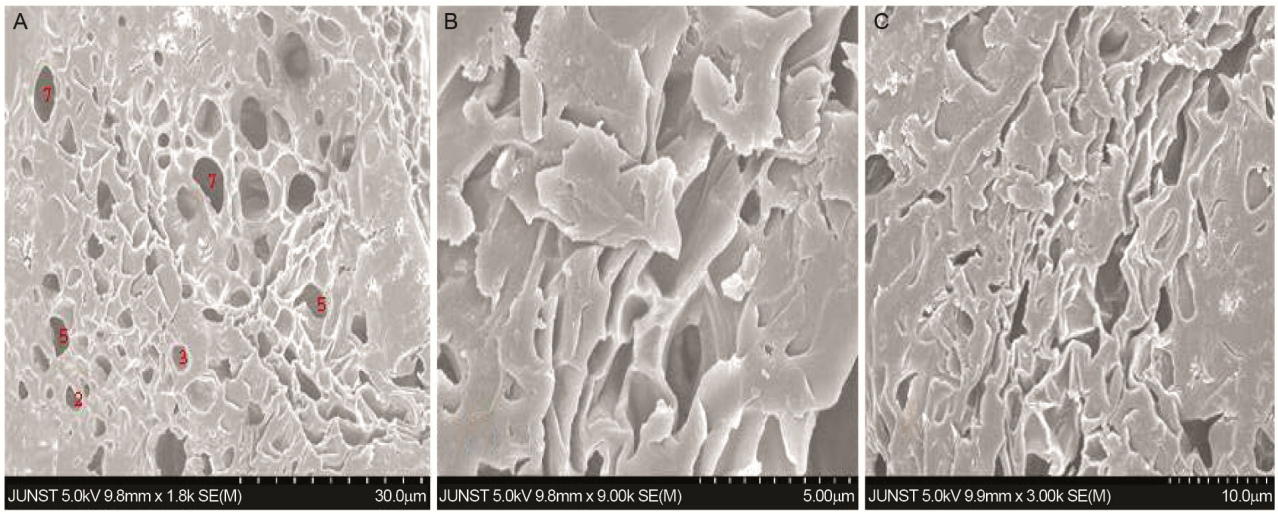

Fig. 2 — FE-SEM micrograph analysis of porous gelatin scaffold

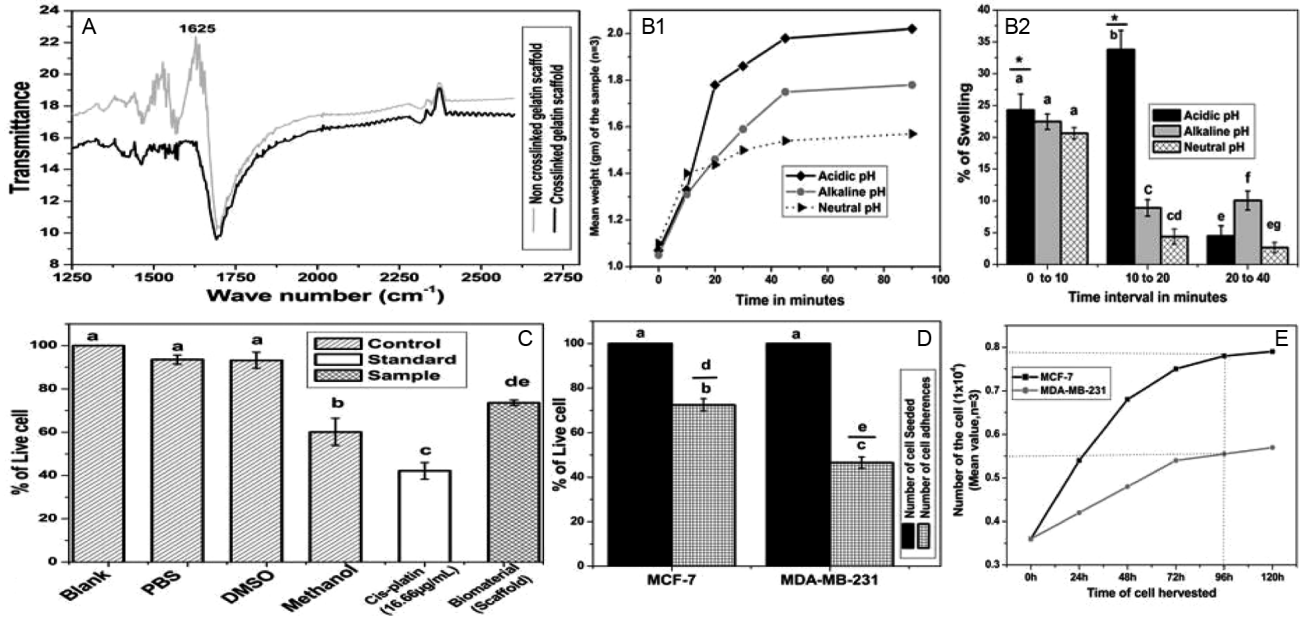

Fig. 3 — Scaffold optimization using FTIR chemical analysis (3A), pH dependent scaffold swelling study (3B1 & 3B2), Cytotoxicity study on PBMCs (3C) and evaluation of anchorage dependent (MCF-7) and independent (MDA-MB-231) cell adherence after 24 h (3D) followed by cell proliferation (3E) on Scaffold. The all experiments were conducted three times , Bar diagram represents Mean ± SD value. Statistical analysis was measured at $P<0.05$ and represented alphabitically. Same letter denotes non significant where as different letters denotes significant diffrence between group. The letter or '*' above the line represent the group analysis

involvement of nucleophilic addition reaction that often forms tetrahedral unstable intermediate called carbinolamine[10]. The protonation of the –OH group followed by loss of a water molecule yields the conjugated Schiff base near 2100 cm^{-1} wave number, which again present after reaction and was absent before reaction. The FTIR analysis indicates no presence of free glutaraldehyde. Therefore the prepared scaffold may not be toxic in nature.

Swelling property of cross linked gelatin scaffold evaluated gravimetrically at different time period. It is found that initially from 0-10 min the rate of swelling are nearly same in all pH medium. After 10 min the rate of swelling increases significantly ($P< 0.05$) in acidic pH medium than alkaline and neutral pH medium (Fig. 3B). Scaffold swelling saturated after 40 min in all medium.

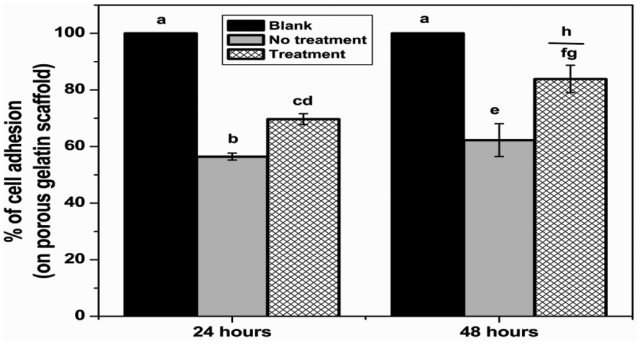

Fig. 4 — *Withania somnifera* (Ashwagandha) root extract induced MCT3T-E1 osteoblast cell adhesion and proliferation on porous gelatin scaffold. The experiment was conducted thrice and the data were represented as Mean ± SD value. Statistical analysis were measured at $P< 0.05$. same letter denotes non significant where as different letters denotes significant diffrence between group. The letter above the line represent the group analysis

As per ASTM (American society for testing material) protocol, the cross linked scaffold was considered hemocompatible as they shows percentage hemocompatibity value 8.29%. The scaffold was also biocompatible as it shows less cytotoxicity on PBMC than the standard cisplatin (16.66 µg/mL) (Fig. 3C).

Honeycomb pad like structure with high porosity provide large surface area that would allow cell growth and cellular distribution. MCF-7 cell adhered into scaffold greater than MDA-MB 231 (Fig. 3D). MCF- 7 has some anchorage proteins which required for adhesions followed by proliferation. MDA-MB 231, being a mesenchymal type of cell do not express any anchorage proteins therefore its proliferation rate was slower than the MCF-7 cell line (Fig. 3E).

MC3T3-E1 osteoblast cell line was used for our experiment. The result (Fig. 4) indicated that the *Withania somnifera* root extract (IC_{50} 24 µg/mL) significantly increases percentage (17 - 22%) cell adhesion compared to no treatment ($P< 0.05$). The rate of cell proliferation increases as the number of adherent cell significantly raised in 48h contrast to both 24 h and the no treatment. Glutamate is the powerful inducer for cell death mechanism[11]. Excessive accumulation of the free radical was responsible for glutamate induced cell death mechanism[5,11]. Our experiment shows that the *Withania somnifera* have high amount of antioxidant activity. Therefore it trapped the free radical and down regulates the glutamate induced cell death mechanism. On treatment it boosts up the MC3T3-E1 osteoblast cell line attachment, adhesion and expantion on porous gelatin scaffold. This designed bone tissue scaffold may be utilized for therapeutic purpose, treating bone tissue malfunction.

Conclusion

The ginseng scaffold was made over a cross linked gelatin. This porous gelatin scaffold shows honey comb pad like structure, chiefly suitable bed for regeneration of bone tissue. The nine major peaks in GCMS indicate nine different compound that acts as a bio-stimulator for cell growth. This designed bone tissue scaffold may be utilized for therapeutic purpose, treating bone tissue malfunction.

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Conflict of interest

All declares no conflict of interest.

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