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Novel Bonegraft Composite using Hydroxyapatite, Egg-Shell Powder and Chitosan Fortified with *Terminalia chebula*

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Abstract

Allogeneic bone grafts are considered to be an integral part of implant dentistry. These grafts have rich osteoconductivity, osteoinductivity and osteogencity and can act as a substrate material to fill the defects on bone surfaces and can augment bone fracture and tooth fracture healing. Based on such principles, this current study fabricated a novel graft composite for bone using egg shell powder (ESP), Hydroxyapatite (HA), Chitosan (CH) and Aqueous extract of fruits of Terminalia chebula (TC). The prepared new bone graft (ESP-HA-CH-Tc) was analyzed using various physico-chemical characterization techniques like FT-IR, XRD, TGA, and SEM to understand the chemical composition, evaluate its surface morphology and stability standard. The aqueous fruit extract of Terminalia chebula (Tc) was analysed for its phytochemical composition by GCMS and invitro pharmacological properties like antioxidant, anti-inflammatory, antimicrobial and the biocompatibility of the graft were assessed by cell culture studies in Breast cancer (MCF-7) cell lines. The incorporation of fruit extract of Terminalia chebula strengthens and augments the ossification property of the graft material and can extend its use in the treatment of fractures and further biomedical applications.

Keywords: *Bone Graft, Cancer Cell Lines, GCMS, In vitro Characterization, Health and well-Being, Novel Method..*

Introduction

Bone is one of peculiar functional connective tissue composed of bone cells with dense intercellular matrix and enormous blood vessels. It provides a skeletal framework for the body, offers mechanical support and also facilitates movements in the human body [1]. Classification of bone includes two types- compact or cortical bone; cancellous or trabecular bone. The cellular components include the osteocytes found on the surface of osteoid seams, for maintenance of bone and osteoclast, found bone surfaces known as resorption pits and involved in bone resorption [2]. Human bone contains two important minerals calcium and phosphorous. Reports suggest that 99% of the calcium in the human body is concentrated in the bones of the

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skeleton in the form of hydroxyapatite crystals and few non-crystalline carbonates and phosphates. Bone Remodeling is peculiar continuous phenomenon that creates and replaces a bone. These processes occur at a fairly accurate turnover rate of 3% in adult human cortical and 26% in trabecular bone annually [1]. Even a healthy bone can break in its continuity when experiencing a high-impact force or stress. Reports suggest that fractures occur in clinical conditions like osteoporosis, Paget's disease or even in a benign or malignant lesion [3]. To replace and treat such fractures many regenerative therapies have been used widely in the form of autologous, allogenic bone substitutes [4].

Bone tissue engineering targets to induce the formation of a functionally regenerated bone regeneration involving a synergistic amalgamation of various biomaterials, cellular products and also factor therapy. Thus, the engineered new bone restored the functions of bone to completely integrated state in support of the adjacent host bone, and thereby functions on par with the native bone. Thus, Bone-engineered preparations have been considered as a potential therapy that can act as substitutes for traditional and conventional bone grafts as they have abundant blood supply and decreased infection rates [5]. Biomaterials commonly used in tissue scaffolding on bone tissue engineering are chitosan, collagen, chitosan, alginate, fibrin, elastin, gelatin, casein etc. owing to their high scaffold nature, good biocompatibility and regenerative and biodegradable properties [6].

Chitosan is a frequent adder to wound dressing preparations owing to its positive charges, gentle gelation capability and film-forming capacity, muco-adhesiveness, antimicrobial, biocompatible and biodegradable properties [7]. Synthetic HA offers strong affinity and binding with the host tissue thereby exhibiting good biocompatible and osteoconductive properties. Eggshell powder is an effective alternative to a bone for treating defects around the implant and other interposition defects due to its biocompatible nature and bonding strength, and mechanical strength to the bony site. It contains 93.7% $CaCO_3$ and so acts as a bone graft material in treatment options in procedures of maxillofacial surgery [8].

Terminalia chebula, commonly called chebulic myrobalan, is a species that belongs to Terminalia and is a native plant of Southern parts of Asia. The tree produces small, ribbed fruits with nuts. From various harvested origins 7 different types of fruits have been identified namely vijaya, putana, rohini, amrita, jivanti, abhaya and chetaki. This plant possesses fracture healing effects. Reports suggest that *Terminalia chebula* in polyherbal formulations effectively treats fractures and also increases the serum calcium and alkaline phosphatase levels depicting a positive effect. Paid to its fracture healing efficacy, the study planned to fabricate of a bone graft substitute material utilizing the aqueous fruit extract of *Terminalia chebula* along with eggshell powder (ESP), Hydroxyapatite (HA), Chitosan (CH) and investigated the osteogenic properties of this TC in the novel composite graft.

Materials and Methods

Extract Preparation from Fruits of *Terminalia Chebula*

Fruits of *Terminalia chebula* (TC) were washed thoroughly, and made to dry. It was powdered in a blender. 10g of TC powder was added to 100ml of distilled water. The mixture was at 60° for 1 hour. The filtrate was obtained using the Whatman filter paper. The obtained extract was maintained at 5° for future characterization studies.

Synthesis of Hydroxyapatite (HAP)

Hydroxyapatite (HAP) was prepared by additional procedures with methods of Bouyer et al. (2000) [9] A 0.5 molar solution of calcium hydroxide in distilled water.

orthophosphoric acid with 0.3 M was further added to it and continuously monitored for pH till it reached 12.5. The prepared mixture was continuously stirred for a period of 24 hours. The obtained mixture was centrifuged at an rpm of 6000 for a period of 15 minutes. The prepared precipitate was gradually collected, thoroughly rinsed with double distilled water and made to dry at a temperature of 100ºC.

Preparation of Chitosan Solution

Chitosan is considered as a weaker base, which on soluble in any dilute acidic solution with a pH less than 6.5, causing conversion of glucosamine units into its soluble forms. 1g chitosan was mixed with 10ml of water. On continuous stirring, chitosan appears in a thick gel form and this can be used in bone graft preparation.

Generation of Powder from eggshells

Eggshells were taken from healthy eggs from a restaurant and completely washed with water. The adjoining membranes in the eggs were manually separated and obtained shells from the eggs were made to dry at 30◦C. Further, the shells were crushed in a blender to obtain an ESP size of particles at 5 µm to 50 µm. The obtained powder of egg shells (ESP) was stored at RT at 30◦C (Figure 1).

Bone Graft Preparation

The base material was prepared using ESP and HA in the ratio of 60:40. 5 g of this ESP mixture was powdered finely in a mortar. 500mg of TC extract was added to the mixture uniformly. 3 ml of 3% chitosan was further used and made into a soft dough. The prepared dough was squeezed out through a 1 cm diameter tube made of glass to form a graft cylindrically namely ESP-HA-CH-TC.

The obtained cylindrical grafts were further powdered and cured at 30 ℃ for a duration of 2 to 3 hrs. The prepared bone graft was then made to dry at 55°C for the whole night and then further stored in sealed polythene packets, maintained in sterilized state using ethylene dioxide in the chamber (Figure 2). The graft material was then analyzed using many physicochemical methods like Fourier Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), Scanning Electron Microscopy (SEM) and X-ray Diffraction (XRD).

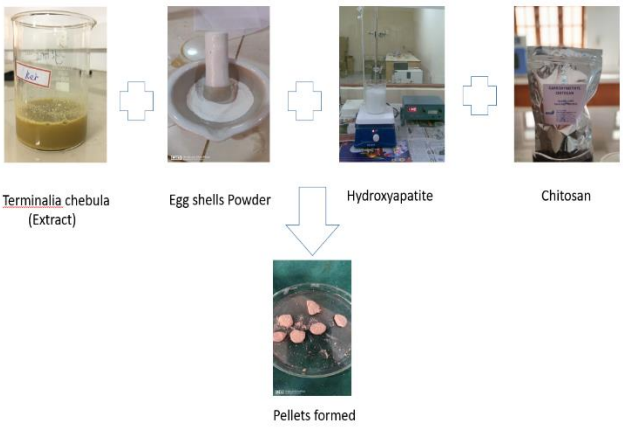

Figure 1. Fabrication of Bone Graft

Sterilization of Bone Graft

The dental implant was sterilized before using further analysis. The graft material were then exposed to methods like wet or dry sterilization. Since the prepared implant constitutes of fruit extracts of TC and biodegradable proteins, chemical method was chosen for sterilization. Ethylene dioxide (ETO) Sterilization is one of the chemical sterilization procedures employed to sterilize products related to medical and

pharmaceutical industries [10]. Thus, ETO sterilization was performed prior to *invitro* studies. Figure 4.2 depicts the ETO chamber and sterilized ESP-HA-CH-TC bone grafts.

Figure 2. ETO Sterilized Bone Grafts

Determination of Total Antioxidant Capacity

The total antioxidant activity of plant extract was assessed by phospho-molybdenum method Prieto et al. (1999) [11]. The invitro anti-inflammatory activity was determined by protein denaturation assay and Human Red Blood Cell (HRBC) membrane stabilization method [12].

Antimicrobial Activity

Sabouraud Dextrose Agar (SDA) and Mueller Hinton Agar (MHA) were prepared. The Test Microorganisms are Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, candida albicans. Antimicrobial activity was determined by Agar well-diffusion method, diameter of the inhibition zone (mm) was eventually measured, and the activity index was also calculated [12].

Characterization of the Dental Implant

The IR spectra of the sample prepared were made to read at 4000 cm^{-1} to 400 cm^{-1} with FTIR spectrophotometer, Nicolet Impact 400 with KBr pellet constituting 1–2 mg of the prepared sample. XRD analysis of the grafted sample was assessed with analytical alpha-1 X'Pert PRO built with RTMS X'Celerator detector. The morphology of surfaces in the graft material was assessed with Supra 55, Zeiss Gemini, SEM and EDX analyses were performed with Oxford instrument X-act. The thermo-gravimetric assessment of the grafts was done with Seiko SSC 5200 H.

In vitro Viability Study – MTT Assay

MCF-7 (breast cancer) cell lines were received from National Centre for Cell Sciences, located in Pune, India. The stock cancer cells were made to culture in DMEM augmented medium containing 10% Fetal Bovine Serum on an inactivated state, penicillin at 100 IU/ml, streptomycin at 100 µg/ml and amphotericin B at 5 µg/ml present humidified atmosphere with 5% Tc. The MTT assay was performed at 37°C until confluent. The sample analysis was performed based on reduced dye at 570 nm with the help of UV spectrophotometer and the proliferation effect of the MCF -7 culture samples were determined as an index of % cell viability [13].

Statistical Analysis

The statistical significance between samples of different concentrations was done using the SPSS analysis, One-way ANOVA and the marked significance present between the groups was determined by Tukey's multi-comparison test. ($p<0.05$ = significant).

Results

Gas Chromatography-Mass Spectrometer (GC-MS)

Phytochemical reports showed that *Terminalia chebula* fruits contain tannins, saponins, terpenoids and phenols in greater

proportions, while cardiac glycosides, and flavonoids in smaller concentrations. The peak 21.439 shows the presence of phenols, peak 39.264 shows Propanoic acid and Peak 46.955 indicates the presence of Vitamin E in extract apart from the other bioactive compounds mentioned above (Figure 3. Table -1).

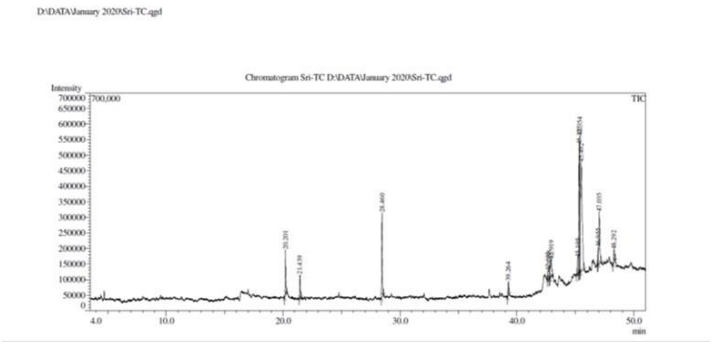

Figure 3. GC-MS Analysis Report of *Terminalia chebula* Fruits.

Table 1. Peak Report TC Extract

S.NO	**R.Time**	**Area**	**Area%**	**Name**
1	20.201	550645	5.08	1-dodecanol
2	21.439	257702	2.38	2,4-bis(1,1-dimethylethyl phenol
3	28.460	1075952	9.92	2-Propenoic acid, pentadecyl ester
4	39.264	180077	1.66	Propanoic acid, 3-mercapto-, dodecyl ester
5	42.635	184076	1.70	4,4-dimethylandrost-5-en-17-ol
6	42.755	180087	1.66	9.beta,10 alpha.Androst-4-en-3-one, 4-brom
7	42.919	486897	4.49	4,4-dimethylandrost-5-en-17-ol
8	45.195	239083	2.20	Cedran-diol, (8S,14)-
9	*45.325*	*1583279*	*14.60*	Lupeol
10	45.354	1573502	14.51	methyl commate b
11	45.492	3187607	29.40	Betulinaldehyde
12	46.955	182696	1.68	Vitamin E

The thirst for antimicrobial sources from natural plant origins has received much focus at current scenario and many researches were undertaken to recognize the natural plant compounds that act as effective antimicrobials agents to substitute for the artificially man made preparations. TC extract possessed significant antimicrobial activity as depicted by increased zones of inhibition against *Salmonella typhimurium (fig 4.1); Malassezia furfur (fig 4.2); Staphylococus aureus (fig 4.3)* (Table 2).

Table 2. Antimicrobial Activity of TC Extract Against Various Pathogens

S.NO	**Selected Pathogens**	**Zone of inhibition in mm**
1	*Salmonella typhimurium.*	23
2	*Malassezia furfur*	25
3	*Staphylococus aureus*	15
4	*Control (*Fluconazole*)*	28

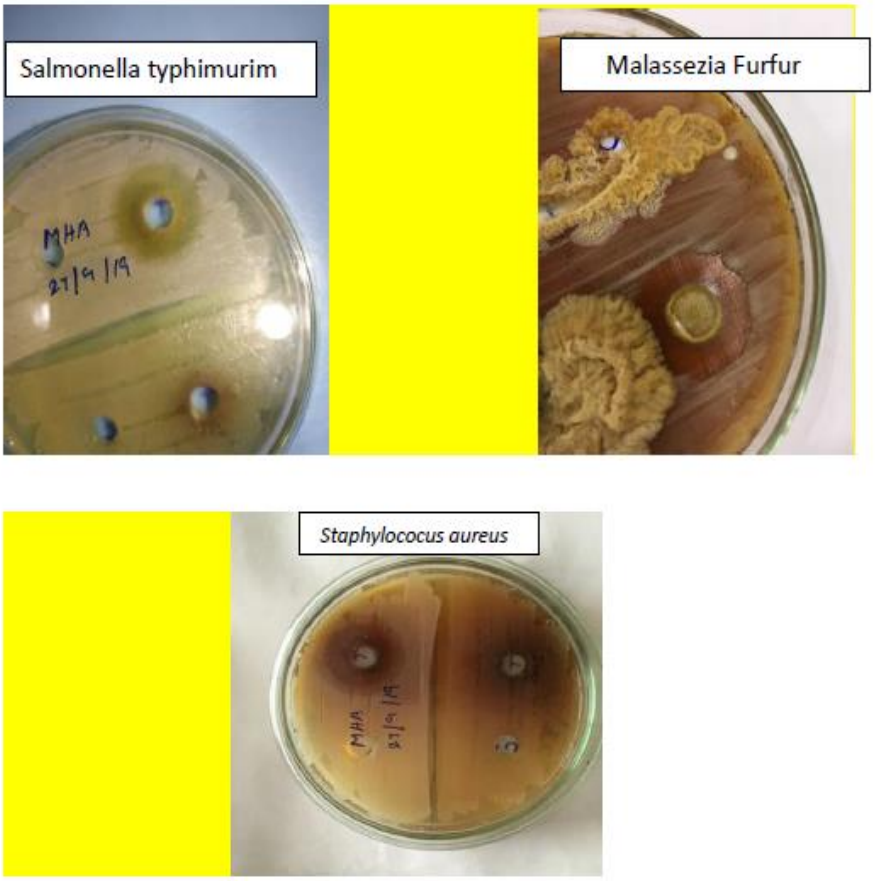

Figure 4. Antimicrobial Activity of *Terminalia chebula* Extract Against Various Pathogens

Determination of Total Anti-oxidant Activity

Table 3 and figure. 5 depicts the antioxidant values (**% Inhibition**) of TC extract showing that there was a dose related higher inhibition percentage of free radicals for both extract and standard ranging from a concentration from 50 to 500 microgram per ml. The maximum anti-oxidant activity was observed at 500 microgram per ml of both extract and standard.

Table 3. Total Antioxidant Capacity of *TC* Extract

Conc (µg/ml)	**Ascorbic acid % Inhibition**	**TC % Inhibition**
50	18.47±1.08	17.98±1.19
100	30.64±1.62	28.87±1.77
200	45.12±1.94	42.25±2.33
300	46.73±1.37	45.33±1.77
400	53.75±1.82	51.12±0.81
500	60.72±1.09	57.94±0.03
IC $_{50}$ (µg/ml)	284	280

Values are Expressed as Mean ± STD

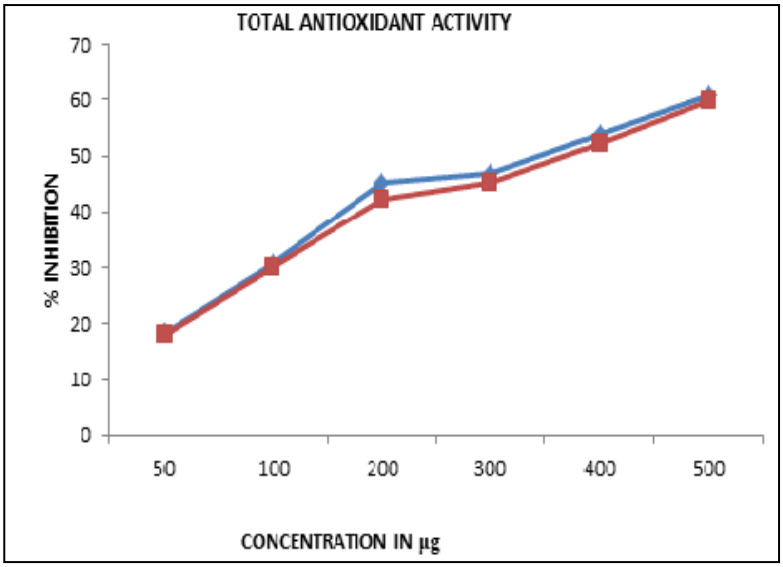

Figure 5. Determination of Total Antioxidant Capacity

Anti-inflammatory Activity

In this study, TC extract showed a dose related higher inhibition percentage of protein denaturation of BSA both extract and standard ranging from 50 to 500 microgram per ml. The maximum inhibition of protein denaturation of extract and standard drug was observed at 500 microgram per ml. TC extract at 50 µg/ml exhibited 26.86% inhibition of protein denaturation. And 1000 µg/ml concentration exhibited 76.29% inhibition on par with the standard diclofenac sodium at 200 µg/ml. This inhibition of denaturation of proteins may be attributed to the alkaloids and flavonoid compounds of the extract (Table 4).

Table 4. Protein Denaturation Study of TC Extract

S. No	Conc. (µg/ml)	Tc Extract (% protein denaturation inhibition)	Diclofenac Sodium (% protein denaturation inhibition)
1	50	26.86	
2	100	38.24	
3	200	52.66	87.61
4	300	58.24	
5	400	63.37	
6	500	69.41	
7	1000	76.29	

HRBC Membrane Stabilization Activity

50 µg/ml concentration of TC extract prevented RBC hemolysis by 21.26%. The inhibition percentage was directly proportional to concentration of TC extract. At conc of 1000 µg/ml, TC extract prevented hemolysis by 79.74% as compared to diclofenac sodium (conc 200 µg/ml) (Table 5).

Table 5. HRBC Membrane Stabilization Study of *TC* Extract

S. No	Conc. (µg/ml)	% inhibition of RBC haemolysis by TC extract (%)	% inhibition of RBC haemolysis by Diclofenac sodium
1	50	21.26	
2	100	36.16	
3	200	49.85	87.61
4	300	58.17	
5	400	63.88	
6	500	68.71	
7	1000	79.74	

Fourier Transform Infrared Spectroscopy (FTIR)

Figure 5.3 shows that a band on 605 cm^{-1} with anti-symmetric based bending motion in ν4 indicative of phosphate groups of HAP. The band at 962 cm^{-1} represents ν1 phosphate bands of HAP and Band at 1044 cm^{-1} and 1087 cm^{-1} is indicative of ν3 phosphate band of HAP (Tas et. al., 2000). The –OH vibrations of HAP are represented by a band at 3435 cm^{-1} (Figure 6).

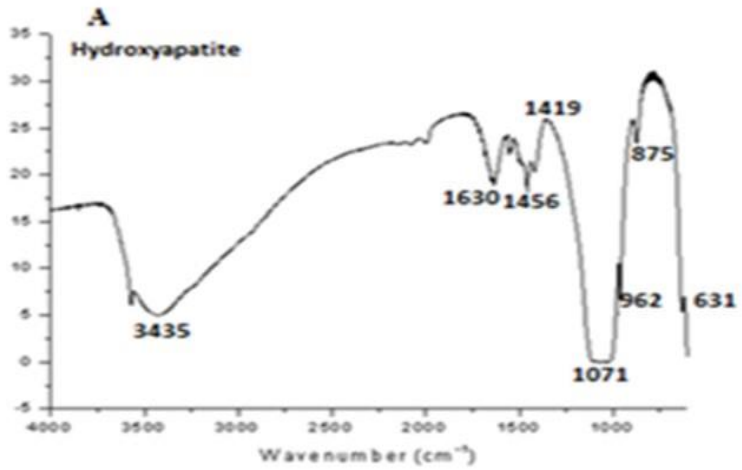

Figure 6. FTIR of HAP

Band at 574 cm-1 is indicative of anti-symmetric based bending motion of groups of phosphate showing hydroxyapatite and eggshell powder. The stretching band of the OH group were found at 3444.11 cm-1. The OH group of chitosan combined with hydroxyapatite were found at 1425.55 cm-1. The spectrum of FTIR showed the peaks of absorption at 2974.05 cm-1(O-H) and (C-H), at 1798.04 cm-1 for (C=O), at 1595.04 for (N-H) and at 1048.72 cm-1 for (C-O) (Figure 7).

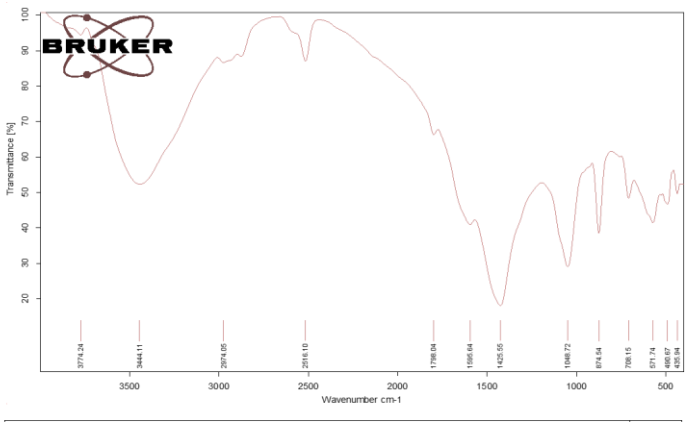

Figure 7. FTIR of ESP-HA-CH-TC Implant

X-Ray Diffraction Studies (XRD)

The absolute peak present at 19.54º; 21.26º; 26.10ºand 28.03º is indicative of the reflections from crystal planes at 111, 202, 002, and 210, respectively thereby showing the presence of HA (Figure 8).

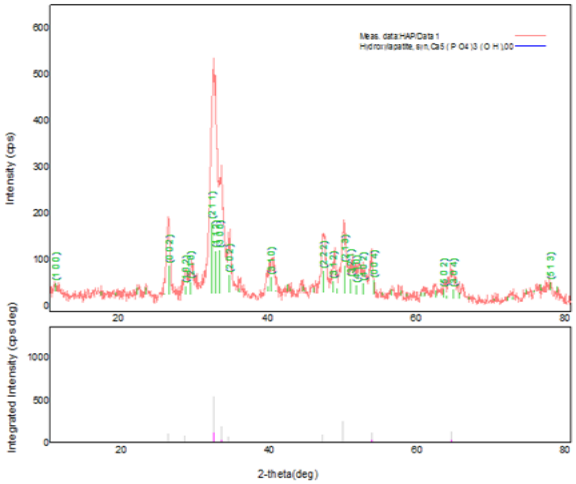

Figure 8. XRD Pattern of HA

Figure 8 and Figure 9 showed the patterns of HA and ESP-HA-CH-TC bone grafts. The 2θ values 28.507° indicate the reflections from crystal planes at 210 are indicative of HA. The absolute peaks at 30.68°, 34.92°, 38.37°, 47.56°, and 57.05° are indicative of reflections from the crystalline planes at 222,112,130 and 315 (Figure 9).

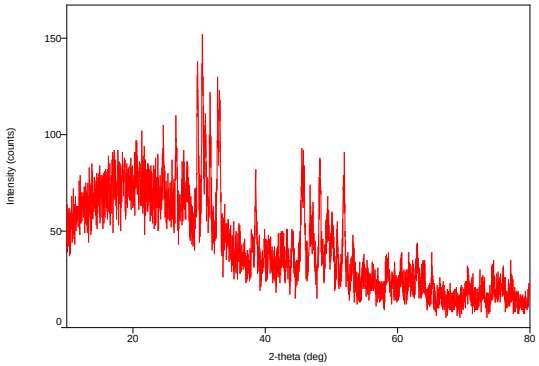

Figure 9. XRD Pattern of ESP-HA-CH-TC

Thermo Gravimetric Analysis (TGA)

TGA analysis of bone graft ESP-HA-CH-TC is depicted in Fig.10. A weight loss, being a single step was absorbed around 33.64°C and 665.59°C leaving a residual value of 9.846 mg. The weight loss in the second stage was absorbed at around 672.57°C and 7961.7°C (Figure 10).

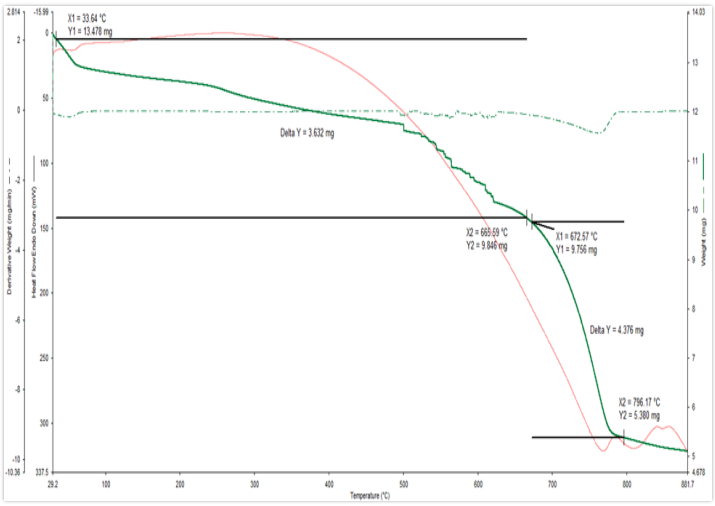

Figure 10. TGA of the Bone Graft ESP-HA-CH-TC

SEM Analysis

Figure 11 indicates the images of SEM by HAP which appear as crystals like rod-shaped and are further aligned as flower-like patterns. The fig. 5.9 shows the SEM image of the ESP-HA-CH-TC composite. Figure 12 (A) shows porosity of the graft and depositions of calcium phosphate crystals of Eggshell and hydroxyapatite on the surface. Figure 12 (B) shows the presence of chitosan in the graft.

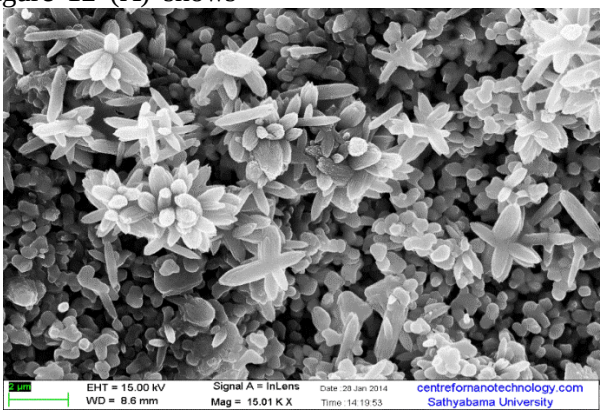

Figure 11. The SEM Image of HA

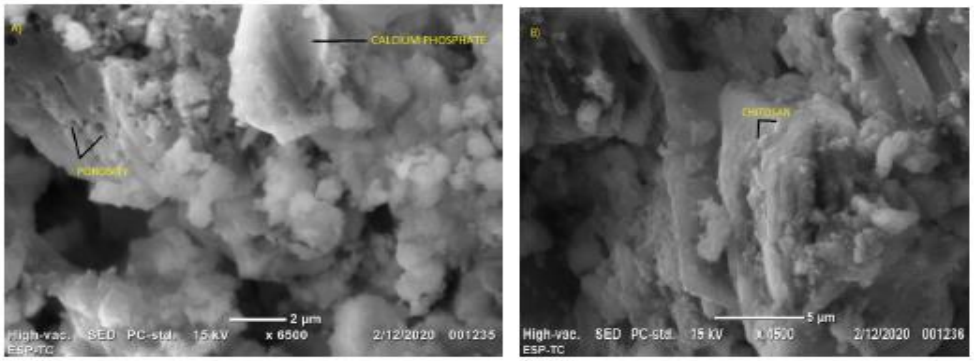

Figure 12. (A) and (B) Shows the SEM Image of the Bone Graft.

Invitro Viability Assay – MTT Assay

Figure 13 shows the MTT assay of TC extract in MCF-7 cell lines. The dose-response graph of the assay is depicted with an increase in concentrations from 0 to 500µg/ml. The IC 50 concentration of TC extract was at 78 µg

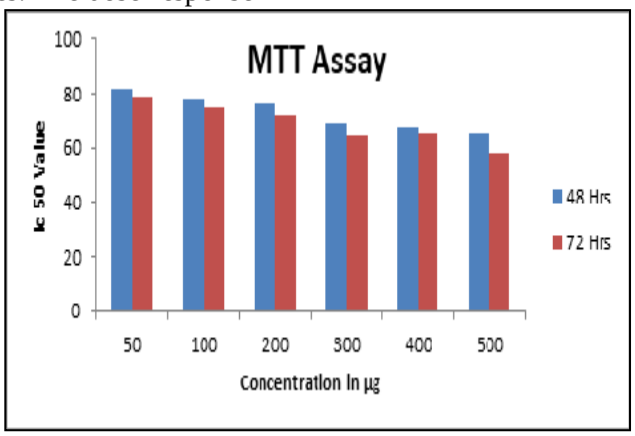

Figure 13. *Invitro* Viability Assay

Discussion

The GC-MS analysis revealed that TC extract contains tannins, saponins, terpenoids and phenols which impart antimicrobial, anticancer and antioxidant properties to the extract. Also, Vitamin E in TC extract offers antioxidant properties to the extract along with phenols. Propanoic acid compounds in TC extract afford an anti-inflammatory property to the graft. Fruit extracts of TC were assessed for antimicrobial activity against some fungi and a few gram-positive and negative bacteria. TC extract was actively resistant to human pathogens namely *Salmonella typhimurium* and *Malassezia furfur* and among all these extracts was highly resistant towards *Staphylococcus aureus*. The antimicrobial property supports the graft in preventing any infection and facilitates the bone repair when placed *in vivo*.

Reports suggest that antioxidants obtained from plant origins such as acids of phenols, tannins, flavonoid components, lignans, proanthocyanins, anthocyanins, quinones, coumarins, etc., owing to their redox properties, can postpone or prevent the occurrence of degenerative diseases. These redox properties present in the bioactive compounds act as donors of hydrogen, effective reducing agents, and scavengers of free radicals generated by oxidative stress [14]. The TC extract possesses flavonoids, anthraquinone, terpenoids, carotenoids and saponins. High concentrations of polyphenols and emodins in TC extract could be the possible reason for exhibiting the anti-oxidant property.

The denaturation of proteins led to the production of auto antigen in many auto-immune diseases [15]. Due to the presence of autoantigens, the electrostatic bondings, hydrogen bondings and disulphide bonds were much affected and the secondary and tertiary structures of the proteins were lost thus resulting in denaturation. The phytochemicals that control the production of autoantigens prevent the denaturation of protein.

TC at concentration 50-1000 µg/ml exhibited an increase in inhibition in a dose dependent manner, depicting HRBC membrane stabilizing potential of Tc extract. When TC extract was made to incorporate in the prepared composite, it was able to show an anti-inflammatory activity at the implant site apart from osteogenic properties when placed *in vivo*.

The free-radical scavenging property of plant flavonoids can be due to the high mobility of the electrons present in the benzenoid nucleus and this property enhances the wound-healing process in rat models.

These wound-healing properties of the skin can be easily utilized for skin-based disorders and ailments because the reduction potential of free radicals effectively prevents the damaging effects on the cutaneous cell structure and their functions. *Terminalia chebula* belonging to the family of Combretaceae possesses various therapeutic applications owing to the presence of various phytochemicals in various parts of the Terminalia plant. The fruits of this plant is reported to possess the effective phytoconstituents that are responsible for its antimicrobial and antioxidant effects [16-18].

Leaves and fruits of this medicinal plant have specifically contributed to wound healing management with a decreasing rate of epithelialization and an improved rate of contraction during skin healing. Incorporation of Hydroxyapatite (HAP) in herbal formulations develops good biocompatibility and osteoinductivity in a graft material and seems much employed in various fields of biomedical applications like orthodontic dentistry, the field of orthopaedics, biomedical and bone tissue engineering and other several medical applications.

Hydroxyapatite is one of the common minerals highly found in tissues of the bone [19]. Owing to its osteoinductive effects, this mineral is widely employed in bone repair and mostly considered as a bone substitute. Wide research has been carried our using this mineral and researchers are still exploring the properties of HAP and its usage in various field of biomedicine. The fabricated a simulated body fluid using hydroxyappatitie and it was considered as a new methological procedure of synthesis [20]. The prepared HAP was found to possess many similarities with the natural human bone structure and its minute mineral composition when compared with other methods of preparation of HAP synthesis. Similar studies discussed about the different methods of preparing HAP and further characterization of HAP derived from many other fabricating procedures and self-explained the properties of HAP [21]. Another preparation synthesized a porous material block using HAP and naphthalene, pore creating agent. These prepared blocks were then characterized by various physico-chemical techniques and discussed the improvement of bone integrity of HA when placed as a graft *in vivo*.

In the present study, fabricated a novel graft composite for bone using eggshell powder (ESP), Hydroxyapatite (HA), Chitosan (CH) and Aqueous extract of fruits of *Terminalia chebula* (TC). This preparation has been an extension of the studies by many researchers who worked in bone graft fabrication. Suprabha et. al. (2009) [22] synthesized novel nano HAP by a different procedure incorporating biomolecules from waste products used in our day today life orange and potato peel, papaya leaf, calendula flower extract, and eggshell. In the study, *insitu*-controlled synthesis of nano-sized HAP was further performed for its use in future preparatory material for the synthesis of new bone grafts and to improve the bone-bonding capacity in the bone graft. Another method by Mojtaba et. al. (2011) [23] prepared a moderately porous scaffold incorporating a nano HAP with nylon 6,6 by salt leaching technique. In this method, HAP well dispersed onto the pore walls of the scaffold bonds and also penetrated the nylon 6, 6 and gave stiffness to the scaffold tissue. Thus, the porous scaffold would be effective as a three-dimensional substrate for bone tissue engineering.

The fabricated a scaffold with macroporous biphasic calcium phosphate and poly methylmethacrylate resin using a method of dual-phase mixing procedure [24]. The prepared scaffold was well compared to other commercially available BCP scaffolds and showed macroporous structure that makes it to facilitate its applications in tissue engineering. Krithiga et al. (2011) [25] fabricated a biocomposite material using biphasic calcium

phosphate that was cross-linked with chitosan, gelatin and extracts of *Terminalia chebula*. The prepared material was further treated with SBF and then physio characterization techniques and proved for its ossification property. A scaffold including bi-phasic calcium phosphate and agarose gel [26]. The material was analyzed for compression behaviour of the scaffold material. The results revealed that Agarose effectively improved the property of biphasic calcium phosphate and imparted elasticity, ductility and toughness to the scaffold material and hence can be implicated in the tissue engineering process.

Another *in vivo* study by Moimas et al. (2006) [27] involved a preparation of bioglass scaffold and used it commercially for perioglas® and also placed *in vivo* by making cortical holes of the bones of the tibia of rabbits and maintained for 6 months. This prepared scaffold was compared with the commercial bioglass that induced new bone formation in the implant site. These results confirmed better findings confirmed with histological studies and tomographic analysis Zhou et al. (2007) [28] fabricated a composite material possessing poly-L-lactide (PLLA) and a bioactive glass using the technique of solvent evaporation. This composite was soaked in SBF for duration of 3 days and allowed for the HAP deposition to occur on the composite. The dried composite was further subjected to various characterization techniques and revealed that bioactivity of the composite was significantly increased which further supported the composite and promoted bone integration when placed *in vivo*.

It has been prepared a composite material with the inclusion of chitosan, HAP and Bioglass. This inclusion of Chitosan in Chitosan -HAP composite was prepared by situ method involving calcium nitrate and ortho phosphoric acid in SBF. Further to the prepared composite bioactive glass was added and tested for in vitro bioactivity and results revealed that the inclusion of chitosan improved the compression strength of the material. Misra et al. (2009) [29] fabricated a bioglass composite film consisting of poly (3-hydroxy butyrate) and vitamin E. The incorporation of vitamin E to enhance the protein adsorption and hydrophilicity on the surface of the biofilm. This composite biofilm was further characterized by physicochemical characterization and can be applied in tissue engineering as a better matrix material for cell adhesion. A bone cement called COOL with inclusions of powder and a liquid phase [30]. The powder phase was made by mixing bioresorbable glass ceramic GB14, TTCP, fluoroapatite, Calcium zirconium phosphate and zinc oxide. In the liquid phase, it contained PMMA dissolved in a mixture of ethanol and ethyl acetoacetate (1:1). The developed COOL bone cement was further checked for its biocompatibility and ALP activity by invitro cell analysis and then compared with commercial bone cement Rifobacin®R. The results revealed that COOL material of bone cement exhibited high biocompatibility and ALP activity compared to the commercial bone cement.

Development of a bone cement using barium sulphate and zirconia as added additives to bone cement to enhance the visualization through X-ray imaging [31]. These incorporation of the additives to the bone cement was helpful to locate the material when placed in the bone defect areas *in vivo* by X-ray imaging technique. It has been reported the rate of degradation of pure collagen and collagen –HAP beads with a use of collagenase enzyme. This enzyme was further able to digest pure collagen compared to the Collagen-HAP gel beads [32]. HAP further provided resistance for quick degradation for the material and thereby provided a longer period and supports the cells to adhere, proliferate and then further differentiate. Chitosan microspheres of size 250-500μm containing high molecular weight chitosan which was able to deliver

phenobarbitone to the target site slowly. It took 3 h to deliver the drug which helps in the slow release of the drug into the target site thereby the activity of the drug was enhanced compared to microporous chitosan molecules with low molecular weight. The high molecular weight chitosan has low solubility and hence forms a viscous layer around the drug and releases it slowly the function of chitosan in drug release by developing a PLLA microspheres by a coating of chitosan for carrying the drug lidocaine [33]. Chitosan with different molecular weights were made to coat the PLLA microspheres. About 50% of licodaine were released in the first hour and 19.2% of licodaine was released by 25th hour by the uncoated PLLA microspheres and CS coated microspheres was released for 14.6% of licodaine at 90th hour. Thus, CS coating helped in slow release of the drug at the target site. De Campos et al. (2001) [34] investigated the effective role of CS in delivering the provided drug at the ocular mucosa. Cyclosporine A was chosen drug that was carried by chitosan and was observed through *in vitro* studies. The results revealed that initial burst occurred at immediately and later followed by gradual slow release of the drug by 24 hours and at the same, it was further confirmed by *in vivo* studies in rabbit animal model. The Cyclosporin A which was loaded with nano CS was topically added in rabbits and they found that a good therapeutic concentration was effectively achieved in external ocular tissues within a period of 48 hours, but negligible concentrations were observed in internal ocular tissues, blood and plasma etc. Fariza et al. (2010) [35] also prepared a artificial bone material with the inclusions of TCP and egg yolk by protein consolidation method. The prepared slurry was then casted into cylindrical implants and further analyzed for its properties by various techniques. The results confirmed the porosity and compression strength of the macroporous material and explains its use in repairing bone defects. In the present study, FTIR results exhibited peaks belonging to HAP and the bone graft ESP-HA-CH-TC confirms the presence of individual components of the graft by the presence of respective peaks for hydroxyl group, amide I and II, Hydrogen bond N-H, and C-H bonding corresponding to HA, ESP, and Chitosan.

The X-ray diffraction studies (XRD)studies revealed that the 2θ values of XRD confirmed the findings of FTIR and depicts the chemical composition of the graft. The 2θ values for HAP, calcium phosphates of ESP and Chitosan were seen in the results and hence it confirmed the composition of the bone graft. This result supports the FTIR and TGA analysis for its inorganic content.

In the Thermo Gravimetric Analysis (TGA), it revealed that the bone graft material was subjected to varied temperatures at which different stages of weight loss occurs. The weight loss in initial stage could be due to loss of water in bone graft. The later weight loss could be mainly attributed to decomposition of chitosan. Residual weight of the bone graft sample could be due to the presence of inorganic contents namely calcium and phosphorous that imparts thermal stability to the prepared bone graft

The SEM images of the bone graft confirms the findings of XRD and FTIR images. The surface morphology of the bonegraft material gives a clear picture on the porosity of the graft that facilitates the diffusion of the TC extracts from the graft into the fracture site. Moreover, the calcium phosphate crystals of ESP and HA affords osteoinduction and osteoconduction through the pores in highly rapid manner. The morphology also shows the impregnation of ESP and HA into chitosan in the matrix for TC and thus helps in repairing the bone defects in a sustained manner. MTT is an important method to evaluate the cell viability and an index for screening therapeutic efficacy of anti-proliferative agents [36]. Fruit extract of TC studies for its anti-

proliferative effect against MCF-7 cell lines, showed that the extract was much effective in a time dependent and dose dependent way and proves its cytotoxicity effect.

There are few reports that promote substantial support to the pharmacological benefits of *Terminalia chebula*. Because *Terminalia chebula* contains several pharmacologically active compounds that exert multiple mechanisms, such as immunomodulatory, cytoprotective, anti-inflammatory, anti-proliferative, and antioxidant and free radical scavenging, treatment with the plant significantly reduced pain and inflammation in dogs while also improving joint cartilage and daily activity. The use of extracts from *Terminalia chebula* may be very beneficial in treating osteoarthritis. [37]

Conclusion

The present study showed that the novel Bone Graft (ESP-HA-CH-TC) exhibited good biocompatibility and promotes bone growth compared to that of a commercial graft preparation. The various characterization techniques namely FTIR, XRD, TGA and SEM analysis performed revealed the chemical composition of the graft, stability and porosity of the composite material. The bioactive compounds of *Terminalia chebula* revealed by GC-MS analysis affords various pharmacological properties to the graft such as ossification ability, anti-microbial, anti-oxidant and anti-inflammatory properties could be to the existence of phytochemicals like flavonoids, alkaloids, triterpenoids, glycosides, phenols, steroids and saponins. This novel combination of bone graft possesses osteoinductive and osteoconductive properties with no side effects and thereby, the (ESP-HA-CH-TC) bone graft can be therapeutically as a remedy for healing fractures, dental implants and other biomedical preparations. The prepared Bone graft (ESP-HA-CH-TC) is economically cheaper than commercial hydroxyapatite and other composite material and hence can be widely used in repairing bone defects after performing animal and human trials.

Conflict of interest

The author hereby declares that there is no conflict of interest.

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