Development of Biopolymeric Scaffolds for Bone Regeneration

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Abstract **- Biomaterial scaffolds are used in bone tissue engineering to provide support repair and regeneration of bone defects. To develop a biopolymeric scaffolds for the treatment of bone regeneration using cellulose from paddy straw. To synthesize bio scaffold, cellulose was extracted from paddy straw. There are two bio scaffolds were prepared using cellulose material and PVA. The scaffold was analysed for their swelling ratio, hemocompatibility, cytotoxicity and antimicrobial analysis were carried out to analyze their efficiency in vitro. Our results showed that, the biosynthesized 1 % and 10 % PS scaffolds were able to swell and found to be nontoxic to Vero cells. The IC50 value of both 1 % and 10 % PS scaffolds were found to be 377.7 µg/ml and 436.2 µg/ml respectively. The hemocompatibility results also confirmed that the PS scaffolds were hemocompatible upto the concentration of 1000 µg/ml. The antimicrobial results of both 1 % and 10 % PS scaffolds were shown to inhibit the S. pyogenes (17 mm), Aspergillus niger (8 mm and 16 mm) microbial growth at the concentration of 500 µg/ml. Taken together, these results confirm that, PS bioscaffolds were found to be highly hemo and cytocompatible and can be used for the treatment of bone regeneration.**

Index Terms **- Tissue engineering, PVA, scaffold, bone regeneration, biopolymers, cellulose.**

I.INTRODUCTION

Bone tissue engineering is a promising therapeutic strategy for bone repair and regeneration. It aims to reconstitute bone tissue with the help of tissue engineering components like cells, growth-stimulating signals and a scaffold [1]. Tissue scaffold provides the microenvironment for cell attachment, proliferation, differentiation, and new tissue formation and it should be porous to allow efficient mass transport of nutrients and oxygen, biocompatible and biodegradable. [2–4] The scaffold should also mimic the composition, structure, morphology, and bioactivity of bone extracellular matrix (ECM) to modulate cell behavior [5,6].

In addition, tissue engineering scaffolds have biomimetic structure and exhibit biological functions that are similar to those of native extracellular matrix (ECM) [7,8]. Artificial ECM provides cells with the mechanical support needed, a broad range of chemical signals that control cell functions [7], and a suitable environment for cell attachment because of physical dimensions similar to those of natural ECM [9–11]. Producing a growth factor-releasing electro spun scaffold remains a challenge, as the growth factor could lose its bioactivity during the harsh process of scaffold fabrication [12].

According to an estimate, around 6 tons of paddy straw are left in the field after harvest of 4 tons of rice by mechanical harvesters [13]. This huge quantity of paddy straw is not being used economically now a days [14]. Because it is a poor feed for the cattle and resists the decomposition when retained in the fields, due to high silica (9-14%) and ash (22%) content [15]. High cost of collection, lack of economically viable options to utilize the straw and a short gap between harvest of rice and sowing of wheat made the farmers to burn it in the fields.

Porous scaffolds fabricated through a various methods including a range of biomaterials, that have been utilized to aid and direct bone regeneration [16-18]. During scaffold manufacture it would therefore seem to be logical and it includes a combination of materials to create a composite scaffold, potentially allowing greater scaffold bioactivity and structural biomimicry have to be achieved.

In general terms, the ideal scaffold is composed of a biocompatible, biodegradable material with similar mechanical properties to the wounded tissues which it is to be implanted in. Scaffolds by design are not intended to be permanent implants and will ideally

facilitates the host cells to deposit extracellular matrix (ECM) and replace the bio-scaffold structures over time. The architecture of the scaffold should be highly porous with an interconnected structure to allow cell proliferation and nutrient migration. The scaffold surface should also be modulated to facilitate cell attachment, proliferation and differentiation [19-22]. Scaffold materials to be nontoxic to tissue cells, easily eliminated from the body and to elicit negligible immune response through their presence.

Moreover, the microarchitecture of bio scaffolds is also centrally important in encouraging cell viability level and fostering tissue in growth. Therefore, the present study aimed to develop and characterize biopolymeric scaffold for bone regeneration.

II. MATERIALS REQUIRED

A. Chemical and reagents

NaCl, KCl, KH2PO4, Na2HPO4, Peptone, NaCl, Yeast extract, Beef extract, Dextrose, agar powder were purchased from Sigma, USA. Sodium citrate, SDS, Nacl was purchased from SRL (India), 1X PBS was from Himedia, (India). 96 well plates were from Tarson (India).

B. Cell culture

Vero (African green monkey kidney cells) cell line was purchased from NCCS, Pune and were cultured in liquid medium (DMEM) supplemented 10 % Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin and maintained under an atmosphere of 5 % CO_2 at 37°C.

C. Preparation of paddy straw-cellulose based bio scaffold

PS bio scaffolds were fabricated by solvent casting/salt leaching technique. In this method, 10 % (w/v) of PVA and PS cellulose (1 % and 10 %) and gentamicin and amphotericin B were carefully dissolved in de-ionized water. The solutions were mixed using a magnetic stirrer for 1 to 2 hours. Proper amounts of this mixture were poured in Petri dishes and air dried. Finally, the mechanically stable bio scaffolds obtained for further experiments.

D. Swelling ratio

In order to measure the swelling degree of biofilm membranes, membrane samples were cut into 2 cm pieces and dried at 50 °C in a vacuum oven for 6 h, the weight of the dried sample was determined (We). The dried samples were soaked in distilled water, maintained and incubated at 37 °C, then weighted (Ws) at specific interval times. The water uptake of biofilm membrane was determined using the following.

Water uptake or swelling ratio (SR) %

[(Ws-We)/Ws] x 100

Small pieces of each film were taken with distilled water in eppendorf tubes. The weight was measured for every 5 min, 10 min, 15 mins.

E. Hemocompatibility Assay

About 5 ml of peripheral blood was collected from the healthy donor in a 3.8 % sodium citrate treated tubes in accordance with institutional guidelines. Then blood was washed with, 10 ml of 0.9 % NaCl solution for 3 times by centrifugation at 1500 rpm for 5 min. The supernatant was replaced with fresh 0.9% NaCl. After centrifugation, the plasma and white Buffy layer was removed using sterile pipette. Finally washed the RBCs with 10 ml of 1x PBS by centrifuging at 1500 rpm at 5minutes. The supernatant was discarded and suspended the erythrocyte in 10 ml of 1x PBS and gently mixed. A 100 µL of 10 % SDS was added as a positive control. The plate was incubated for 1hr at room temperature. Finally, 100 µL of clear supernatant was collected and measured the OD at 540 nm using microplate plate reader (Thermofisher Scientific, USA).

F. MTT Assay

The PS scaffolds (1% and 10 %) were tested for *in vitro* cytotoxicity, using Vero cells by 3- (4,5-dimethylthiazol - 2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured Vero cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1 % antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the PS scaffolds (10 %) and (1 %) sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5 % CO2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for

another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using Graph Pad Prism 6.0 software (USA).

G. Antibacterial activity

The antimicrobial activity was elucidated using agar well diffusion method. Petri plates containing 20 ml nutrient agar medium were seeded with 24 hr culture of bacterial strains (*Streptococcus pyogenes*- MTCC 1928).Totally six wells were cut and different concentration of cc (500 µg/ml, 250 µg/ml, 100 µg/ml and 50 µg/ml) was added along with positive and negative controls. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control.

H. Antifungal activity

The antifungal activity of both 1 % and 10 % SH scaffolds were studied using agar well diffusion method. Petri plates containing 20 ml nutrient agar medium were seeded with 72 hr culture of fungal strains (*Aspergillus niger*). Wells were cut and different concentration of sample PS scaffold (1% and 10%) (500 µg/ml, 250 µg/ml, 100 µg/ml, and 50 µg/ml) was added. The plates were then incubated at 37°C for 72 hours. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B antimycotic solution was used as a positive control.

I. Statistical Analysis

Statistical analysis of the data was performed using using Graph Pad Prism software version 6.00 for Windows Graph Pad Prism software, San Diego, CA. The differences in estimated parameters between the groups was analysed using one-way ANOVA with Bonferroni's test. Data were expressed as mean ±SD. All parameters were analysis at 95% confidence intervals and p values of <0.05 were considered to stastically significance.

III. RESULTS

A. Synthesis of PS scaffold

PS Bioscaffolds were successfully produced through a cellulose extraction method followed by an addition of polyvinylalcohol (PVA) to procure porous hydrogels with different concentrations as 10 % and 1 % PS scaffolds.

Fig 1. Powdered cellulose from paddy straw

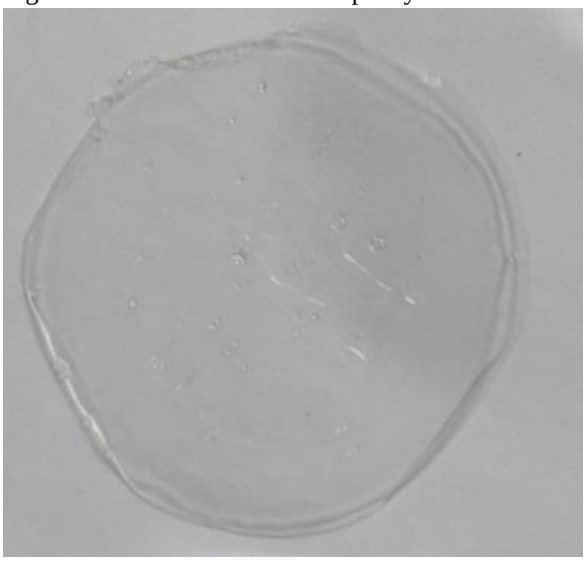


Fig 2. Biosynthesized 1% cellulose base PS bioscaffold

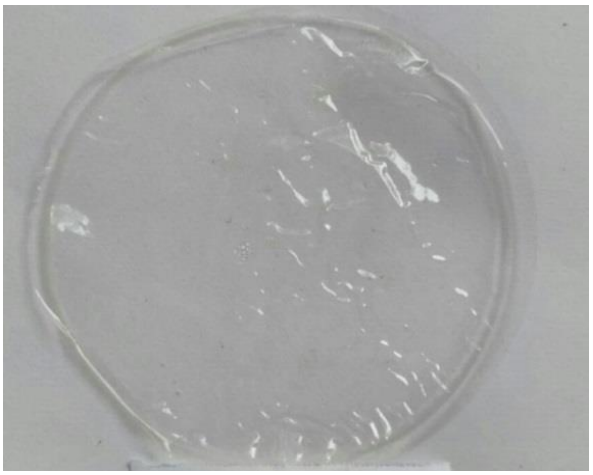


Fig 3.Biosynthesized 10% cellulose base PS bioscaffold

B. Hemolysis

The hemocompatibity of SH scaffold were studied using hemolytic assay. Biomaterials with <2% of hemolysis are classified as hemocompatible. As shown in Figure both 1 % and 10 % PS bioscaffolds were found to be hemocompatible upto the concentration of 900 µg/ml and 1000 µg/ml respectively. The results generated in this study showed that cellulose scaffold can be classified as hemocompatible in nature

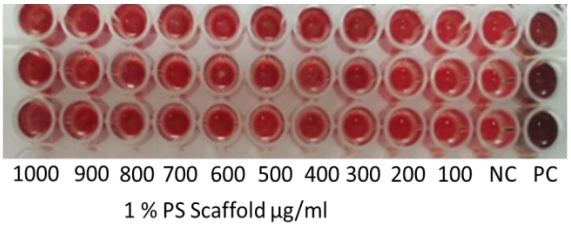


Fig 3. Hemocompatability analysis of 1 % PS scaffolds in human RBC cells at various concentrations 100 µg/ml to 1000 µg/ml along with negative (1X PBS) and positive control (10% SDS).

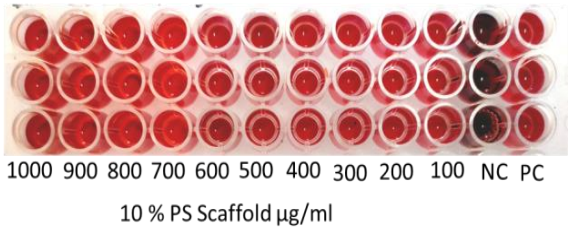
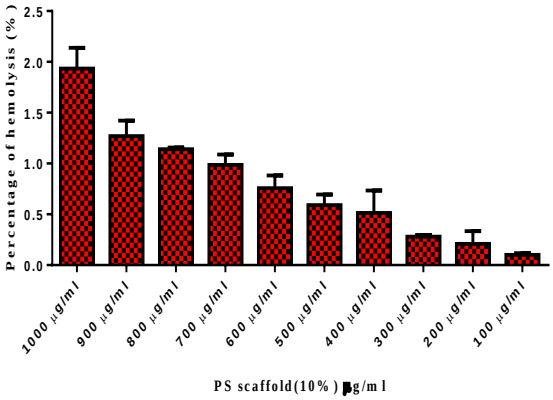
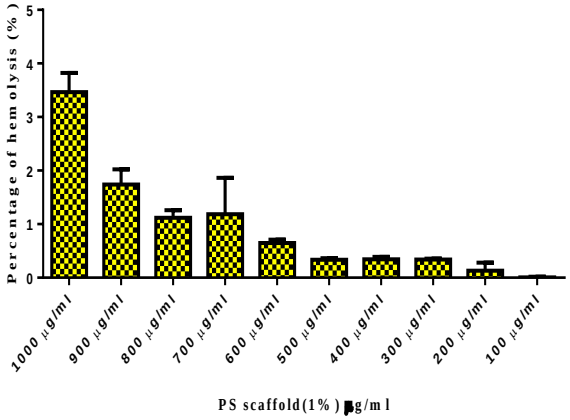


Fig 5. Hemocompatability analysis of 10 % PS scaffolds in human RBC cells at various concentrations 100 µg/ml to 1000 µg/ml along with negative (1X PBS) and positive control (10% SDS).



Graph 1. Hemocompatibility effect of 10 % PS scaffold at various concentration using human RBC cells.

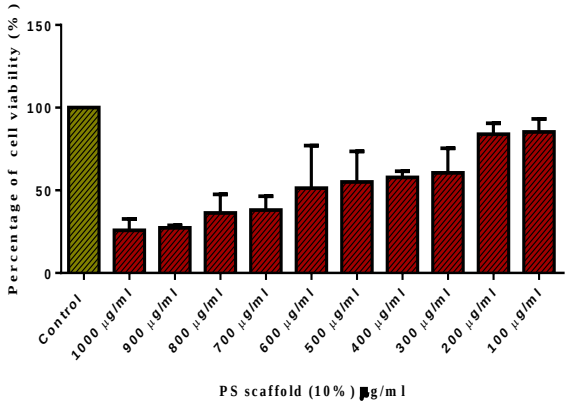


Graph 2. Hemocompatibility effect of 1 % PS scaffold at various concentration using human RBC cells

C. MTT assay

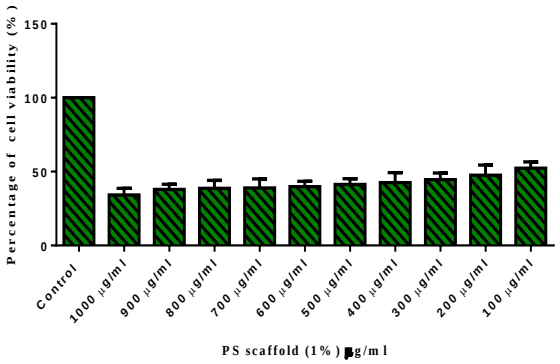
MTT assay was performed to find out the cytotoxicity of PS scaffolds at various concentrations using Vero cells. The positive control was given with 100 % cell viability. The study showed that the number of live cells were significantly greater than the positive control was shown in Fig. Among the several concentrations of PS scaffolds evaluated, the results showed that, the scaffolds found to be nontoxic to the Vero cells was shown in Graph 3 and 4. The IC50 Value of (10 %) PS scaffolds was 436.2 µg/ml. The IC50 Value of (1 %) PS scaffold was 377.7 µg/ml were shown in table 1. Therefore, the PS scaffold 10 % and 1 % were not toxic to Vero cells.

S. No	Name of the test sample	Initial weight	After 5mins	After 10mins	After 15mins
1.	PS scaffold (10%)	0	70.5	64.2	64.2
2.	PS scaffold (1%)	0	96.12	80.7	76.1
3.	Control	0	83.3	83.3	96.15



Graph 3. MTT results showed the cell viability percentage upon treatment with 10 % SPSscaffolds from 1000 µg/ml to 100 µg/ml in Vero cells.

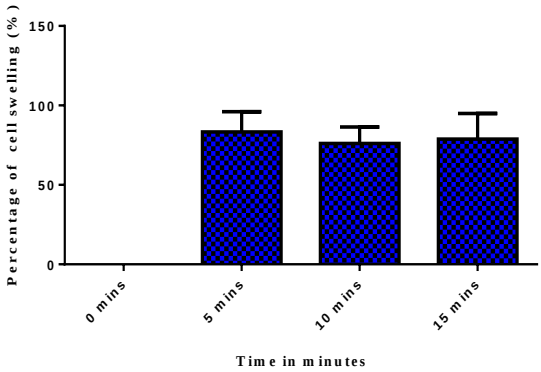
Table 1. Cytotoxicity analysis of both 10 % and 1 % PS scaffolds using MTT assay and their IC50 values.



Graph 4. MTT results showed the cell viability percentage upon treatment with 1 % PS scaffolds from 1000 µg/ml to 100 µg/ml in Vero cells.

D. Swelling ratio

An appropriate gelation time plays an important role in practical applications. This current study shows, the natural derived cellulose-based PS scaffolds have high swelling ratio up to the time point of 15 mins. As shown in table 2 the maximum swelling effect was observed at 15 min in both 1 % and 10 % PS bioscaffolds to the value 96.12 % and 70.5 % respectively.



Graph 5. Swelling effect percentage of 1 % and 10 % PS scaffolds.

S. No	Name of the test Sample	Name of the test Micro organism	Zone of inhibition(mm)				
			500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	AB
1.	PS scaffold (10%)	*Streptococcus pyogenes*	17±1.0	15.6±0.6	10.3±0.3	9.2±0.2	13±1.0
2.	PS scaffold (1%)		17±2.0	15.7±0.7	10.5±0.5	0	16±2.0

Table 2. Percentage of swelling effect by 1 % and 10 % PS caffolds at various time periods.

E. Antibacterial activity

The antibacterial activity of PS scaffolds was tested against *Streptococcus pyogenes* using agar well diffusion method. Both PS scaffolds 10 % and 1 % showed anti-bacterial activity against the *Streptococcus pyogenes* (17 mm) and the maximum zone of inhibition was observed at 500 µg/ml. These results confirm that both 1 % and 10 % bioscaffolds were able to inhibit the bacterial growth maximum at 500 µg/ml was shown in table 3.

S.No	Cell lines	Name of the sample	IC_{50} Value
1.	Vero cells	PS scaffold (10%)	436.2
2.	Vero cells	PS scaffold (1%)	377.7

Table 3. SD ± mean zone of inhibition obtained by sample PS scaffold (10% and 1%) against *Streptococcus pyogenes*.

F. Antifungal activity

Agar well diffusion assay was employed to study the antifungal activity of PS scaffolds was tested against *Aspergillus niger*. PS scaffolds (10 %) showed anti-

fungal activity against the *Aspergillus niger* the maximum zone of inhibition was observed in the concentration of 8 mm at 500 µg/ml. Like-wise, PS scaffolds (1 %) showed anti-fungal activity against the *Aspergillus niger* the maximum zone of inhibition was observed 16 mm at 500 µg/ml was shown in table 4.

Table 4. SD ± mean zone of inhibition obtained by sample PS scaffold (1% and 10%) against *Aspergillus niger*

S. No	Name of the test Sample	Name of the test Micro organism	Zone of inhibition(mm)				
			500 µg/ml	250µg/ml	100µg/ml	50 µg/ml	AB
1.	PS scaffold (10%)	*Aspergillus niger*	8± 1.0	6.6 ±0.6	5.5 ±0.5	4.3 ± 0.3	13± 1.0
2.	PS scaffold (1%)		16 ±1.0	11 ±1.0	9.9 ±0.9	7.5 ± 0.5	16± 1.0

IV. DISCUSSION

Scaffolds are structurally similar to the natural extracellular matrix. The synthetically derived biodegradable polymers can be moulded easily into the desired shapes with proper mechanical strength and they have evaluated the formation of in vivo cartilaginous tissue using rabbit articular chondrocytes seeded in fibrin/PLGA hybrid scaffold [23]. Previous study on bioscaffolds shows naturally derived materials are promising biomedical materials due to their advantages such as similarities to the extracellular matrix, good biocompatibility and inherent cellular interaction. As cellulose fibers resemble the collagen fibers of bone tissue, cellulose has been implicated in bone tissue engineering applications [24].

Therefore, the present study focus on the biosynthesis and characterization of cellulose based bioscaffold for the treatment of bone regeneration. Similar research work regarding to the injectable polysaccharide hybrid hydrogels as scaffolds for burn wound healing [25]. They have assessed the gelation time of hydrogels, a 3 wt % CMC aqueous solution was mixed with a 3% Odex aqueous solution at various volume ratios (1:2, 1:1, and 2:1. The study shows that, the reduction in the gelation time with the increase in the Odex content was likely due to the increase in the crosslinks in the composite hydrogels. The hybrid hydrogel with the CMC/Odex ratio of 2.0 displayed a gelation time (~ 50 s) suitable for burn wound treatments, which may ensure the close contact between the hydrogels and the bone sites. It is noteworthy that an appropriate gelation time plays an important role in practical applications. In the current study, the natural derived cellulose-based PS bioscaffolds have high swelling effect of 96.12 % and 70.5 % at 5 mins of 10% and 1% PS scaffolds respectively.

The previous study on the hemolytic activity of the test against erythrocytes of blood showed the ability of scaffold to cause hemolysis in the blood cells through lysis or cellular eruptions. It was observed that the scaffold presented low hemolytic action on the development of composite scaffolds based on cerium doped-hydroxyapatite and natural gums [26]. Interestingly, our current study shows that the PS scaffold (10 %) was found to be hemo-compatible at 1000 µg/ml and PS scaffold (1 %) was found to be hemo-compatible at 900 µg/ml and <2 % of hemolysis is acceptable universally. Our results showed that the sample 10% PS scaffold is highly hemocompatible.

MTT assay was performed to observe the cytotoxicity of scaffold at serial concentrations against CTM (CC50) and the study showed that among the several concentrations of scaffolds none of them presented toxic characteristics and the cellular viability of the biomaterial is highly large. In the present study, bioscaffolds, results on MTT assay shows that the both PS scaffolds were not toxic to the Vero cells. The IC50 Value of 10 % and 1 % PS scaffold was observed at 436.2 µg/ml and 377.7 µg/ml respectively. These results confirm that both PS scaffolds were cytocompatible in nature and can be used for the regeneration of bone tissues.

The antimicrobial activity of PS scaffolds was studied using agar well diffusion method. The PS scaffolds (10 % and %) showed anti-bacterial activity against the *Streptococcus pyogenes* (17 mm) and the maximum zone of inhibition was observed at 500 µg/ml. Here, our study reveals that our bioscaffoids are highly toxic to bacterial cells without affecting any damage to Vero cells. Both PS scaffolds (1 % and 10 %) showed the anti-fungal activity against *Aspergillus niger* (16 mm)

the maximum zone of inhibition was observed at 500 µg/ml. Eventually, these results showed that paddy based cellulose bioscaffold could be a phenomenal biomaterial for the treatment of bone regeneration.

V. CONCLUSION

Scaffold helps in earlier bone regeneration via contacting with damaged tissues. The acquired scaffolds play a vital role in wound healing, which makes it a promising material for the treatment of bone regeneration. Furthermore, technically invitro and invivo assays will be carried out to ensure the functional effect of PS bioscaffolds on bone regeneration.

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