Original Article

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Dentistry Section

Comparative Evaluation of Titanium Platelet Rich Fibrin Infused with *Triphala indica* and Mangosteen Gels for Osteogenic Cell Viability and Gene Expression on Osteosarcoma Cells (MG-63): An Ex-vivo Study

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ABSTRACT

Introduction: Titanium-platelet Rich Fibrin (T-PRF) is a second-generation Platelet Concentrate (PC) that had a thicker fibrin meshwork, better cellular entrapment and greater with-hold of growth factors. Incorporation of herbal extracts in the form of gels into T-PRF is a novel protocol. To the authors knowledge no study was performed incorporating *Triphala indica* (Ti) gel and Mangosteen Rind (MS) extract into T-PRF and checked for its effect on cell lines so that it can be claimed as Sustained Drug Delivery Systems (SDDS).

Aim: To evaluate the osteogenic cell viability and differentiation of MG-63 cells upon interaction with elutes of T-PRF alone or T-PRF incorporated *Triphala indica* (Ti) or MS.

Materials and Methods: The present ex-vivo study was performed at Department of Periodontics, Saveetha Dental College and Hospital, Saveetha Institute of Technical and Medical Sciences (SIMATS), Chennai, Tamil Nadu, India between February 2025 to April 2025. A 30 years age group healthy volunteer who was willing to take part in the study, without any history of periodontal disease and non-smoker was included. Blood was centrifuged and T-PRF clots were prepared, lyophilised and stored. Further, elutes of Ti and MS were taken and mixed with elute of T-PRF. MTT assay was performed at 1, 3 and 7 days on the cultured MG-63 osteoblastic cell lines. Gene expression was checked for Alkaline Phosphatase (ALP), Bone Morphogenic Protein 2 (BMP 2) and Osteopontin (OSP) at 7th day and Alzarin Red Staining (ARS) for late mineralisation on 21st day. Data was gathered and subjected to statistical analysis. One-way Analysis of Variance (ANOVA) with Post hoc HSD Tukey's multiple comparisons were performed to assess the time changes (1, 3 and 7 days) for MTT assay and qPCR analysis. A p-value <0.05 was considered statistically significant.

Results: T-PRF alone showed significantly better outcomes than T-PRF+Ti and T-PRF+MS at 1st and 3rd day whereas at 7th day there was no statistically significant difference; whereas T-PRF+Ti showed better significant expression of genes (ALP, BMP 2 and OSP) followed by T-PRF+MS and last T-PRF alone. Alizarin Red (AR) staining showed greater staining in order of T-PRF+MS> T-PRF+Ti > T-PRF alone.

Conclusion: T-PRF incorporated with Ti or MS didn't alter the property of T-PRF and also helped in greater release of ALP, BMP 2 and OSP which showed the positivity of osteoblastic differentiation in in-vitro model making T-PRF a SDDS.

Keywords: Drug delivery systems, *Garcinia mangostana*, Herbal, Platelet rich fibrin, Terminalia

INTRODUCTION

Periodontitis is a multifactorial dysbiotic disease that results in attachment loss and bone loss [1]. Restoring the lost periodontal tissues to a fully functional state is one of the most important herculean tasks to a Periodontists [2]. Various treatment strategies such as non-surgical (scaling and root planning) and surgical periodontal therapies (periodontal flap surgery, open flap debridement with and without membranes) have been employed to treat the disease [3]. Biomaterials such as local drug delivery systems (variety of antibiotics line doxycycline, minocycline, tetracycline, metronidazole, clindamycin etc.,) have been employed to concentrate locally at sulcus area [4]. Raw materials like collagen membranes, Cabo polymers, carboxy methylcellulose, poloxamer 123, microspheres, hydrogels etc., have been used as vehicles for SDDS. These materials were lab prepared. With involvement of preservatives and chemical compounds there was always a search for better biomaterial which is easy prepared, less cost, autologous nature and no abnormalities [5-7]. During their search, they got interested to PC.

Leukocyte-platelet Rich Fibrin (L-PRF), Advanced-platelet Rich Fibrin (A-PRF), injectable PRF were some PC's which were used as SDDS by incorporating various antibiotics like doxycycline, metronidazole, vancomycin, penicillin and clindamycin into PC before or after centrifugation. This helped in maintaining good antimicrobial activity on perio-pathogens without disturbing the fibrin structure of the PRF clots [8]. Study done by Esmaeilnejad A et al., 2023 evaluated the A-PRF and L-PRF on cellular activity of pre-osteoblastic MG-63 cell lines and concluded that survival and proliferation of the cells was more in L-PRF and increased with concentrations of the PC's extracts than A-PRF [9]. They also concluded that L-PRF increased proliferation and A-PRF increased the MG-63 cells differentiation. These quoted study results were confirmed by recent systematic review of in-vitro studies by Neimczyk W et al., where they concluded that antibiotic loaded PRF is a good protocol of localised antimicrobial delivery with promising clinical applications in near future [8].

Though L-PRF was autologous it had shorter resorption time of 7 to 11 days with possible silica contamination [10]. Hence, there was a search for a better biomaterial with longer resorption time. During this explore, they got attracted to titanium which was haemo-compatible, corrosion resistant and being regularly used in dental (for

implants) and medical fields. Thus, T-PRF was introduced by Tunali M et al., 2013 [11]. This T-PRF had a greater cellular entrapment with thicker fibrin meshwork and fibrin border when examined under light and scanning electron microscopies. Tunali M et al., 2014 also claimed through their rabbit model study that T-PRF stayed at the surgical site for 21 days indicating a longer resorption time [12]. Bhattacharya HS et al., 2022 also supported the conclusion of Tunali M et al., and stated that T-PRF had a better fibrin meshwork and border with greater cellular entrapment [12,13].

This authenticity of T-PRF made Ercan E et al., 2022, to inject doxycycline hyclate in the T-PRF clots in liquid form [14]. They have studied the kinetics, antimicrobial efficacy and surface morphology of T-PRF injected clots and compared with collagen sponge. They have concluded that T-PRF had a slow and study pace of drug release with greater antimicrobial efficacy against *streptococcus mutans* (Sm) and *pseudomonas aeruginosa* (Pa). Drug impregnation was also recorded through Scanning Electron Microscopy (SEM). Recent studies done by Gummaluri SS et al., 2024 and 2025 in their histological analysis compared T-PRF alone with T-PRF injected with amoxicillin+ clavulanic acid/ metronidazole/ neem gels separately where they concluded that injecting drugs or herbal extract didn't alter the surface structure and impregnated extracts on T-PRF were visualised as surface coatings on SEM [15,16]. There were a thicker fibrin mesh work, fibrin border and good cellular entrapment on Light Microscopy (LM). Thus, a gateway has been established to use T-PRF as a SDDS. Herbal extracts have been used since ages. Because of their reduced side effects, better antioxidant, antimicrobial properties led to their usage in medical and dental fields [17].

Many herbal products such as Aloe vera, Curcumin, *Matricharia chamomile*, *Triphala indica*, *Azadirachta indica*, Mangosteen etc., have been employed in dentistry either as mouth washes or in the form of gels for topical intraoral or extraoral applications [18,19]. Before placing these products directly at the bone site, it is always necessary to check the material interaction on the cell lines. Osteosarcoma MG-63 cell lines were a sought of pure culture to conduct these cell line interaction in-vitro/ex-vivo studies that exactly replicate the human viable bone interaction. As described in previous paragraphs interaction of PRF with osteoblastic cell lines were reported. Rationale behind the conduction of the study was checking the effects Injecting herbal extracts into T-PRF and its interactions on osteoblastic cell lines. To the authors knowledge interaction of T-PRF alone and T-PRF mixed with *Triphala indica* (Ti) or Mangosteen (MS) extracts with that of MG-63 osteosarcoma cells has not been performed. Null hypothesis state that there is no additional benefit of adding Ti and MS into T-PRF and MG-63 cells will no show any cell viability, differentiation and greater gene expression. Alternative hypothesis state that adding Ti and MS to T-PRF will be beneficial and help in MG-63 cells viability, differentiation and greater gene expression.

Hence, the present study aimed to evaluate the osteogenic cell viability and differentiation of osteoblastic MG-63 cells using elutes of T-PRF alone and T-PRF injected with Ti or MS using cell viability (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay) by MTT assay and relative m RNA expression by quantitative Polymerase Chain Reaction (qPCR) for ALP, OSP and BMP-2 for cell differentiation. Final confirmation of mineralisation was assessed using AR staining.

MATERIALS AND METHODS

The present ex-vivo study osteoblastic cell line study was performed to assess the osteogenic viability and differentiation of Ti and MS herbal extracts injected into T-PRF clots against T-PRF alone using MTT assay and qPCR analysis. Study belongs to the Department of Periodontics and sample analysis was performed in White Lab, Saveetha Dental College, Saveetha Institute of Technical and Medical Sciences (SIMATS), Chennai, Tamil Nadu, India. Study was performed during the months of February 2025 to April 2025 Entire protocol was reviewed and approved by scientific review board with a number SRB/SDC/PhD/PERIO-2251/25/024. In cell-based studies, each experimental condition was tested in triplicates across three biological replicates and this is accepted as a minimum standard for establishing reproducibility and determining statistical significance of treatment effects [20]. Hence, in the current study, three groups nine individual experiments (3 per group) and 3-time frames so a total of 27 tests were set for MTT assay. While for qPCR, 1-time frames nine tests were set. The study was according to CRIS (Checklist for Reporting Invitro Studies) protocol to most extent.

Inclusion and Exclusion criteria: A healthy subject aged 30 years age group male without any systemic disease who is willing to take part in the in-vitro analysis, non-smoker, no history of periodontal disease was included in the study for blood sample collection. Whereas individual who is not interested and willing to participate subject who has any history of periodontal or systemic disease, who was under any sought of medication that would alter the platelets metabolism or wound healing were excluded. Further females, pregnant and lactating mothers were also excluded because of their hormonal influence on blood metabolism. Study sample that were not properly prepared or failed to form as intended was classified under the study's exclusion criteria.

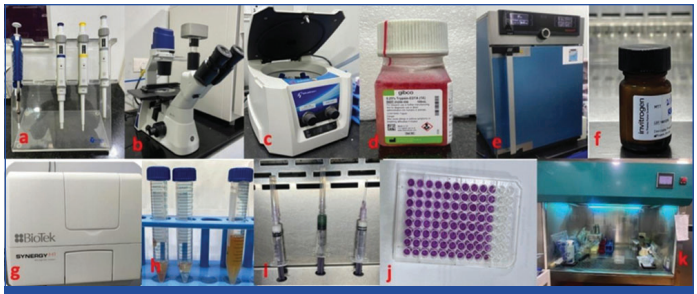
Study Procedure

T-PRF clots preparation and herbal extract gel preparations: T-PRF was prepared according to the modified Tunali M et al., 2014, Gummaluri SS et al., 2024 and 2025 [12,15,16]. Briefly, 20 mL blood was withdrawn from antecubital vein of healthy volunteer and transferred into sterile medical grade titanium tubes (Supra alloys company, Camarillo, USA) and centrifuged at 3500 rotations per minute with 400 × g for 15 minutes (Remi R 8C, New Delhi, India). Further, T-PRF clots were retrieved from the test tubes by separating it from lower blood layer using a sterile tweezer. These clots were transferred to falcon tubes and lyophilised.

MS and *Triphala indica* (Ti) extracts were prepared from Periobiologics™ Lab, Hyderabad, Telangana, India. MS extract was prepared by mixing the rind with 70% ethanol and filtered the solution and extract was concentrated by evaporating the agent. Then this rind extract was incorporated with hydroxyethyl cellulose and mix thoroughly. To this mixture additives like glycerine and phenoxyethanol were added, pH was adjusted to 5.5-6.5 using a pH adjuster (tris-ethanolamine). Homogenisation was maintained with proper blend and later transferred to syringes, subjected to a proper sterilisation and subjected to stability testing at lab prior to usage [21,22]. Thus, 13.2 mg/mL concentration of MS gel was prepared. While coming to Ti, this is a combination of three fruits *Haritaki (Terminalia chebula)*, *Bibhitaki (Terminalia bellirica)* and *Amalaki (Emblica officinalis)*. For preparation of this extract Triphala powder was mixed with distilled water and let it steep for few hours. Further, strain the mixture to obtain a clear extract. This extract was mixed with hydroxyethyl cellulose to obtain a homogenous mixture. Additives such as glycerine and phenoxyethanol were added and pH was adjusted by proper stirring. These were also sterilised packed into syringes and subjected to stability tests prior to the usage [23]. Ti of 18.2 mg/mL concentrated gel was prepared.

Lyophilized T-PRF clots were mixed with the MS and Ti separately in the Dulbecco's Modifed Eagle Medium (DMEM) and incubated to obtain a proper elute which was later transferred to wells containing osteoblastic cells to perform MTT assay at one, three and seven days and q PCR was performed at 7th day so, a total of three groups were formed (T-PRF+Ti, T-PRF+MS and T-PRF alone) [Table/Fig-1].

Osteoblast cell line culture: MG-63 osteoblast cell lines were obtained for National Centre for Cell Science (NCCS), Pune, India. DMEM mixed with 10% Foetal Bovine Serum (FBS) and penicillin-



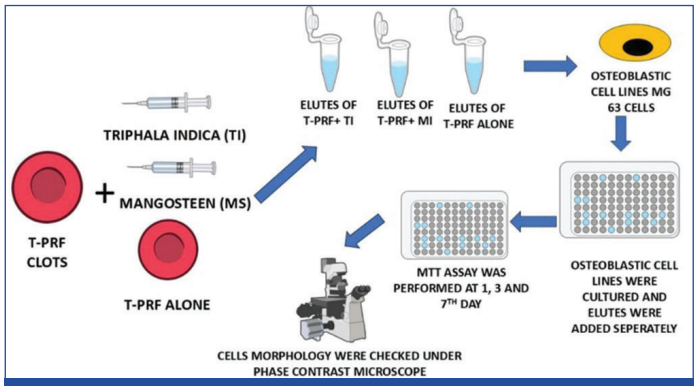
[Table/Fig-1]: The armamentarium where: (a) Shows the micropipettes used; (b) Phase contrast microscope; (c) Microcentrifuge; (d) Trypsin; (e) CO_2 incubator; (f) Microplate reader; (g) T-PRF clot samples; (h) Shows syringes containing T-PRF mixed with Ti and MS gels; (i) Shows the MTT assay plate; (j) Shows the MTT reagent used; and (k) depict the biosafety cabinet used in the current study.

streptomycin concentration of 1% under humidified temperature of 37°C with 5% Carbon Dioxide (CO_2).

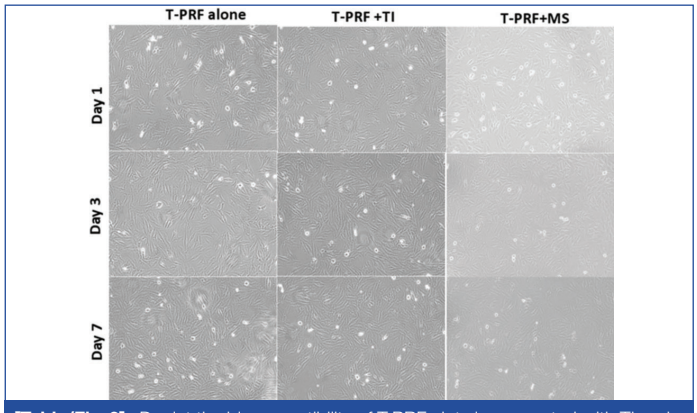
MTT assay procedure: This MTT assay procedure was based on previous study done by Koka P et al., 2018 [24]. Cell proliferation and viability was assessed at one, three and seven days. Initially, 10,000 cultured osteoblast cells per well were seeded in 96 well plates. Further, elute of T-PRF alone and T-PRF injected with Ti/MS individually were added to those wells and were further incubated for 24 hours. These incubated osteoblastic cells were swapped out for 10 µL of MTT stock dye (10 mg/mL) for four hours at 37°C for calculating the viability percentages. Further, Dimethyl Sulfoxide (DMSO) of 100 µL was mixed to dissolve the formazan crystals and checked under a microplate reader (Synergy hybrid Multi-mode Reader (BioTek, Winooski, VT, US)) to record the absorbance values at 570 nm. Percentages were calculated according to the equation:

Cell viability (%)=OD (test sample)-OD (blank)/OD (pc)-OD (blank) * 100.

Further, time durations of three and seven days were also calculated [Table/Fig-2]. Morphology of osteoblastic cells that were interacted with elute of T-PRF alone and T-PRF injected with Ti/MS were assessed and visualised under a phase contrast microscope at 20X magnification [Table/Fig-3].



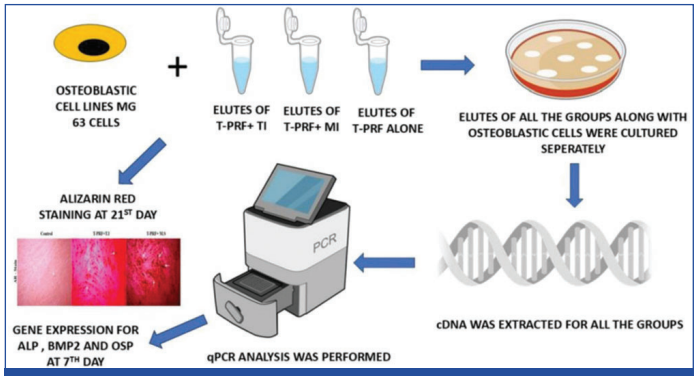
[Table/Fig-2]: Schematic representation of MTT Assay for all the groups.



[Table/Fig-3]: Depict the biocompatibility of T-PRF clots incorporated with Ti and MS along with T-PRF alone separately on osteoblast cells was evaluated by morphological assessment using Phase contrast microscope, at Magnification of 20X.

Quantitative Polymerase Chain Reaction (qPCR) procedure: Total RNA extraction was carried out using TRIZOL reagent (Thermo Fisher Scientifuc, Waltham, MA, USA). A nano drop ND 100 (Thermo Fisher Scientifuc, Waltham, MA, USA) was used to check the quality of DNA obtained at 260 and 280 nm. Then 500 ng of RNA was converted into complementary DNA (cDNA) using the prime script RT reagent (Perfect Real Time, TAKARA, Shiga, Japan). Further, real time PCR equipment (Bio-Rad, Hercules, CA, USA) and KAPA SYBR Fast Master Mix (2x) Universal (Kapa Biosystems, Sigma, Darmstadt, Germany) was used to conduct the qPCR. Cells were seeded at 2.0×10^5 cells/well (6-well plates), treated for seven days with media changes every 48-72 hour. Total RNA (on-column DNase) was isolated; purity and integrity were verified (A260/280=1.8-2.1; A260/230 > 1.8; RIN ≥ 7 where applicable). One microgram RNA was reverse-transcribed (random hexamer/oligo-dT mix). qPCR used SYBR Green with 10 ng cDNA/reaction and primer pairs validated for single melting-peak and efficiency 90-110% from 5-point 1:5 cDNA dilutions. To assess the osteogenesis, three gene marker indicators were used such as ALP, BMP 2 and Osteopontic (OSP) while Beta (β) actin was used as a house keeping gene. Three independent experiments were performed to confirm this osteogenic potential. Briefly it can be explained as follows each condition was performed in three independent biological experiments (different passage and day), each containing three technical replicates per assay and timepoint. Plate maps were randomised, and endpoint reads (absorbance analysis) were performed blinded to group assignment.

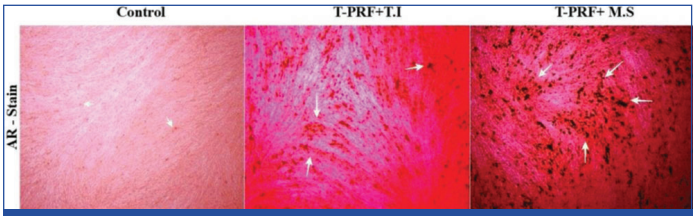
Every plate included no-template and no-RT controls. Single-peak melt curves and efficiency 90-110% were required to accept a primer set. Relative expression is reported with 95% CI from three biological replicates. Primer design was designed using a primer blast software and amplification property maximisation was done using primer sets. OSP, BMP-2 were subjected to a real time PCR reaction at 95°C for three minutes followed by 40 cycles of amplification at 95°C for three seconds and 58°C for 30 seconds whereas for ALP, PCR reaction was run for 95°C for three minutes, 40 amplification cycles at 95°C for three seconds and 60°C for 30 seconds. Beta (β) actin was also triplicated at each time point to normalise the housekeeping genes. Entire information was assessed by Bio-rad CFX management software version 3.0. Relative expression levels of OSP, ALP, and BMP-2 were measured using the 2^−ΔΔCt (double delta Ct) method which was the most convenient way of calculation [Table/Fig-4] [25].



[Table/Fig-4]: Depict the schematic representation of qPCR and Alizarin Red (AR) staining for all the groups.

Alizarin Red (AR) staining: Day 21 AR staining was used to assess late-stage osteogenic differentiation and matrix mineralization. T-PRF alone, T-PRF clots combined with Ti, and MS separately were the four experimental conditions applied to human osteoblasts grown on 24-well plates. Following two PBS washes at the conclusion of the incubation period, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then stained for 20 minutes with 2% AR S solution (pH 4.2). We washed with distilled water to get rid of extra dye.

A 10% cetylpyridinium chloride was used to dissolve the bound dye for quantification, and a microplate reader was used to detect the absorbance at 562 nm for 30 minutes [Table/Fig-5] [26]. To evaluate calcium deposition and osteogenic potential, mineralisation levels of T-PRF+Ti/ T-PRF+ MS were compared with control (T-PRF alone) groups. A calibration curve from known ARS standards was run on each plate. To account for differences in cell number, ARS absorbance was normalised to total Deoxyribonucleic Acid (DNA) (PicoGreen) from sister wells (reported as AU per µg DNA). For imaging, identical exposure/thresholds were used and area fraction quantified in Image J.



[Table/Fig-5]: Depict the Alizarin Red (AR) staining of T-PRF alone, T-PRF+Ti and T-PRF+MS at 21st day of osteoblast cell lines culture with elutes.

STATISTICAL ANALYSIS

Data retrieved was transferred to Microsoft excel spread sheet. Statistical Package for Social Sciences version 22 (SPSS), Chicago Illinois, USA was used to perform the statistical analysis. Data were expressed in mean and standard deviations. Normality was assessed (Shapiro-Wilk) One-way ANOVA with post-hoc HSD Tukey's multiple comparisons were performed to assess the time changes (1, 3 and 7 days) for MTT assay and qPCR analysis. A p-value of <0.05 was considered statistically significant.

RESULTS

The forward and reverse primers of beta actin gene, OSP, ALP and BMP 2 for checking the PCR of the study is shown in [Table/Fig-6]. Regarding MTT assay, on day 1 there was highest cell viability of T-PRF alone followed by T-PRF+ MS and T-PRF+Ti. On day 3 also similar fashion of T-PRF alone showed greater cell viability followed by T-PRF+MS and T-PRF+Ti. There was a record of statistical significance at day 1 (p=0.036*) and day 3 (p=0.047*). For day 7 though numerically values differ, all the groups showed a good cell viability with no statistical significance (0.529#) [Table/Fig-7].

Gene	Forward 5'-3	Reverse 5'-3'
B-actin (housekeeping)	TCGTGTTGGATTCT GGGGAC	ACGAAGGAATAGCCACG CTC
Osteopontin (OSP)	CCTGGCTGAATTCTGA GGGAC	TATAGGATCTGGGTGCAG GCT
Alkaline Phosphatase (ALP)	TGGGCATTGTGACTACC ACTCGG	CCTCTGGTGGCATCTCGT TATCC
Bone Morphogenic Protein (BMP) 2	TGCTAGTAACTTTTGGC CATGATG	GCGTTTCCGCTGTTTGTGTT

[Table/Fig-6]: Show the used forward and reverse primers.
T- thyamine, G-guanine, C-cytosine, A-adenine

Regarding qPCR, there was a significant greater expression of all the three osteogenic gene markers (OSP (p=0.007*), ALP (p<0.001*), and BMP-2 (p=0.042*)) were reported with T-PRF+Ti at 7th day evaluation. Second highest expression was reported for T-PRF+MS and least by T-PRF alone at 7th day evaluation [Table/Fig-8]. During the post-hoc HSD Tukey's multiple comparisons there was statistical significance when T-PRF alone compared with T-PRF+Ti (p=0.031*, p=0.040*) while remaining comparisons showed non-significance at day 1 and 3. While at day 7 all the group comparisons showed non-significant results. While coming to osteogenic gene expressions, for OSP, there was a significant expression when T-PRF alone was compared with T-PRF+Ti/MS (p=0.009*, p= 0.016*), while there was no significance for T-PRF+Ti and T-PRF+MS comparison (p=0.875#). For ALP, comparisons among all the groups showed

Duration	Group	Mean±SD	F-value	p-value
Day 1	T PRF ALONE	100±0	6.088	0.036*
	T PRF+Ti	91.9±4.8		
	T PRF+MS	96.5±1.2		
Day 3	T PRF ALONE	100±0	5.336	0.047*
	T PRF+Ti	96.9±1.3		
	T PRF+MS	98.7±1.6		
Day 7	T PRF ALONE	100±0	0.709	0.529#
	T PRF+Ti	99.2±0.9		
	T PRF+MS	99.4±1.1		

[Table/Fig-7]: Depicts the significant MTT assay comparison of T-PRF alone, T-PRF+ *Triphala indica* elute and T-PRF+ Mangosteen Rind (MS) extract on human osteoblastic cell lines (MG-63) at 1, 3 and 7 days.
Test applied- ANOVA; *indicates statistical significance p<0.05, #indicates non-significant

Gene expression	Group	Mean±SD	F-value	p-value
Osteopontin (OSP)	T PRF+Ti	6.4±2.3	12.448	0.007*
	T PRF+MS	5.8±1		
Alkaline Phosphatase (ALP)	T PRF+Ti	7.2±1.3	42.448	<0.001*
	T PRF+MS	4.8±0.6		
BMP 2	T PRF+Ti	5.4±2.8	5.627	0.042*
	T PRF+MS	4.2±0.7		

[Table/Fig-8]: depict the significant qPCR comparison of T-PRF alone, T-PRF+ *Triphala indica* elute and T-PRF+ MS extract human on osteoblastic cell lines (MG-63) at 7th day post incubation.
Test applied-ANOVA; * indicates statistical significance, # indicates non-significant; BMP: Bone morphogenic protein, p<0.05 considered statistically significant

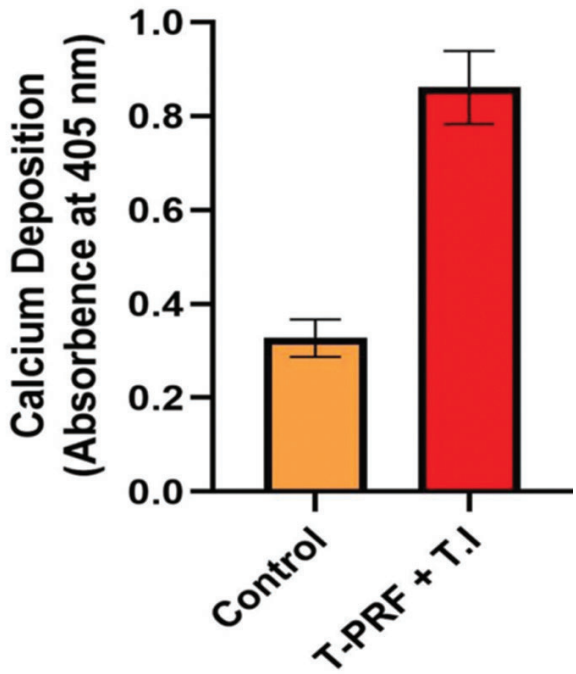
statistical significance indicating a good amount of its expression (p<0.001*, p=0.003*, p=0.028*). Regarding BMP 2, significance was reported when T-PRF alone was compared to T-PRF+Ti (p=0.041*) where as other comparisons were non-significant {T-PRF+MS and T-PRF alone comparison (p=0.120#), T-PRF+MS and T-PRF+Ti comparison (p=0.679#)}. Overall, test groups showed a greater gene expression when compared with T-PRF alone [Table/Fig-9].

Duration	Variable	Comparisons	Mean Difference (I-J)	Sig.
Day 1	T-PRF alone	T-PRF+Ti	8.10864667*	0.031*
		T-PRF+MS	3.45621333	0.363#
	T-PRF+Ti	T-PRF alone	-8.10864667*	0.031*
		T-PRF+MS	-4.65243333	0.194#
	T-PRF+ MS	T-PRF alone	-3.45621333	0.363#
		T-PRF+Ti	4.65243333	0.194#
Day 3	T-PRF alone	T-PRF+Ti	3.13783000*	0.040*
		T-PRF+MS	1.26506000	0.441#
	T-PRF+Ti	T-PRF alone	-3.13783000*	0.040*
		T-PRF+MS	-1.87277000	0.209#
	T-PRF+ MS	T-PRF alone	-1.26506000	0.441#
		T-PRF+Ti	1.87277000	0.209#
Day 7	T-PRF alone	T-PRF+Ti	0.75801000	0.544#
		T-PRF+MS	0.63583667	0.643#
	T-PRF+Ti	T-PRF alone	-0.75801000	0.544#
		T-PRF+MS	-0.12217333	0.983#
	T-PRF+ MS	T-PRF alone	-0.63583667	0.643#
		T-PRF+Ti	0.12217333	0.983#
Osteopontin (OSP)	T-PRF alone	T-PRF+Ti	-5.408838667*	0.009*
		T-PRF+MS	-4.817194000*	0.016*
	T-PRF+Ti	T-PRF alone	5.408838667*	0.009*
		T-PRF+MS	0.591644667	0.875#
	T-PRF+ MS	T-PRF alone	4.817194000*	0.016*
		T-PRF+Ti	-0.591644667	0.875#

Alkaline Phosphatase (ALP)	T-PRF alone	T-PRF+Ti	-6.207674333*	<0.001*
		T-PRF+MS	-3.803151000*	0.003*
	T-PRF+Ti	T-PRF alone	6.207674333*	<0.001*
		T-PRF+MS	2.404523333*	0.028*
	T-PRF+ MS	T-PRF alone	3.803151000*	0.003*
		T-PRF+Ti	-2.404523333*	0.028*
BMP 2	T-PRF alone	T-PRF+Ti	-4.401004667*	0.041*
		T-PRF+MS	-3.224014667	0.120#
	T-PRF+Ti	T-PRF alone	4.401004667*	0.041*
		T-PRF+MS	1.176990000	0.679#
	T-PRF+ MS	T-PRF alone	3.224014667	0.120#
		T-PRF+Ti	-1.176990000	0.679#

[Table/Fig-9]: The post-hoc Tukey's HSD multiple comparison of T-PRF alone, T-PRF+MS and T-PRF+Ti at days 1,3, 7 for MTT assay and it also depict the significance of qPCR of three osteogenic gene expression.
*Indicates statistical significance, #indicates non-significant; p<0.05 considered statistically significant, Test- post-hoc Tukey's HSD multiple comparison

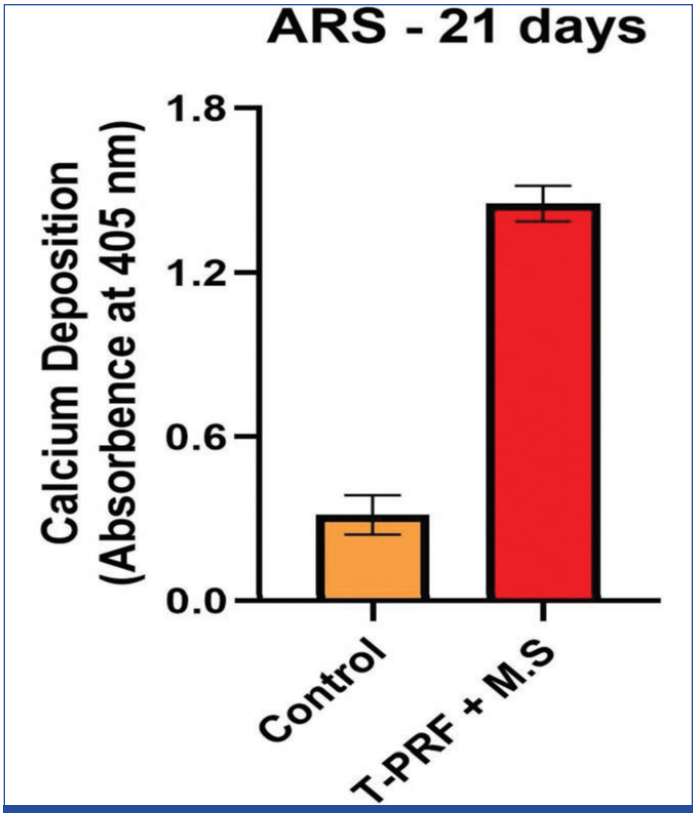
Mineralisation assessment of the present study results was done by AR staining. This staining was performed on the osteoblast cultures incorporated with elutes of T-PRF alone, T-PRF+Ti and T-PRF+MS till 21 days. At the end point results showed increased calcium deposition to T-PRF+MS group followed by T-PRF+Ti and least by T-PRF alone group. This was observed under the microscope, whereas in the graphs test groups showed greater absorbance than T-PRF alone group [Table/Fig-10,11].



[Table/Fig-10]: Graphical representation of Alizarin Red (AR) staining for T-PRF alone and T-PRF+Ti at 21st day.

DISCUSSION

The present study was a primitive ex-vivo study that was performed to check the interaction of the elutes of T-PRF alone or T-PRF+Ti/T-PRF+MS on osteoblastic cell lines MG-63. Current study didn't take negative controls as the focus of interest was to identify the synergistic effects rather than isolated influence of each component. Moreover, including extracts alone as separate entity there will be deviation from the idea of bio-functionalising the T-PRF with the herbal extracts. In the present study systemically healthy male of 30 years was considered for blood drawing because of the previous published study done by Yajamanya SR et al., 2016 where they stated that PRF that was reported has thicker fibrin pattern and



[Table/Fig-11]: Graphical representation of Alizarin Red (AR) staining for T-PRF alone (Control) and T-PRF+MS at 21st day.

also concluded that age always influence the outcome of the quality of PRF [27]. Natural substances always help in maintaining the balance of the body without causing immunological reactions. Herbal extract used in this study were MS and *Triphala indica* (TI). MS has been considered as queen of fruits because of its inherent property of blocking inflammatory process by inhibiting the cyclooxygenase, lipoxygenase, prostaglandins, nuclear factor kappa beta etc. It also controls the secretions of reactive oxygen species and has antibacterial, antiproliferative properties. MS mainly consists of flavonoids, xanthol, saponin, tanin, phenol, B_1-B_2 vitamins, gartanine, garcinon and anthicyanins that help in medicinal properties [28]. These extracts helps in control of gum disease by acting on connective tissue and helps in improving overall oral health.

Hence, in the current study authors have used the elute of these extracts mixing with T-PRF elute to check the interaction on osteoblasts cells regarding cell viability and compatibility and differentiation was checked through qPCR using osteogenic gene expression markers at the 7th day to provide a snapshot of osteogenic activity. Confirmation of mineralisation activity was done using AR staining. As much number of studies was not performed and first of its kind, present study results were compared with the similar existing literature of PC of any types.

In the current study, T-PRF alone value in both MTT assay and gene expression were constant because, fold-change values were calculated using the comparative Ct (2^−ΔΔCt) method as described by Livak KJ and Schmittgen TD (Methods, 2001) [29] and Schmittgen TD and Livak KJ (Nat. Protocols, 2008 [25]). According to this approach, the control group serves as the calibrator, and its expression value is normalised to 1 by definition (2^0=1) for gene expression. Consequently, no variability (error bars) is applicable or plotted for the control, as it represents the reference baseline against which all other groups are compared. Hence, for all experimental groups, fold-change values were computed from biological triplicates, and the mean±SD was represented as error bars in the plots. However, the control group was intentionally shown as a fixed value of one without error bars i.e., (1±0), in accordance with widely accepted qPCR normalisation standards to avoid misinterpretation

of propagated error from the calibration step. Similarly, for MTT assay also control group values were kept constant (100±0). But there was comparison of all the groups on day 3 and 7 hence, baseline values were not removed from the [Table/Fig-7].

Various authors used the Ti on different cell types like cancer cell lines (cervical cancer, triple negative breast cancer cell lines [30], pancreatic cancer cell lines [31]), dermal fibroblasts and keratinocytes [32] which reported good results. Ti had an inhibitory effect of cancer cell lines by activation of programmed cell death by annexin V staining and interfering with reassembly of tubules. On in-vitro Triphala inhibited the growth of human pancreatic cancer cells [31] in both in-vitro and in-vivo model and concluded that inhibitory effect was mainly due to reduction in the activation of p53 and Extracellular Regulated signalling Kinase (ERK). They also stated that Ti will be a potential treatment modality in the treatment of pancreatic cancer. Recent study done by Ramamoorthy R et al., 2023 formulated a polyherbal extract (with Ti mixed with *Acacia catechu*, *Glycyrrhiza glabra Azadirachta indica*, and Coscinium fenestratum medicinal plants) [33]. They concluded that, it helped in rapid wound healing on open wounds. Thus, the above combination was manufactured as poly herbal drug loaded polycaprolactone nanofibrous mats that had a greater efficient of antimicrobial, collagenous and wound healing property. In the present study Ti and MS had shown cell viability on MTT assay indicating that it incorporating these extracts into T-PRF didn't alter the properties of the drug or PC. This might be due to the inherent antioxidant, antimicrobial, anti-inflammatory properties of both the herbal extracts and inherent property of growth factors release, fibrin structure of T-PRF alone had this positivity. The type of cells used in the study also shows an effect of not having an immune-response against the materials and show the activity of viability and osteogenic differentiation. In the current study [Table/Fig-7,8] of MTT assay (1st day) and qPCR (7th day) showed constant values was mainly due to T-PRF elute was alone without any additives.

Present study results were in accordance with a study done by Krithiga G et al., 2014 where they have incorporated terminalia arjuna (TA) bark extract into bone and prepared a bone substitute where they concluded that TA extract had a cell viability of more than 85% on MTT assay and higher ALP activity indicating the osteoblastic cell differentiation and biomineralisation [34]. They also concluded that this substitute acts as a potential alternative to BMP's, as filler bone substitute for fracture areas and as a coating agent on implant surfaces to activate the bone of surgical area.

T-PRF elute alone had a good cell viability than T-PRF+Ti and T-PRF+MS when checked through MTT assay this might be due to inherent stimulative property of T-PRF or the cells that were non-autogenous and are cancer cell lines which have an altered mechanisms of immune response such as non-stimulative major histocompatibility complex etc. There can also be a positivity of addition of drugs also didn't reduce the cell viability and differentiation. But for gene expressions more upregulating expression of ALP, BMP 2 and OSP was reported for T-PRF+Ti followed by T-PRF+MS when compared with T-PRF alone. This might be due to the stimulative effect of these herbal extracts and inherent regenerative efficacious property of T-PRF might have influenced. Moreover clinical studies on Intra bony defects also had a greater bone mineralisation or bone fill might had a positive effect of cell viability and osteogenic differentiation in these tested MG-63 cells.

While coming to MS current results were not in accordance with study done by Nauman MC and Jhonson JJ et al., Yang S et al., and Fan H et al., where they reported that there was an apoptosis of osteosarcoma cells by activating the caspase-3/8 cascade, increased endoplasmic reticulum stress, inhibited wnt/beta-catenin pathway activation and down regulating Fatty Acid Synthase (FASN) in dose dependent manner [35-37]. In the present study, there was a greater cell viability and good upregulated gene expression of ALP, BMP-2 and OSP this might be due to the anti-oxidant property, anti-inflammatory and inherent property of T-PRF in stimulating the cells might have helped and didn't activate the adverse immune responses.

To confirm the mineralisation tendency of osteoblast cells, late-stage mineralisation assay was performed using AZ stain. In the current study, mixed results were produced when AZ staining was compared to MTT assay and qPCR. In AR staining greater mineralisation was reported for T-PRF+MS followed by T-PRF+Ti and least T-PRF alone, whereas, qPCR showed T-PRF+Ti > T-PRF+MS > T-PRF alone. Further, MTT assay showed T-PRF alone had greater cell viability. These variations might be due to the time frame changes where MTT assay was performed till 10th day, q PCR for 7th and AR stain at 21st day. This is in accordance with Wang J et al., 2023 where they used i-PRF interaction with human bone marrow stem cells (hBMSC's) and finalised that i-PRF helped in osteogenic differentiation and proliferation of hBMSC's [38]. Null hypothesis has been rejected and adding Ti/MS helped in cell viability, greater gene expression and mineralisation when compared with T-PRF alone.

Varied restricted end points of assays in the current study altered the outcomes and these endpoints interrogate distinct biological phases. T-PRF provides a fibrin-growth factor milieu (e.g., PDGF, TGF-β) that can enhance proliferation in the early window (higher MTT). Polyphenols in Ti may promote osteogenic commitment (↑RUNX2/ALP/BMP-2 at day 7), even when proliferation is plateauing. Xanthones in MS (e.g., α-mangostin) have been associated with matrix maturation/mineral nucleation, aligning with higher ARS at day 21. Such proliferation-differentiation trade-offs and phase-specific effects are well recognised in osteoblast biology and can yield non-parallel rank-orders across assays, to strengthen integration. In case of future implications current study acts an initial step for further usage of herbal extract incorporations in to biological materials so that they can show their positive effects on humans without adverse effects. Before going to large trials animal experiments should be grounded so that results and out comes will become authentic and this T-PRF SDDS protocol can be applied regularly in the dentistry

Limitation(s)

The present study was an initial preliminary exploratory study where it has experimented regarding the viability, osteogenic differentiation and mineralisation (confirmed by AR staining) which was one of the limitations of the study. MG-63 cells used were non-autogenous origin and they alter the immunomodulatory mechanisms because of which there was no abnormal response in the experimental setup and follow up. Further wound healing tests like scratch test if performed, better biologic process can be established. As study was performed on osteoblast cell lines and scratch test is generally performed for epithelial cells hence it was not performed. Experiments with human osteoblasts and in-vivo trials conduction will be a better way of determining the present invitro conclusions. qPCR was performed only at 1 single point of 7th day (just to show the differentiation of cell lines through gene expression) can also be considered as limitation.

CONCLUSION(S)

Within limitations, T-PRF can be considered as a SDDS. In the present study, T-PRF+Ti and T- PRF+ MS showed similar outcomes of cell viability for MTT assay whereas T-PRF+Ti showed better upregulation of genes like ALP, BMP-2 and OSP followed by T-PRF+MS and T-PRF alone. Whereas mineralisation was recorded in the order of T-PRF+MS > T-PRF+Ti >T-PRF alone confirming the positive nature of the study protocol, this opens a novel treatment protocol of using herbal extracts incorporation into T-PRF that could be placed at surgical site. This would enhance the soft and hard-tissue healing with lower bacterial load and sustained release of the incorporated drug. Thus, T-PRF+Ti/MS gave an additional benefit

for the cell viability and differentiation than T-PRF alone where when added in human trials a better hard-tissue healing can be expected.

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