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Synthesis, Characterization and Biological Evaluation of New Benzamidine Derivatives: Antibiotics for Periimplantitis Causing Pathogen

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Periimplantitis (PI) is complex polymicrobial disease, which destroys implant-supporting tissue. Although facts suggest several synthetic inhibitors of periimplantitis causing bacteria (PCB), but the undesirable side effects of them limits their application. Hence, current investigation was intended to carry out the synthesis, characterization, *in vitro* antimicrobial evaluation and cytotoxicity (cell viability) analysis of new benzamidine derivatives (NBDs) against periimplantitis causing bacteria. Present study involved synthesis of 2-(4-((4-substituted)carbamothioyl)phenoxy)-*N*-(4-substituted benzylidene)acetohydrazide (**4a**-**c**) by treatment of different aromatic aldehydes with 2-(4-carbamothioylphenoxy)acetohydrazide (**3**), that was obtained by hydrazination of ethyl 2-(4-carbamothioylphenoxy)acetate (**2**), the derivative of 4-hydroxybenzothioamide (**1**). The synthesized compounds (NBDs) were subjected to FTIR, ^{1}H NMR, ^{13}C NMR and mass spectrometric characterization. All NBDs were further investigated for their antimicrobial potential (MIC and MBC) against *P. gingivalis* the PCB, using micro-broth dilution method. The NBDs were also tested for their cytotoxicity (cell viability) against HEK 293 cells using MTT assay. The present study successfully synthesized and elucidated the structures of the synthesized NBDs. The NBDs when tested against *P. gingivalis* exhibited MIC ranging between 62.5-500 µg/mL, whereas NBDs **4a** and **4b** exhibited MBC of 125 and 62.5 µg/mL respectively. Also, all NBDs exhibited weak cytotoxicity (cell viability more than 80%) against HEK 293 at 7.81 µg/mL. The significant antimicrobial activity of NBDs and higher cell viability (safety) against *P. gingivalis* supports their potential application in periimplantitis treatment, however these NBDs must be further investigated for the additional *in vivo* and clinical studies.

Keywords: Periimplantitis, Benzamidine, *P. gingivalis,* Cytotoxicity, Hydrazide, Ester, Imines.

INTRODUCTION

The antibacterial activity and osteogenic potential of implant materials are the two major factors that affect the long-term success and development of dental implants [1]. Implants failure due to biological complications of peri-implantitis is a biggest challenge for the implantologists. *P. gingivalis*, Gram-negative anaerobe, is recognized as one of the key pathogen responsible for PI [2]. The prevalence of peri-implantitis is reported upto a range of 28-56% [3]. Reports claimed *P. gingivalis* as the root cause for dental implants failure attributed to peri-implantitis [4]. The current strategy for the treatment of peri-implantitis includes eradication of *P. gingivalis* using surface debridement, followed by antibiotics administration [5].

Evidence suggests the use of imines (Schiff bases) in the peri-implantitis treatment [6]. Emergence of resistance and inhibition of broad spectrum of host proteases due to long term use of conventional antibiotics is a major concern in the treatment of peri-implantitis [7]. However, the most widely used titanium implants currently lack antimicrobial properties [8].

Therefore, it will be of great significance to endow the surfaces of implants with antibacterial properties with short- and long-term pathogen-inhibiting capabilities. Current data, concerning the emerging global threat of antibiotic resistance, have prompted researchers to search for new antimicrobial agents, which can be used as a coating material for dental implants [9]. Fact suggests that use of coatings containing silver, copper, fluoride, zinc, chlorhexidine, cephalothin, gentamycin and amoxicillin in different types of dental implants [10]. Attributed to their strong antimicrobial activities against Gram-positive and Gram-negative bacteria, recently benzamidine analogues have withdrawn high attention of researchers.

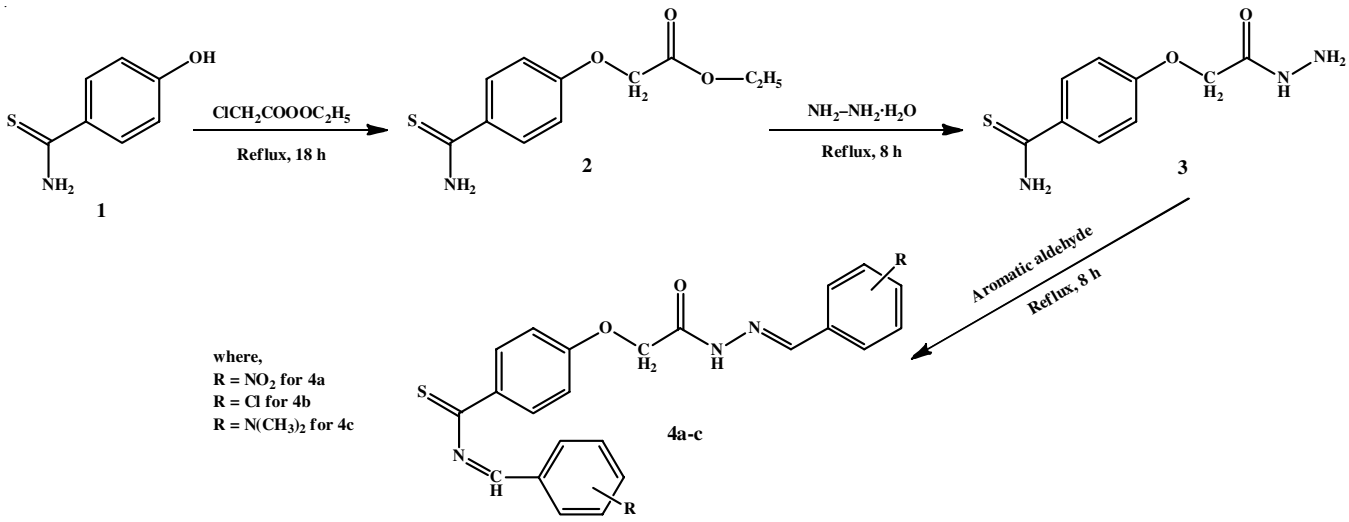
Facts suggest benzamidine analogues to exhibit stronger binding to gingipains (HRgpA and RgpB), the causative agents of *P. gingivalis* that causes periodontitis and peri-implantitis [11]. Evidence suggests esters and hydrazides moieties also exhibit strong antimicrobial activity against *P. gingivalis* [12]. Investigations report that incorporation of ester, hydrazide and imino groups in various organic compounds enhances their antimicrobial activity [13]. A study reported incorporation of urea (amino group) and other polar groups in benzamidine structure offers higher inhibition of HRgpA and RgpB [14]. This means incorporation of some polar group such as amino, imino, nitro, chloro and dimethyl amino group can be a good strategy to enhance the potential of benzamidines against *P. gingivalis* the causative agent of peri-implantitis. Recent studies reported methodologies to convert the phenolic compounds into esters using ethylchloroacetate [15], esters into hydrazides using hydrazine hydrate [16] and hydrazides into imines [17]. Hence based on the complications of peri-implantitis and potential of benzamidines and imines, present study was intended to carry out the synthesis, characterization, cytotoxicity (cell viability) and antimicrobial activity of new benzamidine derivatives (NBDs) against *P. gingivalis* the peri-implantitis causing bacteria.

EXPERIMENTAL

In present study, the chemicals for synthesis of new benzamidine derivatives (NBDs) were purchased from Friendemann Schmidt Chemical (Washington, DC), Sigma-Aldrich Co. (St Louis, MO, USA), Merck KGaA (Darmstadt, Germany), Qrec Chemicals (Rawang, Malaysia) and HmbG® Chemicals (Hamburg, Germany). The purity of NBDs was checked by open capillary tube using SMP11 Analogue equipment and the melting points of all NBDs were calculated. The NBDs ^{1}H NMR and ^{13}C NMR spectra were recorded on δ value scale as downfield chemical shift in ppm against tetramethylsilane (TMS) using NMR 700 MHz ASCENDTM spectrometer and DMSO as solvent. The NBDs IR spectra were recorded on Jasco FT/IR-6700 instrument at wavelength ranging from 400 to 4000 cm^{-1}. The NBDs Mass spectra were recorded using Direct Infusion IonTrap MS (Thermo-Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer). For the elemental analysis of NBDs Perkin-Elmer 240B and 240C instruments were used. The purity of NBDs and monitoring of reactions was done by TLC on aluminum sheets with silica gel 60 F_{254} (0.2 mm) (Merck Millipore, Germany) using SPRECTROLINE® CM-26 UV viewing chamber and CH_3OH:$CHCl_3$ (9:1) as solvent system.

Synthesis of 2-(4-((4-substituted)carbamothioyl)phenoxy)-*N*-(4-substituted benzylidene)acetohydrazide (4a-c): New benzamidine derivatives (NBDs, **4a-c**) were synthesized as per the protocol given in the standard literature with some minor modification [15,18,19]. Briefly, a mixture containing 4-hydroxybenzothioamide (0.01 M), ethyl chloroacetate (0.01 M) in acetone was refluxed for 17 h to yield an intermediary NBD **2**, which was treated with hydrazine hydrate to offer NBD **3**. The NBD **3** was further treated with substituted aromatic aldehyde in equimolar concentration to offer a crude product. The crude product so obtained was recrystallized with methanol using activated charcoal to yield pure NBD **4** (**Scheme-I**).

Ethyl 2-(4-carbamothioylphenoxy)acetate (2): Brown crystals (yield: 76%, m.p. 125 ºC); FTIR (KBr, cm^{-1}): 3270 (N-H), 3119 (=C-H), 2950 (C-H), 1718 (C=O), 1599 (C=S); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.30 (t, 3H, CH_3), 3.41 (br s, 2H, NH_2), 3.80 (q, 2H, OCH_2), 4.35 (s, 2H, CH_2), 6.76-7.86 (m, 4H, Ar-H); ^{13}C NMR (DMSO, ppm) δ: 21.28 (CH_3), 62.22 (OCH_2), 65.13 (CH_2-C=O), 116.77, 131.13, 154.98, 164.47 (Ar-C),



Scheme-I: Synthesis of new benzamidine derivatives (NBDs, **4a-c**)

180.20 (C=S), 182.60 (C=O); and Mass (*m/z*): 239. Anal. calcd. (found) % for $C_{11}H_{13}NO_3S$: C, 55.21 (55.11); H, 5.48 (5.52); N, 5.85 (5.79).

2-(4-Carbamothioylphenoxy)acetohydrazide (3): Yellow crystals (yield: 87%, m.p. 132 ºC); FTIR (KBr, cm^{-1}): 3291 (N-H), 3017 (=C-H), 2955 (C-H), 1602 (C=O), 1586 (C=S); ^{1}H NMR (DMSO-d_6, ppm) δ: 3.41 (br s, 2H, NH_2), 4.36 (s, 2H, CH_2), 6.76-6.79 (m, 4H, Ar-H), 9.05 (Brs, 1H, NH), & 9.07 (Brs, 2H, NH_2); ^{13}C NMR (DMSO, ppm) δ: 65.21 (CH_2-C=O), 116.69, 131.08, 154.92, 164.39 (Ar-C), 180.25 (C=S), 182.58 (C=O); and Mass (*m/z*): 225. Anal. calcd. (found) % for $C_9H_{11}N_3O_2S$: C, 47.99 (48.04); H, 4.92 (4.89); N, 18.65 (18.72).

2-(4-((4-Nitrobenzylidene)carbamothioyl)phenoxy)-N′-(4-nitrobenzylidene) acetohydrazide (4a): Pale yellow crystals (yield: 88%, m.p. 172 ºC); FTIR (KBr, cm^{-1}): 3271 (N-H), 3013 (=C-H), 2935 (C-H), 1515 and 1341 (NO_2), 1605 (C=O), 1515 (C=N), 1505 (C=S); ^{1}H NMR (DMSO-d_6, ppm) δ: 3.40 (Brs, 2H, NH_2), 4.35 (CH_2-C=O), 6.84-7.67 (m, 8H, Ar-H), 9.20 (s, 1H, CH=N), 9.35 (s, 1H, CH=N-N), 9.90 (s, 1H, NH-C=O); ^{13}C NMR (DMSO, ppm) δ: 65.21 (CH_2), 116.71, 124.81, 132.11, 139.26, 150.14, 154.90, 160.04, (Ar-C), 143.26 (N-N=C), 164.44 (C=N), 180.19 (C=S), 182.61 (C=O); and Mass (*m/z*): 491. Anal. calcd. (found) % for $C_{23}H_{17}N_5O_6S$: C, 56.21 (56.18); H, 3.49 (3.52); N, 14.25 (14.29).

2-(4-((4-Chlorobenzylidene)carbamothioyl)phenoxy)-N′-(4-chlorobenzylidene)acetohydrazide (4b): Brown crystals (yield: 84%, m.p. 168 ºC); FTIR (KBr, cm^{-1}): 3272 (N-H), 3026 (=C-H), 2961 (C-H), 1664 (C=O), 1578 (C=N), 1507 (C=S) and 1429 (C=C); ^{1}H NMR (DMSO-d_6, ppm) δ: 3.40 (br s, 2H, NH_2), 4.34 (CH_2-C=O), 6.84-7.67 (m, 8H, Ar-H), 9.20 (s, 1H, CH=N), 9.39 (s, 1H, CH=N-N), 9.91 (s, 1H, NH-C=O); ^{13}C NMR (DMSO, ppm) δ: 65.23 (CH_2), 116.69, 124.79, 128.04, 132.15, 139.96, 150.12, 154.94, 160.01, (Ar-C), 143.19 (N-N=C), 164.41 (C=N), 180.22 (C=S), 182.59 (C=O); and Mass (*m/z*): 469. Anal. calcd. (found) % for $C_{23}H_{17}C_{l2}N_3O_2S$: C, 58.73 (58.69); H, 3.64 (3.59); N, 8.93 (8.89).

2-(4-((4-(Dimethylamino)benzylidene)carbamothioyl)-phenoxy)-N′-(4-(dimethyl amino)benzylidene)acetohydrazide (4c): Light brown crystals (yield: 79%, m.p. 159 ºC); FTIR (KBr, cm^{-1}): 3272 (N-H), 3077 (=C-H), 2961 (C-H), 1664 (C=O), 1578 (C=N), 1507 (C=S) and 1429 (C=C); ^{1}H NMR (DMSO-d_6, ppm) δ: 3.40 (br s, 2H, NH_2), 4.34 (CH_2-C=O), 6.84-7.67 (m, 8H, Ar-H), 9.20 (s, 1H, CH=N), 9.38 (s, 1H, CH=N-N), 9.89 (s, 1H, NH-C=O); ^{13}C NMR (DMSO, ppm) δ: 41.02 ($N(CH_3)_2$), 65.20 (CH_2), 116.62, 124.81, 128.08, 129.91, 132.19, 139.94, 160.11 (Ar-C), 143.21 (N-N=C), 164.41 (C=N), 180.25 (C=S), 182.64 (C=O); and Mass (*m/z*): 487. Anal. calcd. (found) % for $C_{27}H_{29}N_5O_2S$: C, 66.50 (66.48); H, 5.99 (6.01); N, 14.36 (14.29).

Antimicrobial activity: In present study, the minimum inhibitory concentration (MIC) of NBDs against *P. gingivalis* (ATCC 33277) was determined using micro-broth dilution method as per CLSI guidelines [12]. The *P. gingivalis* strains were cultured in blood-enriched tryptic soy agar (eTSA) (Merck KGaA, Germany) maintained at pH of 7.4, supplemented with 5% of L-cysteine (Bio-Basic, Canada), 1% of dithiothreitol and 0.5 mg/mL of vitamin K (Sigma Life Sciences, USA). The NBDs were subjected to two-fold serial dilution aseptically from 1000 to 1.95 μg/mL, using ampicillin as standard. For MIC determination, density of *P. gingivalis* was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/mL). On microtiter plate, 100 μL of *P. gingivalis* culture was added to all wells excluding negative control (containing broth only). Next, plates were incubated for 46 h at 37 ºC into an anaerobic jar (Oxoid, UK) supplemented with gas pack (Merck KGaA, Germany) (which generate 90% N_2, 5% CO_2, H_2) and gas indicator (Thermo-Fisher Scientific, USA). The MIC was determined by measuring absorbance of each well content at 620 nm. To determine the minimum bactericidal concentration (MBC) against *P. gingivalis*, the resultant 20 μL of MIC content of clear wells (in which there was no bacterial growth) of NBDs were aseptically plated on eTSB agar and further incubated for 46 h at 37 ºC in anaerobic jar with gas indicator and gas pack. After incubation, MBC was recorded as the lowest concentration of NBDs with no visible growth of bacteria with clear agar (same as negative control). All the experiments were carried out in triplicate.

Cytotoxicity analysis (cell viability assay): The NBDs were subjected to cytotoxicity analysis (cell viability against normal HEK293 cells) using standard protocol of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazoliumbromide (MTT) assay with minor modifications [11]. Briefly, propagation of HEK 293 cells was done in Dulbecco's modified eagle medium (DMEM) (Corning, USA) supplemented with 5% inactivated fetal bovine serum (FBS) into 5% CO_2 incubator (Heal Force/HF90, China) maintained at 37 ºC with 95% relative humidity. For the cell viability assay, HEK 293 cell were proliferated on 96-well culture plate of 1×10^4 cells density per well and incubation was done over-night for cells attachment. The NBDs and standard were serially diluted in DMEM and added into each of the well to achieve final concentrations of 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 μg/mL, respectively. Next, the plates were incubated at 37 ºC for 24 h, under 5% CO_2. Next, 10 μL MTT solution (Merck, USA) was added into each of the well and again incubated at 37 ºC for 4 h in dark. Next, each of the well contents form microplate was pipetted out and in each of the well DMSO (100 μL) was added for dissolution of crystal formazan. Next, at 570 nm the absorbance was recorded using GloMax Multiple Detection System (Promega, USA). Finally, the percentage cell viability was calculated using following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

RESULTS AND DISCUSSION

The complexity of peri-implantitis is due to *P. gingivalis*, emergence of resistance to available antibiotics and high antimicrobial potential of imines emphasizes the need for imines synthesis [20]. Present study offered 2-(4-((4-substituted) carbamothioyl)phenoxy)-N-(4-substituted benzylidene)-acetohydrazide (**4a-c**) by reacting 2-(4-carbamothioylphenoxy)-acetohydrazide (**3**) with different aldehydes such as 4-nitrobenzaldehyde, 4-chlorobenzaldehyde and 4-dimethylamino benzaldehyde. The NBDs **4a-c** (imines) synthesis followed

Schiff reaction. The NBD **3** (hydrazide), was obtained by amination of ethyl 2-(4-carbamothioylphenoxy) acetate (**2**) using hydrazine hydrate. The precursor NBD **2** (ester) was synthesized by esterification of 4-hydroxybenzothioamide (**1**) using ethylchloroacetate. For esterification reaction to occur easily, dried solvent (ethanol) was used anhydrous potassium carbonate was added in equimolar concentration and obtained reaction product was extracted using ether to offer NBD **2**. During the synthesis, the experiment total anhydrous conditions were maintained and purification of synthesized NBDs was done through recrystallization of all crude with methanol and activated charcoal. Purity of synthesized NBDs was assessed based on the sharp melting point, single spot TLC pattern and elemental analysis.

Structures of the synthesized NBDs were confirmed by the FTIR, ^{1}H NMR, ^{13}C NMR and mass spectrometry data. The NBDs spectral data was characterized based on the literary data [22]. The presence of characteristic FTIR band at 2950 cm^{-1} for C-H stretching, ^{1}H NMR signal at 1.30 (t, 3H, CH_3) & 3.80 3.80 (q, 2H, OCH_2) and ^{13}C NMR signal at 21.28 & 62.22 of CH_3 & CH_2, thereby confirmed structure of NBD **2**; appearance of FTIR band at 1602 cm^{-1} (for C=O), appearance of ^{1}H NMR signals at 9.05 & 9.07 of NH & NH_2 and absence of ^{1}H NMR signals at 1.30 (t, 3H, CH_3) & 3.80 3.80 (q, 2H, OCH_2), also absence of and ^{13}C NMR signal at 21.28 & 62.22 of CH_3 & CH_2, thus confirmed structure of NBD **3**; and appearance of FTIR bands at 2965-2935 cm^{-1}, 3272-3271 cm^{-1}, ^{1}H NMR signal at 6.84-7.67 of Ar-H, 9.20 of CH=N & 9.35-9.39 of CH=N-N and ^{13}C NMR signal at δ 143.19-143.26 ppm of C=N-N consequently confirmed the structure of NBDs **4a-c**. The results of characterization data of NBDs synthesized in the present study were also matched and found to be in agreement with the results of the other studies especially for the ester, hydrazide and imino groups [111,12,21].

Biological activity: The synthesized NBDs were evaluated for their cytotoxicity study (cell viability) by MTT assay on HEK293 cells using 96-well culture microplate using. The percentage cell viability (safety) was determined and statistically analyzed using Graph Pad Prism for Windows, version 9.51 (GraphPad Software Inc., USA). The experimental method for cytotoxicity analysis was carried out as per the standard literature [12]. The cell viability results confirmed that synthesized NBDs were non-toxic and highly safe when compared with standard (chlorhexidine), this is because when all NBDs were administered to HEK 293 the normal human kidney cells, at the dose of 7.81 µg/mL, all NBDs exhibited more than 80.36 ± 1.64% HEK 293 cells viability when compared with standard. The data given in Fig. 1, presents the cell viability data as mean ± standard deviation of the mean with each experiment performed for each NBD in triplicate. The resultant cell viability data reveals that all NBDs are safe on the human kidney cells HEK 293.

Current study involved use of micro broth dilution method to determine the MIC of NBDs against peri-implantitis causing bacteria: *P. gingivalis* (ATCC 33277). Present study revealed that all synthesized NBDs exhibited MIC against tested *P. gingivalis*, with lowest concentration at 62.5 µg/mL and highest concentration at 500 µg/mL (Table-1). Among all the NBDs, compounds **4b** and **4a** exhibited the lowest MIC against *P. gingivalis* that is 125 and 62.5 µg/mL, respectively. Other compounds also exhibited MIC of 500 µg/mL. The MIC content of clear wells (in which there was no bacterial growth) of NBDs when further subjected to MBC experiment, revealed NBDs **4b** and **4a** to exhibit low bactericidal activity that is 62.5 and 125 µg/mL, respectively against *P. gingivalis*, whereas other compounds **2**, **3** and **4c** exhibited no activity (NA).

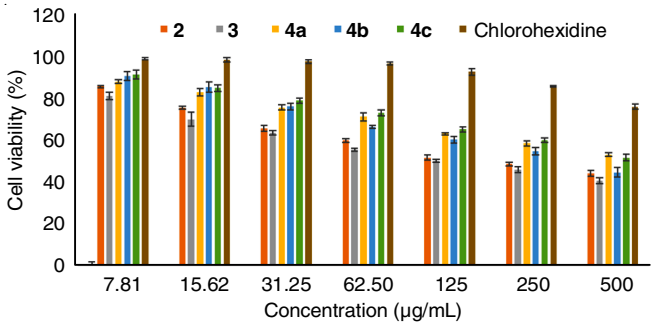


Fig. 1. Cytotoxicity analysis of synthesized NBDs against HEK-293 cells

TABLE-1
ANTIMICROBIAL ACTIVITY OF NBDs AGAINST *P. gingivalis*

NBD	MIC (µg/mL)	MBC (µg/mL)
2	500	NA
3	500	NA
4a	125	125
4b	62.5	62.5
4c	500	NA
Ampicillin	31.25	31.25

The results of antimicrobial and cytotoxicity (cell viability) studies of the synthesized NBDs revealed that substitution of electron withdrawing groups that is nitro in NBD **4a** and Cl in NBD **4b** at *para* position of benzene ring in their chemical structure imparts low MIC and MBC values on one hand and higher safety (cell viability) on the other hand. Results of the present study were also in agreement with the results of other studies on benzamidine derivatives inhibition activity against *P. gingivalis* [11,12,22,23]. Both cytotoxicity and antimicrobial studies data over NBDs supports their high safety and efficacy. However, the synthesized compounds must be further evaluated for the *in vivo* preclinical and clinical significance prior to their use in the treatment of peri-implantitis caused by *P. gingivalis*.

Conclusion

Present study highlights the successful synthesis of new benzamidine derivatives (NBDs) from 4-hydroxybenzothioamide *via* esterification, hydrazination and Schiff's reactions. The structures of synthesized NBDs were further confirmed based on the single spot TLC, sharp melting point, and IR, NMR and mass spectrometric data. Present study concludes that among all the synthesized NBDs, compounds **4a** and **4b** having electronegative group at *para*-position of the benzene ring exhibits not only exhibits good MIC and MBC against peri-implantitis triggering bacteria (*P. gingivalis)* and also

offers higher safety (cell viability) against normal human kidney cells (HEK-293). However, additional *in vivo* and clinical studies are required to further establish the safety and efficacy of NBDs **4a** and **4b** in peri-implantitis treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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