INTRODUCTION

Among the synthetic compounds, the high antimicrobial potential of benzamidines always withdraws the investigators’ attention [1]. Recently the synthesis of benzamidine analogues has become a major subject of interest for many researchers attributed to their antimicrobial properties [2]. Since 1928 of Penicillin discovery, followed by cephalosporins, the β-lactams gained high importance in organic chemistry as antibiotics [3]. β-Lactams are the well-known heterocyclic organic moieties that commonly form the basic nucleus of many commercially available antibiotics such as penicillins, cephalosporins, aztreonam, carumonam, nocardicins and thienamycin. Massive use of β-lactams antibiotics has resulted in development of resistance through mutation and β-lactamase gene transfer [4]. Therefore, continuous development of new β-lactam antibiotics is a prime requirement for organic chemists. β-Lactam is a cyclic azetidine-2-one or β-lactam is a cyclic amide that comprises four atoms in its ring structure (Fig. 1). β-Lactams are named so because the nitrogen atom is attached to the β-carbon atom relative to
the carbonyl and the simplest β-lactam is 2-azetidinone. This β-lactam ring has been proven as the main component of many pharmacophore moieties, which reflects its medicinal and chemical importance for the researchers [3].

Peri-implantitis (PI) is characterized as an inflammatory response that affects the hard and soft tissue, which results in loss of supporting bone and pocket formation surrounding the functioning of osseo-integrated dental implant [5]. The success rates for dental implants has been testified to be 82.9% [6]. Based on the Consensus Report of the Sixth European Workshop in Periodontology, the peri-implantitis incidence ranges from 28-56% [7], leading to implant loss [8]. In peri-implantitis, the biofilm structure changes towards higher counts of more pathogenic microorganisms such as Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans [9]. Among all these microorganisms, P. gingivalis is the most common pathogen which belongs to the red complex bacteria as categorized by Socransky [10]. The strategies for prevention and reduction of clinical signs of peri-implantitis includes plaque control with manual or powered toothbrushes, oral hygiene instructions to the patients and mechanical debridement along with adjunctive measures such as the use of antiseptics, local and systemic antibiotics and air-abrasive devices [11]. Local use of minocycline or doxycycline as an adjunct to mechanical debridement and irrigation with an antimicrobial means has been found to be effective in moderately deep lesions [12].

The use of gingipain inhibitors has been reported, as a new strategy to treat and prevent periodontal disease and other systematic conditions associated with it. Several synthetic compounds have been investigated as a potential gingipain inhibitors but at the same time, they are known to inhibit a broad spectrum of host proteases and offer the detrimental side effects [13]. β-Lactam antibiotics are currently the most widely used class of antibacterial agents and it accounts for 65% of all prescriptions for oral antibiotics in the United States [14]. A major advantage in the treatment of serious infections is attributed to the bactericidal action of β-lactams. For many decades researchers have synthesized several cyclic 2-azetidinones with high antibacterial activity using different synthetic methodologies [15-18]. Hence based on the problems of high prevalence of peri-implantitis, P. gingivalis (associated pathogen), resistance associated with antibiotics and the side effects associated with gingipain inhibitors, the present study was designed to perform the synthesis, characterization, in vitro cytotoxicity analysis and in vitro antimicrobial activity of novel benzamide analogues (NBA) towards P. gingivalis (cause for peri-implantitis).

**EXPERIMENTAL**

In this study, in silico docking experiment was done using AutoDock Vina. The chemicals and biologicals were procured from Friendemann Schmidt Chemical, Sigma-Aldrich, Merck KGaA, Qrec Chemicals and HmbG® Chemicals. The progress of reaction was monitored by thin layer chromatography (TLC) and melting point measurements using Spectroline UV CM-26 and Stuart Analogue Melting Point SMP11, respectively. The chemical structures of the synthesized compounds were confirmed based on attenuated total reflectance-infrared (ATR-IR) spectral, 1H & 13C nuclear magnetic resonance (NMR) spectral and direct infusion mass spectrometry (DIMS) spectral data.

*In silico molecular docking of NBA against Kgp protein of P. gingivalis:* In this in silico docking experiment, the docking of benzamide analogues was carried out to assess their interaction and binding modes with the target protein gingipain K (Kgp) of P. gingivalis (PDB ID: 4RBM) using an Intel i7 with RAM of 16 GB. To prepare protein, structure drawing and conversion to working format software including Discovery studio, ChemDraw and OpenBabel were used [19,20]. All designed chemical structures were modelled using Chemsketch software. The 2D structures of NBA were generated and conversion into respective 3D structures was done using LigPlot. The designed structures of NBA were optimized, followed by energy minimization using AutoDock software and the process of molecular docking [21]. The 3D structures of gingipain K (Kgp) of P. gingivalis (PDB ID: 4RBM) was downloaded from RCSB Protein Data Bank (PDB). The Discovery Studio Visualizer was used to prepare the downloaded Kgp protein by removing the heteroatoms and water molecules. Whereas for addition of hydrogen and assignment of the charges molecular graphics laboratory (MGL) tools were used. AutoDock Vina was used only for defining grid parameters and docking. The docking results were further analyzed using Discovery Studio Visualizer [22].

**Synthesis of N-(3-chloro-2-(substituted phenyl)-4-oxoazetidin-1-yl)-2-(4-(3-chloro-2-(substituted phenyl)-4-oxoazetidine-1-carbonothioyl)phenoxy)acetamide (3a-c):** Novel benzamide analogues (NBA, 3a-c) were synthesized as per the method stated in standard literature with minor modification [23,24]. Briefly, compound (0.0002 M) (previously synthesized by Schiff reaction of compound 1 with 4-nitrobenzaldehyde) was dissolved in 1,4-dioxane and chloroacetyl chloride (0.00025 M), followed by dropwise addition of triethyl amine (0.0003 M) with constant stirring at low temperature (0-5 ºC). Next, the mixture was stirred for 40 h (until the completion of reaction). The reaction was carried out in the presence of molecular sieves. The reaction mixture was added into crushed ice and stirred to obtain the crude product. The crude was recrystallized by dissolving crude product in absolute ethanol and refluxing with activated charcoal for 10 min to yield pure compound 3a. A similar procedure was followed to synthesize compounds 3b and 3c using 4-chlorobenzaldehyde and 4-dimethylaminobenzaldehyde (Scheme-1). The above procedure was repeated three times and optimized by raising the concentration of compound 3a-c, chloroacetyl chloride and triethylamine, from 0.0002 M to 0.002 M, 0.00025 M to 0.0025 M and 0.0003 M to 0.003 M, respectively. The TLC analysis for all the synthesized azetidinones was performed in...
absolute ethanol:chloroform (9.5:0.5 ratio) solvent system and melting points of all the synthesized compounds were recorded.

**N-(3-chloro-2-(4-nitrophenyl)-4-oxazetidin-1-yl)-2-(4-(3-chloro-2-(4-nitrophenyl)-4-oxazetidine-1-carbonothioyl)phenoxo)acetamide (3a):** IR spectrum (cm⁻¹): 3265 (N-H), 3094 (=C-H), 2954 (C-H), 1663 (C=O), 1572 (C=N), 1572 and 1342 (NO2), 1502 (C=S); 1H NMR spectrum (DMSO, ppm) δ: 4.36 (s, 2H, N-CH), 4.82 (s, 2H, CH2-C=O), 5.16 (s, 2H, CH2-Cl), 6.82-7.66 (m, 8H, Ar-H), 9.89 (s, 1H, CONH); 13C NMR spectrum (DMSO, ppm) δ: 60.06 (C-N-C=S), 65.25 (CH2CO), 73.06 (N-N-C), 78.61 (C-Cl), 83.82 (C-CI), 116.62, 124.89, 128.05, 132.17, 139.98, 150.14, 154.97, 160.14, 164.43, (Ar-C), 169.48 (C=O), 173.84 (C=O), 180.2 (C=O); Mass spectrum (m/z): 643 (M⁺).

**N-(3-Chloro-2-(4-chlorophenyl)-4-oxazetidin-1-yl)-2-(4-(3-chloro-2-(4-chlorophenyl)-4-oxazetidine-1-carbonothioyl)phenoxo)acetamide (3b):** IR spectrum (cm⁻¹): 3271 (N-H), 3071 (=C-H), 2954 (C-H), 1663 (C=O), 1572 (C=N), 1572 and 1342 (NO2), 1502 (C=S); 1H NMR spectrum (DMSO, ppm) δ: 4.36 (s, 2H, N-CH), 4.82 (s, 2H, CH2-C=O), 5.16 (s, 2H, CH2-Cl), 6.82-7.66 (m, 8H, Ar-H), 9.90 (s, 1H, CONH); 13C NMR spectrum (DMSO, ppm) δ: 60.06 (C-N-C=S), 65.25 (CH2CO), 73.06 (N-N-C), 78.61 (C-Cl), 83.82 (C-CI), 116.62, 124.89, 128.05, 132.17, 139.98, 150.14, 154.97, 160.14, 164.43 (Ar-C), 169.48 (C=O), 173.84 (C=O), 180.2 (C=O); Mass spectrum (m/z): 640 (M⁺).

**N-(3-Chloro-2-(4-(dimethylamino)phenyl)-4-oxazetidin-1-yl)-2-(4-(3-chloro-2-(4-(dimethylamino)phenyl)-4-oxazetidine-1-carbonothioyl)phenoxo)acetamide (3c):** IR spectrum (cm⁻¹): 3270 (N-H), 3072 (=C-H), 2963 (C-H), 1662 (C=O), 1572 (C=N), 1502 (C=S); 1H NMR spectrum (DMSO, ppm) δ: 2.89 (s, 12H, (CH3)2), 4.36 (s, 2H, N-CH), 4.82 (s, 2H, CH2-C=O), 5.16 (CHCl), 6.83-7.66 (m, 8H, Ar-H), 9.90 (CONH); 13C NMR spectrum (DMSO, ppm) δ: 41.92 (CH3), 60.03 (C-N-C=S), 65.22 (CH2CO), 73.02 (N-N-C), 78.65 (C-Cl), 83.86 (C-Cl), 116.67, 124.93, 128.04, 132.17, 139.97, 143.18 150.09, 154.97, 160.15, 164.39 (Ar-C), 169.48 (C=O), 173.81 (C=O), 180.2 (C=O); Mass spectrum (m/z): 640 (M⁺).

**Scheme-I:** Synthesis of NBA 3a-c

**In vitro antimicrobial activity:** In current investigation, the minimum inhibitory concentration (MIC) of NBA for *P. gingivalis* was assessed through standard micro-broth dilution assay with small changes [25]. Briefly, culturing of *P. gingivalis* was done in soy broth (at pH 7.4), followed by adjustment of *P. gingivalis* culture density (1.5 × 10⁸ CFU/mL), dilution of NBA (from 500 µg/mL to 1.95 µg/mL), addition of *P. gingivalis* culture (100 µL) to wells of microplate, incubation for 46 h (in anaerobic conditions) and finally each well absorbance was recorded at 620 nm to calculate the MIC. Next, the minimum bactericidal concentration (MBC) towards *P. gingivalis*, was determined by further incubating the clear well content (20 µL) in anaerobic conditions for 46 h plated in soy broth agar. Finally, the MBC was determined as NBA concentration with no visible growth. The experiments were commenced three times.

**In vitro cytotoxicity analysis:** After MIC and MBC experiments, the NBA were tested for their safety through cytotoxicity or cell viability towards healthy HEK 293 cells using MTT assay with small changes [26]. Briefly, propagation of HEK 293 cells was done in DMEM in T25 cm² culture flask by incubating at 37 ℃, 5% CO2 and 95% relative humidity; followed by overnight incubation for proliferation on 96-wells plate (10000 cells density), dilution of NBA in DMEM, addition to each well to achieve concentration ranging from 7.81-500 µg/mL, incubation for 24 h, addition of MTT solution (10 µL) into wells, re-incubation in dark for 4 h, pipetting each well content and addition of DMSO (100 µL) to each well, finally NBA absorbance was measured at 570 nm keeping reference at 630 nm on Microplate Reader to calculate the cell viability % using expression given in eqns. 1 and 2.

**RESULTS AND DISCUSSION**

The molecular docking study was performed to establish the binding ability of the NBA to the binding site of gingipain K (Kgp) of *P. gingivalis* (PDB ID: 4RBM) [27]. The docking scores of designed molecules are presented in Table-1.

All the compounds were found to completely occupy the active sites of 4RBM in the target protein Kgp – Gingipain K of *P. gingivalis* (4RBM). Compounds 3a and 3b of all titled compounds found to have highest D-score among all other
<table>
<thead>
<tr>
<th>Docking code number</th>
<th>Docking score</th>
<th>Structure of the compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>L (Parent lead)</td>
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</tr>
<tr>
<td>1</td>
<td>-4.8</td>
<td><img src="image2.png" alt="image2" /></td>
</tr>
<tr>
<td>1a</td>
<td>-5.2</td>
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<tr>
<td>2a</td>
<td>-7.2</td>
<td><img src="image4.png" alt="image4" /></td>
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<tr>
<td>2b</td>
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<td>2c</td>
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</tr>
<tr>
<td>2e</td>
<td>-3.2</td>
<td><img src="image8.png" alt="image8" /></td>
</tr>
<tr>
<td>3a</td>
<td>-7.3</td>
<td><img src="image9.png" alt="image9" /></td>
</tr>
</tbody>
</table>
Compounds 3a and 3b also assumes favourable orientation within the 4RBM binding site. The binding modes of compounds 3a and 3b are represented in 2D ligands interaction diagrams given in Figs. 2 and 4, respectively. The docked pose 3D interaction diagrams of compounds 3a and 3b given in Figs. 3 and 5, respectively, showed the hydrogen bond interaction with 4RBM presenting a probability of compounds to be highly active. This reveals the high inhibitory potential of compounds 3a and 3b and other NBA against \textit{P. gingivalis}. The 2D ligand interaction diagram of compounds 3a and 3b are shown in Figs. 2 and 4, respectively, reveals their significant interactions with specific amino acids of the Kgp. The 2D ligand interaction diagram of compound 3a given in Fig. 2 reveals that a hydrogen bond was formed between the oxo group contained in amide moiety of compound 3a and the leucine amino acid with residue 454 in the chain A; sulphur atom with tyrosine and glycine amino acid of residues 427 and 429; NH group with aspartic acid of residue 452 in chain A; and oxygen atom of NO2 group with aspargine of residue 433 in chain A of the Kgp - Gingipain (4RBM). These interactions are crucial for stabilizing the ligand protein complex and enhancing its binding affinity. The 3D representation of the molecular docking complex confirms the presence of these interactions and provides a view of how compound 3a fits within the active site of the Kgp - Gingipain (4RBM) presented in Fig. 3.

The 2D ligand interaction diagram of compound 3b given in Fig. 4 reveals that hydrogen bonds were formed between the oxo groups of the two azetidine rings present in the compound 3b and the threonine with residue 426 and glycine with residue 427 in the chain A of the Kgp - Gingipain (4RBM). These interactions are crucial for stabilizing the ligand protein complex and enhancing its binding affinity. The 3D representation of the molecular docking complex confirms the presence of these interactions and provides a view of how compound 3b fits within the active site of the Kgp – Gingipain K (4RBM) presented in Fig. 5.
Singh et al. [28] conducted the molecular docking study to evaluate the binding affinity and binding energy of curcumin with gingipain K (PDB ID: 4RBM) of P. gingivalis. The analysis suggests that the curcumin interacts with good affinity with gingipain K protein. Another study was conducted to evaluate the binding affinity of benzamidine derivatives to arginine-specific gingipain from periodontopathogen P. gingivalis. The average binding free energy of about –5 kcal/mol was found between HRgpA and benzamidine derivatives. The average structures of the eight complexes suggest that benzamidine inhibitors interact with Asp387, His435 and Cys468 by hydrogen bonding and with Trp508 by hydrophilic interactions that are essential for the activities of benzamidine inhibitors [29].

Based on the molecular docking results, it was revealed that among in silico docked series of designed NBA (Table-1), compounds L, 1, 1a, 2a-c and 3a-c coded for docking, offered good docking score and hydrogen bonding with the active binding site of Kgp – Gingipain K (PDB ID: 4RBM). Hence based on high docking score of molecules L, 1, 1a, 2a-c and 3a-c, it was decided to carry out the synthesis, optimization and characterization of compounds 3a-c and determine their inhibitory potential (MIC and MBC) against P. gingivalis, followed by cytotoxicity against normal healthy human cells (HEK 293).

Present study successfully synthesized and elucidated the physical and chemical properties of azetidinones, which were also correlated with the other studies [18,23,24]. Similar procedures and results were also highlighted by other studies [30,31]. The IR spectral data of compound 3a-c, revealed the presence of bands near 3229 cm⁻¹ indicating N-H, bands in the region of 3065-3051 cm⁻¹ indicating aromatic C–H, bands between 2998-2917 cm⁻¹ indicating aliphatic C-H and bands between 1682-1653 cm⁻¹ indicating C=O groups; and thereby supported the elucidation of partial chemical structure of compound 3a-c.

The results of the IR for compounds 3a-c (azetidinones) are in accordance with the study done by the Fonkui et al. [31]. The ¹H NMR spectra of compounds 3a-c (azetidinones) exhibited a characteristic δ value signal at 5.16 and between 9.89-9.90 ppm, signifying the presence of 2º amine groups due to treatment of hydrazide with aldehydes. The ¹³C NMR spectra of compounds 3a-c were confirmed due to the presence of CH-Cl groups signifying the presence of β-azetidinone ring formation due to cyclocondensation of imino group with chloroacetyl chloride. The physical properties and the characterization data confirmed and supported the successful synthesis of all compounds.

Biological activity: Novel benzamidine analogues (NBA) were further tested for cytotoxicity (safety or cell viability) towards HEK 293 using MTT assay [26]. The % cell viability was estimated and analyzed statistically by Graph Pad Prism 9.51 Software. The cell viability results confirmed that synthesized NBA are non-toxic and safe in comparison to chlorhexidine (standard). This is based on fact that administration of all NBA (at concentration of 7.81 µg/mL) to healthy cells (HEK 293), exhibited higher than 91% cell viability (Table-2).

Current investigation used micro-broth dilution assay to estimate the NBAs MIC towards P. gingivalis mediated peri-
implantitis. Azetidinone possesses a wide range of biological activities including antibacterial activities against Gram-positive and Gram-negative bacteria. In present study, the newly synthesized azetidinones (compounds 3a and 3b) exhibited a good inhibition activity against *P. gingivalis* with a MIC of 31.25 µg/mL and azetidinone (compound 3c) exhibited a moderate inhibition activity against *P. gingivalis* with a MIC of 250 µg/mL (Table-3). The results of the present study are in accordance with the study performed by Deep *et al.* [32], which showed that azetidinone derivatives of hippuric acid has got the most potent antimicrobial activity. The study performed by Desai *et al.* [33] also highlighted that azetidinone derivatives have the potential to inhibit the growth of Gram-negative microorganisms.

The antimicrobial and cell viability research on novel benzamidine analogues (NBA) indicated that presence of NO₂ and Cl (electron withdrawing groups) in compounds 3a and 3b, respectively on 4th position of benzyl ring offers significant MIC, MBC and safety. Findings of current investigation were also found to agree with the findings of other investigations on inhibitory response of benzamidines towards *P. gingivalis* [1-3,23,24]. Therefore, the current cell viability and antimicrobial research findings supports significant safety and efficacy of NBA.

**Conclusion**

The present study highlights the successful synthesis of novel benzamidine analogues (NBA) from 4-hydroxybenzo-thioamide. The structures of synthesized NBA (3a-c) were further confirmed based on ATR-IR, NMR and mass data. Current investigation concludes that synthesized NBA 3a and 3b bearing electronegative group at para-position on benzene ring offers significant MIC & MBC towards peri-implantitis triggering bacterium (*P. gingivalis*) and exhibits high safety (cell viability) towards HEK-293 (healthy cells). The current study recommends that in future additional preclinical and clinical investigations should be done to establish the efficacy and safety of NBA 3a and 3b to treat peri-implantitis.

**ACKNOWLEDGEMENTS**

The authors are grateful to the AIMST University, Malaysia, for providing student research grant to Dr. Ajay Jain (Ref No.: RMC21/RAVI/PG/AJAY) for conducting this research and assisting in publication. All the authors are also thankful to their respective Departments/Universities for the successful completion of this research.

**CONFLICT OF INTEREST**

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

**REFERENCES**


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**TABLE-2**

*In vitro* CELL VIABILITY ASSAY FOR SYNTHESIZED NBA COMPOUNDS

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.81</td>
<td>91.79 ± 1.75*</td>
<td>91.99 ± 1.56*</td>
<td>92.47 ± 0.83*</td>
<td>97.76 ± 0.55</td>
</tr>
<tr>
<td>15.62</td>
<td>87.17 ± 1.51****</td>
<td>87.92 ± 2.17****</td>
<td>86.4 ± 0.34****</td>
<td>97.39 ± 0.93</td>
</tr>
<tr>
<td>31.25</td>
<td>84.36 ± 1.74****</td>
<td>83.45 ± 1.71****</td>
<td>81.65 ± 2.01****</td>
<td>96.52 ± 0.96</td>
</tr>
<tr>
<td>62.5</td>
<td>78.29 ± 1.89****</td>
<td>74.68 ± 0.52****</td>
<td>74.53 ± 0.58****</td>
<td>95.75 ± 0.73</td>
</tr>
<tr>
<td>125</td>
<td>71.96 ± 0.88****</td>
<td>66.17 ± 0.32****</td>
<td>65.74 ± 1.75****</td>
<td>91.75 ± 1.57</td>
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<tr>
<td>250</td>
<td>63.75 ± 1.43****</td>
<td>55.95 ± 0.90****</td>
<td>57.95 ± 0.81****</td>
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</tr>
<tr>
<td>500</td>
<td>55.73 ± 1.33****</td>
<td>47.32 ± 1.31****</td>
<td>48.16 ± 1.34****</td>
<td>75.18 ± 1.16*</td>
</tr>
</tbody>
</table>

Data is presented as mean ± standard deviation with each experiment performed in triplicate. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test with compared mean standard vs. compounds (3a-c) to determine the source of significant difference between the groups using GraphPad Prism software version 10. Data were expressed as mean ± standard deviation of the mean. Statistical significance is indicated by *p < 0.05, **p < 0.01; ***p < 0.001; ****p < 0.0001.

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**TABLE-3**

MIC AND MBC DATA OF SYNTHESIZED NOVEL BENZAMIDINE ANALOUGES (NBA) AGAINST *P. gingivalis*

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>31.25</td>
<td>62.5</td>
</tr>
<tr>
<td>3b</td>
<td>31.25</td>
<td>31.25</td>
</tr>
<tr>
<td>3c</td>
<td>250</td>
<td>No activity</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>31.25</td>
<td>31.25</td>
</tr>
</tbody>
</table>

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The antimicrobial and cell viability research on novel benzamidine analogues (NBA) indicated that presence of NO₂ and Cl (electron withdrawing groups) in compounds 3a and 3b, respectively on 4th position of benzyl ring offers significant MIC, MBC and safety. Findings of current investigation were also found to agree with the findings of other investigations on inhibitory response of benzamidines towards *P. gingivalis* [1-3,23,24]. Therefore, the current cell viability and antimicrobial research findings supports significant safety and efficacy of NBA.