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Synthesis, Characterization, Cytotoxicity Analysis and Anti-Dengue Activity of Newer Nucleoside Analogues

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World Health Organization (WHO) report suggests that dengue the life-threatening disease currently has no specific medication. Hence, present study was intended to synthesize some new nucleoside analogues (NNAs) to combat the dengue virus (DENV-2). Study involved synthesis of 1-(4-((substituted cyclohexyl)methyl)-2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-1,2,4-triazinan-1-yl)ethanone (**3a-f**) by hydrogenation and acetylation of 4-((substituted-cyclohexa-2,5-dienylidene)methyl)-2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (**3a-f**), that was synthesized by treating uridine derivative (**1**) with various substituted aldehydes. The structures of newly synthesized NNAs were characterized using NMR, FTIR and mass spectrometric data. Effectiveness of the synthesized NNAs against dengue was also evaluated based on their anti-dengue activity using DENV-2 serotype and cytotoxicity evaluation against Vero cells using MTT assay. Present study reports successful synthesis of NNAs **3a-f** with high inhibition potential against DENV-2 and minimal to absence of cytotoxicity. Significant anti-dengue activity and least/no cytotoxicity of NNAs against DENV-2 supports their potential application in dengue treatment. However, synthesized NNAs should be further evaluated for preclinical and clinical significance.

Keywords: Nucleoside, DENV-2, Dengue, Anti-dengue, Uridine.

INTRODUCTION

Dengue fever has become a major threat for the health of 50% of world population [1]. The 2023 facts sheet of World Health Organization (WHO), reports occurrence of 100-400 million cases in each year, of which more than 75% cases are mild and asymptomatic. Dengue is a vector-borne disease that is transmitted by *Aedes aegypti* mosquito. Dengue is a viral infection that occurs in humans by arthropode borne flavivirus called dengue virus (DENV) [2]. There exist four serotypes of dengue (DENV-1, DENV-2, DENV-3 and DENV-4) [3]. Facts suggest DENV-2 associated dengue hemorrhagic fever (DHF) as major cause of serious ailment and eventually death. Surprisingly, till date there is no specific treatment for dengue/or severe dengue [4,5]. At present dengue treatment is limited to supportive care and fluid therapy [6]. Hence, there is urgency to search a potent anti-dengue agent [7].

Today several efficacious anti-DENV drugs are available at different development stages; however, but still none of the drug has been clinically approved for dengue treatment [8]. Few studies highlighted anti-dengue activity of various nucleoside inhibitors, safety of those drugs is a major problem [9-12]. A study reported enamines potential against Aedes aegypti [13]. Hence, evidences over dengue problem, problems of therapeutics for dengue treatment and antiviral potential of nucleoside inhibitors and enamines, current study was intended to perform the synthesis, cytotoxicity analysis and anti-dengue activity of some new nucleoside analogues (NNAs). Thus, in this work, the successful synthesis, characterization,cytotoxicity and anti-dengue activity of new nucleoside analogues *i.e.* 1-(4-((substi-

tuted cyclohexyl)methyl)-2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-1,2,4-triazinan-1-yl)-ethanone (**3a-f**) is carried out.

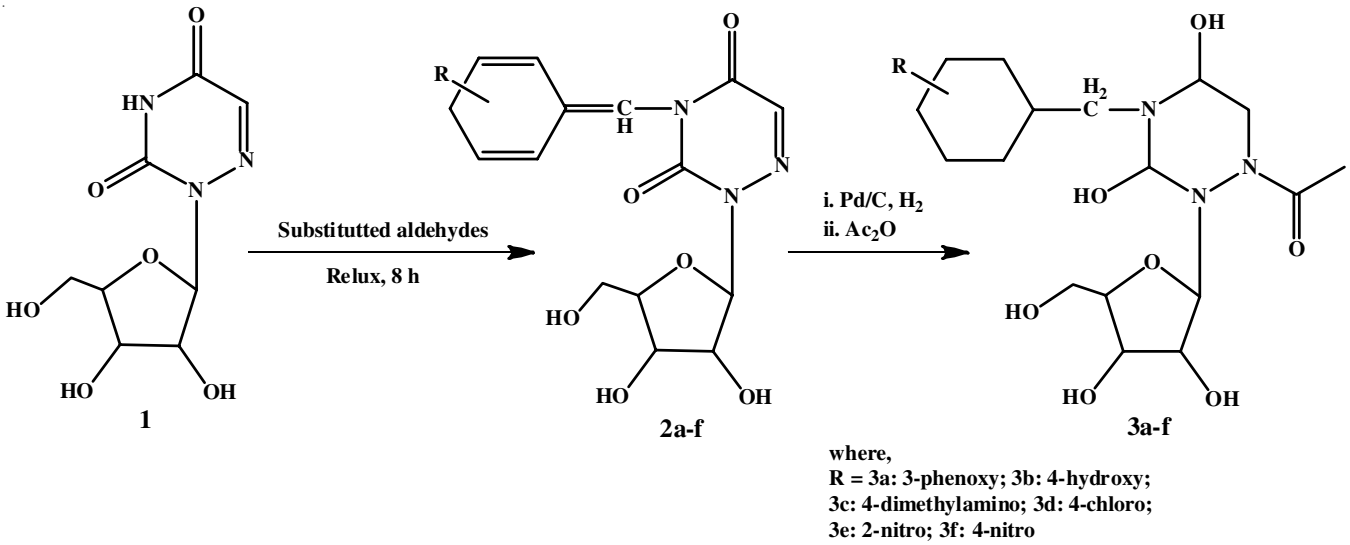
EXPERIMENTAL

The chemicals for newer nucleoside analogues (NNAs) synthesis were procured from Merck KGaA (Darmstadt, Germany), HmbG® Chemicals (Hamburg, Germany), Sigma-Aldrich Co. (St. Louis, USA), Friendemann Schmidt Chemical (Washington, DC, USA) and Qrec Chemicals (Rawang, Malaysia). The synthesized compounds were characterized based on ^{1}H NMR and ^{13}C NMR (Bruker Avance Neo NanoBay NMR 400 MHz spectrometer) signals on δ value scale with tetramethylsilane (TMS) as standard. The FTIR data of NNAs was recorded by Jasco ft/ir-6700 instrument at wavelength of 4000 to 400 cm^{-1}. The mass spectra of NNAs were recorded by Direct Infusion IonTrap MS Full Scan Thermo-Scientific Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer (Waltham, MA, USA). The NNAs purity, was determined by measuring melting points using SMP11 Analogue apparatus. Reaction monitoring was done by TLC on aluminum sheets with silica gel 60 F_{254} (0.2 mm) (Merck Millipore, Germany) using CH_3OH:$CHCl_3$ (9.5:0.5) as solvent system in SPRECTROLINE® CM-26 UV chamber.

Synthesis of 1-(2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-4-((substituted cyclohexyl)methyl)-1,2,4-triazinan-1-yl)ethenone (3a-f): Synthesis of compound **3a-f** (NNAs) was performed as per the standard procedure with minor modifications [14-18]. Briefly, compound **1** and hydrazine hydrate in equimolar concentration were refluxed for 8 h to offer intermediary compound **3a**, that was further hydrogenated (in presence of palladium) followed by treatment with acetic anhydride in the equimolar concentration (1.0 mmol) to offer crude product, that was recrystallized with methanol using activated charcoal to yield:pure compound **3a** (**Scheme-I**). Using similar experimental protocol, compounds **3b-f** were also synthesized.

1-(2-(3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-4-((3-phenoxycyclohexyl)-methyl)-1,2,4-triazinan-1-yl)ethenone (3a): Light brown crystals; yield: 79%, m.p.: 148 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3300-3150 (O-H), 3078 (=C-H), 2955, 2824 (-C-H), 1716 (C=O), 1716 (C=O), 1685 (N-C=O), 1583 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.247 (m, 2H, H3″), 1.523 (m, 2H, H2″),1.677 (m, 1H, H1″), 1.906 (m, 1H, H6″), 1.952 (m, 2H, H4″), 2.000 (s, 3H, CH_3), 2.308 (d, 2H, CH_2-N), 3.229 (d, 2H, H6), 3.331 (m, 1H, H3′), 3.416 (m, 2H, H5′), 3.523 (m, 1H, H4′), 3.633 (m, 1H, H5″), 3.864 (m, 1H, H2′), 4.001 (brs, 1H, OH5′), 4.221 (brs, 1H, OH2′), 5.069 (brs, 1H, OH3′), 5.312 (m, 1H, H5), 5.427 (brs, 1H, OH5), 5.703 (brs, 1H, OH3), 5.880 (d, 1H, H1′), 5.993 (d, 1H, H3), 7.061-7.706 (m, 5H, Ar′-H); and ^{13}C NMR (DMSO, ppm) δ: 20.211 (CH_3), 24.166 (C3″), 37.745 (C1″), 38.104 (C2″), 39.149 (C4″), 40.149 (C6″), 45.208 (CH_2), 51.126 (C5″), 56.104 (C6), 61.970 (C5′), 70.307 (C5″), 72.257 (C2′), 74.316 (C3′), 76.428 (C5), 78.495 (C1′), 84.470 (C4′), 89.309 (C3), 117.352 (C2‴ & 6‴), 122.855 (C4‴), 124.169 (C3‴ & C5‴), 155.786 (C1‴), 166.694 (C=O); and Mass (*m/z*): 481. Anal. calcd. (found) % for $C_{23}H_{35}N_3O_8$: C, 57.37 (57.41); H, 7.33 (7.29); N, 8.73 (8.69).

1-(2-(3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-4-((4-hydroxycyclohexyl)-methyl)-1,2,4-triazinan-1-yl)ethanone (3b): Pale yellow crystals; Yield: 76%, m.p.: 149 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3300-3150 (O-H), 3080 (=C-H), 2957, 2827 (-C-H), 1729 (C=O), 1683 (C=O), 1597 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.538 (m, 4H, H2″ & H6″), 1.739 (1H, m, H1″), 1.925 (m, 4H, H3″ & H5″), 2.004 (s, 3H, CH_3), 2.308 (d, 2H, CH_2-N), 3.226 (d, 2H, H6), 3.339 (m, 1H, H3′), 3.401 (m, 2H, H5′), 3.499 (m, 1H, H4″), 3.522 (m, 1H, H4′), 3.768 (m, 1H, H2′), 4.003 (brs, 1H, OH5′), 4.210 (brs, 1H, OH2′), 5.053 (brs, 1H, OH3′), 5.277 (m, 1H, H5), 5.411 (brs, 1H, OH5), 5.669 (brs, 1H, OH3), 5.880 (d, 1H, H1′), 5.998 (d, 1H, H3); ^{13}C NMR (DMSO, ppm) δ: 20.628 (CH_3), 24.166 (C2″ & C6″), 38.898 (C3″ & C5″), 39.107 (C1″), 45.219 (CH_2), 56.104 (C6), 61.973 (C5′), 72.256 (C2′), 74.316 (C3′), 76.428 (C5), 78.495 (C1′), 80.876 (C4″), 84.569



Scheme-I: Synthetic route of NNA **3a-f**

(C4′), 89.309 (C3), 163.353 (C=O); and Mass (*m/z*): 405. Anal. calcd. (found) % for $C_{17}H_{31}N_3O_8$: C, 50.36 (51.32); H, 7.71 (7.68); N, 10.36 (10.41).

1-(2-(3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-4-((4-(dimethylamino)cyclohexyl)methyl)-3,5-dihydroxy-1,2,4-triazinan-1-yl)ethanone (3c): White crystals; yield: 80%, m.p.: 200 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3350-3150 (O-H), 3080 (=C-H), 2996, 2794 (-C-H), 1729 (C=O), 1678 (C=O), 1585 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.516 (m, 4H, H2″ & H6″), 1.648 (m, 1H, H1″), 1.902 (m, 4H, H3″ & H5″), 2.002 (s, 3H, CH_3), 2.169 (s, 6H, $(CH_3)_2$), 2.496 (d, 2H, CH_2-N), 2.599 (m, 1H, H4″), 3.278 (m, 2H, H6), 3.347 (m, 1H, H3′), 3.385(m, 2H, H5′), 3.498 (m, 1H, H4′), 3.774 (m, 1H, H2′), 4.2222 (brs, 1H, OH5′), 4.642 (brs, 1H, OH2′), 5.037 (brs, 1H, OH3′), 5.429 (m, 1H, H5), 5.519 (brs, 1H, OH5), 5.781 (brs, 1H, OH3), 5.880 (d, 1H, H1′), 5.979 (d, 1H, H3); ^{13}C NMR (DMSO, ppm) δ: 20.429 (CH_3), 26.217 (C2″ & C6″), 30.004 (C3″ & C5″), 39.364 (C1″), 45.219 (CH_2), 49.586 $(CH_3)_2$, 56.104 (C6), 61.965 (C5′), 70.301 (C4″), 72.252 (C2′), 74.199 (C3′), 76.431 (C5), 78.466 (C1′), 84.571 (C4′), 89.299 (C3), 163.353 (C=O); and Mass (*m/z*): 432. Anal. calcd. (found) % for $C_{19}H_{36}N_4O_7$: C, 52.76 (52.82); H, 8.39 (8.44); N, 12.95 (13.01).

1-(4-((4-Chlorocyclohexyl)methyl)-2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-1,2,4-triazinan-1-yl)ethanone (3d): Pale yellow crystals; yield: 75%, m.p.: 134 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3350-3150 (O-H), 3080 (=C-H), 2996, 2794 (-C-H), 1729 (C=O), 1670 (C=O), 1585 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.516 (m, 4H, H2″ & H6″), 1.648 (m, 1H, H1″), 1.902 (m, 4H, H3″ & H5″), 2.103 (s, 3H, CH_3), 2.496 (d, 2H, CH_2-N), 3.399 (m, 2H, H6), 3.341 (m, 1H, H3′), 3.393 (m, 2H, H5′), 3.405 (1H, m, 4″), 3.512 (m, 1H, H4′), 3.765 (m, 1H, H2′), 4.217 (brs, 1H, OH5′), 4.644 (brs, 1H, OH2′), 5.031 (brs, 1H, OH3′), 5.399 (m, 1H, H5), 5.521 (brs, 1H, OH3), 5.778 (brs, 1H, OH5), 5.881 (d, 1H, H1′), 5.982 (d, 1H, H3); ^{13}C NMR (DMSO, ppm) δ: 20.634 (CH_3), 24.212 (C2″ & C6″), 38.890 (C3″ & C5″), 39.099 (C1″), 45.133 (CH_2), 56.122 (C6), 61.965 (C5′), 72.246 (C2′), 74.298 (C3′), 76.433 (C5), 78.502 (C1′), 80.869 (C4″), 84.562 (C4′), 89.301 (C3), 156.504 (C=O); Mass (*m/z*): 423; Anal. calcd. (found) % for $C_{17}H_{30}N_3O_7Cl$: C, 48.17 (47.99); H, 7.13 (7.09); N, 9.91 (9.86).

***N*-(2-((2-(3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-1,2,4-triazinan-4-yl)methyl)-cyclohexyl)acetamide (3e):** White crystals; yield: 84%, m.p.: 156 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3350-3150 (O-H), 3079 (=C-H), 2865 (-C-H), 1729 (C=O), 1677 (C=O), 1529 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.304 (m, 2H, H4″), 1.438 (m, 2H, H5″), 1.547 (m, 2H, H6″), 1.699 (m, 2H, H3″), 2.038 (s, 3H, CH_3), 2.308 (1H, m, H1″), 2.496 (d, 2H, CH_2-N), 3.032 (m, 2H, H6), 3.299 (m, 1H, H2″), 3.344 (m, 1H, H3′), 3.482 (m, 2H, H5′), 3.769 (m, 1H, H4′), 3.998 (m, 1H, H2′), 4.216 (brs, 1H, OH5′), 4.640 (brs, 1H, OH2′), 5.007 (brs, 1H, OH3′), 5.329 (m, 1H, H5), 5.417 (brs, 1H, OH3), 5.632 (brs, 1H, OH5), 5.876 (d, 1H, H1′), 5.895 (d, 1H, H3), 5.992 (NH-N), 10.240 (brs, 1H, NH-C=O); ^{13}C NMR (DMSO, ppm) δ: 20.599 (CH_3), 22.957 (C4″), 25.288 (C5″), 28.909 (C6″), 32.945 (C3″), 36.044 (C1″), 45.008 (CH_2), 52.867 (C2″), 56.128 (C6), 61.969 (C5′), 70.307 (C2′), 72.259 (C3′), 76.912 (C5), 78.667 (C1′), 84.569 (C4′), 89.301 (C3), 189.955 (C=O); Mass (*m/z*): 404; Anal. calcd. (found) % for $C_{17}H_{32}N_4O_7$: C, 50.48 (50.52); H, 7.97 (8.08); N, 13.85 (13.90).

***N*-(4-((2-(3,4-Dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-1,2,4-triazinan-4-yl)methyl)-cyclohexyl)acetamide (3f):** Light brown crystals; yield: 86%, m.p.: 142 ºC; FTIR (KBr, ν_{max}, cm^{-1}): 3350-3150 (O-H), 3079 (=C-H), 2865 (-C-H), 1729 (C=O), 1729 (C=O), 1675 (N-C=O), 1529 (C=N); ^{1}H NMR (DMSO-d_6, ppm) δ: 1.524 (m, 4H, H2″ & H6″), 1.703 (m, 1H, H1″), 1.993 (m, 4H, H3″ & H5″), 2.073 (s, 3H, CH_3), 2.496 (CH_2-N), 3.03 (m, 2H, H6), 3.345 (m, 1H, H3′), 3.398 (m, 2H, H5′), 3.495 (m, 1H, H4′), 3.771 (m, 1H, H2′), 4.008 (m, 1H, H4″), 4.213 (brs, 1H, OH5′), 4.649 (brs, 1H, OH2′), 5.036 (brs, 1H, OH3′), 5.326 (m, 1H, H5), 5.518 (brs, 1H, OH3), 5.739 (brs, 1H, OH5), 5.887 (d, 1H, H1′), 5.986 (m, 1H, H3), 5.992 (NH-N), 10.158 (brs, 1H, NH-C=O); ^{13}C NMR (DMSO, ppm) δ: 20.631 (CH_3), 24.159 (C2″ & C6″), 37.998 (C3″ & C5″), 39.964 (C1″), 45.204 (CH_2), 56.112 (C6), 61.972 (C5′), 72.261 (C2′), 74.309 (C3′), 76.433 (C5), 78.492 (C1′), 80.866 (C4″), 84.575 (C4′), 89.314 (C3), 192.359 (C=O); Mass (*m/z*): 404; Anal. calcd. (found) % for $C_{17}H_{32}N_4O_7$: C, 50.48 (50.42); H, 7.97 (7.93); N, 13.85 (13.81).

Cytotoxicity analysis: The synthesized NNAs **3a-f** were evaluated for their cytotoxicity using standard protocol for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay with minor modifications [19]. Briefly, the Vero cells C1008 (ATCC, USA) were propagated in Dulbecco's modified eagle medium (DMEM) (Corning, USA) and supplemented with 5% heat inactivated fetal bovine serum (FBS) in 5% CO_2 incubator, humidified at 37 ºC. For the study, Vero cells were grown on 96-well culture plate with 1×10^4 cells density per well and incubated over-night for attachment of cells. Serial dilutions of NNAs **3a-f** and standard were prepared in DMEM and added in each well to make the final concentrations of 12.5, 25, 50, 100, 200 and 400 µg/mL, respectively. The plates were subjected to incubation for 72 h at 37 ºC with 5% CO_2, followed by addition of 10 µL MTT solution (Merck, USA) to each well and further incubation for 4 h in dark at 37 ºC. Next, the wells content of plate was pipetted out, followed by addition of DMSO (100 µL) to each well to dissolve crystal formazan. Next, the absorbance was measured using GloMax Multi Detection System (Promega, USA) at 490 nm with reference wavelength of 750 nm and finally percentage (%) cell viability was calculated by applying the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Sample absorbance treated well (mean)}}{\text{Control absorbance untreated well (mean)}}$$

***In vitro* anti-dengue activity:** The *in vitro* anti-dengue activity of the synthesized compounds **3a-f** against DENV-2 was performed as per the established procedures and standard protocol with slight modification [20-22]. The Vero cells were seeded onto 24-well culture plates at cell density of 5×10^4 cells per well and allowed for overnight incubation for cell attachment. The next day, the Vero cells were infected with DENV-2 at multiplicity of infection (MOI) of 0.1 except for the cell

control. Compounds were added in triplicates into each designated well 3 h after infection (post-treatment) in different concentrations. The cells were rinsed twice with PBS. The plates were subjected to incubation for 72 h at 37 ºC with 5% CO_2. The plates were subjected to five freeze-thaw cycle at the end of incubation and the supernatants were collected and stored at -80 ºC until further analysis. The cells supernatant was subjected to RNA extraction using quantitative real-time polymerase chain reaction (qRTPCR) assay [23] was used to determine the DENV-2 viral load. The DENV-2 viral RNA was extracted from the supernatant by using the Favorgen RNA extraction kit (Favorgen Biotech Co., Ping Tung, Taiwan) according to the manufacturer's protocol and the extracted RNA was stored at -80 ºC until analysis. An in-house qPCR utilizing SYBR green dye (Biorad, USA) with primers DENV-2 were developed. The forward primer sequence for DENV-2 is 5′-AGTTGTTAGTCTACGTGGACCGACA while the reverse primer sequence is 5′-CGCCACAAGGGCCATGAACAG, with amplicon size of 251bp. The assay was performed using the Bio-rad CFX 96 under the following conditions: 15 min activation at 95 ºC, followed by 35 cycles of 30 s at 95 ºC, 30 s at 60 ºC and 1 min at 72 ºC and final elongation at 72 ºC for 10 min. Standard curves were prepared by qRTPCR using serial dilution of known copies number of purified amplification product for DENV-2 (10^8 PFU/mL to 10^3 PFU/mL). The experimental data was subjected to statistical analysis so as to determine the IC_{50} of all NNAs **3a-f**.

RESULTS AND DISCUSSION

Treatment of 2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (**1**) with different aldehydes yielded intermediary enamines (**3a-f**), that were hydrogenated followed by acetylation to offer 1-(2-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-3,5-dihydroxy-4-((substituted cyclohexyl)methyl)-1,2,4-triazinan-1-yl)ethenone (**3a-f**). Purity of the synthesized NNAs **3a-f** was determined based on the elemental analysis, single spot thin-layer chromatography (TLC) pattern and the sharp melting point. The elemental analysis revealed that elements of C, H and N were within ±0.4% of theoretical values. The structure of synthesized enamines was characterized using FTIR, ^{1}H NMR, ^{13}C NMR and mass spectrometric data. The spectral data of NNAs was characterized based on the literary facts [24]. The successful synthesis of NNAs **3a-f** was confirmed based on the presence of the characteristic FTIR bands between 3014-3079 cm^{-1}, ^{1}H NMR signal at 9.66-10.88 (s, 1H, =CH-N) and ^{13}C NMR signal at 115.00-115.67 (=C-N).

Biological activity: Cytotoxicity study of NNAs **3a-f** was evaluated by MTT assay on Vero cells using 96-well culture plate. The percentage cell viability was calculated and statistically analyzed using Graph Pad Prism for Windows, version 9.51 (GraphPad Software Inc., USA). The cytotoxicity experimental protocol was based on the standard literature [19]. The cytotoxicity study results suggest that the synthesized NNAs **3a-f** were non-toxic and were much safer when compared to the standard (6-azaudridine), this is because when NNAs **3a-f** were added to Vero cells at the dose of 12.5 µg/mL exhibited more than 84.21 ± 1.65% Vero cells viability, whereas standard exhibited 65.13 ± 6.38 % Vero cell viability (Table-1). 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-f**) to determine the source of significant difference between the groups using GraphPad Prism software version 5. Data were expressed as mean ± standard deviation of the mean. Statistical significance is indicated by $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ (Fig. 1).

The cytotoxicity results of the synthesized NNAs **3a-f**, indicates much higher safety of newly synthesized NNAs when compared with standard. The results of present study were also in agreement with the results of other studies. For example, the study of Park *et al.* [25] also reported 6-azauridine to cause cytotoxicity. Among NNAs **3a-f**, compounds **3d** and **3e** exhibited maximum safety, as exhibited more than 90% cells viability. Relating the cytotoxicity study data and chemical structure of NNAs **3a-f** and standard, revealed that substitution of strong electron withdrawing group for example -NO_2 and -Cl at position 4 in cyclohexyl ring in the structure of NNAs for compounds **3d** and **3f** offers higher Vero cells viability/Safety.

Anti-dengue activity: The anti-dengue activity of NNAs **3a-f** against DENV-2 serotype was done using 6-azauridine as standard [23,26]. The DENV-2 infected Vero cells were treated with different dilutions of NNAs **3a-f**. Among all NNAs, the compounds **3e** and **3f** exhibited half minimal inhibitor concen-tration (IC_{50}) when compared with standard. The IC_{50} of compound **3** was found to be lower than the standard. The IC_{50} data for all NNAs is given in Table-2.

The results of anti-dengue study of NNAs **3a-f** revealed that the substitution of very strong electron withdrawing group that is nitro group at position of 4 and 6 of cyclohexyl ring in the structure of NNAs for **3e** and **3f** offers maximum DENV2 inhibition. Both the cytotoxicity and anti-dengue studies data over NNAs **3a-f** supports their high safety and efficacy. How-

TABLE-1
CYTOTOXICITY VALUES OF SYNTHESIZED NNAs **3a-f**

Conc. (µg/mL)	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	Standard
400	88.67 ± 0.32	88.48 ± 5.62	86.69 ± 4.99	98.77 ± 1.58**	92.39 ± 4.09	87.36 ± 2.93	86.14 ± 2.13
200	88.85 ± 3.41	89.11 ± 3.43	85.1 ± 0.55	98.79 ± 2.24*	90.25 ± 2.23	82.42 ± 9.30	83.12 ± 8.63
100	93.87 ± 4.56	86.35 ± 4.68	83.72 ± 2.77	98.17 ± 2.46*	91.66 ± 1.82	81.22 ± 7.44	86.35 ± 5.16
50	92.05 ± 5.21	84.08 ± 7.29	87.86 ± 2.75	97.7 ± 2.47**	94.29 ± 1.34*	79.89 ± 4,81	81.71 ± 6.27
25	88.82 ± 9.72	83.90 ± 2.90	85.93 ± 3.56	97.52 ± 2.19*	91.14 ± 3.61	83.98 ± 8.29	77.81 ± 4.46
12.5	86.48 ± 6.35***	86.56 ± 1.83***	86.22 ± 7.04***	97.36 ± 2.78****	90.02 ± 3.23****	84.21 ± 1.65***	65.13 ± 3.68

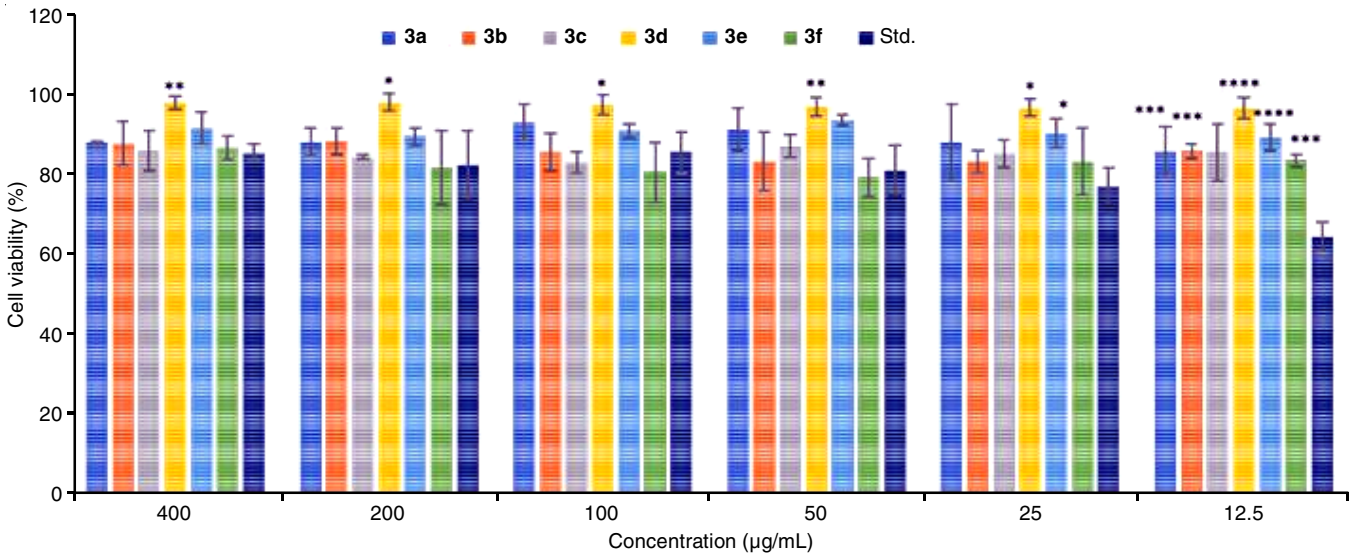


Fig. 1. Cytotoxicity analysis of synthesized nucleoside analogues (NNAs) against Vero cells C1008 (where, $^{*}p < 0.0001$)

TABLE-2
ANTIDENGUE ACTIVITY OF COMPOUND **3a-f**

NNAs	IC_{50} (µg)
3a	190.70
3b	91.02
3c	139.10
3d	142.80
3e	43.54
3f	31.32
6-Azauridine	6.25

ever, the synthesized compounds must be further evaluated for their *in vivo* preclinical and clinical significance.

Conclusion

Based on physical and chemical characterization, melting point, IR spectrum, mass and NMR data, the present study confirmed the successful synthesis of new nucleoside analogues (NNA, **3a-f**). This study claims that synthesized NNAs **3a-f** offers higher safety when compared with 6-azauridine and exhibits significant inhibition of DENV2. Present study offered compound **3f** which possess better IC_{50} and safety when compared with 6-azauridine. The significant antiviral activity of NNAs against DENV2 supports their potential application in the treatment of dengue. However, in preclinical and clinical *vivo* studies are essential to further support these findings.

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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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