Synthesis of Biuret Derivatives as Potential HIV-1 Protease Inhibitors Using (LDHs-g-HMDI-Citric Acid), as a Green Recyclable Catalyst

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In this study, a novel catalyst based on layered double hydroxides (LDHs) attached by hexamethylene-1,6-diisocyanate (HMDI) and citric acid (LDHs-g-HMDI-Citric acid) is reported and used to increase the yield of biurets synthesis. Biuret derivatives **5a–n** were prepared by reaction of several phenyl allophanates (**3a–d**), which prepared from the reaction of phenyl chloroformate and urea derivatives (**2a–d**), with variously substituted amines (**4a–g**) in the presence of LDHs-g-HMDI-Citric acid as a reusable heterogeneous catalyst at reflux condition for 60–180 min. These biurets (**5a–n**) were evaluated for human immunodeficiency virus type-1 (HIV-1) protease inhibitory activity by HIV-1 p24 antigen ELISA kit and six of them (5n, 5i, 5j, 5 m, 5f, and 5a) showed moderate activity on HIV-1 virus with IC_{50} values ranging from 55 to 100 μM compared with the azidothymidine as the reference drug (IC_{50} = 0.11 μM). Results of the *in vitro* test and docking study were in good correlation.

Keywords: Biurets, Synthesis, Layered double hydroxides catalyst, HIV-1 protease inhibitor, Molecular docking

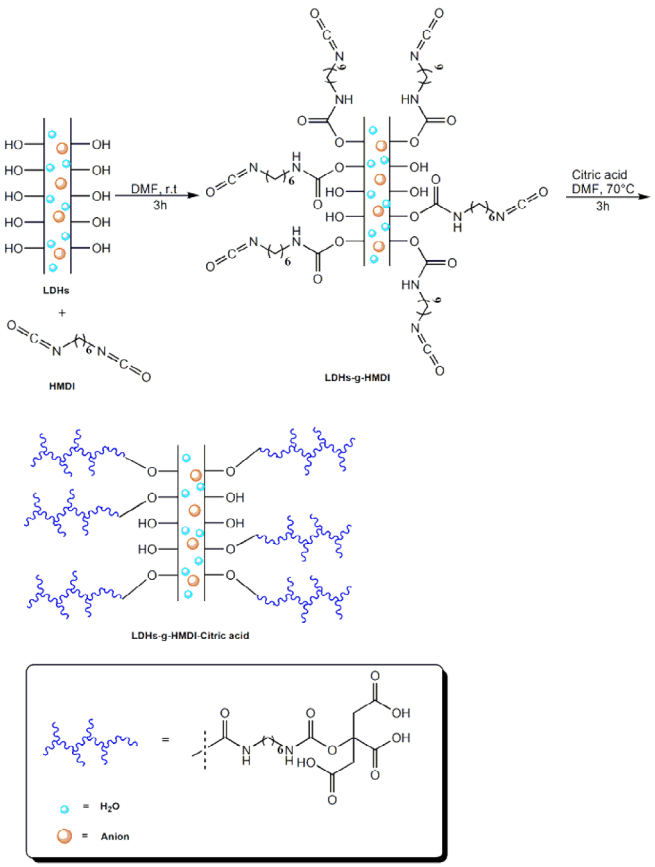
Introduction

Human immunodeficiency virus type-1 (HIV-1) is a retrovirus capable of suppressing the immune system by destroying CD4 lymphocytes.[1,2] Over time, damages to the immune system by HIV infection lead to different pathological problems, including severe infections and certain cancers.[3] HIV-1 protease is an enzyme that is crucial for viral assembly and maturation.[4] Viral RNA is translated into a polypeptide sequence that includes several individual proteins (reverse transcriptase, transcriptase, protease, and integrase). The HIV-1 protease (EC 3.4.23.16) is a viral aspartic protease that can recognize Phe-Pro and Tyr-Pro sequences and cleaves Gag and Gag-Pol viral polyproteins into functional enzymes, which provides a proper situation for HIV-1 survival and assembly.[5,6] Therefore, it is possible to fight HIV-1 infection using the substances that can inhibit HIV-1 protease activity. Inhibitors of HIV-1 protease can lead to the production of new noninfectious viruses with disorganized structures. Therefore, they could be considered as potential therapeutic agents to treat HIV/AIDS. To date, numerous HIV-1 protease inhibitors have been introduced in the literature, and some of them, such as saquinavir, indinavir, and their analogs, are approved by the FDA.[7] However, in long-term treatment, resistance to these drugs and several adverse effects such as insulin resistance, dyslipidemia, cerebrovascular and cardiovascular diseases, and unacceptable toxicity have been identified for these inhibitors which limit their clinical use.[8-10] Therefore, finding novel HIV-proteases inhibitors with a novel structure, improved bioavailability, and low toxicity is compulsory to improve the quality of HIV patients.

Today, anti-HIV-1 peptides (AHPs) have an essential role in the treatment of HIV infection. They mimic many key enzyme substrates or important epitopes. Their specific binding to their targets, which results in relatively few off-target side effects, and also a low potential for systemic toxicity.[11,12] In this regard, biuret derivatives with pseudo peptide structures could be considered as anti-HIV-1 protease peptide-like agents. There are several reports on various pharmacological effects of biuret derivatives, such as inhibition of gastric acid secretion, anti-analgesic, anti-inflammatory, anti-nociceptive, anti-leishmaniasis, antitumor, and hypoglycemic activity.[13-16] However, the activity of these compounds against HIV has not been investigated.

In the past few decades, scientists have shown great interest in developing green chemistry and protecting the living environment.[17] Citric acid is one of the safe and most environmentally benign agents that is widely used as a green biocatalyst in one-step synthesis.[18-22] It exists especially in concentrate lemons and limes, which is applied in the food, beverage, cosmetic, and pharmaceutical industries.[23] Following our previous study on the synthesis of new biuret compounds,[16] an eco-friendly procedure using modified layered double hydroxides (LDHs), as a green recyclable heterogeneous catalyst (Scheme 1), was used for the synthesis of these derivatives in shorter time reaction and higher yield.

LDHs are a class of anionic clay minerals with octahedral layer structure that are represented by the general formula $[M_{1-x}^{2+} M_x^{3+} (OH)_2]^{x+}[A^{n-}]_{x/n}. mH_2O$.[24,25] In this formula, M^{2+} and M^{3+} indicate any divalent and trivalent metal cations, respectively, and x is the metal ratio $M^{3+}/(M^{2+} + M^{3+})$.[24-26] A^{n-} is an interlayer and the exchangeable anion (organic or inorganic) and m is the amount of water present in the interlayer area.[27,28] Due to the ability to combine a wide variety of metal cations and anions and specific structure of LDHs, they have exhibited various applications.[24,25] The applications contain adsorbents, anion exchangers, medical applications ranging from simple antacids to targeted drug delivery mechanisms,[29-33] additives in polymers,[34] anti-wear additives in lubricants like motor oil,[35,36] flame retardants,[37,38] the formation of interesting nano-composite materials,[39-45] as well as catalysts and precursors to mixed metal oxide catalysts.[46-49] In recent years, the development of magnetic LDH-based nanoparticles (MLDHs) with a large surface area as a magnetic nanocatalyst is in great attention.[50,51] There are various methods for preparing LDHs, such as co-precipitation,[52] urea hydrolysis,[53] the sol–gel method,[54] hydrothermal synthesis,[55] reformation,[56] and mechanical milling.[57]



Scheme 1. Preparation of LDHs-g-HMDI-citric acid.

In this paper, the co-precipitation method was used, which is the most common and inexpensive "one-pot" method for the preparation of citric acid grafted hexamethylene-1,6-diisocyanate-g-supported layered double hydroxides (LDHs-g-HMDI-citric acid), as a green recyclable heterogeneous catalyst for the synthesis of biuret derivatives (**5a**–**5n**). The anti-HIV activity of these compounds was assessed *in vitro*. A docking study on the derivatives with the highest inhibitory effect on HIV-1 protease was also carried out.

Experimental

General. All common chemicals for the synthesis step were purchased from Merck (Darmstadt, Germany). Reagents used for cell culture were obtained from Gibco (Waltham, Massachusetts, USA). The single-chain replication (SCR) HIV-1 was supplied by the Pasteur Institute of Iran (Tehran, Iran). HIV-1 p24 antigen ELISA kit was purchased from BioMerieux (Marcy l'Etoile, France). Ultrapure water was used to prepare all of the aqueous solutions. Melting points were determined on a MEL-270 Sibata melting point apparatus and are uncorrected. For Fourier transform infrared spectroscopy (FT-IR) analysis, the FT-IR spectra were recorded on a Nicolet Magna FT-IR 550 spectrometer (Nicolet Instrument Co., WI, USA), using FT-IR grade potassium bromide (KBr). ^{1}H nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a 400 MHz Brucker spectrometer using a deuterated solvent (DMSO-d_6, and $CDCl_3$). Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as internal standard. Mass spectra were obtained using a Finningan TSQ-70 instrument (Thermo Finnigan LLC,San Jose, CA). Elemental analyses for C, H, and N were performed using a Heracus CHN–O rapid elemental analyzer and the results are within ±0.4% of the theoretical values. Qualitative analysis of LDHs-g-HMDI-citric acid was performed by energy-dispersive X-ray spectroscopy (EDX) on a FESEM-SIGMA instrument (Oberkochen, Germany). Scanning electron microscopy (SEM) micrograph was acquired on a VEGA 2 TESCAN SEM operated at 15–20 kV. The curves obtained from thermogravimetric analysis (TGA) were recorded under the air atmosphere using TGA/DTA PYRIS DIAMOND (Pyris Diamond Perkin-Elmer, Waltham, MA, USA).

General Procedure for Synthesis of LDHs-g-HMDI-Citric Acid Catalyst

Preparation of Layered Double Hydroxides (LDHs). The conventional co-precipitation method was used for the synthesis of LDHs in a one-pot reaction.[58] Briefly, an aqueous salt solution was prepared by adding a zinc salt (Zn $(NO_3)_2.6H_2O$) and a chromium Salt ($Cr(NO_3)_3.9H_2O$) with the Zn/Cr molar ratio of 3:1. Subsequently, the mixture was added dropwise to NaOH solution (8 M) in a 250 mL round-bottom flask at 80 °C under vigorous stirring. Then, the pH of the solution was adjusted to 7.0. The solution was stirred for 48 h at 80 °C for crystallization. Then, the product was filtered and washed with deionized water several times and dried overnight at 100 °C.

Anchoring of HMDI onto LDHs Surface (LDHs-g-HMDI). In order to prepare LDHs-g-HMDI, 1 g of LDHs was added into 20 mL of anhydrous DMF and stirred at room temperature for 2 h. Then, 17 mmol of hexamethylene-1,6-diisocyanate (HMDI) in 15 mL anhydrous DMF was added dropwise into the flask, and the mixture was stirred for another 3 h. All unreacted HMDI was removed by filtration and washing with DMF three times. The product was dried under vacuum overnight.

Preparation of LDHs-g-HMDI-Citric Acid. To a stirred solution of prepared LDHs-g-HMDI (1 g) in anhydrous DMF (20 mL) at 70 °C, 15 mL of citric acid solution (20 mmol) in anhydrous DMF (15 mL) was added dropwise with continuous stirring for 3 h and then cooled to room temperature (25 ± 1 °C). The resulting solid product was then filtered and washed with anhydrous DMF several times and then dried at 70 °C.

General Experimental Procedure for the Modified Synthesis of Biuret Derivatives 5a-n Catalyzed by LDHs-g-HMDI-Citric Acid. As in the previous study, urea derivatives **2a–d** were synthesized by the reaction of amine hydrochlorides **1a–d** with potassium isocyanate in water.[59] Phenyl allophanates **3a–d** were prepared by reaction of phenyl chloroformate with ureas **2a–d** in dichloromethane in the presence of pyridine at 0 °C.[16] A mixture of allophanates **3a–d** (1 mmol) and variously substituted amines **4a–g** (1 mmol) in 10 mL anhydrous dichloromethane in the presence of LDHs-g-HMDI-Citric acid as catalyst was refluxed for 60–180 min. After completion of the reaction, which was monitored by thin-layer chromatography, the mixture was allowed to cool at room temperature and then centrifuged and the supernatant was separated from the catalyst. After washing with water (three times), the solvent was evaporated under a vacuum. The products were purified by preparative thick layer chromatography as previously reported (Scheme 2) and the structures of the selected biuret derivatives were admitted on the basis of their physical and spectral data (FT-IR, ^{1}H NMR, and MS).

Spectral Data for the Biuret Derivatives

***N*,N′-Bis Phenyl Imidodicarbonic Diamide (**5a**).** White solid; Yield 91%; mp 212 °C; IR (KBr) ν_{max}: 3386, 3200, 3130, 3024, 1690, 1605, 1550, 1237 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 7.02–7.5 (m, 12H), 9.50 (s, 1H) ppm. MS (EI) m/z: 533.4 (M^+), 402.995, 255, 213, 137, 106, 91. Anal.Calcd for $C_{14}H_{13}N_3O_2$: C, 65.87; H, 5.13; N, 16.46. Found C, 65.75; H, 5.18; N, 16.32.

***N*-Phenyl-N′-(3-phenylpropyl)Imidodicarbonic Diamide (**5b**).** White solid; Yield 91%; mp 89–91 °C; IR (KBr) ν_{max}: 3340, 3308, 3043, 2928, 2861, 1701, 1669 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 1.6–1.9 (m, 2H), 2.5–2.9 (m, 2H), 3.0–3.4 (m, 2H), 6.7–7.4 (m, 10H), 9.5 (br, 1H). MS (EI) m/z: 297(M^+), 193, 117, 93. Anal. Calcd. For $C_{17}H_{19}N_3O_2$: C, 68.67; H, 6.44; N, 14.13. Found: C, 68.47; H, 6.50; N, 14.08.

***N*-Phenyl-N′-[3-(benzo[b]thiazol-2-ylthio)Propyl] Imidodicarbonic Diamide (**5c**).** Yellow solid; Yield 89%; mp 105–109 °C; IR (KBr) ν_{max}: 3352, 3317, 3100, 2944, 1703, 1686, 1602 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 2.01–2.04 (m, 2H), 3.2–3.4 (m, 4H), 7.06–7.45 (m, 7H), 7.77 (d, 1H, J = 8.0 Hz), 7.95 (d, 1H, J = 8.0 Hz), 9.38 (s, 1H). MS (EI) m/z: 386 (M+), 293.9, 254.7, 251, 193.6, 180.0, 166.4, 134.4, 93.1. Anal. Calcd. For $C_{18}H_{18}N_4O_2S_2$: C, 55.94; H, 4.69; N, 14.50. Found: C, 55.98; H, 4.39, N, 14.65.

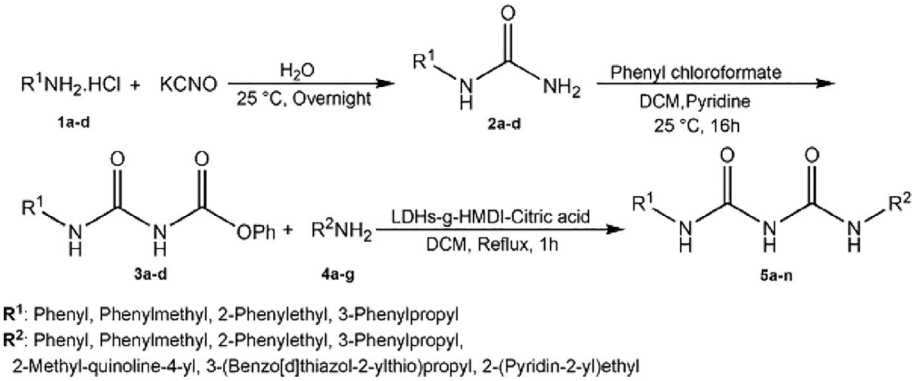
***N*,N′-Bis Phenylmethyl Imidodicarbonic Diamide (**5d**).** White solid; Yield 95%; mp 168–169 °C. Anal.Calcd for $C_{16}H_{17}N_3O_2$: C, 67.83; H, 6.05; N, 14.83. Found C, 67.72; H, 5.07; N, 14.75.

***N*-(Phenylmethyl)-N′-(2-Phenylethyl)Imidodicarbonic Diamide (5e).** White solid; Yield 94%; mp 155–160 °C. Anal.Calcd for $C_{17}H_{19}N_3O_2$: C, 68.67; H, 6.44; N, 14.13. Found C, 68.32; H, 6.55; N, 13.97.

***N*-(Phenylmethyl)-N′-(3-Phenylpropyl)Imidodicarbonic Diamide (**5f**).** White solid; Yield 89%; mp 88 °C; IR (KBr) ν_{max}: 3345, 3310, 3045, 2940, 2865, 1710, 1660 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 1.80–1.90 (m, 2H), 2.65 (t, 2H, J = 8.0 Hz), 3.2 (t, 2H, J = 8.0 Hz), 4.4 (d, 2H, J = 8.0 Hz), 7.17–7.35 (m, 10H), 9.45 (s, 1H). MS (EI) m/z: 311 (M^+), 296, 282, 219, 205, 194, 176, 118, 105, 91. Anal. Calcd. For $C_{18}H_{21}N_3O_2$: C, 69.43; H, 6.80; N, 13.49. Found: C, 69.35; H, 6.85; N, 13.26.

***N*-(Phenylmethyl)-N′-[2-(Pyridin-2-yl))Ethyl] Imidodicarbonic Diamide (**5g**).** White solid; Yield 92%; mp 74 °C; IR (KBr) ν_{max}: 3380, 3266, 3100, 2948, 1698, 1680 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 2.97 (t, 2H, J = 6.5 Hz), 3.56 (q, 2H, J = 6.0 Hz), 4.35 (d, 2H, J = 5.5 Hz), 7.08–7.10 (m, 1H), 7.13 (d, 1H, J = 7.5 Hz),7.21–7.30 (m, 3H), 7.27–7.3 (m, 2H), 7.55–7.58 (m,1H), 8.5 (d, 1H, J = 4.0 Hz), 9.46 (s, 1H). MS (EI) m/z: 298 (M+), 192.1, 149, 121, 106, 93.1. Anal. Calcd. For $C_{16}H_{18}N_4O_2$: C, 64.41; H, 6.08; N, 18.78. Found: C, 64.56; H, 5.95; N, 18.85.

***N*-(Phenylmethyl)-N′-(2-Methylquinolin-4-yl) Imidodicarbonic Diamide (**5h**).** White solid; Yield 90%; mp 161 °C; IR (KBr) ν_{max}: 3415, 3340, 3065, 1702, 1692, 1670, 1600 cm^{-1}. ^{1}HNMR (400 MHz, DMSO-d_6) δ: 2.5 (s, 3H), 4.31 (d, 2H, J = 6.3 Hz), 7.09–7.12 (m, 1H), 7.16–7.21 (m, 4H), 7.31–7.36 (m, 1H), 7.5 (t, 1H, J = 5.72 Hz),



Scheme 2. LDHs-g-HMDI-citric acid catalyzed synthesis of biuret derivatives.

7.78 (d, 1H, J = 6.76 Hz), 7.84 (d, 1H, J = 6.72 Hz),7.98 (s, 1H), 9.16 (br, 1H). MS (EI) m/z: 334 (M+), 316, 291, 283, 270, 200, 184, 176, 158, 106, 91. Anal. Calcd. For $C_{19}H_{18}N_4O_2$: C, 68.25; H, 5.43; N, 16.76. Found: C, 68.04; H, 5.49; N, 16.81.

***N*-(Phenylmethyl)-N′-[3-(Benzo[d]thiazol-2-ylthio)Propyl]Imidodicarbonic Diamide** (5i)**.** Yellow oil; Yield 85%; mp 70–73 °C; IR (KBr) ν_{max}: 3348, 3309, 3080, 2946, 1700, 1688, 1600 cm^{-1}.^{1}HNMR (400 MHz, $CDCl_3$) δ: 1.9–2.07 (m, 2H), 3.3–3.4 (m, 4H), 4.35 (d, 2H, j = 6.4 Hz), 7.0–7.5 (m, 7H), 7.65–7.8 (m, 2H), 9.6 (br,1H). MS (EI) m/z: 400 (M^+), 292, 283, 250, 203, 194, 166, 105. Anal. Calcd. For $C_{19}H_{20}N_4O_2S_2$: C, 56.98; H, 5.03; N, 13.99. Found: C, 56.89; H, 5.24; N, 13.78.

***N*,N′-Bis(2-Phenylethyl)Imidodicarbonic Diamide** (5j)**.** White solid; Yield 96%; mp 115 °C; IR (KBr) ν_{max}: 3359, 3272, 3030, 2944, 1690, 1530, 1230 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 2.78–2.86 (m,4H), 3.38–3.48 (m, 4H), 7.19–7.39 (m, 12H), 9.4 (s, 1H, NH) MS (EI) m/z: 311 (M^+), 219, 205, 190, 162, 147, 119, 105, 91. Anal. Calcd. For $C_{18}H_{21}N_3O_2$: C, 69.43; H, 6.80; N, 13.49. Found: C, 69.22; H, 6.91; N, 13.54.

***N*-(2-Phenylethyl)-N′-[3-(Benzo[d]thiazol-2-ylthio)Propyl]Imidodicarbonic Diamide** (5k)**.** Yellow solid; Yield 95%; mp 70–73 °C; IR (KBr) ν_{max}: 3348, 3308, 3070, 2949, 1701, 1687, 1603 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 2.03 (t, 2H, J = 6.5 Hz), 2.80 (t, 2H, J = 6.5 Hz), 3.28–3.37 (m, 4H), 3.4–3.6 (m, 2H), 7.16–7.2 (m, 3H), 7.24–7.28 (m, 3H), 7.35–7.40 (m, 1H), 7.72 (d, 1H, J = 7.5 Hz), 7.88 (d, 1H, J = 8.0 Hz), 9.6 (br, 1H). MS (EI) m/z: 414 (M+), 309.4, 294.1, 250, 191, 180, 166, 148, 120, 105, 91. Anal. Calcd. For $C_{20}H_{22}N_4O_2S_2$: C, 57.95; H, 5.35; N, 13.52. Found: C, 57.91; H, 5.47; N, 13.75.

***N*,N′-Bis (3-Phenylpropyl)Imidodicarbonic Diamide** (5l)**.** White solid; Yield 93%; mp 85–86 °C. Anal.Calcd.for $C_{20}H_{25}N_3O_2$: C,70.77; H, 7.42; N,12.38. Found: C, 70.47; H, 7.47; N, 12.31.

***N*-(3-Phenylpropyl)-N′-[3-(Benzo[d]thiazol-2-ylthio)Propyl]Imidodicarbonic Diamide** (5m)**.** Yellow oil; Yield 90%; mp 95–96 °C; IR (KBr) ν_{max}: 3349, 3305, 3075, 2949, 1703, 1688, 1605 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 1.84–1.9 (m, 2H), 2.03–2.08 (m, 2H), 2.68 (t, 2H, J = 7.6 Hz), 3.20–3.27 (m, 2H), 3.3–3.5 (m, 4H), 7.15–7.2 (m, 3H), 7.25–7.3 (m, 3H),7.38–7.43 (m, 1H),7.73 (d, 1H, J = 8.0 Hz), 7.9 (d, 1H, J = 8.0 Hz), 9.50 (s, 1H). MS (EI) m/z: 428 (M^+), 350, 336, 308, 294, 268, 250, 224, 194, 181, 166, 136, 108, 91. Anal. Calcd. For $C_{21}H_{24}N_4O_2S_2$: C, 58.85; H, 5.64; N, 13.07. Found: C, 58.56; H, 5.53; N, 12.96.

***N*-(3-Phenylpropyl)-N′-[3-(1-Phenyl-1H-tetrazol-5-ylthio)Propyl]Imidodicarbonic Diamide** (5n)**.** White solid; Yield 88%; mp 90–91 °C; IR (KBr) ν_{max}: 3350, 3310, 3089, 2946, 1700, 1685, 1605 cm^{-1}. ^{1}HNMR (400 MHz, $CDCl_3$) δ: 1–1.22(m, 1H), 1.52–2 (m, 4H), 2.5–2.6 (m, 2H), 3.03–3.35 (m, 5H), 6.9–7.5 (m, 5H), 7.5–7.75 (m, 5H), 9.3 (br, 1H). MS (EI) m/z: 439 (M^+), 296, 277, 236, 219, 205, 191, 178, 166, 145, 132, 118, 106, 91. Anal. Calcd. For $C_{21}H_{25}N_7O_2S$: C, 57.38; H, 5.73; N, 22.31. Found: C, 57.31; H, 5.89; N, 22.25.

Cells and Viruses. Blood samples from three healthy volunteers were collected in heparinized tubes. The volunteers were seronegative for HIV, HBV, and HCV (approved by blood transfusion center, Alzahra hospital, Esfahan, Iran). Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphodex density centrifugation (1800g, 20 min). The cells were cultured in a complete medium, containing Roswell Park Memorial Institute (RPMI) media supplemented with 15% fetal bovine serum (FBS), phytohemagglutinin (PHA), 100 μg/mL penicillin solution, 100 μg/mL streptomycin, and 2 mM L-glutamine. PBMCs were incubated in a moistened atmosphere with 5% CO_2 for 6 h at 37 °C.[60] The virus titers were measured using HIV-1 p24 antigen ELISA kit (BioMerieux, France). The viruses were stored at −70 °C for later use.

HIV Replication Assay. The anti-HIV-1 activity of biuret derivatives was studied by the HIV-1 p24 Antigen ELISA kit according to the manufacturer's protocol.[61] Briefly, 6×10^5 PBMCs were seeded in 24-well plates and infected with 0.01 mol of SCR HIV-1 subtype A, in 500 μL of culture medium. Different concentrations of biuret derivatives (0.1, 1, 10, 50, and 100 μM) were added to each assay sample and incubated at 37 °C for 12 h. The infected cells were

then washed and overlaid with the medium at different concentrations of biuret derivatives. Azidothymidine (AZT) (0.1, 1, 10, 50, and 100 μM) and DMSO (1%) were used as positive control and blank. After 3 days of incubation, the supernatant was collected and transferred to the coated 96-well plate to quantify the HIV-1 p24 core protein. Optical density (OD) of viruses was measured at 450 nm by ELISA reader (Awareness Technology Inc., Florida, USA). The minimal concentration required to suppress the load of P24 by 50% (IC_{50}) was determined by regression analysis of the dose–response curve generated from the data.

Docking Studies. Molecular docking studies were performed using AutoDock 4.2 software (The Scripps Research Institute, La Jolla, CA, USA) to characterize the binding modes of the selected compounds,[62] with HIV-1 protease active site. The crystal structure of HIV-1 protease (PDB code: 1HPV) was derived from the protein data bank (www.rcsb.org). The optimized structure of the protein and ligands were used as the input for the subsequent docking studies. The AutoDockTools program (ADT; version 1.5.6) was utilized to prepare input files as well as to view the results.[62] Water molecules were removed from the PDB file, and polar hydrogens were added to the protein structure. The Kollman-united charges method was assigned for all atoms of the enzyme to calculate the partial atomic charges, and grid maps were generated by AutoGrid 4.2 (The Scripps Research Institute, La Jolla, CA, USA). The chemical structures of the ligands were generated by Marvin Sketch version 5.7 (ChemAxon, Budapest, Hungary).[63] HyperChem software (version 8.0)(Hypercube, Inc, Gainesville, FL, USA) was used to add hydrogen atoms and geometric optimization. The atomic charges were defined based on Gasteiger-Marsili partial charges. Each docking job was carried out by 100 runs. All other parameters and the docking validation were performed using previously published methods.[64] Afterward, the results obtained from the docking simulations were ranked based on the lowest binding free energy and were analyzed to determine the binding modes and orientations of the selected compounds in the active site of the protein.

Results and Discussion

Characterization of LDHs-g-HMDI-Citric Acid Catalyst. As shown in Scheme 1, the procedure of the preparation of LDHs-g-HMDI-Citric acid was accomplished in three steps. LDHs were prepared by co-precipitation of Zn^{2+} and Cr^{3+} salts with the Zn/Cr molar ratio of 3:1. Then, hexamethylene-1,6-diisocyanate (HMDI) as a linker was connected to LDHs to obtain functionalized LDHs-g-HMDI. Finally, the resulting LDHs-g-HMDI was reacted with citric acid to prepare LDHs-g-HMDI-Citric acid as a novel acidic catalyst. The structure of LDHs-g-HMDI-Citric acid catalyst was evaluated precisely using various techniques such as FT-IR, X-ray diffraction (XRD) analysis, energy dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), TGA, and derivative thermal gravimetric (DTG).

FT-IR Spectra. The FT-IR spectra of the catalyst and precursor composites, including LDHs, LDHs-g-HMDI, and

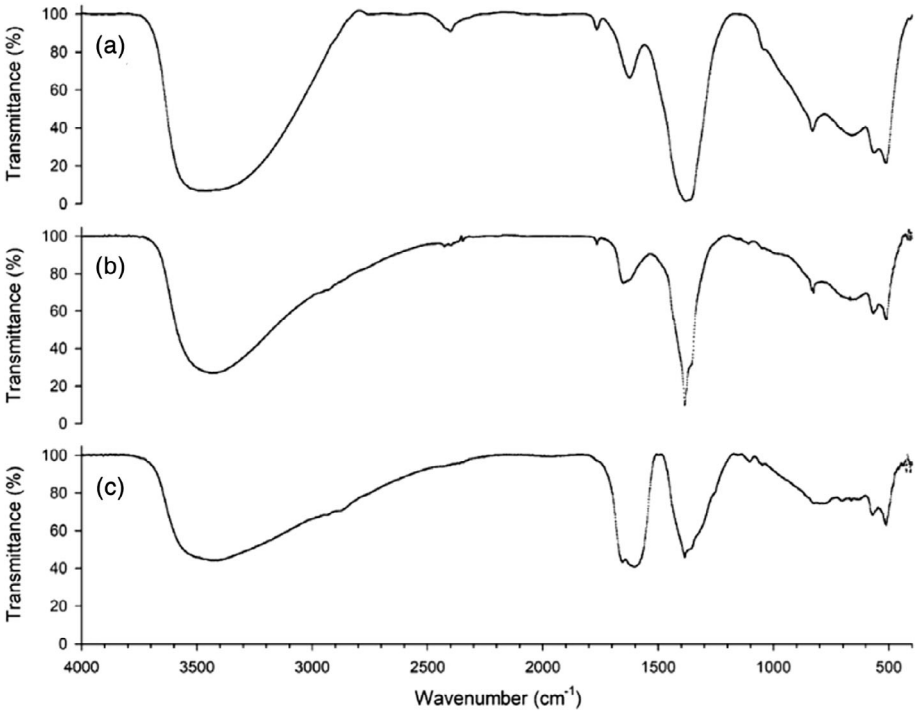


Figure 1. FT-IR spectra of (a) LDHs, (b) LDHs-g-HMDI and (c) LDHs-g-HMDI-citric acid.

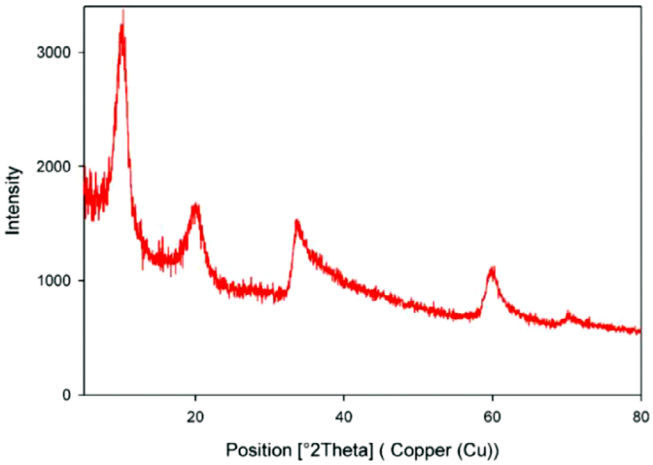


Figure 2. X-ray diffraction pattern of LDHs-g-HMDI-citric acid.

LDHs-g-HMDI-Citric acid, are illustrated in Figure 1. The characteristic peaks are observed in the region 500–800 cm^{-1} declare the vibrating bands belong to the Zn─O and Cr─O groups in Figure 1(a)–(c). As is illustrated in Figure 1(b) and (c), the absorption band at 1384 cm^{-1} is assigned to the nitrate anions that exist between the layers of LDHs. The specific peaks at 1651 cm^{-1} and 1659 cm^{-1} imply the existence of C═O groups in Figure 1(b) and (c), respectively. The appearance of an absorption band at 2400 cm^{-1} in Figure 1(b) is attributed to N═C═O functional group of LDHs-g-HMDI. The fade of this peak in Figure 1(c) further confirms the successful attachment of citric acid to the surface of the HMDI-g-LDHs. Also, the characteristic peaks at 2667 cm^{-1} and 2941 cm^{-1} depicted in Figure 1(b) and (c) can be ascribed to the aliphatic CH_2 groups. Also, the O─H bond of acid group (COOH) absorbs between 2500 and 3300 cm^{-1}, clearly verifies the successful immobilization of citric acid onto the surface of LDHs (Figure 1(c)).

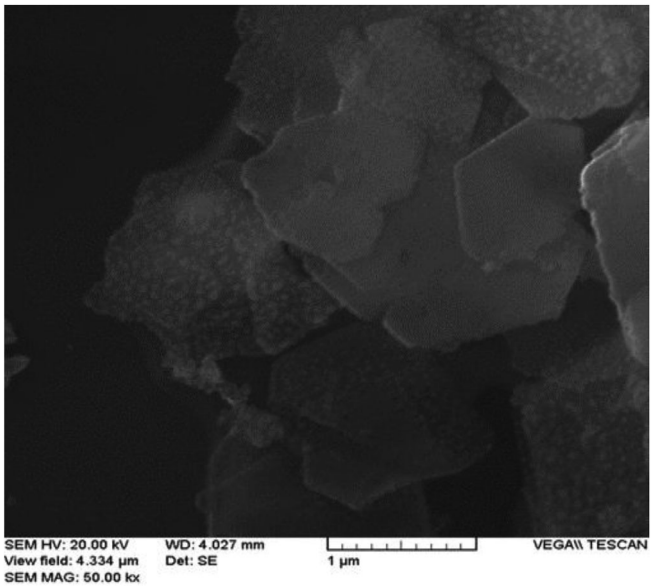


Figure 4. SEM micrograph of LDHs-g-HMDI-citric acid.

X-ray Diffraction Studies. The XRD technique was applied to identify the crystalline structure of synthesized LDHs-g-HMDI-Citric acid. As can be observed in Figure 2, the positions and relative diffraction peaks at around 10.04 (1), 19.91 (2), 33.67 (2), 59.81 (2), 70.20 (5), are attributed to LDHs-g-HMDI-Citric acid. This pattern emphasizes the effective grafting of citric acid on the LDHs-g-HMDI.

Energy Dispersive X-ray Analysis. The composition elements of the catalyst (C, N, O, Zn, and Cr) are shown in the EDX of the typical catalyst sample (Figure 3). According to Figure 3, the results approve the successful grafting of citric acid and hexamethylen-1,6-diisocyanate (HMDI) on the surface of LDHs.

SEM Analysis. The morphology and particle size of the LDH-g-HMDI-Citric acid were observed by SEM analysis

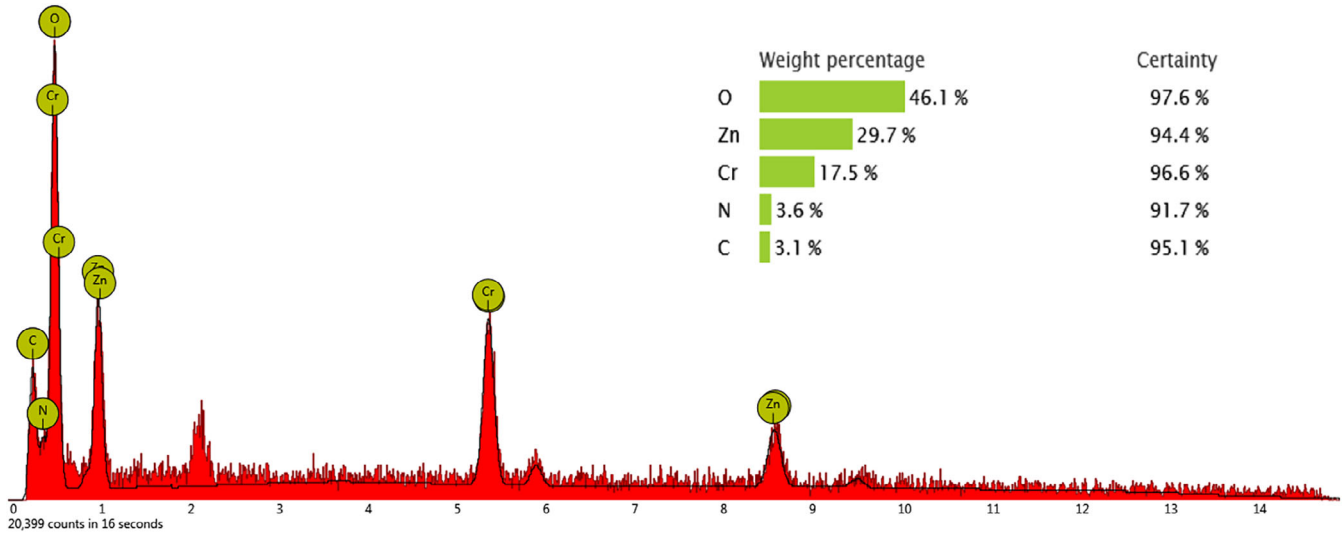


Figure 3. EDX spectrum of LDHs-g-HMDI-citric acid.

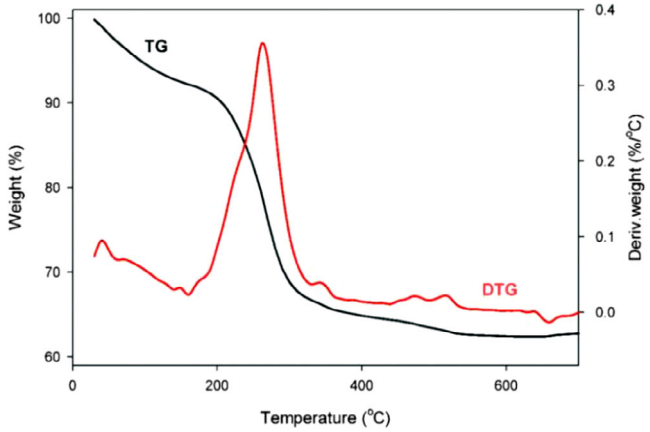


Figure 5. TGA analysis of LDHs-g-HMDI-citric acid.

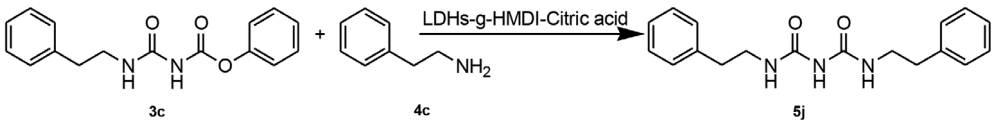
as shown in Figure 4. As can be observed in Figure 4, the SEM micrograph of the LDHs-g-HMDI-Citric acid reveals uniformity and layered structure.

Thermal Gravimetric (TG) and Derivative Thermal Gravimetric (DTG) Analysis. In Figure 5, the first weight loss of ca. 5.5% at 170 °C can be attributed to the removal of residual water attached to the catalyst surface. The second weight loss (17%) at about 200–370 °C is likely related to the removal of the residual organic solvent and immobilized urethane moiety. Also, the third weight loss of about 2.5%, which occurred in the thermal range of 370–500 °C is probably due to the decomposition and removal of the citric acid moiety from the catalyst. The complete decomposition of the particles does not occur even at 700 °C. Therefore, the results confirmed a good grafting of citric acid and HMDI on the surface of LDHs.

The Catalytic Activity of LDHs-g-HMDI-Citric Acid for the Synthesis of Biuret Derivatives. Initially, to establish the optimized reaction conditions, the model reactions between allophanate and phenylethylamine in the presence of LDHs-g-HMDI-Citric acid was investigated. For this purpose, the effects of several parameters such as different amounts of catalyst, type of solvent (THF, DCM, Ethanol, DMF), and temperature on the reaction time as well as yield of the model reaction were assessed. According to the data that are presented in Table 1, the best results for the model synthesis of *N*,*N*′-bis(2-phenylethyl)imidodicarbonicdiamide (**5j**) were obtained when the reaction was carried out in DCM and reflux condition using 5 mg of catalyst for 1 h (entry 7). In addition, increasing the amount of the catalyst to 8 mg showed no progress in the rate and yield of the reaction (entry 8). It should be noted that the reaction under the same conditions and absence of catalyst was carried out, and the product was achieved after a prolonged reaction time with a lower yield (entry 14). The results proved the vital role of LDHs-g-HMDI-Citric acid as a suitable catalyst for the synthesis of biurets.

Furthermore, the reactions of various allophanates and amines were carried out. As can be seen in Table 2, the corresponding products (**5a**–**n**) were obtained with higher yields (28–96%) and shorter times (60–180 min) than the previous study.[16]

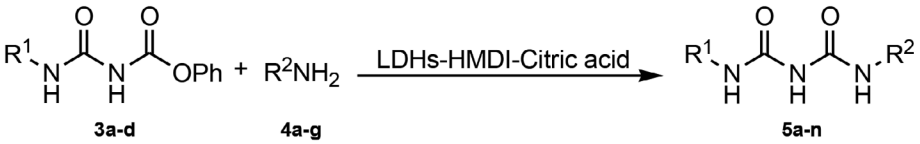
Table 1. Screening the reaction parameters for the production of *N*,*N*′-bis(2-phenylethyl)imidodicarbonic diamide.[a]



Entry	Solvent	Catalyst (mg)	Temperature (°C)	Time (minutes)	Yield (%)[b]
1	THF	3	r.t	180	38
2	CH_2Cl_2	3	r.t	180	59
3	EtOH	3	r.t	180	25
4	DMF	3	r.t	180	12
5	THF	3	60	120	51
6	CH_2Cl_2	3	Reflux	90	87
7	CH_2Cl_2	5	Reflux	60	96
8	CH_2Cl_2	8	Reflux	60	96
9	EtOH	3	60	120	45
10	DMF	3	120	150	33
11	THF	3	Reflux	120	68
12	EtOH	3	Reflux	120	59
13	DMF	3	Reflux	120	41
14	CH_2Cl_2	No catalyst	Reflux	300	Trace

[a] Conditions: phenyl [(2-phenylethyl)aminocarbonyl]carbamate (1 mmol), phenylethyl amine (1 mmol), solvent (10 mL).
[b] Isolated pure yield.

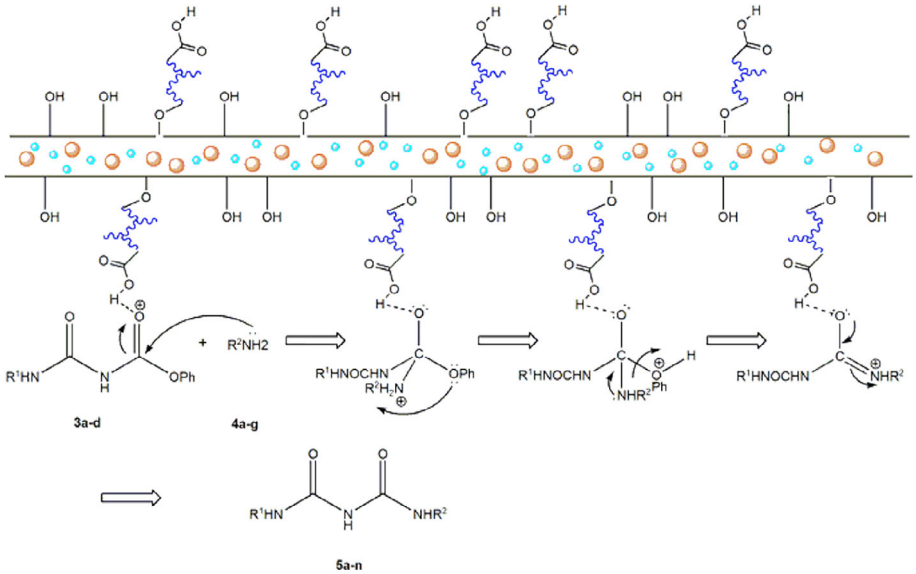
Table 2. Synthesis of biuret derivatives catalyzed by LDHs-g-HMDI-citric acid as catalyst.[a]



Compound	R^1	R^2	Reaction time (minutes)	Melting point (°C) Found	Melting point (°C) Reported[16]	Yield (%)[b] present	Yield (%)[b] Reported[16]
5a	Phenyl	Phenyl	60	212	208–210	91	53
5b	Phenyl	3-Phenylpropyl	60	89–91	92–93	91	57
5c	Phenyl	3-(Benzo[*d*]thiazol-2-ylthio) propyl	60	105–109	105–109	89	35.6
5d	Phenylmethyl	Phenylmethyl	60	168–169	168–169	95	76
5e	Phenylmethyl	2-Phenylethyl	60	155–160	156–159	94	73.4
5f	Phenylmethyl	3-Phenylpropyl	90	88	90–91	89	34
5 g	Phenylmethyl	2-(Pyridin-2-yl)ethyl	100	74	73–74	92	58
5 h	Phenylmethyl	2-Methyl-quinoline-4-yl	120	161	163	90	47.2
5i	Phenylmethyl	3-(Benzo[*d*]thiazol-2-ylthio) propyl	80	70–73	68–72	85	23
5j	2-Phenylethyl	2-Phenylethyl	60	115	110–112	96	84
5 k	2-Phenylethyl	3-(Benzo[*d*]thiazol-2-ylthio) propyl	100	70–73	70–73	95	76
5 L	3-Phenylpropyl	3-Phenylpropyl	100	85–86	85–86	93	68.5
5 m	3-Phenylpropyl	3-(Benzo[*d*]thiazol-2-ylthio) propyl	60	95–96	93–96	90	47
5n	3-Phenylpropyl	3-(1-Phenyl-1H-tetrazol-5-ylthio)propyl	100	90–91	89–92	88	28

[a] Conditions: allophanate (1 mmol), amine (1 mmol), catalyst (5 mg), dichloromethane (10 mL), reflux conditions.
[b] Isolated pure yield.

A plausible mechanism that can be suggested for the reaction of various derivatives of allophanates and amines in the presence of LDHs-g-HMDI-Citric acid is depicted in Scheme 3. At first, due to the interaction between the acidic hydrogen of LDHs-g-HMDI-Citric acid and the oxygen of carbonyl functional group from



Scheme 3. A plausible mechanism for the synthesis of biuret derivatives (**5a**–**n**) catalyzed by LDHs-g-HMDI-citric acid.

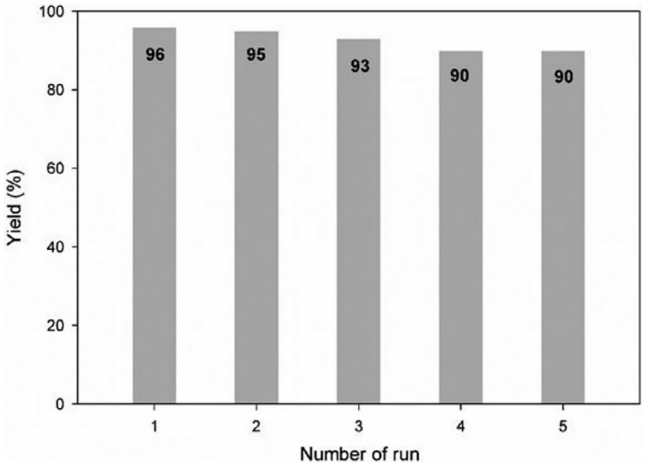


Figure 6. Recyclability test of LDHs-g-HMDI-citric acid.

allophanate, the carbonyl site became more active for nucleophilic addition of amine, and the nucleophilic substitution was easier. In other words, by lowering the HOMO (highest occupied molecular orbital) energy level of amine and approaching its level to the LUMO (lowest unoccupied molecular orbital) energy level of carbonyl, the catalyst causes the reaction to take place at a much higher rate and yield.

At the end of the reactions, the catalyst was isolated by centrifuge (2000*g*, 10 min), and the isolated catalyst was washed with hot ethanol and dried in an oven at 70 °C. The isolated catalyst particles were reused in subsequent reactions at least five times without significant loss of catalytic activity (Figure 6). Also, the exposure of the catalyst to air at room temperature for 2 weeks did not significantly alter its activity. These results demonstrate the exceptional stability and reusability of the LDHs-g-HMDI-Citric acid as a heterogeneous catalyst.

Biological Evaluation. The antiviral activities (IC_{50} values) of the synthesized compounds and azidothymidine (AZT) as a reference drug are presented in Table 3. According to the results, compounds **5n**, **5i**, **5j**, **5 m**, **5f**, and **5a** exhibited antiviral activities (IC_{50} between 55.09 and 100 μM) against the HIV-1 virus. Other compounds did not show any significant antiviral activities at the concentrations higher than 100 μM and therefore considered as inactive compounds. The above six compounds were selected for docking studies.

Table 3. IC_{50} (μM) values of the active compounds.

Compound	IC_{50} (μM)
5a	100.0 ± 1.7
5f	94.3 ± 1.3
5i	64.4 ± 0.7
5j	75.7 ± 1.1
5 m	85.6 ± 1.2
5n	55.1 ± 0.9
Azidothymidine	0.11 ± 0.01

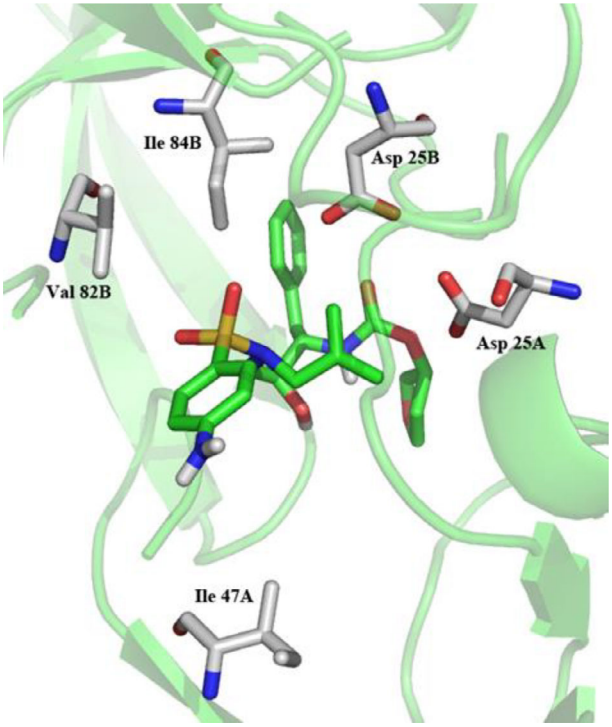


Figure 7. Residues involved in the interaction of VX-478 with HIV-1 protease according to the docking methodology.

Molecular Docking Studies

Evaluation of the Docking Protocol. To verify the validity of the docking protocol, initially, the docking of VX-478, a potent and orally bioavailable inhibitor of the enzyme, over the HIV-1 protease was investigated. The native ligand (VX-478) was extracted from the X-ray structure of HIV-1 protease (PDB: 4XCT) and redocked into the corresponding active site of protein (according to the mentioned protocol in the docking studies section). As it is shown in Figure 7, the obtained results from redocking and the crystallographic structure are in agreement in terms of the ligand interactions, position, and orientation in the active pocket of the enzyme. The root mean square distance (RMSD) of re-docked and co-crystallized ligand over 4XCT was less than 2.0 Å that confirmed the accuracy of docking protocol.[65]

Binding position and interaction of the active compounds. Molecular docking is a popular computational method that is used in the prediction of binding modes of a potential bioactive compound with its biomolecular target.[66] The synthesized biuret derivatives could be considered as potential HIV-1 protease inhibitors due to their pseudo peptide structures. Therefore, the binding energy, position and interaction of the most active compounds with HIV-1 protease were investigated by molecular docking studies. The HIV-1 protease is a homodimeric protease including two identical 99-residue monomers. Each monomer consists of the conserved triads (Asp-Thr-Gly) in positions of 25–27

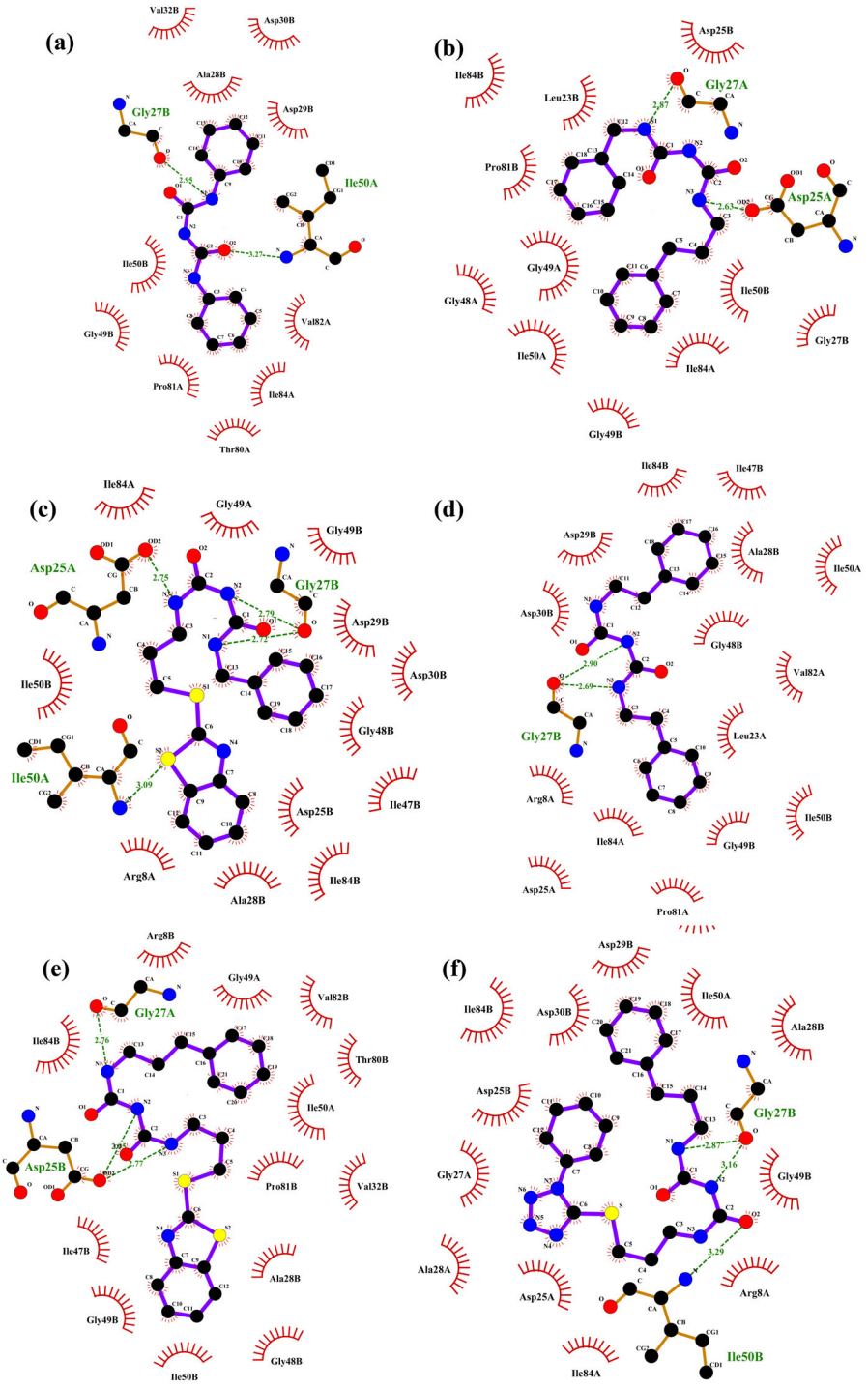


Figure 8. 2D plots of the interaction between selected compounds and HIV-1 protease; olive green arrow: H bond interaction, brick red representation: Hydrophobic site, nitrogen is blue, oxygen is red, carbon is black and sulfur is yellow. (a) **5a**, (b) **5f**, (c) **5i**, (d) **5j**, (e) **5 m**, and (f) **5n**.

and 25′–27′, that aspartate amino acids do all of the catalytic activities.[67] All the selected compounds were successfully docked into the corresponding active site of HIV-1 protease (Figure 8). The binding affinity of the active compounds to HIV1-protease and H- bond interactions with key residues of active site has been shown in Table 4. Each compound has one or more atoms with free electron pairs that able to form hydrogen or electrostatic bond with the binding pocket of the enzyme. Also, the hydrophobic groups, such as benzyl moieties, could occupy the binding pocket by interaction with hydrophobic residues. As shown in Figure 8, the biuret moiety in the majority of the selected compounds participates in hydrogen bonding with Asp 25, Gly 27, and Ile 50. The hydrogen bonding of Asp

Table 4. The docking binding affinity of the selected compounds to HIV1- protease and hydrogen bond interactions.

Compound	Binding energy	Type of interactions
5a	−6.48	Two hydrogen interactions are possible with • Amino acid Gly 27B (H-acceptor) with distance about 2.95 Å • Amino acid Ile 50A (H-donor) with distance about 3.27 Å
5f	−7.56	Two hydrogen interactions are possible with • Amino acid Gly 27A (H-acceptor) with distance about 2.87 Å • Amino acid Asp 25A (H- acceptor) with distance about 2.63 Å
5i	−8.40	Three hydrogen interactions are possible with • Amino acid Gly 27B (H-acceptor) with distance about 2.72 Å • Amino acid Asp 25A (H- acceptor) with distance about 2.75 Å • Amino acid Ile 50A (H-donor) with distance about 3.09 Å
5j	−7.19	Only one hydrogen interaction (H-donor) is possible with amino acid Gly 27B with distance about 2.69 Å
5 m	−8.55	Two hydrogen interactions are possible with • Amino acid Gly 27A (H-acceptor) with distance about 2.76 Å • Amino acid Asp 25B (H- acceptor) with distance about 2.77 Å
5n	−8.94	Two hydrogen interactions are possible with • Amino acid Gly 27B (H-acceptor) with distance about 2.87 Å • Amino acid Ile 50B (H-donor) with distance about 3.29 Å
Azidothymidine	−8.89	Only one hydrogen interaction (H-donor) is possible with amino acid Ile 50B with distance about 3.06 Å

25 with different inhibitors has been previously reported.[67] In summary, docking results revealed that all of the selected compounds could occupy an HIV-1 protease active site. However, compound **5n** showed higher interaction energy. There is a good correlation between docking analysis and anti- HIV activity (IC_{50}). Therefore, selected compounds could serve as lead compounds for developing new potential HIV-1 protease inhibitor candidates.

Conclusion

The LDHs-g-HMDI-Citric acid was introduced as a new, efficient, versatile and heterogeneous catalyst for the synthesis of biuret derivatives and fully characterized by FT-IR, EDX, XRD, SEM, and TGA techniques. The attractive features of the presented catalyst are high yields of the products, short reaction times, simple reaction conditions, efficient recyclability, and reusability of the catalyst. The HIV-1 inhibitory activity of the synthesized biuret derivatives was investigated by *in vitro* assay, followed by the docking study. The results of the *in vitro* assay were in good correlation with those of the docking study. In both studies, compound **5n** showed the best activity. It seems that further exploration of biuret derivatives might lead to compounds with high HIV-1 protease inhibitory activity.

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