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Microwave-Assisted Synthesis of Hydroxybenzylidene-Andrographolides and Its Inhibitory Activity against HIV-1 Protease

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ABSTRACT

Andrographolide, a lacton diterpenoid, due to its many biological activities, was subjected to a semi-synthetic work by reacting andrographolide (**a**) with hydroxybenzaldehyde under microwave irradiation. This reaction led to three new andrographolide analogues, which are 3,19-2-hydroxybenzylidene andrographolide (**b**), 3,19-3-hydroxybenzylidene andrographolide (**c**), and 3,19-4-hydroxybenzylidene andrographolide (**d**), respectively. The yields were 85%, 86%, 86% for compounds **b**, **c**, **d** respectively. These new compounds had already been studied previously by pharmacophore screening and molecular docking simulation, which revealed their affinity to HIV-1 protease. Furthermore, their inhibitory activity against HIV-1 protease was measured by *in vitro* fluorometric method at (Ex/Em) = 330/450 nm which resulted 18.14, 10.72, 9.93, 8.32 µM respectively for IC_{50} value. The increased activity of these compounds may reflect the binding of the hydroxybenzaldehyde moiety with the hydrophobic area of the HIV-1 protease.

INTRODUCTION

HIV-1 protease inhibitors (PIs) have played a critical role in the success of highly active antiretroviral therapy for treatment of HIV-1 infected patients (Anderson *et al*., 2009; Shen *et al*., 2008; Thompson *et al*., 2012). PIs have the highest intrinsic antiviral activity (Jilek *et al*., 2012) and the only antiretroviral drugs that have been successfully used in monotherapy (Perez-Valero and Arribas, 2011). PIs are known to act by preventing cleavage of viral polyproteins into functional subunits, thereby inhibiting maturation of the virus (Swanstrom and Wills, 1997). A recent study has suggested that in mediating their antiviral effects, PIs affect multiple distinct steps in the life-cycle of the virus including both entry and post-entry events explaining their remarkable potency in suppressing viral replication (Rabi *et al*., 2013).

Our previous *in silico* studies indicated that andrographolide ($C_{20}H_{30}O_5$), an α-alkylidene γ-butyrolactone, two olefin bonds at C-8 and C-12 and three hydroxyls at C-3, C-19 and C-14 (Nanduri *et al*., 2004), which has been analyzed by X-ray crystallographic method and defined as 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naphthalenyl]ethylidine]dihydro-4-hydroxy-2(3H)-furanone (Smith *et al*., 1982), interacts with two important aspartate residues (Asp25 and Asp29) in the binding pocket of HIV-1 protease, similarly as its hydroxybenzylidene derivatives. Therefore, andrographolide and its derivatives potential to be developed as PIs for anti-HIV drugs (Megantara *et al*., 2017).

In order to determine the importance of the hydroxyl groups located at C-14 and C-19 combined with the presence of oxygen atom in the lactone ring of andrographolide for aspartic protease inhibitors activity, we modified this particular ligand by protecting its pharmacophores and adding hydroxyl-benzaldehyde moiety to fill in the hydrophobic empty space on the receptor's active site. Therefore, herein we report the semi-synthesis of andrographolide analogues by modifying a synthesis method

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that has been successfully carried out by Hadi Poerwono *et al.* (Poerwono *et al*., 2007) and furthermore an *in vitro* study to determine inhibitory activity against HIV-1 protease enzyme. Microwave-assisted organic synthesis was chosen to obtain a variety of advantages, including shorter reaction time, better yield together with simplicity in processing and handling (Razzaq and Kappe, 2008), and reduce hazardous intermediate products. This method complies on green chemistry, which is also called sustainable chemistry. The term green chemistry is defined as "the invention, design and application of chemical products and processes to reduce or to eliminate the use and generation of hazardous substances by keeping carbon footprint as low as possible. Microwave assisted organic synthesis has emerged as a new "lead" in organic synthesis and has provided the excellent momentum for many chemists to switch to microwave assisted chemistry (Gangrade *et al*., 2015).

MATERIALS AND METHODS

Chemicals

The chemicals were Andrographolide [Sigma-Aldrich], 2-Hydroxybenzaldehyde [Merck], 3-Hydroxybenzaldehyde [Sigma-Aldrich], 4-Hydroxybenzaldehyde [Merck], Pyridinium *p*-toluenesulfonate (PPTS) [Sigma-Aldrich], Triethylamine [Sigma-Aldrich], Sodium sulfate anhydrous [Sigma-Aldrich], Benzene [Merck], Dimethyl sulfoxide (DMSO) [Merck], Chloroform [Merck], Methanol [Merck], Ethyl acetate [Merck], *n*-Hexane [Merck], Lopinavir/Ritonavir 200 mg/50 mg (Aluvia®) as drug standard, and HIV-1 protease inhibitor screening kit [Bio Vision, USA].

Instruments

The synthesis reactions were carried out in an ace pressure tube using a Microwave synthesis reactor [Sineo MAS-II]. Melting point was determined on a Fisher-Johns apparatus (Fisher Scientific, Waltham, MA, USA) (uncorr). TLC Analysis was carried out using GF254 (Merck Millipore, Darmstadt, Germany) under UV Lamp 254/366 nm (CamagTM, Camag Chemie-Erzeugnisse & Adsorptionstechnik AG, Muttenz, Switzerland). FTIR spectra were recorded in KBr powder on a Shimadzu® FT-IR Prestige-21 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Mass spectral data were recorded on MS Mariner BiospectrometryTM (Applied Biosystems, Foster City, CA, USA). ^{1}H and ^{13}C-NMR spectral data were recorded on an Agilent® (Agilent Technology, Santa Clara, CA, USA) at 500 MHz and 125 MHz, respectively. Instruments used for *in vitro* assay were fluorometer microplate readers (Varioskan Flash-Thermo Fisher scientific), black 96-well microtiter plate (Thermo Fisher scientific), plate shaker-thermostat (Biosan), vortex mixer (Julabo Paramix 3), and sonicator (FALC).

Synthesis of hydroxybenzylidene-andrographolide derivatives

Andrographolide (0.1 g, 0.28 mmol) was reacted with either 2-hydroxybenzaldehyde (0.3 g, 2.83 mmol) or 3-hydroxybenzaldehyde (0.3 g, 2.83 mmol) or 4-hydroxybenzaldehyde (0.3 g, 2.83 mmol) in an ace-pressure tube using a Microwave synthesis reactor [Sineo MAS-II] (300W). Each of the mixture was then added by stirring with pyridinium *p*-toluenesulfonate (20 mg) in benzene-dimethyl sulfoxide (4.5:0.5) for 3h. After the reaction was completed (checked by thin layer chromatography), the contents were treated with excess of triethylamine to quench the remaining catalyst. The reaction mixture was diluted with benzene and washed with water (3 times, 3 × 10 mL). The organic layer was separated, dried over sodium sulfate anhydrous, and concentrated by vacuum distillation. The product was purified by column chromatography using chloroform–methanol (20:1) as eluent.

Inhibitory activity *in vitro* study against HIV-1 protease enzyme

The solutions (substrate, standard, enzyme control, inhibitor control, andrographolide and its hydroxybenzylidene derivatives) were prepared according to the manual instruction of HIV-1 protease inhibitor screening kit (Bio Vision, USA). Aluvia® solution was prepared by dissolving the powder of Aluvia® tablet in methanol, by considering both ritonavir and lopinavir are freely soluble in that particular solvent (PDR, 2001; WHO, 2009). The fluorescence of each solution (substrate, standard, enzyme control, inhibitor control, andrographolide and its hydroxybenzylidene derivatives) was measured at 330/450 nm in a kinetic mode for 1-3 hour at 37°C. Each measurement was replicated three times.

RESULTS AND DISCUSSION

In this work, we have successfully synthesized three new hydroxybenzylidene-andrographolide analogues under microwave irradiation as shown in Figure 1.

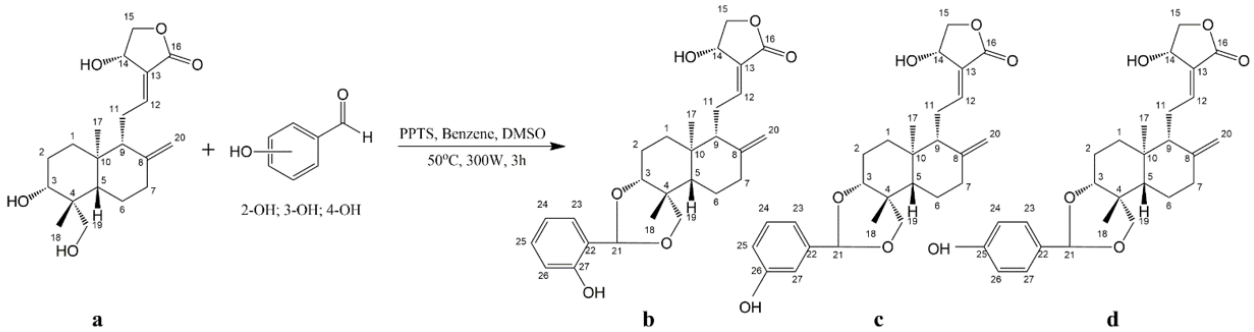

Fig. 1: The synthesis of 3,19-2-hydroxybenzylidene andrographolide (**b**), 3,19-3-hydroxybenzylidene andrographolide (**c**), and 3,19-4-hydroxybenzylidene andrographolide (**d**).

Compound **b** was obtained as a white solid. Yield: 85%, m.p.: 140–142°C. ^{1}H-NMR (500 MHz, MeOH-*d*4): δ (ppm) 0.75 (s, 3H, CH3), 1.22 (s, 3H, CH3), 1.30 (t, 2H, CH2, *J* = 13 Hz), 1,39 (dt, 2H, CH2, *J* = 8.7 Hz, *J* = 4.1 Hz), 1.80 (dt, 2H, CH2, *J* = 6.1 Hz, *J* = 3.5 Hz), 1.90 (t, 1H, CH, *J* = 7.7 Hz), 2.03 (dd, 2H, CH2, *J* = 12.8 Hz, *J* = 6.0 Hz), 2.59 (t, 2H, CH2, *J* = 6.6 Hz), 2.62 (t, 1H, CH, *J* = 3.7 Hz), 4.15 (dt, 2H, CH2, *J* = 8.2 Hz, *J* = 2.0 Hz), 4.47 (dd, 1H, CH, *J* = 6.1 Hz, *J* = 4.1 Hz), 4.67 (s, 2H, CH2), 4.89 (s, 2H, CH2), 5.98 (s, 1H, CH), 6.8 (d, 1H, Ar-H, *J* = 7.5 Hz), 6.85 (t, 1H, Ar-H, *J* = 7.5 Hz), 7.25 (t, 1H, Ar-H, *J* = 7.5 Hz), 7.41 (d, 1H, Ar-H, *J* = 7.5 Hz), 8.95 (s, 1H, Ar-OH). ^{13}C-NMR (125 MHz, MeOH-*d*4): δ (ppm) 15.5, 23.4, 25.2, 25.7, 29.0, 38.1, 39.0, 40.0, 43.7, 56.3, 57.4, 65.0, 66.7, 76.2, 80.9, 102.1, 209.2, 115.0, 120.0, 126.8, 129.8, 137.4, 148.8, 149.4, 154.7, 172.6. MS (EI): [M]+ *m/z* = 454.24(33), 436.26(100), 420.25(21), 284.19(4), 256.19(15). FT-IR 3396, 70 (alcohol O-H); 2927,03 (C-C-H); 1726,32 (Ester C=O); 1674,24 (Aldehyde and ketone C=O); 1647,24 (Aromatic C=C); 1296,19 (Sp^2 C-O).

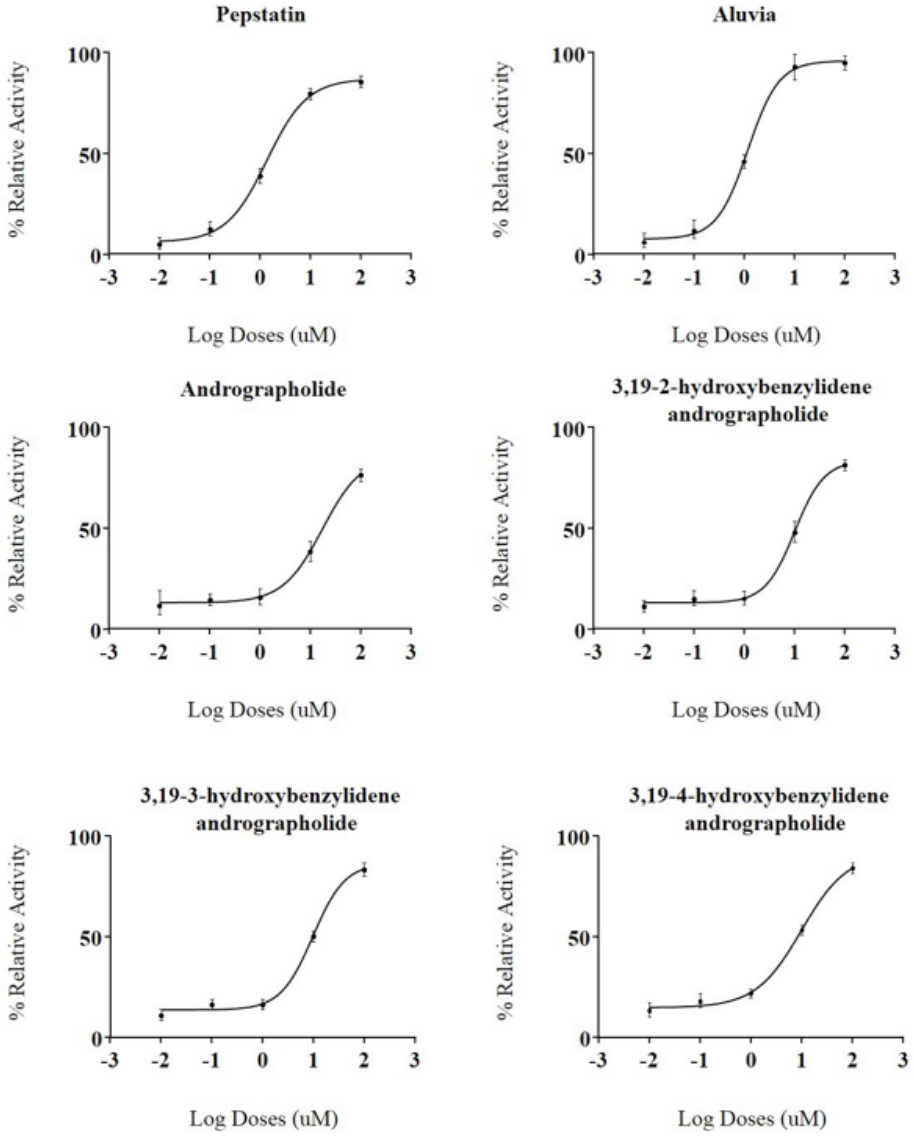

Fig. 2: Relative inhibition curve of the compounds against HIV-1 protease.

Compound **c** was obtained as a white solid. Yield: 86%, m.p.: 167–170°C. ^{1}H-NMR (500 MHz, MeOH-*d*4): δ (ppm) 0.73 (s, 3H, CH3), 1.20 (s, 3H, CH3), 1.29 (t, 2H, CH2, *J* = 13 Hz), 1,35 (dt, 2H, CH2, *J* = 8.7 Hz, *J* = 4.1 Hz), 1.77 (dt, 2H, CH2, *J* = 6.1 Hz, *J* = 3.5 Hz), 1.88 (t, 1H, CH, *J* = 7.7 Hz), 2.02 (dd, 2H, CH2, *J* = 12.8 Hz, *J* = 6.0 Hz), 2.55 (t, 2H, CH2, *J* = 6.6 Hz), 2.60 (t, 1H, CH, *J* = 3.7 Hz), 4.11 (dt, 2H, CH2, *J* = 8.2 Hz, *J* = 2.0 Hz), 4.42 (dd, 1H, CH, *J* = 6.1 Hz, *J* = 4.1 Hz), 4.63 (s, 2H, CH2), 4.85 (s, 2H, CH2), 5.95 (s, 1H, CH), 6.7 (d, 1H, Ar-H, *J* = 7.5 Hz), 6.85 (t, 1H, Ar-H, *J* = 7.5 Hz), 7.25 (d, 1H, Ar-H, *J* = 7.5 Hz), 7.5 (s, 1H, Ar-H), 8.8 (s, 1H, Ar-OH). ^{13}C-NMR (125 MHz, MeOH-*d*4): δ (ppm) 15.2, 23.0, 24.9, 25.5, 28.9, 37.8, 38.7, 39.5, 40.6, 56.0, 57.1, 64.6, 66.2, 76.1, 79.9, 101.7, 208.5, 114.2, 118.6, 126.5, 129.4, 137.2, 147.1, 148.4, 153.3, 170.5. MS (EI): [M]+ *m/z* = 454.20(35), 436.26(100), 420.24(20), 284.18(5), 256.15(17). FT-IR 3396,70 (alcohol O-H); 2927,99 (C-C-H); 1727,26 (Ester

C=O); 1673,28 (Aldehyde and ketone C=O); 1580,69 (Aromatic C=C); 1220,96 (Sp^2 C-O).

Compound **d** was obtained as a white solid. Yield: 86%, m.p.: 192–194°C. ^{1}H-NMR (500 MHz, MeOH-*d*4): δ (ppm) 0.70 (s, 3H, CH3), 1.21 (s, 3H, CH3), 1.32 (t, 2H, CH2, *J* = 13 Hz), 1,31 (dt, 2H, CH2, *J* = 8.7 Hz, *J* = 4.1 Hz), 1.68 (dt, 2H, CH2, *J* = 6.1 Hz, *J* = 3.5 Hz), 1.83 (t, 1H, CH, *J* = 7.7 Hz), 2.11 (dd, 2H, CH2, *J* = 12.8 Hz, *J* = 6.0 Hz), 2.51 (t, 2H, CH2, *J* = 6.6 Hz), 2.48 (t, 1H, CH, *J* = 3.7 Hz), 4.10 (dt, 2H, CH2, *J* = 8.2 Hz, *J* = 2.0 Hz), 4.37 (dd, 1H, CH, *J* = 6.1 Hz, *J* = 4.1 Hz), 4.56 (s, 2H, CH2), 4.78 (s, 2H, CH2), 5.92 (s, 1H, CH), 6.7 (d, 1H, Ar-H, *J* = 7.5 Hz), 6.85 (d, 1H, Ar-H, *J* = 7.5 Hz), 7.25 (d, 1H, Ar-H, *J* = 7.5 Hz), 7.35 (d, 1H, Ar-H, *J* = 7.5 Hz), 8.62 (s, Ar-OH). ^{13}C-NMR (125 MHz, MeOH-*d*4): δ (ppm) 15.0, 22.2, 24.2, 25.5, 28.2, 38.0, 37.7, 38.1, 42.7, 55.5, 56.1, 62.9, 65.4, 74.8, 80.2, 101.2, 208.1, 114.0, 118.2, 124.5, 128.9, 136.2, 146.1, 147.4, 152.5, 170.2. MS (EI): [M]+ *m/z* = 454.20(36), 436.26(100), 420.27(22), 284.20(6), 256.21(19). FT-IR 3217,32 (alcohol O-H); 2927,99 (C-C-H); 1726,32 (Ester C=O); 1674,24 (Aldehyde and ketone C=O); 1598,05 (Aromatic C=C); 1218,07 (Sp^2 C-O).

Inhibitory activity against HIV-1 protease was performed by measuring the amount of product formed in the reaction catalyzed by the HIV-1 protease expressed by IC_{50} value which was calculated from relative inhibition curve (Figure 2).

Most current HIV PIs were designed to mimic the substrate transition state. The hydroxyl group, particularly -CH-COH-CH2- of the inhibitor, interacts with the carboxyl group of the protease active site residues, Asp 25 and Asp 25′, by hydrogen bonds. The inhibitor-contacting residues of HIV protease are relatively conserved, including Gly 27, Asp 29, Asp 30, and Gly 48, but the accumulation of drug-resistance mutations alters the structure of HIV protease and causes treatment failure (Lv *et al.*, 2015). Our previous *in silico* studies indicated that andrographolide interacted with two important aspartate residues (Asp25 and Asp29) in the binding pocket of HIV-1 protease, similarly as its hydroxybenzylidene derivatives (Megantara *et al.*, 2017).

Inhibitory activity in-vitro against HIV-1 protease enzyme was used pepstatin as inhibitor standard due to its powerful inhibitory activity for proteases (Marciniszyn *et al.*, 1976). Besides Pepstatin, we also used Aluvia® tablet, contains lopinavir and ritonavir as inhibitor standard. Lopinavir is a novel protease inhibitor (PI) developed from ritonavir. Coadministration with low-dose ritonavir had significantly improved the pharmacokinetic properties and furthermore increased the activity of lopinavir against HIV-1 protease. Coformulated lopinavir/ritonavir should be regarded as a first-line option when including a PI in the management of HIV-1 infection (Cvetkovic and Goa, 2003). When lopinavir and ritonavir are given simultaneously, ritonavir will inhibit CYP3A4 isoenzyme in the liver that increases the concentration of lopinavir (Kumar *et al.*, 1999). Furthermore, cell-to-cell spread of HIV-1 was potently blocked in the presence of both lopinavir and darunavir at doses corresponding to the maximum plasma concentrations (C_{max}) (14 µM LPV; 12 µM DRV) achieved *in vivo* (Back *et al.*, 2008; Lafeuillade *et al.*, 2002).

Result showed that its IC_{50} was 1.61 ± 0.21 µM, which was close to other report: 2.0 µM (Sarubbi *et al.*, 1993). Aluvia® tablet, contains lopinavir and ritonavir, was used as drug standard and its IC_{50} value was 1.12 ± 0.11 µM. The inhibitory activity of Aluvia® against HIV protease is stronger than pepstatin. The IC_{50} of andrographolide, 3,19-2 hydroxybenzylidene andrographolide, 3,19-3 hydroxybenzylidene andrographolide, and 3,19-4 hydroxybenzylidene andrographolide were 18.14 ± 5.95 µM, 10.72 ± 1.39 µM, 9.93 ± 1.24 µM, and 8.32 ± 1.07µM, respectively. It was noticeable that the IC_{50} of andrographolide and its derivatives was higher than the standards (Figure 3). All hydroxybenzylidene-andrographolide analogues showed better inhibition than its parent compound, but derivate-3 (3,19-4 hydroxybenzylidene andrographolide) is the strongest in inhibiting the enzyme.

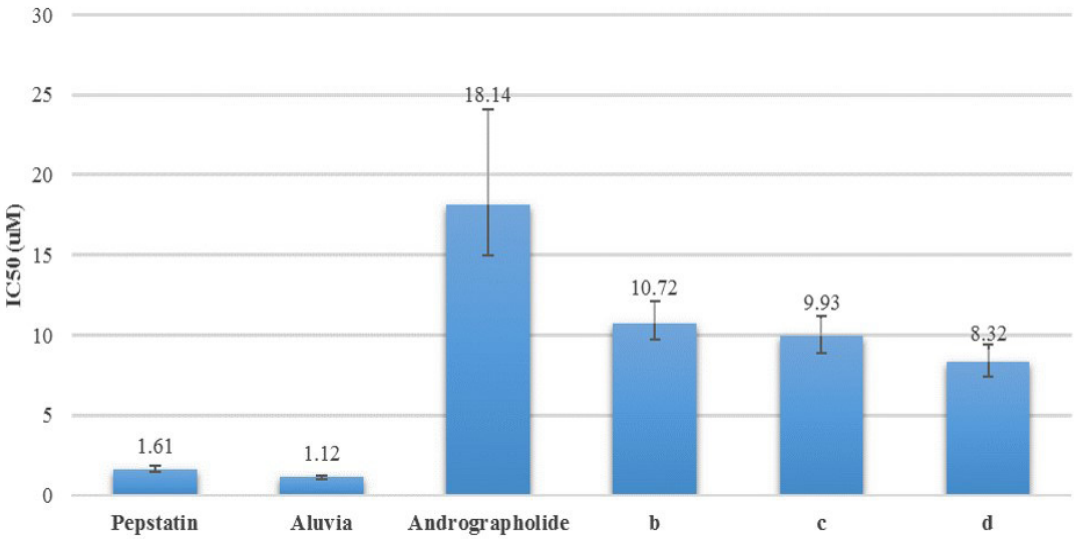

Fig. 3: IC_{50} histogram of the compounds 3,19-2-hydroxybenzylidene andrographolide (**b**), 3,19-3-hydroxybenzylidene andrographolide (**c**), and 3,19-4-hydroxybenzylidene andrographolide (**d**).

CONCLUSION

The microwave-assisted synthesis had resulted three new hydroxybenzylidene-andrographolides with sufficient yields (85-86%). Andrographolide and its hydroxybenzylidene derivatives indicated inhibitory activity against HIV-1 protease, which proves that the replacement of hydroxyl groups at C-3 and C-19 with hydroxybenzaldehyde is responsible for increasing the activity. The increased activity of these compounds may reflect the binding of the hydroxybenzaldehyde moiety with the hydrophobic area of the HIV-1 protease. The strongest activity was showed by 3,19-4 hydroxybenzylidene andrographolide.

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