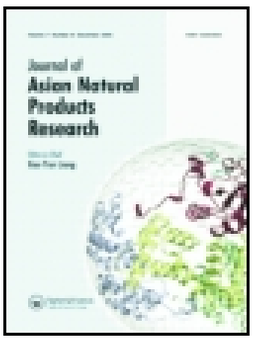

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Design, semisynthesis and cytotoxic activity of novel ester derivatives of betulinic acid-1,2,4 oxadiazoles

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ABSTRACT

Taking into consideration of the biological activity of betulinic acid derivatives containing a oxadiazole ring, the semisynthetic betulinic acid-1,2,4-oxadiazole esters **(14–25)** were synthesized starting from betulinic acid **(1)** and 5-(bromomethyl)-3-aryl-1,2,4-oxadiazoles **(2–13)** and final compounds were tested for cytotoxic activity on three human cancer cell lines *in vitro*. All tested compounds showed good cytotoxic activity. The structures of synthesized compounds are established based on infrared (IR), nuclear magnetic resonance (NMR), and mass spectrometry.

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Betulinic acid; 1,2,4-oxadiazole; coupling; ester; pro drug; cytotoxic activity

1. Introduction

Betulinic acid (BA) is a pentacyclic lupine triterpene with multiple biological activities, such as antibacterial [1], anti-inflammatory [2], antiplasmodium [3], anticancer [4], and anti-HIV [5] activities. Previous reports revealed that compound BA is a melanoma-specific cytotoxic agent [6]; however, recent evidence has indicated that BA possesses a broader spectrum of cytotoxic activity against other cancer types. Moreover, compound BA has been suggested to induce apoptosis via the activation of caspases, regardless of cellular p53 gene status and CD95 activation. This apoptosis inducing ability, the apparent lack of toxicity on normal cells and the favorable therapeutic index have made BA an attractive and a promising anticancer agent [7].

The structures of BA consist of a 30-carbon skeleton that has three available sites for simple chemical modifications at C-3, C-20, and C-28. Modifications of the parent structure of these compounds at said positions can produce potentially important derivatives, which were found to be more effective than the starting ones, thus making them appealing for further development as antitumor drugs [8–11]. Nitrogen-containing derivatives of BA, such as amide derivatives [8], amine derivatives, amino acid conjugates [9], oxime derivatives [10], hydrazine and hydrazone derivatives, N-heterocyclic derivatives [11], and other imidazolide derivatives have been reported to possess anti-proliferative effect against tumor cell lines.

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Recently, the compounds containing the 1,2,4-oxadiazole scaffold have received considerable attention because of their unique chemical structure and broad spectrum of biological properties including histamine H3 antagonism [12], muscarinic agonism, tyrosine kinase inhibition, antitumor [13], anti-inflammation activity [14], and monoamine oxidase inhibition [15]. In addition, anticancer activity of some 3,5-disubstituted-1,2,4-oxadiazoles has recently been reported [16]. The 1,2,4-oxadiazoles are also widely used as heterocyclic amide or ester bioisosteres [17] and in the design of dipeptidomimetics as peptide building blocks [18].

In the last few years, several semisynthetic esters have been synthesized as anticancer agents either in the form of drugs per se or as prodrugs. The interesting cytotoxic activity of betulinic acid analogs and 1,2,4-oxadiazoles encouraged us to investigate the synthesis of novel betulinic acid–oxadiazole esters (**14–25**) via coupling reaction of betulinic acid **(1)** with 5-(bromomethyl)-3-aryl-1,2,4-oxadiazoles **(2–13)**. The use of Cs_2CO_3 in the preparation of esters from betulinic acid and alkyl halides is known [19]. The process has been applied here for the synthesis of betulinic acid–oxadiazole esters (**14–25**). The synthesized compounds were screened for their *in vitro* cytotoxicity against three human cancer cell lines.

2. Results and discussion

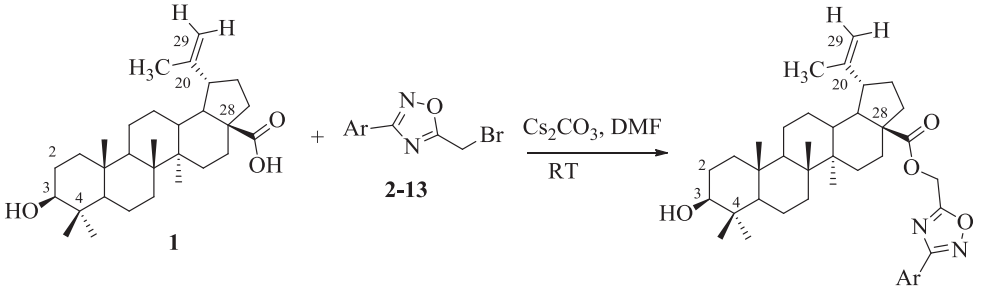
2.1. Chemistry

In the present study, we have synthesized C-28-modified 1,2,4-oxadiazole esters of betulinic acid **(14–25)** via coupling reaction of betulinic acid **(1)** with 5-(bromomethyl)-3-aryl-1,2,4-oxadiazoles **(2–13)** in the presence of Cs_2CO_3 at RT, as shown in Scheme 1.

Initially, 5-(bromomethyl)-3-aryl-1,2,4-oxadiazoles **(2–13)** were synthesized from the reaction of substituted amidoximes with bromoacetyl bromide and immediate reaction in toluene at reflux temperature [20]. Amidoximes were prepared as per literature [21]. C-28 modified 1,2,4-oxadiazole esters of betulinic acid **(14–25)** were prepared in high yields by coupling reaction of betulinic acid **(1)** and 5-(bromomethyl)-3-aryl-1,2,4-oxadiazoles **(2–13)** in the presence of Cs_2CO_3 at RT for overnight.

2.2. Spectral analysis

The structural assignment of the title compounds (**14–25)** has been made on the basis of ^{1}H NMR, ^{13}C NMR, and mass spectra studies. Taking compound **14** for example, structure of **14** is interpreted from spectroscopic data. In the ^{1}H NMR spectrum of **14,** 5′-CH_2, 4″-CH_3 protons appeared at δ 5.31–5.39 (m), 2.42 (s) and aromatic protons at δ 7.27 (d, J = 8.0 Hz, H-2″ & H-6″), 7.94 (d, J = 8.0 Hz, H-3″ & H-5″), Protons for betulinic acid moiety appeared at δ 0.74, 0.77, 0.85 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3), 0.97 (s, 23-CH_3, 27-CH_3), 1.68 (s, 30-CH_3), 0.69–2.28 (all m, remaining protons), 3.01–3.06 (m, H-19), 3.17 (dd, J = 9.1, 4.3 Hz, H-3), 4.58 (d, J = 2.0 Hz, H-29α), 4.73 (d, J = 1.6 Hz, H-29β). In the ^{13}C NMR spectrum of **14**, the newly formed ester carbonyl and oxadiazole carbons appeared at δ 175.0 (-COOCH$_2$), 174.3 (C-3′), 168.4 (C-5′), olefin carbons appeared at δ 150.2 (C-20), 109.7 (C-29). Aromatic carbons appeared at 141.7 (C-4″), 129.5 (C-3″ & C-5″), 127.4 (C-2″ & C-6″), 123.5 (C-1″), betulinic acid carbons appeared at δ 78.9 (C-3), 64.2 (5′-CH_2), 56.8



2, 14) Ar = 4-Methyl phenyl
3, 15) Ar = 4-Methoxy phenyl
4, 16) Ar = 4-pyridyl
5, 17) Ar = 2-Methoxy phenyl
6, 18) Ar = 2-Benzyloxy phenyl
7, 19) Ar = 3-pyridyl
8, 20) Ar = Phenyl
9, 21) Ar = Benzyl
10, 22) Ar = 2-Chloro phenyl
11, 23) Ar = 4-Fluoro phenyl
12, 24) Ar = 2-Methyl phenyl
13, 25) Ar = 4-Bromo phenyl

Scheme 1. Synthesis of C-28 modified 1,2,4-oxadiazole esters of betulinic acid **(14–25).**

(C-17), 56.0 (C-5), 55.3 (C-9), 50.5 (C-18), 49.5 (C-19), 46.7 (C-14), 42.4 (C-8), 40.7 (C-4), 37.1 (C-13), 38.7 (C-10), 38.1 (C-1), 37.1 (C-13), 36.8 (C-22), 34.3 (C-7), 31.9 (C-16), 30.4 (C-21), 29.6 (C-15), 29.5 (C-2), 29.0 (C-12), 27.9 (C-23), 27.4 (C-24), 27.2 (C-11), 25.4 (C-30), 21.5 (4″-CH_3), 20.8 (C-6), 19.3 (C-26), 18.2 (C-25), 16.1 (C-27). Mass spectrum of compound **14** was consistent with the assigned structure, showing $[M + H]^+$ peak at *m/z* 630.

2.3. In vitro *anticancer activity*

The antiproliferative/cytotoxic activities of the newly synthesized BA-1,2,4-oxadiazole ester analogs **(14–25)** were evaluated against three different types of human cancer cell lines, *viz*., human colorectal cancer **(Colo-205)**, human hepatocellular liver carcinoma **(Hep G2)**, and human cervical cancer **(HeLa)** using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, according to the method of Mossman [22]. The cytotoxic potency of the compounds varied between the cell lines suggesting a structural property of these compounds as possible determinants of their biological activity. The cytotoxic investigations were evaluated at CFRD (Osmania University), Hyderabad.

It is evident from the results that all the target compounds have shown significant cytotoxic activity against all the tested cell lines (Table 1). However, **24** and **25** are found to be inactive against Colo-205 and Hep G2 cell lines at 300 μM concentration. Etoposide (a standard drug molecule) was used as a positive control in these assays and the IC_{50} values were recorded as 0.42, 4.77, and 22.5 μM, respectively. Among the derivatives, about 45–50% of the test compounds have shown effective inhibition of growth in all the cell lines at less than 100 μM concentration.

Compound **16** exhibited an excellent antiproliferative activity against all the cell lines (IC_{50} values are less than 50 μM conc.) followed by **17**, **18, 19,** and **20**. Furthermore, results indicated based on the IC_{50} values, the Hep G2 cell lines appeared to be more sensitive than Colo-205 and HeLa cell lines. Nevertheless, the order of relative sensitivity among the cell

Table 1. *In vitro* cytotoxicity of BA-1,2,4-oxadiazole derivatives **(14–25)** against Colo-205, Hep G2 and HeLa human cancer cells by MTT assay expressed in IC_{50} (μM).

	Compound	Colo-205	Hep G2	HeLa
1	14	58.2	237	231
2	15	134	271	246
3	16	35.7	34.1	43.4
4	17	26.1	30.1	61.7
5	18	34.8	36.5	103
6	19	88.5	68.4	65.6
7	20	42.3	34.5	64.8
8	21	212	155	228
9	22	174	105	155
10	23	144	151	123
11	24	NA	NA	181
12	25	NA	NA	61.7
13	Standard [A]	0.42	4.77	22.5

NA indicates that the compound is inactive at 300 μM.
[A]Etoposide was used as positive control.

lines differed, when compared to the activity of etoposide. All the betulinic acid-oxadiazole ester analogs **(14–25)** were found to be effective antiproliferative agents against HeLa cells, with 2- to 10-fold less active than the IC_{50} value of etoposide. Similarly, the analogs **16, 17, 18, 19,** and **20** have exhibited potent antiproliferative activity against Hep G2 cell lines. Similarly, these analogs (excluding **19)** also showed moderate cytotoxicity against Colo-205 cell lines.

3. Experimental

3.1. General experimental procedures

All melting points were obtained on a Polmon instrument, India (model MP96) and are uncorrected. The IR spectra were measured on a Fourier transform infrared spectroscopy Perkin-Elmer 337 (Perkin Elmer instrument company, Massachusetts, USA). ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 MHz, Switzerland using TMS as an internal standard (Chemical shift in δ, ppm). *J*values are given in hertz (Hz). Mass spectral data were obtained with Agilent 6310 ion trap mass spectrometer, USA. All the materials and solvents were used directly unless otherwise stated. All the compounds synthesized were purified by recrystallization or column chromatography on silica gel (60–120 mesh, Spectrochem, Mumbai, India).

3.2. General procedure for the synthesis of C-28 modified 1,2,4-oxadiazole esters of betulinic acid (14–25)

To a suspension of betulinic acid **(1)** (0.3 g, 0.65 mmol) and cesium carbonate (0.27 g, 1.97 mmol) in DMF (3 ml), was added 5-(bromomethyl)-3-p-tolyl-1,2,4-oxadiazole **(2)** (0.25 g, 0.98 mmol). The mixture was stirred at RT for overnight. After completion of the reaction by TLC detection, the reaction mixture was diluted slowly with 30 ml of cold water and the solids formed were collected by filtration to give crude product, which was further purified by column chromatography using 60–120 silica-gel by eluting with petroleum ether/ethyl acetate (6:4), to give pure titled compound as white solid.

3.2.1. 4-Methyl-phenyl-1,2,4-oxadiazole ester of betulinic acid (14)

Yield: 80%; white solid; mp 143–145 °C; IR (KBr) ν_{max}: 1591 (-C=N), 1733 (ester), 3077 (=C-H), 3545 (-OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.77, 0.85, 0.97, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.69-2.28 (all m, remaining protons), 2.42 (s, 3H, 4″-CH_3), 3.01-3.06 (m, 1H, H-19), 3.17 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.58 (d, 1H, J = 2.0 Hz, H-29α), 4.73 (d, 1H, J = 1.6 Hz, H-29β), 5.31-5.39 (m, 2H, 5′-CH_2), 7.27 (d, 2H, J = 8.0 Hz, H-3″ & H-5″), 7.94 (d, 2H, J = 8.0 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.1 (C-27), 18.2 (C-25), 19.3 (C-26), 20.8 (C-6), 21.5 (4″-CH_3), 25.4 (C-30), 27.2 (C-11), 27.4 (C-24), 27.9 (C-23), 29.0 (C-12), 29.5 (C-2), 29.6 (C-15), 30.4 (C-21), 31.9 (C-16), 34.3 (C-7), 36.8 (C-22), 37.1 (C-13), 38.1 (C-1), 38.7 (C-10), 40.7 (C-4), 42.4 (C-8), 46.7 (C-14), 49.5 (C-19), 50.5 (C-18), 55.3 (C-9), 56.0 (C-5), 56.8 (C-17), 64.2 (5′-CH_2), 78.9 (C-3), 109.7 (C-29), 123.5 (C-1″), 127.4 (C-2″ & C-6″), 129.5 (C-3″ & C-5″), 141.7 (C-4″), 150.2 (C-20), 168.4 (C-5′), 174.3 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: m/z 629.5 $[M+H]^+$.

3.2.2. 4-Methoxy-phenyl-1,2,4-oxadiazole ester of betulinic acid (15)

Yield: 80%; white solid; mp 153-154 °C; IR (KBr) ν_{max} 1613 (-C=N), 1732 (ester), 3069 (=C-H), 3540 (-OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.78, 0.86, 0.96, 0.98, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.68-2.25 (all m, remaining protons), 2.93-2.97 (m, 1H, H-19), 3.18 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 3.87 (s, 3H, 4″-OCH_3), 4.59 (d, 1H, J = 1.6 Hz, H-29α), 4.73 (d, 1H, J = 1.6 Hz, H-29β), 5.30-5.38 (m, 2H, 5′-CH_2), 6.97 (d, 2H, J = 8.8 Hz, H-3″ & H-5″), 7.99 (d, 2H, J = 8.4 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.3 (C-27), 15.9 (C-25), 16.0 (C-26), 16.1 (C-6), 18.2 (4″-CH_3), 19.3 (C-30), 21.5 (C-11), 25.4 (C-24), 27.2 (C-23), 27.9 (C-12), 29.0 (C-2), 29.5 (C-15), 29.6 (C-21), 30.4 (C-16), 31.9 (C-7), 34.3 (C-22), 36.8 (C-13), 37.1 (C-1), 38.1 (C-10), 38.8 (C-4), 40.7 (C-8), 42.4 (C-14), 46.7 (C-19), 49.5 (C-18), 50.5 (C-9), 55.3 (C-5), 56.0 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.7 (C-29), 114.2 (C-1″), 118.8 (C-2″), 123.5 (C-6″), 129.1 (C-5″), 134.2 (C-3″), 150.2 (C-4″), 162.1 (C-20), 168.1 (C-5′), 174.2 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: m/z 645.5 $[M+H]^+$.

3.2.3. 4-Pyridine-1,2,4-oxadiazole ester of betulinic acid (16)

Yield: 60%; white solid; mp 185-186 °C; IR (KBr): ν_{max} 1608 (-C=N), 1741 (ester), 3072 (=C-H), 3393 (-OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.66, 0.74, 0.85, 0.96, 0.98, 1.69 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.68-2.25 (all m, remaining protons), 2.91-2.96 (m, 1H, H-19), 3.19 (dd, 1H, J = 4.8, 4.4 Hz, H-3), 4.61 (d, 1H, J = 2.0 Hz, H-29α), 4.73 (d, 1H, J = 1.6 Hz, H-29β), 5.34-5.43 (m, 2H, 5′-CH_2), 7.93 (d, 2H, J = 7.6 Hz, H-3″ & H-5″), 7.99 (d, 2H, J = 7.2 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.3 (C-27), 15.2 (C-25), 16.2 (C-26), 16.4 (C-6), 19.1 (C-30), 21.3 (C-11), 25.3 (C-24), 27.1 (C-23), 27.9 (C-12), 29.0 (C-2), 29.5 (C-15), 29.6 (C-21), 30.9 (C-16), 31.9 (C-7), 34.3 (C-22), 36.8 (C-13), 38.1 (C-1), 38.7 (C-10), 38.8 (C-4), 40.6 (C-8), 42.4 (C-14), 46.7 (C-19), 49.4 (C-18), 50.5 (C-9), 55.3 (C-5), 55.9 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.8 (C-29), 121.3 (C-1″), 133.8 (C-2″ & C-6″), 150.1 (C-3″ & C-5″), 150.6 (C-20), 166.9 (C-5′), 175.0 (C-3′), 175.5 (-$COOCH_2$); ESI–MS: m/z 616.5 $[M + H]^+$.

3.2.4. 2-Methoxy-phenyl-1,2,4-oxadiazole ester of betulinic acid (17)

Yield: 65%; white solid; mp 165-166 °C; IR (KBr): ν_{max} 1603 (-C=N), 1741 (ester), 3069 (=C-H), 3488 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.78, 0.81, 0.96, 0.97, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.65-2.23 (all m, remaining protons), 2.94-2.98 (m, 1H, H-19), 3.17 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 3.98 (s, 3H, 2″-OCH_3), 4.60 (d, 1H, J = 1.6 Hz, H-29α), 4.73 (d, 1H, J = 1.2 Hz, H-29β), 5.37-5.45 (m, 2H, 5′-CH_2), 7.04-7.08 (m, 2H, H-3″ & H-5″), 7.46 (dd, 1H, J = 2.0 Hz, 1.6 Hz, H-4″), 7.98 (dd, 1H, J = 1.6, 1.6 Hz, H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.3 (C-27), 15.8 (C-25), 15.9 (C-26), 16.0 (C-6), 18.2 (4″-CH_3), 19.3 (C-30), 20.8 (C-11), 25.5 (C-24), 27.4 (C-23), 27.9 (C-12), 29.6 (C-2), 30.4 (C-15), 31.9 (C-21), 34.2 (C-16), 36.8 (C-7), 37.1 (C-22), 38.1 (C-13), 38.8 (C-1), 40.7 (C-10), 42.4 (C-4), 46.7 (C-8), 49.4 (C-14), 49.5 (C-19), 50.5 (C-18), 55.3 (C-9), 56.0 (C-5), 56.6 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.7 (C-29), 114.2 (C-3″), 118.8 (C-1″), 123.5 (C-5″), 129.1 (C-4″), 134.2 (C-6″), 150.2 (C-20), 162.1 (C-2″), 168.1 (C-5′), 174.2 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: m/z 645.5 $[M + H]^+$.

3.2.5. 2-Benzyloxy-phenyl 1,2,4-oxadiazole ester of betulinic acid (18)

Yield: 60%; white solid; mp 203-204 °C; IR (KBr): ν_{max} 1602 (-C = N), 1733 (ester), 3066 (=C-H), 3534 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.78, 0.81, 0.96, 0.97, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.65-2.23 (all m, remaining protons), 2.99-3.04 (m, 1H, H-19), 3.16 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.60 (d, 1H, J = 1.6 Hz, H-29α), 4.73 (d, 1H, J = 1.6 Hz, H-29β), 5.28 (s, 2H, 1‴-OCH_2), 5.33-5.43 (m, 2H, 5′-CH_2), 7.04-7.08 (m, 2H, H-3″ & H-5″), 7.27 (dd, 1H, J = 2.0, 1.6 Hz, H-4″), 7.35 (m, 3H, H-3‴, H-4‴ & H-5‴), 7.53 (dd, 2H, J = 1.2, 1.2 Hz, H-2‴ & H-6‴), 8.01 (dd, 1H, J = 1.6, 1.6 Hz, H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.3 (C-27), 15.9 (C-25), 16.1 (C-26), 18.2 (C-6), 19.3 (C-11), 20.8 (C-30), 25.4 (C-23), 27.4 (C-24), 27.9 (C-12), 29.6 (C-2), 30.4 (C-15), 30.9 (C-21), 31.9 (C-16), 34.2 (C-7), 36.8 (C-22), 36.9 (C-10), 37.1 (C-1), 38.1 (C-13), 38.7 (C-4), 38.8 (C-8), 40.6 (C-14), 42.4 (C-19), 46.7 (C-18), 49.4 (C-9), 50.5 (C-5), 55.3 (C-17), 56.0 (5′-CH_2), 70.4 (1‴-CH_2), 78.9 (C-3), 109.7 (C-29), 113.4 (C-3″), 116.1 (C-1″), 120.9 (C-5″), 126.7 (C-6‴), 126.9 (C-4‴), 127.6 (C-2‴), 127.3 (C-5‴), 128.8 (C-3‴), 129.0 (C-4″), 131.4 (C-6″), 136.6 (C-1‴), 150.3 (C-20), 157.1 (C-2″), 166.9 (C-5′), 173.2 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: m/z 721.5 $[M + H]^+$.

3.2.6. 3-Pyridine-1,2,4-oxadiazole ester of betulinic acid (19)

Yield: 65%; white solid; mp 203-204 °C; IR (KBr): ν_{max} 1594 (-C=N), 1739 (ester), 3067 (=C-H), 3474 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.75, 0.85, 0.96, 0.97, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.68-2.35 (all m, remaining protons), 2.92-2.97 (m, 1H, H-19), 3.18 (dd, 1H, J = 4.8, 4.4 Hz, H-3), 4.60 (d, 1H, J = 1.6 Hz, H-29α), 4.72 (d, 1H, J = 1.6 Hz, H-29β), 5.38-5.39 (m, 2H, 5′-CH_2), 7.42-7.47 (m, 1H, H-5″), 8.34-8.37 (m, 1H, H-4″), 8.75 (dd, 1H, J = 1.6, 1.6 Hz, H-6″), 9.30 (d, J = 1.6 Hz, 1H, H-2″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.4 (C-27), 15.5 (C-25), 16.2 (C-26), 16.3 (C-6), 19.2 (C-30), 20.8 (C-11), 25.4 (C-24), 27.3 (C-23), 27.9 (C-12), 29.6 (C-2), 30.3 (C-15), 30.9 (C-21), 31.8 (C-16), 34.2 (C-7), 36.8 (C-22), 37.1 (C-13), 38.1 (C-1), 38.7 (C-10), 38.8 (C-4), 40.6 (C-8), 42.4 (C-14), 46.7 (C-19), 49.4 (C-18), 50.5 (C-9), 55.3 (C-5), 56.0 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.8 (C-29), 122.7 (C-5″), 123.6 (C-1″), 134.8 (C-6″), 148.6 (C-4″), 150.1 (C-20), 152.1 (C-2″), 166.5 (C-5′), 175.1 (C-3′), 175.1 (-$COOCH_2$); ESI–MS: m/z 616.5 $[M + H]^+$.

3.2.7. Phenyl-1,2,4-oxadiazole ester of betulinic acid (20)

Yield: 75%; white solid; mp 152-153 °C; IR (KBr): ν_{max} 1609 (-C=N), 1739 (ester), 3067 (=C-H), 3473 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.74, 0.77, 0.86, 0.96, 0.97, 1.69 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.69-2.37 (all m, remaining protons), 2.92-2.95 (m, 1H, H-19), 3.18 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.60 (d, 1H, J = 2.0 Hz, H-29α), 4.72 (d, 1H, J = 1.6 Hz, H-29β), 5.32-5.41 (m, 2H, 5′-CH_2), 7.48-7.52 (m, 3H, H-3″, H-4″ & H-5″), 8.06 (dd, 2H, J = 1.6, 1.2 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.1 (C-27), 18.6 (C-25), 19.8 (C-6), 20.6 (C-26), 25.6 (C-11), 27.2 (C-30), 27.4 (C-23), 25.4 (C-24), 27.3 (C-12), 27.9 (C-2), 29.6 (C-15), 30.4 (C-21), 31.9 (C-16), 34.2 (C-7), 36.8 (C-22), 37.1 (C-10), 38.1 (C-4), 38.7 (C-1), 38.8 (C-13), 40.6 (C-8), 42.4 (C-14), 46.7 (C-19), 49.4 (C-18), 50.5 (C-9), 55.3 (C-5), 56.0 (C-17), 56.8 (5′-CH_2), 79.0 (C-3), 109.8 (C-29), 126.3 (C-1″), 127.5 (C-2″ & C-6″), 128.8 (C-3″ & C-5″), 131.4 (C-4″), 150.2 (C-20), 168.4 (C-5′), 174.5 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: *m/z* 614.5 $[M + H]^+$.

3.2.8. Benzyl-1,2,4-oxadiazole ester of betulinic acid (21)

Yield: 70%; white solid; mp 176-177 °C; IR (KBr): ν_{max} 1591 (-C=N), 1739 (ester), 3068 (=C-H), 3382 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.75, 0.80, 0.87, 0.96, 0.99, 1.68 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.66-2.31 (all m, remaining protons), 2.92-2.96 (m, 1H, H-19), 3.16 (dd, 1H, J = 4.8, 4.8 Hz, H-3), 4.07 (s, 2H, 3′-CH_2), 4.59 (d, 1H, J = 2.0 Hz, H-29α), 4.71 (d, 1H, J = 1.6 Hz, H-29β), 5.20-5.29 (m, 2H, 5′-CH_2), 7.31-7.32 (m, 2H, H-2″ to H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.8 (C-27), 18.5 (C-25), 19.1 (C-6), 20.1 (C-26), 20.6 (C-30), 20.8 (C-11), 25.5 (C-23), 27.4 (C-24), 27.9 (C-12), 29.6 (C-2), 30.3 (C-15), 30.9 (C-21), 31.8 (C-16), 32.2 (1″-CH_2), 34.3 (C-7), 36.8 (C-22), 37.1 (C-10), 38.2 (C-1), 38.7 (C-4), 38.8 (C-13), 40.7 (C-8), 42.4 (C-14), 46.7 (C-19), 49.4 (C-18), 50.5 (C-5), 55.3 (C-9), 56.0 (C-17), 56.7 (5′-CH_2), 78.9 (C-3), 109.7 (C-29), 127.2 (C-4″), 128.7 (C-3″ & C-5″), 129.0 (C-2″ & C-6″), 135.1 (C-1″), 150.2 (C-20), 169.5 (C-5′), 174.5 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: *m/z* 629.5 $[M + H]^+$.

3.2.9. 2-Chlorophenyl-1,2,4-oxadiazole ester of betulinic acid (22)

Yield: 65%; white solid; mp 155-156 °C; IR (KBr): ν_{max} 1603 (-C=N), 1741 (ester), 3075 (=C-H), 3488 (-OH) cm $^{-1}$; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.75, 0.79, 0.82, 0.97, 0.99, 1.66 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.66-2.24 (all m, remaining protons), 2.93-2.97 (m, 1H, H-19), 3.19 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.61 (d, 1H, J = 1.2 Hz, H-29α), 4.72 (d, 1H, J = 1.2 Hz, H-29β), 5.36-5.44 (m, 2H, 5′-CH_2), 7.05-7.07 (m, 2H, H-3″ & H-5″), 7.47 (dd, 1H, J = 2.0, 1.6 Hz, H-4″), 7.98 (dd, 1H, J = 1.6, 1.6 Hz, H-6‴); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.4 (C-27), 15.7 (C-25), 15.8 (C-26), 16.0 (C-6), 18.4 (4″-CH_3), 19.5 (C-30), 20.5 (C-11), 25.6 (C-24), 27.5 (C-23), 27.8 (C-12), 29.5 (C-2), 30.3 (C-15), 31.8 (C-21), 34.3 (C-16), 36.7 (C-7), 37.2 (C-22), 38.3 (C-13), 38.6 (C-1), 40.6 (C-10), 42.3 (C-4), 46.5 (C-8), 49.5 (C-14), 49.6 (C-19), 50.1 (C-18), 55.4 (C-9), 56.1 (C-5), 56.3 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.6 (C-29), 114.3 (C-3″), 118.9 (C-1″), 123.4 (C-5″), 129.1 (C-4″), 134.2 (C-6″), 150.4 (C-20), 162.2 (C-2″), 168.7 (C-5′), 174.1 (C-3′), 175.2 (-$COOCH_2$); ESI–MS: *m/z* 649.5 $[M + H]^+$.

3.2.10. 4-Fluoro-1,2,4-oxadiazole ester of betulinic acid (23)

Yield: 80%; white solid; mp 163-164 °C; IR (KBr) ν_{max}: 1590 (-C=N), 1733 (ester), 3069 (=C-H), 3542 (-OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.72, 0.77, 0.85, 0.96, 0.98, 1.67 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.69-2.27 (all m, remaining protons), 3.01-3.05 (m, 1H, H-19), 3.18 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.56 (d, 1H, J = 1.6 Hz, 1H, H-29α), 4.72 (d, 1H, J = 1.6 Hz, H-29β), 5.31-5.38 (m, 2H, 5′-CH_2), 7.28 (d, 2H, J = 8.0 Hz, H-3″ & H-5″), 7.96 (d, 2H, J = 8.0 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.2 (C-27), 18.2 (C-25), 19.3 (C-26), 20.8 (C-6), 25.4 (C-30), 27.2 (C-11), 27.4 (C-24), 27.9 (C-23), 29.0 (C-12), 29.4 (C-2), 29.6 (C-15), 30.3 (C-21), 31.9 (C-16), 34.6 (C-7), 36.8 (C-22), 37.1 (C-13), 38.1 (C-1), 38.5 (C-10), 40.7 (C-4), 42.4 (C-8), 46.7 (C-14), 49.5 (C-19), 50.5 (C-18), 55.3 (C-9), 56.0 (C-5), 56.4 (C-17), 64.2 (5′-CH_2), 78.7 (C-3), 109.7 (C-29), 123.5 (C-1″), 127.4 (C-2″ & C-6″), 129.4 (C-3″ & C-5″), 141.7 (C-4″), 150.1 (C-20), 168.8 (C-5′), 174.1 (C-3′), 175.4 (-$COOCH_2$); ESI–MS: m/z 633.5 $[M + H]^+$.

3.2.11. 2-Methylphenyl-1,2,4-oxadiazole ester of betulinic acid (24)

Yield: 69%; white solid; mp 175-176 °C; IR (KBr): ν_{max} 1608 (-C=N), 1742 (ester), 3075 (-C = C), 3483 (-OH) cm $^{-1}$; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.73, 0.76, 0.84, 0.96, 0.98, 1.67 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.63-2.21 (all m, remaining protons), 2.57 (s, 3H, 2″-CH_3), 2.93-2.97 (m, 1H, H-19), 3.19 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.58 (d, 1H, J = 1.2 Hz, H-29α), 4.76 (d, 1H, J = 1.2 Hz, H-29β), 5.39-5.44 (m, 2H, 5′-CH_2), 7.05-7.09 (m, 2H, H-3″ & H-5″), 7.45 (dd, 1H, J = 2.0, 1.6 Hz, H-4″), 7.97 (dd, 1H, J = 1.6, 1.6 Hz, H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 15.1 (C-27), 15.6 (C-25), 15.8 (C-26), 16.0 (C-6), 18.2 (2″-CH_3), 19.3 (C-30), 20.8 (C-11), 25.5 (C-24), 27.4 (C-23), 27.9 (C-12), 29.6 (C-2), 30.4 (C-15), 31.9 (C-21), 34.2 (C-16), 36.8 (C-7), 37.1 (C-22), 38.1 (C-13), 38.8 (C-1), 40.7 (C-10), 42.4 (C-4), 46.7 (C-8), 49.4 (C-14), 49.5 (C-19), 50.5 (C-18), 55.3 (C-9), 56.1 (C-5), 56.6 (C-17), 56.8 (5′-CH_2), 78.9 (C-3), 109.8 (C-29), 114.4 (C-3″), 118.6 (C-1″), 123.2 (C-5″), 129.3 (C-4″), 134.5 (C-6″), 150.5 (C-20), 162.4 (C-2″), 168.2 (C-5′), 174.6 (C-3′), 175.0 (-$COOCH_2$); ESI–MS: m/z 629.5 $[M + H]^+$.

3.2.12. 4-Bromo-1,2,4-oxadiazole ester of betulinic acid (25)

Yield: 84%; white solid; mp 193-195 °C; IR (KBr) cm^{-1}: ν_{max} 1596 (-C=N), 1723 (ester), 3075 (=C-H), 3548 (-OH) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 0.71, 0.75, 0.85, 0.96, 0.98, 1.65 (all s, each 3H, 24-CH_3, 25-CH_3, 26-CH_3, 23-CH_3, 27-CH_3, 30-CH_3), 0.69-2.27 (all m, remaining protons), 3.01-3.05 (m, 1H, H-19), 3.18 (dd, 1H, J = 9.1, 4.3 Hz, H-3), 4.54 (d, 1H, J = 1.6 Hz, H-29α), 4.71 (d, 1H, J = 1.6 Hz, H-29β), 5.31-5.37 (m, 2H, 5′-CH_2), 7.26 (d, 2H, J = 8.0 Hz, H-3″ & H-5″), 7.98 (d, 2H, J = 8.0 Hz, H-2″ & H-6″); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.1 (C-27), 18.2 (C-25), 19.3 (C-26), 20.8 (C-6), 25.4 (C-30), 27.2 (C-11), 27.4 (C-24), 27.9 (C-23), 29.0 (C-12), 29.4 (C-2), 29.6 (C-15), 30.3 (C-21), 31.9 (C-16), 34.6 (C-7), 36.8 (C-22), 37.1 (C-13), 38.1 (C-1), 38.5 (C-10), 40.7 (C-4), 42.4 (C-8), 46.7 (C-14), 49.5 (C-19), 50.5 (C-18), 55.3 (C-9), 56.0 (C-5), 56.4 (C-17), 64.2 (5′-CH_2), 78.7 (C-3), 109.7 (C-29), 123.5 (C-1″), 127.4 (C-2″ & C-6″), 129.4 (C-3″ & C-5″), 141.2 (C-4″), 150.1 (C-20), 168.8 (C-5′), 174.1 (C-3′), 175.4 (-$COOCH_2$); ESI–MS: m/z 693.5 $[M + H]^+$.

3.3. Anticancer activity

3.3.1. Cell lines and cell culture

The cell lines are human colon cancer cell line (Colo 205) and hepatocellular carcinoma cell line (Hep G2), which was derived from the liver tissue of a 15-year-old Caucasian American male and HeLa (Hela or HeLa cell) the oldest and most commonly used human cell line [23]. The cell line was derived from cervical cancer cells taken on 8 February 1951 [24] from Henrietta Lacks, a patient who eventually died of cancer on 4 October 1951. George Gey was able to isolate one specific cell, multiply it, and start a cell line. Gey named the sample HeLa, after the initial letters of Henrietta Lack's name. Colo 205 (Human colon cancer cell line), Hep G2 (Human liver carcinoma cell line) and HeLa (Cervical cancer cell line) cell lines were obtained from the National Centre for Cellular Sciences (NCCS), Pune, India. Cells were cultured in RPMI-1640 media (RPMI – Roswell Park Memorial Institute medium), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM $NaHCO_3$, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained in culture at 37 °C in an atmosphere of 5% CO_2.

3.3.2. Test concentrations

Initially, stock solutions of each test substance were prepared in 100% dimethyl sulfoxide (DMSO, Sigma Aldrich) with a final concentration of 8 mg/ml. Exactly 50 µl of stock was diluted to 1 ml in culture medium to obtain experimental stock concentration of 400 µg/ml. This solution was further serially diluted with media to generate a dilution series of 10 to 200 µg/ml. Precisely, 100 µl of each test concentration was added to 100 µl of cell suspension (total assay volume of 200 µl, and efficacy of the derivatives was evaluated with three different set of experiments) and incubated for 24 h at 37 °C in 5% CO_2.

3.3.3. Cytotoxicity

The cytotoxicity of novel BA-1,2,4-oxadiazole esters **(14–25)** was screened on human colorectal cancer (Colo-205), human hepatocellular liver carcinoma (Hep G2), and human cervical cancer (HeLa) cell lines using etoposide as positive control by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, according to the method of Mossman (1983). Briefly, the cells (2×10^4) were seeded in each well containing 100 µl of medium in 96 well plates. After overnight incubation at 37 °C in 5% CO_2, exactly 100 µl of different test concentrations (10–200 µg/ml) was added to the cell suspension, which is equivalent to 2–40 µg per 200 µl of assay volume. The viability of cells was assessed after 24 h, by adding 10 µl of MTT (5 mg/ml) per well and incubated at 37 °C for additional three hours. The medium was discarded and the formazan blue, which formed in the cells, was dissolved in 100 µl of DMSO. The intensity of color formation was measured at 570 nm in a spectrophotometer (Spectra MAX Plus, Molecular Devices, supported by SOFTmax PRO-5.4). The percent inhibition of cell viability was determined with reference to the control values (without test compound). The data were subjected to linear regression analysis and the regression lines were plotted for the best straight-line fit. The IC_{50} (inhibition of cell viability) concentrations were calculated using the respective regression equation and expressed in µM.

4. Conclusion

Herewith, we report the simple and efficient method for synthesis of novel mixed natural product heterocyclic compounds, C-28 modified 1,2,4-oxadiazole esters of betulinic acid **(14–25)**. Anticancer screening revealed that compound **16** was active against HeLa cell line with an IC_{50} of 43.4 μM and compound **17** was found to be interesting candidate with an IC_{50} of 26.1 and 30.1 μM against Colo-205 and Hep G2 cell lines.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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