Design, Synthesis, Molecular Modelling, and Biological Evaluation of Oleanolic Acid-Arylidene Derivatives as Potential Anti-Inflammatory Agents

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Introduction: Oleanolic acid, a pentacyclic triterpenic acid, is widely distributed in medicinal plants and is the most commonly studied triterpene for various biological activities, including anti-allergic, anti-cancer, and anti-inflammatory.

Methods: The present study was carried out to synthesize arylidene derivatives of oleanolic acid at the C-2 position by Claisen Schmidt condensation to develop more effective anti-inflammatory agents. The derivatives were screened for anti-inflammatory activity by scrutinizing NO production inhibition in RAW 264.7 cells induced by LPS and their cytotoxicity. The potential candidates were further screened for inhibition of LPS-induced interleukin (IL-6) and tumour necrosis factor-alpha (TNF- α) production in RAW 264.7 cells.

Results: The results of in vitro studies revealed that derivatives 3d, 3e, 3L, and 3o are comparable to that of the oleanolic acid on the inhibition of TNF- α and IL-6 release. However, derivative 3L was identified as the most potent inhibitor of IL-6 (77.2%) and TNF- α (75.4%) when compared to parent compound, and compounds 3a (77.18%), 3d (71.5%), and 3e (68.8%) showed potent inhibition of NO than oleanolic acid (65.22%) at 10 μ M. Besides, from docking score and Cyscore analysis analogs (3e, 3L, 3n) showed greater affinity towards TNF- α and IL-1 β than dexamethasone.

Conclusion: Herein, we report a series of 15 new arylidene derivatives of oleanolic acid by Claisen Schmidt condensation reaction. All the compounds synthesized were screened for their anti-inflammatory activity against NO, TNF- α and IL-6. From the data, it was evident that most of the compounds exhibited better anti-inflammatory activity.

Keywords: LPS, natural products, IL-1 β , IL-6, inflammation, RAW 264.7 cells

Introduction

Inflammation is a combination of highly regulated sequences of events provoked by a variety of stimuli which include microbial, allergic, metabolic, autoimmune, constitutive and physical factors. The series of events are distinguished by five classical inflammatory signs, including redness, pain, swelling, and heat as described by Celsius. Virchow, later on, added the fifth sign loss of function during the 19th century. The response to inflammatory stimulus includes a vascular response (dilation and increased permeability) and a cellular response (leukocytes migration, cellular activation), the whole process is regulated by inflammatory mediators. First, there is an increase in the pro-inflammatory mediators and then of the anti-inflammatory mediators. Depending on the etiological factor and the balance between the inflammatory mediators will be the progression towards

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healing or injury.^{1–8} Moreover, inflammation is the body's primary response to different harmful stimuli involving the innate and adaptive immune system. However, this physiological side of inflammation relies on the presence of endogenous suppressors of pro-inflammatory signalling pathways.⁹

Regardless of the fact that drug design and discovery has a high reliance on synthetic chemistry, contribution of natural products cannot be ignored as they played a prominent role in the discovery of various leads for drug development to treat various human diseases. Moreover, WHO list of essential drugs consists of 252 drugs, of which 11% are of plant origin.^{10–22} Proper screening of biologically active natural products results in the identification of various bioactive molecules as well as drugs.^{23–30} One such example is the discovery of the first analgesic, anti-inflammatory drug aspirin from the bark of willow tree by Greeks and Romans since 400 BC. In 1989 aspirin (acetylsalicylic acid) was approved as the first drug for the treatment of rheumatic disease.³¹ Triterpenoids represent a group of C₃₀ compounds which

are biosynthetically derived from the cyclization of squalene.³² Due to the ubiquitous nature and diverse biological activities of triterpenoids, they have been the target of interest worldwide for both chemists as well as biologists. Triterpenoids like ursolic acid, oleanolic acid, betulinic acid, and moronic acid (Figure 1) have been reported to exhibit anti-inflammatory, anti-cancer, anti-allergic, and hepatoprotective properties.^{12,33–42} The presence of the carboxyl group at C-17 position, hydroxyl group at the C-3 position and double bond at C-12 position in pentacyclic triterpenoids make these natural scaffolds susceptible to a variety of chemical transformations.⁴³ Oleanolic acid, a pentacyclic triterpenic acid, is widely distributed in medicinal plants and food and is the most commonly studied triterpene for various biological activities.^{37,38,44} On oleanolic acid, substantial structural modifications have been executed to uncover more potent anti-inflammatory derivatives.^{45–47}

The present study was carried out to synthesize arylidene derivatives of oleanolic acid at the C-2 position to develop more effective anti-inflammatory agents. To

Ursolic acid

Betulinic acid

Moronic acid

Oleanolic acid

Figure 1 Some triterpenes with potential biological activity as an anti-inflammatory agent.

synthesize its various derivatives, oleanolic acid was isolated from *Plectranthus rugosus* by column chromatography of ethyl acetate fraction followed by recrystallization from methanol. Nmr and mass spectrometry confirmed the structures. The derivatives were screened for anti-inflammatory activity by scrutinizing the NO production inhibition in RAW 264.7 cells induced by LPS and their cytotoxicity. The potential candidates were further screened for inhibition of LPS-induced interleukin (IL-6) and tumour necrosis factor-alpha (TNF- α) production in RAW 264.7 cells.

Experimental Section

Materials and Methods

All the chemicals, as well as reagents, are of high purity. Dulbecco's eagle medium (DMEM) and phosphate buffer saline (PBS) were purchased from Sigma, UK. Elisa kits, IL-6, Human TNF- α , were purchased from Invitrogen (USA). From Gibco USA, fetal bovine serum was obtained and lipopolysaccharide (LPS) *E. Coli* from Calbiochem (USA). Griess reagent was purchased from Promega (USA) and MTT from Calbiochem (San Diego CA). Besides, other chemicals that were used were of research-grade. From the central drug house (CDH) Jammu, ferric chloride, glacial acetic acid, chloroform was purchased. Ascorbic acid, ethanol, methanol, picric acid, calcium chloride, sodium chloride, 2,2 diphenyl picryl hydrazyl (DPPH), sucrose, trichloroacetic acid were procured from Merck. From Qualigens, potassium dihydrogen phosphate, hydrogen peroxide and hydrochloric acid were purchased. Ferric nitrate, ethylene diamine tetraacetate, potassium chloride, sodium hydroxide, dimethyl sulfoxide, sodium dihydrogen monophosphate was purchased from HiMedia. From sisco research laboratories (SRL) butylated hydroxytoluene was bought and from Rankem ethyl acetate and hexane was purchased.

Chemistry

In the present study, oleanolic acid (**1**) was isolated from leaf extract of *Plectranthus rugosus* by column chromatography in 60:40 hexane:ethyl-acetate followed by recrystallization from methanol. The solvents were distilled before using in the reaction. All the reactions were monitored by using 0.2 mm-thick, aluminum-backed TLC plates and were visualized under UV light at 254 nm. The structure was confirmed by nmr and mass spectrometry to give pure white amorphous powder having

percentage purity more than 95% mp: 281–283°C [α]_D²¹ +81.3° (CHCl₃; c = 0.6). ¹H NMR (400 MHz, CDCl₃): δ 5.27 (1H, s, H-12), 3.21 (1H, d, J=7.2, H-3), 2.83 (1H, d, J=11.3, H-12), 1.97 (2H, m, H-2), 0.91 (3H, s, H-23), 0.72 (3H, s, CH₃-24), 0.76 (3H, s, CH₃-25), 0.88 (3H, s, CH₃-26), 1.14 (3H, s, CH₃-27), 0.92 (3H, s, CH₃-29), 0.96 (3H, s, CH₃-30). ¹³C NMR (100 MHz, CDCl₃): δ 183.22 (C-28), 143.62 (C-13), 122.62 (C-12), 79.12 (C-3), 55.24 (C-5), 47.68 (C-9), 46.55 (C-17), 45.7 (C-19), 41.65 (C-14), 40.96 (C-18), 39.12 (C-8), 38.8 (C-1), 37.12 (C-10), 33.86 (C-21), 33.09 (C-29), 32.55 (C-22), 32.59 (C-7), 30.66 (C-20), 28.77 (C-23), 27.9 (C-15), 27.8 (C-2), 25.49 (C-27), 23.89 (C-30), 23.44 (C-16), 22.98 (C-11), 18.33 (C-6), 17.38 (C-26), 15.55 (C-25), 15.38 (C-24). ESI-MS m/z 456 [M+H]⁺

Synthesis of Arylidene-Oleanolic Acid Derivatives

The protocol for synthesis of oleanolic acid-arylidene derivatives **3** from the parent compound oleanolic acid **1** involves two steps which includes oxidation with pyridinium chlorochromate (PCC) reagent followed by Claisen Schmidt condensation (Scheme 1). So far, many synthetic strategies for the preparation of oleanolic acid analogs have been reported.^{48–52} In a typical procedure, to a solution of compound **1** (400 mg, 0.87 mmol) in dichloromethane (DCM) at room temperature (rt) few drops of PCC reagent were added. The reaction was monitored by TLC till its completion in 4 h. The reaction mixture was filtered to remove insoluble residue after quenching with cold water. The filtrate was extracted with ethyl-acetate and the organic layer was finally dried over sodium sulphate, concentrated in vacuum and purified by column chromatography to finally give compound **2** (365 mg 91% yield) as pure white amorphous solid; mp 179–183°C; ¹H NMR (400MHz, CDCl₃): δ 5.27 (1H, s, H-12), 3.22 (1H, d, J = 12.0 Hz, H-18), 1.90 (2H, m), 1.82 (4H, m), 1.55 (12H, m), 1.33 (4H, m) 1.05 (3H, s, CH₃-23), 0.94 (3H, s, CH₃-24), 0.86 (3H, s, CH₃-25), 0.84 (3H, s, CH₃-26), 0.82 (3H, s, CH₃-27), 0.79 (3H, s, CH₃-29), 0.65 (3H, s, CH₃-30); ¹³C NMR (100 MHz, CDCl₃): δ 215.7, 181.25, 151.29, 123.32, 53.07, 52.19, 51.18, 50.07, 49.09, 47.29, 46.48, 41.34, 40.22, 39.55, 39.29, 39.18, 39.15, 39.12, 39.08, 38.39, 38.24, 38.18, 36.33, 28.19, 23.37, 21.19, 17.44, 17.78, 16.59, 15.24 ESI-MS m/z 455 [M+H]⁺

Scheme 1 Reagents and conditions (a) PCC, DCMrt, 90% (b) Ar-CHO, LiOH, Ethanol, rt, 80%.

To the solution of compound **2** (365 mg, 0.8 mmol), in ethanol at room temperature lithium hydroxide LiOH (1.2 Eq) was added. To this mixture, various aromatic aldehydes were added ([Table 1](#)) by Claisen Schmidt condensation reaction ([Scheme 1](#)). The crude mixture was extracted with ethyl-acetate, and the organic layer was dried over sodium sulphate, concentrated in vacuum and purified by column chromatography to give pure **3a-3o** in 80–90% yield

3-Oxo-2-[4-Fluorobenzylidenyl]-Olean-12-En-28-Oic Acid (**3a**)

Yield 92%; mp 159–161; ^1H NMR (400 MHz, CDCl_3): δ 7.57 (1H, s), 7.39 (2H, m), 7.37 (2H, m), 5.28 (1H, s), 2.35 (2H, m), 2.27 (1H, d, $j = 8.0$ Hz), 1.93 (4H, m), 1.78 (3H, s, CH3-23), 1.63 (3H, s, CH3-24), 1.45 (3H, s, CH3-25), 1.39 (3H, s, CH3-26), 1.09 (3H, s, CH3-27), 0.85 (3H, s, CH3-29), 0.48 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 207.56, 181.08, 142.24, 138.66, 135.31, 132.19, 132.19, 130.55, 129.22, 129.22, 123.87, 123.13, 55.22, 51.49, 48.89, 47.14, 46.29, 42.33, 40.87, 39.66, 39.25, 38.98, 37.46, 34.22, 33.68, 30.45, 29.18, 26.34, 24.45, 23.97, 23.77, 20.21, 19.34, 18.92, 18.23, 16.45. ESI-MS m/z 561 $[\text{M}+\text{H}]^+$

3-Oxo-2-[3-Bromobenzylidenyl]-Olean-12-En-28-Oic Acid (**3b**)

Yield 93%; mp 148–151; ^1H NMR (400 MHz, CDCl_3): δ 7.48 (1H, s), 7.36 (2H, m), 7.22 (2H, m), 5.32 (1H, s), 2.45 (1H, m), 2.42 (2H, m), 2.23 (1H, $j = 8.0$ Hz), 1.92 (4H, m), 1.72 (3H, s, CH3-23), 1.62 (3H, s, CH3-24), 1.42 (3H, s, CH3-25), 1.33 (3H, s, CH3-26), 1.08 (3H, s, CH3-27), 0.80 (3H, s, CH3-29), 0.44 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 207.91, 183.84, 143.09, 138.43,

137.97, 136.18, 135.34, 134.67, 130.29, 129.96, 128.66, 128.19, 127.97, 127.18, 125.55, 125.08, 64.78, 53.33, 52.88, 48.28, 45.55, 43.94, 42.33, 39.66, 39.34, 39.08,

Table 1 Oleanolic Acid-Arylidene Derivatives Varying at the Aromatic Ring at the C-2 Position

Compound	R	Time of Reaction (h)	Yield (%Age)
3a		03	92%
3b		02	93%
3c		04	83%
3d		2.5	88%
3e		04	92%
3f		03	81%
3g		03	87%

(Continued)

Table I (Continued).

Compound	R	Time of Reaction (h)	Yield (%Age)
3h		02	80%
3i		05	91%
3j		03	86%
3k		03	92%
3L		05	87%
3m		04	88%
3n		02	81%
3o		03	83%

36.87, 36.64, 32.28, 30.85, 29.86, 29.67, 28.17, 24.22, 23.77, 22.94, 21.32, 20.44, 17.27. ESI-MS m/z 622 $[M+H]^+$

3-Oxo-2-[3-Chlorobenzylidenyl]-Olean-12-En-28-Oic Acid (3c)

Yield 83%; mp 178–181; 1H NMR (400 MHz, $CDCl_3$): δ 7.50 (1H, s), 7.31 (2H, m), 7.25 (2H, m), 5.31 (1H, s), 2.43 (1H, m), 2.41 (2H, m), 2.27 (1H, d, $j=8.0$ Hz), 1.96 (4H, m), 1.76 (3H, s, CH3-23), 1.61 (3H, s, CH3-24), 1.46 (3H, s, CH3-25), 1.38 (3H, s, CH3-26), 1.04 (3H, s, CH3-27), 0.65 (3H, s, CH3-29), 0.58 (3H, s, CH3-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.94, 183.86, 143.04, 138.35, 137.97, 136.15, 135.34, 134.65, 130.23, 129.98, 128.63, 128.16, 127.93, 127.93, 127.17, 125.60, 125.05, 64.71, 53.37, 52.81, 48.27, 45.54, 43.95, 42.39, 39.64, 39.33, 39.05, 36.87, 36.64, 32.28, 30.86, 29.88, 29.66,

28.18, 24.27, 23.77, 22.91, 21.37, 20.48, 17.26. ESI-MS m/z 578 $[M+H]^+$

3-Oxo-2-[4-Nitrobenzylidenyl]-Olean-12-En-28-Oic Acid (3d)

Yield 88%; mp 154–156; 1H NMR (400 MHz, $CDCl_3$): δ 7.62 (1H, s), 7.45 (2H, m), 7.31 (1H, m), 7.22 (1H, m), 5.31 (1H, s), 2.41 (2H, m), 2.27 (1H, d, $j=8.0$ Hz), 1.94 (4H, m), 1.79 (3H, s, CH3-23), 1.66 (3H, s, CH3-24), 1.49 (3H, s, CH3-25), 1.29 (3H, s, CH3-26), 1.04 (3H, s, CH3-27), 0.80 (3H, s, CH3-29), 0.43 (3H, s, CH3-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.53, 181.04, 142.22, 137.45, 134.81, 133.44, 129.77, 129.53, 129.42, 128.13, 126.28, 123.02, 55.23, 51.97, 48.92, 47.19, 46.23, 42.77, 40.75, 39.66, 39.38, 38.97, 37.55, 34.44, 33.91, 30.22, 29.18, 26.62, 24.28, 23.91, 23.77, 20.02, 19.44, 18.66, 18.19, 16.82. ESI-MS m/z 588 $[M+H]^+$

3-Oxo-2-[4-Chlorobenzylidenyl]-Olean-12-En-28-Oic Acid (3e)

Yield 92%; mp 183–185; 1H NMR (400 MHz, $CDCl_3$): δ 7.52 (1H, s), 7.33 (2H, m), 7.21 (1H, m), 7.12 (1H, m), 5.41 (1H, s), 2.42 (1H, m), 2.41 (2H, m), 2.28 (1H, d, $j=8.0$ Hz), 1.92 (4H, m), 1.72 (3H, s, CH3-23), 1.60 (3H, s, CH3-24), 1.48 (3H, s, CH3-25), 1.32 (3H, s, CH3-26), 1.12 (3H, s, CH3-27), 0.88 (3H, s, CH3-29), 0.44 (3H, s, CH3-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.52, 181.05, 142.21, 138.66, 135.56, 134.14, 130.52, 129.30, 129.28, 123.09, 55.28, 51.92, 48.94, 47.59, 46.22, 42.77, 40.79, 39.38, 39.33, 38.97, 37.55, 34.44, 33.92, 30.29, 29.19, 26.66, 24.28, 23.92, 23.71, 20.09, 19.66, 18.63, 18.15, 16.89. ESI-MS m/z 578 $[M+H]^+$

3-Oxo-2-[2-Methoxybenzylidenyl]-Olean-12-En-28-Oic Acid (3f)

Yield 81%; mp 171–174; 1H NMR (400 MHz, $CDCl_3$): δ 7.46 (1H, s), 7.32 (2H, m), 7.12 (1H, m), 6.96 (1H, m), 5.32 (1H, s), 3.83 (3H, s), 2.88 (1H, m), 2.43 (2H, m), 2.28 (1H, d, $j=8.0$ Hz), 1.98 (4H, m), 1.79 (3H, s, CH3-23), 1.65 (3H, s, CH3-24), 1.48 (3H, s, CH3-25), 1.39 (3H, s, CH3-26), 1.09 (3H, s, CH3-27), 0.85 (3H, s, CH3-29), 0.48 (3H, s, CH3-30). ^{13}C NMR (100 MHz, $CDCl_3$): δ 207.91, 183.88, 159.71, 138.27, 137.44, 134.33, 129.49, 125.88, 122.82, 116.04, 114.19, 55.49, 53.48, 52.98, 48.29, 45.42, 44.28, 42.43, 39.59, 39.49, 39.07, 36.98, 36.56, 32.31, 30.77, 29.88, 28.27, 24.39, 23.77, 22.99, 21.44, 20.55, 17.33, 17.09, 15.66. ESI-MS m/z 573 $[M+H]^+$

3-Oxo-2-[2-Trifluoromethylbenzylidenyl]-Olean-12-En-28-Oic Acid (3g)

Yield 87%; mp 177–179; ^1H NMR (400 MHz, CDCl_3): δ 7.58 (1H, s), 7.52 (2H, m), 7.41 (1H, m), 7.32 (1H, m), 5.28 (1H, s), 2.48 (1H, m), 2.46 (2H, m), 2.26 (1H, d, j = 8.0 Hz), 1.94 (4H, m), 1.75 (3H, s, CH3-23), 1.62 (3H, s, CH3-24), 1.44 (3H, s, CH3-25), 1.30 (3H, s, CH3-26), 1.19 (3H, s, CH3-27), 0.95 (3H, s, CH3-29), 0.38 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 207.92, 183.84, 159.77, 138.29, 137.47, 134.36, 129.47, 125.86, 122.85, 116.09, 114.16, 55.47, 53.44, 52.96, 48.24, 45.44, 44.27, 42.42, 39.53, 39.45, 39.09, 36.93, 36.52, 32.37, 30.79, 29.82, 28.24, 24.32, 23.79, 22.91, 21.42, 20.59, 17.32, 17.07, 15.61 ESI-MS m/z 611 $[\text{M}+\text{H}]^+$

3-Oxo-2-[4-Bromobenzylidenyl]-Olean-12-En-28-Oic Acid (3h)

Yield 80%; mp 163–166; ^1H NMR (400 MHz, CDCl_3): δ 7.54 (1H, s), 7.32 (2H, m), 7.22 (1H, m), 7.14 (1H, m), 5.43 (1H, s), 2.45 (1H, m), 2.42 (2H, m), 2.26 (1H, d, j = 8.0 Hz), 1.94 (4H, m), 1.74 (3H, s, CH3-23), 1.64 (3H, s, CH3-24), 1.42 (3H, s, CH3-25), 1.34 (3H, s, CH3-26), 1.14 (3H, s, CH3-27), 0.82 (3H, s, CH3-29), 0.42 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 207.51, 181.06, 142.22, 138.68, 135.57, 134.11, 130.53, 129.35, 129.23, 123.04, 55.23, 51.96, 48.93, 47.54, 46.21, 42.78, 40.73, 39.34, 39.34, 38.96, 37.56, 34.47, 33.95, 30.28, 29.11, 26.63, 24.24, 23.95, 23.78, 20.04, 19.68, 18.67, 18.13, 16.82 ESI-MS m/z 622 $[\text{M}+\text{H}]^+$

3-Oxo-2-[Pyridine-4-Ylmethylene]-Olean-12-En-28-Oic Acid (3i)

Yield 91%; mp 138–141; ^1H NMR (400 MHz, CDCl_3): δ 8.57 (1H, s), 8.39 (H, m), 7.48 (2H, m), 7.27 (1H, m) 5.24 (1H, s), 2.39 (2H, m), 2.28 (1H, d, j = 8.0 Hz), 1.98 (4H, m), 1.75 (3H, s, CH3-23), 1.63 (3H, s, CH3-24), 1.45 (3H, s, CH3-25), 1.39 (3H, s, CH3-26), 1.09 (3H, s, CH3-27), 0.85 (3H, s, CH3-29), 0.48 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 205.56, 180.07, 143.27, 139.67, 136.32, 134.18, 134.18, 131.54, 128.21, 128.21, 124.88, 124.15, 56.21, 52.48, 49.88, 48.18, 47.26, 43.33, 41.88, 38.65, 38.23, 36.99, 35.43, 33.24, 32.69, 30.46, 28.19, 27.39, 25.47, 22.99, 23.76, 21.25, 18.36, 17.94, 17.26, 16.43 ESI-MS m/z 544 $[\text{M}+\text{H}]^+$

3-Oxo-2-[Pyridine-3-Ylmethylene]-Olean-12-En-28-Oic Acid (3j)

Yield 93%; mp 148–151; ^1H NMR (400 MHz, CDCl_3): δ 8.77 (1H, s), 8.44 (H, m), 7.22 (2H, m), 7.12 (1H, m) 5.33

(1H, s), 2.35 (2H, m), 2.25 (1H, d, j = 8.0Hz), 1.91 (4H, m), 1.71 (3H, s, CH3-23), 1.61 (3H, s, CH3-24), 1.40 (3H, s, CH3-25), 1.32 (3H, s, CH3-26), 1.02 (3H, s, CH3-27), 0.91 (3H, s, CH3-29), 0.41 (3H, s, CH3-30). ^{13}C NMR (100MHz, CDCl_3): δ 205.46, 180.22, 143.77, 139.66, 136.43, 134.88, 134.88, 131.87, 128.93, 128.93, 124.32, 124.32, 56.44, 52.77, 49.84, 48.39, 47.39, 43.28, 41.89, 38.56, 38.45, 36.89, 35.34, 33.58, 32.79, 30.63, 28.91, 27.28, 25.78, 22.30, 23.69, 21.59, 18.29, 17.92, 17.19, 16.54 ESI-MS m/z 544 $[\text{M}+\text{H}]^+$

3-Oxo-2-[Thiophene-2-Ylmethylene]-Olean-12-En-28-Oic Acid (3k)

Yield 92%; mp 184–186; (thiophen-2-ylmethylene)- ^1H NMR (400 MHz, CDCl_3): δ 8.13 (1H, s), 7.22 (2H, m), 7.12 (1H, m) 5.23 (1H, s), 2.32 (2H, m), 2.20 (1H, d, j = 8.0Hz), 1.92 (4H, m), 1.78 (3H, s, CH3-23), 1.68 (3H, s, CH3-24), 1.48 (3H, s, CH3-25), 1.37 (3H, s, CH3-26), 1.01 (3H, s, CH3-27), 0.82 (3H, s, CH3-29), 0.49 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 204.49, 180.28, 148.79, 142.68, 136.48, 134.80, 134.83, 131.77, 128.85, 128.23, 124.56, 124.39, 54.48, 52.57, 49.36, 48.74, 47.92, 43.93, 41.49, 38.29, 38.19, 36.19, 35.78, 33.49, 32.49, 30.49, 28.29, 27.48, 25.36, 22.22, 23.82, 21.57, 18.29, 17.74, 17.19, 16.49 ESI-MS m/z 549 $[\text{M}+\text{H}]^+$

3-Oxo-2-[2,6-Dichlorobenzylidenyl]-Olean-12-En-28-Oic Acid (3L)

Yield 87%; mp 172–174; ^1H NMR (400 MHz, CDCl_3): δ 7.53 (1H, s), 7.31 (2H, m), 7.12 (1H, m) 5.28 (1H, s), 2.36 (2H, m), 2.26 (1H, d, j = 8.0 Hz), 1.97 (4H, m), 1.75 (3H, s, CH3-23), 1.64 (3H, s, CH3-24), 1.45 (3H, s, CH3-25), 1.32 (3H, s, CH3-26), 1.13 (3H, s, CH3-27), 0.89 (3H, s, CH3-29), 0.40 (3H, s, CH3-30). ^{13}C NMR (100 MHz, CDCl_3): δ 207.59, 181.03, 142.29, 138.56, 135.38, 132.12, 132.10, 130.59, 129.24, 129.24, 123.86, 123.43, 55.58, 51.42, 48.81, 47.19, 46.25, 42.35, 40.81, 39.64, 39.21, 38.94, 37.42, 34.27, 33.63, 30.47, 29.16, 26.32, 24.48, 23.90, 23.79, 20.22, 19.37, 18.97, 18.21, 16.48 ESI-MS m/z 612 $[\text{M}+\text{H}]^+$

3-Oxo-2-[Naphthalene-1-Ylmethylene]-Olean-12-En-28-Oic Acid (3m)

Yield 88%; mp 185–187; ^1H NMR (400 MHz, CDCl_3): δ 8.17 (2 H, m), 7.59 (2H, m), 7.47 (2H, m), 7.29 (1H, m), 7.13 (1H, m) 5.24 (1H, s), 2.31 (2H, m), 2.20 (1H, d, j = 8.0Hz), 1.90 (4H, m), 1.71 (3H, s, CH3-23), 1.68 (3H, s, CH3-24), 1.47 (3H, s, CH3-25), 1.32 (3H, s, CH3-26).

1.19 (3H, s, CH₃-27), 0.86 (3H, s, CH₃-29), 0.44 (3H, s, CH₃-30). ¹³C NMR (100MHz, CDCl₃): δ 207.87, 181.11, 142.29, 138.78, 135.42, 132.17, 132.11, 130.46, 129.31, 129.31, 123.78, 123.33, 55.48, 51.41, 48.82, 47.64, 46.82, 42.59, 40.57, 39.33, 39.28, 38.79, 37.29, 34.19, 33.85, 30.29, 29.18, 26.59, 24.51, 23.75, 23.59, 20.29, 19.29, 18.89, 18.69, 16.54 ESI-MS m/z 593 [M+H]⁺

3-Oxo-2-[Benzylidenyl]-Olean-12-En-28-Oic Acid (3n)

Yield 81%; mp 144–146; ¹H NMR (400 MHz, CDCl₃): δ 7.58 (2H, m), 7.39 (2H, m), 7.37 (1H, m), 7.13 (1H, m), 5.26 (1H, s), 2.38 (2H, m), 2.21 (1H, d, j = 8.0Hz), 1.94 (4H, m), 1.77 (3H, s, CH₃-23), 1.67 (3H, s, CH₃-24), 1.46 (3H, s, CH₃-25), 1.37 (3H, s, CH₃-26), 1.09 (3H, s, CH₃-27), 0.89 (3H, s, CH₃-29), 0.47 (3H, s, CH₃-30). ¹³C NMR (100MHz, CDCl₃): δ 207.51, 181.04, 142.28, 138.64, 135.39, 132.15, 132.14, 130.59, 129.28, 129.28, 123.89, 123.18, 55.23, 51.41, 48.85, 47.13, 46.27, 42.39, 40.81, 39.63, 39.26, 38.91, 37.45, 34.29, 33.64, 30.43, 29.16, 26.33, 24.48, 23.92, 23.73, 20.28, 19.33, 18.99, 18.28, 16.43 ESI-MS m/z 543 [M+H]⁺

3-Oxo-2-[3-Nitrobenzylidenyl]-Olean-12-En-28-Oic Acid (3o)

Yield 83%; mp 161–163; ¹H NMR (400 MHz, CDCl₃): δ 8.30 (1H, s), 8.13 (1H, m), 7.91 (2H, t), 7.61 (1H, m), 7.25 (1H, m), 5.33 (1H, s), 2.46 (1H, m), 2.40 (2H, m), 2.24 (1H, d, j=8.0Hz), 1.91 (4H, m), 1.79 (3H, s, CH₃-23), 1.63 (3H, s, CH₃-24), 1.42 (3H, s, CH₃-25), 1.31 (3H, s, CH₃-26), 1.02 (3H, s, CH₃-27), 0.81 (3H, s, CH₃-29), 0.42 (3H, s, CH₃-30). ¹³C NMR (100 MHz, CDCl₃): δ 207.98, 183.85, 143.05, 138.39, 137.97, 136.19, 135.32, 134.61, 130.29, 129.96, 128.66, 128.18, 127.92, 127.95, 127.13, 125.69, 125.05, 64.79, 53.32, 52.89, 48.22, 45.58, 43.91, 42.35, 39.65, 39.31, 39.07, 36.82, 36.67, 32.27, 30.83, 29.85, 29.62, 28.17, 24.27, 23.75, 22.93, 21.38, 20.42, 17.28 ESI-MS m/z 588 [M+H]⁺

Biological Activity

Evaluation of Cell Viability by MTT Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay was used to study the cytotoxicity of all synthetic compounds against cultured RAW 264.7 cells (purchased commercially from ATCC, Manassas, VA, USA).⁵³ Camptothecin was taken as a reference standard in this study. In brief RAW 264.7 cells at a density of 16,000 cells/well were seeded into 96-well plates and were allowed to adhere in a CO₂ incubator at 37 ° C for a time

period of 24 h. After 24 h of incubation, the cells were treated with different concentrations of oleanolic derivatives (0–10 μM/mL) for another 24 h. Afterwards, 20 μL of MTT (0.5mg/mL in PBS, PH 7.4) was added and kept for incubation for another 4 h at 37°C. Finally, the supernatant was removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals which are formed after the addition of MTT. The absorbance was measured by using the synergy Mx plate reader at 570 nm. The results were expressed as a percentage of cell viability by using LPS-induced group as a control group. Three replicates were carried for each treatment.

NO Assay

For the evaluation of NO production in RAW 264.7 cells, the concentration of nitrite was measured in the supernatant as an indicator using Griess reaction. In brief, RAW 264.7 macrophage cells (2 x 10⁵ cells/well) were treated with different concentrations of synthetic derivatives in the presence or absence of LPS (1μg/mL) 1 h before LPS treatment and then kept in incubation for 24 h. Dexamethasone in different concentrations was used as a positive control. After an incubation period, supernatant (100 μL) was collected by centrifugation at 1000 rpm and mixed with Griess reagent (0.1% N-1-naphthyl ethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) and kept for incubation for 10 min at room temperature in dark. By using the synergy Mx Plate reader, absorbance was measured at 540 nm. With respect to the standard concentration curve of sodium nitrite (NaNO₂), concentration of nitrite was calculated.⁵⁴

The percentage inhibition of NO was calculated with the following formula

$$\text{NO inhibition(\%)} = \frac{(\text{NO}_2)\text{control} - (\text{NO}_2)\text{sample}}{(\text{NO}_2)\text{control}} \times 100$$

Measurement of Cytokine Production in RAW 264.7 Cells

The inhibitory effect of all synthetic derivatives on the production of cytokines (TNF-α and IL-6) was determined by enzyme-linked immunosorbent assay (ELISA) kit. In 96-well plate RAW 264.7 cells were seeded at a density of 2 x 10⁵ cells/well and left for incubation for overnight. The cells were then pretreated with synthetic derivatives for 1hr before stimulation with LPS for 24 h to induce inflammation. To collect the supernatant, the culture plate was

then centrifuged at 1500 rpm and assayed according to the protocol of the manufacturer (Invitrogen) to measure the amount of TNF- α and IL-6 produced in each sample. The whole experiment was carried out in triplicate.

Molecular Docking and Selection of Predicted Interaction Pose

Docking of all the synthetic derivatives of oleanolic acid on TNF- α (PDB ID: 2AZ5) and IL-1 β (PDB ID: 3040) was performed with molecular operating environment (MOE)2019.01 program.⁵⁵ Structure breaks of TNF- α and IL-1 β after retrieved from the RCBS Protein database⁵⁶ were fixed using MOE2019.01. Partial charges and hydrogen atoms were added to both proteins after removing the water molecules. By using the optimized potential for liquid simulations (OPLS) force field in MOE2019.01, the protein structures were minimized. The 3D structure of all the synthetic derivatives of oleanolic acid was prepared and minimized using the MMFF94x force field in MOE2019.⁵⁷ After structure preparation of synthetic derivatives of oleanolic acid and proteins, by using induced-fit docking protocol, the ligands were docked on co-crystallized ligand site in TNF- α and site predicted by MOE and meta pocket 2.0⁵⁸ in case of IL-1 β . All the docked solutions were clustered, and representative solutions of the most populated cluster were selected based on score and subjected to manual selection, based on suitability and binding. The highest scoring solution among manually selected groups of experimentally active compounds were considered as the representative pose. The binding affinity of representative docked pose was calculated using Cyscore.^{59,60}

Statistical Analysis

All the results were expressed as (mean $\pm$ of SD). Graphpad prism eight and microsoft excel was used to calculate SD. P-value of less than 0.5 was considered significant. All the experiments were carried out in triplicates.

Results

Effect on Cell Viability

To find the safety level of all the five potent analogs (**3d**, **3e**, **3L**, **3n**, **3o**), MTT assay was performed. Raw cells were subjected to different concentrations (1–100 μ M) of (**3d**, **3e**, **3L**, **3n**, **3o**), for a time period of 48 hours. At low concentrations (1–10 μ M), we observed that cell viability did not get affected and was more than 80% at 10 μ M (Figure 2).

Figure 2 Cytotoxicity of compounds **1**, **3d**, **3e**, **3L**, **3n** and **3o**: RAW 264.7 cells were treated up to 10 μ M concentrations with **1**, **3d**, **3e**, **3L**, **3n** and **3o** for 48 h, and cell viability checked by MTT assay. The data represent mean $\pm$ SD (n = 3) of the representative experiment.

Therefore, to investigate the anti-inflammatory of (**3d**, **3e**, **3L**, **3n**, **3o**), on RAW 264.7 cells, our data is non-toxic at a concentration of (1–10 μ M).

Effect of Derivatives on NO Production in LPS Stimulated RAW 264.7 Cells

Among 15 new derivatives, compounds **3a** (77.18%), **3d** (71.5%) and **3e** (68.8%) showed more potent inhibition of NO than oleanolic acid (65.22%) at 10 μ M which is comparable to dexamethasone (85.7%) and L-NAME (80.5). Analogs **3c**, **3k**, **3m**, **3g**, and **3L** showed moderate inhibition of NO, respectively. However, analogs **3b**, **3f**, **3h**, **3i** and **3j**, showed poor inhibitory activity (Table 2).

Measurement of Pro-Inflammatory Cytokines (TNF- α and IL-6) in RAW 264.7 Cells

The synthetic analogs were observed for IL-6 and TNF- α inhibition. Compounds **3d**, **3e**, and **3L** showed maximum IL-6 inhibition of 76.5%, 74.9% and 77.2% respectively, which is comparable with oleanolic acid (71.6%) at 10 μ M concentration. Analogs **3b**, **3c**, **3h**, **3j**, **3m**, **3n**, and **3o** exhibit moderate activity (Table 2), whereas, analogs **3a**, **3f**, **3g**, **3i**, **3k** showed weak IL-6 inhibitory activity. In the case of TNF- α , analogs **3e**, **3L** and **3o** showed maximum inhibitions against TNF- α of 79.3%, 75.4% and 77.1%, which are more potent than oleanolic acid (73.2%). Others exhibited either moderate or weak activity than oleanolic acid (Table 2). In LPS untreated cells, the levels of TNF- α and IL-6 were undetectable and served as a control. At 10 μ M concentration, there were no significant changes in cell viability on the treatment of RAW

Table 2 The % Cell Viability and Percentage (%) Inhibition of Compounds 1–15 Against NO, IL-6, and TNF- α in LPS (1 μ g/mL) Stimulated RAW 264.7 Cells

S. No.	% Cell Viability		% Inhibition of NO		% Inhibition of TNF- α		% Inhibition of IL-6	
Codes	1 μ M	10 μ M	1 μ M	10 μ M	1 μ M	10 μ M	1 μ M	10 μ M
1	98.13 $\pm$ 2.5	78.02 $\pm$ 3.6	15.22 $\pm$ 0.8	65.9 $\pm$ 1.9	25.5 $\pm$ 0.1	73.2 $\pm$ 0.9	26.7 $\pm$ 1.9	71.6 $\pm$ 0.5
3a	90.4 $\pm$ 2.8	74.2 $\pm$ 0.7	17.18 $\pm$ 0.9	77.8 $\pm$ 1.4	3.2 $\pm$ 1.8	20.8 $\pm$ 1.9	7.3 $\pm$ 2.4	18.3 $\pm$ 1.8
3b	91.2 $\pm$ 1.8	71.6 $\pm$ 5.7	11.2 $\pm$ 2.4	14.6 $\pm$ 1.3	4.1 $\pm$ 0.1	19.6 $\pm$ 0.8	13 $\pm$ 1.6	33.3 $\pm$ 2.5
3c	81.1 $\pm$ 2.6	68.2 $\pm$ 2.8	32.6 $\pm$ 3.6	41.6 $\pm$ 2.1	9.4 $\pm$ 2.1	17.7 $\pm$ 0.1	11 $\pm$ 1.2	39.5 $\pm$ 2.6
3d	99.2 $\pm$ 4.5	81.7 $\pm$ 1.8	31.5 $\pm$ 6.6	71.5 $\pm$ 2.3	8.7 $\pm$ 2.2	27.7 $\pm$ 0.2	24.4 $\pm$ 1.5	76.5 $\pm$ 0.3
3e	98.22 $\pm$ 2.8	82.9 $\pm$ 3.3	28.8 $\pm$ 4.5	68.8 $\pm$ 3.7	19.1 $\pm$ 0.2	79.3 $\pm$ 0.5	28.5 $\pm$ 1.8	74.9 $\pm$ 2.3
3f	78.6 $\pm$ 1.4	58.3 $\pm$ 2.8	14.3 $\pm$ 1.8	36.5 $\pm$ 3.9	5.6 $\pm$ 2.8	16.7 $\pm$ 2.1	7.7 $\pm$ 1.4	21.3 $\pm$ 2.7
3g	98.8 $\pm$ 2.5	72.6 $\pm$ 4.5	29.4 $\pm$ 2.3	42.8 $\pm$ 3.7	8.4 $\pm$ 0.9	22.5 $\pm$ 0.5	5.3 $\pm$ 2.4	18.9 $\pm$ 0.7
3h	93.6 $\pm$ 3.6	71.1 $\pm$ 2.6	13.4 $\pm$ 2.3	33.6 $\pm$ 3.4	11.3 $\pm$ 1.9	20.1 $\pm$ 1.4	11.2 $\pm$ 1.6	38.6 $\pm$ 0.5
3i	78.4 $\pm$ 1.9	55.9 $\pm$ 4.9	18.2 $\pm$ 2.2	41.4 $\pm$ 0.9	13.6 $\pm$ 0.2	27.5 $\pm$ 0.8	8.9 $\pm$ 0.8	17.6 $\pm$ 1.1
3j	99.3 $\pm$ 4.9	68.4 $\pm$ 3.8	18.3 $\pm$ 3.2	34.6 $\pm$ 0.4	18.8 $\pm$ 0.4	41.7 $\pm$ 0.5	18.6 $\pm$ 0.4	35.9 $\pm$ 1.2
3k	98.1 $\pm$ 2.4	65.6 $\pm$ 3.7	27.2 $\pm$ 2.4	44.2 $\pm$ 3.2	7.2 $\pm$ 2.7	36.7 $\pm$ 2.4	7.8 $\pm$ 1.9	19.6 $\pm$ 2.5
3L	99.5 $\pm$ 4.8	82.3 $\pm$ 3.2	21.4 $\pm$ 2.9	42.4 $\pm$ 2.6	25.5 $\pm$ 2.1	75.4 $\pm$ 0.6	32 $\pm$ 1.5	77.2 $\pm$ 2.9
3m	92.2 $\pm$ 5.7	58.9 $\pm$ 2.7	13.12 $\pm$ 2.5	43.2 $\pm$ 1.9	8.9 $\pm$ 2.4	29.9 $\pm$ 1.6	19.8 $\pm$ 1.6	37.6 $\pm$ 2.5
3n	96.6 $\pm$ 1.8	84.4 $\pm$ 4.7	14.1 $\pm$ 0.8	42.4 $\pm$ 0.5	8.2 $\pm$ 1.7	35.8 $\pm$ 0.6	29.7 $\pm$ 1.4	55.6 $\pm$ 3.2
3o	97.7 $\pm$ 4.1	83.9 $\pm$ 3.6	21.2 $\pm$ 0.9	46.8 $\pm$ 0.4	19.8 $\pm$ 0.8	77.1 $\pm$ 0.4	8.9 $\pm$ 1.5	51.6 $\pm$ 2.6
Curcumin	–	–	43 $\pm$ 0.5	79.5 $\pm$ 0.2	65 $\pm$ 0.1	80 $\pm$ 0.6	–	77.1 $\pm$ 0.9
Dexa	–	–	48.7 $\pm$ 2.8	85.7 $\pm$ 1.2	70.9 $\pm$ 2.4	86.2 $\pm$ 3.2	–	82.3 $\pm$ 2.4
L-NAME	–	–	44.5 $\pm$ 2.3	80.5 $\pm$ 2.1				

Note: Compounds in bold font are active.

264.7 cells. Analogs that exhibit more than 50% inhibition of TNF- α and IL-6 are considered more potent.^{61,62}

Molecular Docking Studies

All the synthetic derivatives were docked on TNF- α and IL-1 β (Figure 3A and B). The top-scoring solutions have been analyzed for the Cyscore score, and ADME properties are mentioned in (Table 3). The docking pose shows the interaction of (3d, 3e, 3L, 3n, 3o) and dexamethasone with TNF- α and IL- β . The binding affinity of analogs and dexamethasone with TNF- α and IL-1 β was calculated using Cyscore 2.^{60,63} From docking score and Cyscore analysis, it shows that the analogs (3e, 3L, 3n) are having a greater affinity towards TNF- α and IL-1 β than dexamethasone which was also verified from in vitro results.

Discussion

In the current study, we investigated the various derivatives of oleanolic acid on the production of pro-inflammatory mediators in RAW 264.7 mouse macrophages stimulated by LPS. In RAW 264.7 mouse macrophages, the analogs were evaluated for their potential to reduce lipopolysaccharide (LPS) induced TNF- α and IL-6 production. RAW 264.7 cells upon activation with LPS releases TNF- α and IL-6.

LPS, a component of a gram-negative bacterial cell wall, induces macrophages and monocytes activation which is having a pivotal role in the innate immune response. Stimulation of RAW 264.7 cells with LPS leads to a series of intracellular events which results in the secretion of cytokines as well as other mediators of inflammation that eventually constitute the pro-inflammatory response. RAW 264.7 cells on pretreatment with the analogs (3d, 3e, 3L, 3o) at various concentrations followed by LPS treatment for 24 h results in the downregulation of pro-inflammatory cytokines (IL-6 and TNF- α). At 10 μ g/mL, the analogs showed potent inhibition of cytokines. In LPS untreated cells, the levels of IL-6 and TNF- α were not detectable and served as a control. MTT assay also showed that up to 10 μ g/mL concentration, the analogs (3d, 3e, 3L, 3n, 3o) did not affect the viability of RAW 264.7 cells. So the binding of potent analogs ((3d, 3e, 3L, 3n, 3o) results in the downregulation of pro-inflammatory cytokines (TNF- α and IL-6) and upregulated the secretion of anti-inflammatory cytokine IL-10.

Moreover, the analogs (3a, 3d, 3e) also inhibit the production of NO also, which is also released during the process of inflammation by activated phagocytes or resident cells to activate various macrophages. With reference to standard drug dexamethasone, the inhibitory potential of

Figure 3 Interaction of active compounds with TNF- α (A) and IL-1 β (B).

Table 3 Docking and Cyscore Score Along with ADME Properties

Compound Name	Docking Score		Cyscore (Binding Affinity)		ADME				
	TNF- α	IL-1 β	TNF- α	IL-1 β	Log P _{o/w}	LogS	Log k _p	TPSA	BA Score
Dexamethasone	-5.171	-5.934	-1.974	-1.319	2.15	3.56	7.32	94.83	0.55
4-nitro benzaldehyde (3d)	-7.113	-7.097	-2.597	3.417	6.91	9.10	3.45	100.19	0.56
4-chloro-Benzaldehyde (3e)	-6.653	-7.253	-2.334	3.257	7.39	9.10	3.26	63.60	0.56
2,6 Di-chloro Benzaldehyde (3L)	-7.157	-7.174	-1.931	3.529	8.22	10.15	2.48	54.37	0.56
Benzaldehyde (3n)	-6.731	-6.641	-3.051	4.004	7.47	9.00	3.06	54.37	0.56
3-nitro-Benzaldehyde (3o)	-7.453	-7.755	-2.382	-3.972	6.94	9.10	3.45	100.19	0.56

Note: Compounds shown in bold font are active.

analogs was studied. Besides, from docking score and Cyscore analysis analogs (**3e**, **3L**, **3n**) showed greater affinity towards TNF- α and IL-1 β than dexamethasone, which was also verified from our in vitro results.

So we have undertaken a research program directed toward the structural modifications of (**1**) at C-2 position to fine tune its biological potential as an anti-inflammatory agent. Compound (**1**) was subjected to oxidation by pyridinium chlorochromate (PCC) reagent at room temperature (rt) that resulted in the formation of (C-3) oxidized derivative (**2**) in almost quantitative yield. To the solution of compound (**2**) (365 mg, 0.8 mmol), in ethanol at room temperature lithium hydroxide LiOH (1.2 Eq) was added. To this mixture, various aromatic aldehydes were added by Claisen Schmidt condensation reaction. The crude mixture was extracted with ethyl-acetate, and the organic layer was dried over sodium sulphate, concentrated in vacuum and purified by column chromatography to give pure **3a-3o** in 80–90% yield. The products were confirmed by ¹H NMR, ¹³C NMR, and MS analysis. By employing the above reaction conditions, a series of oleanolic acid-arylidene derivatives that vary at substitutions on aromatic ring has been synthesized.

Conclusion

Herein, we report a series of 15 new arylidene derivatives of oleanolic acid by Claisen Schmidt condensation reaction. All the compounds synthesized were screened for their anti-inflammatory activity against NO, TNF- α and IL-6. From the data, it was evident that most of the compounds exhibited better anti-inflammatory activity. Molecular docking studies further confirm the anti-inflammatory activity of the derivatives (**3d**, **3e**, **3L**, **3n**, **3o**). Compounds (**3d**, **3e**, **3L**, **3n**, and **3o**) with o-nitro, o-chloro and p-chloro substitutions were found to be the most promising derivatives. The study also encouraged to study the molecular mechanisms involved, which further

demonstrates its anti-inflammatory action at the molecular level.

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Disclosure

The authors declare no conflicts of interest in this work.

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