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Metals Complexes Formed with Oleanolic Acid

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Abstract

The oleanolic acid possesses diverse pharmacological properties, it is considered as a good starting material for creating new compounds. The oleanolic acid isolated of *Plumeria obtusa* leaves was used as raw material to obtained calcium, magnesium, zinc, nickel and copper complexes. The structures of complexes were confirmed by HRMS, ^{1}H NMR, and ^{13}C NMR. Five new compounds were synthesized to promote increased biological activity of oleanolic acid and PCR assays for the different type of cancer.

Keywords

Oleanolic Acid, Complex, PCR

1. Introduction

Oleanolic acid (OA) is a pentacyclic triterpenoid that occurs naturally in several plants either as a free acid or as an aglycone of saponins [1]. OA is found in high concentrations in the leaves, fruits, and oil of *Olea europaea L.* [2] [3]. OA can be extracted from apples [4] or plants such as the *Plumeria obtuse* [5]. OA has been extracted from more than 1620 different plant species and used for food and medicinal purposes [6]. OA has gained significant interest, and several studies have demonstrated the importance of its use in hepatoprotective [7] [8], anti-inflammatory [7] [8], and anticancer activities [9]. OA is also a phosphorylase inhibitor [10]. In China, OA has been used orally to treat liver disorders [11], and it is a registered drug used intravenously in the treatment of hepatitis B and liver cancer [12].

Since OA possesses diverse pharmacological properties, it is considered as a good starting material for creating new compounds [13]. OA comprises three

active points: C3, where hydroxyl is stored; the double bonds present in the ring between the C12 and C13 carbons; and C28, where carboxylic acid is stored (**Figure 1**); these points enable modifications, which lead to the creation of new chemical compounds [14] [15].

However, OA has limited bioavailability and does not provide a good plasma half-life because of its low solubility in water [16] [17]. Several studies have demonstrated that the structural alterations in OA can have a significant impact on biological activities [18]. The improvements in biological activities, such anti-inflammatory, antidiabetic, nephropathy, and cytotoxicity, have been achieved by changing some points in the OA structure [19] [20]. Terpenes complexes were very effective in several diseases [21].

Tabrizi *et al.* [22] showed that ruthenium (II) *p*-cymene complexes of naphthoquinone derivatives worked powerfully to combat melanomas in humans. Ghosh *et al.* [23] demonstrated the antioxidant activity of the quercetin-magnesium complex, and similar benefits were recorded for the complexation of copper with quercetin. The use of the bactericidal properties of calcium and copper complexes against Gram-positive bacteria has also been demonstrated [24].

Taking into consideration the aforementioned and potential medical uses of OA and its derivatives, five new compounds were synthesized using the OA.

2. Experimental

2.1. General Procedures and Equipment

The ^{1}H and ^{13}C experiments were performed on a Bruker 600-MHz NMR spectrometer equipped with an AVANCE III console, and a DCH cryogenically cooled probe. The conditions for ^{1}H experiments were as follows: Bruker pulse sequence = zg30; the number of acquisition points = 65,536; the number of acquisitions = 128; sweep width = 20.55 ppm; recycle delay = 2 s; frequency = 600.13 MHz. The conditions for ^{13}C experiments were as follows: Bruker pulse sequence = zgpg30; the number of acquisition points = 65,536; the number of

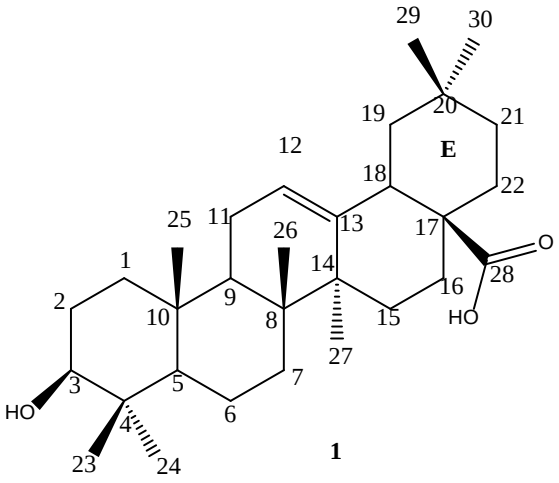


Figure 1. Molecular structure of oleanolic acid.

acquisitions = 4096; sweep width = 238.9 ppm; recycle delay = 2 s; frequency = 150.9 MHz. Deuterated methanol, CD_3OD, was used as the solvent.

The IR spectra were recorded on a Perkin Elmer Paragon 1000 FTIR spectrophotometer in the ATR mode over the range 400 - 4000 cm^{-1}.

The analyses were performed on LC-MS-QTOF-6530 model L-1200 (Agilent brand) with the software Profinder series B.06.00; flow: 0.6 mL/min-C18 column - 1.8 μM, 3 mm × 100 mm. The mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in acetonitrile.

2.2. Isolation of Oleanolic Acid

The leaves of Plumeria alba were collected and dried in a greenhouse, after drying were submitted to extraction with ethanol in the ratio 6:1 solvent for the sample. After 7 days, the material was filtered, and the solvent was removed using reduced pressure. The sample was subjected to the silica gel 60 (0.063 - 0.2 mm/70 - 230 mesh ASTM) filter column [25] [26] (Macherey-Nagel, Germany) and fractionated with one liter of each of the following eluents: hexane, dichloromethane, ethyl acetate, methanol, and water. The solutions obtained were concentrated and analyzed by CCD. The pre-selected extracts were subjected to classical silica gel chromatography and Sephadex® LH-20 for the isolation and purification of compounds.

2.3. Melting Temperature

The melting temperatures were determined on MQAPF 320 equipment from Microquímica Equipamentos Ltda.

2.4. Complex Reaction

A round bottom flask (500 mL) was charged with 1 equivalent of metal sulfate to 2 equivalent of OA and 300 mL methanol. The resulting reaction mixture was vigorously stirred at 57˚C for 6 hours. The solution was cooled to room temperature, and the methanol was removed using reduced pressure to obtain the compounds (**Figure 1**) [27]. The material was measured and calculated the yield the reaction.

2.5. RNA Extraction and qPCR

Total RNA was isolated from H4IIE cells or from tissue from rats maintained with liquid nitrogen, using the TRIzol reagent (Life Technologies). The total RNA was isolated from cells using TRIzol reagent (Life Technologies). RNA was quantified using Synergy H1/Take 3 plate setup (BioTek). The cDNAs were synthesized using 2 μg of RNA for each sample using high-capacity cDNA Reverse Transcription kit (Life Technologies) on an ABI GeneAMP 9700 (Life Technologies).

The resulting cDNA was amplified in duplicate by real-time quantitative PCR (qPCR) using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers

were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: β-actin (housekeeping gene), forward primer: 5'-GGG AAA TCG TGC GTG ACA TT-3', reverse primer: 5'-GCG GCA GTG GCC ATC TC-3'; G6Pase, forward primer: 5'-TGT TCC TCT TAA TCC TGC CCA-3', reverse primer: 5'-CCA ACC TGC ACA AGT TCC CTT-3'. qPCR amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50˚C for 2 min and 1 cycle of 95˚C for 10 min, followed by 40 cycles of 15 s at 95˚C and 1 min at 60˚C. The dissociation curve was completed with 1 cycle of 1 min at 95˚C, 30 s at 55˚C, and 30 s at 95˚C. mRNA expression was analyzed using the $\Delta\Delta$CT method, and normalized concerning the expression of the β-actin using ABI 7500 Fast System SDS Software v1.3.0 (Life Technologies). Amplification of specific transcripts was further confirmed by obtaining melting curve profiles. All results were expressed as fold change from the induced Dex-cAMP controls. For the assays, samples at the concentration of 5.0 mg/100 µl were used with 5.0 µl of the cDNA.

3. Results and Discussion

After the use of three chromatographic columns with different mobile phases and monitoring the separation with thin layer chromatography and with the developers phosphomolybdic acid and vanillin, the oleanolic acid compound was obtained, where it was confirmed according to the ^{13}C and ^{1}H NMR data in Table 1.

The synthesized compounds were analyzed using the NMR technique, and the ^{13}C and ^{1}H data for OA were initially obtained for comparison with the NMR data of the compounds.

The results in Table 1 show that C28 for the compounds with Ca, Mg, Cu, Zn, and Ni has a different displacement value compared to that obtained with OA. Considering C28 as one of the possible reaction points [14] [15], and the data obtained by the IR technique which show the absence of the band for carboxylic acid bound to C28, it is clear that the reaction is formed together with a carboxylic acid group. It is still possible to discern a small variation in the displacement of the C12 and C13 carbons and those carrying the double bond. This suggests that electron displacement is promoted by the withdrawal effect exerted by both metals when they are bound to the hydroxyl oxygen of the carboxylic acid group.

The structures of the synthesized compounds are also supported by the following data.

$C_{30}H_{48}O_3$ (Oleanolic acid) White solid, 0.621 g of the pure compound, m.p. 307˚C - 309˚C, IR (ATR) ν_{max}/cm^{-1}: 3455, 3048, 2932, 2859, 1689, 1460, 1382, 1271, 1031, 954, 658.

$C_{60}H_{94}CaO_6$ It was obtained in 71% yield (0.1150 g, 0.1209 mmol) via reaction of the 1.0 eq. of calcium sulfate to 2.0 eq. of oleanolic acid. White solid, IR (ATR) ν_{max}/cm^{-1}: 3370, 2928, 2865, 1537, 1465, 1387, 1136, 1029, 995. LC-MS $[M+H]^+$ calculate for $C_{60}H_{95}CaO_6$: 951.6755, found 951.6750.

Table 1. ^{1}H and ^{13}C NMR chemical shifts to OA and complexes (δ, ppm) [a]–oleanolic acid isolated.

	OA[a]		Ca–OA		Mg–OA		Cu–OA		Zn–OA		Ni–OA	
C	δC	δH	δC	δH	δC	δH	δC	δH	δC	δH	δC	δH
1	38.43	0.99	38.44	1.01	38.44	1.02	38.43	1.00	38.44	0.99	38.44	0.98
2	26.47	1.79	26.49	1.75	26.49	1.77	26.46	1.76	26.47	1.77	26.5	1.75
3	78.32	3.33	78.41	3.33	78.34	3.33	78.32	3.33	78.36	3.33	78.41	3.33
4	38.43	-	38.48	-	38.44	-	39.02	-	38.47	-	38.49	-
5	55.36	0.84	55.45	0.89	55.41	0.89	55.38	0.88	55.4	0.89	55.57	0.87
6	18.10	1.41	18.17	1.58	18.18	1.57	18.16	1.59	18.17	1.59	18.18	1.58
7	32.41	1.57	32.78	1.53	32.81	1.54	32.78	1.53	32.81	1.55	32.8	1.53
8	39.16	-	39.13	-	39.18	-	39.12	-	39.11	-	39.13	-
9	48.01	1.71	48.02	1.72	48.02	1.71	48.01	1.72	48.03	1.71	48.01	1.72
10	36.77	-	36.81	-	36.82	-	36.81	-	36.8	-	36.82	-
11	22.56	1.92	22.94	1.93	22.88	1.91	22.83	1.93	22.9	1.92	22.98	1.93
12	122.26	5.26	121.08	5.23	121.21	5.22	121.2	5.24	121.16	5.25	120.96	5.23
13	143.81	-	145.25	-	145.18	-	145.25	-	145.29	-	145.39	-
14	41.49	-	42.06	-	41.87	-	41.78	-	41.77	-	42.13	-
15	27.33	1.18	27.38	1.16	27.38	1.17	27.4	1.17	27.52	1.18	27.39	1.16
16	22.65	2.03	23.16	1.93	23.17	1.91	23.03	1.94	23.05	1.95	23.17	1.93
17	46.23	-	46.77	-	46.71	-	46.75	-	47.01	-	46.78	-
18	41.33	3.17	41.62	3.32	41.68	3.32	41.72	3.32	41.73	3.31	41.64	3.28
19	45.84	1.74	46.77	1.87	46.71	1.69	46.77	1.88	46.64	1.87	46.85	1.85
20	30.20	-	30.40	-	30.38	-	30.36	-	30.38	-	30.43	-
21	33.50	1.43	34.12	1.44	34.06	1.44	33.96	1.43	33.4	1.45	34.18	1.44
22	32.15	1.76	32.54	1.87	32.44	1.74	32.42	1.88	32.54	1.87	32.6	1.87
23	27.43	0.96	27.87	1.14	27.75	1.33	27.71	1.13	27.72	1.12	27.91	1.14
24	14.90	0.93	14.92	1.093	14.96	1.03	14.97	1.09	15.08	1.08	14.94	1.09
25	14.47	0.84	14.50	0.96	14.54	0.99	14.52	0.98	14.57	0.97	14.51	0.96
26	16.31	0.80	16.79	1.097	17.00	0.90	17.00	1.09	17.02	1.08	16.84	1.07
27	24.98	1.34	25.08	1.34	25.04	1.35	25.03	1.35	25.14	1.36	25.12	1.37
28	180.43	-	184.45	-	185.65	-	187.63	-	187.89	-	184.82	-
29	32.61	1.33	32.99	0.79	33.21	0.96	33.06	0.78	33.03	0.78	33.05	0.78
30	23.11	0.92	23.16	0.99	23.20	0.80	23.13	0.99	23.15	0.99	23.2	0.99

$C_{60}H_{94}MgO_6$ It was obtained in 68% yield (0.1120 g, 0.1198 mmol) via reaction of the 1.0 eq. of magnesium sulfate to 2.0 eq. of oleanolic acid. White solid, IR (ATR) ν_{max}/cm^{-1}: 3244, 2926, 1526, 1385, 1089, 1029, 771. LC-MS $[M+H]^+$ calculate for $C_{60}H_{95}MgO_6$: 935.6979, found 935.6976 [28].

$C_{60}H_{94}CuO_6$ It was obtained in 70% yield (0.0989 g, 0.1015 mmol) via reaction of the 1.0 eq. of copper sulfate to 2.0 eq. of oleanolic acid. Green solid, IR (ATR) ν_{max}/cm^{-1}: 3395, 2938, 2862, 1537, 1386, 1094, 995, 728. LC-MS $[M+H]^+$ calculate for $C_{60}H_{94}CuO_6$: 973.6346, found 973.6344.

$C_{60}H_{94}ZnO_6$ It was obtained in 68% yield (0.101 g, 0.1036 mmol) via reaction of the 1.0 eq. of zinc sulfate to 2.0 eq. of oleanolic acid. White solid, IR (ATR) ν_{max}/cm^{-1}: 3299, 2940, 2864, 1595, 1461, 1084, 736, 677. LC-MS $[M+H]^+$ calculate for $C_{60}H_{94}ZnO_6$: 974.6342, found 974.6341.

$C_{60}H_{94}NiO_6$ It was obtained in 69% yield (0.0899 g, 0.0928 mmol) via reaction of the 1.0 eq. of nickel sulfate to 2.0 eq. of oleanolic acid. Green solid, IR (ATR) ν_{max}/cm^{-1}: 3320, 2928, 2863, 1534, 1453, 1373, 1029, 772. LC-MS $[M+H]^+$ calculate for $C_{60}H_{94}NiO_6$: 968.6404, found 968.6401.

The shift in displacement promoted by the bonding between the metal (Ca/Mg/Zn/Ni/Cu) with the oxygen of the hydroxyl at C28 indicates the formation of that new bond.

The stretch ν (C=O) at C28 at 1691 cm^{-1} (Figure 2) present to OA is absent in the IR spectra for all complex formed. Also shows the absence of characteristic stretching ν (C=O) band in the wave-number region between 1715 - 1680 cm^{-1}, and the absence of band resulting from the angular deformation of the ν (OH) bond of the carboxylic acid in the region between 955 - 875 cm^{-1} [29]. However, these bands are present in the IR spectrum of OA and reinforce the formation of the complex; therefore, five new complexes with OA are created.

As shown (Figure 3) it is possible to identify the formation of the complexes with the metal and OA, the bond between the metal and the oxygen atom promotes a displacement of electrons, in this way the NMR values (Table 1) increase, such as the values obtained for C28. The same bond, suppresses the band obtained in 1689 cm^{-1} for OA in the IR experiments.

The fold change values show the activity of the compounds isolated in trials where three different genes were used (Table 2). The compound synthesized

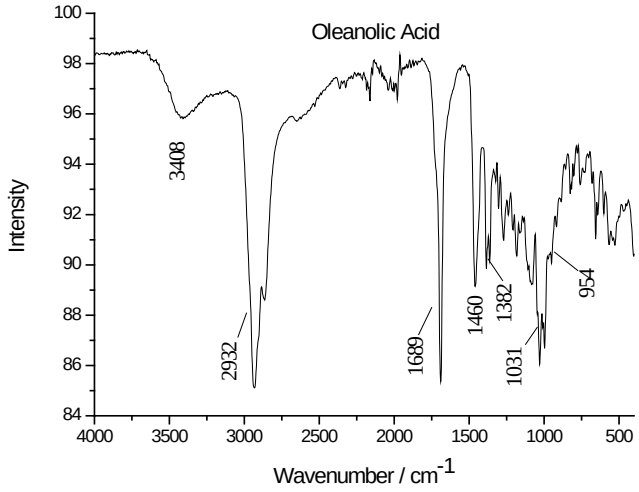
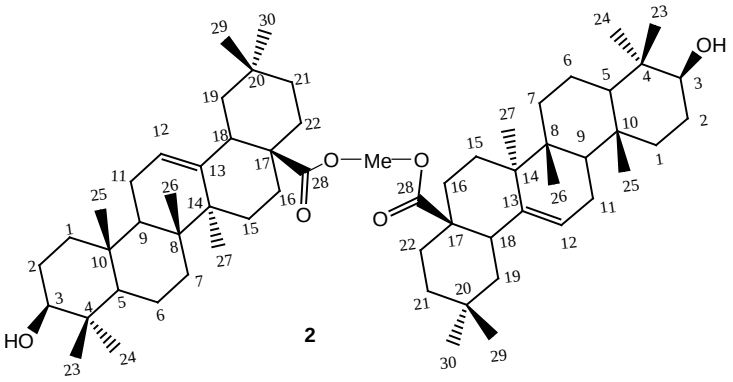


Figure 2. The infrared spectrum of oleanolic acid.



Me = Ca^{+2}, Zn^{+2}, Mg^{+2}, Ni^{+2}, Cu^{+2}

Figure 3. The molecular structure of the complex formed with calcium, magnesium, copper, zinc, and nickel with oleanolic acid.

Table 2. Results of PCR assays for the complexes of calcium, magnesium, and oleanolic acid.

Amostras	G6P	PEPCK	FAS
Oleanolic acid	3.6	3.9	1.8
Complex Ca	0.8	0.9	1.1
Complex Mg	9.0	6.5	3.9
Complex Cu	0.1	0.6	0.3
Complex Zn	0.0	0.5	0.9
Complex Ni	0.7	0.4	0.6

from oleanolic acid and calcium showed little efficiency in the control of these genes, presenting lower values when compared to pure oleanolic acid, that is, there was a decrease in the biological activity of the synthesized compound. Different results were obtained for the compound formed from oleanolic acid and magnesium; the values were higher than the result of the pure oleanolic acid and the compound formed with calcium. Greater success was achieved with the G6P gene reaching a fold change value close to 9.0, for the PEPCK gene, there is also a significant value. Not being so effective for the FAS gene [30] [31].

4. Conclusion

Five novel compounds were synthesized using OA as starting material, the novel and inedited structures were confirmed by NMR, IR and LC-MS experiments. The NMR, IR, and LC-MS data support the literature data for the passive points of reactions in the OA molecule. Among the five new compounds synthesized, the complex formed with magnesium, showed better results for the tests performed.

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