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3 **Analysis of constituents of the eastern Nigeria mistletoe, *Loranthus micranthus* Linn revealed**
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5 **presence of new classes of osteogenic compounds**
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3
4 **Abstract**
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8 **Ethnopharmacological relevance:** Postmenopausal osteoporosis accounts for one of the prevalent
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10 disease conditions in aging population globally. This situation is exacerbated by the lack of osteogenic
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12 therapy. In search for plants of Nigerian origin with osteogenic potential, we evaluated eastern
13
14 Nigerian mistletoe, having ethnotraditional claims of anti-diabetic, anti-hypertensive and anti-cancer
15
16 activities as well as preventive effect in various post-menopausal syndromes.
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20 **Materials and Methods:** Methanolic extracts of mistletoe leaves harvested from three host trees - *Kola*
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22 *acuminata* (KM), *Citrus spp* (CM) and *Garcinia kola* (GKM) - were evaluated for osteoblast viability
23
24 and osteogenic activities using primary rat calvaria culture. Lupeol (**1**) was isolated from the stem bark
25
26 of *Bombax ciba* and its congener, dihydroxylupeol palmitate (**2**) in addition to three other compounds;
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28 3-methoxy quercetin (**3**), 3,4,5-trimethoxy gallate (**4**), and friedelin (**5**) were isolated from the leaves of
29
30 mistletoes species. Following their chemical characterization, the compounds were evaluated for
31
32 osteogenic potential using validated models including alkaline phosphatase (ALP) assay,
33
34 mineralization assay and expression of osteogenic genes - bone morphogenetic protein-2 (BMP2) and
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36 osteoblast transcription factor (RUNX2) - in primary calvarial cultures harvested from neonatal rats.
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38 Uterine estrogenicity of the extracts was tested in adult female Sprague Dawley rats.
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44 **Results:** Methanol extracts of mistletoe from three hosts exhibited increase in ALP activity (a marker
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46 of osteoblast differentiation) at lower concentrations (0.2-0.8µg/ml) and either no or inhibitory effect at
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48 higher concentrations (1.6 and 3.2µg/ml). None of the extract had cytotoxicity to osteoblasts at the
49
50 concentrations tested. Five compounds viz. **1** from *Bombax ciba*, and **2-5** were isolated from the
51
52 mistletoe leaves. Out of these, **5** exhibited significant loss of osteoblast viability and hence it was not
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54 considered further. All four compounds exhibited stimulatory effects on osteoblast differentiation as
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56 assessed by ALP assay and determination of osteogenic gene expression. Compound **2** was relatively
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more potent than its precursor, compound **1** in stimulating BMP2 upregulation. KM did not show uterine estrogenicity.

Conclusion: Methanolic extracts from the three mistletoes species possess *in vitro* osteogenic activity, and from these extracts three new classes of compounds have been found to promote osteoblast differentiation in vitro. In light of these findings, we propose that mistletoe species may be developed as safer alternative(s) in the management of diseases where lack of bone formation is the pathology.

Key words: Osteoblast differentiation, bone morphogenetic protein-2, mineralization, osteoporosis, cytotoxicity.

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1. Introduction

Osteoporosis is a metabolic bone disease that afflicts 200 million people globally out of which 80% are women. WHO defined osteoporosis as a disease characterized by a combination of low bone mass, deterioration of bone architecture and perhaps alterations in other aspects of bone quality leading to increased risk of fracture. Osteoporosis is frequent in women after menopause and in aged men (Kaunitz, et al., 2009). Clinically used agents to reduce fracture risk in osteoporosis include bisphosphonates, selective estrogen receptor modulators (SERMs) and calcitonin (Gerstenfeld and Einhorn, 2003; Delaney, 2006; Gass and Dawson-Hughes, 2006, Trivedi et al., 2010). Parathyroid hormone (PTH 1-34) is the only anabolic (bone forming) agent available for clinical use in postmenopausal osteoporosis (Tashjian and Gagel, 2006) that has recently been recommended by FDA (Food and Drug Administration) to carry a warning label because it is associated with an increased risk of osteosarcoma in rats (John *et al.*, 2002).

Several factors underlie the pathogenesis of osteoporosis. One of the most frequently observed causes of osteoporosis is estrogen (E2) deficiency. E2 deficiency leads to bone resorption by activating inflammatory responses including cytokine and growth factor levels, T cells and free radical production (Johannes et al., 2002; Nadia et al., 2012; Gianoukakis and Smith, 2004). The rise in inflammatory and oxidative stress are while favourable to the bone resorbing osteoclasts, contrariwise detrimental to the function and viability of osteoblasts, the bone forming cells. Collectively, these events lead to net bone loss in bone remodeling cycles of postmenopausal women.

The emerging theory of postmenopausal/aging-induced osteoporosis is that it is caused by a chronically elevated level of systemic and local inflammation due to aberrant immunological response of the body (Kregel and Zhang, 2006). Many phytochemicals known in traditional medicine have the

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3 potential to treat bone diseases however, not much laboratory work has been reported evaluating their
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5 possible development and use. Medicinal plants have traditionally occupied an important position in
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7 the socio-cultural and medicinal arena of rural and tribal sections of Africa. The eastern Nigeria
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9 mistletoe, *Loranthus micranthus* has been employed traditionally in the management of various
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11 ailments including diabetes, high blood pressure and conditions affecting human immune system for
12
13 the past many years. Furthermore, aqueous decoctions or alcoholic extracts of *Loranthus micranthus*
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15 have diverse folklore claims of efficacy in epilepsy, diabetes, hypertension, cardiovascular diseases,
16
17 menopausal syndrome, infertility, rheumatism, agglutination and in conditions generally requiring
18
19 modulation of the immune system. Very recently, the immunomodulatory potentials of *Loranthus*
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21 *micranthus* have been reported (Osadebe and Omeje, 2009; Omeje et al., 2011a; 2011b; 2011c;
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23 2011d).

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32 Continuing with our effort towards exploring additional pharmacological actions of east Nigerian
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34 mistletoes, in particular, the claim of its ability to prevent postmenopausal syndrome of which
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36 osteoporosis is one of the most common diseases, we investigated possible osteogenic effect of
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38 extracts for its potential application in the treatment of osteoporosis from this species parasitic on three
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40 host trees -*Kola acuminata* (KM), *Citrus spp* (CM), *Garcinia kola* (GKM). Identification of bioactive
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42 compounds is another important criterion required to gain insight into the osteogenic properties of
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44 medicinal plants and is relevant to skeletal health. To that effect, we have isolated four compounds
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46 from the extract and studied their osteogenic potentials in vitro.
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3 **2. Materials and methods**
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5 **2.1 Collection of plant materials**
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8 *Loranthus micranthus* Linn. (Loranthaceae) leaves parasitic on the three selected host trees (*Kola*
9
10 *acuminata*, *Citrus spp*, *Garcinia kola*) were collected in October 2011, from different locations in
11
12 Nsukka LGA, Enugu state. The leaves were identified and certified by Mr. A. O. Ozioko, a taxonomist
13
14 of the Bioresources Development and Conservation Programme, Nsukka, Enugu state. Voucher
15
16 specimens were kept at the centre with the numbers BDC-1021-011, BDC-1022-011, and BDC-1023-
17
18 011 for reference purposes.
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25 **2.2 Reagents and Chemicals**
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27 All fine chemicals including cell culture media and supplements were purchased from Invitrogen
28 (Carlsbad, CA) or Sigma Aldrich (St. Louis, MO). Methanol (BDH Ltd., Poole, England), distilled
29
30 water, and other routine reagents were used freshly prepared and standardized.
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37 **2.3 Preparation of crude aqueous-methanol extract**
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39 The leaves of *Loranthus micranthus* parasitic on the three selected host trees (*Kola acuminata*, *Citrus*
40
41 *spp*, *Garcinia kola*) were cleansed and dried under shade for 8 days. They were pulverized in
42
43 mechanized laboratory grinder to fine powder. A total of 3.0 kg, 2.75 kg and 1.2 kg of the respective
44
45 powdered plant materials were macerated repeatedly with distill water (total volume; 12 L, 10 L; 7.5 L
46
47 respectively). The resulting aqueous extracts were lyophilized under vacuum affording dry powdered
48
49 extracts which were weighed and their percentage yields calculated. The dry extracts were placed in
50
51 amber-coloured glass bottles and stored in a refrigerator (4 °C) until use. The yields obtained were
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53 24.12 %, 19.06 % and 9.80 % respectively. The extracts, at predetermined concentrations, were
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3 completely solubilized in DMSO and diluted accordingly with appropriate media to produce a final
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5 DMSO concentration of less than 0.01 % in both vehicle and treatments.
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7 8 **2.4 Isolation of Compounds from mistletoe** 9

10 **2.4.1 Fractionation of crude extracts of mistletoe** 11

12 Based on preliminary evaluated osteogenic potentials of the different crude extracts, exactly 700 g of
13
14 crude extract obtained from mistletoe parasitic on *Kola acuminata* was uniformly dispersed in 1.5
15
16 litres of distilled water and then carefully poured into a stoppered separatory funnel. Then, aliquots
17
18 (500 milliliters) of analar grade hexane was poured into the funnel and vigorously agitated for 5 mins
19
20 to allow for equilibration. The funnel was mounted on a stand to allow for complete separation of the
21
22 solvents into layers. The lower aqueous layer was tapped off and the upper hexane layer collected in a
23
24 glass bottle. This process was repeated severally until the hexane can no longer extract any further
25
26 constituents from the extract dispersion. The hexane fraction was pooled together and concentrated *in*
27
28 *vacuo* (40 °C) to afford the dry hexane fraction. Then, solvents of increasing polarity in the order,
29
30 chloroform<ethyl acetate<butanol<water were used accordingly as described above for hexane to
31
32 afford corresponding fractions. Similarly, the same processes were repeated for approximately 50 g of
33
34 crude extract obtained from mistletoe parasitic on *Garcinia kola* to afford the same set of fractions.
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36 The major fractions were screened for osteogenic activities and active fractions subjected to column
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38 chromatography.
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49 **2.4.2 Isolation of compound 1** 50

51 The air dried powdered of stem bark of (2.0 kg) of *Bombax ceiba* was placed in glass percolator with
52
53 ethanol (10 L) and were allowed to stand at room temperature for about 48 hours. The percolate was
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55 collected and this process of extraction was repeated for five times (5x 10L) successively. The
56
57 combined extract was filtered and concentrated using a vacuum afforded dark brown residue (200 gm).
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3 The crude ethanolic extract was dissolved in distilled H₂O and triturated with hexane using 3x 1L
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5 volume and yielded 25 g hexane fraction (4743-F004) and the insoluble portion was partitioned
6
7 between water and n-butanol and this process repeated three times. Both fractions n-butanol (4743-
8
9 F005) and aqueous (4743-F006) were concentrated under vacuum to yield 100 g and 50 g respectively.
10
11 The n-butanol fraction was further analyzed to isolate active constituents. The n-butanol fraction was
12
13 subjected to gross column chromatography (CC) over silica gel (60-120 mesh, 1.0 Kg). The column
14
15 was eluted using CHCl₃ with increasing amount of MeOH as eluent in the gradient of (95:05 → 05:95)
16
17 and yielded seventeen fractions (200 ml each). On the basis of TLC profile, similar fractions were
18
19 combined and formed 8 major fractions F001-F008. Fraction F001 (CHCl₃: MeOH, 100:0; 400 ml) (25
20
21 g) was re-chromatographed over silica gel (230-400 mesh, 300 g) using n-hexane: ethyl acetate (98:2)
22
23 as eluent system in isocratic manner. Fractions of 100 ml each were collected. Fraction having same
24
25 TLC profile, combined together, concentrated which afforded solid material. It was recrystallized in
26
27 chloroform at room temperature. White microcrystalline solid was obtained and characterized as lupeol
28
29 (1) with melting point of 212°C.
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38 UV λ_{max} nm (ϵ): 250 (11,100), 268 (1890.4), 289 (1160). IR ν_{max}^{KBr} 3167 (OH), 2872 (CH str), 1728
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40 (C=O), 1645 (C=C), 1384 (isopropylene side chain). The MS spectrum showed a molecular ion peak at
41
42 m/z 426 and an M+H peak at 427 in addition to the presence of IR signal for an OH group. This
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44 corresponds to a molecular formula of C₃₀H₅₀O.
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51 ¹HNMR (CDCl₃, 500 MHz): δ (J in Hz) 2.37-71 (1H, m-H1), δ 2.37-71 (1H, m-H2), δ 3.19 (1H, dt,
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53 4.6, 11.5-H3), δ 2.37-71 (1H, m-H4), δ 2.37-71 (1H, m-H5), δ 2.37-71 (1H, m-H6), δ 2.37-71 (1H, m-
54
55 H7), δ 2.37-71 (1H, m-H8), δ 2.37-71 (1H, m-H9), δ 2.37-71 (1H, m-H10), δ 2.37-71 (1H, m-H11), δ
56
57 2.37-71 (1H, m-H12), δ 2.37-71 (1H, m-H13), δ 2.37-71 (1H, m-H14), δ 2.37-71 (1H, m-H15), δ 2.37-
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59 71 (1H, m-H16), δ 2.37-71 (1H, m-H17), δ 2.37-71 (1H, m-H18), δ 2.39 (1H, dt, 4.1, 9.5-H19), δ 2.37-
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3 71 (1H, m-H20), δ 2.37-71 (1H, m-H21), δ 0.96 (3H, s-H23), δ 0.78 (1H, s-H24), δ 0.84 (3H, s-H25),
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5 δ 1.04 (3H, s-H26), δ 0.92 (3H, s-H27), δ 0.80 (3H, s-H28), δ 4.70 (1H, brs-H29), δ 4.58 (1H, brs-
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7 H29), δ 1.70 (3H, s-H30).
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12 ^{13}C NMR (CD_3OD , 400 MHz): δ 38.9 (C1), δ 27.6 (C2), δ 79.2 (C3), δ 39.1 (C4), δ 55.5 (C5), δ 18.5
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14 (C6), δ 34.5 (C7), δ 41.0 (C8), δ 50.6 (C9), δ 38.3 (C10), δ 21.1 (C11), δ 25.3 (C12), δ 37.4 (C13), δ
15
16 43.0 (C14), δ 27.6 (C15), δ 35.8 (C16), δ 43.2 (C17), δ 48.5 (C18), δ 48.2 (C19), δ 40.2 (C20), δ 30.1
17
18 (C21), δ 151.1 (C22), δ 28.2 (C23), δ (15.5 (C24), δ 16.3 (C25), δ 16.2 (C26), δ 14.7 (C27), δ 18.2
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20 (C28), δ 109.5 (C29), δ 19.5 (C30). These spectra data correlates well with available literature data for
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22 lupeol (Jain and Bari, 2010).
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30 **2.4.3 Isolation of compounds 2, 4 and 5**

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33 Exactly 20 g of the hexane fraction was chromatographed on silica gel (100-200, 0.5 kg) packed in a
34
35 glass column (6 X 85 cm) with the bed of 45 cm in height. The elution was performed with gradient
36
37 mixtures of hexane, hexane: ethyl acetate, ethyl acetate. Aliquots of 200 ml were collected and
38
39 monitored by analytical TLC. Twenty-one (21) fractions F1-F21 (1-2, 3-20, 21-28, 29-38, 39-43, 44-
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41 45, 46-57, 58-69, 70-83, 84-93, 94-109, 110-127, 128-147, 148-163, 164-181, 182-185, 186-197, 198-
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43 211, 212-231, 232-234, 235-243) respectively were collected. F5 (39-43; Hexane: ethylacetate, 98:2)
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45 yielded a golden yellow lipophilic liquid and a steroidal compound characterized using NMR, MS and
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47 in comparison with published spectral data as Friedelin, (**5**) (125 mg) which was recrystallized in
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49 acetone.
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3 Compound **5** had white needle-like crystals and showed a melting point of 261-262°C in an
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5 uncorrected Gallenkamp. UV λ_{max}^{nm} (ϵ): 256 (11,140), 275 (1970.5), 299 (1087). The IR ν_{max}^{KBr} was
6
7 only visible at 2879 (CH str) and 1745 (C=O). The MS spectrum exhibited a molecular ion peak at m/z
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9 426 and an $[M+H]^+$ of 427 corresponding to a molecular formula of $C_{30}H_{47}O$.
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16 1H NMR ($CDCl_3$, 400 MHz): δ 1.95, 1.71 (2H, ddd-H1); δ 2.36, 2.27 (2H, ddd-H2); δ 2.26 (1H, q-H4);
17
18 δ 1.72, 1.27 (2H, d-H6); δ 1.49, 1.37 (2H, m-H7); δ 1.38 (1H, dd-H8); δ 1.53 (1H, m-H10); δ 1.45,
19
20 1.27 (2H, m-H11); δ 1.33, 1.34 (2H, m-H12); δ 1.47, 1.27 (2H, m-H15); δ 1.58, 1.35 (2H, m-H16); δ
21
22 1.56 (1H, m-H18); δ 1.37, 1.23 (2H, m-H19); δ 1.50, 1.32 (2H, m-H21); δ 1.51, 0.95 (2H, m-H22), δ
23
24 0.88 (3H, d-H23); δ 0.73 (3H, s-H24); δ 0.87 (3H, s-H25); δ 1.01 (3H, s-H26), δ 1.05 (3H, s-H27); δ
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26 1.18 (3H, s-H28); δ 0.99 (3H, s-H29); δ 0.93 (3H, s-H30).
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34 ^{13}C NMR ($CDCl_3$, 200 MHz): δ 22.5 (C1), δ 41.6 (C2), δ 213.3 (C3), δ 58.4 (C4), δ 42.3 (C5), δ 41.5
35
36 (C6), δ 18.3 (C7), δ 53.2 (C8), δ 37.5 (C9), δ 59.5 (C10), δ 35.7 (C11), δ 30.6 (C12), δ 39.8 (C13), δ
37
38 38.4 (C14), δ 32.5 (C15), δ 36.2 (C16), δ 30.2 (C17), δ 43.0 (C18), δ 35.4 (C19), δ 28.2 (C20), δ 32.7
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40 (C21), δ 39.3 (C22), δ 7.0 (C23), δ 14.8 (C24), δ 18.0 (C25), δ 20.3 (C26), δ 18.6 (C27), δ 32.2 (C28),
41
42 δ 35.2 (C29), δ 32.0 (C30). The above spectra data agree perfectly with literature data for friedelin
43
44 (Mann et al., 2011; Mahato and Kundu, 1994).
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51 F9 (70-83; Hexane: Ethylacetate, 90:10) was oily and afforded very high yield (220 mg) of pure
52
53 needlelike crystalline compound (**4**) was precipitated on standing and recrystallized in acetone. KH
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55 was unambiguously characterized as 3, 4, 5-trimethoxy gallate using MS, NMR and in comparison
56
57 with published spectral data.
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3 Compound **4** was isolated as white crystalline solid with melting point of 83°C. The MS spectrum
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5 showed a strong molecular peak ion at m/z 226 corresponding to a molecular formula of $C_{11}H_{14}O_5$.

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7 1H NMR ($CDCl_3$, 400 MHz): δ 7.05 (1H, s-H2), δ 7.05 (1H, s-H6), δ 3.83, (3H, s-OCH₃), δ 3.83, (3H,
8
9 s-OCH₃), δ 3.83, (3H, s-OCH₃), δ 3.89, (3H, s-OCH₃).

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15 ^{13}C NMR ($CDCl_3$, 400 MHz): 126 (C1), δ 106.5 (C2), δ 153 (C3), δ 106.5 (C6), δ 143.6 (C4), δ 143.5
16
17 (C4), δ 167 (C=O), δ 59.0 (OCH₃), δ 60.0 (OCH₃), δ 58.9 (OCH₃), δ 59.0 (OCH₃). The above spectral
18
19 data agrees well with literature data described for 3, 4, 5-trimethoxy gallate (Kane et al., 1988).

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25 F12 (110-127; Hexane: Ethylacetate, 80:20, 2.5 g) was further chromatographed on silica gel (230-400
26
27 mesh, 0.1 kg) packed in a glass column (1.5 X 105 cm) with the bed of 60 cm in height and eluted with
28
29 Hexane: Ethylacetate (100:00-00:100) to yield eight (8) fractions, F12A-F12H of 50 ml aliquots.
30
31 Similar fractions based on analytical TLC were pooled together. F12C, F12D and F12E were pooled
32
33 together and on concentration, afforded a white amorphous compound **2** (154 mg) which was
34
35 recrystallized in acetone-methanol mixture. Compound **2** was characterized using a combination of
36
37 NMR, MS, and 2D correlation spectroscopy and in comparison with published literature as 7 β , 15 α -
38
39 dihydroxy lupeol palmitate (Omeje et al., 2011a), had white amorphous appearance and m.p = 107.0
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41 °C.
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47 $UV_{\lambda_{max}}^{CHCl_3}$ nm (ϵ): 250 (11,100), 268 (1890.4), 289 (1160). IR ν_{max}^{KBr} 3167 (OH), 2872 (CH str), 1728
48
49 (C=O), 1645 (C=C), 1384 (isopropylene side chain). HREIMS $[M]^+$ m/z : 696.6022 calculated for
50
51 $C_{46}H_{80}O_4$ with 7 double bond equivalence (DBE). $[M+H]^+ [-H_2O]$ m/z : 679.6021 (loss of water),
52
53 $[M+Na]^+$ m/z : 719.5936, $[M+HCOO]^-$ m/z : 741.6045, $[M+Cl]^-$ m/z : 731.5751, $[M+CF_3COO]^-$ m/z :
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55 809.5920, all calculated for $C_{46}H_{80}O_4$. EIMS (relative int.), M^+ (m/z): 696.
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3 ¹HNMR (CDCl₃, 500 MHz): δ 4.66 (1H, d, terminal proton near a double bonded carbon), δ 4.57 (1H,
4
5 br, s, equivalent proton near a double bonded carbon), δ 4.45 (1H, dd, proton near an oxygenated
6
7 carbon), δ 4.14 (1H, dd, proton near an oxygenated carbon), δ 3.79 (1H, dd, proton near an oxygenated
8
9 carbon), δ 3.00 (OH, protons), δ 0.84 (1H, methine proton), δ 1.13 (1H, methine proton), δ 2.26 (2H, t,
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11 methylene protons in a flexible region), δ 0.97 (3H, s, angular methyl), δ 1.06 (3H, s, angular methyl) δ
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13 0.86 and δ 1.07 (3H, s- C-4 dimethyl protons).
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20 ¹³CNMR (CDCl₃, 500 MHz): δ 173.91(ester carbonyl), δ 150.52 (double bonded carbon), δ 109.96
21
22 (double bonded carbon), δ 80.37 (oxygenated carbon), δ 72.71 (oxygenated carbon), δ 68.25
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24 (oxygenated carbon), δ 52.31 (methine carbon), δ 50.39 (methine carbon), δ 49.15 (quaternary
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26 carbon), δ 48.29 (methine carbon), δ 48.05 (quaternary carbon), δ 47.77 (methine carbon), δ 45.89
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28 (methylene carbon), δ 42.78 (quaternary carbon), δ 39.99 (methylene carbon), δ 38.62 (methylene
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30 carbon), δ 37.77 (quaternary carbon), δ 37.63 (methine proton), δ 37.42 (quaternary proton) δ 35.05 (
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32 methylene carbon), δ 14.36 (terminal methyl of fatty acid chain).
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39 DEPT-135 signals observed for 8 methyls, 23 methylenes (overlapping CH₂ observed), 8 methines
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41 carbons. 7 quaternary carbons were not shown in the DEPT. 1H-COSY, HSQC, and HMBC studies
42
43 confirmed all the fragments and connectivities. Positive NOE and NOESY confirmed spatial
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45 arrangements or configurations. Spectral data correlate well with literature data for dihydroxylated
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47 hydroxylated esterified lupane nucleus (lupeol) and was elucidated to be 7 α , 15 β -dihydroxy-lup-
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49 20(29)-en-3 β -O-palmitate (Omeje et al., 2011).
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2.4.4 Isolation of compound 3

Approximately 10.3 g of butanol fraction obtained from the mistletoe extract was chromatographed on silica gel (230-400, 400 g) in a column of dimension, 6 x 60 cm with a bed height of 25 cm. The column was ran on gradient solvent system, Dichloromethane: methanol (100:0 to 0:100) and monitored by analytical TLC. Aliquots in 250 ml were collected and similar fractions pooled together to afford 17 fractions as follows; F1 (1-2); F2 (3-5); F3 (6-13); F4 (14-17); F5 (18-19); F6 (20-28); F8 (29-37); F9 (38-49); F10 (50-55); F11 (56-63); F12 (64-68); F13 (69-74); F14 (75-81); F15 (82-89); F16 (90-91); F17 (92-98); F18 (99-113); F19 (114-122). The fraction F2 (3-5; 900 ml; 95:5; 2.5 g dry weight), on standing, afforded large quantity (150 mg) of yellow amorphous compound which was further purified through repeated precipitation. This compound was characterized using a combination of NMR, MS, and 2D correlation spectroscopy and in comparison with published literature as a 3-methoxy derivative of quercetin (Yoo et al., 2005).

Compound **3** was isolated as pale yellow amorphous solid. UV (MeOH): λ_{\max} (nm):255 and 356. IR (Nujol) cm^{-1} : 1740(C=O), 3370(O-H). The MS spectrum showed an M+H ion peak at m/z 317 corresponding to a molecular formular of $\text{C}_{16}\text{H}_{27}\text{O}_7$.

$^1\text{H-NMR}$ (CD_3OD) δ (ppm):6.20,1H,d,(J=2Hz)H-6, 6.40,1H,d,(J=2Hz) H-8, 6.90,1H,d,(J=8Hz) H-5', 7.60,1H,d,d(J=2, 8Hz) H-6', 7.71,1H,d(J=2Hz), H-2', δ 12.4 (OH at position 5 chelated to the carbonyl at position 4), 3.82,3H,s(OCH_3).

$^{13}\text{CNMR}$ (CD_3OD , 400 MHz): δ 157.8 (C2), δ 139.2 (C3), δ 179.4 (C4), δ 163.2 (C5), δ 99.3 (C6), δ 164.7 (C7), δ 94.5 (C8), δ 162.3 (C9), δ 106.0 (C10), δ 122.5 (C1'), δ 116.0 (C2'), δ 145.4 (C3'), δ 149

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3 (C4'), δ 116.3 (C5'), δ 123 (C6') and δ 59.7 (OCH₃). The above spectral data agree with literature data
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5 for 3-methoxy quercetin (Yoo et al., 2005).
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10 **2.5 Culture of rat calvarial osteoblasts (RCOs)**

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12 Rat calvarial osteoblasts were obtained following our previously published protocol of sequential
13 digestion (Bhargavan et al., 2009; Trivedi et al., 2008; 2009, Kumar et al., 2010). Briefly, calvariae
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15 from 1-2 d old SD rats (both sexes) were pooled. After surgical isolation from the skull and the
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17 removal of sutures and adherent mesenchymal tissues, calvariae were subjected to five sequential (10–
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19 15min) digestions at 37 °C in a solution containing 0.1 % dispase and 0.1% collagenase P. Cells
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21 released from the second to fifth digestions were pooled, centrifuged, re-suspended, and plated in T-
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23 25cm² flasks in α -MEM containing 10% FCS and 1% penicillin/streptomycin (complete growth
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25 medium).
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34 **2.6 Effect of extract on uterine weights of animal: Preliminary estrogenicity test on extracts**

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36 In an attempt to probe the possible estrogenic activity of our extracts on the uterus of the experimental
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38 animals, thirty-two adult Sprawl Dawley (SD) rats (32 females; 180-220 g) were randomly divided
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40 into 4 equal groups of 8 animals each as follows: Vehicle group (gum acacia in distilled water p.o.),
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42 100 mg/kg (p.o.) of extract group; 200 mg/kg (p.o.) of extract group; 400 mg/kg (p.o.) of extract group.
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44 All animals received the above doses daily for 21 days and were closely monitored. The doses were
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46 chosen based on our already established protocols (Osadebe and Omeje, 2008; 2009). Finally, all
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48 animals were sacrificed on the last day to collect the uteri following anesthesia with ketamine. The
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50 uterine weights were recorded. Obvious hypertrophy of the uterus (significant increase in uterine
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52 weights compared to control) was interpreted as preliminarily, potential estrogenicity of the test
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54 extracts (Laws et al., 2000, Saarinen et al., 2006).
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2.7 MTT assay for cell toxicity

To ascertain the safety of the extracts on RCOs, MTT assay was performed. Briefly, RCOs at 70 to 80 % confluency were trypsinized, and 2000 cells/well seeded in a 96-well plate in α -MEM supplemented with 10 % FBS (100 μ l/well). After 24 hours of incubation (37 °C; 5 % CO₂) to allow for proper attachment of cells, media was dumped off and replaced with α -MEM supplemented with 5 % FBS (starvation state). Four (4) hours later, cells were treated in six replicates with appropriate concentrations of extracts, compounds and controls and incubated at 37 °C, 5 % CO₂ for 24 hours. At the end of 24 hours incubation, 10 μ l of MTT solution (5 mg/ml in filtered PBS, pH: 7.4) was added to each well and incubated (37 °C, 5 % CO₂) for 3-4 hours to allow for complete metabolism of MTT by the cells forming formazan. At the end of this incubation period, the media was carefully dumped off and plates thoroughly drained on pieces of paper towels. The formazan in each well was completely dissolved in 100 μ l of DMSO with the aid of gentle shaking (10 rpm for 5 minutes) and optical density at 405 nm taken for each plate.

2.8 Osteoblast differentiation: ALP assay

For determination of alkaline phosphatase (ALP) activity, 2×10^3 cells/ well were seeded in 96-well plates. Cells were treated with different concentrations (.2-3.2 μ g/ml) of the individual extracts or compounds for 48 h in α -MEM supplemented with 5% charcoal treated FCS, 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 1% penicillin/streptomycin (osteoblast differentiation medium). At the end of incubation period, total ALP activity was measured colorimetrically using p-nitrophenylphosphate (PNPP) as substrate at 405 nm (Trivedi et al., 2008; 2009, Kumar et al., 2010).

2.9 Mineralization of RCOs

For mineralization studies 2×10^3 cells/ well were seeded in 6-well plates in differentiation media with 10^{-7} M dexamethasone. Cells were cultured with and without the compounds and controls for 21 days at 37°C in a humidified atmosphere of 5 % CO₂ and 95 % air, and the medium was changed every 48 hours with fresh treatments each time. After 21 days, the attached cells were fixed in 4 % formaldehyde for 20 min at room temperature and rinsed once in PBS. Alizarin red-S stain was used for staining mineralized nodules, followed by extraction of the stain (with 10 % Cetyl pyridinium chloride) for colorimetric quantification at 570 nm (Trivedi et al., 2008; 2009, Kumar et al., 2010).

2.10 RNA isolation and qPCR

Total RNA from each sample was isolated using Trizol (Ambion) according to manufacturer's instructions. Total RNA (1µg) from each sample was reverse transcribed using RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to manufacturer's instructions. mRNA levels of described genes were determined by SyBr green chemistry (Light Cycler 480 SyBr green I master; Roche) using a Light Cycler 480 (Roche) according to manufacturer's instructions. The specificity of the PCR product was documented by LightCycler melting curve analysis and migration on ethidium bromide-stained agarose gel. The relative quantitation for any given gene was calculated after determination of the difference between CT of the target gene and that of the calibrator gene GAPDH (house-keeping gene) using a Δ CT method. Primer sequences used for the osteogenic genes are as shown in **Table 1**.

2.11 Statistical analysis

Data are expressed as mean \pm SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by Newman-Keuls post-hoc multiple comparison test using

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Prism 3.0 version software. Where necessary, Student's 't' test was used to study statistical significance in experiments with only two treatments.

2.12 Theory/calculation

Preliminary screening of the different extracts of mistletoes using osteoblast ALP assay (a differentiation marker) provided an insight and support for detailed investigation of their osteogenic potentials. This formed the main thrust of the present research.

3. Results and discussion

The effects of aqueous-methanol extracts of eastern Nigeria mistletoe harvested from three host trees (*Kola acuminata*, *Citrus spp*, *Garcinia kola*) on the viability (MTT assay) and early differentiation (ALP assay) of primary osteoblasts (rat calvarial osteoblasts, RCO) were studied. In addition, following compounds were isolated and studied - lupeol (**1**) from *Bombax ciba*, and 7 β , 15 α -dihydroxy lupeol palmitate (**2**), 3-methoxy quercetin (**3**), 3, 4, 5-trimethoxy benzoate (**4**) and friedelin (**5**) from the mistletoe leaves (Figure 1). The relative yields of various compounds are provided in Table 2. From the available literature, mistletoes, especially the eastern Nigeria species is known to produce hydroxylated lupeol (**2**) rather than lupeol (Omeje et al; 2011a; 2011b; 2011c). Because **2** is abundantly available in mistletoe leaves, it was deemed reasonable to compare its activity with lupeol (**1**) isolated from another natural source, i.e. stem-bark of *Bombax ciba*. Data show the relative order of abundance of the compounds from the highest to the lowest. Notably, **1** had a very high yield (4%) in the stem-bark of *Bombax ciba* followed by **3** that had a good yield (0.33%) in the leaves of mistletoe, *Loranthus micranthus*. In the leaves of same species, the yields of **2** and **4** were comparable (0.06-0.07%) whereas **5** was the lowest (0.008%).

All three extracts had no effect on the viability of RCO at concentrations ranging from 0.2 to 3.2 μ g/ml (Supplementary Figure 1). At concentrations higher than 3.2 μ g/ml, cell culture medium turned turbid, suggesting precipitation of less soluble constituents of the extracts and thus not included for the study.

In ALP assay (osteoblast differentiation), CM (*Citrus spp*-mistletoe) and KM (*Kola acuminata*-mistletoe) exhibited biphasic effect – increased ALP activity at the lower but decreased at the higher concentrations (Figure 2). Similar biphasic pattern is often reported with phytoextracts not only in bone cells but also other cells (Ososki and Kennelly, 2003; Biju et al., 2012). Although, no definitive explanation for such effect is available, it is surmised that putative inhibitory (cytotoxic) constituents

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3 that are otherwise present in low abundance in the extract reach levels adequate to counteract the
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5 stimulatory effect of the extract when used at higher concentrations. Stimulation of ALP activity by
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7 CM from 0.2 to 0.8µg/ml was comparable to BMP-2 treated (positive control) RCO. At 1.6µg/ml, CM
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9 had no effect whilst at 3.2µg/ml; it inhibited ALP activity compared to control. Like, CM, ALP
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11 activity of RCO was increased by KM from 0.2 to 0.8µg/ml but the extent of stimulation was
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13 significantly lower than BMP-2. At 1.6µg/ml, KM had no effect but at 3.2µg/ml, it inhibited ALP
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15 activity compared to control. GKM (*Garcinia kola-mistletoe*) modestly but significantly increased
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17 ALP activity from 0.2 to 1.6µg/ml but the effect was lacking at 3.2 µg/ml, and unlike CM and KM,
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19 there was no inhibition of ALP activity by GKM in comparison to control (Figure 2C). From these data
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21 it appeared that out of the three extracts, CM at lower concentration was most effective in stimulating
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23 osteoblast differentiation.
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33 We investigated potential cytotoxicity of five compounds in RCO. At concentrations ranging from
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35 100pM (10^{-10} M) to 1µM (10^{-6} M), none except **5** had an effect on RCO viability. As **5** showed
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37 significant decline in RCO viability at 1nM and above (Supplementary Figure 2), it was not included
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39 in further studies.
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46 Effect of **1**, **2**, **3** and **4** on osteoblast differentiation was tested at concentrations ranging from 100pM to
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48 100nM because phytochemicals have poor bioavailability (Setchell et al., 2009; Khan et al., 2013) and
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50 are unlikely to attain micromolar (10^{-6} M) levels in blood following their oral administration. As shown
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52 in figure 3, all four compounds increased ALP activity however, **4** had the strongest effect, increasing
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54 the activity in a concentration-dependent manner and at 1- and 100nM, it doubled ALP activity over
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56 the control. **1** increased this activity at the higher concentrations (10- and 100nM but not lower)
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58 whereas **3** had biphasic effect, increased ALP at the lower concentrations (100pM and 1nM) but
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3 decreased at 100nM. Phytochemicals are known for having biphasic effect-lower concentrations
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5 exerting stimulatory and no/inhibitory biological effect at higher concentrations (Ososki and Kennelly,
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7 2003). **2** had a modest stimulation of ALP activity over the control at 100pM and 10nM but no effect at
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9 100nM (Figure 3).

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16 Based on the efficacy with ALP assay, we selected 1- and 10nM for all four compounds to test their
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18 effects in stimulating osteogenic genes, Runx-2 and BMP-2. All four compounds increased the mRNA
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20 levels of both osteogenic genes although the levels of increase varied between the compounds (Figure
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22 4A,B). Runx2 expression was maximally increased by **1** and **2** and least by **3** whereas the increase with
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24 **4** was in-between compounds **1** or **2**, and **3**. Runx2 is a ‘master’ transcription factor for osteoblast
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26 differentiation and bone formation, and responsible gene for cleidocranial dysplasia (Baumert et al.,
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28 2005). BMP-2 is known to regulate the expression of Runx2 through Smad signalling (Mattahbo et al.,
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30 2006). Together, BMP-2 and Runx2 constitute an important stimulatory axis for osteoblastogenesis.
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32 BMP-2 is secreted from osteoblasts and acts in an autocrine/paracrine mode. BMP-2 mRNA levels
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34 were increased by all four compounds compared to control. The increase in BMP-2 expression was
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36 highest achieved with **2** followed by **4** and **1**, whilst **3** had lesser increase compared to the former two.
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38 Data suggest that stimulation of osteoblast differentiation by compounds **2** and **4** is upstream of Runx2
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40 whilst compound **1** and **3** act at the Runx2 level.
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51 Primary cultures of osteoblasts such as RCO form mineralized nodules (mineralization) when treated
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53 for an extended time (18-21 d) in medium containing β -glycerophosphate and L-ascorbic acid. These
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55 nodules contain nascent Ca^{2+} which is stained with alizarin red-s dye and quantified colorimetrically
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57 after dye extraction (Figure 5A,B). This assay is the most definitive proof of stimulation of osteoblast
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3 function by any agent. At 10nM, all four compounds equally increased the mineralized nodules over
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5 the control. These data suggest in vitro osteogenic effect of all four compounds. It appears that greater
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7 induction of BMP-2 expression by **2** and **4**, and Runx2 which is downstream of BMP-2 by **4** and **3**
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9 translated to comparable stimulatory outcome when osteoblast mineralization was assessed.
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11 Compound **1** (not present in mistletoe) and its congener, **2** (present in mistletoe), had generally
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13 comparable effect on various parameters of osteoblast differentiation with the exception of BMP-2
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15 upregulation, in which **2** had greater stimulatory effect than **1**. A structure-activity relationship
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17 between **1**, **2** and other lupeol derivatives with respect to stimulating BMP-2 production by osteoblasts
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19 may be studied in future.
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29 A secondary concern with the use of plant-derived products for the treatment of postmenopausal
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31 diseases is the risk for endometrial hyperplasia or excessive cell growth in the uterus. Those cells may
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33 occasionally exhibit cancerous growth. The osteoporosis study thus observes participants for
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35 endometrial hyperplasia as well. The extracts, at the tested doses did not exhibit any significant effect
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37 on the uterine weights of female Sprague Dawley rats thus suggesting that they did not possess
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39 estrogen 'like' effect (**Figure 6**).
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46 **Conclusion**

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48 We showed that crude extracts of the eastern Nigeria mistletoe, *Loranthus micranthus* Linn parasitic
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50 on selected host trees and three compounds isolated from the extract have osteogenic effect in vitro. In
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52 particular, 7 β , 15 α -dihydroxy lupeol palmitate (**2**) (a derivative of lupeol) and 3, 4, 5-trimethoxy gallate
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54 (**4**) (a derivative of gallic acid) showed excellent osteogenic properties. To the best of our knowledge,
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56 this is the first report demonstrating osteogenic activity of these compounds. In addition, the analytical
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method that we have established to determine the quantity of these osteogenic compounds would contribute importantly to the quality control of the extracts for future assessment of preclinical efficacy and safety studies. Taken together, our study helps to strengthen the traditional claim of the Nigerian mistletoe as a remedy for post-menopausal disorders as it suggests an anti-osteoporosis promise of the plant. Future studies will address the in vivo bone anabolic effect in preclinical animal models of estrogen deficiency-induced osteoporosis and identify the mechanisms involved in the osteogenic actions of the bioactive compounds.

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6 **Figure 1:** Structures of isolated compounds. Compound **1** (from *Bombax ciba*), **2, 3, 4** and **5** (from
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8 *Loranthus micranthus*) were isolated (**Table 2**) and characterized (see Materials and Methods).
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11 **Figure 2:** Effect of extracts of mistletoes on osteoblast differentiation. Rat calvarial osteoblasts
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44 **Figure 4.** Effect of compounds **1, 2, 3** and **4** on mRNA levels of osteogenic genes. Rat calvarial
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Figure 5. Effect of **1, 2, 3** and **4** on mineralization of RCOs. RCOs (2×10^3 cells) were seeded in 6-well plates and incubated with 1 and 10 nM of **1, 2, 3** or **4** for 21 days. At the end of incubation, cells were fixed and stained with alizarin red-S (upper panel – representative photomicrograph). Stain was extracted and OD measured colorimetrically. Values are obtained from three independent experiments in triplicate/treatment point and expressed as mean \pm SEM; and $***P < 0.001$ compared with control.

Figure 6: Effect of extract from mistletoe harvested from *Kola acuminata* on of SD rats uterine weight. At end point of the experiment did not showed any marked change when compared to vehicle. All values are expressed as mean \pm SEM (n=6).

Table 1. Primer sequence of genes used for qPCR

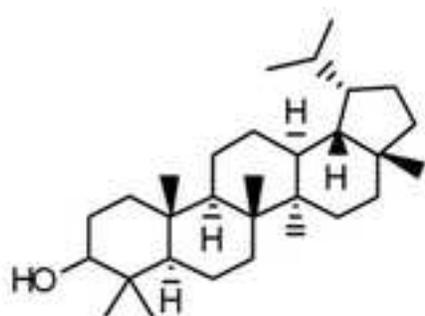
Gene name	Primer sequence	Accession number
Bone morphogenetic protein 2 (BMP2)	F-CGGACTGCGGTCTCCTAA R-GGGGAAGCAGCAACACTAGA	NM_007553.2
Runt-related transcription factor 2 (RUNX 2)	F-CCCGGGAACCAAGAAATC R-CAGATAGGAGGGGTAAGACTGG	AF053956.1
GAPDH	F-TTTGATGTTAGTGGGGTCTCG R-AGCTTGTCATCAACGGGAAG	NM_017008

Table 2. Estimated yield of isolated compounds from the plant materials

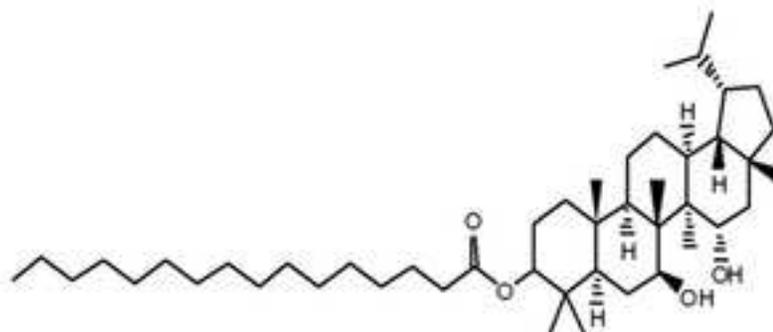
Compounds	Yield (% dry weight of plant material)
1 (LP)	4.00 % (stem bark of <i>Bombax ciba</i>)
2 (DLP)	0.06 % (leaves of <i>Loranthus micranthus</i>)
3 (QD)	0.33 % (leaves of <i>Loranthus micranthus</i>)
4 (KH)	0.07 % (leaves of <i>Loranthus micranthus</i>)
5 (FRD)	0.008 % (leaves of <i>Loranthus micranthus</i>)

Percentage yields were calculated as per the dry weight of the plant materials. Data shows very high yield of LP from the bark of *Bombax ciba* and reasonable quantities of DLP, QD and KH from the dry leaves of *Loranthus micranthus*. The concentration of FRD was the least in the *Loranthus micranthus* plant material.

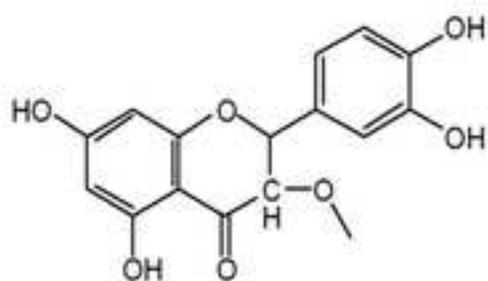
Figure 1



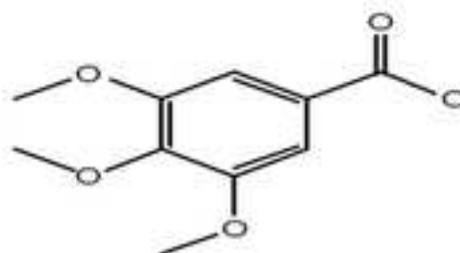
Compound 1: Lupeol (LP)



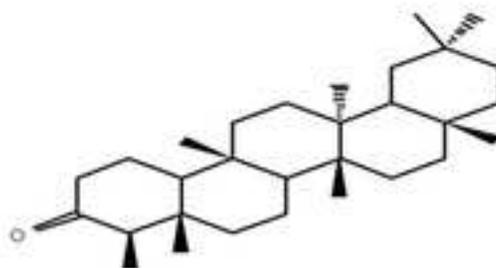
Compound 2: 7 β , 15 α -dihydroxy lupeol palmitate (DLP)



Compound 3: 3-methoxy quercetin(QD)



Compound 4: 3,4,5-trimethoxy benzoate (KH)



Compound 5: Friedelin (FD)

Figure 2

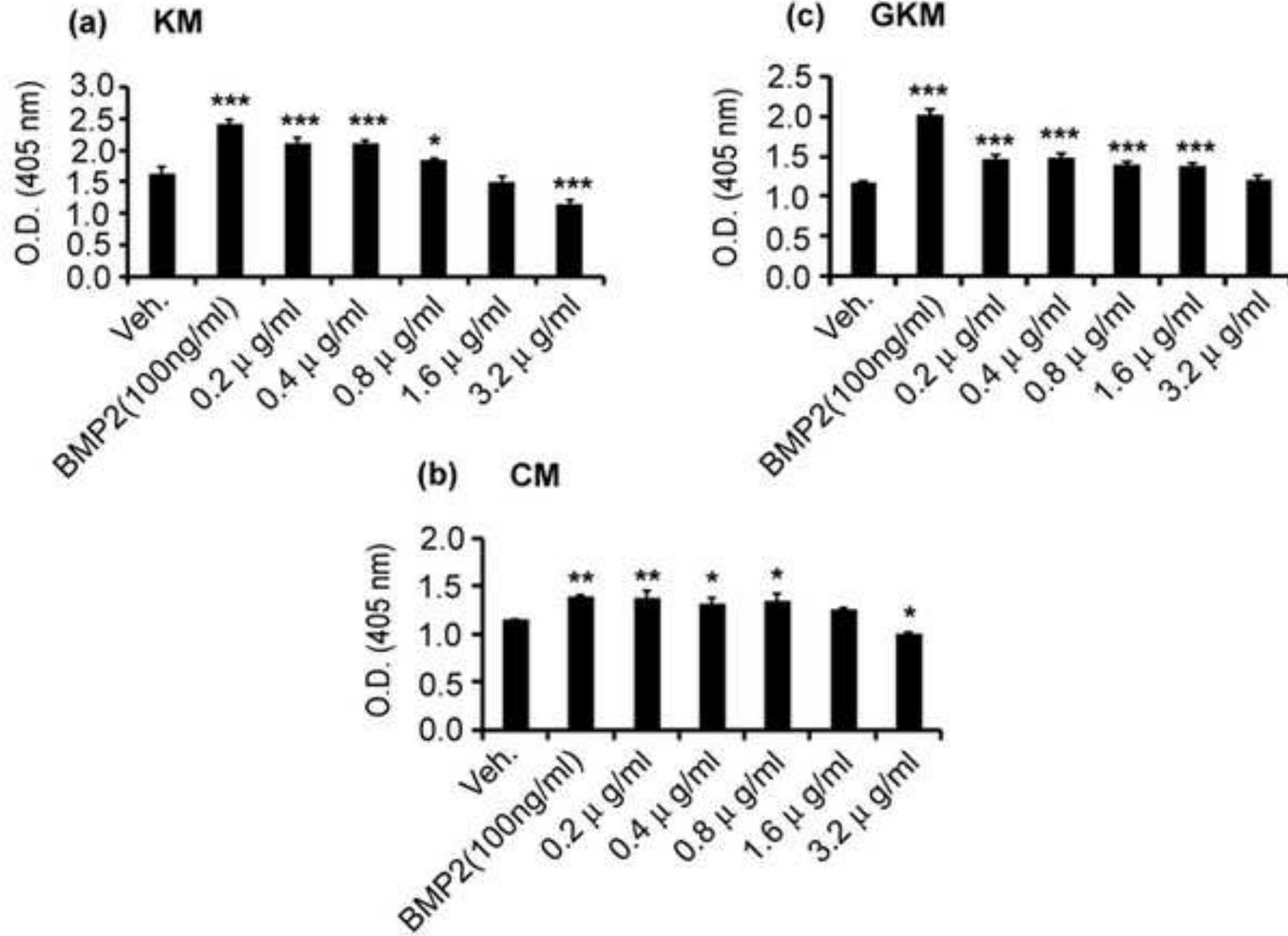


Figure 3

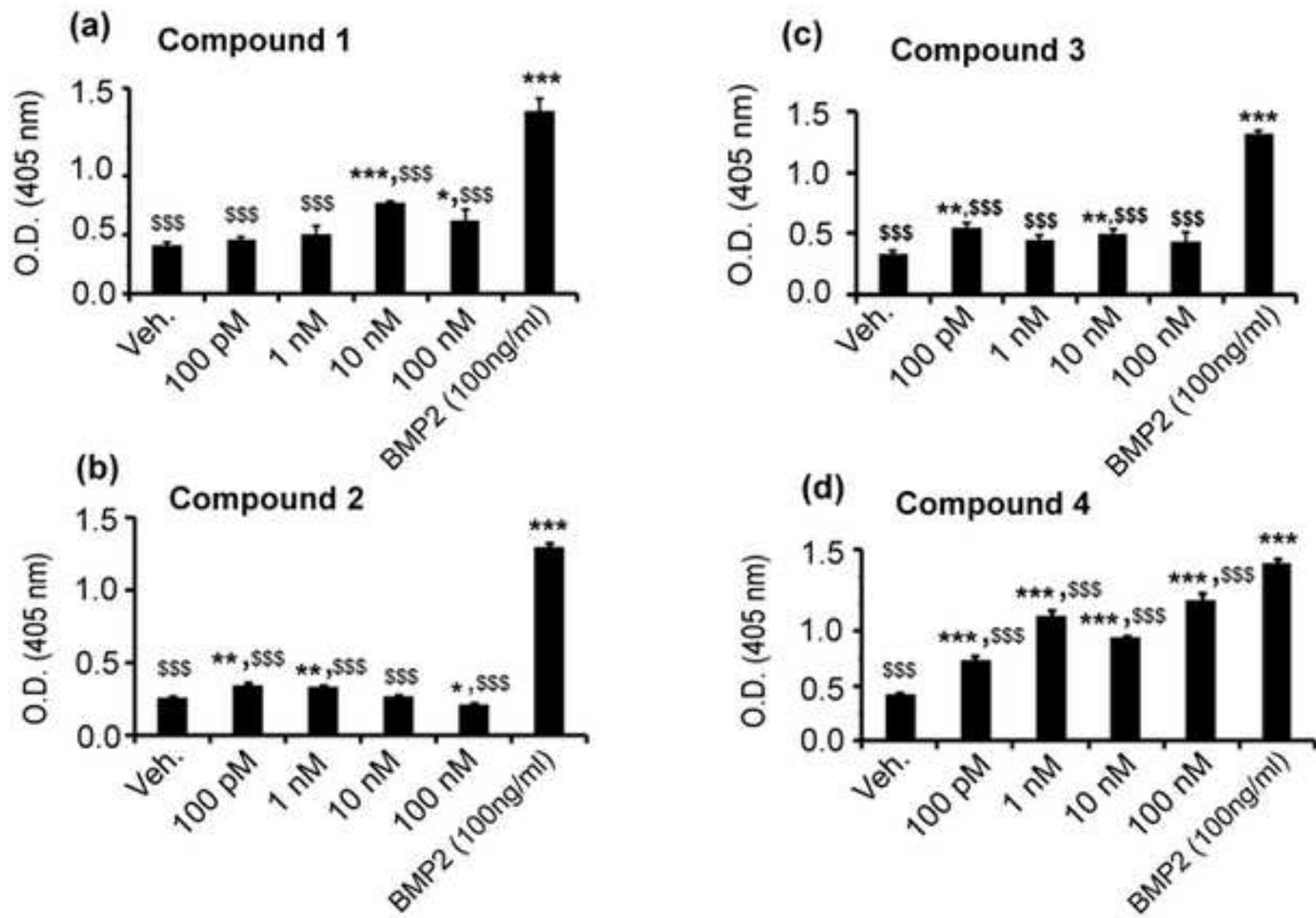


Figure 4

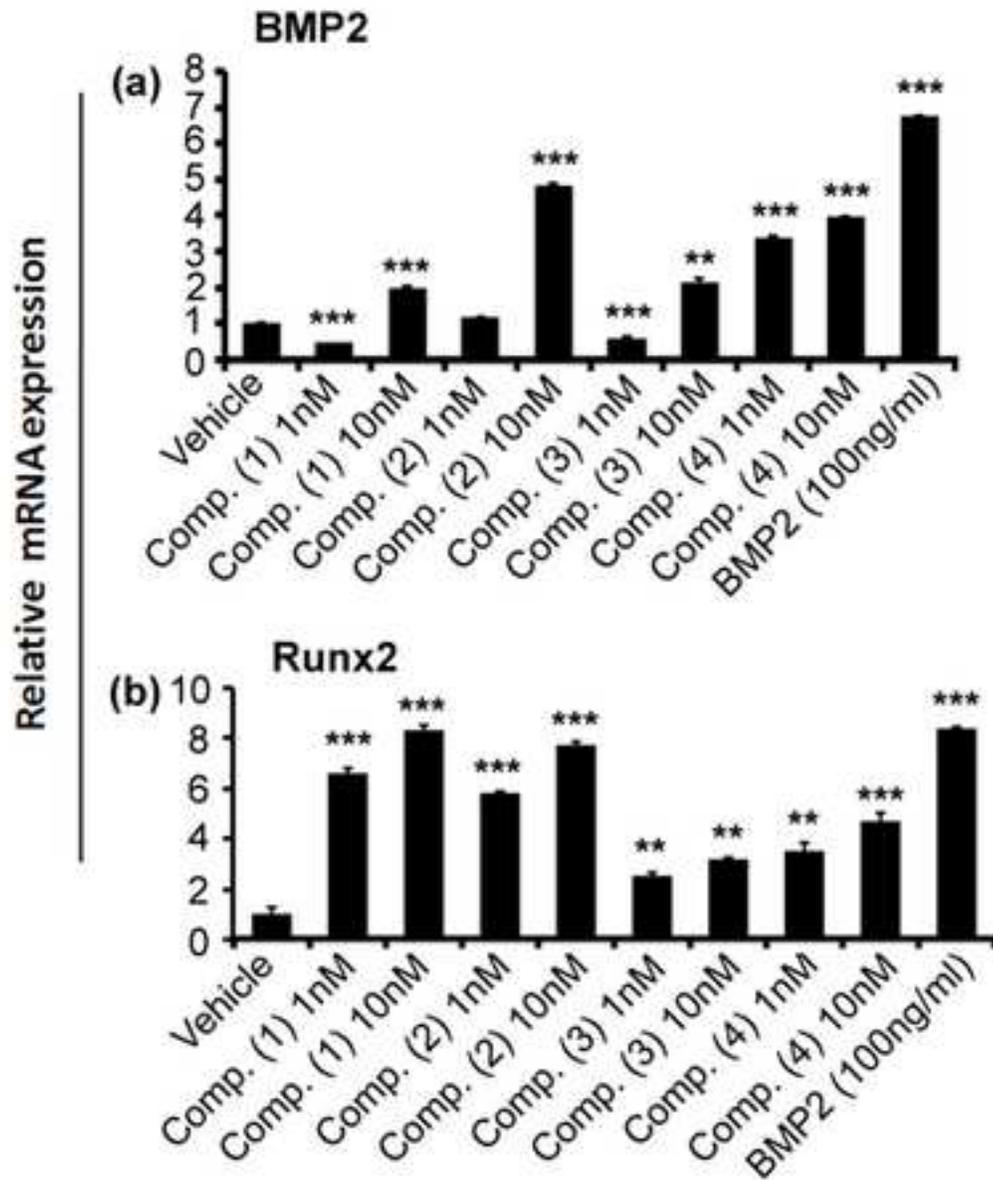


Figure 5

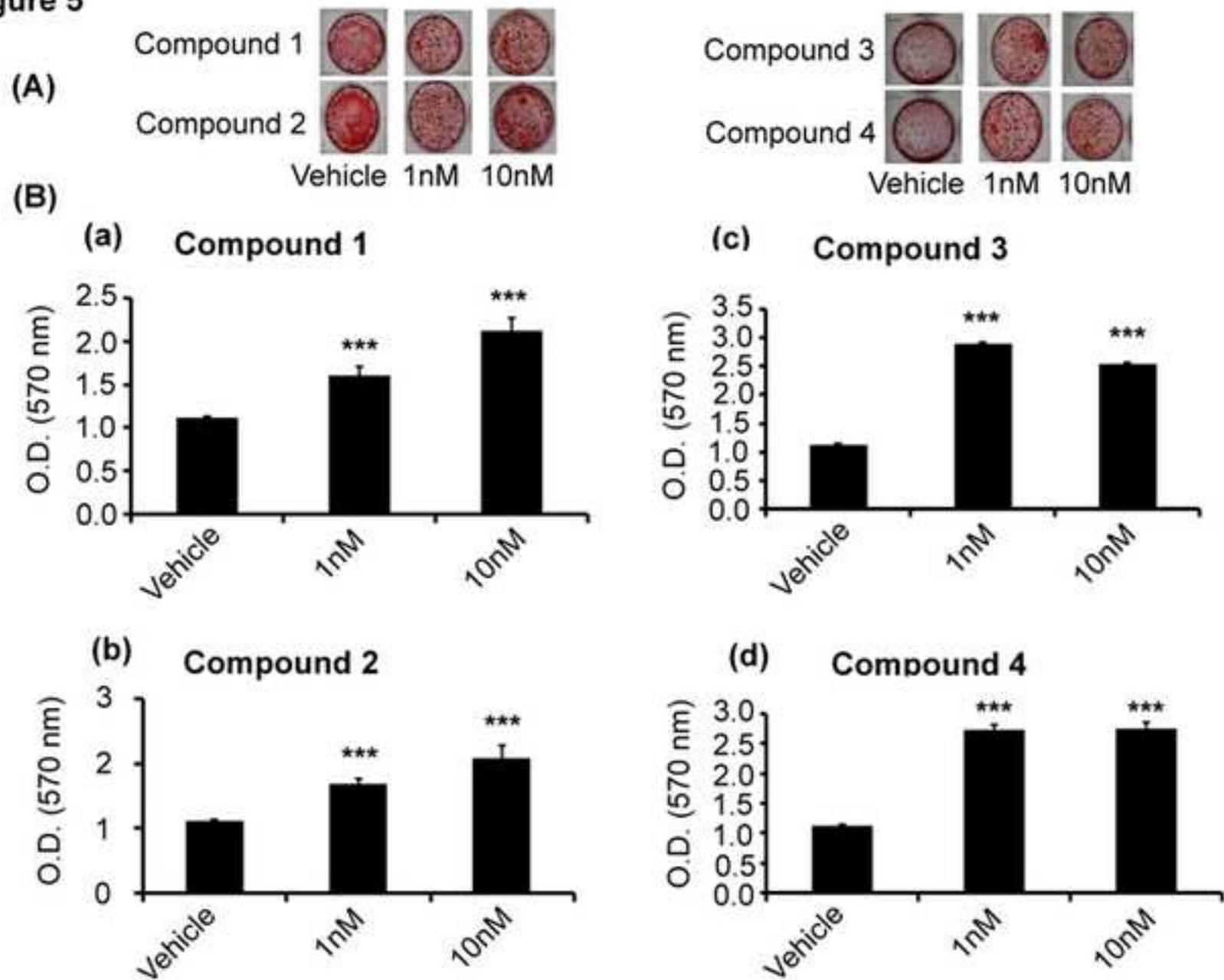
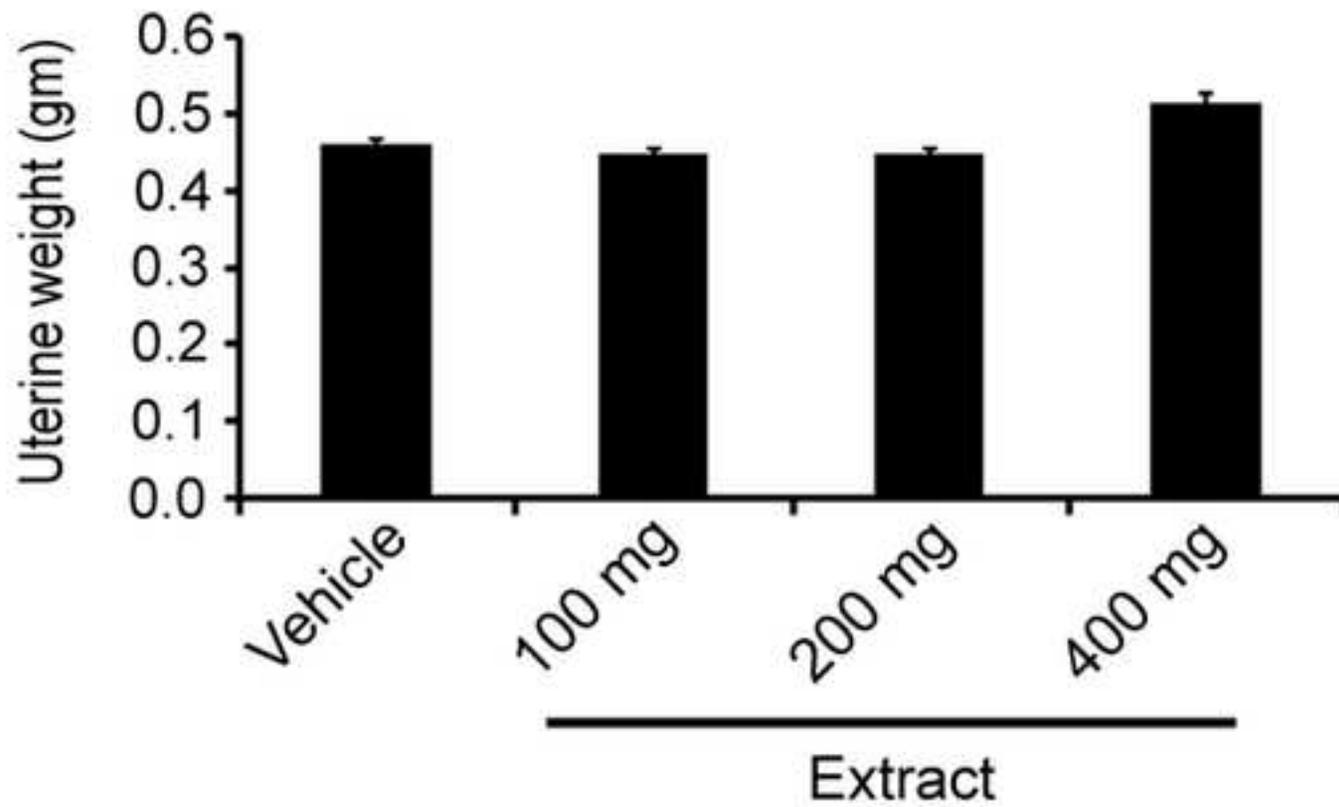


Figure 6



List of legends for Figures

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Figure S1

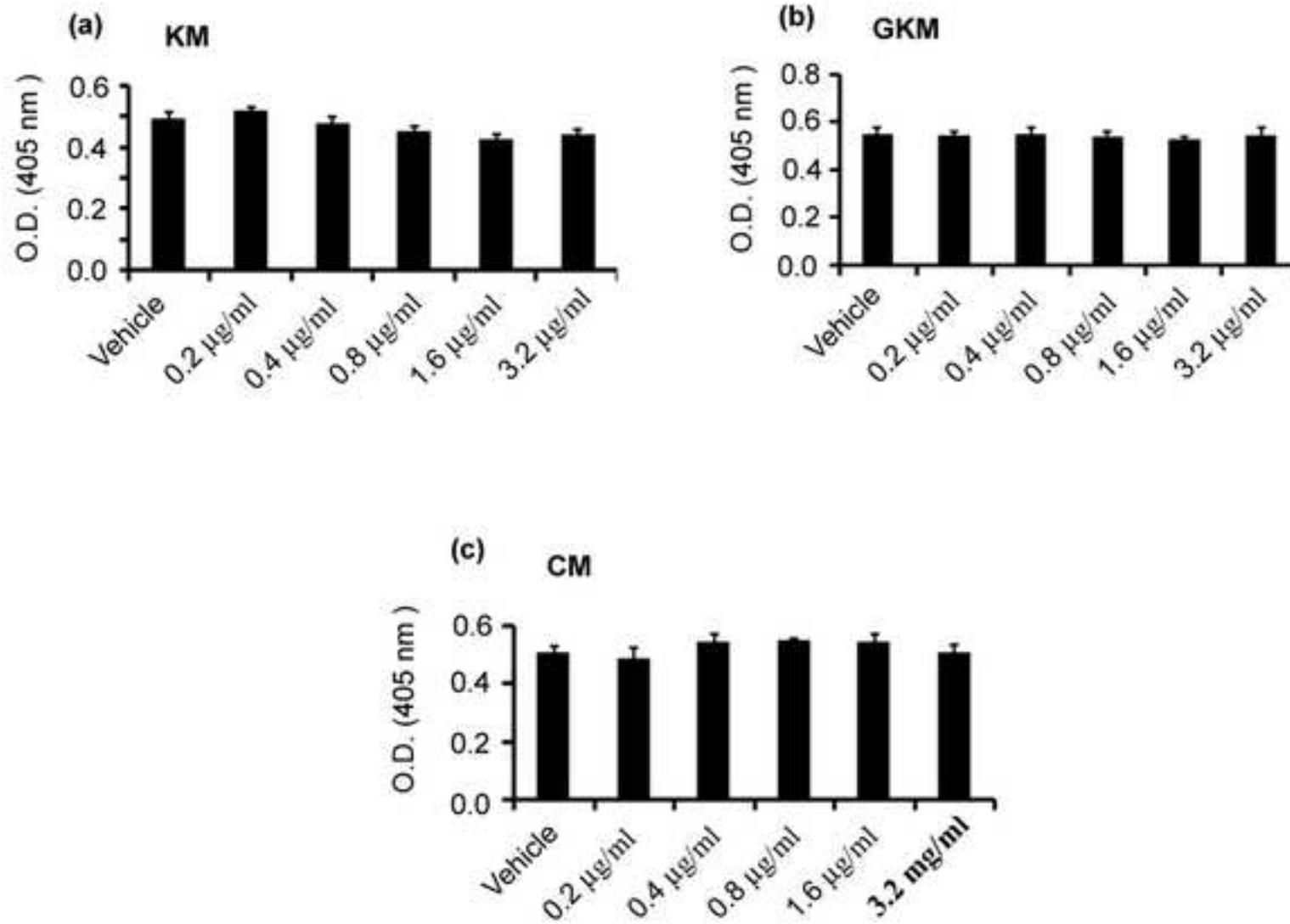
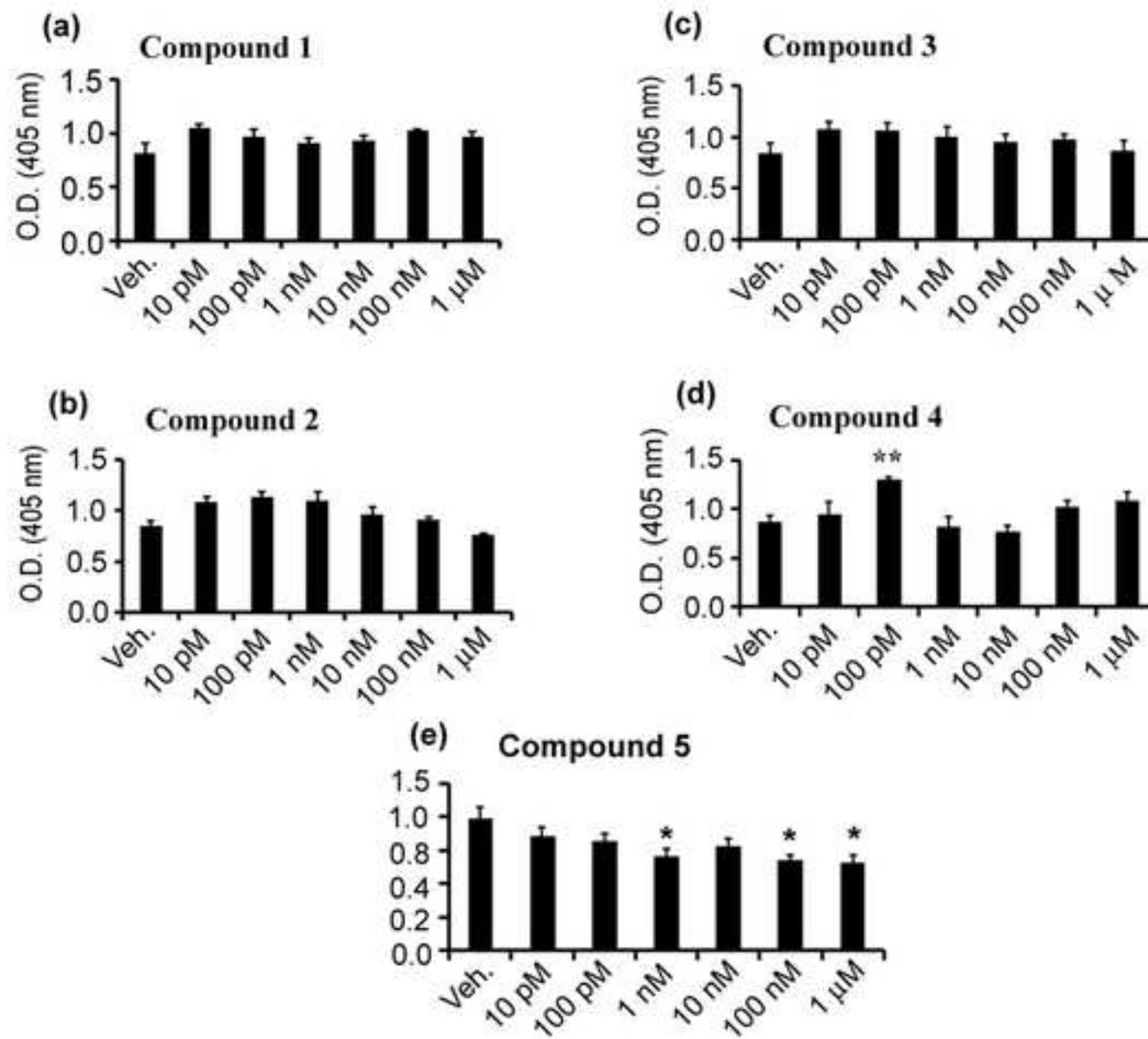


Figure S2



Legends for supplementary figures

Figure S1:Effect of extracts of mistletoes on osteoblast viability. Rat calvarial osteoblasts (2×10^3 cells) were seeded in 96-well plate and treated with increasing concentrations of KM or CM or GKM for 24 h. Viability was measured using MTT assay spectrophotometrically at 405 nm. Values are obtained from three independent experiments in the replicate of six/treatment point and expressed as mean \pm SEM.

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