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Bone mineralization enhancing activity of a methoxyellagic acid glucoside from a *Feijoa sellowiana* leaf extract

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The capability of an aqueous methanol extract obtained from the leaves of *Feijoa sellowiana* Berg. on possible prevention and treatment of osteoporosis has been examined by evaluating its stimulating effect on the two human osteoblastic cell lines HOS58 and SaOS-2. The extract was found to increase significantly the mineralization of cultivated human bone cell, whereby a clear increment (15.3 ± 2.7%) in von Kossa positive area was determined when administering 25 μg/ml leaf extract. A phytochemical investigation of the extract has demonstrated the high phenolic content and led to the isolation and identification of twenty three of them, among which the new 3-methoxyellagic acid 4-*O*-β-glucopyranoside was fully identified. All structures were elucidated on the basis of conventional analytical methods and confirmed by FTMS, 1D- and 2D-NMR data. The new compound was found to cause a significant increase of mineralized area at 20 μg/mL, while at lower concentrations the effect was not significant. However, an increase of the number of mineralized spots (nodules) at all tested concentrations of the compound was observed.

1. Introduction

Due to our interest in biological activity as well as the diverse phenolic metabolites production of the terrestrial plants (Hussein et al. 2006, 2007), we investigated the capability of an aqueous methanol leaf extract of *Feijoa. sellowiana* Berg. for possible prevention or treatment of osteoporosis by evaluating its stimulant effect on the two human osteoblastic cell lines HOS58 and SaOS-2. A phytochemical screening, including color reactions and chromatographic analysis (Harborne 1973) of this extract has shown it to contain mainly phenolic compounds. A comprehensive analysis of the constitutive phenolics of the plant leaves has therefore been undertaken. Twenty three compounds (**1**–**23**), including the new natural product 3-methoxy ellagic acid 4-β-*O*-$^4C_1$-glucopyranoside (**14**) have thus been isolated and identified. All structures were confirmed by FTMS and NMR analysis. The effect of the new compound on *in vitro* mineralization of SaOS-2 human osteosarcoma cells was investigated as well.

Feijoa sellowiana Berg, synonym: *Acca sellowiana* (Berg) Burret, known in English as Brazilian guava, fig guava, guavasteen or horn of plenty, is one of the Myrtaceous species which are known as potential sources of bioactive phenolics (Hussein et al. 2002; 2006, 2007). The plant is a very branchy shrub or small tree, 3–6 m in height with cylindrical trunk and pale grey bark and branches. Its leaves are evergreen. They are thick and leathery, 2–6 cm long and 1–3 cm wide. The flowers are conspicuous with 4 cm wide, bisexual, borne singly or in cluster. The fruits are 5–8 cm long, 3–7 cm in diameter; variable in shape from round to elongated pear shape with dull blue-green to blue or grayish green, sometimes with a red or orange bluish waxy skin (Baily 1958). They are widely consumed because of their characteristic flavor and aroma, which are similar to pineapple (Kolesnik 1992). Extracts of *F. sellowiana* fruits were previously reported to exhibit high antibacterial, antioxidant and cytotoxic activities (Vuotto et al. 2001), strong effects on human phagocytes functions and suppressing effects on cytokine secretion by intestinal epithelium (Manabe and Isobe 2005) while the peel extracts were found to possess, besides their antibacterial activity, an anti-cancer effect on solid and hematological cancer cells (Vuotto et al. 2001; Bontempo et al. 2007). However, the biological activity of the leaves of the plant was poorly examined, albeit they were reported to possess antibacterial activity (Vuotto et al. 2001).

Only one previous phytochemical investigation of *F. sellowiana*, among some other Myrtaceous species, has been reported (Okuda et al. 1982). This investigation was based on HPLC and TLC screening and demonstrated the detection of eight ellagitannins in the leaf extract of *F. sellowiana.* However, it should be mentioned that in a previous report (Khallouki et al. 2007) an isolated compound from the root bark of *Anisophyllea dichostyla* was tentatively identified on the basis of UV spectra, HPLC-ESI-MS and

nano-ESI-MS–MS only, to be 3-methoxy ellagic acid 4-*O*-β-D-glucopyranoside. It is quite obvious that the analytical techniques used in this report could not led alone to the concluded structure, which essentially needs a comprehensive NMR analysis for the determination of the precise structural details, including substituent positions, glycoside configuration, conformation and site of attachment to the aglycone.

2. Investigations, results and discussion

2.1. Influence on human osteoblastic cell cultures

Osteoblastic-mediated bone formation can be divided into three phases: proliferation, matrix maturation and mineralization. We estimated cell vitality in the MTT assay as parameter for the proliferation phase, protein content and ALP activity as indicators for matrix maturation and finally mineralization of the extracellular matrix. In order to increase the validity of our results and to hinder false-positive data, we used two different cell lines.

2.1.1. Cellular protein content and alkaline phosphatase activity of HOS58 cells

The extract reduces the cell proliferation and protein production of HOS58 human osteosarcoma cells with an IC_{50} of 112 μg/ml. There was a transient increase in total cellular protein at doses between 3.1 and 25 μg/ml. Due to lacking cytotoxicity at this concentration, this can be considered as stimulation of cell maturation (Fig. 1). At higher concentration (>50 μg/ml), protein production drops.
Alkaline phosphatase activity (ALP) of HOS58 cells was reduced dose dependently within the non-toxic concentration range tested (3.1–25 μg/ml) to values between 75% and 90% of vehicle treated control (Fig. 2). This supports the proposed triggering of cell maturation by *F. sellowania* in respect to an increased formation of extracellular protein matrix (ECM). This matrix will give support to the subconsequent mineralization. The enzyme alkaline phosphatase plays an important but yet undefined role in the mineralization process and its abundance varies strongly with cell activation. It is usually down regulated when ECM is produced (as it is not necessary for this process). The model substance β-glycerophosphate (2 mM) was used as positive control and led to significant increase of ALP activity within the usual range, thus indicating quite normal cell behavior.

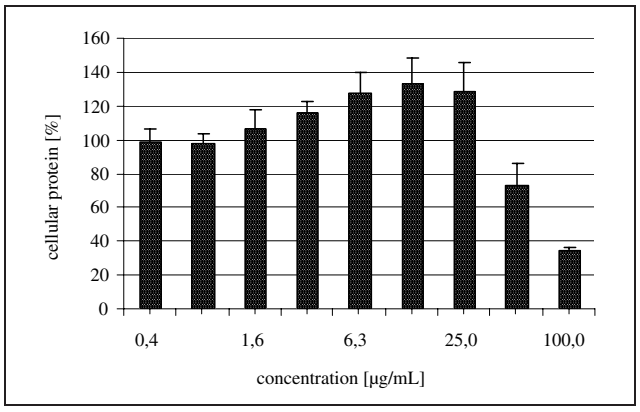


Fig. 1: Total cellular protein of HOS58 human osteosarcoma cells under influence of *Feijoa sellowania* leaf extract. Bars represent mean of 3 experiments plus standard deviation. 100% = control cells treated with vehicle

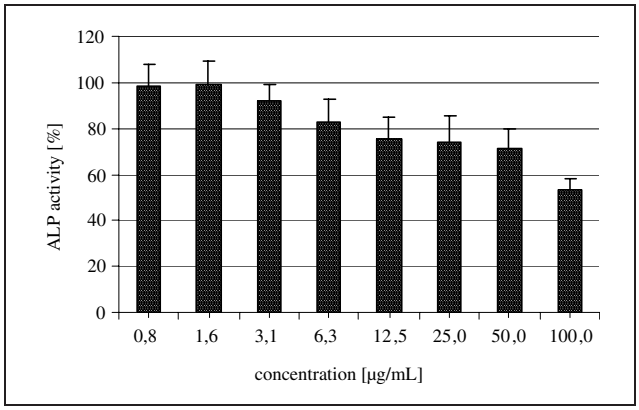


Fig. 2: Alkaline Phosphatase activity of HOS58 human osteosarcoma cells under influence of *Feijoa sellowania* leaf extract. Bars represent mean of 3 experiments plus standard deviation. 100% = control cells treated with vehicle

2.1.2. Mineralization of SaOS-2 cells

2.1.2.1. Enhancing activity of the crude extract

The aqueous methanolic leaf extract of *F. sellowiana* possesses a stimulating effect on the mineralization of SaOS-2 cells. There was a strongly significant increase (15.3 ± 2.7%) in von Kossa positive area (von Kossa 1901) when 25 μg/ml *F. sellowiana* leaf extract were administered (Fig. 3). 5 μg/ml leaf extract did alter von Kossa positive cell mineralization only slightly but significantly to 10.7 ± 2.6% indicating a dose dependent activity. β-Glycerophosphate (2 mM) serves as trigger for mineralization and facilitates SaOS-2 cells to mineralize (9.8 ± 3.1% von Kossa positive area). Unstimulated cells do not show a positive von Kossa staining indicating a lacking calcium deposition.

2.1.2.2. Enhancing activity of 3-methoxyellagic acid 4-*O*-glucopyranoside

Only mineralization with 20 μg/mL of the new compound is significantly higher than control (bGP) with p-values far below 0.001 (SPSS: Dunnetts t-test, LSD, Bonferroni and Tukey's post hoc ANOVA tests) albeit the average of von Kossa positive area at each concentration is higher than control (Fig. 4). There is a slight hint that 5 μg/mL is dif-

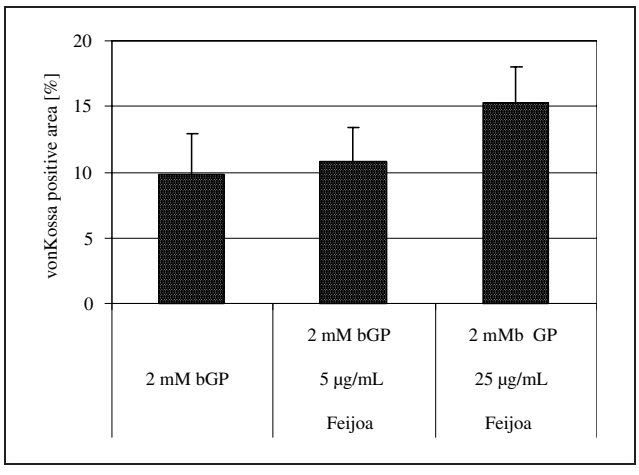


Fig. 3: Mineralization of SaOS-2 cell in vitro under influence of *Feijoa sellowania* leaf extract. Bars represents mean of 2 experiments with 20 samples each plus standard deviation. bGP = β-glycerophosphate

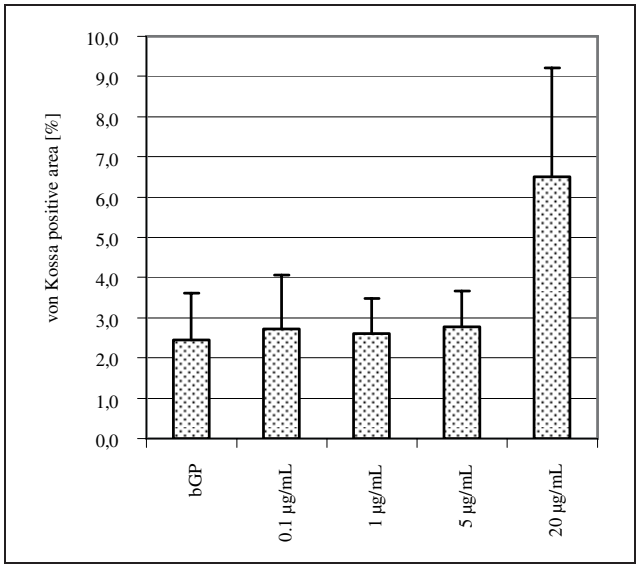


Fig. 4: percent of von Kossa positive area under the effect of different concentrations of the new compound

ferent as well, LSD indicates a p value of only 0.13 but this is not significant ($p = 0.05$). The number of von Kossa positive spots was determined (Fig. 5) to prove an increase of the number of these spots (nodules) at all tested concentrations. This can be taken as evidence for increased cell maturation and osteoblasticity.

In conclusion, the positive effects on the cultivated bone cells indicate that the leaves of *F. sellowiana* or their extract could be candidates for a development of a phytotherapeutic agent for prophylaxis and/or treatment of the symptoms of osteoporosis. The noticed increase of the mineralized area at high concentrations and the increased number of mineralized spots at all tested concentrations of 3-methoxyellagic acid 4-*O*-glucopyranoside leads to the suggestion that this compound is contributing to the significant stimulating effect seen. Still, regarding the strong triggering of mineralization by the crude extract compared to the moderate effect of the tested ellagic acid derivative,

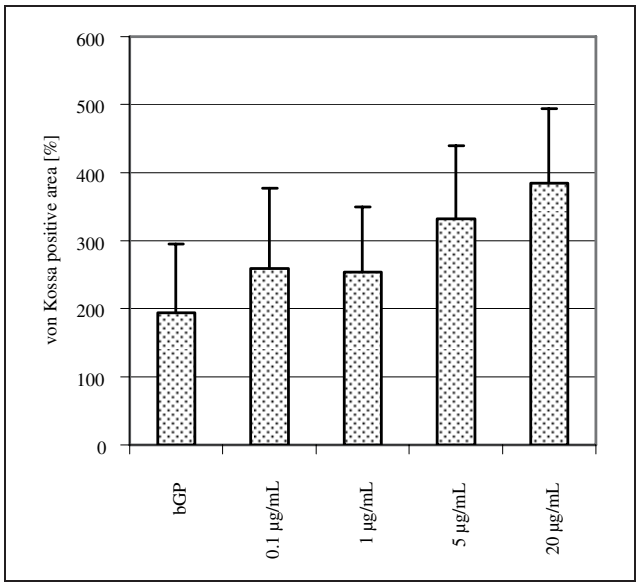


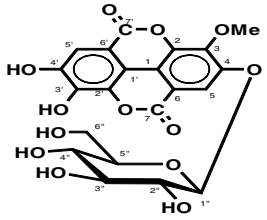
Fig. 5: Number of von Kossa positive spots under influence of the new compound. All Bars are significantly different from control (bGP) as assessed by Dunnetts t-test, LSD, Bonferroni and Tukey's post hoc ANOVA tests. P-values are at least below 0.003. SPSS was used.

other yet undetermined compounds will add their share to the total effect on mineralization. This question must be left for future work to be clarified.

2.2. Isolation and structure elucidation

Following column chromatographic fractionation of the *F. sellowiana* leaf extract, 23 compounds (**1**–**23**) were isolated. Conventional and spectral analysis mainly by NMR spectroscopy and by mass spectrometry indicated that one of these compounds (**14**) is a new natural product.

Compound **14** was obtained as a white amorphous powder. The molecular ion peak at m/z 477.209 $[M-H]^-$ (calc.: 477.217) observed by FTMS and the ^1H, ^{13}C and DEPT NMR data suggested the molecular formula $C_{21}H_{18}O_{13}$. The characteristic chromatographic properties (weak mauve spot on PC under UV light) and UV absorption maxima in methanol suggested that **14** is an ellagic acid derivative. The pronounced red shift of the absorption maxima at 249 and 273 (shoulder) nm of the aromatic chromophors in the molecule of **14** observed on addition of NaOAc + H_3BO_3 to 254, 315 (shoulder) nm, might be attributed to the presence of free di-ortho hydroxyl groups in the aromatic ring(s). Normal acid hydrolysis of **14** yielded glucose (comparative paper chromatography, CoPC), and compound **14a**. The latter was also released on incubating **14** at 37 °C for 24 h, together with β-glucosidase enzyme (Ec 3.2.1.21, from Almonds, chromatographically pure, salt free, lypholized powder, Sigma). Compound **14a** was extracted by EtOAc from the 2 N acidic hydrolysate. It exhibited a molecular weight of 316 as established by EI-MS ($[M]^+$ at m/z = 316), corresponding to a molecular formula of $C_{15}H_8O_8$. Chromatographic properties (yellowish buff spot on PC under UV light) and UV absorption maxima, together with the EI-MS data suggested that **14a** is a monomethoxy-ellagic acid. Comparison of the 1D NMR data of **14a** with those of free ellagic acid (Nawwar et al. 1994), and of 3,3′-dimethoxyellagic acid (Nawwar et al. 1982) indicated that the methoxyl function in **14a** is attached at C-3. Analysis of the ^{13}C spectrum of **14a** (Table 1) was aided by comparison with the ^{13}C data reported for 3,3′-dimethoxyellagic acid (Sato, 1987) and for ellagic acid (Nawwar et al. 1994) as well. These data confirmed the structure of **14a** to be 3-methoxyellagic acid. Consequently, the parent compound **14** is 3-methoxyellagic acid mono-*O*-β-glucopyranoside. Comparison of the 1D NMR data proved that **14** contained a glucoside moiety which revealed its anomeric proton as a doublet at δ ppm 5.2 (J = 7 Hz). This finding, together with the result of hydrolysis with β-glucosidase enzyme proved the β-configuration of the existing glucose moiety. The conformation of the glucose moiety is, therefore $^4C_1$. This follows from the β-configuration just discussed (Nawwar et al. 1984). In the ^{13}C NMR spectrum, the β-configuration was further confirmed by the δ values of the recorded sugar resonances (Table 1, Kalinowski et al. 1984). In the HMBC spectrum



Compound **14**: 3-methoxyellagic acid 4-*O*-β-D-$^1C_4$-glucopyranoside.

Table 1: NMR spectral data of 3-methoxyellagic acid 4-*O*-β-$^4C_1$-glucopyranoside 14 and 3-methoxyellagic acid 14a

	14			**14a**	
	δ_H (*J*, Hz)	δ_C	HMBC$^+$	δ_H (*J*, Hz)	δ_C
1		114.37 s			112.36
2		137.7 s			141.98
3		142.93 s			140.64
4		150.0 s			148.63
5	**7.52 s**	111.47 d (168 Hz)	1, 3, 7, 4_{weak}	7.47 s	111.84
6		111.13 s			112.66
7		158.54 s			159.40
1′		112.8 s			112.98
2′		135.94 s			136.63
3′		141.3 s			140.21
4′		152.5 s			152.63
5′	7.44 s	111.35 d (165 Hz)	1′, 3′, 7′, $4'_{weak}$	7.42 s	110.83
6′		106.8 s			107.85
7′		158.5 s			159.30
OMe	3.99 s	60.84 q (146 Hz)	3	4.00 s	61.45
1″	5.2 (d, J = 7 Hz)	101.1 d (160 Hz)	4		
2″		73.99 d (142 Hz)			
3″		77.91 d (144 Hz)			
4″		70.03 d (146 Hz)			
5″		77.27 d (142 Hz)			
6″		61.29 t (141 Hz)			

of **14**, a ^{3}J correlation of the anomeric glucose proton H-1″ (δ 5.2) to the aromatic carbon C-4 (δ 150) allowed positioning of this moiety at this carbon. The recognizable ^{2}J correlation of the downfield aromatic proton (δ 7.52) to the same C-4 carbon was in accordance with this conclusion. Correlations of the methoxyl protons (δ 3.99) to C-3 (δ 142.93) and of the same downfield aromatic proton (δ 7.52) to the same C-3 carbon confirmed that the site of attachment of the glucoside moiety is at the C-4 position of the methoxy ellagic acid moiety. Furthermore, the measured shift values of the carbon resonances of the glucose moiety confirmed that the sugar core exists in the pyranose form (Kalinowski et al. 1984). The complete structure of compound **14** was, therefore determined to be 3-methoxyellagic acid 4-*O*-β-D-$^1C_4$-glucopyranoside.

3. Experimental

3.1. Instruments and materials

Optical rotations were obtained by a Kruess P 8000 digital polarimeter. ^{1}H NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d_6 and converted to TMS scale by adding 39.5. Typical conditions: spectral width = 8 kHz for ^{1}H and 30 kHz for ^{13}C, 64 K data points and a flip angle of 45. FTMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität zu Berlin). UV recording was made on a Shimadzu UV-Visible-1601 spectrophotometer. Paper chromatographic analysis was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6% HOAc; (3) BAW (n-BuOH−HOAc−H_2O, 4 : 1 : 5, upper layer). Solvent 3 was used for preparative paper chromatography (PPC).

3.2. Plant material

The leaves of *F. sellowiana* Berg. were collected from El-Orman Botanical garden, Cairo, in May 2006 and identified by Dr. M. El Gebali, National Research Centre (NRC), Cairo, Egypt. A specimen of the plant (NRC 633) was deposited at the NRC herbarium.

3.3. Extraction and isolation

The fresh *F. sellowiana* leaves (5 kg) were homogenized in a MeOH−H_2O (3 : 1) mixture (three extractions each with 5 l). The dried filtrate (200 g) of the homogenate was applied to a polyamide 6s (1500 g) (Riedel-De-Haen Ag, Seelze Hannover, Germany) column (150 × 7.5 cm) and eluted with H_2O followed by H_2O−MeOH mixtures of decreasing polarities to yield 10 individual fractions (I–X) which were separately dried in vacuum, and subjected to two dimensional paper chromatographic investigation (2DPC). Compounds **1** (145 mg) and **2** (125 mg) were isolated pure from fraction II (eluted with 20%) by column fractionation (CF) of 3 g material over 25 g Sephadex LH-20 using H_2O for elution. Compounds **3** (88 mg), **4** (102 mg) and **5** (95 mg) were individually separated from 762 mg of fraction III (eluted with 30%) by being fractionated over a Sephadex LH-20 column (150 g) (eluted with H_2O, followed by 50% aqueous MeOH) followed by preparative paper chromatography (PPC), using BAW as solvent, while compounds **6** (75 mg), **7** (78 mg), **8** (63 mg) and **9** (111 mg) were obtained from 620 mg of fraction IV (eluted with 40%) through fractionation on a polyamide column (15 g) using the solvent system methanol: benzene: H_2O (60 : 38 : 2) as an eluent, followed by PPC, using BAW as solvent. Repeated CF of fraction V (1.1 g, eluted with 50%) on polyamide, using solvent system (60 : 38 : 2), followed by PPC, using 6% aqueous acetic acid as solvent yielded pure samples of **10** (122 mg); **11** (79 mg) and **12** (134 mg). Compound **13** (102 mg) was separated pure by being precipitated from a concentrated clear acetone solution of fraction VI (935 mg), (eluted with 60%) by ether (thrice), while compound **14** (87 mg) was isolated pure from fraction VII (1113 mg), (eluted with 70%) by Sephadex LH-20 (10 g) column fractionation, using mixture of H_2O/MeOH for elution. Compounds **15, 16** and **17** were individually isolated from 9.42 g of fraction VIII (eluted with 80%) through acetone/ether precipitation followed by column fractionation of the precipitate (3.67 g) on a Sephadex LH-20 (35 g) column (eluted with EtOH followed by the subsequent gradual addition of acetone/H_2O (1 : 1) mixture to the used EtOH), thus leading to the desorption of three successive subfractions (i – iii). Adsorption column chromatography over polyamide (25 g) of the dried material (265 mg) of subfraction i, using H_2O/EtOH mixtures of decreasing polarities led to the separation of a pure sample of compound **15** (87 mg)**.** Repeated precipitation of the material of subfraction ii (1.2 g) from acetone by ether (thrice) afforded pure sample (107 mg) of compound **16**. Repeated precipitation of the material of subfraction iii (1.3 g) from MeOH by ether (thrice) followed by Sephadex LH-20 (15 g) column fractionation of the precipitate, using n-butanol/isopropanol/H_2O (4 : 1 : 5) as an eluent yielded pure sample (112 mg) of compound **17.** Application of PPC on the material of fraction IX (298 mg) using BAW as solvent led to the separation of three pure samples of compounds **18** (41 mg), **19** (28 mg) and **20** (19 mg). Polyamide (16 g) column fractionation of fraction X (1.53), using EtOAc water saturated for elution afforded individual pure samples of compounds **21** (114 mg), **22** (143 mg) and **23** (160 mg).

3.4. Investigations on human osteoblastic cell cultures

3.4.1. Cell culture

Human osteosarcoma cells HOS58 (H. Siggelkow, Heidelberg, D), and SaOS-2 (DSMZ, Braunschweig, D) were grown as monolayer in Iscove's modified Dulbeccos medium (IMDM; Lonza, Verviers, BE) with 10% fetal

bovine serum (Sigma, Deisenhofen, D), 2 mM L-glutamine, and 1% penicillin – streptomycin solution (penicillin 10000 IE/ml, streptomycin 10000 µg/ml, both Biochrom, Berlin, D). Both cell lines were grown at 37 °C in 95% air humidity and 5% CO_2, and subcultured routinely. All cell culture plastics were provided by TPP (Biochrom, Berlin, D).

3.4.2. Cell maturation assay

The cell maturation assay was used to determine the osteoblasticity of osteoblastic HOS58 cells. Cells were grown to 90% confluence in 96-well plates for 48 h. After washing with Hank's buffered saline (HBSS, Sigma, Deisenhofen, D) twice, medium was changed to IMDM without phenol red supplemented with 0.05% bovine serum albumin (Sigma, Deisenhofen, D), 2 mM L-glutamine, and 1% antibiotics (assay medium). Different concentrations of *Feijoa* extract in assay medium were prepared using a stock solution (10 mg/ml DMSO) and serial dilution with medium. Final DMSO concentration did not exceed 0.5%. Cells were then incubated for 5 days. At the end of incubation time, cells were washed twice with HBSS; lysed by 0.1% Triton X-100 in TRIS buffer and lysate was assayed for protein content (Roti-Nanoquant, Carl Roth, Karlsruhe, D) and alkaline phosphatase activity (cleavage of 4-nitrophenylphosphate at basic conditions). Three independent experiments were carried out with eight replicates each and expressed as mean +SD. Statistical differences were analyzed using single side ANOVA: p-values $\leqq 0.05$ were considered significant.

3.4.3. Mineralisation assay

SaOS-2 cells were seeded into 24 well-plates (10^4 cells/well) in growth medium (see cell culture) and grown to confluence. Medium was discarded and cell maintained in assay medium (IMDM with 5% FBS only, all other additives as described) with or without *F. sellowiana* crude extract (5, 25 µg/ml) for 21 days. Medium was changed every 3 days. Mineralization was triggered by adding 2 mM β-glycerophosphate (bGP) to the medium continuously. At the end of the incubation period, cells were stained for mineral deposition by the von Kossa method (von Kossa 1901). After washing with warm HBSS cells were fixed with 5% buffered glutardialdehyde (Grade II, Sigma, Germany) for 30 min. Cell layers were washed twice with deionised water and incubated with 5% silver nitrate in water under UV radiation for 50 min. Cells were then washed 3 times with deionized water and 5% sodium thiosulphate in water was added for 2 min. After the last wash, cells were examined for stained (black) spots under microscope. For documentation and analysis cells then were photographed using a Canon EOS 20D attached to the microscope. Changes in both the number of mineralization nodules and its areas were quantified using CellExplorer 2001 software (BioSciTec, Frankfurt, D).
In case of mineralization assay each bar represents mean +SD of 20 pictures from two independent experiments.

3.5. 3-Methoxyellagic acid 4-O-β-$^4C_1$-glucopyranoside (**14**)

White amorphous powder, $[\alpha]_D^{25}$ 32.24° (c = 0.068, MeOH), R_f-values: 0.33 (H2O), 0.48 (HOAc), 0.55 (BAW). UV λ_{max} (nm) in MeOH: 249, 273sh., 335 sh., 363; in MeOH + NaOAc + H_3BO_3: 254, 315 sh., 354. HRFIMS: m/z = 477.0784 [M-H], ($C_{21}H_{18}O_{13}$). Normal acid hydrolysis gave glucose and 3-methoxyellagic acid (**14a**) (Co-PC). 3-Methoxyellagicacid (14a): R_f-values: 0.03 (H_2O), 0.10 (HOAc), 0.76 (BAW). UV λ_{max} (nm) in MeOH 251, 348 shoulder, 369; in MeOH A+ NaOAC + H_3BO_3: 257, 315 shoulder, 375. EIMS: [M] + at m/z = 316. For NMR data of 3-methoxyellagic acid 4-*O*-β-glucopyranoside (**14**) & (**14**a) see Table 1.

3.6. Known compounds

Chromatographic behavior, UV spectral, ESI-MS (negative mode), ^1H & ^{13}C NMR data were consistent with those previously reported for, gallic acid (**1**, Nawwar et al. 1982); β-glucogallin (**2**, Haddock et al. 1982); 1-*O*-galloyl-2,3-*O*-hexahydroxydiphenoyl-$^4C_1$-β-glucopyranose (**3**, Latte and Kolodziej 2000); 2,3-*O*-hexahydroxydiphenoyl-(α/β)-glucose (**4**, Tanaka et al. 1993); genkwanin 8-*C*-glucopyranose (**5**, Markham et al. 1975); epicatechin (**6**, Shen et al. 1993); catechin (**7**, Shen et al. 1993); epigallocatechin (**8**, Dawang et al. 1987); gallocatechin (**9**, Dawang et al. 1987); quercetin 3-*O*-β-galactoside (**10**, Nawwar et al. 1984); quercetin-3-*O*-α-arabinofuranoside (**11**, Hussein et al. 2003); quercetin 3-*O*-α-$^1C_4$-rhamnopyranoside (**12**, Agrawal 1989; Markham 1978); 1,3-di-*O*-galloyl-4,6-*O*-hexahydroxydiphenoyl β-glucopyranose (**13**, El Mousallamy et al. 1991); 2,3-di-*O*-galloyl-4,6-*O*-hexahydroxydiphenoyl-(α/β)-$^4C_1$-glucopyranose, tellimagrandin-I (**15**, Okuda et al. 1983); 2,3-*O*-4,6-di-hexahydroxydiphenoyl-glucose, peduncullagin (**16**, Okuda et al. 1983); 1-C-2,3-*O*-hexahydroxy-4,6-*O*-hexahydroxy diphenoyl-5-*O*-galloyl (open-chain)-glucose, casuarinin (**17**, (Okuda et al. 1983); 3,4,3′- trimethoxy ellagic acid (**18**, Khac et al. 1989); 3,3′-dimethoxy ellagic acid (**19**, Nawwar et al. 1982); ellagic acid (**20**, Nawwar et al. 1994); kaempferol (**21**, Nawwar et al. 1984); quercetin (**22**, Nawwar et al. 1984) and flavone (**23**, Bontempo et al. 2007).

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