Original Article

Investigation of phenolic leaf extract of *Heimia myrtifolia* (Lythraceae): Pharmacological properties (stimulation of mineralization of SaOS-2 osteosarcoma cells) and identification of polyphenols

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ABSTRACT: Evaluation of the activity of an aqueous alcoholic extract obtained from the leaves of *Heimia myrtifolia* (Lythraceae) by determining its stimulating effect on two human osteoblastic cell lines HOS58 and SaOS-2 indicated its potential for use in the prevention and treatment of osteoporosis. In addition, the extract was found to significantly increase the mineralization of cultivated human bone cell SaOS-2, in which a strong dose-dependent increase was observed. A phytochemical investigation of the extract also confirmed that *H. myrtifolia* is capable of synthesizing and accumulating appreciable amounts of several phenolics, thus leading to the isolation and characterization of sixteen of these constituents. Identified among these isolates were a new natural product, 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose, and a rare natural product (this marks its second report), 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-*O*-methyl ether). Structures of these isolates were fully elucidated on the basis of conventional methods of analysis and confirmed by ESI/MS and ^{1}H and ^{13}C-NMR analysis.

Keywords: *Heimia myrtifolia*, phenolics, osteoporosis, 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose

1. Introduction

Due to interest in biological activity as well as in the diverse phenolic metabolite production of terrestrial plants (*1-3*), the current authors investigated an aqueous alcoholic leaf extract of *Heimia myrtifolia* Cham. for its potential use in the prevention or treatment of osteoporosis by evaluating its stimulant effect on two human osteoblastic cell lines HOS58 and SaOS-2. Phytochemical screening, including color reactions and chromatographic analysis of this extract, has shown that it contains mainly phenolic compounds (*4*).

A comprehensive analysis of the constitutive phenolics of the plant leave extract was therefore undertaken. Sixteen compounds (compounds **1-16**), including a new natural product, 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose (compound **7**), and a rare natural product (this is its second report in nature), 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-*O*-methyl ether) (compound **9**), were subsequently isolated and identified. Structures were confirmed by electrospray ionization/mass spectrometry (ESI/MS) and nuclear magnetic resonance (NMR) analysis.

H. myrtifolia Cham., known as sun opener or shrubby yellow crest, is one of the Lythraceous species that is native to South America, ranging from Brazil to Uruguay, where it is common along the sides of streams. It is a deciduous shrub growing up to 1 m tall. The yellow flowers are 5 petaled and 1 cm in diameter. The leaves are approximately 2-5 cm long and 1 cm wide and are variably arranged in alternate, opposite, or whorled fashion on the stem. *H. myrtifolia* flowers from August to September. The flowers are hermaphrodite (*5*).

Extracts from *H. myrtifolia* that contain Lythrine and Lythridine alkaloids exhibit diuretic activity (*6*). Strong anti-inflammatory activity of *H. myrtifolia* originates from an alkaloidal fraction containing vertine (Cryogenine) (*7*), while 70% acetone extract of *H. myrtifolia* has a strong cytotoxic effect on human promyelocytic leukemia cells (HL-60) (*8*).

Three biphenylquinolizidine lactone alkaloids were isolated from *H. myrtifolia*, namely Vertine (Cryogenine), Lythrine, and Lythridine (*9,10*). The fatty acid composition of seed lipids consists of linoleic

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acid (18:2) as the primary or dominant fatty acid of *H. myrtifolia*, while palmitic acid (16:0) is the only secondary component, constituting 10% or more of the total fatty acid composition (*11*). Nothing in literature indicated the phenolic content or bone mineralization activity of *H. myrtifolia* Cham. and Schl., thus leading the present study to investigate the aqueous alcoholic extract in depth for its phenolic content and its activity in preventing or treating osteoporosis.

2. Materials and Methods

2.1. *Instruments and materials for phytochemical investigation*

^{1}H-NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer (JEOL, Tokyo, Japan) at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C-NMR chemical shifts to dimethyl sulfoxide (DMSO)-d_6 and converted to the tetramethylsilane (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.

ESI/MS spectra were measured on a Finnigan LTQ-ESI/MS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität zu Berlin). UV recording was done on a Shimadzu UV-Visible-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

Paper chromatographic analysis was carried out on Whatman No. 1 paper (Whatman, Kent, UK) using solvent systems: 1) H_2O; 2) 6% AcOH; and 3) BAW (*n*-BuOH/AcOH/H_2O, 4:1:5, v/v, upper layer). Solvents 2 and 3 were also used for preparative paper chromatography (PPC).

2.2. *Plant material*

The leaves of *H. myrtifolia* Cham. were collected from El-Orman Botanical Garden, Cairo, Egypt, in March 2007 and identified by Mrs. Tereize Labib, Agricultural Engineer, El-Orman Botanical Garden, Giza, Egypt. Vouchered specimens of the authenticated plant were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt.

2.3. *Extraction, isolation, and purification of phenolics from H. myrtifolia*

The air dried plant material (2 kg) was extracted with 70% ethanol. The aqueous alcohol extract was filtered and evaporated *in vacuo* at ~ 45ºC until dry to yield 90 g of a sticky dark brown material. The dried residue was applied to a polyamide 6s column (250 g, 125 cm L × 5 cm D) (Riedel-de Haen AG, Seelze-Hannover, Germany) and eluted with H_2O followed by H_2O/MeOH mixtures of decreasing polarities to yield 36 individual fractions (2 L each) that were examined separately under UV light. Similar fractions were pooled to yield eleven main fractions (I-XI) that were separately dried in a vacuum and subjected to two-dimensional paper chromatographic investigation (2D-PC). Compounds **1-7** were individually isolated from 5.6 g of fraction III (eluted with 20% MeOH), where compound **1** (250 mg) was crystallized from the concentrated fraction and then purified by application to a Sephadex LH-20 column (10 g; GE Healthcare Bio-Sciences, Uppsala, Sweden) eluted with MeOH, while other compounds were separated through column fractionation on a Sephadex LH-20 column (30 g) (eluted with distilled water 100% followed by pure MeOH 100%). This led to the desorption of five successive sub-fractions (III-[i-v]). Sub-fraction III-iv (2.8 g) was fractionated over a Sephadex LH-20 column (20 g) using *n*-butanol saturated with water, leading to the desorption of five successive sub-fractions (III-iv-[ib-vb]). PPC using BAW as a solvent of the dried material (700 mg) of sub-fractions (III-iv-iib) led to the separation of pure samples of compounds **2** (90 mg), **3** (82 mg), and **4** (96 mg), while PPC using BAW as an eluent of the dried material (570 mg) of sub-fractions (III-iv-ivb) yielded the pure compounds **5** (137 mg), **6** (112 mg), and **7** (136 mg). Compounds **8** (1.4 g), and **9** (150 mg) were individually separated from 5.5 g of fraction VI (eluted with 60% MeOH) through initial crystallization of a yellow amorphous powder (1.7 g) from the concentrated fraction followed by purification over a Sephadex LH-20 column (20 g) using *n*-butanol saturated with water as a solvent. Compound **10** (180 mg) was separated from Fraction IX (2.2 g, eluted with 80% MeOH) by purification of (500 mg) using column chromatography with a Sephadex LH-20 column and MeOH as an eluent.

PPC of the material of fraction X (2.9 g, eluted with 90% MeOH) with BAW as a solvent led to the separation of four pure samples of compounds **11** (95 mg), **12** (125 mg), **13** (89 mg), and **14** (119 mg). Polyamide 6s (25 g) column fractionation (1 g) of fraction XI (6.5 g, eluted with 100% MeOH) using EtOAc saturated with water for elution afforded individual pure samples of compounds **15** (570 mg) and **16** (280 mg).

2.4. *Spectral data for the new natural product 1,6-di-O-dehydrotrigalloyl-β-D-$^{4}C_1$-glucopyranose (compound 7)*

The new natural product was a white amorphous powder (136 mg), R_f values (×100): 50 (6% AcOH), 28 (BAW). UV λ_{max} (nm) in MeOH: 273 nm. UV λ_{max} (nm) in MeOH after acid hydrolysis: 273 nm. Negative ESI/MS indicated a molecular ion at $[M-H]^-$, m/z 1,155, which corresponded to a molecular mass (M_r) of 1,156. ^{1}H-NMR (DMSO-d_6) δ (ppm): 7.10 and 6.99 (1H, *d*, *J* = 2.5 Hz, H-2 and H-2'), 6.96 (2H, *s*, H-8 and H-8'), 6.42

and 6.40 (1H, *d*, *J* = 2.5 Hz, H-8b and H-8b'), 6.53 and 6.54 (1H, *s*, H-14 and H-14'), 4.70 (1H, *d*, *J* = 8 Hz, H-1''), 4.40 (1H, *d*, *J* = 12 Hz, Ha-6''), 4.22 (1H, *dd*, *J* = 3.5, 12 Hz, Hb-6''), 3.1-3.8 (*m*, sugar protons) (see the structural formula).

^{13}C-NMR (DMSO-d_6) δ (ppm): 119.95 (C-1 and C-1'), 168.16 (C-1b and C-1b'), 102.79 and 102.93 (C-2 and C-2'), 148.59 (C-3 and C-3'), 138.95 (C-4 and C-4'), 151.07 (C-4a and C-4a'), 138.90 (C-4b and C-4b'), 140.02 (C-5 and C-5'), 140.00 (C-6 and C-6'), 146.05 (C-7 and C-7'), 108.20 (C-8 and C-8'), 115.68 and 115.80 (C-8a and C-8a'), 110.70 and 110.80 (C-8b and C-8b'), 166.27 (C-8c, C-8c', C-9b and C-9b'), 114.74 (C-9 and C-9'), 136.89 (C-10 and C-10'), 141.90 (C-11 and C-11'), 141.92 (C-12 and C-12'), 148.31 (C-13 and C-13'), 109.16 (C-14 and C-14'), 94.53 (C-1''), 74.29 (C-2''), 76.82 (C-3''), 70.33 (C-4''), 73.76 (C-5''), 66.24 (C-6'').

2.5. *Spectral data for the rarely reported natural product 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-O-methyl ether) (compound **9**)*

The rarely reported natural product was a yellow amorphous powder (150 mg), R_f values (×100): 6 (6% AcOH), 48 (BAW). UV λ_{max} (nm) in MeOH: 290, 324 sh; ^{1}H-NMR (DMSO-d_6) δ (ppm): 4.99 (1H, *d*, *J* = 11 Hz, H-2), 4.6 (1H, *d*, *J* = 11 Hz, H-3), 5.96 (1H, *d*, *J* = 2.5 Hz, H-6), 6.10 (1H, *d*, *J* = 2.5 Hz, H-8), 7.47 (2H, *d*, *J* = 8.5 Hz, H-2' and H-6'), 7.05 (2H, *d*, *J* = 8.5 Hz, H-3' and H-5'), 4.04 (3H, *s*, OMe). UV spectral data and ^{1}H-NMR data for compound **9** were identical to those reported in the literature (*12*).

2.6. *Chemicals and biochemicals for biochemical assays*

Cell culture plastics, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine, trypsin, and antibiotics were purchased from Biochrom KG (Berlin, Germany). Bovine serum albumin (BSA; fraction V) and Iscove's modification of Dulbecco's medium (IMDM), with or without phenol red, were purchased from Invitrogen (Karlsruhe, Germany). All other reagents were obtained from Sigma (Deisenhofen, Germany). HOS58 cells were obtained from H. Siggelkow (Heidelberg, Germany). SaOS-2 cells were purchased from DSZM (Braunschweig, Germany). Other chemicals used were of analytical grade. All cell culture plastics were provided by Biochrom KG (Berlin, Germany).

2.7. *Cell culture*

Human osteosarcoma cells HOS58 and SaOS-2 were grown as a monolayer in IMDM with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin solution (penicillin 10,000 IE/mL, streptomycin 10,000 µg/mL). Both cell lines were grown at 37ºC in 95% air humidity and 5% CO_2 and were routinely sub-cultured.

For assays, HOS58 and SaOS-2 cells were grown to 90% confluence in 96-well plates for 48 h. After cells were washed twice with PBS, the medium was changed to IMDM without phenol red supplemented with 0.05% BSA, 2 mM L-glutamine, and 1% antibiotics (assay medium).

Different concentrations of *Heimia* extract in assay medium were prepared using a stock solution (10 mg/mL DMSO) and serial dilution with medium. The final DMSO concentration did not exceed 0.05%. The procedure was further carried out as indicated below.

2.8. *Cell viability and cell proliferation assays (Neutral Red assay)*

The Neutral Red assay was used to measure the cell proliferation rate and cell viability. HOS58 cells (see "Cell culture") were incubated with different concentrations of *H. myrtifolia* (3.9-250 µg/mL) for 43 h. A 0.4% aqueous stock solution of Neutral Red (NR; Sigma) was prepared and an aliquot added to bring the IMDM medium to a final concentration of 50 µg/mL.

Pre-incubating NR-containing medium overnight at 37°C proved advantageous in terms of removing fine precipitate and dye crystals that formed when NR was mixed with medium. Deposition of such precipitated crystals onto the cell cultures during incubation would interfere with the assay. The NR-medium was centrifuged for 10 min at 1,500 × g before use to facilitate removal of crystals. After washing, 0.2 mL of the NR-medium was added to the wells and the plates were incubated at 37ºC in 95% air humidity and 5% CO_2 for a further 3 h, resulting in the uptake of the vital dye into viable cells.

The dye-medium was removed and the cells were washed rapidly with 1% formaldehyde-1% $CaCl_2$ to remove extraneously adhering, unincorporated dye and simultaneously promote adhesion of the cells to the substratum. The formaldehyde had to be left only briefly in contact with the cells since longer exposure would result in extraction of the dye. Removal of the formaldehyde and addition of 0.2 mL of a mixture of 1% acetic acid/50% ethanol to each well then resulted in the extraction of the NR into the solution. After 20 min the trays were placed on a microtiter plate shaker for a few seconds and the absorbance of the extracted dye was measured with a Dynatech microplate reader equipped with a 540-nm filter. Cell viability was calculated as the percent of vehicle control (*13-15*).

2.9. *Cell maturation assay*

A cell maturation assay was used to determine the osteoblastic activity of osteoblastic cells. HOS58 cells (see "Cell culture") were incubated with different concentrations of *H. myrtifolia* for 5 days and the

medium was exchanged on day 3. Cells were then lysed and the enzyme activity of alkaline phosphatase (ALP) was determined in the supernatant.

2.9.1. *Cell lysis*

Upon the conclusion of incubation, cells treated with *H. myrtifolia* extract or vehicle were washed twice with PBS and disrupted (lysed) by adding 100 µL of 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 9.8 (lysis buffer) followed by freeze/thawing and vigorous mixing. The obtained suspension was centrifuged and the supernatant (cell lysate) assayed for protein content (Roti-Nanoquant, Roth GmbH, Karlsruhe, Germany) and ALP activity (cleavage of 4-nitrophenylphosphate under basic conditions).

2.9.2. *Protein quantification*

The total cellular protein was determined using Roti-Nanoquant reagent (Roth GmbH, Karlsruhe, Germany) and a modified Bradford method in accordance with the manufacturer's instructions (*16*). Briefly, 10 µL cell lysates was diluted with PBS (1:4) in a microtiter plate. Roti-Nanoquant (200 µL) reagent was added and mixed and the OD was read out at 405 and 620 nm using a spectrophotomer (Anthos Labtec, Salzburg, Austria). The total protein content was calculated from a standard curve using BSA.

2.9.3. *ALP activity*

Cellular ALP activity was determined by the release of 4-nitrophenol (4-NP) from 4-nitrophenylphosphate (4-NPP). An aliquot of cell lysates was mixed with 0.2 M aminopropanol buffer, pH 9.8 (AMP) and 24 mM 4-NPP in AMP. After incubation at 37ºC, the reaction was stopped by 0.5 M NaOH (50 µL), and the OD was read out at 405 nm. The concentration of 4-NP was calculated utilizing a calibration curve.

Here, for ALP determination assay two independent experiments were carried out with six replicates each and results were expressed as mean ± S.D. Statistical differences were analyzed using single side ANOVA; p values < 0.05 were considered significant.

2.10. *Mineralization assay*

SaOS-2 cells were seeded into 24 well-plates (10^4 cells/well) using growth medium (see "Cell culture") and grown to 90% confluence. The medium was discarded and cells were maintained in assay medium (see "Cell culture") with or without *H. myrtifolia* crude extract (1, 5, and 25 µg/mL) for 21 days. The medium was changed every 3 days. Mineralization was triggered by continuously adding 2 mM β-glycerophosphate (bGP) to the medium, while Zn was added to other cells as a positive control. Upon the conclusion of incubation, cells were stained for mineral deposition by the arsenazo III method for measuring calcium (*17*). After cells were washed with warm PBS, they were fixed with 5% buffered glutardialdehyde (Grade II, Sigma) in cold PBS for 30 min. Cell layers were washed twice with deionized water and Arsen-Azo III dye was added. Cells were then incubated at room temperature for 2 min. After cells were mixed thoroughly, the absorbance at 650 nm was measured for the bluish-purple color formed after complex formation between Ca^{2+} and Arsen-Azo III dye, which is directly proportional to calcium deposition. In the mineralization assay, each bar represents mean ± S.D. of 1 experiment with 4 parallels.

2.11. *Statistical analysis*

For ALP determination assay, two independent experiments were carried out with six replicates each and results were expressed as mean ± S.D. Statistical differences were analyzed using one-way ANOVA; p values < 0.05 were considered significant. In the mineralization assay, each bar represents the mean ± S.D. of 1 experiment with 4 parallels.

3. Results and Discussion

Little phytochemical investigation has been conduced into the phenolic metabolites and biological activities of *H. myrtifolia*. Thus, this study describes the isolation and characterization of different phenolic compounds and the influence of the aqueous alcoholic extract of *H. myrtifolia* on the growth and maturation of human osteoblastic osteosarcoma cell lines, HOS58 and SaOS-2.

3.1. *Isolation and structure elucidation of phenolics*

Phytochemical investigation indicated the presence of complicated phenolic mixtures in the aqueous alcoholic extract of *H. myrtifolia*. Following column chromatographic fractionation of the *H. myrtifolia* leaf extract, sixteen compounds (compounds **1-16**) were isolated. Conventional and spectral analysis mainly by UV, ^{1}H-NMR, and ^{13}C-NMR spectroscopy and by ESI/MS spectrometry indicated that one of these compounds, 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose (**7**), was a new natural product (Figure 1) and that another, 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-*O*-methyl ether) (**9**), was a rare natural product (noted for the second time in the present study) that was isolated as a new natural product in 1992 (*12*) from *Prunus domestica* (family Rosaceae). Compounds **9** and **10** are the first such compounds reported from the family Lytheraceae while the compounds **1-6, 8,** and **11-16** are the first such compounds reported from the genus *Heimia*.

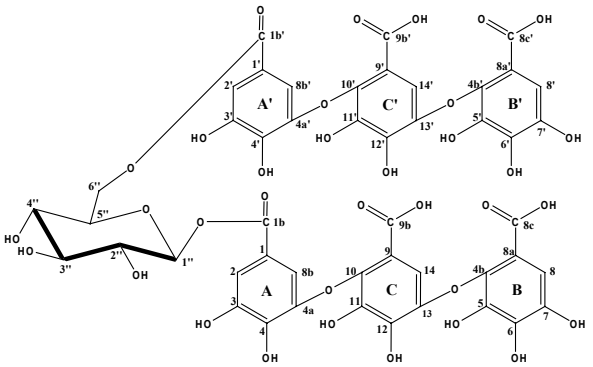

Figure 1. Compound 7: 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose. Numbering of the carbons is for convenience.

3.1.1. *Known compounds*

The present study has described the isolation and structural elucidation of sixteen phenolic compounds where chromatographic behavior, UV spectral, ESI/MS (negative mode), and ^{1}H and ^{13}C NMR data were consistent with those previously reported for apigenin-7-*O*-rutinoside (**1**) (*18-20*); protocatechuic acid (**2**) (*21*); vanillic acid (**3**) (*22*); apigenin-4'-*O*-methyl ether 7-*O*-glucoside (acacetin-7-*O*-β-glucoside) (**4**) (*23,24*); methyl gallate (**5**) (*25,26*); gallic acid (**6**) (*27-30*); apigenin-7-*O*-β-D-$^4C_1$-glucoside (**8**) (*24,31*); 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-*O*-metyhl ether) (**9**) (*12*); dehydrotrigallic acid (**10**) (*32*); 3,4,3'-trimethoxyellagic acid (**11**) (*33*); 3,3'-dimethoxyellagic acid (**12**) (*27,34*); 3-methoxyellagic acid (**13**) (*27,34*); ellagic acid (**14**) (*32*); apigenin (**15**) (*24,35*); and kaempferol (**16**) (*36,37*).

3.1.2. *The new natural product 1,6-di-O-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose (compound **7**)*

The new natural product, compound **7** (Figure 1), was obtained as a white amorphous powder that possesses gallic acid-like characteristics (intense blue color with $FeCl_3$, rosy red color with KIO_3 and UV spectral maximum in MeOH at 273 nm). Negative ESI mass spectral analysis established that compound **7** was a di-dehydrotrigalloyl glucose ($[M-H]^-$, m/z 1,155) with a M_r of 1,156, which on complete acid hydrolysis yielded dehydrotrigallic acid (Comparative paper chromatography (CoPC), UV, and ^{1}H and ^{13}C-NMR spectral analyses) (*32*) together with glucose (CoPC). Partial acid hydrolysis of compound **7** yielded, besides glucose and dehydrotrigallic acid (CoPC), an intermediate (**7a**) that was purified by preparative PC. This was found to have a M_r of 668 (negative ESI mass spectrum: $[M-H]^-$, m/z 667) and UV spectral maximum in MeOH at 273 nm, thus suggesting its structure to be a monodehydrotrigalloyl glucose (*38*).

To determine the site of attachment of the dehydrotrigalloyl moieties in the molecule of the parent compound (**7**), ^{1}H-NMR spectral analysis was then carried out. The spectrum, recorded in DMSO-d_6, revealed a characteristic doublet at δ ppm 4.70 (*d*, *J* = 8 Hz) attributable to the β-anomeric glucose protons in compound **7**. The spectrum also showed two downfield glucose proton resonances at 4.40 (*d*, *J* = 12 Hz) and 4.22 (*dd*, *J* = 3.5 Hz and 12 Hz) attributable to the two methylenic glucose protons at C-6.

In addition, two different patterns of proton signals each belonging to a dehydrotrigalloyl moiety were also noted in this spectrum at δ ppm 7.10 (*d*, *J* = 2.5 Hz); 6.99 (*d*, *J* = 2.5 Hz); 6.96 (*s*) (2H); 6.40 (*d*, *J* = 2.5 Hz); 6.42 (*d*, *J* = 2.5 Hz); 6.53 (*s*); and 6.54 (*s*). This assignment was based on comparison with the ^{1}H-NMR spectrum of free dehydrotrigallic acid, which had signals at δ ppm: 7.02 (*d*, *J* = 2.5 Hz); 6.95 (*s*); 6.48 (*d*, *J* = 2.5 Hz); and 6.42 (*s*) (*32*).

Dehydrotrigalloylation at the anomeric and the 6-positions of glucose in compound **7** was evidenced by an upfield shift of the anomeric carbon at δ ppm 94.53 in the ^{13}C NMR spectrum and the low field of methylenic proton signals in the ^{1}H NMR spectrum in comparison to the corresponding chemical shifts in free β-glucose (*39*).

The weight of evidence described above indicated that compound **7** is 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose. Final proof of its structure was then obtained through ^{13}C-NMR spectral analysis, which resulted in a spectrum containing essentially double signals for most of the dehydrotrigalloyl carbons. In this spectrum, resonances were assigned by comparing them to the ^{13}C-NMR data reported for free dehydrotrigallic acid (*32*) as well as for 1,6-di-*O*-galloyl glucose (*40,41*), β-anomeric carbon signals were readily identified from the characteristic chemical shift values at δ ppm 94.53, attributable to glucose C-1β, and at 66.24, attributable to C-6 glucose carbon. Other resonances in this spectrum exhibited chemical shift values [74.29 (C-2"), 76.82 (C-3"), 70.33 (C-4"), 73.76 (C-5")] that were in accordance with the proposed structure of compound **7**. Furthermore, the measured chemical shift values of the glucose carbon resonances proved that this moiety existed in a pyranose form (*39*), thus confirming the final structure of compound **7** to be 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose, which represents, to the extent known, a new natural product.

3.2. *Influence on human osteoblastic cell cultures*

Osteoblastic-mediated bone formation can be divided into three phases: proliferation, matrix maturation, and mineralization (*42*). Cell vitality was estimated in the Neutral Red assay (*13,14*) as a parameter for the proliferation phase, and protein content and ALP activity were determined as indicators for matrix maturation and finally mineralization of the

extracellular matrix (ECM). In order to increase the validity of the results and to eliminate false positives, two different cell lines, namely HOS58 and SaOS-2 cell lines, were used.

3.2.1. *Cell viability and cell proliferation assay (NR assay)*

Neutral red (3-amino-*m*-dimetyhlamino-2-methyl-phenazine hydrochloride)-based colorimetric assay is one of the best methods to detect mammalian cell survival and proliferation and is frequently used (*13,14*). The NR assay is based on the incorporation of the supravital dye, neutral dye, into lysosomes of viable uninjured cells after incubation of the cell culture with the extract. This weakly cationic dye penetrates cell membranes by nonionic diffusion and binds intracellularly to anionic carboxylic and/or phosphate groups of the lysosomal matrix. Xenobiotics that injure the plasma or lysosomal membrane decrease the uptake and subsequent retention of the dye. Dead cells cannot retain the dye after washing/fixation (*14,15*). After extraction from the lysosomes, the neutral dye is quantified spectrophotometrically and this amount is compared to the amount of dye extracted from control cell cultures (*13-15*). Cytotoxicity testing of *H. myrtifolia* did not indicate any reduction in cell viability (Figure 2). Even at the highest concentration tested (125 µg/mL), HOS58 cells retained their metabolic activity.

3.2.2. *Cellular protein content and ALP activity of HOS58 cells*

The extract was slightly toxic to HOS58 cells up to 125 µg/mL (Figure 2), suggesting that it was tolerated by the cell line prior to this concentration.

Total protein content assay – There was a transient increase in total cellular protein at doses between 15.6 and 62.5 µg/mL. Given a lack of cytotoxicity at this concentration, this can be considered the stimulation of cell maturation (Figure 3). At higher concentrations (> 100 µg/mL), protein production decreased.

ALP activity assay – ALP activity is commonly used as an indicator of osteoblastic cell maturation. The enzyme is considered to mark the middle stage of bone formation and generally appears during the matrix maturation phase (*43,44*). It plays an unclear, but crucial role in matrix mineralization (*44*). The level of cellular ALP activity of HOS58 human osteoblastic osteosarcoma cells was significantly reduced in a dose-dependent manner within the *H. myrtifolia* non-toxic concentration range tested (15.6 and 62.5 µg/mL) to values between 90% and 75% of vehicle treated control (4,100 and 3,200 nmol/min/mg) (Figure 4). A higher concentration (125 µg/mL) of *H. myrtifolia* resulted in an even more pronounced reduction of ALP.

This finding supports the proposed triggering of cell maturation by *H. myrtifolia* in terms of increased formation of ECM. This matrix provides support for subsequent mineralization. The enzyme ALP plays an important but yet undefined role in the mineralization

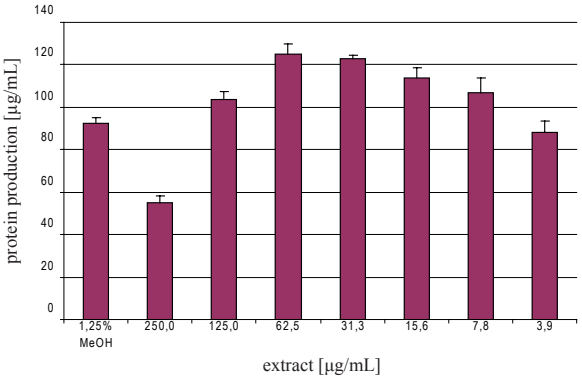

Figure 3. Total cellular protein produced by HOS58 human osteosarcoma cells under the influence of *H. myrtifolia* leaf extract. A dose-dependent increase was observed for medium doses (15.6 to 62.5 µg/mL). Two independent experiments with 6 parallels each were performed.

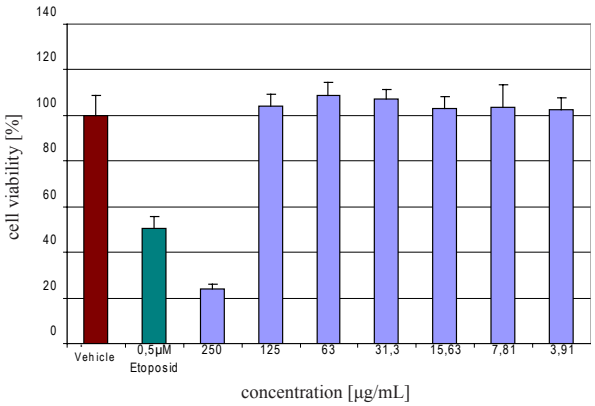

Figure 2. Cytotoxicity on HOS58 human osteosarcoma cells. No significant toxicity was observed up to 125 µg/mL, suggesting that the cells continued to be viable in the experimental setup. Two independent experiments with 6 parallels each were performed.

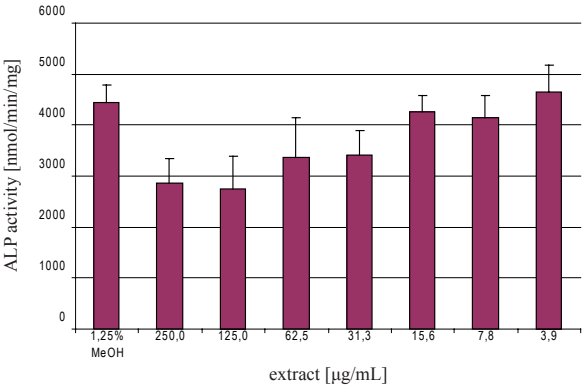

Figure 4. ALP activity of HOS58 human osteosarcoma cells under the influence of *H. myrtifolia* leaf extract. A dose-dependent decrease of enzyme activity was observed. Two independent experiments with 6 parallels each were performed**.**

process and its abundance varies considerably with cell activation. It is usually down-regulated when ECM is produced (as it is not necessary for this process). The model substance bGP (2 mM) was used as a positive control and led to a significant increase in ALP activity within the usual range, thus indicating quite normal cell behavior.

3.2.3. *Mineralization assay of SaOS-2 cells and enhanced activity of the crude extract*

To accomplish the testing of *H. myrtifolia* crude extract, the effect of *H. myrtifolia* on *in vitro* mineralization was studied using SaOS-2 cells. This delicate process is not fully understood but it requires a highly sophisticated ECM, an active ALP (at least in the beginning), and a certain concentration of inorganic phosphorus. Mineralization as the last step of bone formation unambiguously shows the bone character of cells (*43*). It is therefore used as an endpoint for *in vitro* studies of cells with an osteoblastic lineage. After 21 days of cultivation, unstimulated cells (containing no bGP) did lack calcium deposition (data not shown). The addition of bGP (2 mM) was found to be essential for the activation of the mineralization process and led to detectable calcium deposits of SaOS-2 of about 30 µg/mL (data not shown). This finding has been noted by others, and bGP is frequently used to trigger mineralization (*45*). Zn is added as a positive control as it helps in calcium deposition or aids in calcium deposition inside the matrix (*46*). The aqueous alcoholic leaf extract of *H. myrtifolia* has a stimulating effect on the mineralization of SaOS-2 cells. There was a highly significant increase (280 µg/mL) in calcium deposition or mineralization when using 25 µg/mL of *H. myrtifolia* leaf extract (Figure 5). Using 1 and 5 µg/mL of leaf extract did significantly alter positive cell mineralization to 175 and 205 µg/mL, respectively, indicating strong dose-dependent activity (Figure 5).

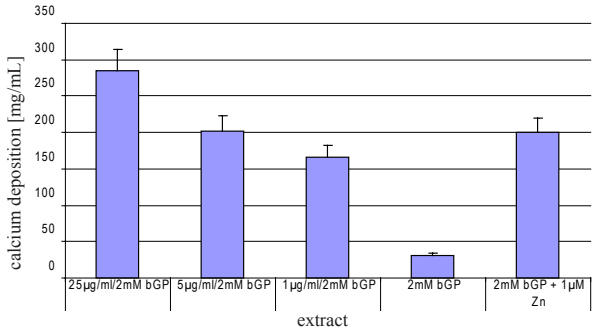

Figure 5. Mineralization (calcium deposition) of SaOS-2 human osteosarcoma cells *in vitro* under the influence of *H. myrtifolia* leaf extract. A strong dose-dependent increase was observed for *Heimia* extract. Calcium deposition was triggered by 2 mM bGP (glycerophosphate). One experiment with 4 parallels each was performed.

4. Conclusion

Sixteen compounds were isolated from the genus *Heimia* for the first time. These compounds included 5,7,4'-trihydroxy-3-methoxyflavanone (dihydrokaempferol-3-*O*-methyl ether) (compound **9**), which was recorded for the second time, and a new natural product, 1,6-di-*O*-dehydrotrigalloyl-β-D-$^4C_1$-glucopyranose (compound **7**).

Further, an aqueous alcohol extract of *H. myrtifolia* was found to stimulate mineralization and protein production of SaOS-2 human osteosarcoma osteoblastic cell cultures for the first time. Utilizing pharmacologically relevant concentrations (< 30 µg/mL), the current data thus show that the *H. myrtifolia* aqueous alcoholic extract enhances the osteogenicity of cultured bone cells. This positive effect on bone cells with comparatively low doses of *H. myrtifolia* extract is, at least partly, attributed to its high phenolic content.

In conclusion, *H. myrtifolia* extract has been found to have a bone-enhancing effect *in vitro*. Its high content of phenolic compounds, which help in calcium deposition in the bone cells matrix, may lead to the extract playing a role in the prevention of osteoporosis.

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