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# Rapid Identification of Bioactive Compounds Reducing the Production of Amyloid β-Peptide (Aβ) from South African Plants Using an Automated HPLC/SPE/HPLC Coupling System

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## Abstract

Automated HPLC/SPE/HPLC coupling experiments using the Sepbox system allowed the rapid identification of four bioactive principles reducing the production of amyloid β-peptide (Aβ) from two South African plants, *Euclea crispa* subsp. *crispa* and *Crinum macowanii.* The structures of biologically active compounds isolated from the methanol extract of *Euclea crispa* subsp. *crispa* were assigned as 3-oxo-oleanolic acid (1) and natalenone (2) based on their NMR and MS data, while lycorine (3) and hamayne (4) were isolated from the dichloromethane-methanol (1:1) extract of *Crinum macowanii.* These compounds were shown to inhibit the production of Aβ from HeLa cells stably expressing Swedish mutant form of amyloid precursor protein (APPsw).

**Key Words:** HPLC/SPE/HPLC, Alzheimer's disease, Amyloid β-peptide, *Euclea crispa* subsp. *crispa*, *Crinum macowanii*

## INTRODUCTION

As one of the most serious health problems worldwide, Alzheimer's disease (AD) is the most common form of age-related dementia (Lim *et al.*, 2006). Previously reported biochemical and genetic data have emerged to suggest that cerebral elevation, accumulation, or failure of clearance of amyloid β-peptide (Aβ) causes synapse loss and neuronal injury in the pathogenesis of AD (Walsh and Selkoe, 2007). Sequential proteolytic cleavage of amyloid precursor protein (APP) by membrane bound β-, and γ-secretases produces two major isoforms of Aβ, Aβ40 and Aβ42. More amyloidogenic Aβ42 is considered as a pathogenic agent (Iwatsubo *et al.*, 1994). Even though there was great success in the understanding of AD, preventive drug is not available yet. Successful therapeutics for prevention or treatment of AD require a potent compound that can efficiently antagonize AD-specific disease progression, *i. e.,* reducing the production of Aβ (Iwatsubo *et al.*, 1994).

Herbal natural products are increasingly utilized by the general population worldwide, and continued to be a rich source of many novel therapeutic agents (John, 2009). However, the classical bioactivity-guided fractionation for the purification and identification of biologically active compounds from plants extracts can be tedious process with long time frames (Clarkson *et al.*, 2006; Harvey, 2007). In addition, the bioassay during the bioactivity-guided fractionation may be obscured by poor solubility of samples or by fluorescent or colored substances in samples. A further complication can be synergistic activity of two constituents which may diminish or disappear upon separation (Li *et al.*, 2009). If the major target-compounds contained in crude extracts are identified in advance or earlier, the isolation efforts could be focused on truly bioactive components, avoiding repeated isolation of inactive or common constituents resulting in an increase in productivity and efficiency (Clarkson *et al.*, 2006). The automated HPLC/SPE/HPLC coupling experiments using a Sepbox system has the potential of providing the information of major target compounds by one step isolation from crude extracts. An extract can be fractionated into 100-300 substances composed of 1-4 compounds by this Sepbox system within less than 30 hours.

*Euclea* species has been used in African traditional medi-

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cine for hypnotics purpose or for alleviating toothache and headache (Joubert *et al.*, 2006). *Euclea* species contain antimicrobial naphthoquinones and pentacyclic triterpernoids (Áurea Cruz Costa *et al.*, 1976; Lall and Meyer, 2000; Schiaffella *et al.*, 2000; Joubert *et al.*, 2006). In particular, essential oils, saponins, terpenoid derivatives and alkaloids as well as flavonoids have been identified from *Euclea crispa* subsp. *crispa* (Pretorius *et al.*, 2003).

*Crinum macowanii* Baker is widespread through the tropical and temperate regions of sub-Saharan Africa, and about 20 species of the genus *Crinum* (Amaryllidaceae) are endemic to southern Africa (Nair *et al.*, 2000). *C. macowanii* has been used for the treatment of sexually-transmitted diseases and backache, or for lactation purpose in Zimbabwe and South Africa (Duri *et al.*, 1994; Nair *et al.*, 2000). The extracts of *C. macowanii* exhibited antiviral and antifungal effects against exotic RNA viruses and *Candida albicans*, respectively (Gundidza, 1986; Duri *et al.*, 1994).

In the course of screening natural resources for small molecules that possess Aβ-lowering effects *in vitro*, we examined the inhibitors produced in the root of *E. crispa* subsp. *crispa* and the bulb of *C. macowanii* on the Aβ42 formation in HeLa cells line stably expressing Swedish mutant form of APP (APPsw). During this survey, we isolated 3-oxo-oleanolic acid (1) and natalenone (2) from the methanolic extract of *E. crispa* subsp. crispa, and lycorine (3) and hamayne (4) from dichloromethane-methanol (1:1) extract of *C. macowanii* using the Sepbox system. These compounds 1-4 exhibited significant Aβ42-lowering (Fig. 1) activity in a dose-dependent manner. Here we first describe the Aβ reducing effects of compounds 1-4 and rapid separation of these bioactive compounds using the automated HPLC/SPE/HPLC technique.

## MATERIALS AND METHODS

### General experimental procedures

The automated HPLC/SPE/HPLC coupling experiments was performed by using a Sepbox 2D-250 (Sepiatec GmbH, Germany). Optical rotations were measured using a 35 polarimeter (PerkinElmer Co., USA). The NMR spectra were obtained on a Varian Unity Plus 500 MHz NMR system (Varian Co., USA). Low-resolution ESIMS data were obtained on an Agilent 1200 HPLC/MS system (Agilent Co., USA). The analytical HPLC data was obtained on a YL9100 HPLC system (Younglin, Korea) equipped with YL9160 PDA detector, ELSD ZAM 3000 detector (Schambeck SFD, GmbH, Germany) and YLClarity version 3.0.2.222 data processing software. An Eclipse XDB C18 column (4.6×150 mm, 5 μm) was used for the analysis of fractions or compounds. Semi-preparative HPLC was performed using a Gilson 321 HPLC system (Gilson Co., France) with a UV/VIS-151 detector (254 nm) and a Shodex RI-101 refractive index detector. A Phenomenex Luna C18(2) column (10×250 mm, 10 μm) was used for the purification of fractions at a flow rate 4 ml/min.

### Plant material

The roots of *Euclea crispa* subsp. *crispa* were collected in the Lebombo mountains of KwaZuluNatal in South Africa. A voucher specimen (No. PRE841058.0) was deposited and identified at the South African National Biodiversity Institute (SANBI).

The bulbs of *Crinum macowanii* were collected on the coastal area of KwaZuluNatal in South Africa. A voucher specimen (No. 679627) was deposited and identified at the South African National Biodiversity Institute (SANBI).

### Plant extraction

The roots and bulbs were separately cut into small pieces and dried in an oven at 30-60°C for 48 hours. Dried mate-

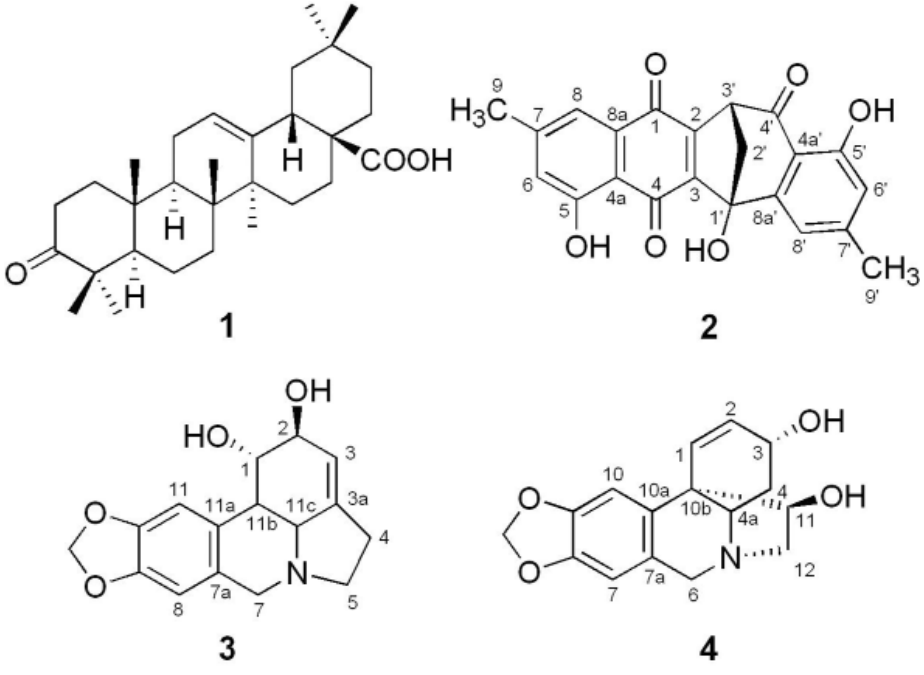


**Fig. 1.** The structures of compounds 1-4. (1) 3-oxo-oleanolic acid, (2) natalenone, (3) Lycorine, (4) Hamayne.

rial was ground to a coarse powder using a hammer mill and stored at ambient temperature prior to extraction. The powdered plant material (300 g) was extracted either with dichloromethane (DCM):methanol (MeOH) (1:1), or MeOH. After filtration, the organic extracts were concentrated using a rotary vacuum evaporator, and then further dried *in vacuo* at ambient temperature for 24 hours to give the methanol extract of *Euclea crispa* subsp. *crispa* (3.05 g) and DCM:MeOH (1:1) extract of *Crinum macowanii* (1.6 g). All extracts were stored at −20°C prior to screening.

## Purification and isolation

The methanol extract of *E. crispa* subsp. *crispa* (300 mg) was absorbed onto C4 reverse-phase resin (800 mg) and packed in a stainless steel HPLC column (10×50 mm). It was then subjected to Sepbox HPLC/SPE/HPLC coupling system equipped with UV (254 nm) and ELSD detectors using the separation conditions shown in Table 1 to obtain 98 subfractions. The subfractions SA28-30 and SA28-92 exhibited relatively significant Aβ-lowering effects (approximately 50% inhibition at 25 μg/ml). The analyses of HPLC data revealed that the SA28-30 contained two compounds and SA28-92

**Table 1.** The fractionation conditions of the methanol extract of *E. crispa* subsp. *crispa* using the Sepbox system

| 1st Separation | | Elution time | Acetonitril/water | Results |
|---|---|---|---|---|
| C4 RP HPLC column<br>20×200 mm, 10 μm, 8 ml/min | | 0-10 min<br>10-47 min<br>47-67 min | 0/100<br>0/100-100/0<br>100/0 | 15 SPE-Trap |
| **2nd Separation** | | **Elution time** | **Methanol/water including 0.05% TFA** | **Results[e]** |
| Step 1 | SPE18 (T[a] 51-54 min) | 0-30 min | 80/20-100/0 | SA28-1 to -7 |
| | Column 2.6[b] | 30-70 min | 100/0 | |
| Step 2 | SPE17 (T 48-51 min) | 0-40 min | 75/25-100/0 | SA28-8 to -22 |
| | Column 2.6 | 40-70 min | 100/0 | |
| Step 3 | SPE16 (T 44-48 min) | 0-40 min | 75/25-100/0 | SA28-23 to -38 |
| | Column 2.6 | 40-70 min | 100/0 | |
| Step 4 | SPE15 (T 41-44 min) | 0-40 min | 65/35-100/0 | SA28-39 to -54 |
| | Column 2.6 | 40-70 min | 100/0 | |
| Step 5 | SPE14 (T 39-41 min) | 0-50 min | 60/40-100/0 | SA28-55 to -68 |
| | Column 2.6 | 50-70 min | 100/0 | |
| Step 6 | SPE12 (T 37-39 min) | 0-55 min | 55/45-100/0 | SA28-69 to -83 |
| | Column 2.5[c] | 55-70 min | 100/0 | |
| Step 7 | SPE11 (T 35-37 min) | 0-55 min | 55/45-100/0 | SA28-84 to -93 |
| | Column 2.5 | 55-70 min | 100/0 | |
| Step 8 | SPE10 (T 33-35 min) | 0-55 min | 50/50-90/10 | SA28-94 |
| | Column 2.5 | 55-70 min | 100/0 | |
| Step 9 | SPE9 (T 31-33 min) | 0-55 min | 45/55-85/15 | SA28-95 |
| | Column 2.5 | 55-70 min | 100/0 | |
| Step 10 | SPE8 (T 29-31 min) | 0-55 min | 40/60-80/20 | SA28-96 |
| | Column 2.5 | 55-70 min | 100/0 | |
| Step 11 | SPE6 (T 27-29 min) | 0-55 min | 35/65-75/25 | SA28-97 |
| | Column 2.4[d] | 55-70 min | 100/0 | |
| Step 12 | SPE5 (T 25-27 min) | 0-55 min | 30/70-70/30 | SA28-98 |
| | Column 2.4 | 55-70 min | 100/0 | |
| Step 13 | SPE4 (T 23-25 min) | 0-55 min | 25/75-65/35 | No peak |
| | Column 2.4 | 55-70 min | 100/0 | |
| Step 14 | SPE3 (T 20-23 min) | 0-55 min | 20/80-60/40 | No peak |
| | Column 2.4 | 55-70 min | 100/0 | |
| Step 15 | SPE2 (T 17-20 min) | 0-55 min | 10/90-50/50 | No peak |
| | Column 2.4 | 55-70 min | 100/0 | |

[a]T: Trapping time for the eluents from 1st separation column, [b]Column 2.6: C18 RP HPLC column (10×150 mm, 5 μm, 3 ml/min), [c]Column 2.5: C18 RP HPLC column (10×250 mm, 5 μm, 4 ml/min), [d]Column 2.4: C18 Aqueous RP HPLC column (10×250 mm, 5 μm, 4 ml/min), [e]The eluents coming from 2nd separation steps were collected in 48 deep well by 6 ml using an automatic fraction collector, and the fractions were then manually re-arrayed into other 48 deep well plates through monitoring HPLC peaks from UV (254) and RI detectors.

was composed of one major compound. The SA28-30 fraction was further purified by C18 reverse phase (RP) HPLC using a refractive index detector and a Phenomenex Luna C18 (2) column (10×250 mm), eluting with a 90% methanol in water (4 ml/min) to afford betulin and compound 1 (2 mg). Compound 2 (0.5 mg) was obtained from the subfraction SA28-92 by C18 RP HPLC using a UV detector (254 nm), a Luna C18 (2) column (10×250 mm) and a methanol-water gradient solvent system (70 to 80% for 40 min, 4 ml/min).

The DCM-MeOH (1:1) extract of *C. macowanii* (300 mg) was absorbed onto C4 reverse-phase resin (800 mg) and packed in a stainless steel HPLC column (10×75 mm). It was subjected to Sepbox purification using the separation conditions shown in Table 2 to obtain 33 subfractions. Two subfractions SA20-17 and SA20-28 showed significant Aβ-lowering effects with approximately 70% inhibition at 25 μg/ml. The fraction SA20-28 contained only one substance, compound 3 (1 mg), and the fraction SA20-17 contained three substances as determined by HPLC analysis. The SA20-17 was further purified by C18 reverse phase (RP) HPLC using a UV detector (300 nm) and a Phenomenex Luna C18 (2) column (10×250 mm), eluting with a 20% methanol in water containing a 0.02% trifluoroacetic (4 ml/min) to obtain compound 3 and compound 4 (0.8 mg).

**3-oxo-oleanoic acid (1):** colorless powder. mp. 220°C; $[\alpha]_D^{20}$ +90° (c 0.5, $CHCl_3$), ESI MS *m/z* 477 $[M+Na]^+$; $^1H$ NMR (500 MHz, $CDCl_3$): δ 0.83 (3H, s, $H_3$-26), 0.92 (3H, s, $H_3$-29), 0.95 (3H, s, $H_3$-30), 1.05 (3H, s, $H_3$-24), 1.06 (3H, s, $H_3$-25), 1.09

**Table 2.** The fractionation conditions of the DCM-MeOH (1:1) extract of *C. macowanii* using the Sepbox system

| 1st Separation | | Elution time | Eluting solvents (Acetonitril/water) | Results |
|---|---|---|---|---|
| C4 RP HPLC column<br>20×200 mm, 10 μm, 8 ml/min | | 0-7 min<br>7-37 min<br>37-48 min<br>48-68 min | 0/100<br>0/100-50/50<br>50/50-100/0<br>100/0 | 17 SPE-Trap |
| **2nd Separation** | | **Elution time** | **Eluting solvents** | **Results[g]** |
| Step 1 | SPE18 (T[e] 51-54 min) | 0-20 min | Methanol/water[d]<br>100/0 | SA20-1 |
| Step 2 | SPE17 (T 48-51 min) | 0-20 min | 100/0 | SA20-2 |
| Step 3 | SPE16 (T 44-48 min) | 0-20 min | 100/0 | SA20-3 |
| Step 4 | SPE15 (T 41-44 min) | 0-10 min | 100/0 | SA20-4 |
| Step 5 | SPE14 (T 39-41 min) | 0-10 min | 100/0 | SA20-5 |
| Step 6 | SPE12 (T 37-39 min) | 0-10 min | 100/0 | SA20-6 |
| Step 7 | SPE11 (T 35-37 min) | 0-10 min | 100/0 | SA20-7 |
| Step 8 | SPE10 (T 33-35 min) | 0-10 min | 100/0 | SA20-8 |
| Step 9 | SPE9 (T 31-33 min) | 0-10 min | 100/0 | SA20-9 |
| Step 10 | SPE8 (T 29-31 min) | 0-10 min | 100/0 | SA20-10 |
| Step 11 | SPE6 (T 27-29 min) | 0-10 min | 100/0 | SA20-11 |
| Step 12 | SPE5 (T 25-27 min) | 0-10 min | 100/0 | SA20-12 |
| Step 13 | SPE4 (T 23-25 min) | 0-10 min | 100/0 | SA20-13 |
| Step 14 | SPE3 (T 20-23 min) | 0-10 min | 100/0 | SA20-14 |
| Step 15 | SPE2 (T 17-20 min) | 0-10 min | MeCN/water, pH3[e] | SA20-15 |
| Step 16 | SPE13 (T 0-4 min) | 0-4 min | 0/100 | SA20-16 to -23 |
| | Column 2.3[b] | 4-20 min | 0/100-10/90 | |
| | | 20-40 min | 10/90-60/40 | |
| | | 40-50 min | 60/40-100/0 | |
| | | 50-60 min | 100/0 | |
| Step 17 | SPE7 (T 0-3 min) | 0-20 min | Water, pH 3/1M $NH4^+COO^-$, pH 3[f] | SA20-24 to -33 |
| | Column 2.2[c] | 20-45 min | 100/0-85/15 | |
| | | 45-55 min | 85/15-0/100 | |
| | | | 0/100 | |

[a]T: Trapping time for the eluents from 1st separation column, [b]Column 2.3: Carbo RP HPLC column (10×250 mm, 5 μm, 3.5 ml/min), [c]Column 2.2: Cation exchange HPLC column (10×250 mm, 5 μm, 3.5 ml/min), [d]Substances trapped into SPE 2 - SPE6, SPE8 - SPE12 and SPE 14 - SPE18 were not subjected to the second separation, but they were washed out of the SPEs with methanol (flow rate 6 ml/min), [e]Solvent mixture of acetonitril and water including formic acid (pH 3), [f]Solvent mixture of water including formic acid (pH 3) and Ammonium formate buffer (pH 3), [g]The eluents coming from SPEs or 2nd separation columns were collected in 48 deep well by 6-7 ml using an automatic fraction collector, and the fractions were then manually re-arrayed into other 48 deep well plates through monitoring HPLC peaks from UV (254) and RI detectors.

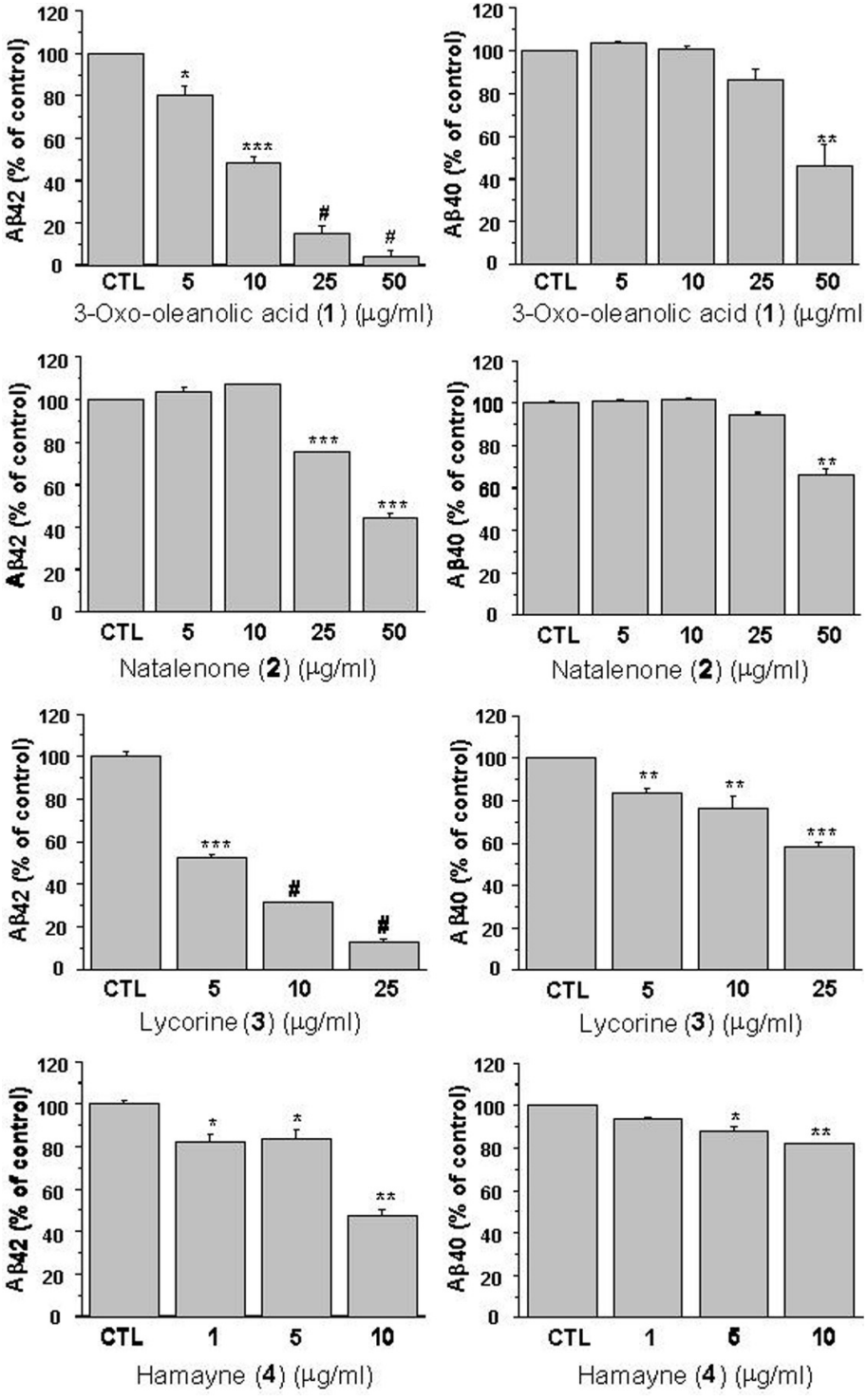


**Fig. 2.** Aβ-lowering effects of 3-oxo-oleanolic acid (1), natalenone (2), lycorine (3), and hamayne (4). Cells were pre-treated with indicated concentration of each compound for 8 h before the levels of Aβ40, and Aβ42 in conditioned media were determined using sandwich ELISA as described in Methods. The values ± s.d. were normalized to the control levels. **p*<0.05, ***p*<0.01, ****p*<0.001, #*p*<0.0001 with student *t*-test.

(3H, s, $H_3$-23), 1.16 (3H, s, $H_3$-27), 1.18 (1H, m, $H_2$-19), 1.65 (1H, m, $H_2$-19), 1.43 (1H, br ddd, *J*=14.0, 11.0, 7.0 Hz, $H_2$-1), 1.90 (1H, br ddd, *J*=14.0, 7.0, 3.5 Hz, $H_2$-1), 2.37 (1H, ddd, *J*=16.0, 7.0, 3.5 Hz, $H_2$-2), 2.55 (1H, ddd, *J*=16.0, 11.0, 7.0 Hz, $H_2$-2), 2.85 (1H, br dd, *J*=14.0, 4.0, H-18), 5.32 (1H, br t, *J*=3.0 Hz, H-12); $^{13}$C-NMR (125 MHz, $CDCl_3$) 15.1 (C-25), 17.0 (C-26), 19.6 (C-6), 21.5 (C-24), 23.0 (C-11), 23.6 (C-16), 25.9 (C-27), 26.5 (C-23), 27.7 (C-15), 30.7 (C-20), 32.2 (C-7), 32.5 (C-22), 33.1 (C-29), 33.9 (C-21), 34.2 (C-2), 36.8 (C-10), 39.2 (C-1), 39.4 (C-8), 41.2 (C-18), 41.8 (C-14), 45.9 (C-19), 46.6 (C-9, C-17), 47.4 (C-4), 55.4 (C-5), 122.4 (C-12), 143.7 (C-13), 183.3 (C-28), 217.6 (C-3).

**Natalenone (2):** Optically inactive yellow powder; mp. 220°C; ESI MS *m/z* 377 $[M+H]^+$; $^1$H NMR (500 MHz, $CDCl_3$)

δ 2.39 (3H, s, H-9'), 2.42 (3H, s, H-9), 3.00 (1H, d, *J*=10.5 Hz, $H_2$-2'a), 3.04 (1H, dd, *J*=10.5, 4.0 Hz, $H_2$-2'b), 4.18 (1H, d, *J* =4.0 Hz, H-3'), 4.84 (1H, s, OH), 6.73 (1H, br s, H-6'), 6.95 (1H, br s, H-8'), 7.04 (1H, br s, H-6), 7.47 (1H, br s, H-8), 11.47 (1H, s, OH), 11.49 (1H, s, OH); $^{13}$C NMR (125 MHz, $CDCl_3$) δ 22.4 (C-9), 22.8 (C-9'), 52.5 (C-3'), 54.2 (C-2'), 113.0 (C-4a), 114.9 (C-8'), 119.1 (C-6'), 122.0 (C-8), 124.7 (C-6), 148.8 (C-7'), 149.5 (C-7), 162.0 (C-5), 164.0 (C-5').

**Lycorine (3):** pale yellowish powder; mp. 237°C; $[\alpha]_D^{20}$ −65° (c 0.2, EtOH); ESI MS *m/z* 288 $[M+H]^+$; $^1$H NMR (500 MHz, $CD_3OD$) δ 2.91-2.74 (2H, m, $H_2$-4), 2.96 (1H, br d, *J*=11.5 Hz, H-11b), 3.50 (1H, m, $H_2$-5), 3.65 (1H, m, $H_2$-5), 3.84 (1H, d, *J* =11.5 Hz H-11c), 4.24 (1H, br s, H-2), 4.22 (1H, d, *J*=14.0 Hz, $H_2$-7), 4.42 (1H, d, *J*=14.0 Hz, $H_2$-7), 4.55 (1H, br s, H-1), 5.77 (1H, br s, H-3), 5.99 (2H, br s, $OCH_2O$), 6.84 (1H, s, H-8), 7.00 (1H, s, H-11).

**Hamayne (4):** pale yellowish powder; mp. 80°C; $[\alpha]_D^{20}$ −72° (c 0.2, EtOH); ESI MS m/z 288 $[M+H]^+$; $^1$H NMR (500 MHz, $D_2O$) δ 2.23 (1H, m, $H_2$-4), 2.34 (1H, m, $H_2$-4), 3.81 (1H, br d, *J*=14.0 Hz, $H_2$-12), 3.98 (1H, dd, *J*=14.0, 7.0 Hz, $H_2$-12), 4.06 (1H, dd, *J*=13.5, 4.0 Hz, $H_2$-4), 4.25 (1H, br d, *J*=7.0 Hz, H-11), 4.33 (1H, d, *J*=15.5 Hz, $H_2$-6), 4.47 (1H, m, H-3), 4.76[a] (1H, $H_2$-6), 5.95 (2H, br s, $OCH_2O$), 6.13 (1H, br d, *J*=10.5 Hz, H-2), 6.23 (1H, d, *J*=10.5 Hz, H-1), 6.69 (1H, s, H-7), 7.01 (1H, s, H-10) [a]overlapping signal with a solvent signal.

### Measurement of Aβ production

HeLa cells line stably expressing Swedish mutant form of APP (APPsw) were maintained in DMEM supplemented with 10% heat-inactivated FBS as described before (Landman *et al.*, 2006). Cells with 60-70% confluence were pre-incubated with extracts or compounds for 8 h before the levels of Aβ40 and Aβ42 in conditioned media were determined using sandwich ELISA (Biosource Int.) according to the manufacturer's protocol.

## RESULTS

One step fractionation of the methanol extracts of *E. crispa* subsp. *crispa* (SA28) and the DCM-MeOH (1:1) extracts of *C. macowanii* (SA20) by the HPLC/SPE/HPLC coupling experiments using the Sepbox system resulted in the isolation of four biologically active subfractions showing inhibition of Aβ production and each containing 1-3 constituents. The subfraction SA20-28 contained predominantly one compound and was analysed by NMR. Subfractions SA28-30, SA28-92 and SA20-17 yielded compounds 1, 2 and 4 via further simple purification steps using semi-preparative HPLC, respectively. Based on the analyses of spectral data including NMR spectra and the literature data, the structures of compounds 1-4 were determined to be 3-oxo-oleanolic acid (1), natalenone (2) isolated from *E. crispa* subsp. *crispa* and lycorine (3) and hamayne (4) isolated from *C. macowanii*. The NMR data of compounds 1-4 were in good agreement with those previously reported in the literature (Ferreira *et al.*, 1977; Evidente *et al.*, 1983; Viladomat *et al.*, 1994; Kwon *et al.*, 1997; Campbell *et al.*, 2000).

Compounds 1-4 lowered the production of Aβ42 with $IC_{50}$ values of approximately 10, 50, 5 and 10 μg/ml, respectively (Fig. 2). Lycorine (3) was most effective in reducing Aβ42 levels. 3-Oxo-oleanolic acid (1) also exhibited significant Aβ42-lowering activity in a dose-dependent manner. The production of Aβ40 was affected by treatment with 3-oxo-oleanolic acid (1) or lycorine (3) only at high concentration. Lycorine (3) was not cytotoxic against cells tested for Aβ-lowering effects. Cell viability was not affected by treatment with 3-oxo-oleanolic acid (1) at concentration up to 10 μg/ml, whereas 25 μg/ml of 3-oxo-oleanolic acid (1) inhibited cell proliferation by up to 60% of vehicle-treated cells.

## DISCUSSION

The Sepbox system has been developed to rapidly separate complex mixtures of natural products to almost pure compounds or mixtures of a few substances, which can then be used directly for biological testing. The method is a combination of HPLC and solid phase extn. (SPE) and is coupled into a HPLC/SPE/HPLC unit with 2 semi-preparative pumps, 7 HPLC columns, 18 collecting columns for SPE, a light scattering detector and UV detectors. In Sepbox-250 system used in this study, a crude extract initially separates into a maximum of 18 fractions using a C4 reverse phase HPLC column. These fractions are transferred to 18 SPE columns which include 15 reverse phase SPE, a hydrophilic carbo SPE, a cation exchange SPE and an anion exchange SPE. A fraction adsorbed into each SPE column was subjected to one of six semi-preparative HPLC columns for the second separation, eluting with a different solvents from those of the first fractionation. In general, the second HPLC columns are composed of a C18 RP column for hydrophobic substances, an ordinary C18 RP column, a C18 RP column for hydrophilic substances, a carbo column for oligosaccharides or water soluble substances, a cation exchange column and an anion exchange column. Users can select one of six HPLC columns for a second separation, considering polarity and solubility of each fraction. Fractionation of a crude extract into 100-300 subfractions including many fairly pure compounds is possible through this HPLC/SPE/HPLC coupling experiments within less than 30 hours.

We were able to rapidly isolate and identify four bioactive compounds namely 3-oxo-oleanolic acid, natalenone, lycorine and hamayne capable of reducing Aβ production, from two South African plants via above mentioned procedure. The biological studies for 3-oxo-oleanolic acid have focused on its anti-tumor effects (Huang *et al.*, 2006). Natalenone is known to be present as a naphthoquinone dimer in isolation studies from *Euclea* species which is used in African traditional medicines (Ferreira *et al.*, 1977), while lycorine and hamayne possesses antitumor and choline esterase inhibitory effects (Campbell *et al.*, 2000; Houghton *et al.*, 2004; Liu *et al.*, 2009). However, the Aβ reducing effects of the compounds 1-4 have not been reported previously.

Aβ is produced by sequential proteolytic cleavages of the β-amyloid precursor protein (APP) by a set of membrane-bound proteases termed β-secretase and γ-secretase (Iwatsubo *et al.*, 1994; Scheuner *et al.*, 1996). Heterogeneous γ-secretase cleavage at the C-terminal end of Aβ produces two major isoforms of Aβ, Aβ40 and Aβ42. While Aβ40 is the predominant cleavage product, the less abundant Aβ42 represents a longer and more amyloidogenic form of Aβ. Moreover, increased cerebrocortical Aβ42 appears to closely correlate with synaptic dysfunction associated with AD (España *et al.*, 2010). The biological study on the action mechanism of compounds 1-4

is in progress.

In summary, our investigation of bioactive principles of two South African plants for the development of Aβ reducing agents using the Sepbox system resulted in the rapid identification of four bioactive compounds reducing Aβ production, for which their structures were determined as 3-oxo-oleanolic acid, natalenone, lycorine and hamayne based on NMR and MS data. 3-Oxo-oleanolic acid and lycorine exhibited significant reducing effects of Aβ42 production in a dose dependent manner. This result demonstrates how biologically active constituents can be isolated and identified rapidly from plants extracts using an automated HPLC/SPE/HPLC coupling system.

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