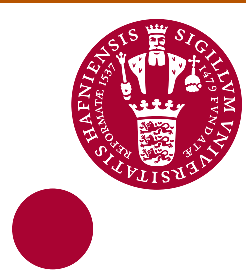
CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Copenhagen University Research Information System

UNIVERSITY OF COPENHAGEN



Serotonin transporter protein (SERT) and P-glycoprotein (P-gp) binding activity of montanine and coccinine from three species of Haemanthus L. (Amaryllidaceae)

Stafford, Gary Ivan; Birer, C.; Brodin, Birger; Christensen, Søren Brøgger; Eriksson, André Huss; Jäger, Anna; Rønsted, Nina

Published in:
South African Journal of Botany

DOI:
10.1016/j.sajb.2013.06.002

Publication date:
2013

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY-NC-ND

Citation for published version (APA):
Stafford, G. I., Birer, C., Brodin, B., Christensen, S. B., Eriksson, A. H., Jäger, A., & Rønsted, N. (2013). Serotonin transporter protein (SERT) and P-glycoprotein (P-gp) binding activity of montanine and coccinine from three species of *Haemanthus* L. (Amaryllidaceae). *South African Journal of Botany*, *88*, 101-106. https://doi.org/10.1016/j.sajb.2013.06.002

Download date: 08. apr.. 2020

South African Journal of Botany 88 (2013) 101–106

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Serotonin transporter protein (SERT) and P-glycoprotein (P-gp) binding activity of montanine and coccinine from three species of *Haemanthus* L. (Amaryllidaceae) ☆

G.I. Stafford [a], C. Birer [b], B. Brodin [c], S. Brøgger Christensen [b], A.H. Eriksson [d,1], A.K. Jäger [b], N. Rønsted [a,*]

[a] Natural History Museum of Denmark, University of Copenhagen, Sølvgade 83, DK-1307 Copenhagen K, Denmark
[b] Natural Products Research, Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark
[c] Drug Transporters in ADME, Department of Pharmacy, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark
[d] Bioneer: FARMA, Department of Pharmacy, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form 2 June 2013
Accepted 5 June 2013
Available online 2 July 2013

Edited by J Van Staden

Keywords:
Haemanthus
Amaryllidaceae
Alkaloids
Serotonin transporter protein
Montanine
Coccinine
Blood–brain barrier
MDCK(II)-MDR1
P-glycoprotein
Calcein-AM

ABSTRACT

The alkaloid rich extracts from an acid/base extraction of bulb material of *Haemanthus coccineus* L., *H. montanus* Baker and *H. sanguineus* Jacq. revealed that two montanine type Amaryllidaceae alkaloids, montanine (1) and coccinine (2) were the major alkaloid constituents. Together these two alkaloids constituted 88, 91 and 98% of the total alkaloid extract from each species respectively. GC–MS analysis revealed that *H. coccineus* and *H. sanguineus* had a relative abundance of coccinine (74 and 91% respectively) to montanine (14 and 7% respectively); whereas *H. montanus* had 20% coccinine and 71% montanine. The three extracts and two isolated alkaloids were evaluated for binding to the serotonin transporter protein (SERT) in vitro. Affinity to SERT was highest in *H. coccineus* ($IC_{50} = 2.0 \pm 1.1$ μg/ml) followed by *H. montanus* ($IC_{50} = 6.8 \pm 1.0$ μg/ml) and *H. sanguineus* ($IC_{50} = 28.7 \pm 1.1$ μg/ml). Montanine ($IC_{50} = 121.3 \pm 3.6$ μM or 36.56 ± 1.14 μg/ml; $K_i = 66.01$ μM) was more active than coccinine ($IC_{50} = 196.3 \pm 3.8$ μM or 59.15 ± 1.08 μg/ml; $K_i = 106.8$ μM), both of which were less active than the total alkaloid extracts of each species investigated. The possible synergistic effects of two coccinine/montanine mixtures (80:20 and 20:80) were investigated, however the mixtures gave similar activities as the pure compounds and did not show any increase in activity or activity similar to the total alkaloid extracts. Thus the considerably higher activity observed in the total alkaloid extracts is not correlated to the relative proportions of coccinine and montanine in the extracts and thus are likely to be due to more potent unidentified minor constituents. Both alkaloids exhibited low binding affinity to P-glycoprotein (P-gp) as demonstrated by low inhibition of calcein-AM efflux in the MDCK-MDR1 cell line. This indicates that P-gp efflux will not be limiting for blood–brain-barrier passage of the alkaloids.

© 2013 The Authors. SAAB. Published by Elsevier B.V. All rights reserved.

1. Introduction

Depression is generally considered to be associated with reduction of monoamine neurotransmitters in the brain. Several antidepressants exert their effect by selective inhibition of serotonin reuptake by binding to the serotonin transporter protein (SERT) (Stahl, 1998). In a screening of plants used for depression-like disorders in traditional medicine in South Africa, an ethanolic extract of leaves and bulbs of *Boophone disticha* (L.f.) Herb. (Amaryllidaceae) has shown affinity to SERT in both in vitro (Nielsen et al., 2004) and in vivo (Pedersen et al., 2008). Four active alkaloids have been isolated from the extract; buphanamine, buphanidrine, buphanisine and distichamine, with IC_{50}-values of 55 μM, 62 μM, 199 μM and 65 μM respectively, in the SERT-binding assay. The alkaloids also showed activity in a functional assay, buphanidrine and distichamine being the most active with IC_{50}-values of 513 μM and 646 μM, respectively (Sandager et al., 2005; Neergaard et al., 2009).

The Amaryllidaceae alkaloids include more than 500 identified compounds, which have been divided into eighteen structural types based on their hypothetical biosynthetic pathways (Jin, 2009). Structurally, buphanamine and buphanadrine belong to the crinine-type and have the benzo-1,3-dioxole moiety in common with the clinically used SSRI, paroxetine, which could explain their affinity to the SERT. A detailed screening of alkaloids isolated from Amaryllidaceae (Elgorashi et al., 2006; Neergaard et al., 2009), suggested that Amaryllidaceae

Abbreviations: AChE, acetylcholinesterase; BBB, blood–brain-barrier; BCRP, breast cancer resistance protein; CNS, central nervous system; P-gp, P-glycoprotein; SERT, serotonin transporter protein; SSRI, selective serotonin reuptake inhibitor.

☆ This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

* Corresponding author at: Natural History Museum of Denmark, Sølvgade 83, Opg. S., DK-1307 Copenhagen K, Denmark. Tel.: +45 33 32 22 48.
E-mail address: nronsted@snm.ku.dk (N. Rønsted).
[1] Current address: DMPK & Safety, LEO Pharma A/S, Industriparken 55, DK-2750 Ballerup, Denmark.

0254-6299/$ – see front matter © 2013 The Authors. SAAB. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.sajb.2013.06.002

alkaloids with affinity to SERT primarily are of the crinine type (Jin, 2009). In a recent study on the Southern African Amaryllidaceae tribe Haemantheae (Bay-Smidt et al., 2011), we found that SERT activity appears to be pronounced and restricted to the genus *Haemanthus* within this tribe. Alkaloid rich extracts of three of the eight *Haemanthus* species tested had IC_{50} < 10 μg/ml. Two of the most active extracts contained primarily montanine type alkaloids, which have previously not been tested for SERT affinity. Several of the closely related white-flowered *Haemanthus* (Bay-Smidt et al., 2011), which are found in the summer rainfall regions of southern Africa, are used in African traditional medicine (Crouch et al., 2005). These include the bulbs and roots of *H. albiflos* Jacq., which are used by the Xhosa in infusions or poultices to promote the healing of broken bones, (Crouch et al., 2005; Dold and Cocks, 2000). *Haemanthus* species are not reported to be used in African traditional medicine to treat mental illnesses, although several closely related Amaryllidaceae genera are (Sobiecki, 2002; Stafford et al., 2008), such as *Scadoxus puniceus* (L.) Friis & Nordal (syn = *Haemanthus kalbreyeri* Baker), *Ammocharis coranica* (Ker-Gawl.) Herb, *Boophone disticha* (L.f.) Herb., *B. haemanthoides* F.M.Leight. and *Pancratium tenuifolium* Hochst. ex A.Rich.

The first study to isolate alkaloids from *Haemanthus* found 10 alkaloids from several species (Wildman and Kaufman, 1955). Coccinine ((2β)-2-*O*-methylpancracine) was found to be the principle component in *H. coccineus* and montanine ((2α)-2-*O*-methylpancracine) the only alkaloid detected in *H. montanus*.

In a study by da Silva et al. (2006), where montanine was isolated from the bulbs of the South American *Hippeastrum vittatum* (L'Hér.) Herb., montanine showed a LD_{50} of 64.7 mg/kg and 67.6 mg/kg for male and female mice, respectively. When given i.p., montanine dose-dependently decreased sodium pentobarbital-induced sleep, protected against pentylenetetrazole-provoked convulsions, increased the number of entries and the time spent in the open arms of an elevated plus maze and augmented the time spent struggling during a forced swimming test. When given immediately after inhibitory avoidance training, montanine did not affect avoidance memory retention in rats. The reported in vivo activity of montanine, particularly in the forced swimming test, supports the possibility that montanine and its beta 2-*O*-methyl-isomer, coccinine (Fig. 1), may serve as leads for SERT binding compounds. Montanine has also been shown to significantly inhibit acetylcholinesterase (AChE) (Pagliosa et al., 2010). An activity utilized in treatment of neurological disorders and neurodegenerative diseases related to the levels of acetylcholine. For this reason galanthamine, an important alkaloid isolated from several Amaryllidaceae, is approved for the pharmacological treatment of Alzheimer's disease.

It has however been estimated that 98% of CNS drug candidates do not enter the brain (Pardridge, 2007). The passage by passive diffusion of compounds with optimal physicochemical properties may be hindered by the activity of efflux transporters, amongst which P-glycoprotein (P-gp; MDR1 gene product) plays a major role. Our understanding of

montanine: $R^1 = H$, $R^2 = OCH_3$ (α-isomer)

coccinine: $R^1 = OCH_3$, $R^2 = H$ (β-isomer)

Fig. 1. Montanine and coccinine.

the substrate-specificity of P-gp is still rather limited (Li et al., 2007). In an evaluation of the interaction of nine potentially CNS-active Amaryllidaceae alkaloids of the crinine, lycorine and galanthamine types with P-gp (Eriksson et al., 2012), structurally similar compounds such as crinine and epibuphanisine showed very different P-gp interaction emphasizing the difficulty in predicting P-gp interaction.

In the present study we investigated affinity of extracts of three *Haemanthus* species, *H. coccineus* L., *H. montanus* Baker and *H. sanguineus* Jacq., and major alkaloid constituents, coccinine and montanine, to the SERT protein. The pure alkaloids were also evaluated with for their interaction with P-gp using the calcein-AM assay. *Haemanthus coccineus* L. (Synonyms: *H. concolor* Herb., *H. moschatus* Jacq., *H. splendens* Dinter, *H. tigrinus* Jacq.) is a perennial geophyte which occurs at an altitude of 15–1200 m in the winter rainfall region of southern Africa from Namibia through the Northern Cape and Western Cape to Eastern Cape of South Africa. *Haemanthus montanus* Baker (synonyms: *H. amarylloides* in sense of Baker, not of Jacq., misapplied name) a perennial geophyte which occurs in the summer rainfall regions of Botswana and Northern West, Griqualand, Mpumalanga, Free State, KwaZulu-Natal, Eastern Cape provinces of South Africa. *Haemanthus sanguineus* Jacq. (synonyms: *H. incarnatus* Burch. ex Herb., *H. rotundifolius* Ker Gawl.) is a perennial geophyte found only in the Western and Eastern Cape of South Africa.

2. Materials and methods

2.1. Plant material

Three species of *Haemanthus* L. (Amaryllidaceae) were obtained from a specialist nursery (www.rareplants.co.uk) for this study. The plant material was identified by NR and GIS and vouchers (Table 1) are deposited at the Herbarium, Botanical Garden and Museum, University of Copenhagen (**C**). Plant material was cultivated in a greenhouse and harvested in February 2011.

2.2. Alkaloid extraction for initial screening

Alkaloids were extracted from 300 mg dried bulb scales. The pulverized plant material was macerated in 400 μl methanol for 5 min. Three milliliters 0.1% H_2SO_4 was added and the material was extracted for 45 min under ultra-sonification followed by shaking for 2 h. The sample was centrifuged for 10 min at 4000 rpm and the supernatant taken off. Extraction was repeated twice more, but the sample was left on the shaker overnight during the second extraction. The three extracts were pooled, centrifuged for 10 min at 4000 rpm, and retained on an ion-exchange SPE (solid phase extraction) column (Isolute SCX, 500 mg, 3 ml, Mikrolab, Denmark). The column was preconditioned with 2 ml methanol and 2 ml 0.1% H_2SO_4. After addition of the alkaloid extract, the column was washed with 2 ml 0.1% H_2SO_4 and 4 ml methanol. The alkaloid extracts were then with 6 ml 25% NH_4OH in methanol, pH 11–12, concentrated under vacuum until dryness and re-dissolved to a concentration of 5 mg/ml in methanol.

2.3. Isolation of montanine and coccinine

The same extraction protocol that was used for the initial screening was performed on 11.97 g dried *Haemanthus coccineus* (fw 71.21 g) which yielded 275 mg extract (23 mg/g dried bulb), 12.00 g dried *H. montanus* (fw 59.53 g) yielded 496 mg extract (41 mg/g dried bulb) and 12.00 g dried *H. sanguineus* yielded 443 mg extract (37 mg/g dried bulb). Two hundred milligrams of the *H. coccineus* and *H. montanus* extracts were suspended in methanol and separated by column chromatography on a silica gel column (170 ml Merck 9385 silica gel, height 80 cm, diameter 2 cm). An eluent system consisting of 80:20 chloroform:methanol was used and 150 fractions collected. The fractions were pooled by TLC profile into 2 fractions for each column.

Table 1
Summary of material used, yield, retention time and composition.

Species voucher number	Material harvested on 23 February 2011	Yield of alkaloid rich extract	Alkaloid composition (Area %)	Retention time (RT, min)
Haemanthus coccineus L. Rønsted 471 (C)	Growing bulb (fw 148.12 g, dw 29.86 g)	41.32 mg/g dried bulb	1.5 unidentified crinanol	21.735
			74.1 coccinine	23.497
			13.8 montanine	24.064
Haemanthus montanus Baker Rønsted 365 (C)	Dormant bulb (fw 71.21 g, dw 11.97 g)	22.93 mg/g dried bulb	2.3 manthine	22.846
			20.7 coccinine	23.414
			71.0 montanine	24.122
			2.9 dihydrolycorine	25.965
			3.0 unidentified lycorenine	26.229
Haemanthus sanguineus Jacq. Rønsted 375 (C)	Growing bulb (fw 99.44 g, dw 16.57 g)	36.89 mg/g dried bulb	1.7 unidentified amide	20.069
			90.5 coccinine	23.737
			7.0 montanine	24.280

2.4. Alkaloid GC–MS profile

Alkaloid profiles of the plant extracts were obtained by gas chromatography–mass spectrometry (GC–MS) using a method developed by Berkov et al. (2008) and modified according to Larsen et al. (2010). The GC–MS spectra were recorded on an Agilent GC–MS system (5973 N Mass Selective Detector, 6890 Network GC-system, 7683 Series Injector and Autosampler, Agilent Technologies, Santa Clara, USA) operating in EI mode at 70 eV. An HP-5MS column (30 m × 0.25 mm × 0.25 μm) was used (Agilent Technologies, Denmark). The temperature program was: 100–180 °C at 15 °C/min, 1 min hold at 180 °C and 180–300 °C at 5 °C/min and 1 min hold at 300 °C. The injector temperature was 280 °C. The flow-rate of carrier gas (Helium) was 0.8 ml/min. The split ratio was 1:20. Samples were filtered through a 0.45 μl Millipore filter before injection of 1 μl into the GC–MS. Limit of detection and quantification determined for galanthamine was 28.4 μg/ml and 94.6 μg/ml respectively. Alkaloids were identified to type by comparison with the NIST 08 Mass Spectral Search Program, version 2.0 (NIST, Gaithersburg, Maryland) and with published spectral data.

2.5. Estimation of SERT-affinity

The alkaloid extracts of *H. montanus*, *H. coccineus*, *H. sanguineus* and the isolated alkaloids, coccinine and montanine, were tested for affinity to SERT using the method described by Plenge et al. (1990) and Nielsen et al. (2004) with modifications (Neergaard et al., 2009). Two mixtures that closely resemble the proportions found in the whole alkaloid extracts of *H. montanus* and *H. coccineus*, of the alkaloids were tested to determine if there are a synergistic effect between montanine and coccinine. Briefly, whole rat brains, except cerebellum, were homogenized with an Ultra Turax homogenizer in 1:10 (w/v) buffer (Tris base 5 mM; NaCl 150 mM; EDTA 20 mM; pH 7.5). The homogenate was centrifuged at 6000 *g* for 10 min and the homogenized tissue pellet washed with the same buffer 1:10 (w/v). The supernatant was discarded, the pellet was suspended in buffer (Tris base 5 mM; EDTA 5 mM; pH 7.5), left for 20 min at 0 °C and centrifuged at 6000 *g* for 10 min. The supernatant was discarded and the pellet was suspended in buffer (Tris base 50 mM; NaCl 120 mM; KCl 50 mM; pH 7.5) and centrifuged at 6000 *g* for 10 min. The supernatant was discarded and the protein pellet finally suspended in 1:10 (w/v) buffer (Tris base 50 mM; NaCl 120 mM; KCl 50 mM; pH 7.5). The tissue homogenate was kept at −80 °C until use. The extracts and compounds were tested in a minimum of six concentrations from 0.05 μg/ml to 5 mg/ml (4.17 ng/ml–417 μg/ml in the assay). Twenty-five microliters of each dilution was mixed with 50 μl of [^{3}H]-citalopram (4 nM, 0.67 nM in the assay) and 225 μl of tissue suspension, respectively. The total binding of [^{3}H]-citalopram was determined with a solvent blank. Paroxetin (120 μM, 10 μM in the assay) was used for the determination of the non-specific binding. All samples were incubated for 2 h at 25 °C and then filtered under vacuum using Avantec GC50-25 mm glass fiber filters. The radioactivity on the filters was determined by liquid scintillation using Ultimo Gold XR as scintillation fluid. Specific binding was calculated as total binding minus unspecific binding. All experiments were done in triplicate. IC_{50}-values were calculated using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA).

2.6. Identification of purified compounds

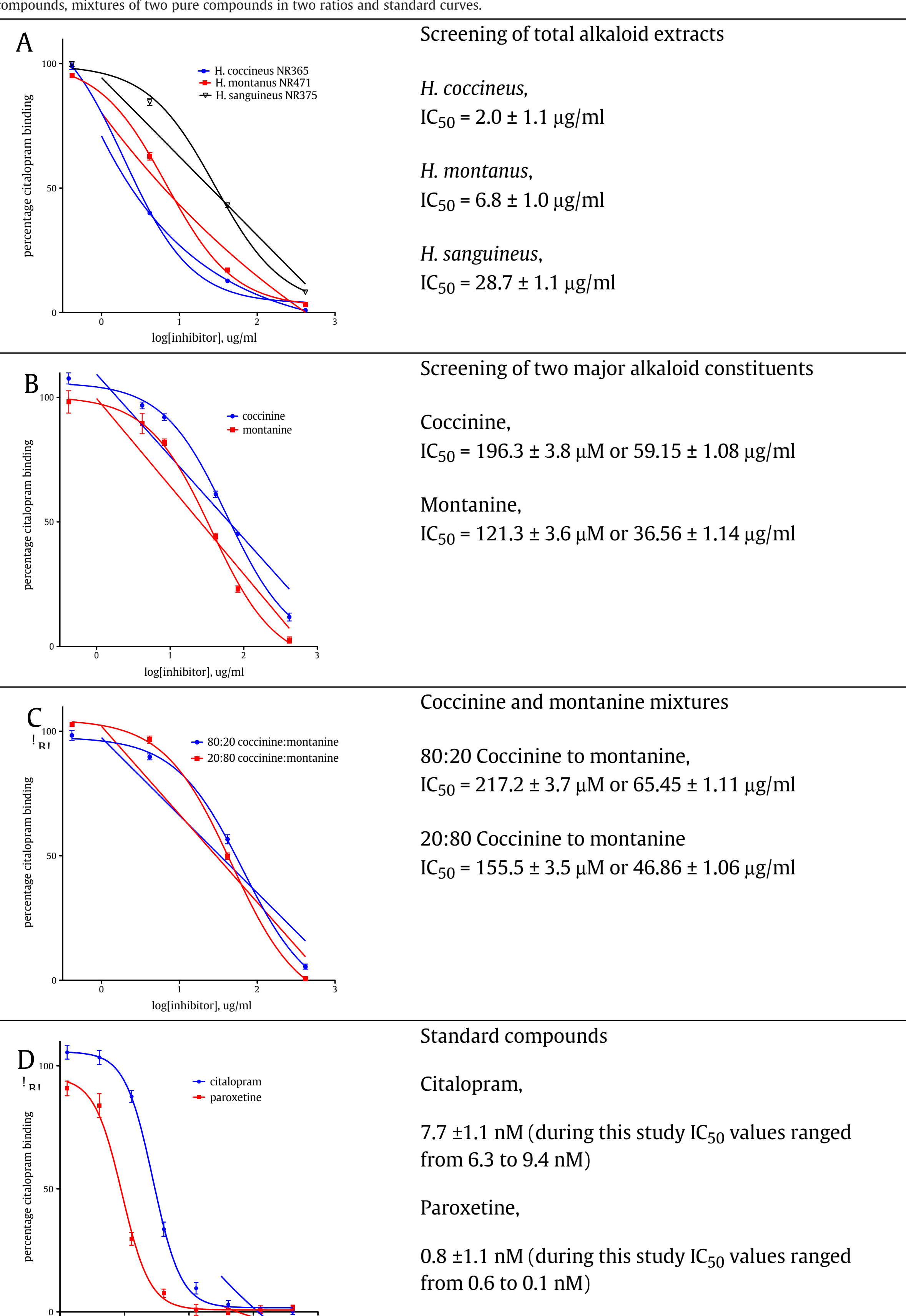
The structural identification of the alkaloids was achieved by a combination of spectroscopic techniques and comparison with literature data. Montanine and coccinine were identified by ^{1}H NMR (Varian *Mercury 300* MHz; $CDCl_3$; Supplementary data Table S1), ^{13}C NMR (150 MHz) and MS corresponding to literature (Ishizaki and Hoshino, 1992; Jin and Weinreb, 1997). Washing of the deuterochloroform solutions of the alkaloids with a 10% solution of sodium carbonate in deuterium oxide enabled analysis of the coupling systems in the ^{1}H NMR spectra.

2.7. Calcein-AM assay for P-gp interaction

The assay was performed as described previously (Eriksson et al., 2012). Briefly MDCK(II)-MDR1 cells were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands). Cells were seeded in culture flasks and passaged in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), 1% L-glutamine and 1% non-essential amino acids. The cells were grown in an atmosphere containing 5% CO_2 at 37 °C. Growth medium was replaced every second or third day. For the experiments, cells were seeded at a density of 2×10^5 cells/cm^2 in 96-well plates in culture medium. All experiments were conducted 3–4 days after seeding. MDCK(II)-MDR1 cells in passages 6–10 were used.

The interaction of test compounds with P-gp was assessed using the calcein-AM assay (Polli et al., 2001). MDCK(II)-MDR1 cells were washed twice with 100 μl HBSS supplemented with 0.05% bovine serum albumin and 10 mM HEPES at pH 7.4, and 50-μl test solutions were added to each well. The cells were incubated at 37 °C for 15 min before the addition of 50 μl of 50 μm calcein-AM in HBSS pH 7.4. The fluorescence (ex/em: 485 nm/520 nm) was subsequently monitored for 60 min at excitation on a NOVOstar plate reader (BMG LabTech, Offenburg, Germany). A background (50 μl HBSS pH 7.4) was included for each round of experiments. The alkaloids were tested in three individual cell passages at six concentrations ranging from 16 to 1600 μM. Experiments were performed in triplicate within each cell passage. The known P-glycoprotein inhibitors cyclosporin A and verapamil were included as positive controls at a concentration range of 0.05–5 μM (cyclosporin A; IC_{50}: 5.1 ± 5.9 μM) and 0.1–100 μM (verapamil; IC_{50}: 6.0 ± 3.2 μM). The fluorescence response in fluorescence units (FU) was linear as a function of time for all samples in the interval 0 to 60 min. The signal curves were plotted as a function of time for each test compound and the slope of the curve was determined. The

Table 2
Log-concentration versus percentage [^{3}H]-citalopram displacement showing serotonin transporter protein (SERT) binding of total alkaloid extracts, pure compounds, mixtures of two pure compounds in two ratios and standard curves.



Graph	Results
A	Screening of total alkaloid extracts *H. coccineus*, IC_{50} = 2.0 ± 1.1 µg/ml *H. montanus*, IC_{50} = 6.8 ± 1.0 µg/ml *H. sanguineus*, IC_{50} = 28.7 ± 1.1 µg/ml
B	Screening of two major alkaloid constituents Coccinine, IC_{50} = 196.3 ± 3.8 µM or 59.15 ± 1.08 µg/ml Montanine, IC_{50} = 121.3 ± 3.6 µM or 36.56 ± 1.14 µg/ml
C	Coccinine and montanine mixtures 80:20 Coccinine to montanine, IC_{50} = 217.2 ± 3.7 µM or 65.45 ± 1.11 µg/ml 20:80 Coccinine to montanine IC_{50} = 155.5 ± 3.5 µM or 46.86 ± 1.06 µg/ml
D	Standard compounds Citalopram, 7.7 ±1.1 nM (during this study IC_{50} values ranged from 6.3 to 9.4 nM) Paroxetine, 0.8 ±1.1 nM (during this study IC_{50} values ranged from 0.6 to 0.1 nM)

slope values (FU/s) were plotted as a function of compound concentration and the data were fitted to a modified Michaelis–Menten equation (Eq. 1) including V_{blank} using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA):

$$V = ((V_{max} \times [I])\ (IC_{50} + [I])) + V_{blank} \quad (1)$$

where V (in FU/s) is the conversion rate of calcein-AM to calcein, V_{blank} represents the baseline rate of conversion observed in the HBSS buffer with no inhibitor present and [I] is the inhibitor concentration. The values reported represent the mean of three individual determinations from separate cell passages.

3. Results and discussion

3.1. Alkaloid isolation and structure elucidation

The alkaloid extracts of *H. coccineus* and *H. sanguineus* were comprised predominantly of coccinine, whereas *H. montanus* contained mostly the alpha *O*-2-methyl-isomer, montanine as previously reported (Wildman and Kaufman, 1955; Inubushi et al., 1960). These two isomers accounted for between 87 and 97% of the total alkaloid extract of the three species investigated. Similar alkaloid composition has been reported for *H. deformis* (Crouch et al., 2005).

The *H. coccineus* and *H. montanus* extracts were subjected to column chromatography and coccinine was the first alkaloid to be isolated from each of the columns followed almost immediately by montanine. It was possible to recover 114.8 mg of coccinine and 15.0 mg of montanine from *H. coccineus*. From *H. montanus* it was only possible to recover 23.3 mg of montanine and 6.1 mg coccinine. The purity of the fractions was confirmed by GC–MS, with coccinine and montanine having retention times of 23.2 and 23.8 min respectively.

3.2. Affinity to the serotonin reuptake transport protein

The total alkaloid extracts showed considerable affinity for the SERT, with IC_{50}-values 2.0, 6.8 and 28.7 μg/ml for *H. coccineus*, *H. montanus* and *H. sanguineus* respectively (Table 2). Although the total alkaloid extracts are comprised of 88–98% of varying ratios of coccinine and montanine, however the individual alkaloids exhibited considerable less affinity for SERT, with IC_{50}-values of 59.2 and 36.6 μg/ml (196.3 and 121.3 μM) respectively. A possible synergistic effect between coccinine and montanine such that one isomer leads to increase in affinity of the other to the SERT was investigated by preparing two mixtures of coccinine and montanine in similar ratios to the ones found the active extracts (8:2 and 2:8 coccinine:montanine). However, no synergistic effect was observed with the mixtures displaying similar activity to the pure alkaloids, 65.5 and 46.9 μg/ml (217.2 and 155.5 μM) respectively (Table 2).

Another possible explanation for the higher activity observed in the total extracts could be that the remaining unidentified constituents making up the 12–2% of the extract that is not coccinine or montanine are responsible of the activity. We consider this a possible explanation as Amaryllidaceae alkaloids with far more potent SERT affinity, such as cherylline (3.4 μM) and epivittatine (12.1 μM) from *Crinum moorei* (Elgorashi et al., 2006), have been reported previously. Furthermore, the majority of the alkaloids that have had an affinity to SERT are crinine-type alkaloids (Elgorashi et al., 2006), in particular β-crinane alkaloids. To date no β-crinane alkaloids have been isolated from the genus, however two α-crinanes, haemanthamine and heamanthidine are known from *Haemanthus* and the closely related genus *Scadoxus*. One might suggest that these or similar compounds may be responsible for the observed activity in the three *Haemanthus* species under investigation. However in a preliminary screening study of SERT activity in tribe Haemantheae (Bay-Smidt et al., 2011), the extracts with the lowest IC_{50} values all contained montanine type compounds, whereas there was no general trend towards low IC_{50} values for extracts containing crinine type compounds.

Further studies to isolate larger quantities of the alkaloids is required to determine if there is indeed more potent compounds with an affinity to the SERT protein present in *Haemanthus*.

3.3. BBB transport

Pure coccinine and montanine were tested in the Calcein-AM assay in order to identify any whether they displayed P-gp-inhibitory activity. The compounds were tested at six concentrations between 17 μM and 1.7 mM. Coccinine and montanine both inhibited Calcein efflux, with IC_{50}-values of 0.96 ± 0.40 mM for coccinine and 0.78 ± 0.16 mM for montanine. For the full kinetic characterization, see Table 3. This interaction was in the same range as previously investigated Amaryllidaceae alkaloids (Eriksson et al., 2012). This range of potency was more than 100 times weaker than the interaction with P-gp by established standard inhibitors as cyclosporine and verapamil. Thus, it would be assumed that P-gp does not play a role in the overall transport. However, this would need to be addressed in future studies of transepithelial transport of coccinine and montanine. Such studies may also elucidate whether other transporters, such as BCRP, are involved in absorption or distribution of coccinine and montanine.

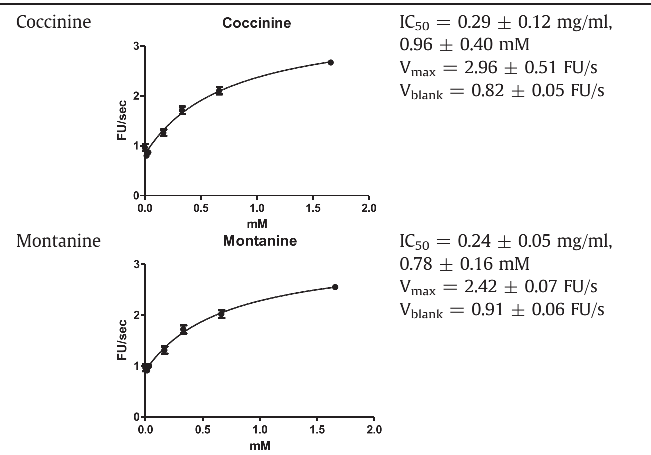
4. Conclusions

Both montanine and coccinine exhibit moderate SERT affinity however this does not explain the higher activity observed in the extracts. Further studies are required to identify the cause of this activity. If it were due to a minor alkaloid this would be very interesting, as it would have to be extremely potent given the relatively small proportion in the total alkaloid extract.

Acknowledgments

The authors would like to acknowledge Mette Frandsen, Eleonora Teonori and Flemming Vangsgaard for help in the laboratory and Sonny Larsson for discussions of the results and suggestion of the experiment for synergy. This research was supported by a Steno grant (No. 272-07-0281) to NR from the Danish Council for Independent Research — Natural Sciences, a Marie Curie International Incoming Fellowship (No. 235167) within the 7th European Community Framework Programme (GIS and NR) and the Predicting Drug Absorption Consortium (the Danish Strategic Research Council).

Table 3
Parameter estimation for montanine and coccinine interaction with P-gp. (Mean ± SE, n = 3).



Coccinine		$IC_{50} = 0.29 \pm 0.12$ mg/ml, 0.96 ± 0.40 mM $V_{max} = 2.96 \pm 0.51$ FU/s $V_{blank} = 0.82 \pm 0.05$ FU/s
Montanine		$IC_{50} = 0.24 \pm 0.05$ mg/ml, 0.78 ± 0.16 mM $V_{max} = 2.42 \pm 0.07$ FU/s $V_{blank} = 0.91 \pm 0.06$ FU/s

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2013.06.002.

References

Bay-Smidt, M.G.K., Jäger, A.K., Krydsfeldt, K., Meerow, A.W., Stafford, G.I., van Staden, J., Rønsted, N., 2011. Phylogenetic selection of target species in Amaryllidaceae tribe Haemantheae for acetylcholinesterase inhibition and affinity to the serotonin reuptake transport protein. South African Journal of Botany 77, 175–183.

Berkov, S., Bastida, J., Viladomat, F., Codina, C., 2008. Analysis of galanthamine-type alkaloids by capillary gas chromatographymass spectrometry in plants. Phytochemical Analysis 19, 285–293.

Crouch, N.R., Pohl, T.L., Mulholland, D.A., Ndlovu, E., 2005. Alkaloids from three ethnomedicinal *Haemanthus* species: *H. albiflos*, *H. deformis* and *H. pauculifolius* (Amaryllidaceae). South African Journal of Botany 71, 49–52.

da Silva, A.F.S., de Andrade, J.P., Bevilaqua, L.R.M., de Souza, M.M., Izquierdo, I., Henriques, A.T., Zuanazzi, J.A.S., 2006. Anxiolytic-, antidepressant- and anticonvulsant-like effects of the alkaloid montanine isolated from *Hippeastrum vittatum*. Pharmacology, Biochemistry, and Behavior 85, 148–154.

Dold, T., Cocks, M., 2000. Indigenous plant use of the amaXhosa people on the eastern border of the Great Fish River Reserve, Eastern Cape. Annals of Eastern Cape Museums 1, 26–53.

Elgorashi, E.E., Stafford, G.I., Jäger, A.K., Van Staden, J., 2006. Inhibition of [^{3}H] citalopram binding to the rat brain serotonin transporter by Amaryllidaceae alkaloids. Planta Medica 72, 470–473.

Eriksson, A.H., Rønsted, N., Güler, S., Jäger, A.K., Sendra, J.R., Brodin, B., 2012. Structurally related, potentially CNS-active Amaryllidaceae alkaloids, show differential interactions with p-glycoprotein. The Journal of Pharmacy and Pharmacology 64, 1667–1677.

Inubushi, Y., Fales, H.M., Warnhoff, E.W., Wildman, W.C., 1960. Structures of montanine, coccinine, and manthine. The Journal of Organic Chemistry 25, 2153–2164.

Ishizaki, M.M., Hoshino, O., 1992. Total synthesis of montanine-type Amaryllidaceae alkaloids which possess a 5, 11-methanomorphanthridine ring system, through cyclisation with sodium bis (2-methoxyethoxy) aluminium hydride: the first stereoselective synthesis of montanine, coccinine, O-acetylmontanine, pancracine and brunsvigine. The Journal of Organic Chemistry 57, 7285–7295.

Jin, Z., 2009. Amaryllidaceae and *Sceletium* alkaloids. Natural Product Reports 26, 363–381.

Jin, J., Weinreb, S.M., 1997. Application of a stereospecific intramolecular allenylsilane iminoene reaction to enantioselective total synthesis of the 5,11-methanomorphanthridine class of Amaryllidaceae alkaloids. Journal of the American Chemical Society 119, 5773–5784.

Larsen, M.M., Adsersen, A.A., Davis, A.P., Lledó, M.D., Jager, A.K., Rønsted, N., 2010. Using a phylogenetic approach to selection of target plants in drug discovery of acetylcholinesterase inhibiting alkaloids in Amaryllidaceae tribe Galantheae. Biochemical Systematics and Ecology 38, 1026–1034.

Li, W.-X., Li, L., Eksterowicz, J., Ling, X.B., Cardozo, M., 2007. Significance analysis and multiple pharmacophore models for differentiating P-glycoprotein substrates. Journal of Chemical Information and Modeling 47, 2429–2438.

Neergaard, J.S., Andersen, J., Pedersen, M.E., Stafford, G.I., van Staden, J., Jäger, A.K., 2009. Alkaloids from *Boophone disticha* with affinity to the serotonin transporter. South African Journal of Botany 75, 371–374.

Nielsen, N.D., Sandager, M., Stafford, G.I., Van Staden, J., Jäger, A.K., 2004. Screening of indigenous plants from South Africa for affinity to the serotonin reuptake transport protein. Journal of Ethnopharmacology 94, 159–163.

Pagliosa, L.B., Monteiro, S.C., Silva, K.B., de Andrade, J.P., Dutilh, J., Bastida, J., Cammarota, M., Zuanazzi, J.A.S., 2010. Effect of isoquinoline alkaloids from two *Hippeastrum* species on *in vitro* acetylcholinesterase activity. Phytomedicine 17, 698–701.

Pardridge, W.M., 2007. Blood–brain barrier delivery. Drug Discovery Today 12, 54–61.

Pedersen, M.E., Szewczyk, B., Stachowicz, K., Wieronska, J., Andersen, J., Stafford, G.I., van Staden, J., Pilc, A., Jäger, A.K., 2008. Effects of South African traditional medicine in animal models for depression. Journal of Ethnopharmacology 119, 542–548.

Plenge, P., Mellerup, E.T., Nielsen, M., 1990. Inhibitory and regulatory binding sites on the rat brain serotonin transporter: molecular weight of the [^{3}H] paroxetine and [^{3}H] citalopram binding proteins. European Journal of Pharmacology 189, 129–134.

Polli, J.W., Wring, S.A., Humphreys, J.E., Huang, L., Morgan, J.B., Webster, L.O., Serabjit-Singh, C.S., 2001. Rational use of in vitro P-glycoprotein assays in drug discovery. The Journal of Pharmacology and Experimental Therapeutics 299, 620–628.

Sandager, M., Nielsen, N.D., Stafford, G.I., Van Staden, J., Jäger, A.K., 2005. Alkaloids from *Boophane disticha* with affinity to the serotonin transporter in rat brain. Journal of Ethnopharmacology 98, 367–370.

Sobiecki, J.F., 2002. A preliminary inventory of plants used for psychoactive purposes in southern African healing traditions. Transactions of the Royal Society of South Africa 57, 1–24.

Stafford, G.I., Pedersen, M.E., Van Staden, J., Jäger, A.K., 2008. Review on plants with CNS-effects used in traditional South African medicine against mental diseases. Journal of Ethnopharmacology 119, 513–537.

Stahl, S.M., 1998. Mechanism of action of serotonin selective reuptake inhibitors: serotonin receptors and pathways mediate therapeutic effects and side effects. Journal of Affective Disorders 51, 215–235.

Wildman, W.C., Kaufman, C.J., 1955. Alkaloids of the Amaryllidaceae. III. Isolation of five new alkaloids from Haemanthus Species. Journal of the American Chemical Society 77, 1248–1252.