Preparative isolation of bio-markers from the leaf exudate of Aloe ferox (‘‘aloe bitters’’) by high performance counter-current chromatography§

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ABSTRACT

One of the most crucial factors determining the safety and efficacy of any herbal medicine or natural product-based formulation is the quality of the raw material. The absence of readily available bio-markers (standards) is one of the hurdles which need to be overcome to develop robust and effective quality control protocols.

Aloe ferox Mill. is a most coveted ethnomedicinally import plant indigenous to South Africa. A. ferox has been used since ancient times in folk medicine and recently it has gained popularity as an ingredient in cosmetic formulations and food supplements. This study aimed to develop a superior method for the isolation of bio-markers from ‘‘aloe bitters’’ (exudate) obtained from A. ferox.

For separation by HPCCC the solvent system comprising of EtOAc/n-BuOH/H_2O (3.5:1.5:5, v/v/v) was used in reversed phase mode. By this method, and only in one run, eight bio-markers were separated and isolated on semi-preparative scale including aloesin, aloeresin C, aloeresin A, 5-hydroxyaloin, aloin B, aloinoside B, aloin A and aloinoside A. The isolation of bio-active molecules from A. ferox (Cape aloes) is presented to illustrate the efficiency and advantages of high performance counter-current chromatography (HPCCC).

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1. Introduction

During the last decades the use of complementary medicines has escalated resulting in a greater research interest and commercialization of natural products. One of the most important factors in the development of phytomedicines and other plant-based consumer products (e.g. cosmetics) is the quality control of the herbal raw material as well as formulated products. Quality control is a fundamental process in the pharmaceutical industry as this process is the basis of safety and efficacy (Calixto, 2000). The tedious isolation process and lack of readily available standards are clearly rate limiting factors in the quality control of herbal products. Although tremendous progress has been made to produce bio-markers for popular herbal medicines the same cannot be said for most of the African traditional medicines.

Extracts from medicinal plants are notoriously complex making the isolation and purification of the bio-markers a challenging and time-consuming process. Several chromatographic methods have been developed to isolate the bio-markers from herbal extracts for further studies. Counter-current chromatography (CCC) is a liquid–liquid separation technique, which makes use of a support-free liquid stationary phase that is held in place by a rotating force field (Ito, 2005). In CCC, both the stationary and mobile phases are liquids while in other types of liquid chromatography, the stationary phases are solid. Thus through using CCC, decomposition and adsorption of compounds by the solid stationary phase do not occur making HPCCC ideally suited for the rapid isolation of natural products which is only one of many advantages HPCCC offers over conventional techniques (Ito, 2005; Marston and Hostettman, 2006).

Aloe ferox Mill. (Xanthorrhoeaceae) commonly known as the ‘‘bitter aloe’’ or ‘‘Cape aloe’’ is a variable species indigenous to the Cape coastal region of South Africa (Glen and Hardy, 2000; Van Wyk et al., 1995). A. ferox has been used since ancient times and has an illustrious history in folk herbal medicine. This plant is one of only few depicted in ancient San rock paintings emphasizing the close interaction between ancient cultures and plants as a source of healing (Reynolds, 1985; Van Wyk, 2008). A. ferox has been used traditionally as a laxative in Africa which is the most important application for this plant. It has also been reported to

have bitter tonic, anti-oxidant, anti-inflammatory, antimicrobial and anticancer properties (Chen et al., 2012). The use of A. ferox in cosmetics and as a food supplement is a recent development that was stimulated by the tremendous commercial success of Aloe vera.

The bio-activity ascribed to A. ferox has in previous studies been directly associated with specific compounds in the leaf exudate. Aloesin decreases the plasma insulin levels (Yimam et al., 2013) and also inhibits mushroom tyrosinase (Wu et al., 2012). The latter property of aloesin has generated tremendous interest in the application of this chromone to treat hyperpigmentation and has been included in cosmetic formulations as a natural and safe alternative to kojic acid (Kim et al., 2012). Aloesin ameliorates intestinal inflammatory responses in a DSS-induced ulcerative colitis rat model (Park et al., 2011) while the structural analogue, aloeresinA, presented a dose dependent a-glucosidase inhibitory activity in vivo (Jong-Anurakkun et al., 2008). The anthrone C-glycosides, aloin A and B exhibited antimicrobial (Coopoosamy and Magwa, 2006; Kambizi et al., 2004), anti-oxidant (Patel et al., 2012) and antitumour properties (El-Shemy et al., 2010). Aloin A and B have been recognized as the responsible compounds for the laxative effect of A. ferox (Breimer and Baars, 1976; Van Os, 1976).

This study aimed to develop a fast and efficient method for the isolation and analysis of the leaf exudate constituents in A. ferox by HPCCC and UPLC, respectively.

2. Results and discussion

2.1. UPLC

Several methods have been published on the HPLC analysis of A. ferox leaf exudate (Patel et al., 2012; Van Der Bank et al., 1995; Viljoen et al., 2001). In this study, an ultra fast UPLC method was used in which separation of all metabolites were achieved in less than 1.60 min (Fig. 1) while the shortest published HPLC method available is 30 min.

2.2. Isolation of bio-markers

Counter-current chromatography (CCC) as a highly efficient liquid–liquid chromatographic method, offers several advantages over conventional methods such as the ability to accommodate a variety of aqueous and non-aqueous organic solvent systems, the interchangeable use of liquid mobile and liquid stationary phases, good resolution and the cost-effectiveness of the technique, since columns are long-lasting and require only limited volumes of solvent. For separation by HPCCC several solvent systems consisting of $CHCl_3$, CH_2Cl_2, MeOH, EtOH, EtOAc, n-BuOH and H_2O were examined according to the polarity of the compounds. Different ratios of two groups of solvents including $CHCl_3$, CH_2Cl_2, MeOH, EtOAc, H_2O (group 1) and MeOH, EtOAc, n-BuOH, hexane, H_2O (group 2) were tested. In each solvent system, the concentration of each compound in upper and lower layers was determined by UPLC analyses and the distribution ratio was calculated based on reversed phase elution (the concentration in upper phase divided by the concentration in lower phase). The different combinations of the chloride systems (group 1) were too polar for isolation of bio-markers in the exudate. According to the partition coefficient values (K_D) for each compound in the second group, the solvent system comprising of EtOAc/n-BuOH/H_2O (3.5:1.5:5, v/v/v) was selected (Table 1). To rule out experimental error the shake-flask test of each solvent system for calculation of the K_D values was carried out in triplicate. Finally, using HPCCC and only in one run eight compounds were isolated from the leaf exudate of A. ferox (Fig. 2).

2.3. Identification of isolated compounds

In mass spectrometry both positive and negative ion modes were investigated and the results showed that higher sensitivities and more information were obtained in positive mode. The MS data of the eight compounds are as followed:

Compound 1: ESI-MS (m/z, rel. intensity %): 395.1 $[M+H]^+$ (100), 377.1 (14), 275.1 (8).
Compound 2: ESI-MS (m/z, rel. intensity %): 703.2 $[M+H]^+$ (100), 541.2 (39), 289.1 (5).
Compound 3: ESI-MS (m/z, rel. intensity %): 541.8 $[M+H]^+$ (100), 395.1 (3).
Compound 4: ESI-MS (m/z, rel. intensity %): 435.1 $[M+H]^+$ (78), 273.1 (41), 255.1 (100), 229.1 (16), 149.0 (13).

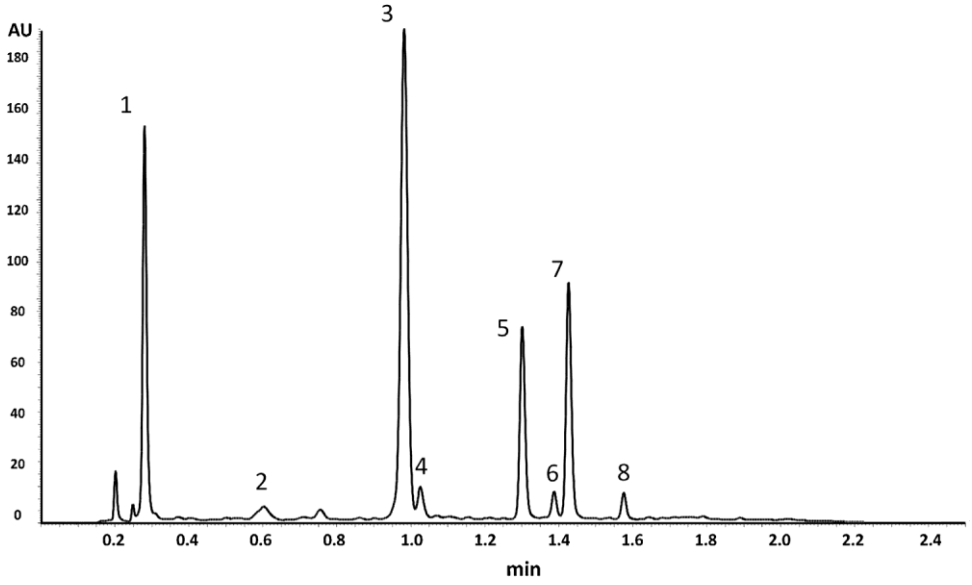

Fig. 1. UPLC chromatogram of the leaf exudate of Aloe ferox. The elusion with the mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 0.60 mL/min at a concentration of 17.5% B that was increased to 32% in 1.3 min and maintained for 1.5 min. Then immediately returned back to the initial concentration and kept for 1 min. The injection volume was 1 mL and the eluent was monitored at 297 nm.

Table 1
Isolated compounds, their retention times in UPLC and HPCCC, their partition coefficient values (K_D) for the selected solvent system and the amounts obtained.

No.	Isolated compounds	UPLC RT[a] (min)	HPCCC RT (from–to, min)	K_D[b]	Amount (mg)
1	Aloesin	0.27	elu[c] 25–28	0.29	46.0
2	Aloeresin C	0.59	elu 36–42	0.51	2.1
3	Aloeresin A	0.98	ext[d] 12–19	9.28	25.9
4	5-Hydroxyaloin	1.03	elu 114–130	2.19	4.1
5	Aloin B	1.30	elu 158–169	2.92	7.6
6	Aloinoside B	1.39	elu 58–62	0.94	2.7
7	Aloin A	1.43	elu 214–230	3.80	15.2
8	Aloinoside A	1.58	elu 84–92	1.52	3.1

[a] Retention time.
[b] K_D values are based on reversed phased mode.
[c] Elution.
[d] Extrusion.

Compound 5: ESI-MS (m/z, rel. intensity %): 419.1 $[M+H]^+$(74), 401.1 (16), 257.1 (43), 239.1 (100), 229.1 (17), 149.0 (14).
Compound 6: ESI-MS (m/z, rel. intensity %): 565.1 $[M+H]^+$(22), 419.1 (100), 401 (7), 239.1 (16), 229.1 (6), 149.0 (6).
Compound 7: ESI-MS (m/z, rel. intensity %): 419.1 $[M+H]^+$(72), 401.1 (13), 257.1 (41), 239.1 (100), 229.1 (18), 149.0 (16).
Compound 8: ESI-MS (m/z, rel. intensity %): 565.1 $[M+H]^+$(24), 419.1 (100), 302 (8), 239.1 (16), 229.1 (13), 149.0 (10).

The standards were analysed in the same condition and the MS data compared with published data (Wu et al., 2013; Zhong et al., 2013). The isolated substances were identified as aloesin, aloeresin C, aloeresin A, 5-hydroxyaloin A, aloin B, aloinoside B, aloin A and aloinoside A. The yields of the isolated compounds are shown in Table 1 and their structures are presented in Fig. 3.

2.4. Isolation of stereoisomers

The isolation of stereoisomers poses several challenges in chromatography especially on a large scale. Among the isolated compounds in this study, aloin A and B as well as aloinoside A and B are stereoisomers (see Fig. 3). Isolation of single compound from a mixture of aloin A and B by counter-current chromatography was reported previously. Tang et al. isolated these two isomers using a solvent system composed of chroloform/methanol/n-butanol/water (4:3:1:2, v/v/v/v) (Tang et al., 2013), but in the present study all four stereoisomers in addition to four other compounds were isolated in a single run.

3. Materials and methods

3.1. Plantmaterial

The spray dried leaf exudate of A. ferox was a donation from Organic Aloe (Pty) Ltd. (Western Cape, South Africa).

3.2. Standards

Aloin A and B were purchased from Sigma (St. Louis, USA), the aloinosides were kindly supplied by Prof. Ermias Dagne (Ethiopia) and the chromones by Prof. G Speranza (Italy).

3.3. UPLC

The exudate and the isolated substances were analysed by UPLC based on a published method for HPLC (Chen et al., 2012). The UPLC

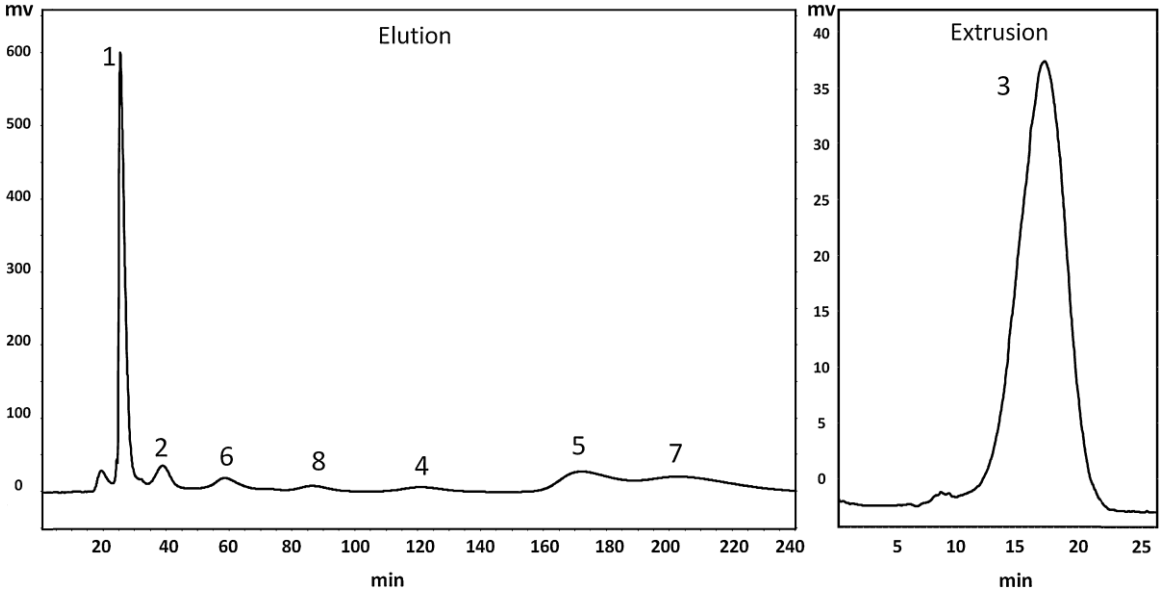

Fig. 2. HPCCC chromatograms of the leaf exudate of Aloe ferox. The isolation of 300 mg of the sample was carried out in reversed phase mode using EtOAc/n-BuOH/H_2O (3.5:1.5:5, v/v/v) at 30 8C. The flow rate was 3 mL/min while the coils were rotating at 1600 rpm. The eluent was monitored at 297 nm.

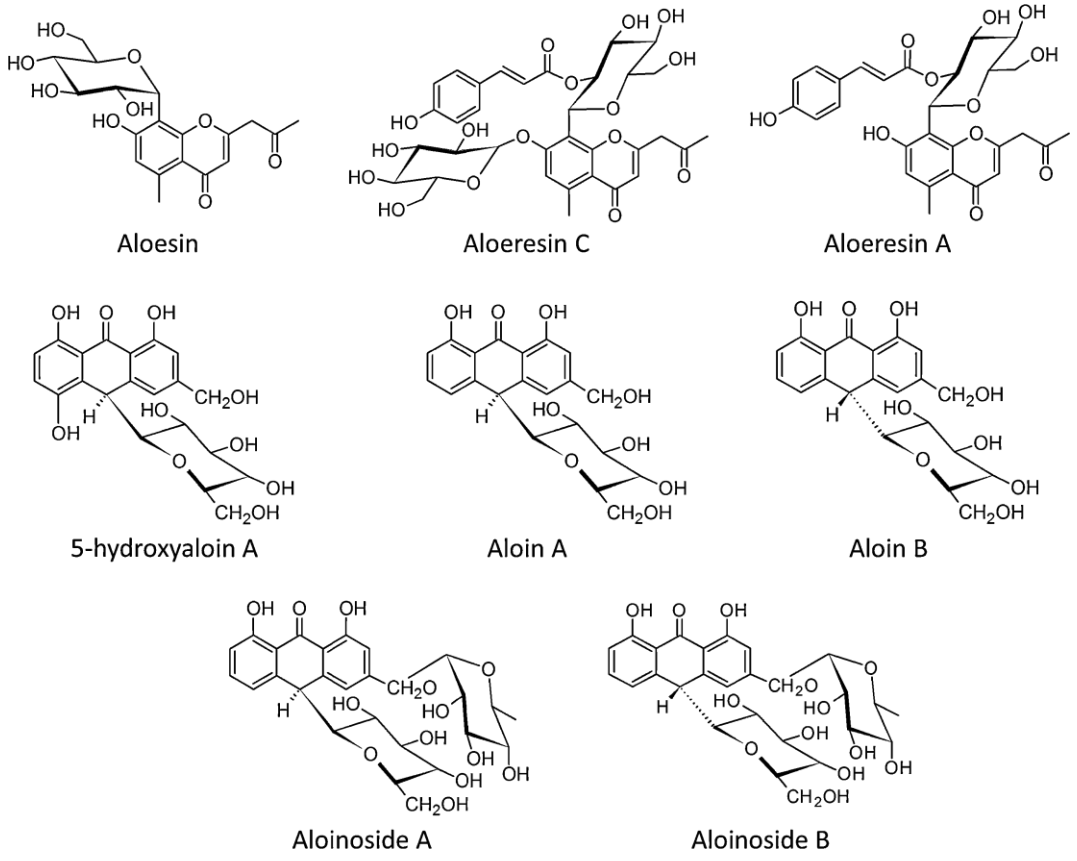

Fig. 3. Structures of isolated compounds.

comprised of a Waters Acquity instrument with an H Class quaternary solvent manager, PDA eλ detector and FTN sampler manager (Waters, Milford, USA) equipped with a BEH C18 (50 × 2.1 mm, 1.7 μm) column (Waters, Milford, USA). The chromatographic conditions were modified to reduce the analysis time to 2.5 min at 25 °C. The mobile phase consisted of water (A) and acetonitrile (B) at a flow rate of 0.60 mL/min. The analysis commenced with an initial concentration of 17.5% B that was increased to 32% in 1.3 min and maintained for 1.5 min; then immediately returned back to the initial concentration and kept for 1 min. The injection volume was 1 mL and the eluent was monitored at 297 nm.

3.4. HPCCC

The HPCCC was performed on a Spectrum instrument (Dynamic Extractions, Berkshire, UK) which is connected to a Quaternary gradient pump (Scientific Systems, Inc., PA, USA) and a Sapphire 600 UV detector (Prague, Czech Republic). The pump and the UV were monitored by an Agilent interface 35900E (Agilent Technologies, Santa Clara, USA) using EZChrom software. The fraction collector was a Gilson FC203B (Middleton, USA). The isolation was carried out in reversed phase mode using EtOAc/n-BuOH/H_2O (3.5:1.5:5, v/v/v) at 25 °C. The semi-preparative columns (total 175 mL) were filled with the upper phase as the stationary phase. The mobile phase (lower phase) was pumped in to the system using a flow rate of 3 mL/min while the bobbins were rotating at 1600 rpm until the instrument was equilibrated with a dead volume (V_{mp}) of 36 mL in 12 min. 300 mg of the sample (dissolved in 6 mL of upper phase/lower phase, 1:1, v/v) was injected after filtration. The elution run time was 240 min and the extrusion was 25 min at the same flow rate. The eluent was monitored at 297 nm and the fractions were collected per minute. Fractions were analysed by UPLC and similar fractions were combined. The solvent was evaporated with a Genevac A EZ-2 Plus (Suffolk, UK) to obtain eight pure compounds.

3.5. LC–MS

The pure compounds obtained from HPCCC as well as the standards were analysed by LC–MS to confirm their identity. For LC–MS analyses, a Waters Acquity UPLC with a binary solvent manager was used interfaced with a Xevo G2QTof mass spectrometer (Waters, Milford, USA). UPLC analysis was performed on the same column (see Section 3.3) with the mobile phase consisting of 0.1% formic acid in water (A) and acetonitrile (B). A gradient elution with the flow rate of 0.6 mL/min was started from 10% B increasing to 32% in 2.5 min and returned back to initial concentration in 0.5 min.

The mass spectrometer was operated in a positive ion electrospray mode. N_2 was used as the desolvation gas. The desolvation temperature was set to 350 °C at a flow rate of 600 L/h and the source temperature was 100 °C. The capillary and cone voltages were set to 2800 and 60 V, respectively. Data were collected between 100 and 1500 m/z.

4. Conclusion

Quality control is the most crucial aspect in the manufacturing of phytomedicines and cosmeceuticals. The process however

cannot be efficiently implemented in the absence of adequate analytical methods and authentic standards. This study showed that HPCCC can be considered a fast and reputable method for the isolation of bio-markers (including stereoisomers) from a crude extract which can easily be scaled up from analytical to preparative processes to yield sufficient quantities of pure molecules for quality control purposes and pharmacological studies.

Conflict of interest

The authors have declared that there is no conflict of interest.

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