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Chromatographic evaluation of phenolic acids in some cultivated Aloë species

Chromatograficzna analiza kwasów fenolowych w uprawianych gatunkach z rodzaju *Aloë*

Aloë genus is composed of about 280 tropical or subtropical plants, which are distributed in South Africa, Latin America, Asia and the Caribbean. The *Aloë* has been used for thousands of years to heal a variety of conditions, most notably burns, wounds, skin irritations and constipation. *Aloe* was one of the most frequently prescribed medicines throughout most of the 18th and 19th centuries and it remains one of the most commonly used herbs [13, 19].

Two basic products from the leaves of *Aloë* species are obtained: a gel of parenchyma cells and *Aloe* latex, a yellow, bitter liquid from the skin of the aloe leaf [16]. The gel is derived from the pulp of the leaves and contains many polysaccharides [10] (including mannan, galactan, glucomannan, arabinorhamnogalactan, pectins and glucuronic acid-containing polysaccharide) [4], phytosterols, organic acids, tannins, vitamins, minerals, enzymes, saponins, fatty acids and other organic and inorganic compounds [3, 11, 21]. Latex (also known as drug aloe) contains anthraquinone gycosides, constituents with strong laxative effects [10, 12]. The phytochemical analysis of the exudate revealed that aloe is comprised of a mixture of phenolic compounds, mainly anthrones (aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives), chromones and phenylpyrones with a low content of polysaccharides and aliphatic compounds [12,20].

Aloe has a broad range of pharmacological properties, including antiinflammatory, antiviral, antioxidative actions [9], antibacterial, immunostimulant [25], antifungal [21], analgesic, antitumor, antidiabetic and inhibition of tumor cells activation and proliferation [3, 6, 24]. *Aloe* preparations are also used in cosmetic industry, to product creams, lotions, cleansing milk, skin tonic and face masks.

Aloe preparations were taken internally as a purgative, acting on the lower bowel. They were used in atonic constipations but the oral use of aloe for constipation is no longer recommended, as anthraquinones present in latex can cause severe side effects [14, 19]. Overdosage can result in gastritis, diarrhoea and nephritis. As *Aloe* products stimulate uterine contractions, they should be avoided during pregnancy. They should also not be taken during lactation because they are excreted to breast milk [19].

The variety of biologically active compounds found in *Aloë* species still draws scientists' attention and remains a rich source of investigations. Within this constituents, phenolic compounds, especially phenolic acids, seem to be poorly investigated [9].

The dried flowers from *Aloë barbadensis* Mill. were analysed verifying chlorogenic, caffeic, 5-p-coumaroylquinic, caffeoylshicimic, 5-feruloylquinic, 5-p-*cis*-coumaroylquinic, p-coumaric and ferulic acid. Flavonoides were also found (e.g. luteolin, apigenin, quercetin, kaempferol, isoorientin, isovitexin). The presence of cinnamic acid, oxycinnamic [23] and salicylic acid [1] in *Aloë vera* and protocatechuic acid in *Aloë berhoma, Aloë rivae, Aloë mgalacantha, Aloë pulcherie* was demonstrated [5]. There are also some reports about the presence of ferulic, cinnamic, caffeic and p-coumaric in estric associations with chromone's derivatives. In industial trials of aloe resin p-coumaric and cinnamic acids were found [2].

Phenolic acids are pharmacologically active compounds. Their occurrence may exert a crucial impact on the herb's activity. That is why, in this research the qualitative evaluation of phenolic acids composition in few *Aloë* species was carried out.

MATERIAL AND METHODS

The study material were a few years' raw leaves of seven *Aloë* species collected in UMCS Botanical Garden in Lublin, in May 2006: 1) *Aloë arborescens* Mill. (1000g), 2) *Aloë ferox* Mill. (100g), 3) *Aloë saponaria* Haw. (50g), 4) *Aloë marlothii* Bgr. (50g), 5) *Aloë speciosa* Bak. (50g), 6) *Aloë bellatula* Reyn. (50g), 7) *Aloë ciliaris* Haw, (50g).

The material was stabilized in 99.8º alcohol to inactivate enzymes released from the damaged cells. Subsequently, the material was extracted three times (1h) by reflux in hot water bath with 95% ethanol and 50% ethanol. Ethanolic and ethanolic-aqueous extracts were filtered, mixed and the solvent was evaporated in the vacuum. The dry remains (1/10 part from *A. arborescens*) were dissolved in 5 ml of 95% ethanol and subjected to chromatographic analysis. The second part (9/10) of *A. arborescens* dry extract was eluted with hot water portions. Water extract was filtered in order to eliminate the ballast compounds and extracted with petroleum ether. Subsequently, fraction A – of free phenolic acids was isolated in the typical way from water layer by extraction with diethyl ether [8]. Bound phenolic acids were liberated using alkaline and acid hydrolysis. The process of acidic hydrolysis was performed in the following conditions: conc. HCl, pH 2–3, 100ºC, 1h. The alkaline hydrolysis: $NaBH_4$ (in the amount of 0.8 g per 100ml) and 1% $Ba(OH)_2$, pH 12–13, 100º C, 15 min. Both hydrolysates were cooled, filtered and extracted with diethyl ether. After that, fractions B and C of phenolic acids released after acidic and alkaline hydrolysis was isolated in the analogous way to fraction A.

The obtained A, B and C *Aloë arborescens* Mill. extracts were analyzed with the use of two dimensional thin-layer chromatography (2D-TLC) [22] and high performance liquid chromatography (HPLC). The extracts of other *Aloë* species were analyzed only with the use of HPLC.

Thin-layer chromatography. In the TLC and 2D-TLC analysis cellulose plates were used (DC-Fertigplatten, Merck, cellulose 100 x 100 x 0.1 mm), which were developed in the horizontal DS-chambers according to the earlier published procedure [22]. After drying, the chromatograms were observed in UV light (λ = 366 nm) before and after treatment with ammonia vapor and in daylight after spraying with the typical reagents [15].

HPLC analysis. The used HPLC system was from Knauer (Berlin, Germany) and it consisted of a pump Model K-1001 and UV/VIS detector, equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20 µl sample loop, computer program Chroma 2000. A Hypersil ODS column (250 x 4.6 mm id; 5 µm, Merck, Darmstadt, Germany) was used. All compounds were detected at λ = 254 nm.

Phenolic acids were separated by isocratic elution with methanol: water (25:75, v/v) containing 1% (v/v) acetic acid at a flow-rate of 1.0 ml/min.

The organic solvents were of HPLC grade (Merck's methanol, J.T. Baker's acetic acid and water). After preparation, the mobile phase was filtered through 0.45 μm filter (J.T. Baker, Phillipsburg, NY, USA). Stock solutions of phenolic acids were prepared by dissolving 5 mg of each compound in 10 ml of methanol. Standard solutions of phenolic acids (Sigma, St. Louis. MO, USA) were prepared in methanol over the concentration range 0.025–0.1 mg/ml. The volumes injected amounted to 10 μl.

Calibration curves were obtained by plotting the peak height (y) against the concentration of standard solutions (x) and they showed linear relationships. All phenolic acids were quantified using the external standard method. The identification of phenolic acids was accomplished by comparing their retention times with those of appropriate standard compounds.

The methanolic extracts for quantitative RP-HPLC analysis were prepared according to the procedure described above. All phenolic fractions (A, B, C) were filtered through 0.45 μm membrane filters and injected into the chromatographic column.

RESULTS AND DISCUSSION

The aim of the study was a quantitative and qualitative analysis of phenolic acids in the leaves of several *Aloë* species. Especially the leaves of *Aloë arborescens* Mill. were thoroughly investigated. This species is cultivated in Poland, in greenhouses in Klęka near Poznań. Plant material obtained in that way is used to produce Polish pharmaceutical preparations (eg: Biostymina, Bioaron). Analysis of the academic literature demonstrated that phenolic acids in *Aloë arborescens* Mill. was poorly examined [17, 18].

In this work a detailed quantitative and qualitative analysis of phenolic acids in raw *Aloë arborescens* Mill. leaves was carried out. TLC and HPLC examinations of free and bound phenolic acids were conducted. As a result of the studies, fractions of free and liberated (after acidic-B and alkaline-C hydrolysis) phenolic acids were isolated. The obtained results of TLC analysis of each *Aloe arborescens* fraction are presented in Table 1.

Due to the performed analysis the following phenolics were identified: p-coumaric, ferulic, homoprotocatechuic, β-phenyllactic, protocatechuic, caffeic, p-hydroxybenzoic p-hydroxyphenylacetic, syringic, o-hydroxyphenylacetic, trans-cinnamic and vanillic. Also, methylic ester of p-coumaric acid and aloeemodin were detected.

All identified phenolic acids (except vanilic acid) were provided for the first time for the analyzed species. For the first time the presence of homoprotocatechuic, β-phenyllactic, syringic, p- and o-hydroxyphenylacetic acid was proved. Also, the occurrence of other phenolic compounds, which have not been identified through a lack of standards, were observed in the leaves of *Aloë arborescens* Mill.

In the majority of cases the identified phenolic acids occurred in free and bound forms. Only p-hydroxyphenylacetic, o-hydroxyphenylacetic and trans-cinnamic acids were observed exclusively in the fraction obtained after alkaline hydrolysis. It may indicate that these acids are present in plant material only in ester form in different compounds.

A quantitative analysis of phenolic acids was carried out (Table 2). A great diversification of the content of particular compounds was demonstrated, from 0.01 mg % (protocatechuic acid, fraction A) to 1.46 mg % (p-coumaric acid *cis* in C fraction), in fresh material and, respectively, 0.26 mg % to 41.81 mg % of dry plant material.

A large amount of p-coumaric and ferulic acids attracts attention, especially an increased amount

of these compounds after acidic hydrolysis, eg. p-coumaric *trans* and *cis*, from 1.5 mg % and 1.37 mg % to 36.97 mg % and 41.81 mg %, respectively. Those result confirmed information signaled before about the presence in *Aloë* leaves estric connections of these acids with chromone's derivatives. It also suggests a great amount of these connections in the examined plant material. The results of the quantitative analysis are shown in Figure 1.

Table 1. The occurrence of phenolic acids in leaves of *Aloë arborescens*

No.	Phenolic acids	TLC analysis		
		A	B	C
1	homoprotocatechuic	+	+	+
2.	β- phenyllactic	+	+	+
3	protocatechuic	+	+	+
4	caffeic *trans cis*	+ +	+ +	+ +
5	p-hydroxyphenylacetic	–	–	+
6	p- hydroxybenzoic	+ +	+	+
7	p-coumaric *trans* *cis*	+ ++	+++ ++	++++ +++
8	vanillic	+	+	+
9	syringic	+	+	+
10	o-hydroxyphenyllacetic	–	–	+
11	ferulic *trans* *cis*	+ ++	+++ ++	+++ +++
12	p-coumaric acid methyl ester *trans cis*	 + +	 + +	 ++ ++
13	Trans-cinnamonic	–	–	+

+ detectable, – not detectable, A – fraction of free phenolic acids, B, C – fractions of bound phenolic acids

The further investigation and RP-HPLC analysis showed the presence of phenolic acids in six Aloë species (Table 3). A comparison of the chromatographic data allowed to form a sequence of species in the order of phenolic acids content (from the highest to the lowest amounts): *Aloë ferox* Mill.→ *Aloë speciosa* Bak. → *Aloë saponaria* Haw. → *Aloë marlothii* Bgr. → *Aloë bellatula* Reyn. → *Aloë ciliaris* Haw.

The highest amount of phenolic acids was found in *Aloë ferox* Mill., the lowest in *Aloë ciliaris* Haw. *Aloë arborescens* Mill. and *Aloë speciosa* Bak. possess a similar quantity of phenolic acids. Other species have a definitely lower content of these compounds.

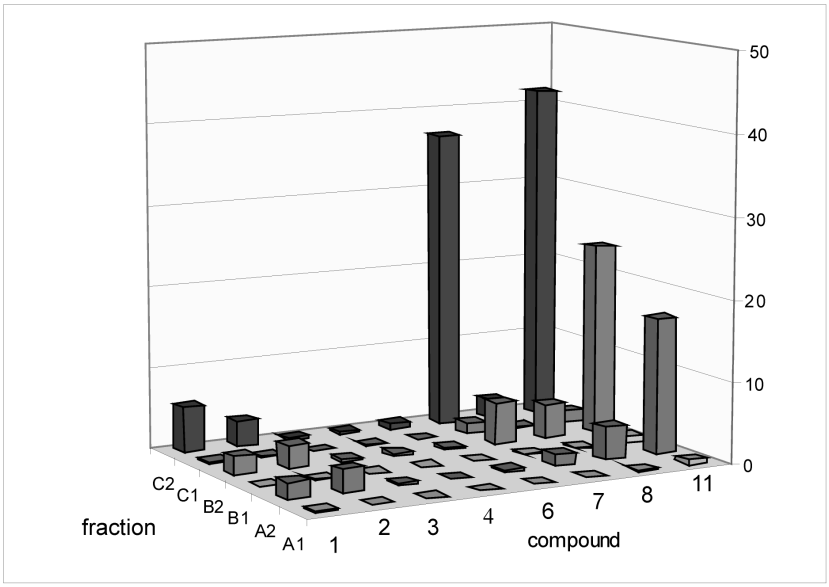

Fig. 1. The occurrence of phenolic acids in *Aloë arborescens* Mill. Explanation see Table 2

Table 2. The content of phenolic acids in *Aloë arborescens* Mill. (mg %). Explanation see Table 1

No.	Phenolic acid	A		B		C	
		1	2	1	2	1	2
1	homoprotocatechuic	0.07	2.02	0.08	2.28	0.20	5.67
2	β- phenyllactic	0.10	2.76	0.10	2.86	0.11	3.22
3	protocatechuic	0.01	0.26	0.01	0.33	0.01	0.30
4	caffeic *trans* *cis*	tr	tr	0.01	0.36	0.01	0.31
6	p- hydroxybenzoic	0.01	0.30	0.01	0.28	0.02	0.60
7	p-coumaric *trans* *cis*	0.05	1.50	0.18	5.22	1.29	36.97
8	vanillic	0.14	4.08	0.15	4.28	0.08	2.32
11	ferulic *trans* *cis*	0.58	16.68	0.83	23.63	1.46 1.42	41.81 40.69

A – fraction of free phenolic acids, B, C – fractions of bound phenolic acids, 1 – content of compound in fraction in mg/100g of fresh material, 2 – content of compound in fraction in mg/100g of dried material, tr – trace amount

Table 3. The occurence of phenolic acids in *Aloë* species

No.	Phenolic acid	*Aloë ferrox*	*Aloë saponaria*	*Aloë marlothii*	*Aloë speciosa*	*Aloë bellatula*	*Aloë ciliaris*
1	p-coumaric	+	+	+	+	+	+
2	ferulic	+	+	+	+	+	+
3	homoprotocatechuic	+	+	+	+	+	+
4	protocatechuic	+	+	+	+	+	+
5	p- hydroxybenzoic	+	+	+	+	+	+
6	vanillic	+	+	+	+	+	+
7	caffeic	+	+	+	+	+	+
8	syringic	-	-	-	+	-	-

The present study demonstrated for the first time the significant presence of p-coumaric, ferulic, homoprotocatechuic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic in *Aloë arborescens* Mill. and other Aloë species, especially *Aloë ferox* Mill. The majority of identified compounds posseses the proved pharmacological activity. Immunostimulant properties of p-coumaric and ferulic acids are well known. Moreover, p-coumaric acids posseses antiviral, antifungal, ferulic acid anti-inflammatory, caffeic acid – antitumor, p-hydroxybenzoic and vanillic antibacterial properties.

Phenolic acids may act synergistically with the main active compounds of Aloë and exert a beneficial impact on the pharmacological activity of the obtained pharmaceutical preparations [9].

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SUMMARY

The *Aloë* genus has been used for thousands of years in the treatment of burns, wounds, skin irritations and constipation. *Aloe* has a broad range of pharmacological properties, including antiinflammatory, antiviral, antioxidative actions, antibacterial, immunostimulant, antifungal, analgesic, antitumor, antidiabetic and inhibition of tumor cells activation and proliferation. Phenolic acids are active compounds and may exert a beneficial impact on the pharmacological activity of *Aloë.* Thus, TLC and HPLC methods were used for quantitative and qualitative evaluation of phenolic acids, both free and liberated (by acidic and alkaline hydrolysis) in the leaves of *Aloë arborescens* and other *Aloë* species. The assay demonstrated the presence of phenolic acids mainly: p-coumaric and ferulic in the material. The leaves of *A. arborescens* also contain 11 other phenolic acids: homoprotocatechuic, β-phenyllactic, protocatechuic, caffeic, p- hydroxybenzoic,

p-hydroxyphenylacetic, syringic, o-hydroxyphenylacetic, trans-cinnamic and vanillic. The content of particular compounds was demonstrated and it ranged from 0.01 mg % (protocatechuic acid, free) to 1.46 mg % (p-coumaric acid *cis,* liberated after alkaline hydrolysis), in the fresh material and, respectively, 0.26 mg % to 41.81 mg % of dry plant material.

STRESZCZENIE

Różne gatunki aloesów są od wieków znanymi i cenionymi roślinami leczniczymi. Rośliny te wykazują m.in. działanie immunostymulujące, przeczyszczające, przeciwbakteryjne, przeciwgrzybicze, żółciopędne, ułatwiające gojenie ran. W pracy metodami chromatograficznymi (TLC, HPLC) analizowano obecność i zawartość kwasów fenolowych w kilku gatunkach aloesów ze szczególnym uwzględnieniem *Aloë arborescens* Mill. W rezultacie w jego liściach stwierdzono występowanie 13 fenolokwasów: ferulowego, p-kumarowego, homoprotokatechowego, β-fenylośminlekowego, protokatechowego, kawowego, p-hydroksyfenylooctowego, p-hydroksybenzoesowego, wanilinowego, syryngowego, o-hydroksyfenylooctowego, trans-cynamonowego oraz estru metylowego kwasu p-kumarowego oraz określono zawartość głównych związków z tej grupy, która kształtowała się w zakresie od 0,01 mg % (dla wolnego kwasu protokatechowego) do 1,46 mg % (kwasu cis -p-kumarowego, uwolnionego w wyniku hydrolizy zasadowej) w świeżym surowcu oraz odpowiednio 0,26 mg %–41,81 mg % w surowcu wysuszonym. Chromatograficznie porównano również obecność kwasów fenolowych w liściach sześciu innych gatunków z rodzaju *Aloë.*