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HPLC Fingerprinting of Sennosides in Laxative Drugs with Isolation of Standard Substances from Some Senna Leaves

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Abstract Senna leaves are one of the oldest medicinal herbs and they are used as laxative. Herbal teas which contain senna leaves are most commonly used to promote weight loss. The quality control of slimming teas which contain Senna leaves and also pharmaceutical preparations including Senna extract enriched by sennoside B was achieved by HPLC fingerprinting method. While the presence of sennoside A and B in laxative drugs was proved, it was seen to be devoid of sennosides in slimming teas. Kaempferol 3-*O*-*β*-D-gentiobioside (**1**), aloe-emodine 8-*O*-*β*-D-glucopyranoside (**2**), rhein 8-*O*-*β*-D-glucopyranoside (**3**), torachrysone 8-*O*-*β*-D-glucopyranoside (**4**), isorhamnetine 3-*O*-*β*-D-gentiobioside (**5**) were also isolated from Senna leaves.

Keywords: Anthraquinones; flavonoids, naphthalenes; chemical fingerprint; HPLC; Senna leaves.

1. Introduction

Senna extracts which contain anthranoids are commonly used for constipation and anthranoids are well known for their laxative properties [1]. Various constituents. which are classified as anthraquinones, dianthrones, flavonoids, naphthalenes were isolated from Senna leaves (*Cassia* spec., Leguminosae) [2-5]. The major constituents of Senna leaves are anthranoid derivatives

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and therefore they have strong laxative effects which originated especially from sennosides A and B. Commercial herbal teas, which have the slimming effect, include Senna leaves. Generally, Senna leaves are imported from India or Sudan to Turkey and usually used for losing weight.

One of the common quality control methods used by industry is analyzing chemical markers which are known to be present in the herbal products. However, variances due to geographical source, cultivation and processing methods affect the chemical composition and clinical efficacy. Therefore, it is necessary to establish a method to control the quality of Senna leaves.

Fingerprinting technique has been widely accepted as a useful method for the evaluation and quality control of herbal materials and their finished products. Several techniques, such as thin layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) [6], infrared spectroscopy (IR) [7], high-performance liquid chromatography (HPLC), ultra-violet spectroscopy (UV), mass spectrometry (MS) and nuclear magnetic resonance (NMR) can be applied for fingerprinting [8].

The aim of this study is the quantitative analysis of sennosides A and B in the herbal slimming teas and laxative pharmaceutical preparations in Turkey.

2. Materials and Methods

2.1. General

The UV (MeOH) spectra were recorded on an Agilent 8453 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury plus 400 MHz for proton and 100 MHz for carbon using TMS as internal standard. The solvents were $CDCl_3$ and CD_3OD. TLC was carried out on precoated silica gel 60 F_{254} aluminium sheets (Merck). For column chromatography (CC) normal phase silica gel 60 (0.063-0.20 mm Merck) and Sephadex LH-20 (Fluka) were used. Compounds were detected by UV fluorescence and/or spraying with vanillin-H_2SO_4 reagent followed by heating 100 ^{o}C for 5-10 min and/or exposure to NH_3 vapor.

2.2. Plant Material and Pharmaceutics

Slimming teas were provided from market. Pharmaceutical preparations were assured from pharmacy

2.3. Reagents and standard solutions

HPLC-grade methanol was provided from Merck. Deionized water was prepared using a Millipore Milli-Q Plus filter systems (Millipore, Bedford, MA, USA). Standards of sennosides A and B were purchased from Fluka (Switzerland). The anthraquinones, rhein-8-*O*-*β*-glucopyranoside, aloe-emodine-8-*O*-*β*-D-glucopyranoside, the flavonoids, kaempherol-3-*O*-*β*-D-gentiobioside, isorhamnetine-3-*O*-*β*-D-gentiobioside, the naphtalenes, torachrysone-8-*O*-*β*-D-glucopyranoside were isolated from the leaves of Senna and their structures were spectroscopically elucidated by us.

2.4. Extraction and fractionation

Senna containing tea mixture (1 g) was extracted with methanol (20 mL) under reflux for 30 min.. After filtration and concentration of extract was diluted with 10% aqueous methanol to 20 ml. Aqueous methanol phase was extracted with 2 × 10 ml chloroform., 2 × 10 mL ethyl acetate and 2 × 10 mL *n*-butanol respectively. *n*-Butanol phase was concentrated and dissolved in 10 mL 70% methanol and then filtered through a nylon membrane filter (0.2 µm, 47 mm). The methanolic

extract solution directly injected into the HPLC.

As a pharmaceutical preparations were used tablets. A tablet was powdered and dissolved 70% methanol and after filtering was completed to 100 mL.

2.5. Isolation of the compounds

Powdered leaves of *Cassia acutifolia* Del. (300 g) were extracted with MeOH (3 × 3.5 L, 45 °C) and the combined extracts were evaporated to yield 44.0 g residue. The MeOH extract was dissolved in water and partitioned with petroleum ether, ethyl acetate and *n*-butanol. *n*-Butanol extract (9.56 g) was fractionated on a Sephadex LH-20 column eluting with 70% methanol to give eight main fractions (Fr. A-H). The fraction C was chromatographed over silica gel eluting with $CHCl_3$-MeOH-H_2O (100:10:5) to give Fraction a (161.3 mg). Further column chromatography was applied on Fr_a using silica gel and eluted with $CHCl_3$-MeOH-H_2O (80:20:2 to 60:40:4). A mixture of **1** and **5** (101.3 mg) was obtained. Fractions (Fr_{a1}) obtained from Fr_a chromatographed over Sephadex LH-20 column with methanol elution to get pure compound **2** (6.3 mg). Compound **4** (14.6 mg) was obtained from Fraction D using Sephadex LH-20 column and eluting with methanol. Fraction D gave compound **3** (8.8 mg) by silica gel column elution with $CHCl_3$-MeOH-H_2O (70:30:3 to 60:40:4).

*Compound (**1**);* Yellow amorphous powder. UV, IR, ^{1}H-NMR (CD_3OD, 400 *MHz*) and ^{13}C-NMR (CD_3OD,, 100 *MHz*) data are in agreement with data given in the literature for kaempferol 3-*O*-*β*-D-gentiobioside [9-11].

*Compound (**2**):* Orange amorphous powder. UV, IR, ^{1}H-NMR (CD_3OD,, 400 *MHz*) and ^{13}C-NMR (CD_3OD, 100 *MHz*) data are in agreement with data given in the literature for aloe-emodine 8-*O*-*β*-D-glucopyranoside [12].

*Compound (**3**):* Yellow amorphous powder.UV, IR, ^{1}H-NMR (CD_3OD,, 400 *MHz*) and ^{13}C-NMR (CD_3OD,, 100 *MHz*) data are in agreement with data given in the literature for rhein 8-*O*-*β*-D-glucopyranoside [13].

*Compound (**4**):* Yellow orange amorphous powder UV, IR, ^{1}H-NMR (CD_3OD,, 400 *MHz*) and ^{13}C-NMR (CD_3OD, 100 *MHz*) data are in agreement with data given in the literature for torachrysone 8-*O*-*β*-D-glucopyranoside [14].

*Compound (**5**):* Yellow amorphous powder. UV, IR, ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD,, 100 MHz data are in agreement with data given in the literature for isorhamnetine 3-*O*-*β*-D-gentiobioside [9-11].

2.6. HPLC-PDA conditions and standard preparation

HPLC separation was conducted on an Agilent 1100 system (USA) with a photodiode array detector (PDA, Agilent 1100 G1315) working at 270 nm. Data acquisition and processing were performed using a chromatography software package (Chemstation version A. 07.01). The compounds were separated on a Dionex C_{18} column (4.6 mm × 250 mm, particle size 5μm). The mobile phase consisted of methanol / water. The linear gradient profiles were as follows: 40:60 (0 min), 40:60 (15 min), 70:30 (30 min), 40:60 (35 min). The flow-rate was 0.5 mL/min and the total run time was 35 min. The injection volume was 20 μL. Identification of compounds was performed by retention time and by spiking with standards and isolated compounds under the same conditions. The UV-VIS

spectral data from the photodiode array detector were collected within 35 min over the 265–320 nm range of the absorption spectrum, and the chromatograms were plotted at 270 nm. Peaks were assigned according to their retention times and by co-elution with authentic standards, as well as based on UV spectra for both the standards and samples under the same chromatographic conditions.

The standard stock solutions (100 µg/mL) were prepared by dissolving 1 mg of each compounds in 10 mL of methanol:water (70:30). These solutions were stored in dark glass bottles at 4 °C.

2.7. Calibration curves

Working standard solutions, which spanned a concentration range from 5 µg/mL to 200 µg/ml for HPLC analysis were prepared.

3. Results and Discussion

Usually, Senna leaves are imported from India or Sudan to Turkey. *Cassia acutifolia* and *Cassia angustifolia* are known as Senna. Determination of the active substances is an important criterion for the standardization of herbal medicine. Phytoequivalence is important to prove the safety and efficacy of herbal medicine. In the last years fingerprint method has been an important method for the standardization of herbal medicines [15]. EMA (European Medicines Agency) and FDA (Food and Drug Administration) both suggest the fingerprint method to evaluate the compatibility of herbal medicine to standard extract. The change of the concentration of components causes a different therapeutic activity, therefore, fingerprint method is a valid and effective method for quality control of herbal medicine. Commercial herbal slimming teas contain senna leaves but it is not clear which *Cassia* species are that.

The quantitative method for the chemical markers confirm the presence of the compounds but it does not confirm the presence of the plant material which contains the chemical markers. In this study, provided materials taken as "*Cassia* sp." were analysed microscopically to determine its species. Nevertheless, it is not possible to decide which one is *Cassia acutifolia* or *Cassia angustifolia*. Therefore determination of chemical markers of Senna is very important for quality.

Firstly, studies were planned to isolate of sennosides from imported "*Cassia* sp." but it was not possible to obtain sennosides A and B. However, kaempferol 3-*O*-*β*-D- gentiobioside (**1**), aloe-emodine 8-*O*-*β*-D-glucopyranoside (**2**), rhein 8-*O*-*β*-D- glucopyranoside (**3**), torachrysone 8-*O*-*β*-D-glucopyranoside (**4**), isorhamnetine 3-*O*-*β*- D-gentiobioside (**5**) (Scheme) were isolated from the leaves of Senna and their structures were identified based on spectroscopic data [^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC (400 MHz, CD_3OD)] and the comparison of spectral data with those reported [9-14] (Table 1 and 2).

Herbal teas show slimming effects because of sennosides of senna leaves. The lack of sennosides, which are responsible for laxative effect, was an interesting result. Therefore further investigations were carried on senna leaves containing herbal teas whether they have sensonides or not.

HPLC analysis of the anthraquinones in Senna was conducted on a reversed-phase (RP) column using organic solvents such as acetonitrile or methanol, and aqueous acetic acid or aqueous phosphoric acid [16]. In the present study, it was aimed to develop a simple, sensitive, and rapid method with a high degree of specificity, to simultaneously determine the anthraquinones, dianthrones, flavonoids and naphthalenes of Senna. Since most of the compounds present in extract are known to be polar compounds, methods were developed based on reversed-phase (RP-18) HPLC. Therefore mixtures of methanol:water and acetonitrile:water in different proportions were tested as mobile phase for HPLC analyses, as were the effects of adding acetic acid to adjust the pH value (pH 4) and solvent strength over the separation. The best results were obtained using gradient elution with methanol:water. A significant improvement in the

separation of the peaks was also observed after adding acetic acid. Gradient analytical HPLC methods coupled with photodiode array detection (PDA) were applied simultaneously to separate flavonoids, anthraquinones, dianthrones and naphthalens. The individual compounds were well separated on a Dionex C_{18} column (4.6mm × 250mm, particle size 5µm reversed-phase column). Sample analysis was processed by Agilent software. The injection volume was 20 µl and flow rate was 0.5 ml/min.

As a result, under the optimised conditions a baseline separation was achieved within 35 min, with symmetrical, sharp and well-resolved peaks for almost all analytes. The peaks for compounds were sequenced according to the retention time, compared to the UV spectrum of known compounds, and their spiking with standards (Figure 1-2).

This is the first report to separate and determine quantitatively rhein 8– *O–β*-D-glucopyranoside, aloe-emodine 8-*O–β*-D-glucopyranoside, kaempferol 3–*O-β*-D- gentiobioside, isorhamnetine 3–*O-β*-D-gentiobioside, torachrysone 8–*O–β*-D- glucopyranoside, sennosides A and B simultaneously by HPLC. However, this method did not separate rhein 8–*β*-glucopyranoside and sennoside B very well. Sennoside B and rhein 8-*O-β*-D-glucoside was seen overlapped in HPLC. Photodiode array detector (PDA) was used for identifying separately of these two compounds.

In this study the amount of hydroxyanthracene derivatives was found very low in slimmimg teas. Such as rhein 8-O-*β*-D-glucopyranoside was 0.006-0.03% and the amount of aloe-emodin 8-O-*β*-D-glucopyranoside was also found to be similar. Slimming teas 2, 3 and 6 did not have aloe emodin. These results are not valid for the mentioned previously. The amount of naphthalene, torachrysone 8–*O–β*- glucopyranoside was found higher than the amount of other substances (Table 3).

According to Pharmacopoeia Europea quantity of hydroxy antracene derivatives is not supposed to be less than 2.5%. *C. alata* has been registered in the Thai Herbal Pharmacopeae and their hydroxy anthracene derivatives which have been calculated over rhein 8-O-*β*-glucoside, are not supposed to be less than 1% [17].

Instead of the presence of sennoside B, rhein 8–*O–β*-D-glucopyranoside was identified in slimming teas. Each chromatogram was checked with their UV spectra and the lack of sennosides was proved completely (Figure 1).

Differences in degradation pathways were observed between the powdered herbal material and the extract, depending on storage conditions and packaging materials. Within the crude plant material, sennosides may be degraded into the monomer of sennoside B which is rhein 8–*O-β*–D-glucopyranoside by enzymatic processes. Forced decomposition of herbal material under high temperature caused oxidative decomposition of the sennosides to rhein 8-*O-β*-D-glucopyranoside [18].

In contrast, in the UV spectra of pharmaceutical preparations sennoside B were observed instead of rhein 8-*O-β*-D-glucosides (Figure 2). Senna extracts containing pharmaceutical preparations are standardized over sennoside B. The three laxative drugs were investigated with the same method to prove whether they contain sennoside A and B or not. The content of sennosides of three laxative drugs belonging to different companies were quantitatively analysed by HPLC-PDA. The sennosides were clearly observed on each chromatogram and corresponding their UV spectra. As a result, it was determined that all samples contained sennoside A and B (Table 3).

Quantities of sennoside B in three laxative drugs were determined and its amount was found in laxative drug 1, laxative drug 2 and laxative drug 3, 4,32 mg/tablet, 1,49 mg/tablet and 5.82 mg/tablet, respectively (Table 3). As seen from the data sennoside B containing pharmaceutical preparations were shown equivalence qualitatively but they didn't equivalent quantitatively.

Table 1. ^{1}H NMR data for compounds **1-5**.

C/H atom	δ_H (ppm), *J* (Hz)	δ_H (ppm), *J* (Hz)	δ_H (ppm), *J* (Hz)	δ_H (ppm), *J* (Hz)	δ_H (ppm), *J* (Hz)
	1	**2**	**3**	**4**	**5**
2		7.26 *s*	7.72 *s*		
4		7.63 *s*	8.10 *s*	7.12 *s*	
5		7.69 *d* (8.0)	8.10 *d* (7.4)	6.76 *d* (2.2)	
6	6.19 *d* (0.7)	7.87 *t* (7.3)	7.73 *dd* (8.0/2.5)		6.19 *d* (0.7)
7		7.86 *d* (8.0)	7.86 *d* (7.7)	6.95 *d* (2.2)	
8	6.40 *d* (1.1)				6.40 *d* (1.1)
3-$\underline{C}H_2OH$		4.63 *s*			
2-$CO\underline{C}H_3$				2.58 *s*	
3-CH_3				2.28 *s*	
OCH_3				4.11 *s*	3.39 *s*
2′	8.10 *dd* (8.5/2.2)				8.08 *d* (2.6)
3′	6.89 *dd* (9.1/2.2)				
5′	6.89 *dd* (9.1/2.2)				6.91 *d* (8.4)
6′	8.10 *dd* (8.5/2.2)				7.62 *dd* (8.4/2.2)
Glu					
1″	5.23 *d* (7.7)	5.15 *d* (7.7)	5.17 *d* (7.7)	5.02 *d* (7.6)	5.37 *d* (7.7)
2″	3.03-3.46*	3.21-3.44*	3.19-3.44*	3.39-3.72*	3.03-3.46*
3″	3.03-3.46*	3.21-3.44*	3.19-3.44*	3.39-3.72*	3.03-3.46*
4″	3.03-3.46*	3.21-3.44*	3.19-3.44*	3.39-3.72*	3.03-3.46*
5″	3.03-3.46*	3.21-3.44*	3.19-3.44*	3.39-3.72*	3.03-3.46*
6″	3.65 *dd* (12.0/6.0)	3.44 *dd* (11.0/6.0)	3.44 *dd* (11.0/6.0)	3.72 *dd* (12.0/6.0)	3.65 *dd* (12.0/6.0)
	3.98 *dd* (12.0/2.0)	3.69 *dd* (11.3/2.0)	3.69 *dd* (11.3/1.9)	3.94 *dd* (12.0/2.0)	3.98 *dd* (12.0/2.0)
Glu					
1‴	4.16 *d* (7.7)				4.16 *d* (8.0)
2‴	3.03-3.46				3.03-3.46*
3‴	3.03-3.46				3.03-3.46*
4‴’	3.03-3.46				3.03-3.46*
5‴	3.03-3.46				3.03-3.46*
6‴	3.59 *dd* (12.0/6.0)				3.59 *dd* (12.0/6.0)
	3.74 *dd* (12.0/2.0)				3.74 *dd* (12.0/2.0)

* Signal patterns are not clear due to overlapping.

When the results were summarized, it was observed that, slimming teas did not have sennoside B and the percentages of the anthranoids were not of a significant value but somehow they showed strong laxative effect like laxative drugs which have quite high sennoside content.

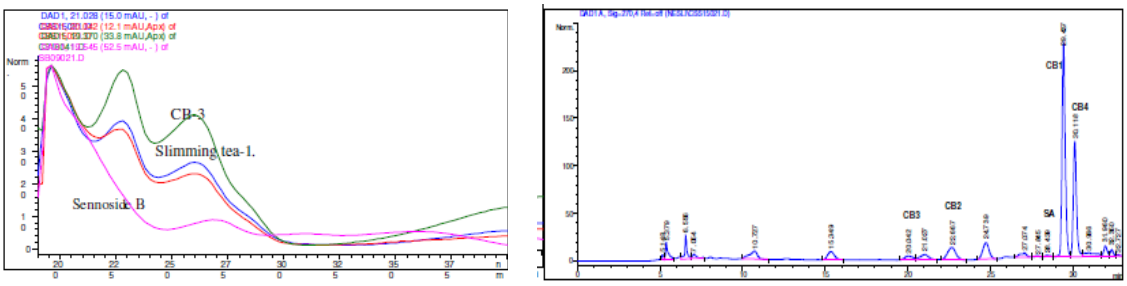
These results make us think of the malpractice for imported herbal teas, namely;

i. Imported "*Cassia* sp." could not be identified whether it was *Cassia acutifolia* or *C. angustifolia* because the powder plant materials have uncharacteristic microscobic

elements.

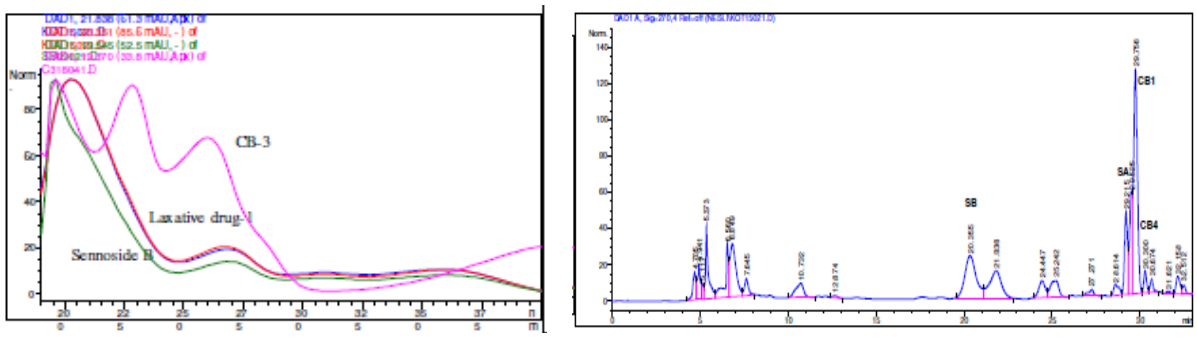
ii. Some *Cassia* sp. like *Cassia auriculata* (Palthe Senna) do not contain anthranoids which are responsible from laxative effect.

iii. *Colutea arborescens* are known as false Senna. From this viewpoint, as a result it could be said that either *Cassia auriculata* or *Colutea arborescens* are content of herbal teas instead of *Cassia acutifolia* or *C. angustifolia* , or some laxative effected unknown substances maybe put in the herbal teas.



(CB-1 and CB-5: kaempferol-3-*O*-*β*-D-gentiobioside and isorhamnetine-3-*O*-*β*-D-gentiobioside, CB-2: aloe-emodine-8-*O*-*β*-D-glucopyranoside, CB-3: rhein-8-*O*-*β*-D-glucopyranoside, CB-4: torachrysone-8- *O*-*β*-D-glucopyranoside, SA: sennoside A)

Figure 1. UV spectrums and HPLC chromatograms of slimming tea-1

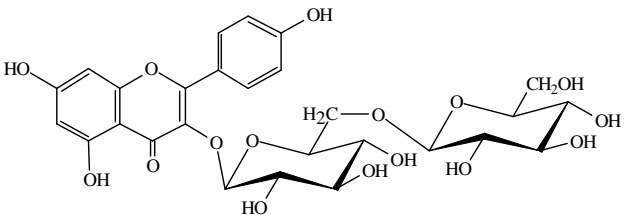


(CB-1 and CB-5: kaempferol-3-*O*-*β*-D-gentiobioside and isorhamnetine-3-*O*-*β*-D-gentiobioside, CB-4: torachrysone-8-*O*-*β*-D-glucopyranoside, SA: sennoside A, SB: sennoside B)

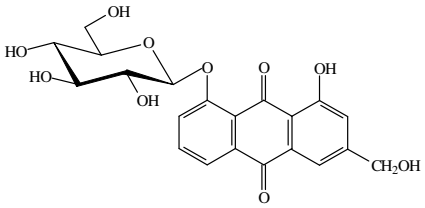
Figure 2. UV spectrums and HPLC chromatograms of laxative drug-1

Table 2. ^{13}C NMR data for compounds **1-5**.

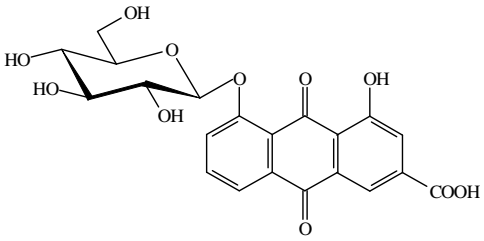
C atom	δ_C (ppm) **1**	δ_C (ppm) **2**	δ_C (ppm) **3**	δ_C (ppm) **4**	δ_C (ppm) **5**
1		162.34	161.86	153.00	
2	157.34	116.17	124.61	122.00	157.34
3	134.36	152.95	140.10	134.48	134.36
4	178.25	121.29	123.16	119.31	178.25
5	161.82	121.44	121.29	98.11	161.82
6	98.78	136.65	136.90	157.94	98.78
7	164.80	116.69	119.79	103.84	164.80
8	93.73	158.93	159.03	157.28	93.73
9	157.79	188.26	188.15	109.39	157.79
10	103.43	182.81	182.42	137.79	103.43
11		132.96	135.46		
12		123.13	118.95		
13		-	121.40		
14		135.50	133.46		
COOH			166.49		
3-CH_2OH		61.29			
2-$COCH_3$				206.69	
2-$CO\underline{C}H_3$				31.32	
3-CH_3				18.99	
OCH_3				55.86	55.81
1′	122.61				121.56
2′	131.17				113.39
3′	115.03				147.23
4′	160.36				134.90
5′	115.03				110.01
6′	131.17				121.80
Glu					
1″	103.31	101.18	101.19	101.08	103.31
2″	73.92	73.97	73.99	73.75	73.92
3″	76.77	77.22	77.23	77.21	76.77
4″	70.21	70.19	70.20	70.35	70.21
5″	76.66	77.95	77.96	77.47	76.66
6″	68.41	62.74	61.29	61.45	68.41
Glu					
1‴	102.93				102.93
2‴	73.88				73.88
3‴	76.62				76.62
4‴’	70.14				70.14
5‴	76.47				76.47
6‴	61.42				61.42



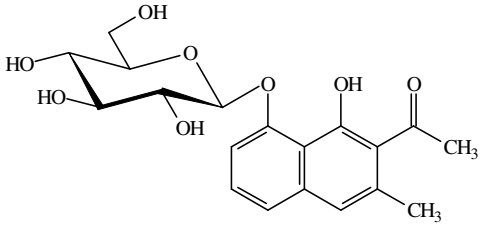
Kaempferol-3-*O*-*β*-D-gentiobioside (**1**)



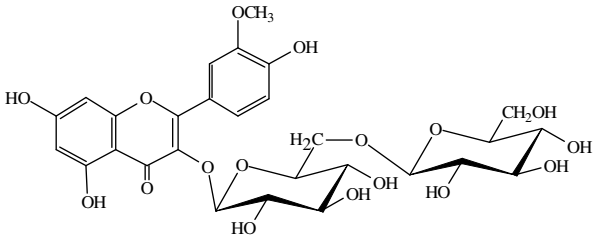
Aloe-emodine-8-*O*-*β*-D-glucopyranoside (**2**)



Rhein-8-*O*-*β*-D-glucopyranoside (**3**)



Torachrysone-8-*O*-*β*-D-glucopyranoside (**4**)



Isorhamnetine-3-*O*-*β*-D-gentiobioside (**5**)

Figure 3. The compounds from the senna leaves

Table 3. Percentages of anthraquinones, naphthalenes and flavonoids in slimming teas and laxative drugs

	%					
Slimming teas-Laxative drugs	**1 and 5**	**2**	**3**	**4**	**Sennoside A**	**Sennoside B**
Slimming tea 1	0.162	0.038	0.0066	0.182	-	-
Slimming tea 2	0.061	-	0.0125	0.166	-	-
Slimming tea 3	0.074	-	0.012	0.155	-	-
Slimming tea 4	0.15	0.007	0.0379	0.271	-	-
Slimming tea 5	0.077	0.0072	0.026	0.25	-	-
Slimming tea 6	0.056	-	0.0101	0.095	-	-
Slimming tea 7	0.051	0.0063	0.0127	0.064	-	-
Laxative drug 1	-	-	-	-	4.107	4.32
Laxative drug 2	0.933	-	-	1.826	3.106	1.49
Laxative drug 3	1.389	-	-	-	5.33	5.82

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