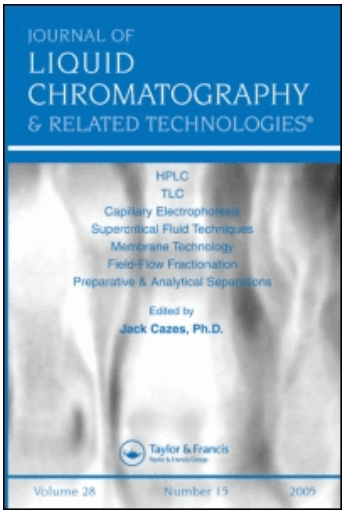

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THE COMPARATIVE STUDIES ON PHENYLPROPANOID GLYCOSIDES OF *VISCUM ALBUM* SUBSPECIES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Viscum L. is a semi-parasitic genus growing on various host plants, such as trees and shrubs. The plant is known as "Ökse Otu" in Turkey. In our country, *Viscum* L. is represented by one species and 3 subspecies. These subspecies are *V.album* ssp. *album, V.album* ssp. *abietis, V.album* ssp. *austriacum*. Comparative investigations were carried on with the active principles, phenylpropanoid glycosides, of the subspecies of *V.album*.

Qualitative and quantitative determinations of the phenylpropanoid glycosides named syringin, coniferin, and kalopanaxin D of 3 *V.album* subspecies were done by using HPLC. We developed a new isocratic mobile phase (Methanol:water: 0.1 N sodium acetate 20:73.5:6.5) for HPLC analysis of these group of compounds.

INTRODUCTION

The medicinal plant genus *Viscum* L. is a member of the family Loranthaceae. In Turkey, the genus is represented by one species, and its three subspecies. They are *V. album* L. ssp. *album, V. album* ssp. *abietis* (Wiesb.) Abromeit, *V. album* ssp. *austriacum* (Wiesb.) Vollmann.[1] In Turkish, *V .album* is known as "Ökse Otu," which means "bird-lime herb."[2]

The European mistletoe, *Viscum album* L. is an evergreen parasitic plant found widely throughout Europe, except in northern areas.[3] The main area of therapeutic applications are; cardiovascular illnesses, especially hypertension and arteriosclerosis, cancer, and arthrosis. A large number of constituents have already been isolated from the mistletoe drug. It is not yet known which compounds are responsible for its reported actions. The flavonoids and phenol carboxyclic acids, together with the phenylpropanes and lignans, as well as the amines, are all possible agents of cardiovascular activity.[4] Several pharmaceutical formulations containing *V. album* are currently available in the market for their antitumoral and antihypertensive effects especially in Germany and Switzerland.[5,6]

In Turkey this plant has been used since ancient times to treat various diseases.[2] In addition, *V. album,* has been used against hypertension in the eastern and southern parts of Anatolia.[7]

The chemical composition of the different *V. album* L. samples collected from various host plants from the view point of main active principles has been previously studied by our group.[8,9] In line with the above study, our qualitative analysis revealed that alkaloids, phenylpropanoids, flavanoids, cardioactive glycosides, lignans, reducing or nonreducing sugars, saponins, cyanogenic glycosides, tannins, and viscotoxins are found as the main constituents of the plant.[9] Continuing our research on these subspecies, comparative investigations were carried out, especially with phenylpropanoid glycosides of the subspecies of *V. album.*[10]

The leaves and stems of the samples belonging to three subspecies were dried and powdered, then extracted with ethanol at room temperature. The concentrated extracts were dissolved in water and extracted with petroleum ether (40°- 60°), diethylether, ethylacetate, and n-butanol/water, respectively. The yield of the fractions of *V. album* ssp. *album* were found higher than the others.

With the controls done by thin layer chromatography, it is defined that the contents of the extracts of n-butanol and ethylacetate were richer than the others, therefore, these extracts were selected for the further investigations.[10]

As a conclusion, the phenylpropanoid glycosides named syringin, coniferin, and kalopanaxin D were obtained from *V. album* ssp. *album*. The structures of all the compounds were elucidated by means of spectral evidence (UV, IR, ^{1}H-, ^{13}C-NMR, FAB-MS).[10]

In this study, qualitative, and quantitative determinations of the phenylpropanoid glycosides of three *V. album* subspecies were done by using High Performance Liquid Chromatography (HPLC).[10]

Additionally, the phenolic compounds were also isolated from *V.album* ssp.*album*. On the other hand, flavanonE and chalconE contents of the plant were not studied using HPLC, due to the isomerisation problems.[11]

MATERIALS

Chemicals

Authentic samples of the phenylpropanoid glycosides (syringin, coniferin, kalopanaxin D) were isolated from *V. album* ssp. *album* during the study. The structures of these compounds were elucidated by UV, IR, ^{1}H-NMR, ^{13}C-NMR, MS. HPLC grade solvents and bidistilled water were used for chromatographic studies. All chromatographic experiments were performed at room temperature.

Plant Materials

The three subspecies of *Viscum album* L. were collected from different regions of Turkey. All specimens are deposited in the Herbarium of Ankara University, Faculty of Pharmacy (Ankara, Turkey). The materials, specimen numbers and their collection sites are described below.

Species

Host Plant	Herbarium no	Locality
Viscum album L. *ssp. album* (*Armeniaca vulgaris* Lam)	AEF 18953	Ankara, Baglum in orchard, June 1995

Host Plant	Herbarium no	Locality
Viscum album L. ssp. *abietis* (Wiesb.) Abromeit (*Abies bornmülleriana* Mattf.)	AEF 18947	Bolu, Gölcük lake side, April 1995
Viscum album L. ssp. *austriacum* (Wiesb.) Vollmann (*Pinus nigra* Arn.)	AEF 18939	Ankara, Kizilcahamam Soguksu forest April 1995

Instrumentation

A Hewlett-Packard HPLC system was used, consisting of the following components: Model 1050 pump, equipped with a Rheodyne Model 7125 valve fitted with a 20 μL loop, a model 1050 UV detector set at 264nm, and HP-3996A integrator.

METHODS

Sample Preparation

The air-dried and powdered leaves, stems and twigs of three *V. album* subspecies were extracted with 80% ethanol at room temperature several times; the process being checked by TLC. After filtration, the filter paper was evaporated to dryness.

The H_2O-soluble part of the EtOH extract was successively extracted with petroleum ether (40°-60°), Et_2O, EtOAc, and BuOH. Each fraction was evaporated to dryness.

We used the EtOAc and the BuOH extracts for this study. The yields and the amounts of the extracts belonging to three *V. album* subspecies were given in the previous study.[12]

The EtOAc and the BuOH extracts of three *V. album* subspecies were weighed accurately into 10 mL volumetric flasks and adjusted with methanol. The suitable dilutions were prepared by transferring aliquots from these stock solutions into 10 mLvolumetric flasks. Then, they were filtered through a Millipore HA (0.45 μm) membrane filter and finally degassed under vacuum before use.

Table 1

Syringin and Resorcinol Ratios in Dilutions Used for Calibration Curve

	Syringin (0.006 mg/mL)	Resorcinol (0.0045 mg/mL)	Total Volume
1. Dilution	1.25 mL	1.00 mL	10 mL
2. Dilution	1.00 mL	1.00 mL	10 mL
3. Dilution	0.75 mL	1.00 mL	10 mL
4. Dilution	0.50 mL	1.00 mL	10 mL

Standard Preparation and Calibration Curve

For this analysis, the internal standard method was used. Resorcinol (Merck., Art.7590) reported in literature was used as an internal standard.[4,14] Resorcinol (5 μL) was injected into column and its retention time was determined as 8.735 min. Syringin (0.06 mg) was weighed accurately into a 10 mL volumetric flask and dissolved in methanol to prepare a 0.006 mg/mL solution. Resorcinol (1 mg) was prepared in this same way. This stock solution was used to prepare a 0.0045 mg/mL solution. The final dilution of resorcinol (1 mL) as an internal standard was then added to the syringin samples. The dilution ratios used for the calibration curve were given Table 1.

Calibration curve of syringin was constructed by triplicate injection of mixture of resorcinol-syringin at concentrations ranging from 0.30 μg/mL to 0.75 μg/mL for syringin and plotting the peak heights versus the concentrations.

At least four standard points were used for the curve and standard linear regression was used to determine the slope and intercept. The regression equations and correlation coefficients determined for the standard were [y=0.63030x+0.00137] (r=0.995137601).

Syringin is a major compound in all of the extracts and its structure is very similar to structures of coniferin and kalopanaxin D.[13] Therefore, the amounts of kalopanaxin D and coniferin were measured according to syringin.

Chromatographic Conditions

HPLC column: LiChrospher RP-18e, 5μm particle size, 4mm i.d.x 250mm (Hewlett-Packard Chemical Industries, Ltd.). Mobil phase: Water-methanol-0.1N sodium acetate (73.5:20:6.5 v/v/v pH:6.60). Flow rate: 0.8 mL/min. Detection: UV, 264 nm. Detection sensivity: 0.005 aufs. Column pressure: 151 barr. Chart speed: 0.3 cm/min.

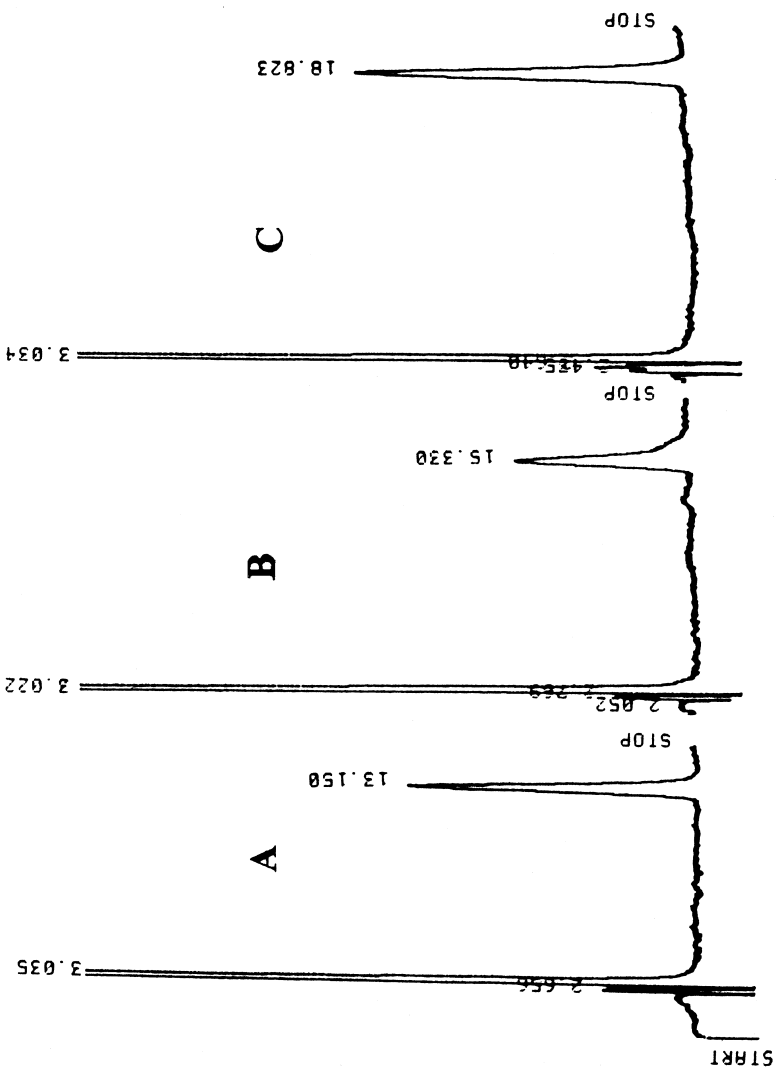

Figure 1. HPLC chromatograms of the standards (A) Coniferin; (B) Kalopanaxin D; (C) Syringin.

Table 2

The Concentrations of Extracts Belonging to *Album* Subspecies* Used in HPLC

Extracts Plant Name	EtOAC	n-BuOH
V. album ssp. *abietis*	0.375	0.250
V. album ssp. *album*	0.125	0.125
V. album ssp. *austriacum*	0.250	0.125

* mg/mL

RESULTS

In this study, the leaves, stems, and twigs belonging to three *Viscum album* L. subspecies were extracted with ethanol at room temperature. The extract was evaporated to dryness and then was extracted with PE, Et_2O, EtOAc, and n-BuOH, respectively.

Three phenylpropanoid glycosides [coniferin (1), syringin (2), kalopanaxin D, (3)] were isolated from the n-BuOH and the EtOAc extracts using chromatographic tecniques.[10] The structures of these compounds were elucidated by spectroscopic analysis (UV, IR, ^{1}H-NMR, ^{13}C-NMR, and FAB-MS).

The n-BuOH extract was fractionated on a silica gel column (VLC) and eluted with $CHCl_3$-MeOH-H_2O [(90:30:1 $\rightarrow$ (61:32:7)] and then with MeOH-H_2O (50:50). The fractions were tested with TLC. The combined fractions were subjected to MPLC on LiChroprep RP-18 using H_2O-MeOH mixtures of increasing amounts of MeOH (20-100 %) to give compound 1, 2, and 3.

Similarly, VLC and MPLC techniques were also applied to the EtOAc extract, and syringin was isolated. In this study, the three phenylpropanoid glycosides obtained from the EtOAc and the n-BuOH extracts belonging to three *V. album* subspecies, were evaluated qualitatively and quantitatively by HPLC.

For qualitative analysis, the retention times of eluted peaks of the n-BuOH and the EtOAc extracts belong to three *V. album* subspecies were compared with the retention times of authentic samples. The structure elucidation of the authentic phenylpropanoid glycosides isolated from *V. album* ssp. *album* were established by spectroscopic methods (UV, IR, NMR, FAB-MS). The retention times of the phenylpropanoid glycosides are given below (see Figure 1).

Table 3

Content of Phenylpropanes in *V. album* Subspecies*

Subspecies	Coniferin		Kalopanaxin D		Syringin	
	EtOAH	n-BuOH	EtOAc	n-BuOH	EtOAc	n-BuOH
ssp. *abietis*	0.0340 ± 0.0058	0.0434 ± 0.0005	Trace amount	0.0617 ± 0.0066	0.2077 ± 0.0189	0.2615 ± 0.0084
ssp. *album*	0.2953 ±0.0243	0.1768 ± 0.0243	Trace amount	0.2444 ± 0.0104	0.7475 ± 0.0127	0.5072 ± 0.0147
ssp. *austriacum*	Trace amount	0.0433 ± 0.0003	Trace amount	0.0792 ± 0.0034	0.2102 ± 0.0012	0.3106 ± 0.0013

* w/w %

Compound	Retention times (min.)
Coniferin	13.150
Kalopanaxin D	15.330
Syringin	18.823

We started HPLC with the concentrations of 0.125mg/mL n-BuOH and the EtOAc extracts. But we were not able to detect some of the compounds in some extracts. Therefore, the concentration of these extracts were increased two or three times and these concentrations were used for analysis.

The concentrations of the extracts used for HPLC analysis are shown in Table 2.

As a result of qualitative analysis, syringin was determined as the major compound in the EtOAc and the n-BuOH extracts belonging to the three *V. album* subspecies.

As shown in Table 3, the highest syringin amount is detected in ethylacetate and n-butanol extracts of *V. album* ssp. *album*. While coniferin is the highest amount in ethylacetate extract of *V. album* ssp. *album*, it is in trace amounts in ethylacetate extract of *V. album* ssp. *austriacum*.

The highest amount of kalopanaxin D is detected in n-butanol extract of *V. album* ssp. *album*. Ethylacetate extract of *V. album* ssp. *abietis* contains both syringin and coniferin, whereas, the presence of kalopanaxin D in this extract was not detected (Figures 2, 3).

DISCUSSION

In the literature, HPLC analysis of phenylpropanoids were chromatographed over Lichrospher 100 CH-18/2 (5 μm) using acetonitrile:water (9:91) (A), acetonitrile:water (25:75) (B) (Gradient) as a mobile phase.[14] But, in this study, this mobile phase did not succeeded for qualitative and quantitative analysis. In 1998, HPLC analysis of phenylpropanoids isolated from *V. album* (*Pyrus caucasica* Fed.) was achived on a LiChrospher RP-18 (4x125 mm, 5 μm) using water:acetonitrile 1 % 0.1 N phosphoric acid (gradient CH_3CN in water: 0% $\rightarrow$ 50%, 1 mL/min, 30 min) as mobile phase.[15]

In this study, we found a new mobile phase (methanol:water: 0.1 N sodium acetate 20:73.5:6.5, isocratic) for HPLC analysis of this group of compounds. It is an isocratic mobile phase. Therefore, it could be evaluated as a new finding for HPLC analysis of this group of compounds in *V. album* subspecies.

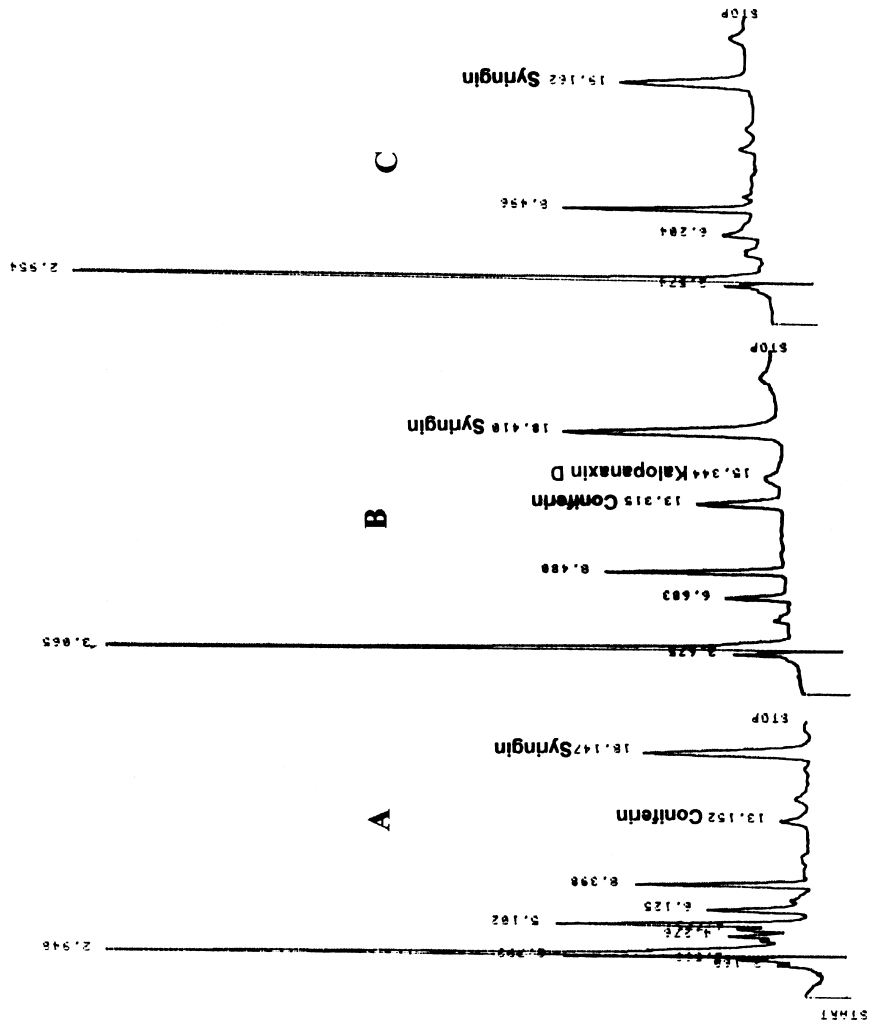

Figure 2. HPLC chromatograms of the ethylacetate extracts belonging to 3 *V. album* subspecies (A) ssp. *abietis; (B) ssp.* album; (c) ssp. *austriacum*.

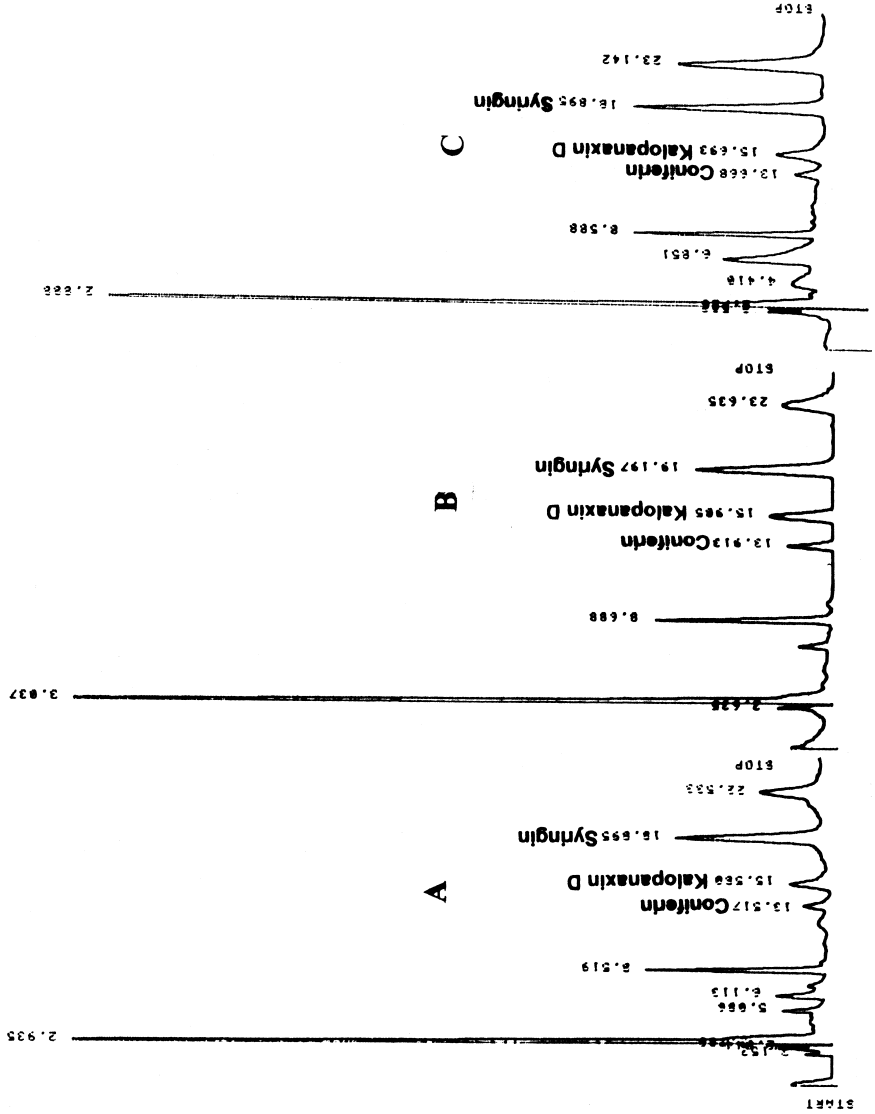

Figure 3. HPLC chromatograms of the n-butanol extracts belonging to 3 *V. album* subspecies (A) ssp. *abietis; (B) ssp.* album; (c) ssp. *austriacum.*

Table 4

Content of Phenylpropanes in *V. Album* Samples[a] Reported in Literature

	Extract		Compounds				
Host Plants	**Alcoholic-Aqueous**	**n-BuOH**	**Coniferin**	**Kalopanaxin D**	**Syringin**	**Syringenin-4-O -[Apiofuranosyl (1→2)]Glucoside**	**Literature**
Malus sp.	+				0.065	0.053	4
Pinus sp.	+				0.210	0.210	4
Pyrus caucasica		+	*	*	*	*	15
Quercus sp.	+				0.059	0.034	4
Tilia cordata							*4*
Leaves	+				0.018	0.034	
Stems	+				0.048	0.067	
Berries	+				0.027	0.009	
Ulmus glabra	+				0.043	0.045	4
Unidentified		+			0.03-0.07♦		3

[a] w/w %. * Detected but quantitative analysis was not done. + Studied extracts. ♦ Total amount.

The subspecies of *V.album* samples were taken into consideration in previous studies (Table 4). But, the subspecies of *V.album* samples were inspected in this study by us. We were unable to determine the presence of syringenin-4-O-[apiofuranosil(1→ 2)]-glucoside. Therefore, we didn't study this compound with HPLC analysis. As a result, the amount of syringin in *V.album* samples (ssp. *album*, ssp. *abietis*, ssp. *austriacum*) collected from Turkey was determined as 5208 - 1.2525 %.

All literature findings showed that the amount of syringin in *V.album* samples collected from Turkey have been found to be the highest of all other studies.

In literature, this standardization of mistletoe extracts which were used for hypertension and arteriosclerosis were done in the aspect of the easily detectable phenylpropanols, lignans, viscotoxins, and lectins. The phenylpropanoid content of different misletoe preparations were determined.[4]

The in vitro experiments done for the determination of biological activity showed that syringin has inhibited cAMP-PDE at 50(g/mL).[14]

The results obtained in HPLC analysis showed that the n-BuOH and the EtOAc extracts, belonging to *V. album* samples collected from three different host plants (*Abies bornmülleriana, Pinus nigra, Armeniaca vulgaris*) have been manifested to be hopeful in the aspect of antihypertonic activity.

Further investigations must be carried out on the antihypertonic activity of mistletoe extracts, to identify the active principles; whether phenylpropane or lignan derivatives, choline and choline esters as well.[4,14, 16] In fact, the detection of phenylpropanoid content of the extracts is not sufficient for antihypertonic activity.

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