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Comprehensive Phytochemical Analysis of Various Extracts of *Kickxia lanigera* Growing in Türkiye

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Abstract

Plants can be used as food additives, nutraceuticals and medicines thanks to the valuable secondary metabolites they contain. In this study, the phytochemical contents of the extracts of *Kickxia lanigera* plant from Baskil district of Elazığ province in Turkey obtained in different organic solvents (hexane, chloroform, ethyl acetate, and butanol) were determined by spectrometric methods such as GC-MS/FID, ESI-LC-MS/TOF, and NMR. The result of GC-MS analysis: Hexane; palmitic acid (19.08%), oleic acid (18.76%), dotriacontane (13.08%), chloroform ($CHCl_3$); linoleic acid (38.13%), oleic acid (34.08%), palmitic acid (13.79%) were determined in high amounts. ESI-LC-TOF/MS analysis showed that the ($CHCl_3$) and butanol (BuOH) extracts had low levels of standard phenols, and the main components in the ethyl acetate (EA) extract (in mg/kg plant) were cinnamic acid (5.92), hesperidin (4.7), apigetrin (4.5) and *p*-coumaric acid (2.8). the ^1H NMR spectrum showed that the $CHCl_3$ and EA extracts were rich in phenols.

Key Words: *Kickxia lanigera*, phytochemical, fatty acid, phenolic, spectrometric analysis

1. Introduction

Plants have been used by humans for centuries to treat diseases for therapeutic purposes due to the secondary metabolites they contain (Başar & Erenler, 2024). The identification of secondary metabolites with bioactive properties in plants has led to the use of plant-derived substances. The plant is rich in phenols, molecules that can serve as antioxidants to treat many diseases (Başar et al., 2024; Yenigün et al., 2024). Phenolic compounds are the most abundant and best-known phytochemicals in all plants (Khoddami et al., 2013).

The presence and detection of secondary metabolites in the structure of plants are determined using spectrometric methods such as HPLC (High-performance liquid chromatography), HPLC-TOF/MS (High-performance liquid chromatography Time-of-light mass spectrometry), GC-FID (Gas chromatography flame ıonization detector), LC-MS/MS (Liquid chromatography-mass spectrometry), GC-MS (Gas chromatography-mass spectrometry), NMR (Nuclear magnetic resonance) (Chaouche et al., 2021).

Gas chromatography has a molecular mass working range from 2 (molecular hydrogen)

to about 1500 mass units (C100 n-alkane). Within this mass range, compounds suitable for chromatography are classified as persistent gases, volatile compounds, and semi-volatile compounds (Marriott et al., 2001). Essential oils range from volatile to semi-volatile compounds. Since they are derived from natural flora, they range from highly volatile alarm compounds that need to disperse rapidly in the ambient air to more waxy compounds that have a lower vapor pressure and represent some of the structural components of a plant (Sahin yaglioglu et al., 2020).

Modern separation techniques such as HPLC–TOF-MS and HPLC–MS/MS are the most powerful and fastest chromatographic methods for chemical profiling studies of plants (Yan et al., 2016). They are widely used due to their high sensitivity for both terminal and fragment ions, their large variety, and their high volume. Phenol content analysis is widely used in areas such as pesticide analysis (Ferrer et al., 2012).

Kickxia is a genus of plants belonging to the Plantaginaceae family and contains several species known as crabgrass and fluellines. *Kickxia lanigera* (DESF.) Handel-Mazzetti is an annual herbaceous plant that reproduces by seed or spores and can survive as a seed in harsh environmental conditions (Pinar, 1973). It flowers from July to September and is common in vineyards, fields, and dry places at an altitude of 0-1200 meters. It grows worldwide in southwest Europe, Asia, and northwest Africa as well as in the Mediterranean region in Turkey (Yousefi et al., 2016; Gül, 2020).

In this study, the chemical contents of hexane, chloroform ($CHCl_3$), ethyl acetate (EA), and butanol (BuOH) extracts of the *K. lanigera* plant were analyzed by spectrometric methods (GC-MS, HPLC-TOF/MS, and NMR). By determining the chemical profile of this plant, the isolation of pure molecules will provide information about its biological activity as well as its usability in cosmetics, food, and pharmacology.

2. Material and Methods

2.1. Preparation of Herbs

The *Kickxia lanigera* plant was collected and identified by Prof. Dr. Lütfi BEHÇET in the Baskil district of Elazığ province. For extraction, the plant was dried in an airy and sunless environment. These dried plants were crushed with liquid nitrogen and prepared for extraction.

2.2. Extraction Process

To separate non-polar substances, 2 kg of powdered plant samples of *K. lanigera* were repeated three times at 2-day intervals in hexane solvent. Then the hexane solvent was removed and allowed to dry. The dried plant samples were macerated with $CHCl_3$ four times at 2-day intervals. The remaining plant pulp was boiled in methanol (MeOH): water (1:1, v:v) mixture in a reflux apparatus for 3 hours. The mixture was cooled to room temperature and filtered. The MeOH solvent in the MeOH: water mixture was removed by rotation. The remaining water phase was extracted with the solvents EA and BuOH. Thus, hexane, $CHCl_3$, EA, and BuOH crude extracts of the plant were obtained.

2.3. GC-MS/FID Analysis

To determine the chemical content of the hexane and $CHCl_3$ extracts, the esterification process was performed on an Agilent Technologies Brand 7890A model GC-MS instrument with an Agilent 5975C inert MSD with Triple-Axis Detector model mass detector. Instrument conditions; column characteristics, 30 m X 320 µm X 0.25 µm; HP-5Ms (5% phenylmethylsiloxane), injection volume; 1 µL, flow rate (He); 1mL/min (constant flow), detector temperature; 230 °C, ionization mode; EI^+ ionization voltage; 70 eV, working mass range; 50-550. GC-MS analysis conditions; initial temperature, 100 °C for 10 min, step 1; 5 °C/min rise to 180 °C for 15 min, step 2; 20 °C/min rise to 300 °C for 25 min, and the analysis time was set to 62 seconds. The esterification process was

carried out with extracts. In the esterification process, 50 mg of the plant extracts were dissolved in 5 mL of hexane, and 5 mL of 1M KOH (dissolved in MeOH) was added and mixed vigorously with a vortex device for 30 seconds. According to the reaction (Figure 1), 1 mL of the upper phases (hexane phase) containing the fatty acid methyl esters formed in the mixtures was taken and filtered through a 0.45-micron filter into vials using a syringe.

Phytochemical analysis of the components in the mixture was performed by feeding it to GC-MS (Gül, 2020).

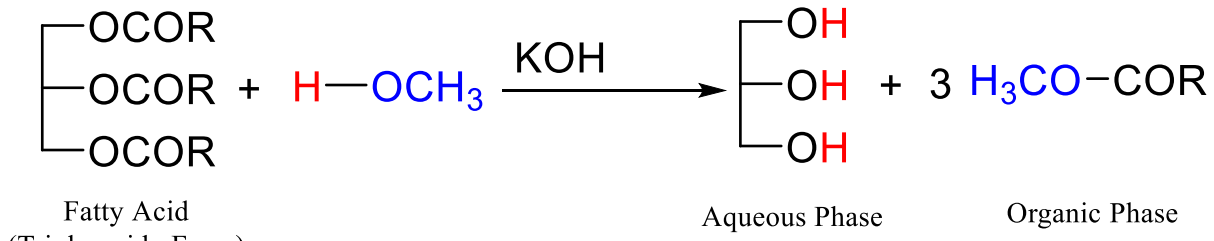


Figure 1. Fatty acid esterification reaction

2.4. ESI-LC-TOF/MS Analysis

The qualitative and quantitative analyses of the phenols were carried out with the 1260 infinity LC, 6210 TOF-MS instrument (injection volume: 10 µL, flow rate 0.6 mL/min, column temperature 35 0C, column model ZORBAX SB-C18 4.6x100mm, 3.5 µm) To determine the phenolic content of the Kickxia lanigera plant, a quantity of dry crude extracts (approx. 1 mg) was taken, dissolved in MeOH to a concentration of 200 ppm of the sample solutions, filtered with a syringe through a 0.45-micron filter into vials and added to the HPLC-TOF instrument. In addition, mixed solutions of 45 phenol standards present at concentrations of 25, 50, 100, 250, 500, 1000, and 2500 ppb were prepared, filtered, and added to the HPLC-TOF/MS instrument. The calibration curves obtained from the solutions of the standards at different concentrations were used to calculate the concentrations of phenols in the samples at a concentration of 200 ppm. These concentrations were used to determine the amount of phenols in the plant. The qualitative analysis of the phenols in the plant was performed by comparing the retention time and m/z values of the phenols. The analytical conditions used in the ESI-LC-TOF/MS analysis of the plant extracts are shown in Table 1.

Table 1. Mobile phases, duration, and concentrations used in HPLC-TOF analysis

No	Time (min)	0.1% Formic acid- water (%)	Acetonitrile (%)
1	0	90	10
2	1	90	10
3	20	50	50
4	23	20	80
5	25	90	10
6	30	90	10

2.5. ^{1}H NMR Analysis

The $CHCl_3$, EA, and BuOH extracts of *K. lanigera* were dissolved with *d*-DMSO. Their contents were determined by Agilent-600 MHz ^{1}H NMR.

3. Results and Discussion

Four different extracts were obtained from the extraction of the *K. lanigera* plant with the solvents hexane, $CHCl_3$, EA, and BuOH. The content analysis of the apolar fraction (hexane, $CHCl_3$) was performed with GC/MS, and the content analysis of the polar fractions (EA and BuOH) was performed with ESI-LC-TOF/MS. In addition, ^{1}H NMR recordings of the extracts obtained were made and a comparison of the contents was carried out (Figure 2).

3.1. GC-MS Analysis Results of *K. lanigera* Extracts

GC-MS is one of the most ideal techniques for the phytochemical analysis of non-polar samples. This technique can be used for components that evaporate before the chemical decomposition temperature or that can pass into the vapor phase by various techniques. When solvents such as hexane and $CHCl_3$ are used for extraction, non-polar substances such as essential oils and fatty acids generally pass from the plants into these organic solvents. Essential oil components can be analyzed directly by GC-MS due to their low boiling point. Fatty acid components are usually not present in free form, but in the form of triglycerides, and since their boiling point is very high, direct analysis cannot be performed with GC-MS. To overcome this obstacle, the conversion of fatty acids in the form of triglycerides to methyl esters by the methylation method is one of the most commonly used techniques. As a result of the methylation techniques performed in this study, the FID chromatograms were obtained from GC-MS analysis of mixtures containing fatty acid methyl esters in hexane and $CHCl_3$ extracts of *K. lanigera* plant (Figure 3). Qualitative analyses of the components in the samples were performed using the retention times of the components in the "Supelco 37 component fame-mix" and the W8N05ST, NIST, and WILEY7N libraries. Quantitative analyses were performed by calculating the areas under the peaks (Table 2).

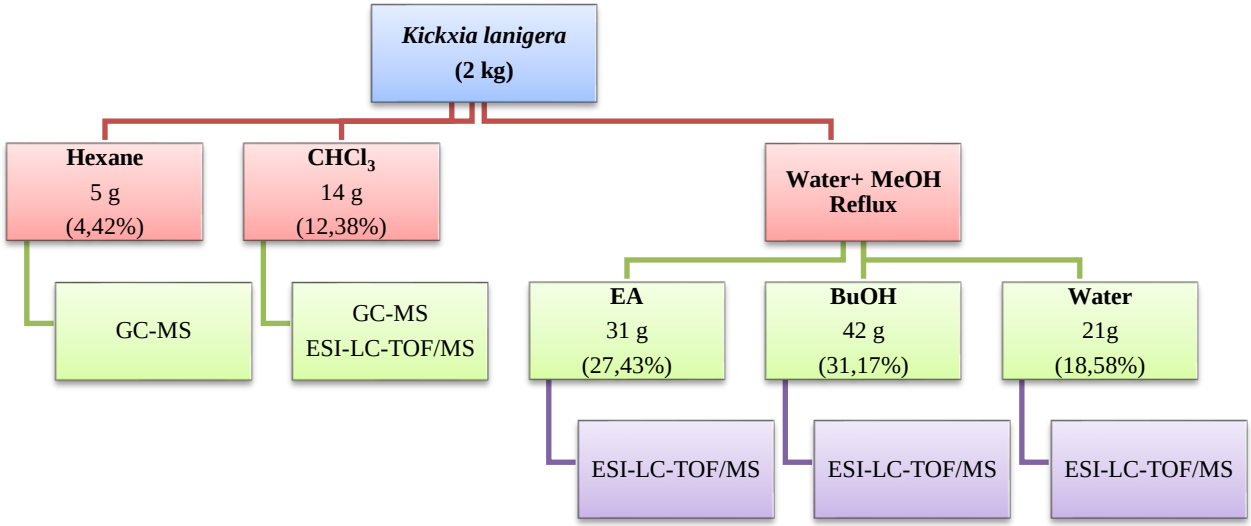


Figure 2. Extraction and analysis scheme of *K. lanigera*

According to the results of the GC-MS analysis, it was determined to be the main constituent; in the hexane extract oleic acid (19.76%), palmitic acid (19.08%), and in the $CHCl_3$ extract linoleic acid (38.13%), oleic acid (34.08%) and palmitic acid (13.79%). It was determined to be palmitic acid (13.79%). According to these ratios, it is seen that the polarity of saturated fatty acid, which has lower polarity than unsaturated fatty acid, is higher in the extract of hexane solvent, which is more non-polar than $CHCl_3$ solvent. In addition, it was determined that hexane (26.5%) was much richer than $CHCl_3$ (1.86) extract in terms of hydrocarbon ratios

3.2. ESI-LC-TOF/MS Analysis Results of *K. lanigera* Extracts

Qualitative Analyzes: The HPLC-TOF/MS technique is a very useful technique for the phytochemical analysis of extracts containing polar compounds obtained from polar solvents such as EA, BuOH, MeOH, and water. For the chromatographic analyses, an SB-C18 column was used, which is called the reverse phase. This column filler is obtained by binding an 18-hydrocarbon to the silica gel used in classical column chromatography. This subsequently added carbon chain changes the physical character of the filler from polar to non-polar (i.e. from

hydrophilic to hydrophilic). This change causes non-polar substances to be retained by the similarly non-polar column filler, while polar substances are less strongly retained and leave the column earlier (i.e. the retention time decreases). Therefore, it can be said that the substances on the left side of the total ion chromatograms (TIC) obtained with the ESI-LC-TOF/MS instrument above are polar, while the substances on the right side are relatively non-polar.

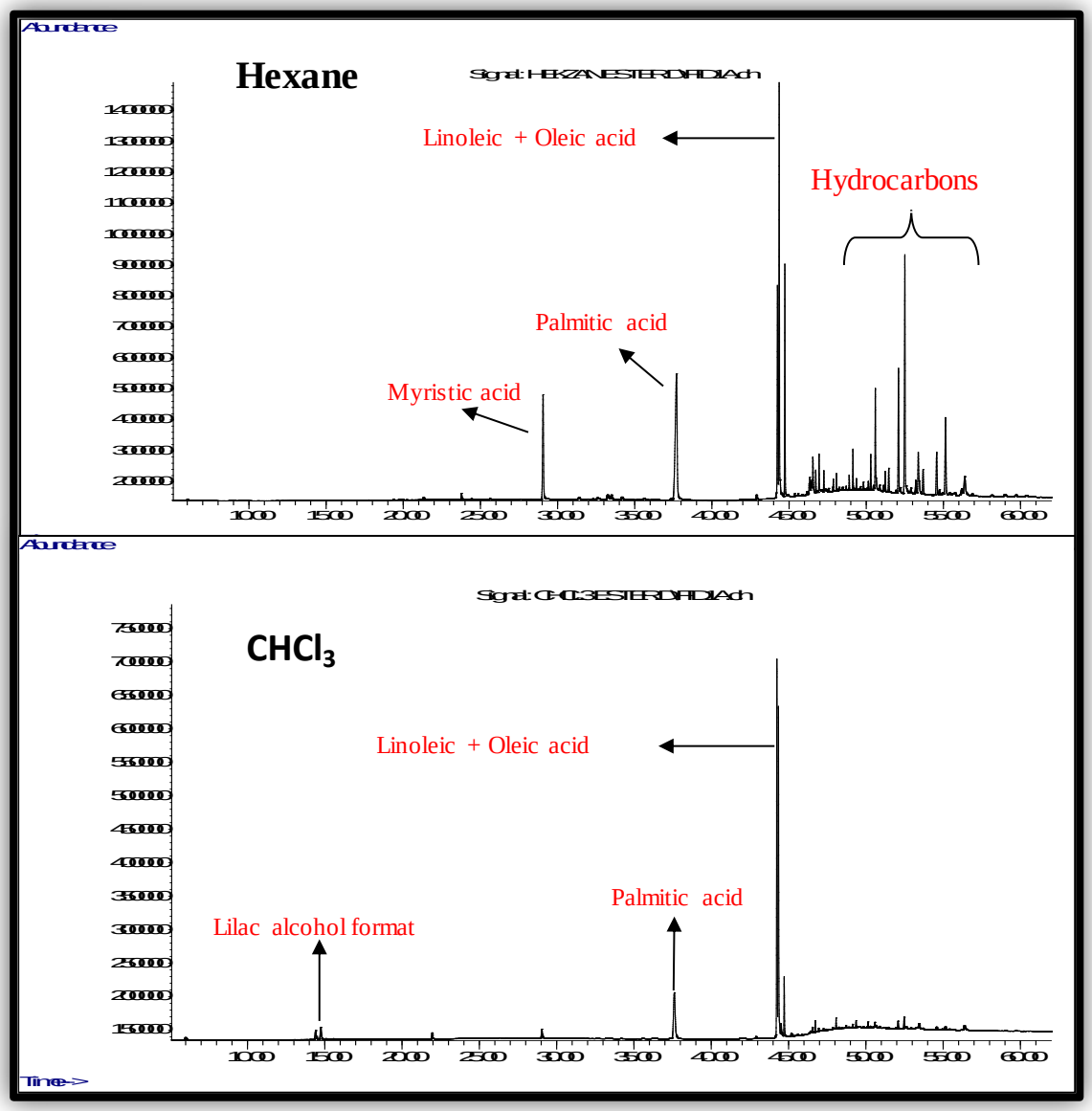


Figure 3. FID chromatograms of hexane and $CHCl_3$ extracts of *K. lanigera*

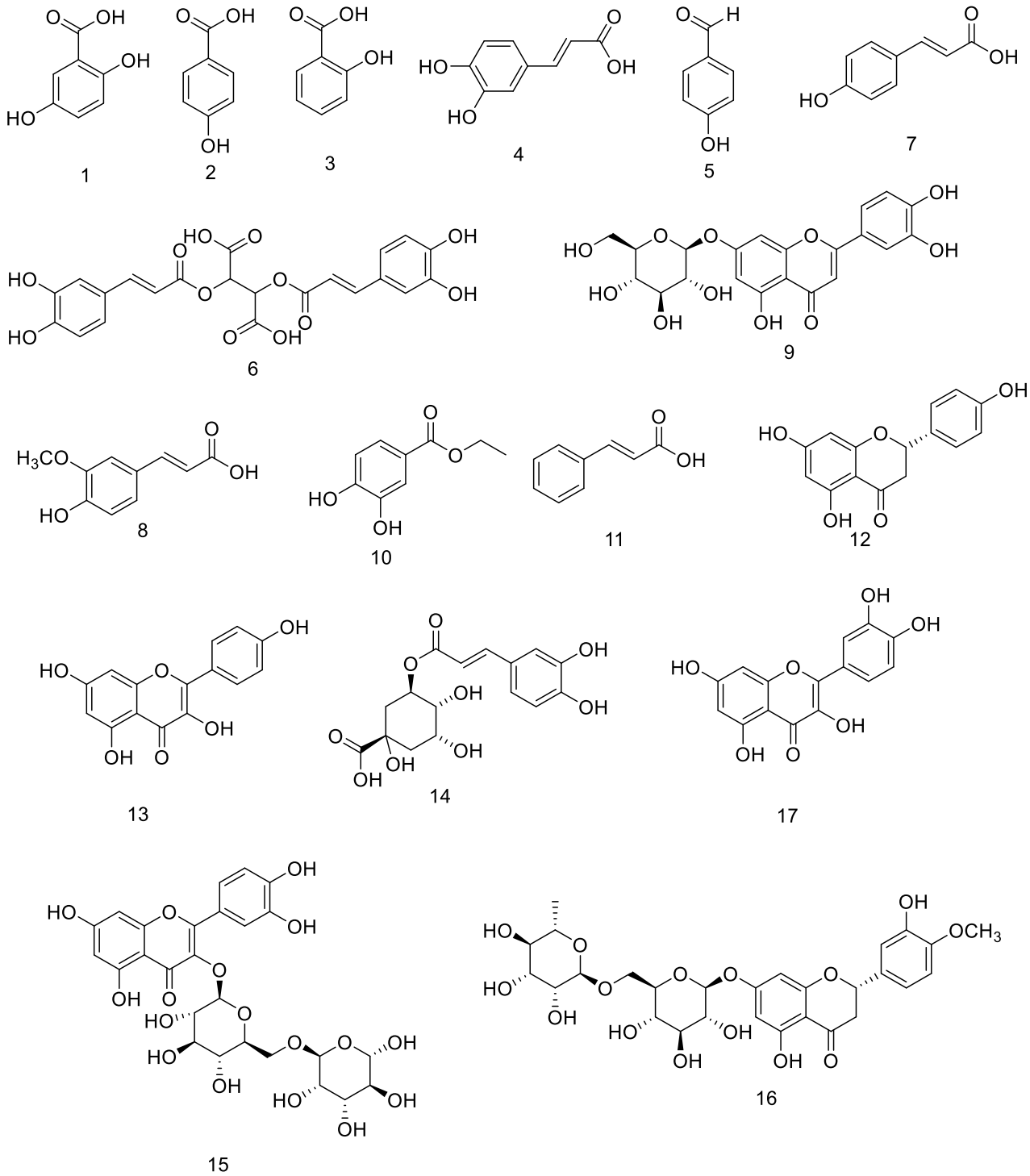
When the TIC chromatograms of the plant extracts were examined as a result of qualitative ESI-LC-TOF/MS analysis of $CHCl_3$, EA, MeOH, and water extracts of the *Kickxia lanigera* plant, the retention time was about 13 minutes. It was found that the compound with m/z ratio (-) 573.2246 was the major component in all extracts. It can be seen that this tendency exists in $CHCl_3$, EA, BuOH, and water extracts, and especially the compounds between 21-24 min in $CHCl_3$ extract are almost absent in the other extracts. From this, it can be deduced that the solvent $CHCl_3$ used in the extraction can extract all substances in this range from the water phase.

Table 2. Components and ratios in hexane and $CHCl_3$ extract of *K. lanigera*

R.T (min)	Compound Name	Hexane	$CHCl_3$
Fatty Acid Methyl Esters			
Saturated			
29.079	Myristic Acid	6.82	1.47
37.725	Palmitic acid	19.08	13.79
44.751	Stearic acid	8.12	5.43
46.754	Eicosanoic acid	-	0.82
	Total	34.02	21.51
Unsaturated			
44.271	Linoleic acid	9.72	38.13
44.385	Oleic Acid	18.76	34.08
		28.48	72.21
	Total	**62.5**	**93.73**
Hydrocarbons			
44.540	3-Octadecene	-	1.86
49.163	Heptacosan	1.08	-
50.617	Nonakosan	5.24	-
52.121	Hentriacontane	6.54	-
52.533	Dotriacontane	13.08	-
	Total	**26.50**	**1.86**
Hydrocarbon Alcohols			
14.419	Lilac alcohol formate C or D	-	1.86
14.757	Lilac alcohol formate C or D (isomer)	-	2.56
46.565	14-Metil-8-Hekzadekin-1-ol	1.56	-
	Total	**1.56**	**4.42**

In addition, it can be seen that the substances in the water and BuOH extracts are not present in the 1-6 minute interval of the TIC chromatogram in the $CHCl_3$ and EA extracts. Another important point is that almost all compounds with the same retention time show different m/z in the chromatograms as a result of a more detailed analysis (Figure 4). In addition, as a result of qualitative analysis, it was determined that 17 different phenolic compounds were present in plant extracts by comparing the molecular ion masses and retention times of phenolic standards (Figure 5).

Quantitative Analyzes: As a result of the qualitative analysis of the $CHCl_3$, EA, and BuOH extracts of *K. lanigera*, the concentrations of phenols detected in the 200 ppm solutions administered to the device were first calculated, and from these concentration values their amounts in the plant extract and the plant (mg phenolic/kg plant) were calculated. It was found that the *K. lanigera* plant is generally not rich in the standard phenolic compounds studied and that the EA extract is richer in available phenolic compounds compared to other extracts, especially cinnamic acid, hesperidin, apigetrin, and *p*-coumaric acid. It was found that small amounts of naringenin, kaempferol, *p*-coumaric acid, and 4-hydroxybenzoic acid were found in the $CHCl_3$ extract and small amounts of hesperidin and routin were found in the BuOH extract. It can be seen that the amounts of cinnamic acid, apigetrin, and hesperidin are much higher in the EA phase than in the other extracts (Table 3).



(**1**) Gentisic acid, (**2**) 4-hydroxybenzoic acid, (**3**) Salicylic acid, (**4**) (Caffeic acid), (**5**) 4-hydroxybenzaldehyde, (**6**) Cisoric acid, (**7**) *p*-coumaric acid, (**8**) *trans*-ferulic acid, (**9**) Apigetrin, (**10**) Protocatechuic acid ethyl ester, (**11**) Cinnamic acid, (**12**) Naringenin, (**13**) Kaempferol, (**14**) Chlorogenic acid, (**15**) Rutin, (**16**) Hesperidin, (**17**) Quercetin

Figure 5. Molecular structures of standard phenolics detected in *K. lanigera* extracts

Table 3. Phenolic content and amount in *K. lanigera* extract (mg phenol/kg plant)

No	Compound Name	$CHCl_3$	EA	BuOH
1	Gentisic acid	tr*	1,2	tr
2	4-hydroxybenzoic acid		1,1	tr
3	Salicylic acid		0,11	tr
4	Caffeic acid		0,20	0,11
5	4- hydroxybenzaldehyde	0,30	0,41	tr
6	Chicoric acid		0,22	0,14
7	*p*-coumaric acid	0,50	2,8	tr
8	*trans-ferulic acid*		0,11	0,13
9	Apigetrin		4,5	0,17
10	Protocatechuic acid ethyl ester		0,01	tr
11	cinnamic acid		5,92	0,10
12	Naringenin	0,60	1,60	0,11
13	Kaempferol	0,50	0,12	0,09
14	Chlorogenic acid	0,15	0,14	2,01
15	Rutin		1,60	0,5
16	Hesperidin		4,7	1,1
17	Quercetin	0,40	0,4	0,08

***tr:** Trace amount

3.2. ^{1}H NMR spectra of the extracts

Since plant extracts generally contain many compounds, their ^{1}H-NMR spectra also appear complex (Figure 6). However, looking at the ^{1}H NMR spectra of *K. lanigera* $CHCl_3$, EA and BuOH extracts, we find that they have relatively simple spectra and provide some important clues about the content of the extracts. A general observation of the spectra shows that the $CHCl_3$ and EA extracts are more intense in the aromatic region (6-8 ppm) than the BuOH extract and that the BuOH extract is more intense in the sugar region (CH peaks around 3.5-4 ppm and OH proton peaks around 5-5.5 ppm). Thus, it can be said that $CHCl_3$ and EA extracts are richer in aromatic compounds, while the BuOH extract is richer in glycosides. The signals at 13 ppm (especially in EA extract) in the proton NMR belong to the protons of the OH group, which have formed intramolecular hydrogen bonds. When the proton signals in the spectra of $CHCl_3$ and EA extracts, which have similar chemical compound classes, are examined more closely, it is found that there are quite a few differences apart from some common signals, especially the many sharp peaks around 4 ppm are more intense than the -OCH_3 peaks and the OH and CH peaks in the sugar regions in the EA extract. Based on these data, it can be said that the proportion of aromatic compounds is higher in the $CHCl_3$ extract and the proportion of compounds containing glycosides is higher in the EA phase. It can therefore be seen that the polarity of the extracted compounds increases depending on the polarity of the solvent. Therefore, it will provide information on the secondary metabolites in the extracts obtained and lead to activity-guided isolations.

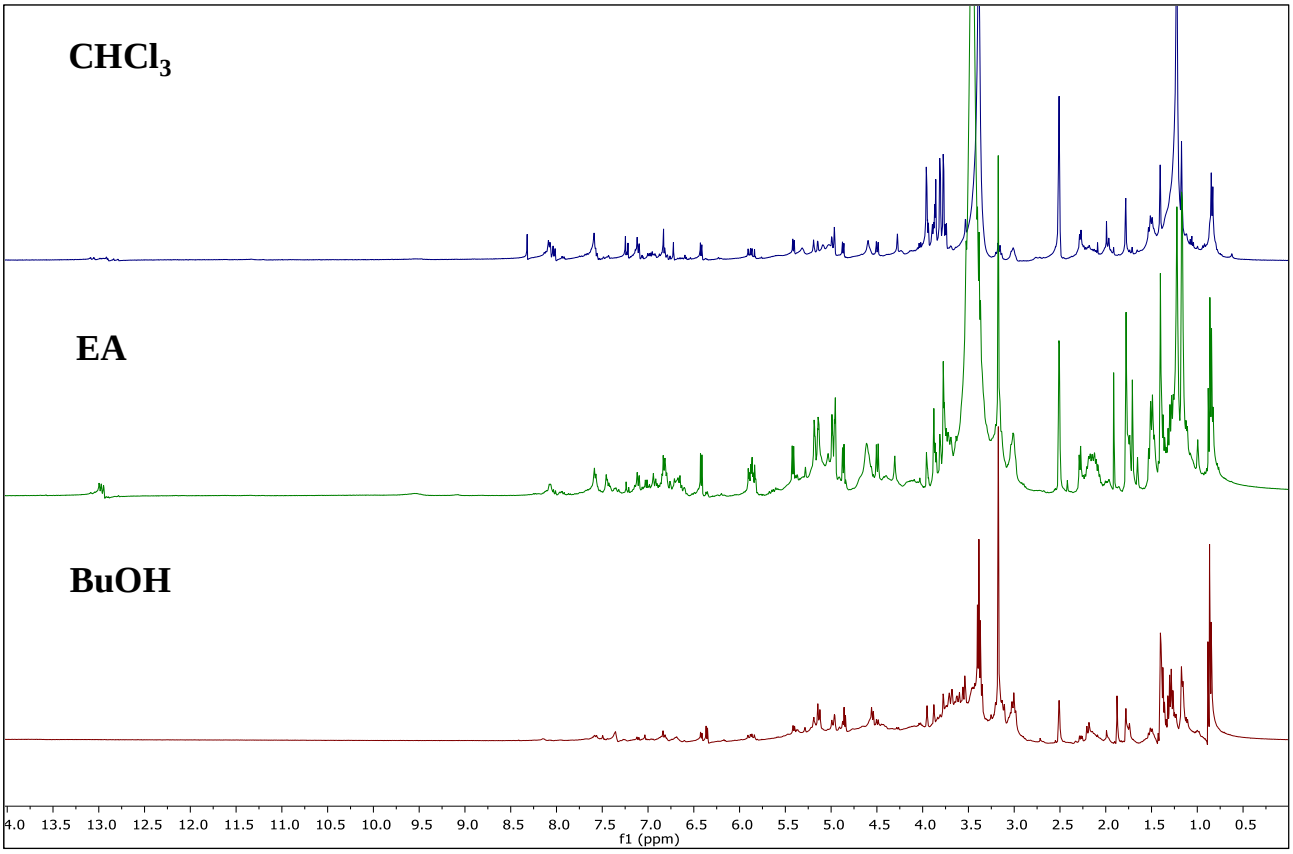


Figure 6. NMR spectra of $CHCl_3$, EA, and BuOH extracts of *K. lanigera*

4. Conclusion

In our study, the phytochemical content (GC-MS and ESI-LC-TOF-MS) of the fractions obtained by different solvent methods of the *K. lanigera* plant was determined. As a result of GC-MS analysis, the hexane, and $CHCl_3$ extracts were found to have a high percentage of fatty acids (65.60% and 93.72%, respectively), with a higher ratio of saturated fatty acids and hydrocarbons in the hexane phase and a higher ratio of unsaturated fatty acids and hydrocarbons in the $CHCl_3$ extract. ESI-LC-TOF/MS analysis revealed that the EA extract was rich in apigetrin, hesperidin, and p-coumaric acid, while the $CHCl_3$ and especially the BuOH extracts had a low number and ratio of phenolic compounds. According to the results of ESI-LC-TOF/MS analysis, it was found that the EA extract was rich in apigetrin, hesperidin, and *p*-coumaric acid, while the $CHCl_3$ and especially BuOH extracts had a low number and ratio of phenolic compounds. Therefore, the results of the constituent analysis of this rich plant are expected to be an important indicator for the isolation of bioactive molecules in the determination of biological activity and their use in food additives and pharmacological fields.

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Author Contribution

Fatih Gül: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Conceptualization, Writing – review & editing. **Yunus Başar:** Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Conceptualization, **Ibrahim Demirtas:** Conceptualization, Funding acquisition, Methodology, Project

administration, Supervision, Writing – review & editing. **Lütfi Behçet:** Resources

Conflicts of Interest

The authors declare no conflict of interest.

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