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Research Article

# The Phenolic Chemistry of *Lappula squarrosa* (Retz.) Dumort., *L. barbata* (Bieb.) Gurke and *L. microcarpa* (Ledeb.) Gurke Species

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**Abstract:** The aim of this research was to study the phenolic chemistry of *Lappula squarrosa* (Retz.) Dumort., *L. barbata* (Bieb.) Gurke and *L. microcarpa* (Ledeb.) Gurke. The specimens were sorted into three groups according to their general morphological features. Nine morphological characters were determined in each group and measured. According to Fisher's test, four of these characters which belong to fruit morphology had a difference of 95 %. Each group was diagnosed and were found to belong to *L. squarrosa, L. barbata* and *L. microcarpa*. The specimens were hydrolyzed in hydrochloric acid and were extracted with isoamyl alcohol, ethanol and ethylacetate. The extracts were analyzed by paper chromatography. It was found that phenolics from fruits can be important characters in the distinction of these species and good evidence was obtained to determine the relationships between these species.

Key Words: *Lappula*, Chemotaxonomy, Phenolic substances, Boraginaceae, Turkey.

## *Lappula squarrosa* (Retz.) Dumort., *L. barbata* (Bieb.) Gurke ve *L. microcarpa* (Ledeb.) Gurke Türlerinin Fenolik Kimyasına Yönelik Bir Çalışma

**Özet:** Bu çalışmanın amacı *Lappula squarrosa* (Retz.) Dumort. *L. barbata* (Bieb.) Gurke ve *L. microcarpa* (Ledek.) Gurke türlerinin fenolik kimyası üzerinde çalışmaktı. Örnekler, genel görünüşlerine ve stereomikroskop altındaki morfolojik incelemelere göre üç gruba ayrılmıştır. Her grupta dokuz morfolojik karakter belirlenmiş ve bu karakterler ölçülmüştür. Fisher testine göre bu karakterlerden meyve morfolojisine ait dört karakter % 95 farklılık göstermiştir. Belirlenen grupların temsil ettiği türler teşhis edilip bu türlerin *L. squarrosa*, *L. barbata* ve *L. microcarpa* oldukları saptanmıştır.

Örnekler, hidroklorik asit ile hidroliz edilmiş; izoamil alkol, etanol ve etil asetat kullanılarak ekstre edilmiştir. Elde edilen ekstratlar kağıt kromatografisi yöntemiyle analiz edilmiştir. Bu cinsin türleri için özellikle meyvede bulunan fenoliklerin tür ayrımında önemli bir karakter olabileceği ve türler arasındaki akrabalığa da iyi bir kanıt teşkil edebileceği gösterilmiştir.

Anahtar Sözcükler: *Lappula*, Kemotaksonomi, Fenolik Maddeler, Boraginaceae, Türkiye.

## Introduction

*Lappula* Fabricius, belonging to the *Boraginaceae* family is represented by seven species in several geographical regions in Turkey. As pointed out by Edmondson it is too difficult to distinguish *Lappula* species from each other due to hybridization (1).

Nutlet morphology is very important in the classification of *Lappula* species. In particular, it has been found that if the specimens in Turkey are not ripe, it is difficult to distinguish *L. microcarpa* Gurke specimens from the *L. barbata* (Bieb.) Gurke specimens. The aim of this research was to examine the phenolic chemistry of the specimens of *L. squarrosa* (Retz.) Dumort., *L. barbata*, and *L. microcarpa*, using morphological characters. Substantial evidence from other sources such as cytology and chemotaxonomy is needed to clarify this problem.

Chemotaxonomy has been a useful tool in solving such problems for decades (2). One of the chemical compound groups used in chemotaxonomy is water soluble phenolic compounds of plant samples (3).

Phenolic compounds were used by Bate-Smith (4-7); Harborne (8-10); Ribereau (11); Cutler (12,13); Rezende and Gottlieb (14); Blatt (15); and Sandor (16) to solve taxonomical problems.

## Materials And Methods

### A. Morphological Study

A total of 97 plant samples were collected from eight locations in the Black Sea Region in June and July 1993. Nine morphological characters of the samples were examined under a stereomicroscope. According to these morphological characters, the samples were divided into three groups. The data obtained by the stereomicroscope were analyzed statistically with a Macintosh 145 microcomputer using the Statview program to determine similarities or differences between the plant groups.

Plant specimens were collected from the following areas and sample numbers of plant specimens on which chemical analysis were carried out are given:

*L. squarrosa* A4 Kastamonu: Ilgaz, Apaydın 21; A5 Sinop: between Boyabat and Taşköprü, Apaydın 23a; A6 Samsun: Kavak, Apaydın 1; A6 Ordu: between Aybastı and Gölköy, Apaydın 30; A7 Trabzon: between Maçka and Sümela, Apaydın 32; A8 Artvin: between Hopa and Borçka, Apaydın 33; A8 Artvin: Murgul, Apaydın 34; A8 Artvin: Ardanuç, Kızılcık environs, Apaydın 37.

*L. barbata*; A4 Kastamonu: Tosya, Yukarıkayı village and Kavakçeşme, Apaydın 17; A5 Samsun: Bafra, Kabuklu village, Apaydın 9; A5 Samsun: Bafra, Meşelitürkmenler district, Apaydın 12; A5 Sinop: between Taşköprü and Boyabat, Apaydın 23; A6 Samsun: Kavak, Apaydın 1b; A6 Samsun: between Hasan Uğurlu Dam and Çamalan, Apaydın 3; A6 Samsun: Ayvacık, Suat Uğurlu Dam, Apaydın 7; A6 Amasya: Gümüşhacıköy, Gümüş district, Apaydın 14; A6 Ordu: between Fatsa and Aybastı, Apaydın 25.

*L. microcarpa*: A6 Samsun: Ayvacık, Suat Uğurlu Dam, Apaydın C7.

*Rochelia disperma* (L. fil.) C. Koch: A4 Çankırı: Çankırı-İstanbul highway, Apaydın 19. This species was used as reference to show the utility of flavonoids to rank the genus and species level.

### B. Chemical Study

Each dried plant leaf sample was ground using a Wiley mill. A certain amount of ground leaf sample (0.2-0.4 g) was weighed and was put into a test tube. In the test tube, the plant material was hydrolyzed by HCl (2 N, 5 ml) for 20 minutes. After the tube content cooled, an aliquot of hydrolyzed solvent was transfered to a small test tube, 5-6 drops of amyl alchol were added and the whole tube content was mixed. After the acid-alcohol phases of the tube contents were separated, the amyl alcohol phase was taken out with a pasteur pipette, and this extract was applied to chromatography paper (Whatman No.1) (4). The fruits of the dried plant samples were also separately ground with a Wiley mill. A certain amount of powder was weighed out and was placed in a test tube. First, this material was extracted with 4 ml of diethyl ether for 24 hours and then the extract was discarded. The remaining fruit powder was subsequently hydrolyzed with 5 ml of 2 N HCl at 100°C for 40 minutes. After the extract cooled, 3 ml of ethyl acetate was added to the tube content. The tube content was mixed well, the mixture was allowed to separate from two liquid phases. The ethyl acetate phase was taken out by a pasteur pipette, and was applied to chromatography paper (17).

Each leaf extract was run in butanol: acetic acid: water (BAW 4:1:5 top phase) solvent. The fruit extracts were also run in the same solvent. In addition to these, three fruit extract (B25, A37 and A23a) were developed by first 6 % acetic acid and then BAW (4:1:5) as the second dimension (17).

Two leaf extracts, representing two different plant groups according to morphological observation were also run by toluene: acetic acid: water (4:1:5) solvent mixture (18).

After completion of chromatographic runs, the Rf values of the compounds separated on the chromatography paper were examined under U.V. light. 1 % aqueous ferric chloride were sprayed on the chromatography paper in order to determined whether the compounds were phenolic or not (19).

## Results

Of the 97 plant samples collected, there were 52 and 43 plant samples belonging to Group A and Group B, respectively. There were only two plant samples belonging to Group C, which were collected from two location (Yukarıkayı, Tosya and Çarşamba, Ayvacık Suat Uğurlu Dam, Samsun).

There were nine morphological characterictics studied under the stereomicroscope. The data obtained by stereomicroscopic measurements were used to determine the statistical differences between the plant groups. Only the data obtained from Group A and Group B were analyzed statistically because the sample number from Group C was too small.

The statistical similarity between Group A and Group B was approximately 44 %. These two groups differed from each other in respect to their glochidiate length, corolla length, style length and corolla radius.

Using the guidelines and the diagnostic characteristics indicated in the Flora of Turkey, each representative plant group was identified as respective *Lappula* species. The samples belonging to Group A were identified as *Lappula squarrosa* (Retz.) Dumort. The plant samples in Group B belonged to *Lappula barbata* (Bieb.) Gürke. The plant samples from Group C were *Lappula microcarpa* (Ledeb.) Gürke.

**Rf Values For *Lappula squarrosa* (Retz.) Dumort.**

A plant extract of *L. squarrosa* (Retz.) Dumort. from Group A (A1) was run in toluene: acetic acid: water (4:1:5) solvent mixture. It was found that only two substances were separated having Rf values of 0.38 and 0.66 (Figure 1).

A leaf sample from Group A extracted with aqueous ethanol (A33) was developed in BAW (4:1:5) and eight glycosides with Rf values of 0.25; 0.30; 0.56; 0.60; 0.65; 0.70; 0.75; 0.86, and five aglycones with Rf values of 0.58; 0.64; 0.69; 0.83; 0.88 were separated (Figure 2).

The fruit extract of the plant sample A21 was developed in BAW (4:1:5) and five glycosides with Rf values of 0.18; 0.46; 0.57; 0.72; 0.83; (Figure 3) and five aglycones with Rf values of 0.60; 0.65; 0.72; 0.83; 0.89 were separated (Figure 5).

However, Rf = 0.46 band belonging to glycoside was not found in A23a, A37, A34 specimens (Figure 3).

In the two-dimensional paper chromatography eight glycosidic phenolic substances were separated from A37 in 6% acetic acid and the Rf values of these substances were 0.64, 0.47, 0.54, 0.34, 0.62, 0.60, 0.53, 0.51 in the first dimension and the Rf values of glycosides were 0.14, 0.38, 0.50, 0.58, 0.60, 0.66, 0.76, 0.83 in BAW solvent system in the second dimension. The A23a specimen did not have any glycosidic phenolic substance with Rf 0.34 in the first dimension and Rf 0.58 in the second dimension in contrast to the A37 specimen (Figure 7).

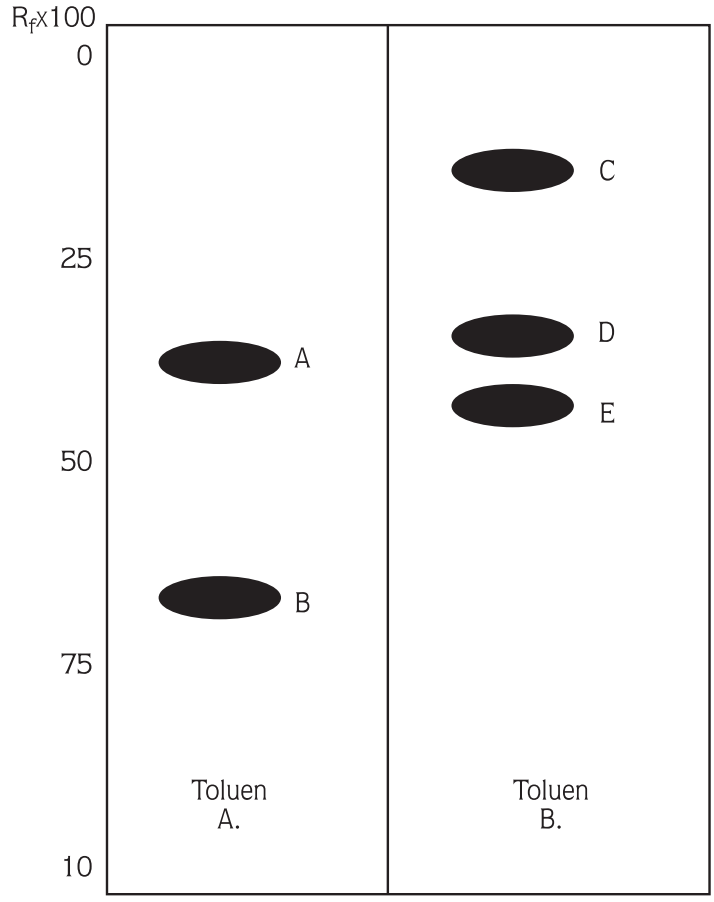


Figure 1. The $R_f$ values produced in Toluen: Acetic Acid: Water (4:1:5) solvent system of hydrolyzed leaf extracts.

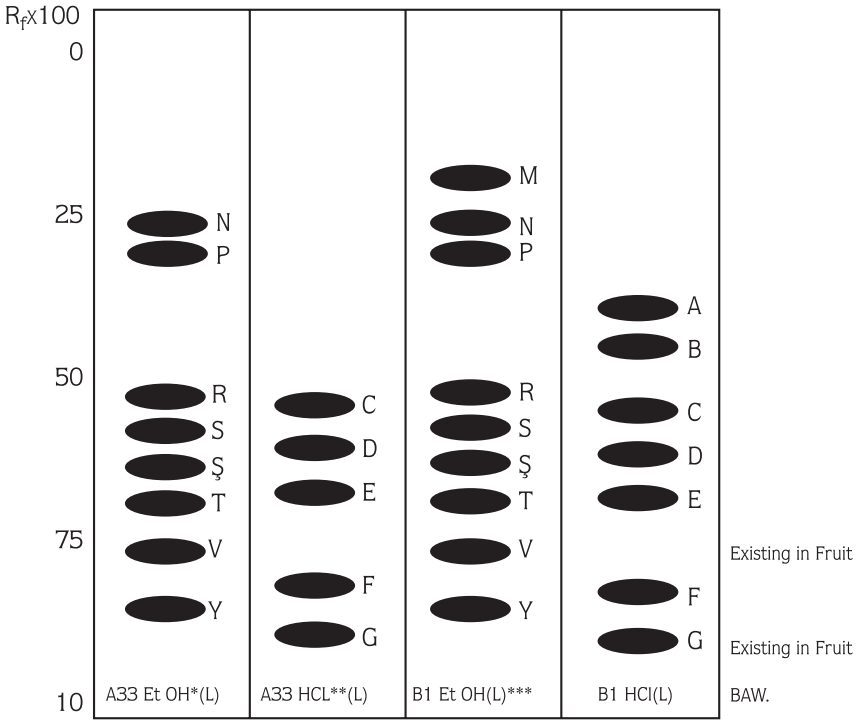


Figure 2. The $R_f$ values produced in BAW (4:1:5) solvent system of hydrolyzed and non-hydrolyzed leaf extracts.
Et OH= Non-hydrolyzed glycoside bands.
HCl= Hydrolyzed aglycone bands
L= Leaf

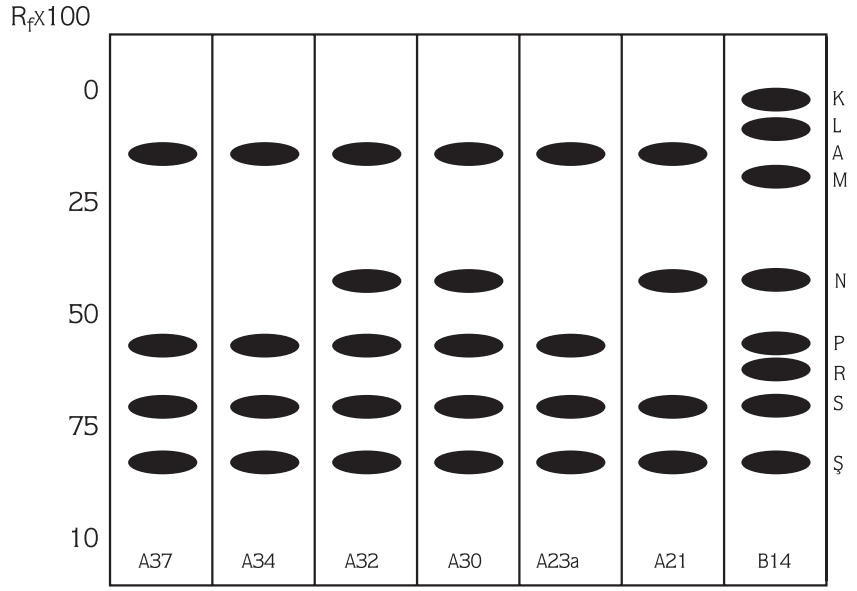


Figure 3. The $R_f$ values produced in BAW (4:1:5) solvent system of non-hydrolyzed fruit extracts.

### Rf Values For *Lappula barbata* (Bieb.) Gurke

Three phenolic substances were separated in toluene: acetic acid: water (4:1:5) solvent system with Rf values of 0.19; 0.34 and 0.44 (Figure 1) respectively.

Leaf and fruit extract were developed separately in BAW (4:1:5) solvent system. Nine glycosides and seven aglycones were separated from the leaf extract (B1). Rf values of the glycosides were 0.20; 0.25; 0.30; 0.56; 0.64; 0.67; 0.70; 0.75 and 0.86 respectively. Rf values of the aglycones were 0.39; 0.47; 0.58; 0.64; 0.69; 0.79 and 0.83 respectively (Figure 2). Eight glycosides and five aglycones were separated from fruit extracts

(B14) and (B9) respectively Rf values of the glycosides and aglycones were 0.10; 0.14; 0.22; 0.46; 0.57; 0.63; 0.72 and 0.83, and 0.60; 0.70; 0.79; 0.83; 0.89 respectively (Fig 3, 5).

In the two-dimensional paper chromatography, five glycosides were separated from B25 in 6 % acetic acid solvent system in the first dimension. Rf values of the glycosides were 0.64; 0.47; 0.51; 0.66; 0.34; 0.60; 0.53; 0.51. In the second dimension Rf values of the substances separated in BAW (4:1:5) solvent system were 0.14; 0.38; 0.43; 0.54; 0.58; 0.66; 0.76; 0.83 (Figure 7).

### Rf Values for *Lappula microcarpa* (Ledeb.) Gurke

Five phenolic aglycones were separated in the BAW (4:1:5) solvent system and the Rf values were 0.60; 0.70; 0.79; 0.83 and 0.89 respectively (Figure 6).

## Discussion

According to the Rf values of the separated substances belonging to all groups some symbols are used for phenolic bands.

When the hydrolyzed leaf extracts shown in Figure 1 were examined it was seen that A and B bands belong to Group A, whereas C, D and E bands belong to Group B, only. A, B and C, D, E bands belong to Group A and Group B respectively.

When the substances separated in BAW (4:1:5) solvent system of hydrolyzed and non-hydrolyzed leaf extracts were examined, it was seen that C, D, E, F and G

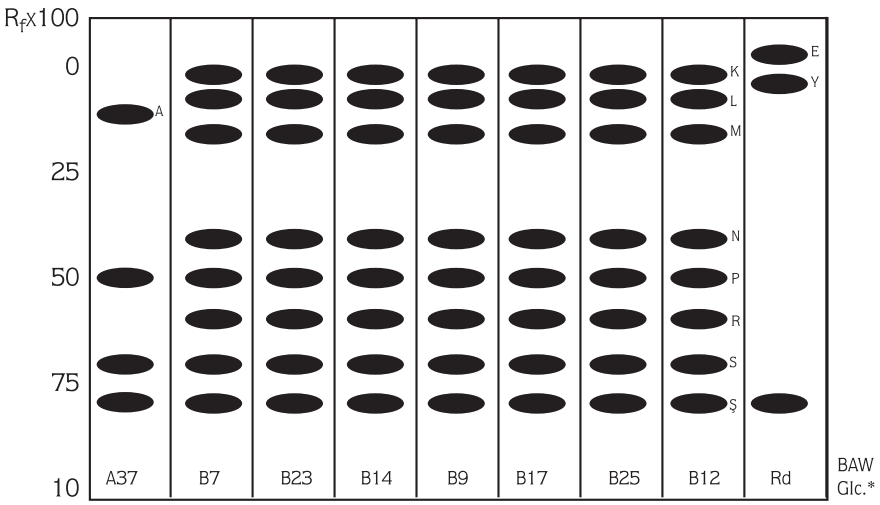


Figure 4. The $R_f$ values produced in BAW (4:1:5) solvent system of non-hydrolyzed fruit extracts. Glc: Glycoside.

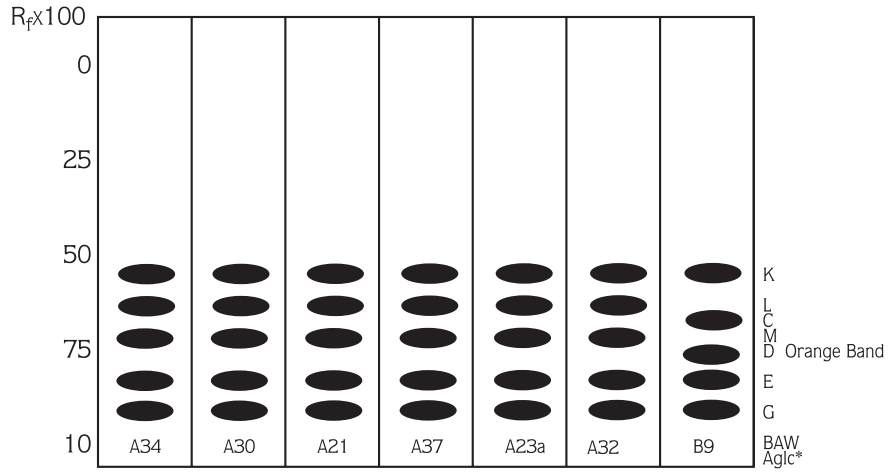


Figure 5. The $R_f$ values produced in BAW (4:1:5) solvent system of hydrolyzed fruit extracts. Aglc: Aglycone.

bands belonging to aglycones were present in Group A and Group B, whereas A and B bands were characteristic only for Group B in HCl solvent system. N, P, R, S, Ş, T, V and Y bands belonging to glycosides were present in Group A and Group B whereas an M band was only characteristic for Group B in EtOH solvent system (Figure 2).

When non-hydrolyzed fruit extracts were examined it was spen that P, S and Ş bands were present in Group A and Group B, whereas K, L, M and R bands were only present in Group B in BAW solvent system. It was determined that an N band was present in all specimens belonging to Group B and some specimens of Group A. However, an N band was not seen in other specimens belonging to Group A, and these specimens seem to have no variation on account of fruit morphology. The specimens with an N band in Group A were investigated in respect to fruit morphology and it was determined that these specimens were similar to Group B specimens due to their style length, glochid length, exertion of style between nutlets, the presence of extra glochids and the presence of dense tubercules. An A band is characteristic for Group A (Figure 3 and 4). As pointed out by Rieseberg (20) morphological information has been augmented with evidence from secondary chemistry, ecological and geographical data. It has been concluded that hybridization may be possible between *Lappula* species although it is often too difficult to determine whether intermediacy for chemical, ecological, geographical and molecular characters actually results from hybridity (3,20). It can be concluded that the variation owing to fruit morphology and the presence of an N band in some specimens belonging to Group A may also indicate hybridization.

Chromatographic bands belonging to *Rochelia disperma* (Rd) which is similar to *L. patula* morphologically were shown in Figure 4 and 6. These bands showed that phenolic substances are quite distinctive characters based on the chemical and taxonomic differences between *Lappula* and *Rochelia*.

When the substances observed in BAW (4:1:5) solvent system were examined, it was seen that E, G and K bands were the same for Group A, B and C, and D band was characteristic for Group B and C, and L and M bands were characteristic for Group A (Figure 5, 6).

When the substances belonging to non-hydrolyzed fruit extracts by two-dimensional paper chromatography in acetic acid (6 %) / BAW (4:1:5) solvent system were examined, it was seen that A, B, M, K and L bands were found in both Group A and Group B. C and E bands were characteristic of Group B. A37 specimen which is similar to Group B on account of fruit morphology has an F band as well. A37 specimen has also D and G bands which is only found in Group A.

The plant specimens belonging to the same species formed the same bands although they were collected from different geographical areas. For example, *L. barbata* specimens collected from different places did not show a clear chemical variation (Figure 6).

It has been suggested that Turkish *L. microcarpa* specimens are difficult to distinguish from *L. barbata* specimens, since ripe fruit samples are needed for identification (3). Several specimens were collected from the distribution range for *L. microcarpa* indicated in the Flora of Turkey (1). However, most of the samples collected from these areas did not belong to *L. microcarpa*. The collected specimens were very similar to the characters of *L. barbata* and only two specimens had exactly the same characters as *L. microcarpa*. This has also been emphasized in the Flora of Turkey (1). The differences were due to some quantitative properties such as fruit length and the number of tubercules. The

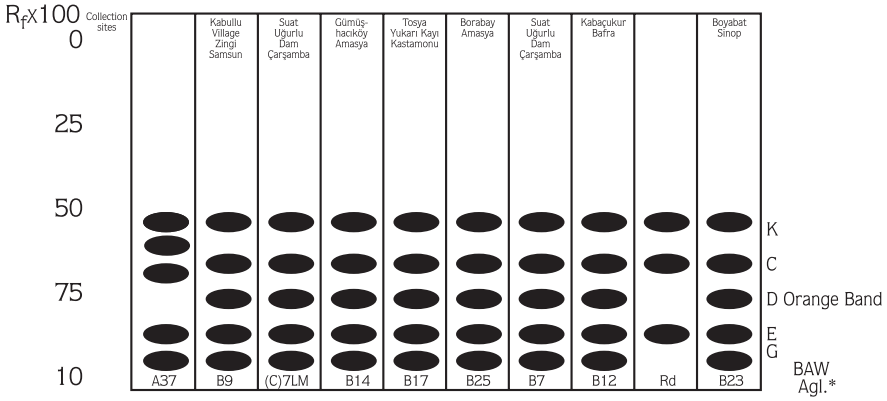


Figure 6. The $R_f$ values produced in BAW (4:1:5) solvent system of hydrolyzed fruit extracts. Aglc: Aglycome.

number of different phenolic bands belonging to *L. squarrosa* and *L. barbata* were higher in BAW (4:1:5) solvent system, whereas *L. microcarpa* and *L. barbata* showed the same phenolic bands in the same solvent system.

Morphological studies and the evaluation of the results by Fisher's test showed that the length of glochids, the length and width of corolla and the length of style may be important characters for the discrimination of species. The length of fruit, pedicel, sepal, stem and leaf and the width of sepal were not important characters for the discrimination of species (Table). Corolla and pistil characteristics were found to be significantly different between populations from different localities in several studies as indicated by Linhart and Grant (21).

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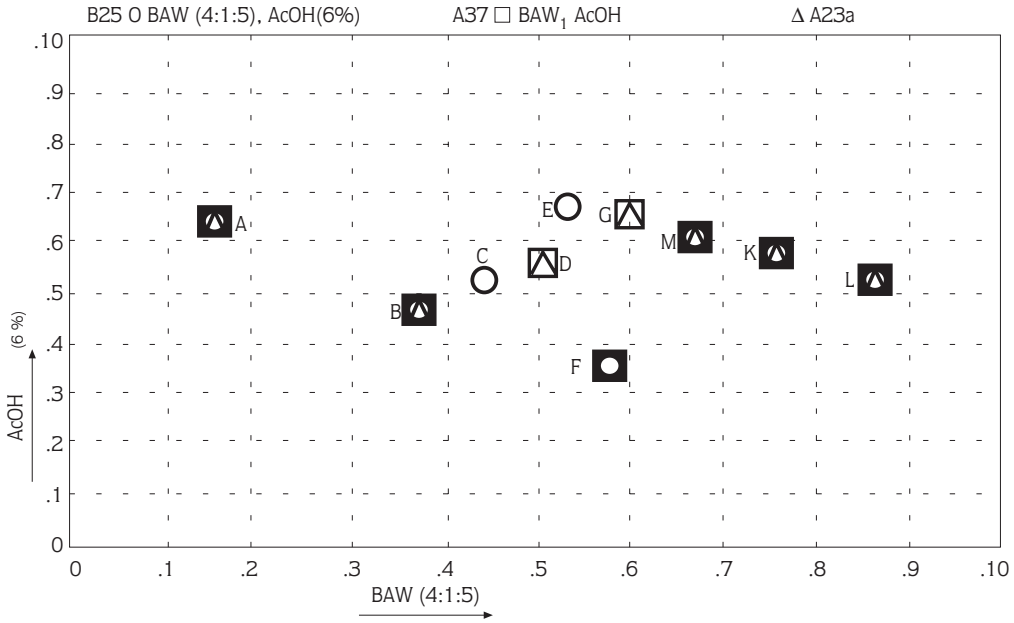


Figure 7. The $R_f$ values produced in acetic acid (6%) / BAW (4:1:5) solvent sysetm and two-dimensional chromatography.

Table. The Similarity Proportions of the Measured Characters According to the Results of Fisher's Test.

| GROUP NUMBER | FEATURE CHARACTERS | DIFFERENCE | ACCORDING TO ALL FEATURES BETWEEN BOTH GROUPS | |
|---|---|---|---|---|
| | | | DIFFERENCE | SIMILARITY |
| A-B | Fruit length | 0 | | |
| A-B | Glochidiate length | + 95 % | | |
| A-B | Pedicel length | 0 | | |
| A-B | Corolla length | + 95 % | | |
| A-B | Sepal length | 0 | 44 % | 56 % |
| A-B | Leaf of the System length | 0 | | |
| A-B | Style length | + 95 % | | |
| A-B | Sepal width | 0 | | |
| A-B | Corolla width | + 95 % | | |

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