Chromatographic Profiles of Fruits and Flowers of Russian Olive (*Elaeagnus angustifolia* L.) Morpho-variants from Ladakh, India

Anup Raj*[1], Faizan Ahmad[2] and Mohd. Mehdi[2]

[2]*Krishi Vigyan Kendra, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, Kargil, Ladakh-194103, Jammu and Kashmir*

[1]*Highland Agriculture Research and Extension Station, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, Kargil, Ladakh-194103, Jammu and Kashmir*

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Russian olive (*Elaeagnus angustifolia*) is a multipurpose tree with fruits and flowers being used by locals since ages for its medicinal value. This species is commonly known as 'Sersing' and represented by five different morpho-variants in the Ladakh region. The present study was carried out to find out phytochemical differences among these five morpho-variants of Russian olive with the help of chromatographic studies. The chemical profiles of fruit and flower extracts obtained using TLC indicate diversity among the variants. Similarly, gas chromatograms of fruit volatiles presented clear evidence of qualitative as well as quantitative differences among the variants. In fruits, presence of stigmasterol was also reported. This study was the first attempt to obtain intra-species diversity through chemical profiling using chromatographic techniques which can be used as fingerprints for identification of these variants.

Key Words: Chemical diversity, Chromatograph, Russian olive, Secondary metabolites, Stigmasterol

Introduction

Ladakh (32º15´-36º N and 75º15´-80º15´ E) located in the Western Trans-Himalayas, is a high altitude desert steppe lying between altitudes 2650-7672 m (Humbert-Droz and Dawa, 2004). Extremely low temperatures (25ºC below freezing point), meager precipitation (<250 mm annual), diurnal pattern of temperature fluctuations and abridged growing season with insignificant precipitation are the characteristic climatic features.

Russian olive (*Elaeagnus angustifolia* L.), a small Eurasian tree, is distributed from Spain in the west to China in the east through western and central Asia (Hooker, 1890). In India, the species grow in Ladakh region of north-western Himalayas and is represented by five different fruit morphotypes (Anup Raj *et al*., 2010). Locally, this species is known as 'Sersing' and the variants as *bee*, *chapacha*, *balti*, *marpo* and *ringmo*. Though, Russian olive grows throughout Ladakh it thrives well in relatively warmer climate of district Kargil within an altitudinal range of 2650-2900 m above mean sea level. The villages where the species grows naturally are Shillikchey, Hardass, Poyen, Mingi, Goma Kargil, Chikten and some parts of the Kargil city. Different parts of the plants are being used in the Amchi system of medicines in Ladakh.

Morphological variations among different varieties of Russian olive have been reported from different regions throughout its distribution range (Musegjan, 1958; Goncharova and Glushenkova, 1990; Lancaster, 1993; Huang and Jiang, 2005). But no chemical data is available which can supplement the morphological data. Thus, in this study chromatographic profiles of fruits and flowers of the five Russian olive variants were analyzed to find out variety-specific differences in chemical composition among them.

Materials and Methods

Fruits and flowers of five different morpho-variants of Russian olive (*Elaeaegnus angustifolia* L.) were collected from Kargil district during December 2010 and June 2011, respectively, and were analysed at Molecular and Structural Biology (MSB) Laboratory, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow for chromatographic studies during July-August, 2011.

Phytochemical Extraction from Plant Tissue Matrix

Pulp from fruits of each variant of Russian olive (viz., *bee, balti, ringmo, marpo and chapacha*) was separated from seed. A known weight (2 g) of fruit pulp and flowers of each variant was ground using mortar-pestle and kept overnight in 50 % aqueous methanol (20 ml). After this

**Author for Correspondence: E-mail:* arajleh@yahoo.co.in

initial extraction, the aqueous methanol extracts were sequentially extracted in equal volumes of chloroform and ethyl acetate, respectively. Each extraction was done thrice for getting maximum yield. Chloroform and ethyl acetate fractions were dried, re-dissolved in HPLC grade methanol and stored at 4°C for TLC analysis.

For HPLC analysis, phytochemicals from fruit pulp were first extracted using 50 percent aqueous methanol. Then the methanol extract was defatted using equal volumes of n-hexane followed by liquid-liquid partitioning with chloroform. The chloroform extract was dried and re-dissolved in HPLC grade methanol and kept at 4°C for TLC and HPLC analysis to find out the presence of stigmasterol in the fruit pulp. Each extraction was done thrice. A pure stigmasterol standard was also prepared in HPLC grade methanol and subjected to TLC and HPLC for comparison.

Thin Layer Chromatography

After spotting the chloroform fraction of fruit and flower extract on pre-coated silica gel plates of 10 X 20 cm (60 F_{254}; MERCK Inc.), five different solvent systems were tested for separation of compounds. Two solvent systems (toluene: ethyl acetate: formic acid; 10:8:2 and ethyl acetate: methanol: water; 100:16.5:13.5) were found suitable for the development of chromatograms. Ultra violet light, iodine vapours and anisaldehyde reagent spray were used for visualization. The chromatograms thus developed were recorded digitally and analysed using Digital Imaging System (Multi Doc-It Imaging System for TLC). Similarly ethyl acetate fractions were also chromatographed and analysed.

Head Space Gas Chromatography (HS-GC) Analysis

A Perkin Elmer gas chromatograph (Clarus 500) equipped with capillary column (50 m x 0.32 mm, Perkin Elmer Inc.) and attached with Turbomatrix 16 headspace sampler was used for HS-GC analysis of fruits and flowers of Russian olive.

Sample: Two g fruits of each variant were filled into a 20 ml vial.

GC conditions: A FID detector was used for the percentage determination of volatile components. Oven temperature was programmed as follows: 40°C for 5 min, ramping to 150°C at 5°C/min, hold at 150°C for 5 min and again ramping from 150°C to 210°C at 7.5°C/min. Injector temperature: 270°C; carrier gas: N_2 with a flow rate of 1 mL/min; detector temperature: 250°C; split ratio: 1:20; and sample size: 1 µl.

HS conditions: Oven temperature – 120°C; needle temperature – 100°C; transfer line temperature – 100°C; sample thermostating – 15 min; pressurising time –5 min.

High Performance Liquid Chromatography Analysis

HPLC (Waters 600) equipped with autosampler (Waters 717 plus) and Photodiode Array (PDA) detector (Waters 2996) was used for HPLC analysis. The column used was Reverse Phase C_{18} column. The mobile phase consisted of two solvents: methanol (A) and water (B), each containing 0.05% acetic acid. The solvent gradient in volumetric ratios of solvents A and B was as follows: from 0 to 30 min, 40 to 60% A; from 30 to 45 min, 60 to 75% A; from 45 to 54 min, 75 to 95% A; from 54 to 55 min, 95 to 100% A at a flow rate of 0.60 ml/min; from 55 to 60 min at 100% A at a flow rate of 1.00 ml/min; from 60 to 64 min, 100 to 40% A and flow rate from 1.00 ml/min to 0.60 ml/min; and finally from 64 to 65 min at 40% A and flow rate 0.60 ml/min. This mobile phase was filtered through a 0.40 µm membrane filter (Millipore), while samples were through Acrodisc Syringe Filter (0.2 µm Supor Membrane Filter), prior to use. Injection volume of l0 µl was used for all the observations. The chromatographic peak of the stigmasterol was confirmed by comparing its retention time and UV spectra with those of the reference standard of stigmasterol. The detector was scanned from 210 to 400 nm.

Results

Results of TLC analysis are presented in Tables 1 and 2 and Figures 1 and 2. A perusal of these data and figures alludes to the qualitative differences present in phytochemical composition among the fruits and flowers of the mopho-variants of Russian olive. For example, in the chloroform extract of fruits, band number 5 (R_f=0.62) and 6 (R_f=0.74) were present only in *chapacha* while band 3 (R_f=0.13) was present only in *bee* and *ringmo* varieties (Table 1). Likewise, bands 6, 7, 10, 11 and 12 present in TLC profile of ethyl extract of fruits were able to discriminate these varieties from one another (Fig.1). Among the 13 bands found in the chloroform extract of flowers, only 1 (R_f=0.02), 8 (R_f=0.41) and 13 (R_f=0.95) bands were common in all the varieties (Table 2). Similarly, qualitative as well as quantitative

Table 1. R_f values of TLC bands representing different compounds separated and visualized in the chloroform and ethyl acetate extract of fruit samples

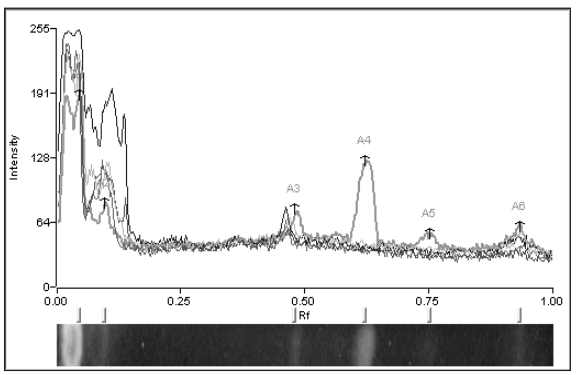
Band	R_f Values				
	Bee	Balti	Ringmo	Marpo	Chapacha
Chloroform extract					
1	0.04	0.04	0.04	0.04	0.04
2	0.09	0.09	0.09	0.10	0.10
3	**0.13**	–	**0.13**	–	–
4	0.47	0.46	0.46	0.46	0.47
5	–	–	–	–	**0.62**
6	–	–	–	–	**0.74**
7	0.94	0.94	0.94	0.94	0.94
Ethyl acetate extract					
1	0.06	0.05	0.05	0.09	0.06
2	0.1	0.11	0.1	0.12	0.11
3	0.16	0.18	0.17	0.17	0.17
4	0.23	0.23	0.23	0.24	0.24
5	0.26	0.26	0.26	0.27	0.27
6	–	**0.33**	–	–	**0.33**
7	–	–	–	–	**0.57**
8	0.63	0.63	0.63	0.63	0.63
9	0.75	0.75	0.75	0.75	0.75
10	**0.89**	**0.88**	**0.89**	–	–
11	–	**0.93**	**0.92**	**0.92**	**0.93**
12	**0.95**	**0.95**	**0.95**	**0.96**	–

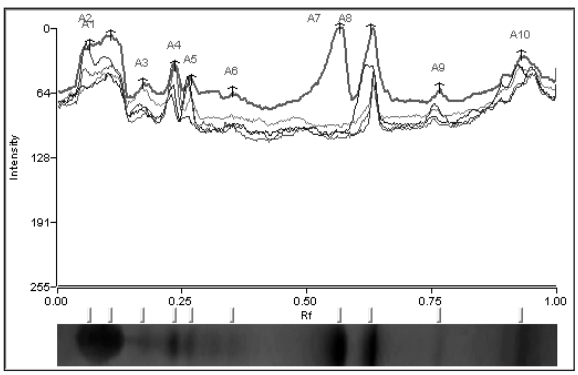
"-" indicates absence

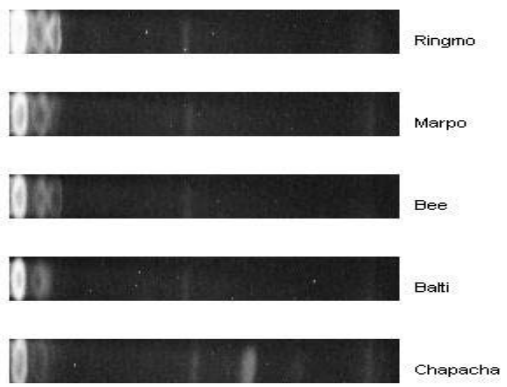
Table 2. R_f values of TLC bands representing different compounds separated and visualized in the chloroform and ethyl acetate extracts of flowers

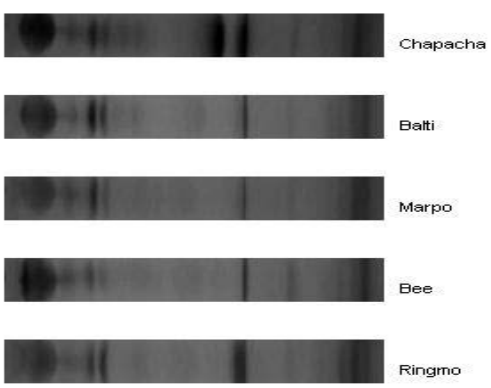
Band	R_f Values				
	Bee	Balti	Ringmo	Marpo	Chapacha
Chloroform extract					
1	0.02	0.02	0.02	0.02	0.02
2	0.15	0.15	–	0.15	0.15
3	–	0.16	–	–	–
4	–	–	–	0.2	–
5	–	–	0.23	–	–
6	–	–	0.27	–	–
7	–	–	0.34	–	–
8	0.41	0.41	0.41	0.41	0.41
9	0.45	0.45	–	0.45	–
10	0.54	0.54	–	0.54	0.54
11	0.63	0.63	–	0.63	0.63
12	0.87	0.87	0.87	–	0.87
13	0.95	0.95	0.95	0.95	0.95
Ethyl acetate extract					
1	0.04	–	0.04	0.04	0.04
2	0.10	0.10	–	0.10	0.10
3	0.18	0.18	0.18	0.18	0.18
4	0.45	–	0.45	0.45	0.45

"–" indicates absence.



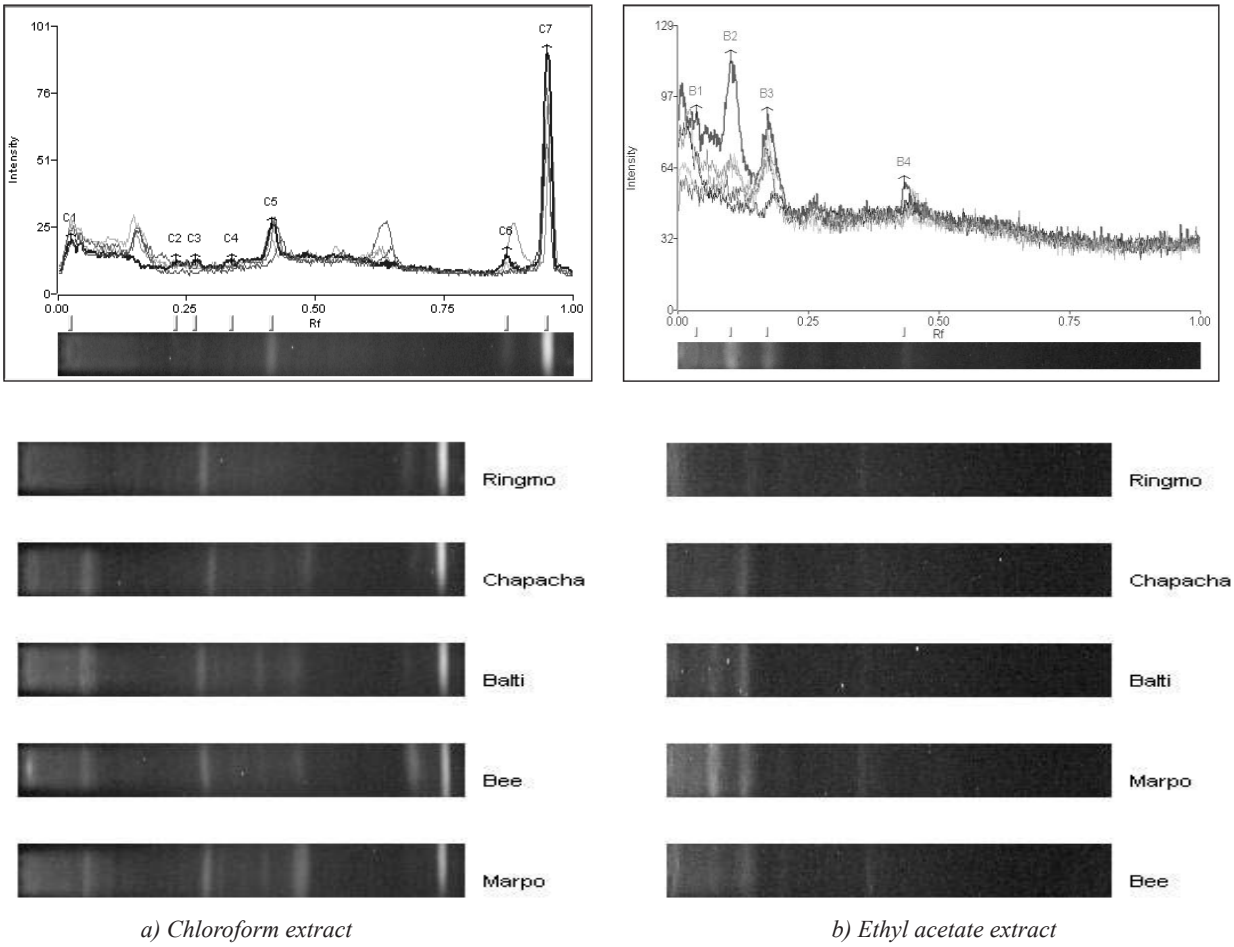






a) Chloroform extract *b) Ethyl acetate extract*

Fig. 1. Thin Layer Chromatogram of a) chloroform and b) ethyl acetate extracts of fruit samples of each variant of Russian olive



a) Chloroform extract *b) Ethyl acetate extract*

Fig. 2. Thin Layer Chromatogram of a) chloroform and b) ethyl acetate extracts of flowers of each variant of Russian olive

differences (relative composition) among the varieties with respect to the volatiles present in fruits of Russian olive were quite evident as indicated by HS-GC analysis (Table 3; Fig. 3). Table 3 depicts the proportion of major fruit volatiles present in Russian olive varieties. These 10 volatiles constituted about 85 % or more of the total volatiles in all these varieties. The compound 1 (RT=2.7 minute) was present only in two variants namely, *ringmo* and *marpo* while the 2nd compound (RT=4.04 minute) was found only in *balti*. Though rest of the volatile compounds were present in each variant, their relative composition was quite different.

The thin layer chromatogram of chloroform extract developed in the solvent system of $CHCl_3$ (70 mL), toluene (24 mL), MeOH (8 mL) and ethyl acetate (4 mL), and anisaldehyde spray (p-anisaldehyde 250 µL, water 40 mL, acetone 10 mL and perchloric acid 5 mL) indicated presence of stigmasterol in only one variant (*bee*) of Russian olive. This result was further confirmed by HPLC analysis using stigmasterol standard (Fig. 4).

Discussion

Many studies in other species, have proved that chemical profiles of secondary plant products based on chromatographic data are valuable in taxonomic studies. Heidi *et al*. (1997), showed marked intraspecific variation in many of their volatiles in two of the species of genus *Narcissus* using headspace collection and GC-MS analysis of flower volatiles. Similarly, in a chemotaxonomic study of genus *Crataegus* using paper chromatography, Arjmandi *et al*. (2009) showed that the genus can be classified into one section, five series, and 13 taxa at species and infra-species level. The result of flavonoid data were well correlated with other taxonomic data. In a study on phenolics, stability in chemical profile of individual trees suggests that quality is tightly controlled

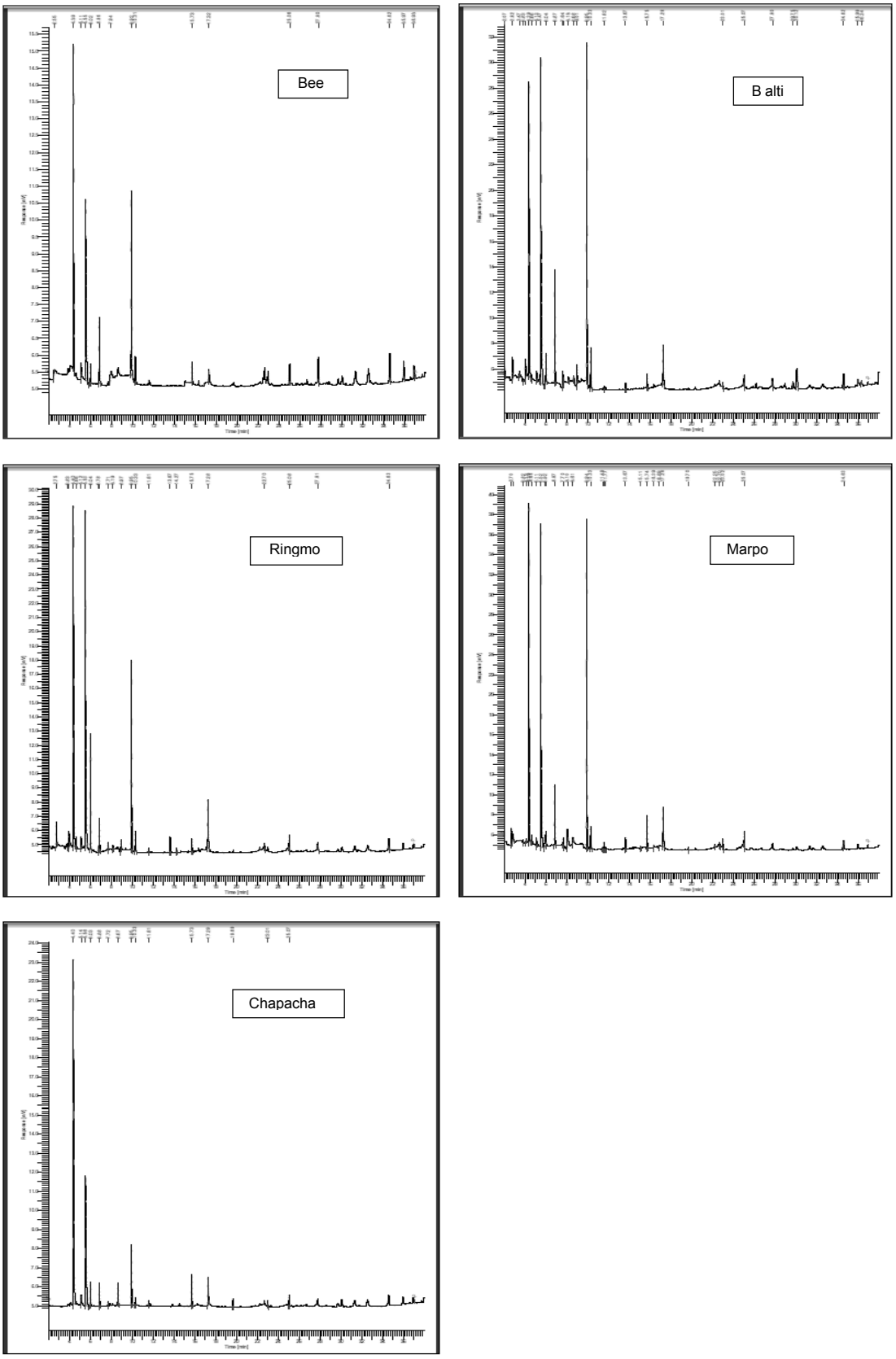


Fig. 3. Gas chromatograms of fruits of Russian olive variants

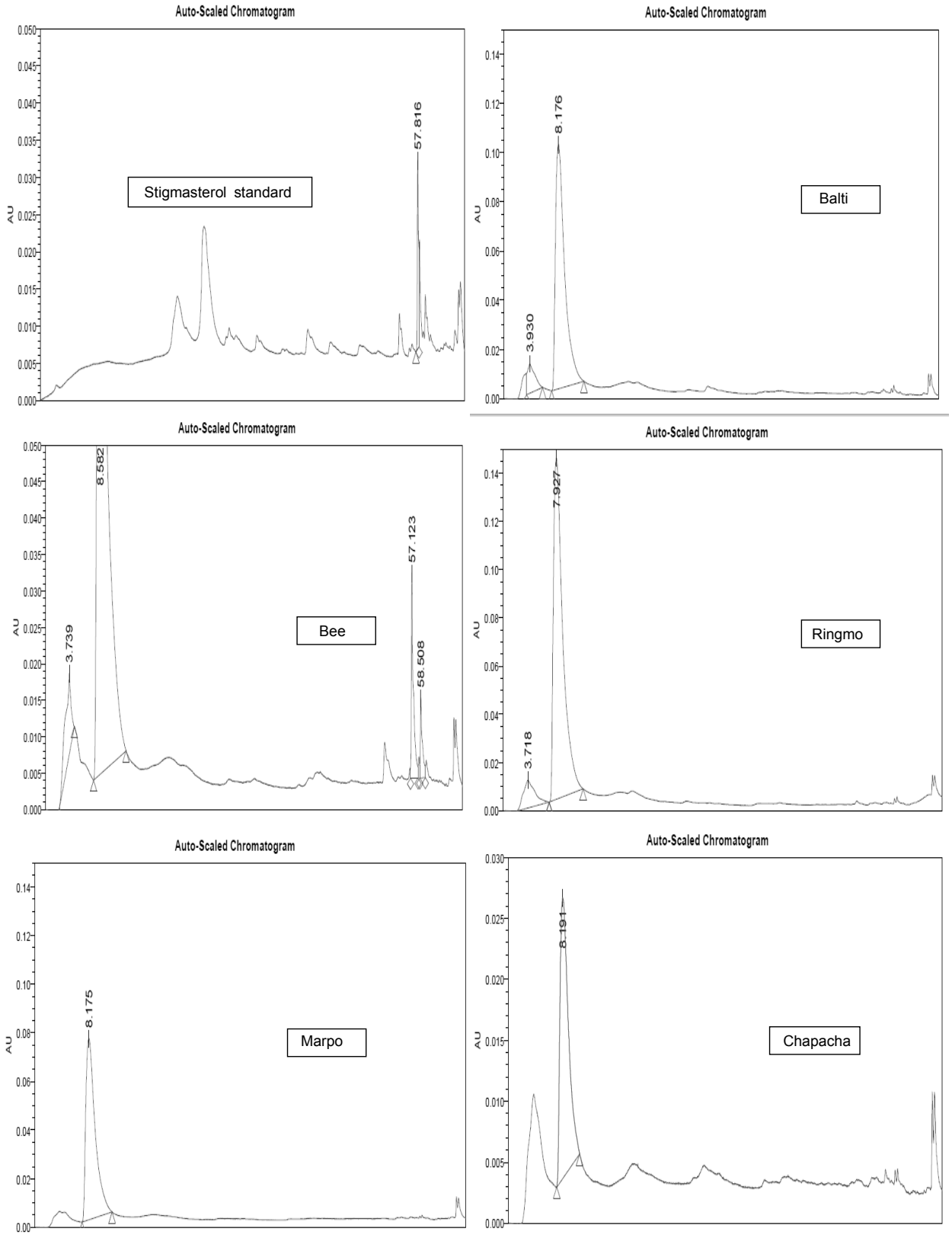


Fig. 4: HPLC plots of stigmasterol standard and fruit extracts of Russian olive variants