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REVIEW ARTICLE

Fractal analysis of flavonoids in complex chemical compositions in extracts of *Chamaedaphne calyculata* (L.) Moench (ericaceae) in oligotrophic swamps of Western Siberia

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Abstract

Evaluated the possibility of organizing the biosynthesis of plant adaptogens-flavonoids from the standpoint of the theory of neutrality. We assumed that the biosynthesis of flavonoids and low-molecular compounds with similar physicochemical properties is neutral with respect to microfluctuations of environmental conditions. Leaf extracts *Chamaedaphne calyculata (L.)* Moench from natural populations oligotrophic swamps the Middle Ob region (Western Siberia) was studied by method HPLC-high performance liquid chromatography. The samples to carry out the HPLC analyses were taken in ecologically equivalent swamp areas. Chromatograms differed in the number of peaks, the time of their release, and the area of particular peaks. The hierarchy constructing procedure from single up to "combined" chromatograms, where peaks from 1, 2, 3, 5, and 9 chromatograms were placed on the common time scale of the peaks release, was carried out for fractal analysis. The number of peaks varied from 21-30 in particular chromatograms up to 76 peaks in a "generalized" united chromatogram. The complex of calculation procedures has shown that all chromatograms obtained from *Chamaedaphne calyculata* plants are stochastic fractals, where the independent variables are the number, the size (area) and release time of each particular peak. In the presence of the fractal properties of the flavonoid system, it is necessary to analyze the possibility of implementing both neutral and deterministic variants of the synthesis of this or that flavonoid.

Keywords: Stochastic fractals, flavonoids, *Chamaedaphne calyculata*, HPLC-High performance liquid chromatography, oligotrophic swamps, Western Siberia

Introduction

In plants from natural ecosystems, combinations of recorded traits or compounds often show the signs of a stochastic process, with weak dependencies on external conditions (Gelashvili et.al 2013). The previous results indicate the fractal nature of the formation of morphological and physiological complexes of *Chamaedaphne calyculata* plants (Usmanov et.al 2016).

Flavonoids are considered the group of polyphenols with numerous physiologically active properties-various

antioxidants, antibacterial, photo- and cryo- protectors, pigments involved in photosynthesis, etc. The biological diversity of flavonoids is very large: nowadays, several thousand of them are described and, according to general opinion, it is only a small part of these compounds.

Flavonoids are low-molecular compounds that can be attributed to the plant metabolome (Bundy et. al 2008). The metabolome is a set of all low-molecular metabolites of a cell, tissue or organism, which is determined by the genome, on the one hand, and, on the other hand, is regulated by adaptive processes under the action of various environmental factors (Goncharov et. al 2015, Smolikova et. al 2015, Rees et al 2017). In the practice of chromatographic studies, it looks like chromatograms with a changing number of peaks, the time of their release, as well as the area of particular peaks. To emphasize the individuality of such chromatograms, the term "fingerprint" was taken from criminalistics (Bundy et. al 2008, Goncharov et. al 2015, Smolikova et. al 2015, Rees et al 2017).

The total number of flavonoids and other low-molecular compounds in metabolomes is estimated in thousands, so in real conditions, all these compounds are either very difficult, or expensive, or simply impossible to identify (Bundy et. al 2008, Goncharov et. al 2015). Therefore, the dynamics of the general mechanisms of metabolomes formation, in particular, the role of stochastic processes, is under investigation currently. In the most common form, the stochasticity of biological objects is revealed with the help of the fractal analysis apparatus (Gelashvili et.al 2013, Mandelbrot 1982).

If the properties of stochasticity are manifested for a wide range of chromatograms of extracts of different plants from different populations, then any chromatograms can be interpreted as stochastic fractals (Mandelbrot 1982). If the formation of chromatograms of extract in biosystems of different levels-from a single plant through coenopopulations-to the areal in some geographical boundaries forms self-similar structures throughout the selected range, the chromatograms are a fractal system. Fractal systems of any nature show "a unique property that allows on the basis of available (almost always incomplete) information about a part of the object make a statistically correct conclusion about the object as a whole..." (Gelashvili et.al 2013).

The work objective of this paper is to assess whether it is possible to consider the chromatograms of flavonoids and compounds with similar physical and chemical properties from *Chamaedaphne calyculata* as fractal objects with stochastic properties.

Materials and Methods

Ecological conditions

Chamaedaphne calyculata was investigated in natural conditions of oligotrophic swamps. The swamp contour with ecologically homogeneous conditions was chosen for the analyses (Lapshina 2010, Ovechkina 2017). Natural homogeneity of conditions was determined by a number of parameters:

1) The drain less swamp without water flow from other landscapes, and the movement of water flow within the borders of the swamp is chaotic, influenced by temperature, wind, melting of snow, etc. Water pH = 3-5;

2) Raised swamps, by definition, have no supply of nutritional agents, with the exception of the removal of the underground substrate. Removal practically stops with the growth of peat, and the plants on the surface get resources from decaying peat masses. Ash content of soils is within 2% to 5% (Ivanov et. al 2017);

3) The vegetation of the selected area is homogeneous. Syntaxonomically, the whole investigated area is covered by plants of the class Oxycocco-Sphagnetea Br.- Bl. et R.Tx., 1946. Dominant species belonging to the Ericaceae. *Chamaedaphne calyculata* is about 18% to 35 % of the plant composition (Ovechkina 2017).

Leaves for analysis were selected in the first ten-day period of July. Individual samples of leaves of plants from 9 different habitats were taken. All plant material was dried to the air-dried basis, crushed to a particle size of no more than 2 mm.

Extraction was carried out with a weighed quantity of 20 mg in stages: with hexane (in three doses with a total volume of the solvent of 100 ml), after evaporation of hexane, the vegetable fiber was extracted with 70% ethanol (in three steps, combining extracts filtered with glass filters). The use of hexane as a preliminary extractant was caused by the necessity to clean the samples from various non-polar organic substances not related to phenolic metabolites. Further on, during the analysis of samples with the HPLC method, these extracts were not analyzed.

HPLC of *Chamaedaphne calyculata* extracts

Sample preparation: Preparation of vegetable raw materials was carried out in two stages: to clean the vegetable raw materials from the fat-soluble fraction, hexane was used. Vegetable raw materials were incubated three times with hexane for 15 minutes with continuous shaking, filter through glass filters. Then, the vegetable raw material was incubated for 45 minutes in 70 % aqueous solution of ethanol three times, filtering and combining the obtained extracts. The extracts were evaporated in a water bath to obtain a concentrated solution; the solution was dried to obtain a constant dry weight.

Flavonoids analysis: For the analysis, the weighed quantity of the extracted sample was taken, dissolving it in eluent 50:50 water-acetonitrile. Chromatography run was carried out by the HPLC method on a chromatograph Waters "Breeze". The chromatography run of extracts of leaf samples was carried out in the inverse phase mode on a column Luna C18 250 × 4.6 mm, 5 µm. Each sample was analyzed three times. Standards and substances in the samples were detected at a wavelength of 360 nm (Fig. 1.).

Fractal analysis: At the first stage of testing for fractality, sampling and scaling are carried out-these are unformulated procedures, at the stage of which the researcher makes a hypothesis what is a self-similar structure, and what hierarchy these structures can form, preserving the property of self-similarity (Gelashvili et.al 2013).

Further on, the fractal properties of the selected hierarchy are evaluated-calculation procedures, on the basis of which the hierarchy under investigation can or cannot be attributed to fractal ones (the calculation stages are given below).

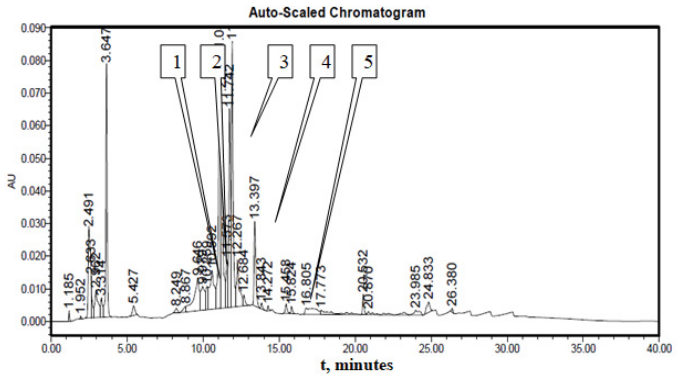

Figure 1. Chromatogram of the Chamaedaphne calyculata leaves extract. On the "X" axis - time, on the "Y" axis signal intensity.1-5 - peaks identified according to the standards. 1. naringin, peak release time - 10,30 min; 2. rutin - 10,59 min; 3. dihydro quercitin, 11,94 min; 4. fisetin - 13,397 min; 5. quercitin - 15,458 min.

The statistical processing was carried out with the help of Excel software package.

Results and Discussion

Fractal analysis of the chromatograms of *Chamaedaphne calyculata* extracts

The main stages of the fractality evaluation are (Gelashvili et.al 2013):

1) Object selection that is considered as "self-similar"

2) Definition of a hierarchical system within which objects retain self-similarity

3) The calculation stage at which it is determined whether the hierarchical system by self-similarity has this property or not

The first stage: Sampling- the initial selective process, during which a kind of unified structure, checked for the properties of self-similarity, is chosen. The chromatogram of the "single" extract of a standard sample of plant material averaged over three analytical runs was taken as an elementary unit of the hierarchy (Fig. 1.). A separate chromatogram was characterized in three parameters:

1) The release time of a single peak in a standard solvent system

2) The number of peaks released in a particular chromatogram

3) The size of peaks as a reflection of the substance concentration

The second stage: Scaling-determination of the scale measure and range of levels of the organization (hierarchy), in which all incoming and hierarchy-forming structures have the properties of self-similarity. Scaling allows determining under what conditions and on what scale the studied chromatogram complexes are considered fractal objects.

For this purpose, a hierarchy is created where the separate elements (in this case, chromatograms) are combined. In this investigation, a hierarchy by creating "combined" chromatograms with an increasing number of combined chromatograms was created. Almost all peaks on the chromatograms were released within 40 minutes; therefore, a single interval from the beginning of the peaks to the 40th minute was chosen for the combined chromatograms. In case of increasing the scale (combination), all the peaks of the summed chromatograms were placed on a single time axis. In the case when the same (in time of release) peaks were detected on both chromatograms, they were summed at the time, characterized for these peaks. If a peak appeared, which is on one, but not on the other chromatogram, the "new" peak was placed on the corresponding point of the time scale of the peaks. Thus, the total number of separate peaks in the combined chromatogram can increase significantly. The entire population, in this case, is the combination of all peaks detected in all chromatograms. It can be seen from Tab. 1. that as the chromatograms are combined, the number of peaks increases.

The procedure of chromatograms combining by hierarchical levels: the bottom three rows are the chromatograms of the particular extracts; the fourth row is the chromatograms combined in three; the top one is the combination of all chromatograms into the entire population of peaks found in all chromatograms. For perception convenience, the combined chromatograms of 2 and 4 levels are not given. The number of peaks is given in Tab. 1.

The investigation was carried out on the analogy of the study of the spatial structures: smaller sites are combined into larger ones, i.e., a hierarchy of scales is created (Mandelbrot 1982). The same method is used in phytosociology: the lists of specific plant species for separate sites are compiled, then these lists are combined on an increasing scale for areas covering biogeocenoses and landscapes, and flora of the corresponding rank (Gelashvili et.al 2013, Mirkin et. al 2012). Instead of individual plants, it seems possible to use individual peaks of chromatograms and their release time, and a separate whole chromatogram-as an analog of the phytosociological description on the site. This approach allows forming populations of realized peaks for a single plant if plant samples were taken from different areas on a single plant. Then, "entire" populations are formed for separate coenopopulations as the sum of selected plants, further on, for arbitrary groups of coenopopulations and, finally, for all studied general list of plants at the region.

In this investigation, all chromatograms of plants with the ecologically homogeneous site of the oligotrophic swamp were combined (Fig. 2. and Tab. 1.).

The assessment of the fractal nature of the analyzed chromatogram selection: On the basis of the constructed hierarchy, a generalizing matrix was created (Tab. 1. and Fig. 2.), on which the following indicators were calculated:

Self-similarity estimate: Mq (N) is the generalized statistical sum of the substance diversity indices (pi) calculated for data with this or that order of distribution (q). The expression recommended by the authors (Gelashvili et.al 2013) was used to calculate this indicator:

$$Mq(N)=\sum_{i=1}^{(N)} p_i^q,$$

where N-total sample size. This indicator ranged from 1 (single sample) to 9 (all investigated sites);

pi-the index of diversity; that is, the part of each compound in the overall picture;

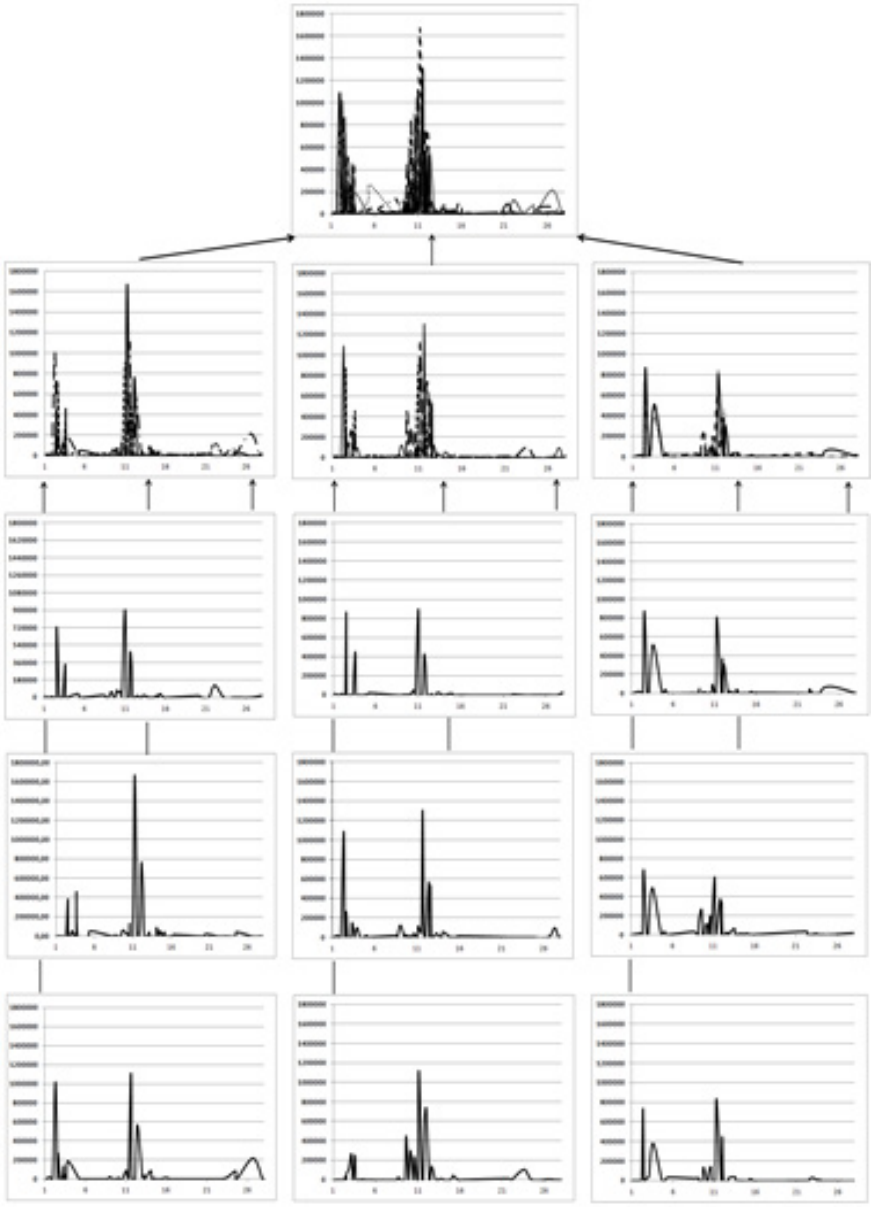

Figure 2. The procedure of chromatograms combining by hierarchical levels.

Table 1. Chromatograms hierarchy for the 5 levels of combination.

	Plants/grounds								
Levels of combining chromatograms	1	2	3	4	5	6	7	8	9
1. The number of peaks in individual chromatograms	36	41	34	33	30	34	32	28	21
2. Combining chromatograms in pairs	44		39			46		41	
3. Combining chromatograms of three		54			49			43	
4. Combining chromatograms in 2 groups			75				69		
5. Total number of peaks for all plants					108				

q-the order of distribution moment. This indicator is in the range recommended by the authors from -3 to 3 and actually sets the range of degrees to which pi of each element should be raised, which, in its essence, the scaling is. This indicator is necessary to represent the data of the generalizing matrix in a wide range of power levels. It is necessary to confirm the self-similarity of the observed pattern in the given range of these levels (Mandelbrot 1982).

At the first stage, a set of moments Mq was calculated for each value of q. The property of self-similarity is considered to be proved if in the whole range q a significant correlation between logMq from log N is seen (Gelashvili et.al 2013). Thus, the property of self-similarity is the first condition for the compliance of the object with the principles of fractal formalism.

However, the same property of self-similarity (according to the authors of the technique) may be characteristic of objects of regular nature, described by relatively simple laws, and in which between the demonstrated indicators, a rigid functional dependence is always seen.

The Akaike Information Criterion (AIC). The authors (Gelashvili et.al 2013) propose to estimate the irreducibility of

Table 2. General results of the fractal analysis of the total chromatograms of *Chamaedaphne calyculata*.

Stages of analysis	Procedure	Results
Sampling	Non-formalized selection of an assumed self-similar structure	the minimal self-similar structure was defined as a separate single chromatogram
Skaling	Estimation of the range of various scales of a set of self-similar structures	All chromatograms from an ecologically homogeneous area (stenosis) from single samples through samples of various sizes to a total population
Estimation of self-simulation	$Mq(N)=\sum_{i=1}^{(N)} p_i^q$	The entire chosen set of chromatograms and their aggregates possess the property of self-similarity, since all the correlations between the logarithms of Mq and N are significant (p>0.05), and the values of the correlation coefficients tend to 1.
Estimation of stochasticity (Akaike Information Criterion (AIC)).	$AIC = ln\frac{RSS}{n} + \frac{n+k}{n-k-2'}$	In all cases, the nonlinear model is better applicable to the observed pattern than the linear one.
Estimation fractal properties of chromatogram's pool	Complete of chromatograms are all are stochastic organized self-similar structures, forming fractal stochastic systems	

the observed pattern to simple mathematical models according to the Akaike Information Criterion (AIC). To assess the nature of the observed pattern of the dependence of log Mq indicators from log N, the authors propose to use two models-"linear" and "quadratic."

This criterion for small samples was calculated by the formula:

$$AIC = ln\frac{RSS}{n} + \frac{n+k}{n-k-2'}$$

where, n- the volume of analyzed data, k- the number of model parameters (for a linear model, it is 3, for a quadratic model-4), RSS- the sum of squared deviations from the values predicted by the models.

The Akaike Information Criterion (AIC) is calculated for both models over the entire range of values used in the calculations of the order of q distribution moments. In the investigated case, the values of the Akaike criterion were closer to the quadratic models.

The more accurate approximation of the value of the Akaike criterion to the quadratic model than to the linear model in the entire range of orders of distribution moments (q) from -3 to +3 was obtained as a result. This indicates the possibility of the object under investigation to have fractal (multifractal) properties. In other words, the Akaike information criterion indicates that the population investigated in the whole range (q) cannot be described by standard curves, but can be described as a stochastic fractal (Mandelbrot 1982).

The presence of self-similarity in investigated samples was estimated according to the reliability of the linear dependence of logarithms of the indices N and Mq, and applicability to the observed pattern of the fractal hypothesis-the results of the comparison of the Akaike criterion for the linear and quadratic models. Thus, all chromatograms obtained from *Chamaedaphne calyculata* plants are stochastic fractals, where the independent variables are the number, the size (area) and release time of each particular peak.

Conclusion

Investigated the variability of HPLC-chromatograms of extracts from leaves of *Chamaedaphne calyculata* in the natural conditions of oligotrophic swamps of Western Siberia. All chromatograms are “self-similar” structures with properties of stochastic fractals, where the independent variables are the number, the size (area) and release time of each particular peak. The hierarchy constructing procedure from single up to "combined" chromatograms, where peaks from 1, 2, 3, 5, and 9 chromatograms were placed on the common time scale of the peaks release, was carried out for fractal analysis. The number of peaks varied from 21-30 in particular chromatograms up to 108 peaks in a "generalized" united chromatogram. In general, there is a low similarity between the individual chromatograms (Tab. 2.).

We consider possible mechanisms for the dependence of the synthesis of flavonoids and compounds with similar physicochemical properties on the micro-distribution of salts in the soil in the zone of the root system. Previously, the data on permanent microfluctuations of the chemical composition of soils and dirt in very different environments: from swamps to steppes and steppe solonchaks were obtained (Usmanov et. al 2014, Usmanov et. al 2016, Usmanov et. al 2016, Usmanov et. al 2017, Usmanov et. al 2018, Aitov 2013). Changes in the concentration of elements of the soil solution and marsh waters act as regulatory factors for certain stages of plant metabolism. It is known that the system of flavonoid biosynthesis allows in some cases to synthesize a particular substance through different metabolic pathways formed through metabolic branches and shunts (Ivanov et. al 2016, Ivanov et. al 2017, Usmanov 1986, Bashirova et. al 1998, Caston et. al 2005). The synthesis of particular compounds in plants is controlled by a large number of endogenous and exogenous factors (Bundy 2008), and the combination of stimulating and inhibitory regulators is constantly changing. Due to this fact, the response of plants to the constant fluctuations of the medium, the composition of the flavonoid metabolome is represented as the formation of a stochastic set, the composition of which continuously fluctuates. It is the evidence in favor of the fact that the chromatograms of the *Chamaedaphne calyculata* flavonoids are fractal objects with stochastic properties. Taking into account the fluctuating nature of the environmental parameters and the high mobility of the element synthesis of flavonoid metabolome, it is possible to expect two variants of case scenarios (Bundy 2008):

a) The real response of the system of flavonoids synthesis has a neutral nature, i.e., it has mechanisms to choose among several equally likely events, which only partly depend on the specific factors of the external environment.

b) The synthesis of this or that flavonoid is strictly determined by the regulators of metabolism operating here and now. In the presence of fractal properties of the flavonoid system, it is possible to realize the implementation of both neutral and deterministic variants of the synthesis of this or that flavonoid. The analysis of this alternative will be the topic of the future investigation.

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