REVIEW

from

Professor Georgi Yankov Papanov, DSc, Department of Organic Chemistry, Faculty of Chemistry, Plovdiv University "Paisii Hilendarski", retired

of dissertation for awarding the scientific degree "**Doctor of Sciences**"

in the field of higher education:	4. Natural sciences, mathematics and computer science
professional field:	4.2. Chemical sciences
scientific specialty:	Organic chemistry

Author: Assoc. Prof. Dr. Petko Ivanov Bozov from the Department of Biochemistry and Microbiology at the Faculty of Biology of the University of Plovdiv "P. Hilendarski ".
Topic: "Clerodane diterpenoids of species of the family Lamiaceae"

I. SUBJECT OF REVIEW

By order № P33-902 of 11. 03. 2021 of the Rector of Plovdiv University "Paisii Hilendarski" (PU) I have been appointed a member of the scientific jury for providing a procedure for defense of a dissertation on "Clerodane diterpenoids of species of the family Lamiaceae" to obtain the degree of "Doctor of Science" in the field of higher education: 4. Natural sciences, mathematics and computer science, professional field: 4.2. Chemical sciences (Organic chemistry). The author of the dissertation is Assoc. Prof. Dr. Petko Ivanov Bozov - Department of Biochemistry and Microbiology at the Faculty of Biology, University of Plovdiv "P. Hilendarski".

The set of materials on paper and electronic media presented by Assoc. Prof. Dr. Petko Ivanov Bozov is in accordance with Art. 45 (4) of the Regulations for development of the academic staff of PU, includes the following documents:

- application to the Rector of the University of Plovdiv for disclosure of the procedure for defense of the dissertation;
- CV in European format;
- a copy of the diploma for the educational and scientific degree "Doctor";

- minutes of department councils related to the opening of the procedure and the preliminary discussion of the dissertation;
- dissertation work;
- abstract;
- списък на научните публикации по темата на дисертацията;
- copies of scientific publications;
- декларация за оригиналност и достоверност на приложените документи;
- reference for fulfillment of the minimum national requirements;

I.1. GENERAL PRESENTATION OF THE RECEIVED MATERIALS AND DOCUMENTS OF ASSOC. PROF. DR. PETKO IVANOV BOZOV

The documents and materials submitted by the candidate are well designed and arranged. The dissertation has applied 28 scientific papers on the topic of the dissertation did not participate in the procedure for acquiring ONS "Doctor". Eighteen of the scientific publications are in scientific journals, referenced and indexed in world-famous databases of scientific information. One article is cited in a scientific journal, referenced and indexed in world-famous databases of scientific information. Nine of the publications are in refereed editions, and one is in full text in a conference proceedings. Seventeen scientific publications are other than those presented in procedures for acquiring ONS "Doctor" and for the academic position "Associate Professor". Of these, 11 publications are in scientific journals, referenced and indexed in world-famous databases of scientific information. All articles are extremely well designed and presented.

I.2. BRIEF PRESENTATION OF THE CANDIDATE FOR THE SCIENTIFIC DEGREE "DOCTOR OF SCIENCES

Assoc. Prof. Dr. Bozov graduated from the University of Plovdiv “P. Hilendarski ”in 1986. with a qualification "chemist, chemistry teacher" with a second specialty "physics". He works as a chemist controlling the quality of essential oils in NIRELK "Bulgarian Rose" in Kazanlak, and since 1989. is a full-time doctoral student at the University of Plovdiv, Department of Organic Chemistry under the supervision of me and Assoc. Prof. Dr. Petar

Malakov. In 1994 he successfully defended the ONS "Doctor" on "Di- and triterpenoids in members of the family Lamiaceae and their biological activity" in the specialized scientific council on "Organic Chemistry and Organic Technologies" of the Higher Attestation Commission". After that he worked at Geo Milev Primary School and high school Hr. Smirnenski "as a teacher of chemistry and English and a part-time lecturer in General and Organic Chemistry at the Medical University - Plovdiv. Since 2010 he has been on an employment contract at the University of Plovdiv, Faculty of Biology.

He has completed a course "Working in a multiethnic environment" and "Basic and specific computer skills of the teacher" at the National Pedagogical Center of the Ministry of Education and Science (MES). He is a member of the Union of Scientists in Bulgaria and the Regional Union of Chemists at NTS - Plovdiv, as well as the Chitalishte Board of Trustees at Chitalishte "Hristo Botev", Plovdiv.

I know him as a student, then as a graduate and doctoral student. He is an extremely hard-working, resourceful and ambitious researcher and lecturer, a precise researcher who constantly studies and masters modern methods for extraction, separation, chemical and spectral characterization and identification of the structure and stereochemistry of complex organic chemical compounds. Assoc. Prof. Dr. Bozov managed not only to transfer phytochemical research in the field of diterpenoids from the Faculty of Chemistry to the Faculty of Biology of Plovdiv University, but also to develop them at a higher level, from which developed the current dissertation for obtaining the scientific degree "Doctor of Science".

I.3. STRUCTURE AND CONTENT OF THE DISSERTATION

The dissertation contains 289 pages, which include:

- content and abbreviations used - 4 pages
- introduction - 3 pages
- literature review (first chapter) - 32 pages
- purpose and tasks of the dissertation - 1 page
- materials and research methods (second chapter) - 12 pages
- results and discussion (third chapter) - 111 pages
- conclusions and contributions - 6 pages

- cited literature - 16 pages
- appendices (1-23) - 91 pages
- scientific papers on the topic of the dissertation, did not participate in the procedure for acquiring ONS "Doctor" - 12 pages

The literature review includes 376 sources, of which only four are in Cyrillic. It is at a modern, critical and comprehensive level, with 28% of publications coming from the last 15 years. A thorough knowledge of the literature and very correct citation of the Bulgarian authors is demonstrated. As a consequence, the purpose and tasks of the dissertation are correctly formulated.

The above-ground parts of the plant material of five genera of the Lamiaceae family were personally collected by him and there was no discrepancy in determining the species from the Departments of Botany at the University of Plovdiv and the Agricultural University, as well as from the Institute of Botany at BAS, which shows that he is well acquainted with the botanical characteristics of the species he has selected and their probable location.

Regarding the extraction of natural organic compounds, purification and separation into individual substances, as well as their physico-chemical and spectral characterization, Assoc. Prof. Bozov has extensive practical experience and has not been an insurmountable problem. Even as a graduate he showed great interest in reading the various spectra, and as a doctoral student he boldly handled their interpretation. He greatly enriched his knowledge in the joint reading, interpretation and use of different techniques in NMR spectroscopy and for this he expressed his gratitude to Prof. Plamen Penchev in his dissertation. Assoc. Prof. Bozov has always been interested in the application of natural organic compounds, which is also observed in these scientific studies.

The text of the dissertation contains 33 tables and 137 figures. The main, most voluminous part of the dissertation "Results and Discussion" is structured in five sections, dedicated respectively to:

1) Diterpenoids of species of the genus *Scutellaria*

Of these, a total of 35 *neo*-clerodane compounds were isolated, of which 15 were novel diterpenoids. They are chemically and spectrally characterized, the structures of which are

presented in Table № 15. In addition to the diterpenoids in the studied species, 2 sterols, 2 iridoids and 2 cleroindicines were identified, which are known organic compounds.

2) Diterpenoids of the genus *Teucrium polium* subsp. *vincentinum* L. and *Teucrium scordium*

subsp. *scordioides* (Schreb.) Maire et Petitmengin

Four fractions were eluted from the bitter fraction of *Teucrium polium* by column chromatography. From the first, a compound was isolated by TLC whose spectral characteristics (IR, ^{1}H, ^{13}C and DEPT NMR spectra, molecular ion peak in HR-ESIMS and the calculated general formula) was characterized by a diterpenoid with the trivial name polyvincin A. After analysis of the recorded 2D NMR spectra (HSQC, COSY, HMBC and NOESY) its structure and stereochemistry are unambiguously determined. It turns out that the data from the proton spectrum are very close to those published by Malakov and co-authors for the acetylated diterpenoid with the trivial name montanin D, isolated from *Teucrium montanum*, for which ^{13}C NMR spectral data have not been published. The proton spectra for it were recorded at 80, 100 and 220 MHz, and for polyvincin A at 600.13 MHz. These and other spectral data complement the spectral characteristics of the product obtained after acetylation of montanin D.

The structure and stereochemistry of the second new diterpenoid polyvincin B was proved by similar studies from the second fraction. Its spectral characteristics are very close to those of teopolin XII isolated from *Teucrium polium* L. by Fiorentino and co-authors in 2011. Differences in the proton spectrum are observed, some additional data from ^{13}C NMR spectra are also given.

From the third fraction was isolated and identified third new *neo*-clerodane called polyvincin C. Its structure and stereochemistry were identified by the mentioned spectral studies. For all three new *neo*-clerodanes, in addition to the spectral data from the applied spectral studies, their physicochemical parameters were determined.

Three compounds were eluted from the bitter fraction of *Teucrium scordium* by chromatographic separation, which after careful spectral studies were identified as furo clerodane dilactones with the trivial names 6-acetyl-teucrin F, teucrin E and 3α-acetoxy-teucvin. The first two compounds were previously obtained semi-synthetically by Malakov et al. In acetylation of the corresponding hydroxyl derivatives isolated from *Teucrium hamaedris*. In the studied new plant material from another location, they were isolated as natural diterpenoids, as well as the third 3α-acetyl derivative of the diterpenoid teucvin, isolated and identified by E. Fujita (Japan).

All three diterpenoids have been identified by the mentioned precise spectral studies, which are presented in the dissertation.

3) Diterpenoids of the genus *Salvia*

From the bitter fraction of *Salvia splendens* Ker.-Gawl. chromatography on a silica gel column eluted four substances and one substance from the aerial parts of *Salvia nemorosa* L. Under crystallization conditions, crystalline products were obtained from the four eluted substances, which were characterized spectrally and their physicochemical parameters were determined. They have been identified as furo *neo*-cleroda dilactone diterpenoids, which turn out to be known compounds. The powdery substance isolated from *S. nemorosa* L. after spectral characterization was identified as sclareol (Labdane type diterpenoid), which is also described in the literature. It was found that in *S. amplexicaulis* LAM. does not contain diterpenoids.

4) Biological activity of extracts and clerodan diterpenoids

4.1. Testing of the anteefidant activity of extracts of species of the genus *Scutellaria* and of clerodane diterpenoids against larvae of *Leptinotarsa decemlineata* Say.

Eight extracts of different types were tested and the obtained results were presented in Table 25. In the Table 26 was presented the test results of 9 *neo*-clerodanes. Eleven *neo*-clerodane diterpenoids isolated from *Scutellaria galericulata* were tested for insect-antifeedant activity against larvae of *Leptinotarsa* decemlineata Say., in optional feeding experiments. The results are presented in Table 27. The insect-antifeedant activity of 14 neo-clerodan diterpenoids isolated from *Scutellaria altissima* was tested. The results obtained are given in Table 28. In these studies, a difference was found in the activity of epimeric pairs with R and S configuration at C-11 and the type of substituents at C-15 or C-19.

The antifeedant activity of 10 diterpenoids of the genus *Teucrium*, isolated from *T. polium* and *T. scordium*, was tested. The results are presented in Table 29.

I fully support the comment and the conclusions from the conducted research on 4.1.

4. 2. Testing of the antimicrobial activity of clerodane diterpenoids against pathogenic and hygienic-indicator microorganism

The antimicrobial activity of 22 clerodane diterpenoids (Fig. 135) isolated from species of the genus *Scutellaria*, *Salvia* and *Teucrium* of the family Lamiaceae against 19 strains belonging to 11 different species of pathogenic bacteria in food and against two strains of yeast was studied. belonging to Candida albicans species. All tested compounds have a clerodane skeleton,

which are conditionally divided into several groups depending on the substructures (A-C) in the C-1 - C-10 clerodane nucleus and the substructures (Ia, Ib, IIa, IIc, IIIc) in C- 11 - C-16 side chain. It is noted what the symbols A, B and C mean, as well as Ia, Iv, IIa, IIc, IIIc. In Table 30 were shown the results of antimicrobial activity. I also fully share the comments and conclusions of the research.

4.3. Testing the cytotoxic activity of *neo*-clerodane diterpenoids against carcinogenic lung tumor cells (H1299) and normal umbilical cord cells (HUVEC)

Twelve *neo*-clerodane diterpenoids were studied, the names and numerical designations of which were also given, as well as from which species of the genus *Scutellaria* they were isolated and tested for this activity. The compounds are divided depending on substituents in the decalin nucleus of groups A and B, and based on C-11 - C-16 substructures into four groups (I - IV). In Table 31 were presented the cytotoxic activity of 7 *neo*-clerodanes against H1299 and HUVEC cell lines. Graphpad Prism experimental results for cytotoxic activity are given in Appendix 22.

I fully share the conclusion that has been made.

5) Isolation and characterization of other organic compounds

5.1. Determination of the chemical composition of the essential oils of *Ajuga laxmanii* Benth, *Salvia amplexicaulis* Lam. and *Stachys cretica* subsp. *bulgarica* Rech. Fil of the family Lamiaceae

The analysis was performed using GC and GC / MS. The essential oil of both species (*Salvia* and *Ajuga*) was studied, for which yield and humidity, qualitative and quantitative composition were determined (Table 32). It has been found that the acetone extract of the species *Salvia* and *Ajuga* does not contain di- and triterpenoids.

The chemical composition of the essential oil of the endemic species *Stachys cretica* subsp. *bulgarica* Rech. Phil. through the GC / MS analysis quantitatively identified 20 components (89.20%) presented in Table 33. Di- and triterpenoids were also not identified.

5.2. Quantitative determination of polyphenolic compounds in *Sculellaria altissima*

The analysis was performed using high performance liquid chromatography. The qualitative and quantitative of 8 polyphenolic compounds, the structure of which are shown in Figure 136, was determined. They were found to be known compounds.

5.3. Isolation and characterization of sterols, cleroindicins and glucoside-related iridoids

In *Scutellaria albida*, a glucoside-linked iridoid, which turns out to be the known iridoid catalpol, was identified by ^{1}H NMR spectrum.

In *Scutellaria altissima* were identified the known *β*-sito- and stigmasterol, as well as the glucoside-linked iridoid globularin. The substances are characterized spectrally and the data for them are presented in Annexes 3 and 4, and their structures in Figure 137.

From *Scutellaria hastifolia* L. are isolated and characterized cleroindicin B and cleroindicin F, whose spectral data and structures are presented in Annex 23. Cleroindicins B and F are new compounds both for the species *Scutellaria hastifolia* L. and for the genus and family Lamiaceae.

I fully support the conclusions of the contributions and the contributions of the dissertation, as they are in full accordance with the conducted phytochemical studies of 15 species belonging to five genera of the family Lamiaceae.

The abstract contains 66 pages, which are in full accordance with the research, described and discussed in the dissertation and published 28 scientific papers, in 16 of which Assoc. Prof. Bozov is the first author, in 17 he is a corresponding author, and in three articles he is an independent author. Most of the scientific reports are published in specialized scientific journals with IF. The calculated total IF of the dissertation is 14.921. There are 5 participations at international scientific forums on the topic of the dissertation, and 7 at national ones.

So far, the scientific papers related to the dissertation, in which Assoc. Prof. Dr. Bozov participates, have been cited 141 times by other authors, of which 76 have not been used in other procedures.

NOTES AND QUESTIONS

On page 107 in the dissertation is written table 18, instead of table 15, on page 144 quotes table 27 instead of table 24, and on page 139 is written page 19 instead of page 24.

1. It is known that there are a large number of species in the different genera of the Lamiaceae family. In which of the genera can be assumed to contain new, undescribed organic compounds?

2. Will you continue the research of the species of the genus *Teucrium* and *Scutellaria*? Do you have any plans for the cultivation of Bulgarian or foreign species that contain natural organic

compounds (diterpenoids, polyphenols) with proven biological activity, with a view to their use for medicinal purposes?

CONCLUSION

The peer-reviewed dissertation has an indisputable scientific contribution, as it contains complete scientific data on 22 new diterpenoids: two with a 19-*nor*-clerodane skeleton and 20 with a *neo*-clerodane skeleton. Their structure and stereochemistry have been proven by spectral studies (proton and carbon NMR, and some newer techniques - 2D spectra have been applied, IR spectroscopy and Mass spectrometry). The physicochemical parameters of diterpenoids are also determined.

These studies have made great scientific contributions in the field of diterpenoids, which are valuable natural biologically active organic compounds.

The conducted studies related to the biological activity of a series of extracts and individual substances (cytotoxicity testing, antifeedant and antimicrobial activity) have a practical focus and value.

The peer-reviewed dissertation in terms of volume and quality meets all the requirements of the Academic Staff Development Act in Bulgaria, the Regulations for its application, as well as the relevant regulations of Plovdiv University "Paisii Hilendarski" and the specific requirements of the Faculty of Biology for obtaining the degree "Doctor of Science".

I find it reasonable to give my positive assessment of the dissertation and to recommend to the esteemed scientific jury to award Assoc. Prof. Dr. Petko Ivanov Bozov the scientific degree "Doctor of Science" in higher education 4. Natural Sciences, Mathematics and Informatics, professional direction 4.2. Chemical sciences (Organic chemistry).

09. IV. 2021 prepared the review:…..…….…… ..

Plovdiv / Prof. DSc Georgi Papanov/