MEDICAL UNIVERSITY VARNA „PROFESSOR DR PRASKEV STOYANOV“
FACULTY OF PHARMACY
DEPARTMENT OF PHARMACOLOGY, TOXICOLOGY AND PHARMACOTHERAPY

S T A N I S L A V Y O R D A N O V M A R C H E V

PHARMACOLOGICAL STUDIES OF STEROID HORMONES, NATURAL PRODUCTS AND NEWLY SYNTHETISED 2H-SUBSTITUTED HYDRASID HYDRASONES IN EXPERIMENTAL MODELS OF EPILEPSY, PAIN AND OSTEOPOROSIS

SUMMARY

OF DISSERTATION
For award of an educational and scientific degree`
DOCTOR
***S*cientific specialty: Pharmacology (incl. pharmacokinetics and chemotherapy)**

Scientific supervisors: Prof. Dr Stefka Valtcheva - Kousmanova, PhD, DSc.
Ass. Prof. Polina Gateva, PhD
Scientific consultant: Corr. member of BAS, Prof. Dr Mila Vlaskovska, PhD, DSc.

V A R N A
2 0 2 0

The PhD student has a master's degree in pharmacy from the Freie Universität Berlin and is a doctoral student in an independent form of study at the Department of Pharmacology, Toxicology and Pharmacotherapy of the Medical University of Varna "Prof. Dr. P. Stoyanov ”. The dissertation was discussed, approved and directed for defense at an extended meeting of the Department, held on 25.06.2020 at the Faculty of Pharmacy of the Medical University of Varna.

The dissertation consists of 129 pages, 11 figures, 24 tables. The reference lists contains 155 papers by foreign authors and 15 publications by Bulgarian authors. The research presented in the dissertation is funded by research projects NSF DN 13/16 21. 12. 2017 and MUS D-74/2017.

The public defence of the thesis will be held on 24.09.2020 (online) by a scientific jury with following members:
Chair: Prof. Dr Petko Penkov Marinov, PhD
Members:
Prof. Dr Ivanka Ilieva Kostadinova, PhD
Prof. Dr Nikolay Damianov Danchev, PhD
Prof. Georgi Tsvetanov Momekov, DSc
Ass. Prof. Dr Maria Delcheva Zhelyazkova-Savova, PhD

The documents and papers related to the defence are available on 55, Marin Drinov Str. as well as on the website of the Medical University, Varna

I. INTRODUCTION

Epilepsy is one of the most serious and widespread neurological diseases following migraine, stroke (cerebrovascular disease) and Alzheimer's disease (Yagielski, A., 2016). It is characterized by high population morbidity, serious medical and psychological consequences for the individual and significant requirements for the state health and social systems. There are currently over 65 million patients diagnosed with epilepsy worldwide, and new 130,000 are added each year (Hesdorffer D. C., et al., 2013). According to the accepted international definition, "Epilepsy is a chronic, detrimental disease that manifests with unpredictable (two or more), recurrent (separated by at least 24 hours) seizures not caused by immediately identified cause" (Reddy DS , 2013). The main and pathognomonic feature of the major types of epilepsy is the appearance of generalized or focal seizures. Treatment of epilepsy is symptomatic and there is currently no effective mechanism-based therapy and/or effective prevention. The development of new antiepileptic drugs (AELs) that are effective in treating patients with refractory forms of epilepsy, destructive personality changes and / or comorbid conditions is of great clinical and therapeutic importance. The fact that 40% of patients with epilepsy suffer from drug-resistant forms of the disease is quite important in this context (Mohanraj, R., Brodie, M. J., 2005).

It is well known that patients with epilepsy who have been on long-term anticonvulsant therapy are at a much higher risk of osteoporotic bone changes and of related bone fractures, as well as various other traumatic complications. Osteoporosis is a progressive systemic disease of the skeletal system characterized by progressive bone loss and changes of bone microarchitectonics (World Health Organization, 1998). The osteoporosis progress leads to decrease of the bone strength and significantly increases the risk of fractures after minimal trauma. In cases of no fracture the “silent” disease could have a single alarming symptom - pain. The pain is an aversive signal of actual or potential damage to the body. It is a dynamic complex of sensory, cognitive and emotional perceptions. In evolutionary terms, pain is an inevitable component of life (Macfarlane, G. J. et al., 2006). According to the definition of the International Association for the Study

of Pain (IASP), "Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage" (Merskey, H., Bogduk, N., 1994).

The oestrogen containing hormone replacement therapy (HRT) is the method of choice for managing symptoms and preventing osteoporotic bone changes during menopause. However, the hormone replacement therapy (HRT) causes serious side effects. Among the most troublesome of them are the increased risk of uterine and breast cancer, thromboembolism, and menorrhagia (Rang, H. P., et al., 2007). As an alternative, HRT with phytoproducts is widely used. The latter is not related with do not cause the potential complications of HRT with native oestrogens. Currently, the soy phytoproducts are the most used. Herbal medicines with Genistein and / or Genistin as an active ingredient are also widely used. The hypothesis that combination therapies help to individualize the treatment of women with severe post-menopausal osteoporosis finds considerable support. In this context, phytoproducts derived from plants, known for their culinary or medicinal properties, might be suitable for phytochemical and pharmacological studies in experimental models of oestrogen-deficient osteoporosis.

We focused on studies of Aronia fruit juice 10 ml / kg and 5 ml / kg evaluating the presence of antiosteoporous action / estrogen-like activity with probable therapeutic potential for the prevention and / or treatment of women with postmenopausal osteoporosis. The fruits of *Aronia melanocarpa* are popular for their organoleptic properties. In recent years, Bulgarian and foreign researchers demonstrated interest reflecting the opportunity to reveal pharmacotherapeutic application of phytoproducts from Aronia to find real (Valcheva-Kuzmanova, S., Belcheva, A., 2006). The performed phytochemical analyses show that the fruits of *Aronia melanocarpa* contain a large number of biologically active substances, which might have favourable therapeutic effects. Studies on other plants should be interpreted in the same context. Experimental and clinical data impose that celery products (*Apium nodiflorum*) can have numerous and varied effects on health. Phytochemical analyses of parts or products of celery have shown that they contain numerous chemical compounds representing different groups of secondary metabolites. Systematic *in vivo* and *in vitro* studies on chronic treatment with *Apium nodiflorum* extract by Vlaskovska et al., present convincing evidences for a favourable effect of

clinical and paraclinical symptoms in experimental models of postmenopausal osteoporosis (Tsakova, A., et al., 2015, Tsakova, A., 2016).

III. AIMS AND TASKS

(The sections here are presented with the numbering they have in the dissertation)

1. 1. Aims of the dissertation

1. 1. Bearing the diversity of clinical and laboratory data, which often are opposite, aim of the dissertation is to study the effect of the suprarenal and gonadal steroids on the intensity, dynamics and latency of the kainic acid induced seizures and lethality in an experimental models of epileptogenesis in rats. In the same direction the effect of a series of newly synthesised hydrazid hydrasones compounds on the nociception will be studied.

1. 2. The second aim is to study the effect of the phytoproducts from *Aronia Melanocarpa* and *Apium Nodiflorum* on the bone mineral density (BMD) and the bone mineral content in animals with experimental oestrogen-deficient “postmenopausal” osteoporosis.

2. Tasks in the dissertation studies in the context of above defined aims

2. 1. Experimental epileptiform syndrome

2. 1. 1. To perform two surgical interventions on each animal aiming to recruit a sufficient number of experimental animals with "zero" levels of corticosteroid and androgen hormones (bilateral suprarenalectomy and total orchidectomy)

2. 1. 2. To validate and practically applied in the study kainic acid-induced convulsive syndrome as a model of experimental epileptogenesis.

2. 1. 3. To apply a new original cumulative scale for quantitative analysis of the intensity of experimental epileptiform syndrome (quantitative assessment of the intensity of somatic and cognitive symptoms)

2. 1. 4. To study the action of steroid female and male gonadal hormones and glucocorticoids on kainic acid-induced convulsive syndrome. The hormones are:

(i) *Corticosterone*
(ii) *Estradiol*
(iii) *Progesterone*
(iv) *5α-Dihydroprogesterone*
(v*) 5α-Dihydrotestosterone*

2. 1. 5. To investigate the effect of a series of newly synthesized hydrazide-hydrazone compounds on the nociception using the “Hot Plate” test and the “Formaline” test. The compounds are: 4a, 4b, 4c, 8a, 8b

2. 2. Experimental estrogen-deficient osteoporosis

2. 2. 1. To perform surgical interventions (bilateral ovariectomy) to recruit a sufficient number of experimental animals with "zero" levels of female sex hormones

2. 2. 2. To be validated and applied in the study an experimental model of estrogen-deficient 'post-menopausal' osteoporosis in rats
2. 2. 3. To validate and apply in the study a methodology for osteodensitometric measurement of the bone mineral density (BMD) and the bone mineral content (BMC) in experimental estrogen-deficient "post-menopausal" osteoporosis in rats

2. 2. 4. To investigate the effect of *Aronia melanocarpa* fruit juice on BMD and BMC in animals with experimental estrogen-deficient "post-menopausal" osteoporosis

2. 2. 5. To investigate the effect of *Aronia melanocarpa* fruit juice on the nociception in animals with experimental estrogen-deficient "post-menopausal" osteoporosis with the "Randall-Selitto" test and the "Hot Plate" test

2. 2. 6. To investigate the effect of *Apium nodiflorum* extracts on BMD and BMC in animals with experimental estrogen-deficient "post-menopausal" osteoporosis

IV. MATERIALS AND METHODS

1. Experimental model of kainic acid-induced epileptogenesis. Methods of experimental pharmacological and cognitive studies

Laboratory animals: Male rats (Wistar, age 10 weeks, body weight 190-210 g) were placed under controlled conditions (20 ± 2° C, 12 hour cycle day / night starting at 8:00 h, at libitum access to water and standard food up to 24 hours before surgery), randomly distributed (at random) 3 animals in a cell in regularly sanitized cells with an area of 0.05 m^2. The body weight of all experimental animals was measured weekly. After completion of the experiments, the animals were exterminated with CO2. All procedures and tests are approved by the Commission on Bioethics of Research at the Medical University, Sofia.

Surgical interventions: To minimize the effect of changes in the level of endogenous sex hormones, the gonads and adrenal glands are removed. All surgical interventions are performed under aseptic conditions under general anesthesia and *intra-operationem* locally applied anesthetic. Extraction of the suprarenal glands is performed through a bilateral incision of the dorsal muscles in the lumbar region. During the second stage of the surgical intervention, en bloc testis, epididymis and vas deferens are excised

bilaterally through an incision of the scrotum. In some animals, these organs remain intact. These animals are included in the so-called sham operated group of controls.

Resource plan-program: In the current studies, 70 male animals from the vivarium of MU Sofia were used. After a 7-day habituation and under general anaesthesia with Thiopental (24 mg / kg, IP) and Ketamine 90 mg / kg, IP), and locally administered 1% Lidocaine (0.4 ml / rat), bilateral 10-15 mm incisions were performed. Through them the suprarenal glands of 60 animals are excised after ligation of the blood vessels. The glands of the other 10 animals were not removed and remained intact. Surgery is continued with a 15 mm medial scrotal incision and en bloc excision of the testis, epididymis and vas deferens of all animals with suprarenal glands removed.

All animals received local Sulfathiazole (20 mg/per animal) and Topocine spray, and followed by a wound closure by layers with single dose Gentamicin (8 mg/kg, *i.m*.). In the postoperative period, the animals were left for 3 days in single cells, after which every 4 animals were randomly grouped into larger cells. All animals had free access to water with added NaCl (4.5 g / l) and standard feed for laboratory animals throughout the postoperative period. After recovery, the operated animals are divided into groups of 10 animals, separately in the sham operated group - a total of 7 groups. Pharmacological treatment is carried out daily for 5 days from postoperative day 7 to postoperative day 11. The studied steroid hormones are Corticosterone, 17β-Estradiol, Progesterone, 5α-Dihydroprogesterone, 5α-Dihydrotestosterone, Sigma-Aldrich. The administered steroids were dissolved in sterile olive oil and administered subcutaneously in a volume of 0.1 ml / 100 g, i.e., at the same time (8: 30-9: 30 h) according to the scheme presented in Table 1. 1.

Table 1. 1. Scheme of the pharmacology treatment

	Group	**Compound**	**Dose**
Г1	*Sham operated* controls	*Olive Oil*	0.1 ml/100g, b.w

Г2	Operated controls	*Olive Oil*	0.1 ml/100g, b.w.
Г3	Operated	*Corticosterone*	30 mg/kg, b.w.
Г4	Operated	*17ϐ-Estradiol*	0.03 mg/kg, b.w.
Г5	Operated	*Progesterone*	75 mg/kg, b.w.
Г6	Operated	*5α-Dihydroprogesterone*	75 mg/kg, b.w.
Г7	Operated	*5α-Dihydrotestosterone*	0.75 mg/kg, b.w.

The resource plan-program optimally assured the time, material and methodological requirements for the implementation of the current research project.

The study: It is generally accepted that kainic acid, pentylenetetrazole, pilocarpine, flurothyl or electroshock are the most appropriate and commonly used epileptogenic stimuli to induce epileptiform seizures in experimental models of epilepsy. In the studies performed, kainic acid-induced seizures in rats were accepted as the most adequate experimental model of epileptic syndrome. Epileptiform seizures were induced by subcutaneous injection of 24 mg / kg, i.e. kainic acid, dissolved in 0.2 ml buffer (pH 7.4) after previous hormonal treatment for 5 consecutive days. Behavioral reactions were reported for 3 hours by two observers who were blinded regarding the hormonal treatment of the animals. Additionally, the time for the onset of seizure activity and the time of exitus was recorded. The intensity of the epileptiform syndrome is assessed according to a new original 6-step scale created by M. Vlaskovska and co-workers presented in Table 1. 2.

Table 1. 2. Cumulative scale for quantitative assessment of the intensity of epileptiform syndrome

LOCOMOTOR BEHAVIOR **Points**

- Locomotor agitation 1

- Myoclonus of the facial muscles and / or convulsions of muscles of the head, and / or contractions of the cervical muscles or the muscles of the forelimbs 2

- Contractions of the masticatory muscles and/or *wet dog* shaking, or clonus of the forelimbs 2.5

- Mild generalized clonic seizure 3

- Severe generalized clonic seizure with straightening of the back limbs and falling to the floor of the cage 4

- Submaximal tonic seizure (tonic flexion of forelimbs) 5

- tonic seizure (tonic flexion of forlimbs and tonic extension of backlimbs) 6

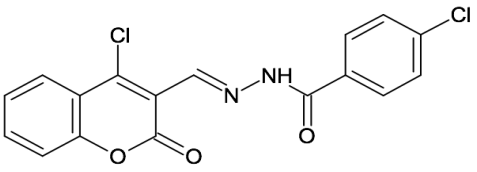
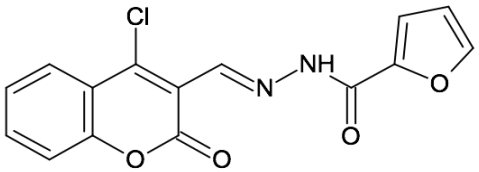
Statistical analysis: The results were presented as Mean ± SEM. The data analysis was performed using ANOVA two way measures with the next Bonferroni multiple comparison test. The differences were considered significant at $P \leq 0.05$.

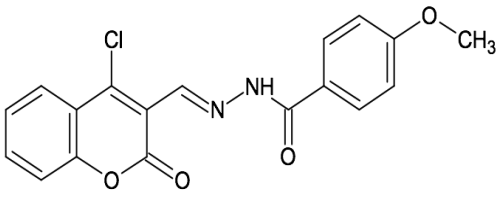
2. Materials and pharmacological methods for studying the analgesic effect of 2H-chromene-substituted hydrazide-hydrazones.

The methods for synthesis of original coumarin and 2H-chromium-substituted hydrazide-hydrazones have been applied in studies conducted at the Faculty of Pharmacy of the Medical University, Sofia, Institute of Organic Chemistry with Center for Phytochemistry at BAS and Institute of Biophysics and Biomedicine at Biomedicine. The entire amount of newly synthesized substances that were used in the studies was obtained from the Faculty of Medicine of the Medical

University of Sofia. Based on pharmacophoric carting and three-dimensional structural analysis aryl-hydrasone derivatives with overt anticonvulsive activity were synthetised (Ulloora, S., et al., 2013). Using a molecular hybridization methodology, teams of Bulgarian researchers synthesized a series of new hydrazide-hydrazone-coumarin derivatives which might exhibit anticonvulsant and analgesic activity (Angelova, V. T., et al., 2016a, 2016b, 2017). The main physicochemical characteristics of the test compounds are presented in Table 1. 1., Section 1. 2. 1. Newly synthesized coumarin and 2H-chromium-substituted hydrazide-hydrazones.

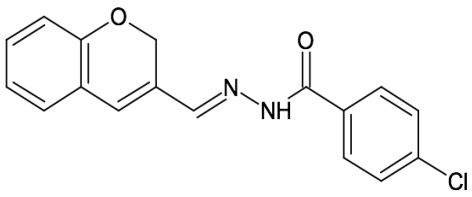
Table 1. 2. 1. Main chemical characteristics of newly synthesized coumarin and 2H-chromium-substituted hydrazide-hydrazones

Compound		**Physicochemical parameters**		
Code	Chemical structure	MW	Log O/B	PSA
4a		361.17	2.18	67.76
	(4-chloro-N- [(E)-(4-chloro-2-oxochromen-3-yl)methylidene]benzohydrazide)			
4b		316.70	0.61	80.90
	(N'-[(E)-(4-chloro-2-oxochromen-3-yl) methylidene]furan-2-carbohydrazide)			

4c 

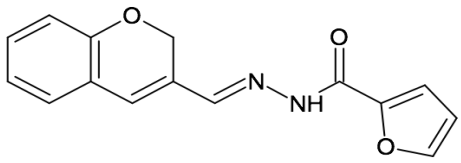
356.76 1.41 76.99

(N'-[(E)-(4-chloro-2-oxochromen-3-yl) methylidene]-4-methoxybenzohydrazide)

8a 

312.75 3.32 50.69

(4-chloro-N'-[(E)-2H-chromen-3-ylmethylidene]denzohydrazide)

8b 

368.27 1.75 63.83

(N'-[(E)-2H-chromen-3-ylmethylidene]furan-2-carbohydrazide)

MT - molecular weight, PSA - polar surface area, Log O / B - octanol / water partition coefficient

After *in silico* physicochemical and NMR analyzes of the synthesized compounds, the authors concluded that these compounds possess essential pharmacophore elements that are believed to determine good anticonvulsant activity (Tripathi, L., Singh, R., Stables, JP, 2011). A consequence of these studies is the initial pharmacological and toxicological screening of these compounds for the presence of anticonvulsant and

analgesic activity in *in vivo* models of convulsive syndrome. The main pharmacological and toxicological characteristics of five compounds that were considered promising are presented in Table 1. 2. 2. Data are compiled fro results, published by Angelova, V. T., et al. (2017).

Table 1. 2. 2. Main pharmacological and toxicological characteristics of newly synthesized coumarin and 2H-chromen-substituted hydrazide hydrazones

Compound		**Pharmacology parameters**		
Code	Test	ED50 (mg/kg)	TD50 (mg/kg)	PI
4a	MES	99.71	> 300	> 3.01
4b	MES	68.66	> 300	> 4.37
4c	MES	81.29	> 300	> 3.69
8a	MES	87.63	> 300	> 3.42
	PTZ	218.50	> 300	> 1.37
8b	MES	12.51	> 300	> 23.98
	PTZ	127.10	> 300	> 2.36

MES - maximum electroshock (50 mA, 50 Hz, 0.2 sec), PI - protective index (TD50 / ED50), PTZ - Pentylenetetrazole (85 mg / kg, sc)

The results of the screening showed that the compounds of codes 4b, 4c and 8b have real pharmacotherapeutic potential as anticonvulsants. Further studies of their analgesic activity are needed, as well as studies to elucidate the mechanism of their action (Angelova, V. T., et al., 2017).

Laboratory animals: Male albino mouses (ICR, body weight 20-25 g) were placed under controlled conditions (20 ± 2o C, 12 hour day / night cycle starting at 8:00 am and access at libitum to water and standard food), pre-distributed at random to 8 animals / cage in regularly sanitized cells with an area of 0.05 m2. After completion of the experiments, the animals were not exterminated. All procedures and tests have been approved by the Bioethics Commission of the Scientific Research of the Medical University of Sofia and the Bioethics Committee of the INB at BAS.

Resource plan-program: The current studies were performed on 96 albino male mice obtained from the vivarium of MU Sofia. Pharmacological treatment is carried out after a 7-day habituation period. Test substances were dissolved in DMSO and administered intraperitoneally at a dose of ED50 in a final injection volume of 0.1 ml / animal in the range of 9: 00-10: 00 h according to the schedule presented in Table 2. 1. The test is performed in an early (up to 5 min) and a late (up to 30 min) period after administration of the respective substance to the same animal.

Table 2. 1. Groups and number of animals in a pharmacological study group
for assessment of the analgesic action of chromium-substituted hydrazide-hydrazones in a dose ED50 via Hot Plate test and Formalin test

Test	**Test (min)**	**Studied compounds**						
type	I иII		4a	4b	4c	8a	8b	DMSO
HP	I - 5 min	(N)	8	8	8	8	8	8
	II - 30 min	(N)	8	8	8	8	8	8
F	I - 5 min	(N)	8	8	8	8	8	8
	II - 30 min	(N)	8	8	8	8	8	8
ED50 (mg/kg) with MES			100	70	80	90	12.5	0.1 ml

I - first measurement, II - second measurement, HP - Hot Plate test, F - Formalin test, MES - maximum electroshock, N - number of animals in a group

Study: Algogenic stimuli with different modalities were applied: nociceptive test with thermal stimulation (Hot Plate test with a temperature of 47 ± 1º C. D 'Amour, FF, Smith, DL, 1941, HP test) and intraplantar injection of 5% formalin solution (Barrot , M., 2012, Test F). The nociceptive threshold determined by the HP test is verified by the latency time (latency, sec) from the moment of stepping on the hot surface to the moment of feeling pain, manifested as a protective reflex of lifting / licking one of the limbs. Intraplantar injection into the hind limb of the animal of 5% formalin solution in a volume of 0.1 ml provokes a biphasic allogeneic reaction. The initial reaction is licking of the injected limb and is reported in the early period after injection. After a latency period of 25-30 minutes, a later lifting / biting reaction of the injected limb is reported. The degree of hyperalgesia determined by test F was verified by the latency time (latency, sec) from the moment of subplantar injection to the moment of onset of early and resp. late allogeneic reaction.

Statistical analysis: The results are presented as mean ± standard deviation and are analyzed by one-way analysis of variation (ANOVA) and Holm – Sidak post hoc test with perceived significance level P <0.05.

3. Experimental model of estrogen-deficient osteoporosis. Osteodensitometric and algometric studies

Laboratory animals: Female rats (Wistar, aged 2.5 months, body weight 180-185 g) were placed under controlled conditions (20 ± 2o C, 12 hour cycle day / night starting at 8:00 h, at libitum access to water and standard food up to 24 h before surgery), divided at random (4 animals / cell) in regularly sanitized cells with an area of 0.05 m2. The body weight of all experimental animals was measured twice a week. After completion of the

experiments, the animals were anesthetized with Pentobarbital (20 mg / kg, IP) and exterminated. The treatment of the animals and the planned experiments are in accordance with the national laws (Ordinance №20 of 01.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for the sites for their use, breeding and / or delivery) and international requirements. (EU Directive, 2010/63 / EU for animal experiments) and are approved by the Commission on Bioethics of Scientific Research of MU Sofia.

Surgical interventions: Surgical interventions are performed a week after the habituation of the animals. All surgical procedures are performed under aseptic conditions and general anesthesia, and intraoperatively applied local anesthetic. After laparotomy, a bilateral ovariectomy is performed, leaving a small number of animals in a control group with intact ovaries, the so-called sham operated group. At the end of the operation, all animals received a single dose of antibiotic. A control group of unoperated animals was kept under the same laboratory conditions. This allows the analysis of osteoporotic changes in ovariectomized, non-ovariectomized and healthy animals to take into account the processes of physiological aging of all animals by about 12 months until the end of the study.

Resource plan-program: The study consists of two experimental settings

First experimental setting: 64 female rats from the vivarium of MU Varna were used. Under general anesthesia with Ketamine 30 mg / kg and Xylazine 30 mg / kg. The animals underwent a medial laparotomy (15 mm) in the pelvic area. Bilateral ovariectomy was performed in 48 animals. In the other 16 animals, which served as a control group, the so-called sham-operated, the ovaries remain intact. Postoperatively, all animals received one dose Cefazolin 200 mg / kg, IP. After a 14-day recovery period, ovariectomized animals were randomly assigned to 3 groups of 16 animals. Pharmacological treatment begins after this recovery period and lasts for 12 weeks according to the scheme presented in Table 3. 1. The test pharmacological agents are administered as a solution

in a volume of 10 ml / kg, ie, through a gastric tube every day between 8:30 and 10:30 am.

Table 3. 1. Scheme of the conducted pharmacological treatment at the first experimental setting

Group	**Ovariectomy**	**Treated**
Control	*sham operated*	distilled water
Osteoporosis	yes	distilled water
Osteoporosis	yes	Aronia 10 ml/kg
Osteoporosis	yes	Aronia 5 ml/kg

Second experimental setting: 30 female rats obtained from the vivarium of MU Sofia were used. After 7 days of habituation and under general anesthesia with Pentobarbital (12 mg / kg, IP) and Ketamine (50 mg / kg, IP), and locally administered 1% Lidocaine (0.4 ml / rat), the animals underwent medial pelvic laparotomy (15 mm) through which a bilateral ovariectomy of 20 animals was performed. In the other 10 animals, which served as a control group, the so-called sham-operated, the ovaries remain intact. Postoperatively, all animals received Gentamicin at a dose of 8 mg / kg, i. m. After a 5-day recovery period, ovariectomized animals were randomly assigned to 2 groups of 10 animals. The studied pharmacological agents were administered as a solution in a volume of 0.2 ml / 100g, i.e., through a gastric tube daily between 8:30 - 10:30 am. The pharmacological treatment began 6 months after the operative interventions and lasts for 12 weeks according to the presented scheme of Table 3. 2.

Table 3. 2. Scheme of the conducted pharmacological treatment in the second experimental setting

Group	Ovariectomy	Treated
Control	*sham operated*	0.9% NaCl
Osteoporosis	yes	0.9% NaCl
Osteoporosis	yes	*Apium extract* 2.5 mg/kg

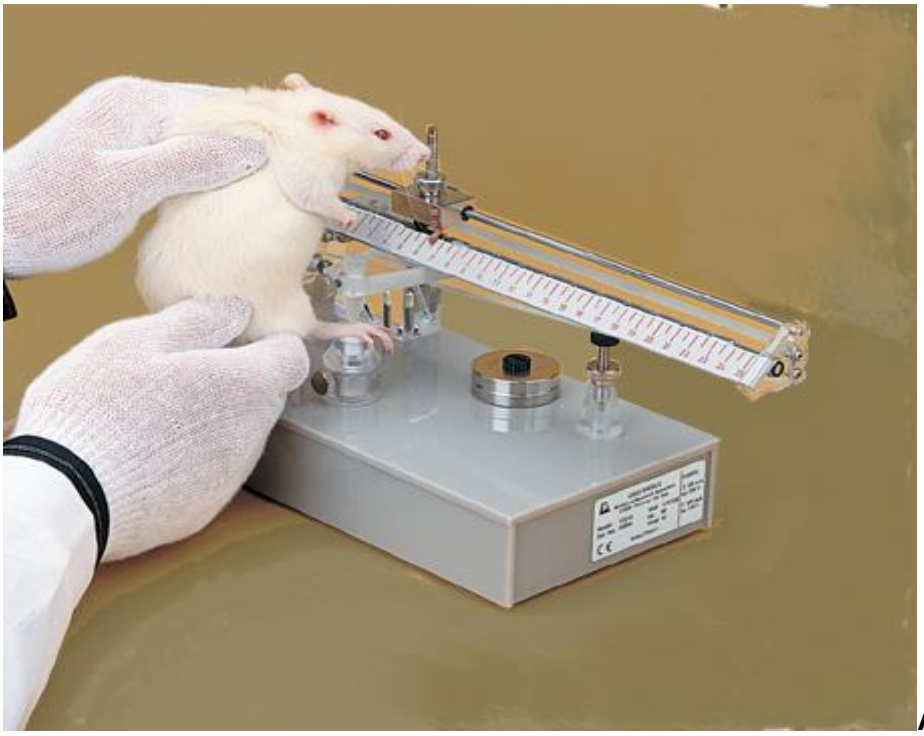
Apium extract 2.5 mg/kg (equivalent to Quercetin 2.4 mg/kg) in 0.9% NaCl

The resource plan-program provided opportunities for studying the changes occurring in osteoporosis in the bone architecture, density and mineralization and the effect of pharmacologically active substances with potential antiosteoporous action.

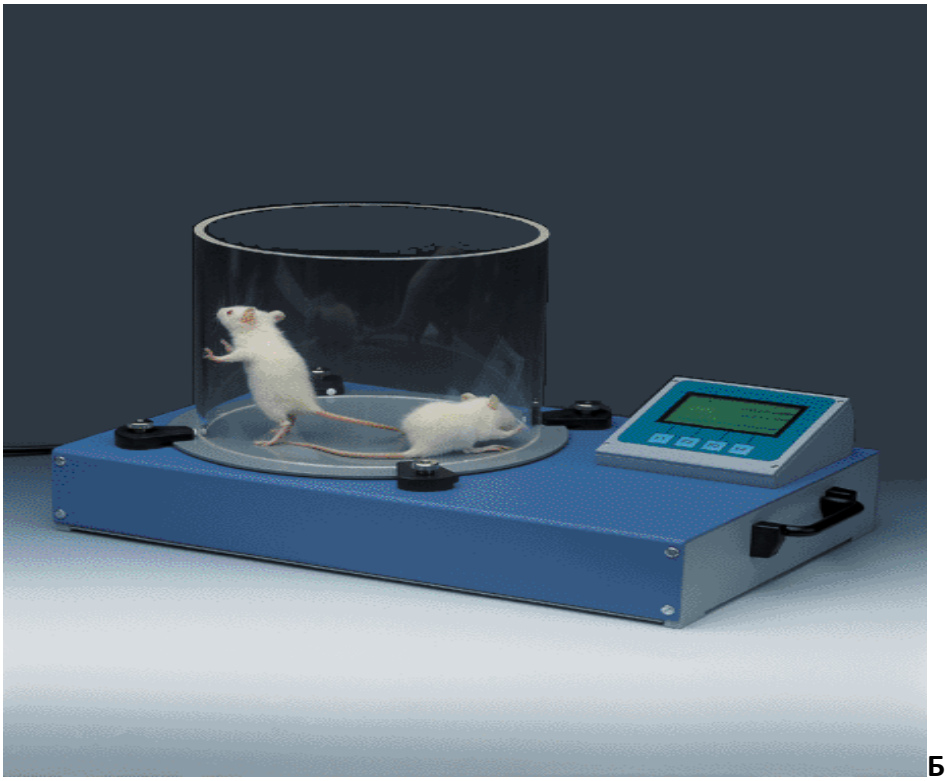
Study: (i) Densitometry: Bone mineral density (BMD), bone mineral content (BMC) of the operated and non-operated animals are examined in the following periods: 2 weeks before, 6 months after surgery and at the end of the experiment. The animals were under general anesthesia with Nembutal (12 mg / kg, IP) and Calypsol (50 mg / kg, IP). To detect mild osteoporotic changes, BMD and BMC are examined *ex situ* in isolated massive long bones. For these studies, the femur and tibia were separated from the right limbs of 4 randomly picked animals from each post mortem group. Densitometric examinations were performed with a DEXA Hologic Discovery, version 13.2: 3, with software for smallanimals. Qualitative and quantitative calibration of the device was performed daily. The studies were performed after adequate phantom calibration for small animals. Anesthetized animals are positioned in the device and the procedure was performed following manufacturer's instructions at technical parameters 140/100 kVp, 2.5 mA avg, 146 sec, 50 Hz, scan length 3.5, scan width 17.9, line splicing 0.1512, point resolution 0.0640, 3.96 x 0.04 coll. Total fat and muscle mass of the animals were also measured. All measurements were performed by single researcher. All 50 densitometric examinations were performed under the guidance of Dr. N. Temelkova at the Center for Osteometry of Alexandrovska University Hospital, Sofia. *(ii) Nociception:* The nociceptive thresholds of each animal were measured according to a schedule: 3 times in the

preoperative period, twice a week in the post-operative period and 3 times a week in the pharmacological treatment period. The experiments were performed starting at 9:00, and on the days of pharmacological treatment immediately after treatment. Allogeneic stimuli with different modalities were applied: nociceptive test (Paw Pressure Test, PP test) by mechanical pressure with an analgesimeter (Randall, LO, Selitto, JJ, 1957) and nociceptive test (Hot Plate) by thermal stimulation with warmed (51 ± 1º) C) plate (D 'Amour, FF, Smith, DL, 1941). The analgesimeter is a device that measures the nociceptive threshold when applying a linearly increasing pressure on the dorsal surface of the hind limb of an animal with a cone with a mass of 74g and a contact area of 2.2 mm2, sliding at a constant speed of 40 mm / s on a vernier. Pain sensitivity, respectively the nociceptive threshold is measured in relative units (RU) at the moment when the force of the applied mechanical stimulus reaches the nociceptive threshold and the animal withdraws its limb. The nociceptive threshold determined by the hot plate test was verified by the latent time (latency, sec) from the time of application of the nociceptive stimulus to the moment of pain sensation, manifested as protective reflexes of lifting / licking the hind limb. Three measurements were made per animal every two hours. Figure 3. 1. illustrates the applied in real conditions test PP (A) and test HP (B).

Statistical analysis : The results are presented as Mean±SEM and were analysed using Student's *t-test* with level of confidence $P \leq 0.05$.



A.



Б.

Figure 3. 1. Test PP (A) and test HP (Б) in real setting

4. *Aronia melanocarpa fruit juice*

The fruit juice of *Aronia melanocarpa* (AMFJ) was prepared by grinding, pressing, squeezing and filtering fresh fruits of *Aronia melanocarpa Elliot,* grown near Troyan. The juice contains 75-80% of the raw material - not less than 13% dry mass (determined by refractometer). AMFJ had a pH of 3.4 and a titratable acidity (such as malic acid) of 0.6%. After reconstitution, AMFJ was preserved by pasteurization at 80 ° C for 10 minutes with added potassium sorbate (1.0 g / l). Until it was used for experiments, it was stored in a refrigerator. The juice thus prepared was tested for polyphenol content and antioxidant activity (Valcheva-Kuzmanova, C. et al., 2014). The tested parameters are presented in Table 4. 1.

1	Total phenols
2	Total proanthocyanidins
3	Glycosides of cyanidin
4	Phenolic acids
5	Capacity to prevent the formation of hydroxyl radicals
6	Absorption capacity of the oxygen radical

Data on the content of biologically active substances in the juice used by Aronia, as well as data on its antioxidant activity are presented in Table 4. 2. The table compiles data from studies by other authors (Valcheva-Kuzmanova, S. et al., 2014).

Table 4. 2. Content of biologically active substances in AMFJ (mg / l) and antioxidant activity

Substance	
Total phenols (EGA/l)	5461
Total proanthocyanidins	3122.5
Cyanidin - galactoside	143.7
Cyanidin - arabinoside	61.7
Cyanidin - glucoside	4.4
Cyanidin - xyloside	11.6

Chlorogenic acid	585
non-Chlorogenic acid	830
HRPC (EGA/l)	30560 μmol TE/l
ORAC 2 (TE/l)	52045 μmol TE/l

EGA/l - *equivalent of gallic acid (μmol/l), HRPC - hydroxyl radicals prevention capacity to prevent the formation of, ORAC - oxygen radical absorption, TE/l - Trolox equivalent/litre*

5. *Apium nodiflorum extract*

The methods have been applied in studies conducted at IOCCP-BAS. The total amount of Apium extract used in the studies was obtained from IOCCP-BAS.

(I) Extraction: Fresh roots of Bulgarian celery (*Apium nodiflorum*) were extracted in methanol for 24 h at 18 ° C. The extracts were then filtered, collected and the entire amount evaporated in vacuo to dryness.

(ii) Phytochemical analysis. The standardization of the *Apium* extract was performed by quantifying the total content of phenols and flavonoids in the dry residue dissolved in saline (10 mg / ml). Flavonoids were quantified according to a standard curve obtained from 0.009, 0.019, 0.037, 0.075 and 0.19 mg / ml Quercetin at an absorption of 425 nm. The phenols were quantified according to a standard curve obtained from 0.009, 0.019, 0.037, 0.075 and 0.19 mg / ml Gallic acid at an absorption of 760 nm. The analysis was performed in triplicate for each concentration.

V. MAIN RESULTS

1. Experimental epileptoformic syndrome

1. 1. Antiepileptic / anticonvulsant studies of steroid suprarenal and gonadal hormones

Experimental data showed that after removal of the suprarenal glands and testicles (group G2) there was a significant shortening of the latency period from the application of an epileptogenic stimulus to the appearance of mild clonic seizures. The latency time was shortened from 130.2 ± 11.5 min in animals with preserved glands (group D1) to 61.2 ± 5.1 min in those with removed glands (group D2), ie the latency was shortened by 53% (Table 1. 1.).

After treatment with 17β-estradiol (group G4) there was a severe exacerbation of the epileptiform syndrome. The results showed that after estrogen treatment, weak clonic seizures appeared significantly earlier than in its absence. The latency time was reduced from 61.2 ± 5.1 min (group G2) to 31.4 ± 4.6 min (group G4), ie the latency was shortened by 49%. An anticipated consequence of these studies was to continue the project with a study of the effect on epileptogenesis of a hormone with opposite physiological action, ie androgenic hormone. Qualitatively analogous, but quantitatively more pronounced was the effect of the androgenic analogue 5α-Dihydrotestosterone. The results revealed that treatment with 5α-Dihydrotestosterone strongly aggravates all clonic components of the induced epileptiform syndrome. The mean latency time for the onset of the first cramps or mild clonic seizures was reduced from 34.0 ± 3.3 min to 19.0 ± 2.2 min, resp. from 61.2 ± 5.1 min to 33.6 ± 5.3 min (group G7), ie the latency for their occurrence was shortened

by 44% and 45%, respectively. The results of the studies showed that substitution of progestogens (group G5 and group G6) or corticosteroids (group G3) did not cause significant changes in the latency time of clonic components in the kainic acid-induced experimental epileptiform syndrome. The results of these studies are summarized in Table 1. 1.

Table 1. 1. Effect of steroid suprarenal and gonadal hormones on clonic components of kainic acid-induced experimental epileptiform syndrome

	Latency time clonic seizures (min)		
Group (1-7)	**Compound**	**First cramps**	**mild seizures**
Sham (N9)	*Olive oil*	31.2±3.9 (N9)	130.2±11.5 [a] (N5)
after surgery (N8)	*Olive oil*	34.0±3.3 (N8)	61.2±5.1 (N6)
after surgery (N8)	*Corticosterone*	30.5±1.8 (N5)	63.2±2.6 (N6)
after surgery (N8)	*17β-Estradiol*	29.0±4.3 [c] (N8)	31.4±4.5 [a] (N8)
after surgery (N9)	*Progesterone*	44.2±10.8 (N9)	66.2±14.2 (N9)
after surgery (N9)	*5α-DHP*	35.2±5.2 (N9)	44.2±5.2 [c] (N4)
after surgery (N9)	*5α-DHT*	19.0±2.2 [b] (N9)	33.6±5.3 [b] (N9)

N - number of animals in the group and convulsive syndrome [a] $P \leq 0.001$, [b] $P \leq 0.01$, [c] $P \leq 0.05$

Experimental data showed that after the removal of the suprarenal glands and testicles (group D2) there was a significant reduction in latency from the application of an epileptogenic stimulus to the appearance of submaximal tonic seizures. The latency time

was shortened from 95.0 min in animals with preserved glands (group G1) to 62.5 ± 18.5 min in animals with removed glands (group G2), ie latency was shortened by 34% (Table 1. 2.). The treatment with 5α-Dihydrotestosterone aggravates all the tonic components of the induced epileptiform syndrome. The mean latency for the onset of mild tonic seizures was reduced from 62.5 ± 18.5 min to 35.0 ± 4.8 min (group G7) - the latency for their onset was shortened by 44%.

In contrast to clonic seizures, treatment with the progestogen analogue 5α-Dihydroprogesterone caused a quantitatively similar but qualitatively opposite effect on maximal tonic seizures. Their latency time increased from 60.0 min (group G2) to 92.0 min (group G6), ie the latency was increased by 35%. Treatment with 17β-estradiol (group G4) did not cause significant changes in the latency period for submaximal and maximal tonic seizures after administration of a kainic acid epileptogenic stimulus. Corticosterone treatment (group G3) completely suppresses the appearance of tonic components in kainic acid-induced experimental epileptiform syndrome. The results of this group of studies are summarized in Table 1. 2.

Table 1. 2. Effect of steroid suprarenal and gonadal hormones on tonic components of kainic acid-induced experimental epileptiform syndrome

Latency time tonic seizures (min)			
Group (1-7)	**Compound**	**Submaximal**	**Maximal**
Sham (N9)	*Olive oil*	95.0 [b] (N1)	- (N0)
after surgery (N8)	*Olive oil*	62.5±18.5 (N2)	60.0 (N1)
after surgery (N8)	*Corticosterone*	- (N0)	- (N0)
after surgery (N8)	*17ϐ-Estradiol*	48.4±8.6 (N7)	82.3±37.9 (N3)
after surgery (N9)	*Progesterone*	80.0±28.2 (N4)	- (N0)

after surgery (N9)	*5α-DHP*	69.1±14.1 (N6)	92.0 [c] (N1)
after surgery (N9)	*5α-DHT*	35.0±4.8 [b] (N4)	- (N0)

N - number of animals in the group and convulsive syndrome [a] $P \leq 0.001$, [b] $P \leq 0.01$, [c] $P \leq 0.05$ vs G2

The appliance of the newly created scale for assessment of convulsive components allows to quantify the motor and cognitive components of the experimental kainic acid-induced epileptiform syndrome. Experimental data clearly showed that after removal of the adrenal glands and testicles (group D2) there was a significant exacerbation of epileptiform syndrome. The intensity of the epileptic symptoms of rats in quasi-physiological status, in which the glands remained intact, the so-called sham-operated animals, was 2.66 ± 0.31 (Table 1. 3.). The intensity of the epileptic convulsive syndrome after removal of the suprarenal glands and testicles becomes 4.0 ± 0.42, ie the clinical manifestations worsened with 50%. Treatment of operated animals with 17β-estradiol caused significant deterioration of the motor component of the epileptiform syndrome. Quantified, this is manifested by more than 31% increase in the values of the intensity of the convulsive syndrome, which increased from 4.0 ± 0.42 in severe hormonal deficiency to 5.25 ± 0.25 after treatment with 17β-Estradiol (group G4).

Unlike with the estrogens, corticosteroid substitution has the exact opposite effect. The administration of Corticosterone reduced the intensity of the convulsive syndrome to 2.75 ± 0.09 (group G3), ie corticosteroids "softened" the epileptiform activity in rats with "total" hormone deficiency to that of group of animals with normal hormone levels(group G1). The administration of gestagens (group G5 and group G6) or androgens (group G7) did not change the intensity of the convulsive syndrome. The results of this group of studies are summarized in Table 1. 3. The effect of the studied hormones on the intensity of convulsive symptoms corresponded to the effect of these hormones on lethality in kainic acid-induced experimental epileptiform syndrome. Removal of the suprarenal glands and testicles caused a threefold increase in mortality from 11.1% to 37.5%. A sharp increase

in mortality in evoked epileptiform syndrome to 87.5% occured after administration of 17β-estradiol (group G4). This contrasted with the complete blockade of mortality in animals after treatment with Corticosterone (group G3)

Предизвиканият тежък хормонален дефицит на оперираните и нетретирани плъхове скъсява достоверно с 24% времето за настъпване на екзитус при развитие на епилептичен статус от 97.0 мин на 74.0±3.5 мин (група Г2). Третирането с естрогени (група Г4) или гестагени (група Г5 и група Г6) не променя времето до настъпване на екзитус. Този ефект контрастира със значителното скъсяване на времето до настъпване на екзитус с 24% след третиране с андрогени. Резултатите показват, че прилагането на *5α-Dihydrotestosterone* скъсява времето за настъпване на екзитус след инжектиране на епилептогенния стимул от 74.0±3.5 мин на 56.5±13.1 мин (група Г7). Резултатите от тези експерименти са обобщени в Таблица 1. 3.

The induced severe hormonal deficiency of the surgically treated and untreated rats significantly shortened by 24% the time of death in overt status epilepticus from 97.0 min to 74.0 ± 3.5 min (group G2). Treatment with estrogens (group G4) or progestogens (group G5 and group G6) did not change the time to death. This effect was opposite to the significant shortening of the time to death by 24% after androgen treatment. The The administration of 5α-Dihydrotestosterone shortened the time to death after injection of the epileptogenic stimulus from 74.0 ± 3.5 min to 56.5 ± 13.1 min (group G7). The results of these experiments are summarized in Table 1. 3.

Table 1. 3. Effect of suprarenal and sex steroid hormones on intensity, lethality and time to death of rats with kainic acid-induced experimental epileptiform syndrome

Substance	**Intensity (RU)**	**Lethality (%)**	**Death (min)**
Г1 Olive oil	2.66±0.31 [b]	11.1 [a] (N1)	97.0 [b] (N1)
Г2 Olive oil	4.0±0.42	37.5 (N3)	74.0±3.5 (N3)

Г3 Corticosterone	2.75±0.09 [a]	- (N0)	- (N0)
Г4 17β-Estradiol	5.25±0.25 [b]	87.5 [a] (N7)	87.3±24.1 (N7)
Г5 Progesterone	3.71±0.43	44.4 [c] (N4)	87.0±32.5 (N4)
Г6 5α-DHP	4.55±0.33	44.4 [c] (N4)	86.5±13.0 (N4)
Г7 5α-DHT	4.11±0.30	44.4 [c] (N4)	56.5±13.1 [b] (N4)

N - number of animals in a group and respectively with the corresponding clinical / pathological-anatomical determinant, "-" not observed; a $P \leq 0.001$, b $P \leq 0.01$,
c $P \leq 0.05$ vs G2

Summary of the experimental data: Removal of the suprarenal glands and testes severely worsens the clinical and pathological parameters of the kainic acid-induced experimental epileptiform syndrome. Basically, treatment with estrogens or androgens has a strong adverse effect on these parameters. In contrast, the use of glucocorticoids could completely antagonize the adverse effects of these hormones on the clinical picture and the pathological parameters of kainic acid-induced convulsive syndrome.

1. 2. Studies of analgesic action of newly synthesized 2H-chromene-substituted hydrazide-hydrazones

In this series, the experiments mainly aimed to investigate the possible analgesic effect of five of the newly synthesized substances previously predicted to show analgesic activity. Test substances were administered intraperitoneally as a 1% solution in DMSO in a final injection volume of 1 ml at a dose of ED50 of each test substance. ED50 values are used from a previous publication (Table 1. 2. 1). These substances are designated as 4a, 4b, 4c, 8a and 8b as coded earlier by the authors (Angelova, VT, et al. , 2017).

Table 1. 2. 1. Doses of intraperitoneal injection of studied substances(mg/kg)

Control	**4a**	**4b**	**4c**	**8a**	**8b**
1% DMSO	100	70	80	90	12.5

The Hot Plate test results revealed that substance 8b had a pronounced analgesic effect, which occured 25-30 minutes after injection. The latency time increased from 22 ± 2 sec in the control group to 53 ± 6 sec in the treated group, ie the increase was more than 2 times. The effect of substances 8a and 4c was significantly weaker. By the power of their analgesic action, the substances can be graded in the following order: 8b> 8a> 4c. The results obtained in these studies are summarized in Table 1. 2. 2.

Table 1. 2. 2. Effect of newly synthesized 2H-chromen- substituted hydrazid-hydrasones on nociception test “Hot Plate”

Time (test)	**Latency time (sec)**					
(min)	**Control**	**4a**	**4b**	**4c**	**8a**	**8b**
0-5	22±3	24±2	21±2	25±3	27±2	24±1
25-30	22±2	28±9	30±4	38±6 [c]	35± 5 [c]	53±6 [b]

[b] $P \leq 0.01$, [c] $P \leq 0.05$ vs control

The hyperalgesia may be a component of a complex clinical picture of epileptiform syndrome. A formalin test was used to examine hyperalgesic changes in nociception. The data from the series of experiments showed that substances 4a and 4c had a well-defined antinociceptive effect in both the earliest and late post-injection periods. Substances 8a

and 8b showed a weaker antinociceptive effect in the late post-injection periods. The results obtained in these studies are presented in Table 1. 2. 3.

Table 1. 2. 3. Effect of newly synthesized 2H-chromen- substituted hydrazid-hydrasones on nociception test “Formalin”

Time (тест)	**Reaction time (sec)**					
(min)	**Control**	**4a**	**4b**	**4c**	**8a**	**8b**
0-5	68±18	18±7 [a]	56±16	24±9 [b]	40±7	50±4
25-30	120±19	31±10 [a]	82±10	56±14 [b]	58±18 [c]	60±11 [c]

[a] *P ≤ 0.001,* [b] *P ≤ 0.01,* [c] *P ≤ 0.05 vs control*

Summary of the experimental results: Our results clearly revealed that in a series of original newly synthesized chromium-substituted hydrazide-hydrazones there were compounds with a pronounced antinociceptive effect. An HP test revealed that three of the newly synthesized chromen-substituted hydrazide hydrazones, 4c, 8a and 8b, had antinociceptive activity. As compare the power of the antinociceptive action verified by the HP assay, these compounds were classified as 8b> 8a> 4c. The Formalin test showed that compounds 4a and 4c exhibited moderate analgesic activity verified as 4a> 4c. The above data together with the previously published anticonvulsant effects (Angelova, V. T., et al., 2017) might be used as a proof that newly synthesized chromium-substituted hydrazide-hydrazones have therapeutic potential and may be of interest for the development of new antiepileptic agents.

2. Experimental model of estrogen-deficient osteoporosis

2. 1. Studies on the antiosteoporotic and analgesic effect of *Aronia* juice 10 ml / kg and *Aronia* juice 5 ml / kg

Bone mineral density *changes*

The Densitometric studies showed that estrogen deficiency, occurring after total ovariectomy, causes osteoporosis and a decrease in bone mineral density (BMD) in the studied long bones of the hind limbs - isolated at random and exterminated by 3-4 animals from each group . The mean values show that the BMD is reduced from 0.297 ± 0.003 g / cm2 to 0.263 ± 0.002 g / cm2 in the femur and from 0.230 ± 0.001 g / cm2 to 0.217 ± 0.002 g / cm2 in the tibia, ie the BMD is reduced by 11%, resp. with 6%. The mean values of BMD of femur from animals with osteoporosis were 0.263 ± 0.002 g / cm2, and of those treated with 10 ml / kg Aronia juice - 0.299 ± 0.01 g / cm2, ie the decrease of BMD by 11%, which was found in osteoporosis, was completely overdone. The effect of Aronia 5 ml / kg juice was similar, but less pronounced than Aronia 10 ml / kg juice. The mean BMD values of the femur were 0.278 ± 0.009 g / cm2, ie the BMD remained reduced by 6% compared to the control group. The results of these measurements are presented in Table 2. 1. 1.

Table 2. 1. 1. Effect of 10 ml / kg and 5 ml / kg of Aronia juice on bone, BMD respectively on the hind limb of rats in the experimental model of osteoporosis

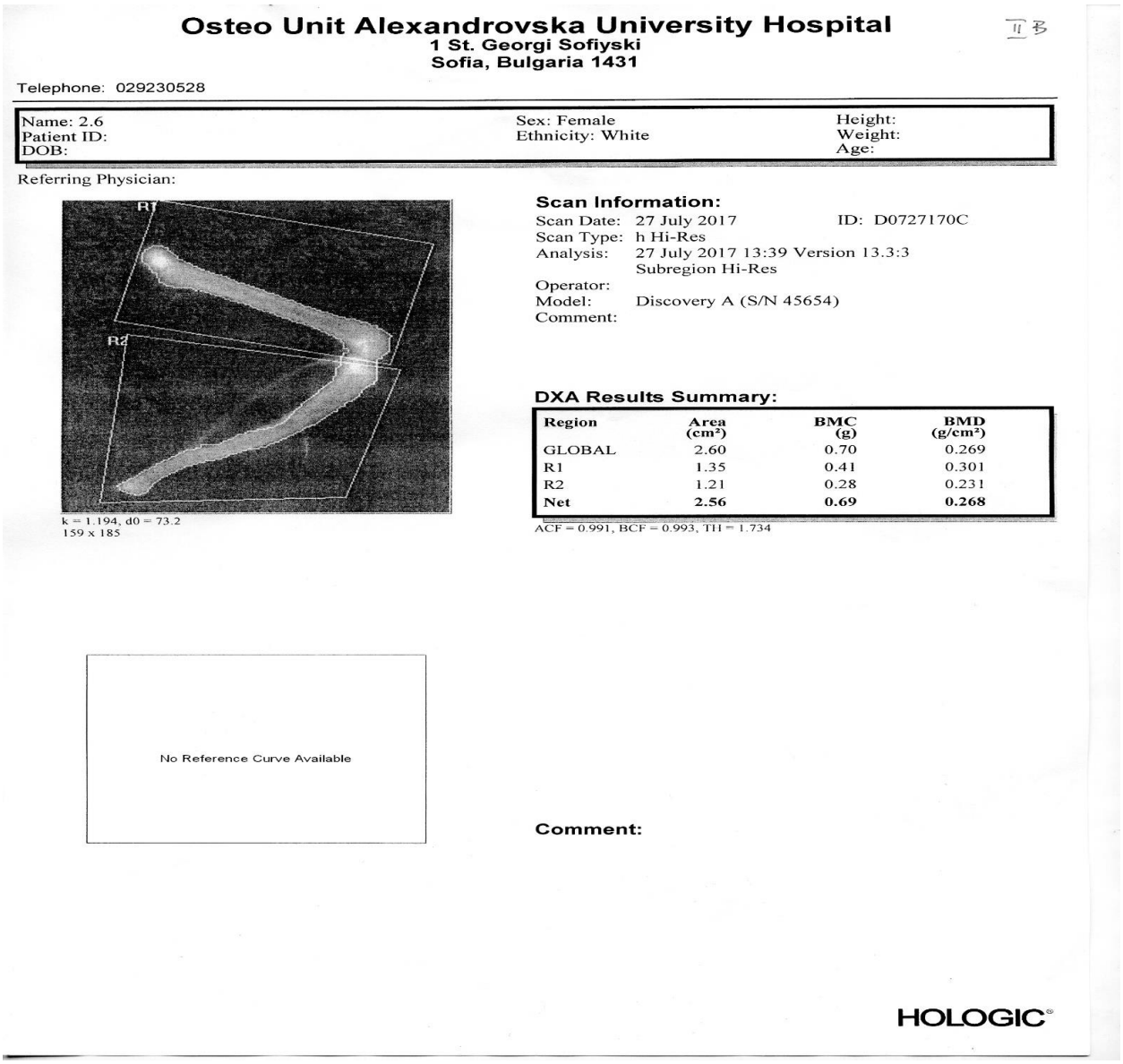
	Bone mineral density (g/cm^2)	
Group	**Femur**	**Tibia**
Control	0.297±0.003 (n4)	0.230±0.001 (n4)
Osteoporosis	0.263±0.002 [a] (n4) (*-11%*)	0.217±0.002 [a] (n4) (*-6%*)
O. Aronia 10 ml/kg	0.299±0.01 (n4) (*+1%*)	ns (n4)

o.	Aronia 5 ml/kg	0.278±0.009 [a] (n4) (*-6%*)	ns (n4)

n - number of animals in each group with post mortem DEXA for BMD; (+/- %) - change vs control, ns - not significant, [a] $P \leq 0.05$ vs control

The densitometric studies indicated that long-term treatment with Aronia juice may prevent the development of osteoporotic changes in the bone structure of the femur. Administration of Aronia juice 10 ml / kg kept the BMD of the femur of ovariectomized animals at levels typical of healthy animals. Figure 2. 1. 1. and Figure 2. 1. 2. shows original densitographs of the examined bones, which are representative of the bone structure and BMD of the animals from the Control group and the Osteoporosis group. Figure 2. 1. 3. and Figure 2. 1. 4. shows original densitographs of the examined bones, which are representative of the bone structure and BMD of the animals from the group Osteoporosis + Aronia 10 ml / kg and the group Osteoporosis + Aronia 5 ml / kg. In contrast to the beneficial effect of osteoporotic changes in BMD in the femur when treated with Aronia extracts in this therapeutic protocol the tibial bones, did not show significant beneficial effect on BMD in osteoporosis.

It might be presumed that static and dynamic load on the tibia are less weaker than on and correspond to a thinner bone structure and fine architecture of this bone compared to the femur which may contribute to some extent to the lack of effect.



Osteo Unit Alexandrovska University Hospital
1 St. Georgi Sofiyski
Sofia, Bulgaria 1431

II B

Telephone: 029230528

Name: 2.6	Sex: Female	Height:
Patient ID:	Ethnicity: White	Weight:
DOB:		Age:

Referring Physician:

k = 1.194, d0 = 73.2
159 x 185

Scan Information:

Scan Date: 27 July 2017 ID: D0727170C
Scan Type: h Hi-Res
Analysis: 27 July 2017 13:39 Version 13.3:3
Subregion Hi-Res
Operator:
Model: Discovery A (S/N 45654)
Comment:

DXA Results Summary:

Region	Area (cm^2)	BMC (g)	BMD (g/cm^2)
GLOBAL	2.60	0.70	0.269
R1	1.35	0.41	0.301
R2	1.21	0.28	0.231
Net	**2.56**	**0.69**	**0.268**

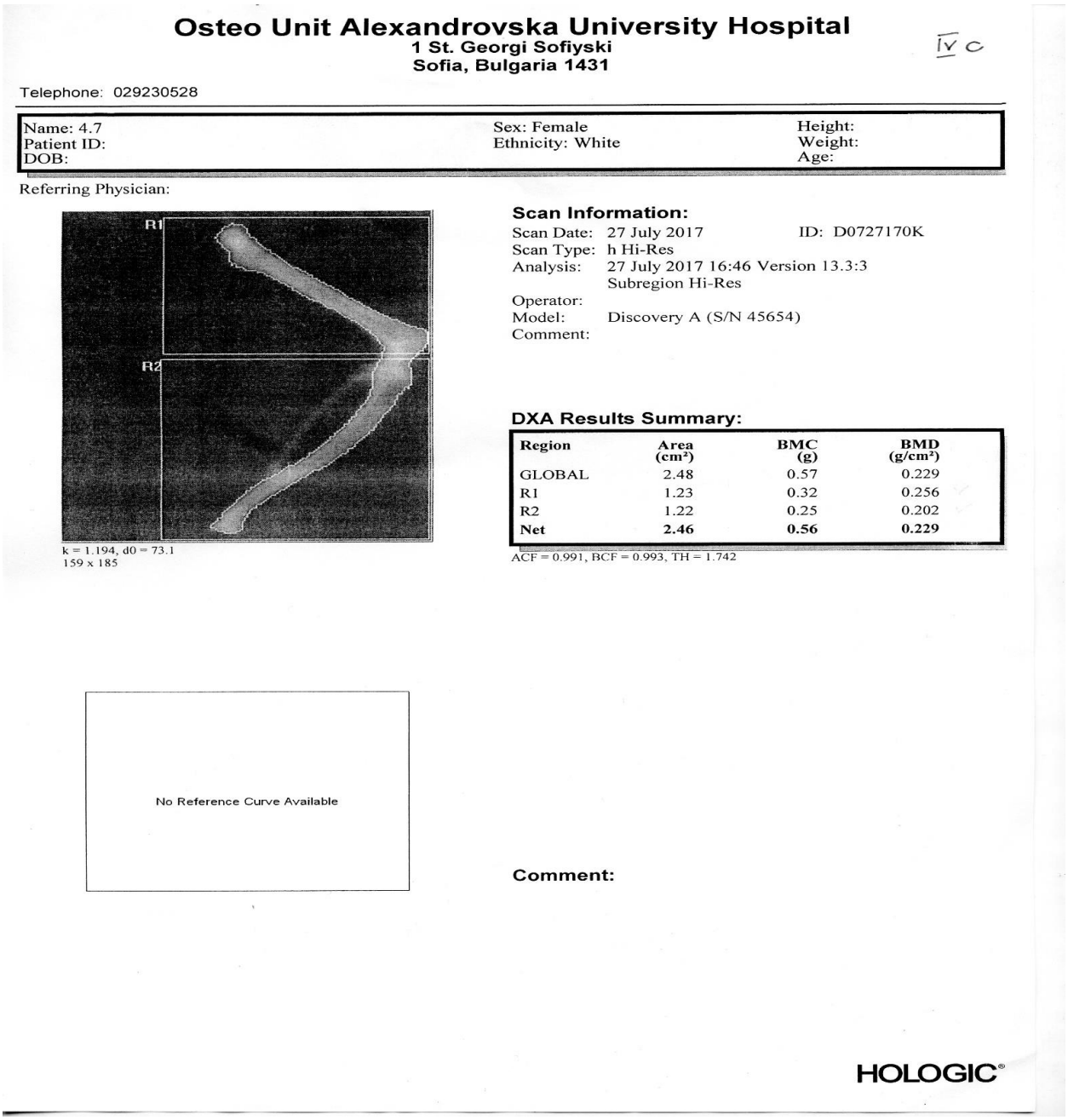
ACF = 0.991, BCF = 0.993, TH = 1.734

No Reference Curve Available

Comment:

HOLOGIC®

Figure 2. 1. 1. Original *post mortem* densitography and BMD of femur (R1) and tibia (R2) of rat II-B from the control group



Osteo Unit Alexandrovska University Hospital
1 St. Georgi Sofiyski
Sofia, Bulgaria 1431

IV C

Telephone: 029230528

Name: 4.7	Sex: Female	Height:
Patient ID:	Ethnicity: White	Weight:
DOB:		Age:

Referring Physician:

k = 1.194, d0 = 73.1
159 x 185

Scan Information:

Scan Date: 27 July 2017 ID: D0727170K
Scan Type: h Hi-Res
Analysis: 27 July 2017 16:46 Version 13.3:3
Subregion Hi-Res
Operator:
Model: Discovery A (S/N 45654)
Comment:

DXA Results Summary:

Region	Area (cm²)	BMC (g)	BMD (g/cm²)
GLOBAL	2.48	0.57	0.229
R1	1.23	0.32	0.256
R2	1.22	0.25	0.202
Net	**2.46**	**0.56**	**0.229**

ACF = 0.991, BCF = 0.993, TH = 1.742

No Reference Curve Available

Comment:

HOLOGIC®

Figure 2. 1. 2. Original *post mortem* densitography and BMD of femur (R1) and tibia (R2) of rat I V-C from group osteoporosis

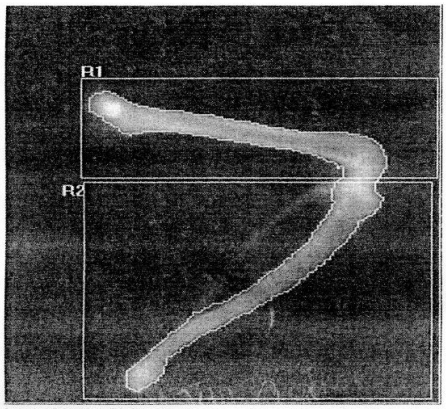
Osteo Unit Alexandrovska University Hospital
1 St. Georgi Sofiyski
Sofia, Bulgaria 1431

VIII A

Telephone: 029230528

Name: 8.5	Sex: Female	Height:
Patient ID:	Ethnicity: White	Weight:
DOB:		Age:

Referring Physician:



k = 1.194, d0 = 73.2
159 x 185

Scan Information:

Scan Date: 27 July 2017 ID: D0727170T
Scan Type: h Hi-Res
Analysis: 27 July 2017 15:37 Version 13.3:3
Subregion Hi-Res
Operator:
Model: Discovery A (S/N 45654)
Comment:

DXA Results Summary:

Region	Area (cm^2)	BMC (g)	BMD (g/cm^2)
GLOBAL	2.47	0.64	0.258
R1	1.19	0.36	0.305
R2	1.26	0.27	0.213
Net	**2.46**	**0.63**	**0.258**

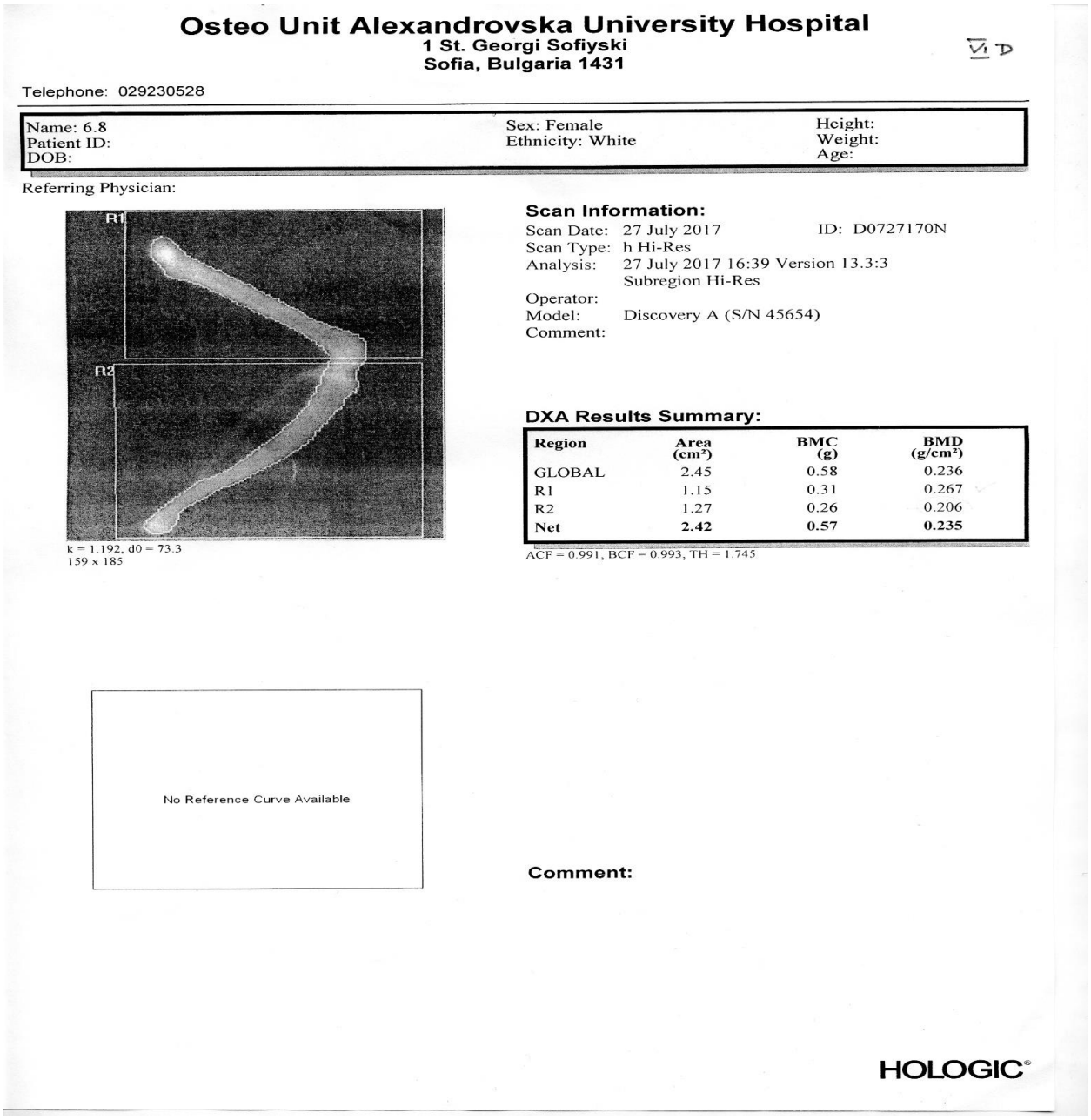
ACF = 0.991, BCF = 0.993, TH = 1.743

No Reference Curve Available

Comment:

HOLOGIC®

Figure 2. 1. 3. Original *post mortem* densitography and BMD of femur (R1) and tibia (R2) of rat VIII-A from group osteoporosis and Aronia 10 ml/kg



Osteo Unit Alexandrovska University Hospital
1 St. Georgi Sofiyski
Sofia, Bulgaria 1431

VI D

Telephone: 029230528

Name: 6.8	Sex: Female	Height:
Patient ID:	Ethnicity: White	Weight:
DOB:		Age:

Referring Physician:

k = 1.192, d0 = 73.3
159 x 185

Scan Information:

Scan Date: 27 July 2017 ID: D0727170N
Scan Type: h Hi-Res
Analysis: 27 July 2017 16:39 Version 13.3:3
Subregion Hi-Res
Operator:
Model: Discovery A (S/N 45654)
Comment:

DXA Results Summary:

Region	Area (cm^2)	BMC (g)	BMD (g/cm^2)
GLOBAL	2.45	0.58	0.236
R1	1.15	0.31	0.267
R2	1.27	0.26	0.206
Net	**2.42**	**0.57**	**0.235**

ACF = 0.991, BCF = 0.993, TH = 1.745

No Reference Curve Available

Comment:

HOLOGIC®

Figure 2. 1. 4. O Original *post mortem* densitography and BMD of femur (R1) and tibia (R2) of rat VI-D from group osteoporosis and Aronia 5 ml/kg

Changes in the bone mineral content *(BMC)*

The densitometric studies showed that estrogen deficiency, occurring after total ovariectomy, causes a negligible decrease in bone mineral content (BMC) in the isolated at random studied long bones of the hind limbs. The average values revealed a tendency to decrease the CMS only in the femur. The average value of bone mineral content decreased from 0.34 ± 0.03 g to 0.32 ± 0.01 g, ie decreased by about 6%. No significant changes in mineral content were found after treatment with Aronia. The results of these measurements are presented in Table 2. 1. 2.

Table 2. 1. 2. Effect of Aronia juice 10 ml / kg and Aronia juice 5 ml / kg on the bone mineral content of the hind limb of rats in an experimental model of osteoporosis

	Bone mineral content (g)		
	Femur	**Tibia**	**Total**
Control	0.34±0.03 (n4)	0.26±0.01 (n4)	0.61±0.04 (n4)
Osteoporosis	0.32±0.01 (n4) (*-6%*)	0.27± 0.01 (n4) ns	0.61±0.02 (n4) ns
o. Aronia 10 ml/kg	0.34±0.01 (n4) ns	0.26±0.006 (n4) ns	0.63±0.006 (n4) ns
o. Aronia 5 ml/kg	0.33±0.006 (n4) ns	0.26±0.03 (n4) ns	0.60±0.008 (n4) ns

n - number of animals in each group with post mortem DEXA for BMC; (+/- %) - change vs control, ns - not significant

Changes in pain sensitivity

The experiments showed reliable results from the studies with the Hot Plate test. The use of the Randall-Selitto test did not prove effective. The results of the measurements are presented in Table 2. 1. 3. We found that in the case of osteoporosis the pain threshold

was significantly reduced. In the Hot Plate test, the pain threshold was reduced by 13% - from 32.55 ± 1.36 sec to 28.26 ± 1.18 sec. Treatment with Aronia juice 10 ml / kg and Aronia 5 ml / kg restores nociception levels to those of control animals, ie it can significantly reduce pain sensitivity in a model of experimental osteoporosis.

Table 2. 1. 3. Effect of 10 ml / kg Aronia juice and 5 ml / kg Aronia juice on pain sensitivity in an experimental model of osteoporosis in rats

Group	**Test Randall-Selitto (RU)**	**Test Hot Plate (sec)**
Control	4.58±0.27 (n32)	32.55±1.36 (n37)
Osteoporosis	4.71±0.20 (n35) ns	28.26±1.18 [a] (n42) (*-13%*)
O. Aronia 10 ml/kg	4.69±0.19 (n39) ns	35.52±0.99 (n45) ns
O. Aronia 5 ml/kg	5.01±0.19 (n33) ns	34.33±1.08 (n39) ns

n - the number of the individual measurements made on the animals of each group in two consecutive days (-%) - change value vs control, ns - not significant, P ≤ 0.05 vs Control

Summary of experimental results:

The densitometric results might serve as a convincing evidence that estrogen-deficient experimental rat osteoporosis can be used as a reliable model of postmenopausal osteoporosis in women. The long-term treatment with Aronia juice 10 ml / kg might delay the development of osteoporotic bone changes. In line with the results of bone mineral density studies are the data on changes in pain sensitivity. In osteoporosis, the nociceptive thresholds measured by the Hot Plate test are significantly lowered. Treatment with Aronia juice 10 ml / kg might be beneficial in relieving the pain of osteoporosis. The applied scheme of pharmacological treatment with juice of Aronia10 ml / kg and Aronia 5 ml / kg however did not cause significant changes in the mineral

content. There was a trend to decrease BMC in the femur, which could stop after treatment with Aronia juice 10 ml / kg.

2. 2. Studies of the antiosteoporotic effects of *Apium* extract

Throughout the postoperative period, the body weight of the operated and non-operated animals was regularly measured. The body weight of non-ovariectomized rats increased by 19.5%. In the preoperative period, the mean body weight in the group with intact ovaries was 231 ± 4.0 g. Six months later it increased by 20% to 276 ± 12 g. In contrast to non-operated rats, the mean body weight in the ovariectomy groups increased by more than 32%. Immediately before surgery, the mean body weight of the animals was 239 ± 8 g. Six months after ovariectomy, the mean body weight of the animals in these groups reached 317 ± 8 g. Measurement data showed that adipose tissue weight in ovariectomized rats increased significantly. That was also confirmed in a post mortem section of a large number of animals. At the end of the experiments, a post mortem measurement of the weight of the uterus of some of the animals in each group was also performed. The mean weight of the uterus of animals from the control group of animals was 0.71 ± 0.1 g (N = 3). The weight of the uterus of animals with ovariectomy was 0.42 ± 0.02 g (N = 3). The data are also valid for Section 2.1.

Changes in the bonemineral density

The densitometric analysis showed that in the model of estrogen deficiency in removed ovaries, changes in bone mineral density were weak or absent. The average data from *ex situ* densitometric measurements show that the bone mineral density was reduced from 0.294 ± 0.003 g / cm2 to 0.278 ± 0.005 g / cm2 in the femur, ie the reduction is 5%, and in the tibia from 0.234 ± 0.004 g / cm2 to 0.224 ± 0.01 g / cm2, ie a reduction of 4%. On the background of these changes in bone mineral density, it had been documented that treatment with Apium extract in this experiment has an uncertain effect. The results of these measurements are presented in Table 2. 2. 1.т

Table 2. 2. 1. Effect of Apium nodiflorum extract on bone mineral density of hind limb bones in an experimental model of post-menopausal osteoporosis

Bone mineral density (g/cm2)		
Group	**Femur**	**Tibia**
Control	0.294±0.003 (n4)	0.234±0.004 (n4)
Osteoporosis	0.278±0.005 [a] (n4) (*-5%*)	0.224±0.01[a] (n4) (*-4%*)
Osteoporosis Apium	0.285±0.005 [a] (n3) (*-2%*)	0.226±0.01 (n3) ns

n - the number of the the animals of each group with post mortem DEXA study in hind limb, (-%) - change value vs control, ns - not significant, $P \leq 0.05$ vs Control

Changes in the bone mineral content

The results of densitometric studies show that estrogen deficiency after removal of the ovaries did not cause significant changes in the mineral content of the femur and tibia. No changes in the mineral content of the studied bones were documented during treatment with Apium extract during this stage. The results of these measurements are presented in Table 2. 2. 2.

Table 2. 2. 2. Effect of Apium nodiflorum extract on bone mineral content of hind limb bones in an experimental model of post-menopausal osteoporosis

Bone mineral content (g)

Group	Femur	Tibia	Total
Control	0.31±0.01 (n4)	0.24±0.01 (n4)	0.57±0.04 (n4)
Osteoporosis	0.28±0.01 [a] (n4) (*-6%*)	0.21±0.02 (n4) ns	0.61±0.02 (n4) ns
Osteoporosis Apium	0.32±0.01 (n4) ns	0.26±0.006 (n4) ns	0.63±0.006 (n4) ns

n - the number of the the animals of each group with post mortem DEXA study in hind limb, (-%) - change value vs control, ns - not significant, P ≤ 0.05 vs Control

Summary of the experimental results:

The presented data show that at this stage of estrogen deficiency with removed ovaries, no significant osteoporotic changes in the bones occur. Pharmacotherapy with Apium extract as an antiosteoporous agent has an uncertain effect on treatment, which is probably due to insufficient duration.

VI. ANALYSIS AND DISCUSSION

Effect of steroid suprarenal and gonadal hormones on experimental kaynic acid-induced epileptiform convulsive syndrome

The data from the conducted studies supplement the existing information about the effect of steroid suprarenal and gonadal hormones on epileptogenesis and do not claim to fully clarify these issues. We found that kainate-induced epileptiform syndrome could be used as an adequate model of epileptogenesis. To achieve "zero" hormonal levels and hormonal substitution on the background of complete deficiency of glucocorticoids and androgens, the model was deliberately created in castrated male animals with removed adrenal glands and with maintained water-salt and nutritional balance. Surgical interventions are an extreme stressor and

drastically aggravate the convulsive syndrome. Other authors also report that in male animals, castration enhances responses to various stressors (Thomas, J., McLean, J. H., 1991).

The data we presented here indicate that administration of Corticosterone could completely suppress the kainate-induced epileptiform syndrome. They are in line with the results of a number of clinical studies showing that the use of corticosteroids significantly alleviates the clinical manifestations of convulsive syndrome in patients with epilepsy (Curian, M., Korff, CM, 2011, Inutsuka, M., et al. , 2006, Verhelst, H., Boon, P., Buyse, G., 2005; Sinclair, DB, 2003). The elucidation of the basic mechanisms of the antiepileptic action of Corticosterone is beyond the scope of this dissertation and might be a subject of future research. It is reasonable to assume that the anti-stress action of corticosteroids at a central and peripheral levels underlies their anti-epileptogenic action.

Our results reveal that Estradiol severely exacerbates kainate-induced epileptiform syndrome. Estrogens have been shown to have a similar pro-convulsive effect in castrated female animals (Reddy, D. S., 2009). It is well known that Estradiol stimulates the release of glutamate and inhibits the synthesis of GABA, which may be a plausible explanation for its pro-convulsive action. We found that the effect of progestogens on kainate-induced epiletogenesis is manifested by a tendency to decrease the latency in weak seizures from Dihydroprogesterone and with a slight increase in latency in the submaximal. The studied gestagens did not have a significant effect on the intensity and lethality of tonic seizures. Experimental and clinical studies have shown that Progesterone has anticonvulsant activity in female individuals (Reddy, D. S., 2013; Reddy, D. S., et al., 2004; Herzog, A. G., 1999).

In vitro experiments have shown that Progesterone and Dihydroprogesterone inhibit amygdala-triggered adhesion activity in the motor neurons of the brain of female animals (Lonsdale, D., Burnham, W. M., 2003). It can be assumed that the weaker effect observed in males compared to the effects described by other authors in females correlates with the lower expression of cerebral progesterone receptors in males. We revealed that Dihydrotestosterone exacerbates the kainate-induced epileptiform syndrome and increases the associated lethality. In clinical and experimental studies, Testosterone has

been shown to potentiate epileptiform seizures after being metabolized to Estradiol by the aromatase enzyme (Reddy, D. S., 2004). It can be assumed that the described effects of Dihydrotestosterone are realized by a similar mechanism.
In the presented studies, the specificity and intensity (somatic and cognitive symptoms) of the clinical symptoms of kainate-induced epileptiform syndrome were for the first time quantified using the quantitative point scale created by Vlaskovska and co-workers. The data obtained in our studies show that the quantitative cumulative scale allows for a detailed and adequate analysis of somatic and cognitive symptoms in an experimental model of epileptogenesis. Applying such a methodological approach would help to avoid uncertain and ambivalent interpretations in assessing the nature and intensity of behavioral responses through the behavioral assessment scales used so far.

Summary

The data presented here showed:

(i) *C* orticosterone had stronger antiepileptic effect than Progesterone
(ii) *Testosterone* had significant pro-convulsive activity. This requires careful dosing in individuals receiving anabolic steroids
(iii) Hormonal imbalance may be significant for manifestations of epileptiform convulsive activity in persons with epilepsy
(iv) The established quantitative cumulative scale allows for a detailed and adequate analysis of somatic and cognitive symptoms in an experimental model of kainate-induced epileptogenesis. An adequate model of convulsive epileptiform syndrome was used

Effect of newly synthesized chromen-substituted hydrazide-hydrazones with anticonvulsant action on nociception

Epileptogenesis is a complex multicomponent process that determines both the development of clinical symptoms of epilepsy and the progression of the disease (Pitkanen, A., Engel, J. Jr., 2014). Epilepsy is a disease also characterised by occurrence

of severe comorbidity and complications, caused by ischemic events, cerebral traumatic injuries, genetic ionic channelopathies, neuronal hyperexcitability, and feed-forward cortical inhibition, which may be the electrophysiological equivalent of the aura (Zarcone, D., Corbetta, S., 2017).

It is well known from the physicians practice that epilepsy and neuropathic pain share many common characteristics, among which are paroxysmal activity, the presence of a provoking agent, aura, familial predisposition. In this study, we have presented data revealing that some of the newly synthesized 2H-chromene derivatives may have antinociceptive activity in correspondence to previously published data on anticonvulsant activity of these compounds (Angelova, VT, et al., 2016) . It can be assumed that the processes of central sensitization, observed in epilepsy and neuropathic pain, cause a progressive increase in the frequency of seizures. The later leads to chronicity of the process, and causes drug resistance and / or addiction.

Postictal headache is observed in 41% of patients with temporal lobe epilepsy, in 40% of patients with frontal epilepsy and in 59% of patients with occipital epilepsy according some clinical observations (Ito, M., et al., 2003). From the results obtained in the present studies, no conclusions can be drawn about the mechanisms of analgesic action of the test compounds. However, there is a strong evidence that 2H-chromene derivatives may act as ligands for adenosine A2A receptors (Areias, F., et al., 2012) and / or as inhibitors of NMDA glutamate receptors (Costa, BM, et al., 2010). Other authors have shown that coumarin derivatives can act as non-competitive agonists of GABAA receptors (Luszczki, JJ, et al., 2009) or modulate the activity of NMDA-ergic neurons in the CNS (Irvine, MW, et al., 2012). . In this context, it can be presumed that some of the neurotransmitter processes cited above are involved in the mechanisms of antinoceceptive action of the test compounds.

Summary

Our data obtained in the presented studies with the “Hot Plate” test and the “Formalin” test clearly show that compounds 4a, 4c, 8a and 8b can reduce the nociception. This could be further used as an additional beneficial feature in the development of new anticonvulsants.

Pharmacotherapeutic potential of Aronia melanocarpa fruit juice in a model of experimental post-menopausal osteoporosis

Using an experimental approach to induce estrogen-deficient osteoporosis in ovariectomized female rats, we validated an adequate model of osteoporosis, representing clinically manifested postmenopausal osteoporosis. It can be reasonably accepted that the pathogenetic mechanisms unfolded in the development of experimental osteoporosis are generally similar to the mechanisms involved in the pathogenesis of destructive bone changes in postmenopausal osteoporosis (Jee, WSS, Yao, W ., 2001, Lelovas, PP, et al., 2008). Methods, which we used for measuring bone mineral density and bone mineral content, are accepted as the best clinical methods for objectification and quantification of bone demineralization and destructive process in osteoporosis. The results of our studies show that when animals are treated with 10 ml / kg of Aronia juice, the development of osteoporosis occurs later and the bone changes are less pronounced. This was verified with significantly improved BMD and CMC parameters after treatment with 10 ml / kg Aronia juice. The data on the beneficial effect of 5 ml / kg of Aronia juice in osteoporosis are unconvincing.

According to clinical practice the most common manifestation of osteoporosis in adults is a fracture of the proximal segment of the femur. The present studies provide us with convincing evidence that supplementation therapy with Aronia juice 10 ml / kg in osteoporosis caused by estrogen deficiency after ovariectomy (experimental model of postmenopausal osteoporosis in women) might have a beneficial effect on bone destruction and a significant improvement of the basic parameters of bone micro-architectonics.

Bone pain is a usual symptom of metabolite bone damages, often as a result of bone destruction in osteoporosis and Paget's disease. So far, the pathogenetic mechanism of the pain associated with these diseases has not been fully elucidated in detail. The most acceptable from the numerous hypotheses is that the pain is due to the sensitization of the periosteal nociceptors in tissue damage or because of inflammatory processes. The structural damage to nerve fibers caused by direct tissue compression is also possible (Gennari, C., 1998). It is well known that inflammatory mediators (bradykinin, histamine, cytokines, PG F2α, nerve growth factor), generated in tissue damage, accelerate the kinetics of transduction processes in peripheral nerve terminals and decrease the threshold for their activation. Inflammatory mediators increase the osteoclasts' activity. Disorders of the bone metabolism lead to microfractures, which might be an acceptable explanation for the occurrence of osteoporotic pain (Orita, S., et al., 2012). The present study shows that the development of osteoporosis is accompanied by severe hyperalgesia (Randall-Selitto Test, Hot Plate Test). Pharmacotherapy with 10 ml / kg of Aronia juice reduced the pain sensitivity of treated animals compared to placebo-treated controls. It can be assumed that Aronia fruit juice 10 ml / kg would be a phytopreparation with therapeutic potential in post-menopausal osteoporosis by improving bone structure and reducing pain.

It is now well documented that estradiol is the major regulator of bone homeostasis through direct action on osteoblast and osteoclast activity. Estrogens block the synthesis and/or release of pro-inflammatory cytokines and the production of immunocompetent cells in the bone marrow (Pacifici, R., 1996; Sipos, S., et al., 2009). There are evidences that pro-inflammatory cytokines such as IL-6, TNFα, RANKL stimulate the development and activity of osteoclasts (Sipos, S., et al., 2005; Ginaldi, L., et al., 2005), the latest being pivotal for the development of post-menopausal osteoporosis. Typically the elevated levels of IL-6 correlate with symptoms of osteoporosis and / or bone pain in the absence of other concomitant pathological conditions affecting the skeletal system (Theoharides, TC, Boucher, W., Spear, K., 2002).

Известно е, че фитоестрогените стимулират естрогеновите ER-β рецептори, но все още цялостният механизъм на тяхното действие не е изяснен напълно. Phytoestrogens are known to stimulate estrogen ER-β receptors, but the complete mechanism of action is not yet fully understood. The numerous hypotheses that represent the basic mechanisms of action of phytoestrogens range from inhibition of DNA II topoisomerase activity through regulation of cellular regulatory centers to anti-angiogenesis and / or antioxidant activity. In recent years, the hypothesis that phytoestrogens can inhibit cellular proliferation by modulating the signaling cascades of transforming growth factor b1 has been discussed t (Kim, H., Peterson, TG, Barnes, S., 1998, Wang, J., Wang, S. , 2012). So far there we did not found any published data on pharmacotherapeutic potential of Aronia juice in post-menopausal osteoporosis, as well as on the antioxidant activity of phyto-formulas from parts of the plant. Our results, as well as data from phytochemical and phytopharmacological studies of fruits and phytoproducts from Aronia by others, gives reasoning that “Aronia melanocarpa may be one of the most useful medicinal plants” (Valcheva-Kuzmanova,S., Belcheva, A., 2006).

Summary

The results of studies on the pharmacological action of Aronia juice in experimental postmenopausal osteoporosis syndrome remind data to be carefully examined for potential therapeutic / prophylactic use of 10 ml / kg Aronia juice.

Pharmacotherapeutic potential of Apium nodiflorum extract in a model of experimental post-menopausal osteoporosis

During our studies in estrogen deficiency, occurring after ovary removal, there was no significant deviations in the densitometric parameters of the bones of ovariectomized animals as compared to the densitometric parameters of the bones of control animals.

The data show that pharmacotherapy with short-acting Apium extract, used to prevent potential osteoporotic changes, has a weak and uncertain effect. Tsakova and colleagues recently (2015 and 2016) presented results of multidisciplinary studies on bone changes in overt osteoporosis at molecular, tissue and systemic level as well as the beneficial effect of Apium on estrogen substitution. The authors revealed that the treatment with Apium extract has a positive effect in advanced osteoporotic bone changes, should the extract is given long-term in case of overt estrogen deficiency and clinical symptoms of postmenopausal osteoporosis. Changes in microarchitectonics and features in the histomorphology of long bones in experimental models of postmenopausal, estrogen-deficient osteoporosis were observed in long-term phytopharmacological substitution with Apium extract or placebo treatment. There were evidences that in osteoporosis, parallel changes in trabecular bone microarchitectonics occur simultaneously in both the pineal and metaphyseal segments of the femur and tibia. Chronic treatment with Apium extract prevents the development of severe destructive changes in bone microarchitectonics. Destructive changes in bone microstructure in both the pineal and metaphyseal segments of the femur and tibia in experimental osteoporosis are consistent with data from histomorphological studies by other authors (Hidaka, S., et al., 2006, Bitto, A., et al., 2011, 2008, Yogesh, HS, et al., 2011). These findings questions the hypothesis that the post-ovariectomy bone changes do not affect the trabecular bone tissue in the pineal gland of long bones (Jee, W. S. S., Yao, W., 2001; Westerlind, K. C., et al., 1997).

Other teams published on anti-inflammatory effects on the Apium extract (Lewis, D. A., 1985). Most likely, the anti-inflammatory and antinociceptive effects of Apium extract have a multicomponent pathogenesis. Presumptively, the components of the extract exhibit pronounced antioxidant activity and inhibit the generation of superoxide radicals. At the same time, they might inhibit the generation and release of inflammatory mediators (histamine, 5-HT, bradykinin, prostaglandins) and / or antagonize the effect of pro-inflammatory cytokines (IL-6).

Celery (*Apium nodiflorum*) shows some of the highest levels of antioxidant activity, which correlates with the content of high levels of total phenols and flavonoids found in plant

phytoproducts (Stankovic, MS, 2011, Morales, P., et al., 2012). The content of total phenols and flavonoids in the *Apium* extract is in the same ranges as these reported in other studies regarding celery stems and roots. *Apium nodiflorum* (celery) is a popular and widespread plant in the country, which can be a cheap and easy to obtain raw material for the pharmaceutical industry.

Summary

The research done in the scope of the presented dissertation was planned in two major aspects. Basically, complex studies of the pharmacotherapeutic effects of phytoproducts from the plants *Aronia melanocarpa* and *Apium nodiflorum*, known for their medicinal and culinary qualities, were performed at two levels: *in vivo* integrative level (nociception) and *ex situ* (post mortem osteodensitometry of femur and tibia). In an applied aspect, we have validated an experimental model "equivalent" of post-menopausal osteoporosis.

VII. CONSLUSIONS

A newly created quantitative scale for assessing the intensity of somatic and cognitive manifestations of experimental epileptiform syndrome has been validated and applied, which further allow detailed and adequate analysis.

Estrogen treatment worsens the clinical and pathophysiological characteristics of experimental kainic acid-induced epileptiform syndrome.

Androgen treatment has a strong pro-convulsive effect.

Glucocorticoid treatment alleviates the course and / or eliminates the life-threatening manifestations of experimental kainic acid-induced epileptiform syndrome.

Hormonal imbalance is a major pathogenetic factor in the development of epileptiform / convulsive activity.

The newly synthesized hydrazide-hydrazone compounds 4a, 4b, 4c, 8a and 8b, which showed an *in silico* profile of anticonvulsants, have moderate analgesic activity.

The estrogen-deficient model of experimental post-menopausal osteoporosis has been reliably validated and applied in a series of pharmacological studies.

Long-term treatment with *Aronia melanocarpa* juice has a protective effect on bone mineral density and at the same time reduces pain sensitivity in experimental "post-menopausal" osteoporosis.

The use of an extract of *Apium nodiflorum* with insufficient duration in experimental "post-menopausal" osteoporosis has an uncertain beneficial effect on bone mineral density.

VIII. CONTRIBUTIONS

It was found that the newly synthesized hydrazide-hydrazone compounds 4a, 4b, 4c, 8a and 8b, which showed an *in silico* profile of anticonvulsants have moderate analgesic activity.

It was found that long term treatment with *Aronia melanocarpa* extract 10 ml / kg might significantly delay the development of osteoporotic bone changes and reduce pain sensitivity in experimental post-menopausal osteoporosis.

A newly developed quantitative scale for quantitative assessment of clinical symptoms in experimental epileptiform syndrome has been validated and applied. The quantitative

cumulative scale allows for a detailed and adequate analysis of somatic / convulsive and cognitive manifestations in experimental "epilepsy".

X. PUBLICATIONS RELATED TO THE DISSERTATION

1. Slavina Surcheva, **Stanislav Marchev**, Roman Tashev, Stilyana Belcheva, Mila Vlaskovska. Action of adrenal and gonadal steroid hormones on kainic acid-evoked seizures in a rat model of epileptogenesis. "Biotechnology & Biotechnological Equipment", Vol. 31, 2017 (6); p. 1226-1230 (IF=1.277)

2. **Stanislav Marchev**, Pavlina Andreeva-Gateva, Roumiana Tzoneva, Slavina Surcheva, Alex Tzonev, Kalina Kamenova, Violina T. Angelova, Jana Tchekalarova, Mila Vlaskovska. Analgesic activity of some aroylhydrazone-based molecular hybrids with antiseizure activity: *in vivo* and *in silico* evaluations. "Biotechnology & Biotechnological Equipment", Vol. 33, 2019 (1); p. 98-107 (IF=1.327)

3. **Stanislav Marchev**, Stefka Valcheva-Kuzmanova, Slavina Surcheva, Pavlina Andreeva-Gateva. Increased risk of osteoporosis in epilepsy; the role of anti-epileptic drugs. Сп. "Наука Фармакология", кн.2, 2019; 19-24

4. Vasilena Kuzmanova, Atanas Kuzmanov, Simeon Todorov, **Stanislav Marchev**, Stefka Valcheva-Kuzmanova. Pain in osteoporosis - causes and pathogenesis. "Варненски медицински форум", т. 9, 2020, бр. 2; стр. 7-11

5. Pavlina Andreeva-Gateva, **Stanislav Marchev**. Melatonin in epilepsy and comorbid conditions - through the eyes of evidence-based medicine. Сп. "Наука Фармакология", кн. 2, 2020; стр. 5-13

XI. NATIONAL AND INTERNATIONAL CONFERENCES AND CONGRESS PARTICIPATIONS RELATED TO THE DISSERTATION

1. P. Andreeva-Gateva, J. Tchekalarova, V. Angelova, **S. Marchev**, Y. Voynikov, N. Vassilev, M. Vlaskovska, S. Surcheva. Preclinical screening of coumarin and 2H-Chromene substituted hydrazyde-hydrazone derivatives, as potenrial anticonvulsants. 13th Congress of the European Association for Clinical Pharmacology and Therapeutics (EACPT), Prague, 24th-27th June 2017. Published in Clinical Therapeutics, v39, 85, E78-E79, August 2017

2. **Marchev, S.**, Temelkova, K., Todorova, M., Eftimov, M., Georgieva, A., Kuzmanova, V., Kuzmanov, A., Surcheva, S., Vlaskovska, M., Valcheva-Kuzmanova, S. Effects of antioxidants isolated from Aronia and Apium on experimental model of osteoporosis. 18th World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. Krakow, Poland, 19-22 April, 2018. Abstract: Osteoporosis International 29 (Suppl. 1), P610. (IF=3.83)

3. Tzonev Alex, **S. Marchev**, P. Andreeva-Gateva, S. Surcheva. Evaluation of the antinociceptive properties of newly synthesized hydrazide-hydrazone derivatives. NVIII International Congress of Medical Sciences, 10-13 May, 2018, Sofia, Abstract book, p.14-15

4. П. Гатева, **С. Марчев**, С. Сурчева, В. Ангелова, К. Каменова. Аналгетична активност на новосинтезирани ароилхидразони – предклинични и *in silico* проучвания. Девета национална конференция за изследване и лечение на болката с международно участие, Хисаря, 07-09 Юни, 2018 г.

5. **Stanislav Marchev**, Pavlina Andreeva-Gateva, Alex Tzonev, Jana Tchekalarova, Violina Angelova, Mila Vlaskovska, Slavina Surcheva. Experimental study on the analgesic and anti-seizure activity of newly synthesised hydrazide-hydrazone derivatives bearing 2H-chromene and coumarin scaffold. 18 th World Congress of Basic and Clinical Pharmacology, 01-06 July, 2018, Kyoto, Japan; P03-1-81

6. S. Valcheva-Kuzmanova, V. Kuzmanova, A. Kuzmanov, M. Eftimov, M. Todorova, A. Georgieva, K. Kuzmanov, **S. Marchev**, M. Vlaskovska. Effects of Aronia melanocarpa fruit juice on metabolic indices in a rat ovariectomy-induced model of bone loss. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases, Paris, France, 04-07 April, 2019. Abstract: Osteoporosis International (2019) 30 (Suppl. 2):S465. (IF=3.83)

7. S. Valcheva-Kuzmanova, A. Kuzmanov, V. Kuzmanova, M. Eftimov, A. Georgieva, M. Todorova, K. Kuzmanov, **S. Marchev**, M. Vlaskovska. Evaluation of lipid metabolism, inflammation and bone turnover in a rat model of ovariectomy-induced bone loss. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases, Paris, France, 04-07 April. Abstract: Osteoporosis International (2019) 30 (Suppl. 2):S501. (IF=3.83)

8. S. Valcheva-Kuzmanova, A. Georgieva, M. Eftimov, M. Todorova, V. Kuzmanova, A. Kuzmanov, **S. Marchev**, K. Kuzmanov, M. Vlaskovska. Effects of polyphenol-rich aroniamelanocarpa fruitjuice on bone mineral density and pain sensitivity threshold in ovariectomized rats. 14 th Congress of the European Association for Clinical Pharmacology and Therapeutics (EACPT), S72-S73, Stockholm, Sweden. Abstract: European Journal of Clinical Pharmacology (2019) 75 (Suppl. 1):S72-S73. (IF=2.997)

9. P. Gateva, S. Surcheva**, S. Marchev**, J. Tchekalarova, V. Amgelova. Antiseizure and analgesic activity of newly synthesized melatonin derivatives bearing aroylhydrazone moiety. IV International Conference on Natural Products Utilization. Albena Resort, 29 May-1 June 2019, PP56

XII. THANKS TO

I would like to express my cordial gratitude to my supervisors Prof. Dr. Stefka Valcheva-Kuzmanova, MD, MD and Assoc. Prof. Dr. Pavlina Gateva, Ph.D. and to my scientific consultant: Corr. member of BAS Prof. Dr. Mila Vlaskovska, MD, MD

I would like to thank for the help and cooperation to Dr. N. Temelkova

I would like to thank to the whole team from MU Varna and MU Sofia

And last but not least to my family for all the support!