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Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVII, Fascicula 4, 2016

DNA ANALYSIS OF LATE BRONZE AGE FUNERARY CONTEXT FROM EASTERN ROMANIA

LUCIAN D. GORGAN[1], MITICĂ CIORPAC[1], FLORICA MĂŢĂU[2], NECULAI BOLOHAN[3]

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Keywords: aDNA, mitochondrial DNA, control region, haplogroup, Late Bronze Age
Abstract: The aim of this study was to identify the corresponding haplogroup for the analyzed sample, presenting also the first results obtained on ancient DNA isolated from a Late Bronze Age funerary context bone remains samples, discovered in Eastern Romania. Also, to identify an efficient and reliable protocol for ancient DNA extraction and to test whether the protocol is efficient and capable of yielding good quality DNA, the phenol:chloroform and DNA IQ protocols have been compared, evaluating the level of PCR inhibitors and the DNA viability.

INTRODUCTION

In the last decade, advances in genomic sequencing have started to provide insight into the complex narrative of human ancestry embedded in human DNA, particularly through genetic variation. Genetic variants are sequences of DNA base pairs that differ from more common ancestral sequences and can be traced to specific human populations, either present or past by ancient DNA (aDNA). The genetic analysis of human ancestry is a search for variants in an individual's genetic code and determine how related that person is to specific ethnic and geographic populations. For males, if the samples are very good preserved, the Y chromosome is sequenced since this allele is always passed down from father to son withal for females, mitochondrial DNA is sequenced, since a daughter inherits the DNA from her mother. Genetic variants found in these sequencing data can be used to construct a paternal or maternal evolutionary tree, showing how certain human populations connect to each other.

The aim of this study is to introduce the first DNA results obtained on a funerary context assigned to a Late Bronze Age archaeological site from Eastern Romania which is located on the Eastern side of the Carpathians, in a hilly region of the Moldavian Plateau integrated in the Berheci River basin (Figure 1).

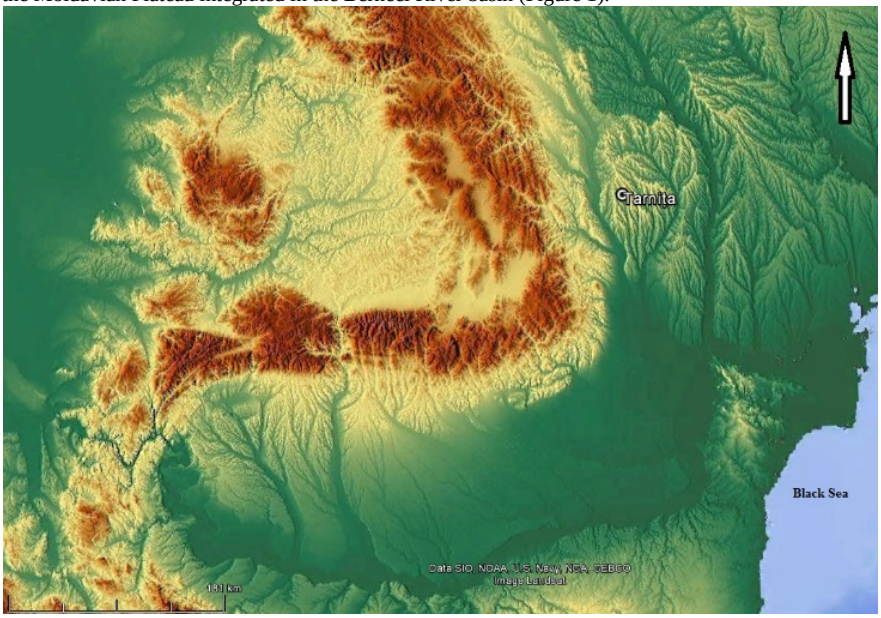

Figure 1 The location of the Late Bronze Age funerary discovery

The funerary discovery was located in the Tarnița village (Onceşti commune, Bacău County) and was largely attributed to the Noua culture (Antonescu 1976) which is usually depicted as being an eastern intrusion of the Sabatinovka culture. The Noua culture reaches a significant area, limited eastward by the Dniester river, westward by the Apuseni Mt., to the north by the Northern Carpathians and the southern extent is represented by the Siret-Prut confluence (Vulpe 2001). The Late Bronze Age Noua communities located North and North-Western area of the Black Sea are considered to be characterized by a highly nomadic way of life sustained and by a stock-breeding economy. Their main burial practices are represented by inhumation in a supine position, with few vessels, and sometimes with jewelry and tools or weapons as grave goods (Dascălu, 2007).

The funerary context from Tarnița (Figure 2) was discovered in 1972 and consists in a pit containing a human skeleton in a left supine position. The left hand was flexed and sustain the skull while the right hand was slightly lodged on the pelvis and the legs were strongly bent on the left side. The osteological remains were in a poor state of preservation. The burial contains two vessels as grave goods which were attributed based on their typological characteristics to the Noua culture (Antonescu, 1976).

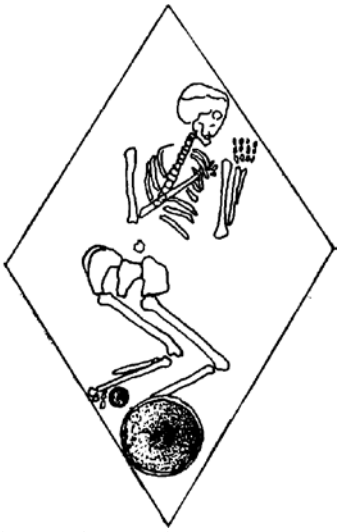

Figure 2 The funerary discovery from Tarnița (drawing by Antonescu 1976)

MATERIALS AND METHODS

Samples preparation

Samples are represented by 1 humerus (Figure 3A) and 1 clavicle (Figure 3B) from an entire skeleton located in the deposits of "Iulian Antonescu" Museum Complex (Bacău County, Romania).

Sample contamination is the major problem in ancient DNA (aDNA) studies and preventing its occurrence is always a priority for researchers. External contamination must be eliminated and several methods have been reported with this aim in the literature: abrasion, treatment with hypochlorite, irradiation with UV, etc. (O´Rourke et al., 2000, Kaestle and Horsburgh 2002, Kemp and Glenn Smith 2005). Extreme measures are usually reported during the procedures of extraction and amplification. The working area must be exclusively employed for ancient specimens and separated from the one where modern DNA is manipulated to avoid that any trace may contaminate samples. UV irradiation of the working area was also used, as well as the sterile and RNA-se/DNA-se free laboratory materials and regents. Masks, gloves and labs coat were used during the samples manipulations. Amplification inhibitors may also be co-purified in the extracts obtained and its presence produces negative results. Two different aDNA isolation protocols have been tested to eliminate substances that may act as inhibitors, so this situation must always be considered and essayed when negative results are systematically obtained (Kemp et al., 2006).

A Humerus B Clavicle

Figure 3. T72 (Tarniţa 1972) osteological samples

The aDNA extraction from bone remains involve two main activities: samples preparation and extraction protocol. The bones were cleaned from debris and washed with distillated water, followed by a drying step. Further, the bones were washed with 10% NaOCl, dried and exposed to UV light for 15 minutes on each side. Finally, with a sterile bone drill a small area of the bone surface was removed and then bone powder was sampled, using a reduced rotation speed, to avoid the temperature increase followed by an accelerate aDNA degradation. Every bone was prepared using the required standard protocols for aDNA analysis (facemask, gloves, and disposable lab coat), sterile and disposable tools in a controlled environment.

DNA isolation

For a higher confidence degree, two extraction protocols have been tested: phenol-chlorophorm-isoamyl alcohol (PCI) and DNA IQ (Promega, USA). For both DNA extractions protocols a total demineralization step was used. Around 200 mg of bone powder was used for PCI protocol and incubated at 56°C overnight with 1 ml of bone incubation buffer (*Tris HCl 50mM pH=8, SDS 10%, EDTA 0.5mM* and *Proteinase K 1mg/ml*), followed in the next day by the classic extraction steps. The DNA IQ extraction was made from 40 mg of bone powder according to the manufacture protocol. For both extraction protocols blank control samples were used to assess the potential reagents or lab contamination.

In order to identify any possible contamination that might have occurred in the different stages of the samples preparation and mainly in the aDNA isolation, at least two extraction blank controls and multiple PCR non-template controls were included in each amplification reaction. The rate of contamination for this analysis was less than 0,4%.

aDNA isolation protocols evaluation by Real-Time PCR

DNA quality and inhibition degree were evaluated using Real-Time qPCR with GoTaq 1-Step RT-qPCR System (Promega, USA), using the HV1 first part specific primers.

PCR and sequencing

The mitochondrial hyper variable region 1 (HV1) was amplified via PCR method using four pairs of primers (Table 1). The PCR was performed in a 25 µL reaction volume using GoTaq® Hot Start Polymerase (Promega, USA). The amplicons were purified using the Agencourt AMPure XP (Beckman Coulter, USA) and direct sequenced using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, USA) in the CEQ 8000 Genetic Analysis System (Beckman Coulter). The sequence analysis was performed using the CEQ8000 instrument software. Multiple sequences have been analyzed and low frequency mutations were considered artefacts resulting from post-mortem aDNA damage.

Table 1 HV1 primers sets

Locus	Pimers	Amplicon Size (bp)	Reference
MPS1A	F 5’-CCC AAA GCT AAG ATT CTA AT-3 R 5’-TAC TAC AGG TGG TCA AGT AT-3’	170	Gabriel et al., 2001
MPS1B	F 5’-CAC CAT GAA TAT TGT ACG GT-3 R 5’-TGT GTG ATA GTT GAG GGT TG-3’	126	Gabriel et al., 2001
MPS2A	F 5’-CCC CAT GCT TAC AAG CAA GT-3 R 5’-TGG CTT TAT GTA CTA TGT AC-3’	133	Gabriel et al., 2001
MPS2B	F 5’-CAC TAG GAT ACC AAC AAA CC-3’ R 5’-GAG GAT GGT GGT CAA GGG AC-3’	143	Gabriel et al., 2001

RESULTS AND DISCUSSIONS

aDNA quantification and inhibition

The first stage of current experiment was the evaluation of the ability to remove the PCR inhibitors and the aDNA recovery efficiency from bone remains by two widely used extraction protocols. In order to assess the aDNA recovery, the extracted DNA was firstly subject to spectrophotometric quantification. By comparing the DNA concentration in both extraction assays, the DNA quantity extracted with PCI protocol was greater, suggesting that the PCI extraction protocol had a better efficiency. Even if, the DNA quantity it's double in PCI case in comparison with DNA IQ (Table 2), for both protocols the amount of aDNA recovered from ~1.8 kya bones is quite low.

Table 2 The amount of aDNA recovered from ~1.8 kya bones using two extraction protocols

Sample ID	PCI aDNA Concentration (ng/ul)	DNA IQ aDNA Concentration (ng/ul)
Bk*	0.1	0.0
R**	0.0	0.0
T72	1.5	0.7

Bk* - blank probe
R** - reagents control, distillated water instead of incubation buffer
All the samples were read in 2 µl volume

Several previous studies shown a dependency between the aDNA extraction protocol and the degree of inhibition (Rohland and Hofreiter, 2007; Jakubowska et al., 2012). Therefore, a RT-qPCR approach was used to evaluate the co-extraction of PCR inhibition. For both protocols the same amount of aDNA was used, 0.6 ng/. Furthermore, an absolute quantification of DNA copies per reaction was made, using a standard quantification curve ranging between 2 and $2x10^5$ copies per reaction. The cycle threshold (Ct) value shown from the real-time PCR is obvious much lower for the DNA IQ protocol (Figure 1) which indicates a higher aDNA template concentration and a correlated low number of cycles for the amplification to start. Also, the RT-qPCR data shown that the DNA IQ protocol it's able to extract about 565 inhibitors free DNA copies/µl while the PCI protocol extract just 13.5 copies/ µl. Also, the RT-PCR amplicons melting curve analysis which is frequently used as a diagnostic tool for assessing qPCR amplicon length with intercalating dye qPCR assays, indicate the presence of just one single product.

Finally, regarding the aDNA quantity and quality we can conclude that the DNA IQ protocol offer better results with a high aDNA purity and viability, compared to the classic PCI protocol, due to a higher amount of PCR inhibitors co-extracted by PCI protocol.

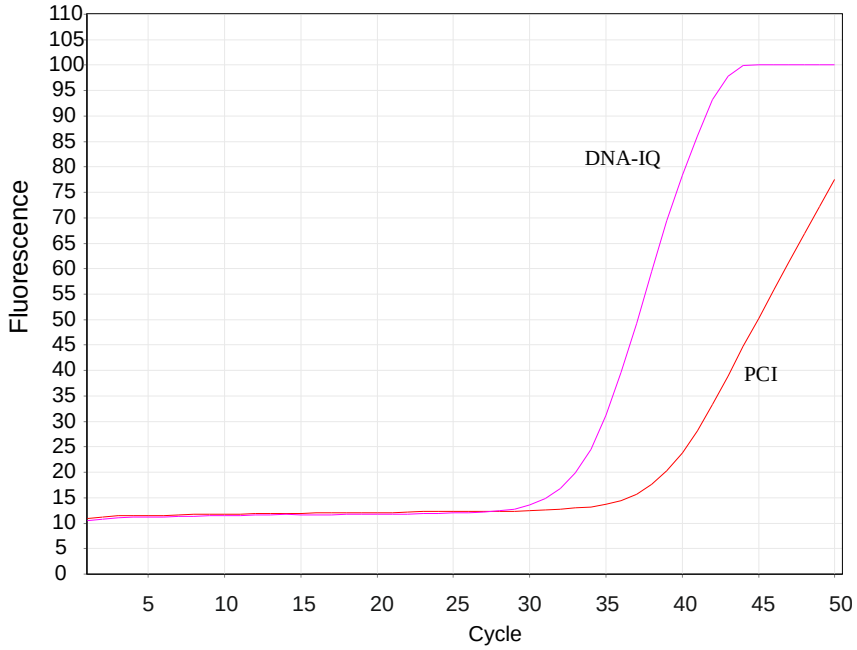

Figure 4A The RT-PCR T72 bone aDNA sample analysis for both tested protocols PCI and DNA-IQ: Ct values

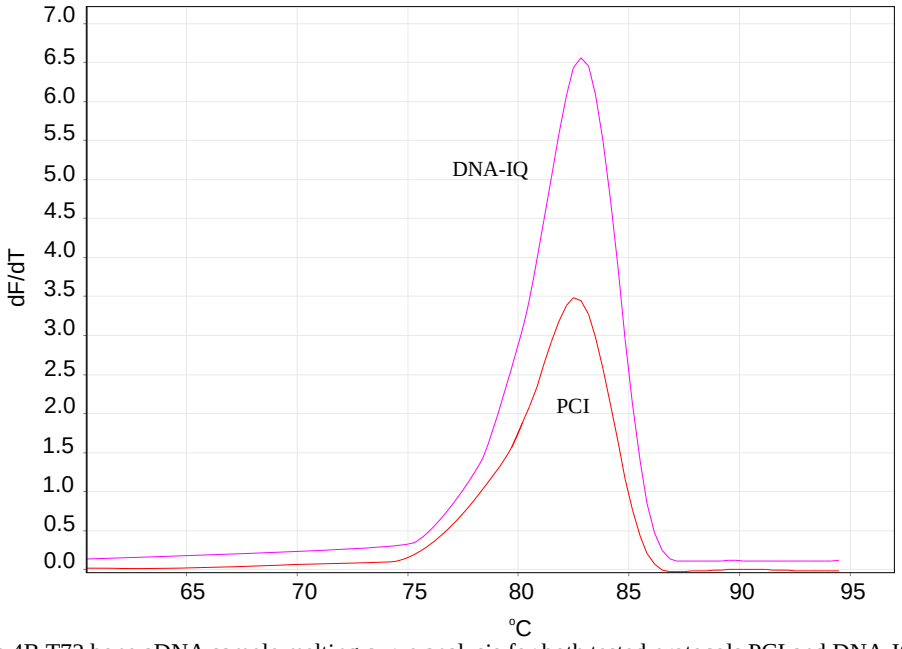

Figure 4B T72 bone aDNA sample melting curve analysis for both tested protocols PCI and DNA-IQ: Ct values

Genetic and cultural context

The sequences were subjected to Nucleotide BLAST (Basic Local Alignment Search Tool, Altschul et al., 1990) to identify the similarities with the previous sequences from data base and haplogroup assignation (Figure 5 and Table 3). Also, the T72 sample shown an identity of 97% with HV1 sequences from a previous study conducted by Lippold et al., 2014.

According to Eupedia database, Haplogroup HV is the most successful maternal lineage in Europe and the Near East with a distribution of over half of the European population and between 25% and 40% of the Near Eastern population. Most Europeans belonging to the HV lineage descend from a branch that was renamed haplogroup H. Another small but substantial European branch was called haplogroup V. The major subclades of HV haplogropup are HV0 and V, HV1, HV2, HV4, HV5, HV12, HV13 and H.

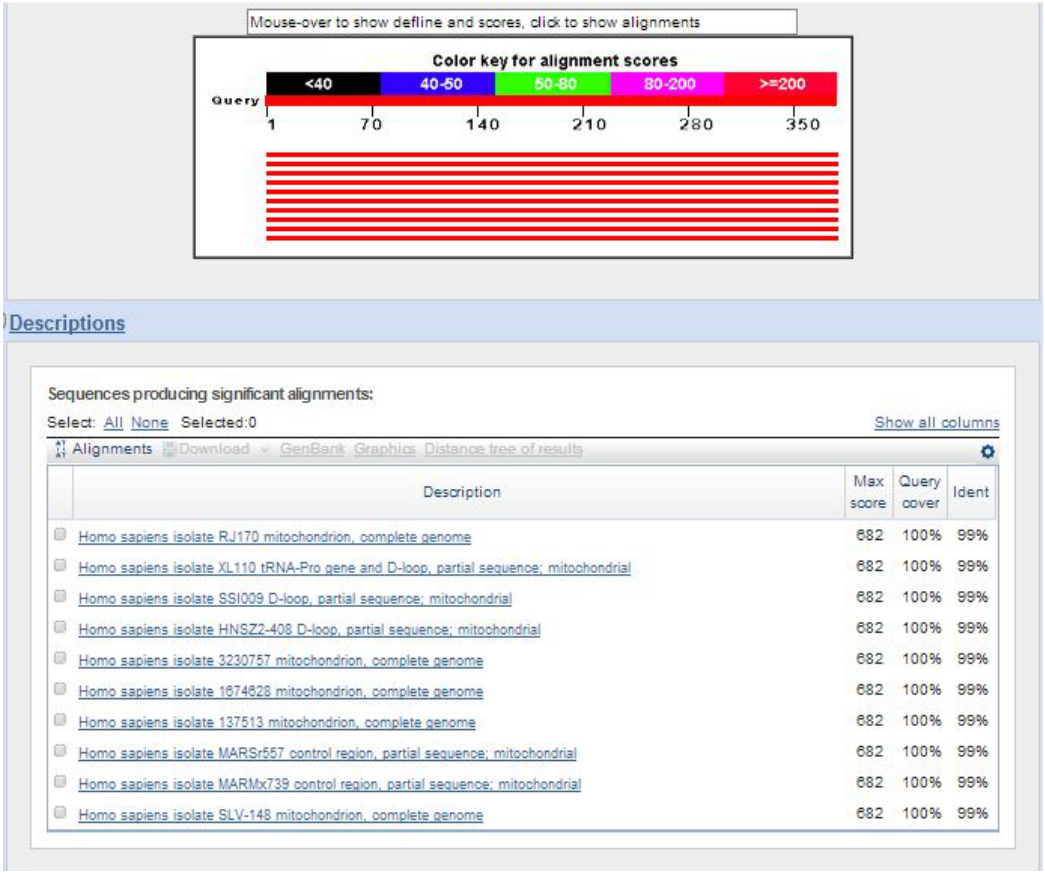

Figure 5 Blast report from NCBI (National Center for Biotechnology Information) indicate a 100% similarity with HV2 haplotype

Table 3 Sample haplogroup prediction based on Alignment Summary: rCRS track view (http://mitomaster.mitomap.org/)

Sequence	Predicted Haplogroup	Total Variants	Variants
T72	HV2	6	T16126C, T16217C, C16218T, T16229d, C16232CT, T16304C

The T72 sequence was aligned with haplogroup HV and HV2 NCBI sequences and used to construct a ML tree, in order to confirm the haplogroup assignation.

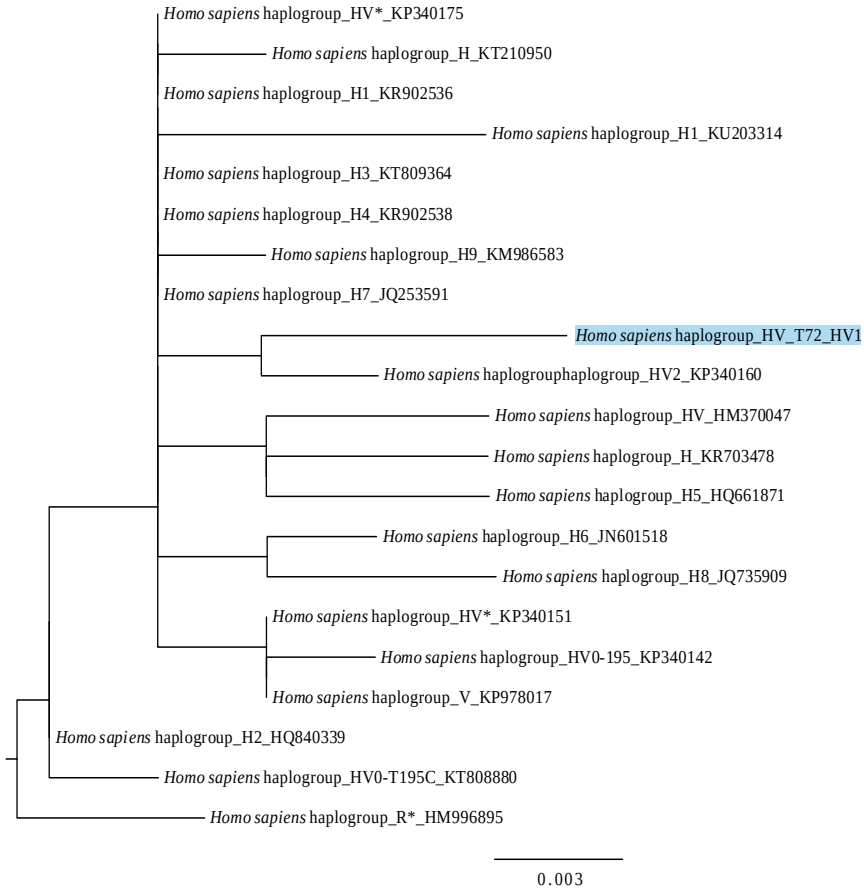

Figure 6 Molecular Phylogenetic analysis by Maximum Likelihood method

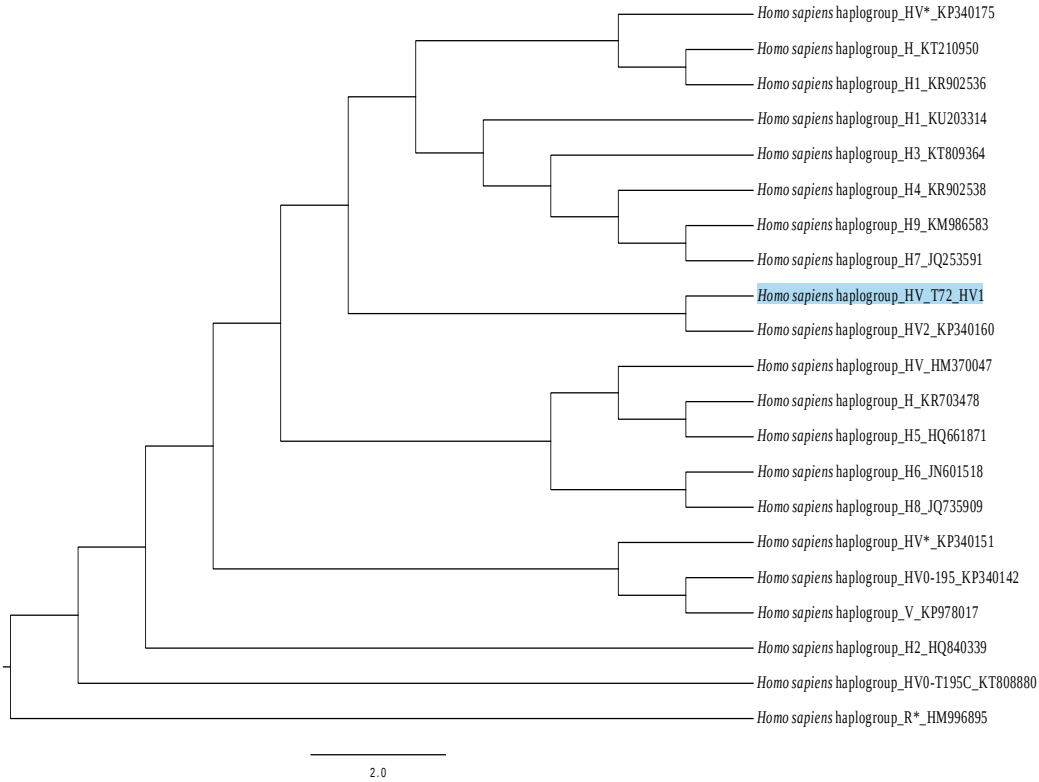

Figure 7 The Cladogram of ML tree

The evolutionary history was inferred by using the Maximum Likelihood (Figure 7) method based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-691.0766) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.1000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 381 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

CONCLUSIONS

The mtDNA is relatively easy to work with, as it has a reduced size, coupled with the conserved arrangement of genes, which means that many pairs of universal primers will amplify regions of the mitochondria in a wide variety of vertebrates and invertebrates.

Even if the arrangement of genes is conserved in the mitochondrial genome, the overall mutation rate is high, may be due partly to the by-products of metabolic respiration and also to less-stringent repair mechanisms compared with those acting on nuclear DNA. The high mutation rates mean that mtDNA generally shows relatively high levels of polymorphism and therefore will often reveal multiple genetic lineages both within and among populations.

mtDNA is effectively a single haplotype that is transmitted from mothers to their offspring, which means that mitochondrial lineages can be identified in a much more straightforward manner than nuclear lineages, which, in sexually reproducing species, are continuously pooling genes from two individuals and undergoing recombination.

The ancient DNA analysis indicate with a maximum confidence, that the T72 sample belongs to HV2 haplogroup.

Genetic information for sample T72 obtained from the amtDNA analysis are in agreement with previously analyzed LBA samples from Romania (Hervella et al. 2015; Bolohan et al. 2016) which were attributed to the Noua culture based on the associated grave goods. The HV clade as a whole has a high occurrence between Western and Central Asia region. Most of the lineages within the HV haplogroup were spread into Europe during the Late Glacial Maximum (27-16 kya). In particular, high HV occurrences are observed in Southern Europe which could have spread into Europe with the amelioration of the climatic conditions (De Fanti et al. 2015).

REFERENCES

Altschul, Stephen; Gish, Warren; Miller, Webb; Myers, Eugene; Lipman, David (1990). "Basic local alignment search tool". Journal of Molecular Biology 215 (3): 403–410.doi:10.1016/S0022-2836(05)80360-2. PMID 2231712.
Antonescu, Silvia (1976) "Săpăturile de salvare de la Tarnița (comuna Oncești, județul Bacău)", Carpica VIII, 37-40.
Bolohan, Neculai, Ciorpac, Mitică, Gorgan, Măţău, Florica, Lucian Dragoş (2016) "DNA analysis of a Noua culture funerary context from Eastern Romania", ArheoVest IV, in print.
Dascălu, Lidia (2007). “Bronzul mijlociu şi târziu în Câmpia Moldovei”. Iaşi.
De Fanti, Sara, Barbieri, Chiara, Sarno, Stefania, Sevini, Federica, Vianello, Dario, Tamm, Erika, Metsaplu, Ene, van Oven, Mannis, Hübner, Alexander, Sazzini, Marco, Franceschi, Claudio, Pettener, Davide, Luiselli, Donata (2015) "Fine Dissection of Human Mitochondrial DNA Haplogroup HV Lineages Reveals Paleolithic Signatures from European Glacial Refugia". PloS ONE, 10 (12): e0144391. . doi:10.1371/ journal.pone.0144391.
Gabriel, M. N., Huffine, E. F., Ryan, J. H., Holland, M. M.,and Parsons, T. J. (2001). "Improved MtDNA sequence analysis of forensic remains using a "mini-primer set" amplification strategy ". Journal of forensic sciences, 46(2), 247-253.
Hervella, Montserrat, Rotea, Mihai, Izagirre, Neskuts, Constantinescu, Mihai, Alonso, Santos, Ioana, Mihai, Lazăr, Cătălin, Ridiche, Florin, Soficaru, Andrei Dorian, Netea, Mihai G., de-la-Rua, Concepcion (2015) "Ancient DNA from South-East Europe Reveals Different Events during Early and Middle Neolithic Influencing the European Genetic Heritage". PloS ONE, 10 (6): e0128810, doi:10.1371/journal.pone.0128810.
Kaestle, F., Horsburgh, K. (2002). "Ancient DNA in Anthropology: Methods", Applications and Ethics. Yearbook of Physical Anthropology, 45: 92-130.
Kemp, B. Glenn Smith, D. (2005). "Use of bleach to eliminate contaminating DNA from the surface of bones and teeth". Forensic Science International, 154:53-61.
Kemp, B. Monroe, Glenn Smith, D. (2006). "Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts". Journal of Archaeological Science, 33: 1680-1689.
Lippold, Sebastian, Xu, Hongyang, Ko, Albert, Li, Mingkun, Renaud, Gabriel, Butthof, Anne, Schröder, Roland, Stoneking, Mark "Human paternal and maternal demographic histories: insights from high-resolution Y chromosome and mtDNA sequences". bioRxiv: 001792.
Nei M. and Kumar S. (2000). "Molecular Evolution and Phylogenetics ". Oxford University Press, New York.
O´rourke, D. Hayes, Carlyle, S. (2000). "Ancient DNA Studies in Physical Anthropology". Annual Review of Anthropology, 29: 217-242.
Tamura, K., Stecher, G., Peterson, D., Filipski ,A., and Kumar, S. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0". Molecular Biology and Evolution 30: 2725-2729.

Vulpe, Alexandru (2001). "Epoca metalelor ", In: Petrescu-Dîmbovița, Mircea, Vulpe, Alexandru. (eds.), "Istoria Românilor, I. Moştenirea timpurilor îndepărtate", Bucureşti, 212-294.

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PAP TEST. PERFORMANCE AND LIMITS

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Keywords: Pap smear, cytodiagnostic, smear

ABSTRACT. Harvesting the smears and the cytodiagnostic interpretation represents the first step in diagnosing the pre-invasive pathology of the cervix. The study lot was made of 1476 patients who came for a specialty consult in two medical units in Iași, in the period of time between 2010 and 2015 and who were harvested Pap smears. The data was statistically processed in order to draw conclusions about the incidence of cervical benign pathology and the usefulness of its detection through this test. If we refer to the whole lot, the cytological results are satisfactory: 83% smears are within normal limits, 10.1% ASCUS, 2.7% L-SIL, 0.8% H-SIL. From all the feminine genital neoplasia, the cervical cancer is the easiest to detect, with low costs, as it benefits from very effective early diagnostic methods: cytology, HPV testing, colposcopy, and biopsy. We estimate that 50-75% of the results that are false negative are due to the harvesting errors.

INTRODUCTION

The cervical-vaginal cytology can be interpreted according to many classifications, starting with Babeș-Papanicolaou and ending with the Bethesda system, but no matter what interpretation way we choose, the quality of the smears and the cytologist’s experience play an important role, too (Davies P., et al., 2006). In conclusion we estimate that, due to the fact that the lack of interest and financial possibilities in our country do not allow an organized action of detecting cervical lesions nationwide, there may be a selection of the risk cases by performing a free Pap test to all the patients addressing a state or private specialized medical unit, provided they respect the protocol of harvesting cervical secretions, preparing the smears and reading them (Cox J.T., 2006).
The natural history of cervical cancer shows that the evolution of a pre-invasive lesion can last over 10 years until it turns into a malignant lesion. The detection of these lesions, followed by a correct treatment, leads to their healing in almost 100% of the cases. Thus, we can state that now cervical cancer is curable, as long it is detected early (Vlădăreanu R., 2006).

PURPOSE AND OBJECTIVES

Everyone admits that the first step in diagnosing the pre-invasive pathology of the cervix is the cytological smear. The routine harvesting performed during a specialized consult would be an important accomplishment, with a real diagnostic and prognostic importance.

MATERIAL AND METHODS

We are presenting a study that was performed on a sample of 1476 cases with cervical cancer, in the period of time between 2010 and 2015, who were harvested smears and were performed a Pap test. They were divided into two groups:

Group 1 – 277 patients with cervical cancer who were consulted at the Family planning clinic from Elena Doamna Clinical Hospital of Obstetrics and Gynaecology Iași as they came for contraceptive advice;

Group 2 – 1199 patients with cervical pathology were consulted without being hospitalized at Elena Doamna Clinical Hospital of Obstetrics and Gynaecology Iași.

The data were expressed under a form that allowed them to be centralized in SPSS 18.0 data bases and processed with appropriate statistical functions that will help formulate conclusions referring to the incidence of cervical benign pathology and the usefulness of its detection with this test.
Cervical-vaginal cytology can be interpreted according to many classifications, starting with Babeș-Papanicolau and ending with the Bethesda system, but regardless of the interpretation way, the quality of the smears and the experience of the cytologist play an important part. The cytologist must be up-to-date, to have the necessary skills and to have the moral and professional qualities that guarantee the quality of their work (Wright T.C.jr., et al., 2002).
The harvesting instruments are recommended to be the Ayre type wooden or plastic spatula (preferably for the xo-cervix and the posterior Douglas pouch) and Cervex-Brush for endo and exo-cervix.
The harvesting conditions must also be respected: prior treatment of inflammatory or local atrophic processes, no sexual intercourse, vaginal douches or digital vaginal examination 48 hours before harvesting. The harvesting should be done after

applying a speculum or two valves (which must be held by a help) showing without traumatizing the cervix (Schneider V., 2000).
The first harvesting is recommended to be done from the surface of the exo-cervix and from the posterior Douglas pouch, in order to avoid an eventual contamination with blood when the brush is used to harvest material from the endo and exo-cervix. Collected secretions are stretched on separate slides for the exo, endo cervix, and the posterior Douglas pouch, then they are fixed by immersing them in 95% alcohol or using spray with polyethylene-glycol (the habit of using air drying can cause cell shrinking). The recommended coloration is Papanicolaou, but the routine one is May-Grumwald Giemsa. (Mc Googan E., et al., 2001). Of course, it is mandatory to register and write a referral note to the lab.
Presently, the use of ThinPrep or Cyto-Rich techniques for preparing the slates in liquid, also called "display in monolayer or thin layer", which increase the sensitivity and specificity of the Pap test (Badea M., 2002), is not possible on a large scale because of the costs and also because of the lack of cyto-technicians and trained specialists. Some authors consider that the future belongs to automatic slide processing and interpreting (Schneider A et al., 1996; Obaid A.T., 2009; Willis B.H., et al., 2005).

RESULTS AND DISCUSSION

The distribution on age groups shows the following aspects (p=0.001):

- lot 1 (277 cases) – cervical pathology appeared mainly in patients aged between 20 and 30 years old (52.7%), followed by the age group 30-40 years old (31.4%);
- lot 2 (1199 cases) – the highest incidence of patients with cervical pathology was found for the age group 30-40 years old (30.5%), followed by the age group 40-50 years old (24.3%) and an incidence of 5.1% for women over 60 years old.

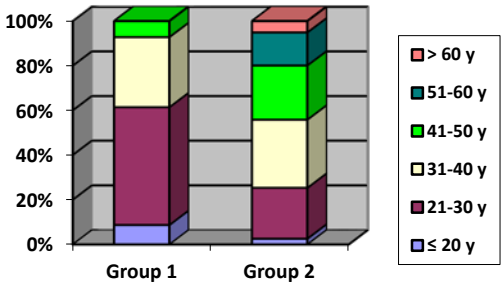

Fig. 1. The distribution of the patients with cervical pathology in study lots depending on age

The mean age was significantly higher for lot 2 (28.98 vs 39.24 y; p=0.05).

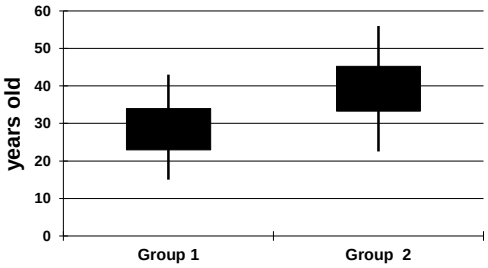

Fig. 2. Mean age of the patients with cervical pathology on study lots

The cases studied did not show significant differences in the social environment, most patients coming from cities and towns (69.7% vs 72.8%; p=0.783).

Cyto-bacteriological smears interpretation (CBS). We notice a higher incidence of coccobacillary flora for both study lots, without significant differences depending on the patient's age.

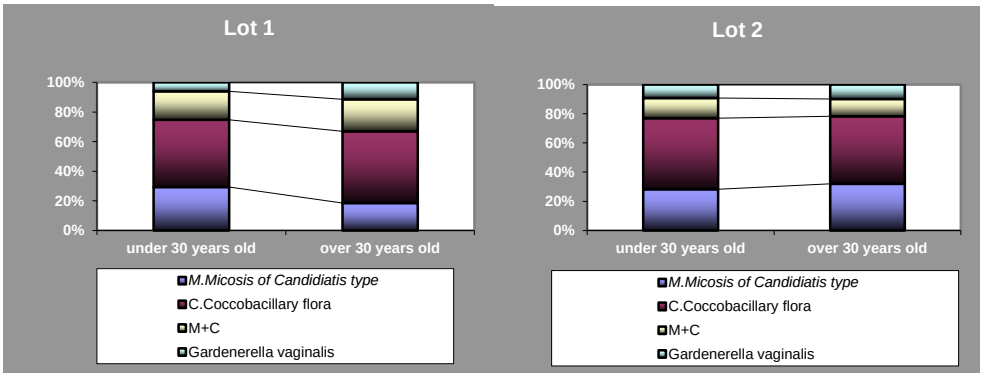

Fig. 3. The distribution of patients depending on the cyto-bacteriological smear

Distribution by cytodiagnostic

In group 1, 60% of the patients under 30 years old had a normal smear, whereas the patients over 30 years old showed a predominance of the benign cellular changes (51.4%) (χ^2=28.71; p<0.001).

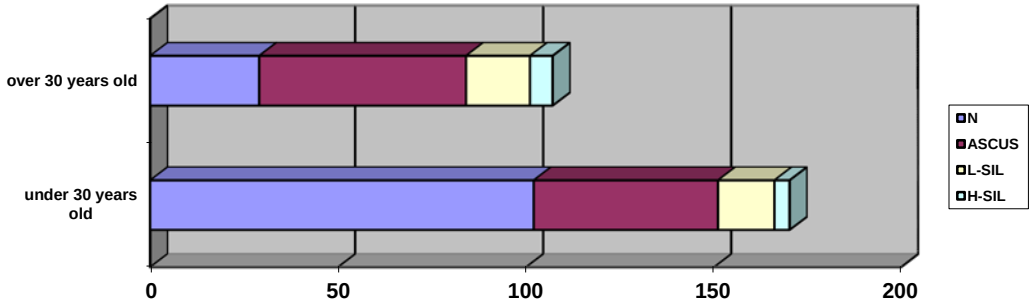

Fig. 3. Distribution of the patients in group 1 by cytodiagnostic

In group 2, 47% of the patients under 30 years old had a normal smear, whereas the patients over 30 years old had a predominance of benign cellular changes (51.7%) (χ^2=65.55; p<0.001).

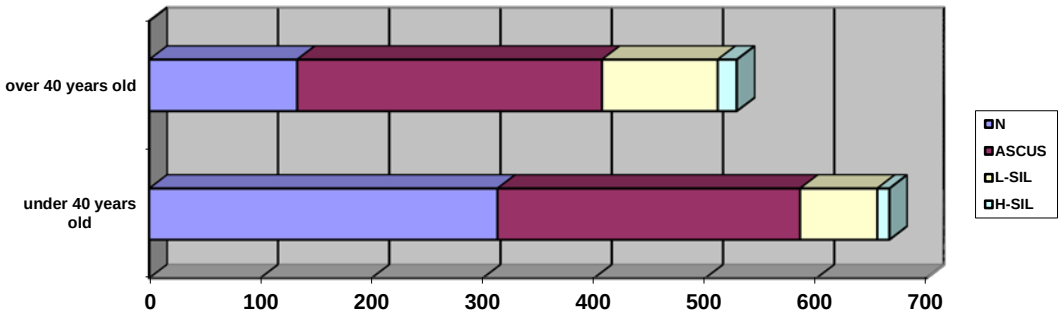

Fig. 4. Distribution of patients in group 2 by cytodiagnostic

The study lot raises a number of comments:

- patients in group 1, who came for a consult regarding contraception or contragestion, in the context of family planning, were young patients who did not show any relevant symptoms, but who were diagnosed with unknown cervical pathology after the consult in 44.8% of the cases (contraception 52.8%, contragestion 36.8%);
- 62.9% of the patients who were consulted for different genital symptoms without being hospitalized (group 2), showed cellular anomalies. The increased frequency might be explained by deficiencies in harvesting and preparing the slides. The fact that only 14.7% of the patients who came for a medical consult were harvested a Pap smear could be explained by patients' lack of interest in having this test done or by the patients' financial shortcomings, as the test must be paid for.

We consider that since the lack of interest and the poor financial possibilities in our country do not allow an organized nationwide activity of detecting cervical lesions, we can make a selection of the risk cases by performing a free Pap test to all the patients who come for a specialized consult in a state or private medical centre, considering they respect the harvesting, preparing the slides and interpreting protocol.

CONCLUSIONS

Based on the cases studied, there are significantly more patients with cervical pathology in the age group 21 to 30 years old and 31 to 40 years old.
The patients with cervical pathology came mainly from the urban area.
The cytological examination showed an increased presence of coccobacillary flora.
For the patients over 30 years old, the benign cellular changes are predominant.
From the feminine genital cancers, the cervical one is the easiest to detect, with low costs, benefiting from effective methods of early diagnostic: cytology, HPV testing, colposcopy, and biopsy. We estimate that 50-75% of the false negative results are due to harvesting errors.
The detection of pre-cancer lesion by screening causes an important decrease of cervical cancer, especially when referring to its advanced forms.

REFERENCES

Davies P., Arbyn M., Dillner J., et al. (2006) *A report on the current status of European research on the use of human papillomavirus testing for primary cervical cancer screening*. Int J Cancer, 118(4): 791-796.
Cox J.T. (2006) *Human Papillomavirus testing in primary cervical screening and abnormal Papanicolaou management*. Obstet Gynecol Sup, 61(6): 15-25.
Vlădăreanu R. (2006) *Obstercă şi Ginecologie Clinică*. Ed. Universitară „Carol Davila” Bucureşti, 367-371.
Wright T.C.jr., et al. (2002) *Consensus Guidelines for the mangement of cervical cytological abnormalitis*. JAMA, 287 (16): 2120-2129.
Schneider V. (2000) *Screening errors and reporting.In: Intrernational Consensus Conference on the fight against cervical cancer*. IUAC. Chicago, 89(6): 529.
Mc Googan E., et al. (2001) *Cell preparation methods and criteria for sample adequancy*. IAC Task Force Summary, 42(1):25-32.
Badea M. (2002) *Sinopsis de patologie preinvazivă*. Ed.Infomed. Bucureşti, 35(3): 176-179.
Obaid A.T. (2009) *Fiftin years after the International Conference on Population and Development: What have we achieved and how do we move forward?* Int J Gyn Obstetr,106: 102-105.
Schneider A., et al. (1996) *New adjunctive methods for Cervical Cancer Screening.* Obstet Gynecol Clin Amer, 23: 657.
Willis B.H., Barton P., Pearmain P., et al. (2005) *Cervical screening programmes: Can automation help? Evidence from systematic reviews, an economic analysis and a simulation modelling exercise applied to the UK.* Health Technol Assess, 9(13):1-236.

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CONTRIBUTIONS TO THE PHYTOCHEMICAL STUDY OF THE POLYPHENOLIC FRACTIONS SEPARATED FROM *THYMUS PULEGIOIDES* L. NATURAL POPULATIONS HARVESTED IN NORTHERN ROMANIA

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Keywords: *Thymus pulegioides* L., polyphenols, HPLC-UV
Abstract: *Thymus pulegioides* L. represents one of the plant species that are part of *Serpylli herba*, a drug used in therapeutics as a stomachic, carminative, expectorant and diuretic and also in nutrition as a flavouring agent. In our study we evaluated the quantitative determination of polyphenolcarboxylic acids and flavonoids from 24 samples of *T. pulegioides* collected from spontaneous flora in North-East Romania. We also aimed for the identification of some components by means of high-performance liquid chromatography. We detected the presence of a high chemical variability of the polyphenolic fraction going up to the level of the different bioactive components. To better control the pharmaceutical quality of the plant material it would be desirable to introduce this species into culture.

INTRODUCTION

Thymus L. is one of the largest genuses of the *Lamiaceae* family, which based on its high polymorphism and its large variety of culture sorts; it still presents some important taxonomic challenges. Some reference publications (Blashek et al., 2006; Heywood and Richardson, 1972) estimated the total number of species for this genus to be about 150 out of which 75 species belong to the European flora.

The *Thymus pulegioides* L. species belongs to the *Serpyllum* (Miller-Bentham) section, it grows spontaneously in the southern zone up to the high plateau of Abyssinia, in the North reaching Greenland and the European and Siberian Arctic, and in the East reaching the Kamchatka Peninsula and Japan.

As a pharmaceutical product, *T. pulegioides* is part of the *Serpylli herba* mixture drug, actually constituted of several species of *Thymus*, namely: *T. pannonicus* All., *T. austriacus* Bernh., *T. dacicus* Borbas, *T. marschallianus* Willd., *T. glabrescens* Willd. and *T. pulegioides* L. The product may contain up to 0.6% essential oil, its composition varying depending on the drug source. It also contains tannins of the labiates type up to 3% and flavonoids (Czygan, 1997; Wagner and Bauer, 1999). A recent paper (Shekarchi et al., 2012) published by a research group from Iran studying 29 species of lamiaceae plants out of which 4 species of *Thymus*, established one of the best methods for selective extraction of rosmarinic acid and its quantitative determination by the high-performance liquid chromatography (HPLC) technique. The authors have identified for *Thymus citriodorus* a significant high concentration of rosmarinic acid, a component with antioxidant and antimicrobial activity.

The essential oil from *Thymus pulegioides* received little attention in the past. However, in the last decade a few research groups have focused on different chemotypes that are present in spontaneous populations of wild thyme (De Martino et al., 2009; Groendahl et al., 2008; Loziene and Venskutonis, 2005; Loziene et al., 2003, Mockute and Bernotiene, 1999; 2001; 2005). Among these, there is a study that refers to the essential oil separated from *Thymus pulegioides* of Romanian origin (Pavel et al., 2010).

In 2012, a chemical and microbiological study on the volatile oil of *Thymus pulegioides* was published by Spanish authors (Pinto et al., 2006), who evidenced that the major components are carvacrol and thymol. Antifungal activity was investigated on seven clinical strains of *Candida*, five of *Aspergillus* and on five human dermatophytes. The essential oil of *Thymus pulegioides* has been proved to develop a clinically relevant inhibitory action of fungi. To investigate the mechanism of action the authors used the flow-cytometry technique to monitor the integrity of cytoplasmic membrane and the level of ergosterol for the studied fungi; the essential oil has damaged the membrane, reducing its ergosterol content.

The *Serpylli herba* product is used in traditional medicine as stomachic, carminative, expectorant, and in the treatment of bladder or kidney infections, but it is also used for food flavoring. As decoctions or tinctures, the plant product is used in phytobalneotherapy for the treatment of rheumatic pain; the tinctures can also be used in the massage therapy. In Romanian folk medicine (Butură, 1979), the mixture of wild thyme, comfrey (*Symphytum officinale*) and common hop (*Humulus lupulus*) was administered internally for common cold and rheumatism.

In this study we aimed to investigate the intraspecific and intrapopulational chemical variability of *Thymus pulegioides* L. specimens collected from northeastet of Moldavia region (Romania).

MATERIALS AND METHODS

Plant material

We collected 24 samples (June, 2012) consisting of the flowering aerial parts (*herba*) of *Thymus pulegioides*, originating from 12 villages (Vama, Valea Putnei, Lunca Putnei, Lunca Broșteni, Mădeni, Frumosu, Farcașa, Dreptu, Galu, Potoci, Doina Arini and Ortoia) from the northern Moldavia region of Romania. Depending on the size of the spreading area we collected one sample or more subpopulation samples. The plant material was collected from clearing sites close to forest edge. Voucher specimens were deposited at the herbarium of the "Stejarul" Biological Research Centre, Piatra Neamt, Romania.

Sample preparation

Powdered plant material (2.5g) was weighed accurately and extracted under reflux 3 times with methanol (30 mL), the extracts were combined into a 100-mL volumetric flask, and finally, the plant residue was washed with methanol, adjusting the volume to 100 mL. For each extract, the absorbance at 660 nm was measured 3 times spectrophotometrically, respectively, then each sample was submitted 3 times for HPLC analysis.

Instrumentation and chromatographic conditions

Thin-layer chromatography was prepared according to the method of H.Wagner and S.Bladt (1996). Spectrophotometric determinations were carried out using a Jenway 6300 VIS spectrophotometer. The spectrophotometric determination of the phenolcarboxylic acids was performed by treating the methanolic extracts in alkaline medium (natrium carbonate) with phosphowolframic acid, which coloured in blue the samples, the absorbance was measured at λ = 660 nm; the results were expressed as caffeic acid equivalents for the whole mass percent. The spectrophotometric determination of the flavonoids mainly aimed the capacity of these compounds to form intense yellow complexes in the presence of Al^{3+} cations, for which the absorbance is measured at λ= 430 nm; the results were expressed as rutoside equivalents for the whole mass percent (according to Romanian Pharmacopoeia).

The cromatographic analysis were carried out using a Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprised of a quaternary solvent delivery system, an on-line degasser, a column temperature controller and UV-photodiode array detector (DAD) coupled with an analytical workstation; Agilent Zorbax Eclipse XDB-C18 reverse-phase column (4.6 × 150 mm, 5 µm); column temperature: 30 °C; detection wavelength: 320 nm; flow rate: 1 mL/min; gradient elution: acetonitrile (solvent A) and 2 mM aqueous sodium acetate solution adjusted to pH 3.5 with glacial acetic acid (solvent B); the initial conditions were 2% A and 98% B; the linear gradient programme: 2-14%A in 20 min, 14-20%A in 20 min, 20-30%A in 10 min, 30-25%A in 10 min, after which we switched back to the initial conditions; sample injection (10 µL) was performed by an autosampler programme. Chromatographic peaks were confirmed by comparison of the values for retention time and the UV spectra of reference substances. Quantitative determination was finalized by means of external calibration method.

RESULTS AND DISCUSSIONS

The study of the polyphenolic fraction was carried out by thin layer chromatography for polyphenolic acids and flavonoids (Wagner and Bladt, 1996), spectrophotometric determination and HPLC analysis.

By thin layer chromatography we detected the presence of small quantities of flavonoid compounds, while the spots of phenolic acids were clearly outlined. The spectrophotometric determination has revealed the existence of intraspecific chemical variability for the polyphenolic fraction and also of intrapopulational variability if one compares each different subpopulations collected from Frumosu, Farcasa, Dreptu or Potoci localities.

Of the analyzed samples, the highest content in phenolic acids was detected in plant material collected from Ortoaia (sample 24), followed by a sample from Dorna-Arini (sample 23). The smallest concentrations were determined for samples from Farcasa (no. 4 and 5), for which we also had two subpopulations rich in this type of active principles.

The flavonoid content, as already observed by thin layer chromatography, was reduced, 6 of the 24 samples showing concentrations below 0.1%.

In order to determine the nature of some of the polyphenols present in the plant material we applied HPLC analysis, by which we could determine, based on available standards, the presence of rosmarinic acid, chlorogenic and caffeic acids, and also the flavonoids apigenol, apigenol-7-O-glucoside and luteolin (Table 1). The data reveals that rosmarinic acid is the component present in the highest amounts in the plant product, while apigenol-7-O-glucoside is the major flavonoid. Our determinations could not reveal the presence in the plant material of rutoside or luteolin-7-O-glucoside. In such case it should be required that the results of spectrophotometric determinations to be expressed as the found reference substances namely, rosmarinic acid and apigenol-7-O-glucoside for more accurate interpretation.

The existence of intraspecific and intrapopulational chemical variability was evidenced by means of spectrophotometry, and confirmed by HPLC analysis.

Table 1. Polyphenolic derivates identified in *Thymus pulegioides* L. samples by means of HPLC analysis

No.	**Population**	**mg/g dried plant material**					
		Chlorogenic acid	Caffeic acid	Rosmarinic acid	Apigenol -7-O-glucoside	Luteolin	Apigenol
1	Vama 1	0.55	0.07	10.36	0.07	0.05	0.08
2	Vama 2	0.20	0.06	12.46	0.07	0.06	0.12
3	Valea Putnei	0.09	0.08	20.16	0.39	0.16	0.13
4	Lunca Brosteni 1	0.09	0.07	18.08	0.34	0.13	0.19
5	Lunca Brosteni 2	0.05	0.06	13.50	0.29	0.11	0.10
6	Mădei	0.20	0.05	17.40	0.38	0.09	0.07
7	Frumosu 1	0.05	0.06	6.40	0.07	0.03	0.04
8	Frumosu 2	0.44	0.05	16.55	0.30	0.09	0.11
9	Frumosu 3	0.08	0.08	7.25	0.05	0.06	0.09
10	Fărcașa 1	0.05	0.05	8.02	0.07	0.05	0.13
11	Fărcașa 2	0.09	0.05	20.19	0.25	0.14	0.14
12	Fărcașa 3	0.14	0.04	16.34	0.22	0.07	0.09
13	Fărcașa 4	0.07	0.05	5.78	0.00	0.05	0.08
14	Fărcașa 5	0.05	0.06	4.19	0.01	0.05	0.14
15	Fărcașa 6	0.06	0.05	8.76	0.22	0.07	0.10
16	Fărcașa 7	0.10	0.06	7.40	0.06	0.09	0.14
17	Dreptu 1	0.23	0.06	15.40	0.24	0.07	0.07
18	Dreptu 2	0.10	0.06	9.76	0.18	0.05	0.07
19	Galu	0.09	0.05	6.53	0.05	0.09	0.16
20	Potoci 1	0.08	0.05	7.63	0.03	0.06	0.11
21	Potoci 2	0.15	0.05	7.85	0.04	0.06	0.05
22	Potoci 3	0.16	0.06	6.99	0.06	0.05	0.11
23	Dorna Arini	0.23	0.09	14.36	0.19	0.09	0.10
24	Ortoaia	0.15	0.08	16.93	0.42	0.14	0.11

By comparing the results of the two determinations, spectrophotometry *versus* HPLC (Table 2), we find that there is a large difference in terms of the values for the quantity assayed by spectrophotometry compared with the results obtained from HPLC analysis, which indicates that other polyphenolic derivates are present in significant amounts in the extracts.

Table 2**.** Polyphenol content determined spectrophotometrically compared with the total phenolic acids and flavonoids identified by HPLC

No.	**Population**	**Polyphenolcarboxylic acids [mg per g]**		**Flavonoids [mg per g]**	
		Spectro	**HPLC**	**Spectro**	**HPLC**
1	Vama 1	15.98	10.98	1.54	0.20
2	Vama 2	16.41	12.73	1.27	0.25
3	Valea Putnei	25.76	20.32	4.00	0.68
4	Lunca Broșteni 1	22.27	18.25	3.13	0.67
5	Lunca Broșteni 2	16.60	13.61	1.71	0.50
6	Mădei	23.82	17.65	3.39	0.54
7	Frumosu 1	13.49	6.50	0.65	0.15
8	Frumosu 2	22.50	17.04	3.27	0.50
9	Frumosu 3	15.75	7.42	1.47	0.20
10	Fărcașa 1	12.10	8.12	0.89	0.24
11	Fărcașa 2	20.40	20.33	3.81	0.52
12	Fărcașa 3	19.63	16.52	1.72	0.38
13	Fărcașa 4	9.85	5.90	0.56	0.14
14	Fărcașa 5	9.46	4.30	0.57	0.21
15	Fărcașa 6	15.28	8.88	1.61	0.39
16	Fărcașa 7	15.51	7.57	1.16	0.29
17	Dreptu 1	16.91	15.69	2.71	0.38
18	Dreptu 2	12.41	9.92	1.03	0.30
19	Galu	10.31	6.67	0.91	0.29
20	Potoci 1	13.18	7.76	0.76	0.19
21	Potoci 2	13.88	8.05	1.09	0.15
22	Potoci 3	10.47	7.20	1.03	0.21
23	Dorna Arini	24.52	14.68	3.62	0.38
24	Ortoaia	26.69	17.16	5.19	0.68
25	Pârâul Cârjei	21.26	16.87	3.23	0.41
26	Pârâul Pintei	16.06	7.21	1.24	0.25

The table shows that the samples from Galu (19) has the lowest content of phenolic acids determined by HPLC, while the plant population of Ortoaia (24) has the highest content. But if we try to ascertain the proportion by which we were able to identify by HPLC the components of phenolic acid type from the total amount determined spectrophotometrically, it presents similar percentages for the two samples: 64.66% in the first case and 64.30% in the second.

When comparing the subpopulations sampled from Vama, Lunca Brosteni, Dreptu, Potoci, Farcasa and Frumosu, the samples with the lowest values proved to be two from Farcasa (13.14), while the richest samples came from Frumosu (8) and Brosteni (4). For these samples, the identification of polyphenolics acids by HPLC as percentages from the total amount determined spectrophotometrically was approx. 60% for Farcasa and 76% for Frumosu, respectively, 82% for Lunca Brosteni.

By means of HPLC analysis we were able to quantify a low content of flavonoids, consisting of apigenol, apigenol-7-O-glucoside and luteolin. Similar to the phenolic acids determinations, the sample from Galu (19) has the lowest content in flavonoids, the total sum of the amount for the

compounds identified by HPLC accounting for only 32%. In the case of the sample collected from Ortoaia (24), it turns out to be among the richest in flavonoid content, yet the percentage of identification by HPLC is just 13%.

From the rest of the samples, the subpopulations of Frumosu and Farcasa stand out. Sample no. 7 representing a subpopulation of Frumosu has a low flavonoid content, the same as samples no. 13 and 14 collected from Farcasa. Calculating for these samples the recovery percent of the three flavonoid components identified, we found out that for the Frumosu sample the value is 23%, while for the Farcasa samples it is 24% and 36%, respectively.

The highest amount of flavonoids are assayed for one sample from Farcasa (11) and one from Frumosu (8), yet the identification proportion by HPLC, is only 14% and 15%, respectively. The results show that the samples analysed contain other flavonoid compounds in significant amounts.

Our data confirm our previous observation (Necula et al., 2011) that the biosynthesis level of polyphenolic derivatives varies within the *Thymus pulegioides* populations (probably depending on pedoclimatic conditions of the place of origin), as observed from determinations of total amounts as well as in the case of each individual component.

CONCLUSION

The phytochemical analysis carried out on polyphenolic fractions from samples of *Thymus pulegioides* L. collected from 12 localities from the north-east of Romania in the year of 2012 has demonstrated the existence, in the plant material sampled from the wild flora, of a high chemical variability concerning the level of bioactive compounds. Important components of the polyphenolic fraction are rosmarinic, caffeic and chlorogenic acids, apigenol, apigenol-7-glucoside and luteolin which also vary from a sample to another in large limits. In this situation, it would be important to cultivate the species in monitored agrotechnical conditions, to offer the patients a medicine vegetal product of a good quality.

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REFERENCES

Blashek W., Ebel S., Hackenthal E., Holzgrabe U., Keller K., Reichling J., Schultz V., (2006): *Hagershandbuch der Drogen und Arzneistoffe*, 7th Edition, Springer, Berlin, Germany

Butură V., (1979) “Enciclopedie de etnobotanică românească” (in Romanian), Editura Ştiinţifică şi Enciclopedică, Bucureşti, Romania, pp. 66-67

Czygan F.C., (1997): *Serpylli herba, in „Teedrogen und Phytopharmaka”*, edited by M. Wichtl, 3rd Edition, WFG, Stuttgart, Germany

De Martino L., Bruno M., Formisano C., De Feo V., Napolitano F., Rosselli S., Senatore F., (2009): *Chemical Composition and Antimicrobial Activity of the Essential Oils from Two Species of Thymus Growing Wild in Southern Italy*, Molecules, 14(11), 4614-4624

“Farmacopeea Română” (Romanian Pharmacopoeia), 10th Edition (in Romanian), Medical Publishing House, Bucureşti, Romania, 1993.

Groendahl E., Ehlers B.K., Keefover-Ring K., (2008): *A new cis-sabinene hydrate chemotype detected in large thyme (Thymus pulegioides L.) growing wild in Denmark*. J Essent Oil Res, 20, 40–41

Heywood V.H., Richardson I.B.K., (1972): *Labiatae, in „Flora Europeea“*, edited by T.G. Tutin, V.H. Heywood, N.A. Burges *et al.*, (Vol.3), Cambridge University Press, Cambridge, UK

Loziene K., Venskutonis P., (2005): *Influence of environmental and genetic factors on the stability of essential oil composition of Thymus pulegioides*, Biochem. Syst. Ecol., 33, 517–525

Loziene K., Vaiciuniene J., Venskutonis P.R., (2003): *Chemical composition of the essential oil of different varieties of thyme (Thymus pulegioides L.) growing wild in Lithuania*. Biochem Syst Ecol, 31,249-59

Mockute D., Bernotiene G., (1999): *The main citral-geraniol and carvacrol chemotypes of the essential oil of thymus pulegioides L. growing wild in vilnius district (Lithuania). J*. Agric. Food Chem 47(9), 3787-90

Mockute D, Bernotiene G., (2005): *Chemical composition of the essential oils and the odor of Thymus pulegioides L. growing wild in Vilnius*. J Essent Oil Res. 17, 415–418

Mockute D., Bernotiene G., (2001): *The alpha-terpenyl acetate chemotype of essential oil of Thymus pulegioides L.*, Biochem. Syst. Ecol., 29(1), 69-76

Necula R., Aprotosoaie A.C., Cioanca O., Gille E., Stanescu U., (2011): *Contributions to the study of the biochemical variability of the polyphenols existing in 8 Thymus Pulegioides populations from Northern Moldavia*; Bulletin of the Transilvania University of Brasov, Series VI: Medical Sciences; Vol. 4 (53); No. 2; 15-22.

Pavel, M., Ristic, M., Stevic, T. (2010): *Essentials oils of Thymus pulegioides and Thymus glabrescens from Romania: chemical composition and antimicrobial activity*, J. Serb. Chem. Soc. 75(1), 27-34

Pinto E., Pina-Vaz C., Salgueiro L., Gonçalves M.J., Costa-de-Oliveira S. *et al.*, (2006): *Antifungal activity of the essential oil of Thymus pulegioides on Candida, Aspergillus and dermatophyte species*. J. Med. Microbiol., 55(Pt 10),1367-73

Shekarchi M., Hajimehdipoor H., Saeidnia S., Gohari A.R., Hamedani M.P., (2012): *Comparative study of rosmarinic acid content in some plants of Labiatae family*. Phcog Mag, 8(29), 37-41

Wagner H., Bauer R., (1999): In *„Arzneidrogen und ihre Inhaltsstoffe“*, 6th Edition, WFG, Stuttgart, Germany, pp.108-110

Wagner H., Bladt S., “Plant Drug Analysis – A Thin Layer Chromatography Atlas”, 2nd Edition, Springer, Berlin, Germany, 1996.

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THE INFLUENCE OF SUBSTRATE AND TEMPERATURE ON LIPASE ACTIVITY IN SOME PLANT SPECIES

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Abstract. *The purpose of this work was to study the lipases coming from six plant sources, to see to what extent the substrate (oil) and/or temperature influence the activity of this enzyme, and which of these sources shows a higher activity. The biological materials, used as lipase sources (provided by Suceava Genebank and by Suceava Agricultural Research and Development Station), have been represented by seeds belonging to the following plant species: sunflower (Helianthus annuus L., hd. Rapid), pumpkin (Cucurbita maxima L., local variety), soy (Glycine max L., var. Turda 6114), peanut (Arachis hypogaea L., var. Dabuleni), corn (Zea mays L., hd. F 376), and walnut (Juglans regia L., var. Bratia). As substrates for enzyme activity, were used the following refined oils: sunflower, pumpkin, soy bean, peanut, corn, and walnut, purchased from supermarkets. The research method has consisted in titrating (with a solution of KOH 0.01 N) of fatty acids released from oils by lipase, in a certain time interval. At pH 5.4 and 20°C the lipase from sunflower seeds has registered the highest activity on soybean oil. At the same pH, but at 40°C, the lipase from walnut kernels has registered the highest activity on walnut oil. On its own substrate, the highest activity was registered, at 20°C, by walnut and sunflower lipase, while at 40°C by walnut lipase. It seems that a higher content of linoleic and linolenic acids of some oils (used as substrates), as well as the enzyme chemical composition and spatial configuration caused an increased activity of sunflower and walnut lipase, as compared to the other enzyme sources analyzed.*

INTRODUCTION

The lipases (triacylglycerol hydrolases, EC 3.1.1.3) are enzymes of the hydrolase class, esterase subclass, which catalyses the hydrolysis of glycerides releasing, finally, fatty acids and organic alcohols. Glycerides are most widespread in the animal kingdom and vegetable fat. They are substances spare that accumulate in fat tissue of animals and the plants, seeds (sunflower, castor, poppy, cotton, pumpkin) and fruit (olive, peanuts, almonds) (*Oprica, 2011*).

Lipases may have various origins (animal, vegetable or microbial), playing an important and vast application potential in: foods, detergents, oleochemicals, pharmaceuticals, fine chemistry, biodisel, cosmetics and fragrances, paper pulp, leather, biosensors and lipid-rich wastewater treatment (*Barros et al*., *2010; Donato et al., 2008; Enujiugha et al., 2004; Freire and Castilho, 2008; Gandhi, 1997; Hasan et al., 2006; Isbilir et al., 2008; Paques and Macedo, 2006; Pastore et al., 2003; Polizelli et al., 2008; Sharma et al., 2001; Yesiloglu and Baskurt, 2008*).

According to *Barros et al.* (2010), lipases are employed in food manufacturing to liberate fatty acids into food products by selective hydrolysis of the fats and oils present in many kinds of food. Depending on the carbon chain length and on the degree of unsaturation, the fatty acid obtained provides the food with flavors, colors and unusual smells, playing an important role in the physical-chemical, organoleptic and nutritional properties of many products (*Freire et al., 2008; Gandhi, 1997; Sharma et al.*, 2001) ct. by *Barros et al*. (2010).

The commercial lipases are generally produced from microorganisms (*Penicillium* spp., *Geotrichum* spp., *Aspergillus* spp., *Rhizomucor* spp., *Candida* spp. or *Pseudomonas* spp.) or animals, such as pancreatic and pregastric tissues of ruminants (*Baumann et al., 2000; Cardenas et al., 2001; Kilcawley et al., 2002; Steenkamp and Brady, 2003*).

The use of microbial lipases on an industrial scale is still restricted due to high production costs, favoring the search for other sources of these enzymes (*Paques and Macedo, 2006*). Seed lipases present advantages over animal and microbial lipases due to some quite interesting features such as: specificity, low cost, availability and ease of purification, representing a great alternative for potential commercial exploitation as industrial enzymes (*Paques and Macedo, 2006, Hellyer et al., 1999; Villeneuve, 2003; Enujiugha et al., 2004; Polizelli et al., 2008*).

Thanks to their many advantages, the seed lipases aroused last years an increasing interest among scientists. That is why, the purpose of this work was to study the lipases coming from six plant sources, to see to what extent the substrate (oil) and/or the temperature influences the activity of this enzyme, and which of these sources shows a higher activity.

MATERIALS AND METHODS

Research materials. The biological materials, used as lipase sources, provided by Suceava Genebank and by Suceava Agricultural Research and Development Station, have been represented by seeds (with moisture content of 10-12%), belonging to the following plant species: sunflower (*Helianthus annuus* L., hd. Rapid), pumpkin (*Cucurbita maxima* L., local variety), soy (*Glycine max* L., var. Turda 6114), peanut (*Arachis hypogaea* L., var. Dabuleni), corn (*Zea mays* L., hd. F 376), and walnut (*Juglans regia* L., var. Bratia). As substrates for enzyme activity, were used the following refined oils: sunflower, pumpkin, soybean, peanut, com, and walnut, purchased from supermarkets.

Research methods. Each *enzyme sample* was prepared in glass bottle with stopper where it mixed 2 g seeds finely divided with two sides ether, then let stand for 2 hours for oil extraction, stirring periodically. It has separated ether, and they have introduced again 6 parts ether over the product partly skimmed, for 2 hours, after which it was separated ether. The skimmed seeds were dried in an oven with fan, at a temperature of 28°C. There were obtained defatted seeds containing lipase.

Since many previous attempts to determine the lipase activity, carried out at different pH (5.4, 7.4 and 8.2) and at 20°C and 40°C, showed that at pH 5.4 were obtained the highest values, the lipase activity was determined at 20ºC and 40ºC, and at pH 5.4 for each temperature. The method has consisted in titrating (with a solution of KOH 0.01 N) of fatty acids released from oils by lipase, in a certain time interval (*Bordei et al., 2007*). The lipase activity (LA) was expressed as fatty acid micromols (µmol), represented by oleic acid, formed, as result of enzyme action, from a gram of product, in one minute

Statistical analysis. The data of experiments, consisting in 4 replicates for each determination, were statistically processed using SAS Version 8.02 (*SAS Institute, 2005*). In orer to analyse the significance of differences among samples, generalised linear model analysis was carried aut, and for multiple comparisons was used Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSIONS

Tables 1-6 reproduce the lipase activity of the six seeds species on the six various substrates (oils).

Table 1. Lipase activity (mean values ± SD) of sunflower seeds on different substrates

SF lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	6.3± 0.8ab*	5.18± 0.39c*	8.70± 0.69a	6.58± 0.47ab	6.64± 0.08ab	7.35± 1.07ab
LA (µmol oleic acid/g/min.)	40°C	5.4	7.02± 0.83ab	5.64± 0.38c	6.9± 1.05ab	7.25± 0.78ab	6.34± 0.62ab	6.63± 0.71ab

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soy bean; PN=peanut; CN=corn; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent ($P<0.05$).

As seen in table 1, at 20°C and pH 5.4 sunflower lipase has registered significant differences ($P<0.05$) between its activity on soybean oil (with highest value), and the activity on walnut, corn, peanuts and sunflower oils (with close values).

At the same pH, but at 40°C, the highest values of sunflower lipase activity were registered, in order, on peanut, sunflower and soybean, corn and walnut oils (with close values), followed by pumpkin ($P<0.05$).

Table 2. Lipase activity (mean values ± SD) of pumpkin seeds on different substrates

PK lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	4.4± 0.32c	4.36± 0.52c	4.35± 0.36c	5.3± 0.42c	4.7± 0.48c	5.37± 0.55c*
LA (µmol oleic acid/g/min.)	40°C	5.4	4.68± 0.41c	3.72± 0.3cd	5.02± 0.45c	4.2± 0.53cd	5.1± 0.45c	6.36± 0.5ab*

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soy bean; PN=peanut; CN=com; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent (P<0.05).

At 20°C and pH 5.4, the activity of lipase from pumpkin seeds has registered no significant differences between samples, even if the highest activity was on walnut and peanut oils, followed by corn, sunflower, pumpkin (own substrate) and soybean (Table 2).

From the same Table 2, one can see that at 40°C the pumpkin seeds lipase had the highest activity on walnut oil, followed by soybean, corn, and sunflower oils, the lowest activity being on peanut and pumpkin oils (P<0.05).

Table 3. Lipase activity (mean values ± SD) of walnut kernels on different substrates

WN lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	6.16± 0.68ab*	6.18± 0.5ab	5.35± 0.52c*	4.93 ±0.4c	5.7± 0.65c	6.35± 0.48ab
LA (µmol oleic acid/g/min.)	40°C	5.4	6.2± 0.64ab	6.59± 0.5ab	5.86± 0.63c	6.1± 0.5ab	6.23± 0.55ab	7.98± 0.67a

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=com; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent (P<0.05).

In the Table 3. at 20°C, and pH 5.4, the lipase from walnut (kernels) had the highest activity on walnut, pumpkin and sunflower seed oils, where it recorded significantly higher values, compared to corn, soybean and peanut seed oils (P<0.05).

At 40°C the walnut lipase registered the highest activity on its own substrate (walnut oil), followed by a significant lower activity on: pumpkin, corn, sunflower and peanut oils (P<0.05).

Table 4. Lipase activity (mean values ± SD) of peanut seeds on different substrates

PN lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	4.25± 0.47cd*	4.9± 0.42c*	4.36± 0.5cd	5.27± 0.44c	4.7± 0.48cd	5.41± 0.63c
LA (µmol oleic acid/g/min.)	40°C	5.4	5.12± 0.43c	5.36± 0.55c	4.7± 0.44cd	6.14± 0.69 ab	5.15± 0.4c	4.37± 0.42cd

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=com; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent (P<0.05).

As seen in Table 4, at pH 5.4 and 20°C the peanut seeds lipase activity has registered significant higher values on walnut, peanut and pumpkin oils, as compared to corn, soybean, and sunflower oils (P<0.05).

At 40°C, the highest value of peanut lipase activity was recorded on peanut oil (own substrate), followed by pumpkin, corn, sunflower, soybean and walnut oils (P<0.05).

Table 5. Lipase activity (mean values ± SD) of corn caryopses on different substrates

CN lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	4.68± 0.52c	3.82± 0.4cd*	4.7± 0.38c*	4.61± 0.54c	4.38± 0.26c	5.4± 0.63c
LA (µmol oleic acid/g/min.)	40°C	5.4	5.03± 0.58c	4.7± 0.42c	4,1± 0.45cd	4.7± 0.39c	5.36± 0.7c	3.68± 0.4cd

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=corn; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent (P<0.05).

Analysing the data of Table 5, one can see that, at 20°C, and pH 5.4, the activity of corn lipase has registered values without significant differences on: walnut, sunflower, soybean, peanut, and corn oils, as compared to pumpkin oil where its activity was lower (P<0.05).

At 40°C and pH 5.4, the corn lipase had the highest activity on corn, sunflower, pumpkin and peanut (with close values), as compared to soybean and walnut oils (P<0.05).

Table 6. Lipase activity (mean values ± SD) of soybean on different substrates

SB lipase activity	Temp.	pH	Refined oils					
			SF	PK	SB	PN	CN	WN
LA (µmol oleic acid/ g/min.)	20°C	5.4	3.75± 0.29cd*	3.8± 0.35cd	3.4± 0.31cd	4.73± 0.44c*	4.1± 0.38cd	4.03± 0.47cd
LA (µmol oleic acid/g/min.)	40°C	5.4	4.02± 0.35cd	4.77± 0.28c	3.6± 0.32cd	3.36± 0.41cd	4.9± 0.51c	5.03± 0.48c

SD=standard deviation; LA=lipase activity; SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=corn; WN=walnut; Temp = temperature ;*Means with different letters are statistically diferent (P<0.05).

In the Table 6, at 20°C and pH 5.5, the soybean lipase had the greatest activity on peanut oil, followed by the other five oils analyzed (including its own substrate), which registered close values, but significantly lower (P<0.05).

At 40°C the soybean lipase registered the highest activity on walnut, corn and pumpkin oils (with close values), followed by sunflower, soybean and peanut oils with significantly lower values (P<0.05).

Fig. 1 reproduces the comparative evolution, at pH 5.4 and 20°C, of the lipase activity from sunflower, pumpkin, soybean, peanut, corn and walnut on the six analyzed substrates (oils).

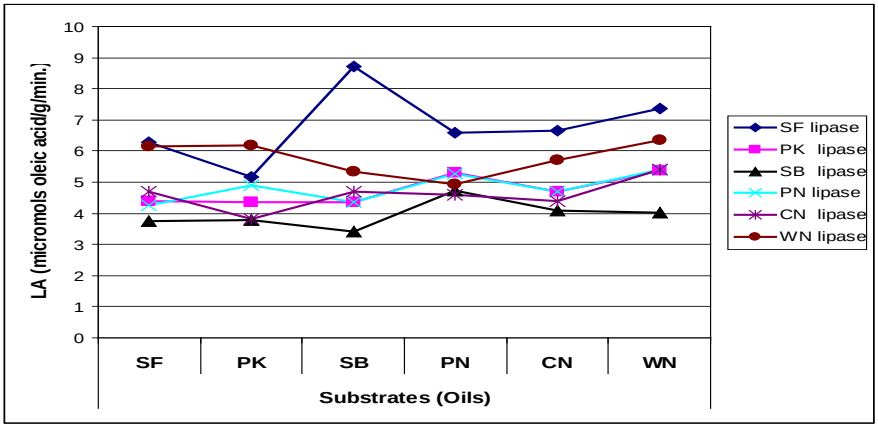

Fig. 1. The evolution of lipase activity values from sunflower, pumpkin, soybean, peanut, corn and walnut, at pH 5.4 and 20°C, on the six analyzed substrates (oils)
SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=corn; WN=walnut

As seen in Fig. 1, at 20°C, compared to the other enzyme sources, the sunflower lipase (on sunflower, soybean, peanut, corn and walnut oils), and walnut lipase (on sunflower, pumpkin and walnut oils), had higher activities, the highest one being registered (by sunflower lipase) on soybean oil.

On its own substrate, the highest activity was registered by walnut and sunflower lipase, with close values, followed by corn, peanut and pumpkin lipase, also with close values, but significant lower ($P<0.05$).

Fig. 2 reproduces the comparative evolution, at pH 5.4 and 40°C, of the lipase activity from sunflower, pumpkin, soybean, peanut, corn and walnut on the six analyzed substrates (oils).

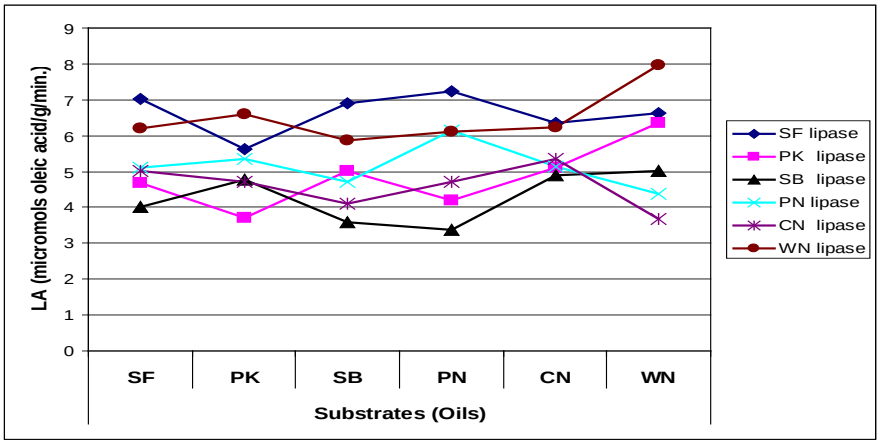

Fig. 2. The evolution of lipase activity values from sunflower, pumpkin, soybean, peanut, corn and walnut, at pH 5.4 and 40°C, on the six analyzed substrates (oils)
SF=sunflower; PK=pumpkin; SB=soybean; PN=peanut; CN=corn; WN=walnut

In the Fig. 2 one can see that, at 40°C, compared to the other enzyme sources, the sunflower lipase (on sunflower, soybean and peanut oils), and walnut lipase (on pumpkin and walnut oils), had higher activities, the highest one being registered by walnut lipase on walnut oil.

On its own substrate, the highest activity was registered by walnut lipase, followed by sunflower and peanut lipase (with close values), but significant lower ($P<0.05$).

Since, under the same conditions of pH, temperature and substrates, the lipases from the other sources (PK, SB, PN and CN) had enzyme activities significantly lower, compared to those of sunflower seeds and walnut kernels, this could be correlated either with chemical composition of analyzed substrates (oils), or with chemical composition and spatial configuration of the enzyme itself, or with the both.

As to chemical composition of substrates, according to some data on vegetable refined oils, published in methods, standards and scientific works (*Orthoefer, 1996; AOCS, 1997; Firestone, 1999; Codex Alimentarius, 1999; Grompone, 2011; Kochlar Prakash, 2011; Tong Wang, 2011*), the linoleic acid content varies between 14 and 74 (wt%), with higher values in sunflower, walnut, soybean, corn and pumpkin oils, and lower ones in peanut, sesame, and almond oils. By the same authors, the linolenic acid ranges between 0,5 to 14 (wt %), with a higher quantity in walnut oil, followed by soybean and sunflower oils, while the oleic acid between 17 and 67 (wt%), with higher values within peanut, sesame and pumpkin oils, and less in corn, sunflower, almond, soybean and walnut.

Thus, an increased content of linoleic and linolenic acid were the substrate (soybean oil) where sunflower seeds lipase had the highest activities at pH 5.4 and 20°C.

It seems that, at 40°C, the chemical composition of oils influenced the lipase activity of the sources analyzed. Thus, the increased content in linoleic and linolenic acids in walnut kernels oil made the lipase of walnut kernels to register the highest enzyme activity.

CONCLUSIONS

Analysing the activity of lipases coming from six plant sources (sunflower seeds, pumpkin seeds, soybean, peanut seeds, corn caryopses and walnut kernels), on six refined oils used as substrates (sunflower, pumpkin, soybean, peanut, corn, and walnut), at pH 5.4, and temperatures of 20°C and 40°C, it could find out a relation between oils chemical composition and the lipase activity.

As compared to the other sources, at pH 5.4 and 20°C the lipase from sunflower seeds has registered the highest activity on soybean oil. At pH 5.4 and 40°C the lipase from walnut kernels has registered the highest activity on walnut oil.

On its own substrate, the highest activity was registered, at 20°C**,** by walnut and by sunflower lipase, and at 40°C by walnut lipase.

It seems that a higher content of linoleic and linolenic acids of some oils (used as substrates), as well as the enzyme chemical composition and spatial configuration caused an increased activity of sunflower and walnut lipase, as compared to the other enzyme sources analyzed.

REFERENCES

AOCS (1997) - *Physical and chemical characteristics of oils, fats and waxes*, in Official Methods and Recommended Practices of the American Oil Chemists' Society, AOCS Press, Champaign, IL

Barros M., Fleuri L.F., Macedo G.A. (2010) - *Seed lipases: sources, applications and properties - a review*, Braz. J. Chem. Eng. vol. **27**, no. 1, São Paulo, Jan./Mar. 2010, On-line version ISSN 0104-6632, http://dx.doi.org/10.1590/S0104-66322010000100002 (2010

Baumann M**.,** Hauer B.H. and. Bornscheuer U.T (2000)- *Rapid screening of hydrolases for the enantioselective conversion of "difficult-to-resolve" substrates*, Tetrahedron: Asymmetry **11**, pp. 4781-4790

Bordei D. (coord.), Bahrim G., Pâslaru V., Gasparotti C., Elisei A., Banu I., Ionescu L., Codină G .(2007) - *Controlul calităţii în industria panificaţiei. Metode de analiză,* Ed. Academica, Galaţi, pp. 280-281
Cardenas F**.,** de Castro M.S., Sanchez-Montero J.M.,. Sinisterra J.V, Valmaseda M., Elson S.W. (2001) - *Novel microbial lipases: catalytic activity in reactions in organic media,* Enzyme Microb. Technol. **28** pp. 145-154
Codex Alimentarius (1999) (FAO/WHO) - *Codex Standard for named vegetable oils*. CODEX STAN 210 1999 (revision and amendments: 2003, 2005)
Donato A.G.A., Antunes O.A.C., Freire D.M.G., Lago R.C.A., Cavalcanti E.D.C. and Sousa J.S. (2008) - *Produção de Ácidos Graxos Catalisada por Lipases não Purificadas de Sementes ou Frutos Vegetais para Subseqüente Esterificação por Catálise Ácida.* PI 0603824-7 A
Enujiugha V.N., Thani F.A., Sanni T.M. and Abigor R.D.(2004) - *Lipase Activity in Dormant Seeds of the African oil bean (Pentaclethra macrophylla* Benth*)*, Food Chemistry, **88**, No. 3, 405
Firestone D. (ed.) (1999) - *Physical and Chemical Characteristics of Oils, Fats and Waxes*, AOCS Press, Champaign, IL
Freire G.D.M. and Castilho F.L. (2008) - *Lipases em Biocatálise. In: Bon et al. (org). Enzimas em biotecnologia: Produção, Aplicação e Mercado,* Rio de Janeiro, Interciência
Gandhi N.G. (1997) - *Application of Lipase,* Journal of the American Oil Chemists' Society, **74**, No. 6, 621
Grompone A. Maria (2011) - *Sunflower oil*, in *Vegetable Oils in Food Technology, Composition, Properties and Uses,* (ed. Gunstone F.D.), 2nd edn., John Wiley & Sons, Ltd., Publication, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK, p. 140
Hasan F**.,** Shah A.A., Hameed A. (2006) - *Industrial Applications of Microbial Lipases. Enzyme Microbial and Technology,* **39**, No. 2, 235
Hellyer S.A., Chandler I.C. and Bosley J.A. (1999) - *Can the Fatty Acid Selectivity of Plant Lipases be Predicted from the Composition of the Seed Triglyceride?,* Biochemica et Biophysica Acta, **1440,** No. 2-3, 215
Isbilir S.S., Ozcan, M.H. and Yagar, H. (2008) - *Some Biochemical Properties of Lipase from Bay Laurel (Laurus nobilis* L*.) Seeds*. Journal of the American Oil Chemists' Society, **85,** No. 3, 227
Kilcawley K.N**.**, Wilkinson M.G. and Fox P.F. (2002) - *Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources*, Enzyme Microb. Technol. **31**, pp. 310-320
Kochlar Prakash S. (2011) - *Minor and speciality oils* in *Vegetable Oils in Food Technology, Composition, Properties and Uses,* (ed. Gunstone F.D.), 2nd edn., John Wiley & Sons, Ltd., Publication, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK, pp. 293-294; 320-321; 329-330
Oprica L. (2011) - *Biochimia produselor alimentare*, Ed. Tehnopress, p.114.
Orthoefer F.T. (1996) - *Vegetable oils*, in *Bailey's Industrial Oil and Fat Products, Vol 1: Edible Oil and Fat Products: General Applications* (ed. Y.H. Hui), 5th edn, John Wiley & Sons, Inc., New York, pp. 19–44
Paques F.W. and Macedo G.A. (2006) - *Lipases de Látex Vegetais: Propriedades e Aplicações Industriais: A Review*. Química Nova, **29**, No. 1, 93
Pastore M.G., Costa V.S. and Koblitz M.G.B. (2003) - *Purificação Parcial e Caracterização Bioquímica de Lipase Extracelular Produzida por Nova Linhagem de Rhizopus* sp. Ciência e Tecnologia de Alimentos, **23**, No. 2, 135
Polizelli P.P. Tiera M.J. and Bonilla-Rodriguez G.O. (2008) - *Effect of Surfactants and Polyethylene Glycol on the Activity and Stability of a Lipase from Oilseeds of Pachiraaquatica*. Journal of the American Oil Chemists' Society, **85**, No. 8, 749
SAS Institute, *SAS User's Guide* (2005) - *Statistical Analysis System Institute*, Cary, NC
Sharma R, Chisti Y., Banerjee U.C. (2001) - *Production, Purification, Characterization, and Applications of Lipases.* Biotechnology Advances, **19**, No. 8, 627
Steenkamp L. and Brady D. (2003) - *Screening of commercial enzymes for the enantioselective hydrolysis of R,S-naproxen ester*, Enzyme Microb. Technol. **32**, pp. 472-477
Tong Wang (2011) - *Soybean oil*, in *Vegetable Oils in Food Technology, Composition, Properties and Uses,* (ed. Gunstone F.D.), 2nd edn., John Wiley & Sons, Ltd., Publication, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK, p. 60
Villeneuve P. (2003) - *Plant Lipases and Their Applications in Oils and Fats Modification*, European Journal of Lipid Science and Technology, **105**, No. 6, 308
Yesiloglu Y. and Baskurt L. (2008) - *Partial Purification and Characterization of Almond Seed Lipase.* Preparative Biochemistry & Biotechnology, **38**, No. 4, 397

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SECTION OF MOLECULAR INTERACTIONS IN THE LIVING WORLD

ABSTRACTS

ANALELE STIINTIFICE
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SEC. II A.
GENETICA SI BIOLOGIE MOLECULARA

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Hall B 339: $12^{00} – 14^{30}$; $16^{00} – 19^{00}$

Moderators:
Senior Researcher I dr. Pincu ROTINBERG
Lecturer dr. Daniela NICUTA

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A PARTICULAR DIFFERENCE BETWEEN HEAG AND HERG CHANNELS COULD PROVIDE A SPECIFIC WAY OF TARGETING ION CHANNELS IN CANCER THERAPY

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The voltage-dependent hEAG and hERG ion channels are members of the same family of potassium channels involved primarily in cellular excitability, having important and potential different roles in regulating cancer cells functions. Due to the high structure similarity of their transmembrane pore-forming domains, hEAG blockers also reduce the hERG conductance, leading to cardiac side effects. A specific blocker of hEAG has not yet been found, although such a compound should have important antitumor activity, as hEAG experimental blockade leads to the inhibition of tumour cells growth and functions. In this study we present a correlation found between two different independent studies which shows that clofilium, an antiarrhythmic agent, has a high potential of being used as a lead compound to find a specific hEAG blocker. The correlation shows that a particular region between the S6 and P helices could provide an alternative binding mode for clofilium derivatives in order to specifically target the hEAG ion channel

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INVESTIGATION OF ANTIOXIDANT POTENTIAL OF SOME PROANTHOCYANIDIN EXTRACTS OBTAINED FROM *VITIS VINIFERA* SEEDS

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Vitis vinifera grape seeds - waste from wine industry - with their high polyphenolic content, represents a generous source for recovery of some bioactive phytocompounds with biomedical, agricultural and ecological capitalization. Among polyphenolic compounds, the proanthocyanidines are chemical structures useful in the detoxification of carcinogenic metabolites, in the scavenging of free radicals, their antioxidant capacity being more effective than other oxidative stress scavengers, as vitamins C, E and beta-carotene. The high throughput technology developed at SCDVV Iasi has led to the separation (by fraction the polyphenolic crude extracts obtained from grape marc after oil removal), the biological characterization of two final bioactive proanthocyanidin phytoproducts and, consequently, waste valorification. The interaction of the fractionated proanthcyanid in phytoproducts with the cell viability, cell apoptosis and cell cycle progression in neoplastic HeLa and Vero normal cells was evaluated in our previous investigations, proving their cytotoxic and/or cytostatic effects with a higher impact on the cancerous cells. Antioxidant property of proanthocyanidin biopreparations ProF-l.f. (laboratory form) and ProF-m.f. (micropilot form) was evaluated in neoplastic HeLa and normal Vero cells by DCFH-DA assay (flow cytometry) and by biochemical assessment of activity levels of catalase, glutathione peroxidase and superoxiddismutase scavenging enzymes. The proanthocyanidin extracts have reduced the levels of the reactive oxygen species, antioxidant potential of the ProF-l.f. beeing slightly higher that of ProF-l.m. both on neoplastic and normal cell cultures. Also, ProF-l.f. was more efficient on neoplastic cells as compared with the normal cells, but the differences weren’t very high. Investigation of the antioxidant enzymes status has revealed a significant increase in the activity of GPx and SOD in HeLa cell cultures with a minor decrease of CAT, while in the normal cells activity of all enzymes was amplified. Amplitude of the enzymatic activity was higher in Vero cells as compared with HeLa cells as well as ProF-l.m. impact on the enzymatic activity was slightly higher than ProF-l.f. Tested biopreparations have exerted an antioxidant effect both in neoplastic and normal cells. By modulating of the ROS scavenging enzymes activity, ProF-l.m. and ProF-l.f. were behave as antioxidant optimizers.

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A GENETICALLY ENGINEERED *ARTHROBACTER NICOTINOVORANS* STRAIN FOR IMPROVED PRODUCTION OF 6-HIDROXY-NICOTINE

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Introduction. The aerobic soil bacterium *Arthrobacter nicotinovorans* is able to utilize D L-nicotine as the sole growth substrate. The ability of this microorganism to metabolize the highly toxic tabacco alkaloid nicotine is linked to the presence in the bacterial cells of the 160 kb catabolic plasmid pAO1. The metabolic intermediate 6-Hydroxy-Nicotine (6HNic) produced by *Arthrobacter nicotinovorans* pAO1 when grown on nicotine containing medium was shown to bind to nAChRs, and by modulating their function, to sustain spatial memory formation in a rat model of Alzheimer’s disease. This paper presents data on the first attempts to produce and isolate 6HNic using a genetically engineered *A. nicotinovorans* pAO1 strain.

Materials and Methods. The growth, the nicotine consumption and the 6HNic accumulation in a nicotine containing medium were compared for two strains: *A. nicotinovorans* pAO1 wild type strain (wt) and a genetically engineered *A. nicotinovorans* pAO1 strain (pART2NDH) containing the genes nicotine-dehydrogenase (NDH) cloned in the nicotine inducible pART2 expression vector. The bacterial growth curves were fallowed spectrophotometrically. The consumption of nicotine and accumulation of 6HNic in the growth medium was quantified by HPLC using a reverse phase column and as mobile phase 1 mM sulfuric acid:methanol 75:25 at a flow rate of 1 ml/min.

Results and Discussions. In the wt strain, the nicotine is quickly depleted from the medium and only low amounts of 6HNic are observed. In case of pART2 NDH, the overexpression of NDH allowed a 5 fold accumulation of 6HNic in the growth medium. From the Brenda database, several inhibitors for 6HLNO – the downstream enzyme in the nicotine metabolic pathway - were selected: methylene blue (MB), $HgCl_2$ and $ZnSO_4$. As the later compound gave the best results in terms of 6HLNO inhibition, different concentrations of $ZnSO_4$ were tested in order to identify the best conditions for a higher accumulation of 6HNic.

Conclusions. The genetically engineered *A. nicotinovorans* pART2NDH strains provides a feasible biotechnological method for 6HNic production. As expected, the usage of chemical inhibitors for the downstream enzyme in the nicotine metabolic pathway increased the 6HNic levels in the growth medium of the strain.

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INTERACTIONS OF ROUNDUP PESTICIDE UPON SOME PHYSIOLOGICAL PROCESSES IN RYE SEEDLINGS

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Extensive use of pesticides might be unreasonable risk to humans, animals, non-target plants, and the environment. Myers et. al. stated that, since the late '70s until 2016, production of glyphosate-based herbicides (GBHs) was more than 100-fold increase, in response to the unprecedented global emergence and wide spread of glyphosate resistant weeds. To understand the phytotoxic effects of some pesticides on crop plants, in this study we aimed to determine the effects of roundup, an organophosphonate herbicide, upon the main physiological processes in rye seedlings. The study was conducted to determine the germination indicators (energy and germinal faculty), the foliar photosynthetic pigments (chlorophyll a and b, and also carotenoids), the intensity of photosynthesis and respiration, dry matter content and total mineral elements, assessed in the fourteenth day of ontogenetic development. *Secale cereale* cv. Flora caryopses were treated for 6 and 12 hours with 0.1%, 0.2%, 0.5%, and 1.0% Roundup solutions (v/v), containing 0.36 mg/ml, 0.72 mg/ml, 1.8 mg/ml, and 3.6 mg/ml glyphosate (a phosphanoglycine active ingredient). At low concentrations of Roundup (0.1% and 0.2%), energy and germinal faculty showed an insignificant decrease, both after 6 hours and 12 hours of treatment. The highest concentration of herbicide (3.6 mg/ml glyphosate) has induced a decline of germination approximately 1.9-2.2 times compared to the control. The amount of the assimilating pigments decreased, regardless of concentration or duration of treatment, and the ratio of them (a/b and a+b/c) was lower than control, proving the existence of an effect that generate reactive oxygen species. There is a direct dose-response relationship regarding the photosynthesis and respiration processes. Inhibition of photosynthesis reported in case of other fungicides is associated with reduction of stomatal conductance and transpiration, prove affecting of photosystem II activity (Bigot et al., 2011). Similar manifestation presents transpiration activity that declined after Roundup treatment. Photosynthesis and transpiration are directly interlinked affecting plant growth and productivity. Regarding dry matter, the inhibitory effect was maintained in all dilutions and pesticide exposure time, being correlated with the corresponding decrease in the biomass production. Also, the rate of mineral elements synthesis increased and total nitrogen content decreased.

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EVALUATION OF DNA SINGLE- AND DOUBLE-STRAND BREAKS INDUCTION IN FIBROBLAST CELLS AFTER PHOTON IRRADIATION USING ALKALINE AND NEUTRAL COMET ASSAY

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Single-Cell Gel Electrophoresis – SCGE or Comet Assay (CA) is a sensitive, very cheap and rapid technique for quantifying and analyzing DNA damage in individual cells, being one of the most used methods in the cancer research area for evaluation of genotoxicity and chemoprevention effectiveness (Srinivasan et al., 2007). One of the main objectives of this study is establishing of the genetic apparatus reactivity in mammalian cells to the photons action. This goal was achieved by assessing the degree of DNA damage, as a consequence of photon irradiation, using alkaline and neutral comet assay. Alkaline version is suitable for radiobiological studies because it has high sensitivity for detecting several types of lesions: single-stranded breaks (SSBs), double-strand breaks (DSBs), alkali-labile sites (ALSs) and incomplete excision repair sites (IERSs). On the other hand, the neutral comet assay reflects mainly double-strand breaks (DSBs). In our experiment, the cells were seeded in 25 cm2 cell culture flasks at an initial density of 3 x 105 cells /flask. After 24 hours from the initiation of the cultures, when the cells have 85-90% confluence realizing the monolayer, the cells were counted and distributed in Eppendorf tubes (1x106 cells/tube, 3 tubes/variant), and then, exposed to the photon flux (1Gy, 2 Gy, 3Gy and 5 Gy). Sham irradiated cells were used as control. The comets were stained with ethidium bromide dye. One hundred comets were analysed from each of three slides/variant using CASP 1.2.2 software (CASP or Comet Assay Software Project, http://www.casp.sourceforge.net). The percent of the DNA in the head decreased varying between 97.92 (control) to 97.26 (2 Gy), without evidence of a dose-effect relationship. Instead, percent of DNA in the tail presented a linear increase from 1 Gy to 3 Gy (2.35-3.25). "Tail moment" parameter, which is the product of multiplication between tail length and the total DNA fractionated in the tail, increased from 0.50 (control) to 3.29 (5 Gy), revealing accumulation of a large number of fragments (single and double DNA breaks) of information material. This index provides the most stable estimates for DNA damage because it has a large degree of uniformity in the fragments dispersion. As expected, the highest mean of tail moment, evaluated using neutral Comet assay, was shown at 3Gy and 5Gy, respectively indicating a large percent of double strand breaks.

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ARTHROBACTER PLASMIDS: MOLECULAR CLASSIFICATION AND CONSERVED GENE CLUSTERS

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Members of the *Arthrobacter* genus are ubiquitous in polluted and toxic soil samples. Despite their potential in environmental biotechnologies, theirs practical applications are hampered due to the scarce availability of useful tools for genetic engineering. More than a decade have past since the sequencing of the most known *Arthrorobacter* plasmid – pAO1, but very little in known about the core functions - replication and partition of *Arthrobacters* plasmids.

In this study, the available *Arthrobacter* plasmids sequences were analyzed by BLAST in order to identify their putative replication origin. Gene syntheny and genome wide comparisons were performed and visualized with progressiveMauve. Evolutionary relationships were inferred using the Maximum Likelihood method. Proteome wide comparations for core-genome plot analysis was performed with CMG biotools.

Based on parA homologs sequence, the *Arthrobacter* specific plasmids have been classified into 4 clades. Iteron like sequences were identified on most of the plasmids indicating the position of the putative *Arthrobacter* specific ori's. A cluster of 12 ORFs predicted to encode the components of a T4-secretion system involved in bacterial conjugation was identified as highly conserved and syngenic among a subset of 14 *Arthrobacter* plasmids. Also, a DNA repeat of about 370 nucleotides was found to be present 5’ to the ORFs of DUF4192-, DprA- and ParB-like proteins on 12 additional *Arthrobacter* plasmids. The DNA repeats contain alternating GC and AT reach sequences, potential protein DNA-binding sites and purine reach stretches. A core-genes common for all the *Arthrobacter* plasmids could not be identified, indicating that the plasmid diversity within this genus exceeds what can be inferred from the study of the available sequences.

It is hoped that the findings presented here will stimulate further experimental work aimed at the elucidation of the ORFs implicated in the regulation of the life cycle of these plasmids.

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PAIN THERAPY PHARMACOGENOMICS: GENETIC POLYMORPHISMS THAT INFLUENCE THE RESPONSE TO PAIN ANALGESIC MEDICATION

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Genetics and genomics are certain to have a large impact in drug development and proper pharmaceutical treatment of subgroups of patients with many specific diseases. We should be able to increase the therapeutic margin for many agents. Genetic variation will also be important in refining estimates of risk from all kinds of environmental agents and in choosing more effective and more cost-effective risk reduction strategies. The linkage of information about genetic variation and information about environmental, nutritional, behavioral, metabolic, medical, and healthcare factors will be necessary to interpret the variation in clinical and public health terms. However, there is a great risk that present efforts to protect confidentiality and privacy of individual genetic information may make such research infeasible. The response of human organism to the treatment with opioid and non-opioid analgesics is genetically determined and is varying from individual to individual; the source of this variation is studied by the pharmacogenomics of pain. The genetic polymorphisms that explain the variability of the analgesic effect of opioid and non-opioid drugs is discussed in the present paper emphasizing the role of cytocrome P450 CYP2D6 polymorphisms for morphine and codeine. Microarray chips are new research tools in pharmacogenetics opening new perspectives for an individualized drug therapy. A microarray chip can be described as a gene expression assay which consist in a glass slide or a micromembrane spotted with DNA from hundreds or even thousands gene probes. Microarray technology is used in pharmacogenetics research to scan the whole human genome for polymorphisms that can alter the normal individual response to drugs; many thousands of polymorphisms can be scanned simultaneously with a single microarray chip. In the present paper, we will try to demonstrate that microarray technology is one of the most important recent advances in molecular genetics and to expose its applications in pain therapy pharmacogenetics.

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INFLUENCE OF PHOTON IRRADIATION UPON V 79 CELLS PROLIFERATION AND GENOMIC STABILITY – PRELIMINARY DATA

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The main objective of this study is to assess the effect of ionizing radiation on the growth and development of V79 cell cultures, both in the first days after irradiation (expression time) and to the descendants of several generations (P0 - P12), in order to establish how occurred mutations are perpetuated and changed from one generation to another. The Chinese hamster lung fibroblasts (V79) were exposed to photon irradiation (1, 2, 3, and 5 Gy, with a flow source at 260.88 cGy min) using VARIAN CLINAC® 2100SC particle accelerator from "St. Spiridon" Hospital Iasi. The control version was maintained under the same conditions, less radiation exposure. The cell viability (made by tripan blue assay) decreases from one generation to another, both in control (from 97.05% - cell culture initiation – P0 to 90.33% – P12) and irradiated variants, without a direct dose-effect relationship. Worthy of mention is the fact that after reducing the number of living cells until P4 level, there was a slight revival of viability maintained throughout the experiment. Regarding the aneuploidy level, the number of chromosomes was ranging from 18 to 22, without a link between the prevalence of a certain number of chromosomes and radiation dose, however, metaphases with 20 chromosomes had the highest frequency. Also, we count triploid and tetraploid metaphases and chromosomes with single or double chromatid breaks. Accordingly, preliminary results of this complex study shows both cytotoxic effect of the photons irradiation on the V 79 cells (expressed by the cell viability and clonogenic assays), and genotoxic effects evidenced by changes in the number of chromosomes and by identification of chromosomal aberrations (mono- and double strand breaks) in metaphase. Although there is no direct dose-effect relationship, corroborating of processed data allow, for this moment, to establish that the cell viability decreases in the first generation after irradiation, finding a restoration of this parameter in subsequent progeny, but without reaches the control values. This process can be attributed to the intervention of cellular repair mechanisms which, up to a particular level, are able to repair or remove (by apoptosis or another cellular death mechanism) alterations occurred in the DNA. Also, increased of aberration rate and DNA structural damages prove harmful action of photon irradiation upon genetic apparatus, alterations that can persist over several generations.

POSTERS

Central hall, 1st floor: 15^{00} – 16^{00}

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TREATMENT RESISTANCE OF TYROSINE KINASE INHIBITORS INDUCED BY THE PRESENCE OF MUTATIONS IN THE TYROSINE KINASE SITUS OF ABL GENE INVOLVED IN TRANSLOCATION WITH BCR-ABL IN CML

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The presence of Philadelphia chromosome resulted from a mutual translocation between chromosomes 9 and 22 is a genetic marker for CML (Chronic Myeloid Leukemia). At molecular level the translocation involves a break point inside the BCR (Breakpoint Cluster Region) gene on the chromosome 22 , and respectively ABL (Abelson) oncogene on the chromosome 9 resulting the BCR-ABL fusion gene. The resulting fusion gene produces a tyrosine kinase (TK) which can be inactivated by administrating tyrosine kinase inhibitors (TKI). The highlight rate of the occured mutation in patients with resistence to treatment is quite low because there are many other possible causes for treatment resistance. The molecular analysis was performed on a lot of 12 patients selected from ones with CML which initially responded to treatment, but then progressed to the accelerated phase and showed early signs of relapse. The method consisted of semi-nested PCR analysis followed by Sanger sequencing. We found two different mutations at 2 patients (24% of pacients): T315I (which causes resistance to all TK inhibitors) and F317L. Our findings are in accordance with other research that showed that in roughly 33% of cases, mutations in BCR-ABL gene, clonal evolution and amplification of the BCR-ABL gene are common causes of treatment resistance. The molecular technique used has an increased sensitivity because the sequenced gene is ABL involved in translocation with BCR-ABL, thus the result is not tainted by the wild type ABL gene.

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“IN VITRO” MULTIPLICATION OF *MENTHA PIPERITA* L.

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The regenerative potential of meristematic explants from *Mentha piperita* L. was evaluated for the establishment of a clonal propagation protocol, as an alternative for biomass production. *Mentha piperita* L. (Lamiaceae) is one of the most economically important aromatic and medicinal plants ,that contains various classes of compounds (volatile oil rich in menthol and flavonoids, phenolic acids) with multiple pharmacological, cosmetic applications.

Meristematic explants, taken from seedlings of *Mentha piperita* L.,germinated in aseptic conditions were tested for their regenerative potential.

The procedure involved shoot tip cultures, followed by rapid shoot multiplication, rooting and finally establishment of plantlets in soil.

Murashige-Skoog medium has been diversified according to hormonal balance, using benzylaminopurine (BAP) in combination with α-naphthaleneacetic acid (NAA). The agar solidified MS medium containing 0,5 mg/l benzylaminopurine and 0,2 mg/l α-naphthaleneacetic acid (NAA) was optimum for shoot proliferation at *Mentha piperita* L. and allowed the development of large number of cloned shoots.

The regeneration of whole plants was obtained in two steps: the shoots were excised and transferred to fresh medium and then rooting of these shoots was achieved on the same medium with 0,5 mg/l benzylaminopurine and 1 mg/l α-naphthaleneacetic acid.

In each variant of MS basal medium studied it has worked on every 10 samples. The samples were kept in growth chamber at 23±1o C and a photoperiod of 16 hours.

The excised shoots were subcultured for roots induction. Regenerated plants were transferred to ex vitro conditions for an acclimatisation period.

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POPULATION GENETIC INFERENCE FROM HSP70 SEQUENCES POLIMORPHISM IN FISHES

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Heat shock proteins (HSPs) or stress proteins, a subset of molecular chaperones, are a superfamily of, intracellular proteins forming a part of the cellular defence. HSPs have an unusually high degree of identity at the amino acid level, among diverse organisms. Molecular chaperones are major cell constituents in all organisms under nonstress conditions and they are essential to ensure proper folding and intracellular localization of newly synthesized polypeptides. The need for molecular chaperones is higher under stressful conditions, as the rate of damage to cell proteins or problems with proper folding increases markedly (Sorensen et al., 2003; Padmini et al., 2008). The heat-shock protein 70 (HSP70) stress protein family consists of several members with similar molecular sizes, some of which are heat inducible and others are constitutively expressed (Morimoto et al., 1990). Both HSP70 and heat-shock cognate protein 70 (HSC70) are cytosolic. HSP 70 have evolutionarily diverged and have been classified by phylogenetic analysis into four distinct clusters corresponding to their intracellular localization, i.e., in the cytoplasm, endoplasmic reticulum, mitochondria, or chloroplasts (Boorstein et al., 1994; Morimoto et al., 1990); however, the evolutionary relatedness among HSP70 and HSC70 proteins in vertebrates has not been fully elucidated (Yamashita et al., 2004).

Despite the intense interest that has been given to HSPs, a few studies have addressed questions about the nucleotide structure and the evolution of these genes. As a result, the aim of the present study was to clone and analyse the stress-associated HSP70 gene structure in *Cyprinus carpio* by direct sequencing and a phylogeny inferring.

Phylogenetic analysis including neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) trees of DNA sequence alignments analysis were conducted using Paup 4.0b10 (Swofford 2000) using PaupUp graphical interface (Calendini & Martin, 2005). Genetic distances used in NJ trees are Kimura two-parameter model distances with a transition : transversion ratio of 2 : 1. Non-synonymous/synonymous substitutions distances (dN/dS) ratios, as described by Nei & Gojobori (1986), were calculated using MEGA7 software (Tamura et al., 2013). Bootstrap analysis was made with 1000 replicates except in ML where only 100 replicates were generated. Hierarchical likelihood ratio tests were conducted using a batch file supplied with MODELTEST 3.7 (Posada & Crandall, 1998) to provide the evolutionary models used in ML and Bayesian analysis.

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PAPANICOLAOU TEST: PERFORMS AND LIMITIES

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Harvesting the smears and the cytodiagnostic interpretation represent the first step in diagnosing the presinvasive pathology of the cervix. The study lot was made of 1476 patients who came for a specialty consult in two medical units in Iasi, in the period of time between 2010 and 2015 and who were harvested Pap smears. The data was statistically processed in order to draw conclusions about the incidence of cervical bening pathology and the usefulness of its detection through this test. If we refer to the whole lot, the cytological results are satisfactory: 83% smears are within normal limits, 10,1% ASCUS, 2,7% L-SIL, 0,8% H-SIL. From all the feminine genital neoplasias, the cervical cancer is the easiest to detect, with low costs, as it benefits from very effective early diagnostic methods: cytology, HPV testing, colposcopy, biopsy. We estimate that 50-75% of the results that are false negative are due to the harvesting errors.

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EFFECTS OF *CRATAEGUS MONOGINA L.*(HAWTHORN LEAVES AND FLOWERS) HYDROALCOHOLIC EXTRACTS ON CELL DIVISION IN *CUCUMIS SATIVUS L.*

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Keywords: Hawthorn, flavonoids, polyphenols, chromosomes mutation, cell cycle, *Cucumis sativus* L. root meristems.

Abstract: Hawthorn (*Crataegus monogyna*) hydroalcoholic extract was prepared by extraction of powdered dried leaves and flowers with ethanol 70% v/v (1:10), by reflux for two hours. Extract was qualitative and quantitative analyzed. This extract was tested for effects on seed germination and cell division cycle of *Cucumis sativus* root meristems.

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DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION VALUES IN PATIENTS WITH SEPSIS WITH ORO-MAXILLOFACIAL PORTAL OF ENTRY

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In patients with risk factors for developing methicillin resistance, first-line treatment options should be reconsidered and antibiotics such as: clindamycin, trimethoprim-sulfamethoxazole, or new cyclins should be administered. In hospital settings, the alternatives include vancomycin, teicoplanin and linezolid. The favorable disease course depended on precocity of etiologic diagnosis and initiation of appropriate therapy. A total of 9036 bacterial strains isolated from patients admitted to the "Sf. Spiridon" Emergency County Hospital during 2013-2016 were tested. Minimum inhibitory concentration (MIC) was determined and CMI 50 and CMI 90 values were calculated for the following antimicrobial agents: gentamicin, oxacillin, rifampicin, kanamycin, erythromycin, trimethoprim-sulfamethoxazole tetracycline, penicillin, ofloxacin, ciprofloxacin, Vancomycin and Tobramycin. Susceptibility categories were established according to the breakpoints recommended by CLSI 2016 (Clinical and Laboratory Standards Institute) for the fully susceptible category (in this study the intermediately susceptible isolates were classified as resistant). The *S. aureus* strains tested in this study demonstrated elevated MIC 90 values (64μg/ml) for kanamycin and tetracycline and high percentage of resistance to kanamycin, erythromycin, trimethoprim-sulfamethoxazole. For penicillin the resistance rate was 94.7%. Resistance to third generation cephalosporins and aztreonam has progressively increased in Romania after their introduction in therapy. The study showed that 45% of all infections could be prevented by vancomycin administration.

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THE NEUROCOGNITIVE EFFECTS OF *HYPERICUM PERFORATUM* L. EXTRACT ON AMYLOID BETA (25-35)-INDUCED MEMORY DEFICITS AND OXIDATIVE STRESS IN LABORATORY RATS

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Hypericum perforatum L., also known as perforate St John's-wort, is one of the most ancient medicinal herbs commonly used for many human ailments. *Hypericum perforatum* L. presents anti-depressant, anxiolytic, anti-inflammatory, antiseptic and sedative effects in the nervous system. In the present study, the effects of the hydroalcoholic extract of *Hypericum perforatum* L. administration (25 mg/kg and 75 mg/kg, i.p., for 7 days) on spatial memory performance were assessed on amyloid beta (25-35)-treated rats. The amyloid beta (25-35)-induced memory impairments were observed, as measured by the Y-maze and radial arm-maze tasks. Decreased activities of superoxide dismutase and glutathione peroxidase were observed in the rat hippocampal homogenates of amyloid beta (25-35)-treated animals as compared with control. Also, production of malondialdehyde significantly increased in the rat hippocampal homogenates of amyloid beta (25-35)-treated animals as compared with control, as a consequence of impaired antioxidant enzymes activities. Additionally, the administration of the hydroalcoholic extract of *Hypericum perforatum* L. at amyloid beta (25-35)-treated animals improved short term memory performance in Y-maze test, but did not exhibit significant improvements in long term memory in radial arm maze test. These findings associated with a decreased oxidative stress level, suggest the antioxidant effects and memory-enhancing proprieties of the hydroalcoholic extract of *Hypericum perforatum* L.. The obtained results indicate that the hydroalcoholic extract of *Hypericum perforatum* L. may be a potential candidate for the development of therapeutic agents to manage memory impairment and oxidative stress associated with Alzheimer’s disease.

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AMELIORATIVE EFFECTS OF *MATRICARIA CHAMOMILLA* L. EXTRACT ON SCOPOLAMINE-INDUCED MEMORY IMPAIRMENT IN RATS: A BEHAVIORAL AND BIOCHEMICAL STUDY

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Matricaria chamomilla L. is one of the most ancient medicinal herbs commonly used for many human ailments, including nervous disturbance. In the present study, the effects of the hydroalcoholic extract of *Matricaria chamomilla* L. administration (25 mg/kg and 75 mg/kg, i.p., for 7 days) on spatial memory performance were assessed in scopolamine-treated rats. Scopolamine-induced memory impairments were observed, as measured by the Y-maze and radial arm-maze tasks. Decreased activities of superoxide dismutase, glutathione peroxidase and catalase along with increase of acetylcholinesterase activity and decrease of total content of reduced glutathione were observed in the rat hippocampal homogenates of scopolamine-treated animals as compared with control. Production of protein carbonyl and malondialdehyde significantly increased in the rat hippocampal homogenates of scopolamine-treated animals as compared with control, as a consequence of impaired antioxidant enzymes activities. Additionally, in scopolamine-treated rats treated with the hydroalcoholic extract significantly improved memory formation and decreased oxidative stress, suggesting memory-enhancing and antioxidant effects. These findings suggest that the hydroalcoholic extract of *Matricaria chamomilla* L. may be a potential candidate for the development of therapeutic agents to manage memory impairment and oxidative stress associated with Alzheimer’s disease through decreasing the activity of acetylcholinesterase and anti-oxidative mechanism.

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EFFECTS OF *HYPERICUM PERFOLIATUM* (ST JOHN`S WORT AERIAL PARTS) HYDROALCOHOLIC EXTRACTS ON CELL DIVISION IN *CUCUMIS SATIVUS L.*

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Key words. St John`s Wort, flavonoids, polyphenols, chromosomes mutation, cell cycle, *Cucumis sativus* root meristems.

Abstract. St John`s Wort (*Hypericum perforatum*) hydroalcoholic extract was prepared by extraction of powdered dried flowering aerial parts with ethanol 70% v/v (1:10), by reflux for two hours. Extract was qualitative and quantitative analyzed. This extract was tested for effects on seed germination and cell division cycle of *Cucumis sativus* root meristems.

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MOLECULAR PHYLOGENY OF *SCARDIUNIUS* GENUS INFERRED BY NUCLEAR RAG1 (RECOMBINATION ACTIVATING GENE 1) GENE ANALYSIS

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“Cyprinid” is the name given to any fish that belongs to the carp family Cyprinidae which includes fishes like the carp, the crucian carp, the zebra fish, the chub, the rudd etc. With over 200 genus and 2000 species, the minnow family is the largest family of freshwater fishes. Within this family, because of the high genetic and morphological variability, the phylogenetic relationships were never completely solved. The rudd, common term used for *Scardinius erythrophthalmus* species is a bentho-pelagic freshwater fish which is most often encountered in nutrient-rich waters with abundant vegetation. It belongs to Scardinius genera which includes a total number of 10 species *(S .erythrophthalmus, S. hesperedicus, S. knezevici, S. plotizza, S. dergle, S. acarnicus, S. elmaliensis, S. graecus, S. racovitzai* and *S. scardafa*) and more than half of them have a status from near threatened to critically endangered on IUCN Red List. This study aims to present the phylogenetic relationships within this genus by analyzing the RAG1 gene, a nuclear protein-coding locus (NPCL) marker that is applicable across diverse taxa and show good phylogenetic discrimination. The total DNA was isolated for the species from Romania (*S. erythrophthalmus* and *S. racovitzai*) using different protocols according to the sample type. RAG1 gene amplification was performed using the RAG1F and RAG9R primers and the amplicons were sequenced using CEQ 8000 Genetic Analysis System (Beckman Coulter) with two other internal primers. The GenBank sequences for *Scardinius* genera species RAG1 gene dataset and *Carassius carassius* as outgroup were used for phylogeny inference. The sequences were aligned using ClustalW method from MEGA 7 software and the phylogenetic trees were constructed using BEAST v1.8 (Bayesian Evolutionary Analysis Sampling Trees). Our results show a complex phylogeny within the *Scardinius* genus and RAG1 gene is a good NPLC marker for the phylogenetic studies of this genera.

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PRELIMINARY DATA ON PHYLOGENY AND PHYLOGEOGRAPHY OF *COBITIS ELONGATOIDES* INFERRED BY CYTOCHROME B GENE ANALYSIS

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Cobitis is a Palearctic genus of ray-finned fish in the Cobitidae family, with more than 80 identified species, however the clasification within family is disputed and remain uncertain. The phylogeny of the genus *Cobitis* remains one of the most interesting problems of the family Cobitidae. The classification within Cobitidae family is still disputed, the number of recognized species varies according to the number of morphological traits considered. Phenotypic species-specific character may be partly modified toward unification due to asexual reproduction, poluploidy and hybridization within the genus *Cobitis*. The most common species for Danube and Dniester River Basins are *Cobitis taenia*, *Cobitis tanaitica*, *Cobitis elongatoides* and belongs to the *Cobitis taenia* hybrid complex. These species have a particular type of reproduction (gynogenesis) that leads to an asexual lineage (diploid, triploid and rarely tetraploid individuals). *Cobitis elongatoides* was exclusively the maternal ancestor of all the *C. elongatoides-tanaitica* hybrids, while the hybridization process was reciprocal within the *C. elongatoides*-taenia complex. *Cobitis elongatoides*, commonly named spined loach, is native to Danube basin, upper Elbe and Odra drainages, and also Dniester River. In the present work, we investigated the variability, phylogenetic and phylogeographic relationships between the *Cobitis elongatoides* individuals from different river basins analyzing the molecular data provided by the mitochondrial marker cytochrome b gene (575bp). Total DNA extraction for the captured individuals from Prut and Dniester Rivers was performed using phenol chloroform isoamyl alcohol protocol. In order to amplify the cytochrome b gene, the L15267 and H15891 primers were used. The amplicons were successfully sequenced using CEQ 8000 Genetic Analysis System (Beckman Coulter). Genetic diversity within and between hydrological basins was assessed in Arlequin (v 3.5) software, computing general molecular indices. Our data reveal a high similarity between *C. elongatoides* individuals and *C. elongatorides* x *C. tanaitica* hybrids that belong to different tributaries of the Danube river basin, pointing that *C. elongatoides* is the maternal ancestor of all *C. elongatorides* x *C. tanaitica hybrids*.

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SERUM SELENIUM AND MAGNESIUM LEVELS VARIATION IN ALZHEIMER'S DISEASE AND MILD COGNITIVE IMPAIRMENT PATIENTS

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The main symptoms of Alzheimer's disease (AD) is memory loss. Due to intense brain degeneration causing memory loss halmark, several metals ions function impairment has been described. In this way, selenium, and magnesium which are both extremely important in brain tissue can be quantified from blood serum. This study aims to quantify and compare selenium and magnesium levels in demented patients. Blood serum samples were collected from Alzheimer`s disease (n=20), and mild cognitive impairment (n=17) patients admitted in Socola Psychiatric Institute, Iasi. Also, samples from age and sex-matched control subjects were collected. Graphite furnace atomic absorption spectrometer with high resolution and continuous source (GF HR CS-AAS) analysis was performed for metal ions concentration determination. Standard statistic analysis was performed (one-way ANOVA). Selenium levels were found increase in AD patients with more than 35%, as compared to healthy controls. Magnesium levels also varied in a significant manner in AD patients, as compared with MCI patients and healthy controls. Thus, serum magnesium and selenium levels significant variation indicates that they may be a useful parameter in AD severity evaluation, but further studies are needed.

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