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REVIEW on PhD Thesis
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Applied study of some lipid extracts from *Mytilus galloprovincialis* Lmk. And *Rapana venosa* molluscs

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Objectives and purpose of the work

Through documentary study and the realized papers, the PhD thesis proposes to bring a contribution at big volume of information's accumulated in the marine resources capitalization domain. Through this desideratum, the subject of the PhD thesis is framed in research traditions of the specialized institutions from the Black Sea Coast.

The documentary study has distinguished the assiduous research of the Black Sea molluscs from different investigation ways, theoretical and applied respectively, based on examination and citation of 194 speciality papers. From selected information's, the lipid extracts are less represented from the applied point of view. Therefore, it was choose the research of some biological active substances from the two *Mytilus galloprovincialis* Lmk. and *Rapana venosa* mollusc's species. The choice was motivated by the fact that the two species are into trofyc relation, which facilitate the collected of the row material and some similarity from the biochemical point of view.

The objectives of the PhD thesis are:

- putting up-to-date and systematization of the data from literature regarding the lipid extracts from the *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs,

- developing the knowledge's by creation of the new original incorporated draft of this marine organisms,

- realization of the producing premise of some pharmaceutical products which could capitalize the bioactive potential of the molluscs taken in the study.

The strategy of this has followed a multidisciplinary approach: chemistry, biology, biochemistry, medical and pharmaceutical sciences, which was accomplish through a complex collaboration in research activity.

Therefore, the realization of the papers was possible through a serried contribution with Faculty of Natural and Agricultural Sciences, Faculty of Pharmacy, Faculty of Medicine and Faculty of Physiscs from "Ovidius" University of Constanta, I.N.C.D.M. "Grigore Antipa" from Constanta, the Phatology Department from the Clinical Emergency County Hospital of Constanta, the Microbiology and Histophatology Departments from Sanitary and Veterinary Public Health, the Microbiology Laboratory from Human Public Health, the Museum Complex of Natural Sciences from Constanza, the Physico-Chemical Laboratory from S.C. Biotehnos S.A., The Trying Phisico-Chemical Laboratory from Polytechnic University of Bucharest, the "Ilie Murgulescu" Institute and the Vectors Fighting, Toxicology and Pesticides Department from Public Health Institute Bucharest from Romania.

The PhD thesis is distributed in 2 principal parts: part I- the knowledge degree and part II – personal contributions.

In the firs part of the PhD thesis is describe the marine organisms like souces of biological active products and substances and those pharmaceutical forms utilized in dermatology medication. By this chapters it was followed the evaluation of the informational essence and the motivation through new direction of applied research.

In the last part of the paper is follow a selection of the analitique methods of row material and lipid extracts characterization; the selection is extend on

quantification methods of those biological active characteristics and those effects. This is continuing with the obtained results from the technological, analitique and pharmaco-chemical point of view by *in vitro* and *in vivo* tests. The research results are presented in 49 tables, 61 figures and 11 original papers.

The conclusions argue the realization of the objectives and also open the new research expectations in marine biochemistry domain, and even more.

Introduction

Exhaustive research conducted over the past decades have shown that marine life offers a much wider range of biologically active than Earth, including specialists forming the belief that marine organisms in general and particularly seaweeds, are an important source of drugs future therapeutic than herbs.

The interest manifested notorious for this research is fully justified, given the existing possibilities and potential for better use of various marine organisms as a source of biologically active substances available. Moreover, the expression "sea medicine 'already iterated is not a mere metaphor, but under some certainties.

It is estimated that in the not too distant future the huge riches of blue continent will be able to suffice mankind with food and raw materials. Certainly the food and pharmaceutical industry will have an important share, being the main beneficiaries, along with other economic sectors.

This type of research requires a huge infrastructure and qualified personnel, research is organized on several levels:

- Recognition by taxonomist's species of marine origin;
- Collection, storage and preservation;
- Extraction of biologically active substances and determining their chemical structure;
- Investigation of pharmacological products, highlighting the therapeutic potential of using and obtaining bio seafood.

Natural products of marine origin have attracted the attention of researchers around the world in the last five decades. Currently, approximately 16 000 bioactive substances of marine origin have been isolated from marine organisms and listed in 6800 publications. In tandem with these publications, there are other publications about 9000 to cover synthesis, biological activity studies, environmental studies, etc.. on this subject. The ocean is considered to be a rich source and contained in the medicament, and potentially others.

Romanian Research at the Black Sea

Romanian activity in the area has kept pace with the history and evolution of this research. In marine research started more than 100 years ago in the Black Sea are found information about biologically active substances from marine organisms related to this field interference with marine animal physiology work [25]. However, research evidence related to assessment and recovery of biologically active compounds from marine organisms have developed after their establishment in a distinct direction in the field of Marine Biochemistry. Practical research comprised the majority of marine organisms existing in the Romanian seaside.

Black Sea molluscs were the most thoroughly studied organisms in different research programs [54]. Thus began highlighting the presence and concentration dynamics of main biochemical compounds in the annual life cycle, research that continued extraction and purification of biologically active substances to developing their technologies. For marine molluscs, have been isolated, purified and characterized fractions of free amino acids, fat fractions, protein and polysaccharide. It revealed the presence and enzymes were isolated as: amylase, lipase, protease, glucosidase, cellulase, catalase and peroxidase. It was isolated, purified and conditioned in an extract Rapana venosa pepsin inhibiting activity which has been conditioned in the form of tablets ulcer [143].

From an organizational perspective, research Romanian Black Sea was founded and grew with the establishment of the Romanian Institute of Marine Research in Constanta in 1970 and was conducted in collaboration with the Institute for Chemical-Pharmaceutical Research Bucharest Medicines Enterprise Biofarm Bucharest Medicines Commission (now the National Medicines Agency) with the Institute for State Control of Medicines and Pharmaceutical Research, Bucharest, including specialized departments of institutes of higher education in Constanta and Bucharest.

From 1979 to 1992 the Marine Research Institute in Constanta, ran a National Programme "Better awareness of marine biological resources for pharmaceutical purposes," and from 1986 to 1991, an international program with the same theme, with the participation of States CMEA: USSR, Germany, Poland, Bulgaria and Romania, the program called "superior capitalization World Ocean resources" under the direction of Dr. Natalia Roşoiu.

Since 1991, research has continued to SC Biotehnos S. A. Bucharest, ending far the manufacture and circulation domestically and for export to all countries in the former Soviet space, a valuable and

osteoprotective antirheumatic drug extracted from marine organisms for human ALFLUTOP injection and injection ALFLUTOP veterinary.

Among the products developed and approved by the Ministry of Health stated:

- MACRONIL extract fillet of mackerel (Scomber scombrus), the effect of reducing the total bacterial flora and Staphylococcus aureus in the airways and
- Product range ALFLUTOP solution for injection for human and veterinary use;

Of patents on biologically active substances and drugs of marine origin, we can mention the following:

- Amylolytic preparations (Patents 68328/1978, 75140/1980) [52, 129];
- Antireumtic injectable medication, osteoprotective, antitumor (Patents 82273/1983; 108765/1994), [130, 131] ALFLUTOP trademark;
- Complex of active substances antipeptică and antacid (Patent 82876/1984) [134];
- Preparations of hyaluronidase activity (Patents 87312/1985, 102703/1990), [135, 136];
- Opoterapică antiulcer composition (Patent 90969/1986) [137];
- Medicinal mouthwash (Patent 92641/1987) [138];
- Ulcer drug (Patent 96784/1988) [139];
- Antirheumatic solution for external use (Patent 98316/1989) [140];
- Injectable thrombolytic drug (Patent 108763/1994), [141];
- Local inflammatory dentifrice (Patent 111017/1996), [142] brand MARADENT registered;
- Massage paste (Patent 111020/1996) [86];
- Lyophilised injectable anti-rheumatic and osteoprotective (Patent 120317/2005), [133] ARMAVITAL trademark, etc.

In all marine life, molluscs table is one of the species in the Black Sea and the Romanian littoral, both in natural populations and by the culture. This marine natural resource is not currently exploited in an organized and specialized industrial level. In general, a small fraction of mussel production is marketed for food, and that recovery is conditioned by their size [65].

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food, and That recovery is conditioned by Their size [65].

Biochemical investigations were carried out in two directions, one to establish links with their lifestyle and to substantiate the physiological, the other leaning applied.

Regarding the overall biochemical composition, two references delayed by a decade [47, 101] (Tables 1 and 2) indicate that seasonal variations have been preserved over time.

Table 1. Seasonal variation of global biochemical composition at *Mytilus galloprovincialis* Lmk. and *Rapana venosa* (Dincu, 1991-1992), [47].

Biochemical composition	*Mytilus galloprovincialis* Lmk.		*Rapana venosa*			
	July	September	May	August	Oct.	Apryl
Water (%)	84.70	81.37	73.17	74.30	67.18	74.80
Mineral elements	6.69	10.75	8.05	5.27	9.84	8.49
Organic substance	93.31	89.25	91.95	94.73	90.16	91.51
Total nitrogen	10.82	7.00	8.54	11.27	4.73	7.80
Proteins	67.63	43.75	53.38	70.45	29.56	48.75
Lipids	8.22	8.66	11.54	10.86	9.28	10.35
Total carbohydrates	17.46	36.64	27.03	13.42	51.32	32.41
Glycogen	18.06	-	-	-	-	30.00

Table 2. Global biochemical composition at *Mytilus galloprovincialis* Lmk. and *Rapana venosa* (Mircea şi col., 2002) [101].

Biochemical composition	*Mytilus galloprovincialis* Lmk.	*Rapana venosa*
Water (%)	79,51	71,15
Mineral elements	8,43	7,45
Organic substance	91,57	92,55
Total nitrogen	7,98	8,74
Proteins	51,57	54,63
Lipids	8,97	8,99
Total carbohydrates	32,80	28,83
Glycogen	-	-

Other documentary sources indicate that there are many substances elaborated by marine molluscs, featuring extracts from these pharmacological properties of mammalian organisms. Such extracts or tissue preparations exert some antitumor, cardioactive, liver, etc. [1, 94]. However, not all of the cases could be identified to date, the chemical nature of these active ingredients [16, 150]. In Table 3 are presented as biologically active substances extracted from Mytilus galloprovincialis Lmk. Rapana venosa and their pharmacological action as well.

Table 3. Biologically active substances extracted from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs.

Species	Biological active substances	Pharmacological action with application in therapeutical	References
Mytilus galloprovincialis Lmk.	Hepatoprotective factor	Protective action on liver	[106, 148]
	Sulfomucopolysaccharides	Protective action on hyperlipidemia and hypercholesterolemia	[106, 148]
	Proteine with adhesive property	Used in surgery Particularly in orthopedics, fractures and tendon and internal medicine	[106, 148]
	Amylase	Used in the treatment of pancreas diseases: entering the composition of medicines called Triferment, festive panzcebil used in the treatment of digestive diseases	[128, 148]
	Cellulase	Digestive disease therapy	[49, 148]
	Proteases (pepsin)	The therapy of various diseases digestive; in the composition of Triferment type drugs, festive, panzcebil	[50, 74]

	Meso-inozitol	Exhibits an lipotropic and interferes with metabolism cholesterol by preventing its accumulation in the liver	[148]
	Insulin	Pancreatic hormone hypoglycaemic; stimulate the biosynthesis of glycerides and inhibits lipolysis, stimulates the biosynthesis of proteins and amino acids, etc.	[148]
	Prostaglandins (Arachidonic acid)	Act as hormones local tissue; involved in the modulation of hormonal stimulates smooth muscle contraction; running a vasodilator; decreases gastric acidity; increases capillary permeability; processes involved in regulating body temperature, etc.. It is used in treatment of cancer, hyperemia, asthma, allergies; prevents thrombus formation, dissolves blood coagulant, scars wounds, stop internal bleeding, calms nerves involved in reproductive processes, etc.	[112]
Rapana venosa	Protease	Therapy of digestive disease; enter into the composition of medicines called Triferment, festive, panzcebil	[148]

	Amylase	Used in the treatment of diseases of pancreas; enter into the composition of medicines called Triferment, festive, panzcebil	[128, 144, 148]
	pepsin inhibitor	The treatment of gastric ulcers, stomach; of Acute gastritis with hyperacidity and chronic, etc.	[128, 144]
	tripsin inhibitor şi chymotrypsin	In the therapy and prophylaxis of traumatic shock, pancreatic, septic, hemorrhagic; prophylaxis of postoperative complications and postrheumatoid.	[128, 148]

Materials and Methods

1.Sanitary characterization of raw material, dry and lipid extracts from Mytilus galloprovincialis Lmk. and Rapana venosa

The feedstock dry matter and in the final extract were determined both the content of heavy metals, pesticides and microbiological load.

1.1.Determination of heavy metals

Heavy metal content analysis was performed on INCDM "Gregory Antipa" Constanta, Laboratory Measurements and Physico-chemical (AML) with atomic absorption spectrometer with graphite furnace - ATI UNICAM type SOLAAR 0.39 Z 939Z [37].

1.2. Determination of pesticides

Pesticide analysis was performed on INCDM "Gregory Antipas' Constanta, laboratory measurements and physicochemical analysis (LMA) with a Hewlett-Packard Gas Chromatograph equipped with ECD detector-Perkin Elmer electron capture calibrated HCB, lindane, heptachlor, aldrin, dieldrin, endrin pp 'DDE, pp'DDD, pp' DDT [38].

1.3. Determination of microbiological load

Samples were made in the Department of Food Microbiology, the Laboratory
Veterinary Constanta (DSV). Within this department are microbiological determinations on animal foods, using traditional working techniques based on standardized methods.

For the determination of microbiological load (coliform bacteria, *Escherichia coli*,
Salmonella pathogen, *Listeria monocytogenes*, pathogen, sulphite-reducing *Clostridia*, Staphylococcus coagulase-positive and total germ NTG) of the samples analyzed from two molluscs (meat, dry lipid extract) were applied techniques specific work as certified methodology.

2. Analytical methods for characterization of quality final products (lipid extract)

2.1. Organoleptic examination included
1. Appearance; 2. Smell; 3. Taste; 4. Colour [100].

2.2. Determination of relative density

To characterize the two mollusks Mytilus galloprovincialis extracts Lmk. and Rapana venosa pycnometer was used [100].

2.3. Determination of refractive index

We worked with an ABBE refractometer (Carl Zeiss Jena, type 389 820) of the Optical Physics Laboratory, "Ovidius" Univeristy of Constanta, according to respective metodologiei [192].

2.4. Determination of iodine index

The iodine index is the amount of iodine in grams that is additional to the double bonds of the unsaturated fatty acids per 100 g fat.
Iodine index is one of the most important chemical constants oils (fats) and for assessing the degree of unsaturation of the oil (fat), its tendency to activity, rancidity and other changes that occur during storage and processing of edible oils and technical or food rich in fat [2].

2.5. Determination of acidity index

The acidity index is the amount in grams of KOH required to neutralize the free fatty acids in one gram of oil. Acidity index of fats and oils depends on how secure and their mode of storage [100].

2.6. Determination of saponification index and unsaponifiable matter content

The saponification index is the number of milligrams of KOH required to neutralization of free and esterified fatty acids present in one gram of fat. Saponification index characterizing the molecular weight of the fatty acids contained in the fat.

The saponification index is also dependent on the presence of the unsaponifiable substances, the lower the index, and the existence of free fatty acids in the fat analysis, the increasing value of the index [2].

2.7. GC/MS Chromatographic determination

The chromatograms were measured with an Agilent composed of a gas chromatograph (GC 6890 N) with an injector (7693 series) and coupled with mass spectrometry (MS 5973) with quadrupole. System and computer-aided data processing is done with Chemstation software provided with the library (spectra) NIST.

Two methods were used, namely the method methylation derivatization method with BSFTA [bis (trimethylsilyl) trifluoroacetamide]. Both processes are part of the current methodology of the Department of Physico-chemical analysis of the SC BIOTEHNOS SA from Bucharest, Romania.

2.8. UV/VIS Spectral measurements

The spectral analyzes were performed with a UV VIS spectrometer GBC CINTRA 5 the quartz cell of 10 mm. Spectra were determined extracts solubilized in n-hexane, with a dilution of 1:2 (v / v).

2.9. IR Analysis

IR spectra were detected with a Bruker the vector 33, device type used as PIKE. ATR method was used - attenuated total reflectance after the current methodology developed Optical Spectroscopy Laboratory Spectroteam Arena Group for Romania and Bucharest.

3. Highlight and evaluation of *in vivo* and *in vitro* methods of biological effects

3.1. Determination of in vitro sun protection factor

Analyses were performed at the National Center for Environmental Consultancy. UPB-CNC holding test laboratory physicochemical RENAR according to EN ISO / IEC 17025/2001, Accreditation Certificate Nr. 141-L, Polytechnic University of Bucharest, Faculty of Industrial Chemistry.

Determination of SPF (sun protection factor) was carried out using a UV

spectrophotometer, JASCO 570, equipped with integrating sphere with corresponding software and a support TRANSPOR 3 TM, having a composition similar to the natural skin was applied to standardized amount of 2 mg/cm2 cream [45].

3.2. Microbiological methods for determining the effects of lipid extracts fromMytilus galloprovincialis Lmk. and Rapana venosa of a bacterial culture of E. coli

The research was conducted in collaboration with the Laboratory of Microbiology of the Department Public Health, Constanta, applying the Kirby-Bauer disc diffusion method. Study of lipid extracts on cultures of Escherichia coli was achieved by determining the microbiological activity of antibiotics within the Microbiology Laboratory of Sanitary Veterinary Directorate from Constanta [27, 192].

3.3. Methods of evaluating the action of skin inflammation lipid extracts (*in vivo*)

Methodology was used Histopathology Laboratory of the Department of Sanitary Veterinary Direction from Constanta, comprising assembly techniques. To evaluate the results using microscope Leica DM L 32, viewing sânge10 × 100; for weaving 20 × 10; 10% formalin fixative solution.

3.3.1. Histological processing technique tissue samples

Object

The procedure is to produce histological tissue to obtain micro-sections for specific histological further processing, in order to obtain the final permanent nedeparafinate histological sections.

Application domain

Cytological diagnosis histopathological / histochemical cyto of various animal diseases.

Methods

Histological processing technique allows the tissue samples citohistochimice examinations and / or citohistopatologice subsequent processing of the harvested tissue fragments in order to obtain specific microsecţiunilor for subsequent histological processing (histologic / histochemical staining) [39].

3.3.2. Hematoxylin and eosin staining for histological and histopathological evidence of tissue structures

Staining of histological sections and highlighting structures is performed specific reagents, hematoxylin and eosin, recipes this protocol. Color may be carried out automatically and / or manually.

Results

Nucleus - dark blue, violet staining with Mayer's hematoxylin and violet - reddish to
Harris hematoxylin staining; Cytoplasm - Pink;
Fundamental substance - Pink; Connective fibers - brick; Muscle tissue - red;
Fibrin - Pink; Hyaline - pink - red; Fibrinoid - pink - red [40].

3.3.3. May-Grunwald Gimsa method for blood

Histological processing techniques and methods include staining of the smears of blood panoptic May-Grunwald Gimsa method for studies of cell morphology blood.
Panoptic staining results: good smear has a weak violet coloration. Examined under a microscope, red blood cells have a reddish color, are light nuclei
violet, pale blue nucleoli, eosinophilic granulations are orange, the blue-black of basophils, neutrophils the dark brown and azurophilic granulations red-purple shade [113].

3.4. Immunohistochemistry and biohistometrice methods

Laboratory methodology was used immunohistochemistry (IHC) in the Constanta County Emergency Hospital, Department of Pathology. Immunohistochemical methods rely on linking an antigen, which is cellular or tissue component and a specific antibody labeled. In the case of antibodies, using peroxidase labeling enzyme extracted from horseradish root (HRP - horseradish peroxidase) and for evidence of antigen-antibody complexes formed, the indirect method using enzyme-conjugated streptavidin-biotin-(streptavidin-biotin Labelled LSBA).

3.4.1. Technique conjugated streptavidin-biotin system LSAB enzyme (HRP)

Making sections for immunohistological studies are done in stages involve treatment with various substances, incubation and repeated washings.

Results

Specific antigens are exposed by the appearance of a brown coloration of the cellular and tissue structures which contain [104].

3.4.2. Working method for sbiohistometric study

Preparations are examined microscopically Nikon E-600 with a Sony video cameras. The images obtained are processed using software analysis-semi-automatic image LUCIA G.

The quantitative data thus obtained are put in the batch tables was calculated parameter of central tendency (mean) and dispersion parameter (standard deviation). Hypotheses validation was carried out by means of Student (t-test), which is considered a statistical significance $p < 0.05$ significant to rejection of the null hypothesis established for each case individually.

In this case, we performed a qualitative interpretation of the images obtained from optical microscope that is measuring the thickness of collagen fibers. Thus, we chose a homogeneous field, measuring the thickness of various fibers, and then calculating the mean and standard deviation for each lot subject to the experiment [23].

4. Extraction techniques of lipids with solvents accepted in pharmaceutical and food industry

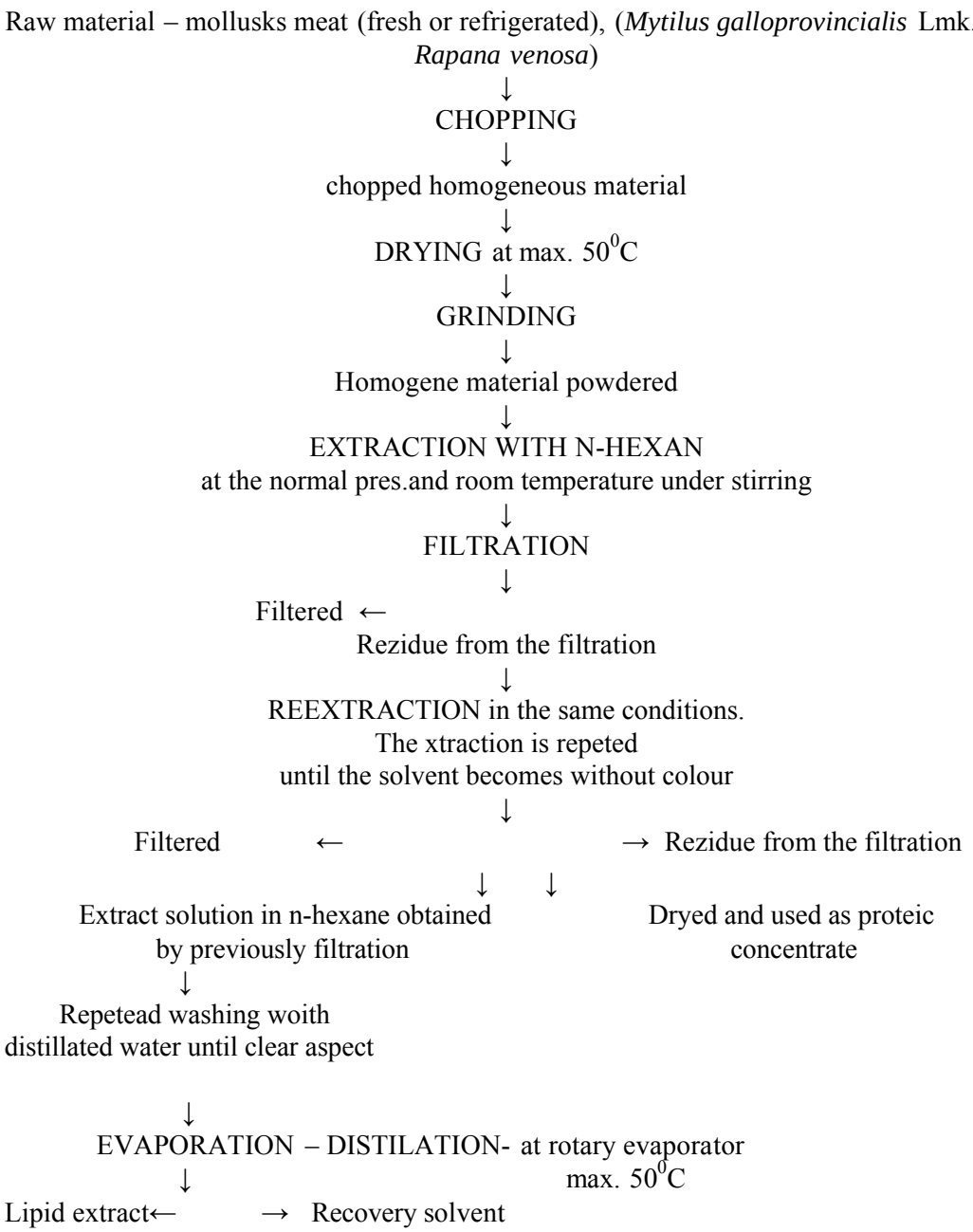
For analytical purposes, lipid extraction of mussels (*Mytilus galloprovincialis* Lmk.) and sea snail (*Rapana venosa*) was achieved by Christie's method [33].

Petrol Extract is a natural product produced by distillation of crude oil, which is actually a mixture of fractions with distillation range between $60\text{-}100^0$; in terms of chemical composition, the percentage saturated aromatics belongs.

N-hexane is a liquid hydrocarbon resulting fractional distillation of gasoline extraction and has the following features: water-insoluble flammable Pf = 68.80 and

d density = 0.6594. We chose n-hexane, because the boiling point quite down and low density as compared to water, the difference in density allows performing washing the extract with distilled water and a clear separation of the aqueous lipid fractions.

According to the process adapted to the sequence of operations is as follows:



5. Methods of evaluation of therapeutic effects of lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* extracts in cutaneous inflammatory processes (*in vivo*)

Number of animals
At least 10 animals are used in the treatment group, and at least 5 in the control group. If you use less than 20 guinea pigs in the treatment group and 10 in control and that it was not possible to conclude that the test substance is a sensitizer, it is advisable to test at least 20 animals in the treatment group and 10 in the control.

Decisive test. Local application
Day 20-22. Control and treatment groups.
Ribs animal control and treatment groups are cleaned hair. A gauze saturated with the test substance is applied to one side of the animal and another gauze soaked only in the vehicle can also be applied on the other side. Packs are held in contact by an occlusive dressing for 24 hours.

Remarks. Control and treatment groups.
-About 21 hours after removing the compress, the test area is cleaned and trimmed with attention and / or depilated if necessary;
-About 3 hours later (approximately 48 hours after the start of application) the skin reaction is observed and recorded according to the degree shown below;
-Approximately 24 hours after the observation, a second observation is made (at 72 hours) and again recorded.
Recommended readings in white both control and treatment groups.

Magnusson and Kligman graduated scale for assessing reactions at compress application

0 = no visible change;
1 = erythema discrete or irregular;
2 = moderate and confluent erythema (adherent);
3 = intense erythema and swelling [116].

Data were recorded in the tables, which was calculated parameter of central tendency (mean) and dispersion parameter (standard deviation SD). Hypotheses Validation was performed by student method (t-test, two-Tailes distribution) which was considered a statistical significance p (t) <0.05 significant for rejection of the null hypothesis formulated for each situation. The results are presented in tables and graphs.

Results and discussion

1.Phisico-chemical and biochemical characterization of lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa*

1.1. Characterization of hygienic-sanitary raw material, row material, intermediary substance and lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* [7].

1.1.1. Heavy metal analysis is shown in Table 4.

Table 4. Heavy metal content of the raw material and the dried lipid extract of the two species *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs (mg/kg).

Entry	Heavy	Lim. Max. mg/Kg	*Mytilus* Raw material	*Mytilus* Dry subst.	*Mytilus* Lipid extract	*Rapana* Raw material	*Rapana* Dry subst.	*Rapana* Lipid extrac
1.	Cd	0.1	0.10	0.46	0.22	0.065	0.51	0.16
2.	Cr	-	0.31	0.40	0.28	0.19	1.70	0.78
3.	Pb	0.5	0.17	0.51	0.29	0.09	0.24	0.10
4.	Cu	5.0	7.31	20.02	11.31	10.25	37.14	19.27
5.	M	-	1.90	3.97	2.10	1.23	4.79	2.02
6.	Fe	-	14.47	40.38	24.04	7.25	29.31	13.24

1.1.2. Analysis of pesticides is shown in Table 5.

Table 5. Pesticide content of raw material, dry substance and lipid extract of the vtwo species *Mytilus galloprovincialis* Lmk. and *Rapana venosa* mollusks (mg/kg).

Entry	Pesticide	Lim. Max. mg/Kg	*Mytilus* Raw material	*Mytilus* Dry subst.	*Mytilus* Lipid extract	*Rapana* Raw material	*Rapana* Dry subst.	*Rapana* Lipid extra
1.	HCB	0.2	0	0	0.0469	0,0045	0	0.0711
2.	Lindan	0.02	0	0	0.1188	0,0004	0	0.0644
3.	Heptaclor	0.2	0.0803	0	0.1200	0	0	0.0268
4.	Aldrin	0.2	0.0370	0	0.2096	0	0	0.0487
5.	Dieldrin	0.2	0	0	0	0	0	0
6.	Endrin	0.05	0.0446	0	0.2323	0	0	0.0563

7.	pp' DDE	1	0.0044	0	0.4161	0	0	0.2013
8.	pp' DDD	1	0.0440	0	0.3320	0	0	0.1455
9.	pp' DDT	1	0.0457	0	0.3620	0	0	0.0739

1.1.3. Analysis of the microbiological load is shown in Table 6.

Table 6. Microbiological load the raw material, dry matter and lipid extract of *Mytilus galloprovincialis* Lmk. and *Rapana venosa* nolluscs (CFU/g).

				Mytilus galloprovincialis Lmk.			*Rapana venosa*		
	Dilution	Limits	Unit	Row material	Dry substance	Lipid extract	Row material	Dry substance	Lipid extract
1. Coliforms bacteria	10^{-1}	100	UFC/g	**3000**	**3000**	<10	**3000**	**3000**	<10
2. *Escherichia coli*	10^{-1}	10	UFC/g	**3000**	<10	<10	**3000**	<10	<10
3. *Salmonella*-pathogen	10^{-1}	-	UFC /25 g	-	-	-	-	-	-
4. *Listeria monocytogenes*-phatogen	10^{-1}	-	UFC /25 g	-	-	-	-	-	-
5. Sulphite reductant Clostridium	10^{-2}	10	UFC/g	<10	<10	<10	<10	<10	<10
6. Coagulase-positive Staphylococcus	10^{-2}	10	UFC/g	<10	<10	0	<10	<10	0
7. Total number of germs	10^{-3}	00.000	UFC/g	**300.000**	**300.000**	30.000	**300.000**	**300.000**	18.900

1.2. The composition of lipid fractions and lipid characteristics indexes

1.2.1.Indices features of lipid extracts

Comparative analysis of lipid extracts from both molluscs analyzed was performed along with a vegetable oil, olive oil, in order to highlight their qualities comparative biochemical and biological active.

The second lipid extracts of molluscs are obviously different olive oil, both in color and in consistency (Table 7). Both features may have originated in the source of food for *Rapana venosa* extract is much lighter (right, yellow brown), while *Mytilus galloprovincialis* Lmk. extract shows a much darker color (center, dark brown) (Figure 1).

Table 7. Organoleptic examination of the two extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs.

Organoleptic examination	*Mytilus galloprovincialis* Lmk.	*Rapana venosa*
Aspect	viscous liquid	Semisolid viscous
Colour	dark brown	yellow brown
Smell	marine organisms	marine organisms

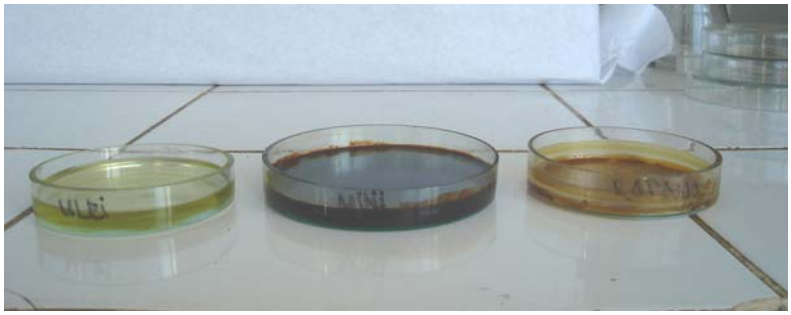


Figure 1. Lipid extracts from the two molluscs *Mytilus galloprovincialis* Lmk. (Center), *Rapana venosa* (right) and olive oil (left).

The data obtained from the analysis of the physico-chemical properties of both Mytilus galloprovincialis Lmk., Rapana venosa lipid extracts are presented in Table 8.

Table 8. Indices features of the two lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* compared with olive oil.

Physico-chemical	*Mytilus galloprovincialis* Lmk.	*Rapana venosa*	Olive oil
relative density	1.077±0.109	0.8353±0.131	0.911±0.0002
Refractive index ($n^{20}D$)	1.482	1.497	1.468
Iodine index (g%)	82.843±0.584	86.546±4.338	66.305±2.832
Acid value (mgKOH/g)	43.268±1.025	38.543±0.532	2.508±0.572
Index of saponification (mgKOH/g)	285.64±5.388	143.55±4.082	112.24±2.875
Unsaponifiable substances (%g)	2.864±0.079	2.539±0.100	2.155±0.154

Changes in characteristics of the indices of the two lipid extracts from *Mytilus galloprovincialis* Lmk, *Rapana venosa* and olive oil is shown in Table 9 and Figures 2,3,4,5 and 6 [5, 14].

Table 9. Indices features variation of the two lipid extracts and olive oil and analyze of their statistical significance.

	Olive oil	*Mytilus galloprovincialis* Lmk.	*Rapana venosa*
Relative density average	0.911	1.077	0.835
DS	0.0002	0.109	0.131
Iodine index average (II)	6 6.305	82.843	86.546
DS	2.832	0.584	4.338
Acidity index average (IA)	2.508	43.268	38.543
DS	0.572	1,025	0,532
Saponification index average (IS)	112.24	285.64	143.556
DS	2.875	5.388	4.082
Unsaponifiable substances average (SN)	2.155	2.864	2.539
DS	0.154	0.079	0.100

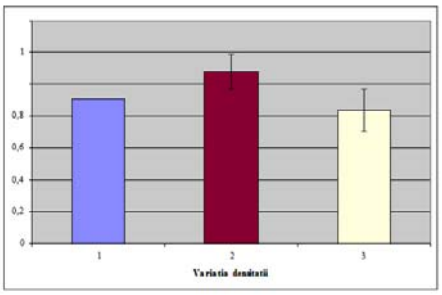


Figure 2.Variation of relative density of lipid extracts from *Mytilus galloprovincialis* Lmk., *Rapana venosa* and olive oil.

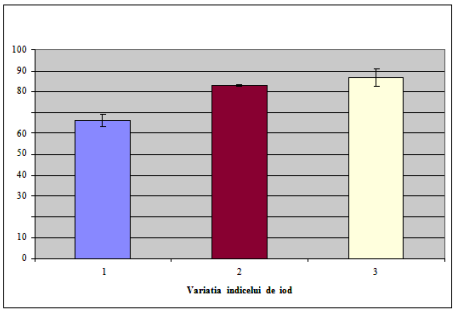


Figure 3. Variation of iodine index from *Mytilus galloprovincialis* Lmk, *Rapana venosa* and olive oil.

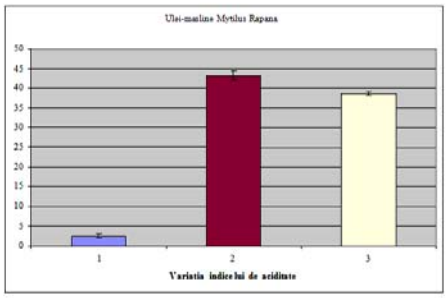


Figure 4.Variation of acidity index of lipid index extracts from *Mytilus galloprovincialis* Lmk., *Rapana venosa* and olive oil.

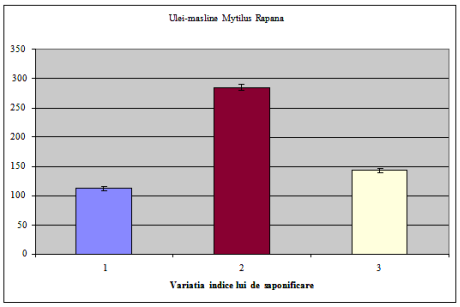


Figure 5. Variation of saponification of lipid extracts from *Mytilus galloprovincialis* Lmk, *Rapana venosa* and olive oil.

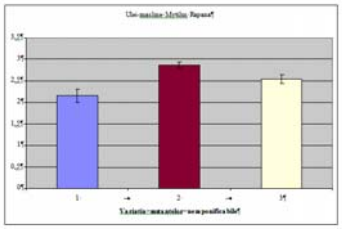


Figure 6. Variation of unsaponifiable substances of lipid extracts from *Mytilus galloprovincialis* Lmk., *Rapana venosa* and olive oil.

The data provided fall within the values provided by Romanian Pharmacopoeia
X for pharmaceutical oils.

By GC/MS analysis, UV / VIS and IR spectra appreciable amounts of valuable lipid fractions were detected in both mollusc species investigated [14].

1.2.2. Chromatography GC / MS.

The spectra obtained by chromatography GC / MS are shown in Figures 7, 8, 9, 10, 11 and 12.

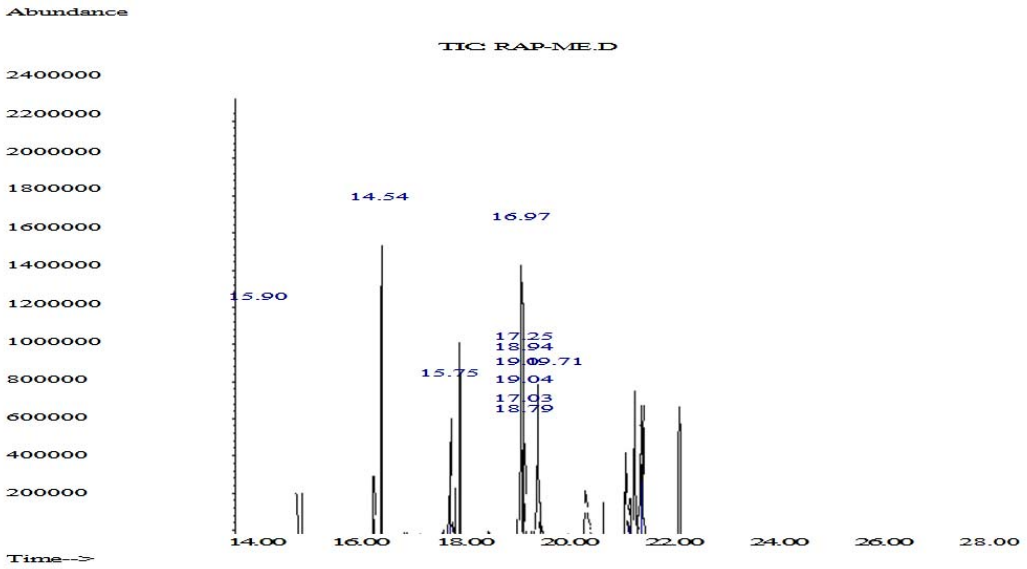


Figure 7. Chromatography GC / MS by the methylation method of lipid extract from *Rapana venosa.*

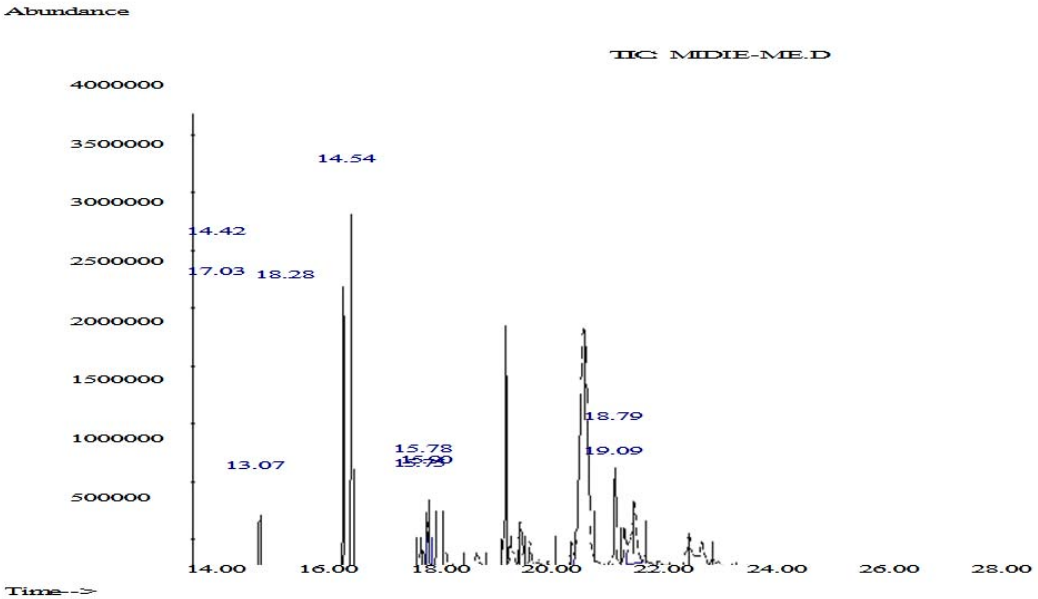


Figure 8. Chromatography GC / MS by the methylation method of lipid extract from *Mytilus galloprovincialis* Lmk.

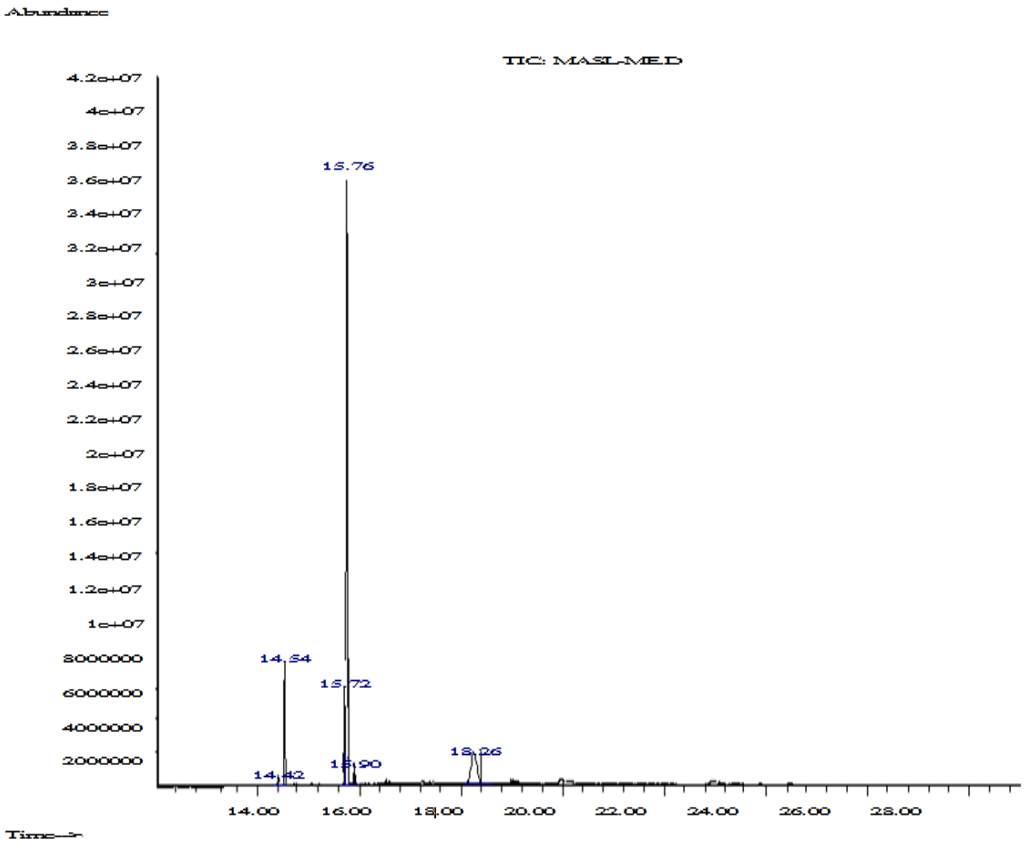


Figure 9. Chromatography GC / MS by the methylation method of olive oil.

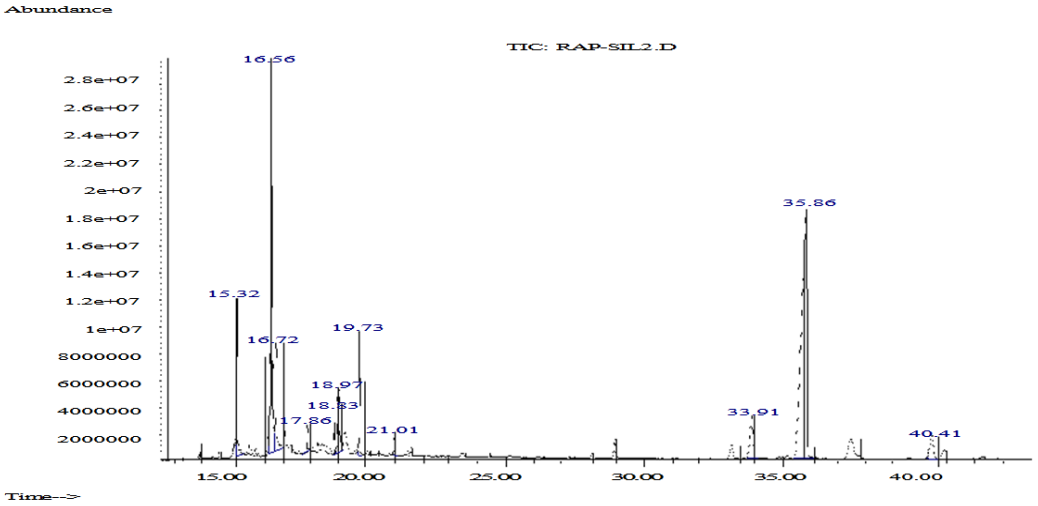


Figure10. Chromatography GC / MS by the method of derivatization

of lipid extract from *Rapana venosa.*

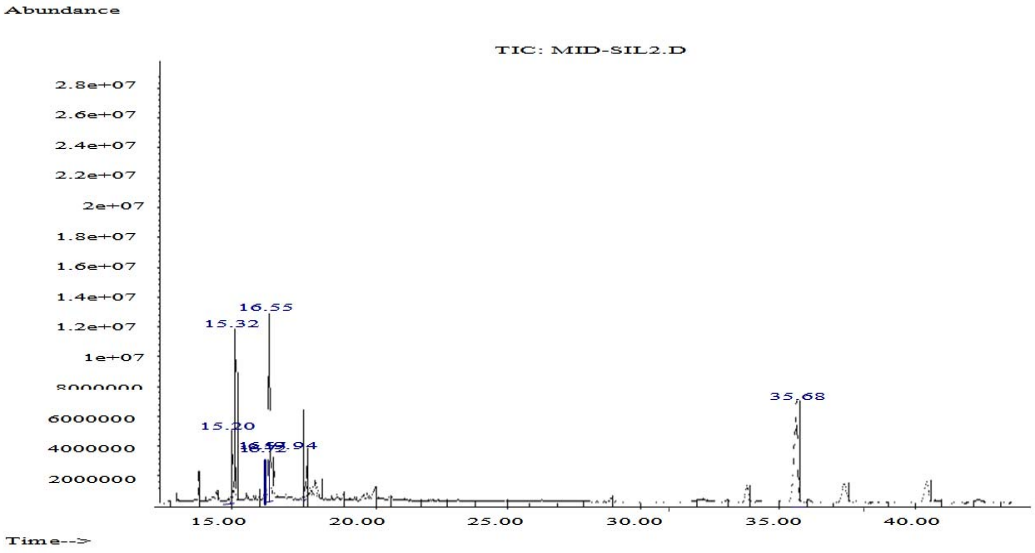


Figure 11. Chromatography GC / MS by the method of derivatization of lipid extract from *Mytilus gallorpovincialis* Lmk.

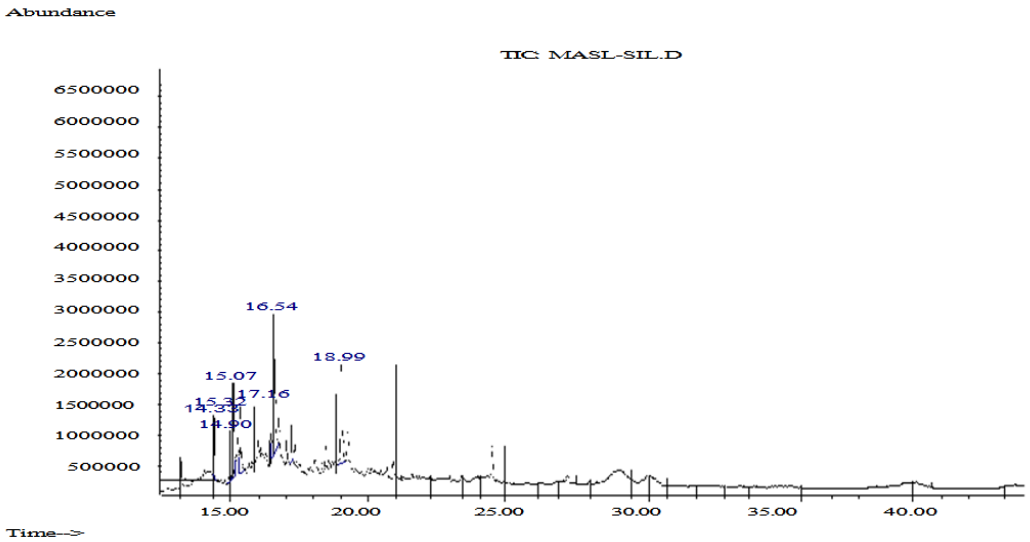


Figure 12. Chromatography GC / MS by the method of derivatization of olive oil.

By applying both methods of GC / MS chromatography, there is a more complex composition of the two species of mollusc extract as compared to olive oil, [5, 14].

1.2.3. UV / VIS analysis.

Figure 13 shows the UV / VIS spectra of *Mytilus galloprovincialis* Lmk., *Rapana venosa* lipid extracts and olive oil.

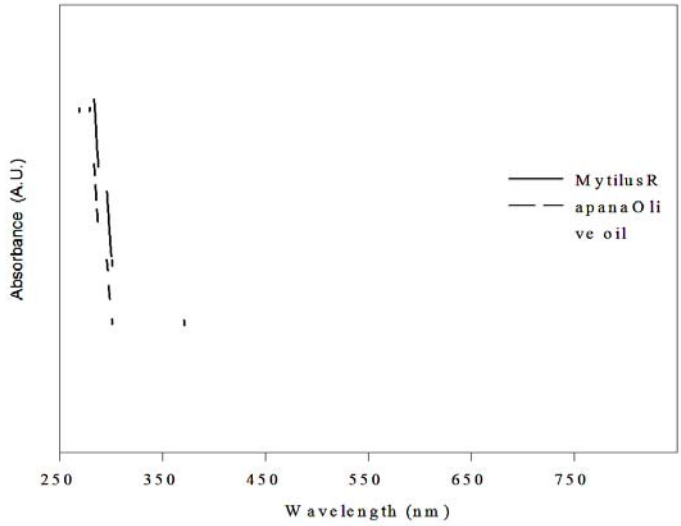


Figure 13. UV-VIS analysis of lipid extracts from *Mytilus galloprovincialis* Lmk., *Rapana venosa* and olive oil.

The compositional diversity of the two molluscs extracts is evident from the olive oil. At the same time, common parts can be seen at the same wavelength but in different concentrations [5, 14].

UV-VIS spectra provide enough information to show the presence of chromophores characteristics of fatty acids, identified by GC / MS chromatography.

1.2.4. IR analysis.

IR spectra (Figures 14 A, B and C) showed significant differences among the three lipid extracts studied.

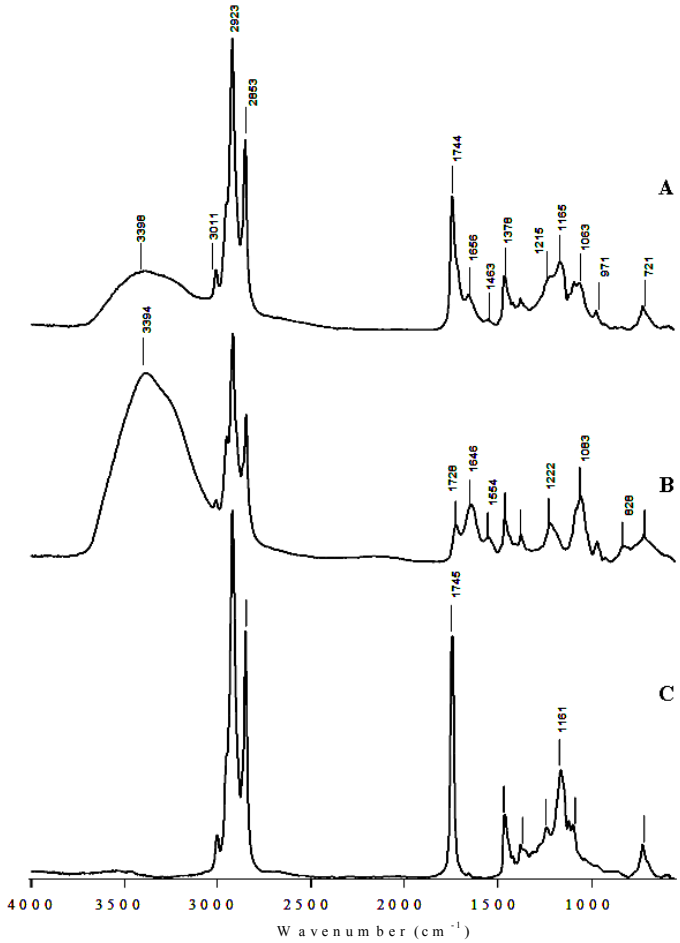


Figure 14. IR spectra of lipid extracts from *Mytilus galloprovincialis* Lmk.(A), *Rapana venosa* (B) and olive oil (C).

Absorption spectra present characteristic frequency of the fatty acids, which confirms the results of the chromatographic GC / MS analysis.

2.1. Action of *Mytilus galloprovincialis* Lmk. şi *Rapana venosa* against the solar radiation

This experiment showed improved sunscreen performance without producing side effects and adverse reactions by replacing synthetic chemicals with lipid extracts of two species of molluscs studied.
It is known that some absorbers of UV radiation can be partially degraded and can
cause deterioration of the skin during UV exposure and of course this

reaction can influence the effectiveness of sunscreen protection. For information on the UV absorber, we investigated *in vitro* SPF parameters of lipid extracts from *Mytilus galloprovincialis* Lmk. şi *Rapana venosa* marine species.
UVA radiation has been measured, the ratio UVA / UVB and SPF *in vitro* by Diffey and Robson method of the following lipid extracts:

1) lipidic extract from *Mytilus galloprovincialis* Lmk.,
2) lipidic extract from *Rapana venosa*,
3) lipidic extracts from *Mytilus galloprovincialis* Lmk.+*Rapana venosa* 1:1 (v/v),
4) lipidic extract from *Mytilus galloprovincialis* Lmk.+absorber UVR 1:1 (v/v)
5) lipidic extract from *Rapana venosa*+absorber UVR 1:1 (v/v).

This experiment was performed to determine the photoprotective capacity of the biologically active substances contained in the lipid extract of *Mytilus galloprovincialis* Lmk. şi *Rapana venosa*.
In the two lipid extracts was introduced an absorber-UVR (Saliform), supported by European standards, to observe its influence on the analyzed samples.
In Table 10 are presented absorbtions of both lipid extracts quantified by UVA domain, UVA / UVB ratio and SPF.

Table 10. UVA radiation, UVA / UVB ratio and SPF of lipid extracts from *Mytilus galloprovincialis* Lmk. şi *Rapana venosa* with and without absorber-UVR.

Nr.	Probe	UVA*	UVA/UVB*	SPF**
1.	lipidic extract *Mytilus galloprovincialis* Lmk.	5	0.81	6.8
2.	lipidic extract *Rapana venosa*	2.0	0.73	2.9
3.	lipidic extracts *Mytilus galloprovincialis* Lmk. +*Rapana venosa* 1:1 (v/v)	3.4	0.60	5.7
4.	lipidic extract *Mytilus galloprovincialis* Lmk. +absorber- UVR (Saliform) 1:1 (v/v)	3.8	0.34	10.9
5.	lipidic extract *Rapana venosa* +absorber -UVR (Saliform)1:1 (v/v)	2.7	0.26	8.8

* relative parameter;
** absolute parameter.

The data obtained show that most UVA absorbance in relative parameter 5 presents *Mytilus galloprovincialis* Lmk lipid extract., in case of the report UVA /UVB with relative parameter 0.81 of the same lipid extract and SPF's where the parameter absolute is 10.9, *Mytilus galloprovincialis* Lmk. + UVR absorber-1:1 (v/v).
As the results of the analyzes performed, the highest absorbance parameters are attributable to *Mytilus galloprovincialis* Lmk lipid extract [6].

2.2. Effect of lipid extracts on microbial cultures of *Escherichia coli*

To highlight the antibacterial effects of two lipid extracts of *Mytilus galloprovincialis* Lmk. and *Rapana venosa* the qualitative Kirby-Bauer diffusion method was applied.
We used a strain of *Escherichia coli* isolated from a urinary tract infection, resistant to most antibiotics. It is known that it has a pronounced susceptibility to imipenen and gentamicin antibiotics. As culture medium it was used standard Mueller-Hinton medium.
In the both test plates the culture is uniformly increased, confirming the strength of the bacteria against the two lipid extracts subject of the experiment.
In order to highlight the effects on cultures of Escherichia coli, a second experiment was performed by disc diffusion method provided by the Romanian Pharmacopoeia X [192]. In this experiment, the witness was the same antibiotics used in the first Kirby-Bauer disc diffusion method (IPM-imipenen and GM-gentamicin).
Using a pure strain of *Escherichia coli*, and a TBX-type culture medium (tryptone ball glucuronidase).
In this case, the result is different, as follows: in the 3 plates subjected to the experiment, the culture is increased uniformly, except plate of *Mytilus galloprovincialis* Lmk lipid extract, in that there is a clear zone, no bacterial colonies, as defined, with a diameter of 5 mm zone of inhibition (Figure 15).

Fig. 15. Plate 2 - TBX medium seeded with *Escherichia coli* and *Mytilus galloprovincialis* Lmk lipid extract.

The antibacterial effect against the action of the bacterium *Escherichia coli* may be quantified (Table 11) based on the sensitivity (the diameters of the inhibition zones) obtained:
- Witnesses: IPM = 9 mm; MW = 7 mm;
- *Mytilus galloprovincialis* Lmk lipid extract = 5 mm;
- And the amount of product used.

Table 11. Comparison of antibacterial effects presented by lipid extract from *Mytilus galloprovincialis* Lmk. and antibiotics IPM (imipenen) and GM (gentamicin).

Probe	Quantity (mg)	The diameter of the inhibition zone (mm)
IPM (imipenen)	0.03	9
GM (gentamicina)	0.01	7
lipidic extract from *Mytilus galloprovincialis* Lmk.	0.3	5

2.3. The therapeutic effect of the lipid extract on **the thermal burn induced in Wistar rats**

In the present experiment, there were investigated the anti-inflammatory and dermorestitutive effects of lipid extracts of *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs using histological techniques and hematological analysis.

This experiment was conducted on a group of 15 rats Wistar, selected from Biobase of Faculty of Natural and Agricultural Sciences, Ovidius University of Constanţa from Romania.

Wistar rats showed the following characteristics:
- Weight 250 ± 10 g and males.

These rats received a thermal burn induced by application to the back of a quantity of 1 ml of water at 90 ± 2^0C, an area of 2.5 cm for 5 seconds, resulting in a second-degree burn.

In view of the fact that all these parameters were constant for all Wistar rats subjected to the experiment, the results could be interpreted on account of the different treatments applied.

The 15 Wistar rats were divided into 5 groups, each group having 3 rats as

follows:
- Group I, Control: Wistar rats with thermal burn untreated;
- Group II: Wistar rats with thermal burns treated with the lipid extracts from *Mytilus galloprovincialis* Lmk.+*Rapana venosa* 1:1 (v/v), applied 2 times / day;
- Group III: thermal burn Wistar rats treated with *galloprovincialis* Lmk.lipid extract., Applied 2 times / day;
Group IV: thermal burn Wistar rats treated with lipid extract from *Rapana venosa*, applied 2 times / day;
- Group V: thermal burn Wistar rats treated with specific burn ointment, applied 2 times / day.

Figures 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25 present the image of the tissues from groups I, II, III, IV and V, treated with the lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* before and after treatment.

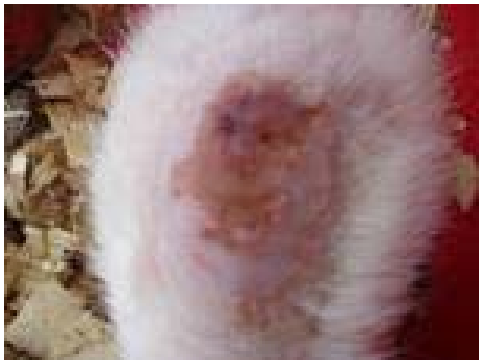


Fig. 16. Group I. Control. Wistar rats, 1 day before treatment.

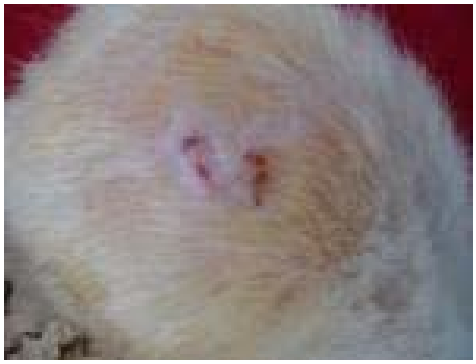


Fig.17. Group I, Control. Wistar rats, 21 days after treatment.

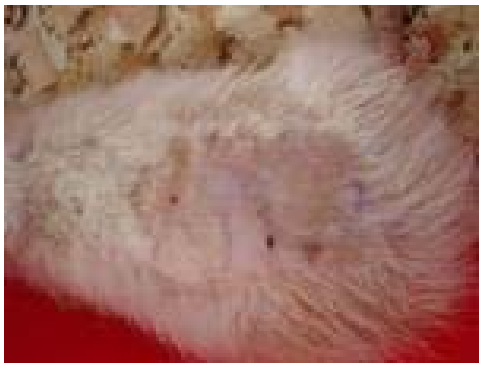


Fig.18. Group II. Wistar rats 1 day before treatment.

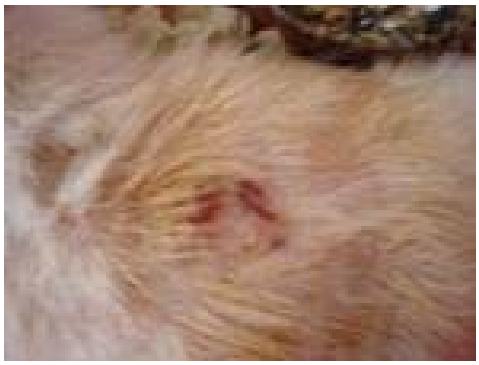


Fig. 19. Group II. Wistar rats, 13 days after treatment.

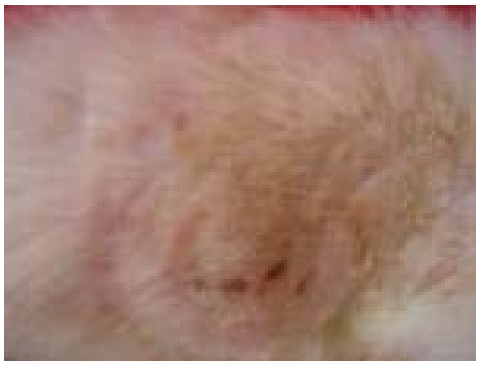


Fig.20. Group III. Wistar rats
1 day before treatment.

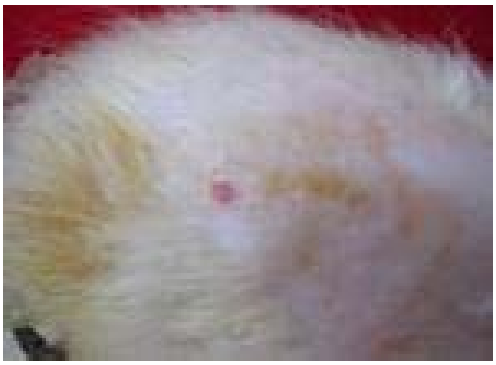


Fig. 21. Group III. Wistar rats, 12 days after treatment.

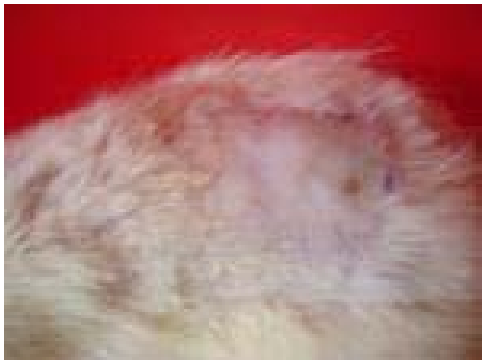


Fig. 22. Group IV. Wistar rats
1 - day prior to treatment.

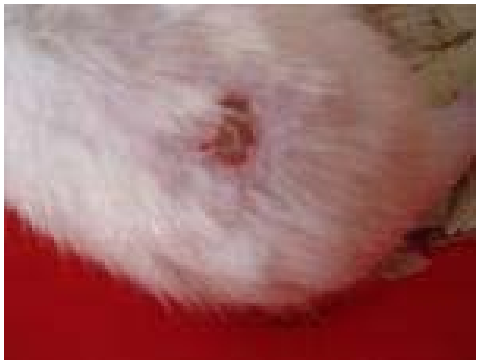


Fig. 23. Group IV. Wistar rats, 14 days after treatment.

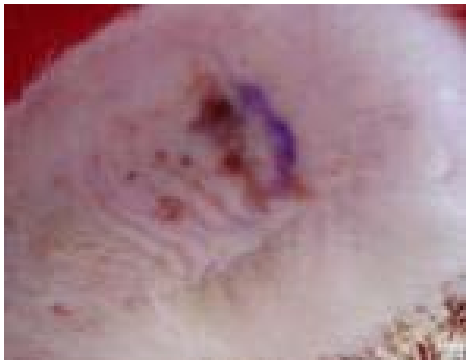


Fig. 24. Group V. Wistar rats, 1day before treatment.

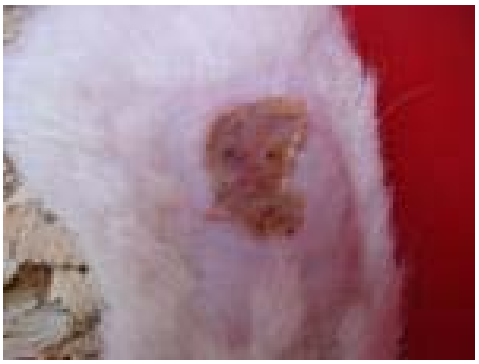


Fig. 25. Group V. Wistar rat, 14 days after treatment.

Treatment of 15 Wistar rats of the 5 groups consisted of lubrication of the tissues affected by the burning heat with pure specific extract 2 times per day.
Thus, we could draw the following conclusions:
Group I, Control: Wistar rats with thermal burns, untreated: the majority of their curing is observed to day 21 (one of three rats) the positive therapeutic effect of 33%.
Group II: Wistar rats with thermal burns treated with the lipid extracts *Mytilus galloprovincialis* Lmk.+*Rapana venosa* 1:1 (v/v), applied 2 times / day: the majority of their cure are observed on the 13th day of treatment (two of three rats) the positive therapeutic effect of 66%.
Group III: thermal burn Wistar rats treated with *Mytilus galloprovincialis* Lmk.lipid extract, applied 2 times / day: their healing is observed in the majority of 12th day of treatment (two of three rats) the positive therapeutic effect of 66%.
Group IV: Wistar rats with thermal burns treated with the lipid extract *Rapana venosa* applied 2 times / day: the majority of their curing is observed in 14-day-treatment (one of three rats) the positive therapeutic effect of 33 %.
Group V: Wistar rats with thermal burns, burns treated with specific ointment, applied 2 times / day: the majority of their curing is observed to day 14 of treatment (two of three rats) the positive therapeutic effect in 66%.

The experiment *in vivo*, shows a remarkable anti-inflammatory and dermorestitutive effect from lipid extract of *Mytilus galloprovincialis* Lmk

2.4. Histology and hematology analysis

For more precise differentiation of the effects exerted by the two lipid extracts were taken tissue samples in order to perform histological analysis and blood samples for hematology.
Both blood and tissues were collected from a Wistar rat, and which has not been subjected to the experiment, are used as control in the histologic analysis sample [8].

Figures 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 and 37 submitted to smears of blood and tissues from five groups of Wistar rats subjected to the experiment.

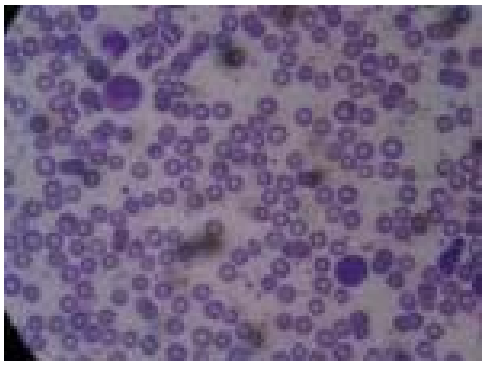


Fig.26. Blood smear-control group, 10x100, MGG.

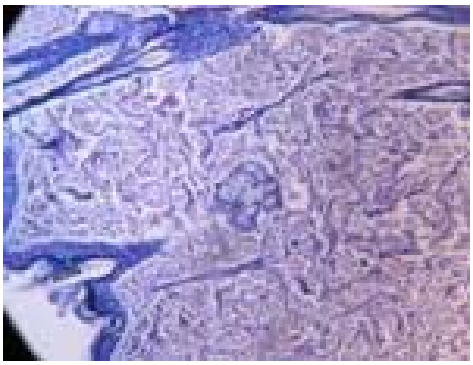


Fig.27. Smear tissue-control group, 20x10, HE.

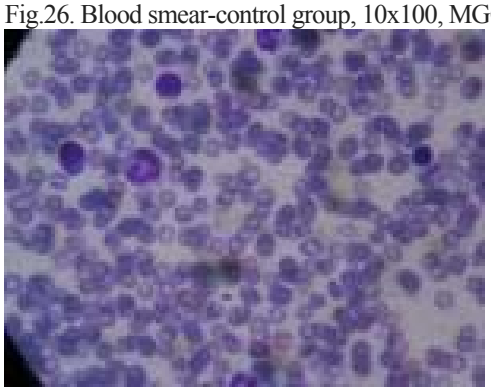


Fig.28. Smear blood-group I, 10x100, MGG.

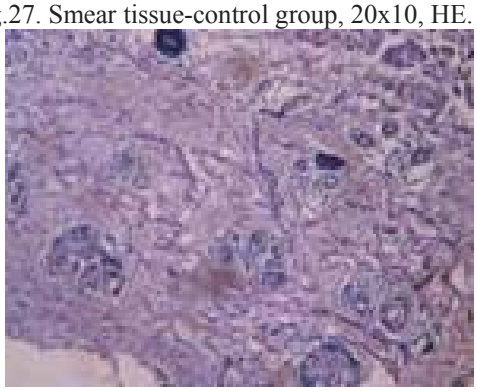


Fig.29. Smear tissue-lot I, 20x10, HE.

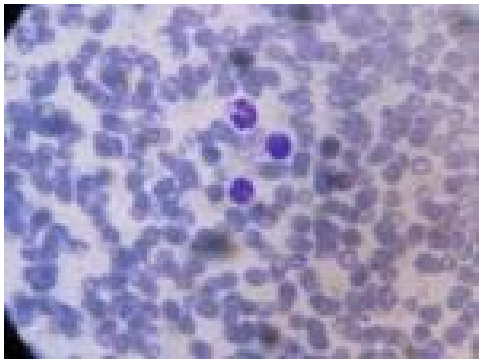


Fig.30. Smear blood-group II, 10x100, MGG.

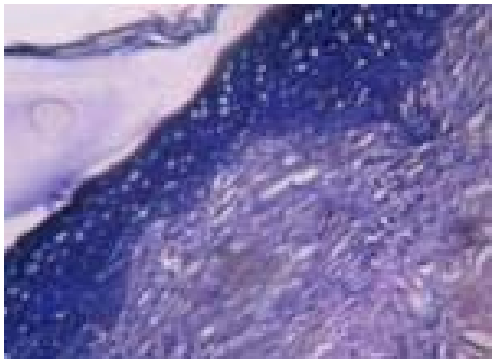


Fig.31. Smear tissue-lot II, 20x10, HE.

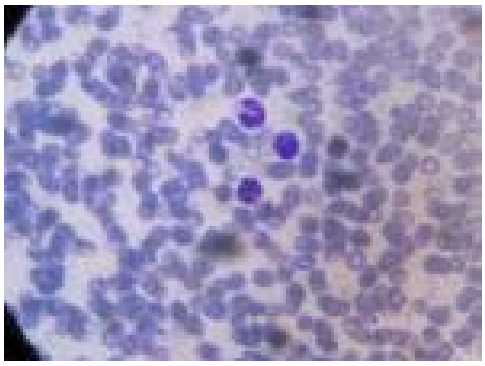


Fig.32. Smear blood-group III, 10x100, MGG.

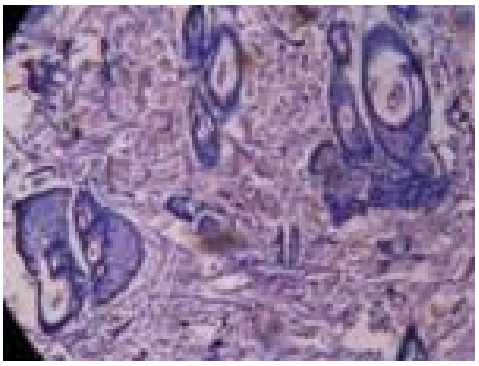


Fig.33. Smear weaving group III, 20x10, HE.

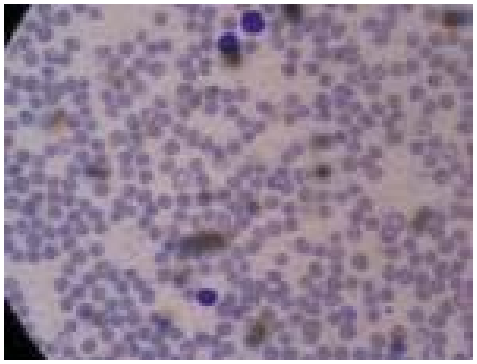


Fig. 34. Smear blood-group IV, 10x100, MGG

Fig.35. Smear weaving group IV, 20x10, HE.

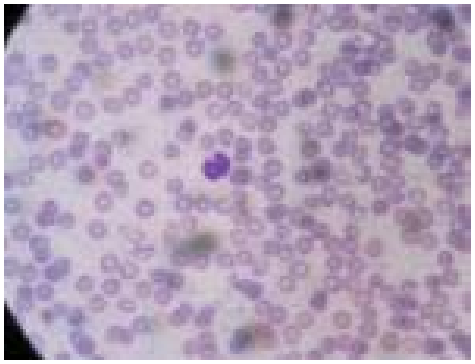


Fig.36. Smear blood-group V, 10x100, MGG.

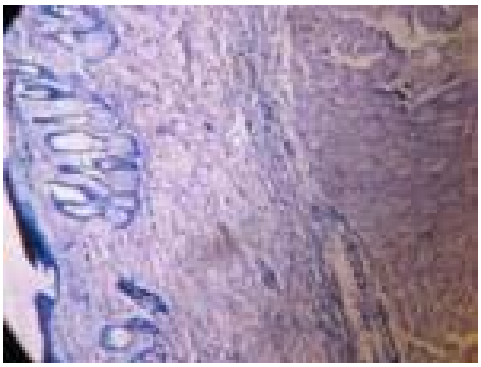


Fig.37. Smear tissue-lotV, 20x10, HE.

Results allow us the following concluding remarks:
- The first tissue recovered on the 12th day after the treatment, occurs in the group III due to *Mytilus galloprovincialis* Lmk. lipid extract.,
- The second tissue recovered on the 13th day after the treatment, is the second group and due to lipid extract of *Mytilus galloprovincialis* Lmk+*Rapana venosa*

1:1 (v / v);
- The third tissue restored in the 14th day after treatment is represented by both the group and the groups IV and V is due to the lipid extract of *Rapana venosa* and specific ointment burns.
- Last tissue restored in the 21th day after treatment, is the group I of Wistar rats with untreated burning [5, 8].

In the view of the fact that the lipid extract were applied as such, without any previous incorporated in an ointment base should be able to take part to some extent in the healing of burns, inflammatory effect has not been calculated in percentage (E%) from the formula of Newbould, but the statistical significance of the results (Table 12 and Figure 38).

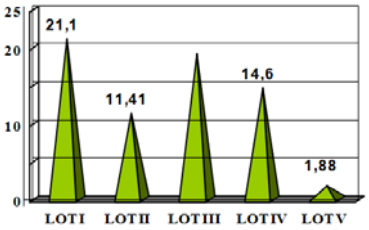


Fig.39. Leukocytes average variations.

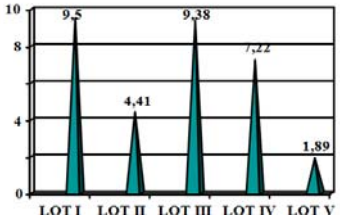


Fig.40 Lymphocytes average variations (LYM).

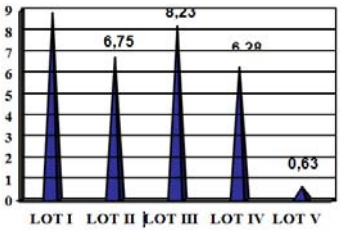


Fig.41. Granulocytes average variations (GRA).

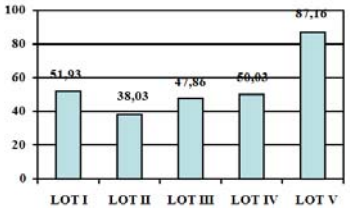


Fig.42. Lymphocytes average variations (LY).

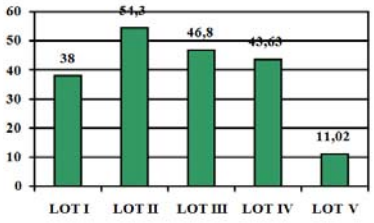


Fig.43. Granulocytes average variations GR).

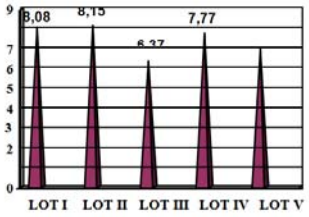


Fig.44 Erythrocytes average variations.

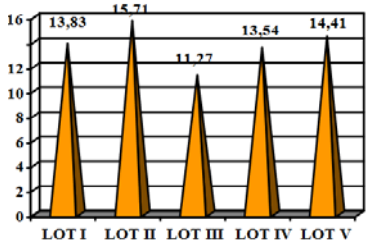


Fig.45. Hemoglobin average variations.

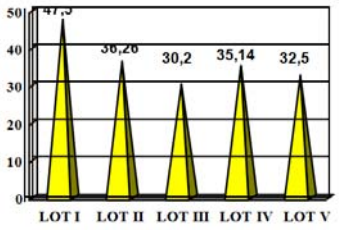


Fig.46.Hematocrit average variations.

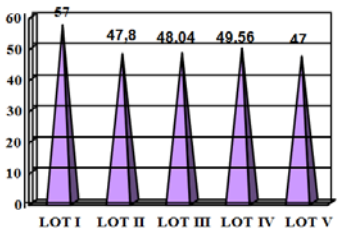


Fig.47. Average erythrocytar volume.

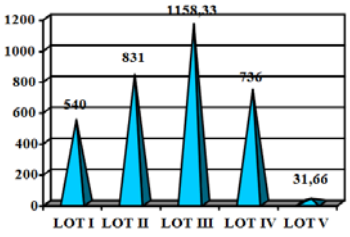


Fig.48. Platelets average variations.

Given the above Figures showing average values of blood components analyzed, the following conclusions are asserted:

- Changes in leukocyte average (Figure 39) shows that the average lowest is recorded in group V, followed by groups II, IV, III and I;
- Lymphocyte average variations (see Figures 40 and 42), tilts the balance of the groups II and III, followed by other groups, namely, IV, I and V;

- Changes in granulocytes average (Figures 41 and 43) shows the presence of the best values average in group V, followed in descending order, groups II, IV, III and I;
- Changes in erythrocyte average (Figure 44), confirms the minimum average in group III, this time following groups V, IV, II and I;
- Changes in mean hemoglobin (Figure 45), plays the same average minimum group III, followed by the groups V, IV, I and II.
- Average hematocrit variations (Figure 46) recorded the lowest average group III, followed by groups V, IV, II and I;
- Changes in average mean corpuscular volume (Figure 47) shows the minimum average value of groups II and III, followed by the groups V, IV and I;
- Changes in platelet average (Figure 48) shows that the average was presented by group V, followed by groups I, IV, II and III [8].

Thus, of the 8 constant blood analyzed, 5 of them (62.5%), confirming the restoration of the third group, represented by Wistar rats with thermal burn treated with lipid extract from *Mytilus galloprovincialis* Lmk. [5, 8].

2.5. Immunohistochemical and biohistometric study

To understand the healing process and to show the difference between tissues restored between all 5 groups of Wistar rats, it was analyzed for its role IV collagen antibody, and more, recovery data of hypodermis, the dermis and epidermis in cases of burns. The study was conducted on 5 groups of Wistar rats subjected to thermal burns of Iind degree and a blank in which the tissue of a Wistar rat, which was not subjected to the experiment.
Collagen IV antibody has been used.

Figures 49, 50, 51, 52, 53 and 54, represent tissue staining with specific antibodies.

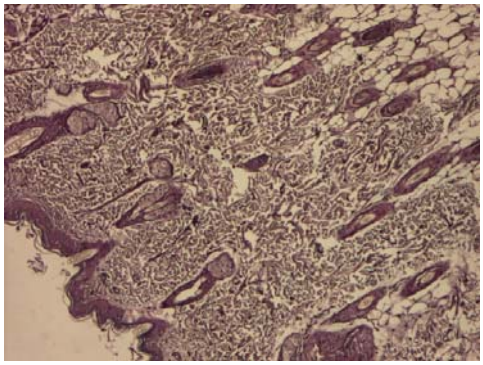


Fig.49. Skin biopsy material - Control,

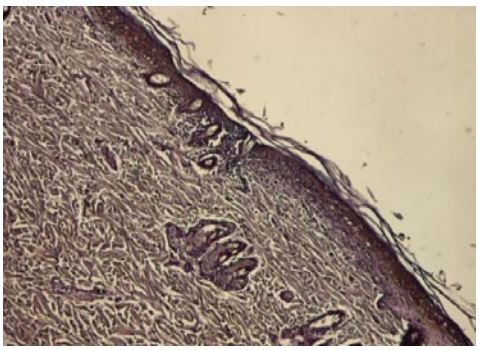


Fig.50. Skin biopsy material - group I,

×40, C IV.

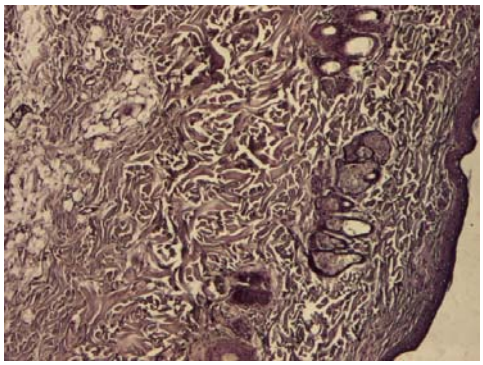


Fig.51. Skin biopsy material - control II, ×40, C IV.

×40, C IV.

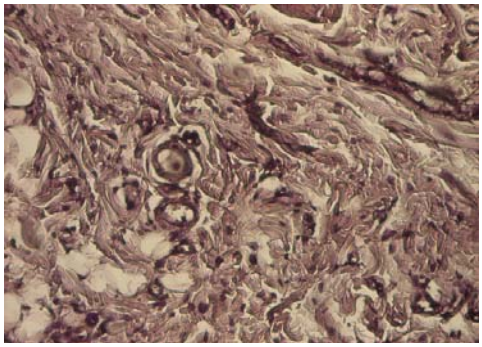


Fig.52. Skin biopsy material - control III, ×100, C IV.

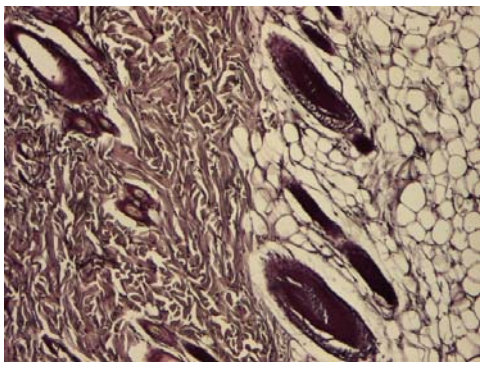


Fig.53. Skin biopsy material - control IV, ×100, C IV.

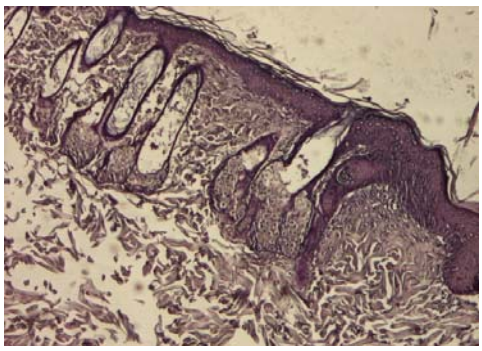


Fig.54. Skin biopsy material - control V, ×40, C IV.

Group I, Control. The tissue of Wistar rats show a moderate positive (+ +) immunoreaction in the epidermis, dermis, vascular wall and sebaceous glands.
Group II. The reaction is weakly positive (+) in the dermis and hair follicles.
Group III. It shows a weak positive reaction (+) in the dermis and hair follicles follicles and highly positive (+ + +) in the hypodermis, and blood vessel muscle cells.
Group IV. Observe well colored portions, moderately positive (+ +) in the dermis, the vascular wall and weak positive (+) in the hair follicles.
Group V. A moderate positive reaction (+ +) in the vascular wall and glands.

As can be seen, group III has high affinity to collagen IV compared with other groups, including almost all layers of connective tissue, which supports the healing process by restoring and regenerating of the skin tissue [9]. In Table 14,

the criteria of differentiation in tissue repair immunostaining are given for the five groups of Wistar rats subjected to the experiment.

Table 14. Criteria of differentiation in tissue repair of the five groups of Wistar rats by IV collagen immunostaining.

Group	Colagen IV (Clone C IV, 22, Dako Cytomation)
Group I- Control	++
Group II	+
Group III	+++
Group IV	+
Group V	++

In order to quantify the results, the study was conducted biohistometric followed by statistical evaluation of the thickness of collagen (Figures 55, 56, 57, 58, 59 and 60).

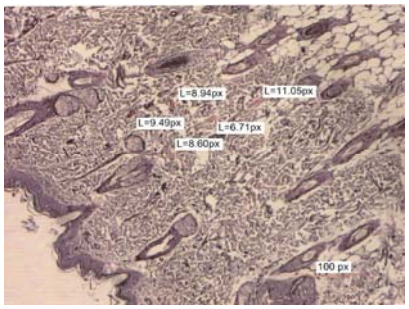


Fig.55. Skin biopsy material - Control, ×40, C IV.

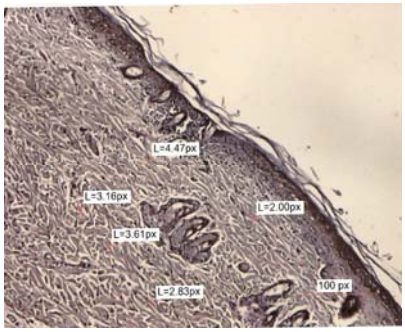


Fig.56. Skin biopsy material - group I, ×40, C IV.

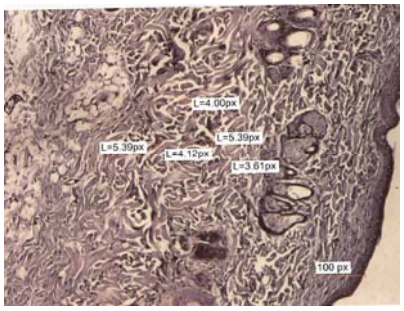


Fig.57. Skin biopsy material - group II,

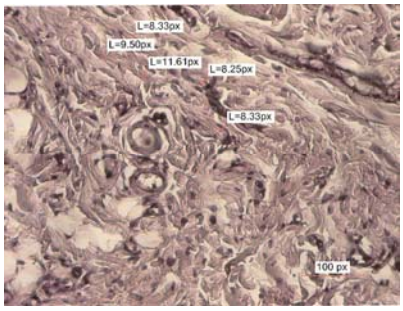


Fig.58. Skin biopsy material - group III,

×40, C IV.

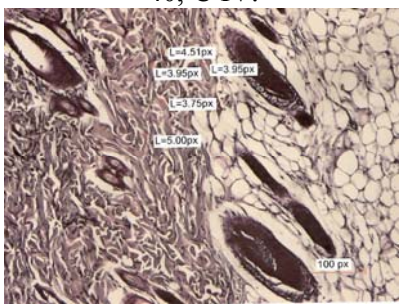


Fig.59. Skin biopsy material - group IV, ×40, C IV.

×40, C IV.

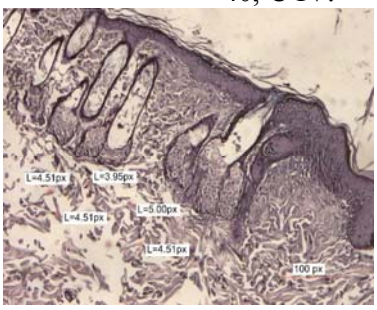


Fig.60. Skin biopsy material - group V, ×100, C IV.

The data are summarized in the table and plotted. The variation of the thickness of collagen is shown in Table 15 and Figure 6.

Table 15. Changes in the thickness of collagen fibers in the skin tissues of the 5 groups of Wistar rats subjected to experiment (px).

No.	Group I, C	Group II	I Group II	Group IV	Group V
1.	2.94	4.72	9.17	4.67	4.42
2.	3.21	4.50	9.20	4.23	4.49
3.	3.06	4.99	9.04	4.15	4.19
Average	3.07	4.74	9.13	4.35	4.37
SD	0.13	0.24	0.08	0.28	0.15
p(t) against Group		0.99	0.99	0.99	0.99
p(t) against Group I, C		0.001	2.16	0.006	0.0004
p(t) against Group			0.99	0.88	0.91
p(t) against Group I			0.0003	0.14	0.10
t against Group III				0.99	0.99
p(t) against Group III				0.0004	1.67
t against Group IV					0.39
p(t) against Group IV					0.92

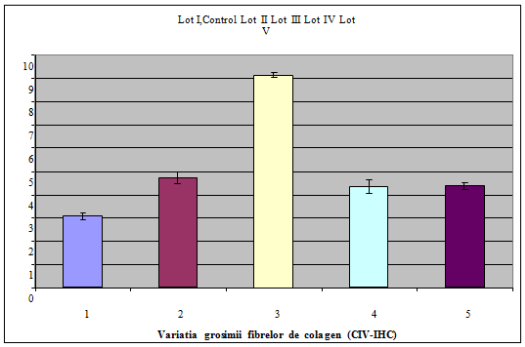


Figure 61. Graph variation thickness of collagen in skin tissues of the 5 groups of Wistar rats subjected to experiment (px).

From the analysis there is a statistically significant difference ($p < 0.05$) in groups II, IV and V compared to group I (control). Also, this difference is observed for the third group, compared with group II and group IV compared to group III [9].

3. Development of the necessary pharmaceutical extracts and incorporation of lipid emulsions and oinments. Their characterization and quality control

There has been created three emulsions in which the active material was the lipid extracts of the two molluscs and their mixtures in a ratio of 1:1 (v / v). The base emulsion consisted of liquid paraffin, Span 60 (sorbitan monostearate), glycerol, nipagin, lavender oil and distilled water.

Quality control of these emulsions was carried out at intervals of 14, 30, 60 and 90 days, consisting of the following measurements: appearance, color, odor, type emulsion based on staining method and the method of dilution; the measurement of electrical conductivity; determination of viscosity; stability determination, determination of pH and electric voltage [10, 61].

In addition, there were made six types of ointments using two different formulations of the pharmaceutical base. Three basic variants consisting in lanolin and petrolatum ointment which compounds the simple ointment and three variants of cetyl alcohol, white wax, lanolin, stearic acid, castor oil, which compounds the complex ointment. On this basis, corresponding lipid extract was added and lavender oil for correcting smell.

This ointment quality control was conducted every 14, 30, 60 and 90 days, consisting of the following measurements: appearance, color, odor, determination of the pH and electric voltage, penetration determining the by the rod method, determination of tensile [11, 61].

4. Making of sensitivity of guinea pig skin tissue with lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa*

As a control substance it was used DNCB value (1-chloro-2,4-dinitro-benzene), vehicle-inducing 80% ethanol and acetone as vehicle challenge.

Animals. Guinea pigs young adult from the biobase provided by the Institute of Public Health, allocated and serialized. Animal conditions were examined before the start of the study and the animals were acclimatized for 5 days before starting the experiment. The ambient temperature was 20-22^{o}C, 45-70% relative humidity, natural light cycle.

Test material administration. The product (corresponding to 2 ml) was applied to each of the 20 animals in contact with the shaved skin, under small glass chambers attached to the side treated with the help of an adhesive tape. Another 10 animals were used for DNCB application (0.5 ml) in ethanol 80%, constituting the control group. Animals were exposed between 9 am and 3 pm.

At 3 in the afternoon (after 6 hours) glass chambers were removed, and the treated flanks were washed with methanol and water.

This treatment was applied to Guinea pigs three times, weekly. After 14 days, the same guinea pigs were treated (challenge response) to another, untreated area (right hip).

Steps:

1. Induction: 10 females and 10 males are treated with the test substance on a shaved hip, for a period of six hours, once every week, three treatments totally.

2. Blank test: 10 animals are treated similarly with DNCB (1-chloro-2, 4 - dinitrobenzene).

3. Challenge / test 14 days after the third induction treatment the animals are treated again with the test substance, but on the other untreated side. 5 untreated females and 5 males constitute a control group, being treated similarly with the test material. It is performed also a DNCB witness control.

Changes in skin irritation were recorded at 24 and 48 hours after each treatment. To assess these changes Draize scale was used:

Erythema and eschar formation	Value
No erythema	0
Very weak erythema (barely perceptible)	1
Well-defined erythema	2
Erythema moderate - severe	3
Severe erythema (deep red) to the formation of bedsores	4

Edema formation

No edema	0
Edem very weak (barely perceptible)	1
Mild edema (edges well defined by swelling)	2
Moderate edema (swollen about 1 inch)	3
Severe edema (swollen more than 1 millimeter)	4

In terms of this study (test report no. 437/2008, testing sensitizing potential in guinea pigs of the three lipid extract from Mytilus galloprovincialis Lmk, Rapana venosa and Mytilus galloprovincialis Lmk. + Rapana venosa 1:1 (v / v), the products led to their classification as **non-sensitized.**

5. Possibilities of recycling and recovery of waste

Processing procedures of raw materials to obtain lipid extracts and to achieve dermofarmaceutical products, consisted of two distinct phases. The first stage generates wastes whose sequence in order of decreasing quantitaties related to raw material is presented in Table 16.

Table 16. Quantities of waste resulting from meat processing of *Mytilus galloprovincialis* Lmk and *Rapana venosa*.

	Mytilus galloprovincialis Lmk	*Rapana venosa*
Shellfish	45-50%	55-60%
Protein residue delipoidated	20-25%	30-35%
Water laundering and processing	depending on the amount	depending on the amount
Spent solvents in the extraction process	depending on the amount extraction	depending on the amount extraction

The second stage of achieving emulsions and ointments does not lead to waste, because all components placed in process are fully consumptioned.
The protein residue derived from our experiments can be used as such or in mixtures, their chemical composition are shown in Table 17 [12].

Table 17. Chemical composition of protein extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa*.

	Proteic extract of *Mytilus galloprovincialis* Lmk.	Proteic extract of *Rapana venosa*
Yield%	24-26	25-29
Dry matter %	13,7-14,8	16,5-18,3
Ash (% of dry matter)	5,3-9,7	6,7-8,84
Organic matter (% of dry matter)	79,4-90,16	83,2-92,14
N_2 total	6,69	8,02-9,31
Total protein (N% x 6.25)	41,81	50,13-58,19

Nutritional qualityties of the two extracts are highlighted by the presence of high concentrations of the eight essential amino acids, namely valine, leucine, isoleucine, threonine, methionine, phenylalanine, lysine and tryptophan (Table 18).

Table 18. Composition of amino acid residues of the two proteins and their quantitative processing, the shellfish *Mytilus galloprovincialis* Lmk. and *Rapana venosa*.

Nr. crt.	Compounds	Concentration (mg%)
1.	Alanine	21,66
2.	Valine	5,50
3.	Leucine	6,60
4.	Izoleucine	3,45
5.	Proline	4,66
6.	Glicyne	7,56
7.	Serine	4,10
8.	Treonine	3,73
9.	Aspartic acyd	5,03
10.	Metionine	2,57
11.	Glutamic acyd	15,26
12.	Fenil-alanine	3,72
13.	Lizine	8,98
14.	Tirozine	3,63
15.	tryptophan	1,53
	TOTAL	97,93

Research in the field [12], have shown some positive effects of behavior and the health status following the introduction of captive wild animals feeding with protein flour derived from *Mytilus galloprovincialis* Lmk and *Rapana venosa*.
Thus, we conducted a *Vulpes vulpes* L. species experiment, captive in Microreserve of the Natural Museum of Natural Sciences (Dolphinarium, Constanta), monitoring the behavior and its physiological functions.
Protein extracts obtained from the two molluscs were administered orally once daily for six months, at a dose of 200 mg. 8 copies of foxes were divided into two groups, five adults and three juniors. We analyzed blood levels of Ca^{2+}, $PO4^{3-}$, TGP and TGO.
Through preliminary sampling administered protein extracts revealed a low percentage of the ratio $Ca^{2+}/PO4^{3-}$ both the junior and adult specimens (Table 19) expressed by blood tests or clinical through a diminished rickets process and a matte aspect of hair.

Table 19. Biochemical analyzes of eight *Vulpes vulpes* L. specimens (before and after six months of treatment).

		Before treatment				After six months of treatment			
Nr crt.	Specimens	Ca^{2+}* (mg/dl)	$PO4^{3-}$* (mg/dl)	TGP* (U/l)	TPO* (U/l)	Ca^{2+}* (mg/dl)	$PO4^{3-}$* (mg/dl)	TGP* (U/l)	TGO* (U/l)
1.	Adult	6,64	2,76	24,6	67,3	8,6	5,3	36,4	29,3
2.	Adult	6,53	3,9	63,2	65,7	9,3	4,2	42,1	27,2
3.	Adult	7,19	4,9	61,8	51,0	10,4	5,7	39,7	31,1
4.	Adult	7,93	5,2	61,3	54,3	11,1	6,2	35,2	28,4
5.	Adult	6,96	3,9	45,1	64,1	9,3	4,5	26,3	22,3
6.	Junior	8	4,2	42,2	53,2	9,7	6,5	19,8	17,3
7.	Junior	7,1	5,8	57,0	46,5	10,7	6,9	21,1	18,7
8.	Junior	7,1	4,5	27,9	30,7	11,3	7,1	18,3	19,3

* Normale values: - Ca^{2+}: 8,5-11,5 mg/dl;
- $PO4^{3-}$: 3,0-7,0 mg/dl;
- TGP: 0-40 U/l;
- TGO: 0-30 U/l [91].

Elevated transaminase values from *Vulpes vulpes* L. specimens studied may be due to problems with non-infectious causes (eg steatosis), infectious (hepatitis Rubarth) or parasitic causes [13].
During the six months of adjunctive therapy was observed normalization of the four biochemical blood parameters investigated, the degree of assimilation is

raised to species of arctic fox, unnoticed as adverse symptoms or abnormal renal excretion clereance's.
The solvents used in the process (n-hexane or benzene extraction) is recovered at a rate of 90-95% in concentration. The amount of solvent used is dependent on the size of the batch in use. Commonly used ratio was 1:1 (v/w) solvent/dry meat powder. The solvent recovered is mixed with fresh solvent in a ratio of 1:2 (v/v) and may be reintroduced into the process. Basically, there are only minor losses by manipulation.

Conclusions

PhD thesis "Study of applications of lipid extracts from Mytilus galloprovincialis Lmk. and Rapana venosa molluscs" address the complex methodological investigation focused on three main areas namely obtaining of lipid extracts and analyzing their biological and physico-chemical properties, in vitro and in vivo studies, making of pharmaceutical products (emulsions and ointments) and assessing the biologically active potential and the possibilities of exploiting the natural resources of marine origin.

In analytical practice, the focus was on Romanian Pharmacopoeia methodology Clinical Laboratory and laboratories specializing in food hygiene practice, human and animal health. Extraction procedures and techniques were taken from the art and adapted to marine raw material novelties. In vitro and in vivo experiments have been carried out by the recently used specialized laboratories methodologies.

There were performed topical pharmaceuticals (emulsions and ointments), but due to inappropriate choice of preservatives, they were not within the rules of the National Medicines Agency (NMA) as of the stability tests. Investigations continue in order to improve conditioning formulations and extend the experiments *in vitro* and *in vivo*.

Thus, the analysis of the results of a comprehensive study of the applicability of lipid extracts from Mytilus galloprovincialis Lmk. and Rapana venosa molluscs, allow us to make the following feedback, comments and conclusions:

1. Hygienic-sanitary characterization of raw material, dry matter and lipid extracts of *Mytilus galloprovincialis* Lmk. and *Rapana venosa*, allowed us to conclude the following:

-High levels of heavy metals and pesticides in the raw material, dry matter and lipid extracts obtained can be attributed to the accumulation in the sample zone (Baia Mamaia $44^0$10'N, $28^0$41'E Romanian sector of the Black Sea) of different microorganisms due to pollution. It is noted, however, that the presence of microorganisms in the lipid extracts decreases than in the raw material which

suggests that processing and production process has the bactericidal effects;

-Given the content in heavy metals and pesticides higher than accepted rules compared to microbiological load can be concluded that the two molluscs samples analyzed have biological risk factors on the use of end products, lipid extracts from Mytilus galloprovincialis Lmk and Rapana venosa natural catches for pharmaceutical purposes.

Thus, it should be used as raw material for the pharmaceutical industry mussels obtained from cultures.

2. Data obtained from the analysis of lipid fractions composition and lipid characteristics indices of the two molluscs compared to olive oil, allow the following concluding remarks:

- The two lipid extracts from *Mytilus galloprovincialis* molluscs Lmk. and *Rapana*
venosa molluscs present greater more complex composition compared to olive oil with a high content of polyunsaturated fatty acids, in particular 4,8,12-trimetildecanoic,
4,7,10,13,16,19-docosahexaenoic acid 10-octadecanoic, 5,8,11,14,17-eicosapentaenoic, arachidonic, 11, eicosanoic, 24-nor-22 ,23-metilcolest-5-en- 3β-ol, 3β-22E-ergosta-7, 22 - dien-3-ol, 3β, 24R-ergost-5-en-3-ol, 25-hydroxy-24-metilcolesterol and 3β, 5α-cholesta-8, 24 - dien-3-ol;

- In *Mytilus galloprovincialis* Lmk. lipid extract there is the presence of vitamin E (α-tocopherol) in an amount of 14μg/ml and the lipid extract of *Rapana venosa* were detected 40 I.U. vitamin D;

- In the three lipid extracts there have been emphasized frequencies that is characteristic of the fatty acids and γCH3,-COO, COOH, νC-OH,-COO-aryl, γCH, γCH I, νC-C γC-C, γCH trans-γCH, νC-OH, νCOOH acid α, β unsaturated νCH2 and I νCH2, νCH2 (CH3) as the combined bands, sometimes covered by bands νCH aliphatic νCO-, νCH3, I νCN and γNH;

- An iodine value of both lipid extracts (*Mytilus galloprovincialis* Lmk. 82.843 g%. and 86.546 g% ***Rapana venosa***) are higher compared to olive oil (66.305 g%), indicating a higher degree of unsaturation;

- Content rich in polyunsaturated fatty acids such as arachidonic acid, the precursor of prostaglandins, vitamins E and D warrants thorough examination of lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs species, in order to capitalize them as dietary supplements and prepared dermofarmaceutics.

3. Results of *in vitro* experiments on action of two molluscs lipid extracts analyzed on the solar radiation, leads to the following conclusions:

- The apparent protective effects of solar radiation exert *Mytilus galloprovincialis*

Lmk. lipid extract. (6.8 to 10.9) followed by lipid extract of *Mytilus galloprovincialis* Lmk. + Rapana venosa 1:1 (v / v), (5.7) *Rapana venosa* lipid extract (2.9-8.8) ;

- Thus, the data we obtained, opening the prospect of replacing sunscreen ingredients of chemical synthesis (different UV-R absorbers) by lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venosa*, which exerts a better protection of skin tissue without adverse / side effects.

4. Regarding the effects of lipid extracts on microbial cultures of *Escherichia coli* compared with antibiotics imipenen (IPM) and gentamicin (GM), we find that:

- The first Kirby-Bauer diffusion method revealed that no lipid extract subjected to the experiment presents antibacterial (sensitivity) of *Escherichia coli* effects;

- The second diffusion method applied by the methodology described by Romanian Pharmacopoeia X, showed a weak antibacterial effect of *Mytilus galloprovincialis* Lmk. lipid extract on *Escherichia coli*.

5. Experimental results in vivo, expressed by the therapeutic effect of lipid extracts of Wistar rats induced thermal burns, blood and histological analyzes and biohistometric and immunohistochemical study shows that the largest anti-inflammatory and dermorestitutiv exercises *Mytilus galloprovincialis* Lmk. lipid extract, which was determined with a high content of polyunsaturated fatty acids, in particular omega-3.

The order of histologically tissue restoration was:

- The first tissue recovered on the 12th day, shown in group III due to *Mytilus galloprovincialis* Lmk. of the lipid extract lipid

- The second tissue recovered on the 13th day and is represented by the group II is due to lipid extract of *Mytilus galloprovincialis* Lmk. + *Rapana venosa* 1:1 (v / v);

- The third tissue restored in the 14th day, is represented as the group IV and group V and is due to lipid extract specific *Rapana venosa* and burn ointment;

- Last tissue restored in a 21-day, is the group I, control (Wistar rats with untreated burning heat);

The effects of lipid extracts from *Mytilus galloprovincialis* Lmk. and *Rapana venous* molluscs species used as a treatment for burns healing of the skin tissue of the four groups of Wistar rats, produced healing of scars nine days earlier compared to group I, control, thermal burn Wistar rats that were not subject to any treatment. Moreover, collagen IV immunostaining and quantification of its fiber thickness, argues restoring of first tissues from batch III; thus, the results indicate that the lipid extracts of molluscs *Mytilus galloprovincialis* Lmk. and *Rapana venosa* are able to initiate the process of recovery and the reduction of scar by

reducing inflammatory infiltration and edema and by by stimulating of the dermis and the epidermis regeneration, the proliferation of fibroblasts and angiogenesis.

6. Regarding the development of topical pharmaceuticals (emulsions and ointments), note the following:

- Three emulsions were made and six types of ointments, simple and complex, with the bioactive lipid extracts of *Mytilus galloprovincialis* Lmk. and *Rapana venous* molluscs and mixtures thereof in a ratio of 1:1 (v / v); processes for preparing emulsions and ointments have been adapted for preparing schemes set out in the literature; ointment bases used to incorporate lipid extracts have been made from components currently used in production technology of emulsions and ointments. According to stability study conducted over 90 days, we obtained pharmaceuticals not within the rules of the National Agency for Medicines and Health Ministry, probably due to inappropriate choice of preservatives;

- Promoting emulsions and ointments obtained as medicinal preparations still requires more thorough testing *in vitro* and *in vivo* for the therapeutic potential and the framing of pharmaceutical bases in accordance with standards established by the National Medicines Agency (NMA) and the Ministry of Health (MoH).

7. **Tissue skin sensitization tests in guinea pigs** showed that the three lipid extracts from *Mytilus galloprovincialis* Lmk., *Rapana venosa* and *Mytilus galloprovincialis* Lmk. + *Rapana venosa* 1:1 (v / v) are non-sensitized products which produced a remarkable anti-inflammatory and dermorestitutive action.

8. Procedures of performing dermo-pharmaceutical topical products from marine molluscs were designed and conducted in compliance with environmental regulations. The scope for recovery of waste will lead to efficient technological process in the case of technology transfer from laboratory scale to pilot or industrial scale.

We believe that the issues addressed in this paper is a modest contribution to broadening the current knowledge of the biochemistry of marine organisms, notorious expressed interest for this research is fully justified, given the existing possibilities and potential for better use of various marine organisms as a source accessible biologically active substances. Deepening and sublimation knowledge of biochemistry of marine organisms will thus lead to crystallization of new ideas and ways on obtaining biologically active substances with definite and possible applications for therapeutic purposes. We believe that developing of this thesis meet such goals, trying to capture the current state of research in the area mentioned and outline some possibilities for therapeutic use in lipid extracts of *Mytilus galloprovincialis* Lmk. and *Rapana venosa* molluscs from the Romanian Black Sea coast.

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