SUMMARY OF THE PhD THESIS

Studies on the antioxidant and antitumor effects of retro-carotenoids in skin cancer

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INTRODUCTION

The important role of carotenoids in various multifactorial processes in animal and human organisms, as well as their ability to interact with active oxygen species, thus reducing their negative effect related to the occurrence and development of several conditions, including a significant number of neoplasms is well known in the scientific world. Also known as European or English yew, *Taxus baccata* is a conifer native to Europe. Carotenoids, especially those with a retro structure such as rhodoxanthin, but also lycopene and zeaxanthin, have been mentioned as some of the essential components of *Taxus baccata* fruits, called aril (TABASZEWSKA et al., 2021; DUMITRAȘ et al., 2022b).

Despite repeated attempts to understand and treat skin cancer, this condition is increasingly frequently diagnosed, becoming one of the leading causes of death, both among humans and animals. Numerous animal studies have reported the spontaneous development of melanoma in various species. Unfortunately, current treatment methods have multiple limitations including an increased number of side effects, the development of resistance to therapy, and, last but not least, the high cost. At the moment, increased attention and an effective study of alternative methods of prevention and therapy of cutaneous melanoma in animals are required to determine the biological effects, the mechanisms of action, and the effectiveness of some phytotherapeutic substances such as carotenoids.

WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

According to the World Health Organization, plants provide 25% of the drugs used in modern medicine, attributed to the phytocompounds they contain (KHAN and AHMAD, 2019). Due to the great advantage that phytocompounds offer, namely, a reduced number of adverse effects compared to chemotherapy used in the therapy of various types of neoplasms, a thorough study of plants with a composition rich in compounds with a possible anti-tumor effect is necessary.

In recent years, many studies have demonstrated the direct relationship between the accumulation of ROS (reactive oxygen species), oxidative stress and the development of cancer. The antioxidant role of carotenoids has sparked particular interest, leading to the hypothesis that they have antitumor activity. Rhodoxanthin, a carotenoid pigment that has received little attention in the scientific literature, is a xanthophyll with a retro-type polyene system. *Taxus bacatta* and *Potamogeton natans* are the only plants in Romania that are rich in carotenoid pigments with a retro-type polyene system: rhodoxanthin, esholxanthin, and esholxanthone (ANDREI et al., 2005).

The **general objective** of this thesis was to study the profile of active biological compounds contained in aril and to carry out *in vitro* and *in vivo* studies with the aim of

establishing the antioxidant and antitumor effect of rhodoxanthin isolated from the aril of *Taxus baccata*.

In order to achieve the formulated main objective, the research was divided into **3 distinct studies**, each of the studies pursuing a specific objective:

- ✓ Characterization of the profile of carotenoids, phenolic compounds, and evaluation of the antioxidant activity of extracts obtained from the aril of *Taxus baccata*:
  - Obtaining the total extract from the aril of *Taxus baccata* for the evaluation of the carotenoid profile through various spectrophotometric and chromatographic techniques;
  - Obtaining a total ethanolic extract from the aril in order to evaluate the polyphenols profile by various techniques;
  - Determination of the antioxidant capacity of the ethanolic extract obtained by two methods: FRAP method (Ferric Reducing Antioxidant Power) and the method of inactivating DPPH radicals;
- ✓ *In vitro* determination of the cytotoxic and antiproliferative activity of rhodoxanthin purified from the aril of *Taxus baccata*, using the two proposed cell lines:
  - Evaluation of the cell viability of the two types of cells of interest by the MTT test following exposure to different concentrations of rhodoxanthin;
  - Evaluation of the cytotoxic effect of rhodoxanthin following the exposure of the two cell lines to various concentrations;
  - Evaluation of the possible protective effect of rhodoxanthin against induced oxidative stress in the cells studied in the presence of H_2O_2 in various concentrations;

- ✓ *In vivo* determination of the potential antitumor effect in the case of B16F10 melanoma in mice of the C57BL/6J line determined by dietary supplementation with rhodoxanthin:
  - Experimental induction of murine malignant melanoma using the B16F10 cell line and mice (*Mus musculus*) of the C57BL/6 line;
  - Investigation of the possible antitumor effect of rhodoxanthin;
  - Histopathological investigation of tumors;
  - Hematology and biochemistry determinations in the case of individuals used in the experiment;
  - Immunochemical determinations in the case of individuals used in the experiment.

STRUCTURE OF THE DOCTORAL THESIS

The doctoral thesis entitled "Studies on the antioxidant and anti-tumor effects of retro-carotenoids in skin cancer" includes 29 figures and 17 tables, being structured

according to technical editing norms in two parts: **Part I - Current state of knowledge** and **Part II – Personal contribution**.

Part I – Current state of knowledge

Part I includes 4 chapters in which the synthesis of the currently known data regarding oxidative stress and its role, the anti-tumor activity of various plant compounds, and the general characteristics of *Taxus baccata* and skin neoplasia in animals is presented.

Part II – Personal Contribution

Part II comprises 6 chapters and represents the personal contribution. This part of the research discusses the working hypothesis and research objectives, the materials and methods used to achieve the proposed goals, the results obtained, the conclusions drawn, as well as the recommendations and originality elements of this study.

RESEARCH RESULTS

Characterization of the profile of carotenoids, phenolic compounds and evaluation of the antioxidant activity of extracts obtained from the aril of *Taxus baccata*

- **The content of bioactive compounds in the aril of Taxus baccata**

The results obtained in the case of quantitative determinations of total carotenoids, respectively total phenols and total flavonoid content are presented in detail in Table 1. Also, the concentration of the major pigment identified at the aril level, rhodoxanthin, is expressed in the table.

Table 1

Phitochemicals content in Taxus baccata aril

Analyzed Samples	Total Carotenoids mg/100 g FW	Rhodoxanthin mg/100 g FW	Total Polyphenols mg GAE/100 g FWF	Flavonoids mg QE/100 g FWF
Sample 1	3.384	2.536	146.69	43.95
Sample 2	3.371	2.582	146.64	45.01
Sample 3	3.379	2.575	143.8	45.26
Average ± SD	**3.378 ± 0.0053**	**2.564 ± 1.0079**	**145.71 ± 22.364**	**44.723 ± 15.425**

The determination of the profile of the majority of carotenoids in the aril of Taxus baccata was carried out by HPLC chromatography, both the total extracts and the fractions separated by column chromatography being analyzed. HPLC chromatograms for total carotenoids extracted from fresh aril (A) and fractions separated on open column (B; C; D) are shown in Figure 1. Five compounds were identified: lutein, β-carotene and three isomers of rhodoxanthin.

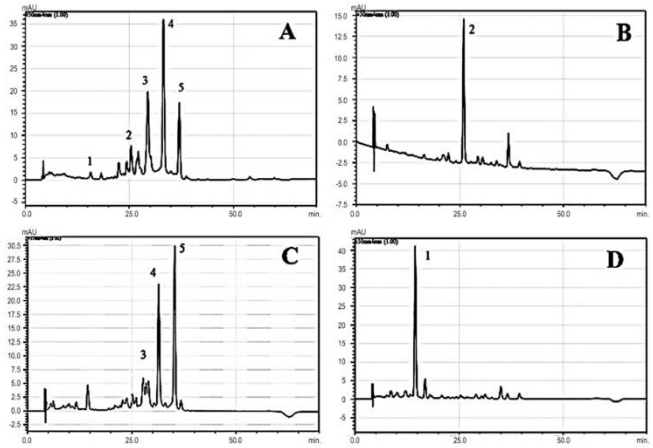

Fig 1. HPLC chromatogram of carotenoids in the total extract obtained by fresh arils of *Taxus baccata* (A) and fractions (fraction 1—(B); fraction 2—(C); and fraction 3—(D)). Five carotenoids are identified: (1) lutein, (2) β-carotene, (3), and (4) and (5) rhodoxanthin isomers

The determination of the profile of polyphenols in the extracts obtained from the aril revealed the presence of 6 compounds from 3 different classes and (Table 2): p-coumarinic acid, protocatechuic acid, hydroxy-caffeic acid, caffeic acid, catechin-glucoside and another compound that did not been identified.

Table 2

Quantification and identification of phenolic compounds (µg/g FWA) by HPLC-DAD-ESI+ analysis

Peak No.	Compound	Retention Time R_t (min)	UV λ_{max} (nm)	$[M+H]^+$ (*m/z*)	Subclass	Concentration (µg/g)
1	NI	3.30	230	381, *219*		42.2
2	p-Coumaric acid-glucoside	6.12	320	327, *166*	Hydroxycinnamic acid	1046.235
3	Protocatechuic acid	9.62	290	155	Hydroxybenzoic acid	149.49
4	Hydroxy-caffeic acid	12.20	322	197	Hydroxycinnamic acid	49.37
5	Caffeic acid	13.78	322	181	Hydroxycinnamic acid	95.76
6	Catechin-glucoside	16.45	280	453, *291*	Flavanol	131.36

NI- not identified

- **Antioxidant activity**

The antioxidant activity of the ethanolic extract obtained by the DPPH method revealed an IC50 value = 68.46 mg/ml, a value that is attributed to moderate antioxidant activity. Regarding the data from the literature regarding the antioxidant activity of the compounds extracted from other fruits, the values obtained by the researchers for black currants, red currants, and gooseberries respectively are lower, but close to the value obtained in our study (LACZKÓ-ZÖLD and et al., 2018). The FRAP test result is expressed in μM TE/100 mL extract. The value obtained by us for aril (10.7 μmol/100 mL) is close to the value reported by DOMÍNGUEZ et al. (2020) for elderberries (10 μmol/100 mL).

In vitro studies on the cytotoxic and antiproliferative effect of rhodoxanthin purified from the aril of Taxus baccata L.

- **Evaluation of the cytotoxic activity of rhodoxanthin on B16F10 murine melanoma cells and human HaCaT keratinocytes**

Following the MTT test, we can highlight the significant difference between the results obtained in the case of the two tested cell lines (Figure 2). In contrast to the case of cells from the HaCAT line exposed to rhodoxanthin where cell viability was between 94.55% and 96.28%, in the case of B16F10 murine melanoma cells, the variation in the percentage of viable cells following rhodoxanthin treatment is significant, compared to the control sample, registering major decreases in cell viability following the use of all concentrations.

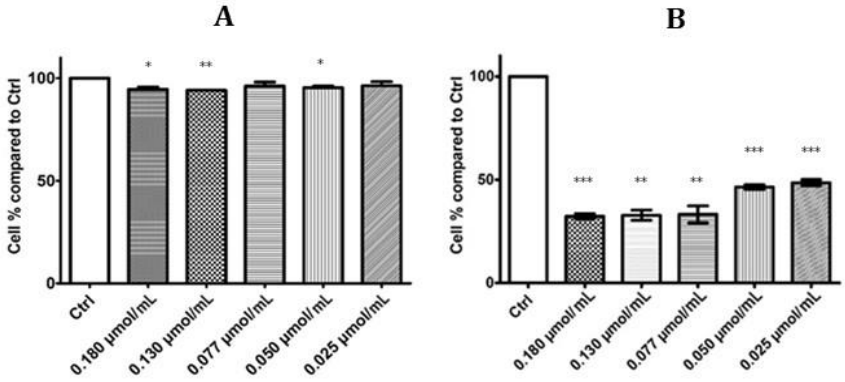

Fig 2. Inhibitory effect of rhodoxanthin at five different concentrations (C1–C5) on HaCaT cells (A) and B16F10 murine melanoma cells (B). Ctrl—untreated cells. Values represent the mean ± SD of three determinations. Statistically significant differences between treated and untreated cells (control): ** p < 0.05; ** p < 0.001; *** p < 0.0001*.

- **Evaluation of the cytoprotective effect of rhodoxanthin against oxidative stress induced by hydrogen peroxide exposure of B16F10 murine melanoma cells and human HaCaT keratinocytes**

In the present study, an MTT assay was performed to explore the protective potential of rhodoxanthin on the same cell lines (HaCaT and B16F10), testing the first three concentrations previously mentioned (C1 = 0.18 µmol/mL rhodoxanthin, C2 = 0, 13 µmol/mL rhodoxanthin and C3 = 0.077 µmol/mL rhodoxanthin). As a result of the pre-treatment of the cells with the 3 concentrations for 24 hours and the subsequent exposure to three different concentrations (200 µm, 500 µm, respectively 1000 µm) of H_2O_2 resulted in the data shown in figures 3 and 4. Exposure to hydrogen peroxide-induced cytotoxicity in HaCaT cells by reducing cell viability by up to 81.30% (200 µM H_2O_2), 78.41% (500 µM H2O2) and 76.89% (1000 µM H_2O_2). Rhodoxanthin treatment demonstrated a cytoprotective effect on HaCaT cells exposed to hydrogen peroxide, with all three concentrations of rhodoxanthin (C1-C3) increasing cell viability by 12.55%, 13%, and 9.66%, respectively.

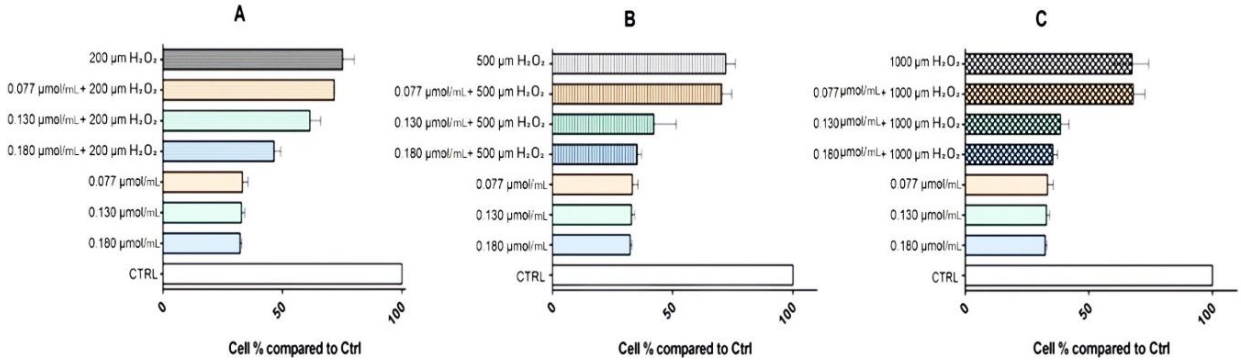

Fig 3. Effects of rhodoxanthin pretreatment on H_2O_2-induced B16F10 cell damage. Cell viability detected by MTT assay. B16F10 cells exposed to H_2O_2 for 2 h. Significance: *p<0.0001* vs. control. B16F10 cells exposed to C1–C3 of rhodoxanthin for 24 h. Significance: *p<0.001*. (A) B16F10 cells exposed to C1-C3 and 200 µm H_2O_2. Significance: *p < 0.05*. (B) B16F10 cells exposed to C1-C3 and 500 µm H_2O_2. Significance: *p<0.05*. (C) B16F10 cells exposed to C1-C3 and 1000 µm H_2O_2. Significance: *p<0.005*.

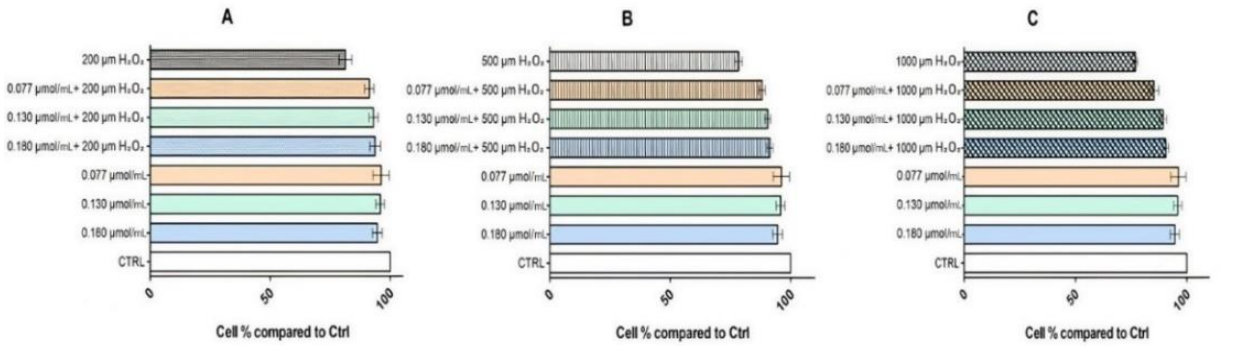

Fig 4. Effects of rhodoxanthin pretreatment on H_2O_2-induced HaCaT cell damage. Cell viability detected by MTT assay. HaCaT cells exposed to H_2O_2 for 2 h. Significance: *p<0.0001* vs. control. HaCaT cells exposed to C1–C3 of rhodoxanthin for 24 h. Significance: *p<0.001*. (A) HaCaT cells exposed to C1-C3 and 200 µm H_2O_2. Significance: *p<0.05*. (B) HaCaT cells exposed to C1-C3 and 500 µm H_2O_2. Significance: *p<0.05*. (C) HaCaT cells exposed to C1-C3 and 1000 µm H_2O_2. Significance: *p<0.005*.

Flow cytometry analysis also revealed a synergistic action of rhodoxanthin and H_2O_2 against B16F10 melanoma cells by effectively reducing the percentage of viable cells (Figure 5).

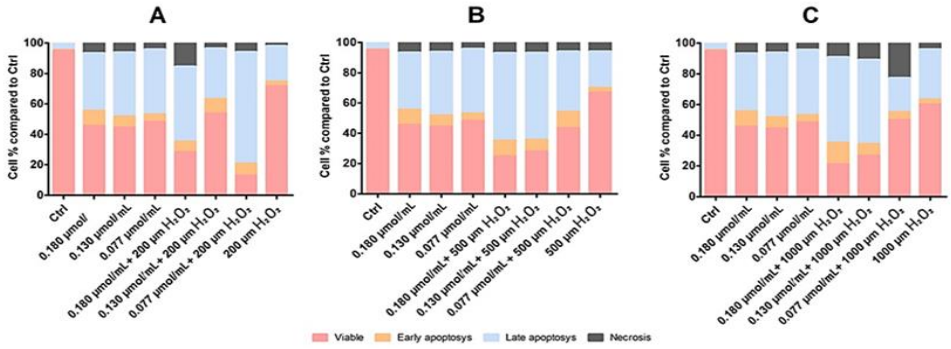

Fig 5. Flow-cytometry analysis of B16F10 cells following rhodoxanthin and hydrogen peroxide exposure. (A) B16F10 cells exposed to C1-C3 and 200 µm H_2O_2. Significance: $p<0.05$. (B) B16F10 cells exposed to C1-C3 and 500 µm H_2O_2. Significance: $p<0.05$. (C) B16F10 cells exposed to C1-C3 and 1000 µm H_2O_2. Significance: $p<0.005$. Values represent the mean±SD of three samples.

Rhodoxanthin showed cytotoxic effects by further reducing cell viability; the higher the concentration of rhodoxanthin, the lower the viability of melanoma cells. The viability rate in the B16F10 control group was 94.9%, a rate that was reduced after exposure to H_2O_2 as follows: 200 µM H_2O_2—71.5%, 500 µM H_2O_2—66.8%, and 1000 µM H_2O_2 —60.1%.

Study of the effects of rhodoxanthin in an experimental model of induction of cutaneous melanoma in C57BL/6J mice

- **The effect of rhodoxanthin administration in mice of the C57BL/6J line**

Following administration of 7 mg/kg/day rhodoxanthin to C57BL/6J mice inoculated with 106 murine malignant melanoma B16F10 cells, no changes were observed in the general condition of the individuals. Manifestations such as decreased mobility, apathy, piloerection or partial closure of the eyes or adverse effects such as diarrhea, common in similar experiments, were not observed in any of the individuals, indicating that rhodoxanthin treatment did not show adverse effects or toxicity. Day 8 was the first day when the first signs of tumor growth could be observed in some of the individuals. Tumors were measured (length and width) at two-day intervals until the end of the experiment. During the course of the study, we observed that although the number of cells inoculated was the same, the growth varied between individuals within the same batch.

Although, both in the case of the melanoma group and in the case of the melanoma+rhodoxanthin group, tumor growth was exponential, the administration of rhodoxanthin visibly slowed this development. Thus, the mean value of tumor volumes determined throughout the study was influenced by rhodoxanthin treatment by a difference of 42.18% between the two major groups studied in favor of the melanoma group. Rhodoxanthin inhibited tumor growth, so that the average of tumor masses after excision, in the case of the treated group, was 15.74% lower than in the case of the untreated group (Figure 6).

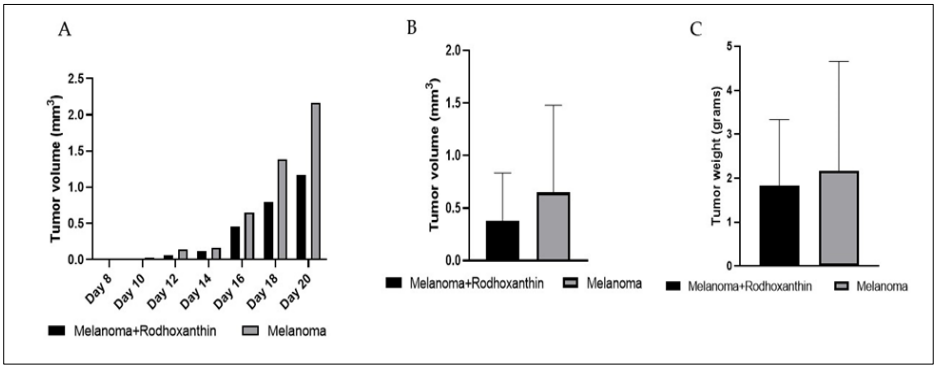

Fig 6. Effect of rhodoxanthin on solid B16F10 melanoma tumor growth in C57BL/6J mice. (A) Tumor volume variation throughout the study. (B) Significant inhibition of tumor volume between the untreated group (0.648 ± 0.829) and the treated group (0.376 ± 0.457). Each value represents the mean ± SD, n = 8). (C) Tumor weight inhibition at a value of 15.48% recorded after the administration of 7 mg/kg/day rhodoxanthin.

- **Hematological analysis**

The variations of the determined parameter values did not show a statistical difference, with a few exceptions (Table 3).

Table 3

Blood count evaluation

Parameters	Control Group	MCT Oil Group	Rhodoxanthin Group	Melanoma Group	Melanoma+Rhodoxanthin Group
Leukocytes (10^9/L)	3,603 ± 0,14	3,977 ± 0,03	5,056 ± 0,04	5,777 ± 0,12	3,626 ± 0,05
Lymphocytes (10^9/L)	3,253 ± 0,03	3,466 ± 0,21	4,166 ± 0,03	4,036 ± 0,07	2,850 ± 0,05
Neutrophils (10^9L)	0,253 ± 0,04	0,476 ± 0,02	0,853 ± 0,04	1,473 ± 0,03	0,793 ± 0,04
Red blood cells (10^{12}/L)	8,593 ± 0,03	10,096 ± 0,02	10,826 ± 0,06	4,332 ± 0,05	8,250 ± 0,05
Hemoglobin (g/dl)	11,210 ± 0,04	13,876 ± 0,08	14,486 ± 0,09	10,943 ± 0,05	11,260 ± 0,03
Hematocrit (%)	38,926 ± 0,41	41,713 ± 0,28	44,210	33,916 ± 0,13	35,010 ± 0,11
Platelets (10^9/L)	252,12 ± 1,42	231,943 ± 0,41	184,5 ± 0,51	91,333 ± 1,52	230,643 ± 0,66

- **Biochemical analysis**

Although tumor cells are producers of large amounts of reactive oxygen species, the value of antioxidant enzymes at the level of tumor cells is generally lower due to their reduced ability to defend against free radicals, so this level is maintained an oxidant-antioxidant imbalance, i.e. the state of oxidative stress. Tables 4 and 5 show the activity values of the antioxidant enzymes determined from the plasma and tissues of the individuals under study.

Table 4

Enzymatic antioxidant activity and total antioxidant status in plasma

Parameters	Control Group	MCT Oil Group	Rhodoxanthin Group	Melanoma Group	Melanoma+Rhodoxanthin Group
Catalase (U/mL)	194,02 ±13,29	203,125 ±292,08	153,8 ±150,38	139,3 ± 608,66	110,2 ±308,46
Superoxide-dismutase (U/mL)	71,72 ± 4,76	68,015 ± 7,72	65,22 ±14,77	89,61 ± 14,89	75,97 ± 1,17
Glutathione-peroxidase	22,86 ± 3,87	21,97 ± 3,93	25,72 ±5,07	20 ± 6,73	25,34 ±0,58
Total antioxidant activity (U/mL)	18,31 ± 1,68	17,92 ± 3,5	29,11±0,43	17,30 ± 0,33	30,33 ± 8,16

Table 5

Enzymatic antioxidant activity and total antioxidant status in tissues

Tissue/parameters	CAT (U/mg protein)	SOD (U/mg protein)	GPx (nmol/min/mg protein)	TAS (U/mg protein)
	Skin			
Control group	1025,11±50,14	48,21±0,21	10,11±0,24	15,21±0,39
MCT oil group	939,16±78,72	46,77±1,18	11,25±2,67	16,03±2,65
Rhodoxanthin group	1491,16±47,12	42,19±1,39	6,47±3,33	15,18±2,44
Melanoma group	846,23±345,21	46,67±5,11	7,95±2,03	11,19±1,96
Melanoma + Rhodoxanthin group	1381,91±250,06	43,37±3,22	5,55±0,40	13,44±0,43
	Liver			
Control group	113,33±10,05	9,77±0,99	0,46±0,05	1,96±0,10
MCT oil group	150,18±27,23	9,86±1	0,60±0,006	2,14±0,16
Rhodoxanthin group	149,44±48,58	13,27±3,05	1,23±0,06	3,02±0,78
Melanoma group	308,53±85,11	21,03±9,83	2,90±0,60	1,74±0,81
Melanoma + Rhodoxanthin group	237,56±4,51	15,79±9,87	1,00±0,35	3,95±1,46
	Tumor			
Melanoma group	368,162±178,48	31,99±3,40	3,74±0,60	7,39±2,99
Melanoma + Rhodoxanthin group	492,22±108,31	22,93±3,61	5,57±1,09	9,07±1,31

Figures 7 and 8 show the effect of rhodoxanthin administration in the individuals studied, on the value of epidermal growth factor, respectively 8-hydroxy-2-deoxyguanosine. In the case of both determinations, a significant decrease was observed, with statistical value ($p<0.05$), both at the level of the skin and at the level of tumors in the case of individuals treated with rhodoxanthin, compared to the values of samples from untreated individuals. This decrease was also found in the analyzed plasma samples. Thus, we can mention the involvement of rhodoxanthin in the slowing of tumor growth, one of the mechanisms being the decrease in the level of EGF and the

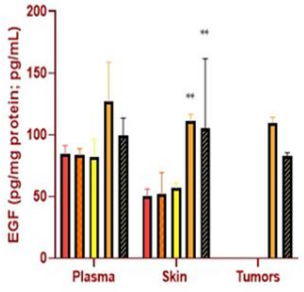

Fig 7. Epidermal growth factor variatio groups.

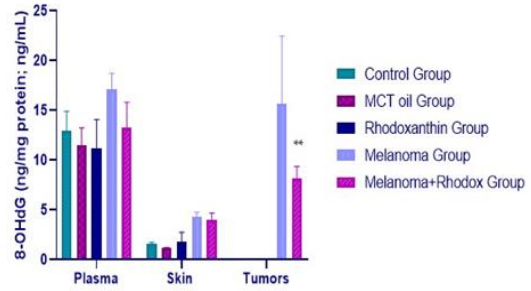

Fig 8. 8-hydroxy-2-deoxyguanosine variation within the groups.

modulation of the level of DNA oxidation by decreasing the concentration of 8-hydroxy-2-deoxyguanosine.

- **Histological analysis**

Following the histological examination of the tumor samples taken from the individuals included in the study and stained with Trichrome Goldner (TG) for examination, a poorly differentiated malignant neoplastic formation was found, located in the hypodermis (Figure 9). The established histopathological diagnosis was poorly melanogenic anaplastic melanoma, with polymorphous cells and numerous intratumoral necrotic microfoci.

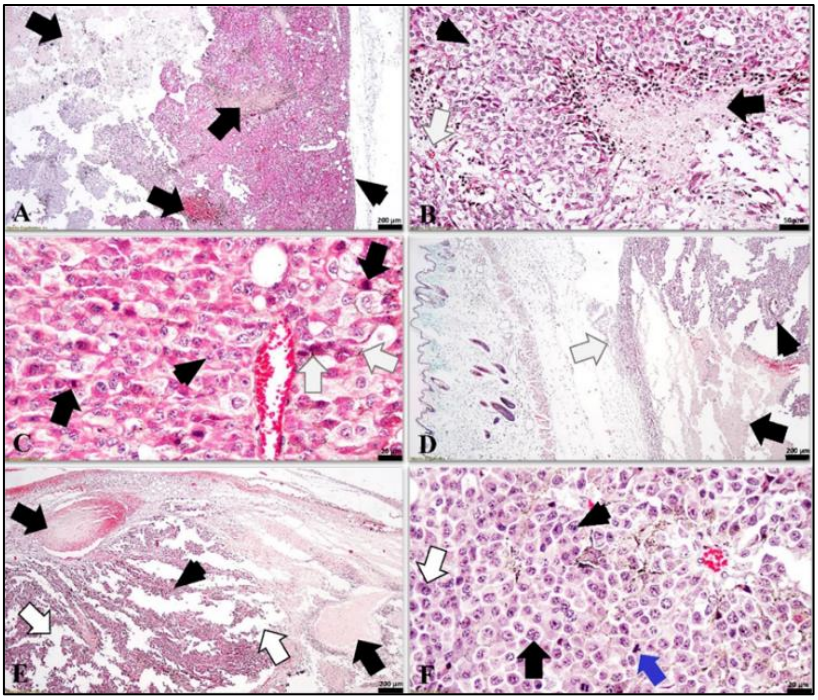

Fig. 9. Cutaneous anaplastic melanoma in mice (Goldner's trichrome stain): (A) (control group): large tumoral mass with extended necrotic areas (arrows) and a discreete capsule (arrowhead); (B) (control group): microscopic features of the melanoma suggesting cellular debris of the necrotized areas (black arrow), tumoral parenchyma (arrowhead) that includes a discrete sustaining tissue (white arrow); (C) (control group): highly cellularised melanoma, with tumoral mela-nocytes displaying anisocytosis, large nuclei and nucleoli (arrowhead), numerous mitotic figures (black arrows), and discreete melanic granules in the cytoplasm of some tumoral cells (white arrows); (D) (group melanoma+rhodoxanthin): the location of the tumoral mass in the hypodermis (arrowhead), which presents large necrotic areas (black arrow) and an ill-defined infiltrated capsule (white arrow); (E) (group melanoma+rhodoxanthin): thrombi in the tumoral mass (arrows), a discrete sustaining tissue identified in between the parenchyma cells (arrowhead) and a low intercellular adhesion suggested by large intercellular spaces (white arrows) suggesting rapid growth and high malignancy; (F) (group melanoma+rhodoxanthin): poorly differentianted melanoma suggested by high cellular pleiomorphism, large nuclei (black arrow) including binucleated melanocytes (white arrow), in many circumstances with macronucleoli (arrowhead), high mitotic index that includes atypic mitoses (blue arrow).

RECOMMENDATION

Considering the limited data available in the specialized literature related to the composition of *Taxus baccata*'s aril composition, the biological effects of its compounds, but especially the action of rhodoxanthin, its potential, and therapeutic value with the aim of in-depth understanding of the value of rhodoxanthin in therapy or prevention various illnesses, especially neoplastic ones, we recommend the following:

- ✓ Carrying out additional studies, in vitro by using various types of tumor cell lines with the aim of determining the biological effects of the administration of various concentrations of rhodoxanthin.
- ✓ Carrying out additional studies in vivo in the case of murine melanoma B16F10 with the aim of determining the optimal dose of rhodoxanthin to be administered and the optimal period of its administration (before and during the induction of melanoma).
- ✓ Carrying out additional studies with the aim of determining the pharmacokinetics of rhodoxanthin.

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