

**"ALEXANDRU IOAN CUZA" UNIVERSITY OF IAȘI
FACULTY OF BIOLOGY**

**STUDIES CONCERNING THE ACTIVITY OF
SOME PLANT AND FUNGAL EXTRACTS ON
SOME HUMAN TUMORAL CELL LINES**

- PhD THESIS SUMMARY-

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INTRODUCTION

Superior mushrooms have a long history of use in chinese, japanese and korean alimentation and traditional medicine. Today we know a big number of mushrooms with therapeutic properties assigned to different compounds from mycelium structure. Substances isolated from *Pleurotus ostreatus*, α - and β -glucans, proved to be very effective in cancer therapy.

One of the well studied plants thanks to his remarkable properties is greater celandine (*Chelidonium majus*). A big number of studies regarding this plant have been published. They highlight the species toxic potential, but also a big number of therapeutic properties, including the antitumor action.

Another plant, well studied regarding his therapeutic actions, is ginger (*Zingiber officinale*). Used from ancient times in oriental medicine, the plant became popular also among western researchers for his therapeutic effects in many diseases, like cancer.

Given these observations, these species can be a valuable study subject on different human cancer types.

Making this thesis was possible thanks to the support of many people.

I give special thanks to my scientific coordinator, Prof. dr. Gogu Gheorghiuță, to the members of Biochemistry and Molecular Biology Department from the Biology Faculty of Bucharest University for the technical support in their laboratory, to the doctoral commission for the amability of analysing my thesis and to my friends and family for all the support during this years of preparation.

PURPOSE

The purpose of this thesis is the analysis of the effects produced by a mushroom extract and some plant extracts on some tumor cell lines. The investigations followed four directions:

1. analysis of the 24 and 72 hours treatment effects of one *Pleurotus ostreatus* polysaccharide extract on Caco-2 human colorectal adenocarcinoma (Caco-2 cell line);

2. analysis of three concentration of an *Chelidonium majus* methanolic extract and sanguinarine on human amelanotic melanoma (C32 cell line);

3. identification of the effects induced by a fresh polyphenolic ginger extract on human skin normal fibroblasts (CCD cell line) comparing to C32 cell line;

4. analysis of three concentration of an autoclaved ginger extract and the phenolic compound 6-shogaol on human amelanotic melanoma (C32 cell line).

I. *Pleurotus ostreatus* Jacq., *Chelidonium majus* L. and *Zingiber officinale* Roscoe and their antitumor action

The abundance of compounds from the structure of *P. ostreatus*, especially polysaccharides, led to the identification of a big effectiveness of this mushrooms extracts in ovary, colon, prostate, breast and bladder cancer.

Scientific literature indicates, in *C. majus* structure, the presence of a big number of alkaloids with important effects in pancreatic, stomach, ovary, pharyngeal, colorectal, mammary, hepatic, ocular and skin cancers and also in different types of leukemia.

The role of ginger in cancer therapy and prophylaxis is undeniably. The plant represents a favorite study object for many researchers. The effects of some polyphenolic compounds extracted from ginger have been studied, among others, through their relationship with the pathology of colon, stomach, intestinal, mouth, pulmonary, prostate, breast cancers and leukemia.

II. Material and methods

II.1. Research material

We used Caco-2 colorectal carcinoma cell line in order to test the effects of a *P. ostreatus* polysaccharide extract. The extract was obtained using the method of Lavi *et al.*, 2006.

Then, we determined the effects of sanguinarine and a methanolic extract of *C. majus* on the C32 amelanotic melanoma cells. The roots of greater celandine (obtained from the Botanical Garden of Iași) were ground, ultrasonicated in a mix of methanol and concentrated HCL and centrifuged.

We also analyzed the influence of 6-shogaol and an autoclaved polyphenolic *Z. officinale* extract on the same cell line. The extract was obtained after autoclaving, drying, pulverization, ultrasonication in methanol and centrifugation.

The research focused also on testing the effects of a fresh *Z. officinale* extract on CCD normal skin fibroblasts and on C32 cells. The extract was obtained after grinding the fresh material followed by centrifugation.

II.2. Research methods

The total polysaccharide content from the *P. ostreatus* extract was calculated using the method of Dubois *et al.*, 1956. Caco-2 cells were then treated for 1 and 3 days, in culture medium with and without bovine fetal serum, with a concentration of 0,5 mg% polysaccharide from the extract.

The content of sanguinarine from the extract of *C. majus* was calculated using the method described by Maiti and Kumar, 2010. C32 cells were then treated for 24 hours with the extract containing 0,5, 1 and 2 μ M sanguinarine and with the same concentration of pure sanguinarine.

Total polyphenols from *Z. officinale* extracts was identified through the method of Singleton *et al.*, 1999. CCD and C32 cells were then treated with 2 și 4 mg% polyphenols from the fresh extract for 24 hours. The autoclaved extract and 6-shogaol with the concentration of 1, 2 și 3 mg% were used only for the treatment for 24 hours of C32 cells.

The toxicity of previously mentioned agents on the three cell lines was done using the method of Mosmann, 1983.

After obtaining the cell lysates, we made the next biochemical analysis: determination of total protein content using the method of Bradford, 1976, of superoxide-dismutase activity (Paoletti method, 1986), catalase (Aebi, 1974), glutathione-peroxidase (Beutler, 1984), glutathione-reductase (Goldberg and Spooner, 1983), glucose-6-phosphate dehydrogenase (Lohr and Waller, 1974) and glutathione-S-transferase (Habig *et al.*, 1974). Glutathione concentration was identified with *Arbor Assays DetectX* kit and the concentration of malone-dialdehyde was identified using the method of Del Rio *et al.*, 2003.

Evaluation of Hsp60, 70, 90, Bcl-2, Bax, p53 and matrix metalloproteinases 2 and 9 from cell lysates was made by Western Blot. The protein expression was calculated after reporting to structural protein β -actine.

All the results were analyzed statistically using Student t test and Microsoft Excel program. The values were expressed as averages \pm standard deviation. $p < 0,05$ values are considered significant (*), $p < 0,01$ distinctly significant (**) and $p < 0,001$ very significant (***)

III. Results and discussion

III.1. Enzyme activity determinations in cell lysates obtained after *Pleurotus ostreatus* extract treatments

Table 1. Biochemical parameters in Caco-2 cells treated with *P. ostreatus* extract

Treatment	Experimental variant	GSH concentration $\mu\text{mol/mg protein}$	MDA concentration $\mu\text{mol/mg protein}$
Without serum	Control zero	9,200 \pm 0,873	0,558 \pm 0,031
	Control 1 day	12,173 \pm 1,181*	0,568 \pm 0,029
	Treatment 1 day	9,702 \pm 1,025	0,643 \pm 0,007*
	Control 3 days	0,536 \pm 0,068**	0,399 \pm 0,023**
	Treatment 3 days	4,186 \pm 0,328**	0,387 \pm 0,011
With serum	Control 1 day	10,274 \pm 0,464	0,631 \pm 0,048
	Treatment 1 day	11,419 \pm 1,310	0,613 \pm 0,013
	Control 3 days	9,675 \pm 0,644	0,540 \pm 0,023
	Treatment 3 days	8,722 \pm 0,651	0,493 \pm 0,034

Table 2. Stress enzyme specific activity in Caco-2 cells treated with *P. ostreatus* extract

Treatment	Experimental variant	SOD U/mg protein	CAT U/mg protein	GPX U/mg protein	GRED U/mg protein	G6PDH U/mg protein	GST U/mg protein
Without serum	Control zero	0,077±0,011	0,033±0,0006	0,266±0,036	0,028±0,0028	0,031±0,0024	0,445±0,022
	Control 1 day	0,105±0,010*	0,042±0,0022*	0,248±0,016	0,015±0,0015**	0,038±0,0010*	0,485±0,023
	Treatment 1 day	0,099±0,007	0,045±0,0063	0,266±0,009	0,019±0,0006*	0,035±0,0006*	0,390±0,018**
	Treatment 3 days	0,078±0,008	0,034±0,0064	0,219±0,019	0,012±0,0011**	0,037±0,0014*	0,369±0,016*
With serum	Control 1 day	0,093±0,005	0,048±0,0036*	0,196±0,016	0,023±0,0010	0,060±0,0062**	0,294±0,021***
	Treatment 1 day	0,101±0,006	0,051±0,0037	0,113±0,003*	0,021±0,0010*	0,064±0,0013	0,332±0,025
	Control 3 days	0,089±0,012	0,046±0,0022**	0,131±0,013*	0,024±0,0007	0,058±0,0013**	0,731±0,027***
	Treatment 3 days	0,072±0,015	0,045±0,0016	0,136±0,009	0,017±0,0009***	0,055±0,0004	0,608±0,103

In medium free treatment, the extract seems to induce the oxidative stress counteraction mechanisms. Considering the inhibition of antioxidant defense systems in untreated cells (as a consequence of protein synthesis inhibition in the absence of serum), it seems that the polysaccharide extract protected tumor cells through stimulation of these systems.

The reduction of superoxide anions and hydrogen peroxide concentration under the influence of *P. ostreatus* polysaccharides identified in previous research and also the reduction of lipid peroxidation observed in this study indicates that the decrease of antioxidant systems in Caco-2 cells treated in the presence of serum was due to direct scavenging activity of the analyzed extract.

III.2. Evaluation of relative protein expression of some molecular parameters after *Pleurotus ostreatus* extract treatments

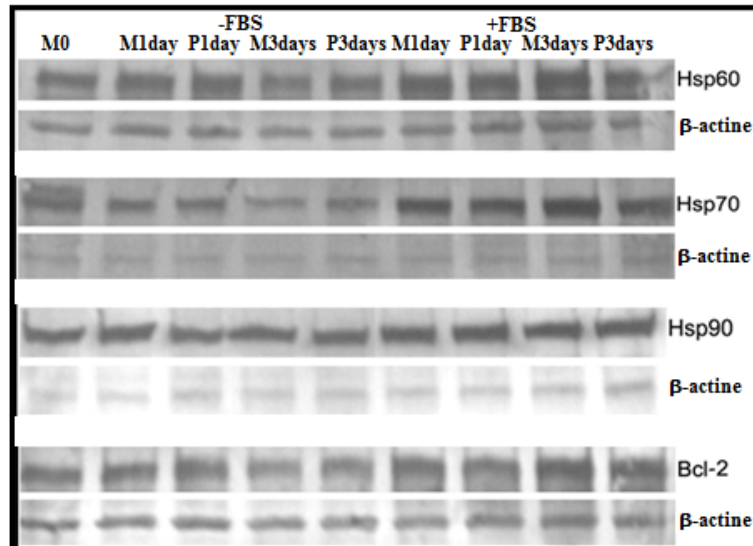


Fig.1. Western blot membranes with Hsp60, 70, 90, Bcl-2 and β-actine spots

The stimulation of Hsp60 and 70 expression and the other antioxidant defense systems in colorectal cancer cells, in the absence of serum, sustains the hypothesis of the protective effect of the extract.

In the cells treated in the presence of serum, underexpression of these proteins and the decrease of the antioxidant

enzyme activity can be indicators of diminished stress conditions.

Hsp90 expression in Caco-2 cells treated in medium with serum has the same profile as Bcl-2 protein expression. Considering the tight relationship between their biological roles, we can conclude that Hsp90 functions towards the stimulation of Bcl-2 expression (antiapoptotic mechanism).

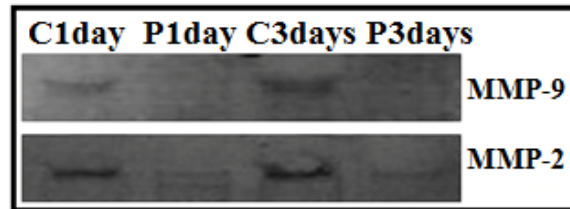


Fig. 2. Western blot membrane with MMP-2 and -9 spots

The decreased expression of MMP-2 and MMP-9 suggests an inhibition of the invasion ability of Caco-2 cells. Although Bcl-2 protein is capable to activate the metalloproteinases (Choi *et al.*, 2005), his overexpression had no effect on this enzymes in treated Caco-2 cells.

III.3. Enzyme activity determinations in cell lysates obtained after sanguinarine and *Chelidonium majus* extract treatments

Table 3. Biochemical parameters in C32 cells treated with *C. majus* extract and sanguinarine

Treatment	Experimental variant	SOD U/mg protein	CAT U/mg protein	GPX U/mg protein	GRED U/mg protein	G6PDH U/mg protein	GST U/mg protein	MDA μmol/mg protein
	Control zero	0,491±0,088	0,011±0,0011	0,157±0,0008	0,0056±0,00006	0,025±0,00075*	0,058±0,0034	0,194±0,006
	Control 1 day	0,354±0,036	0,007±0,0010*	0,162±0,0040	0,0057±0,00019	0,032±0,00048*	0,054±0,0030	0,205±0,015
<i>C. majus</i>	0,5 μM	0,621±0,010	0,017±0,0022*	0,161±0,0045	0,0051±0,00008*	0,003±0,00017*	0,023±0,0011*	0,307±0,011*
	1 μM	1,024±0,012	0,019±0,0015***	0,145±0,0038	0,0047±0,00027	0,0007±0,00005*	0,0126±0,0015*	0,397±0,018*
	2 μM	1,333±0,081*	0,021±0,0017**	0,119±0,0017**	0,0046±0,00039	0,0006±0,0001*	0,010±0,0013*	0,448±0,046
Sanguinarine	0,5 μM	0,905±0,013	0,017±0,0013**	0,161±0,0066	0,0050±0,00011*	0,018±0,00009*	0,054±0,0021	0,379±0,016*
	1 μM	1,167±0,020*	0,019±0,0012**	0,151±0,0172	0,0049±0,00056	0,016±0,00002*	0,032±0,0025*	0,425±0,039
	2 μM	1,587±0,226	0,019±0,0010***	0,134±0,0346	0,0047±0,00041	0,012±0,00007*	0,015±0,0027**	0,463±0,042

The analysis of oxidative stress enzymes suggests the stimulation of this state in C32 cells, which caused the increase in SOD and CAT activity and also of MDA concentration and was manifested through a diminished activity of the enzymes involved in glutathione metabolism.

III.4. Evaluation of relative protein expression of some molecular parameters after sanguinarine and *Chelidonium majus* extract treatments

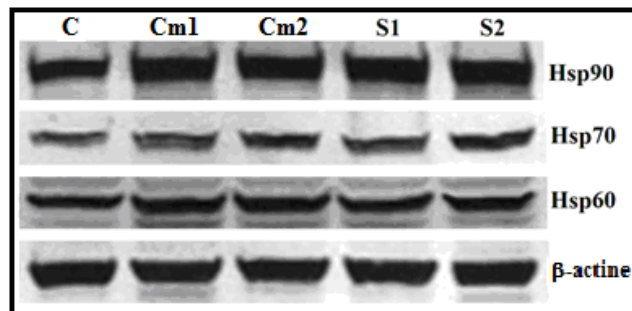


Fig. 3. Western blot membrane with Hsp60, 70, 90 and β-actine spots

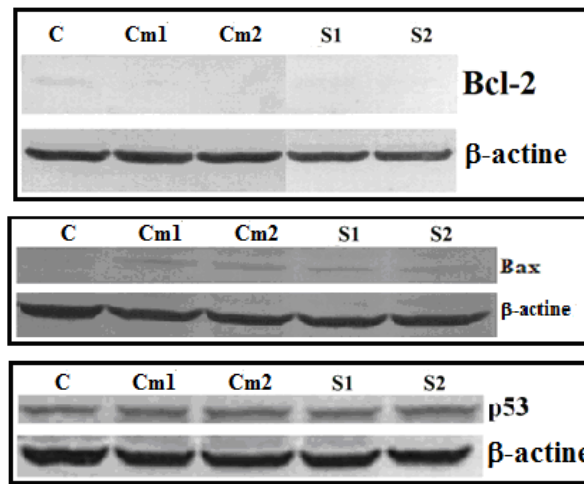


Fig. 4. Western blot membranes with Bcl-2, Bax, p53 and β-actine spots

The increased level of heat shock protein after the exposure to the two agents indicates the initiation of some apoptotic

pathways modulated by these proteins. In C32 cells which survived the treatments, overexpression of chaperon proteins led to cell death prevention through degradation of apoptosis regulation proteins.

Considering the stimulation of Bax and p53 expression, inhibition of Bcl-2 and also the role of Bax in releasing of cytochrome c in cytosol, we deduced that greater celandine extract and sanguinarine can be responsible of cell death induction, as the concentration grow.

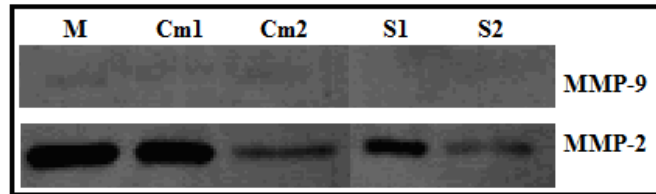


Fig. 5. Spots obtained through MMP-9 and -2 zymography (negative)

Figure 5 indicated that, besides apoptosis induction, greater celandine extract and sanguinarine have also an inhibition effect of tumor aggressiveness (metastasis).

III.5. Citotoxicity of fresh ginger extract on amelanotic melanoma and normal skin fibroblast cell lines

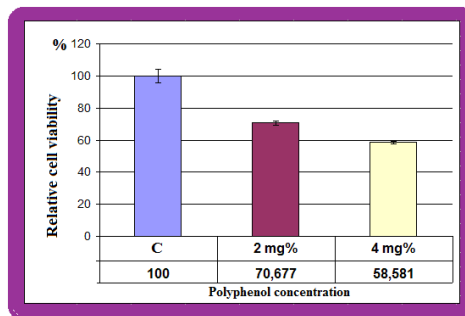


Fig. 6. Relative C32 cell viability after ginger fresh extract treatment

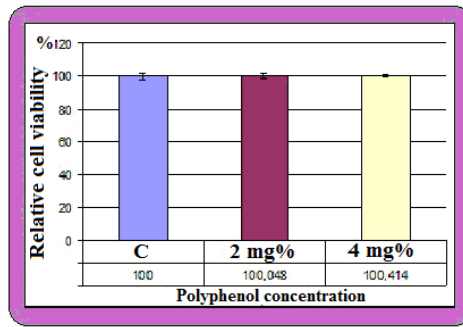


Fig. 7. Relative CCD cell viability after ginger fresh extract treatment

Fresh ginger extract reduces melanoma cells viability depending on the treatment concentration, but maintains normal cell viability to 100%.

III.6. Enzyme activity determinations in cell lysates obtained after 6-shogaol and autoclaved ginger extract treatments

Table 4. Biochemical parameters in C32 cells treated with autoclaved *Z. officinale* extract and 6-shogaol

Treatment	Experimental variant	SOD U/mg protein	CAT U/mg protein	GPX U/mg protein	GRED U/mg protein	G6PDH U/mg protein	GST U/mg protein	MDA μmol/mg protein
	Control 0	0,228±0,0119	0,0027±0,00058	1,468±0,069	0,0020±0,00010	0,0076±0,00035	0,141±0,0057	0,148±0,0086
	Control 1day	0,209±0,0060	0,0021±0,00005	1,557±0,037	0,0021±0,00015	0,0085±0,00046	0,131±0,0022	0,167±0,0083
Ginger	1 mg %	0,264±0,0039*	0,0045±0,00012*	2,727±0,139***	0,0016±0,00008**	0,0066±0,00023**	0,128±0,0052*	0,196±0,0108*
	2 mg %	0,276±0,0050*	0,0048±0,00009*	2,042±0,031***	0,0017±0,00014*	0,0060±0,00030**	0,154±0,0051*	0,204±0,0096*
	3 mg %	0,295±0,0045**	0,0058±0,00015**	1,872±0,059**	0,0024±0,00010	0,0055±0,00022**	0,179±0,0032**	0,223±0,0090*
6-Shogaol	1 mg %	0,225±0,0015	0,0042±0,00005*	4,100±0,101***	0,0018±0,00011*	0,0061±0,00013**	0,163±0,0028*	0,193±0,0079*
	2 mg %	0,254±0,0035	0,0046±0,00004*	2,023±0,081**	0,0025±0,00007	0,0050±0,00012**	0,099±0,0015**	0,155±0,0057
	3 mg %	0,232±0,0046*	0,0057±0,00031**	1,711±0,033*	0,0021±0,00020**	0,0043±0,00019	0,073±0,0014**	0,144±0,0065

The increase of SOD and CAT activity may be due to an increased superoxide radicals content as a consequence of ginger extract action, which determined the activation of defense mechanisms. Progressive increase in GRED activity suggests the acceleration of GSH regeneration. Glutathione regenerated at increased treatment concentrations seems to be used in other mechanisms than GPX activity. NADPH seems to be generated through other pathway than via G6PDH.

The decrease in MDA concentration in 6-shogaol treatment induced also the decrease of GST activity, enzyme that has an important detoxifying role. The presence of other compounds in ginger extract could determine the stimulation of GST activity for the counteraction of the toxic action.

III.7. Evaluation of relative protein expression of some molecular parameters after 6-shogaol and autoclaved ginger extract treatments

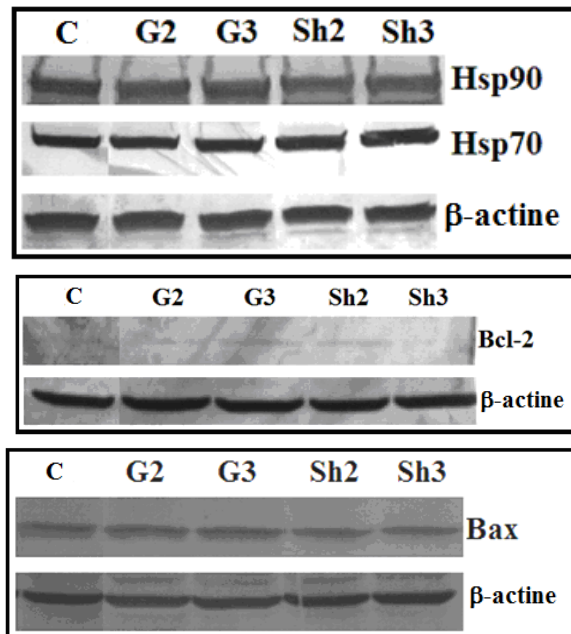


Fig. 8. Western blot membranes with Hsp70, 90, Bcl-2, Bax and β -actine spots

The strong inhibition of Bcl-2 protein expression after the action of 6-shogaol stimulated the activity of the chaperon proteins Hsp70 and 90, for the repair processes completion. In the case of the extract, the cumulative action of a big number of compounds conducted to a less intense Bcl-2, therefore the chaperoning activity was inferior comparing to the control. The in-

crease in chaperon protein expression after 6-shogaol treatment comparing to the extract explains also the reduced rate of Bax synthesis. Maintaining the protein expression over the control suggests though a proapoptotic effect of the both agents.

CONCLUSIONS

After investigating Caco-2 colorectal carcinoma, C32 amelanotic melanoma cell lines and CCD normal skin fibroblasts treated with a polysaccharide fungal extract, respectively with different plant extracts, we concluded:

- Caco-2 cells treatment with 0,5% polysaccharide *P. ostreatus* extract, in the absence of fetal serum, protected tumor cells trough an increase in the activity of antioxidant enzymes, of the enzymes involved in glutathione metabolism, of the reduced glutathione concentration and of the expression of chaperon proteins Hsp60 and 70.
- The treatment in the presence of serum protected tumor cells trough directly scavenging the reactive oxygen species in the presence of the polysaccharides. As a consequence, antioxidant systems and Hsp60 and 70 protein expression were inhibited.
- In both treatment types, an inhibition of apoptosis was observed and was caused by Bcl-2 overexpression, mediated by Hsp90 protein.
- A reduced MMP-2 and MMP-9 expression indicated an inhibition of metastasis by *P. ostreatus* extract.
- In the case of C32 treatment with sanguinarine and greater celandine extract containing 0,5, 1 and 2 μ M sanguinarine, the stimulation of SOD and CAT activity, the increase of MDA concentration and the reduction of the enzymes activities involved in glutathione metabolism suggests a prooxidant effect of sanguinarine and *C. majus* extract which can culminate with apoptosis.

- Heat shock protein expression increased after the treatment, as an effect of oxidative stress installation.
- Sanguinarine and greater celandine extract induced the apoptosis of melanoma cells through Bcl-2 underexpression and overexpression of Bax and p53, the effect being more potent in the case of the extract.
 - The reduced MMP-2 and -9 expressions in treated cells indicated the inhibition of metastasis. The effect was more obvious in sanguinarine treated cells, which suggests the presence in extract structure of some blocking compounds.
 - For the investigations with the fresh ginger extract containing 2 and 4 mg% polyphenols, we showed that this extract doesn't modify normal skin fibroblasts viability, but decrease melanoma cells viability, in a concentration dependent manner.
 - C32 cells treatment with autoclaved ginger extract and 6-shogaol (with 1, 2 and 3 mg% polyphenols) stimulated the protection mechanisms against oxidative stress, through increasing SOD, CAT and GRED activity, in a concentration dependent manner.
 - Autoclaved extract induced lipid peroxidation in C32 cells, which was countered by the increase in GST activity. The response of melanoma cells to 6-shogaol is opposite.
 - 6-shogaol treatment stimulated Hsp70 and 90 expressions, probably as a necessity of repair processes at cellular level.
 - Both types of treatment stimulated C32 cells apoptosis, through underexpression of Bcl-2 and overexpression of Bax, in a concentration dependent manner.

The comparative analysis of the action of the extracts on the three cell lines suggests the following general conclusions:

- *P. ostreatus* extract with a concentration of 0,5 mg% polysaccharides has a protective and antiapoptotic effect on Caco-2 cells, in the absence and in the presence of fetal serum.

Instead, the decrease in metalloproteinases 2 and 9 expression suggests an inhibition of metastasis.

- *C. majus* extract and sanguinarine have a prooxidant, proapoptotic and antimetastase effect on C32 cells in a concentration dependent manner.

- *Z. officinale* fresh extract don't modify CCD cells viability, but decrease melanoma cells viability.

- *Z. officinale* autoclaved extract and 6-shogaol induced antioxidant protection systems and also proapoptotic mechanisms in C32 melanoma cells.

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a) in *ISI journals with impact factor*

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