Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Food Science and Technology
Open Access

The Study of Nutraceutical Effects of the Whey Zonar and of Lyophilized Concentrate Obtained from Zonar in C26 Colon Carcinoma Grafted Subcutaneously in Balb/C Mice

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RESEARCH ARTICLE

Abstract

This paper investigates the nutraceutical properties of the whey Zonar and of lyophilized concentrate obtained from Zonar in C26 tumor bearing BALB/C mice. The experiment was conducted on 30 female mice, divided into 6 groups (n=5). Groups 4, 5 and 6 were injected subcutaneously with $1*10^6$ C26 carcinoma cells. Groups 2,3, 5 and 6 received a diet based on Zonar products. The evolution of body mass and tumor volume was assessed weekly. At the end of the 21-day study, blood samples for hematological, biochemical and oxidative stress analysis were drawn and tumor tissue samples were collected for histopathological examination. After 21 days, a significant *in vivo* reduction of the tumor volume in groups 5 and 6 was recorded. The biochemical analysis showed Zonar's protective muscular effects, due to decreases of the creatine-kinase level in groups 5 and 6. Further investigations revealed an increased level of glutathione in all groups which received Zonar, eliciting its antioxidant potential. Histopathologically, increased necrotic areas highlighted the anti-tumoral effect of the synergism between Zonar and the lyophilized concentrate. The results of this experiment implies that whey Zonar & the lyophilized whey prevents tumor cachexia, as well as other cancer associated adverse effects.

Keywords: Whey Zonar, C26 colon carcinoma, experimental cachexia, nutraceutical properties

Received: 27 January 2022
Accepted: 22 July 2022
Published: 15 November 2022

DOI:
10.15835/buasvmcn-fst:2022.0002

INTRODUCTION

Nutritional modulation by functional and nutraceutical natural foods have the potential to reduce tumour growth rates and cancer associated oxidative stress, inflammation and cachexia. Additionally, it reduces chemotherapy and radiation therapy induced toxicity (Tripathi et al. 2005). Nutritional management of oncological patients is part of a multi-faceted approach therapy. By ensuring proper nutrition and quality of life the treatment response and course may be improved and the chance of survival could increase (Nivya et al. 2012)). There is an important need to establish an *in vivo* system to test the biological potential of a given diet with specific biological properties. Additionally, the gap between the scientific community and the updated knowledge of nutraceuticals and medical staff needs to be filled (Prasad et al. 2010). Colorectal cancer is one of the most studied tumors in terms of understanding the factors that contribute to its development and treatment (Yalcin et al. 2014). Usually, in this type of cancer, cachexia is considered a negative prognostic factor. It interferes with therapy and decreases quality of life due to impaired muscle function (Aulino et al. 2010). An

altered protein metabolism leads to muscle mass loss, so the protein in the diet must be highly digestible and exceed the normal necessary level of a healthy adult (Fearon et al. 2012).

The C26 colon carcinoma cells induce experimental cachexia. In addition, it is highly tumorigenic with high mortality and can even cause lung metastasis. C26 colon carcinoma cells are known to cause hepatic disorders and the loss of adipose and skeletal muscle tissue, providing a suitable model for investigating cancer-associated cachexia and being considered a standardized model of carcinogenesis and cachexia (Aulino et al. 2010).

Whey proteins have received increasing attention in the past decade. Today, whey products such as whey protein concentrates, isolates, hydrolysed whey and demineralized whey, are important dietary supplements with functional properties, suggesting their positive effects on health outcomes (Marshall 2004). Numerous nutritional studies, reports and trials were conducted to identify anticancer properties of whey proteins. Whey proteins have been shown to exhibit antiproliferative effects in colon cancers and other cancers (Bounous 2000., Yalcin et al. 2014). However, these protein sources are expensive. A more nutritive and cheaper source of proteins is highlighted in lactoserum. Relatively less emphasis has been placed on whey-based beverages. The next decade is likely to witness a considerable rise in natural beverage consumption, which has nutraceutical properties and could stand as health boosting agents. Despite this interest, no one to the best of our knowledge has studied the nutraceutical potential of lactoserum, which is a sweet whey-based beverage with the same bioactive components (peptides, essential amino acids, antioxidants, vitamins, minerals, etc.).

This paper aimed to investigate the nutraceutical properties of the lactoserum Zonar and of the lyophilized concentrate obtained from Zonar (LZ) in BALB/C mice bearing C26 tumor, in order to see if the administration of both whey and whey concentrate had anti-tumoral properties.

MATERIALS AND METHODS

Materials

In the present study, we used a commercial formula of whey (lactoserum Zonar, SC Embrion, Satu Mare, Romania) and whey concentrate (LZ) obtained from the same source and from the same lot of whey from cow milk. The lyophilized concentrate was prepared by our collaborators from the Raluca Rapan Institute of Chemistry, Babes-Bolyai University. Both products were characterized with regards to their physico-chemical composition. In order to identify the chemical composition and the concentrations from the LZ, the same amount of material, as in the case of the liquid lactoserum, were used, the sample processing being the same. The concentrate from Zonar (LZ) was obtained by lyophilization using an Alpha 1-4 LDPLUS Model Lyophilizer. The "Zonar" lactoserum was introduced into the freezer at -20 ° C in special plastic pots, half filled, to eliminate the losses by a possible foaming, and then allowed to freeze. The plastic vessels were then placed on the freeze dryer stand where the temperature was set at 40-600C and the pressure 1 bar, for 35 hours. The lactose content was determined by High-Performance Liquid Chromatography (HPLC) using HPLC Jasco Chromatograph (Japan), the method being described by Prodan et al. (Prodan et al. 2018). The total protein content was determined by electrophoresis, according to Prodan et al. (Prodan et al. 2018). Determination of minerals Ca, K, Mg, Na, P, Cu, Fe, Zn, Pb, Ca/P from the lactoserum samples was performed using the dry digestion method and an Optima 2100 inductively coupled optical emission spectrometer (ICP-AES) (Prodan et al. 2017., Prodan et al. 2018).

Diet

A diet based on Zonar, the lyophilized concentrate from Zonar and standard feed was implemented by calculating the daily calories needed for average mice with body weights of 25 g. The caloric requirement to maintain an adult animal is 160kcal / kg / day or 670kJ / kg / day (National Research Council (US) Subcommittee on Laboratory Animal Nutrition, 1995). According to the manufacturer (Cantacuzino Institute), the standard feed has 3.63 kcal / g. According to the analyses made in the Chemistry Laboratory (PP1, Raluca Rapan Institute, UBB Cluj-Napoca), 10 ml of Zonar had 2.35 kcal and 1 g of LZ had 4.13 kcal. Standard food was given once a day orally, and Zonar and Concentrate were administered orally daily twice, for 14 days.

Tumoral Cells

C26 murine colon carcinoma cells (Cell Line Services, 440156) were obtained from Department of Molecular Biology and Biotechnology, Faculty of Biology, UBB, Cluj-Napoca, Romania. The cells were cultured as a monolayer in complete RPMI 1640 medium (Lonza, 09-774F) supplemented with 10 % heat-inactivated fetal bovine serum (HyClone, SV30160.03), at 37 C in 5% Co2 humidified atmosphere. This cell line was authenticated via Real-Time PCR by the company from where it was purchased and tested for mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza, Lt07) The method used was reported by Patras et al. (Patras et al. 2017).

Experimental animals

The experiment was conducted on 30 healthy adult, female, BALB/C mice, aged 8 months with an average body weight of 25±1.95 g. The procedures involving the present project were lead following the guidelines of Directive 2010/63/EU and Romanian national law. The rats were housed in the Establishment for Breeding and use of laboratory animals of UASVN CN, in standard conditions, temperature 23ºC, humidity 55%, and light/dark cycle 12/12h. They were fed standard rodent pellets and had ad libitum access to water for an acclimatization period of 7 days. All the procedures carried out in the present project were approved by the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca Bioethics Committee and the State Veterinary Authority (aut. No. 51/30.03.2017).

Murine tumor model

For tumor induction, 1x 10^6 C26 cells diluted in 100 µl sterile saline solutions were inoculated subcutaneously in the right flank of BALB/C mice. C26 tumors became palpable 7 days after tumor cell inoculation. Tumor size was measured regularly starting with day 7 until the end of the experiment. Tumor volume was calculated as previously reported by Patras et al, according to the formula V= 0.52x a^2 x b, where a is the smallest and b is the largest superficial diameter (mm). Additionally, the body mass of the animals was assessed weekly. The animals were divided in 6 experimental groups (n=5), as follows: G1-reference group; G2-Zonar control (5 ml/ animal); G3-Zonar+LZ (1g/animal) control; G4-C26 control; G5-C26+Zonar (5ml/animal); G6-C26+Zonar+LZ (1g/animal). 2 diets were formulated, one based solely on Zonar whey while the other was based on both zonar whey and concentrate due to the higher protein charge of the concentrate. Our aim was to see which of the diets would be more optimal, from a nutritional standpoint. Based on alternative medicine data, human doctors recommended diets with a higher amount of digestible and bioactive proteins in colon cancer carcinoma, not only to induce an antioxidant effect, but also to prevent cachexia encountered with this pathology (1,2,3). On the 21st day, blood samples were collected in order to determine hematologic, oxidative stress and biochemical parameters. The animals were humanely euthanatized by prolonged narcosis and cervical dislocation. Separately, tumors and the carcass of each tumor-bearing animal were weighed.

Complete blood count and biochemical method

Complete blood counts were determined using the automatic Abacus junior Vet hematology counter. Biochemical analysis was carried out using the UV-VIZ Screen master Touch spectrophotometry analyzer (Hospitex diagnostics, Firenze, Italy), which monitored the following parameters: alkaline phosphatase and creatinine kinase.

Oxidative stress marker

The assessment of glutathione (GSH) in serum was performed by the adapted method described by Sedlak and Lindsay and also Rodrigues et al (Sedlak et al. 1968., Rodrigues et al. 2014). All the samples were measured using UV-Vis Jasco spectrophotometry analyzer (Jasco V-630, Tokyo, Japan).

Histological analysis

The harvested tumor samples were fixed in 10% buffered formalin and then embedded in paraffin. The paraffin cubes were cut with a high precision microtome Leica RM 2125RT, with sections being 5µm thick. The sections were stained by the haematoxylin-eosin method and examined under the Olympus BX 41 microscope, and the images were made using an Olympus UC 30 camera and analyzed with a special software, Olympus Stream Basic.

Statistical analysis

All data is reported as the mean ± SD. To assume Gaussian distribution, normality distribution was checked by the D'Agoustino and Pearson omnibus normality test. One-way analysis of variance ANOVA, followed by Bonferroni's Multiple Comparison test procedure was done for pair-wise comparisons. Statistical significance was set at $p<0.05$ (95% confidence interval). Statistical values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSIONS

Product characterization

Zonar beverage and the lyophilized concentrate from Zonar compositions are described in Table 1.

Table 1. Zonar and LZ protein and mineral content

Chemical compozition	ZONAR	LZ
Total protein g/L	1.517	47.985
BSA g/L	0.216	11.87
α- lactalbumin g/L	0.838	9.57
β-lactoglobulin B g/L	0.233	20.41
β-lactoglobulin A g/L	0.230	6.135
Lactose g/L	53.49	47.4
Ca mg/kg	190.85	3131.48
K mg/kg	901.10	7870.196
Mg mg/kg	47.49	841.64
Na mg/kg	250.42	451.44
Cu mg/kg	0.08	0.6
Fe mg/kg	3.395	16.99
Pb mg/kg	0.039	0.15
Zn mg/kg	0.21	3.24
P mg/kg	287.12	4899.73
Ca/P mg/kg	0.66	0.64

Effect of diets containing Zonar and LZ on animals' body weight and tumor growth

There were no significant changes regarding the body weight between the control groups fed with Zonar, LZ and a standard diet. Weight loss was unaffected in the first 10 days following tumor cell inoculation. Afterwards, the tumor control group's (G4) body weights decreased until the end of the experiment in comparison to the reference group (G1) ($p>0.001$). Further analysis showed that there was a significant increase in the body weights of C26 bearing groups fed with diets based on Zonar and LZ, in comparison to the tumor control group (G4) ($p>0.001$) (Table 2). In G4 group body weight loss was accounted for tumor increase accompanied by carcass loss (Table 3). Remarkably, this correlation is related to tumor mass kinetics, because in the Zonar and LZ groups, tumor decrease was observed, accompanied by non-significant carcass weight loss ($p>0.01$) (Table 3). Additional support to the previous statement comes from the tumor volume analysis, where a significant decrease in G5 (650.52±244.29 mm^3) and G6 (618.45±89.89 mm^3) was observed at 21 days after whey and LZ consumption, in comparison with G4 (1535.68±339.35 mm^3) (Figure 1).

Table 2. Effects of Zonar consumption on the body weight dynamic in experimental groups

Body weight (g)	Day 0	Day 7	Day 10	Day 14	Day 21
Control	19.4±1.81	19.4±1.81	20±1.58	20.2±1.30	21.4±0.89
Zonar	19.8±0.83	20±0.70	20.6±0.54	21.2±0.83	22.2±.0.83
Zonar+LZ group	19.4±0.54	18.4±0.54	20.6±0.89	20.2±0.83	23.4±1.67
C26	19.6±0.54	19.4±0.54	20.4±0.89	18.6±0.54	17.4±0.54***
C26+Zonar	19.4±1.14	20±1	20.8±0.83	21±1.22##	21.2±0.83###
C26+Zonar+LZ	20.2±0.83	20.8±1.09#	20.4±1.81	20.8±1.30#	21.4±1.94###

Note: (mean ± SD). (***= $p>0.001$ as compared to Group 1, (# $p>0.05$, ### $p>0.001$ as compared to Group 4), (5 animals group; two-way ANOVA, Post-test Bonferoni)

Table 3. Effects of Zonar consumption on tumor mass and carcass weight in C26 inoculated groups

Tumour groups	Tumor weight	Carcass weight
C26	0.66±0.20	17.03±0.32
C26+Zonar	0.30±0.12*	20.81±0.40**
C26+Zonar+LZ	0.36±0.04*	21.11±1.62**

Note: (mean ± SD). (* p>0.05, ** p>0.01 as compared to Group 4) (5 animals / group; one way ANOVA, Post test Bonferoni)

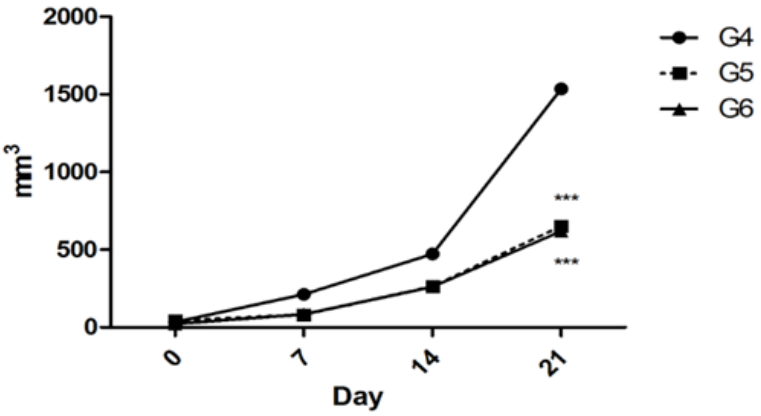

Figure 1. Effects of Zonar consumption on tumor volume gain (mm^3) (mean ± SEM). (***= p>0.001 as compared to Group 4) (5 animals / group; one way ANOVA, Post test Bonferoni)

Effects of Zonar and LZ diet on hematological parameters

The white blood cell count decreased considerably in the untreated C26 group (G4) in comparison to all other groups, controls (G1, G2. G3) and tumoral groups (G5, G6) (Table 3). Although the values remained in the physiological limits of the species, this test revealed an increase in the total white blood cells, based on lymphocyte and monocyte increase in groups fed with Zonar and lyophilized concentrate (G3, G6). In G6 the values are significantly increased in comparison to the reference group and the other controls (Table 4). The erythrogram and thrombogram remain almost unchanged, the values remained within the physiological limits of the species (Table 5).

Table 4. Effects of Zonar consumption on white blood cells, lymphocytes, mid cells and granulocytes in experimental groups

	WBC (10^9/l)	LYM (10^9/l)	MID (10^9/l)	GRA (10^9/l)
Control	7.50±1.79	3.16±1.37	0.37±0.28	2.76±1.18
Zonar group	7.59±1.35	4.78±1.15	0.31±0.20	2.69±0.72
Zonar+LZ group	10.42±2.64	5.03±1.52	1.67±1.83	3.91±1.96
C26 group	4.43±0.82***,††	2.66±0.65***	0.13±0.11	1.63±0.22
C26+Zonar	7.55±1.02###	4.50±0.66###	0.23±0.18	2.75±0.77
C26+Zonar+LZ	10.89±1.50###,††,**	6.40±1.51###,**	0.90±0.50#,*	3.78±1.61

Note: (mean ± SD). (*p>0.05, ** p>0.01, ***= p>0.001 as compared to Group 1, (#p>0.05, ## p>0.01, ### p>0.001 as compared to Group 4, ††p>0.01 as compared to Group 3)(5 animals / group; one way ANOVA, Post test Bonferoni) WBC: 4-12x10^9/l, LYM: 2-14.1x10^9/l, MID: 0-0.98x10^9/l, GRA: 0.1-5.4x10^9/.

Table 5. Effects of Zonar consumption on red blood cells, hemoglobin, packed cell volume and platelet numbers in experimental groups

	RBC (10^{12}/l)	HGB(g/dl)	PCV (%)	PLT (10^9/l)
Control	10.06±1.27	133.4±23.04	41.87±3.52	380.4±125.70
Zonar group	9.95± 0.13	139.6±5.17	42.87±1.10	324.2±46.48
Zonar+LZ group	10.10±0.89	135±9.02	42.85±3.81	341±211.35
C26 group	8.20±4.43	128.4±8.98	43.66±2.02	219.6±86.69
C26+Zonar	10.36±0.45	144.4±8.01	44.71±2.21	279.4±107.18
C26+Zonar+LZ	10.50±0.65	141±4	41.48±2.12	293.8±48.40

Note: (mean ± SD). (5 animals / group; one-way ANOVA, Post test Bonferoni) RBC: 9-15x10^{12}/l, HGB 90-150 mg/dl, HCT: 24-45%, PLT: 250-750x10^9/l

Effects of Zonar and LZ diet on biochemical and oxidative stress parameters

Changes in all tumoral groups who had not received Zonar were seen, specifically, increased creatinine kinase (CK) levels (G4:2023.28±448.80 U/L, G5:1191.24±233.83 U/L, G6:1327.96±241.85 U/L) in comparison to the reference group (463.86±251.18), but further analysis revealed that the CK values were significantly decreased in the groups fed with Zonar diet than the C26 group (2023.28±448.80 U/L) (p>0.001) (Figure 2). No significant difference was identified in the alkaline phosphatase values (ALP) (Figure 2). Figure 3 shows a significant increase in the glutathione levels in G5 (6.15±0.50) (p>0.001) and G6 (6.10±0.31) (p>0.001) groups in comparison to the reference group (3.68±0.53) and C26 tumoral group (3.11±0.32). Additionally, values were high in the control group fed with Zonar and LZ (4.89±0.89) in comparison to the reference group (p>0.001, p>0.01) (3.68±0.53).

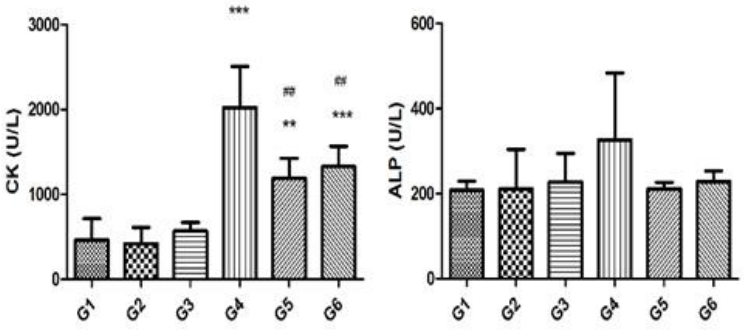

Figure 2. (a) Effects of Zonar consumption on creatinine kinase (CK) in tumor inoculated mice (U/L) (mean ± SD). (**= p>0.01, ***= p>0.001 as compared to Group 1, ## p>0.01 as compared to Group 4) (5 animals / group; one way ANOVA, Post test Bonferoni) (b) Effects of Zonar consumption on alkaline phosphatase (ALP) in tumor inoculated mice (U/L) (mean ± SD). (5 animals / group; one way ANOVA, Post test Bonferoni)

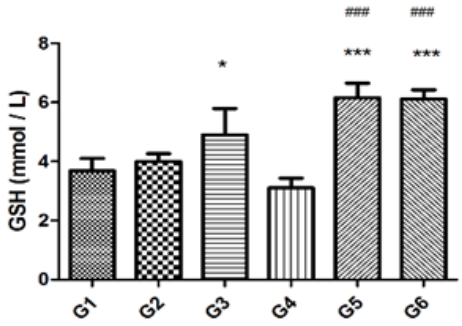

Figure 3. Effects of Zonar consumption on glutathione (GSH) in tumor inoculated mice (mmol/L) (mean ± SD). (**= p>0.01, ***= p>0.001 as compared to Group 1, ### p>0.001 as compared to Group 4) (5 animals / group; one way ANOVA, Post test Bonferoni)

Gross pathology analysis

Macroscopically, there were obvious differences between the various experimental groups in terms of size, weight and appearance on the tumor section. The tumors harvested from the control animals showed the largest size and weight, while the tumors from the treated groups were smaller in size, and on cross section, they had multiple dark-colored necrotic areas. The animals from the group fed with Zonar and LZ presented the most obvious areas of intra-tumoral necrosis.

Histological analysis

Sections from the G4 group showed the presence of an encapsulated anaplastic carcinoma with high cellularity and accentuated nuclear and cellular polymorphism. The tumor was located subcutaneously and compressed the adjacent residual structures, but it did not extend beyond the resection borders. It is a large tumour with a diameter of 1.5x1.5 cm, well delimited, encapsulated, appearing as a solid and expansive neoplastic mass. The tumor was intensely cellular, consisting of poorly differentiated neoplastic cells (cells of epithelial origin), grouped in dense sheets and occasionally in interlacing streams, the cells being separated by a delicate fibrovascular stroma. In the center of the tumor, multiple small areas of intra-tumoral necrosis were observed. The neoplastic cells were large, 35-50 µm in diameter, had a round or oval shape with indistinct cellular borders, finely granular cytoplasm, eosinophilic, slightly vacuolar, and moderate nuclear-cytoplasmic ratio.

The nuclei were round to oval, located paracentral or eccentric, with finely stippled chromatin and had 1-3 distinct nucleoli with irregular contour. Anisocytosis and anisokaryosis were severe and numerous cells had karyomegaly. The tumor was mildly vascularized with rare capillaries. Numerous mitoses were observed on all sections, the mitotic index ranging from 5 to 10 per high power field (Ob 40x) with numerous atypical mitosis (Figure 4).

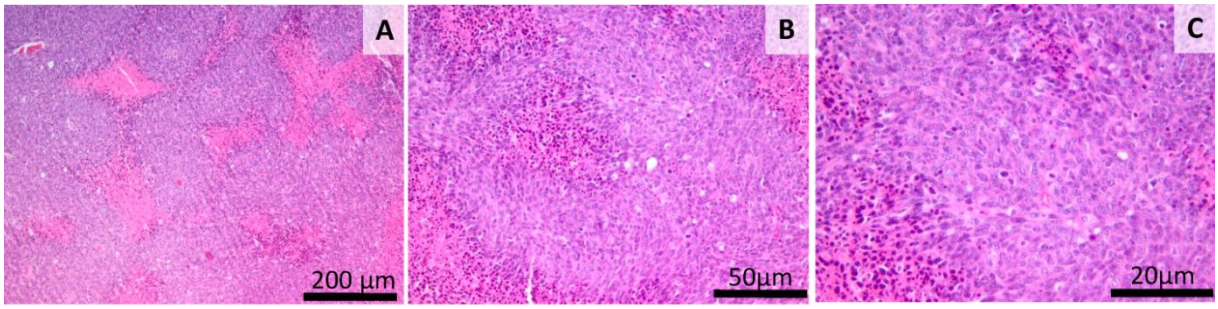

Figure 4. Solid tumor mass microphotograph showing: numerous necrosis icrofiches (A), high cellularity, marked anaplasia, nucleotide / cytoplasm ratio in nucleus, high mitotic index, necrosis microfocus, and many apoptosis (B, C) = 200 µm (A), Bar = 50 µm (B), Bar = 20 µm (C).

Sections from the Zonar group (G5) showed multiple large foci of central necrosis in the tumoral mass. The tumor was well encapsulated without dermal invasion. The neoplastic tissue was lost (lytic necrosis) and replaced by hypereosinophilic cellular debris. Inflammatory infiltrate was discrete, some degenerate neutrophils and rare macrophages being observed. The necrotic foci were associated with a pale eosinophilic homogenous material (oedema) dissociating the collagen fibers and neoplastic cells (Figure 5).

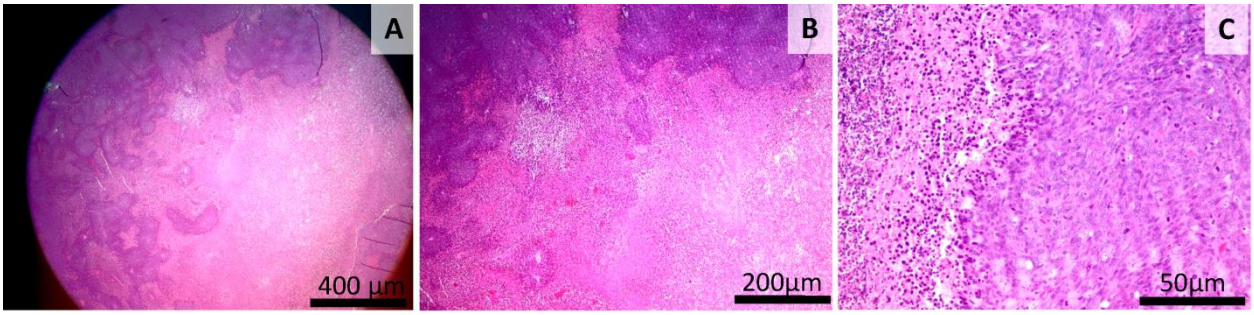

Figure 5. Microphotograph showing the Zonar-treated C26 murine colon carcinoma cell line, the outbreak of intratumoral necrosis, with the presence of eosinophilic cellular debris and intratumoral haemorrhage (A, B); tumor cell interface, necrosis area, mononuclear and rare neurofile inflammatory cells ©, HE staining, Bar = 400 µm (A), Bar = 200 µm (B), Bar = 50 µm (C).

Examination of the sections from the Zonar + LZ group (G6) showed massive intra-tumoral necrosis, viable tumor cells being present virtually only in the subcapsular marginal areas. In one case, numerous blood vessels were seen, both in the vicinity of the necrotic areas and in the peripheral regions with viable tumor cells. The large foci of lytic necrosis had an eosinophilic appearance, containing numerous cell debris, degenerated neutrophils and macrophages, and extravasated red blood cells, as a consequence of vascular necrosis. Also, foci of mineralization have been observed (Figure 6).

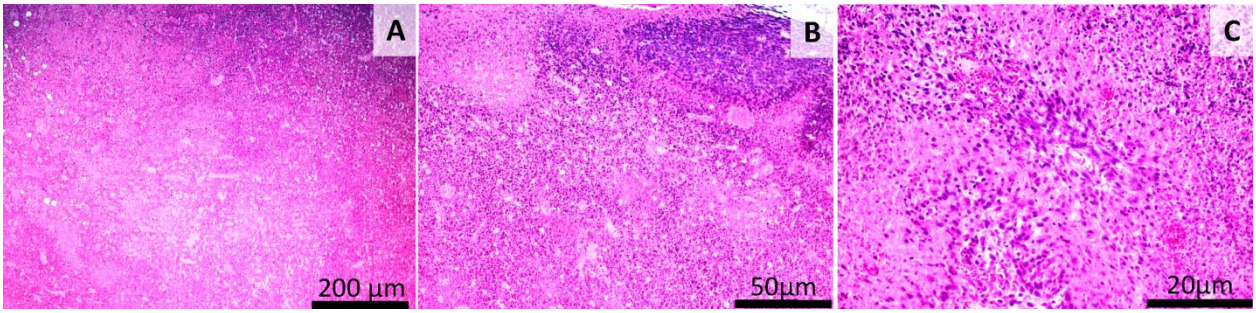

Figure 6. Microphotograph showing Zonar treated batch = Protein concentrate. Massive intratumoral necrosis with the presence of eosinophilic cellular debris (A, B, C); Rare viable tumor cells at the periphery of the formation (A, B), Massive necrosis, bleeding © HE staining, Bar = 200 µm (A), Bar = 50 µm (B), Bar = 20 µm.

We also performed a semi-quantitative assessment of the biological effects of the treatments on tumor morphology, giving a lesion score for several parameters (Table 6). The scores were based on microscopically observed lesions on H & E-stained sections.

Table 6. Effects of Zonar consumption on tumor morphology

Observations	Tumor Control (G4)	Zonar (G5)	Zonar+LZ (G6)
Tumour encapsulation	++++	++++	++++
Dermal invasion	+	+	+
Vascularisation	+	++	++++
Inflammation	+	+++	+++
Tumoral necrosis	++	+++	++++

Note: The scores were noted according to the following algorithm: 0 –absent, + minimum; ++ moderate; +++ high; ++++ extremely high

DISCUSSION

Colorectal cancer is thought to occur due to the interaction between genetic susceptibility and environmental factors, among which diet is the most important (World Cancer Research Fund 2007). Also, oxidative stress caused by the systemic inflammation produced by high fat diets, red meat intake and sugar intake increases risk of cancer (Yalcin et al. 2014). The first aim of this study was to investigate the *in vivo* nutraceutical effect and mechanism of a dietary combination between a whey beverage and the lyophilized concentrate from the same beverage, because to our knowledge it was never described before. This C26 model of carcinogenesis and cachexia helps in investigating the probable action of these products as nutraceutical aliments. A nutraceutical food is defined as a product that provides medical benefits for the prevention or treatment of a pathology (Rajat et al. 2012). Whey proteins have been validated to have the ability to scavenge reactive oxygen species and detoxify the organism of potential cellular carcinogens. Glutathione stimulation is thought to be the primary antitumoral mechanism responsible for immunity stimulation and detoxification (Marshall 2004., Bounous et al. 2003). Animal studies have shown that whey proteins protect against chemical induced colon cancers (Smithers et al. 1998., Tsuda et al. 1998., Bounous 2000., Hakkak et al. 2001., Attaallah et al. 2012) and metastasis (Kuhara et al. 2000). In vitro studies attested whey proteins antitumoral effects as well (Laursen et al. 1990., Tsai et al. 2000., Castro et al. 2009). However, the exact mechanism of action of whey proteins, nevertheless simple whey is not clear (Yalcin et al. 2014).

Chemical analysis of Zonar revealed the presence of bioactive components such as beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, immunoglobulins, amino acids, lactose and minerals. These bioactive components have the potential to modulate the immune response via essential and branched chain amino acids (Marshall 2004), retinol-binding proteins (Guimont et al. 1997), B-lymphocyte function enhancement (Bounous et

al. 1985), etc. The presence of these bioactive molecules might have been responsible for the anti-tumoral effect as well as for the increased glutathione levels in the Zonar dieting groups. The high protein quality and high percent of protein absorption are efficient substrates for enhancing muscle hypertrophy and strength. This could be the responsible mechanism in preventing muscle wasting and cachexia. Zonar's protein content makes it ideal for supporting protein synthesis and muscle preservation. Additionally, in the study was observed a significant positive correlation between tumour volume, tumor mass and carcass evolution. Animals with a decreased tumoral volume and mass had higher carcass mass, hence an approximately normal body weight and a slower tumoral evolution.
Zonar diets observed in this study were able to increase the total white blood cells number, by increasing lymphocytes numbers, thereby minimizing the immunosuppression associated with cancer evolution. This increase show that whey consumption could be used in boosting blood variables during cancer conditions, but further research is needed.

Elevated serum creatine kinase activity occurs with degenerative or necrotizing muscle injury (Goicoechea et al. 2008). Cachexia is a complex metabolic syndrome encountered in cancerous diseases, especially in carcinomas, due to tumor growth and proliferation but also due to the negative effects of chemotherapy (Toledo et al. 2014). Studies conducted on C26 experimental protocol show that the main mechanism involved in cachexia is protein catabolism with protein degradation, dystrophy and muscle atrophy (Aulino et al. 2010). The anti-cachectic treatments studied on murine model of colon carcinoma are varied and include therapies with chemical molecules: indomethacin, ibuprofen (McCarthy et al. 2004) and different diets such as: leucine or fish oil supplementation, administration of a hyperproteic feed, etc. (Van et al. 2009). There was a consistent decrease in the creatin kinase values in the Zonar fed groups, which corroborates with an anti-cachexia property. Additionally, there might have also been a justification for the use of Zonar and LZ for holding back cancer staging, due to normal alkaline phosphatase levels, the enhancement of the alkaline phosphatase enzyme being associated with colon cancer advancement. Generally significant increase of this enzyme occurs in patients with liver metastases (Saif et al. 2005). The protective role of this diet could be attributed to the high content of cystine/cysteine and gamma-glutamyl cysteine which are efficient substrates for glutathione synthesis. Glutathione is the most important thiol in the organism by protecting the body against electrophilic, halogenated structures, epoxides and successfully reduces hydrogen peroxide and other peroxides levels (Anderson 1998). Zonar and LZ diets caused an increase in the systemic glutathione levels that helped in modulating the antioxidant and cellular protective effects hence reducing muscular degradation, atrophy and other cancer associated negative effects. However, the tumoral groups have shown higher glutathione levels. This is mainly due to presence of glutathione itself within the cancerous cells and this mechanism is able to play both protective as well as pathogenic roles. The pathogenic role implies the protection of the cancer cells from reactive oxygen species as a self preservation mechanism. The main role of glutathione, however, remains the removal and detoxification of carcinogens. In order to see this mechanism in action in our experimental lots, we would need to perform further bimolecular analyses to clearly state whether the glutathione in our case is protective or pathological (Kennedy et al. 2020).

Microscopic examination of tumoral tissue showed active tumour cells death represented by a larger intra-tumoral necrosis in groups fed with Zonar diet, but more effectively in the Zonar and LZ diet due to the higher densities of neutrophils and macrophages. A direct association with tumor volume and mass was observed, hence the tumors from the treated groups were smaller in size, and on the section, they had multiple dark-colored necrosis outbreaks. The observed tumoral cellular death could be accounted for both whey proteins antioxidant and the immune stimulating effects, thus further study is needed for establishing the earlier suggested antitumour activities of Zonar and LZ.

CONCLUSIONS

This study suggests that diet supplementation with whey beverage Zonar and the lyophilized concentrate from Zonar enhance the antioxidant capacity of the body by glutathione modulation.

The ability of Zonar diet to prevent protein catabolism, respectively carcass loss and muscular degradation by providing highly digestible and valuable amino acids, peptides and proteins may be a mechanism through which whey consumption becomes an anticachectic agent.

The most pronounced antitumor effect was observed in the group fed with the combination of Zonar with LZ, as evidenced by the increase of necrosis areas and increased cellularity in this group of animals, as well as demonstrated by the significant tumor volume and tumor weight reduction after 14 days of diet consumption.

Zonar and its lyophilisate act as nutraceutical products, the healing potentials being more of a synergistic combination of its content in bioactive peptides.

Authors Contribution: D.A,. Conceived and designed the analysis, article writing; O.S., Designed the *in vivo* experimental protocols; A.P, Collected data and participated in the writing of the article; M.M, Designed the chemical analysis; D.P, Collected the data regarding the chemical analysis of the product; A.N. Collected data, performed

histopathological analysis and supervised the writing of the article; S.B, Collected data and performed the statistical analysis; M.I, Supervised the experimental design and corrected the manuscript; A.T, Collected data, article writing.

Funding Source: This work was supported by a Grant of the Romanian National Authority for Scientific Research & Innovation, CNCS/CCCDI-UEFISCDI, project number PN-III-P2-2.1-BG-2016-0335, within PNCDI III.

Conflicts of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and the writing of the article.

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