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# Biomaterials obtained from probiotic consortia of microorganisms. Potential applications in regenerative medicine

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**ABSTRACT**

The beneficial effects of metabolites resulted from the consortium of probiotic microorganisms are known since the year 220 B.C. when they were used in Asia. The effects of these probiotic consortia were brought into actuality in the last century, after second World War, by researchers of the Moscow Academy of Sciences, during the studies regarding the incidence of cancer in Russia. They have found that at residents of some areas heavily polluted with radiation, the cancer incidence it is practically non-existent. Further investigations revealed that these people were healthy, due to the consumption of a specific mixture, which contains vitamins and minerals (bioproduct), obtained through fermentation, with specific yeasts. Studies have shown that fermented drink contains vitamins from the group B (B1, B2, B3, B5, B6, B7, B9, B12), vitamins A, C, E, polyphenols, amino acids, enzymes, glucuronic acid, usnic acid, caffeine, lactic acid, malonic acid, propionic acid, acetic acid, ethanol (0.5%) and protein (15 g/L). Microbiological analysis of the species involved in the bioprocess showed the existence of the specific symbiosis between several types of microorganisms type: *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Brettanomyces bruxellensis, Bacterium xylinum, Bacterium xylinoides Bacterium gluconicum, Bacterium katogenum, Pichia fermentans Bacterium, Acetobacter xylinum Gluconobacter oxydans, Torulaspora, delbueckii*. The studies performed in vitro with this bioproduct (mixture), have confirmed that the immunomodulatory effect is due to cultivation media, which contain flavanols and catechins.

**KEYWORDS**

consortium of probiotic microorganism; antioxidant properties

## Introduction

The beneficial effects of metabolites resulted from the consortium of probiotic microorganisms (PMC) are known since the year 220 B.C. when they were used in Asia. The effects of these probiotic consortia were brought into actuality in the last century, after second World War, by researchers of the Moscow Academy of Sciences, during the studies regarding the incidence of cancer in Russia. They have found that at residents of some areas heavily polluted with radiation, the cancer incidence it is practically non-existent. Further investigations revealed that these people were healthy, due to the consumption of specific cocktail,

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which contains vitamins and minerals obtained through fermentation, with specific yeast consortium. Asked where they got yeast, residents have said that I know that was brought to Russia by Chinese travelers, and the Chinese have obtained from the Japanese original. The first recordings on this tea consumption were recorded since 220 (b.c) in Manchuria, and from there spread rapidly in India, Africa, Russia, Germany, America, Eastern Europe. The drink is known as "Manchurian Tea", "Kargasok Tea", "Le champion de la charite", "Divine Che", "Tea Fungus" or "Kombucha". Actually research showed that fermented drink contains enzymes, amino acids, beta glucans, antioxidants, antibacterial and antitumor compounds, B vitamins (B1, B2, B3, B5, B6, B7, B9, B12), vitamin A, vitamin C, polyphenols, vitamin E, essential amino acid (lysine), enzymes, glicoronic acid (anticancericene effects) usnic acid (with antiviral and antimicrobial effect) caffeine, lactic acid, malonic acid, propionic acid, acetic acid, ethanol (below 0.5%), protein (15 g / L) [1–2]. Microbiological analysis of the species involved in fermentation of black tea highlighted a symbiosis of several types of bacteria and yeast called probiotics consortium of microorganisms, among the most important are: *Saccharomycodes ludwigii, Schizosaccharomyces pombe, Brettanomyces bruxellensis, Bacterium xylinum, Bacterium gluconicum, Bacterium xylinoides, Bacterium katogenum, Pichia fermentans, Candida stellata, Acetobacter xylinum, Gluconobacter oxydans, Torulaspora delbueckii* [2–6]. Regarding the health effects of the large amounts consumption of this product, although no comprehensive studies or clinical records of hospitals were released, it can be affirmed that this product can become toxic only if it is contaminated with molds or dangerous fungi due to the effect of toxins produced by them. A different kind of toxicity was reported due to accumulation of plumb in the product, the heavy metal being absorbed from the walls of glass vessels in which fermentation is done. An effect of increasing levels of lactic acid in body fluids, or cardiac arrest was observed for tea drinking fermented with probiotic microorganism consortia (PMC). For this reason it is recommended moderate consumption of tea fermented PMC, especially by people with health problems [7–11].

Analysis of tea infusion, reveal that the major components in tea are catechins, flavonols and flavones, theaflavins, thearubigins and other components like theasinensin (Theasinensin A (EGCG dimer), B (EGCG and EGC dimer), C (EGC dimer), and F (EGCG and ECG dimer)). Gallic acid and its quinic acid ester, theogallin, are the major simple polyphenols found in tea [6, 12]. Cinnamic acid derivatives of quinic acid, the coumaryl and caffeoyl-quinic acids have also been identified from tea (Figure 1). Tea water-extractable solids contain about 2–5% caffeine and much smaller quantities of theobromine and theophylline. Tea contains around 17% nitrogenous materials as protein (∼6%) as well as amino and nucleic acids (∼8%). Among the 19 amino acids found in tea, theanine (-N-ethyl glutamine) (Fig. 2) is unique to tea. It is a significant component of both green tea and black tea, comprising about 3% (w/w) of extract solids. Theanine has been associated with antihypertensive effect. Potassium, calcium, magnesium, and aluminum are the predominant minerals found in the tea ash (10–15%) [13].

## Objectives and methodology

Our objective was to evaluate the physico-chemical and biological properties of bioproducts obtained from submerged biosynthesins using consortia of probiotic microorgnisms (PMC). The PMC was inoculated into media which contain dextrose and an aqueous infusion of dried leaves of the *Camellia sinensis*, according to method presented in [14]. After biosynthesis, two bioproduct was obtaining: respectively: 1) supernatant obtained throughout biosynthesis,

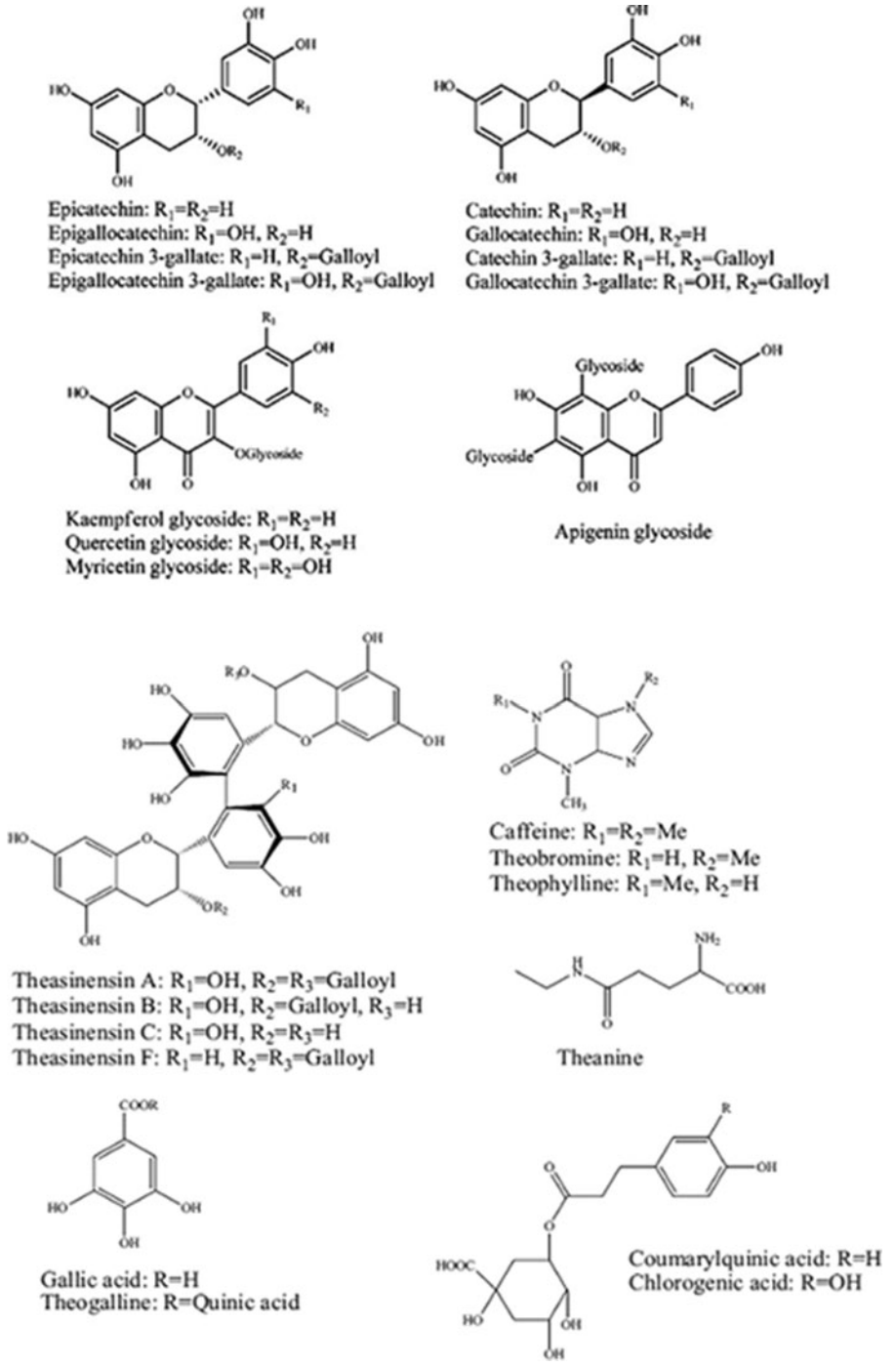


**Figure 1.** Structures of the major other components from tea [13].

using culture media and PMC (PMC supernatant); 2) solid bio product obtained by atomisation of biosynthesis bioproducts of culture media and PMC (PMC powder). For these two bioproducts, we aimed to determine the physical, chemical and biological properties, in order to explain the effects mentioned in the literature. For this purpose, we used the following devices and materials:

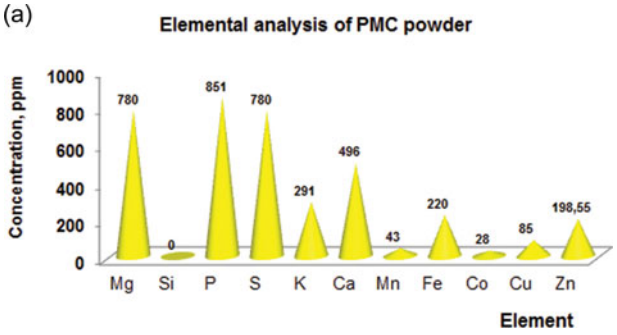


**Figure 2a.** Elemental analysis of PMC powder.

- spectrometer ICP-AES VARIAN Liberty 110;
- spectrophotometer FT-IR GX, Perkin Elmer;
- di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid;
- Folin–Ciocalteu reagent used for determination of the Total Phenolic Content [15];
- human leukemia cell line, type EHEB (DSMZ ACC 67), used to in vitro evaluation of the anticarcinogen effect [16]
- etoposide, named also-16, (4'-Demethyl-epipodophyllotoxin 9-[4,6-O-(R)-ethylidene-beta-D-glucopyranoside], 4' -(dihydrogen phosphate)), used as anti carcinogenic product [17].

Antioxidant and anti-carcinogenic effect was determined *in vitro*, using liquid materials, passed through sterilizing filters Millex GP 0.22 $\mu$m (Millipore). In the case of solid product, was used an aqueous extract and / or acid. Liquid extracts were obtained by homogenizing for 8 hours, under stirring at 200 rpm, of 0.4 g solid in 100 mL of bidistilled water, respectively 0.4 g of solid in 100 mL hydrochloric acid solution 0.5%. In this case, the suspensions resulted, were sterilized by passing on the Millex GP filter $\mu$m 0.22 (Millipore). The Bio-product concentration was chossed by taking into consideration the recommended quantities for human use (respectively 1 capsules on three times /day, each capsules contain 0.25 g atomised powder, Medica Farm Holding). In total in this study we use 4 samples as following:

- sample 1: Culture media: black tea obtained by infusion of 1.5 g black tea at 200 ml hot water, cooled and passed through a sterilizing Millipore filter;
- sample 2: supernatant of PMC powder in water (0.4 g atomized PMC in 100 mL water) passed through a sterilizing Millipore filter.
- sample 3: supernatant PMC powder in 0.5% HCl solution (0.4 g atomized PMC in 100 mL HCL solution 0.5%) passed through a sterilizing Millipore filter.
- sample 4: PMC supernatant, passed through a sterilizing Millipore filter.

## Results and discussion

Besides the major components presented in fig. 1 the elemental analysis of the two bioproducts (Figure 1–2) showed a high content of macro elements such as phosphorus (460 ppm for the product obtained by atomization and 851 ppm for supernatant), sulfur (460 ppm in liquid and 780 ppm in powder) calcium (123 ppm in solution and 496 ppm for solid), magnesium (780 ppm in atomized powder) and potassium (291 ppm in powder). Microelements found in products (Figure 1) were iron (present in concentrations of 50 ppm in solution and 220 ppm in powder), cobalt (found only in atomized powder in a concentration of 28 ppm), copper

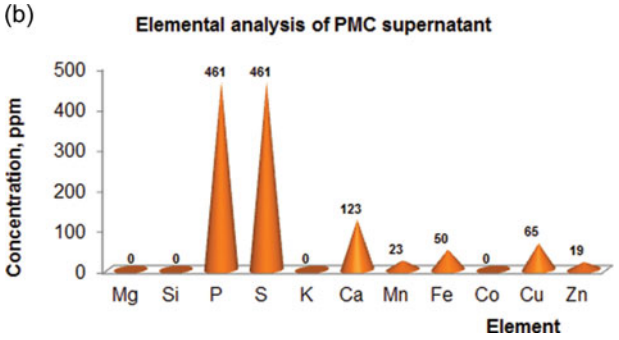


**Figure 2b.** Elemental analysis of PMC supernatant.

(65 ppm in liquid and 85 ppm in powder), manganese (found only in powder obtained by atomization, in concentrations of 43 ppm) and zinc (present in both bioproducts in concentration of 198.6 ppm in solid and 19 ppm liquid). In solid biomaterial, the content of macroelements and microelements is significantly higher than in liquid biomaterial (fig. 2a and 2b), probably due to the conditioning type (after biosynthesis, the resulting slurry was filtered and the clear solution was atomized in vacuum, at low temperature)

Infrared spectroscopy indicated the presence of hydrogen bonded to oxygen, type phenolic or alkyl, and the existence of secondary amine. These statements are supported by the appearance of stretch bands at 3297.65 $cm^{-1}$ (powder) and at 3330.65 $cm^{-1}$ (liquid) (Figure 3, a,b). High intensity of these bands, indicates the presence in these two biomaterials, of a complex compound that contains the hidroxyl group, type phenolic and/or alkyl, in high concentrations [18–19]. The band of the high intensity that occurs in the liquid product at 1637.09 $cm^{-1}$, may be due to existence of aromatic compounds, and to the compounds which contain primary amines such as theanine or derivatives of theanine. The presence of phenolic groups is confirmed by the appearance of the bands at 1417.26 $cm^{-1}$ respectively at 1359.5 $cm^{-1}$, belonging to the phenolic hydroxyl groups or tertiary alcohols, from solid bioproduct.

The phosphorus presence, which exist in the solid biomaterial is indicated by the bands that appear at 1247.78 $cm^{-1}$ and at 1047.98 $cm^{-1}$, due to P-O-C vibration from aromatic phosphates (1247.78 $cm^{-1}$) and of the vibration of the same groups that exist in phosphates

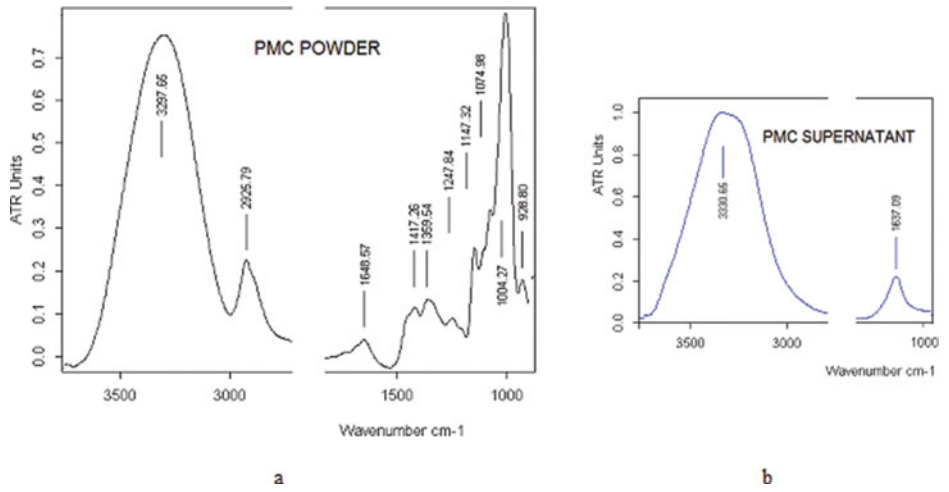


**Figure 3.** Infrared spectra of PMC powder (a) and PMC supernatant (b).

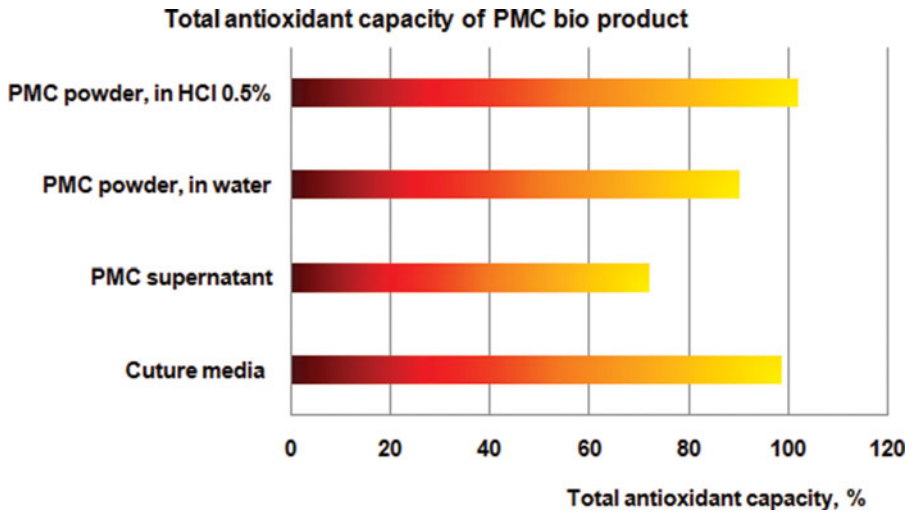


**Figure 4.** Total antioxidant capacity of PMC bio products.

from aliphatic compounds (1047.98 cm $^{1}$). The high intensity band from the 1247.78 $cm^{-1}$ may be also due to the existence of the aromatic ethers (C-O-C) from catechins, flavonols and flavones [13, 18–19].

The existence of the large rings of cyclic ethers and / or substituted alkyl ethers, is confirmed by the appearance of the band at 1147.32 $cm^{-1}$ in solid bioproduct (fig. 3a).

The results concerning total antioxidant activity (AA) (fig. 4) showed that the best bio-products in this regard are the black tea infusion (culture medium) with an AA = 98% and the acidic extract with an AA = 102%. The supernatant has an AA = 70% and the aqueous extract with an AA = 90%. The experiments performed with the Folin Ciocalteu reagent in comparison with Trolox reagent (figure 5), revealed also the fact that the culture medium and the acidic extract have the highest antioxidant activity, namely: 6 mEGallicAcid·$kg^{-1}$;

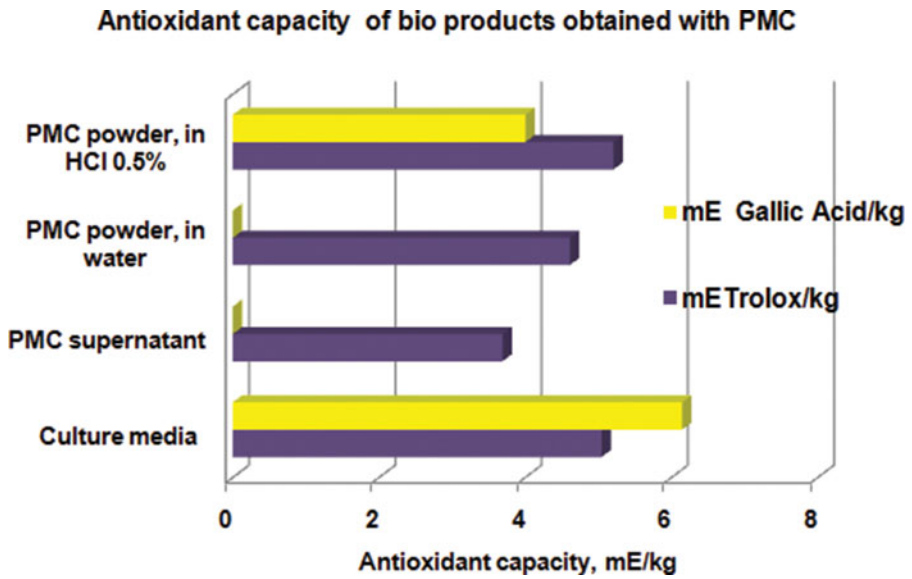


**Figure 5.** Antioxidant capacity of bio products obtained with PMC.

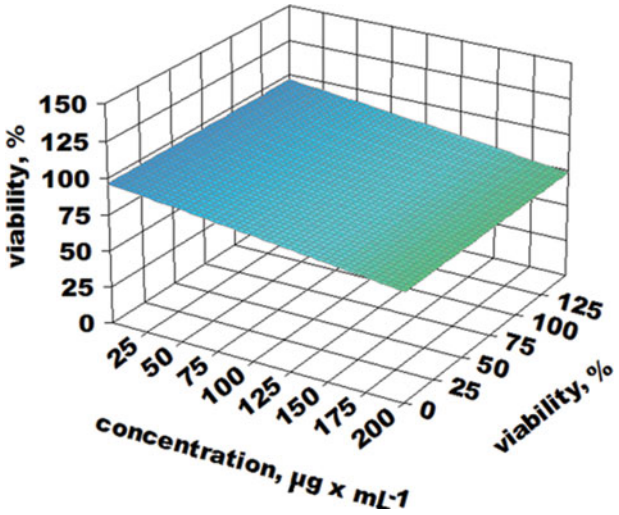


**Figure 6.** Effect of supernatant produced by PMC on EHEB cells line (after 48 h of exposure).

5 mETrolox · $kg^{-1}$ for culture medium; 3.9 mE GallicAcid·$kg^{-1}$ and 5.1 mETrolox·$kg^{-1}$ for acidic extract.

The results obtained in these ways confirm the existence of polyphenolic compounds in the two bio products, thus explaining the immunomodulatory activity.

The experiments carried out on leukemic cell lines showed that after 48 hours of exposure, the development of cancer cells is inhibited by about 25%, for the liquid and for solid bioproduct (Figure 6–7).

However, especially for the solid bioproduct, for a 24 h of exposure and bioproduct concentrations lower than 400 $\mu$g / ml, cell apoptosis is 20% lower compared to the untreated cells, and with cca. 40% lower compared with the results obtained for the cells exposed to etoposide (Figure 10).

For the same time of exposure, but using bioproduct concentrations between 500–1000 $\mu$g / ml, the cell viability seems to be stimulated. Increasing exposure time to 48 hours, reveal the decrease of cell viability, by about 10- 20%. However the viability is lower compared with untreated cells, but is inferior to cells treated with etoposide, where the cells viability is reduced

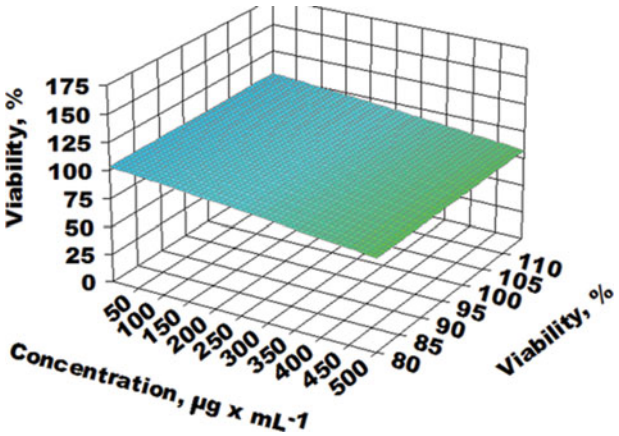


**Figure 7.** Effect of PMC powder extract (aqueous extract) on EHEB cells line (after 48 h of exposure).

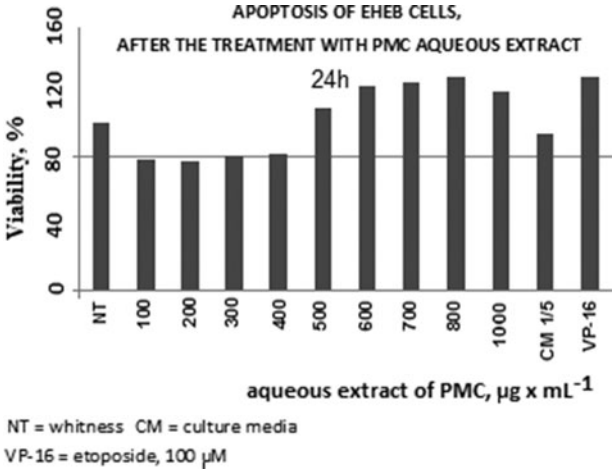


**Figure 8.** Apoptosis of EHEB cells after 24 h of exposure to PMC aqueous extract.

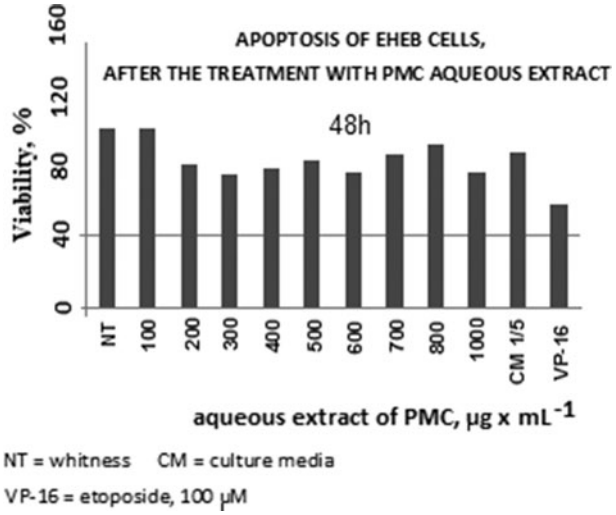


**Figure 9.** Apoptosis of EHEB cells after 48 h of exposure to PMC aqueous extract.

by 50% (Figure 9). *In vitro* determinations reveal a possible apoptotic effect on leukemic cells, effect which apear to be significantly at 48 h after exposure. In all cases, the registered efects are inferior to ectoside reagent which is used as a form of chemotherapy for cancers, that targets DNA topoisomerase (the etoposide blocking the enzyme called topoisomerase 2, which is necessary for cancer cells to divide and grow into 2 new cells [20]).

## Conclusions

The studies performed on the powder and supernantant PMC reveal the presence of macroelements and microelements like Ca, Mg, K, Co, Cu, Fe, Mn, Zn. The antioxidant tests indicate an antioxidant capacity of 3.5 mETrolox/KgPMC for supernatant, respectively of 5,4 mE Trolox/kg PMC for powder.

Total antioxidant capacity ranged betwen 75% for PMC supernatant and 102% for PMC powder in HCl solution (culture media from human digestive system).

Infrared spectra confirm the presence of OH bonds by the presence of the band at 3297,65 $cm^{-1}$ (powder bio product) and respective;at 330.65 $cm^{-1}$, and the presence of C=O in both bioproducts (band from 1648.57 cm-1 from powder PMC respectively the band from 1637.09 cm-1 from supernatant PMC). The studies performed on leukemic cell line treated with the studied bioproducts reveal an decreasing viability with 25% in comparison with the untreated cells.

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