The Effect of Polyphenolics in Extracts from Natural Materials on Metabolic Activity of Metastatic Melanoma WM-266-4 Cells

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Abstract: The importance of natural crops in medicine and pharmacy is growing. Beside bioactive compounds used directly as therapeutic agents, there are also raw materials used for drug synthesis or as a basic model for new biologically active compounds. In this paper, the optimum conditions for material extraction of *Curcuma longa*, *Lycium barbarum*, *Equisetum arvense*, *Vitis vinifera,* and *Rosmarinus officinalis* were investigated to achieve high antioxidant levels. The main aim of this study was to verify the correlation between the content of antioxidants, proanthocyanidins and total phenolic substances for certain extracts from the raw materials (*Curcuma longa*, *Lycium barbarum*, *Equisetum arvense*, *Vitis vinifera* and *Rosmarinus officinalis*) and the reduction of the metabolic activity of skin cancer cells.

Keywords: *Curcuma longa*; *Lycium barbarum*; *Equisetum arvense*; *Vitis vinifera*; *Rosmarinus officinalis*; antioxidant activity; total phenols; proanthocyanidins; metastatic melanoma WM-266-4 cells

1. Introduction

The incidence of malignant melanoma, a type of skin cancer, has been increasing during the last several decades; it is now the fifth most common cancer in males and the seventh in the female population in the United States [1]. The treatment of melanoma remains challenging, as many chemotherapeutic agents show a limited selectivity for tumor cells and cause adverse effects [2]. Moreover, resistance to chemotherapy is frequently observed in melanoma therapy [3]. Many clinically approved anticancer drugs contain ingredients of a natural origin, such as natural products, derivatives or synthesized molecules, which are from natural products [4,5]. Some well-known anti-cancer drugs include ingredients such as cantharanthus alkaloids, colchicine, etoposide, and taxol [6–11]. Plant-based secondary metabolites, such as polyphenol compounds, alkaloids, and terpenes possess diverse pharmacological properties, including cytotoxic and cancer chemopreventative effects [12]. The latter may appear during different phases of malignant tumor development and involve various mechanisms, such as protecting DNA from oxidative damage. Several clinical studies have shown the anti-proliferative effect of natural extracts on different cancer cell lines [13–17]. Most of the surveys were performed in vitro, except those specifically indicated, which were in vivo.

In addition, some studies have shown that various nutritional factors affect cancer rates and even prevent malignancy. Some fruits, vegetables and herbs contain large amounts of nutrient and non-nutrient phytochemicals and appear to be effective antioxidative agents. It has been reported that plant-based extracts were applied to different cells in order to study their antiproliferative activity [18]. For further analysis, different extraction methods were used where the extracts were obtained by methods such as supercritical fluid and pressurized liquid extraction, in addition to conventional procedures [19]. The most frequently utilized solvent was methanol in combination with supercritical carbon dioxide (CO_2). Extracts obtained by supercritical CO_2 extraction have revealed antiproliferative effect, especially equated to Soxhlet extracts [20].

Extracts from Rosemary were applied to several different human cancer cell lines. Active compounds (carnosic acid, rosmarinic acid) and extracts were applied to NCI-H82 (human, small cellular pneumonia, cancer), DU-145 (human, prostate, cancer), Hep-3B (human, breast, adenocarcinoma), PC-3 (human, prostate, adenocarcinoma), MDA-MB-231 (human, breast, liver, carcinoma, hepatocellular), and K-562 (human chronic myeloid leukemia, adenocarcinoma). The MTT test was used, where extracts were shown to have many different cytotoxic effects against cell lines [20]. It was established that extract of rosemary has antiproliferative activity on A2780 human cancer cells and on A2780CP70 daughter-cell line [13].

Pre-clinical studies based on in vitro and animal models of different cancer cell lines, including breast, cervical, colon, gastric, hepatic, leukemia, oral epithelial, ovarian, pancreatic, and prostate, have consistently shown that the curcuma extract, curcumin, possesses anti-cancer activity.

Additionally, the effect of turmeric on A375 melanoma cells was investigated and various concentrations of curcumin were used. The researchers observed cellular morphology and determined migration, invasion, proliferation, and apoptosis of A375 cells in vitro. The final process also revealed that curcumin caused a major change in the morphology of the A375 cells [21]. Other studies indicate an antioxidant, anti-inflammatory, antiangiogenic, and antiproliferative cell line activity, particularly in breast cancer (MCF-7, MDA-MB-231, BT-48), melanoma cell lines (C32, G-361 and WM 266-4), and HNSCC lines (CAL27, UM-SCC14A, UM-SCC1). The highest concentration was 10 mg/mL [16,17].

The arrangement of chemical bonds in the structure of methyl sulfone is almost identical to that of the well-known compound binding the microtubules in cells [22]. DMSO was once considered to be a promising new anti-cancer drug, mostly because it causes induction of polymerization and stabilization of microtubules. Extensive studies have shown that DMSO has no anti-cancer properties [12]. Equisetum (horsetail) is widely found in grasslands and contains high levels of methyl sulfone, and it can be a useful compound with effective and chemotherapeutic impact for metastatic melanoma cells treatment and other possible treatments of metastatic cancers [23]. Horsetail extracts were applied to two human cancer cell lines: HeLa, HT-29, and MCF7. The results of electron spin resonance (ESR) analysis confirmed that the investigated extracts suppressed the formation of lipid peroxyl radicals in a dose-dependent manner. The results show that n-butanol, methanol, ethyl acetate, and water extracts have significant peroxyl radical scavenging activity. Additionally, the chosen extracts repressed the interphase of cells depending on specific cell line and their concentration. Ethyl acetate extract showed the largest outstanding antiproliferative effect without inducing any cell growth stimulation on human tumour cell lines. According to the results obtained, horsetail extracts could be used as a natural source of antioxidants and as potential phytochemicals [14]. The analysis emphasized that some extracts were beneficial antioxidants in comparison to the reference molecules, vitamin E and quercetin. A correlation was found between the total amount of phenolic compounds contained in the extracts and antioxidant effects. Significant effects of the extract on proliferation of melanoma B16 cells were studied and reported [15].

In other studies, phytochemicals present in grapes have also shown potential anticancer activity in preclinical and clinical studies [24–26]. One such phytochemical is resveratrol. Additional extracts attained from various parts of grapes have been tested for their cytotoxic effect on different cell lines: MCF-7 cells, A427 lung cancer cells, gastric adenocarcinoma CRL-1739 cells, A549 and H1299 lung

cancer cells, Cal27 and SCC25 oral squamous cell carcinoma cells, and Jurkat cells. The extracts were also tested for antioxidant activity effect (UV-B induces skin carcinogenesis), anti-inflammatory effect (TNBS induces ulcerative colitis rat model), apoptosis of cells (DU145 and LNCaP), antiangiogenic (MCF-7, MDA-MB-231), and antimetastatic effects (4T1 Breast cancer cells) [27]. A correlation was also found between antioxidant effects and the total amount of phenolic compounds contained from the extracts, which results in a significant effect on the proliferation of melanoma B16 cells [15].

This study analysed the influence of antioxidant activity, proanthocyanidins and total phenols from selected natural materials (curcuma, goji berries, horsetail, rosemary, marc and seeds of grape) on the human melanoma cell line, to which these extracts have never been applied, and the literature concerning similar research is scarce. To determine the metabolic activity (the sum of the chemical reactions that take place within each cell of a living organism and that provide energy for vital processes and for synthesizing new organic material) of these cells, the WST 8 test was employed.

1.1. Curcuma (Curcuma longa)

Curcuma is a tropical plant from the ginger family; it grows under similar conditions. Short stems grow from its pear-shaped outgrowths with lateral shoots. The stalks sprout bright green, petiole-shaped petioles with prominent veins set in the shape of a fish bone. On short flower shoots, clusters of bright yellow double-someric flowers develop [28,29].

Curcuma longa has many diverse effects, for instance antioxidant [30], antimutagenic [31], antimicrobial [32], anti-inflammatory [33], anti-tumour [34], anticarcinogenic [35] wound healing, and gastroprotective activities [36]. *C. longa* and its active compounds (curcumin) (curcumin, 4-hydroxycinnamoyl (feruloyl) methane and bis (4-hydroxycinnamoyl) methane [37]) lessen nephrotoxicity, hepatotoxicity, cardiotoxicity, neurotoxicity, and lung toxicity mainly through the reduction of inflammatory cytokines, antioxidant and antiapoptotic effects [38]. It has been reported that in healthy human subjects, daily intake decreases the amount of total blood lipid peroxides as well as in HDL (high-density lipoprotein cholesterol, also called "good" cholesterol) and LDL (low-density lipoprotein cholesterol, also called "bad" cholesterol)-lipid peroxidation. This reduces the risk for cardiovascular disease. The literature reviewed indicates that curcumin and related plant co-antioxidants are powerful anti-inflammatory substances [39]. Much research has been conducted on the anticancer effects of turmeric. Curcumin and turmeric extract reduce the development of animal tumours, as indicated in evaluated experiments in vitro using tissue culture methods and in vivo in mice using Dalton's lymphoma cells [40,41]. Data also suggests that curcumin may take a role as a suppressor for Sp-1 activation and its downstream genes, including ADEM10, calmodulin, EPHB2, HDAC4, and SEPP1 in a concentration-dependent manner in colorectal cancer cell lines, and that curcumin could also act as a suppressor for Sp-1 activity in bladder cancer and decrease DNA binding activity of Sp-1 in non-small cell lung carcinoma cells [42]. The antiproliferative effects of curcumin include inhibition of several breast tumour cell lines, including hormone-dependent and -independent and multidrug-resistant (MDR) lines. Summarizing the results leads to the conclusion that curcumin is a potent antiproliferative agent for breast tumour cells and could have potential as an anticancer agent [43,44].

1.2. Goji Berry (Lycium barbarum)

Goji berry (*Lycium barbarum*) is a perennial, medicinal plant native to northern China. *Lycium barbarum* shrubs can also be found in Europe, especially in the United Kingdom, Romania and Bulgaria. It is classified as a member of the Solanaceae family, which includes potatoes, tomatoes, peppers, and aubergines. The plant grows up to 3 m tall. Most often, it is grown as a tree with hanging branches. It is a two-sex deciduous plant that blooms with small purple flowers in July. It bears fruit until the first frost and tolerates drought and cold (up to −20 °C) [45–47].

More than 200 different components of the Goji berry have been identified, characterized, and analysed. The most interesting for medicinal purposes are carotenoids, phenylpropanoids,

flavonoids, polyphenols, and polysaccharides [48]. *Lycium barbarum* polysaccharides (LBPs) are the essential compound in the berries and are involved in various biological functions. This bioactive molecule of goji berry consists of a complex mixture of glycoconjugates, with a wide interval of molecular weights (10–2, 300 kDa), which is soluble in water (in our study we used only water) and contains a carbohydrate portion (≥90%) [48,49]. Goji berries possess properties that promote many pharmacological activities because of their polysaccharide content, including antioxidant and antiaging effects, increased metabolism, immune system regulation, neuroprotective properties, reduction of cardiovascular diseases, as well as antidiabetic, antiglaucoma anticancer, and immunomodulatory effects [45,50–53]. According to the literature, ultrasound-enhanced subcritical water is the most effective medium for extraction of polysaccharides from *Lycium barbarum* and it has been reported that is the most effective way to use dried goji berries. Compounds present in *Lycium barbarum* berries are used in cases of treating the onset and progression of cancer in traditional Chinese medicines. Many tests have confirmed pro-apoptotic and antiproliferative activity of compounds present in *Lycium barbarum* against cancer cells. There are reports of significant reduction of lipid peroxidation in mice under the influence of Goji polysaccharide fractions and inhibition of proliferation of liver cancer cells and anticancer properties of polysaccharide fractions. It was acknowledged with in vitro and in vivo studies that LBPs can act at different levels. Proliferation and insulin secretion by pancreatic β-cells, or glucose metabolism by hepatocytes and adipocytes are also increased by LBPs [48–50].

1.3. Horsetail (Equisetum arvense)

Horsetail (*Equisetum arvense*) is an herbaceous perennial species that is spread throughout the temperate zone of the Northern Hemisphere. Its spines have elongated stems from which lateral shoots emerge and give the plant a spindle-like shape. They have small petiole-like leaves. The stem contains quartz, which gives the hinges firmness and brittleness. The sterile stems are 10–90 cm tall and 3–5 mm diameter [54,55].

Equisetum arvense is used in traditional medicines to cure jaundice, loss of hair and for rheumatic diseases [56]. *E. arvense* extracts are important in drug development with various pharmacological properties including antifungal, antioxidant, analgesic, anti-inflammatory, antidiabetic, antitumor, cytotoxic, anticonvulsant activities, and hepatoprotective activity [57–60]. There are several phytochemical compounds in horsetail, such as apigenin, luteolin, equisetumoside A, equisetumoside B and equisetumoside C, nicotine, palustrine, and palustrinine [61,62]. Some of these components are isolated from methanol extract, including two phenolic petrosins, onitin and onitin-9-O glucoside; and four flavonoids, apigenin, kaempferol-3-O-glucoside, luteolin, and quercetin-3-O-glucoside. Phytochemical analysis of the hydroalcoholic extract showed the presence of tannins, saponins, flavonoids, and sterols [63–67]. Several papers examining the antitumor effects of *Equisetum arvense* have been published, including the antitumor effects of peptide extracts on slowly growing mammary adenocarcinoma in CBRB-Rb(8.17)1Iem mice used as a model of breast cancer in humans; peptides from *Hypericum perforatum* and a mixture of *Chelidonium majus* L., *Inula helenium* L., *Equisetum arvense* L., and *Inonotus obliquus* exhibited high activity [68]. Other compounds (onitin, kaempferol-3-O-glucoside) exhibited hepatoprotective activities on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells [67].

1.4. Marc and Seeds of Grape (Vitis vinifera)

Grape (*Vitis vinifera*) is a member of the Vinikov (*Vitaceae* (*Lindley*) or Ampelidae (*Kunth*)) family. *Vitis vinifera* grape species currently consist of between 5000 and 10,000 varieties. There are also some wild grapevines, *Vitis vinifera* ssp. *sylvestris*. The largest number of species is found in areas with moderate subtropical and tropical climates [69,70].

Grape (*Vitis vinifera*) is one of the world's largest fruit crops. Marc and seeds of *Vitis vinifera* possess several medicinal effects, including antioxidant, anti-tumour, antimicrobial, anti-inflammatory, anticancer and antidiabetic activities, as well as hepatoprotective, cardioprotective and neuroprotective

effects [71–74]. *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were exposed to the extracts and tested for antibacterial activity using the pour plate method. Inhibition was successful with Gram-positive bacteria at 850–1000 ppm, and Gram-negative bacteria at 1250–1500 ppm concentration [71]. It has also been reported that grape seed extracts may be useful as antibacterial agents to prevent the deterioration of food products. A large quantity of anthocyanins are found in white grapes, including five anthocyanidin-3-monoglucosides, five anthocyanidin-3-monoglucoside-acetates, five anthocyanidin-3-monoglucoside-p-coumarates, and malvidin-3-monoglucoside-caffeoate [75]. Anticancer effects and induced apoptosis in colon cancer cell lines were noted due to exposure to extract from grape seeds. Among anticancer effect constituents, triterpenoid and betulinic acid inhibited growth by 50% (GI50) in MCF-7 human breast cancer cells. The anticancer effects of resveratrol metabolites, including resveratrol-3-O-sulfate, resveratrol-3-O-glucuronide and resveratrol-4-O-glucuronide on colon cancer cells have been established, and extracts show synergistic chemotherapeutic effects with SN38 and oxaliplatin on metastatic colon cancer cells (SW620) [76,77].

1.5. Rosemary (Rosmarinus officinalis)

Rosemary (*Rosmarinus officinalis*) is a green shrub from the leatherback family. It grows up to 2 m high. It has coniferous, leathery leaves and pale blue or white flowers [78]. Its leaves have a characteristic aromatic odor and an aromatic, slightly bitter taste, and they are used for medical purposes. The main biological activities of *R. officinalis* L. are antitumoral, anti-inflammatory, analgesic, neurodegenerative, endocrinal, anti-infective, and antioxidant [13,79,80].

In the European Union, rosemary extract (E392) has been approved as an effective natural antioxidant for preserved food [81]. Rosemary contains many phenolic acids and there is increasing interest in its therapeutic properties. For example, rosmarinic acid has antibacterial, antiviral, antioxidant, and anti-inflammatory properties [82]. Recent studies have claimed that carnosic acid may be safe and useful as a novel chemotherapeutic agent [83,84]. Carnosic acid exhibits significant growth inhibition and cell cycle arrest in melanoma B16F10 cells, triggers cell cycle arrest at G0/G1 phase, and enhances p21 expression, and it can enhance BCNU- and CCNU-mediated cytotoxicity and cell cycle arrest in B16F10 cells. It also inhibits tumour growth and reduces the values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in vivo [83]. Other studies show that it targets colon cancer cells by increasing intracellular reactive oxygen species and decreasing cell survival mechanisms; it may provide a therapeutic option in colon cancer through joining rosemary compounds with chemotherapeutic drugs [85,86].

2. Materials and Methods

2.1. Materials

This study investigated the following natural materials: Curcuma (*Curcuma longa*), Goji Berry (*Lycium barbarum*), Horsetail (*Equisetum arvense*), Marc and Seeds of Grape (*Vitis vinifera*), and Rosemary (*Rosmarinus officinalis*). The material was obtained from Alfred Galke GmbH (Samtgemeinde Bad Grund, Germany), except the marc and grape seeds, which were obtained from domestic producers. Marc and grape seeds of the *Traminec* species were dried by lyophilization. Other materials were received in the dried form from the supplier; the water content was 10 wt. The material was ground in a grinder before use. The extracts obtained were stored in a freezer at −20 °C. After the extract was obtained, all analyses were performed within 3 weeks.

For extractions, we used water as a solvent because water is present as a solvent in everyday tasks such as cooking. It also acts as a solvent in our bodies, as all biochemical reactions take place in aqueous solutions. Water is at the same time a medium for the transport of nutrients and oxygen to cells and for the removal of metabolic waste. In industries, such as the food industry, water is commonly used as a solvent.

2.2. Determining Optimal Extraction Conditions

Conventional extraction methods were used. After grinding, the naturally dried material was macerated in hot water. Extraction experiments were performed at different temperatures and for different extraction times. Optimal conditions to achieve the highest antioxidant activity were determined with Design Expert Pro 7.0 software, Godward St NE, Minneapolis, 2007 (concentration of the resulting solution was c = 5 mg/mL). The antioxidative activity of the natural material solution was determined by following the exact sequence of the experiments as suggested by the Expert pro 7.0 design program. The experiment was performed at a temperature range from 40 °C to 100 °C and a time range from 5 min to 30 min. For the extract of curcuma and rosemary, the program determined the optimum temperature and time but confirmed that time does not have a significant effect in this time range, while temperature has a visible effect. In the cases of horsetail extract and goji berry extract, both parameters had significant effects. The measurements of the grape extract, according to the program, showed that the temperature and time were significant, but the measured numbers were very similar. Temperatures varied by 5 °C. However, the times varied by 4 min. In cases where time had no significant effect, it was selected at the point where the highest antioxidant activity was achieved.

2.3. Preparation of the Extract

Natural material solutions prepared under optimal conditions were filtered and the raffinate was discarded. Prefiltered natural material solution was lyophilized. After lyophilization, dry extracts were obtained. Before the extracts were applied to the cancer cells, various concentrations of the solutions of the extracts were prepared. Eight solutions of extracts at different concentrations were prepared (water was used as a solvent): c = 100 mg/mL, 20 mg/mL, 10 mg/mL, 5 mg/mL, 1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL.

2.4. Testing of the Cells' Metabolic Activity

Human melanoma cell line WM-266-4 (ATCC® CRL1676™, USA) was cultured in Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003™) containing 1% fetal bovine serum (FBS, ATCC® 30-2021™) and 0.02% MycoZap™ Plus-CL (Lonza, Portsmouth, NH, USA). Cells were cultured at 37 °C, 5% CO_2, ≥90% RH. The cells were plated at a density of 2×10^4 viable cells per well in 96-well culture plates. The cells were cultured in complete medium for 24 h to allow cell attachment. In order to measure the cells' metabolic activity, the cells were exposed to selected concentrations of extracts and cultured for 24 h. Concentrations of extracts were 0.001, 0.01, 0.1, 1, 5, 10, 20, and 100 mg/mL. Cells for controls were cultured for 24 h in the complete medium only, no extracts added. The WST 8 Colorimetric Cell Viability Kit I (PromoKine, PromoCell, Heidelberg, Germany, EU) was used, according the manufacturer's instructions. Absorbance (A) was measured spectrophotometrically at 570 nm (background absorbance at 630 nm) in pentaplicates for all samples. The percentage of the cells' metabolic activity was calculated with the following equation: (A570-A630) test sample value/(A570-A630) control value × 100, where A represents average value calculated from pentaplicates.

In addition, cell morphology was observed with an inverted microscope (DM16000B, Leica) using a digital camera (DFC365 FX Leica, Buffalo Grove IL, USA).

2.5. DPPH Method for the Determination of Antioxidantity

Antioxidant activity was determined according to the DPPH, method as described by Villaño et al. [87]

2.6. Method for the Determination of Total Phenolics

Total phenolics were determined according to the Folin–Ciocalteu, method as described by Gülçin et al. [88].

2.7. Method for the Determination of Proanthocyanidins

Proanthocyanidins were determined using the UV spectrophotometric method (UV-VIS spectrophotometer, BIOTEK SYNEGRY 2) as described by Majhenič et al. [89].

3. Results and Discussion

3.1. Determining Optimal Extraction Conditions

Measurements provided the optimal conditions for achieving the highest antioxidative activity of a single material as shown in Table 1. The antioxidative activity is presented in Figure 1d. Table 1 shows optimal extraction temperatures for all materials at a temperature range from 90 °C to 100 °C, according to the experimental design in the temperature range from 40 °C to 100 °C and the time range from 5 min to 30 min. The extraction times are about 15 min, with a slightly lower optimal extraction time in the case of Horsetail extract, according to the program. A high percentage of inhibitory effectiveness (89%) in rosemary was measured (Figure 1d).

Table 1. Optimal conditions for the highest antioxidative activity.

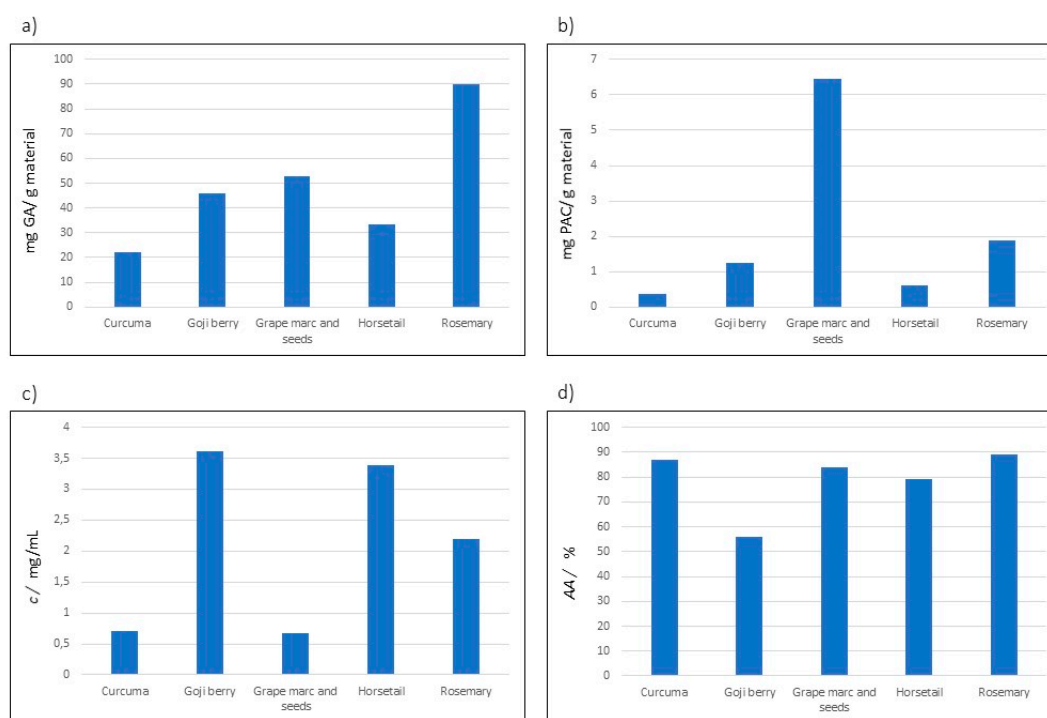| *Material*	$T_{extraction}$ [°C]	$t_{extraction}$ [min]
curcuma (Curcuma longa)	100	15
goji berry (Lycium barbarum)	93	13
grape marc and seeds (Vitis L.)	100	15
horsetail (Equisetum arvense)	100	6
rosemary (Rosmarinus officinalis)	96	20

Figure 1. (**a**) Total phenolic content in selected extracts. (**b**) Proanthocyanidin content in selected extracts. (**c**) Concentrations of extracts required for 50% inhibitory effectiveness. (**d**) Antioxidant activity in selected extracts under optimal conditions. The extracts were obtained by maceration with water at optimum temperature and time: Curcuma (100 °C, 15 min), Goji berry (93 °C, 13 min), Grape marc and seeds (100 °C, 15 min), Horsetail (100 °C, 6 min), and Rosemary (96 °C, 20 min).

Much research has already been done on rosemary, and it has been shown to contain many antioxidants, such as carnosol, rosmanol, carnosic acid, and methyl carnosate [90]. Eighty-seven

perecent inhibitory effectiveness was determined for turmeric, which is assumed to be a component of curcumin. Similar results have been reported in the literature, with most being achieved with added ethanol on extraction; for example, about 70% of the antioxidant activity was achieved on extraction with 50% ethanol at 60 °C and a time of 60 min [91]. High values were obtained with the use of a juice extractor, with antioxidant activity of curcume measured around 65% [92]. Grape extract achieved 84% inhibition, probably because of the high content of proanthocyanidins. The inhibitory value of horsetail extract was measured at 79%. It has been reported that horsetail extracts show high superoxide anion radical-scavenging activities [93]. Goji berries had the lowest inhibitory value, but still above 50%. In the future, it would be interesting to compare the maceration results obtained with supercritical extraction using a co-solvent (water), because supercritical water extraction has been shown to achieve high antioxidant activities. A concentration of 11.3 μg/mL has been measured, corresponding to high antioxidant activities. It has also been found that at lower temperatures (25 °C), the main constituent of the extract is a more polar compound (rosmanol). By increasing the temperature to 200 °C, carnosic acid, carnosol, genquaninin, and carnosic acid have been extracted at 200 °C [90]. Studies show the key impact of different extraction methods on purity, molecular characteristics and biological properties, even in the case of extracted alginates [94]. For example, curcumin-laden alginate—80 nanoparticle polysorbate may be useful in treating various human inflammatory diseases [95].

3.2. Results of Determination of Total Phenols, Proanthocyanidins and Antioxidantive Activity

The concentrations required for 50% inhibition efficiency are shown in Figure 1c. All the extracts achieved high values of inhibitory effectiveness. The visible difference, however, is the amount of extract concentration required to achieve 50% inhibitory potency. For example, curcuma has high percentages of antioxidant activity and low concentration (EC_{50} = 0.708 mg/mL) is required to achieve 50% inhibitory potency. The figures are similar for grapes (EC_{50} = 0.681 mg/mL). According to the literature, to obtain 50% antioxidant activity of curcuma extract, prepared by continuous magnetic stirring in methanol at 25 °C from different types and varieties of turmeric, the required concentrations of extract were measured from 0.03 to 0.23 mg/mL, which comes close to the results of this study [96]. However, rosemary (EC_{50} = 2.199 mg/mL), horsetail (EC_{50} = 3.384 mg/mL), and goji berries (EC_{50} = 3.604 mg/mL) require a slightly higher concentration. The contents of the proanthocyanidins in mg per gram of material selected (mPAC/g material) are shown in Figure 1b, and the total phenol content in mg per gram of the selected material (mg GA/g material) is shown in Figure 1a. The values of proanthocyanidins and total phenols (mg/g material) are higher in cases of higher levels of antioxidant activity. With aqueous extracts of turmeric, methanolic extracts are also approximated in the measurements of total phenols: Methanolic extracts have reached a total phenol content of 43 to 158 mg GA/g extract [96]. Also, a study with ethanol extract confirmed our results on high levels of proanthocyanidins, a type of flavonoid known for its high antioxidant properties. They measured about 1.38 g/L [97].

3.3. The Effects of the Extracts on Cells' Metabolic Activity

Morphological changes of WM-266-4 cells exposed to different concentrations of tested extracts and the control are shown in Figure 2. The effects of the tested extracts on the metabolic activity of WM-266-4 cells are shown in Figure 3.
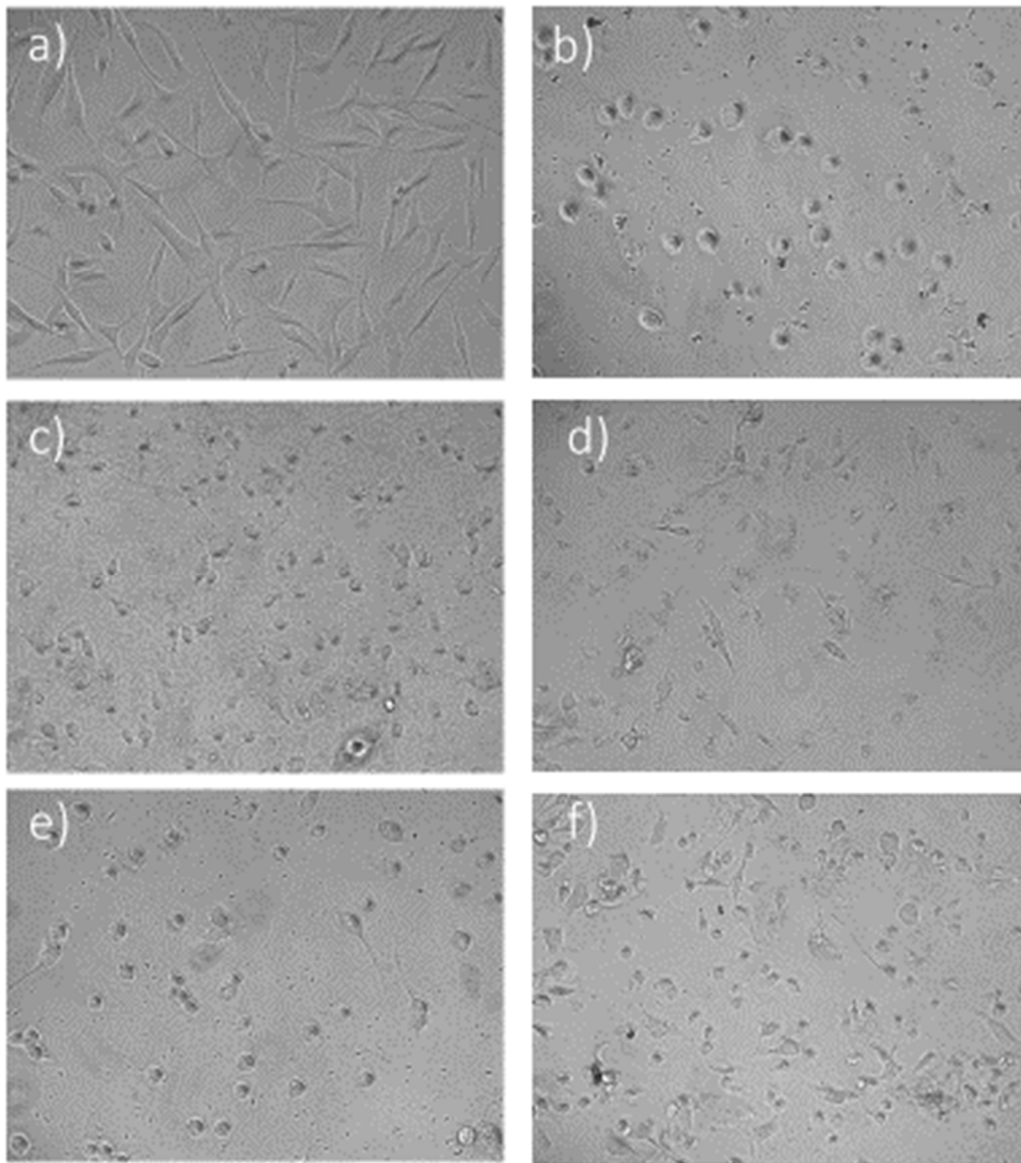
Figure 2. Morphology of WM-266-4 cells when exposed to: (**a**) medium (control); (**b**) extract of curcuma (5 mg/mL); (**c**) extract of goji berry (100 mg/mL); (**d**) extract of horsetail (100 mg/mL); (**e**) extract of grape merc and seeds (20 mg/mL); (**f**) extract of rosemary (5 mg/mL). Magnification 200×.
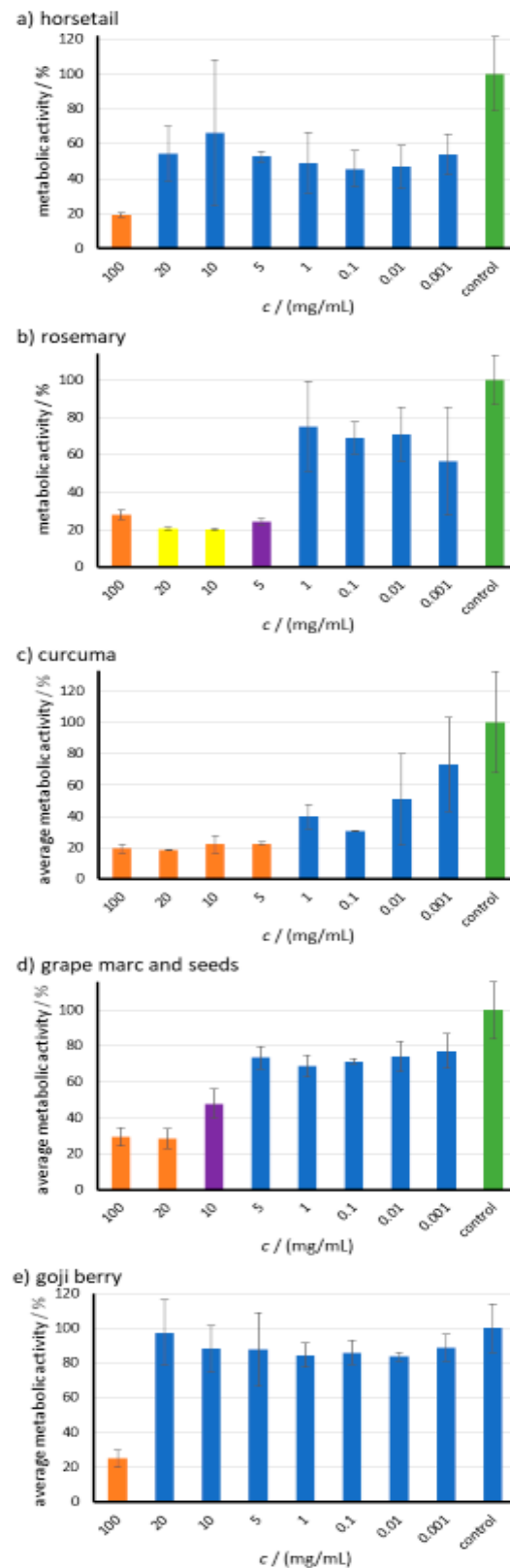
Figure 3. Metabolic activity (decrease in cancer cell activity) at different concentrations of (**a**) curcuma, (**b**) goji berry, (**c**) grape marc and seed, (**d**) horsetail, and (**e**) rosemary.

3.4. Statistical Analysis of the Extracts on Cell Metabolic Activity

To measure metabolic activity, five repetitions were performed at 100, 20, 10, 5, 1, 0.1, 0.01, and 0.001 mg/mL concentrations using five different extracts: curcuma, goji berry, grape marc and seeds, horsetail, and rosemary. The effect of each extract on the metabolic activity of cells was determined by comparison with the control. No test compound was exposed to cell control. In the control, the cells were exposed only to the medium. Each set of measurements was tested for possible outliers using Dixon's and Grubbs's statistical tests [98] and in cases where they were present, they were discarded. Average metabolic activities and corresponding standard deviations at different concentrations of extracts are presented in Figure 3. The change in significance in metabolic activity was accessed using a statistical t-test by comparing two sample means at 95% confidence. This test considers the average values at certain concentrations and its standard deviations. The calculated t-value (t_{calc}) is compared with the critical t-values (t_{crit}) and if $t_{calc} > t_{crit}$, the change in average value was considered as significant. The change in bar colors in Figure 3 represents the transition of significant influence on the metabolic activity with the change in concentration. Similar bar color represents an insignificant influence on metabolic activity with change in concentration. Furthermore, in cases where the same metabolic activity was predicted statistically (using the t-test) for more than two samples, non-significant change between these samples was also confirmed by the analysis-of-variances (ANOVA) at 95% confidence. Curcuma extract shows two concentration regions where metabolic activity drops significantly (Figure 3a). The first concentration region is at 0.001–1 mg/mL (MA ≈ 50%), the second at 5–100 mg/mL (MA ≈ 20%). Goji berry extract has the lowest influence on metabolic activity: an insignificant influence of goji berry extract occurred at up to 20 mg/mL (Figure 3b), but a 100 mg/mL concentration had an influence (MA ≈ 20%). In the case of grape marc and seeds extract, a significant influence on metabolic activity occurs at 0.001–5 mg/mL (MA ≈ 70%) (Figure 3c), followed by a second significant drop at 10 mg/mL (MA ≈ 50%). Grape marc and seed extract had the most significant influence at 20–100 mg/mL concentration (MA ≈ 50%). Horsetail extract showed a significant change in metabolic activity even at the lowest concentration present, 0.001 mg/mL (Figure 3d). However, with an increase in the concentration of horsetail extract up to 20 mg/mL, its influence is statistically insignificant. Increasing the concentration from 20 to 100 mg/mL changed metabolic activity significantly again (MA ≈ 20%). Rosemary extract has a different influence on metabolic activity compared with horsetail extract (Figure 3e). In the range of 0.001–1 mg/mL, the effect is similar; it drops at 5 mg/mL, and again drops at 10–20 mg/mL (MA ≈ 20%). The latter concentration has the most significant effect on metabolic activity. Surprisingly, an increase in rosemary concentration to 100 mg/mL increases metabolic activity.

4. Conclusions

One of the goals of the food industry is to use as few organic solvents as possible. Therefore, our study was limited to water as a solvent. With Design Expert pro 11, optimal parameters for extraction performance were determined to achieve the highest percentage of antioxidant activity. The concentration of extracts required to achieve 50% inhibitory effectiveness, total phenols, and proanthocyanidins was determined.

Furthermore, a study of the influence of extracts on WM-266-4 melanoma cells was performed. A high percentage of antioxidant activity AA (c = 5 mg/mL) = 87% was measured in the curcuma extract and a low concentration (EC_{50} = 0.708 mg/mL) was required to achieve 50% inhibition. Multiple studies have shown that curcuma is a potent antioxidant source that combats free radicals in the body and has significant potential to act as an antitumor agent. Within the frame of our research, we observed that extract of turmeric significantly influences metabolic activity at the lowest extract concentration of 0.001 mg/mL. Curcuma extract shows two concentration ranges where metabolic activity is significantly reduced: at about 50% MA and 20%.

Goji berry extract has the lowest influence on metabolic activity. The highest concentration (100 mg/mL) of goji berry extract had an influence on metabolic activity (MA = 25%). However, there

was no significant change with other concentrations. Grape marc and seed exhibited 84% antioxidant activity. The percentage of total phenols is higher than 50%.

When cell metabolic activity is concerned, compared to the control, it was observed that the extracts inhibited the metabolic activity of the tested cells at all concentrations. Therefore, they reduced or decreased the metabolic activity of cancer cells. Grape marc and seed extract at 20–100 mg/mL concentration have the most significant influence on metabolic activity.

The results showed that horsetail extract causes significant change in metabolic activity even for the lowest concentration present, 0.001 mg/mL. It is assumed that the reason for this is the high content of antioxidants AA (c = 5 mg/mL) = 79% and total phenols GA = 33.49 mg/g in the horsetail extract. Rosemary extract has a similar influence on metabolic activity compared to horsetail extract, because even for the lowest concentration present (0.001 mg/mL), significant inhibition of metabolic activity of the tested cells was noted. The cause of the high metabolic activity of WM-266-4 cells is probably high levels of antioxidants AA (c = 5 mg/mL) = 89% and total GA phenols = 90.10 mg/g extract, as antioxidants and phenols have a beneficial effect or prevent oxidative stress of the cells.

The optimal process conditions for the preparation of extracts from selected natural materials (Curcuma, Goji Berry, Grape marc and seeds, Horsetail and Rosemary) were determined. The obtained extracts decreased the rate of melanoma cell division (WM-266-4). Further, it would be interesting to check in more detail whether the division only stopped or even apoptosis of these melanoma cells may have occurred. We plan to expand the research to at least three other melanoma cell lines to test anti-tumour effects. It would also be interesting to check the content of the potential bioactive compounds in the discharged fractions, since agricultural residues have already been shown to have the potential for an abundant source of natural antioxidants suitable for further development [99]. Furthermore, it would be necessary to check what is the influence of the extracts on the healthy cells. Therefore, this should be a point for further investigation.

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