Total Polyphenol Content and Antiproliferative Activity of Green Tea Extracts Collected from Romanian Pharmaceuticals Market

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Total polyphenol content and anti proliferative activity of five green tea extracts available on the Romanian pharmaceuticals market was analyzed. Results show dose-dependent anti proliferative activity of the selected samples against B164A5 mouse melanoma and A375 human melanoma cell lines. Sample 3, the richest sample in polyphenols showed the highest anti proliferative potential against the screened melanoma cell lines.

Keywords: green tea, polyphenols, melanoma, B164A5, A375

Plant total extracts or isolated and purified active agents continue to yield structural diversity in comparison to standard combinatorial chemistry. Natural compounds represent inexhaustible sources for therapeutic approaches including both prevention and treatment for a wide range of pathologies [1]. Natural products , the old medicine foundations, have nowadays played a vital role in the discovery of new active therapeutic agents [2]. The beneficial effects of green tea, obtained from the plant Camellia sinensis (L.) Kuntze family Theaceae have a rich history dating back almost 5000 years [3]. Depending on the processing steps, from the leaves of this plant may be obtained: white tea, green tea, oolong tea, red tea, black tea [4]. Recent studies have shown that tea is the most consume beverage in the world after water [5]. Among white tea, green tea, oolong tea, red tea, black tea the most beneficial effects in terms of therapeutic efficacy are represented by green tea extracts due to their chemical composition: polyphenols, flavonoids, alkaloids (caffeine, theophylline, theobromine), amino acids, proteins, certain minerals and vitamins. The non-fermented leaves of green tea contain an increased amount of catechins compared to oolong or black tea [3,5].

The health benefits of green tea consummation cover a very wide hat that points towards cardio-vascular diseases, cholesterol-lowering effects, chemoprevention, anti-oxidant, anti-inflammatory, antibacterial, anti-angiogenic, anti-diabetic, neuroprotective, activity [6,5]. The major responsible for the therapeutic activity of green tea extracts are the flavonoids known under the subclass of catechins [7]. Consummation of green tea beverages have been proven to be a good method for the prophylaxis dental decay due to the content in fluor but also due to the antibacterial action of green tea standardized extracts (caffeine and polyphenols) is the use of this natural compounds for the prevention of obesity and weight loss [9].

The aim of this study is to analyze the total polyphenol content and anti proliferative activity on B16 4A5 murine melanoma cell line and A375 human melanoma cell line of green tea extracts collected from Romanian pharmaceuticals market.

Experimental part

Materials and methods

Vegetal extracts

Samples from five companies producing green tea collected from Romanian pharmaceuticals market were analyzed. Samples were assigned as follows: 1.Green tea produced by Dukat; 2.Green tea produced by Vedda; 3.Green tea produced by Fares; 4. Green tea produced by Belin; 5. Green tea produced by Alevia. 1g sample from each group was mixed with 100 ml ethanol 20% and sonicated using a ultrasonic bath Falc LCD series at a frequency of 40Khz, 40 min at 25°C. The solvent was then evaporated by the help of a rotary evaporator until a crystalline solide mass was obtained

Total phenolics (TP) assay

The total phenolic content was quantified using Folin-Ciocalteu reagent according to the method described by Morgenstern et al., 2014 [10]. Sample (25 mg) with 1 mL ethanol 50% was sonicated 15 min using ultrasonic bath (FALC LCD Series). The sample was centrifuged 10 min at 5000 rot/min (EBA 21, Hettich) and 10µL supernatant was mixed with 100 µL ethanol 5% in water, 2 mL Na₂CO₃ 15%, 200µL Folin-Ciocalteu reagent and 1 mL water. After 30 min of incubation at 50°C the absorbance of samples was measured at 750 nm using a UV-Vis spectro-photometer (Analytic Jena Specord 205). The calibration curve was obtained using gallic acid (GA) as standard (concentration range 10–50 mg/mL). The regression equation was: y=1.9224x-0.10164 and the coefficient of correlation R2=0.9138. The results were expressed in mg GA/g dry

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matter (d.m). All determinations were performed in triplicate.

In vitro cell culture and expansion

The anti proliferative effects of the prepared extracts were determined on B164A5 mouse melanoma and A375 human melanoma cell lines. The melanoma cell lines (Sigma-Aldrich Company, Ayrshire, UK) were cultured in complete growth medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 1% Penicillin/ Streptomycin mixture (Pen/Strep, 10,000 IU/mL, FCS; PromoCell), and 2% HEPES (4-(2-hydroxyethyl)-1piperazineethanesulfonic acid; Gibco BRL). The cells were cultured and expanded in standard conditions, at 37°C and 5% CO, atmosphere. Medium replacement was performed every third day and, upon reaching 80 to 90% confluence, the cells were passed using 0.25% Trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Sigma-Aldrich Company) followed by centrifugation (10 min, 300 x g) and were then replated in T75 culture flasks at a density of 10,000 cells/cm² to ensure optimal growth and expansion.

MTT proliferation assay

B16 4A5 murine melanoma cell line and A375 human melanoma cell line were seeded onto a 96-well culture plate at a cellular density of 6,000 cells/well and attached to the bottom of the well overnight. After 24 h, 100 µL of new medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA) and 25µg/mL respective 50µg/mL of the tested extracts (dissolved in dimethyl sulfoxide - DMSO; Sigma-Aldrich Company) were added and incubated for 72 h; the medium was supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 1% Penicillin/ Streptomycin mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). The melanoma cells were then assayed by the addition of 10 μ L of 5 mg/mL MTT solution from the MTT-based in vitro toxicology assay kit (Tox-1; Sigma-Aldrich Company). The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 4 h contact period. The precipitated crystals were dissolved in 100 µL of lysis solution provided by the manufacturer (Sigma-Aldrich Company). Finally, the reduced MTT was spectrophotometrically analyzed at 570 nm, using a microplate reader (Bio-Rad, Hercules, CA, USA); wells with untreated cells were considered as reference for viability, while DMSO, which was used to prepare stock solutions of the tested substances, was also added on the cells for the evaluation of cellular proliferation. All in vitro experiments were carried out on two micro plates in quadruplicates for each tested substance as well as controls.

Statistics

The Prism software package (Graph Pad Prism 4.03 for Windows) was used for data presentation. The experiment was repeated three times and results were presented as mean \pm SD. Paired Student's t tests, one or two way ANOVA were applied to evaluate statistical significance (*, p < 0.05;**, p < 0.01; and ***, p < 0.001).

Results and discussions

Results have shown (fig. 1) that among the tested samples, sample number 3 possess the highest amount of total polyphenols, expressed as mg GA/g dry matter, closely followed by sample 1, 5, 2 and 4. Polyphenols are most

often naturally occurring compounds, but may also refer to structures of synthesis or semi-synthesis, having grafted on the benzene ring one or more hydroxyl groups [11]. This class of chemical active agents have been postulated for their antioxidant, cardio-preventive, chemo-preventive, anti-inflammatory, anti-aging, antimicrobial effects [12]. Polyphenols from green tea have been intensively discussed in the literature due to the fact that they present chemo-preventive activity in an increased number of animal tumor bioassay systems. Beside this effect green tea polyphenols have been awarded the following health benefits: to reduce the risk of cardiovascular disease, protection against stroke, diminished risk of osteoporosis, protection against bacterial and viral infections, against liver disease [13]. The amount of total polyphenols is directly correlated with the method of extraction [14]. In a similar study using commercially available green tea from Argentina it has been showed that the amount of total polyphenols vary from 21.02 ± 1.54 to $14.32 \pm 0.45\%$ of gallic acid equivalents (GAE) [15].



Fig. 1. Total phenolic content mg GAE/g of tested samples

The anti-proliferative activity (fig. 2 and fig. 3) of the tested samples using 25μ g/mL respective $50 \propto$ g/mL and a period of incubation of 72 h is stronger for the A375 human melanoma cell line compared to B164A5 mouse melanoma cell line. Sample 3 presented the most increased anti-proliferative effect for both cell lines, directly correlated with the concentration. At the concentration of 50µg/ml the viability was around 40% for the human melanoma cell line and around 50% for the mouse melanoma cell line. Also for the other samples the activity was directly correlated with the concentration fallowing the next order (which include the intensity of the antiproliferative effect) :2,4,1,5. Presumably the antiproliferative activity is directly correlated with the total polyphenol amount. Specific literature in this field show that both *in vitro* and *in vivo* studies have been pointed towards the protective mechanisms of green tea polyphenols at molecular and cellular level against UV radiation damage, that, in the end ,can lead to different types of skin cancer [16]. The photo protective effect of green tea polyphenols along with its major direct implication, namely the chemo -preventive activity have been reviewed by the group of Yusuf *et al.* [17]. A linked study on this topic have shown that either administered oral or topical applicant green tea extract can prevent non-melanoma skin cancer induced by UVB radiation, and the mechanism involves DNA repair by GTPs [18]. A recent large study including several melanoma cell lines, namely A375, Hs294t, SK-Mel28 and SK-Mel119 have described the capacity of inhibition in a dose-dependent manner of cell proliferation as well as colony formation after treaptment with green tea polyphenols [19]. The capacity of green tea catechines of reducing the invasive potential of Å375 (BRAF-mutated) and Hs294t (Non-BRAF-mutated)

melanoma cell lines with a mechanism that points twords epithelial to mesenchymal transition along with COX-2 and PGE2 targets was depicted by the group of Singh *et al.* [20]. It has been shown that the major polyphenolic component of green tea, epigallocatechin-3-gallate, is responsible for melanoma inhibition [21]. Furthermore, the combination of epigallocatechin-3-gallate with decarbazine profed to be for both *in vitro* and *in vivo* models a good chemo-preventive strategy for melanoma models emploing B16-F3m cells [22]. Results of the present study, namley analyses of the anti-proliferative activity on B16 4A5 murine melanoma cell line and A375 human melanoma cell line of green tea extracts collected from Romanian pharmaceuticals market, assigned with 1,2,3,4,5,as described in the method section are correlated with the data existing in the literature.

A375 human melanoma cell line



Fig. 2. Cell viability of A375 human melanoma cell line after incubation with $25\mu g/mL$ respective $50\mu g/mL$ of tested samples



Fig. 3. Cell viability of B164A5 mouse melanoma cell line after incubation with 25µg/mL respective 50µg/mL of the tested samples

Conclusions

Among the five green tea samples collected from Romanian pharmaceuticals market, sample number 3 presented the highest amount of total polyphenols. All green tea extracts presented anti-proliferative activity in a dose dependent manner. Comparing the two melanoma cell lines, the activity was more potent for the A375 human melanoma cell line. Among the screened extracts, sample number 3 presented the highest anti-proliferative activity presumably to the highest amount of polyphenols.

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