Chemical Properties and *in vitro* Antitumor Effects of *Momordica Charantia* Extracts in Different Solvents

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Momordica Charantia also known as bitter melon, is used in traditional medicine for its antidiabetic, anticancer and antiviral properties. It is known that a large number of pathological damage is caused by reactive oxygen species. Plant polyphenols with antioxidant capacity can neutralize these reactive chemical species preventing the damage of cellular functions. The aim of our study was to determine the antitumor effects, antioxidant activity, phenolic compounds, and protein concentrations of ethanol and aqueous extracts of M. charantia sprouts. The ethanol and aqueous extracts antiproliferative potential was observed in dose – dependent manner. We observed an increase of antioxidant activity in the case of ethanol extract showed by decrease of absorbance. In aqueous extract we did not found significant differences regarding antioxidant activity. The total phenolic content of both extracts increased proportionally with concentration. Analysis by Lowry method showed an increased content of proteins in both aqueous and ethanol extract compared to results obtained by Bradford assay. The study suggests that both extracts may be considered a source with bioactive properties which could be of interest for pharmaceutical use.

Keywords. Bitter melon extracts, antioxidant and antitumor activity, protein concentration

Natural compounds in plants gained increased interest in phytotherapy research. Momordica charantia plant is used in traditional medicine for a number of diseases [1]. It is well known that a large number of pathological damage is caused by reactive oxygen species. The vast majority of the 60 species belonging to the genus Momordica have important medical properties [2]. Plant polyphenols with antioxidant capacity have the ability to neutralize these reactive chemical species preventing the damage of cellular functions [3]. In the literature, different approaches are described and compared for extraction of phytochemicals from plants in order to use them for medical purposes. One attractive approach is also the supercritical CO_2 extraction used in other medicinal plant extracts [4-9]. Phytochemical investigations of M. charantia have revealed that polyphenols are the main components of this plant [10-14]. M. charantia is a plant with hypoglycemic and hypolipidemic effect, used in the treatment of diabetes mellitus type 2. Numerous recent studies suggested the antibacterial, antiviral, immunomodulatory and anticancer effect of this plant. The reported results of different studies are nevertheless contradictory. The antitumoral effect depends on the extracting method and also on the nature of the solvents. The aim of the present study was to evaluate the antioxidant activity, total phenolic content and total protein content of M. charantia sprouts extracts in two different solvents. Also, our study followed the in vitro antiproliferative effect of alcoholic and aqueous *M. charantia* extracts.

Experimental part

Materials and methods

Chemicals. Ethanol (absolute), methanol (HPLC grade), DPPH (di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium), Propyl gallate (Propyl 3,4,5-trihydroxybenzoate), Na_2Tartrate x 2 H_2O (Disodium (2R,3R)-2,3-dihydroxybutanedioate), Cu_2SO_4 x 5 H_2O (Copper sulfate), NaOH (Sodium hydroxide), Na_2CO_3 (Sodium carbonate), Salicylic acid (2-Hydroxybenzoic acid), were ssupplied by Sigma-Aldrich. BSA 7% (Bovine Serum Albumin), was obtained from Ortho Clinical Diagnostics and Folin Ciocalteu Reagent from Merck. Bradford Reagent consisted of: 100 mg Coomassie Brilliant Blue G – 250 (Sigma), in 50 mL ethanol 95%, and 100 mL *o* – Phosphoric acid 85% (w/v) (Sigma - Aldrich), followed by the addition of type I deionized water to a final volume of 1 L. Lowry solution was prepared as follows: Solution A (mixture of NaOH and Na_2CO_3), Solution B (Cu_2SO_4 x 5 H_2O), Solution C (Na_2Tartrate x 2 H_2O), with a ratio (v/v/v) of 100:1:1. For cell culture test all reagent was supplied by Invitrogen.

Plant material. *M. charantia* sprouts were kindly provided by the Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" Timisoara. The plant raw materials were cleaned and dried at room temperature protected from light. Sprouts were ground to a fine powder in order to provide a homogeneous mixture used for the extraction step. Material was maintained at room temperature, in dark until required for analyzes.

Extraction procedure. Powdered dried sprouts of *M. charantia* (10 g) were extracted with 95% ethanol 5%

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methanol (100 mL) under magnetic stirring, in dark at room temperature for 24 h. The obtained supernatant (ethanolic extract) was transferred into a clean flask and evaporated to dryness under reduced pressure. For the aqueous extract the same amount of powder (10 g) was used in 100 mL deionized water under the same conditions as for the ethanol extract. The yield of dried extracts was calculated based on dry weight: yield (%) = $(m_1/m_2) \times 100$, where m_1 was the weight of extract obtained after solvent evaporation and m_2 was the weight of the fresh powdered sprouts.

Antioxidant capacity in *M. charantia* sprout extracts. Antioxidant activity was evaluated by the DPPH test. The assay is considered more selective compared to other techniques [15]. The DPPH test was performed according to El-Agbar et al. with minor modifications [16]. Briefly, DPPH (152 μM), solution in methanol, (2 mL) was mixed with 2 mL aliquot of extract sample by a vortex mixer, then the mixture was allowed to stand for 40 min in dark at room temperature. The absorbance was measured at 515nm. Propyl gallate was used as positive control. Scavenging activity was calculated as:

$$\text{inhibition (\%)} = (A_{control} - A_{sample}/A_{control}) \times 100,$$

where $A_{control}$ is the absorbance of DPPH in methanol (without the sample addition) and A_{sample} is the absorbance in the presence of the sprouts extract in DPPH solution [17]. The percentage of DPPH scavenging activity was plotted against concentration of the extract (mg/mL), in order to obtain the amount of the extract necessary to decrease DPPH radical concentration.

Evaluation of total phenol content (TPC). The total phenol content of M. charantia sprouts extract was determined spectrophotometrically by using Folin Ciocalteu method with some modifications [18]. Salicylic acid was used for the calibration curve. Stock solution, 1 mg/mL and working standard concentration of 10, 25, 50, 100, 250, 500 μg/mL were prepared in type I deionized water. Sprouts extract was diluted with methanol. Different aliquots (200 μL) were transferred into test tubes and mix thoroughly with 1.72 mL type I deionized water. 80 μL Folin-Ciocalteu reagent was added to the mixtures and vortexed, followed by additions of 200 μL 20% (w/v) Na_2CO_3 and 600 μL type I deionized water. Reference was concomitantly prepared, using the same protocol with the addition of water instead of sample extract. The solutions were incubated for 1 hour at room temperature, protected from light. The absorbance was measured at 725 nm, using a spectrophotometer (VIS model 5600, Jenway). Concentration of total phenols was calculated by using the calibration curve of salicylic acid and was expressed as mg salicylic acid equivalent (SAE)/ mL.

Protein determination in crude extracts. Bradford and Lowry assays were used for the determination of the total protein content of aqueous extract. As standard, a stock solution of BSA (1 mg/mL) was used to prepare dilutions for both assays within the interval from 1 to 0.1 mg/mL. In Bradford method 3 mL of Bradford reagent was added to 100 μL extract sample. After vortex mixing, solutions were allowed to stand at room temperature, in dark for 30 min. The absorbance was read by using the spectrophotometer at 595 nm. In Lowry method to 1.5 mL sample 2.1 mL Lowry solution was added. Following mixing, solutions were incubated at room temperature, protected from light for 20 min. Folin reagent (instant fresh) was prepared by adding 6 mL type I deionized water to 5 mL Folin Ciocalteu reagent. After 20 min of incubation, 0.3 mL diluted Folin reagent was added to each tube and vortexed immediately. Solutions were incubated once more at room temp in dark for 30 min and absorbance was measured at 660 nm. Total protein concentration, was determined from the BSA calibration curve.

In vitro test. To evaluate the antitumor properties of *M. charantia* we utilized MCF7 cell line (Human Breast Adenocarcinoma cell line)(CLS). MCF-7 cells were cultured as monolayers in a specific medium: DMEM supplemented with 10% penicillin/streptomycin, 10% heat inactivated FBS (fetal bovine serum) at 37°C in an atmosphere of 5% CO_2. $1x10^5$cells/wells were seeded in 96 wells plate and after 24h the MC extracts were added. Ethanolic and aqueous extracts re-suspended in both, specific culture medium, and DMSO (dimethyl sulfoxide) at the final concentrations of 15, 25, 50 and 75 mg/mL. The cells were incubated 48h with MC extracts. Cytotoxicity of M. charantia on MCF7 cells was performed using Vybrant® MTT Cell Proliferation Assay Kit. The MTT (3 - (4,5 dimethyl thiazol-2-yl) -2,5-diphenyl-tetrazolium bromide) method is based on the conversion of MTT to a insoluble form named formazan by viable cells. Formazan was then solubilized in DMSO and its concentration was determined by optical density at 570 nm.

Results and discussions

Antioxidant activity of the ethanol and aqueous extracts of sprouts of M. charantia was determined by DPPH bleaching assay. Results (fig. 1) showed a concentration - dependent activity for both extracts in the concentration range of 2-10 mg/mL. Above 10 mg/mL concentration changing in activity was much slower and at 75 mg/mL both extracts reached their maximum inhibition percent against DPPH radicals of 73.35% for ethanol extract and 38.75% for water extract. Scavenging activity of ethanol extract was significantly higher. In spite of this the radical scavenging activity of propyl gallate used as positive control showed a clear linear correlation to the concentration (fig. 2).

Total phenolic content was higher in water extract and considerably smaller in ethanol extract, most likely because of the different solubility of polyphenols in the extraction solvent (table I). Relationship between inhibition and total phenolic content, (fig. 3 (a) and (b)) in aqueous and ethanol extracts is expressed by the correlation coefficients $r^2 = 0.9238$ for ethanol extract and $r^2 = 0.9706$ for aqueous extract.

In biochemical work, determination of protein concentration is frequently required. Most protein assays

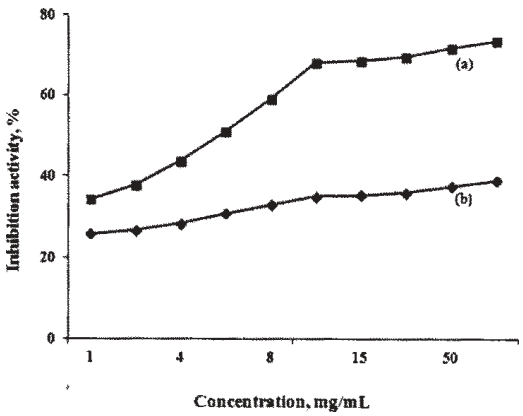

Fig. 1 Free radical scavenging activities of ethanol (a) and water (b) extracts of *M. charantia* sprouts at different concentrations against DPPH radicals. Each value is the mean of 3 independent measurements

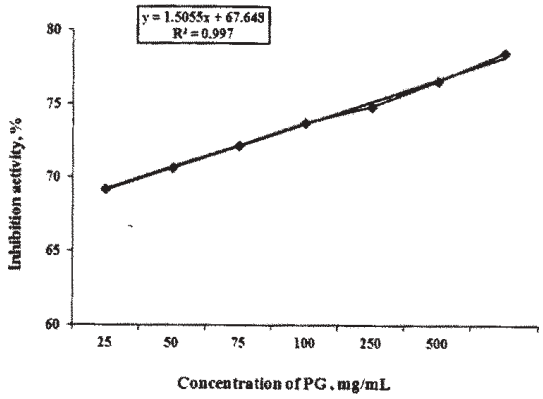

Fig. 2 Calibration curve and equation of DPPH test for positive control. Each sample was measured in triplicates and averaged

Extract	Concentration (mg/mL)	TPC[1](mg/mL) of *M. charantia* sprouts extracts
Water extract	8	5.69 ± 1.56
	10	7.07 ± 1.06
	25	18.76 ± 1.08
	50	39.03 ± 0.94
	75	59.63 ± 0.99
Ethanol extract	8	1.73 ± 1.10
	10	2.38 ± 0.66
	25	4.35 ± 0.76
	50	5.85 ± 1.12
	75	7.98 ± 1.01

[1]mg SA equivalent / mL of crude extract; each value is excpressed as average of three determinations ±SD

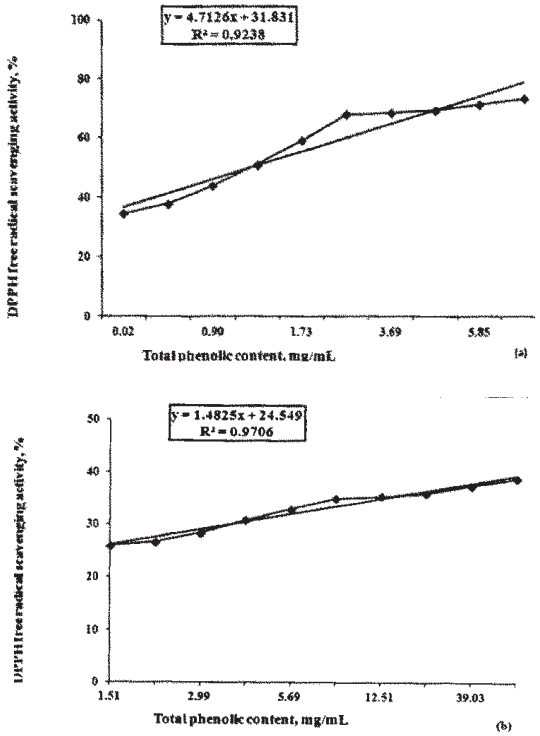

Fig. 3 Linear correlation between inhibition and TPC of *M. charantia* ethanol (a) and aqueous (b) sprouts extracts. Each value represent the average of three replicates

rely on the reaction between a reagent dye and the protein of interest that will conduct to an increase in the absorbance at the specific wavelength. As a general rule, the more protein is in a sample, the higher is the absorbance [19,20]. The calibration curves were used as reference for protein quantification (fig. 4). Bradford and Lowry methods were used to assess whether differential protein concentration are a result of methodical variations. Analysis by Lowry method showed an increased content of proteins in both aqueous (from 3 to 33%) and ethanol extract (only for the first, 9.93% and second, 5.13% dilution of crude extracts of M. charantia sprouts), compared to results obtained by Bradford assay (fig.5). The reported significant differential protein content, suggest that care should be taken when the purpose of protein determination is quantitative, in order to allow an accurate data interpretation.

In the case of MCF-7 cell line we observed a significant decrease of cell proliferation in ethanolic extracts, the inhibition was dose-dependent, and in aqueous extracts the inhibition rate was lower than in ethanolic extracts,

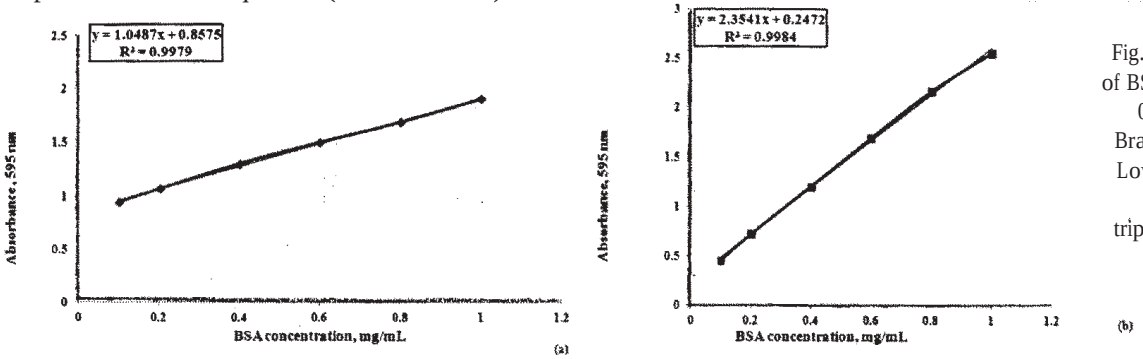

Fig. 4 Calibration curve of BSA within the interval 0.1 to 1 mg/mL by Bradford assay (a) and Lowry assay (b). Each value was run in triplicate and averaged

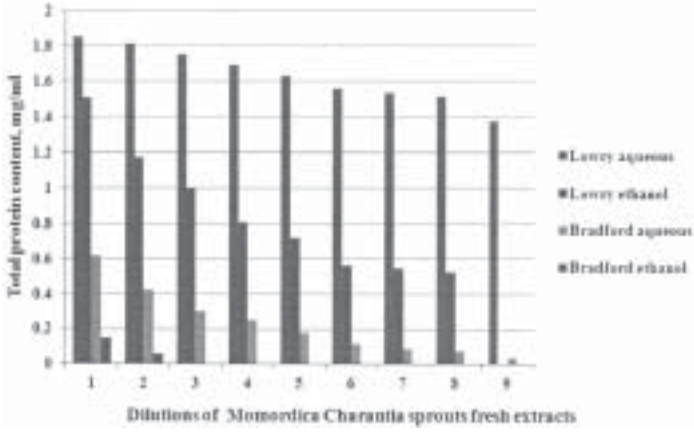

Fig. 5 Total protein content in both aqueous and ethanolic extracts of *M. charantia* sprouts

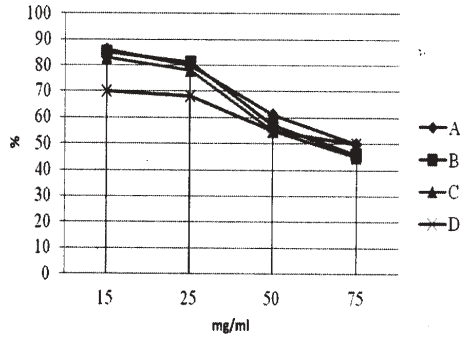

Fig. 6 MCF-7 cell proliferation: (A) aqueous extract re-suspended in medium, (B) aqueous extract re-suspended in DMSO, (C) ethanolic extract re-suspended in medium, (D) ethanolic extract re-suspended in mediu.

but in higher doses cell growth was more significantly decrease (fig.6).

As oxidation is responsible for generating free radicals which cause damages to cell membranes and other structures leading to degenerative conditions such as, inflammation, damage to nerve cells in the brain, certain cancers, increased risk of coronary heart disease, and acceleration of ageing process, the role of the antioxidant is to neutralize those free radicals. The majority of tests made on *M. charantia* extracts evidenced a powerful antioxidant activity of the plant. Semiz and Sen investigated the effects of *M. charantia* fruit pulp, and seed extracts on glutathione S-transferases, cytochrome P450s, and antioxidant enzymes in liver, kidney, and lungs reflecting the antioxidant and protective potency by hepatoprotective effects in CCl_4-intoxicated rats [11]. S Patel et al., reported a higher radical scavenging effect in alcoholic extract of *M. charantia* Linn [21]. Fruit which is in accordance with our results being also marked by the presence of phenolic compounds that potentate the antioxidant activity. Leelaprakash et al. investigated the in vitro antimicrobial and antioxidant activity of *M. charantia* leaves in aqueous and methanol extracts and concluded that it contains a rich source of phytochemicals which has free radicals scavenging activity that can be isolate in order to obtain the natural antioxidant [22]. The cytotoxic effect of the ethanolic or crude water soluble extract of *M. charantia* has been reported in different type of cancer cell line. Crude protein extract of *M. charantia* showed the highest anticancer capacity superior than that of 5-FU in some epithelial derived cell lines [23]. Also, the extracts of *M.charantia* is led to cell growth inhibition of the human neoplastic mammary cell line (MCF7), leukemia cell line (HL-60), cervix cancer cell line (HeLa)[24-26]. However, the biological activity is not compulsory related to the antioxidant capacity [27]. The higher antiproliferative effect of alcoholic extract on MCF7 cell line is positively correlated with the higher scavenging activity, 73.35% found in ethanolic extract compared to 38.75% found in aqueous extract. The data presented in this study demonstrate that it is possible to measure antioxidant activity, total phenolic content and total protein concentration in sprouts of *M. charantia* aqueous and alcoholic extract. Considerable attention was given to sample preparation following analytical step. As noted in the experiments described, operations related to the handling of the extracted constituents, choosing the appropriate technique and the absorbance measurement should be specific. For example, a wrong method for total protein estimation may result in a missed evaluation of protein content, resulting in erroneously concentration values.

The subject was also studied in a previous paper [28].

Cnclusions

It is well known that M. charantia plant could be the source for various compounds with potentially beneficial effects. This study was focused on analyzing ethanol and aqueous extracts of M. charantia sprouts by using various methods including, evaluation of antioxidant capacity, total phenolic, total protein content and antiproliferative effects on MCF7 cell line. The comparative study showed significant differences between the two extracts, suggesting that attention should be taken when the aim involves confirmation of pharmacological and medical use. Total protein evaluation by Lowry and Bradford assays suggest that it is necessary to choose the appropriate analytical technique when the aim involves quantification of proteins. The study and the literature survey also suggests that both extracts may be considered a source with bioactive properties which could be of interest for pharmaceutical use.

Acknowledgments. This project was financially supported by Project UEFISCDI, PN II-Capacităţi, Module III—bilateral projects Romania-Hungary; contract 677/22.04.2013.

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Manuscript received: 13.09.2015