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Antioxidant activity of ethanolic and aqueous extracts of *Uncaria tomentosa* (Willd.) DC.

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Abstract

The antioxidant properties of aqueous and ethanolic extracts of the *Uncaria tomentosa* bark were evaluated. The analysis included trolox equivalent antioxidant capacity (TEAC), peroxyl radical-trapping capacity (PRTC), superoxide radical scavenging activity (SOD) and quantitation of total tannins (TT) and total phenolic compounds (TPC). The obtained results indicate high antioxidant capacity of the studied materials in comparison to the other extracts of fruits, vegetables, cereals and medicinal plants. Higher antioxidant activity and total phenolic compounds of the alcoholic preparations – TEAC = 0.57 mmol of Trolox/g, PRTC = 0.52 mmol of Trolox/g and SOD = 0.39 U/mg than of the aqueous preparation – TEAC = 0.34 mmol of Trolox/g, PRTC = 0.19 mmol of Trolox/g and SOD = 0.10 U/mg were observed. These results might suggest higher medical suitability of alcoholic extracts. However, the highly elevated level of tannins in alcoholic extracts may cause undesirable gastric effects.

Keywords: *Uncaria tomentosa*; Cat's claw; Antioxidant activity; Free radicals; Phenolic compounds; Tannins

1. Introduction

Uncaria tomentosa (Willd.) DC. commonly known as cat's claw or uña de gato, is indigenous to tropical areas of South and Central America liana classified into *Rubiaceae* family (Reinhard, 1999). For at least 2000 years, among many Peruvian tribes, especially Ashaninka, this species has been deeply believed to possess magical healing power and has been extensively used for the treatment of asthma, cancer, cirrhosis, fevers, gastritis, diabetes, rheumatism, dysentery, inflammation of the urinary tract and many other diseases (Keplinger et al., 1999; Falkiewicz et al., 2001; Heitzman et al., 2005). Recently, medical preparations from *Uncaria tomentosa* have become very popular in Europe and America, particularly as an anticancer remedy (De Jong et al., 1999). In majority of the latest studies, high biological activity of cat's claw is attributed to unique tetracyclic and pentacyclic oxindole alkaloids (Phillipson et al., 1978; Laus and Keplinger, 1994; Laus, 1998). However, due to wide spectrum of the uña de gato activity, synergistic participation of other chemical compounds in the healing process must be taken into account (Falkiewicz et al., 2001).

Phenolic constituents, such as flavonoids, phenolic acids, diterpenes and tannins are especially worthy of notice due to their high antioxidative activity (Shahidi et al., 1992; Rice-Evans et al., 1996). Many investigations indicate that these compounds are of great value in preventing the onset and/or progression of many human diseases (Halliwell and Gutteridge, 1989; Halliwell et al., 1992; Willet, 1994; Tsao and Akhtar, 2005). This effect has been explained by the restoration of redox equilibrium disturbed by different factors (e.g. diet, alcohol, some drugs) and, in consequence, by diminishing of damages in cellular structures (Ames et al., 1993).

Therefore, over the past few years, a number of medicinal plants have been extensively investigated for the presence and activity of polyphenols and other antioxidants (Pietta, 1998; Pietta et al., 1998; Sindambiwe et al., 1999; Halvorsen et al., 2002; Naik et al., 2003; Pegg et al., 2005). This study was designed for the evaluation of possible beneficial antioxidative potency of the *Uncaria tomentosa* extracts by employing

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different methods and techniques. The results we report comprise determination of total antioxidant status (TAS), peroxyl radical-trapping capacity (PRTC), superoxide scavenging activity (SOD) and quantitation of tannins and total phenolic compounds (TPC).

2. Materials and methods

2.1. Plant material and chemicals

The analysis was performed on the bark of the *Uncaria tomentosa* originated from Peru and supplied by A–Z Medica, Gdańsk, Poland. The voucher material is deposited at the Laboratory of Phytochemistry, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland.

The standards of oxindole alkaloids were purchased from ChromaDex (Santa Ana, USA). Ammonium hydroxide, sodium carbonate, sulfuric acid, hydrochloric acid, methanol, ethanol, ethylacetate were supported by POCh (Gliwice, Poland). Ferric chloride was supplied from FLUKA (Germany). Acetonitrile (HPLC grade) was purchased from ACROS-ORGANIC (Belgium). Bovine serum albumin (BSA, Fraction V), sodium dodecyl sulphate (SDS) and triethanolamine (TEA) were obtained from Sigma–Aldrich (Germany). Folin-Ciocalteu reagent was provided by MERCK (Germany). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and superoxide dismutase were supplied with Randox Kits No. NX2332 and SD125 (Randox Laboratories Ltd., UK). 2,2′-Azobis(2-amidopropane) hydrochloride (ABAP) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. HPLC-fingerprint analysis of alkaloids in the plant material

To 100 mg of the bark, 15 mL of 2% sulphuric acid solution was added and sonified for 15 min in an ultrasonic bath (Bandelin Sonorex RK 103H). The mixture was then centrifuged 3000 rpm for 10 min and extracted three times with 10 mL of ethylacetate. The aqueous phase was separated and adjusted to pH 10 with 10% NH_4OH and then extracted three times with 10 mL of ethylacetate each. The organic extracts were combined, evaporated to dryness and the residue dissolved in 1 mL of methanol. The qualitative and quantitative content of alkaloids was determined by the HPLC fingerprint analysis [HPLC: L-7100 Intelligent Pump (Merck-Hitachi), L-7200 Autosampler (Merck-Hitachi), L-7450 Diode Array Detector (Merck-Hitachi)]; Software: D-7000 Chromatography Data Station Software version 4.0; Column: LiChrospher® 100 RP-18 (250 mm × 4 mm, Merck); Precolumn: LiChrospher® 100 RP-18 (4 mm × 4 mm, Merck); Solvents: A—phosphate buffer solution (10 mM, pH 6.6), B—methanol: acetonitrile (1:1); Gradient: (60% A and 40% B) to (30% A and 70% B); Time: 30 min; Washing: 20% solvent A and 80% B; Temperature: 21 °C; Flow rate: 1.0 mL/min.; Detection: 245 nm] (Sheng et al., 2000; Stuppner et al., 1992).

2.3. Preparation of samples

2.3.1. Ethanol extract (EX_{ET})

An amount of 0.5 g of the bark was extracted in 5 mL of 50% ethanol for 3 h at 37 °C. Then, the extract was centrifuged (MLW K70D) for 15 min at 4000 rpm. Supernatant was evaporated on Speed-Vac and next exsiccated with P_2O_5.

2.3.2. Aqueous extract (EX_{AQ})

An amount of 0.5 g of the bark was extracted in 5 mL of 0.1 M, pH 7.4 phosphate buffer for 3 h in a tight dark glass ware. The extract was then centrifuged for 15 min at 4000 rpm. Supernatant was evaporated on Speed-Vac and exsiccated with P_2O_5.

The dry preparations (10 mg) were dissolved in 10 mL of adequate solvent (EX_{ET} in 50% ethanol; EX_{AQ} in 0.1 M of phosphate buffer).

2.4. Quantitative determination of tannins

Quantitative determination of tannins was carried out with the protein precipitation method (Hagerman and Butler, 1978). The standard protein solution (1 mg/1 mL) was prepared by dissolving bovine serum albumin (BSA) in 0.20 M of acetate buffer, pH 5.0, containing 0.17 M of sodium chloride. An amount of 2 mL of BSA (1 mg/mL) was added to 1 mL of the sample in a 5 mL glass centrifuge tube. The solutions were carefully mixed and left at room temperature for about 15 min and, then, they were centrifuged for 15 min (Sigma K 300D). The supernatant was decanted and discarded. The surface of the pellet and the walls of the tube were washed with buffer without disturbing the pellet and centrifuged once again. The precipitate was dissolved in 4 mL of the sodium dodecyl sulphate (SDS)–triethanolamine (TEA) solution (1% SDS and 5% (v/v) TEA in distilled water). An amount of 1 mL of the ferric chloride reagent (0.01 M of ferric chloride in 0.01 M of hydrochloric acid) was added and mixed immediately.

Approximately 15–30 min after the addition of the ferric chloride reagent, the absorbance at 510 nm (A_{510}) was measured on Zeiss Spectrophotometer (slit width, 0.03 mm; path length, 1.0 cm; zeroed against air).

2.5. Total phenolic compounds (TPC)

TPC in the EX_{ET} and EX_{PB} samples were determined according to the method of Shahidi and Naczk (1995). An amount of 0.25 mL aliquot of the prepared samples were mixed with 0.25 mL Folin-Ciocalteu reagent (previously diluted with water 1:1 (v/v)) and 0.5 mL of saturated sodium carbonate (Na_2CO_3) solution and 4 mL of deionized water. The mixtures were intensively shaken, left at room temperature for 25 min and centrifuged at 5000 rpm for 10 min. The supernatant absorbance was measured at 725 nm using spectrophotometer (Schimadzu UV-160 1PC). The results were expressed as D-catechin equivalents.

2.6. Trolox equivalent antioxidant capacity (TEAC)

The relative abilities of antioxidants to scavenge radical cation 2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]

$ABTS^{\bullet+}$ were measured by a spectrophotometric technique in comparison with the antioxidant potency of standard amounts of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), according to Miller and Rice-Evans (1996). The determination of TEAC was carried out using the Randox kit. The antioxidant capacity was evaluated by spectrophotometric measurement of radical cation $ABTS^{\bullet+}$ generated from ABTS in a presence of metmyoglobin and hydrogen peroxide (UV-160 1PC with CPS-Controller, Shimadzu, Japan; temperature 37 °C, $\lambda = 600$ nm; calibrated against air). Initial absorbance of the blank sample (20 μl of deionized water mixed with 1 mL of chromogen) and the standard with 1 mL of the chromogen were measured. The absorbance of the preparations was measured successively in the same way (after diluting to concentration of 5 mg/10 ml). After each measurement, 200 μL of substrate were added and exactly after 180 s the final absorbance was determined. The results were compared with those of 1.65 mM of Trolox.

2.7. Peroxyl radical trapping capacity (PRTC)

The measurements were carried out according to Bartosz et al. (1998). The assay was performed as follows. To the cuvettes 90 μL of 5 mM ABTS solution, 20 μL of the extract samples and 300 μL of 200 mM ABAP solution [2,2′-azobis(2-amidopropane)-hydrochloride] were added. The final reaction volumes (3 mL) were adjusted with 0.1 M of sodium phosphate buffer, pH 7.0 previously warmed to 37 °C. The cuvettes were placed in a temperature-controlled recording spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu, Japan) adjusted to reach 37 °C inside the cuvettes; absorbance at 414 nm was monitored for 15 min with automatic measurement every 30 s. The inhibition time of the starting reaction was directly proportional to quantity/activity of antioxidants. The inhibition time should not exceed 15 min, therefore the EX_{ET} sample was diluted with 50% ethanol. The concentrations of the 20 μL samples were 5 mg/10 mL (EX_{AQ}) and 2.5 mg/10 mL (EX_{ET}). The series of standards ($n = 3$): 0.1–2.0 mM were prepared from the stock Trolox solution using the 50% ethanol as a dilutant. The full details in respect to the plant extract assay were described previously (Zieliński, 2002).

2.8. Superoxide radical scavenging activity (SOD)

The superoxide radical scavenging activity of the extracts was measured according to superoxide dismutase kit (Zieliński and Kozłowska, 2000). The superoxide dismutase with the activity of 4.9 U/mL was used as the standard and was supplied as a part of reagent kit. In general, one unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of reduced adenine nucleotides (NADH, NADPH) oxidation of the control by 50%. Then, extrapolation of 50% inhibition values in the samples allows to calculate the SOD-like activity. The percent of reaction inhibition was plotted against $\log_{10}$ of different SOD activities (SOD/mL) giving a standard curve, and then the SOD activity of the sample was calculated on SOD unit/mL of the investigated extract. The superoxide dismutase standard curve for the determination of scavenging activity of phosphate buffered and ethanol extracts were prepared with 0.1 M of phosphate buffer (pH 7.4) and 50% ethanol, respectively. The assays were performed at 37 °C using a Shimadzu spectrophotometer UV-160 1PC. The test required 50 μL of a sample, with a read time of 3 min. All measurements were repeated three times.

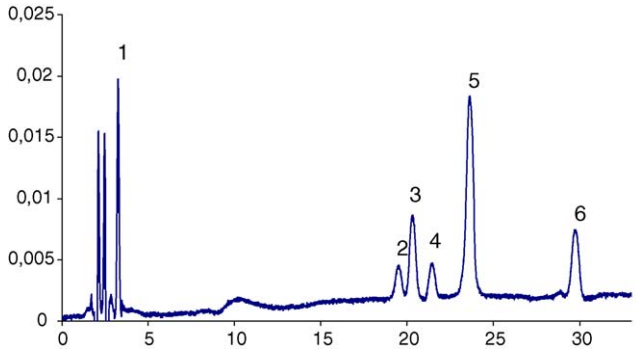

Fig. 1. HPLC-fingerprint analysis of the studied bark of *Uncaria tomentosa*.

3. Results and discussion

3.1. HPLC-fingerprint analysis of alkaloids

The fingerprint chromatogram is showed in Fig. 1. Five pentacyclic oxindole alkaloids, including uncarine F (2), speciophylline (3), mitraphylline (4), isomitraphylline and/or pteropodine (5) and isopteropodine (6) were found. Unfortunately, under the applied conditions isomitraphylline and pteropodine are not separated, what was showed on the standards (non published data). Hence, we are not sure does in our preparation we have one or two alkaloids. The calculated alkaloid content is presented in Table 1. It must be emphasized that total amount of oxindole alkaloids is in agreement with literature data (Lemaire et al., 1999). Moreover, the determinations did not show the presence of any tetracyclic oxindole alkaloids (e.g. rhynhophylline). Several studies have shown that *Uncaria tomentosa* occurs in nature in two different chemotypes characterized by pentacyclic or tetracyclic pattern of indole and oxindole alkaloids. It is suggested that both types of alkaloids appear antagonistic, therefore, pentacyclic and tetracyclic mixtures of alkaloids are unsuitable for medicinal use (Reinhard, 1999). Owing to this, the Asháninka healers used only the plants which represent pentacyclic

Table 1
Content of alkaloids in 100 g of the bark from *Uncaria tomentosa* [mg]

Peak no.	Alkaloid	Content
1	Coffeine[a]	–
2	Uncarine F	67.16
3	Speciophylline	115.25
4	Mitraphylline	67.01
5	Isomitraphylline/pteropodine	371.39
6	Isopteropodine	104.44

The total amount of the main alkaloids is 725.34 mg (~0.72%).
[a] Internal standard.

chemotype (Keplinger et al., 1999) and many pharmaceutical firms standardize their preparations for high dominance of pentacyclic alkaloids (above 95% of all alkaloids) (Falkiewicz and Łukasiak, 2001). Thus, the obtained HPLC data confirm the authenticity and high quality of the used plant material.

3.2. Tannins and total phenolic compounds (TPC)

All obtained results are presented in Fig. 2. For the tannins, higher absorbance of the preparation extracted with ethanol was observed (EX_{ET} = 35.8 A/g; EX_{AQ} = 1.68 A/g). Significantly, minor tannin presence in the sample extracted in phosphate buffer may be explained by less solubility of these compounds in aqueous solvents. Unfortunately, the obtained results are not fully informative. The tannin quantification given in the absorbance units per gram is difficult to compare with the literature data. In case of similar determinations of several varieties of Sorghum vulgare and Vigna sinensis where absorbance has been ranged from 0.2 to 0.4 units per gram of extract (Hagerman and Butler, 1978), the tannin concentration in the preparations can be considered as very high. However, this conclusion should be assumed with caution due to other nature of the analyzed plant material differing by chemical composition of polyphenols.

The content of TPC in ethanol extract from *Uncaria tomentosa* bark (EX_{ET}) was twice higher than in aqueous extract (EX_{AQ}) and amounted to 292 and 111 mg/g, respectively, expressed with D-catechin units. These values are very high in comparison with other TPC concerning cereals (from 0.481 to 0.896 mg/g d.m.), vegetables (for example 11.7 mg/g d.m. for broccoli, 9.9 mg/g d.m. for garlic and 7.6 mg/g d.m. for pepper) and fruits (for example 23.1 mg/g d.m. for blackberry) (Vinson et al., 1998; Wang and Lin, 2000). However, high content of phenolics in both extracts from *Uncaria tomentosa* is not surprising when compared to other medical plants. For example, Amarowicz et al. (2004) found comparable phenolics content for ethanol extracts from horsetail (216 mg/g), bearberry leaves (312 mg/g), narrow-leaved Echinacea root (62 mg/g), senega root (72 mg/g), and wild-licorice root (63 mg/g). Moreover, Joubert et al. (2004) also showed very high phenolics content in aqueous extract from rooibos, an herbal tea prepared from *Aspalathus linearis*, an indigenous South African plant.

3.3. Antioxidant capacity

The graphical presentation of the antioxidant activity evaluated by the TEAC, PRTC and SOD methods are presented in Fig. 2.

The estimated TEAC values based on the relative abilities of the 50% ethanol and the phosphate-buffered uncaria extract to scavenge the $ABTS^{\bullet+}$ in the Trolox equivalents showed higher value for the EX_{ET} extract (0.57 mmol/g) in comparison to EX_{AQ} (0.34 mmol/g). Also, the EX_{ET} extract showed the highest PRTC value (0.52 mmol/g), being over two times higher when compared to the EX_{AQ} (0.19 mmol/g) extract. The results of PRTC are expressed as the number of peroxyl radicals (μmol) trapped by unit weight, based on dry matter of the investigated material. This finding indicates that the 50%

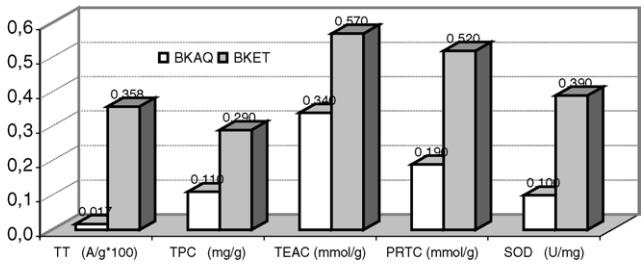

Fig. 2. Total tannins (TT), total phenolic compounds (TPC), trolox equivalent antioxidant capacity (TEAC), peroxyl radical-trapping capacity (PRTC) and superoxide radical scavenging activity (SOD) of the aqueous extract (BK_{AQ}; c = 1 mg/mL) and the 50% ethanolic extract (BK_{ET}; c = 1 mg/mL) originated from *Uncaria tomentosa*.

ethanolic extract was able to scavenge more peroxyl radicals derived from thermal decomposition of the ABAP than the aqueous one. Currently, the TEAC assay is one of the often used analytical strategies of assessment of antioxidant activity and it shows a well correlation with the other ones such as TRAP assay (total radical-trapping antioxidant parameter assay, 2,2′-diphenyl-l-picrylhydrazyl assay (DPPH assay), photochemiluminescence assay (PCL assay), and ferric reducing ability of plasma assay (FRAP assay) (Schlesier et al., 2002). The latest, measuring the reduction of Fe^{+3} to Fe^{+2} in the presence of antioxidants, was used by Halvorsen et al. (2002) to assess total antioxidants in a variety of dietary plants used worldwide. Their results demonstrated that there was more than a 1000-fold difference among total antioxidants in various dietary plants. Since electron-donating antioxidants are also responsible for scavenging the effect of peroxyl radicals or $ABTS^{+\bullet}$ cation radicals used in this study, the antioxidant capacity of both extracts from *Uncaria tomentosa* should occupy the top position within the worldwide dietary plants (TEAC and PRTC of EX_{ET} = 57 mol/g and 52 mmol/g, respectively; TEAC and PRTC of EX_{AQ} = 34 mol/g and 19 mmol/g, respectively).

Similar results to the antioxidant capacity were obtained by measuring the superoxide radical activity (SOD). In this study, the EX_{ET} extract showed higher superoxide radical scavenging activity (EX_{ET} = 0.39 U/mg; EX_{AQ} = 0.10 U/mg). Taking into consideration our previous work on SOD-like activity of different plant material provided in this study, it should be pointed out that the values of SOD-like activity of the uncaria extracts, especially ethanolic extracts, are higher than those originating from cereal grains, legume seeds and cruciferae sprouts (Zieliński and Kozłowska, 2000; Fernandez-Orozco et al., 2002). Superoxide scavenging activity was also provided by Nakamura et al. (1998) for rosmarinic acid and by Joubert et al. (2004) for rooibus flavonoids and tannin, as well as aqueous extracts. It is difficult to compare their data with those obtained in this study because of the determination of IC_{50} value (concentration of the test sample required to inhibit 50% of the superoxide anion radical in 1 mL of the reaction mixture).

The beneficial activity of free scavengers provided by the *Uncaria tomentosa* preparations may be explained as follows. Recently, numerous investigations have suggested that the cat's claw extracts mediate their activity through inhibition of NF-κB. This transcriptor factor regulates expression of TNF-α and

other pro-inflammatory cytokines responsible for many inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease and atherosclerosis (Åkesson et al., 2003). Among several known external and internal signals which activate NF-κB, free radicals seem to be most significant. Therefore, decreasing their concentration in cells by scavengers can be simply associated with low production of TNF-α. Such synergistic participation of the *Uncaria tomentosa* antioxidants may be partially confirmed by the results of Aguilar et al. (2002) who compared anti-inflammatory activity of aqueous and hydroalcoholic (80% of ethanol) extracts of cat's claw and reported that only hydroalcoholic one impaired NF-κB DNA binding.

Much higher antioxidant activity of the alcoholic preparation have given evident assumption that this preparation is more useful than the aqueous one in medical approach (Pietta et al., 1998). Particularly, in case when high activity of preparation is desired, e.g. during anti-cancer therapy. Moreover, such a preparation consists more oxindole alkaloids and a larger spectrum of biologically active constituents. Unfortunately, high concentration tannins in alcoholic tinctures often cause undesirable gastric disorders. The solution of this problem that we would like to suggest is partial precipitation of tannins after binding with whey proteins (non published data). Other possibility is an extraction by less concentrated ethanol solution. The use of polar and non-polar solvents in adequate ratio also gives a great opportunity for pharmacological modification and standardization of the preparations originated from *Uncaria tomentosa*.

The methods employed in this study are easy to use and provided reproducible results showing antioxidant properties of both preparations, particularly for the ethanol extract. Highly elevated TPC and TT contents in ethanolic extracts show that phenolic constituents must be responsible for such properties. It is in agreement with the data of Gonçalves et al. (2005) which indicate that the radical scavenging effectiveness of *Uncaria tomentosa* is related to proanthocyanidins. As it was shown, removing these compounds from cat's claw decoctions leads to a significant loss of antioxidant capacity. The chemical structures of proanthocyanidins include one or more aromatic rings bearing hydroxyl groups that are potentially able to act as reducing agents, as hydrogen donating antioxidants and as singlet oxygen quenchers (Rice-Evans et al., 1996). However, antioxidant activity of non-phenolic compounds should be also taken into account. For example, it has been shown that triterpenes present in *Uncaria tomentosa*, such as ursolic and oleanoic acid are efficient protectors against lipid peroxidation (Falkiewicz and Łukasiak, 2001).

The importance of the above mentioned and other antioxidants being able to remove peroxyl radicals is due to their effectiveness in hydrophobic phase: these are apparently the chain-breaking inhibitors of lipid peroxidation. Peroxyl radical scavengers can also act in the aqueous phase, e.g. when dealing with radicals from DNA, RNA, thiols and proteins. Previous studies on antioxidant activity of cat's claw extracts have shown their potency in the prevention of TBARS and DNA-sugar damages induced by ferrous ammonium sulfate (Deschmarchelier et al., 1997). Simple dependence between antioxidant potency of cat's claw preparations and their protective ability against DPPH and UV irradiation-induced cytotoxicity have also been reported (Sandoval et al., 2000). By protection of different cellular and extracelular components against degenerative damages, they have a great value for redox equilibrium and, in consequence, for maintenance/restoration of healthy status by whole organism.

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