

Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity

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Received 16 April 2004; received in revised form 21 October 2004

Available online 9 December 2004

Abstract

Decoctions prepared from the bark of *Uncaria tomentosa* (cat's claw) are widely used in the traditional Peruvian medicine for the treatment of several diseases, in particular as a potent anti-inflammatory agent. Therefore, the main purpose of this study was to determine if the well-known anti-inflammatory activity of cat's claw decoction was related with its reactivity with the oxidant species generated in the inflammatory process and to establish a relationship between such antioxidant ability and its phenolic composition.

We observed that the decoction prepared according to the traditional Peruvian medicine presented a potent radical scavenger activity, as suggested by its high capacity to reduce the free radical diphenylpicrylhydrazyl, and by its reaction with superoxide anion, peroxy and hydroxyl radicals as well as with the oxidant species, hydrogen peroxide and hypochlorous acid. It also protected membrane lipids against peroxidation induced by the iron/ascorbate system, as evaluated by the formation of thiobarbituric acid-reactive substances (TBARs). The decoction phenolic profile was established by chromatographic analysis (HPLC/DAD and TLC) revealing essentially the presence of proanthocyanidins (oligomeric procyanidins) and phenolic acids, mainly caffeic acid.

Thus, our results provide evidence for an antioxidant mechanism underlying the anti-inflammatory activity of cat's claw and support some of the biological effects of proanthocyanidins, more exactly its antioxidant and radical scavenging activities.

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Keywords: *Uncaria tomentosa*; Decoction; Proanthocyanidins; Antioxidant; Scavenger; Anti-inflammatory activity

1. Introduction

Uncaria tomentosa (Willd.) DC., Rubiaceae, grows in the Amazon forests of Peru, where it is known as “uña de gato” (cat's claw). Decoctions prepared from the bark have been widely used in traditional Peruvian medicine for the treatment of several diseases, in particular as potent anti-inflammatory agents (Desmarchelier

et al., 1997; Sandoval et al., 2000). Although previous studies have supported some of their pharmacological properties, namely the antimutagenic (Rizzi et al., 1993; Sheng et al., 1998) and the anti-inflammatory actions (Aquino et al., 1991; Piscocoya et al., 2001), the molecular mechanisms underlying such activities have not yet been clarified. The capacity of these decoctions to inhibit either the TNF α production, or to a lesser extent the PGE₂ production, may explain some of their anti-inflammatory properties (Sandoval et al., 2000; Piscocoya et al., 2001). Additionally, it has been reported that they possess antioxidant activity, as evaluated by the

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reaction with the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sandoval et al., 2000). Nevertheless, we are aware that a better characterization of this antioxidant capacity requires model systems and methodologies involving the generation of diverse reactive species, namely superoxide anion ($O_2^{\bullet-}$), peroxy (ROO^{\bullet}), and hydroxyl (HO^{\bullet}) radicals, hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl), as well as the evaluation of the potential ability to decrease them (Halliwell, 1997). Although the production of such reactive species occurs during the normal metabolism in all the aerobic organisms, it may be dramatically increased in the inflammatory processes.

It is well known that the central feature of inflammatory activity is the activation of phagocytic cells that synthesize and release large amounts of reactive species causing cell and tissue injury, either directly by oxidative degradation of essential cellular components or indirectly by altering the normal protease/antiprotease balance (Halliwell et al., 1988). Providing that reactive oxygen species may act as toxins, mediators and modulators of inflammatory gene activation, efforts have been directed to investigate antioxidant molecules as potential therapeutic agents (Conner and Grisham, 1996). In this context, polyphenols becoming increasingly important (Korkina and Afanas'ev, 1997; Groot and Rauen, 1998; Packer et al., 1999). Epidemiological studies have suggested a correlation between the consumption of polyphenol-rich foods or beverages and the prevention of diseases (Maxwell, 1995; Arnao et al., 2001). Indeed, polyphenols are reducing agents and like vitamin C and vitamin E they possess potential for protection against oxidative damage (Rice-Evans et al., 1996; Bagchi et al., 2000).

On the other hand, concerning the *Uncaria* chemical constituents, previous studies have been essentially focused on its alkaloid components (Laus and Keplinger, 1994; Mohamed et al., 2000; Muhammad et al., 2001). Thus, the aim of this study was to evaluate the polyphenolic composition and the scavenging properties of an *U. tomentosa* decoction, prepared according to traditional Peruvian medicine and to establish a relationship between phenolic composition and antioxidant activity.

The assessment of its general free radical scavenging ability was got by the interaction with DPPH in solution. To better characterize this antioxidant potential, the interactions of the decoction with the superoxide, hydroxyl and peroxy radicals as well with hydrogen peroxide and hypochlorous acid generated in homogeneous model systems were evaluated. Also, the effect on lipid peroxidation, initiated by Fe^{2+} /ascorbate in sarcoplasmic reticulum (SR) membranes, used as a native membrane model, was assessed by TBARs formation. Attempting to establish a relationship between its polyphenolic constituents and antioxidant capacity, a decoction depleted of tannins was prepared and its reacting

efficiency with DPPH determined. Furthermore, the decoction was fractionated by gel chromatography and analyzed by HPLC and TLC. The proanthocyanidin (condensed tannins) content was also determined.

2. Results and discussion

2.1. Free radical scavenger activity

The general free radical scavenger activity of the decoction was evaluated by its interaction with DPPH in solution. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Because of its odd electron, the methanolic solution shows a strong absorption band at 517 nm (Blois, 1958), which decreases in the presence of free radical scavengers. DPPH scavenging activity has been largely used as a quick and reliable parameter to assess the in vitro general antioxidant activity of plant extracts (Soares et al., 1997; Bonina et al., 2000; Munasinghe et al., 2001), that has been assigned to phenolic compounds namely, phenolic acids and flavonoids (Rice-Evans et al., 1996; Van Acker et al., 1996).

Uncaria tomentosa decoction promptly reacted with DPPH leading to a loss of 70% in absorbance intensity. In contrast, the tannin-free decoction only presented 7% of DPPH reduction capacity (Fig. 1). A correlation between the capacity of DPPH reduction and the proanthocyanidins content (grey bar in the center) was also verified. These results clearly indicate that the loss of proanthocyanidins leads to a significant loss of antioxidant capacity.

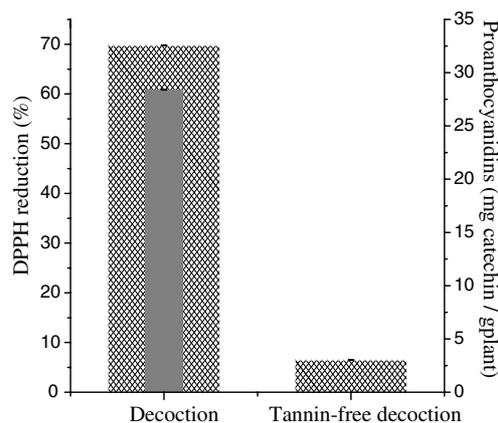


Fig. 1. Relationship between DPPH radical quenching of decoction and tannin-free decoction in terms of percentage of DPPH reduction and proanthocyanidins content, as expressed by mg of catechin/g plant. The great columns (crossed lines) represent the capacity of DPPH reduction of both decoction and tannin-free decoction. Proanthocyanidins were only detected in the decoction as evidenced by the grey column in the center. Bars represent the mean \pm SD of at least three different experiments.

As already referred, the aim of this study was mainly to identify the major constituents of *U. tomentosa* decoction responsible for its antioxidant activity and more specifically for the scavenging capacity of reactive species involved into the inflammatory process.

2.2. Chemical characterization

An HPLC profile of the *U. tomentosa* decoction recorded at 280 and 320 nm is illustrated in Fig. 2. The phenolic profile was established according to the retention characteristics in reversed-phase and/or UV spectra, using HPLC/DAD analyses before and after fractionation on Sephadex LH-20 and tannins elimination (Table 1). The identification of the compounds present in the decoction was tentatively made on the basis of their retention and/or spectral characteristics in comparison with authentic samples.

The confirmation of the presence of protocatechuic acid, catechin, epicatechin, vanillic acid, caffeic acid and syringic acid, was assessed by the addition of authentic samples to the decoction or to the tannin-free decoction. In these cases, no deviations were found neither in the retention times nor in the UV spectra.

HPLC/DAD analyses were consistent with the major presence of flavan-3-ol related compounds (proanthocyanidins). A few ellagic derivatives were found but galates were not detected. Phenolic acids and their derivatives were also found, being caffeic acid the most representative. Yet, some phenolic compounds could not be well identified because its co-elution with others. In order to establish a better characterization of the decoction constituents, fractionation through a Sephadex LH-20 column was carried out. Nine fractions were

separated according to UV behaviour, but only three of them (F_I, F_{II}, and F_{III}) were chemically distinct. As represented in Table 1, the fraction I contained caffeic acid, as the main constituent, protocatechuic acid and catechin, as well as some constituents with spectra suggesting flavonols (quercetin and kaempferol) and phenolic acid derivatives (ellagic and *p*-coumaric). In addition, epicatechin and flavan-3-ol related compounds were detected in small quantities. Higher concentrations of these compounds occurred in fraction II. Fraction III also showed peaks corresponding to flavan-3-ol related compounds. Vanillic and syringic acids could only be identified in the tannin-free decoction, since they were present in low concentrations and co-eluted with the proanthocyanidins.

Complete acid hydrolysis of both fractions II and III led to the disappearance of flavan-3-ol derivatives and to the simultaneous appearance of cyanidin, showing the presence of proanthocyanidins, mainly of the procyanidin type. Concerning the polymerisation degree of proanthocyanidins, TLC analysis revealed that fraction II was constituted by oligomeric procyanidins, essentially dimers and trimers whereas more polymerised proanthocyanidins (oligomers greater than four flavan-3-ol subunits) were detected in fraction III.

The results obtained illustrate that the majority of the detected phenolic compounds are proanthocyanidin-like, suggesting that the radical scavenging effectiveness of *U. tomentosa* decoction can be related to them. Proanthocyanidins have been reported to provide protection against free radicals and oxidative stress, possessing important biological, pharmacological and therapeutic properties (Packer et al., 1999; Bagchi et al., 2000; Santos-Buelga and Scalbert, 2000), namely anti-inflammatory activity (Li et al., 2001).

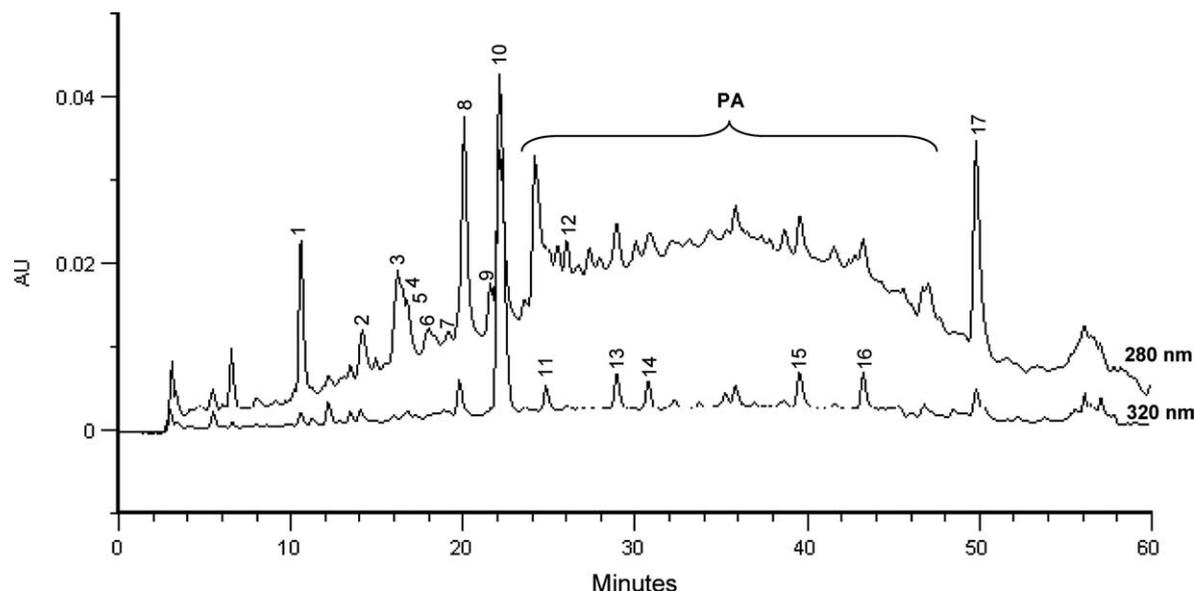


Fig. 2. A typical HPLC profile of *Uncaria tomentosa* decoction recorded at 280 and 320 nm. Peaks identification is shown in Table 1.

Table 1
HPLC phenolic composition of the *Uncaria tomentosa* decoction

Peak number	R_t (min)	λ_{max} (nm)	Compound ^a	F _I	F _{II}	F _{III}	Tannin-free decoction
1	10.6	259; 293sh	Protocatechuic acid	+			+
2	14.1	279	Proanthocyanidin		+		
3	16.2	279	Proanthocyanidin		+		
4	16.4	279	Catechin	+			
5	16.8	279	Proanthocyanidin		+		
6	18.0	279	Proanthocyanidin		+		
7	19.2	279	Proanthocyanidin		+		
8	20.1	279	Epicatechin		+		
8	20.1	279	Proanthocyanidins		+	+	
9	21.6	261; 292	Vanillic acid				+
9	21.6	279	Proanthocyanidins		+	+	
10	22.1	298; 323	Caffeic acid	+			+
11	24.8	280; 336sh	Acylated flavan-3-ol derivative				
12	26.0	275	Syringic acid				+
13	28.9	282; 309sh	<i>p</i> -Coumaric acid derivative				+
14	30.8	281; 309sh	<i>p</i> -Coumaric acid derivative	+			+
15	39.6	258; 266sh; 285sh; 354	Quercetin derivative	+			+
16	43.2	266; 284sh; 346	Kaempferol derivative	+			+
17	49.8	256; 306sh; 356; 370	Ellagic acid derivative	+			+
PA	23.5–47.0	279–280	Proanthocyanidins		+	+	

The peaks profile is shown in Fig. 2. The compositions of their three fractions eluted from a Sephadex LH-20 column (F_I, F_{II} and F_{III}), as well as of the tannin-free decoction are also evidenced. +, compounds present in the fractions; sh, shoulder.

^a Identification tentatively made by comparison of the retention times and/or UV spectra with authentic samples.

To confirm that correlation, the tannin-free decoction, that exhibited a very low DPPH scavenger activity as compared to the decoction (Fig. 1), was analysed by HPLC. Spectra corresponding to the proanthocyanidins were not detected, although other polyphenols were observed, as referred to Table 1, supporting evidence for a relationship between the decoction radical scavenger activity and its proanthocyanidins content.

2.3. Antioxidant activity characterization

2.3.1. Interaction with superoxide anion

The reactivity of decoction towards superoxide anion was evaluated by the decrease in the rate of nitroblue tetrazolium (NBT) reduction induced by $O_2^{\cdot-}$ generated by the enzymatic system xanthine oxidase/hypoxanthine. Any compound that decreases the rate of NBT reduction should react with $O_2^{\cdot-}$ unless itself reacts directly with NBT. The use of superoxide dismutase (SOD), the physiological scavenger of superoxide anion, enabled us to confirm the $O_2^{\cdot-}$ production in the assay, as it inhibited the reaction of NBT reduction in a concentration dependent manner (data not shown) with an inhibition concentration at 50% = 3.47 U ml⁻¹. Fig. 3 shows the results of the reactivities of different amounts of decoction against $O_2^{\cdot-}$ as expressed by SOD equivalent units with evidence for a linear correlation between decoction concentration and $O_2^{\cdot-}$ scavenging activity. Considering that the decoction neither reduced NBT by itself nor inhibited xanthine oxidase, as evaluated by uric acid formation (data not shown),

the results denote the capacity of decoction for reacting with superoxide (Halliwell, 1997).

Although $O_2^{\cdot-}$ is a free radical, its reactivity is limited and it is enclosed in the compartment where it is produced. However, two molecules of superoxide anion rapidly dismutate to hydrogen peroxide and molecular

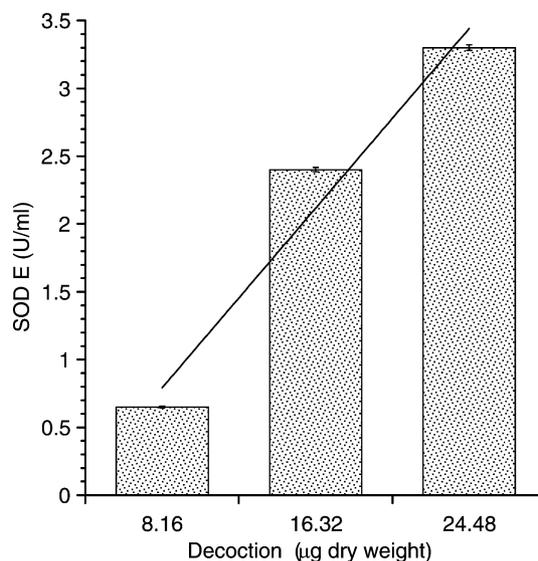


Fig. 3. Superoxide anion scavenging activity of *Uncaria tomentosa* decoction as evaluated by the inhibition of NBT reduction and expressed in terms of superoxide dismutase equivalent units (SOD E). Bars represent the mean \pm SD of at least three different experiments and the line evidences the linear correlation between superoxide anion scavenging capacity and the amount of decoction.

Table 2
Scavenging of hydrogen peroxide, hydroxyl radical and hypochlorous acid by *Uncaria tomentosa* decoction

Decoction (μg dry weight)	Scavenger capacity (%)		
	H_2O_2	$\cdot\text{OH}$	HOCl
10.2		32.4 ± 3.5	
16.32	7.4 ± 0.27		7.4 ± 0.34

Data are expressed as the mean \pm standard deviation of at least three experiments. The scavenger capacity was expressed in percentage of decrease relatively to the control assay, i.e., the assay in the absence of added decoction.

oxygen, spontaneously or in a reaction catalysed by SOD. It can also react with nitric oxide (NO) to give peroxynitrite (ONOO^-), which is a highly cytotoxic species (Nordberg and Arnér, 2001).

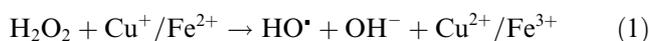
2.3.2. Interaction with hydrogen peroxide

Hydrogen peroxide can be sensitively measured by the conventional peroxidase-based assay where guaiacol is used as an electron donor. The decoction reactivity with H_2O_2 was estimated by the decrease in absorbance at 436 nm. In fact, any compound that reacts with H_2O_2 should decrease the rate of guaiacol oxidation decreasing the absorbance intensity at 436 nm. In the presence of decoction (16.32 μg dry material) there was a slight decrease in absorbance at 436 nm, denoting some interaction between decoction and H_2O_2 , considering that decoction neither oxidized directly guaiacol nor was a substrate for peroxidase (data not shown). This decrease indicated a scavenger capacity of about 7%, as shown in Table 2.

Although hydrogen peroxide is not a free radical it has a great physiological relevance because of its ability to penetrate biological membranes and to act like an intermediate in the production of more reactive oxygen species, namely hydroxyl radical and hypochlorous acid (Nordberg and Arnér, 2001).

2.3.3. Interaction with hydroxyl radicals

Hydroxyl radical is a highly reactive species attacking almost all biological molecules. It is formed from H_2O_2 in a reaction catalysed by metal ions (Fe^{2+} or Cu^+) known as the Fenton reaction (Nordberg and Arnér, 2001), where the superoxide anion plays an important role by reducing the metal ions.



To test the interaction of the decoction with hydroxyl radicals, we used a simple assay where the radicals are formed in solution from the ascorbate/iron ion/ H_2O_2 system and are detected by their ability to degrade deoxyribose that, on heating with TBA, forms a pink chromogen (Payá et al., 1992). *U. tomentosa* decoction competed with deoxyribose for hydroxyl radicals demonstrating in vitro significant protective effect on the

deoxyribose damage assay. Appropriate blanks were performed to rule out the possibility of decoction to affect the measurement of deoxyribose degradation (it had no effect when added to the TBA reagents), to enhance hydroxyl radical formation (it had no pro-oxidant effect) and to react with $\text{HO}\cdot$ giving TBA-reactive substances (no chromogen formation in the absence of deoxyribose). Decoction showed a very good scavenging capacity towards $\text{HO}\cdot$ (Table 2) in comparison with mannitol (10.2 μg of decoction dry material presented a hydroxyl radical-scavenging capacity of about 30% against about 40% for 1.8 mg mannitol). Mannitol was used as a reference scavenger of hydroxyl radicals.

2.3.4. Interaction with hypochlorous acid

Hypochlorous acid (HOCl) is produced in vivo, at inflammation sites by enzymatic oxidation of chloride ions. HOCl is much more toxic than either superoxide or hydrogen peroxide. It is a non-specific oxidising and chlorinating agent that reacts promptly with a variety of biological molecules (Winterbourn and Kettle, 2000). One of the major extracellular targets of HOCl attack is α_1 -antiproteinase, the main circulating inhibitor of serine proteases, as elastase. Thus, elastase activity may be used to evaluate either α_1 -antiproteinase activity or hypochlorous acid scavenging capacity. Actually, in the presence of HOCl the elastase activity is maximal, decreasing when previously we add a compound with hypochlorous acid scavenging capacity.

In the presence of decoction (16.32 μg dry material) the elastase activity was inhibited 7.4% (Table 2) denoting only a slight HOCl-scavenging activity, especially, when compared with *N*-acetylcysteine. This reference compound, in our assay conditions, at the concentration of 54.8 μg , inhibited almost all the elastase activity (99.6%). Control experiments showed that decoction neither inhibited elastase directly nor interfered with the capacity of α_1 -antiproteinase to inhibit elastase.

2.3.5. Interaction with peroxy radicals

Peroxy radicals are formed upon the reaction of carbon-centred radicals with oxygen and its formation is the major chain-propagation step in lipid peroxidation. They are biologically relevant active species with high capacity to damage cellular constituents (Cadenas, 1989).

Peroxy radicals were generated in vitro by thermal decomposition of the hydrophilic azo initiator 2,2'-azobis-(2-amidinopropane hydrochloride) (AAPH) at a defined rate in aqueous solution leading to the immediate decay of the fluorescence intensity of B-phycoerythrin (Niki, 1990). However, in the presence of little amounts of decoction this fluorescence decay was decreased in a non-linear and concentration-dependent manner, as shown in Fig. 4. This non-linear fluorescence decay was similar to that of Trolox, a water soluble vitamin

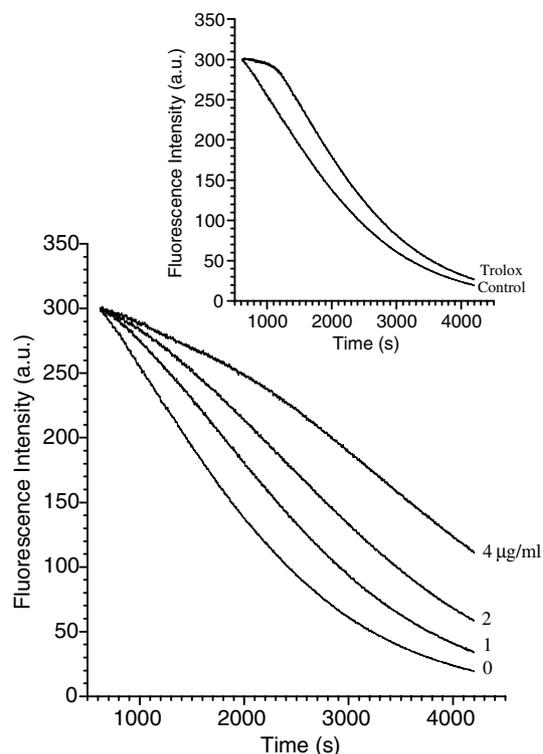


Fig. 4. Scavenging effect of *Uncaria tomentosa* decoction on AAPH-induced peroxyl radicals as assessed by the protection on the fluorescence decay of B-phycoerythrin. B-Phycoerythrin (15 nM) in phosphate buffer pH 7.4 was exposed to AAPH at 37 °C. Decoction, at different concentrations, was added immediately before AAPH as described in Section 4. The recordings are typical assays of at least three different experiments. Inset represents a typical assay where it is evident the effect of 3 µM of Trolox (0.8 µg/ml).

E analogue and a well-known chain-breaking antioxidant (Niki, 1990). It caused an initial inhibition period of B-phycoerythrin fluorescence quenching, followed by a fluorescence decay similar to that of the control assay, as shown in the inset. Decoction did not induce a so clear initial inhibition period, but efficiently protected B-phycoerythrin from peroxy radicals attack during the reaction time. Decoction is a mixture of constituents that probably react with ROO^\cdot in different ways, either as chain-breaking antioxidants, or as retardant antioxidants only decreasing the propagation rate like acetaminophen or salicylate (Dinis et al., 1994).

2.3.6. Inhibition of lipid peroxidation

The impact of decoction on lipid peroxidation was investigated on the basis of the effects on membrane lipid peroxidation induced by Fe^{2+} /ascorbate as evaluated by the production of thiobarbituric acid-reactive substances (TBARs). Lipid peroxidation in SR membranes, used as a model, increased with time, reaching an apparent maximum at 60 min (data not shown). Decoction significantly inhibited membrane lipid peroxidation, decreasing in about 86% the amount of TBARs formed

Table 3

SR membrane lipid oxidation promoted by Fe^{2+} /ascorbate (20 µM/100 µM) in the absence and presence of decoction (12.24 µg dry weight), as measured by TBARs formation

SR membranes in different incubation systems	TBARs	
	nmolMDA/mg protein	% of inhibition protein
Native membranes	n.d.	
Fe^{2+} /ascorbate	15.52 ± 0.32	
Decoction + Fe^{2+} /ascorbate	2.18 ± 0.014	86

Data are expressed as the mean ± standard deviation of at least three experiments; n.d., not detected.

at 60 min relatively to the control assay, as indicated in Table 3. Although the measurement of thiobarbituric acid-reactive substances is a test lacking specificity, it is a very good indicative of SR lipid oxidation as previously demonstrated (Dinis et al., 1993, 1994; Soares et al., 1997).

The potential inhibitory effect of decoction on lipid peroxidation was evaluated by using iron as a metal ion catalyst. It has been suggested that the antioxidant activities of flavonoids depend on their reducing and chelating properties (Van Acker et al., 1996; Korkina and Afanas'ev, 1997). Therefore, the ability of decoction to inhibit lipid peroxidation may be exerted by complexing iron under a form catalytically silent. To clarify the antioxidant potential of *U. tomentosa* decoction in the presence of iron, the metal binding capacity of decoction was tested by assessing its absorption spectra in the absence and presence of iron. The results obtained (Fig. 5) show that the characteristic absorption peak of decoc-

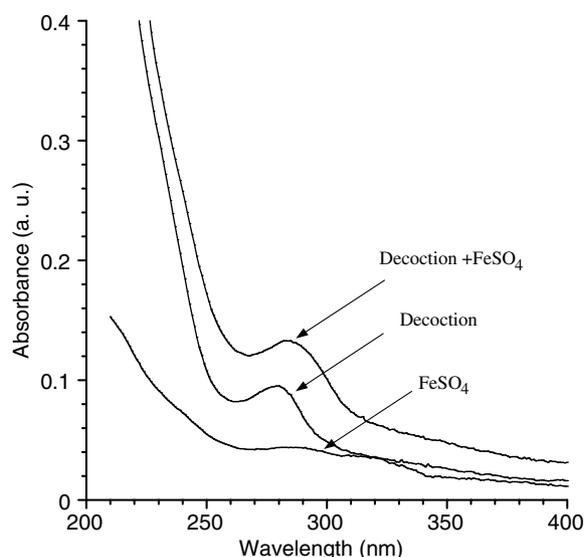


Fig. 5. The characteristic absorbance peak of *Uncaria tomentosa* decoction in the absence (decoction) and presence of iron (decoction + FeSO_4). Ferrous sulphate (FeSO_4) alone does not present any peak at 280 nm, the characteristic absorbance peak of decoction.

tion at 280 nm was shifted to a higher wavelength, suggesting that decoction can bind iron under neutral pH. Thus, the decoction antioxidant capacity, specifically the ability of HO[•] scavenging and the inhibition capacity of lipid peroxidation induced by the transition metal could be in part ascribed to a mechanism of iron binding, as described for the Ginseng extract (Zhang et al., 1996).

3. Conclusion

Despite cat's claw traditional use in various states of inflammation, the biochemical mechanisms underlying such properties remain unclear. Attempting to clarify some of those mechanisms we explored the capacity of reaction of a bark decoction against the oxidant species generated in the inflammatory process, as well as the involved chemical constituents.

Uncaria tomentosa bark decoction shows to be mainly composed by phenolic acids, essentially caffeic acid and proanthocyanidins, namely oligomeric procyanidins. The chemical structures of these phytochemicals 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). Owing to the basic chemical structure of its components, it would be expected that *U. tomentosa* bark decoction possessed a strong antioxidant activity. Actually, our data demonstrate such high antioxidant ability of the decoction, suggesting an important role of proanthocyanidins in its free radical scavenging properties, as clearly evidenced by the different effects of decoction and tannin-free decoction on the DPPH radical quenching (Fig. 1).

Furthermore, our data indicate that the decoction is a very good scavenger of superoxide anion and hydroxyl radical and in a lesser extension of hydrogen peroxide and hypochlorous acid. Additionally, it shows an efficient peroxy scavenging activity when the radicals are formed in hydrophilic environment as well as a strong protective effect on membrane lipids against oxidation induced by the iron/ascorbate system. The decoction also shows some degree of metal-binding capacity which can explain in part its capacity for protecting membrane lipids from oxidation as well as its capacity of interaction with hydroxyl radicals. Noteworthy are the relatively very low amounts of decoction used in all the experiments to minimize the potential interference of decoction constituents with the reagents (8–25 µg decoction dry weight corresponding to 2.4–7.5 µg proanthocyanidins).

In conclusion, our data provide a contribution for a better understanding of cat's claw anti-inflammatory activity and confirm some of the biological effects of

its proanthocyanidins, namely the antioxidant and radical scavenging activities. Although preliminary studies about cytotoxicity have already been performed (Santa Maria et al., 1997), further research is required in order to make *U. tomentosa* decoction an effective anti-inflammatory agent.

4. Experimental

4.1. Chemicals

The azocompound 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) was obtained from Polysciences, Inc. (Warrington, PA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), xanthine oxidase, hypoxanthine, nitroblue tetrazolium, superoxide dismutase (SOD), guaiacol, horseradish peroxidase, deoxyribose, elastase, α₁-antiprotease, elastase substrate, *N*-acetylcysteine and B-phycoerythrin were purchased from Sigma-Aldrich Química S.A. (Portugal). Ascorbic acid was obtained from Fluka (Switzerland). The reference compounds, caffeic acid (minimum 99%), (+)-catechin hydrate (minimum 98%), *p*-coumaric acid, ellagic acid (≈95%), (–)-epicatechin, kaempferol (minimum 90%), protocatechuic acid, quercetin dihydrate (minimum 98%), syringic acid (minimum 98%) and vanillic acid were purchased from Sigma-Aldrich. The cyanidin chloride was obtained from Extrasynthese. All the other chemicals were of grade for biochemical analysis. Deionized water was used throughout and when necessary Ultrapure Milli Q water was also used.

Sarcoplasmic reticulum vesicles were prepared from rabbit white muscles as previously described (Dinis et al., 1993).

4.2. Plant material

Uncaria tomentosa barks (Willd) DC. were kindly supplied by Dr^a Olga Lock De Ugaz, from Pontificia Universidad Católica, Lima, Peru.

Voucher specimen is deposited at the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Coimbra.

4.3. Preparation of *Uncaria* extracts

Decoction was prepared according to the traditional Peruvian medicine, by boiling 0.750 g of powdered bark in distilled water (150 ml) for 40 min. After cooling, the final volume was adjusted to 250 ml and the marc was eliminated by filtration.

A tannin-free decoction was also prepared from aliquots of decoction (10 ml) treated with hide powder (100 mg), according to the guidelines for the tannins determination in *Ratanhiae radix* (Council of Europe,

2002). After centrifugation, the resulting solution was filtered and the free radical scavenging activity and HPLC phenolic profile were evaluated.

In order to establish the phenolic profile of *U. tomentosa* bark decoction and its *in vitro* antioxidant and radical scavenging activity, four decoctions were prepared as described above and mixed together. Seven hundred millilitres were taken and the lipids were eliminated with *n*-hexane (1.5:1, v/v, three times). The aqueous phase was concentrated under reduced pressure almost to dryness and dissolved in methanol–water (2:3, v/v) to a final volume of 5 ml. A standardized extract, containing 30 mg of proanthocyanidins, expressed as mg catechin equivalent by 100 mg of decoction dry weight, was obtained. This extract is referred as decoction along the work.

4.4. Determination of proanthocyanidins

Proanthocyanidins contents were determined by spectroscopy at 500 nm, on the basis of a colorimetric reaction with vanillin-HCl, as described by [Julkunen-Titto \(1985\)](#). The results were expressed as mg of catechin equivalent/g of dry plant material.

4.5. Gel chromatography fractionation

An aliquot of the decoction (4 ml) was fractionated by gel chromatography on a Sephadex LH-20 column (25–100 μm , 170 \times 20 mm), from Sigma–Aldrich. The elution was performed using MeOH–H₂O (1:1) 150 ml, MeOH–H₂O (7:3) 150 ml and acetone–H₂O (7:3) 100 ml. Nine fractions were obtained according to their behaviour under UV observation. All fractions were concentrated under vacuum and dissolved in MeOH–H₂O (1:1) to a final volume of 1 ml.

4.6. Analytical HPLC

HPLC analyses were carried out in a Gilson instrument equipped with a photodiode-array detector (DAD) and an ODS-2 column (5 μm , 250 mm \times 4.6 mm i.d.) from Waters. A mobile phase constituted by methanol (A) and 5% aqueous formic acid, v/v (B) was used. The gradient consisted of 5–15% A, for 10 min, 15–35% A for more 25 min, then from 35% to 50% A, for 15 min and finally 50–100% A for 10 min in a total of 60 min, at a flow-rate of 1 ml min⁻¹. Chromatographic profiles were recorded at 280 and 320 nm. Data treatment was carried out with a software Unipoint^R.

The compounds were identified by their retention times and UV spectra, comparing them with authentic samples. For some compounds, their confirmation was performed by the addition of authentic samples to aliquots of the decoction or tannin-free decoction.

4.7. Thin layer chromatography

TLC was carried out as described by [Escribano-Bailón et al. \(1992\)](#) to classify the proanthocyanidins according to their polymerisation degree. Pre-coated silica gel 60 F₂₅₄ plates from Merck and toluene-acetone-acetic acid (3:7.5:1) were used as stationary and mobile phases, respectively. Detection was made with a solution of 1% vanillin in 70% hydrochloric acid. Catechin was used as a reference compound.

4.8. *In vitro* antioxidant and radical scavenging activity

4.8.1. Measurement of free radical scavenger activity

Free radical scavenging activities of decoction and tannin-free decoction (reported to 280 μg of plant dry weight) were evaluated by their reactivities with an ethanolic solution of 500 μM DPPH from the change in absorbance at 517 nm, according to the method of [Blois \(1958\)](#). The results were expressed in percentage of DPPH reduction, as evaluated by the decrease in the absorbance value at 517 nm as compared to the control assay, i.e., the assay without the sample under study.

4.8.2. Superoxide anion scavenging activity

Superoxide anions were generated enzymatically ([Payá et al., 1992](#)) at room temperature by adding 0.042 U ml⁻¹ of xanthine oxidase to a mixture of 2 ml containing 50 mM KH₂PO₄ pH 7.4, 1 mM EDTA, 100 μM hypoxanthine and 100 μM NBT. The rate of NBT reduction was recorded during 150 s in a Perkin–Elmer Lambda 6 Spectrophotometer, at 560 nm. The decoction was added at several concentrations and the results were expressed as SOD equivalent units.

Control experiments were performed to determine whether the decoction directly reduced NBT or inhibited xanthine oxidase, as described by [Soares et al. \(1997\)](#).

Superoxide dismutase was used as a reference scavenger of superoxide anion.

4.8.3. Hydrogen peroxide scavenging activity

Hydrogen peroxide was measured by the formation of a brown colour, recorded at 436 nm, in a reaction mixture of 1 ml containing 150 mM KH₂PO₄–KOH buffer pH 7.4, 100 μl guaiacol solution (obtained by adding 100 μl of pure guaiacol to 50 ml desionized water) and 5 μl horseradish peroxidase (5 mg/ml in the same phosphate buffer) as described elsewhere ([Aruoma et al., 1989](#)). The reaction was started by H₂O₂ addition. Decoction was pre-incubated with H₂O₂ for 30 min at 25 °C and then, aliquots were removed and assayed for the remaining H₂O₂. Adequate blanks were prepared to determine whether the sample vehicle interfered with the reaction or if decoction was a substrate for peroxidase.

4.8.4. Hydroxyl radicals scavenging activity

Hydroxyl radicals were generated by the ascorbate/iron/H₂O₂ system, essentially as referred elsewhere (Halliwell et al., 1987; Burits and Bucar, 2000). The reaction mixture in a final volume of 1 ml, contained 20 mM KH₂PO₄–KOH buffer pH 7.4, 2.8 mM 2-deoxyribose, FeCl₃–EDTA (100 and 104 μM, respectively), 1 mM H₂O₂ and 100 μM ascorbate. After an incubation period of 1 h at 37 °C the extent of deoxyribose degradation was measured by the thiobarbituric acid method (Buege and Aust, 1978). Decoction, vehicle (blank) or D-manitol (reference compound) were added before ascorbate addition. Solutions of iron salt, H₂O₂ and ascorbate were always prepared just before use.

4.8.5. Hypochlorous acid scavenging activity

Hypochlorous acid (HOCl) was prepared immediately before use and the HOCl scavenging activity was indirectly evaluated by the measurement of elastase activity, as described by Payá et al. (1992). Control experiments were also carried out to eliminate any direct effect of decoction on α₁-antiproteinase or elastase activities. *N*-Acetylcysteine was used as a reference scavenger of HOCl (Haenen and Bast, 1991). Briefly, decoction was mixed with α₁-antiproteinase (0.77 mg) in phosphate buffer and HOCl was added at a final concentration of 75 μM. This reaction mixture was incubated at 25 °C for 1 h and then elastase was added. After incubation for further 30 min, the remaining elastase activity was evaluated by the change in absorbance at 410 nm, using *N*-succinyl-ala-ala-ala-*p*-nitroanilide as substrate.

4.8.6. Peroxyl radicals scavenger activity

To assess the capacity of decoction as a scavenger of peroxyl radicals generated in aqueous phase, we used the water-soluble azo initiator AAPH. AAPH generates peroxyl radicals at a constant rate by thermal decomposition, causing a decrease of B-phycoerythrin fluorescence intensity (Maitra et al., 1995). B-Phycoerythrin (15 nM) was incubated at 37 °C in 2 ml of 0.2 M phosphate buffer pH 7.4 with continuous stirring. Decoction or Trolox (a reference compound) were added 30 s before addition of 10 mM AAPH and the peroxyl scavenger activity was evaluated by the quenching of B-phycoerythrin fluorescence as previously described (Maitra et al., 1995). The fluorescence measurements were carried out at 37 °C during 1 h in a Perkin–Elmer computer controlled LS 50 spectrofluorimeter. The excitation and emission wavelengths were 540 and 575 nm, respectively (band-passes 5 nm).

4.8.7. Antioxidant activity against membrane lipid peroxidation

Sarcoplasmic reticulum membranes were used, as a native membrane model, to assess the effect of decoction

on lipid peroxidation, as previously described (Dinis et al., 1994). Briefly, SR membranes (2 mg protein/ml) in a medium containing 10 mM TRIS, 150 mM NaCl, pH 7.0, were incubated under magnetic stirring at 37 °C, for 90 min, in the presence of 20 μM FeSO₄ and 100 μM ascorbate. Decoction or vehicle was added before the peroxidation inducers. The extent of oxidation and the decoction antioxidant potential were evaluated by the production of TBARs (Buege and Aust, 1978). Butylated hydroxytoluene was added to the TBA reagent to prevent additional chromophore formation during the assay procedure.

4.8.8. Capacity of binding Fe²⁺

The binding of iron ions by the decoction was investigated spectrophotometrically. Spectra of the decoction before and after addition of 80 μM FeSO₄ in TRIS buffer pH 7.0 were recorded at room temperature in a Perkin–Elmer Lambda 6 spectrophotometer, between 210 and 400 nm and compared with that of FeSO₄ recorded in the same conditions.

Acknowledgements

This research was funded in part by the Fundação para a Ciência e Tecnologia, Programa Operacional de Ciência, Tecnologia e Inovação (POCTI) and POCTI/35019/AGR/2000. The authors also thank Prof. Leonor Almeida for her advice and many helpful suggestions.

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