



## Caffeic and chlorogenic acids in *Ilex paraguariensis* extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins

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### ABSTRACT

The present study concentrates on the evaluation of the anti-glycation effect of some bioactive substances present in yerba maté (*Ilex paraguariensis*): 5-caffeoylquinic acid, caffeic acid and a sapogenin (oleanolic acid). Bovine serum albumin and histones were incubated in the presence of methylglyoxal with or without the addition of 5-caffeoylquinic acid, caffeic acid and oleanolic acid. After the incubation period, advanced glycation end product (AGE) fluorescence spectra were performed and protein structural changes were evaluated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. Chlorogenic acid, caffeic acid are the main substances responsible for the anti-glycation effect of maté tea.

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### 1. Introduction

Glycation of proteins is the adduct formation between amino groups and reducing sugars or dicarbonyl compounds that occurs in vivo, leading to important changes in protein structure with consecutive impairment of their biological activity. Glycation is at the same time favored by oxidative processes and is a generator of oxidative stress, and when combined they participate as pathogenic factors in diabetic microvascular complications, atherosclerosis and neurodegenerative diseases [1–4].

Diets rich in fruit and vegetables protect against degenerative disease due to the presence of bioactive substances that exert specific actions in biological targets, including anti-glycation activity [5–9]. Compounds with antioxidant capacity, such as polyphenols, have been proven to possess anti-glycation effects at physiological concentrations [8,10,11], although there are reports indicating that their anti-glycation capacity depends on the model system employed. Plant material presents other more hydrophobic compounds that

also display antioxidant and hypoglycemic activities, such as the sapogenins ursolic and oleanolic acid, which might be at the basis of the anti-glycation behavior observed in non polar extracts from medicinal herbs [6,12].

In a previous study we have shown that *Ilex paraguariensis* extracts have potent anti-glycation activity [9]. In this work we had used the crude extract from *I. paraguariensis*, as it is commonly brewed and drunk: 5 g of dry leaves extracted in 100 ml of water at 80 °C. *I. paraguariensis* (maté tea) is consumed by large segments of the population in Argentina, Brazil, Uruguay and Paraguay. It is gaining acceptance in the USA and has been used in Europe, notably in Germany for many years both as an ethnic beverage and as an herbal supplement. Our first study showed the anti-glycation effect on the crude extracts. It is not known which compounds respond for this activity. The present study aims to determine which of the main components of maté tea extracts is responsible for the effect. Water soluble extracts of *I. paraguariensis* contain caffeic acid, chlorogenic acids (several isomers), saponins and sapogenins [13,14].

Caffeic acid (Fig. 1A) is a naturally occurring phenolic compound, found in many fruits vegetables and herbs, e.g. coffee, artichoke, pear, basil, thyme, oregano and apple [15].

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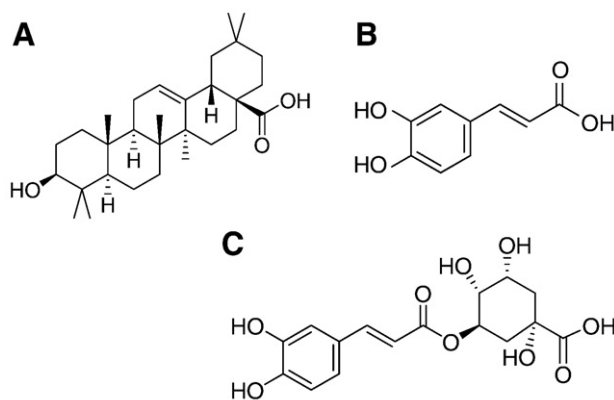


Fig. 1. Chemical structure of tested bioactive substances: A) oleanolic acid, B) caffeic acid and C) 5-caffeoylquinic acid.

Caffeic acid has been shown to be an inhibitor lipoxygenase, which forms leukotrienes from arachidonic acid. This function has been useful to elucidate the roles of leukotrienes in various inflammatory responses. Caffeic acid is also able to protect skin cells when exposed to ultraviolet (UV) radiation [16–18].

Chlorogenic acids (Fig. 1B) are a family of esters formed between certain trans cinnamic acids and (–)-quinic acid and are major phenolics compounds in coffee, strawberries, pineapple, apple, sunflower, blueberries. 5-caffeoylquinic acid (5-CQA) is the only chlorogenic acid commercially available

and has been extensively studied due to its antioxidant activity. Chlorogenic acids are free radical and metal scavengers; may interfere with glucose absorption and has been shown to modulate gene expression of antioxidant enzymes, among other biological activities [15,16].

Ursolic acid and its isomer, oleanolic acid (Fig. 1C), are triterpenoid compounds found across the vegetal kingdom that have anti-inflammatory, anti-arthritic, cytostatic and anti-proliferative effects, hepato-protective effects in mice, and membrane stabilizing properties [19–22]. These compounds are reported to interfere with cholesterol metabolism and to

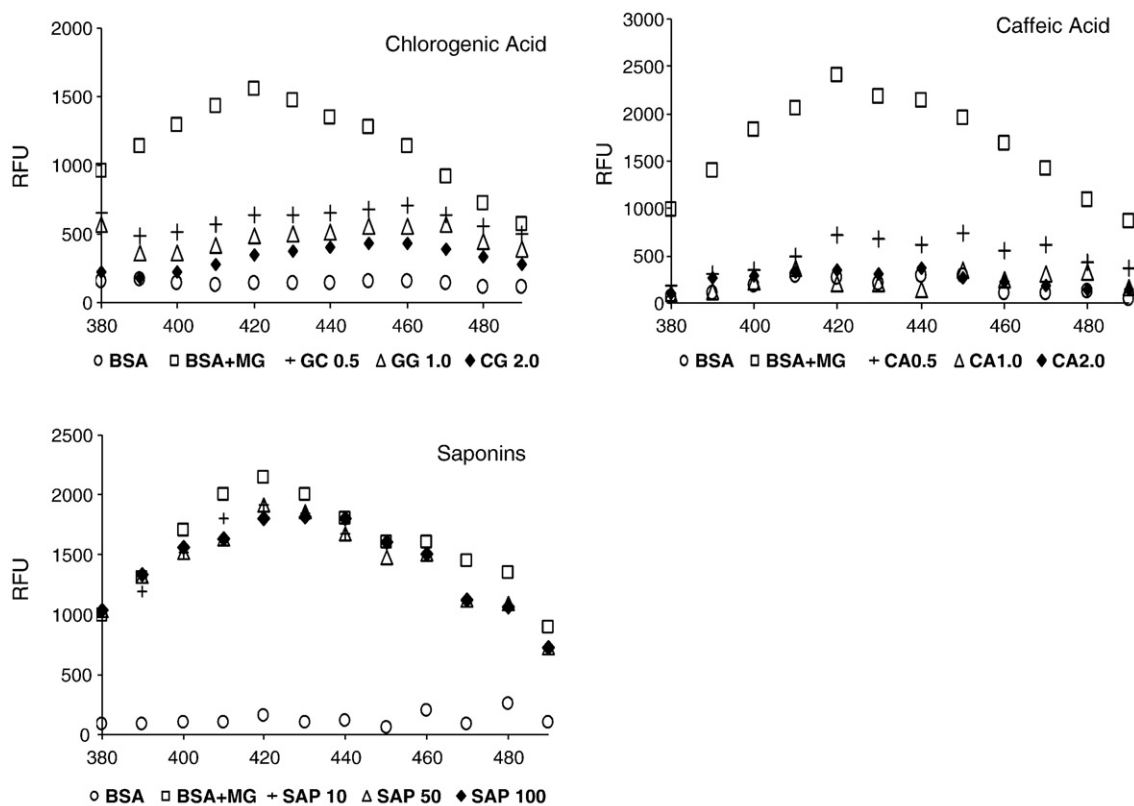


Fig. 2. Fluorescence profile of AGE generated during the incubation of BSA with methylglyoxal for 72 h at 37 °C in the presence or absence of 5-caffeoylquinic acid, caffeic acid or oleanolic acid. The figures show a typical experiment out of 3.

delay the intestinal absorption of dietary fat via inhibition of pancreatic lipase activity [23,24]. Recently oleanolic acid has been noted for its anti-tumoral effect [25]. Oleanolic acid and ursolic acid are relatively non-toxic, and have been used in cosmetics and health products.

The present study concentrates on the evaluation of the anti-glycation capacity of some bioactive substances that are major components of maté tea water extracts: the polyphenols 5-caffeoylquinic acid (chlorogenic acid) and caffeic acid and a sapogenin (oleanolic acid). We employed two different proteins models: bovine serum albumin and histones. Fig. 1 shows the chemical structures of these compounds.

## 2. Experimental

### 2.1. Reagents

All chemicals reagents were of analytical grade. 5-caffeoylquinic acid, caffeic acid, oleanolic acid, bovine serum albumin (BSA) and fetal calf thymus histones (type II S) were purchased from Sigma (St Louis, MO).

### 2.2. Glycation protocol

The glycation protocol was that described earlier [9]. BSA was dissolved in 10 mmol/L PBS buffer, pH 7.4 containing 150 mmol/L NaCl and 0.01% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> to a final protein concentration of 1 mg/mL. It was incubated in the presence or absence of methylglyoxal (5 mmol/L final concentration) at 37 °C for up to 4 days. The times and concentrations were chosen following the data from our previous published work [9,26]. Samples were incubated in the presence or absence of caffeic acid, chlorogenic acid and oleanolic acid in different concentrations or 10 mmol/L aminoguanidine, the standard anti-glycation compound. After incubation, samples were dialyzed against 10 mmol/L PBS buffer, pH 7.4 containing 150 mmol/L NaCl and kept frozen at -80 °C until analysis.

The same protocol was repeated for histones, except that methylglyoxal final concentration was 1 mmol/L. The rationale for this is that histones are very rich in lysine and arginine (preferentially bound by methylglyoxal) and quickly form dimers and polymers when glycated [26]. AGE fluorescence spectra were determined at  $\lambda_{exc}$  340 nm using a SPECTRAMax Gemini XPS spectrofluorometer with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA, USA) [4,9].

### 2.3. Analysis of protein conformation changes by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Electrophoresis was run on 10% gels (reducing conditions). Each lane was loaded with 10  $\mu$ g protein. Equipment used was Mini Gel III from BioRad (BioRad, Hercules, CA). Gels were stained with Coomassie Brilliant Blue. Densitometric analysis was performed after scanning the films using Image J software.

### 2.4. Statistical analysis

Results are expressed as the mean value  $\pm$  SD. For difference analyses, one-tailed *t*-test (unpaired) or paired for the in vitro tests were performed. Differences were considered to be statistically significant if the null hypothesis could be rejected

with 95% confidence. The NCSS statistical software package (NCSS, Kaysville, UT, USA) was employed for all calculations.

## 3. Results and discussion

Fig. 2 depicts the AGE fluorescence spectra after excitation at 340 nm for BSA samples incubated in the presence or absence of MG and inhibitors. Control BSA showed no significant signal. When BSA was incubated with MG, the fluorescence highly increased in the 380–480 nm range. When the compounds under study were added to the reaction media containing BSA and MG or histones and MG, the fluorescence decreased in a dose dependent manner. Caffeic acid was more effective than chlorogenic acid in inhibiting fluorescent AGE generation. Oleanolic acid displayed the weakest anti-glycation activity when compared to the other bioactive compounds.

Fig. 3 shows the effect of each bioactive substance on AGE generation after the incubation of histones or bovine serum albumin (BSA) with methylglyoxal (MG) at 1 mmol/L and 5 mmol/L, respectively during 72 h at 37 °C. Phenolic

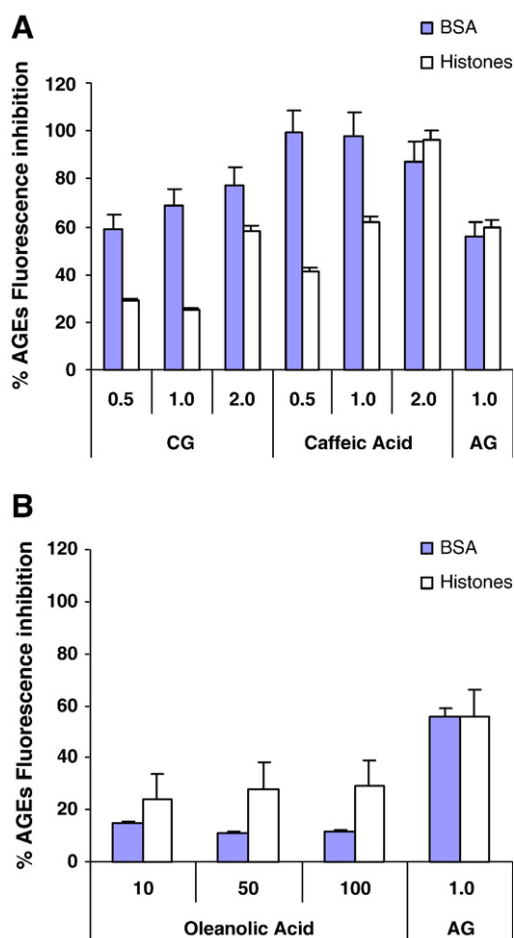


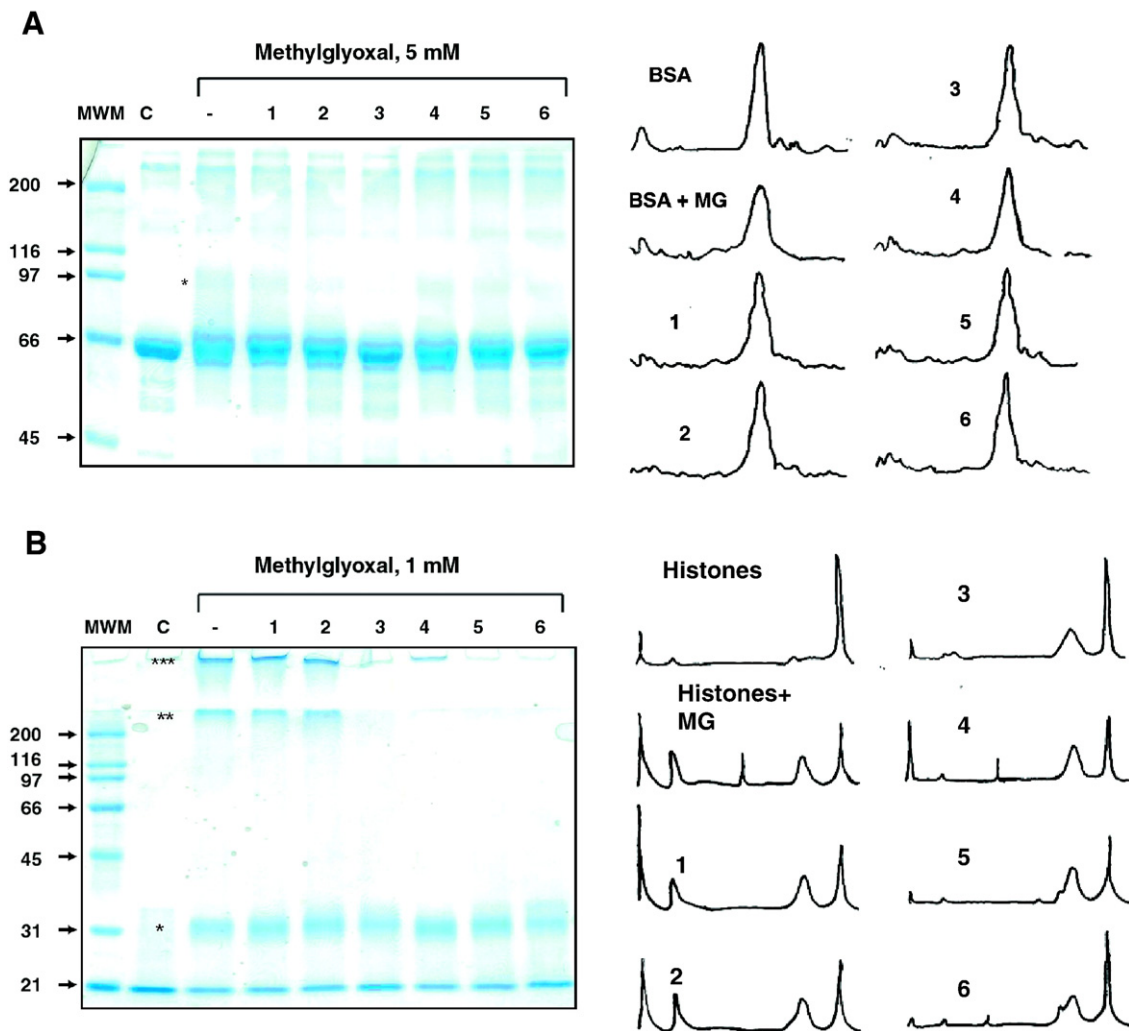
Fig. 3. Percentage AGE inhibition by (A) chlorogenic acid (CG), caffeic acid (CA) at 0.5, 1.0, 2.0 mmol/L or (B) oleanolic acid at 10, 50, 100  $\mu$ mol/L in bovine serum albumin (BSA) and histones incubated with methylglyoxal (MG) (5 mmol/L and 1 mmol/L respectively) in comparison with aminoguanidine (AG) as a standard AGE inhibitor.

compounds were tested at a millimolar concentration range whereas oleanolic acid was tested at the micromolar range, since it is hydrophobic and cannot be dissolved in aqueous medium at higher concentrations. Aminoguanidine (AG) was also tested, as a standard anti-glycation agent at 1.0 mmol/L concentration.

Caffeic acid inhibited more than 95% of the AGEs generation ( $p < 0.0001$ ), at the lower concentration used (0.5 mmol/L), when BSA was the protein present in the model system. When histones were tested, the inhibition was 41% ( $p < 0.0001$ ). Chlorogenic acid (CG) reached 59% of inhibition at 0.5 mmol/L concentration ( $p < 0.0001$ ) for BSA and 29% for histones ( $p < 0.001$ ) as the target proteins, respectively.

Oleanolic acid was more effective in protecting histones than BSA against glycation. Although the protective effect was not as important as that observed for the polyphenols, the inhibition reached 17% (NS) and 24% ( $p < 0.02$ ) for BSA and histones, respectively, at the lowest concentration (10  $\mu\text{mol/L}$ ).

Fig. 4 shows the SDS-PAGE profiles for BSA (Fig. 4A) and histones (Fig. 4B) incubated in the absence (control) or presence of methylglyoxal (MG), and co-incubated with MG and different concentrations of 5-caffeoylquinic acid (5-CQA) or caffeic acid (CA). In both protein models, chlorogenic and caffeic acid displayed a concentration-dependent inhibition of AGE generation. The histone preparation employed contains essentially octameric histones and lacks H1. The band in the control lane corresponds mainly to H2B-H2A, H4 at 14 kDa. MG produces cross-linking of histones to give dimeric forms of an approximate Mr of 30 kDa. MG also induced further aggregation of dimers into high molecular weight polymers, some of which are not able to enter the stacking gel. Histone cross-linking by MG was also inhibited by the addition of 5-CQA in a concentration dependent manner. High molecular weight polymers (over 500 kDa) were almost absent at 5 mmol/L 5-CQA and caffeic acid as compared to the control. MG also produced a crosslink band



**Fig. 4.** SDS-PAGE Coomassie stained gels profile and densitograms of albumin (A) and histones (B) incubated with/without methylglyoxal (5 and 1 mM respectively) in the presence or absence of chlorogenic acid (1,2,3) or caffeic acid (4,5,6) at 0.5, 1 and 2 mmol/L. Densitometric analyses were performed after scanning the gels using Image J software. The figures show a typical experiment out of 3. \*Histone dimers; \*\*high molecular weight histone polymers, \*\*\*very high molecular weight histone polymers.

at 90 KDa in BSA incubations, which was blocked by 5-CQA and caffeic acid.

Since oxidative steps are involved in some of the pathways in glycation, antioxidant compounds present in the diet may act as anti-glycation agents [27]. These substances, at the concentrations commonly found in food are thought to be safe, have no side effects at their achievable systemic levels [28] and could prove to be interesting adjuvants in the treatment diabetes or scaffold structures for the development of anti-glycation drugs. Although the bioavailability of some of these compounds is low, it is not negligible, and sources that contain large amounts and are consumed daily may well be a bioactive source in humans [28,29]. Notably, *I. paraguariensis*, like coffee, contains a high concentration of caffeic acid, mostly esterified as chlorogenic acids. It also contains saponins. Its previously reported potent anti-glycation activity, higher than that of green tea, prompted us to study its individual compounds, which could be used by the pharmaceutical industry in the development of novel anti-glycation drugs.

Many of the compounds found in yerba maté are present in other sources, and therefore, an exploration of this issue can generate data that can be generalized to the other nutritional sources of the compounds.

In this work we measured the glycation of proteins by methylglyoxal, and explored the effects of the putative inhibitors. Endogenously produced carbonyls and dicarbonyls, such as glyceraldehyde and methylglyoxal are involved in numerous pathogenic processes in vivo, including advanced glycation end-product formation. MG is produced in vivo as a byproduct of glycolysis (trioses), from glycation of proteins by glucose, as a product of lipid peroxidation, and from the metabolism of acetone and threonine.

Phenolic acids are among the most important non vitamin antioxidant phytochemicals naturally present in almost all vegetable and fruits. Their biological activity is related to their lipophilicity and is affected by the presence of the ring substituent hydroxyl groups and, in the case of polyhydroxylated phenolic esters, by the length of the ester moiety [30]. Our results show that caffeic acid was more effective as anti-glycation agent than chlorogenic acid in the model systems employed, independent of the protein tested. This behavior could be explained by the differences on either their hydrophobicity or their overall size and polarizability, as already demonstrated by Verna and Hansch [18] in relation to polyphenol antitumor activity. In the BSA model, these phenolic acids were able to inhibit glycation at 0.5 mmol/L concentration in a more efficient way than aminoguanidine (a well known anti glycation agent) at 1.0 mmol/L. There is only one previous publication showing protective effects of chlorogenic acid from the effects of methylglyoxal. In that report the authors showed that the treatment of Neuro-2A cells with phenolic acids suppressed cell apoptosis induced by methylglyoxal [11]. To the best of our knowledge, this is the first work reporting the effect of chlorogenic and caffeic acids in a protein glycation model system.

Steroidal saponins have many biological functions, e.g., anti-carcinogenic, anti-thrombotic, anti-viral, hemolytic, hypocholesterolemic and hypoglycemic. Some saponinins are used as raw material for industrial production of steroidal drugs. Our data show that oleanolic acid, a saponin widespread in nature and present in the ginseng roots, was

a mild anti-glycation agent at micromolar concentrations in the model systems used. Interestingly, this compound showed significant anti-glycation activity only on the histone model. Human albumin (very similar to BSA) has several hotspots for glycation by methylglyoxal which are very exposed on the surface [31]. The histone preparation we employed contains essentially octameric histones that tend to associate. The hydrophobic nature of oleanolic acid might have permitted some dissociation and account for this differential activity. To the best of our knowledge this is the first evidence reporting the anti-glycation activity of isolated saponins or saponinins. The probable mechanism of action might be related to their antioxidant activity [14].

Anti-glycation agents are thought to function as nucleophilic traps for reactive carbonyl intermediates (in our case methylglyoxal) in the formation of AGE. However, alternative mechanisms of actions, such as chelation have been demonstrated for many of them. In previous reports, millimolar concentrations of AGE inhibitors inhibition of AGE formation results primarily from the chelating or antioxidant activity of the AGE inhibitors, rather than their carbonyl trapping activity, and that may well be the case for chlorogenic acids and caffeic acid [27].

Oleanolic acid was evaluated at micromolar concentrations, since it is hydrophobic and precipitates in the reaction media at higher concentration. This compound has low bioavailability and the concentration tested may mimic that actually found in humans.

#### 4. Conclusions

Our results show that chlorogenic acid, caffeic acid, and to a lesser extent oleanolic acid, bioactive compounds present in maté tea, have an anti-glycation effect. In many cases the effect is stronger than the anti-glycation effect of aminoguanidine, a well known anti-glycation agent. Yerba maté has a combination of different classes of substances that may act synergically to explain its potent anti-glycation effect.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fitote.2009.04.007.

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