UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE FARMACOLOGICHE
E BIOMOLECOLARI - DiSFeB
Direttore: Prof. Alberto Corsini

In vitro study on the antibacterial properties of vegetable tanned leathers with natural tannins

This study has been realized by Silvateam S.p.a. in collaboration with

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Abstract

Tannins are high molecular weight polyphenols, naturally synthesized by plants to defend themselves against biotic and abiotic stress factors. Their role as antioxidant, antibiotic and antibacterial agent has been known for many years among agriculture, food, pharma and cosmetics industry.
The antibacterial activity of vegetable tanned leathers with natural tannins, Chestnut, Quebracho and Tara extracts was assessed in comparison with chrome tanned leathers and synthetic materials. The trial was performed *in vitro* by inoculating gram positive (*Staphylococcus aureus) and gram negative* (*Escherichia coli*) bacterial strains.
Vegetable tanned leathers with natural tannins have shown antibacterial activity against all the tested bacteria.

Introduction

Tannins are a complex family of water-soluble polyphenolic compounds, synthesized as secondary metabolites by many plants (Haslam 1981). Tannins are present in numerous types of trees and plants and they can be found in barks, leaves, wood and also in fruits and roots. From a chemical point of view, it is difficult to define tannins due to their heterogeneity in terms of chemical compositions and molecular weight (MW). Traditionally, tannins have been divided into two large groups: hydrolysable and condensed tannins (Haslam, 1996; Scalbert, 1991). Hydrolysable tannins are composed of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids with a MW ranging from 300 to 5,000 Da (Mueller-Harvey and McAllan, 1992). Depending on the substances that are produced following hydrolysis (by acids, basis or certain enzymes), hydrolysable tannins can be classified in gallotannins (yielding gallic acid) or ellagitannins (yielding ellagic acid). Tara pod tannin and Chestnut wood tannin are representative of gallotannins and ellagitannins, respectively. Condensed tannins are oligomers of flavonoids units with a MW ranging from 1,000 to 20,000 Da. Quebracho extract is among the most industrially produced tannin that has been shown to be predominantly composed of oligomers of profisetinidins.

The chemical composition of tannins could be explained taking as representative three of the most industrially processed tannins: chestnut tannin, quebracho tannin and tara tannin.
Chestnut tannin is an ellagic-type hydrolysable tannin according to MALDI-TOF mass spectrometry (Pasch, 2002). Castalagin is the key building block of the chestnut tannin (Fig. 1).

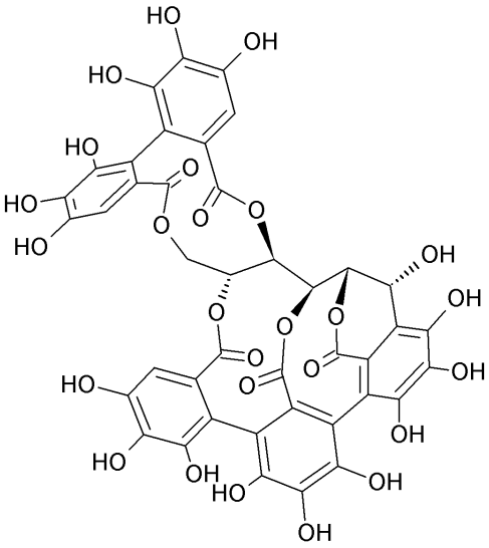

Fig. 1: Castalagin is the main representative substance present in the chestnut tannin.

Castalagin with the isomer vescalagin represent around 30% of the product. It has been shown that these substances and their higher oligomers are present in this tannin and are quite stable because they are coming from the rearrangement of polypentagalloylglucose naturally occurring in the chestnut wood (Pash and Pizzi, 2002). The higher oligomers contain repeating units of polygalloylglucose chain where galloyl groups can be linked differently to each other (Pizzi *et al.*, 2009). The chestnut tannin has been found to be composed also of digalloyl glucose, glucose and gallic acid (Radebe *et al.*, 2013).

The composition of tara tannin has been shown to be based on a polymeric structure where monomers are esters of gallic acid on a core of quinic acid. The predominant structure is a pentagalloyl quinic acid (Giovando *et al.*, 2013). The tannin contained in tara pods, after extraction and purification, is known as tannic acid according to Food Chemical Codex (FCC, 2003). Tara tannin could be represented by the structure in Fig 2.

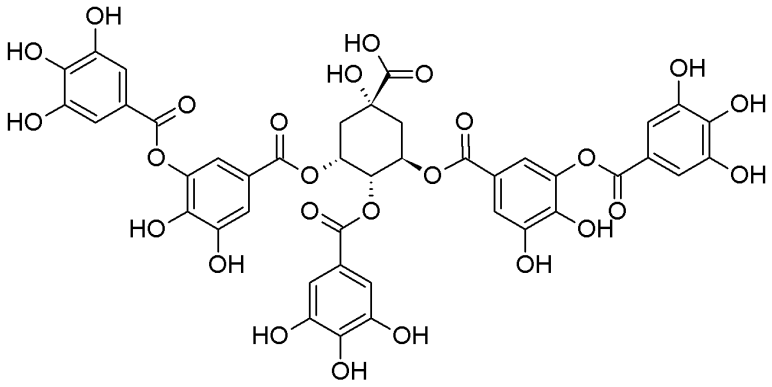

Fig. 2: Pentagalloyl quinic acid, the predominant structure in the tara tannin.

The molecular composition of quebracho tannin is based on polyflavonoids of the profisetinidins type (Pasch, 2001). More researches have reported (Venter, 2012 and Pasch, 2001) that quebracho tannin is a mixture of polyanthocyanidins oligomers consisting of linear structures that could be represented by the trimer shown in Fig. 3.

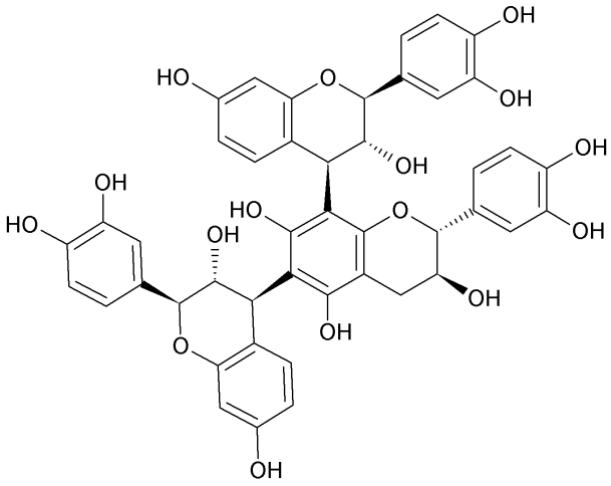

Fig. 3. Fisetinidin trimer is the main structure contained in the quebracho tannin.

The extraction of tannins is one of the first industrial activities related to nature. In fact, tannins have been extracted from the beginning of the industrial era for several applications, starting from inks manufacturing and silk dying to leather tanning and wine stabilization. During the last two centuries several botanical species have been classified and proposed to be used as raw material, but only few have kept this role up to now. Chestnut, quebracho and tara are among these raw

materials. The availability and the tannin content of these plants are key factors to produce cost effective and sustainable extracts.

Normally the production process consists of a hot-water extraction, like making tea or coffee, followed by some purification steps and spry-drying to obtain a product in powder form. Natural tannins act by inhibiting the growth of many microorganisms including bacteria, yeasts and fungi (Landete, 2011). In fact, tannins are the main protection agents against bacteria in the plant kingdom. Their antimicrobial properties have raised particular interest and have been exploited in several fields such as food science and animal nutrition. Indeed, tannins have been used in the food industry as additives, natural colourants and preservatives (Panzella and Napolitano, 2017). More recently tannins are replacing the use of antibiotics in livestock production to enhance animal health and performance due to their ability to improve feed digestion and to protect from microbial caused intestinal disease (Frutos *et al.*, 2004).

The abundance of tannins in nature together with their chemical and biological properties and their ability to form complexes with proteins (mainly) and polysaccharides (Spencer *et al.*, 1988) has led to their widespread use in the leather industry.

Tannins bind to the collagen proteins inside the hide and coat them, causing them to become less water-soluble and more resistant to bacterial attack (Hagerman *et al.*, 1981).

This process also causes the hide to become more flexible. Only tannins are able to impart to tanned leather these unique characteristics that make them so special and distinguishable. The “smell of leather” is something typical and unique.

In today’s leather making processes, tanners use vegetable tannins in both liquid and powder form. The most famous and ancient extract is obtained from the chestnut wood and is mainly used for the production of sole leathers, with high yield in weight, which are compact, firm and flexible, and the retanning of chrome-free leathers. Another tannin comes from the quebracho wood, a tree that grows in the Chaco region in Argentina. In this case the extract imparts to the leathers an unmistakable reddish shade, a warm touch and a bright appearance, required characteristics to make leather goods. In addition to chestnut and quebracho, Tara tannin is mainly used within the car and upholstery industries. This extract provides to the leathers excellent properties, such as fullness, softness, good resistance to light and to heat.

Tannins can also be used in the retannage of chrome tanned leathers to improve their "cold, impersonal feel" characteristics, by imparting the typical warm and natural feeling of vegetable tanned leathers.

Today a retreat towards more sustainable lifestyle and a slowdown to the use of chemicals, favoured by various deep changes in research, make tannins an important natural component in the tanning industry.

Leather has been used since ancient times to make clothes, shoes and other types of clothing that can be worn in direct contact with human skin. To the human skin is associated a complex commensal microbial flora which plays a protective role (Bojar and Holland, 2002). Some body parts such as feet and armpits, exhibiting increased temperatures and glandular secretions (e.g. sweat), support the growth of bigger bacterial populations compared to other body parts. In these areas, microbes belonging to the *Staphylococcus* genus and a few species belonging to *Brevibacterium*, *Micrococcus* and *Kytococcus* genera are thought to be responsible for the biotransformation of odourless glandular secretions into bad smelling compounds (Natsch *et al.*, 2006; Ara *et al.*, 2006; James *et al.*, 2013).

The purpose of the study was the evaluation of the tannin antibacterial activity against the microbes even after being included in a leather during the tanning process. This activity was assessed in leather articles made with natural tannins in comparison with synthetic materials and chrome tanned leathers.

The use of leather tanned with vegetable tannins, due to their antibacterial properties, may offer a solution to prevent or decrease the development of unpleasant odours caused by the microbial fermentation of body secretions in feet.

Material

Shoe lining and insole includes:

1. Synthetic lining

   The lining tested is made of PU and textile hybrid material with high poromeric, absorbent and breathable properties and with a resin / fiber ratio of 1:1. The lining weighs 200 g/sqm and has a thickness of 0.7 mm. The lining has been provided by a shoe manufacturing company and it is one of the most commonly used in the sector.

2. Chrome tanned leather for lining and insole

   Standard chrome tanning with 2% Cr_2O_3 was carried out on South German steer hides, pickled to pH 3.0 and basified to pH 3.8 at equilibrium. The wet blue obtained was shaved to 1.0 mm for lining and 2.0 mm for insole, neutralised to pH 4.8 and fatliquored with a blend of sulphated and sulphited natural and synthetic oils, at a dosage of 6%. No bactericides nor anti-mould agents were used in the process.

3. Chrome tanned leather retanned with natural tannins for lining and insole

   The standard chrome tanned leather previously obtained was retanned with the addition of 10% of a powder blend made of chestnut and quebracho tannins. The retannage with natural tannins was carried out between neutralising and fatliquoring.

4. Vegetable tanned leathers using different tannin materials for lining and insole

   Standard vegetable tanning was carried out on South German steer hides, pickled to pH 3.5 and tanned with 30% of a natural tannin in powder form (without any pretannage).

   Three types of fully vegetable leathers were prepared using different tannins:

   - Chestnut
   - Quebracho
   - Tara

   All leathers were fatliquored in the same way as chrome tanned leathers, using a blend of sulphated and sulphited natural and synthetic oils, at a dosage of 6%. No bactericides nor anti-mould agents were used in the process.

Bacterial strains and growth conditions.

The bacteria used in these trials are: *Escherichia coli* (ATCC 47066) and *Staphylococcus aureus* (ATCC 6538P), representing Gram-negative and Gram-positive bacteria, respectively. The two bacterial strains were grown aerobically at 37°C in LB-Lennox broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) (Difco). LB plates were prepared from LB broth by adding 15 g/L of agar (Difco).

Methods

A standard test was carried out to assess the antimicrobial activity of leaching materials (Shake Flask Method).

Stationary phase cultures were diluted 100 fold in fresh LB medium and bacteria were aerated up to Optical Density at 600nm (OD_{600}) of 0.4 corresponding approximately to 10^8 colony forming unit (CFU) per ml. Bacteria were then diluted in 25 ml of fresh LB medium to obtain a concentration of 10^6 CFU/ml. The samples to be tested (25 mm of diameter) were added to each culture. A culture with no leather or synthetic sample (cells only) was used as negative control. Cultures were incubated and aerated at 37°C for 6 hours. At the start of the test t=0 (t_0) and at t=6h (t_{6h}) 0.1 ml samples were withdrawn from each culture to determine viable bacteria (CFU/ml). Viable bacteria were determined by performing serial dilutions and plating them onto LB plates. Plates were incubated at 37°C for 18h and colonies enumerated.

The percent of bacterial reduction (%R) was calculated as follows:

%R = (A-B)/Ax100, where A = CFU/ml at t_0 and B = CFU/ml at t_{6h}.

To assess the antimicrobial activity of non-leaching materials the standard test (AATCC 100) was slightly modified due to the different absorption properties of the specimens under analysis.

Stationary phase cultures were diluted 100 fold in fresh LB medium and bacteria were grown and aerated up to OD_{600} of 0.4 corresponding approximately to 10^8 CFU/ml. Bacteria were then diluted in fresh LB to obtain a concentration of approximately 10^6 CFU/ml. Each sample (25 mm of diameter) was placed in a sterile Petri dish and 0.6 ml of the diluted bacterial suspension was deposited onto the sample surface. As a control a drop of 0.6 ml (Cells only) was placed in an empty sterile Petri dish. Each sample was analysed as a quadruplicate. At the start of the test t=0 (t_0) one of the four samples was placed in a 50 ml sterile tube and 10 ml of a 0.9% NaCl neutralising solution was added. The sample was vigorously agitated for 10 minutes to remove adhered bacteria. Viable bacteria were determined by performing serial dilutions and plating them onto LB agar plates. The remaining three samples were incubated for 6 hours at 37°C. At t=6h (t_{6h}), each sample was placed in 50 ml sterile tube and 10 ml of 0.9% NaCl neutralising solution was added. The samples were vigorously agitated for 10 minutes to remove adhered bacteria. Viable bacteria were determined by performing serial dilutions and plating them onto LB plates.

The percent of bacterial reduction (%R) was calculated as follows:

%R = (A-B)/Ax100, where A = CFU/ml at t_0 and B = CFU/ml at t_{6h}.

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Results and discussion

The antibacterial activity was assessed during all trials on the following samples

- Synthetic lining (SL)
- Chrome tanned leather (CTL)
- Chrome tanned leather retanned with natural tannins (CTL retanned NT)
- Vegetable tanned leather with Chestnut tannin (VTL Chestnut tannin)
- Vegetable tanned leather with Quebracho tannin (VTL Quebracho tannin)
- Vegetable tanned leather with Tara tannin (VTL Tara tannin)

Initially, it was assessed whether the treated samples showed antibacterial activity due to release of free substances from the material itself. For this purpose, a standard test was carried out to evaluate the antibacterial activity of leaching materials (Shake Flask Method).

As shown in Table 1, none of the tested leather samples presents antibacterial activity against *E. coli* which was used as a model organism. No bacterial reduction (%R) was observed after 6 hours of treatment.

Table 1. Evaluating the antimicrobial activity of leaching specimens

	CFU/ml		
Sample	**t_0[1]**	**t_{6h}[2]**	**% R**
Cells only	$4x10^6$	$4x10^{10}$	0%
SL	$3x10^6$	$2.1x10^9$	0%
CTL	$3.2x10^6$	$3.9x10^9$	0%
CTL retanned NT	$4.1x10^6$	$5x10^9$	0%
VTL Chestnut tannin	$2x10^6$	$1x10^{10}$	0%
VTL Tara tannin	$5x10^6$	$7x10^9$	0%
VTL Quebracho tannin	$5x10^6$	$2x10^9$	0%

[1] t_0, viable bacteria expressed as CFU/ml measured at t=0
[2] t_{6h}, viable bacteria expressed as CFU/ml measured after 6 hours of incubation

Later, the evaluation of the antibacterial activity of the different samples were assessed by using the standard test (slightly modified as described in the Material and Methods) for non-leaching materials. The antibacterial activity was tested at two different time points (3h and 6h) following contact with the bacterial cells. The antimicrobial activity was considered efficient when observing at least 1 log reduction in bacterial viability (%R ≥90%).

As shown in Table 2, vegetable tanned leather with chestnut tannin (VTL Chestnut tannin) showed a fast and very strong antibacterial activity with a 96% of bacterial reduction (%R) already after 3 h of contact with bacterial cells. The antibacterial activity of vegetable tanned leather with Tara and Quebracho (VTL Tara tannin and VTL Quebracho tannin) was not as fast as the vegetable tanned leather with chestnut (VTL Chestnut tannin) but was nevertheless very strong after 6 h of contact with bacterial cell.

In the case of chrome tanned leathers the antibacterial effect is nil, but the same material, if properly retanned with natural tannins (CTL retanned NT), has an excellent activity, with a 2 log reduction after 6 hours.

Table 2. Evaluating the antimicrobial activity of non-leaching specimens against *E. coli* at two different time points

	CFU/ml			%R	
Sample	**t_0**	**t_{3h}**	**t_{6h}**	**3h**	**6h**
Cells only	$7x10^6$	$1.1x10^8$	$1.9x10^9$	0%	0%
SL	$2.5x10^6$	$3.9x10^8$	$4.8x10^8$	0%	0%
CTL	$6.7x10^6$	$3.2x10^6$	$4.5x10^6$	52%	33%
CTL retanned NT	$4.8x10^6$	$7.8x10^5$	$5.1x10^4$	84%	99%
VTL Chestnut tannin	$1.6x10^3$	$7x10^1$	$<1x10^1$	96%	100%
VTL Tara tannin	$1x10^5$	$1.2x10^5$	$3.5x10^1$	0%	100%
VTL Quebracho tannin	$1x10^6$	$4.5x10^5$	$6x10^3$	58%	99%

[1] t_0, viable bacteria expressed as CFU/ml measured at t=0
[2] t_{3h}, viable bacteria expressed as CFU/ml measured after 3 hours of contact
[3] t_{6h}, viable bacteria expressed as CFU/ml measured after 6 hours of contact
The values reported are the mean of two replicates

Based on these initial results it was decided to evaluate the antibacterial activity of the samples only after 6 h of contact with bacterial cells.

Successively, the antibacterial activity was assessed against *Escherichia coli*, representative of Gram-negative bacteria, and against *Staphylococcus aureus*, representative of Gram-positive bacteria.

Each sample was analysed in triplicate in two independent experiments. The antibacterial activity of different samples against *E. coli* is shown in Table 3.

Table 3. Evaluating the antimicrobial activity of non-leaching specimens against *E. coli*

	CFU/ml		
Sample	**t_0[1]**	**t_{6h}[2]**	**%R**
Cells only	$6.5x10^6$	$2.7x10^9$	0%
SL	$2.5x10^6$	$2.2x10^9$	0%
CTL	$8.7x10^6$	$4.8x10^6$	44%
CTL retanned NT	$7.7x10^6$	$1.9x10^5$	98%
VTL Chestnut tannin	$1.2x10^5$	$<1x10^2$	100%
VTL Tara tannin	$1.1x10^6$	$1x10^2$	100%
VTL Quebracho tannin	$2.7x10^6$	$9x10^2$	100%

[1] t_0, viable bacteria expressed as CFU/ml measured at t=0
[2] t_{6h}, viable bacteria expressed as CFU/ml measured after 6 hours of contact
The values reported are the mean of six replicates

All vegetable tanned leather samples (VTL Chestnut, Quebracho and Tara) showed an excellent antibacterial activity against *E. coli*, as witnessed by a 4 log reduction in bacterial viability following 6 hours contact. On the opposite, no antibacterial activity was observed for the synthetic lining (SL) nor for chrome tanned leather sample (CTL). Interestingly, the chrome tanned leather retanned with natural tannins (CTL retanned NT) presented a good antibacterial activity, with more than 1 log reduction in bacterial viability following 6 hours contact.

The antibacterial activity of the above samples was also evaluated against the Gram-positive organism *S. aureus*. However, by applying the modified standard procedure (AATCC 100) it was not possible to recover bacteria at t_0 for the following samples: chrome tanned leather retanned with vegetable tannins (CTL retanned NT) and vegetable tanned leather with Chestnut, Quebracho and Tara tannins (VTL Chestnut, Quebracho and Tara). The inability to recover bacteria at t_0 was specific for the *S. aureus* bacterial suspension. In fact, as shown in Table 4, when the same volume (0.6 ml) of a *S. aureus* or of an *E. coli* suspension was deposited onto two VTL Chestnut tannin samples, it was possible to recover the *E. coli* bacterial suspension but not the *S. aureus* one.

Table 4

Bacterial suspension	CFU/ml	Bacterial suspension + sample	CFU/ml at t_0
E. coli	$1.1x10^6$	*E. coli* + VTL Chestnut tannin	$5.7x10^5$
S. aureus	$5x10^6$	*S. aureus* + VTL Chestnut tannin	$1x10^2$

These results strongly suggested that the antibacterial activity of vegetable tanned leather was stronger and faster on Gram-positive compared to Gram-negative bacteria.

Based on these results, the AATCC 100 procedure was modified when analysing the antibacterial activity against *S. aureus* for the vegetable tanned leather with Chestnut, Quebracho and Tara tannins (VTL Chesnut tannin, VTL Quebracho tannin and VTL Tara tannin). The concentration of the initial bacterial suspension was used to calculate the percent of bacterial reduction (%R*). The results are reported in Table 5.

Table 5. Evaluating the antimicrobial activity of non-leaching specimens against *S. aureus*

Sample	CFU/ml		%R	%R*
	t_0[1]	t_{6h}[2]		
cells only	$2x10^6$	$9x10^9$	0	
SL	$1.3x10^6$	$7x10^9$	0	
CTL	$2.1x10^5$	$<1x10^1$	100%	
CTL retanned NT	$2x10^4$	$<1x10^1$	100%	
VTL Chestnut tannin	$<1x10^1$	$<1x10^1$		100%
VTL Tara tannin	$<1x10^1$	$<1x10^1$		100%
VTL Quebracho tannin	$<1x10^1$	$<1x10^1$		100%

[1] t_0, viable bacteria expressed as CFU/ml measured at t=0
[2] t_{6h}, viable bacteria expressed as CFU/ml measured after 6 hours of incubation
The values reported are the mean of six replicates
%R* is calculated considering as t_0 value the concentration of the initial bacterial suspension.

The vegetable tanned leather samples (VTL Chestnut, Quebracho and Tara tannins) again displayed an excellent antibacterial activity against *S. aureus* as witnessed by the very fast bacterial killing with a 6-7 log reduction in bacterial viability already at t_0. The chrome tanned leather (CTL) and the chrome tanned leather retanned with natural tannins (CTL retanned NT) samples both showed a very good antibacterial activity following 6 hours contact, as observed against *E. coli*.

Conclusions

This work shows that tanning of leather with chestnut, tara and quebracho tannins leads to materials with excellent antibacterial activity, especially against Gram-positives bacteria. All leathers tanned with the vegetable tannins act as non-leaching antimicrobial material, a property that makes these natural substances suitable for the contact with human skin. Therefore, the use of leather tanned with vegetable tannins may offer a solution to prevent or decrease the development of unpleasant odours caused by the microbial fermentation of body secretions in feet.

On the other hand, chrome tanned leather has a partial antibacterial property, only against *S. aureus* a representative of Gram-positive bacteria.

The same chrome tanned leather, if retanned with natural tannins, reaches a complete antibacterial activity against all tested bacteria.

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