

# EXTRACTION OF MULBERRY FLAVONOIDS FOR INDUSTRIAL AND PHARMACEUTICAL USES

Andreoni N.<sup>1</sup>

<sup>1</sup>Dipartimento di Chimica e Biotecnologie Agrarie. Facoltà di Agraria. Università degli studi di Pisa, via del Borghetto 80, 56124 Pisa, Italy.

## ABSTRACT

Mulberry tree, a plant of the family of *Moraceae* and genus *Morus*, has been widely cultivated to feed silkworm for the production of silk. Until the past century, in Italy the mulberry cultivation was ruled by regulations which forbade to cut down this plant. But, after the coming in the market of the synthetic textile fibres, the silk production diminished and so the cultivation of mulberry was almost forgotten. At present, it can be found as ornamental or wild plant in marginal areas. Nevertheless, other utilizations, beyond silkworm feeding, are possible for mulberry. For example, leaves could be used for the preparation of protein concentrates for animal or human nutrition and for extracting compounds with either pharmaceutical activity or textile fibres colouring ability.

This paper describes experiments carried out for the extraction of flavonoids from mulberry, the major responsible compounds for the claimed therapeutic benefits ascribed to mulberry in the folk medicine, in order to study if the recovery of such substances, together with other products, can make suitable the growing, management and processing of mulberry and so allow the conservation of this historical plant.

## INTRODUCTION

There is evidence that domestication, selection and improvement of Mulberry started about 5,000 years ago in China together with sericulture. In Italy, until the past century, the mulberry cultivation was ruled by regulations which forbade to cut down this plant; but, after the coming in the market of the synthetic textile fibres, the silk production diminished and so the cultivation of mulberry was almost forgotten. At present, it can be found as ornamental or wild plant in marginal areas. Wild or cultivated mulberry varieties are spread in countries all over the world from temperate to tropical areas, from sea level to altitudes of 4,000 m and from humid tropics to semi arid lands (FAO, 1990; Tipton, 1994). Beyond silkworm feeding, depending on regions, mulberry is also appreciated for its fruit (fresh, in juice, as preserve), for its medicinal properties (mulberry leaf tea), for landscaping, as a vegetable (leaves and young stems) and as a feed for ruminants and others animals (Zepeda, 1991; Bellini *et al.*, 2000). Due to high percentages of crude protein (15 – 25 %) and in vitro dry matter digestibility (75 – 85 %), together with perennial nature and adaptation to various soil types, mulberry leaves appear an excellent forage for feeding and supplementing ruminants. In fact, there are several places where mulberry leaves are used traditionally as a feed in mixed forage diets for ruminants and there have also been several studies on the use of mulberry for cows and other domestic animals (Sánchez, 2000). A group of nutritional scientists in India has suggested that the powdered leaves of white mulberry (*Morus alba* L.) might make a good, nutritious, non toxic and low cost food ingredient for *paratha*, the traditional food item of breakfast and dinner of the Indian diet (Scrivastava *et al.*, 2003). Moreover, in Korea and in Japan mulberry fruit and leaves are used as functional foods. In Korea is actually made an ice-cream, with agreeable taste, containing powder of mulberry leaves as an ingredient, which has shown to reduce the blood glucose level, instead of rising, as it could be expected for a food with a high sugar content (Kim *et al.*, 1999). The

blood serum glucose reducing is only one of the healthy properties attributed to mulberry already in the old Chinese herbal medicine, from the old Latins and in the folk medicine (Andallu *et al.*, 2001). Other examples are the lowering men's blood cholesterol and lipids levels, fighting arterial plaques and antiphlogistic, diuretic and expectorant effects (Andallu and Varadacharyulu, 2003; Doi *et al.*, 2000; Jang *et al.*, 2002).

Many of these properties have been proved by clinical studies and various compounds present in mulberry (flavonoids, alkaloids, steroids), responsible of such therapeutic benefits, have been also recognized (Asano *et al.*, 2001; Cheon *et al.*, 2000; Doi *et al.*, 2001; Kim *et al.*, 1999; Nomura, 1999). Nevertheless, just these substances can limit the use of mulberry leaves as a food or food ingredient, especially for those people affected by those pathologies which mulberry extracts can help to control, due to interactions with other medicines or to physiological intolerance. Therefore, in order to use mulberry leaves as animal feed or safe human food source, it is necessary to remove these substances. If, by one hand, it rises the processing costs, on the other hand, it could be an advantage by the recovery of such substances, for which industrial (for example as natural dyeing) or medical uses, under controlled conditions, can be hypothesized.

This paper describes experiments carried out for the extraction of flavonoids from mulberry leaves, believed the major responsible for the claimed therapeutic benefits attributed to mulberry in the folk medicine. The aim of this study was to find a process, easily practicable without the use of toxic solvents, for the recovery both of the flavonoids and of the extracted leaf material, to be used as potential food source. In order to evaluate the suitability of the process, the aqueous extracts from leaves were also essayed for dyeing power of textile fibres.

## MATERIALS AND METHODS

Two samples of 0.3 g of fresh, chopped leaves of white mulberry (*Morus alba* L.) were added to 5 ml of water containing 0,5 % sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ). The former sample was immediately boiled for 15 minutes and the latter was let stand, protected from air, for a day and then submitted to same treatment. From each one of the two samples, after centrifugation, were collected four 1 ml fractions of a cloudy aqueous phase.

For qualitative evaluation of the dyeing ability, three fractions of the aqueous extracts, diluted with water to 3 ml, were separately heated at 90 °C for 20 minutes with pieces, 10 cm long, of a white wool thread, respectively treated with solutions of  $\text{Al}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Sn}^{4+}$  as mordants.

For the separation of flavonoids, the remaining fraction of each one of the above mentioned two extracts, diluted with 0.1 N HCl, was eluted through a  $\text{C}_{18}$  column (Isolute, Stepbio Bologna, Italy) activated with ethanol and conditioned with 0,1 N HCl. After washing the column with water, the adsorbed apolar compounds were extracted with 3 ml of ethanol. 2.5 ml of this alcoholic solution were eluted through a cation exchange column SCX-2 (Isolute, Stepbio) activated with alcohol and saturated with  $\text{Al}^{3+}$  ions (Andreoni, 2000; Andreoni e Pereira, 2003). After washing the column with alcohol until phenolic substances were detectable in the eluate, the adsorbed presumed flavonoids were extracted with HCl 1 N. The obtained acid eluate was passed through a  $\text{C}_{18}$  column, which was washed with water until neutrality of the eluate and, finally, extracted with alcohol. The phenols eluted from the SCX-2 column with ethanol and those retained were qualitatively compared by the spots obtained on silica gel chromatographic sheets (Merck) eluted with the mixture ethyl acetate/ethanol/chloroform acetic acid/water = 5.5/3/1.5/1/1, dipped in a diluted Folin-Ciocalteu reagent (Singleton e Rossi, 1965) and, lastly, exposed to ammonia vapours in a closed jar. Flavonoids were recognized on similar chromatographic sheets by the yellow-green fluorescence after dipping in an alcoholic 5 % aluminium chloride solution (Stahl, 1969).

## RESULTS AND DISCUSSION

By the simple treatment of mulberry leaves for a few minutes with boiling water was obtained an aqueous cloudy yellow-brown mixture containing mainly phenolic substances together with other compounds. Such aqueous extract gave positive results in the colouring essays. The obtained colours were respectively yellow, yellow-clear, brown-grey on the wool treated with  $\text{Al}^{3+}$ ,  $\text{Sn}^{4+}$ ,  $\text{Fe}^{3+}$  as mordants.

By eluting a fraction of this aqueous extract through a  $\text{C}_{18}$  column and then by extracting with ethanol, a clear alcoholic solution of mulberry phenols was obtained. The subsequent elution through the strong cation exchange column SCX-2, saturated with ions  $\text{Al}^{3+}$ , allowed the separation of flavonoids (adsorbed on this column) from other phenols and other compounds (released by the column). After the extraction from the SCX-2 column with HCl IN, flavonoids were obtained in an alcoholic solution by the use of a  $\text{C}_{18}$  column. These results were confirmed by TLC (Thin Layer Chromatography). In fact, on the chromatographic sheets blue fluorescent spots were observed under UV light, due to phenolic acids, in the case of phenols released by the SCX-2 column, and green-yellow fluorescent spots, after treatment with  $\text{AlCl}_3$ , characteristic of flavonoids (Ribéreau-Gayon, 1969), in the case of phenols retained by this column. The separation of flavonoids by the elution through the cation exchange column saturated with  $\text{Al}^{3+}$  ions was possible because these substances form complexes with such ions (Ribéreau-Gayon, 1968; Markham, 1975). Similar interactions are possible also when these cations are situated in exchange places of cation exchange resins, provided that there are no conditions (as for example the contact with high ionic strength solutions) which remove the ions from exchange sites (Andreoni e Pereira, 2002). So, by passing through such a column an alcoholic solution of phenols, due to high eluting power of ethanol, all these substances were released, except flavonoids, owing to strong complexes of these compounds with aluminium ions situated in exchange places of the SCX-2 resin. On the contrary, by passing the HCl solution through the column, both aluminium ions and flavonoids were released, because of the removal of metal ions from the exchange places of the resin and the dissociation of the complexes of phenols with  $\text{Al}^{3+}$  ions.

The adsorption of flavonoids on cation exchange resins saturated with  $\text{Al}^{3+}$  ions can be considered similar to dyeing textile fibres by the use of metal ions as mordants fixed on the fibres. So by complexing colouring substances (mainly phenols) with these cations, such substances remain fixed on the fibers. The dyeing power resulted higher in the case of sample let stand with water before boiling, presumably because of hydrolysis of glycosides of flavonoids and other phenols by the endogenous enzymes with the production of free phenols, which could better interact with the mordant fixed on the wool. It suggests that in order to obtain, from mulberry leaves, an extract suitable for dyeing, it may be better to let stand the chopped material and water for some hours so as to allow the hydrolytic enzymes to act, whereas, in order to obtain phenols to be investigated or used for pharmacological purposes it may be better to inactivate hydrolytic enzymes.

From the results of this research it can be concluded that mulberry leaves could receive adequate attention as food ingredient even for human nutrition and that the treatment with boiling water could be a possible way for obtaining more suitable food source by the reduction in content of bioactive compounds, which, on the other hand, could be recovered for potential industrial or medical utilizations. Moreover, the experimented new separation method of flavonoids by the use of cation exchange columns saturated with aluminium ions suggests further separations and easier recognition of bioactive components of mulberry.

## ACKNOWLEDGEMENTS

This research was supported by Fondi di Ateneo (ex 60%) of Pisa University.

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