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New Collagen Fibril Formation and Thermal Stability Activator based on Phytogenic Polyphenols

Keywords: taxifolin, *Larix Sibirica Wood Extract*, fibril formation, collagen, flavonoids, antioxidant

Abstract

A study has been performed of the effect of the bioflavonoid complex *Larix Sibirica Wood Extract* upon type I collagen fibrils' structure and thermal stability. It is shown that *Larix Sibirica Wood Extract* accelerates fibril production, with a characteristic periodic cross striation (banding) of collagen fibrils being restored. Differential scanning calorimetry data are indicative of increased melting temperature in collagen fibrils forming in neutral or slightly alkaline conditions, but not in individual tropocollagen molecules found in acid conditions. It is suggested that some of the aspects of *Larix Sibirica Wood Extract's* biological activity can be attributed to its ability to accelerate fibril production processes and contribute to collagen fibrillar form stability and these particular capabilities of the bioflavonoid complex are expected to be made use of in cosmetic industry as well as in medicine.

Introduction

The positive trend today is towards man's active life period becoming increasingly longer which makes more people try to keep healthy appearance of their skin and look for ways to maintain it. With this object in view intensive investigations are carried out all over the world with the aim of developing safe preparations to check the natural process of skin aging.

Phytogenous preparations containing flavonoids have been used for this purpose for ages. Generally, the action of flavonoids upon a human being can be determined by their ability to protect the human organism from environmental stressors due to their antioxidant activity and heavy metal binding capabilities as well as their effect upon signal systems of cells (1). We must admit that our knowledge about the mechanisms of these substances' action upon the human organism is far from being sufficient (2). This can be illustrated by a recent discovery that despite their positive effects upon human health some products of flavonoid metabolism in the human organism may be toxic (and mutagenic). For example, recently published data indicate that this may be the case with quercetin, one of the best studied and most widely used flavonoid (3).

The non-toxicity of dihydroquercetin (taxifolin) may be due to the absence of C2-C3 double bond [3]. This suggests that research into the properties of taxifolin may be promising. The present paper studies the interaction between taxifolin (INCI Name: *Larix Sibirica Wood Extract*) and collagen and the effects of the said preparation upon the collagen fibril formation process and fibril stability.

Larix Sibirica Wood Extract

Larix Sibirica Wood Extract is produced from the butt log part of the Siberian Larch by means of extraction and after-purification. *Larix Sibirica Wood Extract* is a bioflavonoid complex (produced by Bioflavon Ltd, Obninsk, Russia, under the trade name of Flavocon®) whose main active constituent is taxifolin. In addition to taxifolin the complex includes its biogenetic precursor substances aromadendrene and eriodictyol (2 through 8 percent) which are related flavonoids having similar physicochemical and biological properties.

A taxifolin molecule has two chirality centers and can exist in the form of four stereoisomers. The *Larix Sibirica Wood Extract* taxifolin is one of those stereoisomers, namely the 2R, 3R one. The stereochemical structure determines whether the substance will show any biological activity and to a large extent determines the type of such activity.

Effects of Larix Sibirica Wood Extract upon Type I Collagen

Collagen is one of the most widespread proteins of the human body. Collagen re-

sides in the intercellular space and is organized in the form of fibrils marked by low solubility in water and having great mechanical strength: the tensile strength of collagen fibers is greater than that of steel wires of the same mass. So the function of collagen is to form components of human body tissues that are characterized by great strength, which includes the fibrillar components of vessels and the skin. So far from 12 to 14 collagen types have been identified, but most abundant are type I, type II and type III collagens.

A single type I collagen molecule has a molecular mass of about 285 kDa, is about 14 Å wide and about 3000 Å long. A collagen molecule sometimes referred to as tropocollagen consists of three protein threads which are subunits of proteins – each in the form of a left-handed helix. These three left-handed helices are further twisted around one another to form a major right-handed helix having a larger pitch of strand. The twist in the opposite direction prevents the resulting bundle from getting untwisted when under tension and it also makes it particularly strong.

Some collagen types (including type I collagens) form fibrils cross-striated with transverse bands. The fibrils are made up of sets of parallel-oriented tropocollagen molecules, they represent the next and higher level of collagen structural organization. The exact spatial pattern of collagen molecules in fibrils has not been adequately studied. Of particular interest is the question of what causes the cross striation of fibrils that can be seen with an electron microscope **(Fig. 1)**. The transverse bands have a period D = 640–680 Å, which is considerably shorter than the length of an individual tropocollagen molecule (about 3000 Å). A question arises as to how the cross striation is formed with a period considerably smaller than the length of molecules forming such structure. It is believed that tropocollagen molecules are added to one another following the head-to-tail addition pattern, but there are gaps between them. As a result there are areas in the fibril cross-section where molecules overlap and areas where a certain number of gaps can be seen. The areas where molecules overlap correspond to dark bands and the gaps between the molecules correspond to light areas (about 400 Å).

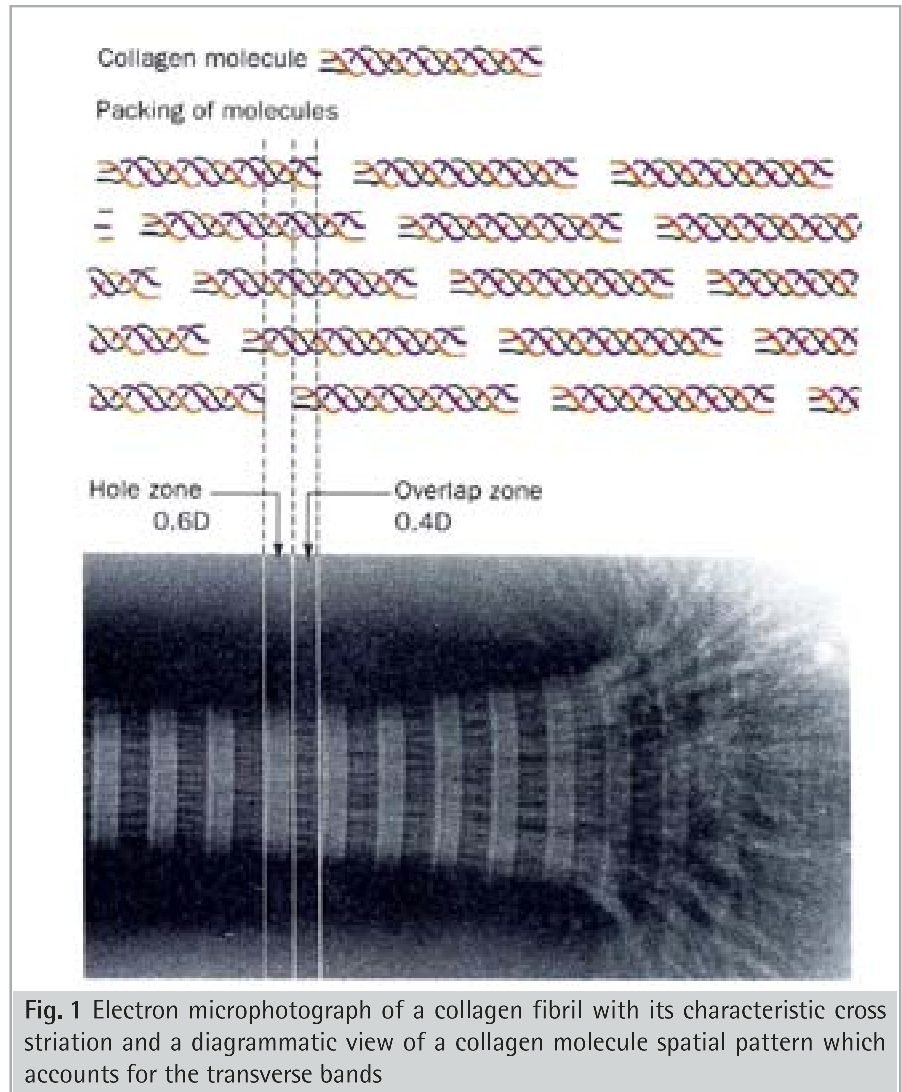

Fig. 1 Electron microphotograph of a collagen fibril with its characteristic cross striation and a diagrammatic view of a collagen molecule spatial pattern which accounts for the transverse bands

It is well known that in acid conditions (acetic acid 100 mM, pH of about 3.0) collagen can be brought into solution whose fundamental units will be individual tropocollagen molecules. As the acidity level is lowered and the solution becomes neutral the tropocollagen molecules become to be able to spontaneously assemble into fibrils, with the characteristic cross striation being restored. The process of fibril formation takes several hours and is susceptible to a number of factors (6). This paper shows the effect of the *Larix Sibirica Wood Extract* complex upon the said process.

In a phosphate buffer collagen molecules form fibrils spontaneously, the rate of the process depending on the temperature. Light scattering variations can be used to determine the fibril formation dynamics (4, 7).

Fig. 2 shows light scattering curves for samples containing different concentrations of the bioflavonoid complex, and control samples. As may be seen from the figure in the control sample that does not contain the preparation the fibril formation process at given conditions starts after about ten minutes of incubation and develops very slowly. In the presence of *Larix Sibirica Wood Extract* the fibril formation process is accelerated and starts earlier as compared with the control. This is indicative of activation of the fibril formation process induced by *Larix Sibirica Wood Extract*.

Results and Discussion

A number of authors reported that some flavonoids could fluoresce when interacting with certain proteins (like serum albumin, for instance) (5, 8, 9). During our experiment we determined that in aqueous solution *Larix Sibirica Wood Extract* did not have pronounced fluorescence capabilities, however, in solutions containing collagen we were the first to discover that *Larix Sibirica Wood Extract* could fluoresce which would allow one to monitor the fibril formation process.

It is well known that in acid conditions collagen does not form fibrils, so the preparation's fluorescence intensity is insignificant **(Fig. 3b)**. In a phosphate buffer at neutral pH values fibril formation does take place and the corresponding samples show a considerable increase in the Larix Sibirica Wood Extract fluorescence intensity **(Fig. 3a)**. It may be conceived that in the process of fibril formation the *Larix Sibirica Wood Extract* interaction with collagen intensifies, which is reflected by increased fluorescence intensity.

Collagen fibril formation is clearly traced by electron microscopy methods. **Fig. 4** shows collagen fibrils formed at different *Larix Sibirica Wood Extract* concentrations.

All of the microphotographs show the characteristic collagen fibril cross striation with a period of about 64 nm. All of the typical structural peculiarities of fibrils are kept very well in the presence of *Larix Sibirica Wood Extract* which is indicative of the fact that *Larix Sibirica Wood Extract* does not lead to any fibril formation abnormality. Moreover, periodicity is more pronounced at large concentrations of *Larix Sibirica Wood Extract*.

The Effect of Larix Sibirica Wood Extract Upon Collagen Molecules' Thermodynamic Characteristics

The scanning calorimetry method was used to determine the effect of *Larix Sibirica Wood Extract* upon the thermodynamic characteristics of collagen molecules and collagen fibrils already formed. With this aim in view we studied collagen fibrils formed in 0.01 M phosphate buffer (pH = 7.4, 20 °C, 24 hours) in the presence of *Larix Sibirica Wood Extract* at different concentrations **(Fig. 5, Table 1)**.

As is seen from the data in the table at 20 °C and physiological values for pH and ionic force of the medium adding *Larix Sibirica Wood Extract* at concentrations

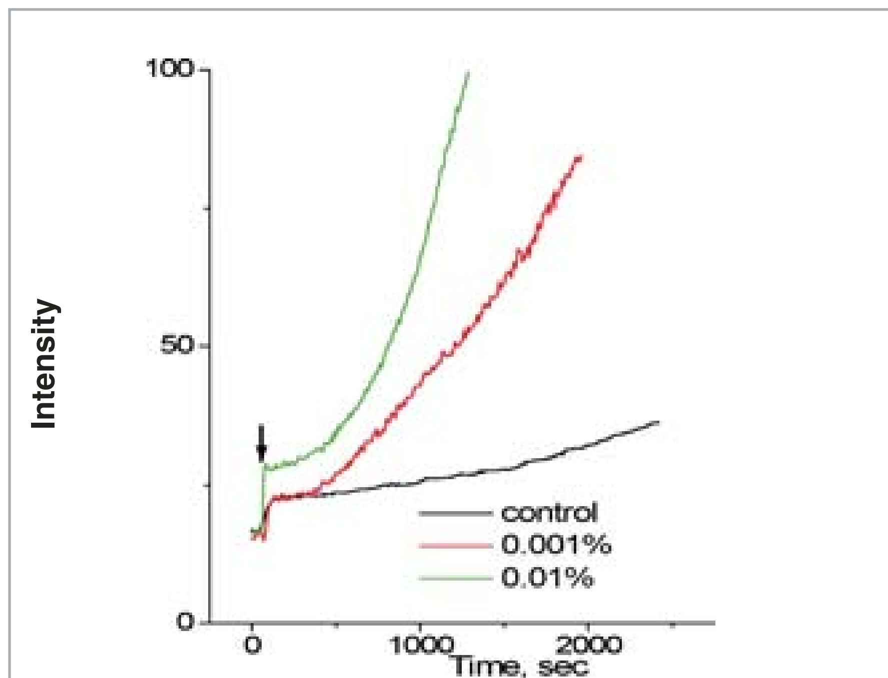

Fig. 2 Variation of intensity of right angle light scattering in a collagen suspension (0.1 mg/ml) at 510 nm in the presence of *Larix Sibirica Wood Extract*.
Incubation medium – 10 mM phosphate buffer, pH = 7.4, 25 °C
The arrow indicates the point in time at which the protein was added

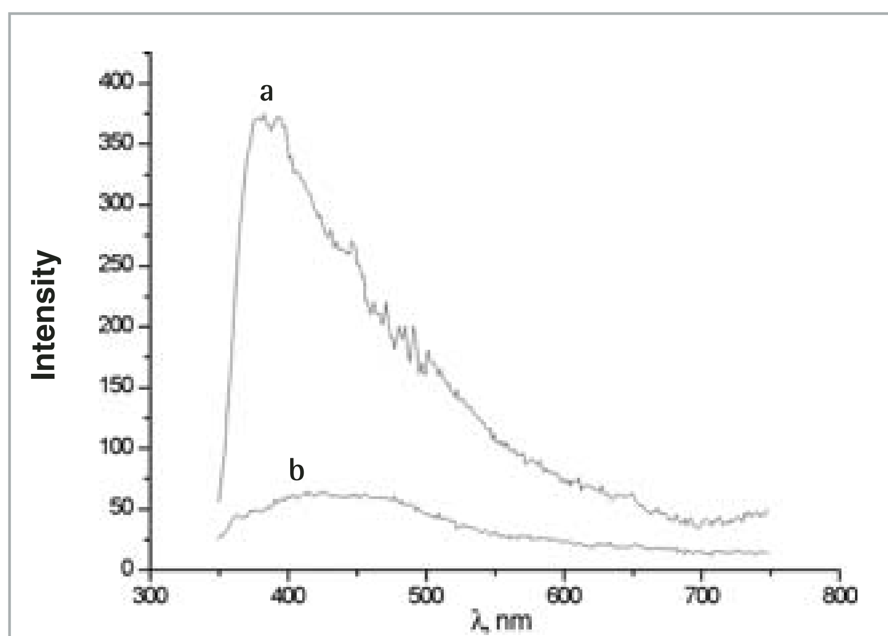

Fig. 3 *Larix Sibirica Wood Extracts* (0. 01%) fluorescence spectrum in collagen (0.1 mg/ml): **a** – at pH = 2.9 (0.1 M acetic acid), **b** – at pH = 7.4 (10 mM phosphate buffer)

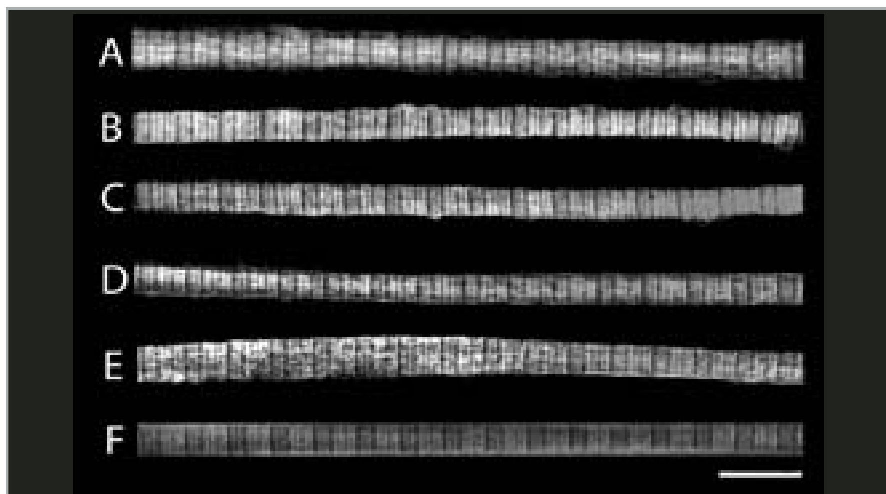

Fig. 4 Electron microphotographs of collagen fibrils formed at different *Larix Sibirica Wood Extrac*t concentrations: 10mM phosphate buffer (pH = 7.4), 20°C.
The duration of the fibril formation process was 24 hours.
The preparation was stained in 1 % phosphotungstic acid and 1 % uranyl acetate.
A – control preparation; B – 0.00001 %, C – 0.0001 %, D – 0.001 %, E – 0.01 %, F – 0.1 % Larix Sibirica Wood Extract. The bar represents 130 nm

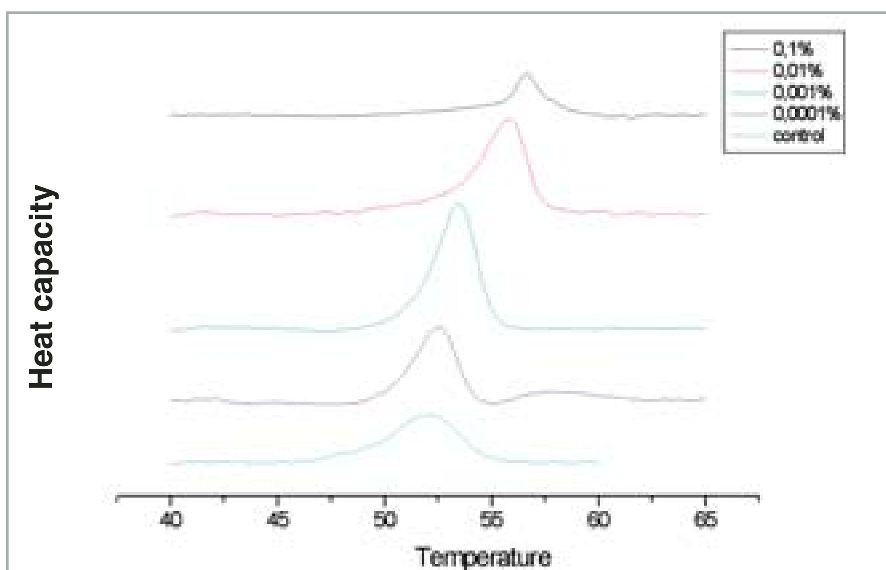

Fig. 5 Microcalorimetric curves for collagen (0.1 mg/ml) melting in the presence of Larix Sibirica Wood Extract at different concentrations. The preparation was made in a 10mM phosphate buffer (pH = 7.4) at 20°C

Larix Sibirica Wood Extract %	Temperature of collagen melting, Tm (°C)	Half-width of transition, $_{1/2}$ (°C)
0	52.0	3.2
0.00001	52.0	3.2
0.0001	52.5	2.1
0.001	53.5	2.2
0.01	55.8	2.3
0.1	56.7	1.0

Table 1 The thermodynamic parameters of collagen fibers assembled in 0.01 M phosphate buffer at 20°C and pH 7.4

higher than 0.0001 % leads to an increase in the phase transition temperature and narrowing of the heat absorption peak in collagen fibrils. This is indicative of both collagen fibril stabilization under such conditions and increased phase transition cooperativity.

The second series of experiments was conducted to study the effect of fibril formation temperature upon the thermodynamic characteristics of collagen fibrils formed in a 0.01 M phosphate buffer (pH = 7.4, 37 °C, 24 hours) in the presence of *Larix Sibirica Wood Extract* at different concentrations. The results of the experiments are shown in **Fig. 6** and in **Table 2.** It follows that adding *Larix Sibirica Wood Extract* at concentrations of 0.0001 % and higher leads to a considerable increase in the fiber melting temperature as compared with the individual collagen molecule melting temperature. It also leads to forming a narrow peak in collagen fibrils' heat absorption, and similarly to the experiments at 20 °C the cooperativity of the phase transition increases considerably when the *Larix Sibirica Wood Extract* concentration is increased. Thus, the extract promotes the fibril formation process and leads to a more uniform fibril arrangement as compared with the control.

It should be noted that at 37 °C and physiological values for pH and ionic force of the medium adding *Larix Sibirica Wood Extract* at concentrations of 0.0001 % and higher leads to a significant increase in the temperature and cooperativity of the collagen fibril melting process. Thus, the stabilizing action of *Larix Sibirica Wood Extract* at 37 °C is considerably stronger than it is at 20 °C **(Fig. 6)**.

Conclusion

When analyzed the results of the study of interaction between *Larix Sibirica Wood Extract* and collagen obtained using a variety of methods point to two important facts, namely, the appearance of fluorescence of the preparation under study and the acceleration of fibril formation.

At 37 °C and physiological values for pH and ionic force of the medium *Larix Si-*

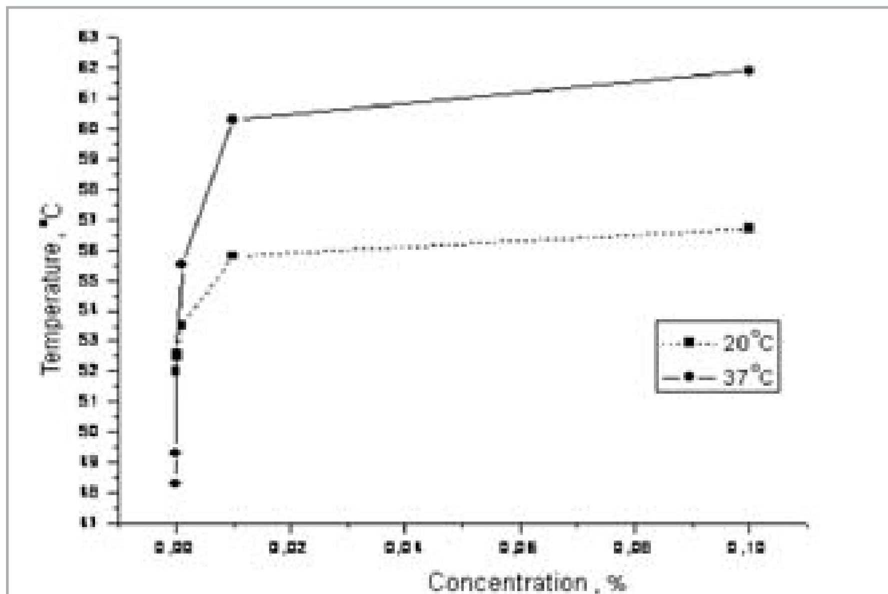

Fig. 6 Phase transformation temperature for collagen fibrils versus *Larix Sibirica Wood Extract* concentration (in the process of fibril formation in a 0.01 M phosphate buffer (pH = 7.4))

Larix Sibirica Wood Extract %	Temperature of collagen melting, Tm (°C)	Half-width of transition, $_{1/2}$ (°C)
0	48.3	2.8
0.00001	49.3	1.8
0.0001	52.6	2.3
0.001	55.5	2
0.01	60.3	1.9
0.1	61.9	1

Table 2 The thermodynamic characteristics of collagen fibers prepared using a 0.01 M phosphate buffer (pH 7,4) at 37 °C for 24 h

birica Wood Extract leads to a significant increase in the temperature and cooperativity of the collagen fibril melting process. It should be remembered that *Larix Sibirica Wood Extract* consists of flavonoids, phytogenous substances belonging to polyphenols, that have positive effect upon human health due to their antioxidant, capillary protective, hepatoprotective, cardioprotective properties and are substantial components of vegetable foods. Therefore the extract can be used not only as an ingredient in cosmetic and dermatologic preparations, but also as a dietary supplement (the »beauty from inside« concept).

The raw material for cosmetics Flavocon® won general recognition at the international BSB European Innovation Prize contest for innovation in the area of 'cosmetics raw materials' where we were placed third (the prize was awarded on April 17 at In-cosmetics 2007 in Paris).

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