J. Chem. Sci., Vol. 121, No. 4, July 2009, pp. 509–514.

Collagen–curcumin interaction – A physico-chemical study

N NISHAD FATHIMA, R SARANYA DEVI, K B REKHA and
ARUNA DHATHATHREYAN*
Chemical Laboratory, Central Leather Research Institute, Council of Scientific and Industrial Research, Adyar, Chennai 600 020
e-mail: aruna@clri.res.in

MS received 2 February 2009; accepted 13 May 2009

Abstract. Curcumin is a widely used therapeutic agent with a wide spectrum of biological and physiological applications like wound healing and interacts with the skin protein, collagen. This work reports the effect of curcumin on various physico-chemical properties of collagen. The results suggest that significant changes in viscosity and surface tension occur on collagen interacting with curcumin. Secondary structure analysis using circular dichroism shows that curcumin does not alter the triple helical structure of collagen. Increasing concentration of curcumin resulted in aggregation of the protein. Further, curcumin imparts high level of thermal stability to collagen with shrinkage temperature of collagen increasing from 60 to 90°C.

Keywords. Collagen; curcumin; circular dichroism (CD); differential scanning calorimetry (DSC), viscosity, surface tension.

1. Introduction

Curcumin [1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a member of the curcuminoid family of compounds, is a yellow-orange pigment derived from the rhizome of *Curcuma longa.*[1,2] It has attracted considerable attention in recent years due to its wide spectrum of biological and pharmacological activities, including antioxidant, antitumor, antiinflammatory, antibacterial, antifungal, antiviral, antiproliferative, anti-invasive, antiangiogenic and anticoagulant activities.[3–7]

It binds to a variety of proteins and inhibits the activity of various kinases. Various pre-clinical cell culture and animal studies suggest that curcumin has potential as a mediator of chemoresistance and radioresistance; as a chemopreventive agent; and as a therapeutic agent in wound healing, diabetes, Alzheimer disease, Parkinson disease, cardiovascular disease, pulmonary disease, hypercholesteremia, pancreatitis and arthritis. Curcumin is capable of scavenging oxygen free radicals such as superoxide anion radicals, hydroxyl radicals[8] and nitrogen dioxide radicals,[7,9] which are the initiators of lipid peroxidation. The lipid peroxidation has a main role in the inflammation, in heart diseases and in cancer. Curcumin protects (52%) haemoglobin from nitrate-induced oxidation to methaemoglobin. It may be used in singlet oxygen-mediated diseases as a pharmacologic agent.

Topical application of turmeric is a household remedy in India for several conditions such as skin diseases, insect bites and chicken pox.[10] It has been reported that there is an improvement in the quality of wound healing by slow delivery of antioxidants (curcumin) to collagen, which also acts as a supportive matrix for the regenerative tissue. The preventive and therapeutic effects of curcumin on the advanced glycation and cross-linking of collagen in diabetic rats have been demonstrated.[11] The various medical uses of curcumin brings it in contact with the important connective tissue protein, collagen. Hence, in this study, we present the changes brought about by curcumin on various properties of collagen at the molecular level. The effect of curcumin on the conformation, viscosity, surface activity and thermal stability of collagen has been investigated.

2. Materials and methods

2.1 *Materials and collagen solution preparation*

Deionized distilled water from Millipore (Milli-Q) (18 MΩ) was used for the experiments. All the flu-

*For correspondence

ids used in the study were HPLC grade and obtained from Merck, India. Curcumin was obtained from Sigma Chemicals, USA. Collagen solutions were prepared from tendons freshly dissected from the tails of 6-month-old male albino rats frozen at –20°C by acetic acid extraction and salting-out with NaCl.[12] The purity of the collagen preparation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis; the bands appearing in the gel corresponded only to type 1 collagen. The collagen concentration in the solutions was determined from the hydroxyproline content.[13] The average molecular weight of collagen was 300,000 Daltons, on the basis of which the molar concentration was determined. The stock concentration of the prepared collagen was 0·2 μM. Collagen solution was treated with curcumin in the increasing molar ratio of collagen to curcumin from 1 : 1 to 1 : 100 and incubated for 24 h under room temperature (25°C).

2.2 *Circular dichroism (CD) measurements*

Purified collagen solution was incubated in the presence of various concentrations of curcumin at 25°C for 24 h and the spectra were recorded at 25°C using a Jasco 715 Circular Dichroism spectropolarimeter. A scan speed of 20 nm per min was used with an average of three scans per sample. A slit width of 1 nm and a time constant of 1 s were used. A 1 mm cell was used for the experiments. A reference spectrum containing acetate buffer was also recorded. The CD spectra of the samples were obtained after subtracting the reference spectrum. Changes in the conformation of collagen on addition of various concentrations of curcumin were recorded.

2.3 *Viscosity measurements*

Viscosity measurements were performed using an Ostwald type viscometer with a flow time for buffer of 47 s at 25°C. The viscometer was placed in a water-bath, which was connected to Julabo thermostat. The same viscometer was used for all measurements and was mounted so that it always occupied the same position in the bath. Viscosity measurements were made from 10°C to 40°C in 5°C intervals. This heat-induced aggregation reverses on cooling. The flow times of collagen samples were measured after a thermal equilibrium time of 30 min. The collagen concentration ($0{\cdot}04 \times 10^{-6}$ M) was fixed and the measurements were carried out with different concentrations of curcumin, $0{\cdot}04 \times 10^{-6}$ M to 4×10^{-6} M. The mixture was then incubated for 24 h at room temperature. The viscosity measurement was based on the flow rate of collagen solution through the capillary of an Ostwald viscometer. In these experiments the viscosity contribution (η) due to collagen was measured as a function of the concentration of curcumin. The flow time was measured with a digital stopwatch at least three times and the average was taken. The viscosity was calculated from the relation, $\eta = (t - t^0)/t^0$, where t^0 is the flow time of buffer (pH 4·2, $l = 2 \times 10^{-2}$ M) and t is the flow time for each sample.

2.4 *Thermal stability measurements*

Rat tail tendons (RTT) were treated with 0·5% curcumin solution at pH 4·2 for 48 h. The native and curcumin treated rat tail tendons (RTT) were blotted uniformly weighed and hermatically encapsulated in aluminium pans. The samples were fused in a differential scanning calorimetric cell of DSC Q 200 TA instruments. The temperature was calibrated using indium as standard. The heating rate was maintained constant at 3°C/min. The denaturation temperature, T_D (in °C) associated with the phase and enthalpy changes for native and fibres treated with curcumin was determined. The hydrothermal stability of curcumin treated tendon was determined by the standard method using micro-shrinkage tester.[14] The treated tendons were cut into small pieces and then mounted on to microscopic slides under wet conditions. The slides were placed on a holder for heating. Shrinkage due to heating was visualized through a microscope placed above the holder.

2.5 *Surface tension measurements*

Surface tension is the intermolecular force of attraction between adjacent molecules, expressed in force per unit width, as dynes/centimeter (dynes/cm) or milliNewtons/meter (mN/m). Water, at ambient temperature, has a high surface tension of about 72 dynes/cm. A NIMA DST 9005 contact angle meter was used to measure the surface tension of the fluids (accuracy $\pm 0{\cdot}01$ mN/m) on glass. DST9005 Dynamic Surface Tensiometer is a sophisticated computer controlled instrument that can measure the forces on samples as they are immersed in the test liquid. Advancing and receding, dynamic and static contact angles can be measured. A temperature con-

trollable stage and stirrer agitator are included for precise, repeatable measurements.

Glass substrates were cover slips (18 mm × 18 mm) with a mean rms value of about 0·16 ± 0·01 nm from ERMA, FRG and were cleaned with warm chromic acid and washed repeatedly with fresh deionized water, dried using a stream of nitrogen and were stored in a desiccator for use. The cover slips were immersed in a Perspex tank where the different sample liquids or their mixtures were kept. Both dynamic and static surface tension measurements were carried out and the results were recorded on a computer.

3. Results

3.1 *Secondary structural changes in collagen on interaction with curcumin*

Circular dichroic (CD) spectra of protein solutions provide information about secondary structure contents of proteins. CD has been used widely to characterize effect of small molecule interaction on conformation of collagen.[15–17] Far UV CD spectra of collagen solutions treated with various concentrations of curcumin at 25°C is shown in figure 1. Addition of curcumin to collagen solution did not alter the CD spectra significantly (figure 1 for θ_{220}) at a given concentration. The ratio of positive to negative peak (Rpn) of the CD spectra, an indicator for conformational changes for native and curcumin treated collagen solution is given in table 1.

3.2 *Hydrodynamic changes in collagen on interaction with curcumin*

Hydrodynamic properties of collagen are important for its biomedical applications. In order to study the effect of curcumin on the viscosity of native and curcumin treated collagen, studies were carried out using Ostwald viscometer varying the collagen : curcumin ratio. Figure 2 gives the relative viscosity vs the concentration plot (η/η_0 vs [curcumin]/[collagen]) with varying temperatures. It is evident from the figure that the relative viscosity of both native and curcumin treated collagen solution increases with increasing concentration. The effect of temperature on the viscosity of collagen before and after treatment with curcumin is given in figure 3. The temperature range between 10 and 40°C was chosen because above 37°C the thermal denaturation of collagen and the proteins show a highly pronounced tendency to aggregate.[18,19]

3.3 *Thermal stability changes in collagen on interaction with curcumin*

Collagen fibre shrinks to one third its original length at a characteristic temperature called the shrinkage temperature when thermal energy is provided. Hydrothermal stability or shrinkage temperature of the collagen fibres is a measure of the stability of the matrix as a whole, which arises due to the long range ordering of the matrix. On shrinking, collagen fibres become translucent and highly elastic. The shrinkage temperature for native collagen is 60°C. Thermal stability of collagen fibres registers a steep increase on crosslinking. Hydrothermal stability of the curcumin treated tendon collagen was found to rise up to 86°C, which indicates that curcumin

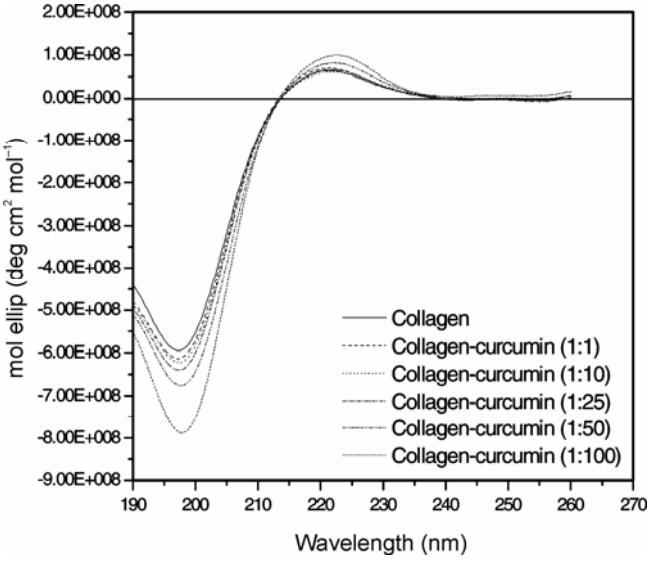

Figure 1. Far-UV CD spectra of collagen (0·04 μM) in the presence of varying concentration of curcumin (0·04 to 4 μM).

Table 1. Rpn (ratio of positive to negative peak) values of native collagen and collagen treated with various concentrations of curcumin.

Sample	Rpn
Native collagen	0·1075
Collagen–curcumin (1 : 1)	0·1128
Collagen–curcumin (1 : 10)	0·0972
Collagen–curcumin (1 : 25)	0·1037
Collagen–curcumin (1 : 50)	0·1202
Collagen–curcumin (1 : 100)	0·1243

imparts high level of thermal stability to collagen by crosslinking. Differential scanning calorimetry gives the denaturation temperature as well as the enthalpy changes involved in this process of shrinkage. Figure 4 gives the DSC thermograms of native and curcumin treated collagen fibres.

3.4 *Surface tension changes in collagen on interaction with curcumin*

In an interaction, any changes which happen at the molecular level will result in the change of surface tension. Measuring surface tension is a direct indicator of the molecular level changes brought about due to any interaction. In order to find out the effect of curcumin on the surface activity of collagen, dynamic and static surface tension measurements were carried out. Figure 5 shows the surface tension measurements of native and curcumin treated collagen solution with respect to time. Three different ratios of collagen and curcumin were taken. The static surface tension values are given in table 2. It can be seen that the surface tension increases after treating with curcumin in the ratio of 1 : 1. However, on further increasing the concentration of curcumin, the surface tension is found to decrease.

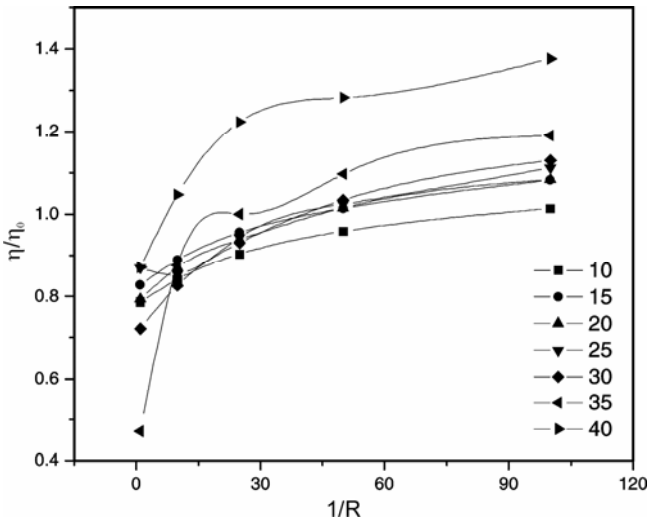

Figure 2. A plot of relative viscosity (η/η_0) against $1/R$ (η and η_0 are the viscosity of collagen in the presence of curcumin before and after treatment. R = [collagen]/[curcumin].

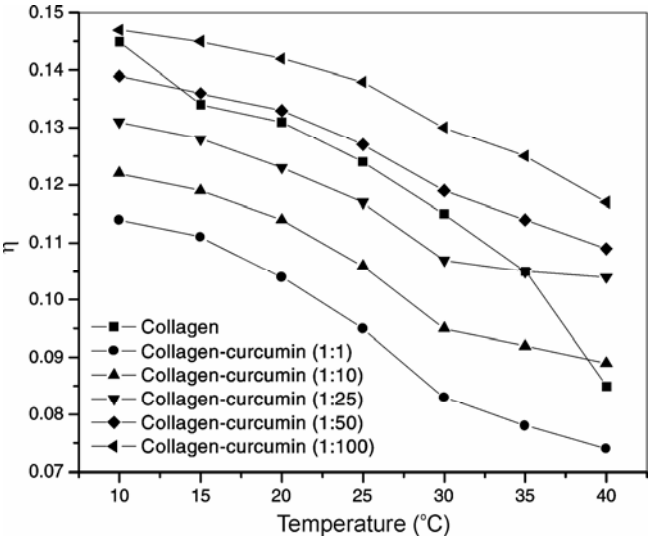

Figure 3. A plot of viscosity η with various temperatures ranging from 10 to 40°C.

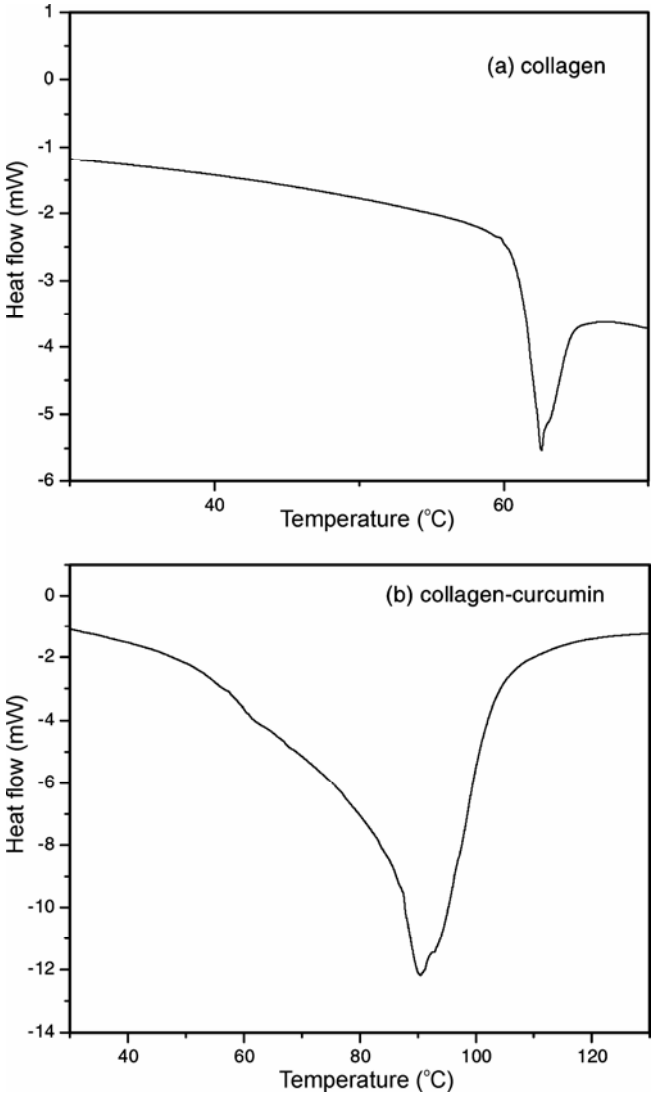

Figure 4. DSC thermograms of native and curcumin treated RTT fibres.

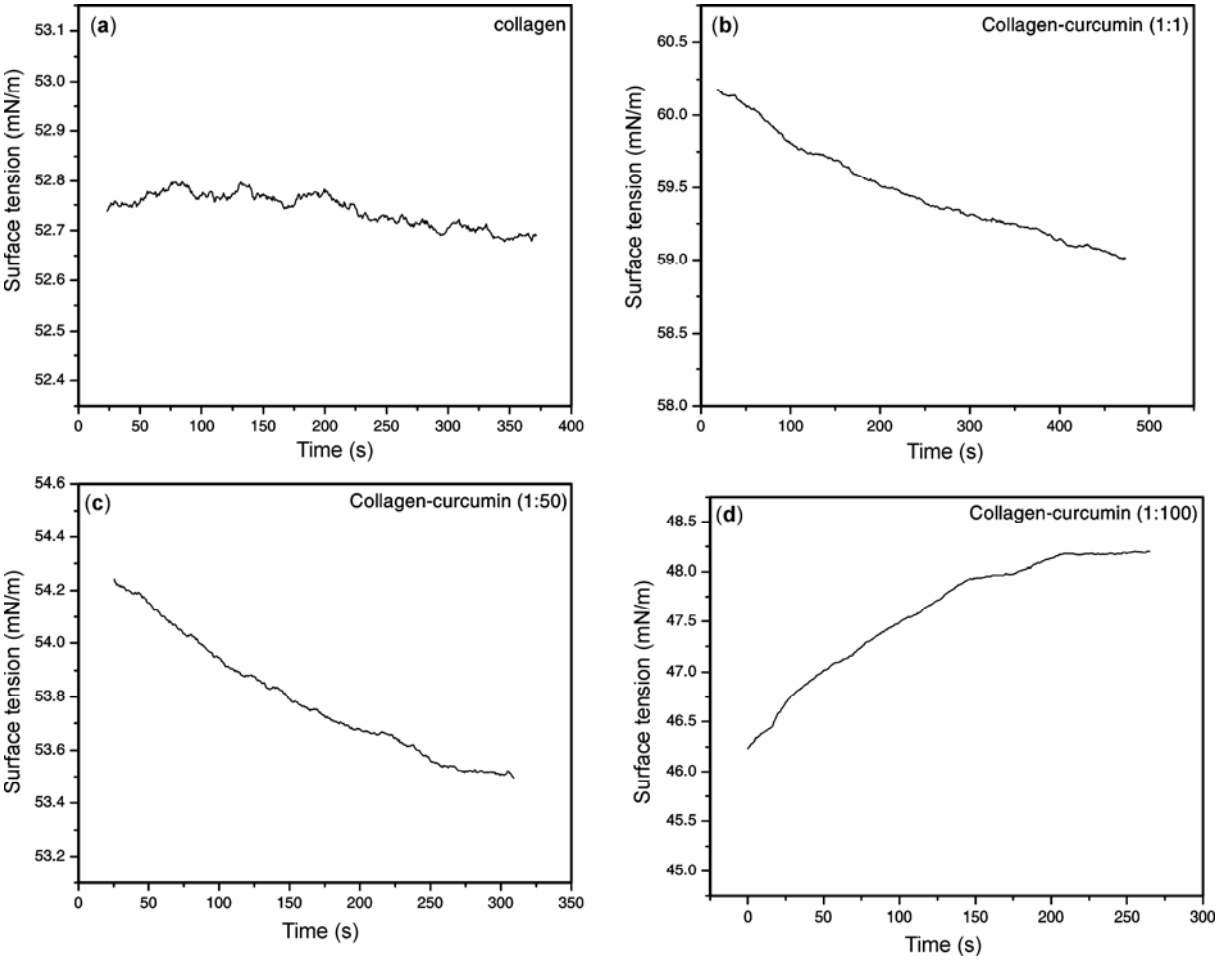

Figure 5. Dynamic surface tension plots for (**a**) native collagen and curcumin treated collagen in ratios, (**b**) 1 : 1, (**c**) 1 : 50 and (**d**) 1 : 100, respectively.

Table 2. Static surface tension values of native collagen and collagen treated with various concentrations of curcumin.

Sample	Static surface tension (mN/m)
Collagen	53·1
Collagen–curcumin (1 : 1)	60·4
Collagen–curcumin (1 : 50)	54·5
Collagen–curcumin (1 : 100)	48·5

4. Discussion

The changes brought about in the physico-chemical properties of collagen on interaction with curcumin has been addressed in this study. The secondary structural studies show that by increasing the concentration of curcumin, a slight decrease in molar ellipticity around 220 nm (positive peak) and an increase around 197 nm (negative peak) is observed (figure 1). It has been reported that on complete denaturation, the positive peak at 220 nm disappears completely and the negative band is red shifted.[20] On addition of curcumin, there is no significant change (red shift) in the negative band and no disappearance of the positive band at 220 nm. This shows that curcumin brings about only very slight change in packing of helices and does not change the conformation of protein. It has been earlier reported that interaction of collagen with small molecules like polyphenols, aldehydes and chromium while influencing the thermal and enzymatic properties, do not alter the secondary structural features of the protein dramatically.[16,21,22] It can be seen from table 1 that after treating with curcumin the Rpn ratio increases initially followed by a decrease up to a value of 1 : 25 after which again an increase is observed. This increase is indicative of aggregation of collagen at that curcumin concentration. Viscos-

ity measurements also indicate the aggregation of collagen on treatment with increasing concentration of curcumin (figure 2). Also, there is decrease in viscosity of collagen on increasing the temperature with the largest change between temperatures 35 and 40°C possibly because of the melting temperature of collagen being 37°C. However, it can be seen from the figure (figure 3) that this decrease is less pronounced after treatment with curcumin. This could be indicative of stability of protein interacting with curcumin.

Curcumin also affects the thermal property of collagen significantly as can be seen from the figure 4 where the denaturation peak for curcumin treated collagen is 90°C as against the 62°C of native collagen. Also, the enthalpy peak for curcumin treated collagen was found to be 881·7 J/g and that of collagen is 13·21 J/g. The reason for the difference between hydrothermal shrinkage measured using micro shrinkage tester and DSC could be due to the difference in moisture levels of the samples. Also, the higher enthalpy for curcumin treated collagen could be due to the merging of denaturation peak and the water peak. It can be seen from the figure 5 that the dynamic surface tension remains almost constant for native collagen, whereas for the collagen treated with curcumin there is a decrease with time. The collagen treated with curcumin in the ratio of 1 : 100 showed an increase in the dynamic surface tension with time. This indicates that though higher concentration of curcumin increases the surface activity of collagen, with time this decreases. This could also be due to changes in local restructuring of water leading to exposure of non polar groups thus increasing the surface activity of collagen. Curcumin increases the surface activity of collagen as seen from table 2.

5. Conclusion

The mechanism of interaction of curcumin with collagen is mainly through hydrogen bonding and charge interactions. It has been shown that curcumin has three p*K*a values at 8·38, 9·88, and 10·51 in aqueous solution, corresponding to deprotonation of the three hydroxyl groups.[23] Therefore, curcumin is fully protonated at pH 4·0 to form the highly positively charged species. Therefore, interaction of this species with the negative charges on the protein is likely to be strong. These attractive interactions allow curcumin to remain in close proximity to the protein and contribute greatly to its stabilization. The additional crosslinks formed through hydrogen bonding increase the thermal stability and viscosity of the protein. The changes in the physico-chemical properties of collagen due to interaction with curcumin thus help in the accelerated wound healing process.

References

1. Sharma O P 1976 *Biochem. Pharmacol.* **25** 1811
2. Vijayalaxmi B 1980 *Mutat. Res.* **79** 125
3. Dickinson D A, Levonen A L, Moellering D R, Arnold E K, Zhang H, Darley-Usmar V M and Forman H J 2004 *Free Radic. Biol. Med.* **37** 1152
4. Ruby A J, Kuttan G and Babu K D 1995 *Cancer Lett.* **94** 79
5. Aggarwal B B, Kumar A and Bharti A C 2003 *Anticancer Res.* **23** 363
6. Jankun E S, McCabe N P, Selman S H and Jankun J 2000 *Int. J. Mol. Med.* **6** 521
7. Sreejayan N, Priyadarsini K I, Devasagayam T P A and Rao M N A 1997 *Int. J. Pharmacol.* **151** 127
8. Reddy A C and Lokesh B R 1994 *Mol. Cell. Biochem.* **137** 1
9. Unnikrishnan M K and Rao M N 1995 *Mol. Cell. Biochem.* **146** 35
10. Nadkarni K M (ed.) 1976 *Curcuma longa* Indian Materia Medica (Bombay: Popular Prakashan Publishing Company) p. 414
11. Sajithlal G B, Chittra P and Chandrakasan G 1998 *Biochem. Pharmacol.* **56** 1607
12. Chandrakasan G, Torchia D A and Piez K A 1976 *J. Biol. Chem.* **251** 6062
13. Woessner Jr J F 1961 *Arch. Biochem. Biophys.* **93** 440
14. Borasky R and Nutting G C 1949 *J. Am. Leather Chem. Assoc.* **44** 830
15. Fathima N N, Madhan B, Rao J R and Nair B U 2003 *J. Inorg. Biochem.* **95** 47
16. Fathima N N, Bose M C, Rao J R and Nair B U 2006 *J. Inorg. Biochem.* **100** 1774
17. Fathima N N, Suresh R, Rao J R and Nair B U 2007 *J. App. Polym. Sci.* **104** 3642
18. Katakam M and Banga A K 1995 *J. Pharm. Pharmacol.* **47** 103
19. Yoshioka S, Aso Y and Kojima S 1997 *J. Pharm. Sci.* **86** 470
20. Brown E M, Dudley R L and Elsetinow A R 1997 *J. Am. Leather Chem. Assoc.* **92** 225
21. Fathima N N, Madhan B, Rao J R, Nair B U and Ramasami T 2004 *Int. J. Biol. Macromol.* **34** 241
22. Gayatri R, Sharma A K, Rajaram R and Ramasami T 2001 *Biochem. Biophys. Res. Commun.* **283** 229
23. Bernabe-Pineda M, Ramirez-Silva M T, Romero-Romo M, Gonzalez-Vergara E and Rojas-Hernandez A 2004 *Spectrochim. Acta* **A60** 1091