Stabilization of Collagen by the Plant Polyphenolics *Acacia mollissima* and *Terminalia chebula*

G. Krishnamoorthy, B. Madhan, S. Sadulla, J. Raghava Rao, W. Madhulatha

Central Leather Research Institute, Adyar, Chennai 600 020, India

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ABSTRACT: The central role of collagen as the major structural fibrous protein in the mammalian extracellular matrix has motivated a significant effort toward the determination of its mechanical properties at all levels, ranging from single monomers and long-chain polymers to a structural element within a biological tissue. However, the stabilization of collagen against collagenolytic degradation finds significance in biomedical and industrial applications. Tannins are plant-derived polyphenols that have the ability to inhibit the collagenase activity at minimum concentration. The inhibitory effect of wattle (*Acacia mollissima*) and myrobalan (*Terminalia chebula*) on the action of collagenase against collagen was probed in this study. The kinetics of the inhibition of collagenase by wattle and myrobalan was deduced from the extent of hydrolysis of 2-furanacryloyl–L-leucyl–glycyl–L-prolyl–L-alanine. Both wattle and myrobalan tannin exhibited competitive modes of inhibition against collagenase. Circular dichroism studies of collagenase on treatment with wattle and myrobalan revealed changes in the secondary structure of collagenase. These results suggest that the tannins of *A. mollissima* and *T. chebula* extracts facilitated collagen stabilization through collagenase inhibition. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 108: 199–205, 2008

Key words: antioxidants; biocompatability; biofibers; biomaterials; biopolymers

INTRODUCTION

Type-I collagen is an important major structural fibrous protein, a major component in mammalian connective tissue, and forms the supporting framework of many body components; it self-associates to form fibrils in skin, bone, tendon, and cornea and binds with ligands, including proteins, proteoglycans, and lipids.[1,2] This type of fibrous collagen can be fabricated into various forms of biomaterial such as a gel, sponge, fibers, and film to serve as a scaffold of great potential in tissue engineering with a low antigenicity and unique biocompatibility and with biodegradable, mechanical, hemostatic, and cell-binding properties.[3,4] In addition to its biological function, because collagen has the ability to persist in the body without producing a foreign-body response that could lead to premature rejection, it has been extensively investigated as a biomaterial for artificial skin, tendons, blood vessels, cartilage, and bone.[5,6] Freeze-dried, lyophilized collagen and collagen-based materials have been successfully used, both clinically and experimentally.[7] However, a subset of burn patients have cells that produce elevated levels of matrix metalloproteinases (MMPs), which can cause the premature degradation of collagen in cultured skin substitutes and lead to graft failure *in vitro*. The high rates of degradation and the deficient mechanical properties of collagen often fail to meet the requirements of specific applications, which, consequently, limits the use of collagen-based materials.[8] Mammalian skin is an excellent source of type-I collagen for biomedical and leather-making uses.[9] Collagens from domestic animals are usually used in industry, and they are chemically modified with proteases, a crosslinking agent, or physical methods to vary and control their function for multiple uses.[10–14]

The stabilization of collagen against collagenolytic degradation finds importance in biomedical applications (in the preparation of biomaterials) and in industrial applications (in leather making). The extracellular degradation of collagens can occur both in nonhelical sites and through a triple-helical cleavage. This is achieved by collagenases, which belong to the family of endopeptidases called MMPs. Collagenase-1 or MMP-1 (interstitial collagenase), collagenase-2 or MMP-8 (neutrophil collagenase), and collagenase-3 or MMP-13 are mammalian enzymes known to be able to initiate the intrahelical cleavage of triple-helical collagen at neutral pH.[15–17] The inhibition of the MMPs, which is one useful strategy for the treatment of many pathological conditions, has been the subject of intense research in recent years. Although

Correspondence to: B. Madhan (bmadhan76@yahoo.co.in) or J. Raghava Rao (clrichem@mailcity.com)

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collagen is usually used as a material for constructing artificial organs, collagen-based biomaterials are usually stabilized either by physical or chemical crosslinking on a macroscopic level to control the rate of biodegradation of the material, to suppress its antigenicity, and to improve the mechanical properties.[18] For example, chemical treatments such as succinylation, methylation, and acetylation have been applied for the modification of collagen molecules to control the rate of proteolysis.[19] However, such chemical modifications result in changes in the collagen structure, which result in poor interaction with the cells. Gluteraldehyde and formaldehyde have been used as collagen crosslinkers.[14,20] Recently, the use of these aldehydes has been discouraged because they elicit carcinogenicity.[21,22] Hence, it is important to have alternative methodologies for the preparation of stable collagenous materials for biomedical applications such as wound healing and bioprosthetics.

Vegetable tannins are plant polyphenols containing sufficient hydroxyl and other suitable groups (i.e., carboxyls) that effectively complex with protein and other macromolecules under suitable environmental conditions.[23,24] They are capable of crosslinking with collagen through the formation of multiple hydrogen bonds.[25,26] In pathophysiological conditions, polyphenol have also been shown to control the breakdown of both aggrecan (aggregated proteoglycans) and collagen in a model of cartilage degradation[27] through MMP inhibition.[28] They have been reported to be involved in the inhibition of the expression, secretion, and activation of MMPs.[29–31] *Acacia mollissima* (black wattle or mimosa) can be classified under catechol or condensed tannins, which are condensed proanthocyanidins based on flavonoids and polyhydroxy flavones.[32] *Terminalia chebula* (myrobalan) are pyrogallol or hydrolysable tannins, which are esters of phenol carboxylic acid with a high nutritious value and which have found use in medicinal applications (traditional medicines); it is now chiefly used in tanning, dyeing, and adhesive manufacture.[33–36]

Just like MMPs, bacterial collagenases such as *Clostridium histolyticum* collagenase (ChC) also degrade extracellular matrix. ChC belongs to the M-9 metalloproteinase family, which is able to hydrolyze triple-helical collagen and an entire range of synthetic peptide substrates under physiological conditions.[37–39] In fact, the crude homogenate of ChC, which contains several distinct collagenase isozymes, is the most efficient system known for the degradation of connective tissue.[40,41]

In this study, we evaluated the stabilization of collagen by plant polyphenolics, namely, wattle (*A. mollissima*) and myrobalan (*T. chebula*) tannin, against collagenase degradation.

EXPERIMENTAL

Materials

All reagents and chemicals were analytical grade. Collagenase (Type IA) and 2-furanacryloyl–L-leucyl–glycyl–L-prolyl–L-alanine (FALGPA) were sourced from [Sigma-Aldrich Corporation (Bangalore, India)] Wattle and Myrobalan extracts were procured from Rallis India, Ltd. (Chennai, India). All other reagents and chemicals used for the study were sourced from SRL, Ltd. (Mumbai, India).

Preparation of polyphenolics from wattle and myrobalan extracts

Wattle and myrobalan extract powders (1 g) were sequentially extracted with 100 mL of methanol/water (50 : 50, v/v) and 100 mL of acetone/water (70 : 30, v/v) for 1 h, in each case. Once centrifuged at 10,000 rpm for 15 min, the combined supernatants from the two previous extractions were concentrated in a vacuum rotary evaporator at 50°C; freeze-dried extracts were stored at −20°C. Total polyphenolic contents were determined according to the Folin–Ciocalteu method.[42] The resulting extract was used in this study.

Preparation of rat-tail tendon (RTT) for collagen degradation studies

Tendons from the tails of 6-month-old male albino rats (wistar strain) were teased and washed with 0.9% NaCl at 4°C to remove the adhering muscles and other soluble proteins. The RTT was subsequently washed extensively in double-distilled water before it was used for the degradation studies.

Monitoring of RTT collagen degradation by the inhibition of collagenase by the plant polyphenols

A known amount of collagenase was incubated with 0, 20, 40, 80, and 160 μ*M* of wattle and myrobalan solution for 18 h in 1 mL of 0.1*M* Tris-HCl (pH 7.4) containing 0.05*M* $CaCl_2$ at 25°C. Subsequently, the RTT collagen fibers were treated with the incubated samples of native and wattle- and myrobalan-treated collagenase. The ratio of RTT collagen to collagenase was maintained at 50 : 1, and the reaction was buffered at pH 7.4 with 0.1*M* Tris-HCl and 0.05*M* $CaCl_2$. The treated samples were incubated at 37°C and after 72 h. The reaction was stopped, and the mixture was centrifuged for 15 min at 10,000 rpm. The supernatant was analyzed for hydroxyproline and percentage collagen degradation was determined. The percentage inhibition by the polyphenols, namely, wattle and myrobalan, was calculated as differences in the percentage degradation of RTT collagen treated with native and polyphenol-treated collagenase:

$$\% \text{ Inhibition} = \left(\frac{\% \text{ COLLEGEN deg radation}_{\text{native collagenase}} - \% \text{ COLLAGEN deg radation}_{\text{polyphenol-treated collagenase}}}{\% \text{ COLLAGEN deg radation}_{\text{native collagenase}}} \right) \times 100$$

Kinetic investigations on the assay of native collagenase

A ChC assay with FALGPA as the substrate was performed according to a method reported earlier.[41] Assays were carried out spectrophotometrically by continuous monitoring of the decrease in absorbance of FALGPA after the addition of ChC. The FALGPA (at concentrations of 0.1–1.6 m*M*) was put in an appropriate amount of Tricine buffer (0.05*M* Tricine, 0.4*M* NaCl, and 10 m*M* $CaCl_2$, pH 7.5) and ChC (100 μL of 0.4 mg/mL) was added, and the final volume was adjusted to 1 mL. We monitored the course of the hydrolysis of FALGPA with a Varian Cary 100 UV–visible spectrophotometer by following the decrease in absorbance at 324 nm when [FALGPA] = 0.1 m*M*. At higher concentrations of FALGPA (0.2 and 0.4–1.6 m*M*), the decreases in absorbance were measured at 338 and 345 nm, respectively. An initial rate treatment was adopted by the treatment of the first 10% of hydrolysis of substrate according to standard methods.[43]

Kinetic investigations of the inhibition of collagenase by the plant polyphenols

The reaction of wattle- and myrobalan-treated ChC with FALGPA was performed under the same conditions mentioned as those used for the assay of native collagenase in this study. The ChC was treated with various concentrations of aqueous solutions of wattle and myrobalan (10, 20, 40, 80, and 160 μM) for 18 h at 25°C. The final concentrations of ChC in all of the treatments were maintained at a constant value (0.4 mg/mL). The FALGPA at concentrations of 0.1–1.6 m*M* was put in an appropriate amount of Tricine buffer (0.05*M* Tricine, 0.4*M* NaCl, and 10 m*M* $CaCl_2$, pH 7.5), polyphenol incubated ChC (100 μL) was added, and the final volume was adjusted to 1 mL. The hydrolysis of the substrate was monitored at corresponding wavelengths (immediately after the addition of plant polyphenol incubated ChC) as done in the case of native collagenase. The concentrations of the substrate (FALGPA) used were in the range 0.1–1.6 m*M*. The rate of hydrolysis was calculated with initial rate methods. The rate data were analyzed in terms of Michaelis–Menten treatment. From the Lineweaver–Burk plots of v^{-1} versus $[S]^{-1}$, the kinetic parameters, such as maximum velocity (V_{max}) and the Michaelis–Menten constant (K_m) of the enzyme, were calculated. The initial velocities were calculated from the slope of the absorbance changes during the first 10% of hydrolysis and converted into units of microkatals (μmol/s) by division to full hydrolysis of the substrate and multiplication by the substrate concentration.

Circular dichroism investigations of the plant polyphenol treated collagenase

The circular dichroism spectrum of type-IA collagenase with a concentration of 0.18 mg/mL in 1 m*M* acetate buffer (pH 4.0) was acquired at 25°C with a Jasco-J715 spectropolarimeter. The rate of nitrogen purging was maintained at 5 L/min up to 200 nm and increased to 10 L/min below 200 nm. A 2-point calibration was done with (+) 10 camphorsulfonic acid. The samples were prepared in double-distilled water. All of the solutions were filtered through 0.25-μm filters to remove suspended particles. A 0.1-cm cell was used for the experiments. A slit width of 1 nm was used. A scanning speed of 20 nm/min was used with an average of five scans per sample. Each spectrum was corrected by a baseline measured with the same solvent used in the sample. A reference spectrum was recorded with acetate buffer. The conformational changes in collagenase on interaction with wattle and myrobalan were investigated after incubation the enzyme with various concentrations (0.6–90 μ*M*) of wattle and myrobalan (average molecular weights of 2000 and 1500 Da were taken for wattle and myrobalan, respectively). The spectra obtained were deconvoluted with G and F and K2D programs, and the mean values of secondary structure components were tabulated.[44–46]

RESULTS AND DISCUSSION

MMP-1 (collagenase) plays an important role in diverse physiological processes, such as development, tissue morphogenesis, and wound repair. Likewise, it seems to be implicated in a variety of human diseases, including cancer, rheumatoid arthritis, osteoarthritis, pulmonary emphysema, and fibrotic disorders. It was of profound interest to us to establish how plant polyphenols (wattle and myrobalan) could inhibit the activity of ChC against collagen.

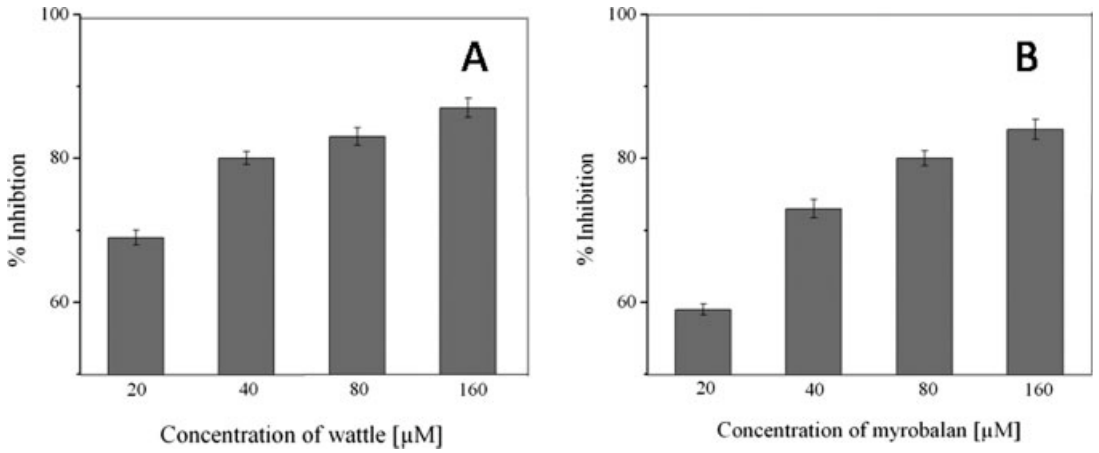

Figure 1 Percentage inhibition of collagen degradation by collagenase incubated with increasing concentrations of plant polyphenols: (A) wattle and (B) myrobalan.

Inhibition of collagenase by wattle and myrobalan polyphenolics against collagen degradation

To establish the effect of wattle and myrobalan in the inhibition of collagenase, RTT collagen fibers treated with native collagenase and collagenase incubated with various concentrations of wattle and myrobalan were studied. Wattle and myrobalan treatment of collagenase resulted in the inhibition of the collagenolytic activity against collagen [Fig. 1(A,B)]. Wattle at a concentration of 20 μ*M* exhibited 68% inhibition of collagenase against the degradation of RTT collagen fibers. The inhibition increased with increasing concentration of wattle, and at a concentration of 160 μ*M*, 88% inhibition was observed. In the case of myrobalan (160 μ*M*) treated collagenase, the inhibition of collagen degradation was observed to be 85%, which indicated that wattle could be more effective than myrobalan in the direct inhibition of collagenase.

Kinetic analysis of the inhibition of collagenase by the wattle and myrobalan polyphenolics

To establish the mechanism of inhibition of enzyme activity by wattle and myrobalan, collagenase treated with various concentrations of plant polyphenols (wattle and myrobalan) were studied for their ability to hydrolyze the synthetic substrate FALGPA at different concentrations. To analyze the inhibition of ChC by wattle and myrobalan, Lineweaver–Burk plots, that is, double-reciprocal plots, for the hydrolysis of FALGPA by wattle- and myrobalan-treated collagenase were determined [Fig. 2(A,B)]. The kinetic parameters K_m and V_{max} were calculated and are given in Table I. K_m obtained for native collagenase was 0.55 m*M* for the substrate FALGPA (25°C, pH 7.5 in 50 m*M* Tricine buffer containing 0.4*M* NaCl and 10 m*M* $CaCl_2$). The K_m value of the FALGPA hydrolysis by collagenase was similar to that obtained earlier.[39] V_{max} was found to be 0.244 ±

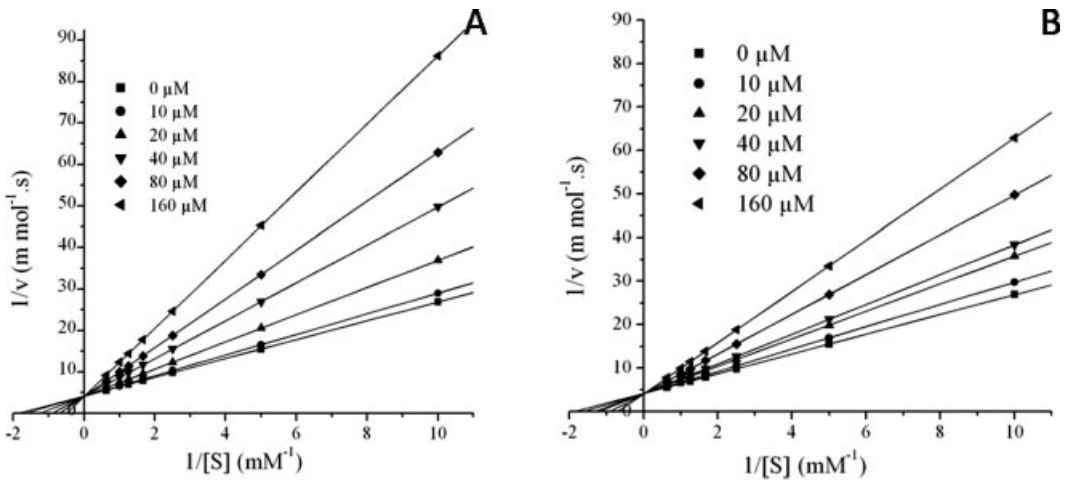

Figure 2 Lineweaver–Burk plots of FALGPA hydrolysis by collagenase in the presence of plant polyphenols. Assays were performed with various concentrations of FALGPA (0.1–1.6 m*M*) and plant polyphenols: (A) wattle and (B) myrobalan at concentrations of 0–160 μ*M*.

TABLE I
Michaelis–Menten Parameters for the Collagenase Hydrolysis of FALGPA at 25°C and pH 7.5 in the Presence of Various Concentrations of Wattle and Myrobalan

Compound	Concentration (μ*M*)	K_m (m*M*)	V_{max} (m mol/s)	Type of inhibition
Control (only collagenase)	—	0.55 ± 0.06	0.244 ± 0.02	
Wattle	10	0.606 ± 0.05	0.2439 ± 0.02	Competitive
	20	0.800 ± 0.11		
	40	1.111 ± 0.06		
	80	1.428 ± 0.08		
	160	2.000 ± 0.07		
Myrobalan	10	0.680 ± 0.06	0.2439 ± 0.02	Competitive
	20	0.790 ± 0.08		
	40	0.880 ± 0.09		
	80	1.110 ± 0.05		
	160	1.430 ± 0.12		

0.02 m mol/s. The Lineweaver–Burk plots obtained for FALGPA hydrolysis by collagenase incubated in the presence of different concentrations of wattle and myrobalan revealed that there was no change in V_{max} compared to the control. Both wattle and myrobalan exhibited dose-dependent inhibition of the activity of ChC. Lineweaver–Burk plots clearly showed a competitive inhibition [Fig. 2 (A,B)] for both wattle and myrobalan. From the K_m values (Table I), it was clear that wattle exhibited a significantly higher inhibition of the activity of ChC compared to myrobalan. The addition of 160 μ*M* wattle and myrobalan to collagenase resulted in K_m values of 2.0 ± 0.07 and 1.43 ± 0.12 mM, respectively. The competitive inhibition exhibited by both wattle and myrobalan may have worked through direct competition with the substrate by binding to the active site or binding to a remote site and causing a conformational change in the enzyme. Both the mechanisms gave identical inhibition kinetic results.

Structural changes in the collagenase on treatment with the wattle and myrobalan polyphenolics

The structural alterations in collagenase on binding with the polyphenols could also bring about the inhibition. The structure of collagenase on treatment with increasing concentrations of wattle and myrobalan was studied with circular dichroism. The structure of collagenase altered significantly after interaction with wattle and myrobalan, which was clearly observed from the circular dichroism spectra [Fig. 3(A,B)]. In the far UV region, collagenase exhibited double minima at about 210 and 220 nm and a maximum at 195 nm with a crossover point at about 200 nm.[47] Collagenase had α-helix, β-sheet, β-turn, and random-coil structures. The CD spectrum of the native collagenase (Fig. 3) exhibited double-negative bands at 208 and 222 nm and a positive band around 195 nm. These results were characteristics of the α-helix, whereas a peptide/protein with a β-sheet conformation alone will exhibit a single nega-

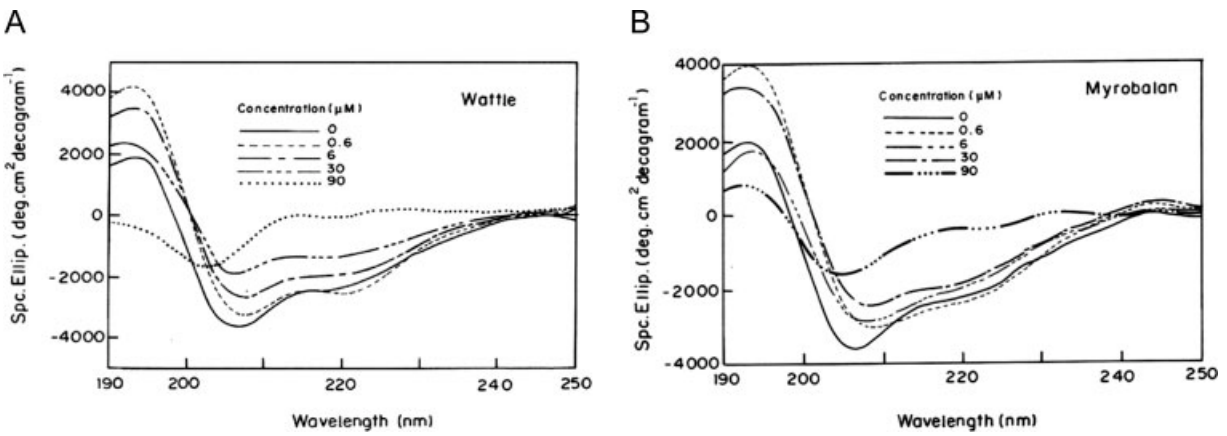

Figure 3 Circular dichroism spectra of collagenase treated with different concentrations of plant polyphenols (0.6–90 μ*M*): (A) wattle and (B) myrobalan.

TABLE II
Secondary Structure Contents of the Collagenase at Various Concentrations of Wattle and Myrobalan

Compound	Concentration (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Native	—	37	36	12	15
Wattle	0.6	26	25	6	43
	6	20	23	5	52
	30	7	9	2	80
	90	2	3	1	94
Myrobalan	0.6	30	33	6	31
	6	26	29	3	42
	30	6	7	1	86
	90	4	5	1	90

tive band between 215 and 225 nm. Collagenase had both α-helix and β-sheet structures almost equally distributed as its secondary structure, which may not have been clearly distinguishable through the CD spectrum. The secondary structural details could be predicted with deconvolution techniques.[44–46] From the analysis of the CD spectra with the CONTINLL package (CDPro, A software package for analyzing protein CD spectra, http://lamar.colostate.edu/~sreeram/CDPro/), we observed that the native collagenase contained an almost equal distribution of α-helix and β-sheet structures with 37 and 36% of each, respectively (Table II). Even treatment with 0.6 μM of wattle and myrobalan resulted in significant changes in the secondary structure of the collagenase. The secondary structure content analysis indicated that the 94% conformation of collagenase was randomized on treatment with 90 μM of wattle, and in the case of treatment with myrobalan, at similar concentration levels, 90% of the structural content was randomized. The structural alterations in collagenase on binding with wattle and myrobalan were clearly demonstrated with the circular dichroism study. Any structural alterations in an enzyme are known to affect the enzyme–substrate recognition process, as the structural fitting of enzyme in binding with the substrate is essential. The structural alterations in collagenase on interaction with the polyphenols was implicated in the inhibition of collagenolytic activity.

CONCLUSIONS

Plant polyphenols extracted from *A. mollissima* and *T. chebula* were shown to be effective in the prevention of collagen degradation against collagenase by inhibition of the latter competitively. Among the two polyphenolics chosen, namely, wattle and myrobalan, wattle exhibited better inhibition. Both wattle and myrobalan were found to alter the conformation of collagenase significantly, and the effect of structural alterations resulting in the inhibition of the collagenolytic activity was not ruled out. These polyphenolic compounds can be effective in the prevention of extracellular matrix degradation and, hence, may find use in the preparation of collagenous biomaterials.

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