doi: 10.53446/actamednicomedia.1627421

Acta Medica Nicomedia

Cilt: 8 Sayı: 1 Şubat 2025 / Vol: 8 Issue: 1 February 2025
https://dergipark.org.tr/tr/pub/actamednicomedia

Research Article | Araştırma Makalesi

BIOCHEMICAL AND HISTOLOGICAL ANALYSIS OF COLLAGEN CONTENT IN LUNG, LIVER AND KIDNEY TISSUES OF RATS TREATED WITH *BETA VULGARIS* L. VAR. CICLA

BETA VULGARIS L. VAR. CICLA VERİLEN SIÇANLARIN AKCİĞER, KARACİĞER VE BÖBREK DOKULARINDAKİ KOLLAJEN MİKTARININ BİYOKİMYASAL VE HİSTOLOJİK ANALİZİ

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ABSTRACT

Objective: Collagen is a fundamental component of the extracellular matrix (ECM) and plays a critical role in organ structure, cellular functions, and wound healing. *Beta vulgaris* L. var. cicla (chard) is known for its diverse bioactive compounds, including vitamins, flavonoids, and nitrates. Chard has been associated with numerous health benefits, such as antioxidant, anti-inflammatory, and antidiabetic effects. This study investigates the impact of chard on collagen content in vital organs, specifically the lung, liver, and kidney.

Methods: The rats divided into two groups: the control and the chard given group. The chard extract was administered to rats at a dose of 100 mg/kg per day for 7 days. On the 8th day, the rats were sacrificed, and tissues from the lung, kidney, and liver were collected. The collagen content was measured using both biochemical and histological analyses.

Results: Chard administration exhibited tissue-specific effects on collagen content: it increased collagen in the lung, decreased it in the liver significantly, and had no effect on kidney collagen. These biochemical changes were supported by histological results in the lung and kidney; however, no significant histological changes were observed in the liver. These varied effects might be related to differences in collagen metabolism and regulatory mechanisms across tissues.

Conclusion: The findings suggest that chard, due to its distinct effects on collagen synthesis and ECM remodeling, holds promise as a potential therapeutic agent for applications such as wound healing, tissue strengthening, and antifibrotic therapy. Further studies on the mechanisms underlying these effects are necessary to fully understand the potential of chard in clinical applications.

Keywords: Chard, collagen content, lung, kidney, liver

ÖZ

Amaç: Kollajen, ekstraselüler matriksin (ECM) temel bir bileşenidir ve organ yapısı, hücresel işlevler ve yara iyileşmesinde kritik bir rol oynar. *Beta vulgaris* L. var. cicla (pazı), vitaminler, flavonoidler ve nitratlar dahil olmak üzere çeşitli biyoaktif bileşenleriyle bilinir. Pazı, antioksidan, anti-inflamatuar ve antidiyabetik etkiler gibi çok sayıda sağlık yararıyla ilişkilendirilmiştir. Bu çalışma, pazının akciğer, karaciğer ve böbrek gibi hayati organlardaki kollajen miktarı üzerindeki etkisini araştırmaktadır.

Yöntem: Sıçanlar iki gruba ayrıldı: kontrol ve pazı verilen grup. Pazı ekstresi sıçanlara 7 gün boyunca günde 100 mg/kg dozda verildi. 8. günde sıçanlar sakrifiye edildi ve akciğer, böbrek ve karaciğer dokuları toplandı. Kollajen miktarı hem biyokimyasal hem de histolojik analizler kullanılarak ölçüldü.

Bulgular: Pazı uygulaması, kollajen içeriği üzerinde dokuya özgü etkiler gösterdi: akciğerdeki kollajeni artırdı, karaciğerdeki kollajeni önemli ölçüde azalttı ve böbrek kollajeni üzerinde hiçbir etkisi olmadı. Bu biyokimyasal değişiklikler, akciğerdeki ve böbrekteki histolojik sonuçlarla desteklendi; ancak karaciğerde önemli bir histolojik değişiklik gözlenmedi. Bu çeşitli etkiler, dokular arasında kollajen metabolizmasındaki ve düzenleyici mekanizmalardaki farklılıklarla ilişkili olabilir.

Sonuç: Bulgular pazının kollajen sentezi ve ECM'nin yeniden şekillenmesi üzerindeki belirgin etkileri nedeniyle yara iyileşmesi, doku güçlendirme ve antifibrotik tedavi gibi uygulamalar için potansiyel bir terapötik ajan olarak umut vadettiğini göstermektedir. Pazının klinik uygulamalardaki potansiyelini tam olarak anlamak için bu etkilerin altında yatan mekanizmalar üzerine daha fazla çalışma gereklidir.

Anahtar Kelimeler: Pazı, kollajen miktarı, akciğer, böbrek, karaciğer

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Submitted/Başvuru: 26.01.2025 • **Accepted/Kabul:** 12.02.2025 • **Published Online/Online Yayın:** 28.02.2025

Introduction

Collagen is an essential component of the extracellular matrix (ECM) in the development of connective tissues such as cartilage, tendons, and ligaments, as well as various organs, including skin, heart, liver, kidneys, lungs, blood vessels and bones.[1] In addition to its structural role, it has important cellular functions including adhesion, migration, autophagy, apoptosis and proliferation.[2] Collagen belongs to a family of fibrous proteins characterized by a triple-helical structure. More than 30 different types of collagen have now been identified and documented.[1] The primary collagen types found in the ECM are collagen types I and III, although types IV, V, VI, and VIII are also present. Fibroblasts are capable of producing collagen in the tissues. Matrix metalloproteinases (MMPs) such as collagenases and gelatinases play a critical role in collagen turnover by breaking down intact and damaged fibrillar collagen, respectively. They occur in development, wound healing, and major inflammatory diseases.[1,3] Under the normal physiological conditions, there is a balance between collagen production and breakdown. While collagen degradation is linked to inflammation, angiogenesis, and re-epithelialization, collagen biosynthesis is linked to the healing of wounds. Since the injury and healing of a tissue requires a tightly regulated process, defects in the collagen turnover lead to pathological diseases, including fibrosis.[1] Wound healing and fibrotic diseases have some common features. Collagen deposition is an essential and usually reversible aspect of wound healing. However, in cases of severe or repeated tissue injuries or disruptions in the healing process, it becomes a key factor in the transition from normal tissue repair to an irreversible fibrotic state.[4] Changes in the original tissue architecture of an organ caused by elevated collagen levels can lead to stiffness and a loss of functional cells, ultimately impairing the organ's function.[5]

Beta vulgaris L. var. cicla, commonly known as chard, is a green leafy, low-cost vegetable whose bioactive compounds have been the subject of research for their health benefits. It is a member of the Chenopodiaceae family and is distributed all over the world, being widely used in many traditional dishes. The leaves can be eaten raw in a salad, cooked separately, or combined with the stems. Chard has many chemical compounds such as fatty acids, phospholipids, glycolipids, polysaccharides, pectins, saponins, flavonoids, phenolic acid, betalain, vitamins A, B, C E, K, calcium, iron, phosphorus, zinc, magnesium, potassium, copper and manganese. These certain bioactive compounds' effects have been shown to be hepatoprotective,[6] anti-cancer and anti-inflammatory,[7] anti-diabetic,[8] anti-acetylcholinesterase and antioxidant[9-12.] The medicinal value of chard is also well documented.[13-15]

As collagen is crucial for healthy organs, the present study examined whether chard has an effect on the amount of collagen in vital organs, such as the lungs, liver and kidneys, through biochemical and histological analyses. The characteristics of bioactive compounds can create challenges for their use as potential therapeutic agents. There is a lack of comparison of the effects on normal groups based on tissue distribution in the body after consumption. The results of the study may help clarify the relationship between chard consumption and health outcomes in terms of collagen content.

Methods

Chemicals

All chemicals used in the experiments were of analytical purity and were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, USA), and Fluka (Buchs, Switzerland).

Plant Extract

Chard leaves were sourced from markets in Istanbul, Türkiye, and authenticated by Prof. Dr. Kerim Alpınar from Istanbul University Faculty of Pharmacy (Voucher specimen number: 67901). The leaves were rinsed with distilled water and air-dried at 25°C. A total of 100 g of dried chard leaves were boiled in 1 L of distilled water for 8 hours, reaching the boiling point. After filtration, the water was removed using a rotary evaporator, and the resulting chard extract was weighed. It was dissolved in distilled water to be administered to the animals.

Animal Groups

The study was carried out with the permission of the Marmara University Animal Experiments Local Ethics Committee (Approval No: 19.2024.mar). The twenty female Sprague-Dawley rats of 3 months old, weighing 250-350 g, were chosen to be used. The experiment consisted of control group (C, n=6) and chard given group (chard, n=6). In the C group, rats received saline (0.9 %NaCl) orally. In the chard group, rats were administered chard extract by gavage at a dose of 100 mg/kg per day for 7 days. On the eighth day, the rats were sacrificed, and lungs, kidneys and liver tissues were collected. Tissues were fixed in 10% formaldehyde for detection of collagen density by histological and biochemical analyses.

Biochemical Analysis of Tissue Collagen

Tissue collagen was measured by the colorimetric method.[16] Five-micrometer-thick lungs, kidney and liver sections were cut from each paraffin block and placed on glass slides. Six slides were collected from each animal, with each slide having 2 tissue sections. An avarage of slides was calculated and used for analysis. The tissue sections were deparaffinized with xylene, rehydrated in a graded series of alcohol solutions, and stained with a saturated solution of picric acid in distilled water containing 0.1% fast green (Sigma-Aldrich F7252) and 0.1% of sirius red (Fluka 43665). Sections were incubated in the dark, at room temperature for 30 minutes. Then, sections were rinsed and transferred to a test tube containing 1 mL of absolute methanol and 0.1 N NaOH (1:1, v/v). The tubes were gently mixed until the color

was eluted completely. Absorbance of the eluted color was read at 540 nm and 605 nm by spectrophotometer. Collagen content of tissues were calculated using the formula below. The results were expressed as a collagen ratio (%).

Non-collagenous protein (mg) = Absorbance at 605 nm / 2.08

Collagen (mg) = [Absorbance at 540 nm - (0.291 x Absorbance at 605 nm)] / 38.4

Collagen ratio (%) = (mg Collagen x 100) / (mg Collagen + mg Non-collagenous protein)

Histological Analysis of Tissue Collagen

After fixation with 10% formaldehyde, tissues were processed routinely for paraffin embedding. Five-micrometer-thick paraffin sections were stained with Masson's trichrome for collagen fiber detection. Stained sections were photographed with a camera (Olympus DP72, Tokyo, Japan) attached to a photomicroscope (Olympus BX51, Tokyo, Japan). To calculate the percentage of the mean area of collagen fiber deposition, five images from five non-overlapping areas in each tissue samples were analyzed, with quantification carried out using ImageJ software (ImageJ, v.2.1, NIH, USA).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9.0.1 (GraphPad Software, San Diego, CA, USA). For every group, the data were presented as mean ± standard error (SE). The results were analysed statistically with the Student's t-test, according to normal distribution. p-values below 0.05 are regarded as significant.

Results

Biochemical Analysis

The collagen content in tissues was shown in Figure 1. No change was observed in kidney tissue. A decrease was found in the liver ($p<0.001$), an increase was found in the lung tissues ($p<0.0001$) in the chard group compared to the control group.

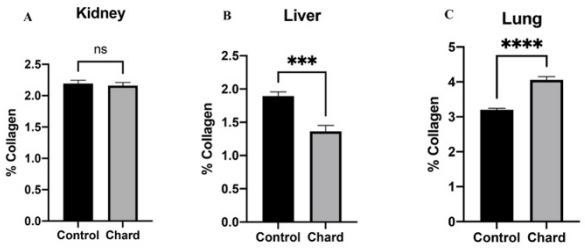

Figure 1. Collagen content in the kidney, liver and lung tissues of the control and chard given group.
Values are given as mean ± standard error. Each group consists of six rats. ***p<0.001, ****p<0.001 means significantly different from control group, ns means not significant.

Histological Analysis

The mean area percentage of collagen fibers in Masson's trichrome stained sections was shown in Figure 2. With the administration of chard, an increase was observed in lung tissue ($p<0.05$), while no statistically significant changes were observed in the liver and kidney tissues.

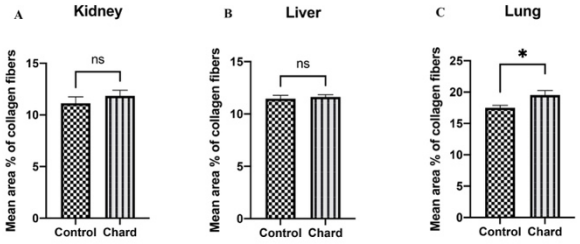

Figure 2. The percentage of the mean area of collagen fiber deposition in the kidney, liver, and lung tissues.
Values are given as mean ± standard error. Each group consists of six rats. *p<0.05 means significantly different from control group, ns means not significant.

In Figure 3, the collagen distribution in the kidney, liver, and lung samples were shown for the control and chard groups. In the kidney tissue sections of the control and chard groups, collagen fibers were detected in the renal corpuscle and tubulointerstitial area. No statistically significant difference in collagen density was found between the control and chard groups. In the liver tissue sections of the control and chard groups, collagen fibers were detected in the parenchyma and portal areas. Similarly, no statistically significant difference was found in collagen density between the control and chard groups. In the lung sections of the control and chard groups, collagen fibers were detected in the inter-alveolar septa, around the bronchioles, and the vessels. The amount of collagen fibers in the chard group was significantly higher than in the control group ($p<0.05$).

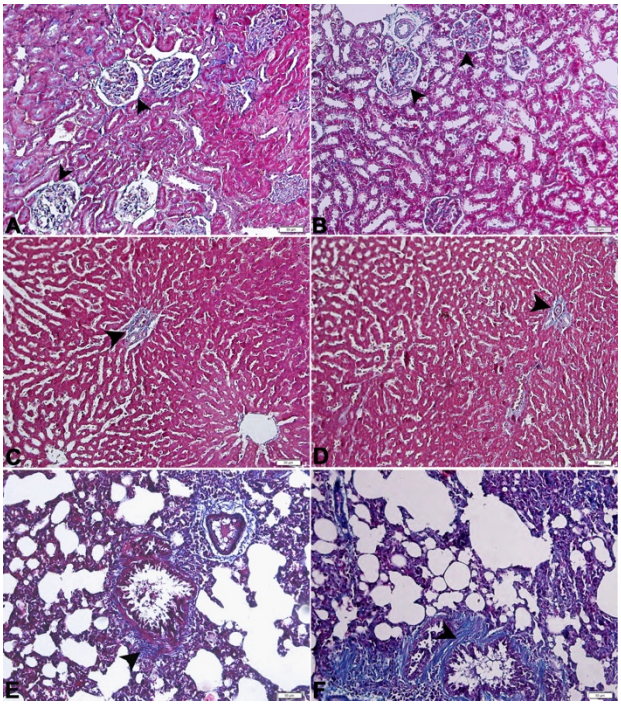

Figure 3. Representative light micrographs of control (A, C, and E) and chard (B, D and F) groups. Collagen fibers (arrowheads) in the renal corpuscle of kidney samples (A and B). Collagen

fibers (arrowheads) in the portal areas of liver samples (C and D). Collagen fibers (arrowheads) around bronchioles in lung samples (E and F). Masson trichrome (with aniline blue) staining. Scale bars: 50μm

Discussion

Collagen is produced and modified by fibroblasts and many other cell types. In general, each tissue has its own distinct ECM with a complex three-dimensional structure.[1] Additionally, fibroblasts can degrade collagen using specialized enzymes known as collagenases. They have critical role in degradation and remodeling of ECM.[17] Collagen molecules are made up of various amino acids, including glycine, proline, hydroxyproline, and alanine. Three polypeptide chains are joined together to form alpha triple helices. Disulfide bonds are established both within individual polypeptide chains and between neighboring chains. Vitamin C and iron are essential for the formation of these bonds. These bonds provide structure and stability to the triple-helix collagen macromolecule. This structure, along with the involvement of modified amino acids, makes collagen biosynthesis a complex process requiring multiple factors both inside and outside the cell.[18] The synthesis of collagen, an essential structural protein in the body, can be supported by a diet rich in amino acids, which constitute the protein's primary structure, along with cofactors such as vitamin C and iron.[19] Chard contains carbohydrates, fats, proteins, fibers, carotenoids, flavonoids, minerals, pigments, non-flavonoid phenolics, and vitamins. In particular, the leaves are rich in fiber, magnesium, iron, flavonoids, and vitamin C, making chard one of the best sources of these nutrients.[13,14]

In addition to collagen's role in supporting tissue structure, excessive collagen accumulation is a hallmark of organ fibrosis. The overexpression of collagen in fibrotic kidneys, liver and lungs is a key factor in tissue dysfunction. As fibrosis advances, the amount and distribution of collagen undergo significant changes.[17,20,21] Inhibiting key enzymes in collagen synthesis or promoting collagen degradation via overexpression of MMPs could help accelerate fibrosis resolution, presenting a potential therapy.[18] Therefore, understanding the impact of chard on collagen levels in various tissues is essential for advancing health insights. The effect that is decreased of cardiac collagen and MMP-1 levels with Swiss chard juices in barium chloride intoxicated rats has been reported.[22] However, there is no study to clarify the effect of chard extract on collagen content in healty animals.

Experimental Design and Duration

The dose and duration of chard were determined based on the study by Ustundag et al.[23] The administered chard dose and treatment period in this study, 100 mg/kg/day for 7 days, align with procedures applied in other animal studies investigating its antioxidant properties[24,25] and are considered an effective dose and appropriate duration to induce metabolic changes. Based on the results of the present study, a 7-day treatment period was sufficient to observe changes in collagen levels, but longer treatment durations may offer further insights into the effects of chard on collagen content.

Kidney

Collagen is a key component of the kidney and is extensively distributed across all kidney tissues. In the context of healthy kidney, type I and III collagen, are the most common collagen in the interstitial matrix of the kidney, type IV collagen is a key component of the glomerular basement membrane, and type VI is found in the interstitium, the intima and adventitia layers of the kidney vasculature.[21] Normal collagen molecules interact with extracellular matrix proteins to create an appropriate microenvironment for renal cells, influencing their physiological functions. Abnormalities in collagen can interfere with the connection between renal cells and matrix molecules, leading to various kidney diseases.[17] Thus, the collagen turnover pathway is a primary target for drugs aimed at addressing the progression of certain types of kidney disease. Our findings showed that kidney collagen content remained unchanged after chard administration. This conclusion is further corroborated by our histological findings. Yanardag et al. showed that chard extracts partially reduced degenerative changes in the kidneys of STZ-induced diabetic rats; however, serum urea and creatinine levels did not differ from those of the control group.[9] The lack of effect of chard on kidney collagen levels, despite its impact on the lungs and liver, likely results from the precise regulation of collagen production in specific tissues and cell types, primarily controlled at the transcriptional level.[26] Another reason could be that collagen turnover (synthesis and breakdown) rates might vary across organs[1] and may make kidney less responsive to the modulatory effects of chard's bioactive compounds. However, studies in the literature indicate that flavonoids impact kidney collagen. A study by Zhou et al. found that curcumin, which was a kind of flavonoid, decreased the accumulation of collagen in the kidney of animals with unilateral ureteral obstruction.[27] Furthermore, Ren et al. showed that quercetin, which was another kind of flavonoid, suppressed collagen deposition in the obstructive kidneys.[28]

Liver

Our current study showed that the effect of chard application on collagen levels varied between different tissues. We found that chard treatment reduced liver collagen levels; however, this reduction could not be demonstrated histologically. This suggested that the liver's collagen turnover might have been altered by chard administration, but the structural changes in the liver may not have become apparent during the experiment. In a healthy liver, interstitial fibrillar collagens like types I, III, and V are mainly found in the space of Disse, the portal tract, and central vein walls. Basement-type collagen, primarily type IV, is located in

the sinusoidal walls, forming a network, as well as around bile ducts. Both types are present in low amounts, just enough for normal function.[18] The collagen content of tissues is influenced by changes in both the synthesis and degradation rates of collagen.[29] Chard, which is rich in vitamin E, may have reduced collagen synthesis. It is known that vitamin E inhibits proliferation and collagen synthesis of hepatic stellate cells.[30] The inhibition or reversal of hepatic stellate cell activity is a potential therapeutic strategy for liver fibrosis.[31] Polyphenolic compounds and polyphenol-rich extracts have been shown to improve collagen homeostasis in the liver.[32] The water extract of chard leaves is rich in phenolic compounds, including vanillic acid, caffeine, ellagic acid and pyrogallol, as well as flavonoids, such as hesperidin, rosmarinic acid, luteolin, and derived from apigenin namely vitexin.[33,34] Treatment with apigenin has been shown to alleviated hepatic fibrosis models through the TGF-β1/Smad3 and p38/PPARα signaling pathways. Protein expressions of collagen 1 and matrix metalloproteinase inhibitor 1 were decreased, while expression of matrix metalloproteinase 2 was found to be increased with apigenin treatment.[35] It has been reported that, in cholestasis-related liver injury, rosmarinic acid suppresses matrix-producing cells and fibrogenic changes by reducing hepatic collagen and hydroxyproline content and inhibiting matrix metalloproteinases and tissue inhibitor of metalloproteinases mRNA expression.[36] Since liver fibrosis can occur in numerous diseases, the discovery of effective anti-fibrotic treatments would represent a major advancement by addressing a critical medical need. Thanks to its high antioxidant properties[37,38] chard may have the potential to ameliorate the initial tissue damage that triggers liver fibrosis, suggesting that its ability to reduce liver collagen could have a positive effect on fibrosis treatment during administration.

Lung

The lung functions as a biomechanically dynamic organ. Collagen in the healthy lungs creates a dense fibrous network throughout the major airways, bronchi, and bronchioles, offering the strength and stability necessary for their proper function. The lung parenchyma, the area responsible for gas exchange, contains as an interstitial matrix primarily composed of collagens I and III. The basement membrane includes collagens IV, VI, and XVII. Collagens in the lung's basement membrane and interstitial space serve as essential molecular frameworks for key physiological processes, including fibroblast proliferation, migration, and adhesion.[20] The load-bearing capacity of lung tissue is attributed to its collagen content, along with elastin fibers and glycosaminoglycans. Lung diseases are partially associated with changes in the composition, quantity, and organization of the extracellular matrix in different compartments of the lung.[39] Collagen provides tensile strength, while elastin enables extensibility and elastic recoil in the airways. Together, they likely influence both bronchoconstriction and the reopening of airways. An increase in collagen and elastic fiber content in the airway walls may contribute to persistent obstruction in asthmatic airways. Nevertheless, it has also been suggested that increased collagen may have a protective role by stiffening the airways, thereby resisting the forces generated by airway smooth muscle contraction.[40] We found an increase in lung collagen after chard was administered and we demonstrated the increase in collagen in the lung histologically. Sacan and Yanardag have explained that chard has high proline content.[10] Findings in the study by Shaw et al. suggest that the presence of proline and vitamin C can enhance collagen production and improve tissue mechanics in engineered ligaments.[41] Vitamin C, which is abundant in chard, is involved in collagen synthesis, helping to preserve the integrity of blood vessels and lung tissue. Vitamin C is thought to support the integrity of the endothelial barrier, which is crucial in preventing fluid leakage into the lungs. This could aid in the repair and regeneration of damaged lung tissue in acute respiratory distress syndrome.[42] It enhances collagen mRNA production in fibroblasts.[43] In the present study, the high proline and vitamin C content in chard may contribute to increased collagen levels in the lungs. Because the lungs are directly exposed to high levels of oxygen, they are more susceptible to oxidative injury; therefore, protecting the lungs is important. Additionally, we suggest that chard's antioxidant and anti-inflammatory properties may help protect collagen from degradation and contribute to maintaining lung integrity. Chard is also high in nitrate. In the oral cavity, bacteria and xanthine oxidase reduce nitrate to nitrite, which is then converted to NO by xanthine oxidoreductase, deoxyhemoglobin, myoglobin, respiratory chain enzymes, vitamin C, polyphenols, and protons.[44] For many years, it has been recognized that nitrates promote bronchial relaxation. Increased intake of nitrate-rich green leafy vegetables, along with dietary nitrate supplementation, has been shown to improve endothelial and cardiovascular function, offering a potential approach to modulate vascular disease development in conditions such as hypertension, diabetes, and atherosclerosis.[45] Hu et al. also reported that dietary nitrate enhanced skin microvascular density in the wound area, encouraging cell expression and collagen fiber deposition.[46] In the present study, the increase in collagen in the lung may also be related to the nitrate content of chard. Collagen accumulation relies on various factors, including the rates of gene transcription and mRNA translation, post-translational modifications, secretion processes, and the degradation of newly synthesized collagen.[47] Collagen metabolism and accumulation are precisely regulated by collagenases and their inhibitors. This study also suggests that increased collagen accumulation in the lung may occur through the collagenolytic pathway, a mechanism that chard may potentially modulate. To clarify this area of study, the effects of chard on MMPs and TIMPs should be thoroughly investigated. Apart from all this, increased collagen in lung tissue should also be considered in relation to pulmonary fibrosis development. Therefore,

individuals at risk of lung fibrosis may need to limit their consumption of chard.

The main limitation of the present study is that tissue biochemical parameters were not estimated. We were also unable to fully explain the most likely mechanisms underlying chard's effects on the differences in collagen metabolism across tissues. However, the aim of the this study was not the investigation of these mechanisms, but, first of all, showing whether the chard, which we consume as a food, affects collagen levels in liver, kidney and lung in healthy individuals. The possible mechanisms of the impacts of chard will be evaluated in further studies.

In conclusion, the use of complementary and alternative medicine, including dietary supplements with plant-derived phytochemicals, is increasingly popular for health promotion and treatment. Vegetables like chard are affordable, widely accessible, and generally more acceptable to patients than conventional drugs, suggesting that their consumption could serve as a potential therapeutic option. Chard, in particular, may have the potential to influence collagen production across various tissues. It increased collagen in the lungs, reduced it in the liver, and had no effect on kidney collagen levels. Given its diverse effects, chard showed promise for development as a therapeutic agent across a range of applications, including wound healing, tissue strengthening, and antifibrotic therapy. Nevertheless, individuals with fibrosis may consider limiting their intake of chard.

Ethical Approval

The study was carried out with the permission of the Marmara University Animal Experiments Local Ethics Committee (Approval No: 19.2024.mar).

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Concept & Design: AY, RY; Data Collection or Processing: BAT, AM, SO, EA, ST; Analysis or Interpretation: BAT, AM, SO, EA; Resources: AY, RY, EA; Writing – original draft preparation: AY, RY, BAT, EA; Writing – review and editing: BAT, AM, SO, EA, ST, RY, AY; Supervision: AY, RY.

Financial Disclosure

The authors declared that this study has received no financial support.

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