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The effect of rosemary and lemon balm extracts and rosmarinic acid on collagen type I metabolism in fibroblasts from Osteogenesis Imperfecta patients

Doctoral dissertation based on a series of scientific publications

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ABBREVIATIONS

4-PBA	4-Phenylbutyrate
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATG	Autophagy-related gene
BiP	Binding immunoglobulin protein
BR	Bortezomib
CHOP	CCAAT/enhancer-binding protein homologous protein
CRTAP	Cartilage-associated protein
CypB	Cyclophilin B
ECM	Extracellular matrix
eIF2α	Eukaryotic translation initiation factor-2α
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
HSP47	Heat shock protein 47
IRE1	Inositol requiring enzyme 1
LAMP2A	Lysosomal-associated membrane protein 2A
LBE	Lemon balm extract
LC3	Microtubule- associated protein 1 light chain 3
LC-PDA-MS	Liquid chromatography-photodiode array detection-mass spectrometry
MMPs	Matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MT-MMP1	Membrane-Type Matrix Metalloproteinase 1
OI	Osteogenesis imperfecta
P4H	Propyl 4-hydroxylase
P4H	Propyl 3-hydroxylase
PDI	Protein disulfide isomerase
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
RA	Rosmarinic acid
RANKL	Receptor activator for nuclear factor κB ligand
RE	Rosemary extract
SDS-urea-PAGE	SDS-urea polyacrylamide gel electrophoresis
SQSTM1/p62	Sequestosome-1
TGF-β	Transforming growth factor beta
UPR	Unfolded protein response
XBP-1s	X-Box binding protein 1

INTRODUCTION

1. Osteogenesis imperfecta (OI)

Osteogenesis imperfecta (OI) is an inherited disease of the connective tissue manifested mainly by defects in the skeletal system (bone fragility, skeletal deformities, reduced bone mineral density, short stature), but also a number of extra-skeletal symptoms such as blue sclera, hearing impairment, skin fragility, joint laxity, muscle weakness, tooth abnormalities (dentinogenesis imperfecta), cardio-respiratory defects and impaired pulmonary function [1-3]. The prevalence of OI has been estimated at 1 in 15 000 to 20 000 live births [1, 2]. The disease is characterized by phenotypic and genotypic heterogeneity [1-6], and variable expression of the same mutation, the causes of which are still not fully understood [7, 8]. Despite the large number of mutations discovered in many different genes, the majority (85-90%) of patients have dominant mutations in *COL1A1* (Online Mendelian Inheritance in Man (OMIM) 120150) and *COL1A2* (OMIM 120160) genes encoding α1(I) and α2(I) chains of type I collagen [1-8].

The classification of OI due to the collagen type I mutations includes four clinically defined types, the phenotype of which ranges from mild non-deforming to the lethal [9, 10]. Patients with absence of deformities of bones and usually blue sclera are classified as mild type I, with the perinatal lethal as type II, with the most severe deforming form as type III and with moderate bone deformities and short stature as OI type IV.

Many other causative mutations in non-collagenous genes, related to either dominant or recessive forms of the disease, code for proteins that are involved in folding and post-translational modifications of type I collagen (*CRTAP*, *P3H1*, *PPIB* and *BMP1*) or in its intracellular trafficking (*FKBP10* and *SERPINH1*), quality control of collagen biosynthesis, and ER stress response (*CREB3L1* and *MBTPS2*). Other genes encode proteins that are secreted by osteoblasts and can bind to collagen in the extracellular matrix (ECM) and influence mineralization during bone formation (*SERPINF1* and *SPARC*). Some are important in the anabolic bone function of the canonical WNT signaling pathway (*LRP5*, *WNT1*, and *MESD*), and other genes encode proteins that influence osteoblasts in ways not fully understood (e.g. *SP7* and *TENT5A*). A new classification based on the causative gene of more rare mutations identifies twenty OI subtypes [1-6, 11-12].

2. Biosynthesis and degradation of type I collagen

Type I collagen is one of the most abundant proteins of the ECM of various connective tissues such as bone, skin, cornea and tendon [13-15]. It is not only a structural scaffold of tissues and organs responsible for their mechanical strength, but also plays an important role in adhesion, migration, growth regulation and metabolism of cells [13-15]. In bone it forms the scaffolding of the bone matrix providing a template for mineralization, balancing bone hardness and elasticity [16]. Type I collagen is a heterotrimer composed of two α1 and one α2 chains. They are is synthesized as a pro-α chains with N- and C-propeptides at both ends. The C-propeptide is involved in the association of the pro-α chains and the formation of a triple helix that goes towards the N-terminus. The triple helix consists of 1014 amino acids, forming the characteristic repeating Gly-X-Y triplets. Glycine as the smallest amino acid is the only one that can fit into the limited space of the central core of the triple helix, while X and Y are often proline and hydroxylated proline, respectively.

Biosynthesis of type I collagen is a very complex process involving intracellular and extracellular steps leading to the formation of mature collagen fibrils [13, 14, 17]. After transcription of *COL1A1* and *COL1A2* genes and then translation and translocation of procollagen chains to the rough endoplasmic reticulum (ER), free chains undergo post-translational modifications before forming the triple helix. These include hydroxylation of lysine and proline residues involving collagen-specific enzymes: prolyl-4 hydroxylase (P4H), prolyl-3 hydroxylase (P3H) and lysyl hydroxylase (LH). Hydroxylysine residues are additionally a place for glucosyl and galactosyl residues attachment. O-glycosylation of hydroxylysine is catalysed by hydroxylysyl galactosyltransferase and galactosylhydroxylysyl-glucosyltransferase. These modifications are of fundamental importance because they determine the process of folding, stability, secretion of procollagen as well as collagen fibrillogenesis, cross-linking, mineralization and collagen-cell interactions [13-15, 17-20].

In modifications, folding and secretion of procollagen are essential chaperones that protect unfolded chains against non-specific interactions and their aggregation as well as catalyse the isomerization of disulfide bonds [19-23]. P4H is composed of two α and two β subunits, where the β subunit as the protein disulfide isomerase (PDI) acts as ER chaperone. PDI catalyzes the formation of disulfide bonds between procollagen chains and prevents their aggregation [21]. The triple helix formation proceeds in a zip-like manner and its rate of formation depends on the cis-trans isomerization of proline residues by peptidyl-prolyl cis-trans isomerases, e.g. FKBP65 acting as a chaperone [21]. It has also been shown that mutations in

the genes encoding chaperone proteins like P3H/cartilage-associated protein (CRTAP)/cyclophilin B (CypB) complex, responsible for 3-hydroxylation of Pro986 in the α1(I), or heat shock protein 47 **(**HSP47) lead to severe or lethal phenotypes of OI [22, 23]. HSP47 was found to bind preferentially to the triple helix and plays the critical role in the protection of the triple helix conformation during the export of procollagen from the ER to the Golgi apparatus [21, 23].

Secretion of procollagen type I is followed by cleavage of N- and C-terminal propeptides by specific procollagen proteinases. After removing the propeptides, collagen molecules spontaneously assemble into quarter-staggered fibers, stabilized by intramolecular and intermolecular cross-linking, catalyzed by the lysyl oxidase [13, 14, 16, 18, 24].

To maintain cell homeostasis, the ECM undergoes a constant turnover in which collagen is degraded and newly synthesized [24-26]. Collagen with a specific triple helix conformation and complex supramolecular structure is highly resistant to degradation by most proteases. Enzymes capable of digesting this structure are captesin K and matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases [25]. In degradation of interstitial collagen fibrils (type I or type III collagen) are involved MMP-1, MMP-8, MMP-13 and gelatinase MMP-2. MMP-1 cleaves triple helix into two distinctive fragments, 1/4 C-terminal and 3/4 N-terminal [25]. MMP-2 gelatinase can also act as a weak interstitial collagenase [26]. The degradation of intracellular collagen can occur through phagocytosis of intact collagen fibrils or endocytosis of already cleaved collagen particles via integrin receptors (e.g. α1β1, α2β1). Initial degradation of fibrous collagen is mediated by membrane-type MMP1 (MT-MMP1). The intracellular degradation of collagen takes place in lysosomes, which contain a number of cathepsins, including cathepsins B, D, K and L which cleave collagen into low molecular weight peptides [24]. The same pathway is used to degrade newly synthesized collagen prior to secretion when the collagen is misfolded in so-called lysosome-dependent autophagy, where lysosomes fuse with vesicles derived from ER or the Golgi apparatus. This process will be discussed later when describing the cell's response to the presence of mutant type I collagen.

3. Mutations in collagen type I genes

Mutations of collagen type I genes, depending on the type, can result in quantitative or structural defect in the collagen type I. The decrease in biosynthesis of structurally normal type I procollagen is caused by a null *COL1A1* allele due to premature stop codons, either directly or through frame shifts mutations [27, 28]. The heterozygous deletions of the entire *COL1A1*

gene was also reported [29]. Reducing the expression of normal collagen by half and the lack of mutated collagen is the cause of non-deforming the mildest type I phenotype. The collagen type I mutations which are the cause of severe and lethal OI include mainly substitutions of glycine residues with another amino acid (80%), but also mutations of the splicing site, as well as small deletion or duplication that shift the register of α chains in the helix [1-8, 30-32]. They are defined as dominant-negative, because even in the presence of two normal and one mutant chains in the collagen molecule, the structure and function of such collagen is disturbed and may be degraded. The abnormal structure of this molecule results from excessive modifications (hydroxylation and glycosylation) of the procollagen chains due to the delay of the triple helix formation from the site of the mutation to the N-terminus [1-3, 5, 6, 30, 33]. The resulting mutant collagen is secreted into the ECM but also accumulates inside the cell, which can cause ER stress. The cell consequently activates the defense system, the unfolded protein response (UPR) to maintain the functional integrity of the ER under stress conditions by improving the conformation and secretion of collagen.

4. ER stress and Unfolded Protein Response

The UPR consists of three major signaling pathways mediated by ER transmembrane receptors: protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) (Figure 1).

All three sensor under normal condition are bound by the chaperone - binding immunoglobulin protein (BiP), while under stressful conditions BiP binds preferentially to misfolded proteins and activates UPR pathways [34, 35]. PERK through phosphorylation of eukaryotic translation initiation factor (eIF2α) inhibits total protein synthesis, but favors the translation of some proteins, such as the activating transcription factor 4 (ATF4), which is involved in cell survival but also in ER stress-dependent apoptosis [36]. During chronic stress, UPR promotes apoptosis by upregulating of pro-apoptotic genes, e.g. the homologous protein of the CCAAT enhancer binding protein (CHOP) [34-36]. ATF6 moves to the Golgi apparatus, where undergoes activation by its proteolytic cleavage and then enters the nucleus and activates the transcription of target genes [34, 35]. Activated IRE1 forms an alternative spliced variant of the transcription factor X-Box binding protein 1 (XBP1s), which increases the expression of chaperones and proteins involved in the proteasomal ER-associated degradation (ERAD) [37].

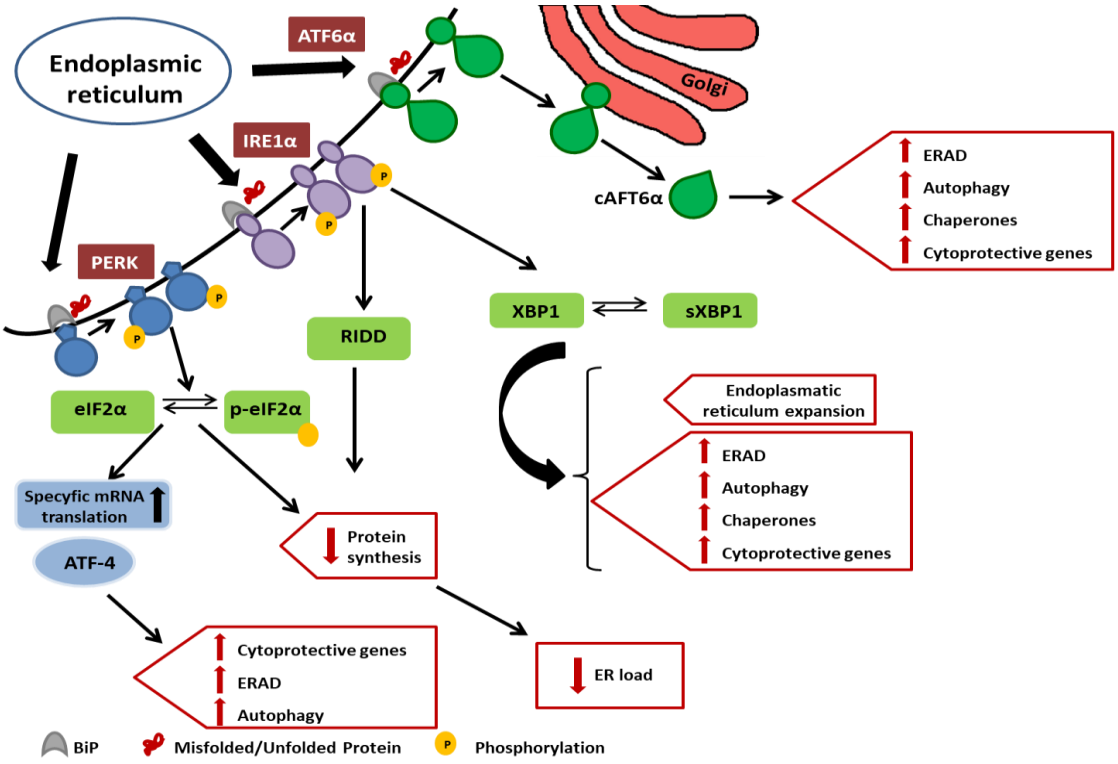

Figure 1. Schematic presentation of unfolded protein response (UPR) activation due to the presence of misfolded protein. PERK- protein kinase R-like endoplasmic reticulum kinase, IRE1α- inositol-requiring enzyme 1 alpha, ATF4 - activating transcription factor 4, ATF6 - activating transcription factor 6, ERAD - ER-associated degradation, XBP1 - X-Box binding protein 1.

If conformation of mutated collagen is not improved by chaperones, it is destined for degradation most often via autophagy [38, 39]. Autophagy is a complex system regulated by more than 30 autophagy-related gene (ATG) proteins. The most used markers of autophagy are beclin 1, a microtubule-associated protein 1 light chain 3 (LC3), and sequestosome 1 (SQSTM1/p62), later referred to as p62 [38, 39]. In case of mutations occurring in C-propeptide, which most affect the trimer assembly, the degradation of misfolded procollagen chains may occur with the involvement of the ERAD pathway [40, 41].

5. Treatment of OI

The treatment of OI is primarily supportive and symptomatic, and requires multidisciplinary management with medications, physical therapy, orthopedic interventions, and multi-specialist consultations. Current treatments and future therapeutic approaches include: anti-catabolic bisphosphonates, denosumab as anti-RANKL (receptor activator for nuclear factor κB ligand) antibody, teriparatide as anabolic recombinant human parathyroid

hormone, sclerostin and transforming growth factor (TGF-β) antibodies [1-4, 12, 42] Various approaches are taken including progenitor cell therapy using healthy progenitor stem cells, transplantation of hematopoietic stem cell, *ex-vivo* expanded mesenchymal and amniotic fluid stem cells [3, 42]. However, they are remained experimental due to inconsistent results, safety and ethical considerations. According to the latest reports, 4-phenylbutyrate (4-PBA), the molecular target of which is ER stress caused by intracellular accumulation of mutant collagen, is of great interest [42-47].

THE AIM OF THE STUDY

OI is a phenotypically and genetically heterogeneous disease of connective tissue, known as “brittle bone” disease caused, in the majority (85–90%) of patients, by mutations in one of the two *COL1A1/COL1A2* genes coding for collagen type I [1-8, 30-32]. The mutations result in qualitative (OI types II, III and IV) or quantitative (OI type I) collagen abnormalities. Although OI is primarily a bone disease, quantitative and structural mutations affecting type I collagen have consequences in all tissues that produce this protein. The extra-skeletal symptoms include blue sclera, excessive mobility ligaments, skin brittleness, muscle weakness, hearing loss and dentinogenesis imperfecta. Most studies on the mechanisms of the pathology of this disease are carried out on patients' skin fibroblasts, because collagen type I as the most abundant protein of bones and skin is similarly expressed in both tissues, while fibroblasts are more accessible than osteoblasts and easier to culture. Due to the important role of this protein in connective tissue, it acts as a scaffold and is responsible for mechanical strength [13-16], collagen reduction by about 50% in OI type I may be related to impairment mechanical properties of the tissue disrupting its proper structure and function. However, the lack of mutated collagen is associated with the mildest symptoms [27-29]. Structural mutations, the most common substitutions for glycine by a larger amino acid, disrupt the structure and secretion of collagen into ECM and are associated with much more severe disease symptoms [1-10].

Earlier studies focused on understanding the effects of mutations on the structure and function of secreted mutant collagen and its interactions with receptors or non-collagen proteins [1, 2, 8, 30]. Recent studies have focused on the importance of mutant collagen that is not secreted due to abnormal structure and accumulates in the cells [43-49]. In some human fibroblasts and osteoblasts and those derived from OI animal models (mice, zebrafish) as the consequences of such collagen accumulation, ER stress was found. The use of the chemical chaperone 4-PBA resulted in part in increasing collagen secretion and reducing ER stress in OI fibroblasts [44, 45]. In OI zebrafish model, it was shown that administration of this chaperone can improve the phenotype [46]. Hence, a new direction of therapy seems to be promising in which ER is a molecular target and the search for other safe drugs preventing the accumulation of mutated collagen is of fundamental importance.

Medicinal plants containing significant amounts of polyphenols with high therapeutic potential have been widely researched for many years. Due to their high biological activity (e.g. antioxidant, anti-inflammatory, antibacterial and antiviral), they are used in the treatment of

many diseases. For years, the Department of Medical Chemistry of the Medical University of Bialystok has been conducting research on the search for natural compounds with beneficial effects on collagen, both in healthy [50-52] and OI [53] human skin fibroblasts.

In the current research it was hypothesized that rosemary (*Rosmarinus officinalis* L.) extract (RE) and lemon balm (*Melissa officinalis* L.) extract (LBE), and rosmarinic acid (RA) as one of their main ingredients, which attract particular attention of pharmacists due to their high therapeutic potential, can improve the quantitative defect of normal collagen type I in OI type I and/or minimize the accumulation of mutant collagen in OI type II and III fibroblasts. Therefore, the aim of these studies was to assess the effect of these extracts and RA, at non-toxic concentrations, on biosynthesis, secretion and degradation of type I collagen in skin fibroblasts of patients with OI type I, II and III.

MATERIALS AND METHODS

The studies were performed on five primary skin fibroblast lines (one OI type I, two OI type II and two OI type III) obtained from the Department of Molecular Biology and Genetics of Medical University of Silesia in Katowice, Poland, carrying the following mutations in α1 chain of collagen type I:

- OI type I (exon 5, c. 459 del T)
- OI type II 1 (exon 38 Gly691Cys);
- OI type II 2 (exon 23, Gly352Ser)
- OI type III 1 (exon 45, Gly901Ser);
- OI type III 2 (exon 52, Gly1448Val)

Normal human skin fibroblast lines CCD25Sk and CRL-1474 as age matched controls for OI types I and III were purchased from the American Type Culture Collection, and as control for lethal OI type II, fibroblasts derived from the foreskin on the 7th day of life of the donor were used. Fibroblasts from skin biopsy of OI patients and healthy control were obtained after informed consent in accordance with the Declaration of Helsinki, and study was approved by Bioethical Committee of the Jagiellonian University in Kraków, Poland (KBET/108/B/2007) collaborated with the Department of Molecular Biology and Genetics of Medical University of Silesia in Katowice.

Rosemary and lemon balm extracts were prepared and analyzed using liquid chromatography-photo-diode array detector-mass spectrometry (LC-PDA-MS), and were provided by the Department of Pharmacognosy of Medical University of Bialystok.

The experiments were carried out under standard conditions (skin fibroblasts were grown in DMEM at 37 °C in a 5% CO_2 incubator). Fibroblasts of OI type I patient were incubated for 24 h with rosemary and lemon balm extracts at the concentrations 0.1-100 μg/mL each, and rosmarinic acid at the concentrations of 0.1-100 μM. Fibroblasts of OI types II and III were treated with rosemary extract and rosmarinic acid at the same concentrations as above, and in addition, inhibitors of autophagy: 50 μM CQ, 50 mM NH_4Cl and 5 mM 3-MA or proteasome: 50 nM BR and 2.5 μM MG132 were used. All reagents were diluted in DMSO, and its final concentration did not exceed 0.1% (v/v).

In this study the following assays were used:

- cell viability test using MTT
- isolation of total RNA, cDNA synthesis and determination of expression of individual genes by quantitative Real-time PCR

- electrophoresis of cDNA in polyacrylamide gel to analyze XBP1 splicing products
- Western blot and densitometry for analysis of protein expression
- SDS-urea-PAGE to study collagen type I migration and identification of mutant collagen
- Enzyme-Linked Immunosorbent Assay (ELISA) to measure the amount of procollagen type I in cell lysate and in the media
- confocal fluorescence microscopy with immunofluorescence staining to study colocalization of collagen type I with lysosomal and autophagy markers
- subcellular fractionation of cell lysate to obtain lysosome fraction
- zymography to measure activity of MMPs (MMP-1, MMP-2, MMP-9)
- determination of proteasome activities using fluorogenic substrates
- statistical analysis of the results was performed using the Statistica 12 software (StatSoft, Tulsa, OK, USA). Statistical differences were estimated by the use of one-way ANOVA followed by Tukey's test and values of $p < 0.05$ were considered as significant.

RESULTS

1. Stimulating effect of RE, LBE and RA on the biosynthesis of type I collagen and inhibiting the activity of MMP in OI type I skin fibroblasts

Detailed phytochemical analysis of the secondary metabolites of rosemary and lemon balm extracts, using LC-PDA-MS method, revealed the presence of 34 different compounds. Among them, there were polyphenols as derivatives of caffeic acid, flavonoids such as luteolin and apigenin derivatives, and related compounds such as diterpenes. The quantitative content of RA as the dominant component of both tested extracts was determined as 27.23 ±0.54 mg/g and 80.26 ±1.24 mg/g for RE and LBE, respectively.

RE and LBE at concentrations of 0.1–100 μg/mL each and RA at concentrations of 0.1–100 μM did not affect cell viability. By using the Human pro-collagen I alpha 1 Simple Step Elisa Kit (Abcam, Cambridge, UK), the decrease by about half of the amount of type I procollagen was detected in cells and medium of OI type I skin fibroblasts, while exposure of OI cells to RA (0.1–100 μM) and extracts: RE (0.1–100 μg mL) and LBE (0.1–100 μg mL) had a significant stimulating effect on type I procollagen biosynthesis. The highest increase in the amount of secreted procollagen was noted at 0.1, 1 and 10 μM of RA and 0.1, 1 and 10 μg/mL of each extract. Moreover, in the presence of LBE at concentrations of 1 and 10 μg/mL, the amount of procollagen type I in the medium was normalized to that in the medium of normal cells. The results were confirmed by Western blot and SDS-urea-PAGE. Electrophoresis of pepsin-digested procollagen in non-reducing conditions allows to identify type III procollagen which was not significantly affected in RE treated OI cells. The quantitative real-time PCR showed the stimulating effect of RA and extracts on collagen biosynthesis at the mRNA level. Densitometric analysis of the active forms of MMPs showed an increase in the activity of MMP-2 and MMP-9 in OI cells compared to the normal cells and no changes in MMP-1 activity, while RA, RE, and LBE at some concentrations exerted an inhibitory impact.

The results of these studies are described in details in the original paper No. 1 [Sutkowska J. et al., 2021].

2. Reduction of accumulation of type I collagen under the influence of RE by inducing autophagy in OI types II and III skin fibroblasts

In untreated skin fibroblasts of two patients with severe type III and two patients with lethal OI type II, the accumulation of collagen type I was found by the use of SDS-urea-PAGE and Western blot. When OI cells were treated with RE at concentrations of 1-100 μg/mL, a significant reduction in intracellular collagen levels was achieved at concentrations of 50 and 100 μg/mL RE in all four cell lines. RA as the main component of the extract, used in a wide range of concentrations (1-100 μM), besides reducing the accumulation of type I collagen in OI II cells of patient 1 (at the highest concentration), it had no effect on other cells, therefore only RE at concentrations of 50 and 100 μg/mL was further investigated.

Since the retained procollagen can activate UPR, the expression of selected proteins such as BiP, PDI and transcription factors ATF4, AFT6 and XBP-1s was assessed. PDI, which catalyzes the formation and isomerization of disulfide bonds and acts as a collagen chaperone, was increased in all OI cells at mRNA level. Expression of the chaperone BiP, as an activator of UPR sensors, was beyond OI II 1 increased in untreated OI cells at both mRNA and protein levels. In turn, expression of ATF4 and AFT6 at the mRNA level was increased in all cells, while at protein level in three OI cell lines except OI II 1. The splicing form of XBP-1 (XBP-1s) was manifested mainly in type III OI cells. Under the influence of 50 and 100 μg/mL RE, the decrease in the expression of UPR proteins was observed in most cases, except for cells with unchanged expression of BiP.

The expression of proteins involved in the autophagy process in untreated and RE treated OI cells was then determined. Although in untreated OI cells there was an increase in the expression of proteins involved in the initial phase of autophagosome formation: ATG5 (in all cell lines) and beclin 1 (except OI II 1), as well as of protein of final autophagosome-lysosome fusion (LC3-II), there was no dynamic autophagy process. The ratio of LC3-II to LC3-I, which is indicator of the autophagic flux, in untreated cells either increased in OI III or remained unchanged in OI II. The expression of p62, which is an indicator of autophagic degradation, was unchanged in OI III and increased in OI II cells. RE significantly induced autophagic flux in all OI cells as indicated by the increase in the LC3-II/LC3-I ratio with concomitant reduction of p62 protein amount. In order to confirm the induction of active autophagy and mutant collagen degradation with involvement of this process, fractionation of the cell lysate was performed and the presence of type I procollagen in the lysosome-enriched fraction was demonstrated. Additionally, using confocal fluorescence microscopy with

immunofluorescence staining, co-localization of endogenous collagen type I with LC3-II as autophagy marker and LAMP2A as a lysosomes marker was showed.

3. Effect of RE on proteasome-mediated degradation of unfolded procollagen chains

In order to check whether type I collagen is also degraded by the proteasome (ERAD pathway), the polyubiquitination of type I procollagen, separated in polyacrylamide gel under non-reducing conditions, was analyzed. Polyubiquitination of unfolded procollagen chains was detected only in OI type III 2 cells with a mutation in C-propeptide (Gly1448Val). The increase in this modification was noted in the presence of RE, which was accompanied by the decrease in the level of unfolded procollagen α1(I) chains. In the presence of BR, a proteasome inhibitor, the increased level of polyubiquitinated procollagen monomers correlated with increased level of unfolded procollagen chains. In OI untreated cells, the decrease in trypsin-like and caspase-like activities, and no change in chymotrypsin-like activity was found, while in the presence of RE all activities decreased compared to the untreated OI cells. The decrease in proteasome activity had no inhibitory effect on the degradation of total proteins because their levels in RE treated cells were comparable to that of normal cells.

4. Effect of RE on MMP-mediated degradation of collagen

Additionally, the expression and activity of MMP-1 and MMP-2, which degrade extracellular collagen I, was tested. Expression of MMP-1 at the mRNA level was significantly increased in untreated OI types II and III cells compared to the normal and decreased in the presence of RE. In turn, MMP-2 mRNA expression was elevated only in OI type II cells and normalized under influence of RE. Moreover, RE at the concentration of 100 μg/mL, with the exception of OI II 1, showed the reducing impact on the activity of MMP-2.

5. The protective effect of RE against apoptosis of OI fibroblasts

Finally, the expression of pro-apoptotic proteins such as Bax and CHOP (at mRNA and protein level) and cleaved caspase-3 at the protein level was assessed. In OI untreated cells, the increase in their expression was shown, while the decrease was observed under the influence of RE at both concentrations (50 and 100 μg/mL).

The results of the studies, included the points 2-5, are described in details in the original paper No. 2 [Sutkowska-Skolimowska J. et al., 2022].

DISCUSSION

Despite intensive research on OI, the continuously discovered mutations in various genes coding proteins which are or are not related to collagen metabolism, confirm the complex molecular pathomechanisms of this disease and make it difficult to develop a universal treatment strategy [1-4, 12, 42]. There are some anti-resorptive treatments with the use of bisphosphonates, but long-term osteoclast inhibition by these drugs can deteriorate bone quality, leading to non-dynamic bone in which not repaired microdamages accumulate and may lead to an overall increase in bone fragility [1, 2]. Children after long-term pamidronate treatment developed atypical femoral fractures and delayed tooth eruption [54]. Other anti-resorptive therapies using Denosumab against RANKL, Romosozumab against sclerostin or Odanacatib against cathepsin K are currently under investigation [2, 3, 5, 42, 55-57]. In order to improve the patient's condition, growth hormone, growth factors or vitamin D supplementation are administered [57].

Our research, for the first time, provides evidence of the beneficial effects of RA and RA-rich extracts (RE and LBE) in OI type I with quantitative collagen type I defect and OI types II and III with the accumulation of mutant collagen.

In OI type I fibroblasts treated with low concentration of RA (0.1-10 μM) or extracts (0.1-10 μg/mL) there was a significant stimulation or even normalization of collagen biosynthesis. The results were confirmed at the both mRNA (by quantitative real-time PCR) and protein (by Western blot, ELISA and silver staining of pepsin-digested procollagen separated by SDS-urea PAGE) levels. It can be assumed that RA as the main component of both extracts, as shown by using LC-PDA-MS method, may be responsible for the stimulating effect of RE and LBE. This assumption is based on the quantitative analysis of the extracts, which showed about three times higher content of RA in LBE (80.26±1.24 mg/g) compared to RE (27.23±054 mg/g), and on the about 3-fold greater LBE-induced stimulation of type I collagen biosynthesis compared to RE, used at the same concentrations. Greater LBE stimulating effect on collagen type I may also be the result of the synergistic action of RA and another component present in LBE - apigenin 7-O-glucoside, which was not found in RE, and whose slightly stimulating effect on collagen type I in OI cells has been shown in previous studies conducted at the Department of Medical Chemistry [53]. Using SDS-urea-PAGE of pepsin-digested procollagen, which allowed the separation of type I collagen from the second essential skin collagen type III, it was demonstrated unchanged type III level, which suggests selective action of tested extracts and RA on affected in OI collagen type I.

In addition, it was investigated whether, apart from the reduction of collagen type I due to *COL1A1* mutation, there is no increased its degradation with the participation of MMPs. The activity of MMP-1 specific for type I collagen in untreated OI cells was similar to normal, while the increased activities of gelatinases (MMP-2 and MMP-9) were reduced in cells treated with extracts and RA.

Recent studies have demonstrated a relationship between the ER stress due to the accumulation of misfolded collagen in cells and the severity of the disease, and developed the ER stress-targeted therapy [43-47]. Administration of 4-PBA to the zebrafish OI model with glycine substitution (Gly574Asp) in *COL1A1* resulted in the reduction of cellular stress and improvement of bone mineralization in larvae and skeletal deformation in adults [46]. Similarly, in osteoblasts derived from two murine OI models with mutations Gly349Cys in *COL1A1* (Brtl mouse) and Gly610Cys in *COL1A2* (Amish mouse), 4-PBA was showed to reduce collagen aggregates and increase collagen incorporation into matrix [43].

In the present study it was proved on fibroblasts from patients type II and III with substitutions of glycine in the α1(I) chain that rosemary extract may be such a stress relieving factor. In contrast to the beneficial effect of RE in type I OI cells exerted at its low concentrations (0.1, 1 and 10 μg/mL), the reduction of mutant collagen accumulation was achieved in OI types II and III cells subjected to higher RE concentrations (50 and 100 μg/mL). The probable mechanism of reducing this accumulation is the intensification of degradation of mutant collagen by RE-induced autophagy.

Autophagy is a dynamic process of the degradation of intracellular components, including soluble proteins, aggregated proteins and non-functional cell organelles, activated to limit cell damage [38, 39]. The process of autophagy is strictly regulated by ATG core proteins, of which LC3 plays an essential role in the formation and maturation of the autophagosome. The cytosolic form of LC3-I is converted into the active, membrane-bound form LC3-II during the formation of the autophagosomes, while the final cargo degradation occurs after autophagosome fusion with lysosomes, which is strictly dependent on the p62 [39, 58, 59]. An increase in the LC3-II/LC3I ratio and an increase in p62 degradation are important markers of effective autophagic flow [39, 58]. In untreated OI cells, despite the increase in the expression of proteins involved in the initial (ATG5 and beclin 1) and subsequent (LC3-II) stages of this process, no activation of this process was demonstrated.

The stimulating effect of RE on the degradation of mutant collagen by the autophagolysosomal pathway was confirmed by the presence of type I collagen in the lysosomal fraction and its increase after inhibition of lysosomal activity with ammonium chloride. In

addition, the use of immunofluorescence microscopy allow to demonstrate the collocation of type I collagen in RE-treated OI cells with both autophagosome (LC3-II) and a lysosome (LAMP2A) markers.

This was accompanied by a reduction in the expression of proteins of UPR activated in three out of four tested OI cells. Similarly, Besio et al. [44, 45] reported that not all tested cells with collagen and non-collagen mutations, demonstrating accumulation of mutant collagen and ER stress, activated this process. The importance of UPR in OI and the way of the activation of this process remains unclear, as activation of the UPR also independent of BiP was observed in OI cells [34, 44, 45, 60]. It is possible that other regulatory mechanisms are involved in the activation of the cell response during ER stress. According to results of Besio et al. [44, 45] PERK pathway with increased ATF4 expression and IRE1α pathway were activated, while there was no activation of ATF6 in fibroblasts with collagen type I mutations and mutations in (P3H1/CRTAP/CypB) complex that impaired hydroxylation of prolyl-3 type I collagen. In contrast, in our study, activation of ATF4 (effector of PERK pathway) and ATF6 as well as expression of spliced forms of XBP1 (effector of IRE1α pathway) was found along with BiP upregulation. XBP1s expression was predominant in OI type III fibroblasts. While it is still unknown how activation of UPR pathways and their effectors affects directly collagen type I, it was reported that forced XBP1s expression in cells with glycine substitution in α1(I) chain enhanced the folding and secretion of mutant type I collagen [37]. ATF4 in the active form can upregulate both the survival e.g. by enhancing autophagy and the apoptosis by upregulation of CHOP gene [36]. In turn, the active ATF6 enhances the expression of chaperones and genes for proteins involved in ERAD [34, 35].

In the next step, it was examined whether the proteasome 26 is involved in the degradation of the unfolded procollagen chains. The increase in the level of procollagen α1 chains was only shown in OI type III with a mutation in C-propeptide (Gly1448Val) according to the previous reports [40, 41]. In RE-treated cells, the polyubiquitination of these chains was increased and the amount of unfolded procollagen chains decreased. The greater accumulation of these chains under the influence of BR (proteasome inhibitor) confirms the proteasome's involvement in removing these chains. It turned out, however, that to a lesser extent than BR, RE also partially inhibits the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome. At this stage of research it is difficult to explain the mechanism RE-induced procollagen chains degradation, it is possible that in the case of partial inhibition of proteasome activity, unfolded chains may be also degraded in the process of RE-activated autophagy. As between the two protein degradation systems (proteasome and autophagy) a crosstalk was

reported [59], it is suspected that as a result of proteasome inhibition, autophagy may be activated to remove polyubiquitinated proteins and promote cell survival. It is possible that the RE-caused decrease in proteasome activity, noted in all cells (results not shown), rather than the activation of UPR, which did not occured in all cells, induced autophagy, which protected the cells from the toxic stress leading to cell apoptosis. The confirmation of the protection of cells against apoptosis by RE was a significant reduction in the expression of pro-apoptotic proteins (Bax, CHOP and active caspase 3).

The cell's response to inhibited intracellular collagen degradation in OI untreated cells could be a significant upregulation of the genes coding extracellular MMP-1 and MMP-2, which was lowered in the presence of RE. Negative correlations between the activity of lysosomal enzymes and MMPs have been reported [61], but our studies mainly focus on the intracellular processes of collagen degradation.

RA and tested extracts (RE and LBE), unlike synthetic drugs that can cause adverse side effects, are characterized by many valuable biological properties (antioxidant, anti-inflammatory, antimicrobial, antiviral, antiangiogenic, anti-depressant, antithrombotic, antihyperglycemic, anti-allergic, anticarcinogenic, and anti-aging), which are constantly gaining the growing interest of researchers and pharmaceutical, food and cosmetic industries [62-75]. They are being intensively studied for their wider use in treating or supporting the treatment of cancer and many other diseases.

It is worth also noting, that in addition to providing new clinically relevant properties of RA and RA-containing extracts (RE and LBE) related to their potential to promote type I collagen expression in OI type I skin fibroblasts and reduce mutant collagen accumulation and improve cell homeostasis in more severe types of OI, the following limitations of this study should be considered. First, our data is limited to the use of a small number of skin fibroblasts with α1(I) mutations. Second, taking into account the pharmacokinetics of RA or other components of the extracts *in vivo*, there may be differences between the results obtained in *in vitro* study compared to the *in vivo* administration. Therefore, it is necessary not only to increase the number of tests on fibroblasts or osteoblasts collected from a larger number of patients, but also to conduct *in vivo* experiments.

CONCLUSIONS

1. RE and LBE at concentrations of 0.1, 1 and 10 μg/mL and RA at concentrations of 0.1, 1 and 10 μM significantly reduced or completely eliminated the quantitative defect of collagen type I in OI type I fibroblasts by their stimulating effect on collagen biosynthesis and inhibiting effect on MMP (MMP-1, MMP-2 and MMP-9) activity.
2. RE at concentrations of 50 and 100 μg/mL significantly reduced the level of accumulated mutant collagen type I in fibroblasts of patients with severe OI type III and lethal OI type II by inducing autophagy. It was evidenced by the increase in LC3-II/LC3-I ratio and degradation of p62 as well as localization of collagen type I in lysosomal fraction and its co-localization with autophagy (LC3-II) and lysosome (LAMP2A) markers by confocal fluorescence microscopy.
3. The RE-induced decrease in intracellular accumulation of mutant type I collagen was associated with the decrease in expression of unfolded protein response proteins suggesting alleviation of cell stress.
4. The decrease in the pro-apoptotic markers (Bax, CHOP and cleaved caspase 3) levels under influence of RE indicates its protective effect on OI fibroblasts.
5. Partial inhibition of proteasome activity by RE did not reduce its effectiveness in degradation of unfolded procollagen chains and did not affect the total protein level.
6. The obtained results provide new clinically relevant properties of RA and RA-rich extracts (RE and LBE), which may have some implications in OI therapy, but need to be confirmed in future experiments *in vivo*.

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SUMMARY

Osteogenesis imperfecta (OI) is an inherited disease of the connective tissue manifested mainly by defects in the skeletal system (bone fragility, skeletal deformities, reduced bone mineral density, short stature), but also a number of extra-skeletal symptoms such as blue sclera, hearing impairment, skin fragility, joint laxity, muscle weakness, tooth abnormalities (dentinogenesis imperfecta), cardio-respiratory defects and impaired pulmonary function. The prevalence of OI is estimated at 1 in 15 000 to 20 000 live births. The molecular mechanisms underlying the disease are also complex. The vast majority of cases (85-90%) are caused by dominant mutations in the *COL1A1* and *COL1A2* genes encoding type I collagen. The classification of OI due to the collagen type I mutations includes four clinically defined types, the phenotype of which ranges from mild non-deforming type I, moderate type IV, severe deforming type III to the lethal type II. Many other recessive mutations in non-collagenous genes code for proteins involved in type I collagen biosynthesis, modifications, quality control, secretion as well as osteoblast differentiation and bone mineralization.

Mutations of collagen type I genes, depending on the type, can result in quantitative or structural defect in the collagen type I. The decrease in biosynthesis of structurally normal type I collagen in OI type I is caused by a null *COL1A1* allele due to premature stop codons. The causative mutations of severe and lethal OI include mainly substitutions of glycine residues with another amino acid (80%) and are defined as the dominant-negative. The resulting mutant collagen is secreted into the ECM but also accumulates inside the cell, which can cause phenotype-related ER stress. The cell can activate the UPR and improve the conformation of mutant collagen or destined for degradation most commonly via autophagy or ERAD pathway. During chronic stress, ER promotes cell apoptosis.

The currently used anti-resorptive treatment mainly uses the administration of bisphosphonates. Many various approaches remain experimental. According to the latest reports, 4-PBA, the molecular target of which is ER stress caused by intracellular accumulation of mutant collagen, is of great interest.

In this study it was hypothesized that rosemary (*Rosmarinus officinalis* L.) and lemon balm (*Melissa officinalis* L.) extracts, and rosmarinic acid, as one of their main ingredients, which attract particular attention of pharmacists due to their high therapeutic potential, can improve the quantitative defect of normal collagen type I in OI type I and/or minimize the accumulation of mutant collagen in OI type II and III fibroblasts with substitutions of glycine in α1(I) chain.

RE and LBE at concentrations of 0.1, 1 and 10 μg/mL and RA at concentrations of 0.1, 1 and 10 μM significantly reduced or completely eliminated the quantitative defect of collagen type I in OI type I fibroblasts by their stimulating effect on collagen biosynthesis and inhibiting effect on MMP (MMP-1, MMP-2 and MMP-9) activity.

RE at concentrations of 50 and 100 μg/mL significantly reduced the level of accumulated mutant collagen type I in fibroblasts of patients with severe OI type III and lethal OI type II by inducing autophagy. Activation of this process was evidenced by the increase in LC3-II/LC3-I ratio and degradation of p62 as well as localization of collagen type I in lysosomal fraction and its co-localization with autophagy (LC3-II) and lysosome (LAMP2A) markers by confocal fluorescence microscopy. The RE-induced decrease in intracellular accumulation of mutant type I collagen was associated with the decrease in expression of UPR proteins suggesting alleviation of cell stress. This was confirmed by the decrease in the pro-apoptotic markers (Bax, CHOP and cleaved caspase 3) levels under influence of RE.

RE, despite partial inhibition of proteasome activity, also increased the degradation of unfolded procollagen chains in OI cells with a mutation in C-propeptide, but did not affect the level of total protein in lysates. The obtained results reveal new clinically important properties of RA and extracts (RE and LBE) that may have some implications in OI therapy, but need to be confirmed in future *in vivo* experiments.

STRESZCZENIE

Osteogenesis imperfecta (OI) to dziedziczna choroba tkanki łącznej objawiająca się głównie defektami układu kostnego (łamliwość kości, deformacje szkieletu, obniżona gęstość mineralna kości, niski wzrost), ale także szeregiem objawów pozaszkieletowych, takich jak niebieska twardówka, zaburzenia słuchu, kruchość skóry, wiotkość stawów, osłabienie mięśni, nieprawidłowości zębów (dentinogenesis imperfecta), wady sercowo-oddechowe i upośledzenie czynności płuc. Częstość występowania OI szacuje się na 1 na 15 000 do 20 000 żywych urodzeń. Mechanizmy molekularne leżące u podstaw choroby są również złożone. Zdecydowana większość przypadków (85-90%) jest spowodowana dominującymi mutacjami w genach *COL1A1* i *COL1A2* kodujących kolagen typu I. Klasyfikacja OI spowodowanej mutacjami kolagenu typu I obejmuje cztery klinicznie zdefiniowane typy, których fenotyp waha się od łagodnego niedeformującego typu I, umiarkowanego typu IV, ciężkiego deformującego typu III do śmiertelnego typu II. Wiele innych mutacji recesywnych występujących w genach niekolagenowych koduje białka zaangażowane w biosyntezę kolagenu typu I, modyfikacje, kontrolę jakości, sekrecję kolagenu oraz różnicowanie osteoblastów i mineralizację kości.

Mutacje genów kolagenu typu I, w zależności od typu, mogą powodować ilościowe lub strukturalne defekty kolagenu typu I. Spadek biosyntezy strukturalnie prawidłowego kolagenu typu I w OI typu I jest spowodowany brakiem ekspresji allelu *COL1A1* z powodu przedwcześnie wprowadzonego kodonu stop. Przyczyną ciężkich postaci choroby są głównie podstawienia reszt glicyny innym aminokwasem (80%) i są definiowane jako dominujące negatywne. Zmutowany kolagen jest wydzielany do ECM, ale również gromadzi się wewnątrz komórki, co może powodować związany z fenotypem stres ER. Komórka może aktywować UPR i poprawić konformację zmutowanego kolagenu lub przeznaczyć do degradacji najczęściej poprzez szlak autofagii lub ERAD. Podczas przewlekłego stresu ER promuje apoptozę komórek.

Obecnie stosowane leczenie antyresorpcyjne polega głównie na podawaniu bisfosfonianów. Wiele różnych podejść pozostaje eksperymentalnych. Według najnowszych doniesień dużym zainteresowaniem cieszy się 4-PBA, którego celem molekularnym jest stres ER wywołany wewnątrzkomórkową akumulacją zmutowanego kolagenu.

W badaniu tym postawiono hipotezę, że ekstrakty z rozmarynu (*Rosmarinus officinalis* L.) i melisy (*Melissa officinalis* L.) oraz kwas rozmarynowy jako jeden z ich głównych składników, które przyciągają szczególną uwagę farmaceutów ze względu na ich wysoki

potencjał terapeutyczny, mogą poprawić ilościowy defekt prawidłowego kolagenu typu I w OI typu I i/lub zminimalizować akumulację zmutowanego kolagenu w fibroblastach OI typu II i III z podstawieniami glicyny w łańcuchu α1(I).

RE i LBE w stężeniach 0,1, 1 i 10 µg/ml oraz RA w stężeniach 0,1, 1 i 10 µM znacząco zmniejszyły lub całkowicie wyeliminowały ilościowy defekt kolagenu typu I w fibroblastach OI typu I poprzez stymulujący wpływ na biosyntezę kolagenu i hamujący wpływ na aktywność MMP (MMP-1, MMP-2 i MMP-9).

RE w stężeniach 50 i 100 µg/ml istotnie obniżał poziom nagromadzonego zmutowanego kolagenu typu I w fibroblastach pacjentów z ciężką postacią OI typu III i śmiertelną OI typu II poprzez indukcję autofagii. O aktywacji tego procesu świadczy wzrost stosunku LC3-II/LC3-I i degradacja p62 oraz lokalizacja kolagenu typu I we frakcji lizosomalnej i jego kolokalizacja z markerami autofagii (LC3-II) i lizosomu (LAMP2A) za pomocą konfokalnej mikroskopii fluorescencyjnej. Indukowany przez RE spadek wewnątrzkomórkowej akumulacji zmutowanego kolagenu typu I był związany ze spadkiem ekspresji białek UPR, co sugeruje złagodzenie stresu komórkowego. Zostało to potwierdzone przez znaczne obniżenie poziomu ekspresji markerów proapoptotycznych (Bax, CHOP i aktywnej kaspazy 3) pod wpływem RE.

RE, pomimo częściowego zahamowania aktywności proteasomu, zwiększał również degradację niesfałdowanych łańcuchów prokolagenowych w komórkach OI z mutacją w C-propeptydzie, natomiast nie wpływał na poziom całkowitego białka w lizatach. Uzyskane wyniki ujawniają nowe klinicznie istotne właściwości RA i ekstraktów (RE i LBE), które mogą mieć pewne implikacje w terapii OI, ale muszą zostać potwierdzone w przyszłych eksperymentach *in vivo*.

The stimulating effect of rosmarinic acid and extracts from rosemary and lemon balm on collagen type I biosynthesis in osteogenesis imperfecta type I skin fibroblasts

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The Stimulating Effect of Rosmarinic Acid and Extracts from Rosemary and Lemon Balm on Collagen Type I Biosynthesis in Osteogenesis Imperfecta Type I Skin Fibroblasts

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Abstract: Rosemary extract (RE) and lemon balm extract (LBE) attract particular attention of pharmacists due to their high therapeutic potential. Osteogenesis imperfecta (OI) type I is a heritable disease caused by mutations in type I collagen and characterized by its reduced amount. The aim of the study was to evaluate the effect of the extracts and rosmarinic acid (RA) on collagen type I level in OI skin fibroblasts. Phytochemical analysis of RE and LBE was carried out by liquid chromatography–photodiode array detection–mass spectrometry. The expression of collagen type I at transcript and protein levels was analyzed by qPCR, ELISA, SDS-urea PAGE, and Western blot. In OI patient's fibroblasts the exposure to the extracts (0.1–100 μg/mL) and RA (0.1–100 μM) significantly increased collagen type I and the best results were obtained with 0.1–10 μM RA and 0.1–10 μg/mL of the extracts. LBE showed a greater stimulating effect than RE, likely due to a higher RA content. Moreover, collagen type III expression and matrix metalloproteinase (MMP-1, -2, -9) activity remained unchanged or decreased. The obtained data support the clinical potential of RA-rich extracts and RA itself in modulating the quantitative defect of type I collagen in type I OI.

Keywords: rosemary extract; lemon balm extract; rosmarinic acid; collagen type I; skin fibroblasts; osteogenesis imperfecta type I

1. Introduction

Medicinal herbs and herbal remedies have been widely used in traditional medicine. Furthermore, their use is growing rapidly in modern therapy of various ailments and diseases. Rosemary (*Rosmarinus officinalis* L.) and lemon balm (*Melissa officinalis* L.) are perennial plants from the Lamiaceae family, naturally occurring in the Mediterranean Sea and West Asia, as well as being commonly cultivated in Europe and North America [1,2]. They are widely used in the pharmaceutical, food and cosmetic industries due to their various biological activities. which include antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, immunomodulating, antidepressant, anticoagulant, antihyperglycemic, antinociceptive, antiulcer, and antitumor [1–5]. For the broad effects of these herbs on human health, a whole range of active compounds are responsible that can act alone or in various combinations. An important component of rosemary extracts (RE) and lemon balm extract (LBE) that independently showed a wide range of pharmacological activities and therapeutic applications is rosmarinic acid (RA), an ester of caffeic acid and 3, 4–dihydroxyphenyl lactic acid (Figure 1).

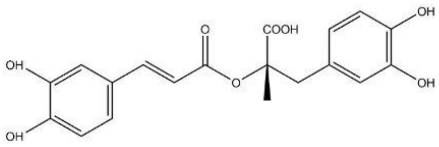

Figure 1. Chemical structure of rosmarinic acid (RA).

The characteristics of the RA have been discussed extensively in the reviews [6–9]. Based on the results of in vitro studies and clinical trials, it can be assumed that it brings many benefits in the treatment of disorders related to the nervous, cardiovascular, gastrointestinal, hepatic, endocrine, respiratory and reproductive systems, cancer diseases, osteoarthritis, allergic rhinitis, periodontal disease, acute pancreatitis, and metabolic syndrome.

The antitumor activity of rosemary and lemon balm extracts, well-studied in vitro and in vivo, is associated with an increase in antioxidant enzyme activity, a reduction in tumor-stimulating inflammation, suppression of tumor angiogenesis, regulation of epigenetic modifications, modulation of immune responses, alteration of hormone signaling, modification of specific metabolic pathways, stimulation of cancer suppressive genes, and cell death programming [3–5,10]. Rosemary and its derivatives can also inhibit the metastasis process and enhance the effect of chemotherapy by reducing the phenomenon of chemoresistance [10].

There are also reports of a beneficial effect of the RA and extracts (RE and LBE) on the skin with particular interest in the field of cosmetology and medicine in supporting the treatment of skin diseases [11–19]. RA has shown its efficacy in treating allergic disorders in clinical trials [11]. Lee et al. [12] in an in vivo study revealed its possible use as a therapeutic agent in atopic dermatitis, one of the inflammatory disorders of the skin. Research on the beneficial effects of RE on the skin, including the protective effect against ultraviolet (UV) radiation and melanoma, reduction of skin damage, anti-aging effect by increasing skin hydration and elasticity, and accelerated wound healing, was described in the review by de Macedo et al. [13]. The mechanisms of the anti-aging action of this extract include the reduction of reactive oxygen species level and expression of p53 protein, prevention of DNA damage, down-regulation of matrix metalloproteinases (MMP-1 and MMP-3) mRNA as well as inhibition of lipid peroxidation [14–16]. Rosemary has been shown to be effective as a preservative in both food and cosmetics, often used in combination with other extracts [15,16]. Rosemary leaf extract is used as skin conditioner and is present up to 10% in hand and body products and 3% in preparations for eye shadows, soaps and detergents [15]. It also prevents herpes infections, dermatomycosis, cellulitis, and pain [15].

LBE also showed many beneficial effects on the skin, including the protective effect against skin damage caused by UVB radiation [17], improving skin elasticity in healthy adults [18] or treatment of acne, and infectious diseases of the skin [19].

Taking into account a number of extremely valuable health-promoting properties of RA and extracts and relying on our previous research on the stimulating effect of polyphenols on type I collagen in human skin fibroblast [20–22], we assumed that they could have a positive impact on the quantitative collagen type I defect in osteogenesis imperfecta (OI) type I. OI is a phenotypically and genetically heterogeneous rare disease of connective tissue originally classified into four types: mild type I, lethal type II, progressive-deforming type III, and moderately mild type IV [23]. Most (85–90%) patients experience qualitative or quantitative abnormalities of type I collagen, due to a mutation in one of two genes *COL1A1*/*COL1A2* encoding this protein [24–27]. Currently, the classification has expanded to 20 types due to the discovery of many other causative genes related more or less closely to collagen type I biosynthesis, modification, secretion or processing [25–27]. Although OI is primarily a bone disease, extra-skeletal defects are described in several patients [25–29]. Abnormalities of collagen type I may manifest as excessive mobility of ligaments, skin fragility, muscle weakness, hearing loss, and dentinogenesis imperfecta. Most of the research on the mechanisms of the pathophysiology of this disease is carried

out on patients' skin fibroblasts because, compared to osteoblasts, they are more accessible and easier to culture. Collagen is the main component of the skin extracellular matrix (ECM) as it constitutes 75% of the dry weight of the skin, and type I collagen accounts for 80 to 90% of the total collagens [30,31]. Due to the significant role of this protein in the skin, it acts as a scaffold and is responsible for mechanical strength; decreasing collagen biosynthesis by approximately 50% in OI type I may be associated with impairment of mechanical properties of the tissue disrupting its proper structure and function [28,29]. Therefore, increasing biosynthesis of collagen type I could at least partially restore the proper composition of the skin ECM and improve its properties in this disease and likely have beneficial effects on bone as well, considering the high amount of collagen type I in this tissue. In addition to demonstrating the beneficial effect of natural compounds on the biosynthesis of collagen type I in skin fibroblasts of a patient with OI type I, their influence on the activity of MMPs involved in collagen and skin homeostasis, such as MMP-1, MMP-2, and MMP-9, was also investigated.

2. Materials and Methods

2.1. Chemicals

Acetonitrile Optima was purchased from Fisher Chemical (Thermo Fisher Scientific, Leicestershire, UK), ultra-pure water (UPW) (resistivity of 18.2 MΩ-cm was obtained using the POLWATER DL3-100 system (Labopol, Kraków, Poland). Formic acid (FA) (Ph. Eur., Merck, Darmstadt, Germany) was used as the mobile phase modifier. Ethanol used to extraction was purchased from POCH Basic (POCH, Gliwice, Poland). Hesperidin and rosmarinic acid were purchased from CAYMAN (Ann Arbor, Michigan, USA). Apigenin (purity > 96%) was isolated from *Lychnis flos-cuculi* herb [32], luteolin 7-*O*-glucoside (purity > 96%) was isolated from *Bidens cernua* [33], hispidulin 7-*O*-glucoside (purity > 96%) was isolated from flowers of *Cirsium rivulare* [34], hispidulin (purity > 96%) was isolated from flowers of *C. rivulare* [35], cirsimaritin (purity > 96%) was isolated from leaf buds of white birch [36]. Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Penicillin, streptomycin, and glutamine were obtained from Quality Biologicals Inc. (Gaithersburg, MD, USA). Radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail (P8340), dimethyl sulfoxide (DMSO), 4-aminophenylmercuric acetate (APMA), magnesium L-ascorbate, and pepsin were provided by Sigma–Aldrich Corp. (St. Louis, MO, USA).

2.2. Preparation of RE and LBE Extracts

Rosemary (*R. officinalis*) and lemon balm (*M. officinalis*) leaves were collected from the garden of medicinal plants from the Medical University of Białystok, Poland in July 2018. Samples of the collected plant material were identified based on the scientific botanical literature and its morphological features by one of the authors (M.T.). Immediately after harvesting, the plant material was dried in the dark and well-ventilated room. Dry, powdered plant materials (each 5.0 g) were sonificated (Sonic-5, Polsonic, Warsaw, Poland) at 40 °C in 50% (*v*/*v*) ethanolic solution (5.0 g sample/50 mL) for 30 min, twice. Filtered supernatants were reduced to dryness under vacuum (Büchi System, Flawil, Switzerland) at a controlled temperature (40 ± 2 °C), further suspended in water, and freeze-dried using a vacuum concentrator (Labconco, Kansas City, MO, USA) until constant weight. With this procedure, the observed yield values were 1.18 g for RE (yield 23.65%), and 1.25 g for LBE (yield 22.5%).

2.3. Liquid Chromatography-Photo-Diode Array Detector-Mass Spectrometry (LC-PDA-MS) Analysis of RE and LBE

The assessment of chemical composition of each extract (RE, LBE) was carried out on a 1260 Infinity chromatograph (Agilent, Santa Clara, CA, USA) consisting of binary pump, a column oven, and photo-diode array (PDA) detector over 55 min period. The separation

was performed using a Kinetex XB C18 column (150 × 3 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA). The mobile phase was 0.1% (*v*/*v*) FA in water (A) and 0.1% FA in acetonitrile (B). The separation was achieved by a gradient of 0–2 min 1% B; 2–20 min 1–20% B; 20–40 min 20–75% B; 40–45 min 75–95% B; 45–48 min 95% B; 48–49 min 95–1% B; 49–55 min 1% B. The flow rate was 0.5 mL/min and the column temperature was maintained at 25 ± 0.8 °C. The UV–Vis spectra was recorded from 190 to 540 nm with selective wavelength monitoring at 280 nm. Mass spectrometry (MS) detection was carried out on a 6230 MS/TOF mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ionization source with Agilent Jet Stream thermal focusing. The parameters used for ionization source were set as follows: drying and sheath gas flow: 12 L/min; nebulizer: 35 psi; source temperature 350 °C; ion spray voltage 4500 V for the positive mode analysis. The data were collected in 115–1900 *m*/*z* range and processing was performed using Mass Hunter qualitative analysis software.

2.4. Preparation of Standard Solution for Quantification

Standard solutions with concentrations of 0.1; 0.3; 0.5; 0.8; 1.0, 3.0 mg/mL were prepared from the stock standard solution by dilution method and were immediately used to determine the standard curve.

2.5. Method Validation

2.5.1. Selectivity

Three solutions were made for both tested extracts to exclude the occurrence of co-elution. Analysis with the use of PDA detector at three selected wavelengths and additional MS detection were used. The presence of other compounds during the retention of the substance being determined was excluded.

2.5.2. Linearity

Standard solutions of RA at 6 concentration levels were analyzed in triplicate. Calibration curves were generated using linear regression on the plots of peak area of each standard versus amount injected to column. Linear regression parameters for the standard curve were determined using ANOVA. Statistical significance has been confirmed. Calculations were made using MS Excel 2016.

2.5.3. Limits of Detection (LOD) and Quantification (LOQ)

In accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) recommendations [37] for the standard used, the detection limit and limit of quantification have been set as equations 3 SD/a and 10 SD/a, respectively. The slope of the calibration curve was taken as standard deviation as the product of the standard error of the intercept and square root number of repetitions (see Table 1).

Table 1. Regression data, limit of detection (LOD) and limit of quantification (LOQ), accuracy and precision obtained during LC-PDA-MS method optimization.

Analyte	Regression Equation [a]	R^2	Linear Range [mg/mL]	LOD [mg/mL]	LOQ [mg/mL]	Accuracy [%]	Precision [%]
RA	y = 4338.7x + 85.712	0.9998	0.1–3.0	0.0694	0.2103	99.88 ± 2.53	1.73

[a]—the value for y corresponds to the peak area and x to the concentration, respectively.

2.5.4. Accuracy and Precision

Accuracy was determined as the theoretical recovery using detector response and regression equation parameters. Precision was determined using six concentration levels analyzed in triplicate and expressed as a coefficient of variation (%CV) (see Table 1).

2.6. Primary Human Skin Fibroblasts

The study was performed on the primary skin fibroblast line obtained from an OI type I patient carrying an out of frame deletion in exon 5 of the *COL1A1* gene (g.2674 del T, c.459delT, p.Gly145Alafs * 111, unpublished data) in accordance with the Declaration of Helsinki and was approved by the Bioethical Committee of the Jagiellonian University in Kraków, Poland (KBET/108/B/2007, 31 May 2011). Normal human skin fibroblast line CCD25Sk was purchased from the American Type Culture Collection and used as control. Fibroblasts were used between passages 2–6 and cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin in an incubator at 37 °C and humidified atmosphere containing 5% CO_2. For the experiments, cells were grown to 90% confluence and 2 h before treatment, culture medium was replaced with fresh serum-free DMEM supplemented with magnesium ascorbate (25 µg/mL). Compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C as the concentrated stock solutions. Fresh dilutions in DMEM were made prior to adding them to cell cultures for the final concentrations: RA (0.1–100 µM), RE (0.1–100 µg/mL) and LBE (0.1–100 µg/mL), with DMSO not exceeding 0.1%.

2.7. Cell Viability Assay

The assay was performed using [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT). After 24 h treatment of cells with the compounds at 37 °C, the culture medium was removed from the wells, and cells were washed three times with PBS. Then 1 mL of MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h at the same temperature. After this time MTT solution was replaced with 1 mL of 0.1 M HCl in absolute isopropanol and subjected to thoroughly shaking to dissolve the resulting formazan crystals. Cell viability was evaluated by the measurements of the absorbance (570 nm) using a microplate reader (TECAN, Männedorf, Switzerland).

2.8. Quantitative Real-Time PCR Analysis

Total RNA of cells was isolated using a Total RNA Mini Plus concentrator (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The concentration of obtained RNA was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of RNA (1 µg) were subjected to the synthesis of complementary DNA (cDNA) with the use of the cDNA Synthesis Kit (Bioline, London, UK). Each cDNA was diluted 10-fold and used as a template for quantitative real-time PCR (qRT-PCR) assay using SensiFAST™ SYBR Kit (Bioline, London, UK). The qRT-PCR was performed in CFX96 real-time system thermal cycler (Bio-Rad, Hercules, CA, USA) in the final volume of 10 µL of the reaction mixture. The sequences of primers (Genomed, Warsaw, Poland) used to analyze gene expression were as follows: *COL1A1*, forward 5′-ATG TCT AGG GTC TAG ACA TGT TCA-3′ and reverse 5′-CCT TGC CGT TGT CGC AGA CG-3′; glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*, forward 5′-CTC TGC TCC TCC TGT TCG AC-3′ and reverse 5′-GCC CAA TAC GAC CAA ATC C-3′. Three replicates in double repeats were conducted for each reaction. The qRT-PCR proceeded according to the following program: 30 s at 95 °C for initial denaturation, followed by 40 cycles (10 s at 95 °C, 10 s at 60 °C and 20 s at 72 °C). The specificity of products of each amplification was verified by the analysis of the melting curves. The relative gene expression level was calculated using the $2^{-\Delta\Delta CT}$ method in the CFX96 real-time PCR system.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA) Measurement for Procollagen Type I

The amount of procollagen type I in cell lysates and secreted by fibroblasts was measured using Human pro-Collagen I alpha 1 Simple Step Elisa Kit (Abcam, Cambridge, UK). After 24 h treatment of cells with tested compounds, the experimental media were collected and centrifuged at 2000× *g* for 10 min. The concentration of protein in cell culture supernatants was determined with Coomassie Plus—The Better Bradford Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA). Cells were washed three times with PBS and harvested with Extraction buffer provided with the assay. After incubation on ice for 20 min samples were centrifuged at 18,000× *g* for 20 min at 4 °C. The concentration of protein in cell lysates was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). A standard curve was prepared using Procollagen I alpha 1 standard in the concentration of 10–1000 pg/mL. Aliquots of samples (50 µL/well) of an appropriately diluted cell lysates and experimental media, containing 0.5–1 mg of total proteins, were added to a 96-well microtitre plate coated with procollagen type I standard and then the manufacturer's protocol was followed. The assays were done in duplicates in three independent experiments. The secretion of procollagen was calculated by dividing the amount of procollagen released into the cultured medium by the sum of medium and intracellular collagen.

2.10. Western Blot

The cultured media collected after experiments were concentrated 10 times, cells were harvested in RIPA buffer (Sigma–Aldrich Corp., St. Louis, MO, USA) supplemented with protease inhibitor cocktail (P8340) (Sigma–Aldrich Corp., St. Louis, MO, USA). An equal amount of protein (20 µg) was loaded on 7.5% polyacrylamide gel and after electrophoresis proteins were transferred onto Immobilon-P Transfer membrane (Merck Millipore Ltd., Tullagreen, Carrigtwohill, County Cork, Ireland). Then membrane was washed in TBS-T (50 mM Tris-HCl, 500 mM NaCl, pH 7.5 containing 0.05% (*v*/*v*) Tween 20) and blocked with 5% (*w*/*v*) non-fat dried milk in TBS-T at room temperature for 1 h. After this time, the membrane was washed three times with TBS-T and incubated overnight at 4 °C with primary antibodies: mouse anti-collagen type I (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit anti- β-actin (1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA) as a loading control. The appropriate secondary anti-mouse immunoglobulin G (IgG) (Sigma–Aldrich Corp., St. Louis, MO, USA) and anti-rabbit horseradish peroxidase conjugated antibodies (Cell Signaling Technology, Massachusetts, USA) at dilution of 1:2000 were added and membrane was incubated for 1 h at room temperature with slow mixing. After washing (3 times for 10 min with TBS-T) membrane was developed using a Westar Supernova Chemiluminescent Substrate for Western Blotting (Cyanagen, Bologna, Italy) and documented with the use of BioSpectrum Imaging System gel documentation apparatus (UVP, Upland, CA, USA). The intensity of the protein bands was measured by densitometry, for which an imaging densitometer (G: BOX, Syngene, Cambridge, UK) was used.

2.11. Steady-State Analysis of Type I Collagen

Confluent fibroblast cultures were incubated for 2 h in serum-free medium containing magnesium ascorbate (25 µg/mL), and after the adding of RA, RE, and LBE incubation was continued for 16 h. Procollagens were harvested from the media and cell layer and precipitated overnight at 4 °C with ammonium sulfate (176 mg/mL). After centrifugation at 37,000× *g*, pellets were dissolved in 0.5 M CH_3COOH. To obtain collagen, procollagen solution was digested with pepsin (50 µg/mL) for 4 h at 4 °C. Procollagen chains and collagen chains were separated on 5% SDS-urea-polyacrylamide gels under reducing and non-reducing conditions, respectively, and visualized by silver staining [38]. The intensity of collagen bands was measured by densitometry (G: BOX, Syngene, Cambridge, UK).

2.12. Zymography

The fibroblast conditioned medium containing an equal amount of total proteins was activated with 1 mM APMA, mixed with four times concentrated sample buffer and load on 10% SDS-polyacrylamide gel containing 1 mg/mL gelatin (Sigma–Aldrich Corp., St. Louis, MO, USA) for detection of MMP-1, MMP-2, and MMP-9 activities, respectively. After electrophoresis, the gels were transferred to a 2.5% Triton X-100 solution to remove SDS. Then the gels were placed in the incubation buffer (50 mM Tris-HCl, pH 8.0, supplemented with 5 mM $CaCl_2$, 5 μM $ZnCl_2$ and 0.02% NaN_3) overnight at 37 °C. For gel staining 0.5% Coomassie Brilliant Blue R-250 in solution containing 40% methanol and 10% acetic acid was used. Finally, they were destained until MMP as transparent stripes on a blue background were visible. Images of the zymograms were subjected to the densitometry (G:BOX, Syngene, Cambridge, UK).

2.13. Statistical Analysis

The results were statistically analyzed using the Statistica 12 software (StatSoft, Tulsa, OK, USA). They were presented as the mean ± standard deviation (SD). Statistical differences were estimated using a one-way ANOVA followed by Tukey's test. Values of $p < 0.05$ were considered to indicate statistically significant differences.

3. Results

3.1. Qualitative and Quantitative Analysis of RE and LBE

Detailed phytochemical analysis of the secondary metabolites present in both tested extracts (RE and LBE) showed the presence of 34 different compounds. The fingerprints of the analyzed extracts were established using LC-PDA-MS method. The analysis revealed constituents comprising polyphenols as caffeic acid derivatives (14, 24), flavonoids such as luteolin (7, 8, 16, 20, 22, 23) and apigenin (10, 25) derivatives, respectively, and related compounds such as diterpenes (29, 31, 32). The LC-MS analysis is summarized in Table 2, Figure 2 and Figure 3. Finally, we determined the quantitative content of the dominant RA component in both tested extracts. Quantitative analysis of its content showed its high concentration of 27.23 ± 0.54 mg/g and 80.26 ± 1.24 mg/g for RE and LBE, respectively (Table 3).

Table 2. Qualitative analysis of rosemary extract (RE) and lemon balm extract (LBE) by liquid chromatography-photodiode array detection-mass spectrometry (LC-PDA-MS).

No	Rt (min)	Tentatively Identified Compounds	λ Max (nm)	$[M + H]^+/[M + H]$ (*m/z*)	Extracts
1	1.50	Quinic acid [a]	290	-/191	RE, LBE
2	9.88	2-hydroxy-3-(3,4-dihydroxyphenyl)-propanoic acid [b]	283	-/197	RE, LBE
3	17.54	Medioresinol [c]	290	-/387	RE
4	20.20	Gallocatechin [d]	280	-/305, 225	RE
5	20.60	Hydroxyjasmonic acid sulphated [b]	275, 325	-/305, 225	LBE
6	21.20	Salvianolic acid derivative isomer 1 [b]	275, 325	-/537, 493, 295	LBE
7	22.68	Luteolin glucosylrhamnoside [b]	280, 330	-/593	LBE
8	23.05	Luteolin 7-*O*-glucoside [s]	282, 333	287, 449/285, 447	RE, LBE
9	23.88	Nepetin 7-*O*-glucoside [a]	272, 345	317, 479/477	RE
10	24.71	Apigenin 7-*O*-glucoside [s]	280, 338	271, 433/-	LBE
11	24.94	Hesperitin-7-rhamnoglucoside [s]	284, 330	303, 610/301, 609	RE
12	25.12	Salvianolic acid B [b]	280, 325	-/717	LBE
13	25.40	Hispidulin 7-*O*-glucoside [s]	274, 334	301, 463/461	RE
14	25.81	Rosmarinic acid [s]	290, 330	361, 343, 505/359	RE, LBE
15	26.12	Sagerinic acid [b]	280, 330	-/719, 359, 161	LBE
16	26.15	Luteolin 7-*O*-glucuronide [b]	268, 340	287, 463/461	RE, LBE
17	26.45	Nepetin derivative [a]	275, 335	317, 685/683	RE
18	27.12	Cirsimaritin derivative [a]	278, 332	315, 477, 655/653	RE
19	27.12	Lithospermic acid isomer 1 [b]	290, 325	-/537, 493, 359	LBE

Table 2. *Cont.*

No	Rt (min)	Tentatively Identified Compounds	λ Max (nm)	$[M + H]^+/[M + H]$ (*m*/*z*)	Extracts
20	27.32	Luteolin 3′-O-(O-acetyl)-glucuronide isomer 1 [a]	268, 335	505/285, 459, 503	RE
21	27.71	Lithospermic acid isomer 2 [b]	290, 325	-/537, 493, 359	LBE
22	27.77	Luteolin 3′-O-(O-acetyl)-glucuronide isomer 2 [a]	268, 335	505/285, 459, 503	RE
23	28.14	Luteolin 3′-O-(O-acetyl)-glucuronide isomer 3 [a]	268, 335	505/285, 459, 503	RE
24	28.32	Rosmarinic acid sulphated isomer [b]	290, 328	-/439, 359	LBE
25	29.90	Apigenin [s]	268, 335	271/269	RE
26	30.15	Salvianolic acid derivative isomer 2 [b]	290, 327	-/715	LBE
27	30.50	Hispidulin [s]	276, 334	301/299	RE
28	30.82	Salvianolic acid derivative isomer 3 [b]	290, 327	-/715	LBE
29	32.04	Rosmanol isomer 1 [a]	284	369/283, 301, 345	RE
30	32.26	Cirsimaritin [s]	275, 335	315/313	RE
31	32.80	Rosmanol isomer 2 [a]	284	369/283, 301, 345	RE
32	33.65	Rosmanol isomer 3 [a]	288	369/283, 301, 345	RE
33	34.00	Genkwanin [a]	268, 334	285/283	RE
34	38.38	Miltipolone isomer [a]	285	345/299	RE

Comparison: [s]—reference substance, [a]—Borrás Linares et al. [39]; [b]—Ozarowski et al. [40]; [c]—Hossain et al. [41]; [d]—Nie et al. [42].

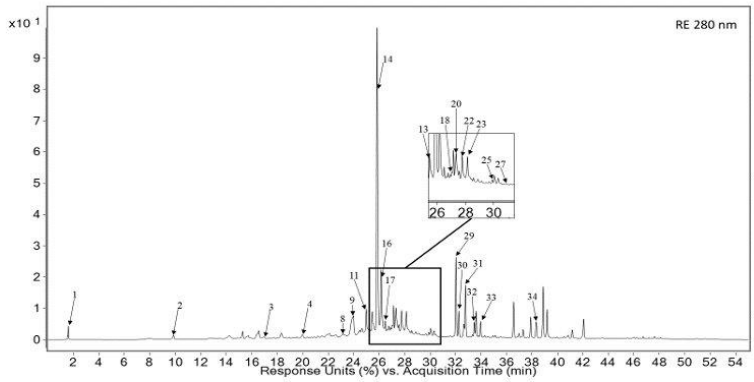

Figure 2. UV spectrum of major constituents of rosemary extract (280 nm).

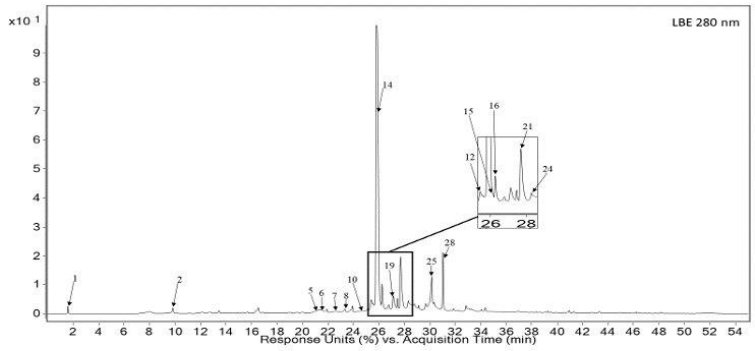

Figure 3. UV spectrum of major constituents of lemon balm extract (280 nm).

Table 3. Concentration of rosmarinic acid (RA) in rosemary extract (RE) and lemon balm extract (LBE).

Sample	RA Concentration [mg/g]	SD [mg/g]	RSD [%]
RE	27.23	0.54	2.01
LBE	80.26	1.21	1.51

SD—standard deviation, RSD—relative standard deviation.

3.2. The Influence of RA, RE and LBE on the Viabiliy of Normal and OI Fibroblasts

After 24 h treatment of normal and OI skin fibroblasts with RA at concentrations of 0.1–100 μM and with extracts (RE and LBE) at concentrations of 0.1–100 μg/mL each, no significant effect on cell viability was found (Figure 4).

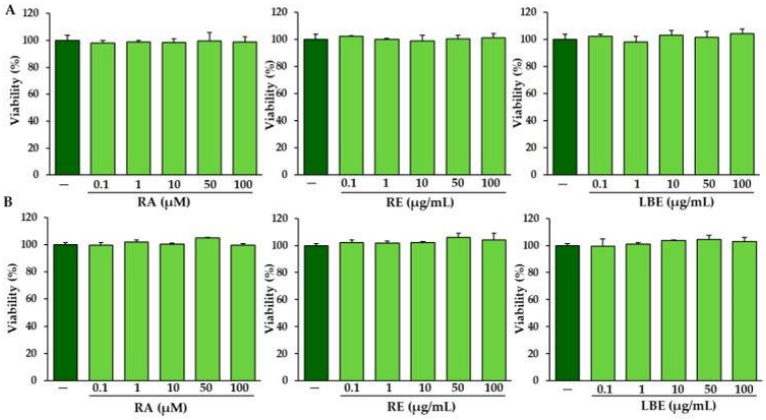

Figure 4. Effects of rosmarinic acid (RA) at concentrations of 0.1–100 μM, and rosemary extract (RE) and lemon balm extract (LBE) at concentrations of 0.1–100 μg/mL each on the viability of normal (**A**) and OI (**B**) skin fibroblasts. Values represent the means of percentage of the untreated cells ± SD ($n = 5$).

3.3. Effect of RA, RE and LBE on the Content of Intracellular and Secreted Type I Procollagen in OI Fibroblasts Determined by ELISA

Human pro-Collagen I alpha 1 Simple Step Elisa Kit (Abcam, Cambridge, UK) was used to estimate the effect of RA and extracts (RE and LBE) on the amount of intracellular and secreted type I procollagen. In OI skin fibroblasts, the amount of intracellular and secreted type I procollagen was reduced by about half compared to normal cells, while the exposure of OI cells to RA at concentrations of 0.1–100 μM, to RE at concentrations of 0.1–100 μg/mL, and to LBE at concentrations of 0.1–100 μg/mL resulted in a significant increase in the secreted procollagen at all concentrations (Figure 5A). The highest increase as compared to untreated OI cells was observed in the presence of RA at concentrations of 0.1, 1, and 10 μM and extracts (RE and LBE) at concentrations of 0.1, 1, and 10 μg/mL each. Moreover, LBE at concentrations of 1 and 10 μg/mL normalized the amount of procollagen type I to the level of this protein in the cultured medium of normal cells. In turn, cells treated with RA and extracts also showed an increase in the content of type I procollagen, except for their highest concentrations (100 μM RA, 100 μg/mL RE, 100 μg/mL LBE), to a lesser extent than in the medium (Figure 5B). Secreted procollagen type I accounted for 82.5% and 79.1% of total collagen in normal and OI cells, respectively, while under the influence of 100 μM RA, 100 μg/mL RE, and 100 μg/mL LBE, it decreased to 74.5%, 71.8%, and 73.6%, respectively. In the presence of LBE at concentrations of 0.1, 1, and 10 μg/mL there was the increase in secretion of type I procollagen in OI cells comparable to that showed in the normal cell (Figure 5C).

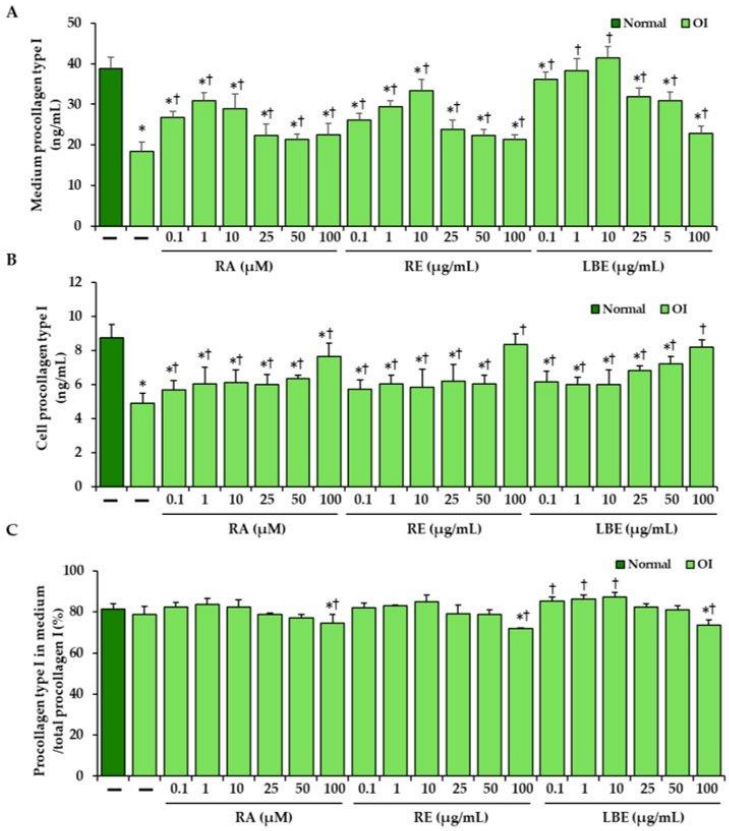

Figure 5. The influence of rosmarinic acid (RA), rosemary extract (RE), and lemon balm extract (LBE) on the amount of secreted (**A**) and intracellularly retained (**B**) type I procollagen in OI cells. (**C**) Secretion expressed as the ratio of secreted type I procollagen to total procollagen I. Values represent the mean ± SD of three experiments done in duplicate. * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells.

For subsequent analyzes, we chose the concentrations of RA (0.1, 1, and 10 μM), RE (0.1, 1, and 10 μg/mL), and LBE (0.1, 1, and 10 μg/mL), at which they had the most beneficial effect on procollagen type I in OI fibroblasts.

3.4. Analysis of Procollagen and Collagen Type I and III in OI Fibroblasts Treated with RA, RE and LBE

SDS-urea polyacrylamide gel electrophoresis (SDS-urea PAGE) was used for the identification and comparison of procollagen and collagen type I and III in treated OI cells. Procollagen type III migrated close to the proα1 of procollagen type I and their levels in OI cultured medium were significantly lower compared to the normal, while an increase is evident after the treatment of OI cells with RA at concentrations of 0.1, 1, 10 μM, and extracts (RE and LBE) at concentrations of 0.1, 1, and 10 μg/mL each (Figure 6A). The increase in the level of type I collagen in the treated OI cells was confirmed after separation of pepsin-digested procollagen under non-reducing conditions, whereas no substantial impact of RA and both extracts (except a slight reduction by 1 μg/mL LBE) on

type III collagen was found (Figure 6B). The intensity of the α1(I) bands in cell layer was comparable in the treated and untreated OI cells or slightly increased at 1 μM RA.

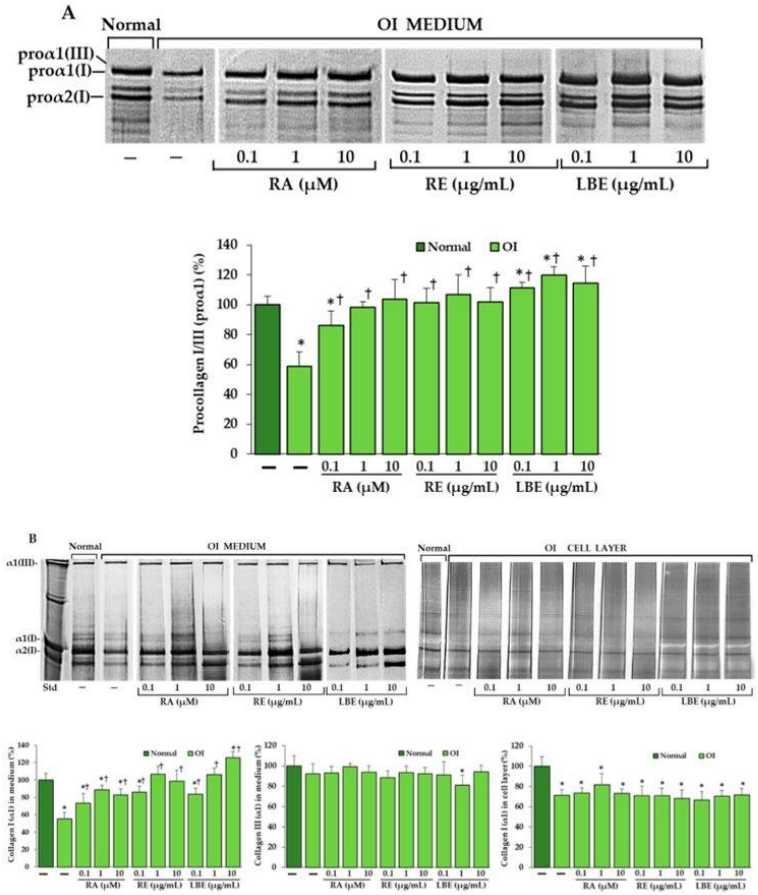

Figure 6. SDS-urea polyacrylamide gel electrophoresis (SDS-urea PAGE) of procollagen type I and III in reducing conditions (**A**) and collagen type I and III in non-reducing conditions (**B**) in OI fibroblasts treated with rosmarinic acid (RA), rosemary extract (RE), and lemon balm extract (LBE); Std—bovine collagen type I (Biocolor Life Science, U.K). Densitometry results represent the mean of three independent experiments; * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the control sample assumed as 100%.

3.5. Western Blot Analysis of Expression of Collagen Type I in OI Fibroblasts

Western blot analysis clearly showed the increase in the secreted type I collagen under the influence of RA and both extracts in OI cells (Figure 7). Densitometric measurements showed the greatest increase, corresponding to the level of collagen secreted by normal cells, in the presence of LBE at concentrations of 0.1, 1 and 10 μg/mL and 10 μg/mL RE. Normalization of intracellular type I collagen to the β-actin showed either no difference in treated versus untreated OI cells or a reduction by RE (1 μg/mL) and LBE (10 μg/mL).

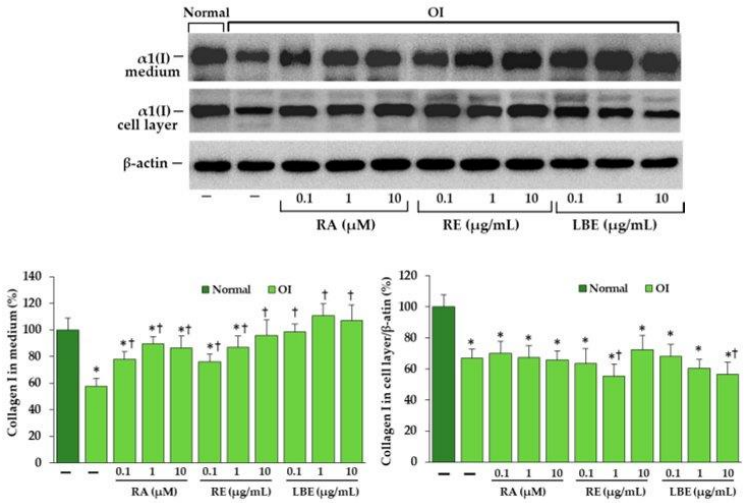

Figure 7. The influence of rosmarinic acid (RA), rosemary extract (RE), and lemon balm extract (LBE) on secreted (in medium) and intracellular type I collagen, β-actin was shown as cell protein loading control. Densitometry results represent the mean of three independent experiments; * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the control sample assumed as 100%.

3.6. Effect of RA, RE and LBE on the Expression of Collagen Type I at mRNA Level in OI Fibroblasts

To examine whether the extracts and RA affect collagen type I at mRNA level, the quantitative real-time PCR was performed. As shown in Figure 8, in OI untreated cells, the expression of *COL1A1* gene encoding α1(I) was reduced by about half, while the significant increase was seen in cells treated with RA and extracts. At 0.1 μM RA and 0.1, 1 μg/mL RE, the transcript level was comparable to normal cells, while in the presence of 1 μM RA and 0.1, 1, and 10 μg/mL LBE, *COL1A1* expression was significantly exceeded compared to untreated OI and normal cells.

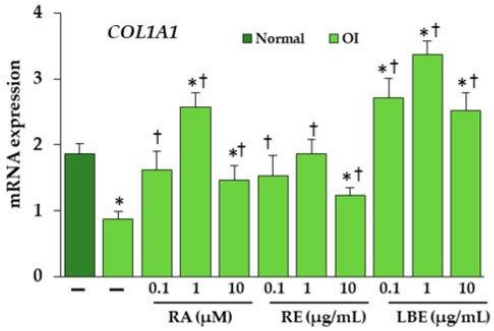

Figure 8. The influence of rosmarinic acid (RA), rosemary extract (RE), and lemon balm extract (LBE) on the expression of *COL1A1* gene encoding α1 of collagen type I in OI skin fibroblasts. Values represent the mean ± SD of three experiments done in duplicate. * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells.

3.7. Effect of RA, RE and LBE on the Activity of MMP-1, MMP-2 and MMP-9 in OI Fibroblasts

By using zymography assay, the presence of proenzymes (pro-MMP-2 and pro-MMP-9) and their active forms (MMP-2 and MMP-9), as well as MMP-1, was demonstrated (Figure 9A). Densitometric analysis of individual active forms of enzymes in OI showed similar MMP-1 and higher activities of MMP-2 and MMP-9, compared to those in normal cells (Figure 9B). MMP-2 activity was inhibited by 10 μM RA, 1 and 10 μg/mL RE, and LBE at all concentrations (0.1, 1, and 10 μg/mL). MMP-9 activity was inhibited by RA and LBE at all used concentrations and by RE at concentrations of 1 and 10 μg/mL. In turn, collagenase I (MMP-1) was only inhibited by the extracts at their higher concentrations (1 and 10 μg/mL).

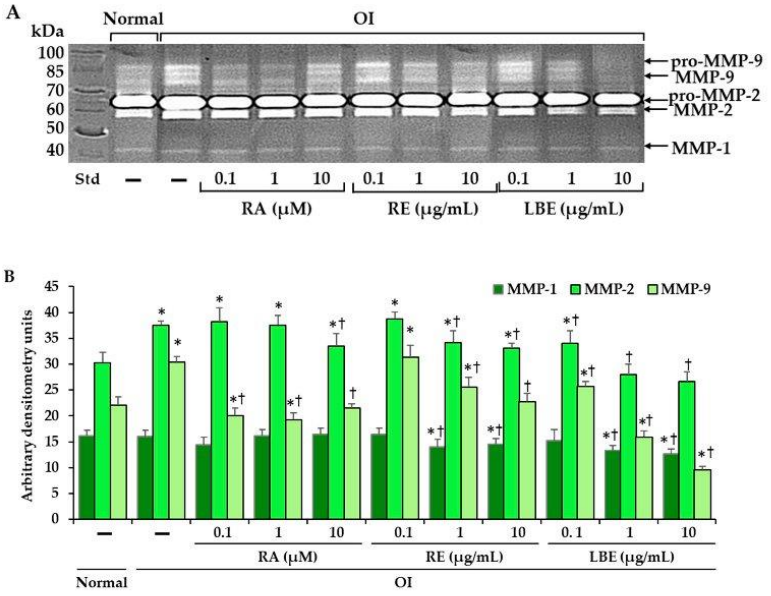

Figure 9. The influence of rosmarinic acid (RA), rosemary extract (RE), and lemon balm extract (LBE) on the activity of MMP-1, MMP-2, and MMP-9 in OI cells determined by zymography (**A**); Std—PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA). Densitometry results represent the mean of three independent experiments (**B**); * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells.

4. Discussion

OI type I is the mildest type of the disease caused by stop or frameshift as well as splicing site mutations in *COL1A1* gene resulting in the haploinsufficiency of the α1 chain of collagen type I [43–45]. These mutations are known as quantitative in contrary to those which alter the amino acid sequence of type I collagen α chains and are called structural mutations [24–27]. The most common structural mutations are glycine substitutions in the triple helical domain of the α1 or α2 chains, which give the wide range of phenotypic severity from mild to lethal [23–29]. The quantitative and structural mutations affecting collagen type I have consequences in all tissues that produce this protein, however, the easy to obtain patients' skin fibroblasts are the cell type commonly used for molecular testing. Apart from the few reports concerning structural mutations, where the expression and thermostability of mutant collagen type I in osteoblasts was slightly different than that of the skin fibroblasts [46,47], most collagen type I defects are similarly expressed and give symptoms not only in the bone but also in other connective tissues rich in this protein.

The greatest amount of collagen type I is found in skin, tendons, ligaments, bones, teeth, and the cornea [30]. Clinical symptoms commonly observed in OI patients include, in addition to abnormal bone formation and fragility, growth deficiency, blue or gray sclera, hearing loss, joint laxity, muscle weakness, dentinogenesis imperfecta, valvular regurgitation, and impaired pulmonary function [25–29]. In the skin collagen type I accounts for about 85–90% of the total collagen, the rest is collagen type III accounting for 10–15% and small amounts of dermal collagen are types IV, V, VI, and VII [30,31]. Collagen type I provides the dermis its structural integrity and has a significant impact on the properties of the skin because it is essentially responsible for the tensile strength and its mechanical properties, and it maintains skin firmness and elasticity. ECM of the skin is not only a structural but also a dynamic structure as collagen type I is a signaling molecule, which transmits the signals to the cell via integrins influencing the shape and behavior of cells [48]. In order to properly perform these functions, it is important to maintain collagen homeostasis. For example, abnormal collagen homeostasis associated with decreased biosynthesis of collagen under the influence of environmental factors, mainly UV radiation, is manifested by thin, fragile skin, which impairs its structural integrity and mechanical properties, and contributes to various skin diseases [31,49]. Abnormal skin structure and function in OI include thinness, translucency, bruising, decreased elasticity, and elastosis perforans serpiginosa [28,29]. Despite the growing research and knowledge of the genetic and molecular mechanisms of collagen biosynthesis and its abnormalities in this complex heterogeneous disease, OI is still an incurable disease. Therefore, research in this area is important to provide patients with the best possible treatments. There are many reviews on the pre-clinical treatment strategies, but existing therapies for OI are mainly based on an intravenous administration of bisphosphonates, however, with undesirable side effects [25–27,50–52]. Long-term inhibition of osteoclasts by bisphosphonates may reduce bone quality, leading to non-dynamic bone in which microdamages are not repaired and accumulate, likely causing an overall increase in bone fragility [25,26]. Unusual femoral fractures and delayed teeth eruption have been reported in children after long-term treatment with pamidronate [50].

Other antiresorptive therapies using monoclonal antibodies, such as Denosumab, against receptor activator of nuclear factor kappa-B ligand, Romosozumab against sclerostin (negative regulator of bone formation) or Odanacatib against cathepsin K (involved in the degradation of type I collagen) are currently under investigation in OI [50–52]. To improve patients' condition growth hormone, growth factors (e.g., transforming growth factor), vitamin D supplementation are used [25–27,50,51].

Our study provides for the first time evidence of the beneficial effects of RA and extracts rich in this compound (RE and LBE) that either significantly reduced the quantitative defect of type I collagen in OI type I skin fibroblasts or, depending on their concentration, normalized to the level found in normal cells. After testing the cell responses to doses of the extracts and RA in the 1000–fold (0.1–100 μg/mL and 0.1–100 μM) range, an optimal stimulatory effect on collagen type I in relatively low concentrations of RA (0.1–10 μM RA) and extracts (0.1–10 μg/mL) was found. The reduction of type I collagen by about a half as a result of loss-of-function mutation in *COL1A1* gene and the increase under the influence of RA and the tested extracts (RE and LBE) were confirmed by assays at the mRNA (qRT-PCR) and protein (ELISA, SDS-urea PAGE, Western Blot) levels. At mRNA level, LBE showed about three-fold greater stimulating effect on the *COL1A1* gene as compared to RE. Taking into account the approximately three times higher concentration of RA in LBE (80.26 ± 1.24 mg/g) compared to RE (27.23 ± 0.54 mg/g) (Table 3), it can be hypothesized that RA as the main component of both extracts may be responsible for their beneficial effect on collagen type I. RA administration itself showed significant increase in collagen type I mRNA expression, the highest at a concentration of 1 μM, which corresponds to its content of approximately 4 μg/mL in LBE and 12 μg/mL in RE that at these concentrations significantly increased collagen type I expression. The quantification of procollagen type I with Elisa also showed the most effective stimulating effect by LBE as

compared to RA and RE; in this case the level of procollagen in OI cells was comparable to the normal cells. Higher stimulating potential of LBE than RE at concentrations containing a comparable amount of RA may suggest that other compounds present in the extracts may be responsible for this effect. As shown in the Table 2, they contain many biologically active substances, some of them are common and others specific for one extract. For example, apigenin has only been identified in RE, whereas its glucoside (apigenin 7-*O*-glucoside) has been identified in LBE. Our previous studies have shown that apigenin had inhibitory while apigenin 7-*O*-glucoside slightly stimulatory effects on collagen biosynthesis in OI cells [21]. Based on these data, it can be assumed that the higher LBE stimulating effect on collagen type I may be the result of the synergistic action of RA and the apigenin 7-*O*-glucoside, although a different type of interaction between these and other compounds cannot be ruled out. A common component of both extracts is luteolin 7-*O*-glucoside (Table 2), and although in our research it was not quantified, other studies suggest significant differences in the content of it in RE and LBE [53,54]. In unpublished studies, we noted the stimulating effect of this flavonoid on type I collagen in normal human skin fibroblasts. If luteolin 7-*O*-glucoside shows a similar effect on OI cells with collagen deficiency, it can contribute to the action of rosemary and lemon balm extracts, but possible to a different degree due to the large differences in the concentration of this flavonoid in both extracts, given by these authors [53,54].

It should also be emphasized that, unlike drugs, the use of which usually involves more or less serious side effects, the use of these compounds of plant origin with many valuable properties, especially antioxidant, may bring a number of additional beneficial effects on the skin. Both RA and the extracts tested by us, at similar low concentrations, at which we revealed the stimulation of collagen type I biosynthesis, have been shown to protect against the effects of free radicals on skin fibroblasts. RA at the concentrations of 0.5 and 1.0 μM had ability to support the long-term lifelong growth of fibroblasts and increased tolerance to cellular stress by reducing the rate of telomere loss and reducing epigenetic molecular markers of cellular aging: 5-methyl-cytosine, 5-hydroxymethylcytosine, and oxidative stress marker 8-hydroxy-2′-deoxyguanosine [55].

Numerous reports indicate that RA has potential as a therapeutic agent in skin diseases caused by reactive oxygen species induced, among others, by solar UV radiation. UV is a very important environmental factor associated with an increased risk of many skin diseases including atrophy and neoplastic changes, and premature aging. It is responsible for many harmful symptoms, including inflammation, wrinkles and sagging skin, discoloration, erythema, swelling, immunosuppression, and hyperplasia [56]. RA at the concentration of 2.5 μM showed a protective effect on skin cells against UV radiation through the activation of the antioxidant enzymes (superoxide dismutase, catalase) and transcription factor (erythroid-derived 2)-like 2) [57]. The results of Rodríguez-Luna [58] suggest that RA at the concentration of 5 μM in combination with 5 μM fucoxanthin could be used as an interesting strategy in the counteracting oxidative damage in the skin induced by UVB. There is also a report of a protective effect of RA against the harmful effects of UVA on the formation of collagen type I fibers in normal human skin fibroblasts, however at a much higher concentration (62.5 μg/mL, which is equivalent to 174.6 μM) [59]. We had previously discovered the protective effect of RA (100–150 μM) against the negative influence of parabens (methyl- and propylparabens) on the skin collagen type I and III and the viability of human skin fibroblasts [22]. Most people are exposed to them throughout their lives due to their widespread presence in cosmetic and personal care products applied daily to the skin, and their excessive use can create a real health risk due to their estrogenic properties [60]. RA shows promise in the treatment of various inflammatory skin diseases such as psoriasis [61] and atopic dermatitis [12] as shown in in vitro and in vivo studies. It has been shown that RA and extracts rich in this compound exhibit anti-aged effects on the skin [13–18,62,63]. Rosemary rich in active antioxidants, microelements, and nutrients, exhibits protective potential against free radical damage and is essential in improving the

quality of the skin. An interesting feature of the lemon balm extract is its melanogenic properties, which can also be used to protect the skin against UV radiation [17].

Skin homeostasis is mediated by the coordinated secretion by fibroblasts of MMPs involved in the degradation of the ECM components, including collagen [49]. Their secretion is stimulated by oxidative stress, UV radiation, and cytokines and they play an important role in in wound healing, skeletal growth, and remodeling as well as various pathophysiological processes, such as photoaging, inflammation, angiogenesis, and cancer [49]. In skin fibroblasts, MMPs are secreted in a latent form and the active forms appear in the medium after culturing for 72–96 h [64]. In our study to test the activity of MMP-1, -2, and -9 after 24 h of cell culture, MMPs were activated with APMA. Depending on the concentrations of RA and extracts, they did not have an effect or showed inhibitory effect, which may be beneficial in many processes including collagen degradation, which they mediate.

In summary, both RA and extracts (RE and LBE) at low concentrations non-toxic to the fibroblasts showed the beneficial effects on type I collagen and MMPs (MMP-1, -2, and -9) involved in collagen and skin homeostasis. Extracts, especially LBE, turned out to be more effective than RA itself in stimulation of the expression of type I collagen at the protein level. It is important to note that the extracts are generally cheaper and more convenient to produce for the use as nutraceuticals. In an in vivo study involving eleven healthy young individuals, it was shown that the intake of a single dose of LBE extract containing 500 mg of RA per day was safe [65]. The goal of this study was to use the extract to treat age-related neurodegenerative disorders; however, long-term safety studies in this population are needed to confirm these results. The stronger effect of the extracts on type I collagen compared to RA demonstrated in our study requires further research to identify compounds other than RA responsible for their more effective action. Despite the identification of a large amount of polyphenolic compounds with broad biological activity, there are little, with the exception of our reports [20–22], research on their influence on this key protein of the human body.

Of note, in data interpretation it is important to take into consideration the following limits of the present study. First, our data are limited to the use of a single OI skin fibroblast line and the test on more OI type I patients cells will be necessary to strengthen the results. Secondly, considering the pharmacokinetics of RA or other components of the extracts (their limited oral absorption or rapid metabolism), there may be large differences between the results obtained from our in vitro study versus in vivo administration. At the target site of action in the body, it is possible that concentrations may be significantly lower than the ones used. However, the concentrations of the tested extracts and RA at which the optimal effect on collagen was obtained in our study can be considered similar or much lower compared to those used in many other studies. Finally, additional data on their pharmacokinetics are necessary in order to use them in the co-therapy of this disease or also other disorders associated to collagen type I deficiency.

5. Conclusions

This study demonstrates new clinically relevant properties of RA and of RA containing extracts (RE and LBE) related to their potential to promote the expression of type I collagen in the skin fibroblasts of type I OI patient. Noteworthy, their lack of toxicity up to a concentration of 100 μM RA and 100 μg/mL of extracts, and their beneficial effect on collagen type I at low concentrations, as well as a number of their other valuable health promoting effects, are of particular relevance for therapy. RA may play a major role in the action of RE and LBE on collagen type I; however, testing the action of other components in future research may better elucidate the mechanism of action of these extracts.

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Abbreviations

APMA	4-aminophenylmercuric acetate
BSA	Bovine serum albumin
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
FA	Formic acid
FBS	Fetal bovine serum
LBE	Lemon balm extract
LC-PDA-MS	Liquid chromatography-photodiode array detection-mass spectrometry
MMPs	Matrix Metalloproteinases
MTT	(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide
OI	Osteogenesis imperfecta
PBS	Phosphate-buffered saline
PDA	Photo-diode array
qRT-PCR	Quantitative Real-time PCR
RA	Rosmarinic acid
RE	Rosemary extract
RIPA	Radioimmunoprecipitation assay buffer
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-urea PAGE	SDS-urea polyacrylamide gel electrophoresis
UV	Ultraviolet
UPW	Ultra pure water

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Rosemary estract – induced autophagy and decrease in accumulation of collagen type I in osteogenesis imperfecta skin fibroblasts

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Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts

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Abstract: Osteogenesis imperfecta (OI) is a heterogeneous connective tissue disease mainly caused by structural mutations in type I collagen. Mutant collagen accumulates intracellularly, causing cellular stress that has recently been shown to be phenotype-related. Therefore, the aim of the study was to search for potential drugs reducing collagen accumulation and improving OI fibroblast homeostasis. We found that rosemary extract (RE), which is of great interest to researchers due to its high therapeutic potential, at concentrations of 50 and 100 μg/mL significantly reduced the level of accumulated collagen in the fibroblasts of four patients with severe and lethal OI. The decrease in collagen accumulation was associated with RE-induced autophagy as was evidenced by an increase in the LC3-II/LC3-I ratio, a decrease in p62, and co-localization of type I collagen with LC3-II and LAMP2A by confocal microscopy. The unfolded protein response, activated in three of the four tested cells, and the level of pro-apoptotic markers (Bax, CHOP and cleaved caspase 3) were attenuated by RE. In addition, the role of RE-modulated proteasome in the degradation of unfolded procollagen chains was investigated. This study provides new insight into the beneficial effects of RE that may have some implications in OI therapy targeting cellular stress.

Keywords: rosemary extract; collagen type I; unfolded protein response; autophagy; proteasome; skin fibroblasts; apoptosis; osteogenesis imperfecta

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1. Introduction

Osteogenesis imperfecta (OI) is a rare hereditary bone disease, with a frequency of 1 in 15,000 to 20,000 live births, characterized by phenotypic and genotypic heterogeneity [1,2]. The most common symptoms include bone fragility, skeletal deformities, reduced bone mineral density, short stature, blue sclera, dentinogenesis imperfecta, hearing impairment, joint hypermobility, skin fragility, muscle weakness, and cardio-respiratory defects [1–3].

Despite recent discoveries of mutations in many genes, the most common are mutations in genes encoding type I collagen, which cause the majority (85%) of cases of OI [1–5]. Mutations in the *COL1A1* or *COL1A2* genes encoding the α1(I) and α2(I) chains of type I collagen, respectively, are dominant and cause quantitative or structural disturbances in collagen. The classification of OI due to collagen mutations includes four types, the phenotype of which ranges from mild (type I) and moderate (type IV) to severe (type III) and perinatal lethal in OI type II [6]. The molecular defect in OI type I is a null

COL1A1 allele due to premature stop codons, either directly or through frame shifts resulting in reduced synthesis of functional collagen type I [4,5,7]. OI types II-IV are mainly caused by substitutions of glycine residues by another amino acid (80%), but also by splicing site mutations as well as small triplet deletion or duplication mutations, which shift the register of α-chains in the helix [2,4,5,8]. Most of them result in the synthesis of mutant misfolded collagen molecules. In recent years, many other causative genes associated with recessive and X-linked forms of the disease have been detected. Most of these genes code for type I collagen-related proteins that play an important role in folding and post-translational modifications, secretion as well as quality control of collagen synthesis. Mutations of proteins unrelated to collagen type I play an important role in osteoblast maturation and bone mineralization [2,3,5,8–10].

Synthesis of type I collagen is a complex process including the intracellular and extracellular steps preceding the formation of mature collagen fibrils. Two proα1 and one proα2 chain are synthesized in the endoplasmic reticulum (ER) and undergo important post-translational modifications prior to triple helix folding, including hydroxylation of proline (at C-4 and C-3) and lysine residues [11,12], which have fundamental importance for the stability of the helix. Proper folding and post-translational modifications in the ER determine the effectiveness of collagen secretion to the extracellular matrix (ECM) [11,12]. Glycine substitutions are responsible for the delay in the formation of the triple helix. Prolonged exposure of procollagen chains to post-translational modifying enzymes leads to increased hydroxylation of proline and lysine residues and glycosylation of hydroxylysine residues, causing the synthesis of collagen molecules with the abnormal structure [2,4,5,8,13]. The mutated collagen is secreted into ECM, but it may be partially retained in the ER causing cellular stress, which may be related to the clinical outcome [14–18]. It has been reported that some OI cells activate an unfolded protein response (UPR) to restore cell homeostasis [14,17–21]. The best-studied three ER membrane receptors of UPR include inositol requiring enzyme 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [19,20,22]. Under normal conditions, the chaperone—binding immunoglobulin protein (BiP) binds all three sensor proteins in their ER luminal domain and keeps them inactive, while under stressful conditions it binds preferentially to misfolded proteins leading to activation of UPR pathways. ATF6 moves to the Golgi apparatus, where it is cleaved by different proteases and then, as an active transcription factor, enters the nucleus and activates the promoter of its related target genes. IRE1 and PERK are activated by autophosphorylation and oligomerization. Activated IRE1 forms an alternative spliced variant of the X-Box binding protein 1 (XBP1s), which, as a transcription factor, increases the expression of various chaperones and proteins involved in the proteasomal of ER-associated degradation (ERAD). The activation of PERK inhibits global protein synthesis through phosphorylation of eukaryotic translation initiation factor (eIF2α) but favors the translation of some mRNAs, such as the activating transcription factor 4 (ATF4), which is involved in both cell survival and ER stress-dependent apoptosis. During chronic stress, ER promotes apoptosis by upregulating genes such as the homologous protein of the CCAAT enhancer binding protein (CHOP) [20,22,23]. If conformation of mutated collagen is not improved by chaperones, it is destined for degradation most often via autophagy [24].

Autophagy is a complex system regulated by more than 30 autophagy-related gene (ATG) proteins. The most studied markers associated with autophagy are beclin 1, a microtubule-associated protein 1 light chain 3 (LC3), and sequestosome 1 (SQSTM1/p62), later referred to as p62 [25]. In some cases, especially the mutations occurring in C-propeptide, that most affect the trimer assembly, the retrotranslocation of misfolded procollagen chains into the cytosol may occur and result in their degradation by the proteasome [26,27].

So far, anti-catabolic bisphosphonates, denosumab as a synthetic parathyroid hormone and growth hormone have been used in the therapy of OI [1,3,10]. Experimental OI therapy strategies such as genetically engineered stem cell transplantation methods,

reprogramming of somatic cells into pluripotent stem cells as well as anti-transforming growth factor (TGF-β) therapy have been summarized by us recently [28]. The major disadvantages of these therapies are their poor efficacy, or cytotoxic side effects. Research is still underway to find new and more effective drugs. According to the latest research, the chemical 4-phenylbutyrate chaperone (4-PBA) is of great interest, the molecular target of which is ER stress caused by intracellular retention of mutant collagen in osteoblasts and fibroblasts [16–18,29,30].

Medicinal plants containing significant amounts of polyphenols are of great interest to researchers due to their high therapeutic potential. Polyphenols are widely studied as regulators of fundamental biological processes, including cell proliferation, apoptosis, and autophagy [31,32]. Rosemary (*Rosmarinus officinalis* L., Lamiaceae) is a rich source of many bioactive polyphenol compounds with a wide range of biological activities, such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic and anticancer [33,34]. In our previous study, we showed a beneficial effect of rosemary extract (RE) on the biosynthesis of type I collagen in OI type I fibroblasts with quantitative type I collagen defect [35]. This time we examined whether RE could reduce the accumulation of mutant collagen in the fibroblasts of two patients with severe type III and two patients with lethal type II OI carrying mutations in α(I) chain. In addition, possible mechanisms involved in the action of RE were also investigated.

2. Results

2.1. Steady-State Collagen Analysis in OI Fibroblasts

To identify mutated collagen, the mobility of the α1(I) and α2(I) bands was analyzed by SDS-urea polyacrylamide gel electrophoresis (SDS-urea PAGE) (Figure 1). All cells with glycine substitution (G910S and G1448V in patients 1 and 2 with severe type III, and G691C and G352S in patients 1 and 2 with lethal type II OI) in the α1(I) showed delayed migration of α1(I) and α2(I) chains and intracellular retention. In OI type II (patient 1) cells, an additional band $[\alpha1(I)]_2$ dimer was identified between the α1(I) monomers and the α1(III) trimer, confirming the substitution of glycine with cysteine (G691C).

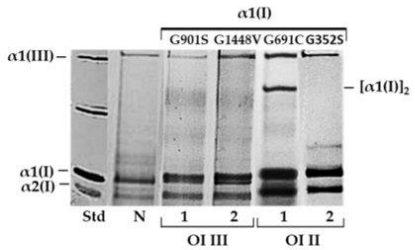


Figure 1. SDS-urea polyacrylamide gel electrophoresis (SDS-urea PAGE), in non-reducing conditions, of collagen type I in fibroblasts of patients 1 and 2 OI types III and II with glycine substitutions in α1(I) chain. To detect collagen after electrophoretic separation, silver staining was used; N—normal cells, Std—standard (bovine collagen type I) (Biocolor Life Science, UK).

2.2. Reduced Accumulation of Collagen Type I in OI Fibroblasts Treated with Rosemary Extract

In untreated OI fibroblasts, the accumulation of collagen type I in all four cell lines was confirmed by Western blot (Figure 2). When OI cells were treated with RE at the concentrations of 1–100 μg/mL, the level of accumulated collagen in cell layers was significantly reduced at 25, 50 and 100 μg/mL RE in OI type III patient 1, and at the concentrations of 50 and 100 μg/mL RE in the rest of the cells. Moreover, in cells of OI III 1 (at 50 μg/mL RE) and OI III 2 (at 50 and 100 μg/mL RE) the level of collagen type I was normalized (Figure 2A). The level of collagen secreted by OI fibroblasts, apart from the

increase in the medium of OI III 1 fibroblasts treated with 100 μg/mL RE, remained unchanged. It should be added that RE at these concentrations did not significantly affect the viability of OI cells (Supplementary Figure S1).

Since rosmarinic acid (RA) is an essential component of RE, we also examined its effect on the mutant collagen retention. RA in a wide range of concentrations (1–100 μM), apart from lowering the accumulation of collagen in OI II 1 cells at its highest concentration (100 μM), had no effect in the remaining cells (Supplementary Figure S2). In the subsequent studies, we focused on the mechanisms of action of RE at the concentrations of 50 and 100 μg/mL.

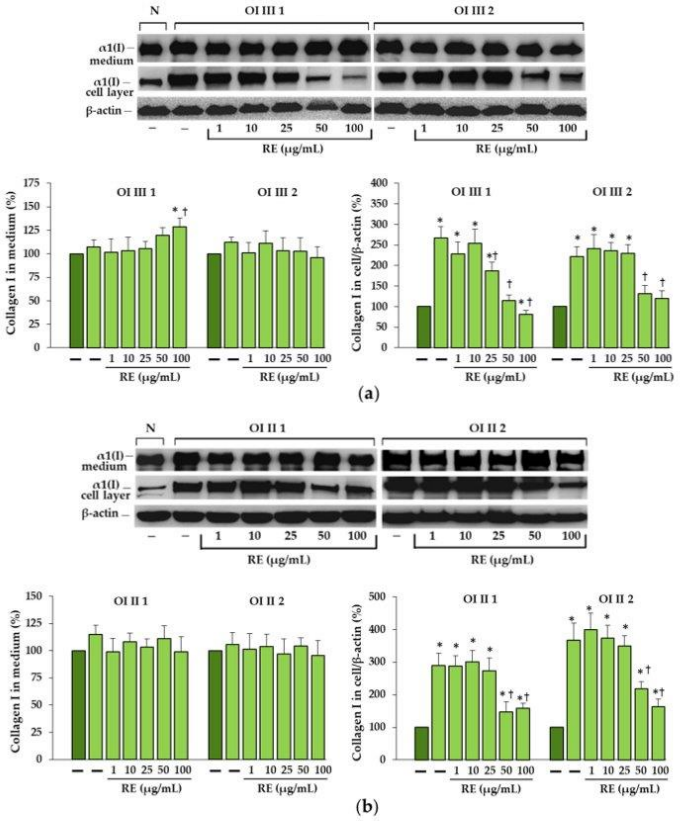


Figure 2. The effect of rosemary extract (RE) on the levels of secreted (in medium) and intracellularly accumulated type I collagen in OI type III (**a**), and OI type II (**b**) cells; β-actin was used as cell protein loading control. The bars represent the results of the gel densitometry as the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal (N) cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent normal and OI, respectively.

2.3. Activation of Unfolded Protein Response in OI Cells

To determine whether retained mutant procollagen activates UPR, the expression of BiP, protein disulfide isomerase (PDI), ATF4, AFT6 and XBP-1s was evaluated (Figure 3).

Expression of the chaperone BiP, which is an activator of UPR sensors, was significantly increased in untreated OI cells at the mRNA level beyond OI II 1 cells where it remained unchanged compared to the normal cells (Figure 3a). PDI, which catalyzes the formation and isomerization of disulfide bonds and acts as a collagen chaperone, was significantly increased in all untreated OI cells at mRNA level (Figure 3a). In the presence of RE (50 and 100 μg/mL), the expression of both chaperone transcripts was mostly reduced to a different extent compared to the untreated OI cells (Figure 3a).

ATF4, which is the effector in the PERK branch, was according to the analysis of its transcript expression, upregulated in untreated OI cells. RE either did not change or it decreased the expression of this factor mRNA, dependently on RE concentration, in comparison to the untreated OI cells (Figure 3a).

Expression of ATF6 was also investigated, and the level of its mRNA in untreated OI cells increased compared to the control. In cells exposed to RE (50 and 100 μg/mL) the decrease in ATF6 gene expression was noted in OI III, no effect in OI II 2 or stimulating effect of 100 μg/mL RE in OI II 1 (Figure 3a).

Activation of the IRE1α branch was studied by determining the level of XBP-1 expression in which splicing is mediated by this protein. In normal cells, the splicing form of XBP-1 (XBP-1s) was absent, while in OI it was manifested mainly in type III OI and was barely detectable in OI II cells (Figure 3b). Under the influence of RE at a concentration of 100 μg/mL, the spliced form disappeared in OI type III and only the unspliced (XBP-1u) form was observed, while in RE-treated OI II cells a slower migrating band appeared, with greater intensity in OI II 1 (Figure 3b). To find out whether the changes in the expression of proteins included in the UPR in OI cells reflect the expression of their genes, Western blots were performed (Figure 3c).

The results of the Western blot analysis revealed a similar increase in BIP at the protein level as was at the mRNA level, and no increase in untreated OI II 1 cells where no upregulation of the BIP gene was shown. Interestingly, RE at both concentrations (50 and 100 μg/mL) increased BIP expression in OI II 1 cells, and in others with upregulation of this chaperone protein, the RE induced decrease in relation to untreated OI cells was noted (Figure 3c). The expression of ATF4 and ATF6 transcription factors at the protein level was increased as at the mRNA level, except for OI II 1 cells, where their genes were upregulated. In RE-treated cells, the normalization of ATF4 level was achieved, while the ATF-6 level was either unaffected or significantly decreased by RE in OI III 1 cells (Figure 3c). XBP-1s at the protein level was almost undetectable in normal age-matched cells for OI type III, while its expression increased almost 6-fold and 4-fold in OI III 1 and 2 cells, respectively, and significantly decreased under the influence of RE. In contrast, in untreated OI II cells, only an upward trend and no RE effect on the expression of this spliced form of the factor was observed (Figure 3c).

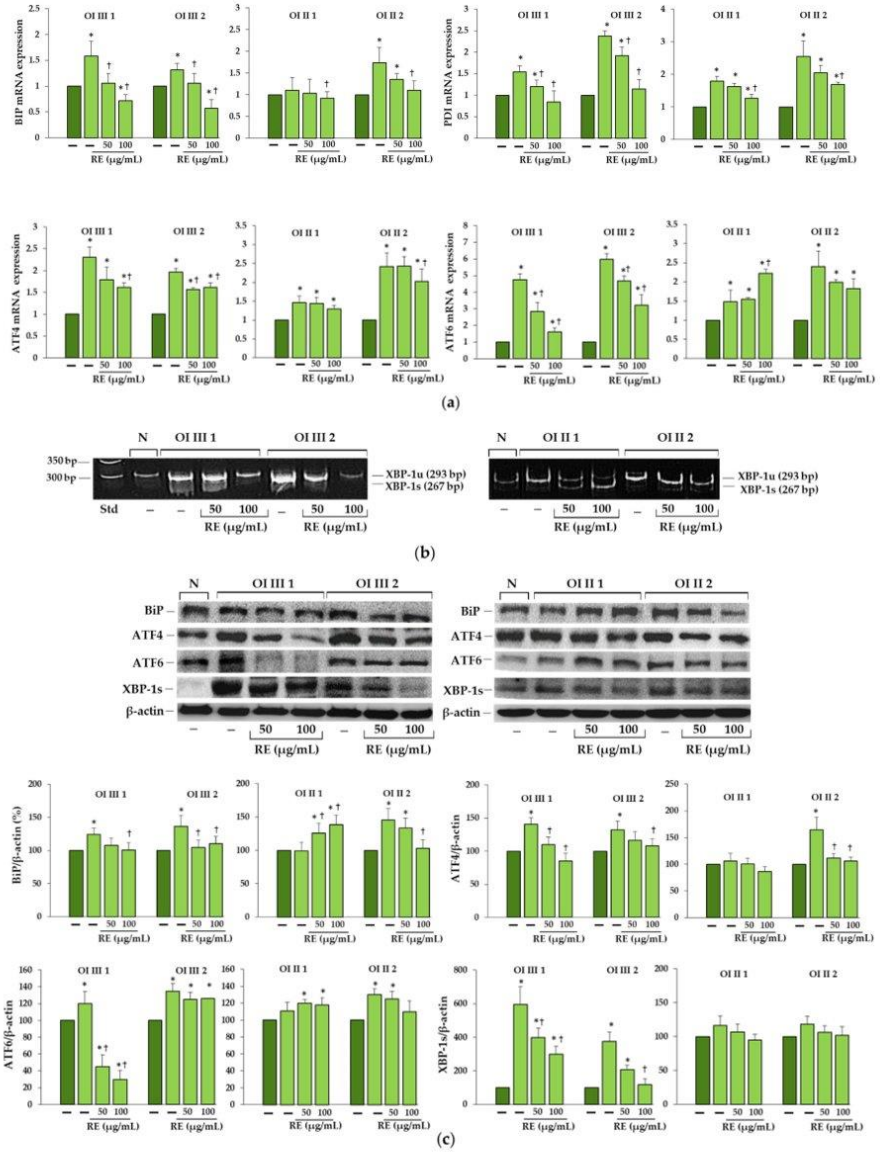


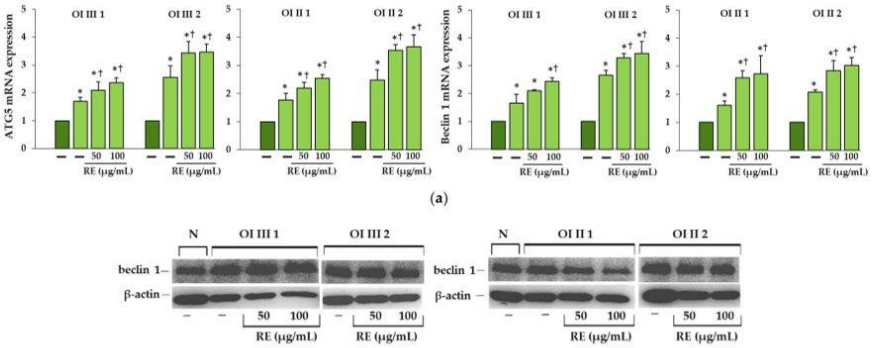
Figure 3. (**a**) The influence of rosemary extract (RE) on mRNA relative expression of binding immunoglobulin protein (BiP), protein disulfide isomerase (PDI), and activating transcription factors 4 and 6 (ATF4, ATF6) in OI types III and II cells. The bars represent the results of the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal cells; $^{\dagger}p < 0.05$, OI treated cells vs. OI untreated cells; dark green and light green bars represent normal and OI, respectively.

(**b**) Unspliced and spliced X-box binding protein 1 (Xbp-1u and Xbp-1s) RT-PCR products were analyzed on 7% polyacrylamide gel. (**c**) Western blot analysis of BiP, ATF4, ATF6 and XBP-1s in OI type III and II cells; β-actin was used as cell protein loading control. The bars represent the results as the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal (N) cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent normal and OI, respectively.

2.4. Rosemary Extract-Induced Autophagy in OI Cells

ATG5 and beclin 1, which are important in initiation of autophagosome formation, increased in all untreated OI cells at the mRNA level and their expression intensified in the presence of RE (Figure 4a). In addition, the expression levels of autophagy related markers were determined by Western blot as well. Beclin 1 level increased in untreated OI III and OI II 2 and even more in RE treated cells consistent with gene expression, while there was no change in untreated and RE treated OI II 1 cells compared to normal cells (Figure 4b). LC3-II as a marker of the final fusion of the autophagosome with the lysosome was increased in untreated OI cells apart for OI III 1. In the presence of RE (50 and 100 μg/mL), no effect in OI III 2 and significant stimulation of LC3-II expression in the remaining cells was demonstrated as compared to untreated cells (Figure 4b).

In order to assess the dynamic process of autophagy, the ratio of LC3-II to LC3-I was determined, which in untreated cells either increased (in OI III 1 and 2) or remained unchanged (in OI II 1 and 2) compared to normal, while RE significantly induced autophagic flow in all OI cells (Figure 4b). The expression of p62, which is an indicator of autophagic degradation, was unchanged in OI III and increased in OI II cells. We examined whether RE facilitates degradation of p62. Treatment of cells with RE significantly decreased the p62 level, which suggests RE-induced autophagic degradation (Figure 4b). Since p62 and LC3-II are also degraded along with the digestion of cell components by autophagy; therefore, in order to further confirm that the increase in LC3-II induced by RE was not indicative of accumulation of this protein, ammonium chloride (NH_4Cl) as a lysosome proteolysis inhibitor was used. In all OI cells with inhibited lysosomal protein degradation, not only the LC3-II level but also p62 were significantly higher than in cells treated with RE alone (Supplementary Figure S3).



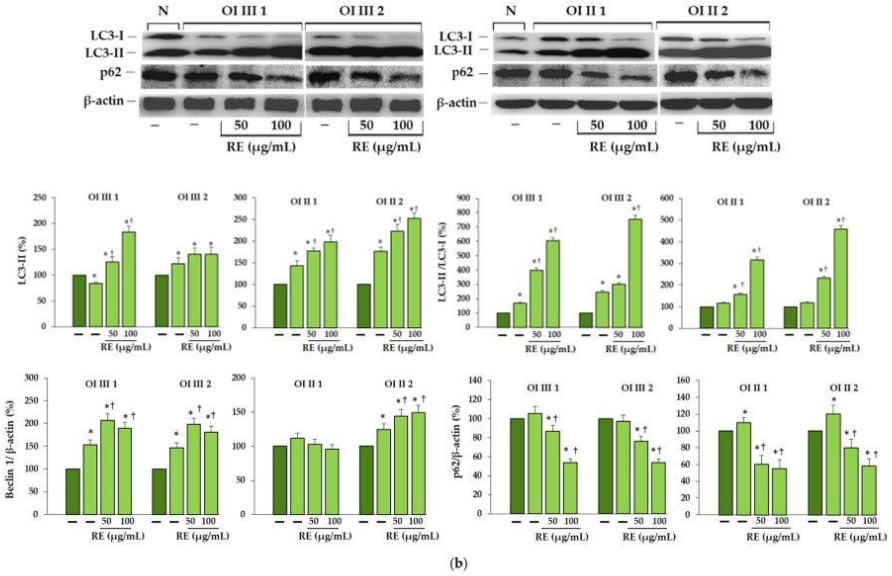


Figure 4. (**a**) The influence of rosemary extract (RE) on mRNA relative expression of autophagy-related gene (ATG5) and beclin 1 in OI types III and II cells. The bars represent the results of the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells; dark green and light green bars represent normal and OI, respectively. (**b**) Western blot analysis of beclin 1, microtubule-associated protein 1 light chain 3 (LC3-I/LC3-II), and sequestosome 1 (SQSTM1/p62) in OI type III and II cells; β-actin was used as cell protein loading control. The bars represent the results as the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal (N) cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent normal and OI, respectively.

To confirm that collagen type I is degraded in the lysosomal pathway, a lysosome-enriched cell fraction was prepared and tested for its level in the presence and absence of lysosomal inhibitor in RE treated cells. We found that collagen type I was localized to a very small extent in the lysosomal fraction of normal cells and to a much greater extent in untreated OI cells (Figure 5). In OI cells treated with RE alone the level of type I collagen was lower than in the presence of inhibitor of lysosomes NH_4Cl, which indicates its lysosomal degradation.

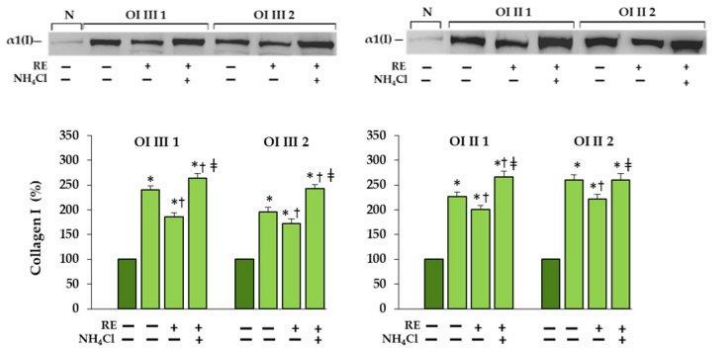
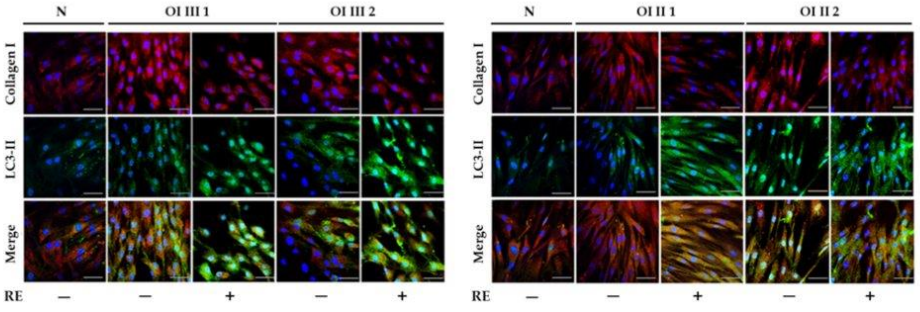
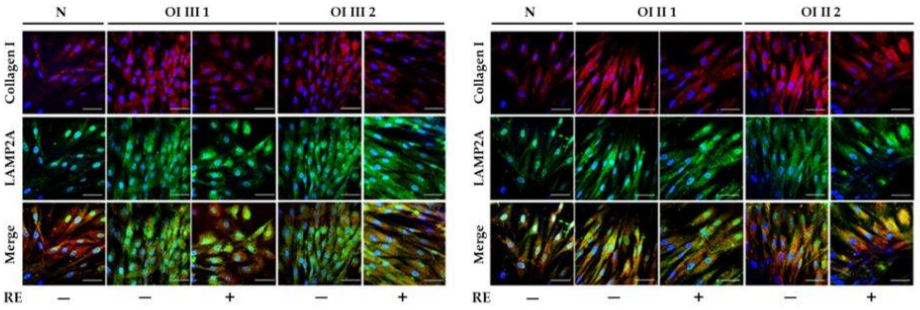


Figure 5. Western blot analysis of type I collagen in the lysosome-enriched cellular fraction, prepared as described in Methods, in OI types III and II cells treated with 50 μg/mL RE and 50 mM NH_4Cl. Equal amounts of proteins were loaded on 10% polyacrylamide gel. The bars represent the results of the gel densitometry as the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal (N) cells; † $p < 0.05$, OI cells treated with RE vs. OI untreated cells; ‡ $p < 0.05$, OI cells treated with RE + NH_4Cl vs. OI cells treated with RE alone. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent normal and OI, respectively.

Using confocal fluorescence microscopy with immunofluorescence staining, representative images of which are presented in Figure 6, we assessed the localization of endogenous collagen I, LC3-II and lysosomal-associated membrane protein 2A (LAMP2A) in normal and OI cells. It was observed that compared to normal cells, collagen type I staining intensity (red) in untreated OI cells increased, and after treatment with RE it decreased, whereas LC3-II (green) inversely decreased in untreated OI cells and increased in the presence of RE. Similar to the Western blot results, collagen type I staining decreased in the presence RE; moreover, it was co-localized with LC3-II as evidenced by the merged images (Figure 6a). We additionally assessed co-localization of collagen type I and LAMP2A, which is a lysosomal marker. As shown in Figure 6b, co-immunofluorescence staining for collagen type I (red) and LAMP2A (green) showed the increase in their co-localization after treatment of OI cells with RE. The above results suggest a stimulating effect of RE on the intracellular collagen degradation mediated by the autophagolysosomes.



(**a**)



(**b**)

Figure 6. Colocalization of collagen type I (red)/LC3-II (green) (**a**), and of collagen type I (red)/LAMP2 (green) (**b**), and changes in the intensity of staining of these proteins in normal (N) and OI types III and II cells, treated with 50 μg/mL rosemary extract (RE), revealed by confocal microscopy analysis. A merge signals of colocalizing collagen type I/LC3-II and collagen type I (/LAMP2 were detectable, with greater intensity in RE-treated cells. Nuclei were stained with DAPI (blue). Scale bar, 50 μm. Representative immunofluorescent images are shown.

2.5. Involvement of Proteasome in Degradation of Unfolded Procollagen Chains in OI III 2 Cells

Since it has been reported that unfolded procollagen chains can be degraded by the ERAD pathway [27], we checked the polyubiquininination of type I procollagen in OI cells following SDS-PAGE under non-reducing conditions. As it turned out, under these conditions, polyubiquitinated proteins corresponding to the molecular weight of procollagen chains were detected only in OI III 2 cells, and the increase in this modification was noted in the presence of RE (Supplementary Figure S4). In order to determine the way of their degradation, a Western blot of polyubiquitinated proteins (Figure 7a) and SDS-PAGE of silver-stained proteins (Figure 7b) in the presence of proteasome and autophagy inhibitors were performed. Three polyubiquitinated protein bands corresponded to monomers, dimers and trimers of type I procollagen. Dimers and trimers are stabilized by the formation of inter-chain disulfide bonds within the C-propeptide. There was the increased level of unfolded proα1(I) chains and a decreased level of trimers in OI cells compared to the control cells (Figure 7b), while the level of monomer polyubiquitination was slightly reduced in OI cells (Figure 7a). In the presence of bortezomib (BR), a proteasome inhibitor, increased levels of polyubiquitinated monomers correlated with increased level of unfolded procollagen chains. Treatment with autophagy inhibitors chloroquine (CQ) and 3-methyladenine (3-MA) remained without effect on the level of polyubiquitination, but interestingly, the level of unfolded procollagen chains in the presence of CQ slightly increased and under the influence of 3-MA decreased as compared to untreated OI cells. Rosemary extract caused the increase in the polyubiquitination of monomers and dimers (Figure 7a), which was accompanied by a marked decrease in the level of unfolded collagen (Figure 7b).

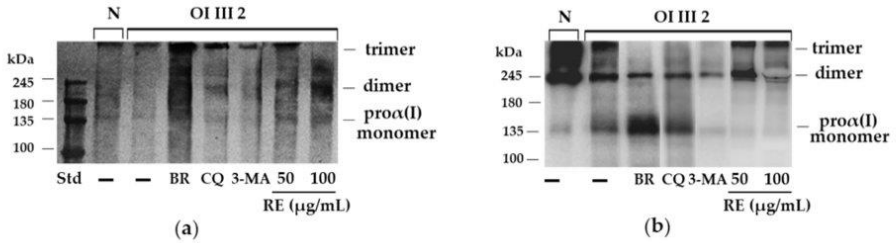
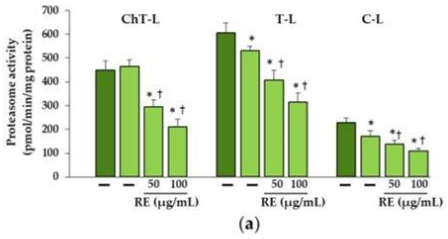
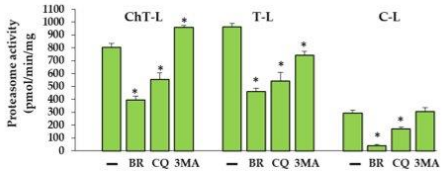


Figure 7. Polyubiquitynation (**a**) and SDS-PAGE of silver-stained (**b**) procollagen type I monomers, dimers and trimers, performed under non-reducing conditions in normal (N) and OI III 2 cells treated with 50 and 100 μg/mL rosemary extract (RE), 50 nM bortezomib (BR), 50 μM chloroquine (CQ), and 5 mM 3-methyladenine (3-MA).

Additionally, the effect of RE on proteasomal activities: chymotrypsin-like (Ch-L), trypsin-like (T-L) and caspase-like (C-L) in untreated and RE-treated OI fibroblasts was examined. As shown in Figure 8a, the decrease in T-L and C-L activities and no change in ChT-L activity were found in untreated OI cells, while in the presence of RE all activities were lowered as compared to untreated cells. The effectiveness of BR was confirmed by inhibitory effect on all proteasome activities (Figure 8b). The use of autophagy inhibitors, in turn, resulted in a slight stimulation of ChT-L activity by 3-MA and an inhibitory effect of CQ on all proteasome activities (Figure 8b).

To check whether the inhibition of proteasome activity by RE does not lead to the accumulation of non-collagen proteins, the level of silver-stained lysate proteins separated on 10% gels under reducing conditions was assessed and additionally the level of polyubiquitination of these proteins (Supplementary Figure S5). The increase in the level of polyubiquitinated total proteins (Supplementary Figure S5a) coincided with the increase in the level of total protein expression in OI untreated cells (Supplementary Figure S5b) compared to the normal, which can be explained by the disruption of the autophagy and proteasome degradation processes. Treatment of OI cells with RE did not lead to the total protein accumulation, as was the case with the use of proteasome (BR and MG132) or autophagy (CQ, 3-MA and NH_4Cl) inhibitors, their levels were comparable to the normal (Supplementary Figure S5b).





(**b**)

Figure 8. Proteasomal activities: chymotrypsin-like (ChT-L), trypsin-like (T-L) and caspase-like (C-L) in OI III 2 cells treated with 50 and 100 µg/mL rosemary extract (RE) (**a**), 5 nM bortezomib (BR), 50 µM chloroquine (CQ) and 5 mM 3-methyladenine (3-MA) (**b**). The bars represent the mean values from three independent experiments. (**a**) *$p < 0.05$, OI cells vs. normal cells; $^{\dagger}p < 0.05$, OI cells treated with RE vs. OI untreated cells; dark green and light green bars represent normal and OI, respectively; (**b**) * $p < 0.05$, OI cells treated with inhibitors vs. OI untreated cells.

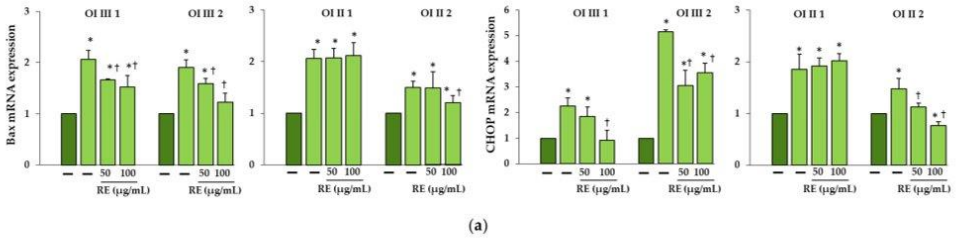
2.6. Expression and Activity of Collagen Type I Degrading MMPs

Additionally, we assessed the expression of matrix metalloproteinases MMP-1 and MMP-2, degrading extracellular collagen I, in untreated and RE exposed OI cells. MMP-1 mRNA in OI cells was significantly upregulated compared to normal cells, while RE significantly decreased it. MMP-2 was upregulated at the mRNA level in OI type II and was normalized in the presence of both RE concentrations (50 and 100 µg/mL) in OI II 1 and at 100 µg/mL in OI II 2 (Supplementary Figure S6a).

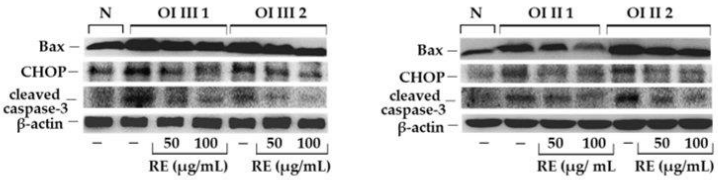
The zymography allowed to identify mainly pro-MMP-2 in media of normal cells and additionally its active form MMP-2 in OI, while RE, beyond OI II 1, at a concentration of 100 µg/mL, showed a lowering effect, the highest in OI III 1 (Supplementary Figure S6b).

2.7. Rosemary Extract Reduces the Expression of Apoptosis Markers in OI Cells

The results presented in Figure 9 showed the significant increase in the expression of proapoptotic proteins such as Bax and CHOP at both mRNA and the protein levels as well as cleaved caspase-3 protein. Under the influence of RE at both concentrations (50 and 100 µg/mL), downregulation of these markers was mostly observed; even if the levels of Bax and CHOP mRNA did not change in OI II 1 cells, a significant decrease was observed at the protein level.



(**a**)



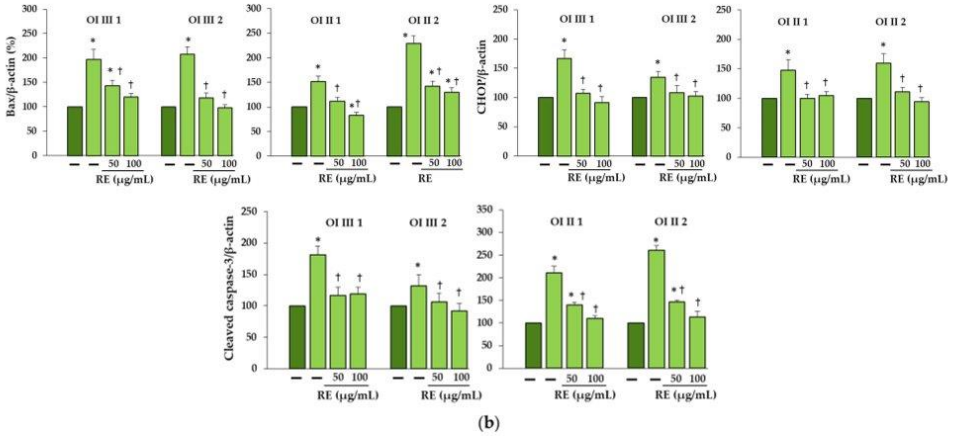


Figure 9. (**a**) The effect of rosemary extract (RE) on mRNA relative expression of Bax and CCAAT enhancer binding protein (CHOP) in OI types III and II cells. The bars represent the results of the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells; dark green and light green bars represent normal and OI, respectively. (**b**) Western blot analysis of Bax, CHOP and cleaved caspase-3 in OI type III and II cells; β-actin was used as cell protein loading control. The bars represent the results as the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal (N) cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent normal and OI, respectively.

3. Discussion

OI is a genetically and phenotypically heterogeneous group of connective tissue disorders caused mainly by an autosomal dominant mutations in collagen type I, which is the most abundant protein in bone and skin ECM [1–10]. The discovery in the last two decades of new causative genes (around twenty) that are involved in the regulation and function of type I collagen, but also in other aspects of bone biology [2,3,5,8–10] confirms the complexity of the underlying mechanisms and complicates understanding the relationship between mutations and phenotype as well as finding an effective and a universal method of treating the disease. Recently, ER stress caused by intracellular retention of mutant collagen in osteoblasts and fibroblasts was found as an attractive target of OI therapy [14–21]. The use of the chemical chaperone 4-PBA, approved by *Food and Drug Administration*, reduced ER stress and restored cell homeostasis in human fibroblasts of OI patients carrying dominant mutations in α1 and α2 collagen type I chains [17] as well as recessive mutations in cartilage-associated protein *(CRTAP)*, prolyl-3-hydroxylase 1 (*P3H1*) and cyclophilin B (*PPIB*) impairing prolyl-3 hydroxylation of collagen type I [18]. Moreover, it was found that 4-PBA normalizes the overproduction of type I collagen and improves the misfolding of the type I collagen helix in OI fibroblasts due to glycine substitution, and also improves the impaired mineralization of osteoblasts differentiated from OI induced pluripotent stem cells [30]. Administration of this drug to the OI dominant zebrafish model, carrying typical glycine substitution G574D in *COL1A1*, alleviated cellular stress and improved bone mineralization in larvae and skeletal deformity in adults. This was accompanied by the reduction of the ER cisternae size and promoting the secretion of collagen [29]. Similarly, in osteoblasts of two murine OI models carrying G349C mutation in *COL1A1* (Brtl mouse) and G610C in *COL1A2* (Amish mouse), 4-PBA pre-

vented collagen type I accumulation through increased its secretion and reduction of aggregates in mutant cells [16]. In addition, increased collagen incorporation into the matrix and improved mineral deposition in osteoblasts was observed in both murine models, which convinces about the influence of ER stress on the phenotype. Therefore, as well as the discovery of the therapeutic potential of this chemical chaperone, finding of other safe compounds to reduce cellular stress and restore cell homeostasis may be a new strategy for treating this disease, or at least some OI cases.

This study provides, for the first time, evidence of the beneficial effects of rosemary extract on fibroblasts with mutations in the collagen triple helix resulting in intracellular collagen accumulation. Moreover, the presented results explain the likely mechanisms of reducing cellular stress, mainly by enhancing the degradation of mutant collagen through autophagy. For our study, we chose glycine substitutions in the α1(I) chains of two patients with severe OI type III (G901S and G1448V), and two with lethal type II (G352S and G691C). As we demonstrated by SDS-urea-PAGE, all cells showed delayed migration of α1 and α2 collagen chains and intracellular accumulation of mutant collagen.

Collagen type I is characterized by a unique right-handed triple helical structure, and each of three left-handed polyproline-like helices contains a repeated sequence (Gly-X-Y) in which X and Y are often proline and hydroxyproline [36]. The triple helical domain is flanked on both sides by N- and C-terminal propeptides. Procollagen folding takes place in the ER, after which the protein is transported to the Golgi apparatus and secreted, where the cleavage of propeptides takes place and mature collagen is formed. Folding is a very complex process involving many chaperones, such as BiP, PDI, prolyl 4-hydroxylase, various peptidyl-prolyl *cis-trans* isomerases, and heat shock protein 47, whose role is to provide stabilization of the structure and to protect aggregation of unfolded chains [11,12,37].

We found increased BiP expression in two OI type III (G901S and G1448V) and one OI type II (G352S) cells, and a consequent activation of UPR pathways, whereas in OI II 1 cells with G691C the expression of this chaperone remained unchanged compared to the normal cells. According to Besio et al. [17] increased BiP was detected in three out of five tested fibroblasts with glycine substitution in α1 and α2 chains. Expression of PDI, which catalyzes the formation and isomerization of disulfide bonds and acts as a collagen chaperone by interacting with collagen α single chains, was upregulated in all α1(I) mutant cells, while in the study of Besio et al. [17] in four out five cells with mutations in α1(I) and it remained unchanged in cells with mutations in α2(I). The same authors reported the activation of mainly the PERK pathway and increased ATF4 expression in cells with mutations in α1 and α2 as well as IRE1α pathway with a predominance in cells with mutations in α2, but there was no difference in the level of activated ATF6 [17]. In contrast, in our study, along with the increase in BiP expression, activation of both transcription factors ATF4 (effector of PERK pathway) and ATF6 in cells with BiP upregulation was found, while expression of spliced forms of XBP1 (effector of IRE1α pathway) was predominant in OI type III as confirmed by real-time PCR and polyacrylamide gel electrophoresis of (reverse transcriptase) RT-PCR product. It should be added that in our studies, the lack of increase in BiP expression in cells with G691C mutation was consistently associated with the lack of activation of UPR proteins, while in the case of some collagen and non-collagen mutations, activation of some UPR pathways was also observed in the absence of BiP [17,18]. It is possible that other factors or regulatory mechanisms are involved in the activation of the UPR during ER stress. In the osteoblasts of the mouse OI model with the substitution of glycine by cysteine (G610C), closely located to the one we studied (G691C) but in α2(I), no conventional UPR was detected also, only enhanced autophagy [15]. Moreover, as it turned out in our studies, the upregulation of genes of transcription factors ATF4 and ATF6 did not coincide with the increased amount of the protein, which means that their expression is regulated by post-transcriptional mechanisms and the expression of the genes themselves cannot be compared without determining the expression at the protein level.

Based on the obtained results, we can say that RE, if not completely eliminated, largely reduced ER stress caused by the accumulation of mutant collagen. This was evidenced by the decreased expression of chaperone proteins BiP and PDI as well as effectors of UPR branches along with the decreased level of intracellular collagen. Interestingly, in OI II 1 cells where we did not detect increased BiP expression and activation of UPR pathways, upregulation of pro-survival factors (BiP, ATF6 and XBP-1s), but not ATF4, was found in RE-treated cells. Activation of the PERK pathway leads to inhibition of global protein translation by inhibition of eIF2α except for ATF4, which in the active form can upregulate both the survival (autophagy) and the apoptotic (CHOP) pathway genes. In turn, the active transcription factors ATF6 a and XBP1s enhance the expression of chaperones, and also ATF6 of genes for proteins involved in ERAD [19,20,22]. While it is still unknown how activation of individual UPR pathways and their effectors directly affects collagen, one study found that forced XBP1s expression in cells with glycine substitution (G425S) in α1 (I) chain enhanced the folding/assembly and secretion of mutant type I collagen [38].

Since the cell response to ER stress caused by intracellular accumulation of mutant collagen is most often autophagy or, less frequently, ERAD, we investigated the activation of these two degradation systems in untreated and RE exposed OI cells.

Autophagy, is a dynamic tightly regulated lysosomal pathway of degradation of intracellular components, including soluble proteins, aggregated proteins and damaged cell organelles. It is an evolutionarily conserved process, capable of responding to stress to limit cell damage. The autophagy process is regulated by several ATG core proteins, of which LC3 plays essential role in the formation and maturation of the autophagosome, [25,39]. Cytosolic form LC3-I is converted into an active membrane-bound form LC3-II during the formation of the autophagosomes, while the final degradation of the cargo takes place after fusion of autophagosome with lysosomes. It is strictly dependent on the p62, which apart from the ratio LC3-II/LC3I, is an important marker of effective autophagic flux [40]. Even though the LC3-II level was increasing in lethal untreated cells as compared to the aged-matched control, the ratio LC3-II/LC3-I remained unchanged, which along with the p62 increase indicated a lack of activation of autophagy. OI III cells showed an increase in the LC3-II/LC3-I ratio, but the p62 level remained unchanged, which also did not suggest an increase in autophagic activity. p62 with LC3 recognition sequence binds to LC3-II and after the formation of the autophagosome and its fusion with the lysosome, is degraded inside the autophagolysosome, that is why the decrease in p62 expression may indicate an active process of autophagy.

A markedly increased autophagic activity was observed in all OI cells after RE exposure, as shown by a dose-dependent increase in LC3-I to LC3-II conversion, along with accelerating p62 degradation. In addition, the stimulation of ATG5 mRNA and beclin 1 mRNA and protein, which initiate autophagosome formation, was demonstrated in RE-treated cells. Another evidence of the stimulating effect of RE on the degradation of mutant collagen with involvement of lysosomal pathway was the confirmation of the presence of collagen type I in the lysosomal fraction and the increase in its level in cells treated additionally with ammonium chloride, which raises the pH and thus inactivates lysosomes. The same results were obtained in the presence of chloroquine—another autophagy inhibitor (not shown). Moreover, the immunofluorescence microscopy studies have showed that collagen type I collocation increase in RE-treated OI cells with both the marker of autophagosome (LC3-II) and marker of lysosomes (LAMP2A). These results clearly indicate the RE-mediated degradation of mutated collagen type I in the autophagolysosomal process, although digestion of collagen by lysosomes regardless of autophagy cannot be ruled out. Omari et al. [41] showed that in addition to autophagy, accumulated in OI collagen type I can be digested in a noncanonical autophagy process.

As reported earlier the proteasome may be involved in the removal of unfolded procollagen chains [27]. Misfolded proteins or unfolded procollagen chains are retranslocated from the ER to the cytosol for degradation by the 26S proteasome after modification with polyubiquitin chains. As expected, polyubiquitination of unfolded proα1(I) chains was

demonstrated in OI III 2 with a C-propeptide mutation (G1448V). The increase in the amount of unfolded procollagen chains in untreated OI cells can be explained by decreased C-L and T-L proteasome activities. The greater accumulation of these chains in the presence of BR, which inhibited to a much greater extent all three activities (ChT-L, T-L and C-L) of the proteasome confirms the proteasome's contribution to the removal of these chains. However, while it turned out that RE also decreased ChT-L, T-L and C-L activities, this decrease did not coincide with the accumulation of unfolded chains. On the contrary, there were lower levels of them than in untreated cells. At this stage of the study, it is difficult to explain the mechanism of action of RE in these cells, but it is very likely that in the case of inhibition of the proteasome activity, unfolded chains may be partially degraded in the process of RE-activated autophagy or by the proteasome, as the activity of the proteasome was only partially inhibited. It was also noted that, despite the reduction in proteasome activity by CQ, which inhibits autophagy, there was no additional accumulation of unfolded chains. On the other hand, another inhibitor of autophagy 3-MA that block autophagy at the initiation and maturation stages by acting on phosphoinositol 3 phosphate kinase (PI3K), caused a decrease in the level of unfolded chains compared to untreated OI cells, perhaps due to its stimulating effect on ChT-L activity. Since the two protein degradation systems (proteasome and autophagy) appear to be mechanically linked, it is suspected that when the proteasome is inhibited, autophagy may be activated to remove polyubiquitinated/unfolded protein aggregates and promote cell survival [42]. It is possible that a decrease in proteasome activity, also noted in other RE-treated OI cells used in this study (results not shown), triggers autophagy, protecting cells from the toxic long-term stress leading to cell apoptosis. The increase in the expression of pro-apoptotic proteins (Bax, CHOP and active caspase 3) in OI cells and their significant reduction or even normalization in the presence of RE, may unequivocally indicate that the accumulation of mutant type I collagen caused such stress and despite the activation of UPR (with the exception of OI II), the degradation processes mediated by autophagy and the proteasome were disturbed. The cell's response to inhibited intracellular degradation processes could be a significant upregulation of extracellular MMP-1 and MMP-2 genes, lowered in the presence of RE, while the importance of these upregulation requires further study. Moreover, the observed increase in the level of extracellular type I collagen only in the presence of 100 μg/mL RE in OI III 1 can be explained by its strong inhibitory effect on the activity of MMP-2 and not by an increase in collagen secretion. Negative correlations between the activity of lysosomal enzymes and MMPs have been reported [43] and are worth studying further, but our research focuses on the intracellular degradation processes.

It is also possible that, under the influence of RE, collagen folding is improved due to reduced over-modification of free procollagen chains as a RE-induced decrease in mRNA expression of one of the enzymes (β(1-O) galactosyltransferase (GLT25D1)) involved in these modifications was observed (data not shown).

Phytochemicals, due to their natural origin, low toxicity, as well as many valuable biological and pharmacological properties, are of wide interest among researchers as potential drugs with a high therapeutic and preventive potential for many diseases. *R. officinalis* is a polyphenol-rich source constituents such as luteolin and apigenin derivatives, caffeic acid derivative (rosmarinic acid) and other such as diterpenes (rosmanol isomers), detailed qualitative analysis of which was presented earlier [35].

In our previous studies, we have shown a stimulatory effect of rosemary and lemon balm extracts, RA as well as some flavonoids on collagen biosynthesis in OI type I [35,44] as well as normal human skin fibroblasts [45–47]. In these studies, the use of RA alone in a wide range of concentrations did not bring the expected effects, which may suggest the participation of other components of the rosemary extract in stimulating autophagy or its other effects. It has been reported by other authors that luteolin 7-*O*-glucoside (one of the identified RE components) protects against damage to the heart muscle induced by starvation by enhancing autophagy through inhibition of mechanistic target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK) signaling pathway [48]. It has

also been shown that apigenin increases the expression of LC3-II, the formation of autophagolysosomal vacuoles and triggers autophagic flow in hepatocellular carcinoma cells [49]. It is widely accepted that a variety of plant extracts and dietary phytochemicals including resveratrol, curcumin, epigallocatechin-3-gallate, punicalagin, oleuropein, myricetin and rosmarinic, norhydroguaiaretic, and ferulic acids may stimulate autophagy [31,50]. These compounds remove protein aggregates, stimulate the antioxidant defense and ameliorate the ER stress, resulting in increased cell survival [31–34,50]. Pierzynowska et al. [51] reported on the removal of mutant huntingtin aggregates in the transfected HEK293 cells via genistein-induced autophagy, which may be the basis for the development of an effective therapy for this inherited neurodegenerative disease. Interestingly, like RE in OI cells, genistein showed significant inhibition of all protesome activities in the fibroblasts of patients with all types of mucopolysaccharidosis, which according to the authors, may lead to the stabilization of lysosomal enzymes and constitute a new approach in the treatment of this genetic disease [52].

A synergistic effect of several different compounds present in the rosemary extract may also be likely. While the biological properties (e.g., antioxidant) of polyphenols were previously related mainly to the structure of these compounds, now a more convincing explanation is modulating the activity and/or expression of key proteins for signaling cascades by interacting with them or modulating epigenetic regulation of gene expression. Therefore, it is believed that pleiotropic mechanisms and specific polyphenol-protein interactions are involved in their beneficial effects [53,54].

Finally, the limitations of this study should also be mentioned. Firstly, experiments were conducted on fibroblasts, which is related to the availability of biological material, and the disease mainly affects the skeletal system. On the other hand, collagen type I is a major component of skin and bone and, with a few exceptions, is similarly expressed in fibroblasts and osteoblasts. It is also worth noting that the activity of the proteasome may vary with age and even tissues or may be different in OI patients; therefore, more detailed studies are needed to understand the molecular mechanisms of RE-induced changes in proteasome activity. Although at this stage of our research we did not focus on explaining the consequences of proteasome inhibition by RE, the lack of accumulation of non-collagen proteins was shown.

4. Materials and Methods

4.1. Chemicals

Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); penicillin, streptomycin, and glutamine were purchased from Quality Biologicals Inc. (Gaithersburg, MD, USA). Radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail (P8340), magnesium L-ascorbate, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), bovine serum albumin (BSA), pepsin, gelatin, NH_4Cl, CQ, 3-MA, and MG132 were provided by Sigma-Aldrich Corp. (St. Louis, MO, USA). BR was a product of Selleck Chemicals (Houston, TX, USA). Proteasome substrates: N-Suc-LLVY-AMC (7-amido-4-methylcoumarin) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), Bz-VGR-AMC and Z-LLE-AMC were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Rosemary extract (RE) was prepared and characterized using LC-MS technique according to procedure described in our previous study [35]. RA was a product of BIOKOM (Warsaw, Poland).

4.2. Fibroblast Culture and Treatment

The study was performed on skin fibroblasts derived from two patients with severe OI type III and mutations in *COL1A1*: Gly901Ser (patient 1) and Gly1448Val (patient 2), and two patients with lethal OI type II and mutations in *COL1A1*: Gly691Cys (patient 1)

and Gly352Ser (patient 2) as well as two age matched normal cells. The normal skin fibroblast line used as a control for OI type III was CRL-1474 obtained from American Type Culture Collection (Manassas, VA, USA), and as a control for OI type II the normal line was derived from the foreskin on the 7th day of life of the donor. Fibroblasts from skin biopsy of OI patients and healthy control were obtained after informed consent in accordance with the Declaration of Helsinki and was approved by Bioethical Committee of the Jagiellonian University in Kraków, Poland (KBET/108/B/2007).

Fibroblasts were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL) at 37 °C in a humidified incubator in atmosphere containing 5% CO_2. For experiments, fibroblasts were grown to 90% confluence and the cultured medium was replaced with fresh DMEM without serum, supplemented with 25 μg/mL of magnesium ascorbate, before addition of compounds. Compounds were stored at 4 °C as the concentrated stock solutions in DMSO and were diluted in medium prior to addition to cell cultures. Fibroblasts were treated with RE at the concentration of 1–100 μg/mL and RA at the concentrations of 1–100 μM for 24 h. In addition, cells were treated with autophagy inhibitors: 50 μM CQ, 50 mM NH_4Cl and 5 mM 3-MA or proteasome inhibitors: 50 nM BR and 2.5 μM MG132, all of which were dissolved in DMSO and appropriately diluted before adding to cell cultures. In all experiments the concentration of DMSO did not exceed 0.05% (*v*/*v*).

4.3. MTT Test to Determine Viability of Treated Cells

Fibroblasts (1×10^4 cells per well) were treated with RE (1–200 μg/mL) for 24 h. Then cells were washed three times with PBS and MTT solution (0.5 mg/mL) was added for 4 h. After removing MTT solution 1 mL of 0.1 M HCl in absolute isopropanol was added to dissolve formazan crystals by thoroughly shaking on a plate shaker (BioSan, Riga, Latvia), and the absorbance at 570 nm was measured using a microplate reader (TECAN, Männedorf, Switzerland).

4.4. Quantitative Real-Time PCR

Total RNA was isolated from cultured cells using a Total RNA Mini Plus concentrator (A&A Biotechnology, Gdynia, Poland) and the concentration of RNA was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The equal amounts (1 μg) of total RNA were used to the synthesis of complementary DNA (cDNA) with the use of cDNA Synthesis Kit (Bioline, London, UK). Quantitative Real-time PCR (qRT-PCR) analysis was performed in the CFX96 Real-Time System thermal cycler (Bio-Rad, Hercules, CA, USA) using the SensiFAST™ SYBR kit (Bioline, London, UK). The expression of desired gene was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and changes were calculated by the ΔΔCt method. The sequences of primers (Genomed, Warsaw, Poland) are shown in Supplementary Table S1. The qRT-PCR parameters were as follows: 30 s at 95 °C followed by 40 cycles: 10 s at 95 °C, 10 s at 60–62 °C and 20 s at 72 °C. The reaction products were verified by analysis of their melting curves.

4.5. XBP1 Splicing Analysis

PCR mixture contained 1 μg of isolated RNA and primers (0.3 μM each): sense (5′-TCAG CTT TTA CGA GAG AAA ACT CAT GGC CT-3′) and antisense (5′-AGA ACA TGT GTG TCG TCC AAG TGT GTC GTC CAA GTG TG-3′) purchased in Genomed (Warsaw, Poland). Samples were incubated 30 min at 50 °C followed by 30 cycles at 94 °C, 60 °C, and 72 °C for 30 s each in the CFX96 Real-Time System thermal cycler (Bio-Rad, Hercules, CA, USA). Reaction products were analyzed by electrophoresis on 7% polyacrylamide gel and visualized with ethidium bromide.

4.6. Western Blot

Cell layers were harvested using RIPA buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) and protease inhibitor cocktail (P8340) (Sigma-Aldrich Corp., St. Louis, MO, USA). The conditioned media were collected and concentrated 10 times with Centrifugal Filter Units (10K) (Merck Millipore Ltd., Carrigtwohill, County Cork, Ireland). The concentration of total protein in cell lysates and media was measured using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and Coomassie Plus—The Better Bradford Assay Reagent (ThermoFisher Scientific, Rockford, IL, USA), respectively. For Western blot an equal amount of protein (20 μg) was loaded on polyacrylamide gel (7.5%, 10% or 12% depending on the molecular mass of protein). Proteins were transferred from gels onto Immobilon-P Transfer membranes (Merck Millipore Ltd., Tullagreen, Carrigtwohill, County Cork, Ireland), which were blocked with 5% (*w*/*v*) non-fat dried milk diluted in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% (*v*/*v*) Tween 20 (TBS-T) for 1 h at room temperature. Then, membranes were washed with TBS-T and incubated overnight at 4 °C with solutions of the following primary monoclonal antibodies: mouse anti-collagen type I (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-ATF4 (1:1000; Abcam, Cambridge, UK), rabbit anti-ATF6 (1.1000; Abcam, Cambridge, UK), rabbit Bax (1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse Beclin-1 (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit BiP (1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse cleaved caspase-3 (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse CHOP (1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-procollagen I (1:1000; Abcam, Cambridge, UK), rabbit LC3 (1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-p62 (1:1000; Abcam, Cambridge, UK), mouse XBP-1s (1:1000; Cell Signaling Technology, Danvers, MA, USA), mouse poly ubiquitinylated proteins, multi ubiquitin chains (1:500; Biomol Int., Plymouth Meeting, PA, USA), and rabbit anti- β-actin (1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA) as a loading control. In the next step the appropriate horseradish peroxidase conjugated secondary antibody: anti-mouse immunoglobulin G (IgG) (whole molecule) (1:2000; Sigma-Aldrich Corp., St. Louis, MO, USA), anti-rabbit antibodies (1:2000; Cell Signaling Technology, Danvers, MA, USA), anti-rabbit immunoglobulin G (IgG), Fc, HRP conjugate antibodies (1:2000; EMD Millipore Corp., Temecula, CA, USA) or anti-mouse IgG (whole molecule)—alkaline phosphatase antibody (1:2000; Sigma-Aldrich Corp., St. Louis, MO, USA) was added for 1 h with gentle shaking. After washing with TBS-T membranes were subjected to Westar Supernova Chemiluminescent Substrate for Western Blotting (Cyanagen, Bologna, Italy) and analyzed by densitometry (G:BOX, Syngene, Cambridge, UK). The intensity of analyzed proteins were normalized to β-actin which was a loading control. The data were expressed as a percentage of the normal sample taken as 100%. Determination of polyubiquitinated proteins was performed using Sigma Fast BCIP/NBT Alkaline Phosphatase Substrate (Sigma-Aldrich, St. Louis, MO, USA) by colorimetric detection (Gel Doc XR and Gel Documentation System; Molecular Imager Gel Doc XR, Bio-Rad Laboratories Inc., Hercules, CA, USA).

4.7. Immunofluorescence

Fibroblasts grown on cover-slips were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, the cells were permeabilized in PBS containing 0.2% Triton-X100 for 5 min and blocked in 5 % normal donkey serum (Sigma-Aldrich Corp., St. Louis, MO, USA) at room temperature for 60 min to block non-specific reactions. Then cells were incubated with mouse monoclonal anti-collagen type I antibody (1:250, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit monoclonal anti-LC3B antibody (1:2000, Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal anti-LAMP2A antibody (1:100, Abcam) for 60 min at room temperature. After incubation, the cells were washed three times with PBS and incubated in donkey anti-mouse IgG con-

jugated with Alexa Fluor 543 (1:200, Molecular Probes) or donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (1:200, Molecular Probes) at room temperature for 1 h. Then, the cells were washed three times in PBS and stained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Corp., St. Louis, MO, USA) for 10 min to indicate cell nuclei. The samples were washed twice with PBS and embedded in fluorescent medium (Medium Coverquick, Hygeco, OH, USA), dried overnight and stored in the dark until assessment. The immune labeled cells were analyzed using Nikon Digital Sight DS-Fi1 camera and a fluorescence microscope Nikon ECLIPSE Ti/C1 Plus, equipped with three filters DAPI (blue), FITC (green), and TRITC (red) (excitation wavelength/emission filter: 405/450 nm, 488/515 nm, 543/605 nm, respectively). No fluorescence signal was detected when cells were incubated with secondary antibodies alone (data not shown). At least five pictures of different areas of each treatment group were taken, independently analyzed and one representative image for each study group was presented.

4.8. Steady-State Analysis of Type I Collagen

Procollagens was extracted from cell lysates by precipitation overnight at 4 °C with ammonium sulfate (176 mg/mL). To obtain collagen, procollagen was subjected to digestion with pepsin (50 μg/mL) for 4 h at 4 °C. For electrophoretic analysis of migration of collagen chains, SDS-urea-PAGE (5% polyacrylamide gel) and silver staining were used.

4.9. Subcellular Fractionation

Cells were suspended in buffer containing 40 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, pH 7.5 for 30 min on ice. They were then homogenized by shearing 30 times through a 28.5-gauge needle and centrifuged at 1000× *g* for 10 min. The pellet was collected as the nuclear fraction, while the supernatant was subjected to centrifugation at 12,000× *g* for 10 min. The lysosome enriched pellet was washed using isotonic buffer (150 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES pH 7.5) and dissolved in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5). The presence of collagen type I in this fraction was analyzed by Western blot.

4.10. Determination of Proteasome Activities

Cells were sonicated in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA,1 mM EGTA, 0.5% Triton X, and centrifuged at 12,000× *g* for 15 min at 40 °C. The total protein concentration in supernatants was determined by the Bradford method in BioPhotometer (Eppendorf, Hamburg, Germany), using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Hercules, CA, USA) with BSA as a standard. The supernatants were diluted to concentration of 1.5 mg protein/mL in the lysis buffer. The reaction mixture (total volume of 50 μL) contained 30 μL of assay buffer (100 mM Tris/HCL, pH 7.5, 1 mM EDTA, 1 mM EGTA pH 7.5), 10 μL of cell lysate supernatant and 10 μL of fluorogenic peptide-AMC substrates: Suc-LLVY-AMC (Sigma Aldrich Corp., St. Louis, MO, USA) for chymotrypsin-like activity, Bz-VGR-AMC (Enzo Life Sciences, Inc., Farmingdale, NY, USA) for trypsin-like activity or Z-LLE-AMC (Enzo Life Sciences, Inc., Farmingdale, NY, USA) for caspase-like activity in a final concentration of 100 μM each [55]. The 96-well black plates (Corning Inc., Corning, NY, USA) were used and the assays were performed at 37 °C in FLUOStar OPTIMA (BMG Labtech Gmbh, Offenburg, Germany) over 30 min with one reading every 2 min, at 355 nm for excitation and 460 nm for emission. One unit of the proteasome activity was expressed as the amount of AMC released from the substrate per minute (pmol/min). The activity was calculated for the amount of total protein (U/mg). All assays were performed in triplicates.

4.11. Zymography for the Determination of MMP Activity

The conditioned media were collected and subjected non-reducing SDS–PAGE. The substrate for MMP was gelatin (1 mg/mL) (Sigma-Aldrich Corp., St. Louis, MO, USA). In

order to remove SDS, gels were incubated in 2.5% Triton X-100 solution for 30 min at room temperature. In the next step, gels were incubated overnight in 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 5 µM $ZnCl_2$ and 0.02% NaN_3) at 37 °C with gentle shaking. After that, they were stained with Commassie blue R-250 solution and destained until the appearance of white stripes on a dark blue background. Images of the zymograms were analyzed by densitometry (G:BOX, Syngene, Cambridge, UK).

4.12. Statistical Analysis

The results were statistically analyzed using the Statistica 12 software (StatSoft, Tulsa, OK, USA) and presented as the mean ± standard deviation (SD). Statistical differences were estimated by the use of one-way ANOVA followed by Tukey's test and values of $p < 0.05$ were considered as significant.

5. Conclusions

This study provides new insight into the effects of rosemary extract on OI fibroblasts with mutant collagen retention. The data presented here shows, for the first time, the pro-autophagy effect and the protective action of RE against the apoptosis of OI fibroblasts. Our findings could have important implications in OI treatment trials as RE removed accumulated mutant collagen and unfolded procollagen chains, improving cell homeostasis as indicated by decreased expression of UPR proteins. Reduction of proteasome activity by RE did not result in additional accumulation of non-collagen proteins. Although the exact mechanisms of the autophagy stimulatory and the proteasome and MMP (-1 and -2) inhibitory effects of RE require elucidation, the obtained results of our research are promising and worth continuing in order to understand the molecular pathways involved in the pathology of OI and beneficial effects of RE.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231810341/s1.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethical Committee of the Jagiellonian University in Kraków, Poland (KBET/108/B/2007, 31 May 2011).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

3-MA	3-Methyladenine
4-PBA	4-Phenylbutyrate
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BiP	Binding-Immunoglobulin Protein
BR	Bortezomib

C-L	caspase-like activity
Ch-L	chymotrypsin-like activity
CHOP	CCAAT enhancer binding protein
CQ	Chloroquine
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
eIF2	Eucaryotic translation initiation factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum (ER)-associated degradation
FBS	Fetal bovine serum
IRE1	Inositol requiring enzyme 1
LAPM2A	Lysosomal-associated membrane protein 2A
LC3	Microtubule associated protein 1 Light Chain 3
MMPs	Matrix Metalloproteinases
NH_4Cl	Ammonium chloride
OI	Osteogenesis imperfecta
P3H1	Prolyl 3-Hydroxylase 1
PBS	Phosphate-buffered saline
PERK	PKR-like endoplasmic reticulum kinase
PDI	Protein disulfide isomerase
RA	Rosmarinic acid
RE	Rosemary extract
RIPA	Radioimmunoprecipitation assay buffer
SDS	Sodium dodecyl sulfate
SDS-urea PAGE	SDS-urea polyacrylamide gel electrophoresis
SQSTM1/p62	Sequestosome-1
TGF-β	Transforming Growth Factor Beta
T-L	trypsin-like activity
UPR	Unfolded protein response
XBP-1s	X-Box binding protein 1

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SUPPLEMENTARY MATERIAL

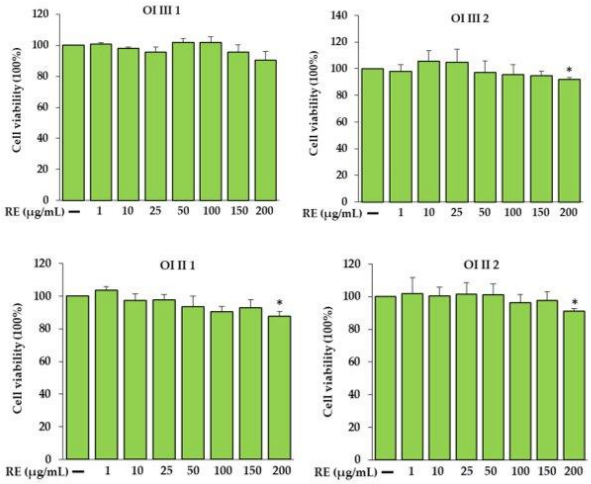


Figure S1. The effect of rosemary extract (RE) at concentrations of 1-200 µg/mL on the viability of OI types III and II fibroblasts of patients 1 and 2. Values represent the mean ± SD of three experiments done in duplicate; * $p < 0.05$, vs. untreated cells. The data are expressed as a percentage of the untreated sample taken as 100%.

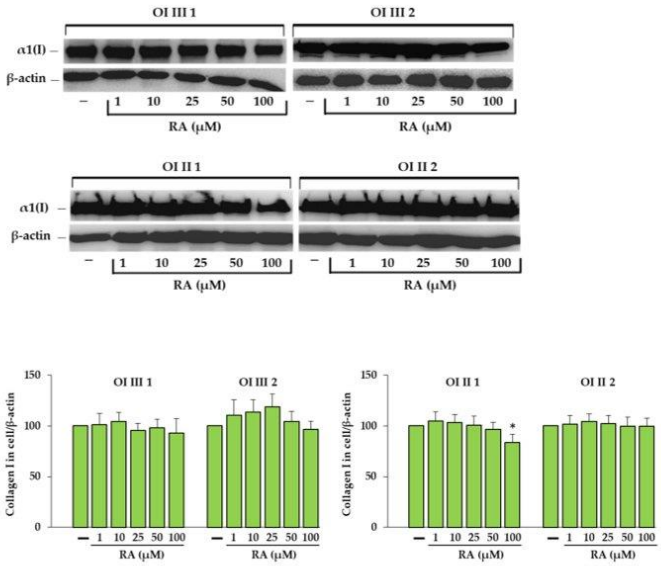


Figure S2. The effect of rosmarinic acid (RA) on the level of intracellular type I collagen in OI types III and II fibroblasts of patients 1 and 2; β-actin was used as cell protein loading control. The bars represent the results of the gel densitometry as the mean values from three independent experiments; * $p < 0.05$, vs. untreated cells. The data are expressed as a percentage of the untreated sample taken as 100%.

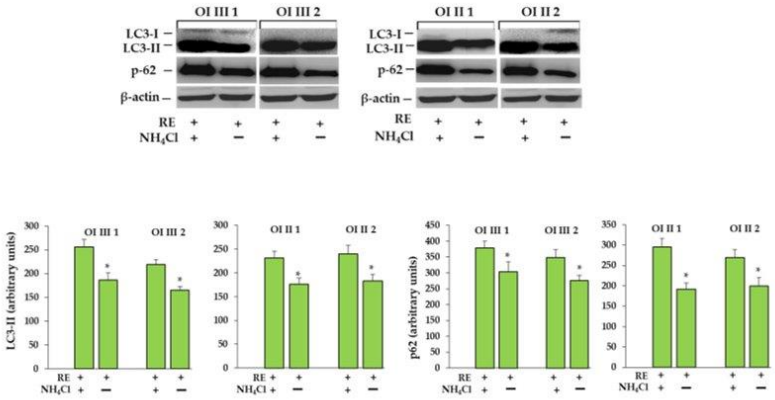


Figure S3. The increase in the level of LC3-II and p62 in the presence of inhibitor of autophagy (50 mM NH_4Cl) in OI cells treated with 50 µg/mL rosemary extract (RE); β-actin was used as cell protein loading control. The bars represent the results of the gel densitometry as the mean values from three independent experiments; * $p < 0.05$, OI cells treated with RE alone vs. OI cells treated with RE and NH_4Cl.

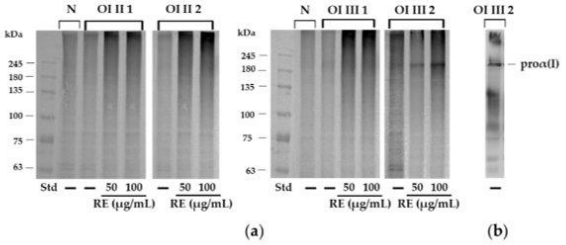


Figure S4. Western blot of polyubiquitynated proteins (**a**) and procollagen type I (**b**) followed SDS-PAGE under nonreducing conditions in normal (N), and untreated and treated with rosemary extract (RE) OI types III and II cells of patients 1 and 2. The arrows indicate the presence of polyubiquitynated high molecular proteins bands only in OI III 2 cells with the mutation in C-propeptide.

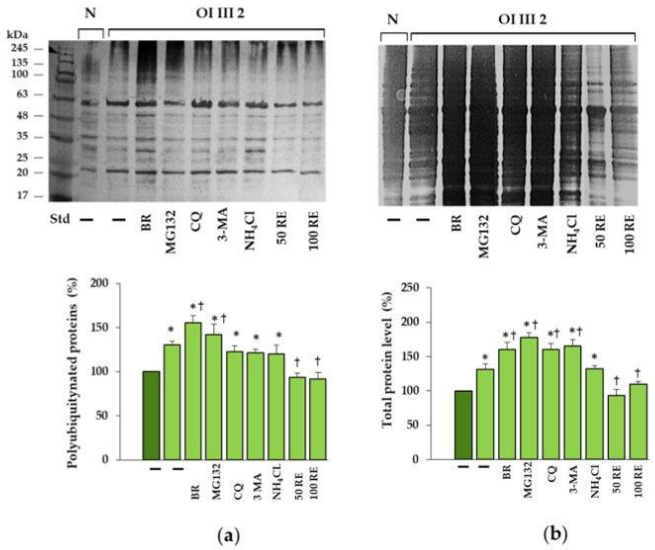
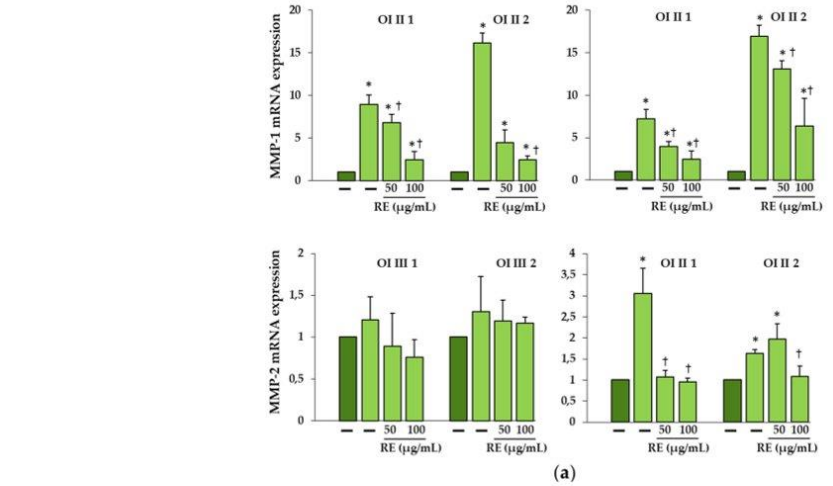


Figure S5. Western blot of polyubiquitinated proteins (**a**) and SDS-PAGE of total lysate proteins separated in reducing conditions and stained with silver salt (**b**) in normal (N) and OI type III cells of patient 2. OI cells were treated with 5 nM bortezomib (BR), 2.5 μM MG132, 50 μM chloroquine (CQ), 5 mM 3-methyladenine (3-MA), 50 mM NH_4Cl, and 50 and 100 μg/mL rosemary extract (RE). The bars represent the mean values from three independent experiments; * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells. The data are expressed as a percentage of the normal sample taken as 100%; dark green and light green bars represent N and OI, respectively.



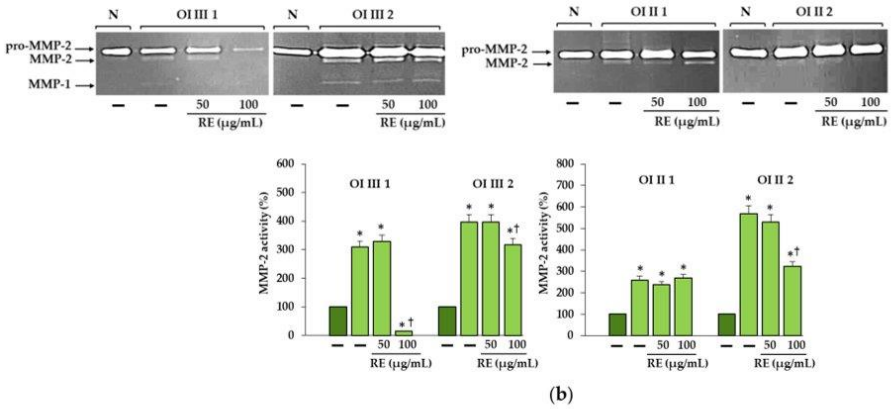


Figure S6. The influence of rosemary extract (RE) on the expression of matrix metalloproteinases (MMP-1 and MMP-2) mRNA (**a**) and MMP-2 activity (**b**) in OI types III and II cells of patients 1 and 2. The expression of MMP genes was assayed by real-time PCR, values represent the mean ± SD of three experiments (**a**). Representative gels of zymography (**b**); densitometry values represent the mean ± SD of three experiments. The data are expressed as a percentage of the normal (N) sample assumed as 100% (**b**); * $p < 0.05$, OI cells vs. normal cells; † $p < 0.05$, OI treated cells vs. OI untreated cells; dark green and light green bars represent N and OI, respectively.

Table S1. Sequences of primers used in the quantitative Real-Time PCR.

Gene		Primer sequence
ATG5	forward	5′-GGGAAGCAGAACCATACTATTTG-3′
	reverse	5′-AAATGTACTGTGATGTTCCAAGG-3′
ATF4	forward	5′-GTTCTCCAGCGACAAGGCTA-3′
	reverse	5′-ATCCTCCTTGCTGTTGTTGG-3′
ATF6	forward	5′ -TGAAGAGCAGGAAGGTGGTAG- 3′
	reverse	5′-AGGCAATGACAAAGAGTAGAAGG-3′
Bax	forward	5′-GGTGCCTCAGGATGCG-3′
	reverse	5′-GGAGTCTGTGTCCACG-3′
Beclin1	forward	5′-AGCTGCCGTTATACTGTTCTG-3′
	reverse	5′-ACTGCCTCCTGTGTCTTCAATCTT-3′
BiP	forward	5′-CGAGGAGGAGGACAAGAAGG-3′
	reverse	5′-CACCTTGAACGGCAAGAACT-3′
CHOP	forward	5′-GCGCATGAAGGAGAAAGAAC-3′
	reverse	5′- TCACAATTCGGTCAATCAGA -3′
PDI	forward	5′-GAGTTCTGCCACCGCTTCCT-3′
	reverse	5′-TGCTTGTCCCAGTCCTCCG-3′
MMP-1	forward	5′-CATTGATGGCATCCAAGCC-3′
	reverse	5′-GGCTGGACAGGATTTTGGG-3′
MMP-2	forward	5′-TGTGTCTTCCCCTTCACTTT-3′
	reverse	5′-GATCTGAGCGATGCCATCAA-3′
GAPDH	forward	5′-CTCTGCTCCTCCTGTTCGAC-3′
	reverse	5′-GCCCAATACGACCAAATCC-3′

STATEMENTS OF AUTHORSHIP/CO-AUTHORSHIP

Białystok, 06.09.2022

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Statement

I declare that my contribution to the preparation of the publication:

1. Sutkowska, J.; Hupert, N.; Gawron, K.; Strawa, J.W.; Tomczyk, M.; Forlino, A.; Galicka, A. The Stimulating Effect of Rosmarinic Acid and Extracts from Rosemary and Lemon Balm on Collagen Type I Biosynthesis in Osteogenesis Imperfecta Type I Skin Fibroblasts. Pharmaceutics 2021, 13, 938, doi: 10.3390/pharmaceutics13070938

which was included in my doctoral dissertation consisted in participation in the research planning, performing most of the experiments, participation in interpretation of results and statistical analysis, and manuscript drafting, which I define as 60% participation in the preparation of the above-mentioned publication.

2. Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23, 10341, doi: 10.3390/ijms231810341

which was included in my doctoral dissertation consisted in participation in the research planning, performing most of the experiments, participation in interpretation of results and statistical analysis, and manuscript drafting, which I define as 60% participation in the preparation of the above-mentioned publication.

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Joanna Sutkowska-Skolimowska
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dr hab. n. med. Anna Galicka (supervisor)

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Medical University of Białystok

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1. Sutkowska, J.; Hupert, N.; Gawron, K.; Strawa, J.W.; Tomczyk, M.; Forlino, A.; Galicka, A. The Stimulating Effect of Rosmarinic Acid and Extracts from Rosemary and Lemon Balm on Collagen Type I Biosynthesis in Osteogenesis Imperfecta Type I Skin Fibroblasts. Pharmaceutics 2021, 13, 938, doi: 10.3390/pharmaceutics13070938

2. Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23, 10341, doi: 10.3390/ijms231810341

which are the part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included supervising the research studies and participation in preparation and critical review of the manuscripts.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

Anne Galicka
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UNIVERSITÀ DI PAVIA
Dipartimento di
Medicina Molecolare

September, 07, 2022, Pavia, Italy

Prof. Antonella Forlino
Department of Molecular Medicine
University of Pavia, Italy

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I confirm that in the article:

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which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included co-participation in preparation and critical review of the manuscript.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

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I confirm that in the article:

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which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included providing the control and osteogenesis imperfecta fibroblasts for this study as well as participation in the final editing of the manuscript.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

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> Sutkowska, J.; Hupert, N.; Gawron, K.; Strawa, J.W.; Tomczyk, M.; Forlino, A.; Galicka, A. The Stimulating Effect of Rosmarinic Acid and Extracts from Rosemary and Lemon Balm on Collagen Type I Biosynthesis in Osteogenesis Imperfecta Type I Skin Fibroblasts. Pharmaceutics 2021, 13, 938, doi: 10.3390/pharmaceutics13070938
>
> Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23.

which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included the preparation and phytochemical analysis of rosemary and lemon balm extracts.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the the doctoral degree in the field of pharmaceutical sciences.

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> Sutkowska, J.; Hupert, N.; Gawron, K.; Strawa, J.W.; Tomczyk, M.; Forlino, A.; Galicka, A. The Stimulating Effect of Rosmarinic Acid and Extracts from Rosemary and Lemon Balm on Collagen Type I Biosynthesis in Osteogenesis Imperfecta Type I Skin Fibroblasts. Pharmaceutics 2021, 13, 938, doi: 10.3390/pharmaceutics13070938

which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included the preparation and phytochemical analysis of rosemary and lemon balm extracts, description of the methods and results of these preparations as well as participation in the final editing of the manuscript.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the the doctoral degree in the field of pharmaceutical sciences.

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I confirm that in the article:

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which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included participation in the preparation of control and osteogenesis imperfecta fibroblasts, stored in the Department of Molecular Biology and Genetics of Medical University of Silesia in Katowice, for providing to the Department of Medical Chemistry of Medical University in Białystok, which were used in this study.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

Hupert

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Katowice, 07.09.2022

Dr hab. n. med. Katarzyna Gawron
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Statement

I confirm that in the article:

Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23

which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included providing the control and osteogenesis imperfecta fibroblasts for this study as well as participation in the final editing of the manuscript.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

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which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included participation in the preparation of control and osteogenesis imperfecta fibroblasts, stored in the Department of Molecular Biology and Genetics of Medical University of Silesia in Katowice, for providing to the Department of Medical Chemistry of Medical University in Białystok, which were used in this study.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the doctoral degree in the field of pharmaceutical sciences.

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> Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23, 10341.

which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included performing confocal fluorescence microscopy with immunofluorescence staining.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the the doctoral degree in the field of pharmaceutical sciences.

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Department of Biology
Medical University of Białystok

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Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary Extract-Induced Autophagy and Decrease in Accumulation of Collagen Type I in Osteogenesis Imperfecta Skin Fibroblasts. International Journal of Molecular Sciences 2022, 23, 10341

which is a part of doctoral dissertation of Joanna Sutkowska-Skolimowska, my contribution included supervising research on the proteasome.

I agree to use this publication by Joanna Sutkowska-Skolimowska in the procedure for awarding the the doctoral degree in the field of pharmaceutical sciences.

KIEROWNIK
Zakładu Biologii
prof. dr hab. Halina Ostrowska
Signature

Kraków, dnia 24.06.2010.
DK/KB/CM/0031/ 689 /2010

UNIWERSYTET
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W KRAKOWIE

Pan
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Komisja Bioetyczna
Uniwersytetu
Jagiellońskiego

Komisja Bioetyczna Uniwersytetu Jagiellońskiego na posiedzeniu w dniu 24 czerwca 2010 roku wyraziła zgodę na przedłużenie badania pt. „Wykorzystanie terapii komórkowej i genowej w leczeniu Osteogenesis Imperfecta typu II i III "
(opinia nr KBET/108 /B/2007) do dnia 31 maja 2011r.

Z poważaniem

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Katowice, 2021 -01- 21

PCN/0022/KB/207/20

Sz. P.
Mgr Agnieszka Fus-Kujawa
Katedra Biologii Molekularnej
i Genetyki SUM

Szanowna Pani Magister,

Odpowiadając na pismo z dnia 09.12.2020 r. w sprawie zapytania, czy na przeprowadzenie badania pt.: ***"Otrzymywanie indukowanych pluripotentnych komórek pnia z fibroblastów oraz ich transfekcja z użyciem polimerów gwieździstych i systempu hodowli na termo-sterowalnych podłożach"***, wymagana będzie zgoda Komisji Bioetycznej Śląskiego Uniwersytetu Medycznego w Katowicach, uprzejmie informuję, że Komisja Bioetyczna SUM zajęła następujące stanowisko.

W świetle *Ustawy z dnia 5 grudnia 1996 r. o zawodach lekarza i lekarza dentysty* (tj. Dz. U. z 2019 r. poz. 537 z późn. zm., badanie molekularne na materiale już pozyskanym nie jest eksperymentem medycznym i nie wymaga oceny Komisji Bioetycznej SUM. Jednocześnie uprzejmie przypominam, że do przeprowadzenia w/w badania konieczna będzie zgoda kierownika Zakładu Biologii Molekularnej, Katedry Biologii Molekularnej i Genetyki.

Brak obowiązku uzyskania zgody Komisji Bioetycznej nie zwalnia wnioskodawcy z obowiązku przestrzegania powszechnie obowiązujących przepisów prawa, w tym wymagań dotyczących ochrony danych osobowych, respektowania praw pacjenta oraz zasad dysponowania ludzkim materiałem biologicznym.

Wykorzystanie danych może być wyłącznie w celu realizacji w/w projektu badawczego.

Z poważaniem

Przewodniczący
Komisji Bioetycznej SUM

Prof. dr hab. Bogdan Okopień

Otrzymują:
1. Adresat
2. a/a

Białystok, 20.04.2022

Joanna Sutkowska-Skolimowska

Zakład Chemii Medycznej

Uniwersytet Medyczny w Białymstoku

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Kierownik

Zakładu Genetyki Klinicznej

Śląski Uniwersytet Medyczny

Dr n. biol. Karolina Bajdak-Rusinek

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W związku z realizacją pracy doktorskiej pt. „The beneficial effect of extracts from Rosemary and Lemon Balm and rosmarinic acid on collagen type I abnormalities in fibroblasts from Osteogenesis Imperfecta patients" zwracam się z uprzejmą prośbą o wyrażenie zgody na wykorzystanie linii komórkowych fibroblastów skóry ludzkiej pacjentów z Osteogenesis imperfecta typu I, II i III (zatwierdzony przez Komisję Bioetyczną Uniwersytetu Jagiellońskiego w Krakowie: KBET/108/B/2007, 31 maja 2011) pozyskanych przez Państwa zakład.

Proszę o pozytywne rozpatrzenie mojej prośby.

Wyrażam zgodę na wykorzystanie linii komórkowych fibroblastów skóry ludzkiej pacjentów z OI typ I, II i III

Z poważaniem

Joanna Sutkowska-Skolimowska

KIEROWNIK
Zakładu Genetyki Klinicznej
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Śląskiego Uniwersytetu Medycznego w Katowicach
dr n. biol. Karolina Bajdak-Rusinek

SCIENTIFIC ACHIEVEMENTS

1. List of publications constituing the doctoral dissertation

- Sutkowska, J.; Hupert, N.; Gawron, K.; Strawa, J.W.; Tomczyk, M.; Forlino, A.; Galicka, A. The stimulating effect of rosmarinic acid and extracts from rosemary and lemon balm on collagen type I biosynthesis in osteogenesis imperfecta type I skin fibroblasts. Pharmaceutics 2021, 13, 938, doi: 10.3390/pharmaceutics13070938. IF: 6,525; MNiSW:100

- Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Botor, M.; Gawron, K.; Galicka, A. Rosemary extract-induced autophagy and decrease in accumulation of collagen type I in osteogenesis imperfecta skin fibroblasts. International Journal of Molecular Sciences 2022, 23, 10341, doi: 10.3390/ijms231810341. IF: 6,208; MNiSW:140

3. List of other scientific publications and monographs

- Galicka, A.; Sutkowska-Skolimowska, J. The beneficial effect of rosmarinic acid on benzophenone-3-induced alterations In human skin fibroblasts. International Journal of Molecular Sciences 2021, 22, 11451, doi: 10.3390/ijms222111451. IF: 6,208; MNiSW: 140.

- Sutkowska-Skolimowska, J.; Galicka, A. ER stress in osteogenesis imperfecta (OI) - causative mutations and potential treatment", In: Advances in Biomedical Research – Cancer and Miscellaneous; Ed.: Młynarczuk-Biały, I.; Biały, Ł. TYGIEL Scientific Publisher, Medical University of Warsaw, Lublin-Warsaw, Poland, 2021, pp. 133–143 https://bc.wydawnictwo-tygiel.pl/publikacja/AA60CB34-1A06-4ECD-748B-83D6A9A5B19C; MNiSW:20

- Chmielecka, A.; Sutkowska, J.; Galicka, A. Evaluation of the effect of synthetic tylophorin analogues on the expression and activity of matrix metalloproteinases MMP-2 and MMP-9 in brain glioblastoma multiforme cells of line LN229. In: Nauka, badania i doniesienia naukowe, 2021, Świebodzice: Idea Knowledge Future, 2021, Ed.: Tobiasz

Wysoczański, Katarzyna Dereń, pp. 32–42, http://www.konferencja-eureka.pl/assets/docs/nauki-przyrodnicze-i-medyczne-2021-cz-1.pdf. MNiSW:5.

3. List of congress reports

Posters:

- Śnietka, E.; Bogdańska, E.; Popławska, P.; Rysiak E.; Sutkowska, J. Diagnostyka kosmetyczna - ocena stanu skóry. V National Conference of Laboratory Medicine Students and Young Diagnosticians "Emerging Diagnostics," Student Session on Clinical Diagnostics. Białystok, Poland, 14.04.2018.
- Sutkowska, J.; Galicka, A. Podłoże molekularne wrodzonej łamliwości kości (Osteogenesis Imperfecta). The 3rd National Scientific Conference "Rare Diseases in the 21st Century. Lublin, Poland, 21.02.2020.
- Sutkowska, J.; Galicka, A. Ocena wpływu kwasu rozmarynowego na biosyntezę i sekrecję kolagenu typu I w fibroblastach pacjenta z wrodzoną łamliwością kości typu I. IV National Scientific Conference Rare Diseases in the 21st century. Lublin, Poland, on-line, 19.02.2021.
- Sutkowska, J.; Strawa, J.W.; Tomczyk, M.; Gawron, K.; Galicka, A. Stimulating effect of rosemary extract and rosmarinic acid on type I collagen biosynthesis in human osteogenesis imperfecta type I skin fibroblasts. International E-Conference on Dermatology and Cosmetology. London, United Kingdom, on-line, 10-11.05.2021.
- Sutkowska, J.; Strawa, J.; Tomczyk, M.; Galicka, A. Assessment of the effect of lemon balm extract on biosynthesis of type I collagen and activity of MMP in human skin fibroblasts of a patient with osteogenesis imperfecta type 1. 13th International Conference on Traditional Medicine and Acupuncture. Singapore, Singapore, on-line, 29.07.2021.
- Sutkowska-Skolimowska, J.; Galicka, A. Evaluation of the effects of benzophenone-3 on collagen expression and survival of human skin fibroblasts. XIII Training and Scientific Conference of the Polish Society of Toxicology. Gdańsk, Poland, on-line, 16-17.2021 - 2nd place in the poster competition
- Sutkowska-Skolimowska, J.; Galicka, A. The effect of benzophenone-3 on the extracellular matrix components of the skin. XXIV Scientific Congress of the Polish Pharmaceutical Society "Salus aegroti suprema lex". Lublin, Poland, on-line, 22-24.09.2021.

- Sutkowska-Skolimowska, J.; Botor, M.; Gawron, K.; Strawa, J.W.; Galicka, A. Rosemary extract decreases accumulation of mutated collagen type I in osteogenesis imperfecta skin fibroblasts. 10th International Conference Agriculture & Food, Burgas, Bulgaria, on-line, 16-19.08.2022.
- Sutkowska-Skolimowska, J.; Strawa, J.W.; Galicka, A. Effect of rosemary extract on the expression of markers of cell stress and apoptosis in osteogenesis imperfecta skin fibroblasts. 10th International Conference Agriculture & Food. Burgas, Bulgaria, on-line, 16-19.08.2022.
- Sutkowska-Skolimowska, J.; Brańska-Januszewska, J.; Ostrowska, H.; Strawa, J.W.; Galicka, A. Rosemary extract-induced decrease in activity of proteasome and increase in autophagy in osteogenesis imperfecta skin fibroblasts. 3rd International Conference on Pharmacology and Toxycology & Nano Medicine and Advanced Drug Delivery. Bangkok, Thailand, on-line, 29-30.08.2022.

4. List of other scientific activities

4.1 Research projects:

- **Co-investigator of the scientific project funded by the Medical University of Bialystok, Poland**
  (Project No: SUB/2/DN/21/003/2213)
  Project title: ,,The effect of bortezomib on autophagy and apoptosis in LN229, LN18 and LBC3 glioblastoma multiforme cell lines".
- **Leader of research grant, Medical University of Bialystok, Poland. (Project (No POWR.03.02.00-00-I051/16 co-funded from European Union funds,**
  PO WER 2014-2020 grant 04/IMSD/G/2021).
  Project title: ,,Evaluation of the therapeutic potential of selected polyphenolic compounds in an in vivo experimental model (Danio rerio) of congenital bone fragility (Osteogenesis imperfecta)."
- **Leader of the statutory project, Medical University of Bialystok, Poland**
  (Project No: SUB/2/DN/19/003/2203)
  Project title: ,,Effects of ultraviolet chemical filters on human skin fibroblasts."
- **Co-investigator of the scientific project funded by the Medical University of Bialystok, Poland**
  (Project No: SUB/2/DN/20/003/2203)

Project title: ,,The effect of bortezomib on the expression of selected integrins, MMPs, collagen and migration of LN229, LN18 and LBC3 glioblastoma multiforme cell lines.”

- **Co-investigator of the scientific project funded by the Medical University of Bialystok, Poland**
  (Project No: SUB/2/DN/19/002/2203)
  Project title: ,,Integrins α2β1, α5β1, α9β1 as targets for anticancer therapy.”

4.2 Scientific internships/training courses:

- National internship at the Silesian Medical University in Katowice, Department of Molecular Biology and Genetics. Katowice, Poland, 18-29.10.2021
- Training ,,Communicating research: paper writing and short presentations", EMBO. Heidelberg, Germany, on-line, 20-21.10.2021
- Pre-conference workshop : "Zebrafish model - future and challenge. Workshop on methods of maintaining and working with the Zebrafish model.". Gdańsk, Poland, on-line, 15.09.2021
- Course: Bioluminescence: Past, Present, Future. On-line, 13.09.2021
- Summer school ,,Scientific discourse and modern research technologies and scientific success”. Medical University of Bialystok, Białystok, Poland, 28.06.2021-02.07.2021
- Course ,,Western blotting - hints and tips". Białystok, Poland, 28.05.2021
- Summer school ,,Biotechnology in medicine and pharmacy”. Vytautas Magnus University, Kaunas, Lithuania, 21-25.09.2020
- Course ,,Selection of the correct serum in the cell culture process”, Merck. Warszawa, Poland, on-line, 26.03.2020
- Course ,,The role of microRNAs in the process of tumorigenesis". Białystok, Poland, 22.10.2020
- Course ,,"Flow of genetic information from gene to protein". Białystok, Poland, 22.05.2020
- Summer school ,,Soft skills and academic success”. Supraśl, Poland, 03-07.06.2019
- Course ,, Applications of spectrometric methods in environmental and forensic research and food”. Ms Spectrum, Białystok, Poland, 26.11.2019

- Course ,,How to write publications in English?". National Representation of PhD Students, Białystok, Poland, online, 26.11.2019
- Course ,,Innovative solutions for mammalian cell culture". Merck, Białystok, Poland, 26.11.2019
- Course ,, Hyaluronic acid in aesthetic fillings - basic level". Warszawa, Poland, 16.07.2017