

Article

Potential of Icariin–Glucosamine Combination in the Treatment of Osteoarthritis by Topical Application: Development of Topical Formulation and In Vitro Permeation Study

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Abstract: The aim of this study was to develop a topically applied formulation with the potential to alleviate arthritis ailments. A combination of two active ingredients, icariin from *Epimedium L.* (Species: *Epimedium Koreanum*) extract as a potential promoter of chondrogenesis and glucosamine sulfate as a precursor of cartilage tissues, was tested. In permeation studies, the potential for skin permeation of both substances was confirmed; however, the in vitro release test did not accurately reflect the degree of skin permeation. The in vitro release of icariin was at a level of 15.0–19.0% for the plant-extract-derived icariin and 29.0–35.0% for the pure substance. The level of glucosamine sulfate release was 38.4% (on average). For icariin of both origins, the release results were higher than those obtained via oral administration (about 12.0%), which shows the potential superiority of topical application. In addition, the physicochemical parameters that affect the in vitro release and performance of topical formulations were addressed. This preliminary research and permeation analysis of the formulation produced a promising picture of its prospects regarding arthritis treatment, although further investigation is needed.

Keywords: icariin; *Epimedium L.* extract; glucosamine sulfate; osteoarthritis; topical application; in vitro release



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1. Introduction

Musculoskeletal disorders are the second most common cause of disability in the elderly worldwide [1]. Among them, the most common is osteoarthritis (OA), which affects approximately 527.81 million people worldwide [2]. One of the effects of this disease is the progressive, permanent loss of cartilage within the joint, whose function is the protection of joint surfaces against frictional, compressive, shear, and tensile loads during movement. The cartilage tissue is composed of chondrocytes suspended in the intercellular matrix consisting of water, hyaluronic acid, minerals, proteoglycan, and collagen fibers, mainly of type II [3]. Due to the lack of blood vessels and nerves, damaged cartilage has only limited self-healing ability [4,5]. The excessive mechanical overloading of cartilage tissue stimulates chondrocytes to produce cytokines and tumor necrosis factor α (TNF- α), which activate the synthesis of metalloproteinases, tissue plasminogen activator, plasmins, and other proteases involved in tissue destruction [6]. As a consequence, a shift in the balance between the production of enzymes and their inhibitors, which regulate the process of cartilage formation, in favor of the destructive effect is observed, which precludes effective regeneration. Attempts to regenerate articular cartilage without removing mechanical loads lead to the formation of tissue with a changed biochemical composition. The decrease in the content of proteoglycans and change in their composition, leading to a shortening of the glycosaminoglycan chains and a weaker ability to bind hyaluronic acid, together with a lack of the ability to aggregate, have the greatest impact on the deterioration of the

biomechanical properties of the tissue. The collagen content usually remains the same, but the fibers are much thinner and, therefore, form looser nets. With time, the volume of the cartilage decreases, and significant defects appear within it; additionally, the bones under the cartilage can also be damaged, which may lead to their remodeling, sclerotization, and the formation of osteophytes [7]. The whole process is accompanied by inflammation of the synovial membrane, which is associated with lympho- and monocytic infiltrates and the phagocytosis of mechanically damaged fragments of articular cartilage. Then, there is general inflammation of all surrounding structures, i.e., the joint capsule, ligaments, tendons, and muscles belonging to the affected joint. The clinical symptoms include, first and foremost, pain associated especially with the commencement of movement, but also limited mobility of the joint, stiffness after immobility, swelling and thickening, and visible changes in the joint's outline [8]. Pain may be caused by damage to various tissues and may be related to the irritation of the nerve endings of the periosteum, damage to the subchondral layer of the bone, prolonged muscle tension, or improper tensioning of other structures to stabilize the joint and may also result from the presence of inflammation [9]. Many years of epidemiological studies conducted on various populations from around the world have allowed for the determination of differences in the incidence and factors increasing the risk of the disease, such as age, gender, or the environment. It has been repeatedly shown that the incidence of osteoarthritis increases with age, and more often affects women than men, while other factors increasing the risk of developing this disease include obesity, smoking, and injuries [2].

This disease leads to the progressive, permanent loss of cartilage within the joint. Due to the lack of blood vessels and nerves, damaged cartilage has only limited self-healing ability [5]. The excessive mechanical overloading of cartilage tissue stimulates chondrocytes to produce cytokines and tumor necrosis factor α (TNF- α), which activate the synthesis of substances involved in tissue destruction [6]. The result is a shift in the balance between the production of enzymes and their inhibitors, which regulate the process of cartilage formation, in favor of a destructive effect; thus, effective regeneration is precluded. The clinical symptoms include, first and foremost, pain associated especially with the commencement of movement, but also limited mobility of the joint, stiffness after immobility, swelling and thickening, and visible changes in the joint's outline. Pain may be caused by damage to various tissues and may be related to the irritation of the nerve endings of the periosteum, damage to the subchondral layer of the bone, prolonged muscle tension, or improper tensioning of other structures to stabilize the joint and may also result from the presence of inflammation. To this day, no effective treatment for causal OA has been developed. The most commonly used therapy includes pain reduction, the inhibition of the disease's progression, and joint function improvement, which are achieved through a combination of pharmacological and non-pharmacological methods and surgical treatment. For this purpose, generally available painkillers are used, and in the case of more severe pain and inflammation, drugs from the group of non-steroidal anti-inflammatory drugs (NSAIDs) are recommended [10]. However, their long-term use is not recommended due to the possibility of a number of side effects [11]. In pharmacological treatment, especially because of low toxicity, an alternative group of substances is used, namely, that of the symptomatic, slow-acting drugs for osteoarthritis (SYSADOA) [12–14]. This group includes both natural and synthetic substances that can be administered orally, intraarticularly, or topically. It has been shown that *Epimedium* L. (Species: *Epimedium Koreanum*) extract has an excellent beneficial impact on bone regeneration and can be involved in the regulation of multiple signaling pathways and biochemical processes [15]. *Epimedium* L. is one of the most commonly used herbs to treat bone fractures and osteoporosis in traditional Chinese medicine, wherein it is assumed that *Epimedium* L. has the ability to "strengthen and regenerate bones" [16]. The *Epimedium* L. herb contains over 60 types of flavonoids, mainly glycosidic flavonoids, phenylpropanoids, alkaloids, polysaccharides, lignins, and sesquiterpenes [17–20], of which epimedin A, B, and C; icarizide_II; and icariin are considered the most important biologically active ingredients and constitute about 52%

of the total content of flavonoids in the plant [21,22]. Pharmacological studies have shown that *Epimedium* L. has a number of anti-inflammatory, antioxidant, and tumor-inhibitory properties [23,24], and clinical studies have suggested a beneficial effect on the treatment of diabetes, depression, cardiovascular diseases, rheumatoid arthritis, osteoporosis, and osteoarthritis [25–28]. Regarding the treatment of diseases of the musculoskeletal system, icariin has been shown to have the greatest impact on bone regeneration from among other constituents of *Epimedium* L. and can be involved in the regulation of multiple signaling pathways and biochemical processes [29]. In a mouse model of osteoarthritis, icariin reduced the destruction of cartilage, promoted chondrocyte differentiation, upregulated the expression of parathyroid hormone-related protein, and down-regulated the expression of Indian hedgehog [30]. Icariin belongs to the glycosyl flavonoids with phytoestrogen activity, and it is the 8-prenyl derivative of kaempferol 3,7-O-diglucoside with the molecular formula of $C_{33}H_{40}O_{15}$. It is also safe, nontoxic, and its price is moderate, which makes it more attractive [31].

Another potentially very helpful substance in OA treatment is glucosamine, which is a naturally occurring aminomonosaccharide and acts as a precursor in the biochemical synthesis of glycosylated proteins or lipids. Of particular importance to this study, it is a constituent of glycosaminoglycans in cartilage and synovial fluids [32]. Glucosamine is an endogenous substance, i.e., a typical constituent of the polysaccharide chains of the cartilage matrix and of glycosaminoglycans in the synovial fluid. In vitro and in vivo studies have shown that glucosamine stimulates the synthesis of physiological glycosaminoglycans and proteoglycans via chondrocytes and hyaluronic acid via synoviocytes [33]. Glucosamine's mechanism of action in humans is still not fully known, which makes it impossible to estimate the time required for the body to respond to the administered preparation. Glucosamine is a relatively small molecule (with a molecular weight of 179.00, while that of glucosamine sulfate potassium chloride is 605.50); it is easily soluble in water and in hydrophilic organic solvents. Only limited information is available on the pharmacokinetics of glucosamine. The commonly sold form of glucosamine that offers significant therapeutic effects is glucosamine sulfate [34–37]; in particular, crystalline glucosamine sulfate is considered highly bioavailable with a proven pharmacological effect [38–40]. The purpose of this study was to design and develop a topical formulation for the treatment of osteoarthritis. This formulation contains a combination of two active ingredients: icariin from *Epimedium* L. (Species: *Epimedium Koreanum*) extract, which may be a potential promoter of chondrogenesis, and glucosamine sulfate as a precursor of cartilage tissue; together, these two ingredients may exhibit multidirectional effects and offer excellent potential with respect to the treatment of osteoarthritis. To test the validity of further studies, including application and clinical trials, permeation tests were conducted as the first step. An in vitro release test was conducted to mirror the permeation study of the active ingredients to confirm their potential for topical application while avoiding the side effects accompanying long-term oral intake [41].

2. Materials and Methods

2.1. Reagents

D-glucosamine sulfate*2KCl (100%) was provided by Biomus Company (Białystok, Poland), Icariin (98.0%) was purchased from Chengdu Biopurity Phytochemicals Ltd. (Sichuan, China), and *Epimedium* L. extract (based on the dried aerial parts of *Epimedium Koreanum* plant) was obtained from The Garden of Naturalsolution Co., Ltd. (Osan-si, Republic of Korea). Basal substances used were as follows: sodium polyacrylate (BASF Care Creations, Germany), *vitis vinifera* L. seed oil, shea butter, xanthan gum, and PEG-40 hydrogenated castor oil (Brenntag Poland Sp. z.o.o., Poland); glyceryl stearate, stearyl alcohol, and Cetareth-25 were purchased from Evonik—Personal Care (Poland). The following permeation promoters were used: propylene glycol (Chemitec, Sosnowiec, Poland); essential oils of rosemary, marjoram, clove, and cinnamon from Avicenna Oil (Wrocław, Poland); and d-menthol and d-camphor, which were provided by PPH Standard sp. z.o.o.

(Lublin, Poland). Phosphate-buffered saline was purchased from Thermo Fisher Scientific (Waltham, MA, USA), methanol clean for analysis was obtained from StanLab (Lublin, Poland), and glacial acetic acid (99.5% clean) for analysis was provided by Chempur (Piekary Śląskie, Poland).

2.2. Preparation of a Formulation Containing Icariin and Glucosamine

The compositions of the formulations are shown in Table 1.

Table 1. The compositions of the formulations proposed.

Prepared Formulations		
INGREDIENT	CREAM (wt.%)	GEL (wt.%)
Water	up to 100.00	up to 100.00
<i>Epimedium</i> L. extract (Species: <i>Epimedium Koreanum</i>)	70.00	70.00
Propylene glycol	3.00	3.00
D-Glucosamine sulfate potassium chloride salt	10.00	10.00
Xanthan gum	0.20	-
Sodium polyacrylate	-	3.00
<i>Vitis vinifera</i> L. seed oil	3.00	-
Glyceryl stearate	3.00	-
Stearyl alcohol	2.50	-
Ceteareth 25	2.00	-
Butyrospermum parkii (shea) butter	1.00	-
PEG-40 hydrogenated castor oil	-	1.00
D-Camphor	0.50	0.50
D-Menthol	0.20	0.20
Alcohol denat.	1.00	1.00
Rosemary essential oil	0.50	0.50
Marjoram essential oil	0.20	0.20
Cinnamon essential oil	0.05	0.05
Clove essential oil	0.20	0.20
Dehydroacetic acid (and) Benzyl alcohol	1.00	1.00

2.2.1. Preparation of Cream

Solid ingredients of the oil phase were melted at a temperature of 75 °C, and the liquid portion of the oil phase was added after melting of the solid portion. Glucosamine sulfate was dissolved in water phase with stirring (IKA RW 20 digital). The aqueous phase containing bioactive components was gradually added to the oil phase, with homogenization (IKA ULTRA TURRAX T25) at 9500–10,000 rpm for 2 min and continuous stirring at 320 rpm for 40 min until the temperature decreased to 40 °C.

2.2.2. Preparation of Gel

Sodium polyacrylate gel was prepared by a cold process. D-glucosamine sulfate potassium chloride salt was dissolved in water phase with stirring (IKA RW 20 digital) at 320 rpm for 10 min. Sodium polyacrylate was added to the water phase and mixed with continuous stirring at 450 rpm for 30 min. Sodium was added until homogenous. The oil phase was gradually added to the water phase upon continuous agitation.

2.2.3. Preparation of Ointment

The constituents of the ointment base were placed together and allowed to melt at 70 °C in water bath. After melting, the ingredients were stirred while gently maintaining temperature of 70 °C for 10 min, and then cooled with continuous stirring at 350 rpm. The active ingredients were added after lowering the temperature of the base to 40 °C and continuing to mix at 500 rpm for 20 min until a homogeneous mass was obtained.

2.2.4. Acceptance Criteria and Specifications

According to specific cosmetic guidelines, each topical preparation must satisfy certain important criteria, such as obtaining/maintaining a specific color, certain pH range, density, viscosity, and homogeneity, and producing no skin irritation effects.

2.3. Physicochemical Tests

2.3.1. Appearance and Homogeneity

The cream and gel bases prepared were inspected visually for clarity, homogeneity, color, and presence of any particles/aggregates.

2.3.2. Determination of pH of Formulations

Metrohm 827pH Lab pH meter (Metrohm Polska, Opacz-Kolonia, Poland) was used for determination of the pH of each formulation in triplicate. The pH meter was calibrated with standard buffer solutions before each use.

2.3.3. Density

Steel pycnometer (Quantachrome, Boynton Beach, FL, USA) with a capacity of 100 mL was used to determine density of the formulations. The pycnometer was filled with the product and measurements were repeated three times. Densities of the formulations were calculated using the following equation (Equation (1)):

$$P_s = 0.99985 \times m_s/v_p + 0.0012 \quad (1)$$

where P_s —formulation density (kg/m^3); m_s —substance mass (kg); v_p —pycnometer volume (m^3) 0.99985—atmospheric pressure factor; and 0.0012—coefficient accounting for air density.

Equation (1) Equation for calculating formulation density.

2.3.4. Dynamic Viscosity

Brookfield LVT rotational viscometer was used to determine dynamic viscosity of the prepared formulations. Viscosity was measured at room temperature (approximately 23 °C) with a spindle no. 3 for creams and no. 4 for gels, and spindle rotation speed of 12 rpm for gels and 1.5 rpm for emulsions.

2.3.5. Assay of Icariin Content in *Epimedium* L. (Species: *Epimedium Koreanum*) Extract—LC-MS

The icariin concentration in the commercially available extract of *Epimedium* L. (Species: *Epimedium Koreanum*) was determined using a Kinetex column 2.6 μm C18 (2.1-id x100 mm). Analysis conditions were as follows: column temperature of 35 °C, injection volume of 3 μL , and mobile phase flow of 0.3 mL/min. The mobile phases were A: water + 0.1 wt.% formic acid, and B: acetonitrile + 0.1 wt.% formic acid. Elution was performed using a stepwise gradient elution shown in Table 2.

Table 2. Gradient mobile phase elution for icariin determination in *Epimedium* L. (Species: *Epimedium Koreanum*) extract via LC-MS.

Time (min)	A (% v/v)	B (% v/v)
0	75	25
1	75	25
15	55	45
16	55	45
17	10	90
22	10	90
23	75	25
28	75	25

2.3.6. Release of Icariin

In vitro release test for icariin from *Epimedium* L. extract (Species: *Epimedium Koreanum*) was conducted with 708-DS Dissolution Apparatus Agilent Technologies Inc. (Santa Clara, CA, USA), synthetic membrane, Acceptor Solution phosphate buffer pH 5.8, and UV-VIS spectrophotometer $\lambda = 270$ nm.

2.3.7. Release of Glucosamine

In vitro release test for glucosamine was conducted with Franz Vertical Diffusion Cell Test System (Copley Scientific TM, Nottingham, United Kingdom). Synthetic membrane was soaked for 30 min prior to the in vitro assay in phosphate-buffered saline (PBS) of pH 7.4. The amount of formulation in each Franz cell and the time it was assembled were recorded. Conditions used were as follows: 32 °C, agitation of 50 rpm, acceptor solution—phosphate buffer of pH 7.4, and the amount of formulation—0.1 g. Samples were taken from the sampling port every hour for five hours. After each sample was collected, the phosphate buffer volume was restored to maintain conditions.

Concentrations of d-glucosamine sulfate in the samples were determined using HPLC-UV (Agilent Technologies) and C18 Column (3.5 μ m, 4.6 mm id \times 150 mm Waters) at temp. of 30 °C and wavelength of $\lambda = 254$ nm. The mobile phase consisted of methanol: water: glacial acetic acid (10:89, 96:0.04, pH 3.5); flow—1 mL/min; and injection volume of 20 μ L. Accumulated amount of glucosamine sulfate was measured after derivatization of glucosamine to phenylthiocarbamyl glucosamine with phenyl isothiocyanate.

3. Results and Discussion

As a chronic degenerative and progressive disease, osteoarthritis affects the lives of people around the world, and to this day, no effective treatment has been developed. The main purpose of this study was to create dermocosmetic products with the potential to alleviate arthritis ailments. Glucosamine is an important precursor in the biochemical synthesis of glycosaminoglycans, which are major components of cartilage [32]. Moreover, icariin, due to its chondrogenic and osteoinductive potential, may be an effective promoter of bone regeneration [42–44]. In vitro studies have shown that icariin has the potential to promote bone engineering by initiating direct and stable chondrocyte differentiation, reducing its apoptosis, and creating an extracellular matrix by chondrocytes—without invoking a hypertrophy effect—due to the fact that it might be an effective accelerant of growth factors for cartilage [42,43]. A potent chondrogenic effect might be caused by the upregulation of the expression levels of aggrecan, collagen II, and SOX9 genes and the downregulation of the collagen I gene. Sun et al. have noted that the protection against cartilage and bone degradation is connected to the ability of icariin to inhibit the protease activity of cathepsin K [44]. Wu et al. have established that icariin regulates articular bone loss, in part by regulating the receptor activator of the nuclear factor-B ligand RANKL (reduction) and osteoprotegerin OPG (enhancement) expression [45,46]. The data from clinical trials concerning the use of icariin in humans are limited. Only one blinded placebo control study has been reported. A randomized, double-blind study of *Epimedium* L. flavonoids that involved the administration of icariin (60 mg/day) to 100 postmenopausal women showed a significantly higher bone mineral density in the treatment group compared to the placebo group in 24 months [47]. It is important to remember that icariin has phytoestrogen activity, so it may disturb the endocrine system and connected pathways through oral administration, but this fact also indicates the superiority of topical application [48,49]. Unfortunately, there are just a few clinical studies that suggest that icariin might be helpful in OA treatment, and especially via topical administration, so this area needs to be further investigated. Both substances (icariin and glucosamine sulfate) are known to exhibit excellent therapeutic effects when used separately; due to their completely different mechanisms of action [33,50], they may also show a synergistic effect, which would be worth examining, especially due to the fact that glucosamine sulfate presents synergistic effects with a few NSAIDs and SYSADOA [40,51]. The determination

of the icariin content in *Epimedium L.* (Species: *Epimedium Koreanum*) extract was essential in order to set the extract concentration in the target product formulation for further tests such that it is within the detection limits of the analytical devices used. The predicted concentration of icariin in the extract was very low based on the information from the manufacturer's specification; the mixture that was provided contained only 5 wt.% of *Epimedium L.* (Species: *Epimedium Koreanum*) extract and a much smaller amount of icariin. The LC-MS method was selected to conduct content analysis due to its high specificity, as only selected ions produced from the analytes of interest are monitored. This analysis allowed for the determination of the quantitative composition of the formulation such that it fell within the limits of detection of the devices used. The calibration function for a standard was made by injecting five different concentrations of reference solution in triplicate. The regression coefficient was 0.999 for the calibration curve, which demonstrates good linearity over the linear range (Figure 1). The LC-MS analysis showed an average of 168.76 ng of icariin per 1 mL of *Epimedium L.* (Species: *Epimedium Koreanum*) extract, which would be under the detection limit while using the standard of 1–5 wt.% of the extract. This result enabled the content of the extract in the product mass, which was set at the level of 70%, to be within the detection limits of icariin for further analysis, and facilitated the determination of the amount of icariin corresponding to the extract in the *in vitro* permeation study.

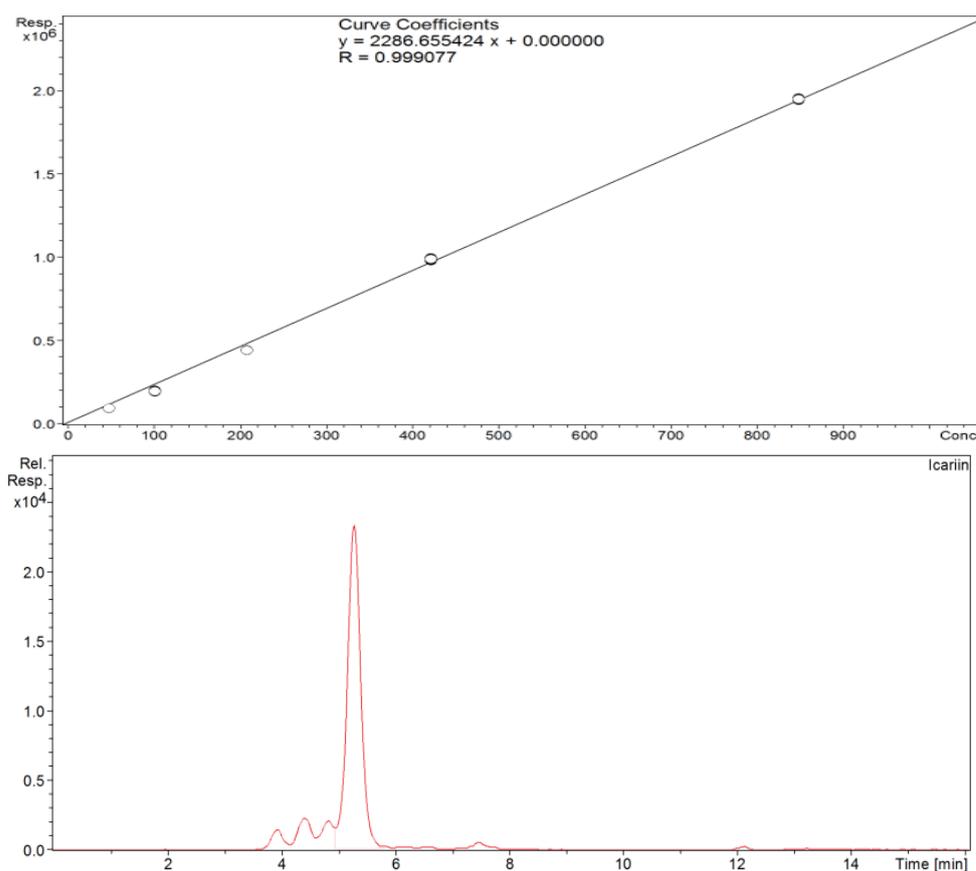


Figure 1. Calibration curve and chromatogram of icariin in *Epimedium L.* (Species: *Epimedium Koreanum*) extract indicating its content of 168.90 ng/mL.

The formulations proposed (cream and gel) for arthritis management were successfully developed so that they met all the acceptance criteria and specifications. All formulations were visually assessed for phase homogeneity, agglomeration, and discoloration. They appeared smooth, homogeneous, free from agglomerates, and initially stable, with no mechanical impurities found in the product masses. This study confirmed that gels and

emulsions are effective matrices with which to hold high concentrations of water-soluble molecules such as glucosamine sulfate and hydrophilic extracts and are able to deliver such molecules across the skin via topical application. The physicochemical parameters of the formulations are shown in Table 3. The rheological and sensorial properties, appearance, consistency, dynamic viscosity, density, and pH were reproducible and met the acceptance criteria.

Table 3. Physicochemical properties of all formulations.

Formulation	Color	Homogeneity and Consistency	pH	Viscosity [mPa × s]	Density
cream 1_1 sample	off-white	creamy, excellent homogenous, no visible impurities	6.32	37.800	0.792
cream 1_2 sample	off-white	creamy, excellent homogenous, no visible impurities	6.29	38.200	0.794
cream 1_3 sample	off-white	creamy, excellent homogenous, no visible impurities	6.33	37.800	0.791
gel 1_1 sample	slightly brown	nontransparent gel, homogenous, no visible impurities	5.19	33.600	0.987
gel 1_2 sample	slightly brown	nontransparent gel, homogenous, no visible impurities	5.17	34.250	0.984
gel 1_3 sample	slightly brown	nontransparent gel, homogenous, no visible impurities	5.18	33.950	0.989

It has been established that some physicochemical parameters can have a significant impact on the degree of skin penetration by particular substances. Malzfeldt et al. [52] have proved that the viscosity of a medium has an influence on the release rate of the active ingredient and that the rate of release is higher in a solution than in a suspension, cream, or ointment. Therefore, the base components were selected such that the lowest possible viscosity of the medium was ensured while maintaining mass stability. The prepared formulations exhibited sufficient viscosity (33,600–38,200 mPa × s) and there was no appreciable change in viscosity over time. Another parameter that may influence the release rate is the pH of the formulations. As the proposed products are cosmetics, it was necessary to follow general guidelines for cosmetics and adjust the pH so that it would not affect the skin's integrity and would not cause any adverse effects such as redness, rash, swelling, or changes in transepithelial water loss. The pH of the carrier may have a significant effect on the permeation of the substance, as it determines the solubility of active ingredients and the formulations' stability during storage. After application, the pH may impact the partitioning of the active substances between the formulation and the skin. In this study, we did not ascertain the effect of pH changes on the ability to release active substances, so further studies are required in order to improve the release profile.

There is little information about the skin permeability of icariin in the literature. Iin et al. have shown that due to its low water solubility, membrane permeability, and low dissolution rate in biological fluids, icariin has low oral bioavailability of about 12% and poor absorption; thus, its clinical applications are limited [53]. The predicted skin bioavailability was poor, as icariin is a relatively large molecule with a molecular weight of 676.7 Da, poor water solubility, and membrane permeability.

In this study, we used absorption enhancers that, in the initial formulation containing the *Epimedium* L. (Species: *Epimedium Koreanum*) extract, enabled the acquirement of an icariin release value of 15–19%. The use of another formulation containing the same amount of icariin as in the extract, but in the form of a pure substance, enabled the acquirement of an icariin release value of 29–35% (Figure 2). The gel matrix was very effective at holding 70% of the hydrophilic extracts and was able to deliver them across the skin via topical application. The results of the study of the in vitro skin permeation of icariin suggest that the use of pure icariin is more effective than the extract, which indicates that, in the case of a formulation with a herbal extract that contains over 260 other substances, there may

be a competitive release of other active ingredients or extract-derived icariin may bond with other ingredients, which would impede permeation [20]. This study also showed potentially higher bioavailability via skin application than oral administration, although this supposition requires proof from further studies as it is based on the accumulated amount of icariin but not the actual level of skin permeation. In view of the fact that Xu et al. have reported a value of released icariin as high as 70%, much higher results may be obtained by the proper modification of the carrier and the use of more appropriate permeation promoters for pure icariin, which is known to display poor solubility both in water and lipid solutions. One group of authors obtained an icariin-loaded nanogel via the reverse microemulsion method, yielding very promising release results [54]. In our study, the challenge was to devise a carrier that is suitable for both substances while accounting for their different natures and that ensures an optimum release profile. This analysis and preliminary characterization of the formulation performed so far have painted a very promising picture of osteoarthritis treatment; however, the skin-penetrating ability, application, and bioavailability enhancement, particularly with respect to icariin, may require further investigation to increase their therapeutic effects. Further studies are underway to improve permeation of the actives, examine the applicational prospects, and confirm the properties of the product.

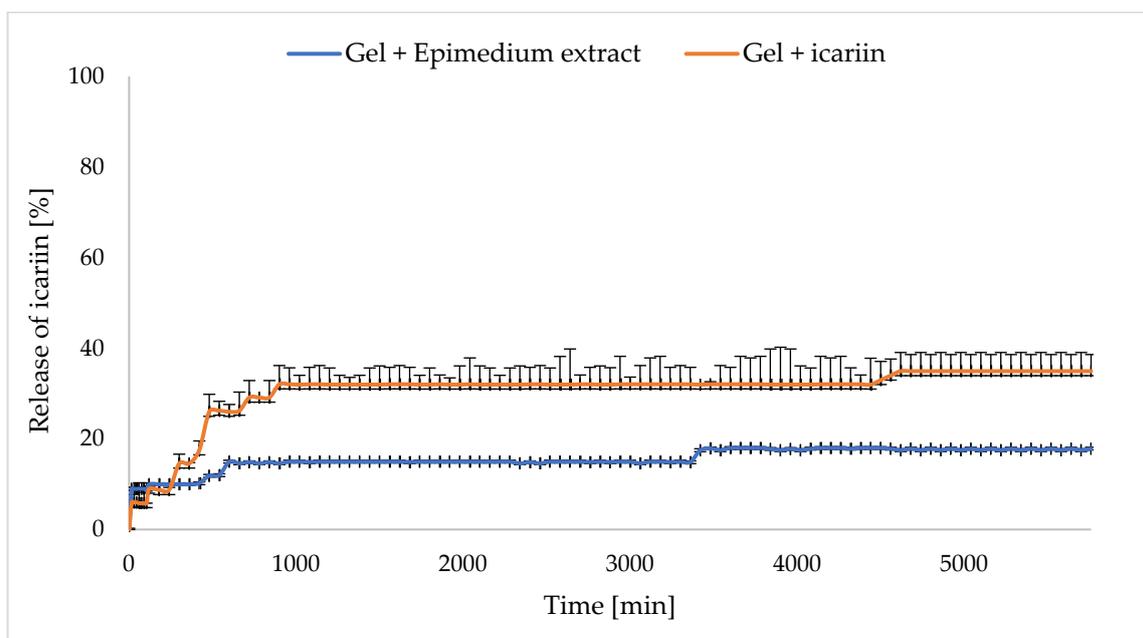


Figure 2. Release of icariin from gel formulation.

Glucosamine is much better known and characterized in the literature than icariin, which facilitated the more precise selection of the analytical conditions. This substance stimulates proteoglycan synthesis via chondrocytes [55] and shows anti-inflammatory activities in different *in vivo* animal models [56,57]. Clinical studies have demonstrated its superior effect compared to a placebo in the short-term treatment of OA pain and movement limitation [36,58–60]; moreover, it has been shown that long-term (3-years) oral treatment with glucosamine sulfate retards the progression of Knee OA [36]. Not all researchers have been enthusiastic about glucosamine with respect to the treatment of OA, and there are some speculations about its low level of bioavailability [61]. Clinical studies conducted by Persiani et al. have shown increased concentrations of glucosamine after oral administration in both healthy subjects and OA sufferers [62,63]. The concentration was measured in plasma and synovial fluid, which indicates a positive influence on cartilage and relatively high bioavailability, but the levels were still lower than that required to positively affect the cartilage. A great deal of hope was elicited by subsequent studies

in which it was shown that much higher concentrations of glucosamine can be absorbed upon topical application compared to the oral route [64], and that it can specifically enter synovial fluid [65]. Lower bioavailability of oral administration of glucosamine is connected to the hepatic first-pass effect [62], which is responsible for drug degradation. The drug's absolute bioavailability is unknown. About 38% of an intravenous dose is excreted in an unchanged form in urine. The absolute bioavailability in humans after a single, oral dose was 25%. In the liver, more than 70% of the administered dose is metabolized. Absorption from the alimentary duct can be of about 90%; approximately 11% of the labeled dose was excreted in the feces [66]. Bypassing the gastrointestinal system is not the only benefit offered by topical administration. It has been widely established that transdermal application offers numerous advantages over oral delivery, especially in terms of targeted drug administration, where its use circumvents most of the undesirable side effects such as nausea, diarrhea, ulcers, and gastric inflammations, and is much safer in long-term treatment [67–69]. Due to its hydrophilic character, glucosamine sulfate is very water soluble, so it can be easily introduced into a medium, which allows for the prediction of its stability under selected conditions and the acquirement of results related to its accumulated amount that are close to the predictions for different carriers. In this study, the best results were obtained for the formulation containing 10 wt.% d-glucosamine sulfate (Table 4). It was also shown that the higher concentration of d-glucosamine sulfate in the formulation led to its higher accumulated amount. The formulations with d-glucosamine salt concentrations above 10 wt.% were not tested because of the difficulty of maintaining their stability. In this study, we used glucosamine sulfate, as it has been reported to exhibit much better effects compared to glucosamine hydrochloride [39]. The findings found in clinical trials with different glucosamine salts indicate the superiority of sulfates over hydrochlorides due to the increase in the sulfur concentration, which is essential for the synthesis of proteoglycans that are important for chondrocyte metabolism [70,71]. In addition, glucosamine sulphate has already shown a synergistic effect when combined with other osteoarthritis treatments [14,40].

Table 4. In vitro cumulative glucosamine release results.

2 g of glucosamine sulfate/100 g of product									
Time [h]	Cream			Gel			Ointment		
	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	45	11.70	2.25	132.00	18.50	6.60	0.00	0.00	0.00
2	104	10.40	5.20	152.50	10.90	7.63	12.00	5.50	0.60
3	236	21.30	11.80	213.50	17.70	10.68	64.50	19.70	3.23
4	335	18.50	16.75	229.00	10.20	11.45	84.00	21.70	4.20
5	355	24.90	17.75	294.50	9.70	14.73	107.50	14.70	5.38
5 g of glucosamine sulfate/100 g of product									
Time [h]	Cream			Gel			Ointment		
	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	156.00	10.80	3.10	326.10	7.60	6.50	25.70	12.10	0.50
2	385.20	3.10	7.70	472.10	6.10	9.40	39.00	15.90	0.80
3	535.60	61.80	10.70	575.20	15.30	11.50	208.40	54.10	4.20
4	1046.40	34.00	20.90	810.70	10.70	16.20	270.90	80.70	5.40
5	1260.90	49.10	25.20	989.70	11.50	19.80	345.20	51.00	6.90

Table 4. Cont.

Time [h]	10 g of glucosamine sulfate/100 g of product								
	Cream			Gel			Ointment		
	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]	average [$\mu\text{g}/\text{cm}^2$]	SD [$\mu\text{g}/\text{cm}^2$]	[%]
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	422.20	27.40	4.20	974.30	108.60	9.70	42.40	11.80	0.40
2	1083.10	9.60	10.80	1137.30	29.40	11.40	82.70	4.10	0.80
3	2479.50	20.50	24.80	1589.00	93.20	15.90	299.90	82.60	3.00
4	3179.20	61.20	31.80	1713.90	15.40	17.10	468.80	24.00	4.70
5	3838.10	39.50	38.40	2173.10	36.60	21.70	603.10	36.10	6.00

The in vitro active ingredient cumulative release from the formulations was on average 38.4% for the creams and 21.7% for the gels after 5 h. The formulations with all the concentrations had similar release profiles; initially, glucosamine was released from the gel formulations much faster, but ultimately, the glucosamine was released from the cream formulation in higher concentrations (Figures 3–5).

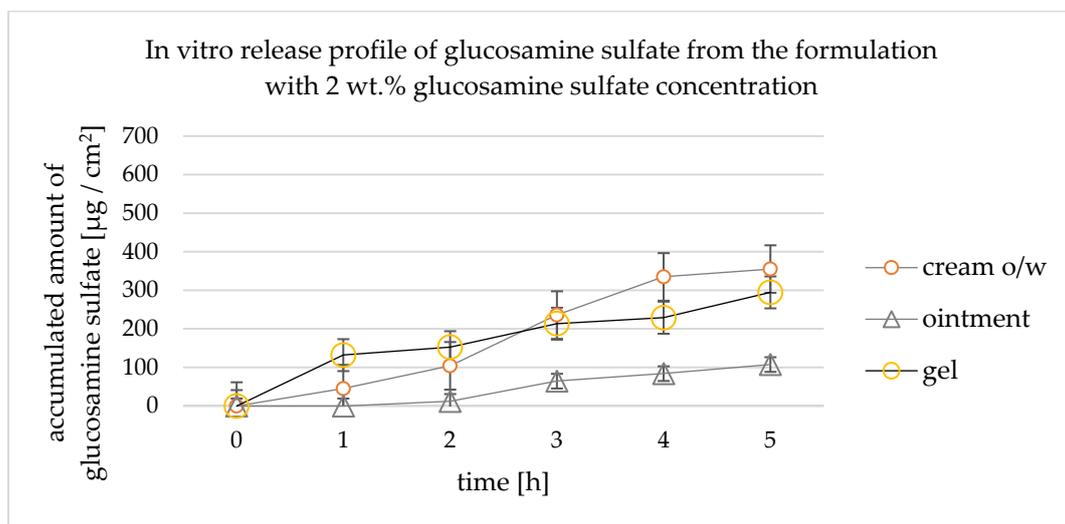


Figure 3. Accumulated amount of glucosamine sulfate—in vitro release study for the formulation with 2 wt.% of glucosamine.

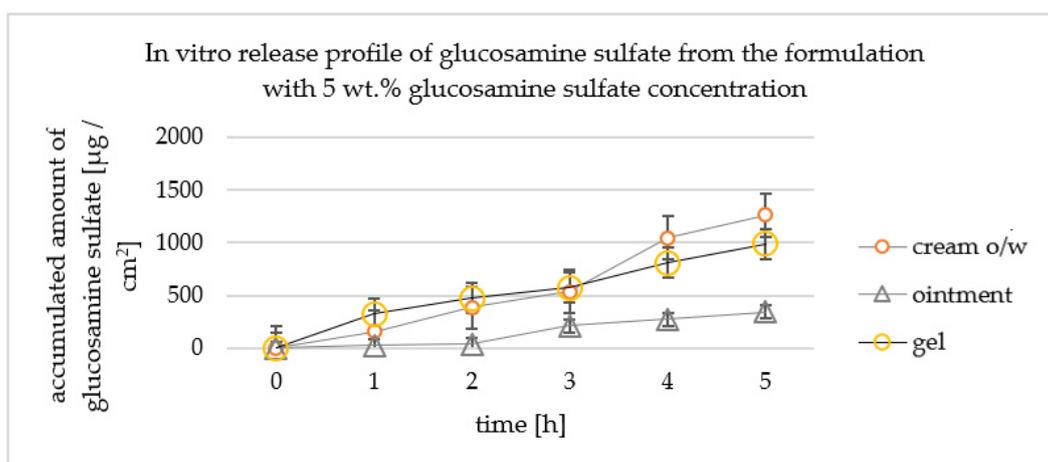


Figure 4. Accumulated amount of glucosamine sulfate—in vitro release study for 5 wt.% formulation.

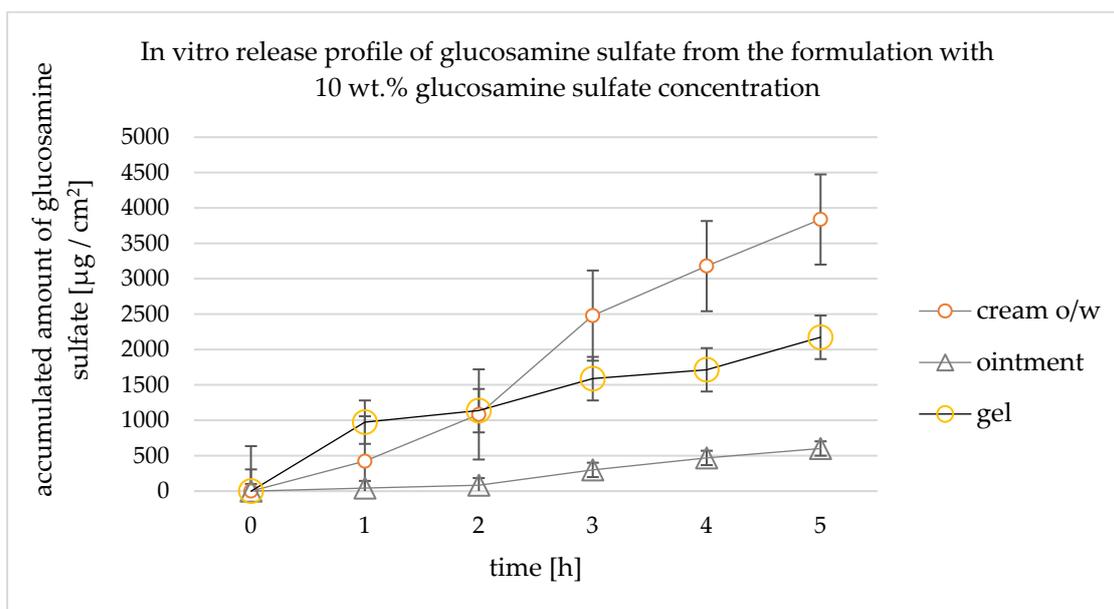


Figure 5. Accumulated amount of glucosamine sulfate—in vitro release profiles of the formulation containing 10 wt.% of glucosamine.

The differences in the in vitro permeation of glucosamine through membranes are correlated with the type of carrier. As the emulsion consists of oil and water phases, it has a greater affinity to the skin than the formulation that consists of only a water phase and a gelling agent. The initial faster release afforded by the gel was probably due to the fact that the active ingredient does not need to partition the two phases and may immediately cross the membrane. The in vitro release study confirmed the potential in an in vitro skin permeation study of both active substances (glucosamine and icariin). Due to the relatively hydrophilic character of glucosamine, the expected in vitro skin permeation was very low especially in the gel formulation. As glucosamine does not contain a chromophore, to make its detection possible via the UV detector used, a derivatization was necessary, which confirmed the results of Kanwischer et al. [72]. A combination of permeation promoters from different groups of chemicals, such as camphor, menthol, propylene glycol, alcohol denat., and essential oils, in the formulations proposed permitted relatively high in vitro release of glucosamine sulfate from the prepared formulation, which was on average 38.4% for the creams after 5 h. A higher permeation result was predicted for the emulsion formulation as it was known that a combination of oil and water phases has greater affinity to the skin and is more compatible with the lipophilic nature of skin, which, eventually, yields a more desirable permeation profile, slower release at the beginning, and much a higher concentration of the active ingredient at the end. A better release profile constitutes a longer duration of action with higher concentrations of the active ingredient.

In permeation studies, the use of different polymeric membranes designed to mimic the human skin, such as polysulfone, polyethersulfone, cellulose, and polydimethylsiloxane, has been suggested by the FDA [73] because they are inert, do not occlude the penetration of the active substances, and provide good permeability [74,75]. Membrane selection is a significant factor in permeation studies as its type should match a given carrier. In this study, synthetic membranes were used to assuage ethical concerns and because of their easier accessibility. Considering the use of semi-liquid carriers in the form of emulsions and gels, it was most advantageous to choose hydrophilic membranes, such as polysulfone and cellulose, which demonstrated significantly better in vitro release results for emulsions and gels than for ointments. These observations confirm that the type and features of the membrane have an impact on the release rate and general accumulation of topical dosage forms. In the case of ointments, the use of a hydrophobic membrane and a carrier that is compatible with it would probably yield much better results. It has been suggested

that polydimethylsiloxane membranes could be used to predict the skin permeability of lipophilic substances such as ointments with adequate results [64,76]. Despite their many advantages, such as the ease with which their size and composition can be adjusted; the ease of their use, acquisition, and storage; and the lack of variability, thus matching the intrinsic properties of the skin, unfortunately, the selected membranes (both polysulfone and cellulose) cannot provide an insight into the interaction of the formulation's ingredients with the skin [77]. This is related to the impossibility of accounting for the lipid perturbation effect, cell metabolism, and the heterogeneous nature of the skin, which are difficult to recreate due to the synthetic nature of the membranes. A validated in vitro release test can provide screening information about the potential of a substance to cross the skin barrier, and the way the structural differences between the tested products may affect the rate and amount of the released substance, so long as the analyses are run under the same conditions. The use of artificial membranes instead of human or animal skin provides information on the release characteristics of a given substance instead of the permeation of the active substances, so further research is required to obtain information on the real degree of permeation. Other factors that can have an impact on the active ingredient's release results are the type and characteristics of the prepared carriers. In the case of the formulation composition, the manufacturing process is crucial. The gel formulation was prepared using a cold process to avoid affecting the stabilities of the active ingredients, but the preparation of the emulsion required 75 °C to melt the solid components of the oil phase, which increased the risk of the decomposition of the active substances. Fortunately, the active substances used were stable at such a temperature and the production process did not affect their concentrations in the formulation. The higher total accumulated amount of the active substance released from the emulsion than from the gel formulation can be explained by the fact that the surfactants in the emulsion reduce surface tension and may also improve the wetting of the membrane surface and increase the contact area with the membrane, as confirmed by Ferreira et al. [78]. An initial faster release from the gel was expected, since, in the gel, the active ingredient does not need to partition the two phases and is immediately able to cross the membrane. It has been well known for many years that the skin is a significant barrier to the ingress of many compounds, and protection against any external factors is its most significant function. The penetration of active substances is hindered mostly by the large species diversity and the lipophilic nature of the lipid layer; therefore, to increase the therapeutic effectiveness of topical application, the use of permeation enhancers is needed. In this study, a combination of permeation enhancers that facilitate the absorption of active substances through the skin by temporarily diminishing the skin's impermeability was used. Dimethyl sulfoxide (DMSO) is one of the most important compounds that enhances the transdermal permeation of a variety of drugs; unfortunately, it is forbidden in cosmetic applications, so the search for other enhancers was necessary. Terpenes, such as menthol and camphor and the terpene constituents of essential oils, increase the solubility of the active substances by forming a eutectic mixture with the penetrating compound and altering the barrier properties of the stratum corneum. The lowering of the melting point affects the solubility of many compounds and thus the degree of skin permeation. The use of ethanol offered several potential advantages. Alcohols act as penetration enhancers by leaching lipids from the stratum corneum, and they also release the sulfhydryl groups in keratin proteins of the stratum corneum, thus significantly increasing the possibility of the penetration of hydrophilic substances. They also act as carriers for the terpenes used to increase the skin penetration and enhance the solubility of icariin [79]. It is important to note that in this study, two main active substances were used: glucosamine sulfate, which is highly hydrophilic, and the aqueous extract of *Epimedium* L. (Species: *Epimedium Koreanum*), which contains hydrophobic icariin. The physiochemical characteristics of the active ingredients' molecules constitute another aspect that is crucial to defining the tendency of their release, and they might have impacted the data obtained in this study. In this research, we considered the use of icariin and glucosamine sulfate in a potential chondrogenic combination. It has been known for many years that the influence

of ethanol on the ability to increase the permeation of hydrophilic compounds is usually connected to a simultaneous reduction in the penetration of hydrophobic ones, but icariin displays poor solubility both in water and lipid solutions. Thus, propylene glycol was added as an additional permeation promoter and co-solvent.

4. Conclusions

Demonstrating the possibility of a topical product's therapeutic effects can be challenging. In this study, the potential of active ingredients to permeate the skin was shown. However, the obtained results of the release study do not reflect the actual degree of skin permeation but refer only to the accumulated amount of the active ingredient, as it was impossible to account for the interaction between the skin components and the lipid barrier that naturally occurs on the skin. We obtained the release of icariin at a level of 15.0–19.0% for the substance derived from the plant extract, and at a level of 29.0–35.0% for the pure substance. In both cases, the release was higher than for oral administration (by about 12.0%).

The average release level of glucosamine sulfate was 38.4%, which is sufficient. Both the cream and gel matrices effectively held 70.0% of the hydrophilic extracts, were stable at a 10.0% glucosamine sulfate concentration, and were able to deliver the substance across the synthetic membrane, thus mimicking topical application.

The lowest results were obtained for the ointment carrier, which is most likely due to the strongly hydrophilic nature of the active substances used. Other parameters that also have an impact on the *in vitro* release of active ingredients and the performance of a topical product were identified, namely, the initial concentration, process parameters, physicochemical properties such as viscosity and pH, the type of selected carrier, and permeation enhancers. This analysis and preliminary research on the formulations created has painted a very promising picture of osteoarthritis treatment, but further investigations are needed to increase their therapeutic effects and verify the actual degree of skin permeation. Due to global demographic changes and gradual lifestyle changes, the number of people suffering from OA is increasing, which, combined with the lack of effective treatments, is mobilizing a constant search for alternative therapies. The idea of using icariin, with its potentially strong ability to activate chondrogenesis and intercellular matrix regeneration by chondrocytes in a hitherto unexplored topical application, provides new insights into the potential use of this active substance and its properties with respect to the treatment of OA. In addition, the novel combination of the two substances (icariin and glucosamine sulfate), which show excellent effects separately due to their completely different mechanisms of action, may exhibit a synergistic effect, which would be worth confirming. Further studies are underway to confirm the properties of the product.

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