Phytochemical studies of plant extracts enclosed in chitosan microparticles and the effect of phytoformulations on skin condition

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ABSTRACT: The cosmetic industry constantly competes in search of new and exotic raw materials, often forgetting the strength in the action of well-known and tested for many years. The main aim of this study was to formulate dermatological preparations containing plant extracts-loaded microparticles and to investigate the effects of their topical application on skin conditions. Extracts were prepared using the Soxhlet apparatus with water and ethanol as solvents with the following common Polish herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome: *Potentilla erecta*. They were characterised by the content of polyphenols, flavonoids and antioxidant capacity (CUPRAC, FRAP and DPPH RSA). Subsequently, extracts were enclosed in chitosan microparticles with studied loading capacity and *in vitro* release profile. The highest level of TPC, TFC and antioxidant activity was noted in aqueous extract from *Sambucus nigra*. Biophysical skin parameters were instrumentally assessed after application to the probands skin of obtained herbal creams and hydrogels. Preliminary studies of the application of phytoformulations revealed that the skin was not irritated, and the skin's barrier permeability was maintained. Moreover, we observed a significant increase in skin hydration. Better short-term hydration properties showed cream containing microparticles with a loaded extract from *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* and hydrogel with free extracts from *Sambucus nigra* and *Viola tricolor* + *Veronica officinalis.* Therefore, prepared herbal dermatological preparations are suitable for skin conditioning.

1. Introduction

There is a growing demand and continuous search for new phytochemicals in the cosmetics market due to consumers' rising expectations worldwide for green and natural products. However, numerous plants commonly occurring in Poland begin to be somewhat forgotten due to the "pursuit" of new, exotic plant materials. Herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*, flowers of *Achillea millefolium, Sambucus nigra,*

Tilia cordata, and rhizome of *Potentilla erecta* have been recognised in Polish traditional folk medicine and herbalism for their beneficial effects on the skin. They have been reported to possess regenerating, soothing, antioxidant, anti-inflammatory, antibacterial, immunosuppressive, and antiproliferative properties [1–7]. These pharmacological activities are attributed to their phytoconstituents, mainly phenolic compounds, a group of small molecules with at least one phenol unit in their structures. They are secondary metabolites synthesised in the shikimic acid of plants and pentose phosphate through phenylpropanoid pathways. Phenolic compounds can be divided into subgroups: phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbens, and curcuminoids. The main flavonoids found in *Viola tricolor* are violanthin, rutin, anthocyanidin [8] and quercetin glycosides [9]. Moreover, numerous studies also reported the presence of cyclotides [1,10] and bisabolol [11]. Dominant compounds among phenolic and sterolic acids content of *Veronica officinalis* were determined as quercitrin, p-coumaric acid, ferulic acid, luteolin, hispidulin and β-sitosterol [12]. *Glechoma hederacea* comprises phenolic acids (chlorogenic, rosmarinic, caffeic, and ferulic acids) and flavonoid O-glycosides (rutin and genistin) [13,14]. Major constituents of *Plantago lanceolata* belong to polyphenols, tannins, flavonoids, alkaloids, terpenoids and iridoid glycosides, such as p-hydroxybenzoic, vanillic, gallic and chlorogenic acids, apigenin, luteolin and luteolin-7-O-glucoside [15,16]. *Achillea millefolium* has a high content of flavonoids (mainly luteolin O-acetylhexoside and apigenin O-acetylhexoside), organic acids (including malic, oxalic and quinic acids), tocopherols (γ-, α- and β-isoforms) and phenolic acids (mainly cis and trans-3,5-O-dicaffeoylquinic acids) [17]. *Sambucus nigra* flowers are a rich source of flavonoids, particularly rutin, quercetin and kaempferol, as well as phenolic acids, such as caffeoylquinic acid [18,19]. Vanillic acid was the dominant phenolic acid, whereas rutoside and (−)-epicatechin (tannin precursor) were major flavonoids found in *Tilia cordata* flowers [20]. *Potentilla erecta* rhizome contains tannins (pyrogallol), ellagitannins (including agrimoniin and pedunculagin), phenolic acids (coumaric, sinapic, caffeic, and gallic acids and their derivatives), flavonoids (kaempferol and quercetin and their derivatives), as well as triterpene saponins [3,21].

The human skin, as the largest organ covering the body, plays an important immunity role in protecting the body against pathogens and excessive water loss. This is assured due to the skin structure comprising three main layers: the epidermis, the dermis, and the hypodermis. The outermost layer of the epidermis – *stratum corneum* – comprises several layers of corneocytes embedded in a lipid matrix, forming a structure similar to "brick and mortar" with keratin-rich corneocytes as "bricks" and intercellular lipids as "mortar". Being a barrier from pathogens, the *stratum corneum* also prevents the penetration of cosmetic active substances to deeper skin layers. However, recent advances in encapsulation technology have significantly improved not only the chemical stability of active substances but also their biocompatibility, skin permeability, and skin cosmetic effects when applied topically [22,23].

Microencapsulation is a technique by which solid, liquid or gaseous active substances can be enclosed within a matrix [24,25]. Microparticles are synthesised, differing from each other in their respective diameters, which range from 1 to 1000 μm, and in their great diversity of spherical shapes, symmetrical or not. Microencapsulation may be achieved by various techniques [26,27] or materials [28]. On the other hand, substances can be microencapsulated for numerous purposes: to keep the material confined for a certain period, to allow its gradual

and controlled diffusion or to launch its release under certain conditions [29,30]. Nowadays, it is a new technology that has been employed in the cosmetics industry as well as in the pharmaceutical, agrochemical and food industries, textiles, being used in drugs, extracts, vitamins, perfumes, oils, proteins, dyes, and bacterial cells, among others [31–40].

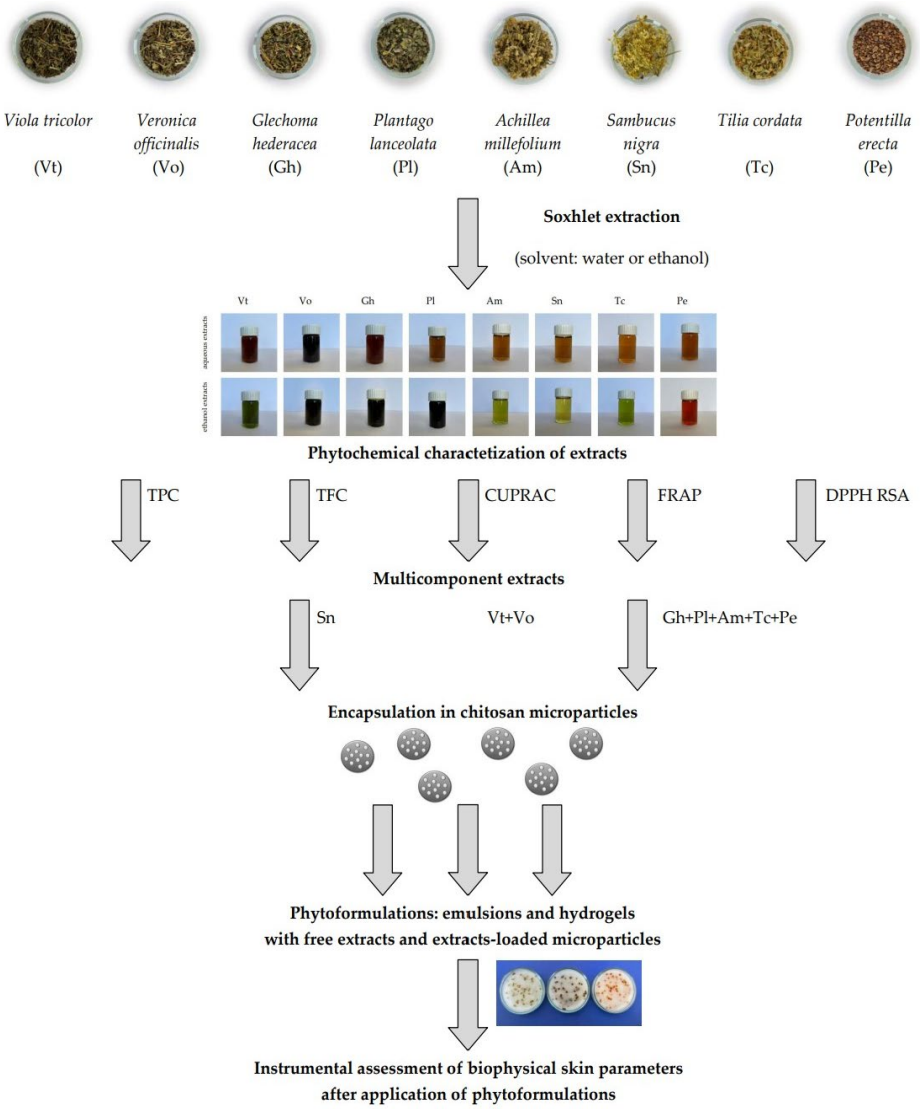


Figure 1. The preparation scheme of extracts, their phytochemical analysis and encapsulation in chitosan microparticles, as well as the preparation of phytoformulations.

This research aimed to develop phytoformulations containing microparticles loaded with plant extracts and to instrumentally assess skin conditions after their topical application (Fig. 1). Herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome: *Potentilla erecta* were extracted using the Soxhlet apparatus with water and ethanol as solvents. In order to formulate a preparation best suited for conditioning the skin, the plant species were selected based on several factors: (I) use in traditional medicine to treat skin disorders, (II) phytochemical composition, and (III) insufficient data on synergistic activity. We characterised their phytochemical profile by the content of polyphenols, flavonoids and antioxidant capacity

(CUPRAC, FRAP and DPPH RSA) individually and in combinations of these extracts. Subsequently, selected extracts were enclosed in chitosan microparticles. The loading capacity and *in vitro* release profile of loaded extracts were examined. Preliminary studies of formulated herbal emulsions and hydrogels were performed on the probands' skin to analyse skin colour, skin surface hydration, and skin barrier quality (manifested as transepidermal water loss—TEWL).

2. Materials and Methods

2.1. Materials

Dry plant raw materials (herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers of *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome of *Potentilla erecta*), tocopherol, *Vitis vinifera* seed oil and *Prunus domestica* seed oil were purchased from herbal wholesaler Nanga (Zlotow, Poland). Folin– Ciocalteu reagent, gallic acid, DPPH (2,2′-diphenyl-1-picrylhydrazyl, free radical), xanthan gum and chitosan (ultra low molecular weight, MW: 20,000 (avg.)) were acquired from Sigma-Aldrich (Poznan, Poland). Sodium carbonate, ethyl alcohol, and paraffinum liquidum were purchased from Stanlab (Lublin, Poland). Sodium phosphate, disodium phosphate, glycerin, and propylene glycol were acquired from Chempur (Piekary Slaskie, Poland). Quercetin, aluminium chloride anhydrous, copper (II) chloride dihydrate, ammonium acetate, neocuproine hemihydrate, iron (III) chloride hexahydrate, 2,4,6-tripyridyl-S thiazine (TPTZ), Trolox® and pentasodium tripolyphosphate (TPP), allantoin, panthenol were supplied by Pol-Aura (Dywity, Poland). Hydrochloric acid, acetic acid, sodium acetate anhydrous and methyl alcohol were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Cetearyl alcohol, caprylic/capric triglicerydes, octyldodecanol, ceteareth-20, isopropyl palmitate, and glyceryl stearate were purchased from CHEMCO (Sobowidz, Poland).

2.2. Extracts Preparation

The plant extraction was conducted using the Soxhlet apparatus. 10 g of each dried plant raw material was extracted using 200 ml water or ethanol as solvents for 3 hours. The extracts from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta* were obtained (Table 1). After characterisation of extract through Total Polyphenols and Flavonoids Content as well as antioxidant activity, aqueous extracts from mixed raw materials were prepared: (I) herbs of *Viola tricolor* + *Veronica officinalis*; (II) herbs of *Glechoma hederacea* + *Plantago lanceolata* + flowers of *Achillea millefolium* + *Tilia cordata* + rhizome of *Potentilla erecta*.

Table 1. Studied medicinal plants.

No	Botanical Name	Popular Name	Abbreviation	Family	Vegetal Part
1	*Viola tricolor*	heartsease, wild pansy	Vt	Violaceae	herb
2	*Veronica officinalis*	heath speedwell, common gypsyweed	Vo	Plantaginaceae	herb

3	*Glechoma hederacea*	ground-ivy	Gh	Lamiaceae	herb
4	*Plantago lanceolata*	ribwort plantain, narrow-leaf plantain	Pl	Plantaginaceae	herb
5	*Achillea millefolium*	yarrow	Am	Asteraceae	flower
6	*Sambucus nigra*	elder, elderberry	Sn	Adoxaceae	flower
7	*Tilia cordata*	small-leaved lime, small-leaved linden	Tc	Malvaceae	flower
8	*Potentilla erecta*	tormentil, septfoil	Pe	Rosaceae	rhizome

2.3. Extracts Characterisation

2.3.1. Total Polyphenols Content (TPC)

Total Polyphenols Content was determined spectrophotometrically using the Folin-Ciocalteu method [41]. Extracts were diluted ten times using extraction solvent (water or ethanol), and then 20 µl of each sample was taken and added to 1.58 ml of distilled water and 100 µl of Folin-Ciocalteu reagent. Subsequently, 300 µl of saturated Na_2CO_3 solution was added to the mixture after 4 minutes and incubated for 40 minutes at 37°C until a characteristic blue colour occurred. The absorbance was measured at a wavelength of 725 nm using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Three measurements were made for each type of extract and calculated based on gallic acid using the standard curve equation in the concentration range of 0–0.50 mg/ml (R = 0.9997). TPC was then expressed as grams of gallic acid equivalents (GAE) per 100 g of dry material weight (DW).

2.3.2. Total Flavonoids Content (TFC)

Total Flavonoids Content was evaluated spectrophotometrically using a method based on forming chelates of Al(III)-flavonoids due to many oxo and hydroxyl groups presented in flavonoids [42]. Each extract was diluted 20 times with extraction solvent (water or ethanol). 800 µl of each extract were added to 80 µl of 5% (v/v) $AlCl_3$ and 1.12 ml of acetic acid and methanol mixture (ratio 1:19). Afterwards, prepared samples were incubated in a dark place for 30 minutes, and the resulting complexes were measured using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at a wavelength of 425 nm. Measurements were performed in triplicate and calculated using the calibration curve for quercetin in the 0-0.025 mg/ml concentration range (R = 0.9997). TFC was demonstrated as a milligrams of quercetin equivalents (QE) per 100 g of dry material weight (DW).

2.3.3. Antioxidant Activity

CUPRAC (CUPric Reducing Antioxidant Capacity) is a method in which the ability to reduce copper (II) ions is tested [43]. Orange–yellow coloured Cu(I)-neocuproine chelate is formed due to the redox reaction between copper-neocuproine and antioxidants, which can be measured via a spectrophotometer. 20 µl of extracts diluted ten times were added to 780 µl of distilled water, 400 µl of 1 M ammonium acetate (pH = 7), 400 µl of 0.01 M $CuCl_2$ and 400 µl of 0.0075 M neocuproine. Obtained mixtures were placed in a dark place for 30 minutes and measured in triplicate at a wavelength of 450 nm using a UV-Vis spectrophotometer (UV-1800,

Shimadzu, Kyoto, Japan). Results were calculated based on the gallic acid calibration curve in the concentration range of 0–0.25 mg/ml (R = 0.9992). CUPRAC results were shown as grams per 100 g of dry material weight (DW).

FRAP (Ferric Reducing Antioxidant Power) measures the ability to reduce iron (III) ions [44]. This method is based on the spectrophotometric measurement of the reduction of the Fe (III)-TPTZ complex (iron-2,4,6-tripyridyl-S thiazine complex) to the Fe (II)-TPTZ complex under the influence of antioxidants. Each extract was diluted ten times, taken out (20 µl) and added to 1.98 ml of a mixture of 0.3 M acetate buffer (pH = 3.6), 0.02 M FeCl3 and 0.01 M TPTZ solution in 0.04 M HCl in ratio 10:1:1, respectively. Samples were incubated in a dark place for 15 minutes. As a result of the reaction, the colourless reagent began to show an intense blue colour measured spectrophotometrically at a wavelength of 593 nm (UV-1800, Shimadzu, Kyoto, Japan). Three measurements were made for each type of extract and calculated based on Trolox® using the standard curve equation in the concentration range of 0–0.25 mg/ml (R = 0.9991). FRAP was expressed as gram per 100 g of dry material weight (DW).

DPPH Radical Scavenging Assay (RSA) was determined using the Brand-Williams method [45] with some modifications [46,47]. Extracts' free radical scavenging activity was analysed by adding 20 µl of each extract to 1.58 ml of ethanol and 400 µl of 300 µM DPPH. Samples were incubated in a dark place for 15 minutes until the discolouration occurred. A control sample served a DPPH solution in ethanol without adding extracts. After incubation, the absorbance was measured spectrophotometrically at a wavelength of 517 nm (UV-1800, Shimadzu, Kyoto, Japan). All of the measurements were replicated three times. The antiradical activity was calculated using the following formula:

$$RSA = (A_{DPPH} - A_{extract})/A_{DPPH} \cdot 100, \quad (1)$$

where A_{DPPH} is the average absorbance of the DPPH solution without adding extracts, and $A_{extract}$ is the average absorbance of the DPPH solution after the addition of tested extracts.

2.4. Microparticles Preparation

Microparticles (MPs) were fabricated via the extrusion method using an encapsulator (B-395 Pro, BÜCHI Labortechnik AG, Flawil, Switzerland) [48]. In order to obtain chitosan microparticles, chitosan in a concentration of 2% (w/w) was dissolved in a solution of 2% (v/v) acetic acid and selected aqueous extracts in a 50:50 ratio. Based on the phytochemical profile, the following aqueous extracts were encapsulated: (I) herbs of *Viola tricolor* + *Veronica officinalis*; (II) herbs of *Glechoma hederacea* + *Plantago lanceolata* + flowers of *Achillea millefolium* + *Tilia cordata* + rhizome of *Potentilla erecta*. The mixture was transferred into the pressure bottle of an encapsulator and forced through a nozzle with a 450 µm diameter. Droplets were separated by an electrical field and shaped by cross-linking in the bath containing 8% (w/w) pentasodium tripolyphosphate (TPP) solution. Collected microparticles were rinsed with distilled water.

2.5. Microparticles Characterization

2.5.1. Morphology and Size

The appearance and sizes of the prepared microparticles were observed by the optical microscope Motic SMZ-171 BLED (Hong Kong, China).

2.5.2. Loading Capacity

The loading capacity (LC) was determined by quantifying the polyphenol content using the Folin–Ciocalteu test [41]. Weighed extract-loaded microparticles were put into 2 ml of 2% (v/v) acetic acid for 1 h, and afterwards, they were centrifuged (10,000 rpm, 10 minutes). The procedure of preparing samples for TPC was reused to evaluate the loading capacity of extracts in microparticles. The absorbance was measured at 725 nm using a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The analyses were performed in triplicate. The presented results were calculated based on the calibration curve for gallic acid as the standard solution and using the following equation [49]:

LC = phenolic concentration (initial – supernatant)/microparticle weigh · 100 (2)

2.5.3. *In vitro* release profile

The microparticles containing plant extracts were weighted (in triplicate), placed in a 12-well polystyrene plate and poured with 2 ml of acetate buffer (pH = 5.4). They were incubated at 37°C for 3 days while the solution was collected after 15 minutes, 30 minutes, 45 minutes, 1h, 1.5h, 2h, 2.5h, 3h, 4h, 5h, 24h, 48h, 72h. The content of polyphenolic compounds was determined by the Folin–Ciocalteu test [41]. The procedure of preparing samples for the loading capacity described above was reused for the *in vitro* release study. The absorbance was measured at 725 nm using a UV–VIS spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The release of extracts from the chitosan microparticles was conducted in triplicate and calculated based on their loading capacity results.

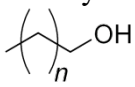
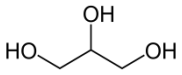
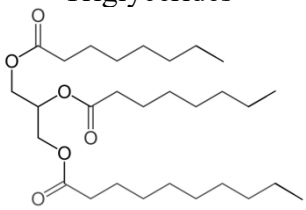
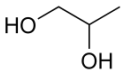
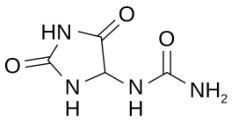
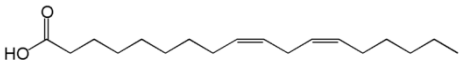
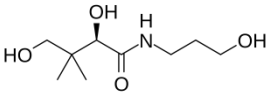
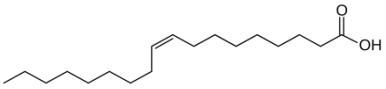
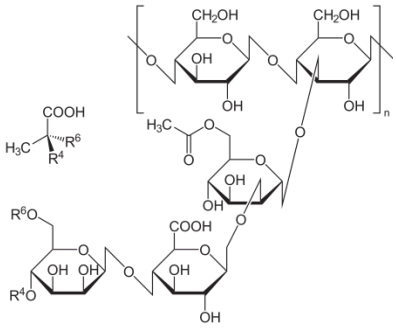
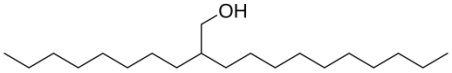
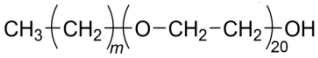
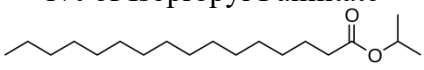
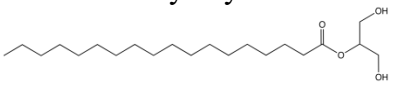
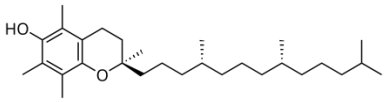
2.6. Dermatological Preparations

Herbal dermatological preparations were formulated as an emulsion (cream) and hydrogel (Table 1). The cream consisted of two phases (oily and aqueous). The oil phase was composed of 7% of cetearyl alcohol, 6% of caprylic/capric triglicerydes, 5% of paraffinum liquidum, 5% of *Vitis vinifera* seed oil, 2% of *Prunus domestica* seed oil, 1.5% of octyldodecanol, 1.1% of ceteareth-20, 1% of isopropyl palmitate, 0.9% of glyceryl stearate. However, the aqueous phase of the cream contained 3% of glycerin, 2% of propylene glycol, 1.5% of allantoin, 1.5% of panthenol, and water up to 100%. In order to prepare the cream, both phases were dissolved and heated to 75-85°C and then combined and mixed with a mechanical stirrer until the formulation cooled down. After cooling down, 0.5% of tocopherol was added to the formulation to prevent its thermal degradation.

Whereas the hydrogel was composed of 3% of glycerin, 2% of propylene glycol, 1.5% of allantoin, 1.5% of panthenol, and 1% of xanthan gum. The components of hydrogel were dissolved in water at room temperature.

Besides the base cream and hydrogel, the preparations containing 5% of aqueous plant extracts and 5% of extract-loaded microparticles were also obtained. They were prepared by the same method as base formulations, except the extracts were added to the mixtures at low temperatures during mechanical stirring along with tocopherol, whereas the microparticles were added to prepared formulations and gently mixed.

Table 1. The composition of prepared cream and hydrogel with chemical structures of added ingrediends.

Cream		Hydrogel
Oil Phase	**Aqueous Phase**	
7% of Cetearyl Alcohol 	3% of Glycerin 	3% of Glycerin
6% of Caprylic/ Capric Triglycerides 	2% of Propylene Glycol 	2% of Propylene Glycol
5% of Paraffinum Liquidum 	1.5% of Allantoin 	1.5% of Allantoin
5% of Vitis Vinifera Seed Oil 	1.5% of Panthenol 	1.5% of Panthenol
2% of Prunus Domestica Seed Oil 	up to 100% Aqua	1% of Xanthan Gum 
1.5% of Octyldodecanol 		
1.1% of Ceteareth-20 		
1% of Isopropyl Palmitate 		up to 100% Aqua
0.9% of Glyceryl Stearate 		
0.5% of Tocopherol 		
The addition of:		
+ 5% of extracts		+ 5% of extracts

+5% of microparticles	+5% of microparticles

2.7. Biophysical Skin Parameters Measurements

Courage + Khazaka probes using MPA software were used to perform preliminary studies of the analysis of the biophysical skin parameters after the application of prepared phytoformulations containing plant extracts [50]. Skin barrier quality (manifested as transepidermal water loss—TEWL), skin surface hydration and skin colour were examined using the tewameter (Tewameter TM 300, Courage + Khazaka, Köln, Germany), corneometer (Corneometer CM 825, Courage + Khazaka, Köln, Germany) and colourimeter (Skin-Colorimeter CL 400, Courage + Khazaka, Köln, Germany), respectively.

Five probands with normal skin (women aged 20–29) participated in the study. Four 4 × 4 cm sections were designated on the volar forearm skin of both arms (Fig. 2). One section served as the control field, and the remaining seven places were covered with different formulations – creams and hydrogels containing plant extracts, plant extract-loaded microparticles, as well as base preparations without extracts. The measurements were performed 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours after applying samples. This test took place in a controlled temperature (20–22°C) and humidity (relative humidity 40–60%).

Before the start of measurements (control) and at each time point, TEWL and skin colour were evaluated in triplicate for each proband. However, corneometric tests require simultaneous testing on the control and treated areas at each point in time. These results show the difference in the corneometer indications (performed five times for each proband at each skin site) between the test field and control field at the appropriate points.

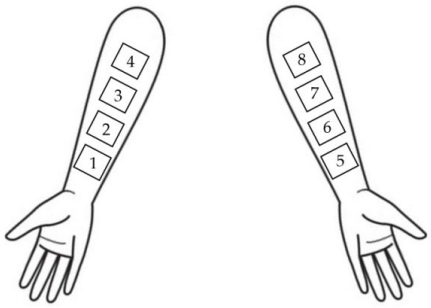


Figure 2. Four application sites (4 x 4 cm) designed on the volar forearm of each arm (1 to 8, including one site serving as a control field for corneometric measurements).

2.8. Statistical Analysis

One-way ANOVA with Tukey's pairwise was performed to statistically compare results. The outcome of the extracts' phytochemical studies (TPC, TFC, CUPRAC, FRAP and DPPH RSA) were compared across all samples extracted with different solvents. The results of skin condition assays (colour and TEWL) for cosmetics were compared to those made for the control field (before the start of the study). Meanwhile, the results of skin hydration were compared to the control samples without herbal preparations. Past 4.09 (PAleontological Statistics Software, Oslo, Norway) was used for all analyses. Data are shown as the mean ± S.D. for each experiment. p-values ≤ 0.05 were considered significant.

3. Results and Discussion

3.1. Extracts Preparation and Characterisation

Figure 3 presents extracts made from Polish herbal plants showing documented healing effects, including herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta* using Soxhlet apparatus with water and ethanol as solvents. Phytochemical characterisation of prepared extracts included the content of polyphenols (TPC), flavonoids (TFC) and antioxidant activity (CUPRAC, FRAP and DPPH) (Table 2).

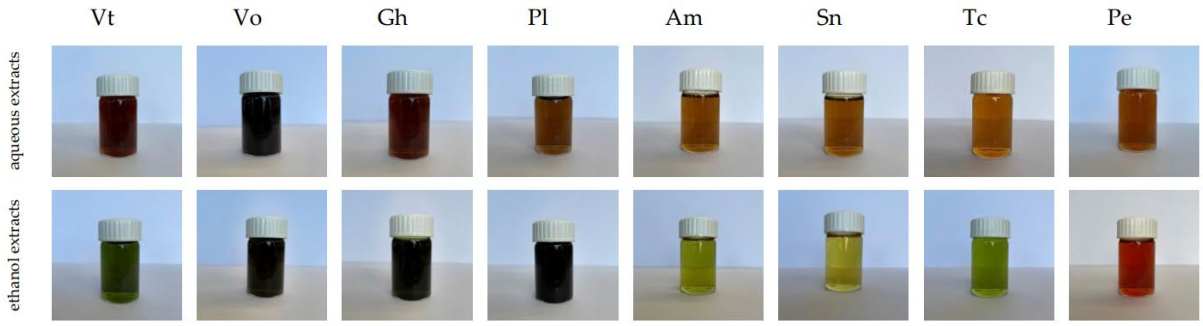


Figure 3. Photographs of prepared aqueous and ethanol extracts from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta*.

Table 2. Results of phytochemical studies – the content of polyphenols, flavonoids and antioxidant activity (CUPRAC, FRAP, DPPH RSA) – of aqueous and ethanol extracts made from herbs: *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers: *Achillea millefolium, Sambucus nigra, Tilia cordata*; and rhizome: *Potentilla erecta*, as well as their mixtures. Different superscript letters in columns presenting the results of different methods for both aqueous and ethanol extracts indicate statistically significant differences ($p \leq 0.05$).

extracts	TPC (g GAE/100g DW)		TFC (mg QE/100 g DW)		CUPRAC (g/100 g DW)		FRAP (g/100 g DW)		DPPH RSA (%)	
	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol	aqueous	ethanol
Vt	0.70 ± 0.06 ^{l}	0.89 ± 0.18 ^{l}	512.2 ± 35.5 ^{i}	651.2 ± 6.7 ^{g,h}	1.17 ± 0.16 ^{k,l,m}	0.92 ± 0.07 ^{m}	2.47 ± 0.45 ^{f,g,h}	1.91 ± 0.08 ^{h}	16.3 ± 4.0 ^{f}	8.6 ± 0.1 ^{f}
Vo	5.75 ± 0.24 ^{b}	4.37 ± 0.23 ^{c}	639.7 ± 27.4 ^{g,h}	979.8 ± 4.5 ^{c}	5.93 ± 0.18 ^{b}	3.71 ± 0.10 ^{c}	6.80 ± 0.43 ^{b}	4.95 ± 0.39 ^{c}	73.6 ± 1.8 ^{a,b}	77.6 ± 3.2 ^{a,b}
Gh	1.61 ± 0.12 ^{j,k}	1.12 ± 0.09 ^{k,l}	376.2 ± 24.8 ^{j}	848.5 ± 3.5 ^{e}	1.74 ± 0.20 ^{h,i,j,k}	1.09 ± 0.07 ^{l,m}	2.99 ± 0.37 ^{e,f}	1.85 ± 0.17 ^{h}	68.1 ± 5.0 ^{b}	31.3 ± 1.5 ^{e}

Pl	1.33 ± 0.10 ^{k,l}	2.24 ± 0.19 ^{h,i,j}	239.2 ± 22.6 ^{k}	1456.5 ± 10.7 ^{a}	1.63 ± 0.32 ^{i,j,k,l}	2.04 ± 0.04 ^{g,h,i}	2.44 ± 0.23 ^{f,g,h}	2.57 ± 0.15 ^{f,g,h}	35.4 ± 3.4 ^{e}	72.2 ± 2.4 $^{a.b}$
Am	3.15 ± 0.04 ^{d,e,f}	1.09 ± 0.06 ^{k,l}	409.2 ± 19.2 ^{j}	430.4 ± 10.1 ^{j}	3.24 ± 0.27 ^{c,d}	1.27 ± 0.10 ^{j,k,l,m}	4.45 ± 0.09 ^{c}	2.19 ± 0.10 ^{g,h}	78.7 ± 1.7 ^{a}	73.6 ± 2.0 $^{a.b}$
Sn	5.35 ± 0.13 ^{b}	2.77 ± 0.18 ^{f,g,h}	1378.3 ± 34.4 ^{b}	916.9 ± 8.8 ^{d}	6.11 ± 0.23 ^{b}	2.28 ± 0.17 ^{f,g,h}	6.10 ± 0.26 ^{b}	3.04 ± 0.17 ^{e,f}	76.3 ± 3.1 $^{a.b}$	75.0 ± 3.6 $^{a.b}$
Tc	3.30 ± 0.03 ^{d,e,f}	2.11 ± 0.30 ^{i,j}	388.7 ± 9.7 ^{j}	597.0 ± 12.8 ^{h}	3.86 ± 0.24 ^{c}	1.46 ± 0.12 ^{i,j,k,l,m}	4.46 ± 0.07 ^{c}	2.81 ± 0.25 ^{f,g}	78.3 ± 2.4 $^{a.b}$	77.3 ± 5.2 $^{a.b}$
Pe	2.38 ± 0.01 ^{g,h,i}	9.41 ± 0.48 ^{a}	136.6 ± 20.7 ^{l}	205.0 ± 1.5 ^{k}	2.85 ± 0.11 ^{d,e,f}	7.31 ± 0.35 ^{a}	4.26 ± 0.21 ^{c,d}	8.29 ± 0.29 ^{a}	78.6 ± 4.2 ^{a}	71.0 ± 2.8 $^{a.b}$
Mixed extracts										
Vt + Vo	3.58 ± 0.24 ^{d,e}	2.97 ± 0.18 ^{e,f,g}	714.1 ± 32.1 ^{f}	610.6 ± 38.3 ^{h}	2.93 ± 0.13 ^{d,e}	2.73 ± 0.19 ^{d,e,f}	3.64 ± 0.11 ^{d,e}	4.54 ± 0.40 ^{c}	47.5 ± 2.3 ^{d}	50.2 ± 3.1 ^{c,d}
Gh + Pl + Am + Tc + Pe	0.85 ± 0.01 ^{l}	3.69 ± 0.45 ^{d}	221.2 ± 12.8 ^{k}	675.1 ± 17.3 ^{f,g}	1.83 ± 0.36 ^{h,i,j}	2.56 ± 0.16 ^{e,f,g}	2.63 ± 0.10 ^{f,g,h}	4.75 ± 0.40 ^{c}	48.7 ± 2.5 ^{c,d}	57.6 ± 4.0 ^{c}

TPC – Total Polyphenols Content; TFC – Total Flavonoids Content; CUPRAC – CUPric Reducing Antioxidant Capacity; FRAP – Ferric Reducing Antioxidant Power; DPPH RSA – DPPH Radical Scavenging Assay

Based on obtained results, we can notice that content of polyphenols, flavonoids, and antioxidant activity significantly depended on the type of used plant, as well as the type of used solvent (Tab. 2). Aqueous and ethanol extracts from *Viola tricolor* showed relatively low content of polyphenols, i.e. 0.70 ± 0.06 g GAE/100 g DW and 0.89 ± 0.18 g GAE/100 g DW, respectively, as well as low antioxidant activity (DPPH was 16.3 ± 4.0% and 8.6 ± 0.1%, respectively). However, the results were inconsistent with those noted in other studies. It can be associated with different extraction methods or solvents and calculations based on different equivalents. Jurca et al. screened three species of the *Violaceae family* (*Viola odorata* L., *Viola tricolor* L. and *Viola wittrockiana* Gams.) in order to assess their polyphenolic contents and antioxidant activities [51]. They extracted flowers with 70% ethanol using a magnetic stirrer and sonicator, which resulted in TPC of 445 mg GAE/100 g DW, TFC of 2.69 mg QE/ml and antioxidant activity: CUPRAC 2.68 mmol Trolox/100 g, FRAP 14.7 g mmol Trolox/ml and

DPPH 74%. They suggested that anthocyanins and phenols presented in *Viola* species flowers play an essential role as antioxidants. Therefore, they can be used as a source of natural antioxidants in pharmaceutical compounds, food processing and human and food medicine. Another study performed by Araújo et al. revealed antioxidant activity, phenolic and flavonoid contents of ethanol extracts of selected edible flowers, including *Viola tricolor* [52]. Extract from *Viola tricolor* had higher polyphenols and flavonoid contents than other studied plants, namely 63.43 mg GAE/g DW and 32.84 mg catechin equivalents (CE)/g DW, respectively.

Veronica officinalis herb extracts prepared with water displayed the highest level of TPC (5.75 ± 0.24 g GAE/100 g DW) and FRAP (6.80 ± 0.43 g/100 g DW), and TFC (639.7 ± 27.4 mg QE/100 g DW) lower only from *Sambucus nigra* extract. The ethanol Vo extract also had a high content of active compounds. TPC (4.37 ± 0.23 g GAE/100 g DW), CUPRAC (3.71 ± 0.10 g/100 g DW) and FRAP (4.95 ± 0.39 g/100 g DW) had high values, lower only from ethanol extract from *Potentilla erecta* and TFC (979.8 ± 4.5 mg QE/100 g DW) lower from ethanol extract from *Plantago lanceolata*. Mocan et al. prepared ethanol extract of three *Veronica* species, including *Veronica officinalis*, in an ultrasonic bath and investigated their chemical composition, antioxidant and antimicrobial effects [12]. Their *Veronica officinalis* extract presented lower than ours TPC values of 32.37 ± 1.27 mg GAE/g DW and TFC (2.52 ± 0.16 mg QE/g DW).

Comparably low content of polyphenols and flavonoids, as well as antioxidant activity, were observed for extracts from *Glechoma hederacea* using both water and ethanol as solvents. TPC were 1.61 ± 0.12 and 1.12 ± 0.09 g GAE/100 g DW, TFC were 376.2 ± 24.8 and 848.5 ± 3.5 mg QE/100 g DW, CUPRAC were 1.74 ± 0.20 and 1.09 ± 0.07 g/100 g DW, FRAP were 2.99 ± 0.37 and 1.85 ± 0.17 g/100 g DW, DPPH RSA were 68.1 ± 5.0% and 31.3 ± 1.5%, respectively for aqueous and ethanol extracts. Our findings were not supported by other research performed by Belščak-Cvitanović et al. [53]. They assessed the aqueous extracts, infusions, macerates and decoctions from several Croatian plants, including *Glechoma hederacea*, against the damaging effects of oxidative stress. They noticed significant differences in polyphenols and flavonoids content related to the used extraction method. Infusions were prepared with warm water (80°C) for a short time (10 minutes), macerates were performed with cold water for a more extended period (72h), whereas decoctions were done with boiling water for 20 minutes. TPC of *Glechoma hederacea* were in a range of 12.45 – 31.82 mg GAE/g DW with a significantly lower value for decoction, which indicated that boiling water may led to phenolic compounds' degradation. TFC ranged from 5.98 mg GAE/g DW for decoction to 20.00 mg GAE/g DW for macerate. Furthermore, FRAP valued from 1.15 mM Fe(II) for macerate to 7.43 mM Fe(II) for extract.

Plantago lanceolata aqueous extract showed a relatively low amount of active substances, i.e. polyphenols content was 1.33 ± 0.10 g GAE/100 g DW, and flavonoids content was 239.2 ± 22.6 mg QE/100 g DW. Their content resulted in antioxidant activity detected via FRAP 2.44 ± 0.23 g/100 g DW, CUPRAC 1.63 ± 0.32 g/100 g DW and DPPH 35.4 ± 3.4%. Moderately higher values of phytochemical analysis were observed for ethanol extracts. TPC was 2.24 ± 0.19 g GAE/100 g DW, whereas TFC had the highest value of all prepared extracts (1456.5 ± 10.7 mg QE/100 g DW). FRAP was similar to the value read for aqueous extract, i.e. 2.57 ± 0.15 g/100 g DW. However, the CUPRAC value was moderate compared to prepared extracts (2.04 ± 0.04 g/100 g DW). DPPH RSA value was similar to other extracts – 72.2 ± 2.4%.

Plantago lanceolata has also been studied by Lukova et al. during the simultaneous extraction of polyphenols and degradation of polysaccharides [54]. TPC after enzymatic hydrolysis was around 40 mg GAE/g DW and DPPH ~70%. They also established its CUPRAC (56 µM Trolox® equivalents (TE)/g DW) and FRAP (92 µM TE/g DW). Jelena Živković et al. obtained *Plantago lanceolata* ethanol extracts using an ultrasonic bath at various temperatures (20-80°C) for different periods (5-65 minutes) [55]. TPC of investigated extracts varied between 24.34 and 42.96 mg GAE/g DW. Moreover, they suggested the following conditions as optimal: extraction time of 64 minutes, ethanol concentration of 45%, solid-to-solvent ratio of 1:49 and extraction temperature of 40°C.

Aqueous extract prepared from *Achillea millefolium* presented moderate values of TPC (3.15 ± 0.04 g GAE/100 g DW), TFC (409.2 ± 19.2 mg QE/100 g DW), CUPRAC (3.24 ± 0.27 g/100 g DW), and FRAP (4.45 ± 0.09 g/100 g DW), whereas the highest value of DPPH (78.7 ± 1.7%). Ethanol appeared to be worse solvent for extraction of *Achillea millefolium* flowers due to relatively low content of extracted polyphenols (1.09 ± 0.06 g GAE/100 g DW) and flavonoids (430.4 ± 10.1 mg QE/100g DW), and hence antioxidant activity: CUPRAC (1.27 ± 0.10 g/100 g DW) and FRAP (2.19 ± 0.10 g/100 g DW). Conversely, DPPH of ethanol extract was high – 73.6 ± 2.0%. Horablaga et al. highlighted the importance of sample preparation and extraction methods on the phytochemical profile of 12 common medicinal plants in Romania, including *Achillea millefolium* [56]. TPC of *Achillea millefolium* extract varied between 14.53 and 52.85 mg GAE/g DW, whereas TFC ranged from 2.50 to 4.27 mg QE/g DW. Antioxidant activity expressed as FRAP were from 6.20 to 43.96 mM Fe^{2+}/100 g. Results depended on the level of plant shredding and extraction method (conventional solvent extraction, ultrasound-assisted extraction and microwave extraction). They noted that values for TPC and antioxidant activity increased significantly with the level of plant shredding (from coarse shredding to fine gridding).

Extracts prepared from *Sambucus nigra* appeared to have a satisfactory level of bioactive compounds compared to other plant extracts. Both water and ethanol were suitable solvents for extraction since their TPC were 5.35 ± 0.13 and 2.77 ± 0.18 g GAE/100 g DW, TFC were 1378.3 ± 34.4 and 916.9 ± 8.8 mg QE/100 g DW, respectively. Antioxidant capacity measured by the CUPRAC method were 6.11 ± 0.23 and 2.28 ± 0.17 g/100 g DW, and by the FRAP method were 6.10 ± 0.26 and 3.04 ± 0.17, while by DPPA RSA were 76.3 ± 3.1 and 75.0 ± 3.6%, respectively for aqueous and ethanol extracts. *Sambucus nigra* was also noticed by other researchers for its beneficial phytochemical characterisation. A study presented by Boroduske et al. compared the phytochemical profile of flower and berries ethanol extracts from cultivated and wild *Sambucus nigra* populations [57]. The highest average TFC of sampled wild *Sambucus nigra* was 9.57 mg rutin equivalents (RE)/g DW for berry extracts and 77.59 mg RE/g DW for flower extracts. Moreover, the highest TPC were 41.31 mg GAE/g DW for berry extracts and 451.72 mg GAE/g DW for flower extracts. Their results indicated that the sampling site significantly affected the phytochemical profile of elderberry fruits and flowers. Another study performed by Viapiana et al. reported slightly lower values for TPC and TFC of *Sambucus nigra* flowers and barriers aqueous infusions [58]. The TPC of berries and flower infusions ranged from 19.81 to 23.90 mg GAE/g DW and from 15.23 to 35.57 mg GAE/g DW, respectively. Meanwhile, the TFC of infusions ranged from 2.60 to 4.49 mg RE/g DW in elderberry and from 5.27 to 13.19 mg RUTE/g DW in elderflower. Furthermore, they assessed

the antioxidant potential of the *Sambucus nigra* infusions via DPPH and FRAP assays. It revealed that the infusions prepared from flowers had higher values of DPPH and FRAP, which correlated with higher TPC and TFC. Another study reported a TPC value of 43 mg GAE/g DW of methanol elderberry fruit extract, a TFC value of 15 mg RE/g DW, and a DPPH of 62.56% [59].

Both aqueous and ethanol *Tilia cordata* extracts had moderate phytochemical profiles compared to other extracts, i.e. TPC were 3.30 ± 0.03 and 2.11 ± 0.30 g GAE/100 g DW, and TFC were 388.7 ± 9.7 and 597.0 ± 12.8 mg QE/100 g DW, respectively. Antioxidant activity evaluated by CUPRAC were 3.86 ± 0.24 and 1.46 ± 0.12 g/100 g DW, whereas FRAP were 4.46 ± 0.07 and 2.81 ± 0.25 g/100 g DW, respectively for aqueous and ethanol extracts. On the contrary, these extracts had high antioxidant activity measured via DPPH RSA (~77-78%). A study presented by Kosakowska et al. compared the content of phenolic acids and flavonoids in extracts from *Tilia cordata* flowers collected from different sites in Poland [20]. The mean phenolic acid content was 0.96 g/100 g DW (it varied from 0.17 to 1.87 g/100g DW), whereas TFC – 0.20 g/100 g DW (from 0.09 to 0.52 g/100 g DW). The phytochemical composition differed significantly depending on the site of small-lived lime populations; however, they did not observe a clear relationship between geographical localisation and the content of active compounds in flowers.

Potentilla erecta ethanol extract had the highest polyphenols content of all prepared extracts (9.41 ± 0.48 g GAE/100 g DW) and antioxidant properties (CUPRAC: 7.31 ± 0.35 g/100 g DW; FRAP: 8.29 ± 0.29 g/100 g DW). Extracts prepared using both water and ethanol as solvents had a high value of DPPH RSA (~71-79%). Based on TPC (2.38 ± 0.01 g GAE/100 g DW), TFC (136.6 ± 20.7 mg QE/100 g DW) and CUPRAC (2.85 ± 0.11 g/100 g DW) results, we can conclude that water was worse solvent for extraction of *Potentilla erecta.* Furthermore, it contained the lowest content of flavonoids regardless of the extraction solvent type. Dróżdż et al. reported that the content of polyphenolics decreases with increasing concentration of alcohol above 60% [60]. They prepared *Potentilla erecta* rhizome extracts using water and ethanol-water (60:40) as solvents due to the higher solubility of active compounds in an alcoholic environment. TPC was higher when extracted with 60% ethanol (111 ± 2.8 mg GA/g) than hot water (74.2 ± 1.90 mg GA/g). Another study performed by Tomczyk et al. revealed that aqueous extract from selected *Potentilla* species, including *Potentilla erecta* herb, had high concentrations of polyphenols such as tannins and phenolic acids, as well as flavonoids (TPC 69.3 mg GAE/g DW and TFC 2.5 mg QE/g DW) [61].

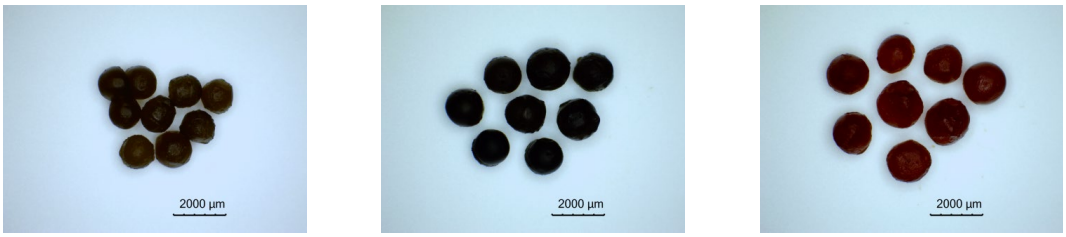
According to these findings, it can be concluded that ethanol is the preferable extraction medium for flavonoids (except *Sambucus nigra* flowers). More polyphenols were extracted with water from *Veronica officinalis, Glechoma hederacea, Achillea millefolium, Sambucus nigra* and *Tilia cordata,* whereas ethanol extracted more polyphenols from *Plantago lanceolata* and *Potentilla erecta*. In the case of antioxidant activity measured by CUPRAC and FRAP methods, more preferable was water for *Veronica officinalis, Glechoma hederacea, Achillea millefolium, Sambucus nigra, Tilia cordata*, while ethanol for *Potentilla erecta.* Higher values for DPPH RSA presented water for *Viola tricolor, Veronica officinalis, Glechoma hederacea, and Achillea millefolium*, while ethanol for *Plantago lanceolata*. Therefore, ethanol turned out to be the preferable solvent for extracting active compounds from *Plantago lanceolata* and *Potentilla erecta*, whereas water – for the rest of the plants.

In this study, we also obtained extracts by mixing plants showing different content of active compounds. Mixing extracts with a high phytochemical content separately led to obtaining new extracts with moderate TPC, TFC and antioxidant activity. Based on the outcome, the obtained multicomponent extracts showed an additive effect. Therefore, in the next steps of this research, extracts from (I) the *Sambucus nigra* flowers; and mixtures from (II) herbs of *Viola tricolor* + *Veronica officinalis*; and (III) herbs *Glechoma hederacea* + *Plantago lanceolata* and flowers of *Achillea millefolium* + *Tilia cordata* and rhizome of *Potentilla erecta* were used.

Other researchers also searched for a synergistic effect of a wide range of bioactives responsible for antioxidant capacity and, hence, potential health benefits. Maleš et al. characterised the antioxidant activity of sage (Salvia officinalisL.), wild thyme (Thymus serpyllumL.) and laurel (Laurus nobilisL.) aqueous extracts and their two- and three-component mixtures [62]. They revealed that individual plant extracts possessed a high content of polyphenolic compounds. However, most two- and three-component extracts have been shown to have additive effects. Moreover, three-component mixtures contained lower TPC than two-component mixtures. Other researchers also did not find synergistic effect of ethanol and water extracts prepared from twelve plants and their combination (*Rosa damascena*, *Viola odorata*, *Matricaria chamomilla*, *Althaaea officinalis*, *Melissa officinalis*, *Elaeagnus angustifolia*, *Foeniculum vulgare*, *Pimpinella anisum*, *Saccharum officinarum*, *Prunus dulcis*, and *Zea mays*) [63]. Manham et al. noticed no additional effect of herbal blends over individual plants – their results for TPC and antioxidant activity fell in the mid-range. It is tough to find a synergistic effect of polyherbal blends. Therefore, it is not always best to have numerous extracts in products intended for skin than one carefully selected.

3.2. Microparticles Characterization

Aqueous extracts from (I) *Sambucus nigra*; and mixtures from (II) *Viola tricolor* + *Veronica officinalis*; and (III) *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* were selected in order to be enclosed in chitosan microparticles due to the lack of potential irritating properties to the skin compared to ethanol. Figure 4 shows the resulting extract-loaded microparticles. They had repeatable size, smooth surface and spherical shape, which minimised the possibility of skin deterioration after applying them to the skin. Fabricated microparticles had a soft polymeric matrix enabling the release of enclosed extracts during their spreading to the skin (mechanical release). The preparation of polymeric microparticles was also studied by other researchers who reported obtaining smooth and spherical chitosan microparticles that can be applied in cosmetics to alleviate skin irritation [64,65].



(a) **(b)** **(c)**

Figure 4. Chitosan microparticles with enclosed aqueous extracts from: (**a**) *Sambucus nigra*; (**b**) *Viola tricolor* + *Veronica officinalis*; (**c**) *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta*.

The formation of chitosan-based microparticles relied on ionic gelation, a widely used method for encapsulating bioactive compounds due to its mild processing conditions. Chitosan is a positively charged, naturally derived polysaccharide containing amino groups ($–NH_2$), which become protonated ($–NH_3^+$) in acidic environments (such as acetic acid). TPP is a multivalent anionic cross-linker composed of phosphate groups linked by oxygen bridges. When chitosan droplets came into contact with the TPP solution, ionic interactions occurred between the protonated amino groups of chitosan and the negatively charged phosphate groups of TPP [66–68]. This led to the formation of a cross-linked network at the droplet surface, stabilizing the structure into spherical microparticles (Fig. 5). Furthermore, the hydroxyl groups (–OH) of the chitosan's glucosamine units might stabilized and enhanced the integrity of the network through hydrogen bonding with nearby chitosan chains or oxygen atoms in phosphate groups (non-bridging $–O^-$ or =O) of TPP [69]. The encapsulated bioactive compounds were thereby entrapped within the polymer matrix. Prepared plant extracts – rich in phenolic compounds, flavonoids, organic acids, and other bioactive constituents – may interact with chitosan through hydrogen bonding and electrostatic interactions [70,71]. Such interactions could partially compete with TPP for the protonated amino groups ($–NH_3^+$) of chitosan, potentially altering the structure of the polymeric matrix.

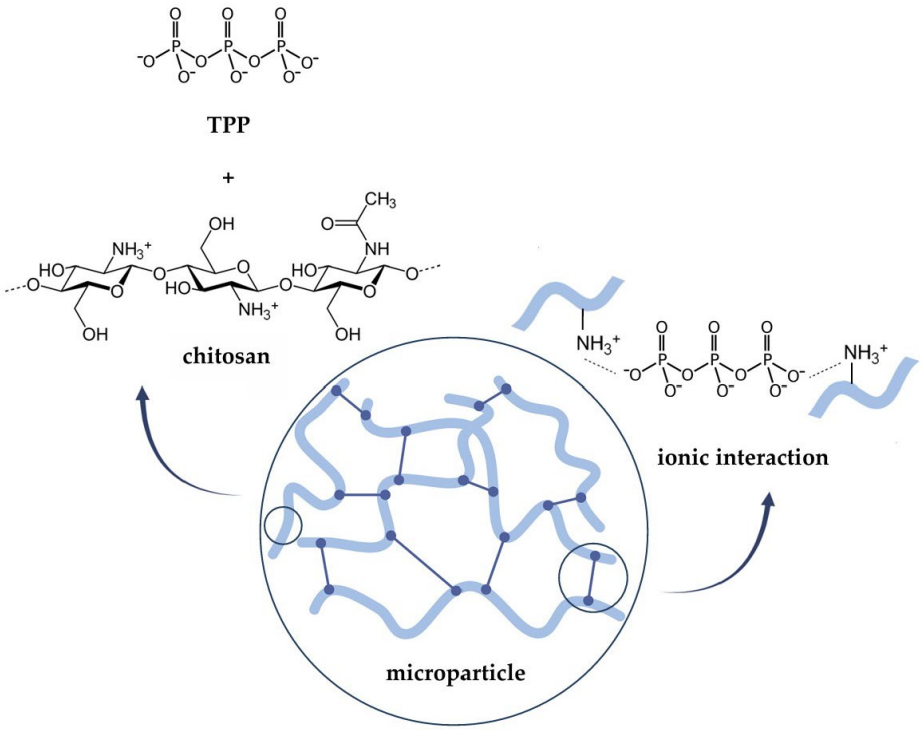


Figure 5. Scheme illustrating the formation of microparticles through cross-linking of chitosan with TPP.

We determined the loading capacity of aqueous extracts enclosed in microparticles. The highest loading capacity was noted for microparticles containing *Sambucus nigra* flower extract (71.4 ± 6.3%). Significantly lower loading capacity was observed for microparticles with the extract from herbs of *Viola tricolor* and *Veronica officinalis* (55.4 ± 2.3%), whereas for microparticles containing Gh + Pl + Am + Tc + Pe extract loading capacity was the lowest – 13.7 ± 0.7%. Based on these results, it can be concluded that plant extracts have been successfully enclosed in chitosan microparticles. The loading capacity of plant extracts in chitosan microparticles is a subject of numerous studies. Other researchers reported loading capacity on different levels: 4.9% for red ginger oleoresin extract [49]; ~13.8-20.7% for pomegranate peel extract [72]; ~70-90% for extracts prepared from *L. cuneifolia, L. divaricata, L. nitida, Z. punctata* and *T. andina* [73].

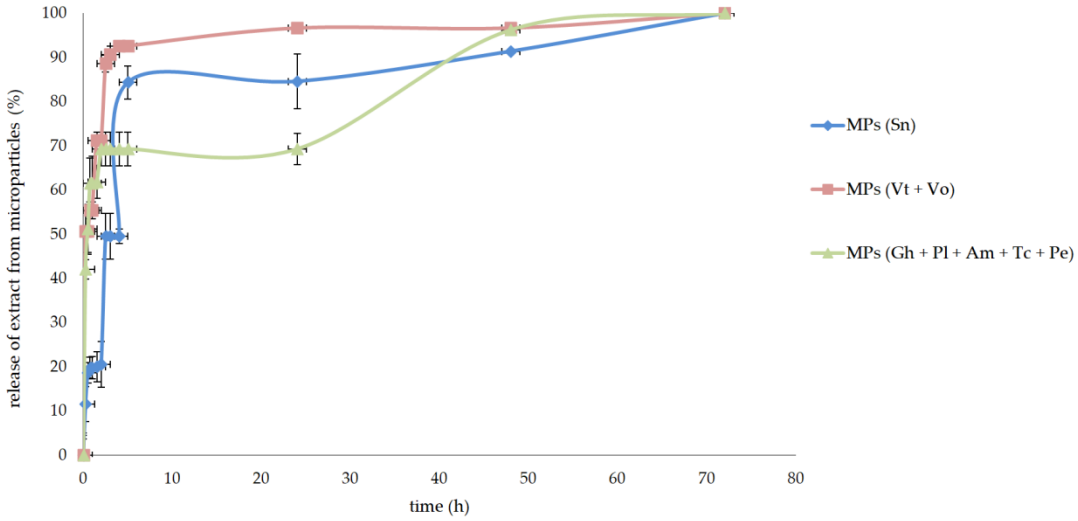


Figure 6. *In vitro* release profile of aqueous *Sambucus nigra* (Sn); *Viola tricolor* + *Veronica officinalis* (Vt + Vo); *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* (Gh + Pl + Am + Tc + Pe) extracts from chitosan microparticles.

In vitro release profiles of extracts from microparticles in acetate buffer (pH = 5.4, 37°C) were also established (Fig. 6). We observed that the extracts were released from the microparticles within three days and occurred in two main stages. The first one lasted 5 hours, and during that time, approximately 70-90% of loaded plant extracts were released from the microparticles with a relatively high release rate. This initial burst release is frequently linked to the presence of active compounds from the outer layer of microparticles, which allows a quick release during the swelling of polymeric microparticles in the release medium [74]. Dissolving the extract in the acidic solution during the preparation of microparticles could also facilitate the initial burst release owing to the better accessibility of some polyphenols in the acidic release medium. In contrast, a sustained release rate characterised the second stage, where bioactive substances must diffuse through the polymeric network. The plant extracts within the chitosan matrix could stabilise formed microparticles, delaying the disintegration of the polysaccharide [75]. The intertwining of polysaccharide chains with the polyphenol molecules and, thus, stabilisation of this system has been attributed to physical interactions, as

well as covalent and hydrogen bonding between both components [76–78]. The release profile was studied in static conditions; however, a mechanical mechanism would apply during the spreading of microparticles to the skin (disruption of chitosan matrix), which fastens the release rate of active substances.

These findings were in agreement with studies performed by other research groups. Moreno et al. obtained chitosan microparticles through electrospraying containing extracts from five medicinal plants (*L. cuneifolia, L. divaricata, L. nitida, Z. punctata* and *T. andina*) [73]. They also established these extracts' *in vitro* release profile (pH = 4.5, 37°C), which had a similar release profile to ours. The rapid release of phenolic compounds occurred in the first 4-5 hours, during which more than 80% of extracts were released. Probably, it was accelerated by the hydration of chitosan microparticles in an aqueous medium. The initial burst release was followed by the constant release rate for the next 12 hours. Nguyen et al. performed release study in pH 1.2, 4.5, 6.8 and 7.8 of carrageenan/chitosan/α–mangostin (xanthone derivative compound extracted from the pericarps of tropical fruit Mangosteen) microparticles [79]. The release depended on the pH of the release medium with better release in a more acidic solution, which was also found by other researchers [80]. They established that active substance was distributed on the surface and inside microparticles. Therefore, an initial burst was observed within the first 2 hours of analysis, followed by a slow release rate due to the release of extracts linked with the polymer matrix. After around six hours, approximately 40-100% of the active substance was released.

3.3. Dermatological preparations

Dermatological formulations in the form of creams and hydrogels containing free extracts and extract-loaded microparticles were obtained, as well as control samples without herbal preparations (Fig. 7). Obtained products were homogenous. Added extract influenced the colour of phytoformulations. Moreover, added microparticles were uniformly distributed through the emulsion and hydrogel. The topical spreading of phytoformulations with microparticles caused the mechanical release of enclosed extracts due to the disruption of soft polymeric matrices.

The structural network of the hydrogel was primarily established by xanthan gum – a polysaccharide composed of 1,4-β-d-glucose backbone with trisaccharide side chains (consisting of mannose–glucuronic acid–mannose) attached to every other glucose residue. Upon hydration, xanthan gum formed a stable three-dimensional matrix through the creation of double-helix structures stabilized by intermolecular interactions [81]. These helices arose from ordered regions within the polymer, where two xanthan chains aligned and twisted together, supported by hydrogen bonding between hydroxyl and carboxyl groups [82]. This helical organization might be accompanied by the physical entanglement of polymer chains and the formation of a gel network. The hydrogel structure was further reinforced by hydrogen bonds formed with water molecules and other hydrophilic components presented in the formulation, including polyols (glycerin, propylene glycol), panthenol, and allantoin [83]. Glycerin and propylene glycol functioned as humectants, enhancing water retention within the hydrogel by forming hydrogen bonds with water molecules and interacting with the hydroxyl groups of xanthan gum. Allantoin, as a humectant, could also participate in weak hydrogen bonding with

hydroxyl groups present in the matrix, potentially aiding in the stabilization of the gel structure. Panthenol might interacted with the aqueous environment and the polymer matrix via hydrogen bonding and van der Waals interactions, contributing to the gel's moisturizing capacity and elasticity. Collectively, these components may interact synergistically to form a hydrogel network, capable of both retaining moisture and delivering bioactive compounds effectively across the skin barrier.

Whereas, oil-in-water (O/W) emulsions were formed through the action of glyceryl stearate and ceteareth-20, which served as non-ionic emulsifiers. These agents stabilized dispersed oil droplets by reducing interfacial tension and forming a coherent interfacial film at the oil-water interface. Cetearyl alcohol functioned as both an emulsion stabilizer and a rheology modifier, contributing to the overall viscosity, consistency, and sensory profile of creams. Caprylic/capric triglycerides and isopropyl palmitate further acted as rheology modifiers, improving the spreadability, texture, and functional properties of the emulsion. Octyldodecanol contributed to emulsion stability and enhanced application properties by promoting uniform distribution on the skin. The aqueous phase, containing humectants such as glycerin, propylene glycol, panthenol, and allantoin, ensured moisture retention. Emulsion stabilization might be achieved through a combination of steric stabilization by non-ionic surfactants, hydrogen bonding within the aqueous matrix, and viscous structuring provided by fatty alcohols.

Phytochemicals present in plant extracts – such as flavonoids, phenolic acids, tannins, alkaloids, and polysaccharides – could significantly influence the physicochemical properties, structural integrity, and functional performance of polymer-based systems. These bioactive compounds may possess multiple functional groups (e.g., –OH, –COOH, $–NH_2$), which allows them to participate in non-covalent interactions with the polymer matrix, including hydrogen bonding with polymer chains (e.g., chitosan or xanthan gum), or electrostatic interactions with charged groups, such as the protonated amino groups ($–NH_3^+$) of chitosan. Therefore, the system's stability and functionality may also be influenced by the incorporation of plant-derived compounds.

Other researchers also investigated the possibility of using chitosan microparticles containing plant extract added to emulsions for cosmetic purposes. Mazutti et al. prepared formulations for topical treatment of cutaneous infections [84]. 5% of chitosan microparticles loaded with *Eugenia dysenterica* leaves extract (EDA) were added to a stable emulsion containing Polawax® Wax, mineral oil, butylhydroxyethyloluene, EDTA, methylparaben, propylparaben, and glycerin. Their study confirmed the possibility of emulsion containing prepared extract encapsulated in chitosan microparticles as a suitable vehicle for topical administration of EDA for treating infections caused by *S. aureus*. Furthermore, Acosta et al. used chitosan microspheres to enclose olive leaf extract [85]. They prepared three o/w and w/o emulsions containing eucalyptus water, roses water, olive oil, calendula oil, jojoba oil, avocado oil, olivem® 1000, sodium stearoyl lactylate, beeswax, glycerol stearate, xanthan gum, shea butter, candy dye, chlorophyll dye, and 0.2% of chitosan microspheres. Based on the extract's suitable *in vitro* release profile, they stated that these microspheres may be applied in cosmetic moisturisers.

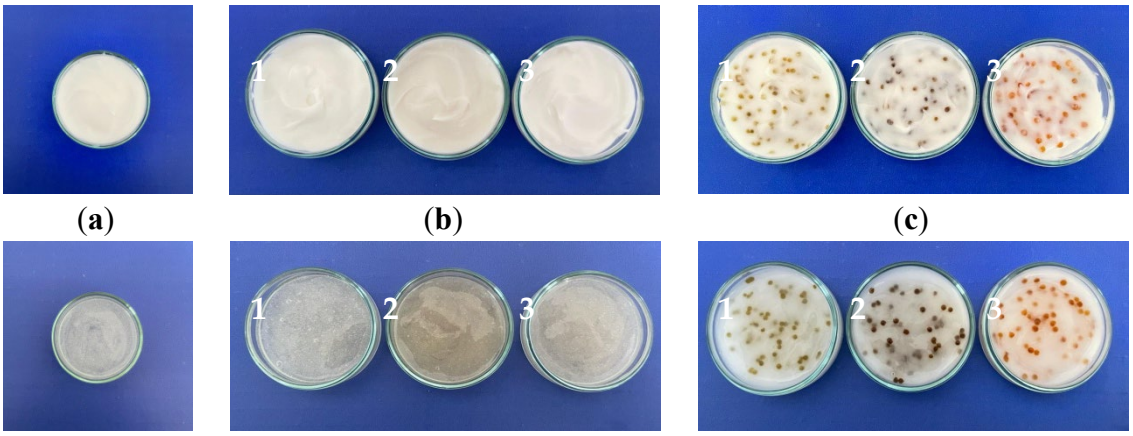


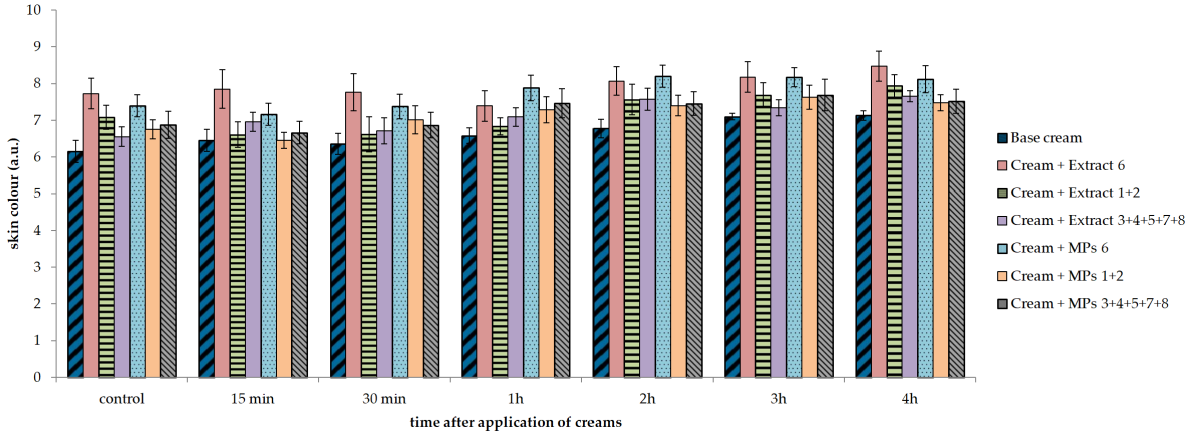
Figure 7. The pictures of prepared base samples: (**a**) cream and (**d**) hydrogel, as well as creams with the addition of (**b**) extracts and (**c**) microparticles, and hydrogels with the addition of (**e**) extracts and (**f**) microparticles containing: (**1**) Sn; (**2**) Vt + Vo; (**3**) Gh + Pl + Am + Tc + Pe.

3.4. Biophysical Skin Parameters

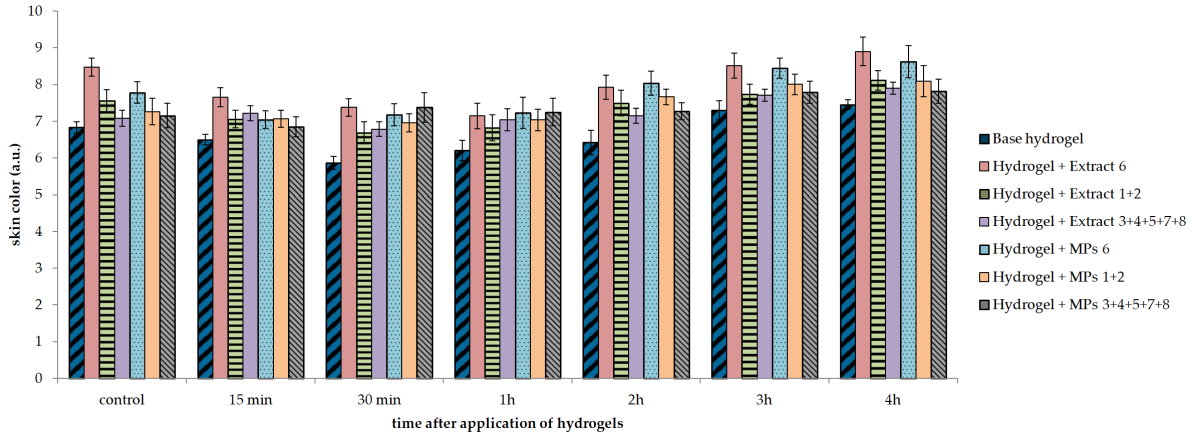
The biophysical skin parameters – skin colour, skin surface hydration and skin barrier quality – were examined using Courage + Khazaka probes. The results of preliminary studies of these analyses after application to the skin of herbal-based preparations are shown in Figures 8–10.

Colour space L*a*b* coordinates express the values of skin colour measurements. Skin brightness is expressed as L*, whereas the location of measured values on the red-green axis is expressed as a* and on the blue-yellow axis – b*. Skin pigmentation is described by b* coordinate, while skin redness, microcirculation and erythema – by a*. Therefore, only a* parameter was considered in this study because prepared herbal dermatological products were not designed to affect the skin's brightness and pigmentation.

Preliminary to the application of products to the skin, control measurements were conducted. Samples were then applied and left to absorb to the skin. The colourimetric indications after applying dermatological preparations to the skin were compared to the control field (skin areas before the test) to assess significant changes in skin redness before and after the application of preparations to the skin. Due to its complex composition, plant extracts may cause adverse skin effects, such as allergic and irritant contact dermatitis [86]. As seen in Figure 8, the application of obtained herbal preparations did not damage or irritate the skin or cause a statistically significant change in skin redness (erythema).



(**a**)

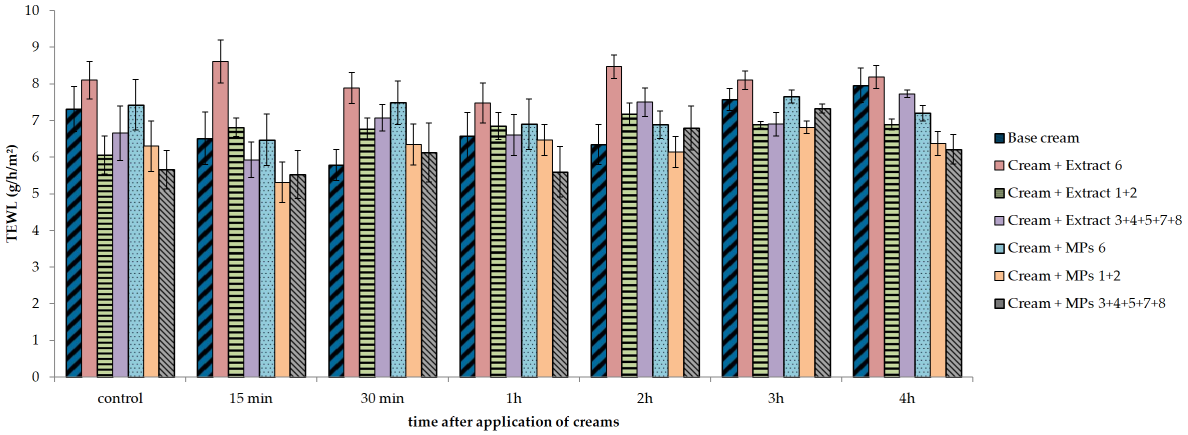


(**b**)

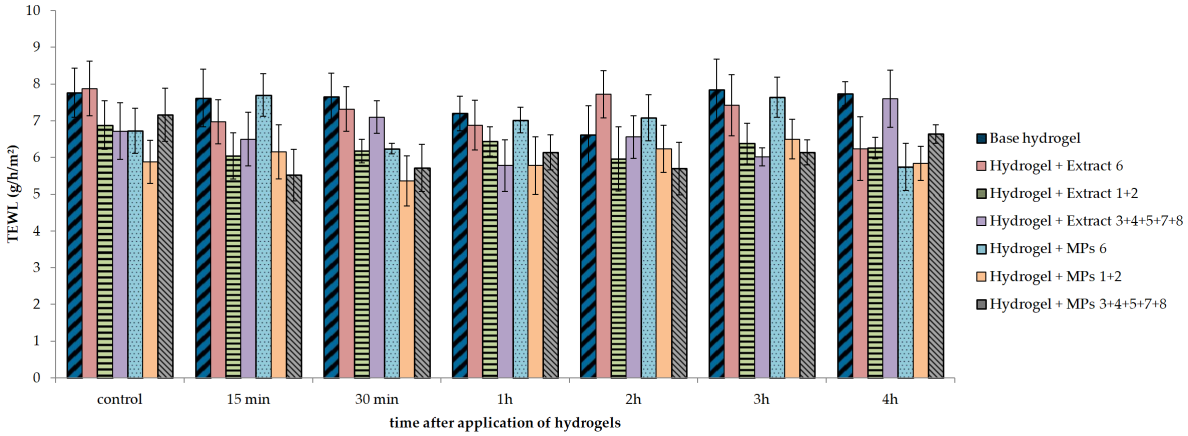
Figure 8. Colourimetric measurements of skin before (control) and after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

Water constantly evaporating from the human skin is an integral part of the body's metabolism. The level of transepidermal water loss (TEWL) indicates the permeability barrier function of skin since even slight damage to the barrier function causes a rise in the water loss. Therefore, this parameter is essential to evaluate the efficiency of topical applied products. Tewametric measurements after 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours of application of the herbal preparations were compared to the results taken for the skin area with applied base samples.

Based on the obtained outcome (Fig. 9), we can conclude that the application of prepared dermatological preparation did not cause deterioration of the epidermal permeability barrier function, and, hence, skin barrier integrity was maintained. Tewameter indicators have not significantly changed throughout this study, resulting in the TEWL values maintaining a stable level (approximately 6-7 g/h/m^2). Therefore, obtained phytoformulations showed slight occlusive effects, preventing the skin's moisture from evaporating in short-term experiments.



(**a**)



(**b**)

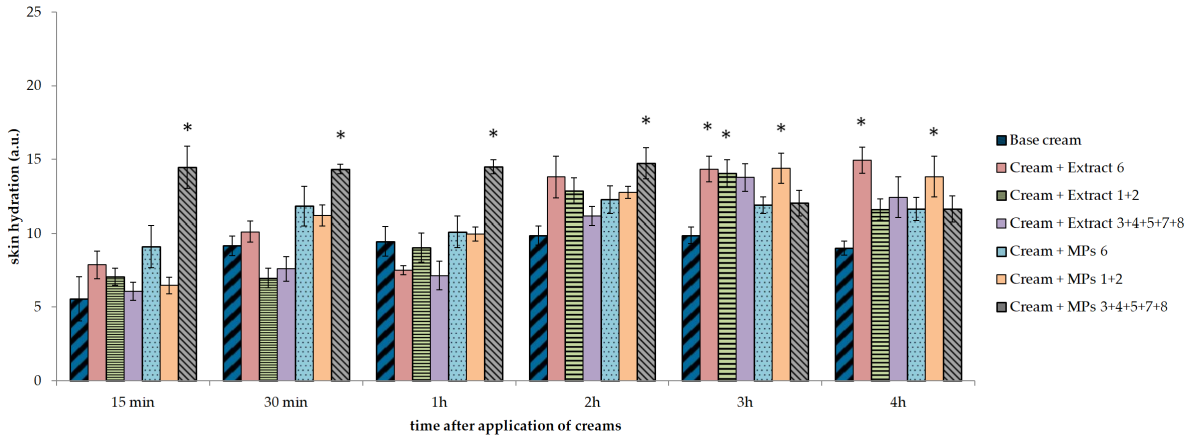
Figure 9. Tewametric measurements of skin before (control) and after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. * indicates a difference at $p < 0.05$ between the results at an appropriate time compared to those made for the control field.

The *stratum corneum* hydration indirectly determines the skin moisture. The coreometer measurement depth is minimal, reaching solely to the *stratum corneum* to eliminate the impact of deeper skin layers. Moreover, due to electrical capacitance measurement, substances on the skin, such as salts or residues of topically applied products, have minimal influence on the corneometer indications. Skin surface hydration was measured before and then 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours and 4 hours after application of dermatological preparations. The results presented are the difference measurements between the test field and control field (non-treated by the products) at each time point (Fig. 10).

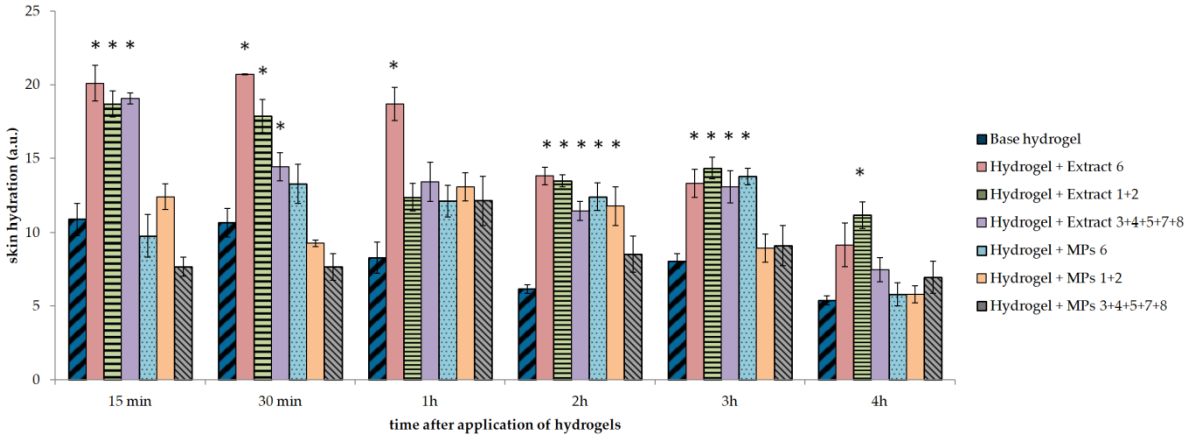
One can see that the application of all samples led to higher skin water content detected in the outermost skin layer. However, it did not occur in the same way for creams and hydrogel. After the application of creams, the corneometer indications were lower (~7 a.u.), and they increased over time (~12 a.u.). However, the sample containing microparticles with a loaded extract from *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* resulted in significantly higher skin hydration values (~14.5 a.u.). After 3 and 4 hours, emulsions containing elderflower extract (~15 a.u.) and microparticles with

Viola tricolor + *Veronica officinalis* extract (~14 a.u.) had significantly higher corneometric values compared to the control sample (base cream) application (~9 a.u.). In contrast, the skin hydration level was higher after applying hydrogels and decreased during the measurements. The introduction of extracts to the hydrogels increased its short-term skin hydration efficacy. Hydrogels containing all three extracts added directly to the hydrogel caused the highest rise in skin hydration values (~19 a.u.). However, over time, it decreased but remained higher (~13 a.u.) than for the base hydrogel application site (~7 a.u.). Therefore, phytoformulations prolonged skin hydration to at least 4 hours in a single application.

Plant extracts are a source of numerous substances that may moisturise the skin. Prepared extracts were rich in polyphenols and flavonoids, containing several hydroxyl groups within their molecules that can form a hydrogen bond with water, thus binding water in the epidermis [87]. This contributed to herbal creams and hydrogels' higher skin hydration properties than base formulations.



(**a**)



(**b**)

Figure 10. Corneometric skin measurements after topical application of prepared (**a**) creams and (**b**) hydrogels with and without extracts and extract-loaded microparticles. The results show differences in the corneometer indications between the test field and control field at the appropriate point. * indicates a difference at $p < 0.05$ between the results of tested samples compared to the base (**a**) cream and (**b**) hydrogel.

Provided benefits on the skin conditioning result not only from prepared extracts but also from the entire composition of formulations. The emulsion contains a mixture of emollients (cetearyl alcohol, caprylic/ capric triglicerydes, paraffinum liquidum, *Vitis vinifera* seed oil, *Prunus domestica* seed oil, octyldodecanol, isopropyl palmitate, glyceryl stearate) creating a temporary occlusive layer on the skin surface, which prevents excessive evaporation of water from the surface (providing an indirect moisturising effect), thereby conditioning the skin [88]. Tocopherol (vitamin E) has the ability to be incorporated into the lipid structures of cell membranes and the intercellular cement of the *stratum corneum*, thus strengthening the epidermal barrier [89]. Strengthening the epidermal barrier not only hinders the penetration of foreign substances and prevents irritation but also inhibits TEWL, thus improving skin hydration. These emulsions also contained humectants (glycerin, propylene glycol) that attract water, which is subsequently occluded by the film formed by emollients. Glycerin and propylene glycol, presented in all creams and hydrogels, are hydrophilic moisturising substances. Moreover, they have the ability to penetrate the *stratum corneum*, thanks to which they act as a penetration enhancer - thus facilitating the transport of other substances deep into the deeper skin layers [90]. Another hydrophilic moisturising substance – allantoin – provides soothing, irritation-relieving, anti-inflammatory effects and supports skin regeneration properties [91]. Panthenol also has an anti-inflammatory effect, accelerates the regeneration processes of the epidermis, soothes irritations and moisturises. Furthermore, it penetrates well into the epidermis, transforming into biologically active vitamin B5, which activates the division of epidermis and dermis cells and stimulates cell renewal [92].

Other studies applied similar methods to assess the effect of topical application of plant extract formulations to the skin using Courage + Khazaka skin probes. *Castanea sativa* leaf ethanol:water (7:3) extract was reported to positively impact the skin barrier integrity [93]. Application of creams containing different plant extracts, such as *Curcuma* and *Acerola* fruit extracts [94], olive leaf extract [95], *Hydrangea serrata* extract [96], *Centella asiatica, Momordica cochinchinensis* and *Phyllanthus emblica* extracts [97], led to increase in corneometric and decrease in tewametric indications and, hence, improving skin condition. However, serum with raspberry leaf cell culture extract, potentially hydrating and moisturising skin, did not cause a significant improvement in skin moisture or TEWL [98]. Hydrogel based on hydroxyethylcellulose with *Cannabis sativa* L. herb extract positively affected skin condition, indicating the protective effect against water loss from the epidermis during a short-time study performed on forearm skin [99]. De Melo et al. evaluated the impact on skin parameters of the immediate film-forming effect of hydrogel and cream containing *Kappaphycus alvarezii* and *Caesalpinia spinosa* extracts [100]. Their results showed that film formed on the skin surface reduced TEWL and improved *stratum corneum* on forearm skin after 1 hour of application. Moreover, their extracts revealed more pronounced effects when added to the emulsion formulation instead of a gel.

Emulsion containing chitosan microparticles loaded with *Eugenia dysenterica* leaves extract was also obtained by Mazutti et al. [84]. They additionally performed *in vitro* skin penetration using Franz diffusion cells on the skin from porcine ears. It was established that active substance was delivered to the *stratum corneum* and the deeper skin layers. Chitosan has been reported to provide an enhancer effect by secondary structure changes of keratin and water content in *stratum corneum* [101]. However, the emulsion formulation loaded with chitosan

microparticles significantly increased the amount of active substance that penetrated the deeper skin layers, which could be ascribed to the effect of the emulsifier presented in the emulsion on the *stratum corneum* lipid arrangements [102,103].

5. Conclusions

In this research, we obtained aqueous and ethanol extracts from eight medicinal plants common in Poland (herbs of *Viola tricolor, Veronica officinalis, Glechoma hederacea, Plantago lanceolata*; flowers of *Achillea millefolium, Sambucus nigra, Tilia cordata*; rhizome of *Potentilla erecta*), as well as their blends. We noticed that aqueous extract from *Sambucus nigra* flowers (elderflower) and aqueous and ethanol extracts from *Veronica officinalis* herbs showed the highest level of TPC, TFC and antioxidant properties. Moreover, *Potentilla erecta* ethanol extract had the highest polyphenols content and antioxidant properties but the lowest content of flavonoids regardless of the extraction solvent type. Selected extracts with established phytochemical profiles (TPC, TFC, antioxidant activity: CUPRAC, FRAP, DPPH RSA) were successfully enclosed in chitosan microparticles with loading capacity ranging from 13.7 to 71.4%. The *in vitro* release profile of extract from microparticles revealed initial burst release lasting 5 hours, followed by a sustained release rate of up to 3 days. Afterwards, dermatological preparations in the form of emulsion and hydrogel containing plant extracts and chitosan microparticles loaded with extracts were obtained. Prepared extracts were protected in chitosan microparticles and mechanically released during the spreading to the skin. Preliminary studies of biophysical skin parameters were instrumentally conducted after the application of phytoformulations. Applying herbal preparations did not cause skin irritation or disintegrity of skin barrier function, but it led to higher hydration of the outermost skin layer. Emulsion with microparticles containing *Glechoma hederacea* + *Plantago lanceolata* + *Achillea millefolium* + *Tilia cordata* + *Potentilla erecta* aqueous extract exhibited the best short-term properties of *stratum corneum* hydration. In terms of hydrogels – the sample containing free extracts from *Sambucus nigra* and *Viola tricolor* + *Veronica officinalis* showed better hydration of the *stratum corneum*. The application of hydrogel resulted in a higher outermost skin layer hydration level compared to emulsions during the first 30 minutes. However, after that time, creams had better *stratum corneum* hydration properties until the end of these short-term preliminary studies. Therefore, developed phytoformulations may find an application as skin conditioning products.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

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