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ORIGINAL ARTICLE / ÖZGÜN MAKALE

Development and Microbiological Evaluation of Natural Diaper Rash (Diaper Dermatitis) Cream Formulations

Doğal Bebek Bezi Pişiği (Bebek Bezi Dermatidi) Krem Formülasyonlarının Geliştirilmesi ve Mikrobiyolojik Değerlendirilmesi

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Abstract

Backround: Rosa damascena Mill, released into the environment as waste in rose products production facilities, contains antioxidant, antimicrobial, and antiseptic phenolic components. It is the development of an effective natural cream formulation for baby diaper rash by taking advantage of the antimicrobial properties of rose pulp and adding natural ingredients (zinc oxide ZnO, natural oils, and beeswax).

Material and Methods: The emulsification method was used to prepare diaper rash cream formulations. Rotational type viscosity determination was performed to examine the rheological behavior of the formulations. In selecting the optimum formulation, pH, viscosity, hydrophilic-lipophilic balance, and physical appearance of the product were considered. To examine its stability properties, its stability was examined in three different environments in line with ICH directives for 6months. The optimum DR-C-7 formulation was subjected to physicochemical and stability tests.

Results: It was observed that the DR-C-7 formulation had a viscosity between 9,820 and 26,130 (Pa.s) in terms of rheological properties. As a result of the challenge test, no microbiological units were found. At the end of a 6-month stability study under different conditions, it was observed that it retained all its features.

Conclusion: It was concluded that R. damascena pulp, which has important phenolic contents such as phenylethyl alcohol, flavonoids, and terpenoids, can be used for thick products such as diaper rash cream with its antioxidant antimicrobial properties.

Keywords: Dermocosmetics, Rose Damescana Pulp, Baby Rash, Emulsion, Microbiological Analysis

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Öz

Amaç: Gül ürünleri üretim tesislerinde atık olarak çevreye salınan Rosa damascena Mill, antioksidan, antimikrobiyal ve antiseptik fenolik bileşenler içermektedir. Gül posasının antimikrobiyal özelliklerinden yararlanılarak ve doğal bileşenler (çinko oksit ZnO, doğal yağlar ve balmumu) eklenerek bebek pişikleri için etkili bir doğal krem formülasyonunun geliştirilmesidir.

Gereç ve Yöntem: Pişik kremi formülasyonlarını hazırlamak için emülsifikasyon yöntemi kullanılmıştır. Formülasyonların reolojik davranışını incelemek için rotasyonel tip viskozite tayini yapılmıştır. Optimum formülasyonun seçiminde pH, viskozite, hidrofilik-lipofilik denge ve ürünün fiziksel görünümü dikkate alınmıştır. Stabilite özelliklerini incelemek için, ICH direktifleri doğrultusunda üç farklı ortamda 6 ay boyunca stabilitesi incelenmiştir. Optimum DR-C-7 formülasyonu fizikokimyasal ve stabilite testlerine tabi tutulmuştur.

Bulgular: Reolojik özellikler açısından DR-C-7 formülasyonunun 9,820 ile 26,130 (Pa.s) arasında bir viskoziteye sahip olduğu gözlenmiştir. Zorlama testi sonucunda herhangi bir mikrobiyolojik birime rastlanmamıştır. Farklı koşullar altında yapılan 6 aylık stabilite çalışması sonunda tüm özelliklerini koruduğu gözlenmiştir.

Sonuç: Feniletil alkol, flavonoidler ve terpenoidler gibi önemli fenolik içeriklere sahip olan R. damascena pulpunun antioksidan antimikrobiyal özellikleri ile pişik kremi gibi kalın ürünlerde kullanılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Dermokozmetik, Rose Damescana Pulpası, Pişik, Emülsiyon, Mikrobiyolojik Analiz

INTRODUCTION

Diaper dermatitis, commonly referred to as diaper rash, is a skin problem that occurs as a result of closure, moisture and irritation in and around the perineum, sub-perineum (1, 2) It is the most common skin disease with a rate of 7-35% in infants and usually develops in the 9-12th months (1). In adults, the skin acts as a protective barrier against all kinds of external factors. However, proper and effective protection in infants usually occurs after 1 year of age. Compared to adult skin, infant skin is thinner, less pigmented, less tolerant to heat and less thermoregulated. Prevention of nappy rash involves strengthening the skin barrier and eliminating the factors that cause inflammation. In this context, diaper rash cream was developed using the antioxidant, antimicrobial and antiseptic phenolic components of Rosa damascena Mill pulp, which is left to the environment as waste in rose product production facilities (3, 4). Microorganisms alone are not effective in the development of nappy dermatitis. However, the interaction of other factors facilitates the access of microorganisms to the epidermis through the damaged stratum corneum (SC) layer, which increases the risk of secondary infections caused by fungi and bacteria (5). When secondary infection develops, the course of gland dermatitis becomes more severe (6). In addition, the use of broad-spectrum antibiotics for various reasons increases the risk of developing gland dermatitis (2). Microbiological

contamination of cosmetic products is important in terms of both posing a risk to consumer health and causing economic loss due to changes that may occur in the product (odour and gas formation, colour and viscosity changes, etc.) (7). Studies show that cosmetic products are mostly exposed to contamination during use by the consumer. Using the products after the expiry date specified on the label, use by more than one person, wetting with saliva, inserting a finger or contaminated object, being in contact with air are among the most important reasons for contamination during use. Microorganisms that frequently cause contamination in cosmetic products are reported as P. aeroginosa, S. aureus, Enterobacter sp., E. coli, K. pneumonia, S. epidermidis, C. albicans, Aspergillus sp. (8). The presence of these microorganisms poses a danger to the health of users. Therefore, in order to prevent microbial growth, some substances with different chemical structures are added as protection against contamination (9). Today, preservative efficacy tests are carried out according to different methods determined by organisations such as the United States Pharmacopoeia (USP) and the British Pharmacopoeia (BP) (10). Microbiological evaluations of these products offered to the market are mandatory for the health of the target audience. There are many methods (pouring, smearing and dripping) for microbiological analysis (11). The aim of this study was to develop an effective natural cream formulation for baby rash by utilising the antimicrobial properties of rose pomace and adding natural ingredients (zinc oxide ZnO, natural oils and beeswax).

MATERIALS AND METHODS

Rose Pomace (Local Producer, Türkiye), Zinc Oxide (Galenik, Türkiye), White Beeswax (Galenik, Türkiye), Sorbitan monooleate (Galenik, Türkiye), Olive Oil (Talya, Türkiye), Lanolin (Doğa Pharm., Türkiye), Polysorbate 80 (Galenik, Türkiye), Devices used: Milwaukee MW150 max (Szeged-Hungary), Rotational Viscometer PCE-RVI 10(Meschede-Germany), Elektromag M5040 PS (Çerkezköy – Türkiye), Mechanic Stirrer (Isolab, Germany), Heated magnetic stirrer (Isolab, Germany).

Method

The main component of the formulation is *Rosa damascena* Mill pulp. It was prepared as a water/oil type emulsion system by adding water phase to the oil phase. The oil phase ingredients (olive oil, almond oil, lanolin, beeswax, calendula oil, emulsifiers) were weighed from low to high on an analytical balance and melted at 70°C until completely liquefied. Water phase materials (magnesium sulphate, EDTA, glycerin, water) were weighed, heated to 70°C and slowly poured over the oil phase. The mixture was homogenised in a high speed homogeniser for 5 minutes. Zinc oxide and vitamin E were then added to the mixture and homogenised at 2000 rpm for 5 min (12). The formulation components are given in Table 1. Eight different formulations were prepared by changing the systems in the diaper rash cream. The distribution of the formulations was checked for colour, rheological properties and other physicochemical properties. Microbiological analysis of the resulting formulation was performed using the pour plate method. Total bacterial and fungal analyses for *Staphylococcus aureus*,

aerobic mesophilic (bacteria, yeast moulds), *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* were reported as CFU/g.

Formulation study

Cream formulations are classical emulsion formulations. Emulsion components include oil phase, water phase and emulsifiers. While developing rose pulp nappy rash creams, it was aimed to prepare suitable formulations in which the rose pulp could be completely emulsified in the cream. The oil phase components were selected in different concentrations and the optimum concentration ratio was tried to be determined for the water phase where the active components of the emulsion are present. Antioxidant, chelating agent and preservative concentrations were kept constant during formulation design. Olive oil and almond oil were used in three different concentrations, zinc oxide, beeswax and lanolin were used in two different concentrations. In addition, the effectiveness of emulsifiers was evaluated by using concentrations in accordance with and opposite to the HLB calculation. In total, eight different formulations were developed and physicochemical studies were carried out to select the optimum formulation (Table 1).

Table 1. Composition of eight different diaper rash cream formulations containing *Rosa Damescana*

Ingredient	DR-C-1	DR-C-2	DR-C-3	DR-C-4	DR-C-5	DR-C-6	DR-C-7	DR-C-8
Olea Europa oil	10.00	15.00	20.00	10.00	15.00	20.00	15.00	15.00
Almond Oil	10.00	10.00	10.00	20.00	20.00	20.00	15.00	15.00
Beeswax	4.00	4.00	4.00	5.00	5.00	5.00	5.00	5.00
Lanolin	1.00	1.00	1.00	2.00	2.00	2.00	2.00	2.00
Sorbitan monooleate	3.50	4.24	4.99	5.23	6.07	7.20	5.23	2.17
Calendula officinalis extract	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Zinc oxide	10.00	10.00	10.00	10.00	10.00	5.00	5.00	5.00
Rose pulp	2.00	2.00	2.00	5.00	5.00	5.00	5.00	5.00
Polysorbate 80	1.50	1.76	2.01	1.97	2.33	2.20	2.17	5.23
Magnesium sulphate	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
BHT	0.05	0.05	0.05	0.05	0.05	0.05	1.00	1.00
EDTA	0.05	0.05	0.05	0.05	0.05	0.05	1.00	1.00
Ethylhexylglycerin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Glycerin	5.00	5.00	5.00	5.00	5.00	10.00	10.00	10.00
D.Water	46.90	40.90	34.90	32.70	26.50	20.50	30.60	30.60

Microbiological analyses

The formulation developed for microbiological analysis within the scope of the study was disinfected with 70% ethanol. To dissolve the product, 5 g/L polysorbate 80 was added to 90 ml TSP (Buffered Sodium Chloride Peptone), 10 g of sample was added and left to dissolve in a water bath for 10-15 minutes. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) were prepared by transferring 1 ml of the sample suspension to 9 ml of TSP using the pour plate method. The dilutions were repeated twice by inoculating 1 ml of the diluted tubes into a 90 mm petri dish. Then 15-17 ml of agar medium cooled to 45°C in a water bath was poured into the petri dishes and left to freeze. Tyriptic Soy Agar (TSA) was used for the total number of aerobic mesophilic microorganisms which were left at 30-35°C for 3-5 days. SDA medium was

used for total yeast and mould counts and the media were incubated at 20-25°C for 5-7 days. In case of growth, the calculation formula is used to count colonies visible to the naked eye. This formula is as follows:

CFU/ml = Total number of colonies obtained x dilution factor/ Sample volüme

Enrichment

Ten g of the sample dissolved in buffered sodium chloride peptone was transferred to 90 ml of Tyriptic Soy Broth (TSB). This medium contains lecithin and polysorbate required for neutralisation and is a general producer medium. After thorough shaking, the medium was incubated at 30-35°C for 18-24 hours (maximum 72 hours). After incubation, a selective medium was used. Enrichment was performed for *E. coli, P. aeruginosa* and S*. aureus. For C. albicans,* 10 ml (1 g or ml) of the sample dissolved in TSP was transferred to 90 ml Sabouraud Dextrose Broth (SDB). After shaking well, it was incubated at 30-35°C for 72 hours (maximum five days). After incubation, a selective medium was used.

Investigation of Aerobic Mesophilic Bacteria

After enrichment, 1 ml of TSB medium was taken and placed in sterile petri dishes. 5 ml of medium was added to Tryptone Glucose Extract Agar (TGEA) medium cooled to 45°C, mixed and inoculated with the sample in duplicate and allowed to solidify. After solidification, it was incubated at 37°C for 48 hours. In case of growth at the end of incubation, the number of colonies formed is calculated taking into account the dilution factor.

Investigation of The Presence of Escherichia Coli

After enrichment, 1 ml of TSB medium was taken and placed in sterile petri dishes. Then 5 ml of medium was added to Macconkey Agar (MCA) medium, mixed and the medium was inoculated with the sample in duplicate and allowed to solidify. Incubated at 30-35°C for 24 hours (maximum 48 hours).

Investigation for The Presence of Staphylococcus Aureus

After enrichment, 1 ml of TSB medium was taken and placed in sterile petri dishes. 5 ml of medium was added to Mannitol Salt Agar (MSA) medium cooled to 45°C, inoculated with the sample in duplicate and allowed to solidify. Incubated at 30-35°C for 24 hours (maximum 48 hours).

Investigation of The Presence of Pseudomonas Aeruginosa

After enrichment, 1 ml of TSB medium was taken and placed in sterile petri dishes. 5 ml of medium was added to Cetrimide Agar (CA) medium cooled to 45°C, mixed and the medium was inoculated with the sample in duplicate and allowed to solidify. Plates were incubated at 25˚C for 5 to 7 days.

Rheological Studies

Apparent viscosity was determined using a PCE-RVI-10 rotational viscometer (PCE Instruments, Hamburg, Germany). Rotational rheometer measures viscosity by calculating the torque required to rotate the spindles immersed in the fluid. The applied torque is related to the viscous friction on the shaft and therefore the viscosity of the fluid. Samples were measured with an L2 spindle at 6 rpm for 120 sec. was carried out throughout. The slip rate is calculated taking into account the dimensions of the shaft,

the rotation speed and the gap between the shaft and the container (formulation 1-2). The gap between the shaft and the container was set to 1.25 mm, resulting in a ratio of 1.2 between the container diameter and the shaft(13). The sliding speed was calculated according to the formulation 1-2. Whether the difference between the values of the formulae was significant or not was evaluated using One-Way Anova test.

$$y = 2 \times \frac{2 \times \pi \times Ni}{60} \times \frac{R_0^2}{R_0^2 - Ri^2} \quad (1)$$

In the equation, "y" is the sliding speed in s-1, "Ni" is the rotation speed in rpm, and "R0" and "Ri" are the radius of the cup and shaft in mm, respectively. Apparent viscosity values were plotted as a function of shear rate and fitted to Eq x according to the Ostwald-de Waele relationship (14).

$$\eta = K.\gamma^{n-1} \quad (2)$$

Here, K is the viscosity coefficient n is the flow behavior index calculated from exponential regression (14).

Calculation of The İnterval of Safety (Mos)

The safety margin value (MOS) of the diaper rash cream formulation is determined in the Turkish Medicines and Medical Devices Agency (TITCK) Cosmetic Guide 3, as specified in the skin surface area (860 cm2), application frequency (1/day) and daily exposure level (according to the product type). It was taken as 2.16 g/day). Systemic exposure dose (SED) was calculated with formula 3 and safety margin values were calculated with formula 4. When calculating dermal absorption, calculations were made assuming that 100% of the product would be absorbed as the worst exposure scenario for all raw materials. MoS value is expected to be higher than 240 in baby products. In this study, these criteria were taken into consideration since the formulation of baby nappy rash cream was carried out.

$$SED = \frac{DAa\ (\mu g/cm2) \times 10\text{-}3mg/\ \mu g \times SSA\ (cm2) \times F(gün\text{-}1)}{60} \quad (3)$$

$$MoS = \frac{PODsys}{SED*\%kons.} \geq 100 \text{ (yetişkinler için)} \quad (4)$$

Stability Study

Stability studies were checked for appearance, color, pH, viscosity changes and microbiological growth in the refrigerator, room and oven (45 ^{0}C) for 6 months, in accordance with ICH ant TITCK directives (18).

RESULTS

Baby barrier cream formulations were tested in terms of physical-chemical, stability and harmful microorganisms on the skin of infants and it was concluded that they comply with microbiological limit values. As a result of the study, characterisation and safety assessment were completed and it was decided that DR-C-7 formulation was the most suitable formulation among eight formulations in terms of appearance, flow properties and stability.

Microorganism Analysis Result

No growth was detected in our tested shampoo product. No growth was observed in this product on the 14_{th} and 28_{th} days following the effect of the preservative

(Table 2).

Table 2. Microbiological test results of cosmetic sample

Microorganisms	Conclusion
Total number of aerobic mesophilic microorganisms (Bacteria, mold, yeast)	≤1 x 102 cfu/ g or ml
Yeast and mold	≤10^2 cfu
Escherichia coli	1 g ve ml not found
Pseudomonas aeruginosa	1 g ve ml not found
Staphylococcus aureus	1 g ve ml not found
Candida albicans	1 g ve ml not found

Physicochemical test results

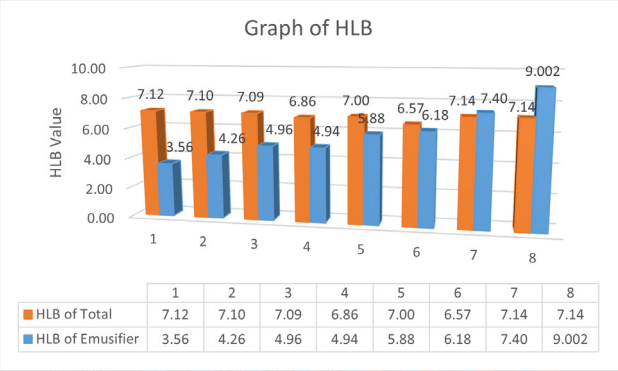
The following table presents the HLB calculation results and measurements of viscosity, yield stress, and flow rate, which are rheological properties, for formulations suitable for infant skin with a skin pH range of 6.2-7.5. Whether the difference between the values of the formulae was significant or not was evaluated using One-Way Anova test. According to the results of statistical analysis, it was found that there was no significant difference between the formulations in terms of pH values ($p>0.05$). HLB values and viscosity values of the formulations were statistically significant ($p<0.05$).

Table 3. Appearance, pH, HLB and rheological results of the formulations (n=3).

Formulation code	Appearance	pH Measurment Result	Oil Phase HLB	Emulsifier HLB	Viscosity (Pa.s)	Shear Rate (1/sec)	Shear Stress (D/cm²)
DR-C-1	Homogenous Cream	7.201±0.10	7.52	3.76	31.23±5.67	0.14	4.37±1.12
DR-C-2	Homogenous Cream	7.243± 0.12	7.43	4.46	34.54±5.22	0.11	4.83±1.03
DR-C-3	Homogenous Cream	7.320±0.14	7.37	5.16	35.45±4.18	0.12	4.96±0.82
DR-C-4	Homogenous Cream	7.137 ± 0.09	7.22	5.2	30.62±5.78	0.12	6.38±1.14
DR-C-5	Homogenous Cream	7.212 ± 0.08	7.26	6.1	37.51±5.22	0.11	6.09±1.03
DR-C-6	Homogenous Cream	6.949 ± 0.11	6.81	6.4	48.20±6.21	0.14	6.74±1.22
DR-C-7	Homogenous Cream	6.992 ± 0.13	7.43	5.5	38.2±4.37	0.12	5.34±0.86
DR-C-8	Homogenous Cream	-	7.43	8.78	-	-	-

HLB calculation results

The calculation of the amount of emulsifier to be used in cream formulations was carried out based on the HLB values of the lipophilic and hydrophilic emulsifiers used. In order to verify the HLB calculation of DR-C-7 and DR-C-8 creams with the same ratio of oil phase and water phase in the formulations, a reverse amount of emulsifier experiment was performed. As a result, it was observed that the phases were not dispersed in each other and phase separation was observed.



Graph 1. HLB calculation graph for all cream formulations.

According to the above results, it is seen that the emulsifier used in the 8th formulation was incorrectly selected. Considering the HLB values in the other 7 formulations, it

was determined that the emulsifier selection was correct and no phase separation was observed.

Rheological Studies Result

The DR-C-7 optimum diaper rash cream formulation resulted in n<1, showing shear thickening according to the Ostwald-de Waele and exhibited Pseudo-Plastic flow. According to the results, the shear rate is between 0.12 s^{-1} and 24.64 s^{-1}, the shear stress is between 271.01 and 2419.35(D/cm^2), and the viscosity values are 220 and 9.82 Pa.S.

Table 4. Viscosity, shear stress, shear rate results of the optimum formulation DR-C-7

Viscostiy (Pa.s)	Shear Rate (1/sec)	Share Stress (D/cm^2)
220±14.64	0.12	0.27±0.02
120±10.50	0.25	0.30±0.03
65.2±8.95	0.62	0.40±0.06
45.3±4.76	1.23	0.56±0.06
38.2±4.37	2.46	0.94±0.11
25.95±1.06	4.93	1.28±0.05
18.04±9.11	12.32	2.22±1.12
9.82±4.50	24.64	2.42±1.11

Graph 2. Graph of viscosity and shear stress versus shear rate of the optimum formulation GP-C-7 (n=3)

Safety Interval Results

The safety assessment results of the DR-C-7 formulation were calculated according to fromulation 3 and 4 and are given below in the table. According to the cosmetic legislation, the MoS (safety limit) value should be equivalent to or above 240 in the evaluations made with the ratios of the concentrations of the ingredients in it, since it is foreseen to be used in infants. Olive oil, Almond Oil, Rose pulp, Shea Butter, Calendula Oil, Calendula Oil, Beeswax do not have NO(A)EL values since the raw materials are natural and used as food. In this case, they are considered safe for topical applications regardless of their concentration of use.

Table 5. MoS values of the components of DR-C-7 fomulation

İngredient	POD_{sys}	MoS
Lanolin	5000	3472>240
Sorbitan monooleate	2600	502>100
Zinc oxide	7950	1104>100
Polysorbate 80	5000	1328>240
Magnesium sulphate	1029	1429>240
Antioxidant (BHT)	2000	5555>240
EDTA	800	1111>240
Ethylhexylglycerin	2000	13889>240
Glycerin	12600	1750>240

Stability Results

Stability studies were carried out in refrigerator, room and oven (45 ^{0}C) for 6 months in accordance with ICH directives and TİTCK cosmetic regulation. The appearance, colour, pH, viscosity changes and microbiological growth were checked. Rose pulp nappy rash cream maintained its physicochemical properties in all environments. After organoleptic controls, it was observed that the specific appearance and colour remained the same from day 0 to 6 months. It was observed that the pH value was in the range of pH 5.5-5.7 under all conditions in accordance with the pH value of the skin, which did not leave any burning sensation for the skin. For the control of microbiological growth, the protective efficacy test (45^{0}C) was carried out in an oven and no growth was found.

Table 6. Stability results; Appearance, color, odor, and microbiological growth results as of Day 0, Month 3, and Month 6

Stability Condition	Control Period	View	Colour	Viscostiy(Pa.S)	Mıcrobıologıcal Growth (45°C) In Incubator
Room Conditions	0 .Month	Homogenıc Cream	Specıfic	25,95±1,06	No Reproductıon
	3 .Month	Homogenıc Cream	Specıfic	25,55±1,31	No Reproductıon
	6 .Month	Homogenıc Cream	Specıfic	25,34±1,36	No Reproductıon
Incubator (45°C)	0 .Month	Homogenıc Cream	Specıfic	22,05±2,10	No Reproductıon
	3 .Month	Homogenıc Cream	Specıfic	21,55±1,12	No Reproductıon
	6 .Month	Homogenıc Cream	Specıfic	20,95±2,16	No Reproductıon
Refrigerator	0 .Month	Homogenıc Cream	Specıfic	26,25±0,96	No Reproductıon
	3 .Month	Homogenıc Cream	Specıfic	26,42±0,78	No Reproductıon
	6 .Month	Homogenıc Cream	Specıfic	26,75±0,96	No Reproductıon

DISCUSSION

Diaper rash is the most common skin problem in childhood, which can be seen in all nappied infants (15). In the literature, it is reported that the incidence is affected by many factors and varies between 7% and 35% (6). The cause of diaper rash is irritation of the skin as a result of excessive moisture and friction, and the pH of the skin changes from acidic to alkaline and becomes colonised with Candida albicans and bacteria (1). The cosmetic manufacturer must ensure that the devices and materials to be used in production are clean and the products are free from pathogenic microorganisms in accordance with Good Manufacturing Practices (GMP) and Microbiological Quality Management. In addition, procedures should include microbiological control of raw materials, bulk and finished products, materials used in packaging, personnel, equipment, preparation and storage rooms (16). The reason for diaper rash is irritation of the skin as a result of excessive moisture and friction. As a result of not changing the diaper frequently, the in contact with urine changes from acidic to alkaline and becomes colonized with microorganisms. Alkaline pH damages the stratum corneum layer by activating the protease and lipase enzymes in the stool (17). In preventing diaper rash; Changing the baby's diaper frequently, ventilating the diaper, choosing diapers with high absorbency capacity, not tying the diaper tightly, not using alcohol-containing cleansing wipes, instead cleaning the area with warm water after each defecation, applying a thin layer of protective creams at each diaper change and avoiding damaging the respiratory tract. It is of great importance to avoid the use of powder because it can cause damage (18). In our study, when the content of diaper rash protective creams was examined; Zinc oxide, lanolin were most commonly used for this purpose (20). In our study, opening the sample, taking the appropriate amount and transferring it to TSP was performed under aseptic conditions in a biosafety cabinet. This type of cream forms a lipid layer on the skin and protects the baby's skin from harmful microorganisms and irritants. Zinc oxide, which has low toxicity, kills microbes on the skin surface with its antiseptic properties, improves the general appearance of the skin and helps to reduce irritation caused by allergic reactions (21). In our study, zinc oxide was used in 8 different formulations,

researches were carried out on its physical and chemical properties, diaper rash creams were prepared and it was aimed to reach the optimum formulation. All formulations were water/oil type emulsions. In the studies, it was found that the capacity of water/oil type emulsions to carry the oil phase and zinc oxide gave very successful results compared to other systems (22). For this purpose, the most suitable formulation in terms of pH, viscosity, HLB (hydrophilic lipophilic balance) and microbiological quality was revealed. In DR-C-8 formulation, phase separation was observed due to the fact that the emulsifier HLB value was not selected suitable for the oil phase. In DR-C-1/2/3 formulations, it was observed that lanolin was used less and therefore the spread was less. In DR-C-4/5/6 formulations, excessive use of almond oil caused a decrease in the resistance to slipping. In this way, it was thought that it would have a negative effect on the removal of the nappy area, which is a mobile area, and on the elimination of nappy rash. DR-C-7 formulation was selected as the optimum formulation because it is a formulation that does not create high raw material costs in terms of its rheological properties and content. When all parameters between the formulations were compared, DR-C-7 was selected as the optimum formulation containing natural ingredients such as olive oil, lanolin, bees wax, zinc oxide. In our country, the Product Safety Assessment Report is among the documents required by TİTCK during cosmetic product notification and checked by experts. This report presents the evaluation of the finished product, taking into account the toxicological character, chemical structure and exposure levels of the product components, and the specific exposure characteristics of the target group or the area where the product will be applied. Information on the microbiological quality of cosmetic products, verification of the effectiveness of the preservative system and verification of the specified minimum duration of the cosmetic product stored under normal conditions and the duration of use of the finished product after opening are important for product safety. The report must include the results of microbiological quality tests and preservative efficacy tests of the cosmetic product (19). The microbiological analysis of the formulation product obtained in our study was tested and it was concluded that it complied with the microbiological limit values.

CONCLUSION

The antioxidant, antiseptic, anti-inflammatory and antibacterial wound healing purposes of R. damascena, which has phenolic contents such as phenylethyl and flavonoid terpenoids, have been achieved. As a result of the study, no growth was detected in any of the prepared formulations.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

Developing Natural Shampoo Formulation Against Hair Loss with Microbiologically Tested Herbal Extracts and Essential Oils

Mikrobiyolojik Olarak Test Edilmiş Bitkisel Ekstraktlar ve Esansiyel Yağlar ile Saç Dökülmesine Karşı Doğal Şampuan Formülasyon Geliştirilmesi

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Abstract

Objectives: Shampoo is the most frequently used cosmetic product. Given the importance placed on cleanliness for hair health and the increasing importance placed on hair beauty and care, it is evident that synthetic shampoos damage hair when used regularly. In this context, the objective was to produce a natural shampoo and conduct antimicrobial analysis using *Urtica dioica* (nettle), wheat germ oil, *Rosa damascena Mill* (Isparta rose), and *Allium sativum* (garlic).

Material and Methods: All necessary processes, including formulation design, characterization, and final product tests, were carried out within the scope of the study until the product was packaged and became the final product. Physicochemical tests (pH, viscosity, dandruff effect, microbiological tests) and stability tests (color, odor, appearance, and pH control) were performed on the developed formulation.

Results: The analysis revealed that the pH value of the formulations was approximately 5.5, which is suitable for the skin. The selected NI-S-7 exhibited high viscosity (26.43 ± 0.75), foaming index (250), and high cleaning efficiency among all formulations. This led to developing a shampoo containing active herbal ingredients that nourish and repair hair. Furthermore, the microbiological analysis of the resulting formulation product was conducted and found to comply with the established limit values.

Conclusion: The transformation of agricultural products into commercial products has the potential to bolster the economy. The shampoo in development will be derived from naturally sourced plants cultivated in Turkey and utilized for medical conditions with an aromatherapeutic effect while preserving the typical scent of the rose plant.

Keywords: Herbal Shampoo, *Urtica dioica, Rosa damascena mill, Allium sativum,* Microbiological analysis

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Öz

Amaç: Şampuan en sık kullanılan kozmetik üründür. Saç sağlığı için temizliğe verilen önem nedeniyle saç güzelliğine ve bakımına verilen önem de giderek artmaktadır. Sentetik şampuanlar düzenli kullanıldığında saçlara zarar verir. Bu kapsamda *Urtica dioica* (ısırgan otu), buğday tohumu yağı, *Rosa damascena Mill* (Isparta gülü) ve *Allium sativum* (sarımsak) kullanılarak doğal bir şampuan üretilmesi ve antimikrobiyal analizlerin yapılması amaçlandı.

Gereç ve Yöntem: Ürün paketlenip nihai ürün haline gelinceye kadar gerekli tüm süreçler (formülasyon tasarımı, karakterizasyon ve son ürün testleri) çalışma kapsamında gerçekleştirildi. Geliştirilen formülasyon üzerinde fizikokimyasal testler (pH, viskozite, kepek etkisi, mikrobiyolojik testler) ve stabilite testleri (renk, koku, görünüm ve pH kontrolü) yapıldı.

Bulgular: Analiz sonucunda formülasyonların pH değerinin cilde uygun olan 5,5'e yakın olduğu belirlendi. Seçilen NI-S-7, tüm formülasyonlar arasında yüksek viskozite (26,43± 0,75), köpüklenme indeksi (250) ve yüksek temizleme verimliliği gösterdi. Saçı besleyen ve onaran bitkisel etken maddeler içeren bir şampuan elde edildi. Ayrıca ortaya çıkan formülasyon ürününün mikrobiyolojik analizi yapılmış ve Türkiye İlaç ve Tibbi Cihaz Kurumu (TITCK) tarafından yayınlanan kozmetik ürünlerde güvenlilik kılavuzına göre sınır değerlere uygun olduğu görülmüştür.

Sonuç: Ülkemizde yetiştirilen ürünlerin ticari ürüne dönüştürülmesi ekonomiye destek olacaktır. Geliştirilecek şampuan, gül bitkisinin tipik kokusunu koruduğu için Türkiye'de yetişen doğal kaynaklı bitkilerden elde edilecek ve aromaterapi etkisiyle tıbbi rahatsızlıklarda kullanılması beklenmektedir.

Anahtar Kelimeler: Bitkisel Şampuan, *Urtica dioica, Rosa damascena mill, Allium sativum,* Mikrobiyolojik analiz

INTRODUCTION

As the economy improves and people become more interested in beauty, the demand for health and skincare products also increases. For centuries, herbs have been popular for these purposes. Cosmetics and foods that contain new bioactive ingredients for skincare and beauty are constantly being researched and developed. The skin is affected by several factors such as ultraviolet rays, stress, hormones, and aging, which can cause loss of elasticity, changes in pigmentation, and wrinkles. Many medicinal plants have been shown to have beneficial effects on the skin, with *Rosa damascena* Mill, *Urtica dioica* L, and *Triticum vulgare* being among the most notable (1,2,3). Research in the field of cosmetology has proven the effect of Rosa **damascena** on rehydrating the skin, reducing scars and stretch marks, managing acne, reducing skin pigmentation, and delaying wrinkles. It is recommended as a skin-revitalizing agent (1). It has been determined that these products may be contaminated with microorganisms found in the environment during production (4). Shampoos are one of the most widely used cosmetic products. To have healthy hair, herbal sources are used as active ingredients to nourish and protect the hair. However, they can be used for different needs formulations that contain high amounts of synthetic chemicals, which can cause a lot of damage to the hair with regular use (5). Aqueous cosmetic products provide a suitable environment for the growth of fungi

and bacteria due to the moisture they contain. Microbiological analysis is carried out on the shampoo for this particular purpose (6). *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, and Aspergillus spp* are microorganisms that frequently cause contamination in cosmetic products. Water and organic/ inorganic compounds are susceptible to microbial contamination under appropriate physicochemical conditions, including those encountered in the production of cosmetics (7,8). To prevent microbial growth, various substances with different chemical structures are added to protect against contamination (9,10). The objective of this study was to develop eight distinct natural herbal hair shampoo formulations with the potential to prevent hair loss and dandruff. The formulations were created using extracts and oils of *Rosa damascena*, *Urtica dioica*, and *Triticum Vulgare* (Wheat), which are native plants of Turkey. Following the completion of the characterization and safety evaluation, it was determined that the AHL-NS-7 shampoo formulation exhibited the most promising characteristics among the eight formulations. The formulation exhibited no stability issues, rendering it the most secure option for consumers.

MATERIAL AND METHODS

Material

The composition of the product includes *Rosa damascena* Extract (Talya), Urtica dioica Leaf Extract (Talya), *Triticum vulgare* (Wheat) Germ Oil (Talya), Guar Gum (BASF), Ammonium Lauryl Sulfate (Sabunaria), Cocamide MEA (Sabunaria), Betaine (Sabunaria). Additionally, the product contains Ethylhexylglycerin (Ashland), Laureth-7 Citrate (BASF), and all other ingredients of cosmetic quality. The following instruments were employed: a Milwaukee MW150 max (Szeged, Hungary), a rotational viscometer PCE-RVI 10 (Meschede, Germany), a mechanical mixer (Isolab, Germany), an incubator Elektromag M5040 PS (Cerkezkoy, Turkey), and an Ultraturrax (VELP Scientifica, Italy).

Methods

A formulation was developed through polymer hydration and high-speed homogenization at elevated temperatures to enhance the performance of shampoos. Physicochemical tests (pH, viscosity, dandruff effect, microbiological tests) and stability tests (color, odor, appearance, and pH control) were conducted on the developed formulation. The pour plate method was employed to conduct total bacterial and fungal analyses for *Staphylococcus aureus*, aerobic mesophilic bacteria and yeast mold, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, with the results expressed in colony-forming units per milliliter (CFU/ml). By the established standards, the pathogens that should not be present in 1 **g** or 1 ml of the cosmetic product were identified as *E. coli*, S. *aureus*, *P. aeruginosa*, and *C. albicans* (ISO 17516, 2014). Selective media were employed to detect the pathogens.

Microbiological Analysis

The formulation developed for microbiological analysis was disinfected with 70% ethanol within the scope of the study. To dissolve the product, 5 g/L polysorbate 80 was added to 90 ml TSP (Buffered Sodium Chloride Peptone), and 10 g of sample was added and left to dissolve in a water bath for 10-15 minutes. Serial dilutions (10-2, 10-3, and 10-4) were prepared by transferring 1

ml of the sample suspension into 9 ml of TSP using the pour plate method. One milliliter was then taken from the dilution tubes and transferred to a 90 mm petri dish, which was repeated twice. Subsequently, 15-17 ml of agar medium, cooled to 45°C in a water bath, was poured into petri dishes and allowed to solidify. The total count of aerobic mesophilic microorganisms was determined using Tyriptic Soy Agar (TSA) at 30-35°C for 3-5 days. For total yeast and mold counting, SDA medium was employed, with the medium incubated at 20-25°C for 5-7 days. In the case of breeding, the calculation formula is used to count colonies visible to the naked eye. This formula is as follows: The number of Colony Forming Units per milliliter (CFU/ml) is calculated by multiplying the total number of colonies obtained by the dilution factor and then dividing by the sample volume.

Enrichment

A 10 g sample was dissolved in a buffered peptone solution and transferred to a 90 ml Tryptic Soy Broth (TSB) medium. This medium contains lecithin and polysorbate, which are necessary for neutralization and are suitable for use as a general growth medium. After thorough shaking, the sample was incubated at 30-35°C for 18-24 hours (up to a maximum of 72 hours). After incubation, a selective medium was used. Enrichment for *E. coli*, *P. aeruginosa*, and *S. aureus* was applied. For *C. albicans*, 10 ml (1 g or ml) of the sample dissolved in TSP was taken and transferred to a 90 ml Sabouraud Dextrose Broth (SDB) medium. After shaking well, it was incubated at 30-35°C for 72 hours (maximum five days). Subsequently, a selective medium was employed for incubation.

Investigation of Aerobic Mesophilic Bacteria

Following enrichment, 1 ml of TSB medium was transferred to sterile petri dishes. Five milliliters of the medium were added to Tryptone Glucose Extract Agar (TGEA) medium cooled to 45 °C, mixed, and inoculated with the sample in duplicate. The inoculated medium was then allowed to solidify. After solidification, the inoculated medium was incubated at 37 °C for 48 hours. In the event of reproduction after the incubation period, the number of colonies formed is calculated by taking the dilution factor into account.

Investigating The Presence of Escherichia coli

Following enrichment, 1 ml of TSB medium was transferred to sterile petri dishes. Thereafter, 5 ml of MacConkey Agar (MCA) medium was inoculated with the sample in duplicate and allowed to solidify. The inoculated plates were incubated at 30-35°C for 24 hours (up to a maximum of 48 hours).

Investigating The Presence of Staphylococcus aureus

Following enrichment, 1 ml of TSB medium was transferred to sterile petri dishes. Five milliliters of cooled (to 45 °C) Mannitol Salt Agar (MSA) medium was inoculated with the sample in duplicate and allowed to solidify. The inoculated MSA was incubated at 30-35 °C for 24 hours (with a maximum incubation period of 48 hours).

Investigating The Presence of Pseudomonas aeruginosa

Following enrichment, 1 ml of TSB medium was transferred to sterile petri dishes. Five milliliters of medium was added to the Cetrimide Agar (CA) medium that had been cooled to 45 °C, and mixed, and the medium

was inoculated with the sample in duplicate. The inoculated medium was then allowed to solidify. The plates were incubated at 25 °C for 5 to 7 days.

Formulation Study

Guar gum was combined with two-thirds of the water at 400 rpm for five minutes. A citric acid solution was then added under stirring. Surfactants and foaming agents were subsequently incorporated into the mixture. Herbal extracts and essential oils were dissolved in Laureth-7 Citrate at varying concentrations and added to the mixture under 400 rpm. The pH value of the mixture was then regulated with 1M NaOH. Consequently, eight distinct formulations with varying consistencies and active ingredients were developed (Table 1, 2).

Table 1. Components and concentrations of shampoo formulations

Contents	BSF-1
Rosa damascena Extract	0.5-1
Urtica Dioica Leaf Extract	0.2-0.4
Allium Sativum Bulb Oil.	0.2
Triticum Vulgare (Wheat) Germ Oil	0.2-0.4
Guar Gum	0.1-0.9
Amonyum Lauril Sulfate	5-6.5
Amonyum Lauril Eter Sulfate	6-7.5
Cocoamide Mea	4-5.5
Betain	5-6.5
Dimethicone	1-3
Glycerine (Humectant)	1-3
Laureth-7 Citrate	1
Ethylene Glycol Distearate	0.5
Butyl Hydroxy Toluene	0.05
EDTA (Etilendiamin tetraase-tik asit)	0.3
Evans	0.01
Water	k.m.

Table 2. Ingredients and quantities used for eight different shampoo formulations

Ingredients	**NI-S-1**	**NI-S-2**	**NI-S-3**	**NI-S-4**	**NI-S-5**	**NI-S-6**	**NI-S-7**	**NI-S-8**
Rosa Damascena Extract	0.5	0.5	0.5	0.5	1	1	1	1
Urtica Dioica Leaf Extract	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4
Allium Sativum Bulb Oil.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Triticum Vulgare (Wheat) Germ Oil	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4
Guar Gum	0.1	0.3	0.6	0.9	0.1	0.3	0.6	0.9
Amonyum Lauril Sülfat	5	5.5	6	6.5	5	5.5	6	6.5
Amonyum Lauril Eter Sülfat	6	6.5	7	7.5	6	6.5	7	7.5
Cocoamide Mea	4	4.5	5	5.5	4	4.5	5	5.5
Betain	5	5.5	6	6.5	5	5.5	6	6.5
Dimethicone	1	2	3	4	1	2	3	4
Glycerine (Humectant)	1	2	3	4	1	2	3	4
Laureth-7 Citrate	1	1	1	1	1	1	1	1
Ethylene Glycol Distearate	0.5	0.5	0.5	0.5	1	1	1	1
Butyl Hydroxy Toluene	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
EDTA	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Essence	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Water	74.95	70.95	66.65	62.35	73.75	69.55	65.25	60.95

Control of Physicochemical Properties

Following the shampoo's retention in a standard room environment, its visual characteristics, including color, were evaluated.

Foaming Power

The cylinder shaking method was employed to assess the foaming capacity of the sample. Five milliliters of the solution was extracted from the shampoo and placed in a 25-ml graduated cylinder. The cylinder's opening was sealed manually and the sample was agitated for 10 seconds. After that, 1-5 ml were transferred to 5 distinct tubes at varying concentrations and the tubes were closed and agitated for 10 seconds. The total volume of the foam contents was recorded 1 minute after shaking. The foam volume was calculated exclusively. Following the initial shaking, the foam volume was recorded in centimeters at one-minute intervals for four minutes, and the foaming index was calculated (11). This process was repeated three times and documented.

Wetting Strength

The wetting ability of a solution is a function of its concentration. For an effective solution, the wetting time should be as low as possible. In other words, the less the wetting time, the more efficient the solution. Table 2 shows that the soaking time of all three shampoos is minimal, indicating that they are of good quality.

Cleaning Power Test

Two drops of shampoo are added to 10 mL of water, and one drop of ink is added to the test tubes. The mixture is then shaken 10 times. The amount of ink present in the foam is rated as none, light, medium, or heavy.

Rheological Studies

The apparent viscosity was determined by using a PCE-RVI-10 rotational viscometer (PCE Instruments, Hamburg, Germany). The rotational rheometer measures viscosity by calculating the torque required to rotate the spindles immersed in the fluid. The applied torque is related to the viscous friction on the shaft and was measured with an L2 spindle at 6 rpm for 120 sec throughout. The slip rate is calculated by the rotation speed and the gap between the shaft and the container (Equation X). The gap between the shaft and the container was set to 1.25 mm, resulting in a ratio of 1.2 between the container diameter and the shaft. The sliding speed was calculated according to the formulation x.

$$y = 2 \times \frac{2 \times \pi \times Ni}{60} \times \frac{R_0^2}{R_0^2 - Ri^2} \quad (1)$$

In the equation, "y" indicates the sliding speed in s-1, "Ni" indicates the rotation speed in rpm, and "R0" and "Ri" indicate the radius of the bowl and shaft in mm. Apparent viscosity values were plotted as a function of shear rate and fitted into Equation x according to the Ostwald-de Waele relationship (13).

$$\eta = K.\gamma^{n-1} \quad (2)$$

In this context, K represents the viscosity coefficient, while n is the flow behavior index, which is calculated from exponential regression (14).

Calculation of the interval of safety (MoS)

The systemic exposure dose (MOS) of the shampoo formulation was taken as 10.46 g/day, with application frequency (1/ day) and daily exposure level according to

skin surface area (1440 cm2) and product type, as specified in cosmetic guideline 3. Systemic exposure dose was calculated with the formula x and safety margin values were calculated with the formula x. Looking at the results, the exposure doses and MoS values of the components in all formulations were found to be>100.

$$SED = \frac{DAa\ (\mu g/cm2) \times 10\text{-}3mg/\ \mu g \times SSA\ (cm2) \times F(gün\text{-}1)}{60} \quad (3)$$

$$MoS = \frac{PODsys}{SED*\%kons.} \geq 100\ (yetişkinler\ için) \quad (4)$$

Stability Study

Stability studies were carried out to evaluate the product's appearance, color, pH, viscosity, and microbial growth in three different conditions: refrigerated, at room temperature, and in an oven set at 45°C for six months. These studies were conducted following the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) directives and TITCK cosmetic regulations.

RESULTS

Microorganism Analysis Result

The shampoo product was tested and no growth was observed. Table 1 shows that no growth was observed on the 14th and 28th days following the effect of the preservative.

Table 3. Microbiological test results of cosmetic sample

Microorganisms	Conclusion
Total number of aerobic mesophilic microorganisms (Bacteria, mold, yeast)	≤1 x 102 cfu/ g or ml
Yeast and mold	≤10^2 cfu
Escherichia coli	1 g ve ml not found
Pseudomonas aeruginosa	1 g ve ml not found
Staphylococcus aureus	1 g ve ml not found
Candida albicans	1 g ve ml not found

Physicochemical Test Results

Table 4 presents the rheological properties, foaming index, cleaning effect, color, and appearance results of the formulations with a skin pH value of 5.5 suitable for the scalp.

Table 4. Appearance, pH, texture, foam index, and rheological results of the formulations (n=3).

Formulation code	Appearance	Color	Texture	Foaming index	Direct Dispersion Test	Ph Measurement Result	Viscosity (Pa.s)	Glide Speed (1/sec)	Glide Voltage (D/cm²)
NI-S-1	Homojen	Opaque-White	Smooth	500	Light	5.612±0.12	12.89±0.30	0.11	117.15±2.77
NI-S-2	Homojen	Opaque-White	Smooth	250	Light	5.343±0.13	14.39±0.36	0.11	130.82±3.27
NI-S-3	Homojen	Opaque-White	Smooth	250	None	5.435±0.11	23.30± 0.90	0.12	194.17±7.52
NI-S-4	Homojen	Opaque-White	Smooth	125	None	5.342±0.10	35.39±0.65	0.11	321.70±5.93
NI-S-5	Homojen	Opaque-White	Smooth	500	Light	5.453± 0.09	13.15±0.18	0.12	109.58±1.47
NI-S-6	Homojen	Opaque-White	Smooth	250	Light	5.498± 0.11	15.19±0.28	0.13	116.87±2.81
NI-S-7	Homojen	Opaque-White	Smooth	250	None	5.567± 0.12	26.43± 0.75	0.12	220.22±6.26
NI-S-8	Homojen	Opaque-White	Smooth	125	None	5.601± 0.12	37.32±0.77	0.13	287.08±5.89

Rheological Study Result

The anti-dandruff and hair-strengthening natural shampoo formulation demonstrated shear thickening according to the Ostwald-de Waele model and exhibited pseudo-plastic flow. The results indicate that the shear rate is between 0.15 and 30.59 s-1, the shear stress is between 32.58 and 249.34 (D/cm2), and the viscosity values are between 213 and 81.50 Pa.s (Table 5, Fig. 1).

Table 5. Rheological properties of optimum formulation NI-S-7

Viscosity (Pa.s)	Glide Speed (1/sec)	Glide Voltage (D/cm²)
215.00±5.86	0.15	33.06±0.85
134.00±7.81	0.31	40.08±0.81
90.57±6.06	0.76	69.16±0.06
54.77±5.53	1.53	80.80±4.30
38.43±4.41	3.06	118.44±13.33
25.03±1.50	6.12	155.69±6.78
13.83±3.06	15.30	216.85±22.66
8.12±1.41	30.59	247.99±9.18

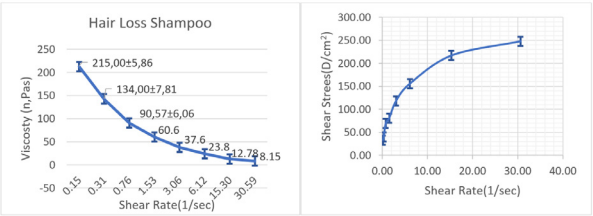

Figure 1. Viscosity results versus shear rate for NI-S-6 (n=3)

Confidence Interval Results

Upon examination of the results. it was determined that the exposure doses and MoS values of the components in all formulations were greater than 100.

Stability Results

The physicochemical properties of shampoo formulations prepared with extracts obtained from natural herbal sources were found to be preserved under all stability conditions. Following organoleptic controls, it was observed that the specific appearance and color remained unchanged from day 0 to 6 months. It was also observed that the pH value was within the range of pH 5.3-5.8 under all conditions, which is compatible with the acceptable pH value for the skin. The viscosity of the shampoo formulation was monitored over time at different temperatures. The viscosity values were found to vary between 23,800 and 26,382 (Pa.s). To control microbiological growth, a protective effectiveness test (45°C) was carried out in an oven, and no growth was detected (Table 6).

Table 6. Stability results; Appearance, color, smell, and microbiological growth results as of Day 0, Month 3, and Month 6

Stabılıty Condıtıon	Stabılıty Condıtıon	Appearance	Colour	Smell	Mıcrobıologıcal Growth In The Oven (45°C)
Room Conditions	0 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	3 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	6 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
In the oven (45°C)	0 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	3 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	6 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
Refrigerator	0 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	3 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth
	6 st month	Homogeneous-Cream	Specıfic	Specıfic	NO growth

DISCUSSION

In recent years, shampoo formulations have focused on products with functional features, such as strengthening hair, preventing dandruff, and accelerating hair growth. However, research indicates that products are frequently contaminated during consumer use (15). The use of cosmetic products without consideration of their expiration date, the sharing of products with multiple individuals, the insertion of contaminated fingers, and the wetting of products with saliva are among the causes of contamination (16). It has been established that cosmetic products with a high water content are more susceptible to microbial growth if contaminated during use (17,18).

A review of the literature reveals that several microorganisms commonly found in cosmetic products include *P. aeruginosa*, *S. aureus*, *Enterobacter spp.*, *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Candida albicans*, and *Aspergillus spp.* Some microorganisms that cause contamination metabolize some substances in the product with their hydrolytic enzymes. As a result, various changes may occur gas formation, viscosity and color changes, taste changes, turbidity, precipitation, and membrane formation (19,20,21).

Intact skin and mucous membranes serve as a physical barrier for microorganisms. Damage to these structures and disruption of their integrity for various reasons allow microorganisms to enter the body. It is well documented that many microorganism species detected as contaminants in cosmetic products are pathogenic and can cause serious infections (19,22). *Pseudomonas aeruginosa* is a microorganism that is frequently implicated in the contamination of cosmetics. It is widely distributed in the

natural environment, particularly in soil and water. As an opportunistic pathogen, it can cause serious infections, including those of the respiratory, urinary, wound, and burn tracts, meningitis, and septicemia. These infections are particularly prevalent in individuals with immunodeficiency. Pseudomonas aeruginosa is among the nosocomial infection factors for which treatment is challenging due to its ability to rapidly develop resistance to antimicrobials (22). It is imperative that cosmetics, which are non-sterile pharmaceutical products, be delivered to consumers in an effective, safe, and high-quality manner. This is guaranteed in our country by the legislation published by TITCK. Contamination of cosmetic products with microorganisms can have detrimental effects on consumer health and cause significant economic losses. Research indicates that cosmetic products are often exposed to contamination during use by the consumer. The most significant causes of contamination during use are the use of products after the stated expiration date, use by multiple individuals, insertion of fingers or contaminated objects into the product, and contact with air. In a study, different cosmetic product groups were evaluated in terms of microbiological contamination. The first group was used by a single individual for its usage period, which had not yet expired. The second group was used by more than one individual for its usage period, which had not yet expired. The third group was used after the expiration date. The results indicated that the second group exhibited a higher contamination rate than the first group, and the third group exhibited a higher contamination rate than both groups (17, 23, 24). In the European Union, green agreement processes and sustainability studies tend to favor the use of natural ingredients in the preparation of formulations. Among the eight different formulations prepared in this study, the NI-S-7 formulation is particularly noteworthy in that it meets these needs, containing *Rosa damascena* Mill, *Urtica dioica* L, and *Triticum vulgare*. Furthermore, an examination of the formulation's physicochemical properties revealed that an increase in the concentration of guar gum directly affected viscosity, preventing fluidity from occurring in the bottle. Formulations NI-S-1, NI-S-4, NI-S-5, and NI-S-8 were eliminated because they did not have the desired flow properties. It was observed that the active ingredient was high in the formulations NI-S-2, NI-S-3, NI-S-6, and NI-S-7, which were suitable in terms of flow properties. However, the NI-S-7 formulation was thought to be most suitable due to its low Guar gum content and acceptable flow properties. All formulations were found to be suitable for application in terms of pH values. The strongest formulations in terms of foam index were identified as NI-S-4 and NI-S-8. The remaining formulations, in descending order of foam strength, were NI-S-2, NI-S-3, NI-S-6, and NI-S-7. The formulations with the least foam strength were identified as NI-S-1 and NI-S-5. The NI-S-7 formulation was found to be a suitable foam index, as the surfactants used exhibited a low index value, which was deemed acceptable.

A comparison of the cleaning properties of the two formulations, NI-S-7 and NI-S-6, revealed that NI-S-7 was the more effective of the two. Consequently, NI-S-7 was selected as the optimal formulation for microbiological stability.

CONCLUSION

The transformation of products grown in our country into commercial products will support the economy. The shampoo to be developed will be obtained from naturally sourced plants grown in Turkey and can be sold in pharmacies for medical disorders with its aromatherapy effect as it preserves the typical scent of the rose plant. To prevent microbiological contamination in cosmetic products, microbiological controls must be carried out starting from the raw material, and production must be carried out under hygienic conditions by Good manufacturing practice (GMP) rules. The legislation stipulates that preservatives should be added to products at risk of contamination, which can protect the product in a way equivalent to the expected shelf life plus the period of use. The preservatives should have a broad spectrum of action and not have any allergic, toxic, or irritating effects on the consumer. After production, the product's shelf life and usage time after opening should be determined through stability tests.

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ORIGINAL ARTICLE / ÖZGÜN MAKALE

Development of an Inhibition-Based Colorimetric Method For Glutathione Determination

GSH Tayini için İnhibisyon Temelli Kolorimetrik Bir Yöntemin Geliştirilmesi

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Abstract

Objectives: Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine), one of the major cellular antioxidants, is an important non-protein intracellular physiological antioxidant with sulphhydryl groups for detoxification of reactive oxygen species (ROS) in all living organisms. GSH deficiency has been shown to be associated with many human diseases, including cardiovascular, immune and ageing diseases, arthritis and diabetes. Therefore, the development of an accurate, reliable and sensitive method for the determination of GSH in biological fluids is essential for the understanding of GSH homeostasis in medicine and biochemical research

Material and Methods: In this study, a very inexpensive, practical, rapid, sensitive, and highly specific colorimetric method for the determination of glutathione (GSH) that can be detected by the naked eye was developed. This method is based on the inhibition of horseradish peroxidase (HRP) by GSH. As the concentration of glutathione increases, a pink coloured compound consisting of 4-chlorophenol, H2O2 and 4-aminoantipyrine (4-AAP) decomposes as a result of the reaction catalyzed by HRP, thus reducing the intensity of the colour.

Results: While the linear range of the developed method was found to be between 15.6-1000 mM, the intra- and inter-day repeatability % coefficient of variation values of the method were less than 15%. The effect of potential interfering substances on the developed method was tested, and no interference was found, except for cysteine. Cysteine increased GSH response by 10%. The developed method was used for the determination of GSH in commercial serum samples, and results were obtained between 91-106%.

Conclusion: In conclusion, this study has developed a very simple, inexpensive and unique colourimetric method for the determination of GSH.

Keywords: Glutathione, Horse radish peroxidase, 4-AAP, Inhibition based

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Öz

Amaç: Başlıca hücresel antioksidanlardan biri olan glutatyon (GSH, L-γ-glutamyl-L-cysteinyl-glycine), tüm canlı organizmalarda reaktif oksijen türlerinin (ROS) detoksifikasyonu için sülfhidril grupları içeren önemli bir protein olmayan hücre içi fizyolojik antioksidandır. GSH eksikliğinin kardiyovasküler, bağışıklık ve yaşlanma hastalıkları, artrit ve diyabet dahil olmak üzere birçok insan hastalığı ile ilişkili olduğu gösterilmiştir. Bu nedenle, biyolojik sıvılarda GSH tayini için doğru, güvenilir ve hassas bir yöntemin geliştirilmesi, tıpta ve biyokimyasal araştırmalarda GSH homeostazının anlaşılması için gereklidir.

Gereç ve Yöntem: Bu çalışmada, glutatyon (GSH) tayini için çıplak gözle tespit edilebilen çok ucuz, pratik, hızlı, hassas ve oldukça spesifik bir kolorimetrik yöntem geliştirilmiştir. Yöntem, horseradish peroksidazın (HRP) GSH tarafından inhibisyonuna dayanmaktadır. Glutatyon konsantrasyonu arttıkça, 4-klorofenol, H2O2 ve 4-aminoantipirinden (4-AAP) oluşan pembe renkli bir bileşik, HRP tarafından katalize edilen reaksiyon sonucunda ayrışır ve böylece rengin yoğunluğu azalır.

Bulgular: Geliştirilen yöntemin doğrusal aralığı 15,6-1000 mM arasında bulunurken, yöntemin gün içi ve günler arası tekrarlanabilirlik % varyasyon katsayısı değerleri %15'in altında bulunmuştur. Potansiyel interferans maddelerinin geliştirilen metot üzerindeki etkisi test edilmiş ve sistein dışında herhangi bir interferansa rastlanmamıştır. Sistein, GSH için yanıtı %10 oranında artırmıştır. Geliştirilen yöntem ticari serum örneklerinde GSH tayini için kullanılmış ve %91-106 arasında sonuçlar elde edilmiştir.

Sonuç: Sonuç olarak, bu çalışmada GSH tayini için çok basit, ucuz ve benzersiz bir kolorimetrik yöntem geliştirilmiştir.

Anahtar Kelimeler: Glutatyon, Yabani turp peroksidazı, 4-AAP, İnhibisyon temelli

INRODUCTION

Glutathione (GSH) is a tripeptide consisting of the combination of glutamate, cysteine and glycine and containing sulfhydryl groups commonly found in animals. As an important metabolic substance in the body, it participates in the tricarboxylic acid cycle and glucose metabolism, can activate various enzymes to promote sugar, fat, and protein metabolism (1), and participates in many important biochemical reactions (2). Based on its antioxidant properties, it can remove free radicals and toxins, protect the sulfhydryl groups of important enzyme proteins from oxidation and inactivation, and ensure the normal operation of molecular physiological functions, such as proteins and enzymes (3). Abnormal GSH levels in the body are directly associated with specific diseases including cancer, human immunodeficiency virus (HIV), liver damage, and neurodegenerative diseases (3–5). Therefore, determination of GSH levels is very important for in vivo detection and biological diagnosis systems.

In the last 20 years, several methods have been proposed for GSH determination, such as methods based on high-performance liquid chromatography (HPLC) (6), UV-Vis spectrophotometry (6), fluorescence spectroscopy (7), and mass spectrometry (8). Despite acceptable sensitivity, some of these methods suffer from practical disadvantages, such as the need for expensive and high-tech instrumentation, time-consuming processes, and complex sample preparation processes. Therefore, it

is essential to develop a method for the rapid, inexpensive, and practical determination of glutathione.

Colorimetry is one of the most widely used methods to determine the concentration of a compound by measuring its colour or optical density (9) . In recent years, there has been an increasing interest in the colorimetry technique, which has advantages such as simplicity, low cost, and recognition even with the naked eye. This is because smartphones have become portable photometers with their advanced cameras and colour measuring software (10,11). In other words, when colorimetric methods are developed, the need for an extra device is reduced, unlike methods such as chromatographic and electrochemical methods.

Horseradish peroxidase (HRP) (E.C.1.11.1.7) is an enzyme that has been widely used and investigated for analytical purposes (12). It catalyses the reduction of hydrogen peroxide in the presence of a reducing compound. Methods involving peroxidases described in the literature are based on colorimetry, chemiluminescence, fluorescence and amperometric measurements (13,14). If the reducing agent is selected to produce a coloured product or to further react with a suitable chromogen to produce a coloured product, then hydrogen peroxide (or an oxidase substrate if HRP is combined with a hydrogen peroxide-producing oxidase) can be determined spectrophotometrically. A wide variety of reducing HRP substrates (such as phenol, aminophenols, indophenols, diamines and a number of other compounds) have been used in the spectrophotometric determination of H_2O_2. Trinder et al. (15) developed a method for the determination of H_2O_2 using HRP-catalysed oxidation of phenol in the presence of 4-aminoantipyrine. This method is the working principle of many oxidase-enzyme-based colorimetric kits. One of these is the glucose oxidase/peroxidase system, which was developed for glucose determination. In this reaction, glucose is catalyzed by glucose oxidase and converted to gluconic acid and H_2O_2. The resulting H_2O_2 reacts with 4-aminoantipyrine (4-AAP) and phenol to produce a pink-red-colored compound in the presence of HRP (16).

Some of the most important criteria for analytical method development are that the method should be simple, rapid, and inexpensive, without expensive equipment. GSH levels are directly related to specific diseases, including cancer, human immunodeficiency virus (HIV), liver damage, and neurodegenerative diseases. The inhibitory properties of the analyte to be determined also form the basis of some methods (17,18). Literature data indicate that Reduced glutathione is an HRP inhibitor (19). The aim of this study was to develop a fast, practical, inexpensive, and colorimetric method for the determination of glutathione, one of the most important cellular antioxidants, which can be detected even with the naked eye. Since HRP will be inhibited in the presence of glutathione, the intensity of the pink-red colour produced by the enzymatic reaction of 4-AAP, 4-chlorophenol and H_2O_2 with HRP will decrease. The decrease in absorbance constitutes the principle of determination of glutathione. No colourimetric method based on HRP inhibition for the determination of glutathione has been found in the literature. This is a unique aspect of this study.

MATERIALS AND METHODS

Chemicals and instruments

HRP (1200 U/L) was obtained from Biolabo (France), and 4-AAP, 4-chlorophenol, H2O2, potassium phosphate dibasic, glutathione, and other chemicals were obtained from Sigma Aldrich (USA). The solutions were prepared using ultrapure water. Spectrophotometric measurements were carried out with Multiscan FC Thermo Scientific device.

An Ethics Committee Approval Certificate is not required for studies to be conducted on commercially sold human cadavers, cadaver parts and other biological materials» (ETHICS COMMITTEE APPROVAL DOCUMENT INFORMATION NOTE 2020, n.d.). We declare that our study, the information of which is included above, is among the studies that do not require ethics committee permission due to the use of commercially purchased

Chemicals Required in the Study

Working buffer: 100 mM pH:7 Phosphate Buffer containing 5 mM 4-chlorophenol and 0.25 mM 4-AAP

Substrate: 0.5 mM H2O2 was prepared using water.

HRP: Prepared in 1200 U/L working buffer.

Glutathione: A 20 mM stock solution was prepared in artificial serum.

Preparation of artificial serum: 111 mM NaCl, 2.9 mM $NaHCO_3$, 2.2 mM K_2HPO_4, 0.8 mM $MgCl_2$, 2.5 mM urea, 5 mM KCl are mixed and the pH is adjusted to 7.4 (20).

Principle of measurement

The working principle of this method is illustrated in Figure 1. Accordingly, in the absence of GSH, the enzymatic reaction will take place with high efficiency and the pink-red colour of quinoneimine formed as a result of the reaction will be apparent. In the presence of GSH, HRP is partially inhibited (depending on the concentration), and therefore, the intensity of the color decreases. A decrease in the intensity of the color causes a decrease in absorbance. GSH can be determined from the graph drawn between the decrease in absorbance and GSH concentration.

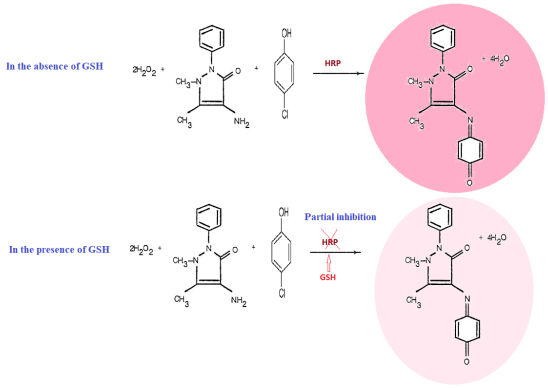

Figure 1. Working principle of the inhibition-based colorimetric glutathione determination method

Test protocol

100 mL HRP solution + 50 mL GSH + 50 mL (0,5 mM) H_2O_2 is added to an ependorf tube and left to incubate at room temperature and in the dark. The reaction was complete within 1 min. The absorbance of the formed color was measured using a spectrophotometer at a wavelength of 504 nm.

Determination of linear range

In order to determine the linear range of determination, standards are prepared by serial dilution from 4 mM GSH. A calibration graph was drawn with 16 points between 0-4 mM, and the range of linearity was determined as the linear range of determination.

Determination of intra-day and inter-day repeatability

The repeatability experiment was performed in 6 replicates at 3 levels, low (0.0625 mM), medium (0.25 mM) and high (1 mM) GSH concentrations, both within and between days, and the mean (x), standard deviation (S.D.±) and % coefficient of variation (% CV) values were calculated.

Determination of substrate specificity and interference effect

In order to determine the substrate specificity of the developed colorimetric GSH method, cysteine, alanine, glycine, glutamic acid, serine, tryptophan, and histidine substrates were used as 0.5 mM instead of GSH. The GSH response was accepted as 100% and compared with the GSH responses of other substrates. The data are plotted as a column graph.

In the interference effect experiment, the same substrates were added separately at the same concentration as that of the sample containing 0.5 mM GSH. The GSH response alone was accepted as 100%, compared with the responses to other substrates + GSH, and graphed.

Recovery

In the recovery experiment, commercially purchased serum samples were spiked with standard additives at 3 levels: low (0.0625 mM), medium (0.25 mM), and high (1 mM) GSH concentrations, and the percentage recovery was calculated.

RESULT AND DISCUSSION

Findings related to the working principle of the developed method

Colorimetric methods allow quantitative measurements to be performed using the relationship between the concentration and absorbance of a solution. To perform these measurements, a spectrum scan was first performed, and the wavelength with the maximum absorbance was selected for quantitative measurements. For this purpose, a spectrum scan was performed between wavelengths of 400-680 nm, including wavelengths in the visible region, and the results are shown in Figure 2. When examining the spectrum scan and the results of the solution obtained from the reaction in the absence of GSH and in the presence of 1 mM GSH (Figure 2), the wavelength at which the maximum absorbance was observed was 504 nm. Absorbance measurements were performed at this wavelength for further optimization and characterization. In addition, the principle of the method developed in this study is to determine GSH by the decrease in intensity of the pink colour formed by HRP activity and the inhibition of HRP by GSH. A careful analysis of Figure 2 shows that the decrease in the red peak is due to the presence of GSH. This confirmed the principles of this study. Optimization of the pH, temperature, buffer type, and concentration of the studied HRP-catalyzed method has been extensively studied (15,16). Therefore, in this study, the measurement conditions were established based on the literature information without repeated optimizations.

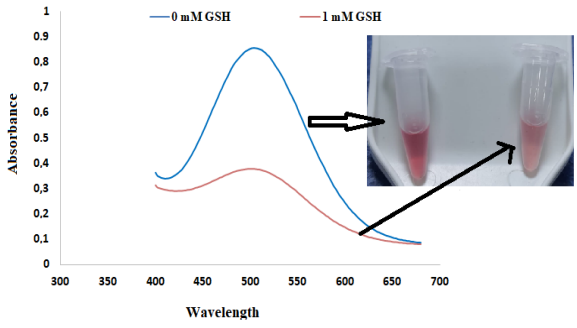

Figure 2. Colourimetric responses obtained in the absence and presence of 1 mM GSH

Results on the linear range of the developed method

The linear range of the developed method is one of the most important factors determining its limitations and performance. The developed method also aims to analyze serum. Therefore, calibrators were obtained by adding a standard to the artificial serum. Samples containing 4 mM GSH were serially diluted, and 16 calibration points between 0.245 m 4 mM were subjected to the test procedure and measured by spectrophotometry. The absorbance was plotted against concentration, and the results are shown in Figures 3 and 4. Figure 3 shows an image of the solutions formed as a result of the working method on the plate, the spectral scan of these solutions, and the GSH concentration-absorbance graph drawn using the absorbances at 504 nm. As shown in Figure 3, no decrease in colour intensity was observed between 0-7.8 mM GSH concentrations. Above 1 mM, the colour disappeared almost completely. The range in which the method showed linearity between concentration and absorbance was found to be between 15.6 mM-1 mM. The equation of the graph plotted in this range was y=0.0006x + 0.0301, and the R^2 value of the graph was 0.9945. The R^2 value of the graph is 0.9945, which is not only acceptable, but also close to perfect. Serum levels of GSH are in mM levels(21), and the linear determination range of the method we developed in this study includes serum GSH levels.

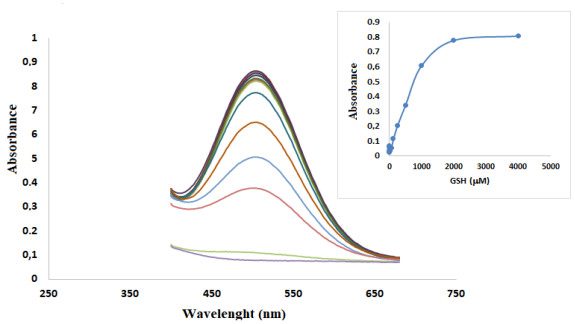

Figure 3. The resulting colours obtained in the presence of 0-4 mM GSH and their spectrum scan between 400-680 nm wavelength

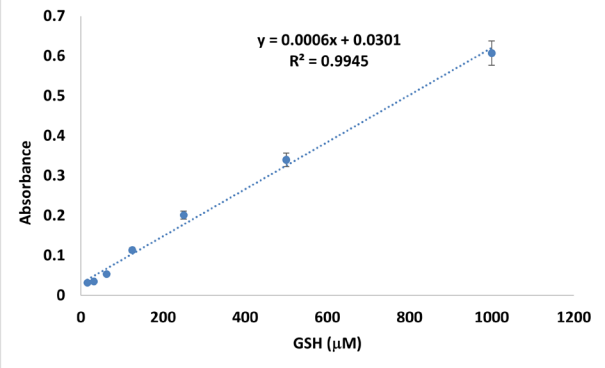

Figure 4. Standard graph for GSH concentrations between 15.6-1000 mM

Selectivity and interference effect of the developed method

To determine the selectivity and interference effect of the colorimetric method developed for GSH determination, cysteine, alanine, glutamic acid, serine, tryptophan, glycine, and histidine were tested at the same concentration (0.5 mM) as GSH. Although these compounds were added alone in the selectivity assay, they were added together with GSH (0.5 mM) in the interference effect assay. The response obtained in the presence of GSH (0.5 mM) was considered 100% and the response obtained from other compounds was plotted as a ratio to the GSH response. The results are shown in Figure 5. According to this, only cysteine gave a response of 13% among the compounds tested, whereas no response was obtained

with the other compounds. In the interference effect experiment, only cysteine increased the response of the method by 11%, whereas the other compounds had no significant effect on the GSH response. These results show that there was no interference effect on the developed method, except for cysteine.

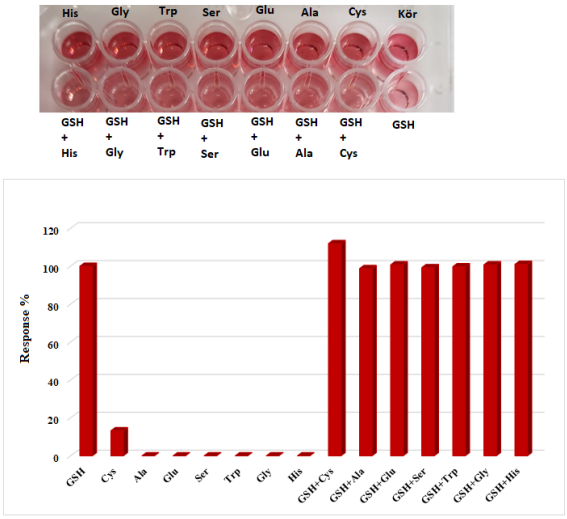

Figure 5. Findings on selectivity and interference effects of the developed method. Cys: Cysteine; Ala: Alanine; Glu: Glutamic acid; Ser: Serine; Trp: Tryptophan; Gly: Glycine; His: Histidine

Accuracy and precision test of the developed method

Intraday and inter-day repeatability and % recovery tests were performed at three levels: low (62.5 mM), medium (250 mM), and high (1000 mM). The repeatability study showed the precision of the method, and the recovery study showed the accuracy of the method. The results are presented in Tables 1 and 2. The repeatability of the method was lower at low concentrations and higher at medium and high concentrations. However, in all cases the repeatability of the method is below the acceptable limit of 15%. This shows that the results of the method are of acceptable precision. The retrieval results were between 91.6-106.9%. These values indicate that the accuracy of the method was close to 100%. The % recovery experiment was performed by standard addition to serum samples obtained commercially from Bio-Rad. The standard was plotted for both the artificial serum and real serum samples (Figure 6). Although the linearity of the graphs plotted with both matrices is good, the slopes of the graphs show a reduction of approximately 33% in the real serum matrix (0.006 vs. 0.004). This suggests that the blank used when studying serum samples should be a real serum sample.

Table 1. Findings related to intra-day and inter-day repeatibility of the developed method (n=6)

GSH (mM)	Intraday repeatibility CV%	Inter-day repeatibility CV%
62.5	11.75	14.55
250	6.83	7.41
1000	3.63	4.45

Table 2. Results on the recovery of the developed method in commercial serum sample (n=3)

Added GSH (mM)	Found (mM)	Recovery %
62.5	57.25±6.81	91.6
250	267.25±21.55	106.9
1000	924.75±36.52	92.47

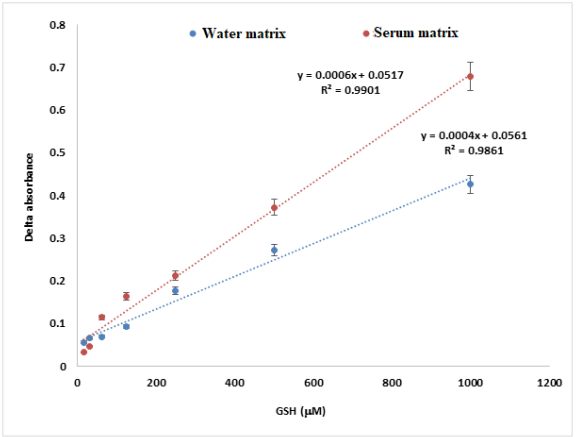

Figure 6. Standard plots obtained by standard addition to both artificial serum and real serum samples

CONCLUSION

In summary, this study demonstrated a colorimetric method for the determination of GSH using the mechanism of HRP inhibition by GSH. Since HRP will be inhibited in the presence of glutathione, the intensity of the pink-red colour produced by the enzymatic reaction of 4-AAP, 4-chlorophenol and H_2O_2 with HRP will decrease. The resulting decrease in absorbance constitutes the principle of glutathione determination. The developed method does not require any complex pretreatment process, does not require expensive equipment, substrate, and enzyme systems, allows rapid determination, is inexpensive, and very practical. Only a spectrophotometer or even smart phones with colour measuring software is sufficient for the method to work. The method showed linearity between concentration and absorbance within the range of serum GSH concentrations. Cysteine exhibited 13% response, whereas the other compounds showed no response. In the interference tests, only cysteine elicited an 11% increase in method response, indicating minimal interference, except for cysteine. The repeatability of the method was lower at low concentrations but improved at medium and high concentrations, remaining below the acceptable 15% limit in all cases, indicating acceptable precision. The recovery results fall between 91.6-106.9%, suggesting a method accuracy close to 100%. % recovery experiments using standard addition to commercially obtained serum samples from Biorad show satisfactory linearity in both artificial and real serum matrices. However, the slopes of the graphs for real serum were reduced by approximately 33%, indicating the necessity of using real serum as a blank in serum sample studies. Table 3 shows a comparison of GSH determination methods. Accordingly, when compared with the literature, it can be said that the determination method developed in this study has a wide determination range and an ideal sensitivity. At the same time, the time required for the method developed in this study was very short, that is, 1 min. Although the sensitivity of the developed method is ideal, the limit of determination can be reduced by using nanoparticles.

The limitations of the study are that it was not compared with a reference method, and it was not integrated into a mobile phone. With the software installed on the mobile phone, it is possible to easily measure glutathione through color measurement.

Table 3. Comparison of GSH determination methods

Method	Prob	Linear range	Reference
Colorimetric	Naphthalimide-capped AuNPs	0.025-2.28 mM	(22)
Colorimetric	Carbon nanodots	0-7 mM	(23)
Colorimetric	Cu-S nanoparticles	0.5-10 mM	(24)
Fluorescence	N-Doped Carbon Dots	0.2-1000 mM	(25)
Colorimetric	Cobalt oxyhydroxide nanosheets	0.1-300 mM	(26)
Colorimetric & Fluorescence	Red-emmiting N-doped CDs	12.5-800 mM	(27)
Colorimetric	TEMPO/ $Cu(acac)_2$/TMB	1-100 mM	(28)
Colorimetric	4-AAP, 4-Klorofenol ve H_2O_2	15.6-1000 mM	This study

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