T.C.
YEDİTEPE UNIVERSITY
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STUDIES ON HERBAL DYE FORMULATIONS (BİTKİSEL BOYA FORMÜLASYONLARI ÜZERİNDE ÇALIŞMALAR)

MASTER OF COSMETOLOGY THESIS

BEYZA DOĞAN

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ONAY

Bu tez Yeditepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliğinin ilgili maddeleri uyarınca yukarıdaki jüri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu'nun 11/09/2017 tarih ve 2017/11-01 sayılı kararı ile onaylanmıştır.

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ABBREVIATIONS

Residual Pigment Contribution: (RPC)

ME-PPD: 2-methoxymethyl-p-phenylenediamine

PPD: p-phenylenediamine

PTD: p-toluenediamine

SCCP: Scientific Committee on Consumer Products

EDTA: Ethylenediaminetetraacetic acid

SCEs: Sister Chromatid Exchanges

IARC: International Agency for Research on Cancer

FDA: The Food and Drug Administration

HPLC: High Performance Liquid Chromatography

FM: Final Mixture

SEM: Scanning Electron Microscope

CFU: Colony Forming Unit

SD: Standard Deviation

MD: Mean Diameter

USP 30 – NF 25: The United States Pharmacopeia 30 and The National Formulary 25

S. spp.: Salmonella Enterica

E.coli: Escherichia coli

ABSTRACT

This study aims to investigate the performance of newly developed dye formulations with using different herbal extracts on various hair strands. For this purpose first of all, the water extracts were prepared either alone or combinations of various powdered leaves and flowers of dye yielding plants such as Orchanet root (A) (*Alkanna tinctoria* (L.), Walnut fruit pericarp (W) (*Juglans regia L.),* Black okra flower calyx (B) (*Hibiscus sabdariffa L.),* Henna leaves (H) (*Lawsonia inermis*). These extracts were used to obtain different colors as natural colorants for hair strands. Natural and synthetic dyes performances on hair were assessed by various methods such as Light microscopy, Scanning Electron Microscopy (SEM), measuring mechanical strength (by Instron® tester), formulation study and microbial safety test. Hair strands used in this study were obtained from volunteers who were in different age and gender. The obtained hairs were encoded as natural white, brown, blonde (NW, NB and NY), respectively. They were then treated with dye formulations and were encoded as brown, blonde, red hairs (SB, SY and SR), respectively. Finally, the reconstituted powder form of herbal dye formulation was developed with herbal extracts and their successful combinations. Natural hair dyes provided from herbal extracts were evaluated certain methods as described below. First of all, the extracts were performed to the hair and color changes were examined in certain periods of time (during 30 days). The cuticle layer of the hair was observed by light microscopy in order to compare synthetic and natural hair dyes for efficacy test. The coloring power and stability of dyes were examined by naked eye and light microscopy method after washing the hair with shampoo and drying at several times. The mechanical strength and elongation of treated or natural hair strands which applied dyes from certain plant extracts and synthetic dyes were evaluated by Instron® tensile test device. Superficial appearance and changes in the diameter of the dye treated or natural certain hair strands were also observed by SEM microscopy and taken their photos by camera. Finally, pharmacopeia microbial safety test (USP 30 - NF 25) was performed only to formulation of natural dye extracts. According to sterility results, all formulation of dye extracts met the requirement limit of microbial safety test. The most dramatic color change was observed the combination of Walnut fruit pericarp and Henna extracts (W+H) when apply to all hair type. The cuticle of synthetic dye applied hair (especially blond hair with treated synthetic dye (SY-2)) appeared damage. Our findings were also confirmed with SEM analysis. The resconstitutable powder natural dye formulation could be potentially efficient, safe new hair dye candidate for future.

ÖZET

Bu çalışmada değişik bitki ekstreleriyle geliştirilen yeni formülasyonlar farklı saç tellerine uygulanarak etkinliği in vitro metotlarla değerlendirilmiştir. Bu amaçla, seçilen bitkilerin toz edilmiş çiçek ve yaprak kısımlarından hazırlanan hava civa kökü, ceviz kabuğu, kara bamya çiçeği, kına yaprakları su ekstreleri yalnız ve farklı kombinasyonları şeklinde çeşitli renkler elde edilmek üzere hazırlanmıştır. Doğal ve sentetik boyaların saçtaki performansı ışık mikroskobu, taramalı elektron mikroskobu, mekanik gücün ölçülmesi (Instron cihazıyla), formülasyon çalışması ve mikrobiyal güvenlik testi gibi yöntemlerle değerlendirilmiştir. Bu çalışmada kullanılan saç örnekleri doğal beyaz, kahverengi, sarı (NW, NB, NY) şeklinde kodlanmıştır. Boyalı saçlar sırasıyla kahverengi, sarı, kırmızı saçlar (SB, SY ve SR) şeklinde kodlanmıştır. Son olarak bitki ekstreleri ve onların başarılı kombinasyonlarından yeniden yapılandırılan toz şeklinde bitkisel boya formulasyonu geliştirilmiştir. Bitki ekstrelerinden hazırlanan doğal saç boyaları aşağıda tanımlanan bazı metotlarla değerlendirilmiştir. İlk olarak, ekstrelerin saçlar üzerindeki performansı ve renk değişimleri belirli zaman aralığında 30 gün boyunca çalışılmıştır. Saçın kütikül tabakası sentetik ve doğal saç boyalarının etkinliği karşılaştırılmak üzere ışık mikroskobuyla gözlemlenmiştir. Saç boyasının kalıcılığı ve gücü çıplak gözle ve ışık mikroskobuyla saçların şampuanla birkaç kez yıkaması ve kurutulmasını takiben incelenmiştir. Bitki ekstreleri ve sentetik boyalarla işlem görmüş saç ve doğal saçlarla mekanik güç ve uzama Instron cihazıyla değerlendirlmiştir. Ayrıca, taramalı elektron mikroskobuyla ve kamera ile alınan fotoğraflarla boya ile işlem görmüş ve doğal saç tellerinin yüzeysel görünümü ve çapındaki değişiklikleri gözlemlenmiştir. Son olarak, sadece bitkisel boya ekstrelerinin formülasyonuna farmakopedeki mikrobiyal güvenlik testi(USP 30 - NF 25) uygulanmıştır. Tüm boya ekstreleri yapılan mikrobiyal güvenlik testine uygun bulunmuştur. En belirgin değişiklik tüm saç tiplerine uygulanan ceviz ve kına karışımlarından elde edilmiştir. Sentetik boya uygulanmış saçın kütikülünde (özellikle işlem görmüş sarı saça sentetik boya uygulanmış saçta) hasar ortaya çıktı. Buluşlarımız SEM analiziyle onaylanmıştır. İlerisi için, yeniden yapılandırılan toz şeklinde doğal boya formülasyonumuz etkili, güvenli saç boyası adayı olabilir.

1. INTRODUCTION AND AIM

Almost every human has changed their hair color and hascolored his-her hair in a certain period of his-her lives. This situation has occured since the formation of human communities. The basis for hair dyeing dates back to ancient times. In history, it is known that Persians, Israelis, Greeks and Romans had dyed their hair by using minerals or natural materials. The first historical record on the use of natural herbal dyes was encountered in old Egyptian documents about 4000 years ago. Roman men had dyed their greying hairs with the help of lead acetate, today known as a metallic hair dye as a result of the lead particules be soaked in sour wine. While Roman women had got the benefit of sun light in order to lighten their hair.

The studies on hair coloring technology developed with the discovery of synthetic dyes. With being applicable of hair dyes at home, the image of the woman and, consequently the effect on social life has started to change. Hair dyes have a significant place in cosmetic products applied to the hair. Today, 50 % of womenhave dyed their hair. Half of those have dyed to close their greying hair, and the rest of them dyed to give their hair gleam and create an unnatural color.

In this study, vegetable dyes have been handled with being natural origin so as to determine the effects and duration of usage because of very little penetration to hair cuticle of prepared herbal dyes. Synthetic dyes carries risks and side effects to human health and they caused abrasion and denudation in cuticle so herbal hair dye is preferred prepared authenticated herbs that not to contain any harmful ingredient and also they don’t damage cuticle. The water extracts of Orchanet, Black okra, Walnut, Henna plants both separately and in combinedforms were dissolved in water and then applied to hair (healthy or damaged hair) to identify various color shades. The stability of herbal hair dyes was analyzed during 30 days. The cuticle layer of hair was examined to compare synthetic hair dye and herbal hair dye for long lasting efficacy test. Instron tensile test, Scanning Electron Microscopy (SEM) and Light Microscopy techniques were taken into account to use in terms of the tensile strength, stability, and morphological properties of coloring power at dyed hair with these extracts.

1.1. HAIR

A hair is stated as a slender, fibrous structure growing from follicle in the skin of mammals. Hair strand basically is made up of keratin and amino acid composition of cuticle and cortex of hair keratin differs through cuticular layers. The cuticle of hair keratin contains large amounts of amino acids of serine, glycine, and proline so it acts as hydrophobic character. [1] The keratin is insoluble complex protein structure found in epidermal structure. The keratin classified as amorph and crystal plays an important role interms of detecting the structure and properties of hair. The protein chain of keratin is configured by hydrogen bonds, disulfur bonds, salt bonds, peptit bonds, van der waals forces. [2]

The human scalp, eyebrows, and lashes include long, thick, pigmented terminal hair shafts, and also the body consists of short, thin and often unpigmented vellus hairs. In human, totally 5 million hair follicles of which 80,000 to 150,000 are located on the scalp is estimated. The elongation of scalp hair follicles shows between 0.3 and 0.5 mm per day. Its growth rate changes with the proliferation and follicular differentiation of the matrix-keratinocytes in the hair bulb. The thickness of the hair shaft associates with the size of hair bulb dictated by the volume of mesenchymal component of hair follicle. [3]

All hair follicles in human are formed when a fetus is five months old. A person has approximately 150 000 hair. This ratio is only three percent of hairs in human body. As distinct from the hairs in the body, the hair strand also includes phospholipids, cholesterol, and fatty acids. The weight of the hair constitutes approximately its 65-96 % from proteins, its 3 % water, the rest of it from free amino acids, pigments, lipids, trace elements. [3]

1.1.1. Morphological Properties of Hair

There are three major segments to the hair shaft taking place from the outside to the inside; the cuticle, cortex, and medulla. [4]

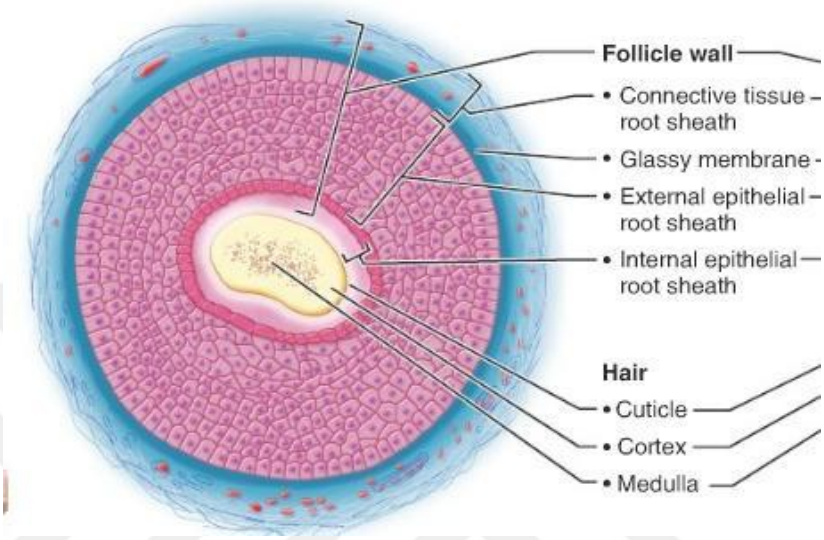


Figure 1.1. The human hair follicle: concentrical layers, main regions, and structure.

The cuticle is the translucent outer segment of the hair shaft containing scales. It acts as a protective covering of the hair shaft due to the water repelling of shaft. The scales found in the cuticles contribute to detect shine, health and also the ability of hair becoming tangled. When permanent hair color products are applied, they must penetrate the cuticle to alter hair color.

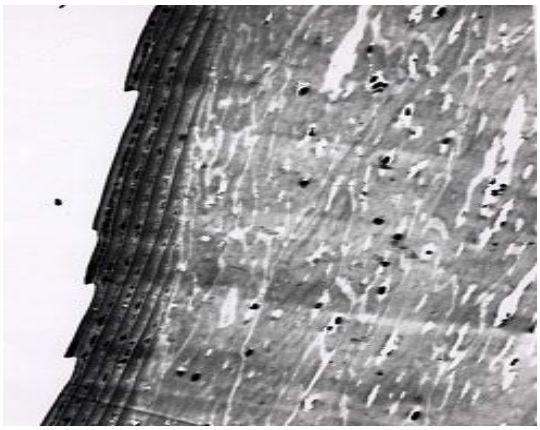


Figure 1.2. Scanning Electron Photomicrograph of Hair

The cortex is the middle segment of the hair shaft consisting of protein chains. It functions as hydrophilic and absorbs water. It may include pigment granules, cortical fusi, and ovoid bodies is oval-to-round-shaped structures. The pigment detecting the natural color of the hair is involved in cortex. During perm and color services, permanent changes are performed in cortex.

The medulla is the hollow central core of the hair shaft and it may not be found in all hair types. When it is filled with air, it shows as a opaque structure by transmitted light or as a white structure by reflected light, but after filling it with mounting medium or some clear substance, structure indicates clear in transmitted light. The medulla has generally amorphous in appearance in human hairs, its structure is well defined in animal hairs.

Figure 1.3. Diagram of Medullas

1.1.2. Hair Types

It is crucial to analyze the hair type during consultation so as to perform hair color on anyone's hair. Detecting the density, texture, and curl pattern provide convenience in deciding what to use for hair color and application.

The hair is evaluated and examined how many hairs are in each square inch on the head in order to detect the density of the hair.

Hair could be:

- Thin
- Average
- Thick

The density of the hair is determined to able to pull it back into a ponytail. In this photograph, the density is thin.

Figure 1.4. An example of a person with thin hair.

Figure 1.5. An example of a person with average density.

It is necessary to detect the density of the hair to count how many hairs head per square inch.

Thick hair is something more people would like to have. Whether your hair is long or short, having thick hair just seems to look better. In colder climate hair tends to be thinner, while in warmer regions hair is often thicker.

Figure 1.6. An example of a person with thick hair.

1.1.3. Function of Hair

Hair found in all mammals possesses various functions. It regulates mainly body temperature and also remains the body warm by insulating. It serves as temperature regulator related to the muscles in the skin. When the outside temperature is cold, these muscles pull the hair strands to the upright to form pockets trapping air. Trapped air acts

as insulator next layer to the skin by warming. In addition, it reduces friction to protect aganist sunlight by taking charge as sense organ. [5]

1.1.4. Structure of Hair

Human hair basically consists of alpha keratin proteins found in hair fibres. Hair structure constitutes three main morphological components the multicellular cuticle sheath, the medulla, and fibrous cortex. The cuticle, protective layer keeping the core of the fibers composed of beta keratins. These have a scaled structure between seven and ten superimposed layers and cuticle edges. Epicuticle coating the cysteine-rich exocuticle covers the scale cells of the cuticle. Finally, there is endocuticle taking part in the interface of the cortex and it is responsible for the remaining cell organelles. [3]

1.1.5. Types of Hair Color

1.1.5.1. Temporary Hair Color

It covers only cuticle layer and doesn't change the natural pigment. It lasts approximately one shampoo and doesn't mix the developer. There some examples: hair color sprays, color styling mousses, color intensifiers, color-enriching shampoos.[4]

1.1.5.2. Semi-Permanent Hair Color

It coats the cuticle and penetrates the outer layer of the cortex. It doesn't modify natural pigment and doesn't mix the developer. It lasts approximately four to five shampoos and uses large direct dyes. [4]

1.1.5.3. Deposit-Only Hair Color

It penetrates the cuticle and the multiple segments of the cortex. It doesn't lighten natural pigment and mix with low volume developer. It utilizes a combination of coupling dyes and direct dyes and acts excellently as gray coverage. [4]

1.1.5.4. Permanent Hair Color

It penetrates totally the cortex and permanently modifies natural pigment. It cannot be washed out and mix with any volume developer. It also lighten hair and functions as gray coverage. [4]

1.1.5.5. Bleaches

These lighten natural hair color and apply the toner to the hair or decolorize unnatural pigment by decolorizing the natural pigment through oxidation. [4]

1.1.5.6. Metallic Dyes

When the metallic dyes are performed daily, they cause the gradual coloration of gray hair. These are not compatible with professional chemical services. Today, on the markets, lead acetate solutions are commonly used as metallic dyes.[4]

1.1.6. Color Theory

There are main three colors (red, yellow, and blue) on the hair to provide natural-looking colors changing degrees. A secondary color (green, violet, and orange) will be obtained, if hair is minus a primary. Neutral color is get by combining equally three primary colors. Color tones neutralizes each other in complementary colors. [4, 7]

Color wheel represents all colors in the rainbow: red, orange, yellow, green, blue, to violet. Any color can be obtained from color wheel that detects each color pigment. [4]

All hair color being natural and artificial appear from red, yellow, and blue known as primary colors. Red and yellow constitute warm tones, but blue is the coolest tone. [4]

Secondary colors are obtained from mixture of primary colors. They are green, violet, and orange that can give us more choices with coming it to color. [4]

1.1.7. Level System and Residual Pigment Contribution (RPC)

Level system analyzes whether it is light, dark or medium and shows numerical values for level. Hair color undergones the chemical process to break down the natural pigment called melanin in the hair. It is removed from cortex and the natural pigment left in the hair at the ideal level is called as Residual Pigment Contribution (RPC). [4]

1. 2. HAIR DYES

1.2.1. Types of Hair Dyes

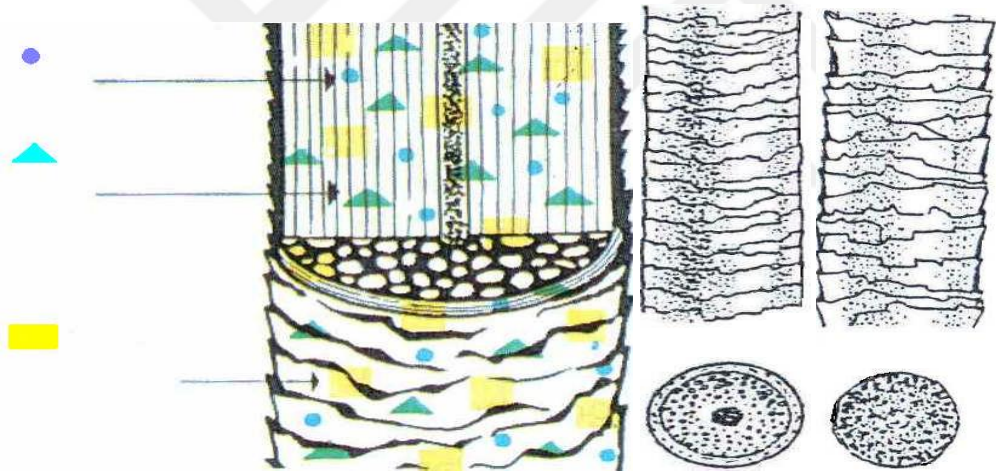


Figure 1.7. Entering of the hair dye builders to the hair. Healthy hair. Absorbent and porous hair.

Hair dyes are cosmetics that can be used to mask new hair growth with regular application. These are classified according to the origin: Vegetable hair dyes (henna, chamomile, and cinchona). They are non toxic and last only a short time. Mineral or metallic hair dyes (silver nitrate). They gradually darken or lighten hair for daily use. These dyes lasting for weeks or months are potentially toxic and aren't compatible with hydrogen peroxide. Synthetic hair dyes are classified according to degree of permanence and wash fastness as follows. [8]

1.2.1.1. Temporary Non-Oxidative Hair Dyeing

The temporary non-oxidative dyeing with high molecular weight maintains on the surface of the cuticle. [8] It provides specific properties to add colorful reflections, to remove the yellowish effects of the white hair, and to coat a small quantity of white hair. [9] It also contributes to deposit on the hair strands by dyeing of hair involving up to 15 % white hair. [10]

1.2.1.2. Semi-Permanent Non-Oxidative Hair Dyeing

These dyes are formulations consisting of basic (cationic) dyes and possess a low molar mass with high affinity for hair keratin. They last from three to six washes. In these hair dyeing process, oxidation reaction doesn't occur, so it is basic application. There are some available products in the market: lotions, emulsions, shampoos, mousses. These products must contain ideal viscosity so that they can not flow during application. Since the product opening cuticle has generally high pH value, they penetrate slightly in the cortex with their low molar mass.[10]

1.2.1.3. Permanent Oxidative Hair Dyeing

These dyes lasting longest are the most versatile. They include up to 6 % peroxide and utilize ammonia as alkalizing agent. They can represent the widest range of hues and they can either darken or lighten natural hair color. [8]

1.2.1.4. Hair bleaches

Hair bleaches include hydrogen peroxide, ammonia and persulfates so that their effectiveness and processess can speed up. Hydrogen peroxide functions as the oxidizing agent releasing oxygen from the hair shaft and lightening the hair related with the oxygen amount required. [8]

1.2.2. Their Mechanisms of Action

Hair dyes has specific mechanisms of action depending on color resistance, into temporary, semipermanent, demipermanent, and permanent. Semipermanent dyes penetrate a little into the cortex while temporary serve through dye deposition on cuticles. Demipermaent and permanent dyes are associated with the oxidation dyes and their interactions build the ultimate shade with an oxidizing agent as distinct from the alkalizing agent. Molecules diffuse intensely into the cortex in oxidation systems to detect a longer color resistance. [10]

1.2.3. Herbal Dyes

1.2.3.1. Orchanet plant (*Alkanna tinctoria* (L.) Tausch)

Figure 1.8. Orchanet plant

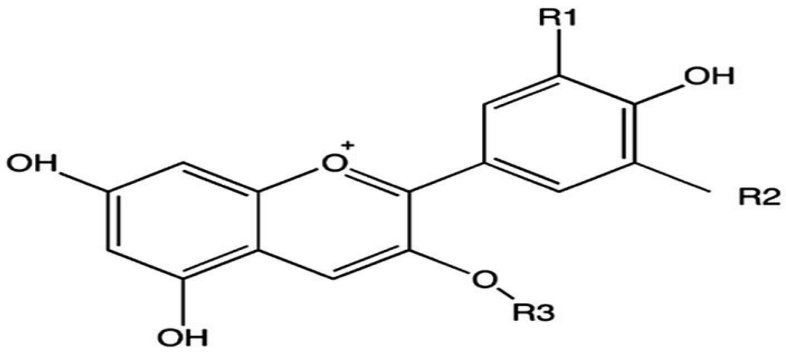
Orchanet is classified in the team Tubiflore of the familia Boraginaceae (Hodangiller) of the type of *Alkanna.* The roots of Orchanet plants include in 5-6 % of the coloring agent called alkanes. Alkannin consists of anchusic acid and alkannic acid as the derivative of antresan. It is obtained by consuming with benzen or ether. ***Alkanna tinctoria* (L.) Tausch** composes of wax, tannin, naphtaquinone derivatives like alkannin acting as red coloring agent. The roots of this plant known as Radix alkannae rubrae, Radix alkannae spuriae are majorly utilized in the wool dyeing.

1.2.3.2. Walnut (*Juglans regia L.)*

Figure 1.9. Walnut

Walnut takes place in class Dicotiledoneae, the team Juglandales, the family Juglandaceae, and the type Juglans. It generally consists of 3.5 % water, 15-30% protein, and 55-77 % fatty oil 1.5 to 3 % ash, and 5-15 % or carbohydrate. It also provides minerals like Ca, P, Mg, Fe, Na, K and vitamins such as A, B1, B2, B6, C. Green fruits before hardening their shell are used in the food and drug industries firstly and then their leaves, shells, and roots are used tannin and dye industries. Its shells contain the coloring agent called as juglone. Walnut is capable of dyeing wool directly and after dyeing dark brown color is obtained.

1.2.3.3. Black okra (*Hibiscus sabdariffa* L. (Hs, roselle; Malvaceae)



Cyanidin-3-sambubioside (R1= OH; R2= H; R3= Sambubioside)
Delphinidin-3-sambubioside (R1= OH; R2= OH; R3= Sambubioside)
Cyanidin-3-glucoside (R1= OH; R2= H; R3= Glucose)
Delphinidin-3-glucoside (R1= OH; R2= OH; R3= Glucose)

Figure 1.10. Chemical structures of main anthocyanins.

Figure 1.11. Black okra

The flowers of ***Hibiscus sabdariffa L.*** include Anthocyanine pigments changing color relating with pH as an environmental indicator, being safe for user's health and being cost effective. ***Hibiscus sabdariffa L.*** made up of polyphenols of the flavonol and its type in polymerised form. Hs extracts possess some flavonoids such as; hibiscitrin (hibiscetin-3-glucoside), luteolin, gossytrin and other gossypetin glucosides, quercetin and sabdaritrin.

1.2.3.4. Henna (*Lawsonia inermis*)

Figure 1.12. Henna

Henna takes place in the type of Lawsonia of the family Lythraceae. It has the coloring agent known as Lawson, giving red and orange color to wool fibers. It includes hennotannic acid, a dye leaving behind red color by bonding keratin of hair and finger nails with the collagen in skin cells.

1.3. SYNTHETIC and NATURAL DYES

1.3.1 Synthetic dyes

The dyeing includes an aqueous solution of the hair coloring amount of the dye that possesses the formula A-NR~(CH2),,-NHR'. Radical groups are obtained from the group consisting of hydrogen, lower alkyl and lower alkoxy, n is an integer of 2-6 and A is selected from the compound forming anthroquinone, azo and benzene. [12]

Natural dyes are obtained from natural sources associating with their source of origin; such as plant, animal, mineral, and microbial dyes. Actually, plants functions as the major sources of natural dyes. [13]

The utilities of some vegetal pigments are analyzed by dyeing tests on yak hair and using distinct natural components. Yellow, blue, and brown shades are beared in mind as two different sources for red. As red pigments, alizarin serves better than mulberry fruits extracts due to the short time required. Anthocyanin pigments derived from mulberry fruits with iron salts forms blue material intensely as distinct from azulene derivatives. Curcumin, p benzoquinone and juglone can be examined to confer to hair brown shades. [14]

1.3.2. Chemistry

Benzoic hair dyes contains variations except for the cited 5,6 dihydroxy indole examples cited. A group of oxidation synthetic hair dyes related topyridine derivatives must be preferred like 2,6-diaminopyridin, 2,6-dihydroxypyridin, and 2,5-diaminopyridine and its derivatives. The pyridine oxidation dyes can be used to modify commercially inthe marketed packs. They provides advantages to dermatological compatibility, better stability at pH and fast washing. [15]

Two colorless products are identified in synthetic dyes amino indamine and amino indoaniline. Transformation is inhibited by oxygen. Indamine is irradiated in nitrogen bubbled isopropanolic solution so as to approve the reduction mechanism. [16].

Natural dyes acts sensitively interms of water quality. Softwater is an ideal in all cases for washing, scouring, mordanting, and dyeing. Sodium exameta phosphate contributes to soften hard water. Rainwater is the best in terms of the most natural dye, but it may be more contaminated than tap water. Tap water including the largest amount of contaminants is the last choice. [17]

1.3.3. Application

2-Methoxymethyl-p-phenylenediamine (ME-PPD) is the dye intermediate of synthetic dye that can decrease the allergy risk. It has an excellent color performance compared to p-phenylenediamine (PPD) and p-toluenediamine (PTD) due to distinct coloring behavior. In this research, four reactions carry out with p-toluenediamine (PTD) and 2-methoxymethyl-p-phenylenediamine (ME-PPD) serving in shading role as an information source with 2-methoxymethyl-p-phenylenediamine (ME-PPD). It allows to make decisions about the data whether the research and development activities related to new primary requires to start. [18]

Natural color dyed hair is less damaged than the synthetic dyed hair when observing the cuticle by microscopic. In the efficiacy test, the cuticle of synthetic dyed hair leads to damage strongly than the natural color dyed hair. [15]

Fourteen species of Aphyllophorales (Basidiomycotina) from the natural dyes are detected for the presence of dyes. *Phaeolus schweinitzii* obtains the greatest dye between five colors involved with excellent intensity. The formed dye colors of this species are olive brown, olive- grey, dark brown, brownish – grey, and linoleum - brown thanks to all excellent intensity. These colors can be formed with 1:1 ratio of wool fibre and fungus. *I. radiatus* and G. *Resinaceum*are dyeing material produces in brown-base colors. [20]

1.4. TOXIC EFFECTS & HUMAN HEALTH RISK OF DYES

1.4.1. Acute Toxicity / Poisoning Accidents

All main hair dye ingredients like p-phenylenediamine (PPD), p-toluenediamine (PTD), or resorcinol show low acute toxicity properties. Human poisoning's accidental cases due to hair dye substances may rarely occur. Some cases related human poisoningby hair dyes are determined forp-phenylenediamine (PPD), but they are generally resulted from suicide or homicide. The standard hair dyes are formulated as max 2 % p-phenylenediamine (PPD) in 100 ml dye solution in the industrialised countries and so the possibility of accidental poisoning are negligible. Dermal toxicity tests shows that hair dye substances are non- toxic in the external applications. [21]

The dyes and cosmetics producers are responsible for total responsiveness of products safety assessment strategy. In the Scientific Committee on Consumer Products (SCCP), the time of application, product pH, dyeing compounds applied and its precursors mixture must be beared in mind with the toxicological analysis. Nitrophenylenediamines and nitroaminophenols from hair dyes penetrate through epidermal barriers according to their chemical structure bondings form to limit the permeation ability with lipophilic structures. [22]

When precursors and peroxide diffuse into hair shaft, reaction is in permanent hair dyes. Dye precursors oxidized by H_2O_2 to p-benzoquinone diamine may lead to anaphylaxis and mutation because of further oxidization to a trimer. There are several poisonous ingredients in permanent dyes such as: p-phenylenediamine (PPD), resorcinol, propylene glycol, Ethylenediaminetetraacetic acid (EDTA) sodium. [23]

1.4.2. Contact Allergy

P-phenylenediamine (PPD) is known as the most common basic intermediates of oxidative hair dyes. It has derivatives calledp-toluenediamine(PTD) stimulating contact allergy. To illustrate, the North Contact Dermatitis Group explained a decreasing 'Significance Prevalence Index Number' for allergenic reactions to p-phenylenediamine (PPD) interms of the clinical importance of contact allergens through the population.

This allergy can be minimized by enhancing with occupational safety, wearing protective gloves, and decreasing the hair dye with the scalp during application. [21]

Figure 1.13. Examples of hair dye products branded as 'natural' or similar, categorized as (a) oxidative, (b) non-oxidative, and (c) raw plant material.

The preservatives in some products like phenoxyethanol and parabens lead to preservative related contact allergy at small proportion. Henna powder (*Lawsonia inermis)* is the botanical product that can be positive in case of contact allergy. It is responsible for some cases of allergic reactions. [24]

1.4.3. Genetic Toxicity

Most of vitro genotoxicity investigations related to the hair dyes reveal to able to minimize hydrogen peroxide that is mutagenic substance of oxidative dyes. There are some genotixicity studies in humans published in the scientific literature. For instance, two studies in female volunteers exposed to up 13 cumulative hair dye applications detected no increased incidence in sister chromatid exchanges (SCEs) or chromosome breaks in peripheral lymphocytes. There is no increase in sister chromatid exchanges (SCEs) over control values in lymphocytes of 13 female volunteers at 6 h or 7 days after commercial hair dye. [21]

1.4.4. Carcinogenicity

According to the International Agency for Research on Cancer (IARC), some hair dyes beared in mind mutagenic and carcinogenic in exposed human populations. There are some epidemiological studies that hairdressers exposed to hair dyes carry a higher risk of bladder cancer. Genotoxic effects can be associated with the azo structure found in the structure of the dyes, known as carcinogenic. [9]

Aromatic amines, the carcinogenic potential of the main hair dye ingredients like benzidine 4-aminobiphenyl and 2-naphthylamine are known as human carcinogens. They lead to increase the bladder cancer in the dye industry. [21]

1.4.5. Human Systemic Exposure to Hair Dyes

The health risk of topical human exposure to a substance shows its systemic toxicity with the human systemic exposure and the dose-response of its toxic effect. Thus a toxic potential of the substance is associated with occurence of systemic exposure. [21]

Hair dyes stimulate anti-inflammatory pathways and they act as skin sensitizers by activating inflammatory T cells. It can refer why many consumers can utilize hair dyes continuously without forming allergies. [25]

1.5. FORMULATION & EVALUTION OF NATURAL HAIR DYES

The word ‘natural’ on the bottle of hair color doesn’t refer chemical-free. Some brands of natural products utilize peroxide for preparing the hair colorants or rely on harsh chemicals like 1-3 % phenylenediamine. These chemicals damage and weaken the hair shaft over time because they proceed harmful processing of oil or coal based components. The Food and Drug Administration (FDA) has conducted a monograph approving henna as the agent of hair colorant. Henna is accepted one of a select group from the natural products by The Food and Drug Administration (FDA) recognition. The dye including only plant products can be formulated thanks to their safe use. [26]

Permanent hair colorants consist of two components called the dye precursors and peroxide. The dye precursors in alkaline soap is one component while the other component is stabilized solution of hydrogen peroxide. The two components are mixed to utilize and apply to the hair. The precursors and peroxide diffuse into the hair shaft in which color forms. [27]

It has been examined that indigo leaves are be able to give more color intensity for ageing while henna powder can’t show color intensity on ageing. This natural hair colorant provides one advantage by not showing any irritation. [28]

High Performance Liquid Chromatography (HPLC) method is simple and practical to identify the direct and temporary dyes in preparations related with the plant extracts. In addition, this method doesn’t require extraction step during the analysis of these components. According to some chromatographic and preparation conditions, vegetable extracts and their basic components: Henna (*Lawsonia inermis*) extract and Lawsone; Chamomile (*Matricaria Chamomilla*) extract and Apigenine; (Nut-hull *Juglans regia*) extract and Juglone; Natural Indigo. [29]

The surfactant used in the products leads to dry scalp by losing the fat under the skin. After that, the hair follicle damages and falls off slowly. The plant pigments in the plant extracts through dyeing of the hair, penetrate into the cortex region without breaking the hair follicles. Some hair dyeing treatments consisting of powdered the

plant extracts by natural products are useful and effective at alkaline pH without hair damge and skin irritation. [30]

The hair have some main properties as macroscopic and microscopic. Macroscopic properties include length, color, and curliness, while microscopic properties contain the model of the medulla, pigmentation of the cortex, and types of scales on the cuticle. Medullary index is measured during viewing hair between 40 time and 400 times for typical magnification. A specifically useful microscope for hair analysis is known a comparison microscope providing distinct samples to view simultaneously. [5]

The dyeing activities of the final mixture (FM) extracts containing powder blend and henna on various fibers have been examined and formulated. Cold extract of final mixture (FM) and FM powder in 2 % carbopol gel have indicated good dyeing effect but hot extract of FM and gel formulations of FM extracts have indicated poor dyeing effect on hair. According to this study, heat stimulates loss of the dyeing characteristics while it doesn't affect the liquid forms of aqueous extracts of the plants as dyeing materials.

The marketed natural hair colorant served as the paste possesses disadvantages such as; messy application, lengthy soaking time, difficult rinsing off. Gel based formulations related with plant extracts are stable to utilize and are excellent interms of dyeing properties and rinsability. It also prevents the hair greying and is safe and eco friendly. [31]

Coconut oil and tila oil (1:1 ratio) have been utilized as vehicle to prepare hair dye. Sesame oil is preferable thanks to good stability interms of natural antioxidants like sesamin and sesamol. Coconut oil known as common hair oil acting strengthing hair. The oil formulation may show dyeing action associating with the reaction of'Lawsone' (thiol group) of mehendi (orange red pigments) and 'Indigotin' of Nili (violet) with keratin of hair. In other study, lawsone and indigotin have penetrated with deep into the medulla area resulting in darker colour grade. Tannins in amla forms affinity between adjective and hair. The color grade and penetration have been observed by microscopic

evaluation. The hair dye formulation from herbs may include synthetic harmful substance like p-phenylenediamine (PPD) leading to toxicity as compared with herbal and synthetic marketed dyes. The oil herbal hair dye prepared with composition is simple and its formulation is stable at room temperature. As oil hair dye utilizes regularly, the intensity of hair color increases and also reduces hair damage. The oil hair dye prepared by Taila pak vidhi, gets natural herbal hair formulations from dark brown to burgundy hair color. It allows the hair soften, condition, promote the growth of the hair calm due to amla and maka through darkening of hair. [32]

1.6.EXTRACTION TECHNIQUES for NATURAL DYE

Fresh or dried plant materials of plant samples like roots, leaves, barks, fruits, and flowers are extracted. Grinding and drying are prepared to preserve the phytochemicals in the final extracts. Extraction means that plants are separated to their active portions by utilizing selective solvents and standard protocols. All extraction process aims to separate the soluble plant metabolites by leaving behind the insoluble cellular residue.

Plant Tissue Homogenization: Dried plant materials are grinded in the blender to fine particles in specific amount od solvent and shaken for 5-10 min and then left 24 hour after filtering the extract. The filtrate is dried by reducing pressure and dissolved again for detecting the concentration.

Serial exhaustive extraction: It is the main type of the extraction including successive extraction with solvent of rising polarity from a non-polar to a more polar solvent.

Soxhlet extraction: When the wanted compound contain a limited solubililty in a solvent and the impurity isn’t soluble in its solvent, this extraction is needed. Simple filtration is performed to separate the compound from the insoluble substance while the compound has been containing high solubility in the solvent.

Maceration: Before dissolving soluble matter, powdered plant-drug is kept in contact with the solvent in the container for a certain period with continuous agitation.

Decoction: It allows water soluble and heat constant constituents from crude drug to extract by boiling it in water for 15 minutes, cooling, passing, straining efficient cold water through the drug for necessary volume.

Infusion: It consists of diluted solution with soluble substances of crude drugs. The solids are macerated to prepare infusions for a short time with either cold or boiling water.

Digestion: During the maceration extraction process heat is performed. If moderately elevated temperature isn't objectionable, it will be used.

Percolation: The solid substances are moistened with the certain menstruum to provide it to stand for 4 h in closed container after packing the mass and closing the top of perculator. The mixture is macerated for 24 h to produce a shallow layer by putting an additional menstruum. After opening the outlet of perculator, the liquid is dripped slowly. Menstruum is added until the percolate measures three quarters of necessary volume of the finished product. The marc is pressed and liquid is put to percolate. The required volume is produced by adding efficient menstruum and the mixed liquid is clarified by filtrating.

Sonication: It utilizes the ultrasound with frequencies varying from 20 kHz to 2000 kHz. This procedure forms cavitation and increases the permeability of the cell walls.

1.7. NATURAL DYE FORMULATION: CHITOSAN

Low molecular weight chitosan is on the confines of chitosan and chitosan oligosaccharide. Its absorptivity and bioactivity is obviously higher than normal chitosan. This natural polymer derived from crab shells and shrimp can have its properties adjusted, most notably water solubility, by controlling the number and order of acetylated units and deacetyleated units. In cosmetics products, chitosan based materials are widely used owing to their fungicidal, fungistatic nature, antioxidant property, and most importantly cationic nature. Such materials are performed generally in cosmetics of hair care, skin care and oral care. Chitosan based material can reduce skin irritation in case of skin care products. [11]

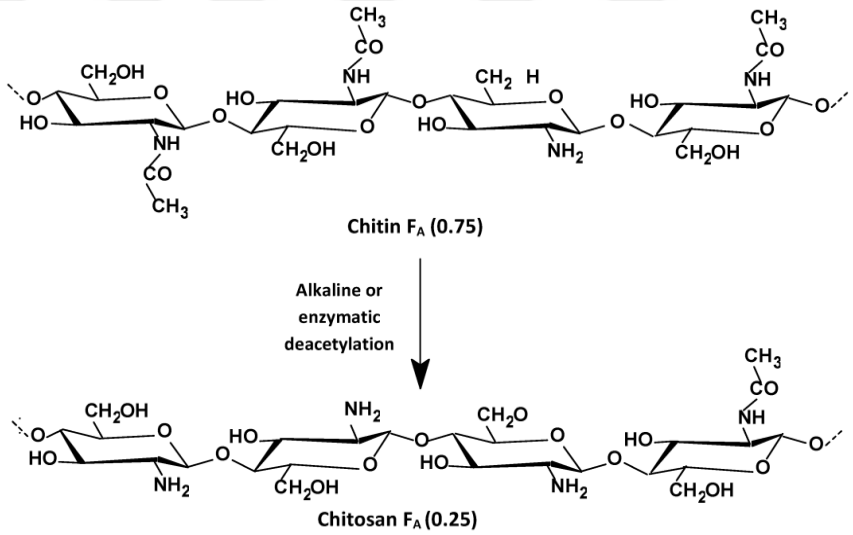


Figure 1.14. The structure of chitosan

2.MATERIALS AND METHODS

2.1.Material

2.1.1.Plant Materials

Black okra (flower calyx), Orchanet(root), Walnut (fruit pericarp), Henna (leaves) were obtained from Arifoğlu Co..Plant has been identified by Erdem Yeşilada.

2.1.2. Lab Devices

Lyophilizer-CHRIST LCG, LYO CHAMBER GUARD 121550 PMMA, Drying Oven-Binder, Light Microscope-Carl Zeiss Axio Lab A1 11 300 531, Refrigerator (-20^{o}C and -80^{o}C), Precision Scales–Ohaus, Explorer, Sonicator-Bandelin Sonorex Supet, Mixer-KKSS01 digital KIKA-WERKE, Hot Plate, Laboratory Vacuum System-HEI-VAP VALUE G1 Heidolph 2, The decisive source of water-GFL, Vacuum pressure pump-Model No: DOA-P730-BN, Grinder-KA A11 Basic Model A11B5000, Dryer Machine, Instron machine (Instron 3382J, Norwood, MA, USA), SEM(Scanning Electron Microscope) Zeiss Euo 40, Vortex MS1 Minishaker were utilized.

2.1.3. Laboratory Supplies

Petri plates, beakers, lab vessels, glass bottle, erlenmayer flask, graduated cylinder, parafilm, filter paper, droppers, glass funnel, pincer, glass pipette with pressure pump, vials, weighing spoons, the experimental set- footed, siever with 0.50 mm, automatic pipette (1000 mL) were used.

2.1.4. Plant Materials and Their Sources

Plant materials were obtained from Yeditepe University and Turkish market (Arifoğlu co.). The plant's latin names, families, sources and their coloring agents as seen in **Table 2.1. .**

Table 2.1.Plants name families, sources and their coloring agents

Plant Names	Latin Names	Family	Sources	Coloring agents
Orchanet	*Alkanna tinctoria (L.)* Tausch	Boraginaceae	Root	Naphtaquinones (Alkannins, shikonins)
Black okra	*Hibiscus sabdariffa L.*	Malvaceae	Flower calyx	Anthocyanine
Walnut	*Juglans regia L.*	Juglandaceae	Fruit pericarp	Naphtaquinones (Juglone)
Henna	*Lawsonia inermis*	Lythraceae	Leaves	Naphtaquinones (Lawsone)

In this study, the hair strands obtained from people who were different age and gender. The obtained hair strands were respectively; the natural white (Age: 61, male), natural blonde (Age: 13, female) and natural brown hair (Age: 25, female). The obtained hair strands with treated dye were respectively; brown colored hair treated with synthetic dye (Age: 24, female), blonde colored hair treated with synthetic dye (Age: 36, female), red colored hair treated with synthetic dye (Age: 30, female). All plants, hair materials and their the codes were tabulated as seen **Table 2.2. .**

Table 2.2. Dye materials & Codes of natural hairs

Dye materials & Codes	Natural white hair	Natural blonde hair	Natural brown hair
Orchanet root water extract (A)	NW + A	NY + A	NB + A
Black okra flower water extract (B)	NW + B	NY + B	NB + B
Henna water extract (H)	NW + H	NY + H	NB + H
Walnut fruit pericarp water extract (W)	NW + W	NY + W	NB +W
Orchanet root water extract + Black okra flower water extract (A + B)	NW + A + B	NY + A + B	NB + A + B
Orchanet root water extract + Henna water extract (A + H)	NW + A + H	NY + A + H	NB + A + H
Walnut fruit pericarp water extract + Black okra flower water extract (W + B)	NW + W + B	NY + W + B	NB + W + B
Walnut fruit pericarp water extract + Henna water extract (W + H)	NW + W + H	NY + W + H	NB + W + H

Table 2.3.Dye materials& Codes of synthetic colored hair (Professional applications)

The implementation of synthetic dyes (after 30 days)	First application of synthetic brown dye	Second application of synthetic brown dye	First application of synthetic blonde dye	Second application of synthetic blonde dye	First application of synthetic red dye	Second application of synthetic red dye
Synthetic dyed hair	SB-1	SB-2	SY-1	SY-2	SR-1	SR-2

2.2.METHODS

2.2.1. Preparation of the Plant Extracts

All plants were extracted using with alcohol (99 %) and purified water. It was calculated obtained amount of extract then study was performed only using water extract of plants because of enough amount of extract yield.

2.2.1.1.Preparation of Water Extracts of the Plants and Implementation of these Extracts to Brown Hair

5 g from each of Henna(leaves) (H), Black okra (flower calyx) (B), Walnut (fruit pericarp) (W), Orchanet (root) (A), Chamomile, Pomegranate flower powders were weighed following the grinding process and 90 mL boiled purified water were added. Then, 90 mL of boiled purified water was added into each of the plants powders and 15 minutes was soaked and were filtered by using vacuum pump device. Plant powder solution was refrigerated at -80 ^{o}C. The frozen plant powders were placed to the lyophilizer for 2 nights. The obtained extracts were weighed and labelled. 0.1 g of those extracts were weighed and was placed into the beaker.10 mLof purified water was added into theeach beaker. Plant extracts were dissolved by using sonicator in 5 minutes. Then 0.14 g of the natural brown color hair was placed into the graduated cylinder of extract solution 10 mL. Then hairs were treated with plant extracts during 30 days.

Additional studies were performed by implementing the extracts of Black okra flower calyx (B), Walnut leaves (W), and Orchanet root (A) to the blonde hair bleached with hydrogen peroxide. Also the extracts of Black okra flower calyx (B), Walnut leaves (W), and Orchanet (A) were applied of the hair treated with laurel leaf oil.

2.2.1.2.The Implementation of the Extracts of Black Okra Flower Calyx, Walnut Leaves, and Orchanet Root to the Blonde Hair Bleached with Hydrogen Peroxide

5 g from each of Black okra flower (B), Walnut leaves (W) and Orchanet root (A) were weighed. A little strand of the blonde hair was bleached with hydrogen peroxide was put to each of three 100 mL beakers. 5 g from each of these plants' powders was added to them and 10 mL water was added slowly by dropper. They were boiled at 100 ^{o}C on the hot plate and then they were soaked about for 5 minutes. The blonde hair bleached with hydrogen peroxide was examined to determine its stability depending on dyeing performances of these plants at certain time intervals. Then hairs were treated with plant extracts during 30 days.

2.2.1.3.Combination of the Extracts of Walnut Fruit Pericarp, Henna Leaves, Orchanet Root, Black Okra Flower Calyx for the Application of Brown Color Hair

0.05 g of the extracts of Walnut fruit pericarp (W) and Henna leaves (H) were weighed and mixed in first small beaker. 0.05 g of the extracts of Walnut fruit pericarp (W) and Black okra flower (B) were weighed and mixed in the second small beaker. 0.05 g of the extracts of Orchanet root (A) and Henna (H) were weighed and mixed in the third small beaker. 0.05 g of the extracts of Orchanet root (A) and Black okra flower (B) were weighed and mixed in the fourth small beaker. 10 mL distilled water was added to each of them and 0.14 g natural brown color hair (NB) was placed to them to treat each other. Then hairs were treated with plant extracts during 30 days.

2.2.1.4.The Application of Laurel Leaf Oil to the Hair Treated with the Extracts of Walnut Fruit Pericarp, Henna, Orchanet Root, Black Okra Flower Calyx

0.05 g of the prepared water extracts of Walnut fruit pericarp (W), Henna leaves (H), Orchanet root (A), and Black okra flower calyx (B) were put to four small beakers. 30 drops of laurel leaf oil was added to each of them. These extracts were thoroughly mixed with laurel leaf oil. A little strand of natural brown color hair (NB)

were placed to each of them. The hairs applied with these extracts mixed with laurel leaf oil were observed at certain time intervals. Then hairs were treated with plant extracts during 30 days.

2.2.1.5.The Implementation of the Extracts of Walnut Fruit Pericarp, Henna Leaves, Orchanet Root, Black Okra Flower Calyx to White Hair

0.1 g of each of these extracts were weighed and was put into the graduated cylinder. 10 mL dwater was added into the graduated cylinder for each. They were dissolved well by sonicator. After that, 0.14 g of the natural white color hair (NW) was placed into the dissolved extracts of each of the plants. Then hairs were treated with plant extracts during 30 days.

2.2.1.6.Combination of the Extracts of Walnut Fruit Pericarp, Henna Levaes, Orchanet Root, Black Okra Flower Calyx for the Application of White Color Hair

0.05 g of the extracts of Walnut pericarp (W) and Henna leaves (H) were weighed and mixed in first small beaker. 0.05 g of the extracts of Walnut pericarp (W) and Black okra flower calyx (B) were weighed and mixed in the second small beaker. 0.05 g of the extracts of Orchanet root (A) and Henna (H) were weighed and mixed in the third small beaker. 0.05 g of the extracts of Orchanet root (A) and Black okra flower (B) were weighed and mixed in the fourth small beaker. 10 mL distilled water was added to each of them and 0.14 g natural white color hair (NW) was placed to them to treat each other. Hair were treated with combination of plant extracts during 30 days.

2.2.1.7.The Implementation of the Extracts of Walnut Fruit Pericarp, Henna, Leaves, Orchanet Root, Black Okra Flower Calyx to Blonde Hair

0.1 g of each of these extracts were weighed and was put into the graduated cylinder. 10 mL dwater was added into the graduated cylinder for each. They were dissolved well by sonicator. After that, 0.14 g of the natural blonde color hair (NY) was placed into the dissolved extracts of each of the plants. Hairs were treated with combination of plant extracts during 30 days.

2.2.1.8.Combination of the Extracts of Walnut Fruit Pericarp, Henna Leaves, Orchanet Root, Black Okra Flower Calyx for the Application of Blonde Color Hair

0.05 g of the extracts of Walnut fruit pericarp (W) and Henna leaves (H) were weighed and mixed in first small beaker. 0.05 g of the extracts of Walnut fruit pericarp (W) and Black okra flower calyx (B) were weighed and mixed in the second small beaker. 0.05 g of the extracts of Orchanet root (A) and Henna (H) were weighed and mixed in the third small beaker. 0.05 g of the extracts of Orchanet root (A) and Black okra flower calyx (B) were weighed and mixed in the fourth small beaker. 10 mL distilled water was added to each of them and 0.14 g natural blonde color hair (NY) was placed to them to treat each other. Hair were treated with combination of plant extracts during 30 days.

2.2.1.9.Preparation of Alcohol Extracts of the Plant Materials

5 g from each of Henna leaves (H), Black okra flower calyx (B), Walnut fruit pericarp (W), Chamomile, Orchanet root (A), Pomegranate flower extracts was weighed after the grinding process and 90 mL alcohol was utilized. After weighing, 90 mL alcohol was added into each of the plants powders. They were mixed by the mixing device for an hour. After that, filtration was made by filter paper and their alcohols were blown by the rotavapor. The alcohol extracts of the plants were frozen at the refrigerator of -80^{o}C for one night. The frozen plant powders were placed to the lyophilizer for 2 nights. The obtained extracts were weighed and labelled. 0.1 g of each of those extracts were weighed and was placed into the beaker. 10 mL dwater was added into the each beaker. Plant extracts were dissolved by using sonicator in 5 minutes. Then 0.14 g of the natural brown color hair (NB) was placed into the graduated cylinder of extract solution 10 mL. Hairs were treated with plant extracts during 30 days.

2.2.2. Light Microscopy Method (Carl Zeiss Axio Lab A1 11 300 531)

At this study the dye treated hair were examined by light microscopy, to describe the morphological apperance, thickness and shape etc. Hairs were treated with different plant extracts during 30 days; Henna leaves (H), Black okra flower calyx (B), Walnut fruit pericarp (W), Orchanet root (A). Through observation, the hairs were washed, dried and photomicrographs were taken bylight microscope (Carl Zeiss Axio

Lab A1 11 300 531). The dye treated hairs (synthetic and natural) were determined in terms of appearance, thickness and shape. Additionally, the hair photos also were taken by camera (mobile phone) to examine the changes or differences of hair color before and after of dye process.

2.2.3. Mechanical Strength of Hair (Instron® Bluehill machine)

Mechanical strength of the hair can be measured by suitable equipment intended for measuring strength properties or elasticity of hair fiber due to tension force or load. Hair fibers have elastic and plastic property. Plastic property of the hair fiber is measurable when a force is applied; the hair fiber extends in part, due to this force and effect, stretching about 2 % of its original length. Hair samples were analyzed and evaluated the tensile strength of the human hair by Instron® Bluehill machine. While being calibrated this machine, the force was balanced, the length was reseted and then the mechanism put the hair strand was placed by selecting the tensile strength method. Strain graphs were prepared depending on charge and elongation of the hair strands to compare their tensile strength. The chemical bonds were rearranged without observing structural change.

2.2.4.Scanning Electron Microscopy (SEM) (Zeiss Euro 40)

Scanning electron microscopy (SEM) is an electron microscope capable of producing highly amplified and sharp images (up to 300,000 times from 3 to 20 nm, according to the equipment being used) of sample surfaces of hair by scanning them with focused electron beams, under vacuum. The scanning electron microscopy (SEM) was utilized to analyze hair strands. This technique provided observations of thickness of hair strands under an electron beam by detecting the size of their component particles. SEM allowed the hair strandsto magnificate in evaluating hair surface morphological conditions. Images were taken at certain angle to hair length in distinct magnifications and then cuticular scale's width was measured per square width of each hair through hair diameter.

Working Principle of SEM (Zeiss Euro 40); when current was applied, the filament got hot. It consists of an electron beam by standing on the opposite end of filament. Under the gun, there was the anode plate. The electron was down because

electron was minus and anode is plus. Electromagnetic coils took place on the side of the device. The objective of its device was to bring perpendicular electrons to samples. Electron broke off weak bond electrons of the samples. Non elastic collision came true without energy transfer. Secondary dedector was worked as electron dedector. Secondary electron beam collected kicked electrons and then gave the image. In this device, it is necessary that the vacuum runs and the samples are solid and dry. After making gold-plating to the samples, they are placed into the device. Resolution settings are made and then the images are taken.

2.2.5.Formulation of Herbal Hair Dye

Walnut fruit pericarp (W), Henna leaves (H), Orchanet root (A), and Black okra flower calyx (B) plants'powders were sieved size to 500 μm. Then the final powders were weighed to 8 g . Boiled purified water was added onto the plant powders and the final dispersion were completed 150 mL. The extracts were soaked 20 minutes and filtered by vacuum pump device. Plant powder dispersions were freeze dried during two days. The obtained extracts were weighed and labelled. 0.1 g of each plant' extracts were weighed and put into the eppendorfs. 0.5 mL purified water were added to onto to them and then vortexed. The dye process were established by hanging the hair on the rope. The extracts were Orchanet root (A), Walnut fruit pericarp (W), Black okra flower calyx (B), Henna leaves (H). And also the combination extracts were Orchanet root with Henna (A+H), Orchanet root with Black okra flower (A+B), Walnut fruit pericarp with Henna (W+H), and Walnut fruit pericarp with Black okra flower (W+B). The all extracts and their combinations were applied to the natural brown hair (NB) and bleached hair with peroxide respectively.

2.2.5.1. Final Natural Dye Formulation

Natural powder hair dye formulation was prepared. The each plant extracts and combination extracts were mixed with 0.01 g chitosan with 0.025 %. Powder mixture was reconstituted with purified water before dye applications to hair.

2.2.6. Microbial Safety Test of Natural DyeExtracts

The water extracts of Black okra (flower calyx) (B), Orchanet (root) (A), Walnut (fruit pericarp) (W), Henna (leaves) (H), were dissolved into the purified water. Then plate method (USP 30 – NF 25) was performed as mentioned above.

2.2.6.1. Preparation of the Sample

The method (Microbiological examination of nonsterile products: Microbial enumeration tests) was performed for determination of sterility of solution dye samples. [32]

At this purpose for sample preparation depends upon the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed. [32]

***Dye Solutions Preparation (Water-soluble Products)*:**

Dissolve or dilute (0.1 g in 10 mL) the four different samples of dye extract solutions to be examined for sterility. The four extracts were Orchanet root (A), Black okra flower calyx (B), Walnut fruit pericarp (W), Henna leaves (H).

Buffer Solution: Buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. If necessary, adjust to pH 6-8. Further dilutions, where necessary, were prepared with the same diluent.

Plate-count methods were performed at least in duplicate for each medium and the mean count of the result was used.

2.2.6.2. Pour-plate Method

1 mL of the each sample of dye solution and 15 mL of casein soya bean (digest agar) or Sabourraud-dextrose agar was added to the dish (petri dishes were around 9 cm in diameter). Both media being at not more than 45 °C. If larger petri dishes were used, the amount of agar medium is increased accordingly. The plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar were incubated at 20-25 °C for 5-7 days. The arithmetic mean per culture medium of the counts and calculate the number of colony forming unit (CFU) per gram or per milliliter of product were taken.

3. RESULTS

3.1. Results of Preparation of Water and Alcohol Extracts of the Plant Materials

Alcohol and water extacts were prepared for each powdered plants Orchanet root (A), Walnut fruit pericarp (W), Black okra flower calyx (B), Henna leaves (H) as mentioned **Section 2.2.1.**

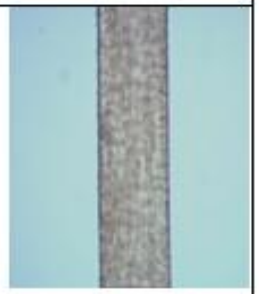
Alcohol extracts were not obtained enough amount (not much yield) for hair dyeing process. So the all study were performed using all plant's water extracts and also Chamomile, Pomegranate flower extracts were not showed enough performance. The combination of Orchanet root with Henna leaves (A+H); Orchanet root with Black okra flower calyx (A+B); Walnut fruit pericarp with Henna leaves (W+H); and Walnut fruit pericarp with Black okra flower calyx (W+B) were studied and evaluated in order to dye performance. Plants' extract performance was also evaluated by following studies; light microscope method, SEM images, mechanical test (Instron®), and Microbial safety test.

3.2. Results of Light Microscope Method

The performance of natural color was evaluated the efficacy of the dyeing power. Color observations were analyzed after repeated application of these plant extracts by light microscopy by measuring the diameter of the hair strand and color performance. The observations were also performed by camera (mobile phone).The most significant color change was observed in natural white hair (NW) applied with Henna extract (H) and Walnut fruit pericarp extract (W) as shown in **Table 3.2.** and **Table 3.4.** respectively. The color dramatically changed when the Black okra flower extract combination with Orchanet root extract (A+B) applied to all different hair types; NW, NY, NB (**Table 3.6**.). The diameter thickness of hair strand was increased approximately 10.38 μm - 6.40 μm when the combination of Orchanet root and Henna extract (A+H) applying to natural white and blonde hair (NW and NY) respectively as seen in **Table3.5.** .The diameter thickness of hair strand decreased dramatically when the combination of Walnut fruit pericarp and Black okra flower extracts (W+B) applying to brown hair (NB) (**Table 3.8**.) .The diameter thickness of hair increased

(from fourteenth to thirtieth day) when the combination of Orchanet root and Henna extracts (A+H) applying to white and blonde hair (NW and NY) and also Walnut fruit pericarp and Henna extracts (W+H) applying to all hair types (NW, NY andNB) respectively as seen in **Table 3.5.** and **Table 3.7.** .The diameter thickness of hair strand decreased approximately 0.9 μm-4.8 μm when the combination of Orchanet root and Black okra flower extracts (A+B) and also Walnut fruit pericarp and Black okra flower extracts (W+B) applying to white and brown hair (NW and NB) respectively as seen in **Table 3.6.** and **Table 3.8. .**

Table 3.1. The images of application of Orchanet root extract (A) to natural white hair (NW), natural blonde hair (NY) , natural brown hair (NB)

Natural hair	Natural white hair		Natural blond hair		Natural brown hair	
Day/ Plant extract	4th day	30th day	4th day	30th day	4th day	30th day
Air mercury root extract(A)						
pH: 4.51	NW + A **MD*:** 98.4 µm **SD**:** ± 2.5	NW + A **MD*:** 99.7 µm **SD**:** ± 5.6	NY + A **MD*:** 70.4 µm **SD**:** ± 9.4	NY + A **MD*:** 77.1 µm **SD**:** ± 3.7	NB + A **MD*:** 66.5 µm **SD**:** ± 10	NB + A **MD*:** 79.7 µm **SD**:** ± 5.0
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

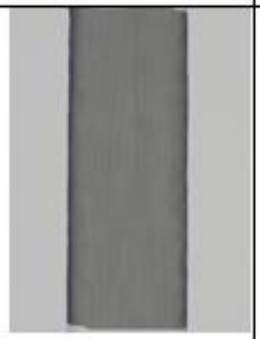
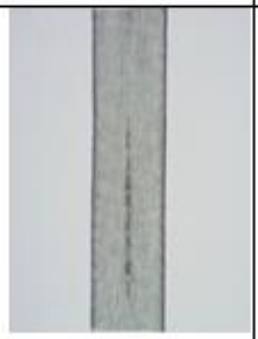
Table 3.2. The images of application of Walnut fruit pericarp extract (W) to natural white hair (NW) , natural blonde hair (NY) , natural brown hair (NB).

Walnut fruit pericarp water extract(W)						
pH: 5.08	NW + W **MD*:** 86.0 µm **SD**:** ± 16.2	NW + W **MD*:** 84.2 µm **SD**:** ± 8.7	NY + W **MD*:** 83 µm **SD**:** ± 6.9	NY + W **MD*:** 76.2 µm **SD**:** ± 0.0	NB + W **MD*:** 72.2 µm **SD**:** ± 68	NB + W **MD*:** 69.6 µm **SD**:** ± 15.7
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

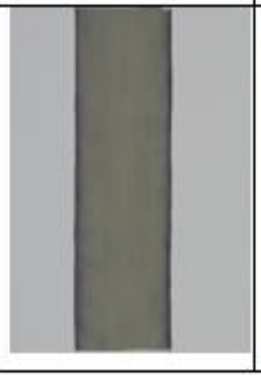
Table 3.3. The images of application of Black okra flower extract (B) to natural white hair (NW) , natural blonde hair (NY) , natural brown hair (NB).

Black okra flower calyx water extract(B)						
pH: 2.6	NW + B **MD*:** 82 µm **SD**:** ± 6.9	NW + B **MD*:** 79.3 µm **SD**:** ± 6.8	NY + B **MD*:** 62.5 µm **SD**:** ± 4.3	NY + B **MD*:** 76.2 µm **SD**:** ± 0.0	NB + B **MD*:** 90.1 µm **SD**:** ± 16.7	NB + B **MD*:** 87 µm **SD**:** ± 17.5
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

Table 3.4. The images of application of Henna extract (H) to natural white hair (NW), natural blonde hair (NY), natural brown hair(NB).

Henna water extract(H)						
pH: 4.51	NW + H **MD*:** 83.8 µm **SD**:** ± 15.7	NW + H **MD*:** 102.8 µm **SD**:** ± 17.5	NY + H **MD*:** 76.2 µm **SD**:** ± 1.2	NY + H **MD*:** 78.0 µm **SD**:** ± 3.7	NB + H **MD*:** 74.0 µm **SD**:** ± 8.1	NB + H **MD*:** 88.2 µm **SD**:** ± 14.4
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

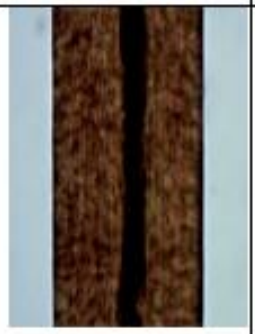
Table 3.5. The images of application of Orchanet root (A) and Henna extract (H) to natural white hair (NW), natural blonde hair (NY), natural brown hair (NB).

Natural hair	Natural white hair		Natural blond hair		Natural brown hair	
Day / Plant extract	4th day	30th day	4th day	30th day	4th day	30th day
Air mercury root + Henna water extract (A + H)						
pH: 7-8	NW + A + H **MD*:** 88.6 µm **SD**:** ± 5.0	NW + A + H **MD*:** 102.4 µm **SD**:** ± 10.6	NY + A + H **MD*:** 70 µm **SD**:** ± 7.5	NY + A + H **MD*:** 76.7 µm **SD**:** ± 4.3	NB + A + H **MD*:** 75.8 µm **SD**:** ± 14.4	NB + A + H **MD*:** 71.8 µm **SD**:** ± .
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

Table 3.6. The images of application of Orchanet root (A) and Black okra flower extract (B) to natural white (NW), natural blonde (NY), natural brown hair (NB).

Air mercury root + Black okra flower calyx water extract (A + B)						
pH: 4-5	NW + A + B **MD*:** 99.7 µm **SD**:** ± 11.9	NW + A + B **MD*:** 66.5 µm **SD**:** ± 16.2	NY + A + B **MD*:** 67.8 µm **SD**:** ± 18.4	NY + A + B **MD*:** 71.2 µm **SD**:** ± 2.8	NB + A + B **MD*:** 94 µm **SD**:** ± 32.6	NB + A + B **MD*:** 71.3 µm **SD**:** ± 15.6
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

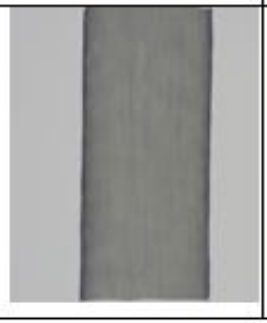
Table 3.7. The images of application of Walnut fruit pericarp (W) and Henna (H) extract to natural white hair (NW), natural blonde hair (NY), natural brown hair (NB).

Walnut fruit pericarp + Henna water extract (W + H)						
pH: 7-8	NW + W + H **MD*:** 102.4 µm **SD**:** ± 0.5	NW + W + H **MD*:** 102.8 µm **SD**:** ± 8.7	NY + W + H **MD*:** 71.5 µm **SD**:** ± 8.0	NY + W + H **MD*:** 75 µm **SD**:** ± 7.2	NB + W + H **MD*:** 83.3 µm **SD**:** ± 3.7	NB + W + H **MD*:** 95 µm **SD**:** ± 3.1
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

Table 3.8. The images of application of Walnut fruit pericarp (W) and Black okra flower (B) extract to natural white hair (NW), natural blonde hair (NY), natural brown hair (NB).

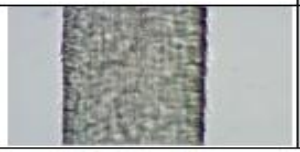
Walnut fruit pericarp + Black okra flower calyx water extract (W + B)						
pH: 3-4	NW + W + B **MD*:** 88.6 µm **SD**:** ± 12.5	NW + W + B **MD*:** 87.7 µm **SD**:** ± 12.5	NY + W + B **MD*:** 71.8 µm **SD**:** ± 1.27	NY + W + B **MD*:** 76.2 µm **SD**:** ± 6.3	NB + W + B **MD*:** 71.8 µm **SD**:** ± .	NB + W + B **MD*:** 67 µm **SD**:** ± 8.1
Microscope images (Magnitude 40X)						

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

The performance of synthetic color were evaluated the efficacy of the dyeing power using Light microscopy method (**Table 3.9. - 3.10.**). It was observed that the diameter of hair was increased only synthetic red dye. When the other dyes applied to the hair, the diameter of hair strand were decreased (**Table 3.9.**). The observation 30 days later, the diameter of hair was increased only synthetic red dye (SR) (probably dye was combined with henna) but the other dyes were decreased the hair thickness oppositely. (**Table 3.10.**)

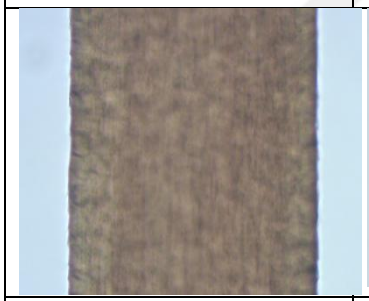
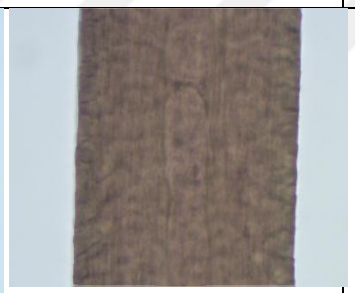
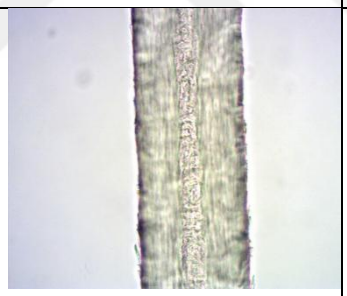
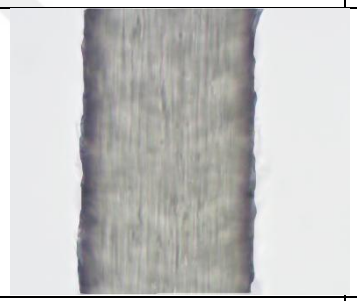
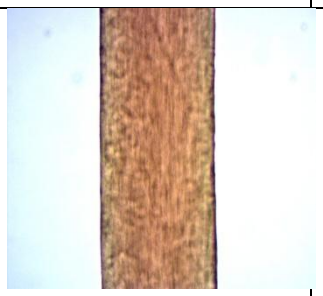
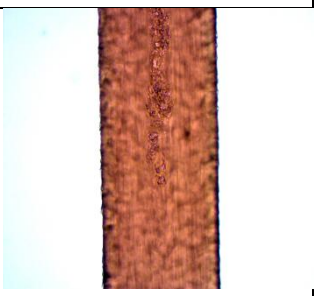
Table 3.9. The images of application of synthetic dyes to dyed hairs.

Application of synthetic brown hair dye	to brown hair	Application of synthetic blonde hair dye	to blonde hair	Application of synthetic red hair dye	to red hair
Before	After	Before	After	Before	After
					
(SB) **MD*:** 113 (µm) **SD**:** ± 21.9	(SB) **MD*:** 111.3 (µm) **SD**:** ± 4.38	(SY) **MD*:** 91.7 (µm) **SD**:** ± 18.1	(SY) **MD*:** 87.7 (µm) **SD**:** ± 5.0	(SR) **MD*:** 86 (µm) **SD**:** ± 3.8	(SR) **MD*:** 119 (µm) **SD**:** ± 2.5
					

The hair sample photos by camera (above); photomicrographs- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

Table 3.10. The images of application (1st and 30 th day) of synthetic dyes to treated hairs.

Application Image The first day (1st) (synthetic brown SB to dyed hair)	**Application Image The 30 th day (synthetic brown SB to dyed hair)**	**Application Image First day (1st) (synthetic blonde SY to dyed hair)**	**Application Image The 30 th day (synthetic blonde SY to dyed hair)**	**Application Image The First day (1st) (synthetic red SR to dyed hair)**	**Application Image The 30 th day (synthetic red SR to dyed hair)**
					
(SB) **MD*:**117.0 (µm) **SD**:** ±2.5	**(SB)** **MD*:**88.2 (µm) **SD**:** ±27	**(SY)** **MD*:**90 (µm) **SD**:** ± 11.8	**(SY)** **MD*:**83.8 (µm) **SD**:** ±4.3	**(SR)** **MD*:**97.1 (µm) **SD**:** ±11.8	**(SR)** **MD*:**108.1 (µm) **SD**:** ±3.7

The hair sample photos by camera (above); photomicrograps- microscope images by light microscope (below)

The arithmetic average of hair strand of thickness (µm) and standard deviation (σ)Mean Diameter (MD) (µm); **Standard Deviation (SD) (σ)

3.3.Results of Mechanical Strength (Instron® Bluehill Machine)

The hair strands of elongation beahavior versus load capacity (N) were examined using Instron (Bluehill Machine) method following the application of plantextracts and synthetic dye to the hair strands (**Figures 3.1.-3.3.**) . Tensile strength was obtained 0.6 N for natural blonde hair (NY) (**Figures3.1.-3.2.**). Furthermore it was obtained that the hair strength was decreased (less than approxiamately 0.3 N) following natural dyeing process (Black okra flower calyx (B) and Walnut fruit pericarp (W)) as seen in (**Figures 3.1. -3.2.).**). Beside the hair strength was slightly increased following synthetic (brown and blonde) (SB and SY) dyeing process (0.62 - 0.8 N) as seen in (**Figures 3.1. -3.2.**).

According to Instron® test analysis, the most hair strand deformation was observed after applying of synthetic yellow dye to blonde hair (SY-1), the elongation was almost twice as much as higher than others (around 40 mm) (**Figures3.1.-3.3.**). After dyeing process with the Black okra flower calyx (B) and Walnut fruit pericarp (W) the hair strand showed almost 15 mm elongation. Beside the natural blonde hair (NY) strand (without dye treatment) showed 17 mm elongation. (**Figures 3.1. -3.2.**).

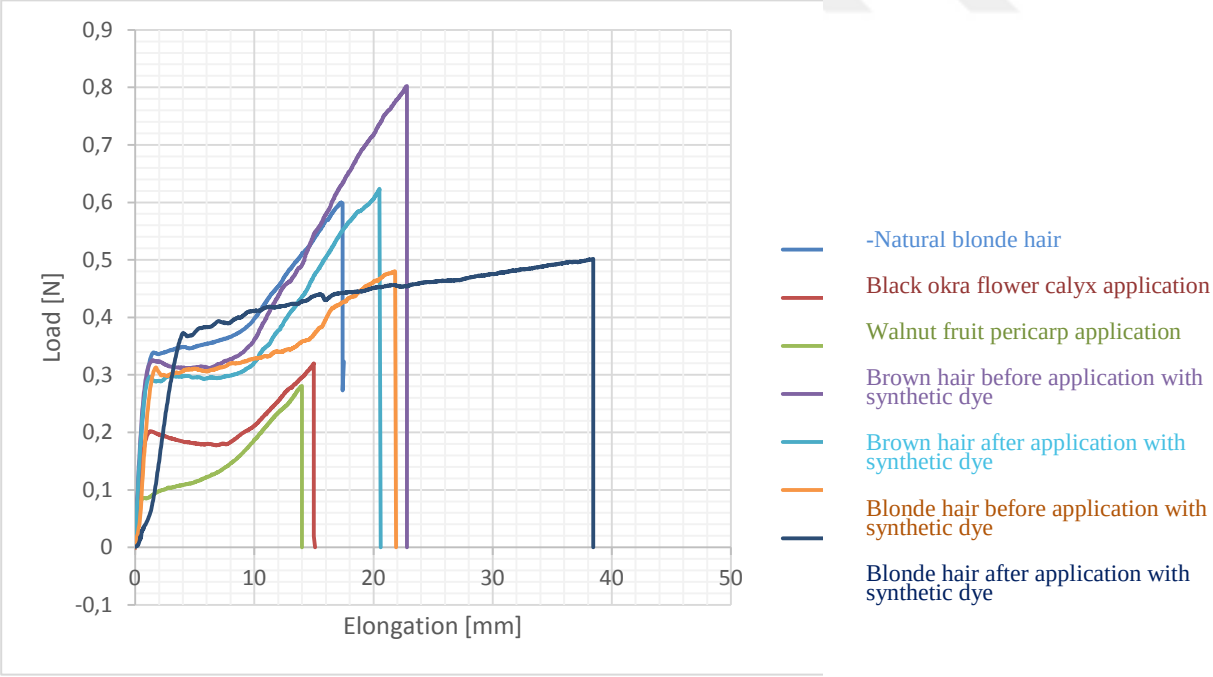


Figure 3.1. The total graph of load versus elongation for natural and hair with synthetic dye by both application.

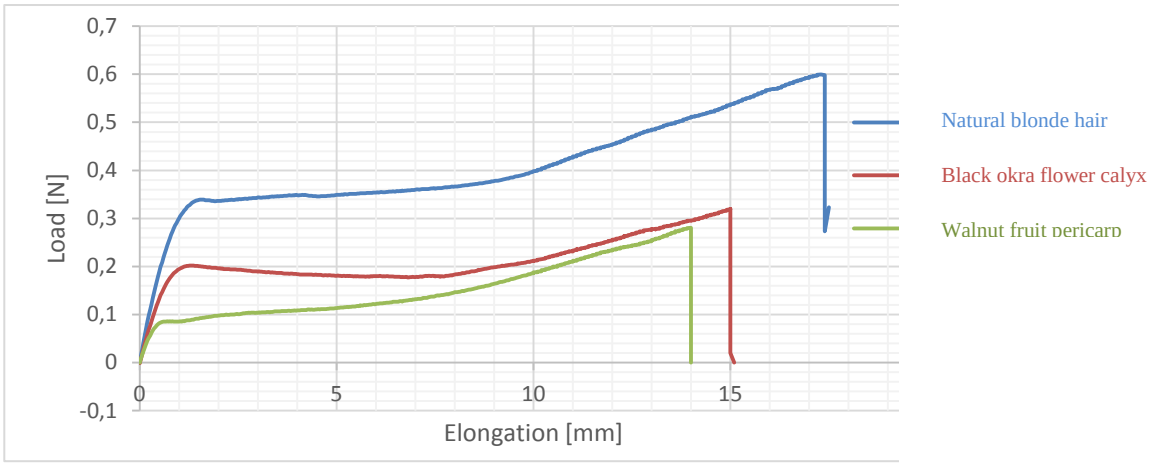


Figure 3.2. The graph of load versus elongation for natural hair by application of these plant extracts.

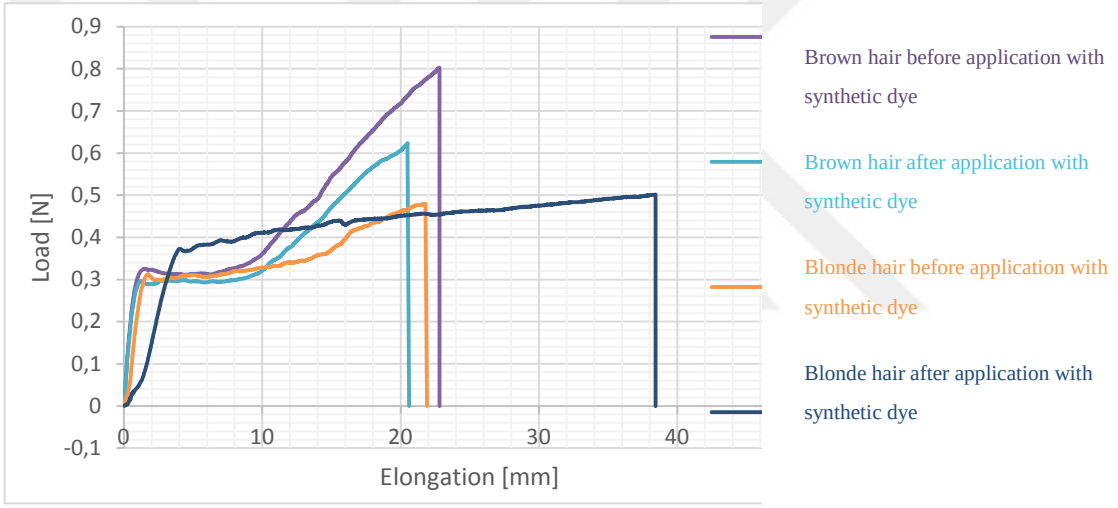


Figure 3.3. The graph of load versus elongation of application of synthetic dye to synthetic dyed hair.

3.4. Results of SEM (Scanning Electron Microscope) Method

Scanning electron micrograph (SEM) were performed to Black okra flower calyx (B) and Walnut fruit pericap (W) extracts to natural hair and synthetic blonde (SY-2) and brown (SB-2) dye applications to treated hair (**Figures 3.4. -3.10.**).

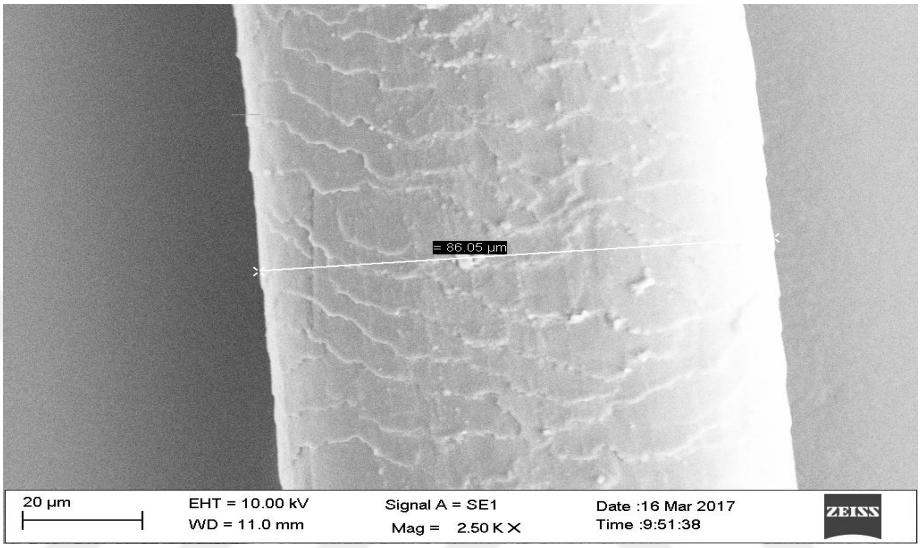


Figure 3.4. The image in close mode of synthetic brown dye immediate applications to brown hair (diameter of hair strand is 86.05 µm).

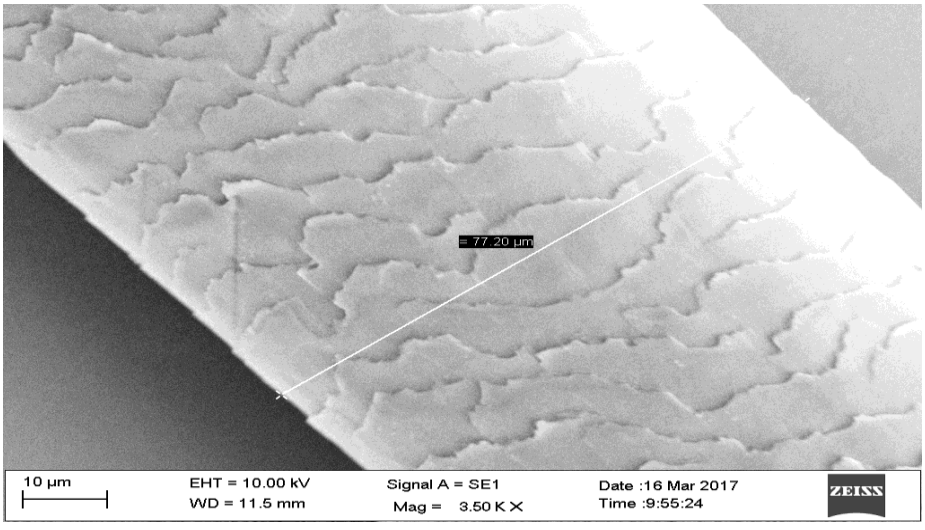


Figure 3.5. The image of hair strand synthetic brown dye applications to brown hair 30 days later (diameter of hair strand is 77.20 µm).

When the synthetic brown dye applied to treated hair, the diameter of hair strand was decreased to 8.8 μm and it was observed that the hair was partially damaged (**Figures 3.4. - 3.5.**).

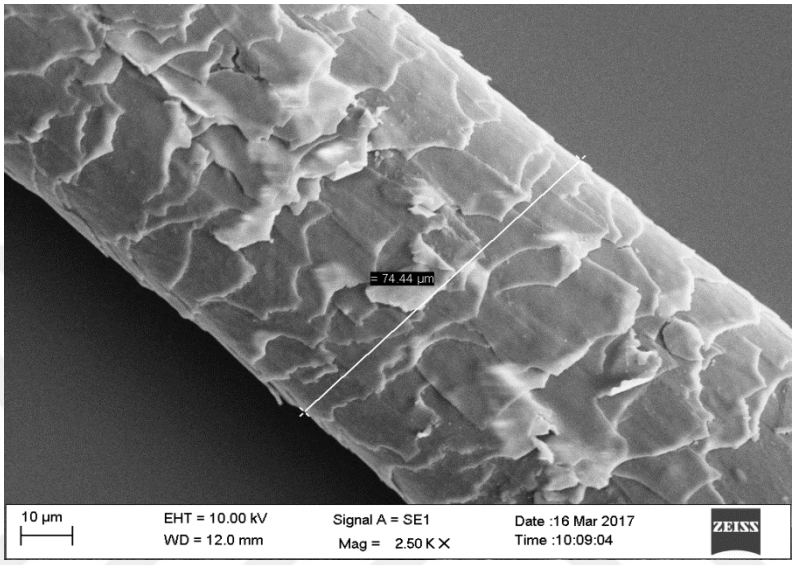


Figure 3.6. The image of hair strand synthetic blonde dye immediate applications to treated hair (diameter of hair strand is 74.44 μm).

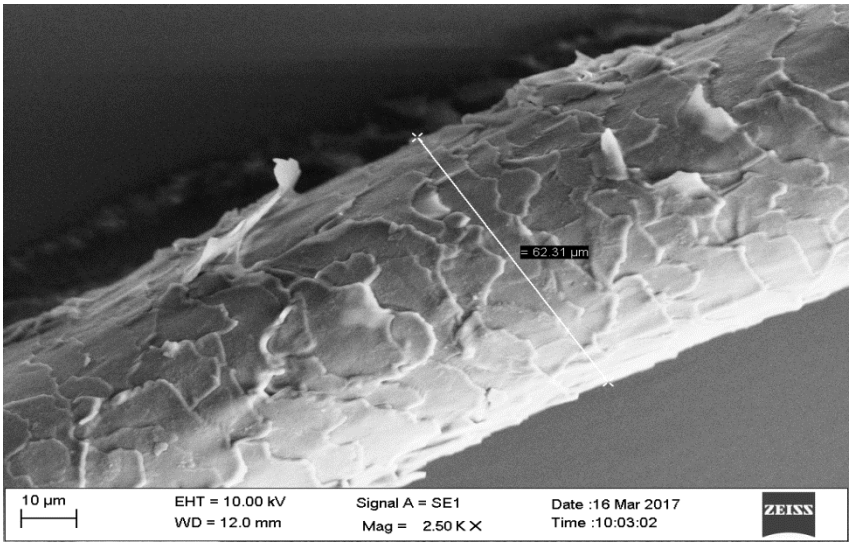


Figure 3.7. The image of hair of synthetic blonde dye applications to treated hair 30 days later (diameter of hair strand is 62.31 μm).

When the synthetic blonde dye applications to treated hair it was observed that the diameter hair strand was slightly decreased around 12.13 μm. The hair was badly damaged as seen in **Figures 3.6. - 3.7.** .

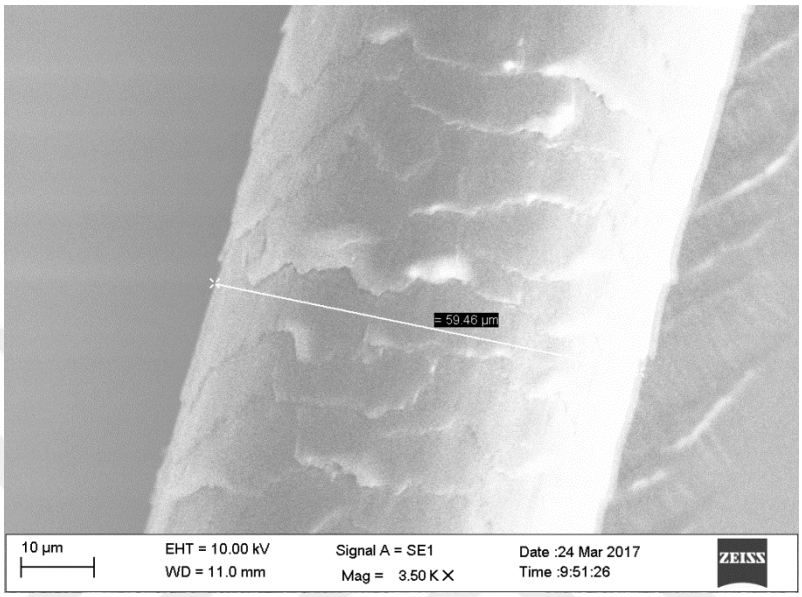


Figure 3.8. The image of natural blonde hair (diameter of hair strand is 59.46 μm).

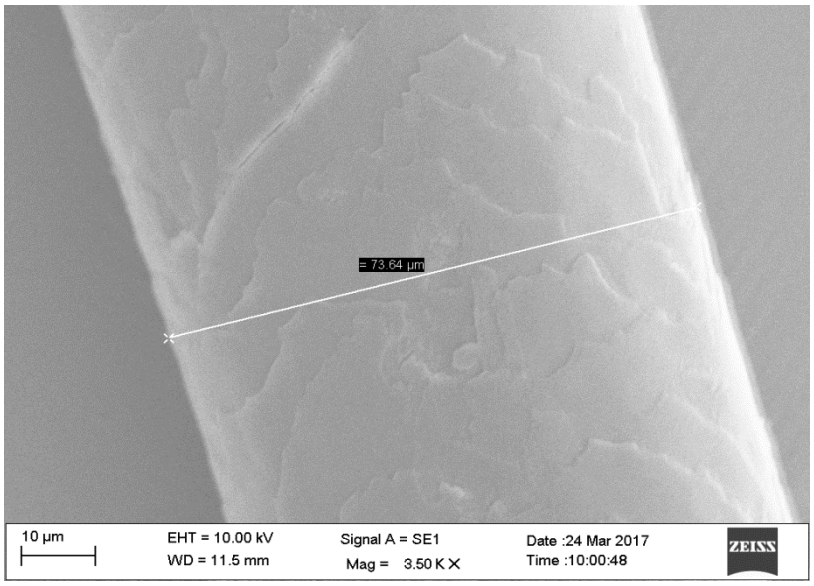


Figure 3.9. The image of hair the Black okra flower (B) extracts applications to natural blonde hair 30 days later (diameter of hair strand is 73.64 μm).

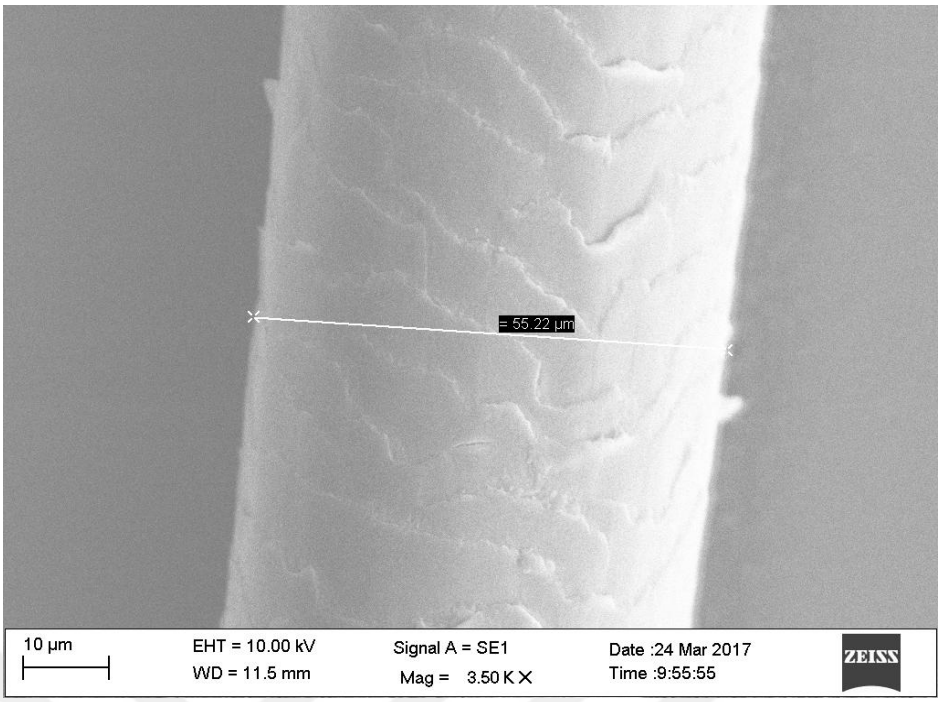


Figure 3.10. The image of hair strand in Walnut fruit pericarp (W) extracts applications to natural blonde hair 30 days later (diameter of hair strand is 55.22 µm).

Following Black okra (B) dye application to natural blonde hair(NY), it was obtained that the diameter of the hair strand was slightly increased around 14.18 µm (**Figures 3.8.- 3.10.)**. According to SEM images (**Figures 3.9.- 3.10.**) it was observed, the diameter of hair strand was slightly decreased (4.24 µm) (in another words the diameter of hair strand do not change clearly). Following the both the Black okra (B) and Walnut fruit pericarp (W) extracts applications to natural blonde hair, according to SEM images any clear damage of hair was not observed (**Figures 3.8.- 3.10.**) .

3.5. Results of Formulation of Herbal Hair Dye

The all natural dye extracts were formulated as described **Section 2.2.5**. The dye formulation of all extracts and their combination were reconstituted with water (**Section 2.2.5.1**). Then the dye process were established by hanging the hair on the rope. Then the Orchanet root (A), Walnut fruit pericarp (W), Black okra flower calyx (B), Henna leaves (H) water extracts and their combinations were applied to either blonde hair (NY) or bleached hair with peroxide and also natural blonde hair (NY) respectively. The hair dye process were evaluated at the end of the 7^{th}, 15^{th}, 30^{th} days after washing of hair with shampoo and drying process. In application with all extracts and combined extracts, generally the color change of natural blonde hair (NY) was more noticable than the ones applied individually. Furthermore, the bleached blonde hair with with peroxide, the dye was more permanent when the applications of dye of combined extracts (NY + A + B, NY + A + H, NY + W + B, NY + W + H) . Generally the applications of each dye with combined extracts were more permanent than the ones applied individually (**Figures 3.11.- 3.16.**) **.**

Figure 3.11. The application of these plants' extracts to natural blonde hair at 1st day.

Figure 3.12. The application of these plants' extracts to the hair with peroxide at 1st day.

Figure 3.13. The application of these plants' extracts to natural blonde hair at 15th day.

Figure 3.14. The application of these plants' extracts to the hair with peroxide at 15th day.

Figure 3.15. The application of these plants' extracts to natural blonde hair at 30th day.

Figure 3.16. The application of these plants' extracts to the hair with peroxide at 30th day.

3.6.Results of Microbial Safety Test of Natural DyeExtracts

The microbial safety test of the extract dye solutions were performed four different samples as mentioned **Section 2.2.6.** The microbial safety test results were evaluated according to (USP 30 – NF 25) method as seen in **Table 3.11.**

Table 3.11. Microbial Growth Ranges of Plant Extracts

Extract (0.01g/5ml)	Total Aerobic Microbial Count (cfu/g)	Total Combined Yeast/Mold Count (cfu/g)	*Salmonella* spp.	*E.coli*
Orchanet root	$< 10^3$	$< 10^2$	-	-
Walnut pericalp	$< 10^3$	$< 10^2$	-	-
Black okra flower calyx	$< 10^3$	$< 10^2$	-	-
Henna	$< 10^3$	$< 10^2$	-	-
Sterile purified water	-	-	-	-
Microbial limit	10^4	10^3	-	-

(-): No Growth

As seen in table **Table 3.11.** , the each extracts were meet the requirement of microbial safety test (USP 30 - NF 25). For each extracts, total aerobic microbial count (cfu/g) was less than 10^3 and total combined yeast/mold count (cfu/g) less than 10^2.

4. DISCUSSION

Natural hair dyes currently being marketed are based on plant pigments such as logwood, chamomile, henna and several other plants which contain lawsone; 2-hydroxy-1,4-naphthoquinone etc. [33, 34, 35]. Due to their low stability of these materials they can not be marketed in ready to use formulations for applications. Generally, these are provided in powdered forms and mixed with warm water before applications. [36] At this study, the selected plant extracts containing similar coloring materials were used respectively; naphtaquinones-alkannins, shikonins, (Orchanet root), anthocyanine (Black okra flower calyx), naphtaquinones-juglone (Walnut fruit pericarp.), naphtaquinones-lawsone (Henna leaves).

4.1. Preparation of Water and Alcohol Extracts of the Plant Materials

At the beginning of this study, it was examined that the final yield of the all extracts and their combinations (Orchanet plant root (A), Walnut fruit pericarp (W), Black okra flower calyx (B), Henna leaves (H), Orchanet root with Henna leaves (A+H); Orchanet root with Black okra flower calyx (A+B); Walnut fruit pericarp with Henna leaves (W+H); and Walnut fruit pericarp with Black okra flower calyx (W+B). The extract of Orchanet root(A) and Henna leaves (H) were found the most efficient yield than others. Generally, dye performance of all extracts and their combinations were evaluated by other tests as mentioned below.

4.2.Light Microscopy Method

The image analysis of dye treated hairs with all extracts were performed using by light microscopy and scanning electron microscopy (SEM). The obtained hair samples were variable color and the hair pigmentation within the shaft were usually uniform. It means that samples of hair strand used for microscopy studies were found healthy**.**

According to literature human scalp hair fibers have diameters within the range 30-100 μm depending upon age and racial group. [18] At this study, it was observed that the size of hair strands were around 60-100 μm. In terms of light microscopy test (**Section 2**and **Table 3.1. -3.8.).**

Morphological light microscopical examination of scalp hair is an inexpensive, rapid, and non-invasive investigation, which can also provide valuable information about color performance. [43] At this study, the obtained photos and photomicrographs of hair strand provided their color performance about natural and synthetic dyes. Our findings have shown that the most significant color change was observed with dye of Henna extract (H) application to all hair type (**Table 3.4. and Section 3.2.).** The most dramatic color change was observed the combination of Walnut fruit pericarp and Henna extracts (W+H) applying to all hair types as seen in **Table 3.7. and Section 3.2. .** Observation with the naked eyes also supported those findings.

However, the repeated applications of natural extracts (3-4 times)enhances the color retaining property. [18, 30] Microscopical observations were recorded during 30 days and photos were taken after repeated application of these plant extracts. The size of hair strands was analyzed and measured the diameter of the hair fiber by using light microscopy. In this study, extracts of Black okra flower calyx (B),Orchanet root (A), Henna leaves (H) and Walnut fruit pericarp (W) when used individually it was exhibited a faint coloring on human hair samples. When it was applied to hair strand as combination of extracts they gave more various and significant coloring. For instance, Henna (H) when used alone as colorant, gave an unappealing orange-red color, but a combination of Henna and the extract of Walnut fruit pericarp (W+H) indicated appealing reddish brown color with good dyeing performance. The color lasted for about 30 days after washing with shampoo.

4.3. Mechanical Strength of Hair (Instron®)

Natural and sythetic hair dyes performance were evaluated both the performance of hair strength and elongation by using Instron® machine. Hair strength has been equated with health (or lack of damage). The force required to stretch and break individual hair strand has been measured using the Instron® tester.

The instrumental measurement of hair properties were tested by Instron®. The evaluated parameters were hair' elongation and strength after applications of synthetic and natural hair dyes. According to our results (**Figures 3.1.-3.3.**) for applying synthetic yellow dye (SY-2) to blonde hair (SY-1), the elongation was observed around 40 mm. This value was found as twice as much as higher than other measurements. The dramatic hair deformation was also observed with applications of synthetic yellow dye. The hair generally behaves like a piece of elastic; after extending slightly, it returns to its original length. The assuming elastic deformation of dye treated hair should be range of 5-25 % and that range is elastic limit of regular hair strand. This is also due to the structure of the keratin molecule. But after dying process the hair elongation was dramatically changed for synthetic blonde hair dye as seen in **Figures 3.1.- 3.3.** .

According to mechanical strength study, the tensile strength of natural blonde hair (NY) was obtained around 0,6 Newton (N). It was also observed that the value of tensile strength of hair was slightly increased following synthetic (brown and blonde) (SB-2), (SY-2) dyeing process. The tensile strength of hair was obtained 0.62- 0.8 Newton (N) (**Figures 3.1.-3.2.**). Furthermore, it was obtained that the tensile strength of hair was dramatically decreased (less than approxiamately 0.3 N) following the natural dyeing process with Black okra flower calyx (B) and Walnut fruit pericarp (W) as seen in **Figure 3.2.** . Measurement of breaking strength are particularly prone to variability even different sections of the same hair will often break at different strength. According to results the tensile strength of hair determine whether natural dyes (Black okra flower calyx (B) and Walnut fruit pericarp (W)) weaken of hair strength. In contrast the applications of synthetic dyes seems strengthen to hair. Synthetic dyes generally strengthen hair because of after synthetic hair dye applications in hairdresser, the hair is well-nourished and hydrated with the other hair care products so the hair is became strengthen.

An important consideration in Instron® tests, the effect of moisture should be considered on the tensile and elastic properties of hair. Feughelman and Robinson worked with the effect of moisture on the mechanical properties of hair. [39] But our study we could not have chance to determine the effect of moisture on the tensile properties of dye treated hair.

In this study it was also found that the most deformation of hair strand had been after application of synthetic blonde dye to synthetic blonde colored hair (SY-2). The hair strand applied with Black okra flower calyx extract (B) showed that it was found approximately 16 mm elongation of hair strand with application of Walnut pericalp extract (W) and 15 mm. with application of natural blonde hair (NY). That means the dyeing process with plant extracts to hair generally less harmed than synthetic dyes (**Figures 3.1.- 3.2.).**

However, scientists can show that hair from the three ethnic groups behaves different mechanical properties intrinsically comparable fashion and can thus confirm a common structure for all the world's hair. The African hair seems to be the most fragile and an elongation of 40 %. Asian hair is the strongest and an elongation of 55 %. For either of these features Caucasian hair occupies an intermediate position. The Turkish populations are generally Caucasian. These properties vary greatly depending on the shape of the hair. [36, 39, 40] In our study we could not have chance to evaluate the ethnic group differences.

4.4.SEM (Scanning Electron Microscope) Method

SEM is very efficient method to evaluate hair surface morphological conditions. The couple of samples of synthetic and natural dyes applied to the hair strands were examined by SEM images so as to show damaged edges from those hair strands. [37] So far, it was obtained limited samples of SEM images at this study (**Figures 3.4. - 3.10.**).

According to our limited SEM images, whenthe dye of synthetic blonde was applied to treated hair, it was observed that the diameter hair strand was slightly decreased. The hair was badly damaged as seen in **Figures 3.6. - 3.7. .**When the dye of synthetic brown was applied to treated hair, it was found that the diameter of hair strand was decreased. (approximately 8.8 μm). It was also observed that the hair was partially damaged as seen in **Figures 3.4. - 3.5. .** Generally the hair showed a compact architecture interms of the scales on the surface of hair strand and macrofibrils were tightly attached to each other according to SEM images (**Figures3.8. , 3.9. and 3.10**.) .

According to SEM images, there were not observed any clear damage when the extracts of Black okra (B) and Walnut fruit pericarp (W) were applied to hair strands of natural blonde (NY) as seen in **Figures 3.8.-3.10. .** Following the synthetic Black okra (B) dye application to natural blonde hair (NY), it was obtained that the diameter hair strand was also slightly increased around 14.18 μm (**Figures 3.8.- 3.10.**).

In the analysis with SEM, there were some differences in the surface details between application of synthetic dye to hair and natural dye of used plant extracts. The cuticle of synthetic dye applied hair (especially blond hair with treated synthetic dye (SY-2)) appeared damaged and there were some abrasion and denudation in these cuticle. Our findings were also confirmed with references. [11, 41]

4.5. Formulation of Herbal Hair Dye

Hair dyes' ingredients that carries their risk to human health may be developed interms of their safety assessment by applying toxicity tests giving preference to topical administration route.[21]

This formulation contains water extracts of the plants that are quitely environment friendly. Further, it could penetrate into cortex region without damaging the hair follicles. When it wasutilized regularly, the intensity of hair color increased day by day. The natural polymer Chitosan (low molecular weight) solution (0.025 %) was used as a carrier and viscosity enhancer in our herbal hair dye formulation. Because it is positively charged, it repairs and polishes by connecting to negatively charged the hair, thanks to its structure similar to the keratin. It prevents harmful mold and bacteria and repairs microorganism-induced damages formed in hair.

4.6.Microbial Safety Test

Evidence of microbial contamination in dye solution was obtained by appropriate Pharmacopeial method(USP 30 – NF 25). The extracts of Black okra (B), Orchanet(A), Walnut (W), Henna (H) were meet the requirements of the microbial safety test in pharmacopeia (USP 30 - NF 25) as seen in **Table 3.11.** So the test performed during week and according to results of sterility analysis, no microbial growth was examined.

Furthermore, the mold formation was observed in extract of Black okra flower calyx (B) in a couple of days later. The pHof this extract solution was obtained around 2.6 as seen in **Figure 3.3.** . According to literature the molds most will grow well over the pH range 3-7. Some such as *Aspergillus niger* and *Penicillium funiculosum* can grow at pH 2 and below.[42, 43]. Additionally, according to microbial safety test results it was found some survival microorgamisms for all extracts but in the limit range of pharmacopeia. **Table 3.11. .**

So we decided to prepare final formulation with powdered dye extracts. The powdered extract was reconstituted by purified waterbefore applications (**Section 2.2.5.1.** and **Section 3.5**.) .

5. CONCLUSION

Our study proposes to investigate the performance of newly developed dye formulations with using different herbal extracts on various hair strands. For this purpose, first of all, the water extracts were prepared either alone or combinations of various powdered leaves and flowers of dye yielding plants such as Orchanet root (A) (*Alkanna tinctoria* (L.), Walnut fruit pericarp (W) (*Juglans regia* L.), Black okra flower calyx (B) (*Hibiscus sabdariffa* L.), Henna leaves (H) (*Lawsonia inermis*). These extracts were used to obtain different colors as natural colorants for hair strands. Natural and synthetic dyes' performances on hair were assessed by various methods such as light microscopy, scanning electron microscopy (SEM), measuring mechanical strength (by Instron® tester), formulation study and microbial safety test.

The evaluating efficacy of dye formulation with natural plant extracts in vitro involves use of normally high accurate equipment. Tests are generally specific and suitable assay for study with hair strands. The tests should be provided information of only hair strand. As a future work, the newly developed formulation of natural dye extracts will be applied to volunteers and evaluated with similar methods like SEM, Instron® etc. .

The most dramatic color change was observed the combination of Walnut fruit pericarp and Henna extracts (W+H) when apply to all hair types. The resconstitutable powder dye formulation could be potentially efficient, safe new hair dye candidate for future.

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