Natural Antioxidants in Cosmetics

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Chapter 15

Natural Antioxidants in Cosmetics

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INTRODUCTION

Cosmetics are pharmaceutical products that are used for improving skin appearance and body odor. These products are available in various forms, ranging from lotions, creams, powders, and so forth. Cosmetics are used for cleansing, protecting, and moisturizing the skin. Generally, consumers prefer to choose cosmetics that have less harmful effects on their skin. "Cosmeceutical" products have now been developed by many pharmaceutical industries. According to Duroja *et al.*, a cosmeceutical is the combination of a topical cosmetic and a pharmaceutical that is used for enhancing beauty through ingredients that have biological functions related to the skin [1]. The topical cosmetic, which contains antioxidants, is used to protect human skin against the damage caused by ultraviolet radiation (UVR) and by free radicals [2].

Antioxidant compounds are very interesting for pharmaceutical industries. Antioxidants can be added to cosmetic preparations because of their activities against free radicals. Unfortunately, most antioxidants are not stable, and they can cause many problems in the formulation of cosmetics. The selection of antioxidants and their concentrations in cosmetic formulations must be optimized. Nowadays, the use of plant-derived natural antioxidants in cosmetics is

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preferred over synthetic antioxidants [3]. Extracts of plant-derived antioxidants generally contain a mixture of natural compounds, which could have synergetic effects; therefore, they can have better effects and less toxicity [3].

Due to their instability, keeping constant the activities of antioxidants in formulations during their claimed shelf life is often problematic. For this reason, the concentration of each antioxidant should be determined and controlled for its chemical stability to ensure that the consumer gets a product that has the claimed activity. Activities assays of plant derived antioxidant are not simple because the compounds are usually very complex.

The aim of this chapter is to review all aspects of the use of (natural) antioxidants in cosmetics. Free radicals, human skin defense systems, the use of antioxidants in cosmetics, determination of antioxidants' activity/capacity, and important natural antioxidants and their mechanisms are summarized and discussed.

FREE RADICALS AND REACTIVE OXYGEN SPECIES

Most of the biological molecules are nonradicals, which are characterized by two outer orbital electrons (pair-electron). When a chemical reaction breaks the bonding of these electrons, they become unpaired and are called free radicals. Free radicals are very unstable and reactive due to their capability to lose or gain electrons [4–6]. They can be formed by hemolytic bond fission or via electron transfer [7]. Free radicals can be continuously formed by cellular metabolism and by the induction of external factors such as UVR, chemicals, air pollutants and cigarette smoke, drugs, pesticides, anesthetics, and industrial solvents [5,8–10].

Due to their instability, free radicals will always be transformed into their stable forms, making their lifetimes very short. Free radicals can initiate continuous chain reactions that could become very dangerous because of their damaging effects. Reactions of free radicals with important components of cells or the cell membrane can be very hazardous because it can affect the cell functions or even kill the cells [5]. Free radicals can cause metabolism disorder by forming covalent bonds with enzymes, receptors, and membrane components, as well as by disturbing transport processes [7].

Cell components that are very sensitive to oxidation are lipids, proteins, DNA, and carbohydrates. The interaction of free radicals with the target molecule can cause irreversible damage(s) to it or alter its function(s). Chain reactions take place when free radicals interact with lipids to form lipid peroxidation, which results in an increase in cell permeability and eventually causes cellular damage [5,7,11]. Interactions of free radicals with protein residues affect the functions of essential proteins such as immunoglobulin, albumin, and hemoglobin [6]. Reactions of free radicals with thiol groups affect the activities of the enzymes [7], while the reaction with DNA can lead to irreparable damage or inaccurate repairs [5], which can trigger cytotoxicity or mutation [7].

Reactive oxygen species (ROS), reactive nitrogen species, atomic hydrogen, many heavy transition metals, chlorine, certain drugs, ionizing radiation, and environmental wastes are different types of free radicals [5]. Generally, free radicals are formed dominantly by ROS [12,13]. ROS can be formed via the by-products of various metabolic pathways, which are located in different cellular compartments [14,15].

The toxicity of ROS depends on its half-life. ROS needs some time to reach its target from the site of formation. The toxicity of ROS is determined more by the time needed to reach the target than its reactivity. The interaction of ROS and the target molecules can be inhibited by the antioxidants that are available at the site of ROS formation [15,16]. Hydroxyl radicals are very reactive, and these radicals directly react at the site of formation [17]. Tocopheroxyl-, ascorbyl-, and certain semiquinones-type radicals are intermediate radicals that will cause damage only under suitable conditions [17]. One of the persistent radicals that are present in the skin and have physiological effects is the melanin radical [17].

ROS can affect the structure and function of skin endogenously and exogenously [12,13]. Almost 80% of ROS that affect the skin are produced by UVR, which comprises 95–98% of UVA and 2–5% UVB [18]. The main target of UVR is O_2 that occurs in the mid-lower epidermis. Donation of one electron can transform O_2 into superoxide anion ($O_2{*}^{-}$), which is very unstable and reactive. For transformation into stable molecules, it initiates reactions that yield other free radical *OH. Carbon-centered radicals are formed in the process of lipid peroxidation, while sulfur-centered radicals are formed in the oxidation of thiol compounds [17].

Interaction of UVR and skin surface can result in free radicals or ROS and biochemical transformation in collagen, elastin, and connective tissues. They can affect skin firmness and elasticity, making the skin dry and appear wrinkled. The UVB can cause erythema and sunburn. In comparison to UVB, UVA can penetrate the skin deeper; indirectly, it can penetrate 50% of the exposure through the skin even in the shade, and UVA cannot be filtered by glass. UVA is also more potent in inducing pigment darkening and tanning than UVB. UVA causes lipid peroxidation 10 times more potent than UVB. UVA is more cytotoxic than UVB [9,18–22].

SKIN DEFENSE MECHANISM

It is known that skin is the outermost human organ, so it is often influenced by endogenous and exogenous free radicals [12,23]. To deal with the adverse effects of the free radicals, skin has its own defense mechanism, which derives from endogenous and exogenous antioxidants. Endogenous antioxidants originate from melanin, while exogenous antioxidants derive from antioxidants that are administrated orally and topically. This defense mechanism is related to the structure and function of constituent skin layers [23].

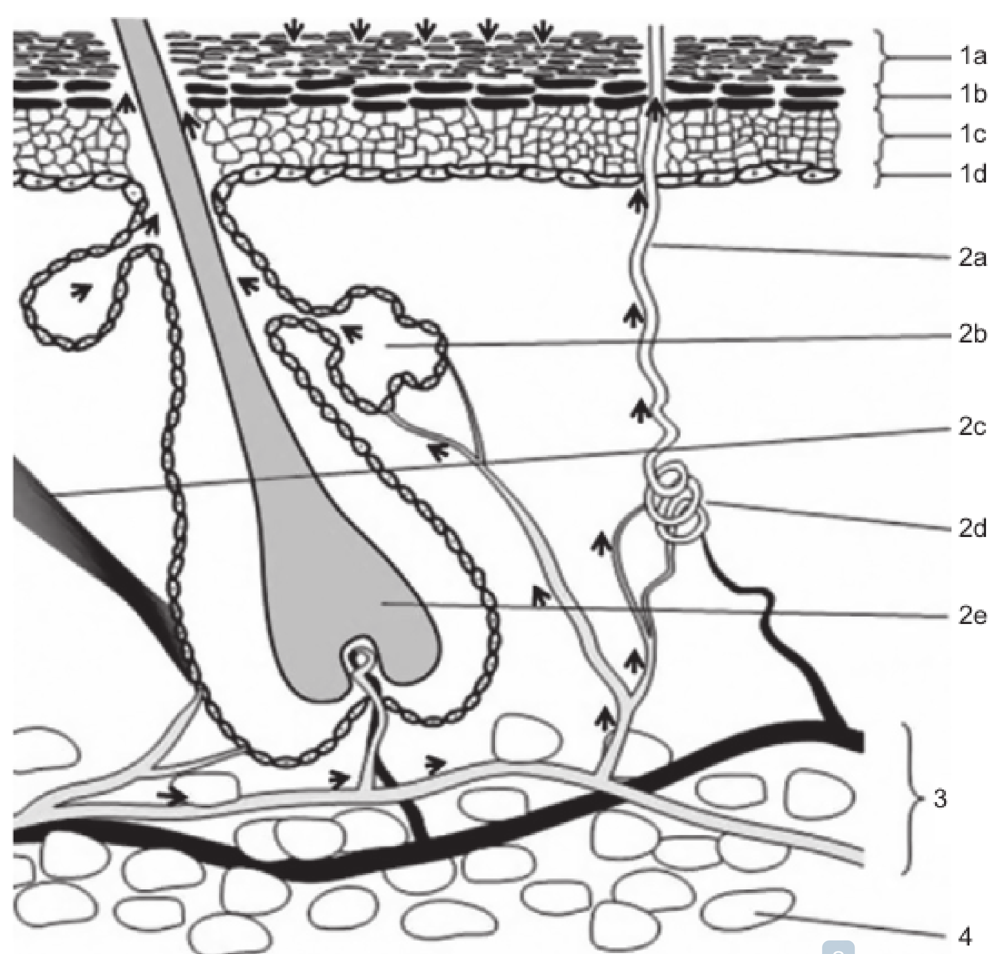

FIGURE 1 Diffusion and accumulation of antioxidants in human skin. (1a) Stratum corneum, (1b) stratum granulosum, (1c) stratum spinosum, (1d) stratum basale, (2a) sweat duct, (2b) Sebaceous gland, (2c) arrector pili muscle, (2d) sweat gland, (2e) hair follicle, (3) blood vessels, (4) fat lobules. Arrows show the diffusion of oral antioxidants and accumulation of topical antioxidants in the epidermis. *Modified from Ref. [12].*

Skin comprises three layers (Fig. 1). The uppermost layer is the epidermis, which contains corneocytes, keratinocytes, and melanocytes; they are cells responsible for melanin synthesis. The epidermis, which comprises the stratum corneum, stratum granulosum, stratum spinosum, and stratum germinativum/basale, has no blood vessels. The stratum basale is composed of keratinocyte and melanocyte cells. Melanocytes synthesize melanin, which can absorb UVR. The stratum corneum is a layer surrounded by the epidermis, which is composed of nondividing cells or dead cells named corneocytes or horny cells, and has *ceramide* as its main component. Ceramide has an important role in water retention and keeping the skin moisturized; it plays an important role in maintaining the integrity of the skin defense. Ceramide is the primary lipid, while the other lipids are fatty acid and cholesterol. These lipid bilayers have amphiphilic characteristics. Intrinsic and extrinsic proteins such as enzymes are also tucked inside these bilayers. A healthy stratum

corneum is the best protection for the epidermis, and a determinant of healthy skin appearance [10,24,25].

The next layer is the dermis, which keeps the characteristics of the skin and serves as a water reservoir for the skin. This layer contains fibroblasts, collagen, elastin, and hyaluronic acid. The hypodermis is the deepest tissue layer that contains collagen and extracellular matrix (as adipocytes and used as fat storage) [10,24,25]. Skin defense systems against ROS comprise enzymatic and nonenzymatic mechanisms, and both of these could deactivate ROS. Enzymatic defense systems are done by superoxide dismutase, catalase, and glutathione (GSD) peroxide [26], while nonenzymatic mechanisms are done by low-molecular weight antioxidants [27]. However, antioxidant defense mechanisms are taking place in a comprehensive way between enzymatic and nonenzymatic antioxidants [28,29]. Concentrations of enzymatic and nonenzymatic antioxidants vary in the different skin cells. Fibroblasts have a higher concentration of antioxidant enzymatic antioxidants than keratinocytes, while melanocytes have no enzymatic antioxidants. Cells with high levels of antioxidants are more resistant to oxidative stress [30].

First, the enzyme defense system converts ROS into hydrogen peroxide and oxygen; subsequently, hydrogen peroxide is transformed into water and oxygen. Nonenzymatic reactions are performed by vitamin C, vitamin E, vitamin B, vitamin A, and other antioxidants. These molecules scavenge the free radicals and delay the oxidation process [31]. Very complex interactions between antioxidants occur; therefore, changes in the redox status or concentration of the antioxidant will affect other antioxidants in the system [28,29].

Nonenzymatic defense systems cannot be provided by the human body; they can be supplied through oral or topical preparations that contain vitamins, carotenes, or other antioxidants. These antioxidant molecules are generally accumulated in the epidermis through diffusion processes from adipose glands, blood and lymph, and via secretion by sweat glands or sebaceous glands to the epidermis, as presented in Fig. 1 [12].

The *stratum corneum* contains hydrophilic and hydrophobic antioxidants. The concentration of antioxidants in the epidermis is higher than in the dermis [23]. The activity and capacity of the antioxidants in the epidermal layer are higher than in the dermis, although the thickness of the epidermis is only 10% of the skin. This is due to the early defense system of the epidermal layer. The skin antioxidant capacity is also higher than that of other organs [23,28,29]. Consumption of rich antioxidant-containing foods or application of topical antioxidants can overcome the occurrences of oxidative stress caused by the inequilibrium between ROS and the skin defense system [12,32]. Skin can naturally defend itself against ROS by the use of antioxidants [21].

Two mechanisms of antioxidants are stabilizing the free radicals by scavenging and slowing the oxidation; by these mechanisms, the formation of further free radicals can be suppressed [33,34]. The main defense systems of the skin dealing with the UV inductions are melanogenesis and enhancement of

DNA repair [23]. ROS can stimulate lipid peroxide, which can generate hyperpigmentation and melanogenesis, and this could develop into skin darkening. Enzyme tyrosinase can convert tyrosine into melanin; melanin, a skin pigment, can absorb UVR [35]. Melanogenesis, which stimulates the abnormal production of melanin, can be overcome by increasing the antioxidant defense capacity of the skin. A correlation is observed between the defense system of the epidermis and skin pigmentation. The addition or use of antioxidants in cosmetics is one way to maintain the amount of antioxidant pool in the skin [21,26].

The major target of UVR that penetrates into the skin is mitochondrial DNA. Oxidation of DNA can cause various damages to it, such as DNA strand break. Self-cellular defense systems can carry out DNA repair. Antioxidants might increase the capability of the repair enzyme systems by posttranscriptional gene regulation of the transcriptional factor [23].

Healthy skin has intrinsic antioxidants, which can protect it against exposure to free radicals. On the contrary, the defense system of old skin is impaired, so an external supply of both oral and topical antioxidants is needed. Antioxidants have the ability to donate a hydrogen atom for stabilizing the radical electron, so it can delay or minimize the propagation or initiation of chain reactions and ultimately prevent skin damage [36].

For preventing skin from the damages of oxidation reactions, the amount of endogenous antioxidants must be balanced with the amount of ROS. Under certain conditions, when there is very high exposure to ROS, the body's antioxidant defense systems are not sufficient to maintain the balance. That is why exogenous antioxidants should be administrated either orally or topically [12,21,32,37].

Exogenous antioxidants include vitamins, trace elements, and phyto-antioxidants [32]. GSD and vitamin C are hydrophilic antioxidants, while vitamin E and ubiquinol-10 are lipophilic antioxidants [21,32]. Vitamin E (tocopherol) can inhibit lipid peroxidation to form tocopheryl stable compounds, which can stop the chain reaction of membrane lipid peroxidation [32]. Vitamin C can react with tocopheryl radicals for restoring and regenerating vitamin E. Trace elements such as selenium, copper, zinc, manganese, and iron are a cofactor of the enzymatic antioxidants [38]. Phyto-antioxidants are compounds of plant extract mixtures usually containing terpenes and polyphenols [32]. Antioxidants from plant extracts, which contain a mixture of compounds, are now used in cosmetic preparations [39]. The use of mixtures of compounds as antioxidants is preferable on account of its advantages, that is, the combined effects of various compounds contained therein either as biologically active compounds or as protectors of other ingredients [32].

NATURAL ANTIOXIDANTS

Based on their function, antioxidants can be divided into primary or natural and secondary or synthetic antioxidants. Primary antioxidants comprise

mineral antioxidants (such as selenium, copper, iron, zinc, and manganese), vitamins (C and E), and phyto-antioxidants. Generally, a mineral antioxidant is a cofactor of enzymatic antioxidants. The functions of secondary or synthetic antioxidants are capturing free radicals and terminating the chain reaction. Some examples of secondary antioxidants are butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and metal chelating agent, tertiary butylhydroquinone, and nordihydroguaiaretic acid [38,40].

The increasing application of plant antioxidants could replace the application of synthetic antioxidants [32,41,42]. Natural antioxidants can be a single pure compound/isolate, a mixture of compounds, or plant extracts; these antioxidants are now widely used in cosmetic products [39]. Natural antioxidants function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, and enzyme inhibitors [32,41,42].

Phyto-antioxidants consist mostly of polyphenols and terpenes; this differentiation is based on their molecular weight, polarity, and their solubility. Polyphenols have —OH groups attached to the benzene ring. Their activity as antioxidants is determined by the number and the position of —OH groups on the benzene ring. Phenolic groups modulate protein phosphorylation by their ability to inhibit lipid peroxidation (as chain-breaking peroxyl radical scavengers). Flavonoids and stilbenes are the largest group of polyphenols, while the largest group of terpenes are carotenoids that serve as singlet oxygen quenchers [32]. A summary of some natural antioxidants generally used in cosmetic preparations and their mechanisms of action are presented in Table 1. The chemical structures of the pure natural antioxidants are presented in Fig. 2.

APPLICATION OF ANTIOXIDANTS IN COSMETICS

Antioxidants are responsible for the chain-breaking of radical scavengers and for inhibiting the oxidation reaction; by these mechanisms, antioxidants can prevent oxidative damage [56,57]. In cosmetic preparations, antioxidants have two functions, that is, as the active ingredients and as protectors of other ingredients against oxidation [38].

Currently, the application of antioxidants in cosmetics is increasing; however, to obtain the desired activities, some strategies should be considered. The short life of ROS can be overcome by using antioxidants that have high reactivity and capacity. Antioxidants must not be transformed into their radicals such as ascorbyl- or tocopheryl radicals; this will trigger the chain reaction. Antioxidants should remain stable in the product; they must not react with the other ingredients and should be protected from oxygen radicals. The selection of antioxidants that can be used in cosmetics depends on their hydrophobic or lipophillic characteristics. Unfortunately, sometimes the selection of antioxidant(s) (by pharmaceutical industries) used in cosmetic products is not based on scientific judgment, but rather on their price.

TABLE 1 Summary of Natural Antioxidants Which Have Been Used in Cosmetics

Antioxidants	Sources	Use In Cosmetics as	Mechanism of Action of the Antioxidants	Products (™/®)	References
α-Tocopherol	Wheat germ, sunflower	Pure compound or isolate, plant oils	Via glutathione pathway, and chain breaking lipid peroxidation	Estee Lauder Re-Nutriv Ultimate Youth Creme Reviews, Body Lotion, Cosmetics Bakery, Singapore	[18,43]
Aloesin (low-molecular weight glycoprotein)	*Aloe vera*	Plant extracts, pure compound	*Tyrosinase* inhibitor (at DOPA-oxidation site)	Constance Cosmetic, Aloe Vera Jelly (aloe therapy and oily skin moisturizer), The Intensive Whitening Cream	[44,45]
Anthocyanin, proanthocyanin	Berry species (*Vaccinium* sp.)	Plant extracts, pure compound	Radical scavenger, Inhibition of low density lipoprotein oxidation	Revale Skin (day cream, night cream), DDF Doctor's Dermatologic Formula Protect	[46–49]
Arbutin	*Vaccinium vitisidaea*	Pure compound or isolate	Inhibition of tyrosinase and melanosome, but without any cytotoxicity effects on melanocytes	Buhna W Quinone Cream 15 g (Arbutin cosmetics)	[44]
Ascorbic acid	Rosehip oil	Pure compound or isolate, plant oils	Quenching UV-induced free radicals, and regenerating vitamin E	100% Pure Argan Oil Hydration Facial Moisturizer SPF 30	[18,19]
Azelaic acid	*Pityrosporum ovale*	Pure compound or isolate	Inhibition of thioredoxin reductase, so the toxicity effects of melanocytes are hampered	Ampleur Luxury White Cream AO	[44]

Carotenoid (lycopene, β-carotene, lutein)	*Lycopersicon esculentum, Daucus carota, Hippophae rhamnoides*	Carotenoid, plant extracts, pure compound	Breaking chain in lipid peroxidation, decreasing UV-induced erythema, and sunburn cell formation	Ilike Rich Carrot Moisturizer, Dharpin's Products	[19,43,50]
Carnosic acid	*Rosmarinus officinalis*	Plant oil	Scavenging lipid free radicals	Natural skin facial toner, wildcrafted herbal product	[32,51]
Curcumin	*Curcuma longa*	Pure compound or isolate	Radical scavenging	EWG's Skin Deep, Gentle Earth Products Virgin Coconut Rose Face Cream	[19]
Epigallocatechin-3-gallate (EGCG)	Green tea, black tea (*Camellia sinensis*)	Plant extracts, pure compound	Radical scavenging, sunscreen	Olay, Regenerist Perfecting Cream, Sunday Riley's products	[19,52]
Licorice, flavonoid liquertin, isoliquertin.	*Glycyrrhiza glabra*	Plant extracts	Inhibiting tyrosinase without interfering with DNA synthesis	YUKIMIZU Natural Skin Cream	[44]
Kojic acid	*Aspergillus* spp., *Penicillium* spp.	Pure compound or isolate	Inhibiting copper binding to tyrosinase, so it can be used as a lightening agent	100% Pure Skin Brightening Facial Cleanser	[44]
Pycnogenol (proanthocyanidin)	*Pinus pinaster*	Bark extract	Converting vitamin C radical into its active form, and increasing the levels of glutathione and other radical scavengers	Dermae Pycnogenol Cream	[44,53]
Quercetin (flavonol)	Contained in various fruits, vegetables,	Pure compound or isolate	Iron chelator, which maintains and protects the	EltaMD UV Physical Broad-Spectrum, SPF 41	[19,32,54,55]

Continued

TABLE 1 Summary of Natural Antioxidants Which Have Been Used in Cosmetics—Cont'd

Antioxidants	Sources	Use In Cosmetics as	Mechanism of Action of the Antioxidants	Products (™/®)	References
	beverages, and herbs, Leaf of *Psidium guajava*		enzymatic antioxidant activity, sunscreen	EltaMD by Swiss-American Products	
Resveratrol (stilben)	Contained in many grapes and berries	Plant extracts, pure compound	Lowering generation of H_2O_2 by UVB, inhibiting the increase in lipid peroxidation, it can ward off free radical damage (caused by UV rays and pollution), and encourages cell renewal, so it can reduce wrinkles and dark spots	Moisture Reservoir Hand Cream, Love Life Skin, 46 Graphic Place Moonachie, NJ Estee Lauder Re-Nutriv Ultimate Youth Creme Reviews	[19,43]

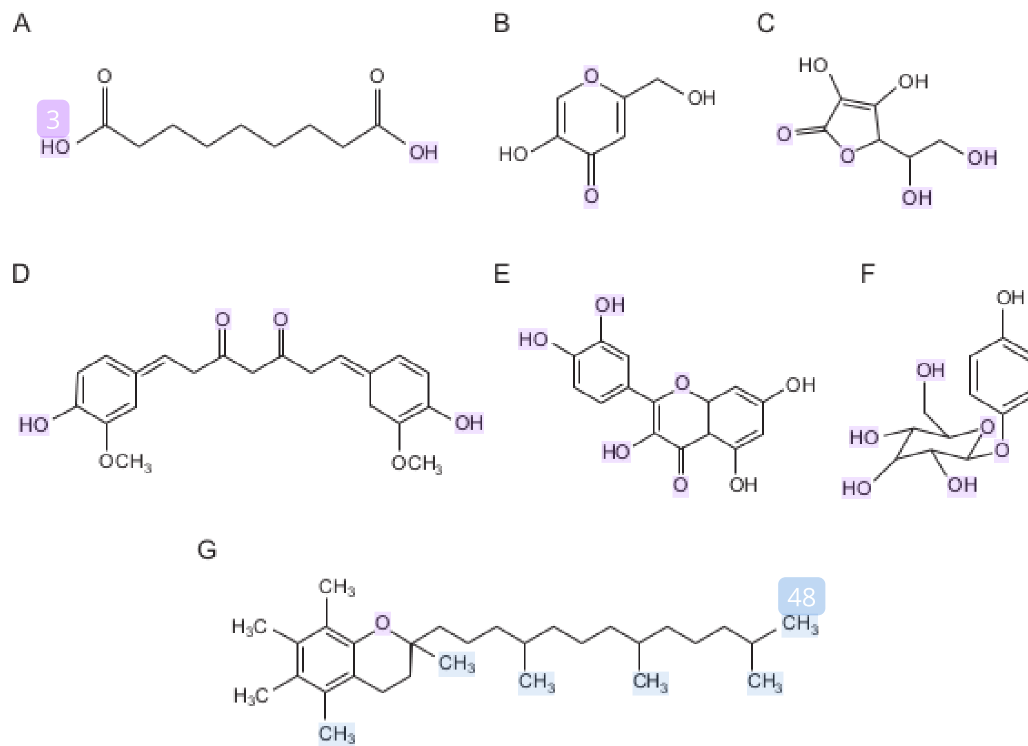

FIGURE 2 Chemical structures of pure natural antioxidants. (A) Azalaic acid, (B) kojic acid, (C) ascorbic acid, (D) curcumin, (E) quercetin, (F) arbutin, (G) α-tocopherol.

Generally, antioxidant by nature are unstable, deeply colored, and susceptible to hydrolysis and photodegradation in the presence of oxygen; that is why it is very difficult to have good cosmetic formulations and to maintain their aesthetic validity and acceptability. Modifying the chemical structure of the antioxidant such as substitution wiht its esters (e.g., tocopheryl acetate, ascorbyl palmitate), or by shortening the lipophilic chain of CoQ10, may be able to improve its stability, but unfortunately, it reduces its activity.

For being active, a stable antioxidant is needed, but unfortunately, antioxidants are generally unstable compounds. This instability can cause many problems. In a cosmetic formulation, the concentration of antioxidants must be stable for achieving the desired activity. Their color should not change in the production processes and storage, so that their antioxidant activities remain constant and the product retains an aesthetic appearance. All this raises many problems in the formulation of cosmetic products [12,21,32]. That is why a valid method is needed for determining the antioxidant's capacity to evaluate its activity [32].

Application of the relatively new "lipid-based delivery system" technology could protect and maintain the stability of the antioxidants. This technology also has protective effects against skin dehydration. Lipid carriers can increase the skin penetration of the antioxidant, so its desired activity can be guaranteed. Various lipid carriers such as nanoparticle emulsion, various vesicular systems (liposomes, phytosomes, transfersomes, etosomes, niosomes, and

FIGURE 3 SEM of the liposomes of quercetin (5000×). *Cited from Ref. [55].*

nanotopes), and particulate systems (lipid microparticles and lipid nanoparticles) have been developed and are being used. The stability of ascorbyl palmitate and vitamins K and A in cosmetics can be enhanced by using lipid nanocarriers. Phytosomes of green tea and grape seed can improve their free radical scavenging and UV protection activity. Skin penetration of vitamin E acetate was increased by using Nanotop™. The antiaging effect of vitamin E acetate and CoQ10 was improved by the application of nanoemulsions [36,56–60].

We have now developed various lipid carrier systems, such as liposomes, phytosomes, and lipid nanoparticles for the natural antioxidants pycnogenol, quercetin, squalene, and *p*-metoxycinnamic acid, which will be used in UV-protector preparations. Figure 3 shows liposomes of quercetin, which were prepared in our laboratory; the liposomes were viewed by using SEM. These liposomes can increase the permeation of quercetin into human skin, so it will have the desired photoprotective activity [55]. This part of our work is still in progress.

ANTIOXIDANT CAPACITY; METHODS OF DETERMINATION AND QUALITY CONTROL

The determination of the antioxidant capacity of a sample can be used for getting information regarding its resistance to oxidation, the quantitative contribution of its antioxidant substances, and its antioxidant activity. For example, the antioxidant capacity of food depends on the colloidal properties, the condition and stages of oxidation, localization of antioxidants in different

phases, and the free radical generator or oxidant used [61]. Jung used the other term "antioxidant power"; this term is similar in meaning to the term antioxidant capacity [36].

In determining an antioxidant's capacity, several constraints have to be faced, particularly for phytoantioxidants, which may contain thousands of different compounds that often work synergistically. It is known that cosmetic products usually have a mixture of ingredients, which may have different characteristics (hydrophilic and hydrophobic), so they need to have a mixture of antioxidants that have the same characteristics. This gives rise to problems in the determination of the total activities of the antioxidants [38].

Several methods can be used for measuring antioxidant capacity (activity). Generally, spectrometric methods are used for the determination of *in vitro* antioxidant activity; these methods are based on the reaction of antioxidants with free radicals. Antioxidants donate their hydrogen atom to the free radicals. Electrochemical methods are also widely used.

Generally, antioxidant capacity (activity) evaluations can be classified according to the chemical reactions:

1. Based on electron transfer (ET-based) that involves a redox reaction [32] and a single electron transfer mechanism [38]. This assay is based on measurement of the capacity of an antioxidant in the reduction of an oxidant. The oxidant compound will change its color when it is reduced, and the degree of the color change depends on its concentration in the sample; examples of this assay are [61] as follows:

a. DPPH (2,2′-diphenyl-1, 1′-picrylhydrazyl) assay:

DPPH is a free radical that is unstable at room temperature; it can react by reducing the hydroxyl group(s) of the antioxidant molecules. The percentage of the scavenging activity of DPPH generally depends on the concentrations of the antioxidants [42]. This method is suitable for antioxidants that are soluble in organic solvents, especially alcohol. Light, oxygen, pH, and solvent type could lead to biased interpretations of the results. The disadvantages of this method are the narrow linear range and steric hindrance [38]. Recently, Olech *et al*. reported the application of an HPTLC–DPPH method; this method, combined with imaging processing software, can be used to evaluate the antioxidant activities of the chemical constituents of plant materials [62]. A schematic picture of the DPPH reaction is shown (Fig. 4).

b. TEAC (trolox equivalent antioxidant capacity) assay:

A TEAC assay is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) [42,65]. Reaction between ATBS with peroxidase and H_2O_2 will yield the colored ABTS radicals. The occurrence of antioxidants in the sample will inhibit the formation of colored ABTS radicals. This method can be used for evaluating both hydrophilic and

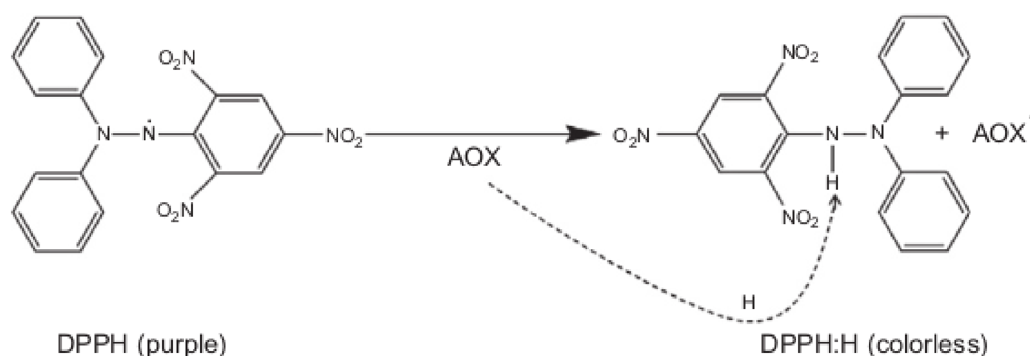

FIGURE 4 Schematic of DPPH assay: Donation of hydrogen from AOX to DPPH molecule can lead to a decrease in the absorbance (decolorization). DPPH radical scavenging activity can be measured spectrophotometrically at 517 nm against methanol. AOX, antioxidant; ●, odd electron. *Modified from Refs. [63,64].*

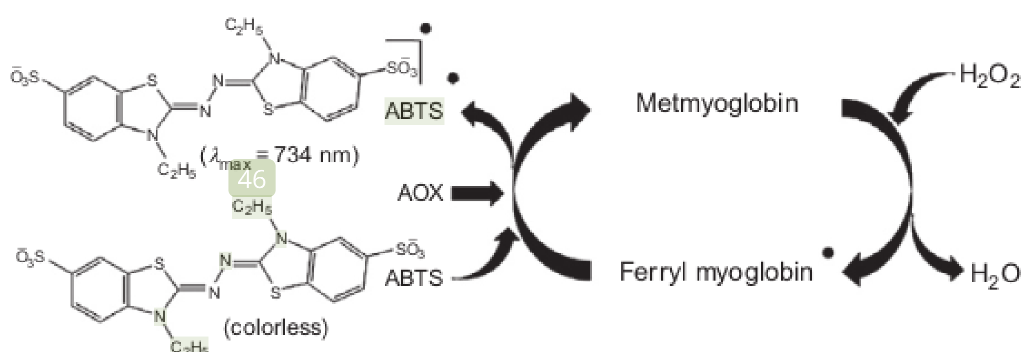

FIGURE 5 Schematic of TEAC reaction (for details see text). AOX, antioxidant; ●, odd electron. *Modified from Ref. [65].*

lipophilic antioxidant samples [38,66]. The TEAC reaction might be too fast, so it could contribute to the reduction of the ferryl myoglobin radical, and the addition of hydrogen peroxide could oxidize the antioxidants in the sample before it is measured. ABTS radicals are not ROS, so the method does not show the actual antioxidant activity. This method only indicates specific oxidant-reducing power [67]. Figure 5 shows the schematic reaction of a TEAC assay.

2. Based on hydrogen atom transfer (HAT-based). The method involves antioxidants' ability to donate hydrogen atoms [32]; this can measure the neutralization capacity of the free radicals [38]. In this case, antioxidant and substrate compete for thermally generated peroxyl radicals via decomposition of azo compounds; examples of this assay are described in brief [61].

a. ORAC (oxygen radical absorbance capacity) assay:
ORAC measures antioxidant scavenging activity against peroxyl radicals induced by AAPH (2,2-azobis-(2-amidino-propane)-dihydrochloride) at 37 °C (15 min). It measures the ability of the antioxidant to inhibit the declining of the fluorescence of β-phycoerythrin (B-PE) or FL (fluorescein or 3′,6′-dihydroxy-spiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one).

The loss of fluorescence of B-PE or FL is a sign of the extent of damage caused by its reaction with peroxyl radicals. The protective effect can be measured by comparing an area of the sample under fluorescent decay with antioxidants and without antioxidants (blank sample). The limitation of B-PE was its variability in different lots. It seems that FL is a better probe for this assay. Trolox, a water-soluble analog of vitamin E, is used as a control antioxidant standard. ORAC can be used for biological samples, hydrophilic and liphophylic emulsions, and commercial products. Detailed methods have been described [37,40,47,67–70]. The schematic of this assay is presented in Fig. 6.

b. TRAP (total radical trapping antioxidant parameter) assay:
According to Sies, TRAP can be defined as "a quantitative measure of the total secondary antioxidant content of a biological fluid" [70]. This method is based on luminol (3-amino phthal hydrazide)-enhanced chemiluminescence (CL) for determining the antioxidant capacity of substances. Alkyl peroxyl radicals from the decomposition of AAPH in the presence of luminol will produce luminescence, which is quenched by the addition of antioxidants. The TRAP value is determined by the duration of the time period (T_{sample}) in which the sample quenches the CL signal in the presence of antioxidants. Generally, Trolox (8 nM) is used as reference inhibitor (T_{trolox}):

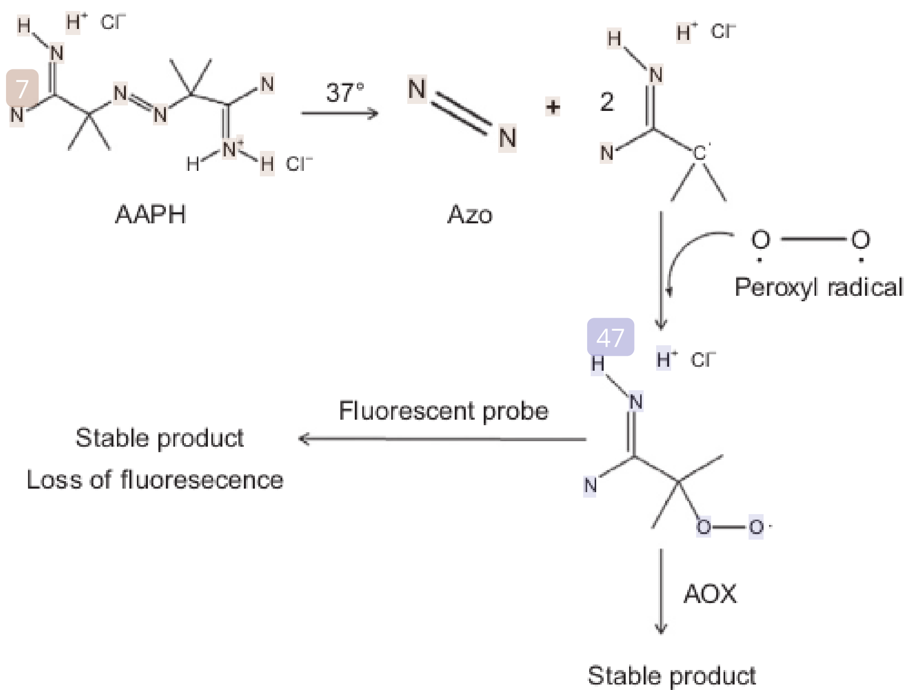

FIGURE 6 Schematic of ORAC assay: Peroxyl radical is more reactive to antioxidant (AOX) compared to the fluorescence probe (e.g., B-PE), so the decline in the fluorescence of B-PE can be inhibited. *Modified from Ref. [61].*

$$\text{TRAP value} = 2.0[\text{Trolox}]T_{\text{sample}}/fT_{\text{trolox}}$$

where *f* is the dilution of the sample and 2.0 is the stoichiometric factor of trolox (the number of peroxyl radicals trapped per molecule of trolox). This method is suitable only for nonenzymatic antioxidants [69]. It can measure antioxidant capacity in hydrophilic and lipophilic compartments of plasma and tissue homogenate [71]. The detailed method can be referred to in previous publications [47,70,72–75]. This assay is presented schematically in Fig. 7.

3. Based on electrochemical characteristics.
This method can be used to measure either a single compound or a mixture. It is based on the determination of the redox capacity, which requires neither reagents nor dependent absorbance. Cyclic voltammetry methods have been adapted to the quantification of the overall reducing capacity of antioxidants in different biological fluids [32,38,76]. Validation procedures for measuring global antioxidants in the stratum corneum by cyclic voltammetry have been described in detail by Ruffien-Ciszak *et al.* [77].

It seems very difficult to choose an appropriate method for the determination of the antioxidant capacity in samples because each different antioxidant has its own type of reaction mechanisms. No single method can be used to

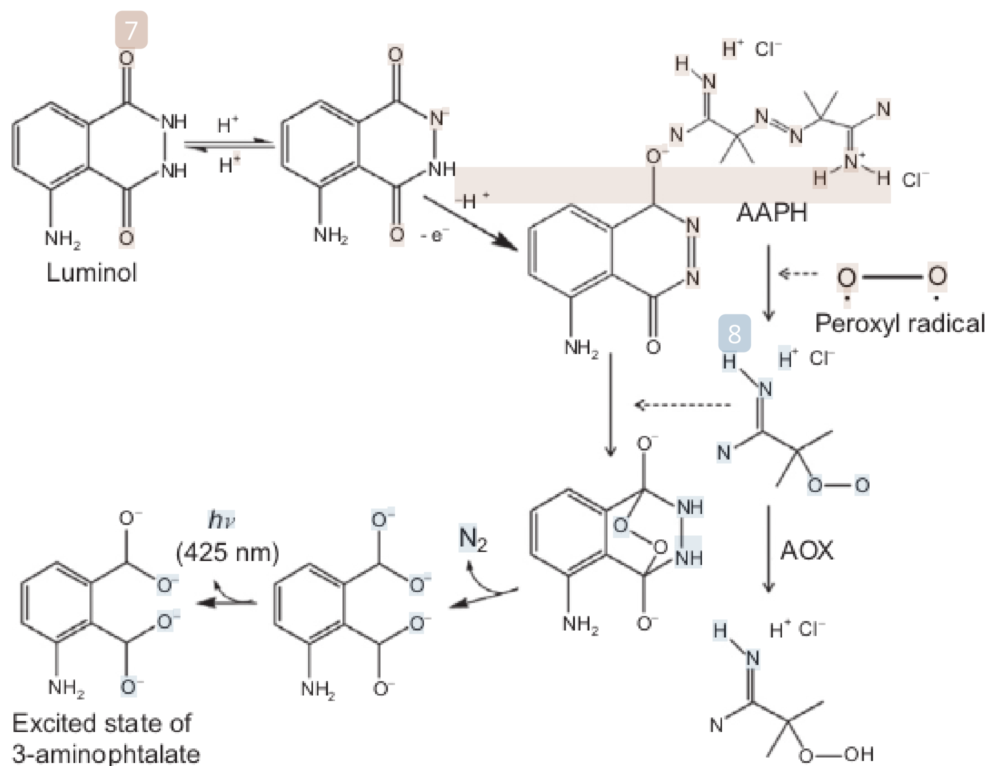

FIGURE 7 Schematic of TRAP assay: Luminol is oxidized by peroxyl radicals; in neutral or alkaline conditions, luminol is oxidized to anion form, and further oxidation by H_2O_2 yields the excited state of 3-aminophtalate; then, it returns to its ground state by emission of characteristic luminescence (CL) at 425 nm. Antioxidant (AOX) can quench CL. *Modified from Refs. [61,74].*

describe the different modes of action of the different antioxidants. An *in vitro* assay only shows the activity for a given reaction system, and unfortunately, its correlation with *in vivo* systems is uncertain. It is recommended to perform more than one type of *in vitro* antioxidant assay, and it will be better if the assay could be correlated with the *in vivo* system. Comprehensive discussions on these aspects have been published by Badarinath *et al*. [69].

The selected method of antioxidant assay must be validated first before being routinely used; this is because the ingredients in cosmetics are very complex. The method should have the capability to determine all types of antioxidants. The activities of each antioxidant must be determined before evaluating the activity of the finished products [66]. Due to the complexity of the composition of plant extracts, food, and cosmetics, both the antioxidant capacity and the concentrations of each of its active ingredients including the antioxidants need to be determined [42].

Unfortunately, for quality control purposes in the pharmaceutical industry in Indonesia, only the content of the active ingredients is determined quantitatively, and bioactivity evaluations are generally not performed. For determination of the concentrations of each active ingredient including the antioxidant compounds in samples, it is recommended to evaluate the accuracy profiles of each of the active ingredients including the antioxidants, instead of performing the validation method using classical methods [78].

Due to the possibility of variations in the content of secondary metabolites, including antioxidants caused by different conditions at the sites of cultivation, metabolite profiling studies of the plant extracts are recommended. It is well known that the conditions at the site of cultivation, the method of harvesting and drying, etc. can influence the content of the secondary metabolites in plants both qualitatively and quantitatively.

In addition, in cosmetics that have plant extracts as one of their active ingredients (see Table 1), the possibility of contamination with some toxic materials such as heavy metals, toxic microorganisms, microbial toxins, pesticides, and residual solvents must be evaluated; the content of all these toxic components must be lower than their maximum permitted levels [79].

CONCLUDING REMARKS

At present, the market development of cosmeceuticals is growing very rapidly worldwide. This might be due to the increasing demands of consumers for safe and effective cosmetics.

For relatively good, stable, acceptable cosmeceutical formulations, the selection of the antioxidants, other active ingredients, and the excipients must be optimized. A total quality control of the cosmetics must be performed. This is not being done as of now.

The routine quality control procedures should be able to determine both the bioactivities of all the active ingredients in products (including antioxidant

capacity) and their concentrations before the products are released to the market. This is very important on account of the instability characteristics of known antioxidants.

In conclusion, to provide the consumer with an effective and safe product, it is important that metabolite profiling studies of plant extracts are performed. For effectivity, it is of extreme importance to ensure the stability of the plant extracts or pure compounds to be able to support clinical results, and for safety reasons, it is important to investigate the concentrations of certain toxic components such as heavy metals. The cosmetic industry has undertaken several measures to date to increase the stability of active ingredients using conjugates to change the molecular characteristics as well as the addition of synthetic antioxidants such as BHT/BHA. The issue with this development is that the antioxidant capacity is often reduced and the additive synthetic compounds could have irritancy and sensitizing properties [80].

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ABBREVIATIONS

AAPH	2,2-azobis-(2-amidino-propane)-dihydrochloride
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AP	antioxidant power
BHA	butylated hydroxyl anisole
BHT	butylated hydroxytoluene
B-PE	β-phycoerythrin
CL	chemiluminescence
CoQ10	coenzyme Q10
DNA	deoxyribonucleic acid
DPPH	2,2′-diphenyl-1,1′-picrylhydrazyl
EDTA	ethylenediaminetetraacetate
ET	electron transfer
FL	fluorescein
GSD	glutathione synthetase deficiency
HAT	hydrogen atoms transfer
HPTLC	high-performance thin layer chromatography
NDGA	nordihydroguaiaretic acid
ORAC	oxygen radical absorbance capacity
PG	propyl gallate
RNS	reactive nitrogen species
ROS	reactive oxygen species

SEM scanning electron microscope
SOD super oxide dismutase
TBHQ tertiary butylhydroquinone
TEAC trolox equivalent antioxidant capacity
TRAP total radical trapping antioxidant parameter
UVR ultraviolet radiation

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