OPTIMIZATION EXTRACTION CONDITIONS AND CHARACTERIZATION OF ANTI-HYPERPIGMENTATION POLYSACCHARIDE FROM CALLISIA REPENS

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OPTIMIZATION EXTRACTION CONDITIONS AND CHARACTERIZATION OF ANTI-HYPERPIGMENTATION POLYSACCHARIDE FROM CALLISIA REPENS

by

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LIST OF SYMBOLS

&	And
~	Approximately equal
°C	Degree Celsius
=	Equal
<	Less than
>	More than
%	Percentage
±	Plus-minus
∑	Sigma
×	Times
λ	Lambda
E	Epsilon
-	Minus
η	Eta
Pa	Pascal

LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
cm	Centimeter
mm	Millimeter
ANOVA	Analysis of variance
BBD	Box Behnken design
CCD	Central composite design
cm^{-1}	Inverse centimeters
Da	Dalton
$CuSO_4$	Copper (II) sulfate
g	Gram
h	Hour
H_2O	Water
HPLC	High-performance liquid chromatography
I	Holar intensity spectrum
i.e.	*Id est* (that is)
kDa	Kilo Dalton
L-DOPA	3,4-Dihydroxy-L-phenylalanine
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MW	Molecular weight
nm	Nanometer
OH	Hydroxyl group
pH	Potential of hydrogen
PMP	1-Phenyl-3methyl-5pyrazolone
rpm	Revolutions per minute
RSM	Response Surface Methodology
s	Second

TFA	Trifluoroacetic acid
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction
USA	United States of America
v/v	Volume per volume
W	Watt
w/v	Weight per volume
w/w	Weight-to-weight
SPF	Sun protection factor
DESs	Deep eutectic solvents
Fig.	Figure
U	International unit of enzyme activity
DPPH	2,2-Diphenyl-1-picrylhydrazyl
L-tyrosine	Tyrosinase
EDTA	Ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
UV	Ultraviolet
LC-MS	Liquid chromatography-mass spectrometry
FTIR	Fourier-transform infrared spectroscopy
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
UK	United Kingdom
US	United States of America
HCl	Hydrochloric acid
KH_2PO_4	Potassium dihydrogen phosphate
ACN	Acetonitrile
OH	Hydroxyl group
SD	Standard deviation
DHA	Dehydroascorbic acid
mPa.s	Milli Pascal-second
Pa.s	Pascal-second
DMSO	Dimethyl sulfoxide
CA	Citric acid
AA	Aspartic acid

HPSCE	High Pressure Size Exclusion Chromatography
IC_{50}	Half-maximal inhibitory concentration
C.repens	*Callisia repens*
CRP	*Callissia repens* polysaccharide

PENGOPTIMUMAN KONDISI PENGESTRAKAN DAN PENCIRIAN POLISAKARIDA ANTI-HIPERPIGMENTASI DARI CALLISIA REPENS

ABSTRAK

Pelarut eutektik dalam (DES) adalah media hijau yang menjanjikan untuk pengekstrakan. Kajian ini bertujuan untuk mensintesis DES berasid novel daripada asid aspartik dan asid sitrik, mencirikan sifat fizikal dan kimianya, menilai fitotoksicitinya, dan meneroka kesesuaiannya untuk pengekstrakan polisakarida. Dua kaedah pemanasan, iaitu pemanasan konvensional dan gelombang mikro, dikaji untuk pembentukan DES. Hanya pemanasan gelombang mikro pada 270 W selama 6 minit dengan nisbah molar 3:1 (asid sitrik:asid aspartik) berjaya membentuk DES. DES yang disintesis berwarna jingga, mempunyai ketumpatan 1.44 $g \cdot ml^{-1}$ dan kelikatan tinggi iaitu 11665 Pa·s. Ia larut dalam pelarut kutub dengan suhu disosiasi 119.0 ℃. Menariknya, tindak balas Maillard berlaku semasa sintesis DES melebihi ikatan hidrogen, di mana analisis LC-MS mengesahkan kehadiran hasil sampingan Maillard. Indeks pengperangan dan penurunan pH mengesahkan perubahan kimia di luar pembentukan DES konvensional. DES tidak konvensional ini tidak menunjukkan fitotoksisiti berdasarkan percambahan biji gandum dan mempunyai sifat pelarutan polisakarida yang baik. Kajian ini mendedahkan bahawa komponen DES klasik seperti asid organik (asid sitrik) dan asid amino (asid aspartik) boleh mengalami tindak balas tidak boleh balik (iaitu Maillard) di bawah keadaan pemanasan gelombang mikro, mencadangkan bahawa DES tidak semestinya pelarut lengai. Oleh itu, kajian ini menekankan kerangka berhati-hati dalam mereka bentuk pelarut hijau yang reaktif. Selain itu, kajian ini bertujuan untuk mendapatkan polisakarida daripada *Callisia repens* yang mempunyai aktiviti anti-tirosinase, faktor perlindungan matahari (SPF),

dan aktiviti antioksidan. Polisakarida daripada *C. repens* diekstrak menggunakan kaedah DES asid sitrik-asid aspartik berbantukan gelombang mikro, dengan keadaan optimum (kuasa gelombang mikro 180 W, masa 4 minit, dan kepekatan DES 3%) ditentukan menggunakan reka bentuk Box-Behnken untuk memaksimumkan perencatan tirosinase, SPF, dan aktiviti antioksidan. Respons yang diperoleh ialah: (a) aktiviti perencatan monofenolase = 98.8%, (b) aktiviti perencatan difenolase = 81.6%, (c) aktiviti pemunahan radikal bebas DPPH = 84.7%, (d) SPF = 6.05, dan (e) hasil = 7.2%. Polisakarida yang diekstrak kemudian dicirikan. Kandungan protein yang rendah menunjukkan ketulenan tinggi polisakarida tersebut. Spektrum inframerah menunjukkan kumpulan berfungsi polisakarida, mengesahkan kejayaan pengekstrakan. Analisis monosakarida menunjukkan ia terdiri terutamanya daripada glukosa, galaktosa, dan arabinosa. Analisis kromatografi penelapan gel (GPC) menunjukkan berat molekul rendah iaitu 22.3 $\pm$ 0.3 kDa. Berdasarkan analisis pengkelatan kuprum, spektrum UV-Vis, serta kajian kinetik, dicadangkan bahawa aktiviti-aktiviti ini disumbang oleh ekstrak polisakarida kasar, yang bertindak sebagai perencat kompetitif tirosinase. Nilai IC_{50} bagi perencatan monofenolase dan difenolase masing-masing ialah 0.67 dan 2.80 mg/mL. Berdasarkan keputusan ini, hubungan struktur-sifat disahkan, dan polisakarida ini berpotensi digunakan dalam aplikasi kosmetik.

OPTIMIZATION EXTRACTION CONDITIONS AND CHARACTERIZATION OF ANTI-HYPERPIGMENTATION POLYSACCHARIDE FROM CALLISIA REPENS

ABSTRACT

Deep eutectic solvents (DESs) are promising green media for extraction. This study aimed to synthesize a novel acidic DES from aspartic acid and citric acid, characterize its physical and chemical traits, assess its phytotoxicity, and explore its suitability for polysaccharide extraction. Two heating methods, i.e., conventional and microwave heating were investigated for DES formation. Only microwave heating at 270 W for 6 min using 3:1 molar ratio (citric acid : aspartic acid) successfully formed the DES. The synthesized DES appears orange, has a density of 1.44 $g.ml^{-1}$ and high viscosity of 11665 Pa.s. It is soluble in polar solvents, with a dissociation temperature of 119.0 ℃. Interestingly, a Maillard reaction occurred during DES synthesis beyond hydrogen bonding, in which LC-MS analysis confirmed the presence of Maillard by-products. Browning index and pH reduction further confirmed the chemical changes beyond conventional DES formation. This unconventional DES showed no phytotoxicity based on wheat seed germination and provided good polysaccharide-solubilizing properties. Furthermore, the synthesized DES was used to obtain polysaccharides from *Callisia repens* with anti-tyrosinase activity, sun protection factor (SPF), and antioxidant activity. Polysaccharides from *C. repens* were extracted using a citric acid-aspartic acid deep eutectic solvent (DES)-microwave-assisted approach, and the conditions (i.e., microwave power of 180 W, time of 4 minutes, and DES concentration of 3%) were optimized using the Box–Behnken design to maximize tyrosinase inhibitory activity, SPF, and antioxidant activity. The responses

obtained were: (a) monophenolase inhibitory activity = 98.8%, (b) diphenolase inhibitory activity = 81.6%, (c) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity = 84.7%, (d) SPF = 6.05, and (e) yield = 7.2%. The extracted polysaccharides were then characterized. Low protein content indicated a high purity of the extracted polysaccharides. The infrared spectrum showed the functional groups of polysaccharides which indicate that polysaccharide has been successfully extracted. Monosaccharide analysis showed that it was mainly composed of glucose, galactose, and arabinose. Gel permeation chromatography (GPC) analysis indicated that it had a low molecular weight of 22.3 ± 0.3 kDa. Based on copper chelating and ultraviolet-visible (UV-Vis) spectral analyses as well as kinetic study, it was suggested that the activities were contributed by this crude polysaccharide extract, which were found to be competitive inhibitors of tyrosinase. IC_{50} values of 0.67 and 2.80 mg/mL for monophenolase and diphenolase inhibitory activities were also obtained. Based on these results, structure-property relationships were confirmed, and the polysaccharides could be used in cosmetic applications.

CHAPTER 1

INTRODUCTION

1.1 Background of study

In recent years, with the advancement of human civilization and growing awareness of health and wellness, the beauty and skincare industries have seen a surge in demand for natural and botanical ingredients. Consumers have become increasingly aware of the potential risks associated with synthetic chemicals and are seeking sustainable, eco-friendly products. This has sparked strong interest among scientists to study bioactive compounds such as polysaccharides (Al-Ajalein et al., 2023; Zhang et al., 2023; Abd et al., 2020; Kanlayavattanakul & Lourith et al., 2015; Yao & Xu et al., 2022).

Polysaccharides are carbohydrate macromolecules made up of chains of more than ten monosaccharides linked together by glycosidic bonds. They are significant biological macromolecules (Delattre et al., 2011) and are abundantly available in nature, found in a variety of plants, animals, and microbes. Numerous studies have indicated that natural polysaccharides have great application potential in food, health products, medications, and skincare products (Delattre et al., 2011; Cao et al., 2020; Meneguin et al., 2021; Elnashar, 2011; Tan & Gan, 2016; Tseng et al., 2010). Bioactive polysaccharides are characterized by their water retention, absorption, antioxidant, anti-inflammatory, anti-collagenase, anti-elastase, and anti-melanogenic or anti-tyrosinase activities. Recently, the use of natural polysaccharides for skin applications has gained more attention due to their promising efficacy in wound healing, moisturizing, anti-aging, and whitening (Yao & Xu, 2022). However, reliable natural reagents are in short supply because of issues such as the instability of natural components, low efficacy, and biosafety concerns (Hu et al., 2020; Tseng et al., 2021).

Deep eutectic solvents (DESs) are recognized as environmentally acceptable green solvents due to their simple preparation, low cost, low toxicity, and high biodegradability (Shafie, Yusof & Gan, 2019). They have gained increasing attention as efficient and sustainable media for extracting a wide range of bioactive compounds from natural sources. Notably, DESs have been successfully applied to the extraction of plant-based metabolites such as flavonoids, polysaccharides, lignin, and proteins (Cai et al., 2020; Saravana et al., 2018; Shang et al., 2021). Beyond extraction, DESs are also utilized in diverse fields including environmental remediation, nanomaterials synthesis, and biocatalysis (Omar & Sadeghi, 2020; Ni et al., 2019; Gotor-Fernández & Paul, 2019).

Of particular interest are acidic deep eutectic solvents (ADESs), which have attracted growing research attention due to their tunable physicochemical properties—such as acidity, viscosity, and density—and their potential for application in acid-catalyzed reactions (Qin et al., 2020). These properties enable ADESs to be tailored for specific extraction processes, offering a versatile and sustainable alternative to conventional solvents.

Undoubtedly, citric acid has been used as an important DES component. Wide range of citric acid-based DESs have been produced and characterized (Shafie, Yusof & Gan, 2019; Kurtulbaş et al., 2022; Yusoff, Gan & Shafie, 2023; Bai et al., 2023). On the other hand, amino acids have attracted immense attention lately as in this aspect. Apart from low toxicity, biodegradability, and low cost, amino acids have unique structure by having carboxy and amino groups, which enables them to function as both HBA and HBD (Guimarães et al., 2022). To meet the requirements of green chemistry and the needs for safety, citric acid-amino acid based DESs have become popular recently. Yi Hu et al. demonstrated that amino acid-based DESs (e.g., glycine – citric

acid, alanine – citric acid, arginine – citric acid) exhibit notable antibacterial activity and biosafety, supporting their potential use in functional foods (Hu et al., 2023). However, aspartic acid, a dicarboxylic amino acid containing both carboxyl (-COOH) and amino ($-NH_2$) groups, remains unexplored as a DES component. These functional groups enable strong hydrogen bonding, allowing aspartic acid to serve as either a hydrogen bond donor or acceptor, thereby enhancing its capacity to form stable DESs (Scheiner, Kar & Gu, 2001). Furthermore, its high aqueous solubility, attributed to favorable dipole interactions, suggests promising solvation properties (Sing et al., 2025). Consequently, aspartic acid is a promising candidate for formulating acidic DESs with broad applicability, particularly in extraction processes.

Cai et al. stirred HBAs and HBDs (molar ratio 1:1) at room temperature for 24 h to prepare temperature-responsive DES (TRDES) for extracting polysaccharides from Ganoderma lucidum. The yield was 92.35 mg/g under the extraction conditions of 50 min and 60 °C. By analysing the extraction ability of recycled extractant, the molecular weight and so on, the cyclic studies demonstrated that TRDES had good cyclic stability (Cai et al., 2020). On the other hand, Saravana et al. obtained DES by stirring choline chloride and glycerol at 80 ℃ for 60 min, the molar ratio is 1: 2. Then using subcritical water-DES extraction method to extract polysaccharide from brown seaweed for 25 min at the ideal conditions of 150 ℃, 19.85 bar, 70% water content and L/S ratio of 36.81 mL/g. The result showed the alginate yield is 28.12% and the unprocessed polysaccharides has displayed antioxidant activity (Saravana et al., 2018). Shang et al. used microwave-assisted DES to extract polysaccharides from bladder wrack. At 168 ℃ and 35 minutes of extraction time, the polysaccharide extraction rate was 11.633% and the antioxidant activity results show that, the extracted bladder-wrack polysaccharides have outstanding in vitro antioxidant properties, including

DPPH and ABTS radical-scavenging activity (Shang et al., 2021). Wu et al. prepared DES by using choline chloride and ethylene glycol in a molar ratio of 1:2 as the extraction solvent to extract polysaccharides from lotus leaves. The best extraction conditions were 60% water content in DES, 90 ℃, a liquid-solid ratio of 30 mL/g and a period of 120 minutes received an extraction rate of 5.38%, which higher than hot water extraction (yield, 3.22%) (Wu et al., 2021).

The microwave-DES-assisted extraction (MDAE) of polysaccharides from sweet tea leaves also conducted by the team of Wu. The greatest extraction yield (4.16%. w/w) of sweet tea polysaccharides extracted by MDAE was achieved in comparison to hot water extraction (P-W). The optimized MDAE method has a lower extraction time but a higher extraction yield when compared to P-W. Furthermore, the fundamental chemical structure of the extracted polysaccharides was unaltered by the MDAE approach (Wu et al., 2022). Shafie et al. achieved an extraction yield of 14.44% by applying Box-Behnken design to optimize the extraction parameters of pectin from Averrhoa bilimbi with DES. The pectin extracted in this experiment had strong free radical scavenging ability (41.46%) and decreasing antioxidant power (1.15 mM) of ferric (Shafie et al., 2019). The above results showed that DES has great potential as a green chemical solvent in extracting natural active plants.

1.2 Problem statement

As a common ornamental plant, *Callisia repens* is widely appreciated for its drought tolerance and ease of cultivation, yet its medicinal potential remains largely underexplored. While traditional use in regions such as Taiwan and Lingnan suggests its applicability in managing diabetes, and existing studies highlight the antioxidant and anti-diabetic properties of its water extract (Tseng et al., 2010; Leng et al., 2019),

the polysaccharides—its major bioactive constituents—have not been systematically investigated for their extraction, structural features, or potential anti-pigmentation effects. This study aims to address this research gap by developing an efficient extraction process for these polysaccharides and evaluating their applicability in skincare.

Hyperpigmentation disorders — such as melasma, age spots, and post-inflammatory hyperpigmentation — pose significant dermatological and cosmetic concerns, often impacting self-esteem and quality of life. These conditions arise from overproduction of melanin, primarily regulated by the enzyme tyrosinase. Current tyrosinase-inhibiting products frequently rely on aggressive chemicals including heavy metals and hormones, which can cause skin irritation, allergic reactions, and long-term health risks. Hence, there is a growing demand for safe, natural alternatives for hyperpigmentation treatment.

Conventional extraction methods using organic solvents or water are often inefficient, time-consuming, and prone to degrading thermolabile compounds. Although ionic liquids have emerged as alternative solvents, their toxicity and environmental impact raise sustainability concerns. Deep eutectic solvents (DESs) represent a greener alternative, offering low toxicity, biodegradability, simple preparation, and low cost. However, their application in extracting bioactive polysaccharides from *C. repens* has not been reported. This study seeks to leverage DES-based extraction to obtain polysaccharides with anti-tyrosinase potential, thereby supporting the development of natural and effective skincare ingredients.

1.3 Significance of study

This study could fill the research gap on *C. repens* polysaccharide and the development of extraction method and solvent. This work also provides scientific basis for its application in the field of skin care as well as new ideas and possibilities for the research and development of natural skin care products in the future based on polysaccharide research that promotes the development of skin care products industry to a more sustainable and healthy direction.

1.4 Objectives

This study focused on the extraction, structural characterization and anti-pigmentation activity of polysaccharides from *C. repens*, aiming to find new natural and effective ingredients for skin care products and contribute to the sustainable development of the skin care industry through in-depth exploration of this natural plant extract. The specific aims of this study are stated as follows:

1.4.1 Objectives 1

To develop and characterize a novel deep eutectic solvent (DES) for the extraction of water-soluble polysaccharides from *C. repens*, focusing on simplicity and effectiveness.

1.4.2 Objectives 2

To optimize the extraction process using the synthesized DES and evaluate the anti-hyperpigmentation activity of the extracted polysaccharides.

1.4.3 Objectives 3

To formulate a skincare product incorporating the polysaccharides extracted from *C. repens*.

CHAPTER 2

LITERATURE REVIEW

2.1 Callisia repens and its Traditional Uses

Callisia repens (Jacq.) L. with the common names of creeping basketplant, turtle vine or, Bolivian jew, is a small evergreen plant that belongs to the *Commelinaceae* family Fig.1(a). It is a low-growing, creeping, perennial succulent plant. The stems are thin, fleshy, and trailing, often forming dense mats. In addition, the stems can root at nodes when they touch the soil. The leaves are small (up to 4 cm long and 2 cm wide) and oval-shaped (acute at the tip and rounded at the base) with thick foliage. In terms of color, the leaves are green and tinged with purple or pink on the underside. Its flowers are borne in clusters in the leaf axils, small and white. This species thrives in shaded, stony, or pebbly areas in subtropical to tropical woodlands, and it is native to the French Antilles (Guadeloupe and Martinique), Argentina, and Southeastern America (Texas and Florida). It spreads via stem segments and seeds, allowing for simple and quick development in a variety of conditions (Tseng et al., 2010). As a result, this plant has spread widely in many countries, such as China, Malaysia, and Australasia. Scientists have also examined *C. repens* as a green roof plant in large cities to assist in alleviating urban warming and promoting urban greening due to its rapid growth and high drought and heat resistance (Feitosa, Castiglia & Wilkinson,2018; Silva et al., 2018; Cao et al., 2019; Zheng et al., 2016; Zheng et al., 2009; Ouyang et al., 2022). People in rural areas of China, such as Fujian Province, often drink water boiled with *C. repens* to help manage diabetes (Leng et al., 2019). However, the extract from *C. repens*, especially the polysaccharides, for skincare applications has not yet been reported. *Callisia*, derived from the Greek word "*kallos*," meaning beauty, has been used by indigenous people for skin treatments.

Previous work suggests that this plant contains a high content of carbohydrate polymers, leading us to hypothesize that these polysaccharides contribute to its skin-protective effects, including anti-aging and whitening (Sk et al., 2024).

Figure 2.1 Callisia repens

2.2 Hyperpigmentation

In human skin, melanocytes are regularly distributed in the dermis/epidermis margin and are responsible for melanin synthesis. Melanocytes in the basal layer of the epidermis interact and cooperate with the fibroblasts in the dermis and the keratinocytes in the overlying epidermis in a specific way to regulate skin function (Yamaguchi et al., 2007). The overactivity of melanocytes leads to the synthesis of melanin which results in visible skin pigmentation. The types of melanin production

depend on the function of melanogenic enzymes and the availability of substrates (Yamaguchi et al., 2007). Common pigmentation disorders include melasma, post-inflammatory pigmentation, macula, macula, etc. Skin pigmentation is a common skin disease that results from changes in the color of the skin due to various internal and external factors such as hormonal changes, inflammation, injuries, acne, eczema, certain medications, and UV exposure (Perez-Bernal, Muñoz-Pérez & Camacho, 2000). The key biological process in skin pigmentation is melanin synthesis, also known as melanogenesis. Melanin bodies were produced by melanocytes and exist in the basal layer at the junction of the dermis and epidermis (Duval et al., 2014). Also, melanin bodies were responsible for the production and storage of skin pigments such as melanin. These pigments are further distributed to adjacent keratinocytes, giving the skin its colour (Yamaguchi & Hearing, 2009). The synthesis of melanin is primarily regulated by tyrosinase, which catalyses the conversion of the amino acid tyrosine to dopa quinone, the precursor of eumelanin (brown to black pigment) and fusomelanin (yellow to red pigment). This process takes place in the melanosomes and involves a variety of enzymatic reactions. Pigment synthesis results in visible pigmentation on the skin (Yamaguchi & Hearing, 2009). Given the central role of tyrosinase activity, melanin synthesis, and melanosome transfer in pigmentation, targeting these processes is crucial for treating hyperpigmentation disorders.

Tyrosinase is key to initiating melanin production, and overactivity of tyrosinase promotes the production of more melanin, leading to skin hyperpigmentation (Al-Ajalein et al., 2023). Tyrosinase has the critical rate-limiting activity of converting tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and Dopaquinone by hydroxylation. In the presence of cysteine, the nascent Dopaquinone can produce a soluble yellow, red pheomelanin through a complex biochemical

reaction (Hennessy et al., 2005; Liu et al., 2005). When cysteine is depleted, the excess Dopaquinone spontaneously undergoes a complex biochemical reaction to form a dark brown or black high molecular weight insoluble polymer, known as DHI-melanin (5,6-dihydroxyindole-melanin). However, if Dopachrome tautomerase (DCT) is present, Dopachrome will undergo a complex biochemical reaction to form another lighter, soluble called DHICA-melanin (Ito, 2003). These are the three types of melanin usually contained in human skin, which are mixed in different proportions on the surface of human skin to form visible skin pigmentation (Wakamatsu et al., 2006).

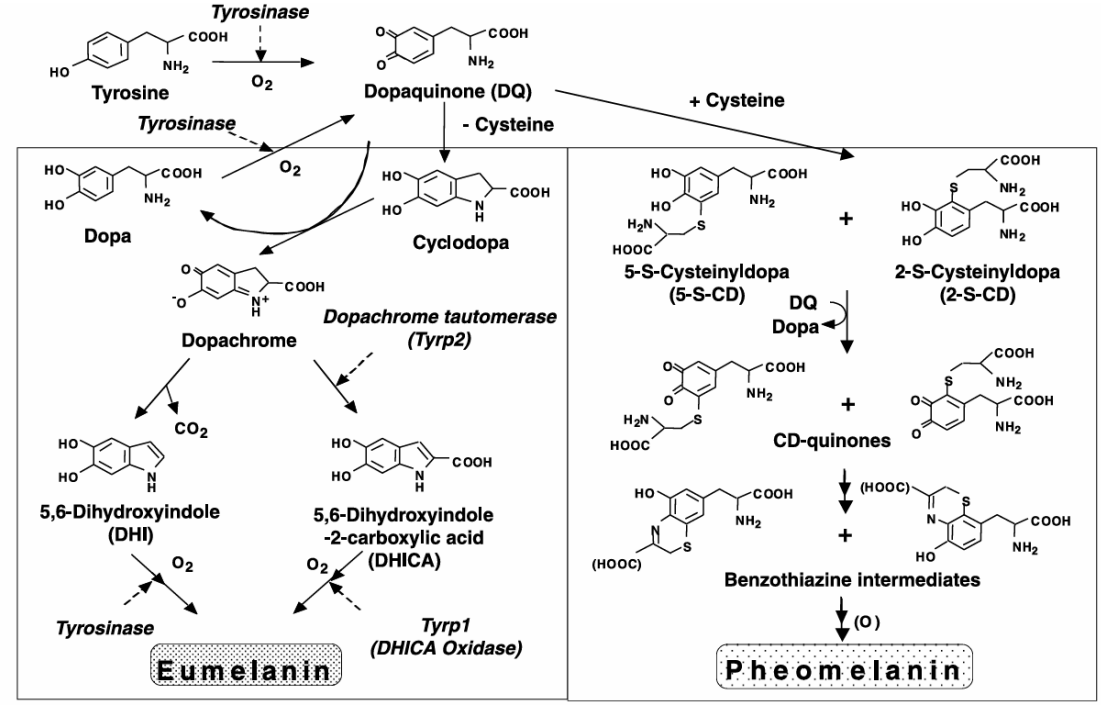

Figure 2.2 Pathways for production of eumelanin and pheomelanin in melanocytes (Adapted from (Wakamatsu et al., 2006))

The pursuit of healthy, flawless and radiant skin has been an enduring desire in both modern dermatology and skincare industries. However, due to the influence of many internal and external factors such as genetics, ultraviolet radiation, hormonal fluctuations, and aging, skin pigmentation is triggered, resulting in skin discoloration (Yamaguchi & Hearing, 2009; Rathee et al., 2021). Tyrosinase is key to initiating

melanin production, and overactivity of tyrosinase promotes the production of more melanin, leading to skin hyperpigmentation (Al-Ajalein et al., 2023). While skin pigmentation is a natural and important function that helps protect the skin from UV damage, excessive pigmentation can lead to a variety of cosmetic and dermatological problems, including melasma, post-inflammatory hyperpigmentation, freckles, and lentigines (Smit, Vicanova & Pavel, 2009). These conditions not only affect a person's appearance but can also have a profound effect on self-esteem and overall well-being.

However, according to the results of the survey, 51.8% of the respondents said they found side effects after using whitening products (Al-Sarraf, 2022). A recent meta-analysis of 67,665 participants worldwide had found the lifetime prevalence of skin bleaching was 27.7% ($p < 0.01$) (Sagoe et al., 2019). There are many skincare products with whitening and freckle slogans on the market often add excessive heavy metals, hormones, etc., and consumers are prone to skin allergies, pain, peeling, skin ulceration and other problems after use. Although the whitening effect of such products may be more obvious for a while, it has buried no small hidden dangers for the health of consumers. Hydroquinone, mercury and corticosteroids added to cosmetics are used to enhance the whitening effect, but prolonged use in large skin areas under hot and humid conditions can cause even fatal complications. Such as exogenous ochronosis, impaired wound healing, fish-odor syndrome, kidney disease, steroid addiction syndrome, susceptibility to infection, corticosteroid widespread cutaneous and endocrine complications (Olumide et al., 2008). With the improvement of people's knowledge level and the strong pursuit of healthy lifestyle, accompanied by the promotion of herbal products, people increasingly believe in and tend to buy and use products containing organic or natural ingredients (Chinomona, 2018). The plant's natural chemicals have a variety of cellular effects on a variety of skin diseases,

providing an opportunity to develop new formulations to treat pigmentation problems (Kaur, Aggarwal & Nagpal, 2019). Therefore, the extraction of natural, safe and effective substances, such as polysaccharide, from plants that are cheap and easy to obtain as effective agents of whitening skin care products has great application prospects and market economic benefit potential.

2.3 Polysaccharides as Bioactive Compounds

Polysaccharide is a carbohydrate polysaccharide macromolecule. It is a polysaccharide chain with more than ten monosaccharides linked together by a glycoside bond and it is also a significant macromolecule with biological action. Polysaccharides have a high density of -OH functionalities, giving them a hydrophilic nature and a large capacity to form H-bond networks. Therefore, except some of them, polysaccharides are generally hydrosoluble. Because polysaccharides have different structures, they have a wide variety of biological functions and properties, such as: energy storage (starch, glycogen), cell wall structure (cellulose, pectin) or gel formation (mucus). In addition, many studies have shown that polysaccharides and oligosaccharides have biological functions and are directly involved in biological reactions (Delattre, Fenoradosoa & Michaud, 2011). Starch is the most common carbohydrate polymer selected by evolution as a plant store, followed by fructan and cell wall polysaccharides (galactomannan, xylodextran and galactosan). Starch and fructan have the advantage of being composed of glucose and mainly fructose, respectively. These carbohydrates are quickly incorporated into energy-producing metabolism, which eventually rapidly produces ATP and provides carbon for the biosynthesis of most biomolecules in plant cells. As a polysaccharide particularly suited for storage function, starch has a unique mechanism that can be mobilized by

hydrolysis or through a mechanism that directly phosphorylates terminal glucose residues (Buckeridge, dos Santos & Tiné, 2000). The unpacked state of fructans is more permeable than starch, making them useful in controlling the osmotic potential of cells (Bieleski, 1993). Polysaccharides are abundantly available in nature since they are present in a range of plants, animals, fungi and microbes.

Because of the health benefits of polysaccharides, the extraction of polysaccharides from plants has been most developed as part of the history of herbal ingredients (Albuquerque, 2022). Numerous reported literatures have indicated that natural polysaccharides have a significant amount of application potential in food and health products, medication and skin care products (Cao et al.,2020; Meneguin et al., 2021; Elnashar, 2011; Tan & Gan, 2016; Tseng et al., 2010). At present, plant polysaccharides have been reported to have a variety of biological activities, such as significant anti-tumor, anti-oxidation, anti-coagulation, anti-diabetes, radiation protection, antiviral, hypolipidemia and immune regulation, and have a wide range of medicinal value (Table 2.1) (Chen et al., 2023). Bioactive polysaccharides are characterized by the ability of water retention, water absorption, antioxidant, anti-inflammation, anti-collagenase, anti-elastase, anti-melanogenic or anti-tyrosinase. Recently, the use of natural polysaccharides for skin applications has gained more attention because of their promising potent efficacy in wound healing, moisturizing, anti-ageing, and whitening (Zhang et al., 2023). However, reliable natural reagents are currently in short supply because various issues, such as the instability of natural components, low efficacy, and bio-safety concerns (Hu et al., 2020; Tseng et al., 2021).

Table 2.1 List of reported bioactive polysaccharides from different sources

No.	Source	Type of polysaccharide	Activity	Reference

1	Momordica charantia	Acidic heteropolysaccharide	Antioxidant, α-amylase inhibitory and ACE inhibitory activities	(Tan & Gan, 2016)
2	Nostoc commune	Heteropolysaccharide	Wound-healing and anti-allergic	(Tseng et al., 2021)
3	Ganoderma lucidum	Heteropolysaccharide	Antioxidant	(Cai et al., 2020)
4	Brown seaweed	Alginate and Fucoidan features (Sulfated polysaccharides)	Antioxidant	(Saravana et al., 2018)
5	bladder-wrack	Heteropolysaccharide	Antioxidant and anticancer	(Shang et al., 2021)
6	Lotus leaves	Heteropolysaccharide	Antioxidant, hypoglycemic, and immunomodulatory	(Wu et al., 2021)
7	Lithocarpus litseifolius (Hance) Chun	Heteropolysaccharide	Antioxidant, anti-diabetic, antiglycation, and prebiotic effects	(Wu et al., 2022)
8	Averrhoa bilimbi	Heteropolysaccharide	Antioxidant	(Shafie, Yusof & Gan, 2019)
9	Cinnamomum cassia bark	Heteropolysaccharide	Antioxidant and anti-hyperpigmentation	(Al-Ajalein et al., 2023)
10	Momordica charantia	Acidic heteropolysaccharide	Antioxidant, α-amylase and angiotensin converting enzyme inhibitory	(Tan & Gan, 2016)
11	Leonurus japonicus Houtt	Heteropolysaccharide	Antioxidant, anti-MMPs, anti-tyrosinase	(Zhang et al., 2023)
12	Nostoc commune	Sulfated polysaccharides	Antioxidant, wound-healing and anti-allergic	(Tseng et al., 2021)
13	Gracilaria lemaneiformis	Heteropolysaccharide	Anti-pigmentation.	(Feng et al., 2023)
14	Ganoderma lucidum	Heteropolysaccharide	Immunomodulatory, anti-tumor and antioxidant	(Jiang et al., 2019)

15	Bei Chaihu	Homogeneous	Antioxidant and hepatoprotective	(Zhao et al., 2012)
16	Glycyrrhiza glabra	Glycogen-like	Antioxidant	(Mutaillifu et al., 2020)
17	Astragalus	Acidic heteropolysaccharides	Anti-cancer and antitumor	(Li et al., 2020)
18	Grifola frondosa	Heterogeneous	Antitumor immunomodulatory	(Li et al., 2018)
19	Monostroma angicava	Rhamnan-type sulfated	Anti-thrombin	(Li et al., 2017)
20	Lycium barbarum L. leaves	Heterogeneous	Anticoagulant and antiplatelet	(Lin et al., 2019)
21	Ginseng	Heteropolysaccharide	Immunoregulatory and radioprotective	(Feng et al., 2024)
22	Potentilla anserina L	Heteropolysaccharide	Anti-radiation	(Shi et al.,2023)
23	Padina tetrastromatica	Sulfated olysaccharides	Antiviral	(Karmakar et al., 2010)
24	Pholiota nameko	Heteropolysaccharide	Hypolipidemic	(Li, Zhang & Ma, 2010)
25	Lonicera japonica	Homogeneous	Hypoglycemic and hypolipidemic	(Wang, Zhao & Liu, 2017)
26	Sea buckthorn leaves	Sulfated polysaccharide containing uronic acid	Antioxidant activity and immunological activity	(Liu, 2023)
27	Sargassum tenerrimum	Heteropolysaccharide	Hypolipidemic	(Sinha et al., 2010)
28	Mulberry leaf	Heteropolysaccharide	Anti-diabetic activity	(Zhang et al., 2014)
29	Lepidium meyenii Walp.	Heteropolysaccharide	Anti-fatigue	(Li et al., 2017)

30	Brassica rapa L.	Heteropolysaccharide	Anti-fatigue and antioxidant activity	(Zhao et al., 2021)
31	Pleurotus eryngii residue	Heteropolysaccharide	Antitumor activity	(Ma et al., 2014)
32	Astragalus membranaceus	Heteropolysaccharide	Anti-inflammatory activity	(Chen et al., 2023)

2.4 Anti - hyper pigmentation Polysaccharides

As a widely used medicinal and edible spice since ancient times, Al-Ajalein et al. used citric acid as extraction solvent and microwave assisted to extract polysaccharide form cinnamon. Box-Behnken design was used to optimize the extraction process and the optimize monophenololase inhibition activity and diphenolase inhibition activity of 97.5% and 99.4% was found from the obtained cinnamon polysaccharide respectively. This method not only obtained a high yield of 13.5% for cinnamon polysaccharide, but also with SPF value of 6.1. The functional groups, molecular weight and composition of polysaccharides were analyzed and determined by using FTIR, gel permeation chromatography technique and high-performance liquid chromatography (HPLC). The characterization results have showed that the extracted polysaccharides were pectinoid polysaccharides with low molecular weight and low esterification and contained trace phenols (Al-Ajalein et al., 2023).

Gracilaria lemaneiformis is a commercial edible red alga with high polysaccharide content. Feng et al. had used 2% H2SO4 at a ratio of 1:40 (w/v) for 2 h pretreatment on Gracilaria lemaneiformis dry sample and then used distilled water at a ratio of 1:100 (w/v) for 3 h at 90℃ to extract polysaccharide. The structure of heteropolysaccharides (GLHP) isolated from Gracilaria lemaneiformis was characterized by gel chromatography (GCP), infrared spectroscopy (FT-IR) and

nuclear magnetic resonance spectroscopy (NMR). The results showed that the molecular weight of GLHP was 47,600 Da, which was mainly composed of galactose (62.08%) and glucosamine (14.72%). In addition, GLHP had a significant inhibitory effect of hyperpigmentation in UVA-stimulated melanoma cells and keratinocyte cells via α-MSH/MC1R pathway and reduced the melanin contents in zebrafish tail models. Hence, GLHP could be as a novel cosmeceutical product for anti-pigmentation (Feng et al., 2023).

Ganoderma lucidum has been an expensive Chinese medicine since ancient times. Modern science and technology had found that Ganoderma lucidum polysaccharide (GLP) is the main active component of Ganoderma lucidum, which has immunomodulatory, anti-tumor and significant antioxidant effects. Jiang et al. have reported that GLP can inhibit UVB-induced melanin production by antagonizing cAMP/PKA and ROS/MAPK signaling pathways and is a potential natural safe whitening sunscreen additive (Jiang et al., 2019).

Jesumani et al. Had extracted polysaccharide from Sargassum horneri (ShoP) by hot water extraction. The crude polysaccharide was characterized by HPLC and FTIR, which showed that the polysaccharide was fucose-containing sulfated polysaccharide with not only strong inhibitory effects on tyrosinase (64.72 ± 0.46%) and total antioxidant capacity (80.93 ± 0.17%), but also scavenging ability on superoxide free radicals (67.4 ± 0.42%) (Jesumani et al., 2019).

Sangthong et al. had investigated the cosmetic activity and in vivo efficacy of Volvariella volvacea (VVP) as a multifunctional active cosmetic ingredient. The polysaccharide was extracted by hot water shaking was found to present the highest inhibitory effects toward lipid peroxidation (IC50 of 0.0378 mg/mL), tyrosinase (51.46 mg KAE/g), and elastase (604.21 ± 73.66 mg EGCG/g). The chemical compositions

of the hot water extracted polysaccharide for moisture content was 8.15 ± 0.28, and the carbohydrate content was 43.16 ± 0.38%, protein content was 19.40 ± 0.29%, crude fat content was 2.49 ± 0.18, ash content was 11.71 ± 0.28, and fiber content was 15.10 ± 0.16 (Sangthong et al., 2022).

Tao et al. used Viscozyme-assisted extraction method to extract and finally added 2L ethanol to obtain polysaccharide solution of purslane (VPOP3). VPOP3 extracted from Purslane is a low molecular weight acid heteropolysaccharide. Its structure was characterized by Fourier transform infrared spectroscopy, molecular weight and monosaccharide composition. The molecular weight was mainly 0.71 kDa, and the main monosaccharide composition was arabinose. High concentration of VPOP3 can significantly down-regulate the expression of tyrosinase (Tyrosine-related protein-1 and tyrosine-related protein-2 in the melanin generation signaling pathway, reduce the production of melanin, and thus achieve whitening effect (Tao et al., 2023).

Wang et al. used Celluclast to hydrolyze Hizikia fusiforme and then separated the sulfated polysaccharide (HFPS) by ethanol precipitation method. Fourier transform infrared spectroscopy (FT-IR) was used to characterize the structure of HFPS, and the existence of sulfate esters was proved. The cell experiments had found that HFPS can inhibit α-MSH-stimulated melanin production by inhibiting MITF expression, down-regulating tyrosinase, TRP-1 and TRP-2 levels (Wang et al., 2020). Kamilijiang et al had dried and degreased Saussurea involucrata using petroleum ether (1:10, w/v), followed by air drying and decolorization. The polysaccharide was gained after 2 hours' extraction by using 80 ℃ hot waters with an extraction yield of 11.37%. Saussurea involucrata polysaccharide not only has a significant in vitro radical scavenging activity but also can inhibit melanin biosynthesis by downregulating melanogenesis-related proteins and factors (Kamilijiang et al., 2022).

According to a study by Liu et al., Cuscuta chinensis polysaccharide (CPS) was extracted by hot water method and enzymatically hydrolyzed cuscuta chinensis polysaccharide (ECPS) was prepared by mannase enzymatic hydrolysis method. The molecular weight distribution of polysaccharides was determined by SEC-MALLS RI. The weighted average molecular weight of CPS and ECPS were 434.6 kDa and 211.7 kDa, respectively. Bioassay results showed that ECPS exerted anti-melanin activity by down-regulating the expression of tyrosinase, MITF, and TRP-1 without cytotoxic effects in B16F10 melanoma cells (Liu et al., 2018).

As a conclusion, it was suggested that certain polysaccharides could exhibit anti-hyperpigmentation via inhibiting tyrosinase activity and/or inhibiting MITF expression, down-regulating TRP-1 and TRP-2 levels in the signaling pathways. In this case of study, Callisia repens was used as the source of polysaccharide.

2.5 Mechanisms of Anti-hyperpigmentation by Polysaccharides

Polysaccharides have garnered significant interest for their bioactive properties, including their potential to treat hyperpigmentation disorders. Hyperpigmentation results from the overproduction and uneven distribution of melanin, often driven by excessive tyrosinase activity, melanosome synthesis, and transfer. Research has shown that certain polysaccharides possess anti-hyperpigmentation effects by inhibiting key pathways involved in melanin synthesis and melanosome transfer. This section reviews both in vitro and in vivo studies exploring the efficacy of these polysaccharides, providing insights into their mechanisms of action and potential therapeutic applications. Pigmentation is an umbrella term that includes various skin discoloration, pigmentation, and diseases associated with darkening, as mentioned in Section 2.2.

Recent research highlights the potential of polysaccharides to modulate these pathways, providing a promising approach to reducing excessive pigmentation and promoting even skin tone (Al-Ajalein et al. 2023; Feng et al., 2023; Jiang et al., 2019; Jesumani et al., 2019; Sangthong et al., 2022; Tao et al., 2023). Studies have demonstrated that polysaccharides derived from traditional medicinal plants can downregulate tyrosinase activity, leading to reduced melanin production (Al-Ajalein et al., 2023; Feng et al., 2023). This effect was often linked to the ability of polysaccharides to interact with the enzyme's active site or modulate its expression at the genetic level. Beyond tyrosinase inhibition, polysaccharides can also disrupt melanosome synthesis and transfer. Melanosome transfer from melanocytes to keratinocytes is a critical step in visible skin pigmentation, certain polysaccharides have been shown to interfere with this transfer process. For an example, Ganoderma lucidum polysaccharide can inhibit paracrine action of keratinocytes and fibroblasts through IL-6/STAT3/FGF2 pathway, thereby reducing melanogenesis production, which providing multiple mechanisms for their anti-hyperpigmentation effects (Jiang et al., 2019). Research also indicated that polysaccharides exert their anti-hyperpigmentation effects through antioxidant activity. By scavenging free radicals and reducing oxidative stress in the skin, polysaccharides from Achatina fulica and Heimiella retisporacan indirectly influence melanin synthesis, as oxidative stress is known to stimulate melanogenesis (Kao et al., 2019). This multi-faceted approach makes polysaccharides promising candidates for treating hyperpigmentation disorders.

Numerous in vitro studies have demonstrated the efficacy of polysaccharides in inhibiting melanin synthesis and reducing hyperpigmentation. For an example, Tao et al. reported that polysaccharides isolated from portulaca oleracea L. exhibited potent tyrosinase inhibitory activity when tested on B16-F10 melanoma cells. The study

showed a significant decrease in melanin content and tyrosinase activity after polysaccharide treatment, indicating their capacity to modulate melanogenesis at the cellular level (Tao et al., 2023). Additionally, sulfated polysaccharides from marine algae extracts have shown promise by reducing melanin production through the downregulation of microphthalmia-associated transcription factor (MITF), which controls tyrosinase expression (Kamilijiang et al., 2022). Such cell-based assays provide essential insights into the mechanisms and bioactivity of polysaccharides, serving as a foundation for further preclinical studies.

In vivo studies further corroborate the anti-hyperpigmentation effects of polysaccharides observed in vitro. Research by Tao et al. demonstrated that different concentrations application of polysaccharide-rich extracts (VPOP3) on α-MSH-induced hyperpigmented zebrafish embryos models significantly reduced melanin deposition, melanin content and TYR activity. At a concentration of 50 μg/ml, VPOP3 had an anti-melanogenic effect comparable to arbutin (Tao et al., 2023). Similarly, in vivo skin whitening experiments evaluated the efficacy of the polysaccharide liposome formulation and found that it had better skin whitening activity than the arbutin formulation group (Lv et al., 2024). These findings illustrate the translational potential of polysaccharides for treating hyperpigmentation.

The demonstrated anti-hyperpigmentation properties of polysaccharides highlight their potential for clinical and commercial applications. Polysaccharides could be developed into novel cosmeceutical formulations, targeting hyperpigmentation disorders such as melasma, age spots, and post-inflammatory hyperpigmentation. Additionally, their natural origin, biocompatibility, and multifaceted action make polysaccharides attractive for use in skincare products, which enhancing formula efficacy while maintaining good skin tone (Baptista &

Freitas, 2021). Future research and development may focus on optimizing polysaccharide extraction and formulation methods to enhance efficacy, stability, and consumer accessibility, paving the way for effective therapeutic and preventive solutions in dermatology.

2.5.1 Other anti-hyperpigmentation properties - antioxidant and protection factor

Up to 5% of the oxygen in the atmosphere is active in producing activated oxygen or reactive oxygen species (ROS). On the one hand, ROS regulates homeostasis in the human body, including epidermal keratinocyte proliferation, on the other hand, but also aggravates skin aging and inflammation, leading to pigmentation. In addition to the oxidation caused by ROS in the air, as the largest organ of the human body, if exposed to ultraviolet light, it will also promote the production of reactive oxygen species, causing pigmentation and other problems caused by oxidative aging of the skin (Nakai & Tsuruta, 2021). Therefore, the antioxidant capacity is usually used as one of the indicators to judge the anti-pigmentation ability of polysaccharides. In most studies, the antioxidant properties of polysaccharides are often judged by carrying out scavenging free radical tests, such as DPPH, FRAP and hydroxyl free radicals. On the other hand, sun protection factor (SPF) is a general measure that describes the effectiveness of a product or substance against UV rays. It can also be used to determine the UV resistance of polysaccharides that with potential applications as sunscreens.

For example, Al-Ajalein et al. found that Cinnamomum cassia polysaccharides extracted by microwave-assisted DES method had good antioxidant properties, with ferric reducing antioxidant power of 4.4 mM and SPF of 6.1 (Al-Ajalein et al., 2023).

Tao et al. used enzyme-assisted extraction to collect polysaccharide from Portulaca oleracea L., and it was found that the attained polysaccharide, VPOP3, showed the greatest antioxidant capability with the DPPH and hydroxyl radical scavenging rates of 52.44% and 59.76%, respectively (Tao et al., 2023). Polysaccharides from polygonatum sibiricumhave have showed a certain scavenging effect on DPPH, hydroxyl radical, superoxide anion radical and a particular effect on the chelating ability of ferrous which was reported by Wang et al. (Wang et al., 2022). Yan et al. used four different extraction methods: hot water extraction (HWE), ultrasonic assisted extraction (UAE), microwave assisted extraction (MAE) and enzyme assisted extraction (EAE) to extract water-soluble polysaccharides from Amomum villosum and found out polysaccharide attained by ultrasonic assisted extraction had showed the best antioxidant activity (Yan et al., 2015).

In the study of Jesumani et al., three kinds of seaweed (*Sargassum vachellianum* (SvP), *Sargassum horneri* (ShoP) and *Sargassum hemiphyllum* (SheP) were used as raw materials for polysaccharide extraction, and the scavenging ability, reducing ability and total antioxidant activity of three kinds of polysaccharides at different concentrations were studied. The DPPH, superoxide radical scavenging, reducing power and total antioxidant activity results showed that SheP and SvP had the highest DPPH scavenging capacity, which were $74.56 \pm 0.10\%$ and $74.13 \pm 0.36\%$ (1000 μg/mL), respectively. However, SheP exhibited maximum total antioxidant ($80.93 \pm 0.17\%$) and superoxide radical scavenging activity ($67.4 \pm 0.42\%$), as well as better anti-tyrosinase activity (Jesumani et al., 2019). Wang et al. reported that PBP, a sulfated polysaccharide isolated from Padina boryana, had antioxidant effects and application potential in the cosmetic industry. It was found that PBP had obvious protective effect on skin cells stimulated by UVB. PBP inhibits the death of human