Polyhydroxynaphthoquinone Pigment From Vietnam Sea Urchins as a Potential Bioactive Ingredient in Cosmeceuticals

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Abstract
In this study, valuable polyhydroxynaphthoquinone (PHNQ) pigments were recovered from sea urchin food waste and were investigated as a potential bioactive ingredient for cosmeceuticals. The crude PHNQ pigment extract from 4 Vietnam sea urchins, Diadema setosum, Diadema savignyi, Stomopneustes variolaris, and Tripneustes gratilla, exhibited effective 2,2-diphenyl-1-picryl-hydrazyl-hydrate scavenging activity, tyrosinase inhibitory activity, and antibacterial activity. The moisturizing cream with 0.5% of PHNQ pigments from D. setosum and Tripneustes gratilla sea urchins showed no dermal irritation over 14 days of mouse skin test. Four major active components in PHNQ were identified via high-performance liquid chromatography coupled with diode array detector and mass spectrometry. Echinochrome A contributed considerably to the antioxidant activity of the extracts while those containing echinochrome A and spinochrome E were significantly active against various bacteria. The promising results laid the foundation for establishing a novel process from food waste to innovative biomaterial and formulating eco-friendly skincare products with PHNQ components from sea urchins as precious ingredient.

Keywords
polyhydroxynaphthoquinone, quinonoid pigment, sea urchin, cosmeceutical, biological activity

Received: September 18th, 2020; Accepted: October 20th, 2020.

Cosmetics were defined as care substances for cleansing, enhancing attractiveness, and changing the human appearance without altering the body’s structure or process.¹ For a long time, the utilization of synthetic chemicals in manufacturing cosmetics posed undesired issues. The inappropriate formulation of composite based on synthetic components induced product instability and generated side effects like skin irritation, photodermatitis, comedogenicity, carcinogenicity, and eczema.²⁻⁴ Thus, the use of natural active ingredients possessing clear origin, compositions, interactive mechanisms with the skin, and supplemental health benefits garnered widespread attentiveness in current cosmetic industry. Kligman in his study introduced the term “cosmeceuticals” as cosme(tic) products with pharma(ceutical) benefits.⁵ The cosmeceuticals such as creams, lotions, and ointments contained bioactive ingredients (eg, phytochemicals, essential oils, vitamins, and antioxidants), which not only improved skin beauty but were mainstay of treating, mitigating, and preventing skin diseases.⁶ Rising demand for cosmeceuticals has promoted manufacturers to replace synthetic chemicals by naturally derived ingredients in production process. Among the published bioactive ingredients in cosmeceuticals (eg, ulvan,⁷ sulfate galactofucan,⁸ curcumin⁹), phenolic compounds attracted considerable attention. The compounds found popularly in anti-aging and whitening skin products were considered to enhance antioxidative activity and skin protection against ultraviolet (UV) radiation.¹⁰⁻¹² In recent years, hundreds of phenolic compounds were determined and quantified from plant species. Flavonoids and gallic acid were phenolic compounds extracted from terrestrial species while marine organisms like algae contained mainly phlorotannin class of polyphenol compounds.¹³ Interestingly, phenolics extracted from shells and spines of sea urchins had structures similar to those extracted from

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terrestrial plants. Typically, the quinonoid pigments called polyhydroxynaphthoquinones (PHNQs) in many sea urchin species were reported to possess several valuable bioactivities such as antimicrobial, antioxidative, and cardioprotective activity. From *Scaphechinus mirabilis*, the authors successfully isolated echinochrome A (Ech A), which is the active substance of the new drug Histochrome for the treatment of myocardial infarction and eye diseases. Besides, Powell et al in their study also reported that quinonoid pigments from *Pseudechinus mildari* urchins displayed high anti-UV activity.

In Vietnam, the urchin species were diverse and distributed widely across the sea. In Khanh Hoa province, *Diadema setosum* (Leske), *Tripneustes gratilla* (Linnaeus), *Stomopneustes variolaris* (Lamarck), and *Diadema savignyi* (Audouin) were the most abundant species. According to a national survey program, production of edible urchins in Vietnam in the period of 1996-2000 reached over 500 tons per year. This corresponded to the fact that huge amount of waste materials (e.g., shell, spine) containing valuable quinonoid substances were discharged into the environment.

In this research, valuable quinonoid pigments were recovered from shell and spine of 4 different sea urchin species and were studied for the first time as a potential bioactive ingredient in cosmetics. Quinonoid compounds were fractionated and the compositions were determined by high-performance liquid chromatography coupled with diode array detector and mass spectrometry (HPLC-DAD-MS). PHNQ pigments were examined for their antioxidative activity by 2,2-diphenyl-1-picryl-hydrazyl-hydra te (DPPH) radical scavenging test, antimicrobial activity against *Candida albicans* yeast and 7 Gram-negative and Gram-positive bacteria, and inhibition of tyrosinase enzyme activity. The moisturizing creams with high bioactive PHNQ pigments were tested for dermal irritation on mouse skin. The results provided database for further formulating novel skincare products with quinonoid pigments from sea urchins.

### Experimental

#### Materials

The sea urchin species *D. setosum* (Leske, 1778), *D. savignyi* (Audouin, 1809), *Tripneustes gratilla* (Linnaeus, 1758), and *Stomopneustes variolaris* (Lamarck, 1816) were collected at a depth of 5 m (Vietnam, Khanh Hoa province, Nhatrang Bay, 12°11' n, 109°14' E and 12°15' n, 109°20' E). These species were identified by morphological method.

The reagents utilized for this study were all purchased from Sigma-Aldrich Co., which included sulfuric acid (H2SO4), chloroform (CHCl3), ethyl acetate (EtOAc), ethanol (EtOH), and DPPH, and tested microorganisms are *Bacillus cereus* (ATCC-11778), *Staphylococcus aureus* (ATCC-27853), and *Candida albicans* (ATCC-10231). Tested mice were BALB/c strain.

#### Extraction of Quinonoid Pigments

The internal organs of the urchins were eliminated. The shells and spines were cleaned with tap water, dried in shade, and extracted exhaustively with ethanolic acid sulfuric 10% (v/v) at ambient temperature. The ethanol extract was concentrated in vacuo. The residue in water was partitioned respectively by chloroform (CHCl3) and ethyl acetate (EtOAc). After that, the CHCl3 and EtOAc fractions containing PHNQ were then combined, and concentrated under reduced pressure to collect crude PHNQ extract.

#### Determination of PHNQ Content

The total PHNQ content was quantified by UV absorbance method at 470 nm wavelength (UV-1800, Shimadzu, Japan) using a quartz cuvette (1 cm path length). Ech A was used as standard with a concentration range of 10-50 µg/mL. All the experiments in this study were performed with 3 replicates.

#### PHNQ Pigment Identification

The crude PHNQ extract was dissolved in ethanol. The ethanol solution was filtrated by 0.2 µm cellulose acetate membrane (Advantec MFS, Inc., California, USA) prior to HPLC-DAD-MS analyses on an LC-MS-2020 instrument (Shimadzu, Japan) with a Discovery-HS-C18 column (3 µm size, 150 × 2.1 mm). A 0.5% solution A (acetic acid) and a solution B (acetonitrile containing 0.5% acetic acid) were applied following gradient elution as follows: 10%-40% B (0, 6 minutes), 40%-100% B (6, 11 minutes), 100% B (11, 12 minutes), 100%-10% B (12, 13 minutes) vía 10% B (13, 17 minutes). The flow rate and the injection volume were fixed at 0.2 mL/min and 2 µL, respectively. The spectra were recorded on a diode array detector at λ = 254 nm.

#### DPPH Free Radical Scavenging Activity

The crude PHNQ extract was dissolved in methanol to obtain a stock solution (800 mg/L) for testing antioxidative activity. The DPPH radical scavenging capacity was performed pursuant to the method of Yen and Chen. The UV absorption coefficient at 516 nm was determined using an ultraviolet-visible (UV-Vis) spectrophotometer (UV-1800, Shimadzu, Japan). Scavenging activity was calculated following the formula as below:

\[
SC (%) = \left[1 - \frac{A_{m} - A_{c}}{A_{c}}\right] \times 100\%
\]  

where \(A_c\) is the absorption of comparative sample (DPPH in ethanol solution), \(A_m\) the absorption of sample (DPPH and...
Inhibition of Tyrosinase Activity

The crude PHNQ extract was dissolved in ethyl acetate at a concentration of 25 mg/mL for testing antimicrobial activity. The PHNQ solution was screened based on a paper disc-diffusion assay following to El Masry HA et al. In short, the PHNQ solution was infused at a concentration of 500 µg/disc onto sterile Whatman discs (6 mm diameter). After 1 hour under air hood, EtOAc solvent was evaporated. The antimicrobial activity was assessed against 8 human pathogens namely Gram-positive bacteria (Bacillus subtilis ATCC-11778, Staphylococcus aureus ATCC-25923, and Listeria monocytogenes ATCC-19111), Gram-negative bacteria (Klebsiella pneumonia ATCC-700603, Salmonella typhi ATCC-14082, Escherichia coli ATCC-25922, and Pseudomonas aeruginosa ATCC-27853), and yeast (Candida albicans ATCC-10231). These tested microorganisms were grown up on bacto nutrient agar, and the turbidity of suspensions was adjusted to 10⁸ cells/mL utilizing a UV-Vis spectrophotometer (UV-1800, Shimadzu) at 625 nm wavelength. Similar procedure with pure EtOAc solvent and ampicillin was performed for negative and positive control, respectively. The plates were incubated for 24 hours at 37 °C and then the diameter of the inhibition zones was measured for activity comparison.

\[
\frac{1}{Y} = a + b \ln(X)
\]  

where \(Y\) is the reagent concentration and \(X\) the Sc% value.

Result and Discussion

Chemical Composition of PHNQ Pigment From Vietnam Sea Urchin Species

The composition and content of PHNQ pigment extracted from 4 species of Vietnam sea urchins are exhibited in Table 1. As shown in Table 1, the total PHNQ content from the sea urchins distributed in the range of 1910 ± 340-6150 ± 510 µg/g. The PHNQ content from the urchin species in this study was much higher than those of the species in Okhotsk sea, Pacific Ocean14 and E. mathaei species in Madagascar sea.25 Besides differences in extraction conditions, it was supposed that the climatic conditions can affect the biosynthetic ability of the urchins in producing PHNQ pigments.

Structural identification of the compounds contributing to PHNQ pigment was determined based on comparison of the retention time of the HPLC peaks, UV-Vis \(\lambda_{\text{max}}\) of PHNQ sample in this paper with those of published PHNQ pigment (see Supplemental Table S2 for more details of referenced peaks). As the result, the peaks 1, 2, 3, and 4 were assigned to the compounds spinochrome E (Sp E), spinochrome D (Sp
It is noticeable that the PHNQ pigment from *D. setosum* reported by Anderson et al. contained not only Ech A and Sp E but also Sp D and Sp A. Moreover, according to Brasseur et al., there was no Sp E in composition of PHNQ pigment from *D. savignyi* while PHNQ pigment from *Tripneustes gratilla* contained Sp D and Ech A rather than Sp A. Despite diversity in composition due to growing in different geographical regions, the major component of PHNQ pigment in each urchin species remains unchanged, ie, Ech A in *Diadema* species and Sp E in *Tripneustes gratilla*.

### Antioxidant Activity of PHNQ Pigment From the Sea Urchin Species

PHNQ pigment extracted from shells and spines of the sea urchins contained the phenolic compounds with several hydroxy groups, which was considered to exhibit highly antioxidant activity. According to Da-yong Zhou et al., the activity intensity was contingent on the ratio of the components in PHNQ. In this research, the antioxidant activity of PHNQ pigments was investigated based on DPPH free radical scavenging assay and the results were displayed in Supplemental Table S3 and Figure 1.

It can be observed from Supplemental Table S3 that the PHNQ pigment from *Tripneustes gratilla* displayed the lowest antioxidant activity among the 4 sea urchins with an EC$_{50}$ value of 58.47 μg/mL. Highest DPPH scavenging capacity belonged to the PHNQ pigments from the species of *Diadema* genus (EC$_{50}$ = 28.07 μg/mL for *D. savignyi* and EC$_{50}$ = 26.82 μg/mL for *D. setosum*).

There existed some published works on DPPH scavenging capacity of PHNQ extracted from different sea urchin species. To be specific, the PHNQ pigment extracted from *Echinometra mathaei* species with diethyl ether solvent included Sp A, Sp B, Sp C, and Ech A components and exhibited higher DPPH scavenging capacity than the commercial phenolic antioxidants butylated hydroxytoluene. Li et al. in their work found that the PHNQ pigment extracted from *Glyptocidaris crassispina* contained Sp B, Sp D, and Sp E in composition, and was more active in DPPH scavenging than *Strongylocentrotus intermedius* PHNQ, which contained only Sp B (IC$_{50}$ = 60 μg/mL compared to IC$_{50}$ = 80 μg/mL respectively). Vasileva et al. were successful in isolating the components in PHNQ extracted from the urchin species namely *Scaphechinus mirebeltis*, *Strongylocentrotus pallidus*, and *Mesocentrotus nudus*. The components were examined for DPPH scavenging activity, which followed the order of echinamine B (EC$_{50}$ = 6.5 μM) >Ech A >spinamine E >echinamine A >Sp E >α-tocopherol. Thus, it was supposed from our work that PHNQ pigments from *D. setosum* were significantly more antioxidative to those from other sea species via the highest contribution of Ech A in composition.

### Antimicrobial Activity of PHNQ Pigment From the Sea Urchin Species

The antimicrobial activity of PHNQ pigment from the sea urchin species against tested bacteria is exhibited in Table 2 and Supplemental Figure S4. Larger inhibition zone diameter demonstrated a more effective antimicrobial activity. The crude extract from the sea urchins demonstrated activity against all 7 bacteria but was ineffective against yeast *Candida albicans*. PHNQ extracted from *D. setosum* showed the highest performance inhibiting 6 per 7 bacteria except *Bacillus cereus*, whereas PHNQ extracted from *D. savignyi* expressed the lowest antibacterial activity against all the bacteria except *Staphylococcus aureus*. The outcome would originate from high contribution of Ech A in composition of the extracted PHNQ pigment (87% in PHNQ from *D. setosum* and 59% in PHNQ from *D. savignyi*). Component Ech A was reported previously by Service et al. that displayed strong activity inhibiting the growth of *Staphylococcus aureus*.
Pseudomonas strain 111, Vibrio fischeri, and Micrococcus sp. As stated by Stekhova et al in their study, Ech A also exhibited outstanding performance against the bacteria species namely Trichophyton mentagrophytes and Staphylococcus aureus compared to Sp B, Sp C, and Sp D. The result suggested further orientation of producing natural antimicrobial products containing PHNQ pigment with Ech A as the major ingredient.

In addition, it was noticeable that the PHNQ pigment extracted from Stomopneustes variolaris inhibited significantly Bacillus cereus and PHNQ from Tripneustes gratilla acted markedly against Listeria monocytogenes and Escherichia coli. One common point from PHNQ extracted from the 2 urchins above was the high proportion of Sp E in their composition; thus, the component Sp E was assumed here to possess variable effect depending on bacteria strains. The result was consistent with the report of Brasseur et al. in which the isolated Sp E were strongly active against E. coli (EC$_{50}$ = 28.53 µM) with a minimum inhibitory concentration of 7.25 µg/mL but not relatively effective in case of Caloplaca marina and Shewanella oneidensis. In our study, the crude PHNQ extract from the sea urchins, in which 2 main components were Ech A and Sp E, exhibited antimicrobial activity against all the tested bacteria and were very promising in application as an antibacterial ingredient in cosmeceutical products.

Tyrosinase Inhibition Activity of PHNQ Pigment From the Vietnam Sea Urchins

When human skin was exposed to UV radiation, the melanocytes produced a protective pigment called melanin to block the UV light. However, redundancy of melanin in skin was considered to cause negative symptoms such as hyperpigmentation, tanning, age spots, and freckles. Tyrosinase is a rate-limiting enzyme involving in controlling the melanin generation. Thence, in manufacturing process of skin care products, inhibitors of tyrosinase enzyme were commonly

![Figure 1. DPPH free radical scavenging activity of PHNQ pigment extracts from the four Vietnam sea urchins. DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; PHNQ, polyhydroxynaphthoquinone.](image)
implemented to manipulate the formation of melanin. In this study, the PHNQ crude extracts were tested for tyrosinase inhibition with a fixed concentration of 250 µg/mL and the results are shown in Table 3.

The most effective inhibitors seemed to be the PHNQ pigment extracted from *Diadema setosum*. The pigment can restrain 87.9% ± 4.1% of tyrosinase enzyme, which nearly reached the activity of whitening substance Kojic acid (88.5% ± 3.3%). In contrast, PHNQ pigment from *Tripneustes gratilla* showed the lowest performance when only 68.5% ± 2.9% of tyrosinase was inhibited. The result was correlated well with the antioxidant effect when the contribution of Ech A in the composition of PHNQ pigment was likely to determine the antioxidant and tyrosinase inhibition activity of the PHNQ pigments.

### Skin Irritation Assessment

In order to evaluate the safety level of using PHNQ crude extracts in cosmeceuticals, 2 cream samples with PHNQ pigment in ingredient were assessed of skin irritation. Sample KM1 with PHNQ pigment extracted from *Diadema setosum* and sample KM2 with PHNQ pigment extracted from *Tripneustes gratilla* represented 2 components Ech A and Sp E, respectively. Erythema and edema symptoms were tracked at time intervals and the results are displayed in Supplemental Table S4, S5 and Figure S5.

From Supplemental Tables S4 and S5, the mouse expressed no irritation indication (skin erythema or edema) after treating with 0.2 mL of cream containing 0.5% of PHNQ pigment. After 14 days of assessment, the primary irritation index grade was still 0.

### Conclusion

In this research, valuable PHNQ pigments were recovered successfully from food waste of the 4 most popular sea urchin species in Vietnam namely *Diadema setosum, D. savignyi, Tripneustes gratilla*, and *Stomopneustes variolaris*. HPLC-DAD-MS analysis was effective to determine the active composition of PHNQ pigments. The 4 crude PHNQ extracts all exhibited promising bioactivities without causing skin irritation, which were promising to be applied for replacing the synthetic chemicals in cosmeceuticals.

For the active components in PHNQ pigments, the PHNQ pigments with Ech A in composition were determined to exhibit highly antioxidant activity and those containing echinochrome A and Sp E were significantly active against various bacteria. The PHNQ comprising Ech A component can inhibit the growth of tyrosinase enzyme to a great extent. The PHNQ pigment from *Diadema setosum* species with Ech A as the major component was exceptional among those from other species when it exhibited the highest performance in all bioactivity tests. The interesting results were a promising suggestion for employing specific components of PHNQ into specific types of cosmeceuticals.

### Statement of Human and Animal Rights

Experiments were performed in accordance with Vietnamese ethical laws and European Communities Council Directives of November
24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research is funded by Vietnam Academy of Science and Technology (Project number VAST06.01/19-20).

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Supplemental Material
Supplemental material for this article is available online.

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