Antioxidant Activity of the Sea Bird Nest (Eucheuma Cottonii) and Its Radical Scavenging Effect on Human Keratinocytes

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***Abstract*—The potential of *Eucheuma cottonii* (EC) to be a novel source of antioxidants and protection against photoageing is increasingly evident but largely unexplored. This study aimed to evaluate the antioxidant activity and radical scavenging capacity of EC extracts on human keratinocytes. Aqueous and methanol extracts from EC were evaluated in a series of *in vitro* assays on the HaCaT keratinocyte cell line. Antioxidant activity was determined via the DPPH assay, while MTT was used to evaluate the cytotoxicity of EC extracts up to 72h exposure. Quantitative and qualitative DCFH-DA fluorescence assays assessed intracellular reactive oxygen species (ROS) levels in UV-irradiated cells. EC extracts at concentrations from 10 µg/ml were found to possess significant antioxidant activity ($p<0.05$). Interestingly, the aqueous extract compromised cell viability at high concentrations, while the methanol extract was relatively non-toxic. Intracellular ROS levels significantly decreased with increasing concentration of EC extract treatment ($p<0.05$). In conclusion, EC extracts demonstrated antioxidant activity and protective effects against UV-induced ROS degeneration in keratinocytes, thus underlining its potential in nutraceutical research to promote skin rejuvenation.**

***Index Terms*—*Eucheuma cottonii*, sea birdnest, antioxidant, keratinocytes**

I. Introduction

Intense ultraviolet radiation exposure on skin keratinocytes leads to photoageing and undesirable cosmetic consequences. A major cause for photoageing is the accumulation of toxic free radicals and cellular damage induced by UV-B exposure [1]. *Eucheuma cottonii*, known in some communities as *Kappaphycus alvarezzi* or the 'sea bird nest', is an edible red/brown rhodophyte seaweed of the order Gigartinales. A potent source of K-carrageenan, a gelling agent and stabiliser [2], this commercial crop is farmed in tropical waters of South-East Asia for applications in the food and cosmetic industries. It is claimed to possess properties analogous to that of edible swiftlet nest in skin rejuvenation, although evidence on its effectiveness and mechanism of action is scarce.

Currently hailed as a health-promoting dietary supplement, *E. cottonii* is gaining recognition as a cardioprotective agent in murine models, exhibiting the most significant antihyperlipaemic and *in vivo* anti-lipid peroxidation effects among a host of other tropical seaweeds [3]. A recent study showed that the polyphenol-rich extract of *E. cottonii* inhibited erythrocyte lipid peroxidation in cancer-induced rats [4]. The effect was attributed to flavonoids and other possible natural antioxidants in seaweed, including phlorotannins. The presence of sulphur moieties in carrageenan [5] also may contribute to its free-radical scavenging activities.

Although the anti-lipid peroxidation effect of *E. cottonii* has been suggested in animal studies, the antioxidant and radical scavenging capacity of *Eucheuma* sp. on epithelial cells has not been studied. Thus, we aimed to investigate the radical scavenging potential of *E. cottonii* on keratinocytes. Upon establishing its potential antioxidative activity on keratinocytes, this knowledge may be realised in cosmetic and medical applications for the alleviation of photoageing in the population.

II. Materials and Methods

A. Preparation of E. cottonii Extracts

Raw dry *E. cottonii* (EC) were purchased from a local supplier in Kuala Lumpur, Malaysia. EC was rinsed thoroughly with reverse osmosis water to eliminate epiphytes and debris, and homogenised into fine (5 mm) sections using a blender. The resultant EC homogenate was then allowed to dry at 60 ℃ for 24 hours.

Methanol extract: The dry sample was blended into a fine powder and immersed in methanol (50 g/250 mL, w/v) and incubated at ambient temperature in the dark for four days. The resultant solvent was then filtered and concentrated using a rotary evaporator. Final drying was at 60 ℃ overnight before storage in a desiccator at ambient temperature.

Aqueous extract: The dried homogenised sample was immersed in ultrapure water at 5˚C for 24 hours (15 g/600 mL, w/v). The sample was boiled at 100˚C for 30 minutes. The aqueous extract was filtered with Whatman's filter paper, freeze-fried and stored at 4˚C.

The extracts then were reconstituted in DMSO and serially diluted into concentrations from 5 µg/mL to 2560 µg/mL for downstream applications.

Manuscript received July 2, 2014; revised December 12, 2014.

doi: 10.12720/jomb.4.6.461-465

B. DPPH Radical Scavenging Assay

The antioxidant potential of EC extracts was determined by measuring its capacity to reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Absolute ethanol, DPPH (Sigma-Aldrich, USA) and ascorbic acid (Fisher Scientific, UK) were used as blank, negative and positive controls respectively. DPPH reagent was dissolved in absolute ethanol to a concentration of 0.5 mM, and added into each well containing the extracts and controls (except the blank). The plate was then incubated at ambient temperature in the dark for 40 minutes before measuring the absorbance using a microplate spectrophotometer (Tecan, Switzerland) at 540 nm. The percentage of antioxidant activity was determined via the following formula:

$$Activity\ (\%) = [1 - (A_{sample} - A_{blank})/(A_{control})] \times 100 \quad (1)$$

With $\boldsymbol{A_{sample}}$ and $\boldsymbol{A_{control}}$ representing the absorbance of EC extract and negative control respectively.

C. Cell Culture

Human keratinocytes (HaCaT), a generous gift from Dr. Chee-Onn Leong, were maintained at standard culture conditions (37 ℃ and 5% CO_2) in DMEM medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (Gibco, Life Technologies, USA). Cells were seeded at 80% confluence into multi-well formats for the viability and radical scavenging assays.

D. MTT Assay

HaCaT cells were seeded at a density of 3.5×10^4 cells/well and serum-starved with DMEM supplemented with 1% FBS for 24 hours. Treatment with EC extracts were performed at concentrations from 5 µg/mL to 1280 µg/mL for 24, 48 or 72 hours. Treated cells were incubated with 10 µL/well of 5000 µg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (CalBiochem, USA) for 4 hours. The medium was removed and 200 µL DMSO was added and incubated for further 10 minutes. Absorbance was measured with a microplate spectrophotometer (Tecan, Switzerland) at 570 nm. The percentage of cell viability was then computed using the following formula:

$$Cell\ viability\ (\%) = [A_{sample}/A_{control}] \times 100\% \quad (2)$$

With $\boldsymbol{A_{sample}}$ and $\boldsymbol{A_{control}}$ representing the absorbance of EC-treated cells and negative control (cells without treatment) respectively.

E. Intracellular ROS Scavenging Activity Assay

Intracellular reactive oxygen species (ROS) levels of EC-treated cells were determined using the dichlorofluorescin diacetate (DCFH-DA) redox dye method. Experiments were repeated with cells exposed to ultraviolet light irradiation (UV lamp: Uvitec, UK) at 312 nm and intensity of 0.74 mW/cm^2 for 20 seconds.

Qualitative Assay: HaCaT cells were treated with 10, 100 or 1000 µg/mL of EC extract. Positive control cells were incubated with 10 µM H_2O_2 alone for 15 minutes. Non-treated cells were negative control. The cells were incubated for 24 hours before washing with PBS and staining with freshly prepared 25 µM DCFH-DA (Sigma-Aldrich, US). The cells were incubated for another 30 minutes in the dark and washed twice with PBS. Bright field and corresponding fluorescence images were captured using a fluorescence microscope (Nikon, Japan) with FITC filter.

Quantitative Assay: HaCaT cells were seeded into 24-well plates and treated with EC extract (concentrations and controls as above). The cells were then incubated for 24 hours before trypsinisation and transfer to a 96-well fluorescence assay plate. Cells were stained with 10 µM DCFH-DA and incubated for 30 minutes in the dark. Absorbance was measured using a fluorescence micro-plate spectrometer (Dynex, Opsys MR) with emission and excitation wavelengths of 485 nm 535 nm respectively.

F. Statistical Analysis

Results from the DPPH assay and DCFH-DA assay (quantitative) were analysed using GraphPad Prism 6 software. One-way ANOVA along with Sidak's and Dunnett's multiple comparisons test were used to determine significant differences between various treatment concentration groups. All experiments were performed in independent triplicates and expressed as mean ± standard deviation. Results were considered significant at $p<0.05$.

III. Results

A. Antioxidant Activity of EC Extracts

EC extracts demonstrated the capacity to quench DPPH free radicals with increasing concentrations, with methanol extract exhibiting a stronger antioxidant activity. The antioxidant activity of each extract increased proportionally in a dose dependent manner, as did the ascorbic acid (Fig. 1). The difference in antioxidant activity between each treatment concentration of both groups were statistically significant ($p<0.05$) from concentrations 20 µg/mL to 2560 µg/mL. Nonetheless, compared to positive control (up to 98.17%), the antioxidant activity of EC extracts were substantially weaker (up to 34.83%/43.09%).

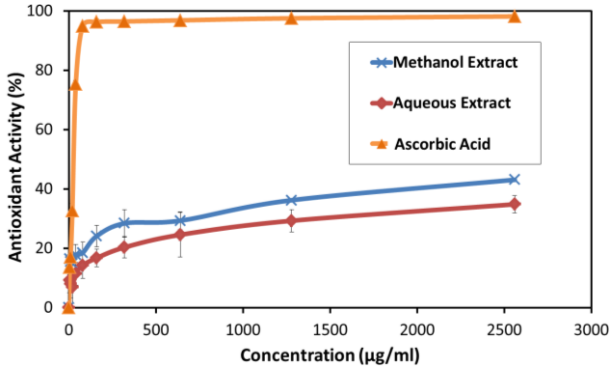


Figure 1. Antioxidant activity of EC extracts (5 µg/mL to 2560 µg/mL) compared with ascorbic acid (positive control). Negative control and blank were DMSO and (DMSO + ethanol) respectively. Results are expressed as mean ± standard deviation of triplicates from three independent experiments.

B. Effect of EC Extracts on Cell Viability

Viability for cells treated with the aqueous EC extract declined with increasing concentration and duration (fig. 2A). IC_{50} values were 1756.59 µg/mL, 917.87 µg/mL, and 781.31 µg/mL for 24, 48 and 72 hours respectively.

For the methanol extract, viability of the EC-treated cells maintained above 50% (Fig. 2B). Hence, the absence of an IC_{50} value suggests that the extract was non-toxic to the HaCaT cells. The results suggest that the aqueous extract exerted a mild cytotoxicity to keratinocytes at high concentrations and extended exposure duration.

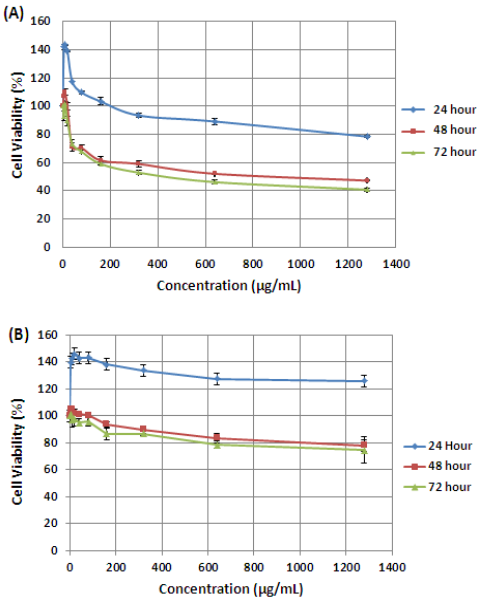
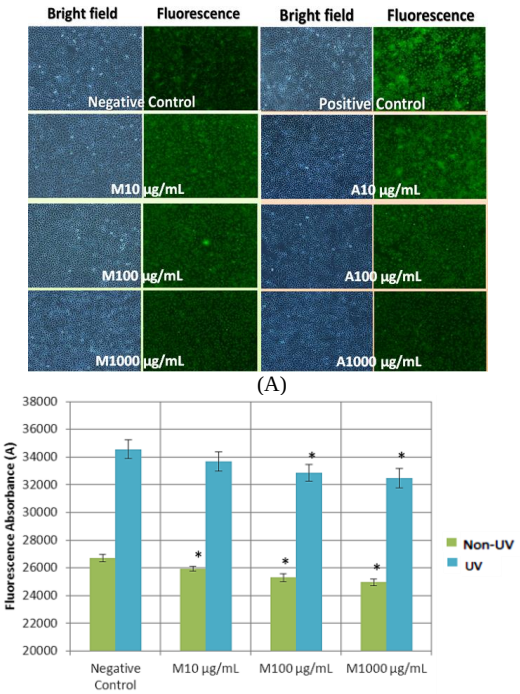
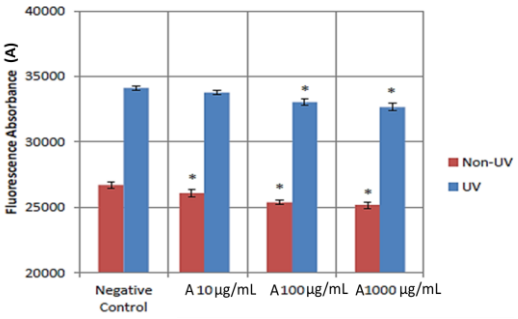


Figure 2. Viability of HaCaT cells with increasing concentrations of EC extracts (5 µg/mL – 1280 µg/mL) over 24, 48 and 72 hours. (A) aqueous extract; (B) methanol extract. Non-treated cells were the negative control. Values are means ± standard deviation of triplicates from three independent experiments.





(B)

Figure 3. Qualitative (A) and quantitative (B) DCF fluorescence of UV and non-UV irradiated cells at 485/535 nm, after treatment with 10, 100 or 1000 µg/mL of EC extracts for 24 hours. Negative controls are non-treated UV (10 mJ/cm^2)/non-UV irradiated cells indicating baseline levels of ROS, and positive controls are UV/non-UV irradiated cells treated with H_2O_2. Quantitative data is expressed as mean ± SD of triplicates from 3 independent experiments. M10 µg/mL: cells treated with 10 µg/mL methanol extract; A10 µg/mL: cells treated with 10 µg/mL aqueous extract; *p<0.05 compared with negative control. Photomicrographs were captured at 100x mag.

C. ROS Scavenging Activity of EC Extracts in Human Keratinocytes

Intracellular ROS levels of keratinocytes were determined via DCFH-DA staining, whereby the fluorescence intensity is directly proportional to ROS levels. The fluorescence intensity in UV-irradiated cells was higher than that of non-UV irradiated cells, which demonstrates that irradiation increased baseline ROS levels in the cells. Our findings revealed that EC treatment (both methanol and aqueous extracts) reduced oxidative activity for UV and non-UV irradiated cells in a dose-dependent manner (Fig. 3).

IV. Discussion

In the current study, antioxidant properties and reactive oxygen species scavenging ability of *Eucheuma cottonii* (EC) extracts were investigated. A simple colourimetric assay to screen for free radical-scavenging ability of antioxidants in bioactive compounds is the DPPH assay. 1,1-diphenyl-2-picrylhydrazyl (DPPH) exists as a stable free radical with delocalisation of a spare electron contributing to the intense violet colour which is converted to pale yellow upon reduction [6]. Our results provide evidence that even dried EC, as opposed to fresh plants, exhibited substantial antioxidant activity.

EC is a rich source of polyphenols, phytochemicals, proteins, carrageenan, pigments, polyunsaturated fatty acids, minerals, and vitamins [7], [8]. Polyphenolic compounds were found to be closely associated with free-radical scavenging properties [9]. The methanol fraction, which demonstrated a higher level of antioxidant activity, may have yielded a significant amount of polyphenols. This is substantiated by reports that seaweeds contain more polar compounds [10]–[12]. Κ-carrageenan, a water-soluble sulphated linear polysaccharide, is also a major component of EC. However, only approximately 1-2% is present in the crude aqueous extract [13]. Nonetheless, phlorotannins in the aqueous fraction of

brown seaweed may also contribute to its antioxidant activity [14].

Antioxidants are purported to protect body tissues from oxidative stress and many pathological conditions such as cancer and heart disease by neutralising harmful free-radicals such as ROS. ROS induces cell death, skin ageing and tissue injury from the action of oxidising biomolecules [4], [10]. In its natural habitat, the presence of sunlight and oxygen molecules exposes seaweeds to free radicals. However, absence of structural damage to the seaweed components suggested that they may possess an anti-oxidative defense mechanism by producing compounds that protects against oxidation [12], [15]. EC and seaweeds such as *S. binderi*, *C. lentillifera*, and *C. racemosa* are known to be potent sources of antioxidants [3], [11], [15]–[17]. Furthermore, EC consumption enhances gluthionine (GSH) production [18] and exhibited better antihyperlipaemic and *in vivo* antioxidant effects as compared to *C. lentillifera* and *S. polycystum* seaweeds [7].

The sun emits a wide spectra of electromagnetic radiation, including ultraviolet (UV) rays which are deleterious to cellular components, particularly collagen [19]. UVB radiation (in the 295-315 nm wavelength range), the most damaging UV light, causes sunburn, undesirable cosmetic effects, and even cancer [19]–[21]. Our results show that UV-irradiated human keratinocytes developed higher levels of intracellular ROS compared to non-UV irradiated cells, as visualised through the conversion of non-fluorescent 2', 7'-dichlorofluorescin diacetate (DCFH-DA) to fluorescent 2', 7'-dichlorofluorescein (DCF). Previous studies have demonstrated that human keratinocytes exhibit cellular toxicity upon exposure to UVB irradiation, thus elevating ROS levels leading to premature photoageing [22], [23].

Our study also established that EC extracts reduced ROS levels dose-dependently in cells with or without exposure to UV irradiation. This correlated with findings from the DPPH antioxidant assay, thus reaffirming the radical-scavenging capacity of EC on human keratinocytes.

We assessed the potential cytotoxicity of EC extracts using MTT which measures the formazan formed in metabolically active mitochondria. Our findings suggest that methanol EC extracts had little or no toxicity towards HaCaT cells as the percentage of cell viability remained above 50%, even at higher concentrations. EC is known to exert anti-proliferative and cytotoxic effects on cancer cell lines but is non-toxic to normal or primary cell types [4], [18]. Additionally, *in vivo* administration of EC extract enhanced epithelial tissue healing in Sprague-Dawley rats [11]. EC had also been widely consumed by indigenous populations without reports of ill effects. The reason behind the mild toxicity of the aqueous extract is unclear, perhaps to be elucidated with further fractionation and purification of compounds within the extract.

To our knowledge, this is the first report of EC exerting antioxidant activity against UV-induced ROS levels in keratinocytes. Similar studies on other seaweeds have revealed comparable findings with an attempt to elucidate its underlying mechanisms. Phlorotannins and cytokinins are thought to be responsible for seaweed's antioxidant capacity [24]–[26]. *A. nodusum* was reported to increase the activity of superoxide dismutase which scavenges superoxide [27]. Another study demonstrated *G. lanceolata*'s antioxidant effect in inhibiting lipid accumulation and ROS level by reducing several mRNA transcription factors, while *G. birdie* was found to enhance glutathione levels [28], [29]. Therefore, EC may well be a promising source of antioxidants with a potential application in the alleviation of dermal pathology related to UV-mediated ROS injury.

V. Conclusion

Aqueous and methanol EC extracts exhibited significant antioxidant activity and reduced the level of UV-induced ROS in HaCaT human keratinocytes; perhaps attributed to its polyphenolic compounds. The methanol extract exerted a stronger antioxidant activity compared to the aqueous fraction. Although the aqueous extract exerted slight cytotoxicity at high concentrations, implications of this are unclear. Further analysis is necessary to identify the bioactive constituent of EC which would contribute to the understanding of its ROS scavenging effect.

Acknowledgment

This study was funded by the International Medical University Biomedical Science and Bachelor of Medical Sciences undergraduate research project grants. The authors would also like to thank A/P Dr. Shar Mariam, A/P Dr. Chee-Onn Leong and their graduate students for usage of their equipment and technical assistance.

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