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BIOACTIVITIES OF THE HYDROUS ETHANOL EXTRACT FROM MARINE GREEN ALGA *CHLAMYDOMONAS* SP. W80

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ABSTRACT: For the simultaneous productions of bioenergy and high value coproducts by green algae, the 50% ethanol extract of the marine green alga *Chlamydomonas* sp. W80, which is a potential hydrogen-producing algal strain, was examined for various bioactivities, such as antioxidant, anti-inflammatory, anti-aging, hair growth promotion, immune stimulatory and skin lightning. The skin lightning effect of the extract was found with three assay methods; anti-melanin formation, inhibition of endothelin-1 (ET-1) and stem cell factor (SCF) productions. The hair growth promotion effect was also found with two assay methods; dermal papilla cells propagation and inhibition of 5α-reductase.

Key Words: Green algae, Bioenergy, High valuecoproduct, Skin lightning, Hair growth promotion

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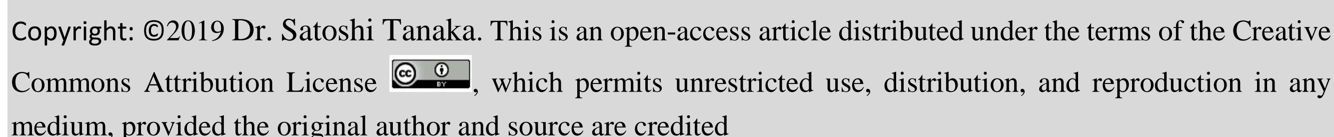

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INTRODUCTION

Biofuel from microalgae is a renewable and carbon neutral alternative to fossile fuels [1 to 5]. Since fuel price is generally quite low, simultaneous productions of value added co-products, such as health supplements, cosmetics and fine chemicals, by microalgal cells make the algal biofuel production processes economically more feasible [6]. The marine green alga *Chlamydomonas* sp. W80 (*C.* W80) produces hydrogen [7] and also shows a surprisingly high tolerance against oxidative stress caused by methyl viologen, which is reduced by the photosynthetic apparatus generating highly toxic superoxide (O_2^-) [8]. We have been intensively making researches on the isolation of useful genes of *C.* W80 and successfully isolated various anti-stress genes, such as ascorbate peroxidase [9], glutathione peroxidase [10-11], type 3 late embryogenesis abundant protein [12] and BBC1 [13]. We also found that the extract of *C.* W80 has some radicals cavenging activity [14].

C. W80 is a stress tolerant and fast-growing strain, thus co-productions of energy and bioactive compounds by this strain can be a promising biofuel production process. In this study, we examined the bioactivities, such as anti-inflammatory, immuno stimulatory, skin lightning, anti-aging and hair growth promotion effects of *C*. W80 cell extract with various assay methods.

MATERIALS AND METHODS

Preparation of the extract from *Chlamydomonas* W80

The green alga *Chlamydomonas* W80 used in this study was isolated from the coastal area of Wakayama, Japan and identified as a *Chlamydomonas* species as described previously [15]. To obtain a large amount of *C*. W80 cells, the cells were grown in an outdoor open pool (a circular pool with 20 m diameter and 8 cm water depth) under sunlight with the artificial sea water for the culture medium. Cells were harvested by low speed centrifugation, washed with water and freeze-dried. Approximately 100 g of dried sample was extracted with one liter of ethanol-water 1:1 (w/w) under reflux at 80°C for 2 hours. After filtration, the extracts were dried in vacuum at 60°C. The yield of extract was 18.7% of the dry weight.

Cultured cells for bioassay

Murine B16 melanoma cells were obtained from Riken Cell Bank (Tsukuba, Japan). Normal human epidermal keratinocytes (NHEK) and keratinocytes growth medium, Humedia-KG2 (KGM) were purchased from KURABO INDUSTRIES LTD. (Osaka, Japan). Human follicle dermal papilla cells (DPC) and HFDPC growth medium, PCGM were purchased from TOYOBO Co., LTD. (Tokyo, Japan).

Melanogenesis assay with B16 melanoma cells

The B16 melanoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 35 µg/mL penicillin and 70 µg/mL streptomycin, at 37°C in a humidified, CO_2-controlled (5%) incubator. Melanin content measurement was based on the previously reported method [16]. The B16 melanoma cells (4.0 x 10^5 cells per dish) were inoculated into DMEM containing 10% FBS and 1 mmol/L theophylline in 60-mm tissue culture dishes and cultured for 8 hours. Then, the *C.* W80 extract dissolved in DMEM containing 10% FBS and 1 mmol/L theophylline was added to the culture and the cells were incubated for 4 days. The medium without *C.* W80 extract was added to the control experiment cultures. After incubation, the cells were harvested with trypsin treatment and cell number was counted. The cells were collected by centrifugation (2,500 x *g* for 6 min. at room temperature), lysed in 2 mL of 1 mol/L NaOH containing 10% DMSO and sonicated. The absorbance of lysate was measured at 475 nm to determine the melanin content.

Enzyme-linked immunosorbent assay (ELISA) for endothelin-1 (ET-1) and stem cell factor (SCF) protein

NHEK were cultured in Humedia-KG2 with growth supplements. NHEK (2.0 x 10^5 cells per well) were inoculated in KGM into 96-well plates and incubated for 24 hours. The cells were washed two times with Hanks buffer and suspended in 1 mL of Hanks buffer. The cells were then irradiated with 30 mJ/cm^2 of UV-B. Buffer was removed and the cells were treated with the *C.* W80 extract dissolved in KGM. After 48 hours incubation, the concentrations of endothelin-1 (ET-1) and Stem Cell Factor (SCF) in the culture media were measured by ELISA. ET-1 was analyzed by ELISA kit for human ET-1 (Endothelin, Human, EIA Kit; CAYMAN CHEMICAL, Michigan, USA) (Awano, et al., 2006). SCF was measured using an ELISA kit for human SCF (SCF, Human, ELISA Kit, Quantikine; R & D systems, NE, USA) [17].

MTT assay

DPC were cultured in collagen coating flask, in PCGM with growth supplements. Cell proliferation was determined by MTT assay [18]. DPC (2.0 x 10^5 cells per well) were inoculated into DMEM containing 10% FBS in collagen-coated 96-well plates and cultured for 3 days. After incubation, culture medium was removed and 0.2 mL of *C.* W80 extract dissolved in DMEM was added to each well. Cells were incubated at 37 ◦Cin a 5% CO_2 incubator for 5 days. After incubation, 20 μl of MTT (5 mg/mL) was added to each well and cells were incubated for 4 h at 37◦C. The supernatant was then removed and 200 μl of dimethyl sulfoxide was added to dissolve for mazan products. The cell numbers were estimated by comparing the optical density at 570nm of the samples to that of control sample and expressed as a percentage of the control.

Testosterone 5α-reductase assay

The enzyme suspension of testosterone 5α-reductase was prepared from rat liver homogenate (S-9) mixture as described previously [19]. Twenty μL of testosterone (4.2 mg/mL) dissolved in propylene glycol and 825 μL of NADPH (1 mg/mL) dissolved in Tris-HCl buffer (5 mmol/mL) were mixed in a test tube. Then 80 μL of the *C.* W80 extract dissolved in 50% ethanol and 75 μL of S-9 were added and incubated at 37°C for 30 min. Reaction was stopped by adding 1 mL methylene chloride. The mixtures were centrifuged (1,600 x *g*, 10 min) and methylene chloride phase were analyzed by gas chromatography (Shimazu GC-7A, KYOTO, JAPAN).

IC_{50} value calculation and statistical analysis

The half maximal inhibitory concentration (IC_{50}) was calculated from the values of the inhibitory activities at several concentrations by using a linear regression analysis. Statistical analysis was done by Student's t test. Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

The bioactivities of the 50% ethanol extract of *C*. W80 were examined by 29 assay methods. Table 1 shows the list of assay methods. We examined the effects of antioxidant (4 assay methods), anti-inflammatory (5 assay methods), immunostimulatory (2 assay methods), skin lightning (4 assay methods), anti-aging (10 assay methods) and hair growth promotion (3 assay methods). Among these 29 assay methods, the extract of *C*. W80 showed the effectiveness in 3 skin lightening (B16 melanoma cells, ET-1and SCF) and 2 hair growth promotion (inhibition of testosterone 5α-reductase and proliferation of dermal papilla cells) assays. No effect was observed in other 24 assays (data not shown).

Table 1. Screening for bioactivities of extracts of *Chlamydomonas* W80

Antioxidant (4 assays)	**Skin lightning (4 assays)**
SOD-like activity	Inhibition of tyrosinase
Hydrogen peroxide elimination	Inhibition of melanin production in B16 melanoma cells
DPPH radical elimination	Endothelin-1 (ET-1) assay
Beta-carotene assay	Stem cell factor (SCF) assay
Anti-inflammatory (5 assays)	**Anti-aging (10 assays)**
Suppression of nitric oxide (NO) production	Elastase inhibition
Suppression of tumor necrosis factor-α production	MMP-1 inhibition
Hyaluronidase inhibition	Estrogen-like activity
Inhibition of beta-hexosaminidase secretion	Enhancement of production of laminin-5
Platelet aggregation inhibition	Enhancement of skin fibroblasts growth
	Promotion of growth of human epidermal keratinocytes (NHEK)
Immunostimulatory (2 assays)	UV damage recovery
Enhancement of nitric oxide (NO) production	Increase in catalase activity
Enhancement of production of tumor necrosis factor-α	Suppression of damage caused by hydrogen peroxide
	Enhancement of production of transglutaminase-1
Hair growth promotion (3 assays)	
Inhibition of testosterone 5α-reductase	
Proliferation of dermal papilla cells	
Androgen receptor antagonist	

Skin lightning effects of *C*. W80 extract

The inhibitory effect of *C*. W80 extract on melanogenesis were assayed with the B16 melanoma cell model. The B16 cells were treated with the *C*. W80 extract for 96h, and the inhibition of UV-B induced melanogenesis was evaluated by measuring the intracellular melanin content. The melanin levels were lowered in concentration-dependent manner by the extract from *C*. W80 (Table 2) and the IC_{50} value was calculated to be 58 µg/mL. This value is smaller than those of *Glechoma hederacea* extract (ca. 500 µg/mL) [20] and *Schinus terebinthifolius* extract (250 µg/mL) [21] and is comparable to the extract of non seeds (78.8 µg/mL) [22].

The inhibitory effects of *C*. W80 extract on ET-1 and SCF productions were also examined. UV-B light stimulates the secretion of cytokines such as α-melanocyte stimulating hormone [23], ET-1 [24,25] and SCF [26] from keratinocytes. These cytokines induce melanogenesis and ET-1 and SCF have synergic effects on skin pigmentation [26]. Table 3 shows the effects of *C*. W80 extract on ET-1 production in normal human epidermal keratinocytes (NHEK). At the concentrations of 12.5 µg/mL and 50 µg/mL, the inhibitory effects of *C*. W80 extraction ET-1 production were 26.3% ($p < 0.05$) and 33.2% ($p < 0.01$), respectively, and the IC_{50} value for ET-1 inhibition was calculated to be 141 µg/mL. This value is lower than the value of *Ipomoea aquatic* extract (250 - 500 µg/mL) [27]. Table 4 shows the effects of *C*. W80 extract on SCF production in NHEK. SCF is generated in the epidermis, and is involved in pigmentation after melanogenesis.

At the concentrations of 12.5 μg/mL and 50 μg/mL, the inhibitory effects of *C*. W80 extraction SCF production were 17.1% and 43.2% ($p < 0.01$), respectively, and the IC_{50} value for ET-1 inhibition was calculated to be 59.7 μg/mL. This value is much lower than the value of *Vigna angularis* extract (189 μg/mL) [28]. These results indicate that the *C*. W80 extract influences multiple cytokines related to skin pigmentation and has skin-lightning effect.

Table 2. Effect of the extract of *C*. W80 on melanin production

μg/mL	melanin content (% of control)
25	35.9
50	35.6
100	89.1

Table 3. Effect of the extract of *C*. W80 on ET-1 production

μg/mL	ET-1 assay (% inhibition)
12.5	26.3 ± 5.2*
50.0	33.2 ± 3.6**

Mean±S.E., n=3, *:p < 0.05, ** : p < 0.01 Comparison with the UVB irradiation control

Table 4. Effect of extract of *C*.W80 on SCF production

μg/mL	SCF assay (% inhibition)
12.5	17.1 ± 13.4
50.0	43.2 ± 11.4**

Mean±S.E., n=3, ** : p < 0.01 Comparison with the UV-B irradiation control

Hair growth promotion effects of *C*. W80 extract

The hair growth cycle includes repeating steps of growing, involutional and resting phases. Dermal papilla cells appear on the skin at the root and play an important role in hair growth regulation. It is reported that dermal papilla cells are propagated in the growing phase of hair cycle [29]. To evaluate the effect of the extract from *C*. W80 on cell proliferation of human hair follicles, the dermal papilla cells were treated with various concentrations of the extract from *C*. W80. *C*. W80 extract was found to promote proliferation of dermal papilla cells in dose-dependent manner (Table 5). The extract of *C*. W80 significantly ($p < 0.001$) increased the proliferation of dermal papilla cells by 119.2% at 25 μg/mL concentration compared with the control. The value of proliferation increase (119.2%) obtained with *C*. W80 was as good as or slightly higher compared with the values of previous studies, such as 116% at 10 μM of minoxidil [30] and 111.5% at 25 μg/mL of *Prunus lannesiana* extract [3], indicating that *C*. W80 extract has hair-growth promoting effect *via* the proliferation of dermal papilla cells.

Testosteron, one of the androgenic hormone, is converted by 5 α -reductase to dihydrotestosterone (DHT) [32]. Because the accumulation of DHT in sculp induces the hair loss, suppression of DHT production through inhibition of 5α-reductase activity is thought to be effective to treat alopecia. As shown in Table 6, *C*. W80 extract inhibited 5 α-reductase activities in dose-dependent manner. The IC_{50} value of 5 α-reductase activity was calculated to be 285μg/mL. This value is comparable to the values of extract from *Psidium guajava* (160 μg/mL) [33] and *Acer palmatum* (340 μg/mL) [34], indicating that *C*. W80 extract can potentially be used for the treatment of androgenetic alopecia (AGA) *via* the 5α-reductase inhibition.

Table 5. Effect of extract of *C*.W80 on Dermal papilla cells proliferation

μg/mL	Cell proliferation (% of control)
1.56	102.2 ± 1.5
6.25	106.9 ± 2.3
25.0	119.2 ± 1.3***

Mean±S.E., n=6, ***: $p < 0.001$

Table 6 Effect of extract of *C*.W80 on testosterone 5alpha-reductase activity

μg/mL	Inhibition activity (%)
200	38.3
400	63.3
1000	79.7
3000	88.2

The present study indicates that the 50% ethanol extract of *C*. W80 has the bioactivities to promote skin lightning and hair growth. Although further investigations are needed to identify the active principles of *C*. W80 cell extract for these bioactivities, the simultaneous productions of value-added co-products and fuels, such as hydrogen, by *C*. W80 can be an economically promising process for biofuel production. As far as we know, the present study is the first example of comprehensivesurvey for bioactivities in fuel producing microalgae and suggesting the possibilities of simultaneous co-production of value added materials and biofuel in other many unexploited algal strains.

CONCLUSION

The present study revealed that the 50% ethanol extract of the marine green alga *Chlamydomonas* sp. W80, which is a potential hydrogen-producing algal strain, has both skin lightning and hair growth promotion effects, indicating the possibility of the simultaneous productions of bioenergy and value added coproducts by this green algae.

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