Vol.62: e19180198, 2019
http://dx.doi.org/10.1590/1678-4324-2019180198
ISSN 1678-4324 Online Edition

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BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

Article - Human and Animal Health

The Anti-melanogenesis Activities of Some Selected Brown Macroalgae from Northern Coasts of the Persian Gulf

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Received: 2018.01.23; Accepted: 2019.03.23.

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HIGHLIGHTS

- *P. boergesenii* exhibited higher in vitro anti- tyrosinase activity compared to other algae.
- *P. boergesenii* inhibited melanin synthesis higher in comparison to other tested algae.
- The type of algae, place and time of collection can affect the inhibitory activities.

Abstract: Melanogenesis is a biological process which led to the synthesis of melanin pigment. Abnormal melanin production results in melasma, solar lentigo, post inflammatory melanoderma, etc. In this study, we examined the potential inhibitory effects of 17 brown macroalgae from Persian Gulf on melanogenesis. The effects of various concentrations (100, 250 and 500 µg/mL) of methanolic extracts of macroalgae belonging to four genera (including: *Padina*, *Colpomonia*, *Cystoseira* and *Sargassum*) were studied on oxidation of L-Dopa by mushroom tyrosinase. Subsequently, the activity of macroalgae with high

inhibitory effect on monophenolase activity of mushroom tyrosinase and zebrafish was investigated using L-tyrosine as a substrate. Anti-melanogenesis effects of algae extracts were studied on zebrafish as an alternative in vivo model. Kojic acid was used as a positive control. All the tested macroalgae showed inhibitory effect on activities of diphenolase and monophenolase (of mushroom tyrosinase). *P. boergesinii* exhibited the most in vivo anti-tyrosinase activity compared with other samples.
P. boergesenii inhibited zebrafish tyrosinase more potent than kojic acid (83% vs 50% inhibition for kojic acid). Moreover, it reduced melanin synthesis in zebrafish 42% (kojic acid: 50%).
Keywords: Algae; Persian Gulf; melanogenesis; mushroom tyrosinase; zebrafish.

INTRODUCTION

Melanin is one of the most widely distributed pigments in bacteria, fungi, plants and animals. The skin and hair color of mammals are determined by several factors. One of the most important factors is the degree and distribution of melanin pigments [1, 2]. The role of melanin is protecting the skin against UV radiations and reactive oxygen species (ROS) damages.

Tyrosinase (EC 1.14.18.1) is the key enzyme in the synthesis of melanin that catalyzes hydroxylation of L-tyrosine into L-Dopa (monophenolase activity) and its oxidation to dopaquinone (diphenolase activity). Multiple skin disorders, such as melasma, freckles, age-related and chemical-spots, resulted in excessive accumulation of melanin pigmentation in the skin [3]. There are various mechanisms for inhibiting melanogenesis. Tyrosinase inhibition is the most common method for reducing pigmentation in the skin[4].

In addition to the formation of melanin pigment, tyrosinase plays a role in browning of fruits, vegetables and sea foods. It oxidizes the phenols to the quinones and is responsible for the enzymatic browning of the damaged fruits during handling and storage. The produced quinones, in addition to the unpleasant odors and colors, may react irreversibly with amine and sulfide groups of proteins. The protein-quinone reaction decreases the digestibility of proteins and the amount of available essential amino acids, such as lysine and cysteine [1, 5, 6]. Furthermore, tyrosinase plays an important role in insect defense, wound healing, parasite capsule formation and external skeletal formation [7]. The available tyrosinase inhibitors such as hydroquinone have toxicity and low efficacy [8]. Therefore, researchers are looking for safer and more effective inhibitors like natural tyrosinase inhibitors for use in cosmetic and food industries to prevent or treat hyperpigmentasion disorders and browning reactions and as an alternative method for controlling insect pests.

The skin care effects such as whitening, sun protection, antioxidant, antibacterial, antifungal, moisturizing, anti-aging and anti-wrinkle were reported from brown algae[9]. Rucky coasts and proper environmental conditions in northern coasts of the Persian Gulf have created a suitable habitat for the growth and spread of macroalgae. Therefore, various species of brown macroalgae can be found in tidal zones of these areas [10]. Due to lack of reports on the effect of brown algae on the control of melanogenesis in Iran, the aim of this

study was to investigate the beneficial skin care and inhibitory effects of the selected brown algae extracts on melanogenesis.

MATERIAL AND METHODS

Chemicals

Mushroom tyrosinase (3130 unit/mg), L-DOPA (powder, ≥98.0 %), L-tyrosine (powder, ≥98.0 %), Synthetic melanin, DMSO (Dimethylsulfoxide) and methanol were purchased from Sigma Chemical Co (St.louis, MO, USA). Pro-prep and Pro-measure protein solutions were purchased from Intron Biotechnology (Korea). Kojic acid was obtained from Fluka and the other chemicals used were of analytical grades.

Macroalgae Collection

The brown seaweeds were collected at low tide time from the coastal areas of Bushehr. After harvesting, sands, salts and epiphytes were removed with fresh water and then, cut into appreciate size and were air-dried at room temperature with good-controlled air condition carefully. Samples were milled into powder and kept at -80°C for further analysis. The voucher specimens were pressed and stored in 5% formol for identification. Morphological and anatomical examinations of cell structures were done for accurate identification with the aid of identification keys in the taxonomic publications [11-14].

Algal Extracts Preparation

Dried algal powder (200 mg) was extracted with 6 mL 80% methanol in an ultrasonic bath for 30 min, vortexed and then was kept at room temperature and dark place for 48 h. After one more time vortexing, the extracts centrifuged at 10000 g for 15 min, filtered through watman No.1 filter paper. After drying the extracts, the weight of dried extracts were calculated. Then, dried extracts were dissolved in 3% DMSO and final dilutions of 100, 250 and 500 µg/mL were made.

In Vitro: Mushroom Tyrosinase Inhibitory Assay

The inhibitory effects of brown macroalgae were examined by cell-free mushroom tyrosinase assay as described by chan *et al.* [15] with minor modifications. The tyrosinase inhibitory activity was determined using L-Dopa and L-tyrosine as substrates. In brief, 100 µL of 200 unit/ml of mushroom tyrosinase in 25 mM phosphate buffer (pH 6.8) was added to 50 µL of various dilutions of extracts (100, 250 and 500 µg/mL) in 96-well plate and the absorbance of wells were recorded at 70- second intervals (10 cycles) at 475 nm with microplate reader (Tecan sunrise, Switzerland). This experiment was performed for measuring and correcting the interference of the excess absorbance of the phenolic compounds in the extract. Then, 100 µL of L-Dopa (2.5 mM) or L-tyrosine (1.5 mM) were added to mixture reaction and absorbance was read at 70-second intervals (20 cycles) at 475 nm. Kojic acid (50, 100, 250 and 500 µg/mL) and 3% DMSO were used as positive and negative controls, respectively.

Inhibitory activity of tyrosinase was calculated as:

$$\% \text{ Inhibition} = \{[(A-B)-(C-D)]/(A-B)\} \times 100$$

A: Absorbance of the enzyme, substrate and DMSO solution.

B: Absorbance of the substrate and DMSO solution.
C: Absorbance of the enzyme, substrate and extract solution.
D: Absorbance of the substrate and extract solution

In Vivo Assay

Origin and Maintenance of Parental Zebrafish

Adult zebrafish were purchased from a commercial dealer. Male and female zebrafish were kept in separate acrylic tanks at 28.5°C under the light/dark cycle of 14/10 h. Zebrafish were fed 6 days a week and 3 times a day, with bloodworm food and supplemented with daphnia. In the evening, 6 fishes in 1: 2 ratio of female: male groups were transferred to 5 L tanks that have been marbled and filled with fresh water. By turning on the light in the morning, natural spawning was induced. The lucid fertilized eggs were collected for further experiments.

Tyrosinase Inhibitory Activity Assay

Tyrosinase inhibitory effects of brown macroalgae were determined spectrometrically as described previously by Choi et al. and Cha *et al.*[16, 17] with some modifications. In a 6-well plate, 100 embryos of the same age were added to each well containing 6650 µL of sterile fresh water and were kept in incubator at 28.5°C. After 9 hpf (hours post fertilization), 350 µL of the algal extracts (100 µg/mL) was added. Replacing the medium was done once a day to ensure uniform distribution of the compounds in the wells. After 48 hpf, zebrafish embryos were collected and sonicated in 6 mL pro-prep protein extraction solution in 15 mL falcon tube for extraction of enzyme and melanin. Lysate was removed by centrifuging at 10000 g for 5 min. Supernatant was aspirated and pellet put aside for determination of melanin content. After quantitation of supernatant by pro-measure kit, the samples were adjusted by pro-prep solution to 250 µg pr/ 100 µL. Then, 100 µL 1.5 mM L-tyrosine was added to 100 µL of samples in 96-well plate and subsequently was incubated at 28°C for 60 minutes. Absorbance of samples was recorded at 475 nm. The blank (containing 100 µL of pro-prep solution and 100 µL of 1.5 mM L-tyrosine) absorbance was eliminated from each absorbance value. Kojic acid (100 µg/mL) and 0.1 % DMSO were used as positive and negative controls, respectively. The final activity was expressed as a percentage to the negative control.

Melanin content of zebrafish embryos

The pellet was dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min. The mixture was intensely vortexed to solubilize the melanin pigment. The absorbance of the supernatant was read at 490 nm. NaOH was used as blank. Standard curve for synthetic melanin was obtained at concentration ranges between 1-300 µg/ml (concentrations 1, 5, 10, 25, 50, 100, 200, 300 µg/ mL). The melanin content was calibrated by protein amount, and expressed as a percentage to negative control.

Statitical Analysis

Experiments were carried out in triplicate and the data were expressed as the mean ± standard error. One-way ANOVA was used to compare the mean values of each treatment. A value of $P < 0.05$ was considered as statistically significant in all experiments.

RESULTS

In this study, 17 samples of brown algae were collected. Algae were identified using valid sources and keys by morphological characteristics. The species, location and time of collection are listed in Table 1.

Table1. Seaweeds and their location and collection time.

Herbarium Code	Sample Code	Species	Location	Collection time
B100211	Pa	*Padina australis*	Halileh	December
B131111	Pb	*Padina boergesenii*	Jofreh	February
B131311	Pd	*Padina distromatica*	Gheshm	May
B131312	Pt	*Padina tetrastromatica*	Gheshm	May
B110611	Cs1	*Colpomenia sinuosa*	Haleh	March
B110211	Cs2	*Colpomenia sinuosa*	Halileh	March
B131411	Cs3	*Colpomenia sinuosa*	Southern Ouli	February
B131121	Cs4	*Colpomenia sinuosa*	Jofreh	February
B100311	Ct1	*Cystoseira trinodis*	Rostami	December
B131131	Ct2	*Cystoseira trinodis*	Jofreh	February
B100322	Sa1	*Sargassum angustifolium*	Rostami	December
B131211	Sa2	*Sargassum angustifolium*	Nirue havaee	February
B110312	Sc	*Sargassum crassifolium*	Rostami	October
B110311	Sg	*Sargassum glaucescens*	Rostami	October
B100321	Ss1	*Sargassum swartzii*	Rostami	December
B130311	Ss2	*Sargassum swartzii*	Rostami	February
B110313	St	*Sargassum tenerrimum*	Rostami	October

The percent of inhibition of algae on L-Dopa oxidation by mushroom tyrosinase was calculated at three concentrations of 500, 250 and 100 µg/ mL. *P. boergesinii* showed the highest inhibitory activity compared to other seaweeds, but its activity was lower than that of kojic acid as positive control. The percentage of inhibition of samples is shown in Table 2.

Table 2. Percent inhibitory effects of algae on oxidation of L-Dopa by mushroom tyrosinase. Each value is expressed as the mean ±SE (n=3).

Sample Code	Species	Conc (µg/mL)		
		100	250	500
Pa	*P. australis*	3.83±0.21	9.80±0.42	12.63±0.82
Pb	*P. boergesenii*	17.63±0.56	36.68±1.03	51.75±0.69
Pd	*P. distromatica*	8.52±1.02	9.57±0.99	10.02±0.54
Pt	*P. tetrastromatica*	8.43±0.84	12.35±0.39	18.48±1.24
Cs1	*C. sinuosa*	7.66±1.13	10.88±0.93	12.81±0.65
Cs2	*C. sinuosa*	7.87±1.01	9.92±0.73	10.10±0.81
Cs3	*C. sinuosa*	12.34±0.78	13.84±1.02	23.25±0.91
Cs4	*C. sinuosa*	15.90±1.02	17.61±0.93	20.27±1.53
Ct1	*Cystoseira trinodis*	17.21±1.33	15.10±0.72	15.22±0.69
Ct2	*Cystoseira trinodis*	14.45±0.78	15.31±1.08	18.46±0.79
Sa1	*S. angustifolium*	5.23±0.94	10.35±0.67	10.62±0.45
Sa2	*S. angustifolium*	10.74±0.6	12.58±1.03	13.28±0.91
Sc	*S. crassifolium*	5.71±0.72	8.78±0.11	13.12±0.89
Sg	*S. glaucescens*	3.68±0.47	5.64±0.82	10.52±0.71
Ss1	*S. swartzii*	13.38±1.02	20.04±1.31	20.55±0.54
Ss2	*S. swartzii*	6.79±0.88	7.95±0.23	10.60±0.67
St	*S. tenerrimum*	11.61±0.96	11.99±0.77	14.35±1.05
	Kojic acid	81.85±1.52	92.33±0.87	93.54±0.67

P. boergesenii, *C. sinuosa* (Cs3, Cs4) and *S. swartzii* (Ss2) showed the highest inhibitory effects on L-Dopa oxidation by mushroom tyrosinase, and therefore, were selected to investigate the inhibitory effect on monophenolase activity of mushroom tyrosinase and in vivo studies.

All algae showed anti-tyrosinase activity on monophenolase activity less than kojic acid. *S. swartzii* did not inhibit tyrosinase activity and *P. boergesenii* exhibited the highest percentage of tyrosinase inhibition (Table 3).

Table 3. Percent inhibitory effects of selected algae on hydroxylation of L-tyrosine by mushroom tyrosinase. Each value is expressed as the mean ±SE (n=3).

Species	Tyrosinase inhibition			
	50µg/mL	100 µg/mL	250 µg/mL	500 µg/mL
P. boergesenii (Pb)	NT [1]	46.46±1.29	78.58±1.12	99.61±0.51
C. sinuosa (Cs3)	NT	11.80±1.06	13.97±1.41	20.51±1.23
C. sinuosa (Cs4)	NT	9.44±0.59	11.25±1.01	13.61±1.31
S. swartzii (Ss2)	NT	NI [2]	NI	1.10±0.10
Kojic acid	94.55±1.52	99.65±0.42	100	100

[1] NT: Not Tested

[2] NI: No Inhibition

To ensure the consistency of the in vitro assay results (mushroom tyrosinase) with in vivo model, the inhibitory effects of selected algae were investigated on melanin synthesis and tyrosinase activity of zebrafish. The percentage of inhibition of algal extracts at 100 µg/ mL was calculated by using L-tyrosine as a substrate. *P. boergesenii* exhibited the highest inhibitory activity compared to kojic acid and the other algae (83.09±1.53 vs 50.45±0.86 for kojic acid), followed by two samples of *C. sinuosa* (Cs3, Cs4) that were close to that of kojic acid (Table 4). Significant difference ($P<0.05$) was observed between kojic acid and all tested algae on melanin synthesis reduction (Table 4).

Table 4. Percent inhibitory effects of algae on monophenolase activity of zebrafish tyrosinase and melanin synthesis at 100 µg/mL. Each value is expressed as the mean ±SE (n=3).

Species	Tyrosinase activity	Melanin synthesis
P. boergesenii (Pb)	83.09±1.53	41.95±1.74
C. sinuosa (Cs3)	43.28±1.59	27.74±1.06
C. sinuosa (Cs4)	41.98±0.78	18.31±1.06
S. swartzii (Ss2)	24.37±1.53	19.73±0.89
Kojic acid	50.45±0.86	50.21±1.27

DISCUSSION

In this study, we investigated anti-melanogenesis of 17 brown macroalga using in vitro and in vivo models. *P. boergesenii* exhibited higher in vitro anti- tyrosinase activity (monophenolase and diphenolase activities of mushroom tyrosinase) compared to other algae. Its ability to inhibit melanin synthesis was lower than that of kojic acid (42% versus 50%)but was higher in comparison to the other tested algae. In previous studies, the anti-tyrosinase effect of *Padina* seaweed have been reported. Quah *et al.*, studied on ethanolic extract of *P. tenuis* in all various tested concentrations (10-1000 µg/ mL) which showed tyrosinase's diphenolase inhibitory activity. They also reported that the hexane fraction of the *P. tenuis* extract was able to inhibit HEM (human epidermis melanocyte) proliferation in vitro and reduce melanin synthesis in guinea pigs exposed to UVB radiation

without any dermal irritation during the treatment [18]. Tyrosinase's diphenolase inhibitory activity of *P. distromatica* and *P. australis* in present work showed some similarity with that of *P. teunis* in the mentioned work. *P. boergesenii* inhibited the monophenolase activity of mushroom tyrosinase more than diphenolase activity (46.46% vs 17.63%). Algae that can inhibit both monophenolase and diphenolase tyrosinase activities are more potent inhibitors. Cha *et al.* reported that extract of *P. arborescens* (100 µg/ mL), has inhibited monophenolase activity of tyrosinase enzyme by 17%. In our study, *P. boergesenii* (100 µg/ mL), inhibited monophenolase tyrosinase activity by 46%, indicates that *P. boergesenii* is a stronger inhibitor in comparison to *P. arborescens*[16]. In previous studies, antioxidant, antibacterial, antifungal and anti-melanogenesis effects of *P. boergesenii* have been reported [19-22]. The anti-melanogenic effect of *P. boergesenii* is probably due to the presence of fucoxanthin, fucoidan, phenolic and terpene compounds. Fucoidan had a competitive inhibitory effect on the tyrosinase enzyme in the oxidation of tyrosine. It also has anti-aging effects[23].

UV is the main cause of damage to cellular components and some skin diseases such as hyperpigmentation, weakness, wrinkles, erythema and skin cancer. Over exposure to UV radiations results in ROS production, leading to enhance melanin synthesis, DNA damage, proliferation of melanocytes, inflammatory reactions and skin angiogenesis. Thus, ROS scavenging compounds and redox agents like antioxidants are effective in the treatment of hyperpigmentation and other UV-mediated disorders[24]. Heo *et al.* showed that fucoxanthin significantly reduced the production of ROS by UVB rays in human fibroblasts. Fucoxanthin increases cell survival and reduces cellular damage, suggesting that fucoxanthin can protect the skin from UVB damage [25]. Shimode *et al.* showed that fucoxanthin reduced tyrosinase activity, melanogenesis in melanoma cells, and UVB-induced pigmentation. It also significantly reduced the expression of COX-2, endothelin A receptors, p75 neurotrophin, melanocortin-1 and tyrosinase-related protein 1[26]. Urikura *et al.* exhibited that fucoxanthin significantly inhibited the UVB-induced epidermis hypertrophy, which may lead to wrinkles, as well as the vascular endothelial growth factor and the expression of the matrix metalloproteinase-13. Also, their results showed that topical treatment of fucoxanthin reduced the aging of the skin caused by sunlight and wrinkles in mice exposed to UVB radiation [27]. Compounds with reduction potential like antioxidants can have anti-tyrosinase effects through two ways: by interacting with *O*-quinones, and suppression of the oxidative polymerization of melanin intermediates or by tyrosinase inhibition through reacting with copper in the active site of the enzyme[28]. Fucoidan is a sulfated polysaccharide that could react with copper through sulfide atom. The antioxidant effect of *P. boergesenii* is another mechanism involved in reducing the synthesis of melanin [23].

In this study, the anti-tyrosinase potential of seven samples of the seaweed *Sargassum* was investigated. *S. swartzii* showed most inhibitory effect on L-Dopa oxidation compared to other algae in this genus. Other algae had approximately the same effects on inhibition of the enzyme (3-14% in the range of tested concentrations). Concentrations of 250 and 500 µg/ mL of *S. swartzii* exhibited relatively similar inhibitory effects (Table 2). Several studies have been shown the anti-tyrosinase effects of *Sargasum* Sp [15-16, 18, 29-30]. For instance, Cha *et al.*, reported that *S. siliquastrum* was one of four strong algae among 43

tested algae, with a relatively low IC_{50} value of 19.85 µg/ mL. *S. siliquastrum* also inhibited tyrosinase and melanin synthesis in melanocyte B16 cells. *S. siliquastrum* (100 µg/ mL), inhibited monophenolase tyrosinase activity and melanin synthesis in zebrafish by 50%. Propylthiouracil (PTU) (0.2 mmol/ L) and arbutin (20 mmol/ L) exhibited anti-tyrosinase activity by 69% and 57% and anti-melanogenesis potential by 72% and 61% respectively in zebrafish [16]. In our study, kojic acid (100 µg/ mL) reduced monophenolase tyrosinase activity and melanin synthesis in zebrafish by 50.45% and 50.21%, respectively. *S. swartzii* showed minimum inhibitory activity among the other seaweeds (24.37% and 19.73% at concentration of 100 µg/ mL, respectively). *S. swartzii* inhibited the monophenolase activity of zebrafish tyrosinase more than mushroom tyrosinase at 100 µg/mL (24 ٪ vs no inhibition). Chan *et al.* showed that ethanolic extract and hexane, ethyl acetate and water fractions of *S. polycystum* exhibited little activity on mushroom tyrosinase, but the ethanolic extract and hexane fraction significantly inhibited the B16F10 mouse melanoma cells tyrosinase. In addition, hexane fractions reduced the production of melanin in B16F10 cells [15]. The ethanolic extracts and hexane fractions of *S. polycystum* had a higher toxicity on HEM cells than normal cells. Local administration of *S. polycystum* cream for three weeks reduced the production of melanin in Indian guinea pigs [18].

S. swartzii collected from Rostami in December (Ss1) showed more inhibitory activity when compared with the sample collected in February (Ss2). Farasat *et al.* showed that the sampling time had a significant effect on antioxidant capacity, content of phenol and flavonoids of green seaweeds. Temperature variations activate the defensive compounds such as antioxidant enzymes and molecules such as ascorbic acid. Environmental changes can affect the metabolism and the type of bioactive compounds of algae [30]. Likewise *padina*, the anti-tyrosinase effect of *Sargasum* seaweed is probably related to the compounds such as fucoidan and fucoxanthin.

The results of the anti-tyrosinase activity of *C. sinuosa* collected from different places in February and March indicate that the collection time has more effect on secondary metabolites compared to collection places. In a study by CHA *et al.*, *C. sinuosa* collected from Jeju Island in February to May at concentration of 100 µg/ mL inhibited monophenolase tyrosinase activity by about 31%, while in our study, the algal samples from Jofreh and Southern Ouli in February showed inhibitory effects 9-12%. These results show that the location of collection due to the different environmental conditions, the type of bed, etc., influence on inhibition potency [16]. Farasat *et al.* showed that the collection site significantly affects the antioxidant capacity, and also the amount of phenols and flavonoids. Therefore, the time and place of collection, can affect the anti-melanogenic activity through changing the antioxidant capacity [31].

Two samples of the same species, *C. sinuosa* from two locations (Cs3, Cs4) showed relatively similar inhibitory effects on hydroxylation of L-tyrosine by the zebrafish tyrosinase enzyme. Their effects on synthesis of melanin were slightly different. *C. sinuosa* (Southern Ouli, Cs3) showed more inhibitory effect on melanin synthesis in comparison to *C. sinuosa* (from Jofreh, Cs4). The tyrosinase inhibitory effect of *C. sinuosa* may be due to the presence of phlorotanins. The antioxidant effect of *C. sinuosa* through the reduction of quinone products can play a role in inhibiting the synthesis of melanin [32]. In general, these results

indicate that the type of algae as well as the place of collection or even collection time can affect the inhibitory activities.

In this study *P. boergesenii* showed the most anti-tyrosinase activity that can be considered as a promising seaweed for using in skin care formulations, especially whitening agents. However, further investigations on *P. boergesenii* may lead to identify bioactive compounds responsible for anti-tyrosinase effects.

Funding: This research received no external funding.
Acknowledgments: The authors are grateful to the Marine Pharmaceutical Research Center of Ahvaz Jundishapur University of Medical Sciences.
Conflicts of Interest: We have no conflict of interest.

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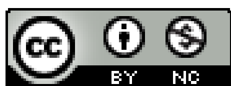

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