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ORIGINAL ARTICLE

Impact of retort process on characteristics and bioactivities of herbal soup based on hydrolyzed collagen from seabass skin

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Abstract Impact of retort processing on the characteristics and bioactivity of herbal soup, based on hydrolyzed collagen from seabass fish skins, as sterilized health drink in glass bottles, was investigated. Retort processing was conducted at either 115 °C or 121 °C for 5, 7, 9 or 11 min (F_0 values) and compared to no retort processing. All retort processing conditions yielded sterile soups, but some differences in moisture content, pH, viscosity, UV-absorbance, browning index, fluorescence intensity, color, α-amino group and total reducing compound contents were observed, compared to those without retort processing. Retort processing enhanced antioxidative activity of herbal hydrolyzed collagen (HHC) soups, regardless of conditions. HHC soups with F_0 value of 7 at 115 °C (115/7) and 121 °C (121/7) showed significantly higher ABTS and DPPH radical scavenging activities, ferric reducing antioxidant power and H_2O_2 scavenging activity, compared to others. Retort processing had no significant ($p > 0.05$) effect on the appearance, color, odor, viscosity, flavor, taste and overall perception of HHC soups. The 115/7 and 121/7 samples stimulated cell proliferation and enhance collagen production of L929 mouse fibroblast cells. It was therefore concluded that retort processing could be used for preparing sterilized HHC soup as a ready-to-serve functional drink that is both healthy and safe.

Keywords Hydrolyzed collagen · Herbal soup · Retort · Antioxidative activity · Fibroblast proliferation · Collagen production

Introduction

The growing demand for medicinal plants and herbs as healthy food supplements, health drinks and tonics have fueled the development of numerous products enriched with bioactive components. Traditional herbs have been used from time immemorial in health care in China and many other countries especially in Asia including Thailand. Herbal products with health benefits are sold in several forms including tablets, tea, liquid extracts and soups (Ekor 2013). Many soups and water extracts are commercialized and marketed as diet supplements and health foods and many herbal soups are considered as bioactive functional foods. Maillard reaction products (MRPs) and the formation of colloidal nanostructures of self-assembling single molecules extracted from plants and the flesh of animals generally occur during the preparation of these soups due to extended boiling. These reactions have been shown to affect the chemical compositions and bioactivity of herbal soup (Ke et al. 2012).

With increasing consumer demand for health promoting foods and drinks, fish protein hydrolysate has been shown to be important because of its nutracuetical properties and Thiansilakul et al. (2007) and Gómez-Guillén et al. (2011) produced fish skin hydrolysates with bioactivity. To enhance bioactivity of fish skin hydrolysate, Karnjanapratum et al. (2016) employed the Maillard reaction and the

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resulting hydrolysate showed increased antioxidative activity. Benjakul et al. (2017; 2018) developed seabass skin hydrolyzed collagen (HC), with negligible fishy odor, using enzymatic hydrolysis. To increase its nutraceutical value in herbal soup, selected herbs could be used as the source of active compounds to supplement the peptides in the hydrolysate. Several herbs are rich in phenolic compounds, which have been known to possess a wide range of bioactivities, particularly antioxidant activity. Radicals generated can cause a number of diseases. The intake of drink rich in polyphenols has been considered to prevent several diseases such as cancer, neurodegeneration and inflammation (Halliwell 2006; Huang et al. 2010; Ferguson 2010). Hydrolysate from fish skin has been proven to nourish the skin via the induction of fibroblast cell proliferation and collagen production (Benjakul et al. 2018). Thus, the development of herbal soup containing hydrolyzed collagen could bring about the nutraceutical product for health and skin care.

Retort processing is a major preservation techniques widely used in thermal processing for shelf-life extension of foods particularly in the production of ready-to-serve products (Gokhale and Lele 2012). A sterilization temperature above 100 °C is used for production of low acid foods in hermetically sealed containers and in a pressure cooker. Temperatures usually range from 110 to 121 °C, depending on the pressure used and the type of product. Sterilization is generally carried out at 121 °C but the temperature of 115 °C is commonly used for heat sensitive material (Heinz and Hautzinger 2007). Therefore, the retort conditions must be optimized for specific products. An effective thermal process could be designed by varying the sterility level using different times (F_0) and temperatures. Nevertheless, thermal conditions could affect the taste, appearance and nutrition as well as bioactivity of final products (Majumdar et al. 2015). Therefore, it is necessary to determine the optimum conditions to ensure the safety, nutritive value and bioactivity of each product.

The objective was to produce soup that had high nutritional content, derived from healthy natural sources, which are safe to eat. To achieve this objective the physical, chemical, sensory and antioxidative characteristics of herbal soup based on seabass skin HC, produced under different retorting conditions was studied. In addition the effects of the soups prepared from different retorting conditions were tested on cell proliferation and collagen production enhancement in an in vitro cell culture system.

Materials and methods

Chemicals and cell lines

Alcalase (EC 3.4.21.62) from *Bacillus licheniformis* and papain (E.C. 3.4.22.2) from papaya (*Carica papaya*) latex were gifted from Siam Victory Chemicals Co, Ltd. (Bangkok, Thailand). 2,4,6-trinitrobenzenesulphonic acid (TNBS), bathophenanthrolinedisulphonic acid, 2,2′-azino-bis (3-thylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was procured from Corning (Mediatech Inc., Manassas, VA, USA). L929 cells (mouse fibroblast, subcutaneous connective tissue) were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Fetal bovine serum (FBS) was obtained from Invitrogen (Paisley, Scotland). All chemicals and reagents for cell culture were obtained from Sigma Chemical Co. All chemicals were of analytical grade.

Preparation of hydrolyzed collagen from seabass skin

Hydrolyzed collagen (HC) from seabass skin was prepared following the method described by Benjakul et al. (2017) with a slight modifications. Briefly, skins of seabass (*Lates calcarifer*) weighing 2.5–3.0 kg were cleaned by removing the remaining flesh and washing with cold tap water (≤ 10 °C). The samples were placed in polyethylene bags and stored at − 20 °C until used.

Frozen skins were cut into pieces (3.0 × 3.0 cm) using an electric sawing machine (Union Kitchen & Service Co., Ltd., Bangkok, Thailand). The cut frozen skins were thawed and pretreated with alkaline (0.10 M NaOH) and acid (0.10 M citric acid) solutions as described by Benjakul et al. (2017). The resulting pretreated skins were then used for preparing the HC.

Two-step hydrolysis was carried out using 3% papain (pH 7.0) at 40 °C for 3 h, followed by 2% Alcalase (pH 8.0) at 50 °C for 2 h according to the method of Benjakul et al. (2017). After enzyme inactivation, the resulting mixture was filtered through two layers of cheesecloth and two layers of fiberglass filter to remove the debris. The filtrate was then fed into the filter unit, which consisted of a 5μ pleated polypropylene sediment filter (4.5 inch diameter × 20 inches long) (PF10TT, Treatton, Taiwan) at a feed flow rate of 2 L/min using a diaphragm pump (R/O-

450, Treatton, Taiwan). The filtered HC was collected and used for preparation of herbal HC soup.

Preparation of herbal HC soup

Herbal HC (HHC) soup was prepared using a traditional Thai process. The dried herbs used in the formulation were as follows (based on total weight): 19.5% Chinese yam tubers (*Dioscorea opposita* Thunb.), 18.0% Longan fruit (*Dimocarpus longan* Lour.), 15.5% Gojiberry fruit (*Lycium barbarum* L.), 13.0% Rehmannia root (*Rehmannia glutinosa* Libosch.), 10.0% Solomon's seal (*Polygonum multiflorum* Thunb.), 8.5% Codonopsis root (*Salvia miltiorrhiza* Bge), 6.5% Milkvetch root (*Astragalus mongholicus* Bge.), 5.0% Dong quai [*Angelica sinensis* (Oliv.) Diels.] and 4.0% Szechuan Lovage root (*Ligusticum chuanxiong* Hort.). The mixed herbs (120 g) were added to 2 L of deionized water and the mixture was heated at 95 °C for 30 min using a hot plate (C-MAG HS 7, IKA Works Inc., Wilmington, NC, USA) and then filtered through two layers of cheesecloth and the filtrate was transferred manually into 75 mL pasteurized glass bottles with screw caps (5.2 cm diameter × 45 cm high) and was termed 'herbal extract'. The total reducing compound content was determined by Folin–Ciocalteu reagent (Sato et al. 1996) and shown to be 531.72 mg Gallic equivalent/L. For comparison HC was used instead of deionized water for preparing the HHC soup, in which the same extracting conditions were used. After filtration, the resulting HHC soup was poured into the glass bottles and capped as described above.

Retort process for sterilization

Screw-capped glass bottles containing HHC soup were subjected to thermal processing using a pilot-scale horizontal stationary retort system (Water Spray Retort AT2000, Owner Foods Machinery Co., Ltd., Bangkok, Thailand). Two retort temperatures, 115 and 121 °C, and four retorting periods (F_0 values) 5, 7, 9 and 11 min were used. Pressure was maintained at 1.63 kg/cm^2 for 115 °C and 1.83 kg/cm^2 121 °C throughout the process using a hot water–air mixture.

All glass bottles were placed on perforated trays and then transferred into a retort vessel. Thermocouples (22 × SSA12080E700TS probes, E Val Flex Module, ELLAB, Denmark) were inserted into five of the bottles for measurement of sample temperatures during the thermal process. Data were recorded using a data logging device (E Val Flex System, ELLAB, Denmark) and temperature input data were converted into corresponding process lethality values. These process lethality values were expressed as F_0 values. After reaching the required F_0

value, the glass bottles were cooled rapidly, by recirculating water, to 45 °C. All glass bottles containing samples were subsequently stored in a dry place at room temperature (28–30 °C) and the samples were subjected to the following analyses.

Analyses

Sterility test

Sterility tests of all the samples with different retort processing conditions were conducted by ADCET (Agro-Industry Development Center for Export, Prince of Songkla University). Total viable aerobic and anaerobic counts of mesophilic and thermophilic microorganisms were determined at 35 and 55 °C, respectively, according to the method of BAM (FDA 2001).

Physicochemical characteristics

Physicochemical characteristics of retort processed HHC (R-HHC) soup, non-retorted processed HHC (NR-HHC) soup, HC and herbal extract were determined as follows.

Moisture content and pH value The samples were analyzed for moisture content using analytical method No. 950.46 (AOAC 2000) and pH using a pH meter (Sartorius PB-10, Göttingen, Germany).

Viscosity measurement Viscosity of the samples was determined at 4 and 25 °C using a Brookfield Viscometer Model RV-DV II Pro$^+$ (Brookfield Engineering Inc, Middleborough, MA, USA) at 200 rpm. The UL cylindrical spindle (ULA-15E) was used along with Brookfield Ametek UL adapter.

Measurement of UV-absorbance and browning index Prior to measurement, appropriate dilution of the samples was made using distilled water and the absorbance was measured at 294 and 420 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) for determining UV-absorbance and browning intensity, respectively (Karnjanapratum et al. 2016).

Measurement of fluorescence intensity Fluorescence intensity of MRP samples with an appropriate dilution was measured at an excitation wavelength of 347 nm and emission wavelength of 415 nm using a RF-1501 Fluorescence Spectrophotometer (Shimadzu, Kyoto, Japan) (Karnjanapratum et al. 2016).

Determination of color Color was measured in a Hunter Lab Colorimeter (C04-1005-631 colorFlex, Reston, VA, USA). The values were expressed as $L*$ (lightness), $a*$ (redness/greenness) and $b*$ (yellowness/blueness). Total difference in color ($\Delta E*$) and chroma ($\Delta C*$) were then calculated according to Eqs. (1) and (2), respectively (Gennadios et al. 1996; Jangchud and Chinnan 1999).

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \qquad (1)$$

$$\Delta C^* = \sqrt{\Delta a^{*2} + \Delta b^{*2}} \qquad (2)$$

where ΔL^*, Δa^* and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard ($L^* = 93.65$, $a^* = -0.95$ and $b^* = 0.52$).

Determination of α-amino group content The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). To diluted samples (125 μL), 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution was added. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite and mixture was cooled at room temperature for 15 min. L-leucine standard solutions with concentrations ranging from 0.5 to 5.0 mM were used. The absorbance was read at 420 nm and α-amino group content was expressed in term of L-leucine.

Total reducing compound content Total reducing compound content was measured using the Folin–Ciocalteu method described by Sato et al. (1996) with slight modifications. Briefly, 100 μL of sample were transferred into a test tube and 0.75 mL of Folin–Ciocalteu reagent (twofold diluted with deionized water) was added and mixed well. The mixture was allowed to stand at room temperature for 5 min. Then, 0.75 mL of 2% (w/v) sodium carbonate was added to the mixture and mixed gently. After standing at room temperature for 90 min, the absorbance was read at 725 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid solutions (0.01–0.05 mg/mL) were used for standard curve preparation.

Antioxidative activity

All samples were determined for antioxidative activity using different assays. The activities determined in all the assays were expressed as μmol TE/mL sample.

ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) radical scavenging activity was determined by the method described by Binsan et al. (2008). A standard curve was prepared from Trolox ranging from 50 to 600 μM.

FRAP (ferric reducing antioxidant power) was determined according to the method described by Benjakul et al. (2017). The standard curve was prepared using Trolox ranging from 0 to 500 μM.

DPPH (2,2-diphenyl-1-1picryl-hydrazyl) radical scavenging activity was quantified as described by Benjakul et al. (2017). The standard curve was prepared using Trolox (0–60 μM).

Hydrogen peroxide (H_2O_2) scavenging activity was assayed according to the method of Wettasinghe and Shahidi (2000). Trolox (0–10 mM) was used as the standard.

Sensory evaluation

Sensory characteristics of R-HHC and NR-HHC soups were evaluated by 50 untrained panellists. All panellists were asked to evaluate samples for appearance, color, odor, viscosity, flavor, taste and overall likeness using a nine-point hedonic scale, using a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely. The samples were labelled with random three-digit codes. Panellists were instructed to rinse their mouth with water after each sample evaluation and the order of presentation of the samples was randomized (Meilgaard et al. 1999).

Impact of the HHC soup on cell proliferation and enhancement of collagen production of mouse fibroblast cells (L929)

The R-HHC soups that had high antioxidative activity and high likeness score were selected to evaluate their impact on cell proliferation and enhancement of collagen production of L929 cell line, in comparison with herbal extract, HC and NR-HHC soup.

Cell culture

L929 (mouse, subcutaneous connective tissue) cells were cultured in 25 cm^3 Corning bottles, with DMEM, at 7.2–7.4 pH. The medium was supplied with 10% FBS. All the culture bottles containing the cell lines were maintained at 37 °C in a 5% CO_2 flux incubator (Binder Model C 170, Binder Inc., Johnson Ave Bohemia, NY, USA).

MTT assay: proliferative assays

Triplicate L929 cell lines were seeded at a density of 2×10^4 cells/mL (100 μL) in 96 well plates and 100 μL of the sample solution, at different concentrations, was added to the cell lines. After incubation at 37 °C for 72 h, cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in a MTT I Proliferation Kit (Roche Diagnostics; Burgess Hill, West Sussex, UK) according to the manufacturer's instructions. Absorbance was read at 570 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Offenburg, Germany).

Enhancement of collagen production

The selected samples were used to study the collagen production enhancement of mouse fibroblast cell line

(L929) using the soluble collagen assay (Biocolor Ltd., UK). L929 cells (100 μL, 2×10^4 cells/mL) were seeded in 96 well plates and incubated at 37 °C for 24 h. The selected samples (100 μL) were then added to the cell lines and consecutively incubated at 37 °C for 48 h. The supernatant was collected (100 μL) and used for measuring the soluble collagen content according to the manufacturer's instructions. Absorbance was read at 540 nm using a FLUOstar Omega microplate reader. The standard curve was prepared using bovine collagen (0–500 μg/mL).

Statistical analysis

Experiments were carried out in triplicate. Data were subjected to analysis of variance (ANOVA) and comparison of means was carried out using the Duncan's Multiple Range Test (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussion

Microbiological quality of HHC soups

The microbiological qualities of HHC soups, treated with different retort processing conditions, were evaluated to ensure the safety using a sterility test. There were no total viable aerobic and anaerobic counts for both mesophile and thermophile in all samples that had been subjected to the retorting process, irrespective of time and temperature combination used. This indicated that the thermal processes used could yield HHC soup in glass bottle with microbial safety. Jang and Lee (2012) and Shah et al. (2017) reported similar results for several processed foods that had been retorted for their safety for consumer and shelf-life extension.

Physicochemical properties of HHC soups

Solid content, pH value and viscosity

The effects of various retort processing conditions on composition and properties of HHC soups were tested. Solid content, pH and viscosity of HHC soups from retort processing (R-HHC soup) using different temperatures (115 and 121 °C) and F_0 values (5, 7, 9 and 11 min), HC, herbal extract and HHC soups that were not retorted (NR-HHC soup) are shown in Table 1. Solids content of R-HHC soup were not significantly different ($p < 0.05$) to NR-HHC soup, but herb extract had 3.01% solids content, which was significantly higher ($p < 0.05$) than the solids content of HC (0.76%). Herb extract was mildly acidic (pH 5.82) (Table 1), which might be due to the presence of some acids or acidic compounds in the herbs. When HC (pH 7.53) was used for extraction of herb, the resulting HHC soup had decreased pH and R-HHC soup had a significantly lower ($p < 0.05$) pH than of NR-HHC soup.

Table 1 Solid content, pH value and viscosity of HHC soups with different retort processing conditions

Samples	F_0	Treatment time (min)	Solid content (%)	pH	Viscosity (cP)	
					25 °C	4 °C
HC		–	0.76 ± 0.01d	7.53 ± 0.09a	2.06 ± 0.01Bc	2.15 ± 0.02Ac
Herbal extract		–	3.01 ± 0.00c	5.82 ± 0.03f	2.23 ± 0.01Bb	2.30 ± 0.02Ab
NR-HHC		–	3.61 ± 0.01b	7.26 ± 0.02b	2.29 ± 0.04Ba	2.46 ± 0.01Aa
R-HHC						
115	5	22	3.64 ± 0.00ab	6.86 ± 0.02cd	2.32 ± 0.01Ba	2.48 ± 0.01Aa
	7	30	3.66 ± 0.03ab	6.82 ± 0.02cd	2.32 ± 0.01Ba	2.46 ± 0.01Aa
	9	37	3.61 ± 0.01b	6.78 ± 0.09cd	2.30 ± 0.02Ba	2.47 ± 0.01Aa
	11	46	3.63 ± 0.08ab	6.54 ± 0.02e	2.30 ± 0.01Ba	2.45 ± 0.01Aa
121	5	5	3.64 ± 0.03ab	6.92 ± 0.01c	2.30 ± 0.02Ba	2.47 ± 0.02Aa
	7	9	3.68 ± 0.02a	6.71 ± 0.28d	2.31 ± 0.01Ba	2.45 ± 0.01Aa
	9	13	3.67 ± 0.03a	6.79 ± 0.06cd	2.29 ± 0.01Ba	2.47 ± 0.01Aa
	11	16	3.65 ± 0.03ab	6.52 ± 0.03e	2.31 ± 0.01Ba	2.46 ± 0.01Aa

Data are mean ± standard deviation (n = 3)

115 and 121 indicate the temperature used in retort process at 115 and 121 °C, respectively

HC, hydrolyzed collagen; Herb extract, herbal extract using water as medium; NR-HHC, non-retort processed herbal hydrolyzed collagen soup; R-HHC, retort processed herbal hydrolyzed collagen soup

a, b, c, d, e in the same column indicate the significant difference ($p < 0.05$)

A, B indicates significant difference of viscosity measured at different temperature ($p < 0.05$)

Notably, R-HHC soup, with higher F_0 values, showed significantly ($p < 0.05$) lower pH levels, especially those with the F_0 value of 11 min, for both temperatures used. However, there was no significant difference ($p > 0.05$) in pH value between retort processed samples at 115 and 121 °C when the same F_0 was used. Jeong et al. (2004) and Juániz et al. (2016) had previously shown that heating could liberate low molecular weight phenolic compounds from high molecular weight polymers. Jeong et al. (2004) reported that 5-hydroxyvaleric acid, vanillic acid and ferulic acid were detected when sufficient heat treatment was applied in preparing antioxidative phenolic compounds from fruit peel. Karnjanapratum et al. (2016) reported that the decrease in pH of unicorn leatherjacket fish skin (*Aluterus monoceros*) hydrolysate was in accordance with the formation of MRPs. Thus MRPs formation might be related to the decreased pH levels of HHC soup subjected to retort process.

Viscosity of HHC soups as affected by different retort processing conditions were measured at 25 and 4 °C (Table 1). Higher viscosity was observed for NR-HHC soup, compared to those of herbal extract or HC ($p < 0.05$). It was noted that there was no significant difference ($p < 0.05$) in viscosity between R-HHC and NR-HHC soups. Nevertheless, the viscosity of HHC soups at 4 °C was significantly higher ($p < 0.05$) than that of 25 °C. At the lower temperature, the peptides might be favorably localized or aligned together due to the lower enthalpy. Similar result was reported by Bozdogan (2015) who studied viscosity of bitter orange juice as affected by temperature and found that progressively lower viscosity was observed as the temperature increased; therefore temperature was another factor that could affect physical property or viscosity of HHC soups.

Browning index, absorbance at 294 nm and fluorescence intensity

The impact of retort processing conditions on the Maillard reaction in HHC soups was monitored by measuring browning index including the absorbance at 294 nm and fluorescence intensity. Browning index A_{420} and A_{294} of HHC soup with different retort processing conditions are presented in Fig. 1. A_{420} has been used as a measure of browning index of final products and A_{294} represents intermediate products of the Maillard reaction. A_{420} (Fig. 1a) and A_{294} (Fig. 1b) of NR-HHC were significantly higher ($p < 0.05$) than those of HC and herbal extract. The result suggested that MRPs could be generated during preparation of HHC soup. Carbonyl compounds in herbs might undergo glycation with free amino group of HC, especially at higher temperatures. Generally, A_{420} increased with increasing F_0. The lower A_{294} was observed when F_0 values increased at both the temperatures tested.

This result suggests that the generation of MRPs took place while the intermediate products were converted to final MRPs.

A similar trend was also found for fluorescence intensity (Fig. 1c), in which HHC soups showed significantly higher intensity ($p < 0.05$) than HC and herbal extract. Jing and Kitts (2002) reported that fluorescence in the Maillard reaction occurs prior to the generation of brown pigments and can be used to indicate the reaction rate and MRPs formation. NR-HHC soup showed significantly higher fluorescence intensity ($p < 0.05$) than HC and herbal extract. In addition, the retorting process enhanced the fluorescence intensity of resulting HHC soups (R-HHC soups). Fluorescence intensity was increased as F_0 increased for both the temperatures used. Samples retorted at 121 °C showed significantly lower fluorescence intensity ($p < 0.05$) than those retorted at 115 °C. The differences in MRPs, including intermediates and final products, might govern the varying bioactivity of samples obtained from different retort processing conditions. Benjakul et al. (2005) showed a correlation between antioxidative activity and fluorescence intensity or browning intensity in sugar-amino acid model systems. The Maillard reaction was associated with improved antioxidative activity of hydrolysate from unicorn leatherjacket fish skin (*Aluterus monoceros*) and silver carp muscle (*Hypophthalmichthys molitrix*) (Karnjanapratum et al. 2016; You et al. 2011).

Color

The effect of retort processing conditions used on color of HHC soups was determined. Color of HHC soups was evaluated in comparison with HC and herb extract (Table 2). NR-HHC soup showed the marked decrease in lightness (L^*) that coincided with increased yellowness (b^*). Total color difference (ΔE^*) and difference in chroma (ΔC^*) of HHC soups were significantly different ($p < 0.05$) from those of HC and herb extract. Darker and browner colors were found in HHC soup, compared to HC, which was in agreement with the higher browning index (Fig. 1) of HHC soup. Ke et al. (2012) showed that non-enzymatic glycation (Maillard reaction) and formation of nanostructures distinguish soups from their raw materials, yielding soup with bio-functions. In general, there were no significant differences ($p > 0.05$) in A_{420}, L^*, a^*, b^*, ΔE^* and ΔC^* values between NR-HHC and R-HHC soups. Furthermore, different retort processing conditions in the ranges tested did not affect the color of the HHC soups.

α-amino group

Changes in peptides in HC due to different retort processing conditions were monitored by measuring free α-

Fig. 1 A_{420} (**a**), A_{294} (**b**) and fluorescence intensity (**c**) of HHC soups with different retort processing conditions. HC, hydrolyzed collagen; Herbal extract, herbal extract using water as medium; NR-HHC, non-retort processed herbal hydrolyzed collagen soup; R-HHC, retort processed herbal hydrolyzed collagen soup. Bars represent standard deviation (n = 3). Different letters on the bars indicate significant differences ($p < 0.05$). Numbers denote F_0 values (5, 7, 9 and 11 min)
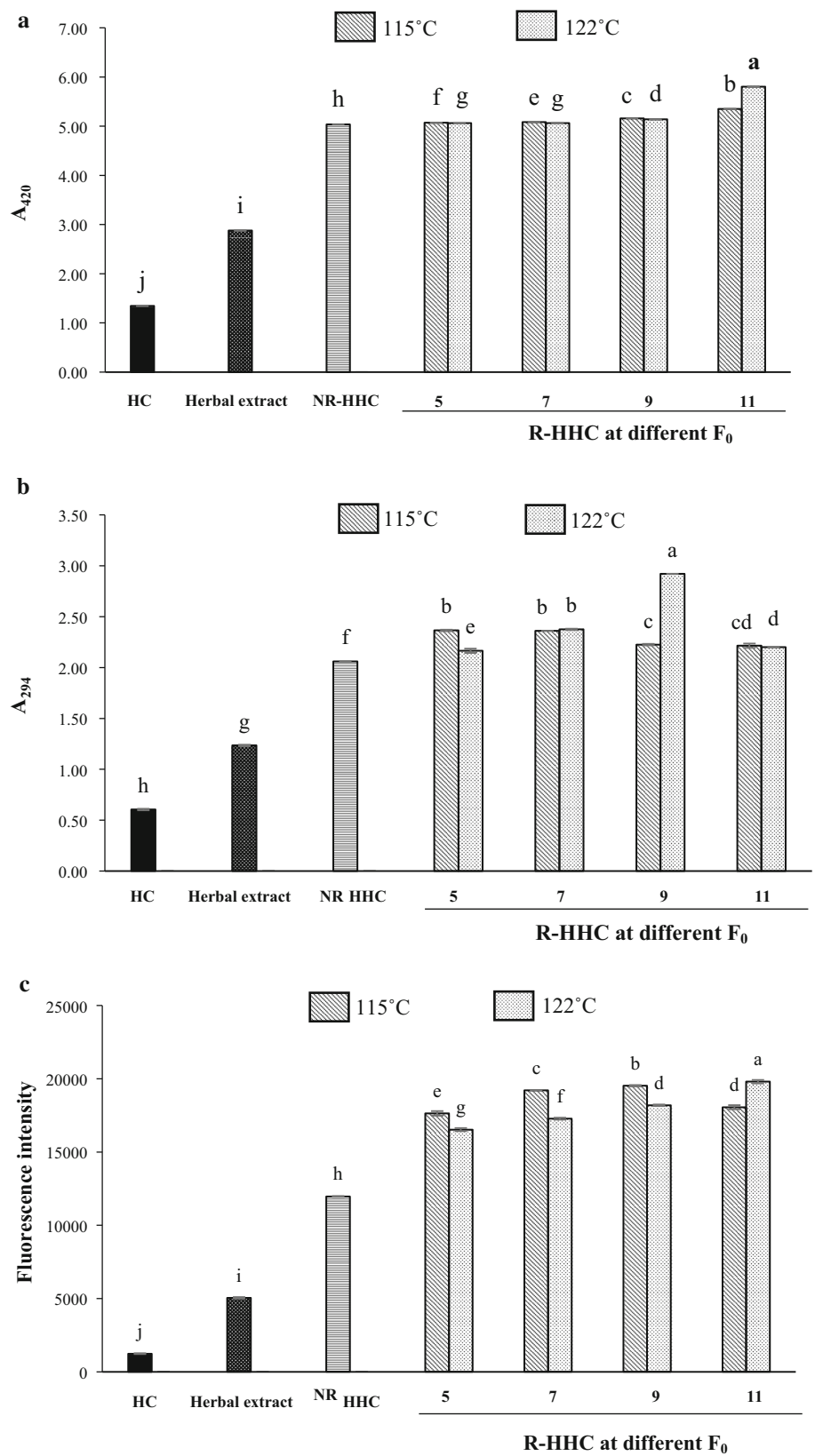
Table 2 Color profile and α-amino group content of HHC soups with different retort processing conditions

Samples	F_0	Color					α-amino group content (mmol/L sample)	Total reducing compound content (mg/L sample)
		L*	a*	b*	ΔE*	ΔC*		
HC		36.27 ± 0.03a	5.89 ± 0.07c	18.59 ± 0.08b	61.79 ± 0.02c	11.07 ± 0.04b	76.38 ± 1.84a	315.96 ± 2.57e
Herb extract		31.59 ± 0.32b	19.04 ± 0.13a	42.71 ± 2.96a	77.10 ± 1.88b	45.32 ± 2.73a	2.60 ± 0.92d	531.72 ± 4.22d
NR-HHC		12.14 ± 0.31c	10.52 ± 0.12b	12.83 ± 0.60c	79.81 ± 0.24a	8.28 ± 0.254c	75.32 ± 2.76a	865.60 ± 21.71c
R-HHC								
115	5	12.47 ± 0.40c	10.12 ± 0.83b	12.79 ± 0.69c	79.50 ± 0.60a	8.15 ± 0.15c	66.30 ± 6.44b	930.09 ± 9.29b
	7	12.21 ± 0.42c	10.11 ± 0.72b	12.7 ± 0.20c	79.67 ± 1.20a	8.29 ± 0.25c	67.89 ± 4.60b	979.18 ± 16.95a
	9	12.21 ± 0.71c	10.17 ± 0.53b	12.72 ± 0.56c	79.87 ± 0.33a	8.19 ± 0.31c	62.05 ± 3.68bc	928.64 ± 13.88b
	11	12.12 ± 0.36c	10.18 ± 0.29b	12.80 ± 0.46c	79.79 ± 0.56a	8.21 ± 0.29c	62.05 ± 4.01bc	943.50 ± 11.71b
121	5	12.37 ± 0.53c	9.93 ± 0.83b	12.76 ± 0.56c	79.81 ± 0.64a	8.25 ± 0.33c	57.80 ± 2.79c	922.48 ± 35.96b
	7	12.43 ± 0.55c	10.20 ± 0.95b	12.73 ± 0.55c	79.50 ± 0.50a	8.28 ± 0.41c	58.33 ± 1.84c	922.30 ± 17.27b
	9	12.13 ± 0.81c	10.26 ± 0.69b	12.75 ± 0.35c	79.71 ± 0.94a	8.26 ± 0.53c	61.52 ± 4.01bc	932.99 ± 8.75b
	11	12.15 ± 0.40c	10.20 ± 0.20b	12.75 ± 0.88c	79.74 ± 0.98a	8.23 ± 0.27c	66.30 ± 4.60b	1002.01 ± 10.35a

Data are mean ± standard deviation (n = 3)

115 and 121 indicate the temperature used in retort process at 115 and 121 °C, respectively

HC, hydrolyzed collagen; Herb extract, herbal extract using water as medium; NR-HHC, non-retort processed herbal hydrolyzed collagen soup; R-HHC, retort processed herbal hydrolyzed collagen soup

a, b, c, d, e in the same column indicate the significant difference ($p < 0.05$)

amino group content of HHC soups in comparison with those of HC and herbal extract (Table 2). NR-HHC soup had similar α-amino group contents to HC that were not significantly different ($p < 0.05$). Nevertheless, very low α-amino group content was found in the herbal extract, indicating that they contained negligible free amino groups. HC was rich in free amino groups. There were significantly decreased ($p < 0.05$) levels of α-amino group content in R-HHC soups compared to NR-HHC soup but no significant differences ($p > 0.05$) were observed in α-amino group content with different F_0 values when the retort temperature was 115 °C. Nevertheless, the significant increase ($p < 0.05$) in α-amino group content was found in R-HHC soups with F_0 of 11 min at 121 °C, but the α-amino group content tended to decrease with increasing F_0 at 115 °C, which was in accordance with the increase in browning index and fluoresces intensity (Fig. 1). The results suggested that α-NH_2 or ε-NH_2 groups in the HC are covalently attached to the carbonyl group of herbal extract to form advanced glycation end products to a greater extent with increasing heating time (F_0 values). Budryn et al. (2016) had previously shown that phenolic-amino acid interactions could take place during food processing, leading to the reduction of free amino acids and peptides in protein hydrolysates. For soup prepared with a retort temperature of 121 °C, the increase in the free amino group content might be due to the formation of the low

molecular weight peptides at high temperature (Karn-janapratum and Benjakul 2015). Therefore, the retorting process used in the present study might have induced some chemical reactions, particularly Maillard reaction and phenolic compound-peptide complex, which might be related to bioactivity of resulting HHC soup.

Total reducing compound content

Total reducing compound contents of herbal extract and HHC soups with and without being subjected to the retort process were also investigated using the Folin–Ciocalteu (F–C) reagent (Table 2). F–C reagent was reactive towards reducing compounds including phenols, thiols, proteins and amino acids (Everette et al. 2010). HC contained the reducing compounds of 315.86 mg/L, while higher total reducing compound content was noted for W/O-Retort (865.60 mg/L). This was attributed to the reducing power of both HC and herbal extract (531.72 mg/L). Slight increases in total reducing compound content were observed after being subjected to the retort process, but different lethality levels used had no significant effect ($p < 0.05$) on total reducing compound content for both the temperatures tested. Among all the samples, the HHC soups obtained from F_0 at 7 and 11 min with the retort temperatures of 115 and 121 °C showed the highest reducing compound contents. It was postulated that an

increase in total reducing compound content might correlate with the changes in phenolic compounds or peptides, in which the fragmentation of high molecular weight polyphenols and peptides occurred and generated new low molecular weight derivatives (Lee et al. 2003; Jeong et al. 2004; Juániz et al. 2016; Karnjanapratum and Benjakul 2015). On the other hand, Budryn et al. (2016) found that the decrease in reducing power might be governed by the formation HC-phenolic complex. In general, the generation of new complexes could empower the bioactivity more effectively than the innate ingredients (Ke et al. 2012). Therefore, total reducing compound content of HHC soups generally determined the bioactivity of HHC soups.

Antioxidative activities of HHC soups

The impact of the retort processing used on antioxidative activities of HHC soups was investigated using different in vitro chemical assays. ABTS and DPPH radical scavenging activities, ferric reducing antioxidant power and hydrogen peroxide scavenging activity of HHC soups with different retort processing conditions are shown in Fig. 2. ABTS radical scavenging activity (Fig. 2a) of HC and

herbal extract samples were 89.81 and 1126.85 μmol TE/L sample, respectively and significant increases in activity ($p < 0.05$) were found for NR-HHC soup indicating that the herbal extract could contribute to the increased activity in HHC soup. Moreover, the generation of new compounds, especially MRPs in the presence of herbal extract and peptides in HC, could result in the increased ABTS radical scavenging activity of the HHC soups. As the F_0 values increased the ABTS radical scavenging activity significantly increased ($p < 0.05$) in R-HHC soups prepared at the retort temperature of 121 °C. Nevertheless, there was no significant difference ($p > 0.05$) between samples prepared at 151 °C with different F_0 values. Similar results were observed for FRAP (Fig. 2b). FRAP of NR-HHC soup were significantly higher ($p < 0.05$) than those of HC and herbal extract. FRAP increased in all the R-HHC soups. Increased F_0 values could enhance the activity of R-HHC soups, particularly those with retort temperature of 121 °C. Chotphruethipong et al. (2017) showed that phenolic compounds act as antioxidants not only because of their ability to donate hydrogen or electrons but also their stable radical intermediates. MRPs formed in R-HHC soups also contributed to the increased
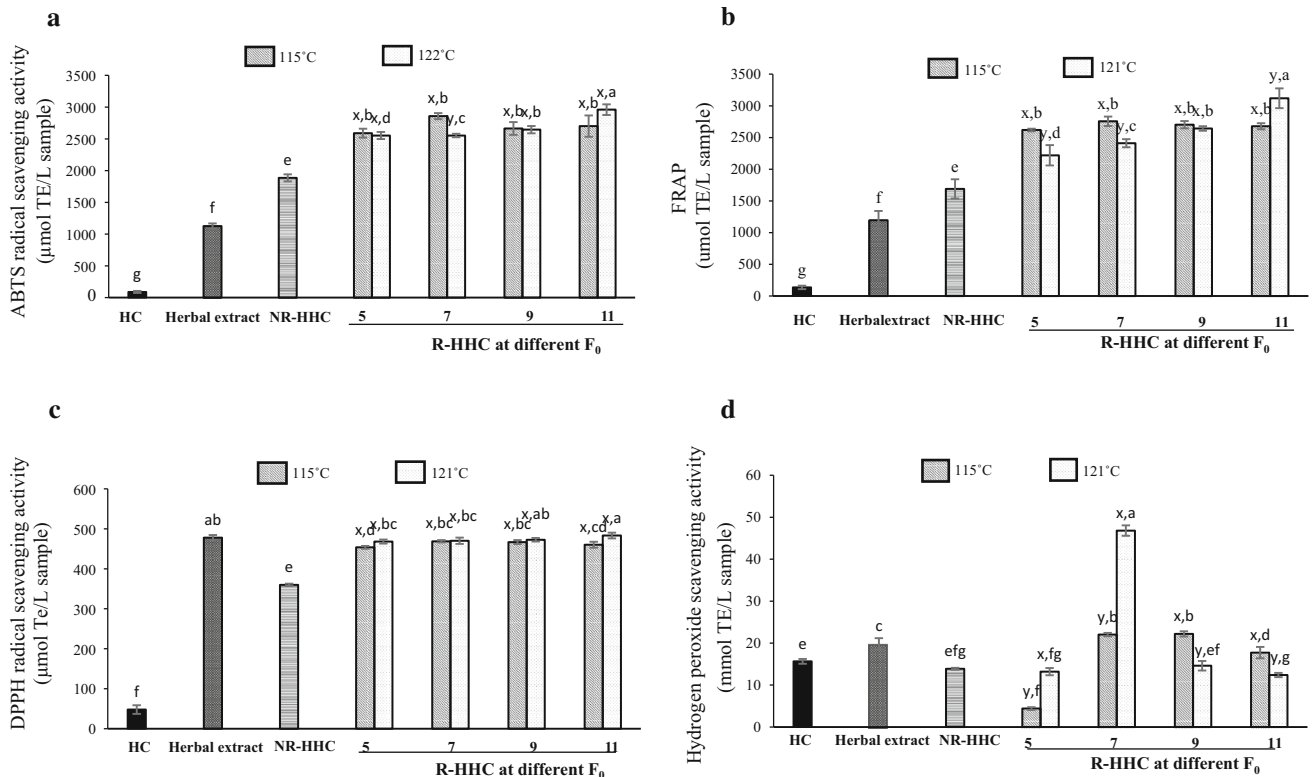

Fig. 2 Antioxidative activities of HHC soups with different retort processing conditions. HC, hydrolyzed collagen; Herbal extract, herbal extract using water as medium; NR-HHC, non-retort processed herbal hydrolyzed collagen soup; R-HHC, retort processed herbal hydrolyzed collagen soup. Bars represent standard deviation (n = 3). [a,b,c,d,e,f,g]Significant differences ($p < 0.05$). [x,y]Significant differences within the same F_0 value ($p < 0.05$). Numbers denote F_0 values (5, 7, 9 and 11 min)

antioxidative activity. In general, ABTS radical scavenging activity and FRAP correlated well with browning index (Fig. 1a) and fluorescence intensity (Fig. 1c). The correlations between antioxidative activity and MRPs have been previously reported (You et al. 2011; Wang et al. 2016). Due to high ABTS radical scavenging activity and reducing power, HHC soups produced using the retorting process would have enhanced antioxidative activity thus increasing their health benefits.

DPPH radical and H_2O_2 scavenging activities of different samples are shown in Fig. 2c, d, respectively and both activities of NR-HHC soup were significantly lower ($p < 0.05$) than those of herbal extract. However, the retort process used showed the potential enhancement of activities. It was noted that the highest DPPH radical scavenging activity was observed at the highest F_0 value used, particularly at 121 °C, which was significant at $p < 0.05$. No significant ($p > 0.05$) increase in DPPH radical scavenging activity was observed in R-HHC soups prepared at 115 °C when the F_0 value increased. H_2O_2 scavenging activity of R-HHC soups varied, depending on retort conditions (Fig. 2d). Among all samples, the soup prepared at 121 °C with an F_0 value of 7 min showed the highest activity, which was significantly higher at $p < 0.05$. Karnjanapratum and Benjakul (2015) had shown that thermal processing affected antioxidative activity of fish skin hydrolysate. Phenolic compounds also contributed to H_2O_2 scavenging activity (Juániz et al. 2016). The decreased H_2O_2 scavenging activity was observed for autoclaved fish skin hydrolysates (Karnjanapratum and Benjakul 2015). H_2O_2 has been known to be generated in vivo by several oxidizing enzymes, such as superoxide dismutase. Together with reactive oxygen species, it can be broken down to hydroxyl radical ($^.OH$) and singlet oxygen ($O_2^{.-}$) via the Fenton reaction in the presence of Fe^{2+} (Wang et al. 2007). $^.OH$ and $O_2^{.-}$ can initiate lipid peroxidation and be toxic to cells. Therefore, it is crucial for cells to remove H_2O_2 via an antioxidant defense (Wang et al. 2007). The results indicated that the retort process used for shelf-life extension could influence the antioxidative activities of HHC soups in different ways.

Sensory characteristics

To investigate the effect of retort processing conditions on sensory properties, a nine-point hedonic scale was used to evaluate the likeness of different HHC soups along with R-HHC soups in comparison with NR-HHC soup (Table 3). All samples showed acceptability with the score for likeness being greater than or equal to 7 (moderately like) for all attributes tested. In general, there was no significant difference ($p > 0.05$) in likeness score for all attributes between R-HHC and NR-HHC soups. All samples had similar scores for viscosity (Table 1) and color

Table 3 Sensory characteristics of HHC soups with different retort processing conditions

Characteristics	NR-HHC	R-HHC							
		115				121			
		5	7	9	11	5	7	9	11
Appearance	7.84 ± 1.21	7.84 ± 0.83	7.68 ± 1.11	7.74 ± 0.93	7.89 ± 1.05	7.68 ± 0.82	7.68 ± 1.11	7.58 ± 0.90	7.79 ± 0.85
Color	7.79 ± 1.32	7.63 ± 1.54	7.53 ± 1.68	7.37 ± 1.74	7.63 ± 1.71	7.63 ± 1.38	7.68 ± 1.73	7.47 ± 1.71	7.53 ± 1.58
Odor	7.63 ± 0.96	7.42 ± 1.30	7.58 ± 1.12	7.37 ± 1.26	7.79 ± 1.23	7.42 ± 1.12	7.42 ± 1.68	7.42 ± 1.30	7.63 ± 1.30
Flavor	7.26 ± 0.99	7.26 ± 1.48	7.16 ± 1.21	7.26 ± 1.15	7.37 ± 1.46	7.21 ± 1.51	7.11 ± 1.79	7.42 ± 1.46	7.47 ± 1.31
Viscosity	7.21 ± 1.32	7.00 ± 1.11	7.11 ± 1.10	7.00 ± 0.88	7.00 ± 1.00	7.00 ± 1.05	7.05 ± 1.13	7.00 ± 1.05	7.00 ± 1.05
Taste	7.00 ± 1.00	7.32 ± 1.06	7.21 ± 1.27	7.37 ± 1.07	7.53 ± 1.02	7.37 ± 1.30	7.00 ± 1.41	7.42 ± 1.22	7.32 ± 1.00
Overall	7.16 ± 1.17	7.42 ± 1.02	7.26 ± 1.10	7.21 ± 0.92	7.42 ± 0.84	7.47 ± 0.90	7.05 ± 1.31	7.32 ± 0.95	7.47 ± 1.12

Data are mean ± standard deviation (n = 3)

115 and 121 indicate the temperature used in retort process at 115 and 121 °C, respectively

5, 7, 9, 11 indicate F_0 value of retort process used

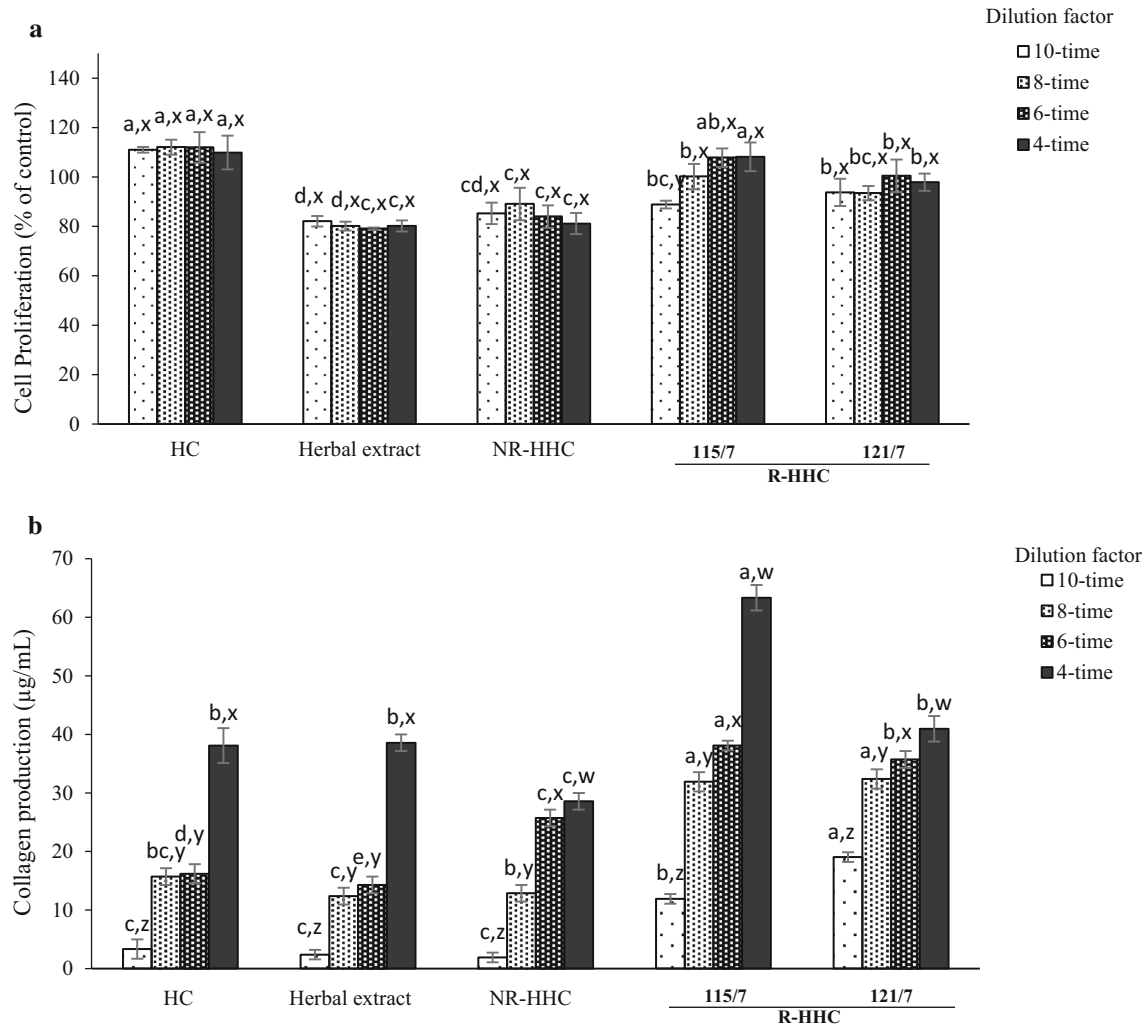NR-HHC, non-retort processed herbal hydrolyzed collagen soup; R-HHC, retort processed herbal hydrolyzed collagen soup

Fig. 3 Effect of HHC soups with different retort processing conditions on cell proliferation (**a**) and enhancement of collagen production (**b**) of mouse fibroblast L929 cells. HC, hydrolyzed collagen; Herb extract, herbal extract using water as medium; NR-HHC, non-retort processed herbal hydrolyzed collagen soup; 115/7, retort processed herbal hydrolyzed collagen soup with F_0 value of 7 min at 115 °C; 121/7, retort processed herbal hydrolyzed collagen soup with F_0 value of 7 min at 121 °C. Bars represent standard deviation (n = 3). [a,b,c,d]Significant differences ($p < 0.05$). [x,y]Significant differences within the same sample ($p < 0.05$)

(Table 2) among all the samples, which were not significantly ($p > 0.05$) different indicating that the conditions of retort process used in the present study had no detrimental effect on sensory properties of HHC soup. Shah et al. (2017) reported similar results for retort processed meat curry in which there was no difference in sensory properties of products at 121 °C with different retorting times (F_0 of 7–11 min).

Effect of selected HHC soups on cell proliferation of L929 cells

Since R-HHC soups from both retort temperatures with an F_0 value of 7 min possessed high activity for all antioxidative assays tested and had high acceptance by the sensory panel, these two treatments were selected for a

comparison of their effect on cell proliferation and collagen production of L929 mouse fibroblast cells using a MTT test in comparison with HC, herbal extract and NR-HCC soup at various concentrations (Fig. 3a). None of the samples showed toxicity to L929 cell viability. More than 80% of cell viability was found at all the concentrations tested, compared with that of control that had 100% cell proliferation. HC showed the stimulatory effect on cell growth with the highest cell proliferation (110–112%), regardless of concentration. Collagen peptides in HC might serve as a good nutrient for cell growth but lower cell viability was observed for herb extract, which might be related to its acidity since its pH was less than 6 (Table 1). Consequently, slightly decreased pH levels of the culture medium (pH 6.2–6.5) were obtained, but herbal extract showed no cytotoxic effect on L929 cell proliferation under the

concentration range tested. It was noted that the retort processing used could increase the potential of R-HHC soup in cell growth stimulation as evidenced by the increased cell proliferation. The activities of samples retorted at 115/7 and 121/7 were not significantly different ($p < 0.05$) at the same concentration tested. Nevertheless, 115/7 samples, especially at 6-times and 4-times dilution, showed comparable activity to those of HC and were not significantly different ($p < 0.05$), which resulted in the highest cell proliferation. Antioxidative compounds have previously been shown to possess various bioactivities such as potential to stimulate proliferation of L929 cells (Pitz et al. 2016) and antitumor activity (Chen et al. 2013). Liu et al. (2014) reported, using flow cytometry scanning, that collagen peptides markedly increased osteoblast growth by promoting DNA synthesis. Thus, the stimulation effect of R-HHC soups on L929 cell proliferation, especially for 115/7 sample, was confirmed.

Effect of selected HHC soups on collagen production of L929 cells

The impact of selected HHC soups on collagen production of L929 cells was investigated using in vitro cell culture. Collagen production enhancement of R-HHC soups (115/7 and 121/7 samples) is shown in Fig. 3b. Collagen production of L929 mouse fibroblasts was enhanced in a dose dependent manner in NR-HHC, both R-HHC samples and both retorting processes (115/7 and 121/7). HC showed similar ($p > 0.05$) activity to those of herb extract. NR-HHC soup, particularly at 6-times dilution, had significantly higher potential ($p < 0.05$) than HC and herbal extract at the same concentration used. Nevertheless, significantly lower activity ($p < 0.05$) of NR-HHC soup was observed when the highest concentration (4-time dilution) was applied. Thus, retort processing augmented the effects of HHC soup in inducing collagen production of L929 cells. The 115/7 sample showed the significantly higher potential ($p < 0.05$) in enhancing collagen production in L929 cells, which was in accordance with its cell proliferation stimulation (Fig. 3). Schwarz (2015) found that in high collagen producing cells, particularly fibroblasts, the pathway is controlled by post-transcriptional regulation, which requires feedback control between secretion and translation rates and is based on the helical structure of the procollagen molecule and additional tissue-specific modifications. Shibuya et al. (2014) found that fish collagen peptide could activate in vitro fibroblast cell growth and transcriptionally up-regulate type I collagen in vivo. Therefore, HHC soups, especially those subject to 115/7, promoted collagen production in L929 fibroblast cells by regulating/stimulating DNA synthesis.

Conclusion

Retort processed HHC soups in glass bottle were prepared using different retort processing conditions. Retort process generally caused some changes in physicochemical properties, while enhancing antioxidative activities of resulting HHC soups. The sensory evaluation panel found that R-HHC soups had high acceptance, which was comparable to NR-HHC soup. High antioxidative activities were found in R-HHC soups with F_0 value of 7 min at both 115 and 121 °C. These HHC soups could induce cell proliferation as well as enhance collagen production of L929 cells. Therefore, it is concluded that R-HHC soup in glass bottles could be a new ready-to-serve functional drink that has health benefits.

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