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EFFECT OF EXTRACTION METHODS ON THE YIELD, FUCOSE CONTENT AND PURITY OF FUCOIDAN FROM *Sargassum* sp. OBTAINED FROM PULAU LANGKAWI, MALAYSIA

(Kesan Kaedah Pengekstrakan Fukoidan Terhadap Hasil, Kandungan Fukosa dan Ketulenan Fukoidan daripada *Sargassum* sp. dari Pulau Langkawi, Malaysia)

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Abstract

The objective of this study was to determine the effect of extraction methods on the yield, fucose content and purity of fucoidan that had been isolated from brown seaweed, *Sargassum* sp. The extraction of fucoidan was carried out based on the factorial design assigned, which involved three different parameters including two types of extraction (acid and alkaline), three different extraction temperatures (45, 65 and 85 °C) and three different extraction time (1, 3 and 5 hours). Results obtained showed that the best extraction parameter that gave the highest yield of fucoidan extract was the alkaline extraction at 65 °C for 3 hours. Alkaline extraction also produced higher purity and better quality of fucoidan compared to the fucoidan extracted by hydrochloric acidic.

Keywords: acidic extraction, alkaline extraction, fucoidan, fucose content

Abstrak

Objektif kajian adalah untuk mengkaji kesan kaedah-kaedah pengekstrakan terhadap hasil, kandungan fukosa dan ketulenan ekstrak fukoidan yang diperoleh daripada rumpai laut perang, *Sargassum* sp. Pengekstrakan fukoidan dilakukan berdasarkan reka bentuk eksperimen berfaktor, di mana tiga parameter yang berbeza telah ditetapkan termasuklah dua jenis pengekstrakan (berasid dan beralkali), tiga suhu pengekstrakan (45, 65 dan 85 °C) dan tiga masa pengekstrakan (1, 3 dan 5 jam). Keputusan kajian menunjukkan bahawa kaedah pengekstrakan terbaik yang memberi hasil fukoidan paling tinggi adalah melalui pengekstrakan beralkali pada suhu 65 °C selama 3 jam. Pengekstrakan beralkali juga menghasilkan ekstrak yang lebih tulen dan berkualiti berbanding dengan fukoidan yang diekstrak menggunakan asid hidroklorik.

Kata kunci: pengekstrakan berasid, pengekstrakan beralkali, fukoidan, kandungan fukosa

Introduction

Seaweeds are usually classified into three groups including brown (Phaeophyceae), red (Rhodophyceae) and green seaweeds (Chlorophyceae). Red algae is the most abundant group (6000 species), followed by brown (2000 species) and green (1200 species) [1]. They are excellent sources of various bioactive compounds such as polyphenols, carotenoids and polysaccharides [2-5]. These seaweeds have traditionally formed part of the oriental diet, especially in Japan, China and Korea [6]. The major use in Western countries has mainly focused on the extraction of active

compounds used by pharmaceutical, cosmetics and food industries as the source of phycocolloids (polysaccharides extracted from both fresh and marine algae), thickening and gelling agents.

In Malaysia, there is an abundant supply of seaweeds consisting of the three groups. Brown and red seaweeds are mainly used as the sources of phycocolloids, i.e. carrageenan and alginate while green seaweed is normally consumed fresh. According to Phang [7], Malaysia is rich in wild species of brown seaweed such as *Sargassum, Laurencia, Fucus, Ascophyllum* and many others. Among these brown seaweeds, *Sargassum* contributes the highest amount of phytocolloids. There are 21 species of *Sargassum* recorded in Malaysia as reported in literature [7]. *Sargassum binderi* is a type of brown seaweed (Phaeophyceae) that is commonly found in Malaysia. Phang [7] also reported that the phytocolloids extracted from *Sargassum binderi* consist of fucoidan, alginate and laminaran.

Ponce et al. [8] claimed that fucoidan is the major bioactive compound found in *Sargassum*. Fucoidan is a water soluble polysaccharide containing mainly L-fucose and sulphate, together with minor amount of glucuronic acid and xylose. It is a high molecular weight (approximately about 20000 Da) water soluble dietary fibre or polysaccharide. It comes from a family of sulphated homo and heteropolysaccharides composed mainly of α-(1/2)-, α-(1/3)- and/or α-(1/4)-linked α-L-fucose pyranose residues [9]. The main monomer for bioactive components is the fucose, which is one of the eight essential biological sugars. In addition, fucoidan also contains galactose, mannose, xylose, and glucoronic acid residue [10]. The chemical structures of fucoidans from different brown seaweeds vary in terms of types of population species, level of maturity, geographical location and climate [11]. Besides, these particular factors also exhibit different biological activities of fucoidan. Biological activities of fucoidan mainly occur due to its degree of sulphation, structural peculiarities and molecular weight [12]. Fucoidan has a variety of biological functions such as antibacterial, antiviral, antioxidant, anti-cancer, anti-tumour as developed in nutraceutical products and dietary supplements [13]. Lim et al. [9] reported that fucoidan extracted from *Sargassum sp*. that was harvested in Semporna Sabah, Malaysia gave some different characteristics compared to other species of brown seaweeds such as *Padina sp*.

There are many extraction methods that can be used to extract fucoidan from seaweeds. Most of the extraction methods tend to extract fucoidan as a multicomponent crude form of fucoidan, commonly referred as the crude fucoidan [14]. Ion-exchange chromatography or filtration process can be performed to obtain a purified fucoidan. Initially, pre-treatment of the seaweed is commonly performed using a mixture of methanol/chloroform/water (4:2:1 v/v/v) to remove lipids, terpenes and phenols [15]. Raw seaweeds are usually extracted with acid or base solutions as the solvent [13, 16]. Each method of extraction gives different yield and purity of the extracted fucoidan. Hence, this study was carried out to determine the effects of extraction methods (types of extraction, time and temperature) on the fucoidan yield and its fucose content.

Materials and Methods

Chemicals and reagents

Absolute ethanol, methanol and chloroform were obtained from Merck, Germany. Hydrochloric acid (HCl), sulphuric acid (H_2SO_4), calcium chloride ($CaCl_2$), sodium hydroxide (NaOH), sodium iodide (NaI) and Cetavlon were acquired from Fischer Scientific, USA, whereas L-cysteine was obtained from Sigma Aldrich, USA.

Sample preparation

Brown seaweed, *Sargassum* sp. from Pulau Langkawi, Kedah, Malaysia was collected to be used as the study sample. The sample was intentionally planted in an isolated coastal area which did not mix with other genus of seaweeds. The seaweed sample was cleaned and washed with filtered water to remove the sand, debris, epiphytes and other foreign matters prior to storage of sample in an ice cooler box (4 °C). This was to done to ensure low temperature and to preserve the structure of the sample throughout the transportation to the laboratory in UKM, Malaysia. Upon arrival, the sample was again rinsed with distilled water for several times to ensure that it was free from sand and debris. The cleaned seaweed sample was oven-dried at 60 ºC for three days, until the sample achieved a constant weight. The dried sample was ground into powder form by using a high power grinder (C7104, Panasonic, Japan) before being tightly-packed in a glass jar and stored at -18 ºC until further analyses.

Experimental design
The design of this study was a factorial, consisting of three factors that represent the type of extraction, extraction temperature and time. There were two types of fucoidan extraction i.e. acid and alkaline extraction using both 0.15 M of HCl and $CaCl_2$ respectively, at 45, 65 and 85°C for 1, 3 and 5 hours.

Extraction of fucoidan: Acid extraction
The extraction of fucoidan using acid solution, HCl was performed according to the procedures as described [17]. Pre-treatment of the sample was initially conducted to remove lipids, coloured matter and low molecular weight components by refluxing approximately 20 g of the ground sample in 200 mL absolute ethanol at 80 °C for 2 hours. The sample solution was then centrifuged at 18,500×*g* for 10 minutes and the supernatant was removed. The defatted sample was then left to dry overnight at room temperature before being weighed again. Sample was extracted with 200 mL of 0.15 M HCl solution at 45 °C for 1 hour with continuous mechanical stirring (350 rpm). This step was consecutively repeated four times to ensure a complete fucoidan extraction. The sample was centrifuged at 18,500×*g* for 10 minutes after extraction and 3 M sodium hydroxide, NaOH was slowly added into the collected supernatant while being slowly stirred to neutralize the extract solution. The neutralized extract solution was stored overnight at 4 °C. Four volume of absolute ethanol was added into the extract solution and was left to stay overnight at 4 °C to precipitate the fucoidan, prior to centrifugation at 18,500×*g* for 10 minutes to recover fucoidan. The fucoidan obtained was then washed with ethanol and dried at room temperature until constant weight was achieved, and kept in an air tight sample bottle. These procedures were repeated by following different parameters i.e. extraction temperature (65 and 85 °C) and extraction time (3 and 5 hours).

Alkaline extraction
Alkaline extraction of fucoidan was conducted by referring to the method reported in literature [18]. Approximately 20 g of ground seaweed sample was pre-treated with 200 mL of mixture solvent consisting of methanol, chloroform and distilled water (4:2:1; v/v/v) with continuous mechanical stirring at 350 rpm overnight. This was to remove lipids, coloured matter and low molecular weight components from the sample. The suspension was then filtered, and the sample residue was collected. This step was repeated several times until clear solution was obtained to ensure the removal of unwanted components. The defatted seaweed was re-weighed prior to drying using a vacuum oven (Vacucell MMM, Munich, Germany) at 50 °C for 24 hours. Sample was then heated with 200 mL of 0.15 M $CaCl_2$ solution at 45 °C for 1 hour to extract water-soluble polysaccharides. This step was consecutively repeated four times to ensure a complete fucoidan extraction.

Sample solution was mixed thoroughly with 30 mL of 10% hexadecyltrimethylammonium bromide/Cetavlon solution (in excess) and left overnight at 4 °C after being filtered. The solution was centrifuged at 18,500×*g* for 10 minutes using an *Eppendorf 5810R* centrifuge and the supernatant was removed. The precipitate obtained was washed with distilled water and mechanically stirred with 50 mL of 20% ethanolic sodium iodide, NaI (Fischer Scientific) solution overnight at room temperature. This step was repeated for four consecutive days. The mixture solution was rinsed with ethanol to remove excess sodium iodide and dissolved in water. The fucoidan solution was then dialysed against distilled water for several days using a Visking tube with a molecular weight cut-off (MWCO) of 2000 Da, before being lyophilised to obtain crude fucoidan and kept in an air tight sample bottle. These procedures were repeated by following different parameters i.e. extraction temperature (65 and 85 °C) and extraction time (3 and 5 hours).

Quantitative analyses: Determination of extraction yield
The collected fucoidan extract was weighed and the extraction yield was calculated as the following equation 1:

$$\text{Yield of fucoidan (\% of dry weight)} = \frac{\text{Weight of fucoidan (g)}}{\text{Weight of ground seaweed (g)}} \times 100\% \quad (1)$$

Determination of fucose content
Approximately 1 mL of fucoidan extract and 4.5 mL of diluted sulphuric acid, H_2SO_4 solution (1:6, H_2O:H_2SO_4) were pipetted into respective test tubes that had been placed in an ice-water bath. All test tubes were placed into a

boiling water bath for 10 minutes after 1 minute of cooling in the ice-water bath. Aliquots in the test tubes were then left to cool to room temperature prior to the addition of 0.1 mL of 3% L-cysteine (Sigma Aldrich) solution and the solutions were allowed to stand for 30mins to ensure thorough mixing as reported by Lim et al. [9]. Absorbance reading of all the samples was measured in a 96-well plate at 396 nm and 427 nm by using a BioTek Epoch microplate spectrophotometer (Vermont, USA) [19]. Calibration curve ranging from 0.002-0.02% (w/v) of a commercial fucoidan (F_{ysk}) was used to determine the content of fucoidan in the sample extracts. This spectrophotometric analysis was also used in order to calculate the purity of fucoidan based on its content and the initial concentration of sample used (0.02%). n =3 replication was conducted to ensure more accurate results.

Statistical analysis

All analyses were conducted in triplicates (n=3) and collected data were expressed in terms of mean and standard deviation. The statistical analysis was performed using both t-test and one-way ANOVA followed by Duncan's multiple range test (DMRT) using SPSS software for Windows Version 22. Difference in values of mean was considered significant when $p < 0.05$.

Results and Discussion

Yield of fucoidan extract

Techniques of extraction greatly influenced the yield of fucoidan extract. Different mechanical and chemical processes such as the solvent extraction and steam distillation are normally used for the extraction of some bio-compounds from plant sources. The selection of methods differs according to the nature of soluble polysaccharides to be extracted (fucoidan) to obtain its maximum yield. Based on the study that has been conducted, it showed that the acid extraction (fucoidan extracted by HCl) of all parameters gave lower yield in terms of its dry weight percentage (% w/w) compared to that of the alkaline extraction (Figure 1). The alkaline extraction of fucoidan at 65 °C for 3 hours yielded statistically the highest amount of fucoidan, 3.81±0.73% compared to other extraction parameters. In contrast, the least yield of fucoidan extracted by HCl at 85 °C for 5 hours was 0.13±0.05%. Similar result was reported by Ale that the alkaline extraction yielded a significant higher amount of fucoidan compared to acidic extraction [20].

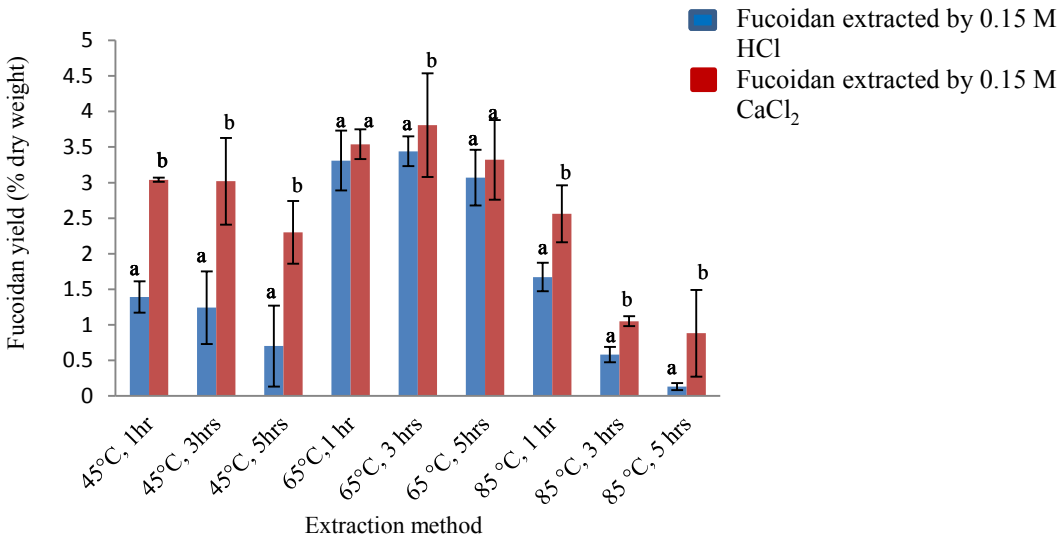

Figure 1. Graph of fucoidan yield (dry weight percentage) against the extraction method. [a-b] Different letters on fucoidan yield indicate a statistically significant difference ($p< 0.05$)

Both acid and alkaline extraction methods showed a significant difference in the fucoidan yield due to its chemical properties. Both solvents used during the process influenced the yield of fucoidan due to their pH values. Protons or hydroxide ions interfere with the hydrogen bonds between the various polysaccharides, releasing them into the solution resulted in an increased yield of extract [15]. The acidic properties of HCl during the extraction of fucoidan might cause the breaking of chemical bonds between the structures of fucose, resulted in the failure of fucose detection [21]. In addition, the extraction of fucoidan at higher temperature (more than 70 °C) and longer extraction time might lead to a degradation of fucose chain. Lower temperature tends to provide slightly low yield of fucoidan as the extraction process might be less efficient than that of the intermediate temperature (65 °C) used (Figure 1). Theoretically, temperature of a substrate or solvent affects the rate/kinetics of a chemical reaction [11]. The higher the temperature applied, the rate and kinetic become higher and faster. In this study, the extraction temperature of 45 °C might not be suitable to extract fucoidan from the seaweed. This explained the result obtained where the yield of fucoidan at 85 °C was lower although longer extraction time was used.

Fucose content of fucoidan extract

Screening of fucoidan content from the raw seaweed sample played the role as the pre-confirmatory analysis in this study. The method used was defined as the colorimetric method in which specific colour reactions occur between fucose with the mixture of L-cysteine and sulphuric acid. This colorimetric method was applied with two different wavelengths, 396 nm and 427 nm. Both absorbance readings were recorded and the change in absorbance between those wavelengths (396 nm and 427 nm respectively) differentiates the presence of fucose content from that of other hexoses [9]. Usov et al. claimed that the absorbance of other hexoses remains the same at both wavelengths at a point of time [22].

Based on the analysis done, fucoidan extracted by 0.15 M $CaCl_2$ at 65 °C for 3 hours showed the significantly highest ($p< 0.05$) fucose content compared to that of all extraction methods that was 3.80±0.91%. Figure 2 also showed the significantly lowest ($p< 0.05$) fucose content was found to be in the fucoidan extracted by 0.15 M HCl at 85 °C for 5 hours which was 0.22±0.7%. The result obtained was reliable as brown seaweeds do not contain other 6-deoxyhexoses which may be found in other seaweeds [22]. Hence, the colour reaction was due to fucose from the brown seaweed sample itself, excluding other sugars by the colour measurement at two wavelengths.

According to recent studies by Rioux et al. [11], fucoidan content in various seaweed species ranges from 0.4% to 21%. This showed that the fucoidan content highly depends on the species of seaweed itself. In addition, he claimed that the fucoidan content in seaweed is also influenced several other factors including season, geographic location, chemical composition of fucoidan as well as the maturity of the plant [11]. Recent study on the fucoidan content in brown seaweed samples collected from several regions in Japan stated that they contain fucoidan ranging from 2.2% to 10.4% [23]. Therefore, it is clearly observed that the fucoidan content in brown seaweed (*Sargassum sp.*) from Pulau Langkawi, Malaysia is within the range. Studies also reported that the fucose composition also varies among species of seaweeds as well as its technique of fucoidan extraction [12]. As Ponce et al. reported, fucoidan been extracted at room temperature and at 70°C possessed completely different chemical compositions [8].

Structure of the plant itself also affects the fucose content in the brown seaweed. Scientifically, it is determined that fucose can highly be contained in the holdfast of the seaweed [24]. Blade-derived fucoidan tend to possess lower fucose content compared to that obtained from the holdfast part. Previous research proved that the sporophyll part of the seaweed plant contains higher quantity fucoidan than that from the blade [25]. Moreover, Chopin and Sawhney [26] claimed that maturity of the seaweed plants also relates positively with the fucose content. The more mature is the seaweed plant, the higher is its fucose content. Hence, there are many internal and external factors which will influence the fucose content in brown seaweeds. Several related factors will be taken into account and discussed in the further analyses of this study.

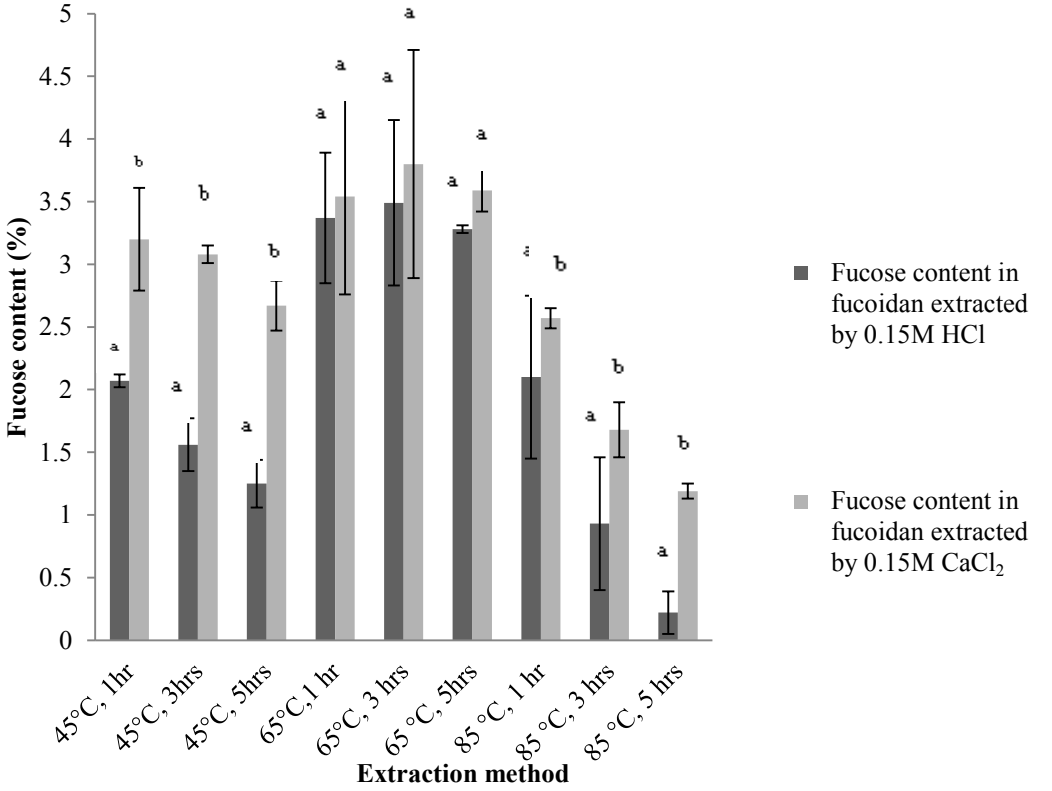

Figure 2. Graph of fucose content (%) against the extraction method. [a-b] Different letters on fucoidan yield indicate a statistically significant difference ($p < 0.05$)

Fucoidan purity

The quality of the extracted fucoidan is normally determined by its purity. The colour of fucoidan also affects the quality of fucoidan in terms of its purity [27]. Purity of the fucoidan extract can be determined visually as lighter colour of fucoidan indicates that it tends to have higher purity, thus higher quality. On the contrary, darker colour of fucoidan extract shows that the extract has a low purity. However, the purity of fucoidan can be measured more accurately by using the colorimetric method as described [19].

In this study, fucoidan obtained from both extraction methods were compared in terms of their purity by using colorimetric method, followed with *t*-test analysis. Based on the results shown in Table 1 below, fucoidan obtained from alkaline extraction (using $CaCl_2$) had a significantly higher purity ($p< 0.05$) than the fucoidan obtained from acidic extraction. This might be due to the existence of other soluble polysaccharides especially alginate that contained in the extract from acidic extraction. During the extraction of fucoidan by HCl, simultaneous extraction of alginate occurred, causing the alginate to be present as the alginic acid. Presence of alginic acid in the fucoidan extract significantly affected the purity of fucoidan when tested. On the contrary, the presence of alginic acid in the fucoidan extracted by alkaline solvent, $CaCl_2$ was determined to be in the form of alginate (insoluble).

Table 1. Percentage of purity of the fucoidan extract

Type of extraction	Purity (%)
Acidic extraction	36.5[a]
Alkaline extraction	82.7[b]

[a-b] Different letters on fucoidan yield indicate a statistically significant difference ($p < 0.05$)

Conclusion

The best parameter for fucoidan extraction was at 65 °C for 3 hours, as it produced the highest yield experimentally. However, there was no significant difference when extracted for 5 hours at lower temperature (45 and 65 °C). Alkaline extraction exhibited higher purity and better quality of fucoidan obtained compared to that of the acid extraction.

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