Study of fucoidan digestibility via an *in vitro* digestion system

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A thesis submitted to

Auckland University of Technology

in partial fulfilment of the requirements for the degree

of Master of Applied Science (MAppSc)

2015

School of Applied Science

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Yuanyang Cao

Abstract

Fucoidan is a known health product mainly consumed orally. It is important to investigate the digestibility of fucoidan in order to understand how fucoidan exerts its health benefits.

Our objective for this project is discussed under the following sub-headings: To study the digestion pattern of fucoidan using *in vitro* models. And to compare different *in vitro* models for a digestibility study of fucoidan.

Sample digested through the two different digestion models showed an increase of the reduced sugar which infer to the breakdown of the polymer to smaller units, such as glucose and galactose. Sulfate levels which contribute to the biological activities of fucoidan decreases significantly during the digestion process. It has also been observed that the level of uronic acid, which is linked to anticoagulant and antitumor property of fucoidan, increases during the digestion process. Also, the level of monosaccharides in fucoidan is increased during digestion process. This may be due to the anti-peptic activity of fucoidan, which breaks down the sulfate-fucose bonds and bind the sulfate compounds with protein.

In order to determine the reaction through different stages of digestion, GC-FID assay was used to test the individual monosaccharides from different reactors during the digestion process. Results showed that the oral digestion process and gastric environment without enzyme almost had no influence on the digestibility of fucoidan. But the pepsin worked in the stomach reactor, significantly increased the level of monosaccharides (mainly fucose) in fucoidan digesta. These changes may relate to the protein contained within the fucoidan structure, enzymolysis by pepsin and contribute to the broken down of polymer.

Overall, the results showed that fucoidan is digestible. The digestibility of the polymer is probably related to the anti-peptic activity of fucoidan and enzymolysis of

protein within fucoidan.

In the future, it should be investigated as to, how we can increase or maintain the high sulfate level in digested fucoidan as biological activities of fucoidan is related to a high sulfate content of fucoidan.

Acknowledgements

This study would not have been possible without the support from so many people.

Firstly, I would like to express my sincerest gratitude to my primary supervisor, Dr. Michelle Yoo. My achievement would not have been possible without her idea and help on this topic. She was always patient with my study and experiment, and gave me assistance and encouragement when I needed. Her help has driven me to work at my very best throughout the year. She has given me support in experimental design and thesis writing. One could not wish for a better advisor.

Deepest gratitude is also to my secondary supervisor, Associate Professor. Jun Lu, about the technical guidance, knowledge on the instrumental analysis, ideas on the analysis of data and advices on the design of the experiment. My study would not have been possible without the support from him.

I convey thanks to Associate Professor. Lindsey White. My achievement would not have been possible without his advice on this topic.

Special thanks also to all my seaweed group friends, Loretta White and April Wang for sharing the literature and invaluable assistance.

In addition, I am indebted to Roy Ma, who supported me for the assays and statistical analysis.

Thanks to the chemistry technicians especially Dr. Chris Pook in the School of

Applied Sciences for helping me source chemicals and providing knowledge on the use of equipment that I needed for my studies.

Finally I am truly grateful to my parents for the financial support, my friends Cathy Guo, Frank Zhang, Jennifer Wu for their support in my Master Degree study in the Auckland University of Technology

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List of Abbreviations

U. pinnatiFIDa	*Undaria pinnatiFIDa*
aPTT	activated Partial Thromboplastin Time
TT	Thrombin Time
HSV	Herpes Simplex Virus
SMC	Smooth Muscle Cell
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
GIT	Gastrointestinal Tract
TIM	TNO's gastrointestinal model
HGS	Human Gastric Simulator
SIVDP	Static *In vitro* Digestion Protocol
TCA	Trichloroacetic acid
FT-IR	Fourier Transform Infrared Spectroscopy
DCM	Dichloromethane
GC-FID	Gas chromatography-Flame ionization detector
UV	Ultraviolet
TFA	Trifluoroacetic Acid
w/w	weight/weight

Chapter 1 Introduction

Consumers are becoming increasingly aware of products derived from plants and animals (Liu & Yao, 1994), to pursue wellbeing and a healthy life (Mak, Hamid, Liu, Lu, & White, 2013). Polyphenols from berry fruits (González, Domínguez, Moreno, & García, 2010) and, squalene from the hound fish (Kim & Mendis, 2006), *citrus* genus from lemon (Ulber & Lang, 1999), and bioactive compounds from marine ecosystems (Zhang et al. 2005), are examples of some of the bioactive compounds with validated health benefits (Guan & Li, 2010; Holtkamp, Kelly, Szajdek & Borowska, 2008).

Recently, there has been a growing interest in fucoidan extract from seaweeds. Fucoidan is composed of α-1,3-linked and α-1,4-linked L-fucose residues, sulfate compounds, small amounts of uronic acid and monosaccharides including galactose, mannose, xylose and glucose (Zhang, Zhang, Wang, Shi, & Zhang, 2009).

Over the past decade, fucoidan has been extensively studied for its chemical structure, concentration in different species of seaweeds and characteristics of its major constituents. The relationship between its inherent structure and different properties of fucoidan like antioxidant (Zhang, Zhang, Wang, Shi, & Zhang, 2009), anticoagulant (Cumashi et al., 2007), antiviral (Adhikari et al., 2006), antitumor (Maruyama, Tamauchi, Hashimoto, & Nakano, 2002), anti-inflammatory (Cumashi et al., 2007) and antithrombotic (Millet et al., 1999; Ngo, Wijesekara, Vo, Van, & Kim, 2011) properties has also been widely investigated (Hemmingson, Falshaw, Furneaux, & Thompson, 2006; Li, Wei, Sun, & Xu, 2006).

Whether fucoidan contains polysaccharides that are digestible by humans and how the bioactive composition changes during the digestion process remains unknown. In this

project an *in vitro* digestion model has been used to investigate the digestion process of fucoidan extracted from the brown seaweed, *U.pinnatiFIDa.* In *in vitro* digestion model, which is based on human anatomy and physiology, is developed to simulate the oral and gastric pH, temperature, secretion and motility conditions. The model has been widely used for resolution of pharmaceutics (Blanquet et al., 2004) and to determine bioavailability of nutrients in food items (Beer, Wood, Weisz, & Fillion, 1997). The different digestibility changes involved in target food items are also studied using an *in vitro* digestion model (Tedeschi, Clement, Rouvet, & Valles-Pamies, 2009). The model is widely used because it is simple to operate, cheaper in comparison to *in vivo* trials, and robust in producing data with high repeatability without ethical concerns (Oomen et al., 2002).

Aim

There are no published literatures on the digestibility of fucoidan using an *in vitro* digestion system. Therefore, the aim of this project is to investigate the quantitative and qualitative changes of monosaccharides and polysaccharides from fucoidan during the simulated digestion process.

In addition, bioactive compounds in fucoidan, such as sulfate constituents and uronic acid, are studied under the simulated digestion process. The study anticipates to determining the efficiency of bioactive compounds upon digestion.

Chapter 2 Literature review

2.1 Seaweed

Seaweed is a loose colloquial term encompassing macroscopic, multicellular, benthic marine algae (Abowei & Ezekiel, 2013) which are not included in plant kingdom (Kandale et al., 2011). Seaweeds can also be classified by their applications, for example as foods, medicine, fertilizer and for use in hydrocolloids industry or for treating industrial effluents through the recovery of heavy metals.

Seaweeds are farmed or foraged from the wild (Abowei & Ezekiel, 2013). They are mainly consumed by coastal people, particularly in East Asia, for example, Brunei, Japan, China, Korea, Taiwan, Singapore, Thailand, Cambodia, and Vietnam. They are also consumed in South Africa, Indonesia, Malaysia, Belize, Peru, Chile, the Canadian Maritimes, Scandinavia, South West England, Ireland, Wales, California, Philippines, and Scotland (McHugh, 2003).

In Asia, sheets of dried *Porphyra*, also known as Nori (Japan), Zicai (China), and Gim (Korea), are used in soups or to wrap sushi (Choi et al., 2014). *Chondrus crispus* (commonly known as Irish moss or carrageenan moss) is red algae used in producing various food additives (Van de Velde & De Ruiter, 2005), along with *Kappaphycus* and various gigartinoid seaweeds. *Porphyra* is red algae used in Wales to make laver (Abowei & Ezekiel, 2013). Laverbread, made from oats and laver, is a popular dish of Wales. In northern Belize, edible seaweeds are combined with milk, nutmeg, cinnamon, and vanilla to make a common beverage called "Dulce" (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011).

Table 1. A table summarizing different types of seaweeds based on lists of pigments and ecosystem (Smith,1944)

Genus	Colour	Ecosystem	Cultivated as food
Caulerpa	Green	Under water	Not clear
Fucus	Brown	In intertidal zones on rocky shores	Not clear
Gracilaria	Red	Under water	Yes
Laminaria	Brown	8–30 m under water	Yes
Macrocystis	Brown	Giant kelp, forming floating canopies	Not clear
Porphyra	Red	Intertidal zones in temperate climate	Yes

U.pinnatiFIDa is a type of brown seaweeds, which is native to Japan, China and Korea. It is also cultivated in France, Spain, Italy, Argentina, North and South America, Australia and New Zealand (Neill, Heesch, & Nelson, 2008). Circumstantial evidence shows that the Japanese and Korean fishing vessels carried *U. pinnatiFIDa* to New Zealand from 1979 to 1987 (Hay & Luckens, 1987). In 2010, New Zealand Biosecurity altered its policies and a permit was issued to cultivate *U. pinnatiFIDa* and to study its chemical composition in detail. The cultivation of *U. pinnatiFIDa* was restricted to highly infested areas (Wang, 2012).

U.pinnatiFIDa is a very invasive species. The fresh *U.pinnatiFIDa* contains over 70% water. It is capable of invading native seaweed communities and replacing them as it reproduces very quickly. The reproduction cycle of *U.pinnatiFIDa* is dependent on locations subjected to significant and consistent wave action. The *U.pinnatiFIDa*

population is increasing at a very fast pace in New Zealand (Russell, Hepburn, Hurd, & Stuart, 2008). Hence it becomes very important to study *U.pinnatiFIDa* and elucidate changes during the digestion; discovery of new products with less side effects from *U.pinnatiFIDa*, as it supposed to have beneficial health effect on humans.

U.pinnatiFIDa, on the basis of structure, is divided into blade and sporophyll as shown on Figure 1. Fucoidan extracted from sporophyll of New Zealand *Undaria* species has been shown to have high antioxidant activity by Fung, Hamid & Lu (2013).

Past studies have demonstrated that the month of harvest affects the composition of fucoidan in seaweeds. The *U.pinnatiFIDa* harvested in September is known to contain the highest amount of crude fucoidan. (Mak, Hamid, Liu, Lu, & White, 2013; Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010).

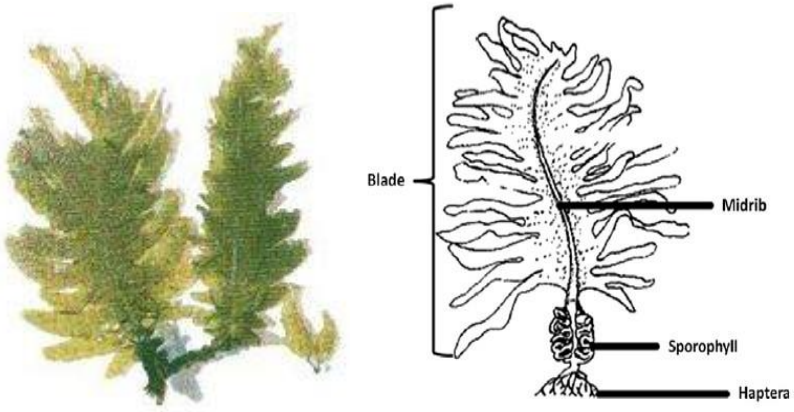


Figure 1. A diagram showing the structure of *U.pinnatiFIDa* (Adah, Hamid & Lu 2013).

The blade is lanceolate and broad with a prominent midrib, and translucent with color ranging from green to yellowish-brown to dark brown. The blade could also be described as triangular and lobed. The appearance of the blade evolves and changes over time; it is initially simple, flattened, and broad with a pronounced or distinct

midrib. The stipe of *U.pinnatiFIDa* is wavy or corrugated above the holdfast. The stipe is usually short (10 to 30 centimeters in length and up to 1 centimeter in diameter). In mature plants there are convoluted wing-like reproductive outgrowths or frills (sporophyll). The amount of crude fucoidan that could be extracted from *U.pinnatiFIDa* rose markedly from April to June–July (from 3.2 to 16.0% dry weight) as the plant matures (Skriptsova, Shevchenko, Zvyagintseva, & Imbs, 2010).

2.2 Fucoidan from seaweed

Fucoidan is a heterogeneous sulfated polysaccharide extracted from seaweeds (Colliec, Boisson-vidal, & Jozefonvicz, 1994), which is commonly found in brown seaweeds or marine invertebrates. Table 2 shows the fucoidan extract from different brown seaweeds.

Table 2. A table showing the fucoidan extract from different brown seaweeds

Brown seaweed	Chemical composition	References
Fucus vesiculosus	fucose, sulfate	Nishino, Nishioka, Ura, & Nagumo, 1994
Fucus evanescens	fucose:sulfate:acetate (1:1.23:0.36)	Cumashi et al., 2007
Fucus distichus	fucose:sulfate:acetate (1:1.21:0.08)	Cumashi et al., 2007
Fucus serratus	fucose:sulfate:acetate (1:1:0.1)	Synytsya et al., 2010
Lessonia vadosa	fucose:sulfate (1:1.12)	Chand á & Matsuhiro, 2008
Macrocytis pyrifera	fucose:galactose (18:1), sulfate	Ale, Mikkelsen & Meyer, 2011
Pelvetia wrightii	fucose:galactose (10:1), sulfate	Maruyama & Yamamoto, 1984
Undaria pinnatiFIDa	fucose:galactose (1:1.1), sulfate	Synytsya et al., 2010
Ascophyllum nodosum	fucose, xylose, GlcA (4.9:1:1.1), sulfate	Berteau & Mulloy, 2003
Padina pavonia	fucose, xylose, mannose, glucose, galactose, sulfate	Holtkamp, Kelly, Ulber, & Lang, 2009
Laminaria angustata	fucose:galactose:sulfate (9:1:9)	Maruyama & Yamamoto, 1984
Ecklonia kurome	fucose, galactose, mannose, xylose, GlcA, sulfate	Nishino, Nishioka, Ura, & Nagumo, 1994

Fucoidan is composed of α-1, 3 and α-1,4-linked L-fucose residues, with sulfated groups as major constituents along with small amounts of uronic acid and other sugars such as galactose, mannose, xylose and glucose (Table 3) (Li, Lu, Wei, & Zhao, 2008).

2.2.1 Structure of fucoidan

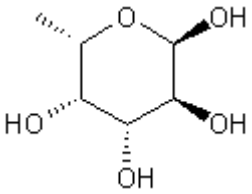
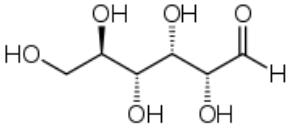
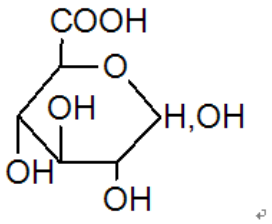
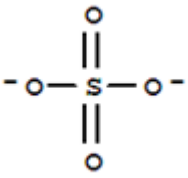
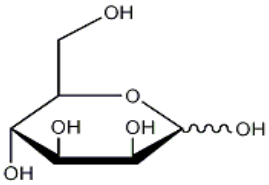
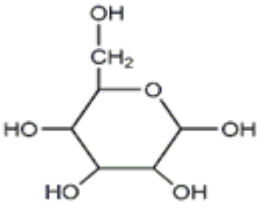
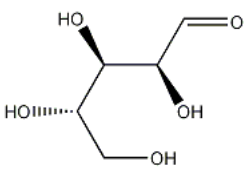
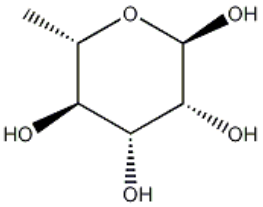
Fucoidan is over 100 kilodalton (KDa) large in average size. The degree of sulfation and the native structure of fucoidan cannot be identified directly because the polymer is combined by complex and volatile compounds. The structure of fucoidan elucidated so far can explain some of its important biological activities. A study by Percival and McDowell (1967) proposed that the core region of fucoidan is primarily made of α-l-3-linked fucose polymer. A revised structure of fucoidan is shown in figure 2.

Figure 2. Revised structure of fucoidan by Patankar (Patankar, Oehninger, Barnett,

Williams, & Clark, 1993) Fuc1-alpha-4 means α-1, 4-linked L- fucose.

But according to Côté (1959), Fucal-SFuc, Fucal-3Fuc, and Fucal-4Fuc disaccharides are obtained from acetolysates of fucoidan (Côté, 1959). Later on, Patankar et al. (1993) proposed that terminal fucose at branch points is attached via α-l→2 or α-l→4 linkages. That means the linkage of fucoidan is changeable which depends on the species of seaweed and the growing environment of seaweed (Holtkamp, Kelly, Ulber, & Lang, 2009). So there is still no specific model for the fucoidan and no detail structure information on the fucoidan extracted from *U.pinnatiFIDa*.

Table 3. A table showing the major constituents of fucoidan from *U.pinnatiFIDa.*

name	Structure	name	structure
L-fucose		Glucose	
Uronic acid		Sulfate	
Mannose		Galactose	
Xylose		Rhamnose	

2.2.2 Extraction of fucoidan from *U. pinnatiFIDa*.

The amount and composition of crude fucoidan depends on two crucial factors. Firstly, the part of the seaweed considered for extraction of fucoidan and secondly, the method of extraction involved (Mak, 2012). There are three types of extraction procedures depending on solvents (calcium chloride, hydrochloric acid and water) used for extraction.

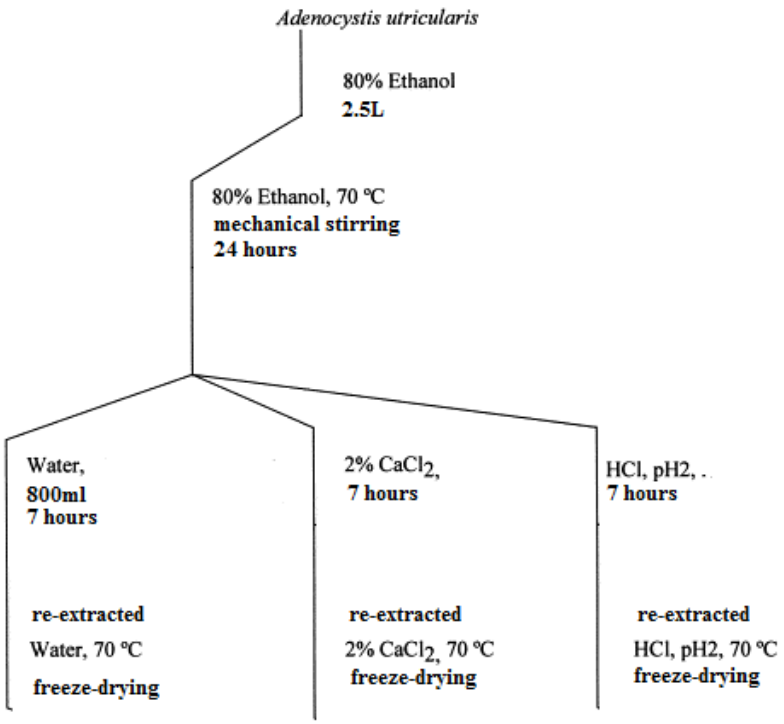


Figure 3. Three different extraction assays (Ponce, Pujol, Damonte, Flores, & Stortz, 2003)

According to figure 3, the dried seaweed is washed in 2.5 L 80% ethanol, at 70^{o}C with mechanical stirring for 24 hours. Then the samples were collected and moved to 800

mL water or 2% $CaCl_2$ solution or HCl (pH = 2) solution at 70^{o}C for 7 hours for extraction. After re-extraction, the sample were freeze-dried and stored at 4^{o}C.

Extraction with acidic solvents such as hydrochloric acid (HCl) gives higher yields of crude fucoidan but could also result in the extraction of undesirable products such as alginic acid and metals and may cause degradation of fucose chains.

Water, as a solvent, is considered the best for extraction of uncontaminated fucoidan. But this procedure does not give high yield of fucose and sulfate (Mak, 2012).

Table 4. A comparison of different constituent of fucoidan from different part and different procedure involved in extraction from *U.pinnatiFIDa* (Mak, 2012).

Extraction method	Fucose	Sulfate	Uronic acid	Protein
$CaCl_2$	16.37 ± 0.29	28.01 ± 0.09	4.91 ± 0.03	0.193 ± 0.001
Water	9.52 ± 0.25	27.88 ± 0.03	2.09 ± 0.12	0.092 ± 0.006
Acid	4.26 ± 0.31	22.09 ± 0.68	3.75 ± 0.04	0.161 ± 0.006

According to table 4, the yield of fucose, sulfate, uronic acid and proteins retrieved from the blade part of *U.pinnatiFIDa* by $CaCl_2$ extraction assay is higher than the other two assays. Hence the $CaCl_2$ extraction assay is the most efficient among the three assays.

2.2.3 Biological activities of fucoidan

Lately biological activities of fucoidan have been studied extensively. Fucoidan is therapeutically very important. The various biological properties of fucoidan that are of importance are anticoagulant activity, anti-proliferative activity, antitumor activity, anticancer activity, immunomodulatory activity, anti-inflammatory activity, and antiviral activity. The biological activities of fucoidan are dependent on various factors such as, the degree of sulfation, molecular mass of fucoidan, and molar ratio of fucose to sulfate (Wijesinghe & Jeon, 2012). The biological activities fucoidan are discussed in the following sections.

2.2.3.1 Anticoagulant activity

The potent anticoagulant also called antithrombotic action of fucoidan is by far the most widely studied in comparison to the other biological activities of fucoidan. Anticoagulants (antithrombotic) are classes of drugs that work to prevent the coagulation (clotting) of blood which can help prevent deep vein thrombosis, pulmonary embolism, myocardial infarction and ischemic stroke. Anticoagulant activity occurs naturally in leeches and blood-sucking insects including mosquitos (Ribeiro 1987).

The anticoagulant activity of fucoidan is closely related to the sulfate content and position. It is also dependent on molecular weight and composition of saccharides (Li, Lu, Wei, & Zhao, 2008). Fucoidan from *E. kurome* and *H. fusiforme*, which are naturally high in sulfate content, have high anticoagulant activity. This is further supported by chemical sulfation of native fucoidan to increase the overall sulfate content of fucoidan (Nishino, Nishioka, Ura, & Nagumo, 1994). Qiu et al (2006) reported that the chemically over-sulfated fceceucoidan showed four times higher anticoagulant activity. When fucans were chemically over-sulfated, activated partial

thromboplastin time (aPTT) and thrombin time (TT) have increased to 110–119% and 108–140% of the original values (Nishino & Nagumo, 1992).

However, the position of sulfate groups on the saccharide residues of fucoidan also has an influence on the anticoagulant activities. The activities are related to the concentrations of C-2 sulfate and C-2, 3 disulfated saccharide residues (Chevolot et al., 1999).

Anticoagulant activities of fucoidan are determined by the amount of sulfate and as well as the position of sulfate. Duarte, Cardoso, Noseda, & Cerezo. (2001) reported that the anticoagulant properties of fucoidan are mainly determined by the fucose sulfated chains, in particular, by the disulfated fucosyl units. Silva et al (2005) reported that 3-O-sulfation at C-3 of 1-4-α-L-fucose units was responsible for the anticoagulant activity of fucoidan from *Padina gymnospora*. However there are also some conflicting theories also. When the sulfate content of the fucans increases, the anticoagulant activities decrease (Dobashi, Nishino, Fujihara, & Nagumo, 1989; Nishino, Yokoyama, Dobashi, Fujihara, & Nagumo, 1989). Mourao (2004) disclosed that linear sulfated α-L-fucans and sulfated α-L-galactans are the critical components to anticoagulant activity and it depends critically on the pattern of sulfation and monosaccharide composition.

The molecular weight of fucoidan may differ in terms of extraction method, species and which part of the seaweed the fucoidan is extracted from. Fucoidan has to bind thrombin to express anticoagulant activity. Hence molecular weight must be large enough to achieve such bioactivity. Fucoidan from *Lessonia vadosa*, with molecular weight of 320kDa, showed good anticoagulant activity in its natural form (Chandía & Matsuhiro, 2008). However when depolymerized into 32kDa fractions, anticoagulant activity decreases (Chandía & Matsuhiro, 2008). Even a small decrease in the molecular size of the sulfate-fucose linkage compounds is able to reduce its anticoagulant activity. When fucoidan is hydrolyzed into smaller fractions via chemical modification, anticoagulant activities have also been shown. According to

Mauray et al. (1995), fucoidan with molecular weight of 20kDa, extracted from *Ascophyllum nodosum*, has shown anticoagulant properties. Millet et al. (2007) reported that low-molecular-weight (8 kDa) fucoidan obtained by acid hydrolysis from the high-molecular weight fucoidan, showed a significant anticoagulant effect on New Zealand rabbits.

Some studies have shown that sugar composition, including fucose, galactose, glucose, xylose, mannose and ribose, of fucoidan may be related to anticoagulant activity (Dobashi, Nishino, Fujihara, & Nagumo, 1989; Nishino, Yokoyama, Dobashi, Fujihara, & Nagumo, 1989). The influence of sugar composition on anticoagulant activity is of lesser importance than when compared to the content and position of sulfates. Uronic acid does not contribute towards anticoagulant activity, but it is able to enhance the anticoagulant activity by making the sugar chain more flexible (Li, Zhao, & Wei, 2008).

The mechanisms by which sulfated galactans contribute to anticoagulant activity investigated by Pereira, Melo, & Mourão (2002). Potentiation of plasma cofactors, which are the natural inhibitors of coagulation proteases lead the sulfated polysaccharides have the function of anticoagulant activity. As heparin is the most generally used anticoagulant medicine, a comparation was established in order to demonstrate the activity involve with the charge density of sulfate saccharide and the distribution of sulfate groups and on monosaccharide composition of naturally hexatomic polysaccharide contribute to the complex interaction. In order to achieve anticoagulant activity, the sulfated galactans require longer chain than heparin. The anticoagulant activity requires a molecular size of sulfated galactans over 45 kDa (Pereira, Melo, & Mourão, 2002). Due to fucoidan's anticoagulant property, it can be utilized for making anticoagulant drugs, or functional foods or as a medicinal material (Mauray et al., 1995).

2.2.3.2 Antiviral activity

Recent studies show that fucoidan has the potential to restrict viral activity. For decades, sulfated polysaccharides, including fucoidan, have been demonstrated to exhibit antiviral activities with *in vivo* and *in vitro* trials. Li & Yao (1994) reported that development of a cytopathic effect could be prevented by fucoidan extracted from *Laminaria japonica,* which in turn has significantly reduced the infections caused by *poliovirus III, adenovirus III, ECHO6 virus, coxsackie B3 virus* and *coxsackie A16*.

Ponce et al. (2003) examined the effect of content and position of sulfate in fucoidan and its antiviral activity. Hemmingson et al. (2006) summarized that galactofucan sulfates contribute to the antiviral activity. The lower uronic acid and/or higher sulfate fractions reduce the survival rate of virus and increase the antiviral activity significantly. The high sulfate fractions in fucoidan alone are not a good indicator of antiviral activity. Mandal et al. (2007) further showed the importance of the position of sulfates. Sulfate located at C-4 of (1→3)-linked fuco pyranosyl units have been shown to contribute to the anti-herpetic activity.

Many studies have indicated that herpes, which is an infection caused by a herpes simplex virus (HSV), can be treated by fucoidan from *Adenocytis utricularis* (Ponce et al., 2003), *U.pinnatiFIDa* (Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004), *Stoechospermum marginatum* (Adhikari et al., 2006), *U.pinnatiFIDa* (Hemmingson et al., 2006), *Cystoseira indica* (Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008) and *U.pinnatiFIDa* (Mandal et al., 2007). Lee et al. (2004) have shown to suppress the viral activities against HSV-1 and HSV-2 without being cytotoxicity on Vero cell cultures. The low cytotoxicity of fucoidan adds value to its potential as an antiviral treatment. Inhibitory actions against the replication of enveloped virus, such as human immunodeficiency and human cytomegalovirus, have been shown with

fucoidan from *Adenocystis utricularis* by Ponce et al. (2003). Yet, there is no evidence of a virucidal assay to show that fucoidan has a direct effect on deactivating virions.

According to Mandal et al. (2007), the antiviral mechanism of fucoidan is to inhibit viral sorption in order to inhibit viral-induced syncytium formation. Tobacco mosaic virus with added fucoidan showed agglutinated virions when observed on scanning electron microscope (Lapshina, Reunov, Nagorskaya, Zvyagintseva, & Shevchenko, 2006). Hayashi et al. (2008) proposed that antiviral activity of fucoidan was through direct inhibition of viral replication by oral administration. Oral administration of fucoidan stimulated both innate and adaptive immune defense functions.

2.2.3.3 Antitumor activity

Cancer is identified as a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body which is known as tumor or neoplasm. There are over 100 different cancers that affect human (Stewart, Kleihues, 2003).

Several studies reported that fucoidan from different seaweeds possessed antitumor properties (Itoh et al., 1992; Kwak, 2014; Maruyama & Yamamoto, 1984; Maruyama et al., 2002; Synytsya et al., 2010). Fucoidan has been reported to be able to inhibit breast cancer cells, prostate cancer cells, and lungs cancer cells. The anticancer mechanism of fucoidan has not been fully elucidated. The anticancer property of fucoidan is linked to its structure and sulfate content (Boo et al., 2013; Li, Lu, Wei, & Zhao, 2008; Shirahata, Zhang, Yoshida, Eto, & Teruya, 2013).

Alekseyenko et al. (2007) showed that low molecular-weight sulfate compounds of fucoidan have the antitumor activity on rodent model (2-3-month-old C57BL/6) with transplanted Lewis lung carcinoma. During the 20 days of treatment, the tumor weight was reduced by 10 % and the lungs were repaired by 10%.

In addition to directly inhibiting the growth of tumor cells, fucoidan can also restrain the development and diffusion of tumor cells by means of enhancing the body's immunomodulatory activities (Li, Lu, Wei, & Zhao, 2008)

2.2.3.4 Antioxidant activity

An antioxidant is a molecule that inhibits the oxidation of other molecules. Nearly all organisms are able to repair oxidative damage in their body and the negative effects that free radicals have on our bodies are well recognized. Antioxidants can delay or prevent the oxidative stresses applied on our organs (Mak, 2012).

Fucoidan extracted from seaweeds is a potential antioxidant, in the form of food additives to treat free radical-mediated disease (Sies, 1997). Fucoidan has been reported to have significant antioxidant activity in both *in vitro* and *in vivo* experiments. From the studies, fucoidan can act as an excellent natural antioxidant with no side effect.

Li et al (2002) indicated that the increase of lipid peroxide in serum, liver and spleen of diabetic mice could be prevented by fucoidan extracted from *L. japonica*. However the treatment of spontaneous lipid peroxidation of tissue homogenates caused by $FeSO_4$ *in vitro* with fucoidan showed no inhibition effect.

The low molecular weight and high sulfate content of fucoidan contribute to the anti-oxidant activity of fucoidan. Zhao et al. (2005) indicated that fucoidan from *L. japonica* contain highly sulfated fractions and had excellent scavenging capacities on the superoxide radical and hypochlorous acid. Li et al. (2006) reported that both molecular mass and sulfate content of fucoidan related to the effects on the azo radical's 2-2-Azobis (2-amidinopropane) dihydrochloride induced LDL oxidation. These studies express that the ratio of sulfate-fucose is an effective indicator to antioxidant activity.

2.2.3.5 Therapeutic potential in surgery

The low molecular weight fucoidan potentiates basic fibroblast growth factor activity, mobilizes the stromal cell-derived factor 1, and facilitates angiogenesis in a rat model. Schepetkin and Quinn (2006) and Luyt et al. (2003) suggested that fucoidan could be an alternative for conventional treatment in critical ischemia.

Fucoidan can reduce rat smooth muscle cell (SMC) proliferation *in vitro* in a more intensive manner than heparin. Low molecular weight fucoidan with high affinity for SMC and sustained plasma concentration markedly reduces intimal hyperplasia, suggesting its use for the prevention of human in-stent restenosis (Luyt et al., 2003)

Luyt et al. (2003) identified that the low molecular weight fucoidan potentiates the effect of FGF-2 on [3 H] thymidine uptake and obtained a promising new assay for identify the hind limb ischemia by the promotion of revascularization. And the fucoidan has no direct antithrombin effect which means it could be used as clinical application without hemorrhagic side effect.

2.3 The Human digestive system

The human digestive system involves various processes as intake of food, decomposition of the ingested food to nutrients, absorption of nutrients into the blood stream, as well as excretion of undigested fractions out of the body (Dorcas, Peterson, & Flint, 1997). The digestive system is made of the digestive tract and digestive glands. Digestive tract includes the mouth, pharynx, esophagus, stomach, small intestine (duodenum, jejunum, and ileum) and large intestine (caecum, colon, rectum and anal canal) (Furness & Costa, 1987). Five digestive glands (Thompson, Ratcliffe, & Bayne, 1974) include;

- Salivary glands are responsible for secretion of saliva. Salivary amylase breaks down starch into maltose in mouth.
- Gastric gland used for secretion of gastric juice. Gastric pepsin digests proteins to peptides in stomach.
- Liver secretes bile. Bile is stored in gallbladder and it is involved in breaking down lipids in small intestine.
- Pancreas secretes pancreatic juice which is used to digest proteins, lipids and carbohydrates in chyme.
- Intestinal glands responsible for secretion of intestinal juice to further digest proteins, lipids and carbohydrates into amino acids, glycerol and fatty acids, and glucose, respectively.

Most of the daily foods contain starch, protein and fats. The digestibility is described in brief as followed:

- Starch is initially digested to maltose by saliva in the mouth. In the small intestine by intestinal juice, and pancreatic juice further digest the starch into glucose; this was absorbed by the capillaries.

- Protein: is initially digested in the stomach by gastric juice to peptides. Intestinal juice and pancreatic juice further digest them to amino acids.

- Fat: some of the fat could be digested in oral part where some short chain lipids could be broken down to diglycerides by lingual lipase. The fat is mainly digested in the small intestine through the pancreatic lipase from the pancreas and bile from the liver contributes to the emulsification of fats for absorption fatty acids. One molecule of a triglyceride results in 3 fatty acid molecules and one glycerol molecule.

2.3.1 Oral cavity, pharynx and esophagus

The mouth, also known as oral cavity, is the opening of the respiratory and digestive tract, which is covered with a mucous membrane layer. Tongue and salivary glands are present in the mouth. The tongue is to taste the food and help stir saliva with food to form bolus. Food is cut up by incisors and chewed into smaller particles by molar teeth to assist digestion. Salivary gland secretes saliva, which contains digestive enzymes which include pepsin and lipase, to the particle surface to start the digestion. When not eating, the flow of saliva washes away the bacteria which can cause tooth decay, and inflammations. Saliva contains antibodies and enzymes, such as lysozyme, which can decompose protein and directly kill bacteria (Franceschi et al., 1990).

The esophagus is a long tube connecting the throat and stomach, which is covered with a mucous membrane layer. During swallowing, a small piece of muscle (epiglottis) is shut off to prevent food from entering the airway (trachea) through to the lungs, while the top part in mouth will rise (the soft palate) in order to prevent the food from entering the nasal cavity. The bolus is pushed down the esophagus, not by

gravity, but by a series of muscle contractions and relaxations which is called peristalsis.

When the bolus is swallowed and passed through the oesophagus, upper esophageal sphincter will open and a series of peristaltic movements will push the foods into the stomach (Mosher, 1927). The mucosal secretion grow on the wall of the oesophagus will lubricate and accelerate the transfer of bolus. Other than mucus secretion, there is no digestive secretion happening in the esophagus.

2.3.2 Stomach

Stomach is a large, broad bean shaped with muscular cavity which can be sub-divided into three parts: Cardia where the contents of the esophagus empty into the stomach; The gastric body: The main, central region of stomach; gastric antrum: the region of the stomach that connects to the duodenum. (Wilson, 2008). The bolus enters the stomach from esophagus when the sphincter is open. The sphincter is usually closed to prevent the stomach contents from refluxing into the esophagus. The shape and structure of human stomach is shown in figure 4.

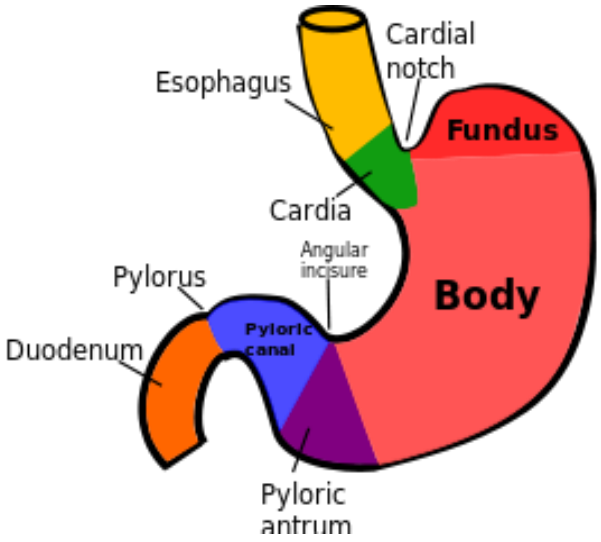


Figure 4. Stomach model of human digestion system (Moore, Dalley, & Agur, 2013)

Peristaltic motility in the stomach is composed of rhythmic contractions and relaxations. This keeps the bolus combined with the digestive enzymes (Freeman, 1981). The frequency of the contractions is approximately 3 cycles per min. 2 waves consist of a cycle of gastric peristalsis. The 1st cycle starts from the stomach wall and spread towards the antrum, mixing and forcing the antral contents toward the pylorus, the 2nd cycle works in the opposite direction (Kong & Singh, 2010). Stomach cells on the surface secrete mucus, hydrochloric acid and pepsinogen. Pepsinogen is a precursor of pepsin, activated by the acidic pH of hydrochloric acid. Mucus covers the surface of the stomach in order to protect it from damaging the wall by hydrochloric acid and enzyme (Park & Robinson, 1985).

Stomach juice, which is composed of hydrochloric acid, provides a highly acidic environment (Smith, 2003). Stomach acid can also destroy most bacteria and serve as a barrier against infection (Erickson, Medina, & Hubbard, 2000). But too much stomach acid may destroy the mucosal defense mechanisms and diseases as ulceration or indigestion. The regulation of stomach acid is accomplished by an interaction among hormonal, paracrine, and neural pathways (Schubert, 2008). The main hormone responsible for stimulating acid secretion is gastrin which is released by enterochromaffin-like cell and histamine transfer through the bloodstream (Wallmark, Larsson, & Humble, 1985). When the gastrin reaches the target, it will reduce the releasing of stomach acid in order to reduce the concentration of gastric juice. Pepsin is able to break down about 10% of proteins present in the food. Only a few substances such as alcohol and aspirin can be directly absorbed by the stomach, but only at a small amount (Charlton & Bothwell, 1983). All the mechanical and chemical processes in the stomach break down the food into chyme. The stomach also has the function to store and maintain the temperature of the yellow greenish chyme which contributes to the high effective absorption of the small intestine.

2.3.3 Small intestine

The small intestine acts as the main absorption organ of human body. Protein, fats, carbohydrates could be digested and absorbed in small intestine within 3-8 hours.

Stomach delivers food to the first section of the small intestine which is referred to as duodenum (Case, 1917). The amount of food which enters the duodenum through pyloric sphincter is controlled by hormones.

Gastrin and cholecystokinin are stimulant of intestine peristalsis. Glucagon, secretin and adrenaline will reduce the intestine peristalsis. If the duodenum is full and its wall stretched, the stomach will slow down its activity through a nerve impulse. Generally a well-balanced meal lasts four hours for the stomach to empty. Too much fat in the meal could take six or more hours for the stomach digestion.

The duodenum gets bile from liver and pancreatic enzymes from pancreas (Corazziari, Shaffer, Hogan, Sherman, & Toouli, 1999). The digestive juices travelling through the pyloric sphincter into the duodenum play an important role in digestion and absorption. Peristalsis stirs foods in the gut, mixes it with intestinal fluids and can also contribute to digestion and absorption of food (Minocha & Carroll, 2003).

The first 10cm of duodenal surface is smooth. The rest of the duodenum has creases, small protrusions and microvilli. They significantly increase the surface area of duodenum to enhance the absorption of nutrients (Minocha & Carroll, 2003).

The duodenum is connected to jejunum, which is connected to ileum. The former is primarily responsible for the absorption of fat and other nutrients (Nunez, Ayudarte, Morales, Suarez, & Gil, 1990). Similarly, intestinal folds, the villi and microvilli on the surface lead to the huge surface area and greatly enhance the absorption of nutrients. Small intestinal wall has rich blood supply. The blood capillaries transfers the nutrient absorbed in the intestine via the portal vein to the liver. The intestinal wall

secretes mucus to lubricate gut and this helps to dissolve nutrients. Small intestines also releases small amount of enzymes to digest the protein, sugar and fat.

The consistency of intestinal contents gradually changes with progression through the small intestine peristalsis. In the duodenum, duodenal juice is quickly pumped in order to dilute hydrochloric acid in gastric juice. The intestinal contents become thinner when they flow through the small intestine, due to the action of water, mucus bile and pancreatic enzyme (Kararli, 1995).

2.3.4 The pancreatic gland

The pancreatic fluid is secreted which contains digestive enzymes and transfer to the small intestine. These enzymes which include erepsin, maltase, lactase, sucrase contribute to the further breakdown of the carbohydrates, proteins and lipids in the chyme. Pancreas tissue includes two basic components: Pancreatic acini and hormone islet (Gold & Freedman, 1965). Trypsinogen is produced by the pancreas acini, then packed into the pancreatic duct through a variety of tubular and drains directly into the duodenum (Slack, 1995).

Pancreas is controlled by the hormones gastrin, cholecystokinin and secretin, which are hormones secreted by cells in the stomach and duodenum (Tortora & Derrickson, 2008).

Pancreatic enzymes can digest proteins, carbohydrates and fats (Ganong & Barrett, 2005; Liddle, Green, Conrad, & Williams, 1986). The protein enzymes are secreted in inactive form, which are only activated when they reach the lumen. The pancreas also secretes large amounts of bicarbonate, and this leads to a neutralization of hydrochloric acid in order to maintain the pH (Ganong & Barrett, 2005).

There are three kinds of pancreatic secretion of hormone: insulin, used to maintain the levels of blood sugar (glucose); glucagon, used to elevate blood sugar levels; and somatostatin, used to inhibit the release of the above two hormones (Zulewski et al., 2001).

2.3.5 Large intestine

The large intestine is made up of the ascending colon (right), transverse colon, descending colon (left) and the sigmoid colon, which is connected to rectum (Bjarnason, Hayllar, MacPherson, & Russell, 1993; Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). The appendix is a small tube, prominent in the ascending colon near the large intestine and small intestine connection parts. The large intestine also secretes mucus, and it is mainly responsible for the absorption of water and electrolyte in feces and urine. Digestion in the large intestine takes almost 16 hours. The pH of large intestine is 8.3-8.4.

Intestinal contents that reach the large intestine are in liquid form. Through absorption of water and electrolytes, solid feces are formed (Fordtran & Locklear, 1966). Numerous bacteria are present in the large intestine to further assist with digestion. It also helps with the absorption of nutrients. Colonic bacteria also produce some important materials, such as vitamin K. These bacteria are critical to healthy intestinal function.

2.3.6 Digestion of polysaccharides

Several complex carbohydrates are digestibilable (Grabitske & Slavin, 2008). They do comprise of important dietary elements for humans. Dietary fiber is a large polysaccharide; digestion among other benefits (Eastwood, 1992). Soluble fiber binds bile acids in the small intestine, making them less likely to enter into the body; this in

turn lowers cholesterol levels in the blood (Kumar et al., 2012). Soluble fiber also attenuates the absorption of sugar, reduces sugar response after eating, normalizes blood lipid levels and, once fermented in the colon, produces short-chain fatty acids as by-products with wide-ranging physiological activities. Insoluble fiber is associated with reduced diabetes risk, but the mechanism is not clear (Schröder, 2007).

2.3.7 Digestion of fucoidan

Fucoidan is referred to a type of "sulfate polysaccharide" (Berteau & Mulloy, 2003). The solubility of polysaccharide compound is reasonably high in water. Fucoidan has limited solubility in organic solvents such as methanol and ethanol (Fitton, 2011; B. Li et al., 2008). Fucoidan is found in various species of brown algae and brown seaweed such as mozuku, Kombu, bladderwrack, wakame, and hijiki (variant forms of fucoidan have also been found in animal species, including the sea cucumber). Fucoidan shows more complex and heterogeneous composition and structure than the related polymers found in marine invertebrates. For example, another polymer found in seaweeds called fucan-sulfate is composed of fucose and sulfate groups only (Balboa, Conde, Moure, Falqué & Domínguez, 2013; Pomin & Mourão, 2008).

The extraction of low weight molecular compounds can be achieved using water assay, calcium chloride assay, and acid assay. Hydrolytic, enzymes, ultrasound microwave, precipitation and purification are used during the extraction. This process is called fractionaion. The depolymerisation of crude fucoidan could be extracted through chemical, physical, enzymatic, and radical induced processes.

The reason fucoidan needs a fractionation is that the low molecular weight fractions are usually more biocompatible and active than the crude extract and preservation of the original structure and sulfation pattern is desirable (Anastyuk et al., 2012), however there is no consensus on the optimal molecular size nor definitive correlation between structures.

2.4 *In vitro* digestion system

In vitro digestion models refers to physicochemical models in lab settings, which is used to study the digestion process of food products, viability of probiotics and dissolution of drugs (Chen, Hébrard, Beyssac, Denis, & Subirade, 2010). Often these models are accompanied with chemical or physical analyses of samples at different digestion steps to study changes in compositions and concentrations of test materials. *In vitro* digestion model is divided into different sections, which is very similar to human digestion system. The different sections are mouth, and small intestine.

Table 5. The comparison between *in vivo* and *in vitro* digestion systems are as followed (SHIME, TIM, HGS) (Oomen et al., 2002)

Sections	*In vivo*	*In vitro*
Mouth	Foods are chewed and formed into a soft bolus that can be swallowed	Mostly omitted
Stomach	Meal affects secretion and gastric emptying	The ratios used during digestion are often greater than *in vivo*
Intestines	Absorption, motility and secretion are controlled	Omit absorption of nutrients in the intestines. Physical processing and temporal changes in luminal conditions are omitted

2.4.1 Static and dynamic *in vitro* digestion models

A static model is defined as a model in which digestion output is not removed during the digestion process and which does not mimic the physical processes that occur *in*

vivo (Amidon, Lennernäs, Shah, & Crison, 1995). Good static models are particularly useful where there is limited digestion, e.g. stomach, but are less applicable for total digestion studies. These types of models are predominately used for digestion studies on simple foods and on isolated or purified nutrients. Hence they are ideal for assessments of the digestibility of, for example, isolated allergenic proteins. Many of these models are quite crude and simply involve homogenization of the food, acidification with hydrochloric acid, addition of gastric enzymes followed by a varying delay simulating gastric residence time, neutralization with sodium carbonate or hydroxide and the addition of pancreatic enzymes and bile salts whilst stirring at 37^{o}C.

Dynamic models may or may not remove the products of digestion but have the advantage that they include the physical processing and time changes in luminal conditions that mimic conditions *in vivo* (Wickham, Faulks, & Mills, 2009). This is particularly useful where the physical condition of the digest changes over time, e.g., viscosity, particle size reduction. It also takes into account some time effects not otherwise considered, e.g., unstirred layers, diffusion, creation of colloidal phases, partitioning of nutrient between phases.

2.4.2 Available *in vitro* digestion models in the literature

Simulator of Human Intestinal Microbial Ecosystem (SHIME) has been developed by Molly et al (1994). The stimulating parameters include the body temperature, pH, salivary, gastric and intestinal mixing with peristaltic movements, secretions and absorption of water and small molecules.

2.4.2.1 SHIME

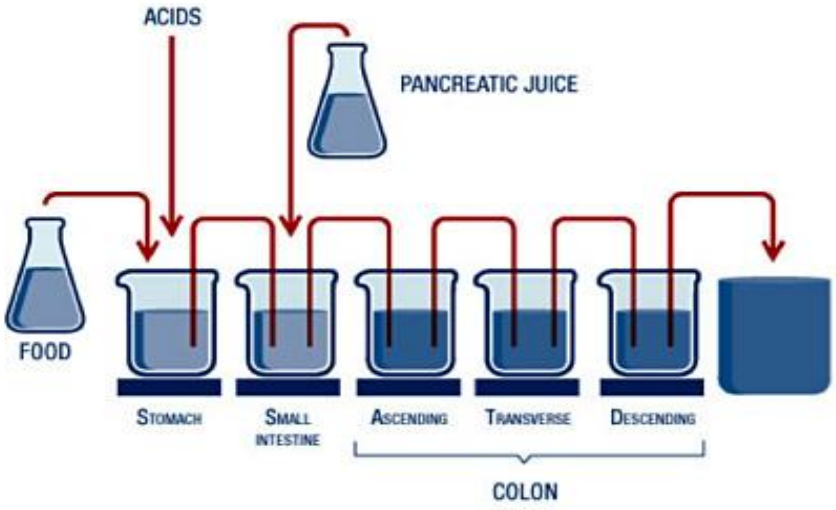


Figure 5. A pictorial description of SHIME (Oomen et al., 2002)

According to figure 5, the SHIME model has six computer controlled multi-chamber reactors in order to simulate the conditions of the GIT including duodenum, jejunum, ileum, caecum; ascending colon, transverse colon and descending colon. In SHIME the first two reactors are used to simulate food uptake and gastric digestion in the stomach and duodenum/jejunum. The colon microbiota is simulated through the last three reactors of the SHIME (De Boever et al, 2000).

The SHIME method can be applied for numerous studies, such as nutritional studies (Alander et al., 1999), pharmaceutical studies (Wedel, Quinlan, & Iaizzo 1995), general safety assessments and micro-ecological studies (De Boever, Deplancke, & Verstraete, 2000). One drawback of the SHIME model is it over-simplies the dynamic feature of the GIT. The buffering capacity and absorption mechanisms of metabolism are omitted (Yoo, 2009).

2.4.2.2 TNO (gastro-) Intestinal Models (TIM)

TNO (gastro-) Intestinal Models (TIM) is a validated and a relatively inexpensive *in vitro* model which does not involve intensive labor and ethical constraints. Results can be deduced even if there is small numbers of test and control products. Large samples analyse can be achieved in short periods with reasonable reproducibility and accuracy.

The TIM model is controlled by four computer control chambers. TIM is used to study the GIT of humans and monogastric animals (Kong & Singh, 2008).

The advantages of TIM include simulation of peristaltic movements, controlled squeezing, absorption of nutrients and water in the small intestine compartments and simulations of gastric emptying rates and intestinal transit times. Gastric, bile and pancreatic secretions are infused to control the pH, body temperature, concentrations of electrolytes, concentrations of enzymes and bile with physiological relevance.

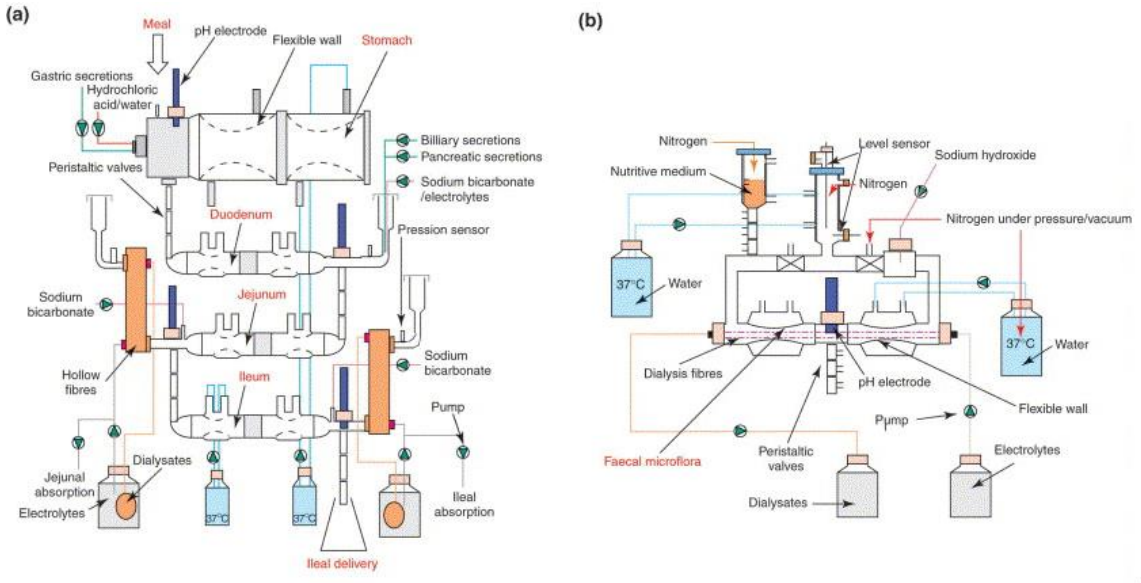


Figure 6. TIM model. TIM-1 (a) mainly simulates condition similar to stomach, duodenum, jejunum and ileum. TIM-2 (b) stimulates large intestines part also.

2.4.2.3 HGS

The Human Gastric Simulator (HGS) is developed to mimic the continuous peristaltic movement of stomach walls, with similar amplitude and frequency of contraction forces as *in vivo*.

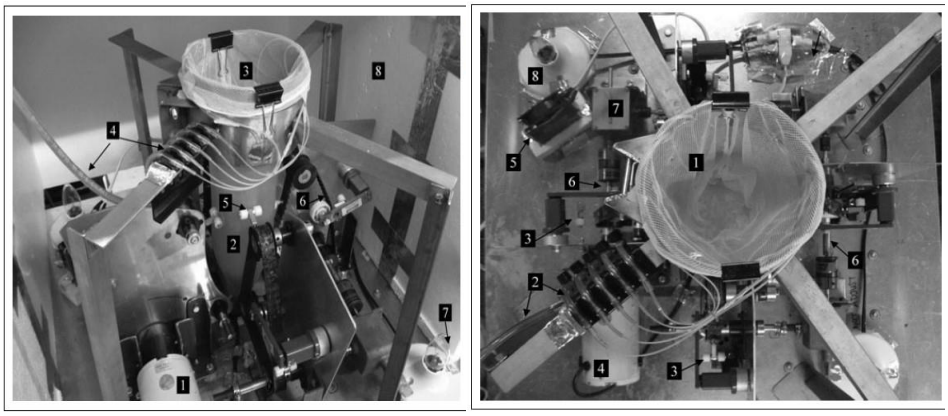


Figure 7. HGS model (Kong & Singh, 2010). (1) Motor; (2) latex lining; (3) mesh bag; (4) secretion tubing; (5) roller; (6) belt; (7) light bulb for temperature control; (8) plastic foam insulation.

The HGS mainly contains a latex lining chamber to mimic stomach chamber, 12 rollers secured on belts are driven by a motor assembly, gastric secretion and emptying systems and temperature control in order to push the stomach walls. This system simulates a pattern of mechanical forces and make HGS model an excellent simulation of digestion process which is similar to *in vivo* (Kong & Singh, 2010). The HGS provides a detailed investigation of the changes in physical and chemical properties of ingested foods. But the gastric reactor size of HGS (5.7 L) is bigger than human stomach (1-1.6 L) (De Bolós, Garrido, & Real, 1995).

Table 6. A table summarizing the different characteristics of *in vitro* digestion models.

		SHIME	TIM	HGS
	Static/Dynamic	static	dynamic	dynamic
General	Shape and container	Glass beaker	Glass jacket with flexible inner wall	Round Cylindrical
	Temperature	37 ℃	37 ℃	37 ℃
	Sequential	no	yes	yes

	control of pH			
	Anaerobic	yes	yes	no
Oral cavity	Saliva	no	yes	yes
	volume of saliva	flowing	50 mL	20 mL
	pH	6.9	5.0	
	Motility	Mechanical stirring, 150rpm	Peristaltic movement	Peristaltic movement
	Transit time	15 min	5 min	2 min
Stomach	Volume of gastric juice	25 mL	250 mL	70 mL
	pH	4.0	5.0 initially then decrease with time	4.27
	Motility	Mechanical stirring	Peristaltic movement	Peristaltic movement
	Transit time	3 h	depend on content	2 h

Chapter 3 Materials and methods

3.1 Extraction of fucoidan from *U.pinnatiFIDa*

Following steps were involved in the extraction of fucoidan from *U.pinnatiFIDa*

The collected sample (*U.pinnatiFIDa*) was washed with distilled water. Then the washed sample was dried at 60℃ for 7-12 h in an oven (Sanyo Convection Oven MOV-112F). The sample was crushed by mortar and pestle for 10 min to obtain powder (Prabhasankar et al., 2009). 20 g of the powdered sample was taken and refluxed with 200 mL of 80% v/v ethanol (BDH Laboratories) at 75℃ for 1h to remove lipophilic pigments (chlorophylls, fucoxanthin and carotenes) and low molecular weight proteins.

The sample was centrifuged (Eppendorf Centrifuge 5810R V3.1) at 1800 g for 10 min and dried at room temperature in lab for 3hours. 10g of the dried biomass was extracted with 100 mL of 0.2 M HCl (BDH Laboratories) at 60℃ with constant high speed stirring by the vortex (SCHASS 213) for 1 h.

The extraction was centrifuged at 5000 g for 10 min and the supernatant was collected. 400 mL of 99% v/v Ethanol (BDH Laboratories) was added into the supernatant to obtain the final ethanol concentration of 75% v/v, and the solution was stored at 4℃ for 7-14 h. Sample was centrifuged again at 5000 g for 10 min, and the precipitate was collected (crude fucoidan), and washed with 75% v/v ethanol (BDH Laboratories) and acetone (BDH Laboratories). The sample was dried at room temperature in the lab overnight and stored in a sealed container for further analysis.

3.2 Static *In vitro* Digestion Protocol (SIVDP)

The static *in vitro* digestion protocol (SIVDP) utilized in this study was modified according to Hur, Lee and Lee 2015 (Hur et al., 2015). The digestion process is shown in Figure 8. In SIVDP, the digestion process occupying in oral and gastric process occur in the same reactor.

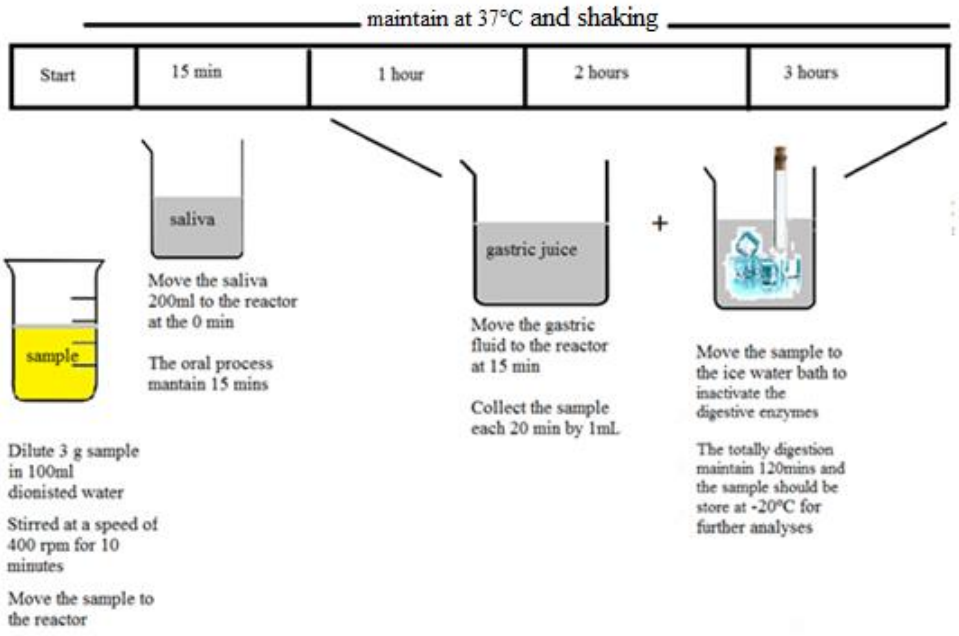


Figure 8. Schematic diagram of Static *in vitro* digestion model.

3.2.1 Oral digestion process

Artificial saliva was obtained by mixing 2.00 ± 0.02g α-amylase powder (*Aspergillus oryzae* SIGMA-A3176, SIGMA) in 200 mL of distilled water (Yoo, 2009). The pH of the saliva was 7.0 ± 0.1.

A 3.0 ± 0.1g dried sample from extracted crude fucoidan was weighed and dissolved in 100 mL deionized water mixed with 60 mL artificial saliva were swirled (150 rpm) in a 500 mL glass bottle with glass lid and it was placed in a water bath which has

shaking function (GYROTORY water bath SHARKER Model G76) to simulate and mimic the chewing process.

The oral digestion in the mouth reactor runs for 15 min. Every 5 min, 2 mL of sample was collected in a test tube for further analysis. A pH meter and a thermometer was used to control and monitor the pH and temperature of 37 ℃ in order to keep the activity of enzyme.

Each sample collected was placed in an ice water bath for 15 min in order to stop further enzymatic reaction. And then the samples were stored at -20 ℃ for further analysis.

3.2.2 Gastric digestion process

The gastric fluid was prepared according to Hur, Lee & Lee (2015). The pH of the gastric fluid was 1.5 ± 0.5. The composition is given in Table 7.

120 mL of gastric fluid were mixed with digests and it was swirled (60 rpm) in a 500 mL glass bottle which was placed in a shaking water bath (GYROTORY water bath SHARKER Model G76) to simulate the motility of the GIT for 2h at 37 ℃. The gastric process maintains 105 min and sample of 2 mL was collected for every 20 min during the stomach process. A pH meter and thermometer was used to monitor and detect the pH value and temperature of 37 ℃ in order to keep the activity of enzyme. The sample was immediately treated to inactivate the digestive enzymes by placing it in an ice bath for 15 min. The supernatant was stored at -20 ℃ for further analyses.

3.2.3 Control experiment

Fucoidan, approximately 3g was dissolved in 0.15 N HCl (BDH Laboratories) to make up a solution of 100 mL. This was maintained at 24 ℃ and to this solution, no

digestive enzyme remained. This acts as a control for the digestibility study. Afterwards the control sample was placed in the glass reactor at 37 ℃ and it rotated in a shaking water bath for 180 min.

Table 7. Chemical composition of gastric juice

Chemical	Amount
KCl	2.2 g/L
HCl	0.15 N
Pepsin (Pepsin from porcine gastric mucosa, SIGMA-P6887)	1 g/L
NaCl	5 g/L
CaCl2	0.22 g/L

3.3 Dynamic *in vitro* digestion protocol

The dynamic *in vitro* digestion model was adopted from Yoo (2009) and is pictorially described in figure 9.

Artificial saliva and gastric juice were prepared as described below.

2.00 ± 0.02 g of α-amylase powder (A3176, SIGMA) was mixed with 200 mL of deionized distilled water to obtain artificial saliva, and the pH maintained at 6.9. Artificial gastric juice was prepared as mentioned in table 7.

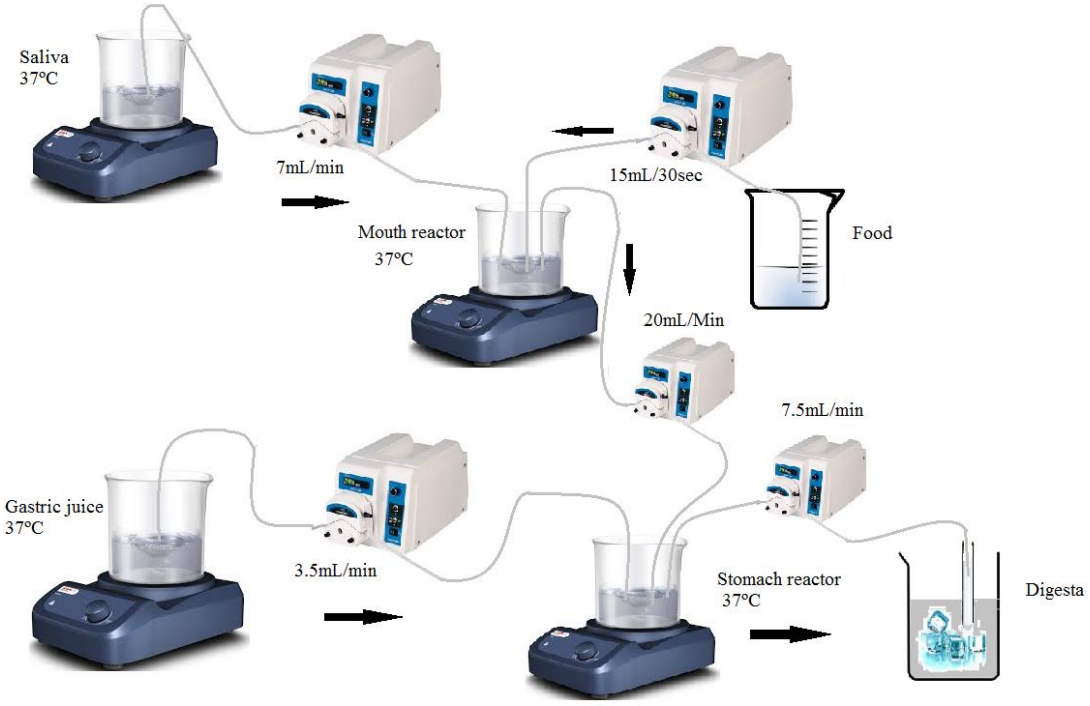


Figure 9. Schematic diagram of Dynamic *in vitro* digestion protocol

3.3.1 Oral digestion process

3g fucoidan was dissolved in 100 mL of water and was placed in a beaker and stirred at 400 rpm for 10 min. Artificial saliva was placed in a 500 mL beaker and the temperature maintained at 37 ± 0.5 ℃ on a hot plate magnetic stirrer (Microspin, PTFE) and stirred at 150 rpm. The saliva solution was continuously delivered into the oral reactor through silicon peristalsis tubing with 0.6 mm internal diameter (RS component Ltd) by using a pump (505U, Watson Marlow) at a rate of 7.0 mL/min, the temperature was maintained at 37 ± 0.5 ℃

In the mouth part, a homogenizer (L5M-A Laboratory Mixer, Silverson®) was used to mimic the mechanical grinding by teeth and to reduce potential blockage during the sample delivery in the peristaltic pump tubings.

At the beginning of the digestion process, 10 mL of fucoidan solution (37℃) was injected into the mouth reactor, every 30 seconds for 5 min. The oral part of digestion which occurs in mouth part of reactor takes 15 min. Samples of 1 mL was collected and placed in a test tube every 5 min during the oral process. A pH meter and thermometer was used to control and detect the pH value and temperature of 37 ℃ in order to keep the activity of enzyme.

Each sample collected was placed in ice water bath for 15 min, in order to stop further enzymatic reaction and stored at -20 ℃ for further analyses.

3.3.2 Gastric digestion process

A 1 L beaker was equppied with a 4cm x 0.5cm diameter stir bar at a speed of 150 rpm to mimic the gastric motility at 37 ± 1 ℃. The beaker was covered by a disc of polystyrene; with the thickness of 2.0 ± 0.1 cm and holes for maintaining the pH and thermcouples (0.6 mm ID, RS component Ltd.).

The gastric secretion was maintained at 37 ± 1 ℃ in a container and delivered to the stomach reactor via a peristaltic pump (505U, Watson Marlow) at a flow rate of 3.5 mL/min to simulate the gastric phase (Mainville et al., 2005).

A peristaltic pump (MUD01, Major Science) was used to connect the mouth part with the stomach part of the static *in vitro* digestion model. 20 mL/min of above mixture was pumped out from the mouth into the stomach. The temperature was maintained at 37 ± 0.1 ℃.

1 mL of digest was collected every 20 min. The total collecting time was 3 hours. The collected samples were placed in an ice water bath to inactivate the digestive enzymes.

3.3.3 Control experiment

Fucoidan, around 3g was dissolved in 0.15 N HCl to make up a solution of 100 mL. This was maintained at 24 ℃ and it was without any digestive enzyme. This acted as a control for the digestibility study. Afterwards the control sample was placed in the mouth reactor at 37 ℃ and ran through the whole dynamic *in vitro* digestion system as showed in 3.3.

3.4. Analysis of samples

A total of 12 samples, 1 mL each were collected from each of the *in vitro* digestion method. Collected samples analyzed by phenol-sulfuric acid assay, Somogyi Nelson method, Gas Chromatography-Flame Ionization detector assay, Millipore separation assay, FT-IR assay, sulfate assay, uranic acid assay and total soluble carbohydrate assay.

3.4.1 Phenol sulfuric acid assay

The phenol sulfuric acid assay was used to identify the amount of total water-soluble carbohydrates in order to indicate whether in soluble carbohydrates exist during digestion (Dubois et al., 1956).

A standard curve was made by D-glucose. 100 mg D-glucose (Fisher chemicals) was weighed and dissolved in 10 mL of deionized water to make a glucose stock solution (10 mg/mL). A stock solution was prepared and from it the following working concentrations were prepared, 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL and 0.10 mg/mL. 3 mL of concentrated sulfuric acid was added to 1 mL of diluted

sample. Glucose standards were prepared for calibration. Addition of sulfuric acid was followed by vigorous shaking at high speed in a vortex mixer.

50 µL of 80% (w/w) phenol (Fisher BioReagents) was pipetted into the mixture and maintained at 90℃ for 10 min in a water bath. The mixture was vortex at high speed for 30 seconds and left at room temperature for 5 min to cool down. The absorbance was read using UV spectrophotometer (Ultraspec 7000, Biochrom Ltd) at 490 nm.

3.4.2 Somogyi Nelson Method

Somogyi-Nelson method is an extensively used highly accurate method for determining the amount of reducing sugars (Hatanaka & Kobara, 1980; Yoo, 2009).

12 g of sodium potassium tartrate and 24 g of anhydrous sodium carbonate was weighed and mixed with 250 mL of distilled water. 4 g of copper sulfate pentahydrate and 16 g of sodium bicarbonate was weighed and added to 200 mL of distilled water. 180 g of anhydrous sodium sulfate was prepared and mixed in 500 mL of boiling distilled water.

The prepared solutions were combined and diluted to 1 L to make a low alkalinity copper reagent. The arsenomolybdate reagent was made by mixing 25 g of ammonium molybdate to 450 mL of distilled water.

21 mL of concentrated sulfuric acid and 25 mL of distilled water containing 3 g of disodium hydrogen arsenate heptahydrate was mixed with the ammonium molybdate solution with stirring. Continuously stir the mixture for 24 h at 37 ℃ by stirrer at 150 rpm and stored in a brown glass bottle stand to use.

1 mL of the diluted (factor of 100) sample and 1 mL of the low alkalinity copper reagent was pipetted in a test tube and vigorously mixed by a vortex mixer at a high speed for 30 s. The test tube was placed in a boiling water bath (100 ℃) for 10min, and cool at room temperature for 5min. 1 mL of the arsenomolybdate reagent was added and the mixture was vortexes at a high speed for 30s. Read absorbance of sample at 500 nm. 1 mL of distilled water acted as blank.

3.4.3 Uronic acid assay

3.4.3.1 Materials

Sodium tetraborate (0.025 M) solution (sulfuric acid reagent), 1.016g 99% Sodium tetraborate ($Na_2B_4O_7$: MW 201.22 g/mol, Sigma-Aldrich) were dissolved in concentrated sulfuric acid (H_2SO_4) with constant stirring and vortexing.

0.05 g Carbazole (Sigma-Aldrich - $C_{12}H_9N$, MW: 167.21 g/mol) was dissolved in 40 mL of absolute ethanol in a brown glass bottle and stored at 4 ℃. Benzoic acid was added to 200 mL deionized water till precipitation starts, the amount of benzoic acid should be 0.58 g/mL and maintained the solution at 25 ℃. 2 mg of fucoidan or sample was dissolved in 0.4 mL of benzoic acid saturated deionized water in a 1.5 mL Eppendorf tube

D-glucuronic acid standard stock solution (100 μg/mL), 1 mg D-glucuronic acid ($C_6H_{10}O_7$ MW: 194.14 g/mol) was added in 10 mL benzoic acid saturated deionized water.

3.4.3.2 Method

In order to prepare the standard curve, 4 mL of uronic acid (D-glucuronic acid) standards (0.5, 1, 5, 10, 20, 30, 40 and 50 μg/mL, 8 standard solutions) were prepared, in a 10 mL centrifuge tube.

Triplicates of 6.5 mg of each sample were weighed and dissolved in 0.4 mL saturated benzoic acid solution in a 1.5 mL Eppendorf tube. 50 µL of the sample were dispensed into heat resistant 96 well plates. 200 µL of sodium tetraborate (0.025 M) solution was then added and gently mixed with a pipette tip. The plate was heated for 10 min at 100 ℃ in an oven. Then samples were cooled at room temperature for 15 min. 50 µL of 0.125% carbazole solution was moved to the oven for another 10 min at 100 ℃. Samples were cooled at room temperature for 15 min then the wavelength was read at 550 nm (Cesaretti, Luppi, Maccari, & Volpi, 2003).

3.4.4 Sulfate assay

3.4.4.1 Materials

Gelatin solution, 1 g of gelatin (Sigma-Aldrich) was dissolved in 200 mL of deionized water and heated at 60-70 ℃ and then stored at 4 ℃ 7- 12 h overnight. Barium chloride – gelatin reagent, 1 g of $BaCl_2$ (Sigma-Aldrich) was dissolved in 200 mL of gelatin solution and maintained for 2-3 h. K_2SO_4 stock solution (1 g/L), 90.625 mg of K_2SO_4 were dissolved in 50 mL of deionized water in a centrifuge tube (50 mL). 3% (w/v) trichloroacetic acid (TCA) (Sigma-Aldrich), 6 g of TCA powder/crystal was dissolved in 200 mL of deionized water in a glass bottle.

3.4.4.2 Method

In order to prepare the standard curve, K_2SO_4 (174 g/mol) standards (5, 25, 50, 100, 150, 200 µg/mL) were prepared.

5mg of each sample was weighed into glass ampoules. 1 mL of 1 M HCl was added to ampoule. Then the samples were sealed with an oxy-gas flame. Then the ampoules were then incubated for 16 hours at 105 ℃.

40 μL samples were transferred from each ampoule to 1.5 Eppendorf tube. 760 μL of TCA (3% w/v) and 200 μL of $BaCl_2$ – gelatin was added reagent to make 1 mL total volume. The mixture was left at room temperature for 10 min. 250 μL of all sample, blanks and standards were pipetted in duplicates into 96 welled plates. Duplicates of standards, as well as a blank were pipetted into the 96-well plate. The wavelength of sample was read at 420 nm (Mak, 2012) and within an hour of sample preparation (Dodgson, 1961).

3.4.5 Infrared Radiation (IR) assay

In this study, the level of sulfate and hydroxyl were used to identify the digestibility of fucoidan through *in vitro* digestion(Alexander et al,1999).

Samples of 5 mL was pipetted in centrifuge tube (15 mL) and centrifuged (400e, Labofuge) at 4000 rpm for 20 min. Supernatant liquid was removed into another centrifuge tube and freeze dried using a Christ LOC-1M freeze dryer for 10 h. Freeze dried powder was collected and stored at 4 ℃.

The samples were placed in sample compartment of infrared spectrophotometer (Nicolet was 10 FT-IR spectrophotometer, Thermo Scientific, USA). The spectra of the sample were recorded on the computer. The wave length of sample range was used from 4000 cm-1 to 525 cm-1 accoridng to FT-IR Raman of Sigma-Aldrich. The crude fucoidan powder was also examined as control experiment.

3.4.6 Gas Chromatography- Flame ionization detector assay

3.4.6.1 Preparation of sugar standards: erythritol, fucose, galactose, xylose, mannose, glucose, erythritol.

20mg of the dried standards was weighted and placed into individual vials and 1 mL Milli-Q purified was added water to each vial. 0.5 mL solution from each monosaccharide solutions was collected and moved into a test tube. The vials of sugar standards were stored at – 20 ℃ for anti-bacteria and made a less noisy blank standard curve.

Table 8. A retention time table of the 6 sugars. These sugars were selected as they were the main monosaccharides found in fucoidan.

Name	Retention. Time min
Erythritol	7.393
Rhamnose	9.743
Fucose	9.336
Xylose	10.06
Mannose	12.874
Glucose	13.279
Galactose	13.745

3.4.6.2 Preparation of crude fucoidan for GC FID analysis

Fucoidan was hydrolyzed and digestd in 0.5 mL of 2 M trifluoroacetic acid (BDH Laboratories) at 121℃ in sealed glass tubes flushed with nitrogen (BDH Laboratories).

After cooling, 25 µL of 20mg/mL erythritol (BDH Laboratories) was added as the internal reference. Then filtered through a 0.2 µm Phenex-RC syringe filter into clean glass tubes and evaporate to dryness with stream of filtered air.

3.4.6.3 Reduction of monosaccharides to corresponding alditols

40 µL aqueous samples were placed in a 4 mL vial. 8 µL of 15 M ammonia (BDH Laboratories) was added to each tube. 400 µL of 0.5 M sodium borohydride (BDH Laboratories) was added in DMSO (BDH Laboratories) to each tube. The tubes was capped and vortexed to mix.

The sample was incubated for 90 min at 40 ℃. Using either a heating block or a water bath for incubation. 40 µL of 18 M acetic acid (BDH Laboratories) was added to each tube. Vortexed to mix. The mixture should effervesce as sodium borohydride was removed.

3.4.6.4 Acetylate the alditols

The GC-FID cannot identify the peaks of sugars directly but the sugar-alditols. Acetylate process was necessary for the GC treatment.

80 µL of 1-methylimidazole (BDH Laboratories) was added. The 1-methylimidazole was stored over silica gel at 4 ℃.

800 µL acetic anhydride (BDH Laboratories) was added to each tube and vortex to mix. The sample was incubated for 10 min at room temperature. 2 mL milliQ water (BDH Laboratories) was added to each tube to destroy the excess acetic anhydride. Vortex to mix. The sample was incubated for 10 min at room temperature or until cool. 400 µL dichloromethane (DCM) was added to extract the alditol acetates. Vortex to

mix (the tubes can not be capped, as the glue holding Teflon liners is soluble in DCM).

The phase was allowed to separate to the DCM phase (lower part) was transfeered to a clean 2 mL glass vial using a glass Pasteur pipette. This process was done by first expelling the air from the Pasteur pipette and then lowering it through the aqueous phase and into the DCM phase. Care was taken to ensure that very little or preferably none of the aqueous phase enters the pipette. (The DCM is volatileand tends to squirt out of the pipette; hold both tubes together in one hand so that it was possible to transfer the DCM quickly without losses.)

Another 400 μL of DCM was mixed into the original solution (aqueous phase) and the extraction process was repeated. If necessary, the sample was centrifuged at slow speed for 2 min to separate the phases. Remove the lower DCM phase and combined the DCM extracts.

1 mL milliQ water and DCM extract and were mixed. Upper aqueous phase was removed and discard. Washing procedures were repeated for additional three times. After the last wash, it was centrifuged and the lower DCM phase was collected to a clean glass vial (caps should be Teflon-lined).

The DCM was evaporated to using a dryness centrifugal concentrator and 500 μL of DCM was added. The sample was then analysed by GC-FID (Melton & Smith, 2001).

Alditol acetates were analyzed by GC (GC-2010, Shimadzu) using a ZB-5 capillary column (30 m × 0.25 mm) coupled to a flame ionization detector. The detector temperature was held at 280 ℃ while the injector temperature was set at 250 ℃. Nitrogen was used as the carrier gas at a flow rate of 1.5 mL/min and a split ratio of 10. The oven temperature was set at 40 ℃ for 1 min, increased to 240 ℃ at 50 ℃ /min, further increased to 250 ℃ at 2 ℃ min, and then held for 5 min at the final temperature.

3.5 Digestibility flow chart

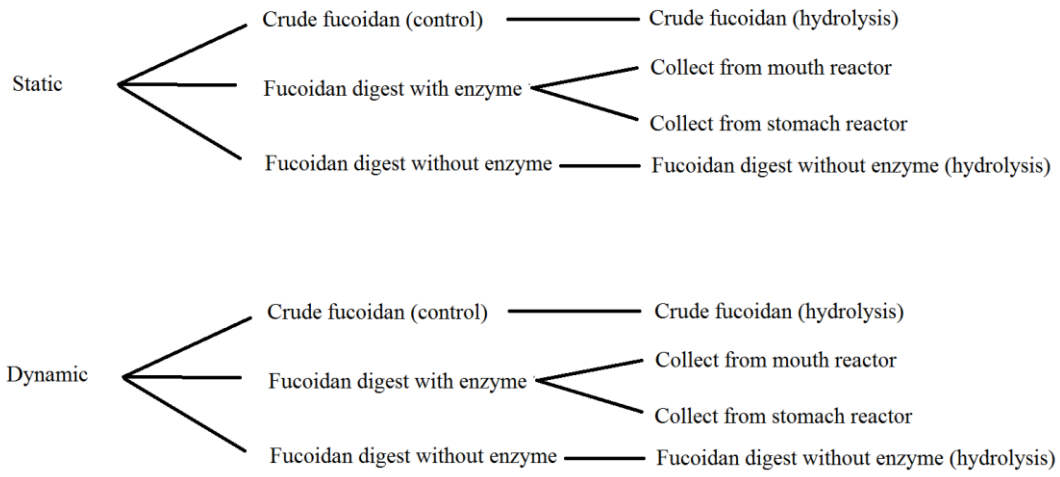


Figure 10. Flow Chart

According to figure 10, samples were collected during the sample preparation and digestion. The sample prepared in different conditions were used for eliminate the interference from samples and digestion models and determine the digestibility of fucoidan.

3.6 Statistical Analysis

Analysis on digested samples and controls was conducted at least in triplicates. Results were expressed by means of values and standard deviation of three separate determinations. One way analysis of variance (ANOVA) was examined for significance. All statistical analyses were performed using XLSTAT-MX version 2012.4.02 (Addinsoft, New York, NY, USA).

Chapter 4 Results and discussion

In this chapter, the digestibility of fucoidan in terms of total carbohydrates and reducing sugars is discussed firstly. This is then followed by a discussion on the changes of concentrations of L-fucose, sulfate compounds and uronic acid from digested fucoidan through UV spectrophotometer assays. And the last part is focused on the discussion of changes in concentrations of monosaccharides from digested fucoidan.

4.1 Total water soluble carbohydrates and reducing sugars in samples

Total water soluble carbohydrates and reducing sugars were estimated using the phenol-sulfuric acid assay and Somogyi-Nelson Method. The residual enzyme from fucoidan extraction, mainly in the form of enzymes added during the *in vitro* digestion process, may influence the results (Hatanaka & Kobara, 1980). The digested samples were treated with ice-water bath in order to inactivate the digestive enzymes.

Fucoidan is a water soluble polymer with low concentration (about 2-10%) of monosaccharides including the reducing sugars (fucose, galactose and glucose) (Hatanaka & Kobara, 1980). The change in the concentrations of reducing sugars can be interpreted as break down of the polymer to smaller units such as glucose and galactose. The concentration of total water soluble carbohydrates indicates the availability of total water soluble proportion of the sample at the time of measurement. Because fucoidan is a water soluble carbohydrate, any loss of sample should be monitored throughout the digestion process.

Table 9. A table showing soluble carbohydrates results obtained from the phenol-sulfuric acid assay with fucoidan samples digested with digestive enzymes and samples digested without digestive enzymes for 3 h of digestion period in the dynamic model.

Time (hour)	Concentration of total water soluble carbohydrates without enzyme (w/w %)	Concentration of total water soluble carbohydrates with enzyme (w/w %)
0	77 ± 3.3	77 ± 3.3
1	76 ± 3.2	80 ± 4.8
2	80 ± 2.4	78 ± 2.5
3	78 ± 4.3	80 ± 2.1

Table 10. A table showing soluble carbohydrates results obtained from the phenol-sulfuric acid assay with fucoidan samples digested with digestive enzymes and samples digested without digestive enzymes for 3 h of digestion period in the static model.

Time (hour)	Concentration of total water soluble carbohydrates without enzyme (w/w %)	Concentration of total water soluble carbohydrates with enzyme (w/w %)
0	82 ± 2.1	82 ± 2.1
1	80 ± 1.0	74 ± 4.4
2	79 ± 2.3	79 ± 1.0
3	77 ± 3.1	80 ± 1.0

The concentration of total water soluble carbohydrates of crude fucoian taken before

any chemical treatment or digestion and those from the digested samples at each hour are summarised in Table 9 and Table 10. There was no significant difference found, which indicates that throughout the digestion process, either with digestive enzymes or without digestive enzymes, no significant loss of sample took place in both static digestve and dynamic digestive systems.

Table 11. Results of reducing sugars obtained from the Somogyi-Nelson method in dynamic digestive system.

Time (hour)	Concentration of reducing sugars without enzyme (w/w %)	Concentration of reducing sugars with enzyme (w/w %)
0	12 ± 3.3^{a}	12 ± 3.3^{a}
1	12 ± 2.1^{a}	20 ± 1.1^{b}
2	14 ± 2.0^{a}	26 ± 0.8^{b}
3	15 ± 4.2^{a}	25 ± 0.56^{b}

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

Table 12. Results of reducing sugars obtained from the Somogyi-Nelson method in static digestive system.

Time (hour)	Concentration of reducing sugars without enzyme (w/w %)	Concentration of reducing sugars with enzyme (w/w %)
0	15 ± 2.1^{a}	15 ± 2.1^{a}
1	15 ± 1.1^{a}	19 ± 3.2^{b}
2	15 ± 1.2^{a}	27 ± 0.2^{b}
3	15 ± 1.1^{a}	27 ± 1.7^{b}

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

According to the tables 11 and 12, there was a significant increase on the concentrations of reducing sugars in digested fucoidan with digestive enzymes compared with the samples from digestion without enzymes in both dynamic and static digestive models. The concentrations of reducing sugars from samples collected from digestion without digestive enzymes remained the same throughout the 3 hr trial. The values were not significantly different from one another when compared to the initial crude fucoidan sample, and also when compared at different time points.

However the digested samples with digestive enzymes have shown significant differences from the initial crude fucoidan sample which means break down of the fucoidan polymer was related to the digestive enzymes. From the results, reducing sugars including rhamnose, fucose, xylose, mannose, glucose and galactose may have been released from the fucoidan polymer. And this was further examined through GC-FID and shown in section 4.3.

The increase in the level of reducing sugars through simulated digestion was

consistent with the study by Hu, Nie, Min and Xie (2013). A non-starch polysaccharide from the seeds of *Plantago asiatica* was digested with artificial saliva, gastric and intestinal juice. According to the study, the reducing sugars in polysaccharide from the seeds of *Plantago asiatica L* were significantly increased and the molecular weight of the polysaccharide decreased due to the breakdown of glycosidic bonds. From Somogyi-Nelson method, only the amount of reducing sugars was able to be determined. To further assess whether the fucoidan was digested due to the breakdown of glycosidic bonds or not, samples require to be analysed by NMR.

4.2 Change in level of sulfate compounds using UV spectrophotometer and Infrared Radiation spectra.

Fucoidan is mainly composed of L-fucose, sulfate compounds, and uronic acid. The biology activity for example, the anti-oxidant activity (Boo et al., 2013; Li, Lu, Wei, & Zhao, 2008) and therapeutic potential in surgery (Shirahata, Zhang, Yoshida, Eto, & Teruya, 2013), anti-cancer (Yang et al., 2008), anticoagulant and antithrombotic activity (Millet et al., 1999) coincides with high concentration of sulfate compounds in fucoidan. The amount of sulfate compounds was measured using UV spectroscopy and Fourier Transform Infrared Radiation spectra (FT-IR). The change of sulfate compounds from digestion would be the assertive evidence for the level of biological activity of fucoidan.

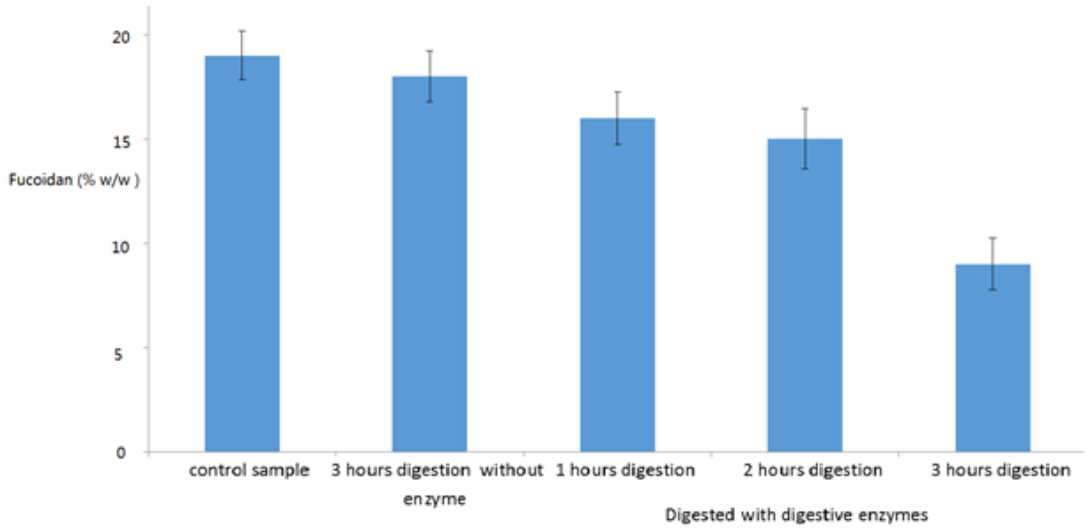


Figure 11. A graph showing sulfate concentration, in a control fucoidan sample containing fucoidan and distilled water, fucoidan digested with no-digestive enzymes and fucoidan digested with digestive enzymes in the dynamic model, over a 3hr digestion period, analysed with a UV spectrophotometer at 420nm. Error bars indicate standard deviation and the values are means of triplicate analysis.

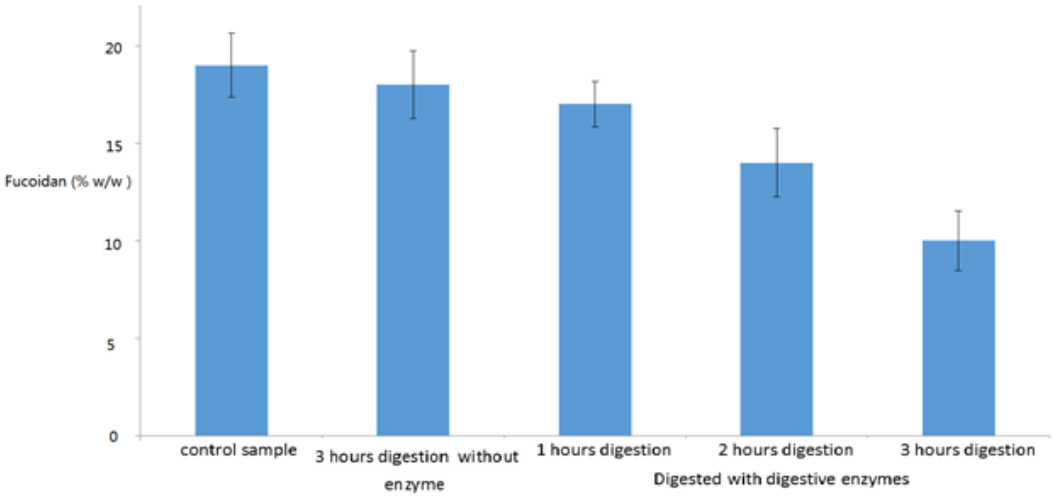


Figure 12. A graph showing sulfate concentration, in a control fucoidan sample containing fucoidan and distilled water, fucoidan digested with no-digestive enzymes and fucoidan digested with digestive enzymes in the static model, over a 3hr digestion period, analysed with a UV spectrophotometer at 420nm. Error bars indicate standard deviation and the values are means of triplicate analysis.

As shown in Figue 11 and 12, the amount of sulfate compounds decreased by almost a half duirng the 3hr digestion period. Sulfate compounds are closely connected to the long chain polymers inside fucoidan as suggested by Patankar's stimulated fucoidan structure shown on figure 14 (Patankar et al., 1993). The linkage of fucose-sulfate bonds provide a potential reasons for the digestibility of fucoidan. The removal of sulfate from the long chain structure of fucoidan contribute to the unstable molecule of the side chain, this leads to fomation of monosaccharides and oligosaccharides. And there is no significant difference between static and dynamic models for sulfate compounds comparison during digestion process. According to figure 13, the result shows proofed that the peak height of sulfate is decreased after digestion.

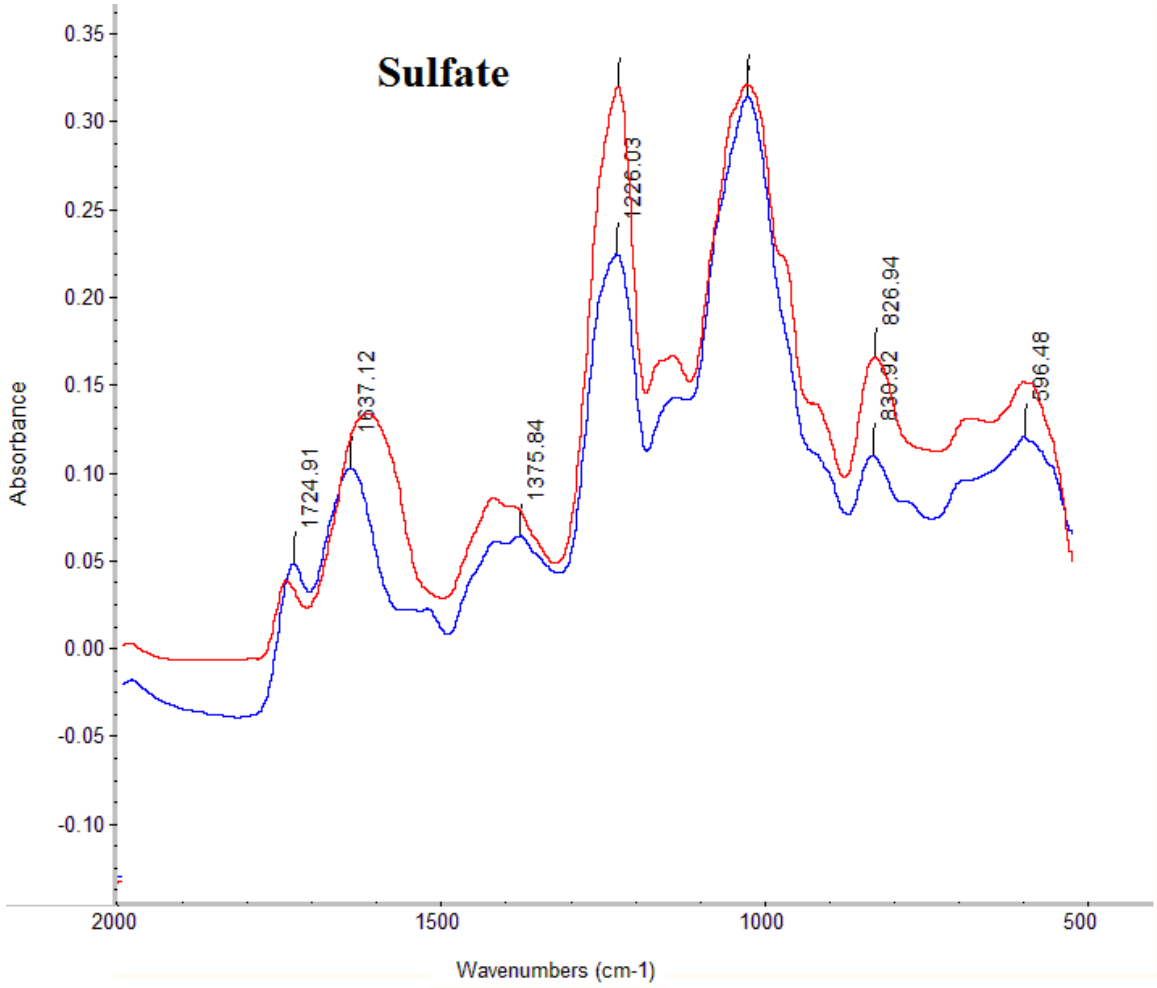


Figure 13. FTIR spectrum showing sulfate content (1226 cm^{-1}) between the crude fucoidan (blue) and digested sample from dynamic model at 3 hours (red). The crude fuoidan (blue) is set as the base line.

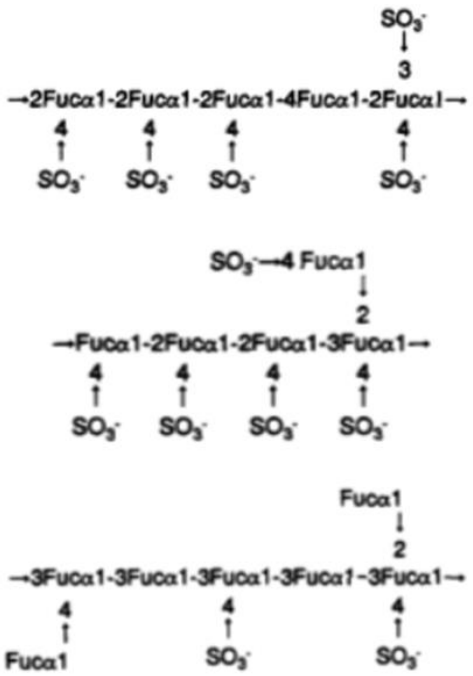


Figure 14. Patankar's hypothetical structure of fucoidan (Patankar et al., 1993). Fuc1-alpha-4 means α-1, 4-linked L- fucose.

The decrease in sulfate compounds over the digestion period was seen when fucoidan was digested with digestive enzymes. The decrease in sulfate concentration is also supported by GC result. As shown in figure 13, the wavelength 1226 nm shows significant quantitatively difference on between before and after digestion in sulfates. The decrease in content of sulfate compounds from FTIR is consistent with the results from UV spectrophotometer. Ths results indicate that the digestive enzymes have an influence in reducing the concentration of sulfate compounds. No similar study on the digested fucoidan was done to date.

According to Shibata et al. (2000) and Juffrie et al. (2006) fucoidan from *Cladosiphon okamuranus tokida* has the function of preventing the anti-peptic digestion. This effect was also observed using other sulfated polysaccharides such as dextran sulfate, carrageenan, heparin, and Fucus fucoidan, but not dextran or mannan. The anti-peptic effects of sulfated polysaccharides was believed to be due to their anionic charge and

ionic binding to the protein substrate. This was supported by the lack of inhibition with a low molecular weight synthetic substrate, N-acetylphenylalanyl diiodotyrosine, proving that the effect is not on the pepsin itself. The study indicated that the binding of the sulfated polysaccharides might influenced by molecular charge or conformation. The mechanism by which it does so is unclear, but our study is consistent with results from Shibata et al. (2000) and Juffrie et al. (2006).

Considering the application of fucoidan in anti-oxidant (Wang, Zhang, Zhang, & Li, 2008), anti-cancer (Yang et al., 2008), anticoagulant and antithrombotic activities (Millet et al., 1999), the decrease in sulfate content would suggested reduced efficacy over the course of digestion.

However, low level of sulfate in fucoidan has been shown to reduce blood lipids (Huang, Wen, Gao, & Liu, 2010), hence not all of the health benefits from consuming fucoidan is lost. Further implications of the results could be that intake of foods high in proteins should be avoided with fucoidan to minimise the infuence of the anti-peptic activity of fucodian to the overall digestion process. Targeted delivery of fucoidan may reduce the anti-peptic activiy and enable the biological value of fucoidan to be retained.

4.3 Change in level of uronic acid using UV spectrophotometer and Infrared Radiation spectra.

The uronic acid concentration of fucoidan is summarized in figure 15 and 16, Sample digested with digestive enzymes has significantly higher level of uronic acid than the sample digested without digestive enzymes and control. Ponce et al., (2003) indicated a similar result that the level of uronic acid increased after fraction treatment through an anion-exchange column chromatography which was used to break down the long chain polysaccharide to short chain compounds. The high level of uronic acid is

benificial for the anticoagulant and antitumor activity according to Usui, Asari, and Mizuno (1980). The results showed that the anticoagulant and antitumor activity of fucoidan is enhanced during the digestion process.

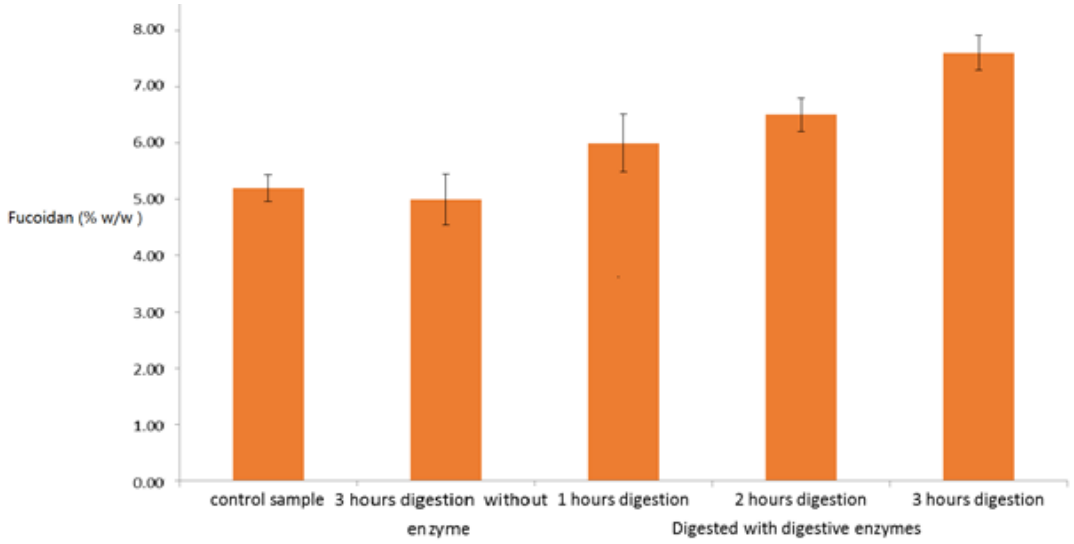


Figure 15. A graph showing uronic acid concentration of fucoidan a control fucoidan sample containing fucoidan and distilled water, fucoidan digested with no-digestive enzymes and fucoidan digested with digestive enzymes, over a 3hr digestion period in dynamic model, analysed with a UV spectrophotometer at 550nm. Error bars indicate standard deviation and the values are means of triplicate results.

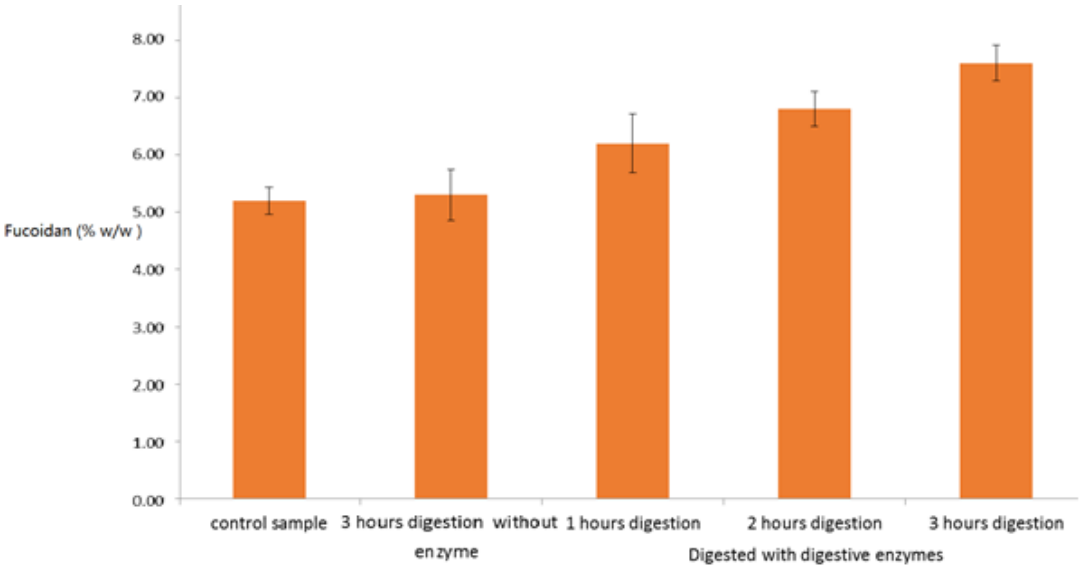


Figure 16. A graph showing uronic acid concentration of fucoidan a control fucoidan sample containing fucoidan and distilled water, fucoidan digested with no-digestive enzymes and fucoidan digested with digestive enzymes, over a 3hr digestion period in static model, analysed with a UV spectrophotometer at 550nm. Error bars indicate standard deviation and the values are means of triplicate results.

There is no significant difference on the level of uronic acid of fucoidan between the dynamic and static models.

4.4 Change in level of hydroxyl group using Infrared Radiation spectra.

According to figure 17, the peaks at wavelengths 3380 nm and 2928nm have significant difference in hydroxyl group between the crude fucoidan and the digested sample collected at the end of the 3hr digestion with digestive enzymes. This indicates that the content of monosaccharides with Hydroxyl groups, including L-fucose, galactose, xylose, mannose and glucose, may have been produced. These results relate to the break down of long chain polysaccaride to the short chain monsaccharides which provide an evidence of the digestiblity of fucoidan. The increase in monsaccharides has no influence on the bio-acitivty of fucoidan.

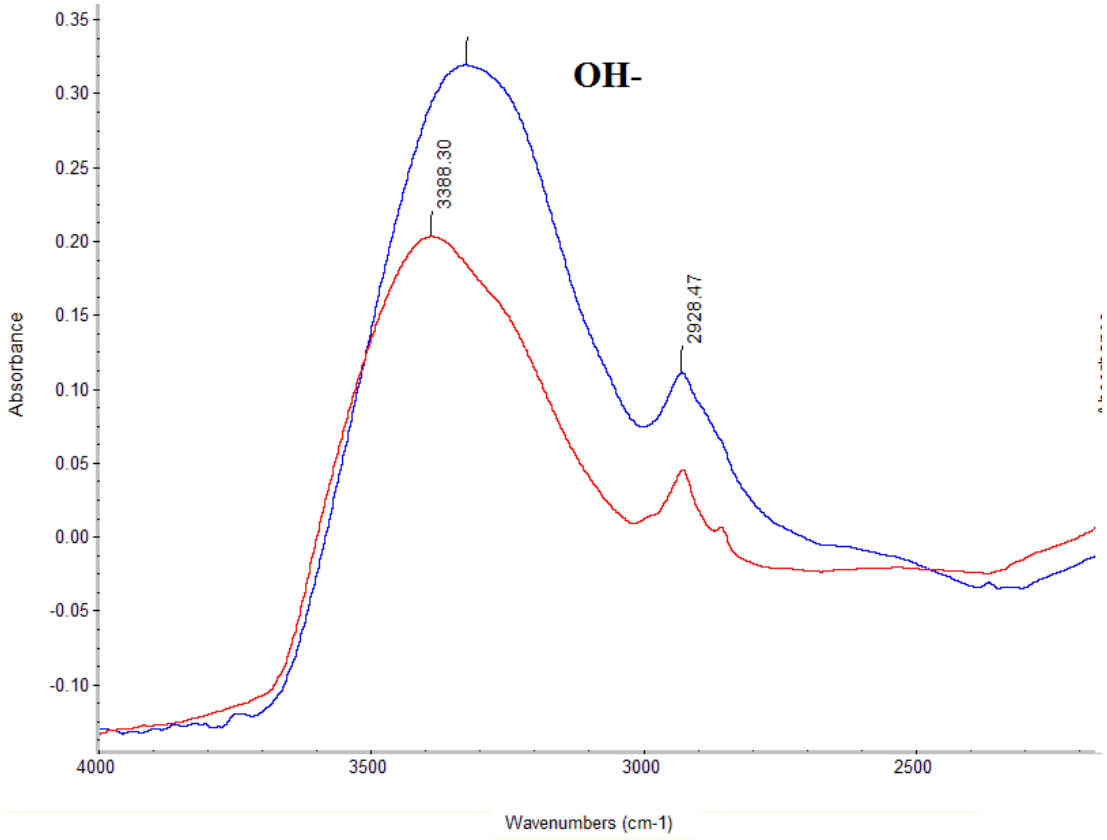


Figure 17. FT-IR spectrum showing Hydroxyl content between the crude fucoidan (blue) and 3 hours digesta (red).

4.5 Analysis of monosaccharides, using GC-FID

4.5.1 Trifluoroacetic Acid (TFA) assay

Melton and Smith (2001), has shown that hydrolysis of non-cellulosic polysaccharides is achieved by incubating in 2M trifluoroacetic acid (TFA) at 121 ℃ for 1h. The TFA hydrolysis assay is known as an effective preparatory step for analysing polysaccharides by GC. TFA can be removed by evaporation by steam of air or nitrogen. As fucoidan is a non-cellulosic polymer, TFA hydrolysis seemed a suitable preparatory step. Due to the complex compounds and structure of fucoidan, the length of hydrolysis time was examined in order to ensure complete hydrolysis of fucoidan. The crude fucoidan sample was hydrolysed for 1h, 2h and 3h and analysed through GC-FID.

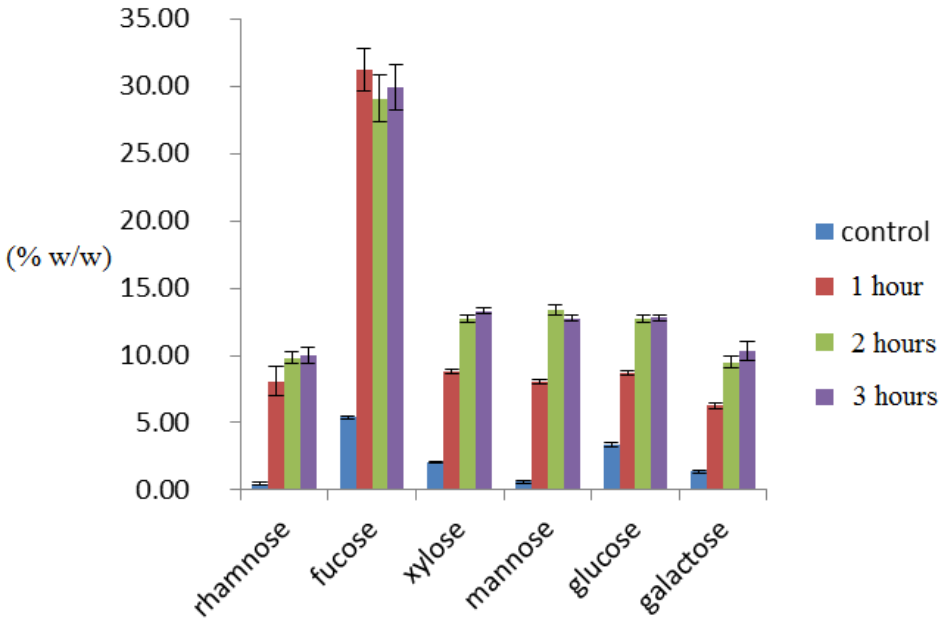


Figure 18. Comparison of fucoidan hydrolysis using TFA assay at different time intervals. Error bars indicate standard deviation and the values are means of triplicate results.

Table 13. Comparison of fucoidan hydrolysis using TFA assay at different time intervals.

Control	rhamnose	fucose	xylose	mannose	glucose	galactose
0h	0.0048 ± 0.06 [b]	0.0538 ± 0.14 [b]	0.0206 ± 0.06 [b]	0.0061 ± 0.14 [b]	0.0337 ± 0.20 [b]	0.0137 ± 0.11 [b]
1h	0.0091 ± 0.010 [a]	0.310 ± 0.015 [a]	0.080 ± 0.0017 [a]	0.080 ± 0.0013 [a]	0.086 ± 0.0014 [a]	0.063 ± 0.0019 [a]
2h	0.010 ± 0.006 [b]	0.290 ± 0.017 [a]	0.130 ± 0.0026 [b]	0.120 ± 0.0017 [b]	0.130 ± 0.0026 [b]	0.100 ± 0.0069 [b]
3h	0.0098 ± 0.004 [b]	0.290 ± 0.017 [a]	0.120 ± 0.0025 [b]	0.130 ± 0.0035 [b]	0.120 ± 0.0025 [b]	0.090 ± 0.0044 [b]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way

ANOVA.

As shown in figure 18 and table 13, the concentrations of rhamonose, xylose, mannose, glucose and galactose after 1h of TFA hydrolysis of crude fucoidan were significantly different when compared with those from hydrolysis after 2 hours and 3 hours.

There was no significant change in the concentrations of all monosaccharides during the second hour and the third hour of hydrolysis. Thus, 1h hyrdolysis was not enough for the fucoidan polymer to totally breakdown to monsaccharides for further analysis by GC-FID. Hence all of the samples, including control sample, samples digested with digestive enzymes and digested without digestive enzymes, were hydrolysed for 2h with TFA.

4.5.2 Level of monosaccharides in crude fucoidan and hydrolysed crude fucoidan from TFA assay.

Several monosaccharides exist in fucoidan (Li et al., 2008). GC-FID analysis was performed to examine the change in concentrations of monosaccharides include rhamnose, fucose, xylose, mannose, glucose and galactose in order to investigate the breakdown of the fucoidan polymer during the simulated digestion.

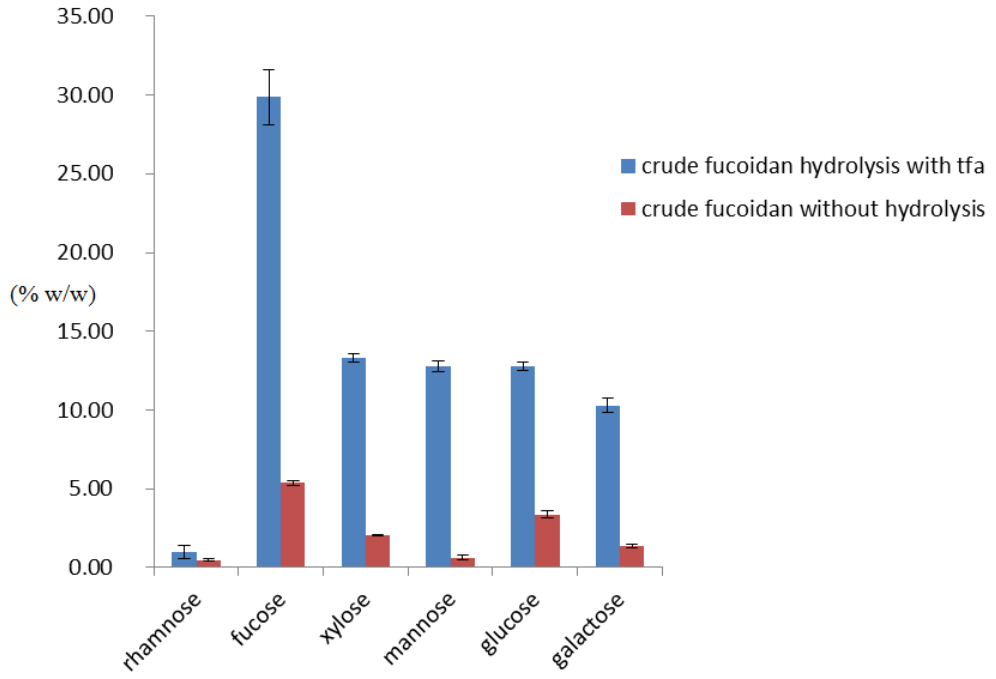


Figure 19. Comparison of hydrolyzed fucoidan and crude fucoidan before hydrolysis. Error bars indicate standard deviation and the values are means of triplicate results.

Table 14. Comparison of hydrolyzed fucoidan and non-hydrolyzed fucoidan.

w/w crude fucoidan ± SD	rhamnose	Fucose	xylose	mannose	glucose	galactose
Crude fucoidan tfa 2hours (%)	0.98 ± 0.41 [a]	29.88 ± 1.75 [a]	13.31 ± 0.25 [a]	12.77 ± 0.35 [a]	12.79 ± 0.26 [a]	10.29 ± 0.45 [a]
Crude fucoidan without hydrolysis (%)	0.48 ± 0.06 [b]	5.38 ± 0.14[b]	2.06 ± 0.06[b]	0.61 ± 0.14[b]	3.37 ± 0.20 [b]	1.37 ± 0.11 [b]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

From the results shown on Figure 19 and Table 14, it is evident that monosaccharides are naturally present in fucoidan at low concentrations (below 1%). All of the 6 monosaccharides chosen for this study have increased in concentration by undergoing

2h of TFA hydrolysis. This indicates that there were large amount of hydrolysable polymer present in fucoidan in its natural form.

4.5.3 Level of monosaccharides in crude fucoidan self-extracted compare with the Sigma – crude fucoidan.

The fucoidan which is used in the study is treated with water : ethonel (7:3) extract method from *U.pinnatifida*. Monosaccharides obtained by TFA hydrolysis of crude fucoidan was analysed by GC-FID. This was comparedwith the crude fucoidan from Sigma.

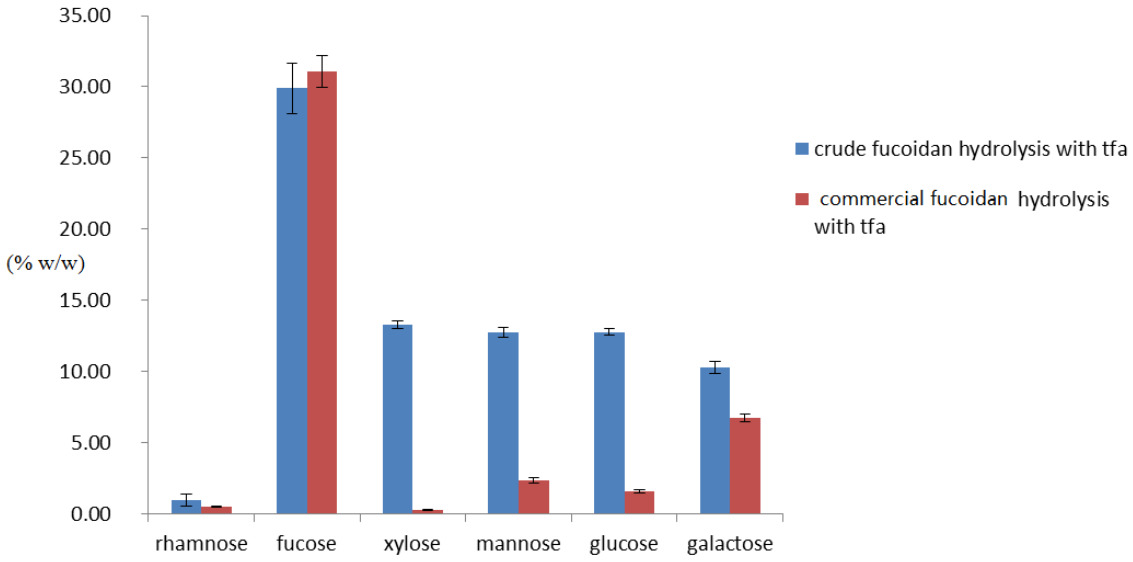


Figure 20. Comparison of monosaccharides in the crude fucoidan used in the study vs. commercially available fucoidan from Sigma Aldrich. Error bars indicate standard deviation and the values are means of triplicate results.

Table 15. Comparison of the crude fucoidan used in the study and commercially available fucoidan from Sigma Aldrich when hydrolyzed for two hours using TFA assay.

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	galactose
crude fucoidan used in the study	0.98 ± 0.41[a]	29.88 ± 1.75 [a]	13.31 ± 0.25 [a]	12.77 ± 0.35 [a]	12.79 ± 0.26 [a]	10.29 ± 0.45 [a]
commercial fucoidan	0.55 ± 0.03 [a]	31.10 ± 1.12 [a]	0.31 ± 0.02 [b]	2.40 ± 0.19 [b]	1.63 ± 0.10 [b]	6.74 ± 0.28 [b]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

According to figure 20 and table 15, the concentrations of xylose, mannose, glucose, galactose, fucoidan from sigma fucoidan were lower than that of the crude fucoidan we have used. And the concentration of fucose from sigma was similar to what was used throughout the study. Ale, Mikkelsen and Meyer (2011) and Mak et al. (2013), found that the concentration and composition of fucoidan may change with the extraction method, extraction part of the seaweed and type of seaweed. The concentration of monsaccharides found from this study for Sigma fucoidan was consistent with the findings of Zhang et al. 2009. This indicates that two hours of hydrolysis using TFA was a sufficient preparatory step for GC-FID and analysis of monosaccharides using the proposed GC-FID was valid and comparable to the previously reported values.

4.5.4 Monosaccharides degradation examined through GC-FID Assay

Bermudez-Soto, Tomas-Barneran and Garcia Conesa (2007) raised a concern that the use of an *in vitro* digestion system may led to a total loss of fucoidan and its monosaccharides. Possible route of loss included the trasfer process. Hence sample

recovery of digestibility study using both the static and dynamic digetion systems was tested.

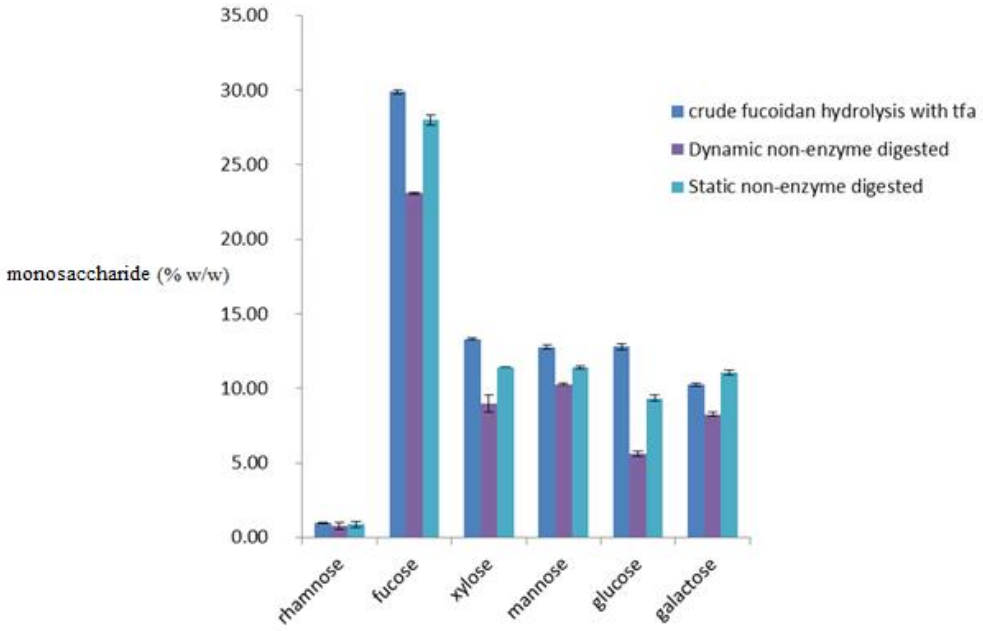


Figure 21. Comparison of crude fucoidan hydrolyzed by TFA and fucoidan run through the two digestive systems (with the same solution only without enzyme) then hydrolyzed by TFA. Error bars indicate standard deviation and the values are means of triplicate results.

Table 16. Comparison of hydrolyzed crude fucoidan and acid-digested fucoidan (with the same solution only without enzyme and hydrolysis)

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	Galactose
Crude fucoidan	0.98 ± 0.41	29.88 ± 1.75 [a]	13.31 ± 0.25 [a]	12.77 ± 0.35 [a]	12.79 ± 0.26 [a]	10.29 ± 0.45 [a]
Static model with no-enzyme	0.89 ± 0.038	27.99 ± 0.56 [a]	11.43 ± 1.83 [a]	11.38 ± 0.87 [a]	9.34 ± 0.64 [b]	11.07 ± 0.6 [a]
Dynamic model with no-enzyme	0.78 ± 0.037	23.10 ± 1.51 [b]	9.00 ± 0.23 [b]	10.29 ± 0.42 [b]	5.60 ± 0.14 [b]	8.26 ± 0.25 [b]

Different letters indicate significant differences at $p < 0.05$ by one-way ANOVA followed by the Tukey's post hoc comparison test. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

By comparing the 3 samples in figure 20 and table 16, there is a loss of fucoidan and its monosaccahride by the TFA hydrolysis of crude fucoidan and TFA hydrolysis of crude fucoidan digested through the static and dynamic model (10% loss in static and 22% loss in dynamic) during the digestion process. During dynamic digestion there is more loosing of sample because the dynamic flow of solvents and solution transfer through the peristaltic tube. When fucoidan was dissolved in water, it becomes sticky, and there is some loss of sample and it may have taken place by adsorption on the glass wall of the reactors used or by stucking to the silicon peristalsis tubing with 0.6 mm internal diameter used in the dynamic digestion model.

Compared to the static model, the continuous flow of digestive fluids would have increased the exposure of sample to result in more effective simulation of digestion.

4.5.5 The study of the crude fucoidan digested in the *in vitro* digestion models

The digestiblity of fucoidan was investagated using the *in vitro* digestion systems. Crude fucoidan was digested through both the static and dynamic digestion systems in non-enzyme and the normal enzymatic conditions. In order to determine the change of digested fucoidan compared with the crude sample, crude fucoidan was also analysed as control. All of the digested and the control crude fucoidan samples were hydrolysed by TFA assay for 2 h before injecting into GC-FID.

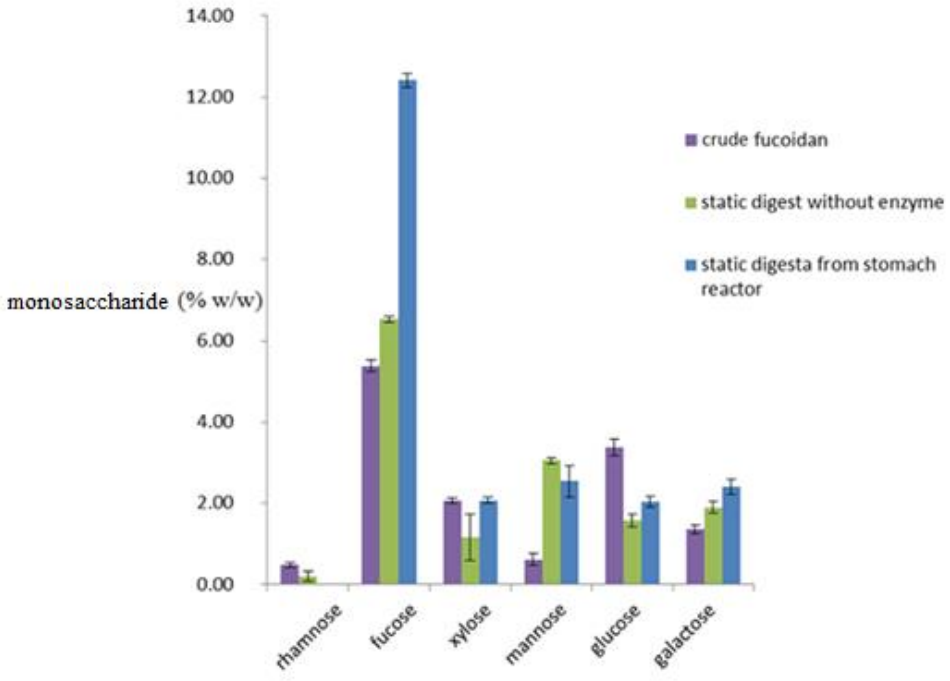


Figure 22. Comparison of crude fucoidan with digested samples from static *in vitro* digestion. Error bars indicate standard deviation and the values are means of triplicate results.

Table. 17 Comparison of crude fucoidan with digested samples from static *in vitro* digestion.

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	galactose
Crude fucoidan (%)	0.48 ± 0.06	5.38 ± 0.14 [a]	2.06 ± 0.06 [a]	0.61 ± 0.14 [a]	3.37 ± 0.20 [a]	1.37 ± 0.11 [a]
Static digesta from stomach reactor %	-	12.41 ± 0.18 [b]	2.07 ± 0.07 [a]	2.54 ± 0.38 [b]	2.04 ± 0.13 [a]	2.40 ± 0.18 [b]
Static digest without enzyme (%)	0.20 ± 0.012	6.53 ± 0.07 [a]	1.17 ± 0.58 [b]	3.06 ± 0.07 [b]	1.57 ± 0.16 [a]	1.91 ± 0.14 [a]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

According to figure 22 and table 17, the level of fucose contained in digested fucoidan is twice that of the crude and Static digest without enzyme samples which means the polysaccharide is assured broken down to the short chain monosaccharide in static digestion model. The other monosaccharides include mannose and galactose which show a significant increasing in the digested sample than the crude sample. And there is almost no rhamnose in the digested sample. By comparing the level of fucose between the static digesta from stomach reactor and static digest without enzyme, the level change of fucose is caused by the enzymes include α-amylase and pepsin we used. In order to determine which enzyme and which digestion process contribute to the digestibility of fucoidan, the samples collected from mouth reactor and stomach reactor were compared in section 4.5.6. Rhamnose from sample of static digesta from stomach reactor was too low to be detected by GC.

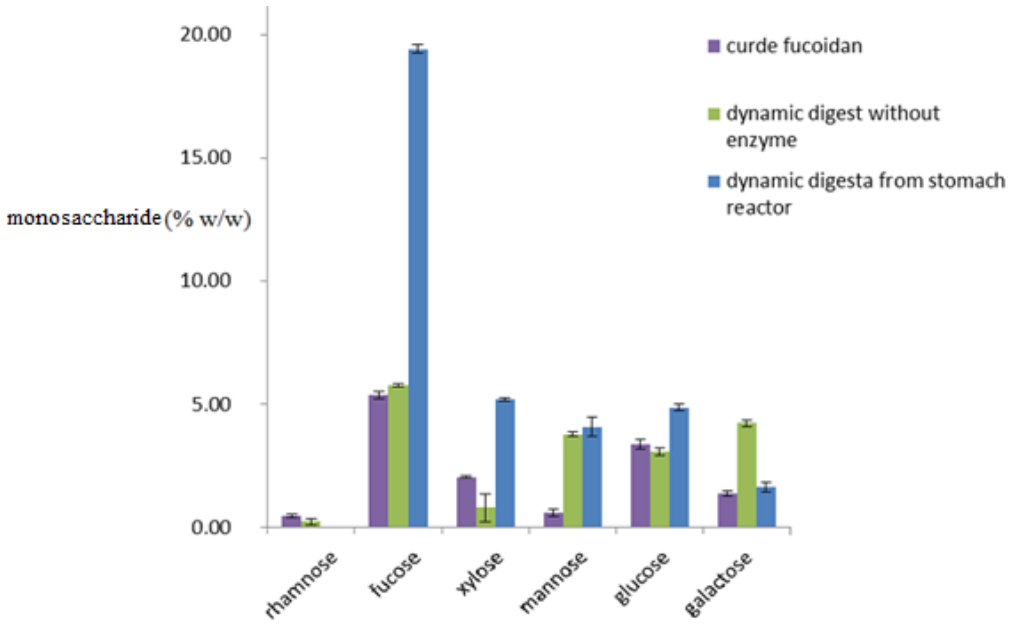


Figure 23. Comparison of crude fucoidan with samples from dynamic *in vitro* digestion. Error bars indicate standard deviation and the values are means of triplicate results.

Table 18. Comparison of crude fucoidan without hydrolysis with samples from dynamic *in vitro* digestion.

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	galactose
Crude fucoidan (%)	0.48 ± 0.06 [a]	5.38 ± 0.14 [a]	2.06 ± 0.06 [a]	0.61 ± 0.14 [a]	3.37 ± 0.20 [a]	1.37 ± 0.11 [a]
Dynamic digesta from stomach reactor (%)	-	19.42 ± 0.10 [b]	5.21 ± 0.19 [b]	4.10 ± 0.13 [b]	4.88 ± 0.37 [b]	1.64 ± 0.46 [a]
Dynamic digest without enzyme (%)	0.25 ± 0.01 [a]	5.77 ± 0.36 [a]	0.80 ± 0.02 [c]	3.79 ± 0.10 [b]	3.08 ± 0.22 [a]	4.23 ± 0.15 [b]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA followed by the Tukey's post hoc comparison test. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

According to figure 23 and table 18, the level of fucose contained in digested fucoidan is triple times than the crude and static digest without enzyme samples. And the other monosaccharides include mannose; glucose and galactose show a significant increasing in the digested sample than the crude sample. Same result is indicated according to section 4.5.5.1.

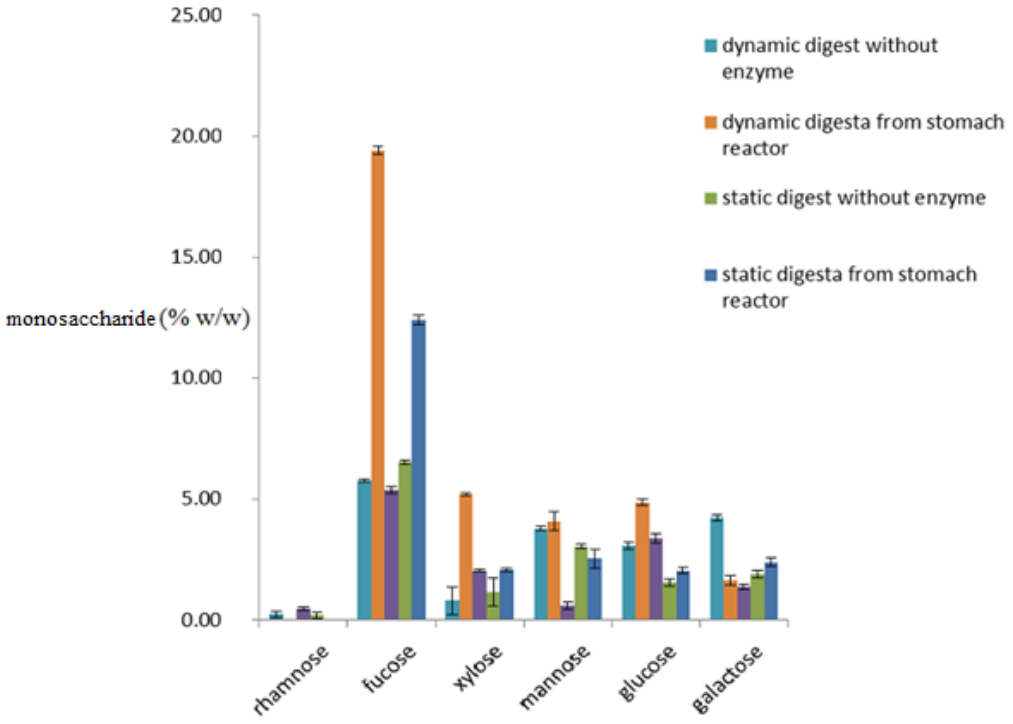


Figure 24. Comparison of crude fucoidan without hydrolysis with samples from dynamic and static *in vitro* digestion w/w fucoidan means grams of monosaccharides contain in per grams fucoidan. Error bars indicate standard deviation and the values are means of triplicate results.

Table 19. Comparison of crude fucoidan without hydrolysis with samples from dynamic and static *in vitro* digestion

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	galactose
Crude fucoidan (%)	0.48 ± 0.06 [a]	5.38 ± 0.14 [a]	2.06 ± 0.06 [a]	0.61 ± 0.14 [a]	3.37 ± 0.20 [a]	1.37 ± 0.11 [a]
Static digesta from stomach reactor %	-	12.41 ± 0.18 [b]	2.07 ± 0.07 [a]	2.54 ± 0.38 [b]	2.04 ± 0.13 [b]	2.40 ± 0.18 [b]
Static digest without enzyme (%)	0.20 ± 0.012 [a]	6.53 ± 0.07 [c]	1.17 ± 0.58 [b]	3.06 ± 0.07 [b]	1.57 ± 0.16 [b]	1.91 ± 0.14 [b]
Dynamic digesta from stomach reactor (%)	-	19.42 ± 0.10 [d]	5.21 ± 0.19 [c]	4.10 ± 0.13 [c]	4.88 ± 0.37 [c]	1.64 ± 0.46 [b]
Dynamic digest without enzyme (%)	0.25 ± 0.012 [a]	5.77 ± 0.36 [a]	0.80 ± 0.02 [b]	3.79 ± 0.10 [c]	3.08 ± 0.22 [a]	4.23 ± 0.15 [c]

Different letters (a, b, c, and d) indicate significant differences at $p < 0.05$ by one-way ANOVA followed by the Tukey's post hoc comparison test. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA.

According to figure 24 and table 19, by comparing the crude fucoidan with the fucoidan digesta from stomach reactor and digest without enzyme samples, we can understand that the level of fucose from dynamic model is 5% higher than the static model. Cause that the flowing solutions in the dynamic model increases the contact area between the sample and enzyme in the solution and lead to high effective of digestion. The concentration of rhamnose from samples of static digesta from stomach reactor and dynamic digesta from stomach reactor is too low to be analyzed.

The *in vitro* digestion process didn't breakdown all the polysaccharides to monosaccharides. It is speculated that there may be some structural change during the process of digestion. Further study should focus on the structure change of the digesta through the use of NMR.

4.5.6 Analysis of fucoidan at different stages of digestion

The *in vitro* digestion systems are divided into different reactors hence there is an advantage of studying each individal digestion steps. The samples collected from the mouth part and the stomach part have been analysed by GC-FID assay to obtain the possible changing include the change of concentration through the two digestion process. As a control of these experiments, the crude fucoidan was also examined.

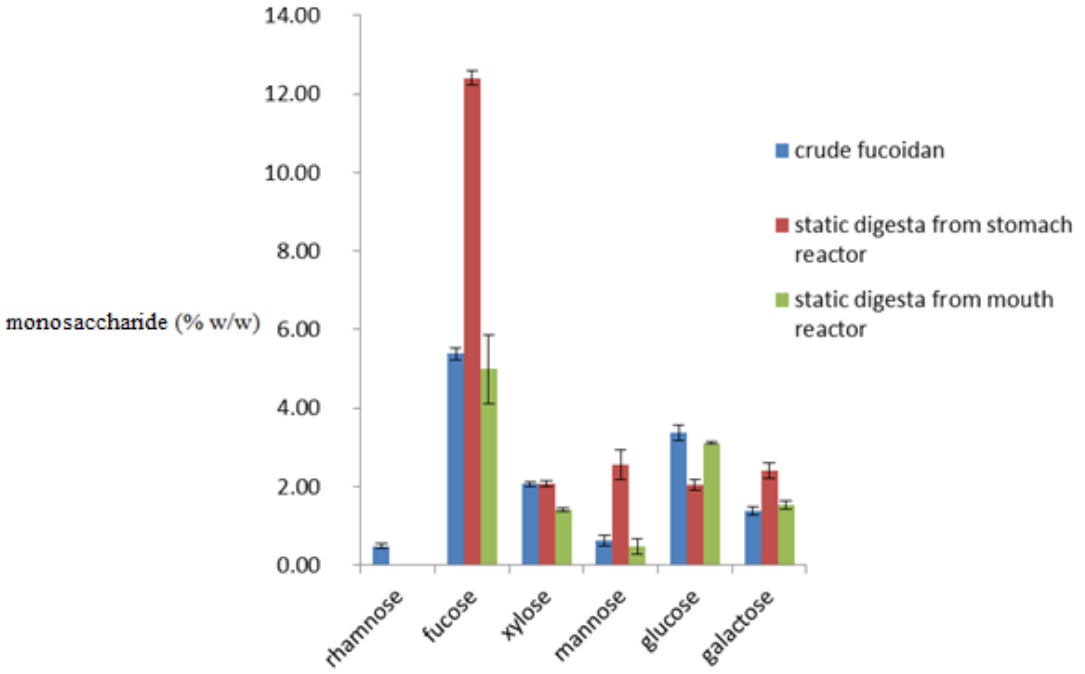


Figure 25. Comparison of crude fucoidan without hydrolysis with samples from static stomach part and mouth part. Error bars indicate standard deviation and the values are means of triplicate results.

Table 20. Comparison of crude fucoidan without hydrolysis with samples from static stomach part and mouth part.

w/w crude fucoidan ± SD	rhamnose	Fucose	xylose	mannose	glucose	galactose
Crude fucoidan (%)	0.48 ± 0.06 [a]	5.38 ± 0.14 [a]	2.06 ± 0.06 [a]	0.61 ± 0.14 [a]	3.37 ± 0.20 [a]	1.37 ± 0.11 [a]
Static digesta from stomach reactor (%)	-	12.41 ± 0.2 [b]	2.07 ± 0.07 [a]	2.54 ± 0.38 [b]	2.04 ± 0.13 [b]	2.40 ± 0.18 [b]
Static digesta from mouth reactor (%)	-	4.99 ± 0.87 [a]	1.42 ± 0.04 [b]	0.48 ± 0.20 [a]	3.10 ± 0.03 [a]	1.53 ± 0.10 [a]

Different letters (a and b) indicate significant differences at $p < 0.05$ by one-way ANOVA followed by the Tukey's post hoc comparison test. Same letters indicate no significant differences at $p \geq 0.05$ by one-way ANOVA. The highlight data has significant difference between other two groups of data.

According to figure 25 and table 20, by comparing the crude fucoidan with the fucoidan digesta from stomach reactor and fucoidan digesta from mouth reactor, we can gain that the level of most monosaccharides except rhamnose (cause the concentration is too low to determine) have no significant difference between the crude fucoidan and fucoidan digesta from mouth reactor which refer to the digestion environments in mouth reactor has no influence on the digestibility of fucoidan. The concentration of rhamnose from samples of static digesta from stomach reactor and static digesta from mouth reactor is too low to be analyzed.

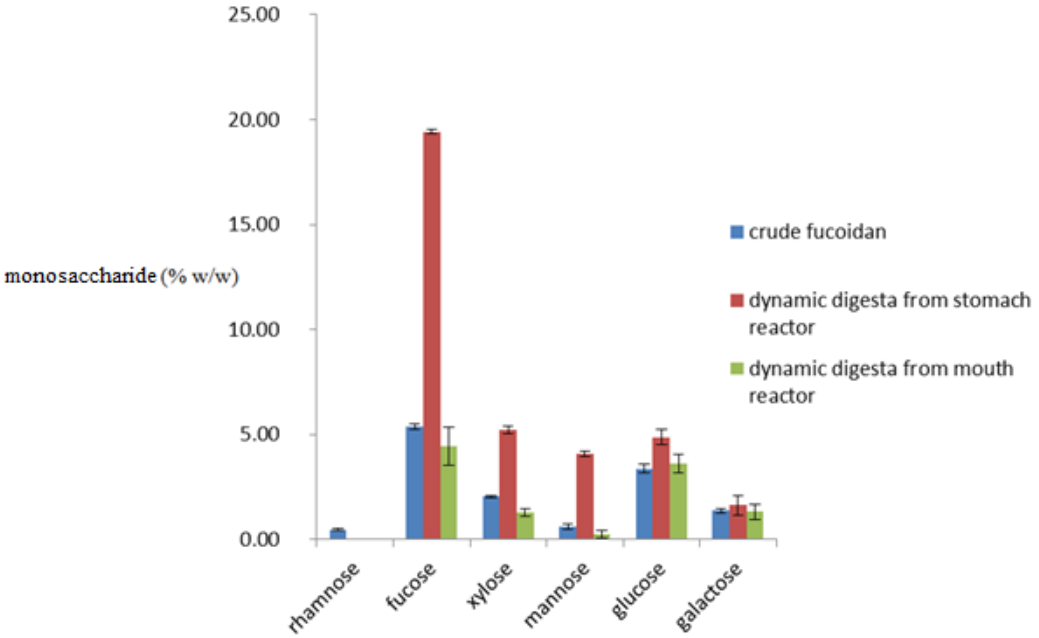


Figure 26. Comparison of crude fucoidan without hydrolysis with samples from dynamic stomach part and mouth part. Error bars indicate standard deviation and the values are means of triplicate results.

As seen in the figure 26 and table 21 the concentrations of all monosaccharides in the digested samples from the mouth reactors are similar to the crude fucoidan sample before hydrolysis, indicating that alpha amylase from artificial saliva was not effectively breaking down the polysaccharides in fucoidan into monomer units. However there was a significant increase in the concentration of monosaccharides from the fully digested samples, once they past the stomach reactors.

Table 21. Comparison of crude fucoidan without hydrolysis with samples from dynamic *in vitro* digestion.

w/w crude fucoidan ± SD	rhamnose	fucose	xylose	mannose	glucose	galactose
Crude fucoidan (%)	0.48 ± 0.06 [a]	5.38 ± 0.14 [a]	2.06 ± 0.06 [a]	0.61 ± 0.14 [a]	3.37 ± 0.20 [a]	1.37 ± 0.11 [a]
Dynamic digesta from stomach reactor (%)	-	19.42 ± 0.10 [b]	5.21 ± 0.19 [b]	4.10 ± 0.13 [b]	4.88 ± 0.37 [b]	1.64 ± 0.46 [a]
Dynamic digesta from mouth reactor (%)	0.25 ± 0.012 [a]	5.77 ± 0.36 [a]	0.80 ± 0.02 [c]	3.79 ± 0.10 [b]	3.08 ± 0.22 [a]	4.23 ± 0.15 [b]

These results indicate that the digestion environment in the stomach reactors and pepsin have influenced the breakdown of fucoidan polymer to monosaccharides. And combined with the reducing concentration of sulfation in UV and IR experiments (section 4.2), the digestion of fucoidan may relate to both the level of sulfation and pepsin. The result is consistent with the study of anti-peptic activity on fucoidan discussed in section 4.2.

Jufferie et al (2006) investigated that fucoidan is a type of sulfated polysaccharide which has the anti-peptic activity due to the anionic change and to the ionic binding to the protein substrate. According to the Shibata's result, the sample mixed with fucoidan affected the digestion ability of pepsin and decrease the amount of pepsin by 10% (Shibata et al., 2000). Patankar (1993) determined the fucoidan structure using NMR. It was shown that the sulfate compounds are connected with the main chain of polymer as branched-chain links with other molecules within the fucoidan. Some plant proteins which occur naturally in fucoidan is also known to influence the digestibility of fucoidan (Varenne, Gareil, Colliec-Jouault, & Daniel, 2003). The

proteins linked on fucoidan can be enzymatically hydrolysed by pepsin. This leads to breakdown of high molecular weight polymer and formation of unstable structure. Thus the binding of sulfate with proteins must have broken down the fucoidan polymer to short chain compounds in my study. Further study using NMR would be necessary to conclude what structural changes have been made during the simulated digestion process at different stages of digestion.

According to the research of Hu, Nie, Min and Xie (2013), a potential reason for the non-starch polysaccharide breaking down to monosaccharide may due to the breakdown of glycosidic bonds among the long chain structure. This could be proofed by NMR as well.

Chapter 5 Conclusion

5.1 Conclusion

Fucoidan extracted from *U. pinnatiFIDa* has undergone significant chemical changes during the gastric digestion period in *in vitro digestion* system. The concentrations of reducing sugars and monosaccharides including glucose, galactose, xylose, mannose, and fucose, have significantly increased after 3 h of digestion when compared with the raw extracted sample. This indicated that the polysaccharide structure of the fucoidan polymer has broken down to shorter chain compounds. The concentration of sulfated groups or compounds in fucoidan has reduced after digestion. Reduction in sulfated groups is undesirable as high concentration of sulfate contribute towards anti-cancer or anti-oxidant activity. Quantitatively, the most significant change in the concentration of sulfate was observed during the gastric digestion period. The result may be caused by anti-peptic activity of fucoidan. The anti-peptic effects of sulfated polysaccharide are known to be caused by anionic exchange and ionic binding to the protein substrate (Juffrie, Rosalina, Damayanti, Djumhana, & Ahmad, 2011).

It is speculated that the change in linkage is cuased by the breakdown of glycosidic bonds which are present among the fucose-sulfate branched chains, during the digestion process. This may have further brokendown the long chain polymer structure of fucoidan. Hence, further study is required to examine the structural characteristics among the sulfation, protein and pepsin. This would allow further correlation between digestibility of fucoidan and pepsin in order to understand the breakdown of polymer. Furthermore, it would help to concentrate further the sulfate composition of fucoidan and contribute to better effectiveness of bio-activity of fucoidan. Fucoidan was separated based on different molecular weight using Millipore centrifugal Filter which ranged from 10 kDa to 100 kDa but cannot be identified by GC-FID assay. The separated fractions should be further examined by NMR in order to identify the structural change.

5.2 Limitations of this research

The monosaccharides which include glucose, galactose, mannose, rhamnose and fucose were qualitatively identified through GC-FID by a acetylate alditols treatment. But the structural changes of the oligosaccharide and polysaccharide during the digestion period can only be identified through NMR. As a result comparisons across the *in vitro* digestion could not indicate all the reaction of digestibility of fucoidan. It would also have been better if the structure of sulfate group linked with the polymer could be identified through the NMR to reduce variability in the results.

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