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THE POTENTIAL OF CAROTENOIDS FROM MARINE TROPICAL MICROALGAE IN THE HEALING PROCESS OF GASTRITIS

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Abstract: Carotenoids which are important towards the biological activities are produced by all higher plants, some bacteria and algae. Marine microalgae can produce a variety of substances including carotenoids and it has a commercial interest as production of microalgae in controlled condition is affordable. Three species of marine microalgae; *Chlorella* sp., *Dunaliella* sp. and *Isochrysis* sp. were cultured and carotenoids were extracted for testing. The total carotenoid present in dry weight of *Chlorella* sp., *Dunaliella* sp. and *Isochrysis* sp. were 55.1%, 99.3% and 29.4% respectively. The carotenoids from the microalgae were tested for toxicity level against MCF7 cancer cells, with *Chlorella* sp., *Dunaliella* sp. and *Isochrysis* sp. showing IC50 value 0.25mg/ml, 0.3 mg/ml and 0.32 mg/ml from crude extract respectively. Antioxidant di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) scavenging activity were present in all the three types of microalgae. *In-vivo* gastritis studies on model mice showed healed result from *Dunaliella* sp. but not from *Chlorella* sp. and *Isochrysis* sp. From the three microalgae species, *Dunaliella* sp. has the highest ability to promote gastric healing as it contains antioxidant and antimicrobial properties.

Keywords: Carotenoid, microalgae, *Helicobactor pylori, Dunaliella* sp., *Chlorella* sp., *Isochrysis* sp.

Introduction

Health professionals have consistently advocated the daily consumption of five portions of fruit and vegetables as a means of protecting against the pathogenesis of degenerative diseases including coronary heart disease, cancers and other free radical-mediated conditions (Kritchevsky *et al*., 1999). Fruit and vegetables contain a wide range of compounds including the antioxidant vitamins C and E, minerals, phenolics and carotenoids. Carotenoids are natural pigments, providing orange, yellow, red and purple colours throughout the natural world. These pigments are produced by all higher plants, along with some bacteria and algae (Britton *et al*., 1995). They are derived from the terpenoid family and are biosynthetically related to other secondary metabolites such as tocopherols and ubiquinones. Within plant tissue they are associated with photosynthetic membranes and are involved in photo-protection and light energy assimilation (Britton *et al*., 1995).

Carotenoids possess a range of important and well documented biological activities. They are potent antioxidants and free radical scavengers (Grassmann *et al.*, 2002), and can modulate the pathogenesis of cancer (van Poppel and Goldbohm, 1995) and coronary heart disease (Kritchevsky *et al*., 1999). Lycopene intake in particular is associated with a decreased incidence of prostate cancer (Giovannucci, 1999), while high levels of β-carotene are correlated with a reduction in the risk of developing lung cancer (Block *et al*., 1992). A number of carotenoids including α-carotene, β-carotene and β-cryptoxanthin have pro-vitamin A activity, since they are converted to retinal by mammals. This role is of particular importance, especially in developing countries where the dietary deficiency of vitamin A can lead to blindness and premature childhood mortality (Mayne, 1996). The xanthophylls (oxo-carotenoids), lutein and zeaxanthin are also known to provide protection against age-related macular degeneration, mediated by their ability to quench single oxygen and blue light in the retina (Landrum and Bone, 2001).

Marine microalgae comprise the largest group of living organisms in the oceans,

constituting an estimated 10,000 species. At present, microalgae offer great possibilities for the isolation of natural substances of significant commercial interest such as pharmaceuticals, alimentary or cosmetic products. Other than that, microalgae can be produced in controlled condition with a low cost, and that they are adapted to a wide variety of environments favors to an exceptional biochemical production. This fact has made microalgae raw materials with a great deal which aimed at eradication of *Helicobacter pylori* infection in human.

With all the resources and benefits that are within our grasp, it is important to explore the potential of the tropical marine microalgae which can contribute greatly in the field of biotechnology.

Methodology

Mass Culture and Optimization

In order to achieve the concentration desire for extraction, a biophotoreactor was used to maximize the microalgae count. The biophotoreactor was prepared for mass culture of microalgae with a specific set of physical water parameters. The cells were grown photoautotrophically by bubbling through the cell suspension air supplemented with 1% (v/v) CO_2 with a flow rate of $100L^{-1}$ culture h^{-1}. The microalgae were cultured and optimized in the chemically defined medium (F2 medium) until it has reached the concentration of 10^9. The cells was then harvested by centrifugation (2500 x g, 10 min), and then lyophilized for storage and extraction process.

Ultrasound Assisted Solvent Extraction

Microalgae obtained from the mass culture were used in the ultrasound assisted solvent extraction (UASE). A lyophilized microalgae sample (100 mg) was suspended in 5 mL N,N-dimethylformamide (DMF). The suspension was sonicated for 3 min in ultrasound apparatus and stored for 24h at 4^OC and centrifuged to separate the extract from the microalgae pellet. Then the supernatant was filtered through a 0.22 µm filter and stored at 4^OC in the absence of light. The extraction was repeated for three times until the solvent being discolored (Vasantha *et al.,* 2011).

Total Carotenoids Concentration

From the extraction, the total carotenoids concentration which include the measurement of chlorophyll and carotenoids' absorbance using spectrophotometer; was determined using the equation proposed by Wellburn. The equation proposed by Wellburn for the determination of carotenoid and chlorophyll concentration in the samples of microalgae has more parameters than other equations thus allows the determination of chlorophyll b contained in the samples. The concentration of total carotenoids was calculated using the following equation:

Chlorophyll a (Ag/ml)= $11.24\ A_{661.6} - 2.04\ A_{644.8}$
Chlorophyll b (Ag/ml)= $20.13\ A_{644.8} - 4.19\ A_{661.6}$
Total carotenoids (Ag/ml) = $(1000\ A_{470} - 1.90$ Chla- 63.14 Chlb)/ 214.
(Lichtentaler and Wellburn 1985)

Detection and Identification of Carotenoids

Samples dissolved in acetone were centrifuged to discard particulate residues prior to injection into the liquid chromatography, and that separation was performed on a C18 column containing dimethyloctadecylsiyl bonded amorphous silica, protect with a guard cartridge. The pigments were eluted with acetonitrile, methanol and ethyl acetate in the ratio 80:18:2 at a rate of 1 ml min^{-1}, and detected by measuring the absorbance at 360-700 nm, using a programmable photodiode-array detector. Standard of beta-carotene was obtained as comparison standards (Tee and Lim, 1992).

Cytotoxicity Test

In order to determine the cytotoxicity of the total carotenoids on human cell lines, sterility total carotenoid extract was determined first. Then MTT cytotoxicity assay was performed to test the effect of the extract on normal cell and cancer cell (3T3 and MCF7). The 96-well plate was incubated in the CO_2 incubator

at 37^{O}C for 1 hour with 20µl/well of MTT (3-(4,5)-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) and the absorbance was measured at 570nm.

In-vivo Study

Female mice (6-8 weeks old) disease-model mice were obtained to observe the effects of total carotenoids on human gastric problem. Five mice were kept in a light-dark cycle (12 h/ 12h) cage at a constant temperature of 22^{O}C and at 60% humidity, and fed with normal laboratory mice food. Later the mice were inoculated intragastrically through a sterile gastric gavage with 10^{9} colony forming units (CFU) of *H. pylori* strain in 0.15 ml sterile PBS (pH 7.4, 0.1 M) three times at 2-day intervals.

Two weeks after inoculation, the 40 infected mice were randomly assigned to three groups. The first group (n=20) was used for infection analysis. One month after the last bacteria inoculation, five mice were killed and samples of stomach and spleen were collected. This procedure was repeated 2, 3 and 4 months after the last administration of *H. pylori*. The second group (n=10) was designated as the infected, treated group. The 10 infected mice were treated orally with the total carotenoids extract from the microalgae (100 mg/kg body weight per day, 0.15-0.18 ml in corn oil) for 2 weeks. The third group was designated as the infected, untreated group. These mice received 0.15 ml corn oil every day for 2 weeks. Four weeks after the last algal extract or corn oil treatment, all 20 mice of the second and third group were sacrificed. Stomach and spleen were removed and prepared for further histopathology analysis.

Antimicrobial Assay

Apart from observing the total carotenoids through histology study, we also looked at the antimicrobial assay which might inhibit the growth of bacteria. The total carotenoid extract was used for antimicrobial assay. With a total of 50µl crude at 30µg/ml concentration, it was diffused in a sterile paper disc and air dried. Common pathogenic bacteria were used as the targeted bacteria, which was diluted at a concentration of 0.5 Mc Farland Standard and swabbed on Mueller Hinton agar. Paper disc with different crude concentration was placed onto the swabbed agar plate and commercial antibiotic disc (Streptomycin, kanamycin, penicillin, vancomycin) were used as positive control. Agar plate was incubated at 37^{O}C for 18 hours. Zone of inhibition was measured post incubation.

Antioxidant Assay: DPPH Stable Free Radical Scavenging Activity

Extending from *In-Vivo* and antimicrobial assay, we also looked at the antioxidant activity from the total carotenoids extract. A total of 50µl solution of the total carotenoid extracts (10mg/ml) was placed in curvettes and 2 ml of methanolic DPPH (Sigma, Germany) solution (6 x 10^{-5}M) was added. The absorbance was determined at 515nm using spectrophotometer. Measurements were recorded immediately as the absorbance at t=0. Then the test solution was incubated in the dark for 30 minutes before reading and recording the absorbance at t=30. The determinations were performed in three replicates. Quercertin (Sigma, Germany). The radical scavenging activity of the tested sample was expressed as inhibition percentage of the DPPH radical scavenging activity, calculated using the following formula:

$$\% \text{ Inhibition} = [(Abs_{t=0} - Abs_{t=30})/Abs_{t=0}] \times 100$$

Where, $Abs_{t=0}$ is the absorbance at t=0 and $Abs_{t=30}$ is the absorbance at t=30.

Results and Discussion

Comparison of the three species, chlorophyll a and b concentrations are highest in *Dunaliella* sp., followed by *Isochrysis* sp. and *Chlorella* sp. As for the total carotenoids concentration, *Chlorella* sp. possesses a higher amount (3.058 Ag/ml) out of the three microalgae where the total carotenoid is higher than the chlorophyll a concentration (Figure 1a).

The carotenoid percentage in dry weight of the microalgae (Figure 1b) showed that

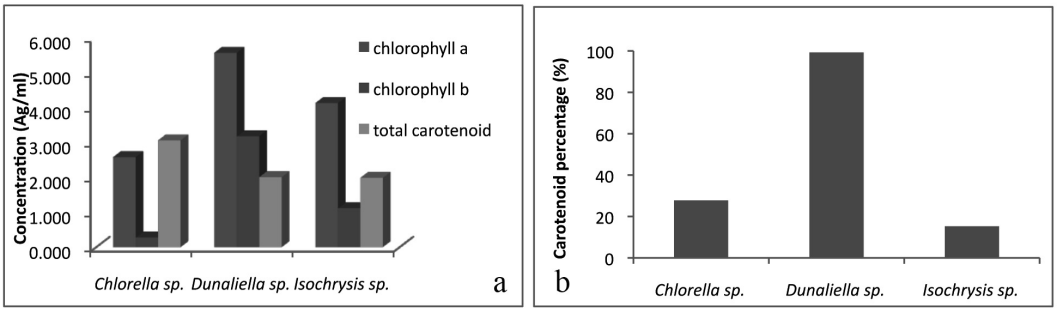

Figure 1: a) The concentration of chlorophyll a, chlorophyll b and total carotenoids of three microalgae species. b) Total percentage of carotenoids present in dry weight of *Chlorella* sp., *Dunaliella* sp. and *Isochrysis* sp.

Dunaliella sp. possess higher number of carotenoid by six folds compared to *Isochrysis* sp. *Chlorella* sp. has carotenoids of 27.8% of total dry weight while *Isochrysis* sp has 15.27%. Both *Chlorella* sp. and *Dunaliella* sp. are of green microalgae while *Isochrysis* sp. is of brown microalgae. However, difference in carotenoid percentage of dry weight between *Chlorella* sp and *Dunaliella* sp. was contributed by the concentration of chlorophyll a and b in the microalgae. Apart from that, the presence of carotenoid is directly proportional with the stress such as nitrogen stress, light stress, UV-B radiation stress, high temperature stress and salinity stress. During stress carotenoid production changes where the carotenoids increase significantly with decreasing of chlorophyll as supported by Dipak and Lele (2004) where the chlorophyll synthesis gets adversely affected while β-carotene increased. Carotenoid increases as a protection of the cell during stress condition where excessive formation of free radicals occurs and damage the cell. Most carotenoids have antioxidant properties that quench excessive free radicals thus restoring the physiological balance in the cell (Edge *et al*., 2007).

Similarly to the study done by Fatimah *et al*., (2001), mentioning that different composition of certain element in media will give a different effect on the algae production, it is prove through a study that a type of algae called cyanobacteria react differently towards different composition of nutrient where the growth as well as the growth rate increased with concentration of carbon and phosphorus but decreased and even disappear when nitrogen content is high in the media (Fatimah *et al*., 2001). A limited nutrient is unreliable to support a large number of cells, so the cell number of algae was reduced. A study by Bhosale (2004) have mention that when an algae is introduce to nitrogen starvation it will enhance the production of the β-carotene and the production of β-carotene is proportional to the decreasing of cell density.

The depletion of major nutrient such as nitrogen theoretically will cause a stress towards the algae, hence triggers certain reaction to encounter the problem. The depletion in nitrogen in the medium of growth will affect the physiology of the algae (Geider and MacIntyre, 2002). Basically an organism under stress will produce a lot of free radical which is a reactive oxygen species (ROS) (Jones and Smirnoff, 2005), This ROS is important for the cell but excess of it may resulting in cell damage or cell death. Organism like algae will enhance the production of carotenoid that act as an antioxidant which will encounter the excessive ROS product.

Carotenoid is not affected by decreasing of the cell because it can still produce by the cell during stress. When the nitrogen element is absent, the cell was stressed and produce ROS (Jones and Smirnoff, 2005), where this ROS was produced to protect the cell. It has to be balanced in the production of ROS, this is because too much of ROS can cause damage to the cell. In order to protect the cell from too much of ROS, carotenoid was produced to

balance up the production of ROS. In this case, carotenoid and fatty acids are two examples for non-enzymatic classes of substances which are able to protect the cell from oxidative damage (Marxen *et al*., 2007).

Detection and Identification of Carotenoid

Table 1 showed that *Chlorella* sp. have more components of fatty acid presence compared to *Dunaliella* sp. Five components of fatty acids had been detected in *Chlorella* sp. while four components of fatty acids presence in *Dunaliella* sp. All fatty acids presence in *Chlorella* sp. can be detected in *Dunaliella* sp. such as Myristic, Palmitic, Palmitoleic and Oleic while cis-5,8,11,14,17-Eicosapentaenoic can only be detected in *Chlorella* sp. All the fatty acids listed in Table 1 are carotenoid fatty acids. Fatty acid methyl esters properties were determined by length of carbon chain, degree of unsaturation and the alcohol content of composed fatty acids (Damiani *et al.,* 2010). Accumulation of cellular β-carotene in *Dunaliella salina* correlates with accumulation of specific fatty acid species like palmitic and oleic acid. However, it should be noted that other components like DHA, EPA, and AA were equally important for the microalgae itself (Becker W., 2004).

Dosage Determination

The effect of total carotenoids towards MCF-7 showed that for all microalgae species, the lowest concentration tested inhibit more than 50% of the cell vitality.

This finding showed that total carotenoids is toxic towards MCF-7 cell lines where between the microalgae species, *Isochrysis* sp., has higher ability to inhibit the cell growth at all tested concentration followed by *Chlorella* sp. and *Dunaliella* sp. LC_{50} value for *Dunaliella* sp. is 0.34ug/ml, while for *Chlorella* sp. is 0.25 ug/ml and *Isochrysis* sp. at 0.32 ug/ml.

As for normal cell, the total of carotenoids from all the three microalgae species are toxic. Figure 3 showed that from the concentration range of 0.47ug/ml to 30ug/ml, *Dunaliella* sp. inhibited more normal cell compared to the other microalgae. Carotenoid from *Isochrysis* sp. is more toxic compared to *Chlorella* sp., however at 15ug/ml, the number of cell vitality is more or less the same. The LC_{50} for all microalgae species are 0.25ug/ml for *Chlorella* sp.; 0.23ug/ml for *Dunaliella* sp. and 0.24ug/ml for *Isochrysis* sp.

However there is a study done by Hardwick *et al.,* (1999) shows that at a higher β-carotene

Table 1: Fatty acid components of *Dunaliella* sp., *Chlorella* sp. and *Isochrysis* sp.

***Dunaliella* sp.**		***Chlorella* sp.**		***Isochrysis* sp.**	
Peak	Fatty Acid	Peak	Fatty Acid	Peak	Fatty Acid
C14:0 (8)	Myristic	C14:0 (8)	Myristic	C14:0 (8)	Myristic
C16:0 (12)	Palmitic	C16:0 (12)	Palmitic	C16:0 (12)	Palmitic
C16:1 (13)	Palmitoleic	C16:1 (13)	Palmitoleic	C16:1 (13)	Palmitoleic
C18:1n9c (17)	Oleic	C18:1n9c (17)	Oleic		
		C20:5n3 (30)	Eicosapentaenoic		

Table 2: β-carotene in *Dunaliella* sp., *Chlorella* sp. and *Isochrysis* sp.

Microalgae sp.	*β-carotene*
Dunaliella sp.	Present
Chlorella sp.	Present
Isochrysis sp.	Absent

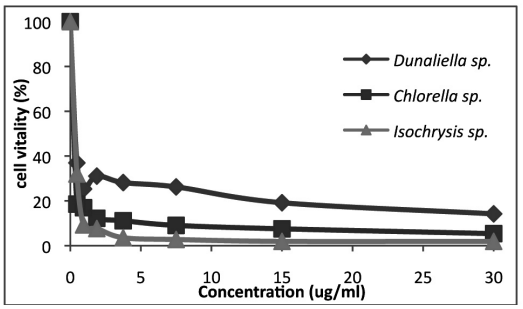

Figure 2: Toxicity effect of total carotenoids from *Dunaliella* sp., *Chlorella* sp. and *Isochrysis* sp. towards breast cancer cell lines (MCF-7)

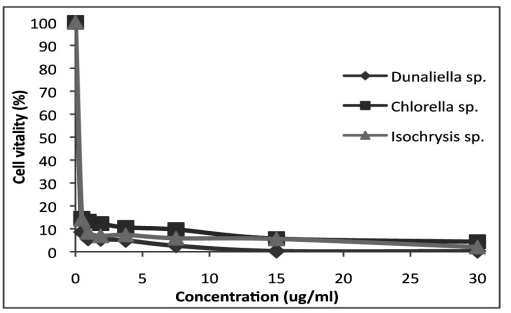

Figure 3: Toxicity effect of total carotenoids from *Dunaliella* sp., *Chlorella* sp. and Ischrysis sp. towards normal cell

content, which is 47 µg/mL will become toxic to normal muscle cell and for other type of carotene was at concentration higher then 23Mm. The study by Hardwick *et al.,* (1999), supports the result of this experiment on the effect of the carotenoid crude extract on normal muscle cell. As in this study the carotenoid was being used to be tested on the cells, the exact concentration of each type of carotenoid is still unknown and there is a probability that the β-carotene content within the carotenoid crude extract is higher than 47 µg/mL or other type of carotene content is higher than 23 µg/mL.

A study done by Cui *et al.,* (2007) on the MCF-7 cancer cells have discovered that β-carotene were able to decrease MCF-7 survival at LC_{50} of 65 µg/mL. Another study by Fornelli *et al*., (2007) also give the same result where using concentration of 50 µg/mL of lycopene to achieve LC_{50} towards the MCF-7 cancer cell. There is a reason that we can relate to this result, when the extraction are done maybe there are also some other pigments that had been extracted together with the carotenoids that are toxic to human cells.

In-vivo Study

The surface epithelium of the stomach was denudated after three days causing the gastric pit to be disrupted with increased in depth (Figure 4b). The lamina propria was swollen on day seven (Figure 4c) due to accumulation of tissue fluid. The presence of gastric gland were observed and characterized by the hyperplasia condition. The swollen of lamina propria was observed until day 14 with the absence of gastric gland hyperplasia. This showed that the histological changes of gastric due to infection started with the denudation of the surface epithelium that affected the gastric pit, followed by the gastric gland hyperplasia and lamina propria swelling.

For treatment groups, *Dunaliella* sp. and *Isochrysis* sp. showed the same histology with the induced group. Hyperplasia was observed at the gastric gland and lamina propria was swollen for *Dunaliella* sp. treatment group. For *Isochrysis* sp. treatment group, the hyperplasia was present in gastric gland; however lamina propria was swollen at certain area only. Gastric pit was disrupted especially at the lower part of Figure 6d where the surface epithelium was denudated.

Gastric gland in Figure 5c was round in shape compared to Figure 5d where the gastric gland was elongated. However the number of gastric gland is higher in group treated with *Dunaliella* sp. *Chlorella* treatment group (Figure 5b) showed disruption of gastric pit, while the lamina propria was at normal condition and absent of gastric gland hyperplasia.

After 14 days of treatment, Figure 6b showed hyperplasia of the gastric gland with swelling of the lamina propria. *Dunaliella* sp. treatment group showed absent of hyperplasia gastric gland, reduced swelling of lamina propria and normal gastric pit condition. *Isochrysis* sp. treatment groups showed reduced in gastric gland hyperplasia and number, reduced lamina

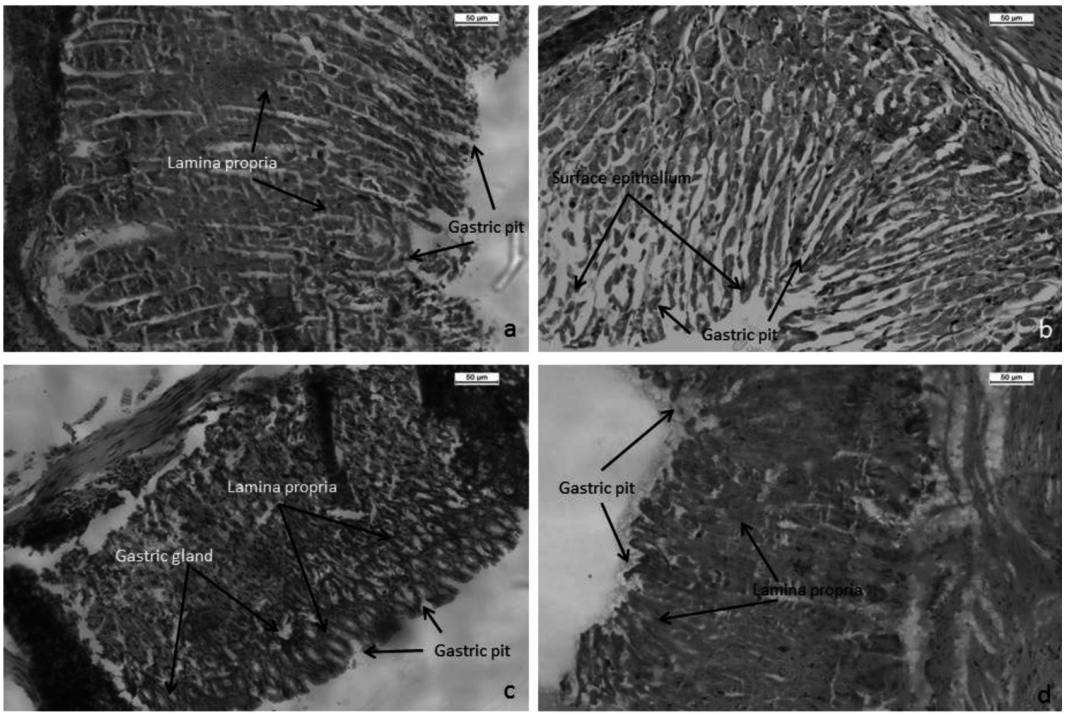

Figure 4: Histological analysis of stomach under 20x magnification for (a) normal group and induced gastritis group after (b) 3 days, (c) 7 days and (d) 14 days

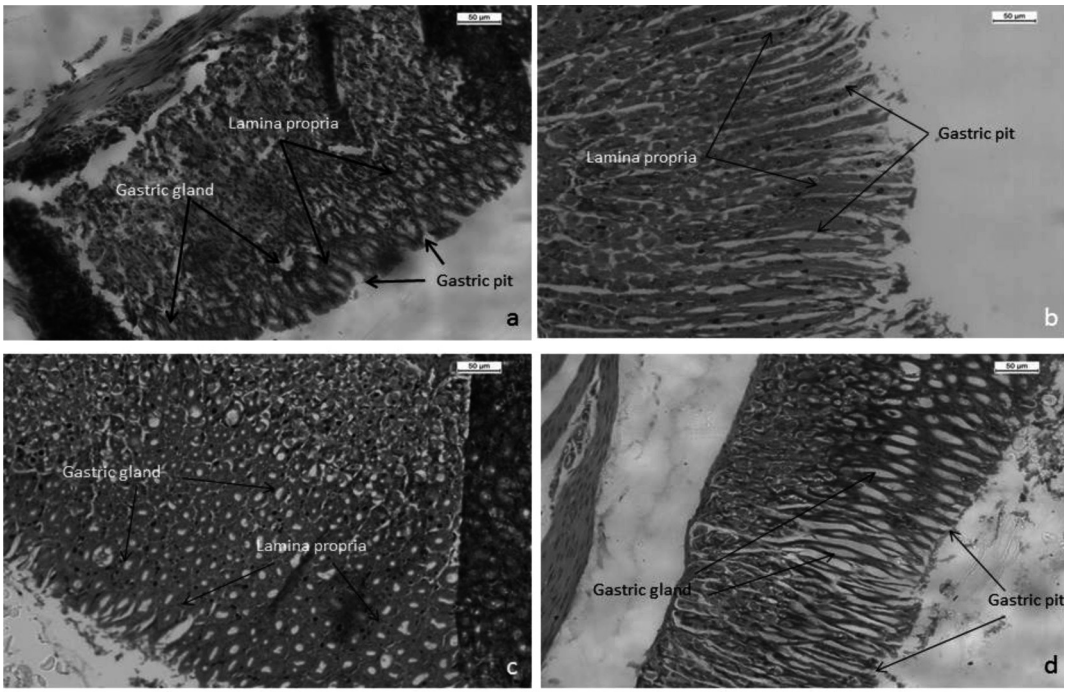

Figure 5: Histological analysis of stomach under 20x magnification for (a) induced group and treatment groups; (b) *Chlorella* sp. treatment, (c) *Dunaliella* sp. treatment and (d) *Isochrysis* sp. treatment after 7 days

propria swelling and minor disruption of gastric pit. Comparison of both treated group with the induced group showed that treatment groups' lamina propria has reduced in swelling.

For *Chlorella* sp. treatment group, after seven days of treatment, the histological changes showed that the gastric pit was disrupted while the lamina propria was at normal condition. After 14 days, gastric gland hyperplasia was present and the lamina propria was swollen. Comparing with gastric condition (Figure 7b), the histological changes suggest that *Chlorella* sp. treatment group delayed the gastric process.

After seven days of treatment, *Dunaliella* sp. treatment group showed the gastric gland was increased in number and hyperplasia due to the swelling of lamina propria. The gastric pit has return to normal condition after 14 days of treatment. The gastric gland was absent and the lamina propria was not swollen. These suggest that the *Dunaliella* sp. treatment has improved the histological changes to normal condition in 14 days.

After seven days of treatment with *Isochrysis* sp., there were two conditions of the gastric pit; first, the gastric pit was disrupted due to the denudation of the surface epithelium causing increased in depth and second, the depth of the gastric gland was reduced. Gastric gland was hyperplasia with elongated shape. Lamina propria was swollen at the upper part of Figure 9c. After 14 days, the gastric pit was back to normal condition with hyperplasia of the gastric gland and lamina propria swellings were reduced.

In induced gastritis, the histological changes started with denudation of the surface epithelium. This affected the gastric pit by increasing the depth, followed by the gastric gland hyperplasia and lamina propria swelling. During this condition, ROS was activated and triggered the inflammatory signaling. This inflammatory signaling was then deactivated by β-carotene by reducing the iNOS and COX-2 expression with suppressing the ROS inflammation (Jang *et al*., 2009). Histological changes involving the *Chlorella* sp. treatment can delay the effect of gastritis where the denudation of the surface epithelium and hyperplasia of the gastric gland were observed on day 14 after treatment. For induced condition, the changes of the surface

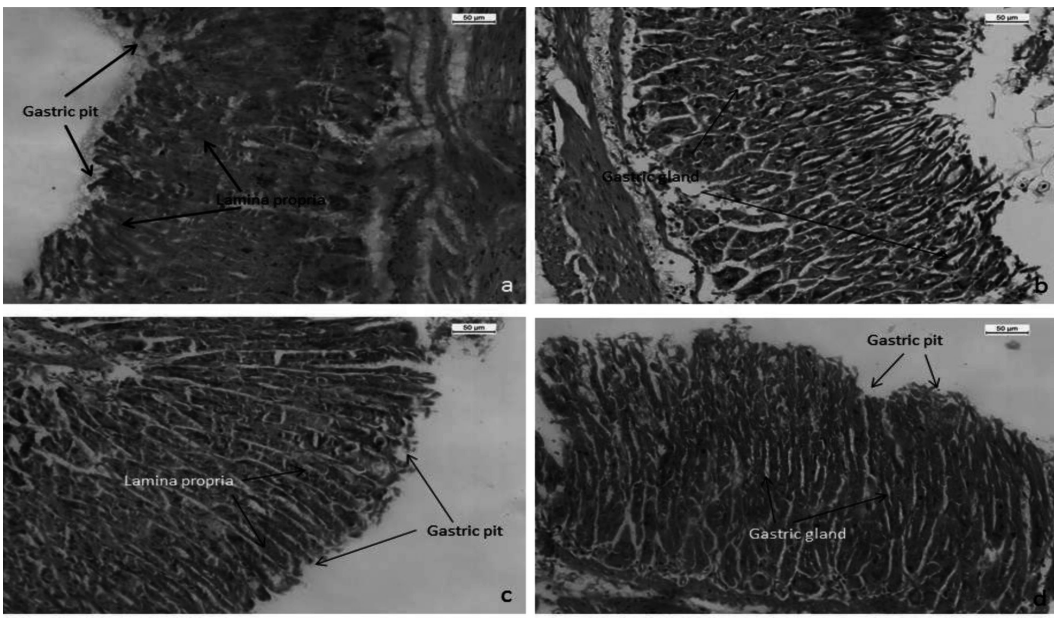

Figure 6: Histological analysis of stomach under 20x magnification for (a) induced group and treatment groups; (b) *Chlorella* sp. treatment, (c) *Dunaliella* sp. treatment and (d) *Isochrysis* sp. treatment after 14 days

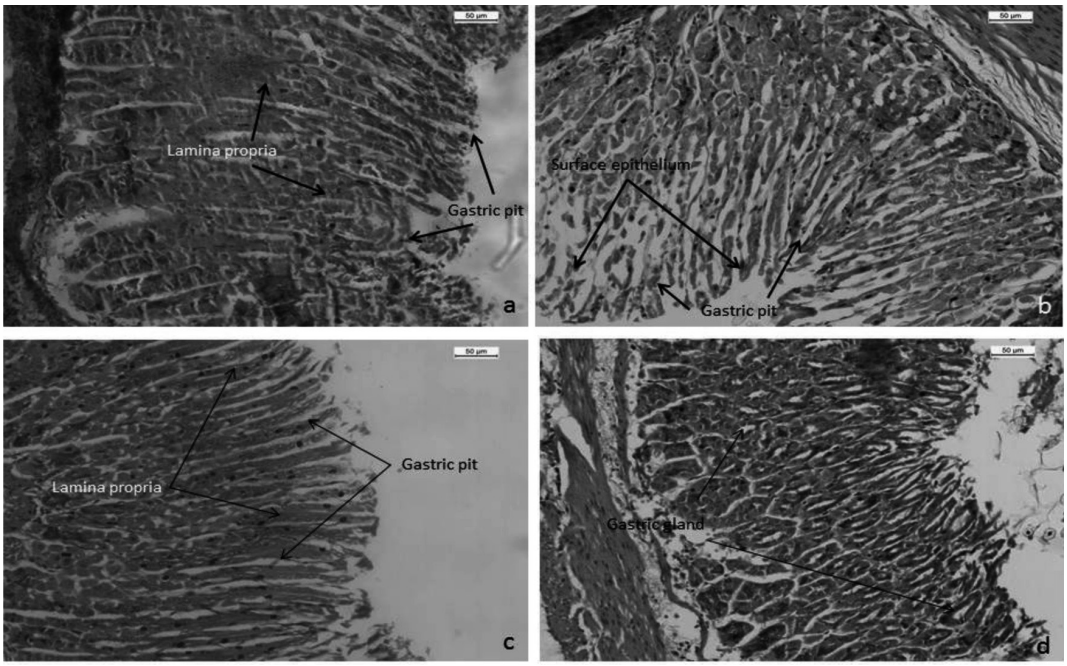

Figure 7: Stomach histological comparison of (a) normal, (b) gastric and *Chlorella* sp treatment group at (c) day 7 and (d) day 14 under 20x magnification using hematoxylin and eosin stain

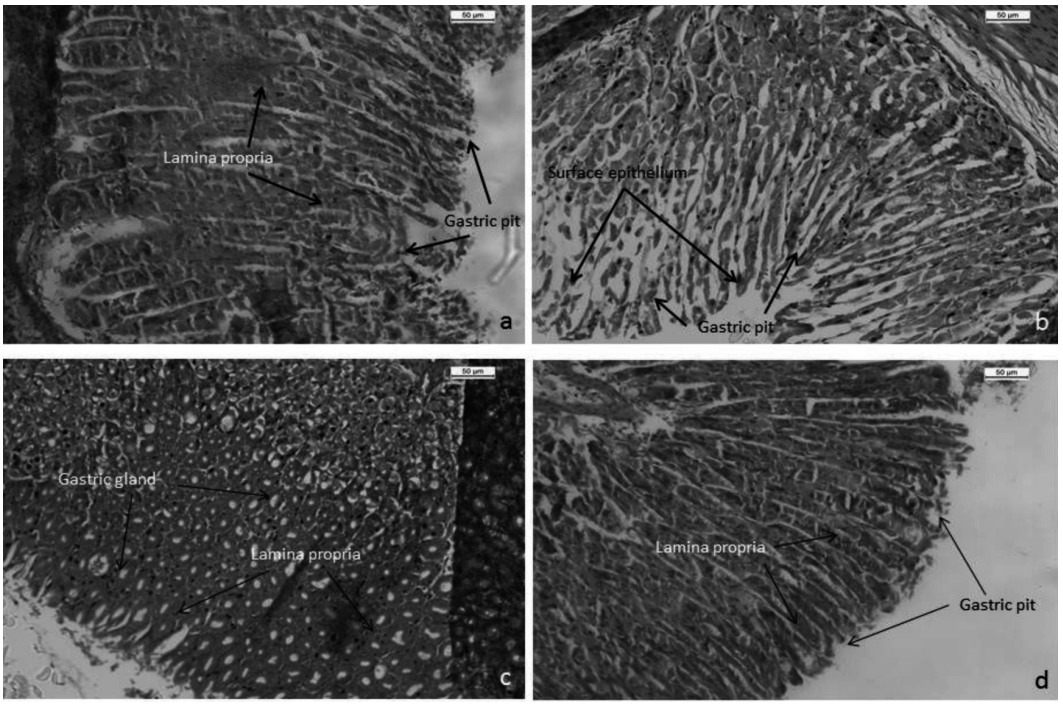

Figure 8: Stomach histological comparison of (a) normal, (b) gastric and *Dunaliella* sp. treatment group at (c) day 7 and (d) day 14 under 20x magnification using hematoxylin and eosin stain

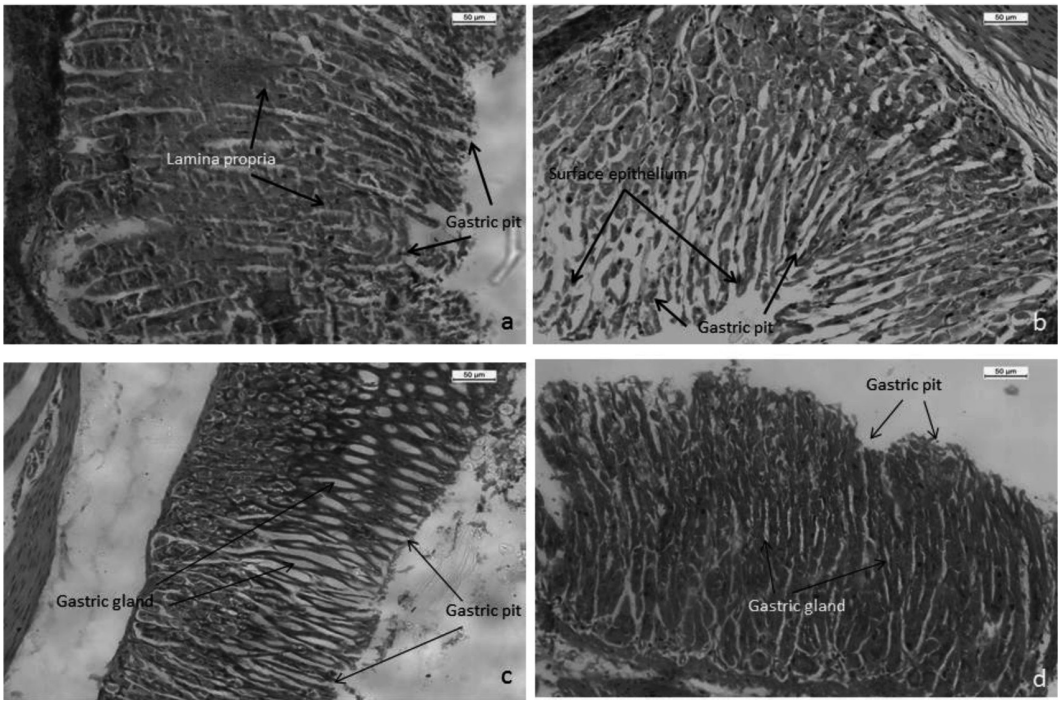

Figure 9: Stomach histological comparison of (a) normal, (b) gastric and *Isochrysis* sp. treatment group at (c) day 7 and (d) day 14 under 20x magnification using hematoxylin and eosin stain

epithelium and gastric gland can be observed after three days. However, *Dunaliella* sp. and *Isochrysis* sp. did not delay the gastric effect but both microalgae can promote gastric healing in 14 days after treatment. Between these two microalgae, *Dunaliella* sp. treatment can heal the stomach by restoring the normal histology faster than *Isochrysis* sp.

A high intake of carotenoids such as astaxanthin, has been proposed to prevent the development of gastric by lowering colonization levels of *H. pylori.* Apart from that Wang *et al*., (2000) suggested that antioxidants may be a new strategy for treating *H. pylori* infection in humans that caused gastritis.

Antimicrobial Assay

Between the three microalgae, *Isochrysis* sp. showed no activity towards all the tested pathogens while for *Dunaliella* sp. and *Chlorella* sp., activities were detected. *Dunaliella* sp. does not have activity towards *E. coli* and *Salmonella* sp. at all concentration while for *P. aeruginosa*, probiotic activity was observed at 100mg/ml only. All concentrations of *Dunaliella* sp tested for *B. cereus* showed positive result for antimicrobial test while *Klebsiella* sp. growth was inhibited by *Dunaliella* sp crude extract at concentration of 100 mg/ml, 50 mg/ml and 25 mg/ml.

Chlorella sp. crude showed positive antimicrobial activity against *P. aeruginosa* at 50 mg/ml and 100 mg/ml. However, at same concentration tested against *E. coli* and *Klebsiella* sp., probiotic activity was observed. At 25 mg/ml against *E. coli* also showed probiotic condition. As for the other pathogens, no reaction was observed.

Between these three microalgae species, *Dunaliella* sp. possess higher antimicrobial activity as it can inhibit the growth *B. cereus* and *Klebsiella* sp. followed by *Chlorella* sp. *Isochrysis* sp. does not have antimicrobial activity towards the tested pathogens.

Table 3: Antimicrobial activity of three microalgae species towards common pathogens

Sample	Concentration (mg/ml)	*E.coli*	*Klebsiella* sp.	*Salmonella* sp.	*P. aeruginosa*	*B. cereus*
Chlorella sp.	100	=	=	-	+	-
	50	=	=	-	+	-
	25	=	-	-	-	-
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-
Dunaliella sp.	100	-	+	-	=	+
	50	-	+	-	-	+
	25	-	+	-	-	+
	12.5	-	-	-	-	+
	6.25	-	-	-	-	+
Isochrysis sp.	100	-	-	-	-	-
	50	-	-	-	-	-
	25	-	-	-	-	-
	12.5	-	-	-	-	-
	6.25	-	-	-	-	-

(=): Probiotic; (+): Inhibition; (-): Negative

As for *Salmonella* sp. and *E. coli* there were no reactions observed might due to resistance. *E. coli* has two mechanisms of protection which are O polysaccharides and K antigen which is an acid polysaccharides secretion (Todar, 2011). As for *Salmonella* sp., two defense mechanisms are present, which is possession of polysaccharides capsule and Vi antigen (Todar, 2011).

Even though *Klebsiella* sp. and *B. cereus* growth were inhibited, the inhibition area is not so distinct. *B. cereus* susceptibility towards antibiotic is not fully understand (Logan and Diaz, 2006) but it is known that this pathogens produce *beta*-lactamase in order to react towards *beta*-lactam which is present in many antibiotics such as penicillin (Forsyth and Logan, 2000). Hence, this study suggested that *Dunaliella* sp. might have a compound that is able to inhibit the growth of *B. cereus*. On the other hand *Klebsiella* sp. has one mechanism of protection which is the polysaccharides capsules (Todar, 2011) and this mechanism is greater when the temperature is lower than 30°C (Hart, 2006). Since the incubation temperature is higher than 36°C, it will decrease the capsule synthesis and hence ease the reaction of *Dunaliella* sp. crude towards the bacteria.

The resistance and the serotypes in which this bacteria will occur differs geologically (Old D. and Threlfall E.J., 1998; Stephen and Peter 2005). In this experiment, the crude extract does not show any reaction on this bacterium, only the gentamicin antibiotic show the inhibition towards this *Salmonella* sp.

E.coli resistance can be acquired via plasmids or drug efflux systems. The chromosomal multiple antibiotic resistance locus in *E.coli,* designated *marA*, influences the expression of the *acrAB* efflux pump and other chromosomal genes, resulting in resistance to a range of antibiotics including tetracycline and many unrelated antibiotics including chloramphenicol, β-lactams, and nalidixic acid (Stephen and Peter., 2005). The influence to the chromosomal genes of *E.coli* can change the antibiotic agent for these bacteria to a probiotic. Where more than to be resistance to the antibiotic maybe these bacteria can grow more when the antimicrobial agent is introduced. The mutation to these bacteria can cause this action happen to some of the new antimicrobial agent.

The presence of antibiotic resistance plasmids are also of assistance in allowing *Klebsiella* sp. to grow in the tissues of patients

receiving antibiotics. The tissues damaged by this bacteria is probably because both release toxic molecules by the bacteria and the host's response, in particular frustrated activated phagocytes releasing toxic oxygen radicals (Stephen and Peter., 2005). *P.aeruginosa* which is low cell-wall permeability conferring intrinsic resistance, the production of extracellular chromosomal and plasmid-mediated β-lactamases, aminoglycosidases and cephalosporinades, an alteration in antibiotic-binding protein sites and an active efflux mechanism which pumps out antibiotic from the cell. However, this resistance rates have changed over the last two decades and today remain around 12% for these agents (Stephen and Peter, 2005). So it is not possible if the result obtained show even if the inhibition is low, the bacteria resistance towards this crude extraction is also low.

Antioxidant Assay

Dunaliella sp. has higher antioxidant activity compared to the other two microalgae, followed by *Chlorella* sp. and *Isochrysis* sp. The antioxidant activity of *Dunaliella* sp is the highest because the concentration needed to inhibit 50% of the free radical is the lowest (13.13 mg/ml), which means that small amount of the crude is sufficient to inhibit the production of free radicals. As for *Isochrysis* sp., the IC50 does not achieve and the highest inhibition was 16.03% at the concentration of 14.5 mg/ml.

Free radicals are molecules that possess a single electron in an outer shell to make it very reactive (Verma *et al*., 2011). For most biological structure, free radical damage is closely associated with oxidative damage which could be prevented by enzymatic and non-enzymatic antioxidant such as vitamin C, vitamin E, quibiquinone and others.

Lipid protein and DNA are all susceptible to be attacked by free radical. Antioxidants are able to counter react against oxidative stress by scavenging the free radicals.

It is said that in a study a substance act as an antioxidant if it has an ability to reduce ROS donating hydrogen atom (Verma *et al.*, 2011). The reducing properties of pigment extract of *Dunaliella* sp. implies that it is able to accept hydrogen atom in a dose dependant manner.

By referring to table 2 of the High Performance Liquid Chromatography (HPLC) result on the carotenoid content in the pigment extract of *Dunaliella* sp. it shows that beta carotene is present in the crude extract and the study of Telfer (2002) states that beta carotene is able to accept electron. A study by Edge *et al*., (2007), showed that a carotene which has a long conjugation structure such as beta carotene and lycopene or having a carboxyl group in its

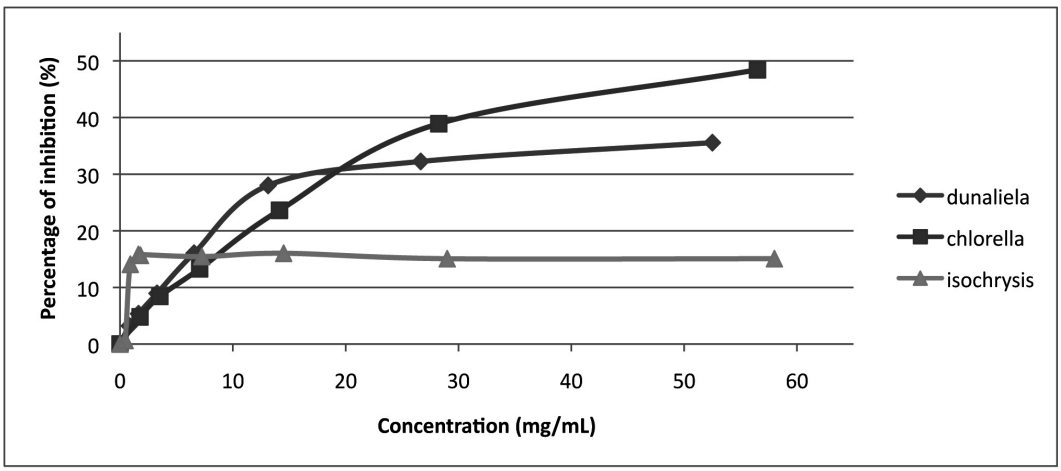

Figure 11: Antioxidant activity of *Dunaliella* sp., *Chlorella* sp. and *Isochrysis* sp.

structure such as astaxanthin and canthaxanthin will have a high chance to accept one electron. However in this study the scope did not touch on each and every carotenoid that is present in the crude but instead this study focused more on the effect of the total crude towards the DPPH compound. This shows that the crude of *Dunaliella* sp. can become a potential antioxidant.

Some of the examples of ROS are superoxide, hydroxyl, peroxyl and alkoxy radicals. Different environment stress factors like pollution, drought, temperature, excessive light intensities and nutritional limitation are able to increase the production of ROS (Marxen *et al*., 2007). As been stated before carotenoids and fatty acids are two examples for non-enzymatic classes of substance which are able to protect the organism from oxidative damage (Marxen *et al*., 2007) where carotenoids and fatty acids are known to process antioxidative properties. The EC50 value is to determine the amount of microalgae extract that is necessary to decrease the absorbance of DPPH by 50% (Marxen *et al*., 2007).

Most organisms that involve uptake of oxygen in their metabolism usually produce ROS as natural by products (He and Hader, 2002; Apel and Hirt, 2004). ROS are like superoxide, hydroxyl or peroxyl and mostly, the production of ROS is in equilibrium under non-stressed condition. Oxidative stressed will cause unstable and very reactive radicals (Fang *et al*., 2002). Highly reactive radicals can cause degenerative disease in human like cancer and cardiovascular disease. These reactive radicals also can cause oxidative damages to proteins DNA and lipids (Jacob and Burri, 1996) in both humans and microorganisms. Based on research done by Seis H. and Stahl W. (1995) stated that carotenoids and fatty acids are two examples for non-enzymatic classes of substances which are able to protect the organism from oxidative damage.

Based on figure 4.3 the crude show antioxidant activity, but the activity occurs at lower rates and below EC_{50}. Based on the study done by Edge *et al*., (2007) stated that the presence of hydroxyl groups on the rings of carotenoids will decrease the reduction potential. While the presence of carbonyl groups will increase the reduction potential means that the radical anion become less reducing. Thus it can be concluded that *Isochrysis* sp. contain carotenoids with more hydroxyl groups that lower the antioxidant property.

Conclusion

Dunaliella sp. contain the highest carotenoid percentage per dry weight (99.27%) followed by *Chlorella* sp. and *Isochrysis* sp. The carotenoids are toxic towards MCF-7 and normal cell where the LC_{50} value for all the microalgae is lower than 0.35ug/ml for MCF-7 and less than 0.26ug/ml for normal cell. *Chlorella* sp is effective to inhibit the growth of MCF-7 at lowest concentration while for normal cell, *Dunaliella* sp is toxic at lowest concentration. The effect of carotenoids on known disease showed that *Chlorella* sp. can delay the process of gastritis, while *Dunaliella* sp and *Isochrysis* sp. promote healing process during gastritis. However, *Dunaliella* sp. can restore the normal histology of the stomach faster than *Isochrysis* sp. This is supported by the antioxidant finding where *Dunaliella* sp. carotenoid contain high antioxidant activity which can reduce the inflammation and hence accelerate the gastric healing process. Apart from that, the ability of the microalgae carotenoids towards inhibiting the microbial activity showed that *Dunaliella* sp. has higher activity, followed by *Chlorella* sp. while *Isochrysis* sp. does not possess the antimicrobial activity. As a conclusion, *Dunaliella* sp. has highest carotenoids content that is toxic towards MCF7 cancer cell and can promote gastric healing as it contains antioxidant and antimicrobial properties.

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