MINISTRY OF EDUCATION AND TRAINING

VIETNAM ACADEMY OF SCIENCE AND TECHNOLOGY

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Research on using a number of advanced techniques and integrated technology to comprehensively process brown seaweed into useful products

Major: Chemical Engineering

Code: 9 52 03 01

SUMMARY OF CHEMICAL DOCTORAL THESIS

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PREAMBLE

1. The urgency of the thesis

Brown seaweed is determined to have many valuable ingredients such as alginic acid, fucoidan, fucoxanthin and phlorotanin... However, the exploitation and processing of brown seaweed in Vietnam is currently limited, brown seaweed is mainly processed into food at the household scale. The extraction of valuable products from brown seaweed only stops at a laboratory or pilot scale. Some studies have applied advanced techniques (ultrasonic extraction, enzyme-microbiological extraction, extraction using Microwave...) but only stopped at extracting specific ingredients, not yet providing a comprehensive processing process for brown seaweed.

2. Research objectives of the thesis

Research and evaluate the effectiveness of applying and integrating advanced technologies (microwaves, enzymes, ultrasound...) to extract useful substances from brown seaweed such as fucoxanthin, phlorotanin, fucoidan and alginate ...

Develop a comprehensive technological process for processing brown seaweed into high-value products.

Research and survey seaweed raw materials of the brown seaweed family to evaluate the content of alginate, fatty acids, lipid layer...

3. Main research contents of the thesis

Building a comprehensive technological process for processing Sargasum seaweed. sp. into high-value products such as fucoxanthin, phlorotanin, fucoidan and alginate using advanced techniques (enzymes, ultrasound, 3-phase centrifugation and membrane filtration).

Research on optimization of phenolic extraction technology from brown seaweed using microwave method.

Develop a technological process for extracting alginate from seaweed using the integrated ultrasound and enzyme method, evaluate biological effects, and develop basic product standards.

Research predicts the ability of some compounds extracted from seaweed to inhibit the enzyme Tyrosinade.

Research investigating alginate content, fatty acid content and lipid layer, predicting the skin whitening ability of some compounds isolated from brown seaweed.

Research and treatment of by-products of the processing process.

CHAPTER 1. OVERVIEW

1.1. Introducing Brown seaweed

1.1.1. General introduction

The phylum Brown algae (Phaeophyta or Ochrophyta), includes the class Phaeophyceae, including multicellular brown algae with many different morphologies and sizes, including 16 orders with about 285 genera and about 2040 species, of which about 1500 species have been discovered. identified worldwide. Except for a few freshwater genera (less than 1%), most brown seaweeds are marine and largely grow in subtidal areas. Of these, 95% of brown seaweed species are widely distributed in cold to temperate waters. In Vietnamese waters, authors Hau Le Nhu and Tu Nguyen Van have listed 827 species of seaweed, of which the Brown seaweed industry has 147 species.

The benefits of seaweed in general and brown seaweed in particular are huge. According to FAO statistics (1976), the estimated resource of the Brown seaweed industry in different geographical areas is 14,600,000 tons with a total exploitation output of 1,315,000 tons. They are used to produce seaweed glues such as alginate, agar or processed as pet food or fertilizer. Many species of brown seaweed are also important food sources for humans.

1.1.2. Chemical composition, activity and applications

Like other seaweeds, Brown seaweed contains basic substances such as carbohydrates (4-70%CK), proteins (3-24%CK), lipids (0.3-4.8%CK), and ash (14% CK). -45%CK). Seaweed in general and Brown seaweed in particular contain highly biologically active compounds such as pigments (carotenoids), polysaccharides (alginate, fucoidan), storage lipids, vitamins,... with potential applications. highly used in the medical and pharmaceutical field.

1.2. Brown seaweed processing technologies

1.2.1. Traditional technology

1.2.2. Modern technology

- Ultrasound assisted extraction (UAE)

- Enzyme assisted extraction (EAE)
- Microwave assisted extraction (MAE)
- Some other modern methods:
+ Pressurized liquid extraction (PLE)
+ Extrusion assisted extraction (ExEA)
+ Supercritical fluid extraction (SFE)

CHAPTER 2. RESEARCH SUBJECTS AND METHODS

2.1. Ingredient

Composition research sample: collected by direct sampling method and deep diving method. Seaweed samples were collected from seaweed beds, trash removed, washed with sea water, then dried to a humidity below 35%, then stored at the Institute of Natural Products Chemistry before processing. Samples were identified by Associate Professor. Dr. Dam Duc Tien – Institute of Marine Resources and Environment.

2.2. Research Methods

2.2.1. Method for determining total phenolic content

2.2.2. Method for determining alginate

2.2.3. In vitro screening method

2.2.4. Method for determining lipid content and composition

2.2.5. Method for determining the composition and content of fatty acids

2.2.6. Determination of Fucoidan content by colorimetric method

2.2.7. Determination of fucoxanthin content

2.3. Methods to evaluate biological effects

2.3.1. Method for evaluating safety indicators

2.3.2. Method for determining acute toxicity

2.3.3. Study on semi-chronic toxicity

2.3.4. Method to evaluate the effect of heavy metal elimination

2.3.5. Method to evaluate the ability to prevent osteoporosis

2.3.6. Test method for antioxidant activity

2.3.7. Test method for cytotoxic activity

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Using advanced techniques and integrated technology to process brown seaweed

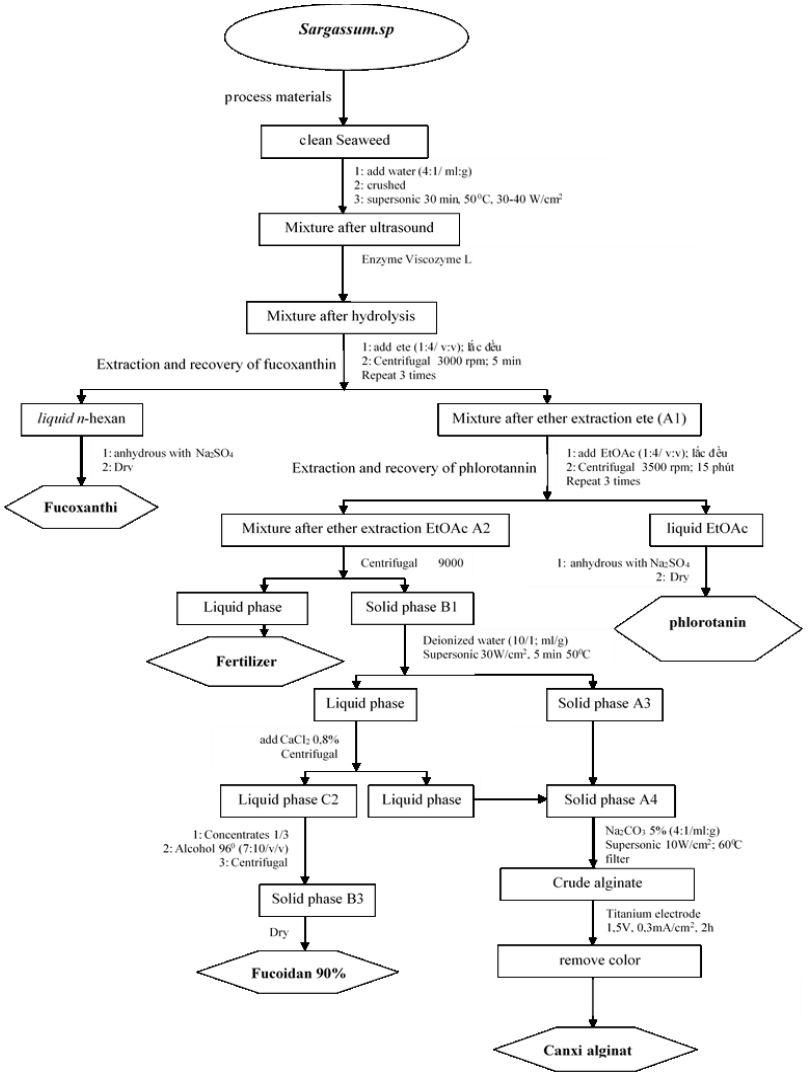

Figure 3.1. Process diagram using advanced techniques and integrated technology to process seaweed

The thesis has proposed a comprehensive technological process for processing seaweed Sargasum sp. into high-value products such as fucoxanthin, phlorotanin, fucoidan and alginate using the integrated enzyme-ultrasound method and advanced techniques (enzyme, ultrasound, 3-phase centrifugation and membrane filtration). The process is described as effective with the ability to recover synchronously and with high efficiency of products and minimize waste from abundant raw materials available in nature.

In addition, the thesis surveys a number of options with the aim of studying the influence of integrating different advanced techniques on the efficiency of the extraction process to create products. The results show that the method of using ultrasound and enzyme integration gives the most optimal results, simultaneously obtaining fucoxanthin, phlorotanin, fucoidan and alginate products with high content of active ingredients, thereby reducing time, solvents, wasting machinery and equipment, thereby reducing product costs and bringing high economic value. In addition, studies on the integrated use of 3-phase centrifugation and membrane filtration techniques have reduced product collection time by 30% compared to conventional methods.

3. 2. Results of research and survey of some raw brown seaweed samples

3.2.1. Research and survey of alginate content of some types of seaweed harvested in Vietnam's sea

Through the results of analyzing the Alginic acid content in Brown seaweed species that fluctuates by month, some authors show that: The accumulated Alginic acid content in different seaweed species has clear differences. The appropriate time to harvest Apricot seaweed is April and May every year.

Results of analysis of the main chemical components of 3 seaweed species show that protein content in seaweed samples fluctuates between 1.7 and 7.0%, lipid content < 2%, and ash content from 20%. .4% to 46.3%. High ash content proves that the seaweed

material contains high mineral salt content, so before processing it is necessary to wash away the minerals still attached to the seaweed plant.

The simultaneous presence of alginate and biopolymers such as fucoidan, laminaran and polyuromanan in brown seaweed shows that brown seaweed is not only a source of raw materials for alginate production but also a raw material for the production of biopolymers. To effectively exploit seaweed resources, it is necessary to build a complex technological process to simultaneously obtain the polysaccharides contained in them.

3.2.2. Research to investigate the content of fatty acids and lipid classes

The lipid content obtained in seaweed samples reached 0.07-2.11%. The main samples with concentrations above 0.5% accounted for more than half of the total research samples. There are 5 seaweed samples with total lipid content at an average level of 0.5-1% and 3 samples with levels as high as 1-2%, namely *Dictyota dichotoma* (RB04 VM-RD); *Sargassum sp. 2* (RB 03 LS); *Turbinaria turbinata* (RB 08 LS) with a content of 1.28%; 2.11% and 1.58%.

Researching the composition and fatty acid content of the total lipids of seaweed samples, we found that there is a diversity of fatty acids in the total lipid composition of the research samples, commonly from C14-C24. In particular, the brown seaweed sample RB 03 LS *Sargassum sp. 2* has the appearance of hexacosanoic saturated fatty acid (26:0) with a content of 0.41%. Fatty acids appear in the lipid composition of seaweed samples, including some fatty acids present in almost all research samples such as 14:0 saturated fatty acids; 15:0; 16:0 and 18:0 or unsaturated fatty acids such as 16:1n-9; 18:2n-6; 18:1n-9; 20:4n-6; 20:3n-6. However, the content of fatty acids is not uniform but is concentrated mainly in 14:0 acids; 16:0; 18:2n-6; 18:1n-9 and 20:4n-6 with the highest content reaching from 14.66-79.43%.

3.2.3. Research predicts the skin whitening ability of some compounds isolated from brown seaweed

Through synthesis of documents, there are 71 substances extracted from Seaweed that have been researched and their structures

determined by groups. The formulas and symbols of these compounds are shown in the appendix. In this study, we used the compound tropolone, which has been shown to inhibit tyrosinase, as a standard.

Compounds 3, 11, 16, 18, 38 and 45 were determined to have the potential to create strong binding affinity for the enzyme tyrosinase with binding energy values of -14, respectively. 06; -13.62; -12.02 and -12.13; -12.15 and -13.99 kcal/mol. In particular, the binding free energy of three substances 3, 11 and 45 exceeds the value obtained with the standard ligand tropolone.

Analyzing the formation interactions of the three compounds with the strongest binding affinity to the target biological target. The data obtained show that the majority of important amino acids form Therefore, the active site of the enzyme (His259, His263, Met280, Ser282, Ala286) participates in binding with the standard inhibitor tropolone.

Two-dimensional and three-dimensional interaction images of compounds 3, 11 and 45. The results showed that, among the three compounds showing strong binding affinity to sEH, only compounds 11 and 45 have the potential to inhibit the activity of this enzyme through direct interactions with amino acids. important in constituting the active site of the enzyme. Compound 3 was observed not to form hydrogen bonds with the tyrosinase enzyme, weak interactions participating in the interaction between the ligand and the enzyme include His61, His244, Val248, His263, Phe264, Val283. Therefore, it can be concluded that compound 3 is not a potential inhibitor of the studied receptor target.

3.3. Technique for extracting phenolics from brown seaweed

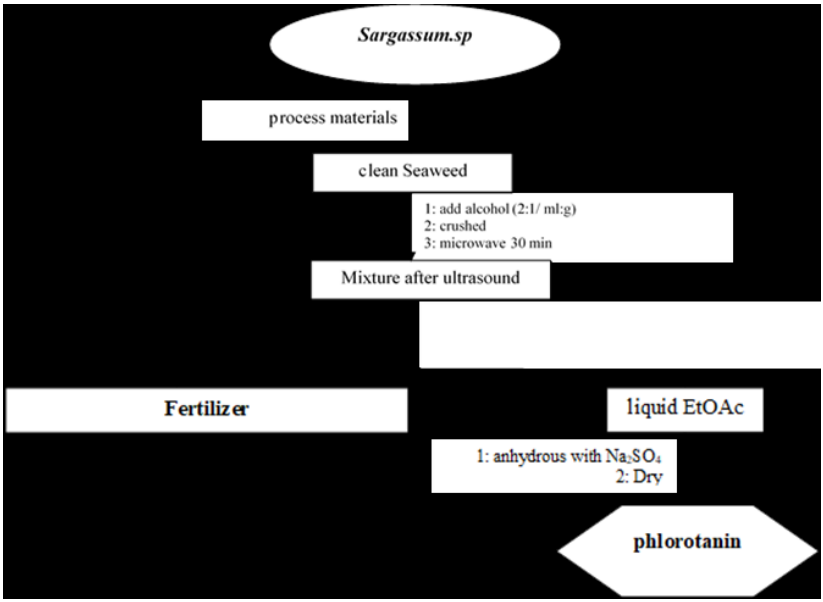

Figure 3.2. Phenolic extraction process from brown seaweed

Results of research on factors affecting the extraction of phenolics from brown seaweed using the microwave extraction method:

Conducting experiments to investigate factors affecting the phenolic extraction process from brown seaweed, respectively solvent concentration (ethanol 0%, 30%, 50%, 75%, 96%), solvent/raw material ratio. material (1/15, 1/20, 1/25, 1/30, 1/35, 40/1), extraction time (15, 30, 45,60, 75, 90 minutes), microwave power (80, 240, 400, 560, 800 W)

Selection results: Ethanol concentration 60%, solvent/material ratio 35/1, extraction time 60 minutes, extraction power 400W.

Results of optimizing phenolic extraction conditions from seaweed using response surface method (RSM):

The optimal total phenolic content of the extract is predicted to be 1.52 mgGAE/g, the optimal extract mass is predicted to be 8.6 mg. The optimal parameters of the extraction process are predicted to be: ethanol concentration 54.5%; Solvent/raw material ratio is 34.6; Extraction time is 64.28 minutes and microwave power is 473.6W. Conduct extraction experiments under conditions of 55% ethanol concentration, solvent/material ratio = 35; Extraction time is 65 minutes and microwave power is 640W and evaluation of phenolic content and extracted extract mass shows: experimentally obtained phlorotannin content is 1.50 ± 0.12 mgGAE/g; The extracted mass was 8.52 ± 0.11 mg, which was not significantly different from the prediction.

Evaluation of the activity of phenolic extract:

Table 3.1. Results of antioxidant activity assessment

No	Sample	SC_{50} (µg/mL)
1	Control (+) [ascorbic acid]	12.6
2	Control (-) [DPPH/EtOH+ DMSO]	-
3	Microwave sample	683
4	Ultrasound sample	590

Table 3.2. Results of evaluating the cytotoxic activity of phenolic extract samples from Padina crassa seaweed

No	Sample	Concentration inhibits 50% of cells (IC_{50}, µg/mL)		
		MCF-7	HeLa	PC3
1	Ultrasound sample	12.80	27.83	57.40
2	Microwave sample	12.78	42.68	67.25

3.4. Research on the process of fucoidan absorption

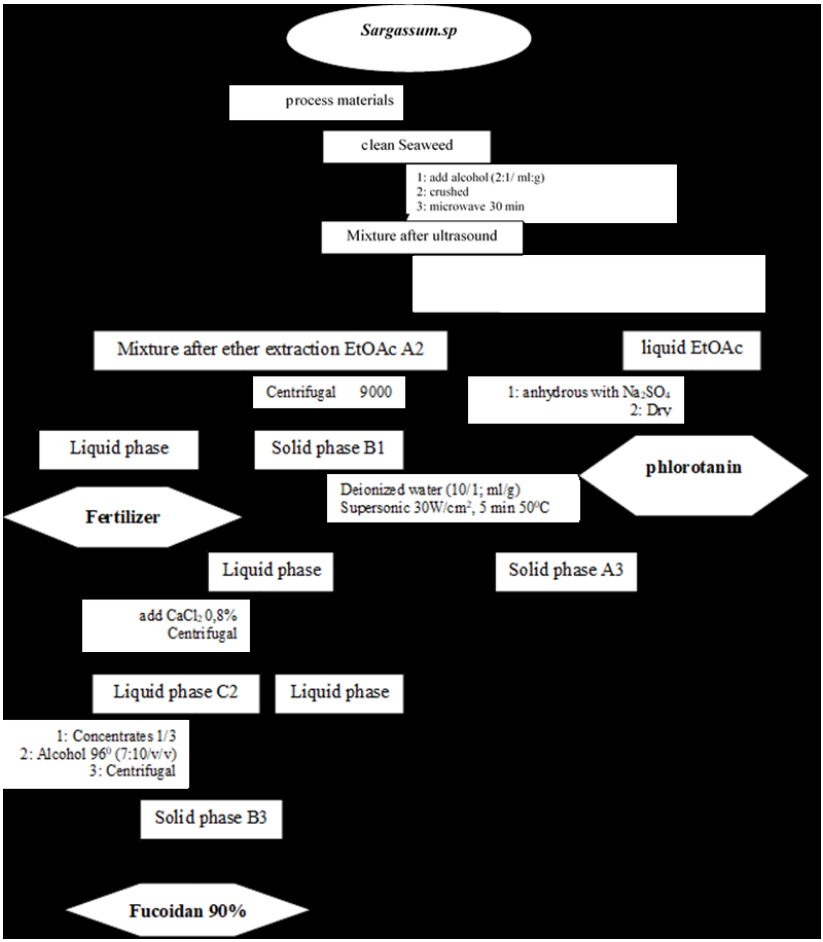

Figure 3.3. Fucoidan extraction process from brown seaweed

Research has been conducted on factors affecting the fucoidan extraction process, including: (i) Determining the solvent for fucoidan extraction; (ii) Effect of solvent: raw material ratio; (iii) Effect of temperature; (iv) Effects of time and ultrasound intensity; (v) Effect of CaCl2 content on the purity of fucoidan; (vi) Effect of ethanol concentration on the ability to precipitate fucoidan from the filtrate.

Optimal results show:

- Reduced water was chosen as the solvent to extract fucoidan from seaweed using ultrasound because it is easy to produce, non-toxic, low cost and no difference in performance compared to 0.1N HCL solvent.

- The solvent:raw material ratio is 8 (1/kg) suitable for extracting fucoidan from brown seaweed residue using ultrasound waves, helping to ensure process efficiency as well as optimize economic efficiency (reducing raw material costs). materials, energy...).

- The parameters of the ultrasound process to extract fucoidan from seaweed are: 40^{o}C, ultrasound intensity 58 w/cm^2, time 3 minutes.

- The $CaCl_2$ content to precipitate alginic acid in the extract is 0.75 g $CaCl_2$/liter of extract.

- Ethanol concentration of 60% is suitable to precipitate and recover the active ingredient fucoidan from the filtrate.

3.5. Research on alginate extraction techniques from brown seaweed

Figure 3.4. Alginate extraction process from brown seaweed

It was determined that enzymatic pretreatment of raw materials significantly increased alginate recovery efficiency. The following conditions are best for cell wall cellulose degradation using Htec2 enzyme: pH = 4.5 - 5.0; Hydrolysis temperature: 45oC; Htec2/substrate concentration was 1.5%; Hydrolysis time: 6-8 hours.

The conditions for obtaining alginate from seaweed residue using ultrasound have been determined: determine the alginate content in raw materials, seaweed residue is treated with a ratio of 0.1 M HCl solution: seaweed residue is 10 (w/w). At room temperature (temperature 25°C), the conditions for alginate extraction were determined by ultrasonic extraction at a temperature of 60°C, ultrasound intensity 58 w/cm2 (frequency 20 kHz)/ 6 minutes , the ratio of 2% Na2CO3 solution: seaweed residue is 10 (l/kg). Determining the concentration of 10% CaCl2 with the corresponding ratio CaCl2: Alginate = 2 (w/w) was chosen appropriately to precipitate and recover Ca(Alg)2 from NaAlg, bleaching the Ca(Alg)2 product with solvent. 5% NaOCl solution with 2% H2O2 equivalent to Ca(Alg)2 precipitate gives Ca(Alg)2 product, light yellow to ivory white.

3.6. Evaluation of the quality and some activities of calcium alginate from brown seaweed

3.6.1. Evaluation of the quality of calcium alginate

The research sample has a moisture value of 6.82%; Ash content reaches 31.08% and Alginic content reaches 20.67%. Besides, the calcium content in alginate powder is very high from 7-10%, showing that alginate powder is very good for bones and joints.

The results of the analysis of microbial criteria showed that in the research sample, *Coliform bacteria, S. Aureus, C. Perfringens, B. Cereus* were present with a content of ≤10CFU/g and two types of bacteria were absent. diseases are *E. Coli* and *Salmonella*. This result meets food safety standards issued by the Ministry of Health.

According to the analysis results presented in Table 3.31, Cu and Zn content have values ≤10 ppm; The content of heavy metals Pb, Cd, and Hg is much lower. The values of heavy metal content are

within the allowable threshold according to food safety regulations of the Ministry of Health.

3.6.2. Evaluation of anti-osteoporosis activity

Calcium alginate at a concentration of 20 µg/ml showed the ability to enhance collagen synthesis compared to the negative control, the percentage of collagen synthesized was 111.10%. At a concentration of 4 µg/ml, the test sample had the ability to stimulate mineral formation slightly compared to the negative control, the percentage of minerals synthesized was 115.42% ($P<0.05$).

3.6.3. Results of safety research

- Gelalginate preparation does not cause acute toxicity to mice orally with a maximum tested dose of 2.5 g/kg body weight.

- Gelalginate preparation at a dose of 500 mg/kg/day when given semi-chronically for 28 days did not affect the weight gain of experimental mice compared to the control ($p>0.05$), did not affect the weight gain of experimental mice compared to the control ($p>0.05$). affects hematological parameters compared to the control group ($p>0.05$), does not affect the weight of liver, kidney, spleen compared to the control group ($p>0.05$), does not increase the AST index, Liver ALT compared to control, did not affect basic kidney enzyme index compared to control group ($p>0.05$).

- Gelalginate preparation at a dose of 1000 mg/kg/day when given semi-chronically for 28 days did not affect the weight gain of experimental mice compared to the control ($p>0.05$), did not affect the weight gain of experimental mice compared to the control ($p>0.05$). affects other hematological parameters compared to the control group ($p>0.05$), does not affect the weight of liver, kidney, spleen compared to the control group ($p>0.05$), does not increase the AST index , Liver ALT compared with control.

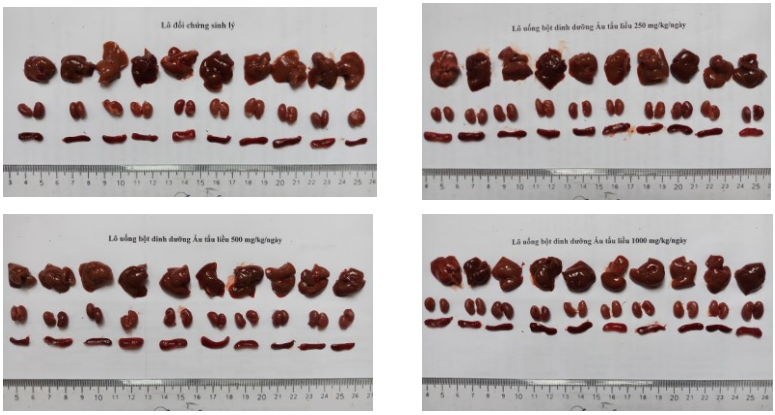

Figure 3.5. Images of liver, kidney, and spleen of mice after semi-chronic toxicity experiment

3.6.4. Evaluation of the heavy metal removal effect of alginate preparations

Calcium algiante is effective in supporting heavy metal excretion in animals with heavy metal poisoning. Specifically, for the results of in vitro experiments on experimental animals mentioned above. Using calcium alginate continuously for 9 weeks at a dose of 0.1g/kg body weight/day helps eliminate heavy metal content from the animal's body by 60 - 70% depending on the specific part. Prompt use of calcium alginate salt immediately after heavy metal poisoning will result in more effective detoxification.

3.7. Research on treatment of by-products of the processing process

By-products of seaweed processing after alginate extraction are mainly seaweed residue and some waste. These ingredients have a quite high pH (~ 10), so we process them using microbial products to ferment to create organic fertilizer.

The selected preparations are EMZEO, EM-fert 1 and S.EM. The results showed that the three selected microbial products were all effective in treating organic compounds from brown seaweed residue

after extracting fuicoidan and alginate. The cellulose degrading group works to assimilate substrates and is the basis for other microorganisms to function and grow. S.EM preparation has the best C/N ratio of 12.33 among the three preparations for further research.

Results of monitoring the process of composting seaweed residue with S.EM product show that: After 6 weeks of composting, the seaweed residue after extracting alginate and fuicoidan has a brown, slightly yellow color and turns into black, loose organic fertilizer. spongy, soft to the touch, no unpleasant odor.

Under the effect of the microorganism in the organic waste treatment probiotic S.EM, the physicochemical properties of seaweed residue after extracting fuicoidan and alginate have certain changes. OC content decreased to NTS contents; P2O5 and K2O increase.

Table 3.3. Some criteria to evaluate seaweed residue before and after incubation of S.EM product

No	**Evaluation criteria**	**Unit**	**Unit**	**After incubation**
1	Sensory		Light brown color, tough, hard seaweed residue	Black, spongy. soft
2	OC	%	67.23	37.12
3	N_{TS}	%	1.34	3.01
4	P_2O_5	%	0,05	0,20
5	K_2O	%	1,77	2,15

CONCLUSIONS AND RECOMMENDATIONS

1. The thesis has proposed a comprehensive technological process for processing Sargasum seaweed. Sp. into high-value products such as fucoxanthin, phlorotanin, fucoidan and alginate by a method that integrates advanced techniques (enzymes, ultrasound, 3-phase centrifugation and membrane filtration) effectively with synchronous recovery and high performance products and minimize waste from abundant raw materials available in nature.

2. A technological process has been developed to extract phenolics from brown seaweed using the microwave method; Research on influencing factors and optimization of phenolic extraction conditions from brown seaweed using response surface method (RSM); evaluated the in vitro antioxidant and cytotoxic activity of phenolics at moderate levels.

3. Researched the process of integrating a number of advanced technologies to obtain alginate; Research on anti-osteoporosis activity, evaluate the safety and effectiveness of calcium alginate gel preparations. The results showed that no toxicity was observed in the preparations at the tested doses. At concentrations of 20 µg/ml and 4 µg/ml, calcium alginate showed the ability to enhance ALP enzyme activity, enhance collagen synthesis and stimulate mineral formation at a statistically significant level compared to the negative control. , specifically: % stimulation of ALP activity is 124.41% (20 µg /ml) and 118.16% (4 µg /ml); % collagen synthesized 111.10% (20 µg /ml); % mineral stimulation 115.42% (4 µg/ml). The alginate gel preparation recorded the effect of eliminating heavy metals in rats when used starting from a test dose of 0.1g/kg of rats.

4. Surveyed and evaluated brown seaweed species in Vietnamese waters, the results showed that: (i) The genus Mo seaweed of the Brown seaweed family has great potential in terms of reserves as well as quality for isolating algianate with high content. The average amount of alginate is up to over 30%; (ii) Algae samples have total lipid content from 0.07 to 2.11%; All contain saturated fatty

acids, monounsaturated fatty acids, and especially polyunsaturated fatty acids (PUFA).

5. Research has been conducted to predict the ability of some compounds extracted from seaweed to inhibit the enzyme Tyrosinade, thereby orienting the development of skin whitening products from active ingredients extracted from brown seaweed.

Recommendations: (1) Continue research and orientation to create some other valuable products from brown seaweed (extracting phlorotanin compounds used in cosmetics, extracting trace minerals, vitamins, lipids... for application). used in the production of functional foods...); (2) Create a finished product from alginate gel to detoxify metals.

NEW CONTRIBUTIONS OF THE THESIS

- For the first time, integrated use of advanced techniques (ultrasonic extraction, microwave extraction, enzyme extraction, membrane filtration and 3-phase centrifugation) and integrated technical technology (ultrasound - enzyme, membrane filtration, centrifugation) 3-phase center) simultaneously creates valuable products from a research object.

- For the first time, conducting research on optimizing phenolic extraction conditions from brown seaweed using the microwave method using response surface method.

- For the first time, we have developed a deep technological process to extract alginate from seaweed using the method of integrating ultrasound and enzymes; Studying the alginate absorption process.

- For the first time, the anti-osteoporosis activity, safety and effectiveness of the calcium alginate gel preparation and the effect of eliminating heavy metals in mice have been evaluated with good results.

- Phenolic compounds from seaweed have been screened for good skin whitening effects through molecular docking, guiding further experimental research.

LIST OF PUBLISHED PROJECTS

1. Tran Quoc Toan, **Tran Duy Phong**, Dam Duc Tien, Nguyen Manh Linh, Nguyen Thi Mai Anh, Pham Thi Hong Minh, Le Xuan Duy, Do Huu Nghi, Hai Ha Pham Thi, Pham Tri Nhut, Ho Sy Tung, and Nguyen Quang Tung; Optimization of Microwave-Assisted Extraction of Phlorotannin From *Sargassum swartzii* (Turn.) C. Ag. With Ethanol/Water; Natural Product Communications. 2021, Vol 16(2): 1–11. Doi: 10.1177/1934578X21996184.

2. Trần Quốc Toàn; Phạm Quốc Long; Hoàng Thị Bích; **Trần Duy Phong**; Phạm Minh Quân; Processing process of seaweed (Sargassum.SP) to obtain Fucoxanthin, Phlorotanin, Fucoidan and Alginate products by using high-intensity ultrasound combined with enzymes; GPHI belongs to Utility Solution Patent No. 2606, issued under Decision No. 3750w/QD-SHTT, March 8, 2021, Vietnam Intellectual Property Office.

3. Đặng Thị Phương Ly, **Trần Duy Phong**, Trần Quốc Toàn, Đoàn Lan Phương, Trịnh Thu Hương, Đặng Thị Minh Tuyết, Đào Thị Kim Dung, Lại Phương Phương Thảo, Hoàng Thị Bích, Phạm Minh Quân, Đàm Đức Tiến, Lưu Văn Huyền, Phạm Quốc Long; Initial assessment of Lipid content and fatty acid composition of some brown seaweed species in the North and Central Coast region - Vietnam; Collection of scientific reports National Science Forum, 2019, 579-585.

4. Trịnh Thị Thu Hương, Đào Thị Kim Dung, Phạm Thu Huế, Lê Tất Thành, Đỗ Thị Thảo, Nguyễn Thị Cúc, Phạm Minh Quân, Trần Quốc Toàn, **Trần Duy Phong,** Phạm Quốc Long; Evaluation of biochemical indicators and anti-osteoporosis activity of the active ingredient calcium alginate from Vietnamese seaweed; Collection of scientific reports National Science Forum, 2019.