International Journal of GEOMATE, Aug., 2016, Vol. 11, Issue 24, pp. 2391-2396
Geotec., Const. Mat. & Env., ISSN: 2186-2982(Print), 2186-2990(Online), Japan

DETERMINATION OF PHYTOCHEMICAL COMPOUND FROM *Spirogyra* sp. USING ULTRASONIC ASSISTED EXTRACTION

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ABSTRACT: Currently, the use of phytochemical compounds from macro algae for cosmetic and pharmaceutical purposes is popular. This study aimed to evaluate ultrasound assisted-extraction (UAE) method on phytochemical components (gallotannin, total flavonoids content, total saponin content and total phenolic compounds) in *Spirogyra* sp.. The ultrasonic devices with same input power of 5 watts and varying 3 frequencies (45 kHz, 210 kHz and 1 MHz) of UAE coupled with 3 solvents (ethanol, methanol and acetone) were investigated. Treated samples were collected after sonication for 120 min. The results revealed that UAE method showed the highest performance in yield extraction compared with hot water. The highest yields of gallotannin and total flavonoids content were obtained by sonication at 45 kHz with 2.7 mg/g and 3.5 mg/g acetone but the highest yield of total phenolic compounds was found when sonicated at 45 kHz with methanol and total saponin content was found with ethanol. Thus, UAE at 5 watts, 45 kHz. showed the best result in yields extraction of those phytochemical compounds. While the suitable solvents for phytochemical compounds extraction differed and depended on the purpose and phytochemical type. UAE in combination with acetone had a suitable effect on *P. digitatum* inhibition while UAE in combination with acetone, ethanol and methanol showed non significantly different on *E.coli* inhibition.

Keywords: Ultrasound assisted-extraction, Phytochemical, Spirogyra sp., Escherichia coli, Penicilium digitatum

1. INTRODUCTION

Ultrasound-assisted extraction (UAE) has proved to be a particularly effective extraction method to reduce the extraction temperature, amount of solvent and the extraction time. This method is especially useful for the extraction of bioactive compounds. Usually, hot water treatment at 80°C for 4 hr. has been used for extraction of *Spirogyra* sp. [1]. However, hot water extraction in polysaccharides is associated with long duration and high temperature which lead to the degradation. [2] found that using ultrasonic at a frequency of 3 2 0 KHz with water extraction of polysaccharides in *Codinopsis pilosula* was effective to increase polysaccharide.[3] investigated different solvents in phytochemical extraction of *Canna indica* by UAE method. It was shown that acetone extract contained highest antioxidant activity, high amount of flavonoid and phenolic compounds. Combination of high frequency with extraction method is the technique of using ultrasonication combine with organic solvent or water solution to extract the substances from proposed materials. Ultrasonic enhances the high frequency through the carrier likes water or organic solvent. This leads to bubbles formations which form the shrinking and expanding of bubbles cycle. During bubble expansion, the substances were pulled out from materials then dissolved in the extracted solvent. Also, the bubble collapse causes high pressure and high temperature which leads to cell destruction which better increases antioxidant extraction by the solvent. There are many factors afferhing the efficiency of this method such as ultrasonic frequency, time and extraction solvent.

Tao (*Spirogyra* sp.) is a genus of filamentous freshwater green algae in the Division Chlorophyta, Order Zygnematales, Family Zygnemataceae. It is named after the helical or spiral arrangement of the chloroplasts. There are more than 400 species of *Spirogyra* in the world [4]. The algae grow in the clean standing water. For moderate quality of clear water, the turbidity would not exceed 10 NTU, temperature 15-27°C and pH 6-7.8. *Spirogyra* sp. is consumed by people in the north and northeast of Thailand as a traditional food. It contains high amount of nutritional compositions including basic nutrients such as carbohydrates, fats, proteins, multivitamins, minerals and antioxidants [5]. *Spirogyra* sp. was screened against three bacteria:

Pseudomonas solanacearum, *Escherichia coli* and *Clavibacter michiganense* and three plant pathogenic fungi: *Fusarium oxysporum*, *Curvularia* species and *Aspergillus niger*. Its antimicrobial property was found to be effective against the entire test organisms [4]. Moreover, *Spirogyra* phytochemical components (alkaiods, steriods, flavonoids, tannins, terpanoids) exhibit antimicrobial activity against *Escherichia coli* and *Candida albicans* [6].

In this study aimed to evaluate ultrasound assisted-extraction (UAE) methods on phytochemical components (gallotannin, flovoniods and total phenolic compounds) in *Spirogyra* sp.

2. MATERAILS AND METHODS

2.1 *Spirogyra* sp. preparation

Fresh *Spirogyra* sp. were collected from the low flowing stream located at SobPerng, MaeTeang, Chiang Mai Provinces and transported to the Postharvest Physiology Research Laboratory, Chiang Mai University. The algal samples were cleaned with distilled water and dried by hot air oven at 60 ℃ for 24 hr.

2.2 Preparation of extracts

Five gram of each *Spirogyra* sp. sample was out roughly and extracted by different methods as the following; hot water (95˚C), UAE (45 kHz, 210 kHz and 1 MHz) with same ultrasonic input power of 5 W in combination with various solvents (ethanol, methanol and acetone). Treated samples were collected at 120 min. during extraction period. Then, the extract of each sample was analyzed for the phytochemicals components.

2.3 Phytochemical analysis

2.3.1 Determination of Gallotannin

Algal extracts (50µl), in test tubes were made up to 1 ml with distilled water. One hundred µl of 0.4 N sulphuric acid and 600 µl of rhodanine were added to the diluted extracts. After 5 min, 200 µl of 0.5 N potassium hydroxide was added followed by 4 ml distilled water after a further 2.5 min. The mixtures were left for an additional 15 min at room temperature and evaluated by UV-vis spectrophotometer (520 nm) which methanol was used as a blank. The mixtures were analyzed in triplicates to measure gallotannin concentration by gallic acid equivalents (GAE) Makkar (1999) [7].

2.3.2 Determination of Total Saponin content

Algal extract (0.25 ml) was pipetted and put into a test tube then, 0.25 ml of vanillin reagent and 2.5 ml of 72% H_2SO_4 were added (in ice bath). The mixture was heated in water bath at 60˚C for 10 min, and then cooled. The absorbance of mixture was measured with spectrophotometer at 544 nm. Diosgenin was used as a reference standard. The test was performed in duplicate. Determination of total sapogenin was carried out according to the method of Hiai *et al.* [8].

2.2.3 Determination of Total phenolic content

Total phenolic content was determinated by the Folin–Ciocalteu colorimetric method, based on the procedure, gallic acid was used as standard phenolic compound. Briefly, 50 µl (two replicates) of the filtered extracts were mixed with 450 µl of distilled water and 2.5 ml of 0.2 N Folin–Ciocalteu reagent. After 5 min, 2 ml of saturated sodium carbonate (75 g/l) was added. The blue-colour solution was measured by spectrophotometer at 765 nm after incubation at 30˚C for 1.5 h with intermittent shaking. Quantitative measurements were performed, related to standard calibration curvet 20, 100, 200, 300, 400, 500 mg/l of gallic acid in 80% methanol. The total phenolic content was expressed by gallic acid equivalents (GAE) as milligrams per game of dry material.

2.2.4 Determination of Total Flavonoid content

Total flavonoid content was measured by aluminum chloride colorimetric assay. An aliquot (1 mL) of the extracts or standard solution of (+)-catechin (20, 40, 60, 80 and 100 mg/L) was added to 10 mL volumetric flask, containing 4 mL of distilled deionized water (dd H_2O). The flask was added with 0.3 mL 5% NaNO2. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At the sixth minute, 2 mL 1 M NaOH was added and the total volume was made up to 10 mL with dis trilled water. The solution was mixed and the absorbance was measured against by UV-VIS Spectrophotometer Lambda 5 (510 nm). 5. Total flavonoid contents were expressed as milligrams of (+)-catechin equivalents (CE) per 100 g dry weight (mg CE/100 g dw). All samples were analyzed in triplicate.

2.4 Antibacterial activity

Antibacterial activity was evaluated using gram negative bacteria *E.coli* 0157:H7 as a propose bacterial. First, bacteria strains were sub-cultured

(37˚C, 24 hr). The effects of various extracts against bacterial strains were determined by zone of inhibition (ZOI) method. The $1x10^6$ of cell suspension per ml was spread in the plates then drilled by sterile cork borer (3 mm in diameter). After that, each drilled plate was filled with 100 µl of *Spirogyra* sp extracts after UAE in combination with different solutions (ethanol, methanol and acetone) extraction method. Amoxicillin was used as a positive control while water was used as a negative control. Then, plates were incubated at 37˚C for 24 hr. The diameter of zone inhibition was measured in millimeter (mm.). All experiments were performed in triplicate.

2.5 Antifungal activity

7-days-old *Pennicillium digitatum* spore on potato dextrose agar (PDA) plate was washed then suspended in the sterile distilled water to produce a final concentration of $1x10^6$ spores per ml. The volume of 20 µl spore suspension was spread on PDA with the mixture of 5000 mg/l *Spirogyra* sp. extracts. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

3.1 Phytochemical analysis

3.3.1 Effect on Gallotannin Yield

Table 1 Phytochemical yields (wt.%) of *Spirogyra* sp. extract by Ultrasound assisted extraction

Frequency of UAE	Solvents	Gallotannin Contents	Total Saponin Contents	Total Phenolic Content	Total Flavonoids Contents (mg/g)
	Hot water	0.03a	0.44a	0.002a	1.52a
	Ethanol	0.65b	2.55b	0.001b	1.62a
45 kHz	Methanol	0.62b	2.38b	0.001b	1.52a
	Acetone	0.51b	2.23b	0.001b	1.25a
	Ethanol	0.87bc	2.02bc	0.004a	3.12c
210 kHz	Methanol	0.81bc	1.97bc	0.004a	2.21b
	Acetone	0.70bc	1.88c	0.003a	2.03b
	Ethanol	2.74d	0.88d	0.002ab	2.62bc
1 MHz	Methanol	2.17d	0.78d	0.001b	2.48b
	Acetone	1.93d	0.74d	0.001b	2.31b

The data followed by the same letter within the column are not significantly different (*P = 0.05)

Gallotannin yield by UAE in combination with different kind of solvents were determined. All UAE treatments at 5 watt, 45 kHz for 120 min. showed higher performance on gallotannin extraction than hot water treatment. Sonication affected cell membrane breakdown reduced on extraction time and increased the yield increased the UAE produced cavitation that gave highest efficiency at low frequency range (20 to 100 kHz) and high power or high intensity ultrasound [9]. In addition to UAE, the phytochemical yields depended on solvents (Table 1). UAE combined with acetone treatment showed the highest yield on gallotannin extraction (2.7 mg/g). UAE extraction combined with methanol and ethanol treatments (0.87 mg/g and 0.65 mg/g.) showed no significant difference. (Fig. 1)

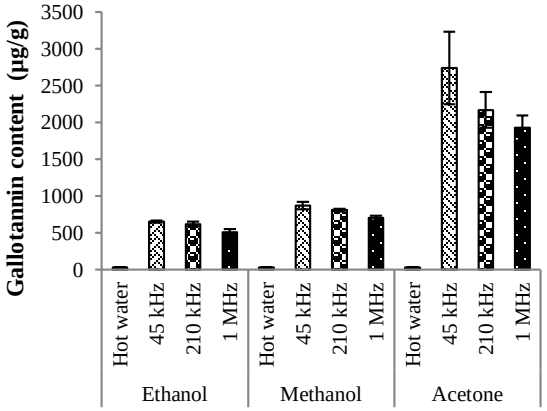

Fig. 1 Gallotannin yields of *Spirogyra* sp. extracted by UAE

3.3.2 Effect on Total Saponin Contents

For most of the *Spirogyra* sp. samples, the highest yield (2.5 g/g) of total saponin contents was UAE in combination with ethanol at 5 watt, 45 kHz for 120 min. (Fig. 2) followed in UAE in combination with methanol and acetone, the yields were 2.0 and 0.8 mg/g. effectively total saponin contents from UAE treatment was higher these treatment with than hot water The UAE extraction technique incombination with ethanol was developed for the fast extraction of saponins from *Eclipta prostrasta* L. [10] UAE has an advantage of accelesating the extraction process, causing less damage to the structural and molecular properties of the samples. Moreover, UAE require shorter time, less solvent and fine, higher exraction yield.

3.3.3 Effect on Total Phenolic Content

UAE combined with methanol treatment at 5 watt, 45 kHz for 120 min. had the best performance on yield extraction as 3.6 mg/g which was significantly different when compared with ethanol and acetone combination treatments ($p<`0.05$).

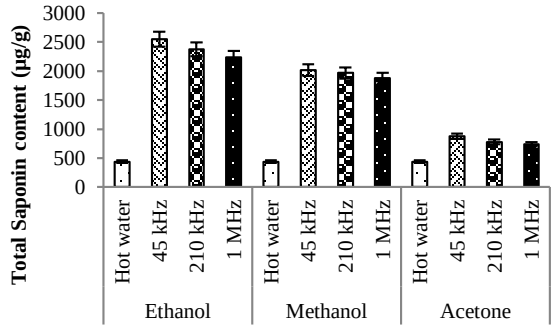

Fig. 2 Total saponin content of *Spirogyra* sp. extracted by UAE.

While UAE combined with ethanol and acetone treatments showed 1.4 and 1.7 mg/g respectively on yields extraction with no significant difference on the frequency of UAE treatments (Fig. 2). Methanol is one of the most frequently used solvent for extraction. It has a polarity of 6.6 compare with 5.2 for ethanol and 5.1 for acetone. This difference in polarity has demonstrated the amount of total phenolic content in the extract [11]. In this case, [9] reported that methanol had higher ability on total phenol extraction for *Mesembryanthemum edule* L. *Aizoaceae* shoot than ethanol at the levels of 104.7 and 74.2 mg GAE.g^{-1}DW respectively.

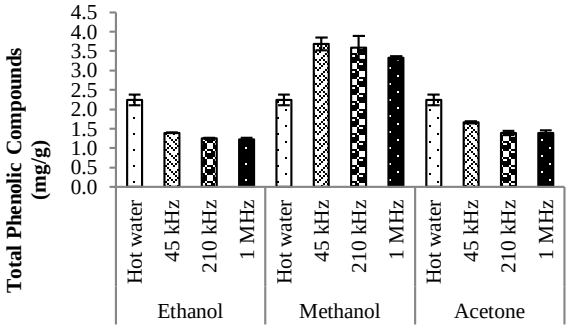

Fig. 3 Total phenolic compounds yield in *Spirogyra* sp. extracted by UAE

3.3.4 Effect on Total Flavonoids Contents

Flavonoids are strong antioxidants capable of reacting with scavenging oxygen species because of their phenolic hydroxyl groups [12]. The highest yield of total flavonoids was 3.1 g/g by UAE at 5 watt, 45 kHz for 120 min. in combination with methanol. While UAE in combination with acetone and ethanol gave the yield of 2.6 and 1.6 g/g respectively. All of the frequencies showed no significant difference on yield extraction of total flavonoids contents (Fig. 4).

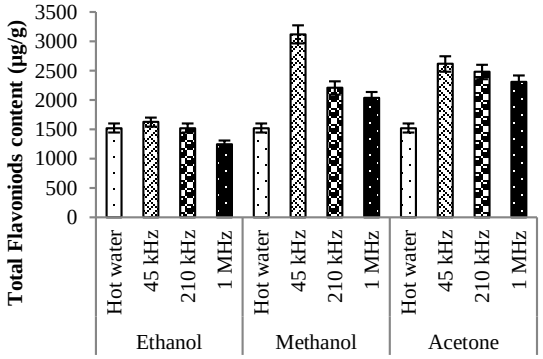

Fig. 4 Total flavonoids content of *Spirogyra* sp. extracted by UAE

3.4 Antibacterial activity

The effect of *Spirogyra* sp. extracts on *E.coli* 0157:H7 growth inhibition showed that *Spirogyra* sp. extracts by UAE (45 kHz, 5 watt for 120 min.) in combination with different solutions (ethanol, methanol and acetone) extraction method inhibited the growth of *E.coli* 0157:H7. The *Spirogyra* sp. extracted from UAE in combination with ethanol and acetone showed the highest results on *E.coli* 0157:H7 growth inhibition as 11.7 mm next is UAE in combination with methanol as 11.0 mm while the *Spirogyra* sp. extract from hot water method showed the negative result on *E.coli* 0157:H7 growth inhibition (Fig. 5) This is relevant to Rutikanga *et al.* [6] who reported that *Spirogyra* sp. extract from methanol extraction method showed the best inhibition on *E. coli* and *Candida albicans* growth rate. Opposite with the *Spirogyra* sp. extract from hot water method.

3.5 Antifungal activity

The effect of *Spirogyra* sp. extracts on *P. digitatum* growth inhibition showed that *Spirogyra* sp. extracts by UAE (45 kHz, 5 watt for 120 min.) in combination with different solutions (ethanol, methanol and acetone) extraction method inhibited the growth of *P. digitatum*. The *Spirogyra* sp. extract from UAE in combination with acetone showed the best results on *P. digitatum* growth inhibition with non-microbial growth. Next is UAE in combination with methanol and ethanol with the number of colony forming at 134 and 286 cfu respectively. While the *Spirogyra* sp. extracts from hot water method showed microbial growth rate similar to control treatment with no significantly different in results. (Fig.6)

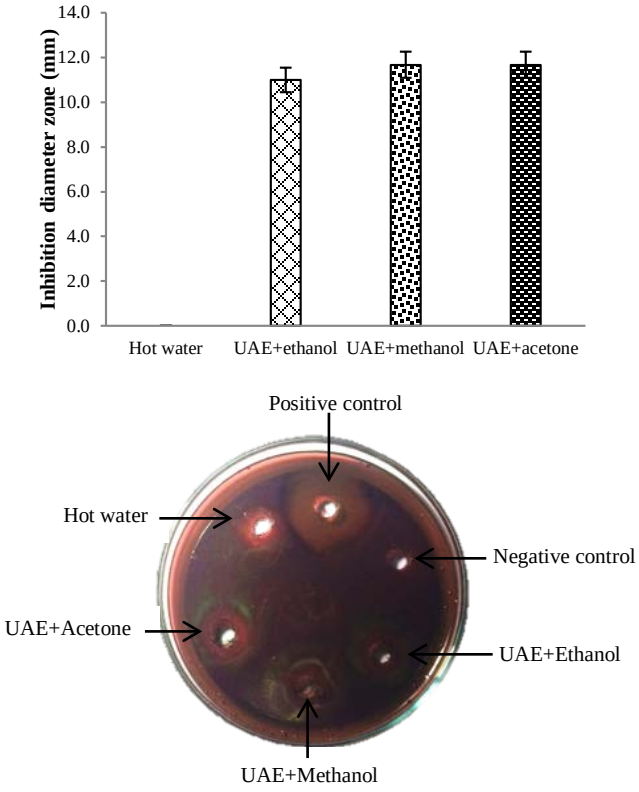

Fig. 5 Antimicrobial activity of *Spirogyra* sp. extracted by UAE in combination with deference solvents on *E.coli* 0157:H7

Phytochemical has the effective on antibiotic. [13] reported that flavonoid and phenolic compound inhibited the growth of *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Escherichia Coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus aureus* and *Staphylococcus epdermidis*. Furthermore, type of solvent is one of the important factor that optimize the extraction ability in many plant bioactive [14]. The result showed the relevant between type of solvent and UAE extraction method. The solvents that have best result on tannin and saponin extraction also gave the positive results on microbial inhibition these because saponin and tannin are bioactive substances which have the effective on microbial inhibition

4. CONCLUSION

Ultrasound Assisted-Extraction (UAE) with the electric power of 5 watt, frequency of 45 KHz for 120 min is the optimum condition for phytochemical extraction in *Spirogyra* sp. Methanol is the appropriate solvent for total phenolic extraction.While acetone is the appropriate solvent for gallotanin and flavonoid extraction in *Spirogyra* sp. UAE in combination with acetone had an suitable effect on *P. digitatum* inhibition while UAE in combination with acetone, ethanol and methanol showed had no significantly different on *E.coli* inhibition.

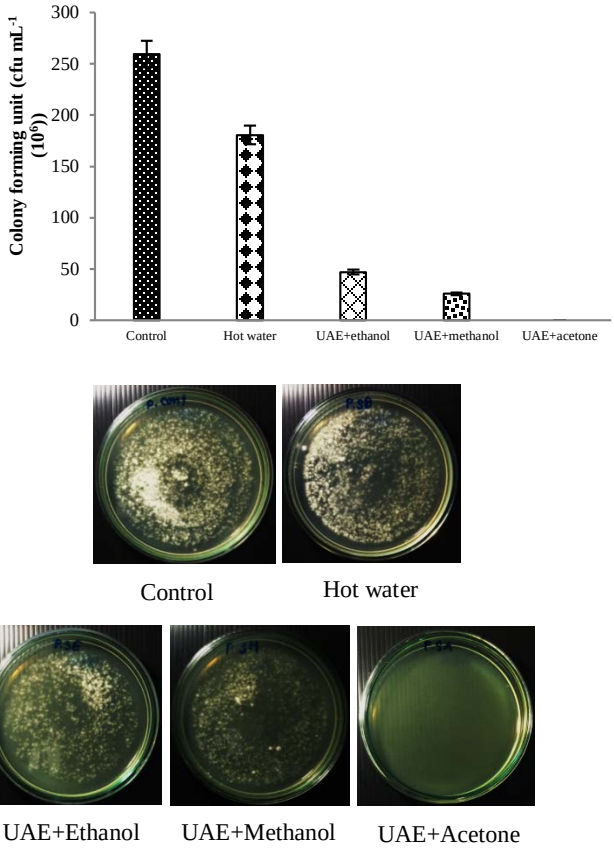

Fig.6 Antifungal activity of *Spirogyra* sp. extracts by UAE in combination with deference solvents on *P. digitatum*

5. ACKNOWLEDMENTS

This work was supported by Graduate School Chiang Mai University and National Innovation Agency. The authors are sincerely grateful to Department of Biology, Faculty of Science Chiang Mai University, Faculty Engineering and Ago-Industry, Maejo University and School of Life and Environmental Sciences, University of Tsukuba for providing the laboratory facilities.

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International Journal of GEOMATE, Aug., 2016, Vol. 11, Issue 24, pp. 2391-2396.
MS No. 5127j received on July 30, 2015 and reviewed under GEOMATE publication policies.
 Pertinent discussion including authors' closure, if any, will be published in April 2017 if the discussion is received by Oct. 2016.
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