International Journal of Agricultural Technology 2017 Vol. 13(7.3): 2529-2537
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Toxicological, Phytochemical and Antioxidant Activity Evaluation of *Nemalionopsis shawii* Skuja from Thailand

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Wanninee C., A. Doungporn, L. Narissara and S. Jantana (2017). Toxicological, phytochemical and antioxidant activity evaluation of *Nemalionopsis shawii* Skuja from Thailand. International Journal of Agricultural Technology Technology 13(7.3): 2529-2537.

The freshwater red alga *Nemalionopsis shawii*, is attached on the rocks in shallow stream located various places in Thailand. Pharmacological studies have not been investigated the toxic and biological activities for utilization. Therefore, the objectives were to examine acute toxicity, total phenolic, flavonoid contents and antioxidant activity. The crude extracts were tested for DPPH scavenging activity antioxidant activities, total phenolic content (TPC) and flavonoid contents (FC) were investigated by using Folin-Cioealteu and colorimetric aluminum chloride assays, respectively. Acute toxicity was tested with a single oral administration of the extract at a dose of 2 and 5 g kg^{-1} body weight. Mortality, behavior, amount of food intake, body weight, and any abnormalities of the visceral organs, were observed. The result showed that, the extract caused neither mortality, nor abnormalities. The *Nemalionopsis shawii* extracted shown the antioxidant activities in all extracts. The highest TPC, FC and DPPH were in ethyl acetate (20.967 ± 0.677 mgGAEg^{-1}extract, 58.326±0.857 mg QE g^{-1}extract and IC_{50} = 0.408±0.025 mg mL^{-1},respectively). The total phenolic and flavonoid contents in the extracts were determined and correlated with the antioxidant activity. Results suggested that this algae possess antioxiant potential which could be considered for future applications in dietary supplements or cosmetics industries.

Key words: red algae,acute toxicity, flavonoid,phenolic

Introduction

Freshwater red macroalgae were considered to be any algae visible to the naked eyes and recognizable in the field. Freshwater red algae constitute only about 3.4 % of the total number of the division Rhodophyta (Sheath, 1984). The genus, *Nemalionopsis* (Thoreales, Rhodophyta) contains approximately 2 species worldwide (Kumano, 2002). The red macroalga, *Nemalionopsis* is occurring mostly in warm and hard water from Asia and North America (Necchi, 2016). In Thailand, *Nemalionopsis shawii* was found in Mae Sa stream, Doi Suthep-Pui National Park, Chiang Mai province (Kunpradid and Peerapornpisal, 2001), Ping river and Nan river

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(Kunpradid, 2005), Kham watershed area, Chiang Rai province (Inthasotti, 2006) and four provinces in the southern Thailand (Nualcharoen *et al.*, 2007).

Algae provide the important sources, which includes the primary and secondary metabolites. The algae can create various collections of bioactive products. They are biologically agile and are used in pharmaceutical industries worldwide. However, a small number of algae are highly toxic when consumed (Ouellette and Wilhelm, 2003). In the past decade, the search for natural antioxidant compounds has gained considerable attention. Antioxidant compounds play an important role against various diseases and ageing processes, which explains their considerable commercial potential in medicine, food production and cosmetic industry. Moreover, interest in employing antioxidants from natural products and concern about the potential toxic effects of synthetic antioxidants (Safer and al-Nughamish, 1999). Macroalgae possess a variety of biological activities, including anti-inflammatory, anticancer, anti-HIV, anti-mutagenic and scavenging of free radicals (Shalaby, 2015). The bioactive compounds in various algae such as carotenoids, phenolics and sulphated polysaccharides have been shown to have antioxidant activities. Presently, many researchers have become more interested in natural source from algae, which could block or reduce free radicals. However, the study on freshwater red algae in South-East Asia is not as well documented in terms of utilization data. Therefore, the more studies on utilization of red macroalgae are needed for red macroalgae management in the future. Thus, this study aimed to evaluate toxicity of aqueous extract of *N. shawii* on rats and antioxidant capacity, total phenolic and flavonoids contents of this species for possible applications cosmetics or dietary supplements.

Materials and methods

Collection and alga material preparation

The freshwater alga extracts use in this study was prepared from *N. shawii*, it was collected from Songkla province, southern Thailand. This alga was hand–picked and thoroughly washed to remove all the unwanted impurities, adhering sand particles and epiphytes. Morphologically distinct thalli of algae was placed separately in new bags and kept in ice box containing and transported to the laboratory. Samples were identified following the Kumano (2002) and Necchi (2016). The dry material was preserved at a temperature of -20 ^{O}C until it used.

Acute toxicity test

Preparation of alga extracts

The dry material (50 g.) was extracted in 500 ml. of distill water at 60 ^{O}C for 24 hours in water bath. The total extracts was filtered and the crude extract was concentrated in rotary evaporator at 40 ^{O}C. Next, the extracts were lyophilized to obtain dried powder for water extract.

Animals

The study was performed on healthy male and female, Sprague Dawley (*Rattus norvegicus*) rats and body weight of 160-200 g. The animals were purchased from the National Laboratory Animal Center, Thailand. The protocols and procedures involved with all animals were performed in accordance with the rules and regulations by the Animal Research Committee of Faculty of Medicine, Chiang Mai University, Thailand. They were allowed to acclimatize in the departmental animal facility for 1 week before the start of the experiment. The study room was maintained at approximately 25 $\pm$ 2 ℃ in 12 hours light dark cycle.

Acute toxicity test

The acute oral toxicity of the crude aqueous extracts of *N. shawii* was evaluated in rat using the procedures described by Organization for Economic Co-operation and Development (2001). A total of 15 animals were divided two dosage groups with 5 animals per dose. The control group was given distilled water. The second and third groups were given with a single dose of 2,000 and 5,000 mg kg^{-1} body weight of dried extract, respectively. Body weight, food and water consumption were monitored daily. Animals fasted approximately 12 hours prior to dosing. Following administration of a single dose of algae preparation, the animals were observed for behavioral changes and general toxicity signs. Results were recorded for the first 5, 15, and 30 min. and at hourly intervals for the next 24 hours and thereafter for a total of 14 days.

Total phenolic, total flavonoid contents and Antioxidant activity

Preparation of the crude extract

The material was dried at 50℃ and then milled. The milled algae were subsequently used for preparing the extracts. As modified from those of Chew *et al*. (2008), the milled algae was extracted with ethyl acetate, ethanol and methanol by continuously shaking for 24 hours, and the water extract was extracted at 60 ^{O}C for 24 hours. The extracts were filtered through filter paper Whatman No.2. The solvent was evaporated and the filtrate was concentrated by rotary evaporator. The extracts were lyophilized to obtain dried powder for water extract**.**

Measurement of total phenolic and total flavonoids content

Total phenolic content of crude extracts were estimated by using Folin-Ciocalteu method as described by Lopez *et al*. (2011). Briefly, 0.5 ml of diluted Folin-Ciocalteu reagent (1:9 v/v; Folin-Ciocalteu reagent: distilled water) was mixed with 100 µl of sample and was left at room temperature for 60 min. Absorbance of all the sample solutions was measured at 720 nm using microplate reader (Ez Read, 2000). Gallic acid was used as the standard, and the total phenolic content was expressed in terms of mg Gallic acid equivalents (GAE) per 1 g of extract.

Total flavonoid content of crude extract were determined by following colorimetric method (Chang *et al*., 2002) Briefly, 20 µl of each algae extracts was separately mixed with 20 µl of 10% aluminum chloride, 20 µl of 1 M potassium acetate and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm using microplate reader (Ez Read, 2000). Quercetin was used as the standard, and the total flavonoid content was expressed in terms of mg quercetin equivalents (GAE) per 1 g of extract.

Measurement of antioxidant activities

The free radical scavenging activity of the freshwater extracts was measured by 1,1-diphenyl-1-picrylhydrazyl (DPPH) following the method of Shimada *et al*. (1992).This method is based on the reduction of stable DPPH radical antioxidants in a methanol solution. Briefly, used as a reagent, 3.9 ml DPPH solution (0.1 mM) was added to 100 µl of algae extracts at different concentrations. After 30 minutes, absorbance was measured at 517 nm using microplate reader (Ez Read, 2000). The measurements were measured in triplicates and the percentage scavenging was calculated as shown by the formular: Scavenging (%) = $[(A_{control} - A_{sample} / A_{control}) \times 100]$, where $A_{control}$ is the absorbance of DPPH alone, and A_{sample} is the absorbance of the reaction mixture containing DPPH and sample. IC_{50}, which stands for the concentration required for 50 % scavenging activity, was then calculated from the equation. Ascorbic acid and BHT (butylated hydroxytolueneso) were also used as control.

Statistical analysis

Data were expressed as the means $\pm$ SD and three measurements and analyzed using one-way ANOVA by DMRT test. Differences in mean values were considered significant when $p < 0.05$.

Results and Discussion

Acute toxicity test

The acute toxicity study showed that animals fed by oral gavages tolerated the limit dose of 5,000 mg kg^{-1}body weight of aqueous extract of *N. shawii*. Mortality, behavior and the amount of water intake, food intake and body weight and abnormalities of visceral organs were then observed. The results showed that the extract cause neither mortality, nor abnormalities. The eating, drinking habit, body weight and behavior of all tested animals were comparable to normal (Fig. 1 and Fig. 2)

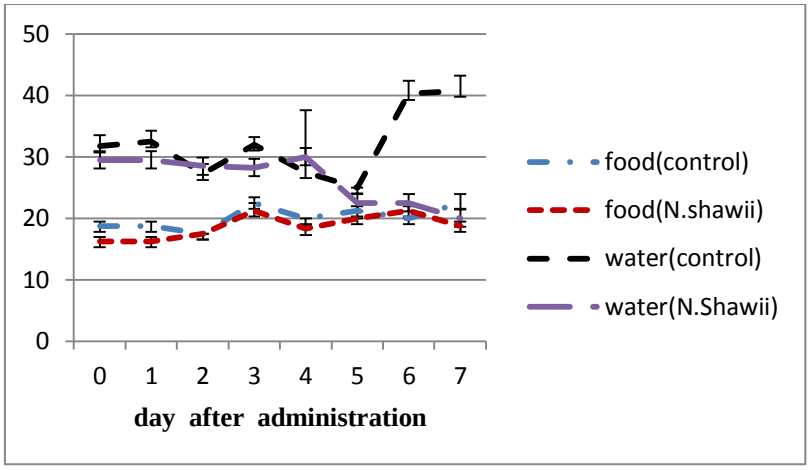

Fig 1. Average of water (ml day^{-1}) and food intake (g day^{-1}) of rats in acute toxicity study of *N. shawii*.

Determination of total phenolic and total flavonoid content

Yield of extracts

The yield of the various solvent of *N. shawii* were shown in Table 1. Significant variations in extraction yield were found among different solvent.The highest extraction yield was recorded for the aquous extract (27.80 %), followed by methanolic extract (7.06 %) and ethanolic extract (5.75 %) respectively. Ethyl acetate extract had the lowest yield.

Phenolic compounds are considered as one of the most important classes of natural antioxidants. These compounds such as flavonoids, phenolic acids, and tannins are considered to be main contributors to the antioxidant activities (Manach *et al*., 2004). The content of phenolic compounds of extracts varied from 2.387±0.135 to 20.967±0.67mgGAE g^{-1} dry extract and significantly different ($p<0.05$). Among them, the ethyl acetate extract showed the highest amount of total phenolic content at

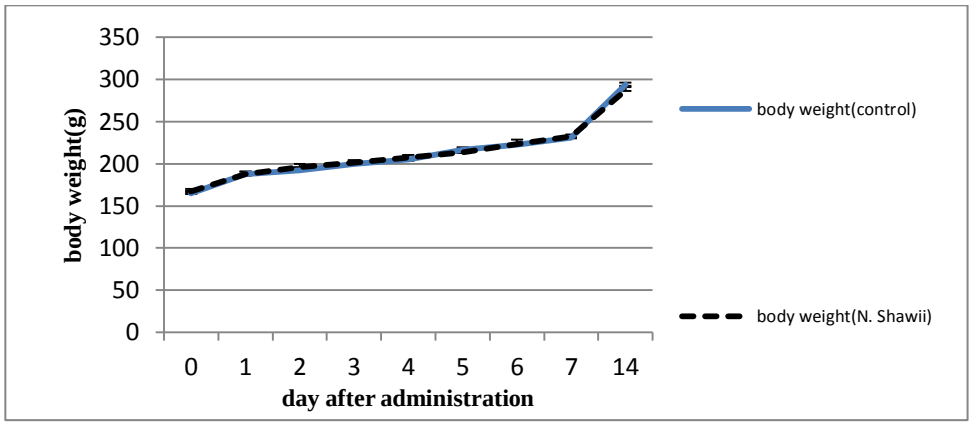

Fig 2. Average of body weight (g.) of rats in acute toxicity study of *N. shawii*

20.967±0.677 mg GAE g^{-1} dry extract (Table 1). There are different amount of phenolic content in the freshwater macroalgae in Thailand, for example, in this results revealed red macroalgae contained lower amounts of polyphenols (aqueous) than aqueous extract of freshwater green alga, *Cladophora* sp., which the total phenolic were 15.95 mg GAE g^{-1} dry extract (Amornlerdpison *et al*., 2015).

As shown in Table 1, the total flavonoid content of algae extracts, varied from 9.363±0.214 to 58.326±0.857 mg QE g^{-1}extract and also significantly different from other solvent ($p<0.05$). In comparison of same ethanolic extract, revealed that this species contained higher of flavonoid than freshwater blue green alga, *Nostoc commune*(1.1±0.14mgQEg^{-1}extract) (Yucharoen *et al*., 2015).

Table1 The yields of extracts, total phenolic and flavonoid contents in *N.shawii* obtained by the different solvents.

extracts	yield (%)	total phenolic content (mg GAE g^{-1} extract)	total flavonoid content (mg QE g^{-1} extract)
ethyl acetate	0.79	20.967 ± 0.677^{d}	58.326 ± 0.857^{d}
ethanolic	5.75	2.387 ± 0.135^{a}	9.363 ± 0.214^{a}
methanolic	7.06	4.742 ± 0.033^{b}	12.472 ± 0.290^{b}
aqueous	27.80	12.753 ± 0.410^{c}	52.042 ± 1.460^{c}

Values are expressed as mean ± sd, n=3, different superscript alphabets, mean significantly different; $\alpha = 0.05$

DPPH radical scavenging activity

The antioxidant activity of the ethyl acetate, ethanolic, methanolic and aqueous extracts of *N. shawii* were investigated by the ability of the extract to scavenge hydroxyl radicals. This is very important because of the fact that hydroxyl radicals were mentioned as the major active oxygen species causing lipid oxidation (Kulisic *et al*., 2007).The $DPPH^{\bullet}$ test is

based on the exchange of hydrogen atoms between the antioxidant and the stable $DPPH^{\bullet}$ free radical. All the extracts show a higher $DPPH^{\bullet}$ radicals scavenging activity after incubation with a free radical solution. $DPPH^{\bullet}$ antioxidant activity values of the investigated extracts slightly differs depending on the solvent applied. The reduction capability of DPPH radical was determined by the decrease induced by antioxidative compounds and those results are shown in Table 2. Similar to the phenolic and flavonoid content, the radical scavenger level varied with extraction method. Among them, the ethyl acetate extract of *N. shawii* showed the highest scavenging activity (IC_{50}= 0.408 ± 0.025 $mg.mL^{-1}$). This study, indicated that the antioxidant compounds were significantly different ($p<0.05$) depending on the extraction method. Moreover, of the tested samples, BHT and ascorbic acid, positive control recorded a little of IC_{50}, lower IC_{50} value indicated higher antioxidant activity, which indicated that all % DPPH scavenging activities observe were significantly lower than positive control at the same concentration. The high scavenging property of this algae may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as radical scavenger. In addition, the selection of the extracting solvent is an important factor for obtaining active compounds in algae.

Table 2 DPPH radical scavenging activity expressed in IC_{50} ($mg\ mL^{-1}$) for *N. shawii* extract.

extracts	***Nemalionopsis shawii***
ethyl acetate	0.408 ± 0.025^{b}
ethanolic	2.689 ± 0.084^{e}
methanolic	2.427 ± 0.056^{d}
aqueous	0.742 ± 0.029^{c}
ascorbic acid	0.009 ± 0.001^{a}
BHT	0.009 ± 0.000^{a}

Values are expressed as mean $\pm$ sd, n=3, different superscript alphabets, mean significantly different; $\alpha = 0.05$

The Pearson's correlation coefficients between the variables are presented in Table 3. The results showed that there were significant and negative correlations between the total phenolic, flavonoid contents and IC_{50} ($p<0.01$). The negative correlations showed that the antioxidant activity of *N.shawii* species were in accordance with their amount of phenolic and flavonoid contents. The higher content of the phenolic and flavonoid contents resulted in higher antioxidant activity with low IC_{50}.These results indicate that antioxidant capacities of this species are determined by their phenolic and flavonoid contents. These results are in agreement with Yanthong and Towatana (2004); Praiboon and Chirapart (2002) who found that *Porphyra veitnamensis*, *Caulerpa macrophysa*, *Sargassum* sp. and nine

species of brown seaweed,were significantly correlated between the DPPH scavenging activity and the phenolic contents, respectively.

Table 3 Pearson's correlation coefficients between the variables.

	DPPH (IC_{50})	**Phenolic content**	**Flavonoid content**
DPPH (IC_{50})		-0.958**	-0.999**
Total phenolic contents	-0.958**		
Total flavonoid contents	-0.999**		

**correlation is significant at the 0.01 level (2 tailed)

Conclusion

The result of acute toxicity study indicate *N. shawii* as safe. This species exhibited, high phenolic compound, total flavonoid contents and also activity of antioxidants. However, this is the first report of investigation on acute toxicity test and antioxidant capacity and phenolic as well as flavonoid content of *N. shawii*. Therefore, it is suggested that further works should be performed on the isolation and identification of the antioxidant component in this algae.

Acknowledgement

This work was supported with grants from the National Research Council of Thailand; Rajamangala University of Technology Srivijaya (2017). We would like to thank the students from fisheries department, faculty of Agriculture, Rajamangala University of Technology Srivijaya for field assistance.

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(Received 25 October 2017; accepted 25 November 2017)