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Cultivation, Quantification and Pharmacognostic Study of methanolic extract of *Thalassiosira weissflogii*

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ABSTRACT: *Thalassiosira weissflogii* is a unicellular microalgae which was found under marine environment. It was cultivated using Guuillard and Ryther F/2 medium in transparent plastic bottle under continuous light at 20ºC for 5 days. It is then harvested and dried under shade. The dried powdered was then mixed with methanol and used for further analysis. Preliminary phytochemical constituents, primary and secondary metabolites were assessed for methanolic extract of *Thalassiosira weissflogii*. Pharmacognostic and physiochemical parameters were also detected to confirm the quality of the drug. Phytochemical compound such as alkaloids, flavonoids, tannins were present but glycosides and saponins were absent. They also yield appreciable amount of primary and secondary metabolites. Based on the pharmacognosy studies, it was found that the drug was pure which can be stored to do further studies. Thus, the methanolic extract of *Thalassiosira weissflogii* can be used for both *in vivo* and *in vitro* studies.

KEYWORDS: Cultivation, Microalgae, Primary and Secondary metabolites, *Thalassiosira weissflogii*

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I. INTRODUCTION

Microalgae are simple photosynthetic organisms which are widespread in nature, playing key roles as primary producers in marine, freshwater and sub-aerial terrestrial systems (Graham L E *et al.,* 2009). Beside their remarkable environmental importance, some economic uses of microalgae have been recognized for decades (Spolaore P *et al.,* 2006). Algae biomass contains 20-30% carbohydrate, 10-20% lipid and 40-60% protein (Wang J and Yin Y, 2018). Individually, diatoms do not appear in a highly visible color, but in groups they can be seen due to the presence of photosynthetic plastids (Nurachman Z *et al.,* 2012).Microalgae are a high-potential source of biomass for the production of food, industrial materials, pharmaceuticals and energy (Wang J and Yin Y, 2018).

Thalassiosira weissflogii is a species of centric diatoms, a unicellular microalgae. It is found in marine environments and also in inland waters in many parts of the world. It is actively studied because it may use C4-plant style strategies to increase its photosynthetic efficiency (Roberts K *et al.,* 2007). *Thalassiosira weissflogii* is a short cylinder in shape and varies in size from 4 to 32 µm in diameter. It tends to be larger in winter, typically 15 µm in diameter, but smaller in summer (5 µm). It occurs both singly and in groups and may be embedded in a gelatinous matrix. There is a silicaceouscell wall with two frustules or valves, a larger epivalve and a smaller hypovalve. The face of the valves varies in shape but is basically irregular rings with one labiate and two or more central processes. Other features that may be present include an irregularly shaped areola or pore, further processes on the rim of the valves, marginal spines, striations and thick radial ribs (Fryxell G and Hasle, 1896). In aquaculture, *Thalassiosira weissflogii* is used to feed to shrimp and shellfish larvae in hatcheries. Thus, in present study the process of cultivation, phytochemical constitutes and pharmacognosy studies were performed for *Thalassiosira weissflogii.*

II. MATERIALS AND METHODS

1. Cultivation of *Thalassiosira weissflogii*

The primary *Thalassiosira weissflogii* were collected from Marakanam, Tamil Nadu. The water used for cultivation was collected from the Kovalam Beach, Chennai. Cultures of *T.weissflogii* were grown under continuous light at 20ºC for 5 days. The growth medium f/2 was prepared as mentioned below.

Growth f/2 medium preparation

This is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation, termed "f Medium" (Guillard and Ryther 1962), has been reduced by half. To prepare, begin with 950 mL of filtered natural seawater and add the following components. The trace element and vitamin solutions are provided below. Bring the final volume to 1 liter with filtered natural seawater. If the alga to be grown does not require silica, then it is recommended that the silica be omitted because it enhances precipitation and autoclave (Guillard and Ryther 1962, Guillard 1975).

Component	**Primary Stock Solution**	**Quantity**	**Molar Concentration In Final Medium**
NaNO3	75 g/L dH_2O	1 ml	8.82 x 10-4 M
NaH2PO4 H2O	5 g/L dH_2O	1 ml	3.62 x 10-5 M
Na2SiO3 9H2O	30 g/L dH_2O	1 ml	1.06 x 10-4 M
trace metal solution	(see recipe below)	1 ml	-------
vitamin solution	(see recipe below)	0.5 ml	-------

f/2 Trace Metal Solution

To prepare, begin with 950 mL of dH_2O, add the components and bring final volume to 1 liter with dH_2O and autoclave. Note that the original medium (Guillard and Ryther 1962) used ferric sequestrene; we have substituted $Na_2EDTA \cdot 2H_2O$ and $FeCl_3 \cdot 6\ H_2O$.

Component	**Primary Stock Solution**	**Quantity**	**Molar Concentration In Final Medium**
$FeCl_3\ 6H_2O$	-----	3.15 g	1.17×10^{-5} M
$Na_2EDTA\ 2H_2O$	-----	4.36 g	1.17×10^{-5} M
$CuSO_4\ 5H_2O$	9.8 g/L dH_2O	1 ml	3.93×10^{-8} M
$Na_2MoO_4\ 2H_2O$	6.3 g/L dH_2O	1 ml	2.60×10^{-8} M
$ZnSO_4\ 7H_2O$	22.0 g/L dH_2O	1 ml	7.65×10^{-8} M
$CoCl_2\ 6H_2O$	10.0 g/L dH_2O	1 ml	4.20×10^{-8} M
$MnCl_2\ 4H_2O$	180.0 g/L dH_2O	1 ml	9.10×10^{-7} M

f/2 Vitamin Solution

First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add 1 mL of the primary stocks and bring final volume to 1 liter with dH_2O. Filter sterilize. Store in refrigerator or freezer(Guillard and Ryther 1962, Guillard 1975).

Component	**Primary Stock Solution**	**Quantity**	**Molar Concentration In Final Medium**
Thiamine HCl (vit. B1)	-----	200 mg	2.96×10^{-7} M
Biotin (vit. H)	1.0 g/L dH_2O	1 ml	2.05×10^{-9} M
Cyanocobalamin(vit. B12)	1.0 g/L dH_2O	1 ml	3.69×10^{-10} M

2. Dry Harvesting

The cultures at stationary phase of growth were harvested and collected by centrifuging at 10,000 rpm for 3 min. The collected *T. weissflogii* pellets were dried under shade and made into a coarse powder with mechanical grinder for further use.

3. Preparation of *Thalassiosira weissflogii* extracts

The obtained algal biomass was subjected to centrifugation (2500 r/min) for the time duration of 10 min so as to partially dehydrate it. About 25 g algal biomass was subjected to extraction for a time period of 30 min using Soxhlet apparatus, using 150 mL of organic solvent, i.e methanol. Respective solvents were used to test phytochemical compound and primary as well as secondary metabolites (Adhoni A S *et al.,* 2016).

4. Preliminary Phytochemical Screening

Different extract of *T. weissflogii* were subjected to qualitative tests for the identification of phytochemical constituents such as carotenoids, alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, reducing sugars, volatile oil, fatty acid, amino acids and carbohydrates according to standard

procedures (Martinez Nadal N G *et al.,* 1963; Hornsey I S and Hide D, 1974; Yao J and Moellering R, 1995; Benkendroff K *et al.,* 2005).

5. QUANTITATIVE DETERMINATION OF PRIMARY METABOLITES

a. Determination of carbohydrate

100 mg of dried powder sample *T. weissflogii* was hydrolysed in a boiling tube with 5 ml of 2.5 N HCl in a boiling water bath for a period of 3 hours. It was cooled to room temperature and solid sodium carbonate was added until effervescence ceases. The contents were centrifuged and the supernatant was made to 100 ml using distilled water. From this 0.2 ml of sample was pipette out and made up the volume to 1 ml with distilled water. Then 1.0 ml of phenol reagent was added followed by 5.0 ml of sulphuric acid. The tubes were kept at 25-30°C for20 min. The absorbance was read at 490 nm (Geetha T S and Geetha N, 2014).

b. Estimation of total chlorophyll content

100 mg of *T. weissflogii*were soaked in 10 ml of DMSO: acetone mixture (1:1) for overnight incubation(in the dark) and absorbance read at 663 and 645 nm and total chlorophyll content was calculated using the following equations.

Chlorophyll a (Ca) = (12.25 × OD at 663) - (2.79× OD at 645) ×10/ (1000×wt)

Chlorophyll b (Cb) = (21.50 × OD at 645) - (5.10× OD at 663) ×10/ (1000×wt)

Total Chlorophyll (C) = (7.15 × OD at 663) + (18.71× OD at 645) ×10/ (1000×wt)

c. Determination of protein

The dried and powdered sample of *T.weissflogii* was extracted by stirring with 50 ml of 50% methanol (1:5 w/v) at 25°C for 24 h and centrifuged at 7,000 rpm for 10 min .0.2 ml of extract was pipette out and the volume was made to 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent was added to all the tubes and allowed it to stand for 10 min. Then 0.5 ml of Folin's Ciocalteau reagent was added and incubated in dark for 30 min. The intensity of the colour developed was read at 660 nm 23 (Geetha T S and Geetha N, 2014).

d. Estimation of total lipid content

10 gm of powdered *T. weissflogii* was used to extract lipids with 150 ml of petroleum ether for 16 hr, at a solvent condensation rate of 2–3 drops/sec according to AOAC approved method with minor modifications of sample size and extraction time. The obtained extract was concentrated and evaporated at room temperature to dryness. The weight of extract gives the total lipid content which was expressed as mg/g dry matter (Geetha T S and Geetha N, 2014).

6. QUANTITATIVE DETERMINATION OF SECONDARY METABOLITES

a. Determination of Total Phenolics and Tannins

The total phenolic content was determined according to the method described by Siddhuraju P and Becker K, 2003. Ten microliter aliquots of the extracts (2 mg/2 ml) was taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution(20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as tannic acid equivalents. Using the same extracts the tannins was estimated after treatment with polyvinyl polypyrrolidone (PVPP). One hundred milligrams of PVPP was placed in a test tube and to this 1 ml distilled water and then 1ml of the sample extracts were added. The contents were vortexed and kept in the test tube at 4°C for 4h. Then it was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along withthe PVPP). The phenolic content of the supernatant was measured as mentioned by Siddhuraju P and Becker K, 2003 and expressed as the content of non-tannin phenolics (tannic acid equivalents) on a dry matter basis. From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

b. Determination of Total flavonoids content

The flavonoids content was determined by the use of a slightly modified colorimetric method. A 0.5 ml aliquot of appropriately (2 mg/2 ml) diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5 % $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$solution was added and allowed to stand for 6 min, and then 2 ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

7. DRUG STANDARDISATION

1. Pharmacognostic Study

Pharmacognostic study was carried on the basis of Morphological characters such as color, odor, taste, size, fracture, texture etc. using the sensory organs of our body (Srivastava S *et al.,* 2014).

2. Determination of foreign matter

Weigh 10 g to 50 g of the substance to be examined, or the minimum quantity prescribed in the monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×).Separate foreign matter and weigh it and calculate the percentage present.

3. Physicochemical test

Solubility

The presence of adulterant in a drug could be indicated by solubility studies with various solvents(Gautam A*et al.*,2010).

i. Alcohol

5 gm of powdered material along with 100 ml of alcohol are shaken well occasionally for the first 6 hours and kept undisturbed for 18 hours. The liquefied extract thus obtained was concentrated in an vacuum oven and the percentage was calculated with the weight of the drug powder taken.

ii. Water

The procedure adopted for solubility percentage of alcohol is used with water instead of alcohol to get the water solubility.

iii. Determination of moisture /Loss on drying procedure

Weigh about 1.5g of the powdered drug into a weighed flat and thin Porcelain dish. Dry it in the oven at 100°C or 105°C. Cool in desiccators and watch the loss in weight is usually recorded as moisture(Ahmed T*et al.*,2013).

iv. Determination of Ash

a. Total ash

About 2gm of powdered drug was weighed accurately into a tarred silica crucible. Incubated at 450°C in a muffle furnace until it is free from carbon. The crucible was cooled and weighed. Percentage of total ash was calculated with reference to air-dried substance (Ahamed T *et al.*,2013).

Determination of total ash value formula:

Total ash value of the sample = 100 (z-x)% / y.

X= weight of empty dish.

Y= weight of the drug taken.

Z= weight of the dish + ash (after complete incineration).

b. Acid insoluble ash

Ash obtained from the total ash was boiled with 25ml of 2N HCl for a few minutes. Filtered through an ashless filter paper. The filter paper was transferred into a tarred silica crucible. Incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of acid insoluble ash was calculated with reference to air-dried substance.

c. Water soluble ash

Ash obtained from the total ash was boiled with 25 ml of distilled water for a few minutes and filtered through an ash less filter paper. The filter paper was transferred into a tarred silica crucible. Incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of water-soluble ash was calculated with reference to air-dried substance.(Ahamed T *et al.*, 2010)

v. Extractive value

a. Determination of alcohol soluble extractive value

About 5gms of air dried coarse powdered drug was weighed and macerated with 100ml of 90% alcohol in a closed flask for 24 hours, shaking frequently during the first 6 hrs and these allowed standing for 18 hrs. Thereafter it was filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The %age of the alcohol soluble extractive values was calculated with reference to the air-dried drug.

b. Determination of water soluble extractive value

About 5gm of air-dried powdered drug was taken & macerated with 100 ml of chloroform water in a closed flask for 24 hrs shaking frequently during the first 6 hrs and then allowed to stand for 18 hrs. Thereafter, it was filtered rapidly taking precautions against loss of the solvent..25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the water soluble extractive value was calculated with reference to the air-dried drug.

c. Determination of pH range

The powder sample was weighed to about 5g and immersed in 100 ml of water in a beaker. The beaker was closed with aluminium foil and left behind for 24 hours in room temperature. Later the supernatant solution was decanted into another beaker and the pH of the formulation was determined using a calibrated pH meter.

III. RESULTS

1. Cultivated *Thalassiosira weissflogii*

After cultivation period, the diatom *Thalassiosira weissflogii* was viewed under microscope to confirm the structure and size which was shown in **Figure 1.**

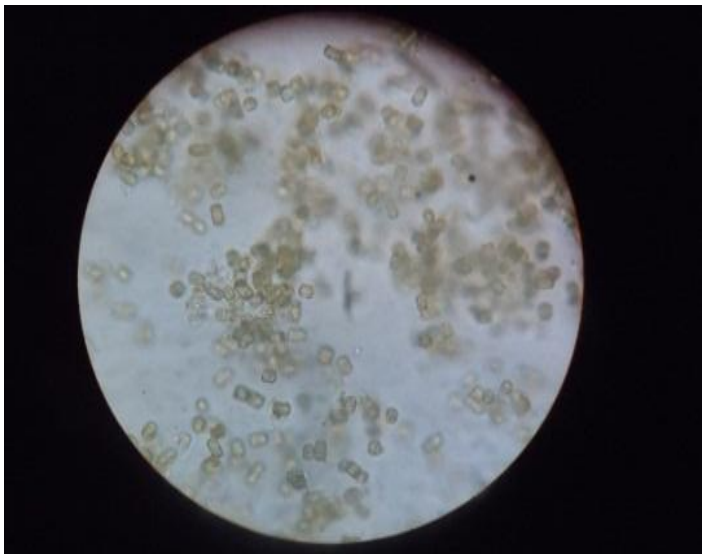

Figure 1: Microscopic image of cultivated *Thalassiosira weissflogii*

2. Preliminary phytochemical screening

Table 1 Phytochemical screening of different extract of *Thalassiosira weissflogii*

Phytochemical constituents	Test name	Methanol Extract
Alkaloids	Wagner's test	+
Flavonoids	Shimoda, Lead acetate test	+
Phenolics & Tannins	Lead actetate test,	+
	Ferric chloride test	+
Steroids & Sterols	Salkowski test	+
Carbohydrates	Fehlings test,	+
	Benedicts test	+
Saponin	Honey comb test,	-
	Foam test	-
Glycosides	Glycosides test	-
Protein & amino acid	Biuret test,	+
	Ninhydrin test	+
Anthraquinone	Borntragers test	-

Methanolic extract of *T. weissflogii* was subjected to the preliminary phytochemical analysis. The phytochemical constituents of methanol extract of *T. weissflogii* shows the presence of alkaloids, flavonoids, phenols, tannins, sterols, carbohydrates, protein and amino acid whereas saponins and glycosides was found to be absent (**Table 1**).

3. Quantification of Primary Metabolites

Table 2 Primary Metabolites of methanolic extract of *Thalassiosira weissflogii*

S.NO	Primary metabolites	Weight (mg/g dw)
1	Carbohydrates	32.57 ± 0.35
2	Chlorophyll	11.20 ± 0.74
3	Protein	25.48 ± 0.57
4	Lipids	20.61 ± 0.19

Primary and secondary metabolites were tested for methanolic extract of *T. weissflogii.* The primary metabolites such as carbohydrates, chlorophyll, protein and lipid were founded to be 32.57 ± 0.35, 11.20 ± 0.74, 25.48 ± 0.57 and 20.61 ± 0.19(mg/g dw) respectively (**Table 2**).

4. Quantification of Secondary Metabolites

Table 3 Secondary Metabolites of methanolic extract of *Thalassiosira weissflogii*

S.NO	Secondary metabolites	Weight (mg/g dw)
1	Total phenolic	16.45 ± 0.26
2	Tannin	4.26 ± 0.61
3	Total flavonoids	11.06 ± 0.32

Quantitative analysis of secondary metabolites in methanolic extract of *T. weissflogii* shows that (Table 2), total phenolic was found high (16.45 ± 0.26 mg/g) followed by total flavonoids (11.06 ± 0.32 mg/g) and then tannin (4.26 ± 0.61 mg/g) (**Table 3**).

5. Drug standardisation

Table 4: Pharmacognostic Study

S.No	Properties	*Thalassiosira weissflogii*
1	Colour	Light green
2	Odour	Musty
3	Taste	Tasteless
4	Texture	Rough
5	Fracture	Heavy fracture
6	Size	10 -15µm

Table 4 demonstrated the pharmacognosy studies of *T. weissflogii.* The colour, odour, taste, texture, fracture and size was noted to be light green, musty, tasteless, rough, heavy fracture with 10-15 µm in diameter.

Table 5: Physicochemical Parameters of *Thalassiosira weissflogii*

Content	Amount g/100g
Moisture content	0.9
Total Ash	12.9
Acid insoluble ash	9.2
Water soluble ash	6.4
Water soluble extractive	2.4
Alcohol soluble extractive	11.4
Solubility (water)	1.4
Solubility (alcohol)	5.5
PH	8.0

Physicochemical parameters such as Total Ash (12.9 g/100g), Acid Insoluble Ash (9.2 g/100g), Water Soluble Ash (6.4 g/100g), Water soluble extract (2.4 g/100g), Alcohol Soluble extract (11.4 g/100g) and pH 8.0was observed which are useful to detect adulteration in *T. weissflogii* powdered sample (**Table 5**).

IV. DISCUSSION

Phytochemical analysis is of paramount importance in identifying new source of therapeutically and industrially valuable compounds having medicinal plants have been chemically investigated (Ambasta S P *et al.*,1986). Different phytochemicals have been found to possess a wide range of medicinal properties, which may help in protection against various diseases. Primary metabolites like chlorophyll, proteins, carbohydrates and lipids are useful in flavouring, fragrances, insecticides, sweeteners and natural dyes (Kaufman P B *et al.,* 1999). Chlorophyll is the most indispensable class of primary compounds as they are the only substances that capture sunlight and make it available to plant system for its cultivation on photosynthesis (Murray A P *et al.,* 1986). Secondary metabolite analysis is necessary for extraction, purification, separation, crystallization, identification of various phyto compounds. Earlier reports revealed that phenolic compounds including flavonoids are potent antioxidants with reported antimutageneic and anticarcinogenic effects (Middleton E and Kandaswami C, 1994). Flavonoids are have been reported to possess many useful properties, including anti-inflammatory, antimicrobial, enzyme inhibition, oestrogenic, antiallergic, antioxidant and anti-tumour activity

(Harborne J B and Williams C A, 2000). Tannin contributes various medicinal properties such as antimicrobial, anti-inflammatory and astringent activity (Chung K T *et al.,* 1998).

In our studies, the presence of phytochemical constitutes and quantitative analyses of both primary and secondary metabolites were assessed. This was correlated with findings of previous research using *Chlorella vulgaris*, the microalgae. The investigation of Bhuvana P *et al.,* 2018 was carried out to assess the presence of different bioactive constituents and quantification of primary and secondary metabolites in different solvent extracts of *Chlorella vulgaris*. The presence of phenolics, flavonoids, carbohydrates, saponin and protein were confirmed by routine qualitative studies. The methanolic extract contains highest concentration of both the primary and secondary metabolites when compared with other extracts. Hence, the present study shows very high amount of both primary and secondary metabolites when compared with the study of Bhuvana P *et al.,* 2018.

Thus, it has been reported that the presence of phytoconstituents such as flavonoids, tannins and polyphenols help in preventing a number of disease through free radical scavenging activity (Vasanthi H R *et al.,* 2006). Antitumor and antioxidant properties have been attributed to the flavonoids based on *in vitro* and *in vivo* studies in both humans and animals (Cody V *et al.,* 1988).

V. CONCLUSION

Bioactive compound present in microalgae play a major role in preventing many diseases. In this paper, it confirm the presence of primary and secondary metabolites in methanolic extract of *Thalassiosira weissflogii* and the standard of drug was also found to be in good form. Further, the study can be extended to test the antioxidant, antimicrobial activity in various microorganisms which have promising effect in medical applications.

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Conflicts of interest

The author declares that have no conflict of interests

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