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Pharmacognostic Standardization and Phycochemical Evaluation of Green Seaweed *Codium Flabellatum*

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Abstract: *Codium flabellatum*, a green seaweed belonging to the Codiaceae family, is renowned for its therapeutic effects, which may be attributed to the number of bioactive compounds it contains. Pharmacognostic standardization and phytochemical evaluation establish the identification of a specific plant and are valuable for authentication and the prevention of adulteration and substitution. In the current study, pharmacognostic studies were conducted through microscopic and macroscopic observations, and three different extracts (ethanol, ethyl acetate, and *n*-hexane) of *Codium flabellatum* were subjected to preliminary phytochemical tests for carbohydrates, lipids, tannins, flavonoids, gelatin, and proteins; a physiochemical test was also performed according to WHO guidelines. Macroscopic study showed that *Codium flabellatum* was green in color, with a thallus 25–30 cm in height and 1–3 cm in width, with a finger-like projection and a spherical air bladder. Microscopic evaluation revealed clavate utricles, 190–260 µm broad, cylindrical in shape, and 750–850 µm long when young, with one or two long hairs just below their tips. Gametangia were found laterally on the utricles, 300–400 µm in length and 100–175 µm in width. The different extracts revealed that all classes of compounds were present. Physiochemical tests showed that the highest extractive values were for chloroform and methanol at 96% and 80.95%, respectively. The moisture and ash content were 94.55% and 84.15%, respectively. This study of *Codium flabellatum* will contribute to authenticating its identity and will also help to evaluate its nutritional potential.

Keywords: *Codium flabellatum*, green seaweed, pharmacognostic standardization.

绿海藻的生药标准化及植物化学评价

摘要：鞭尾草是一种属于鼠尾草科的绿色海藻，以其治疗效果而闻名，这可能归因于它所含的生物活性化合物的数量。生药标准化和植物化学评估确定了特定植物的鉴定，对于鉴定和防止掺假和替代具有重要价值。在目前的研究中，通过微观和宏观观察进行生药研究，并对三叶草的三种不同提取物（乙醇、乙酸乙酯和正己烷）进行了碳水化合物、脂质、单宁、黄酮类化合物、明胶、和蛋白质；还根据世界卫生组织的指南进行了物理化学测试。肉眼研究表明，鞭毛虫呈绿色，菌体高 25-30 厘米，宽 1-3 厘米，有指状突起和球形气囊。显微镜检查显示，椭圆囊宽

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190-260 微米，圆柱形，年轻时长 750-850 微米，尖端下方有一或两根长毛。在椭圆囊的侧面发现配子体，长 300–400 微米，宽 100–175 微米。不同的提取物表明存在所有类别的化合物。理化测试表明，氯仿和甲醇的萃取值最高，分别为 96%和 80.95%。水分和灰分含量分别为 94.55%和 84.15%。这项对鞭尾草的研究将有助于验证其身份，也将有助于评估其营养潜力。

关键词：鞭尾草，绿色海藻，生药标准化。

1. Introduction

Seaweeds, commonly known as macroalgae, are classified according to their color into three different classes: chlorophyta (green), phaeophyta (brown), and rhodophyta (red). Marine algae grow in benthic and littoral habitats. The color of seaweed is due to various pigments, such as xanthene, chlorophyll, fucoxanthin, phycoerythrin, and phycocyanin. Natural products obtained from marine resources contain a large number of chemical constituents, some of which have shown tremendous action on human physiology. Their complex and sometime novel structures have attracted researcher to create new leads [1]. Marine biologically active constituents can protect the organism from external hazards such as ultraviolet radiation, herbivorous animals, and stress.

Seaweeds are a source of nourishment for humans, particularly in East Asia. They are also used to manufacture a variety of food additives such as alginates and carrageenan; the latter is used as a vegetarian alternative to gelatin in cooking and baking. Many seaweeds are utilized in traditional medicine. Alginates are used in wound dressings and the manufacture of dental molds, whereas agar is extensively employed in microbiology to aid in the growth of bacterial cultures. Seaweeds are utilized in cosmetics, toothpaste, and paints, as well as paper coatings, adhesives, dyes, explosives, gels, and many other industrial items [2]. They are a good source of vitamins A, Bl, B12, C, D, and E, as well as niacin, folic acid, pantothenic acid, and riboflavin. They also include minerals, such as calcium, sodium, potassium, and phosphorus. Worldwide, researchers are attracted by marine algae as they produce a wide range of polyunsaturated fatty acids (PUFAs) [3]. Fatty acids play a major role in cellular disorders such as arthritis, thrombosis, cancer, and atherosclerosis, based on the mechanism of action in which fatty acids are metabolically transformed into prostaglandin, leukotrienes, and thromboxane [4].

Chlorophyceae, commonly referred to as green algae plants, includes both freshwater and marine green algae. There are approximately 425 genera and 6,500 species in the Chlorophyceae class. It is a broad group with considerable differences in vegetative structure, distribution, and reproductive strategies [5].

Codium flabellatum is a green marine macroalgae that was first identified by Nizamuddin as per its identification and micro assay test [6]. It grows abundantly in Pakistan from November to March, firmly attached to the mid- and lower littoral rocks at Karachi. Globally, almost fifty species of *Codium* have been found so far [7]. Numerous pharmacological activities of this seaweed, including anti-bacterial, anti-leishmanial, and antifungal activities, have been reported [8, 9]. The popularity of natural therapies for various diseases is growing day by day. Medicinal herbs have been utilized for centuries, and their use and popularity are growing because they have few adverse effects and are easily available. As a result, standardization and quality control standards for plants utilized as sources of natural medicine must be established. These will verify the natural medicine's validity and effectiveness and protect against malpractices such as adulteration and substitution. Hence, it is crucial to conduct pharmacognostic investigations of marine algae. For this purpose, the current pharmacognostic study conducts phytochemical and physiochemical evaluations of *Codium flabellatum* seaweed obtained from the coastal area of Karachi, Pakistan.

2. Materials and Methods

2.1. Collection and Authentication of the Seaweed

The green seaweed *Codium flabellatum* was collected in March 2021 from the coastal areas of Karachi. An already prepared herbarium sheet was obtained from the library of the Center of Excellence in Marine Biology (CEMB) at the University of Karachi for the identification of seaweed. *Codium flabellatum* was found to be similar in both morphology and taxonomy [6]. The voucher number for this seaweed is CYF-06. After identification, the seaweed was cleaned of any adulterants and dried under shade. The dried algae were sieved and placed in an amber glass container for further study.

2.1.1. Chemicals Required

All the required chemicals, including ethanol, ethyl acetate, n-hexane, molish reagent, iodine reagent, barfoed reagent, benedict reagent, sudan dye, bile salt, biuret test, million reagent, nin-hydrin and lead acetate, were purchased from the Sigma-Aldrich Company and were of an analytical grade.

2.1.2. Extraction

Extraction was carried out by the maceration process using three different solvents: ethanol, ethyl acetate, and n-hexane. These solvents were selected for their different polarities to obtain the maximum number of extracts and chemical constituents. For this purpose, 80 grams of seaweed were put into three different glass bottles, and 500 ml of one of the solvents (ethanol, ethyl acetate, or n-hexane) was added to each glass bottle to soak the algae. After the maceration process, pure extracts were obtained by using the rotary evaporator Model JE-RE-2. Glass vials with the correct labeling were used to store pure and dried extracts. They were then used for analysis.

2.2. Pharmacognostic Study

Pharmacognostic study was done by macroscopic and microscopic examinations.

2.2.1. Macroscopic Studies

Macroscopic examination was carried out to observe the size, color, shape, odor, and textures of seaweed, and pictures were taken at different angles with a digital camera.

2.2.2. Microscopic Studies

Microscopic examinations were conducted using thin slices of various parts of algae. The lignification of the thin sections was verified after they were cleaned with water and mounted in glycerin, and pictures were taken with the help of a digital microscopic camera (10x, 40x) [10].

2.3. Physicochemical Analysis

2.3.1. Ash Value Determination

This test was performed to calculate the number of organic substances present within seaweed. Seaweed powder was put in a crucible, weighed, and considered W1. Then the crucible was put into a muffle furnace at a temperature of 100°C. The temperature was increased by 60°C every 30 minutes throughout the procedure until the crude drug was totally converted into ash. The crucible was then removed from the furnace and put into a desiccator for cooling. We again weighed the crucible considered as W_2, and the ash value was calculated [11].

2.3.2. Moisture (Water) Content

This test was considered a physicochemical parameter to evaluate the water content present within algae at the time of collection. Fresh seaweed was collected and washed with tap water to remove any dirt. Once the water was removed, the seaweed was weighed and placed in a glass slide. The seaweed was put under the shade for seven days and then weighed again. A reduction in the weight indicated the moisture content [8, 9].

2.3.3. Extractive Value Determination

This evaluation technique was used to determine the efficacy of solvents used for extraction purposes. A 5 g measure of sample drug was macerated with 100 ml of different solvents for 24 hours with continuous shaking. After 24 hours, the contents were filtered and allowed to dry in a water bath or hot plate. The percentage was then calculated through the formula [12].

2.4. Preliminary Phytochemical Analysis

Dried algae powder was subjected to qualitative analysis to detect macromolecules, including carbohydrates, proteins, lipids, tannins, flavonoids, and gelatin. The presence and absence of any macromolecules were detected through (+), (++), (+++), and (-). The procedure was followed as prescribed [13].

2.5. Chemical Analysis for Carbohydrates

2.5.1. Molisch Test

Three different test tubes contained 2–3 ml each of the sample solution. A few drops of Molisch reagent were added to the tubes. Then 2 ml of concentrated sulfuric acid was slowly added into the test tubes.

2.5.2. Iodine Test

The iodine solution was added by dropper to the three extracted sample solutions until a blue color appeared, indicating the existence of starch.

2.5.3. Benedict's Test

Benedict's reagent is comprised of copper sulfate and sodium hydroxide. This biochemical test was used for the detection of reducing sugar. A 1 ml measure of Benedict reagent was added to 4 ml of sample solution and heated to boiling.

2.6. Chemical Analysis for Lipids

2.6.1. Sudan IV Test

A lipid-soluble dye always appears with a red stain when mixed with lipids. Five test tubes were added, one filled with water and the other with three extract

solutions. Add Sudan IV dye dropwise and observe the results in either test tube.

2.6.2. Grease Spot Test

It is a very easy way to detect a sample's lipid content, as lipids can produce greasy spots on white paper. Add a few drops of each sample solution on white paper and observe the results.

2.6.3. Emulsification Test

2 ml of each sample solution were taken in two sets of test tubes and added to bile salt in one test tube and tween/soap in another test tube. Then, we observed the results.

2.7. Chemical Analysis for Proteins

2.7.1. Biuret Test

In 2 ml of each sample, the solution was placed in a dry and clean test tube, and a biuret reagent was added dropwise. Because of the proximity of the peptide bond, the solution's blue color changes to violet in a short period.

2.7.2. Millon's Test

This phytochemical test is used to determine the presence of the phenolic hydroxy group in a compound. Various amino acids, such as tyrosine and phenylalanine, contain the same functional group, so they give a positive result on Millon's test with a pink to dark red color. About 1–2 mL of each sample solution is added, followed by a small amount of the million reagents. When the mixture boils, a white precipitate forms. The precipitate changes to a red color on boiling.

2.7.3. Ninhydrin Test

This phytochemical test is used to analyze the existence of alpha and free amino acids in protein content. In three sample extract solutions, add a small amount of ninhydrin reagent; the color of the mixtures will appear either pale yellow or deep blue due to the development of a complex structure between ninhydrin and the nitrogen of free amino acids.

2.7.4. Lead Sulfide Test

This method is used for the analysis of amino acids that contain sulfur in their chemical composition, like cysteine. When sulfide reacts with lead acetate, it produces a black precipitate. Add a small quantity of three sample extracts to each test tube, then add 2 mL of 10% sodium hydroxide solution along with a few drops of lead acetate solution. Vigorously mix the solution and boil it in a water bath. If a black precipitate appears, then the sample must possess sulfur-containing amino acids.

2.8. Chemical Analysis for Gelatin

2.8.1. Soda-Lime Test

Gelatin always produces ammonia gas whenever it reacts with a solution of soda-lime. The results are observed after about 2 mL of sample solutions are taken and a few drops of soda-lime are slowly added.

2.8.2. Precipitation Test

Add about 1–2 mL of each sample solution into two individual test tubes, then add the picric acid solution into one test tube and the tannic acid solution into the other and observe the results.

2.9. Chemical Analysis for Flavonoids

2.9.1. Ammonia Test

Initially, 1 ml of each sample was dissolved in an alcoholic solution and spread on the filter paper, then exposed to the vapors of ammonia. As a result, yellow spots appear on the filter paper, meaning the sample has flavonoids.

2.9.2. Vanillin HCL Test

Slowly add vanillin hydrochloric acid to 2 ml of each sample solution and observe the results. The color of the mixtures turns pink, which determines the presence of flavonoids.

2.9.3. Shinoda Test

Magnesium pieces were added to each of the samples. Then, we slowly added diluted hydrochloric acid dropwise. The red color appears within the solution. It indicates the presence of flavonoid glycosides.

2.10. Chemical Tests for Tannins

2.10.1. Test with Iron Salts

Three extract samples were treated with various salts, such as potassium ferrocyanide or ferric chloride, in the presence of ammonia. Bluish-black or greenish-brown color indicated the presence of tannins.

2.10.2. Goldbeater's Skin Test

A small piece of goldbeater skin was used and put into 2% diluted hydrochloric acid, then washed with distilled water and soaked with sample solutions, again washed with distilled water. After a few minutes, add a 1% solution of ferrous sulfate. The brownish-black color indicates the existence of tannins.

2.10.3. Bromine Water Test

This test is used to evaluate condensed tannin. In

three sample solutions, bromine water was added.

3. Results

3.1. Macroscopic Characteristics of *C. Flabellatum*

Macroscopic characteristics of *C. flabellatum* are given in Fig. 1. *C. flabellatum* possesses a flat, large thallus, dichotomously branched, with a height of 25–30 cm and a width of 1-3 cm, with green color. The texture was spongy velvet-like. It has a salty taste and fishy odor. The air bladder was present and spherical, and a finger-like projection as it grows and is attached to rocks by a tiny basal disc. The upper portion is always flat and the apices blunt, while the lower portion of the thallus is sometimes cylindrical.

Fig. 1 Macroscopic feature of *Codium flabellatum*

3.2. Microscopic Studies

Microscopic characteristics of *C. flabellatum* are given in Fig. 2-4. The utricles are clavate, 190-260 µm broad, cylindrical, 750–850 µm long when young, with one or two long hairs just below their tips. Two medullary filaments, or sometimes three, are located at the base of each utricle, which measures 25–40 µm in diameter.

Fig. 2 T.S reproductive utricles with gametangia

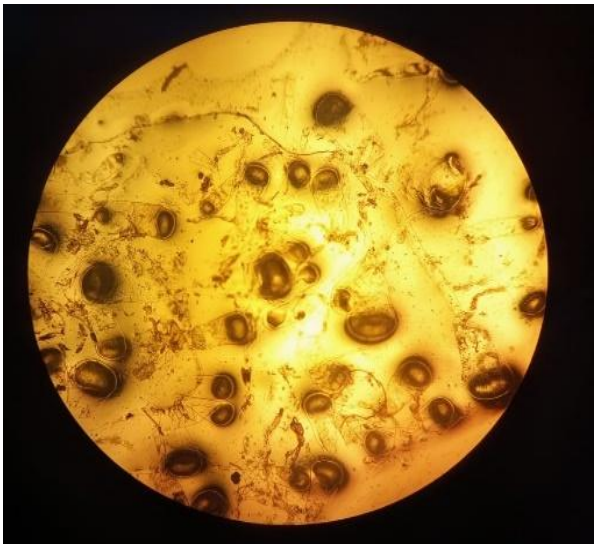

Fig. 3 Different types of utricles

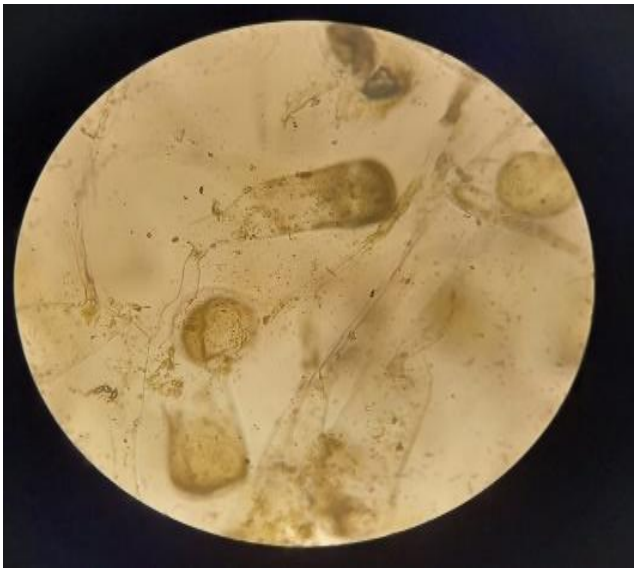

Fig. 4 Different parts of the thallus

Gametangia are found laterally on the utricles. The size ranges between 300–400 µm in length and 100-175 µm in width. Transverse division frequently divides the contents of the gametangia in half.

3.3. Physicochemical Analysis

The physicochemical parameters of crude powder of *Codium flabellatum* were carried out as shown in Table 1. The moisture (water) content for the said seaweed was 94.55%. The maximum soluble extractive value was found in the chloroform and methanol, 96% and 80.95%, while the minimum soluble extract was found in the hexane, 54.54%.

Table 1 Physicochemical parameters of *Codium flabellatum*

S. No.	Parameters	Values (%)
01	Moisture (water) content	94.55
02	Total ash value	84.15
03	Inorganic ash	15.85

Continuation of Table 1		
03	Extractive value (n-hexane)	54.54
04	Extractive value (ethanol)	69.84
05	Extractive value (methanol)	80.95
06	Extractive value (butanol)	76
07	Extractive value (chloroform)	96
08	Extractive value (ethyl acetate)	74.28

3.4. Phytochemical Analysis

A qualitative phytochemical analysis of the various extracts of *C. flabellatum* was carried out, and carbohydrates, proteins, lipids, gelatin, flavonoids, and tannins were evaluated. Details of the macro-molecules were present in a polar solvent, some in semi-polar, while others were shown in non-polar solvents, as mentioned in Table 2.

Table 2 Preliminary phytochemical analysis of *Codium flabellatum* by using various solvents

Preliminary phytochemical analysis	**Ethanol**	**Ethyl acetate**	**N-hexane**
		Carbohydrates	
Molish test	+++	+	-
Iodine test	+++	+++	+++
Benedict test	++	---	---
		Lipid	
Sudan IV test	-	+	+++
Grease spot	+	++	+++
Emulsification	+	+	+++
		Protein	
Biuret test	+	+	+
Millon test	_	+	+
Nin-Hydrin	+	+	+
		Gelatin	
Soda-lime	--	--	+
Precipitation test	+	+	+
		Flavonoids	
Ammonia test	-	++	-
Shinoda test	-	-	+
Vanillin Hcl	_	_	_
		Tannin	
Iron Salt	+	+	+
Gold beater skin test	-	-	-
Bromine test	+	_	_

Notes: (+++) means more potent, (++) moderate amount, (+) less amount/traces (-) absent

4. Discussion

Globally, the significance of marine algae is increasing due to the presence of secondary metabolites obtained from them, and these valuable constituents are useful for therapeutic purposes. Besides health benefits, marine seaweeds are also used in numerous industries such as pharmaceuticals, nutraceuticals, fertilizer, cosmetics, and cattle feed can be used as fuel. Marine algae are used by space technology, and other environmental departments as an indicator for changes in environmental conditions and algae possess the capability to filter the pollutants and essential nutrients from the waste and contaminated water. Seaweeds are also used to prepare vegetable oils in abundant quantities compared to terrestrial plants. Biodiesel, bioethanol, and Butanol can also be obtained from marine algal flora. Seaweeds are also reported to perform biological and pharmacological activities. So, for the safer consumption of marine algae in medicines, it is necessary to evaluate the quality and standardization of algae that ensure the authenticity of any seaweed for biological applications. Such studies should be conducted before evaluating any other activity recommended by the world health organization [14].

Codium flabellatum was first identified by [15], but, due to incorrect identification, it was reinvestigated by [6]. He described its taxonomy for the first time. After 25 years, we have performed macroscopic and microscopic features under a digital microscopic camera to study the morphological characteristics of drugs. Physiochemical parameters like water content, total ash, and extractive values were determined to be assessed a drug's purity and quality. The resultant total organic ash was found to be 84.15%, and the inorganic ash was 15.85%. The ash content of a drug sample is usually determined by the number of inorganic salts contained in the sample and the residue left after incineration [16]. The mineral content of marine algae has been observed to range between 8% and 40%. Marine algae are a source of iodine and calcium. Dry matter accounts for 65 to 85% of organic substances and 30 to 35% of ash [8, 9, 17, 18]. Moisture content also has a significant role in chemical reactions and serves as a bridge between components used in food production and pharmaceuticals. Fresh seaweed contains 75-85% water. In our study, 94.55% of the water content was observed. The extractive values give information on the constituents present in a specific solvent and aid the researcher in further analysis. The result suggests that the extractive value is higher in chloroform and methanol, which proves the compounds' solubility in both polar and semi-polar solvents.

The results of phytochemical screening on three different extracts (ethanol, ethyl acetate, and n-hexane) revealed the occurrence of carbohydrates, protein, lipid, gelatin, and tannin. Among three extracts, all compounds are present, while only flavonoids are absent in ethanol.

A literature survey proved the presence of carbohydrates, protein, tannin, and flavonoids in the same species and other sister species [8, 9, 19, 20]. These preliminary phytochemical studies give an idea regarding the use of seaweed for a particular biological activity. Seaweed is a rich source of carbohydrates and structural polysaccharides, including pectin, gums, and guars, performing strong activity against hypocholesterolemic and hypoglycemic conditions. Protein and flavonoids, among other macro and micronutrients, are also abundant in algae. The food and animal feed industries have long used these compounds because of their nutritional importance. They are a source of vital amino acid-rich

proteins. Non-digestible carbohydrates from seaweed are a source of dietary fiber [21]. Due to the presence of tannins, they might have been employed as antiviral, antiulcer, and antibacterial agents. Tannin-containing medications are often used to treat piles, burns, inflammation, and as an astringent [23]. Flavonoids showed their presence in n-hexane and ethyl acetate. Flavonoids are one of the largest groups of phenolic compounds, and studies have exhibited the potential beneficial effects of flavonoids in fighting disease [8, 9, 22]. In the current results, an ample amount of lipid content has been shown in hexane extract, whereas ethanol and ethyl acetate contain minute quantities. Seaweed lipids vary according to species, season, geographical location, temperature, light intensity, salinity, and species type, as well as combinations of these parameters. Very little research has pointed out the therapeutic qualities of the green seaweed *C. flabellatum.* Further investigation of the extracts is in progress to isolate, identify, and elucidate the bioactive chemicals responsible for their pharmacological action.

5. Conclusion

Marine algae are considered a rich source of macro- and micro-molecules that can be consumed as drugs or starting materials for developing a number of products and medicines. Hence, it is important to evaluate physicochemical, phytochemical, and pharmacognostic features as diagnostic aspects aiding in authenticating and identifying the crude drug *C. flabellatum.* In addition, these characteristics may be used as a reference standard for this species and can also aid in developing a monograph.

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