

Qualitative Tests of Secondary Metabolite Compounds in Ethanol Extract of *Spirulina platensis* from Karimun Jawa Sea, Indonesia

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Abstract. *Spirulina platensis* is a spiral green-blue algae that is abundant in the subtropical and tropical water bodies. *S. platensis* has several potentials to be used in the medical fields due to its wound healing and antitumor potentials. This research aimed to evaluate the secondary metabolite compounds contained in the ethanolic extract of *S. platensis* from Karimun Jawa Sea, Indonesia. The dried *S. platensis* was macerated and extracted using ethanol, then was filtered using Whatman filter paper. A series of tests has been done to determine the secondary metabolites in the ozonated oil using several chemistry tests and reagents in the ethanol extract of *S. platensis*. The tests found that *S. platensis* microalgae harvested from Karimun Jawa sea contains alkaloid, saponin, flavonoid, and quinone. As *S. platensis* from different region contains different substances, this research shows the substances contained in *S. platensis* from Karimun Jawa sea. With this research, we knew that *S. platensis* from Karimun Jawa islands contains alkaloid, saponin, flavonoid, and quinone which can be used as a medicinal ingredient.

Key words: Extraction; Secondary Metabolite; *S. platensis*

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INTRODUCTION

Spirulina platensis is a species of filamentous photoautotrophic green-blue algae that belongs to cyanobacteria class (Saranraj & Sivasakthi, 2014; Suratno, 2010; Wollina et al., 2018a). Cyanobacteria are one of the oldest forms of living things found in the Earth. They share similarities to the primitive bacteria due to their lack of cell walls and with plants because of their photosynthesis ability. *Spirulina* is one of the edible cyanobacteria along with some other species such as *Nostoc* and *Aphanizomenon* (Saranraj & Sivasakthi, 2014).

The filaments of *Spirulina* is small, measuring about 0.1 mm, coiled in variable tightness and number of spirals (Saranraj & Sivasakthi, 2014). Under the light microscope, *Spirulina* can be seen as small (6-12 micrometer wide) trichomes which are coated in a thin layer of wall. The trichome has greenish-blue color (hence its name), and has the ability to move freely (Vonshak, n.d.). Several environmental factors, including temperature and physicochemical conditions can affect its growth. *S. platensis* can be found in tropical and subtropical areas with warm temperature, as it grows optimally at the 30-37 degrees Celcius temperature area with alkaline pH and high bicarbonate content (S. K. Ali & Saleh, 2012; Saranraj & Sivasakthi, 2014). Salinity is

also one of the factors affecting the growth of *Spirulina*. *Spirulina* is mostly found in areas with high concentration of natrium chloride (NaCl) and bicarbonate, which resulted in higher content of its phycocyanin pigment (Christwardana et al., 2013; Saranraj & Sivasakthi, 2014).

Recently, *S. platensis* is being studied because of its high nutritional content which can be used in the medical field (Saranraj & Sivasakthi, 2014). Its interest is getting higher since a lot of people are trying to use "back to the nature" concept of using natural ingredients as medical therapeutic agents (Ergina & Pursitasari, 2014). However, further development of drugs and medicinal products are engineered towards the active contents contained in the medicinal product ingredient; in this case *S. platensis*. Therefore, it is important to determine what are the substances contained in the *S. platensis* which can be used as a basis for further drug development. *S. platensis* is mostly composed of proteins (around 50-70% of the dry weight). The essential amino acids contained in *Spirulina* include leucine, valine, and isoleucine (Sotiroudis & Sotiroudis, 2013). *Spirulina*'s green color comes from phycobiliproteins contained within *Spirulina* cells which serves as a light-collecting pigment for its photosynthesis activity. Phycobiliproteins consist of three pigments i.e. allophycocyanin, phycocyanin, and

phycoerythrin. From these three pigments, phycocyanin is the pigment that is mostly studied due to its potential benefits in the medicine field (Saranraj & Sivasakthi, 2014).

Several studies have found health benefits of *S. platensis*. *S. platensis* are found to have antifungal activity against several kinds of fungi, including *Aspergillus fumigatus*, *Mucor vulgaris*, *Penicillium expansum*, *Fusarium solani*, and *Fusarium oxysporum*; all of which contributes to the invasive mycoses infection (Otlu & Rudic, 2016). Phycocyanin pigment of *S. platensis* has been found to be able to reduce edema, histamine release, myeloperoxidase activity of macrophages, and reduces prostaglandin E2 and leukotriene LTB4 in inflammatory lesions (Wollina et al., 2018b). *Spirulina* is also found to be able to improve wound healing by increasing fibroblast proliferation and migration, enhancing wound closure rate, and inhibiting colonization of *Staphylococcus aureus* in wounds (Wollina et al., 2018a). Anticancer activities can also be found in *S. platensis*. One research found that *S. platensis* has anti skin tumor effects in mice that is exposed to ultraviolet B (UVB) rays (Yogianti et al., 2014).

As with many other living organisms, *S. platensis* also produces secondary organic metabolites. Secondary metabolites are substances that are produced as a result of metabolism through secondary reactions of primary organic substances (carbohydrates, fats, and proteins). These precursor substances that are used in the biosynthesis of secondary metabolites came from primary metabolism process (Mardani, 2013). Secondary metabolites are small, specific and unique metabolites. Not all organism will have the similar configuration and amount of secondary metabolites. Secondary metabolites has the potential to be used as a lead compounds that can be used in the drug development (Ergina & Pursitasari, 2014).

Secondary metabolites are classified into three groups: phenolic, alkaloid, and terpenoids. Most common secondary metabolites which can be found in plants are alkaloid, flavonoid, steroid, saponin, terpenoid, and tannin (Ergina & Pursitasari, 2014). Terpenes are substances formed by a chain of isoprenes, which carbon structure is formed by a link of two or more C5 carbons. Terpenes consist of several substances such as monoterpenes and sesquiterpenes which are volatile substances, diterpenes which are semi-volatile, and triterpenes and sterols which are non-volatile (Khotimah & Minarno, 2016). Terpenoids are a group of secondary metabolites that is effective to be used as plants-originated insecticides (Mardani, 2013).

Alkaloids are basic substances that contains at least one nitrogen atom which form heterocyclic chain. Most alkaloids are colorless and has active optic properties. Flavonoids are secondary metabolites which are synthesized from pyruvate acids through the metabolism of amino acids. Flavonoids are mostly found in the form of glycosides on one or more groups of phenolic hydroxyls (Khotimah & Minarno, 2016) There are several kinds of flavonoids which can be identified, including anthocyanin, proanthocyanidins, flavanols, flavone, glycoflavone, biflavonyle, chalcone, aurone, flavanone, and isoflavone (Khotimah & Minarno, 2016). This experiment aimed to determine the secondary metabolites contained in *S. platensis* extract, such as alkaloid, saponin, phenol, flavonoids, quinone, triterpenoid, and steroid. We hoped that this research helps scientists and pharmacists to discover the contents of *S. platensis* from Karimun Jawa and enables the usage of *S. platensis* from Karimun Jawa islands to be used as a pharmaceutical product.

METHODS

This research was done in Chemistry and Biochemistry Laboratory, Faculty of Medicine Diponegoro University, Indonesia. The *S. platensis* algae were obtained from Karimun Jawa Sea, Central Jawa, Indonesia. All of the tests done to analyze the presence of the secondary metabolites were done with *S. platensis* extract as the test sample using several tests. In this research, we analyzed the presence alkaloid, saponin, phenol, flavonoids, quinone, triterpenoid, and steroid in the *S. platensis* extract.

This experiment began with the maceration of *S. platensis* sample. The maceration process was done to extract the active ingredients contained within the *Spirulina* cells, so that they can be analyzed once they are no longer trapped inside the cells. The liquid then was filtered using Whatman filter paper to separate the solute from the solids, before further evaporation to obtain a thick extract of *S. platensis*.



Figure 1. Microscopic image of *Spirulina platensis* (Whitton, 2012)

Extraction of *S. platensis*

The raw *S. platensis* algae gathered from the Karimun Jawa Sea were dried. The dried *S. platensis* were crushed into fine powder. The powdered *S. platensis* microalgae was macerated in 95% ethanol solution with 1:10 concentration (one part of *S. platensis* powder macerated in 10 parts of 95% ethanol solution). The maceration process was done for five days in a glass container. The glass container was stirred every day to make sure the uniformity of the maceration process. After five days, the solution was filtered through Whatman filter paper, and was evaporated using rotary evaporator machine at the ethanol boiling point temperature until a thick extract was obtained. This extract of *S. platensis* was used as the material in the subsequent tests.

Identification of alkaloid

The identification of alkaloid in this study were done by diluting a few drops (around 1 milligram in weight) of *S. platensis* extract into five milliliters of 2 N sulfuric acid. The mixture of *S. platensis* extract and 2 N sulfuric acid were subsequently tested using 5 drops of both Mayer's and Liebermann–Burchard reagents on separate test tubes (Khotimah & Minarno, 2016; Suratno, 2010). The formation of white precipitation at the bottom of the test tube after the reagent was added shows the presence of alkaloid substances (Akerina & Sangaji, 2019; Ergina & Pursitasari, 2014; Rasyid, 2012).

Identification of saponin

Identification of saponin were done using foam test. *Spirulina* extract was diluted into 9 ml of water and then heated for 5 minutes. The solution then was agitated vertically for 10 seconds. The agitation process will result in the formation of foam layer on top of the solution. Afterwards, one drop of 2 N hydrochloric acid was added into the solution. The presence of saponin can be confirmed by the formation of foams (at least 1 cm high) that remains on the top of the solution for at least 10 minutes and does not subside with the addition of 2 N HCL (Rasyid, 2012; Suratno, 2010).

Identification of phenolic substances

The identification of phenolic substances in this study were done using FeCl_3 solution as the reagent. One milliliter of *Spirulina* ethanol extract was mixed with 2 drops of 5% FeCl_3 in a test tube. The presence of phenols can be identified by the formation of green

or greenish-blue color (Akerina & Sangaji, 2019; Suratno, 2010).

Identification of flavonoids

The presence of flavonoids in the ethanol extract of *S. platensis* were identified using Willstatter test. This test uses magnesium strips and hydrochloric acid-ethanol mixture as the test reagent. One milliliter of *S. platensis* ethanol extract was put into a test tube. Subsequently, 0.1 mg of magnesium and 0.4 ml of amyl alcohol (a mixture of 37% hydrochloric acid and 95% ethanol with similar volume) was added into the test tube. Positive result can be seen by the color change of the solution, which will turn dark yellow or orange (Khotimah & Minarno, 2016; Suratno, 2010).

Identification of quinone

The identification of quinone in this study was evaluated using sodium hydroxide (NaOH). One milliliter of sample was mixed with 1N NaOH and then was evaluated for color changes. Positive result can be seen by the formation of yellow or yellowish-green color resulted from the reaction (Suratno, 2010).

Identification of triterpenoids and steroids

The presence of triterpenoid and steroid in this study were evaluated using chloroform, sulfuric acid, and anhydrous acetate. A few drops of sample were added into a test tube containing 2 mL chloroform. Subsequently, 10 drops of anhydrous acetate and 3 drops of sulfuric acid were added into the test tube. The positive results can be seen from the color change of the sample: initially, the color will turn into red; afterwards it will change into blue or green (Suratno, 2010). The red color indicates the presence of terpenoids, while the green color indicates the presence of steroid (Ergina & Pursitasari, 2014).

RESULTS AND DISCUSSION

In this research, we conducted the tests explained in the above paragraphs to our *S. platensis* sample from Karimun Jawa sea. The positive result from the Mayer's reagent showed that *S. platensis* extract contains alkaloid substance. However, alkaloid identification using Liebermann-Beurchard reagent did not give positive result. This result was similar to the previous identification result of two different researches by Mane & Chakraborty (2018) and Suratno (2010).



Figure 2. Positive alkaloid test using Mayer's reagent on *S. platensis*

After agitation of the mixture, a layer of foam was present on the top part of the mixture, which was present until 10 minutes were elapsed. The foam did not subside upon the addition of one drop of hydrochloric acid. Therefore, this result indicated that *S. platensis* extract contains saponin. This result were in line with previous researches (Fithriani et al., 2015; Mane & Chakraborty, 2018; Saputra, 2018; Suratno, 2010) which found that *Spirulina* sp. contains saponin.



Figure 3. Positive saponin test using foam test on *S. platensis*

Phenolic substance identification test results in color change from green to yellow after the addition of FeCl_3 was observed. Therefore, this result showed that the sample of *S. platensis* does not contain phenolic substances.



Figure 4. Negative phenolic identification test using FeCl_3 reagent on *S. platensis*

We noticed a color change from dark green to yellow was observed in our flavonoid test. This result showed that the *S. platensis* sample positively contains chalcone, aurone, and flavone content. Several previous researches also found similar results regarding the identification of *S. platensis* extract from another region, including in India (Fithriani et al., 2015; Mane & Chakraborty, 2018).



Figure 5. Positive flavonoid test result using magnesium and hydrochloric acid-alcohol reagent on *S. platensis*

We observed a greenish-yellow color in our quinone test, which confirmed that the *S. platensis* sample in this research contains quinone. This result was similar to the previous research conducted by Suratno, (2010), but not with the other researches.



Figure 6. Positive quinone test using sodium hydroxide reagent on *S. platensis*

The triterpenoids and steroids identification test, the color was initially brown, which at the end of the observation period turned into yellowish-green color. This result showed that our sample of *S. platensis* does not contain triterpenoids and steroids.



Figure 7. Negative triterpenoid and steroid identification test using chloroform, anhydrous acetate and H_2SO_4 reagent on *S. platensis* sample from Karimun Jawa Sea, Indonesia.

In this research, the sample of *S. platensis* microalgae that we harvested from Karimun Jawa sea contains alkaloid, saponin, flavonoid and quinone. This result is mostly similar to the previous research conducted by (Suratno, 2010), where he found that the *S. platensis* sample from Lamongan, East Jawa contains alkaloid, saponin, phenol, and quinone.. Another result conducted by (Saputra, 2018) found that ethanolic extract of *S. platensis* contains flavonoids, saponin, phenolic substances, and steroids. A study conducted using laboratory-grown *Spirulina* sp. sample which was planted in sterile water medium found that *Spirulina* sp. contains tannin, flavonoids, steroids, glycosides, alkaloids, and saponin (Fithriani et al., 2015).

The active metabolites of *Spirulina platensis* are tightly affected by the environment they are grown with. (Notonegoro et al., 2018) Results of phytochemical analysis of *S. platensis* extract from

Rankala Lake, Kolhapur, India was slightly different from our study. They found that their *S. platensis* ethanolic extract contains alkaloids, terpenoids, steroids, saponins, flavonoids, phenols, and coumarins (Mane & Chakraborty, 2018). The phytochemical contents were differing from one solvent to another; they found that methanol can extract every single metabolite from *S. platensis* powder, and the other solvents (ethanol, petroleum ether, acetone, and aqueous extract) does not have the same yield (Mane & Chakraborty, 2018). A quantitative study on *S. platensis* extract from Egypt found that *S. platensis* contains relatively high phycobiliproteins, phenolic substance, alkaloids, and terpenoids (Ali et al., 2014).

Saponin is a glycoside that can be found in many organisms. The saponin contents of an organism is highly affected by the variety and stage of growth. The function of saponin are most likely as a storage form of carbohydrate or might be related to defending mechanism against insects. The test for determining the presence of saponin glycosides involves the ability of the shaken extract in the water to form a stable foam that does not subside after a brief period; after all, saponins itself are characterized by their ability to form strong foams in an aqueous solution (Man et al., 2010; Nurwidayati, 2012) Saponins are known to exhibit several medicinal properties, for example as an antidermatophytic agent and antimicrobial activity. Saponins also has the properties to reduce *Malassezia furfur* growth, which can be used as an anti-dandruff formula in shampoo. (Tamura et al., 2012) Saponins also exhibited anti-cancer properties in terms of inhibiting tumor angiogenesis. It works by suppressing cancer angiogenesis inducer in blood vessel endothelial cells and also prevents the adherence, invasion, and metastasis of tumor cells. (Man et al., 2010)

The principle that was used in the alkaloid tests are precipitation reaction caused by ligand exchange. The nitrogen atoms that has free electrons in the alkaloid can replace the iodo ion in the reagent (Khotimah & Minarno, 2016) Most alkaloids are not soluble in water, except if they reacted with acid which causes them to form water-soluble alkaloid salt complex (Khotimah & Minarno, 2016). The white precipitation resulted from the reaction is potassium-alkaloid complex. The nitrogen in the alkaloid will react with the potassium ion from the potassium tetraiodomercurate(II) which forms potassium-alkaloid complex that precipitates in the bottom of the reaction tube (Ergina & Pursitasari, 2014; Suratno, 2010).



Figure 8. Reaction of alkaloid identification (Ergina & Pursitasari, 2014)

The flavonoid test in this experiment was done using magnesium and hydrochloric acid. The addition of magnesium and hydrochloric acid was used to reduce the benzopyrone which is contained in the flavonoid structure. The result of this reaction is the formation of flavylium salts, which has the red or orange color. Flavonoid itself is a substance that contains two aromatic rings with more than one hydroxyl groups (Ergina & Pursitasari, 2014).

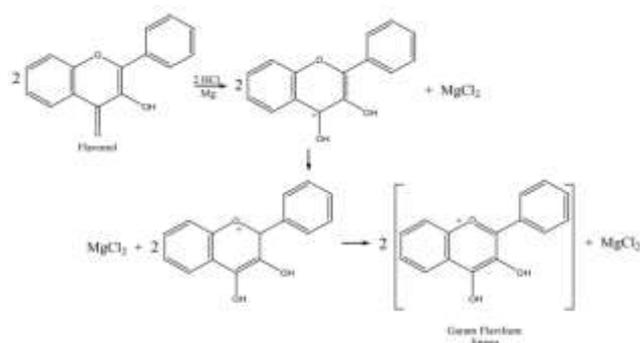


Figure 9. Reaction of flavonoid with magnesium and hydrochloric acid (Ergina & Pursitasari, 2014)

The *S. platensis* sample in our study contains quinone. The principle of quinone identification test were based on the colorimetric reaction. The reaction of quinone identification test involves reciprocal reduction which converts quinone into a colored substance which can be identified with naked eye. This reduction reaction will subsequently be reverted if the solution were exposed to the air (Khotimah & Minarno, 2016).

This research enables *S. platensis* from Karimun Jawa Islands, Indonesia to be used as an ingredient for herbal preparation which needs alkaloids, saponin, flavonoids, and quinone. Alkaloids has several medicinal properties, including analgesic, anti-cancer, anti-arrhythmic, anti-hyperglycemic, and anti-psychotropic effects (Hussain et al., 2018). *S. platensis* saponins are known to be cytotoxic to cancer cells and has the potential to decrease cancer cellular viability; thus enables its use as an anticancer candidate (Akbarizare et al., 2019). Alkaloids and quinones are also can be utilized as medicinal ingredient. Different region and different growth conditions results in different substances contained in the *S. platensis* algae. Therefore, this result shows the contents of *S. platensis* from Karimun Jawa islands. We hope that this research increases the use of *S.*

platensis as an ingredient of healthy foods and medicines with potent efficacy.

CONCLUSION

S. platensis microalgae that we harvested from Karimun Jawa sea contains alkaloid, saponin, flavonoid, and quinone.

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