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Chemical Profiles and Biological Activities of Microalga *Chlorella* sp. from Southeast Sulawesi

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Abstract

Microalgae Chlorella sp. grows in the seas of South-East Sulawesi, Indonesia. However, information on the chemical and pharmaceutical aspects of this species from this region is still limited. Therefore, this research aims to explore the chemical contents and biological activities of Chlorella sp. The sample was collected from the Kali Biru and Nambo waters, Kendari. The microalgae were isolated by the agar plating technique and extracted by ethanol. Chemical content was analyzed by phytochemical screening, Liquid Chromatography-tandem Mass Spectroscopy (LC-MS/MS), Total Phenolics Content (TPC) and Total Flavonoids Contents (TFC). Biological activities evaluation includes antioxidant, toxicity, cytotoxicity and antimicrobial. Antioxidant potency was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). Toxicity and cytotoxicity properties were analyzed by BSLT (Brine Shrimp Lethality Test) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays against Breast cancer (MCF-7) cell lines. Antimicrobial potency was tested towards E. coli, S. aureus and C. albicans. The result showed that ethanol extract of Chlorella sp. (EEC) contained phenolics, flavonoids, alkaloids and terpenoids/steroids which were supported by LC-MS/MS data, TPC and TFC with value 150.33 and 33.18 mgGAE.g^{-1} extract. Seven of ten identified major compounds caused this extract to have antioxidant and anticancer including xanthin, 1,1-Diethyl-3-oxo-2- triazanolate, Azelaic acid, ruspolinone, 6-keto prostaglandin F1a and glycol stearate. In general, the biological activity of EEC is less active than the positive controls for each assay. However, the EEC is more potential to be developed as antioxidant such as sunscreen and anticancer especially breast cancer with IC_{50} value 151-200 ppm and 100-500 ppm, respectively.

Keywords: *Chlorella sp., Ethanol extract, Chemical contents, Biological Activities, Southeast Sulawesi*

Introduction

Southeast Sulawesi is an archipelagic province, has 651 islands, 74.25% of its territory is the ocean (Bangwilsultra, 2016). One of the important and abundant marine natural resources is microalgae or microscopic plants (ø= 3-30 μm). Microalgae have the potential to be developed in the fields of health, food industry, energy, nutraceuticals, pharmaceuticals and cosmetics as well as commercial industries in general (Andrade, 2018; Abdel-Karim *et al*., 2020; Andriopoulos *et al*., 2022). Microalgae resources obtained (isolated) from the waters of Southeast Sulawesi have not been touched much by research activities, both spatially and temporally. Currently, the microalgae research in Southeast Sulawesi is focused on the diversity, distribution and population. In the waters around Kendari City (Toronipa Beach) and Konawe Regency (Batu Gong Beach), 16 types of microalgae have been found, but only 2 types have been identified, namely *Skeletonema* sp., and *Nannochloropsis* sp. (Indrayani *et al*., 2018).

Marine microalgae have attracted the attention of researchers for decades due to their biomolecule content which has various bioactivities. Its development has reached the stage of utilization in various fields such as the functional food industry, nutraceuticals, pharmaceuticals, cosmetics and food

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supplements (Andrade, 2018), as well as the feed industry for aquaculture biota (Andriopoulos *et al*., 2022). Abdel-Karim *et al*. (2020), stated that microalgae have been widely used as food, medicine and cosmetics because they contain many secondary metabolites. Erb *et al*. (2020) and Thangaraj *et al*. (2022) stated that one of the secondary metabolites synthesized by microalgae is phenolic compounds. Phenolic compounds have various bioactivities that can be utilized for human benefit. Phenolics from marine microalgae have antimicrobial, antiviral, anticancer, antidiabetic, antioxidant or anti-inflammatory properties (Azaman *et al*., 2017). Phenolic compounds are also known as polyphenols, namely organic compounds that chemically consist of one or more phenolic rings. Phenolic compounds are considered an important group of natural antioxidants because they can protect living cells from damage caused by free radicals (Andrade, 2018).

The secondary metabolic in microalgae consist of terpenoids, flavonoids, alkaloids, phenolics and saponins. Apart from that, it also contains the pigments chlorophyll, phycobilin, EPA, DHA and carotenoids. Carotenoid pigments consist of carotene and xanthophyll. Carotene is a precursor of vitamin A which is useful for the human body which is metabolized from β-carotene via enzymes. This diversity of bioactive compounds makes microalgae ideal candidates for wide applications, including the pharmaceutical industry, as a source of new drugs and cosmetics that can be used as raw materials in the form of powder, tablets, cream or gel. Microalgae that are rich in phenolic compounds and other secondary metabolite compounds include the green algae *Chlorella* sp. (Azaman *et al*., 2017).

Chlorella sp. contains the highest amount of chlorophyll compared to other plants and β-carotene (Jayshree *et al*., 2016), and considered as an alternative in phytoremediation and also a potential renewable energy source, such as biodiesel because of its high lipid content (Varfolomeev and Wasserman, 2011). Apart from being a food supplement, the microalgae *Chlorella* sp., has been proven to have antibacterial activity in vitro against gram-positive and gram-negative bacteria (Renukadevi *et al*., 2011).

Several Chlorella, like *C. pyrenoidosa*, produce carotenoids such as β-carotene, α-carotene, lutein, zeaxanthin, astaxanthin, and neoxanthin. Each gram of the dry cell mass contains a total of 7 mg carotenoids (3.5 mg lutein, 0.5 mg α-carotene, 0.6 mg β-carotene) and 35 mg chlorophyll (Iwamoto, 2004). The main carotenoids from *C. ellipsoidea* consist of violaxanthin, antheraxanthin and zeaxanthin, while the carotenoids from *C. vulgaris* consist almost entirely of lutein (Cha *et al*., 2008). These compounds, apart from being used in cataract medicine, also function as anti-aging agents (Iwamoto, 2004). *C. vulgaris* also contains flavonoids (Jayshree *et al*., 2016) which act as antioxidants by chelating metal ions, preventing radical formation and increasing the endogenous antioxidant system (Dai and Mumper, 2010). Various potential Chlorella sp. as an antioxidant, cytotoxic and the chemical compound content described above is an important basis for carrying out this research in the waters of Southeast Sulawesi on chemical and pharmaceutical aspects.

In continuing our study on chemical and pharmaceutical aspects of marine natural resources, microalgae especially *Chlorella* sp. was chosen as the research sample. Before research on microalgae, we had worked with sponges (Sahidin *et al*., 2018; 2020; Wahyuni *et al*., 2019; Fristiohady *et al*., 2019, 2020), soft coral (Sadarun *et al*., 2022; Sahidin *et al*., 2023a,b) and microalgae (Haslianti *et al*., 2023).

Based on the literature review (searching) in Science Direct and Springer Link, no information has been found regarding the study of chemical and pharmaceutical aspects of *Chlorella* sp. from South East Sulawesi (Indonesia). This article explains the study of antioxidant, toxicity, cytotoxicity against cancer cell lines especially breast cancer cells lines (MCF-7), antimicrobial potency as well as the study of the chemical content using LC-MS/MS of the ethanol extract of *Chlorella* sp. that grows in Southeast Sulawesi.

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Materials and Methods

The sample was collected Kali biru and Nambo Beach (Kota Kendari). Isolation and incubation *Chlorella* sp. used procedure outlined by Anderson and Kawachi (2005). The culture and microalgae identification used standard method (*Haslianti et al*., 2023)

Fresh *Chlorella* sp. (2 g) was extracted in ethanol (3x10 mL, 24 h each time) at room temperature to get 0.5 mg ethanol extract of *Chlorella* sp. (EEC). Each ethanol extract was collected and concentrated under reduced pressure and stored at 4°C for future analysis in an amber bottle.

The presence of alkaloids, flavonoids, tannins, terpenoids, steroids, and saponins in the selected samples were determined using phytochemical screening methods (Harborne, 1973). The EEC was prepared for LC-MS/MS analysis using the standard operating procedure of this instrument (Sahidin *et al*., 2020).

Total Phenolics Content (TPC), Total Flavonoids Content (TFC) and biological activities

The TPC of the samples were determined using the Folin and Ciocalteu reagent, following Singleton and Rossi's method with minor adjustments and the total flavonoids of the samples were determined using Chang *et al* (Chandra *et al*., 2014). Antioxidant assay of the samples was assessed using the DPPH radical (Sahidin *et al*., 2020). The MTT (3-(4,5-di methylthiazol –2-yl)-2,5-diphenyltetrazoliumbromide) assay was used to assess cytotoxicity in MCF-7 cells in vitro (Asasutjarit *et al*., 2021) and toxicity was tested by BSLT (Brine Shrimp Lethality Test) (Sukardiman and Pratiwi, 2004). Antimicrobial potency of the EEC was evaluated against *Staphylococcus aureus*, *Escherichia* coli (bacteria) and *Candida albicans* (fungi) (Balouiri *et al*., 2016).

Result and Discussion

Extraction of microalgae *Chlorella* sp. (2 g) produced 0.5 g the extract (25 % yield). Phytochemical screening indicated that the EEC contained phenolics, flavonoids, alkaloids and terpenoids/steroids compounds. The presence of phenolics and flavonoids in the extract was supported by TPC and TFC values that are 150.3±1.22 and 33.2±1.88 mgGAE.g^{-1} ext. respectively, which is displayed in Table 1.

The existence of these compounds is also supported by LC-MS/MS data. The UPLC chromatrogram in Figure 1 shows that more than 100 reported compounds consisting of compounds that have been identified and unidentified or are not in the database or are possible new compound candidates. The ten main compounds are outlined in Table 2.

Table 2 shows a comparison of the data obtained from LC-MS/MS (MS^1 and MS^2) with the reference. So it can be concluded that the 10 main compounds that have been identified consist of Xanthine, 1,1-Diethyl-3-oxo-2-triazanolate, D(+)-Phenyllactic acid, Azelaic acid, Ruspolinone, (15Z)-9,12,13 – Trihydroxy – 15 - octadecenoic acid, 6 -keto Prostaglandin F1α, 3-Hydroxypentadecanoic acid, Methyl-3-hydroxy palmitate, and Glycol stearate. The structure of the compound is shown in Figure 2.

The presence of alkaloids in phytochemical screening was supported by the of xanthine, 1,1-diethyl-3-oxo-2-triazanolate, and ruspolinone in LC-MS/MS data. Phenolic/aromatic compounds were represented by D(+)-phenyllactic acid and ruspolinone. The 6-keto Prostaglandin F1α can be an indicator of the presence of terpenoids/steroids compounds in the EES. Meanwhile, flavonoids are not included in the ten main compounds even though the presence of this compounds group can be seen from the results of phytochemical and TFC screening.

Table 1. Chemical profile, TPC and TFC of EEC

	Phenolic	*Flavonoids*	*Alkaloids*	*Terpenoids/ steroids*	*Saponin*	TPC (mgGAE.g^{-1} extact)	TFC (mgGAE.g^{-1} extract)
EEC	+	+	+	+	-	150.3±1.22	33.2±1.88

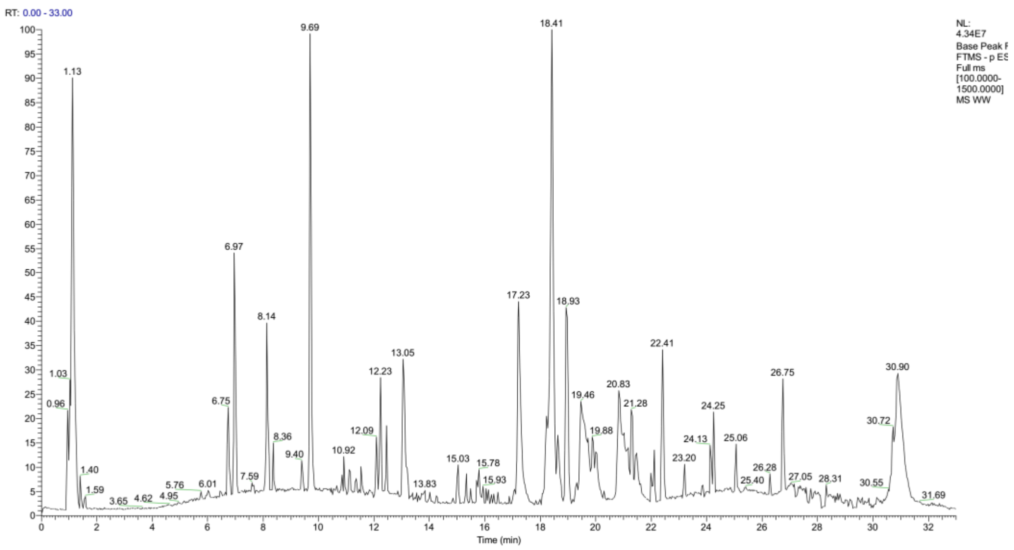

Figure 1. UPLC Chromatogram of compounds from *Chlorella* sp.

Table 2. Compounds of *Chlorella* sp. based on LC-MS/MS data

No	RT (min)	Observed MS^1 [M-H]$^-$ (m/z)	MS^2 Fragmentation	Molecular Weight (Da)	Formula	Component Name	Ref
1	1.13	151.02525	108.02; 94.92; 92.93	152.0334	$C_5H_4N_4O_2$	Xanthine	(Rukdee *et al*., 2015)
2	6.97	131.0703	130.01	132.0773	$C_4H_{10}N_3O_2$	1,1-Diethyl-3-oxo-2-triazanolate	(Gong *et al*., 2021)
3	8.14	165.05486	147.04; 119.05; 72.99	166.0630	$C_9H_{10}O_3$	D(+)-Phenyllactic acid	(Hou *et al*., 2021)
4	9.69	187.09686	143.10652; 125.09619	188.1049	$C_9H_{16}O_4$	Azelaic acid	(Garelnabi *et al*., 2010)
5	12.23	288.12910	165.07416; 103.05409	249.1365	$C_{14}H_{19}NO_3$	Ruspolinone	(Eze *et al*., 2019)
6	13.05	329.23343	229.14516; 171.10185; 139.11203	330.2406	$C_{18}H_{34}O_5$	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	(Lesmana *et al.,* 2021)
7	18.41	369.1010	351.22107	370.2355	$C_{20}H_{34}O_6$	6-keto Prostaglandin F1α	(Enzler *et al.,* 2012)
8	22.41	257.21213	211.20627	258.2195	$C_{15}H_{30}O_3$	3-Hydroxy pentadecanoic acid	(Uhlig *et al*., 2016)
9	24.25	285.24350	147.48150	286.2508	$C_{17}H_{34}O_3$	Methyl-3-hydroxy palmitate	(Kai *et al*., 2015)
10	26.75	327.29037	129.53635	328.2977	$C_{20}H_{40}O_3$	Glycol stearate	(Kim *et al*., 2015)

Table 3. Biological Activities of EEC

Parameter(s)	Sample(s)	Value(s)
Radical Scavenger against DPPH (IC_{50} in mg.L^{-1})	EEC	177.2±2.33
	Ascorbic Acid (positive control)	7.99±0.78
Acute Toxicity against *A. salina* (LC_{50} in mg.L^{-1})	EEC	275.8±2.15
	Potassium Dichromate (positive control)	13.96±0.66
Anticancer Activity against MCF-7 (IC_{50} in mg.L^{-1})	EEC	295.5±2.01
	Fluorouracil (positive control)	5.00±0.22
Antimicrobial Activity against *E. coli* (MIC in mg.L^{-1})	EEC	250.00±1.66
	Chloramphenicol (positive control)	4.00±0.56
Antimicrobial Activity against *S. aureus* (MIC in mg.L^{-1})	EEC	250.00±2.64
	Chloramphenicol (positive control)	2.00±0.44
Antimicrobial Activity against *C. albicans* (MIC in mg.L^{-1})	EEC	250.00±1.88
	Nystatin (positive control)	16.00±1.12

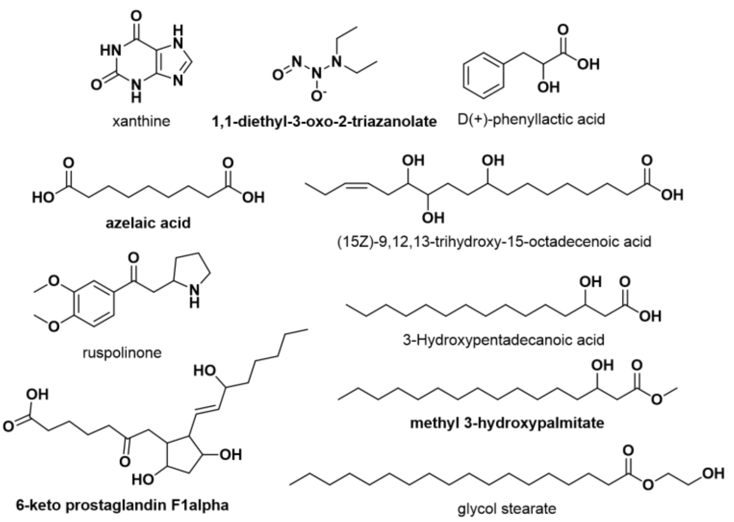

Figure 2. Identified compounds of *Chlorella* sp.

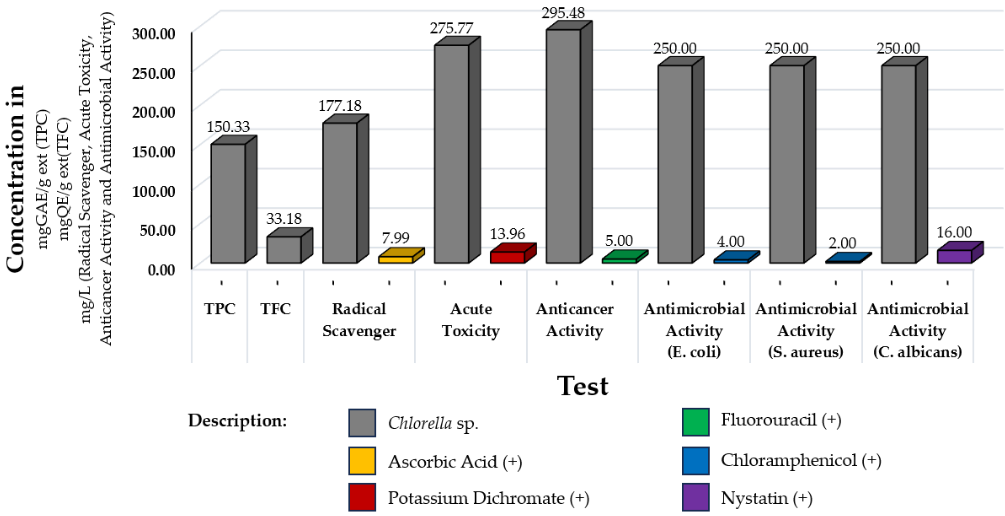

Figure 3. TPC, TFC and Biological Activities of EEC

The diversity of compounds produced by the microalga *Chlorella* sp., will correlate with its biological activities. This evaluation needs to be carried out to utilize these microalgae in the pharmaceutical field. The biological activities that have been evaluated in this research include antioxidant, toxicity, anti-cancer, antibacterial and anti-fungal. The results of the study can be seen in Table 3 and Figure 3.

The main chemical constituents identified by LC-MS/MS from EEC in Table 2 and Figure 2, which support the phytochemical screening results in Table 1, cause EEC to have a variety of biological activities. Although in general the biological activity of EEC is less active than the positive control for each assay. However, the more potential efficacy of the microalgae *Chlorella* sp. can be seen in Table 3 and Figure 3. The antioxidant potential of EEC with the DPPH test system is in the weak category with an IC_{50} value between 151-200 ppm (Sadarun *et al*., 2022), and is toxic with an LC_{50} value <1000 ppm (Meyer *et al*., 1982). Cytotoxic potency of EES towards breast cancer MCF-7 cell line was categorized as moderately active, the IC_{50} value is between 100-500 ppm (Weerapreeyakul *et al*., 2012). Antimicrobial activity against both fungi and bacteria are in the weak category with an MIC value ≥ 32 μg.mL^{-1} (CSLI, 2013). Based on this information, the development of *Chlorella* sp. in the pharmaceutical field, it is more suitable as an antioxidant such as sunscreen (cosmetics) and anticancer.

This is supported by the presence of xanthin which can care for body and skin health, such as anti-aging, eliminating black spots and fighting free radicals and also 1,1-Diethyl-3-oxo-2-triazanolate has radical antidote properties (Michalak *et al*., 2021) Azeleic acid is active anti-inflammatory, tyrosinase inhibitor, anti-acne (Safitri *et al.,* 2020), Ruspolinone can prevent acne, stimulate collagen production, reduce hyperpigmentation and 6-keto Prostaglandin F1a as an anti-inflammatory (Xi *et al*., 2019) and glycol stearate functions to maintain skin moisture.

Conclusion

Ethanol extract of microalgae *Chlorella* sp. (EEC) growing in Kendari waters contains phenolics, flavonoids, alkaloids and terpenoids/steroids. Based on LC-MS/MS data, the presence of xanthin, 1,1-Diethyl-3-oxo-2- triazanolate, Azeleic acid, ruspolinone, 6-keto prostaglandin F1a and glycol stearate causes this extract to have antioxidant and anticancer properties against cancer cells breast MCF-7, Thus, EEC can be developed as an antioxidant such as sunscreen (cosmetics) and anticancer.

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