Assessment of antioxidant and anticancer properties with phytochemical analysis of *Chlorella vulgaris* (Beijerinck 1890) methanolic extract

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ABSTRACT: Algae are highly significant in various fields due to their capability to replace chemical treatments and avoid their associated side effects. They have consistently shown strong efficacy against numerous diseases, regardless of whether they are micro or macroalgae. This research investigated the antioxidant and anticancer properties of a methanol extract from *Chlorella vulgaris.* The growth metrics revealed that *C. vulgaris* reached its peak optical density on the 8th day, its highest growth rate on the 18th day, and its maximum pigment content on the 12th day. The methanol extract displayed in vitro anticancer potential by inhibiting the proliferation of breast cancer (MCF-7) and colon cancer (HCT-116) cell lines, with a stronger effect against colon cancer, achieving inhibition rates of 80.57 ±0.81% and 76.43 ±0.74%, and IC50 values of 14.8 ±0.39 µg and 21.7 ±0.09 µg respectively. The extract's antioxidant activity was highlighted through DPPH and FRAP assays, showing the highest scavenging activity at a concentration of 5 mg/ml. Phytochemical screening identified phenols, alkaloids, flavonoids, glycosides, and saponins as active compounds. In conclusion, the methanol extract of *C. vulg*aris exhibited strong antioxidant and anticancer properties.

Keywords: *Chlorella vulgaris*, antioxidant, anticancer, Breast cancer, Colon cancer, cytotoxicity, DPPH, FRAP

INTRODUCTION

Microorganisms, like unicellular microalgae, can be found in both fresh and saltwater (Garima 2024). Numerous investigations have already been carried out to examine the microalgal byproducts to determine their biological activity as well as to search for components that may have uses for people in several significant fields (Wang et al. 2024). They contain various physiological effects, including antioxidants, antimicrobial, enzyme-inhibiting, cytotoxic, and algicide activity. These effects include both primary and secondary metabolites (Taskin et al., 2007). According to current trends in natural medicinal research, algae have promise as a source of novel biochemically active compounds (Ghaliaoui, et al., 2024). In the meantime, a cooperative technique for exploring the promising effects of these metabolites is the screening of extracts or the isolation of metabolites from different microalgae (Herrero et al., 2013).

C. vulgaris belongs to the family Chlorophyceae. Many nutritional components like pigments, soluble vitamins, choline, dietary fiber, mineral salts like iron, and other bioactive components are all present in good amounts in this species (Spínola et al., 2023). According to Wizilla et al. (2024), it is recognized as a nutritious food source. Reports suggest that *Chlorella vulgaris* microalgae positively influences children's development and enhances body cell growth. (Lee and Lee, 2002). Because it also includes a wide variety of reductants and bioactive secondary metabolites, the microalga is regarded as a good source of antioxidants (Coulombier et al., 2021). Numerous investigations have been conducted to determine the promising roles of C*hlorella*, including increasing animal development, strengthening the immune system, preventing ulcers brought on by stress, acting as an antidiabetic, and treating dyslipidemia brought on by a high-fat diet (Chia, et al., 2013). An imbalance in the ability of the living cells to recover or neutralize the damaging effects of these ROS might result from an excess of them (Verma et al., 2024). Such ROS cause stress leading to lipid peroxidation, protein denaturation, or DNA conjugation, which are all signs of oxidative damage to biological components and ultimately lead to cell death (Yavuz et al., 2003). Any organ's aberrant cell proliferation can lead to cancer, a deadly illness. Finding efficient medications to treat different forms of cancer is challenging, in addition to the high expense of anticancer treatments and their detrimental side effects on the entire body. These problems all make it necessary to create fresh, efficient treatment (Patel et al., 2020). Phytochemical exploration for several promising bioactive components of *C. vulgaris* methanolic extract (CME) will be examined in this work, along with an assessment of its anticancer and antioxidant properties.

EXPERIMENTAL

C. vulgaris culturing conditions

Axenic cultures of *C. vulgaris* (Chlorophyta) were obtained from the Phycology Laboratory at the Faculty of Science, Tanta University, Egypt. The species was first identified using standard

morphological and taxonomical methods as per Desikachary (1959) and Prescott (1962). Algae Base (http://www.algaebase.org) was used to confirm the identification of *Chlorella vulgaris* Beyerinck (Beijerinck). A standard bacterial test was then conducted on a regular basis by inoculating one drop of *C. vulgaris* culture at the start and end of the test into sterilized 10 ml vials containing a bacterial growth medium (GM), F. medium (Feeley et al. 1978). 200 ml of the chosen medium in Bold's Basal Medium (BBM) was added to 500 ml Erlenmeyer flasks (EF) to cultivate *C. vulgaris* axenic cultures. Under carefully monitored laboratory settings (28 °C temperature, 80 µmol m−2S−1 light intensity), the experiment was conducted in a culturing chamber. According to Ginzburg and Ginzburg (1981), this temperature was selected. A cycle of 16 hours of light and 8 hours of darkness was used to enlighten the cultures. To separate adherent *C. vulgaris* cells, each culture flask was manually swirled every day. There were eighteen days throughout the research period. The same growing conditions were used to prepare a large-scale cultivation to obtain adequate biomass for bioactivity assessments.

Growth measurements

Growth was estimated using *C. vulgaris's* optical density, growth rate, which was calculated according to Guillard (1979) equation:

$$\text{Growth rate} = \left(\log_2 \frac{O.D._2}{O.D._1}\right).\left(\frac{1}{t_2 - t_1}\right)$$

Where OD1 was estimated at t_1, time at the beginning of the experiment, while OD2 was estimated at t_2, time at the end of the experiment and pigment content (chlorophyll *a* and carotenoids). For the next eighteen days, these analyses were performed every two days. According to Robert (1979), a Perkin Elmer (Lambda 1) Visible–UV (ultraviolet) spectrophotometer was used to measure the optical density (OD) at 450 nm. Absorption of chlorophyll pigment was observed at 664, 647, and 630 nm wavelengths. Chlorophyll a and b were calculated using the trichromatic equation of Jefrey and Humphrey (1975). The spectrophotometric approach suggested by Jensen and Liaaen (1959) was used to estimate the carotenoids at 450 nm.

***C. vulgaris* mass production and collection**

Prior to this, a *C. vulgaris* culture was cultivated in a 2 L Erlenmeyer flask with 1400 ml of GM and 50 ml of a known aliquot (OD 0.07 at 450 nm). Five groups of 280 ml each of this aliquot were created, and each aliquot was then placed in a 2L EF that had 1400 ml of autoclaved GM. This resulted in five flasks. The indoor airlift cultures and batch cultures were cultivated similarly. For aeration, air bubbles were expelled from the flask bottoms using an air injection device attached to a glass pipe with a 0.2 m sterile bacterial filter. To guarantee adequate culture mixing by the upward migration of air bubbles, the air-flow rate was changed. To harvest the algal cells, centrifugation was performed at 3606 g for 20 minutes. Obtained cells were utilized to create the methanol extract (ME) after the supernatants were eliminated.

***C. vulgaris* methanol extract**

One liter of 100% methanol was used to extract 100 grams of *C. vulgaris* biomass. For three days, the mixes were maintained at room temperature in a shaking incubator. The materials were separated at 7200 g for 20 min after three days. A lyophilizer was used to dry the clear extract after it had been concentrated using a rotary evaporator at 40 °C and reduced pressure (72 mbar). According to Chernane et al. (2014), the pellets were gathered and subsequently dissolved in 5 mg/ml Di-methyl sulfoxide (DEMSO).

Phytochemical analysis of Chlorella Methanol Extract (CME)

The following standard methods, as outlined by Andima et al. (2014), were applied to CME for phytochemical components' screening as follows:

Alkaloids: After adding 3 milliliters of 1% concentrated hydrochloric acid to 3 milliliters of CME in a steam bath, Wagner's reagent was added. Alkaloids existence is demonstrated by the precipitation's turbidity.

Tannins: After adding equal amounts of CME and distilled water while stirring, a few drops of $FeCl_3$ were added. Tannins existence was shown by the production of a green precipitate.

Saponins: In a test tube, five milliliters of extract and five milliliters of distilled water were mixed vigorously and then heated. Stable formation was interpreted as a sign that saponins were present.

Flavonoids: One milliliter of extract was mixed with one milliliter of 2N NaOH solution. The yellow precipitate's development was interpreted as a flavonoid test result.

Terpenoids: After dissolving 2 ml of the CME in 2 ml of chloroform, the mixture was dried by evaporation. After adding two milliliters of concentrated sulfuric acid, the mixture was heated for approximately two minutes. Terpenoids are indicated by a greyish tint.

Steroids: After dissolving two milliliters of the extract in two milliliters of chloroform, two milliliters of strong sulfuric acid were added. The presence of steroids was detected by the formation of a red color in the bottom chloroform layer.

Carbohydrates: Two milliliters of Molisch's reagent were combined with three milliliters of CME, and the mixture was thoroughly shaken. After that, two milliliters of concentrated H_2SO_4 were cautiously poured down the test tube's side. The presence of carbohydrates is shown by a violet ring near the interphase.

Glycosides: The Keller-Kiliani technique was applied to explore Glycosides existence via adding one drop of $FeCl_3$ solution to two milliliters of glacial acetic acid to dissolve two milliliters of CME, then transferred into a test tube that held one milliliter of concentrated H_2SO_4. The presence of a deoxy sugar, which is typical of cardenolides, is indicated by a brown ring in the interphase.

Phenolics: The Folin-Ciocalteau technique was used to determine the total phenolic concentration (Cox et al., 2010). This procedure involved mixing 100 µl of CME with 2.0 ml of 2% Na2CO3 and letting it stand at room temperature for two minutes. Next, 100 µl of the 50% phenol reagent from Folin Ciocalteau was applied. After 30 minutes of room temperature incubation away from light, the absorbance at 720 nm was measured. The usage of gallic acid served as positive control. The amounts of gallic acid equivalent per gram dry weight (mg GAE/g DW) were used to express the samples' total phenolic contents.

Cytotoxic against MCF-7 and HCT-116 cells

MCF-7 and HCT-116 cells from the ATCC Culture Collection were used. Trypan blue dye, crystal violet, and DMSO were all sold by Sigma (St. Louis, Mo., USA). DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin, 0.25% Trypsin–EDTA, and fetal bovine serum were all supplied by Lonza. A Whatmann No. 1 filter paper is used to filter the 0.5% (w/v) crystal violet and 50% methanol that make up the 1% crystal violet stain (Mosmann 1983). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 g/ml gentamycin, 10% heat-inactivated foetal bovine serum, 1% L-glutamine, and HEPES buffer. Every cell was cultivated 2 times a week in a moisted environment with 5% CO_2 at 37°C.

Viability assay

In Corning® 96-well tissue culture plates (96-WTCP), tumor cell lines were suspended in the medium at a density of 5×10^4 cells/well, with 24 hours incubation. Then, to reach 10 concentrations, CME was applied to 96-WTCP in three duplicates, then, six vehicles containing media or 0.5% DMSO that operated as a control. The number of viable cells following a 24-hour incubation period was ascertained using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT test). With ODt standing for the mean optical density of wells treated with CME and ODc for the mean optical density of untreated cells, the viability percentage was computed as [(ODt/ODc)]×100%. Plotting the relationship between surviving cells and medication concentration yields the survival curve for each tumor cell line following CME treatment. Graphical representations were done using Graphpad Prism software (San Diego, CA, USA) were used to determine the 50% inhibitory concentration (IC50), or the concentration needed to produce harmful effects in 50% of intact cells (Mosmann 1983).

Evaluation of antioxidant activity

The free radical scavenging properties were tested using the antioxidant chemical that stops DPPH from oxidizing (Blois 1958). The ability of CME to scavenge DPPH radicals was determined using the following formula:

DPPH radical scavenging activity= $A_{control} - A_{sample} / A_{control} \times 100$

We measured the hydroxyl radical scavenging activity according to Elizabeth and Rao's (1990) methodology. The following method was used to estimate the inhibition percentage:

The inhibition percentage (%) = test – control / control × 100

According to Banerjee and Maulik (2002) and Sutharsingh et al. (2011), ferric reducing antioxidant power (FRAP) is used to assess the antioxidant ability of CME. The antioxidant molecule in CME donates an e-, which transforms ferric cyanide into ferrous form and provides the reducing power that measured at 700 nm.

RESULT AND DISCUSSION

Measures of growth measurements of *C. vulgaris* growth by OD, GR and pigment contents (carotenoid content, and chlorophyll a and b) are shown in Figures 1 and 2. For all pigments, the highest values of these measures were recorded on the eighth, eighteenth, and twelfth days of cultivation, respectively. Consequently, the 18th day of cultivation was chosen as the harvest date to maximize the number of algal cells produced. *Chlorella vulgaris* screening using phytochemicals. In accordance with Abdel-Karim et al., 2020, who used a different solvent for the extraction of *C. vulgaris* and discovered that the observed phytochemical groups are present, Table 1 showed that some groups were not detected.

Cytotoxic activity of CME

Table 2 showed that CME inhibit MCF-7 and HCT-116 with inhibitory percentages range from 2.14±0.25%–76.43±0.74% and 6.11±0.12%–80.57±0.81%, respectively, indicating that CME exhibits cytotoxicity at all concentrations. The anticancer activity increased as the extract concentration increased gradually. CME was therefore more successful in preventing colon cancer than breast cancer. The anticancer effect of CME is demonstrated by the fact that geraniol, an alkaloid molecule, has been extensively studied as a possible anticancer drug (Gabriel et al., 2022; Makhlof et al., 2023). Hong et al. 2021 explored that Quercetin, a flavonoid compound, and gallic acid, a phenolic substance, have also been connected to the development and prevention of certain cancer types.

Antioxidant capacity

Figure 3 illustrates CME's antioxidant activity in DPPH scavenging and FRAP. At minimum quantities (0.001 mg/ml), the activity peaked, and as the concentration of CME rose, it declined. At the maximum CME conc. (5 mg/ml), the strongest FRAP and DPPH scavenging occur. Flavonoids, alkaloids, and phenolic compounds are the main sources of CME's reducing power (Table 1), which is corroborated by Xu et al. (2019) that examined the antioxidant activity of geranoil (an alkaloid), gallic acid (a phenolic), and quercetin (a flavonoid), with encouraging findings. Saponins are used in medicine to treat hypercholestrolemia, hyperglycemia, weight reduction, antioxidants, anticancer, and anti-inflammatory conditions. Additionally, it has antifungal qualities (Mandal et al., 2005).

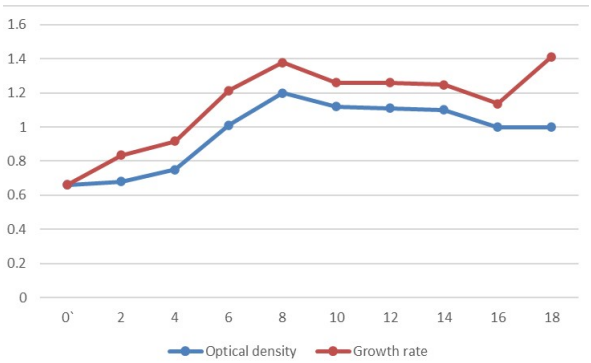

Figure 1. Growth measured as optical density and growth rate of *C. vulgaris* cultured for 18 days.

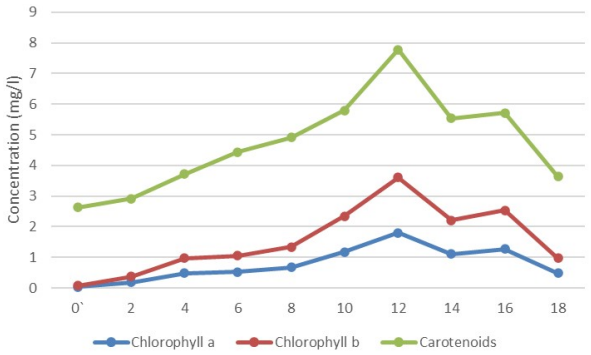

Figure 2. Chlorophyll a, Chlorophyll b, and Carotenoids content of *C. vulgaris* cultured for 18 days.

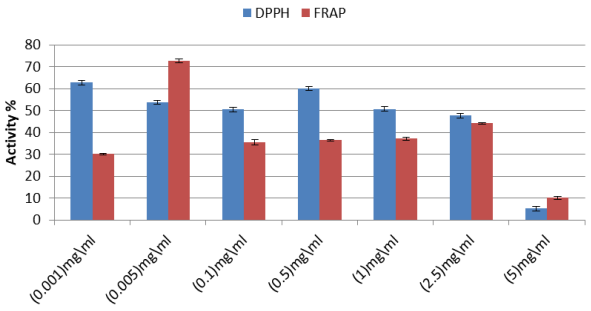

Figure 3. The antioxidant activity of *C. vulgaris* methanol extract in DPPH scavenging and FRAP

Table 1. Qualitative phytochemicals screening of *Chlorella vulgaris* methanol extract

Phytochemical group	Result
Alkaloid	+
Phenol	+
Flavonoid	+
Tannins	-
Glycoside	+
Saponins	+
Steroid	-
Terpenoids	-

Table 2. Cytotoxic activity of *Chlorella vulgaris* methanol extract against (HCT-116 and MCF-7) cell lines

Sample conc. (µg/ ml)	HCT-116 cell line IC50=14.8±0.39 µg		MCF-7 cell line IC50=21.7±0.09 µg	
	Viability%	Inhibitory%	Viability%	Inhibitory%
0	100	0	100	0
1.56	93.89	6.11±0.12	97.86	2.14±0.25
3.125	80.14	19.86±0.23	90.68	9.32±0.15
6.25	71.93	28.07±085	84.74	15.26±0.85
12.5	49.12	50.88±1.02	69.53	30.47±1.02
25	30.25	69.75±0.85	42.98	57.02±0.78
50	19.43	80.57± 0.81	23.57	76.43±0.74

CONCLUSION

Following the required clinical research, *C. vulgaris* may show promise as a therapy for oxidative damage disorders and cancer. The results show that the methanolic extract of *C. vulgaris* exhibits promising antioxidant and cytotoxic effects on MCF-7 and HCT-116 cell lines. Optimizing growth variables may boost the quantitative and qualitative efficacy of the obtained chemicals because every algal extraction technique has different characteristics. Many investigations recommended to be done using *C. vulgaris* as a promising alga

REFERENCE

Abdelkarim, O.; Gheda, S.; Ismail, G.; Abo-Shady, A. (2019). Phytochemical Screening and antioxidant activity of *Chlorella vulgaris*. 41. 76-86. 10.21608/*djs*.2020.139231.

Andima, M., Bernard, S., Owor, R., and Edward, A. (2014). Preliminary qualitative analysis of phytochemical constituents of the Endemic *Aloe tororoana Raynolds* in Tororo, Eastern Uganda. *Global Advanced Research Journal of Agricultural Science*, 3(3), 2315-5094.

Banerjee S. K., Maulik S. K. (2002) Effect of garlic on cardiovascular disorders. *Nutr J* 19:1–4

Blois M. S. (1958) Antioxidant determinations by the use of a stable free radical. *Nature* 181:1199–1200

Chernane H., Mansori M., Latique S., ElMaoua M. (2014) Evaluation of antioxidant capacity of methanol extract, and its solvent fractions obtained from four moroccan macro algae species. *Europ Sci J* 10(15):35–48

Chia, M. A., Lombardi, A. T., Melao, M. D. G. G. (2013). Growth and biochemical composition of *Chlorella vulgaris* in different growth media. *Anais da Academia Brasileira de Ciências*, 85(04), 1427-1438.

Coulombier N., Jauffrais T., Lebouvier N. (2021) Antioxidant Compounds from Microalgae: A Review. *Mar Drugs*. 28;19(10):549. doi: 10.3390/md19100549. PMID: 34677448; PMCID: PMC8537667.

Cox, S., Abu-Ghannam, N., and Gupta, S. (2010). An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. International Food Research Journal, 17, 205-220.

Desikachary, T. V. (1959). Cyanophyta: Indian Council of Agriculture Research.

Elizabeth K., Rao M.W.A. (1990) Oxygen radical scavenging activity of Curcumin. *Int J Pharm* 58:237–240

Feeley J.C., Gorman G.W., Weaver R.E., Mackel D.C., Smith H.W. (1978) Primary isolation media for Legionnaires disease bacterium. *Clin Microbiol* 8:320–325

Gabriel D.S.E.S., Joane N.d.J.M., Emily P.M.L., Carolina M.B., Érico T.C., Mauricio F.S. (2022) Review of anticancer activity of monoterpenoids: Geraniol, nerol, geranial and neral. *Chemico-Biol Inter* 362:109994. https://doi.org/10.1016/j.cbi.2022.109994

Garima, K. (2024). 2 Microbial Planktonic. Handbook of *Aquatic Microbiology*, 12.

Ghaliaoui, N., Hazzit, M., Mokrane, H. (2024). Seaweeds as a Potential Source of Bioactive Compounds. *Research in Biotechnology and Environmental Science*, 3(1), 1-8.

Ginzburg M., Ginzburg B.Z. (1981) Interrelationships of light, temperature, sodium chloride, and carbon source on growth of halotolerent, and halophilic strains of *Dunaliella*. *Brit Phycol J* 16:313–324

Guillard R. R. L. (1979). Growth measurements. Division rate. In R.J. Stein (Ed). Handbook of Physiological methods, Culture methods and growth measurements. Cambridge Univ. Press, Cambridge. 29: 311.

Herrero M., Mendiola J.A., Plaza M., Ibañez E. (2013) Screening for bioactive compounds from algae. *Advanced biofuels, and bioproducts*. Springer Science and Business Media, New York. https:// doi.org/10.1007/978-1-4614-3348-435

Hong Y., Lee J., Moon H., Ryu C.H., Seok J., Jung Y.S., Ryu J., Baek S.J. (2021) Quercetin induces anticancer activity by upregulating ProNAG-1/GDF15 in diferentiated thyroid cancer cells. *Cancers* 13(12):3022–3035

Jefrey S.W., Humphrey G.F. (1975) New spectrophotometric equations for determining chlorophyll a, b, c1, and c2 in higher plants, algae, and natural phycoplankton. *Biochem Physiol Pfanzen* 167:191–194

Jensen A., Liaaen S. (1959) Quantitative paper chromatography of carotenoids. *Acta Chem Scand* 13:1863–1868

Lee, Y., Lee, H. (2002). Industrial uses of algae. *Korean Journal of Applied Microbiology and Bioengineering*, 15, 19- 24.

Makhlof, M. Ghareeb, D. El-Kenany, E. (2023). Assessment of anticancer, antibacterial, antifungal, and antioxidant activities of *Micractinium reisseri* (Chlorophyta, Trebouxiophyceae) methanolic extract. *Rendiconti Lincei. Scienze Fisiche e Naturali*. 34. 10.1007/s12210-023-01139-3.

Mandal, P., Sinha Babu, S. P., and Mandal, N. C. (2005). Antimicrobial activity of saponins from *Acacia auriculiformis*. *Fitoterapia*, 76(5), 462-465.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 65:55–63

Patel, M., Joshi, G., and Sawant, K. K. (2020). "Nanotechnology in oral drug delivery: Salient aspects, state of art, and applications," in Functional bionanomaterials, nanotechnology in the life sciences. *Cham*: Springer, 165–184.

Prescott, G. W. (1962). Algae of the Western Great Lakes Area: 2nd ed. Wm. C. Brown Co., *Dubuque*, Iowa 977 pp.

Robert R.L.G. (1979) Growth measurements, Division rate. In: Stein RJ (ed) Handbook of phycological methods, culturing methods, and growth measurements. *Cambridge Univ Press*, Cambridge, pp 289–311

Spínola M.P., Costa M.M., Prates J.A.M. (2023) Enhancing Digestibility of *Chlorella vulgaris* Biomass in Monogastric Diets: Strategies and Insights. *Animals*. 13(6):1017. https://doi.org/10.3390/ani13061017

Sutharsingh R., Kavimani S., Jayakar B., Uvarani M., Thangathirupathi A. (2011) Quantitative phytochemical estimation, and antioxidant studies on aerial parts of *Naravelia zeylanica* DC. *Int J Pharmaceut Stud Res* 2(2):52–56

Taskin E., Ozturk M., Taskin E., Kurt O. (2007) Antibacterial activities of some marine algae from the Aegean Sea (Turkey). *Afr J Biotech* 6(24):2746–2751

Verma, M. K., Paul, A., Roy, M. (2024). Antioxidant Defense: Key Mechanism of Lead Intolerance. In Lead Toxicity Mitigation: Sustainable Nexus Approaches (pp. 263-275). *Cham*: Springer Nature Switzerland.

Wang, P., Li, D., Sun, M., Yin, J., Zheng, T. (2024). Microalgae enhanced co-metabolism of sulfamethoxazole using aquacultural feedstuff components: Co-metabolic pathways and enzymatic mechanisms. *Journal of Hazardous Materials*, 470, 134279.

Wizilla J. J., Zarirah Z., Mohd S. K., Natrah I., Yong K. Ch., Md Y. I., Fatimah M. Y.(2024), Live-food enriched with *Chlorella vulgaris* as a potential supplemental diet to enhance performance and immune response of Tor tambroides larvae (Bleeker 1854), *Aquaculture*, Volume 580, Part 1, 740276 ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2023.740276. (https://www.sciencedirect.com/science/article/pii/S0044848623010505)

Xu D., Hu M.J., Wang Y.Q., Cui Y.L. (2019) Antioxidant activities of quercetin and its complexes for medicinal application. *Molecules* 24(6):1123–1237

Yavuz, O., Cam, M., Bukan, N., Guven, A., and Silan, F. (2003). Protective effect of melatonin on β-cell damage in streptozotocin-induced diabetes in rats. *Acta Histochemica,* 105(3), 261-266.