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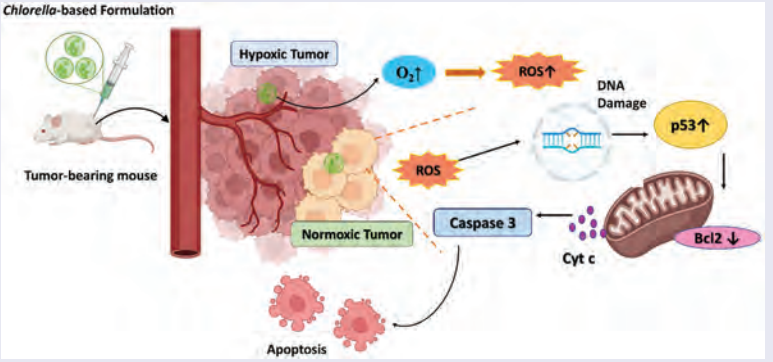
Chlorella spp. as an Emerging Source for Anticancer Remedy and Nutraceuticals: An Advance Study

Umme Tamanna Ferdous[a], Shakeel Ahmad Khan[b], Adnan Shakoor[a,c], Shihab Uddin[a,d], Muhammad Farhan Nazarudin[e], and Abdul Wasy Zia[f]

[a]Center for Biosystems and Machines, King Fahd University of Petroleum & Minerals, Dhahran, Saudi Arabia; [b]Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong; [c]Department of Control & Instrumentation Engineering, King Fahd University of Petroleum & Minerals, Dhahran, Saudi Arabia; [d]Department of Bioengineering, King Fahd University of Petroleum & Minerals, Dhahran, Saudi Arabia; [e]Aquatic Animal Health and Therapeutics Laboratory (AquaHealth), Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; [f]Institute of Mechanical, Process, and Energy Engineering (IMPEE), School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, UK

ABSTRACT

Microalgae are becoming popular to exploit in industrial usage because of their diversified array of phytochemicals, prompt growth rate, ability to grow in season or extreme ambience, and the fact that they do not require arable land and fresh water. They are an excellent reservoir of precious and pharmaceutically important phytochemicals, like carotenoids, polyphenols, lipids, phycobiliproteins, and vitamins. These compounds have shown *in vitro* and *in vivo* anticancer activities towards various cancer cells. Consequently, because of its excellent anticancer, antioxidant, and anti-inflammatory properties, *Chlorella* spp. are also getting attention in pharmaceutical and nutraceutical companies, which this review discusses. Along with their cytotoxicity mechanism, other major bioactivities, especially antioxidant and immunomodulatory effects with possible mechanisms, have also been discussed. Recent advances in elucidating various *Chlorella* species' anticancer potential have also been documented. Moreover, a bibliometric analysis has been performed to evaluate the current trend in *Chlorella* anticancer research. The information provided in this review will effectively identify cutting-edge research trends on *Chlorella* and maximize the potential of new technology for pharmaceutical and nutraceutical industries.



KEYWORDS

Anticancer; antioxidant; bibliometric analysis; *Chlorella*; immunomodulatory; microalgae; nutraceuticals

CONTACT Adnan Shakoor adnan.shakoor@kfupm.edu.sa Center for Biosystems and Machines, King Fahd University of Petroleum & Minerals, Dhahran 31261, Saudi Arabia; Abdul Wasy Zia a.zia@hw.ac.uk Institute of Mechanical, Process, and Energy Engineering (IMPEE), School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh EH14 4AS, UK

Introduction

Cancer is considered a societal and health burden worldwide. In 2022, more than 20 million people were diagnosed with cancer, and 9.7 million deaths were reported. By 2050, cancer case is predicted to be increased to 35 million.[1] Globally, cancer is responsible for 1 in 6 deaths, mainly in low and middle-income countries.[2] Cancer mortality is related to five substantial behavioural and dietary risks: high body mass index, unhealthy food habits, lack of exercise, and tobacco and alcohol use.[3] There are many types of cancer treatment, such as surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, or a combination of these therapies. Though conventional chemotherapeutic treatment in combination with surgery and radiotherapy is effective in managing many cancer patients, almost 50% of cases are unmanageable with serious health complications and side effects, healthy tissue damage, severe infections, autoimmune diseases and other toxicities.[4,5] This increased toxicity and decreased response to many chemotherapeutic drugs engender the need for searching for novel anticancer biomolecules from natural sources, as more than 60% of clinically useful anticancer drugs were developed.[6] Natural products are significant sources of cancer chemotherapeutics, either naturally occurring or synthetically modified forms.[7]

Microalgae are a rich source of valuable phytochemicals like polysaccharides, polyunsaturated fatty acids, sterols, vitamins, carotenoids, phenolic compounds, phycobiliprotein, etc., which have a profound impact on human health and the treatment of diseases.[7–9] Microalgae contain up to 0.2% carotenoids, which can act as antioxidants by scavenging and deactivating free radicals.[10,11] One major class of carotenoids is fucoxanthin, which has antioxidant, anti-inflammatory, anticancer, antidiabetic, cardioprotective and antimalarial activities.[12–14] Polyphenol compounds, including phenolic acids and flavonoids, are another group of valuable bioactive substances from marine microalgae, and they also have potent antioxidant and anticancer properties.[15] Besides these compounds, polyunsaturated fatty acids (PUFA), especially Eicosapentaenoic acid (EPA)[16] are produced by many microalgae such as *Chlorella spp*. These species are mainly used in aquaculture industries as fish feed due to their high content of PUFA. However, these PUFA, especially EPA, can also be a potential source of antioxidant and antitumor activity, as studies showed that EPA exhibited significant cytotoxicity and substantial antioxidant activities.[15,17] *Chlorella* spp. are also getting attention from nutraceutical companies. The global market for *Chlorella* products is increasing at a very high speed. The market of Chlorella products is anticipated to reach USD 639.7 million by the end of 2031. The compounded annual growth is 6.3%, clearly showing its high demand for nutraceuticals.[18] Besides its usage as a dietary supplement, *Chlorella* can be used as a food additive. *Chlorella sorokiniana* fortified gluten-free bread has more nutritional value in carotenoids, protein, and fatty acids than regular gluten-free bread.[19] Due to their richness of valuable bioactive metabolites, *Chlorella* spp. are now under the exploration of their anticancer properties. Many studies have been conducted to show their excellent anticancer capacity. This review article summarizes the health benefits of *Chlorella* spp., their *in vitro* and *in vivo* anticancer activities and the bioactive compounds present in *Chlorella* spp.

Chlorella spp.

Chlorella came from the word 'Chloros', which means green and the Latin suffix 'ella', which means small. *Chlorella* (Chlorophyta) is a single-celled eukaryotic green microalga.[20] *Chlorella vulgaris*, the first pure algal culture, was discovered by a Dutch microbiologist, M.J. Beijerinck, in 1890. Then, after an extended period, in 1919, Otto Warburg mentioned the usage of *Chlorella* in plant physiology-related studies. As an experimental procedure, in the 1940s, Jorgensen and Convit experimented on 80 patients in a leper treatment colony in Venezuela. They gave those patients *Chlorella* soup to observe the effect. The results showed significant health improvement in those patient groups, rendering the

Table 1. The major types of bioactive compounds found in *Chlorella* spp.

Bioactive Compounds	Species	Relative abundance	Major b ioactivities	References
Chlorophyll	*Chlorella* spp.	1.01 mg/g dry weight	Antioxidant	[23,24]
Violaxanthin	*C. ellipsoidea; C. vulgaris*	3.70 ± 0.45 mg/dry weight	Anti-inflammatory; Anti-proliferative	[25–27]
Lutein	*C. vulgaris; C. sorokiniana; C. pyrenoidosa*	3.20–7.14 mg/g dried biomass	Anti-biofilm, antidiabetic, anticancer	[28,29]
B-carotene	*C. vulgaris;C. pyrenoidosa.*	1.01 mg/g dry weight	Antimicrobial	[24,30]
Exopolysaccharide	*C. vulgaris;C. pyrenoidosa*	208.4–364.3 mg/L	Antioxidant, anticancer, antidiabetic	[31,32]
Chlorella growth factors and other peptides	*C. vulgaris; C. sorokiniana; C. pyrenoidosa*	43–67% of dry weight	Antioxidant; Anticancer	[33,34]
Fatty acids	*C. vulgaris; Chlorella* sp.	84.32 mg/g dry weight	Antioxidant; Anticancer	[35,36]
Polyphenols	*C. vulgaris; C. pyrenoidosa*	25–60 mg Gallic acid equivalent/g dry weight	Antioxidant; antimicrobial, cytoxicity	[37]

successful use of *Chlorella* as a food supplement. Chlorella was investigated as a food supplement in Japan in the early 1950s.[21]

To date, 44 species of *Chlorella* are recognized. They are 2–10 µm in diameter and don't have flagella. *Chlorella* contains the green photosynthetic pigments chlorophyll-a and b in its chloroplast, which is the highest in amount compared to any known plant. It is a nutrient-dense superfood containing a high level of proteins (50 to 70 % of dry matter), amino acids, vitamins, minerals, carotenoids, tocopherols, phenolics, flavonoids, phycobiliprotein and other phytochemicals.[22] The antioxidant and anticancer activity of *Chlorella spp*. are mainly attributed to the presence of these phytochemicals Table 1.

Health benefits of *Chlorella* spp.

Chlorella spp. are produced currently on a large scale as a health supplement. They have several health benefits. They produce lutein, a carotenoid with an anti-cataract capacity, which helps treat macular disorders. It also helps lower blood pressure and control blood cholesterol levels. Chlorella powder has excellent wound-healing capacity. Now, they are not only confined to supplement companies; cosmetics companies also make use of these microalgae. *Chlorella* has anti-wrinkle properties, which help prepare *Chlorella*-infused face cream. *Chlorella* spp. also has other health benefits, like helping manage premenstrual syndrome (PMS), combat bacteria, improve immune regulation, and many more.[21]

Many investigations have been carried out to determine the anticancer property of *Chlorella* spp. and its underlying mechanism. *Chlorella* spp. has several bioactive components which show anticancer activity. *Chlorella* is a photosynthetic organism rich in chloroplasts and can generate a significant amount of oxygen through photosynthesis. Oxygen is vital in killing solid tumours in hypoxic tumour conditions. Chlorophyll is abundant in *Chlorella* and can act as a natural photosensitizer that can produce ROS in significant amounts under irradiation. Increased ROS levels induce apoptosis in cancer cells.[38] They also have a water-splitting ability that helps them decompose water content in the tumour interstitium. This decomposition generates oxygen, helps in deeper penetration and alleviates tumour hypoxia. Moreover, they can continuously consume glucose inside the tumour microenvironment, which augments tumour starvation therapy[39](Fig. 1).

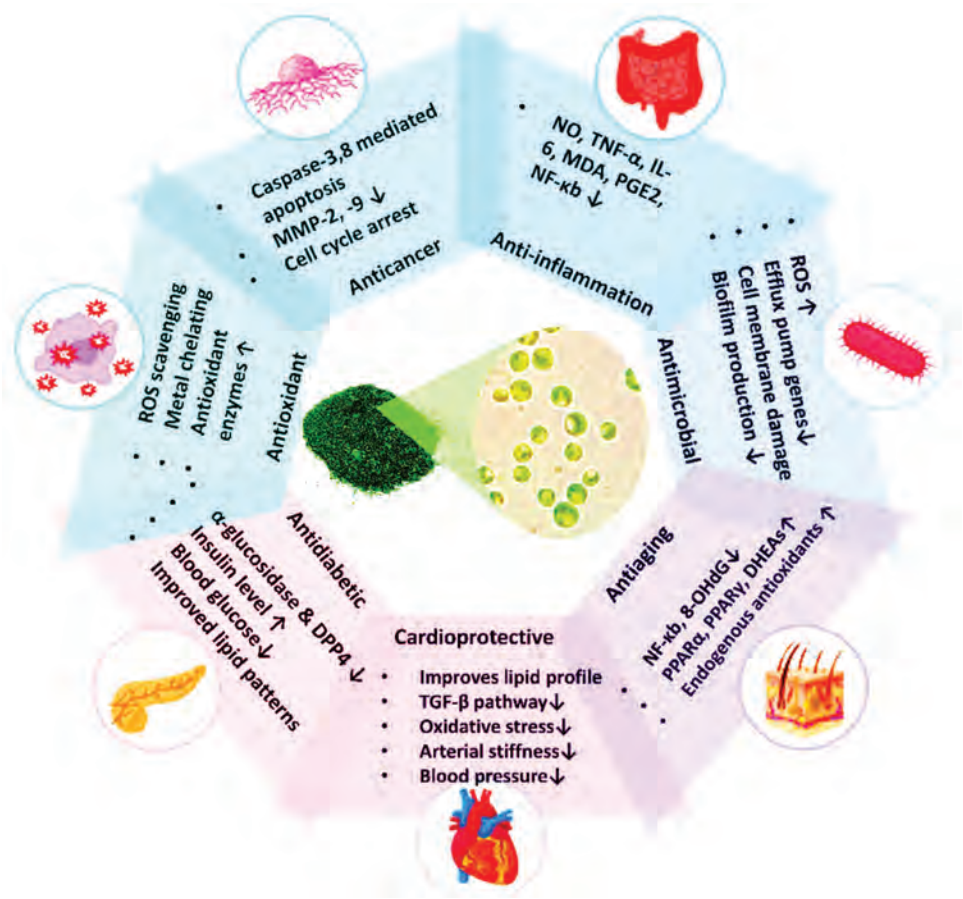


Figure 1. Major bioactive properties of *Chlorella* spp. [created in BioRender.com].

Anticancer activity of *Chlorella* spp.

Chlorella spp. can destroy cancer cells *via* apoptosis and halt cancer cell proliferation, invasion of other cells and formation of blood vessels (Fig. 2).

Apoptosis

Apoptosis is the preferred mode of cell death over necrosis. It causes minimal tissue damage and is considered the main factor in the advancement of specific cancer treatments.[40] This process consists of a complicated interplay between the intrinsic and extrinsic or death receptor pathways. Binding death ligands (FasL, TNF) to their respective receptors (FasR, TNFR) can initiate the extrinsic pathway. This binding activates Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD), which activates caspase 8 from pro-caspase 8.[41] On the contrary, the intrinsic pathway is activated by different stress like endoplasmic reticulum (ER) stress, ROS or DNA damage which in turn activates tumor suppressor p53 protein. This protein upregulates the expression of pro-apoptotic genes (Bax/Bak/Bid) and downregulates the anti-apoptotic gene (Bcl-2) which helps in releasing cytochrome c (cyt c) from the mitochondria. Cyt c binds with apoptotic protease-activating factor-1 (APAF-1) and forms an apoptosome which leads to the activation of caspase 9. In both pathways, a caspase cascade that consists of executioner caspases (caspase-3, −6, and −7) leads to apoptosis.[42]

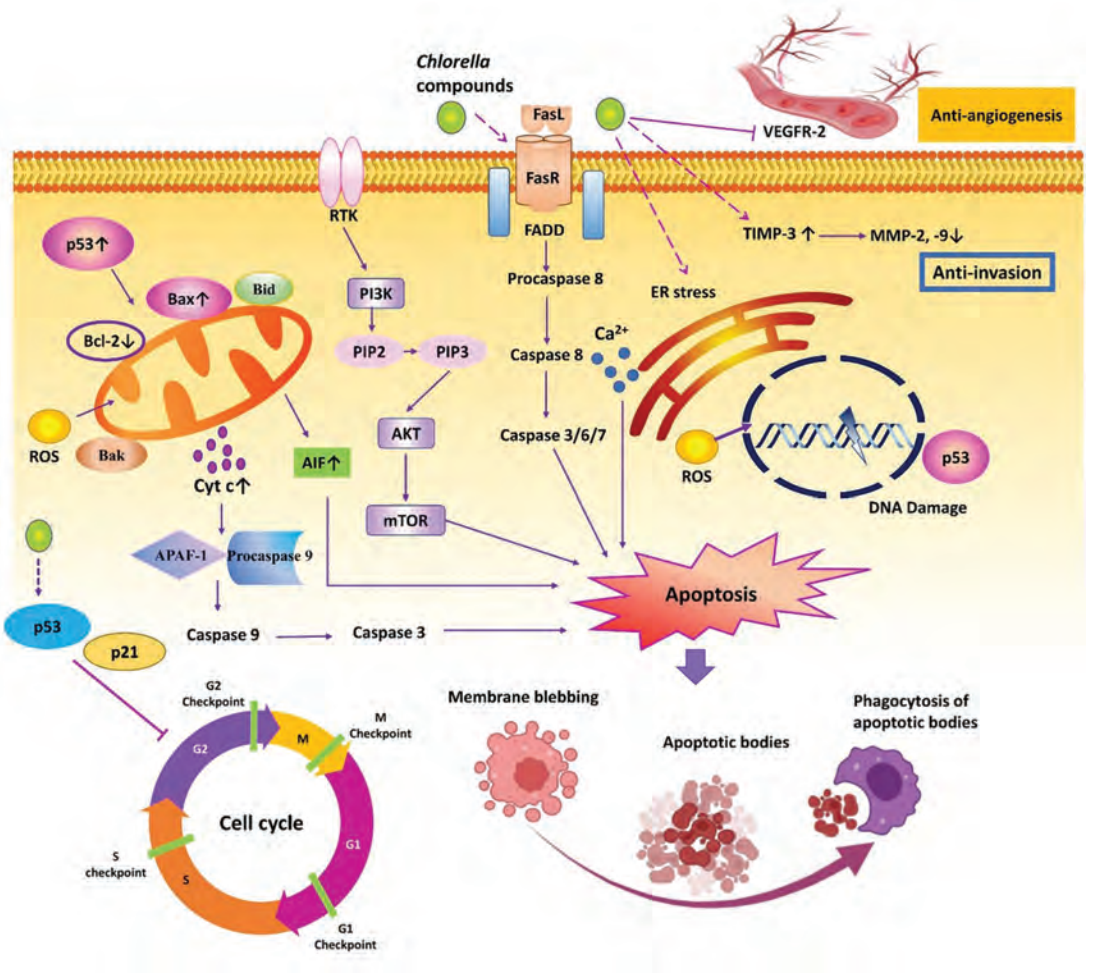


Figure 2. The possible mechanism of anticancer activity of *Chlorella* spp. [created in BioRender.com]. *Chlorella* can induce apoptosis in cancer cells through extrinsic or intrinsic pathways. The extrinsic pathway is triggered by coupling the death receptors with their respective ligands (FASL). Adaptor proteins FADD attaches to these death receptors and activates caspase-mediated apoptosis. In the intrinsic pathway, cellular stress activates p53, initiating BAX/BAK//BID insertion in the membrane of mitochondria. Then cytochrome c releases from mitochondria and couples with APAF-1 and activates caspase 9, subsequently activating caspase 3 and 7, eventually leading to apoptosis. Activation of p53 also induces p21 which results in cell cycle arrest. *Chlorella* also inhibits angiogenesis through modulation of vascular endothelial growth factor receptor (VEGFR) and hinders invasion through downregulating MMPs.

Chlorella spp. can cause apoptosis in cancer cells by triggering both pathways. Ethanol extract of commercial powdered *Chlorella sorokiniana* hindered proliferation of hepatocellular carcinoma cells at 500 µg/mL. This extract induced apoptosis inside the treated cells through DNA damage, increased superoxides and cytoplasmic Ca2 + ions, and decreased mitochondrial membrane potential. Apoptosis was also carried out by enhancing the cytochrome c, AIF, caspases-3, −8, −9, Fas and Fas ligand levels while reducing Bcl-2.[43] Methanol extract of *C. sorokiniana* induced 66% apoptosis in murine lymphoma L5178Y-R cells with DNA fragmentation and caspase induction. Besides, this extract showed no cytotoxicity against normal lymphocytes.[44]

Cell cycle arrest

The cell cycle is a highly regulated cellular process for cell division and DNA duplication with four distinct phases, namely the G1 (gap 1) phase, S (synthesis) phase, G2 (gap 2) phase, and mitosis (M) phase. This sequential process is regulated by four checkpoints and controlled by cyclin-dependent kinase (CDK) proteins. When any checkpoint triggers, CDKs become inactivated, leading to cell cycle arrest. If the DNA repair mechanism fails to repair damaged DNA, apoptosis is triggered by these checkpoints. This checkpoint is regulated by several factors, for instance, CDK1/cyclin B complex and p53 gene regulation. Chemotherapeutic agents can activate p53, which induces p21 to stop cell cycle progression at the G1/S or G2/M checkpoints.[45]

Peptide fractions purified from algal (*C. vulgaris*) protein waste exhibited dose-dependent cytotoxicity against human gastric cancer (AGS) cells with an IC_{50} of 70.7 ± 1.2 μg/mL. These peptides arrested the AGS cells in the post-G_1 phase of the cell cycle.[46] Algal lycopene (AL) from *C. marina* impeded the proliferation of human prostate cancer (PC-3) cell lines by 54% at 20 μM and 61% at 50 μM. AL induced apoptosis through DNA damage and arresting cell cycle at G_0/G_1 phase.[47]

Active cytotoxicity

A partially purified extract of marine microalga *Chlorella ellipsoidea* was reported to show cytotoxicity towards human colon cancer (HCT-116) cells with an IC_{50} of 40.73 ± 3.71 μg/mL. Extract of freshwater microalga *C. vulgaris* hindered the growth of this HCT-116 cells with an IC_{50} of 40.31 ± 4.43 μg/mL. HPLC analysis revealed that violaxanthin and lutein were the major carotenoids present in the extracts of *C. ellipsoidea* and *C. vulgaris*, respectively.[25] In another study, chloroform extract of *C. vulgaris* effectively impeded breast cancer (MCF7) cells' growth with a concentration of 89 μg/ml (IC_{50}) while not inhibiting the normal hepatic (WRL-68) cell line.[48] *Chlorella sp._PR1*, an Indian marine microalga, was extracted with dimethyl sulphoxide, and the extract stopped 50% of B16F10 murine melanoma cells with 5.5 μg/mL concentration.[49] Mexican freshwater *C. sorokiniana* was extracted in methanol solvent, and this solvent inhibited 61.89% ± 3.26% of murine L5178Y-R lymphoma cells at 500 μg/mL, where the IC_{50} was 362.9 ± 13.5 μg/mL. *Chlorella* sp. QUCCCM3, an indigenous microalga from the Qatar desert area, was extracted with hexane, and this extract displayed anti-proliferation capacity against leukaemia K562 cell line with an IC_{50} of 21.37 ± 2.98 μg/mL.[50] In another study, *Chlorella* sp. SRD3 inhibited 72% proliferation of the laryngeal cancer cell line (Hep2) at an IC_{50} of 327 μg/mL and 77% of MCF-7 breast cancer cells at an IC_{50} of 323.3 μg/mL.[51] Methanol extract of marine *Chlorella* sp. inhibited MCF-7 cells to 67.93% at a concentration of 100 μg/mL and major metabolites found in that extract were diterpene and fatty acid esters.[52] But interestingly, the addition of vitamins in methanol extracts of *C. vulgaris* significantly enhanced the cytotoxicity. Methanol extract with thiamin supplementation inhibited 89.3% of HCT-116 cells, whereas methanol extract only killed 68.9%.[53] Extract prepared from *C. vulgaris* C-C using supercritical CO_2 fluid extraction method showed antiproliferative activity against non-small cell lung cancer (NSCLC) cell lines. It also inhibited metastasis in NSCLC cell lines.[54] In another study, polypeptide separated from *C. pyrenoidosa* has been reported to hinder the growth of human liver cancer HepG2 cells with an IC_{50} of 426 μg/mL.[55] Similarly, water extract of *C. vulgaris* also showed cytotoxicity against HepG2 cells.[56]

Partially purified exopolysaccharides from *C. pyrenoidosa* FACHB-9 caused 35.9% inhibition of HCT8 cells at a concentration of 0.6 mg/mL.[57] These authors also studied the partially purified exopolysaccharides from *C. zofingiensis* and *C. vulgaris* with the same concentration and against the same cell line. Exopolysaccharides from *C. zofingiensis* and *C. vulgaris* showed anticancer activity with IC_{50} of 1.70 and 3.14 mg/mL, respectively.[31] Methanol extract of *C. vulgaris* was documented to show a cytotoxic effect against MCF-7 and reduces cell viability to 84.11% at 100 μg/mL concentration.[58] Methanolic extract of *C. vulgaris* has also been reported to kill half of the breast cancer (MCF-7) cells at 23.45 μg/ml. This activity was attributed to the presence of high flavonoid contents in that extract.[59] (Table 2).

Table 2. Major *in vitro* cytotoxic activity of *Chlorella* spp.

Species	Active compounds	Cell lines	Method used	IC_{50}	Reference
C. sorokiniana	Crude methanol extract	Prostate cancer (PC3)	MTT assay	106.5 µg/mL	[60]
C. sorokiniana	Methanol extract	Murine L5178Y-R lymphoma	MTT assay	362.9 µg/mL	[44]
C. sorokiniana	Ethanol extract	Hepatoma cell line (HepG2)	MTT assay	36.2% at 500 µg/mL	[43]
C. sorokiniana	Aqueous ethanol	Lung cancer cell line (A549)	MTT assay	41.49 µg/ml	[61]
C. sorokiniana TH01	Lutein	Human papilloma (KB)	SRB assay	58.07 µg/ml	[62]
C. ellipsoidea	Violaxanthin	Human colon cancer (HCT116)	MTT assay	40.73 µg/mL	[25]
C. vulgaris	Lutein	HCT116	MTT assay	40.31 µg/mL	[25]
C. vulgaris	Flavonoids	Human breast cancer (MCF7)	Trypan blue test and MTT assay	23.45 µg/mL	[59]
C. vulgaris	Peptides	Human gastric cancer (AGS) cell line	MTT assay	70.7 ± 1.2 µg/mL	[46]
C. vulgaris	Phytol	HeLa	MTT	4.38 µg/ml	[63]
C. vulgaris	Hot water extract	HepG2	BrdU proliferation assay	1.6 mg/ml.	[64]
C. vulgaris	Methanol extract + Thiamin	HCT116	MTT	89.3% at 100 µg/mL	[53]
C. pyrenoidosa	Polypeptide	HepG2	MTT	426 µg/mL	[55]
C. pyrenoidosa	XQZ3, polysaccharide	Adenocarcinoma (BxPC-3)	–	0.05 mg/mL	[65]
C. pyrenoidosa AS-6	Methanol extract	MCF-7	MTT	87 µg/mL	[66]
C. zofingiensis	Exopolysaccharides	Human colon cancer (HCT8)	–	1.70 mg/mL	[57]
C. marina	Lycopene	Human prostate cancer (PC-3)	–	61% at 50 µM	[47]
Chlorella sp.	EtOH extract (Gallic acid & Lutein)	Cholangiocarcinoma	PrestoBlue™ reagent cell viability assay	0.30 mg/mL	[67]
Chlorella sp. QUCCCM3	Hexane extract	Leukemia K562	MTT assay	21.37 µg/mL	[50]
Chlorella sp. _PR1	DMSO extract	Murine melanoma B16F10	MTT assay	5.5 µg/mL	[49]
Chlorella sp.	Methanol extract	MCF-7	MTT assay	67.93% at 100 µg/mL	[52]
Chlorella sp., SRD3	Methanol extract	Laryngeal cancer (Hep2)	MTT assay	327 µg/mL	[51]

*"%" indicates cytotoxicity; MTT denotes 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; SRB denotes sulforhodamine B.

Anti-metastasis

Matrix metalloproteinases (MMPs) play a significant role in cancer metastasis. MMP-2 and MMP-9, also known as gelatinase A and B, degrade the extracellular matrix components, gelatine and collagen. Thus, cancer cells can escape from the primary tumor site and spread to other organs. These MMPs also activate vascular endothelial growth factors, which help form new blood vessels and thus promote cancer cell survival.[68]

Ethanol extract of commercial powdered *Chlorella sorokiniana* exhibited anti-invasive capacity by reducing matrix metalloproteinases-2 and −9.[43] Increased amounts of *C. minutissimma* protein extract can upregulate tissue inhibitors of metalloproteinases-3 (TIMP-3), which can downregulate the expression of matrix metalloproteinases, MMP-2 and −9. Kunte et al. (2018) reported this mechanism in human breast and liver cancer cells. At a concentration of 15 µg/ml, expression levels of MMP-2 and −9 were inhibited in the Hepatoma cell line (HepG2), which is an indication of the anti-invasive capacity of *C. minutissimma* extract.[69]

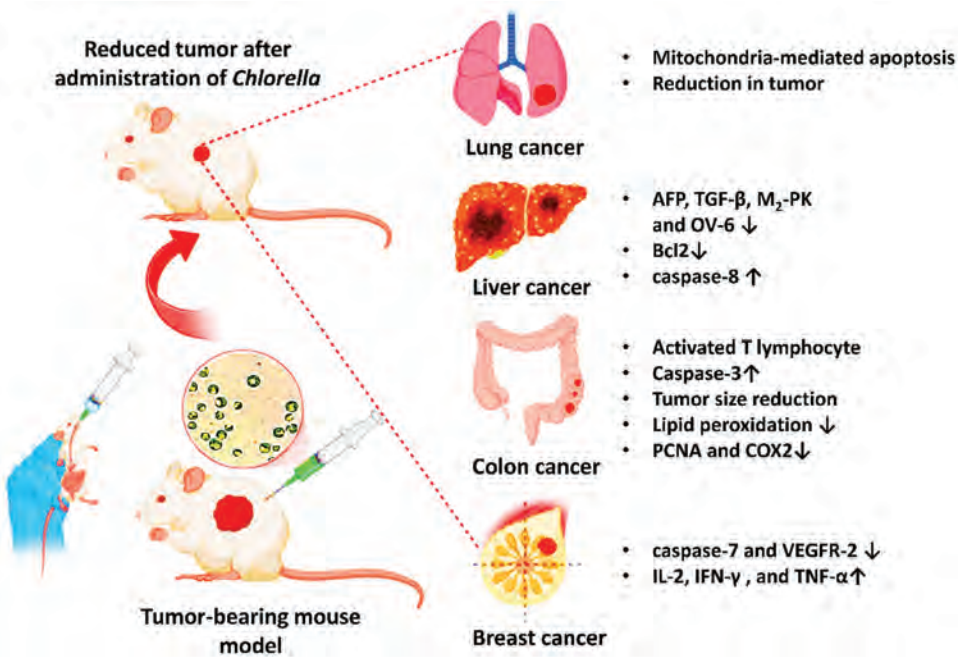


Figure 3. A proposed mechanism of *in vivo* anticancer activity of *Chlorella* spp. after administration of *Chlorella* supplement [created in BioRender.com].

Anticancer activity of *Chlorella* sp. in animal model

Extracts of *Chlorella* spp. were also tested in animal models. *C. sorokiniana* and *C. vulgaris* extracts were mainly used to investigate the *in vivo* anticancer activity (Fig. 3). Chlorella membrane factor from *C. sorokiniana*, inhibited colon carcinoma growth in a murine model. A dose of 10 or 30 mg dry weight/kg in alternative days induced apoptosis in tumour cells and activated T lymphocytes, which rendered tumour inhibition.[70] In another study, daily oral intake of *C. sorokiniana* extract at a dose of 50 mg/kg body weight has been reported to impede the growth of lung adenocarcinoma (CL1–5) cells' growth and lessen the tumour size.[71] Powder of *C. pyrenoidosa* at a concentration of 3% was administered in mammary carcinogenesis-induced rats with a 30 g/kg dose. Immunohistochemical data revealed increased caspase-7 and reduced VEGFR-2, indicating antiangiogenic activity.[72]

Chlorella extract can also ameliorate chemotherapeutic drug-induced cytotoxicity. In a study, extract of *C. sorokiniana* was tested in different concentrations (25–100 µg/mL) daily, combined with cisplatin, a chemotherapeutic drug. *Chlorella* extract decreased the apoptosis rate in leukaemia cells (HL-60) and secured normal myeloid cells from the adverse effect of cisplatin. In a mouse model, *Chlorella* extract, at a concentration of 9.6 mL/kg/day, saved the cellularity of bone marrow from cisplatin-induced hypocellularity.[73] Arifin et al. reported that *C. vulgaris*, at different concentrations (50–300 mg/kg/day), exerted chemopreventive action, which was revealed by reducing the expression of tumour markers AFP, TGF-β, M_2-PK and OV-6. These tumour markers were elevated during hepatocarcinogenesis in mice.[74] In a similar study, Azamai et al. reported reduced Bcl-2 expression, which is an anti-apoptotic protein, while augmenting pro-apoptotic caspase-8 expression in hepatocarcinogenesis-induced rats with the same dose of *C. vulgaris*.[75] Moreover, *C. vulgaris* increased the survivability of tumour-bearing mice at a concentration of 50 mg/kg/day. This extract supported the proliferation of progenitor cells and also elevated the expression of antitumor cytokines, IL-2, IFN-γ, and TNF-α, which in turn hindered tumour growth.[76] *C. vulgaris* also showed chemopreventive properties against colon cancer in rat models. A daily *C. vulgaris* dose of 50 mg/Kg body weight in 1, 2-dimethylhydrazine dihydrochloride (DMH) induced rats exhibited a decrease in lipid peroxidation

Table 3. *In vivo* anticancer activity of *Chlorella* spp.

Species	Active fraction	Study group	Number	Dose	Target	Outcome	Reference
Chlorella sorokiniana	Chlorella membrane factor	Female Balb/c mice	6	10 or 30 mg dry weight/kg body	Colon carcinoma	Enhanced antitumor immunity	[70]
C. sorokiniana	Water extract	female BALB/c nu/nu mice	5	50 mg/kg	Non-small cell lung cancer	Mitochondria-mediated apoptosis	[71]
C. vulgaris	Whole-cell	Male Wistar rats	6	50–300 mg/kg	Hepatocellular carcinoma (HCC)	Decreased liver tumor markers, M2-PK, OV-6, AFP and TGF-β	[74]
C. vulgaris	Whole-cell	Male BALB/c mice	6	50 mg/kg	Ehrlich ascites tumor (EAT)	Enhanced number of immune cells and antitumor cytokines	[76]
C. pyrenoidosa	Whole-cell	Female rats	25	30 g/kg (conc. 3%)	Mammary cancer	Increased Caspase-7, decreased VEGFR-2	[72]

level. Notably, elevated lipid peroxidation level is a sign of cell oxidative damage. Free radicles produced after DMH administration were reduced by *C. vulgaris* treatment through its radicle scavenging activity. It also augmented caspase-3 expression, decreasing proliferating cell nuclear antigen (PCNA) and inflammatory marker COX2, which confirmed apoptosis in those colon cancer cells[77] (Table 3).

Anti-inflammatory and immunomodulatory activities

All of the procedures used to alter or control the immune response for therapeutic purposes can be referred to as immunomodulation, where the immune system is activated to mitigate inflammatory responses and combat maladies like cancer.[78] *Chlorella* spp. possess anti-inflammatory and immunomodulatory effects (Table 4) (Fig. 4). The anti-inflammatory activity of *C. vulgaris* methanol extract was tested using different assays. The data revealed that the extract at 500 µg/mL inhibited 68% of protein denaturation and 63% proteinase activity compared to commercial drug aspirin, and also inhibited 71% hemolysis compared to diclofenac and 73.8% lipoxygenase activity compared to Indomethacin, which proved its anti-inflammatory activity.[81] In a clinical trial, chlorella supplementation ameliorated the dysmenorrhea pain by reducing serum prostaglandins, PGE2, PGF2a, malondialdehyde (MDA), and c-reactive protein (hs-CRP).[88] In another study, a pigment-protein mixture was extracted from *C. pyrenoidosa*, which hindered the production of TNF-α, IL-6, and nitric oxide.[83] *C. miniate* also showed anti-inflammatory activity by inhibiting xanthine oxidase (37.07%) and hyaluronidase (65.39%).[82] Exopolysaccharides from *C. vulgaris* containing α-Larabino-α-L-rhamno-α,β-D-galactan group in the structure showed anti-inflammatory activity by elevating IL-12, IL-10 and INF-γ levels and by reducing TNF-α release in bronchoalveolar lavage fluid in the animal model.[86] *C. ellipsoidea* produced a polysaccharide and protein-rich fraction of 237.0×10^3 g/mol, which showed a high nitric oxide-releasing effect on murine macrophage (RAW264.7) cells due to enhanced expression of iNOS mRNA. This fraction also exhibited an immunostimulating effect by upregulating the expression of pro-inflammatory cytokines, IL-1β, IL-6, and anti-inflammatory cytokines IL-10 and IL-12. This immunostimulating effect was exerted by activating NF- κB and MAPK pathways since the active fraction induced JNK, ERK, and p38 phosphorylation.[84] In a randomized and controlled trial, supplementation with *Chlorella* tablets enhanced the natural killer cells' activity and augmented serum levels of IFN-γ, IL-1β and IL-12 after 8 weeks.[85] Zhou et al. (2023) reported pressurized liquid extraction of *Chlorella* sp. and the extract showed a reduction of NF-κB signaling activation.[79]

Cheng et al.[87] reported that *C. vulgaris* supplementation exerted chemopreventive effects in the immunocompromised mice induced by the cytotoxic drug cyclophosphamide. This supplementation

Table 4. Anti-inflammatory and immunomodulatory effect of active fractions of different *Chlorella* spp.

Species/ supplements	Active fractions	Concentration/ dose	Type of Study	Model/cell type	Mode of action	Reference
Chlorella sp.	Aqueous extract (rich in polyphenols)	10% (v/v)	Cell-based assay	TNF-α stimulated HT-29 cells	Inhibition of NF-κB	[79]
Chlorella sp.	Chlorella-11 peptide	0.009–0.0038 mm	Cell-based assay	LPS-stimulated RAW 264.7 macrophages	Inhibition of NO, TNF-α, MDA and PGE2 production	[80]
C. vulgaris	Methanol extract	500 µg/ml	Biochemical assays	Enzyme inhibition	Inhibition of albumin denaturation, proteinase, lipoxygenase, and hemolysis activities	[81]
Chlorella miniata	Ethanol extract	0.9–10.5 mg/mL	Biochemical assays	Enzyme inhibition	Inhibition of xanthine oxidase and hyaluronidase	[82]
C. pyrenoidosa	Pigment-protein complex	200–400 µg/ml	Cell-based assay	LPS-stimulated RAW 264.7 macrophages	Decrease in TNF-α, IL-6, and nitric oxide	[83]
C. pyrenoidosa	Polysaccharide	6.25–25 µg/ml	Cell-based assay	RAW 264.7 macrophages	Increase in NO and cytokines, IL-1β, IL-6, IL-10, and IL-12; activation of NF-κB and MAPK via JNK, ERK, and p38 phosphorylation	[84]
Chlorella tablets	Whole-cell	5 g/day	Clinical trial	Healthy human participants	Increase in IFN-γ, IL-1β and IL-12; enhanced NK cell activity	[85]
C. vulgaris	Exopolysaccharides	50 mg/kg	Animal study	Allergen-induced guinea pig	Increase in IL-12, IL-10 and INF-γ level and decrease in TNF-α	[86]
C. vulgaris	Whole-cell	6–24% (v/v)	Animal study	CYP treated mice	Increase in IL-2, IL-12, TNF-α and IFN-γ production and NK cell activity; increase in lymphocyte proliferation and phagocytic activities of macrophages	[87]
Chlorella soft gel capsule	Whole-cell	1500 mg/day	Clinical trial	Young women with primary dysmenorrhea	Decrease in serum PGE2, PGF2a, MDA, hs-CRP level	[88]
Chlorella stigmatophora	Polysaccharides	18 mg/kg	Animal study	Paw Edema model	Reduction of edema	[89]

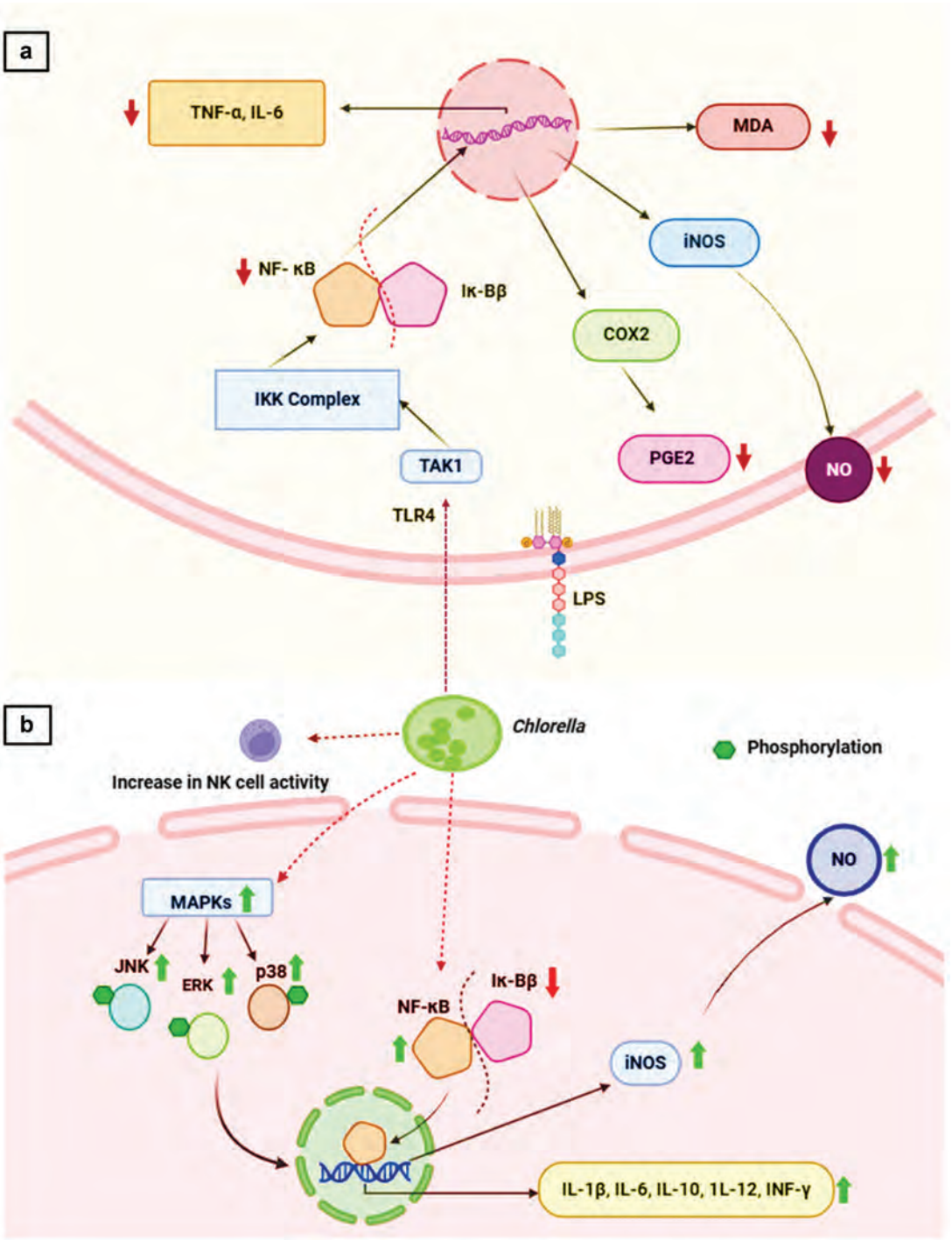


Figure 4. Possible anti-inflammatory and immunomodulatory mechanism of *Chlorella* spp. In [a] LPS-induced macrophage and [b] non-induced macrophage; the red arrow denotes reduction and the green arrow denotes upregulation or increase in the amount. [a] in LPS-induced macrophage, *Chlorella* spp. help decrease the nitric oxide level and thus reduce inflammation. [b] they can also show immunomodulatory effects by elevating NO levels, which enhance the activity of macrophages [created in BioRender.com]. [abbreviations: LPS-Lipopolysaccharide; MDA- Malondialdehyde; COX-Cyclooxygenase; PGE2-prostaglandin E2; iNOS-inducible nitric oxide synthase; TAK-Transforming growth factor beta-activated kinase 1; TLR-Toll-like receptor; NO-Nitric oxide; MAPK- Mitogen-activated protein kinase; JNK- c-jun N-terminal Kinases; ERK- Extracellular signal-regulated Kinases; IKK- inhibitor of nuclear factor-κB (IκB) kinase].

decreased lymphocyte proliferation and phagocytic activities of macrophage while increasing expression of IL-2, IL-12, TNF-α and IFN-γ. Besides, this supplementation enhanced the cytotoxic activity of NK cells in the mouse model. The anti-inflammatory effect of *C. stigmatophora* was assessed by a paw edema test in a rat model. The study showed that crude polysaccharides from this species significantly reduced edema and paw thickness, proving their anti-inflammatory effect.[89] A peptide named Chlorella-11 peptide was isolated from *Chlorella* sp. and tested for its anti-inflammatory effect. Chlorella-11 peptide suppressed nitric oxide production and inhibited the expression of iNOS, TNF-α and NF-κB.[80]

Antioxidant activity

Chlorella spp. have excellent antioxidative properties, which have been evaluated in previous studies. The antioxidant activity of *Chlorella* spp are mainly regulated through free radical scavenging and metal chelation (Fig. 5). A study by Gharehbeglou et al., 2024 highlighted the copper and iron chelating capability of *Chlorella* proteins, which is highly related to its antioxidation capacity. Besides, these proteins showed NO scavenging activity.[90] Besides, this green microalga also showed its antioxidant activity via increased oxidative stress-related enzymes and expression of primary antioxidant genes. Polysaccharides from *C. vulgaris* helped in expanding the lifespan of oxidative

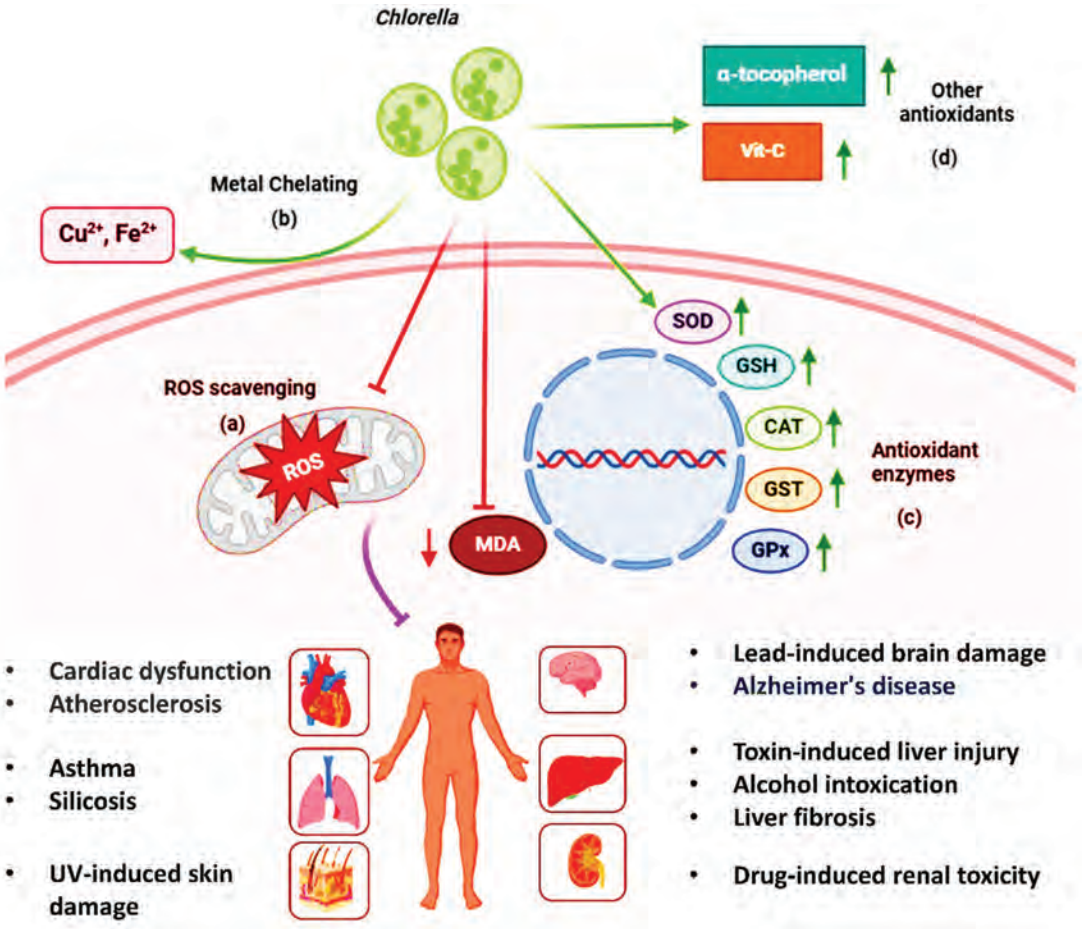


Figure 5. Possible mechanism of antioxidative activity of *Chlorella* spp. (a) Direct free radicle scavenging; (b) copper and iron chelating; (c) upregulation of antioxidant enzymes and (d) increasing activity of other antioxidants [created in BioRender.com].

stressed *Caenorhabditis elegans* as well as increased catalase (CAT) and superoxide dismutase (SOD) enzymes which are related to stress resistance.[91] Supplementation with *C. vulgaris* enhanced the CAT, SOD and reduced glutathione (GSH) levels and also upregulated the genes related to primary antioxidants, *sod1* and *gpx*.[92] This supplement also ameliorates the antioxidant status in cadmium-intoxicated fish by enhancing hepatic CAT, SOD, glutathione peroxidase (GPx) and glutathione-S-transferase (GST) levels.[93]

In a clinical trial, 6-week supplementation with *C. vulgaris* in male smokers significantly augmented the plasma Vit-C and α-tocopherol levels along with increased erythrocyte SOD, CAT and GPx levels.[94] Fatty acids from *Chlorella* sp. S14 also augmented the CAT and GSH levels, inhibiting lipid peroxidation by reducing malondialdehyde (MDA) levels.[36]

Other bioactivities

Besides anticancer, antioxidant and anti-inflammatory activities, *Chlorella* spp. protect against cardiovascular, neurodegenerative and hepatic diseases. These species also showed excellent bioactivities against harmful microorganisms and health protective effects like anti-ageing, anti-obesity and antidiabetic. Some recent preclinical and clinical studies of these bioactivities from different *Chlorella* species are highlighted in Table 5.

Studies have shown that *C. vulgaris* can kill microorganisms by modulating cellular antioxidation defence, disrupting cell membrane integrity, and damaging cellular components.[95,96] In diabetic mice model, *Chlorella* spp. showed amelioration of insulin resistance along with improved hypoglycemic and low lipid profile status. This treatment strategy also boosted gut microbiota and reduced fatty liver symptoms.[97,98] The hepatoprotective effect is also reported in another study where *C. vulgaris* reduced the level of liver enzymes and repaired damaged liver tissue.[104] Besides the antidiabetic effect, *Chlorella* showed wound healing capacity by reducing the inflammatory response and promoting the regeneration of new blood vessels.[107,108] *Chlorella* polysaccharides can downregulate the ageing-related gene expression and improve the cellular antioxidant enzyme level, which can be attributed to their anti-ageing effect. Besides, β-carotene from *Chlorella* sp. showed anti-wrinkle effect in UV-radiated dermal fibroblast cells by increasing collagen expression and reducing the expression of gelatinase enzymes.[101–103] Moreover, *Chlorella* exerted neuroprotective activity, both in vitro and in vivo, by reducing reactive oxygen species and cell loss and ameliorating cognitive memory.[105,106] In some clinical trials on obese people, *C. vulgaris* whole-cell supplementation reduced the total body fat percentage and decreased the level of adipokines, which helped manage obesity.[109,110]

Recent advances in using *Chlorella* spp in cancer therapy

The hypoxic tumour microenvironment hinders the therapeutic efficacy of radiotherapy, blocking the irradiation, which in turn gives rise to the radioresistant tumour and damage to healthy cells. Bioengineered *Chlorella* offers a solution to such resistance. Since this is a photosynthetic microalga, photosynthesis in the hypoxic site can alleviate hypoxia and produce oxygen, which elevates ROS level and, ultimately, apoptosis. *Chlorella* cells can be coated with calcium phosphate (CV@CaP) as a protective layer to facilitate effective delivery to the tumour site. The engineered *Chlorella* not only stops tumour growth but also hinders metastasis. A pilot toxicity study reveals that this CV@CaP is biocompatible and showed no organ damage or noticeable toxicities over 30 days of incubation.[111] Qiao et al. coated *C. vulgaris* cells with red blood cell membranes to be delivered to hypoxic tumour sites to generate *in situ* oxygen and enhance the efficacy of radiotherapy and photodynamic therapy in killing cancer cells.[112] This combinatory approach augmented the ROS level, leading to apoptosis and tumour regression. Incorporating local hyperthermia can increase the efficacy of combination therapy many times. Hydrogel made with *Chlorella* and gold nanorod maximized the therapeutic activity of doxorubicin in breast cancer models. Upon exposure to 808-

Table 5. Bioactivities from *Chlorella* spp.

Bioactivities	Species	Active fractions /formulation	Model	Mechanism	References
Antimicrobial	*C. vulgaris*	AgCl-NPs	*in vitro*	Modulation of SOD, CAT, and GSH; cell wall damage & alteration in chromosomal DNA arrangement	[95,96]
Antidiabetic	*Chlorella* sp.	Whole-cell	Alloxan-induced male Sprague-Dawley albino rats	higher insulin; lower blood glucose; improves lipid pattern and body weight	[97]
	C. pyrenoidosa	*C. pyrenoudosa, Ganoderma lucidum*, and *Panax ginseng* with chromium-enriched yeast	Streptozocin-induced type 2 diabetic mice	Reduction in liver deformation, cecum injury, and jejunal inflammation and improved gut microbiota	[98]
Anti-hypertensive	*C. sorokiniana*	Protein hydrolysate	spontaneously hypertensive rats	Reduction in blood pressure	[99]
Cardioprotective effect	*Chlorella pyrenoidosa*	Whole-cell	Clinical trial	Reduced total cholesterol and LDL; improved vit-D level	[100]
Anti-ageing	*Chlorella* sp.	polysaccharides	*Caenorhabditis elegans*	Increased anti-stress and longevity genes and decreased ageing-related gene	[101]
	C. pyrenoidosa	polysaccharides	*Drosophila melanogaster*	Increased Nrf2 signalling and antioxidant enzymes	[102]
Anti-wrinkle	*Chlorella* sp. HS1	β-carotene	(UV)-irradiated dermal fibroblasts	Increase in collagen type 1 and 3 mRNAs and decrease in MMP1 and MMP3 mRNAs	[103]
Hepatoprotective effect	*C. vulgaris*	Whole-cell	$CdCl_2$-induced male Sprague-Dawley rats	Decrease in hepatic enzymes, ALT, AST, ALP, and urea; repaired the hepatic tissue damage.	[104]
Neuroprotective effect	*Chlorella* sp.	Methanol extract	*In vitro*	Decreased ROS and lipid peroxidation; increased antioxidant enzymes HO-1, NQO1 and CAT	[105]
	C. pyrenoidosa	Peptides	Aβ1–42-induced Alzheimer's disease mice model	Improved spatial cognition and learning memory; decreased cell loss	[106]
Wound healing	*C. vulgaris*	*Chlorella* hydrogel	female Swiss albino mice	higher collagen deposit and skin appendages; lower fibroblast and inflammatory cells	[107]
	Chlorella sp.	*Chlorella* hydrogel	Streptozocin-induced diabetic mice	Reduced inflammatory response, blood vessel regeneration, depleted glucose & ROS	[108]
Anti-obesity	*C. vulgaris*	Whole-cell	Clinical trial on obese men	Increased adiponectin and Nrg-4 level	[109]
	C. vulgaris	Whole-cell	Clinical trial on obese women	Reduce body fat percentage, increased PGC-1α level and body water	[110]

nm near-infrared laser, gold nanorod increased the surrounding temperature in the hypoxic tumour site to 41–42 °C which also helped in expanding the tumour vasculature and thereby easing the delivery of doxorubicin and *Chlorella*, ultimately abolishing breast cancer cells in Balb/c mice.[113] Zhang et al.[39] prepared an alginate gel with *C. sorokiniana* and glucose oxidase enzyme (ACG gel). Glucose oxidase catalyzed the glucose consumption reaction,

which improved the O_2 supply from *C. sorokiniana* cell. This process accelerated tumour starvation therapy and generated an immunopermissive status in the microenvironment, provoking antitumor immunity and hindering tumour recurrence.

In the sonodynamic therapy (SDT), Gao et al.[114] coated *Chlolrella* with macrophage membrane (MChl) to enhance biocompatibility and efficient delivery to the tumour microenvironment. They conjugated this MChl with a dual drug (chloroquine phosphate CQ and hematoporphyrin HP) liposome. The formed MChl-CQ-HP-NP inhibited autophagy and provided local oxygenation, which improved the therapeutic efficacy of the SDT against melanoma.

Anticancer bioactive compounds from *Chlorella* spp.

Anticancer compounds mainly found in *Chlorella* spp. are carotenoids, such as violaxanthin, lutein, and lycopene.[25] (Fig. 6) Other than these, phenolic compounds and flavonoids are abundant in

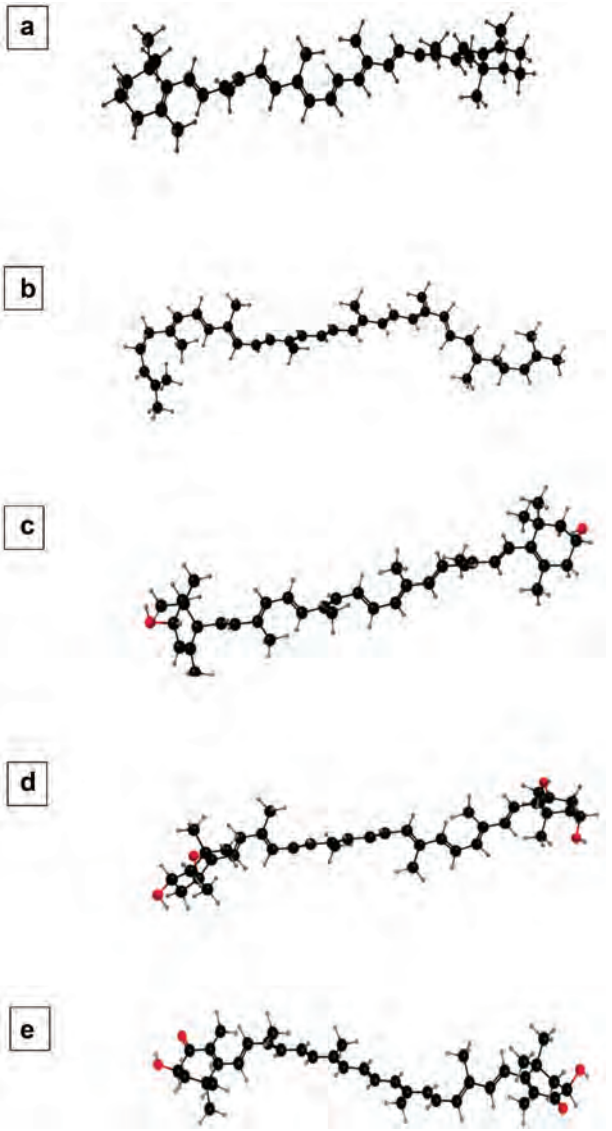


Figure 6. 3D structures of carotenoids found in *Chlorella* spp., a. β-carotene; b. lycopene; c. lutein; d. violaxanthin and e. astaxanthin [created in KingDraw].

Chlorella spp. They are also considered a rich source of polysaccharides and peptides. These polysaccharides and peptides are reported to show anticancer activity.[59] Other than these phytochemicals, different organic solvent extracts showed antitumor activities, which need to be investigated.

Carotenoids

Chlorella spp. produce different kinds of carotenoids, namely lutein, zeaxanthin, violaxanthin, astaxanthin, cryptoxanthin, canthaxanthin, diatoxanthin, echinenone antheraxanthin, and neoxanthin.[115] Bazarnova et al.[116] also reported the presence of fucoxanthin along with lutein and β-carotene, about 12% of total extracted carotenoids. Pantami et al.[117] also reported the presence of another carotenoid, vulgaxanthin I, for the first time. These carotenoids have excellent antoxidation properties and show cytotoxicity towards various cancer cells. In a clinical study on 11 healthy male individuals, a single dose of *Chlorella vulgaris* (6 mg) significantly augmented the carotenoid content, especially β-carotene, in plasma within just 3 days, which proved the bioavailability of carotenoids and the health-promoting capability of *Chlorella* supplement.[118]

β-carotene

C. vulgaris, *C. sorokiniana, C. pyrenoidosa* and C. *zofingiensis* are good sources of this carotenoid. A comparison of multiple studies revealed that *C. pyrenoidosa* can produce more β-carotene (2.2 mg/g dry weight) than other *Chlorella* species. *C. vulgaris* comes next in the list, producing 0.35 mg/g dry weight.[115] This carotenoid can kill cancer cells via p53 upregulation, ROS generation, and growth signal inhibition while protecting healthy cells.[119]

Lycopene

Lycopene is classified under the carotenoids family and is mainly found in tomato and tomato products. It has not only antioxidant activity but also strong anticancer activity. The anticancer activity of lycopene is mostly evaluated in prostate cancer cell lines. Soares et al. reported increased apoptosis in human prostate cancer cells when treated with tomato products at 500–5000 µg/mL concentrations. Lycopene can also arrest cells in different phases of the cycle.[120]

Lutein

Lutein is also classified under the carotenoids family and is mainly found in high amounts in fruits, vegetables and marigolds. Lutein found from alfalfa showed almost similar anticancer activity to the chemotherapeutic drug, doxorubicin, with an IC_{50} of 3.10 ± 0.47 µg/ml in MCF-7 cell line and exhibited good cytotoxicity in liver cancer cells, HepG2 with an IC_{50} of 6.11 ± 0.84 µg/ml.[121] The anticancer activity of lutein from different studies indicated that lutein has cytotoxicity against various cell lines and is less toxic to normal cells.[122] The mechanism of cell killing was apoptosis via ROS-mediated upregulation of pro-apoptotic protein and downregulation of anti-apoptotic protein. Interestingly, it also showed antitumor activity in animal studies.[9,102]

Violaxanthin

Violaxanthin is orange in color and found in fruits and algae. *C. vulgaris* is a prolific producer of natural violaxanthin. Chemical mutagenesis in *C. vulgaris* can even augment the production by 3.18 times more than the wild type.[27] Violaxanthin also has antitumor activity, as described earlier. Violaxanthin from microalgae *Dunaliella tertiolecta* was cytotoxic to MCF-7 cells and induced apoptosis even at lower concentrations (0.1 µg/mL).[123] Violaxanthin-rich extract from *Characiopsis aquilonaris* showed anticancer activity against MCF-7 at an IC_{50} of 96.6 µg/mL.[124]

Astaxanthin

Among all *Chlorella* species, C. *zofingiensis* is the prolific producer of astaxanthin. This microalga is getting attention for astaxanthin production because of its easy culture, high cell density and fast

growth rate.[125] Astaxanthin, known as the "king of carotenoids", has antitumor activity and an excellent ability to sensitize tumour cells to chemotherapeutic drugs, making it a good choice for combining therapy for cancer. Like other carotenoids, this carotenoid also has less toxicity.[126]

Polysaccharides

Polysaccharides are one of the most studied bioactive compounds from *Chlorella* spp. Different types of polysaccharides with distinct structural features are frequently found in them, including homo-polysaccharides (starch-like glucan), heteropolysaccharides (arabinomannan and arabinogalactan), acidic heteropolysaccharide (A-P-E-6, sulfated polysaccharide) and amino polysaccharides (glycosaminoglycan).[127] Dietary polysaccharides have been studied extensively for their anticancer activity since they showed less toxicity towards healthy cells. Their cytotoxic mechanism in different cancer cells mainly exploits the immunomodulatory activity by enhancing immune cells' activity, macrophages and NK cell production and increasing overall immune function.[128] They can also induce apoptosis in cancer cells by regulating multiple proteins involved in signalling pathways. Moreover, they can regulate the cell cycle-related proteins and halt the cell cycle progression, thus stopping cancer cell proliferation.[129]

Phenolics and flavonoids

Though the polyphenols are mainly found in plants, algae are now also getting attention for their higher production of phenolics and flavonoids. Plant and algae polyphenols have long been studied for their anticancer activity and exhibited cytotoxicity against different cancer cells. Flavonoids, a polyphenol class, are also considered strong anticancer agents. Flavonoids like quercetin, genistein, epigallocatechin gallate, and anthocyanidins showed high anticancer activity in different cell lines, such as breast, prostate, liver and colon.[130] They also showed promising antitumor activity in animal models.[9,110] Flavonoids induce apoptosis by downregulating Bcl-2 expression, activating the caspase cascade. Besides, they can hinder cancer cell proliferation by inhibiting telomerase reverse transcriptase expression and halting cell cycle advancement through modulation of the NF-κB pathway. Apart from these activities, they can also hinder angiogenesis and metastasis by decreasing the expression of VEGF and epithelial-mesenchymal transition markers.[131] Though several studies report the presence of polyphenols from *Chlorella* spp., there is a lack of studies regarding the isolation and purification of polyphenols from these microalgae. A recent study reported the possible identification of some phenolic and flavonoid compounds from *Chlorella* sp., which includes 2,4,4',6'-tetrahydroxy-benzophenone, campneoside, syringaldehyde, 3-hydroxy-5,7,8,3',4'-pentamethoxy flavon, kushenol, and glabrol.[132]

Chlorella *peptides*

Chlorella spp. produce many bioactive peptides, which can be extracted through different methods, such as freeze-thawing, enzymatic digestion, microwave extraction, or sonication. *C. pyrenoidosa* and *C. vulgaris* have been reported to produce many novel peptides that showed anticancer activities toward liver, gastric, and breast cancer cells.[133] These species and *C. ellipsoidea* also have good radicle scavenging activity. *Chlorella* spp. derived peptides also showed a cardioprotective effect by inhibiting enzymes that convert angiotensin. *Chlorella*-derived peptides also showed protective effects against UV radiation, inflammation and atherosclerosis.[134]

Polyunsaturated fatty acids

All *Chlorella* species are good sources of fatty acids (FA), both saturated and unsaturated. Pantami et al.[117] quantified the fatty acid methyl esters from C. vulgaris and the study revealed that the fatty acids content in this microalga mainly consists of unsaturated fatty acids (71.2%) where omega-6 is the

prominent FA, comprising 60% of the total FA. Another study also showed the same quantification of unsaturated FA content from the same species and the most abundant FA were palmitic acid, linoleic acid and linolenic acid.[135] Vilakazi et al.[36] measured the FA level in *Chlorella* sp. S14 and found out that polyunsaturated FA (52.87%) are more dominant than monounsaturated FA. Besides, omega-3 fatty acids are the abundant FA in this species. Several studies have reported that polyunsaturated FA, especially omega-3 and omega-6 FA, possessed anticancer activity along with anti-inflammatory and other health-protective effects.[136] Polyunsaturated FAs exert anticancer activity via apoptosis through mitochondrial dysfunction and activation of caspase pathways. They also stop cell proliferation by modulating Raf/MAPK pathway, ERK1/2/Akt/mTOR/NF-κB pathways, ASK1-MKK4/7-JNK/p38MAPK and signalling pathways.[137]

Other bioactive compounds

Among other bioactive compounds, *Chlorella* is rich in Chlorophyll and different vitamins. Many studies have shown that different vitamins like A, C, D and K showed cancer cell-killing capacity.[10] Chlorophyll can generate more ROS in tumour microenvironments, killing the tumour cells.[38]

Anticancer activity of *Chlorella*-synthesized nanoparticles

Biologically synthesized nanoparticles can effectively treat cancers due to their efficacy and low cytotoxicity towards non-cancerous cells.[138] Nanoparticles (NPs) are now being synthesized from different *Chlorella* species. These *Chlorella*-synthesized NPs have excellent bioactive properties including anticancer activity (Table 6). *Chlorella*-synthesized silver NPs exhibited cytotoxicity (63%) towards liver cancer cells, HepG2, after 48 hours of treatment at 4.7 µg/mL.[139] $ZnFe_2O_4$-Ag nanocomposite synthesized from *C. vulgaris* showed higher cytotoxicity by killing 50% of breast cancer cells at 28 µg/mL compared to standard drug cisplatin (84 µg/mL). It also exhibited apoptotic cell death through ROS generation, chromatin condensation, DNA fragmentation, and arresting cells at the S phase.[141] Hussein et al.[140] synthesized silver (Ag) nanoparticles and tested the anticancer activity with *Chlorella* ethanol (ETH) extract. AgNPs-*Chlorella* sp.-ETH inhibited the proliferation of MCF-7 cells with an

Table 6. Anticancer activity of biosynthesized nanoparticles from *Chlorella* spp.

Species	Nanoparticles /nanocomposites	Particle size	Toxicity dose	Target	Toxicity dose to non-cancerous cells	Mechanism	Reference
Chlorella sp.	Ag	9 nm	37% at 4.7 µg/mL	Hep-G2	-	-	[139]
Chlorella sp.	Ag with ethanol extract	20 to 100 nm	IC_{50}: 10.47 µg/mL	MCF-7	IC_{50}: 72.44 µg/mL	Apoptosis, cell cycle arrest at G0/G1 phase	[140]
C. vulgaris	$ZnFe_2O_4$-Ag	14–52 nm	IC_{50}: 28 µg/mL	MCF-7	IC_{50}: 154 µg/mL	ROS generation, cell cycle arrest at S phase, DNA degradation	[141]
C. vulgaris	Au/cellulose	114 nm	IC_{50}: 4.67µg/ml	A549	IC_{50}: 182.75 µg/ml	Upregulation of p53 expression; downregulation of Raf-1	[142]
C. vulgaris	Nano CV with GE extract	20.9 nm.	IC_{50}: 0.5 mg/ml	MCF-7	-	-	[143]
C. variabilis	Microalgal oil	212.7 nm	<50% at 500 µg/ml	AGS	55.3 % at 500 µg/ml	-	[144]
C. minutissima	Ag	10 to 30 nm	IC_{50}: 12.42 µg/ml	Hep-G2	IC_{50}: 120 µg/mL	Cellular apoptosis, nuclear condensation	[145]

*Ag = silver, Au = gold, CV = *C. vulgaris*.

IC_{50} of 10.47 µg/mL after 72 hours of incubation, whereas IC_{50} for tamoxifen was 12.2 µg/mL. After incubation, the same sample inhibited the non-cancerous kidney cell line with an IC50 of 72.44 µg/mL. In MCF-7 cells, this coapplication of Ag nanoparticles arrested the cell cycle at G0/G1 phase and inhibited the cells through late apoptosis Ag NPs from *C. minutissima* inhibited Hep-G2 cells after 48 hours of incubation with an IC_{50} of 12.42 ± 1.096 µg/ml but showed less toxicity toward kidney cells. From fluorometric analysis, the authors predicted that the mechanism of cell death was apoptosis which was evident from the nuclear condensation and formation of apoptotic bodies.[145] Gold/cellulose nanocomposite was synthesized from *C. vulgaris* which exhibited cancer-specific cytotoxicity towards lung cancer cells A549 with an IC_{50} of 4.67 µg/ml through upregulation of apoptotic gene p53 and downregulation of Raf-1 gene, which is related to cancer growth. This synthesized nanocomposite showed less cytotoxicity towards non-cancerous lung cells with an IC_{50} of 182.75 µg/ml.[142]

Research trend on anticancer properties of *Chlorella* spp.

A bibliometric analysis has been carried out to identify the prospect of anticancer research on *Chlorella* species (AC) and the findings are documented in this review. For systemic literature search and bibliometric analysis, the data was collected from the Web of Science database on October 22, 2024, and the VOSviewer software was used to analyze the data. The systematic search was based on the "Topic" field and keywords "Chlorella" AND "anticancer" OR "antitumor" OR "cytotoxicity" OR "anti-proliferation". Only original articles published between 2001–2024 were documented. Articles published in English were selected only for final analysis. A total of 265 articles were obtained and exported in tab-delimited format and finally imported to VOSviewer software for citation and co-occurrence analysis. The AC research was minimal till 2012. Since 2013, the number of publications per year has continued to increase, which was the highest in 2020. Figure 7 shows a decline in the publication trend for the next three years and starts to rise again in 2024, which means this research

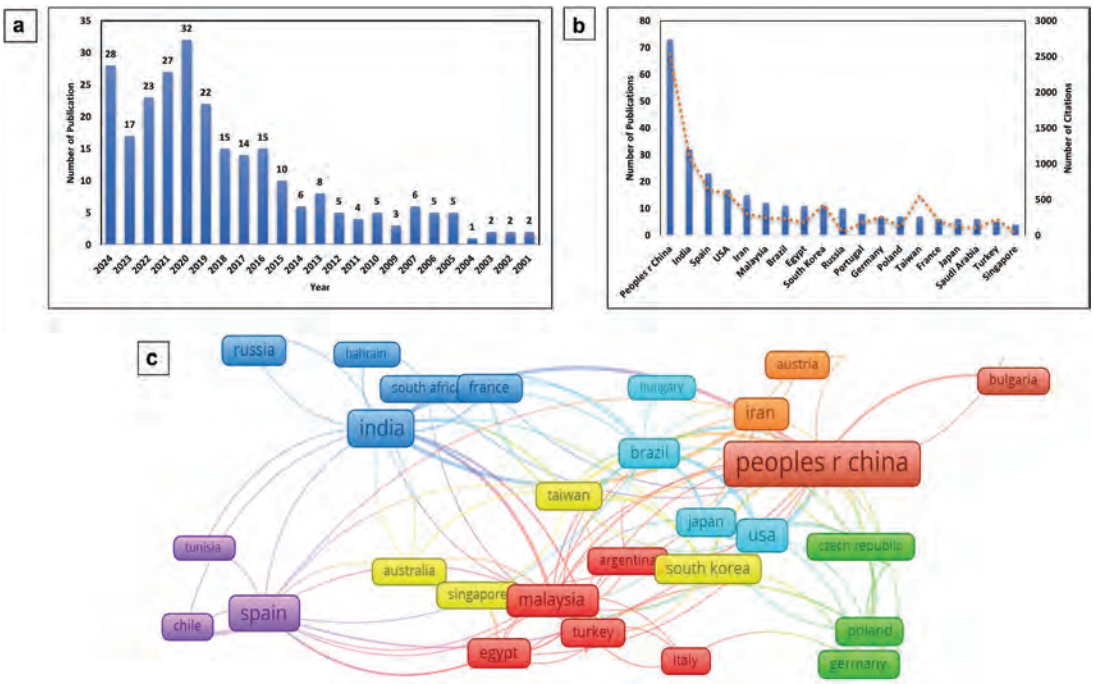


Figure 7. A. number of publications from 2001–2024; B. Number of publications and citations per country (top 19); C. Network map of collaborating countries on the *Chlorella* anticancer research (created in VOSviewer).

trend is becoming popular again. Authors from 53 countries contributed to AC research, where authors from the People's Republic of China contributed the highest number, a total of 73 articles until now, followed by India ($N = 32$), Spain ($N = 32$), USA ($N = 23$), Iran ($N = 15$) and Malaysia ($N = 12$). Fig. 5b shows the top 19 countries contributing to AC research between 2012 and 2022 and their total citations. The People's Republic of China also has the highest citations ($N = 2532$), followed by India ($N = 1113$) and Spain ($N = 630$). Taiwan has only seven publications but has achieved more citations ($N = 554$) than Iran ($N = 301$).

Keyword occurrence analysis is another crucial aspect of any bibliometric analysis, which often provides summarized key findings from a study. In this study, 265 papers were analyzed for all keywords and 1729 keywords were retrieved. Out of 1729 keywords, only 200 keywords were selected based on the minimum occurrence number of 3. Fig. 8 shows the network and overlay occurrence map, which was created based on different clusters with different node sizes equivalent to the repetition of cited keywords. The most frequently cited keywords are "cytotoxicity" (99 occurrences), "microalgae" (53), "chlorella-vulgaris" (51), oxidative stress (35), apoptosis (27), antioxidant (25), chlorella (24) and nanoparticles (22). This keyword analysis proclaims that the therapeutic efficacy of *Chlorella* is pertinent to its potential use in the healthcare industry. Fig. 7b shows a map based on the term average year, indicating the trending topic undergoing intense study. Recently cited keywords are coloured in yellow. This map demonstrates that nanoparticle synthesis from *Chlorella* species and evaluating their cytotoxicity has been getting broader attention since 2019. Therefore, the current research trend has shifted to the biosynthesis of Chlorella-based nanoparticles, especially zinc oxide nanoparticles, and the determination of different bioactivities such as anticancer, antioxidant, and antimicrobial activities.

Challenges and future prospects

Microalgae-based drug discovery is a promising area of research, but it is still in its infancy. Though a lot of research has been conducted on the anticancer potential of *Chlorella* spp., both *in vitro* and *in vivo*, there is still insufficient data to translate it to the clinical phase. Some factors need to be considered before introducing *Chlorella*-based drugs in clinical trials. The bioavailability and safety profile of *Chlorella* products should be studied thoroughly and well documented. Though some reports are available on the biocompatibility and bioavailability of *Chlorella*, most of these in vivo studies are on small animals.[91,92] Therefore, before clinical transformation, preclinical studies are required on large animals regarding their safety and bioavailability. The exact compounds exerting the anticancer potential need to be addressed with their detailed mechanism of action.

Another major challenge using *Chlorella* is the standardization of growth conditions, the extraction methods, and the overall cost. Though the growth rate of *Chlorella* is very fast, the production and extraction costs may limit its use on a large scale. Therefore, a cost-effective production method must be developed to ensure its economic feasibility in healthcare industries.

However, *Chlorella* spp are rich in anticancer phytochemicals and have oxygen-supplying capacity, making them an excellent choice for cancer therapy. Also, *Chlorella* showed other bioactivities, such as antioxidative, anti-inflammatory, and protective effects against many diseases. Engineered *Chlorella* species showed promising efficacy in killing cancer cells without significant side effects and gained greater attention in future research in this particular field.

Conclusion

Chlorella spp. are a gold mine of excellent bioactive compounds. These compounds are reported to have anticancer and other bioactivities. Active fractions from different Chlorella species can hinder the proliferation of various cancer cells through multiple mechanisms. However, more studies are also required to explore marine *Chlorella* spp., since marine microalgae have diversified phytochemicals with more anticancer capacity. Besides, more investigations need to be carried out in terms of their

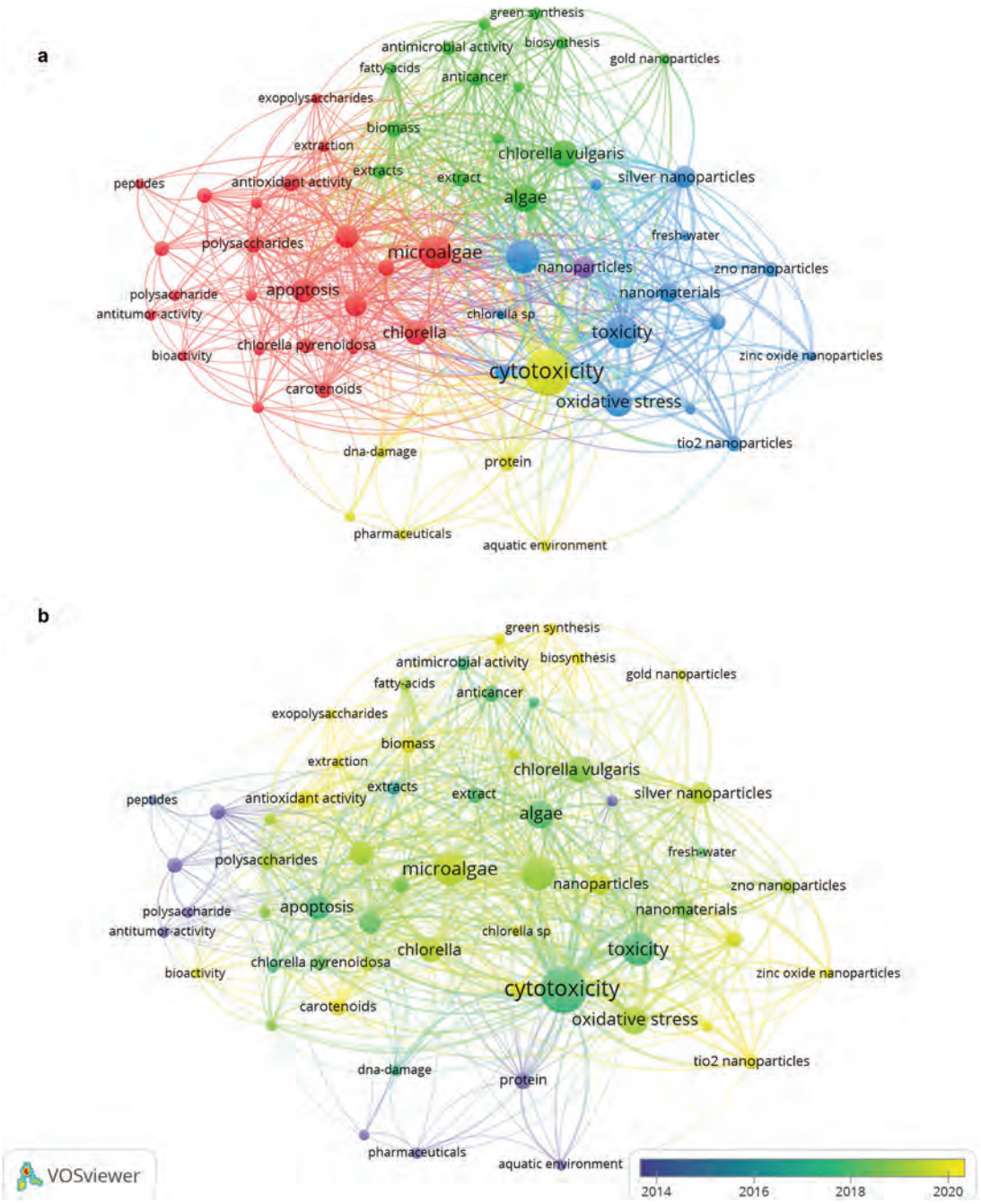


Figure 8. Keyword occurrence analysis, (a) Network occurrence; (b) Overlay occurrence.

exact mechanism in various cancer cells and the exact compounds responsible for the anticancer activity when using these microalgae in cancer treatment. However, bioavailability is a major concern when it comes to drug designing with *Chlorella*. To mitigate this problem, *Chlorella* synthesized nanomaterials are now being investigated widely as an efficient way of drug delivery. Further studies are needed in the animal model as well to ensure bioavailability, effective dose, specific cancer targeting and safety information. Current research trends are also aligned with the use of engineered

Chlorella formulation as a fast, safe and effective treatment option for cancer. A thorough investigation of the safety and regulatory assessment of the *Chlorella* drug will pave the way to successful clinical trials.

Highlights

- *Chlorella* spp. showed *in vitro* and *in vivo* anticancer activities against different cancer cells
- Besides anticancer activity, *Chlorella* spp. have other bioactivities like antioxidant, immunomodulatory, antimicrobial, antidiabetic, antiaging, and protective effects
- Bioengineered *Chlorella* can easily reach hypoxic tumor sites, generate oxygen, and stop tumor growth
- Chlorella-based drug showed excellent biocompatibility and bioavailability in the preclinical studies
- The current research trend focuses mostly on the biosynthesis of nanoparticles from *Chlorella* and elucidating their bioactive properties

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Disclosure statement

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Author contributions

UTF – Conceptualization, data analysis, visualization, Writing- original draft, review & editing. **SAK** – Software, data analysis, Writing- review & editing. **AS** – Data analysis, supervision, Writing- review & editing. **SU** – Conceptualization, visualization, Writing- review & editing; **MN** – Visualization, Writing- review & editing, **AWZ** – Writing- review & editing.

Data statement

All the data is presented within this article.

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