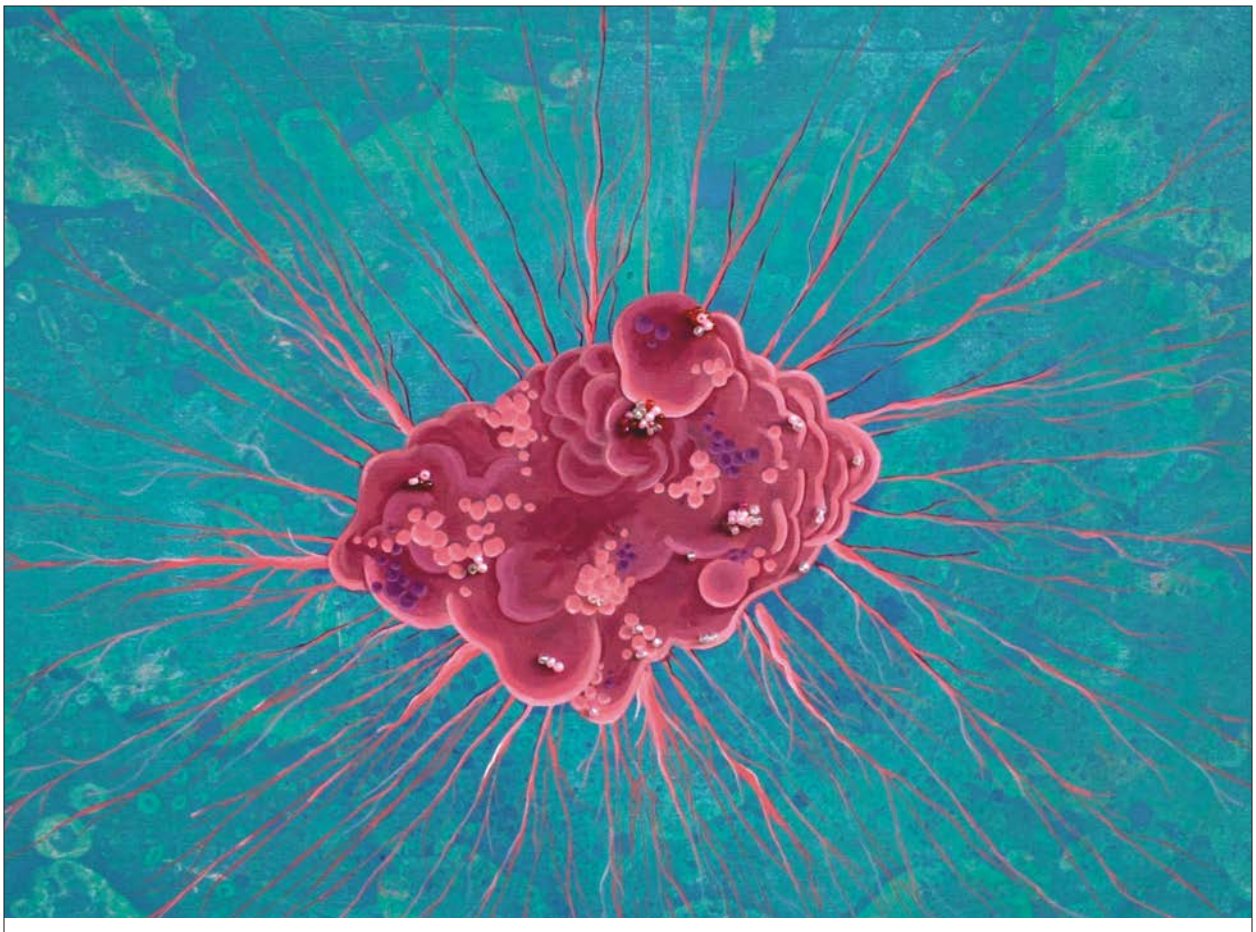

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Research Article
In vitro Anti-oxidant and Anti-cancer Activity of *Tetradesmus acuminatus* Microalgae Extract on MCF-7 Human Breast Cancer Cell Line

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Abstract

Background and Objective: Microalgae are the vital constituents in food chains of aquatic ecosystems and have been used for human consumption as food and as medicines. The wide diversity of compounds synthesized from different metabolic pathways of fresh water algae provides promising sources of secondary metabolites in the form of phytochemical constituents. These metabolites are very interesting source of producing an herbal medicine to treat diseases like cancer which is a major public health concern. The study aims to determine the phytochemical constituents, antioxidant and anti-cancer activities of *Tetradesmus acuminatus* algal extraction MCF-7 human breast cancer cell line. **Materials and Methods:** The dried and dehydrated algae biomass was subjected to extraction by cold maceration method using 5 solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (aqueous). Phytochemical screening was done using different biochemical tests. Quantitative analysis for phenol was determined by Folin-Ciocalteu reagent method. The antioxidant activity was tested using 2, 2-diphenyl-1-picrylhydrazyl, ferric ion reducing power assay. *In vitro* anti-cancer activity on MCF-7 human breast cancer cell line was evaluated by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay. **Results:** The phytochemical analysis revealed broad spectrum of bioactive compounds including flavonoids, glycosides, phenols, tannins, fats and oils. Methanol and aqueous extracts exhibited higher phenolic content as compare to ethanol extract. Antioxidant capacities were shown highest in methanol and ethanol aqueous based on the test performed. The methanol and aqueous extracts were found to be selectively cytotoxic *in vitro* to on MCF-7 human breast cancer cell line with IC_{50} values 468.31±24.15 and 598.12±12.18 μg mL^{-1} for MCF-7, respectively, while it had no cytotoxic effect on normal mice embryo fibroblast cells. **Conclusion:** The results indicate that *Tetradesmus acuminatus* was a promising antioxidant and anti-cancer agent for MCF-7 human breast cancer cell line. However, further studies are needed to conclude its therapeutic use.

Key words: Phytochemical screening, *Tetradesmus acuminatus*, anti-oxidant, MCF-7, human breast cancer cell line, carcinogenesis, antimetastatic activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer causes the highest mortality around worldwide, particularly in undeveloped countries[1]. Chemotherapy drugs are still standard cancer treatments despite their toxicity against normal cells and tissues. Natural products are now being used as a newer approach to develop medication to cure cancer in many pharmaceuticals industries across the globe. The treatment of herbal medicine does not contain any toxic chemicals and does there is no side effects post treatment[2]. Uncontrolled proliferation is a universal property of tumour cells. Investigation of the cellular growth control mechanism has contributed to the understanding of carcinogenesis and to the identification of compounds with specific anti-tumoral activity. Freshwater microalgae have significant contents of bioactive compounds which work as anti-inflammatory, anti-microbial, antiviral and anti-tumor drugs. *In vitro*, the antioxidant activities of the polysaccharides substances extracted from seaweeds inhibit the proliferation of cancer cells[3]. Algal biomass has long history of use in the treatment of cancer and several studies have been conducted on fresh and marine water algae under a multitude of botanical grounds[4]. Secondary metabolites of algae and plants and their semi-synthetic derivatives continue to play an important role in anti-cancer drug therapy and apoptosis of the cancer cells through the compounds derived from microalgae[5,6]. These include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin and paclitaxel (taxol) and the concentration of different phytochemical isoforms in the microalgae is very high such as fatty acids, polysaccharides and carotenoids[7]. Many natural product derivatives containing anti-cancer properties which pose promising interests into lead molecule are in the end phase of clinical development, however, there are limited literature available on its implementation in the clinical practice[8]. About 60% of currently used anti-cancer agents are derived in one way or another from natural sources. Use of fresh and marine water algal biomass for medicinal remedies is an integral part of the Indian cultural life and this is unlikely to change in the years to come[5]. Many traditional healers and herbalists in the India have been treating cancer patients for many years using various species. Solvent extracts derived from the microalgae biomass have shown significant antimetastatic activity against different cancer cells[9]. The present work will open avenues and prospects of freshwater microalgae which are undermined in comparison to the marine algae. Besides this it will add up to the existing information on the pharmacological applications from the extracts of the microalgae. The work is novel, as to the best of our knowledge till date there are limited studies where an algal source is used to treat anti-cancer *in vitro* model. In this study, we have explored 5 solvent extract of algae biomass of *Tetradesmus acuminatus* for the prevention and treatment of MCF-7 human breast cancer cell line at *in vitro* model system.

MATERIALS AND METHODS

This research study was carried out in the year 2018 within 9 months in the Department of Biotechnology and Microbiology, Karnatak University, Dharwad, India and part of the work was completed in the Department of Urology, KLES Kidney Foundation, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi, India.

Isolation and identification: *Tetradesmus acuminatus* was isolated from freshwater lake in Dharwad and enumeration of was done as per the standard methods[10]. Identification was done by the standard algal monographs[11]. The algal cells were observed under bright field microscope. After identification and selection, a single cell was picked using a pasteur pipette and transferred to fresh autoclaved 100ml of BG-11 medium and incubated at 25°C temperature with 1.2±0.2 lux light intensity and 16:8 light dark cycle for 2 weeks to obtain pure cultures. Wet biomass was obtained by centrifugation. The pure cultures were maintained by continuous sub culturing in BG-11 broth media and CHU-13 agar slants.

Cultivation and mass production: *Tetradesmus acuminatus* was grown in two different culture media viz. BG-11 and NPK. The cultures were grown autotrophically in the batch culture of 1000 mL Erlenmeyer flasks and were kept on the rotary shaker at the rate of 140 rpm at 28°C. Large scale cultivation of alga was done in 20 L capacity indigenously made photobioreactor[3]. The photobioreactor is made up of high density polyethylene (40 cm height, 26 cm diameter). The agitation in the culture medium was carried out by sparging filtered air (using 0.2 mL syringe filter) from the bottom using silicon tubing. The photobioreactor was kept under the indirect sun light at an approximate light intensity of 8000 lux at a temperature of 28°C. Biomass was filtered by muslin cloth, dried and weighed.

Extraction: The dried coarsely powdered biomass was subjected to extraction by cold maceration method. In maceration (for fluid extract), the algal powder was kept in contact with the solvent in a stopper bottle for a 7 days with frequent agitation[12,13]. In cold maceration method, 3 g of algal dry mass was extracted sequentially extracted with 30 mL of cold hexane, chloroform, methanol, ethanol and aqueous solution. This involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (aqueous) to ensure that a wide polarity range of compound could be extracted.

Phytochemical analysis: The freshly prepared solvent extracts of *T. acuminatus* were qualitatively tested for different phytochemicals present namely alkaloids, flavonoids, glycosides, phenols, saponins, sterols, tannins and reducing sugar by following the standard procedure of Deepti *et al.*[14].

Determination of total phenolic content: Estimation of total phenolic content of *Tetradesmus acuminatus* was determined using the Folin-Ciocalteu reagent method of Wolfe *et al.*[15] with slight modification. A volume of 200 µL of extract is mixed with an equal volume of Folin-Ciocalteu reagent and incubated for 10 min. About 1.25 mL of aqueous sodium carbonate is added and the reaction mixture is incubated for 90 min at 37°C after addition of 1 mL distilled water. The absorbance of the blue colour was read at 760 nm spectro-photometrically using distilled water as a blank. Gallic acid is used as standard and the total phenolic content was expressed as mg g^{-1} gallic acid equivalent (GAE).

***In vitro* antioxidant activity:** In DPPH assay different concentrations of the extracts and MnNPs were used. A volume of 2.5 mL of 0.04% DPPH solution was mixed with 0.5 mL of all the concentrations of all the extracts and MnNPs separately[16]. After 30 min incubation at room temperature in the dark, the absorbance was read at spectrophotometer at 517 nm in triplicates for each concentration. Ascorbic acid was used as a standard. The percentage inhibition of free radical formation was calculated by the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

where, Ac is the absorbance of the control reaction (100 µL of ethanol with 100 µL of the DPPH solution) and At is the absorbance of the test sample.

The experiment was done in triplicate and the results were expressed as Mean±SE. The results were analysed in triplicate. The IC_{50} value is the concentration of sample required to inhibit 50% of the DPPH free radical.

Antioxidant activity by hydrogen peroxide scavenging assay: The antioxidant activity of methanol and aqueous extracts of *Tetradesmus acuminatus* by hydrogen peroxide scavenging assay was carried out using ascorbic acid as a standard reference[17]. About 0.6 mL of 4 mM H_2O_2 solution in phosphate buffer (pH 7.4) was added to 0.5 mL of known concentration of standard ascorbic acid and to tubes containing different concentrations ranging from 100-500 µL of algal extracts in phosphate buffer (pH 7.4). Absorbance of the solution was measured at 230 nm after 10 min against the blank solution containing phosphate buffer without hydrogen peroxide. Control was prepared by replacing the sample or standard with phosphate buffer. All samples were assayed in triplicates. The percentage of inhibition was calculated by using equation:

$$\text{Inhibition (\%)} = \frac{\text{Ac} - \text{At}}{\text{Ac}} \times 100$$

where, Ac is the absorbance of the control reaction and At is the absorbance of the test sample.

Culturing of cell lines: The cell lines MCF-7 and MEF-L929 were procured from the National Centre for Cell Science, Pune, India. The cells were sub-cultured in Dulbecco modified eagle medium supplemented with 10% foetal bovine serum, 1% penicillin–streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere. After trypsinization, the cell count was done and the cell viability was tested by Trypan blue using a haemocytometer. A known number of cells (2×103 cell/well in 100 µL of medium) were seeded into 96-well plates respectively for carrying out a MTT assay[18].

Treatment groups: The MCF-7 and MEF-L929 cell lines were treated with *Tetradesmus acuminatus* methanol and aqueous extract (5 mg mL^{-1}). Desired concentrations of test compounds were prepared in di-methyl sulfoxide prior to the experiment. The reactant mixtures were diluted with media and cells were treated with different concentration ranges of the extract (3.125-200 µg mL^{-1}) and incubated for 72 h, respectively, which was the optimal treatment time of the

extracts in each of the cell lines. The effect induced was also compared with the standard drugs used, namely, paclitaxel for MCF-7 and MEF-L929 cell lines human breast cancer cell lines[19]. The following treatment groups are set up of the study. Negative control: cells alone, Positive control: cells+paclitaxel, Test groups: cells+methanol extract and cells+aqueous extract. The same treatment group was followed for mice embryo fibroblast (MEF-L929) normal cell lines.

MTT cell viability assays: After 72 h, the media of treated cells (100 µL), were removed and the cell culture were incubated with 50 µL of MTT at 37°C for 4 h. After incubation, the formazan produced was then solubilized by the addition of 100 µL di-methyl sulfoxide. The suspension was placed on a microvibrator for 5 min and then the absorbance was recorded at 540 nm by an enzyme-linked immunosorbent assay reader[20,21]. The results were analysed in triplicate and the percentage was calculated.

Statistical analysis: The results were expressed as mean±standard deviation. Descriptive statistics was used to analyze the mean, standard deviation, variation and level of statistical significance between groups. When $p<0.05$ and $p<0.01$, was considered statistically significant for analysis of percent inhibition of cell growth.

RESULTS

Isolation and identification: *Tetradesmus acuminatus* is a unicellular green microalgae, which exists in both fresh and marine water. It was confirmed using algal monographs when observed under light microscope which revealed small chains of 4 cells (Fig. 1). The dry biomass content of *Tetradesmus acuminatus* recovered from the BG11 media was 8 g/20 L.

Total phenol content: In the present study, total phenolic content of different extracts of *Tetradesmus acuminatus* was determined by the Folin–Ciocalteu reagent method and expressed as GAE g^{-1} of plant extracts. Methanol extract exhibited the maximum amount of phenolic content among the extracts, i.e., 64.21±0.18 mg g^{-1} GAE followed by 62.14±0.15 mg g^{-1} GAE in ethanol extract, 68.24±0.17 mg g^{-1} GAE in aqueous extract and 77.74±0.42 mg g^{-1} GAE in standard gallic acid. All the calculations were made using the r^2 values from the graphs (Table 1).

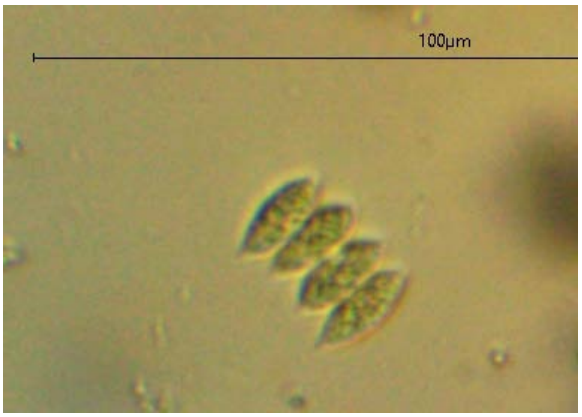

Fig. 1: Microscopical view of *Tetradesmus acuminatus*

Table 1: Total phenolic contents of different solvent extracts

Extracts	TPC	Units equivalent	r^2 values
Methanol extract	68.24±0.17	mg g^{-1} GAE	$R^2 = 0.9867$
Ethanol extract	62.14±0.15	mg g^{-1} GAE	$R^2 = 0.9807$
Aqueous extract	64.21±0.18	mg g^{-1} GAE	$R^2 = 0.9865$
Gallic acid (reference)	77.74±0.42	mg g^{-1} GAE	$R^2 = 0.9957$

Table 2: Inhibition (%) of DPPH free radical of *Tetradesmus acuminatus* extracts

Concentration (µg mL^{-1})	Methanol extract	Aqueous extract	Standard ascorbic acid
100	68.17±0.17	55.96±0.48	77.78±0.17
200	71.78±0.29	73.25±0.46	83.65±0.23
300	76.31±0.99	75.26±0.35	87.20±0.30
400	78.05±0.48	80.58±0.35	90.80±0.35
500	81.54±0.48	82.82±0.24	93.91±0.35

Anti-oxidant assays

Antioxidant activity by 1, 1-diphenyl, 2-picrylhydrazyl (DPPH) radical scavenging ability assay: There was decrease in the concentration of DPPH radical due to the scavenging ability of the soluble constituents present in the methanol and aqueous extracts of *Tetradesmus acuminatus*. The results revealed that the antioxidant activity of methanol and aqueous extracts of *Tetradesmus acuminatus* exhibited significant scavenging activity with 68.17±0.178 and 55.96±0.48 at 100 µg mL^{-1}, respectively whereas, standard ascorbic acid showed highest scavenging activity of 77.78±0.17. A direct positive relation was seen between antioxidant activity and increasing concentration of the extracts (Table 2).

Hydrogen peroxide radical scavenging assay: Hydrogen peroxide radical scavenging assay revealed that methanol and aqueous extracts showed significant scavenging activity i.e., 63.70±0.42 inhibition and 43.86±0.21, respectively whereas, the standard exhibited 74.46±0.13 of inhibition (Fig. 2).

Anti-cancer activity

Effect of *Tetradesmus acuminatus* methanol, ethanol and aqueous extracts on MCF-7 cell line: The result of MTT assays revealed that the methanol, ethanol and aqueous extract of *Tetradesmus acuminatus* extract decreased the percent viability of all the cells but to different extent. Methanol and aqueous extract was found to induce more cytotoxicity than ethanol towards cancer cell lines MCF-7 cell line. The MEF-L929 mice embryo fibroblasts were used as control and it was seen that the extracts had no effect on these cells. The effect of paclitaxel, the standard drug used in the treatment of breast cancer and the extracts was similar. The amalgamation of the extracts and standard drug showed higher degree of cytotoxicity in malignant cells. The IC_{50} values 468.31±24.15 and 598.12±12.18 μg mL^{-1} for MCF-7, respectively (Table 3), while it had no cytotoxic effect on normal mice embryo fibroblast cells. These results revealed morphological changes and shrinkage of cells leading to cell death induced by the extracts in the breast cancer cell lines (Fig. 3, 4).

Table 3: IC_{50} values of cell proliferation inhibition of *Tetradesmus acuminatus* methanol, ethanol and aqueous extracts on MCF-7 breast cancer cell lines (μg mL^{-1})

Cells	Methanol	Ethanol	Aqueous
MCF-7	468.31±24.15	787.19±27.09	517.10±17.08
Standard: Paclitaxel	0.17 μM mL^{-1}		

Values presented are Mean±Standard deviation, n = 3, results were analyzed using descriptive statistics

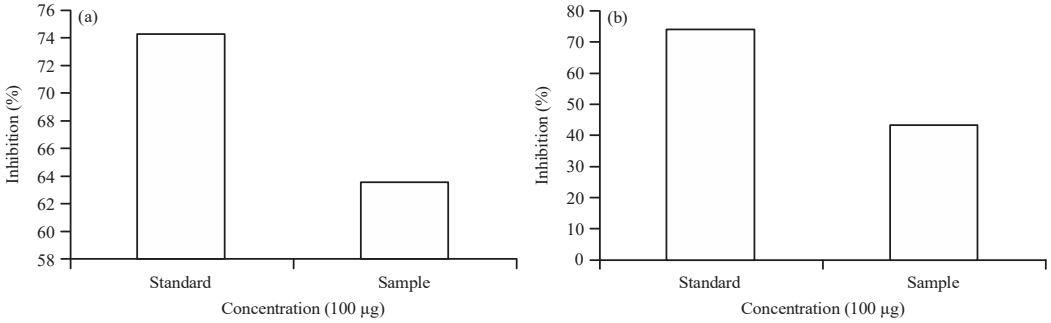

Fig. 2(a-b): H_2O_2 assay of (a) Methanol and (b) Aqueous extracts of *Tetradesmus acuminatus*

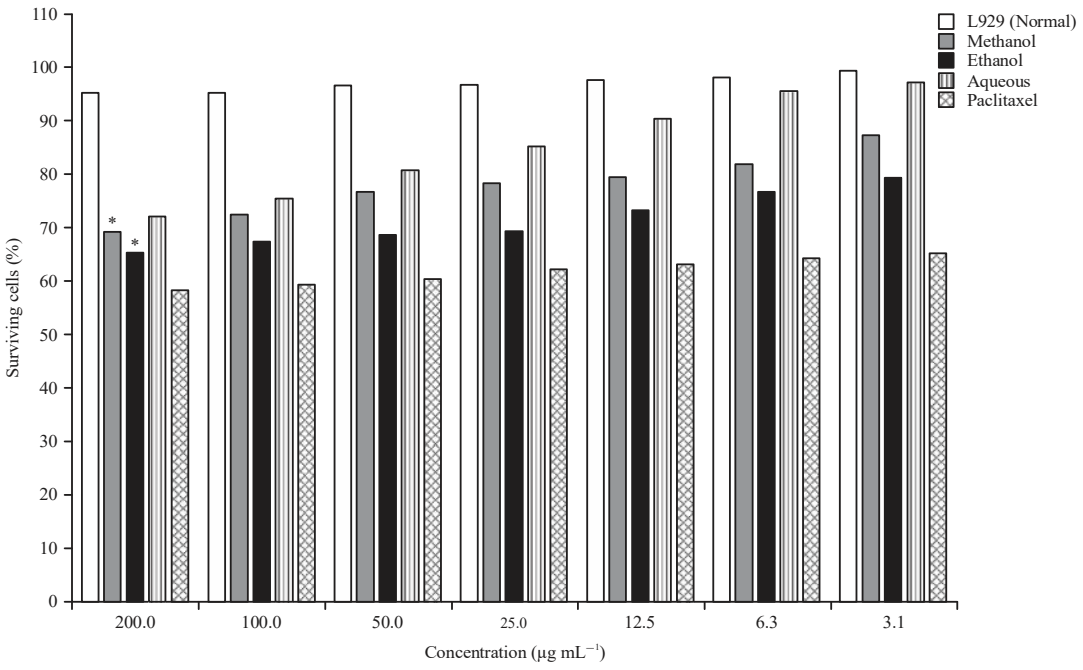

Fig. 3: Effect of anti-cancer activity of *Tetradesmus acuminatus* methanol, ethanol and aqueous on MCF-7 breast cancer cell line and MEF-L929 normal cell line

*$p \leq 0.05$

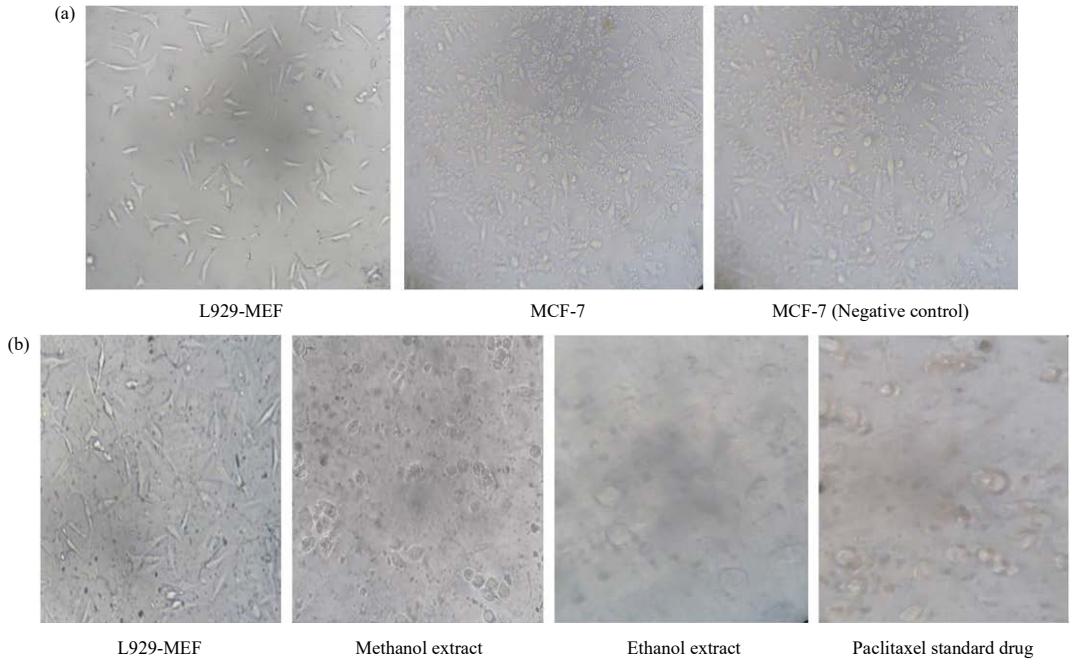

Fig. 4(a-b): Morphological changes showing inhibition of MCF-7 breast cancer cell lines, (a) Before treatment and (b) After treatment

No inhibition was observed on MEF-L929 normal cell line for 72 h

DISCUSSION

In the present study, classical morphology based methods were used for the identification of *T. acuminatus* which was isolated from fresh water lake of Dharwad district, Karnataka, India. According to the characteristics of the cells designated as strain S1, 10 µm long cells differed from ovoid to sub spherical in shape and had band shaped chloroplasts, this isolate was regarded as *T. acuminatus* (Fig. 1). Lipids as important storage compounds of microalgae are mostly synthesised during the stationary phase of growth[22]. Microalgae has become a good candidate for sources of natural antioxidants as revealed by a number of recent studies[23]. *Tetradesmus acuminatus* species was isolated, biomass obtained and dried biomass subjected for phytochemical analysis which revealed a broad spectrum of secondary metabolites. The phytochemicals of *Tetradesmus acuminatus* were qualitatively analysed from the solvent extracts, based on which the current study to evaluate its potential against cancer cell lines was undertaken. This study has revealed the presence of an array of medically potential phytochemicals including, flavonoids, glycosides, phenols, sterols, tannins, reducing sugars and volatile oils were present in the samples. The studied phytochemicals of *Tetradesmus acuminatus* extracts are pharmaceutically important and different phytochemicals such as the phenolic compounds, carotenoids and fatty acids have gained attention of different areas of applications such as pharmaceutical, health, food and cosmetic industries[3]. The bioactive compounds such as phenols are known to be responsible for the antioxidant activities[5]. These compounds are widespread in the plant kingdom as part of our daily diet and are attractive as natural antioxidants. In this study, the phenolic content was studied in *Tetradesmus acuminatus* wherein the methanol extract exhibited the highest total phenolic content 64.21 ± 0.18 mg g^{-1} GAE followed by 62.14 ± 0.15 mg g^{-1} GAE in ethanol extract, 68.24 ± 0.17 mg g^{-1} GAE in aqueous extract.

Reactive oxygen species are thought to play a vital role in many human diseases. Radical scavenging activities are very essential due to the toxic role of free radicals in organic systems. Many secondary metabolites like phenols, polyphenols and flavonoids serve as sources of antioxidants and perform scavenging activity[24]. Reactive oxygen species readily combine and oxidize biomolecules such as carbohydrates, proteins and lipids and thus making them indolent with sub-sequent damage to cells, tissues and organs leading to cancer progression[25]. In the present study, two methods were used to evaluate the total antioxidant capacity of methanol, ethanol and aqueous extracts of *Tetradesmus acuminatus* were evaluated for their antioxidant activity by

DPPH assay and hydrogen peroxide scavenging assay. The dried biomass was processed by the cold maceration method. The solvent extracts were then used for antioxidant assays. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain of reactions that may damage cells[26]. Antioxidants are classified into 2 broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (lipophilic). Meta-regression analysis, meta-analysis and trial sequential analyses of the effects of supplementation with β-carotene, vitamin A and vitamin E singly or in different combinations on all-cause mortality[27].

Antioxidant activity by DPPH assay revealed that methanol and aqueous extracts of *Tetradesmus acuminatus* decrease in the concentration of DPPH radical formation due to the scavenging ability of the chemical constituents present in alga[28]. The antioxidant activity of methanol and aqueous extracts of *Tetradesmus acuminatus* exhibited significant scavenging activity with 68.17±0.178 and 55.96±0.48 at 100 μg mL^{-1}, respectively whereas, standard ascorbic acid showed highest scavenging activity of 79.78±0.17 (Table 2). A direct positive relation was seen between antioxidant activity and increasing concentration of the extracts[29]. Hydrogen peroxide radical scavenging assay revealed that methanol and aqueous extracts of *Tetradesmus acuminatus* possessed activity with 63.70±0.42 inhibition and 43.86±0.21, respectively whereas, the standard exhibited 84.46±0.13 of inhibition (Fig. 2).

The evaluation of the anti-cancer activity of *Tetradesmus acuminatus* extracts is essential for safe treatment[30]. It enables identification of the intrinsic toxicity of the plant and the effects of acute overdose. The MTT assay is used in screening the crude extracts as well as in the isolated compounds to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested algal extracts[31]. The MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. It is frequently used as an *in vitro* model system to measure cytotoxic effects of variety of toxic sub-stances and plant extracts against cancer cell lines[30]. *In vitro* cytotoxicity test using MCF-7 breast cancer cell line was performed to screen potentially toxic compounds that affect basic cellular functions and morphology. The 3 extracts (methanol, ethanol and aqueous) of *Tetradesmus acuminatus* extracts showed *in vitro* growth inhibition effects on the MCF-7 breast cancer cell line, while there was no effect on the growth of normal cells (MEF-L929). Such selective effects were concentration as well as, incubation time period dependent. With respect to concentration 3.125, 6.25, 12.5, 25, 50, 100 and 200 μg mL^{-1} of each extract was evaluated in triplicates by serial dilution. Among these seven concentrations, 200 μg mL^{-1} of methanol and aqueous extract was the most effective in producing percentage growth inhibition. The ethanol extract showed less effect throughout the range of tested concentrations in MCF-7 breast cancer cell line for a single time point of 72 h. However, the standard paclitaxel drug showing significant inhibition on the cancer cell lines. The results showed that methanol and aqueous extract significantly inhibited the MCF-7 breast cancer cell line and was the most potent extract with IC_{50} values 468.31±24.15 and 517.10±17.08 μg mL^{-1} for MCF-7, respectively (Table 2), while it had no cytotoxic effect on normal mice embryo fibroblast cells. The results also confirmed the differential effect induced by the extracts and standard drug in cancerous and normal cells (Fig. 3, 4). Therefore, the inhibition of cell growth by *Tetradesmus acuminatus* extracts might be due to the power of the solvent in surpassing effect of several bioactive constituents, the presences of phenolic compounds like gallic acid and other antioxidant agents that are present in *Tetradesmus acuminatus.*

SIGNIFICANCE STATEMENT

Microalgae biomass has been recognised to have great potential as a source of novel bioactive compounds with industrial as well as health promoting applications in human, animal and aquatic lives. Among them are the species of the genus *Tetradesmus* (previously known as *Scenedesmus)*. Although it is more commonly known as source of food for herbivorous zooplankton and in biofuel production because of its high lipid content, *Scenedesmus* has exhibited the potential of being a source of high-value compounds. In the present study also the selected solvent extracts of *Tetradesmus acuminatus* have shown the presence of phenol content and also exhibited observable antioxidant activity as well as anti-cancer activity against selected breast cancer cell line MCF-7. The results suggest the strong anti-proliferative properties and support the ethno-medical claims for the algae. It is hope that the present results will provide an initial point for investigations aimed at exploring new natural antioxidant substances present in the extracts of algae *Tetradesmus acuminatus* with potent anti-cancer activity.

CONCLUSION

In conclusion, the present study, a fresh water microalgae *Tetradesmus acuminatus* was isolated and mass cultured, further dried algal mass was subject for sequential solvent extraction. It was observed that the microalgae *Tetradesmus acuminatus* contains a wide variety of secondary metabolites that hold strong antioxidant capacity based on the experiments performed which add scientific evidence to conduct further studies, explore the chief compounds present in the algal biomass of *Tetradesmus acuminatus*, evaluate its anti-cancer potential on *in vivo* animal models and put forward an attempt to carry out trails on human beings.

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