Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 2s, https://carcinogenesis.com/

Anti-Malignant Potential of Crude Ethanol Extract Of Microalgae Scenedesmus Dimorphus Against Lung Cancer By Exploring Diary Industrial Wastewater As Cultivation Medium

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ABSTRACT

Cancer is one of the leading causes of death in the modern world, and about 10 million people died in 2020, with an important stake being lung cancer on this issue. This has led to the increase in the need of sustainable therapeutics. Against this background, the study of microalgae has gained more attention because of their ability to produce bioactive metabolites with pharmaceutical particles. In the proposed investigation, the anti-cancer potential of crude ethanol extract of Scenedesmus dimorphus cultivated in 50% dairy industry wastewater was determined against A549 human lung carcinoma cell line. The microalga S. dimorphus (UTEX 1237) was grown in proteose peptone medium with 50% dairy industry wastewater under optimum growth conditions viz., algal inoculum of 1x107cells/ml, a pH of 7.0, the temperature of 20°C, and the light intensity of 15,000 lux for 28days with 12D/12L photoperiodicity in a special designed novel photobioreactor. After 28 days, the biomass extracted with ethanol and evaluated for anti-cancer activity on A549 cell lines revealed dose-dependent cytotoxic effect with an IC50 value of 57.49 g/ml which is close to that of the positive control, cisplatin (IC50 value of 15g/ml). The GC-MS profiles of crude ethanol extract of S. dimorphus revealed the achievement of compounds containing anticancer-antioxidants properties like dibutyl phthalate, hexadecane, and eicosane. The findings as a whole encompass the dual importance of valorizating dairy wastes as well as producing pharmaceutical compounds and S. dimorphus becomes an attractive nutraceutical and pharmaceutical agent

Keywords: *Scenedesmus Dimorphus, Dairy Industry Wastewater, A549 Lung Cancer Cells, Ethanol Extract, GC-MS Analysis*

How to Cite: Mahadevi Narasanagi, Lingayya Hiremath, (2025) Anti-Malignant Potential of Crude Ethanol Extract Of Microalgae Scenedesmus Dimorphus Against Lung Cancer By Exploring Diary Industrial Wastewater As Cultivation Medium, *Journal of Carcinogenesis*, *Vol.24, No.2s*, 77-82

1. INTRODUCTION

Cancer is a health issue of growing concern affecting an uncontrolled cell growth and genetic mutation taking place in the body, causing death around the globe. One in six deaths globally was cancer-related in 2020 and lung cancer is ranked amongst leading cancer-related causes of deaths [1]. One of the most common in vitro models used for testing the anticancer agent is A549 human lung adenocarcinoma cell line that has well characterized genetic and phenotypic characteristics [2]. Although this phenomenon has been successfully treated with the use of chemotherapeutics like cisplatin, side effects of the treatment process are usually associated with being extreme and occurrence of drug resistant cancer phenotypes [3]. This has fuelled the need of new, natural and sustainable anticancer drugs. In this respect, microalgae have been getting some attention because of the broad variety of their biological activities, such as anti-inflammatory, antibacterial, antioxidant, anti-diabetic, and anticancer ones [4]. Microalgae are one of the rich sources of various types of bioactive compounds like carotenoids, polyunsaturated fatty acids (PUFAs), phenolics and phycobiliproteins which have shown therapeutic potential [5].

Of these, Scenedesmus dimorphus, a green microalga belonging to the Chlorophyceeae class stands out as especially promising. It has a high rate of growth, flexibility to diverse nutrient environment, as well as the potentiality to produce bioactive molecules that have pharmaceutical potential [6]. Noteworthy, its growth on other sources of nutrients e.g. industrial wastewater, particularly dairy effluent is providing an environmental friendly approach to generate bioactive

compounds and causing a decrease in environmental pollution [7]. The proposed study will determine the effect of ethanol extract of S. dimorphus grown on dairy wastewater to kill A549 lung cancer cells. The work has considered an innovative photobioreactor setup in optimized biomass production, and it describes the chemical profile of the extract of S. dimorphus through GC-MS. The results point out the combined value of waste bioutilization and production of new anticancer agents. Narasanagi & Hiremath have constructed a novel glass photobioreactor to maximize biomass as well as metabolites production of S. dimorphus under optimum cultivation conditions. Optimum conditions regarding pH (7.0), temperature (20°C), intensity of light (15000 lux) and inoculum concentration (1-2 x 10^7 cells/ml) were adopted and marked high biomass obtainment especially on 50% dairy wastewater. Those environmental conditions enhanced the rate of cell doubling and bioactive compounds production to indicate the efficiency of the photobioreactor under industrial conditions, i.e., biofuel and pharmaceutical industries [7]..

A pharmaceutical maxim of extracting substances in ethanol and conducting a GC-MS profiling procedure on dairy wastewater cultivated S. dimorphus by Narasanagi et al. were carried out on S. dimorphus grown on dairy wastewater. The ethanol extract of S. dimorphus showed a high degree of anti-diabetic and anti-oxidant activities with significant IC_{50} value compared to respective standard drugs. GC-MS showed new peaks that could not be identified in NIST libraries and identifiable dominant bioactives dibutyl phthalate, eicosane, and dodecane. It means that S. dimorphus has a potential to be used as a new source of pharmacologically active molecules [8].

Geethalakshmi & Sarada documented the phenomena that the components of essential oils of Trianthema decandra hexane extract and tetracosane, the fundamental oils were indicated to stalk antimicrobial and antioxidant activity similar to conventional antibiotics, such as chloramphenicol. The obtained evidence substantiates the applicability of substances found in the extract of S. dimorphus and their possible anticancer effect, by means of inhibition of oxidative stress [9]. Pushpakumari et al. through an experiment on the adaptability of S. dimorphus to alternative source of waste water and biodiesel production implicated the high lipid content (34 & 35%) as an essential factor. The study determined the dual role of microalgae in waste reduced and in power generation [10]. Armaini et al., focused on the importance of microalgae in nutraceuticals with specific reference to the presence of astaxanthin, lutein, beta-carotene, and Omega-3 fatty acids. These compounds play a role in immune modulation and due to this exercise significant therapeutically prospect toward lifestyle diseases like cancer, diabetes and heart diseases [11].

The present study focuses on the possibilities of using dairy wastewater as a nutrient medium to grow Scenedesmus dimorphus to produce anticancer metabolites. Although the properties of antioxidant and antimicrobial activities of extracts of microalgae have been estimated so far, very few studies have been conducted on their cytotoxicity in cancer especially lung cancer with algal biomass that were grown under waste-utilizing conditions. The novel photobioreactor designed in the present research benefits with constant supply of aeration, light and nutrient mixing which results in an optimal biomass production. Additionally, it can be named among the few investigations to discover bioactive compounds through the GC-MS including unknown metabolites specific to the wastewater-grown S. dimorphus pointing at the importance of the stress-related metabolism change as a mechanism of improving therapeutic value.

2. EXPERIMENTAL DETAILS

Cultivation of S. dimorphus

Axenic culture of S. dimorphus (UTEX 1237) was obtained at University of Texas Algal Collection. The algal strain was first grown on proteose peptone agar petri-plate incubated in 20ºC and 12/12 h dark and light hours respectively in a dark box. In order to perform experimental cultivation, glass photobioreactors with a volume capacity of 2.5 L and containing a medium of proteose peptone with an addition of 50% of sterilized dairy wastewater were prepared. The reactors were set to 20ºC, pH 7.0 with 15,000 lux of monochromatic LED light which was complemented by aeration and agitation to get uniformity of the reaction and prevent clumping of the biomass [7].

Biomass Harvesting and Extraction

Green algal biomass was collected by a 28 days cultivation period followed by filtration and shade-drying. That is around 192.8gm of dry biomass was produced in 50 L of medium. The biomass was crushed to a fine powder and the powder was put into ethanol overnight. Evaporation of the solvent was carried out in a room temperature environment to give crude ethanol extract that can be preceded further to the bioassay and GC-MS qualification.

Chemical Investigation and GC-MS Profiling

The presence of alkaloids, carbohydrates, proteins, oils, fats and glycosides was indicated by phytochemical screening. The concentration of extract was analyzed with the help of GCMS analysis with Perkin ELM Turbo Mass Spectrometer and RTx5MS column. The helium was used as carrier gas and injection was accomplished in split mode. The temperature gradient was fixed as 100-300 ºC. Dibutyl phthalate, tetracosane, eicosane, hexadecane, and new undeterminable constituents (RT 13.598 and 13.781) were identified [8].

Anti-cancer Assay Against A549 Cells

The A549 human lung carcinoma cell line was grown in DMEM supplemented with 10% FBS, and 1% penicillin-streptomycin. The seeding density of cells in a 96-well plate was as follows; 10^4cells/well, after 24 h incubation. S. dimorphus was exposed to 20, 40, 60, 80, and 100 µg/ml ethanol extract and left to incubate 48 h. As the positive control, cisplatin (15µg/ml) was used. The MTT assay was used in cell viability measurement. The microplate reader was used to observe absorbance at 570 nm. It is based on the IC_{50} value which was obtained by non-linear regression of the % viability of cell lines on the concentration.

3. RESULTS AND DISCUSSIONS

3.1. Cytotoxicity of S. dimorphus Extract Against A549 Cell Line

In order to assess the anti-cancer potential of S. dimorphus ethanol extract based on cytotoxicity, in vitro cytotoxicity assays were done against the A549 human lung adenocarcinoma cell line. Increasing concentrations of extract (20, 40, 60, 80 and 100 µg/ml) were used to treat the cells over a period of 48 hours. The MTT assay was used to evaluate the cell viability and the findings are highlighted in **Table 1**. There was a dose-related reduction in cell viability which signified a high anti-tumour activity.

As depicted in **Table 1**, optical density (O.D) of the 570 nm has been reduced with increase in the ethanol extract concentration of S. dimorphus that demonstrate diminished metabolic activity and therefore demonstrated enhanced cytotoxicity. The extract had a growth inhibition in a concentration of 77.06% at its highest concentration (100 µg/ml) when compared with an untreated control. IC_{50} value of S. dimorphus extract was determined to be 57.49µg/ml, whereas the chemotherapeutic agent, cisplatin at the concentration of 15 µg/ml had an inhibition rate of 92.8% with an IC_{50} value of 15 µg/ml. The findings indicate that the extract is less active than the cisplatin; the extract however has a considerable anticancer activity whose potency is relatively lesser against the normal cells.

Table 1: Cytotoxic activity of S. dimorphus ethanol extract on A549 cells after 48 h

Conc. S. dimorphus extract (µg/ml)	Anticancer activity S. dimorphus extract		
	Mean (O.D at 570nm)	Anticancer activity (%)	IC50 valve (µg/ml)
20	0.350±0.002	78.59	**57.49**
40	0.269±0.001	59.45	
60	0.219±0.004	47.56	
80	0.157±0.004	32.81	
100	0.116±0.006	22.94	
15 (Cisplatin)	0.052±0.004	7.84	

3.2. Morphological Assessment of Treated A549 cells Cells

The A549 cells images taken using the microscope after being treated by exposing them to different concentrations of S. dimorphus extract showed obvious indications of cytotoxic injury. In untreated control cells (**Figure 1**), morphology was significantly normal epithelium and normal nuclei and monolayers were in high density. On the other hand, the cells at the concentrations that were greater than 60 µg/ml indicated evidence of membrane blebbing, cell rounding, detachment and nuclei condensation as indicative of apoptosis. **Figure 1(a-b)**: The change of morphology of A549 cells cultured with the extract of S. dimorphus. a: untreated control; b: 40 µg/ml; c: 60 µg/ml; d: 100 µg/ml. Note how much apoptosis and cell shrinkage there is in the treated groups. These morphological effects denote that the algal extract has caused programmed cell death and not necrosis which is ideal when considering an anticancer agent as it causes less inflammation [3,5].

3.3. GC-MS Based Identification of Bioactive Compounds

The Gas Chromatography Mass Spectrometry (GC-MS) was used to analyse the ethanol extract to explain the compounds that act as anticancer agents. The analysis returned 11 large peaks, two of which did not coincide with any compounds in NIST library, indicating new bioactive molecules. Some of the major chemicals identified in this extract were hexadecane, tetracosane, dibutyl phthalate, dodecane, eicosane, and 2,4-Di-tert-butylphenol which are reported as having a cytotoxic, antioxidant, or antimicrobial effect [7,8]. GC-MS chromatogram of ethanol extract of S. dimorphus grown in dairy wastewater contains the Major: 1- Hexadecane, 2- Dodecane, 3- Eicosane, 4- Dibutyl phthalate, 5- Unknown compound A, 6- Unknown compound B. Dibutyl phthalate has been reported earlier in Medicinal plant as Begonia malabarica and Ipomoea carnea which exhibited high anticancer and anti-larvicidal action [9]. Cytoskeleton alterations Hexadecane and

tetracosane have also been reported to cause oxidative stress-mediated apoptotic death to cancer cells [11]. The effect of 2,4-Di-tert-butylphenol supports it more, as it is known to have anticancer activity through ROS formation and depolarization of the mitochondrial membrane [9]. In addition, the presence of dodecane and eicosane that are typical components of petroleum fractions indicate that the environmental stress condition of an individual that resulted to dairy waste may have triggered the biosynthesis of biosynthesis of rare hydrocarbon-like compounds as a result of dairy waste that exert a powerful bio-activity [8]. In comparing the S. dimorphus extract used in the current study with other microalgae extracts, notably Euglena tuba whose IC_{50} value was reported to be 92.14 µg/ml against A549 cells [8], the IC_{50} value in the former is lower and this portends it to have higher potential. Tetraselmis suecica, in its turn, was observed to be active at significantly lower doses (5 µg/µl), but its effects were more of cell restoration and anti-inflammatory properties than direct DNA-damaging activity [8]. Similar results have been obtained with the algal extract with the effect in relation to Chlorella vulgaris ethanol extract 200 µg/ml which had 75 % inhibition of A549 cell proliferation [8]. Thus, the S. dimorphus extract has a weak-moderate potential as an anticancer agent with comparatively low values of concentrations at which the activity is established, which further proves its pharmaceutical prospects. The probable mechanism of action, according to the data in the compounds profile and morphological findings, is the reactive oxygen species (ROS)-induced apoptosis. It has been demonstrated in several studies that such compounds as dibutyl phthalate and aliphatic hydrocarbons regulate expression of apoptotic markers such as caspase-3/7, Bax, and Bcl-2 [9,11]. Moreover, 2,4-Di-tert-butylphenol, which is a type of phenolic compound, are also known to interfere with mitochondrial membrane potential inducing the intrinsic pathway of apoptosis [5, 9].

Since there are no signs of cytotoxicity in the untreated controls and the extract selectively inhibits the growth of cancerous cells, its extract seems to be of tumour specific nature- a valuable aspect of anticancer agents [3,5]. The excess stress levels due to the 50% dairy waste water medium would have contributed to better biosynthesis regarding the secondary metabolites. Increased levels of nitrogen, phosphates and organic-carbon can induce oxidative stress events in algae causing a higher synthesis of protective compounds including carotenoids, lipids as well as phenolics [7,10].

Narasanagi et al. [8] reported the existence of two unidentified peaks at retention times of 13.598 and 13.781 minutes only in the extracts of dairy wastewater-grown biomass. The identified peaks, that were not observed in algae cultivated under control conditions, might be new anticancer compounds, and thus, require additional structural studies. Besides the anticancer activity, an earlier work carried on the same extract reported significant anti-diabetes (IC_{50} = 177.8 µg/ml) and anti-oxidant (IC 50 = 68.3%) capabilities [8]. This multifactorial bioactivity means that S. dimorphus can be a perfect choice as a functional food, nutraceutical, and adjunctive cancer treatment agent. Further, as opposed to toxic synthetic chemotherapies such as cisplatin, which are associated with nephrotoxicity, ototoxicity and neurotoxicity [3]. The current approach to anticancer algae-derived therapies offers a safer alternative, which is eco-sustainable and has fewer off-target effects. The reuse of dairy wastewater as a nutrient medium fulfils two aspects, which are the pollution reduction and the biomass augmentation. Dairy effluent has very high BOD/ COD load and has a potential of causing harm to the aquatic ecosystems. Its valorisation with the help of microalgae can in addition to green pharmaceutical development get financial advantages due to decreasing culture media cost [7,10]. Scalability is further enabled by the cost-effective ability to cultivate S. dimorphus in a photobioreactor constructed of glass which is a broadly accessible and even inexpensive source. In contrast to the synthetic media, the utilization of waste streams follows the principles of the circular bioeconomy advancing the environmental resilience [7].

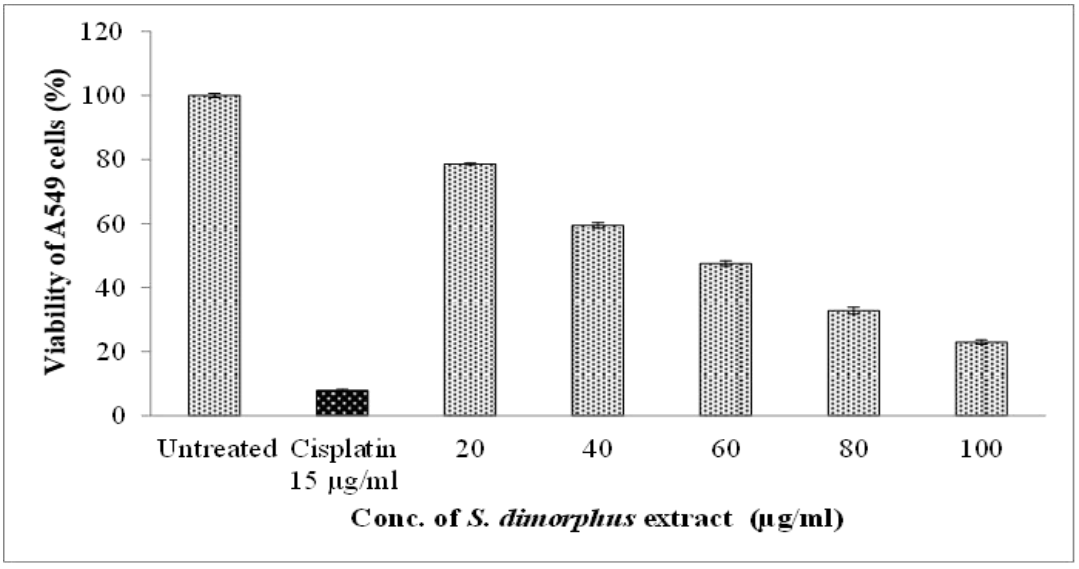
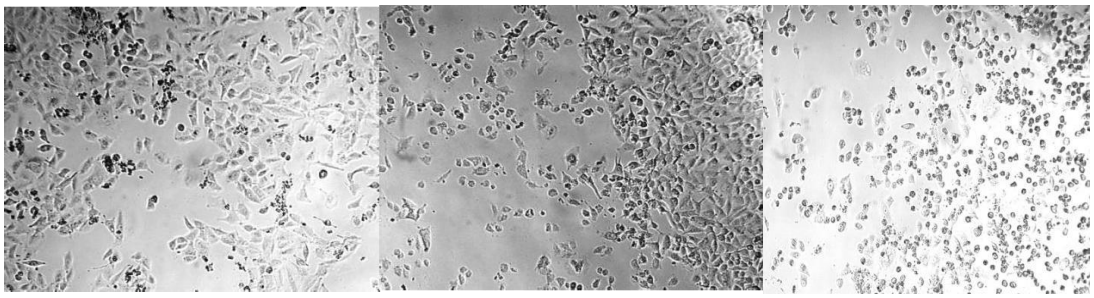
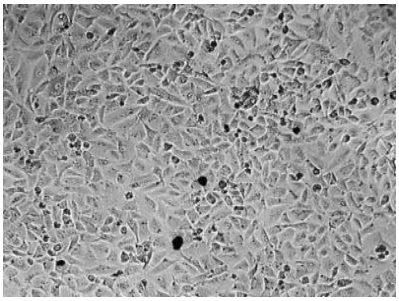


Figure 1(a): Anti-proliferation potential of ethanol extract of S. dimorphus against A549 cells.

20µg/ml 40µg/ml 60µg/ml



80µg/ml 100µg/ml Ciplatin, 15µg/ml



Untreated

Figure 1(b): Morphology of A459 cell line treated with different conc. of S. dimorphus Extract

4. CONCLUSIONS

Following conclusions were drawn from the present investigation.

Low concentrations of 50% dairy wastewater were used to grow S. dimorphus that accumulated a large amount of biomass rich in therapeutic metabolites in a new photobioreactor.

There was a dose-dependent cell death cytotoxicity of A549 lung cancer cells using ethanol extract with an IC_{50} = 57.49 µg/ml.

GC-MS analysis indicated the existence of compounds having known anticancer and antioxidant activities.

The article approves the two-fold application of dairy waste bioutilization and generation of therapeutic compounds.

S. dimorphus can be suggested as an ecofriendly source of new nutraceutical and pharmaceutical usage

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