

# In Vitro Stimulation of Ergosterol from Coelastrella Terrestris by Using Squalene and Studying Antioxidant Effect

Altaf AL-Rawi, Fikrat M. Hassan\* and Bushra M.J.Alwash

Department of Biology, College of Science for Women, University of Baghdad, Baghdad – Iraq

\*Corresponding author: [fikrat@csw.uobaghdad.edu.iq](mailto:fikrat@csw.uobaghdad.edu.iq)

## ABSTRACT

Ergosterol is one of the most important chemicals produced by algae, specifically by microalgae, and the Squalene is the commonly known as a precursor for biosynthesis of ergosterol. *Coelastrella terrestris* was isolated from sediment sample collected from the banks of Tigris River and the modified Chu 10 culture medium was used for algal growth and determining the optimum growth condition (25 °C and 268  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ). In an attempt to further maximize ergosterol production by *C. terrestris*. The optimal temperature and light growth conditions 30 °C and 300  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$  were tested under of different Squalene concentrations treatments (0.1, 0.25, 0.5 and 1%). This combined treatment of optimal culture conditions and Squalene was caused an extremely a highest ergosterol production recorded ( $533.3 \pm 15.92$  ppm) at 1% squalene in phase 2, while the lowest production ( $54.3 \pm 2.48$  ppm) was at 0.10% Squalene in phase3. The present study has further investigated the potential antioxidant activity of *C. terrestris* crude extract and ergosterole by the ability to scavenging free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results observed that antioxidant activity of algal crude extract was lower antioxidant as compared with the ergosterol and ascorbic acid (control), whereas antioxidant activity reached to 100% when used with crude extract the concentration (2000  $\mu\text{g}/\text{ml}$ ), ergosterol (50  $\mu\text{g}/\text{ml}$ ) and ascorbic acid (150  $\mu\text{g}/\text{ml}$ ). This study concluded that selectively combined culture conditions of 30°C and 300  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$  could maximize the biomass of the newly recorded *C. terrestris* and production of ergosterol. This study is the first test of the ability of *C. terrestris* to produce ergosterol and antioxidant activity.

**Keywords:** Algae, Chlorophyceae, *Coelastrella*, Squalene, Secondary products, Antioxidant.

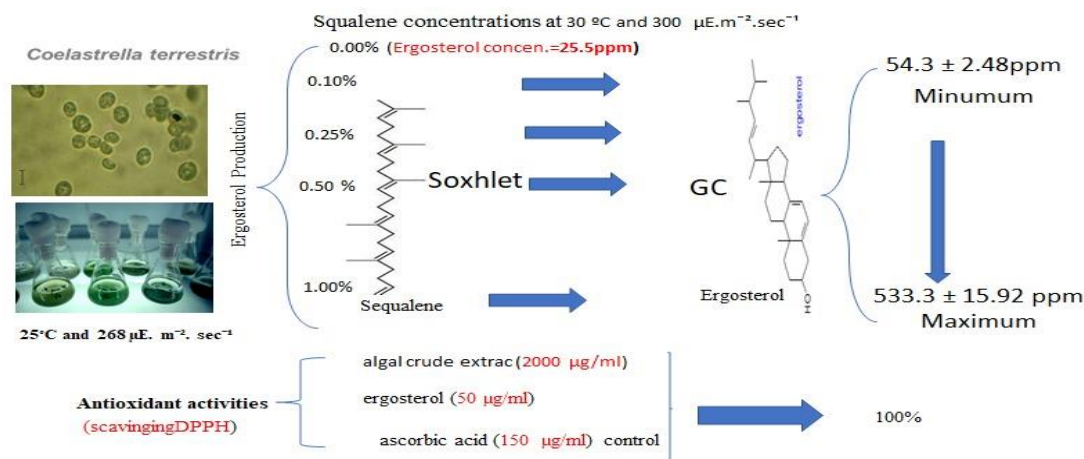
## Correspondence:

Fikrat M. Hassan

Department of Biology, College of Science for Women, University of Baghdad, Baghdad 10070 – Iraq.

Email: [fikrat@csw.uobaghdad.edu.iq](mailto:fikrat@csw.uobaghdad.edu.iq)

## Graphic Abstract



High production of ergosterol obtained from *Coelastrella terrestris*. Squalene precursor induced the ergosterol production from alga. The optimum temperature and light intensity (25°C and 268  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ) used to produce high ergosterol. Antioxidant activity of crud extract and ergosterol produced from *C. terrestris* noticed

## INTRODUCTION

Secondary metabolites are a diverse group of low molecular weight natural products synthesized by animals, plants, bacteria, fungi and algae, but are not required to survival of these organisms (1). Many recent studies investigated secondary products extracted from algae, also known as bioactive compounds, such as steroids, vitamins, alkaloids, phenols and carotenoid (2). Microalgae are described as the greatest natural sources of bioactive molecules due to their potential to produce these compounds in their cultures that are more difficult

to produce by synthetic chemical compounds (3). Secondary products have varied biological activities, such as antioxidant, anticancer, antimicrobial and bioremediation potential (4). Therefore, in medical practice, many of these active compounds are used to treat various diseases such as cancer, pain, inflammation, viral, bacteria and fungi infection and many other diseases, this fact has led to commercial production of some algal secondary products by fermentation technology, for example, *Chlorella* species are commercial utilized to produce 1,3- $\beta$ -glucan, that is

used to reduce blood lipids, whereas Permeah *et al.* (5) showed that the brown algae and two sea cucumber species are potentially presented as the marine sources of antioxidant compounds.

Ergosterol, cholesterol, chondrillasterol, poriferasterol, 28-isofucosterol, and other sterols are synthesized by the Chlorophyta (6). Sterols were first reported in green algae by Patterson *et al.* (7) who identified four major components in *Chlorell ellipsoidea*. Microalgae have ergosterol, 5 $\alpha$ -ergost-7-en-3 $\beta$ -ol, 22-trans-ergosta-5,8,22-trien-3 $\beta$ -ol and ergosta-5,8(9)-dien-3 $\beta$ -ol. As the most common sterol, which plays a key role in cell membrane and other cellular components (8). In addition to its important uses as a parameter in ecology and industry (9), ergosterol exerts crucial biological activities as antioxidant, anti-inflammatory and anticancer compound (10). Precursors addition are attempts to improve growth and productivity of secondary metabolites in plants and other microorganism such as algae (10). When the precursors are fed to culture media, they are incorporated into the biosynthesis pathway of secondary metabolites (11). The time of adding the precursor and its concentration are to be considered when applying precursors to the culture media (12). Several recent studies involved the effect of the precursor on plants such as Jiao *et al* (13), who investigated the effect of the precursor on alkaloids accumulation, AL-Mukhtar [14] showed that medium supplemented with 60 mg. L-1 tyrosine achieved the highest weight of morphine and papaverine in *Papaver somniferum* callus cultures. Another study by AL- Jubori (15) which indicated that by adding anthranilic acid as a precursor to the medium at different concentrations, this led to increasing the alkaloid accumulation dianthalexin in *Dianthus caryophyllus* callus cultures. While most of the research on precursor influence on steroid production was conducted on plants, very rare studies have been addressing such effects on algae in general and microalgae in particular.

The biosynthesis of ergosterol, as with other steroids, start from a linear precursor called "Squalene" that is formed by condensation of six isopentyle units (16) in a complex biosynthetic pathway that can involve more than thirty enzymes, Squalene together with cyclortanol, are important intermediary compounds in sterol biosynthesis (17).

Antioxidants are our first line of defense against free radical damage, with low concentration of them is able to prevent or delay the oxidation of substrate (18). While oxygen is one of the most essential components for living, oxygen is a highly reactive atom that is capable of becoming part of a potentially damaging molecules commonly called "free radicals"(19).Free radicals are able to attack the healthy cells and cause oxidative damage of proteins, DNA and other molecules that may lead to several diseases such as cancer, decline in brain function, heart diseases, and Alzheimer disease, among others (20). The high reactivity of these radicals is due to the presence of one unpaired electron which tends to donate it or to obtain another electron to attain stability. The study of antioxidant, the researchers used the synthetic free radical like 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as free radical scavenging activity, it has a violet color depending on unpaired electron and change to yellow that was caused by the ability of antioxidant substance to donate hydrogen to DPPH in order to scavenge the free radical (21)

The applications of synthetic antioxidants in medicine and food have been prohibited because of the possible risks posed to humans safety, the toxicity of these compounds can lead to significant damage affecting human health and safety, Therefore the need to find novel natural compounds that can inhibit oxidation is clear and increasing, since they lead to no risks or side effects(22). The natural antioxidant compounds found in many algae are more essential bioactive compounds that play a good role against various diseases and ageing through protecting the cell from oxidative reactions (23) Steroids were extracted from the fresh algae *laminaria obtus* and  $\beta$ -sistosterol showed quite strong antioxidant activity (24) Similar activities have been also demonstrated using aqueous extracts of sterols from the red algae *Acanthophors spicifera* and *Bryothamanion triquetrum* (25). The flavonoid and phenolic contents of methanol extracts of the fresh green algae *Chlorella vulgaris* and *Chlorella pyrenoidosa* species demonstrated obvious antioxidant, antibacterial and anticancer activities (26). *Spirulina platensis* protein hydrolysates can be used as nutritional food with antioxidant properties (27). The green alga *C. terrestris* contains carotenoids with high importance, highlighted by the fact that they are natural antioxidants used for protection against oxidative stress (28). In addition, ergosterol extracted from the microalgae *Chlorella pyranoidosa* (29), *Dunaliella tertiolecta* (30), and *Schizochytrium aggregatum* (31), showed antioxidant activities. The present study aimed at provid in *C. terrestris* to produce ergosterol under different concentration of Squalene as a precursor. By doing so, further investigations of the bioactive, including antioxidant, activities of this product can be conducted.

## MATERIALS AND METHODS

*C. terrestris* was isolated from sediment sample collected from the banks of Tigris River and the modified Chu 10 culture medium was used for algal growth and determining the optimum growth condition (25°C and 268  $\mu\text{E.m}^{-2}.\text{sec}^{-1}$ ) (32). Several factors were reported to affect growth and biomass of alga. In this study, effects of precursor were selected. The temperature 25°C and light intensity 268 $\mu\text{E.m}^{-2}.\text{Sec}^{-1}$  were used for cultivation of isolated alga, these levels were considered as control since they were reported to induce optimum growth of all microalgae, 30°C is the optimal temperature and 300  $\mu\text{E.m}^{-2}.\text{Sec}^{-1}$  is the optimal light intensity(33), they were selected as treatment interaction of 30°C and 300  $\mu\text{E.m}^{-2}.\text{Sec}^{-1}$  to stimulate production of microalgal steroid.

### Steroids Induced by Precursor

Squalene precursor (0.1, 0.25, 0.5, and 1%) was selected to be added to the media at both control treatment (25°C and 268  $\mu\text{E.m}^{-2}.\text{Sec}^{-1}$ ) and best interaction treatment (30°C, 300 $\mu\text{E.m}^{-2}.\text{Sec}^{-1}$ ).

### Harvesting of Sample

From day 20 of culture, the samples were harvested at three times during the growth period. The first stage of harvesting was at the last two days of exponential phase, which was named phase 1, while the second stage was at the first two days of stationary phase, which was called phase 2, and the third stage was at the last days of stationary phase which was called phase 3. Limitations to these stages depended on temperature degrees and light intensities used in each experiment.

### Extraction of Steroids

The harvested algae samples were filtered by filter papers (Millipore 0.1m) and the fresh weight of each sample was taken. The filtered sample was then dried in the desiccator apparatus at 37°C for 24 hours until having stable weight, followed by taking the dry weight for each sample [34].

### Soxhlet extraction

About 1g of powdered algal material was placed in the soxhlet thimble with 150 ml of methanol (99%) in 250 ml flat bottom flask. The apparatus was then connected with the water supply to the condenser. The process was carried out for 6-8 hours for each sample. The extract was transferred to petri plates and the solvent was allowed to evaporate. Each extract material was filtered and evaporated to dryness and weighted, then finally stored in the refrigerator for further use [35]. Steroid analysis

### GC analysis

Qualitative and quantitative analysis of ergosterol was performed by using Gas Chromatography (GC) system model (Shimaduz, 2010, Japan) in the Department of Water Treatment Technology- Ministry of Science and Technology, as previously described [36].

The samples were diluted in clean vials in 5 ml of methanol solvent with micropipettes. The sample vials were put in injector vial tray. 1 ml of each sample was injected into GC by an auto-injector and capillary column (DB-5ms×0.25 nm). The elements were detected in flame ionization detector reagent user (FID) and the temperature of reagent 310°C and pressure 100 kpa, over temperature was 150-290 °C and increased 10°C per minute. Identification of the component, in the algae was performed using retention time (Rt) which is defined as the time that the sample takes for passing through the system. Sample concentration was calculated using the area under the peak, according to the formula below.

Concentration of compound (ppm) = (peak area of compound / peak area of standard)  $\chi$  concentration of standard  $\chi$  dilution factor.

### Statistical analysis

The statistical analysis system SAS [37] was used to determine the effects of the studied factors with

different qualities. Least significant difference (LSD) test was used to compare mean values.

## RESULTS AND DISCUSSION

### Effects of Squalene Precursor on Ergosterol Production

After the determination of optimal temperature and light conditions (30°C and 300  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ) for highest growth and ergosterol production, the same conditions were used together with different concentrations of the steroid precursor, squalene, in order to define best conditions to maximize ergosterol production. The same squalene treatments were also tested in combination with control conditions (25°C and 268  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ).

### Effects of Squalene and Control Growth Conditions (268 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ + 25°C) on Growth of *C. terrestris*

The effect of different concentrations of squalene on the mean algal ergosterol concentrations are shown in **Table 1**. The highest ergosterol production (258.8  $\pm$  11.63) was recorded at 1% squalene in phase 2, while the lowest production (28.5  $\pm$  1.67) 0.10% in phase 3. All three phases results recorded a significant increase in mean of algal ergosterol concentrations with increase squalene concentrations, the ergosterol concentration in phase 1 increased from 123.2 ppm at 0.10% squalene to 203.6 ppm at 1% squalene, but in phase 2 the ergosterol concentration increased from 165 ppm at 0.10% to 258 ppm at 1% squalene. A significant decrease in ergosterol concentration was recorded in phase 3 compared with other phases.

The ergosterol concentration was performed by GC analysis during three growth phases at treatment 268  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$  + 25°C (control) and under the effect of different concentrations of Squalene. The results of GC during only phase 2 because the highest concentration of ergosterol was at phase 2 (figure 1). The highest concentration of ergosterol was at 1% Squalene with retention time 8.853 minutes, while the lowest was at 0.1% with retention time 8.874 minutes in the same phase. The Rt which obtained at identical chromatographic conditions of analysis samples was compared with ergosterol standard.

**Table 1:** Effect of different concentrations of Squalene (%) on ergosterol concentration (ppm) of *C. terrestris* during three growth phases at 268  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$  + 25°C. (Mean  $\pm$  SE).

Concentration Squalene %	Ergosterol concentration (ppm) in phase (1)	Ergosterol concentration (ppm) in phase (2)	Ergosterol concentration (ppm) in phase (3)	LSD value	Concentration Squalene %	Ergosterol concentration (ppm) in phase (1)
0.1	123.2 $\pm$ 5.74 c	165.0 $\pm$ 7.21 b	28.5 $\pm$ 1.67 c	19.72 *	0.1	123.2 $\pm$ 5.74 c
0.25	154.5 $\pm$ 6.91 b	192.1 $\pm$ 8.59 b	68.1 $\pm$ 3.42 b	23.85 *	0.25	154.5 $\pm$ 6.91 b
0.5	203.2 $\pm$ 9.42 a	229.5 $\pm$ 10.35 a	81.2 $\pm$ 3.92 a	29.31 *	0.5	203.2 $\pm$ 9.42 a

1	203.6 ± 8.87 a	258.8 ± 11.63 a	91.7 ± 3.77 a	22.94 *	1	203.6 ± 8.87 a
LSD value	24.671 *	33.724 *	12.636 *	---	LSD value	24.671 *

\* (P<0.05). Means having with the different letters in same column differed significantly. Phase 1 = The last two days of log phase. Phase 2 = The first two days of stationary phase. Phase 3 = The last days of stationary phase.

#### Effects of Squalene and Optimal Growth Conditions (300 µE. m<sup>-2</sup>.sec<sup>-1</sup> +30°C) on Weight of *C.terrestris*:

The effect of different concentrations of Squalene on the mean algal ergosterol concentrations are shown in Table 2, the highest ergosterol production (533.3 ± 15.92) was recorded at 1% Squalene in phase 2, while the lowest production (54.3 ± 2.48) at 0.10% Squalene in phase 3. All three phases results recorded a significant increase in mean of algal ergosterol concentrations with increase squalene concentrations, the ergosterol concentration in phase1 increased from 149.2 ± 6.79 ppm at 0.10% squalene to 273.2 ± 9.57 ppm at 1% squalene, but in phase 2 the ergosterol concentration increased from 153.7 ± 6.04 ppm at 0.10% to 533.3 ± 15.92 ppm at 1% squalene. A significant decrease in ergosterol concentration was recorded in phase 3 compared with other phases. The results showed significant differences between all phases and all treatments. The ergosterol concentration was performed by GC during three growth phases at treatment 300µE.m<sup>-2</sup>. sec<sup>-1</sup>+30°C and under effect of different concentrations of squalene. The highest concentration of ergosterol was at 1% squanele with retention time 8.853 minutes, while the lowest was at 0.1% with retention time 8.874 minutes in the phase2 (figure 2). The Rt obtained at identical chromatographic conditions of analysis samples was compared with ergosterol standard.

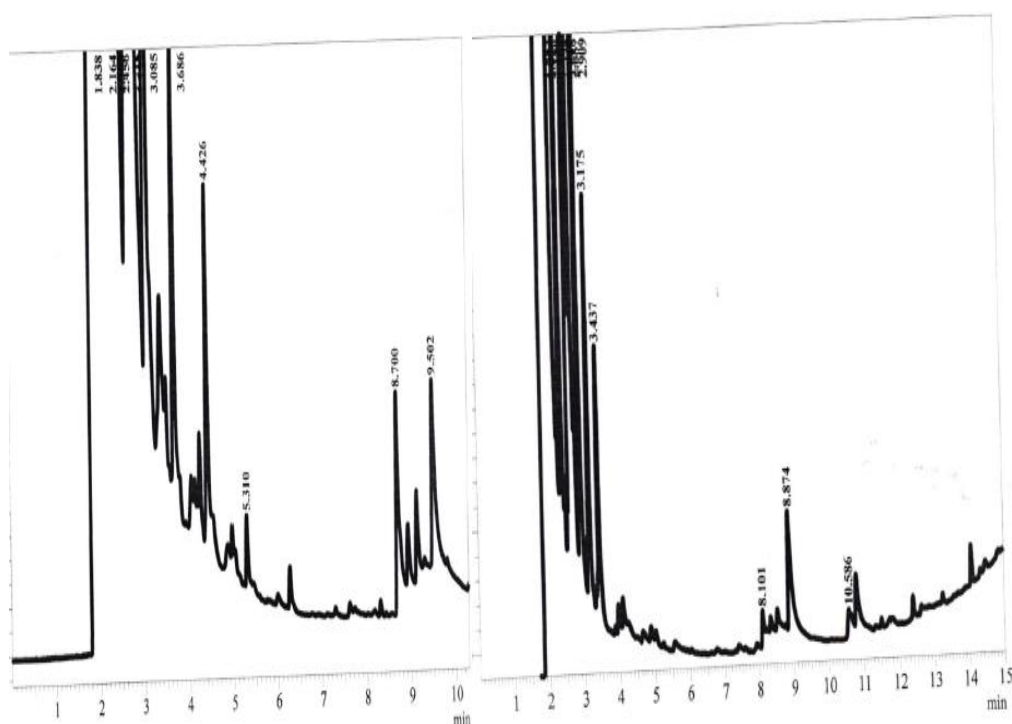
Precursors are defined as molecules that directly interfere in the synthesis of the secondary metabolites. After the addition of precursors to the culture medium,

they incorporate into biosynthetic pathways of these active metabolites (38).

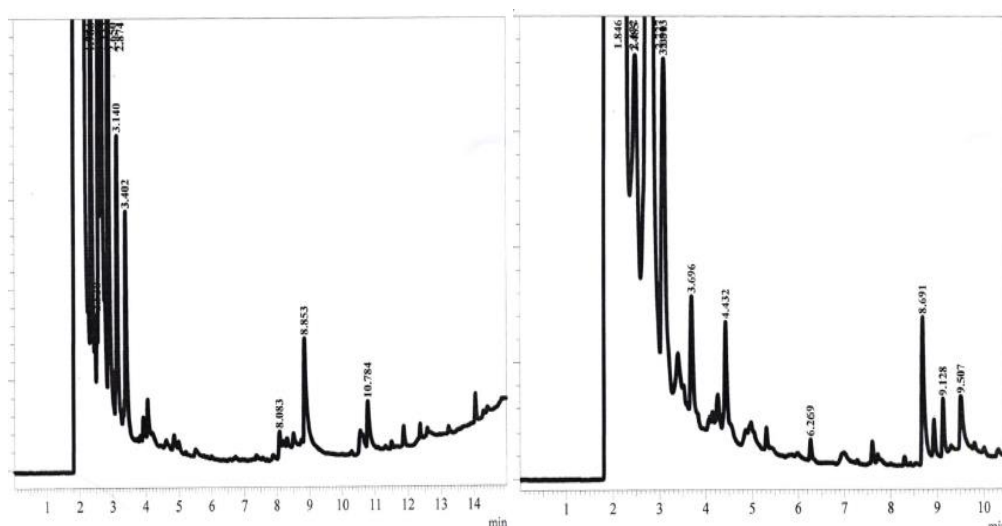
Squalene is a linear triterpene formed via the MVA or MEP biosynthetic pathway and is widely distributed in bacteria, fungi, algae, plants, and animals. Metabolically, squalene is used not only as a precursor in the synthesis of complex secondary metabolites such as sterols, hormones, and vitamins, but also as a carbon source in aerobic and anaerobic fermentation in microorganisms (39).

Squalene is in fact an essential intermediate in the biosynthesis of cholesterol, steroid hormones, and vitamin D. Optimum conditions such temperature was investigated for squalene production of the strain of *Aurantiochytrium sp.* Squalene production by this strain was optimum at 25 °C (40), that agrees with this study. Many chemicals were used as precursors to Enhanced production of organisms such as study by Wong (41) who pointed on that the effects of 2, 4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda*. Chemicals directly as metabolic precursors applying chemical triggers or enhancers to improve cell growth and the accumulation of bioproducts in algal cultures.

The main effects of precursors may back to effect on photosynthesis and chlorophyll-a synthesis which lead to increase the metabolites activity of algal cell and increased lipids contents which considered essential primary metabolites of steroids compounds synthesis. The study of squalene as a precursor in algae is very rare because it is the first study of its kind in Iraq.







**Figure 1:** Effect of different concentrations of squalene (%) on ergosterol concentration (ppm) of *C. terrestris* during growth phase 2 at  $268 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1} + 25^\circ\text{C}$  by GC analysis. A=0.1%, Rt= 8.700. B=0.25%, Rt=8.874. C= 0.5%, Rt=8.853. D=1%, Rt =8.691min

**Table 2:** Effect of different concentrations of squalene % on ergosterol concentration (ppm) of *C. terrestris* during three growth phases at  $300 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1} + 30^\circ\text{C}$ . (Mean  $\pm$  SE).

Concentration squalene %	Ergosterol concentration (ppm) in phase (1)	Ergosterol concentration (ppm) in phase (2)	Ergosterol concentration (ppm) in phase (3)	LSD value
0.10	149.2 $\pm$ 6.79 c	153.7 $\pm$ 6.04 c	54.3 $\pm$ 2.48 d	20.73 *
0.25	211.0 $\pm$ 8.25 b	247.4 $\pm$ 9.63 b	87.4 $\pm$ 3.71 c	26.82 *
0.50	204.2 $\pm$ 6.96 b	284.0 $\pm$ 12.67 b	100.7 $\pm$ 3.85 b	19.44 *
1	273.2 $\pm$ 9.57 a	533.3 $\pm$ 15.92 a	189.1 $\pm$ 7.51 a	34.97 *
LSD value	31.734 *	55.821 *	11.403 *	---

\* (P<0.05). Means having with the different letters in same column differed significantly Phase 1 = the last two days of log phase. Phase 2 = the first two days of stationary phase. Phase 3 = the last days of stationary phase.

One of the most interesting findings of the present study is the observation that combination of optimal culture conditions ( $300 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1} + 30^\circ\text{C}$ ) with the addition of optimal concentration of the precursor (1%) could increase the production of ergosterol to more than 15-fold. This clearly implies that squalene could easily facilitate production of large quantity of ergosterol, through its integration as a precursor in the metabolic biosynthetic pathway of this active biocompound. In the present study, optimizing culture conditions in terms of temperature and light could maximize the biomass of the newly recorded *C. terrestris* and, consequently, production of ergosterol. Interestingly, the addition of squalene as a precursor could further maximize ergosterol production to 15 folds.

As an explanation, it is probable that optimal heat and light conditions could initially bring the cell division and algal biomass to maximum yield, which naturally resulted in higher yield of ergosterol. Further, optimal culture conditions could bring enzymatic activities to maximum level, since enzymes are proteins that favor optimal levels of physiological conditions to reach optimal reactions. Finally, the addition of optimal squalene concentration contributed to even further increase in ergosterol production, since the addition of the precursor would normally result in higher

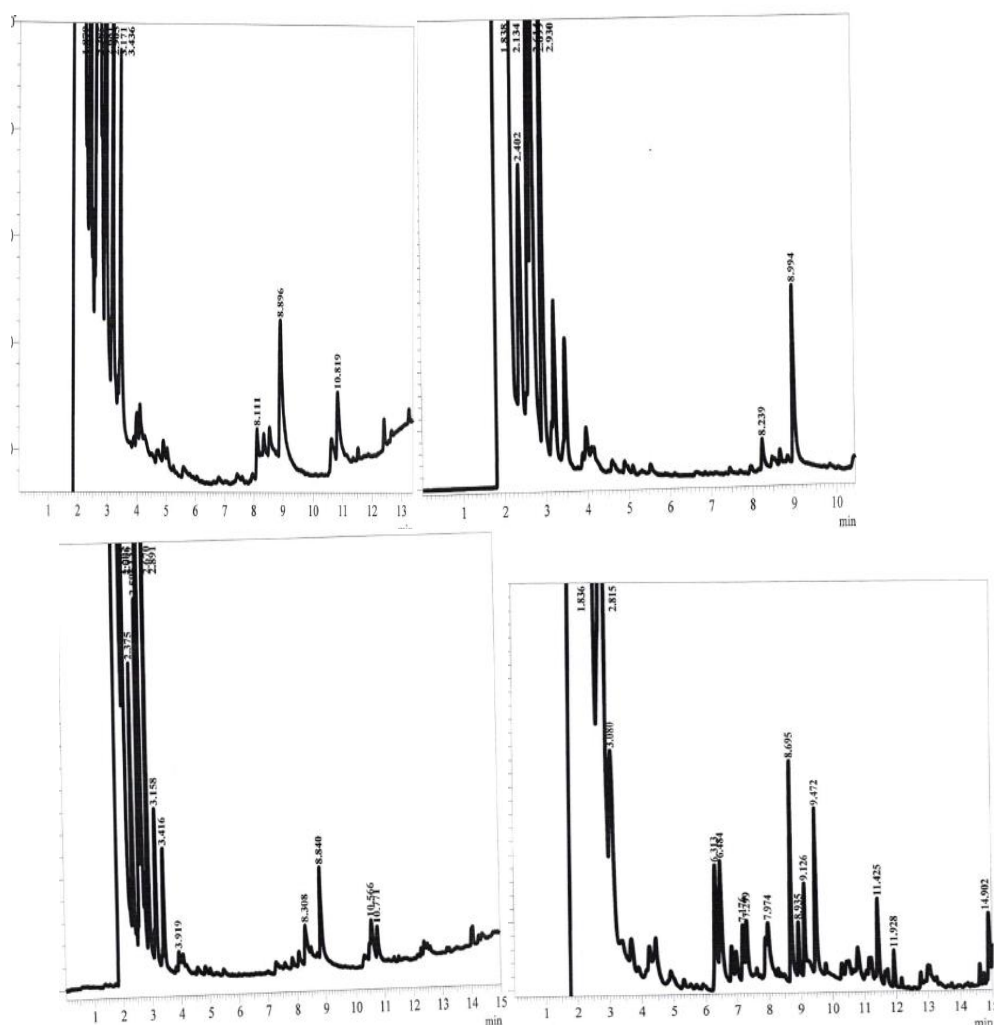
production of the final product. In conclusion, it seems that both optimal culture conditions and precursor concentration acted in a synergistic manner to lead to maximum hormone production; squalene provided higher amount of raw material needed for biosynthesis, whereas temperature and light brought enzymes to maximum activity that could invest and cover the increase in the amount of the precursor.

Therefore, it is valid to claim that the conditions provided by this study were optimal for ergosterol production by *C. terrestris*. In other words, squalene in even higher concentrations could not lead to maximum ergosterol production, because the culture conditions provided best environment to maximize enzymatic activities that is only sufficient to deal with certain or optimal amount of precursor. The present results confirm the previously reported advantage of squalene as a precursor for biosynthesis of various bioactive compounds in different organisms from different taxonomic levels.

**Antioxidant Activities of the Algal Crud Extract and the Standard of Ergosterol** The antioxidant activity of different concentrations of *C. terrestris* crud extract and ergosterol standard was measured by scavenging ability to DPPH, whereas ascorbic acid in similar concentrations was used as a control for antioxidant activity. The algae

crude extracts showed significantly lower antioxidant activities ( $p < 0.05$ ) when using the concentrations of 125, 250, 500 and 1000  $\mu\text{g/ml}$  ( $23.2 \pm 1.06$ ,  $44.6 \pm 2.48$ ,  $64.2 \pm 2.75$ ,  $83.2 \pm 3.19$ , respectively) as compared to similar concentrations of the control ( $89.6 \pm 2.53$ ,  $96.6 \pm 1.27$ ,  $100 \pm 0.00$ ,  $100 \pm 0.00$ , respectively). However, treatments with highest crude concentrations of 1500 and 2000  $\mu\text{g/ml}$  didn't show significant differences as compared to the control (**Table 3**).

Treatment of DPPH with the algal extracted ergosterol showed significantly higher antioxidant activities ( $p < 0.05$ ) when using the concentrations of 12.5, 25, 50 and 100  $\mu\text{g/ml}$  ( $85.3 \pm 2.57$ ,  $99.6 \pm 1.64$ ,  $100 \pm 0.00$ ,  $100 \pm 0.00$ , respectively) as compared to similar concentrations of the control treatment ( $73 \pm 2.42$ ,  $79 \pm 2.96$ ,  $81 \pm 3.07$ ,  $85 \pm 3.29$ , respectively). However, treatment with highest ergosterol concentrations of 125  $\mu\text{g/ml}$  didn't show significant differences as compared to the control (**Table 4**).



**Figure 2:** Effect of different concentrations of squalene % on ergosterol concentration (ppm) of *C. terrestris* during growth phase 2 at  $300 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1} + 30^\circ\text{C}$  by GC analysis. A=0.1%, Rt= 8.994. B=0.25%, Rt=8.700. C= 0.5%, Rt=8.840. D=1%, Rt =8.965.

**Table 3:** Antioxidant activities % of different concentrations of algae crude extracts of *C. terrestris*. Measured by scavenging ability to DPPH. (Mean  $\pm$  SE).

Concentration ( $\mu\text{g/ml}$ )	% Antioxidant activity		LSD value
	Crud extract	Ascorbic acid	
125	$23.2 \pm 1.06$	$98.96 \pm 2.53$	8.631 *
250	$44.6 \pm 2.48$	$100 \pm 1.27$	8.037 *
500	$64.2 \pm 2.75$	$100 \pm 0.00$	7.881 *
1000	$83.2 \pm 3.19$	$100 \pm 0.00$	8.922 *

<b>1500</b>	94.1 ± 2.65	100 ± 0.00	6.776 NS
<b>2000</b>	100 ± 0.00	100 ± 0.00	0.00 NS
<b>LSD value</b>	9.372 *	8.955 *	---

\* (P&lt;0.05)

**Table4:** Antioxidant activities % of different concentrations of ergosterol from *C. terrestris*. Measured by scavenging ability to DPPH. (Mean ± SE).

Concentration (µg/ml)	% Antioxidant activity		LSD value
	Extracted Ergosterol	Ascorbic acid	
<b>12.5</b>	85.3 ± 2.57	73 ± 2.42	7.163 *
<b>25</b>	99.6 ± 1.64	79 ± 2.96	6.981 *
<b>50</b>	100 ± 0.00	81 ± 3.07	7.506 *
<b>100</b>	100 ± 0.00	85 ± 3.29	7.214 *
<b>125</b>	100 ± 0.00	100 ± 0.00	1.00 NS
<b>LSD value</b>	8.179 *	8.648 *	---

\* (P&lt;0.05)

The present work is the first published report on antioxidant potential of active compounds produced by *C. terrestris*. However, at the genus level, only one previous study reported that algal extract from *C. striolata* var. *multistriata* included carotenoids with an antioxidant potential (42). The results of this study referred that ergosterol had the ability to scavenging free radicals when compared with crude extract and ascorbic acid (control), and this is represent by donating their hydrogen atom to the free radicals of DPPH, these results agree with the study of Shao *et al.*(43)who postulated that the ergosterol content in *Button Mushrooms* positively correlated with the antioxidant activities. A few studies Dehpour *et al.* (44) and Marinova and Batchvarov(45) found that DPPH was a stable nitrogen centered free radical changing of the color from violet to yellow. These results were caused by the ability of crude extract, ergosterol and ascorbic acid which donate hydrogen or electron atom to DPPH in order to scavenge the free radicals. This process was done by measuring bleaching process from the purple color of methanol solution of (DPPH) and convert it into the yellow color (46). Table 4 shows an increase in the antioxidant effect of the crude extract with increased its concentration while this effect was lower in compared with ascorbic acid at the same concentration. These results might be the crude extract of *C. terrestris* containing on active compounds along with steroids compounds such alkaloids, saponins, terpenoids, and flavonoids which have antioxidant activity but did not show higher effect because of their low concentrations. The ergosterol treatment showed higher antioxidant activity compared to ascorbic acid in the same concentration, this is due to their higher concentration without any another active compounds (43). The ergosterol compound which used in this technique was standard because of the purification process of ergosterol from crud extract wants long time. The antioxidant potential of natural products from microalgae, including phytosterols, is

extremely essential because synthetic antioxidants such as propyl gallate and butylated hydroxytoluene applied to food and medical products raised wide concerns due to their reported risks to human health(47).There are several recent studies that agree with this study which referred to importance of Sterols (steroid with OH group) such Stigmasterol and  $\beta$ -sitosterol as antioxidant properties [48].The results of this study as antioxidant disagree with study of Yen *et al.* (49) who declared that the ergosterol showed no antioxidant activity on the peroxidation of linoleic acid.

Conclusion the combination of optimal culture conditions with the addition of optimal concentration of the precursor could increase the production of ergosterol to more than 15-fold. The ergosterol produced by the identified and cultured species could show a significant antioxidant activity, but not when used in high concentrations. To the best of the author's knowledge, this is the first record of *C. terrestris* in Iraq and this is the first report of high production of ergosterol and antioxidant activity by this species of algae.

## REFERENCES

1. Wink M. Modes of Action of Herbal Medicines and Plant Secondary Metabolites. *Medicines* 2015 Sept; 2: 251-286. doi:10.3390/medicines2030251.
2. Lutz G A,Turgut Dunford N. Interactions of microalgae and other microorganisms for enhanced production of high-value compounds. *US NIH*.2018 Mar; 23:1487-1504.
3. Priyadarshani I, Rath B. Bioactive compounds from microalgae and cyanobacteria: utility and applications. *IJPSR*.2012October; 3(11): 4123-4130.
4. Baroty G S,Moussa M Y, Shallan M A, Ali M A ,Sabh A Z, Shalaby E A. Contribution to the Aroma, Biological Activities, Minerals, Protein, Pigments and Lipid Contents of the Red Alga: *Asparagopsis taxiformis* (Delile)Trevisan. *JASR*. 2007Mar; 3(12):1825-1834.

5. Perme P, Gohari AR, Saeidnia S, Jamili S, Mostafavi P G, Mirnezami T. Antioxidant Activity Of Two Sea Cucumber Species And a Brown Algae From Persian Gulf. 2nd National Congress on Medicinal Plants. 2013 May).
6. Patterson GW, Thompson M J, Dutkyw S R. To new sterols from *Chlorella ellipsoidea*☆. *Phytochemistry*. 1974; 13(1): 191-194.
7. Patterson GW, Thompson M J, Dutkyw S R. To new sterols from *Chlorella ellipsoidea*☆. *Phytochemistry*. 1974; 13(1): 191-194.
8. Tardieu D, Bailly JD, Benard G, Guerre R. Comparison of two extraction methods for ergosterol determination in vegetal feeds. *Rev Med Vet (Toulouse)*. 2007;158(8-9):442-446.
9. Hippelein M, Rugamer M. Ergosterol as an indicator of mould growth on building materials. *Int. J. Hyg. Environ. Health*. 2004 Sept; (4): 379-385. DOI: 10.1078/1438-4639-00303
10. Shahid I, Rizwan M, Baig DN, Saleem RS, Malik KA, Mehnaz S. Secondary Metabolites Production and Plant Growth Promotion by *Pseudomonas chlororaphis* and *P. aurantiaca* Strains Isolated from Cactus, Cotton, and Para Grass. *J Microbiol Biotechnol*. 2017 Mar; 27(3):480-491. doi: 10.4014/jmb.1601.01021.
11. Al-Mafargi K. I. R. Study the effect of some biotic and abiotic factors On enhancement of essential oil and rosmarinic acid in rosemary *Rosmarinus officinalis* L. In vitro. Thesis, Department of biotechnology, Al-Nahrain University. 2010.
12. Matern U. *Dianthus* species (carnation): In vitro culture and the biosynthesis of diantalexin and other secondary metabolites Medicinal and Aromatic Plants. *Agriculture*. 1994; 28:170-184.
13. Jiao C., Song C., Zheng S. Y., Qing Z., Cai Y., Lin Y. Metabolic profiling of *Dendrobium officinale* in response to precursors and methyl jasmonate. *Int. J. Mol. Sci*. 2018 Mar; 19 (3):728-739. <https://doi.org/10.3390/ijms19030728>
14. AL-Mukhtar S. A. Influence of polyethylene glycol and tyrosine on morphine alkaloids production from callus of *Papaver somniferum* in vitro. *J. Pharm. Sci. and Res*. 2018;10(2):282-287.
15. AL- Jubori S. H. Induction of alkaloid in callus culture of *Dianthus caryophyllus* L. plant and association with chromosomal variations. Thesis Depart. of Biology, Baghdad University, Iraq. PhD. (2018).
16. Kannenberg E. L., Poralla K. Hopanoid biosynthesis and function in bacteria. *Naturwissenschaften*. 1999 April; 86(4): 168-176.
17. Cardozo K. H. 1., Guaratini T., Barros M.P., Falcão V.R., Tonon A.P., Lopes N.P., et al. Metabolites from algae with economic impact. *Comp Biochem Physiol C Toxicol Pharmacol*. 2007 Jul; 146(1-2):60-78.
18. Vinay R., Prakash R., Sushil S. Antioxidant Activity of Some Selected Medicinal Plants in Western Region of India. *Adv Biol Res* 2010; 4 (1): 23-26.
19. Marinova Wahlqvist M.L. Antioxidant relevance to human health. *Asia Pac J Clin Nutr*. 2013;22 (2):171-176. <https://doi.org/10.6133/apjcn.2013.22.2.21>
20. Batchvarov G., V. Evaluation of the methods for determination of the free radical scavenging activity by DPPH. *BULG J AGRIC SCI*. 2011 Febr; 17(1):11-24 Agricultural Academy.
21. Michalak K. Chojnacka I. Algae as production systems of bioactive compounds. *Eng Life Sci*. 2015 Dese; 15(2):160-176. <https://doi.org/10.1002/elsc.201400191>
22. Plaza M., Cifuentes A., Ibaez E. In the search of new functional food ingredients from algae. *Food Sci. Technol*. 2008 Janu ; 19(1): 31-39. <https://doi.org/10.1016/j.tifs.2007.07.012>
23. Weng X. C., Wang W. Antioxidant activity of compounds isolated from *Salvia plebeian* Food chem. 2000 Dese; 71(4): 489-493. [https://doi.org/10.1016/S0308-8146\(00\)00191-6](https://doi.org/10.1016/S0308-8146(00)00191-6)
24. Vasconcelos J. B., Vasconcelos E.R.T.P., Urrea-Victoria V., Bezerra P.S., Reis T.N.V., Navarro D.M.A.F., et al. Antioxidant activity of three seaweeds from tropical reefs of Brazil: potential sources for bioprospecting [Internet]. *J. Ap. Phyco*. 2018. Available from: <http://dx.doi.org/10.1007/s10811-018-1556-5>
25. Lee S. H., Jeon Y. J. Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. *Fito. terapia*. 2013 April; 86: 129-136. <https://doi.org/10.1016/j.fitote.2013.02.013>
26. Guedes A. C., Amaro H. M., Malcata F. X. Microalgae as sources of carotenoids. *Mar. drugs*. 2011; 9 (4): 625-644.
27. Mendiola J. A., Jaime L., Santoyo S., Reglero G., Cifuentes A., Ibañez E. Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chem*. 2007 December; 102(4):1357-1367
28. AL-Rawi A., Alwash B. M., Hassan F. m.. A new record of *Coelastrella terrestris* (REISIGL) Hegwald & N. Hanagata, 2002 (Sphaeropleales, Scenedesmaceae) in IRAQ. *Bull. Iraq Nat. Hist. Mus*. 2018; 15(2): 153-161. <https://doi.org/10.26842/binhm.7.2018.15.2.0153>
29. Klosty M., Bergmann W. Sterols of Algae. III. The occurrence of ergosterol in *Chlorella pyranoidosa*. *J. Am. Chem. Soc*. 1952 March; 74 (6): 1601-1601. <https://doi.org/10.1021/ja01126a513>
30. Francavilla M., Colaianna M., Zotti M., Morgese M., Trotta P., Tucci P. et al. Extraction, Characterization and in vivo neuromodulatory activity of phytosterols from microalga *Dunaliella tertiolecta*. *Curr. Med. Chem*. 2012; 19(18): 3058-3067.
31. Lv J., Yang X, Ma H, Hu X, Wei Y, Zhou W., et al. The oxidative stability of microalgae oil (*Schizochytrium aggregatum*) and its antioxidant ability after gastrointestinal digestion: relationship with constituents. *Eur. J. Lipid Sci. Technol*. 2015; 117(2): 1928-1939. <https://doi.org/10.1002/ejlt.201400588>
32. AL- Rawi A., Alwash B. M., Hassan F.M.. In vitro stimulation of *Coelastrella Terrestris* (REISIGL) Hegwald & N. Hanagata, 2002 (Sphaeropleales, Scenedesmaceae). *Biochem. Cell. Arch*. 2019, 20(1):1693-1702.
33. Mittal S. A. Thin layer chromatography and high pressure liquid chromatography profiling of plant extracts of *Viola odorata* Linn. *Int. J. Pharm Bio Sci*. 2013; 4(1):542-549.
34. Mendiola J. A., Jaime L., Santoyo S., Reglero G., Cifuentes A., Ibañez E. Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chem*. 2007 December; 102 (4): 1357-1367. <https://doi.org/10.1016/j.foodchem.2006.06>



35. Sathish A., Sims R.C. Biodiesel from mixed culture algae via a wet lipid extraction procedure. Bio. Tech. 2012Augustes; 118: 643-647. [https://doi: 10.1016/j.biortech.2012.05.118](https://doi.org/10.1016/j.biortech.2012.05.118). Epub 2012 May 29
36. Arita M, Asai K, Nishioka T. Finding precursor compounds in secondary metabolites. Kyoto University, Kyoto, Japan, Review 2000January; 3(2): 606-856.
37. SAS (Statistical Analysis System), User's Guide. Statistical. V. Inst. Inc. Cary. N.C. A. 9.Sathish, Ashik, and Ronald C Sims 2012.
38. Ghimire G. P., Thuan N. H., Koirala N., Sohng J. K.Advances in biochemistry and microbial production of squalene and its derivatives. J. Micro. and Biot. 2016; 26(3) 441-451.
39. Bora B., Zhou J., Defretin S., Vandewalle inventors X, Freres R. Strain of microalga that produces squalene. United States patent US 9,476,074. 2016 Oct 25.
40. Nakazawa A., Matsuura H., Kose R., Kato S., Honda D., Inouye I., *etal*. Optimization of culture conditions of the *thraustochytridaurantiochytrium* sp. strain 18W-13a for squalene production. Bioresour Tech. 2012 April; 109:287-291.
41. P. K. Wong. Effects of 2,4-D, glyphosate and paraquat on growth, photosynthesis and chlorophyll-a synthesis of *Scenedesmus quadricauda* (Berb) 614. Chemosphere. 2000 July;41(1-2):177-182.
42. Abe K, Hattori H., Hirano M. Accumulation and antioxidant activity of secondary carotenoids in the microalga *Coelastrella striolata* var. *multistriata*. Food Chem. 2005 December; 100(2):656-661. <https://doi.org/10.1016/j.foodchem.2005.10.026>
43. Shao S, Hernandez M., Kramer J. K, Rinker D.L., Tsao R. Ergosterol profiles, fatty acid composition, and antioxidant activities of button mushrooms as affected by tissue part and developmental stage. J. Agri. F.Chem. 2010 November;58(22):11616-25. <https://doi.org/10.1021/jf102285b>
44. Dehpour A. A., Ebrahimzadeh M. A., Fazel N. S., Mohammad N. S. Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. Grasas y Acities. 2009April; 60(4): 405-412. <https://doi.org/10.3989/gya.010109>
45. Marinova G., Batchvarov V. Evaluation of the methods for determination of the free radical scavenging activity by DPPH. Bulgarian. J. Agr.Sc. 2011February;17(1):11-24.
46. X. Luo, P. Su, W. Zhang. Advances in microalgae-derived phytosterols for functional food and pharmaceutical applications. Mar Drugs. 2015 July; 13(7):4231-54.
47. Shanthakumar B., Sathish M., Suresh A. J. In Vitro Antioxidant Activity of Extracts and Stigmasterol from Leaves of *Clerodendrum inerme* Linn. Research J. Pharm., Bio. Chem. Scie. 2013 October-December; 4(4) :1411-1418.
48. Alwash B. M, Hamad S. F. The effect of biotic and abiotic elicitors on Dianthalexin production from the callus of *Dianthus caryophyllus*. Inter. J. Chem. Tech. Rese. 2017; 10 (6): 25-30.
49. Chang Yen G., Y., Wen S. Antioxidant activity and active compounds of rice koji fermented with *Aspergillus Candidus*. Food Chem. 2003 Oct 1; 83(1):49-54.